

The Influence of Chitin on the Immune Response to the House Dust Mite Allergen Blo t 12

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Key Words

Blomia tropicalis · Blo t 12 · Chitin · Recombinant allergens · House dust mites · Immunoglobulin E

Abstract

Background: Information about the biological properties of *Blomia tropicalis* allergens is scarce. It is predicted that Blo t 12, an allergen with two described isoforms, contains a chitin-binding domain, similar to that found in peritrophins. Th2 adjuvant properties have been described for chitin. Therefore, it is feasible that binding to this carbohydrate influences its allergenicity. We aimed to evaluate the chitin-binding activity of Blo t 12 isoallergens and its effect on airway inflammation and antibody responses in a murine model of allergen sensitization. **Methods:** Chitin-binding assays were conducted with the recombinant isoallergens Blo t 12.0101 and Blo t 12.0102. BALB/c mice were sensitized via i.p. with any of the two isoforms (alone, with chitin or alum) and then challenged intranasally. Methacholine-induced bronchial hyperreactivity was tested by whole-body plethysmography and lung sections were stained with hematoxylin and eosin and periodic-acid Schiff. Total IgE and allergen-specific IgE, IgG1 and IgG2 levels were measured by ELISA. **Results:** The two isoforms bound chitin, but Blo t 12.0101 showed a stronger binding capacity. Both isoforms induced

total and allergen-specific IgE, airway hyperreactivity, bronchial inflammation and mucus secretion without any adjuvant; however, when administered with chitin, Blo t 12.0101 induced higher total IgE levels. The IgG1/IgG2a ratio was significantly higher in mice immunized with Blo t 12.0101 than those immunized with Blo t 12.0102. As peritrophins, Blo t 12 was detected in mite feces. **Conclusions:** Blo t 12 isoforms are chitin-binding proteins that induce airway inflammation and bronchial hyperreactivity. However, for Blo t 12.0101, chitin reinforces its effects on total IgE production.

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Introduction

Asthma is becoming a global public health concern due to increasing prevalence and difficulties in clinical management [1]. House dust mite (HDM) allergens are the most important cause of sensitization in asthmatic patients worldwide [2–4]. The IgE response to *Blomia tropicalis*, a geographically-restricted allergenic source in tropical and subtropical environments [5, 6], is very frequent and is associated with clinical manifestations of allergy [7]. Its cross-reactivity with *Dermatophagoides pteronyssinus* is low to moderate [8]; thus, species-specific allergens are to be expected in both sources. In spite of a

high rate of cosensitization in tropical countries, differences in the immune response toward these HDM species have been described, such as genetic determinants in humans [9, 10] and patterns of airway inflammation in animal models [11]. Therefore, molecular characterization of purified components is necessary to obtain a clearer understanding about their allergenic peculiarities to facilitate improvement of the clinical management of respiratory and cutaneous allergies in the tropics.

Knowledge about the biological properties of allergens provides helpful information for understanding allergenicity. The biochemical activities of the major HDM allergens of *D. pteronyssinus* promote allergic inflammation [12], i.e. the proteolytic function of Der p 1 and the lipopolysaccharide (LPS)-binding activity of Der p 2 are directly involved in the development of experimental asthma [13–15]. Moreover, anatomical location seems to be relevant in allergenicity, with mite feces being the main carriers of allergenic molecules [16] probably due to their small size which facilitates antigen delivery into the lower respiratory tract.

Data about the biological functions of *B. tropicalis* allergens are far from complete. The biochemical identity of many of these allergens has been predicted [17], but it has been experimentally demonstrated only for Blo t 13, a fatty-acid-binding protein [18]. Empirical confirmation of biochemical properties is essential to avoid misclassification. For example, Bla g 2 was previously predicted to be aspartyl-protease, but was then experimentally defined as a ligand-binding protein [19].

Blo t 12 is polymorphic allergen group with no described homolog members in *Dermatophagoides* sp. and also one the most epidemiologically relevant allergens in the tropics [20]. It has a predicted chitin-binding domain (CBD) [21], but binding of ligands has not been confirmed experimentally. Since chitin exhibits immunomodulatory properties [22, 23], it is possible that the ability of binding chitin influences the allergenicity of some molecules. It has been shown that this carbohydrate can induce airway inflammation in animal models [24]. When coadministered with ovalbumin, it behaves as an adjuvant, boosting IgE production [25]. Chitin is sensed by different innate immune receptors in mammals, including TLR-2 and mannose receptors found in dendritic cells and macrophages [22]. Thus, it may modulate antigen presentation of allergenic molecules. In this study, we evaluate the chitin-binding properties of two Blo t 12 recombinant isoforms and the influence on the antibody response and development of airway inflammation in a murine model of allergen sensitization.

Material and Methods

Allergens

Blo t 12.0101 and Blo t 12.0102 isoforms were previously isolated from two different cDNA libraries of *B. tropicalis* (published elsewhere [20]). Codon-optimized gene sequences were synthesized and subcloned into pET45b+ (Genscript, Piscataway, N.J., USA). Recombinant plasmids were transformed into *Escherichia coli* (DE3) Origami cells by electroporation. Protein expression was induced by adding IPTG to a mid-log phase culture at 0.1 mM final concentration. His-tagged allergens were purified from the *E. coli* lysate under native conditions by affinity chromatography using Ni-NTA resin (Qiagen, Hilden, Germany) for protein capture and 250 mM imidazole, 50 mM NaH₂PO₄, 300 mM NaCl (pH 8.0) for elution.

Mite bodies and feces from *B. tropicalis* were supplied by Immunotek (Madrid, Spain). Lyophilized mites or feces were resuspended 1:20 in PBS (pH 7.4) and incubated overnight at 4°C with continuous stirring. After centrifugation for 30 min at 6,000 r.p.m., the supernatant was filtered through 0.8-, 0.45- and 0.2-μm filters. Extracts were dialyzed against water in a 3.4-kDa pore dialysis tube and then lyophilized and stored. Protein concentration was determined by the Bradford method.

Sequence Analyses

Allergen sequences (Blo t 12 isoallergens, Der p 15 and Der p 23) were compared with domains deposited at the conserved domain database (CDD) at the National Center for Biotechnology Information (NCBI) and the Pfam database at the Sanger Institute by using the CD-Search tool at the NCBI [26]. Identity/similarity percentages between sequences were calculated by BLAST (bl2seq). Multiple alignments were done in Bioedit v7.0 (Carlsbad, Calif., USA).

Chitin Binding Assays

Affinity assays were performed to evaluate the binding capacity of Blo t 12 isoforms to chitin [27]. A positive control (New England Biolabs, Ipswich, Mass., USA) and the isoallergens were incubated with 100 μl of chitin beads (New England Biolabs) or 10 mg of powdered chitin from crab shells (Sigma, St. Louis, Mo., USA) diluted in Tris 20 mM pH 8.0, NaCl 150 mM (Buffer A) in a final volume reaction of 400 μl for 60 min, at room temperature (RT) and with orbital shaking. After centrifugation (at 15,000 g for 5 min) supernatants were harvested and the chitin pellets were washed 5 times with Buffer A. Proteins were eluted by incubating the chitin pellet with Laemmli buffer plus 5% β-mercaptoethanol at 95°C for 5 min. Supernatants were lyophilized and reconstituted in the original volume. The contents of the pellets and supernatants were visualized in a 15% SDS-PAGE. Since chitin is an insoluble material, protein binding is confirmed when it is recovered from the pellet.

To compare the affinity of the isoforms to chitin, they were incubated in different amounts (20, 10, 5, 2.5 and 1.25 μg) with the carbohydrate. To increase sensitivity for detection of the allergens at the lowest concentrations, the protein content remaining in the supernatants was measured by dot-blot analysis and compared with the expected content before incubation. Briefly, proteins were spotted on a nitrocellulose membrane, blocked with PBS 5% defatted milk, incubated with anti-Blo t 12 rabbit polyclonal antibody and then with horseradish-peroxidase goat anti-rabbit IgG. A che-

miluminescence reaction was developed by covering the membrane with SuperSignal West Femto Maximum Sensitivity Substrate™ (Thermo Scientific, Rockford, Ill., USA). Images were obtained with a chemiluminescence image detector (G:BOX, Syngene, UK).

Detection of Blo t 12 in B. tropicalis Extracts

Anti-Blo t 12 pAb were produced by Vivotecnica (Madrid, Spain). Two New Zealand rabbits were immunized three times with recombinant Blo t 12.0101 over 2 weeks. The final bleeding from each rabbit was used for allergen immunodetection in *B. tropicalis* extracts by ELISA technique. Briefly, wells were coated with 3 µg of allergen extracts (prepared from mite feces or mite bodies), blocked with PBS 3% BSA and incubated for 1 h with purified IgG from rabbit polyclonal antibody serum before (preimmune serum) and after immunization at different dilutions. Alkaline phosphatase-conjugated goat anti-rabbit IgG (Sigma) was added to each well in a 1:2,000 dilution and incubated for 1 h. Wells were washed with PBS 0.05% Tween 20 after each incubation. Colorimetric reaction was developed with p-nitrophenyl-phosphate. Absorbance was read at 405 nm in a MultiScan EK spectrophotometer (Thermo Fisher Scientific Inc.).

Reagents for Animal Models

Preparation of Chitin Particles

The proallergenic properties of chitin have been described for intermediate size fragments (40–70 µm). In our study, this chitin fraction was obtained as described previously [23]. Chitin powder, derived from crab shells (Sigma), was treated with 0.1 M of NaOH for 30 min at RT to remove endotoxins [28]. Chitin was precipitated by centrifugation and washed twice with sterile PBS. The PBS-suspended chitin (10 mg/ml) was sonicated at 30% output for 5 min and then filtered with nylon cell strainers (BioRad, Hercules, Calif., USA) of different pore diameters (100, 70 and 40 µm). The 40–70 µm fraction was collected for experiments.

Preparation of Allergens

For endotoxin depletion, allergens were treated with Triton X-114 at 1% final concentration. Samples were incubated at 4°C for 40 min on a rotor, then for 10 min at 37°C and centrifuged at 20,000 g for 20 min. After that, two liquid phases were visible and the upper one was recovered and treated again with the detergent under the same conditions for a total of three cycles [29, 30]. LPS removal was confirmed by a quantitative and modified Limulus assay (Bio Whittaker, Walkersville, Md., USA), following the manufacturer's instructions. Endotoxin levels were on average 0.35 pg of LPS/µg of protein.

Sensitization and Challenge of BALB/c Mice

Six-week-old female BALB/c mice were obtained from the National Institute of Health (Bogota, Colombia). Housing and care were under standard conditions in the animal facilities at the University of Cartagena with a 12:12-hour dark-light cycle. Bedding and food were autoclaved; all procedures involving animal manipulation were performed inside a laminar flow hood by gloved, gowned and masked personal. Experiments were approved by the local ethical committee of the University of Cartagena. Each experimental group included 5–6 mice.

Mice were sensitized via i.p. injections (days 0, 7 and 14) with 20 µg of Blo t 12.0101 or Blo t 12.0102 alone, with chitin (25 µg) or

adsorbed with alum (2 mg). Seven days after the last immunization, mice were anesthetized with ketamine/xylazine and challenged intranasally over 3 consecutive days with 10 µg of the respective sensitizer isoform. An additional group was immunized with *B. tropicalis* whole extract plus alum as a positive control of the pattern of airway inflammation. Control mice received PBS during sensitization and challenge.

Measurement of Airway Hyperreactivity

After the last nasal challenge, lung function was assessed by methacholine-induced airflow obstruction, using a whole-body plethysmograph (Buxco Electronics, Troy, N.Y., USA). Methacholine was diluted in water at different concentrations (50, 25, 12.5, 6.13 and 3.1 mg/ml) and administered in aerosol (50 µl per chamber for 90 s). Bronchial hyperreactivity was evaluated in conscious animals by means of enhanced pause (Penh) values [31].

Histology

Euthanasia of animals was carried out by using a lethal dose of anesthesia. The lungs were extracted and immersed in 10% neutral-buffered formalin. For processing, lung tissues were first embedded in paraffin, and then cut into sections 4-µm-thick and stained with HE or PAS. Slides were visualized by light microscopy to evaluate lung inflammation and mucus production.

To determine the severity of inflammatory cell infiltration, peribronchial and perivascular inflammatory cell counts were performed blind, based on a modification of the 5-point scoring system described by Myou et al. [32], graduating peribronchial and perivascular inflammation independently according to the number of inflammatory cells: 0 = normal, 1 = few cells, 2 = a ring of inflammatory cells 1–2 cells deep, 3 = a ring of inflammatory cells 3–5 cells deep and 4 = a ring of inflammatory cells >5 cells deep. The total lung inflammation was defined as the sum of peribronchial and perivascular inflammation scores. The extent of mucus production was determined by blind quantification of the abundance of PAS-positive goblet cells in each airway (identified by a magenta-colored stain), using the 5-point grading system described by Tanaka et al. [33], obtaining numerical scores: 0 = 0–5% PAS-positive cells, 1 = 5–25%, 2 = 25–50%, 3 = 50–75% and 4 = 75%. Both scores, for inflammation and mucus production, were calculated in an average of six fields in their respective lung tissue sections. Images were captured at ×400 and ×100 final magnification on an Eclipse 400 microscope connected to a DS-Fi1 camera (Nikon, Japan). Images were processed with NIS-Elements-3.01 software (Nikon).

Antibody Determinations

Total IgE and allergen-specific IgE, IgG1 and IgG2a levels were determined by ELISA. Total IgE was measured using a commercial kit following manufacturer's instructions (BD OptEIA™, BioRad). For specific immunoglobulin determinations, Maxisorp™ microtiter plates were coated with the respective allergens at 5 µg/ml by overnight incubation at 4°C. Wells were then blocked with PBS 1% BSA 0.05% Tween 20 for 3 h at RT. Plasma samples were incubated overnight at 4°C. After 5 washes, wells were incubated with biotin labeled anti-mouse IgE, IgG1 or IgG2a (diluted 1:1,000 in blocking buffer) for 1 h at RT. After 5 washes, alkaline-phosphatase streptavidin (diluted 1:2,000 in 50 mM Tris, 1% BSA and 1 mM MgCl₂, pH 8.0) was

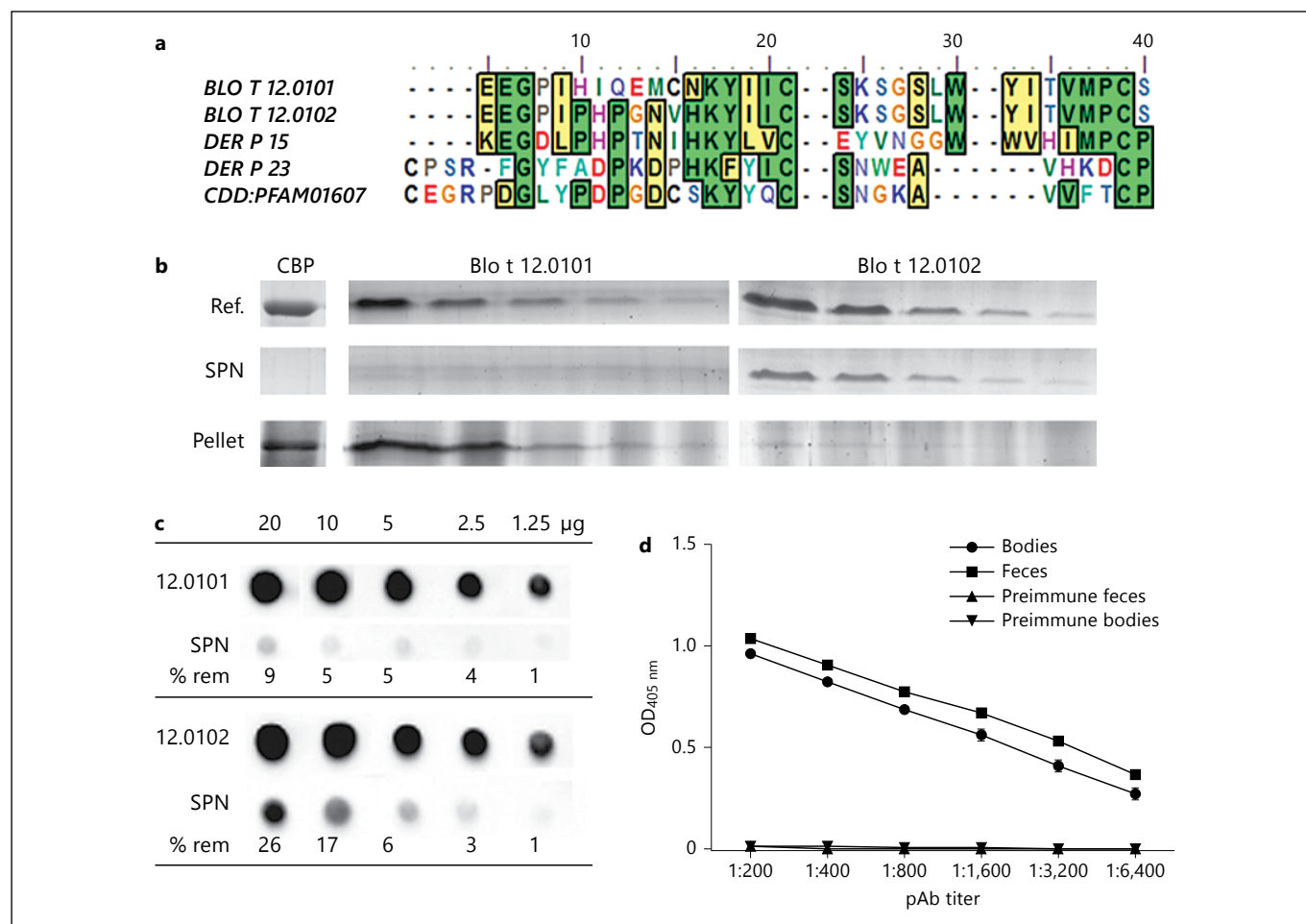


Fig. 1. Blo t 12 isoallergens exhibit chitin-binding activity. **a** Sequence alignment of CBDs from Blo t 12 isoforms, other chitin-binding allergens and CBM14 (PFAM01607). Identical and similar residues are shadowed. **b** SDS-PAGE showing the results of chitin-binding assays with Blo t 12 isoallergens at different concentrations and a positive control (CBP). Contents of supernatants (SPN) and pellets are shown. The amount of protein used for each condi-

tion is shown above and labeled as 'Ref'. **c** The relative amount of protein remaining in the supernatants (% rem) was measured by dot-blot analysis. **d** Immunodetection of Blo t 12 with an allergen-specific rabbit polyclonal antibody (pAb) or the corresponding preimmune serum in *B. tropicalis* extracts prepared from feces or mite bodies. OD = Optical density.

added to the wells and incubated for an additional hour. Paranytrophenil diphosphate, dissolved in 10% diethanolamine 0.05 mM MgCl₂ at 1 mg/ml, was used as substrate solution. Optical densities were obtained by reading the plates at 405 nm in a spectrophotometer. To increase the sensitivity of IgE ELISA, avoiding the competitive effect of IgG for antigen binding, this immunoglobulin was depleted by incubation with protein G sepharose [34].

Statistics

Statistical analysis was done with IBM SPSS v.20 (IBM Corp., USA). Results were expressed as means and their SEM. The Student t test and one-way ANOVA, including a Tukey post hoc analysis were used for comparisons among groups. $p \leq 0.05$ was considered significant.

Results

Blo t 12 Isoforms Are Chitin-Binding Allergens

Both Blo t 12 isoallergens contain a single copy of a CBD (CBM14, PFAM01607) in their C-terminal region, frequently found on proteins that compose the peritrophic matrix of insects and animal chitinases [35–38]. Sequence similarity between CBM14 and the CBD of each isoallergen was 44%. Homology to this domain has been predicted for other HDM allergen sequences, such as Der p 23 and Der p 15. In spite of there being no global sequence homology among Blo t 12 isoallergens and these mite allergens, conservation of key residues for ligand interaction were detected (fig. 1a).

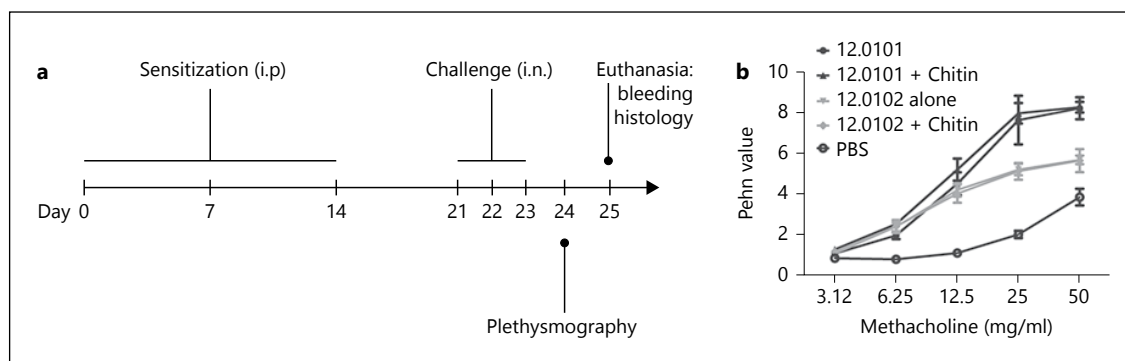


Fig. 2. Airway responsiveness to methacholine in the Blo t 12 sensitization model. **a** Scheme of the experimental protocol used in the animal model of asthma. **b** Methacholine-induced bronchial hyperreactivity was measured at day 24 by whole-body plethysmography. Mean and SEM for each point are shown.

The chitin-binding activity of the isoallergens was tested experimentally with two different chitin preparations. Both isoforms showed lower binding to the chitin beads than to the powdered chitin isolated from crab shells (data not shown); therefore, dose-response experiments were performed with the latter, which was also used in the animal model of allergen sensitization. Although both isoforms bound chitin; compared to Blo t 12.0101, the variant Blo t 12.0102 was recovered from the pellet in smaller quantities (fig. 1b). The protein content remaining in the supernatants was significantly greater for Blo t 12.0102 (fig. 1c).

Blo t 12 was detected in the feces of *B. tropicalis* with stronger recognition by the specific polyclonal antibody than in a preparation enriched with mite bodies, as could be expected due to its phylogenetic relationships with peritrophins (fig. 1d).

Effects of Chitin on Blo t 12 Sensitization *in vivo*

The effect of chitin binding on airway inflammation and sensitization was assessed in a murine model of asthma with the two Blo t 12 isoallergens (fig. 2a). By whole-body plethysmography, a more intense methacholine-induced airflow obstruction, as indicated by higher Pehn values, was observed in mice receiving Blo t 12.0101 (alone or with chitin) than in those receiving Blo t 12.0102 (fig. 2b). Chitin did not have an important effect on bronchial hyperreactivity; at the 12.5-mg/ml dose, the group receiving the variant 12.0101 plus chitin tended to have higher Pehn values, but the difference was not significant ($p = 0.10$).

Lung tissue sections from mice that were sensitized with any of the Blo t 12 isoforms (alone, plus chitin or alum) showed a rich tissue-inflammatory response around the peribronchial and perivascular zones, medi-

ated by lymphocytes, neutrophils and eosinophils (to a minor degree); a patchy interstitial infiltrate was also observed (fig. 3). This inflammatory pattern was associated with marked goblet cell hyperplasia and mucus hypersecretion within the bronchi and airway occlusion by mucus plugs, as demonstrated by PAS staining (fig. 4). A similar pattern of inflammation and mucus production was observed in mice sensitized with alum-adsorbed complete *B. tropicalis* extract. No signs of tissue inflammation or mucus hyperproduction were found in the groups receiving chitin alone or PBS (fig. 3, 4).

Effects of Chitin on Humoral Responses to Blo t 12 Isoforms

Animals sensitized with any of the isoallergens showed higher levels of total IgE and allergen-specific IgG1 and IgG2a than those receiving PBS or chitin alone (fig. 5). As observed in figure 4a, mice receiving Blo t 12.0101 plus chitin showed greater total IgE values than those sensitized with the isoallergen alone ($p = 0.03$) or with Blo t 12.0102 alone ($p = 0.02$) or with chitin ($p = 0.03$). Chitin or alum coadministration did not show any adjuvant effect on total IgE production in Blo t 12.0102-sensitized animals. Administration of chitin alone did not increase total IgE production ($840 \text{ ng/ml} \pm \text{SD: } 722.04$), obtained levels were similar to the control group receiving PBS (mean difference: 442.92 , $p = 1.0$). Allergen-specific IgE values were not significantly different among immunized groups, except in those receiving Blo t 12.0102 plus alum, which showed higher levels (fig. 5b).

Administration of chitin did not increase allergen-specific IgG1 or IgG2 production to any of the two isoallergens. It was also found that Blo t 12.0102 induced stronger IgG1 or IgG2 allergen-specific responses than Blo t 12.0101,

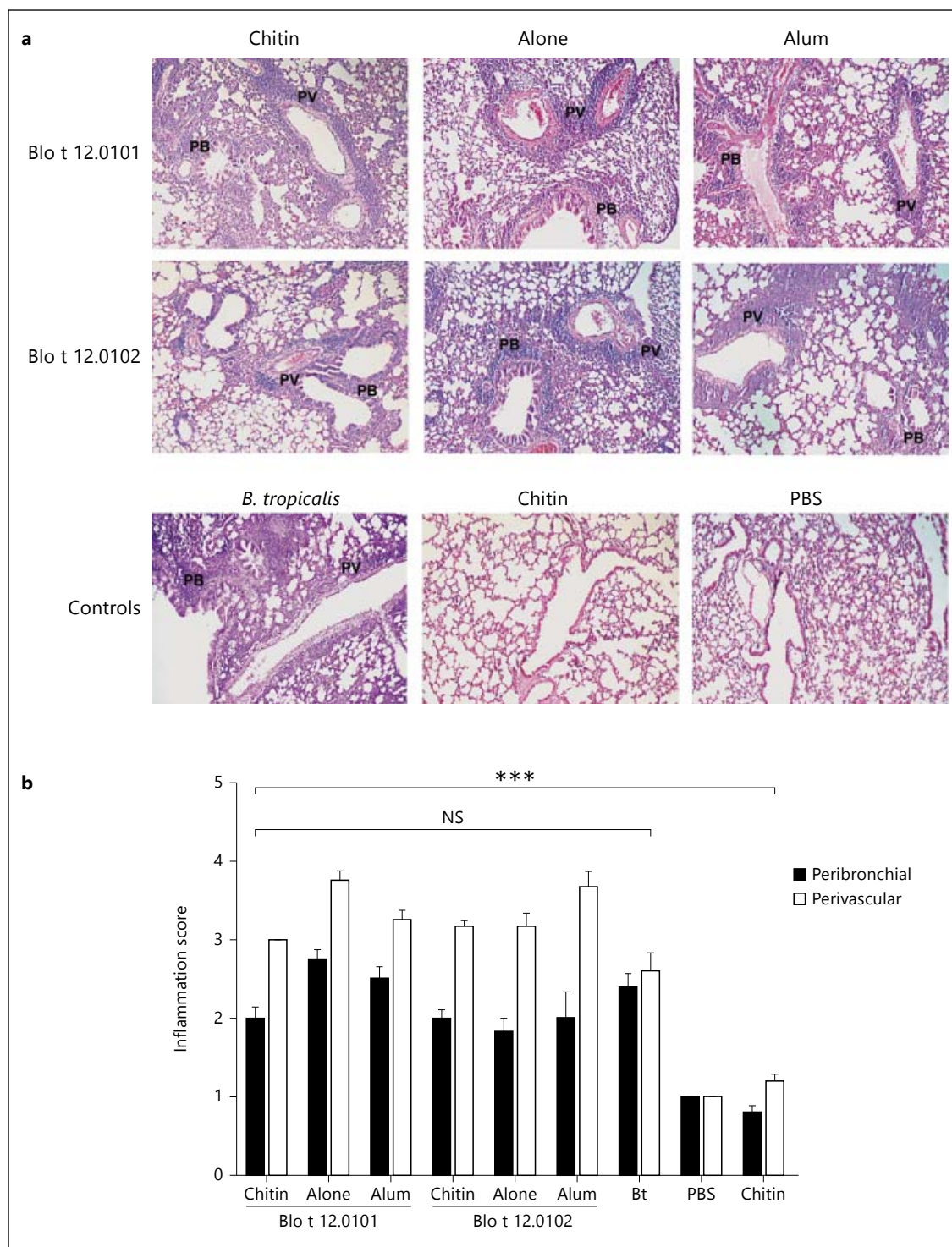


Fig. 3. In vivo effects of Blo t 12 isoforms and chitin administration in mice. **a** HE stain of lung sections from each experimental group. Areas with perivascular (PV) or peribronchial (PB) inflammation are labeled. **b** Inflammation scores obtained are shown in bars. NS = Not significant. *** $p < 0.0001$.

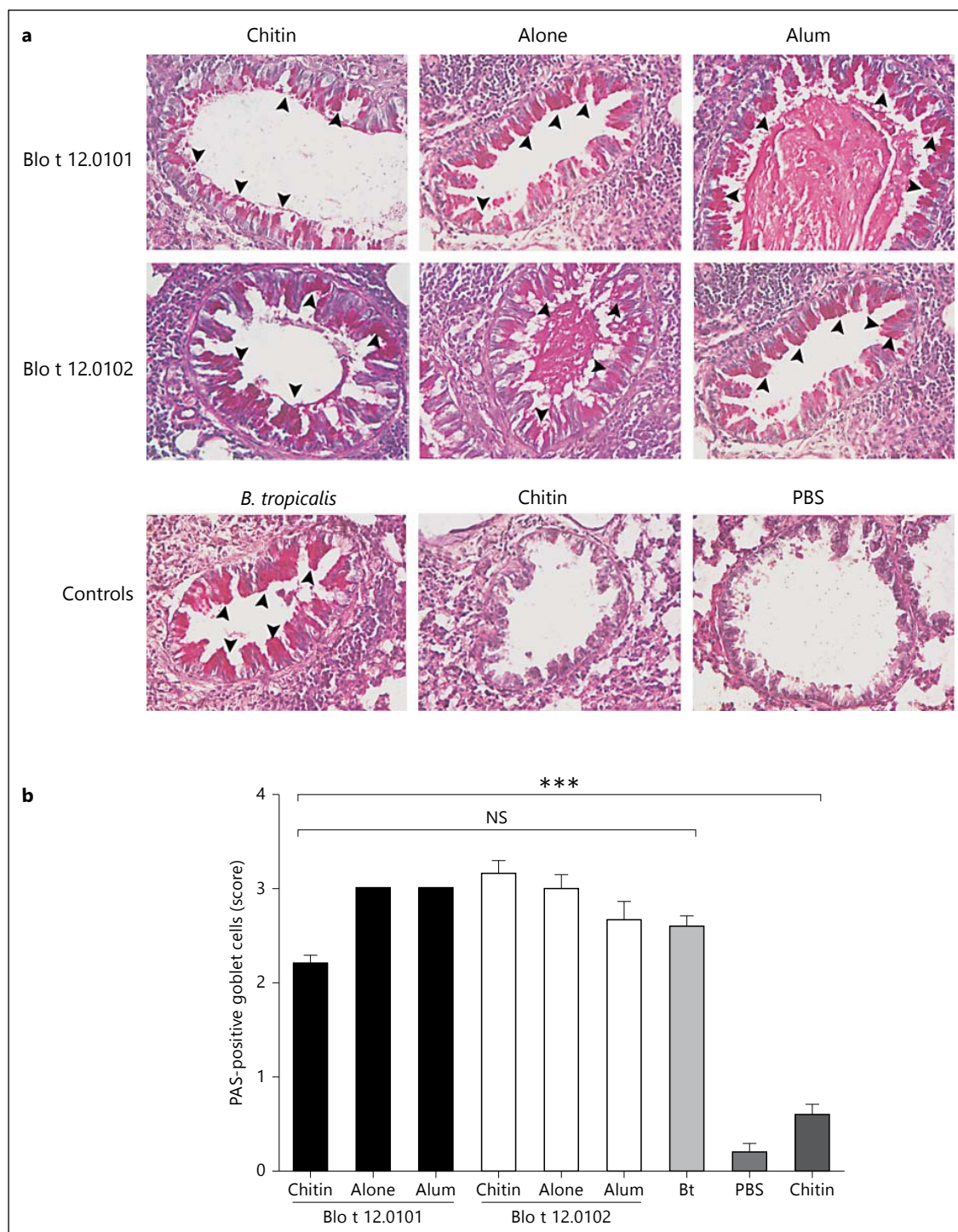


Fig. 4. Evaluation of mucus production in lung sections. **a** PAS stain of lung sections from each experimental group. PAS-positive cells are observed in magenta (indicated by arrowheads). **b** Goblet cell hyperplasia scores are shown in bars. NS = Not significant. *** $p < 0.0001$.

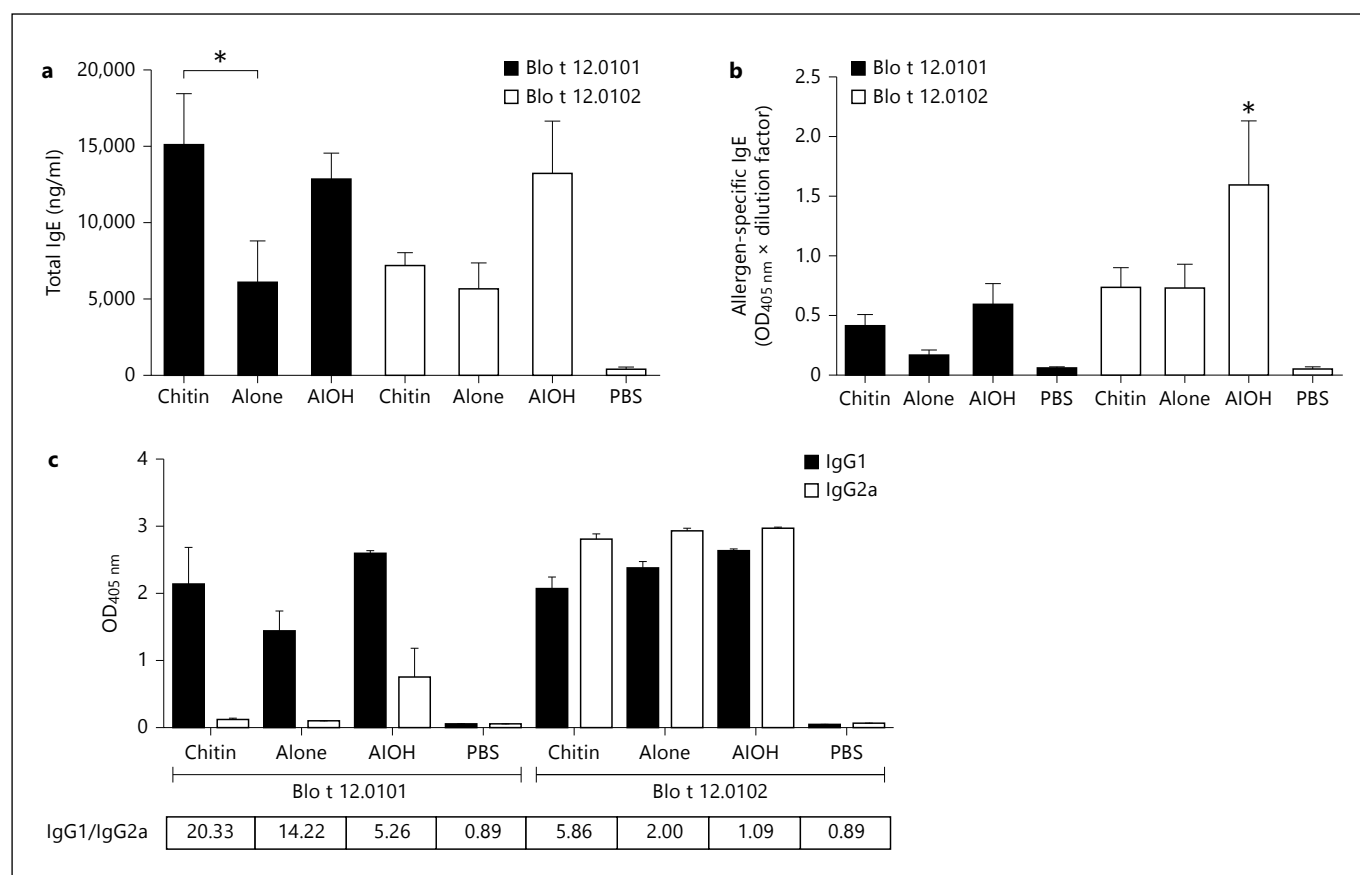


Fig. 5. Humoral responses to Blo t 12 isoallergens. **a** Total IgE levels in each experimental group. * $p < 0.05$. **b** Allergen-specific IgE values in each group. * $p < 0.05$, mice receiving Blo t 12.0102 plus alum showed significantly higher levels than the other groups.

c Allergen-specific IgG1 and IgG2a values in each group are displayed in bars. IgG1/IgG2 ratios appear inside a table below each respective bar. OD = Optical density.

but the ratio of IgG1/IgG2a was higher in Blo t 12.0101-sensitized mice receiving the allergen alone ($p < 0.001$) or with chitin ($p < 0.001$), suggesting a more Th2-polarized response in animals receiving this isoform (fig. 5c).

Discussion

Chitin binding potential is a common feature of several allergens from different sources, including arthropods and plants [39, 40], but this biological property and its importance for allergenic sensitization had never been evaluated experimentally for a HDM allergen. Here we demonstrate, experimentally, that Blo t 12 isoforms bind chitin. Interestingly, it was found that these isoallergens have an intrinsic potential to induce airway inflammation and bronchial hyperreactivity, independently of the presence of this carbohydrate or another adjuvant. However,

chitin coadministration was associated with greater total IgE production in animals sensitized with Blo t 12.0101, the variant with the highest affinity to this carbohydrate.

Although Blo t 12 isoforms are phylogenetically distant from other chitin-binding proteins [41], they conserve key residues for ligand interaction, as we have experimentally confirmed. Since most amino acid variations between the isoforms are located inside the CBD, it is not surprising to find differences in binding capacity. In fact, modeling of their CBD shows differences in their tertiary structure, close to the predicted interaction site with chitin [20]. It has been described that cysteines are relevant for domain folding; one of these amino acids is replaced by a valine in Blo t 12.0102. In addition, other replacements that seem to be relevant in the differential binding activity are two proline residues, only present in the second isoform, which are known to influence loop formation. Other HDM allergens such as the peritrophin

Der p 23 [37] and the chitinase Der p 15 [42, 43] also contain a CBD, but their sequence similarity with Blo t 12 was rather low. This is in agreement with the lack of reactive components to anti-Blo t 12-specific polyclonal and monoclonal antibodies in *D. pteronyssinus* extracts (Zak-zuk et al., unpubl. data). Like the other important mite allergens (Der p 1 [16], Der p 2 [44], Blo t 5, Blo t 21 [45] and Der p 23 [37]), Blo t 12 is present in mite feces; this facilitates exposure to the allergen and may explain its frequent IgE recognition by atopic individuals in several tropical populations [20].

We investigated if intermediate chitin fragments had proallergenic adjuvant effects in the context of the coadministration of Blo t 12 with chitin-binding allergens. In our model, this carbohydrate did not strongly potentiate the immune responses to any of the two isoforms; although it did induce significantly higher total IgE levels in those mice receiving Blo t 12.0101, the isoform with higher ligand affinity, supporting Th2 promoting effects. The presence of the allergen was necessary for increasing total IgE since chitin alone did not boost its production. Interestingly, no increase was observed with the isoform 12.0102, probably because it induced a more Th1-polarized response (as suggested by a lower IgG1/IgG2a ratio). Total and allergen-specific IgE production are supposed to involve common and particular determinants and pathways [46, 47]; further studies are needed to elucidate the mechanisms underlying the effect of Blo t 12.0101-chitin on total IgE. Since chitin administration did not modify allergen-specific IgE production, it cannot be regarded as an adjuvant of allergenicity.

Our findings differed from those reported by da Silva et al. [25] in an ovalbumin airway hypersensitivity model where chitin coadministration was necessary to induce airway allergic inflammation and sensitization. Since Blo t 12 isoforms without any adjuvant elicited strong humoral responses, airway hyperreactivity and inflammation, it seems that the marked proallergenic effects of chitin described previously [24, 25] were masked by the intrinsic inflammatory potential of this allergen group, which is not observed in immunologically 'inert' molecules such as ovalbumin when administered alone [48]. Intrinsic capacities to induce allergic airway inflammation or Th2 activation have been described for a few allergens and have been related to their biochemical properties [15, 49, 50]. Although the immunogenic/proinflammatory potential of Blo t 12 was independent of its chitin-binding properties, the presence of a CBD in its structure may be involved in these effects. The relationship of chitin recognition and degradation in mammals with the develop-

ment of allergic responses has been extensively explored [51]; members of the 18-glycosyl hydrolase family such as the acid mammalian chitinase (AMCase) and some chitolectins, i.e. YKL40 and BPR-30, which also contain CBM14 domains, have been linked to asthma in humans [52, 53] and allergic airway inflammation in animal models [53, 54]. Some biological effects of AMCase associated with its CBD have even been observed in a chitin-free model [55], and similar results have been described for chitolectins [54]. Therefore, an endogenous ligand mediating the Th2-promoting properties of these molecules have been proposed [54, 56]. We hypothesize that, in an analogous manner, Blo t 12 may directly activate inflammatory routes through its CBD, mimicking host-intrinsic signaling factors, as reported for other allergens such as Der p 2 [57]. Furthermore, variations in the Th2-polarizing potential of the two isoallergens, as suggested by a higher IgG1/IgG2a ratio and stronger methacholine-induced bronchoconstriction in Blo t 12.0101-sensitized animals, may be due to the predicted variations in the architecture of their CBD. Interestingly, more intense skin test reactivity to Blo t 12.0101 than to Blo t 12.0102 has been found in asthmatic subjects living in the tropics [20].

Administration of Blo t 12 isoforms induced a strong inflammatory pulmonary infiltration, rich in neutrophils and lymphocytes. Interestingly, in agreement with other reports, this was also observed when mice were exposed to the whole *B. tropicalis* extract [58, 59]. Although there were important differences in methacholine response, a similar level and pattern of inflammation was found among the groups receiving any of the two isoforms. In this sense, it is possible that the functional variations are related to a differential cellular activation (i.e. IL-13 production) or to the participation of cell subpopulations not identified by our histology analysis.

In summary, we have described some biological properties of the allergen Blo t 12. We confirmed experimentally that Blo t 12 isoallergens are chitin-binding proteins, excreted in mite feces. In addition, both isoforms induce a strong inflammatory reaction and airway hyperreactivity, especially Blo t 12.0101, without the need of an adjuvant. The biological activity of binding chitin seems to have a modest effect on the immune responses to this allergen, increasing total IgE production.

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