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**Characterization of a hybrid protein designed with segments of allergens
from *Blomia tropicalis* and *Dermatophagoides pteronyssinus***

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Highlights

- ► We generated a novel genetically engineered molecule that combines IgE epitopes contained in several allergens from the house dust mite *D. pteronyssinus* and *B. tropicalis*

- ► The protein produced in *E. coli* and tested with human serum samples has a lower IgE reactivity and allergenicity compared to those of house dust mite (HDM) extracts and purified allergens.
- ► In mice it induced allergen-specific IgG antibodies, that block the IgE reactivity against HDM in sera from allergic patients.
- ► This protein may be particularly applicable to the development of HDM allergy vaccine.

ABSTRACT

Background: Sensitization to allergens of the house dust mites *Dermatophagoides pteronyssinus* and *Blomia tropicalis* is an important risk factor for asthma and allergic diseases. Allergen-specific immunotherapy is currently based on natural allergen extracts, however, in the last years recombinant allergens with different modifications have shown promising immunological properties that may be advantageously applied for developing novel allergy vaccines.

Methods: A hybrid molecule (MAVAC-BD-2) containing epitopes of *B. tropicalis* (Blo t 5, Blo t 8 and Blo t 10) and *D. pteronyssinus* (Der p 1, Der p 2, Der p 7 and Der p 8) allergens was constructed, expressed in *Escherichia coli* and purified by affinity chromatography. Its folding was analyzed by circular dichroism. Antibody reactivities were evaluated by ELISA and non-denaturing dot blot assays using a battery of sera from mite allergic patients and non-allergic subjects. ELISA inhibition and dot blot assays with monoclonal antibodies were used to detect B-cell epitopes. Human basophil activation and induction of IgG-blocking antibodies in mice immunized with the hybrid protein were also evaluated.

Results: MAVAC-BD-2, expressed as a 22.8 kDa protein, showed a lower frequency and strength of IgE reactivity compared to Blo t 5, Der p 1, Der p 2 and the extracts of *B. tropicalis* and *D. pteronyssinus*. MAVAC-BD-2 inhibited 26% of IgE reactivity to

Der p 2 and Blo t 5, reacted with anti-Der p 1 and anti-Der p 2 monoclonal antibodies and did not induce relevant basophil activation. MAVAC-BD-2 immunized mice produced specific antibodies that reacted against mite extracts and the purified allergens, as well as IgG antibodies that blocked the human IgE reactivity to mite extracts.

Conclusion: MAVAC-BD-2 has hypoallergenic characteristics and in mice induces IgG antibodies that block the human IgE reactivity to mite extracts.

Keywords: specific immunotherapy, recombinant allergen, hybrid molecule, allergy vaccine, IgE.

1. Introduction

The house dust mite (HDM) species *Dermatophagoides pteronyssinus* and *Blomia tropicalis* are the main inducers of mite allergy in tropical and sub-tropical regions [1-4]. More than 25 components with different allergenic activities have been characterized from these species. Allergen specific immunotherapy (SIT) is currently based on the use of allergen extracts, which are heterogeneous mixtures of allergenic and non-allergenic materials [5]. Several approaches for SIT have been developed to overcome the limitations of immunotherapy with allergen extracts [6]. Immunotherapy trials with recombinant allergens, as well as modified allergens with reduced IgE-binding capacity have been performed and have shown promising results. These allergens have been obtained with different methods such as oligomerization, hybrid technology, fragmentation and site-specific mutagenesis, among others [7,8].

The hybrid protein approach has been scarcely explored on mite allergens [9-11]. We think that vaccines based on recombinant molecules containing several antigenic regions from prevalent allergens may allow a more effective SIT. Der p 1 and Der p 2 are among the most studied HDM allergens, being 50 to 90% of *D. pteronyssinus* allergic individuals sensitized to them [12,13]. Besides, Der p 7 is recognized by 30 to 50% allergic patients sensitized to this mite [12-14]. Blo t 5 is a major allergen of *B. tropicalis* with unknown biological function and it has shown IgE reactivity between 70 and 80% in asthmatic patients [15-19]. The group 8 of mite allergens comprises proteins from the glutathione S transferase (GST) family. The IgE frequency of sensitization against mite GSTs varies according to geographical areas. For instance in Malaysia it is about 75%, in Singapore 65% and 19% in Europe [20,21]. In Colombia Blo t 8 showed 76.9% IgE reactivity in a small sample of *B. tropicalis* allergic patients [22]. Blo t 10 and Der p 10 are HDM tropomyosins that show cross-reactivity with other arthropods. Frequency of sensitization against these allergens in mite allergic patients ranges from 2% and 29% in Singapore and European countries [23,24], whereas in Colombia the frequencies of sensitization to Der p 10 and Blo t 10 are 34.6% and 41% respectively [25]. In this study we show the design, production and characterization of a hybrid protein containing segments of the above-mentioned allergens.

2. Materials and Methods

2.1. Serum samples

Sera from one hundred HDM allergic patients and ninety non-allergic subjects who attended a Social Security Clinic and public health centers in Cartagena, Colombia, [26] were obtained for this study. The patients were asthmatic and sensitized to *B. tropicalis* and *D. pteronyssinus* as determined by positive skin prick test (SPT) and specific IgE determination using ELISA. Asthma was defined based to the Global Initiative for Asthma (GINA) criteria, and confirmed by a physician belonging to the research staff, sustained on a clear clinical history with clinical symptoms. All controls were subjects without allergy history, negative SPT and negative IgE levels to allergen extracts. For basophil activation test we selected five allergic patients and one non-allergic control from the study population. This investigation was approved by the Bioethics Committee of University of Cartagena (Approval minutes 47-2012) and written informed consent was obtained from all patients and the non-allergic controls.

For ELISA Inhibition assay, three serum pools were prepared as follows: pool A composed of four sera with high levels IgG reactivity to *B. tropicalis* and the hybrid protein, which was used in the inhibition of IgG reactivity to *B. tropicalis* by MAVAC-BD-2. Pool B composed of six sera with positive IgE reactivity to Blo t 5 and Der p 2, used for the inhibition of IgE reactivity to these allergens by MAVAC-BD-2. Pool C composed by six sera with positive IgE reactivity to *B. tropicalis* and the hybrid protein, this pool was used for the inhibition of IgE reactivity to *B. tropicalis* extract by MAVAC-BD-2 (Table 1).

2.2. Natural allergen extracts and other allergens

Whole mite cultures of *D. pteronyssinus* and *B. tropicalis* were kindly donated by Dr. Enrique Fernandez-Caldas and used to prepare the allergen extracts as previously described [27]. Natural Der p 1 (NA-DP1) and natural Bet v 1 (NA-BV1-1) were obtained from Indoor biotechnologies (Charlottesville, VA, USA). Recombinants Der p 2 and Blo t 5 were produced as His-tagged proteins and purified by affinity Ni-NTA chromatography as described elsewhere [28].

2.3. Construction of the HDM hybrid protein MAVAC-BD-2

Different amino acid segments containing IgE epitopes [24,29-32], were selected from three *B. tropicalis* allergens (Blo t 5, Blo t 8, Blo t 10) and four *D. pteronyssinus* allergens (Der p 1, Der p 2, Der p 7 and Der p 8). The segments were assembled into a single molecule, from the N-terminal to the C-terminal ends, in the following order: amino acids 63 to 108 of Blo t 5, amino acids 216 to 233 of Blo t 10, amino acids 158 to 177 of Der p 8, amino acids 71 to 83 of Blo t 8, amino acid 141 to 155 of Der p 1, amino acid 64 to 108 of Der p 2 and amino acid 94 to 124 of Der p 7. A single molecule containing the selected regions was designed and the 3D structure modeled using I-TASSER [33]. The Pymol program was used to visualize structures and generate images [34]. The synthesis of the nucleotide sequence coding for the designed protein was ordered from GenScript, (Piscataway, NJ, USA) with codon optimization for efficient expression in *E. coli*. The sequence was subcloned into the vector pET45b+ using the Kpn I and Pml I restriction sites, with the N-terminal 6xHis-tag next to the MAVAC-BD-2 sequence for affinity purification. This expression vector was named pET45b+/MAVAC-BD-2.

2.4. Expression and purification of the hybrid protein

For protein cloning competent *Escherichia coli* (*E. coli*) BL21 (DE3) cells (Invitrogen Corporation, Carlsbad, CA, USA) transformed by electroporation with pET45b+/MAVAC-BD-2 were selected on Luria Broth with 0.1 mg/mL ampicillin (LBA) plates. Positively transformed colonies were confirmed by PCR using T7 promoter and T7 terminator primers. For expression, a single colony was grown in LBA overnight (ON) at 37°C, an aliquot was diluted 1:20 in LBA and incubated until an optical density (OD) of 0.5 was reached. Induction was performed with 1 mM isopropyl- β -D-thiogalactopyranoside incubation continued for 4 hours. The hybrid protein was mainly obtained from the inclusion bodies fraction, which was solubilized by incubating the pellet in denaturing binding buffer (8 M urea, 100 mM NaH₂PO₄, and 10 mM Tris, pH 8.0) for 2 hours at room temperature (RT) and sonication on ice using an ultrasonic homogenizer. Insoluble debris were removed by centrifugation and the supernatant was loaded to a Ni-NTA affinity column under denaturing conditions following the instructions from the manufacturer (Invitrogen, Carlsbad, CA, USA). The protein was eluted in 8 M urea, 20 mM sodium phosphate and 0.5 M NaCl, pH 4.0 buffer; for refolding, it was dialyzed against renaturalization buffer (10 mM EDTA, 0.5 M L-Arginine, 5 mM L-Cysteine, 1 mM Cystine, 100 mM Tris pH 8.0), using an 8 kDa cut-off membrane (Spectra/Dialysis membranes, Houston, TX, USA) at 4 °C. The hybrid protein was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with 15% polyacrylamide separation gels using Miniprotean II System (BioRad, USA)

under reducing conditions according to Laemmli [35], and visualized by Coomassie staining. For stability studies MAVAC-BD-2 was stored under physiological conditions at 4°C and samples taken at different time points (0 days to 21 days) for analysis with a 12% SDS-PAGE. The protein content was determined by the Bradford method (Bio-Rad, Palo Alto, CA, USA), using BSA as standard. The dialyzed protein was lyophilized and stored at 4°C. Purified MAVAC-BD-2 was subjected to an affinity chromatography step using the ToxinEraser™ Endotoxin Removal resin Kit (Piscataway, NJ, USA). The LPS concentration tested using the ToxinSensor Chromogenic LAL Endotoxin Assay kit (Piscataway, NJ, USA) was lower than 0.125 EU/mL.

2.5. Structural Analyses

The circular dichroism (CD) spectra of the purified recombinant protein were measured in the UV-range from 190-260 nm on Jasco J-715 CD-spectrometer (Japan Spectroscopic Co., Tokyo, Japan). Measurements were carried out at protein concentrations of 0.3 mg/mL in a cylindrical quartz cuvette with a path length of 0.02 cm; five independent measurements were performed and averaged. The spectra were baseline corrected by buffer spectra subtraction and converted to mean residue ellipticities (Θ) at given wavelengths. The calculation of the secondary structure content was performed with Dichroweb [36].

2.6. IgE and IgG reactivity of MAVAC-BD-2

Dot blot, ELISA and ELISA inhibition were performed to analyze antibody reactivity of the hybrid protein, purified allergens and mite extracts.

For non-denaturing dot blot experiments, 1 µg of MAVAC-BD-2, Blo t 5, Der p 1, Der p 2 and 5 µg of *B. tropicalis* were dotted onto 0.45 µm nitrocellulose membrane. The dot-blot was blocked with phosphate-buffered saline (PBS), 0.1% Tween 20 (PBS-T), 5% skimmed milk for 1 hour at RT. Twenty eight individual allergic sera and fourteen non-allergic sera were diluted to 1:5 in PBS-T and incubated with the dot-blot ON at RT. After washing with PBS-T the membranes were incubated with alkaline phosphatase-conjugate anti-human IgE (Sigma A3525) diluted 1:500 in PBS-T for 3 hours at RT. The bound IgE was developed with p-nitro-blue-tetrazolium (NTB-Bio-Rad) and 5-bromo-4-chloro-3-indoyl-phosphate (BCIP-Bio-Rad), dissolved in alkaline-phosphatase buffer (pH 9.5) during 5 min.

For ELISA, microtiter plate wells (Immulon-4 Dynatech, Chantilly, VA, USA) were coated with 0.5 µg of MAVAC-BD-2 or 5 µg of mite extract in sodium carbonate/bicarbonate buffer at 4°C ON. After washing with PBS-T, wells were blocked with PBS-T, 1% BSA, 0.02% sodium azide (PBS-T-BSA) for 3 hours at RT. After washing, 100 µL of human serum diluted 1:5 or 1:100 in PBS-T-BSA for IgE and IgG respectively, were added and incubated ON at RT. Then the plate was washed and the bound IgE and IgG antibodies were detected by incubating with 100 µL of alkaline phosphatase-conjugate anti human IgE (Sigma A3525) diluted 1:500 or anti-IgG (Sigma A3187) diluted 1:20,000, respectively for 2 hours at RT. After a final washing the wells were incubated with 100 µL of substrate *p*-

nitrophenyl phosphate (Sigma) diluted in 10% diethanolamine, 0.5 mM MgCl₂ at RT for 30 min. The OD was measured in an ELISA reader (Spectra MAX 250, Molecular Device, Sunnyvale, CA, USA) at 405 nm. The cut-off for positive IgE levels was defined as an OD greater than 0.12 (mean OD plus 3 standard deviation of ELISA results from 34 non-allergic sera). All samples were assayed by duplicate.

ELISA inhibition assay. For inhibition of IgG reactivity to *B. tropicalis* by MAVAC-BD-2, ELISA plates were coated with *B. tropicalis* extract and blocked as described for the ELISA assay. Then Pool A diluted 1:100 in PBS-T-BSA was absorbed ON at 4°C with tenfold increasing concentrations (0.1 - 100 µg/mL) of hybrid protein, *B. tropicalis* or BSA, and added to each well. Bound human IgG was detected as described in IgG reactivity by ELISA. For inhibition of IgE reactivity to Der p 2 and to Blo t 5 by MAVAC-BD-2, ELISA plate wells were coated with 0.5 µg of Blo t 5, Der p 2 and blocked as described in the ELISA. Pool B diluted 1:5 in PBS-T-BSA was absorbed ON at 4°C with two concentration (0.1 and 100 µg/mL) of hybrid protein, Der p 2, Blo t 5 or BSA. For inhibition of IgE reactivity to *B. tropicalis* by MAVAC-BD-2, ELISA plates were coated with *B. tropicalis* and blocked as described in the ELISA. Pool C diluted 1:5 was absorbed with tenfold increasing concentrations (0.1 - 100 µg/mL) of hybrid protein, *B. tropicalis* extract or BSA. ON at 4°C. Bound IgE antibodies were detected with alkaline phosphatase-conjugated anti-human IgE (Sigma A3525) diluted 1:500 in buffer. The OD was measured as described above. In all the inhibitions, BSA was used as control. Each experiment was performed by duplicate. The percentage of inhibition

was calculated as follows: percent of inhibition = $\frac{\text{OD without inhibitor} - \text{OD with inhibitor}}{\text{OD without inhibitor}} \times 100$.

2.7. Dot-blot assay with monoclonal antibody

Two μg of MAVAC-BD-2, Der p 1, Der p 2 and BSA were dotted onto 0.45 μm nitrocellulose membrane, which was blocked with PBS-T, 5% skimmed milk, for 45 minutes at RT, washed three times with PBS-T and incubated with anti-Der p 1 monoclonal antibody (Clone MA-4C1-1, Indoor Biotechnologies Inc., Charlottesville, VA, USA) or anti-Der p 2 monoclonal antibody (Clone Dpx-A9, Indoor Biotechnologies Inc., Charlottesville, VA, USA) ON at RT. The bound antibodies were detected by incubation with anti-mouse IgG (Sigma A1418) diluted 1:2,000 in PBS-T for 1 hour at RT, and then read out using BCIP/NBT substrate.

2.8. Basophil Activation Test

Peripheral blood was collected from five mite allergic patients with IgE reactivity to *D. pteronyssinus*, *B. tropicalis* and MAVAC-BD-2 (Table

2). Basophil activation was measured by flow cytometry using the Allergenicity Kit (Beckman Coulter, Inc. CA, USA) following instructions from the manufacturer. One hundred μL of blood was incubated with Der p 2, Blo t 5, MAVAC-BD-2 or mite extracts, for 15 minutes at 37°C. Basophils were gated

based on the expression of CRTh2 marker and activation was assessed by detection of the activation marker CD203c using phycoerythrin-conjugated CD203c mAb 97A6 in a Dakocytomation cytometer (Beckman Coulter, Inc. CA, USA). Data were analyzed using Summit 4.3 software (Beckman coulter, Inc CA, USA). At least 500 basophils were counted in each assay. Allergen-induced upregulation of CD203c was calculated using mean fluorescence intensities (MFI) obtained with stimulated (MFI_{stim}) and unstimulated ($MFI_{control}$) cells and expressed as the Stimulation Index (SI), ($SI = MFI_{stim} / MFI_{control}$). SI of ≥ 2.0 was considered indicative of a positive response.

2.9. Immunization of mice and evaluation of the antibody response

Six to eight-week-old female Balb/c mice were obtained from the National Institute of Health (Bogotá, Colombia). Housing and care were under standard conditions in the animal facilities at the University of Cartagena with 12:12 hours dark – light cycle. Bedding and food were autoclaved. All procedures involving animal manipulation were performed inside a laminar flow hood by gloved and masked personal. Experiments were approved by the ethical committee of the University of Cartagena (No. 42-2012). The protocol for antigen administration was performed as described elsewhere [37]. Briefly, groups of eight mice were intraperitoneally immunized on 0, 7 and 14 days with 40 μ g of MAVAC-BD-2 adsorbed to aluminum hydroxide. Seven days after the last immunization the mice were anesthetized with ketamine/xylazine and challenged intranasally with 40 μ g MAVAC-BD-2 for 3 consecutive days. The control group was sensitized and challenged with PBS adsorbed with aluminum hydroxide. On day 24, mice were

sacrificed and blood collected by heart puncture. Pre-immune sera were obtained in tail veins on day 0. Samples were stored at -20°C until antibody analysis.

Specific IgE, IgG1 and IgG2a responses induced by MAVAC-BD-2 were analyzed by ELISA as follow: Maxisorp plate wells coated with $1\text{ }\mu\text{g/mL}$ hybrid protein were incubated with $100\text{ }\mu\text{L}$ of diluted sera (1:20 for IgE, 1:20,000 for IgG1 and IgG2a). After washing bound antibodies were detected by adding biotin conjugated anti-mouse IgE (BD-13-5992-82), IgG1 (BD-533441) or IgG2a (BD-553504) followed by incubation with avidin peroxidase diluted 1:2,000 for 1 hour at RT. Then plates were incubated with $100\text{ }\mu\text{L}$ of tetramethylbenzidine (TMB, BD Biosciences) for 5 min. Then $50\text{ }\mu\text{L}$ of $2\text{N H}_2\text{SO}_4$ were added and the plate read at 450 nm (Spectra MAX 250, Molecular Device, Sunnyvale, CA, USA). We also evaluated whether IgG antibodies induced by immunization reacted with mite extract and purified allergens by dot blot assay, as described above. Two μg of Der p 2, Blo t 5, MAVAC-BD-2, Bet v 1 and $5\text{ }\mu\text{g}$ of *B. tropicalis* extract were dotted onto $0.45\text{ }\mu\text{m}$ nitrocellulose membrane and blocked with PBS-T, 5% skimmed milk for 1 hour at RT. The membranes were incubated ON at RT with serum pool mouse anti-MAVAC-BD-2 or pre-immune serum. Bound antibodies were detected with 1:1,000 diluted biotin-conjugated anti-mouse IgG2 (BD-553504) diluted in PBS-T for 1 hour at RT, then avidin-peroxidase and 3,3'- diaminobenzidine were added for developing the assay.

2.10. Inhibition of human IgE binding to mite extract by IgG from immunized mice

Maxisorp plate wells were coated with 5 µg of mite extract in 100 µL of sodium carbonate/bicarbonate buffer (pH 9.6) ON at 4°C, and blocked with PBS-T-BSA for 2 hours. The plates were incubated ON at 4°C with 100 µL of serum pool diluted 1:25 from immunized mice or pre-immune sera. To inactivate IgE, the serum pool was previously incubated in a water bath at 56°C for 1 hour [38] and inactivation confirmed by ELISA. After washing wells were incubated ON at 4°C with individual sera diluted 1:5 from seven HDM allergic patients followed by incubation with 100 µL alkaline-phosphatase-conjugated anti-human IgE (Sigma A3525) diluted 1:500. After washing the reaction was developed by incubation with 100 µL of *p*-nitrophenyl phosphate at RT for 30 minutes. Absorbance was determined at 405 nm using the Spectra MAX 250 (Molecular Devices, Sunnyvale, CA, USA). The percentage of inhibition of human IgE binding after incubation with mouse sera was calculated as follow: $100 - [\text{ODI}/\text{ODP}] \times 100$, where ODI and ODP represent the absorbance using immune serum and pre-immune serum, respectively.

2.11. Statistical analysis

The Mann–Whitney U-test or the Wilcoxon matched-pairs signed-rank tests were used for analysis of differences when appropriate. P-values less than 0.05 were considered statistically significant.

3. Results

3.1. Expression and purification of the hybrid protein

A 3D model of the hybrid molecule was generated by I-TASSER using as templates the three-dimensional structures of Blo t 5 [31], Der p 2 [30] and Der p 7 [32,39]. The image was generated with the PyMOL program indicating the positions of assembled segments. Figure 1A shows surface patches of the different allergen segments and Figure 1B shows the position of each segment in the cartoon representation of the model.

MAVAC-BD-2 was expressed in *E. coli* as an N-terminal hexahistidine-tagged protein that accumulated in inclusion bodies. The hybrid protein was recovered and refolded from the inclusion bodies by successive dialysis against L-Cysteine-Cystine buffer including L-Arginine. The protein was purified to homogeneity and SDS-PAGE analysis show that under reduced conditions it migrates as a monomer of 22.8 KDa (Figure 2 A). To prove the stability of the protein stored under physiological conditions samples of MAVAC-BD-2 were taken on consecutive days and analysed with SDS-PAGE (Fig 2 B), showing no visual degradation over extended periods. The CD spectrum of the hybrid molecule shows a maximum at 192 nm and a minimum at 208 nm (Figure 2 C), indicating a partially folded protein.

3.2. MAVAC-BD-2 has lower IgE binding capacity than extracts and purified allergens

One hundred sera from allergic patients were used to compare the IgE reactivity between MAVAC-BD-2 and HDM extracts. Forty seven were randomly selected for comparison of IgE reactivity to MAVAC-BD-2 with those to Blo t 5, Der

p 1 and Der p 2 by ELISA. The frequency of IgE reactivity to MAVAC-BD-2 by ELISA in the 100 sera from allergic patients was 40%. The antibody levels to the hybrid protein were significantly lower than those to *B. tropicalis* ($p < 0.001$), *D. pteronyssinus* ($p < 0.001$), Blo t 5, Der p 1 and Der p 2 ($p < 0.001$), Figures 3A and 3B. IgG reactivity was detected in all sera from allergic and non-allergic individuals, being its levels significantly higher in the allergic group vs. non-allergic group (mean OD 4.4 vs. 3.1, $p = 0.001$).

The IgE reactivity to the hybrid protein, the allergens Blo t 5, Der p 1 and Der p 2 and *B. tropicalis* extract was also analyzed by non-denaturing dot-blot assay using 28 sera from the HDM allergic patients and 14 non-allergic subjects. All sera of allergic patients showed IgE reactivity to *B. tropicalis*. Eight sera (number 2, 12, 13, 14, 16, 17, 18 and 19) showed very weak IgE reactivity to MAVAC-BD-2 and one sera (No. 20) showed reactivity comparable to that to the extract. However, all sera reacted with Der p 2 and some of them also to Blo t 5 or Der p 1. None of the other sera showed detectable IgE reactivity to MAVAC-BD-2 (Figure. 4). The membranes incubated with sera from non-allergic individuals (NC) and PBS-T without serum (BC) did not show IgE reactivity to any of the dotted proteins (Figure 4).

3.3. The hybrid protein contains B – cell epitopes of mite allergens

In the dot blot assay the anti-Der p 1 and anti-Der p 2 monoclonal antibodies showed reactivity against the hybrid protein (Figure 5), suggesting that it conserved the epitopes from the native allergens. ELISA inhibition showed that at the highest

concentration the hybrid protein inhibited the IgE reactivity to Der p 2 and Blo t 5 by 26.3% and 26% respectively (Figure 6A, B). On the other hand, the hybrid also inhibited the IgE reactivity to *B. tropicalis* by up to 20% (Figure 6C) suggesting that some IgE epitopes from Der p 2, Blo t 5 and the mite extract are represented on the hybrid molecule. The hybrid inhibited the IgG reactivity to *B. tropicalis* extract up to 41% (Figure 6D).

3.4. MAVAC-BD-2 shows lower allergenic activity than recombinant allergens and mite extracts

Basophil activation test using 10 µg/mL of antigen showed that samples from all patients were activated upon stimulation with Blo t 5, Der p 2, *B. tropicalis* and *D. pteronyssinus*. In contrast, MAVAC-BD-2 did not induce activation in patients # 1, 2 and 3 (Figure 7), furthermore, the degree of activation induced by the hybrid protein in patients # 4 and 6 was lower than that induced by mite allergens and extracts. No activation was observed in the sample from the non-allergic subject. Anti-human IgE antibody and not PBS induced up regulation of CD203c expression in all patients.

3.5. Immunization with MAVAC-BD-2 induced IgG antibodies that react with the mite extracts and the purified allergens

Mice immunized with MAVAC-BD-2 produced allergen-specific IgE, IgG1 and IgG2a. Interestingly, the levels of IgG2a were significantly higher than those of IgG1 and IgE (Figure 8). The ratio of IgE/IgG2a serum levels was 0.64 and the ratio of IgG1/IgG2a was 0.46, suggesting that MAVAC-BD-2 may potentially induce a biased immune response to a Th1 and/or regulatory profile. Furthermore, the

MAVAC-BD-2 specific IgG produced by the immunized animals showed reactivity against Der p 2, Blo t 5 and *B. tropicalis* extract and no reactivity to the unrelated allergen Bet v 1. No reactivity of the pre-immune sera against the different allergens was detected (Figure 9).

3.6. MAVAC-BD-2 induced IgG blocking antibodies that inhibit patients' IgE binding to *D. pteronyssinus* and *B. tropicalis* extracts

We evaluated whether IgG antibodies induced by mice immunization with the hybrid protein were able to inhibit the binding of human IgE to allergenic extract by ELISA inhibition. When *D. pteronyssinus* extract was on solid phase, the incubation of coated wells with mice immune sera reduced the IgE-binding capacity between 54% and 96%. This reduction ranged from 33% to 90% for *B. tropicalis* extract and less than 11% when the non-allergic serum was used (Table 3).

4. Discussion

The major allergens of *B. tropicalis* and *D. pteronyssinus* Blo t 5, Der p 1 and Der p 2 have a frequency of IgE-binding higher than 60% in patients sensitized to HDM; therefore, they are essential for formulating an efficient allergy vaccine. MAVAC-BD-2 contains segments of these allergens and also segments of group 7, 8 and 10 mite allergens that are relevant in different geographical areas. We think that the combination of IgE and IgG epitopes from these allergens in a single molecule may result advantageous for treating allergy to clinically relevant HDM allergens. The importance of Der p 23 in the design of a protein for mite allergen immunotherapy has been suggested in recent publications [38,40]. However, in

spite MAVAC-BD-2 does not contain any segment of this component, most people allergic to HDM would benefit when using it considering that around 95% of mite allergic patients can be diagnosed by using the combination Der p 1/Der p 2 [41] and that the diagnostic value can be improved by adding Der p 10 [19,42].

The inclusion of Blo t 5 and Blo t 8, adds complementary value to the hybrid molecule, as these allergens have been shown to be important inducers of allergy in the tropical and subtropical regions [4,16,22,25]. SIT with allergen extracts is the only treatment that prevents the progression of the allergic disease with successful clinical outcomes, however, problems have been associated with this approach and the use of modified recombinant allergens has been proposed to provide a safer and more effective treatment. For instance clinical trials evaluating the recombinant Bet v 1 allergen for treating pollen allergy have shown promissory results [43,44]. For HDM allergy the combination of Der p 1 and Der p 2 [10,45] and modified Der p 2 and Der p 23 [38,46] are currently under investigation.

MAVAC-BD-2 is the first genetically engineered molecule that combines IgE epitopes contained in several allergens from both *D. pteronyssinus* and *B. tropicalis*. It has lower IgE binding and allergenic activity than the mite extracts and the allergens Blo t 5, Der p 1 and Der p 2, as detected by ELISA, non-denaturing dot-blot and basophil activation tests. Several reasons may explain these results; as a consequence of the partial refolding detected by circular dichroism, the protein may have a local relative reorientation of the IgE epitopes that led to a different conformation of the binding sites, as a consequence there could be less affinity for

antibody binding, cross-linking of the FcεRI and lower activation of the allergic cascade. In addition, since not all the B-cell epitopes from the mite allergens are represented in the hybrid protein, a lower IgE reactivity compared to the natural allergens and the mite extracts can be anticipated. However, the reactivity of monoclonal antibodies and the ELISA inhibition results when using the human serum pool, indicate that the molecule has relevant B-cell epitopes in accessible areas.

Studies in animal models have shown the beneficial effect of blocking antibodies for successful immunotherapy [47,48] and that these IgG antibodies are associated with clinical improvement of the allergic symptoms in humans [49-52]. Moreover, these antibodies might inhibit the mast cell and basophil degranulation, the IgE-facilitated allergen presentation and IgE production [53,54]. Mice immunized with MAVAC-BD-2 produced high titer of specific IgG2a that reacted against the extracts and the purified allergens (Figure 9) and were able to highly inhibit the specific IgE from allergic patients (Table 3). These results show that the hybrid protein shares common epitopes with the natural allergens and has the structural requirements to induce an adaptive immune response that could protect against further exposure to natural allergens, an important aspect for a potential allergy vaccine. Therefore, under an appropriate SIT approach this hybrid protein may induce specific IgG blocking antibodies countering mast cell degranulation and IgE mediated inflammation in HDM allergic patients. However, further studies are needed to better understand the immune response induced by this molecule,

including the detection of T cell epitopes and the role that antigen presenting cells play in this event [55,56].

MAVAC-BD-2 may be particularly applicable to the development of a HDM vaccine given the following reasons: It is a construct that incorporates segments of seven allergens from the two main HDM species involved in the onset and triggering of allergic diseases in tropical and subtropical regions. In addition it contains known B-cell epitopes of the allergens used for its design, which in the fold of the hybrid protein scarcely react with allergic patient's serum IgE, but induce relevant IgG blocking antibodies. Furthermore, due to its low allergenic activity higher doses could be used and with reduced risk of anaphylactic reactions.

T cell populations play a key role in the regulation of the allergic response [57,58] therefore, modifications intended to generate a hypoallergenic derivative should retain the ability to stimulate these cells for obtaining specific tolerance. Although MAVAC-BD-2 was able to induce an antigenic response in mice and contains some T cell epitopes reported for the allergens included in its structure, more studies are necessary to define the predominant T cells and B cell response induced by this molecule.

In summary, we present a new strategy for designing single molecules composed of several fragments of clinically relevant environmental allergens. The hybrid protein exhibits low allergenic activity and ability to induce IgG-blocking antibodies.

Conflict of interest

All authors declare that they do not have any actual or potential conflict of interest including any financial, personal or other relationships with other people or organizations.

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Legends for figures

Figure 1. A) 3D structural model of the MAVAC-BD-2 with surface color representation of the segments of allergens included in its structure, and schematic representation of the order of the segments in the primary structure. B) Cartoon representation illustrating the position of each allergen segment in the context of the proposed 3D structure.

Figure 2. A) Electrophoresis analysis of MAVAC-BD-2. MW: Molecular weight marker. Lane 1: sample of cell culture without induction. Lane 2: the affinity purified hybrid protein. B) Stability of MAVAC-BD-2 upon extended storage (0 - 21 days). C) Circular dichroism spectrum of MAVAC-BD-2.

Figure 3. IgE levels to MAVAC-BD-2, HDM extracts and purified allergens, by ELISA. A) IgE levels to hybrid protein and extracts in 100 serum samples from allergic patients. B) IgE levels to hybrid protein and purified allergens in sera from 47 mite allergic individuals. (**p < 0.001).

Figure 4. IgE reactivity by nondenaturing dot-blot. Dot-blotted proteins were exposed to sera from 28 HDM allergic patients (lanes 1-28), serum from a non-allergic subject (NC), or buffer without serum (BC).

Figure 5. Dot-blot assay with monoclonal antibodies. Hybrid protein, Der p 1, Der p 2 and BSA were dotted onto nitrocellulose membranes and incubated with monoclonal antibodies as indicated (Protein/antibody): A) MAVAC-BD-2 / anti Der p 1, B) MAVAC-BD-2 /anti Der p 2, C) Der p 1/anti-Der p 1, D) Der p 2/anti – Der p 2, E) BSA/ anti Der p 1.

Figure 6. ELISA inhibition assay. Bar graphics shown the percentages of Inhibition of IgE reactivity of serum pools by the hybrid protein to Der p 2 (A), Blo t 5 (B) and *B. tropicalis* (C), and inhibition of IgG reactivity to *B. tropicalis* (D). Homologous inhibitor and BSA were used as controls.

Figure 7. Basophil activation test results. Stimulation index obtained in samples from five HDM allergic patients exposed to 10 µg/mL of hybrid protein, Der p 2, Blo t 5, *D. pteronyssinus* (Dp) or *B. tropicalis* (Bt) extracts.

Figure 8. Allergen-specific IgE, IgG1 and IgG2a levels in sera from Balb/c mice immunized with MAVAC-BD-2.

Figure 9. Reactivity of IgG antibodies induced by immunization of Balb/c mice with MAVAC-BD-2. Proteins were dotted on nitrocellulose membranes as indicated: A) Hybrid protein, B) *B. tropicalis* extract, C) Der p 2, D) Blo t 5, E) Hybrid protein, F) Bet v 1. The membranes were incubated with a serum pool of mice immunized with MAVAC-BD-2 (A-D and F), or with pool of pre-immune sera as control (E).

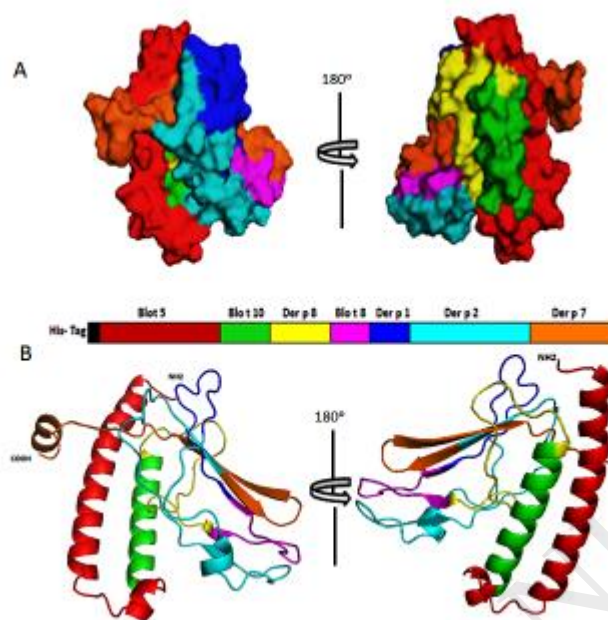


Figure 1

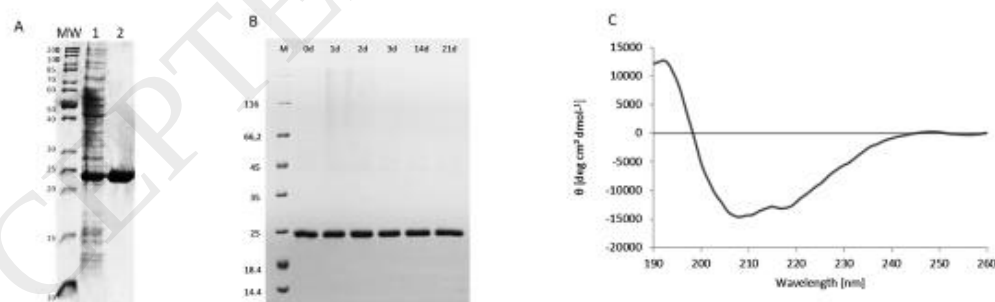


Figure 2

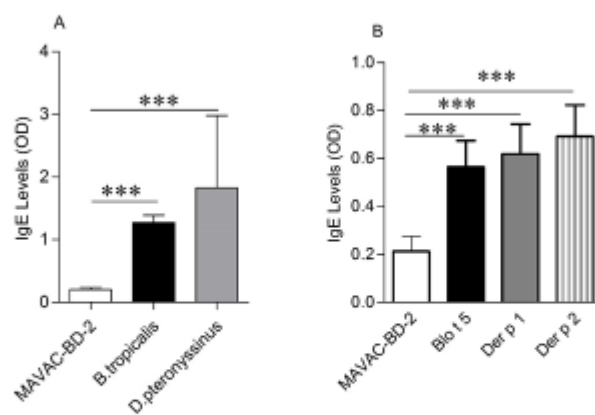


Figure 3

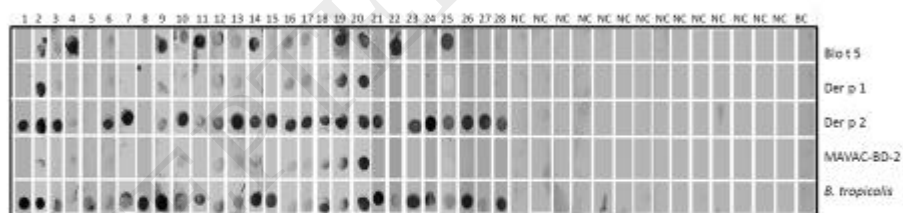


Figure 4

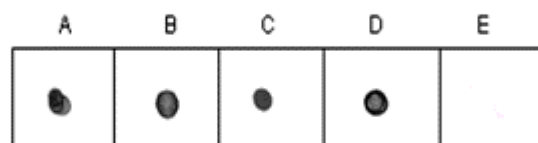


Figure 5

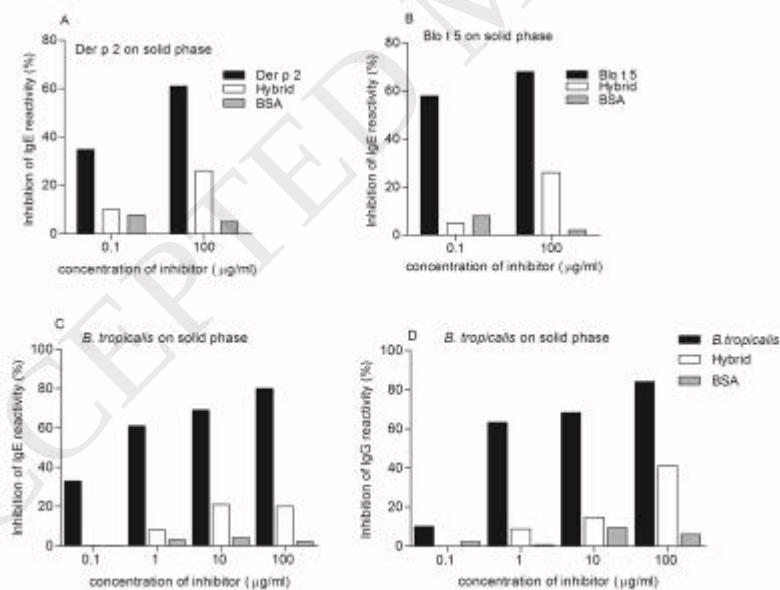


Figure 6

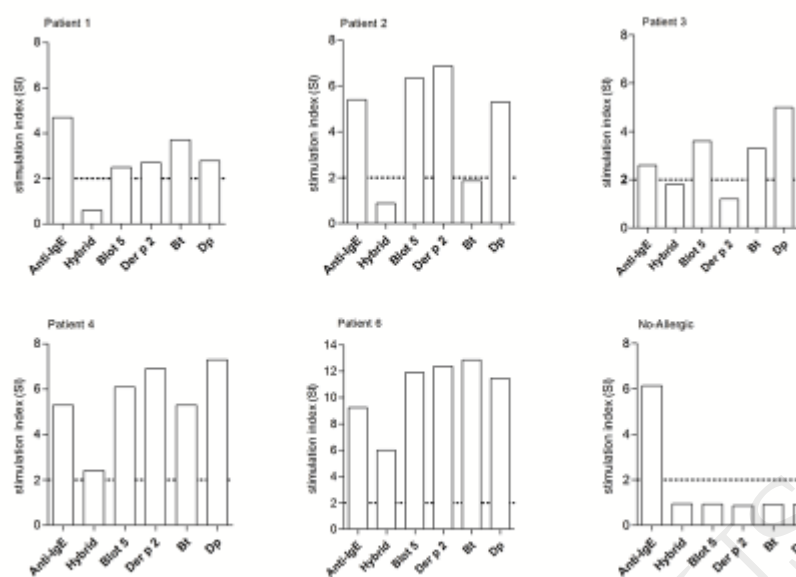


Figure 7

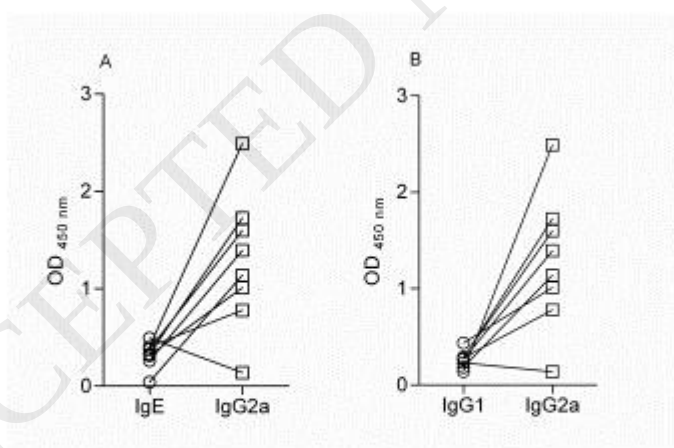


Figure 8

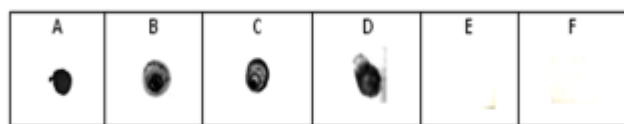


Figure 9

Table 1 . Characteristics of sera used for preparation serum pools

Patient	IgG Levels (OD)	
	MAVAC-BD-2	<i>B. tropicalis</i>
A441	2.02	0.71
A530	2.56	0.68
A598	3.11	1.01
A640	2.54	1.33

Serum Pool A

Patient	IgE levels (OD)		
	MAVAC-BD-2	Der p 2	Blo t 5
A33	2.37	0.17	0.25
A39	2.00	0.29	0.26
A43	2.13	0.21	0.17
A84	2.13	0.76	0.18
A90	0.22	0.36	0.19
A92	0.53	0.35	0.52

Serum Pool B

Patient	IgE Levels (OD)		
	MAVAC-BD-2	<i>B. tropicalis</i>	<i>D. pteronyssinus</i>
A33	2.37	3.32	2.73
A39	2.00	2.26	2.63

A43	2.13	2.31	2.41
A44	2.13	0.50	3.29
A45	2.01	0.69	2.62
A92	0.53	1.02	0.74

Serum Pool C

OD: optical density

Table 2. Immunological characteristics of sera used in basophil activation

Patients	Sex	Prick Test > 3mm	IgE Levels (OD)				
			<i>B. tropicalis</i>	<i>D. pteronyssinus</i>	MAVAC-BD-2	Der p 2	Blo t 5
1	M	Bt, Dp	0.92	2.33	0.16	0.25	0.12
2	F	Bt, Dp	1.20	0.58	0.12	3.08	0.12
3	M	Bt,Dp	0.17	0.78	0.12	0.14	0.42
4	M	Bt, Dp	0.70	0.70	3.28	0.14	1.79
5	M	Bt, Dp	3.29	0.83	0.22	0.73	0.40
6	F	Bt, Dp	0.20	0.37	0.25	0.76	0.18
No-Allergic	F	Neg	0.07	0.07	0.09	0.07	0.08

Table 3. Inhibition of patients' IgE binding to *D. pteronyssinus* and *B. tropicalis* by mouse IgG antibodies induced by immunization with hybrid protein.

<i>D. pteronyssinus</i> on solid phase				<i>B. tropicalis</i> on solid phase		
Serum levels IgE (OD)						
Preincubated with				Preincubated with		
Patients	pre-immune	Immunized	% of Inhibition	pre-immune	Immunized	% of Inhibition
1	2.60	0.30	88	3.32	0.33	90
2	2.71	0.13	95	2.45	0.32	86
3	2.66	0.10	96	0.40	0.15	62
4	2.74	0.35	86	0.72	0.48	33
5	2.52	0.25	89	1.94	0.98	49
6	2.58	0.10	95	0.23	0.15	35
7	1.54	0.69	54	0.29	0.17	41
Mean	2.48	0.28	86	1.34	0.37	57
No-Allergic	0.075	0.08	10	0.08	0.09	11

OD: optical density