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1 **Immunodominant conformational and linear IgE epitopes lie in a single**  
2 **segment of Ara h 2**

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5

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13

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17

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26

27 **Abstract**

28

29 **Background:** Contribution of conformational epitopes to the IgE-reactivity of peanut

30 allergens Ara h 2 and Ara h 6 is, at least, as important as that of the linear epitopes.

31 However, little is known about these conformational IgE-binding epitopes.

32 **Objective:** To investigate the distribution of conformational epitopes on chimeric 2S-

33 albumins.

34 **Methods:** Recombinant chimeras were generated by exchanging structural segments

35 between Ara h 2 and Ara h 6. Well-refolded chimeras, as verified by circular dichroism

36 analysis, were then used to determine the epitope specificity of monoclonal antibodies

37 (mAb) by performing competitive inhibition of IgG-binding. Furthermore, we delineated the

38 contribution of each segment to the overall IgE-reactivity of both 2S-albumins by measuring

39 the chimeras' IgE-binding capacity with sera from 21 patients allergic to peanut. We finally

40 assessed chimeras' capacity to trigger mast cell degranulation.

41 **Results:** Configuration of the conformational epitopes was preserved in the chimeras.

42 Mouse IgG mAb, raised against natural Ara h 6, and polyclonal human IgE antibodies

43 recognized different conformational epitopes distributed all along Ara h 6. In contrast, we

44 identified human IgG mAb specific to different Ara h 2 linear or conformational epitopes

45 located in all segments but the C-terminal one. The major conformational IgE-binding

46 epitope of Ara h 2 was located in a segment located between residues 33 and 81 that also

47 contains the major linear hydroxyproline-containing epitope. Accordingly, this segment is

48 critical for the capacity of Ara h 2 to induce mast cell degranulation.

49 **Conclusions:** Chimeric 2S-albumins provide new insights on the conformational IgE-binding  
50 epitopes of Ara h 2 and Ara h 6. Proximity of the immunodominant linear and  
51 conformational IgE-binding epitopes probably contributes to the high allergenic potency of  
52 Ara h 2.

53

54 **Key Messages:**

- 55 - Conformation of the IgE-binding epitopes is preserved in chimeric 2S-albumins
- 56 - Distribution of the conformational IgE-binding epitopes and the relative contribution  
57 of each segment to the IgE-reactivity differ between Ara h 2 and Ara h 6
- 58 - Proximity of the immunodominant linear and conformational IgE-binding epitopes  
59 probably contributes to the high allergenic potency of Ara h 2.

60 **Capsule Summary**

61 IgE-binding capacity of chimeric peanut 2S-albumins suggests that non-cross-reactive  
62 immunodominant epitopes of Ara h 2 are mostly located in one segment whereas they are  
63 more evenly distributed in Ara h 6.

64 **Keywords:** Peanut allergy, 2S-albumin, IgE, conformational epitope, monoclonal antibody,  
65 Ara h 2, Ara h 6

66 **Abbreviations used:** IgE: Immunoglobulin E; RBL: Rat Basophilic leukemia; P<sup>OH</sup>:  
67 Hydroxyproline; mAb: monoclonal antibody; DSB: disulfide bridge; AChE:  
68 Acetylcholinesterase; r/a: reduced and alkylated; OIT: oral immunotherapy.

69

70 **Introduction**

71 IgE-mediated peanut allergy is a life-threatening disease with an increasing prevalence that  
72 requires effective diagnostics and therapies.<sup>1,2</sup> Ara h 2 and Ara h 6 are the most potent peanut  
73 allergens for triggering mast cell activation.<sup>3-5</sup> Accordingly, IgE levels to these allergens are equally  
74 good predictors of clinical reactivity to peanut.<sup>6-9</sup>

75 2S-albumins Ara h 2 and Ara h 6 share similar structural properties including five  $\alpha$ -helices,  
76 four conserved disulfide bridges (DSB) and a sequence identity of 59%.<sup>10-12</sup> Their compact structures  
77 confer a high resistance to proteolysis and explain the persistence of conformational IgE-binding  
78 epitopes in the digestive tract. Nevertheless, Ara h 2 differs from Ara h 6 by an insertion of 14 or 26  
79 residues (isoforms Ara h 2.01 and 2.02, respectively). These insertions form a flexible surface loop  
80 that contains 2 or 3 repetitions of the hydroxyproline-containing motif DPYSP<sup>OH</sup>S, previously  
81 identified as an immunodominant linear IgE-binding epitope.<sup>13-15</sup> We recently showed that these  
82 motifs accounted on average for half of Ara h 2 IgE-binding capacity.<sup>16</sup> Ara h 6 contains also an  
83 additional DSB linking its C-terminus to the core structure. Although Ara h 2 and Ara h 6 are expected  
84 to cross-react extensively, we reported that 2S-albumin IgE-reactivity was mostly due to non-cross-  
85 reactive epitopes.<sup>16</sup>

86 Linear IgE-binding epitopes of peanut allergens have been exhaustively investigated and their  
87 profiling by peptide microarrays or bead-based epitope assays offers promising developments for  
88 diagnosis.<sup>17-20</sup> However, this strategy excludes IgE-binding to conformational epitopes, which may  
89 also be highly clinically relevant.<sup>15, 21</sup> Indeed, Tscheppe *et al.* reported that two thirds of peanut-  
90 allergic patients preferentially recognized conformational epitopes and Otsu *et al.* proposed that  
91 conformational epitopes are most important for patients with relatively severe reactions.<sup>12, 22</sup>  
92 Moreover, 2S-albumin allergenic potential is dependent on the conformation since unfolding induced  
93 by DSB reduction drastically decreases their resistance to proteolytic digestion, their IgE-reactivity  
94 and their ability to trigger mast cell degranulation.<sup>14, 23, 24</sup>

95 Characterization of conformational epitopes remains particularly challenging because it  
96 requires preservation of protein folding.<sup>25, 26</sup> X-ray crystallography or nuclear magnetic resonance  
97 spectroscopy of allergen-antibody complex can identify the residues involved in a conformational  
98 epitope but are applicable only to monoclonal antibodies (mAb). Recently, phage display technology  
99 has been employed to identify mimotopes of conformational epitopes.<sup>27</sup> The mimotopes were  
100 mimics of both Ara h 2 and Ara h 6 epitopes and were mapped to overlapping surface patches on  
101 both Ara h 2 and Ara h 6.<sup>27</sup> Another strategy is the grafting of surface areas from an allergen on a  
102 homologous non-allergen protein, by replacing segments of the recipient protein with IgE-binding  
103 segments.<sup>28</sup> This approach demonstrated that close proximity of IgE-binding epitopes on a protein  
104 enhanced IgE-dependent mediator release.<sup>29</sup> Similarly, we generated chimeras between caprine and  
105 bovine  $\beta$ -caseins in order to identify epitopes recognized by IgE antibodies from patients allergic to  
106 goat's milk and tolerant to cow's milk.<sup>30</sup>

107 In the present study, we aimed to investigate the conformational epitopes of peanut 2S-  
108 albumins by generating chimeric proteins between Ara h 2 and Ara h 6. The correct exposure of the  
109 conformational epitopes in these chimeras was assessed by using complementary solid- and fluid-  
110 phase assays of antibody binding. In order to establish their functionality, chimeras were first used to  
111 determine the epitope specificity of different mAb, either obtained after immunization of mice to  
112 natural Ara h 6 or produced from Ara h 2-specific memory B cells isolated from patients undergoing  
113 peanut oral immunotherapy.<sup>31</sup> We delineated then the immunodominant conformational epitopes  
114 recognized by IgE antibodies from allergic patients.

115

## 116 **Methods**

### 117 **Human sera**

118 Sera were collected from 15 French and 6 US peanut-allergic subjects. French patients were  
119 recruited from the Paediatric Allergy Clinic of Hospital Necker (Paris) after informed consent from  
120 parents (Table E1, sera 1 to 15).<sup>15, 32</sup> All samples were collected during routine clinical practice and  
121 were studied in accordance with the purpose of the initial study.<sup>32</sup> US peanut-allergic patients had a  
122 strong history of peanut-induced immediate hypersensitivity and peanut-specific IgE  $\geq$  13 KAU/L  
123 (ImmunoCAP, Thermo Fisher Scientific, Waltham, MA, USA) in serum (Table E1, sera 16 to 21). All  
124 adult patients and the parents or guardians of minors signed informed consent. Minors who were  $>6$   
125 years of age, signed an assent. The University of Colorado Denver Institutional Review Board (IRB)  
126 approved use of the sera for this study.

127

### 128 **Monoclonal antibodies against Ara h 6**

129 The IgG2 mAb specific to natural Ara h 6 were obtained in mice. They were used in different  
130 assay formats for epitope blockage or Ara h 6 quantitation in biological fluids and food matrices.<sup>33-35</sup>

131

### 132 **Monoclonal antibodies against Ara h 2**

133 The mAb were generated from Ara h 2-specific B cells isolated from peripheral blood  
134 obtained from children, aged 7-21, enrolled in a single-center, open-label randomized trial of peanut  
135 oral immunotherapy (OIT, NCT01324401), 1-2 months after starting therapy.<sup>31</sup> The Massachusetts  
136 General Hospital IRB approved this study. Briefly, Ara h 2-specific B cells were affinity selected using  
137 an Ara h 2 fluorescent multimer from peripheral blood mononuclear cells.<sup>31</sup> Single B cells underwent  
138 heavy and light chain amplification, with subsequent cloning for expression as IgG1 antibodies.<sup>36</sup>  
139 Monoclonal IgG1 antibodies were affinity purified using agarose protein G beads (Thermo Fisher  
140 Scientific), quantitated by Human IgG cytometric bead arrays (BD Biosciences, Franklin Lakes, NJ,  
141 USA) and validated using ImmunoCAP specific to Ara h 2 (Thermo Fisher Scientific).<sup>36</sup>



142

**143 Design of chimeric 2S-albumins**

144 Recombinant 2S-albumins Ara h 2.01 (Swiss-Prot accession number Q6PSU2-2) and Ara h 6  
145 (Q647G9) were produced in *E. coli* by expression of synthetic genes (Genscript USA Inc., Piscataway,  
146 NJ, USA) subcloned into plasmid pET9c, between *NdeI* and *BamHI* restriction sites (Novagen-Merck,  
147 Damstadt, Germany). Along codon optimization, three restriction sites were added. Insertions of *PstI*,  
148 *AlwNI* and *DraI* sites were silent in rAra h 2 while *AlwNI* insertion induced a mutation of Ile30 in Ara h  
149 6 to Leu, the corresponding residue present in Ara h 2 (Fig. 1). This conservative mutation did not  
150 affect the IgE-binding capacity of rAra h 6 (Fig. E1). Chimeras were generated by restriction of Ara h  
151 2/6 genes and ligation of the complementary fragments. As the presence of unpaired Cys residue  
152 could impair chimera's refolding, unpaired Cys residues at position 84 in chimera C-6662 and position  
153 136 in chimera C-2226 were replaced by a Ser residue by site-directed mutagenesis.<sup>23</sup> The variant  
154 rAra h 2.Δ was obtained by replacing the sequence GRDPYSP<sup>OH</sup>SQDPYSP<sup>OH</sup>SP of Ara h 2.01 by the  
155 dipeptide DS occurring in Ara h 6.<sup>15</sup> Bacterial expression, purification and refolding of recombinant  
156 proteins were performed as previously described.<sup>15</sup> Refolding of recombinant proteins was assessed  
157 by circular dichroism spectroscopy. 3D-structures of the chimeras and Ara h 2.01 (Fig. 1), were  
158 generated with SWISS-MODEL Homology modeling Workspace, using Ara h 6 template 1w2q and  
159 DeepView-Swiss-PdbViewer.<sup>37, 38</sup>

160

**161 IgE-binding measurement by solid-phase assay**

162 The chimeras' IgE-binding capacity was quantified using indirect ELISA with recombinant  
163 allergens (1 µg/mL) adsorbed on a solid phase.<sup>16, 32</sup> After overnight incubation with sera (50 µL/well,  
164 diluted 20- to 400-fold), plates were washed and IgE-binding was revealed by addition of an anti-  
165 human IgE mAb (clone BS17)<sup>39</sup> labeled with acetylcholinesterase (AChE, 2 Ellman Unit (EU)/mL). AChE  
166 activity was then revealed after addition of Ellman's reagent and absorbance was measured at 414  
167 nm. The IgE-binding to each chimera was also evaluated in sera depleted of IgE antibodies

168 recognizing either Ara h 6 or Ara h 2 (see online repository).<sup>16</sup> After overnight incubation with sera in  
169 plate coated with one 2S-albumin (1 µg/mL), depleted sera were not washed away but transferred  
170 into another plate (45 µL/well) coated with a chimera (1 µg/mL). Residual IgE-binding was revealed  
171 as described above.

172

### 173 **Competitive inhibition of IgG and IgE-binding**

174 Competitive inhibitions were performed by using assays that measure the binding of  
175 biotinylated 2S-albumins by the tested antibodies, which were first captured by specific antibodies  
176 immobilized on the solid phase.<sup>15, 33, 40</sup> For characterization of mouse IgG2 mAb, human IgG1 mAb or  
177 human IgE antibodies, plates were coated with a goat anti-mouse IgG polyclonal antibody (Jackson  
178 ImmunoResearch), a mouse anti-human IgG1-Fc-CH2 mAb (clone NL-16, Bio-Rab Laboratories Inc.) or  
179 a mouse anti-human IgE mAb (clone LE27),<sup>39</sup> respectively. Fifty µL/well of purified mAb (0.5 to 10  
180 ng/mL) or sera (diluted 20- to 400-fold) were incubated overnight at 4°C. After washing, 25 µL of  
181 inhibitors (i.e. increasing concentrations of recombinant chimera or peptides, ranging from 3.2 pM to  
182 100 nM)<sup>15</sup> and 25 µL of biotinylated 2S-albumins (0.5 nM) were added and incubated for 4h at RT.  
183 After washing, neutravidin labeled with AChE (2 EU/mL) was added for 15 min. Binding inhibition  
184 induced by a known concentration of inhibitor was then calculated as the ratio B/B0. B0 and B  
185 represent the amount of labeled tracer measured in the absence or the presence of a known  
186 concentration of inhibitor, respectively.

187

### 188 **Mediator release assay**

189 Degranulation assays were performed with rat basophilic leukemia (RBL) SX-38 cells as  
190 previously described.<sup>4</sup> Cells were passively sensitized with total IgE antibodies from single sera,  
191 affinity purified by using anti-IgE mAb (equimolar mix of LE27 and BS17 clones, 1 mg/mL), coupled to  
192 CNBr-activated Sepharose™ 4B (GE healthcare, Uppsala, Sweden).<sup>4</sup> Mediator release was induced  
193 by incubation with different concentrations of recombinant 2S-albumins and was measured by

194 assaying the  $\beta$ -hexosaminidase activity in culture supernatants. Results were expressed as  
195 percentage of the reference release, determined for each serum and induced with anti-human IgE  
196 (LE27 clone; 100 ng/mL).

197

#### 198 **Statistical analysis**

199 Data on IgE-binding to coated chimeras were analyzed using the non-parametric Friedman's  
200 test with Dunn's multiple comparison test. Statistical analyses were performed with GraphPad Prism  
201 8.3.0 software. A  $p < 0.05$  was considered to be significant.

202

## 203 **Results**

### 204 ***Chimeras exhibit 2S-albumin conformation***

205 Design of the chimeric proteins led to the delineation of 4 segments (Fig. 1). Chimeras between Ara h  
206 2 and Ara h 6 were then generated by substituting segments of one 2S-albumin by those of the other  
207 2S-albumin (Fig. 1C). After production in *E. coli*, purification (Fig. E2A) and refolding, CD-spectroscopy  
208 confirmed the presence of  $\alpha$ -helical secondary structures characteristic of native 2S-albumins for all  
209 chimeras (Fig. E2B). The chimeras' conformations were further probed by testing their capacity to  
210 inhibit the binding of IgE antibodies from sera displaying a strong IgE cross-reactivity between Ara h 2  
211 and Ara h 6 (Fig. 2). Correct refolding of the chimeras was thus ascertained by their inhibitory  
212 capacities comparable to those of rAra h 6 and rAra h 2. Conversely, unfolded 2S-albumins, after DSB  
213 reduction and alkylation (r/a), displayed no inhibitory capacity.

214

### 215 ***Monoclonal antibodies recognize conformational and linear epitopes in chimeras***

216 Chimeras were first applied to investigate the epitope specificity of mAb against Ara h 6  
217 (mouse IgG2 mAb, Fig. 3A and E3A) or Ara h 2 (human IgG1 mAb, Fig. 3B and E3B). The segment  
218 recognized by each mAb was determined by identifying the chimeras able to inhibit IgG-binding. For  
219 example, mAb 641 was specific of Ara h 6 segment I since only chimeras containing this segment (*i.e.*  
220 C-6222, C-6622 and C-6662) inhibited mAb-binding (Fig. 3A). Likewise, mAb 614, mAb 215 and mAb  
221 201 recognized segment II, III and IV, respectively. Most mAb recognized a conformational epitope  
222 since r/a rAra h 6 did not display any inhibitory capacity. Only binding of mAb 614 and 637 was  
223 partially inhibited by r/a Ara h 6, but with a 25-fold higher half-maximal inhibitory concentration than  
224 the folded allergen (Fig. 3A and E3A).

225 We also identified human mAb specific of the first three segments of Ara h 2 (Fig. 3B and  
226 E3B). The mAb U2 was specific of segment I. As r/a Ara h 2 was able to inhibit mAb U2-binding, we  
227 further showed that this mAb recognized pep1-21, a peptide covering Ara h 2 N-terminus, which  
228 contains linear IgE-binding epitopes.<sup>13, 14</sup> The segment II of Ara h 2 was recognized by two groups of

229 mAb that differed by their capacity to bind r/a Ara h 2. The mAb M6 (and M7) recognized only nAra h  
230 2 and r/a nAra h 2, but not rAra h 2 or r/a rAra h 2. By testing pep2P<sup>OH</sup> peptide, we showed that these  
231 two mAb were specific of the DPYSP<sup>OH</sup>S motifs. In contrast, peptide pep2P, without hydroxyproline,  
232 did not inhibit mAb M6-binding (Fig. 3B and E3B). Conversely to mAb M6, mAb P31 (and S4)  
233 recognized a conformational epitope that did not involve any residues of the DPYSP<sup>OH</sup>S motifs.  
234 Indeed, rAra h 2 and the variant lacking the DPYSP<sup>OH</sup>S motifs, rAra h2.Δ, displayed similar inhibitory  
235 capacities. Finally, three mAb recognized a conformational epitope located in the segment III. These  
236 mAb also recognized Ara h 6, but with varying affinities. The difference between inhibitory capacities  
237 of Ara h 2 and Ara h 6 was higher for mAb M3 than for mAb M33 or M17 (Fig. 3B and E3B). No mAb  
238 specific to segment IV was identified.

239

#### 240 ***Contribution of each segment to 2S-albumin IgE-reactivity differs between Ara h 2 and Ara h 6***

241 Chimeras were then used to profile the specificity of polyclonal IgE antibodies from allergic  
242 patients. First, we compared the IgE-binding to chimeras passively adsorbed on microplates (Fig. 4).  
243 Despite a high interindividual variability, the level of IgE-binding to rAra h 2 and to chimeras  
244 containing Ara h 2 segment II (*i.e.* C-6222, C-2266 and C-2226, in shades of red, Fig. 4A) was  
245 significantly higher than to rAra h 6. The chimera C-2266, which combined Ara h 2 N-terminal half  
246 and Ara h 6 C-terminal half, even exhibited a higher IgE-binding capacity than rAra h 2 itself. This  
247 result thus showed that the contribution of each segment to the overall IgE-reactivity differs among  
248 patients but also between the two 2S-albumins.

249 In order to further investigate the non-cross-reactive epitopes of Ara h 2 and Ara h 6, IgE-  
250 binding to a 2S-albumin was evaluated with sera depleted of IgE antibodies recognizing the other 2S-  
251 albumin (Fig. 4B). After depletion of Ara h 2-specific IgE antibodies, IgE-binding to rAra h 2 was  
252 expectedly minimal. The IgE-binding capacity of the chimeras then progressively increased with the  
253 sequential replacement of Ara h 2 segments by corresponding Ara h 6 segments, thus indicating that  
254 each segment of Ara h 6 contains IgE-binding epitopes.

255 In contrast, after depletion of Ara h 6-specific IgE antibodies, we did not observe a similar  
256 increase of IgE-binding when Ara h 6 segments were progressively replaced by those of Ara h 2 (Fig.  
257 4C). Here, all chimeras containing Ara h 2 segment II displayed an IgE-binding capacity comparable to  
258 that of rAra h 2, thus indicating that the non-cross-reactive IgE-binding epitopes were mostly located  
259 in segment II.

260

### 261 ***Conformational IgE-binding epitopes are distributed along Ara h 6***

262 The chimeras' immunoreactivity was further investigated by competitive inhibition of IgE-  
263 binding to rAra h 6. In this assay format, the profile of chimeras' inhibitory capacities was highly  
264 variable among patients thus confirming that all segments could contribute significantly to Ara h 6  
265 IgE-reactivity (Fig. 5A and E4A). For example, Ara h 6-specific IgE antibodies of serum 2 recognized  
266 principally the segment I since only chimeras containing this segment inhibited IgE-binding. Segment  
267 I and II contributed the most to Ara h 6 IgE-reactivity for serum 9 whereas chimeras containing Ara h  
268 6 segments II and III retained the highest inhibitory capacities for serum 12. Finally, for serum 17, all  
269 chimeras could inhibit a significant fraction of IgE-binding to Ara h 6 but the segment IV contributed  
270 to Ara h 6 IgE-reactivity slightly more than the other segments (Fig. 5A). Of note, r/a rAra h 6  
271 displayed no inhibitory capacity.

272

### 273 ***Ara h 2 segment II contains an immunodominant conformational IgE-binding epitope***

274 Competitive inhibition of IgE-binding to rAra h 2, instead of nAra h 2, was then performed to  
275 investigate specifically the conformational epitopes of Ara h 2. Indeed, the absence of hydroxyproline  
276 in rAra h 2 results in a 100- to 1000-fold decrease of IgE-binding affinity to the DPYSPS motifs in this  
277 fluid-phase assay.<sup>15</sup> Thereby, high-affinity IgE-binding to the conformational epitopes of labeled rAra  
278 h 2 outcompetes low-affinity IgE-binding to the DPYSPS motifs. The absence of significant IgE-binding  
279 to the linear motifs was confirmed with rAra h 2.Δ and rAra h 2 displaying similar inhibitory capacities  
280 (Fig. E5). Accordingly, the unfolded r/a rAra h 2 displayed no inhibitory capacity (Fig. 5B and E4B).

281           The profile of chimeras' capacities to inhibit IgE-binding to Ara h 2 was then more consistent  
282 among patients than the one observed for Ara h 6. Indeed, despite the absence of IgE-binding to the  
283 DPYSPS motifs, contribution of segment II to Ara h 2 IgE-reactivity remained largely predominant. For  
284 most sera, chimeras containing Ara h 2 segment II displayed the highest inhibitory capacities, thus  
285 demonstrating the presence of an immunodominant conformational epitope in this segment (Fig. 5B  
286 and E4B). Accordingly, chimeras displaying Ara h 6 segment II exhibited a marked reduction of  
287 inhibitory capacity. It can be also noted that, in addition to segment II, segment I and III could also  
288 contribute significantly to Ara h 2 IgE-reactivity such as for sera 11 and 14 (segment I) and for serum  
289 10 (segment III). The lowest inhibitory capacity was observed with C-6662 and the highest one with  
290 C-2226 thus suggesting that segment IV minimally contributed to Ara h 2 IgE-reactivity.

291

#### 292 ***Ara h 2 segment II is critical for mast cell degranulation***

293           Finally, we wanted to confirm the importance of rAra h 2 segment II for triggering the  
294 degranulation of RBL SX-38 cells sensitized with total IgE antibodies from allergic patients (Fig. 6). For  
295 sera 7 and 11, Ara h 6 displayed an allergenic potency markedly lower than that of Ara h 2 and as  
296 expected, chimeras containing Ara h 2 segment II were the most potent in triggering cell  
297 degranulation (Fig. 6A). For sera 9 and 14, rAra h 2, rAra h 6 and the chimeras, displayed comparable  
298 allergenic potencies, thus incidentally confirming that chimeras were functional (Fig. E6). The RBL  
299 cells were then passively sensitized with total IgE depleted of anti-Ara h 6 antibodies. In these  
300 conditions, rAra h 2, but not rAra h 6, and only the chimeras containing Ara h 2 segment II could  
301 efficiently trigger RBL cell degranulation (Fig. 6B).

**302 Discussion**

303           Given our previous finding that IgE-reactivity of Ara h 2 and Ara h 6 was mediated mainly by  
304 non-cross-reactive antibodies,<sup>16</sup> we generated chimeras between Ara h 2 and Ara h 6 in order to  
305 investigate their conformational IgE-binding epitopes.

306           First, we confirmed that conformation of the IgE-binding epitopes was preserved in the  
307 chimeric 2S-albumins. We further showed that most of the tested mAb recognized a conformational  
308 epitope since unfolding of the 2S-albumins prevented mAb-binding. Chimeras enabled then the  
309 identification of the different structural segments recognized by each mAb. Nevertheless, in line with  
310 the unstructured surface loop lying between  $\alpha$ -helices 2 and 3, in Ara h 2 and Ara h 6,<sup>10,11</sup> some mAb  
311 were still able to bind to r/a Ara h 2 (mAb M6) or to r/a Ara h 6 (mAb 614). It is noteworthy that  
312 binding of mAb M6 to the DPYSP<sup>OH</sup>S motifs in Ara h 2 required the presence of hydroxyproline  
313 residues, as previously described for IgE-binding.<sup>15</sup> Moreover, cross-reactivity between Ara h 2 and  
314 Ara h 6 was not observed with mAb specific to segment I or II, but only with mAb specific to segment  
315 III or IV, where the highest level of sequence identity occurs between Ara h 2 and Ara h 6.

316           We then profiled the conformational epitopes recognized by human IgE polyclonal  
317 antibodies. Using solid-phase assay and selective depletion of IgE antibodies, we determined the  
318 distribution of non-cross-reactive epitopes for both 2S-albumins. Considering Ara h 2, the non-cross-  
319 reactive epitopes were mostly located in segment II. This result was partly expected since this  
320 segment contains the DPYSP<sup>OH</sup>S motifs, which accounted on average for about 50% of the IgE-binding  
321 to Ara h 2.<sup>15</sup> However, contribution of segment II was higher than anticipated since it accounted for  
322 about 90% of the IgE-binding to Ara h 2 (Fig. 4B). This result suggested the presence of other  
323 immunodominant epitopes in segment II. Accordingly, our use of human mAb demonstrated the  
324 presence of a conformational epitope bound by mAb P31. Competitive inhibition of IgE-binding to  
325 rAra h 2 and mast cell degranulation assay finally confirmed that the conformational epitope in the  
326 segment II was immunodominant. Thus, Ara h 2 segment II contains at least two immunodominant  
327 IgE-binding epitopes: one linear with the DPYSP<sup>OH</sup>S motifs and one conformational, probably



328 involving residues in the third  $\alpha$ -helix lying between residues 64 and 74 (Fig. 1). The close proximity  
329 of these two immunodominant epitopes in segment II certainly contributes to the outstanding  
330 allergenic potency of Ara h 2, as previously described by Gieras *et al.*<sup>29</sup> Interestingly, mAb M6 (and  
331 M7) has been shown to inhibit 40% to 60% of the IgE/Fc $\epsilon$ R1 cross-linking induced by Ara h 2 but only  
332 20 to 30% of the IgE/Fc $\epsilon$ R1 cross-linking induced by a crude peanut extract.<sup>41</sup> This difference was  
333 probably due to the fact that M6 and M7 were highly specific to Ara h 2. In contrast, mAb M33,  
334 which binds to both Ara h 2 and Ara h 6, was able to inhibit with the same efficiency (30% to 40%)  
335 the IgE/Fc $\epsilon$ R1 cross-linking induced by Ara h 2 or the crude peanut extract. Nevertheless, mAb M3  
336 and M17, which also recognized the segment III of both 2S-albumins, inhibited only 17% and 3% of  
337 the IgE/Fc $\epsilon$ R1 cross-linking by Ara h 2, respectively.<sup>41</sup> These differences should be indicative of the  
338 mAb affinity for Ara h 2 or of steric hindrance phenomena that could block IgE-binding to other  
339 segments. Considering the structural homology between segments III/IV of both 2S-albumins,  
340 inhibition of IgE/Fc $\epsilon$ R1 cross-linking by mAb targeting the segment III/IV should be also more efficient  
341 with sera displaying a high IgE cross-reactivity between Ara h 2 and Ara h 6, such as sera 3 and 13.

342 Surprisingly, we identified human mAb specific to the first three segments of Ara h 2 but  
343 none to the segment IV, which also exhibited the lowest contribution to the IgE-reactivity of Ara h 2.  
344 Considering that these human mAb were generated from B cells of patients undergoing OIT, their  
345 variable regions would have been influenced by OIT. Furthermore, our results are in agreement with  
346 recent studies of linear epitopes, reporting an overlap between the IgG and IgE repertoires induced  
347 by OIT.<sup>42,43</sup> Future studies will be needed to evaluate how the application of chimeric 2S-albumins  
348 may be also useful to monitor OIT-induced changes to epitope recognition and its relationship to  
349 clinical outcomes.

350 Distribution of conformational IgE-binding epitopes on Ara h 6 was quite different from that  
351 of Ara h 2. This was also partially expected since conformational epitopes have been previously  
352 reported to be more important for Ara h 6 than for Ara h 2.<sup>12,15,42</sup> Moreover, mimotopes identified  
353 by phage display screening mapped to a higher number of surface patches on Ara h 6 than on Ara h

354 2.<sup>27</sup> In this study, we identified sera where each of the four segments could contribute significantly to  
355 Ara h 6 IgE-binding capacity. For segment IV, the presence of conformational epitopes in Ara h 6, but  
356 not in Ara h 2, is probably due to the fifth DSB that structurally stabilizes the C-terminus of Ara h 6.

357 In conclusion, chimeric 2S-albumins provided new insights on conformational IgE-binding  
358 epitopes of Ara h 2 and Ara h 6. We showed that distribution of the conformational IgE-binding  
359 epitopes and their relative contribution to the overall IgE-reactivity were substantially different  
360 between the two 2S-albumins. Despite the known heterogeneity of individual IgE responses to  
361 peanut allergens, the outstanding allergenic potency of Ara h 2 is probably explained by the close  
362 proximity of two immunodominant epitopes in the segment II of Ara h 2: one linear and one  
363 conformational. In this regard, mAb specific to this segment may have potential in the development  
364 of immunomodulatory treatments, for example by blocking the IgE-mediated cascade of allergic  
365 inflammation.

366

### 367 **Acknowledgements**

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- 491
- 492

493 **Figure legends**

494

495 **Figure 1:** 3D-structure of Ara h 2 (A) with the four segments as shown on the sequence alignment  
496 between Ara h 2 and Ara h6 (B). Identical residues are indicated by asterisks and  $\alpha$ -helices  
497 correspond to underlined sequences. Substitution of Ile residue at position 30 in Ara h 6 with Leu  
498 residue, as found in Ara h 2, is indicated by a green asterisk. Sequences of the synthetic peptides  
499 pep1-21 and pep2P<sup>OH</sup> are shown. Chimeras were obtained by replacing progressively segments of  
500 one 2S-albumin by those from the other 2S-albumin (C). Conserved disulfide bridges and the  
501 supplementary one in Ara h 6 are indicated in plain and dotted lines, respectively.

502

503 **Figure 2:** Conformation of the IgE-binding epitopes is preserved in chimeric 2S-albumins. Competitive  
504 inhibition of IgE-binding to rAra h 2 (A) or rAra h 6 (B). The assay was performed with two sera  
505 displaying a high level of IgE cross-reactivity between Ara h 2 and Ara h 6 (serum 3 and 13, see Table  
506 E1). r/a : reduced and alkylated (i.e. unfolded).

507

508 **Figure 3:** Epitope specificity of IgG monoclonal antibodies. (A) Competitive inhibition of mouse mAb  
509 binding to rAra h 6 and (B) of recombinant human mAb binding to nAra h 2. Segments recognized by  
510 the tested mAb, i.e. present in chimeras exhibiting the highest inhibitory capacities, are boxed in  
511 green. (C) Schematic representation of the segments recognized by the different mAb.

512

513 **Figure 4:** IgE-binding to chimeras with sera from patients allergic to peanut (n=18). Chimeras were  
514 passively adsorbed on the solid-phase of microplates. (A) IgE-binding to chimeras are expressed in %  
515 of IgE-binding to rAra h 2; (B) IgE-binding to chimeras after depletion of anti-Ara h 2 IgE antibodies  
516 (results are expressed in % of IgE-binding to rAra h 6); (C) IgE-binding to chimeras after depletion of  
517 anti-Ara h 6 IgE antibodies (results are expressed in % of IgE-binding to rAra h 2). Statistical analyses



518 were performed with the nonparametric Friedman test for multiple comparisons: a,  $p < 0.05$  vs rAra h  
519 2 and b,  $p < 0.05$  vs rAra h 6.

520

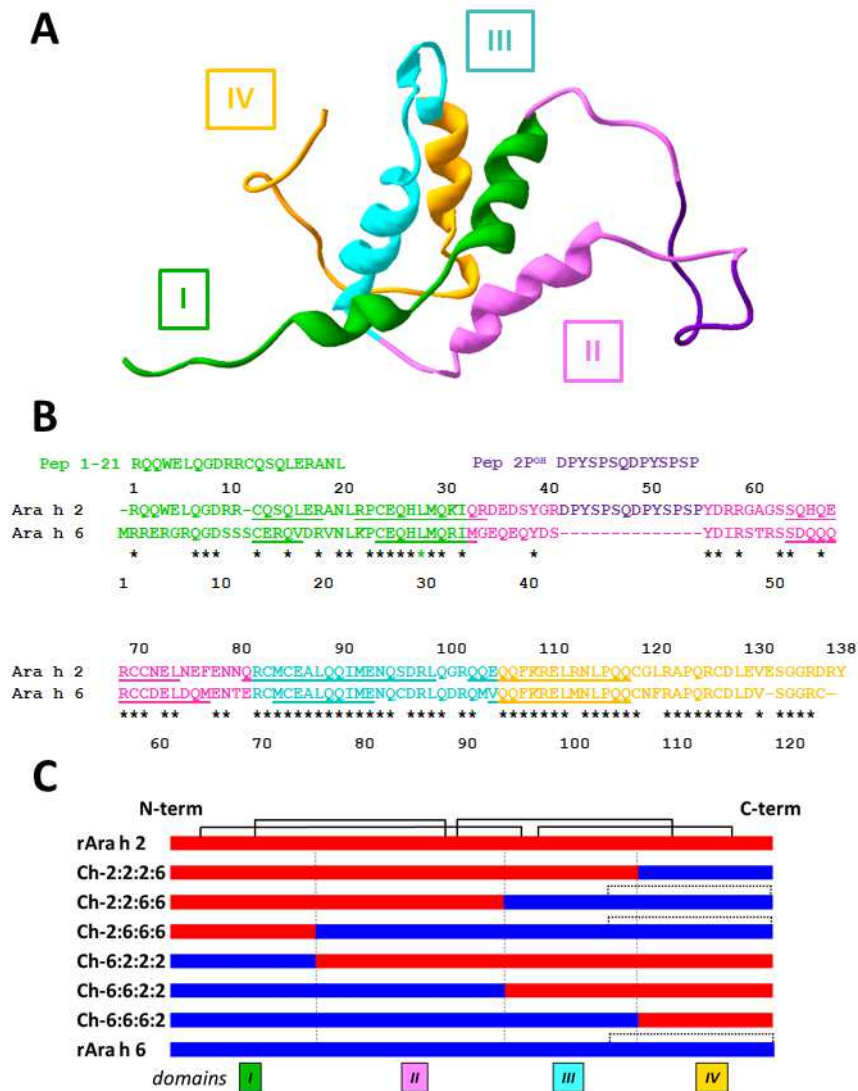
521 **Figure 5:** Competitive inhibition of IgE-binding to rAra h 6 (A) and rAra h 2 (B) by the different chimeras.  
522 Chimeras containing the segment II of Ara h 6 are shown in shades of blue and chimeras containing  
523 the segment II of Ara h 2 are shown in shades of red. Unfolded r/a rAra h 6 and r/a rAra h 2 (green  
524 symbols) displayed no significant IgE-binding capacity. Segments contributing the most to IgE-  
525 reactivity, i.e. present in chimeras exhibiting the highest inhibitory capacities, are boxed in green.

526

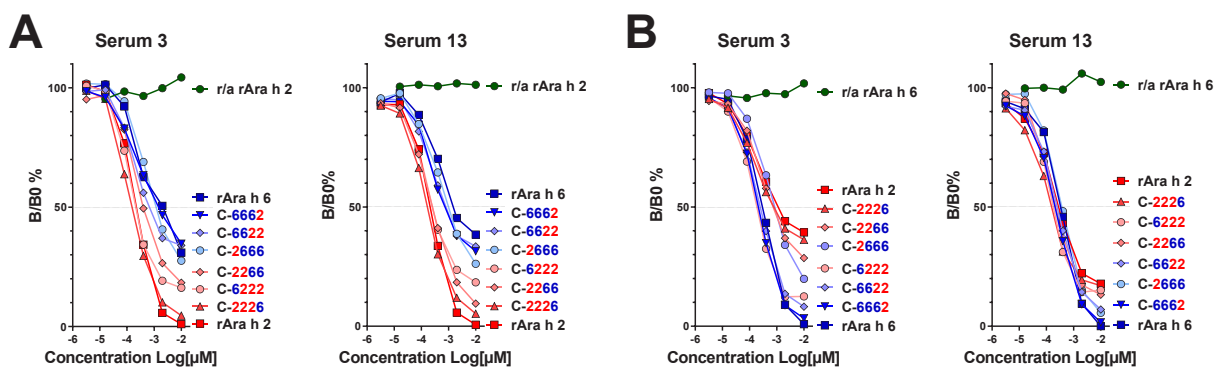
527 **Figure 6:** Mediator release assay with RBL SX-38 cells sensitized with immunopurified total IgE  
528 antibodies from 4 patients. (A) For serum 7 and 11, as Ara h 2 triggered RBL degranulation more  
529 efficiently than Ara h 6, allergenic potencies of chimeras were directly compared. (B) For serum 9 and  
530 14, rAra h 2, rAra h 6 and the chimeras exhibited comparable allergenic potencies (see Fig. E6). In order  
531 to compare chimeras' potencies, RBL cells were sensitized with immunopurified total IgE depleted of  
532 anti-Ara h 6 antibodies. The x-axis shows the concentration of the tested molecule and the y-axis shows  
533 the percentage of the reference release induced with the anti-human IgE mAb LE27.

534

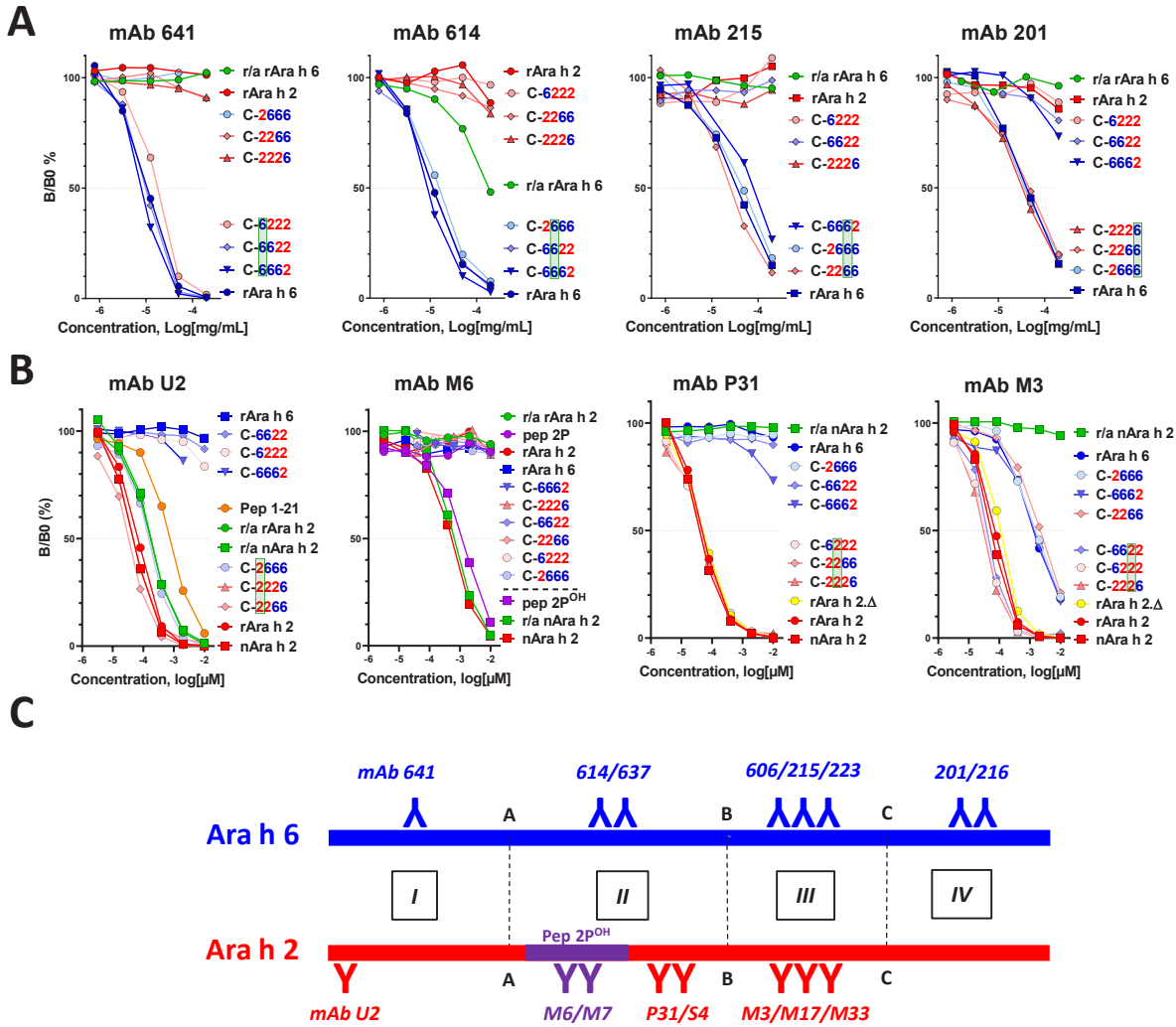
# Figures



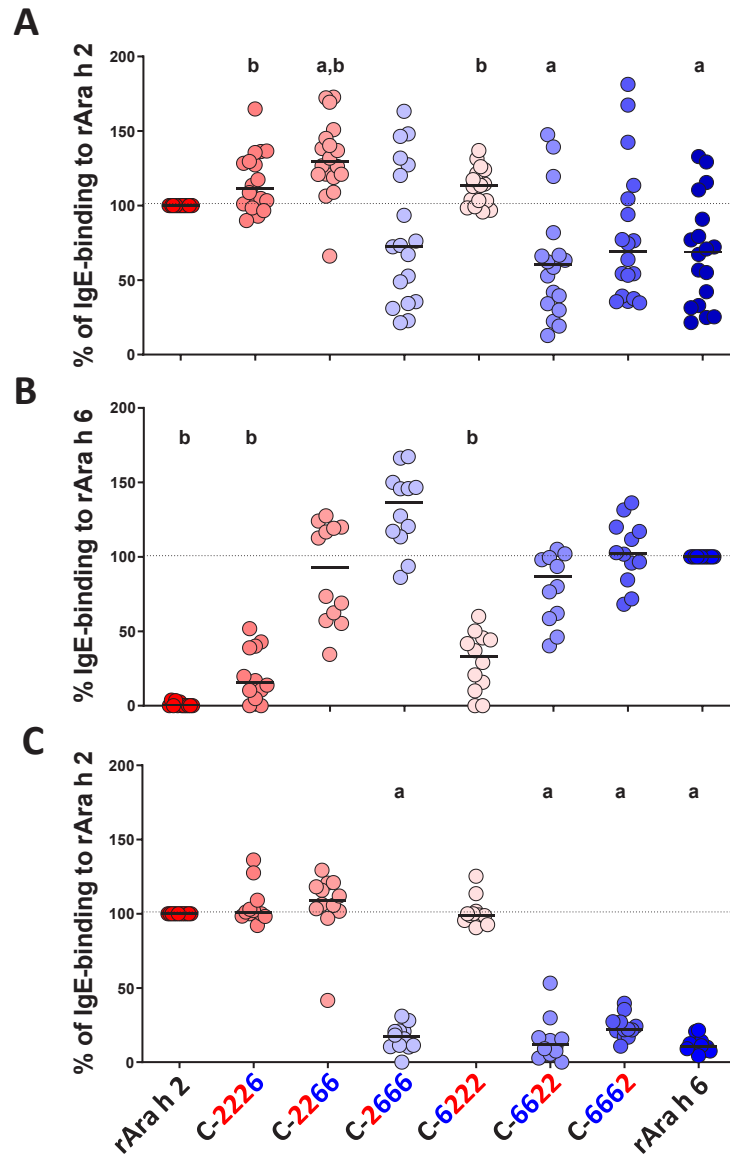
**FIG 1:** 3D-structure of Ara h 2 (A) with the four segments as shown on the sequence alignment between Ara h 2 and Ara h6 (B). Identical residues are indicated by asterisks and  $\alpha$ -helices correspond to underlined sequences. Substitution of Ile residue at position 30 in Ara h 6 with Leu residue, as found in Ara h 2, is indicated by a green asterisk. Sequences of the synthetic peptides pep1-21 and pep2P<sup>OH</sup> are shown. Chimeras were obtained by replacing progressively segments of one 2S-albumin by those from the other 2S-albumin (C). Conserved disulfide bridges and the supplementary one in Ara h 6 are indicated in plain and dotted lines, respectively.



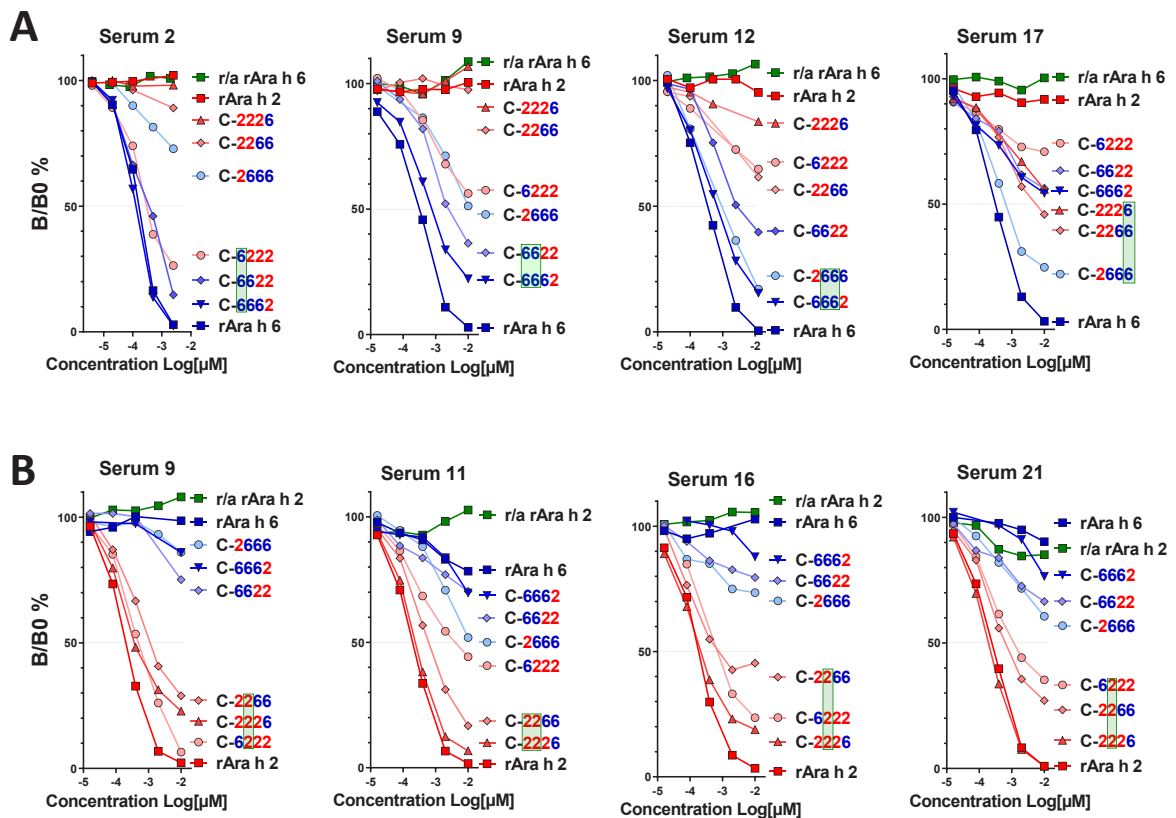
**Fig. 2: Conformation of the IgE-binding epitopes is preserved in chimeric 2S-albumins.** Competitive inhibition of IgE-binding to rAra h 2 (A) or rAra h 6 (B). The assay was performed with two sera displaying a high level of IgE cross-reactivity between Ara h 2 and Ara h 6 (serum 3 and 13, see Table E1). r/a : reduced and alkylated (i.e. unfolded).



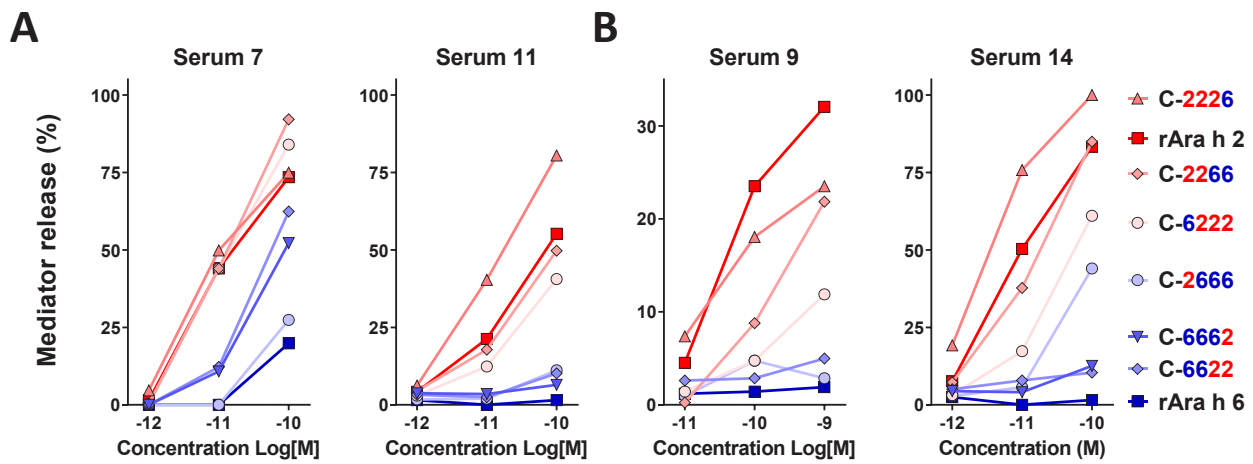
**FIG 3:** Epitope specificity of IgG monoclonal antibodies. (A) Competitive inhibition of mouse mAb binding to rAra h 6 and (B) of recombinant human mAb binding to nAra h 2. Segments recognized by the tested mAb, i.e. present in chimeras exhibiting the highest inhibitory capacities, are boxed in green. Schematic representation of the segments recognized by the different mAb.



**FIG 4:** IgE-binding to chimeras with sera from patients allergic to peanut (n=18). Chimeras were passively adsorbed on the solid-phase of microplates. (A) IgE-binding to chimeras are expressed in % of IgE-binding to rAra h 2; (B) IgE-binding to chimeras after depletion of anti-Ara h 2 IgE antibodies (results are expressed in % of IgE-binding to rAra h 6); (C) IgE-binding to chimeras after depletion of anti-Ara h 6 IgE antibodies (results are expressed in % of IgE-binding to rAra h 2). Statistical analyses were performed with the nonparametric Friedman test for multiple comparisons: a,  $p < 0.05$  vs rAra h 2 and b,  $p < 0.05$  vs rAra h 6.



**FIG 5:** Competitive inhibition of IgE-binding to rAra h 6 (A) and rAra h 2 (B) by the different chimeras. Chimeras containing the segment II of Ara h 6 are shown in shades of blue and chimeras containing the segment II of Ara h 2 are shown in shades of red. Unfolded r/a rAra h 6 and r/a rAra h 2 (green symbols) displayed no significant IgE-binding capacity. Segments contributing the most to IgE-reactivity, i.e. present in chimeras exhibiting the highest inhibitory capacities, are boxed in green.



**FIG 6:** Mediator release assay with RBL SX-38 cells sensitized with immunopurified total IgE antibodies from 4 patients. (A) For serum 7 and 11, as Ara-h-2 triggered RBL degranulation more efficiently than Ara-h-6, allergenic potencies of chimeras were directly compared. (B) For serum 9 and 14, rAra h 2, rAra h 6 and the chimeras exhibited comparable allergenic potencies (see FIG E11). In order to compare chimeras' potencies, RBL cells were sensitized with immunopurified total IgE depleted of anti-Ara h 6 antibodies. The x-axis shows the concentration of the tested molecule and the y-axis shows the percentage of the reference release induced with the anti-human IgE mAb LE27.

## Supplementary methods

### Indirect ELISA

The IgE levels to native 2S-albumins (Table E1) were quantified using an indirect ELISA in which purified native antigens (2.5 µg/mL) were passively adsorbed on microtiter plates as previously described.<sup>1</sup> After overnight incubation with 2 or 3 dilutions of sera (50 µL/well, diluted 10- to 400-fold) in order to be in the range of the standard curve, IgE-binding was revealed by the addition of a labeled anti-human IgE monoclonal antibody (clone BS17, produced in-house)<sup>2</sup>. BS17 tracer was prepared by covalent linkage of the monoclonal antibody to the tetrameric form of acetylcholinesterase (AChE)<sup>3</sup> and added at a concentration of 2 Ellman's Unit (EU)/mL. After washing, Ellman's reagent was used as the enzyme substrate and absorbance was measured at 414 nm. Quantification of specific IgE was performed by comparison with dose–response curves obtained with a total IgE assay performed with human IgE standards (WHO Reference Regent serum IgE, NIBSC). The corresponding immunometric assays of total and specific IgE were performed in parallel, under identical conditions, an anti-human IgE mAb (clone LE27)<sup>2</sup>, complementary to the BS17-AChE tracer, being used as capture antibody for the total IgE assay, instead of the immobilized allergen used for specific IgE-binding measurement by solid-phase assay.<sup>1</sup>

The IgE-binding capacity of the chimeras was quantified using indirect ELISA in which recombinant allergens (1 µg/mL) were passively adsorbed on the solid phase. After overnight incubation with sera (50 µL/well, diluted 20- to 400-fold, approximately 0.05 IU (specific IgE)/well), plates were washed and IgE-binding was revealed by addition of BS17-AChE.

The IgE-binding to each chimera was also evaluated in sera depleted of IgE antibodies recognizing either Ara h 6 or Ara h 2.<sup>4</sup> Here, after overnight incubation with sera (50 µL/well, diluted 20- to 400-fold, approximately 0.05 IU (specific IgE)/well) in microtiter plate coated with rAra h 2 or rAra h 6 (1 µg/mL), incubated/depleted sera were not washed away but transferred instead into another plate (45 µL/well) coated with either the same allergen (to confirm the depletion efficiency)



or with one chimera (to measure the residual non-cross-reactive IgE-binding). The initial plate is then washed and IgE-binding is revealed after incubation with BS17-AChE. Results from the initial plate set the “100%” of IgE-binding to Ara h 2 or Ara h 6 (Fig. 4). After ON incubation with the depleted sera, the plates are washed and non-cross-reactive IgE-binding to the chimera is revealed as described above.

Of note, for RBL SX-38 cell degranulation, depletion of IgE recognizing Ara h 6 from immunopurified total IgE was done by using the same protocol performed in 24 wells of a plate coated with rAra h 6 (1 µg/mL). After overnight incubation, corresponding depleted sera were pooled and stored at -20°C before performing the degranulation assay.

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## Legends of supplementary figures

**Fig. E1:** Comparison of the capacity of nAra h 6, rAra h 6 and rAra h 6 I30L to inhibit IgE-binding to nAra h 6. No significant difference was observed between native and recombinant forms of Ara h 6.

**Fig. E2:** (A) SDS-PAGE analysis of purified recombinant chimeras (A, 1 µg protein/lane, on NuPage™ 4-12% Bis-Tris gels). (B) Circular dichroism analysis of the chimeras. nAra h 2, rAra h 2, nAra h 6, rAra h 6 and the chimeras exhibited spectra characteristic of 2S-albumin structure, with two minima at around 208 and 222 nm indicative of a high content of α-helix. In comparison, reduced and alkylated (r/a) Ara h 6 displayed a spectrum characteristic of unfolded 2S-albumin with a minimum at 200 nm. rAra h 2 and rAra h 2.Δ were previously shown to exhibit similar CD spectra (Bernard *et al.*, JACI, 2015).

**Fig. E3:** Epitope specificity of IgG monoclonal antibodies directed against Ara h 6 (A) or Ara h 2 (B). (A) Competitive inhibition of mouse mAb binding to rAra h 6 and (B) of recombinant human mAb binding to nAra h 2. Segments recognized by the tested mAb, i.e. present in chimeras exhibiting the highest inhibitory capacities, are boxed in green.

**Fig. E4:** Competitive inhibition of IgE-binding to rAra h 6 (A) and rAra h 2 (B) by the different chimeras. Chimeras containing the segment II of Ara h 6 are shown in shades of blue and chimeras containing the segment II of Ara h 2 are shown in shades of red. Denatured r/a rAra h 6 and r/a rAra h 2 (green symbols) displayed no significant IgE-binding capacity. The domains contributing the most to Ara h 6 or Ara h 2 IgE-reactivity for the tested serum, i.e. present in chimeras exhibiting the highest inhibitory capacities, are boxed in green.

**FIG E5: A,** Because of the absence of hydroxyproline residue, competitive inhibition of IgE-binding to rAra h 2 did not evidence IgE-binding to the linear DPYSP motifs and only revealed IgE-binding to the conformational epitopes of rAra h 2. Therefore rAra h 2 and rAra h 2.Δ displayed similar capacities to inhibit IgE-binding to rAra h 2. **B,** Inhibition of IgE-binding to the DPYSP<sup>OH</sup> motifs can be evidenced only when performing competitive inhibition of IgE-binding to nAra h 2 (with naturally occurring post-translational modifications), not to rAra h 2.

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**Table E1.** Clinical features and serum IgE concentrations from patients with peanut allergy

Patients no.	Age/sex	Symptoms	Specific IgE levels (IU/mL)		
			WPPE	Ara h 2	Ara h 6
1	6/M	A, R, U	190.0	77.8	45.0
2	3/F	AD	0.9	0.6	1.2
3	9/M	U	261.0	114.0	66.4
4	11/M	QO, GU, V	323.0	90.7	43.3
5	8/F	A	463.0	215.6	71.6
6	8/M	A,U,V	60.0	30.3	25.6
7	6/M	GU, V	155.0	52.2	26.4
8	2/M	CP	5.4	0.1	4.0
9	7/M	A, R	94.0	32.4	20.7
10	9/F	A, AP	20.6	12.6	0.9
11	6/M	GU	28.0	15.6	2.2
12	4.5/M	U	25.6	4.7	19.0
13	6/M	LO, U, V	348.0	122.8	88.9
14	5/M	LO, AS	338.0	122.0	23.0
15	9/F	LO, GU, V	629.0	293.0	124.0
16	13/M	V	156.0	40.2	54.2
17	15/M	GU, LO, A	64.0	19.2	17.2
18	13/F	GU, A	66.1	35.8	25.4
19	7/M	U, A	285.0	140.7	146.8
20	11/F	A, AO, AP, V	43.2	30.5	6.1
21	23/F	GU, A, AS	34.0	13.6	12.5

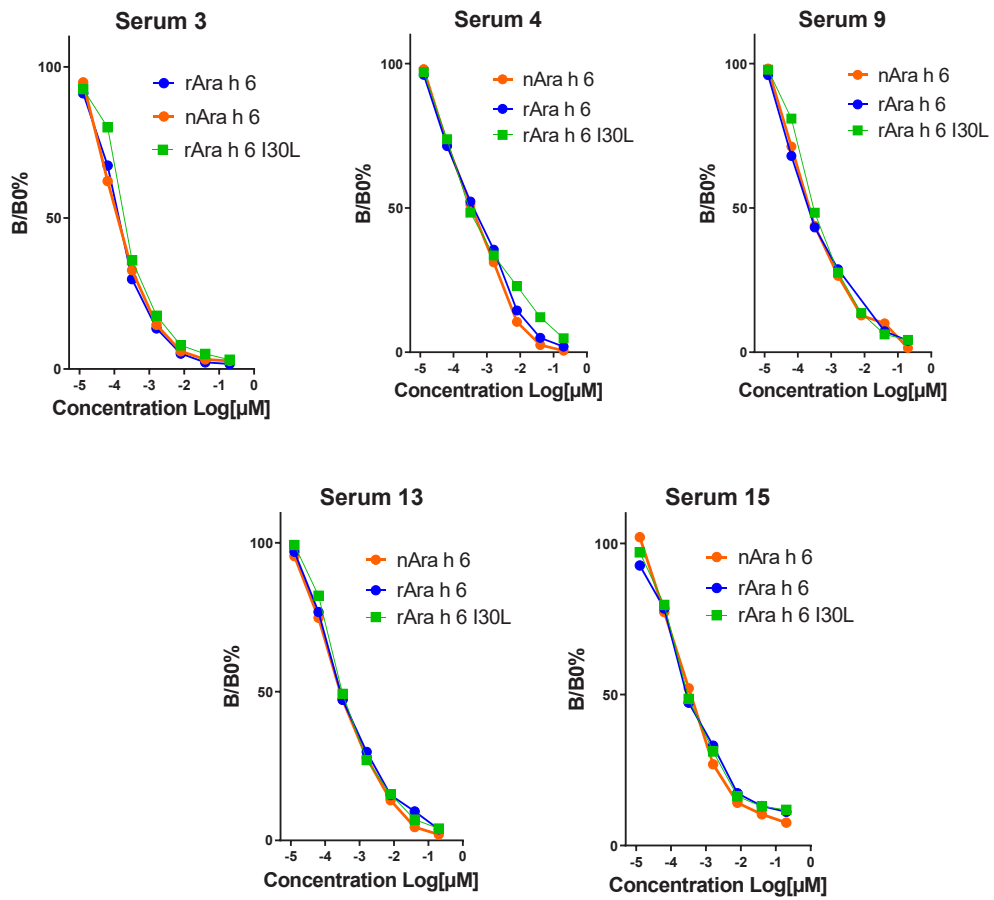
A, Asthma; AD, atopic dermatitis; AO, angioedema; AP, abdominal pain; AS, anaphylactic shock; CP, cutaneous pruritus; F, female; GU, generalized urticaria; LO, laryngeal edema; M, male; QO, Quincke edema; R, rhinitis; RC, rhino-conjunctivitis; U, urticaria; V, vomiting; WPPE, whole peanut protein extract.

## Supplementary data

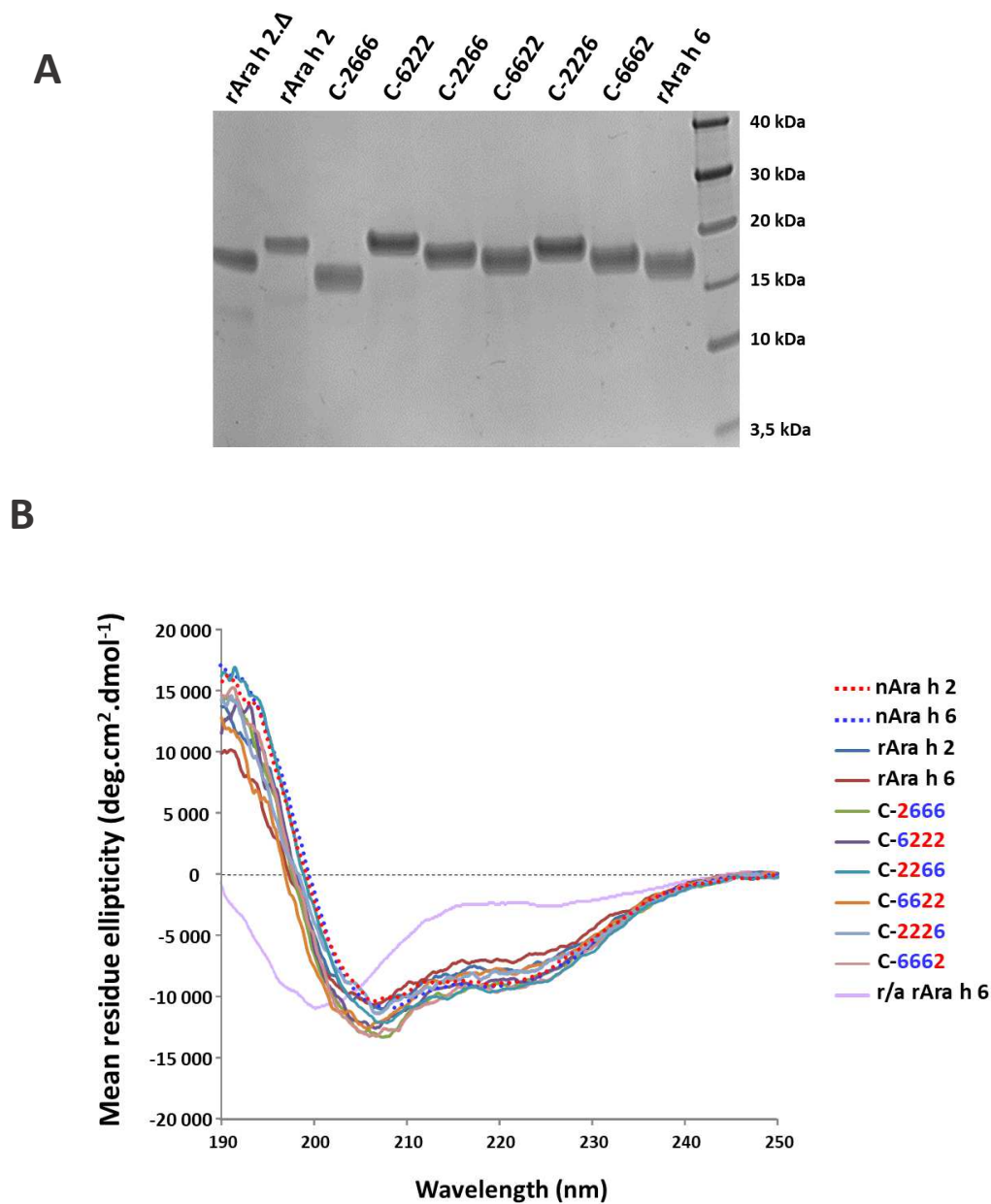
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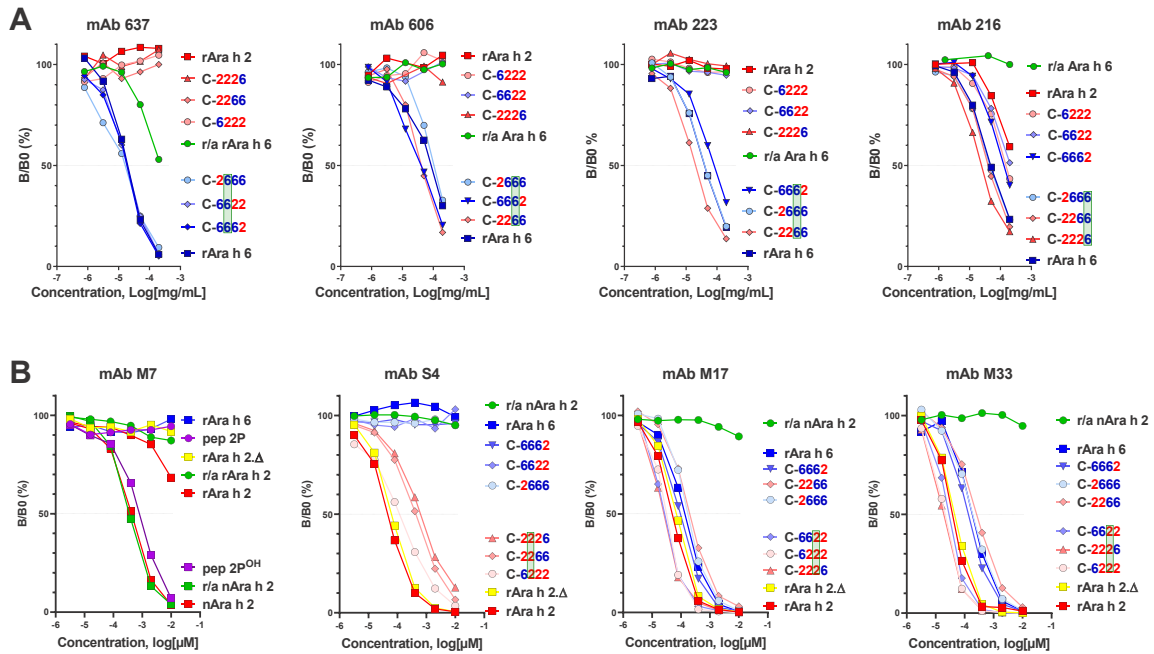
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**Fig. E1:** Comparison of the capacity of nAra h 6, rAra h 6 and rAra h 6 I30L to inhibit IgE-binding to nAra h 6. No significant difference was observed between native and recombinant forms of Ara h 6.

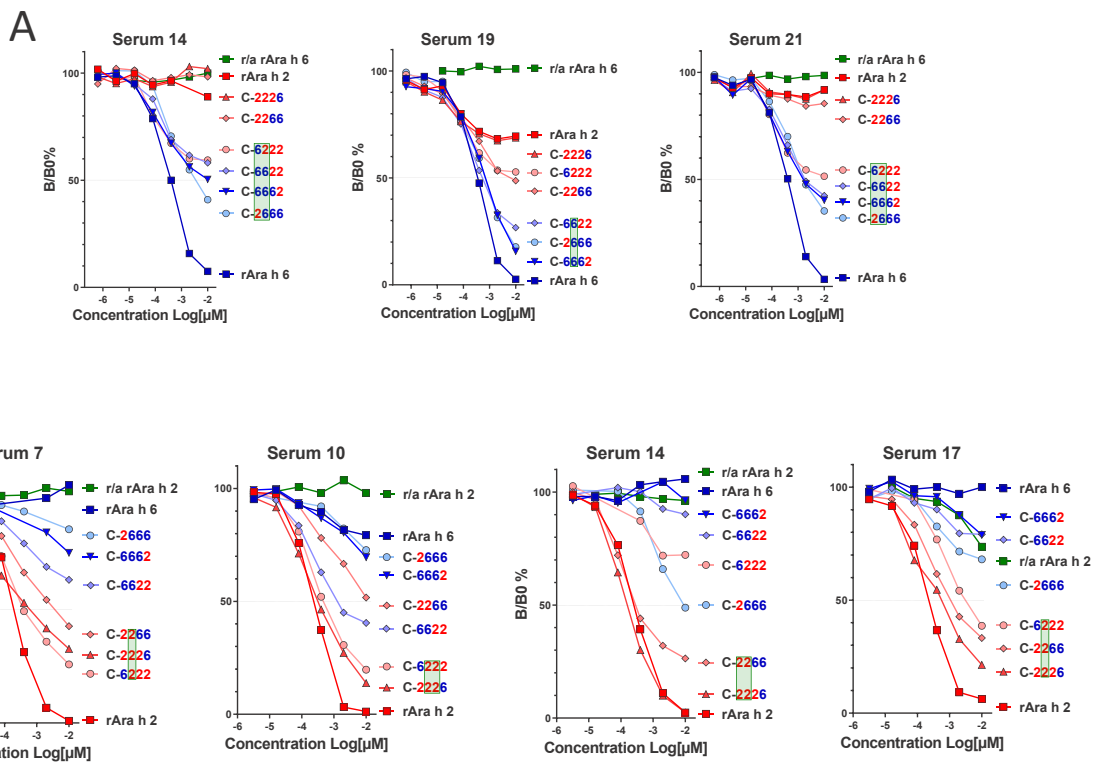


**Fig. E2:** (A) SDS-PAGE analysis of purified recombinant chimeras (A, 1  $\mu\text{g}$  protein/lane, on NuPage™ 4-12% Bis-Tris gels). (B) Circular dichroism analysis of the chimeras. nAra h 2, rAra h 2, nAra h 6, rAra h 6 and the chimeras exhibited spectra characteristic of 2S-albumin structure, with two minima at around 208 and 222 nm indicative of a high content of  $\alpha$ -helix. In comparison, reduced and alkylated (r/a) Ara h 6 displayed a spectrum characteristic of unfolded 2S-albumin with a minimum at 200 nm. rAra h 2 and rAra h 2.Δ were previously shown to exhibit similar CD spectra (Bernard *et al.*, JACI, 2015).

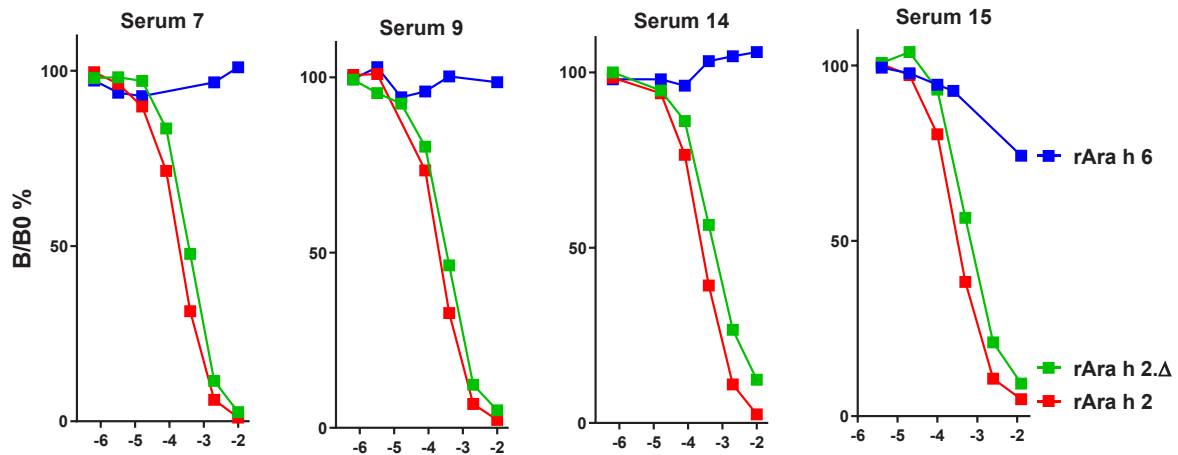
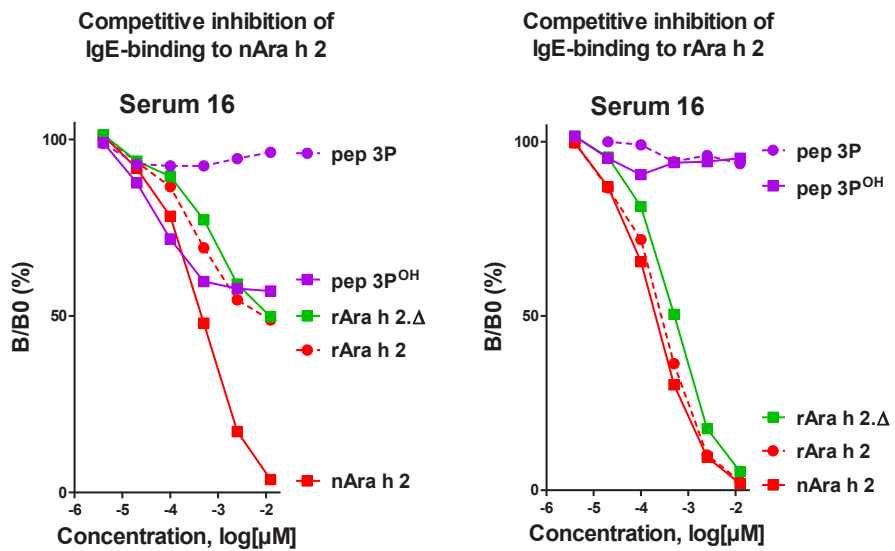


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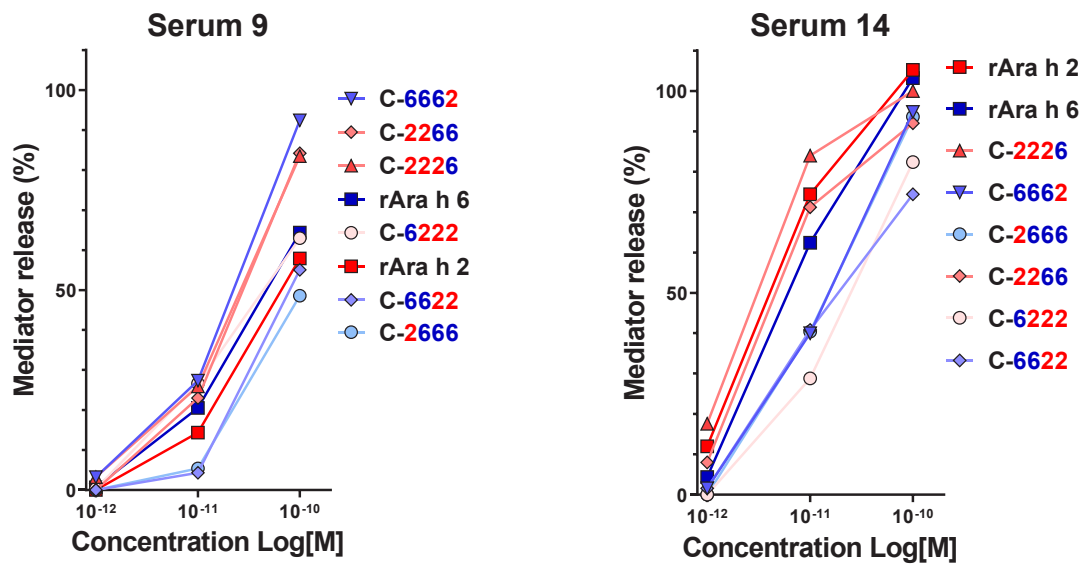




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**A****B**

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