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1 **The minor house dust mite allergen Der p 13 is a fatty acid binding protein and an**
2 **activator of a TLR2-mediated innate immune response**

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32 **Running title: Allergenic determinants of house dust mite allergen Der p 13**

33

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45 binding protein, TLR2.

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47

48 **Abstract (N= 243)**

49

Background: The house dust mite (HDM) allergen Der p

70 **Introduction**

71 Sensitization to house dust mites (HDM) can trigger strong allergen-induced inflammation of
72 the skin and airway mucosa resulting in atopic dermatitis as well as allergic rhinitis and
73 asthma (1). In the most studied HDM species, *Dermatophagoides pteronyssinus* (Der p) and
74 *Dermatophagoides farinae* (Der f), protein allergens have been classified as being major,
75 intermediate, or minor allergens according to their IgE binding frequencies in HDM allergic
76 patient cohorts (2). On the basis of IgE binding frequency as a hypothetical criterion for
77 allergenicity, allergens from groups 1 and 2 have been proposed to be significant inducers of
78 IgE responses, unlike those in groups 4, 5, 7, 21 and 23, which are targets of low to
79 intermediate IgE binding frequencies (3). However, a growing literature points towards a
80 critical role for innate immune pathways in the initiation of HDM allergic responses. It has
81 consequently been proposed that a new allergen classification is warranted, based on their
82 propensities in activating innate responses (4). For instance, the HDM serine protease
83 allergens Der p/f 3, Der p/f 6 and Der p/f 9, despite having low IgE binding frequencies,
84 activate innate immune cells to initiate or prolong the HDM-induced allergy pathogenesis,
85 possibly through the protease-activated receptor 2 (PAR-2) (5).

86 Lipid binding capacity is another potential allergenic determinant commonly observed in
87 proteins triggering allergic responses, including Bet v 1-like molecules, non-specific lipid
88 transfer proteins (nLTP), and lipid-binding lipocalins (6). Lipids may also influence the early
89 stages of the allergic response through TLR4- and TLR2-dependent mechanisms. Several
90 HDM allergens, Der p/f 2, Der p/f 5, Der p/f 7, Der p/f 21 have been considered to be lipid-
91 binding proteins that may have allergenic potential, in cellular assays, or in-vivo experiments
92 using TLR4 and MD2-deficient mice (7-11). Group 13 mite allergens display clear sequence
93 similarities with cytoplasmic fatty acid binding proteins (FABPs) and consequently could
94 also be of importance in innate immune signaling through associations with mite or microbial

95 lipids (2). With the exception of the *B. tropicalis* allergen Blo t 13, which has been shown to
96 bind fatty acids (12), the capacity of other group 13 mite allergens to stimulate innate
97 immunity through their lipid cargo remains to be assessed. The goal of the present study was
98 to investigate a recombinant form of Der p 13 in terms of IgE reactivity, lipid binding
99 propensity, and airway epithelial cell activation.
100

101
102 **Materials and methods**

103 All the methods used are described in the online supporting information of this article.

104

105 **Results**

106 **Physico-chemical analysis of rDer p 13 and detection of natural Der p 13 (nDer p 13) in**
107 **mite bodies.**

108 **SDS-PAGE profiles of rDer p 13 were shown to be similar under both reducing**
109 **(Fig.1.A) and non-reducing conditions (data not shown) and confirmed that the product**
110 **was homogeneous and displayed the expected relative mobility (M_r) around 15kDa.**
111 **Dynamic light scattering (DLS) experiment confirmed that purified rDer p 13 is**
112 **monomeric, with no or only trace amounts of polymers or aggregates (data not shown).**
113 **NanoLC-MS analysis of the intact protein confirmed the expected mass at 15276.9 Da,**
114 **corresponding to the mature Der p 13 sequence (amino acids 2-131) with four extra N-**
115 **terminal amino acids derived from the expression vector (Glu-Ala-Val-Ala) (Fig. 1.B).**

116 Far UV circular dichroism (CD) analysis of recombinant (r)Der p 13 yielded a spectrum
117 typical of a folded protein with ²-sheet structure predominating (~~Supplementary~~ **Fig. 1.C**).
118 Estimates of the secondary structure content using CDpro software indicated 37.5% ²-sheet,
119 12.5% ±-helix, 19% turns and 31% unstructured. We also confirmed by immunoblotting that
120 mouse polyclonal antibodies to rDer p 13 react with the natural molecule present in
121 commercially-available mite bodies but not in mite faeces (Fig. 1.**D**), which is consistent with
122 Der p 13 belonging to the FABP family of proteins that are usually confined to cell cytoplasm
123 and not secreted (13).

124

125 **IgE reactivity to rDer p 13 and basophil degranulation assays**

126 Using 224 sera from HDM-allergic patients (Dpt ImmunoCap Class 3 or higher values)
127 suffering from allergic rhinitis or asthma, the IgE reactivity to rDer p 13 was determined by
128 sandwich ELISA and compared with the IgE binding capacity of rDer p 2. Fifteen out of 224

I suggest removing Fig 1A – the test is sufficient and theer is otherwise no extra need.

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No need to have this sentence.

residues

C-terminal of the alpha mating factor signal peptide (EAVA

Such incomplete signal sequence cleavage commonly occurs during recombinant protein secretion in (REF)

138 patients displayed specific IgE to rDer p 13 (7%), whereas 75% (169/224) of the same cohort
139 exhibited specific IgE to rDer p 2. Our data therefore confirm that Der p 13 is classifiable as a
140 minor allergen in that it is a target of IgE antibody in a low proportion of the HDM-allergic
141 population. There remains, however, the possibility that a complex of Der p 13 and its
142 resident lipid from HDM could be involved in allergic sensitization to itself and other HDM
143 allergens. We therefore ~~investigated~~ **compared the allergen activity of rDer p 2 and rDer p**
144 **13** ~~activity~~ in a rat basophil degranulation assay using the RBL SX-38 cells (rat basophil
145 leukemia cells expressing the human FcμRI receptor) preloaded with sera from patients
146 sensitized to **nDer p 2 or** nDer p 13 (14). **Regardless of the sera used, similar levels of cell**
147 **activation, were observed (P>0.5) by addition of polyclonal anti-human IgE (0.1 μg/ml),**
148 **causing around 50% of the total hexosaminidase release**. Cell incubation with rDer p 13
149 triggered allergen concentration-dependent basophil degranulation following a typical bell
150 shape curve, with a maximum reached with 0.01 μg/mL (Fig. 2). Using the same allergen
151 concentration, rDer p 2 triggered significantly higher levels of degranulation (P<0.05),
152 indicating that Der p 2 exhibits more allergen activity than Der p 13.

153

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cross-linking (P>0.5)
was achieved

154 **Lipid binding by Der p 13**

155 Lipid-binding assays were performed using different fluorophore-conjugated or intrinsically
156 fluorescent fatty acids (bodipy-C16, 11-([5-dimethylaminonaphthalene-1-sulfonylamino])
157 undecanoic acid (DAUDA), dansyl-DL-±-aminocaprylic acid (DACA), *cis*-parinaric acid
158 (cPnA) and dehydroergosterol (DHE)) as well as probes for apolar surfaces and binding
159 pockets (1-anilinonapthalene-8-sulfonate (ANS), bis-ANS). As shown in Fig. 3A, the
160 fluorescence emission of the natural, non-conjugated fluorescent fatty acid cPnA was
161 significantly enhanced when mixed with rDer p 13, indicating the entry of the fatty acid into

DAUDA
DACA
ANS,

a non-polar site. No binding by the fluorophore-conjugated fatty acids bodipy-C16, DAUDA or DACA was observed, although control experiments with a lipid-binding protein as positive controls showed binding as expected (bovine serum albumin; data not shown). Interactions between Der p 13 and ANS were observed, but only minimally with bis-ANS. Addition of oleic acid to pre-formed Der p 13:cPnA complexes failed to show any significant competitive displacement, whereas control experiments with cPnA and a well-characterized fatty acid binding protein, ^{2}-lactoglobulin, showed efficient dose-dependent competitive displacement (**Fig.3.B.**~~ data not shown~~). These results could be interpreted as meaning that lipid ligands bearing bulky fluorophore adducts (DAUDA, DACA and bodipy-C16) or molecules of relatively large diameters (bis-ANS) are excluded from Der p 13's binding site. The enhancement of cPnA's fluorescence emission when mixed with Der p 13 is indicative of entry to a binding pocket removed from polar solvent water. The relatively poor displacement of cPnA by oleic acid from that binding site is indicative of Der p 13's affinity for fatty acids, but selectively so. Fluorescence titration experiments showed that binding of cPNA to rDer p 13 was saturable with a dissociation constant K_d of 0.4 μM, which is typical of a lipid transporter protein (12), and with an apparent stoichiometry consistent with a 1:1 binding (**Fig.3.C** ~~B~~).

Modeling of the Der p 13 tertiary structure and its complex with cPNA

The analysis of the empirical structure of Der f 13 has previously shown that it, in common with other family 13 members, shares close structural similarities to cytoplasmic FABPs (15). FABPs adopt an apo form in the absence of ligand, lipid binding then inducing detectable conformational changes. In human muscle FABP, the main difference between *apo* and *holo* forms is the orientation of residue Phe57 (16), and other slight changes in the portal residues of the binding cavity involving Val25, Thr29, Lys58, Ala75 and Asp76. Interestingly, all these residues are conserved in Der p 13, with the exception of position 29, where there is a

Val. MODELLER 9.14 software (salilab.org) was used to predict the 3D structure of an *apo* form of Der p 13 based on the NMR structure of Der f 13 (PDB code 2A0A) (15) and of a *holo* form using as template the structure of myelin P2 protein in complex with ligand (PDB code 1YIV) (17) (Fig. 4.A and 4.B, respectively). Comparison of Figs. 4.A and 4.B shows an opening of the region around residue Phe57 in the model of the *holo* form. A central cavity that could accommodate an apolar ligand such as a fatty acid is present in both models, with an estimated volume of 590 Å^3 by CASTp (18). To investigate whether the highly conjugated fatty acid cPNA could theroretically interact with the hydrophobic pocket of Der p 13, the protein-lipid docking with both models was simulated using the AutodockVina software. The results show that cPNA cannot enter the cavity of the Der p 13 apo model in which the portal is obscured by Phe57, but could be ligated to the exterior surface of a small cavity which represents the portal of entry to the hydrophobic pocket. The computed Gibbs free energy of binding of cPNA at the protein surface is -5.2 kcal/mol, which corresponds to a binding affinity ($K_{dissociation}$) of about 250 µM, which is weak for a protein:ligand interaction. This inaccessibility of the fatty acid to the internal cavity is a consequence of the conformation change of the Phe57 residue in the static template apoFABP (16), which is probably not realistic. Assuming a similar mechanism in Der p 13, we docked cPNA in the model of the holo form, in which Phe57 is angled away from the presumed portal, obtaining a complex in which cPNA docks readily into the predicted hydrophobic pocket (Fig. 4.C). The computed Gibbs free energy in this case was -6.7 kcal/mol, which brings the theoretical $K_{dissociation}$ to about 12 µM. This value is closer to, but still weaker, than the value obtained from the empirical protein:cPnA titration analysis (see above). This suggests that cPNA binds preferentially in the cavity rather than at the surface of Der p 13, as expected in other FABPs (19, 20), and is consistent with the spectrofluorometric finding that cPnA's fluorescence

Fatty acids bound to FABPs usually orientate so that the carboxylates are positioned inside the cavity, tethered by amino acid side chains such as Arg and Tyr (through a water bridge) – does this apply in the docking simulations?

225 emission is enhanced to a degree expected for its removal from solvent water into an apolar
226 protein environment.
227

228 **Der p 13 activates airway epithelial cells through TLR2**

229 To investigate whether Der p 13 could activate the airway epithelium through
230 proinflammatory cytokine induction, human bronchial epithelial BEAS-2B cells were
231 incubated with different rDer p 13 concentrations **under serum-free conditions**, followed
232 by measurement of IL-8 and GM-CSF levels in culture supernatants. As a positive control,
233 cells were stimulated with the Pam$_3$Cys$_4$ TLR2 ligand. When compared with the control
234 medium, rDer p 13 elicited the production of IL-8 and GM-CSF in a concentration-dependent
235 manner (Fig. 5.A and B). Supernatants from methanol-induced wild-type KM71 cells that
236 had been treated as for the rDer p 13 purification protocol did not stimulate the release of
237 these cytokines, thereby assuring that the allergen-induced IL-8/GM-CSF expression was not
238 due to trace contaminants in the protein preparation.
239 Given the propensity of Der p 13 to transport specific lipids, we hypothesized that the Der p
240 13-induced cytokine release might be mediated by TLR2, this receptor being activated by
241 microbial lipoprotein/lipid ligands (21). As shown in Fig. 5C, preincubation of cells with
242 blocking anti-human TLR2 mAb drastically reduced the Der p 13-induced IL-8 secretion
243 from BEAS-2B cells. This TLR2 dependence was not replicated by the isotype control
244 antibody. **To confirm the specificity of such TLR2 activation, similar assays were**
245 **performed with purified recombinant allergens rProDer p 1 (REF) and rDer p 23**
246 **(REF), two proteins produced in that have known lipid binding activities.**
247 **Under the same experimental conditions, rProDer p 1 was unable to stimulate the**
248 **production of IL-8 (data not shown) whereas rDer p 23-induced cytokine production**
249 **was TLR2-independent (Fig.5.C).**

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Given that TLR2 engages with the MyD88 adaptor protein (22), the involvement of MyD88 in the rDer p 13-induced cell activation was also investigated using a dominant negative MyD88 expression plasmid (DN-MyD88) to down-regulate MyD88 activity. Treatment of BEAS-2B cells transfected with DN-MyD88 drastically reduced IL-8 secretion (Fig.5.D), but no effect was observed with the control plasmid.

Since NF-º B and MAP kinases are known to contribute to cytokine production in stimulated airway epithelium (23), we next examined the role of ERK, p38 and JNK and NF-kB activation on the production of IL-8 from BEAS-2B cells in response to rDer p 13, by using specific pharmacological inhibitors. The blockade of MAPK pathways through cell pretreatments with U0126 (MEK1/2 inhibitor), SB203580 (p38 MAPK inhibitor) or SP600125 (JNK inhibitor), respectively, reduced the IL-8 up-regulation caused by rDer p 13 (Fig. 5E). Similar reduction of IL-8 release was observed with IkB-± phosphorylation BAY-11-7082 and proteasome MG132 inhibitors (Fig.5.E), suggesting that the three MAPK signaling cascades and NF- B were essential for the IL-8 production by rDer p 13-stimulated BEAS-2B cells. **The observed inhibition of rDer p 13-induced IL-8 expression did not result from cytotoxicity of these inhibitors because the total number of cells and cell viability at the end of culture period for each experiment were similar among all culture conditions (data not shown).**

Finally, to investigate the potential involvement in rDer p 13-induced cell activation of lipid present in the allergen's apolar pocket, we measured the IL-8 production when BEAS2-B cells were treated with rDer p 13 that had been extensively digested with trypsin. Treatment with trypsin at 37°C for 1h (rDer p 13:trypsin ratio 20:1) degraded the recombinant allergen (Fig.5.F). Strikingly, such rDer p 13 hydrolyzate was shown to retain its capacity to trigger the IL-8 release in BEAS-2B cells (Fig.5.G). The cytokine production was not due to the presence of trypsin because digested Der p 13 was treated prior to cell exposure with

278 immobilized benzamidine, a matrix that traps and depletes this protease. The efficacy of

279 trypsin removal was confirmed by the absence of cell activation with trypsin preincubated

280 with the benzamidine matrix. These results suggest that **a** ~~the~~ lipidic ligand **of yeast origin**

281 **and transported by** ~~in~~ Der p 13 may be influential in the allergen's effect on the airway

282 epithelial cells.

283

from

285 **Discussion**

286

287 We previously demonstrated that the HDM allergens Der p 2 and Der p 23 triggered allergic
288 sensitization in a large percentage of Thai HDM-allergic patients with frequencies similar to
289 those measured in western countries (24). To determine whether the level of sensitization to
290 minor HDM allergens were similar among Thai and western populations, the present study
291 focused on Der p 13, a member of group 13 mite allergens that has been poorly investigated
292 to date. **To our knowledge, only one publication partially described the production of**
293 **rDer p 13 in bacteria and, moreover, the characterization was restricted to its IgE**
294 **reactivity (REF).** ~~Studies that surveyed IgE reactivities to~~ **R**ecombinant group 13 mite
295 allergens, including rBlo t 13, rTyr p 13, rAca s 13, rLep d 13 and rDer p 13 produced in *E.*
296 *coli*, ~~demonstrated that they typically~~ **were shown to display** a low prevalence of
297 sensitization in the HDM allergic population (25-29). IgE binding frequencies against Der p
298 13, Blo t 13, Tyr p 13, Lep d 13 and Aca s 13 reached 6%, 7-11%, 6%, 13% and 23%,
299 respectively. Because these data were generated using recombinant allergens produced in *E.*
300 *coli*, some of which were expressed as inclusion bodies, there is a possibility of
301 underrepresentation because of inappropriate folding. Assessments of recombinant allergen
302 folding are rarely performed, with the exception of the characterization of the Blo t 13
303 secondary structure (12) and the elucidation of the Der f 13 structure by NMR (15).

304 We produced Der p 13 in *P. pastoris* as a secreted protein, and CD analysis indicated that it
305 exhibits secondary structure content typical of members of the FABP family. Using this
306 recombinant allergen we found that, while the IgE binding frequency of rDer p 2 reached
307 75% (169/224 patients), reactivity to rDer p 13 was only 7% (15/224 patients) **in this thai**
308 **HDM allergic population but reached around 20% in HDM allergic patients living in**
309 **other areas (REF).** Such low frequency of reactivity to Der p 13, as with other members of

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317 group 13 mite allergens (25-29), may be due to the fact that cytoplasmic FABP allergens are
318 restricted to mite bodies and not present in the faeces (3). Whereas fragmented mite body
319 parts together with fecal pellets represent the main allergenic source, the deep penetration of
320 particles with allergen cargo into the lung must be size-dependent (30). Consequently, the
321 HDM allergens transported within mite fecal pellets (10 µm average diameter) should trigger
322 airway inflammation more readily than mite body parts. To support this hypothesis, it has
323 recently been demonstrated that HDM allergens detectable only in mite bodies display weak
324 IgE reactivity in sensitized population with respiratory symptoms, but represent major
325 allergens in patients suffering from atopic dermatitis (31). The HDM allergics in our cohort
326 suffered only from allergic rhinitis or asthma, so it would be interesting to determine whether
327 HDM allergics with atopic dermatitis have similar or higher rates of IgE to Der p 13.

328 It is widely accepted that HDM allergies may be initiated through activation of innate
329 immunity (4), such that any mite component capable of stimulating innate immune signaling
330 could be influential. Pertinent to the role of specific lipids in immune activation, we
331 demonstrated, using environment-sensitive fluorescent lipid probes, that rDer p 13 binds fatty
332 acids and that the protein's binding to hydrophobic ligands is selective. **Indeed, although**
333 **Der p 13 and Blo t 13 share 80% amino acid sequence identity and display similar K_d**
334 **values for cPNA (0.4 10^{-6} M versus 1.31 10^{-6} M, respectively), displacement of cPNA**
335 **with oleic acid was ineffective for Der p 13, unlike with Blo t 13 (ref.). This may**
336 **therefore mean that, despite their sequence and structural similarities, the precise**
337 **binding propensities of the two proteins differ.**

338 Computer-based docking experiments performed with two different models of Der p 13 (one
339 based on the *apo* form of Der f 13, (15) the other based on the *holo* form of myelin P2
340 protein, (17)) with the lipid ligand cPnA known to bind to the protein, predicted that the
341 accessibility of the hydrophobic pocket could be controlled by the side chain orientation of

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'homology' is a frequently used but erroneous term here.

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The tighter binding observed with cPNA could suggest that this highly conjugated fatty acid is more representative of the Der p 13 natural ligand.

353 Phe57. This residue is located immediately beside the portal of entry to the binding pocket of
354 some FABPs (16). A similarly-positioned side chain is found in several FABPs (32), where it
355 is speculated to regulate the entry of ligand to the proteins' binding pockets (20). The
356 identification of the lipid ligand(s) naturally present in the Der p 13 hydrophobic pocket
357 remains to be determined. Der p 13, like all FABPs other than those of nematodes (33), lacks
358 a leader sequence, and is therefore probably confined to the cytosol of mite cells. The lipids it
359 may present are therefore likely to be cytoplasmic lipids, though we cannot exclude the
360 possibility that Der p 13 transports lipid ligands from endosymbiotic bacteria or microbes in
361 house dust.

362 Based on the finding that Der p 13 binds fatty acids, we hypothesized that this allergen could
363 activate TLR2 signaling in airway epithelium. This pathogen-associated molecular pattern
364 receptor forms a heterodimer with either TLR1 or TLR6 and interacts with lipids/fatty acids
365 or lipoprotein (34). The activation of TLR4 in combination with MD2 and CD14 (another
366 receptor recognizing microbial lipidic ligands) by Der p 13 was not evaluated because the
367 BEAS-2B airway epithelial cells were found to be hyporesponsive to the LPS needed as a
368 positive control (data not shown). Indeed, such cells were shown to express MD2 poorly
369 whereas intracellular localization of TLR4 is nevertheless apparent (35).

370 To determine whether rDer p 13 enhances innate responses through TLR2, proinflammatory
371 cytokine production was measured from stimulated BEAS 2B. We focused on both IL-8 and
372 GM-CSF secretion because these cytokines are important chemoattractants and activators for
373 immune cells such as neutrophils, basophils, eosinophils and dendritic cells (36). Our results
374 showed that rDer p 13 stimulated the production of IL-8 and GM-CSF by airway epithelial
375 cells in a time- and concentration-dependent manner. Through a combination of blocking
376 antibodies, specific inhibitors, and depletion of MyD88, we found that Der p 13 triggers
377 airway epithelial cell activation through TLR2-MyD88-NF- B and MAPK-dependent

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mechanisms. Strikingly, this cell activation was shown to be independent of the persistence of intact Der p 13, thereby implicating the protein's lipid cargo. This result supported the hypothesis that Der p 13 facilitates the transfer of immunomodulatory fatty acid/lipid to TLR2 or to a TLR2 co-receptor such as CD14 or CD36 to trigger innate immune signaling.

To our knowledge, this is the first study to reveal the allergenic propensity of a group 13 mite allergen as well as its potential mechanism of action. Together with group 2 allergens and Der p 21 (37, 11), Der p 13 is the third TLR2 stimulator to be identified in HDM. Notably, the presence of hydrophobic cavities in the Der p 5 dimer and Der p 7 structures that potentially bind apolar ligands suggests that HDM allergens transporting lipid cargo could act either synergistically or in a redundant fashion to stimulate TLR2 signaling (9, 10). Immunomodulatory activities exhibited by Der p 5 and Der p 7, however, await direct experimentation. Although it was demonstrated initially that TLR2 ligands reduced Th2-biased allergic responses (38), recent studies indicated that TLR2 signaling could be critical for the development of HDM allergic rhinitis and asthma (39-42). In that context, Der p 13 could represent an important factor in the initiation of the HDM allergic response because TLR2 engagement led to the activation of the epithelial NF- B, which comprises an orchestrator of the HDM-induced airway inflammation, hyperresponsiveness, and fibrotic remodeling (43).

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In conclusion, Der p 13, through its ability to bind lipid and trigger TLR2-dependent innate immune signaling, must be considered as a potential contributor to the induction of the HDM allergic response. **Although Der p 13 appears strictly to be confined within fragmented mite bodies, we speculate that TLR2 activation could occur following deposition of mite fragments onto the lung surface with consequent allergen release. It must be**

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by the unknown action of a dissolving media in the lung the epithelial lining fluid.

423 **pointed out that Der p 2 is also mainly present in mite bodies yet is a major allergen**
424 **able to activate TLR2 and TLR4 (REF).** Because the lipid environment in the mite, house
425 dust or the *P. pastoris* yeast used to produce the allergen may differ considerably, it is
426 important ultimately to identify the natural Der p 13 ligand(s) and to characterize its effects
427 on immune cells. Also, the lipid transfer mechanism involved in the Der p 13-mediated
428 enhancement of TLR2 signaling remains unclear, although FABPs of the sub-family to which
429 Der p 13 belongs are known to interact directly with membranes in the transfer of lipid cargo
430 (44). Nevertheless, our results demonstrate that the HDM allergen hierarchy, based
431 essentially on IgE reactivities, needs further refinement in order to take into account the
432 capacity of allergens to stimulate innate immunity. Consequently, minor HDM allergens such
433 as Der p 13 require further consideration in order to elucidate their abilities to activate airway
434 epithelial cells as well as keratinocytes.
435

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4. Phramongkutklao College of Medicine: Tadech Boonpiyathad

Conflict of interest

Authors declare there are not conflicts of interests.

Author contributions

A.J. designed the study; S.P. performed the experiments in collaboration with D.G., Su. Pi. and E.N.; M.L.M. performed mass spectrometry experiments; J.W. helped with ImmunoCap assays, N.S., P.C., M.V., T.R., A.S. and K.R. helped in the sera collection. A.J., E.N., D.G.

and M.W.K. provided supervision and analysed the data for the spectrofluorometric analysis carried out in Glasgow. S.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.

Figure legends

See notes added to the PowerPoint slides

Figure 1. Detection of nDer p 13 in HDM allergen extracts from

Immunoblot detection of nDer p 13 using anti-rDer p 13 mouse polyclonal antibodies. Lane 1: *D. pteronyssinus* fecal pellet extract, Lane 2: *D. pteronyssinus* mite bodies extract (Stallergenes Greer), Lane 3: purified rDer p 13.

Figure 2. RBL-SX38 cell degranulation by rDer p 13 and rDer p 2.

Cells were primed for 16 h with sera from three HDM allergic patients, containing rDer p 13- (Panel A) or rDer p 2-specific IgE (Panel B).and subsequently stimulated with serial dilutions of purified rDer p 13 or rDer p 2 for 30 min. Degranulation was measured through ²-hexasominidases activity. Percentage of degranulation was presented as subtraction of spontaneous released over total lysis with Triton X-100.

Figure 3. Hydrophobic ligand binding activity of rDer p 13.

Panel A: Fluorescence emission spectra of cis-parinaric acid (cPnA, Ex_{max} = 319nm) bound to purified rDer p 13. The competitive binding of oleic acid used at different concentrations (7.9µM, 79µM, 790µM) is also shown. Curve A: PBS, curve B: cPnA alone, curve C: cPNA + rDer p 13, curve D: cPNA:rDer p 13 complex + 7.9 µM oleic acid, curve E: cPNA:rDer p 13 complex + 79 µM oleic acid, curve F: cPNA:rDer p 13 complex + 790 µM oleic acid. RFI = relative fluorescence intensity. One representative experiment out of 3 is shown.

Panel B. Titration curve of cPnA binding to rDer p 13.#Change in relative fluorescence intensity of cPNA following addition of increasing rDer p 13 concentrations. The solid line represents the theoretical binding curve for a allergen:ligand complex formation with a K_d of

492 0.4μM and and an apparent stoichiometry consistent with one binding site per protein
493 monomer unit.
494
495 **Figure 4. Structural model of Der p 13**
496 Panel A: model of the apo form of Der p 13 based on the NMR structure of Der f 13 (PDB
497 code 2A0A). Residue 57 is in yellow. Panel B: model of Der p 13 structure based on the X-
498 ray structure of myelin P2 protein from equine spinal cord (PDB code 1YIV). Residue 57 is
499 in light pink. Panel C: surface representation of the structure of the complex between the
500 model of the Der p 13 holo form (in purple) and cPNA (in red sticks), obtained by docking
501 simulations.
502
503 **Figure 5. rDer p 13 activates airway epithelial cells through TLR2 signaling pathway**
504 Stimulation of BEAS-2B cells by rDer p 13 triggers IL-8 (Panel A) and GM-CSF (Panel B)
505 cytokine production in a concentration dependent manner. BMMY medium from cultured
506 wild-type P.pastoris and purified according to the purification protocol of rDer p 13 (WT
507 KM71) as well as Pam_3Cys were used as negative and positive controls respectively. To
508 assess the importance of TLR2 signaling in the rDer p 13-induced activation, cells were also
509 preincubated with blocking anti-TLR2 antibody or isotype control (Panel C) prior to allergen
510 treatment. In another set of experiments,cells were transfected with dominant negative
511 MyD88 or control plasmid followed by the rDer p 13 stimulation (Panel D). BEAS-2B cells
512 were preincubated with SP600125, SB203580, U0126 (MAPK pathway inhibitors) or
513 MG132, BAY 11-7082 (NF-°B pathway inhibitors) before treatment with rDer p 13 (Panel
514 E). rDer p 13 was extensively digested with Trypsin at 37C (Panel F, lane 3). As control,
515 undigested rDer p 13 (lane 1) was also preincubated at 37C (lane 2). Prior to cell activation
516 by the different rDer p 13 forms (Panel G), trypsin was removed using immobilized

benzamidine matrix. To control the removal of trypsin, cells were also activated with trypsin alone which was previously applied onto the same benzamidine beads. Data show mean cytokine concentrations + SEM and are representative of three independent experiments. ** P< 0.05, *** P<0.001.

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