Full Report

Allergenicity of house dust mite allergens in Thai population

RSA. 5680045

Assoc Prof. Alain Jacquet Chulalongkorn University

From 17/06/2013 to 31/12/2015

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TRF Grant and Chulalongkorn University Grant

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รูปแบบ Abstract (บทคัดย่อ)

Project Code : RSA. 5680045

Project Title : Allergenicity of house dust mite allergens in Thai population

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Project Period : 17/6/2013-31/12/2015

Whereas a large amount of studies examined the sensitization profile to HDM allergens in western cohort of allergic patients (children as well as adults), a deep characterization of the prevalence of House Dust Mite (HDM) allergy in Thailand remained to be elucidated, mainly because the diagnosis is performed through Skin Prick Test (SPT) and HDM extracts. The aim of our study was to evaluate the prevalence of the IgE reactivity to a set of purified recombinant HDM allergens in a cohort of HDM allergic subjects from different regions in Thailand in order to determine the first component-resolved diagnosis of HDM allergy in Thailand. The recombinant HDM allergens Der p 1, Der p 2, Der p 5, Der p 7, Der p 13, Der p 21 and Der p 23 were produced in Pichia pastoris whereas Der p 10 was expressed in bacteria. Together with these allergen productions, we set collaboration with different hospitals from Bangkok to screen by skin prick test the patients sensitized to HDM allergens and to collect sera from this population for the detection of HDM allergen-specific IgE. The demographic data related to these populations were collected and analized by the clinicians from the Department of Allergy, Chulalongkorn Hospital. ELISA assays were optimized to detect the presence in each serum of specific IgE directed to the different purified recombinant HDM allergens. To set a threshold of positivity, ELISA assays were conducted with negative sera. Our results showed that the pattern of sensitivity to HDM allergens in thai HDM allergic population was similar to the ones from Europe/USA: the highest level of IgE reactivities were against Der p 1, Der p 2 and Der p 23, followed by those to Der p 5, Der p 7 and Der p 21. The IgE binding frequencies of Der p 10 and Der p 13 were very low confirming these two allergens triggered poor levels of sensitizations. Our results confirmed that component-resolved diagnosis of HDM allergy in Thailand would greatly improve the design of tailor-made specific immunotherapies to treat HDM allergy.

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ถึงแม้ว่าจะมีการศึกษาหลายงานเกี่ยวกับรูปแบบการทำให้เกิดภูมิแพ้ในสารก่อภูมิแพ้ไรฝุ่นของคนไข้ที่มีอาการแพ้ในประเทศแถบตะวันตก (ทั้งในคนไข้เด็กและผู้ใหญ่) แต่กระนั้นการศึกษาลักษณะพิเศษแบบเจาะลึกถึงความชุกของโรคภูมิแพ้ไรฝุ่นในประเทศไทยยังคงไม่ชัดเจน เนื่องจากการวินิจฉัยโรคนี้มาจากการทดสอบภูมิแพ้ทางผิวหนังและสารสกัดไรฝุ่นแบบสกัดรวมเพียงเท่านั้น จุดประสงค์ของงานวิจัยครั้งนี้คือ การประเมินความชุกของโรคภูมิแพ้ไรฝุ่นต่อชุดของสารก่อภูมิแพ้ไรฝุ่นในรูปแบบของรีคอมบิแน้นทโปรตีนสกัดบริสุทธิ์ต่างๆ ในกลุ่มคนไข้ที่แพ้ไรฝุ่นจากต่างภูมิภาคในประเทศไทย เพื่อตรวจสอบการวินิจฉัยโรคโดยใช้ส่วนประกอบของสารก่อภูมิแพ้เป็นรายแรกในประเทศไทย รีคอมบิเน้นท์โปรตีนสกัดบริสุทธิ์ของสารก่อภูมิแพ้ไรฝุ่นชนิด Der p 1, Der p 2, Der p 5, Der p 7, Der p 13, Der p 21, และ Der p 23 ถูกผลิตในยีสต์ Pichia pastoris ในขณะที่ Der p 10 ถูกผลิตในแบคทีเรีย สารก่อภูมิแพ้ทั้งหมดนี้ได้ถูกผลิตและนำไปทดสอบภายใต้ความร่วมมือของโรงพยาบาลต่างๆในกรุงเทพ เพื่อคัดแยกการทดสอบภูมิแพ้ทางผิวหนังในคนไข้มีประวัติการแพ้ไรฝุ่น และจัดเก็บเซรั่มจากคนไข้กลุ่มนี้เพื่อการตรวจดู IgE จำเพาะต่อสารก่อภูมิแพ้ไรฝุ่น ข้อมูลทางสถิติที่เกี่ยวข้องกับคนไข้กลุ่มนี้ได้ถูกจัดเก็บและวิเคราะห์โดยผู้เชี่ยวชาญทางคลีนิกจากภาควิชาภูมิแพ้ โรงพยาบาลจุฬาลงกรณ์ เทคนิคการตรวจหาความจำเพาะเจาะจงของสารก่อภูมิแพ้โดยใช้แอนติบอดี้(ELISA) ได้ถูกปรับให้สามารถตรวจพบ IgE จำเพาะต่อรีคอมบิแนนท์โปรตีนสกัดบริสุทธิ์ของสารก่อภูมิแพ้ไรฝุ่นชนิดต่างๆ เพื่อการหาค่าประเมินความเสี่ยงในการแพ้ไรฝุ่น เทคนิคการตรวจหาความจำเพาะเจาะจงของสารก่อภูมิแพ้โดยใช้แอนติบอดี้ ได้ถูกคำนวณโดยค่าจากเซรั่มในคนไข้ที่ไม่แพ้ไรฝุ่น ผลการศึกษาได้ชี้ให้เห็นว่ารูปแบบการแพ้สารก่อภูมิแพ้ไรฝุ่นในคนไข้ในประเทศไทยเหมือนกับผลการศึกษาในคนไข้จากประเทศในแถบยุโรปและอเมริกา โดยค่าจำเพาะต่อ IgE ระดับสูงที่สุดพบใน Der p 1, Der p 2, และ Der p 23 รองลงมาคือ Der p 5, Der p 7, และ Der p 21 และระดับต่ำพบใน Der p 10 และ Der p 13 นอกจากนี้ผลการศึกษายังได้รับรองว่า การวินิจฉัยโรคภูมิแพ้ไรฝุ่นด้วยส่วนประกอบของสารก่อภูมิแพ้ในประเทศไทยนี้ จะสามารถส่งเสริมและสนับสนุนให้การออกแบบวิธีการรักษาด้วยภูมิคุ้มกันบำบัดในภูมิแพ้ไรฝุ่นมีความสำคัญและพัฒนามากยิ่งขึ้น

Keywords : จำนวน 3-5 คำ House dust mite, allergen, IgE, ELISA, diagnosis

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Research Content

1)Introduction

Sensitizations to House dust mite (HDM) allergens commonly trigger allergic diseases as allergic asthma, rhinitis and dermatitis through the development of exarcerbated allergen-specific Th2 immune responses characterized notably by the production of HDM allergen-specific IgE (1). The prevalence of HDM allergy is highly spread in the industrialized countries, affecting more than 20% of the population. And such level of sensitization is also reached in Thailand (2-6).

Together with a case history, the diagnosis of allergy is commonly determined by the sensitization profile to allergens through the detection of allergen-specific IgE in *in-vitro* ImmunoCap assays (solid phase) or *in-vivo* skin prick tests (7). But even whether such tests were first performed with standardized HDM allergen extracts, the accurate identification of the respective HDM allergens responsible for the allergic sensitizations was up to now impossible using HDM allergen extracts. Indeed, such allergenic sources are poorly defined, containing the allergens but also non-allergenic mite proteins (8). Moreover, it was clearly evidenced that, although more than 20 HDM allergens were identified and cloned, such allergenic proteins are not all present in any HDM allergen extracts but their concentrations can greatly differ with the source of the extracts (9,10). Thanks to the isolation of the natural allergens together with the production of highly purified recombinant forms of these molecules, the component-resolved diagnosis, i.e. the contribution of each individual allergen in the allergic response, tremendously improved the characterization of HDM sensitization and consequently, the diagnosis of HDM allergy (11).

Whereas a large amount of studies examined the sensitization profile to HDM allergens in western cohort of allergic patients (children as well as adults) (12,13), a deep characterization of the prevalence of HDM allergy in Thailand remained to be elucidated, mainly because the diagnosis is performed through Skin Prick Tests (SPT) using total HDM allergen extracts. It must be pointed out that the detection of HDM-specific IgE in plasma/sera by ImmunoCap is not systematic analysis performed in any clinical laboratories from hospitals in Thailand. The most abundant mite species in Thailand was shown to be *D.pteronyssius* and SPT clearly established that HDM is the major sensitizer in thai allergic patients.

2) Goal of the project

The aim of our study was to evaluate the prevalence of the IgE reactivity to a series of well characterized recombinant HDM allergens in a cohort of HDM allergic subjects from different regions in Thailand. Such component-resolved diagnosis of HDM allergy in Thailand would

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greatly improve the design of tailor-made immunotherapeutic treatment (personalized medicine) of HDM allergy through the unique selection of HDM allergens inducing sensitizations in any specific patients.

3) Methods

-) Production and purification of recombinant HDM allergens

Recombinant *Pichia pastoris* yeast strains expressing rDer p 1, rDer p 2, rDer p 5, rDer p 7, rDer p 13, rDer p 21 and rDer p 23 were cultured in glycerol-based medium to reach an appropriate cell density. The expression of the different HDM recombinant allergens was induced by the replacement of glycerol-based medium with methanol-based medium to activate the promoter of alcohol oxidase to drive protein expression. For each individual allergen production, the following parameters were optimized to obtain the highest protein expression level: the yeast strain, the culture density during the induction, the methanol concentration and the duration of the induction. The yeast supernatant, containing the individual secreted HDM allergens were collected by centrifugation, diluted to reduce the salt concentration and subsequently applied onto a S sepharose ion-exchange matrix equilibrated in a sodium acetate buffer pH4. The column was extensively washed with the equilibrating buffer to remove unbound materials. Protein elution proceeded by addition of increasing concentrations of NaCl in the buffer. The fractions containing the individual allergens were analyzed by SDS-PAGE after coomassie blue staining. These materials were pooled, concentrated by ultrafiltration. The purification of each individual allergen was achieved by the loading of the concentrated sample onto a Superdex 75 gel filtration column equilibrated in PBS. Fractions containing purified allergens were pooled, assayed for protein concentration and aliquoted and stored at -20C.

Recombinant Der p 10 was expressed as a (His)6-tagged molecule in *E. coli* and then purified following bacteria sonication by using a Ni2+ sepharose high performance column (GE Healthcare) through elution with increasing imidazole concentrations.

-)Quality control of purified HDM recombinant allergens

Each recombinant HDM allergens were characterized by using the following techniques:

SDS-PAGE under reducing and non-reducing conditions, MS analysis of the intact protein, MS/MS analysis of tryptic peptides, protein stability under different storage temperatures, Circular dichroism for the estimation of the secondary structure contents.

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-)Sera collection

To set a cohort of HDM allergic patients, several hospitals from Bangkok were contacted to select some of their HDM allergic patients with a defined case history to agree to participate to the present project:

The King Chulalongkorn Memorial, Children, Ramathibodi and Phramongkutklao hospitals respectively. Moreover, two open days were also organized by the King Chulalongkorn Memorial hospital to measure HDM sensitization through SPT assays in any person attending the meeting. Each participant filled as well a questionnaire related to any allergy previously diagnosed as well as the clinical manifestations of such allergies. Written informed consent was obtained for each case. The study was approved by the Ethic Committees from the Faculty of Medicine, Chulalongkorn University (IRB 023/55), Children hospital (IRB 195/2556), Faculty of Medicine, Ramathibodi hospital, Mahidol University (IRB 03-56-34) and Phramongkutklao College of Medicine (IRB S039Q/57_EXP). In total, 660 patients diagnosed by SPT were initially considered as HDM allergic positive. As negative controls, a cohort of 67 non-allergic subjects (HDM SPT negative) were also enrolled in the study.

Serum sample from each patient was obtained through blood collection, aliquoted and stored at -80C until further analysis.

-)ELISA assays for the detection of specific IgE

For the respective IgE binding assays, ELISA microplates were coated with 500 ng/well of rDer p 1, rDer p 2, rDer p 5, rDer p 7, rDer p 10, rDer p 21 or rDer p 23 at 4°C for overnight. The plates were then washed with PBS-Tween 20, 0.05% (PBS-T) and blocked with PBS-T containing 1% BSA (PBS-T-BSA) for 1 h at 37°C. Serum samples were diluted at 1/8 in PBS-T-BSA and incubated at 37°C for 1 h. The plates were then washed again with PBS-T and further incubated with 1/5000 dilution of goat biotinylated anti-human IgE at 37°C for 1 h. Next, the plates were washed with PBS-T and incubated with 1/2000 dilution of streptavidin-peroxidase for 1 hour at room temperature. The allergen-antibody complex was detected with TMB substrate and the reaction was stopped with 0.5 M sulfuric acid. Optical density (OD) was determined at 450 nm using iMark microplate reader. A patient serum was considered as positive when the measured OD value was higher than the cut off value, established as the mean OD values of negative control sera plus 2 standard deviations.

For the detection of anti-Der p 13 specific IgE, ELISA microplates were coated with goat anti-human IgE (1/1000 dilution) at 4°C for overnight. The plates were then washed with PBS-Tween 20, 0.05% (PBS-T) and blocked with PBS-T containing 1% BSA (PBS-T-BSA) for 1 h at 37°C. Serum samples were diluted at 1/8 in PBS-T-BSA and incubated at 37°C for 1 h. The plates were then washed again with PBS-T and further incubated with rDer p13 (500

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ng/mL) at 37°C for 1 h. Following another typical washing step, the wells were incubated with purified anti-rDer p 13 mouse polyclonal antibodies (these antibodies were generated in our laboratory through mice immunization with purified rDer p 13, 3.5 ng/well) at 37 C for 1h. Next, the plates were washed with PBS-T and incubated with goat HRP-conjugated anti-mouse 1/5000 dilution for 1 hour at room temperature. The allergen-antibody complex was detected with TMB substrate and the reaction was stopped with 0.5 M sulfuric acid. Optical density (OD) was determined at 450 nm using iMark microplate reader

4)Results and Discussion

-)Characterization of purified recombinant HDM allergens

SDS-PAGE analyses confirmed that all purified recombinant HDM allergens exhibited the expected size, with a purity >80%, as measured with the Image Quant TL software (GE Healthcare) (Fig.1). The identity of each allergen was confirmed by liquid chromatography combined with tandem mass spectrometry analyses (LC-MS/MS). The circular dichroism analysis further confirmed that the different HDM allergens were correctly folded as their corresponding secondary structure contents were similar to the predicted ones (for rDer p 10, rDer p 13 and rDer p 21) or the ones estimated thanks to their crystallographic structures (for rDer p 1, rDer p 2, rDer p 5, rDer p 7 and rDer p 23).

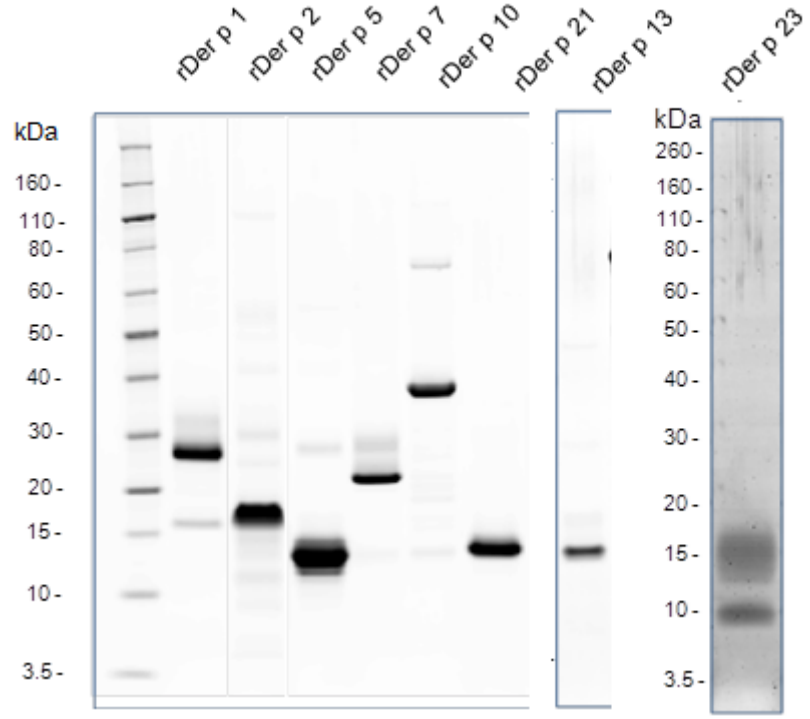

Fig.1: SDS PAGE analysis of purified allergens used in the different ELISA assays.

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Purified recombinant HDM allergens were obtained as described in methods. Following SDS-PAGE, under reducing conditions, proteins were stained with Sypro Ruby. The purity of the allergens ranged from 80 to 100%. MS analysis of Der p 23 confirmed that the 2 bands are related to the allergen.

-)Selection of the HDM allergic cohort

As described in the Methods section, 660 patients were diagnosed with HDM allergy through a questionnaire (declaring allergic manifestations as allergic rhinitis or asthma) as well as a SPT test. The positivity threshold commonly adopted was characterized with a being a wheal of ≥ 3 mm. Ideally, the diagnosis of HDM allergy is further confirmed through the detection of allergen-specific IgE by ImmunoCap analysis. Such analysis was conducted at Chulalongkorn University (Center of Excellence in Immunology and Immune Mediated Diseases, Division of Immunology, Department of Microbiology, Faculty of Medicine) including the 67 sera considered as SPT "negative".

According to the ImmunoCap guidelines, we can consider 6 levels of IgE quantification (Fig.2), considering that 1 kU/L specific IgE is equal to 2.4 ng/mL

Class	Specific IgE (kUA/L)	Level	Clinical Correlation
0	<0.35	Undetectable	Consider non-allergic causes
1	0.35-0.69	Low	Uncertain clinical relevance; weak IgE antibody response may be a risk factor for future sensitization
2	0.70-3.49	Moderate	Probably a contributing factor to total allergic load
3	3.50-17.49	High	Clinically relevant
4	17.50-49.99	Very High	Highly clinically relevant
5	50.00-100.0	Very High	Highly clinically relevant
6	>100	Very High	Highly clinically relevant

Fig.2. ImmunoCap rating defining the different level of specific IgE concentration in an atopic patient.

As expected the 67 SPT negative sera were ImmunoCap negative and belonged to the Class 0 (Specific HDM IgE concentration <0.35 kU/L). The ImmunoCap results obtained with the 660 SPT positive sera evidenced some false positive sera (SPT positive but ImmunoCap negative) (Fig.3). Very surprisingly, the highest level of false positive sera was detected in the sera collected from the open days (140/365), suggesting clearly that the use of sera collected

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from cohort of patients registered to hospitals is much preferable for our study. Moreover, amongst the 185 positive sera from this cohort, the positivity to HDM allergen extracts was quite low as the vast majority of the sera were from Classes 1 and 2 (< 3.50 kU/L). As only the sera from Class III to 6 are clinically relevant of HDM allergy (Fig.2), we decided not to include the sera obtained from the open door days and to select the sera from the “hospital” cohorts with positive ImmunoCap value higher than 3.5 kU/L.

Centre	N	False Positive (Class 0)	True Positive Class 3 to 6 (>3.5 kU/L)
Chulalongkorn	96	5	**77**
Phramongkutklao	14	0	**10**
Ramathibodi	99	9	**60**
Children Hospital	86	5	**70**
Negative Sera	67	True Negative: 67	

Fig.3 Number of False positive sera and positive sera with Class 3-6 ImmunoCap values from the different cohorts.

It must be pointed that the demographic data of our cohorts which includes also the history case as well as the type of clinical allergic manifestations are under analysis by the Department of Allergy which centralized all the files from the patients.

-)IgE reactivity to the individual recombinant HDM allergens.

Following the different ELISA assays performed with the 217 positive sera from ImmunoCap Class 3 to 6 (see Fig.3), the pattern of sensitization can be summarized through the Table 4

	Chulalongkorn	Phramongkutklao	Ramathibodi	Children Hospita
rDer p 1	58/77 = 75%	8/10 = 80%	30/60 = 50%	55/70 = 78%
rDer p 2	64/77 = 83%	7/10 = 70%	46/60 = 77%	51/70 = 73%
rDer p 5	28/77 = 36%	6/10 = 60%	22/60 = 37%	23/70 = 33%
rDer p 7	15/77 = 19%	3/10 = 30%	N.A.	N.A.
rDer p 10	26/77 = 34%	2/10 = 20%	N.A.	10/70 = 14%
rDer p 13	4/77 = 5%	0/10 = 0%	8/60 = 13%	3/70 = 4%
rDer p 21	6/21* = 29%	5/10 = 50%	21/60 = 35%	18/70 = 26%

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rDer p 23	53/77 = 69%	7/10 = 70%	31/60 = 52%	39/70 =56%

*Fig. 4. IgE binding frequencies of different recombinant HDM allergens in cohort of HDM allergic patients from different hospitals of Bangkok. *= only 21 sera out of 77 sera from Chulalongkorn Hospital were tested due to limitations. N.A. = not available as not tested.due to serum availability.*

Our results clearly identified Der p 1, Der p 2 and Der p 23 as the major allergens responsible for the highest percentages of sensitizations. These results are similar to those observed in Europe/USA/Japan (12,13). The mid-tier HDM allergens are Der p 5, Der p 7, Der p 21 and Der p 10 whereas Der p 13 is a poor sensitizer according to the very low IgE reactivity.

It must be pointed out the combination of rDer p 1 and rDer p 2 can detect HDM allergen sensitizations in 196 out of 217 patients which indicates than 90% of the HDM sensitizations can be diagnosed using just these two HDM allergens. Amongst the 21 sera which do not contain Der p 1- and Der p 2-specific IgE (Der p 1/Der p 2 double negative), 8 are positive for Der p 23. The combination of Der p 1 + Der p 2 + Der p 23 covers 94% of the HDM sensitizations, suggesting that the addition of Der p 23 for the diagnosis of HDM allergy does not greatly improve the results obtained only with Der p 1/Der p 2.

The Figure 5 describes the percentage of sera from Chulalongkorn or Phramongkutklao hospitals displaying specific IgE against the 8 HDM allergens but classified according to the number of sensitizations (from 0 to 8). The same percentage was calculated for the sera from Ramathibodi or Children Hospital but based on the use of 6 and 7 allergens respecitively (the data for the Der p 7 sensitizations were not available for the sera from Ramathibodi hospital whereas the Der p 7/Der p 10 sensitizations were not available for the sera from the Children Hospital) (Fig.6 and 7).

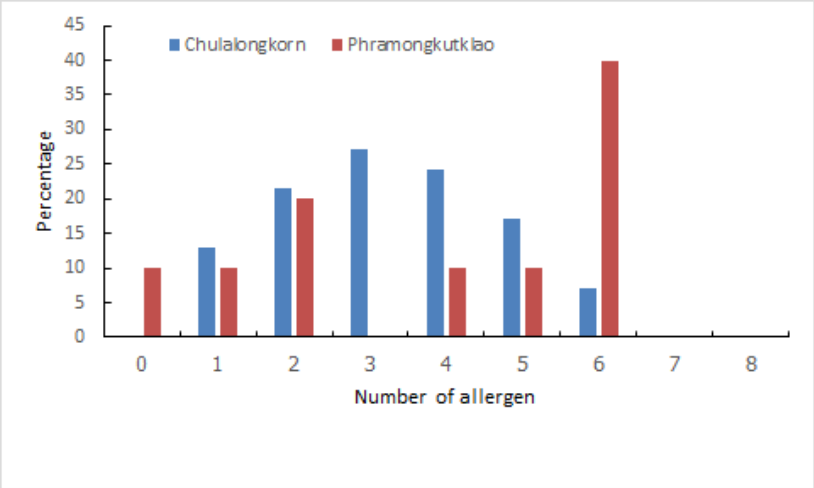

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Fig.5. Percentage of sensitizations against 0 to 8 HDM allergens detected with the sera from from Chulalongkorn or Phramongkutklao hospitals.

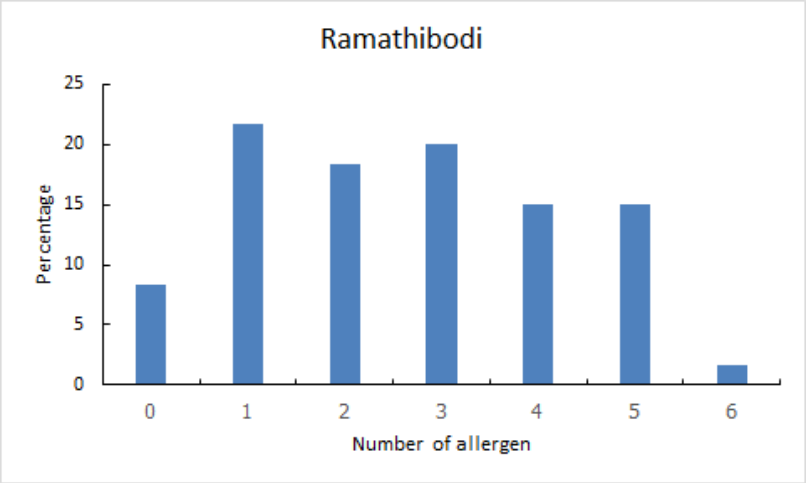

Fig.6 Percentage of sensitizations against the 0 to 6 HDM allergens detected with the sera from Ramathibodi hospital.

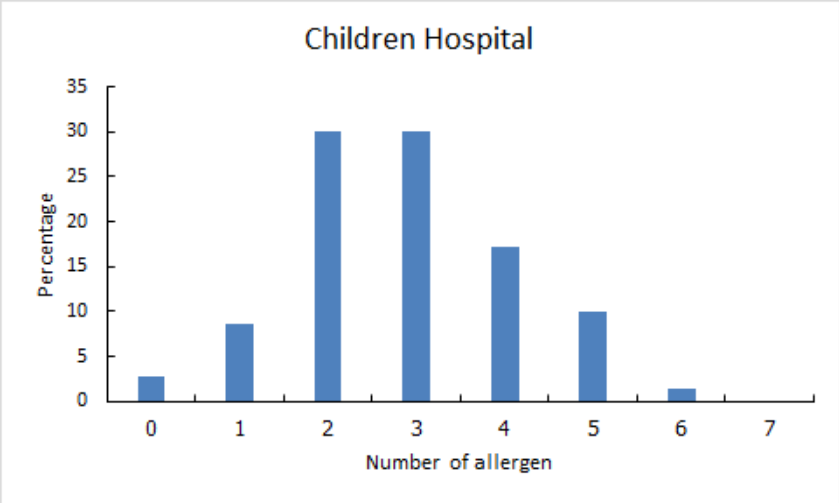

Fig.7 Percentage of sensitizations against the 0 to 7 HDM allergens detected with the sera from Children hospital.

From the figures 5-7, we can discuss the following points:

-)Some sera do not contain any IgE against 8 different HDM allergens, including the major ones as Der p 1, Der p 2 and Der p 23. This result is quite surprising. These patients could display sensitizations against other HDM allergens as there are more than 20 identified HDM

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allergens. Or another interpretation is that the ImmunoCap value obtained for such sera was incorrect and reflected false ImmunoCap positive.

-)With very rare exceptions, it is clear that the HDM allergic patients from our cohort did not develop sensitizations against all the 6, 7 or 8 HDM allergens used in our study. Most commonly, the patients developed mainly sensitizations against 3 to 4 HDM allergens. Similar results were obtained with a cohort of HDM allergic patients from Singapore (14)

Once again, the demographic data with the history case for each patients would be critical to discuss more in details such results with a specific focus on the type of allergic manifestations.

In conclusion, our results confirmed that thai HDM allergic patients developed mainly sensitizations against Der p 1, Der p 2 and Der p 23. As it appeared that the patterns of sensitizations differ largely from patients to patients, such component-resolved diagnosis of HDM allergy is critical to carefully select the allergen cocktail used of the desensitization of the patients through immunotherapeutic treatments.

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Output จากโครงการวิจัยที่ได้รับทุนจาก สกว.

1. ผลงานตีพิมพ์ในวารสารวิชาการนานาชาติ (ระบุชื่อผู้แต่ง ชื่อเรื่อง ชื่อวารสาร ปี เล่มที่ เลขที่ และหน้า) พร้อมแจ้งสถานะของการตีพิมพ์ เช่น submitted, accepted, in press, published

1: Satitsuksanoa P, Kennedy M, Gilis D, Le Mignon M, Suratannon N, Soh WT, Wongpiyabovorn J, Chatchatee P, Vangveravong M, Rerkpattanapipat T, Sangasapaviliya A, Piboonpocanun S, Nony E, Ruxrungtham K, Jacquet A; Mite Allergy Research Cohort (MARC) study team. The minor house dust mite allergen Der p 13 is a fatty acid-binding

protein and an activator of a TLR2-mediated innate immune response. Allergy. 2016 Mar 28. doi: 10.1111/all.12899.

2: Soh WT, Le Mignon M, Suratannon N, Satitsuksanoa P, Chatchatee P, Wongpiyaboron J, Vangveravong M, Rerkpattanapipat T, Sangasapaviliya A, Nony E, Piboonpocanun S, Ruxrungtham K, Jacquet A; Mite Allergy Research Cohort (MARC) Study Team. The House Dust Mite Major Allergen Der p 23 Displays O-Glycan-Independent IgE Reactivities but No Chitin-Binding Activity. Int Arch Allergy Immunol. 2015;168(3):150-60.

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2. การนำผลงานวิจัยไปใช้ประโยชน์
   - เชิงพาณิชย์ (มีการนำไปผลิต/ขาย/ก่อให้เกิดรายได้ หรือมีการนำไปประยุกต์ใช้โดยภาคธุรกิจ/บุคคลทั่วไป)
   - เชิงนโยบาย (มีการกำหนดนโยบายอิงงานวิจัย/เกิดมาตรการใหม่/เปลี่ยนแปลงระเบียบข้อบังคับหรือวิธีทำงาน)
   - เชิงสาธารณะ (มีเครือข่ายความร่วมมือ/สร้างกระแสความสนใจในวงกว้าง)

   The component resolved diagnosis of HDM allergy could interest NSTDA/BIOTEC for future development. We are currently in discussion with this agency.

   - เชิงวิชาการ (มีการพัฒนาการเรียนการสอน/สร้างนักวิจัยใหม่)

   During the period of this project, two students (one master and one PhD) working respectively on recombinant Der p 23 and Der p 13 were graduated. Their respective studiess represented some parts of the present project.

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3. อื่นๆ (เช่น ผลงานตีพิมพ์ในวารสารวิชาการในประเทศ การเสนอผลงานในที่ประชุมวิชาการ หนังสือ การจดสิทธิบัตร)

International meeting presentation

-) XXXII Congress of the European Academy of Allergology and Clinical Immunology-World Allergy Organization Joint Meeting (EAACI-WAO 2013, Milan, Italy, June 2013).
Poster Presentation
Characterization of the house dust mite allergen Der p 21 produced in P.pastoris. M. Theeraapisakkun, E. Nony, P. Pulsawat, M. Le Mignon, J. Wongpiyabovorn, K. Ruxrungtham, A.Jacquet

-) XXXIII Congress of the European Academy of Allergology and Clinical Immunology (EAACI 2014, Copenhagen, Denmark, June 2014).
Poster Presentation
Proteomic analysis of the house dust mite allergen Der p 23 produced in Pichia pastoris. W. T. Soh, E. Nony, M. Le Mignon, K. Ruxrungtham, A.Jacquet

-) XXXIV Congress of the European Academy of Allergology and Clinical Immunology (EAACI 2015, Barcelona, Spain, June 2015).
Poster Discussion Presentation
Physico-chemical and lipid-binding characterization of the house dust mite allergen Der p 13 produced in Pichia pastoris.
P. Satitsuksanoa, E. Nony, P. Pulsawat, S. Piboonpocanun, M. Kennedy, A. Jacquet

-) XXIV World Allergy Congress (WAC 2015, Seoul, South Korea, October 2015)
Poster presentations
-)Der p 23: a major house dust mite allergen in spite of limited release from fecal pellets and prominent protease sensitivity.
W. T. Soh, E. Nony, M. Le Mignon, K. Ruxrungtham, A. Jacquet.

-)The fatty acid binding protein Der p 13 is a minor house dust mite allergen able to activate innate immunity.
P. Satitsuksanoa, N. Suratannon, J. Wongpiyabovorn, P. Chatchatee, K. Ruxrungtham, A. Jacquet, HDM IgE mapping consortium.

Local meeting presentation

Thai Allergy Society (Allergy, Asthma and Immunology Association of Thailand) Meeting, Pattaya, October 2014. Oral presentation.

Component-resolved diagnosis of HDM allergy in Thai population: Preliminary results

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Characterization of the house dust mite allergen Der p 21 produced in *Pichia pastoris*

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ABSTRACT

Background: The development of recombinant house dust mite (HDM) allergens opened the way for the in-depth characterization of these molecules but also provided new opportunities to refine the diagnostic procedures of HDM allergy as well as the allergen-specific immunotherapy through tailor-made treatments.
Objective: In the present study, the HDM allergen Der p 21 was expressed in *Pichia pastoris* under a secreted form. The physico-chemical as well as the allergenic characterizations of recombinant Der p 21 (rDer p 21) were performed.
Methods: Purified rDer p 21, secreted from recombinant *P. pastoris* was characterized by CD and MS analysis and the frequency of IgE reactivity was determined by ELISA using 96 sera of HDM-allergic patients from Bangkok. The direct airway epithelial cell activation by rDer p 21 was also evaluated.
Results: rDer p 21 was highly expressed under a secreted form in *P. pastoris*. The physico-chemical characterization of purified rDer p 21 showed that the allergen displayed appropriate α-helix secondary structure content although a two amino acids truncation at the N-terminus of the protein was evidenced by MS. The prevalence of IgE reactivity to rDer p 21 reached 25% in the cohort of the HDM-allergic patients. rDer p 21 could trigger IL-8 production in airway epithelial cells through TLR2-dependent signaling.
Conclusion: Properly folded rDer p 21 produced in *P. pastoris* is appropriate for HDM allergy diagnosis as well for future recombinant allergen-based specific immunotherapy.

Introduction

House dust mites [2](HDM; *Dermatophagoides* sp.) are one of the commonest sources of airborne allergens worldwide and HDM allergy affects more than 15–20% of the population from industrialized countries [1]. To date, 19 and 27 different HDM allergen groups eliciting specific IgE responses in sensitized patients were identified in *Dermatophagoides farinae* and *Dermatophagoides pteronyssinus* respectively [2,3]. Based on the strict IgE reactivity, group 1 and 2 HDM allergens are the most important allergenic proteins (the prevalence of IgE to these allergens reaches 80–90% in HDM allergic populations) followed by groups 4, 5, 7 and 21 which react with IgE in 30–50% of sera from HDM allergic patients [4].

The characterization of purified natural HDM allergens was roughly limited to groups 1 and 2, more likely because the concentration of other allergen groups is too low to achieve their purification.

With the elucidation of the cDNA sequence, a large panel of recombinant forms of HDM allergens was successfully produced from different heterologous expression systems and purified to homogeneity. The development of recombinant HDM allergens opened the way for the in-depth characterization of these molecules not only at the level of the T- or B-cell epitope mapping but also for the elucidation of their biological activities in order

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[1] Both authors contributed equally to the work.
[2] *Abbreviations used*: HDM, house dust mites; rDer p 21, recombinant Der p 21; LPS, lipopolysaccharide; GRAS, generally recognized as safe; OD, optical density; DMEM, Dulbecco's modified Eagle's medium; ELISA, enzyme-linked immunosorbent assay; Pfu, proofreading activity.

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to identify their allergenic determinants [5]. Strikingly, recombinant HDM allergens provide new opportunities to refine the diagnostic procedures of HDM allergy but also the allergen-specific immunotherapy through tailor-made treatments according to individual sensitivities [6]. In both cases, the well defined and consistent recombinant allergen preparations would replace poorly characterized HDM natural extracts which are difficult to standardize and which contain allergens inducing new sensitizations in treated patients.

Der p 21 is a relatively newly identified HDM allergen and is present in the gut epithelial cells of HDMs [7]. Whereas the Der p 21 cDNA codes for a 14.6 kD mature protein, small angle X-ray scattering experiments demonstrated that the solution structure of recombinant Der p 21 (rDer p 21) is dimeric [7]. Although the allergenic determinants of Der p 21 remain to be identified, this allergen displayed sequence homologies with Der p 5, a putative lipid-binding protein [8]. Consequently, Der p 21 could transport lipids/fatty acids which trigger allergic response through the activation of innate immune pathways. According to the mid-range prevalence of IgE reactivity to Der p 21 (around 30%, [7]), this allergen could be incorporated in the HDM allergen panels used for specific immunotherapy. rDer p 21 was expressed in the *Escherichia coli* bacteria. Although such heterologous expression system was commonly used for a very large panel of recombinant allergens, *E. coli* not only lacks appropriate post-translational modification machinery but also produces large amount of pyrogenic lipopolysaccharide (LPS) which represents important issues particularly when the produced allergens are used for *in vitro* immune cell activation assays as well as *in vivo* applications such as immunotherapy or skin-prick testing. Whereas Der p 21 does not contain disulfide bridges (absence of cysteine residues) or consensus N-glycosylation sites, this protein could carry other post-translational modifications essential for its biological activity. Consequently, the yeast *Pichia pastoris* expression system may be preferred through its "generally recognized as safe" (GRAS) status (notably by the absence of LPS) and its capacity to perform protein modifications at the post translational level.

In the present study, we successfully expressed rDer p 21 in *P. pastoris* under a secreted form. The physico-chemical characterization as well as the IgE reactivity measurements clearly indicated that such method of production is not only appropriate for future Der p 21-based diagnosis and treatment of HDM allergy but also for the determination of its allergenicity.

Material and methods

Human sera and rProDer p 1 and Der p 5

Ninety six sera were obtained from patients followed at Chulalongkorn Hospital, with a positive case history indicative for HDM allergy and *D. pteronyssinus*-specific IgE antibodies (0.98–90 kU/L) as determined with the ImmunoCAP System (Phadia). Each patient expressed written informed consent. As negative controls, sera from non-allergic subjects were also collected (<0.35 kU/L). The sera collection was approved by the Chulalongkorn University Ethic Committee (ref 327/2012). rProDer p 1 was expressed and purified as previously described [9]. rDer p 5 was expressed and purified following the same cloning and purification strategies as used for rProDer p 1.

Cloning of Der p 21 cDNA into P. pastoris expression vector

A 363 bp DNA fragment encoding leader-less Der p 21 (aa 20–140 from full length Der p 21 sequence) was generated by PCR amplification from a *D. pteronyssinus* cDNA library and using specific primers carrying *Xho*I and *Not*I restriction sites (underlined); forward primer: 5′-CTCGAGAAAAGAGAGGCTGAAG TCTTTATTGTTGGTGACAAAAAAGAAG-3′; reverse primer: 5′-GCGGCCGCTTAATAATATTCATCCGGATTTACAGC-3′. The amplified DNA piece was digested with *Xho*I and *Not*I and cloned into an expression vector pPICZα A (Invitrogen) restricted by the same enzymes. The recombinant pPICZ α A-Der p21 plasmid (5–10 μg) was linearized with *Bgl*II to transform the yeast *P. pastoris* strains KM71 and X-33 (Invitrogen) by electroporation using a Gene Pulser Xcell™ (Bio-Rad) at 1500 V, 25 μF, 200 Ω in 1 mm electrode gap cuvette. The transformants were selected on YPD agar plate (1% yeast extract, 2% peptone, 2% dextrose, 2% agar) containing 100 μg/ml Zeocin (Invitrogen) at 30 °C for 2–3 days.

Expression of rDer p 21 in P. pastoris

A single Zeocin-resistant colony was inoculated in 20 ml of YPD medium and grown overnight at 30 °C under shaking conditions. Ten percent of the inoculum was transferred in 500 ml of BMGY medium at 30 °C under shaking conditions until the optical density at 600 nm (OD_{600nm}) reached of 5–9 (approximately 14–15 h). The cells were then harvested by centrifugation at 5000*g* for 5 min and the cell pellet was resuspended in 500 ml of BMMY medium containing 0.5% methanol to induce rDer p 21 expression at 30 °C. Every 24 h, methanol was added to maintain the final concentration of methanol to 0.5%. Aliquots of culture supernatants were collected by centrifugation at 13000*g* for 10 min stored at −20 °C until used.

Purification of rDer p 21 from P. pastoris supernatant

The culture media containing secreted rDer p 21 were diluted ten times with MilliQ water and the pH of the solution was adjusted to pH 4. This material was applied onto a S Sepharose XL column (5 × 2.6 cm, GE Healthcare Lifesciences) equilibrated with 20 mM sodium acetate pH 4.0. After washing the unbound proteins from the column, elution proceeded through stepwise increase of NaCl concentration in the equilibration buffer (from 0 to 1000 mM NaCl). The fraction containing rDer p 21 (500 mM NaCl elution) was concentrated by ultrafiltration using a Amicon Ultra-15 10 K membrane (Cut-off 10 kD) and then further purified by gel filtration onto a Superdex 75 HR column 10/30 (GE Healthcare Lifesciences), equilibrated with PBS pH 7.3. The fractions containing purified rDer p 21 were pooled and stored at 4 °C for further analysis. Protein concentration was determined using the microBCA protein assay kit (Pierce) with bovine serum albumin as a standard. The endotoxin content in rDer p 21 preparations, determined using LAL Chromogenic Endotoxin Quantitation Kit (Pierce), was lower than 0.05 U/ml.

UHPLC–UV–ESI-MS

Accurate mass measurement was performed on a Maxis 4G mass spectrometer (Bruker Daltonics) coupled with a RSLC Ultimate 3000 (Dionex). Purified rDer p 21 was desalted and concentrated for 4 min onto a Acquity BEH300 C_4 column (Waters) equilibrated at 70 °C with 98% solvent A (0.1% trifluoroacetic acid); 2% solvent B (100% acetonitrile/0.1% trifluoroacetic acid) at a flow rate of 0.4 ml/min. Protein was eluted directly into the mass spectrometer at a flow rate of 0.4 ml/min with a linear gradient from 2% to 60% solvent B over 20 min. Mass spectra were acquired in ESI mode using the following instrument settings: capillary voltage 4500 V; End plate offset voltage: −500 V; Nebulizer: 4.0 L/min; Dry heater: 180 °C; Scan rate: 1 s; Quadrupole collision energy: 8 eV. Mass spectra were acquired in positive mode at *m/z* 300–3000. Mass accuracy was achieved by infusing the ESI-L low concentration tuning mix solution (at 100 μL/h) for 4 min at the beginning of the

acquisition. Mass spectra deconvolution was performed using MaxEnt algorithm, and the precise average mass of rDer p 21 was determined using Data Analysis software (Bruker Daltonic, version 4.0).

Circular dichroism

A J-815 CD spectrophotometer (Jasco France) was used to assess the secondary structure of purified rDer p 21. Briefly, the sample was analyzed in a 1 mm cell, at a concentration of 190 μg/ml (based on microBCA assay), at 20 °C, with a wavelength range of 250–200 nm, a 2 nm band width and a scanning speed of 100 nm/min. Eight independent spectral scans were acquired and averaged using Spectra manager v2 software (Jasco).

Serum IgE levels to recombinant HDM allergens

ELISA microplates were coated overnight at 4 °C with rProDer p 1, rDer p 5 or rDer p 21 at 500 ng/well. Plates were washed with PBS-Tween 20 0.05% (PBS-T) and subsequently blocked with PBS-T containing 1% BSA (PBS-T-BSA) for 1 h. Serum samples were then incubated at 1/8 dilution in PBS-T-BSA for 1 h at 37 °C. Plates were washed with PBS-T and incubated with biotinylated secondary anti-human IgE (1/1000 dilution, KPL) for 1 h at 37 °C, followed by streptavidin–peroxidase (1/1000 dilution, BD Biosciences) for 30 min at 37 °C. The allergen-antibody complex was detected by addition of TMB substrate (BD Biosciences). The color development was stopped by addition of sulfuric acid and optical density (OD) was measured at 450 nm using iMark microplate reader (Bio-Rad). A serum was considered as positive when the OD value was higher than a cut off value, established as the mean OD values of negative control serum plus 3 standard deviations.

To evaluate cross-reactivities between Der p 21 and Der p 5, ten rDer p 21 and rDer p 5 double-positive sera were used for inhibition experiments. Sera (1/10 dilution) were preincubated overnight with rDer p 5 or rDer p 21 (10 μg/ml) at 4 °C. The IgE reactivities to rDer p 21 and rDer p 5 were measured as described above, coated rDer p 21 and rDer p 5 being incubated with rDer p 5-and rDer p 21-adsorbed sera respectively.

Cell activation assays

The human bronchial epithelial BEAS-2B cells (ATCC) were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin in 24-well plates. When the culture reached 80% confluency, cells were then incubated in serum-free DMEM for further 24 h. One hour before stimulation, the growth medium was replaced by fresh serum-free DMEM. Cells were stimulated with different concentrations of rDer p 21 (0.5–20 μg/ml) for 16 h in serum-free DMEM. As controls, cells were also incubated with medium alone or with the Pam3CSK4 TLR2 ligand (200 ng/ml, Invivogen). When appropriate, BEAS-2B cells were treated for 1 h at 37 °C with anti-hTLR2 Mab (TLR2.5, eBiosciences, 10 μg/ml) or isotype control prior to the addition of rDer p 21 or Pam3CSK4. Culture supernatants were collected, centrifuged for 5 min at 10,000*g*, and assayed for IL-8 with enzyme-linked immunosorbent assay (ELISA) kit (BD Biosciences) according to the manufacturer's protocol. IL-8 concentrations were determined by interpolation from a standard curve performed with purified human IL-8.

Statistical analysis

Student's *t*-test was used to compare data between different treatments. Differences were considered significant for *P*-values < 0.05.

Result

Cloning of Der p 21

Using two specific primers derived from the sequence DQ354124 (GenBank accession number, [7]) and a *D. pteronyssinus* cDNA library, we successfully cloned the cDNA encoding the mature form of Der p 21 (aa 20–140 from full length Der p 21 sequence). The DNA sequence analysis evidenced three nucleotide substitutions in the cloned cDNA compared with the DQ354124, two of them were silent whereas the third one induced the point mutation M19T (mature Der p 21 numbering). It must be pointed out that similar results were obtained using a polymerase with proofreading activity (Pfu) for the PCR amplification. To express rDer p 21 in the yeast *P. pastoris*, the mature Der p 21 cDNA was cloned into the pPICZ α A expression vector and directly downstream to the α-mating factor leader sequence for the secretion of the expressed allergen. This cloning strategy could lead to the production of authentic Der p 21 without any addition of extra amino acids at the N-terminus and any tag at the C-terminus.

Expression of rDer p 21 in P. pastoris

The resulting plasmid was used to transform two *P. pastoris* strains: the wild type X33 strain with an active AOX1 gene giving fast growth on methanol (Mut^+ phenotype) and the strain KM71H with a deletion in AOX1 gene resulting in slow growth on methanol (Mut^S phenotype). After selection on agar plates containing zeocin, the presence of Der p 21 cDNA in recombinant *P. pastoris* was confirmed by colony PCR. Expression of rDer p 21 was induced by addition of methanol in the culture of recombinant *P. pastoris* in shake flasks at 30 °C. Both KM71 and X-33 recombinant strains, cultured and induced at the same cell density, were able to express and secrete rDer p 21 which migrated onto SDS–PAGE as a 14kD protein (Fig. 1A). Such molecular weight estimation was close to the expected mass for mature rDer p 21 (AA 1–120 with M19T mutation: 14564 Da, UniProt accession number Q2L7C5). No statistically significant difference in the rDer p 21 expression level was observed after 24, 48 or 72 h of methanol induction and between the two tested *P. pastoris* strains. The recombinant KM71 strain was used for the subsequent production and purification of the allergen.

Purification and MS analysis of rDer p 21

Recombinant Der p 21 was purified from the medium of a 48 h-induced shake flask culture of the KM71 strain and using a combination of cation exchange and gel filtration chromatographies. The purification yield was estimated to 10 mg purified rDer p 21 per liter of culture medium (Table 1). Onto SDS–PAGE, purified rDer p 21 migrated as a monomeric 14 kD band (Fig. 1B). However, the UHPLC–UV–ESI-MS analysis revealed the absence of intact rDer p 21 with a corresponding mass of 14563.57 Da but the presence of two main forms at 14304.50 and 14205.40 Da (Fig. 2). These two forms could represent truncated rDer p 21 with N-terminal cleavages at residue V3. Several methionine oxidations (1 to 4, +16 Da) were detected, which is consistent with the presence of five methionine residues within the amino acid sequence.

CD analysis of rDer p 21

The far UV CD spectrum of rDer p 21 showed a maximum around 200 nm and double minimum at 208 and 222 nm (Fig. 3), indicating that rDer p 21 is homogeneously folded, essentially through α helix secondary structure. Based either on the Yang or Reed algorithm [10,11], the α helix content in rDer p 21 was

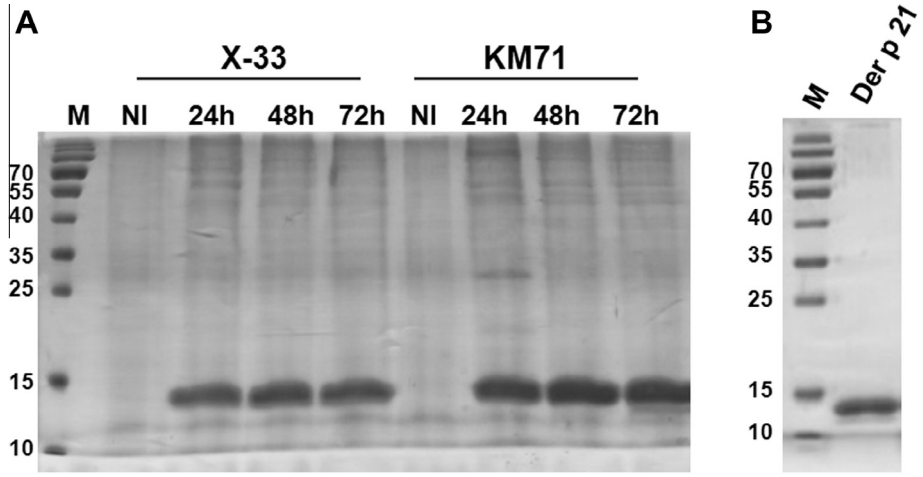

Fig. 1. Der p 21 expression and purification from transformed *P. pastoris* strains. Panel A. X33 and KM71 *P. pastoris* strains were transformed with pPICZ α A vector encoding Der p 21. Recombinant strains were cultured and the Der p 21 expression was induced by methanol for 24, 48 and 72 h. NI: non induced. The secretion of expressed rDer p 21 was monitored by SDS–PAGE and coomassie blue staining. Panel B: SDS–PAGE analysis of purified rDer p 21.

Table 1
Purification of rDer p 21.

Purification step	Total protein (mg)	rDer p 21 (mg)*	Yield (%)	Purification factor
Culture medium (100 ml)	90	9.5	100	1
S Sepharose (20 ml)	5	3.4	36	6.6
Ultra 10 kD (5 ml)	3.7	2.4	25	6.1
Sephadex 75 (3 ml)	1.1	1	10	8.7

* The Der p 21 concentration was estimated by densitometric analysis of SDS–PAGE using a Der p 21 protein standard and through the use of the Quantity One 1-D Analysis software (Bio-Rad).

estimated to 72% whereas less than 28% of the structure corresponded to β-sheets. Such secondary structure quantification was consistent with predictions based on the Der p 21 primary structure and using the GOR4 algorithm [12] or the Chou and Fasman [13] method, that both predict that 80% of the residues will be structured as α helices.

IgE reactivity to rDer p 21

Using a panel of 96 sera from HDM-allergic patients, the IgE reactivity to rDer p 21 was determined by direct ELISA and com-

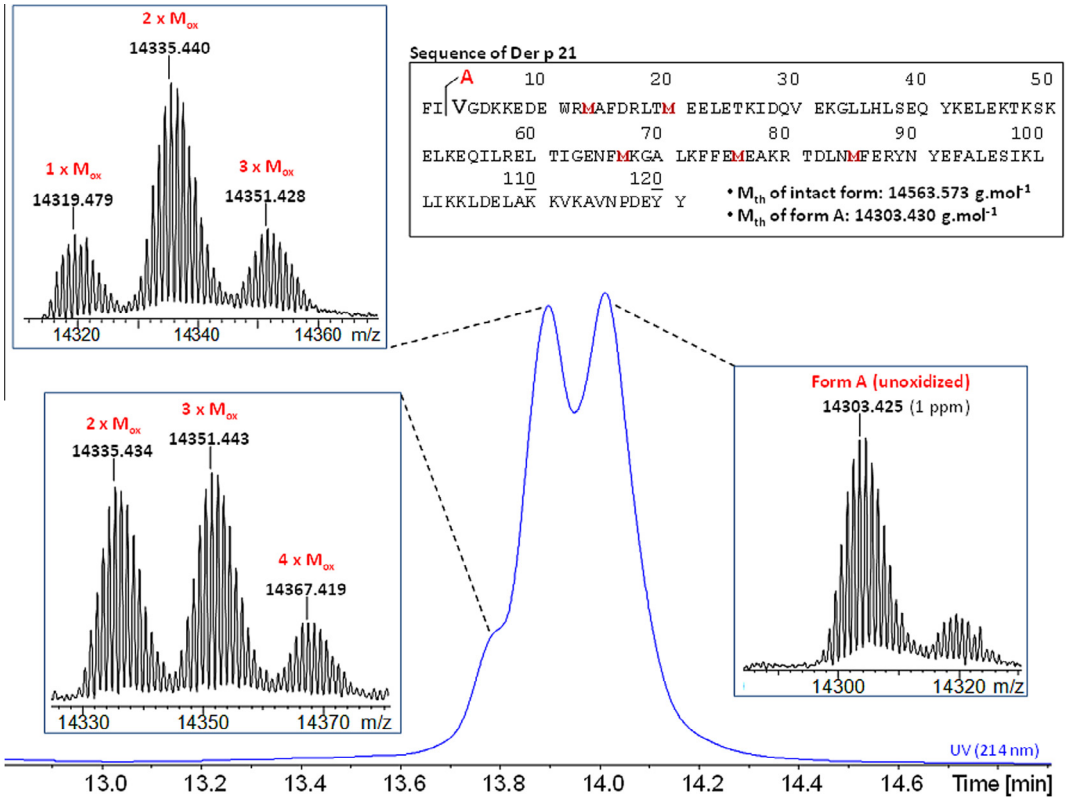

Fig. 2. rDer p 21 UHPLC–UV chromatogram and corresponding mass spectra. Purified allergen was eluted from an Acquity BEH300 C_4 column coupled with a Maxis 4G ESI-Q-ToF MS for accurate mass measurement. Right panel shows the mass spectrum of non-oxidized rDer p 21 (form A) eluted after 14 min with a mass accuracy of 1 ppm. Left panels show the separated oxidized forms. N-terminus truncation and Met oxidation are illustrated within the sequence panel.

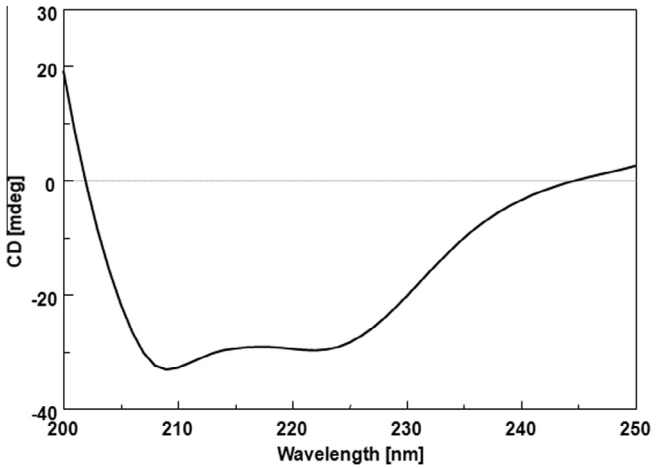

Fig. 3. UV-Circular dichroism spectrum of purified rDer p 21. Purified rDer p 21 was analyzed in a 1 mm cell, at a concentration of 190 μg/ml, at 20 °C using a J-815 CD spectrophotometer.

pared with the IgE binding capacity of rProDer p 1 and rDer p 5. Twenty-four patients displayed specific IgE to rDer p 21 (25%) whereas IgE reactivity to rProDer p 1 and rDer p 5 was detected in 69 (72%) and 66 (69%) in mite-allergic individuals. It must be pointed out that more than 85% of the patients developing anti-rDer p 21 IgE displayed reactivity to rDer p 5 (21 sera) and rProDer p 1 (22 sera). Inhibition assays using sera displaying both IgE to rDer p 5 and rDer p 21 clearly confirmed the absence of cross-reactivities between these two allergens. Indeed, whereas serum preincubation with rDer p 5 or rDer p 21 fully inhibited the IgE binding to the corresponding allergen, no inhibition of IgE reactivity to rDer p 21 was induced by rDer p 5 and vice versa (data not shown).

Biological activity of rDer p 21

To confirm that rDer p 21 produced in *P. pastoris* can also be appropriate to characterize its allergenicity, preliminary experiments were conducted using human bronchial epithelial cells as airway epithelium is the first cellular point of contact for airborne allergens. rDer p 21 was shown to trigger IL-8 secretion from BEAS-2B cells in a concentration-dependent manner when compared with control medium (Fig. 4A, $P < 0.05$). As Der p 21 displays homologies with fatty acid/lipid binding proteins, this allergen could trigger cell activation through TLR2 signaling. To evaluate the TLR2 dependence of the rDer p 21-induced IL-8 production in BEAS-2B cells, we compared the IL-8 secretion in cells preincubated with a blocking antibody to TLR2 [14]. As expected, this pretreatment abolished the IL-8 production trigger by the TLR2 ligand Pam3CSK4 (Fig. 4B). Our results showed also clearly that the cytokine production by BEAS-2B stimulated with rDer p 21 was drastically reduced by the anti-TLR2 blocking antibody (Fig. 4A). Consequently, our preliminary data suggest that rDer p 21 could activate TLR2 signaling.

Discussion

Although numerous recombinant allergens were produced in *E. coli*, the *P. pastoris* expression system could display several advantages over the production in bacteria. First, the downstream processing using *P. pastoris* is facilitated by the localization of the expressed allergen in the supernatant (secretion), contrary to the intracellular production of allergen in bacteria which needs mechanical or chemical cell disruption to initiate the purification. Second, this yeast expression system allows appropriate post-translational modifications of the allergens including disulfide formations, glycosylations and consequently facilitates the appropriate folding [15]. Third, the absence of contaminating endotoxins in yeast reduces drastically the risk of side effects which could occur along allergen-specific immunotherapeutic treatments or allergy diagnosis through skin-prick tests.

In this context, we proceeded to the production, the purification and the characterization of mature rDer p 21 from engineered *P. pastoris*. Using two different *Pichia* strains, we demonstrated that the rDer p 21 expression level was roughly independent on the duration of the methanol induction as comparable production was measured after 24, 48 or 72 h of induction. Consequently, the scaling-up of the rDer p 21 production could be easily shortened through the methanol induction of the allergen expression in 24 h.

The purification of rDer p 21 was successfully achieved by cation exchange and gel filtration chromatographies as estimated by SDS–PAGE analysis. However, the MS characterization of purified rDer p 21 evidenced the absence of authentic protein N-terminus by the truncation of the first two amino acids F1 and I2. N-terminal truncation is a common phenomenon in proteins expressed by *P. pastoris* and likely due to extra cleavages by the yeast proteases Kex2 and STE13 during the α mating factor leader sequence removal [16]. Another modification of rDer p 21 detected by MS was the oxidation of methionine residues. Variability in the amount of methionine oxidation was observed in different preparations as unoxidized rDer p 21 or protein with one to four oxidized methionine residues could be detected. The oxidation phenomenon, commonly observed in proteins produced in *P. pastoris*, could be due to the presence of hydrogen peroxide in the medium produced during the methanol metabolism [17]. Although

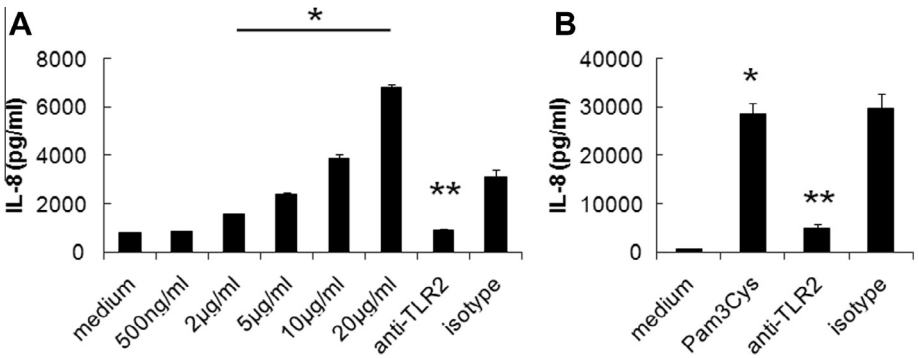

Fig. 4. rDer p 21-induced IL-8 secretion in BEAS-2B cells. Serum-starved BEAS-2B cells were incubated for 16 h with medium alone or with increasing concentrations of rDer p 21 (500 ng–20 μg/ml) (Panel A). As positive control, cells were also activated with Pam3CSK4 (200 ng/ml) (panel B). Cells were also preincubated with blocking anti-TLR2 antibody or isotype control before rDer p 21 (10 μg/ml) or Pam3CSK4 (200 ng/ml) treatment. IL-8-specific ELISA was used to quantify levels of IL-8 protein in culture supernatants. All data are expressed as the mean ± S.E. from at least two independent experiments performed in triplicate.

an extensive MS characterization of rDer p 21 produced in bacteria was not achieved, such protein alteration could not be present as rDer p 21 expressed in *E. coli* displayed a molecular mass in agreement with the theoretical mass from the deduced amino acid sequence [7]. However, it must be pointed out that rDer p 21 produced in bacteria does not carry authentic N-terminus too through the presence of initiation Methionine. We speculate that these two rDer p 21 modifications when produced in *P. pastoris*, N-terminal truncation as well as methionine oxidation, do not interfere with the protein stability and folding. First, CD analysis confirmed that rDer p 21 displayed appropriate exclusive and homogenous α - helix content which was comparable with that measured with rDer p 21 produced in *E. coli* and consistent with the secondary structure predictions [7,12,13]. Second, rDer p 21 produced in *P. pastoris* or *E. coli* exhibited comparable IgE binding capacities (25%) using a panel of sera from HDM allergic patients in Thailand. This frequency is in agreement with the measured IgE reactivity to this allergen performed in Austria (26%) [7] and Singapore (20%) [18] and using rDer p 21 prepared from *E. coli* [7]. These results not only confirmed that the rDer p 21 preparation from *P. pastoris* is similar to that produced in bacteria at the level of IgE binding epitope content but that Der p 21 is an important HDM allergen which could be incorporated into the HDM allergy diagnosis and treatment. Our data from IgE inhibition assays confirmed also that rDer p 21 produced in yeast, similarly to the form produced in bacteria does not share common IgE binding epitopes with Der p 5, although the amino acid sequence of Der p 21 and Der p 5 exhibit 30% identity [8]. Through small angle X-ray scattering experiments, rDer p 21 produced in *E. coli* displayed a dimeric structure, similarly to Der p 5 [7]. Under native conditions, gel filtration chromatography of rDer p 21 produced in *P. pastoris* clearly indicated that the recombinant allergen was monomeric. Our results are in agreement with those recently obtained from the comparison between Der p 5 and Der p 21 through modeling and bioinformatics analysis [8]. Notably, according to the Der p 5 X-ray structure, the glycine at position 30 is critical for the formation of a kink in the N-terminal helix leading to an entangled dimeric structure whereas a **v**aline zipper formed by residues 70 and 73 contributes to the dimer stability [19]. The G30V, V70A and V73F substitutions in the Der p 21 sequence could impair the dimeric formation. Nevertheless, our data clearly demonstrated that rDer p 21 was able to activate airway epithelial cells through TLR2 signaling suggesting that the Der p 21 allergenicity is mediated by interactions with lipid/fatty acid ligands triggering innate immunity. It must be pointed out that Der p 2, a LPS-binding protein [20], as well as Der p 5 were shown also to directly activate airway epithelial cells [21,22]. Moreover, the TLR2-dependence of airway smooth muscle activation by Der p 2 was also evidenced [23]. Although further experiments are needed to confirm the lipid binding activity of rDer p 21 as well as the innate immune activation by this HDM allergen, these preliminary results validated once again the quality of recombinant HDM allergen production in *P. pastoris*.

In conclusion, *P. pastoris* is at least as efficient as *E. coli* to produce monomeric and correctly folded rDer p 21. Through the convenient and effective downstream processing and the absence of endotoxins, the *P. pastoris* expression system could be a generic method for the production of recombinant HDM allergens.

Acknowledgments

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ORIGINAL ARTICLE **Allergens**

Development of recombinant stable house dust mite allergen Der p 3 molecules for component-resolved diagnosis and specific immunotherapy

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Clinical & Experimental Allergy

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Summary

Background The allergen Der p 3 is underrepresented in house dust mite (HDM) extracts probably due to autolysis. Recombinant stable molecule of the allergen is thus needed to improve the diagnosis of allergy and the safety and efficacy of immunotherapy.

Objective The current study reports the immunological characterization of two recombinant molecules of the HDM allergen Der p 3 as useful tools for diagnosis and immunotherapy.

Methods Recombinant mature (rDer p 3) and immature (proDer p 3) Der p 3 and their corresponding S196A mutants were produced in *Pichia pastoris* and purified. The stability, IgE-binding capacity and allergenicity of the different proteins were analysed and compared with those of the major mite allergen Der p 1 used as a reference. Additionally, the immunogenicity of the different allergens was evaluated in a murine model of Der p 3 sensitization.

Results Compared to the IgE reactivity to recombinant and natural Der p 3 (nDer p 3), the mean IgE binding of patient's sera to rDer p 3-S196A (50%) was higher. The poorly binding to nDer p 3 or rDer p 3 was due to autolysis of the allergen. Contrary to Der p 3, proDer p 3 displayed very weak IgE reactivity, as measured by sandwich ELISA and competitive inhibition, rat basophil leukaemia degranulation and human basophil activation assays. Moreover, proDer p 3 induced a T_H1-biased immune response that prevented allergic response in mice but retained Der p 3-specific T-cell reactivity.

Conclusion rDer p 3-S196A should be used for the diagnosis of HDM allergy elicited by Der p 3, and proDer p 3 may represent a hypoallergen of Der p 3.

Keywords Der p 3, diagnosis, hypoallergen, inactive enzyme, recombinant mite allergen, specific immunotherapy

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Introduction

House dust mite (HDM) allergens are one of the major sensitization factors in the development of allergy and asthma worldwide [1, 2]. In the USA and Europe, allergens from *Dermatophagoides* (*D.*) species are prevalent [3]. Currently, complex *Dermatophagoides* natural extracts are largely employed for subcutaneous immunotherapy (SCIT) or sublingual immunotherapy (SLIT) as well as for the diagnosis of allergy [4, 5]. However, despite the development of standardized methods to measure allergen content [6–8], these extracts are highly variable in allergen composition and content, which can jeopardize the outcome of diagnostic tests [9] but also the safety and efficacy of immunotherapy [5, 10]. For these reasons, recombinant allergens and hypoallergens are increasingly used for component-resolved diagnosis and therapy, respectively [11–16].

Between 70% and 100% of allergic patients exhibit high levels of IgE against Der p 1 and Der p 2, which are therefore considered as the two major allergens from *Dermatophagoides pteronyssinus* [17, 18]. However, other allergens, such as the serine proteases Der p 3, Der p 6 and Der p 9, may also play an important role in allergy [18]. Indeed, several studies have reported the enzymatic activity of mite proteases as a potential adjuvant factor in the development and chronicity of allergy [19–21]. There is evidence for the interaction of the protease Der p 3 with the respiratory epithelium, although the protease's allergenicity remains poorly investigated [22–25]. Furthermore, the prevalence of anti-Der p 3 IgE is controversial and varies between 10% and 100%. This finding could be due to the heterogeneity of the preparations of the natural allergen but could also be the result of the high susceptibility of Der p 3 to autolysis, hampering diagnosis performed with the purified natural allergen [17, 26, 27]. Therefore, a more stable and homogeneous Der p 3 allergen is highly desirable to ascertain the real prevalence of sensitization to this allergen.

In a previous study [28], we expressed the precursor form of Der p 3 (proDer p 3) as a soluble recombinant protein in *Pichia pastoris*. Fully mature, active Der p 3 was obtained from the cleavage of the prosequence by the major mite allergen Der p 1. We obtained a homogeneous preparation of recombinant Der p 3 with the biochemical and structural characteristics similar to those of the natural Der p 3 enzyme. We showed that, as for its natural counterpart, the half-life of rDer p 3 is tightly controlled by the autodegradation of the protein. Although proDer p 3 was shown to be enzymatically less active than Der p 3 and thus less sensitive to autolysis, the proteases' secondary structures appeared to be similar [28].

To explore the involvement of the proteolytic activity of Der p 3 in the protease's autolysis, we generated an S196A mutant in which the active serine was replaced by an alanine. The immunogenicity and allergenicity of rDer p 3, its more stable precursor form proDer p 3, and the corresponding inactive counterparts, rDer p 3-S196A and proDer p 3-S196A, respectively, were then investigated. Considering that the propeptide of the recombinant proform of Der p 1 (proDer p 1) was shown to block certain IgE epitopes on Der p 1 and that proDer p 1 produced in *Escherichia coli* could represent a hypoallergenic variant of Der p 1 and was a candidate for mite allergy vaccination [29, 30]; the hypoallergenic characteristics of proDer p 3 were also analysed.

Methods

Human sera and blood samples and allergens

Sera were obtained from patients with an HDM allergy and from non-allergic, non-asthmatic subjects from the unit of Immunology and Allergology of the Centre Hospitalier de Luxembourg, Luxembourg, and the Asthma clinic, Centre Hospitalier Universitaire (CHU), Liège, Belgium. EDTA-anticoagulated peripheral blood samples were obtained from patients with HDM allergy and non-allergic, non-asthmatic subjects from the Asthma clinic, CHU, Liège, Belgium. The diagnosis of HDM allergy was based on a positive case history, positive skin prick test results and serum IgE antibodies against HDM extracts [ImmunoCAP, Phadia, Uppsala, Sweden, IgE (kU/L) $\geq$ 1.4–100]. The samples were collected after informed consent was provided and were analysed in an anonymized manner with the approval of the ethics committee of the CHU of Liège (B707201214995).

Unglycosylated proDer p 1 and proDer p 3 and the corresponding catalytically inactive molecules proDer p 1C34A and proDer p 3-S196A, respectively, were expressed in *P. pastoris*, purified and activated into mature rDer p 1 and rDer p 3 and their inactive counterparts, rDer p 1-C34A and rDer p 3-S196A, respectively, as previously described [28, 31–33]. Natural Der p 3 (nDer p 3) was purified to homogeneity from HDM extracts (see below). Endotoxin levels of purified allergens were determined using the limulus amebocyte lysate assay (Hycult Biotech, Uden, the Netherlands) according to the manufacturer's instructions. The endotoxin levels of the allergens used for the immunization were < 20 U/mg protein.

Purification of nDer p 3

For purification, 1 g of whole *D. pteronyssinus* culture was stirred overnight at 4°C in a phosphate buffer saline pH 7.5 (PBS). Insoluble material was removed by ultracentrifugation at 130 000 g for 1 h. Supernatants containing solubilized proteins were dialysed at 4°C against 20 mM Tris-HCl buffer, pH 7.5 (buffer A). The solution was loaded onto a Q-HP-Sepharose column (60 mL, 2.6 × 10 cm; GE Healthcare, Uppsala, Sweden) previously equilibrated with buffer A. The elution of bound proteins was performed with a linear gradient of buffer A added to 1 M NaCl. The resultant fractions were analysed for Der p 1, Der p 3, Der p 6 and Der p 9 activities using specific chromogenic substrates, as previously described [28]. The fractions containing Der p 3 activity (and also Der p 6 and Der p 9 activities) were applied onto a benzamidine Sepharose affinity column equilibrated with buffer A. nDer p 3 and nDer p 6 were eluted from the column with 100 mM acetate buffer, pH 4. Finally, to separate Der p 6 and Der p 3, fractions containing nDer p 3 were dialysed against buffer A and applied onto a heparin affinity column (GE Healthcare) equilibrated in buffer A. Adsorbed material was eluted with a linear gradient of buffer A with 1 M NaCl. Purified nDer p 3 was concentrated by ultrafiltration (cut-off: 10 kDa) and stored at −20°C.

Circular dichroism measurements

Far UV circular dichroism (CD) spectra were recorded at 25°C in a 1-mm-pathlength cell using a Jasco J-810 CD spectropolarimeter (Jasco Inc., Easton, MD USA). Experiments were performed in 20 mM Tris pH 8.5 at a final protein concentration of 0.13 mg/mL. Five spectra were acquired at a scan speed of 3 nm/min, and were averaged and corrected for baseline contribution of the buffer.

Fluorescence measurements

The intrinsic fluorescence spectra of proteins (4 μM) were recorded at 25°C in a 1-cm-pathlength cell on a Perkin Elmer LS 50 B spectrofluorimeter (Perkin Elmer, Wellesley, MA, USA) using a scan rate of 350 nm/min. The excitation wavelength was 280 nm, and emission spectra were recorded from 300 to 420 nm. Five spectra were recorded, averaged and corrected as explained above.

Enzymatic activity measurements

The rDer p 3, proDer p 3, rDer p 1 and proDer p 1 allergens were incubated at 37°C for various lengths of time in PBS, pH 7.4. Allergens were analysed by SDS-PAGE, and Der p 3 or Der p 1 activities were measured in 10-fold diluted aliquots using 150 μM QAR-AMC as substrate on a Perkin Elmer LS 50 B spectrofluorimeter as previously described [28, 33].

IgE-binding activity assay

NDer p 3, rDer p 3, rDer p 3-S196A and recombinant Der p 1 (rDer p 1) were coated overnight (500 ng/well) at 4°C on immunoplates. Plates were washed with PBS containing 0.01% Tween-20 and then saturated for 1 h at 37°C with PBS–Tween-20 supplemented with 1% BSA (Sigma, Bornem, Belgium). Sera from 22 patients with an HDM allergy and from 8 healthy donors were used at a 1 : 5 dilution and were then incubated for 1 h at 37°C. After washing with PBS–Tween-20, the allergen–IgE complexes were detected with a mouse anti-human IgE antibody (ImTec Diagnostics) coupled to alkaline phosphatase (dilution 1 : 500). The enzymatic activity was detected using the 4-nitrophenyl phosphate disodium salt hexahydrate (Sigma) dissolved in TRIS buffer (pH 9.4). OD 405_{nm} was measured in Bio-Rad Novapath ELISA reader (Nazareth Eke, Belgium). ODs ≥ 3 times the control values were considered to be ELISA-positive results.

For IgE inhibition assays, plates were coated with rDer p 3-S196A (500 ng/well) and 6 sera were previously incubated with increasing concentrations of nDer p 3, rDer p 3, rDer p 3-S196A, proDer p 3 or proDer p 3-S196A (0, 0.25, 0.5, 2.5, 5, 10, 25 and 50 μg/mL). The sera were then incubated on ELISA plates, and IgE-binding activity was measured as described above.

Sandwich ELISA for IgE-binding activity

Plates were coated with a mouse anti-human IgE monoclonal antibody (BD Pharmingen San Jose, CA, USA) (250 ng/well) for 16 h at 4°C, washed with Tris-buffered saline–0.01% Tween-80 and then saturated with the same buffer supplemented with 1% BSA. Sera (dilution 1 : 5) were incubated for 1 h at 37°C. After washing, rDer p 1, proDer p 3 or rDer p 3S196A (500 ng/well) was added to saturation buffer and incubated for 1 h at 37°C. The plates were then washed and incubated with rabbit anti-proDer p 3 or anti-proDer p 1 antibodies for 1 h at 37°C. After washing, a donkey anti-rabbit biotinylated Ig (Amersham, Roosendaal, the Netherlands) (dilution 1 : 5000) was added for 1 h at 37°C. The assay was developed using a streptavidin–HRP conjugate (Amersham) (dilution 1 : 1000) and 3,3′,5,5′-tetramethylbenzidine (Sigma) as the substrate. The reaction was stopped by adding 0.4 M H_2SO_4, and the absorbance was measured at 450 nm. ODs ≥ 3 times the control values were considered to be ELISA-positive results.

Long-term stability assay

The rDer p 3 and rDer p 1 allergens and their corresponding precursor forms (5 μM) were incubated at 37°C. Samples were taken over time and analysed by 15% SDS-PAGE and for Der p 1 and Der p 3 activity using the fluorogenic substrate Boc-QAR-7-Amino-4-methylcoumarin (AMC) (10 μM), as previously described [28, 31].

Rat basophil leukaemia (RBL) cell mediator release assay

The five sera used, coming from patients with HDM allergy (see above), were selected by ELISA to be positive to Der p 1 and Der p 3. RBL-SX-38 cells (a kind gift of Professor Kinet, Beth Israel Deaconess Medical Center, Boston, MA, USA), expressing human FcεRI, were sensitized with sera (dilution 1 : 5) for 17 h. Triggering of RBL cells was induced by adding various concentrations (0.0001–1 μg/mL) of nDer p 3, rDer p 3, rDer p 3-S196A, proDer p 3, proDer p 3-S196A and rDer p 1 for 30 min at 37°C. As a control, cells were also incubated in the absence of allergen to measure spontaneous release. Total release was obtained by adding 0.5% Triton X-100 to the medium. The release of β-hexosaminidase was analysed by incubating

supernatants (50 μL) with 50 μL p-nitrophenyl-N-acetyl-β-D-glucosaminide (2 mM in 0.2 M citrate buffer, pH 4.5; Sigma) for 3 h at 37°C. The reaction was stopped by adding 150 μL 1 M TRIS-HCl, pH 9, and the absorbance at 405 nm was measured. Results were expressed as percentage of the total release minus the spontaneous release.

Human basophil activation assay

This assay was performed on EDTA-anticoagulated peripheral blood samples obtained from patients with HDM allergy and non-allergic, non-asthmatic subjects from the Asthma clinic, CHU, Liège, Belgium (see above) using an Allergenicity Kit (Beckman Coulter ZA Paris, France) according to the manufacturer's instructions. The nDer p 3, rDer p 3, rDer p 3-S196A, proDer p 3, proDer p 3-S196A or rDer p 1 allergens were used at different concentrations (0.0001–10 μg/mL). An anti-IgE antibody and PBS were used as positive and negative controls, respectively. Leucocytes were analysed using a Navios flow cytometer (Beckman Coulter). Basophils (700 target events) were gated as the $CRTH_2$-positive/CD3-negative population. Responses were quantified as percentages of CD203c-expressing basophils in the Q2 region. This region was adjusted to contain 5% of basophils (i.e. activated basophils) in the unstimulated sample (negative control). A fivefold increase in the number of activated basophils (> 25%) compared with the negative control (5%) was considered to be a positive response [34].

Animals

Female Balb/c mice (6 weeks old) were obtained from Harlan (Horst, the Netherlands). Animal care and experimental procedures were performed in accordance with local institutional guidelines (laboratory licence no. LA1500474).

Immunization protocol

Groups of five Balb/c mice were immunized three times at 2-week intervals with 10 μg rDer p 3, rDer p 3-S196A, proDer p 3 and rDer p 1 adjuvanted with alum (ratio of allergen/adjuvant of 1/200; intraperitoneal injection). As a control, two groups of mice were immunized with adjuvant alone (Naïve and IN). The mice were bled on days 0, 28, 42 and 47, and sera were collected. Except for the naïve group that was sensitized with adjuvant alone, airway inflammation was induced by challenging the mice 14 days after the last sensitization (day 42) by the intranasal instillation of 100 μg of crude *D. pteronyssinus* extract (Greer, Lenoir, NC, USA) for 3 consecutive days. For each mouse, serum, bronchoalveolar lavage fluid (BAL) and the spleen were collected on day 47.

Proliferation and cytokines assays

The rDer p 3- and rDer p 1-specific T-cell proliferative responses were measured. Spleen cells (4×10^5/well/ in triplicate) from immunized mice were stimulated with rDer p 1, rDer p 3, proDer p 3 or with rDer p 3-S196A (1.25 μg/mL). As control, cells were incubated with RPMI medium only. After 72 h, cells were pulsed with [^{3}H]-thymidine (Amersham) for 16 h. Cells were harvested, and [^{3}H]-thymidine uptake was measured by scintillation counting. Proliferate responses were expressed as stimulation index (SI), which corresponds to the ratio of counts per min (CPM) measured in the culture stimulated with the allergen to the CPM measured in the culture medium (RPMI). A stimulation index value > 2 was considered positive.

Production of IFNγ and IL-5 in supernatant from restimulated splenocytes was measured using ELISA kits supplied by BD Pharmingen.

Bronchoalveolar lavage

One day after the final challenge, mice were bled. The lungs were immediately washed via the trachea cannula with 1 mL PBS, which was instilled and gently recovered by aspiration three times. The lavage fluid was centrifuged and the cells were counted in a Thoma hemocytometer (Sigma). Cytospin preparations from 50 μL aliquots were stained with May–Grünwald–Giemsa stain for differential cell counts.

Measurement of Der p 3-specific IgG1 and IgG2a

Immunoplates were coated overnight with rDer p 3-S196A (500 ng/well) at 4°C. Plates were then washed 3 times with Tris-buffered saline–0.01% Tween-80 (TBS-T) and saturated for 1 h at 37°C of the same buffer supplemented with 1% BSA. Serial dilutions of sera in saturation buffer were incubated for 1 h at 37°C. Plates were washed five times with TBST-T buffer. Antigen-bound antibodies (IgG1, IgG2a) were detected with anti-mouse subclass IgG1 or IgG2a monoclonal antibodies (dilution 1 : 7000, Biosource). Phosphatase alkaline conjugated to streptavidin (dilution 1 : 1000, Amersham) was added to each well and the enzymatic activity was measured as described above.

ODs ≥ 3 times the control values were considered to be ELISA-positive results.

Statistical analysis

Group data were compared using a Student's *t*-test. Differences were considered significant for $P < 0.05$.

Results

Characteristics of allergens

The purity of the preparations of allergens was assessed by silver-stained SDS-PAGE analysis (Fig. 1a). As previously shown, no Der p 1 could be detected in Der p 3 preparations [28]. The intrinsic fluorescence and far UV-CD analysis showed that rDer p 3 and its inactive counterpart rDer p 3-S196A exhibit a similar tertiary and secondary structure content, respectively (Figs 1b and c). In contrast, proDer p 3 and proDer p 3-S196A exhibited a blue shift of their maximum emission wavelength (333 nm) compared to those of rDer p 3 and rDer p 3-S196A (350 nm) (Fig. 1b) [33].

The proteolytic activity of Der p 3 reduces its stability

To investigate the effect of the enzymatic activity of Der p 3 on its stability, the active protease, rDer p 3, and its less active precursor form, proDer p 3, were incubated at 37°C, and their catalytic activities were quantified at multiple time points. After 6 h of incubation, rDer p 3 lost approximately 70% of its activity (Fig. 2a), whereas more than 90% of the weak activity exhibited by proDer p 3 was maintained after 24 h. In comparison, rDer p 1 and proDer p 1 conserved more than 75% of their initial activity after 24 h of incubation (Fig. 2b). The higher stability displayed by proDer p 3, rDer p 1 and proDer p 1 was confirmed by SDS-PAGE analysis (data not shown). In contrast, after incubation of 4 h at 37°C, the band corresponding to rDer p 3 (25 kDa) continuously decreased in intensity with time, leading to the appearance of low molecular weight degradation fragments (Fig. 2c). Importantly, the inactive mutant rDer p 3-S196A did not undergo autolysis, even after 24 h of incubation (Fig. 2d). These results clearly show that due to its enzymatic activity, rDer p 3 is far less stable than rDer p 1 and that the propeptide plays an important role in the protection of Der p 3 against spontaneous hydrolysis. Moreover, mutation of the active serine in the enzyme's catalytic site completely abolishes the phenomenon of autolysis.

Underestimation of the IgE-binding capacity of Der p 3 due to autolysis

To determine the impact of the autolysis of Der p 3 during the indirect ELISA ($N = 22$), the IgE-binding capacities of rDer p 3 and nDer p 3 were compared with the capacity of the catalytic mutant rDer p 3-S196A, using rDer p 1 as a reference. Interestingly, both natural

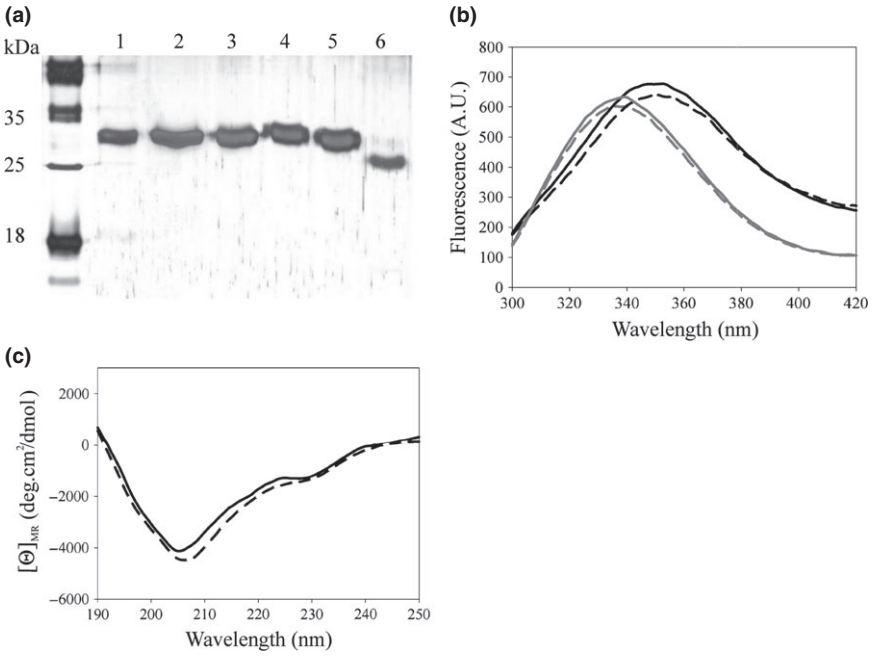

Fig. 1. Characteristics of purified allergens. (a) SDS-PAGE (15%) silver-stained analysis of 4 μM purified 1: nDer p 3, 2: rDer p 3, 3: rDer p 3-S196A, 4: proDer p 3, 5: proDer p 3-S196A, 6: rDer p 1; (b) Intrinsic fluorescence spectra of rDer p 3 (solid black), rDer p 3-S196A (dashed black), proDer p 3 (solid grey) and proDer p 3-S196A (dashed grey); (c) Far UV-CD spectra of rDer p 3 (solid black) and rDer p 3-S196A (dashed black).

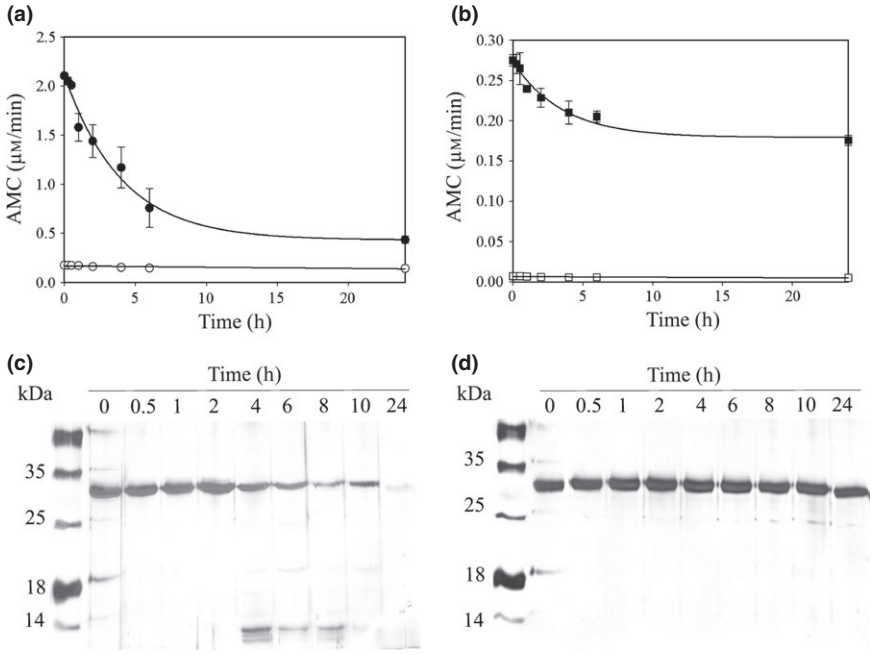

Fig. 2. The proteolytic activity of Der p 3 reduces the protease's stability. (a) For each incubation at 37°C, the residual activity of (a) rDer p 3 (●) and proDer p 3 (○) and (b) rDer p 1 (■) and proDer p 1 (□) was measured in 10-fold diluted aliquots. Standard deviations are < 10%. SDS-PAGE (15%) silver-stained analysis of 4 μM (c) rDer p 3 and (d) rDer p 3-S196A incubated at 37°C during increasing times.

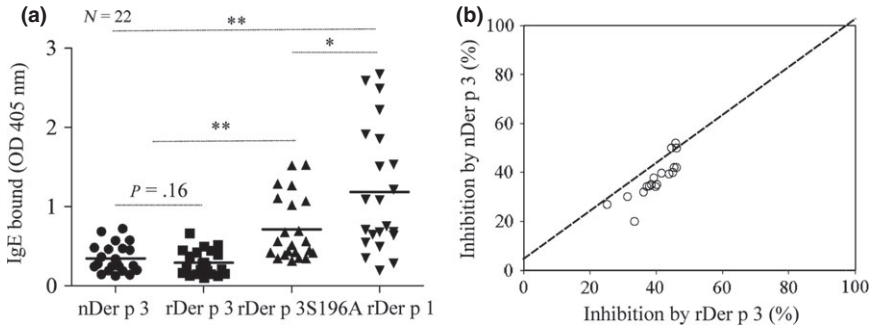

Fig. 3. The autolysis of Der p 3 reduces the protease's IgE reactivity. (a) The IgE reactivity of nDer p 3, rDer p 3, rDer p 3-S196A and rDer p 1 was evaluated by indirect ELISA using positive sera against *Dermatophagoides pteronyssinus* ($N = 22$). *$P < 0.01$ and **$P < 0.001$; (b) Inhibition ($N = 20$) ELISAs were performed using ImmunoCAP-positive sera against *D. pteronyssinus*. The binding of IgE to rDer p 3-S196A was inhibited by nDer p 3 and rDer p 3. The percentage inhibition is shown as 100 – (% IgE binding in the presence of inhibitor/% IgE binding in the absence of inhibitor). The dashed line represents the diagonal.

and recombinant forms of Der p 3 displayed similar IgE-binding reactivity with all of the tested sera (Fig. 3a).

As expected, the IgE reactivity to the stable allergen rDer p 3-S196A (ranging from 0.3 to 1.5; median value of 0.7) was two times higher than the rate measured for rDer p 3 or nDer p 3 (ranging from 0.2 to 0.7 and from 0.1 to 0.7, respectively; median values of 0.3) (Fig. 3a). The negative controls obtained from experiments realized with sera coming from non-allergic patients exhibited values ranging from 0.02 to 0.04.

If we consider approximately 10% and 25% of the tested sera to be Der p 3 positive when considering the active nDer p 3 and rDer p 3 IgE-binding activities, respectively, which is in agreement with the findings of Thomas's group [17], 50% of sera should be Der p 3 positive when considering the IgE response to rDer p 3-S196A.

To evaluate the possibility that Der p 3 may digest IgE during test, sera previously incubated with active Der p 3 during increasing times were analysed by Western blot using an anti-IgE antibody. Noteworthy, no IgE degradation was observed (data not shown) confirming that the discrepancy of IgE-binding rate observed between the active and inactive Der p 3 forms is due to allergen autolysis.

The IgE reactivity of nDer p 3 and rDer p 3 were next analysed by competitive inhibition assays using the same sera pre-incubated with nDer p 3 or rDer p 3 before incubation with solid phase-bound rDer p 3-S196A. The two allergens inhibited the binding of patients' IgE to rDer p 3-S196A in a comparable manner (Fig. 3b; $R = 0.89$). However, strikingly, no more than 60% inhibition was observed. These results indicate again that rDer p 3 retains the relevant IgE epitopes of nDer p 3 and that the IgE-binding capacity of the catalytic mutant rDer p 3-S196A is higher than the capacity of the two active forms.

The recombinant catalytic mutant of Der p 3, rDer p 3-S196A, could thus represent a valuable tool for addressing the underestimation of the prevalence and titre of IgE against Der p 3 in HDM allergy diagnosis.

proDer p 3 has weak IgE reactivity and allergenicity in vitro

To test the hypoallergenic potential of the precursor form of Der p 3, the IgE reactivity of proDer p 3 was evaluated by sandwich ELISA and inhibition assay. The IgE-binding capacity of proDer p 3, estimated by sandwich ELISA, was drastically reduced compared with the capacity of rDer p 3-S196A (Fig. 4a, $P < 0.01$). In the inhibition assay, the IgE reactivity of rDer p 3-S196A was analysed using six patient sera pre-incubated with various concentrations of proDer p 3 or rDer p 3-S196A. The inhibition capacity of proDer p 3 was also drastically reduced in this assay compared with the capacity of rDer p 3-S196A (Fig. 4b). Those results were confirmed using 20 other sera incubated with a fixed proDer p 3 or rDer p 3-S196A concentration (50 μg/mL) (Fig. 4c).

Mediator release assays were performed with RBL cells expressing the human FcεRI receptor passively sensitized with five sera from patients with a *D. pteronyssinus* allergy and stimulated with the different forms of Der p 3 or with rDer p 1, used as a positive control. Overall, the induced degranulation levels were similar for nDer p 3, rDer p 3, rDer p 3-S196A and rDer p 1 and were much higher than those levels induced by proDer p 3 (Fig. 5a). Note that no autolysis of Der p 3 occurred during this rapid test which requires a short incubation of 30 min at 37°C. This finding indicates that the reduced IgE-binding capacity of proDer p 3 is correlated with lower allergenic activity.

To confirm this result in human cells, blood basophils from allergic donors were also stimulated with different concentrations of the different forms of Der p 3 or with rDer p 1. Importantly, CD203c was significantly upregulated (Fig. 5b) in the same manner in basophils stimulated with nDer p 3, rDer p 3, rDer p 3-S196A or rDer p 1, whereas the precursor forms of Der p 3, up to a concentration of 10 μg/mL, did not activate IgE-sensitized

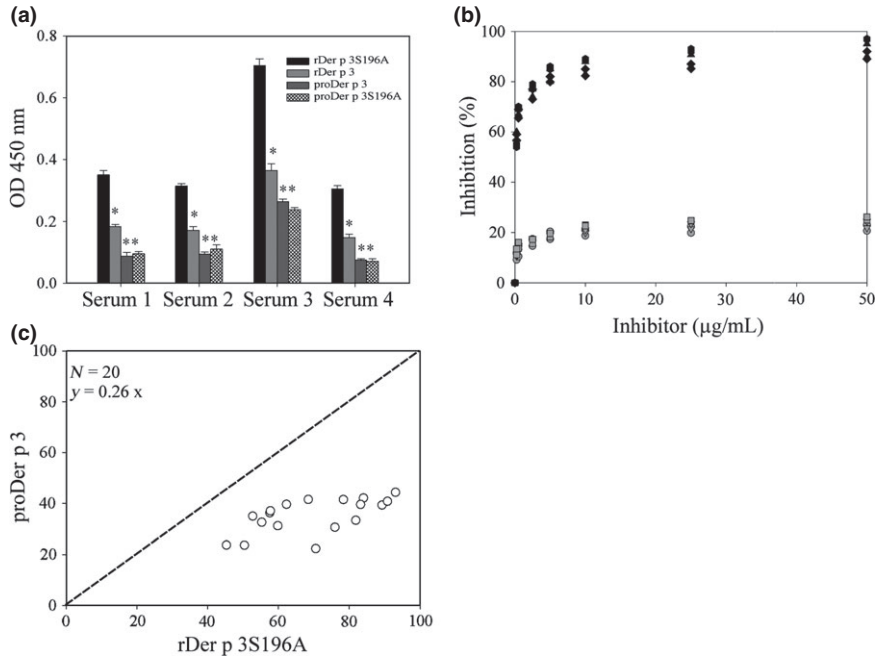

Fig. 4. proDer p 3 exhibits weak IgE reactivity. (a) IgE reactivity measured with a sandwich ELISA using positive sera against *Dermatophagoides pteronyssinus* ($N = 4$). *$P < 0.05$ and **$P < 0.01$. The binding of IgE to rDer p 3-S196A was inhibited by incubating (b) positive sera ($N = 6$, the average curve is shown) with increasing concentrations of rDer p 3-S196A (black panel) or proDer p 3 (grey panel) or by incubating (c) positive sera ($N = 20$) with a fixed concentration (50 μg/mL) of rDer p 3-S196A or proDer p 3.

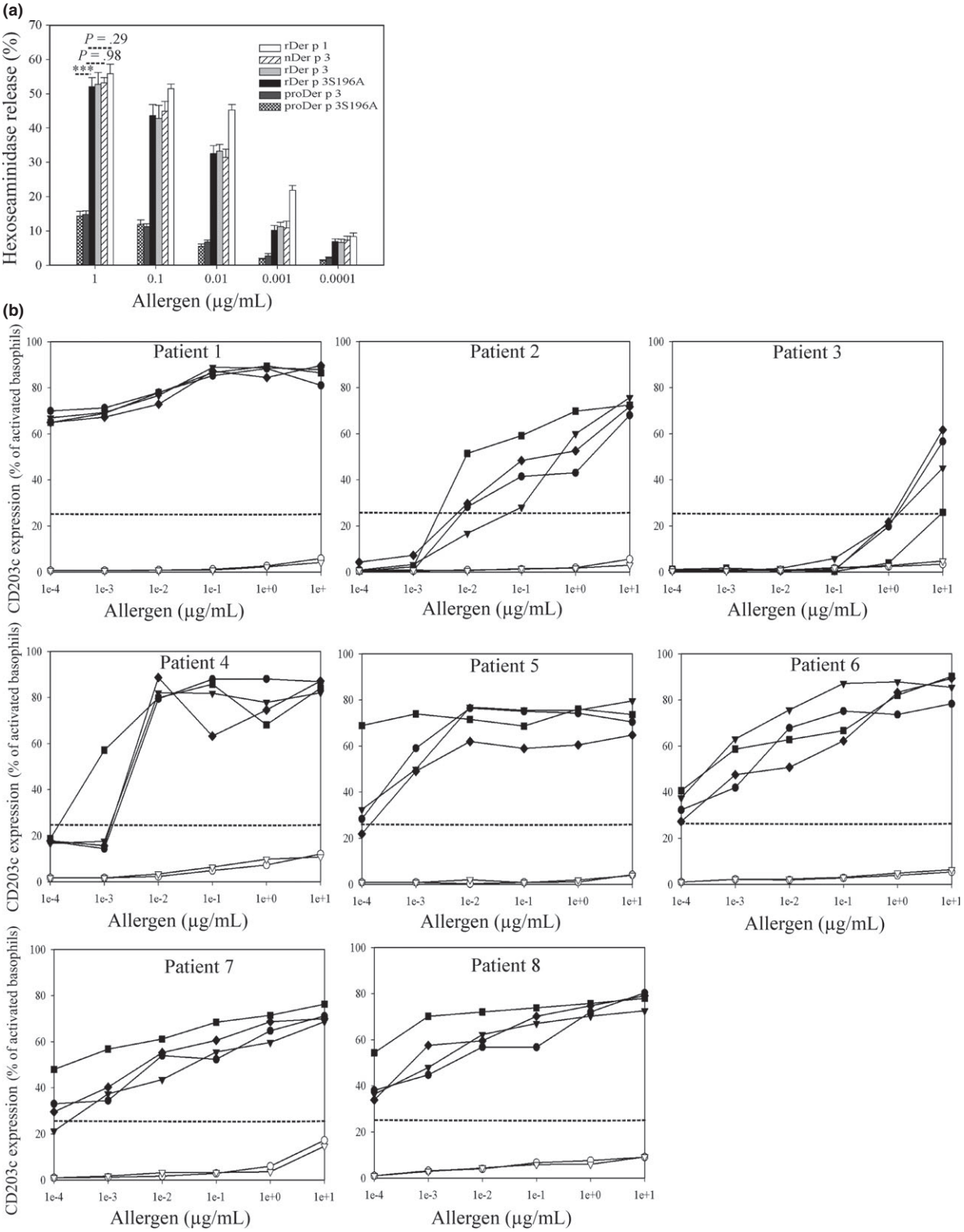

Fig. 5. proDer p 3 displays very weak allergenicity *in vitro*. (a) Mediator release of humanized rat basophil leukaemia (RBL) stimulated with different concentrations of allergens. The results are expressed as the percentage of the total release of β-hexosaminidase. ***$P < 0.001$. (b) CD203c expression at the surface of human blood basophils stimulated with different concentrations of rDer p 1 (■), nDer p 3 (♦), rDer p 3 (●), rDer p 3-S196A (▼), proDer p 3 (○) and proDer p 3-S196A (▽). The percentage of activated basophils is defined as CRTH2/CD203c positive cells.

basophils. Similarly, basophils from a non-allergic individual did not express CD203c when stimulated with the different allergens (data not shown).

In vivo *evaluation of Der p 3 and proDer p 3 allergenicity*

Balb/c mice were sensitized with rDer p 3, rDer p 3-S196A, proDer p 3 or rDer p 1 adjuvanted with alum. As a control, two groups of mice were sensitized with adjuvant alone (Naïve and IN). The pretreated mice (except for the Naïve group) subsequently received HDM extracts by intranasal instillation to provoke airway inflammation (Fig. 6a).

Mice sensitized with rDer p 3 or rDer p 3-S196A exhibited high titres of specific IgE (Fig. 6b) and anti-Der p 3 IgG_1 antibodies (Fig. 6c), whereas the titres of anti-Der p 3 IgG_{2a} antibodies were very low (Fig. 6d). These results demonstrate that rDer p 3 and rDer p 3-S196A induce a typical allergic T_H2-type response, similar to rDer p 1. In contrast, the specific IgE and IgG_1 responses induced by proDer p 3 were significantly lower than those responses induced by rDer p 3 (Fig. 6c, $P < 0.001$). Moreover, proDer p 3 induced the significant production of anti-Der p 3 IgG_{2a} (Fig. 6d, $P < 0.001$). These results clearly suggest that proDer p 3 induces a T_H1-biased immune response, even in the presence of a pro-T_H2 adjuvant such as alum.

Splenocytes isolated from mice sensitized with proDer p 3, rDer p 3 or rDer p 3-S196A were stimulated *ex vivo* with rDer p 3. The cells's proliferation levels were similar for the different groups of mice (Fig. 6e). Moreover, after their stimulation with rDer p 3, proDer p 3 or rDer p 3-S196A, the stimulus index was similar (data not shown), thus suggesting that proDer p 3 retains relevant Der p 3-specific T-cell epitopes. By contrast, splenocytes isolated from mice that were sensitized with different forms of Der p 3 and stimulated *ex vivo* with rDer p 1 and vice versa did not proliferate, thereby serving as a negative control.

Concomitantly, supernatants from cultured stimulated lymphocytes were harvested and analysed for IFN-γ and IL-5. A typical T_H2 cytokine profile, characterized by the high production of IL-5 and low or no secretion of IFN-γ, was elicited in rDer p 3- and rDer p 3-S196A-sensitized mice (Fig. 6f). Again, the T_H1 bias of the immune response induced by proDer p 3 was confirmed because animals sensitized with proDer p 3 secreted high and low amounts of IFN-γ and IL-5, respectively, upon restimulation (Fig. 6f, $P < 0.001$).

Finally, animals sensitized with rDer p 3 and challenged with HDM extracts developed pronounced airway eosinophilia in the BAL fluids (Fig. 6g, $P < 0.01$). This hypereosinophilia was similar to that observed in mice sensitized with rDer p 1. In contrast, eosinophils were not observed in BAL from mice sensitized with proDer p 3.

Discussion

Der p 1 and Der p 2 are described as the two major allergens of *D. pteronyssinus* because these allergens correspond to 70–100% of the total IgE-binding activity of HDM extracts [17, 18, 35]. However, the IgE-binding activity of these extracts could be biased due to the lack or to the poor stability of several allergens [9]. This bias is observed for mite serine proteases that are highly sensitive to degradation, which can compromise diagnosis [26, 27]. In particular, our group and others have shown that nDer p 3 and rDer p 3 undergo to autolysis after 4 h of incubation at 37°C [26, 28]. Here, we investigated the allergenicity of both forms and compared the allergenicity to that of the catalytically inactive mutant rDer p 3-S196A. Thus, for the first time, we evaluated the role of the degradation of an allergenic protease in the context of HDM allergy diagnosis.

Indirect ELISAs and inhibition assays showed that the IgE reactivities of nDer p 3 and rDer p 3 are highly similar, suggesting that the main IgE-binding epitopes of nDer p 3 are present in the protease's recombinant form. Accordingly, the two forms induced a similar release of β-hexosaminidase by humanized basophils in RBL assays. More interestingly, the forms also induced a similar upregulation of CD203c expression on the surface of blood basophils from allergic donors. These results, together with those findings obtained in our previous study, indicate that rDer p 3 has the same structural, biochemical and allergenic properties as its natural counterpart [28].

However, ELISAs and competitive inhibition assays performed with rDer p 3 and rDer p 3-S196A revealed that the rDer p 3's instability during tests due to its proteolytic activity drastically decreases its IgE-binding capacity. It is thus likely that the IgE-binding activity of Der p 3 was underestimated in the past due to the autolysis of nDer p 3 during testing. Using rDer p 3-S196A in ELISAs, we estimated a new prevalence of Der p 3 of approximately 50%. Thus, this catalytic mutant, rDer p 3-S196A could be considered as a suitable alternative for the diagnosis of HDM allergy in the future.

Moreover, due to its proteolysis, the concentration of nDer p 3 in the HDM extracts must decrease with time. Therefore, in the context of immunotherapy with HDM extracts, it would be of interest to stabilize nDer p 3 protein using specific serine protease inhibitors treatment. Additionally, rDer p 3-S196A variants could be used to develop a robust and accurate ELISA allowing the quantification of Der p 3 in the HDM extracts. Such

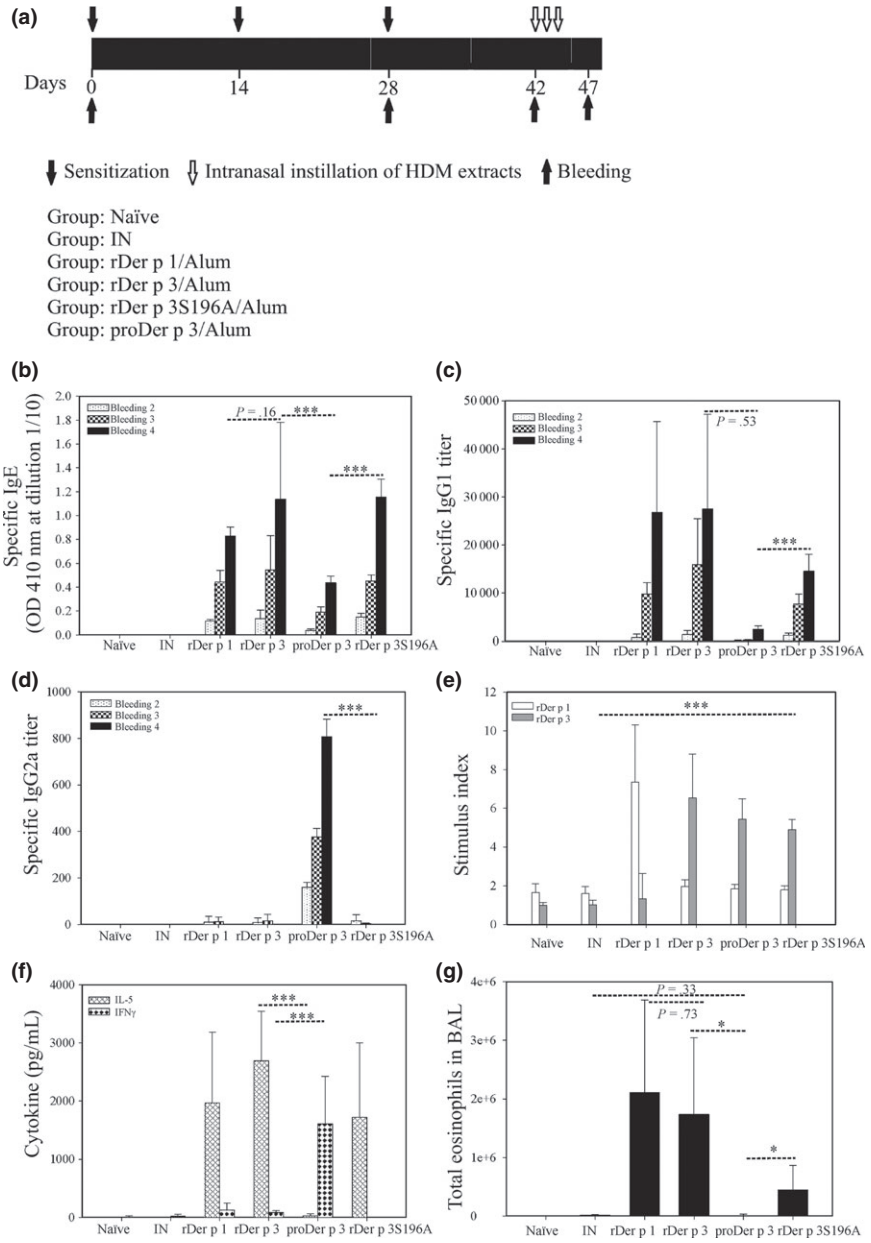

Fig. 6. Evaluation of experimental house dust mite (HDM) allergy by sensitization with proDer p 3. (a) Experimental protocol for the evaluation of immune responses in naïve and sensitized mice. Antibody titres [(b) IgE, (c) IgG_1 and (d) IgG_{2a}], (e) the lymphoproliferative response and (f) the splenocyte cytokine production of *in vitro*-stimulated splenocytes. (g) Airway eosinophilia. The results are expressed as the means of individual titres ± SD from five mice.

an assay could also be applied to assess the stability of the extracts over time and contribute to the standardization of HDM materials.

If the broader use of rDer p 3-S196A reveals that the allergenic role of Der p 3 has been underestimated in allergic subjects, the development of Der p 3-based hypoallergens would be interesting. Here, we describe the immunological characterization of recombinant proDer p 3 as a hypoallergen of nDer p 3. Although the secondary structures of nDer p 3 (24.9 kDa) and proDer p 3 (26.4 kDa) were previously shown to be similar [28], the intrinsic fluorescence spectra of these proteins, which are characteristic of the proteins' tertiary structures, are different [33]. Indeed, when compared with the precursor proDer p 3, the mature forms, nDer p 3 and rDer p 3, exhibit a red shift of their maximum emission wavelength, suggesting the rearrangement of the catalytic domain of the protease after maturation. This phenomenon, affecting the loops but not the secondary structures, has been reported during the activa-

tion of trypsin [33]. The role of the Der p 3 propeptide in allergenicity was thus evaluated. For all tested sera, proDer p 3 showed less allergenicity than each form of Der p 3 (n, r or S196A) in sandwich ELISAs and in competitive inhibition, RBL and human basophil activation assays, as shown for the proDer p 1-Der p 1 couple [29]. The mechanisms behind the weak IgE-binding activity of proDer p 3 are not clear. This mechanism could be related to the blocking of conformational IgE-binding sites on the surface of the protease domain by the N-terminally located propeptide, as was demonstrated for proDer p 1 [29], or to the increase of epitopes accessibility after protease activation.

We thus compared the immunogenicity of proDer p 3 and the mature forms of Der p 3 in mice. Mice sensitized with rDer p 3 exhibited a typical T_H2-biased immune response, as described for Der p 1 [30], characterized by high titres of Der p 3-specific IgG_1 and IgE and the absence of IgG_{2a} production. In contrast, proDer p 3 induced a T_H1-biased immune response characterized by the production of specific IgG_{2a} and the weak production of specific IgE antibodies over time. The cytokine pattern of T-cell proliferation confirmed that proDer p 3 is a T_H1 inducer because the drastic reduction of IL-5 production was accompanied by enhanced IFN-γ secretion. proDer p 3 immunization also drastically reduced the infiltration of eosinophils in the BAL fluids, which is most likely correlated with low IL-5 secretion and reduced IgE-dependent mast cell activation in proDer p 3-sensitized mice after intranasal instillation with HDM extracts [36]. Together with the maintenance of the Der p 3-specific T-cell reactivity, these data clearly increase the interest for the use of hypoallergenic proDer p 3, as a safe recombinant vaccine in allergen-specific immunotherapy. It is noteworthy that the rDer p 3 and rDer p 3-S196A allergens induced both a similar humoral and cellular immune response *in vivo*; which confirms that the IgE epitopes of Der p 3 are maintained in the catalytically deficient mutant. However, the infiltration of the eosinophils in the BAL fluids was reduced for rDer p 3-S196A, which indicates that the proteolytic activity of Der p 3 is important to trigger this inflammation mechanism. Indeed, it was previously shown that Der p 3 induces the activation of the PAR-2 receptor and that PAR-2 mediates eosinophil infiltration [25, 37]. Finally, the treatment of asthmatic mice with serine protease inhibitors decreased the eosinophilia [38].

In conclusion, we report the first example of the underestimation of the IgE-binding capacity of an allergen due to its instability. We suggest that the variant rDer p 3-S196A should be used to correctly evaluate Der p 3-specific IgE binding in the context of HDM allergy diagnosis. Moreover, based not only on a murine model but also on human blood basophil experiments, our data clearly show that proDer p 3 could be used as a hypoallergenic variant of Der p 3 suitable for allergen-specific, systemic immunotherapy.

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Conflict of interests

The authors declare no conflict of interest.

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The House Dust Mite Major Allergen Der p 23 Displays O-Glycan-Independent IgE Reactivities but No Chitin-Binding Activity

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Key Words
Allergen · Chitin · Der p 23 · House dust mite · IgE reactivity · O-glycosylation

Abstract
Background: The in-depth characterization of the recently identified house dust mite (HDM) major allergen Der p 23 requires the production of its recombinant counterpart because the natural allergen is poorly extractable from fecal pellets. This study aimed to provide a detailed physicochemical characterization of recombinant Der p 23 (rDer p 23) as well as to investigate its IgE reactivity in a cohort of HDM-allergic patients from Thailand. ***Methods:*** Purified rDer p 23, secreted from recombinant *Pichia pastoris*, was characterized by mass spectrometry and circular dichroism analyses as well as for its chitin-binding activity. The IgE-binding frequency and allergenicity of Der p 23 were determined by ELISA and RBL-SX38 degranulation assays, respectively. ***Results:*** Purified intact rDer p 23 carried O-mannosylation and mainly adopted a random coil structure. Polyclonal antibodies to rDer p 23 can detect the corresponding natural allergen (nDer p 23) in aqueous fecal pellet extracts, suggesting that both forms of Der p 23 share common B-cell epitopes. Despite its homologies with chitin-binding proteins, both natural Der p 23 and rDer p 23 were unable to interact in vitro with chitin matrices. Of 222 Thai HDM-allergic patients tested, 54% displayed Der p 23-specific IgE responses. Finally, the allergenicity of rDer p 23 was confirmed by the degranulation of rat basophil leukemia cells. ***Conclusion:*** Our findings highlighted impor-

The members of the MARC Study Team are listed in the Appendix.

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tant levels of Der p 23 sensitizations in Thailand. Our study clearly suggested that rDer p 23 is likely more appropriate for HDM allergy component-resolved diagnosis than HDM extracts.

Introduction

The house dust mite (HDM) *Dermatophagoides pteronyssinus* is recognized as one of the most common sources of indoor airborne allergens triggering allergic sensitizations in approximately 20% of the population from industrialized countries [1, 2]. Up to now, 17 different *D. pteronyssinus* allergen groups have been referenced and classified as major, intermediate or minor allergens [3]. Der p 1, a cysteine protease, and Der p 2, a LPS-binding protein, are considered as the most potent HDM allergens. Indeed, these two proteins are not only abundant in the bodies and feces of mites [4] but their IgE reactivities can reach up to 80–90% in HDM-allergic patients [5, 6]. A new 8-kDa HDM allergen from fecal pellets, namely Der p 23, was recently classified as a major allergen. Indeed, in a European study, 74% of the HDM-allergic patients tested developed Der p 23 sensitization [7]. Such high IgE-binding frequency was unexpected because natural Der p 23 (nDer p 23) has been shown to be present only in tiny amounts in fecal pellets and in house dust and also poorly extracted under aqueous conditions. This limited nDer p 23 release could likely be explained by the putative association of the allergen to the peritrophic matrix of mite feces, a semipermeable structure rich in proteins and chitin, which can protect the mite mid-gut from damage by food particles during digestion [8]. The difficulty of sourcing nDer p 23 could be circumvented through heterologous expression of recombinant Der p 23 (rDer p 23) which could provide virtually unlimited amounts of this allergen. Although the characterization of Der p 23 was mainly performed based on rDer p 23 produced in *Escherichia coli* [7], a detailed analysis of the Der p 23 amino acid sequence suggested that this protein could contain post-translational modifications via the presence of (1) a N-terminal leader sequence, (2) two regions rich in threonine residues prone to O-glycosylation and (3) a putative C-terminal chitin-binding domain containing 4 cysteine residues involved in 2 disulfide bonds. Such modifications could be important for both the folding as well as the biological and allergenic properties of Der p 23.

We report here the expression of rDer p 23 in *Pichia pastoris*. Contrary to *E. coli*, this yeast strain contains chitin and can express and secrete O-glycosylated proteins with appropriate disulfides. Following expression and purification, the prevalence of IgE reactivity to rDer p 23 has, for the first time, been examined in a cohort of HDM-allergic subjects from Thailand.

Materials and Methods

Cloning of rDer p 23 in P. pastoris

The mature Der p 23 coding gene sequence (207 bps corresponding to aa 22–90, with the numbering based on L7N6F8 Uniprot sequence) was amplified from total cDNA of *D. pteronyssinus* by PCR. Primers used were designed to incorporate *Xho*I and *Not*I enzyme restriction sites (underlined) at the 5′- and 3′-end of the amplicon, respectively; forward primer: 5′-GGCTCGAGAAAAGAGAGGCTGAAGCTGCCAATGATAATGATGATGATCCTACCAC-3′; reverse primer: 5′-CCGCGGCCGCTTAAGTGCATGTTTCTTCATCTTC-3′. The purified PCR product was subsequently double-digested with *Xho*I and *Not*I and cloned into the yeast expression vector pPICZαA (Invitrogen) restricted by the same enzymes. The *P. pastoris* KM71 strain was subsequently transformed with the recombinant plasmid, pPICZαA-Der p 23, previously linearized with *Bgl*II, by electroporation using the Gene Pulser Xcell™ (Bio-Rad) at 1,500 V, 200 Ω in 1-mm electrode cuvette. The transformants were spread on YPDS agar plate (1% yeast extract, 2% peptone, 2% dextrose, 1 M sorbitol and 2% agar) containing 100 μg/ml Zeocin (Invitrogen) and incubated at 30°C for 2–3 days.

Expression and Purification of rDer p 23

A colony PCR-positive clone was selected to inoculate an overnight culture in BMGY broth at 30°C under shaking conditions. The cells were subsequently pelleted and resuspended in BMMY containing 2% methanol to a final $OD_{600\,nm}$ of 5 to induce the protein expression. Methanol was added every 24 h to maintain the 2% concentration. For the purification of rDer p 23, the culture supernatant was collected by centrifugation after 48 h of induction and directly diluted 10 times with MilliQ water prior to pH adjustment to pH 3. The medium was subsequently applied to a S Sepharose XL column (GE Healthcare Life Sciences) equilibrated with 50 mM glycine-HCl (pH 3). Elution was carried out in step-wise increases of NaCl concentration. The eluted fractions were immediately neutralized with 1 M Tris-HCl (pH 9). Fractions containing rDer p 23 (200 mM NaCl) were concentrated by ultrafiltration with a 3-kDa cut-off membrane (PALL) followed by gel filtration onto a Superdex 75 HR column 10/30 (Life Sciences) equilibrated with PBS (pH 7.3). The fractions containing purified rDer p 23 were pooled and stored at –20°C for further analysis. Protein concentration was determined using a BCA protein assay kit (Pierce).

Circular Dichroism

Purified rDer p 23 was first dialyzed against 10 mM NaH_2PO_4 buffer (pH 4.8). Secondary structure composition of 0.2 mg/ml rDer p 23 was measured with Jasco J-815 circular dichroism (CD) spectrometer in a 0.1-cm-path-length quartz cuvette. CD spectra from 190–260 nm were recorded with 1 nm resolution at a speed of 50 nm/min for 5 cycles. Data were converted to mean residue

weight ellipticity $(\theta)_{mrw}$ (degrees · cm^2 · $dmol^{-1}$) and then analyzed with CDPro software.

Mite Feces Extraction and Immunoblot

Purified feces (36 mg), separated by sequential sieving of *D. pteronyssinus* culture were extracted in 200 µl of PBS (137 mM NaCl, 2.7 mM KCl, 10 mM phosphate buffer, pH 7.3) at 4°C for 16 h under shaking conditions. Soluble extract was collected by centrifugation at 13,000 rpm. nDer p 23 was detected in the fecal extracts by Western blot following 15% SDS-PAGE and protein transfer onto a nitrocellulose membrane (Bio-Rad). The membrane was then blocked with PBS, 1% (w/v) BSA and 0.05% (v/v) Tween 20 at 4°C for 16 h. Next, the membrane was incubated with mouse anti-rDerp 23 polyclonal serum (the serum was produced through animal immunizations with purified rDer p 23 formulated with Alum adjuvant; data not shown) at a 1/2,000 dilution. As a control, the membrane was also incubated with preimmune serum. The membrane was further incubated with goat anti-mouse antibodies conjugated with horseradish peroxidase (KPL) at 1:5,000 dilution. The immunoreactive bands were detected using the chemiluminescent horseradish peroxidase substrate (Millipore) and X-ray film exposure (Kodak).

Nano-Electrospray Ionization Mass Spectrometry Analysis: Direct Infusion

Prior to the nano-electrospray ionization mass spectrometry (nanoESI-MS) analysis, 20 µg of rDer p 23 was desalted with a C_4 ZipTip (Merck Millipore) and was eluted in 50 µl of 50% acetonitrile/0.1% formic acid. Next, the sample was infused at 1 µl/min into an Impact HD mass spectrometer equipped with a Captive-Spray source (Bruker Daltonics). Acquisitions were performed in positive mode with the end-plate offset and capillary voltages set at –500 and 1,200 V, respectively. The drying gas flow rate and dry heater were set at 4.0 liters/min and 180°C, respectively. MS spectra were acquired over the m/z range of 50–3,000 with a scan rate of 1 Hz. MS calibration was performed using the internal lockmass at m/z 1221.9906 (Agilent). Charge state deconvolution was performed using the maximum entropy algorithm.

Chitin-Binding Assay

Chitin beads (New England Biolabs) and shrimp chitin (Sigma) were used in the affinity binding assay. The insoluble chitin (50 µl of chitin bead suspension or 1 mg of shrimp chitin) was equilibrated in PBS and incubated with 20 µg rDer p 23, rDer p 5 (as a negative control), wheat germ agglutinin (WGA, as a positive control, Vector) or 100 µl of HDM fecal extracts at room temperature for 60 min with orbital shaking. Supernatants were collected by centrifugation at 13,000 rpm for 5 min and the chitin pellets were washed 5 times with PBS. Bound proteins were then eluted by incubating the chitin pellet in Laemmli buffer at 95°C for 5 min. Both supernatant and eluted fractions were analyzed on 15% SDS-PAGE and Coomassie blue staining, whereas the HDM fecal extracts were detected by immunoblot with specific rDer p 23 antibodies.

Alpha Mannosidase Digestion

Purified rDer p 23 was digested with Jack bean α-mannosidase (Sigma-Aldrich) at a 1:20 enzyme/substrate ratio in a pH 4 buffer at 37°C for 16 h. The solution was neutralized with 1 M Tris-HCl pH 9 and ultrafiltrated through a 30-kDa cut-off membrane (Vivaspin, GE Healthcare) to remove mannosidase. The flow-through containing rDer p 23 was subsequently concentrated through a 3-kDa cut-off membrane (Vivaspin, GE Healthcare). The filtrate was collected and the protein concentration was determined using a BCA protein assay kit (Pierce).

IgE Reactivity to rDer p 23

Sera from 222 patients with HDM-associated allergic rhinitis or asthma were obtained from the King Chulalongkorn Memorial Hospital (n = 95), Children Hospital (n = 81), Ramathibodi Hospital (n = 32) and Phramongkutklao Hospital (n = 14), respectively. The *D. pteronyssinus*-specific IgE antibodies (0.36–100 kU_A/l) were assayed with the ImmunoCAP System (Phadia). Each patient provided written informed consent. As negative controls, sera from non-allergic subjects were also collected (<0.35 kU/l, n = 67). The study was approved by the Ethics Committees of the Faculty of Medicine, Chulalongkorn University (IRB 023/55), the Children Hospital (IRB 195/2556), the Faculty of Medicine, Ramathibodi Hospital, Mahidol University (IRB 03-56-34) and the Phramongkutklao College of Medicine (IRB S039Q/57_EXP).

For the IgE-binding assays, ELISA microplates were coated with 500 ng/well of rDer p 23 or deglycosylated rDer p 23 or rDer p 2 at 4°C for overnight. The plates were then washed with PBS-Tween 20, 0.05% (PBS-T) and blocked with PBS-T containing 1% BSA (PBS-T-BSA) for 1 h at 37°C. Serum samples were diluted at 1/8 in PBS-T-BSA and incubated at 37°C for 1 h. The plates were then washed again with PBS-T and further incubated with a 1/5,000 dilution of goat biotinylated anti-human IgE (KPL) at 37°C for 1 h. Next, the plates were washed with PBS-T and incubated with a 1/2,000 dilution of streptavidin-peroxidase (BD Biosciences) for 1 h at room temperature. The allergen-antibody complex was detected with TMB substrate (BD Biosciences) and the reaction was stopped with 0.5 M sulfuric acid. OD was determined at 450 nm using an iMark microplate reader (Bio-Rad). Patient serum was considered positive when the measured OD value was higher than the cut-off value, established as the mean OD values of negative control sera plus 2 standard deviations.

Rat Basophil Degranulation Assay

Genetically modified rat basophil leukemia (RBL) cell line expressing human FcεRI receptor, RBL-SX38, were cultured in 96-well culture plate at a cell density of 1.5×10^5 cells/well in RPMI 1640 medium supplemented with 10% FBS at 37°C for 3 h [9]. Cells were subsequently primed for 16 h with human sera (positive or negative for rDer p 23 or rDer p 2-specific IgE) at a 1/10 dilution. Cells were then washed with PBS and incubated with phenol-free RPMI 1640 containing 1 mg/ml BSA for 15 min before the addition of serial rDer p 23 or rDer p 2 dilutions (0.00001 up to 1 µg/ml) for 30 min. To evaluate the maximum degranulation, control cells were treated with 5% (v/v) Triton X-100. The β-hexosaminidase release was measured following incubations of culture supernatant (50 µl) into the same volume of prewarmed 2.5 mM p-nitrophenyl N-acetyl-β-D-glucosaminide for 3 h. Finally, the reaction was stopped with 150 µl of 1 M Tris-HCl (pH 9) and $A_{415\ nm}$ was subsequently read with an iMark microplate absorbance reader (Bio-Rad).

Statistical Analysis

Data were compared using Student's t test. $p < 0.05$ was considered to be significant.

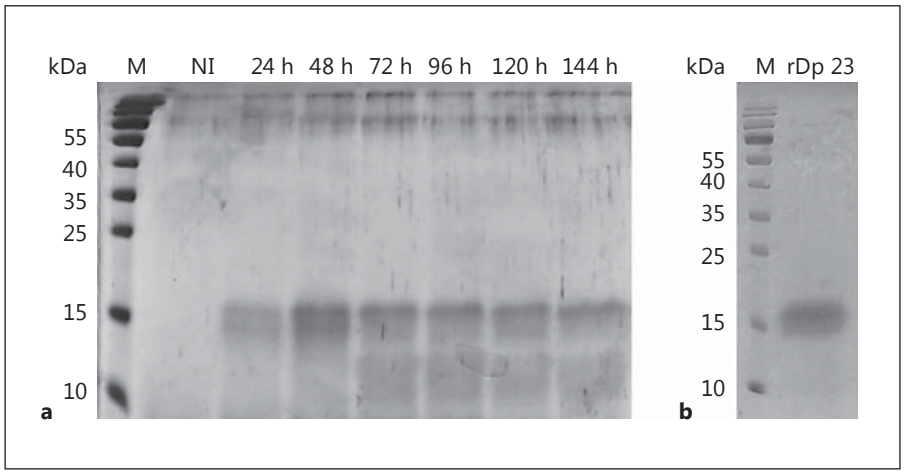

Fig. 1. Expression and purification of rDer p 23 from transformed KM71 *P. pastoris*. **a** SDS-PAGE analysis of rDer p 23 secretion profile under 2% methanol induction at 24, 48, 72, 96, 120 and 144 h. NI = Non-induced. **b** Purified rDer p 23 after ion exchange and gel filtration chromatography. M = Molecular weight markers.

Results

Cloning, Expression and Purification of Mature rDer p 23

Using 2 specific primers derived from the sequence EU414751.1 (GenBank accession No. [7]), we successfully amplified the cDNA encoding the mature form of Der p 23 (aa 22–90) from a *D. pteronyssinus* total cDNA preparation. The DNA sequence of the amplicon perfectly matched the one from the GenBank database. The mature Der p 23 coding sequence was subsequently cloned into *P. pastoris* pPICZαA expression vector, directly downstream to the yeast α-mating factor leader sequence for the secretion of the allergen without any additional amino acids.

Following the transformation of the *P. pastoris* KM71 strain with the recombinant linearized plasmid, zeocin-resistant colonies were screened for the presence of Der p 23 cDNA by colony PCR. Positive colonies were cultured in shake flasks at 30°C and expression of rDer p 23 was triggered by the addition of methanol into the culture medium at a final concentration of 2%. The methanol induction was shown to stimulate the production of secreted rDer p 23, which migrated as a 15-kDa protein on SDS-PAGE (fig. 1a). The expression was shown to reach a maximum after 48 h of induction. For a longer expression period, Der p 23 degradation products migrating at about 10 kDa were observed (fig. 1a). MS analysis of SDS-PAGE bands confirmed that both the 10- and 15-kDa bands contain rDer p 23-related sequences, although the mature Der p 23 theoretical molecular weight deduced from the amino acid sequence was 7.9 kDa. The 15-kDa band corresponded with the intact mature rDer p 23 bearing hexose residues (8–16 residues) with a mass ranging from 9–10.5 kDa, while the 10-kDa band corresponded with truncated rDer p 23 with no hexose and with a mass of approximately 6.5 kDa (data not shown). rDer p 23 was purified to homogeneity from the culture supernatants by a combination of cation exchange and size-exclusion chromatographies (fig. 1b). The SDS-PAGE profile of purified rDer p 23 did not show the presence of a dimeric form as previously reported by Weghofer et al. [7] for rDer p 23 produced in bacteria.

Der p 23 Produced in P. pastoris Is an O-Glycoprotein

The nanoESI-MS characterization of purified rDer p 23 demonstrated the presence of N-terminal sequence heterogeneity, but no host cell proteins were detected. Together with a large percentage of intact, full-length, mature rDer p 23 (aa 22–90, 77%), 4 truncated forms of rDer p 23 were detected, i.e. A (aa 33–90, 5%), B (aa 36–90, 4%), C (aa 37–90, 10%) and D (aa 39–90, 3%; fig. 2a). Noteworthy, no mass increment associated with hexose residues (162.05 Da) was observed on these fragments. Only the intact form of rDer p 23 carried such oligosaccharide structures, suggesting that threonine residues at positions 30–32 could represent an O-glycosylation site (fig. 2a). Quadrupole-time-of-flight MS investigations also established that rDer p 23 has 2 intra-disulfide bonds through the determination of the protein experimental mass (7,972.37 Da; fig. 2a), which fitted with the nonreduced theoretical monoisotopic mass of Der p 23 (aa 22–90, 7,972.37 Da).

As proteins produced in yeast are commonly O-glycosylated through mannose residues [10], we then treated rDer p 23 with Jack bean α-mannosidase which

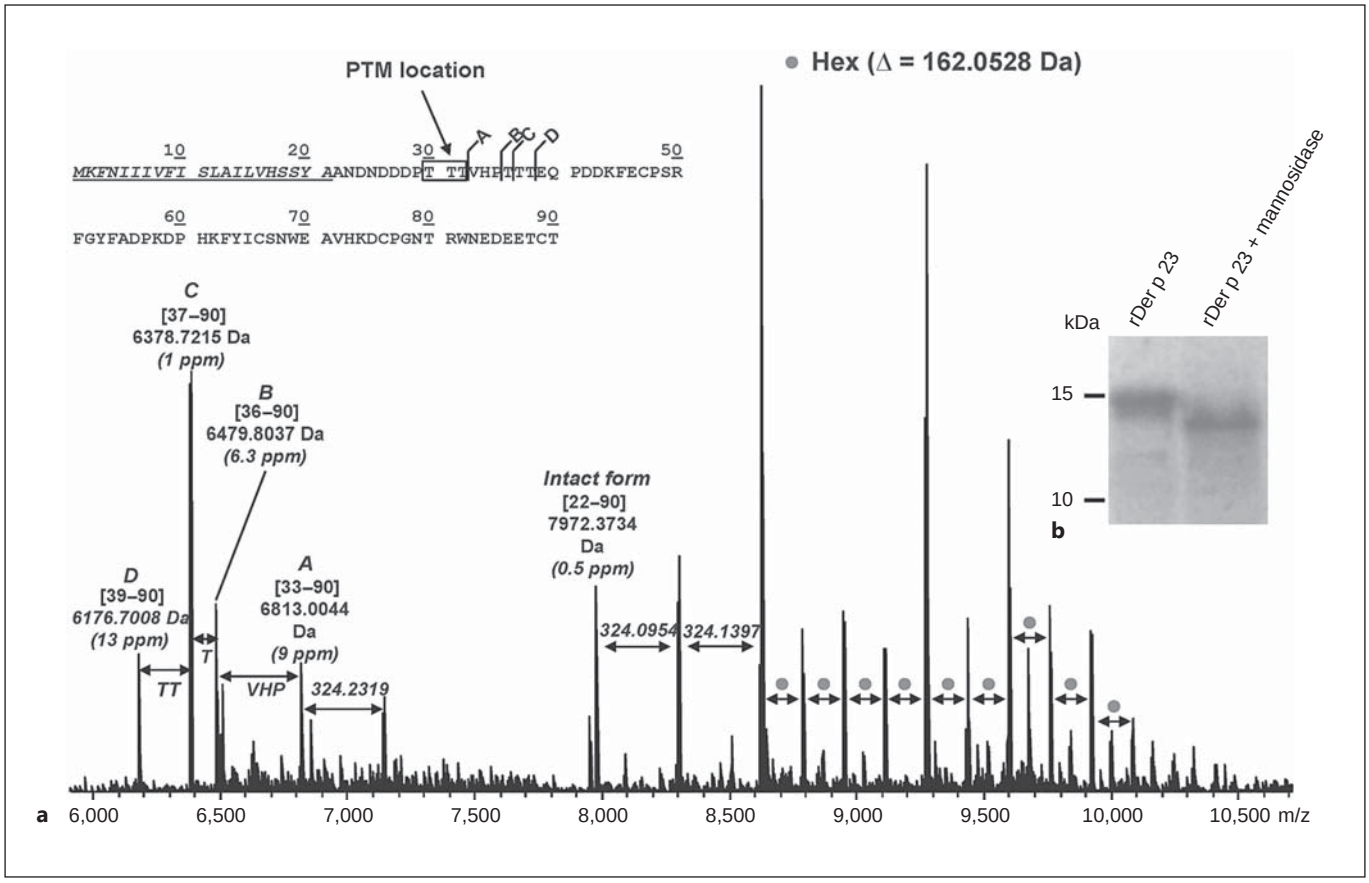

Fig. 2. NanoESI-MS analysis and deglycosylation of rDer p 23. **a** Deconvoluted mass spectrum of purified rDer p 23. The full-length amino acid sequence of Der p 23 is shown on the top left corner. Amino acid sequence 1–21 (underlined) is the signal peptide while mature intact form of rDer p 23 consists in stretch 22–90. Four truncated forms of rDer p 23 are labeled: A (aa 33–90), B (aa 36–90), C (aa 37–90) and D (aa 39–90). Hexose residues are represented with dots. **b** Purified rDer p 23 was incubated with α-mannosidase overnight at 37°C and analyzed by SDS-PAGE. Purified rDer p 23 was also incubated at the same condition as controls. PTM = Post-translational modification.

cleaves α-D-mannose residues [11]. As shown in figure 2b, the molecular weight of rDer p 23 treated with the mannosidase was reduced by 2–3 kDa, confirming that the hexose structures are actually due to O-mannosylation. Altogether, our data indicated that rDer p 23 produced in *P. pastoris* is O-mannosylated at the Thr stretch 30–32.

CD Analysis of Purified rDer p 23

Far-UV CD analyses showed that rDer p 23 is composed of 6% α-helix, 11% β-sheet, 22% turn and 61% random coil structure (fig. 3a). Near-UV spectra of rDer p 23 under reducing conditions with DTT showed structural changes compared to the non-reduced samples, most likely because of the presence of 2 disulfides (fig. 3b).

Detection of nDer p 23 in HDM Feces Extracts

Through chemiluminescence detection, the specific antibodies raised to rDer p 23 were also able to detect nDer p 23 from aqueous extracts of purified HDM fecal pellet preparations (fig. 4). Our results suggest that the recombinant and natural forms of Der p 23 share common B cell epitopes.

IgE Reactivity to rDer p 23

The IgE-binding frequency to rDer p 23 was evaluated in a cohort of Thai HDM-allergic patients (n = 222) by indirect ELISA. All the selected sera displayed an ImmunoCap *D. pteronyssinus*-specific IgE value >0.35 kU_A/l. Of the HDM-positive sera (n = 119), 54% showed specific IgE reactivity to rDer p 23 whereas IgE reactiv-

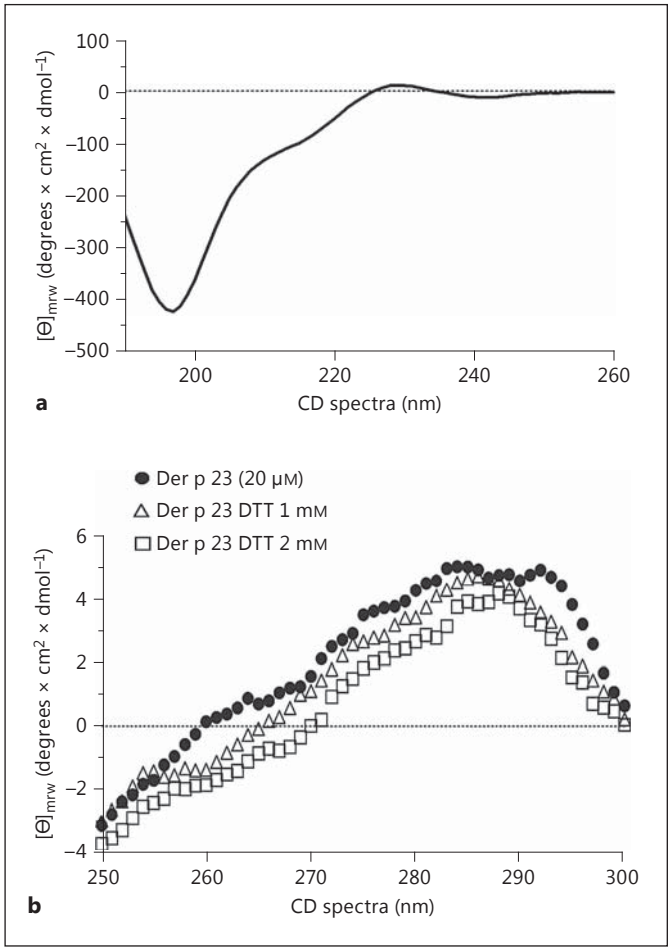

Fig. 3. UV-circular dichroism analysis of purified rDer p 23. **a** CD spectra of untreated purified rDer p 23 at 25°C. **b** rDer p 23 was treated with 1 or 2 mM DTT. All the analyses were done using a J-815 CD spectrophotometer.

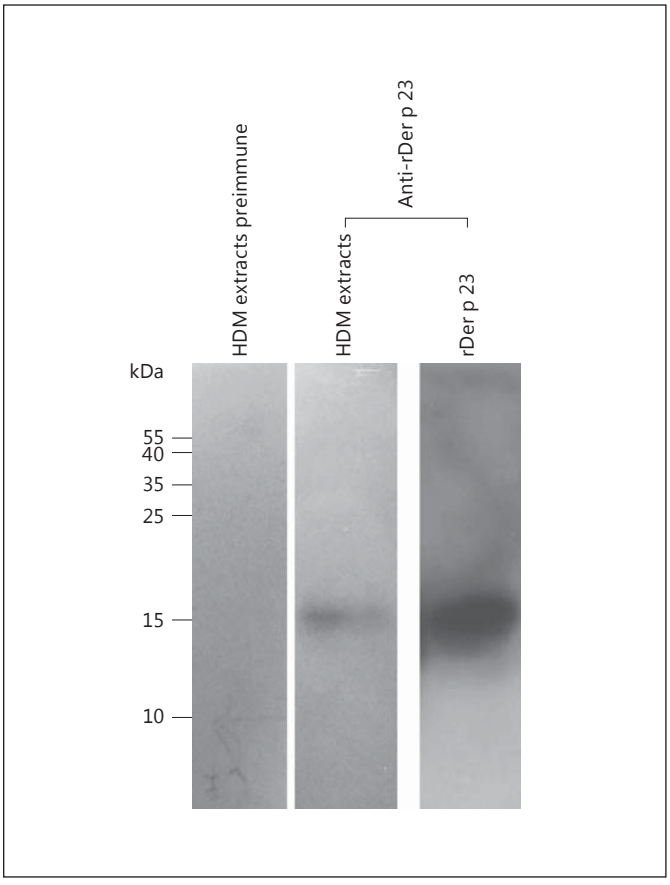

Fig. 4. Detection of Der p 23 in HDM feces from *D. pteronyssinus*. Nitrocellulose-blotted feces extracts were incubated with mouse polyclonal antibody against rDer p 23 and preimmune serum of a Der p23-immunized mouse. Bound antibodies were detected with rabbit anti-mouse-HRP and the luminescence signal was captured by radiography film.

ity to rDer p 2 was detected in 67% of the same mite-allergic individuals (n = 148), confirming that Der p 23 is a major allergen. A significant positive correlation between OD values to Der p 23 and to Der p 2 was demonstrated, even though several patients were monosensitized to one of these two allergens (Pearson's correlation; $p < 0.0001$; data not shown). We further evaluated the influence of rDer p 23 oligosaccharides on its IgE-binding properties. By comparing the OD values of Der p 23-positive sera obtained from the ELISA against rDer p 23 with deglycosylated rDer p 23, we confirmed that the glycan structure of rDer p 23 did not impact the IgE binding (online suppl. table 1; for all online suppl. material, see www.karger.com/doi/10.1159/000442176; $p = 0.8762$).

Allergenicity of rDer p 23

To demonstrate that rDer p 23 can trigger basophil degranulation through interactions with FcεRI-bound IgE, a basophil degranulation assay was performed using RBL-SX38 cells. The allergenic activity of rDer p 23 was demonstrated using 3 representative sera from HDM-allergic patients that contained Der p 23-specific IgE and comparing these with rDer p 2 allergenic activity (fig. 5). rDer p 23 was able to induce basophil degranulation at concentrations ranging from 0.0001 to 1 μg/ml. Such allergenic activity was comparable to that displayed by rDer p 2. No significant degranulation was observed with buffer only, rDer p 23 only or with serum that was non-HDM-allergic.

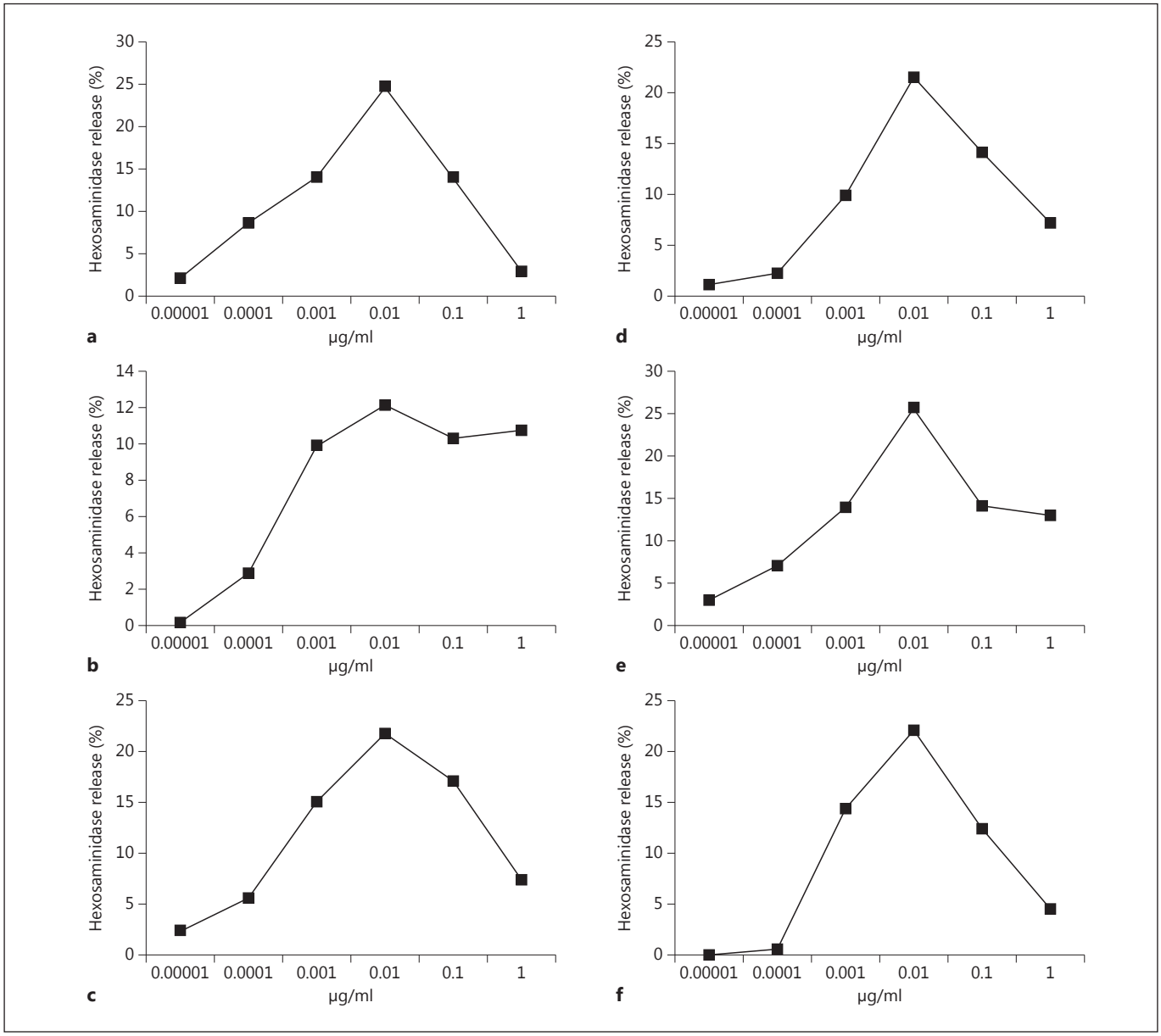

Fig. 5. RBL-SX38 degranulation by rDer p 23 and rDer p 2. The cells were primed for 16 h with sera from 3 HDM-allergic patients, containing rDer p 23- (**a–c**) or rDer p 2-specific IgE (**d–f**) and subsequently stimulated with serial dilution of purified rDer p 23 or rDer p 2 for 30 min. Degranulation was measured via β-hexosaminidase activity. Percentage of degranulation was presented as a subtraction of spontaneous released over total lysis with Triton X-100.

rDer p 23 Does Not Display Chitin-Binding Activity

We evaluated the chitin-binding activity of rDer p 23 using two different sources of insoluble chitins: chitin beads and shrimp-shell chitin. Under our experimental conditions, rDer p 23 as well as nDer p 23 from fecal pellet extracts could not bind to the aforementioned forms of chitin resins (fig. 6). By contrast, the positive control, wheat germ agglutinin (WGA), a well-known chitin-binding protein, bound efficiently to the two chitin-based matrices. On the other hand, rDer p 5, which does not display sequence homology to the chitin-binding domain, was used as negative control. To confirm that putative traces of sugars in the Der p 23 preparation could impair the protein binding to chitin by competition, we

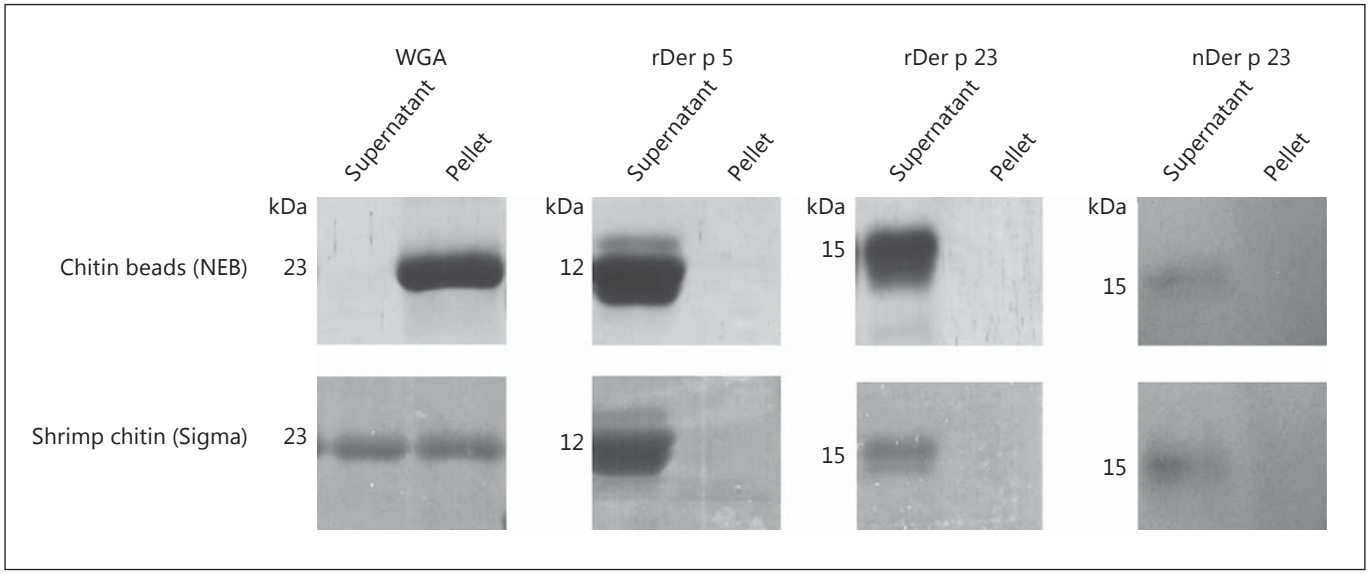

Fig. 6. Chitin-binding assay. Chitin-binding activity of rDer p23 produced in *P. pastoris* was assessed by incubating the protein with insoluble chitin bead and shrimp chitin. The fractions were then analyzed on SDS-PAGE, except nDer p 23 was analyzed by immunoblot. WGA was used as a positive control and rDer p5 as a negative control.

demonstrated that WGA can still bind chitin in the presence of *P. pastoris* culture medium (data not shown). It must also be pointed out that a GST-Der p 23 fusion protein produced in bacteria was also not able to bind to chitin under our experimental conditions (data not shown). Finally, to exclude that the absence of binding was not related to differences in chitin structures between mite feces and chitin beads or shrimp chitin, we performed the same assay using a suspension of HDM fecal pellets. The staining of fecal pellets with calcofluor demonstrated clearly the presence of the peritrophic matrix made in the chitin structures (online suppl. fig. 1). Once again, rDer p 23 did not interact with HDM fecal pellets under our experimental conditions where WGA could be associated with these particles (online suppl. fig. 1). Altogether, our data suggest that Der p 23 alone does not display any chitin-binding activity.

Discussion

A recent paper provided evidence of Der p 23 as a new major *D. pteronyssinus* allergen. The IgE reactivity to a recombinant form of Der p 23 produced in *E. coli* was comparable to those raised to Der p 1 and Der p 2 (74% in European cohorts of HDM-allergic patients) [7]. This high IgE-specific frequency is somewhat challenging. Indeed, Der p 23 has been shown as mainly an unfolded protein, present in very tiny amounts in house dust. Moreover, its aqueous extraction yield from fecal pellets is very low, probably because it is found next to the peritrophic matrix surrounding the feces. Such semipermeable matrix, produced in the mite mid-gut during food digestion to prevent the epithelium from damage by abrasive food particles and pathogens, is mainly composed of proteins and chitin fibrils [12]. According to the capacity of chitin to trigger innate immunity to shape a Th2-biased cytokine environment, the potent allergenicity of Der p 23 could result from its association with chitin [13, 14]. Although the Der p 23 C-terminal region displays homologies with a chitin-binding domain type 2, the interactions between the allergen and chitin structure have still to be shown.

In this study, we selected the *P. pastoris* expression system to produce and characterize a recombinant mature form of Der p 23. The rationale for this selection was based on the presence in the Der p 23 primary structure of a leader peptide for secretion, 2 threonine stretches which could represent O-glycosylation sites and 4 cysteine residues in the putative C-terminal chitin-binding domains which could be involved in 2 disulfides. All 3 of these post-translational modifications, which could be

critical for the allergenic properties and stability of Der p 23, can be performed by yeast. It must be pointed out that HDM allergens could be naturally glycosylated because glycan structures such as mannosylations were detected in natural Der p 1; such modifications could not take place when proteins are produced in bacteria [15]. Full-length mature and O-mannosylated rDer p 23 was successfully secreted by *P. pastoris*. The purified protein migrated as a 15-kDa band onto SDS-PAGE, whereas the calculated mass by MS analysis was 10.1 kDa (full-length, longest mannose chains). This abnormal SDS-PAGE migration could likely result from the proline content in Der p 23 because proline-rich proteins commonly display a decreased electrophoretic mobility [16]. Under our experimental conditions, N-terminal truncations of aa 11–17 were observed, particularly when the methanol induction exceeded 48 h. We speculate that these truncations/degradations could already have occurred via the yeast secretory pathway because stability studies clearly indicated that purified rDer p 23 is highly stable, even when the protein is stored at 37°C for 14 days (data not shown). It must be pointed out that MS analysis of aqueous extracts from fecal pellets also demonstrated the sensitivity of Der p 23 to proteolysis because, although peptide fragments can be clearly identified, the detection of the full-length natural allergen was difficult to achieve (data not shown). The secondary structure analysis by CD confirmed that, similarly to the recombinant form produced in bacteria, Der p 23 expressed in yeast is mainly folded, with a random coil structure and disulfides present, suggesting that Der p 23 likely displays more linear than conformational antibody-binding epitopes on the allergen surface [17]. This is in agreement with the capacity of purified specific polyclonal antibodies to detect rDer p 23 under native (ELISA) or denaturing (immunoblot after SDS-PAGE) conditions.

Using a cohort of sera from 222 Thai HDM-allergic patients, we confirmed that Der p 23 is an important allergen because the frequency of Der p 23-specific IgE reached 54%. Noteworthy is that IgE reactivity was lower than that measured for the typical major allergen Der p 2 (67%) using the same sera. To our knowledge, this is the first time that the IgE-binding frequencies of Der p 2 and Der p 23 have been determined in atopic patients from Thailand. However, RBL degranulation assays produced clear evidence of the potent allergenic activity of Der p 23. Higher IgE reactivity to Der p 23 (74%, n = 347), using a recombinant form produced in *E. coli*, was observed in European cohorts of HDM-allergic patients [7]. It must be pointed out that, based on seroepidemiological data from 1,300 HDM-allergic patients from Canada, Europe, Japan and the USA, around 80% of these patients had IgE reactivity to Der p 2 but around 47% reacted to Der p 23 (our currently unpubl. data). Thus, we believe that the IgE-binding frequencies found within the Thai population for Der p 23 (54%) and Der p 2 (67%) are comparable those observed in other populations. The lower prevalence of Der p 23 sensitivity in Thai HDM-allergic patients cannot be explained by the O-mannosylations of the N-terminal domain of yeast-expressed allergen, as we demonstrated that the glycan structure of rDer p 23 did not influence IgE binding. Moreover, it was recently demonstrated that major IgE epitopes of Der p 23 are present at the C-terminal part corresponding to the putative chitin-binding domain [17]. There is increasing evidence that the allergenicity of HDM allergens can be mediated by their interactions with microbial compounds which are able to activate innate immune signaling pathways, leading to the induction of Th2-biased allergen-specific adaptive responses [18]. In this context, the allergenic activity of Der p 23 could result from association with chitin structures, according to the presence of the putative chitin-binding domain at the C-terminus of the protein. Using two different sources of chitin, our results suggest that rDer p 23 and nDer p 23 are not capable of binding to this sugar polymer. Similar results were obtained with a recombinant GST-Der p 23 fusion protein produced in bacteria (data not shown), suggesting that O-glycosylation could not interfere with any chitin binding. The putative chitin-binding domain of Der p 23 contains only 4 cysteine residues whereas the canonical typical peritrophin-A and chitin-binding 2 domains contain ≥6 conserved cysteines. Moreover, chitin-binding activity has been commonly demonstrated in proteins containing at least 2 of these domains [19, 20]. Some proteins could display chitin-binding activity through a domain containing only 5 cysteine residues as Blo t 12 [21]. To our knowledge, the interaction between chitin and proteins harboring a domain with only 4 cysteines was not demonstrated up to now. As we also demonstrated that rDer p 23 could not bind to chitin structures present on the surface of mite fecal pellets, we speculate that the localization of Der p 23 into the peritrophic matrix of the mite could result from interactions with molecular partners other than chitin, or from the existence of oligomeric forms of the allergen exposing the appropriate 4-cysteine-based chitin-binding domains to interact with chitin. It must also be pointed out that the Der f 23 amino acid sequence was shown to contain a putative chitin binding domain with 5 cysteine residues; 4/5 cysteine residues are conserved in Der p 23

[22]. However, the chitin-binding properties of Der f 23 have yet to be elucidated.

The role of glycosylation in the recognition and uptake of allergens by the innate immune system through C-type lectin receptors was also shown to be important for the initiation of the allergic response [23]. In this study, we observed an O-glycosylation site made of a stretch of threonine residues in the N-terminus of Der p 23. Although the presence of O-glycans has still to be demonstrated in natural Der p 23, O-glycosylation of HDM allergens can be performed in mites, as previously shown for the HDM chitinase Der f 15 [24]. Moreover, on Western blot, nDer p 23 and rDer p 23 had the same molecular weight, indicating that nDer p 23 could also be modified.

In conclusion, the HDM major allergen rDer p 23 displays O-glycan-independent IgE reactivities but no chitin-binding activity. Our findings confirm that Der p 23 displays potent allergenic activity, despite the fact that it is a protease-sensitive and poorly extractable allergen from mite fecal pellets. Recombinant forms of Der p 23 should be included in the panel of allergens for the component-resolved diagnosis of HDM allergy as well as in the design of specific immunotherapeutic treatments. Whereas rDer p 23 produced in *E. coli* and *P. pastoris* could be interchangeable for the in vitro detection of Der p 23 sensitizations, we believe that allergen production in yeast, due to the absence of contaminating endotoxins, would be more convenient for allergen-specific immunotherapeutic treatments or allergy diagnosis by skin-prick testing. Further studies are needed to evaluate the release of Der p 23 from the fecal pellets at the level of the airway epithelium and, notably, the contribution of the HDM chitinases Der p 15 and Der p 18 in the process. The elucidation of the Der p 23-induced innate immune signaling pathways would shed a new light on the allergenic determinant(s) of Der p 23.

Appendix

The Mite Allergy Research Cohort (MARC) Study Team consisted of Pattarawat Thantiworasit and Pinya Pulsawat at the King Chulalongkorn Memorial Hospital, Tassalalpa Daengsuwan at the Children Hospital, Wannada Laisuan, Malinee Tongdee, Nizchapha Dchapaphapeaktak at the Ramathibodi Hospital and Tadech Boonpiyathad at the Phramongkutklao College of Medicine.

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Disclosure Statement

The authors declare that there are no conflicts of interests.

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Patterns of IgE sensitization in house dust mite-allergic patients: implications for allergen immunotherapy

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Keywords
allergen immunotherapy; *Dermatophagoides*; house dust mite; IgE sensitization.

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Abstract

Background: Understanding patterns of IgE sensitization in *Dermatophagoides*-allergic patients living in various geographical areas is necessary to design a product suitable for worldwide allergen immunotherapy (AIT).
Methods: Using a *HIFI Allergy* customized microarray assay, IgEs specific for 12 purified allergens from *Dermatophagoides pteronyssinus* or *D. farinae* were assessed in sera from 1302 house dust mite (HDM)-allergic patients living in various areas. Comprehensive mass spectrometric (MS) analyses were conducted to characterize HDM extracts, as well as purified bodies and feces.
Results: Patterns of IgE reactivity to HDM allergens are comparable in all cohorts of patients analyzed, encompassing adults and 5- to 17-year-old children, as well as American, Canadian, European, and Japanese patients. Overall, >70% and >80% of HDM-allergic patients are sensitized to group 1 and group 2 allergens, respectively, from *D. pteronyssinus* and/or *D. farinae* species. Furthermore, 20–47% of patients also have IgEs to allergens from groups 4, 5, 7, 13, 15, 21, and 23. All patients have IgEs to allergens present in mite bodies and feces. MS-based analyses confirmed the presence of mite allergens recorded by IUIS in *D. pteronyssinus* and *D. farinae* extracts, with groups 2, 8, 10, 11, 14, and 20 prominent in bodies and groups 1, 6, 18, and 23 well represented in feces.
Conclusions: Mite-specific AIT should rely upon a mixture of *D. pteronyssinus* and *D. farinae* extracts, manufactured from both feces and bodies. Such a combination is appropriate to treat children and adult *Dermatophagoides*-allergic patients from Asia, Europe, and North America.

Abbreviations
2D-DiGE, Two-dimensional differential gel electrophoresis; AIT, Allergen immunotherapy; CHAPS, 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate; ESI-Q-ToF, Electrospray ionization quadrupole time-of-flight; HDM, House dust mite; IAU, Intensity arbitrary unit; ISAC, Immunosolid-phase allergen chip; ISU, ISAC standardized unit; LC, Liquid chromatography; MS/MS, Tandem MS; MS, Mass spectrometry; SDS-PAGE, Sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

House dust mites (HDM) represent a major source of allergens affecting more than 500 million people throughout the world (1–3). Although mites encompass more than 40 000 distinct species, the two common Pyroglyphidae *Dermatophagoides pteronyssinus* and *D. farinae* species are responsible for more than 90% of HDM allergies worldwide (2, 3). In sensitized patients, such HDM can induce allergic rhinitis, asthma and/or atopic dermatitis. Whereas allergy symptoms associated with mite exposure can be alleviated by pharmacological

drugs, allergen immunotherapy (AIT) is the only etiologic treatment providing sustained efficacy for moderate-to-severe allergic rhinitis to HDM (1, 3, 4). Some approaches based on recombinant allergens or peptides are under development (5–8), but AIT is currently based on the repeated administration of aqueous mite allergen extracts prepared from either bodies or whole mite cultures, the latter comprising as well fecal particles (9–15). Historically, mites have been grown using various culture media containing either human scales, dried daphnia, cereals, mold cultures as well as fish or dog food (16). However to manufacture pharmaceutical-grade extracts for the treatment of allergic patients, mites cannot be cultured using a medium containing any protein of human or animal origin. In this context, we have developed a well-defined culture medium made of synthetic amino acids, wheat germ and yeast, which allows to produce HDM with similar characteristics to the ones grown on human scales (17).

As AIT aims to reeducate the immune system in a specific manner by eliciting tolerance to the sensitizing allergens (18, 19), the design of AIT products must take into account the patterns of IgE sensitization of allergic patients, with as a final goal to recapitulate at a molecular-level (i.e., in terms of allergen composition) natural exposure conditions. In this context, we conducted seroepidemiological studies in *Dermatophagoides*-allergic pediatric and/or adult patient populations from Canada, Europe, Japan, and the United States. In light of the obtained data, together with an extensive molecular characterization of HDM extracts, we conclude that immunotherapy should rely upon extracts made from both *D. pteronyssinus* and *D. farinae*, providing allergens expressed in bodies and fecal particles from the two species.

Materials and methods

Analysis of IgE reactivity in sera from house dust mite-allergic patients

Blood samples were obtained after informed consent from 503 European adults living in seven distinct countries (*i.e.*, Czech Republic, France, Germany, the Netherlands, Poland, Slovakia, and Spain) (20), 423 pediatric (5–17 years old) patients originating from nine distinct countries (*i.e.*, Denmark, France, Germany, Hungary, Ireland, Romania, Slovakia, Spain, and Ukraine), as well as 350 HDM-allergic Canadian adult patients residing in Ontario with an history of HDM-related allergic rhinoconjunctivitis for at least 1 year, a positive skin prick test to *D. pteronyssinus* and/or *D. farinae* with a wheal diameter >3 mm and IgE titers to *D. pteronyssinus* and/or *D. farinae* ≥0.7 kU/l, as determined using the Immulite 2000 Immunoassay system (Siemens, Munich, Germany) or ImmunoCAP assay (Thermo Scientific, Uppsala, Sweden). Titers of serum IgE to Der p 1, Der p 2, Der f 1, and Der f 2, as well as Der p 5, Der p 7, and/or Der p 10, were assessed in parallel using immunosolid-phase allergen chip (ISAC) assays with natural or recombinant purified allergens (Thermo Scientific), as described elsewhere (21). Results were expressed in ISAC standardized units (ISU) and considered positive when anti-allergen IgE levels were ≥0.3 ISU. Whereas our focus was on allergic rhinoconjunctivitis, a small percentage (i.e., 7.9%) of the patient population had concomitant atopic dermatitis or eczema.

In addition, sera from 28 HDM-allergic Japanese patients were purchased from Trina Bioreactives (Nänikon, Switzerland) without any specific requirement regarding the living place of the donors. These sera were selected on the basis of a clinical history suggestive of HDM allergy and a positive IgE reactivity to a *D. pteronyssinus* extract with specific IgE levels >3.5 kU/l, measured by ImmunoCAP. Sera from 35 HDM-sensitized US individuals were also purchased from PlasmaLab (Everett, WA, USA) without any specific requirement regarding the living place of the donors. Such sera were selected on the basis of a positive IgE reactivity to *D. pteronyssinus* and/or *D. farinae* extracts, with specific IgE levels >1 kU/l, as measured by ImmunoCAP. No information was available regarding potential mite-related symptoms in this group. Serum samples from four individuals unsensitized to HDM allergens were included as negative controls in the *HIFI Allergy* microarray experiments.

Multiplexed IgE quantification assays were carried out using the high-throughput *HIFI* assay platform (AXO Science, Villeurbanne, France) with an EVO100 equipment (Tecan, Männedorf, Switzerland) and a 96-head automated washer as described elsewhere (22) and summarized in Data S1. Highly significant correlations ($P < 0.0001$) were found between IgE levels measured by the *HIFI Allergy* assay and ImmunoCAP or ISAC methods, for all allergens tested (Table S1). A direct comparison of results obtained with the three assays confirmed that the cut-off value for a positive signal with the *HIFI Allergy* array is around 0.35 kU/l or 0.3 ISU (data not shown).

Mite extracts, bodies, and feces, natural and recombinant purified mite allergens

Mites of the *D. pteronyssinus* and *D. farinae* species were grown for 75 and 85 days, respectively and harvested as described elsewhere (17). *D. pteronyssinus* or *D. farinae* source materials were incubated for 24 ± 2 h at +5°C ± 3°C in 4 g/l ammonium bicarbonate. Extracts were obtained after centrifugation, clarifying filtration of the supernatant on 0.22-μm membrane filters, concentration and diafiltration on a 5-kDa cut-off membrane, addition of 2% (mass/mass) mannitol, freeze-drying, and sieving. Mite bodies were purified by using a flotation method whereas feces were purified by sequential sieving as described elsewhere (23) and summarized in Data S1. 2D-DiGE and mass spectrometry (MS) analyses of proteomes and allergomes are described in Data S1.

The WHO/IUIS allergen nomenclature (available on www.allergen.org) was used in the present study. Production and purification of natural (n) Der p 1, Der f 1, Der p 2, Der f 2, and Der p 4 molecules and recombinant (r) Der p 5, Der p 7, Der p 10, Der p 13, Der p 15, Der p 21, and Der p 23 allergens are described in Data S1. The quality of these purified allergens was confirmed as described in Data S1 and Fig. S1.

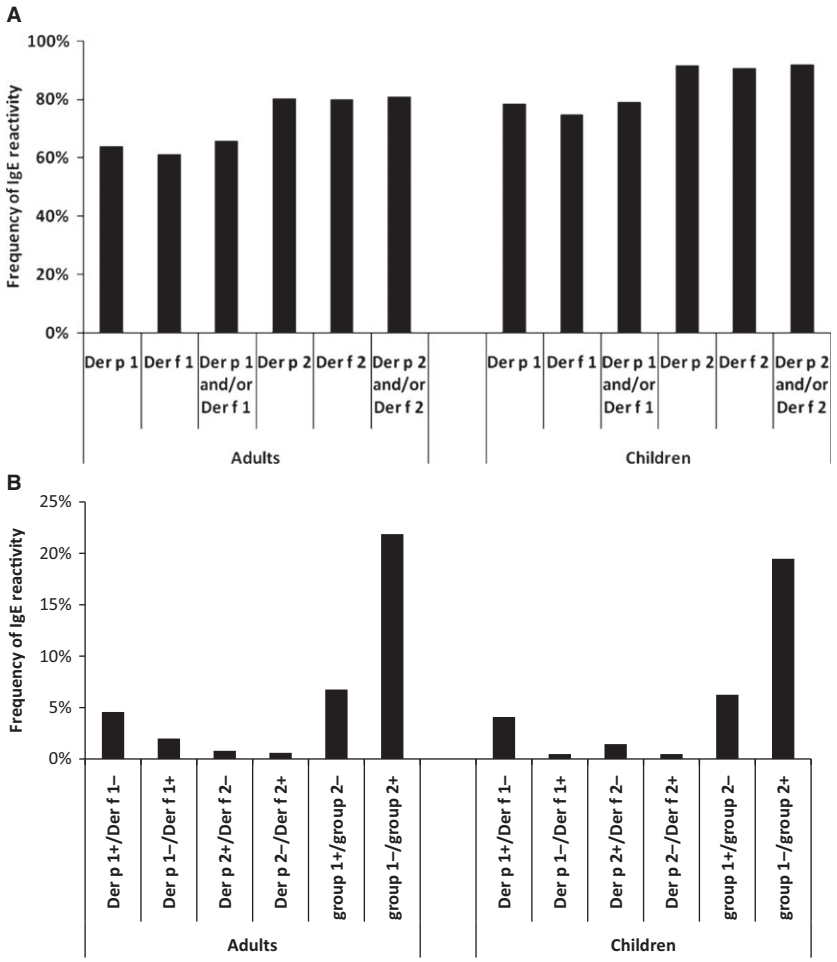


Figure 1 Frequencies of IgE responses to group 1 and group 2 allergens from *D. pteronyssinus* and *D. farinae* in pediatric and adult patient populations. IgE responses were measured by using the ISAC assay in sera from 503 European adult and 416 pediatric patients allergic to HDM. Results are presented either globally (A) or in combination (B). For instance, 'Der p 1+ / Der f 1-' indicates patients who have IgEs to Der p 1 but not to Der f 1, and 'group 1+ / group 2-' indicates patients who have IgEs to Der p 1 and/or Der f 1 but not to either Der p 2 or Der f 2.

Statistical analyses

Analyses were performed with the Microsoft Excel software. Correlation analyses were performed by using the nonparametric Spearman test with the GraphPad Prism 5 software (GraphPad Software, Inc, La Jolla, USA); P-values <0.05 were considered significant.

Results

Patterns of IgE sensitization to major and minor allergens from *D. pteronyssinus* and *D. farinae* in HDM-allergic patients

In a first set of experiments, we compared the IgE reactivity in sera from HDM-allergic children (5–17 years old, $n = 416$) and adults ($n = 503$) using group 1 and group 2 allergens purified from both *D. pteronyssinus* and *D. farinae* species. Results obtained using the ISAC assay demonstrated a comparable pattern of IgE reactivity with mite major allergens in the two cohorts (Fig. 1). Specifically, both group 1 and group 2 allergens from *D. pteronyssinus* and *D. farinae* are relevant in the two populations, as 79% of children and 66% of adults have IgEs recognizing group 1 allergens (i.e., Der p 1 and/or Der f 1), whereas 92% of children and 81% of adults have IgEs directed to group 2 allergens (i.e., Der p 2 and/or Der f 2) (Fig. 1A). Overall, 53–70% of patients exhibit serum IgE reactivity to all of these four major allergens (not shown). Noteworthy, 6–7% of patients have IgEs recognizing group 1 but not group 2 allergens, whereas 19–22% of them have IgEs reacting with group 2 but not group 1 allergens (Fig. 1B). We estimate that ≥5% of children and adults have IgEs reactive with major allergens from only one of the two mite species.

We subsequently analyzed IgE sensitization profiles in a total of 1302 sera from *Dermatophagoides*-allergic patients

from Canada, Japan, and the USA, including as well the ones from Europe (adults and children) tested above. Using a customized multiplexed (*HIFI Allergy*) microarray assay described in Data S1, we assessed not only the IgE response to group 1 and 2 major allergens, but also to minor or mid-potency allergens belonging to groups 4, 5, 7, 10, 13, 15, 21, and 23 obtained as purified natural or recombinant molecules. Analyses conducted with the *HIFI Allergy* assay established that patterns of IgE sensitization assessed both in terms of prevalence and relative contribution to total IgE reactivity are highly comparable in the different cohorts of patients analyzed, irrespective of their geographical origin (Fig. 2A, B). Altogether, those analyses confirmed that allergens from groups 1 and 2 from *D. pteronyssinus* and *D. farinae* represent major allergens, with more than 65% of tested patients having IgEs to each of them (Fig. 2A). Furthermore, group 4, 5, 7, 13, 15, 21, and 23 allergens might be important for selected patients, as they are recognized by IgEs from ≥20% of patients in the various populations (Fig. 2A). In contrast, irrespective of the population considered, ≤11% of patients displayed serum IgEs to Der p 10 (Fig. 2A), establishing the latter as a very minor allergen. In light of these results, we subsequently assessed the pooled data obtained from the 1302 patients regarding the overall prevalence of IgE reactivity with allergens found predominantly in either mite bodies or feces, respectively (see below). As shown in Fig. 2A, a majority of patients are sensitized to allergens well represented in mite bodies (e.g., Der p 2 and Der f 2), but also prominent in fecal particles (such as Der p 1, Der f 1, and Der p 23).

Characterization of major and minor allergens in *D. pteronyssinus* and *D. farinae* whole extracts, bodies, and feces

Having documented a standard pattern of IgE sensitization for HDM-allergic patients of various origins, we investigated the allergen composition of *D. pteronyssinus* and *D. farinae* extracts intended for AIT. We first compared extracts from the two species using a 2D-differential gel electrophoresis (2D-DiGE) analysis of mixed proteins from *D. pteronyssinus* highlighted in green and those from *D. farinae* in red (Fig. 3A–C). Allergens from the two species were localized by spot picking and MS/MS sequencing (Fig. 3C, group 1: white arrows, group 2: blue arrows). This comparative analysis confirmed that proteomes and allergomes from *D. pteronyssinus* and *D. farinae* species are different, with only very limited overlay (yellow spots) in merged pictures (Fig. 3C). A comprehensive MS analysis of those mite extracts subsequently demonstrated the presence of group 1–11, 14, 15, 18, 20, 21, and 23 allergens in *D. pteronyssinus* extracts (Table 1) and of group 1–3, 6–8, 10, 11, 13–16, 18, 20–22, and 25–33 allergens in *D. farinae* extracts (Table 2). These results obtained by LC-MS/MS were further corroborated by immunoblot, using available allergen-specific antisera confirming the presence of mite allergens (including group 1–10, as well as group 13) in *D. pteronyssinus* (17) and *D. farinae* extracts (Fig. S2).

To determine the origin of these allergens, a characterization of the allergomes was further performed in bodies and feces purified by flotation and sedimentation, respectively, from cultured *D. pteronyssinus* or *D. farinae* mites. Using 2D-DiGE, we first confirmed that proteomes from bodies (protein spots highlighted in green) and feces (spots highlighted in red) are significantly different for both *D. farinae* (Fig. 3D–F) and *D. pteronyssinus* (Fig. 3G–I), with only partial overlay (yellow spots) when the figures were merged. MS-based analyses confirmed that mite feces from *D. pteronyssinus* and/or *D. farinae* are rich in groups 1, 6, 18, and specifically for *D. pteronyssinus* in group 23 (Tables 1 and 2). In contrast, bodies contain more group 2, 8, 10, 11, 14, and 20 allergens in both *D. pteronyssinus* and/or *D. farinae* species. Der f 18 previously identified in the gut using immunostaining (24) was also detected in feces by MS.

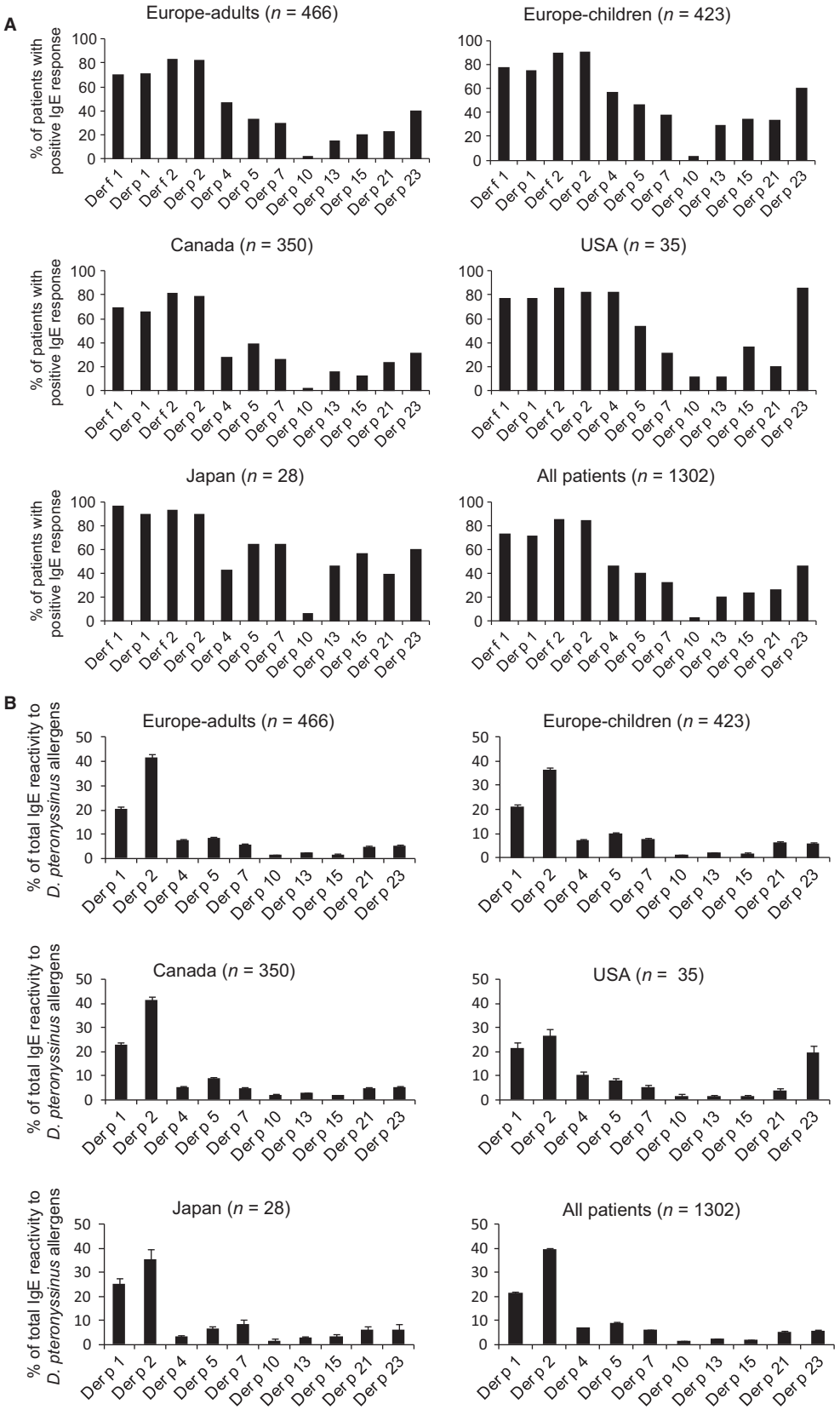
Characterization of culture medium-derived proteins in *D. pteronyssinus* and *D. farinae* extracts

Lastly, we investigated the potential presence of residual components from the mite culture medium used to grow *D. pteronyssinus* and *D. farinae* in allergen extracts. To this end, enzymatic digests of *D. pteronyssinus* and *D. farinae* extracts were analyzed *vs* an in-house data base encompassing mite allergens as well as *Saccharomyces cerevisiae* and *Triticum aestivum* proteins. Whereas numerous peptides originating from mite proteins (2200 and 1454 peptides for *D. pteronyssinus* and *D. farinae* extracts, respectively) were identified in those analyses, only 13 and nine peptides found in *D. pteronyssinus* and *D. farinae* extracts, respectively, were derived from yeast or wheat germ, with none of those corresponding to known wheat or yeast allergens. No gluten was detected in whole mite extracts.

Discussion

Mite-allergic patients in temperate countries are classically co-exposed and cosensitized to allergens from both *D. pteronyssinus* and *D. farinae* species (2, 3). Thus, to design allergen products suitable to treat HDM-allergic patients in various parts of the world, it is critical to understand the patterns of IgE sensitization to allergens from such common *Dermatophagoides* species. To this aim, we performed a

Figure 2 Frequencies and relative contribution of IgE responses to HDM allergens. IgE responses were measured in 466 European adults, 423 European children (aged 5–17 years), 350 Canadian adults, 35 American adults, and 28 Japanese adults allergic and/or sensitized to *Dermatophagoides* species by using a customized multiplexed mite microarray (HIFI Allergy). (A) Patterns of IgE sensitization and (B) contribution to IgE binding of each purified allergen from *D. pteronyssinus* relative to total IgE reactivity against all *D. pteronyssinus* allergens measured with the HIFI Allergy microarray. Data are shown as means + SEMs.


A
Europe-adults (n = 466)
Europe-children (n = 423)
Canada (n = 350)
USA (n = 35)
Japan (n = 28)
All patients (n = 1302)
% of patients with positive IgE response
Der f 1
Der p 1
Der f 2
Der p 2
Der p 4
Der p 5
Der p 7
Der p 10
Der p 13
Der p 15
Der p 21
Der p 23
B
% of total IgE reactivity to D. pteronyssinus allergens
Europe-adults (n = 466)
Europe-children (n = 423)
Canada (n = 350)
USA (n = 35)
Japan (n = 28)
All patients (n = 1302)

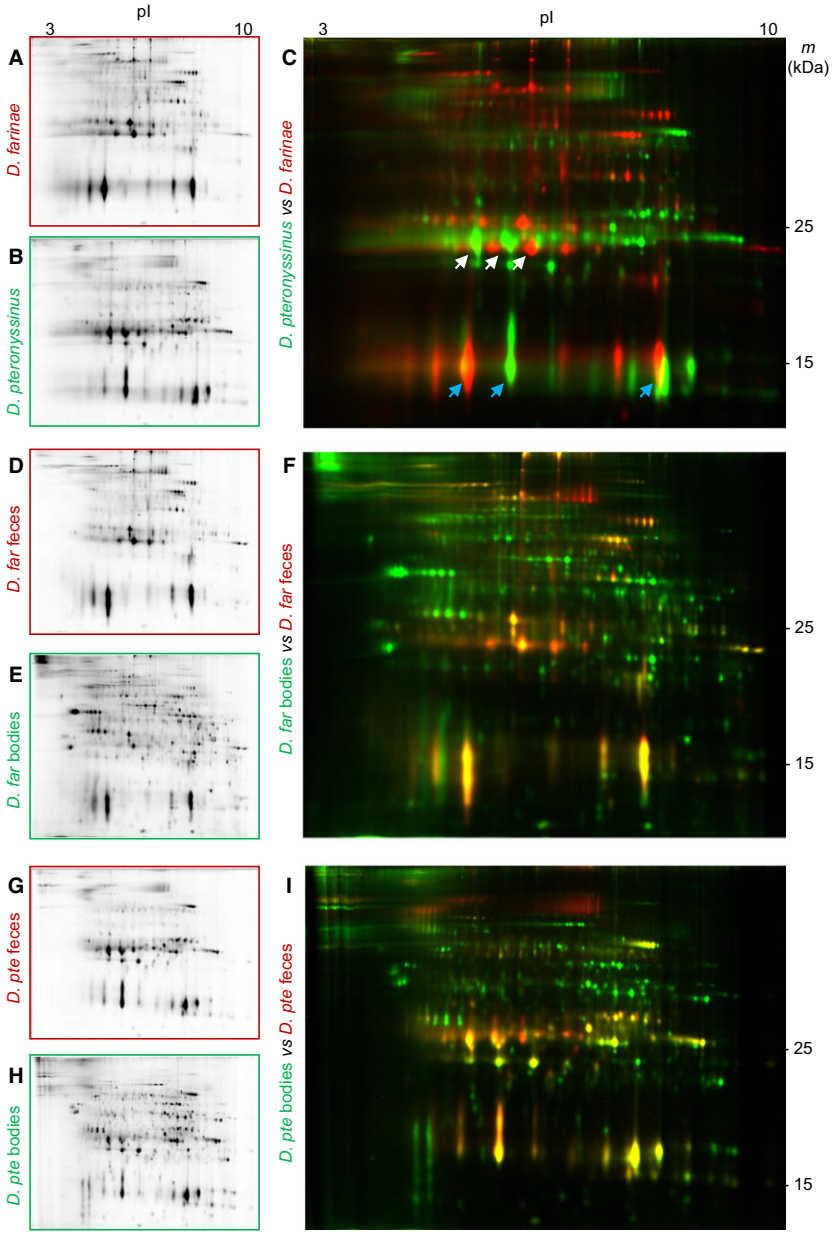


Figure 3 Comparative analyses of mite proteomes by 2D-DiGE. Proteins from *D. pteronyssinus* and *D. farinae* were labeled using distinct fluorescent dyes, mixed and analyzed by 2D-gel electrophoresis. 2D gels from (A) whole *D. farinae* extract (in red), (B) whole *D. pteronyssinus* extract (in green) and (C) the overlay of both. 2D gels from *D. farinae* (D) purified feces (red), (E) purified bodies (green) and (F) the overlay of both. 2D gels from *D. pteronyssinus* (G) purified feces (red), (H) purified bodies (green) and (I) the overlay of both. Yellow spots correspond to proteins present in both compared samples. White and blue arrows indicate main spots corresponding to group 1 and group 2 major allergens, respectively.

seroepidemiological study enrolling 1302 *Dermatophagoides*-allergic patients from Canada, Europe, Japan, and United States.

This study established that (i) patterns observed in 5- to 17-year-old children and adults are similar, and (ii) all patients exhibit comparable patterns of IgE sensitization, irrespective of their geographical location. Among mite allergens, group 1 and 2 allergens are clearly the most important ones, both in terms of prevalence and relative contribution to total IgE reactivity against HDM allergens. Our results are broadly in agreement with the literature (3, 25–29) with evidence that many additional minor allergens from *Dermatophagoides* are recognized by IgEs from HDM-allergic patients, including group 4, 5, 7, 13, 15, 21, and 23 allergens

Table 1 Summary of MS/MS identification of allergens recorded by IUIS within whole extracts, purified bodies or feces from *D. pteronyssinus*

Allergen group	Isoallergen	Avg. mass (kDa)	Whole extracts		Bodies		Feces	
			Coverage (%)	#Peptides	Coverage (%)	#Peptides	Coverage (%)	#Peptides
1	Der p 1.0101	36	48	53	42	23	50	38
2	Der p 2.0101	16	64	23	61	13	71	17
3	Der p 3.0101	28	62	24	53	12	62	15
4	Der p 4.0101	57	77	29	43	14	36	13
5	Der p 5.0101	17	12	1	n.a.	n.d.	n.a.	n.d.
6	Der p 6.0101	30	72	39	54	14	71	21
7	Der p 7.0101	24	40	25	5	1	5	1
8	Der p 8.0101	26	18	4	13	3	n.a.	n.d.
9	Der p 9.0101	29	34	14	30	7	31	9
10	Der p 10.0101	33	18	5	28	11	n.a.	n.d.
11	Der p 11.0101	102	n.a.	n.d.	23	18	n.a.	n.d.
14	Der p 14.0101	191	7	9	23	36	n.a.	n.d.
15	Der p 15.0101	61	47	21	n.a.	n.d.	n.a.	n.d.
18	Der p 18.0101	52	42	16	11	3	26	6
20	Der p 20.0101	40	25	7	39	13	n.a.	n.d.
21	Der p 21.0101	17	18	2	n.a.	n.d.	n.a.	n.d.
23	Der p 23.0101	10	44	8	21	2	57	6

Avg. mass, theoretical average molecular mass of the allergen; coverage, allergen amino acid sequence coverage; n.a., not applicable; n.d., not detected; #peptides, number of peptides identified. Data shown are compiled from the analysis of three batches of whole extracts, and 1 batch each of purified bodies or feces. Der p x.0101 isoallergens were selected as comparators to determine the sequence coverage, even if the presence of multiple isoallergens was detected for most allergens. Numbers of peptides identified by MS are only partially indicative of the abundance of corresponding allergens in the extracts. IgE reactivity of 2D blots of *D. pteronyssinus* extracts using a pool of sera from mite-allergic patients confirmed allergen integrity (data not shown).

(Fig. 2). In our study, 46.5% of patients have serum IgEs specific to Der p 23, but the latter only contributes to 6% of total mite-specific IgE reactivity, with variability among patients suggesting that it may be clinically relevant for selected patients. The low prevalence of IgE reactivity to Der p 10 observed herein confirms previous reports concluding that it represents a minor allergen (25, 30, 31). Some differences are observed between US patients and other cohorts in terms of IgE reactivity with group 4 and 23 allergens. Those differences could be explained by both a few outliers, the small size of the US cohort as well as differences in the patients' selection.

Interestingly, while most patients have IgEs recognizing group 1 and/or group 2 allergens from both *D. pteronyssinus* and *D. farinae,* we estimate that ≥5% of them have IgEs directed to group 1 and/or group 2 allergens from only one of those mite species (Fig. 1). This confirms that despite a well-known amino acid sequence homology, each of Der p 1, Der f 1, Der p 2, and Der f 2 bears species-specific IgE epitopes. Although not shown, we also observed similar differences in terms of IgE reactivity between allergens from the two mite species, for each of the group 3–9 and 21 minor allergens (data not shown). Interspecies differences in the immunogenicity of mite allergens have been reported by others, both in terms of T- and B-cell epitopes (32–34). Altogether, these results strongly suggest that when patients have IgEs to both *D. pteronyssinus* and *D. farinae* allergens, this reflects not only IgE cross-reactivity, but also a *bona fide* co-exposure and sensitization (Fig. 1B). From those observations, we infer that a proper product for AIT should contain both *D. pteronyssinus* and *D. farinae* extracts, in agreement with our demonstration in a murine experimental model that such a combination is indeed more efficacious than each of *D. pteronyssinus* or *D. farinae* single extracts for alleviating asthma symptoms in animals exposed to the two mite species (35).

Using MS to characterize aqueous extracts from *D. pteronyssinus* and *D. farinae,* we confirm the presence of all allergens currently recognized by IUIS (with the exception of Der f 17 and Der f 24) (Tables 1 and 2). This result is consistent with our previous analysis by immunodot blotting establishing that *D. pteronyssinus* extracts contain Der p 3 to Der p 10, Der p 13, and Der p 14 (17). Using the same method, we confirm herein that *D. farinae* extracts contain Der f 3 to Der f 10 as well as Der f 13 (Fig. S2). We document as well the presence of the newly characterized group 25–33 allergens (36) by MS in *D. farinae* extracts (Table 1). In the absence of validated assays to measure these allergens, quantitative data regarding such minor allergens are not yet available. We established that some allergens are predominantly associated with mite feces, such as allergens from group 1, 6, 18, and 23 allergens, while others are more abundant in mite bodies, including group 2, 8, 10, 11, 14, and 20 allergens, in agreement with other studies (23, 37, 38). Interestingly, we detected group 2 and group 18 allergens by MS, not only in bodies as previously described, but also in feces,

Table 2 Summary of MS/MS identification of allergens recorded by IUIS within whole extracts, purified bodies or feces from *D. farinae*

Allergen group	Isoallergen	Avg. mass (kDa)	Whole extracts		Bodies		Feces	
			Coverage (%)	#Peptides	Coverage (%)	#Peptides	Coverage (%)	#Peptides
1	Der f 1.0101	36	61	50	46	20	63	69
2	Der f 2.0101	14	88	26	71	15	78	16
3	Der f 3.0101	28	64	25	45	9	68	17
6	Der f 6.0101	31	34	14	22	6	31	10
7	Der f 7.0101	24	42	26	53	14	n.a.	n.d.
8	Der f 8.0101	23	26	5	39	6	n.a.	n.d.
10	Der f 10.0101	35	33	9	48	27	n.a.	n.d.
11	Der f 11.0101	81	n.a.	n.d.	36	28	n.a.	n.d.
13	Der f 13.0101	15	n.a.	n.d.	31	5	n.a.	n.d.
14	Der f 14.0101	41	21	6	53	18	n.a.	n.d.
15	Der f 15.0101	63	46	27	37	15	46	22
16	Der f 16.0101	55	n.a.	n.d.	20	10	n.a.	n.d.
18	Der f 18.0101	52	48	18	16	6	8	5
20	Der f 20.0101	40	12	6	48	21	n.a.	n.d.
21	Der f 21.0101	16	24	6	32	3	n.a.	n.d.
22	Der f 22.0101	17	49	23	37	7	30	8
25	Der f 25.0101	27	34	5	29	4	6	1
26	Der f 26.0101	18	7	1	69	17	n.a.	n.d.
27	Der f 27.0101	48	21	6	3	1	3	1
28	Der f 28.0101	72	12	7	12	6	5	3
29	Der f 29.0101	18	40	4	52	8	9	1
30	Der f 30.0101	20	49	5	57	9	n.a.	n.d.
31	Der f 31.0101	17	21	2	50	8	n.a.	n.d.
32	Der f 32.0101	34	10	2	51	9	n.a.	n.d.
33	Der f 33.0101	52	n.a.	n.d.	3	1	n.a.	n.d.

Avg. mass, theoretical average molecular mass of the allergen; coverage, allergen amino acid sequence coverage; n.a., not applicable; n.d., not detected; #peptides, number of peptides identified. Data shown are compiled from the analysis of three batches of whole extracts, and 1 batch each of purified bodies or feces. Der f x.0101 isoallergens were selected as comparators to determine the sequence coverage, even if the presence of multiple isoallergens was detected for most allergens. Numbers of peptides identified by MS are only partially indicative of the abundance of corresponding allergens in the extracts. IgE reactivity of 2D blots of *D. farinae* extracts using a pool of sera from mite-allergic patients confirmed allergen integrity (data not shown).

possibly because MS provides a more comprehensive allergen detection encompassing all isoallergens, whether present in a native or denatured form, when compared with immunological methods. Given that most patients have IgEs to allergens expressed in bodies and feces, we conclude that to provide a broad spectrum of mite allergens and derived isoforms, immunotherapy should be performed using extracts made from both bodies and feces of the two *D. pteronyssinus* and *D. farinae* species.

The potential presence in mite extracts of culture medium-derived components is a theoretical concern, most particularly if it were to include potential allergens. By using western blotting and HPLC, we previously demonstrated that *D. pteronyssinus* and *D. farinae* extracts obtained using our culture conditions do not contain potential marker proteins nor allergens from wheat germ and yeast included in the culture medium (17). By using sensitive nanoLC-MS/MS, we corroborate those findings, with only few peptides corresponding to nonallergenic proteins from yeast or wheat germ detected in *D. pteronyssinus* and *D. farinae* extracts. The latter is also in line with our previous observation that no IgE sensitization to culture components was observed over a 2-year follow-up period when HDM-allergic patients were treated with sublingual tablets containing extracts from the two mite species (39).

Altogether, seroepidemiological results combined with comprehensive characterization data of HDM allergen extracts lead to the conclusions that (i) the same product can be used for AIT of *Dermatophagoides*-allergic patients, irrespective of their age and geographical location; (ii) this product should rely upon a mixture of *D. pteronyssinus* and *D. farinae* extracts, produced with corresponding source materials encompassing both bodies and feces.

Acknowledgments

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Conflict of interest

This work was funded by Stallergenes. TB, VB-B, AM, MLM, PL, KJ, SM, SH, HC, EN, and PM are employed by

Stallergenes. The other authors declare no competing financial interest.

Authors' contributions

TB wrote the manuscript, supervised part of the people involved in the study, and interacted with others. VBB supervised the seroepidemiological study. AM and MLM performed LC-MS analyses. PL purified mite bodies and feces and characterized allergens. KJ performed the 2D-DiGE experiments and characterized allergens. SH contributed to seroepidemiological studies. SM, HC, WTS, PS, and AJ produced and purified allergens. CH supervised the ISAC seroepidemiological studies. CAM and BPC performed analyses conducted using *HIFI Allergy*. FTC supervised the dot blot analysis. EN and PM supervised the study and wrote the manuscript. All authors read and approved the final manuscript.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Data S1. Material and Methods.

Figure S1. SDS PAGE analysis of purified allergens used in the *HIFI Allergy* customized multiplexed mite microarray.

Figure S2. Immunodot blot analysis of representative *D. farinae* extracts.

Table S1. Correlations between allergen-specific IgE levels measured by using either ImmunoCAP, ISAC assay, or *HIFI Allergy* customized multiplexed microarray.

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

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Abstract

Background: The house dust mite (HDM) allergen Der p 13 could be a lipid-binding protein able to activate key innate signaling pathways in the initiation of the allergic response. We investigated the IgE reactivity of recombinant Der p 13 (rDer p 13), its lipid-binding activities, and its capacity to stimulate airway epithelium cells.
Methods: Purified rDer p 13 was characterized by mass spectrometry, circular dichroism, fluorescence-based lipid-binding assays, and *in silico* structural prediction. IgE-binding activity and allergenic potential of Der p 13 were examined by ELISA, basophil degranulation assays, and *in vitro* airway epithelial cell activation assays.
Results: Protein modeling and biophysical analysis indicated that Der p 13 adopts a β-barrel structure with a predominately apolar pocket representing a potential binding site for hydrophobic ligands. Fluorescent lipid-binding assays confirmed that the protein is highly selective for ligands and that it binds a fatty acid with a dissociation constant typical of lipid transporter proteins. The low IgE-binding frequency (7%, $n = 224$) in Thai HDM-allergic patients as well as the limited propensity to activate basophil degranulation classifies Der p 13 as a minor HDM allergen. Nevertheless, the protein with its presumptively associated lipid(s) triggered the production of IL-8 and GM-CSF in respiratory epithelial cells through a TLR2-, MyD88-, NF-kB-, and MAPK-dependent signaling pathway.
Conclusions: Although a minor allergen, Der p 13 may, through its lipid-binding capacity, play a role in the initiation of the HDM-allergic response through TLR2 activation.

Sensitization to house dust mites (HDM) can trigger strong allergen-induced inflammation of the skin and airway mucosa resulting in atopic dermatitis as well as allergic rhinitis and asthma (1). In the most studied HDM species, *Dermatophagoides pteronyssinus* (Der p) and *Dermatophagoides farinae* (Der f), protein allergens have been classified as being

major, intermediate, or minor allergens according to their IgE-binding frequencies in HDM-allergic patient cohorts (2). On the basis of IgE-binding frequency as a hypothetical criterion for allergenicity, allergens from groups 1 and 2 have been proposed to be significant inducers of IgE responses, unlike those in groups 4, 5, 7, 21, and 23, which are targets of low-to-intermediate IgE-binding frequencies (3). However, a growing literature points toward a critical role for innate immune pathways in the initiation of HDM-allergic responses. It has consequently been proposed that a new allergen classification is warranted, based on their propensities in activating innate responses (4). For instance, the HDM serine protease allergens Der p/f 3, Der p/f 6, and Der p/f 9, despite having low IgE-binding frequencies, activate innate immune cells to initiate or prolong the HDM-induced allergy pathogenesis, possibly through the protease-activated receptor 2 (PAR-2) (5).

Lipid-binding capacity is another potential allergenic determinant commonly observed in proteins triggering allergic responses, including Bet v 1-like molecules, nonspecific lipid transfer proteins (nLTP), and lipid-binding lipocalins (6). Lipids may also influence the early stages of the allergic response through TLR4- and TLR2-dependent mechanisms. Several HDM allergens, Der p/f 2, Der p/f 5, Der p/f 7, and Der p/f 21, have been considered to be lipid-binding proteins that may have allergenic potential, in cellular assays, or *in vivo* experiments using TLR4- and MD2-deficient mice (7–11). Group 13 mite allergens display clear sequence similarities with cytoplasmic fatty acid-binding proteins (FABPs) and consequently could also be of importance in innate immune signaling through associations with mite or microbial lipids (2). With the exception of the *B. tropicalis* allergen Blo t 13, which has been shown to bind fatty acids (12), the capacity of other group 13 mite allergens to stimulate innate immunity through their lipid cargo remains to be assessed. The goal of this study was to investigate a recombinant form of Der p 13 in terms of IgE reactivity, lipid-binding propensity, and airway epithelial cell activation.

Materials and methods

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

Results

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

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

Moreover, MS analysis identified tryptic peptides covering 90% of the rDer p 13 sequence (Fig. S1). Far UV circular dichroism (CD) analysis of rDer p 13 yielded a spectrum typical of a folded protein with β-sheet structure predominating (Fig. 1C). Estimates of the secondary structure content using CDPro software indicated 37.5% β-sheet, 12.5% α-helix, 19% turns, and 31% unstructured. We also confirmed by immunoblotting that mouse polyclonal antibodies to rDer p 13 react with the natural molecule present in commercially available mite bodies but not in mite feces (Fig. 1D), which is consistent with Der p 13 belonging to the FABP family of proteins that are usually confined to cell cytoplasm and not secreted (13).

IgE reactivity to rDer p 13 and basophil degranulation assays

Using 224 sera from HDM-allergic patients (Dpt ImmunoCap Class 3 or higher values) suffering from allergic rhinitis or asthma, the IgE reactivity to rDer p 13 was determined by sandwich ELISA and compared with the IgE-binding capacity of rDer p 2. Fifteen of 224 patients displayed specific IgE to rDer p 13 (7%), whereas 75% (169/224) of the same cohort exhibited specific IgE to rDer p 2. Our data therefore confirm that Der p 13 is classifiable as a minor allergen in that it is a target of IgE antibody in a low proportion of the HDM-allergic population. There remains, however, the possibility that a complex of Der p 13 and its resident lipid from HDM could be involved in allergic sensitization to itself and other HDM allergens. We therefore compared the allergen activity of rDer p 2 and rDer p 13 in a rat basophil degranulation assay using the RBL SX-38 cells (rat basophil leukemia cells expressing the human FcεRI receptor) preloaded with sera from patients sensitized to nDer p 2 or nDer p 13 (14). Regardless of the sera used, similar levels of cell activation were observed ($P > 0.5$) by addition of polyclonal anti-human IgE (0.1 μg/ml), causing around 50% of the total hexosaminidase release. Cell incubation with rDer p 13 triggered allergen concentration-dependent basophil degranulation following a typical bell-shape curve, with a maximum reached with 0.01 μg/ml (Fig. 2). Using the same allergen concentration, rDer p 2 triggered significantly higher levels of degranulation ($P < 0.05$), indicating that Der p 2 exhibits more allergen activity than Der p 13.

Lipid binding by Der p 13

Lipid-binding assays were performed using different fluorophore-conjugated or intrinsically fluorescent fatty acids (BODIPY-C16, 11-([5-dimethylaminonaphthalene-1-sulfonyl amino]) undecanoic acid (DAUDA), dansyl-DL-α-aminocaprylic acid (DACA), *cis*-parinaric acid (cPnA), and dehydroergosterol (DHE)) as well as probes for apolar surfaces and binding pockets (1-anilinonapthalene-8-sulfonate (ANS), bis-ANS). As shown in Fig. 3A, the fluorescence emission of the natural, nonconjugated fluorescent fatty acid cPnA was

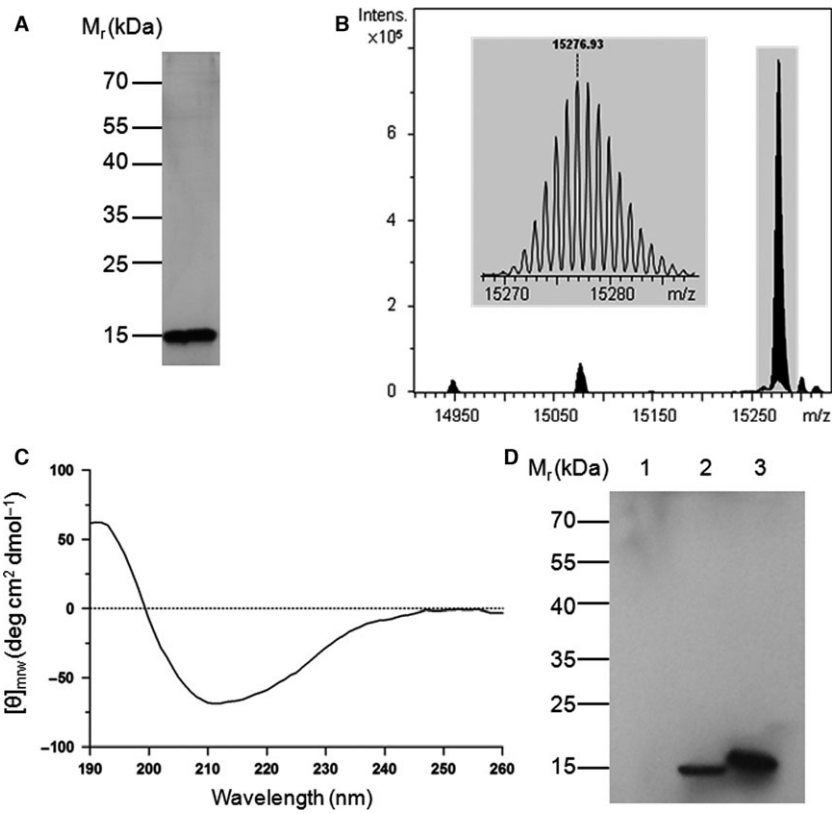

Figure 1 Physico-chemical characterization of rDer p 13 and detection of nDer p 13 in HDM allergen extracts from *D. pteronyssinus*. SDS-PAGE analysis of purified rDer p 13 under reducing conditions (Panel A). Deconvoluted mass spectrum of purified and reduced rDer p 13 obtained using nano LC-ESI-Qq-ToF MS (Panel B). Far UV CD spectrum of purified rDer p 13 at 25°C (Panel C). Immunoblot detection of nDer p 13 using anti-rDer p 13 mouse polyclonal antibodies (Panel D). Lane 1: *D. pteronyssinus* fecal pellet extract, Lane 2: *D. pteronyssinus* mite bodies extract (Stallergenes Greer), Lane 3: purified rDer p 13.

significantly enhanced when mixed with rDer p 13, indicating the entry of the fatty acid into a nonpolar 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 preformed 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, β-lactoglobulin, showed efficient dose-dependent competitive displacement (Fig. 3B). 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. 3C).

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

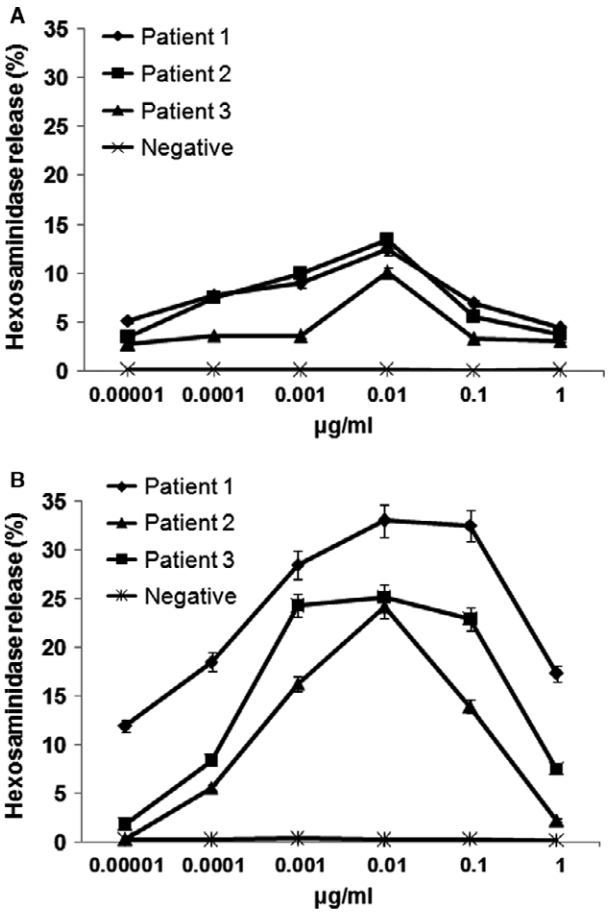

Figure 2 RBL SX-38 cell degranulation by rDer p 13 and rDer p 2. Cells were primed for 16 h with sera from three HDM-allergic patients, containing rDer p 13- (Panel A) or rDer p 2-specific IgE (Panel B) and subsequently stimulated with serial dilutions of purified rDer p 13 or rDer p 2 for 30 min. Degranulation was measured through β-hexosaminidases activity. Percentage of degranulation was presented as subtraction of spontaneous released over total lysis with Triton X-100.

protein in complex with ligand (PDB code 1YIV) (17) (Fig. 4A and B, respectively). Comparison of Fig. 4A and 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 theoretically 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. 4C). 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 emission is enhanced to a degree expected for its removal from solvent water into an apolar protein environment.

Der p 13 activates airway epithelial cells through TLR2

To investigate whether Der p 13 could activate the airway epithelium through proinflammatory cytokine induction, human bronchial epithelial BEAS-2B cells were incubated with different rDer p 13 concentrations under serum-free conditions, followed by measurement of IL-8 and GM-CSF levels in culture supernatants. As a positive control, cells were stimulated with the Pam_3Cys_4 TLR2 ligand. When compared with the control medium, rDer p 13 elicited the production of IL-8 and GM-CSF in a concentration-dependent manner (Fig. 5A and B). Supernatants from methanol-induced wild-type KM71 cells that had been treated as for the rDer p 13 purification protocol did not stimulate the release of these cytokines, thereby assuring that the allergen-induced IL-8/GM-CSF expression was not due to trace contaminants in the protein preparation.

Given the propensity of Der p 13 to transport specific lipids, we hypothesized that the Der p 13-induced cytokine release might be mediated by TLR2, this receptor being activated by microbial lipoprotein/lipid ligands (21). As shown in Fig. 5C, pre-incubation of cells with blocking anti-human TLR2 mAb drastically reduced the Der p 13-induced IL-8 secretion from BEAS-2B cells. This TLR2 dependence was not replicated by the isotype control antibody. To confirm the specificity of such TLR2 activation, similar assays were performed with purified recombinant allergens rProDer p 1 (22) and rDer p 23 (23), two proteins produced in *P. pastoris* that do not have known lipid-binding activities. Under the same experimental conditions, rProDer p 1 was unable to stimulate the production of IL-8 (data not shown), whereas rDer p 23-induced cytokine production was TLR2 independent (Fig. 5C).

Given that TLR2 engages with the MyD88 adaptor protein (24), 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 downregulate MyD88 activity. Treatment of BEAS-2B cells transfected with DN-MyD88 drastically reduced IL-8 secretion (Fig. 5D), but no effect was observed with the control plasmid.

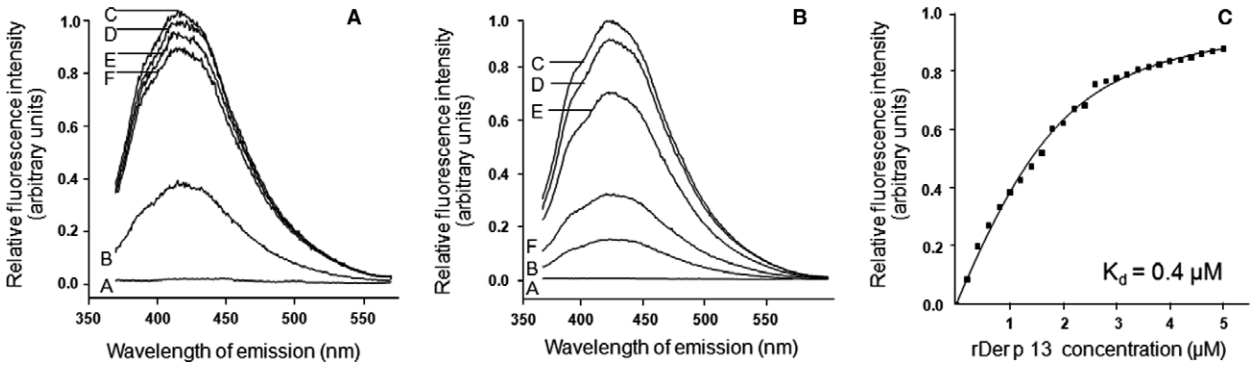

Figure 3 Hydrophobic ligand-binding activity of rDer p 13. Fluorescence emission spectra of cis-parinaric acid (cPnA, Ex_{max} = 319 nm) bound to purified rDer p 13 (Panel A) and β-lactoglobulin (Panel B). The competitive binding of oleic acid used at different concentrations (7.9 μM, 79 μM, 790 μM) is also shown. Curve A: PBS, curve B: cPnA alone, curve C: cPNA + protein, curve D: cPNA: protein complex + 7.9 μM oleic acid, curve E: cPNA: protein complex + 79 μM oleic acid, curve F: cPNA: protein complex + 790 μM oleic acid. One representative experiment out of 3 is shown. Titration curve of cPnA binding to rDer p 13 (Panel C). 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 0.4 μM and an apparent stoichiometry consistent with one binding site per protein monomer unit.

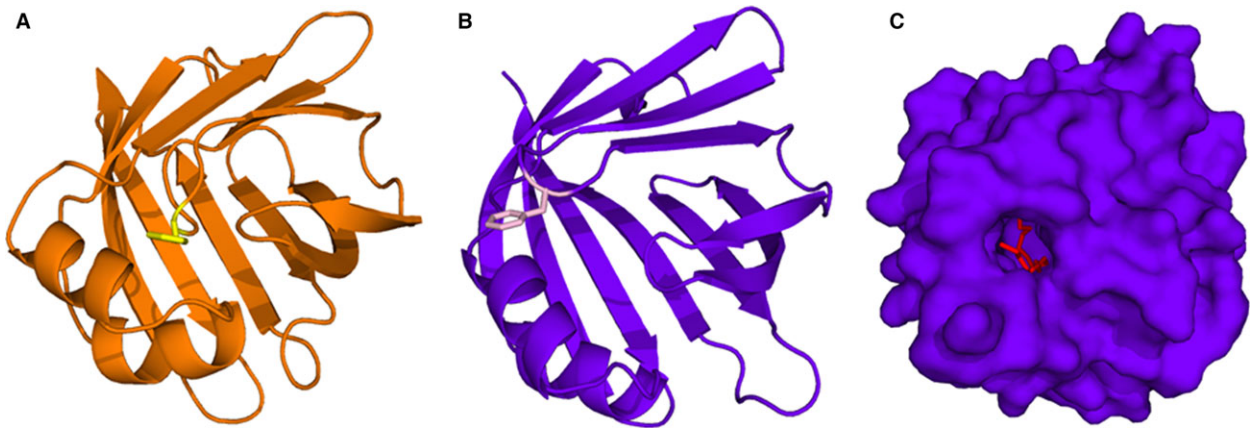

Figure 4 Structural model of Der p 13. Panel A: model of the apo form of Der p 13 based on the NMR structure of Der f 13 (PDB code 2A0A). Residue 57 is in yellow. Panel B: model of Der p 13 structure based on the X-ray structure of myelin P2 protein from equine spinal cord (PDB code 1YIV). Residue 57 is in light pink. Panel C: surface representation of the structure of the complex between the model of the Der p 13 holo form (in purple) and cPNA (in red sticks), obtained by docking simulations.

As NF-κB and MAP kinases are known to contribute to cytokine production in stimulated airway epithelium (25), 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 upregulation caused by rDer p 13 (Fig. 5E). Similar reduction of IL-8 release was observed with IkB-α phosphorylation BAY-11-7082 and proteasome MG132 inhibitors (Fig. 5E), suggesting that the three MAPK signaling cascades and NF-κB 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 1 h (rDer p 13:trypsin ratio 20 : 1) degraded the recombinant allergen (Fig. 5F). Strikingly, such rDer p 13 hydrolyzate was shown to retain its capacity to trigger the IL-8 release in BEAS-2B cells (Fig. 5G). The cytokine production was not due to the presence of trypsin because digested Der p 13 was

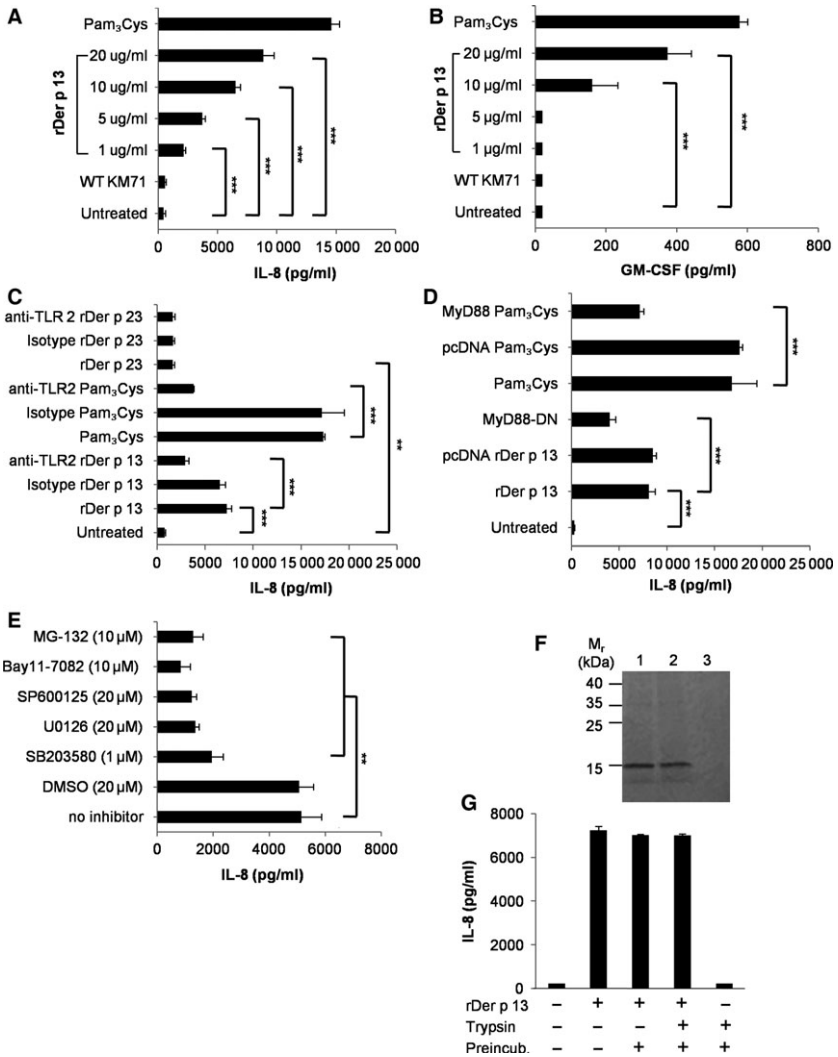

Figure 5 rDer p 13 activates airway epithelial cells through TLR2 signaling pathway. Stimulation of BEAS-2B cells by rDer p 13 triggers IL-8 (Panel A) and GM-CSF (Panel B) cytokine production in a concentration-dependent manner. BMMY medium from cultured wild-type *P. pastoris* and purified according to the purification protocol of rDer p 13 (WT KM71) as well as Pam_3Cys were used as negative and positive controls, respectively. To assess the importance of TLR2 signaling in the rDer p 13-induced activation, cells were also pre-incubated with blocking anti-TLR2 antibody or isotype control (Panel C) prior to allergen treatment (rDer p 23 was also used as a negative control of TLR2 activation). In another set of experiments, cells were transfected with dominant negative MyD88 or control plasmid followed by the rDer p 13 stimulation (Panel D). BEAS-2B cells were pre-incubated with SP600125, SB203580, U0126 (MAPK pathway inhibitors), or MG132, BAY 11-7082 (NF-κB pathway inhibitors) before treatment with rDer p 13 (Panel E). rDer p 13 was extensively digested with Trypsin at 37C (Panel F, lane 3). As control, undigested rDer p 13 (lane 1) was also pre-incubated at 37C (lane 2). Prior to cell activation 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. $^{**}P < 0.05$, $^{***}P < 0.001$.

treated prior to cell exposure with immobilized benzamidine, a matrix that traps and depletes this protease. The efficacy of trypsin removal was confirmed by the absence of cell activation with trypsin pre-incubated with the benzamidine matrix. These results suggest that a lipidic ligand of yeast origin and transported by Der p 13 may be influential in the allergen's effect on the airway epithelial cells.

Discussion

We previously demonstrated that the HDM allergens Der p 2 and Der p 23 triggered allergic sensitization in a large percentage of Thai HDM-allergic patients with frequencies similar to those measured in Western countries (23). To determine whether the level of sensitization to minor HDM

allergens was similar among Thai and Western populations, the present study focused on Der p 13, a member of group 13 mite allergens that has been poorly investigated to date. To our knowledge, only one publication partially described the production of rDer p 13 in bacteria and, moreover, the characterization was restricted to its IgE reactivity (26). Recombinant group 13 mite allergens, including rBlo t 13, rTyr p 13, rAca s 13, rLep d 13, and rDer p 13 produced in *E. coli*, were shown to display a low prevalence of sensitization in the HDM-allergic population (26–30). IgE-binding frequencies against Der p 13, Blo t 13, Tyr p 13, Lep d 13, and Aca s 13 reached 6%, 7–11%, 6%, 13%, and 23%, respectively. Because these data were generated using recombinant allergens produced in *E. coli*, some of which were expressed as inclusion bodies, there is a possibility of underrepresentation because of inappropriate folding. Assessments of recombinant allergen folding were rarely performed, with the exception of the characterization of the Blo t 13 secondary structure (12) and the elucidation of the Der f 13 structure by NMR (15).

We produced Der p 13 in *P. pastoris* as a secreted protein, and CD analysis indicated that it exhibits a secondary structure content typical of members of the FABP family. Using this recombinant allergen, we found that while the IgE-binding frequency of rDer p 2 reached 75% (169/224 patients), reactivity to rDer p 13 was only 7% (15/224 patients) in this Thai HDM-allergic population but reached around 20% in HDM-allergic patients living in other areas (3). Such low frequency of reactivity to Der p 13, as with other members of group 13 mite allergens (26–30), may be due to the fact that cytoplasmic FABP allergens are restricted to mite bodies and not present in the feces (3). Whereas fragmented mite body parts together with fecal pellets represent the main allergenic source, the deep penetration of particles with allergen cargo into the lung must be size-dependent (31). Consequently, the HDM allergens transported within mite fecal pellets (10 μm average diameter) should trigger airway inflammation more readily than mite body parts. To support this hypothesis, it has recently been demonstrated that HDM allergens detectable only in mite bodies display weak IgE reactivity in sensitized population with respiratory symptoms, but represent major allergens in patients suffering from atopic dermatitis (32). The HDM allergics in our cohort suffered only from allergic rhinitis or asthma, so it would be interesting to determine whether HDM allergics with atopic dermatitis have similar or higher rates of IgE to Der p 13.

It is widely accepted that HDM allergies may be initiated through activation of innate immunity (4), such that any mite component capable of stimulating innate immune signaling could be influential. Pertinent to the role of specific lipids in immune activation, we demonstrated, using environment-sensitive fluorescent lipid probes, that rDer p 13 binds fatty acids and that the protein's binding to hydrophobic ligands is selective. Indeed, although Der p 13 and Blo t 13 share 80% amino acid sequence identity and display similar K_d values for cPNA (0.4×10^{-6} M *vs* 1.31×10^{-6} M, respectively), displacement of cPNA with oleic acid was ineffective for Der p 13, unlike with Blo t 13 (12). This may therefore mean that, despite their sequence and structural similarities, the precise binding propensities of the two proteins differ.

Computer-based docking experiments performed with two different models of Der p 13 (one based on the *apo* form of Der f 13, (15) the other based on the *holo* form of myelin P2 protein, (17)) with the lipid ligand cPnA known to bind to the protein, predicted that the accessibility of the hydrophobic pocket could be controlled by the side chain orientation of Phe57. This residue is located immediately beside the portal of entry to the binding pocket of some FABPs (16). A similarly positioned side chain is found in several FABPs (33), where it is speculated to regulate the entry of ligand to the proteins' binding pockets (20). The identification of the lipid ligand(s) naturally present in the Der p 13 hydrophobic pocket remains to be determined. Der p 13, like all FABPs other than those of nematodes (34), lacks a leader sequence and is therefore probably confined to the cytosol of mite cells. The lipids it may present are therefore likely to be cytoplasmic lipids, though we cannot exclude the possibility that Der p 13 transports lipid ligands from endosymbiotic bacteria or microbes in house dust mite.

Based on the finding that Der p 13 binds fatty acids, we hypothesized that this allergen could activate TLR2 signaling in airway epithelium. This pathogen-associated molecular pattern receptor forms a heterodimer with either TLR1 or TLR6 and interacts with lipids/fatty acids or lipoprotein (35). The activation of TLR4 in combination with MD2 and CD14 (another receptor recognizing microbial lipidic ligands) by Der p 13 was not evaluated because the BEAS-2B airway epithelial cells were found to be hyporesponsive to the LPS needed as a positive control (data not shown). Indeed, such cells were shown to express MD2 poorly, whereas intracellular localization of TLR4 is nevertheless apparent (36).

To determine whether rDer p 13 enhances innate responses through TLR2, proinflammatory cytokine production was measured from stimulated BEAS 2B. We focused on both IL-8 and GM-CSF secretion because these cytokines are important chemoattractants and activators for immune cells such as neutrophils, basophils, eosinophils, and dendritic cells (37). Our results showed that rDer p 13 stimulated the production of IL-8 and GM-CSF by airway epithelial cells in a time- and concentration-dependent manner. Through a combination of blocking antibodies, specific inhibitors, and depletion of MyD88, we found that Der p 13 triggers airway epithelial cell activation through TLR2-MyD88-NF-κB- and MAPK-dependent 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 coreceptor 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 (11, 38), 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 (39), recent studies indicated that TLR2 signaling could be critical for the development of HDM-allergic rhinitis and asthma (40–43). 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-κB, which comprises an orchestrator of the HDM-induced airway inflammation, hyper-responsiveness, and fibrotic remodeling (44).

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 *in vivo* following deposition of mite fragments onto the lung surface with consequent allergen release. It must be pointed out that Der p 2 is also mainly present in mite bodies yet is a major allergen able to activate TLR2 and TLR4 (7, 38). Because the lipid environment in the mite, house dust, or the *P. pastoris* yeast used to produce the allergen may differ considerably, it is important ultimately to identify the natural Der p 13 ligand(s) and to characterize its effects on immune cells. Also, the lipid transfer mechanism involved in the Der p 13-mediated enhancement of TLR2 signaling remains unclear, although FABPs of the subfamily to which Der p 13 belongs are known to interact directly with membranes in the transfer of lipid cargo (45). Nevertheless, our results demonstrate that the HDM allergen hierarchy, based essentially on IgE reactivities, needs further refinement to take into account the capacity of allergens to stimulate innate immunity. Consequently, minor HDM allergens such as Der p 13 require further consideration to elucidate their abilities to activate airway epithelial cells as well as keratinocytes.

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Appendix 1
Mite Allergy Research Cohort (MARC) study team

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2 Children Hospital: Tassalalpa Daengsuwan
3 Ramathibodi Hospital: Wannada Laisuan, Malinee Tongdee, Nizchapha Dchapaphapeaktak
4 Phramongkutklao College of Medicine: Tadech Boonpiyathad

Conflict of interest

The authors declare that they have no conflicts of interest.

Author contributions

AJ designed the study; SP performed the experiments in collaboration with DG, Su Pi and EN; MLM performed mass spectrometry experiments; JW helped with ImmunoCap assays, NS, PC, MV, TR, AS, and KR helped in the sera collection. AJ, EN, DG, and MWK provided supervision and analyzed the data for the spectrofluorometric analysis carried out in Glasgow. SP drafted the manuscript with input from AJ, MWK, DG, and EN. All authors contributed to and approved the final version of the manuscript.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. MS sequence coverage of rDer p 13 tryptic digestion Amino acid sequence of expressed rDer p 13, the residues in bold corresponding to the potential trypsin cleavage sites. Grey highlighted sequence represents peptides unambiguously identified by MS/MS.

Data S1. Materials and methods.

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