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**Assessment of the allergenic potential of the main egg white
proteins in BALB/c mice**

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17 ABSTRACT: This work aimed to assess the contribution of the major egg white
18 proteins, ovalbumin, ovomucoid and lysozyme, to the induction and elicitation of
19 allergenic responses. For this purpose, BALB/c mice were orally administered either the
20 individual egg allergens or a mixture of the three proteins in the same proportion, in
21 order to evaluate their relative allergenicity avoiding their different abundance in egg
22 white. Cholera toxin was used as a Th2-polarizing adjuvant. Ovomucoid and lysozyme
23 triggered the most severe anaphylaxis reactions upon oral challenge. As compared with
24 ovalbumin and ovomucoid, lysozyme was a more active promotor of early IgE and
25 IgG1 production and it stimulated stronger Th2-biased responses from both mesenteric
26 lymph node and spleen cells. These results indicate that lysozyme is highly
27 immunogenic and should be considered as a major allergen whose clinical usefulness in
28 the diagnosis, prognosis and therapeutic approaches of egg allergy deserves further
29 consideration.

30 KEYWORDS: *BALB/c, egg allergy, lysozyme, ovalbumin, ovomucoid*

31 INTRODUCTION

32 Eggs are, together with milk, the foods most commonly linked to food allergy in
33 children, with a prevalence based on objective estimates that ranges between 0.5 and 2.5
34 %.¹ Egg allergy mainly affects young children, but even though, in most cases, it
35 resolves within the first years, a significant proportion of patients retains egg allergy
36 through life.²

37 Food proteins cause allergic reactions due to a failure to develop tolerance,
38 although it is not known exactly what triggers an inappropriate immune response
39 towards a particular protein.³ The major egg allergens are contained in the egg white:
40 ovomucoid (OM, Gal d 1, 28 kDa, 11% w/w of the egg white protein content),
41 ovalbumin (OVA, Gal d 2, 45 kDa, 54% w/w), ovotransferrin (OTf, Gal d 3, 78 kDa,
42 12% w/w) and lysozyme (LYS, Gal d 4, 14.3 kDa, 3.4% w/w).⁴ The primary sequence
43 and structural characteristics of these proteins are well known, however, beyond the fact
44 that epitope recognition varies broadly among allergic individuals, there are no common
45 IgE- and T-cell epitopes specifically designed to induce immune responses.⁵ Therefore,
46 it is difficult to find distinct features, except for a relatively high structural stability that
47 makes them resistant to digestion and/or heat treatment.⁶ Several studies point at a
48 predominant role of OM in egg allergy, although no clear consensus has been reached
49 as to the relative allergenicity of these proteins.⁷⁻¹⁰ On the other hand, the allergenic
50 potential of OTf and LYS has not been studied in depth, but while IgE reactivity to OTf
51 is low and OTf-specific antibody levels are found without clinical relevance for defining
52 the sensitizing profile of children with egg allergy, IgE reactivity to LYS is very
53 common.^{11,12} Furthermore, LYS from egg white is broadly used as an antibacterial
54 additive in food and pharmaceutical products, with the consequent risk of sensitization
55 or of development of adverse reactions in already sensitized patients.¹³⁻¹⁵

This paper addresses the capacity of the main egg allergens to sensitize and trigger the manifestations of food allergy. To this aim, the ability of native OVA, OM and LYS to promote sensitization and elicitation of allergic reactions was compared in BALB/c mice which were orally administered the individual egg allergens or a mixture of the three proteins in the same proportion, together with cholera toxin (CT) as a Th2-polarizing adjuvant. Equivalent quantities of each protein were used, regardless of their different molecular masses, in order to provide mice with comparable amounts of T cell and IgE-binding epitopes. Antibody and cytokine responses, as well as development of clinical signs upon challenge were assessed.

MATERIALS AND METHODS

Animals and proteins

Adult (6 weeks old) BALB/c mice were from Charles River Laboratories (Saint Germain sur l'Arbresle, France). All protocols involving animals followed the European legislation (Directive 2010/ 63/UE) and were approved by the CSIC Bioethics Committee and the Comunidad de Madrid (Ref PROEX 089/15).

OVA grade V, OM type III-O and LYS were purchased from Sigma (Sigma-Aldrich, St. Louis, USA). Their protein content was analysed by the bicinchoninic acid assay (BCA Pierce Protein Assay Kit, Thermo Scientific, Waltham, USA), yielding, respectively, 98.58, 98.64 and 99.98%. Except for OVA, which contained 446.0 EU/mg of lipopolysaccharide, as quantified by the Pierce LAL Chromogenic Endotoxin Quantitation Kit (Thermo Scientific), all the protein samples showed levels below 1.0 EU/mg. Consequently, commercial OVA was purified to less than 3 EU/mg by size exclusion chromatography.¹⁶

Sensitization and challenge protocols

The murine model of anaphylaxis followed Li et al.¹⁷ Mice were distributed in 8 groups of 5 mice. Three groups were sensitized by oral gavage by the administration of 1 mg per mouse of either OVA, OM or LYS, plus 10 µg of CT (List Biologicals, Campbell, USA), and another 3 groups were poly-sensitized with a mixture of 1 mg of each of the three proteins, plus 10 µg of CT, during 3 consecutive days on the first week and once per week during the following 6 weeks. There were two control groups: sham-sensitized mice, which received 10 µg of CT, and naïve mice, which just received PBS (Supporting Figure 1).

One week after the last sensitization dose, OVA, OM and LYS-sensitized mice were challenged with 20 mg of OVA, OM or LYS per mouse, respectively. Each group of poly-sensitized mice was also challenged with OVA, OM or LYS. Sham-sensitized and naïve mice were administered PBS. Anaphylactic responses were evaluated by measuring the body temperature decrease with a rectal thermometer (Panlab, Cornellá, Spain) and scoring clinical signs 30 min after challenge with the following scale: 0= no signs; 1= scratching nose and mouth less than 10 times in 15 min; 2= puffiness around eyes and mouth, scratching nose and mouth more than 10 times in 15 min; 3= wheezing and labored respiration, cyanosis around the mouth and tail, diarrhea and difficulty in walking normally; 4= no activity after prodding, and 5= death. Mice were then sacrificed by CO₂ inhalation.

Measurement of antigen-specific immunoglobulins and mouse mast cell protease-1

Blood samples (250 µL) were extracted by cheek puncture 1 day after the fifth and last sensitization doses (days 15 and 43, respectively), centrifuged at 1500 *x g* for 15 min to

104 collect sera and kept at -20°C until analysis. Protein-specific IgE and IgG1 antibodies
105 were quantified by indirect ELISA.¹⁸ Briefly, 96-well plates were coated with 5 µg/mL
106 (for IgE) or 2 µg/mL (for IgG1) of protein (OVA, OM or LYS). Blocked plates were
107 incubated overnight at 4°C with serum samples (1/25 diluted for IgE and 1/1000 or
108 1/5000 diluted for IgG1). Subsequently, plates were incubated with biotin rat anti-
109 mouse IgE or IgG1 and streptavidin-HRP (BD Biosciences). Colorimetric reactions
110 were read at 405 nm in a plate reader (Multiskan FC, Thermo Scientific) after addition
111 of 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) as substrate (Roche,
112 Mannheim, Germany). Mast cell degranulation was evaluated after challenge by
113 measuring serum concentrations of mast cell protease-1 (MCP-1) with a commercial kit
114 (eBioscience, San Diego, USA).

115 **Histology**

116 Samples of duodenum (1 cm) were collected, fixed with formalin, dehydrated using
117 graded series of alcohol and embedded in paraffin wax. Four-micrometre-thick sections
118 were cut from each sample and stained with hematoxylin and eosin. The histological
119 analyses were carried out by direct microscopic examination with a DM2500
120 microscope, equipped with a DFC420 camera, using the Application Suite software (all
121 from Leica Microsystems, Wetzlar, Germany).

122 **Intestinal gene expression**

123 Segments of duodenum (10-15 mg) were freshly and individually isolated from each
124 group of mono-sensitized and naïve mice. RNA was extracted by using NucleoSpin
125 RNA Kit (Macherey-Nagel GmbH & Co., Düren, Germany) and cDNA was synthesized
126 using PrimeScript RT reagent kit (TaKaRa Bio Inc., Shiga, Japan). qPCR was
127 performed in a real-time thermocycler (ViiA7 Real-Time PCR System, Applied

128 Biosystems, Foster, USA) using SYBR Premix ExTaq II (TaKaRa). Relative gene
129 expression of IL-33 and TSLP was normalized to that of the reference gene β -actin and
130 expressed as fold increase compared with the levels measured in naïve mice. Primer
131 pairs and optimized thermal cycling conditions are described elsewhere.¹⁹ All the
132 amplification reactions were performed in triplicate.

133 **Cell culture and quantitation of cytokines**

134 Immediately after sacrifice, spleen and mesenteric lymph node (MLN) cells were
135 collected and processed under sterile conditions. Splenocytes from individual animals
136 and MLN cells pooled from each group were cultured in 48-well plates (4×10^6
137 cells/mL) and stimulated in duplicate with concanavalin A (2.5 μ g/mL), as a positive
138 control, RPMI-1640 medium, as a negative control, OVA, OM or LYS (200 μ g/mL).
139 Supernatants were collected after 72 h at 37°C in 5% CO₂, and stored at -80°C until
140 analysis of cytokines (IL-4, IL-5, IL-13, IL-10 and IFN- γ) by ELISA, using commercial
141 kits (eBioscience).

142 **Statistical analyses**

143 All data are means $\pm$ SEM, except for clinical signs, which are expressed as medians.
144 Differences were determined by applying one-way ANOVA followed by Tukey post-
145 hoc test or by Mann-Whitney U test (clinical sign scores and gene expression data).
146 Changes in mean immunoglobulin levels over different time points were assessed by
147 repeated measures ANOVA. p values <0.05 were considered statistically significant.
148 Statistical analyses were performed using GraphPad Prism v5 (GraphPad Software Inc.,
149 San Diego, USA).

150

151 RESULTS

152 Sensitizing capacity of the major egg allergens

153 Naïve and sham-sensitized mice produced negligible amounts of egg-protein specific
154 serum IgE and IgG1 during the sensitization period (Figure 1). LYS induced higher
155 specific serum IgE levels than OVA and OM 15 days after the starting point of the
156 sensitization phase, although the concentration of LYS-specific IgE antibodies
157 significantly decreased thereafter and it was the lowest at the end of the sensitization
158 protocol (day 43). Specific IgE antibodies were higher in the sera of animals from the
159 OVA group than in those from OM group at day 15, although, at day 43, they showed
160 equivalent serum concentrations of OVA- and OM-specific IgE, respectively. LYS also
161 induced the highest serum concentrations of IgG1 at the beginning of the sensitization
162 period, although, at day 43, the amount of specific IgG1 in the animals of the LYS
163 group was similar to that of the OM group. In general terms, there were no differences
164 in the specific IgE and IgG1 levels in the sera of mono- and poly-sensitized animals
165 (Figure 1).

166 Eliciting capacity of the major egg allergens

167 One week after the last sensitization dose, mice sensitized to OVA, OM and LYS were
168 orally challenged with the respective allergen, while mice poly-sensitized to these
169 proteins were divided in three groups and challenged with each of the allergens. A
170 significant decrease in rectal temperature, with respect to sham-sensitized or naïve mice,
171 was only detected in mice sensitized to OM and LYS and challenged, respectively, with
172 these proteins, as well as in the poly-sensitized mice challenged with LYS (Figure 2a).
173 All the sensitized mice experienced significant clinical signs following oral challenge.
174 The most severe reactions were observed in mice sensitized and challenged with OM

175 and LYS (Figure 2b). Serum MCP-1 values were the highest in the LYS-sensitized
176 group, followed by the OM- and OVA-groups. Mast cells from mice mono-sensitized to
177 OM and LYS responded more strongly than poly-sensitized mice to the administration
178 of these proteins (Figure 2c). Similarly, hematoxylin and eosin staining of duodenal
179 sections showed signs of intestinal inflammation in mono-sensitized mice, but also in
180 sham-sensitized mice, even though in this group nor diarrhoea or high specific IgE and
181 MCP-1 levels were detected (Supporting Figure 2). The degree of inflammatory
182 infiltrate in the lamina propria was of similar intensity in sensitized and sham-sensitized
183 animals, but more evident in LYS-sensitized mice.

184 **Regulation of intestinal cytokines**

185 Analyses of IL-33 and TSLP expression were conducted in the duodenum of the mono-
186 sensitized mice and changes relative to the naïve group were calculated. Sensitization to
187 the 3 proteins significantly upregulated IL-33 and mice sensitized to OM and LYS had
188 significantly higher duodenal expression levels of IL-25 than naïve mice (Supporting
189 Figure 3).

190 **Immunostimulating capacity in mesenteric lymph node and spleen cells**

191 MLN and spleen cells were stimulated with the proteins used for sensitization. Within
192 the mono-sensitized animals, maximum production of the Th2-cytokines IL-4, IL-5 and
193 IL-13, as well as of IL-10, was detected in the MLN cells from mice belonging to the
194 LYS-sensitized group following stimulation with this protein (Figure 3). Production of
195 IFN- γ by MLN cells was negligible in all cases (not shown). Similarly, LYS induced
196 the highest spleen cell production of IL-4, IL-13 and IFN- γ (Figure 4). In addition,
197 LYS, as well as OVA, caused a more abundant release of IL-5 and IL-10 by the
198 splenocytes, compared with OM. In fact, stimulation of the spleen cells of mice mono-

199 sensitized to OM with this protein did not significantly increase the production of any of
200 the cytokines tested with respect to the sham-sensitized mice. Likewise, IL-4, IL-13 and
201 IFN- γ levels were not statistically different in OVA-stimulated spleen cells of OVA-
202 sensitized and sham-sensitized mice (Figure 4). In general terms, MLN and spleen cells
203 from poly-sensitized mice produced comparable or lower cytokine levels than those
204 from the mono-sensitized groups upon stimulation, with similar responses to the
205 different allergens (Figures 3 and 4).

206 **DISCUSSION**

207 This work aimed to assess the relative allergenicity of the egg white proteins regarded
208 as major allergens, OVA, OM and LYS. To accomplish this, we used the BALB/c
209 mouse model to compare the capacity of these proteins to induce oral sensitization and
210 elicitation of allergic reactions. Because the oral route is not inherently sensitizing, CT
211 was used as a Th2-driving agent. It should be highlighted that, although the use of CT
212 has been reported to level the allergic response to different allergens due to its strong
213 adjuvant potential,²⁰ this experimental model proved useful to discriminate among the
214 immunogenicity of the three proteins under study, as it was already found for native and
215 heated egg and milk allergens.^{18,21,22} Mice were sensitized either to the native individual
216 egg allergens or to a mixture of the three proteins in the same proportion. Because
217 numerous T cell and IgE-binding epitopes, evenly distributed along the structure of
218 these allergens, have been described,⁶ we opted for using the same amount of each
219 protein for sensitization and challenge, in order to provide mice with equivalent epitope
220 loads, following an approach similar to other comparative studies.^{21,22}

221 While OVA is the most abundant egg white protein, OM is regarded as the
222 immunodominant component.⁷ In our study, OM-sensitized BALB/c mice experienced
223 higher temperature drops and clinical scores than OVA-sensitized mice following oral

challenge, with similar serum levels of specific IgE and ex vivo spleen cell production of IL-4, IL-13 and IFN- γ upon stimulation, which is in agreement with the results of Martos et al.²¹ in C3H/HeJ mice. In addition, as compared with OVA, oral administration of OM plus CT resulted in a higher concentration of IgG1 antibodies, which can also mediate anaphylaxis in this model,^{18,23} and increased allergen-specific ex vivo IL-4 and IL-13 responses in the MLNs, indicative of a more pronounced Th2-bias.

As well as OM, LYS triggered severe anaphylactic responses despite, before challenge, the concentration of circulating LYS-specific IgE was lower than those of OM- and OVA-specific IgE. A lack of correlation between serum allergen specific IgE levels and clinical reactivity to egg allergens has already been reported in mice and humans.^{24,25} In this respect, Jiménez-Saiz et al.²⁶ demonstrated that murine mast cells remain loaded with clinically-relevant levels of IgE in the absence of detectable serum IgE, being able to produce anaphylactic reactions to allergens for a further 100 days. Thus, it is possible that the depletion of circulating LYS specific IgE concentration detected during the sensitization period reflected a redistribution of the available serum IgE pool to immunologically arm mast cells and basophiles. Indeed, up-regulation of mast-cell Fc ϵ RI surface expression is considered an amplification loop of the allergic disease in cases of high concentrations of circulating IgE.²⁷

In our model, we found no major differences in the gene expression of Th2-driving intestinal cytokines among mice sensitized to OVA, OM or LYS, although the lowest expression of TSLP corresponded to mice sensitized to OVA, which, in general terms, behaved as the least immunogenic and allergenic protein. Sensitization to LYS caused a distinct Th2 biasing effect compared with OVA and OM, as judged by the Th2-cytokine responses generated ex vivo on MLN and spleen cells of sensitized mice. In line with this observation, a recent study showed that LYS induces a higher and more

249 persistent production of IL-5 and IL-13 than the other egg proteins on peripheral blood
250 mononuclear cells (PBMCs) from patients with IgE-mediated egg allergy.²⁸
251 Nevertheless, our study indicates that LYS also increased the production of the Th1-
252 cytokine IFN- γ by spleen cells, suggesting that it enhanced, to a greater extent than the
253 other proteins, the adaptive immune priming of CT, which induces mixed T cell
254 responses, characterized by the significant production of IL-4, IL-13, IFN- γ and IL-17.²⁹

255 It is documented that LYS partially resists pepsin digestion at pH ≥ 2 and that it
256 subsequently precipitates under simulated duodenal conditions because of its
257 electrostatic interaction with bile salts, which helps it to avoid digestion by pancreatic
258 enzymes.^{30,31} Therefore, it can be speculated that LYS aggregation may promote its
259 delivery through Peyer's patches, with consequent transport to the draining MLN and
260 increase in immunogenicity.²² The observation that LYS orally administered to mice is
261 capable of causing a high degree of sensitization, as shown by the enhanced early
262 production of antibodies and release of Th2 cytokines, as well as strong anaphylactic
263 reactions, suggests that both aggregated LYS and either soluble LYS or LYS-digestion
264 products, which may gain access through the absorptive intestinal epithelial cells to
265 effector cells, contribute to the different phases of the allergic response.

266 In egg white-sensitized mice, we previously found serum levels of specific IgE
267 in the order OM $\geq$ OVA $>$ LYS, with LYS also promoting an early production of specific
268 IgE antibodies which subsequently declined, and comparable concentrations of OVA-,
269 OM- and LYS-specific IgG1, despite these proteins are present in egg white in quite
270 different proportions.¹⁸ Equivalent results were obtained in the present study in mice
271 that were poly-sensitized to the same amount of the three proteins, in an attempt to
272 assess their relative allergenicity avoiding their different abundance in egg white. In
273 general terms, poly-sensitized mice behaved similarly to mono-sensitized ones in

antibody and anaphylactic responses induced by the respective allergen, although MLN and spleen cells from poly-sensitized mice responded with a lower production of Th2-cytokines to the stimulation with LYS than those from LYS-sensitized mice. Altogether, these results indicate that LYS is highly immunogenic and able to elicit anaphylactic reactions upon oral challenge, even if its contribution to egg white allergenicity may be concealed by its low relative abundance.

Whereas it has been suggested that sensitization to egg allergens may depend on individual susceptibility rather than the nature of the antigen, the measurement of specific antibodies to individual egg white components, mainly OM-specific IgE, has proved useful to predict different clinical patterns of egg allergy.³²⁻³⁴ Since the contribution of LYS to the allergic response to egg has been largely ignored, analysis of LYS-binding antibodies is not usually included in the characterization of the sensitization profile.^{12,35} In this respect, even if its value as an indicator of clinical reactivity needs to be investigated in detail,³⁶ a comprehensive allergen-based microarray study has shown that LYS is the fourth food allergen most frequently recognized by a cohort of 16,408 subjects with IgE reactivity (above OVA and OM); as well as that with the highest prevalence within egg white allergens in patients exceeding 16 years of age.¹¹ Furthermore, PBMC responses to LYS in 4 month-old children have been shown to significantly predict egg allergy at 12 months.²⁸ Another relevant aspect concerns its medicinal or antimicrobial use. In fact, allergic reactions to LYS-containing drugs have been described not only in egg allergic patients, but also in children that had never previously eaten egg.¹³

In conclusion, the present work shows that LYS is an active promotor of sensitization and elicitation of the allergic reaction in a mouse model of egg allergy. The clinical usefulness of this protein in the diagnosis, prognosis and therapeutic approaches

299 of egg allergy, as well as the assessment of the allergic risk posed by products
300 containing LYS as a preservative, deserves further investigation.

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304 MECD (Spain), respectively.

305 **Notes**

306 The authors have no financial conflicts of interest.

307 **ABBREVIATIONS USED**

308 CT, cholera toxin; LYS, lysozyme; MCP-1, mast cell protease-1; MLN, mesenteric
309 lymph nodes; OVA, ovalbumin; OM, ovomucoid; PBMCs, peripheral blood
310 mononuclear cells

311

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424

FIGURE LEGENDS

Figure 1. Protein-specific immunoglobulin levels in the sera of mice sensitized to egg proteins on days 15 and 43 of the sensitization period. Mice were orally sensitized to OVA, OM or LYS plus CT, or to a mixture of the three proteins in the same proportion (poly-sensitized mice). Sham-sensitized (identified as CT) and naïve mice were used as controls. Data are expressed as means $\pm$ SEM ($n= 5$). Different lowercase letters indicate statistically significant differences ($p< 0.05$) between mouse groups at each time point and different uppercase letters indicate statistically significant differences ($p< 0.05$) between day 15 and 43 within each group.

Figure 2. Anaphylaxis in mice sensitized and challenged with egg proteins. Mice were orally sensitized to OVA, OM or LYS plus CT, and challenged with 20 mg per mouse the respective proteins, or sensitized to a mixture of the three proteins in the same proportion (poly-sensitized mice), and challenged with each of the proteins. Sham-sensitized (identified as CT) and naïve mice were challenged with PBS. Anaphylaxis was assessed by rectal temperature (a), clinical signs (b), and serum concentration of MCP-1 (c). Data are expressed as means $\pm$ SEM (a, c) or medians (b) ($n= 5$). Different letters indicate statistically significant differences ($p< 0.05$).

Figure 3. Cytokine production by MLN cells of mice sensitized to egg proteins, following stimulation with OVA, OM or LYS. Mice were orally sensitized to OVA, OM or LYS plus CT, or to a mixture of the three proteins in the same proportion (poly-sensitized mice). Sham-sensitized (identified as CT) and naïve mice were used as controls. Data are expressed as means $\pm$ SEM (pooled MLN from 5 mice stimulated in duplicate). Different letters indicate statistically significant differences ($p< 0.05$).

448 **Figure 4.** Cytokine production by spleen cells of mice sensitized to egg proteins,
449 following stimulation with OVA, OM or LYS. Mice were orally sensitized to OVA,
450 OM or LYS plus CT, or to a mixture of the three proteins in the same proportion (poly-
451 sensitized mice). Sham-sensitized (identified as CT) and naïve mice were used as
452 controls. Data are expressed as means $\pm$ SEM (n= 5). Different letters indicate
453 statistically significant differences ($p < 0.05$).

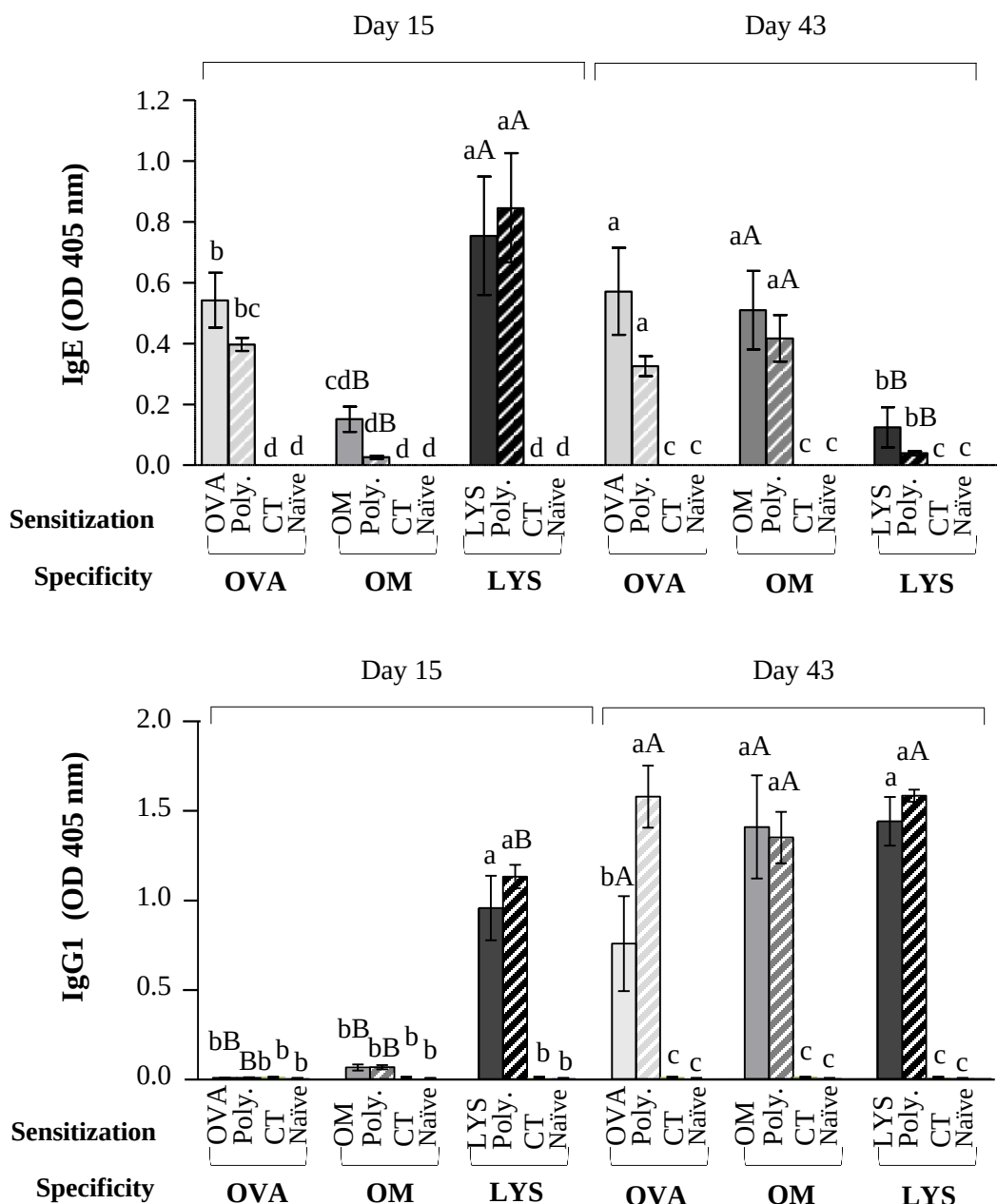
Figure 1.

Figure 2.

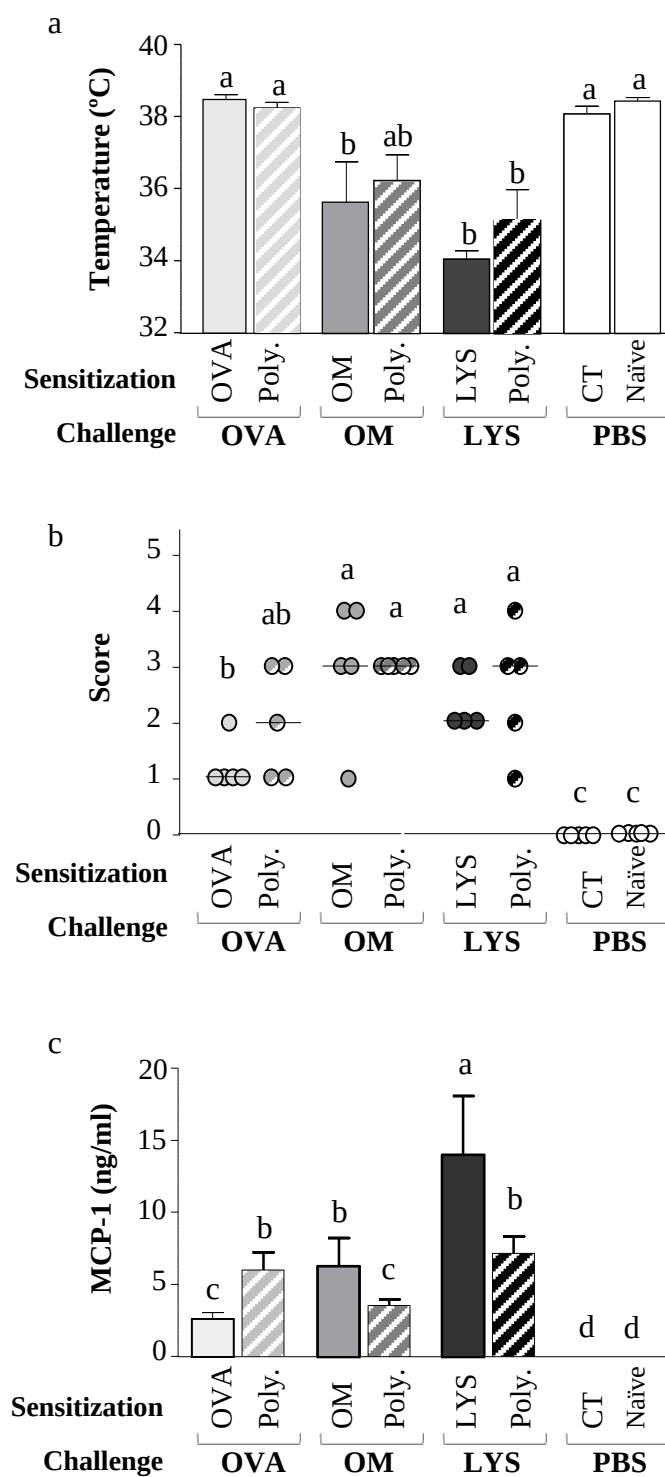


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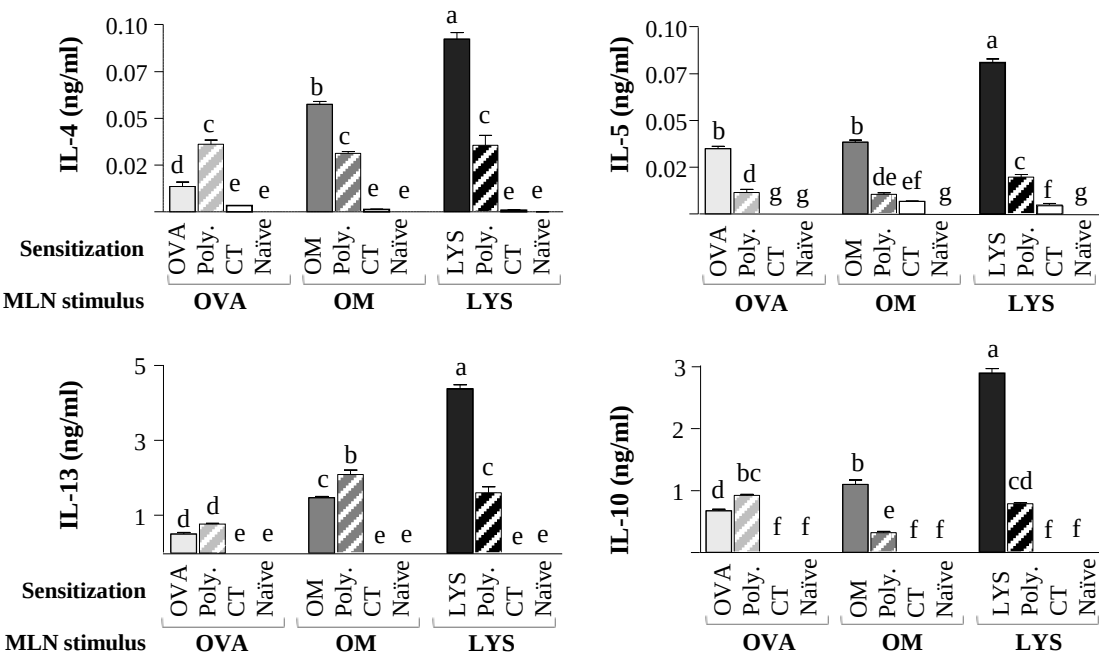


Figure 4.