Tropomyosins in mosquito and house dust mite cross-react at the humoral and cellular level

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INTRODUCTION

Dermatophagoides pteronyssinus allergens are one of the main causes of IgE-mediated allergies world-wide.1 House dust mites (HDM) contain several major allergens as well as panallergens, including tropomyosins.2 A high degree of cross-reactivity has been demonstrated between HDM and crustaceans,3 mollusks4 and insects.5 IgE cross-reactivity between HDM and mosquitoes has also been

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Funding information: Inmunotek, S.L; Austrian Science Funds, Grant/Award Number: SFB F4610, F4604, F4602

Summary

Background: Aedes aegypti and Dermatophagoides pteronyssinus contain important allergens including cross-reactive tropomyosins. However, the functional and clinical relevance of their cross-reactivity is still debated.

Objective: To analyse the humoral and cellular cross-reactivity of recombinant Aed a 10.01, Aed a 10.02 and Der p 10.

Methods: Sera from 15 Austrian house dust mite-allergic, Der p 10-sensitized individuals were tested for IgE reactivity to recombinant tropomyosins in ELISA, inhibition ELISA and basophil activation tests. BALB/c mice were immunized with Aed a 10.01 or Aed a 10.02, and their sera were assessed for reactivity to all tropomyosins. Splenocytes were stimulated with all tropomyosins and synthetic peptides representing the amino acid sequence of Aed a 10.01.

Results: IgE antibodies of Der p 10-sensitized patients cross-reacted with both tropomyosins from A. aegypti. Aed a 10.01 was a more potent inhibitor of IgE binding to Der p 10 and a stronger activator of basophils sensitized with Der p 10-specific IgE than Aed a 10.02. Murine antibodies raised against Aed a 10.01 and Aed a 10.02 cross-reacted with Der p 10. Aed a 10.01-specific antibody showed stronger cross-reactivity with Der p 10 than Aed a 10.02-specific antibody. Splenocytes from both groups of mice proliferated similarly to all tropomyosins. Five cross-reactive T cell-activating regions were identified.

Conclusion and Clinical relevance: Tropomyosins from D. pteronyssinus and A. aegypti show humoral and cellular cross-reactivity, involving 5 potential T cell-activating regions. The more pronounced cross-reactivity of Aed a 10.01 and Der p 10 matched the higher sequence similarity of both proteins.

KEYWORDS

cross-reactivity, house dust mites, mosquito, T-cell epitopes, tropomyosin
demonstrated. HDM and mosquitoes are evolutionarily related as part of the Phylum Arthropoda. The mosquito species Aedes aegypti is also a source of several allergens and contains Aed a 10 (tropomyosin). We previously demonstrated that natural and recombinant A. aegypti tropomyosins cross-react with tropomyosin from HDM.

Tropomyosins belong to a two-stranded alpha-helical coiled coil protein family, which can induce allergic sensitization by ingestion (seafood), inhalation (mites, cockroaches) or parasite infection. In developed countries, sensitization to tropomyosin is low. However, in some areas in Japan and Africa, the sensitization rate against Der f 10 and Der p 10 is higher than 80% and 55%, respectively. In Colombia, sensitization to tropomyosins from mite and Ascaris ranges from 34.6% to 47.7%. The molecular aspects of allergenic cross-reactivity among the different tropomyosins have been studied. Ayuso et al. described eight peptides which correspond to five putative cross-reactive IgE-binding epitopes sharing 56%-98% of homology with tropomyosins from shrimp, lobster, HDM and cockroach. These regions coincided with five IgE-binding mimotopes described by Leung et al. T-cell epitopes from shrimp and cockroach tropomyosins restricted to multiple MHC class II alleles have also been described.

Aedes aegypti contains two variants of tropomyosin, Aed a 10.0101 and Aed a 10.0201, which slightly differ in their primary structure. Aed a 10.0101 has higher homology to other allergenic tropomyosins and higher conservation of the putative IgE-binding epitopes described by Ayuso et al. Based on ELISA competition experiments and sequence analyses, we previously suggested that Aed a 10.0101 contains a higher number of IgE-binding epitopes, is more allergenic and may be more cross-reactive than Aed a 10.0201.

To confirm this hypothesis, we analysed the molecular, humoral and cellular cross-reactivity of recombinant (r) tropomyosins from A. aegypti (Aed a 10.0101 and Aed a 10.0201) and from HDM (Der p 10). The humoral cross-reactivity was addressed by immunoassays using sera from Der p 10-sensitized HDM-allergic patients and mice immunized with either Aed a 10.01 or Aed a 10.02. The capacity to induce mediator release was tested by basophil activation tests (BAT). The cellular cross-reactivity and identification of T cell-activating regions were addressed using splenocytes from mice immunized with mosquito-derived tropomyosins.

2 METHODS

2.1 Human sera

In total, sera from 15 patients (9 female, 6 male, median ages of 25 years, Table 1) allergic to HDM were included. All individuals suffered from perennial rhinoconjunctivitis and showed positive skin prick tests to HDM extract (ALK Abello, Hørsholm, Denmark), and >0.35 kUA/L IgE specific for HDM and Der p 10 as determined by ImmunoCAP (Thermo Fisher Scientific, Phadia AB, Uppsala, Sweden) (Table 1). The study was approved by the local ethics committee (EK1263/2014).

2.2 Allergens

Recombinant (r) Der p 10.0101 (Der p 10) was expressed as described. Whole body extract of A. aegypti, and recombinant tropomyosins of A. aegypti, Aed a 10.0101 and Aed a 10.0201, termed rAed

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TABLE 1 IgE responses of HDM-allergic patients to tropomyosins from HDM and Aedes aegypti

| Patient no. | Age (y) | Sex | Specific IgE (kUA/L) | Specific IgE (O.D.) |
|------------|---------|-----|----------------------|---------------------|
|            |         |     | HDM | rDer p 10 | rDer p 10 | rAed a 10.01 | rAed a 10.02 |
| 1          | 51      | F   | 41  | 1.1       | 0.263     | 0.130       | 0.236       |
| 2          | 9       | M   | 385 | 6.3       | 0.857     | 0.778       | 0.368       |
| 3          | 8       | M   | 1156| 16.8      | 1.961     | 1.924       | 1.215       |
| 4          | 27      | F   | 443 | n.d.      | 1.141     | 0.919       | 1.015       |
| 5          | 58      | M   | 763 | 1.9       | 0.322     | 0.279       | 0.219       |
| 6          | 30      | F   | 694 | 6.9       | 0.871     | 0.860       | 0.314       |
| 7          | 30      | F   | 33  | 11.2      | 1.279     | 1.282       | 1.073       |
| 8          | 10      | M   | 303 | n.d.      | >2.5      | >2.5        | >2.5        |
| 9          | 25      | F   | 242 | 2.1       | 0.385     | 0.306       | 0.247       |
| 10         | 64      | F   | 54  | 55.1      | >2.5      | >2.5        | 0.851       |
| 11         | 12      | M   | 14.9| 27.5      | 0.755     | 0.536       | 0.331       |
| 12         | 7       | F   | 4.1 | 10.6      | 0.444     | 0.483       | 0.161       |
| 13         | 15      | F   | 46.9| 57.0      | 1.894     | 2.081       | 0.499       |
| 14         | 8       | F   | 0.4 | 1.5       | 0.114     | 0.113       | 0.092       |
| 15         | 40      | M   | 1.5 | 7.7       | 0.353     | 0.460       | 0.310       |

HDM, house dust mite; M, male; F, female; n.d., not determined; O.D. optical density. O.D. of 0.065 for rDer p 10, 0.074 for rAed a 10.01 and 0.068 for rAed a 10.02 were used as cut-off value.
a 10.01 and rAed a 10.02 in the following, were produced as described.\textsuperscript{8}

### 2.3 | Circular dichroism (CD)

Far-UV CD spectra of rDer p 10 (280 μg/mL PBS), rAed a 10.01 (250 μg/mL 0.1 mol/L Tris-HCl) and Aed a 10.02 (500 μg/mL PBS) were collected on a Jasco J-810 spectropolarimeter (Japan Spectroscopic Co., Tokyo, Japan) using a 1-mm path length quartz cuvette. Measurements were taken between 250 and 190 nm, with 0.5 nm resolution at a scanning speed of 50 nm/min. Three independent measurements were recorded and averaged for each spectral point. The final spectra were baseline corrected by subtracting the corresponding buffer spectrum. Results were expressed as the mean residue ellipticity (θ) at a given wavelength.

### 2.4 | Detection of human IgE

ELISA plates (Nunc Maxisorp, Thermo Fisher Scientific, Waltham, MA, USA) were coated overnight at 4°C with A. aegypti extract (250 μg/mL) or recombinant allergens (0.5 μg/mL) in carbonate buffer (pH = 9.6). After washing twice with PBS containing 0.05% Tween 20 (PBS-T), nonspecific binding was blocked by incubation for 6 hours at RT with PBS-T supplemented with 1% HSA. Sera were diluted 1:4 in PBS-T containing 1% HSA and incubated ON at 4°C. After washing, bound IgE was detected with alkaline-phosphatase-conjugated anti-human IgE Ab (BD Pharmingen, San Jose, CA, USA) diluted 1:2000 in PBS-T containing 1% HSA and incubated ON at 4°C. After washing, bound IgE was detected with alkaline-phosphatase-conjugated anti-human IgE Ab (BD Pharmingen, San Jose, CA, USA) diluted 1:2000 in PBS-T containing 1% HSA for 60 minutes at 37°C and 60 minutes at 4°C. After washing, the substrate PNPP was added and the reaction was measured at 405 nm. All experiments were performed in duplicate. Sera from three nonallergic individuals were used as negative controls. The mean values from these samples plus five standard deviations were used as the cut-off for positive IgE levels. For inhibition experiments, sera were preincubated with the indicated concentrations of the recombinant tropomyosins.

### 2.5 | Basophil activation test

PBMCs isolated from nonallergic donors were stripped in ice-cold lactic acid buffer (0.13 mol/L KCl, 0.05 mol/L NaCl, 0.01 mol/L Lactic acid, pH = 3.9). After washing twice with PBS, cells were resuspended in 80 μL of PBS or 80 μL of sera from HDM-allergic individuals and incubated for 1 hour at 37°C. Nonstripped basophils served as negative control. After washing, resensitized cells were incubated with different concentrations of each tropomyosin in Hepes Calcium Buffer pH 7.4 containing IL-3 (2 ng/mL) for 15 minutes at 37°C. Medium alone served as negative control. For positive controls, we employed N-formyl-L-methionyl-L-leucyl-phenyalanine (fMLP, Peprotech, Rocky Hill, NJ, USA) and anti-IgE (1 μg/mL, Nordic Immunological Laboratories, Maidenhead, UK), respectively. The reaction was stopped with Heps/EDTA (20 mmol/L) buffer, and cells were stained with CD123-FITC, CCR3-APC and CD63-PE (all BioLegend, San Diego, CA, USA), followed by erythrocyte lysis. The percentages of CD63⁺CD123⁺CCR3⁺ cells ranged from 32.8% to 55.7% (mean value 36%) for fMLP and from 21% to 53.8% (mean value 41%) for anti-IgE. All negative controls were negative (data not shown). For inhibition experiments, tropomyosins (200 and 20 ng in 2 μL buffer) were incubated with pooled mouse sera (5 μL) for 1 hour at 37°C.

### 2.6 | Immunization of mice

Female 6- to 8-week-old BALB/c mice were immunized intraperitoneally with either A. aegypti extract, rAed a 10.01 or rAed a 10.02, or PBS adsorbed to Alum (Brenntag, Mülheim, Germany). In total, the animals received four ip injections of 25 μg of antigen adsorbed to 2 mg Alum in a total volume of 250 μL every 2 weeks. Fifteen days after the last immunization, blood was collected and mice were killed and the spleens were removed under aseptic conditions. All animal experiments in this study were performed under a protocol approved by the Institutional Animal Care and Use Committee of the “Hospital Clínico San Carlos” (Madrid, Spain).

### 2.7 | Murine antibody responses

ELISA plates were coated and saturated as described above. Sera were diluted 1:500 and incubated overnight. Bound antibodies were detected with rat anti-mouse IgG1 antibodies (BD Pharmingen), followed by a HRP-conjugated goat anti-rat IgG (GE Healthcare, Vienna, Austria). ABTS was added, and the reaction was measured at 405 nm.

### 2.8 | Murine cellular responses

Splenocytes (2 × 10⁵ cells/well) from mice immunized with either rAed a 10.01 or Aed a 10.02 were incubated in round bottom 96-well plates (Nunc) with rDer p 10, rAed a 10.01, rAed a 10.02 (each 100-12.5 ng/well) or medium alone for 4 days at 37°C. Concanavalin A (0.5 μg/well; Sigma Aldrich, USA) served as positive control. In addition, splenocytes were incubated with 28 synthetic 20-mer peptides (1 μg/well) overlapping for 10 residues and representing the complete amino acid (aa) sequence of Aed a 10.01 (Thermo Fisher Scientific, Waltham, MA, USA). During the last 16 hours, [3H]-labelled thymidine (0.5 mCi/mL) was added. Stimulation indices (SI) were calculated as the ratio between counts per minute (cpm) obtained in cultures containing splenocytes plus tropomyosins and cpm obtained in cultures containing splenocytes and medium alone.

### 3 | RESULTS

#### 3.1 | IgE cross-reactivity of rAed a 10.01, rAed a 10.02 and rDer p 10

Fifteen HDM-allergic patients sensitized to rDer p 10 showed IgE reactivity to rAed a 10.01 and rAed a 10.02 (Table 1). We observed a tendency for a more pronounced IgE reactivity to rAed a 10.01 than to rAed a 10.02. However, no statistical differences were found.
Sera from patients no. 11, 12, 13 and 15 were individually preincubated with increasing concentrations of rDer p 10, rAed a 10.01 and rAed a 10.02. Thereafter, the inhibition of IgE binding to each tropomyosin was assessed (Figure 1). IgE binding to rDer p 10 was best inhibited by rDer p 10, followed by rAed a 10.01 and rAed a 10.02. IgE binding to Aed a 10.01 was best inhibited by Aed a 10.01 followed by rDer p 10 and rAed a 10.02. Notably, IgE binding to rAed a 10.02 was best inhibited by rDer p 10 and Aed a 10.01.

3.2  rAed a 10.01 and rAed a 10.02 induce activation of basophils resensitized with rDer p 10-specific IgE

To assess whether the IgE cross-reactivity of the tropomyosins of *A. aegypti* and rDer p 10 resulted in effector cell activation, stripped basophils were resensitized with sera from patients 4, 7, 8 and 10 containing high levels of rDer p 10-specific IgE and incubated with titrated amounts of rAed a 10.01 and rAed a 10.02, respectively (Figure 2). rDer p 10 served as positive control. In all patients, rAed a 10.01 induced a dose-dependent basophil activation, although to a lower extent than rDer p 10. In contrast, the same amounts of rAed a 10.02 showed limited activity in this assay.

3.3  Tropomyosins of *A. aegypti* and HDM have a similar secondary structure

Amino acid sequence alignment revealed that rDer p 10 shares 78.9% of identity with rAed a 10.01 and 62.7% with rAed a 10.02 (Figure 3). To evaluate whether these allergens also shared a similar secondary structure, the far-UV CD spectra of rDer p 10, rAed a 10.01 and rAed a 10.02 were recorded at room temperature. All spectra showed a maximum at about 191 nm and two minima at about 208 and 222 nm (Figure 3). These results indicated that rAed a 10.01 and rAed a 10.02 are folded and present an α-helical structure similar to rDer p 10.

3.4  Murine antibodies specific for rAed a 10.01 or rAed a 10.02 cross-react with rDer p 10

We immunized BALB/c mice with rAed a 10.01 and rAed a 10.02, respectively, and analysed whether their sera reacted with the different recombinant tropomyosins. Indeed, IgG1 antibodies raised against both *A. aegypti*-derived tropomyosins bound rDer p 10 (Figure 4). Moreover, antibodies induced by immunization with rAed a 10.01 reacted with rAed a 10.02 and vice versa. Finally, antibodies raised against the recombinant tropomyosins also reacted with their natural counterparts in mosquito extract (Figure 4A). We also assessed whether murine sera were able to inhibit basophil activation by the tropomyosins. Briefly, different concentrations of rDer p 10, rAed a 10.01 and rAed a 10.02 were preincubated with the sera from mice immunized with PBS, rAed a 10.01 or rAed a 10.02 and subsequently added to stripped human basophils resensitized with rDer p 10-specific IgE (Figure 4B). Compared to sera from PBS-mice, sera from rAed a 10.01-immunized mice strongly inhibited basophil activation of all tropomyosins. Sera from rAed a 10.02-immunized mice completely inhibited basophil activation by Aed a 10.02 and showed inhibitory potential for rAed a 10.01 and rDer p 10, although clearly less pronounced than sera from rAed a 10.01-immunized mice.

3.5  Murine T cells specific for *A. aegypti* tropomyosins cross-react with rDer p 10 involving 5 T cell-activating regions

Proliferative responses of splenocytes from mice immunized with either rAed a 10.01 or rAed a 10.02 to different concentrations of all recombinant tropomyosins were assessed (Figure 5A,B). rAed a 10.01-immunized mice showed stronger proliferative responses to rAed a 10.01 than to rAed a 10.02 or rDer p 10. In contrast, splenocytes from rAed a 10.02-immunized mice responded similarly to the three tropomyosins. We stimulated the splenocytes with a panel of 28 overlapping peptides representing the entire aa sequence of Aed a 10.01 (Figure 5A,B). Splenocytes from mice immunized with Aed a 10.01 reacted with peptides covering aa 11-40, 81-100, 111-130, 161-190 and 221-270 (Figure 5A). Splenocytes from mice immunized with Aed a 10.02 responded to peptides covering aa 21-50, 81-140, 161-180 and 211-285 (Figure 5B). These data indicated that both isoforms share five regions containing cross-reactive T-cell epitopes, namely Aed a 10.01-21-40, Aed a 10.01-81-100, Aed a 10.01-111-130, Aed a 10.01-161-180 and Aed a 10.01-221-270 (Figure 5C).

4  DISCUSSION

This study indicates a pronounced immunological cross-reactivity among the tropomyosins Aed a 10.01 and Aed a 10.02 from the mosquito *A. aegypti* and Der p 10 from the house dust mite *D. pteronyssinus* based on high amino acid sequence similarity and the resulting similar fold. Even though the mosquito species *A. aegypti* is not abundant in Austria, 15/15 Austrian HDM-allergic Der p 10-sensitized patients displayed IgE reactivity with the mosquito tropomyosins. In particular, rAed a 10.01 induced the activation of basophils sensitized with Der p 10-specific IgE. Together, these results imply that exposure to the tropomyosins from mosquitoes may trigger IgE-mediated clinical reactions in Der p 10-sensitized patients. To study cross-reactivity resulting from primary sensitization to mosquito allergens, we immunized BALB/c mice with either Aed a 10 molecule. The induced murine antibodies recognized rDer p 10 confirming that cross-reactivity among the tested tropomyosins occurs independently from the primary sensitizer. Moreover, the murine antibodies raised against the mosquito tropomyosins inhibited IgE-mediated effector cell activation by rDer p 10. We noticed that antibodies from mice immunized with Aed a 10.01 were more potent inhibitors of basophil activation by all tropomyosins than antibodies raised against Aed a 10.02. Together with the less inhibitory
Patient 11

Solid phase: rDer p 10

Solid phase: rAed a 10.01

Solid phase: rAed a 10.02

% Inhibition

Concentration (µg/mL)

Patient 12

Patient 13

Patient 15
**FIGURE 1** IgE inhibition experiments. Sera from four HDM-allergic patients were incubated with indicated amounts of rDer p 10, rAed a 10.01 or rAed a 10.02. The inhibition of IgE reactivity to each tropomyosin was assessed by ELISA.

**FIGURE 2** Allergenicity of recombinant tropomyosins. Basophils resensitized with sera from four HDM-allergic patients were activated with different concentrations of rDer p 10, rAed a 10.01 and rAed a 10.02. The expression of CD63 was assessed by flow cytometry.

**FIGURE 3** Similarity of rDer p 10, rAed a 10.01 and rAed a 10.02. A, Amino acid sequences alignment performed with the Constraint-based multiple alignment tool (COBALT) and visualized using the graphical user interface for multiple sequence alignment and molecular phylogeny “SeaView.” Hydrophobic amino acids (VILMFWA) are coloured in blue, negatively charged (DE) in magenta, positively charged (KR) in red, aromatic (YH) in teal, polar (NSTQ) in green and C and G in orange (B) circular dichroism spectra of the recombinant tropomyosins.
activity of Aed a 10.02 in ELISA, these observations provided evidence that Aed a 10.01 and Der p 10 have more common epitopes than Der p 10 and Aed a 10.02. Previously, five highly conserved cross-reactive B-cell epitopes had been identified in tropomyosins from various sources.16-18,22,23 Our previous studies have suggested that these regions are conserved in Der p 10 and Aed a 10.01, but to a lesser extent in Aed a 10.02.9 Along these lines, in all experiments performed in the present study, Aed a 10.02 was less reactive with Der p 10 than Aed a 10.01. These findings may be relevant for the diagnosis of patients sensitized to both A.aegypti and D. pteronyssinus. While Aed a 10.01 represents an important cross-reactive allergen in regions where sensitization to tropomyosins is high, such as Colombia, Japan or some African countries,11,14,15 Aed a 10.02 may be considered as less relevant. The latter is additionally supported by the fact that Aed a 10.02 isoforms are only expressed by mosquito species but not by other arthropod species.

**FIGURE 4** Humoral cross-reactivity of tropomyosins. A, Sera from mice immunized with rAed a 10.01 or rAed a 10.02 were tested for reactivity to recombinant tropomyosins and extract from Aedes aegypti; B, 20 ng (a) or 200 ng (b) of either rDer p 10, rAed a 10.01 or rAed a 10.02 were incubated with the sera from mice immunized with PBS, rAed a 10.01 or rAed 10.02 and used to activate basophils resensitized with Der p 10-specific IgE. The expression of CD63 was assessed by flow cytometry.
FIGURE 5  Cellular cross-reactivity of tropomyosins. Splenocytes from mice immunized with rAed a 10.01 (A) or rAed 10.02 (B) were stimulated with different concentrations of rAed a 10.01, rAed a 10.02, rDer p 10 or 28 synthetic 20 mer peptides representing the complete primary sequence of Aed a 10.01. Proliferation is shown as stimulation indices (SI). C, Peptide sequences inducing proliferation in A are highlighted in grey in the aa sequence of Aed a 10.01, and peptides inducing proliferation in B are underlined in the aa sequence of Aed a 10.02. Overlapping regions are framed.
We also employed BALB/c mice to get a hint on T-cell epitopes possibly involved in cellular cross-reactivity of Aed a 10.01, Aed a 10.02 and Der p 10, respectively. We found that all tropomyosins cross-reacted at the cellular level and identified five cross-reactive T cell-activating regions, namely Aed a 10.0121-40, Aed a 10.0181-100, Aed a 10.01111-130, Aed a 10.01161-180 and Aed a 10.01221-270. Certainly, the approach in BALB/c mice does not mimic the T-cell response of humans which involves the presentation of processed peptides by various HLA class II molecules. Nevertheless, the T cell-activating regions Aed a 10.0121-40, Aed a 10.01111-130, Aed a 10.01161-180 and Aed a 10.01221-270 corresponded to T cell-activating regions in shrimp tropomyosin previously identified in humans.19 Furthermore, these regions are highly conserved within the amino acid sequences of all tropomyosins and show 55%-100% of sequence identity (70%-100% of similarity) between Aed a 10.01, Aed a 10.2 and Der p 10. Therefore, we conclude that T cells specific for epitopes located in these regions may cross-react with tropomyosins from various allergen sources. Thereby, exposure to Aed a 10 may stimulate the T-cell response initially mounted against allergenic tropomyosins from mosquitoes or any other arthropod species as previously suggested for allergens from HDM and Ascaris.24

In summary, we have demonstrated that tropomyosins from HDM and A. aegypti display a distinct antibody and T-cell cross-reactivity. The latter involves five potential T cell-activating regions. Moreover, the degree of IgE cross-reactivity accorded with the degree of homology of the tropomyosins; for example, Aed a 10.01 and Der p 10 share 79% of homology and cross-reacted more pronounced than Aed a 10.02 and Der p 10 which share 63%. Moreover, compared to Der p 10, Aed a 10.01 has more secondary structure content than Aed a 10.02. Accordingly, in particular, Aed a 10.01 has the potential to induce allergic symptoms and exacerbate the immune response against a broad spectrum of tropomyosins.

ACKNOWLEDGEMENTS

Jose Fernando Cantillo was awarded with a Ph.D. scholarship from the Administrative Department of Science, Technology and Innovation of the Colombian Government (Colciencias)-Colombia and a research fellowship from the European Academy of Allergy and Clinical Immunology (EAACI). This work was supported by Austrian Science Funds, projects SFB F4610, F4604 and F4602, Vienna, Austria, and Inmunotek, S.L.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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How to cite this article: Cantillo JF, Puerta L, Fernandez-Caldas E, et al. Tropomyosins in mosquito and house dust mite cross-react at the humoral and cellular level. Clin Exp Allergy. 2018;48:1354–1363. https://doi.org/10.1111/cea.13229