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ORIGINAL ARTICLE

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A hybrid of two major *Blomia tropicalis* allergens as an allergy vaccine candidate

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Abstract

Introduction: Allergen-specific immunotherapy (AIT) represents a curative approach for treating allergies. In the tropical and subtropical regions of the world, *Blomia tropicalis* (Blo t 5 and Blo t 21) is the likely dominant source of indoor allergens.

Aim: To generate a hypoallergenic Blo t 5/Blo t 21 hybrid molecule that can treat allergies caused by *B tropicalis*.

Methods: Using in silico design of *B tropicalis* hybrid proteins, we chose two hybrid proteins for heterologous expression. Wild-type Blo t 5/Blo t 21 hybrid molecule and a hypoallergenic version, termed BTH1 and BTH2, respectively, were purified by ion exchange and size exclusion chromatography and characterized by physicochemical, as well as in vitro and in vivo immunological, experiments.

Results: BTH1, BTH2 and the parental allergens were purified to homogeneity and characterized in detail. BTH2 displayed the lowest IgE reactivity that induced basophil degranulation using sera from allergic rhinitis and asthmatic patients. BTH2 essentially presented the same endolysosomal degradation pattern as the shortened rBlo t 5 and showed a higher resistance towards degradation than the full-length Blo t 5. In vivo immunization of mice with BTH2 led to the production of IgG antibodies that competed with human IgE for allergen binding. Stimulation of splenocytes from BTH2-immunized mice produced higher levels of IL-10 and decreased secretion of IL-4 and IL-5. In addition, BTH2 stimulated T-cell proliferation in PBMCs isolated from allergic patients, with secretion of higher levels of IL-10 and lower levels of IL-5 and IL-13, when compared to parental allergens.

Conclusions and Clinical Relevance: BTH2 is a promising hybrid vaccine candidate for immunotherapy of *Blomia* allergy. However, further pre-clinical studies addressing its efficacy and safety are needed.

KEYWORDS

allergen-specific immunotherapy, allergenicity, house dust mite, hypoallergen, immunogenicity, vaccine candidate

1 | INTRODUCTION

As an important chronic disease, allergies have an increasing prevalence in both developed and low-/middle-income countries, particularly in large cities of Latin America.[1-5] So far, the only therapeutic approach that can produce long-term suppression of allergy symptoms is allergen-specific immunotherapy (AIT). Emerging over a century ago,[6] fundamental goal of AIT is inducing immune tolerance by stimulating the immune system with gradually increasing doses of an allergen extract.[6,7] However, the use of crude allergen extracts in AIT raises major concerns regarding safety and efficacy of treatment.[8,9] Currently, the possibility of using recombinant proteins as an alternative to crude allergen extracts can significantly impact current AIT protocols. Thus, an assortment of recombinant allergens has already been produced for AIT from different allergenic sources, such as pollen, animal dander, mites and various foods.[10-14]

In recent technological assessments published by our group, we discussed the potential of AIT vaccines based on recombinant allergens, in particular using hypoallergenic derivatives.[15,16] Clinical trials are on-going with some hypoallergenic proteins, especially for the treatment and prophylaxis of allergic asthma and rhinitis caused by pollen, which is the main source of allergens in Europe.[9,17] In Latin America, house dust mites are the most important source of indoor allergens.[18,19] In fact, our group has shown that *Blomia tropicalis* (*B tropicalis*) was the most frequent mite in residences in the city of Salvador, northeast Brazil.[20] These data highlight the significant need for permanent research on *B tropicalis* allergens in our region, as well as developing novel and more efficacious treatment strategies, such as AIT using hypoallergens.

Among the 14 *B tropicalis* IgE-binding proteins acknowledged by the International Union of Immunological Societies (IUIS) and another six recently identified by our group,[21] Blo t 5 and Blo t 21 are considered major allergens.[22,23] Despite sharing an extremely similar three-dimensional (3D) structure, these allergens do not cross-react, although co-sensitization is a common feature.[24-28] Previously, our group found a reactivity higher than 80.0% to recombinant versions of Blo t 5 and Blo t 21 in sera of children allergic to *B tropicalis*.[28] Considering the structural similarity of these two allergens, co-sensitization in our region and the high presence of IgE towards these allergens in other populations,[24,29] the present study aimed to generate a hypoallergenic Blo t 5/Blo t 21 hybrid molecule that can potentially treat allergic reactions caused by the house dust mite *B tropicalis*.

2 | METHODS

2.1 | In silico design of *Blomia tropicalis* hybrid proteins

Fragments of Blo t 5 and Blo t 21 were combined in silico, preserving the previously reported Blo t 5-immunogenic regions.[30,31] Also, the IEDB analysis resource Consensus tool was used to predict MHC-II binding on Blo t 21, using the PDB entry 2LM9.[27,32-34] The N-terminal flexible motif of Blo t 5 was removed, since we previously showed that this removal may increase immunogenicity.[35] The software MAESTRO® was used to scan the most stabilizing n-point mutations[40,41] in the wild-type hybrid allergen (BTH1). The mutant with the best score was chosen and was termed BTH2. More details on hybrids' design are given in Appendix S1.

2.2 | Heterologous expression and purification

The recombinant versions of the native allergens (shortened Blo t 5 and Blo t 21) and the hybrid proteins were produced following established protocols. Purification was performed using steps of anion exchange chromatography and size exclusion chromatography. A full description is given in Appendix S1.

2.3 | Investigation of physicochemical characteristics of recombinant proteins

Amino acid analysis (AAA) and mass spectrometry (MS) were used to evaluate identity, quantity and primary structure of the purified proteins.[36,37] The thermal stability and secondary structure element content were verified using circular dichroism (CD) and Fourier transform infrared spectroscopy (FTIR).[38] Dynamic light scattering (DLS) was performed to assess the aggregation behaviour of the proteins in solution.[39]

2.4 | In vitro assessment of proteolytic stability of BTH1 and BTH2

Evaluation of endolysosomal proteolysis was performed in vitro, as minutely described in Appendix S1.

2.5 | Donors and sera

In the present study, non-asthmatic and asthmatic individuals enrolled in the Bahia State Program for the Control of Asthma and Allergic Rhinitis (ProAR)[40] were classified into atopic and non-atopic. This classification was made considering (a) positive skin prick test (SPT) results using *B tropicalis* extract, (b) presence of specific IgE (sIgE) to *B tropicalis* (Phadia Diagnostics AB, Uppsala, Sweden) and (c) a positive clinical history. Table S1 details the reaction profile of the donors/patients included in the study. Other details, such as clinical phenotypes, are described in Appendix S1. All sera donors and ProAR patients signed an informed consent, as indicated and approved by the Ethics Committee on Research of the Faculty of Medicine of the Federal University of Bahia (CAAE 45376814.0.0000.5577).

2.6 | IgE binding and IgE avidity index evaluations

Appendix S1 delineates in detail the ELISA experiments that were performed to determine the avidity indexes (AI) and IgE reactivity.

2.7 | Mediator release assay

The allergenic activity of the hybrid allergens was assessed by mediator release assays, as detailed in Appendix S1.

2.8 | Culture of peripheral blood mononuclear cells

Secreted cytokines and T-cell reactivity profiles induced by the hybrid proteins were evaluated in peripheral blood mononuclear cells (PBMC), as described in Appendix S1.

2.9 | In vivo experiments

BALB/c mice were immunized with 10 μg of BTH2 for 42 days. Spleens were collected, and splenocytes were restimulated with 40 μg of parental allergens and hybrid proteins during 48 hours. Cytokine production was analysed by OptEIA™ Set ELISA (BD Biosciences), and antibody (IgG1, IgG2a and IgE) responses, avidity indexes of these antibodies and production of blocking antibodies were detected by indirect ELISA. A detailed description of all mice related experiments is given in Appendix S1. All animal 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).

3 | RESULTS

3.1 | Construction of Blo t 5 and Blo t 21 hybrid molecules

Blo t 5 and Blo t 21 structurally resemble a three-helical bundle,[25-27] though these proteins share low sequence similarities, which may lead to problematic interfaces between the rearranged helices in a hybrid molecule. So, we firstly constructed a 3D model of the hybrid protein (BTH1) built from the wild-type Blo t 5 and Blo t 21 sequences and then analysed it with the MAESTRO® software tool (Figure S1). The predicted parameters for the N-terminally truncated Blo t 5 (2MEYtrunc) were as follows: (a) average MAESTRO® score = −5.83; (b) ΔG = 8.61 kcal/mol; and (c) T_m = 72.16°C. For the hybrid BTH1, the values were the following: (a) average MAESTRO score = −5.83; (b) ΔG = 9.43 kcal/mol; and (c) T_m = 71.81°C. These values indicate that the stability of BTH1 is comparable to the Blo t 5 (N-terminally truncated). Starting with the ten-best scoring BTH1 models, we further modified the protein surface, aimed at interfering with potential IgE epitopes. Only mutations that increased the stability of the protein were included during the greedy search. The best ranking mutant sequence included the mutations D26Q, D98K, R60E and R64E and is hereafter referred as BTH2 (Figure S1).

3.2 | Expression, solubility and purification of hybrid proteins

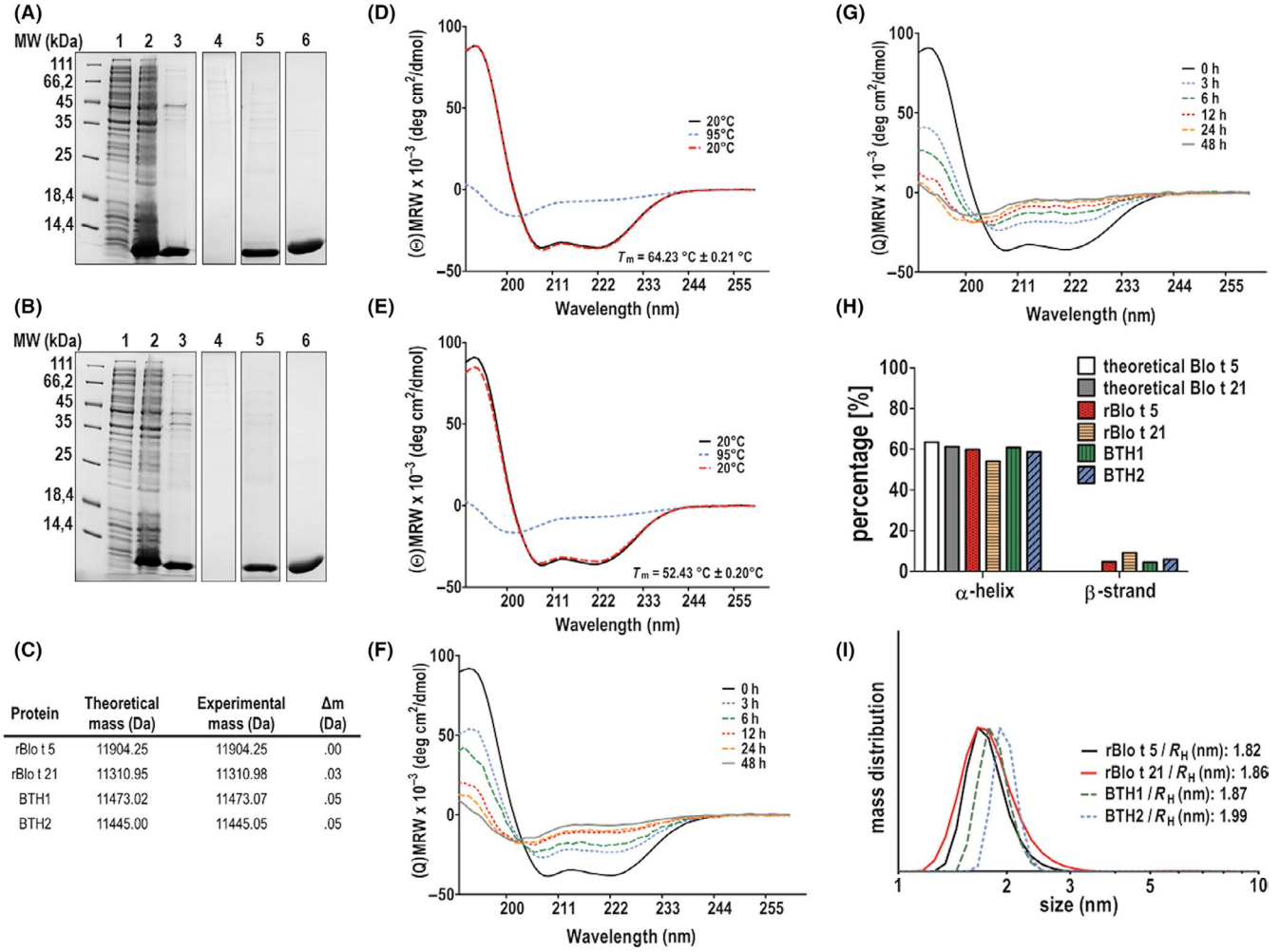
The expression profiles of the non-fusion hybrid proteins BTH1 and BTH2 are shown in Figure 1A,B. The recombinant versions of the parental allergens (rBlo t 5 and rBlo t 21) are presented in Figure S1B,C. Both hybrid allergens were obtained completely in the soluble fraction (Figure 1A,B, Lane 3) similar to the parental allergens (Figure S2A,B). BTH1 and BTH2 migrated as monomeric proteins, with molecular weights of 11 kDa. Chromatographic purification results for the hybrid allergens are also presented in Figure 1 (Lanes 5 and 6).

3.3 | Structural features of BTH1 and BTH2

Primary structures and protein identities were confirmed using MS and AAA. The intact mass measurements of BTH1 and BTH2 resulted in molecular weights of 11 473.07 Da and 11 445.05 Da, respectively, that are identical to their respective theoretical values (Figure 1C). Likewise, rBlo t 5 and rBlo t 21 presented experimentally measured masses identical to the theoretical ones (Figure 1C). AAA revealed that the experimental number of amino acid residues was in accordance with the predicted numbers for rBlo t 5, rBlo t 21, BTH1 and BTH2 (Figure S2A,B,D,E).

The CD spectra of both hybrid proteins showed two typical minima at 208 nm and 222 nm (Figure 1D,E, BTH1 and BTH2, respectively), which were in line with the results found for the wild-type allergens (Figure S1F,G). Remarkably, both hybrid allergens after denaturation at 95°C almost completely refolded at 20°C.

In addition, the thermal stability of BTH1 was increased with a T_m of 64.23 ± 0.21°C, when compared to rBlo t 5 and rBlo t 21 (T_m of 56.26 ± 0.12°C and T_m of 56.87 ± 0.36°C, respectively). However, BTH2 showed a reduced T_m of 52.43 ± 0.20°C. We performed a temporal stability analysis, whose results are shown in Figure 1F,G, S1H,I, S2C,D. Clearly, a slower decrease in folding was observed in the hybrid proteins compared to both parental allergens, when the proteins were kept at 20°C for 48 hours. FTIR measurement confirmed the correct folding, as indicated by a high content of α-helices in the hybrid proteins. This result was not significantly different from the theoretical values as well as from the wild-type allergens (Figure 1H). The hydrodynamic radius (RH) observed in DLS experiments showed that the hybrid allergens were monomeric in solution similar to wild-type allergens (Figure 1I).



Protein	Theoretical mass (Da)	Experimental mass (Da)	Δm (Da)
rBlo t 5	11904.25	11904.25	.00
rBlo t 21	11310.95	11310.98	.03
BTH1	11473.02	11473.07	.05
BTH2	11445.00	11445.05	.05

FIGURE 1 Expression, purification and physicochemical features of *Blomia tropicalis* hybrid recombinant proteins. A, Expression and purification of BTH1. B, Expression and purification of BTH2: Lane 1: before IPTG induction; Lane 2: after IPTG (0.5 mmol/L), 5 h induction; Lane 3: soluble fraction of bacterial extract; Lane 4: solubilized-bacterial pellet (6 mol/L urea); Lane 5: representative sample from anion exchange chromatography; and Lane 6: pooled fractions from size exclusion chromatography. C, Mass spectrometry was used to determine the average protein masses shown in the table. D and E, Circular dichroism spectra of BTH1 (D) and BTH2 (E) recorded at 20°C, after heating to 95°C, and cooling to 20°C. F and G, Circular dichroism spectra of BTH1 (F) and BTH2 (G) recorded at 20°C for 48 h. Six out of nineteen time measurements are shown (see all time points in Figure S2). H, Secondary structure elements of recombinant wild-type allergens and hybrid proteins. Fourier transformed infrared spectra (Figure S2) were used to calculate the secondary structure elements. The theoretical values were based on the sequence available in Uniprot, ID: O96870 and A7IZE9, Blo t 5 and Blo t 21, respectively. I, Dynamic light scattering measurements of the recombinant parental and the hybrid proteins. R_H: hydrodynamic radius

3.4 | Pattern of endosomal/lysosomal proteolysis of BTH1 and BTH2

SDS-PAGE analysis revealed that the hybrid BTH1 was not completely degraded after 48 hours. In fact, BTH1's resistance towards endolysosomal degradation was visibly higher than rBlo t 21, which was also not completely degraded after 48 hours. On the other hand, even though rBlo t 5 and BTH2 showed similar proteolytic stabilities when compared to each other, both presented lowered resistances to proteolysis compared to rBlo t 21 and BTH1 (Figure 2A). Through densitometric evaluation, we calculated half-lives of 8.7, 17.4, 39.4 and 8.5 hours for rBlo t 5, rBlo t 21, BTH1 and BTH2, respectively (Figure 2B). In addition, protein integrity analysis showed that rBlo t 5 and BTH2 started to degrade after 1 hour of endolysosomal proteolysis, whereas rBlo t 21 and BTH1 only started to significantly degrade after 3 hours of microsomes' exposure (Figure 2C).

Tandem mass spectrometry analysis showed differences in the peptides' clusters generated by proteolysis of the hybrid proteins (Figure 3 and S3) compared to recombinant wild-type allergens. The predominant peptide fragments in rBlo t 5 and rBlo t 21 are shown in Figures S4 and S5. The predominant peptide fragments of BTH1 were (a) ANHAIEKGEHQL and (b) QHQLDELNENKSKELQEKIIRE (Figure S3), which were also detected among the four most predominant peptide fragments of rBlo t 5 (Figure S4). We note that the mutant hybrid BTH2 shows peptide fragments shared with both parental allergens: (a) QHQLDELNENKSKELQEKIIRE; (b) MIEGAQGALERELK; (c) TGDLLLKDLKA; and (d) ANHAIEKGEHQL (Figure 3). Taken together, our data suggest that the N-terminus region of the proteins appears

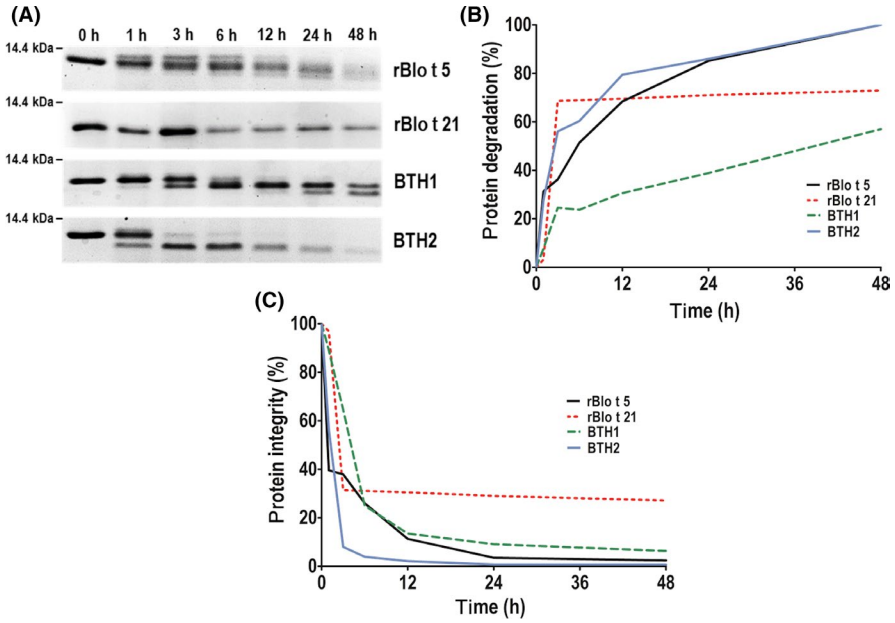


FIGURE 2 Endolysosomal proteolysis profile of *Blomia tropicalis* wild-type and hybrid recombinant proteins. A, SDS-PAGE analysis of degradome assays of wild-type and hybrid recombinant proteins. B, Line chart representation of the densitometric analysis of the SDS-PAGE results presented as percentage of degradation. C, Line chart representation of the densitometric analysis of SDS-PAGE results presented as percentage of integrity. Note that only the intact bands at the molecular weight of non-degraded protein were considered

initially cleaved, leading to the notion of a potential cathepsin cleavage site at the amino acid residues 20-23 (LYL) in the hybrid proteins.

3.5 | Binding of donors' IgE to BTH2 is significantly reduced

When we measured the IgE reactivity of double reactive (Blo t 5 and Blo t 21) sera by ELISA, we observed a statistically significant decrease in IgE binding to BTH2 compared to both the parental allergens and BTH1 in allergic rhinitis (AR) and asthmatic donors (Figure 4A,B, respectively). Single reactive sera (sera #5 and #4 reactive only either to rBlo t 5 or rBlo t 21, respectively) were included in another analysis, shown in Figure S6, although an identical pattern was observed regarding their reduced IgE binding to BTH2. Figure S6 also shows the global IgE reactivity and the cut-off line for each protein.

To verify whether the reduced IgE binding to BTH2 was due to a reduction in antibody avidity, we evaluated the IgE avidity index (AI) of some reactive sera. As shown in Figure 4C, the KSCN concentration required to elute 50% of the allergen-antibody interaction in BTH2 was significantly lower when compared to the other molecules, even in the asthmatic donors who had shown a strong reaction to BTH2. The IgE AI of donors' sera were calculated and are shown in Table S2. We then assessed basophil degranulation. BTH2 presented a reduced capacity to trigger the release of mediators from huRBL cells passively sensitized with patients' serum IgE (Figure S7). In fact, compared with the reactive wild-type allergens, the reduction in allergenicity of BTH2 was statistically significant (Figure 4D). The mean concentrations of rBlo t 5 and rBlo t 21 required to trigger the half-maximal mediator release were 0.0239 ± 0.0521 ng/mL and 0.0035 ± 0.0026 ng/mL, respectively. On the other hand, equivalent levels of huRBL stimulation by BTH1 and BTH2 were only obtained at the concentrations of 5.5229 ± 12.8309 ng/mL and 22.7637 ± 39.8759 ng/mL, respectively.

3.6 | BTH2 shows T-cell priming capacity, inducing higher levels of IL-10 and lower levels of IL-5 and IL-13 than parental allergens and mite extract

Stimulation index of BTH2-stimulated PBMC in comparison with paternal allergens and BTH1 (Figure S8) shows that the hypoallergenic hybrid preserved T-cell epitopes of both proteins. The cytokine profile showed in Figure S9 corroborates this finding and determined the optimal concentration (12.5 µg/mL) used in the final experiment with PBMC. In this case, BTH2 induced significantly higher levels of IL-10 when compared to *B tropicalis* extract (BtE) and parental allergens stimuli (Figure 5A). BTH2 reduced the production of Th2 cytokines (IL-5 and IL-13) as compared to that observed with other stimuli (Figure 5B,C). Although BTH2 induced Th1 cytokine (IFN-γ and TNF) production in comparison with unstimulated cells (Figure 5D,E), significant reduction of TNF was observed in comparison with rBlo t 21 stimuli, while for IFN-γ, there was no difference between the different stimuli (Figure 5D).

3.7 | Different response profiles among asthma phenotypes and allergic rhinitis (AR) donors

Using the Mann-Whitney test, we evaluated the differences in IgE reactivity between the group phenotypes. We found a significantly higher IgE reactivity for parental allergens in asthmatic donors compared to the AR group. In contrast, no significant differences were found in the hybrid proteins. Table S3 shows the *P* values for this analysis. Based on these results, we performed a Kruskal-Wallis test with a Dunn's post hoc test that analysed the two asthma phenotypes compared to the AR group. We confirmed that the severe asthma (SA) group showed a significantly higher IgE reactivity for the parental allergens compared to the AR group (Table S4). Interestingly, while the SA group displayed a higher IgE reaction to BTH1 ($P < .05$) compared to the AR group, we

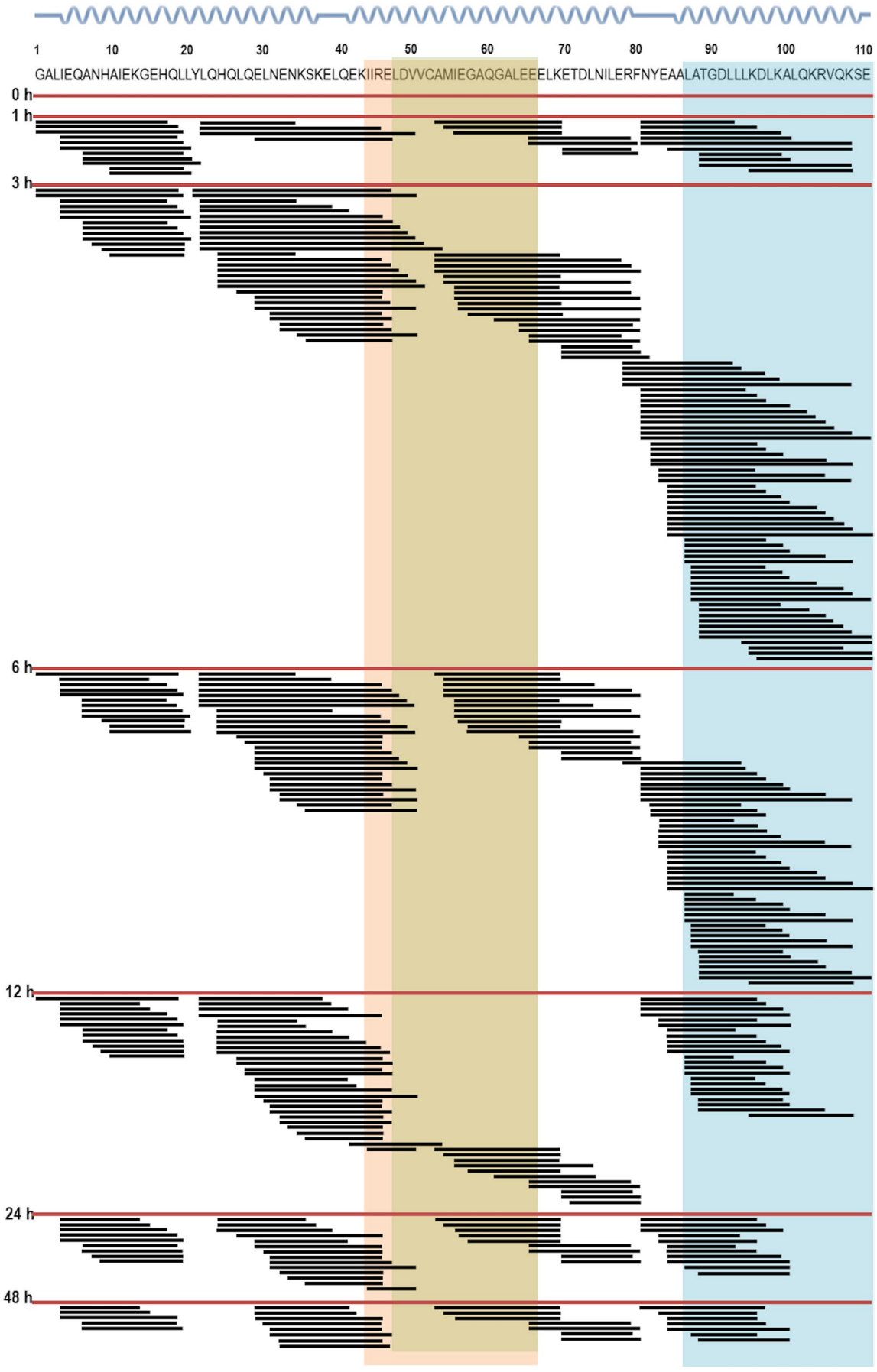

1 10 20 30 40 50 60 70 80 90 100 110
GALIEQANHAIEKGEHQLLYLQHQLQELNENKSKELQEKIIRELDVVCAMIEGAQGALEEELKETDLNILERFNYEAALATGDLLLKDLKALQKRVQKSE
0 h
1 h
3 h
6 h
12 h
24 h
48 h

FIGURE 3 Pattern of BTH2-derived peptide clusters using the degradome assay. Peptides sequenced by tandem mass spectrometry after 48 h of in vitro digestion are shown. Grey bars indicate T-cell epitopes of Blo t 5 and Blo t 21. IIRELDVVCAMIEGAQGAL was earlier considered a T-cell epitope of Blo t 5,[30,31] while T-cell epitope of Blo t 21 (LATGDLLLKDLKALQKRVQDSE) was predicted using IEDB analysis resource Consensus tool.[32-34] Positions of alpha-helical motifs are shown on the top

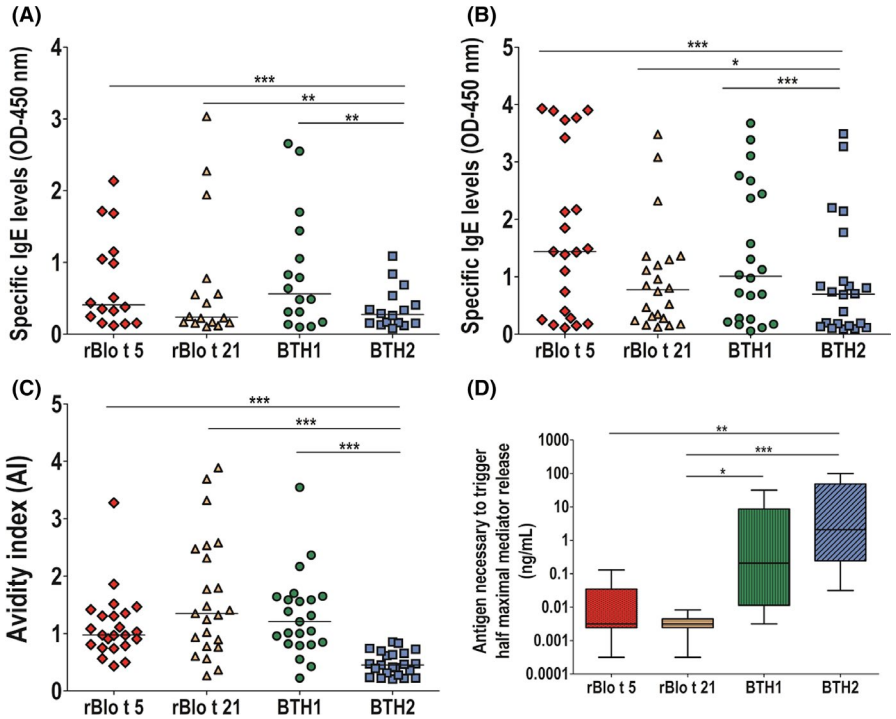


FIGURE 4 Immunological characterization of parental and hybrid recombinant allergens. A, Specific IgE reactivity determined by ELISA using double reactive sera from donors with allergic rhinitis (n = 16). Mean optical densities: rBlo t 5:0.7206 ± 0.6516; rBlo t 21:0.6957 ± 0.8981; BTH1: 0.8596 ± 0.8270; and BTH2: 0.3586 ± 0.2905. B, Specific IgE levels of double reactive sera from asthmatic donors (n = 22). Mean optical densities: rBlo t 5:1.7231 ± 1.4375; rBlo t 21:0.9695 ± 0.9293; BTH1: 1.3542 ± 1.1923; and BTH2: 0.9107 ± 1.0221. C, Concentration of KSCN required to elute 50% of allergen-antibody binding (n = 23). Wilcoxon signed-rank test was used for the comparisons. D, Half-maximal release of inflammatory mediator in huRBL cells. Six titration curves were used to calculate the 50% release (see Figure S6). After logarithmic transformation, one-way ANOVA with Bonferroni's Multiple Comparison Test was used. The median value of the group results is represented by short horizontal solid lines; **P* < .05; ***P* < .01; ****P* < .0001; ns: non-significant

did not observe this outcome for BTH2 (*P* = .2158). These results suggest that the majority of the donors in the SA group showed a similar decrease in IgE binding to BTH2 as in the AR group. We found no differences in Dunn's multiple comparison test when comparing the mild asthma (MA) group with both the AR group and the SA group. Regarding IgE avidity, only BTH1 avidity indexes presented significant differences when asthmatic donors (two merged endotypes) were compared to the AR group (Table S3) using the Mann–Whitney test.

3.8 | BTH2 induces IgG antibodies blocking IgE-allergen interaction, higher levels of IL-10, and lower levels of IL-5 and IL-4 in mice

According to the immunization schedule (Figure 6A), mice immunized with BTH2 produced rBlo t 5- and Blo t 21-specific IgE, IgG1 and IgG2a at significantly higher levels than non-immunized mice (Figure 6B,C). However, the levels of specific IgG1 (sIgG1) and specific IgG2a (IgG2a) were higher than levels of sIgE. While there was no significant increase in BTH2-sIgE, the levels of sIgG2a and sIgG1 were significantly higher than non-immunized mice (Figure 6D). Additionally, Figure 6E-G show that all sIgG antibodies had higher AI than sIgE antibodies. We verified that sIgG antibodies of BTH2-immunized mice inhibited the binding of human IgE to the parent allergens. When rBlo t 5 was coated in plate, pre-incubation with BTH2 immune sera yielded a mean 75.20% reduction of the IgE-binding capacity of human sera reactive to rBlo t 5 (Table 1). In the case of rBlo t 21, the inhibition of IgE-binding was on average 65.17% using mice immune sera (Table 2). In both cases, the reduction range was consistently higher than the ranges of non-allergic sera, which were less than 15.05%.

Splenocytes of mice immunized with BTH2 were stimulated by the adding protein antigens, after which the levels of IL-4, IL-5, IL-10 and IFN-γ in the culture supernatant were assayed. Restimulation with BTH2 could induce significant production of IL-10 compared with unstimulated cells, which yielded similar levels to the parent allergens

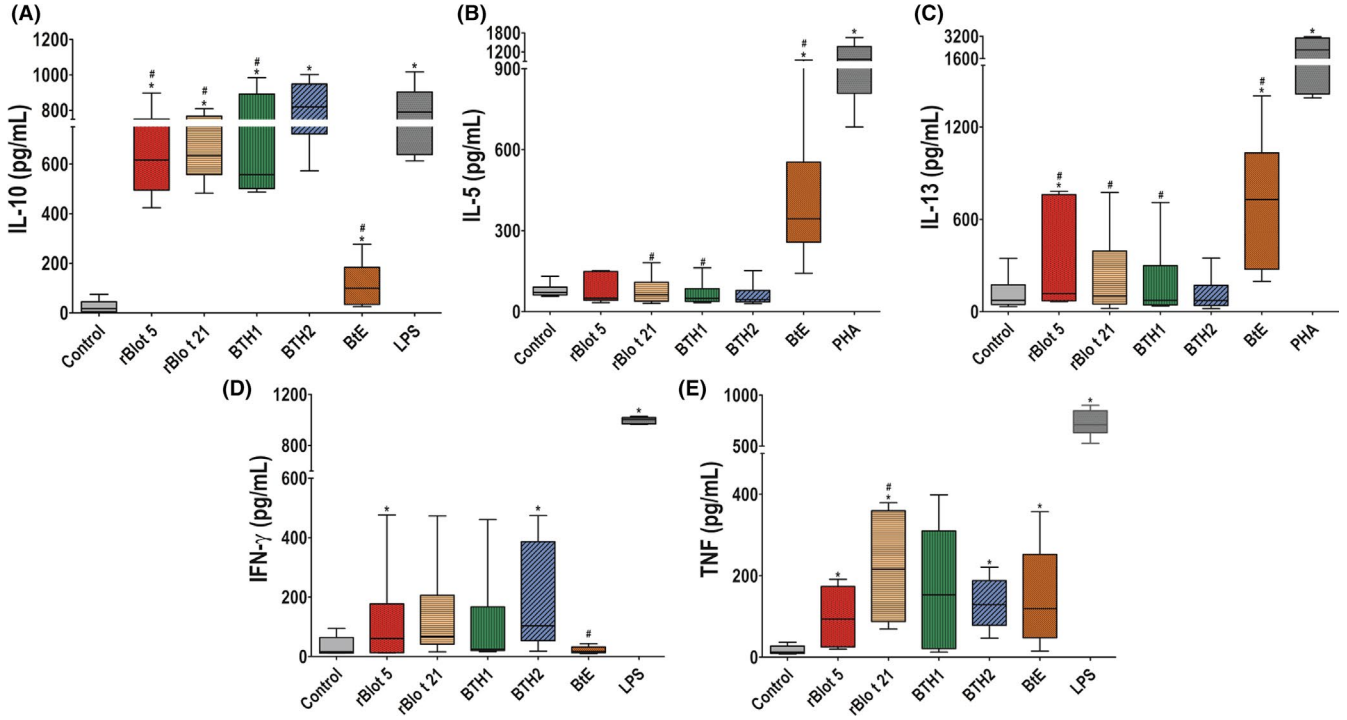


FIGURE 5 Cytokines in peripheral blood mononuclear cell supernatants. The amounts of (A) IL-10, (B) IL-5, (C) IL-13, (D) IFN-γ and (E) TNF in PBMC cultures of six *Blomia tropicalis*-atopic donors are shown in box plot. Wilcoxon signed-rank test was used for the comparisons between control (non-stimulated cells) and the other stimuli (*symbol indicates statistical differences). In addition, the same test was used to compare BTH2 and its parental allergens and BtE (# symbol indicates statistical differences). $^{*,\#}P < .05$

(Figure 7A). In contrast, IL-5 was significantly reduced by BTH2 in comparison with both non- and rBlo t 21-stimulated cells (Figure 7B). Although IL-4 was not significantly decreased upon BTH2 stimuli compared to unstimulated cells, this reduction was significant compared to BTH1-, rBlot 5- and rBlo t 21-stimulated cells (Figure 7C). There were significant differences in IFN-γ production following rBlo t 5, BTH1 and BTH2 stimuli (Figure 7D).

4 | DISCUSSION

The design of recombinant hypoallergens is an innovative approach to improve current AIT protocols with respect to safety, efficacy and convenience for the patients.[8,12,17,41] Nevertheless, rigorous preclinical and clinical studies are necessary before the approval of such engineered hypoallergens for routine use in AIT..[8,9,15-17]

Due to the increasing epidemiological prevalence of allergic diseases caused by *Blomia tropicalis* allergens in developing countries, considerably greater research efforts to develop novel vaccine candidates are urgently needed.[1,5,42]

Here, we described the successful expression and purification of soluble parental (rBlo t 5 and rBlo t 21) and hybrid (BTH1 and BTH2) recombinant allergens and their detailed physicochemical characterization. Unlike other hypoallergens,[14,41] BTH1 and BTH2 did not show drastic changes in CD spectra and secondary structure elements. All proteins displayed refolding capacity but different thermal stabilities, with increased Tm values for rBlo t 21 and BTH1. Interestingly, all investigated proteins exhibited a higher Tm and folding stability than full-length Blo t 5.[35] Previously, we showed that deletion of the N-terminal unstructured motif of rBlo t 5 increased its thermal and fold stability.[35] This effect may be due to (a) decreased dynamic at the N-terminal region; (b) the compactness of the shortened molecule; and[43,44] (c) increased conformational rigidity and modifications in the flexibility of the protein.[43-46] However, currently, we have no clear explanation of why BTH1 has a higher T_m than BTH2.

The aggregation behaviour is an important parameter to be assessed in hypoallergens for AIT, as dimerization and/or aggregation of allergens may directly affect allergenicity.[36,39] All recombinant proteins investigated here showed monomeric behaviour in solution. Thus, the hypoallergenic nature of BTH2 is not linked to aggregation. These results are in agreement with in silico analyses showing that Blo t 5 does not have the tendency to form dimers.[47] Similarly, other members of the group 21 allergens were also reported not to form dimers or aggregates.[47] Moreover, these results further support the notion that the hydrophobic contacts between valine residues and the arrangement of the N-terminal helix may contribute to dimer formation by creating a hydrophobic zipper domain, as described for Der p 5.[48] In line with these observations, both BTH1 and BTH2 hybrid proteins contain the truncated N-terminal region of Blo t 5 featuring alanine instead of valine residues in their sequences.

In addition to detailed physicochemical studies, immunological characterization of candidate AIT vaccines is essential to evaluate improvement of safety and efficacy in AIT.[8,36] In this regard, in vitro

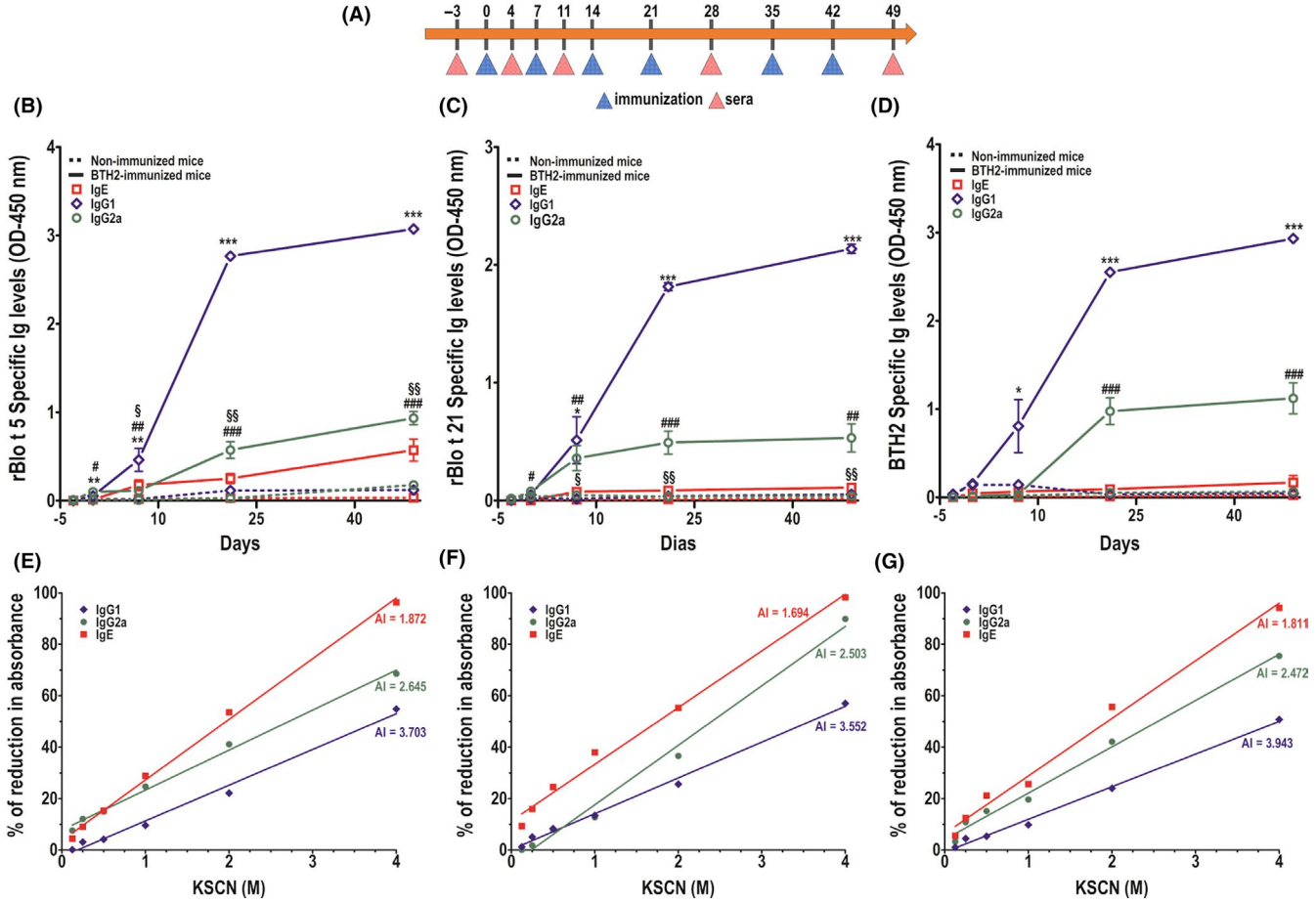


FIGURE 6 In vivo responses of hypoallergenic hybrid BTH2. (A) Schematic representation of immunization schedule; rBlo t 5-specific (B), rBlo t 21-specific (C) and BTH2-specific (D) IgG1, IgG2a and IgE determined by ELISA; *t* test was used to verify statistical differences between BTH2-immunized mice and non-immunized mice. * Comparisons between sIgG1, $^{*}P < .05$; $^{**}P < .01$; $^{***}P < .0001$; # Comparisons between sIgG2a, $^{\#}P < .05$; $^{\#\#}P < .01$; $^{\#\#\#}P < .0001$; * Comparisons between sIgE, $^{\S}P < .05$; $^{\S\S}P < .01$; $^{\S\S\S}P < .0001$; (E) Concentration of KSCN required to elute 50% of rBlo t 5-antibodies (sIgE, IgG1 and IgG2a) binding; (F) Concentration of KSCN required to elute 50% of rBlo t 21 antibodies (sIgE, IgG1 and IgG2a) binding; and (E) Concentration of KSCN required to elute 50% of BTH2-antibodies (sIgE, IgG1 and IgG2a) binding

assays for immunogenicity, such as the degradome assay, emerged as powerful methods to select immunogenic vaccine candidates for AIT.[49] Our in vitro assessment of endolysosomal proteolysis demonstrated that all investigated proteins showed increased resistance towards endolysosomal degradation than the full-length Blo t 5, which was completely degraded within 12 hours.[35] Machado et al discussed that increased fold stability correlates with a decreased susceptibility to endolysosomal proteolysis.[50] However, these authors concluded that proteins with too high fold stability could result in reduced antigen presentation.[50] Thus, we speculate that the stronger resistance of BTH1 compared to the parental allergens may lead to inefficient antigen presentation. However, further experiments are needed to corroborate this assumption. When comparing the peptide clusters of the hybrid allergens generated in the degradome assay, a more diverse pattern was observed for BTH2 than for BTH1. In fact, a prominent region within the rBlo t 5 (QHQLDELNENKSKELQEKIIRE) sequence appeared in all studied time points of BTH2 degradation, while for BTH1, this fragment was only found in the last three time points. The same was observed for the region MIEGAQGALERELK, which is considered an immunogenic region of Blo t 5.[30,31] Interestingly, the predicted T-cell epitope of Blo t 21 was more prominent in the BTH2 than in the BHT1 or rBlo t 21 degradomes. The degradome experiments also uncovered another possible immunogenic region (corresponding to the sequence THEVDDLEKTGNKDEKAR) in the Blo t 21 allergen. However, T-cell proliferation assays are necessary to confirm the importance of these peptides as T-cell epitopes. Taken together, the in vitro degradation assays support the selection of BTH2 as a better candidate for AIT.

The IgE reactivity patterns of the hybrid proteins were evaluated using serum samples from patients with severe asthma (SA) and allergic rhinitis (AR). In allergic asthma, we have identified a multitude of clinical phenotypes associated with elevated Th2 cytokine production (IL-4, IL-5 and IL-13).[51] Nevertheless, reduced IgE reactivity to BTH2 as well as weak IgE cross-linking and mediator release was generally observed in serum samples from asthmatic donors. The avidity index

TABLE 1 Inhibition of *Blomia tropicalis*-allergic donors' IgE binding to rBlo t 5 with mouse IgG antibodies induced by immunization with BTH2

Donor number	Allergic manifestation phenotype	Human IgE levels (OD 450 nm)		
		Pre-incubated with sera from mice		
		Non-immunized	BTH2-immunized	% of inhibition
#02	AR	1.47	0.33	77.60
#15	AR	0.53	0.11	80.06
#19	AR	1.38	0.18	87.16
#35	AR	0.59	0.10	84.01
#36	AR	0.33	0.05	85.09
#39	AR	0.59	0.08	86.14
#43	AR	0.13	0.04	67.61
#75	MA	0.21	0.05	75.62
#79	MA	2.00	1.11	44.37
#10	SA	1.13	0.21	81.00
#11	SA	1.70	1.02	39.78
#13	SA	1.94	0.65	66.57
#65	SA	1.45	0.15	89.61
	Mean	1.03	0.31	74.20
#46	NA	0.01	0.01	9.84
#48	NA	0.01	0.01	15.05

Abbreviations: AR, allergic rhinitis; MA, mild asthma; NA, non-allergic and non-asthmatic; O.D, optical density; SA, severe asthma.

TABLE 2 Inhibition of *Blomia tropicalis*-allergic donors' IgE binding to rBlo t 21 with mouse IgG antibodies induced by immunization with BTH2

Donor number	Allergic manifestation phenotype	Human IgE levels (OD 450 nm)		
		Pre-incubated with sera from mice		
		Non-immunized	BTH2-immunized	% of inhibition
#02	AR	0.53	0.13	76.36
#15	AR	0.54	0.10	82.05
#19	AR	0.71	0.20	71.67
#35	AR	0.14	0.09	35.32
#36	AR	0.75	0.29	61.11
#39	AR	0.35	0.10	71.83
#43	AR	0.34	0.16	52.90
#75	MA	0.11	0.01	94.64
#79	MA	1.60	0.82	48.53
#10	SA	0.37	0.17	53.07
#11	SA	1.09	0.37	65.92
#13	SA	0.65	0.24	63.31
#65	SA	0.58	0.17	70.49
	Mean	0.60	0.22	65.17
#46	NA	0.06	0.05	14.94
#48	NA	0.02	0.02	12.33

Abbreviations: AR, allergic rhinitis; MA, mild asthma; NA, non-allergic and non-asthmatic; O.D, optical density; SA, severe asthma.

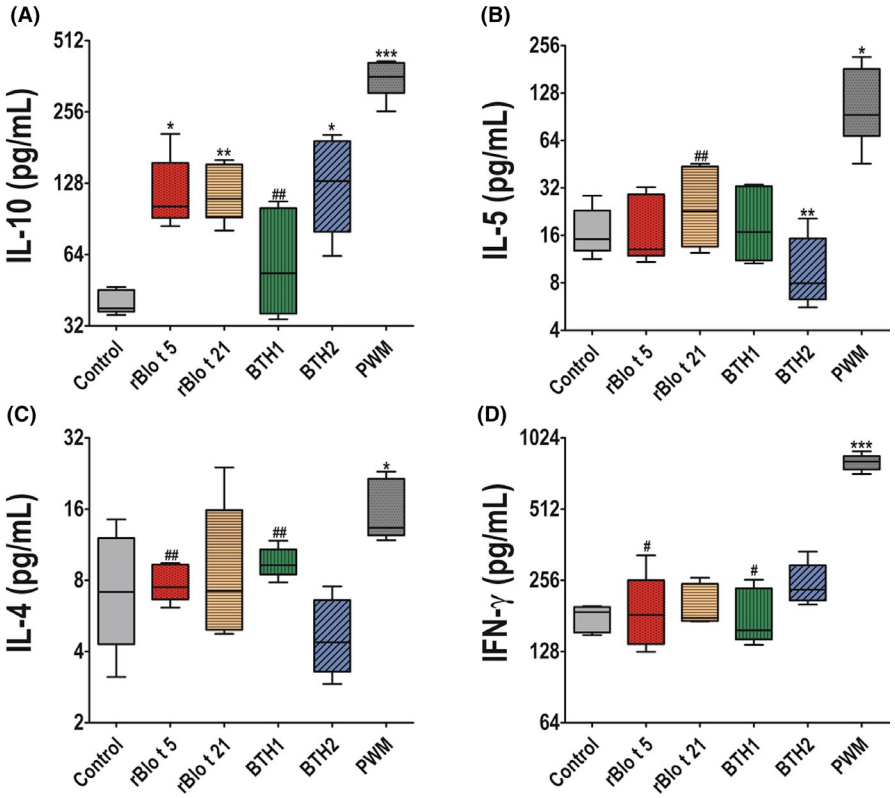


FIGURE 7 Cytokine profile from splenocytes of BTH2-immunized mice. Splenocytes of immunized animals cultured in the presence or absence of parental allergens or hybrid proteins. Supernatants of cultures were analysed by capture ELISA to measure IL-4 (A), IL-5 (B), IL-10 (C) and IFN-γ (D). Statistical analyses were performed by using paired *t* test. * Comparisons with NC, $^{*}P < .05$; $^{**}P < .01$; $^{***}P < .0001$; # Comparisons with BTH2 stimuli, $^{\#}P < .05$; $^{\#\#}P < .01$; $^{\#\#\#}P < .0001$; NS, non-stimulated cells; PWM, pokeweed mitogen

(AI) of BTH2 was significantly changed compared to the parental allergens and to BTH1. In contrast, no differences in the avidity indices of BTH1, rBlo t 5 and rBlo t 21 could be detected. Of note, BTH1 has a higher density of Blo t 5- than Blo t 21-derived IgE epitopes. Therefore, higher mediator release could be expected for BTH1 with sera from donors with higher levels of Blo t 5-specfic IgE antibodies. Similarly, lower levels of mediator release could be expected for BTH1 with sera from donors displaying higher levels of Blo t 21-specific IgE. Taken together, the point mutations in BTH2 may have caused structural remodelling leading to a significant reduction in IgE-binding and IgE-mediated basophil degranulation, when compared to the parental allergens and BTH1.

Another advantage of BTH2 over BTH1 as a vaccine candidate is underscored by its higher capacity to stimulate T-cell proliferation, to induce higher levels of IL-10 and lower levels of Th2 cytokines in PBMC from *B tropicalis*-atopic donors. It is well established that the induction of T-cell responses is an important mechanism in successful AIT, since these cells play a crucial role in regulating allergic responses.[7,14,52-54] One of the important indicators of T-cell regulation is the production of IL-10, which has strong anti-inflammatory and regulatory functions.[55] In this respect, it has been shown that only IL-10-specific Treg cells remained present at high frequencies for three years during house dust mite AIT.[53]

Although in vivo immunization of mice with BTH2 induced allergen-specific IgE, the avidity index of immunization-induced IgE was lower compared to specific IgG1 and IgG2a antibodies. Previous studies reported the induction of high IgG (predominantly IgG1) antibody titres upon immunization with hypoallergenic derivatives.[12,14,41,56-58] In our in vivo study, we showed that BTH2 induces increasing levels of high-avidity IgG antibodies with the capacity to block human IgE-allergen interaction.[7,8,15,59,60] Our results indicate that relevant structures of Blo t 5 and Blo t 21 are retained in the BTH2 hybrid molecule, which is capable to induce an adaptive immune response in mice with the production of cross-reactive IgG antibodies. Thus, it is possible that vaccination with BTH2 may protect individuals against reactions triggered by natural exposure to *Blomia*. Future studies in mouse models using BTH2 as treatment will demonstrate the benefits of its hypoallergenic e immunogenic features.

Stimulation of splenocytes indicated that the T-cell epitopes of the parental allergens are partially preserved in BTH2. High levels of IL-10 and IFN-γ together with reduced levels of TH2 cytokines were observed upon stimulation of splenocytes from BTH2-immunized mice. These observations suggest that BTH2 may potentially induce a Th1-biased and/or a regulatory immune response, as described in other studies.[14,41,58,60]

In conclusion, our results indicate that knowledge-based structural modifications and in silico point mutations are powerful approaches to produce hypoallergenic derivatives. The BTH2 hybrid molecule showed reduced capacity to bind human IgE and to trigger mediator release, was able to stimulate T-cell proliferation in PBMCs from allergic donors, as well as to induce the production of murine IgG antibodies with the capacity to block human IgE-allergen interactions. BTH2 combines two major allergens within a single molecule, thus facilitating the recombinant production of a multicomponent vaccine with the potential to treat co-sensitized patients. Future pre-clinical studies are still required to further confirm the potential of BTH2 as a novel AIT vaccine candidate.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

F. Ferreira, NM and Alcântara-Neves designed the study and supervised the project. M. Wallner and CS Pinheiro were involved in conception and co-supervision of the project. ES Silva significantly contributed to perform the present study, wrote the manuscript and conducted the laboratory assays. L. Aglas and S. Huber performed laboratory assays and manuscript revision. EF Silveira and RT Torres actively participated in animal experiments. EMMA Belitardo helped with laboratory assays and animal experiments and contributed with analysis tool. P. Lackner and J. Laimer performed the in silico analysis assays and manuscript revision. P. Briza acquired and analysed the MS data. F. Ferreira, NM Alcântara-Neves, M. Wallner, CS Pinheiro, LGC Pacheco, AA Cruz and P. Briza analysed and interpreted the data. ES Silva, F. Ferreira, NM Alcântara-Neves, M. Wallner and CS Pinheiro drafted the manuscript. All authors read and approved the final manuscript.

DATA AVAILABILITY STATEMENT

All the data used in this manuscript are available to the public.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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