

Purified Timothy grass pollen major allergen Phl p 1 may contribute to the modulation of allergic responses through a pleiotropic induction of cytokines and chemokines from airway epithelial cells

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Summary

By definition, allergens are proteins with the ability to elicit powerful T helper lymphocyte type 2 (Th2) responses, culminating in immunoglobulin (Ig)E antibody production. Why specific proteins cause aberrant immune responses has remained largely unanswered. Recent data suggest that there may be several molecular paths that may affect allergenicity of proteins. The focus of this study is the response of airway epithelium to a major allergen from *Phleum pratense* Phl p 1. Instead of focusing on a few genes and proteins that might be affected by the major allergen, our aim was to obtain a broader view on the immune stimulatory capacity of Phl p 1. We therefore performed detailed analysis on mRNA and protein level by using a microarray approach to define Phl p 1-induced gene expression. We found that this allergen induces modulation and release of a broad range of mediators, indicating it to be a powerful trigger of the immune system. We were able to show that genes belonging to the GO cluster 'cell communication' were among the most prominent functional groups, which is also reflected in cytokines and chemokines building centres in a computational model of direct gene interaction. Further detailed comparison of grass pollen extract (GPE)- and Phl p 1-induced gene expression might be beneficial with regard to the application of single components within diagnosis and immunotherapy.

Keywords: airway epithelial cells, cytokines, grass pollen extract, microarray, Phl p 1

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Introduction

Allergens are defined as proteins that have the ability to elicit powerful T helper lymphocyte type 2 (Th2) responses, culminating in immunoglobulin (Ig)E antibody production. Why specific proteins cause such an aberrant T and B cell response has remained largely unanswered. Recent data suggest that there may be several molecular paths that may affect allergenicity of proteins. These paths seem to depend on some intrinsic biological activity of the allergens, indicating that allergens can be more than passive players whose sole function is to cross-link IgE-molecules on the cell surface of mast cells.

Proteolytic activity as a general feature of major allergens has been proposed to be involved in the pathogenesis of allergies by enabling the passage of allergens through the epithelial barrier, cleaving adhesion molecules and affecting the functions of various cells and immune responses [1–4].

Recently it has become apparent that some allergen-binding lipids may contribute to the allergenicity of these

proteins by affecting the response of cells to these lipids. For instance, Der p 2 was identified to resemble an important co-factor for Toll-like receptor (TLR)-4 signalling (MD-2), not only structurally but also functionally [5,6]. Several other members of the MD-2-like lipid-binding family are major allergens [7], referring to a more general importance of major allergens being able to bind lipids. Furthermore, a broad role for complex carbohydrates, in particular glucans, in allergen-associated Th2 responses is emerging [8].

However, the allergenicity of these proteins might be independent of their intrinsic biological function. Co-exposure of a tolerogenic protein with a protease can induce allergic sensitization so that environmental proteases derived from bacterial or viral origin might have accessory roles. Furthermore, microorganism-derived components, usually present in allergen extracts, could act as adjuvants with regard to the induction of immune responses.

Due to recent publications there is a growing awareness of the role of structural cells concerning allergen-induced immune responses. In particular, respiratory epithelium is

likely to be important within the process of sensitization. It is accepted to be an active player in the response to the biological activity of aeroallergens, where they contribute to the immunological response by generating a microenvironment for the attraction and regulation of the activity of immune competent cells [9–12].

The focus of this study is the response of airway epithelium to the purified major allergen Phl p 1. Instead of focusing on a few genes and proteins that might be affected by the major allergen, our aim was to obtain a broader view on the immune-stimulatory capacity of Phl p 1. Furthermore, the detailed investigation of the gene expression and mediator release induced by the single major allergen might provide new insights into the contribution of the major allergen to the grass pollen extract (GPE)-induced response of airway epithelium. We therefore performed a detailed analysis on mRNA and protein level by using a microarray approach to define Phl p 1-induced gene expression to define the contribution of the purified major allergen to the induced allergic response.

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 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.

Experimental set-up

NCI-H292 cells were cultured in six-well plates to 80% confluence. Before stimulation, culture medium was removed and the cells preincubated with Hanks's balanced salt solution (HBSS) for 24 h. Cells were then stimulated with grass pollen major allergen Phl p 1 (5 µM) diluted in HBSS or with HBSS alone; 24 h after stimulation supernatants were collected and stored for further analysis. The cells were used for RNA isolation.

Preparation and purification of Phl p 1

Timothy grass pollen major allergen Phl p 1 was purified from pollen (ARTU Biologicals, Lelystad, the Netherlands), as described previously [13,14].

RNA isolation

Total RNA from each sample was extracted using Trizol (Life Technologies, Inc., Gaithersburg, MD, USA), according to

manufacturer's instructions, followed by purification by nucleospin RNA II (Machery-Nagel, Düren, Germany). Concentration of extracted RNA 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) was used to analyse Phl p 1-induced gene expression. The technical handling 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, as described previously [15].

Microarray data analysis and statistics

Experiments were performed in triplicate and simultaneously. Gene chip images and data sets were uploaded into the server of the Netherlands Bioinformatics Center using the Rosetta Resolver Biosoftware package, which was also used for statistical analysis. Triplicates were compared in a factorial design using an error-weight one-way analysis of variance (ANOVA). 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 glyceraldehyde 3-phosphate dehydrogenase (GAPDH), 3'/5' ratios beta-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 [16]. Statistical analysis for differential gene expression was performed using ANOVA (MAANOVA package, version 0.98.8 [17]). The permutation-based Fs test was used for hypothesis testing [18] and all *P*-values were adjusted for false discovery rate correction [19]. For gene ontology and network interaction analysis, GeneSpring GX11 (Agilent Technologies) was used. Correlations between observed fold changes within the microarray data and real-time PCR data were determined using Pearson's correlation.

Real-time PCR

Quantitative real-time PCR was performed with the Light Cycler 2.0 System (Roche Diagnostics, Mannheim, Germany) using LightCycler® Fast Start DNA Master^{PLUS}, according to the manufacturer's instructions. Relative cDNA amount was calculated compared to the expression of the housekeeping gene hypoxanthine-guanine phosphoribosyltransferase (HPRT).

Protein multiplex Luminex Bio-Plex assay

Cell free supernatants of Phl p 1- and control-treated cells were stored at -20°C until analysis. Cytokine levels in the supernatants of the treated 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. Fold changes (FC) were calculated compared to 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 basis of the detection limit for the respective mediator.

Results

Phl p 1-induced changes in gene expression level in airway epithelial cells

The overall gene expression ratio between HBSS and Phl p 1-stimulated cells was measured and calculated using the Rosetta resolver software. Expression was considered to be expressed significantly differentially with $P \leq 0.01$ after one-way Bonferroni multiple test correction. The Affimetrix U133 plus 2.0 contains 54 675 transcripts, representing about 38 500 genes. Stimulation of NCI-H292 cells with Phl p 1 induced a huge response. In total, 7218 transcripts showed a significant regulation of gene expression. The subsequent analysis of transcripts, that were changed by more than threefold, revealed 86 transcripts to be up-regulated and 16 transcripts to be down-regulated, corresponding to 54 and 11 known genes, respectively.

For validation of the microarray data we first investigated the influence of stimulation with Phl p 1 on the expression of housekeeping genes. Analysing the expression of these genes revealed that their regulation is not affected by stimulation with Phl p 1. For example, the average expression ratio between control and stimulated condition for GAPDH has been $1.03 (\pm 0.00)$, and beta-2 microglobulin (B2M) showed a fold change of $0.90 (\pm 0.01)$. To confirm further the observed expression levels, we chose 11 genes from the 65 known genes that showed an up- or down-regulation by more than threefold for confirmatory real-time polymerase chain reaction (PCR). In Table 1 the ratios calculated from the microarray data and the real-time PCR derived expression ratios are shown. Expression levels derived from real-time PCR were calculated as fold change between control and Phl p 1-stimulation and normalized to GAPDH and HPRT. The real-time PCR data strongly resemble the ratios calculated on the basis of the microarray data (see Fig. 1). Statistical analysis of the two sets of data revealed a high correlation ($R = 0.79$ with $R^2 = 0.63$), pointing towards a correlation of 63% between the microarray data and the results of the real-time PCR.

Table 1. Validation of the Phl p 1-induced expression profile of selected genes using real-time polymerase chain reaction (PCR). All values are given as mean ($\pm$ standard deviation). Real-time PCR values are stated as fold change between control and Phl p 1-stimulated cells and normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and hypoxanthine-guanine phosphoribosyltransferase (HPRT).

Gene	Microarray ratio	Real time-PCR (fold induction)
CCL 20	10.9 (± 0.1)	50.2 (± 7.8)
IL-8	7.4 (± 0.1)	8.9 (± 4.2)
IL-13RA2	12.8 (± 1.0)	n.d.
SERPINF4	7.3 (± 0.2)	7.1 (± 1.9)
TNFAIP6	3.0 (± 0.1)	14.3 (± 1.8)
COL8A1	11.9 (± 1.2)	22.1 (± 3.3)
VTCN1	-3.4 (± 0.3)	-8.0 (± 1.8)
PTGS2	4.8 (± 0.2)	8.9 (± 0.6)
CXCL2	6.4 (± 0.1)	9.0 (± 3.1)
CXCR4	6.0 (± 0.2)	9.5 (± 3.0)
TGF-B2	3.7 (± 0.05)	3.6 (± 0.8)

After successful validation of the microarray data we continued with sorting the genes that were regulated at least threefold up or down after stimulation with Phl p 1 into functional classes. Using their ascribed gene ontology, genes were assorted by their molecular function or the biological process in which they are involved (Table 2). After correcting the assorted gene categories for the group 'unclassified genes', we found 10 genes (16.4%) to be in the group 'immunity and defence', four genes in the group 'receptor activity', 'enzyme inhibitor activity' (two) and 'ion binding' (three). Comparable to previous results we obtained with GPE the groups 'cell communication', representing cytokines, chemokines and growth factors, and 'metabolism' appeared to be the most prominent ontology groups, with 23 genes (38%) and 16 genes (26.2%), respectively. All genes identified to be single members of a certain ontology group were pooled within the category 'miscellaneous'.

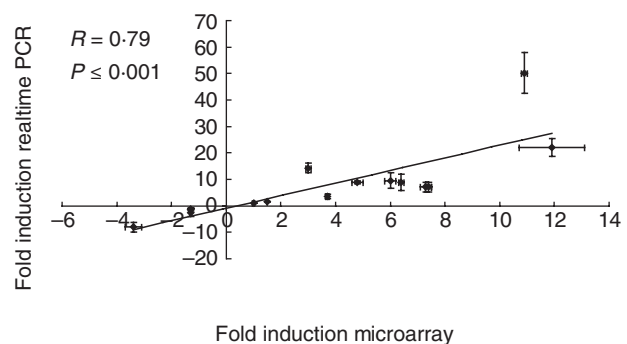


Fig. 1. Correlation plot of real-time polymerase chain reaction (PCR) data and microarray results. Correlations between observed fold changes within the microarray data and the real-time PCR data were determined using Pearson's correlation.

Table 2. Genes expressed differentially in NCI-H292 cells after stimulation with Phl p 1, sorted by ontology into functional groups. Stated are gene aliases, fold changes calculated in relation to control-stimulated cells, gene names, *P*-values and accession numbers.

Gene alias	Fold change	Gene name/description	<i>P</i> -value	Accession no.
Cell communication				
COL8A1	11.9	Collagen, type VIII, alpha 1	1.54E-06	226237_at
IL6	11.2	Interleukin 6 (interferon, beta 2)	4.48E-08	205207_at
CCL20	10.9	Chemokine (C-C motif) ligand 20	5.91E-09	205476_at
IGFBP5	8.0	Insulin-like growth factor binding protein 5	1.65E-08	211959_at
IL8	7.4	Interleukin 8	2.88E-08	211506_s_at
EREG	7.2	Epiregulin	8.94E-08	205767_at
CXCL2	6.4	Chemokine (C-X-C motif) ligand 2	4.48E-08	209774_x_at
NRP2	5.9	Neuropilin 2	3.46E-06	222877_at
CXCL1	5.5	Chemokine (C-X-C motif) ligand 1	3.00E-08	204470_at
IGFL1	5.3	IGF-like family member 1	5.54E-07	239430_at
RCAN1	3.9	Regulator of calcineurin 1	1.25E-07	208370_s_at
BCL2A1	3.9	BCL2-related protein A1	2.07E-06	205681_at
TGFB2	3.7	Transforming growth factor, beta 2	1.16E-07	209909_s_at
PRKAR2B	3.6	Protein kinase, cAMP-dependent, regulatory, type II, beta	1.54E-06	203680_at
IL1A	3.6	Interleukin 1, alpha	2.78E-07	210118_s_at
IL1B	3.6	Interleukin 1, beta	4.95E-08	39402_at
MCAM	3.4	Melanoma cell adhesion molecule	2.65E-07	211340_s_at
GJB2	3.3	Gap junction protein, beta 2, 26 kDa	1.25E-05	223278_at
BIRC3	3.3	Baculoviral IAP repeat-containing 3	4.94E-07	210538_s_at
CXCL3	3.2	Chemokine (C-X-C motif) ligand 3	4.83E-07	207850_at
RAB27A	3.2	RAB27A, member RAS oncogene family	2.63E-07	210951_x_at
TSHZ2	3.1	Teashirt zinc finger homeobox 2	9.65E-05	232584_at
BOLA3	-3.6	BolA homologue 3 (<i>Escherichia coli</i>)	7.78E-08	227291_s_at
Immunity and defence				
TNC	6.3	Tenascin C (hexabrachion)	2.30E-08	201645_at
SAA2	5.7	Serum amyloid A2	4.95E-08	208607_s_at
KLRC2	5.3	Killer cell lectin-like receptor subfamily C, member 2	5.63E-06	206785_s_at
SGK1	3.5	Serum/glucocorticoid regulated kinase 1	1.90E-06	201739_at
KLRC3	3.3	Killer cell lectin-like receptor subfamily C, member 3	1.24E-05	207723_s_at
TNFAIP6	3.0	Tumour necrosis factor, alpha-induced protein 6	2.02E-05	206026_s_at
OAS1	3.2	2',5'-oligoadenylate synthetase 1, 40/46 kDa	5.92E-06	205552_s_at
DEFB1	-3.1	Defensin, beta 1	4.34E-06	210397_at
DTL	-3.2	Denticless homologue (<i>Drosophila</i>)	1.11E-05	218585_s_at
VTCN1	-3.4	V-set domain containing T cell activation inhibitor 1	7.74E-06	219768_at
Receptor activity				
IL13RA2	12.8	Interleukin 13 receptor, alpha 2	2.34E-07	206172_at
CXCR4	7.8	Chemokine (C-X-C motif) receptor 4	1.10E-07	217028_at
ITGB8	3.5	Integrin, beta 8	2.87E-06	205816_at
BDKRB1	3.1	Bradykinin receptor B1	3.57E-07	207510_at
Enzyme inhibitor activity				
SERPINF4	10.0	Serpin peptidase inhibitor, clade B (ovalbumin), member 4	5.91E-09	211906_s_at
SERPINF3	4.0	Serpin peptidase inhibitor, clade B (ovalbumin), member 3	7.74E-08	209719_x_at
Metabolism				
AKR1C2	16.0	Aldo-keto reductase family 1, member C2	6.42E-08	1562102_at
AKR1C1	6.6	Aldo-keto reductase family 1, member C1	5.91E-09	204151_x_at
AKR1B1	5.4	Aldo-keto reductase family 1, member B1 (aldose reductase)	2.30E-08	201272_at
PTGS2	5.5	Prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)	1.27E-06	1554997_a_at
SOD2	4.8	Superoxide dismutase 2, mitochondrial	1.65E-08	215223_s_at
MRPS6	4.7	Mitochondrial ribosomal protein S6	2.58E-05	213167_s_at
HS3ST1	4.1	heparan sulphate (glucosamine) 3-O-sulphotransferase 1	8.05E-06	205466_s_at
FST	3.8	Follistatin	6.03E-08	207345_at
PGM2L1	3.7	Phosphoglucomutase 2-like 1	3.00E-08	229256_at
DIO2	3.6	Deiodinase, iodothyronine, type II	8.39E-05	203699_s_at

Table 2. *Continued*

Gene alias	Fold change	Gene name/description	P-value	Accession no.
SLC5A3	3.4	Solute carrier family 5 (inositol transporters), member 3	1.90E-07	1553313_s_at
SEPP1	-3.1	Selenoprotein P, plasma, 1	3.57E-07	201427_s_at
EMP1	-3.2	Epithelial membrane protein 1	1.64E-06	201324_at
ALDH1L2	-3.4	Aldehyde dehydrogenase 1 family, member L2	1.39E-06	231202_at
CLYBL	-5.0	Citrate lyase beta-like	3.57E-07	238440_at
KRT4	-5.3	Keratin 4	1.43E-07	213240_s_at
Ion binding				
TCHH	9.6	Trichohyalin	6.00E-09	213780_at
KCNK3	7.2	Potassium channel, subfamily K, member 3	1.82E-06	205952_at
TESC	5.4	Tescalcin	1.83E-07	218872_at
Miscellaneous				
TMEM46	5.3	Transmembrane protein 46	5.71E-08	230493_at
IER3	5.8	Immediate early response 3	2.79E-08	201631_s_at
FRMD3	3.7	FERM domain containing 3	5.55E-06	230645_at
Unclassified				
LOC387763	10.0	Unknown	2.30E-08	227099_s_at
1555854_at	8.4	Unknown	5.66E-08	1555854_at
229242_at	6.9	Unknown	7.53E-09	229242_at
C20orf175	4.7	Chromosome 20 open reading frame 175	1.14E-05	227654_at
226560_at	4.4	Unknown	1.24E-07	226560_at
C8orf4	3.7	Chromosome 8 open reading frame 4	1.21E-07	218541_s_at
LOC645638	3.6	Similar to WDNM1-like protein	6.03E-08	229566_at
CXorf58	3.4	Chromosome X open reading frame 58	1.04E-05	1553391_at
243509_at	3.4	Unknown	3.14E-05	243509_at
C13orf15	3.3	Chromosome 13 open reading frame 15	7.42E-06	218723_s_at
C6orf155	3.2	Chromosome 6 open reading frame 155	1.46E-05	220324_at
230356_at	3.2	Unknown	2.81E-07	230356_at
C11orf70	-3.2	Chromosome 11 open reading frame 70	9.07E-07	224463_s_at
228661_s_at	-3.4	Unknown	3.18E-06	228661_s_at
KANK4	-3.5	KN motif and ankyrin repeat domains 4	1.82E-06	229125_at
1558605_at	-3.5	Unknown	4.34E-06	1558605_at
222288_at	-3.6	Unknown	1.32E-07	222288_at
RCSD1	-4.6	RCSD domain containing 1	4.41E-06	225763_at
238632_at	-4.6	Unknown	4.31E-06	238632_at

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

Network analysis and Phl p 1-induced significant signalling pathways

To be able to identify interactions within genes that displayed an altered expression after stimulation with Phl p 1, we performed a network analysis. The evaluation of genes with a significant change in expression level by at least threefold up or down revealed 50 genes to interact directly, with 31 genes showing Phl p 1-induced changes in expression (Fig. 2). Resembling the observation that cell communication was identified to be among the most prominent GO groups, a predominant proportion of the genes in the network are related to chemokines and cytokines, with interleukin (IL)-1A, IL-1B, IL-6 and IL-8 forming hubs within the computational model.

Next we analysed signalling pathways induced significantly after stimulation of NCI-H292 cells with Phl p 1. We therefore

screened all significantly Phl p 1-regulated probe sets independent of their fold change for their participation in specific signalling cascades. As shown in Table 3, the following pathways appeared to be regulated by Phl p 1: epidermal growth factor receptor 1 (EGFR1), IL-1, IL-2, IL-3, IL-4, IL-6, IL-7, Notch, Wnt, transforming growth factor beta receptor (TGF- β R), tumour necrosis factor (TNF)- α , and nuclear factor kappa-light-chain enhancer of activated B cells (NF- κ B).

Phl p1-induced protein expression

GO analysis indicated the group 'cell communication' to be the dominant functional group among Phl p 1-regulated genes. We therefore continued with the determination of Phl p 1-induced release of mediators in general. As shown in Table 4, measurement of the release of a panel of chemokines, cytokines and growth factors showed a massive

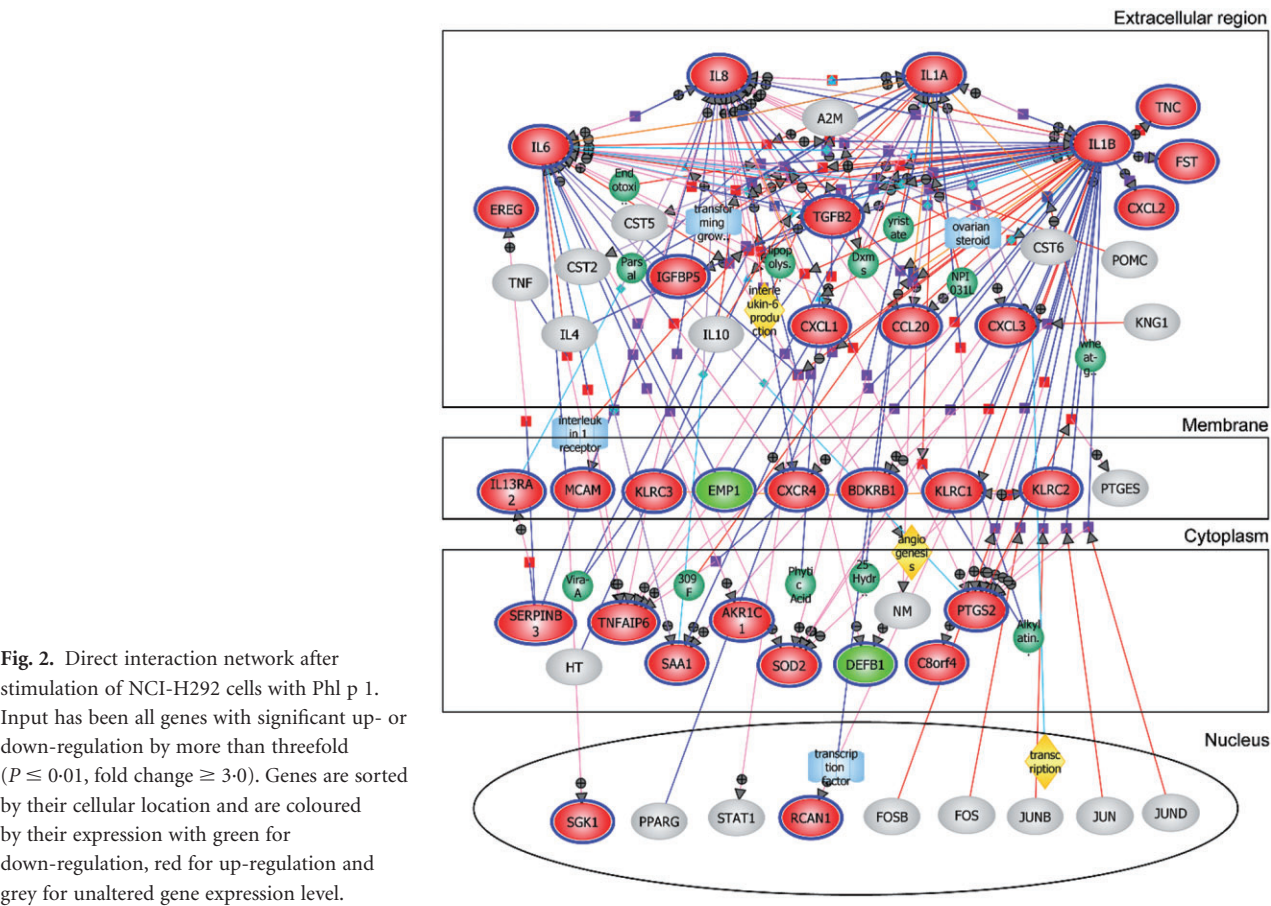


Fig. 2. Direct interaction network after stimulation of NCI-H292 cells with Phl p 1. Input has been all genes with significant up- or down-regulation by more than threefold ($P \leq 0.01$, fold change ≥ 3.0). Genes are sorted by their cellular location and are coloured by their expression with green for down-regulation, red for up-regulation and grey for unaltered gene expression level.

Phl p 1-induced increased release of IL-1RA, IL-6 and IL-8, which was also reflected by a clear increase in gene expression levels. Albeit at rather low level, vascular endothelial growth factor (VEGF) showed an increase in release and also a moderate Phl p 1-induced up-regulation of gene expression. Granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor

(G-CSF), epidermal growth factor (EGF), interferon gamma-induced protein IP-10 and monokine induced by gamma interferon (MIG) showed a moderate increase on mediator levels, with gene expression levels remaining unaltered upon stimulation. Although the monocyte chemotactic protein-1 (MCP-1) showed an increased release of 5.4-fold, the corresponding gene showed no altered expression upon stimulation, which is likely to result from the fact that 24 h were chosen for stimulation. IL-12 also showed a clear enhanced release upon stimulation, with an increase of detectable IL-12p40/p70 by fourfold. Interestingly, this was not reflected in the Phl p 1-induced expression level of the IL-12 gene, with IL12A (p35) and IL12B (p40) gene expression remaining unaltered. For IL-1 β we were not able to detect an increased release, but the stimulation with Phl p 1 resulted in a clear up-regulation of gene expression by 3.6-fold, indicating IL-1 β to be more probably part of a secondary response induced by Phl p 1. A similar picture emerged for TNF- α , IL-15 and fibroblast growth factor (FGF)- β that were also undetectable within the supernatant of Phl p 1-stimulated airway epithelial cells (AECs), with gene expression levels being only slightly up-regulated. IL-2R was identified to be released on basal level after stimulation with Phl p 1 and the gene expression level remained unaltered.

Table 3. Phl p 1-induced signalling pathways.

Pathway	Involved genes*	Genes regulated by Phl p 1	P-value
EGFR1	178	67	4.26E-05
IL-1	24	13	5.90E-03
IL-2	58	28	3.96E-04
IL-3	71	26	1.13E-02
IL-4	49	17	3.01E-02
IL-6	52	23	7.32E-04
IL-7	16	9	6.32E-03
NOTCH	74	27	1.55E-02
TGFBR	137	49	9.98E-04
TNF- α /NF- κ B	193	74	2.69E-07
Wnt	105	37	4.08E-03

*Genes matched with Agilent technology. IL, interleukin; TNF, tumour necrosis factor; EGF, epidermal growth factor; NF- κ B, nuclear factor kappa B.

Table 4. Mediator release from NCI-H292 cells after stimulation with Phl p 1 for 24 h. After stimulation cell free supernatants were collected and analysed for the presence of chemokines and cytokines. The stated values represent the average ($\pm$ standard deviation) of a triplicate experiment and are given in pg/ml; with detection limits in brackets for each of the mediators. Fold change (FC) was calculated in relation to level of control-stimulated cells.

Mediator	HBSS (ctrl)	Phl p 1	FC ELISA	FC microarray
IL-1RA (14.2)	165 ($\pm$ 53)	1563 ($\pm$ 462)	9.5 ($\pm$ 4.1)	1.4*
IL-1 β (5.8)	11.7 ($\pm$ 0)	13 ($\pm$ 2.2)	1.1 ($\pm$ 0.2)	3.6*
IL-2R (10.7)	28 ($\pm$ 3)	36.7 ($\pm$ 16.4)	1.3 ($\pm$ 0.6)	1.1 [†]
IL-6 (3.2)	39 ($\pm$ 10.4)	1855 ($\pm$ 686)	48 ($\pm$ 22)	11.2*
IL-8 (2.3)	180 ($\pm$ 81)	10160 ($\pm$ 0)	56.5 ($\pm$ 25.3)	7.6*
IL-12 (4.8)	16.2 ($\pm$ 2.4)	64 ($\pm$ 4.3)	4 ($\pm$ 0.6)	1.1 [†]
IL-15 (8.5)	n.d.	n.d.	–	1.3*
TNF- α (2.6)	n.d.	n.d.	–	1.2*
MCP-1 (3.9)	204 ($\pm$ 57)	1095 ($\pm$ 39)	5.4 ($\pm$ 1.5)	1.1 [†]
GM-CSF (9.4)	19 ($\pm$ 0)	29.5 ($\pm$ 9.5)	1.6 ($\pm$ 0.5)	1.1 [†]
G-CSF (6.2)	22.2 ($\pm$ 9.4)	43.7 ($\pm$ 8.7)	2.0 ($\pm$ 0.9)	1.0 [†]
VEGF (4.6)	n.d.	16.1 ($\pm$ 1.5)	3.5 ($\pm$ 0.3)	1.6*
FGF- β (8.2)	n.d.	n.d.	–	1.3*
EGF (5.4)	n.d.	15.5 ($\pm$ 5)	2.9 ($\pm$ 0.9)	1.0 [†]
IP-10 (1.1)	n.d.	3.7 ($\pm$ 0.5)	3.4 ($\pm$ 0.5)	1.0 [†]
MIG (1.3)	8.0 ($\pm$ 0.3)	12.2 ($\pm$ 3.9)	1.5 ($\pm$ 0.5)	1.1 [†]

* $P \leq 0.01$. [†]Not significant; n.d., not detectable; ELISA, enzyme-linked immunosorbent assay; HBSS, Hanks's balanced salt solution; IL, interleukin; TNF, tumour necrosis factor; MCP, major chemoattractant protein; GM-CSF, granulocyte-macrophage colony-stimulating factor; VEGF, vascular endothelial growth factor; FGF, fibroblast growth factor beta; EGF, epidermal growth factor; IP, interferon gamma-induced protein; MIG, monokine induced by gamma interferon; FC, fold change.

Discussion

Although in this experimental set-up we stimulated AECs with a single component derived from GPE only, we still observed a huge number of Phl p 1-regulated genes and significant Phl p 1-induced release of mediators. In total, 7218 transcripts showed a significant regulation of gene expression, with 71 genes showing a Phl p 1-induced altered gene expression level of at least threefold. The observed extent of this response might result partially from the usage of the cell line NCI-H292. In previous studies we have already observed more pronounced fold changes and a higher number of affected genes in stimulated NCI-H292 cells when compared to the response induced in primary nasal epithelial cells [20]. Nevertheless, we decided to use this established model, as it offers the advantage of limited experimental variation and lack of potentially contaminating cells. The absence of intra-individual differences enables the detection of small changes in gene expression levels and therefore might add valuable information.

Phl p 1 has already been shown to induce an increased expression of IL-6, IL-8 and TGF- β from AECs on both mRNA and protein levels [13], but our data show the true extent of this response. Subsequent analysis of prominent GO clusters showed that the group 'cell communication' belongs to the most prominent Phl p 1-induced groups, reflecting the ability of the major allergen to induce the release of mediators that, in turn, can attract and influence the activity of immune-competent cells. On the assumption that the response of NCI-H292 cells has been shown to

rather reflect the healthy epithelial immune response towards allergens [20], our data support the observation that epithelial cells from healthy donors release high levels of cytokines and chemokines upon stimulation with house dust mite extract (HDM) or birch pollen allergens [21,22].

Network analysis of direct interactions within genes that showed a change in gene expression level of at least threefold revealed central roles for IL1A, IL6, IL8, but also for IL1B. Phl p 1 induced a pronounced up-regulation of the expression of this proinflammatory cytokine (3.6-fold). Interestingly, polymorphisms within IL1B are associated with allergic rhinitis [23], confirming the importance of this mediator within atopic diseases. Vroiling *et al.* already investigated the response of AECs to HDM allergens in detail on both a respiratory epithelial cell line and primary nasal epithelial cells from healthy donors and allergic individuals [20,24]. Network analysis of the aforementioned samples revealed a set of overlapping genes, describing an epithelial core-response towards HDM. Interestingly, some of these genes (namely IL1B, CXCL1, CCL20, IL8, CXCL2, PTGS2 and SERPINB3) were also regulated by Phl p 1 and part of the interaction network created upon genes that showed Phl p 1-induced alterations in expression level by at least threefold (Fig. 2). It is tempting to speculate about the existence of a general epithelial core-response towards aeroallergens, with genes being part of the core-response and genes showing changes in expression level upon stimulation with the respective allergenic extract that are specific for the source of allergens. Mutations within genes that are part of the core-response or mutations in genes that are induced specifically might explain

why individuals become mono- or polysensitized. However, further experiments are required to follow this hypothesis.

Interestingly, the chemokine receptor CXCR4 displayed a pronounced up-regulation for both, GPE- and Phl p 1-stimulation of AECs. This receptor has been shown to be expressed on Th2 cells and is relevant during Th2-type allergic airway responses [25]. Besides its role as major co-receptor for HIV-entry [26], CXCR4 was also shown to be expressed in human intestinal epithelial cells and is up-regulated in inflammatory bowel diseases [27]. CXCR4 is functionally expressed on the cell surface of various immune cells and plays a role in cell proliferation and migration. This G-protein-coupled receptor (GPCR) is endocytosed upon activation. Furthermore, it has been shown that blocking of CXCR4 has a significant effect in down-regulating the inflammation of allergen-induced immune response [28]. Taken into account that agonist-occupied CXCR4 undergoes clathrin-dependent endocytosis in human epithelial cells [29,30], it is tempting to speculate that the massive up-regulation of CXCR4 we observed after stimulation of AECs with GPE (11.5-fold up) or Phl p 1 (7.8-fold up) might contribute to the epithelial uptake of grass pollen allergens. Furthermore, after stimulation of epithelial cells from allergic donors with the birch pollen major allergen Bet v 1 an over-representation of the GO terms 'response to viruses' and 'cell receptors' could have been detected [31,32]. However, further experiments are required to investigate this possible mechanism.

Many major allergens seem to possess functional attributes which may, at least partially, explain their allergenicity. It might well be that the immune activating molecules are not identical to the proteins triggering the allergic response, although this would still raise the question of why those particular proteins are recognized among the many present during exposure. Extracts from different pollen have been shown to cause detachment of airway epithelial cells dependent on a proteolytic activity of the extracts [33]. Also, the activating capacity of HDM extract and HDM allergens is caused probably by a proteolytic activity of the allergens with, for example, Der p 1 identified to be a serin-protease. Der p 1 has been shown to degrade tight junction proteins, thereby inducing the release of mediators and facilitating the route of the allergen through the epithelial barrier [34–36]. Although we were not able to observe any detachment of Phl p 1-stimulated AECs, members of the family of serinprotease inhibitors (SERPIN) showed an increase in gene expression level and CDH1, encoding for cadherin-1, a calcium-dependent cell–cell adhesion glycoprotein, appeared to be down-regulated upon stimulation with GPE by more than threefold. Phl p 1 is discussed to exert proteolytic activity, with a recombinant in *Pichia pastoris* expressed form acting as protease, whereas natural Phl p 1 failed to act as protease both *in vitro* and *ex vivo* [13]. In contrast to GPE-stimulated AECs, Phl p 1 only induced a moderate down-regulation of CDH1 (–1.5-fold), which

might allow the suspicion that proteases potentially present within the whole extract might allow the passage of allergens (including Phl p 1) through the epithelial barrier by disrupting cell–cell adhesion.

However, regardless of the speculated ability to act as a protease itself, this major allergen induces the up-regulation and release of a broad range of mediators, indicating it to be a powerful trigger of the immune system. Taken together, we were able to show that the major allergen Phl p 1 induces a huge response in AECs, both on the gene expression level, as well as on the level of mediator release. Genes belonging to the GO cluster 'cell communication' were among the most prominent functional groups, which is also reflected in IL1A, IL1B, IL6 and IL8 building centres in a model of direct gene interaction of genes showing an altered gene expression level of at least threefold. Further detailed comparison of GPE- and Phl p 1-induced gene expression might be beneficial with regard to the application of single components within diagnosis and immunotherapy.

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Disclosure

None of the authors has any conflict of interest related to this manuscript.

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