

ORIGINAL ARTICLE

Timothy grass pollen major allergen Phl p 1 activates respiratory epithelial cells by a non-protease mechanism

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Summary

Background Group 1 allergens from grass pollen (e.g. Phl p 1, the major allergen of timothy grass *Phleum pratense*) cause IgE reactivity in about 95% of allergic subjects and exist in all grass species. The respiratory epithelium represents a first line of contact of the immune system with airborne allergens, functions as physical barrier and is an important immunological regulation system.

Objective The aim of this study was to investigate the interaction of Phl p 1 with human respiratory epithelium to elucidate the contribution of epithelial cells to the development of allergic reactions.

Methods Purified Phl p 1 was used to stimulate A549 cells and transiently transfected HEK293 cells. mRNA level of different mediators were investigated by real-time PCR, release of the mediators was determined by ELISA. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test and an *ex vivo* model of the murine trachea were used to investigate a potential proteolytic activity of Phl p 1.

Results Phl p 1 activates respiratory epithelial cells as measured by induction of IL-6, IL-8 and TGF- β mRNA and release. Phl p 1, in contrast to Der p 1 from the house dust mite, does not exert proteolytic activity, as investigated by microscopic observation and MTT test. In an *ex vivo* model of the murine trachea we were able to show that Der p 1, in contrast to Phl p 1, enhances the transportation velocity of particles by the trachea, presumably by ATP released from the injured epithelium.

Conclusion We conclude that under physiological conditions Phl p 1 affects tracheal epithelial cells through a non-proteolytic activity. Enhancement of TGF- β expression induced by Phl p 1 together with the increased release of IL-6 and IL-8 might provide an indirect mechanism through which the allergen may cross the epithelial barrier and attracts immunocompetent cells.

Keywords allergy, Der p 1, epithelial barrier, grass pollen, group 1 allergens, inflammatory mediators, innate immunity, Phl p 1, protease, respiratory epithelium

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Introduction

The prevalence of allergic disorders like hayfever or *asthma bronchiale* is increasing world-wide [1]. In allergic individuals the contact with allergens results in an allergen-specific T-helper type 2 (Th2) immune response leading to IgE-mediated clinical manifestations. The respiratory epithelium represents a first line of contact of the immune system with the environment including airborne allergens. It functions as physical barrier and serves as important immunological regulation system, and is also involved in

allergic reactions like asthma. Upon activation respiratory epithelial cells release inflammatory mediators and cause the attraction of immunocompetent cells.

Over the last years many environmental allergens have been characterized in detail, but the precise mechanisms of the interaction of these allergens with human respiratory epithelium and the contribution of epithelial cells to the development and initiation of allergic reactions still remains poorly understood. Several cysteine and serine protease allergens have been identified and there is clear evidence that these allergens function as Th2 adjuvants,

which could explain their association with asthma. Der p 1, a cysteine protease from *Dermatophagoides pteronyssinus*, and Der p 9, a serine protease, both enhance the release of cytokines from primary cultures of human bronchial epithelium and respiratory epithelial cell lines. This activating capacity of the allergens seems to depend on the proteolytic activity of the allergens as shown by the use of protease inhibitors [2]. Sun et al. [3] were able to show that Der p 3, which functions as a serine protease, and Der p 9 interact with protease-activated receptors (PARs) expressed on human pulmonary epithelial cells, particularly PAR-2. Proteases secreted from *Aspergillus fumigatus* induce IL-6 and IL-8 production in airway epithelial cell lines [4] and Pen ch 13, an alkaline serine protease from *Penicillium chrysogenum*, has been shown to induce the release of prostaglandin-E₂ (PGE₂), IL-8 and TGF- β 1 from human respiratory epithelial cells. Similar to the above described properties of the house dust mite (HDM) major allergens, Pen ch 13 induces IL-8 expression in human airway epithelium by activating PAR-1 and PAR-2 [5].

Extracts from pollen and fungi have been shown to exert proteolytic activity and to cause mediator release from respiratory epithelial cells. Extracts from *Alternaria alternata* and *A. fumigatus* have been shown to induce morphologic changes, cell desquamation and production of IL-6 and IL-8 from the human alveolar type II epithelium-like cell line A549 [6]. Diffusates from different pollen (*Lolium perenne*, *Poa pratensis*, *Acacia longifolia* and *Casuarina distyla*) were shown to cause detachment of murine epithelial cells under serum-free conditions which could partially be blocked by protease inhibitors, indicating an essential role of proteases released from pollen [7]. HDM extracts induce cytokine release and cell detachment in cultured airway epithelial cells, which was largely dependent on the protease activity, whereas the pollen extracts seemed to induce cytokine release without cell detachment, indicating a protease-independent mechanism [8].

About 30% of the population of industrialized countries is suffering from type I allergies. Group 1 allergens like Phl p 1, the major allergen of timothy grass *Phleum pratense* used in this study, cause IgE reactivity in about 95% of the allergic individuals and exist in all grass species. Phl p 1 is a glycoprotein of about 30 kDa and reveals a carbohydrate content of 5% [9, 10]. Grass group 1 allergens constitute the β -expansin subfamily of expansins [11]. They function as mediators of acid-induced cell wall loosening in plants and are essential for fruit ripening [12], fertilization [13] and differentiation [14]. Grobe et al. [15] were able to show that the recombinant β -expansin/allergen of *P. pratense*, expressed in *Pichia pastoris*, catalysed the degradation of a synthetic substrate containing a papain cleavage site, indicating an enzymatic property of the allergen. In contrast to these

observations Li and Cosgrove [16] found that purified native group 1 allergens from timothy grass, ryegrass and maize (Phl p 1, Lol p 1 Zea m 1) possess substantial wall loosening activity, but no detectable proteolytic activity.

Pollen grains do not only function as allergen carriers, but do also contain bioactive lipids that attract cells involved in allergic inflammation. Soluble factors from birch (*Betula alba*) pollen activate human dendritic cells (DC) and aqueous pollen extracts, containing phytoprostanolins, selectively inhibited IL-12p70 production of lipopolysaccharide (LPS)- and CD40L-activated DC, resulting in an increased Th2 and reduced Th1 cell polarizing capacity [17, 18].

The aim of this study was to investigate the ability of Phl p 1 to activate human respiratory epithelial cells and to figure out whether this activating capacity is driven by protease-dependent mechanisms or by contaminating components like bacterial or viral products.

To address this question, human respiratory epithelial cells have been stimulated with purified natural Phl p 1. To exclude the relevance of factors like pollen-associated lipid mediators or bacterial/viral components in our system, highly purified Phl p 1 has been used and the induction of inflammatory mediators was measured on both mRNA and protein level. In different experimental setups a direct comparison with the known cysteine protease Der p 1 was used to further investigate the active principle of Phl p 1.

Our results clearly show that Phl p 1 induces the expression and release of IL-8, IL-6 and TGF- β in alveolar epithelial cells, as well as the activation of the mitogen-activated protein kinases (MAPK) p38 and Erk. Bacterial contaminants could be excluded to be responsible for these effects and in contrast to the known cysteine protease Der p 1 Phl p 1 does not exert proteolytic activity under physiological conditions.

Methods

Reagents

For cell culture usage Dulbecco's modified Eagle's medium GIBCO-LG (DMEM), penicillin-streptomycin (PS), L-glutamine (LG), sodium pyruvate and Hepes buffer were obtained from Invitrogen (Karlsruhe, Germany). RPMI-1640 with stable L-glutamine was obtained from PAA (Germany). Applied foetal calf serum (FCS) (Linaris, Wertheim-Bettingen, Germany) was heat inactivated. LPS from *Salmonella enterica* serovar *Friedenau* was a kind gift from Prof. Dr H. Brade (Department of Immunochimistry and Biochemical Microbiology, Research Center Borstel) and showed no TLR2-mediated activity. The synthetic lipopeptide Pam₃C-SK₄ was synthesized and analysed by EMC microcollections GmbH (Tübingen, Germany). The inhibitors SB 203580 and PD98059 and

the substances PMA and anisomycin were obtained from Calbiochem (Merck, Stuttgart, Germany). The synthetic lipid IVa analogue compound 406 was kindly provided by S. Kusumoto (Suntory Institute for Bioorganic Research, Osaka, Japan). Recombinant Der p 1 was kindly provided by R. Suck (Allergopharma, Reinbek, Germany).

Purification of Phl p 1

Phl p 1 was purified from Timothy grass pollen (ARTU Biologicals, Lelystad, the Netherlands) as described by Suck et al. [19]. Briefly, 10 g pollen were incubated for 30 min in Tris/HCl (20 mM, pH 8.0) at 37 °C and under agitation (225 r.p.m.) centrifuged at 4 °C and 16 000 g and filtered (pore size 0.45 µm). The extract was adjusted to 1 M ammoniumsulphate and a hydrophobic interaction chromatography was performed using a phenyl sepharose high-performance matrix followed by size exclusion chromatography with Superdex 75 (GE Healthcare Life Sciences, Uppsala, Sweden). After desalting, potential endotoxins were removed using EndoTrap blue (Profos AG, Regensburg, Germany) according to the manufacturer's instructions. After lyophilization Phl p 1 was stored at –20 °C until use.

Determination of endotoxin

Levels of endotoxin were determined using the EndosafePTS test system together with 0.1–10 EU/mL cartridges according to the manufacturer's instructions (Charles River Laboratories, L'Arbresle, France). Results were considered valid when the spike recovery was within the range of 50–200%. Endotoxin levels of the used Phl p 1 preparations ranged from 0.1 to 6 pg endotoxin/µg allergen.

Cell culture

HEK293 cells, a human embryonic kidney cell line (ATCC) and A549 cells, a human alveolar type II epithelium-like cell line (DSMZ ACC 107), were cultured in DMEM supplemented with 10% FCS, 1% PS and 1% LG. Cells were seeded at 0.5×10^6 and 0.3×10^6 cells/10 mL in 75 cm³ flasks and cultured at 37 °C, 5% CO₂ for 3 and 4 days, respectively. NCI-H727 cells derived from human lung non-small cell carcinoma (ATCC) were cultured in RPMI supplemented with 10% FCS, 1% PS and 1% LG. Cells were seeded at 0.5×10^6 and 0.7×10^6 cells/10 mL in 75 cm³ flasks and cultured at 37 °C, 5% CO₂ for 3 and 4 days. Cell-free supernatants were harvested and analysed for IL-8, IL-6 and TGF-β production (ELISA, Biosource, San Jose, CA, USA) according to the manufacturer's instructions.

Transient transfection of HEK293 cells

HEK293 cells were seeded at 0.2×10^5 cells/150 µL in 96-well culture dishes and transfected after 24 h using

PolyFect transfectant reagent according to the manufacturer's instructions (Qiagen GmbH, Hilden, Germany). The cells were transiently transfected with 200 ng of human TLR2 construct together with 25 ng of CD14 construct, or with 200 ng TLR4 construct together with CD14 and MD-2 construct, 25 ng each or left untransfected.

Twenty hours after transfection cells were stimulated for 20 h and the harvested cell-free supernatants were analysed for IL-8 production using commercial ELISA (Biosource).

RNA isolation and cDNA synthesis

A549 cells were seeded at 0.5×10^6 cells/mL in six-well culture dishes and cultured overnight. The cells were stimulated for the indicated time-points with Phl p 1 (5 µM) or LPS (100 ng/mL) or left untreated as control. RNA was obtained using Absolutely RNA Miniprepkit as recommended by the manufacturer (Stratagene, La Jolla, CA, USA). DNase digestion was performed on column (RNase-Free DNase Set, Invitrogen, Leek, the Netherlands). One microgram of total RNA was taken for cDNA-synthesis with the SuperScript III Reverse Transcriptase System (Invitrogen) for subsequent real-time PCR. PCR was performed with primers specific for huHPRT (sense: 5'GTCAGGCAGTATAATCCAAAGA, antisense: 5'GGGCATATCCTACAACAAACT), huIL-6 (sense: 5'GATGAGTACAAAAGTCCTGATCCA, antisense: 5' CTGCA GCCACTGGTTCTGT), huIL-8 (sense: 5'TTGCCAAGGAGTGC TAAAGAA, antisense 5'CAACCCTACAACAGACCCACAC) and huTGF-β (sense: 5'GCAGCACGTGGAGCTGTA, antisense 5'CAGCCGGTTGCTGAGGTA).

Real-time polymerase chain reaction analysis

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 with the expression of the house-keeping gene HPRT.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide test

Cell detachment was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich, St Louis, MO, USA) test. The cleavage of MTT to a blue-coloured product (formazan), which is indicative of mitochondrial succinate-dehydrogenase activity in viable cells, was quantified by spectrophotometric analysis. Briefly, after stimulation cells were incubated for 4 h with 40 µL/well MTT solution. The supernatants were discarded and formazan was diluted in 100 µL/well DMSO/HCL/SDS. Absorbance was measured at 570 nm in a plate reader.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis/Western blotting

SDS-PAGE was performed as described by Laemmli [20] on homogenous gels ($T = 15\%$, $C = 2.6\%$) under reducing conditions. Five micrograms protein/cm was loaded onto the gel. After SDS-PAGE the proteins were subsequently transferred to nitrocellulose membrane ($0.45\ \mu\text{m}$ pore size, Schleicher & Schüll, Dassel, Germany) by semi-dry blotting for 30 min at $0.8\ \text{mA}/\text{cm}^2$ [16]. After blotting, the nitrocellulose membranes were treated with $0.1\ \text{M}$ Tris-buffered saline, pH 7.4 containing 0.05% (v/v) Tween 20 (TBST) to block free protein-binding sites. For detection of the IgE-reactive proteins, the membrane was cut into strips and incubated with patients' sera (1 : 20 in TBST). After washing incubation was performed with the secondary antibody, an alkaline phosphatase-conjugated monoclonal anti-human IgE (1 : 2000) (Allergopharma, Reinbek, Germany). Binding patterns were visualized by substrate solution containing nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate, potassium salt in $0.1\ \text{M}$ Tris-buffered saline, pH 9.5 [17].

Immunoblotting

Lysates of A549 cells were obtained by lysing the cells in Laemmli sample buffer ($60\ \text{mM}$ Tris-HCl, 2% SDS, 5% glycerol, $100\ \text{mM}$ dithiothreitol, 0.001% bromophenolblue). Twenty microlitres to $50\ \mu\text{L}$ per lane were subjected for electrophoresis to NuPage Novex Bis-Tris gels (Invitrogen) and blotted on nitrocellulose membranes (Schleicher and Schüll, Dassel, Germany). Non-specific binding sites were blocked with Odyssey's blocking buffer (Li-Cor, Bad Homburg, Germany). The blots were incubated with polyclonal rabbit anti-human phospho-JNK (pJNK, 1 : 1000), polyclonal rabbit anti-IkB (1 : 1000) or polyclonal rabbit anti-pp38 (1 : 1000) in PBS/1% Tween. Monoclonal anti-pp38 (1 : 1000) was used to control the protein load (all antibodies were obtained from Cell Signaling Technology, Beverly, MA, USA). MAP kinase phosphorylation was visualized with IR dye 800-labelled polyclonal goat anti-rabbit IgG (Li-Cor) or Alexa Fluor 680-labelled polyclonal goat anti-mouse IgG (Molecular Probes, Invitrogen, 1 : 100 000) and analysed by use of an Odyssey infrared imager (Li-Cor).

Preparation of the trachea and imaging

C57/Bl6 mice of both sexes were used in this study. The experiments were performed according to the German guidelines for the care and use of laboratory animals.

Mice were killed by inhalation of isoflurane (Baxter, Unterschleißheim, Germany). The thorax was opened and the submandibular gland and the infrahyal musculature were removed. The trachea was cut caudal to the larynx

below the bifurcation. Then, the trachea was removed and transferred to a Delta T culture dish (Bioprotechs, Butler, PA, USA) whose bottom was covered with a thin film of Sylgard polymer (Dow Corning, Wiesbaden, Germany) and that was filled with $2\ \text{mL}$ Hepes-Ringer solution. Connective tissue and blood vessels were removed, and the trachea was oriented with the m. trachealis facing upward and fixed with two insect needles.

The trachea was cut exposing the epithelial lining. Then, the trachea was rinsed using Hepes-Ringer to remove mucus and the dish was filled with $2\ \text{mL}$ fresh buffer and transferred to the Delta T Stage holder and was heated to $30\ ^\circ\text{C}$. During the experiment this temperature was kept constant. Imaging was done with an EHD SMX-150M CMOS camera (EHD Imaging, Damme, Germany) mounted on an Axioskop 2 FS (Zeiss, Göttingen, Germany).

Before measurements, $4\text{--}8\ \mu\text{L}$ of a polystyrene bead suspension (mean diameter $4.5\ \mu\text{m}$; Dynal Biotech GmbH, Hamburg, Germany) was added. Then, the epithelial surface of the trachea was imaged in bright field mode using a $\times 20$ water immersion objective (Zeiss). Transported dynabeads were identified by their appearance and their brownish colour. For each time-point 200 images were taken with a frame rate of 12 images/s. After 15–30 min for baseline velocity Phl p 1 ($5\ \mu\text{M}$) or rDer p 1 ($1\ \mu\text{M}$) were diluted in HEPES/Ringer, added to the trachea and relative bead velocity was measured first every 2 min, later every 5 min. After 90 min ATP ($100\ \mu\text{M}$) was added to proof the vitality of the ciliated tracheal epithelium.

Particle tracking

To track individual particles, Image Pro Plus (Media Cybernetics, Bethesda, MD, USA) was used. The following image processing was performed: to remove non-moving objects in the images, an average image of the 200 images of an individual series was calculated and subtracted on a pixel-by-pixel basis from each image in the series. Then, the built-in automated tracking procedure was used to determine the individual speed of each particle. For each time-point, the mean speed of all tracked particles was calculated for each time-point.

Results

Phl p 1 activates respiratory epithelial cells

To determine whether purified Phl p 1 is able to activate alveolar epithelial cells, we investigated the direct effect of Phl p 1 on IL-6 and IL-8 mRNA induction. For this purpose A549 cells were incubated for different periods with the allergen. The gene expression was quantified via real-time PCR. As shown in Figs 1a and b, Phl p 1 strongly induced IL-6 and IL-8 gene expression with a maximum

after 2 h of stimulation. Furthermore, the Phl p 1-induced gene expression displayed a different kinetic compared with LPS-induced IL-6 and IL-8 mRNA level. After incubation with Phl p 1 also the induction of TGF- β mRNA was observed (Fig. 1c).

To confirm these results on release level, A549 cells were stimulated with Phl p 1 for 20 h and the supernatants were assayed for IL-6 and IL-8 via ELISA, or stimulated for 6, 24 and 48 h to investigate the induction of TGF- β . Figure 2 shows that Phl p 1 caused an increase of IL-8 and IL-6 release in A549 cells (Figs 2a and b), as well as an increased TGF- β release compared with control-stimulated cells (Fig. 2c). As investigated by SDS-PAGE and Western blotting (data not shown), Phl p 1 did not show any structural alterations after incubation for up to 20 h. Because the highest cytokine release was observed after 20 h of stimulation, we used these conditions for further experiments.

A549 cells, as a representative cell line for alveolar cells, do exert only a weak responsiveness to LPS, but stimulation with the lipopeptide Pam₃C-SK₄ resulted in a considerable increase of IL-6 and IL-8 release.

Specificity of the Phl p 1 induced activation of respiratory epithelial cells

Even highly purified Phl p 1 contains immunologically relevant concentrations of endotoxin. We therefore had to

exclude that still existing low levels of endotoxin or other bacterial/viral contaminations are responsible for the observed increased mediator release. We first used HEK293 cells, which were transfected with the pattern recognition receptors (PRR) TLR2/CD14 or TLR4/CD14/MD2 to determine the presence of lipopeptides or LPS, respectively. As shown in Fig. 3a none of the stimuli induced an IL-8 release from untransfected HEK293 cells. As expected the control stimuli Pam₃C-SK₄ and LPS only activated HEK293 transfected with the relevant PRR. Phl p 1 induced an increased IL-8 secretion only from TLR4-transfected HEK293 cells, suggesting the presence of residual endotoxin. We also tested a variety of PRR, namely TLR3, TLR5, TLR7, TLR8, TLR9, NOD1 and NOD2, concerning the potential of our Phl p 1 preparations to activate transfected HEK293 cells via these receptors, but there was no increased IL-8 release detectable (data not shown).

The presence of TLR4 reactivity in Phl p 1 preparations required us to exclude that the epithelial cell activating property of Phl p 1 is mediated by a TLR4 ligand, namely contaminating LPS. For this purpose A549 cells were pre-incubated with compound 406, a TLR4 blocking LPS antagonist, followed by incubation with the indicated stimuli. The increase in IL-8 release after stimulation with LPS can be reduced to control level by compound 406 (Fig. 3c), whereas the activation induced by the TLR2

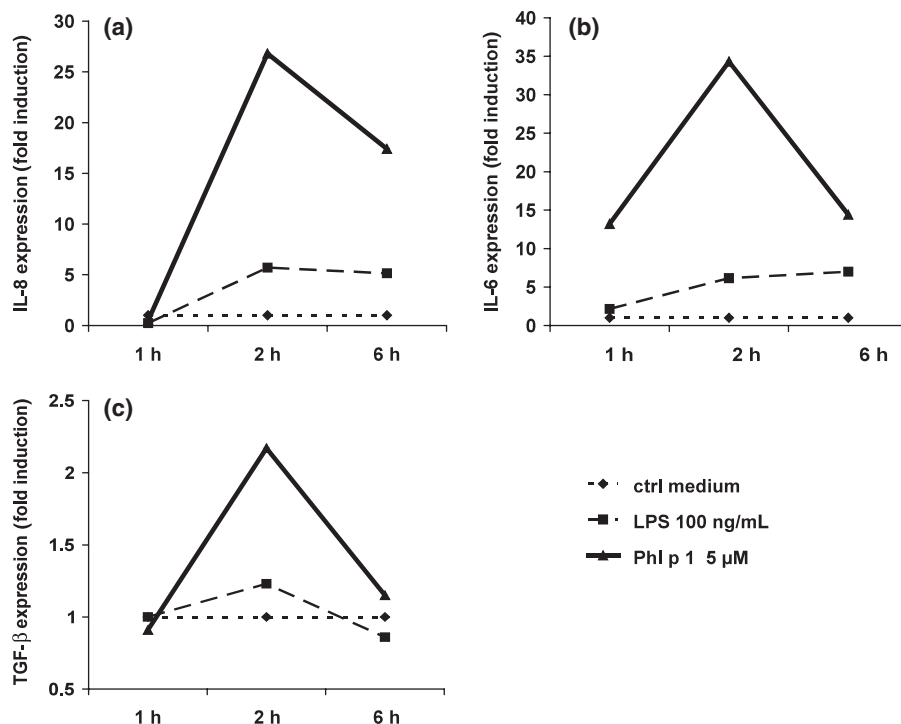


Fig. 1. Activation of respiratory epithelial cells after stimulation with Phl p 1. A549 cells were incubated with the indicated stimuli for 1, 2 and 6 h. After RNA isolation and cDNA synthesis quantitative real-time PCR was performed. Relative cDNA amount of IL-8, IL-6 and TGF- β was calculated compared with the expression of the house-keeping gene HPRT. Representative results from at least three independent experiments are shown. LPS, lipopolysaccharide.

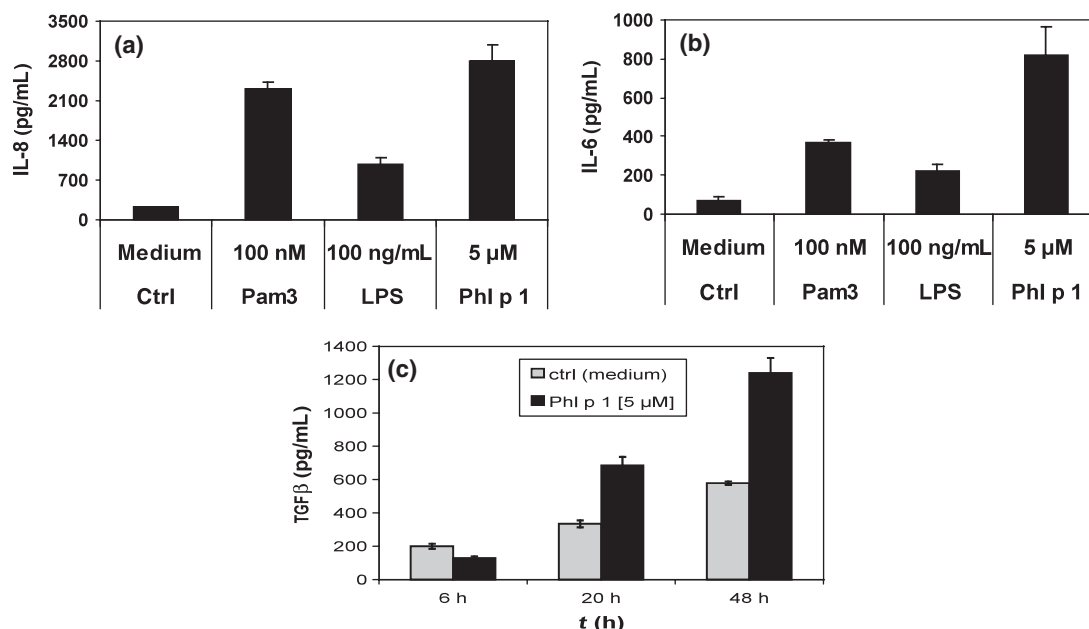


Fig. 2. Activation of respiratory epithelial cells after stimulation with Phl p 1. A549 cells were incubated with the indicated stimuli. After stimulation for 20 h or 6, 20 and 48 h (for TGF- β release) the cell culture supernatants were harvested and assayed by ELISA for IL-8 (a), IL-6 (b) and TGF- β (c) release. Values represent the mean and SD of duplicate cultures. Representative results from at least three independent experiments are shown. LPS, lipopolysaccharide.

ligand Pam₃C-SK₄ is not influenced. The Phl p 1 induced increase of IL-8 secretion remained unaffected by compound 406, indicating that the activating capacity does not result from endotoxin. We decided to show the data obtained with 2 μ M of Phl p 1, as in this case the induced IL-8 release is similar to the LPS-induced sample. The used concentration of compound 406 should have been sufficient to inhibit the IL-8 release induced by the allergen, if this effect was induced by the low level of endotoxin. In addition, an antagonist is more efficient at low concentration of the agonist, and, therefore, at this low concentration of Phl p 1 the results are more valid than at high concentrations.

Also, pre-incubation of A549 cells with monoclonal antibodies blocking TLR2 or CD14 did not inhibit Phl p 1-induced mediator release (Fig. 3b), whereas the activation of the epithelial cells by lipopeptide or LPS could be reduced to background levels after pre-incubation with the respective mAb.

Active principle of nPhl p 1

To show that the protein part of Phl p 1 is responsible for the induction of mediator release from respiratory epithelial cells we performed heat denaturation of the allergen for 10 min at 95 °C before stimulating the cells. In contrast to TNF- α , whose stimulating capacity could be completely destroyed, the Phl p 1-induced IL-8 release was not affected (data not shown). Even after heat denaturation

for 1 h at 95 °C the allergen-induced activation of the cells was only slightly reduced (Fig. 4). To analyse the importance of the protein portion more specifically, we digested Phl p 1 with proteinase K and inactivated the enzyme by heat denaturation before stimulating A549 cells. As shown in Fig. 5a, Phl p 1 has been completely digested by proteinase K and the resulting fragments do not show any remaining immunological reactivity with respect to the recognition by allergen-specific mAb or patient sera (Figs 5b and c). As shown in Fig. 5d, control stimulation with PMA/anisomycin and also LPS-induced IL-8 release were not affected after digestion with proteinase K. In contrast to the control stimuli, Phl p 1 no longer activated A549 cells after digestion with proteinase K.

For several major allergens it has already been shown that they possess proteolytic properties which can disrupt the barrier function of epithelial cells. We, therefore, investigated the potential enzymatic activity of Phl p 1. For this purpose A549 cells were incubated under serum-free conditions with either control stimuli (medium and trypsin), Phl p 1 or Der p 1. As shown in Fig. 6a no cell damage could be observed after incubation with medium, whereas the cells that were incubated with trypsin showed detachment and cell shrinking (Fig. 6b). Der p 1 (Fig. 6c) caused a clear detachment of A549 cells, while Phl p 1 did not influence the integrity of the epithelial cells (Fig. 6d). After the microscopical observation the cells were incubated with MTT and formation of formazan performed only by viable cells was measured at OD₅₇₀. Trypsin and

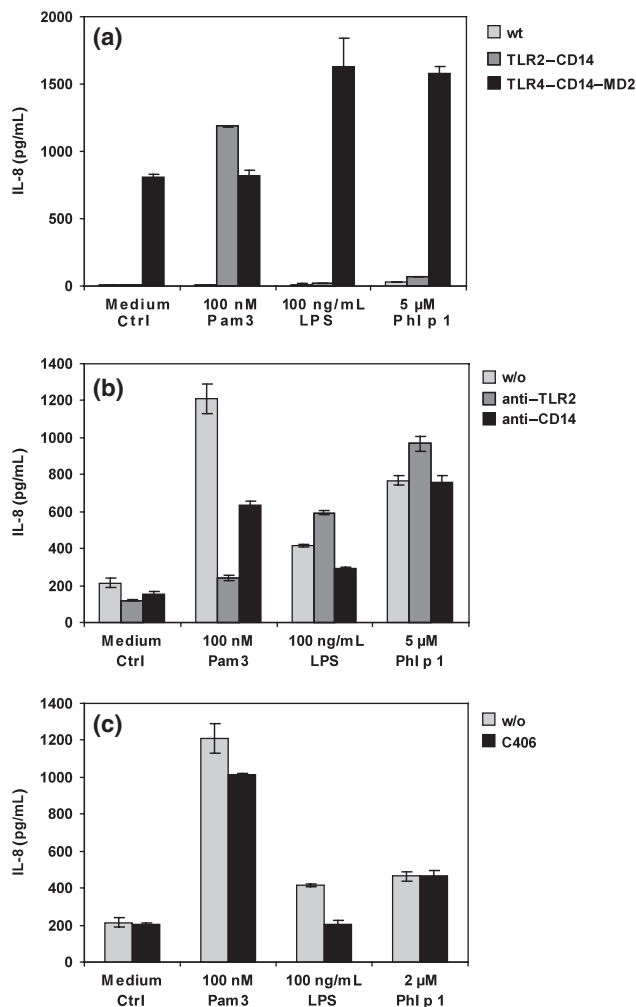


Fig. 3. Specificity of Phl p 1-induced mediator release. HEK293 cells (a) were transiently transfected with human TLR2 together with CD14 (grey bars), or with TLR4 together with CD14 and MD-2 (black bars) or left untransfected (light grey bars). Twenty hours after transfection cells were stimulated with the indicated reagents for 20 h. IL-8 release was measured by ELISA. A549 cells were pre-incubated with compound 406 (c), mAb against TLR2 or CD14 (b), 10 µg/mL each, for 30 min or left untreated and then stimulated for 20 h with the indicated stimuli. IL-8 release was determined by ELISA. Values represent the mean and SD of duplicate cultures. Representative results from at least three independent experiments are shown. wt, wild type; w/o, without; LPS, lipopolysaccharide.

Der p 1 caused reduction of formazan formation due to the observed detachment of cells. In contrast, cells incubated with Phl p 1 showed no decreased cell viability, confirming the microscopical results (Fig. 6e).

To further confirm the hypothesis that Phl p 1 interacts with respiratory epithelial cells independent from any proteolytic properties of the allergen, we used an *ex vivo* model to compare Phl p 1 and Der p 1. For this purpose murine tracheae were prepared from mice and the influence of the allergens on the velocity of particle transport by the ciliated tracheal epithelial cells was determined.

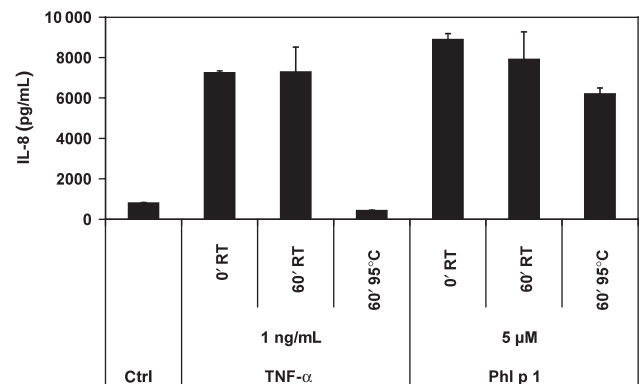


Fig. 4. Heat-treatment of Phl p 1. A549 cells were incubated with either untreated TNF-α and Phl p 1, TNF-α and Phl p 1 incubated for 1 h at RT, or heat-denatured TNF-α and Phl p 1 (1 h at 95 °C). Twenty hours after stimulation the culture supernatants were assayed by ELISA for IL-8 release. Values represent the mean and SD of duplicate cultures. Representative results from at least three independent experiments are shown.

After stabilizing the temperature, the tracheae were stimulated with Phl p 1 or Der p 1 and the relative velocity of Dynabeads resulting from the beating of the ciliated tracheal epithelial cells was determined. In control experiments the tracheae remained untreated. As shown in one representative control experiment (Fig. 7a) the relative velocity of the beads remained stable during the whole experiment. At the end of each experiment ATP was added to proof the vitality of the tracheae. After stimulation of murine tracheae with Phl p 1 (Fig. 7b) no change in bead velocity could be observed. In contrast Der p 1-stimulated tracheae showed an increase in bead velocity after 30 min of stimulation (Fig. 7c).

Signal transduction

To get a first indication which kind of signal transduction pathways may be activated in respiratory epithelial cells after stimulation with Phl p 1, we analysed the activation of MAPK. As shown in Figs 8a and b Erk as well as p38 were phosphorylated after stimulation with Phl p 1 with a maximal phosphorylation after 5–10 min. Medium-treated cells showed no MAPK phosphorylation, while the positive control (PMA/anisomycin-stimulated A549 cells) showed a clear phosphorylation of both Erk and p38. In additional experiments A549 cells were pre-incubated with PD-98059, an inhibitor that blocks the phosphorylation of Erk and SB-203580, which represses the activity of p38. As shown in Fig. 8c, both inhibitors mediated a clear decrease in cytokine release indicating an involvement of the respective MAPK pathways.

Discussion

The aim of this study was to determine the ability of purified Phl p 1 to activate human respiratory epithelial

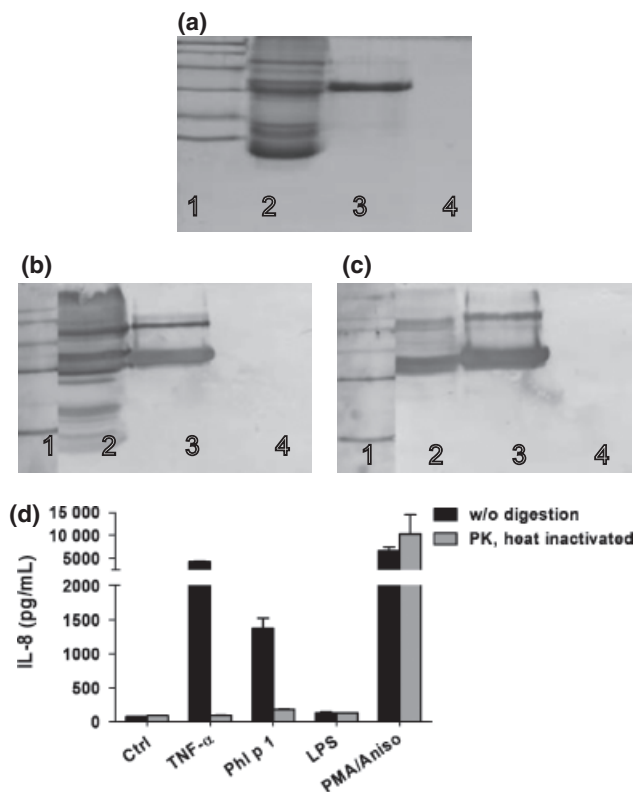


Fig. 5. (a–d) Digestion of Phl p 1. Phl p 1 has been digested for 1 h with proteinase K and the enzyme has been inactivated by heat treatment for 10 min at 95 °C. Lane 1 represents the low molecular weight marker, lane 2 shows whole pollen extract from *Phleum pratense*, in lane 3 untreated Phl p 1 has been applied and lane 4 shows Phl p 1 after digestion with Proteinase K. (a) Shows a Coomassie-stained SDS gel. (b and c) Represent immunoblots with B after incubation with a pool of patients sera and C after incubation of the blot with a mixture of 3 mAb against Phl p 1. (d): A549 cells were incubated with either untreated TNF- α , Phl p 1, LPS and PMA/anisomycin or with proteinase K-treated stimuli. Inactivation of proteinase K has been performed by heat denaturation. TNF- α and Phl p 1 that were incubated for 1 h at RT, or heat-denatured TNF- α and Phl p 1 (1 h at 95 °C). Twenty hours after stimulation the cell culture supernatants were assayed by ELISA for IL-8 release. Values represent the mean and SD of duplicate cultures. Representative results from at least three independent experiments are shown. w/o, without; LPS, lipopolysaccharide.

cells. We have demonstrated that Phl p 1 is able to induce the release of IL-6, IL-8 and TGF β from the pneumocyte type-II-like cell line A549 when stimulated with a concentration of 5 μ M. An activation of A549 cells can be achieved already with 1 or 2 μ M Phl p 1 (data not shown). When we started to investigate the influence of major allergens from timothy grass pollen on respiratory epithelial cells, we compared the influence of Phl p 1, 2/3 and 13. To refer to a stoichiometric relationship, we used the allergens in micromolar concentrations. The applied concentration appears to be high, but *in vivo* the local (punctual) allergen concentration may also be high, when the pollen grain releases its content on the epithelium.

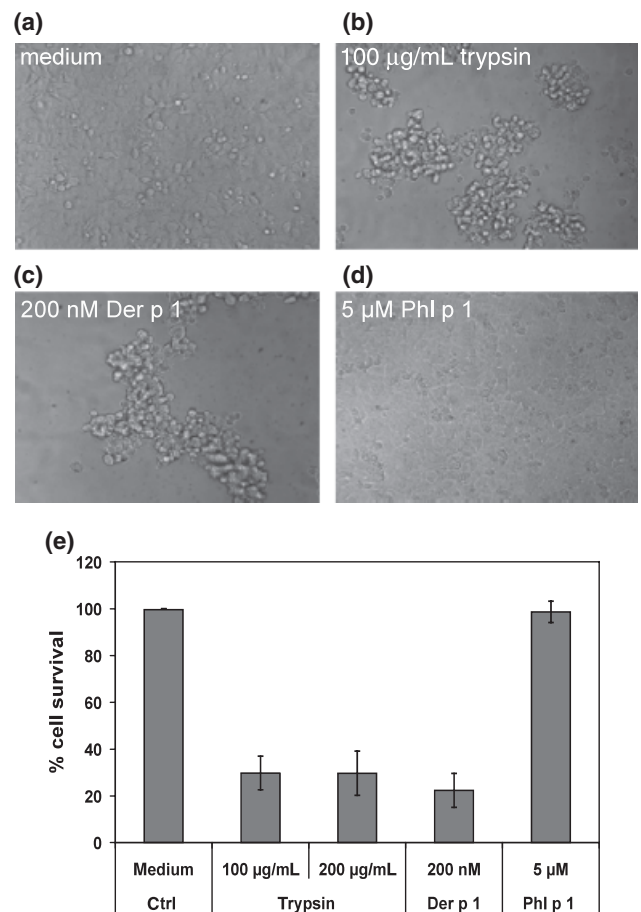


Fig. 6. Proteolytic activity of Phl p 1. A549 cells were incubated for 16 h under serum-free conditions with medium as a control (a), 100 μ g/mL trypsin (b), 200 nM Der p 1 (c) or 5 μ M Phl p 1 (d). After stimulation microscopical images were taken. The cells treated as described were incubated with MTT for 4 h. After incubation the formazan crystals were solved and OD₅₇₀ was measured. Values represent percentage cell vitality compared with medium treated cells (e). Representative results from at least three independent experiments are shown.

However, the allergen concentration *in vivo* is not known. Concerning the change of encounter, grass pollen grains are too large to reach the lower airways, but it has been shown that grass pollen grains release starch granula with attached allergens. Once released these microparticles (0.6–2 μ m) can be inhaled and reach the lower airways [21, 22].

IL-6 is known to be a potent mediator for neutrophil recruitment and activation [23] and IL-8 (CXCL8) is a key chemokine that attracts monocytes/macrophages to inflammatory loci and recruits neutrophils. IL-8 levels are increased in asthmatic bronchial epithelium and lavage fluid [24]. The Phl p 1 induced IL-6 and IL-8 release from respiratory epithelial cells may therefore contribute to the induction of allergic responses by attracting immunocompetent cells.

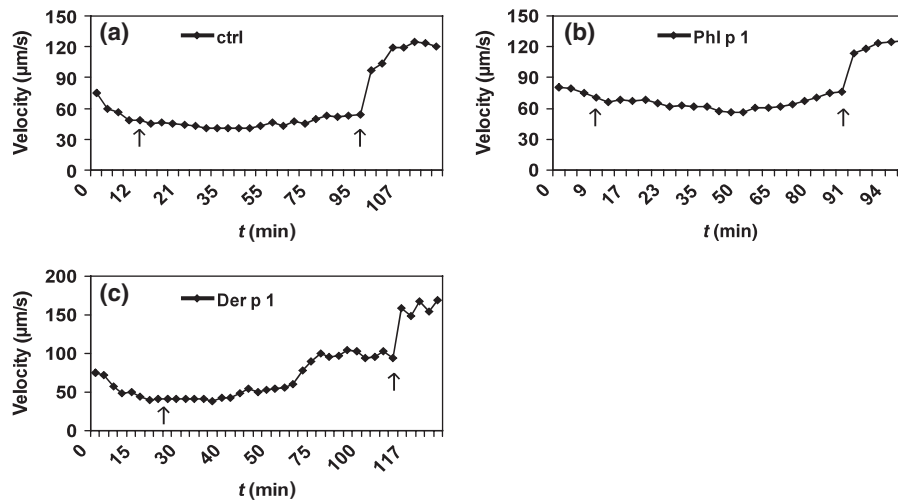


Fig. 7. Proteolytic activity of Phl p 1. Murine tracheae have been prepared as described. After 15–30 min for baseline velocity of particle transport, substances have been applied as follows (first arrow): 200 μ L Hepes-Ringer solution (control, a), 5 μ M Phl p 1 (b) or 1 μ M Der p 1 (c). At the end of each measurement 100 μ M ATP was added (second arrow) to proof the vitality of the ciliated epithelial cells. Representative results from at least three independent experiments are shown.

In contrast to a study from Salib et al. [25], in which allergens from the HDM but not grass pollen increased TGF- β gene expression, we were able to show that stimulation with Phl p 1 leads to an up-regulation of TGF- β mRNA (Fig. 1c) and induction of TGF- β release (Fig. 2c). TGF- β pathways are involved in various cellular processes. For example, TGF- β induces epithelial-mesenchymal transition including loss of apical-basal polarity [26]. TGF- β , expressed in the airways in asthma, has the potential to induce peribronchial fibrosis and there are evidences that TGF- β contributes to allergen-induced airway remodelling [27, 28]. An important role for TGF- β in airway remodelling is also suggested from studies in which TGF- β levels in the lung of asthmatic individuals were reduced by depleting eosinophils that express TGF- β [29]. Other publications have shown that bacterial recognition by TLR2 leads to activation of p38 MAPK and TGF- β signalling that induces a disruption of the integrity of the barrier required for transepithelial migration. However, the precise mechanism by which activation of this pathway results in the loss of epithelial barrier integrity remains to be revealed [30]. Besides the induction of key cytokines, the p38 MAPK cascade is involved in endothelial cell barrier dysfunction and actin remodelling [31, 32].

According to these findings, an enhancement of TGF- β gene expression and phosphorylation of p38 and Erk1/Erk2 MAPK induced by Phl p 1, accompanied by the increased release of IL-6 and IL-8, might provide an indirect mechanism by which the allergen may cross the epithelial barrier and lead to the attraction of immunocompetent cells. Although we were not able to show proof of an epithelial disintegration caused by the allergen Phl p 1, microlesions caused by the allergen cannot be ex-

cluded. The allergen-induced release of TGF- β might lead to a disassembly of the tight junctions, thereby promoting an allergen transfer through the epithelial barrier.

Self-collected timothy grass pollen as well as commercially available pollen is colonized by a variety of microorganisms ranging from Gram-positive over Gram-negative bacteria to moulds (unpublished observations). We addressed the question whether the activating properties of Phl p 1 preparations result from bacterial components, which are known to be strong immunomodulatory agents. It is known that the TLR4 ligand LPS shows a strong adjuvant potential regarding allergen-specific Igs [33] and TLR2-activating lipopeptides also enhance specific antibody levels [34]. The TLR2/6 agonist MALP-2 is even capable to reverse the Th2 skew in an *in vitro* allergy model [35]. As concluded from transfected HEK293 cells (Fig. 3a), LPS was the only relevant microorganism-derived component and its contribution during the stimulation of epithelial cells by Phl p 1 could be excluded by pre-incubation of the cells with substances blocking TLR4 signalling (Figs 3b and c). The still existing stimulatory capacity of the major allergen Phl p 1 results from the protein itself, which was confirmed by the deletion of the biological activity after digestion with proteinase K. Heat denaturation only slightly reduced the allergen-induced mediator release which indicate a high structural stability of Phl p 1. As already mentioned, we were not able to observe any synergistic, additive or adjuvant effect after simultaneous stimulation of respiratory epithelium with Phl p 1 and LPS or lipopeptide, respectively.

Many major allergens are known to exert proteolytic activity, which is suggested to facilitate the transport of

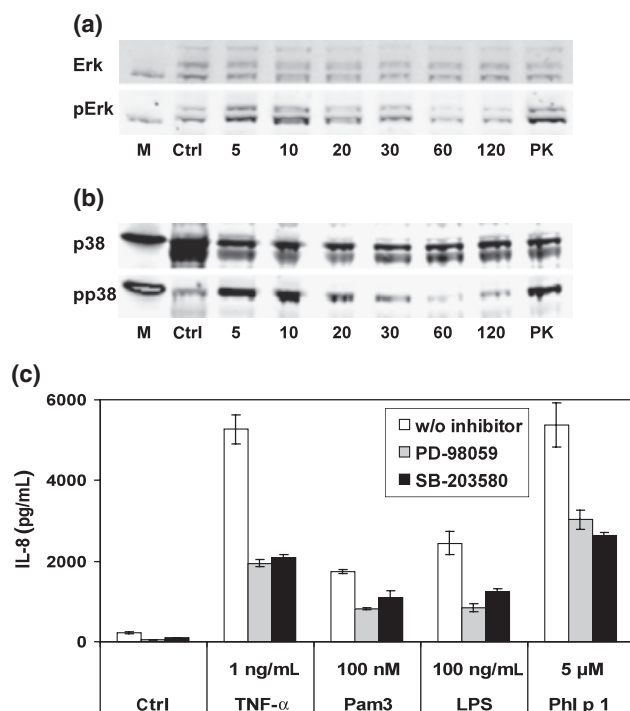


Fig. 8. Activation of MAP kinases in A549 cells upon stimulation with Phl p 1. A549 cells were stimulated with 5 μ M Phl p 1 for the indicated times. Unstimulated cells served as negative control (ctrl). Cells stimulated for 30 min with PMA and anisomycin (30 ng/mL each) served as positive control (PK). Lysed cells were subjected to SDS-PAGE and immunoblotted for phosphorylated p38 and Erk. p38 and Erk served as loading control. (Fig. 6c) A549 cells were pre-incubated with PD-98059, which blocks the phosphorylation of Erk, or SB-203580, which blocks the activity of pp38. Inhibitors were used with a concentration of 10 μ M each. Cells were pre-incubated for 30 min or left untreated and then stimulated for 20 h with the indicated stimuli. IL-8 release was determined by ELISA. Values represent the mean and SD of duplicate cultures. Representative results from at least three independent experiments are shown. w/o, without; LPS, lipopolysaccharide.

the allergen through the epithelial barrier. Potential enzymatic properties of group 1 allergens from grass pollen are controversially discussed and we therefore focussed on the question whether Phl p 1 displays any proteinase activity. Microscopical observations after incubating A549 cells with Phl p 1 or Der p 1 showed that detachment and shrinking of the cells only occur after incubation with the known cysteine protease Der p 1 (Fig. 6c), confirming the described proteolytic properties of this allergen. In contrast, our data clearly demonstrate that Phl p 1 does not possess proteolytic activity under physiological conditions. In contrast to native Phl p 1, proteinase activity has been found in recombinant Phl p 1 expressed in *P. pastoris* [15]. However, the assay conditions (strong reducing milieu and a low pH in the activation buffer) for investigating this potential proteolytic activity were far away from physiological conditions in the lung. In addition we performed an APIzym test to cover a wider

substrate specificity of the potential proteolytic activity of Phl p 1. Again, Phl p 1 exerted no proteolytic activity under physiological conditions (data not shown).

We used commercially available pollen, which was collected and afterwards stored at 4 °C. Nevertheless we saw no degradation or changes in the molecular weight indicating a structural damage, which might influence a potential proteolytic activity of the allergen. Fresh timothy grass pollen is nearly impossible to get because of the low pollen concentrations. However, we performed comparable experiments with freshly collected maize pollen, which contains a Phl p 1 homologous protein (Zea m 1) and did not determine any protease activity in zymograms (data not shown).

A different response to Der p 1 and Phl p 1 was also found *ex vivo* in murine tracheae. Using this model, the influence of compounds on the frequency of the cilia beating and the velocity of added beads in the living ciliated tracheal epithelium *ex vivo* could be investigated.

In direct comparison with Der p 1, timothy grass pollen major allergen Phl p 1-stimulated tracheae resembled the control experiments and no change in bead velocity was observed (Figs 7a and b). The higher bead velocity at the beginning of every experiment presumably results from mechanic stress during preparation. In contrast to these findings, stimulation with Der p 1 resulted in an increase in bead velocity at about 30 min after addition (Fig. 7c). It could be possible that the cysteine protease Der p 1 causes damage to the ciliated epithelium, leading to the induction of ATP release from these cells. This in turn increases the ciliary beat frequency as nucleotides, besides their ability to increase mucus secretion from goblet cells, also increase the ciliary beat frequency of airway epithelial cells [36].

Taken together we could show that Phl p 1 induces the release of IL-6 and IL-8 from respiratory epithelial cells, which would allow the recruitment of immunocompetent cells. Furthermore we could exclude a proteolytic property of the allergen to be responsible for the observed mediator release. This allows the conclusion that in contrast to the cysteine protease Der p 1 the crossing of the epithelial barrier seems not to be promoted by interaction of the allergen with PARs on the epithelial surface. We propose that the enhanced TGF- β gene expression and release, as well as the phosphorylation of p38 MAPK induced by Phl p 1 might provide an indirect mechanism by which the allergen disrupts the epithelial integrity required for the uptake.

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Supporting Information

Additional supporting information may be found in the online version of this article:

Figure S1. Apizym-test for the determination of protease activity. The test was performed according to the manufacturer's instructions (BioMerieux, Lyon, France). 1 = control, 6 = leucinarylamidase, 7 = valinarylamidase, 8 = cystinarylamidase, 9 = trypsin, 10 = α -chymotrypsin. Phl p 1 (5 μ M) was diluted in PBS (pH = 7,4, S1B) or in activation buffer (0,1 M glycine, 5 mM DTT, 10 mM cysteine, 10 mM Ca^{2+} , pH = 3,6, S1D) and incubated for 12 h at 37 °C. In S1A only PBS was tested, S1C represents the activation buffer only.

Figure S2. Investigation for self-degradation by SDS-PAGE. Phl p 1 (S2A) and Der p 1 (S2B), 5 μ g per setting, were diluted in PBS (pH = 7,4) and incubated for 0, 0,5, 1, 2 and 4 h. After incubation the samples were run on a gel and Coomassie stained. M = low molecular weight marker, r = recombinant allergens, I = protease inhibition by addition of α -complete.

Figure S3A. Investigation of isolated nPhl p 1 for protein contaminations by SDS-PAGE and Silver staining. Low molecular weight marker (M, lane 1), whole extract from *Phleum pratense* (10 μ g, lane 2) and purified Phl p 1 (5 μ g, lane 3) were run on a SDS-PAGE gel and silver stained.

Figure S3B and C. Determination of time and concentration dependent IL-8 release induced by Phl p 1. A549 cells were stimulated with the indicated stimuli and for the stated time points. Cell-free supernatants were harvested and determined by ELISA for IL-8 release. Representative results from at least 3 independent experiments are shown.

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S1

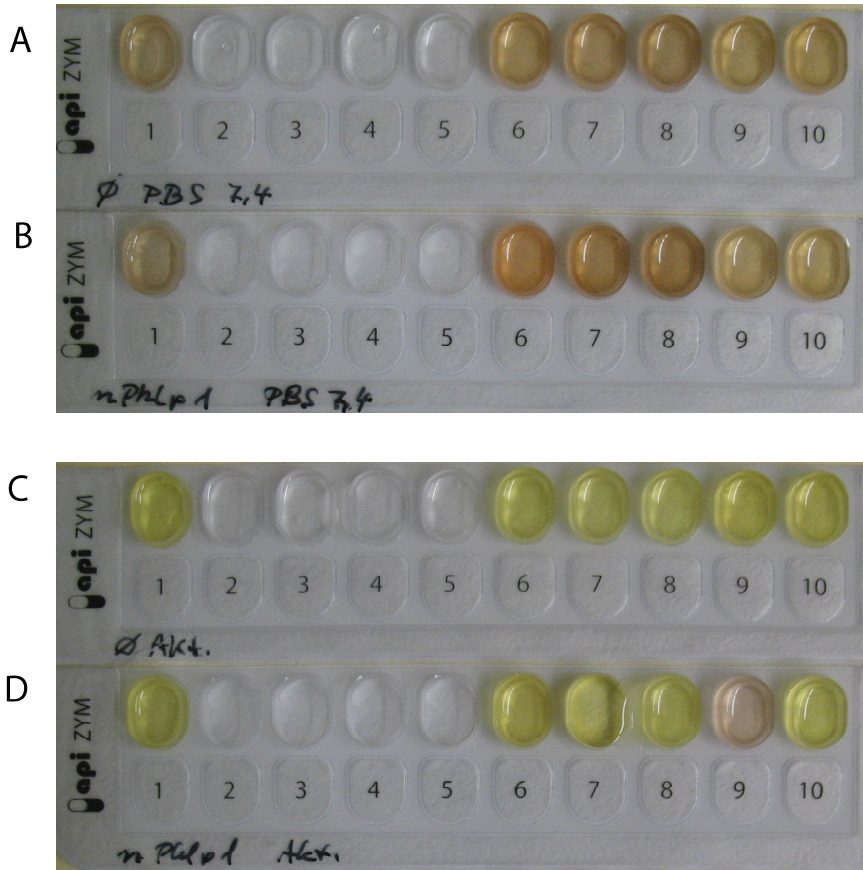


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S2

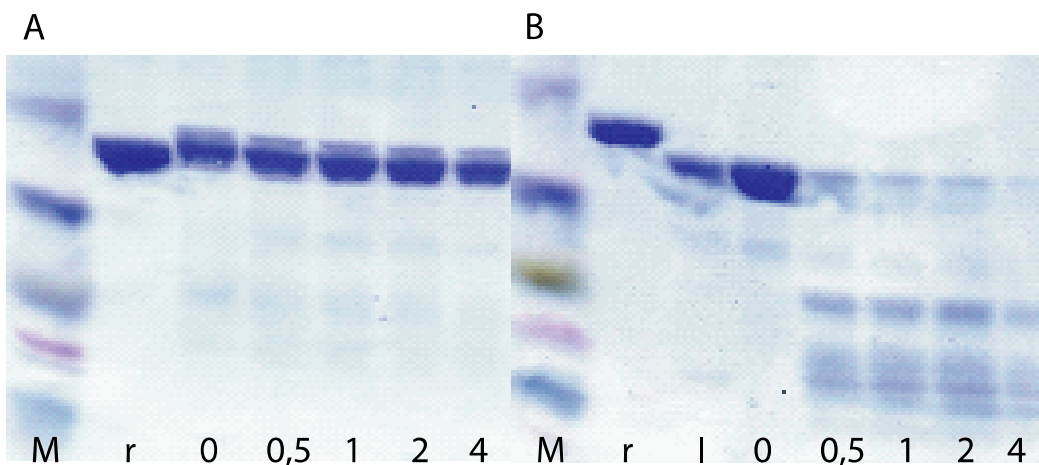


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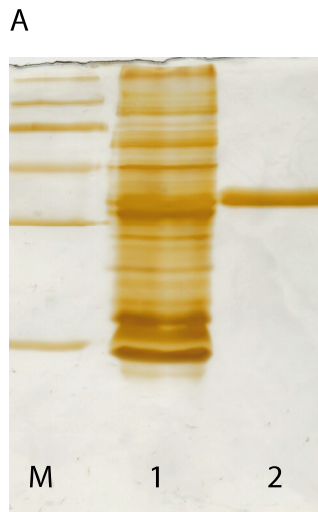


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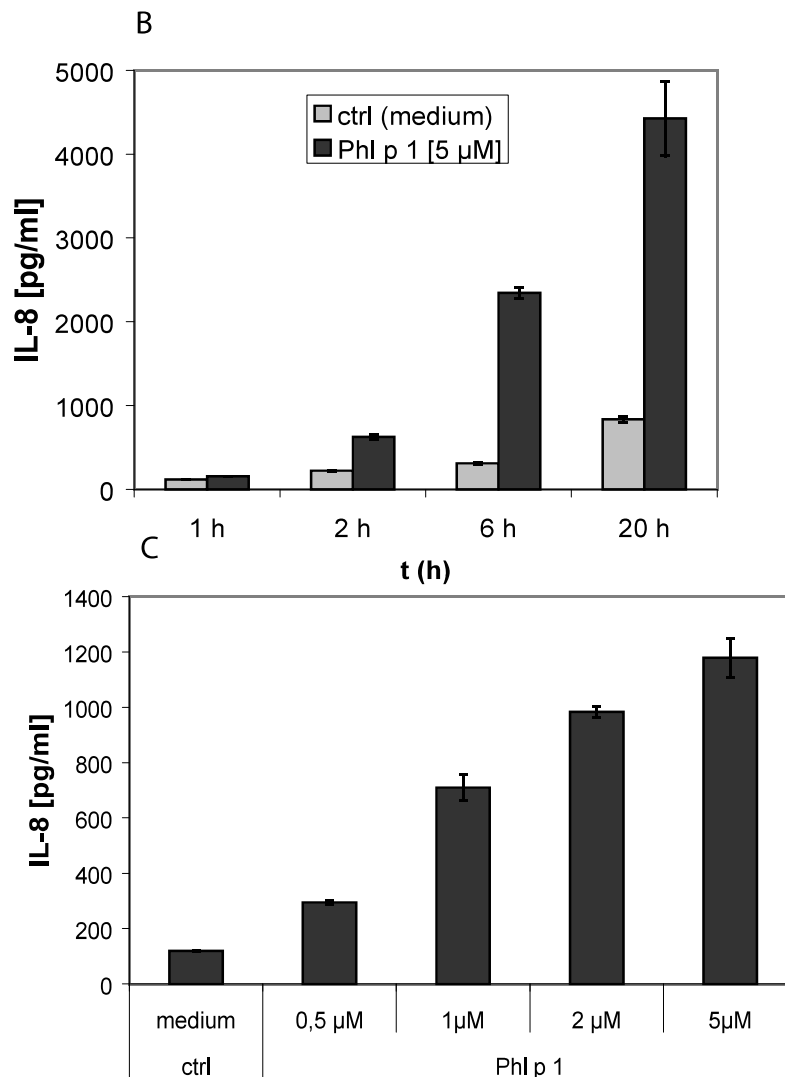


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