Expression and Characterization of Natural-Like Recombinant Der p 2 for Sublingual Immunotherapy

Véronique Bordas-Le Floch Laetitia Bussires Sabi Airouche Aurlie Lautrette
Julien Bouley Nathalie Berjont Stphane Horiot Axelle Huet Karine Jain
Pierrick Lemoine Henri Chabre Thierry Batard Laurent Mascarell
Vronique Baron-Bodo Sophie Tourdot Emmanuel Nony Philippe Moingeon

Stallergnes SA, Antony, France

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Abstract

Background: Recombinant allergens with a native conformation represent an alternative to natural extracts for immunotherapy and diagnostic purposes. **Methods:** We produced the Der p 2 mite allergen in *Pichia pastoris* and *Escherichia coli*. After purification by cation exchange chromatography, recombinant molecules were compared to their natural counterpart based upon structural (disulfide bonds, secondary structure, thermal stability) and immunological properties (antibody reactivity, basophil and T cell activation, tolerance induction in a murine sublingual immunotherapy model). **Results:** The Der p 2.0101 isoform was confirmed to be prevalent in *Dermatophagoides pteronyssinus* extracts. It was then produced as a secreted molecule in *P. pastoris* or refolded from *E. coli* inclusion bodies. The yeast-expressed rDer p 2 molecule exhibits a natural-like disulfide bridge distribution and secondary structure, whereas the *E. coli*-derived rDer p 2 presents some heterogeneity in cysteine bonds and a lower stability following thermal stress.

The two recombinant as well as natural Der p 2 molecules exhibit comparable IgE recognition and activate basophil and CD4⁺ T cells. Sublingual immunotherapy of nDer p 2-sensitized mice using either one of the rDer p 2 molecules efficiently decreases airway hyperresponsiveness as well as Th2 responses. **Conclusions:** Natural and recombinant Der p 2 molecules produced in *P. pastoris* and *E. coli* exhibit comparable immunological properties despite distinct structural features. Natural-like cysteine pairing is a critical parameter to identify stable, well-folded and homogenous proteins appropriate for immunotherapy and diagnostic purposes.

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Introduction

Specific immunotherapy with natural allergens is established as a safe and efficacious treatment for respiratory allergies, both in adults and children [1–10]. Current vaccines are based on complex allergen extracts that are not easy to standardize. In this context, recombinant allergens represent a valid alternative for immunotherapy, as they can be produced using good manufacturing practice-compliant systems in large quantities in the form of

well-defined molecules [11–14]. In this regard, numerous approaches are focusing on hypoallergenic forms with disrupted IgE binding sites, with the aim of producing safer vaccines with a preserved capacity to induce protective immune responses [12, 15, 16]. However, when considering either diagnostic applications or the sublingual route of immunotherapy, allergens in the most natural conformation are preferred [17]. Indeed, maintaining IgE recognition is thought to facilitate allergen addressing to FcεRI-expressing tolerogenic dendritic cells in oral mucosal tissues [18–21].

With the aim of developing a recombinant vaccine to treat allergies to the *Dermatophagoides* mite species, group 1 and 2 allergens represent highly clinically relevant molecules, as more than 80% of house-dust-mite (HDM)-allergic patients exhibit elevated seric IgE titers against each of these molecules [22–25]. Given the high-sequence homology and thus substantial IgE cross-reactivities between allergens from the closely related species *D. farinae* [24, 26–28], a vaccine comprising only *D. pteronyssinus* allergens could be considered to treat allergies to either one of these two prominent mite species.

In this study, we focused on the expression and characterization of the Der p 2 allergen for potential pharmaceutical applications. Although Der p 2 has been previously produced in a recombinant form in a variety of expression systems [29–32], as of today, none of these studies has documented a bona fide natural conformation of the molecule to a level fulfilling the requirements for human administration. As Der p 2 exists as a variety of isoforms/variants [33–35], we first selected a prominent isoform following a mass spectrometry (MS) analysis of mite extracts. The Der p 2.0101 isoform was thus produced as a recombinant molecule in both *Pichia pastoris* (rDer p 2_{pp}) and *Escherichia coli* (rDer p 2_{ec}) and compared with natural purified Der p 2 in terms of structural and immunological properties.

Materials and Methods

Natural Der p 2 Purification

Natural Der p 2 was purified from *D. pteronyssinus* mites cultured at our facilities [36]. Allergen purification was first performed from mite extracts by hydrophobic interaction chromatography using a phenyl sepharose column (GE Healthcare, Saclay, France) and 20 mM sodium phosphate, 1.8 M ammonium sulphate pH 6.2, as well as 20 mM sodium phosphate, 5% isopropanol pH 7.5 as binding and elution buffers, respectively. Natural Der p 2 in 10 mM sodium acetate, 0.5 M NaCl, pH 6 was then loaded onto a SP XL column (GE Healthcare) for further purification, and eluted with 10 mM sodium acetate, 0.5 M NaCl pH 6.

Expression and Purification of Recombinant Der p 2 in *P. pastoris*

A DNA sequence encoding Der p 2.0101 (P49278) was cloned flush with an α-factor signal sequence in pPICZα (Invitrogen, Cergy Pontoise, France) using *Xho*I and *Not*I restriction sites. Yeast GS115 and X33 strains were transformed with the resulting plasmid by electroporation using a Gene Pulser Xcell apparatus according to the manufacturer's instructions (Bio-Rad, Marne-la-Coquette, France). Transformants selected on 100 μg/ml zeocine YPD plates were grown overnight at 30°C in buffered glycerol-complex medium yeast extract pH 6 medium until reaching an OD_{600nm} of 2–6. Cells were then diluted in buffered methanol-complex medium yeast extract pH 6 induction medium to an initial OD_{600nm} of 1 and further grown for 1–6 days at 30°C. Culture supernatants were harvested by centrifugation and stored at –20°C until use.

Supernatants were dialyzed against a 10 mM NaCl, 10 mM sodium acetate, pH 5 binding buffer and applied onto an SP XL column (GE Healthcare). Recombinant Der p 2_{pp} was eluted with a 500 mM NaCl, 10 mM sodium acetate, pH 5.5 gradient. Fractions containing rDer p 2_{pp} were pooled and concentrated using an Amicon cell with a 5-kDa cutoff membrane (Millipore, Saint-Quentin-en-Yvelines, France). Endotoxin levels in rDer p 2_{pp} solution (10 μg/ml), measured by a Limulus Amoebocyte Lysate assay, were under 1 EU/ml.

Expression, Refolding and Purification of Recombinant Der p 2 in *E. coli*

A DNA sequence encoding mature Der p 2.0101 with a DIS mutation was cloned in pET24a(+) (Novagen, Nottingham, UK) using *Nde*I and *Not*I restriction sites. The plasmid was then introduced in BL21 (DE3) cells according to the manufacturer's instructions (Invitrogen) and transformants were selected in the presence of 30 μg/ml kanamycine.

Small-scale expression was performed as previously described [37]. Briefly, overnight starter cultures were diluted in fresh LB medium and further grown until reaching an OD_{600nm} of 0.4–0.8. Der p 2 expression was then induced for 4 h with 1 mM isopropyl β-D-1-thiogalactopyranoside. Cell pellets were collected by centrifugation and stored at –20°C until use.

Fermentation was further performed at a 2-liter scale in a Biostat Bplus bioreactor (Sartorius Stedim, Aubagne, France). An overnight starter culture was used to inoculate a complex rich-growth medium. During the biomass accumulation phase, cells were manually fed with a glucose-containing feeding solution until reaching an OD_{600nm} of 15. Der p 2 expression was then induced for 4 h with 1 mM isopropyl β-D-1-thiogalactopyranoside. Cells were pelleted by centrifugation and stored at –20°C until use.

Cell lysis was performed by freeze/thaw cycles and sonication. Inclusion bodies were recovered and washed as described elsewhere [37]. After solubilisation in 8 M urea, refolding was done by rapid dilution in 50 mM Tris pH 8, 1 mM EDTA, 50 mM NaCl, 5 mM reduced glutathione and 500 mM L-arginine. Refolded Der p 2 was dialyzed against 50 mM Tris, 1 mM EDTA, pH 8 buffer and 10 mM sodium acetate, pH 5.5. The protein was subsequently loaded onto a SP FF column (GE Healthcare) and eluted with 10 mM sodium acetate, 240 mM NaCl, pH 5. Endotoxin levels in 10 μg/ml rDer p 2_{ec} solutions, measured by a Limulus Amoebocyte Lysate assay, ranged from less than 1 to 88 EU/ml.

Western Blots

Purified allergens were quantified using Bradford assays. Following SDS-PAGE on 4–12% Bis-Tris acrylamide gels (Invitrogen), Western blots were performed and revealed with either the Dpx-A9 mouse anti-Der p 2 monoclonal antibody (0.2 µg/ml; Indoor Biotechnologies Limited, Warminster, UK) or a pool of sera obtained from HDM-allergic donors, as previously described [37]. Recombinant Bet v 1 (200 ng) [38, 39] was used as a negative control.

Circular Dichroism

The higher-order structure of Der p 2 molecules was examined by circular dichroism (CD). Spectroscopic profiling was carried out using a Jasco J-815 CD spectrophotometer (Jasco, Bouguenais, France) against a buffer blank (10 mM sodium acetate, 150 mM NaCl, pH 5.0). Eight independent spectral scans were recorded in the 200–260 nm wavelength range, in 1-mm cuvettes with a scanning speed of 100 nm/min, a 1-nm bandwidth and a 0.2-nm data pitch, and averaged. The Yang reference algorithm [40] was used to estimate the relative proportion of α -helical, β -sheet, turn and random coil contents. For thermal denaturation experiments, protein samples were heated from 20 to 90°C in 10°C-incremental steps, and three CD spectra were averaged.

Reversed-Phase Liquid Chromatography-UV-Mass Spectrometry Analyses

Mass calculation by reversed-phase liquid chromatography-UV-MS analyses was performed on each of 3 batches of recombinant Der p 2_{ec} and Der p 2_{pp} molecules. To this end, 5 µg Der p 2 were injected onto a C₁₈ HypersilGold (2.1 × 200 mm, 1.9 µm) column (ThermoScientific, Courtaboeuf, France), thermostated at 65°C and connected to a rapid separation liquid chromatography (RSLC) system (Dionex, Voisins Le Bretonneux, France). An acetonitrile gradient containing 0.1% v/v formic acid was applied over 15 min using an U3000 RSLC chromatographic instrument (Dionex) with a 400 µl·min⁻¹ flow rate to ensure proper UV detection at 214 nm. A quadrupole-time of flight mass spectrometer (Maxis, Bruker Daltonics, Wissembourg, France) was connected to the RSLC for accurate mass measurement. Mass spectra deconvolution was performed using a MaxEnt algorithm and the following parameters: 13,000–16,000 Da, data point spacing auto and resolution 35,000.

For disulfide bridge mapping, proteins (15 µg) were alkylated for 30 min with 15 mM iodoacetamide in a 75 mM ammonium bicarbonate buffer, then digested with trypsin (Sigma, Saint-Quentin Fallavier, France) using a 1/25 (m/m) trypsin/Der p 2 ratio. After a 16-hour digestion at 37°C, the reaction was stopped by adding 0.5% TFA. Digests were separated on a C₁₈ Acquity (BEH130, 100 × 2.1 mm, 1.7 µm) column (Waters, Guyancourt, France) with a 2-step linear gradient from 0 to 55% acetonitrile (0.1% TFA) over 50 min, using a U3000 RSLC chromatographic apparatus (Dionex). Tandem MS acquisition was performed on a quadrupole-time of flight mass spectrometer (Maxis).

Basophil Activation Tests

Basophil activation was assessed with an FC500 cytometry apparatus (Beckman Coulter, Villepinte, France) using the Allergen-icity kit (Beckman Coulter) to measure the surface expression of the activation marker CD203c, as previously described [41]. Whole blood samples treated with acid citrate dextrose were ob-

tained from 6 HDM-allergic donors and incubated at 37°C for 15 min in the presence of increasing concentrations (0.064–1,000 ng/ml) of natural or recombinant Der p 2 molecules. Whole blood cells incubated with either PBS or anti-IgE antibodies were used as negative and positive controls, respectively. Activated basophils were detected after the exclusion of T lymphocytes (CD3+ cells) as CRTH2⁺ CD203^{high} cells.

ELISA Inhibition Assays

The ability of the various Der p 2 molecules to inhibit IgE binding to nDer p 2 was examined using ELISA inhibition with a pool of sera obtained from 12 HDM-allergic patients. Purified natural Der p 2 (10 µg/ml) was coated overnight in a 0.1-M sodium carbonate-bicarbonate pH 9.5 buffer in microtiter plates. The serum pool was mixed with increasing concentrations of either natural or recombinant Der p 2 molecules (ranging from 0.002 to 175 µg/ml) and incubated overnight at 4°C with nDer p 2-coated plates. After 5 washes with PBS 0.1% Tween, bound IgEs were detected using a 1:1,000 dilution of a horseradish peroxidase-conjugated anti-human IgE antibody (Calbiochem, Nottingham, UK) and OPD substrate (Sigma). The reaction was stopped with 3 M HCl, and plates were read at 492 nm on a Multiskan ascent spectrophotometer (Labsystems, Saint Herblain, France). Percentages of inhibition were calculated as $100 \times [(OD_{NI} - OD_I)/OD_{NI}]$ with OD_{NI} and OD_I being the ODs measured with the non-inhibited and inhibited sera pools, respectively.

Sublingual Immunotherapy of nDer p 2-Sensitized Mice

BALB/c mice sensitization was performed according to the protocol previously described [42]. They were sensitized by 2 intraperitoneal injections at 14-day intervals with 10 µg nDer p 2 adsorbed on 2 mg of alum, then exposed daily to *D. pteronyssinus* extract aerosols (corresponding to 1-mg Der p 2 doses) for a week. Sensitized mice were randomized into treatment groups with 6 mice per group, respectively. Unsensitized healthy mice were used as an additional control group. The mice were treated sublingually twice a week for 8 weeks with either vehicle (PBS only), rDer p 2_{ec} or rDer p 2_{pp} (50 µg in 50 µl PBS). Following treatment, they were challenged with *D. pteronyssinus* extract (using doses corresponding to 1 mg Der p 2 per aerosol) on two consecutive days. Airway hyperresponsiveness (AHR) was assessed 24 h after the last challenge as described elsewhere [42], using whole body plethysmography (Buxco Europe Ltd, Winchester, UK) in the presence of either 50 mg/ml methacholine or PBS, and expressed as a Penh Index. Penh indexes were calculated as ratios with baseline Penh after exposure to nebulized PBS. Statistical significance was evaluated with Mann-Whitney tests using 95% confidence intervals.

Assessment of Immune Responses in the Lungs

Bronchoalveolar lavage was performed by flushing out the lungs with 1.2 ml PBS via a tracheal cannula. Lavage fluids were then centrifuged at 800 g for 5 min at 4°C. Cell pellets were resuspended in 0.6 ml PBS, spun onto glass slides by cytocentrifugation, fixed and stained with May-Grünwald Giemsa (Réactifs RAL, Martillac, France) prior to differential cell counts.

To assess T cell responses, lung cells from nDer p 2-sensitized mice were recovered after treatment with either PBS, rDer p 2_{ec} or rDer p 2_{pp} and cultured (1×10^6 per well) for 72 h at 37°C, in 5% CO₂/95% air, in RPMI 1640 medium supplemented with glutamine (PAA laboratories, Les Mureaux, France), penicillin/strepto-

Table 1. Isoform polymorphism and disulfide mapping within natural and recombinant Der p 2 molecules

	Isoform	Molecular mass (Da) (theoretical)	Sequence variation ^a	N-terminal sequence	Secondary structure	Expected disul- fide bridges ^c	Mispaired disulfide bridges
nDer p 2	2.0101	14,115.168 (14,115.177)		DQVDVK	α-helix: 34.2% β-sheet: 50.6% turn: 1.8 % random coil :13.4%	yes	none detected
	2.0105	14,115.168 (14,115.177)	V40L T47S				
	2.0109	14,082.190 (14,082.223)	M111L D114N T47S				
	2.0104	14,096.181 (14,096.234)	V40L T47S M111L D114N				
rDer p 2 _{ec}	2.0101	14,087.169 (14,087.182)	D1S ^b	S ^b QVDVK	α-helix: 52.7% β-sheet: 46.7% turn: 0% random coil: 0.6%	yes	30 to 70% e.g. C21-C119 C8-C78 C73-C119
rDer p 2 _{pp}	2.0101	14,115.208 (14,115.177)		DQVDVK	α-helix: 36.1% β-sheet: 59.6% turn: 0% random coil: 4.3%	yes	none detected

Summary of experimental masses, sequence polymorphisms, mutations, amino-terminal sequence and cysteine bridge distribution obtained for natural and recombinant Der p 2 molecules. Results are compiled from experiments conducted with 11 batches of purified nDer p2 and 3 batches each of rDer p 2_{ec} and rDer p 2_{pp}. The relative amount of isoform 2.0101 within 3 independent batches of nDer p 2 was found to be more than 50%. Percentages of secondary structure elements were estimated on CD spectra using Yang's algorithm.

^a Taking isoform 0101 sequence as a reference. Amino acid substitutions were determined by MS.

^b a D/S mutation was introduced to facilitate cleavage of the amino-terminal methionine.

^c C21-C27, C8-C119, C71-C78, with numbering relative to the amino-acid sequence of mature Der p 2.

mycin (Fisher Bioblock, Illkirch, France) and 10% fetal calf bovine serum (Gentaur, Brussels, Belgium) in the presence of 10 µg/ml of either recombinant, natural Der p 2 or purified nDac g 1 allergen and medium as controls. After 3 days, IL-5, IL-10 and IL-13 levels were measured in culture supernatants by cytometric bead arrays (BD Biosciences, Le Pont de Claix, France) according to the manufacturer's recommendations. Statistical significances were evaluated with Mann-Whitney tests using 95% confidence intervals.

Results

Expression of Recombinant Der p 2 in P. Pastoris and E. coli

Natural Der p 2 was purified from a *D. pteronyssinus* extract by hydrophobic, then ion exchange chromatography and further analyzed by MS to investigate isoform variability and prevalence. As shown in table 1, at least four distinct isoforms were concomitantly found in the mite extract, with isoform 2.0101 being one of the most prominent; it was thus selected as a reference natural

molecule. Corresponding cDNAs were expressed in *P. pastoris* and *E. coli* as described in Methods, leading to rDer p 2_{pp} and rDer p 2_{ec}, respectively.

rDer p 2_{pp} was produced as a secreted molecule, whereas rDer p 2_{ec} was expressed as inclusion bodies. After solubilisation, the latter was refolded by a rapid dilution method. Both recombinant molecules were purified by cation exchange chromatography as described in Methods, with a purity superior to 90%. Yields were in the order of 60–70 mg/l Der p 2 after purification and concentration in both expression systems. Antibody recognition of these recombinant Der p 2 molecules was tested by immunoblot using both specific murine monoclonal antibodies directed against natural Der p 2 and human IgEs from HDM-allergic patients. In these experiments, natural Der p 2 and rBet v 1 were used as positive and negative controls, respectively. All molecules reacted similarly with Der p 2-specific monoclonal and IgE antibodies as a single 14 kDa band (fig. 1).

Physicochemical and Structural Characterization of Recombinant Der p 2 Molecules

Precise masses of rDer p 2 molecules were determined by MS and were confirmed to be in good agreement with the theoretical mass, with less than 3 ppm mass differences (table 1). Furthermore, exact mass data indicated that N-terminal ends of the two recombinant proteins were correctly processed and that the amino-terminal methionine was removed in *E. coli*.

We subsequently compared secondary structures of the three Der p 2 molecules using CD. As illustrated in figure 2a, nDer p 2 and rDer p 2_{pp} displayed similar CD spectra. The *E. coli*-expressed rDer p 2_{ec} molecule was also clearly folded, albeit with a different secondary structure, including most particularly a lower β -sheet content (table 1), when compared with nDer p 2.

Thermal denaturation experiments were conducted between 20 and 90°C to compare the stability of the molecules. As shown in figure 2b and c, respectively, nDer p 2 and rDer p 2_{pp} spectra did not change significantly when the temperature was raised, confirming a high stability for these two molecules. In contrast, thermal denaturation of rDer p 2_{ec} resulted in substantial changes in the CD spectrum, demonstrating a loss of structure above 70°C (fig. 2d). In addition, CD spectra acquired before heating and after cooling rDer p 2_{ec} back to 20°C were clearly distinct (fig. 2e), indicating that the molecule could not refold properly following heat denaturation.

We further explored the distribution of disulfide bridges by liquid chromatography tandem MS. The three expected disulfide bonds (C8-C119, C16-C27, C73-C78) reported in the literature [43, 44] were present in both the natural and the two recombinant allergens. However, some cysteine mispairings (representing 30–70% of cysteine bridges) were also found in the *E. coli*-expressed molecule, thus indicating a heterogeneous folding when compared to rDer p 2_{pp} and nDer p 2 (table 1). Noteworthy no intermolecular bridges were detected. Consistent with the latter observation, the two rDer p 2 as well as nDer p 2 were confirmed to be mainly monomeric, when tested by size exclusion chromatography (data not shown).

Immunogenicity of Recombinant Der p 2 Molecules

The two rDer p 2 molecules were compared with purified nDer p 2 for their capacity to activate peripheral blood basophils obtained from mite-sensitized patients (n = 3). The upregulation of the CD203c activation marker was measured by FACS after incubating basophils with serial concentrations (0.064–1,000 ng/ml) of each of the allergens. In agreement with the IgE reactivity patterns

Fig. 1. Immunoblot analysis of Der p 2 produced in *E. coli* and *P. pastoris*. Western blot analysis of purified natural and recombinant Der p 2 (200 ng/lane) was performed under nonreducing conditions with the specific DpX-A9 murine monoclonal antibody and IgEs from a pool of sera obtained from HDM-allergic patients. Purified rBet v 1 (200 ng) was used as a negative control.

reported in figure 1, the two rDer p 2 molecules were able to trigger basophil activation in a comparable manner to nDer p 2 (fig. 3a).

We further compared the ability of recombinant Der p 2 molecules to inhibit IgE binding to nDer p 2. A pool of sera obtained from HDM-allergic donors was incubated with nDer p 2-coated plates in the presence of increasing concentrations of either rDer p 2_{pp}, rDer p 2_{ec} or nDer p 2, prior to the detection of bound IgEs. As shown in figure 3b, such inhibition experiments established that rDer p 2_{ec} and rDer p 2_{pp} display similar IgE binding, albeit lower than their natural counterpart. Similar experiments performed with individual sera further confirmed a higher reactivity of nDer p 2 when compared to the recombinant molecules (data not shown). This superior reactivity of nDer p 2 is likely a consequence of the IgE recognition of multiple isoforms distinct from isoform 2.0101.

The T cell recognition of the three Der p 2 molecules was also compared. To this end, lung cells were recovered from nDer p 2-sensitized mice and restimulated in vitro for 3 days with either 10 μ g/ml nDer p 2, rDer p 2_{ec} or rDer p 2_{pp}, prior to measuring IL-5, IL-10 and IL-13 levels in culture supernatants. As illustrated in figure 4, all Der p 2 molecules induced a readily detectable production of the three cytokines by T cells. Natural Der p 2 consistently induced the highest cytokine levels, likely due to the recognition of multiple isoforms beyond isoform 2.0101.

Fig. 2. Secondary structure analysis by CD spectroscopy. Natural and recombinant Der p 2 (300 µg/ml) were submitted to CD spectroscopy (a). Thermal stability of nDer p 2 (b), rDer p 2_{pp} (c) and rDer p 2_{ec} (d) spectra were recorded at 20°C as the average of 8 scans in the wavelength range of 200–260 nm. e Refolding after

thermal denaturation of rDer p 2_{ec} was assessed by measuring CD spectra after heating the protein with 10°C-incremental-steps and cooling back to 20°C. Resulting spectra were obtained as the average of 3 scans.

Tolerance Induction by Sublingual Immunotherapy in a Murine Asthma Model

We subsequently assessed whether sublingual immunotherapy (SLIT) with rDer p 2_{ec} or rDer p 2_{pp} could desensitize nDer p 2-allergic mice. Mice were first sensitized with natural purified Der p 2 via the intraperitoneal route prior to challenges with aerosols of *D.*

pteronysinus extracts, as described in Methods. Animals were subsequently treated sublingually twice a week for 8 weeks with PBS or 50 µg of either recombinant Der p 2 molecule per dose. As illustrated in figure 5 (a, f), AHR was significantly reduced following SLIT with either rDer p 2_{ec} or Der p 2_{pp}, respectively, when compared to vehicle treatment ($p < 0.05$), although not to baseline levels ob-

Fig. 3. a Immunological characterization of rDer p 2. IgE binding was investigated by basophil activation and ELISA inhibition assays. Percentages of activated basophils were measured after exposure to varying concentrations of recombinant or natural Der p 2 (0.064–1,000 ng/ml). Data are represented as mean percentages of activated basophils obtained from 6 HDM-allergic donors. Differences at each dilution point are not statistically significant (Mann-Whitney statistical test with 95% confidence intervals).

b IgE binding to nDer p 2 was inhibited by natural and recombinant Der p 2 molecules. A pool of sera obtained from 12 HDM-allergic donors was mixed with increasing amounts of natural or recombinant allergens (ranging from 0.002 to 175 μ g/ml) and incubated with plates coated overnight with nDer p 2 (10 μ g/ml). Results are expressed as percentages of IgE binding inhibition and are representative of 2 independent experiments.

Fig. 4. T cell recognition of Der p 2 molecules. Secretion of IL-5 (a), IL-10 (b) and IL-13 (c) cytokines by lung T cells recovered from 6 Der p 2-sensitized mice were measured by cytometric bead array assays, after a 3-day in vitro restimulation with 10 μ g/ml of the various Der p 2 molecules. Restimulations with growth medium and the irrelevant natural purified Dac g 1 allergen (10 μ g/ml) were used as negative controls. Data are expressed as the mean $\pm$ SEM values obtained from 6 Der p 2-sensitized mice and are representative of 2 independent experiments. n.s. = Not significant. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$, using Mann-Whitney statistical tests with 95% confidence intervals.

Fig. 5. Desensitization of nDer p 2-allergic mice by sublingual treatment with either rDer p 2_{ec} or rDer p 2_{pp}. Mice were sensitized twice with 10 µg purified nDer p 2 adsorbed on alum followed by aerosol challenges then treated sublingually with vehicle (PBS) or 50 µg rDer p 2_{ec} or Der p 2_{pp}, twice a week for 8 weeks. After challenges with aerosols of *D. pteronyssinus* extract, AHR, lung inflammation and rDer p 2-specific T cell responses were investigated. Results obtained with rDer p 2_{ec} and rDer p 2_{pp} are presented in upper and lower panels, respectively. **a, f** AHR to 50 mg/ml methacholine was assessed by whole body plethysmography and expressed as a Penh Index. **b, g** Eosinophilia was measured in

bronchoalveolar lavage. Lung cells recovered from mice after SLIT with vehicle, and either rDer p 2_{ec} or rDer p 2_{pp} were restimulated in vitro with 10 µg/ml nDer p 2 or medium alone. IL-5 (**c, h**), IL-10 (**d, i**), and IL-13 (**e, j**) secretion was analyzed in supernatants by cytometric bead array assays. Data are expressed as mean $\pm$ SEM values obtained from groups of 6 sensitized and 5 healthy mice, respectively. Data are representative of 2 independent experiments for rDer p 2_{ec} (irrespective of endotoxin content) and 1 for rDer p 2_{pp}. n.s. = Not significant. * $p < 0.05$ and ** $p < 0.01$ compared with PBS-desensitized mice using Mann-Whitney statistical tests with 95% confidence intervals.

served in unsensitized (i.e. healthy) animals used as controls in these experiments. This reduction of AHR was associated with a significant decrease of eosinophils in bronchoalveolar lavage (fig. 5b, g). Cytokine secretion was measured in lung T cells recovered from mice after SLIT with PBS, rDer p 2_{ec} or rDer p 2_{pp} as described in Methods. IL-5, IL-10 and IL-13 secretion by T cells decreased in mice treated sublingually with either one of the recombinant Der p 2 molecules when compared to PBS (fig. 5c–e, h–j).

Discussion

Allergen-specific immunotherapy is established as a safe and efficacious treatment for type I allergies against venoms, pollens and mites [1–10, 45]. Although natural extracts are currently used for immunotherapy, recombinant allergens are being considered as an alternative

[11, 12, 46] with some advantages over natural allergens. They can be produced in a reproducible way as highly pure and well-defined molecules with a high batch-to-batch consistency. Also, recombinant technologies allow the production of modified (e.g. hypoallergenic) forms as well as natural-like allergens. The latter represent a preferred option for both diagnostic and SLIT purposes [17, 47].

With the aim of developing a recombinant sublingual mite allergy vaccine, we focused on the expression of Der p 2 with characteristics similar to its natural counterpart. Although at least 14 natural Der p 2 isoforms diverging by only a few point substitutions have been described so far [33, 35], our analysis by MS established Der p 2.0101 as a main isoform in *D. pteronyssinus* extracts besides variants 0104 and 0109, in agreement with previous reports [33, 48]. We thus, produced two recombinant Der p 2.0101 molecules in *P. pastoris* and *E. coli* and compared them after purification with respect to their structural

and immunological properties, using natural purified Der p 2 as a reference.

Der p 2 expression in *E. coli* resulted in the formation of inclusion bodies, whereas the expression in *P. pastoris* yielded a soluble protein. Both recombinant molecules were similarly recognized by Der p 2-specific murine monoclonal antibodies as well as IgEs from HDM-allergic patients. Furthermore, they were comparable in their capacity to trigger basophil activation. The three Der p 2 molecules were also recognized by murine Th2 cells, albeit at various levels, possibly reflecting differences in isoform content or capture/processing of these molecules by antigen-presenting cells. Moreover, the sublingual administration of rDer p 2_{ec} or rDer p 2_{pp} significantly desensitized nDer p 2-allergic mice, as indicated by a decrease in both AHR and established allergen-specific Th2 responses.

Other studies reported a successful expression of recombinant Der p 2 in *E. coli*, alone or as a fusion protein with glutathione-S-transferase or Der p 1 [29, 37, 49–51] as well as in eukaryotic hosts such as *Saccharomyces cerevisiae* [30, 52] and tobacco cells [32]. Some of these molecules were found to resemble the natural counterpart in terms of antigenic properties. However, no detailed analyses were conducted to document molecular homogeneity and stability, which both represent critical properties to warrant pharmaceutical development. More specifically, engagement of the six Der p 2 cysteine into three disulfide bonds residues (C21–C27, C8–C119, C71–C78) [43, 44] is critical to establish the 3-dimensional structure of the molecule and as such to stabilize antigenic determinants [53, 54].

In our study, the mapping of cysteine bridges revealed that Der p 2_{pp} and Der p 2_{ec} recombinant molecules pos-

sess the expected disulfide bonds. Nonetheless, rDer p 2_{ec} presents some heterogeneity, with evidence for additional mispaired disulfide bonds. In agreement with these results, secondary structure and thermal stability analyses by CD spectroscopy confirmed that all molecules exhibit a comparable secondary structure, but that rDer p 2_{pp} and nDer p 2 are more stable than rDer p 2_{ec}. Altogether, our data demonstrate that the rDer p 2 produced in *P. pastoris* more closely resembles nDer p 2 when compared to the *E. coli*-derived rDer p 2. These results differ from those obtained in a recent study indicating that Der p 2 secreted by *P. pastoris* has a mainly unordered structure [31]. Explanations for such discrepancies are unclear but may originate from differences in yeast strains as well as the expression and purification protocols used.

One important observation in our study is that immunoreactivity profiles are not very discriminating, even when significant structural differences are detected between molecules. Along with other authors, we reported previously that the immunoreactivity and allergenicity of the Der p 2 molecule are still preserved to some extent despite incorrect folding [31, 37, 51, 53], likely due to the presence of linear IgE epitopes [55]. Our results confirm a direct link between correct cysteine pairing and both molecular homogeneity and stability evaluated by resistance to heat denaturation. Thus, we conclude that *P. pastoris* is a better expression host than *E. coli* for the production of natural-like Der p 2. In general, documenting proper cysteine pairing is critical for selecting recombinant allergens to be used for immunotherapy and diagnostic purposes in humans, with specific requirements regarding native structure, stability and molecular homogeneity.

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