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The house dust mite allergen Der p 5 binds lipid ligands and stimulates airway epithelial cells through a TLR2-dependent pathway

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Abstract (242 words)

Background

Protein crystallographic studies suggest that the house dust mite (HDM) allergen Der p 5 potentially interacts with hydrophobic ligands. Der p 5, in association with its ligand(s), might therefore trigger innate immune signaling pathways in the airway epithelium and influence the initiation of the HDM-allergic response.

Objective

We investigated the lipid-binding propensities of recombinant (r)Der p 5 and characterized the signaling pathways triggered by the allergen in airway epithelial cells.

Methods

rDer p 5 was produced in *Pichia pastoris* and characterized by mass spectrometry, multi-angle light scattering and circular dichroism. Its interactions with hydrophobic ligands were investigated in fluorescence-based lipid binding assays and *in-silico* docking simulations. Innate immune signaling pathways triggered by rDer p 5 were investigated in airway epithelial cell activation assays *in vitro*.

Results

Biophysical analysis showed that rDer p 5 was monomeric and adopted a similar α-helix-rich fold at both physiological and acidic pH. Spectrofluorimetry experiments showed that rDer p 5 is able to selectively bind lipid ligands, but only under mild acidic pH conditions. Computer-based docking simulations identified potential binding sites for these ligands. This allergen, with putatively associated lipid(s), triggered the production of IL-8 in respiratory epithelial cells through a TLR2-, NF-kB- and MAPK-dependent signaling pathway.

Conclusions & Clinical Relevance.

Despite the fact that Der p 5 represents a HDM allergen of intermediate prevalence, our findings regarding its lipid binding and activation of TLR2 indicate that it could participate in the initiation of the HDM-allergic state.

Introduction

It is broadly accepted that the activation of innate immune signaling pathways is critical for the development of house dust mite (HDM) allergic response (1). Such events would occur mainly at the level of the airway epithelium when this cell layer encounters inhaled mite body debris or fecal pellets. The resulting cross-talk between the activated airway epithelium, innate lymphoid type 2 cells (ILC2s), and dendritic cells might orchestrate Th2 polarization leading to the differentiation and proliferation of allergen-specific Th2 cells, and consequent production of allergen-specific IgE (2). Whereas the mechanism of this initial step remains to be fully elucidated, innate immune responses of airway epithelium to HDM are known to be mediated through LPS/TLR4, beta-glucan/Dectin-1/TLR2, and chitin-dependent pathways (3,4). LPS, beta-glucan and chitin are commonly associated with HDM as they are abundant in house dust through the presence of Gram-negative bacteria, molds or fungi, in addition to mite chitinaceous skeletons or fecal pellets and associated microbes. Such stimulation may trigger the release of large amounts of epithelial-derived proinflammatory cytokines, chemokines including GM-CSF, IL-25, IL-33 and TSLP, thereby triggering the activation of ILC2 cells and DCs to promote Th2-biased airway inflammation (5).

The direct contribution of HDM allergens to the activation of epithelial innate signaling pathways has been investigated, though such studies have been limited to a few allergen proteins. Although it is well-established that the cysteine protease activity of Der p 1 triggers

the production of a several proinflammatory cytokines and the degradation of epithelial tight junctions, the receptor(s) and the signaling pathways involved in such epithelial cell activation remain to be fully identified (6). That the serine proteases Der p 3, 6 or 9 can stimulate the PAR-2 receptor of the airway mucosa, and the drastic reduction of the HDM allergic response in PAR-2-deficient mice, suggests that such stimulation of the airways could represent a key step in allergic sensitization to HDM allergens (6). A potential role for an allergen's biochemical activity has also been sought for the HDM allergens Der f 12, Der p/Der f 23 display amino acid sequence similarities to chitin binding proteins and consequently could also transport chitin (7). However, chitin binding assays using recombinant forms of Der p 23 did not show evidence of such interactions (8). Whereas the chitin binding activity of Der f 12 remains to be demonstrated, the corresponding allergen from *Blomia tropicalis* mite, Blo t 12, does interact with chitin (9).

The major HDM allergen Der p 2, through the presence of a large hydrophobic pocket in its structure, is able to transport LPS to activate TLR4 signaling pathways (10). Interestingly, the Der p 2/LPS complex can substitute for MD2/LPS association to trigger TLR4. Moreover, it has also been shown that Der p 2 also stimulates airway epithelial cells in a TLR2-dependent manner, suggesting that this allergen is also able to transport a variety of lipids, such as fatty acids (11).

We recently showed that Der p 13 and Blo t 7 are lipid binding proteins that are able to stimulate TLR2 of airway epithelial cells, and that its lipid cargo appears to be influential in this activity (12, 13). These data, together with those demonstrating the ability of Der p 21 to induce TLR2-dependent activation (14), suggest that lipid carriage could represent a key determinant shared by several HDM allergens that contributes to initiation of allergic sensitization.

With the exception of a study showing that Der p 5 up-regulates the production of IL-6 and IL-8 in human airway epithelial cells (15), the contribution of this mid-tier HDM allergen (16) in the stimulation of innate signaling pathways remains to be fully explored. Physico-chemical characterization of a recombinant form of Der p 5 showed that, in solution, this α-helical protein was predominantly monomeric at neutral pH and low concentration (17,18). However, oligomerization of this allergen was observed under acidic pH conditions and high concentration. The elucidation of the X-ray crystal structure of Der p 5 revealed a dimeric form with a large hydrophobic pocket laying at a dimer interface that is potentially able to associate with lipid ligands (18). But, the propensity of Der p 5 to interact with hydrophobic ligands and its potential for stimulating innate signaling pathways through its lipid cargo both remain to be demonstrated

We report herein that recombinant Der p 5 produced in *Pichia pastoris* binds lipid ligands, only under acidic conditions, and that the allergen along with its putative ligand(s) trigger TLR2-dependent signaling pathways following exposure of airway epithelial cell to it *in vitro*.

Materials and methods

Cloning, expression and purification of recombinant Der p 5

A cDNA encoding the mature form of Der p 5 (amino acids 20-132, leader-less) was synthesized with optimized *Pichia pastoris* codon usage (Geneart, Germany and based on GenBank accession number S76337). The synthetic cDNA was flanked with 5' end SnaB I and 3' end Avr II restrictions sites. Following a double digestion by SnaB I and Avr II, the Der p 5-encoding cDNA was subsequently cloned into the pPIC9K vector (Invitrogen) cut

with the same enzymes, directly downstream of the sequence encoding the α-mating factor prepropeptide.

P. pastoris SMD1168 cells were transformed by electroporation with the pPIC9K-Der p 5 vectors previously linearized by Sac I digestion. For the selection of clones transformed with Der p 5 cDNA, transformants were first selected for histidinol deshydrogenase-auxotrophy (His+) followed by a screening for geneticin (G418) resistance through plating His+ clones on agar containing increasing concentrations of the antibiotic (250 to 2000 µg/mL). For a typical rDer p 5 expression assay, we followed the same protocols as previously described for rProDer p 1 and rDer p 13 (12). Methanol concentrations of 0.5% were added to the BMMY medium for the induction of rDer p 5 expression. After 24 h, methanol was added to maintain the inducer concentration. After 48 h, the corresponding supernatants were collected by centrifugation and subsequently diluted ten times with MilliQ ultrapure water and the pH was adjusted to 4. This material was applied to a S sepharose XL column (5 x 2.6 cm, GE Healthcare Lifesciences) equilibrated with 20 mM sodium acetate pH 4.0. After washing the unbound proteins from the column, elution was performed through stepwise increase of NaCl concentration in the equilibration buffer (from 0 to 1000 mM NaCl). The fractions containing rDer p 5 (200 mM and 500 mM NaCl elutions, respectively) were concentrated by ultrafiltration and then purified by gel filtration on a Superdex 75 HR column 10/30 (GE Healthcare Lifesciences), equilibrated with PBS pH 7.3. The fractions containing purified rDer p 5 were pooled and stored at -20 °C until further analysis. Protein concentration was determined using the microBCA protein assay kit (Pierce) with bovine serum albumin as a standard. The purity of rDer p 5 was monitored by SDS-PAGE with Coomassie blue staining as well as by immunoblotting using polyclonal antibodies obtained after rabbit immunizations with rDer p 5 (Eurogentec).

Mass spectrometry (MS) of rDer p 5

Liquid chromatography-mass spectrometry (LC-MS) analysis was performed using an Ultimate 3000RSLC chromatography system (Thermo Fisher Scientific) hyphenated to a Maxis 4G mass spectrometer (Bruker). Briefly, 3 µg of rDer p 5 were loaded onto an AcquityUPLC Protein BEH C_4 column (C_4, 1.7 µm, 300 Å, 2.1 x 100 mm, Waters) equilibrated at 75 °C and eluted with a linear gradient from 95% solvent A (5% acetonitrile/ 0.1% formic acid) and 5% solvent B (80% acetonitrile/ 0.1 % formic acid) to 60% solvent B over 15 min at a flow rate of 400 µL/min. The capillary voltage, the end plate offset voltage and the dry gas temperature of the mass spectrometer were set to 4500 V, -500 V and 180 °C, respectively. MS spectra were acquired in positive mode, over the m/z range 300–3000 with a scan rate of 2 Hz. Calibration was performed using an internal lockmass calibration at 1221.9906 atomic mass unit (amu). MS spectra were deconvoluted using the MaxEnt algorithm.

Circular dichroism (CD) analysis

The secondary structure content of purified rDer p 5 was assessed by far-UV circular dichroism (CD) with a Jasco J-815 CD spectrometer at pH 7.3 and pH 4.2. To investigate the secondary structure content under acidic pH, the recombinant protein was previously dialyzed against 20 mM Na acetate buffer pH 4.2. Spectra were acquired using a 1 mm path length quartz cuvette from 190 to 260 nm with 1 nm resolution at a scan rate of 100 nm/min for 8 cycles.

Size-exclusion chromatography and multi-angle light scattering (SEC-MALS)

Recombinant Der p 5 prepared in Na phosphate (pH 7.3) or Na acetate (pH 4.2) buffers was analyzed by SEC-MALS using an Ultimate 3000 liquid chromatography system (Thermo Fisher Scientific) coupled to a µDAWN (Wyatt Technology) multi-angle light scattering (MALS) system. Three micrograms of proteins were loaded onto an Acquity UPLC BEH125 SEC column (Waters) thermostated at 25 °C. Samples were eluted using 0.150 M Na phosphate, pH 7.3 or 0.2 M Na acetate, pH 4.2 buffers (pre-filtered through 0.1 µm pore size filters) with a flow rate of 0.25 mL/min. Proteins were detected by UV absorbance (214 and 280 nm) and MALS. Chromeleon v6.80 software (Thermo Fisher Scientific) and ASTRA v6.1.1.5.22 software (Wyatt Technology) were used to analyze LC-UV and MALS data, respectively.

Spectrofluorometric lipid binding assay

Ligand binding by rDer p 5 was investigated spectrofluorometrically at pH 7.3 and 4.2 as previously described (12,19-21) using a set a fluorescent lipid probes, the most useful of which proved to be the naturally fluorescent fatty acid *cis*-parinaric acid (cPnA, Invitrogen), the fluorophore-tagged fatty acids 11-([5-dimethylaminonaphthalene-1-sulfonylamino]) undecanoic acid (DAUDA, Invitrogen) and dansyl-DL-α-aminocaprylic acid (DACA, Merck), as well as the fluorescent non-specific probe for exposed hydrophobic surfaces in proteins, 1-anilinonapthalene-8-sulfonate (ANS, Merck). The bis form of ANS was also used but the results using it were essentially the same as for ANS. Competition binding experiments were performed using oleic acid (Merck) at different concentrations as competitor. Proteins used as positive controls were bovine serum albumin (BSA, Merck), which binds DAUDA, DACA, cPnA and ANS, and bovine β-lactoglobulin (Sigma–Aldrich),

which is highly selective for fatty acids and only binds cPnA among the probes we used. Titrations were carried out by incremental addition of rDer p 5 (final concentrations of 0 to 2.5 μM) directly to 2 mL cuvette containing fluorescent DAUDA (1 μM) in 20 mM Acetate Na pH 4.2. Fluorescence emission intensities (recorded at 417 nm) were corrected for dilution and fluorescence of free ligand, and fitted using a standard nonlinear regression equation (using Microcal ORIGIN software) to a single noncompetitive binding model to give estimates of the dissociation constant (K_d) (19,22)

Protein-lipid docking simulations

Autodock vina (23) was used to dock DAUDA and oleic acid to the dimeric and monomeric structures for Der p 5. From the Der p 5 structure resolved by X-ray crystallography (PDB Code 3MQ1, 18), we selected the dimer made by chains A and B. Molecular dynamics (MD) simulation on chain A of 3MQ1 was performed considering an aqueous environment to obtain the monomeric structure. Whereas the N-terminal helix is kinked in the dimeric Der p 5 structure, the N-terminal helix of chain A was found to pack against the two other helices to form a compact monomeric structure following 1.2 ns MD simulation.

The docking simulations of DAUDA and oleate were performed under simulation conditions of pH 7.3 and at pH 4.2. For the acidic conditions, any histidine and acidic amino acids with a solvent accessibility greater than or equal to 50%. were protonated. The solvent accessibility of an amino acid, X, was defined as the solvent accessible area of the amino acid in the structure divided by its maximum solvent accessible area, the latter being the solvent accessible area of the amino acid in a tripeptide Gly-X-Gly adopting an extended conformation (24). The N- and C-terminal amino acids were capped with an acetyl and a methyl group, respectively. The protonations and the cappings were performed using the

Charmm-gui webserver (25). The carboxylic acid groups of DAUDA and oleate were also protonated at pH 4.2.

Hundred docking simulations were performed for each condition with Autodock Vina, the best solution of each simulation was selected, according to the Vina binding energy calculation. For each pH and each ligand, 100 ligand poses were obtained and the solutions that binds in a same region at the surface of the monomeric and dimeric Der p 5 structures were clustered.

Human bronchial epithelial cell activation.

The study was approved by the Bioethic Committee from the Faculty of Medicine, Chulalongkorn University (CU-IBC 01/59). BEAS-2B human bronchial airway epithelial cells (ATCC) were cultured in Dulbecco's modified Eagle's medium (DMEM Glutamax; Invitrogen) supplemented with 10% fetal calf serum. Once the cells reached approximately 80% confluency they were incubated in serum-free DMEM for a further 24 h. Prior to any cell stimulation, the culture medium was replaced with fresh serum-free DMEM. Cells were then treated with different concentrations of rDer p 5 (1–20 μg/mL) for 16 h in serum-free DMEM as well as with a wild-type *P. pastoris* BMMY culture medium fractionated using the rDer p 5 purification protocol as a control, or with the Pam_3CSK_4 TLR2 ligand (100 ng/mL, InvivoGen). In another set of experiments, rDer p 5 was pre-incubated at pH 4.2 for 15 min, the buffer was subsequently exchanged into PBS by diafiltration (3kD cut-off membrane, Pall) or by desalting through Sephadex G-25 column (GE Healthcare Lifesciences) before any cell activation assays. The different cell supernatants were collected for the detection of IL-8 by ELISA assays (BD). In some experiments, cells were then exposed to 10 μg/mL rDer p 5 for different periods of time in serum-free DMEM. To evaluate the TLR2 activation,

BEAS-2B cells were pre-incubated with anti-human TLR2-blocking antibody (Mab TLR2.5, 10 µg/mL, eBiosciences) or an isotype control (10 µg/mL, eBiosciences) prior to the addition of rDer p 5. For inhibition of intracellular signaling, cells were transiently transfected for 48 h with plasmid expressing a dominant negative version of human MyD88 (pDeNy-hMyD88, InvivoGen) or with pcDNA control vector (Invitrogen) before stimulation with rDer p 5. When appropriate, cells were pretreated for 1 h with mitogen-activated protein kinase (MAPK) (U0126, 20 µM; SB203580, 1 µM; SP6001125, 20 µM) or inhibitors specific for NF-κB (BAY-11–7082, 10 µM; InvivoGen). For digestion of rDer p 5, the recombinant allergens were treated at pH 7 with trypsin (1/20 enzyme/substrate ratio; Gibco) for 1 h at 37 ºC. As controls, recombinant allergens or trypsin alone were incubated under the same conditions. Prior to cell activation, the protein solutions were treated with HiTrap benzamidine FF beads (GE Healthcare Lifesciences) to remove trypsin (12).

HEK TLR2 reporter cell assays

HEK-Blue hTLR2 cells, expressing human CD14 and TLR2 (InvivoGen) as well as the control parental HEK-Blue Null1 cells (InvivoGen) were cultured in DMEM Glutamax supplemented with 10% FBS. Both cell lines expressed the secreted embryonic alkaline phosphatase (SEAP) gene under the control of a promoter inducible by NF-κB and activator protein 1 (AP-1). After the cells reached approximately 80% confluency, cells were incubated in serum-free DMEM for a further 24 h. Cells were then stimulated with different preparations of rDer p 5 (purified, trypsin-digested or acidic pH pre-incubated), Pam_3CSK_4 (100 ng/mL, InvivoGen) or TNF-α (100 ng/mL) for 16 h at 37 °C. As negative controls, stimulations were also performed with rDer p 23. Twenty µL of different cell supernatants were then collected and mixed with 180 µL of QUANTI-Blue™ detection medium

(InvivoGen) and incubated at 37 °C for 1 h to assess the SEAP activity. Optical density (OD) at 650 nm was measured using iMark microplate reader (Bio-Rad).

Total, nuclear and cytoplasmic protein extraction

rDer p 5-treated BEAS-2B cells at different time points (0 to 120 min) were washed with ice-cold PBS, detached by flushing and collected by centrifugation at 300 g for 5 min. For the total protein extraction, cell pellets were directly lyzed with Laemmli sample buffer (60 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 1% β-mercaptoethanol, 0.01% bromophenol blue). The cytoplasmic and nuclear extracts were obtained by a first treatment of the cell pellets with 100 µL lysis buffer (10 mM HEPES pH 7.5, 10 mM KCl, 0.1 mM EDTA, 1 mM DTT, 0.5% Nonidet-40, 0.5 mM PMSF and 1X protease inhibitor cocktail (cOmplete ULTRA tablet, Roche)), on ice for 20 min. The lysates were subsequently centrifuged at 12,000 g, 4 °C for 10 min. The supernatants were collected, corresponding to the respective cytoplasmic protein extracts. The residual pellets, washed three times with the cell lysis buffer, were next resuspended with nuclear extraction buffer (20 mM HEPES pH 7.5, 400 mM NaCl, 1 mM EDTA, 1 mM DTT, 1 mM PMSF and 1X protease inhibitor cocktail) and incubated on ice for 30 min. The supernatants, collected after centrifugation at 12,000 g, 4 °C for 15 min, represented the nuclear protein extracts. The protein concentration in the cytoplasmic and nuclear extracts was estimated using the microBCA kit.

Western blotting analysis

Equal amounts of proteins from the different extracts were analyzed by SDS-PAGE and western blotting. Polyclonal antibodies specific for both phosphorylated and non-phosphorylated forms of ERK1/2, p38 or JNK MAP kinase as well as for p65 NF-κB subunit (Cell Signaling) were used for specific detections in total protein extracts, as described previously (26). The presence of p65 in cytoplasmic and nuclear extracts was detected by anti-p65 polyclonal antibodies (Thermo Scientific). The β-actin detection with specific polyclonal antibodies was also performed as a loading control. The immunoreactive bands were detected by chemiluminescence and band intensities were analyzed by Image Lab software (Bio-Rad).

Statistical analysis

The data were analyzed using Prism software (version 5.0; GraphPad). The cell activation experiments were repeated three times and a representative experiment is presented in which results from triplicate wells are expressed as means + the standard deviation (SD). For comparison between the two treatments, a Student t test was used from which $P < 0.05$ was considered statistically significant.

Results

Physico-chemical and immunological characterization of rDer p 5.

The purification of secreted rDer p 5 from the yeast culture supernatants was achieved by a combination of cation exchange and size exclusion chromatographies. Following SDS-PAGE and Coomassie blue staining, rDer p 5 migrated as a doublet of around 13-14 kDa (Fig.1.A). These two bands, together with a band migrating around 28 kDa, were detected by anti-

rDer p 5 polyclonal antibodies (Fig.1.B), suggesting that a minor proportion of rDer p 5 migrates as a dimer. The mass of rDer p 5 was checked by MS. The most abundant form, eluting at 15 min, was 13848 Da, corresponding to the mature Der p 5 sequence (amino acids 20-132) with two extra N-terminal amino acids derived from the expression construct (Tyr-Val) (Fig. S1). The MS analysis also shows minor forms at 13864, 13880, 13896 and 13912 Da, which correspond to rDer p 5 with one to four methionine oxidations (+16 Da), respectively. Such minor modifications were also observed for the HDM allergen rDer p 21 produced under identical conditions as rDer p 5 here (14). A slightly truncated form (amino acids 25-132) of rDer p 5 was also identified. Far UV circular dichroism (CD) analysis of rDer p 5 at pH 7.3 and 4.2 yielded spectra typical of a protein comprising predominantly α-helix structures (Fig. 1.C). rDer p 5 exhibited very similar CD spectra at both pH. Finally, the recombinant allergen was also characterized by SEC-MALS to assess its oligomerization state at pH 4.2 and 7.3. In both buffer conditions the apparent molecular masses measured for species contained in the main chromatographic peak were around 16-18 kDa, suggesting that rDer p 5 was predominately monomeric in the bulk phase under either the pH conditions (Fig.1.D.E.), and that the dimer form seen in crystals of the protein might not be representative of the protein in solution. Finally, to validate further our rDer p 5 preparation, we confirmed that rDer p 5 was capable of triggering an allergen concentration-dependent degranulation of RBL SX-38 cells (rat basophil leukemia cells expressing the human FcεRI receptor) preloaded with sera from patients sensitized to Der p 5 (Fig. S2). The dose response followed a typical bell shaped curve, with a maximum reached with 0.01 μg/mL.

Lipid binding by rDer p 5

Fluorescent fatty acid probes (DAUDA, DACA, cPnA) that have been widely used to investigate ligand binding by fatty acid binding proteins were used to investigate lipid binding by rDer p 5 at both pH 7.3 and pH 4.2. We also used a non-specific probe for solvent-exposed hydrophobic regions on proteins ANS. Of these, only ANS was found to bind to the protein at pH 7.3 (data not shown; Fig. S3.A and B for DAUDA and cPnA binding, respectively), indicative of a surface-exposed hydrophobic patch or cavity. At pH 4.2, however, fluorescence emission by cPnA, DAUDA and DACA were all enhanced substantially (Fig. 2.A and B for DAUDA and cPnA binding, respectively), as continued to be the case for ANS (not shown). Under these acidic conditions, the fluorescence emission by both DAUDA (Fig. 2.A) and DACA (not shown) underwent an identical blue shift in their fluorescence emission maximum wavelengths from 532 nm to approximately 487 nm, indicating the transfer of these probes to a highly apolar environment (19). Checking the specificity of this interaction by the usual competition experiments with natural ligands (such as oleic acid;19) failed because fatty acids are considerably less soluble at low pH, and our fluorescent probes tended to partition into the aggregates formed by fatty acids at low pH. But, both DAUDA and cPnA have been found to be highly selective of their binding to fatty acid-binding proteins with contrasting structures (27). Concerning DAUDA and DACA, the dansyl fluorophore is attached to the fatty acid at the alpha and omega ends, respectively. The blue shift in their emission peaks were found to be identical, indicating that the molecules are taken into the binding site in their entirety and shielded from solvent water. It is unlikely that the attached dansyl fluorophore itself contributes significantly to binding because we did not find that a control dansylated ligand, dansyl-glycine, underwent any change in fluorescence emission in the presence of Der p 5 at either pH 4.2 or pH 7.3 (data not shown). Titration of rDer p 5 into a fixed concentration of DAUDA produced an excellent saturation binding

curve (Fig. 2.C) that yielded an apparent dissociation constant K_d of 0.07 µM, which is of the order of magnitude found for lipid transporter proteins. We therefore conclude that Der p 5 undergoes a structural and/or charge change(s) upon movement from pH 7.3 to pH 4.2 that allows access to, and binding within an apolar binding site for fatty acids at least.

Docking of ligands into monomeric or dimeric Der p 5 structures

We performed docking simulations of DAUDA and oleic acid on the dimeric and monomeric Der p 5 structures, at pH 4.2 and 7.3. Although SEC-MALS demonstrated that rDer p 5 is mainly monomeric in solution, we cannot exclude the presence of traces of dimeric forms in the rDer p 5 preparation, albeit at low relative concentrations. Moreover, the Der p 5 dimeric structure displays an apolar cavity at the interface formed by the entanglement of the two monomers and this cavity has been suggested to be the main hydrophobic ligands binding site (18).

Among the 100 poses collected by docking simulation of DAUDA on the Der p 5 dimer, all were located in this hydrophobic cavity at pH 7.3 and 95% at pH 4.2 (Fig.S4.A), with an average computed binding energy of -7.99 kcal/mol and -7.81 kcal/mol, respectively. Oleic acid is also mainly bound in the cavity but with a less favorable binding energy (75 and 63 out of 100 poses, -5.65 kcal/mol and -5.55 kcal/mol, at pH 7.3 and 4.2, respectively).

In the Der p 5 monomeric structure, the docking of DAUDA and oleic acid was, in contrast, more heterogeneous, and distributed into four areas on the protein's surface (Fig.S4.B and C; Table 1) and the computed binding energies were also less favorable than those obtained on the dimeric structure. DAUDA, at pH 7.3, was docked mostly in areas 1 and 2 (45% and 25% of the poses respectively), with corresponding average binding energies of -5.33 and -4.58

kcal/mol, whereas at pH 4.2, the partition of ligand populations within these areas was more balanced. Although the average binding energy for docking in area 1 did not change at pH 4.2, that calculated for area 2 shifted from -4.58 kcal/mol to -5.15 kcal/mol (Table 1). As shown in Table 1, oleic acid poses were preferentially observed in areas 1 and 4 at pH 7.3. At pH 4.2, whereas the population of poses remained stable in area 4, the population of oleic acid located in area 1 decreased and area 3 became effective.

rDer p 5 activates airway epithelium through TLR2-dependent signaling pathways.

To determine whether rDer p 5 could play a role in the airway epithelium activation accompanying exposure to HDM allergens, the BEAS-2B bronchial epithelial cells were cultured for 16 h with various concentrations of rDer p 5 under serum-free conditions, and IL-8 levels were measured in the supernatants by ELISA. As a positive control, cells were stimulated with the Pam_3Cys_4 TLR2 ligand. Fig. 3.A shows that the presence of rDer p 5 elicited IL-8 secretion from BEAS-2B cells in a concentration-dependent manner when compared with control medium or supernatants from methanol-induced wild-type SMD1168 cells that had been fractionated following the rDer p 5 purification protocol ($p < 0.05$).

Given that rDer p 5 can associate with small hydrophobic ligands, it may carry lipids that stimulate TLR2 signaling pathways as recently observed for the HDM fatty acid binding protein Der p 13 and Blo t 7 (12, 13). Consequently, we compared the IL-8 secretion in response to rDer p 5 in BEAS-2B cells pretreated or not with anti-TLR2 blocking antibodies. The TLR2 blockade drastically reduced the rDer p 5-induced IL-8 release from BEAS-2B whereas similar pre-incubation with an isotype control antibody did not impair the cytokine production (Fig.3.B). The TLR2 activation by rDer p 5 was further confirmed by the use of HEK-TLR2 reporter cells, which expressed TLR2 on the cell surface (28) (Fig.3.C). As

TLR2 activation commonly triggers a signaling cascade through the MyD88 adaptor protein (29), the role of MyD88 in the rDer p 5-induced cell activation was examined using a dominant negative MyD88 expression plasmid (MyD88 DN) to down-regulate MyD88 activity. The IL-8 secretion stimulated by rDer p 5 in BEAS-2B cells transfected with MyD88 DN was drastically reduced (Fig.3.D), but no effect was observed with the control plasmid.

Finally, since we previously demonstrated that the fatty acid binding allergen Der p 13 activates innate signaling pathways through its lipid cargo, we investigated whether the rDer p 5-induced TLR2 stimulation is similarly mediated by a lipid ligand potentially associated with the allergen. For that purpose, rDer p 5 was digested with trypsin at 37 ºC for 1 h, leading to extensive allergen proteolysis yielding peptides too small to be detected on SDS-PAGE (Fig.3.E). To prevent the contribution of trypsin in the potential residual cell activation (through, for example, PAR-2), the rDer p 5 hydrolysate was incubated with immobilized benzamidine to deplete trypsin activity. We previously demonstrated that BEAS-2B are completely unresponsive when similar amounts of trypsin were pre-incubated with the benzamidine matrix (12). Remarkably, when compared with intact rDer p 5, the hydrolysis of the allergen did not modulate the IL-8 release in BEAS-2B cells (Fig.3.F). These results suggested that the rDer p 5-induced airway epithelial cell activation is not mediated by the protein backbone alone, but is more likely dependent on an associated lipid ligand of yeast origin. Given that the lipid probes DAUDA and cPNA can bind to rDer p 5 only at acidic pH, we hypothesized that rDer p 5, under this pH condition, could release the lipid ligand(s) of yeast origin, leading to the decrease of its capacity to activate TLR2. To test this assumption, rDer p 5 was pre-incubated at pH 4.2 and the pH was readjusted to 7.3 by dialfiltration or desalting, methods likely able to separate released lipid ligands from the protein backbone. As a control, rDer p 5 was directly diafiltrated or desalted against PBS at pH 7.3. Our results

clearly showed that the acidic pretreatment of rDer p 5 followed by diafiltration or desalting significantly reduced the capacity of rDer p 5 to stimulate the IL-8 production in BEAS-2B cells but also TLR2 activation in HEK reporter cells (Fig.3. G.H). Taken together, our data indicated that rDer p 5 stimulates TLR2 signaling involving its lipid cargo

Since TLR2 signal transduction is correlated with MAPK and NF-κB activation, we next examined the effect of rDer p 5 on these signaling molecules through the detection in stimulated cells of NF-κB p65 and MAP kinases phosphorylation (Fig.4). Western blot analysis indicated that rDer p 5 transiently triggered the phosphorylation of p65 in treated BEAS 2B cells, reaching a maximum at 10min to decline for longer periods (Fig.4.A). The time-dependent translocation of p65 into the nucleus following cell treatment with rDer p 5 confirmed the activation of NF-κB pathway by the allergen (Fig.4.B). rDer p 5-induced phosphorylation of ERK, p38 and JNK was also observed (Fig.4.C). Whereas p38 and JNK activation by rDer p 5 followed the same kinetics, with a maximal at 5-10 min, delayed phosphorylation of ERK was observed, which was maximal at 30 min. Using specific inhibitors, we examined the role of ERK, p38 MAPK, and JNK activation on the production of IL-8 from BEAS-2B cells in response to rDer p 5. Blockade of p38 MAPK or JNK pathways by SB203580 (a p38 MAPK inhibitor) or SP600125 (a JNK inhibitor), respectively, markedly reduced the rDer p 5-induced IL-8 production (Fig.4.D). On the other hand, pretreatment of cells with U0126 (a MEK1/2 inhibitor) failed to affect the secretion of IL-8 in response to rDer p 5. These data thus indicate that, among the three MAP kinases analyzed, p38 and JNK appear to be the only ones involved in the enhanced production of IL-8 in response to rDer p 5. A similar reduction of IL-8 release was observed with IκB-α phosphorylation inhibitor BAY-11-7082, strengthening the role of NF-κB in the rDer p 5-induced IL-8 production.

Discussion

It is increasingly accepted that the allergenic determinants of allergens are related to their abilities to stimulate innate signaling pathways which are essential for the initiation of the allergic response. In this context, the HDM allergens displaying lipid/fatty acid binding activities could play a key role through their ability to activate TLR4 or TLR2 signaling in the airway epithelium and other innate immune cells. It must be pointed that LPS from HDM allergen extracts can trigger IL-1β release by infiltrating monocytes, which drives the IkBζ-dependent expression of GM-CSF in lung epithelial cells (30).

Whereas group 2 mite allergens Der p/f 2, by carrying LPS, stimulate TLR4 (10,31), members of group 7 (Blo t 7) as well as group 13 (Der p 13, Blo t 13) can transport lipid ligands to activate TLR2 (12,13,32). The present study indicates for the first time the allergenicity of Der p 5 (which is considered to be a clinically relevant intermediate HDM allergen (also known as 'mid-tier' in allergen literature, referring to the proportion of allergics who develop specific IgE to it) probably depends on its lipid-binding (16). In this study, we examined a role for ligand binding by Der p 5, for which we used a recombinant form of the protein produced in *P. pastoris* as it is impossible to obtain natural form. The *P.pastoris* expression system allows the protein production free of contaminating endotoxins, which prevents any LPS interference in innate immune cell activation assays. We considered that our rDer p 5 preparation represents a faithful alternative to the native allergen or rDer p 5 produced in *E. coli.* Indeed, the proportion of HDM-allergics that respond to rDer p 5 produced in yeast corresponded to the level found with allergen produced in *E. coli* (around 30%, data not shown, 16). Moreover, our rDer p 5 protein could trigger degranulation of basophils pre-loaded with IgE from Der p 5-reponsive individuals (Fig. S2). The key results are that rDer p 5 demonstrably binds lipid ligands, and that it can stimulate TLR2-NF-κB-MAPK-dependent signaling pathways to trigger proinflammatory cytokine production in an

airway epithelial cell line. Our findings are similar to the recently elucidated capacity of Der p 21, a HDM allergen sharing a common three α-helix bundle structure with Der p 5 (33), to elicit the production of proinflammatory cytokines in airway epithelial cells through TLR2 (14). A relationship between lipid binding and direct immunological activities of group 5 and group 21 HDM allergens may indicate the existence of a common theme in triggering the onset of the allergic response. Although all the cell activation assays were performed under serum-free conditions, BEAS-2B cells were first grown in the presence of serum, a condition which can induce differentiation into squamous cells if sufficient time is allowed (34). However, the limited serum usage in our study should not present this as a problem since it was shown that BEAS-2B cells behave the same when activated with HDM extracts in serum or in serum-free conditions (30). It must be pointed out that, under our experimental conditions, rDer p 5 had to be used at concentrations ranging from 5 to 20 μg/mL in order to measure an effect. Similar or even higher allergen concentrations were previously needed to provide any airway epithelial cell activation by purified, non-proteolytically active allergens, such as rDer p 2, rDer f 2 or rDer f 31 (35-37). The known difficulties in detecting natural, intact Der p 5 in HDM allergen extracts questions the physiological relevance of the rDer p 5 concentration used in our assays (38). But the point is that, during natural sensitization conditions, lower Der p 5 concentrations could be sufficient to activate the airway epithelium through co-activation by contaminating DAMPs/PAMPs present in the mite fecal pellets.

Although we have not yet been able to establish the nature of the lipid ligand(s) present in *P. pastoris*-derived recombinant allergen, it is likely that the protein is selective in that it binds lipid probes that have previously been found to limit their engagement to specialized fatty acid-binding proteins. The similar level of cell activation we observed with degraded rDer p 5 is consistent with the idea that the TLR2 activation by rDer p 5 is mediated by the protein's lipid cargo from *P. pastoris*. The reduction of the capacity of rDer p 5 to activate TLR2 when

the protein was incubated under acidic pH and diafiltrated or desalted with PBS suggested that the lipid cargo from yeast could at least partially release from rDer p 5 under these acidic pH conditions. Whereas the interaction of natural Der p 5 with a wider array of potential lipid ligands remains to be examined, our results suggest that the propensity of Der p 5 to bind lipid ligand could be critical to its allergenicity.

Both the empirical spectrofluorimetry as well as computer simulations of protein:ligand docking indicate that the accessibility of rDer p 5's binding site is pH-dependent. The structural or charge changes involved are as yet unknown, but our simulations have provided possibilities that could be investigated by site-directed mutations of the protein. The effect of pH could be directly relevant given that natural Der p 5 is secreted by the mite into an acidic environment, the *D. pteronyssinus* midgut (18,39). The loading of Der p 5 with the lipid(s) for which it is selective could therefore occur before secretion or in the environment of the midgut itself. In the latter case, loading with lipids from the mite's microbiome could occur, which would have direct relevance to subsequent interaction with TLR2. Lipid loading and exchange at acidic pH distinguishes Der p 5 (and perhaps other group 5 HDM allergens) from allergens that also bind lipids but at neutral pH.

The selective binding of DAUDA to Der p 5 at pH 4.2 could result from the displacement of the lipid ligand originating from *P. pastoris* and/or charge difference at the level of the Der p 5 surface. To further explore the pH-dependence of the binding of DAUDA to Der p 5, computer-based docking simulations were performed at pH 4.2 and 7.3 using the monomeric and dimeric structure of the allergen. Under the conditions we used in cell activation assays (neutral pH and low concentrations), the protein is predominately monomeric although a minor proportion of dimers were detected. The docking of DAUDA with dimeric Der p 5 was predicted to occur in a hydrophobic cavity at the dimer interface, and these interactions were calculated to occur with similar free energies of binding at neutral or acidic pH. For the

monomer, binding was predicted to occur at two apolar patches on the surface of the monomer, the distribution of DAUDA binding between these sites 1 and 2 shifting from 45%/25% at pH 7.3 to 31%/38% at pH 4.2. The difference in the docking proportion was concomitant with a minor increase, and a decrease, in binding free energy for the DAUDA docking in area 1 and 2, respectively. Protonation of DAUDA's carboxylic groups at pH 4.2, and also the acidic amino acid side chains surrounding area 2 in particular of monomeric Der p 5 could be responsible for the change in the docking proportion and for the decrease of the binding energy in area 2 The absence of DAUDA binding to rDer p 5 at pH 7.3, even when the protein was pretreated to displace the lipid ligand of *P. pastoris* (acidic pH incubation and desalting with PBS, data not shown) supported the hypothesis that the selective binding of DAUDA to rDer p 5 at pH 4.2 is mainly mediated by the protonation of the Der p 5 apolar patches and/or DAUDA.

Because of the aggregation of fatty acids (such as oleic acid) at low pH, we were unable to observe the displacement of DAUDA by competition, which is routinely used to investigate binding by natural lipid ligands to other fatty acid-binding proteins (19). However, our docking simulations predicted that the binding energy of the oleic acid:Der p 5 complex would be higher than for the DAUDA:Der p 5 association in both monomeric and dimeric structures, the first complex being thus less stable than the latter. Moreover, the docking of oleic acid was predicted to take place in two other areas in addition to areas 1 and 2 in the monomeric structure. Taken together, the experimental and simulation data indicate that Der p 5's interactions with lipids is complex, and that the lipids for which it is selective may be more elaborate in structure than simple fatty acids.

In summary, our results demonstrate for the first time that Der p 5 binds lipid ligands and activates TLR2 in airway epithelial cells. Such properties are strategically placed to contribute to the induction of the allergic state in the respiratory tract, even if the subsequent

IgE response in an individual does not target the inciting allergen(s). Similar results observed with Der p 2, Der p 13, Der p 21, and Blo t 7 suggest that TLR2 activation may represent a common feature of HDM allergens that exhibit lipid binding activities. This hypothesis is seemingly at odds with results obtained with TLR2 agonists or TLR2-deficient mice that argue against a role for TLR2 in HDM allergy (4,40-42). Our findings nevertheless indicate the existence of an extra dimension in the induction phase of the allergic state, namely the involvement of lipids delivered by lipid-binding allergens in the initiation of allergy, even if the lipid-carrier itself does not become the target of an IgE response, as has also been considered for allergens from other sources (43).

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Conflict of interest

Authors declare there have no conflicts of interests.

Author contributions

A.J. designed the study; P.P., P.S., T.S. and S.K. performed the experiments in collaboration with A.J., D.G., E.N. and M.W.K.; M.L.M. performed mass spectrometry experiments. A.J., E.N., D.G. and M.W.K. provided supervision and analysed the data for the spectrofluorometric analysis carried out in Glasgow. P.P. drafted the manuscript with input from A.J., M.W.K., D.G. and E.N. All authors contributed to and approved the final version of the manuscript.

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Legends to Figures

Figure 1: Characterization of purified rDer p 5

Panel A: SDS-PAGE of purified rDer p 5 under reducing conditions, stained with Coomassie blue. Panel B: Immunoblot detection of rDer p 5 by polyclonal anti-rDer p 5 antibodies. Panel C: Far UV CD spectrum of rDer p 5 at 25 ºC and at pH 7.3 (solid line) and pH 4.2 (dashed line). The calculated proportions of secondary structures at both pH values are also given, and also root mean square (RMS) deviations. Panels D and E: Molecular mass determination of rDer p 5 from size-exclusion chromatography with in-line multi-angle static light scattering (SEC-MALS) at pH 7.3 (panel D) and 4.2 (panel E). MALS calculated molar mass (black, MM) are shown.

Figure 2: Hydrophobic ligand binding by rDer p 5.

Panel A: binding of the dansylated fatty acid DAUDA by rDer p 5 in acetate buffer at pH 4.2. Addition of rDer p 5 elicited a dramatic enhancement of fluorescence emission by DAUDA and a blue shift of its wavelength of peak emission from 532 nm to 487 nm, which is indicative of the fluorophore moving from solvent water to a highly apolar protein environment. Panel B: binding of the unmodified, naturally fluorescent fatty acid *cis*-parinaric acid (cPnA) to rDer p 5 at pH 4.2. This probe is also environmental-sensitive and its fluorescence emission increases dramatically when bound to a lipid-binding protein, but undergoes only a very slight shift in wavelength of peak emission. Panel C: titration with increasing quantities of rDer p 5 into DAUDA in acetate buffer at pH 4.2. The fluorescence emission data were corrected for dilution and fitted with a standard hyperbolic/non-cooperative binding function as previously described (17,18). The calculated dissociation

constant (K_d) for the rDer p 5-DAUDA complex was 0.07 μM and the stoichiometry (n=0.66) was consistent with one binding site per protein monomer unit. The data shown are representative of three independent experiments.

Figure 3 : TLR2-dependent cellular activation by rDer p 5.

Panel A: Production of IL-8 in culture supernatants from BEAS-2B airway epithelial cells treated with different concentration of rDer p 5. BMMY medium from cultured wild-type P. pastoris and purified according to the purification protocol of rDer p 5 (WT KM71) as well as Pam3Cys were used as negative and positive controls, respectively. Panel B: Effect of anti-TLR2 blocking antibodies on the release of IL-8 by rDer p 5-treated BEAS-2B cells. Panel C: Activation of NFkB-SEAP signaling HEK-hTLR2 and parental HEK-Null1 cells stimulated by rDer p 5. As controls, cells were also stimulated with TNF-a, rDer p 23 or Pam3Cys. The SEAP activity was determined 16 h post-activation. Panel D: rDer p 5-induced IL-8 production by BEAS-2B cells previously transfected with a plasmid expressing a dominant negative MyD88 or control plasmid. Panel E: Extensive proteolysis of rDer p 5 following treatment with Trypsin at 37 °C for 1h (rDer p 5: trypsin ratio 20:1, lane 3). As control, purified rDer p 5 stored at -20 °C (lane 1) was also incubated at 37 °C in the absence of trypsin for the same period of time (lane 2). Panel F: IL-8 production by BEAS-2B cells treated with extensively trypsinized rDer p 5. As controls, cells were treated with rDer p 5, or with the allergen pre-incubated for 1h at 37 °C without trypsin. Prior to cell activation, the three rDer p 5 samples were incubated with immobilized benzamidine matrix. To control the removal of trypsin, cells were also stimulated with trypsin alone but previously loaded onto the same benzamidine beads. Panel G: IL-8 production by BEAS-2B cells activated by rDer p 5 pre-incubated at pH 4.2 and diafiltrated or desalted with PBS pH 7.3 (3kD or desalt. 4>>7).

As controls, cells were treated with rDer p 5 directly diafiltrated or desalted with PBS (3kD or desalt. 7>>7). Panel H: Activation of NF-kB-SEAP signaling HEK-hTLR2 and parental HEK-Null1 cells stimulated by rDer p 5 pre-incubated at pH 4.2 and diafiltrated or desalted with PBS pH 7.3 (3kD or desalt. 4>>7). As controls, cells were treated with rDer p 5 directly diafiltrated or desalted with PBS (3kD or desalt. 7>>7). Data show mean IL-8 concentrations or SEAP activity + SD from triplicates and are representative of three independent experiments. *: $P < 0.05$.

Figure 4: Airway epithelial NF-κB and MAPK activation by rDer p 5.

Panel A: BEAS-2B cells were incubated with rDer p 5 (10 µg/mL) for the indicated periods of time. NF-κB activation was determined by the western blot detection of phosphorylated p65. As loading control, unphosphorylated p65 as well as the housekeeping protein β-actin were detected in the same samples. Panel B: Cells were treated with rDer p 5 (20 µg/mL) for the indicated time points. Nuclear and cytosolic extracts were prepared and subjected to western blotting with antibodies specific for p65 translocation as well as β-actin. Panel C: MAPK activation was analyzed by Western blotting with anti-phospho-ERK1/2, anti-phospho-JNK, or anti-phospho-p38 antibodies. As loading control, unphosphorylated p65, ERK1/2, JNK or p38 as well as β-actin were detected in the same samples. The different western blots are representative of three independent experiments. Quantitation was performed using the Image Lab software. Numbers displayed below the bands from Panels A and C represent fold increase over unstimulated. Panel D: Production of IL-8 in rDer p 5-stimulated BEAS-2B cells which were pre-incubated with SP600125, SB203580, U0126 (MAPK pathway inhibitors) or BAY 11-7082 (NF-κB pathway inhibitor). Data show mean

IL-8 concentration + SD from triplicates and are representative of three independent experiments. *: $P < 0.05$.

Table 1. Docking simulations of DAUDA and oleic acid (OLE) on the Der p 5 monomeric structure, at pH 4.2 and 7.3.

The percentage of poses found in the different areas, their average binding energy and the standard deviation are provided. See Figure S4 for the localization of the areas.

Table 1

Ligand	pH	Area 1	Area 2	Area 3	Area 4
DAUDA	7	45% -5.33 ± 0.18 kcal/mol	25% -4.58 ± 0.09 kcal/mol	<15%	<15%
	4	31% -5.24 ± 0.45 kcal/mol	38% -5.15 ± 0.18 kcal/mol	<15%	<15%
OLE	7	30% -4.11 ± 0.39 kcal/mol	<15%	<15%	39% -3.89 ± 0.30 kcal/mol
	4	21% -4.20 ± 0.32 kcal/mol	<15%	25% -4.07 ± 0.21 kcal/mol	39% -3.89 ± 0.26 kcal/mol

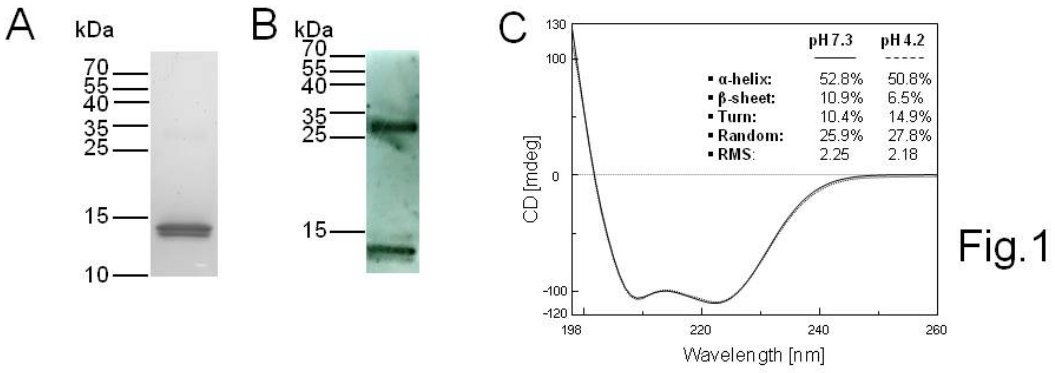
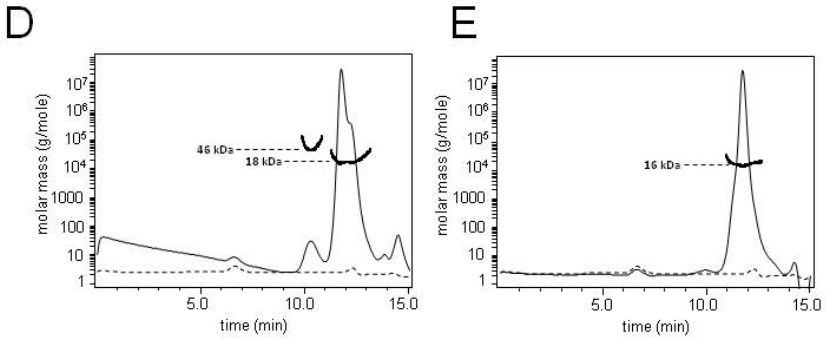


Fig.1



pH 7.3 pH 4.2

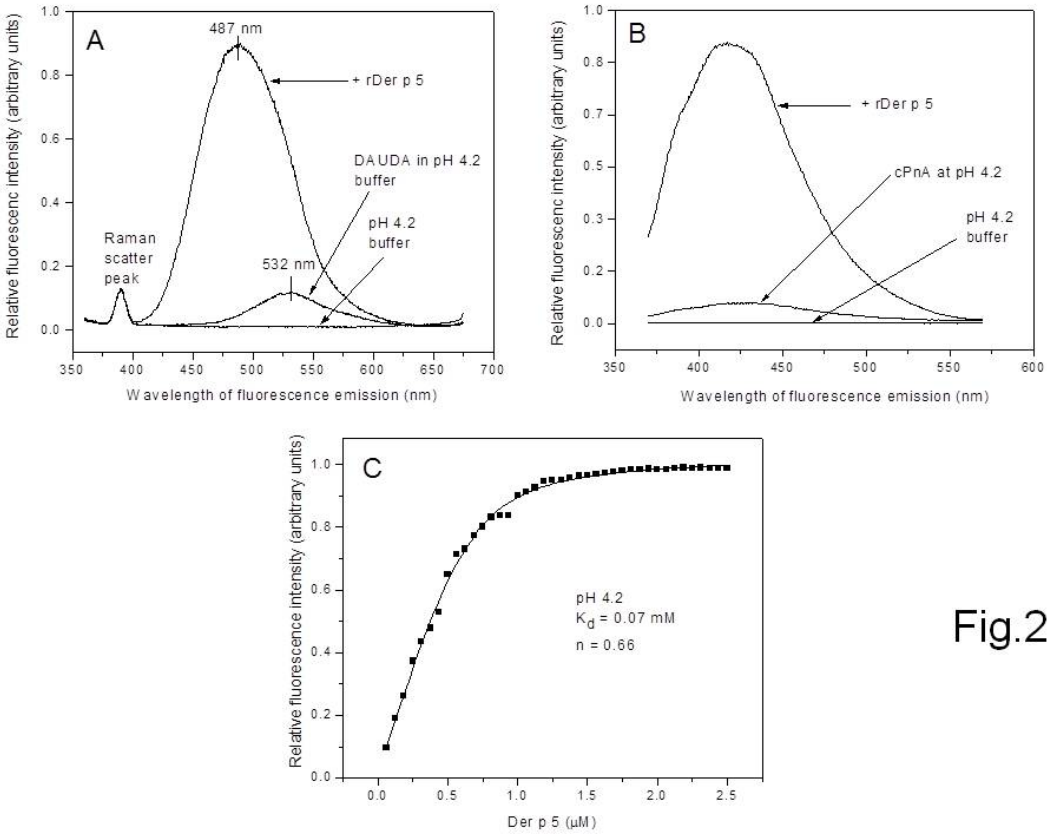


Fig.2

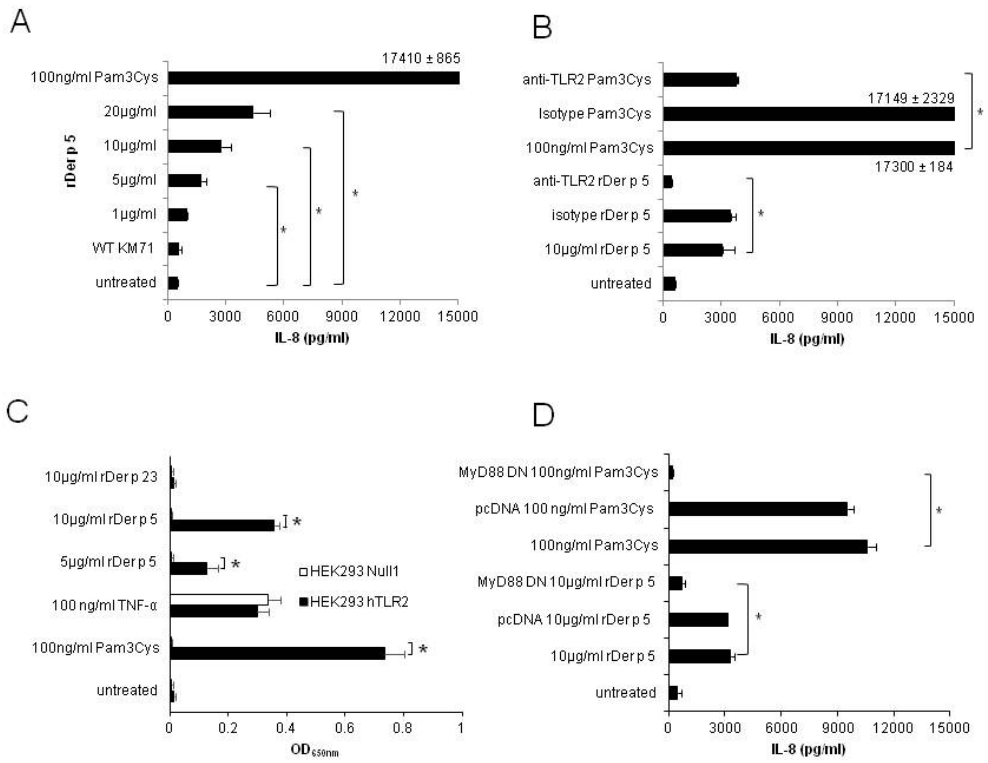


Fig.3

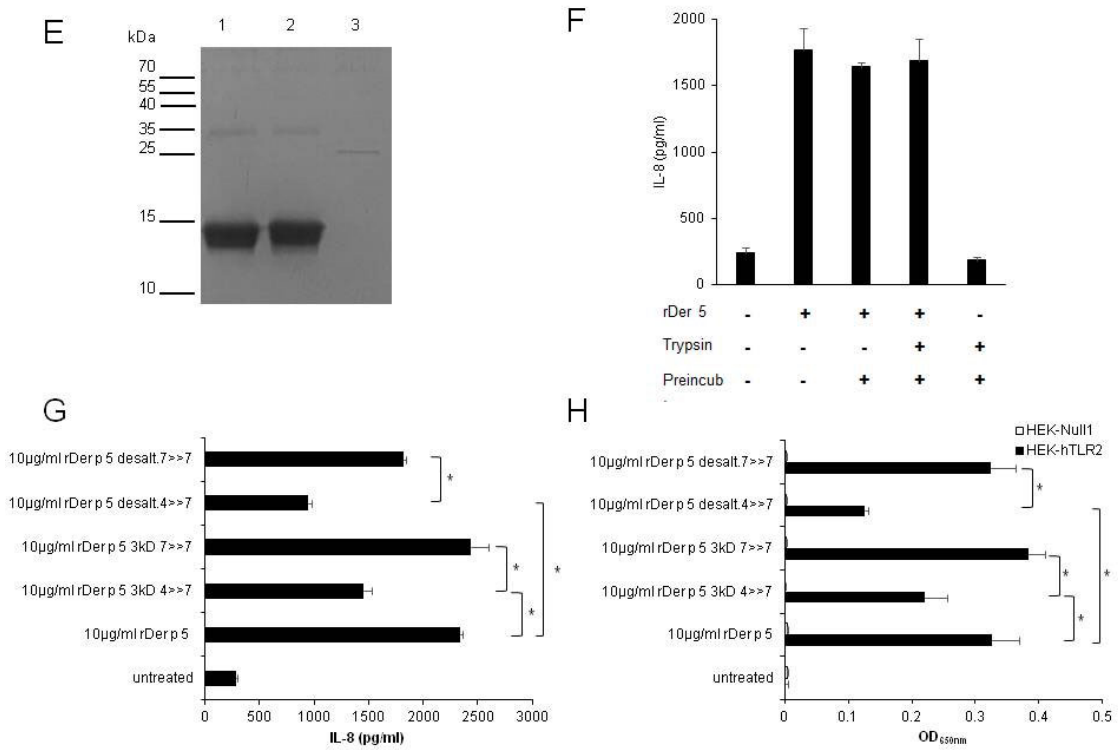


Fig.3

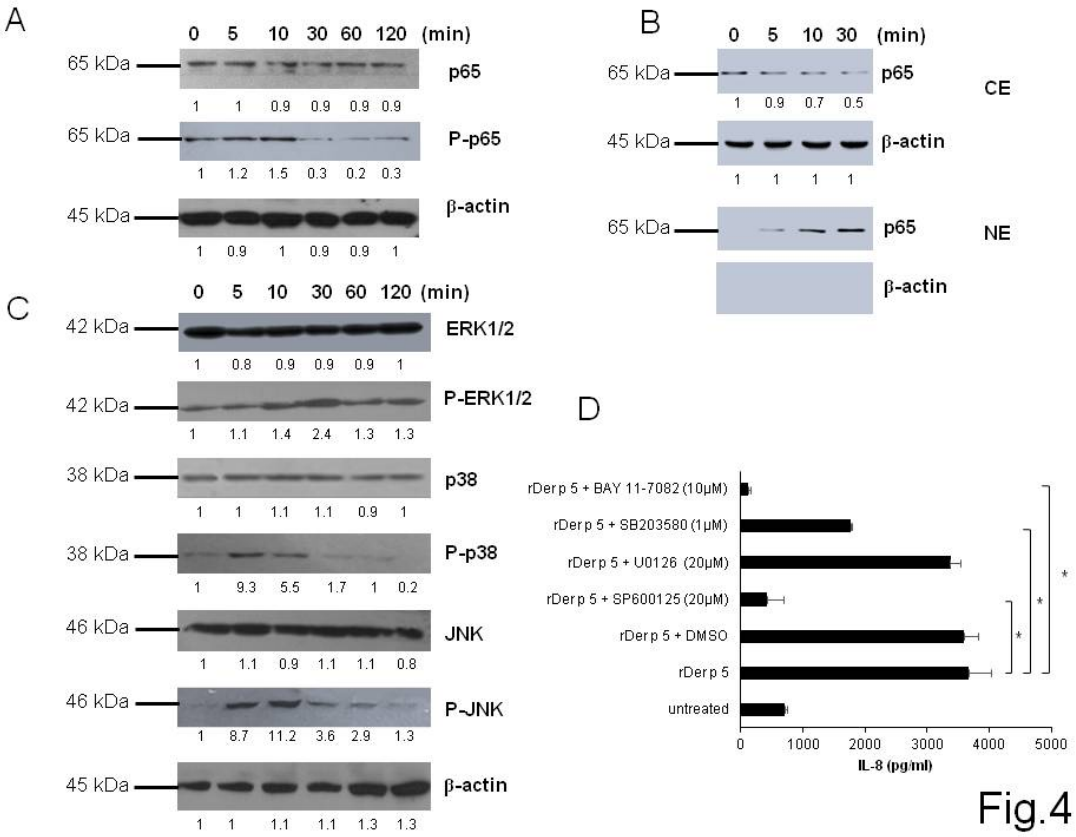


Fig.4