

ORIGINAL ARTICLE Basic Mechanisms in Allergic Disease

Comparison of Timothy grass pollen extract- and single major allergen-induced gene expression and mediator release in airway epithelial cells: a meta-analysis

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Summary

Background Seasonal allergic rhinitis (AR) is a global health problem and its prevalence has increased considerably in the last decades. As the allergic response with its clinical manifestations is triggered by only a few proteins within natural extracts, there is an increasing tendency for single-component-resolved diagnosis and immunotherapy.

Objective As natural exposure is not to single proteins, but to complex mixtures of molecules, we were interested in comparing the activation of respiratory epithelial cells induced by the purified major allergen Phl p 1 with the induction caused by a complete extract of Timothy grass pollen (GPE).

Methods NCI-H292 cells were exposed to GPE or Phl p 1 for 24 h, isolated RNA and cell culture supernatants were used for microarray analysis, multiplex enzyme-linked immunosorbant assay (ELISA) and subsequent analysis.

Results We found 262 genes that showed a GPE-induced change of at least 3-fold, whereas Phl p 1-stimulation resulted in 71 genes with a fold induction of more than 3-fold. Besides genes that were regulated by both stimuli, we also detected genes displaying an opposite response after stimulation, suggesting that GPE might be more than purified major allergens with regard to induced immune responses.

Conclusions and Clinical Relevance Additional components within GPE and the resulting modulation of general processes affecting gene transcription and signalling pathways might be crucial to maintain/overcome the diseased phenotype and to induce the influx of cells contributing to late-phase allergic responses. When the initial process of sensitization is the matter of interest or late-phase allergic responses, one might miss important immune modulatory molecules and their interaction with allergens by applying single components only.

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Introduction

Seasonal allergic rhinitis (AR) is a global health problem and its prevalence has increased considerably in the last decades. Besides the loss of quality of life for the affected individuals the associated socioeconomic costs are significant as AR affects school performance, socialization and work productivity [1]. Given these considerations, it is important to have a fast and correct diagnosis of the disease, as well as an efficient treat-

ment strategy. However, both diagnosis and treatment are hampered by the complexity of the allergens that we encounter in daily life. When we take into account that being allergic to grass pollen could mean that we could have developed IgE specific antibodies to any of many different proteins contained within this allergic mixture, we must make sure that in skin prick testing for instance this particular protein is included. Fortunately, for most allergic mixtures (grass pollen, tree pollen, house dust mite etc.), the IgE response is usually

elicited against a few so called major allergens [2, 3]. However, batch-to-batch differences for a single supplier or between suppliers could mean that differences in protein contents may lead to a missed diagnosis in some individuals that have developed an IgE response for an allergenic protein that is missing within the mixture used for diagnosis.

The batch-to-batch variation has also impacted immunotherapy and for reasons of safety have led to a global interest in developing treatment regimes based on recombinant proteins. Current knowledge of the molecular mechanism behind these approaches states that the administration of large amounts of allergen, which are processed in the lymph nodes by antigen presenting cells, induces the activation of regulatory T cells (Treg), which secrete regulatory cytokines (IL-10, IFN- γ , TGF- β) that are able to inhibit IgE-production, decrease the activation and migration of effector cells like eosinophils, favour Th1-proliferation and switch the synthesis of the antibody-producing B cells towards allergen-specific IgG4 [4]. The quality of the allergen extract used is crucial for both efficacy and safety of any form of IT. For this reason, there is a strong need to standardize allergen mixtures with high degree of batch-to-batch reproducibility and stable amounts of allergenic proteins [4]. Furthermore, natural extracts have been shown to contain a variety of substances that promote the development of allergic immune responses or even systemic reactions [5]. As the allergic response with its clinical manifestations is triggered by only a few proteins of many components within natural extracts, there is an increasing tendency for single-component-resolved diagnosis and immunotherapy to identify the individual allergy-eliciting molecules. The use of defined allergens in diagnostic tests is stated to enable specificity-resolved analysis of the patient's IgE reactivity profile [6, 7]. Interestingly, although the majority of allergens will encounter the respiratory epithelium within a complex mixture, allergen-specific IgE is produced only for a few major allergens. However, when crosslinking of IgE is regarded as a rather passive role of the allergens, although inducing a mechanism with highly relevant immunological outcome, it recently became accepted that allergens themselves show biological activity [8–15].

As natural exposure is not to single, purified proteins, but to complex mixtures of molecules, we were interested in comparing the activation of respiratory epithelial cells induced by the purified major allergen Phl p 1 with the induction caused by a complete crude extract of Timothy grass pollen (GPE). Airway epithelial cells (AECs) are the first to encounter inhaled allergens and pathogens and have been shown to be both a target of inflammatory and mechanical triggers, as well as effectors of ongoing inflammation [16–20]. Apart from

only providing a physical barrier towards inhaled substances, epithelial cells have been shown to actively contribute to airway inflammation by responding to respective stimuli and by interacting with cells of the immune system [20–22]. Airway epithelium plays a central role within the development of immune responses in the lung [23]. Many of the cytokine, chemokines and antimicrobial mediators secreted by AECs in response to allergen exposure or encounter with microbial components have the ability to initiate and regulate immune responses and to recruit cells of the innate and adaptive immune system [24, 25]. Furthermore, recent studies have strengthened the idea that aberrant expression of genes within the epithelium might be a key driver of the immune response, confirming the important role of structural cells like AECs within the initiation of complex disorders like allergy or atopic dermatitis [19, 26–28].

Furthermore, besides mast cells, AEC might as well play a role within the process of the induction of early- and late-phase allergic reactions. A comparison of the response induced by GPE and purified Phl p 1 might be beneficial with regard to the use of single components or whole extracts within diagnosis and specific immunotherapy.

Materials and methods

As this is a meta-analysis of already published data, detailed descriptions of the methods and material used can be found within the respective publications [21, 29].

Briefly, NCI-H292 cells were cultured in six-well plates to 80% confluence. Before stimulation, culture medium was removed and the cells pre-incubated with Hanks' balanced salt solution (HBSS) for 24 h. Cells were then stimulated with grass pollen major allergen Phl p 1 (5 μ M) or GPE (250 μ g/mL), both diluted in HBSS or with HBSS alone (control). To guarantee a proper induction showing concentrations of IL-8 comparable to GPE-induced IL-8 release, we chose a concentration of 5 μ M Phl p 1 for the experimental setup. Twenty-four hours after stimulation, cell-free supernatants were collected and stored for further analysis. The cells were used for RNA-isolation.

As described before, Human Genome U133 Plus 2.0 Genechip Array (Affymetrix Inc., Santa Clara, CA, USA) representing 47 000 transcripts, including 38 500 well-characterized genes was used to analyse grass pollen allergen-induced gene expression. The 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) using 3 µg of purified total RNA as template per reaction. For the reaction, the One-Cycle cDNA Synthesis Kit (Affymetrix) was used. The Array images were acquired using a GeneChip Scanner 3000 (Affymetrix) and analysed with Rosetta Resolver (Rosetta Biosoftware, Seattle, WA, USA).

Data analysis

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 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 by using the robust multi-array average (RMA) algorithm [30]. A linear model was used for differential gene expression analysis [31], Empirical Bayes test statistics was used for hypothesis testing [32] and all p-values were corrected for false discoveries [33]. For Gene Ontology and network interaction analysis, GeneSpring GX11 (Agilent Technologies, Netherlands) was used. Cluster analysis on all significantly regulated genes was done by transforming the mean expression value for a gene in the three groups (NCI-H292 cells stimulated with GPE, Phl p 1 or HBSS only) to Z-scores and using unsupervised K-means clustering based on

correlation by using SpotfireDecisionSite Functional genomics.

Results

Comparison of GPE- and Phl p 1-induced gene expression and mediator release

To get a global impression of potential differences in the expression profiles after exposing AECs to GPE or Phl p 1, we first used principal component analysis (PCA). Figure 1a shows that the profiles in the replicates of GPE-induced gene expression (green circles) and of Phl p1-induced gene expression (red circles) are both distinct from the baseline samples (blue) and from each other. The tight clustering of the replicates shows that the variation between the three experimental groups is much larger than between individual samples of an experimental group. Figure 1b indicates that the contribution of PC1 and PC2 to the overall variability of the data is large, with PC1 contributing 64.2% and PC2 19.6%.

These global differences are also reflected by the number of genes that are induced under the different experimental conditions. With about 11 800 probe sets regulated by GPE and around 7200 probe sets showing a change in gene expression levels after stimulation with Phl p 1. Corresponding to this, 262 genes showed a GPE-induced change of at least 3-fold, whereas Phl p 1-stimulation resulted in 71 genes with a fold induction of more than 3-fold. Gene ontology (GO) analysis revealed the group *cell communication*, representing cytokines, chemokines and growth factors, to be the most prominent GO cluster induced by both stimuli. Therefore, we compared the GPE- and Phl p 1-induced release of mediators (Table 1). The Phl p 1-induced mediator release in general resembles the release of cytokines, chemokines and growth factors, we observed

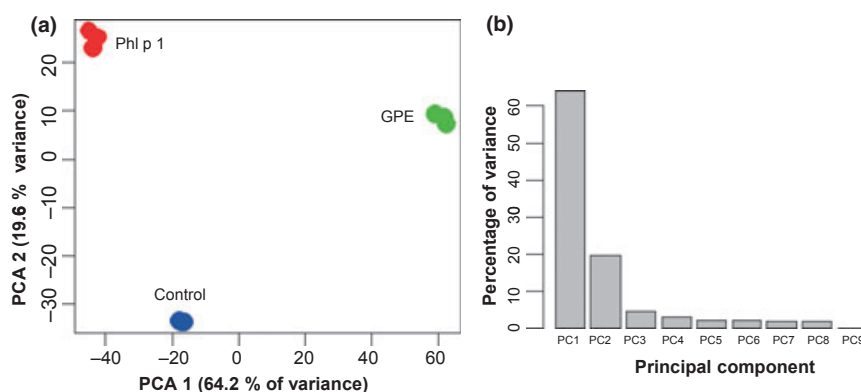


Fig. 1. Principal component analysis. (a) scatter plot of all nine arrays and their individual relative contribution to principal component 1 (PC1) and principal component 2 (PC2). (b) relative contribution to variance for the first 10 principal components.

Table 1. Comparison of mediator release from NCI-H292 cells after stimulation. Mediator release from NCI-H292 cells after stimulation with GPE or Phl p 1 for 24 h. 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. n.d. = not detectable (below detection limit). Fold change (FC) was calculated in relation to level of control-stimulated cells

Mediator	FC ELISA		FC Microarray	
	GPE	Phl p 1	GPE	Phl p 1
IL1-RA	9.8 ($\pm$ 3.3)	9.5 ($\pm$ 4.1)	2.8 [†]	1.4 [†]
IL1- β	-	1.1 ($\pm$ 0.2)	1.8 [†]	3.6 [†]
IL-2R	1.6 ($\pm$ 0.7)	1.3 ($\pm$ 0.6)	1.0 [#]	1.1 [#]
IL-6	54.4 ($\pm$ 14.3)	48 ($\pm$ 22)	8.7 [†]	11.2 [†]
IL-7	-	-	2.4 [†]	1.1 [#]
IL-8	35.6 ($\pm$ 6.8)	56.5 ($\pm$ 25.3)	3.6 [†]	7.6 [†]
IL-12	3.2 ($\pm$ 1.2)	4 ($\pm$ 0.6)	1.3 [†]	1.1 [#]
IL-15	-	-	1.5 [#]	1.3 [†]
IL-17	-	-	1.5 [†]	1.1 [#]
TNF- α	-	-	1.3 [†]	1.2 [†]
IFN- α	2 ($\pm$ 0.2)	-	1.0 [#]	1.0 [#]
MCP-1	4.4 ($\pm$ 1.1)	5.4 ($\pm$ 1.5)	1.0 [#]	1.1 [#]
MIP-1 α	1 ($\pm$ 0.4)	-	1.2 [#]	1.1 [#]
MIP-1 β	2.2 ($\pm$ 0.1)	-	1.4 [†]	1.2 [#]
RANTES	3.2 ($\pm$ 0.4)	-	1.0 [#]	1.1 [#]
GM-CSF	1.8 ($\pm$ 0.2)	1.6 ($\pm$ 0.5)	1.0 [#]	1.1 [#]
G-CSF	1.5 ($\pm$ 1)	2.0 ($\pm$ 0.9)	1.1 [#]	1.0 [#]
VEGF	4.3 ($\pm$ 0.3)	3.5 ($\pm$ 0.3)	-1.3 [†]	1.6 [†]
FGF β	-	-	1.2 [†]	1.3 [†]
EGF	2.7 ($\pm$ 0.2)	2.9 ($\pm$ 0.9)	1.0 [#]	1.0 [#]
IP-10	3.6 ($\pm$ 0.3)	3.4 ($\pm$ 0.5)	1.1 [#]	1.0 [#]
MIG	1.1 ($\pm$ 0.2)	1.5 ($\pm$ 0.5)	1.1 [#]	1.1 [#]

[†] $P \leq 0.01$.

[#]not significant.

after stimulation of AECs with GPE. However, the release of IFN- α , MIP-1 α , MIP-1 β and RANTES showed no increase after stimulation with the single major allergen, whereas GPE induces the release of these mediators, albeit at low levels.

Comparison of GPE- and Phl p 1-induced models of direct gene interaction

To identify potential differences and commonalities in cellular processes affected by exposure to both stimuli, we first determined the direct interaction between the genes that are regulated by GPE or Phl p 1. In this computational model, the Phl p 1-induced interaction network includes 31 of the 71 Phl p 1-regulated genes, while after GPE-stimulation, 80 genes of the 262 could be detected to have a direct interaction [21]. Interestingly, in both models IL1A, IL6 and IL8 hold central positions. While the transcription factor (TF) FOS is highly down-regulated by GPE and also builds a nodal

point within the GPE-induced gene interaction model, the expression of FOS remains unaltered after stimulation with Phl p 1. Another global difference was found concerning the expression of IL-1 β . This mediator showed a 3.6-fold up-regulation after stimulation with Phl p 1 with a central position in the respective gene interaction scheme, whereas GPE-stimulation leads to a less pronounced up-regulation of IL-1 β gene expression (1.8-fold). Furthermore, within the gene interaction scheme based on GPE-induced genes, IL-1 β does not hold a central position.

In a next step, we generated an overlay of these two interaction networks to identify a possible GPE- and Phl p 1-specific regulation of genes. As shown in Fig. 2, many genes appeared to be regulated by both stimuli (blue ovals). Genes highlighted in green were exclusively regulated by GPE, whereas Phl p 1-stimulation did not alter the expression levels. Surprisingly, albeit to a lesser extent, we also found genes that were regulated by Phl p 1 only (red ovals).

Comparison of GPE- and Phl p 1-induced signalling pathways

We continued with investigating the regulation of certain pathways induced by both stimuli. Previously, we were able to show that GPE-stimulation of AECs resulted in a significant regulation of the following pathways: epidermal growth factor receptor 1 (EGFR1), IL-3, IL-6, IL-7, Notch, Wnt, Transforming growth factor beta receptor (TGFB β), Tumor necrosis factor- α (TNF- α), and nuclear factor kappa-light-chain-enhancer of activated B cells (NF κ B)[21]. Interestingly, the comparison with Phl p 1-induced signalling pathways revealed an additional regulation of IL-2 and IL-4 signalling by the single major allergen[29].

K-means clustering of GPE- and Phl p 1-induced transcripts

Illustrating GPE- and Phl p 1-induced transcripts by Venn diagram (Fig. 3) shows that 4093 probe sets were regulated by both stimuli with only 1009 transcripts showing identical changes in gene expression levels. The 3084 transcripts were regulated by both stimuli, but with different changes in expression levels. To be able to compare this group of genes that share specific expression pattern, we performed K-means clustering of the expression levels of the three groups (control, GPE- and Phl p 1-stimulated). The 3084 transcripts, representing 2179 genes, which showed a changed expression level, could be separated into eight clusters (Fig. 4). In cluster 3, 5 and 8, GPE- and Phl p 1-induced expression shows the same tendency, with Phl p 1-induced changes showing a less prominent expression level when compared to the

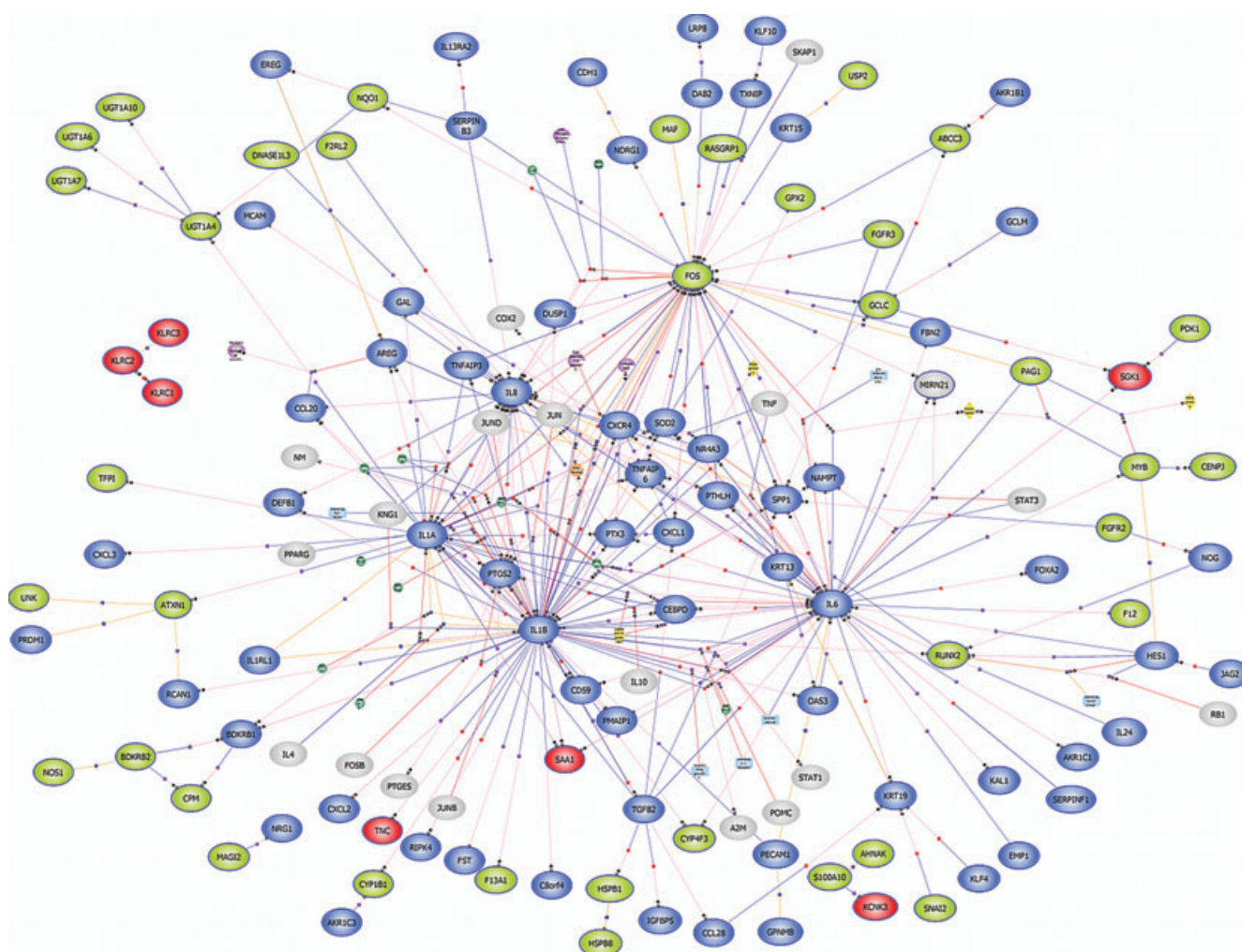


Fig. 2. Overlay of GPE- and Phl p 1-induced interaction network models. Genes that were regulated by more than 3-fold after stimulation with GPE or Phl p 1 were combined in an overlay. Genes that showed an altered expression level after stimulation with both stimuli are highlighted in blue. GPE-specific genes were marked in green, whereas Phl p 1-specific genes are shown in red. The respective specificity of induction has been checked within the whole data sets and has been regarded as specific when the other stimulus did not alter the gene expression level significantly (with $P \leq 0.05$).

influence of GPE-stimulation, likely to result from additional bioactive components within the extract. More detailed analysis of these clusters showed that the Wnt-pathway was significantly induced in cluster 3 with $P = 9.4\text{E-}04$ and the metallothionein-pathway was significantly induced within the regulated probe sets that were sorted into cluster 8 ($P = 2.1\text{E-}09$). In cluster 5, we could not detect any significant pathways. Furthermore, cluster 8 showed a significant enrichment of the GO terms *cadmium ion binding* (GO:0046870) and *copper ion binding* (GO:0005507) with $P = 8.9\text{E-}11$ and $P = 1.1\text{E-}05$, respectively. In cluster 3 and 5, no significantly enriched GO terms could have been detected.

We also found clusters, namely cluster 2 and 4, which display a more pronounced response to Phl p 1-stimulation. Here, the stimulation with the purified

allergen induced a higher increase of gene expression level when compared to the response of AECs to GPE, which might result from the used concentration of the single allergen, which is higher than its regular distribution within GPE. A screening of the probe sets sorted into these cluster did not result in the identification of significant pathways. However, GO analysis revealed that the following GO terms showed significant enrichment in cluster 2: *cell chemotaxis* (GO:0060326, $P = 2.17\text{E-}04$), *leucocyte chemotaxis* (GO:0030595, $P = 2.2\text{E-}04$) and *neutrophil chemotaxis* (GO:0030593, $P = 2.17\text{E-}04$).

To our surprise, we also detected genes that displayed an opposite response after stimulation with GPE or Phl p 1 (cluster 1, 6 and 7). Table 2 shows a selection of genes from cluster 1, 6 and 7. Cluster 1 is composed of

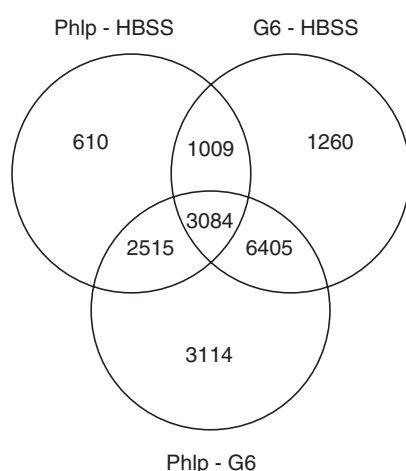


Fig. 3. Venn diagram of GPE- and Phl p 1-induced changes in gene expression levels.

562 genes, which show an up-regulation of gene expression after Phl p 1-stimulation, whereas GPE induced a down-regulation of these genes. Among others, this cluster contains the mucosa-associated epi-

thelial chemokine CCL28 or the melanoma cell adhesion molecule MCAM. Further analysis of the probe sets sorted into cluster 1 based on their expression level upon stimulation revealed an overrepresentation of transcription factors (TFs) within a model of direct gene interaction (data not shown). This was reflected in the GO groups *chromatin* (GO:0000785/GO:0005717) and *nucleosome* (GO:0000786/GO:0005718) showing a significant enrichment with $P = 9.6\text{E-}07$ and $P = 3.9\text{E-}05$ respectively. Furthermore, we found the following signalling pathways to be significantly regulated within cluster 1: IL-6, EGFR1 and TGFBR with $P = 1.05\text{E-}05$, $P = 4.7\text{E-}03$ and $P = 3.8\text{E-}03$ respectively.

Cluster 6 represents 509 genes that displayed a down-regulated expression profile after stimulation of AECs with Phl p 1 and an increased gene expression as response to GPE-stimulation. In this cluster for example we found LRP8, a member of the low density lipoprotein receptor (LDL) family, which is involved in the cellular recognition of lipoproteins, to be 5-fold up-regulated by GPE and slightly down-regulated by Phl p 1. Also, the intracellular adhesion molecule 2 (ICAM-2) was classified to belong to cluster 6, and stimulation

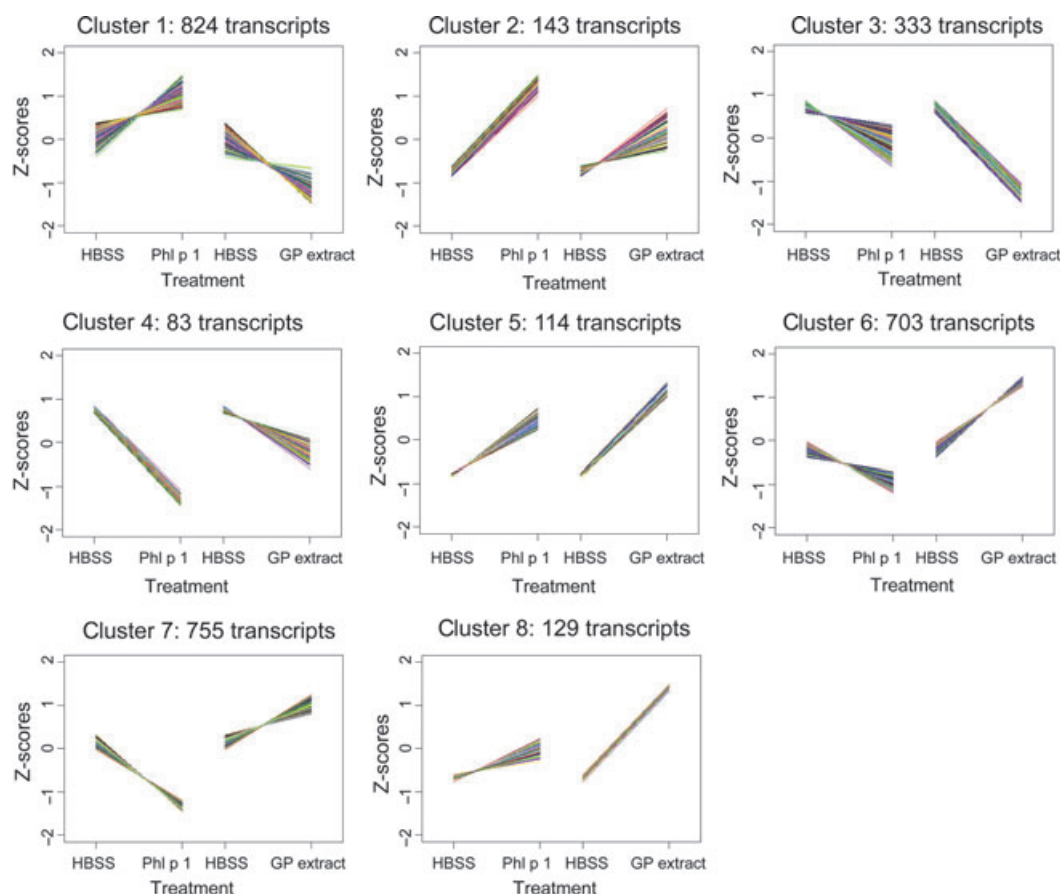


Fig. 4. K-means clustering. Regulated genes were transformed to Z-scores and organized in eight clusters according to the level of regulation using K-means clustering.

Table 2. Selection of genes from cluster 1, 6 and 7. Genes differentially expressed in NCI-H292 cells after stimulation with GPE and Phl p 1. Stated are number of the cluster, accession number, gene symbol, gene name fold change after stimulation with GPE with corresponding *P*-value and fold change after stimulation with Phl p 1 with corresponding *P*-value

Cluster	Gene ID	Gene symbol	Gene name	fc GPE	P-value	fc Phl p 1	P-value
1	1554878_a_at	ABCD3	ATP-binding cassette, sub-family D (ALD), member 3	-3	3.51E-08	1.3	7.73E-04
1	239167_at	AFF1	AF4/FMR2 family, member 1	-5	5.28E-07	1.3	1.84E-02
1	202834_at	AGT	Angiotensinogen (serpin peptidase inhibitor, Clade A, member 8)	-2.9	2.26E-07	1.6	9.08E-05
1	212286_at	ANKRD12	Ankyrin repeat domain 12	-1.8	1.28E-05	2.4	2.50E-06
1	224027_at	CCL28	Chemokine (C-C motif) ligand 28	-3.6	1.56E-07	1.6	2.18E-04
1	207172_s_at	CDH11	Cadherin 11, type 2	-1.6	4.20E-05	2.5	1.68E-06
1	201278_at	DAB2	Disabled homolog 2, mitogen-responsive phosphoprotein (Drosophila)	-2.8	6.90E-08	1.3	2.24E-03
1	201041_s_at	DUSP1	Dual specificity phosphatase 1	-4.3	3.62E-08	1.5	2.39E-04
1	215501_s_at	DUSP10	Dual specificity phosphatase 10	-2	5.81E-05	1.8	2.86E-04
1	205014_at	FGFBP1	Fibroblast growth factor binding protein 1	-2.1	2.91E-05	2.3	3.64E-05
1	204948_s_at	FST	Follistatin	-2	9.59E-07	3.8	2.55E-07
1	212196_at	IL6ST	Interleukin 6 signal transducer (gp130, oncostatin M receptor)	-2	3.35E-05	1.4	3.49E-03
1	209086_x_at	MCAM	Melanoma cell adhesion molecule	-2	3.04E-07	2.6	4.04E-06
1	200632_s_at	NDRG1	N-myc downstream regulated gene 1	-4.1	4.21E-09	1.3	6.58E-04
1	1552309_a_at	NEXN	Nexilin (F actin binding protein)	-2.5	5.81E-06	2.3	6.43E-05
1	1554717_a_at	PDE4D	Phosphodiesterase 4D, cAMP-specific (phosphodiesterase E3 dunce homolog, Drosophila)	-6.4	1.49E-07	2.6	2.76E-05
1	1553313_s_at	SLC5A3	Solute carrier family 5 (inositol transporters), member 3	-1.6	2.78E-05	3.4	1.90E-07
1	227697_at	SOCS3	Suppressor of cytokine signalling 3	-2.3	4.42E-07	1.6	9.01E-05
1	213780_at	TCHH	Trichohyalin	-1.4	4.12E-04	9.6	6.00E-09
1	204731_at	TGFBR3	Transforming growth factor, beta receptor III	-2	2.23E-06	1.7	2.59E-05
1	206907_at	TNFSF9	Tumour necrosis factor (ligand) superfamily, member 9	-3	1.18E-07	1.5	3.15E-04
6	207623_at	ABCF2	ATP-binding cassette, sub-family F (GCN20), member 2	3.1	1.70E-07	-1.3	9.79E-03
6	229309_at	ADRB1	Adrenergic, beta-1-, receptor	2.3	7.91E-05	-1.7	3.56E-03
6	231202_at	ALDH1L2	Aldehyde dehydrogenase 1 family, member L2	5.2	5.42E-08	-3.5	1.39E-06
6	205623_at	ALDH3A1	Aldehyde dehydrogenase 3 family, member A1	7.1	1.52E-07	-1.6	4.40E-03
6	220132_s_at	CLEC2D	C-type lectin domain family 2, member D	2.3	2.79E-05	-1.7	1.20E-03
6	213620_s_at	ICAM2	Intercellular adhesion molecule 2	1.4	5.98E-04	-1.4	2.17E-03
6	205176_s_at	ITGB3BP	Integrin beta 3 binding protein (beta3-endonexin)	1.9	2.50E-06	-1.7	1.92E-05
6	208433_s_at	LRP8	Low density lipoprotein receptor-related protein 8, apolipoprotein E receptor	5	3.45E-09	-1.3	3.17E-04
6	206343_s_at	NRG1	Neuregulin 1	3.1	4.76E-06	-1.7	2.37E-03
6	229839_at	SCARA5	Scavenger receptor class A, member 5 (putative)	1.6	3.48E-04	-1.5	1.89E-03
6	1560739_a_at	UBE3C	Ubiquitin protein ligase E3C	2.4	1.41E-05	-1.9	3.22E-04
7	204942_s_at	ALDH3B2	Aldehyde dehydrogenase 3 family, member B2	1.5	1.21E-04	-2	5.64E-06
7	227291_s_at	BOLA3	bolA homolog 3 (E. coli)	1.5	4.62E-05	-3.7	7.78E-08
7	205500_at	C5	Complement component 5	2.3	3.27E-05	-2.3	8.75E-05
7	210397_at	DEFB1	Defensin, beta 1	1.5	9.28E-04	-3.2	4.34E-06
7	244680_at	GLRB	Glycine receptor, beta	1.5	7.5E-03	-1.9	7.51E-04
7	227163_at	GSTO2	Glutathione S-transferase omega 2	1.7	1.12E-05	-2.3	1.84E-06
7	202431_s_at	MYC	v-myc myelocytomatosis viral oncogene homolog (avian)	1.4	2.0E-04	-1.6	3.56E-05
7	235737_at	TSLP	Thymic stromal lymphopoietin	1.9	3.45E-05	-1.9	9.25E-05

with GPE resulted in an increased gene expression, whereas Phl p 1 induced a moderate down-regulation of ICAM-2. In addition, we analysed direct gene interaction among genes that are represented within cluster 6. Interestingly, genes that are known to be involved in cell cycle and DNA repair (e.g. ATM, BCL2 or CCNA2) hold central positions within this computational model

(data not shown). The investigation of prominent GO groups within this cluster revealed that the GO terms *M phase* (GO:0000279), *cell cycle process* (GO:0022402), *response to DNA damage stimulus* (GO:0006974/GO:0034984) and *DNA repair* (GO:0006281) showed a significant enrichment with $P = 7.7E-19$, $P = 7.9E-16$, $P = 3.1E-15$ and $P = 7.3E-15$. After screening, the

probe sets of this cluster for significant pathways, we identified TNF α /NF- κ B signalling with $P = 0.04$.

Cluster 7 displays the expression profile of cluster 6 with a more pronounced down-regulation of gene expression after stimulation with Phl p 1. This cluster contains 525 genes and among others, the complement component 5 (C5) appeared to be down-regulated by more than 2-fold after stimulation with Phl p 1, whereas GPE induced an up-regulation of more than 2-fold. Also, the thymic stromal lymphopoietin (TSLP) shows a 2-fold up-regulation with GPE-stimulation, Whereas Phl p 1 leads to a 2-fold down-regulation of this allergy-associated cytokine. Further analysis of these transcripts for direct gene interaction displayed central hubs for the TF MYC, but also for the cell cycle-related gene CDK4 or the apoptosis repressor NOL3 (data not shown). A more detailed investigation revealed the presence of many mitochondria-related genes within this interaction model, which was reflected in the GO term *mitochondrion* (GO:0005739) belonging to the most prominent GO groups with $P = 7.04\text{E-}10$. Furthermore, the GO groups *translation* (GO:0006497) and *oxidoreductase activity* (GO:0016491) showed a significant enrichment in cluster 7 with $P = 8.17\text{E-}05$ and $P = 6.9\text{E-}05$ respectively.

Discussion

The use of isolated or recombinant major allergens instead of complex allergen extracts is becoming increasingly popular, as it offers some important advantages for quantitative measures in diagnosis and research. In general, the use of standardized extracts for diagnosis or immunotherapy is still topic of discussion. It is stated that the use of natural allergen extracts has a number of disadvantages such as that they contain many different, also non-allergenic proteins and various ill-defined substances. Also, the standardization of natural allergen extracts might be difficult as the allergen contents and compositions of the extracts might vary from manufacturer to manufacturer and from batch to batch [34, 35]. It has also been published that extracts can lack certain allergenic proteins, which might have got degraded during the process of production and storage and that they can be contaminated with proteins from other allergen sources, which can deliver false diagnostic results [36]. Specific immunotherapy (SIT) is traditionally based on the administration of extracts of allergenic sources. To optimize the efficacy of SIT, the concept of component-resolved therapy (CRT) has been proposed, where compositions of single molecules/purified natural or recombinant allergens should match the patient's molecular profile of IgE-sensitization [7]. By using discrete allergenic molecules, new relevant information such as specific

IgE-concentrations and individual pattern of reactivity can be achieved [37]. Purified major allergens like Phl p 5 have been shown to be effective in SIT [38] and with a combination of the purified HDM-allergens Der p 1 and Der p 2 more than 97% of HDM-allergic patients in Europe could have been diagnosed [39]. In contrast to this, a study comparing the diagnostic performance of allergen molecules and allergen extracts with regards to allergen-specific IgE showed that with HDM extract, a significantly higher sensitivity could have been obtained when compared to the single molecules [40]. Furthermore, a comparison of HDM extract and isolated major allergens with respect to their ability to induce early and late asthmatic responses and bronchial hyperreactivity (BHR) showed that the major allergens Der p 1 and Der p 2 induced less severe late asthmatic responses than HDM extract after similar early asthmatic responses induced by both allergen sources [41]. The authors pointed out that components of the HDM-extract, other than Der p 1 and Der p 2, with no significant influence on IgE-mediated early asthmatic response contribute significantly to the allergen-induced late asthmatic response and BHR. Although the early phase of allergic responses is clearly IgE-dependent with mast cells and basophiles as main players, the late-phase reaction is characterized by the contribution of the release of pre-formed or *de novo* synthesized mediators to the recruitment of inflammatory or immune competent cells.

Grass pollen immunotherapy (IT) has been shown to result in increased expression of IL-10 within respiratory mucosa only during pollen season, implying that mucosal contact with the relevant allergens is required for the recruitment and/or activation of immune competent cells. Furthermore, conventional IT with pollen resulted in an increase in the ratio of IFN- γ :IL-5 mRNA-expressing cells within the nasal mucosa, with no influence of IT on cytokine and proliferative response of peripheral T cells [42, 43].

Thus, the induction of local microenvironments under the involvement of AECs might be decisive for the attraction of immune competent cells and for the overall outcome of the immune response. Therefore, the aim of this study was to compare the induction of gene expression and mediator release from AECs after stimulation with a crude extract from Timothy grass pollen with the response induced by the purified major allergen Phl p 1. As a model we used NCI-H292 cells, which have been shown to rather reflect the response of healthy respiratory epithelium upon stimulation with an allergenic trigger [44]. Confirming the importance of AECs within allergic reactions, it has been shown that nasal epithelium of healthy individuals shows a strong response in terms of released mediators upon stimulation with HDM, whereas this response is decreased or

even absent in allergic individuals [19]. Also for Bet v 1, a major allergen from birch pollen, binding to conjunctival epithelial cells could have been demonstrated only in allergic patients and not in healthy individuals. Furthermore, the entry and transport of Bet v 1 was only evident in allergic patients and not in healthy control subjects [45, 46].

In our experimental setup, we choose to stimulate the cells for 24 h with Phl p 1 or GPE to be as close to the *in vivo* situation as possible, mimicking prolonged exposure during pollen peaks in season. Therefore, we cannot exclude secondary responses or different mediator kinetics to be responsible for the effects we observed, as we only took a snapshot in time.

We have been able to show that in general more transcripts are regulated by GPE, but to our surprise, we still observed a huge response in the number of genes that respond to stimulation of AECs with purified Phl p 1. The overlay of direct gene interaction models after stimulation with GPE or Phl p 1 showed that many genes belonging to the GO cluster *cell communication* were induced by both stimuli, which might contribute to the induction of late allergic responses in terms of the induction of influx of immune competent cells. Moreover, several genes were identified to be exclusively regulated by GPE, which is likely to result from the multicomponential character of the extract. To our surprise, we also found genes to be regulated by Phl p 1 only. As the major allergen is also present in the whole extract, this regulation pattern might result from the high concentration used in this experimental setup or from the absence of otherwise interfering components within GPE. During peaks in season, the pollen load can be relatively high, with up to 500–1000 grains/m³ for birch or grass pollen [47, 48]. Calculating with 500 grains/m³, considering an average respiratory volume of about 500 mL, and a frequency of about 12 breaths/min, this would result in 8640 inhaled pollen grains per day. Pollen grains are typically 20–40 µm in diameter and contain approximately 0.2 ng of major allergens [49]. Considering this, inhaling 8640 pollen grains per day would be equal to 1.7 µg major allergen. For Der p 1, a major allergen from the house dust mite *Dermatophagoides pteronyssinus*, it has been shown that although the quantity inhaled might in general be low (for example 10 ng/day), the concentration in the particles might be up to 0.2 ng per sphere, which is described to be roughly equal to 2 mg/mL [49]. If we now adapt that estimation to the concentration of allergen used in our experimental setup, we end up with 140 µg/mL of purified Phl p 1, and thus are in a realistic range.

By analysing the regulated genes and transforming the values into Z-scores, the modulated probe sets were sorted into clusters according to their expression pat-

tern (Fig. 4). We should stress that K-mean clustering, like all clustering methods, has some limitations. Most notably, this method assumes a spherical distribution of the data analysed, which gives the small samples size we are unable to verify. Secondly, we did check the impact of changing the number of seeds used for our clusters, so that we would not over- or under-sample our data.

In most of the clusters were the expression pattern showed the same tendency after stimulation of AECs with GPE or Phl p 1, we did not find any enriched GO terms, indicating a dispersed distribution of genes over these clusters. However, in cluster 2, we found an enrichment of the GO groups *cell chemotaxis*, *leukocyte chemotaxis* and *neutrophil chemotaxis*, suggesting that to a certain extent both stimuli do induce genes that are relevant for the induction of chemotaxis, and thus the influx of immune competent cells.

In addition, we also observed opposite expression patterns (cluster 1, 6 and 7), with genes being up-regulated after Phl p 1-stimulation, whereas GPE induced a down-regulation of the same genes and *vice versa*. On the level of single genes that showed altered expression upon stimulation, we found a number of specific factors with a specific impact on immune responses. For instance, the CC chemokine ligand 28 (down-regulated 3.6-fold by GPE and 1.6-fold up-regulated Phl p 1) is recognized by CC chemokine receptors CCR3 and CCR10, which are expressed by Th2 cells and effector cells, such as basophils, mast cells and eosinophils [50]. Taken this into account, the major allergen Phl p1 might be held partially responsible for the influx of immune competent cells, although this effect seems to be counteracted in the whole pollen extract GPE.

In contrast to the prior observation, a panel of immunological relevant genes appeared to be up-regulated by GPE, whereas Phl p 1 induced a moderate down-regulation, indicating the relevance of additional components within the extract for the induction of an immune response. ICAM-2 for example was detected to be 1.4-fold up-regulated by GPE, whereas Phl p 1 induced a down-regulation of this adhesion molecule by 1.4-fold. ICAM-2 has been shown to have a functional role in transepithelial migration of leucocytes and is essential for the egression of T cells from the lung [51], suggesting a role for GPE components and not for the purified Phl p 1 within the clearance of a potential immune response.

Also for TSLP, we detected an opposing expression pattern. GPE induced a 2-fold up-regulation, whereas Phl p 1-stimulation resulted in a 2-fold down-regulation of TSLP. This cytokine has been shown to be expressed by lung epithelial cells and it showed the capacity to skew a developing immune response towards a proallergic state through its direct action on

DCs [52]. This might allow the conclusion that one would have missed this potentially Th2-promoting feature of the whole extract when applying the single allergen only.

If we shift the observed differences between GPE- and Phl p 1-induced gene expression pattern on a meta level, significant variations appear within general processes and signalling pathways that are affected. In cluster 1 for example, where we observed an up-regulation of probe sets by Phl p 1, while GPE-stimulation resulted in a down-regulation of the very same transcripts, we were able to identify an overrepresentation of TFs within a model of direct gene interaction, which was reflected by the fact that the GO term *chromatin* was apparent to be significantly enriched. Furthermore, a direct comparison of gene interaction models based on genes that are regulated by more than 3-fold after stimulation with GPE or Phl p 1 clearly showed that more TFs are regulated by the whole extract. Several TFs have been implicated in the inflammatory process in asthma and allergy [53–55] and in previous experiments, we have been able to show that nasal epithelial cells from house dust mite (HDM) allergic patients show a different expression pattern of prominent TFs when compared to healthy individuals [19]. More precisely, we found that TFs of the NF- κ B family are already up-regulated in allergic individuals prior to stimulation, whereas members of the AP-1 family of TFs failed to be up-regulated upon stimulation with the relevant allergens in contrast to healthy controls. The failure of up-regulation of these TFs could be responsible for the prolonged activation of the allergic response and reducing this activated state may result in direct consequences with regard to complaints of affected individuals, by for example reducing the influx of effector cells. Thus, diligent regulation of TF-activation and feedback loops among TFs might well explain both pathological de-regulation and healthy phenotype and the use and/or application of single components might bear the risk of passing over these processes.

With regard to general cellular processes that are affected differentially after stimulation of AECs with GPE or Phl p 1, we observed a significant enrichment of probe sets in cluster 7 that belong to the GO terms *mitochondrion* and *oxidoreductase activity*. Pollen grains have been shown to have intrinsic NADPH oxidases and upon encountering AECs hydration causes the production of reactive oxygen species (ROS), which in turn induce oxidative stress, resulting in increased chemokine and cytokine production, as well as exacerbation of airway inflammation [56, 57]. As the probe sets in cluster 7 appeared to be up-regulated by GPE and down-regulated after stimulation with the purified major allergen, the induction of ROS and subsequent

mediator release might be limited to the whole extract with consequences to the induction of the allergic response.

Interestingly, the comparison of GPE- and Phl p 1-induced mediator release from AECs revealed that although at low levels, MIP-1 α , MIP-1 β and RANTES were released upon GPE-stimulation, whereas Phl p 1 did not induce the release of these mediators. MIP-1 α expression has been shown to be up-regulated in allergen-induced late-phase skin and bronchial reactions [58] and it promotes migration of cells relevant to allergic inflammation like T cells, macrophages, eosinophils and neutrophils [59, 60]. Also, RANTES has been pointed out as one of the major chemokines in recruiting circulating eosinophils to allergic airway tissue and its up-regulation in AECs may contribute to prolonged eosinophilic inflammation [61, 62]. According to this, by applying the single major allergen only these mediators would not have been induced, which might have consequences for the subsequent induction of influx of immune competent cells. The whole grass pollen extract with all its different allergens and additional components might be important especially for the induction of an influx of immune competent cells with regards to the induction of the late-phase allergic response. Natural exposure will always be to the whole extract including inhibitory or activating components, or rather to the pollen grains, releasing their content rapidly upon hydration, which also might be important for IgE-crosslinking.

In summary, this study might contribute to the conclusion that the crude extract might be more than the sum of purified major allergens with regard to induced immune responses. Although AECs are rather not regarded as primary target cells for SIT, additional components within the whole extract and the resulting modulation of general processes affecting gene transcription and signalling pathways might be crucial to maintain/overcome the diseased phenotype and to e.g. induce the influx of cells contributing to the late-phase allergic response. Recombinant or natural single allergens have been shown to be suitable for diagnosis or immunotherapy, especially when the focus lies on the determination of an exact grass sub-species the patient would be allergic to, but when the initial process of sensitization is the matter of interest or late-phase allergic responses one might miss important immune modulatory molecules and their interaction with allergens, or even interactions among allergens themselves by applying single components only.

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Disclosure

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

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