



Purification and characterisation of the dimeric group 12 allergen from *Blomia tropicalis* heterologously expressed by *Escherichia coli* Top10F'

Eduardo Santos da Silva¹ · Luis Gustavo Carvalho Pacheco¹ · Antônio Márcio Santana Fernandes¹ · Claudia Asam² · Elisânia Fontes Silveira¹ · Carina da Silva Pinheiro¹ · Neuza Maria Alcantara-Neves^{1,3}

Received: 6 January 2021 / Accepted: 16 April 2021
© The Author(s), under exclusive licence to Springer Nature B.V. 2021

Abstract

Successful research in the wide-ranging field of allergy is usually achieved by definition not only of physicochemical and immunological properties of natural, but also recombinant allergens. *Blomia tropicalis* mite is a well-known source for various groups of hypersensitivity-causing proteins. The goal of the present work was to produce, purify and characterise by in silico, biochemical and immunological methods the recombinant group-12 allergen of *B. tropicalis*. The recombinant Blo t 12 aggregation capacity as well as the affinity to antibodies from BALB/c immunised mice and *B. tropicalis*-sensitised human donors were investigated through in silico analyses, dynamic light scattering, SDS-PAGE, ELISA and Western blot. The presence of Blo t 12 within *B. tropicalis* extracts was also determined by ELISA and Western blot. High concentrations of dimeric rBlo t 12 were detected through SDS-PAGE next to other aggregates and the results were confirmed by data from DLS and Western blot. The YITVM peptide was predicted to be the most aggregation-prone region. The IgE-reactivity of rBlo t 12 was not completely abolished by aggregate formation but it was significantly decreased compared to rBlo t 5, or *B. tropicalis* extracts. Natural Blo t 12 may naturally dimerise, but it was detected in non-delipidified *B. tropicalis* extracts in low amounts. Given that this allergen may be a specific marker for *B. tropicalis* allergy, the recombinant Blo t 12 herein obtained is characterised as a mid-tier allergen in Brazilian atopic patients and may be useful for the improvement in precision allergy molecular diagnostic applications.

Keywords Aggregation · rBlo t 12 · nBlo t 12 · Dimer · House dust mite · Mite extract

Introduction

During the last decades, healthcare systems in developed and low-/middle-income countries have been burdened with the increasing number of patients with allergic diseases [1, 2]. Among the triggers of atopic sensitisation, house dust mites (HDM) represent one of the key sources of allergens

and risk factors for different allergic illnesses [3]. Like other sources, their allergens can be classified as major, mid-tier and minor, when sera from sensitised patients show >50%, 30–50%, and <30% of immunoglobulin-E (IgE) reactivity, respectively [4, 5]. Although such classification is important, minor allergens may be clinically relevant, and several were related to innate immune mechanisms and have been associated with mild to severe allergic symptoms [4–6]. Recently, a fatty acid-binding protein family from HDM, frequently considered minor allergens in many countries, was identified as direct interactors of serum amyloid A1 proteins, promoting type 2 immunity during acute-phase, with a key role of the innate cytokine, IL-33 [7].

Blomia tropicalis is considered an important HDM species, particularly in tropical and subtropical regions of the globe [3, 8, 9]. Fourteen *B. tropicalis* allergens have been officially recognized by the International Union of Immunological Societies (IUIS) nomenclature subcommittee,

✉ Neuza Maria Alcantara-Neves
neuzalcantara@gmail.com

¹ Department of Biointeraction, Federal University of Bahia, Salvador, Brazil

² Department of Molecular and Cellular Anatomy, University of Regensburg, Regensburg, Germany

³ Laboratório de Alergia e Acarologia, Instituto de Ciências da Saúde, Universidade Federal da Bahia, Avenida Reitor Miguel Calmon, sem nº, Canela, CEP – 40110-100, Salvador, Bahia, Brazil

including the well-established major allergens Blo t 5 and Blo t 21 [9–11]. Interestingly, some *B. tropicalis* allergens are molecule-binding proteins [12–15], displaying unique IgE-binding features, such as Blo t 12 [15]. In fact, patients' IgE-reactivity to this chitin-binding allergen has been shown to be dependent on the geographical area [15, 16]. Despite the clinical importance of this mid-tier/major allergen has already been demonstrated in Colombia and Singapore [15, 17], no other research group confirmed these data or investigated other structural features of Blo t 12 to date. These two research groups have described recombinant expression of Blo t 12 isoforms, using the yeast *Pichia pastoris* and *E. coli* Origami (DE3) strain as hosts [13, 15, 17, 18]. Other biochemical and immunological features of rBlo t 12 have been previously shown; however, no clear information on monomeric, dimeric, or aggregating behaviour of these purified proteins were observed in earlier reports [15, 17]. Moreover, more recently, the same authors stated that Blo t 12 is one of *B. tropicalis* allergens in need of further characterisation [16]. Table 1 summarizes the current physicochemical characterisation and molecular properties of recombinant allergens of *B. tropicalis*, whose list clearly displays rBlo t 12 in the group of allergens lacking characterisation. The present study recombinantly produced this allergen, in order to investigate its presence in *B. tropicalis* extract and gaining more knowledge on its aggregation behaviour and antibody reactivity, especially IgE binding in sera, from Brazilian allergic donors.

Material and methods

Pilot heterologous expressions

Blo t 12.0101 mature coding sequence (GenBank accession no. U27479), without the signal peptide, was codon-harmonized for optimal expression in *Escherichia coli* and was synthesized by ATUM (Newark, CA, USA) into a pD444-NH vector. After transformation of different strains (BL21(DE3)pLys S, BL21(DE3) and Top10F') by thermal shock, the induction of heterologous expression was started by addition of 1 mM isopropyl- β -D-thiogalactopyranoside (IPTG; Thermo Fisher, Waltham, MA, USA) at an OD₆₀₀ of 0.7. These expressions were performed in 5 mL of LB medium at 37 °C, and the strain with higher heterologous expression yield was chosen for posterior scale-up expression. A 12% SDS-PAGE was used to evaluate the expression of the allergen in these three strains. Given difficulties in confirm the visualization of the allergen band in the gel, Western blot analysis was performed using the samples from 3 h of expression. Specifically, Western blot was performed following the transferring of SDS-PAGE bands from the bacterial extract to a nitrocellulose membrane (Merck

Millipore, Billerica, MA, USA). The membrane was blocked with non-fat dried milk diluted in 1 × PBS. Then the membrane was incubated for 1 h with anti-HIS IgG antibody (1:4000, GE Healthcare, Chicago, IL, USA) as a primary antibody, followed by incubation for 1 h with a secondary anti-mouse IgG antibody conjugated with alkaline phosphatase (1:2500, Invitrogen, Carlsbad, CA, USA). The reaction was developed using nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) substrates diluted in alkaline phosphatase buffer.

Scale-up expression and purification

Scale-up expression of Blo t 12.0101 was performed in the strain Top10F'. A pre-inoculum (10 mL of LB medium) of the transformed strain was performed for 16 h at 37 °C. Then, a dilution (1:100) of this pre-inoculum in 1L of LB medium was performed for the scale-up expression. For that, the induction started by addition of 1 mM IPTG at an OD₆₀₀ of 0.7; however, due to the observed dimeric and aggregating band in the first Western blot analysis, we decided to test if the expression for 16 h at 16 °C would change this behaviour. After this time, the cells were harvested at 4000 g, 20 min, 4 °C. The bacterial pellet was resuspended in 20 mM sodium phosphate (NaP) buffer (30 mL), pH 8 and lysed by six freezing/thawing cycles. The protein suspension was centrifuged at 13,000 g, 20 min, 4 °C and the supernatant was collected. The lysate was initially resuspended (30 mL) in NaP buffer containing 3 M urea, centrifuged again and this supernatant was collected. Then the pellet was again resuspended in the same volume, but in NaP buffer with 6 M urea, centrifuged and another supernatant was collected. Given the pilot expression assays resulting in dimerisation and aggregation, a 18% SDS-PAGE and Western blot analysis were performed to confirm the presence of rBlo t 12 and soluble or insoluble fraction of the extract.

The urea-solubilized extracts were purified by affinity chromatography using 1 mL of Ni-NTA agarose resin (Qia-gen, Hilden, Germany) for 10 mL of protein suspension. Different concentrations of imidazole, ranging from 20 mM to 500 mM, were used for elution, with fractions of 2 mL each. Considering that 60 mL of urea-solubilized extracts were initially obtain, six rounds of purification were needed to complete obtain purified fractions. Purity assessment was performed using 12% SDS-PAGE. The eluted samples were pooled, dialysed and concentrated on a three kDa Vivaspin column (GE Healthcare, Chicago, IL, USA). To better visualize possible monomeric purified bands of rBlo t 12, 10 µg of the concentrated samples was evaluated in a 18% SDS-PAGE using three different conditions: (i) standard loading buffer (62.5 mM Tris-HCl pH 6.8, 2.5% SDS, 0.002% Bromophenol Blue, 5% β -mercaptoethanol, and 10% glycerol), with heating at 100 °C for 5 min; (ii) standard loading

Table 1 Summary for heterologous expression of the *Blomia tropicalis* allergens

Allergen	Host	Purification steps	Characterisation tests						References
			Stability*	Purity#	Folding	Protein activity (Expected function)	Aggregation	Allergenicity	
Blo t 1	<i>E. coli</i> <i>P. pastoris</i>	ASP + AEC NiAC	None	None	Crystallography	None (Cysteine protease)	None	Dot blot ELISA SPT WB	[9]
Blo t 2	<i>E. coli</i>	ACP + CEC NiAC + SEC	Degradome	MS SDS-PAGE	CD FTIR	ADA (NPC2 family)	None	Dot blot ELISA MRA WB	[9, 14]
Blo t 3	<i>E. coli</i>	GSC NiAC + AEC	None	None	None	None (Trypsin)	None	ELISA SPT	[9]
Blo t 4	<i>P. pastoris</i>	GSC NiAC	None	SDS-PAGE	None	None (α -Amylase)	None	SPT WB	[9]
Blo t 5	<i>E. coli</i> <i>P. pastoris</i> CHO cells	AAC AEC + SEC GSC NiAC	CD Degradome	MS SDS-PAGE	CD FTIR NMR	None (Unknown)	DLS	Dot blot ELISA MRA SPT WB	[9, 11, 22]
Blo t 6	<i>E. coli</i> <i>P. pastoris</i>	AEC NiAC	None	SDS-PAGE	None	None (Chymotrypsin)	None	ELISA SPT WB	[9, 17]
Blo t 7	<i>P. pastoris</i>	CEC + SEC	None	SDS-PAGE	None	ADA (BPI protein)	None	ELISA	[10]
Blo t 8	<i>E. coli</i>	GSC	None	SDS-PAGE	Crystallography	None (Glutathione S-transferase)	None	ELISA SPT WB	[9, 13]
Blo t 10	<i>E. coli</i>	GSC	None	None	None	None (Tropomyosin)	None	ELISA SPT WB	[9]
Blo t 11	<i>E. coli</i> Sf9 cells	CEC GSC	None	SDS-PAGE	None	None (Paramyosin)	None	Dot blot ELISA WB	[9]
Blo t 12	<i>E. coli</i> <i>P. pastoris</i>	AEC NiAC	None	SDS-PAGE	NMR	Chitin-binding assay (Chitin-binding protein)	None	ELISA	[13, 15, 18]
Blo t 13	<i>E. coli</i> <i>P. pastoris</i>	GSC NiAC	None	SDS-PAGE	None	ADA (Fatty acid-binding protein)	None	ELISA WB	[7, 9, 12]
Blo t 19	Unknown	Unknown	None	None	NMR	None	None	None	[9]
Blo t 21	<i>E. coli</i>	AEC + SEC NiAC	CD Degradome	MS SDS-PAGE	CD FTIR NMR	None (Unknown)	DLS	ELISA MRA SPT WB	[9, 35]

Only allergens deposited in the International Union of Immunological Societies database for allergens were considered for this review

AAC antibody affinity chromatography, ACP acidic precipitation, ADA ANS (8-Anilino-naphthalene-1-sulfonic acid) displacement assay, AEC anion exchange chromatography, ASP ammonium sulphate precipitation, BPI bactericidal permeability-increasing, CD circular dichroism, CEC cation exchange chromatography, DLS dynamic light scattering, ELISA enzyme-linked immunosorbent assay, FTIR Fourier-transform infrared spectroscopy, GSC glutathione Sepharose chromatography, MRA mediator release assay, using basophil lineages or patient-isolated basophils, MS mass spectrometry, NiAC nickel-based affinity chromatography, NMR nuclear magnetic resonance spectroscopy, NPC2 NPC Intracellular Cholesterol Transporter 2, SDS-PAGE sodium dodecyl sulphate–polyacrylamide gel electrophoresis, SPT skin prick test, WB western blot

*The protein shelf time in freezing or room temperature verified by CD or SDS-PAGE, or proteolytic in vitro assays using cell lines and MS tandem verification of the generated peptides (degradome)

#The display of any indication of purity level, either by SDS-PAGE pictures or MS spectra

buffer, with heating at 70 °C for 20 min; and (iii) stronger reducing buffer (200 mM Tris, 5% SDS, 0.1 mM EDTA, 8 M Urea, 100 mM DTT, and 0.1% Bromophenol Blue) and heating at 37 °C for 10 min.

Dynamic light scattering

The aggregation behaviour of the purified rBlo t 12 in solution was assessed by dynamic light scattering (DLS). The experiment was performed on a Zetasizer ZS90 (Malvern Instruments, Malvern, UK) with samples as follows: (i) sample at 0.5 mg/mL in 20 mM NaP buffer pH 8, room temperature (RT); (ii) addition of 3 M urea in the buffer; (iii) addition of 6 M urea in the buffer. Ten measurements were accumulated for evaluation using Zetasizer software and displayed as mass weighted distribution of the hydrodynamic radius (R_H) using GraphPad Prism 7 (San Diego, CA, USA). Data was normalized taking into account the measurements of the buffers alone.

In silico assessment of aggregating domains

Bioinformatics were applied to identify aggregation-prone domains, using PASTA 2.0 and AGGRESKAN3D servers [19, 20]. The amino acid sequence of Blo t 12 was uploaded to the PASTA 2.0 server, choosing 100 top pairing energies and energy threshold of 1.192 kcal/mol. For AGGRESKAN3D, the tertiary structure of the Blo t 12 chitin-binding domain (CBD) [19, 21] was used as input. This 3D structure is based on the sequence of Blo t 12.0102 [21], so we mutated the CBD accordingly to obtain the scores related only to Blo t 12.0101 CBD.

In vitro IgE-binding assays

Human IgE antibody reactivity to rBlo t 12 was measured by indirect ELISA according to a protocol previously published by our group [11]. We performed rBlo t 12 concentration curves (1.25 µg/mL to 80 µg/mL) and different biotin anti-human IgE dilutions (1:62.5 to 1:500) in order to verify the level of decreasing in IgE-binding. We also used *B. tropicalis* extract (BtE) and rBlo t 5 as control antigens, using the optimal concentrations and dilutions of our published data [11, 22]. For this assay, eight sera were obtained from atopic donors living in Salvador, BA, Brazil. Following this pilot assay, we performed another ELISA using 30 atopic, and 10 non-atopic donors. The IgE cut-off was defined by the mean plus two standard deviations of the results obtained using sera from non-atopic donors. Atopy was characterised by positive results of anti-BtE IgE in the ImmunoCAP assay (d201, Phadia, Upsala, Sweden) and skin prick tests (SPT) to BtE extract, as described [11]. Donors signed an informed consent, which was approved by the Ethics Committee on

Research of the Faculty of Medicine of the Federal University of Bahia (CAAE 45376814.0.0000.5577).

In vivo immunisation and IgG-binding assay

The main goal of mice immunisation was to obtain polyclonal anti-rBlo t 12 IgG antibodies that could be used to detect the native allergen (nBlo t 12) in BtE. Therefore, six to eight-week-old male BALB/c mice were subcutaneously immunised with 25 µg of rBlo t 12 on days 0, 15, 30 and 45. rBlo t 12 was diluted in 50 µl of PBS plus 50 µl of Complete Freund's Adjuvant in the first immunisation and 50 µl of Incomplete Freund's Adjuvant in the subsequent immunisations. Sera were taken two days preceding the first immunisation and two days after each immunisation. The kinetics of IgG antibodies against rBlo t 12 was measured by ELISA. Mice experiments were approved by the Ethical Committee for Use of Experimental Animals of the Institute of Health Sciences of Federal University of Bahia (CEUA-ICS 137/2018).

Evaluation of Blo t 12 presence in *B. tropicalis* extracts

The extraction procedure of *B. tropicalis* antigens was done as described in previous publications by our group [11, 23]. Due to the hydrophobic nature of chitin, we took different samples during the delipidification steps in the presence of diethyl ether (PA ACS; Hexis Científica, Jundiaí, SP, Brazil). Samples were obtained following this profile: (i) sample from the first centrifugation (11,000 g for 15 min, 10 °C) without diethyl ether incubation; (ii) sample from the first cycle of incubation for 30 min with diethyl ether and centrifugation; and (iii) sample from the third and last cycle of diethyl ether incubation and centrifugation. Twenty-five micrograms of each sample and 2 µg of rBlo t 12 as control were used for SDS-PAGE and then transferred to a nitrocellulose membrane (Merck Millipore, Billerica, MA, USA). The membrane was blocked with non-fat dried milk diluted in 1 × PBS. Incubation with mice sera (1:1000) was performed at RT for 1 h. The three last time points of immunisation were evaluated in this assay. As detection antibody, an alkaline phosphatase-conjugated anti-mouse IgG antibody (1:4000) was incubated with the membrane for 1 h at RT. Reactions were visualised using NBT and BCIP as substrates and the optical density (OD) for ELISA assays was read at 450 nm in a spectrophotometer (Multiskan, Thermo Fisher, Waltham, MA, USA).

Statistical analysis

GraphPad Prism 7 (San Diego, CA, USA) was used for data plotting and statistical analyses. Kolmogorov–Smirnov test

was chosen for checking the normality of data. Mann Whitney test was used to compare the IgE reactivity among the different antigens in each conjugate dilution in both IgE reactivity assays. Pearson's or Spearman's rank correlation coefficient was determined to verify possible correlations between ELISA and ImmunoCAP® results. Friedman analysis with Dunn's post-test was used to determine differences between IgE binding of BtE samples. *P* values ≤ 0.05 were considered statistically significant.

Results

Recombinant expression of Blo t 12 in *E. coli* induces formation of dimers and aggregates

rBlo t 12 was small-scale expressed in three different *E. coli* strains, being the best expression results that of the strain Top10F' (Fig. S1a, S1b, S1c). As shown in Fig. S1a and S1b, we could only clearly detect in the SDS-PAGE possible recombinantly expressed protein in Top10F' strain (Fig. S1b), but the bands migrated as an approximately 28 kDa dimeric protein band. This outcome led us to perform a Western blot analysis for confirmation, using the samples of the bacterial pellets from before and 3 h after IPTG induction (Top10F' strain), as well as adding a sample of BL21(DE3) pLys S of 3 h induction. Surprisingly, the BL21(DE3)pLys sample displayed bands, under reducing conditions, with molecular weight (MW) of approximately 45 kDa, even though with weak reaction (Fig. S1c). Top10F', differently, showed stronger bands, which also migrated mostly as an approximately 28 kDa dimeric protein band. This data differs from Blo t 12 theoretical molecular weight (MW) of approximately 15 kDa (Fig. S1c). Regarding the Top10F' scale-up expression at 16 °C, the same dimeric bands were found and the solubility test confirm that rBlo t 12 was obtained in both soluble and urea-solubilized fractions of the bacterial extract (Fig. S1d and S1e). However, given the stronger presence of the recombinant protein in the urea-solubilized fractions (Fig. S1e), these samples were chosen for purification. The highest protein elution was at 200 mM imidazole and dimers were present in all eluted fractions (Fig. 1a). Western blot analyses revealed that the use of lower heating temperature and stronger reducing conditions in the SDS-PAGE may reduce the aggregation, but no monomeric band was observed (Fig. 1b and c). DLS experiments showed that purified rBlo t 12 was dimeric (8.73 nm to 8.87 nm) and mostly aggregated (>24.88 nm) in solution at RT (Fig. 1d). In the sample without urea, size distribution was determined to be between 8.87 nm and 235.25 nm, but these values were slightly decreased to 8.84 and 202.75 as well as 8.73 and 125.15 upon addition of 3 M and 6 M urea, respectively (Fig. 1d).

Amino acid residues YITVM may be a key region of aggregation

Results of in silico analysis of primary structure of Blo t 12 are displayed in Fig. 2. The most aggregation-prone sequences were identified inside CBD, between residues 91 and 106 (Fig. 2a). The top paring values determined by PASTA 2.0 server are depicted in Fig. S2a. Ordered and disordered regions were also identified (Fig. 2a). Paring probability plot revealed that, among the residues between 91 and 106, LWYITVM residues exhibited the highest pairing and linear probability (Fig. 2b). Aggregation and disorder profile and aggregation free energy profile are plotted in Figs. 2c and S2b, respectively, confirming aggregation proneness of LWYITVM sequence. However, AGGRESCAN3D scores shortened this sequence to YITVM residues (Fig. 2d).

While the aggregation-prone region showed β -strand high probability (Fig. 3a), only moderate coil probability was found during PASTA 2.0 analysis (Fig. 3b). Figure S2c confirms no helix probability and Fig. S2d and S2e portray 3D-structures using the two servers. In Fig. 3c, other residues with high A3D scores (Fig. 2e) are closely located to YITVM residues after folding.

rBlo t 12 forms IgE-reactive aggregates

Our comparative analysis determined lower IgE reactivity to rBlo t 12, compared to rBlo t 5 and different BtEs (Fig. 4a). The optimum concentration for rBlo t 12 for coating seems to be 40 $\mu\text{g/mL}$. Lower dilution of secondary conjugate antibody did have some effect on increasing the IgE reaction to rBlo t 12 and control antigens as well. Delipidification of BtE significantly decreased IgE binding, comparing the lipidified BtE with the thrice-delipidified BtE. But there was no significant difference between lipidified and one step delipidified BtE (Fig. 4a). The panel of 30 sera from donors with BtE-reactive IgE antibodies displayed IgE reactivity percentages of 46.67% (14 sera out of 30) for rBlo t 12 and 83.33% (25 out of 30) for rBlo t 5 (Fig. 4b). Twelve out of thirty (40%) sera were reactive to both recombinant allergens, whereas 6.67% (2 out of 30) and 43.33% (13 out of 30) were mono-sensitised to rBlo t 12 and rBlo t 5, respectively. Moreover, the lower IgE reaction to rBlo t 12 was significant compared to rBlo t 5 and BtE (Fig. 4b). However, in ELISA, rBlo t 12 IgE-reactive sera showed stronger and more significant correlation with sIgE in ImmunoCAP (Fig. 4c) than rBlo t 5 and BtE reactive sera (Fig. S3a and S3b).

Natural Blo t 12 is faintly present in *B. tropicalis* non-delipidified soluble extract

Mice immunisation with rBlo t 12 produced IgG antibodies against Blo t 12, as it is shown in sera dilution

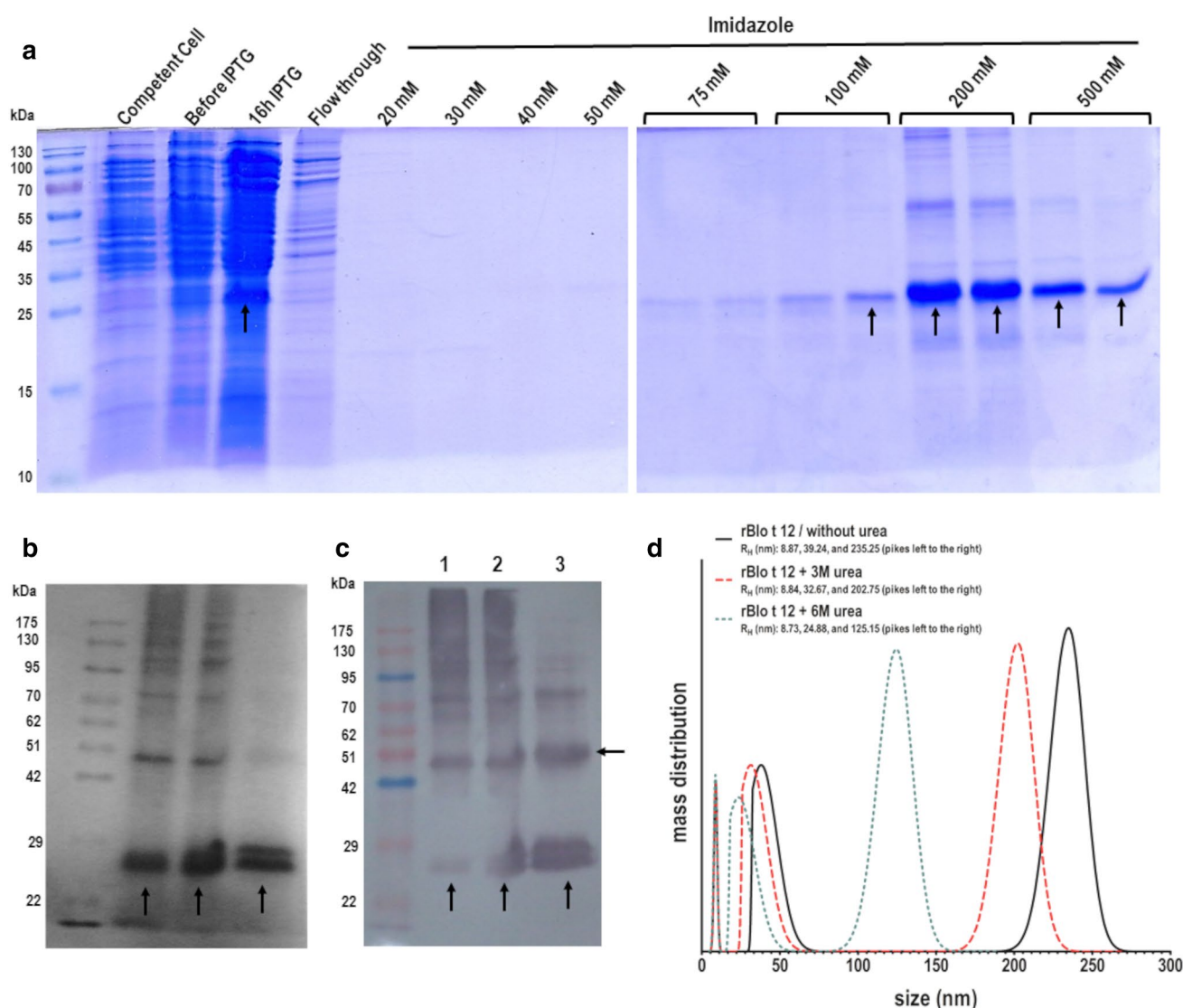


Fig. 1 Expression, purification and aggregation behaviour of rBlo t 12. **a** Scale-up expression and purification. Above each lane is indicated the sample added. The molecular weight maker is shown in the left. Arrows indicate the band for rBlo t 12. **b** Migration and aggregation verification by 18% SDS-PAGE using different sample buffers. **c** Western blot analysis comparing different reducing conditions. In both **b** and **c** two μg of purified rBlo t 12 were added to the wells as

follows: 1 – Standard SDS-PAGE sample buffer, heating at 100 °C for 5 min; 2 – Same buffer as in 1, but heating at 70 °C for 20 min; and 3 – More reducing SDS-PAGE sample buffer, heating at 37 °C for 10 min. **c** Dynamic light scattering measurements of purified rBlo t 12. Each coloured curve represents a sample and is indicated above the plot. R_H hydrodynamic radius. (Color figure online)

curves in Fig. 5a. The optimal concentration of antigen for coating was 2.5 $\mu\text{g}/\text{mL}$ and 1:1600 was the optimal sera dilution. Antibody kinetics revealed that the production of IgG antibodies occurred from day 15 on (Fig. 5b and c). Additionally, faint bands were observed only in non-delipidified BtE, especially when sera after the 3rd and 4th of immunisation were used (Fig. 5c). Two bands were identified between MW of 25 kDa and 30 kDa, and 50 kDa and 60 kDa for natural Blo t 12, but only using the 4th immunised sera (Fig. 5c).

Discussion

New data on the relevance of structural features of mite allergens are essential for improvement of diagnosis and treatment of allergic illnesses [3, 4, 22]. Given the increasing epidemiological prevalence of allergies caused by *B. tropicalis* allergens in Latin America and Asia [3, 8, 24], the search for new allergens, or further studies on their hypersensitivity potential on different populations and

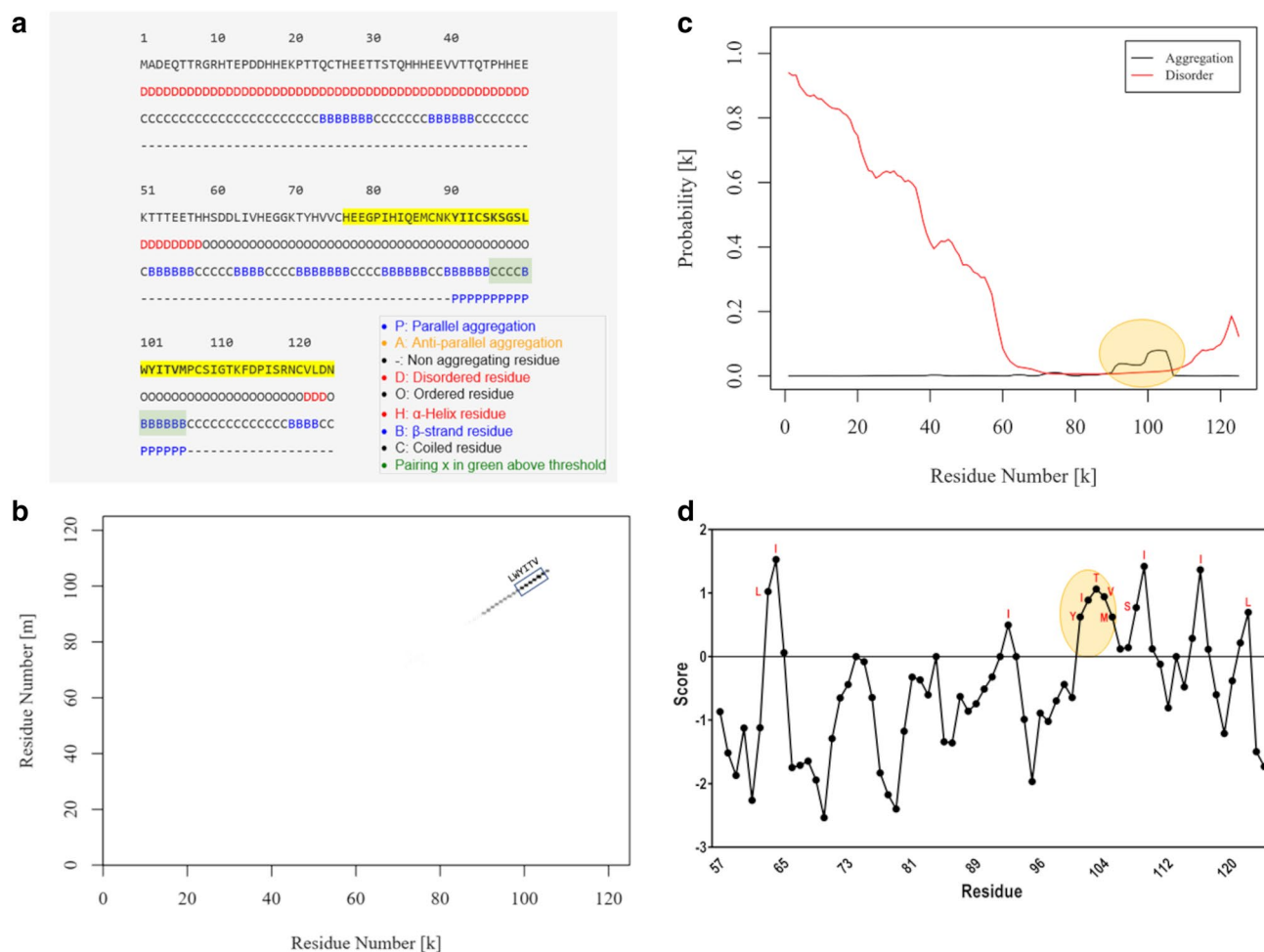


Fig. 2 In silico analysis of primary sequence of Blo t 12. **a** Residue assignment of disorder, α -helix, β -strand, coil and a parallel aggregation region according to PASTA 2.0 server [20]. Note the legend. CBD were highlighted in yellow and the prone-aggregation residues in bold. Among those, coil and β -strand residues were highlighted in green. **b** Pairing and linear probability profiles as a function of the

residue position [20]. A square with the high-score residues was added. **c** Aggregation and disorder profile [20]. **d** AGGRESCAN3D plot. A3D scores were retrieved from the server [19] and used to produce a GraphPad plot, allowing the identification of residues (in red) with high scores. A yellow oval was added in the Figures to highlight the importance of the data. (Color figure online)

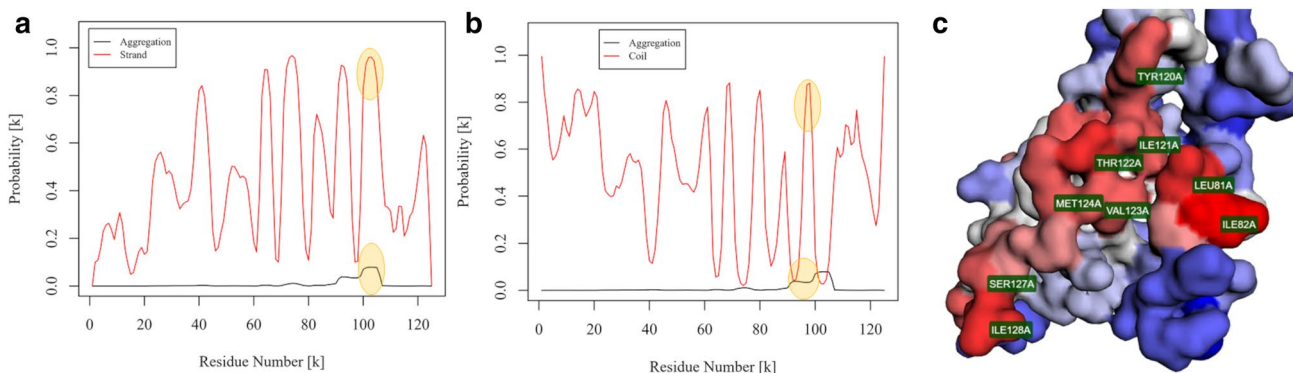


Fig. 3 Secondary and tertiary elements contribution on Blo t 12 aggregation. PASTA 2.0 and AGGRESCAN3D servers were used for the analyses [19, 20]. **a** Aggregation strand profile from PASTA 2.0. **b** Aggregation coil profile from PASTA 2.0. **c** Modelled 3D structure

retrieved from AGGRESCAN3D, focusing in the prone-aggregating region, which was zoomed and all A3D scores residues (all in red) were labelled. (Color figure online)

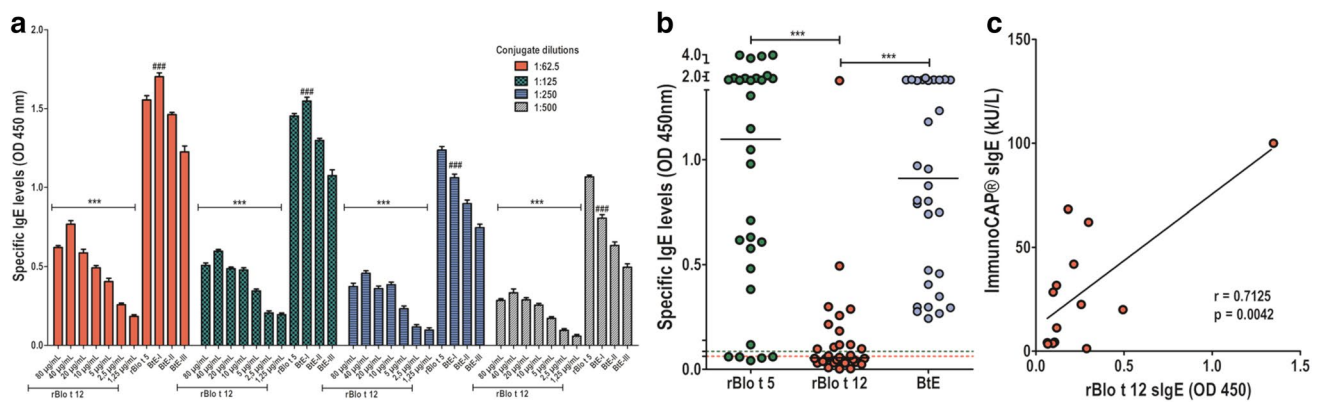


Fig. 4 Human antibody binding assays. **a** Human specific serum IgE levels against rBlo t 12, rBlo t 5 and three different *B. tropicalis* extracts (BtE) was determined by ELISA. The concentration curve for rBlo t 12 is indicated in the graph. The concentrations used for coating of rBlo t 5 and BtE were the same as previous studies [11]. ***: $p < 0.001$ using Mann Whitney test to compare rBlo 12 different concentrations and rBlo t 5 and BtE. ####: $p < 0.001$ Friedman analysis with Dunn's post-test to compare BtE-I and BtE-III. **b** Allergenicity

of rBlo t 12 compared to rBlo t 5 and BtE. Each symbol represents the result obtained with an individual serum. Short horizontal solid lines represent the median value of the group results. The cut-offs are represented by horizontal dotted lines (red 0.062 for rBlo t12 assay, and green 0.086 for rBlo t 5 assay). ***: $p < 0.001$ using Mann Whitney test to compare rBlo 12 and the other antigens. **c** Pearson's correlation of rBlo t 12 ELISA results and sIgE from ImmunoCAP assay ($n = 14$). (Color figure online)

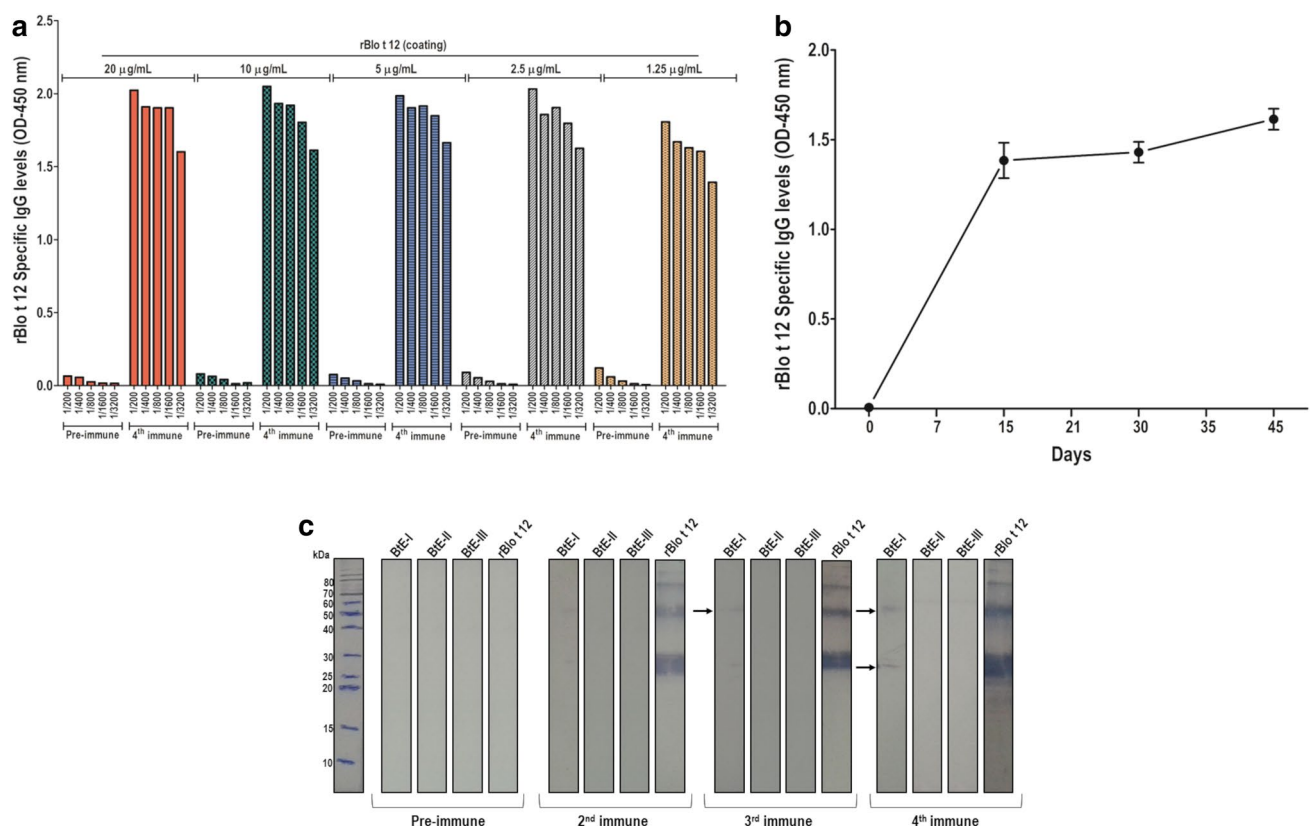


Fig. 5 Detection of the natural allergen in mite extract by using IgG antibodies of rBlo t 12-immunised mice. **a** Pilot assay to define the optimal concentration of rBlo t 12 and mouse sera dilution for anti-rBlo t 12 IgG detection. **b** Kinetics of IgG antibodies of sera from rBlo t 12-immunised mice. **c** Western blot for detection of nBlo t 12 in

BtE. The sample added is indicated above each lane. BtE-I: sample without delipidification. BtE-II sample from the first cycle of incubation with diethyl ether. BtE-III: sample from the third cycle of diethyl ether incubation

specific-characteristics of new allergens are currently needed. Although we recently identified through proteomic analysis six *B. tropicalis* allergen not yet been recognized by IUIS [23], the studies focusing in some *B. tropicalis* allergens are still scarce [9], highlighting the need of confirmation or further investigation on their physicochemical and immunological features [16].

Herein, we described expression and purification issues during the recombinant production of group 12 *B. tropicalis* allergen using *Escherichia coli* as host. Although we managed to express this allergen in a bacterial host system, which has intrinsic advantages over eukaryotic systems [25], the recombinant protein was prone to dimerisation and aggregation. Although the first reports on recombinant expression of Blo t 12 did not showed SDS-PAGE images [15, 17], later on isoform Blo t 12.0101 was used as a control assay, which clearly showed that the recombinant protein produced in *P. pastoris* had also migrated as a dimeric protein, with MW around 28 kDa [13]. Additionally, the authors, in due course, stated that although *P. pastoris* expressed-isoform Blo t 12.0101 displayed IgE-binding capacity (50% of IgE reactivity), it would not allow the obtention of an optimal recombinant for allergy diagnosis, due to excessive glycosylation of the expression in this host [26]. The same isoform posteriorly expressed in *E. coli* Origami (DE3) showed similar MW in reducing conditions, and high MW aggregates in non-reducing conditions [18, 26]. Therefore, our aggregation outcome is not an exclusive issue of *E. coli* Top10F'expressed-rBlo t 12, and this behaviour may be an intrinsic property of the allergen. We then investigated the influence of this on the recombinant allergenicity.

DLS measurements confirmed dimerisation and aggregation of rBlo t 12. Similar pattern was already observed in other heterologously expressed or native proteins, including some allergens, with $R_H > 200$ nm [27–30]. In silico analyses revealed that a C-terminus organized region inside Blo t 12 CBD may be responsible for this aggregation. The YITVM residues indeed display similar hydrophobic characteristics to other key residues in dimeric HDM allergens, such as Der p 5, Der f 5, Der p 21, and Der f 21 [31–34]. Interestingly, in these reports, Val residues were essential to create a stabilizing hydrophobic surface and dimerisation occurred due to the creation of a zipper domain [32, 33]. Ile residues showed the highest A3D scores, and, after folding, they were localized in the proximity of the most aggregation-prone region. We believe this may add hydrophobicity to the region, and, in turn, increases the tendency for aggregation. However, in future studies, the mutation of such residues and expression of a mutated Blo t 12 would be needed to confirm such hypotheses. Nevertheless, we considered these in silico predictions important scientific tools, after all they have been previously essential, for instance, to decrease the number of bench experiments in the production of hypoallergenic

derivatives [4, 35]. Furthermore, bioinformatics has also served as an earlier method not only to identify potential dimeric regions, but to determine the cross-reactivity among allergens, reducing the number of recombinant allergens or peptides needed for bench experiments [33, 36].

Additionally, allergen aggregates can may take a part in the degree of allergenic responses, sometimes leading to decreased IgE binding [29, 37]. Our results evidenced a lower IgE-binding of rBlo t 12 in comparison with rBlo t 5 and BtE. In fact, the critical residues for IgE binding of Blo t 12.0101 were identified as Y121, I122 and T123 [15], all of them are inside the aggregating-prone sequence. Therefore, either dimers or aggregates may possibly be hiding the IgE epitopes, resulting in low IgE reactivity. In addition, absorbance signals for rBlo t 12 were significantly lower than those for rBlo t 5, even though the antigen coating concentration was higher [11]. Antigen titration curves are usually used as way to define the optimal concentration for coating, based in the strength of the absorbance signal [11, 38]. In our case, using data from a previous report as basis [11], we decided for seven concentrations in a titration curve that would include rBlo t 5 optimal concentration, 5 µg/mL [11]. With that in mind, the strongest absorbance signal for rBlo t 12 was the coating antigen concentration of 40 µg/mL, considering that the signal decayed in 80 µg/mL.

Nevertheless, our results indicate that the dimeric rBlo t 12 is a mid-tier allergen in Brazil, a different finding from a previous Singaporean report [17]. However, the IgE reactivity of our rBlo t 12 was close to the one reported in Colombia [15, 16]. It is important to note that these rBlo t 12 IgE-binding findings are the first in a set of Brazilian allergenic patients, something that lead us to believe this mid-tier allergen should be included in personalized medicine tools, such as the one our group recently published [39]. Even with aggregation explaining the lower reaction of sera from *B. tropicalis*-sensitised donors, specific-sensitisation patterns may also be a reason, since isoforms of Blo t 12 already displayed different reaction profile in patients from different countries [15, 17]. Remarkably, rBlo t 12 reactive sera in the present study correlated better with sIgE detected by ImmunoCAP® than rBlo t 5 reactive sera, indicating that the chitin-binding allergen may be useful in diagnosis of *B. tropicalis* allergy. This allergen has been previously stated as a possible specific marker of *B. tropicalis* sensitisation, considering that its *Dermatophagoides* spp. counterpart was not identified yet [15, 16, 26]. A World Allergy Organization consensus document clearly summarized the importance of recombinant allergens for precision allergy molecular diagnostic applications [40]. Hence, the continued study on structural and allergenic features of a recombinant allergen, which is a possible specific marker for *B. tropicalis* allergy, is highly desirable to improve such diagnostic tools in the future [39, 40].

We only found small signs of nBlo t 12 in non-delipidified BtE, showing that soluble extracts may not always identify the whole set of allergic response in patients. A possible explanation for this finding resides in the possibility of a different isoform, which may be present in higher amounts in our extract. It has been previously observed that different isoforms are absent in mites collected from different regions of the globe [15–17]. Besides this possibility of allergic polymorphisms in mites, cultivation conditions, such as storage temperature and humidity, type of feeding, and extraction procedures were described having impacted allergens' content before [23, 41]. Therefore, the expression of nBlo t 12 in the extract of the present study may have been lower for these reasons. However, in a previous proteomic study of our group, we could not find Blo t 12 in any of our 11 soluble BtE, as well as in a commercial extract [23]. Considering the sensitiveness of proteomic analyses, the absence of Blo t 12 in an indicative that the allergen may be weakly expressed in general. Nonetheless, the hydrophobic nature of the chitin usually bound to the allergen should be also taken into account.

A previous study showed that while isoform 0102 binds less to chitin, the isoform used in the present study (0101) had a higher affinity to chitin and was in lower amounts in the soluble supernatant [18]. However, using an ELISA assay, this group detected isoform 0101 in mite bodies and faeces from a commercial extract. Moreover, their two-dimensional gel results revealed that nBlo t 12 indeed aggregated, with spots of MW around 40 kDa [26]. Although their extraction method is different from ours [18, 23], explaining perhaps the low detection of nBlo t 12 in our extract, previously published reports of our group confirm the quality of our method, which allow us to identified, at a proteomic level, new allergens of *B. tropicalis* as well as confirm atopy in several studies [11, 23, 35, 42]. Furthermore, we demonstrated a significant reduction in IgE reactivity of BtE after delipidification, underscoring that not only some allergenic components are being lost, but also allergens with hydrophobic molecule-binding features may be currently under investigated in allergy diagnosis [4, 6]. Therefore, two major possibilities should be considered for the weak presence of nBlo t 12 in BtE: (i) mite from our city may be polymorphic distinct, something that was previously observed in cities such as Singapore and Colombia [15, 17]; and (ii) the allergen was lost due to the delipidification steps or other purification and cleaning procedures of protein extracts [23, 41]. In fact, a thorough study on commercial extracts revealed the absence of not only major allergens, but also minor and mid-tier HDM allergens [41]. Hydrophobic molecule-binding allergens, such as Der p 2 and Der p 5 were absent in some extracts, leading to false-negative diagnostic test results in a few asthmatic and allergic rhinitis patients [41]. These data emphasize both the importance of minor and

mid-tier allergens in atopic reactions as well as the need of quality control of allergenic extracts currently used in allergy diagnosis.

Up to now, the importance of a molecule from allergenic organism is mostly given by its capacity to induce IgE reactivity and, in turn, stronger value is given to the classifications minor, mid-tier or major allergen. This notion is changing, as some authors recently stated how it is time to let go this once useful classification in allergology [5]. We agree and also think that molecules considered minor/mid-tier allergens must be reviewed to investigate their capacity to induce IgE-independent inflammatory responses through the innate immune response, as it has been performed in some cases [7, 9, 10, 16]. Particularly, Group 2 innate lymphoid cells (ILC2) and invariant natural killer T cells (iNKT cells) have been involved in allergic reaction, mainly in asthmatic patients [43–45]. These innate cells can be activated by hydrophobic molecules, lipids for instance [43, 46]. Therefore, it is possible to infer that there is a continuous activation of the innate immune system by hydrophobic molecule-binding allergens. Concomitantly with that, the adaptive immune responses induced by other allergens, considered major, may lead to more symptoms in patients. Considering this, it will be important, in futures studies, to investigate if Blo t 12 and other minor/mid-tier allergens may have a role in the innate responses against *B. tropicalis* and other important allergenic organisms.

Conclusion

In view of these results, *E. coli* Top10F' may be an alternative expression vector for the recombinant production of Blo t 12, with high yields of expression and isoform 0101 is a mid-tier allergen in *B. tropicalis*-sensitised patients from Brazil. Considering these findings, we believe this allergen should be included in personalized diagnostic tools as well as in the set of critical allergens for *B. tropicalis* allergy diagnosis. Additionally, our data indicate that this chitin-binding allergen may indeed naturally dimerise, with suggestion of specific residues causing this outcome. Future studies shall be performed, testing Blo t 12 capacity for activation of innate immune cells, as well as producing mutated rBlo t 12 and purifying nBlo t 12 from BtE by affinity chromatography in order to better understand the importance of some amino acid residues on the aggregation behaviour of Blo t 12.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s11033-021-06361-6>.

Acknowledgements ESS was the recipient of a scholarship from the Research's Foundation of the State of Bahia (FAPESB grant BOL0449/2015). LGCP and NMAN are recipients of research

fellowships from National Council for Scientific and Technological Development of Brazil (CNPq).

Author contributions NM Alcantara-Neves designed the study and supervised the project. ES Silva significantly contributed to perform the present study, wrote the manuscript and conducted the in vitro and in vivo laboratory assays, as well as in silico analyses. CS Pinheiro co-supervised the project, helped with data analyses and manuscript revision. LGC Pacheco was involved in conception the project, revision/validation of in silico analysis assays and manuscript revision. AMS Fernandes performed IgE reactivity assay and helped with other experiments. C. Asam performed expression and purification pilot assays and manuscript revision. E.F. Silveira helped with laboratory assays, particularly DLS analyses. NM Alcantara-Neves and CS Pinheiro also drafted the manuscript. All authors read and approved the final manuscript.

Funding Coordination for the Improvement of Higher Education Personnel (CAPES), Project No. 077/2012.

Declarations

Conflict of interest The authors have no conflicts of interest.

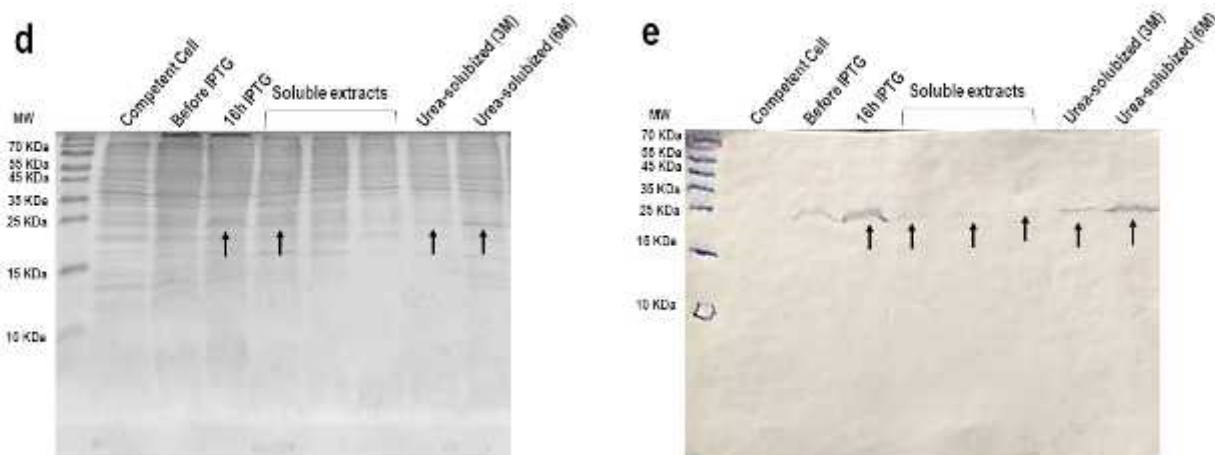
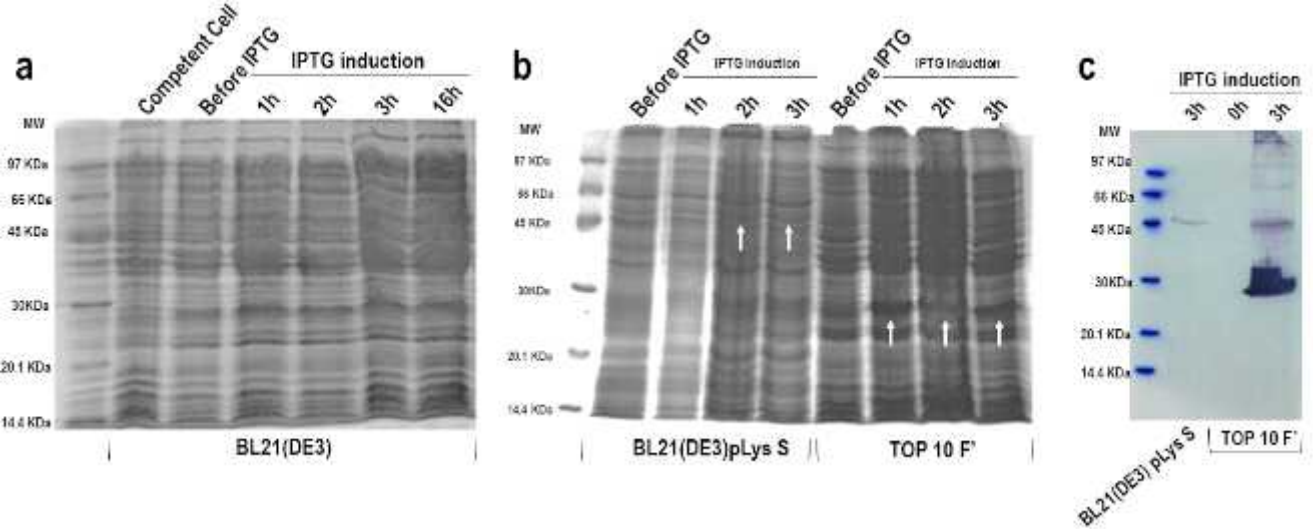
Ethical approval Ethics Committee on Research of the Faculty of Medicine of the Federal University of Bahia (CAAE 45376814.0.0000.5577) approved human sera use. Ethical Committee for Use of Experimental Animals of the Institute of Health Sciences of Federal University of Bahia (CEUA-ICS 137/2018) allowed mice experiments

References

- Pawankar R (2014) Allergic diseases and asthma: a global public health concern and a call to action. *World Allergy Organ J* 7(1):12. <https://doi.org/10.1186/1939-4551-7-12>
- Enilari O, Sinha S (2019) The global impact of asthma in adult populations. *Ann Glob Health*. <https://doi.org/10.5334/aogh.2412>
- Miller JD (2019) The role of dust mites in allergy. *Clin Rev Allergy Immunol* 57(3):312–329. <https://doi.org/10.1007/s12016-018-8693-0>
- Silva ESD, Pinheiro CS, Pacheco LGC, Alcantara-Neves NM (2020) Dermatophagoides spp. hypoallergens design: what has been achieved so far? *Expert Opin Ther Pat* 30(3):163–177. <https://doi.org/10.1080/13543776.2020.1712360>
- Caraballo L, Valenta R, Acevedo N, Zakzuk J (2021) Are the terms major and minor allergens useful for precision allergology? *Front Immunol*. <https://doi.org/10.3389/fimmu.2021.651500>
- Gonzalez-Perez R, Pineda F, Poza-Guedes P, Matheu V, Sanchez-Machin I (2018) Minor allergens in moderate-severe allergic rhinitis: group 4 mite amylase (Blo t4) and geographical variations. *J Allergy Clin Immunol* 141(2):AB286. <https://doi.org/10.1016/j.jaci.2017.12.911>
- Smole U, Gour N, Phelan J, Hofer G, Kohler C, Kratzer B, Tauber PA, Xiao X, Yao N, Dvorak J, Caraballo L, Puerta L, Roskopf S, Chakir J, Malle E, Lane AP, Pickl WF, Lajoie S, Wills-Karp M (2020) Serum amyloid A is a soluble pattern recognition receptor that drives type 2 immunity. *Nat Immunol* 21(7):756–765. <https://doi.org/10.1038/s41590-020-0698-1>
- Caraballo L, Zakzuk J, Lee BW, Acevedo N, Soh JY, Sanchez-Borges M, Hossny E, Garcia E, Rosario N, Ansotegui I, Puerta L, Sanchez J, Cardona V (2016) Particularities of allergy in the Tropics. *World Allergy Organ J* 9:20. <https://doi.org/10.1186/s40413-016-0110-7>
- Santos da Silva E, Asam C, Lackner P, Hofer H, Wallner M, Silva Pinheiro C, Alcantara-Neves NM, Ferreira F (2017) Allergens of *Blomia tropicalis*: an overview of recombinant molecules. *Int Arch Allergy Immunol* 172(4):203–214. <https://doi.org/10.1159/000464325>
- Soongrung T, Mongkorntanyatip K, Peepim T, Buaklin A, Le Mignon M, Malainual N, Nony E, Jacquet A (2018) The *Blomia tropicalis* allergen Blo t 7 stimulates innate immune signalling pathways through TLR2. *Clin Exp Allergy* 48(4):464–474. <https://doi.org/10.1111/cea.13098>
- Carvalho KA, de Melo-Neto OP, Magalhaes FB, Ponte JC, Felipe FA, dos Santos MC, dos Santos LG, Cruz AA, Pinheiro CS, Pontes-de-Carvalho LC, Alcantara-Neves NM (2013) *Blomia tropicalis* Blo t 5 and Blo t 21 recombinant allergens might confer higher specificity to serodiagnostic assays than whole mite extract. *BMC Immunol* 14:11. <https://doi.org/10.1186/1471-2172-14-11>
- Munera M, Martinez D, Labrada A, Caraballo L, Puerta L (2019) Identification of B cell epitopes of Blo t 13 allergen and cross-reactivity with human adipocytes and heart fatty acid binding proteins. *Int J Mol Sci*. <https://doi.org/10.3390/ijms20246107>
- Acevedo N, Mohr J, Zakzuk J, Samonig M, Briza P, Erler A, Pomes A, Huber CG, Ferreira F, Caraballo L (2013) Proteomic and immunochemical characterization of glutathione transferase as a new allergen of the nematode *Ascaris lumbricoides*. *PLoS One* 8(11):e78353. <https://doi.org/10.1371/journal.pone.0078353>
- Reginald K, Pang SL, Chew FT (2019) Blo t 2: Group 2 allergen from the dust mite *Blomia tropicalis*. *Sci Rep* 9(1):12239. <https://doi.org/10.1038/s41598-019-48688-y>
- Zakzuk J, Jimenez S, Cheong N, Puerta L, Lee BW, Chua KY, Caraballo L (2009) Immunological characterization of a Blo t 12 isoallergen: identification of immunoglobulin E epitopes. *Clin Exp Allergy* 39(4):608–616. <https://doi.org/10.1111/j.1365-2222.2008.03193.x>
- Fernandez-Caldas E, Puerta L, Caraballo L, Iraola V, Lockey R (2020) Mite allergens. In: Lockey RF, Ledford DK (eds) *Allergens and allergen immunotherapy*, 6th edn. CRC Press, FL USA, pp 213–235
- Shek LP, Chong AR, Soh SE, Cheong N, Teo AS, Yi FC, Giam YC, Chua KY, Van Bever HP (2010) Specific profiles of house dust mite sensitization in children with asthma and in children with eczema. *Pediatr Allergy Immunol* 21(4 Pt 2):e718–722. <https://doi.org/10.1111/j.1399-3038.2010.01019.x>
- Zakzuk J, Benedetti I, Fernandez-Caldas E, Caraballo L (2014) The influence of chitin on the immune response to the house dust mite allergen Blo T 12. *Int Arch Allergy Immunol* 163(2):119–129. <https://doi.org/10.1159/000356482>
- Zambrano R, Jamroz M, Szczasiuk A, Pujols J, Kmiecik S, Ventura S (2015) AGGREGSCAN3D (A3D): server for prediction of aggregation properties of protein structures. *Nucleic Acids Res* 43(W1):W306–313. <https://doi.org/10.1093/nar/gkv359>
- Walsh I, Seno F, Tosatto SC, Trovato A (2014) PASTA 2.0: an improved server for protein aggregation prediction. *Nucleic Acids Res*. <https://doi.org/10.1093/nar/gku399>
- Naik MT, Kung CCH, Huang T (2014) NMR solution structure of chitin-binding domain from dust mite group XII allergen Blo t 12. doi:<https://doi.org/10.2210/pdb2MFK/pdb>
- da Silva ES, Huber S, Alcantara-Neves NM, Asam C, Silveira EF, de Andrade Belitardo EMM, Aglas L, Wallner M, Gadermaier G, Briza P, Karner I, Torres RT, Alvarez JRU, Wuenschmann S, Chapman M, Ferreira F, Pinheiro CS (2020) N-terminal peptide deletion influences immunological and structural features of Blo t 5. *Allergy* 75(6):1503–1507. <https://doi.org/10.1111/all.14176>
- Santos da Silva E, Marques Ponte JC, Barbosa da Silva M, Silva Pinheiro C, Carvalho Pacheco LG, Ferreira F, Briza P,

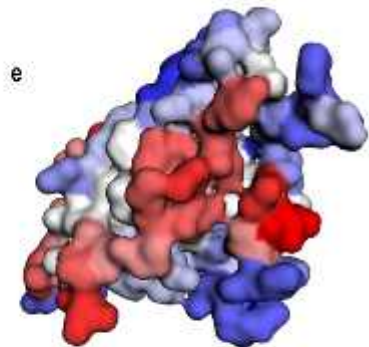
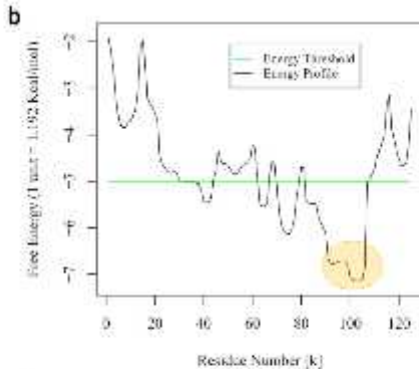
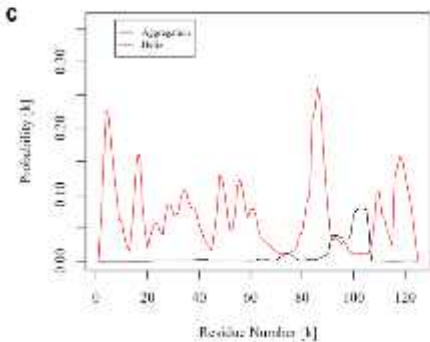
- Alcantara-Neves NM (2019) Proteomic analysis reveals allergen variability among breeds of the dust mite *Blomia tropicalis*. *Int Arch Allergy Immunol* 180(3):159–172. <https://doi.org/10.1159/000501964>
24. Pang KA, Pang KP, Pang EB, Tan YN, Chan YH, Siow JK (2017) Food allergy and allergic rhinitis in 435 Asian patients – a descriptive review. *Med J Malaysia* 72(4):215–220
 25. Rosano GL, Ceccarelli EA (2014) Recombinant protein expression in *Escherichia coli*: advances and challenges. *Front Microbiol* 5:172. <https://doi.org/10.3389/fmicb.2014.00172>
 26. Zakzuk J (2012) Immunological and biological characterization of *Blomia tropicalis* allergens. University of Cartagena Cartagena, Colombia
 27. Rodriguez-Bolanos M, Miranda-Astudillo H, Perez-Castaneda E, Gonzalez-Halphen D, Perez-Montfort R (2020) Native aggregation is a common feature among triosephosphate isomerases of different species. *Sci Rep* 10(1):1338. <https://doi.org/10.1038/s41598-020-58272-4>
 28. Ferrari E, Corsini R, Burastero SE, Tanfani F, Spisni A (2020) The allergen Mus m 1.0102: cysteine residues and molecular allergology. *Mol Immunol* 120:1–12. <https://doi.org/10.1016/j.molimm.2020.01.022>
 29. Najafi N, Hofer G, Gattinger P, Smiljkovic D, Blatt K, Selb R, Stoecklinger A, Keller W, Valent P, Niederberger V, Thalhamer J, Valenta R, Flicker S (2019) Fusion proteins consisting of Bet v 1 and Phl p 5 form IgE-reactive aggregates with reduced allergenic activity. *Sci Rep* 9(1):4006. <https://doi.org/10.1038/s41598-019-39798-8>
 30. Golub N, Meremyanin A, Markossian K, Eronina T, Chebotareva N, Asryants R, Muronets V, Kurganov B (2007) Evidence for the formation of start aggregates as an initial stage of protein aggregation. *FEBS Lett* 581(22):4223–4227. <https://doi.org/10.1016/j.febslet.2007.07.066>
 31. Weghofer M, Dall'Antonia Y, Grote M, Stocklinger A, Kneidinger M, Balic N, Krauth MT, Fernandez-Caldas E, Thomas WR, van Hage M, Vieths S, Spitzauer S, Horak F, Svergun DI, Konarev PV, Valent P, Thalhamer J, Keller W, Valenta R, Vrtala S (2008) Characterization of Der p 21, a new important allergen derived from the gut of house dust mites. *Allergy* 63(6):758–767. <https://doi.org/10.1111/j.1398-9995.2008.01647.x>
 32. Mueller GA, Gosavi RA, Krahn JM, Edwards LL, Cuneo MJ, Glesner J, Pomes A, Chapman MD, London RE, Pedersen LC (2010) Der p 5 crystal structure provides insight into the group 5 dust mite allergens. *J Biol Chem* 285(33):25394–25401. <https://doi.org/10.1074/jbc.M110.128306>
 33. Khemili S, Kwasigroch JM, Hamadouche T, Gilis D (2012) Modelling and bioinformatics analysis of the dimeric structure of house dust mite allergens from families 5 and 21: Der f 5 could dimerize as Der p 5. *J Biomol Struct Dyn* 29(4):663–675. <https://doi.org/10.1080/073911012010525018>
 34. Pang SL, Ho KL, Waterman J, Rambo RP, Teh AH, Mathavan I, Harris G, Beis K, Say YH, Anusha MS, Sio YY, Chew FT, Ng CL (2019) Crystal structure and epitope analysis of house dust mite allergen Der f 21. *Sci Rep* 9(1):4933. <https://doi.org/10.1038/s41598-019-40879-x>
 35. da Silva ES, Aglas L, Pinheiro CS, de Andrade Belitardo EMM, Silveira EF, Huber S, Torres RT, Wallner M, Briza P, Lackner P, Laimer J, Pacheco LGC, Cruz AA, Alcantara-Neves NM, Ferreira F (2020) A hybrid of two major *Blomia tropicalis* allergens as an allergy vaccine candidate. *Clin Exp Allergy* 50(7):835–847. <https://doi.org/10.1111/cea.13611>
 36. Lahiani S, Dumez ME, Khemili S, Bitam I, Gilis D, Galleni M (2019) Cross-Reactivity between Major IgE Epitopes of Family 5 Allergens from *Dermatophagoides pteronyssinus* and *Blomia tropicalis*. *Int Arch Allergy Immunol* 178(1):10–18. <https://doi.org/10.1159/000492871>
 37. Zaborsky N, Brunner M, Wallner M, Himly M, Karl T, Schwarzenbacher R, Ferreira F, Achatz G (2010) Antigen aggregation decides the fate of the allergic immune response. *J Immunol* 184(2):725–735. <https://doi.org/10.4049/jimmunol.0902080>
 38. Shah K, Maghsoudlou P (2016) Enzyme-linked immunosorbent assay (ELISA): the basics. *Br J Hosp Med (Lond)* 77(7):C98–101. <https://doi.org/10.12968/hmed.2016.77.7.C98>
 39. Pinheiro CS, Silva ES, de Andrade Belitardo EMM, Pacheco LGC, Aguiar ERGR, Alcantara-Neves NM, Gadermaier G, Ferreira F (2021) En route to personalized medicine: uncovering distinct IgE reactivity pattern to house dust mite components in Brazilian and Austrian allergic patients. *Clin Trans Allergy*. <https://doi.org/10.1002/ct2.12004>
 40. Steering Committee A, Review Panel M (2020) A WAO - ARIA - GA(2)LEN consensus document on molecular-based allergy diagnosis (PAMD@): update 2020. *World Allergy Organ J* 13(2):100091. <https://doi.org/10.1016/j.waojou.2019.100091>
 41. Casset A, Mari A, Purohit A, Resch Y, Weghofer M, Ferrara R, Thomas WR, Alessandri C, Chen KW, de Blay F, Valenta R, Vrtala S (2012) Varying allergen composition and content affects the in vivo allergenic activity of commercial *Dermatophagoides pteronyssinus* extracts. *Int Arch Allergy Immunol* 159(3):253–262. <https://doi.org/10.1159/000337654>
 42. Alcantara-Neves NM, Veiga RV, Ponte JC, da Cunha SS, Simoes SM, Cruz AA, Yazdanbakhsh M, Matos SM, Silva TM, Figueiredo CA, Pontes-de-Carvalho LC, Rodrigues LC, Fiaccone RL, Cooper PJ, Barreto ML (2017) Dissociation between skin test reactivity and anti-aeroallergen IgE: determinants among urban Brazilian children. *PLoS One* 12(3):e0174089. <https://doi.org/10.1371/journal.pone.0174089>
 43. Chandra S, Gray J, Kiosses WB, Khurana A, Hitomi K, Crosby CM, Chawla A, Fu Z, Zhao M, Veerapen N, Richardson SK, Porcelli SA, Besra G, Howell AR, Sharma S, Peters B, Kronenberg M (2018) Mrp1 is involved in lipid presentation and iNKT cell activation by *Streptococcus pneumoniae*. *Nat Commun* 9(1):4279. <https://doi.org/10.1038/s41467-018-06646-8>
 44. Mindt BC, Fritz JH, Duerr CU (2018) Group 2 innate lymphoid cells in pulmonary immunity and tissue homeostasis. *Front Immunol* 9:840. <https://doi.org/10.3389/fimmu.2018.00840>
 45. Iwamura C, Nakayama T (2018) Role of CD1d- and MR1-restricted T cells in asthma. *Front Immunol* 9:1942. <https://doi.org/10.3389/fimmu.2018.01942>
 46. Doherty TA, Broide DH (2018) Lipid regulation of group 2 innate lymphoid cell function: Moving beyond epithelial cytokines. *J Allergy Clin Immunol* 141(5):1587–1589. <https://doi.org/10.1016/j.jaci.2018.02.034>

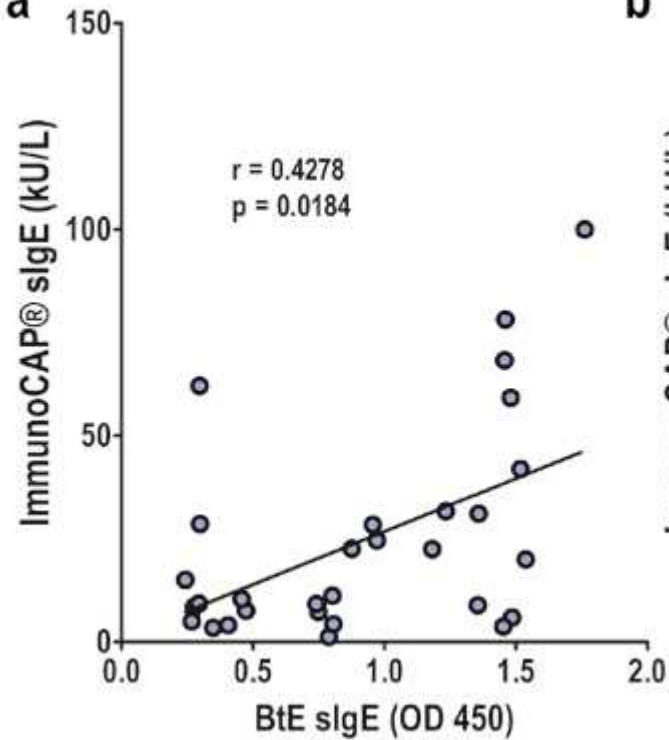
Publisher's Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.



a

Pairing panel 1: Energy -6.337997 pairing segments 101-106 and 102-108 (size 7)
 Pairing panel 2: Energy -6.146068 pairing segments 101-105 and 102-106 (size 6)
 Pairing panel 3: Energy -6.105715 pairing segments 91-106 and 91-105 (size 16)
 Pairing panel 4: Energy -5.927087 pairing segments 91-106 and 91-105 (size 15)
 Pairing panel 5: Energy -5.87475 pairing segments 92-106 and 92-105 (size 15)
 Pairing panel 6: Energy -5.862095 pairing segments 92-106 and 92-105 (size 8)
 Pairing panel 7: Energy -5.574273 pairing segments 101-106 and 101-105 (size 6)
 Pairing panel 8: Energy -5.455122 pairing segments 92-106 and 92-105 (size 14)
 Pairing panel 9: Energy -5.482657 pairing segments 91-106 and 91-105 (size 7)
 Pairing panel 10: Energy -5.352655 pairing segments 101-106 and 101-105 (size 5)



a**b**