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1 # Hydrolysates of egg white proteins modulate T- and B-cell responses in
2 mitogen-stimulated murine cells

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20 This work assessed the effects of hydrolysates of ovalbumin (OVA), lysozyme (LYS),
21 ovomucoid (OM) and whole egg white (EW) on cytokine secretion, antibody
22 production, oxidative stress and proliferation of murine spleen and mesenteric lymph
23 node cells stimulated with T- (concanavalin A -ConA-) or B-cell mitogens
24 (lipopolysaccharide -LPS-). The hydrolysates of OVA, LYS and EW with alcalase
25 reduced ConA-stimulated lymphocyte proliferation, production of Th2-biased
26 cytokines, such as IL-13 and IL-10, and decreased the secretion of the Th1 cytokine
27 TNF-$\alpha$. In addition, these hydrolysates considerably inhibited IgG1-class switching
28 induced by LPS and counteracted the release of reactive oxygen species. EW peptides
29 modulated the immune responses of murine cells to mitogen stimuli, revealing potential
30 activities that could be used for different purposes as Th1- or Th2-skewing mediators.

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32 # 1. Introduction

33 During the past few years, increasing evidence has pointed out the existence of peptides
34 which specifically act on the immune system. The immunomodulating activity of these
35 peptides comprises effects on cell migration, survival and proliferation, as well as
36 stimulation of antimicrobial and immune signaling molecules. Thus, various peptides
37 have shown immunosuppressive properties, of interest in tissue transplantation,
38 autoimmune and inflammatory conditions, as well as immunostimulating properties,
39 paving the way for new immunomodulatory therapies.[1]

40 Although existing studies on the effects of immunomodulating peptides have
41 focused mainly on cells of the innate immune system, there is evidence that they alter T
42 and B lymphocyte functions and could contribute to a balanced Th1 and Th2 response.[2]
43 The modulation of the interaction between Th1- and Th2-type immunity has strong
44 implications in various clinical conditions. Th1 cells are activated in the course of
45 immune responses against pathogens, releasing cytokines, such as IL-2, IFN-γ and
46 TNF-α, which trigger cellular defense mechanisms and also induce B cells to produce
47 opsonizing antibodies belonging to certain IgG subclasses; while Th2 cells mainly
48 release IL-4, IL-5 and IL-13, responsible for the maturation of B cells into antibody-
49 producing plasma cells, including IgE, which mediates parasitic defense and allergic
50 reactions. Other lymphocyte T subsets, such as IL-17-producing Th17 cells, which
51 amplify inflammatory responses, and regulatory T cells (Treg), that exert direct
52 inhibitory effects and release anti-inflammatory factors, such as IL-10 and TGF-β,
53 contribute to complement and equilibrate effector responses against microbial infections
54 and tumor cells, allergy and oral tolerance to antigens, and autoimmune diseases.[3,4]



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55 Whereas the use of known bioactive peptides as templates to synthetize
56 optimized peptides with the ability to modulate the immune response has proved
57 effective in the formulation of new sequences with better properties, the high cost
58 associated with generating synthetic peptides on a large scale is an important obstacle to
59 their production.[1] In this respect, hydrolysis of food proteins appears as an attractive and
60 safe alternative for a reproducible and standardized production of immune-active
61 peptides with low costs that would allow their consumption in the form of functional
62 foods. However, while a number of potentially immunomodulating peptides have been
63 described as arising from food proteins, such as milk whey proteins, contradictory
64 results are frequently reported regarding their biological properties, which could be due
65 to the use of assay systems that fail to correlate with a specific immune response or to
66 the contamination of the peptide preparations with immunostimulating substances.[5,6]

67 Egg white protein hydrolysates are good candidates for non-pharmacological
68 approaches to prevent and treat complex diseases by virtue of their multifunctional
69 properties, which comprise antihypertensive, antioxidant, antimicrobial and lipid-
70 lowering activities.[7,8] The aim of this work was to search for immunomodulating
71 enzymatic hydrolysates of egg proteins using murine cell cultures. For this purpose, the
72 effects of the hydrolysates on cytokine secretion and antibody production, as well as on
73 oxidative stress and proliferation, by spleen and mesenteric lymph node cells stimulated
74 with T- or B-cell mitogens were assessed.

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80 ## 2. Materials and methods

81 ### 2.1. Production of enzymatic hydrolysates of egg white proteins

82 Ovalbumin grade VI (OVA), lysozyme (LYS) and ovomucoid (OM) were purchased
83 from Sigma-Aldrich (St. Louis, MO, USA). Egg white (EW) was separated from fresh
84 eggs, lyophilized, and its protein content was determined by the Kjeldahl method. The
85 lipopolysaccharide (LPS) level of the proteins was quantified by the Pierce LAL
86 Chromogenic Endotoxin Quantitation Kit (Thermo scientific, Waltham, MA, USA),
87 according to the instructions of the manufacturer. The LPS contamination of LYS, OM
88 and EW was lower than 1 UE/mg and they were used as such; while OVA was purified
89 from LPS using size exclusion chromatography, as described by Brix et al.[5] This
90 procedure reduced the LPS content of OVA from 445.8 UE/mg to 3 UE/mg.

91 Protein solutions (5 mg/ml) were prepared in either MilliQ water adjusted to pH
92 1.5 (for hydrolysis with pepsin) or in phosphate buffer pH 7.0 (for hydrolysis with
93 Neutrase and alcalase). Protein solutions were pre-incubated at the reaction temperature
94 for 10 min prior to the addition of either 172 U/mg protein of porcine pepsin (EC
95 3.4.23.1, 3440 U/mg, Sigma-Aldrich), 0.025 U/mg of Neutrase or 0.005 U/mg of
96 alcalase (both from Novozymes A/S, Bagsvaerd, Denmark). Hydrolyses were
97 conducted, under continuous shaking, at 37ºC for 24 h with pepsin or at 50ºC for 60 min
98 with Neutrase and alcalase. The reaction with pepsin was neutralized to pH 7.0 and all
99 the enzymes were inactivated by heating at 95ºC for 15 min. The samples were then
100 centrifuged at 5000x*g* for 10 min (4ºC) and the supernatants were stored at -20ºC prior
101 to subsequent analyses. The protein content of the hydrolysates was determined using
102 the bicinchoninic acid assay (Pierce BCA Protein Assay Kit, Thermo Scientific) and the
103 resulting values were used to standardize their concentration for further experiments.



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104 **2.2. RP-HPLC**

105 The hydrolysates were analyzed in a Waters 600 HPLC system (Waters, Milford, MA,
106 USA) using a RP318 column (250x4.6mm, Bio-Rad). The operating conditions were:
107 flow rate, 1 ml/min; injection volume, 50 µL; solvent A, 0.37 mL/L trifluoroacetic acid
108 (TFA) in Milli-Q water; and solvent B, 0.27 mL/L TFA in HPLC grade acetonitrile
109 (ACN). Elution was performed with a linear gradient of solvent B in A going from 0 to
110 70% in 75 min, followed by 100% B for 30 min. Absorbance was recorded at 214 nm
111 with a Waters 2487 dual detector and the software Empower 2000 system data (Waters)
112 was used.

113 **2.3. MALDI-TOF mass spectrometry**

114 Peptide mass distribution of the hydrolysates was analyzed by MALDI-TOF using a
115 Bruker AUTOflex Speed spectrometer (Bruker Daltonik GmbH, Bremen, Germany).
116 The hydrolysates (0.5 µL dissolved at a concentration of 5 µg/mL) were loaded on a dry
117 2.5-dihydroxybenzoic acid (DHB) matrix spot (0.5 µL of 20 mg/mL DHB in
118 ACN/methanol, 70/30%, containing 1% TFA) onto a Bruker Anchorchip target. All
119 mass spectra were initially calibrated with Peptide Calibration Standard and Protein
120 Calibration Standard I (Bruker Daltonik). Mass spectra were acquired in positive
121 reflection, by summing 50 laser pulses at a fixed slide target position, using a 337 nm
122 nitrogen laser and an acceleration voltage of 20 kV.

123 **2.4. Spleen and mesenteric lymph node cell cultures**

124 All animal procedures were approved by the CSIC Bioethics Committee and followed
125 the current EU legislation (Directive 2010/63/EU on the protection of animals used for
126 scientific purposes). Nine 6-week-old female BALB/c mice, obtained from Charles



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127 River (St-Constant, Canada), were sacrificed by $CO_2$ inhalation. Whole spleens and
128 mesenteric lymph nodes (MLN) were excised, separately pooled in 3 groups (which
129 were used to perform biological replicates) and processed as described by Jiménez-Saiz
130 et al.[9] Splenocytes and MLN cells were cultured in RPMI 1640 supplemented with 10%
131 of fetal bovine serum, L-glutamine (2 mM), penicillin (50 U/mL) and streptomycin (50
132 μg/mL) (all from Biowest SAS, Nuaillé, France), at a cellular density of $4 \times 10^6$ cells/mL
133 in 48-well plates (non-stimulated control), and incubated with either 1 μg/mL
134 concanavalin A (ConA) or 1 μg/mL LPS (both from Sigma-Aldrich) and different
135 concentrations (20-200 μg/mL) of the EW proteins and their hydrolysates.[10] The effect
136 of the heat-inactivated enzymes was evaluated as described above in a separate
137 experiment, using splenocytes isolated from 4 mice pooled in 2 groups and incubated
138 with a concentration of enzyme equivalent to that present in 200 μg/mL of the
139 hydrolysates. The supernatants were collected after 72 h of culture in a 5% $CO_2$
140 incubator at 37ºC, and stored at -80ºC until their analyses, which were carried out at
141 least in duplicate.

142 **2.5. Cytokines and IgG isotypes**

143 The levels of IFN-γ, TNF-α, IL-13 and IL-10 were quantified in the supernatants of
144 ConA-stimulated splenocytes using ELISA kits according to the manufacturer's
145 instructions (eBioscience, San Diego, USA). The levels of IgG1 and IgG2a in the
146 supernatants of LPS-stimulated splenocytes and MLN cells were determined by ELISA.
147 Briefly, 96-well plates were coated with rat anti-mouse IgG1 and IgG2a (BD
148 Biosciences, San Diego, USA) and, after an overnight incubation at 4ºC, plates were
149 blocked and incubated at 4ºC with the culture supernatants for 12 h. Plates were then
150 incubated with biotinylated rat anti-mouse IgG1 and IgG2a, followed with avidin-



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151 horseradish peroxidase (HRP) (all from BD Biosciences). A signal amplification
152 system, based on the subsequent addition of tyramide-biotin and streptavidin-HRP, was
153 used following the instructions of the manufacturer (ELAST ELISA amplification
154 system, Perkin-Elmer Life Sciences, Waltham, MA, USA). The reactions were
155 developed using ABTS as substrate (Roche, Mannheim, Germany) and read at 405 nm
156 in a plate reader (Multiskan FC, Thermo Scientific). The results of the cytokines and the
157 IgGs isotypes were expressed in pg/mL.

158 **2.6. Intracellular reactive oxygen species**

159 The intracellular formation of reactive oxygen species (ROS) was assessed using the
160 dichloro-dihydro-fluorescein diacetate (DCFH-DA) assay. After 72 h of incubation,
161 spleen and MLN cell cultures, incubated with 1 µg/mL LPS and 200 µg/mL of the EW
162 proteins and their hydrolysates, were labeled with 2.5 µM DCFH-DA in Hanks
163 balanced salt solution (Biowest SAS) and incubated in dark for 45 min at 37ºC.
164 Afterwards, cells were washed twice with PBS and subjected to chemical oxidative
165 stress with *tert*-butyl hydroperoxide (*t*-BOOH) (Sigma-Aldrich) for 15 min.
166 Fluorescence emission due to the oxidation of DCFH-DA by intracellular ROS was read
167 at excitation and emission wavelengths of 485 nm and 528 nm, respectively, in a
168 Polarstar Galaxy plate reader (BMG Labtechnologies GmbH, Offenburg, Germany).
169 The results were expressed as percentage of the value corresponding to the LPS-
170 stimulated cells.

171 **2.7. Cell proliferation and viability**

172 The proliferation assays were carried out as described by Quah and Parish,[11] with some
173 modifications. The splenocytes were previously labeled with carboxyfluorescein

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174 succinimidyl ester (CFSE) (5 µM in non-supplemented RPMI culture medium) using a
175 CellTrace CFSE cell proliferation kit (Life Technologies, Carlsbad, USA) and
176 incubated at 37ºC for 10 min. The reaction was stopped by adding 5 volumes of
177 supplemented RPMI culture medium. Cells were then incubated at 4ºC for 10 min,
178 followed by 3 washes with PBS. Splenocytes were cultured, as described, with 1 µg/mL
179 ConA or 1 µg/mL LPS and 200 µg/mL of the samples, for 72 h. Cells were harvested
180 and analytical flow cytometry was performed using a Gallios instrument (Beckman
181 Coulter, Krefeld, Germany). Statistical analysis of CFSE distributions was based on
182 histogram profiling using Overton subtraction as applied by the FlowJo software
183 version 9.1 (Treestar Inc., Ashland, OR, USA). The results were expressed as the
184 percentage of cells in the final population that divided at least once.

185 Cytotoxicity of the hydrolysates was determined on spleen cell cultures using the
186 3-[4,5-dimethylthiazol-2-yl]-2-,5-diphenyltetrazoliumbromide (MTT) method described
187 by Hansen et al.,[12] with modifications. Non-stimulated (RPMI), ConA- (1 µg/mL) and
188 LPS (1 µg/mL)-stimulated splenocytes were cultured with 200 µg/mL of the samples, as
189 described above, in 96-well plates. After 72 h of incubation, 150 µL of 1 mg/mL MTT
190 (Sigma-Aldrich) were added to each well and incubated for a further 3 h. The plates
191 were centrifuged at 500x*g* for 10 min and the supernatants discarded. The formazan
192 precipitate was dissolved by the addition of 50 µL of dimethyl sulfoxide and the
193 absorbance of each well was measured at 570 nm. Cell viability was expressed as
194 percentage of the value of the corresponding control depending of the stimulus.

195 **2.8. Statistical analysis**

196 Data were subjected to one-way ANOVA analysis followed by *post hoc* multiple-
197 comparison using the Tukey's test. In order to examine the influence of the enzyme



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198 used to produce the hydrolysates, one-way ANOVA was also used, followed by the
199 Fisher's Least Significant Difference (LSD) test to compare the mean values of the
200 effects of the hydrolysates produced with pepsin, Neutrase and alcalase on cytokine and
201 antibody secretion, ROS production and cell proliferation. In all cases, $P$ values < 0.05
202 were considered to be statistically significant. Statistical calculations were performed
203 using the STATISTICA programme for Windows (Version 7.1; StatSoft Inc., 2006,
204 www.statsoft.com).

## 205 3. Results

### 206 3.1. Egg white protein hydrolysates with alcalase are characterized by low 207 molecular mass peptides

208 Individual EW proteins (OVA, LYS and OM) and whole EW were hydrolyzed with
209 pepsin, Neutrase and alcalase. The hydrolysis conditions were optimized to obtain a
210 complete degradation of the substrates and the absence of intact proteins was confirmed
211 by RP-HPLC (please, see Electronic Supplementary Information, Fig. S1). As expected,
212 OVA was quite resistant to pepsin action,[13] and its hydrolysis required 24 h at 37ºC.
213 LYS and OM also disappeared after 24 h of hydrolysis with pepsin at 37ºC and pH 1.5.
214 OM is usually readily digested by pepsin, but it gives rise to proteolysis fragments
215 linked by disulfide linkages, which is also the case of pepsin-treated LYS, because these
216 proteins are stabilized by 9 and 4 disulfide bridges, respectively.[14,15] Neutrase and
217 alcalase hydrolyzed the EW proteins in less than 1 h at 50ºC, yielding similar
218 chromatographic profiles.

219 The peptide mass distribution of the hydrolysates was analyzed by MALDI-TOF
220 (Table 1). Molecular masses lower than 10 kDa were found in all cases, although,



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221 comparatively, alcalase led to the smallest fragments: 85-97% of the peptides were
222 lower than 1500 Da, with more than 50% of them between 601 and 900 Da. Almost
223 30% of the peptides present in the hydrolysates produced with pepsin and Neutrase had
224 molecular masses higher than 1500 Da.

225 **3.2. Egg white protein hydrolysates modulate T cell responses in ConA-**
226 **stimulated splenocytes**

227 Incubation of splenocytes from BALB/c mice with ConA, a mitogen able to stimulate
228 mouse T lymphocytes,[10] induced a vigorous T cell proliferation, as assessed by CFSE
229 labelling (Table 2), and secretion of Th1 (IFN-γ and TNF-α, Fig. 1a and 1b) and Th2
230 cytokines (IL-13 and IL-10, Fig. 1c and 1d). The concomitant addition of the
231 hydrolysates of OVA, LYS and OM with pepsin, Neutrase and alcalase did not
232 significantly affect the release of IFN-γ by ConA-stimulated cells (Fig. 1a). However,
233 the hydrolysates modified the production of TNF-α in a dose-dependent manner, with
234 the lowest dose (20 μg/ml) usually reducing the ConA-induced release of TNF-α, which
235 subsequently increased with the hydrolysate concentration (particularly in the
236 hydrolysate of OVA with pepsin, as well as the hydrolysates of OVA, LYS and OM
237 with Neutrase). A low TNF-α production was detected as a result of the addition of the
238 hydrolysate of LYS with pepsin, although intact LYS and OM also decreased the
239 release of TNF-α at the three concentrations tested (Fig. 1b). Regarding Th2 cytokines,
240 the ConA-induced secretion of IL-13 decreased with increasing concentrations of the
241 hydrolysates of OVA, LYS and OM with alcalase and LYS with pepsin, while the
242 highest concentration of the hydrolysates of OVA and OM with pepsin (200 μg/ml) led
243 to an increase in the release of IL-13 (Fig. 1c). IL-10 secretion by ConA-stimulated



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244 splenocytes decreased following incubation with the hydrolysates of OVA with alcalase
245 and LYS with pepsin, as well as with intact LYS and OM at the highest concentrations.

246 The effects exerted by whole EW hydrolysates were then assessed. As shown in
247 Fig. 1a, the levels of IFN-γ were not significantly influenced by the addition of the
248 hydrolysates. However, the highest concentrations of the hydrolysate of EW with
249 Neutrase increased the ConA-induced production of TNF-α, IL-13 and IL-10, while the
250 hydrolysate with alcalase reduced the production of TNF-α, IL-13 and IL-10 (Fig. 1b-
251 d).

252 Altogether, these results indicate that, while intact OVA increased TNF-α, intact
253 LYS and OM reduced the levels of TNF-α, IL-13 and IL-10 and whole EW, those of
254 IL-13 and IL-10; the hydrolysates produced by Neutrase enhanced Th1 responses (ON,
255 LN, MN and EN; Fig. 1b) and, to a lesser extent, Th2 responses (EN; Fig. 1c) and those
256 produced by alcalase reduced Th2 responses (OA, LA, MA and EA; Fig. 1c). On the
257 other hand, the hydrolysate of LYS with pepsin exerted a suppressive effect on both
258 Th1 (TNF-α) and Th2 mediators (IL-13 and IL-10), similar to that produced by intact
259 LYS (LP; Fig. 1b-d). When the effect of the highest concentration of the hydrolysates
260 (200 μg/ml) on the proliferation of ConA-stimulated splenocytes was assessed, a
261 significant decrease was observed after the addition of those of OVA, LYS and EW
262 with Neutrase and alcalase, which also exerted a pronounced influence on Th1 and/or
263 Th2 cytokines (ON, LN, EN, OA, LA and EA; Table 2). The MTT assay showed no
264 significant effects of EW proteins or their hydrolysates on the viability of non-
265 stimulated, ConA- or LPS-stimulated splenocytes, ensuring that inhibition of
266 proliferation was not due to toxicity of the hydrolysates (Table 2). It should be
267 mentioned that the inactivated enzymes did not have any impact on proliferation or



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268 cytokine secretion by ConA-treated splenocytes, except for Neutrase, which
269 significantly enhanced the production of TNF-α and IL-10 (please, see Electronic
270 Supplementary Information, Fig. S2).

271 **3.3. Egg white protein hydrolysates influence B cell responses induced by LPS**
272 **and reduce oxidative stress in spleen and MLN cells**

273 LPS was next used to stimulate murine spleen and MLN cells and estimate the effect of
274 the addition of the EW protein hydrolysates on antibody production and oxidative
275 status. LPS is an immunostimulatory product of Gram-negative bacteria that signals
276 through Toll-like receptor 4, acknowledged as a crucial pathogen recognition receptor in
277 innate responses, as well as in triggering adaptive immunity.[16] Toll-like receptors have
278 been mainly identified in immune cells, especially monocytes, macrophages and
279 dendritic cells, as well as in other cell types, where they participate in responses
280 associated to oxidative stress and inflammation. LPS is also as a potent mouse B
281 lymphocyte mitogen which promotes expansion, class switch recombination and
282 immunoglobulin secretion; and it is known that LPS-activated B cells acquire
283 differential modulatory effects on T-cell polarization.[17]

284 LPS enhanced spleen cell proliferation (although less than ConA; Table 2) and
285 secretion of IgG1 (Fig. 2) and IgG2 (not shown), which are, respectively, Th2 and Th1-
286 biased antibodies, from spleen and MLN cells. All the individual EW proteins (but not
287 whole EW) and their hydrolysates with pepsin, Neutrase and alcalase, reduced the
288 production of IgG1 by LPS-stimulated spleen cells, although the lowest levels, which
289 were in turn significantly different from those produced by the intact proteins, were
290 induced by the hydrolysates of OVA, LYS and OM with alcalase (Fig. 2a). Likewise,
291 these hydrolysates, as well as that of LYS with pepsin, reduced IgG1 secretion from



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292 MLN cells (Fig. 2b). Incubation of spleen and MLN cell cultures with the EW proteins
293 and their hydrolysates also decreased the LPS-induced secretion of IgG2, although, in
294 this case, there were no significant differences between them (not shown).

295 The inhibiting effect of the hydrolysates produced by alcalase and that of LYS
296 with pepsin on IgG1 secretion by LPS-stimulated spleen and MNL cells (OA, LA, MA
297 and LP, Fig. 2) is consistent with the reduction observed in the secretion of Th2-related
298 cytokines by the cells stimulated with ConA in the presence of these hydrolysates,
299 indicated above (Fig. 1c). In addition, these hydrolysates also significantly decreased
300 LPS-induced splenocyte proliferation (OA, LA, MA and LP; Table 2). Furthermore,
301 while, in general terms, all the hydrolysates inhibited ROS generation in *t*-BOOH-
302 stimulated spleen and MLN cells, the highest antioxidant activity corresponded to those
303 produced by alcalase (Fig. 3). The inactivated enzymes did not affect the proliferation
304 or the production of IgG1 and ROS by LPS-stimulated spleen cells (please, see
305 Electronic Supplementary Information, Fig. S3).

306 When the mean values of the effects exerted by the highest concentration (200
307 μg/ml) of hydrolysates with pepsin, Neutrase and alcalase on cytokine and antibody
308 secretion, oxidative stress and cell proliferation were compared, the hydrolysates of EW
309 proteins with alcalase differed from the hydrolysates with Neutrase ($P < 0.05$) by virtue
310 of their decreasing effects on the production of TNF-α, IL-13, IgG1 in MLN, and ROS
311 in splenocytes and MLN cells (please, see Electronic Supplementary Information Table
312 S1).

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315 ## 4. Discussion

316 This work showed that peptides released by enzymatic hydrolysis of EW proteins can
317 modulate the immune responses of murine cells to mitogen stimuli in different ways,
318 revealing potential activities that could be used for different purposes as Th1- or Th2-
319 skewing mediators. Considering the traditional division of the role of Th1 and Th2
320 responses in cell-mediated and antibody-mediated immune reactions, Th1-type immune
321 activation involves the secretion of IFN-γ, which acts as an inductor of anti-microbial
322 and anti-tumoral defense mechanisms through the production of pro-inflammatory
323 cytokines and cytotoxic ROS by effector cells; while Th2-type cells synthetize
324 cytokines that help B lymphocytes to develop into certain types of IgG and IgE
325 antibody producing cells. Both types of T cells, with the contribution of other T
326 lymphocyte subsets, such as Th17 and Treg, cross-regulate each other, so that an
327 imbalanced response leads to oxidative stress and abnormally amplified inflammatory
328 signals, or to food allergy and asthma.[3,18]

329 The hydrolysates exerted effects different from those of the intact proteins from
330 which they derived, with OVA stimulating mainly Th1 cytokines (TNF-α), LYS and
331 OM inhibiting the release of both Th1 and Th2 cytokines (TNF-α, IL-13 and IL-10),
332 and whole EW mainly inhibiting Th2 mediators (IL-13 and IL-10). The hydrolysates of
333 OVA and LYS with alcalase reduced ConA- and LPS-stimulated murine lymphocyte
334 proliferation, production of Th2-biased cytokines, such as IL-13 and IL-10, and, at low
335 concentrations, also decreased the secretion of the Th1 cytokine TNF-α. In agreement
336 with these observations, these hydrolysates considerably inhibited IgG1-class switching
337 induced by LPS, which is part of the IL-4-mediated Th2 response, and counteracted the
338 release of ROS induced by an oxidant agent such as *t*-BOOH. Likewise, the hydrolysate



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339 of EW with alcalase decreased ConA-induced proliferation, as well as both Th1 (TNF-
340 α) and Th2 (IL-13, IL-10) responses and ROS generation in spleen and MNL cells
341 challenged with *t*-BOOH, an effect similar to that exerted by the hydrolysate of LYS
342 with pepsin that can be interpreted as immunosuppressive. In contrast, the hydrolysates
343 of OVA, LYS and EW with Neutrase also decreased the proliferative response to ConA,
344 but increased the level of TNF-α, leading to less pronounced antioxidant effects.

345 It is noteworthy that the most potent immunomodulating hydrolysates in this
346 experimental system, those produced by alcalase and Neutrase, suppressed the mitogen-
347 induced proliferation of lymphocytes even if they exerted distinct effects on the
348 cytokines released. Unlike our results, other studies reported that single food peptides[19]
349 or food protein hydrolysates[20-24] promoted lymphocyte proliferation induced by ConA,
350 which was interpreted as a stimulatory effect on cell-mediated immunity. However,
351 those reports assessed cell viability, the number of healthy cells in a sample, rather than
352 cell proliferation, that is, the number of dividing cells. In fact, the EW protein
353 hydrolysates assayed in the present work did not change the number of viable cells
354 (which allows discarding possible toxic effects); despite they decreased mitogen-
355 induced proliferation as part of their immunoregulating role. In the cells treated with the
356 hydrolysates produced by alcalase, reduced cytokine and antibody secretion followed
357 the pattern of T-cell proliferation, whereas in the case of those produced by Neutrase, it
358 cannot be excluded that the enzyme preparation itself exerted an immunostimulating
359 effect, as judged by the increased release of TNF-α and IL-10, which would have
360 masked the influence of the peptides.

361 The existence of EW protein-derived antioxidant peptides with radical
362 scavenging activities, or able to inhibit low density lipoprotein oxidation and ameliorate



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363 the blood lipid profile and the oxidative status *in vivo* has already been described.[25-28]
364 However, while it is recognized that antioxidants, such as polyphenols or omega-3 fatty
365 acids, can improve the immune function, to the best of our knowledge, there are no
366 previous reports on the capacity of EW hydrolysates to modulate the immune response
367 in murine cell cultures. Furthermore, while the potential immunomodulating use of
368 peptides has traditionally focused on their ability to prevent and repair the damage
369 caused by oxidative stress and inflammatory reactions[29-34] by enhancing Th2-type
370 responses,[19,21,29,31,35] or to stimulate the secretion of Th1 cytokines without an effect on
371 the release of Th2 cytokines and, thus, to play a role in fighting infections;[23,33] the
372 present results suggest that other uses are possible. Thus, the hydrolysates produced
373 with alcalase, able to combine Th2-supresive properties with a remarkable antioxidant
374 capacity, could be of interest as immunotherapeutic approaches against excessive Th2
375 responses and inflammatory conditions, such as those typical of food allergies.[18]

376 Even if it is now well known that, in addition toTh2-cytokine producing T cells
377 and antibody-producing B cells, other cells of the innate and adaptive immunity play a
378 role in the induction and regulation of the inflammatory processes that drive the
379 development of allergy, the screening techniques used appeared useful to evaluate the
380 immunomodulating and antioxidant properties of the EW protein hydrolysates and to
381 discriminate between the impact of those produced with various enzymes on effector
382 lymphocyte activation. It is well documented that hydrolysates of the same protein
383 produced with different enzymes differ in the immunological response they generate in
384 cultured cells;[20,33] even though, due to the complex peptide composition of most food
385 protein hydrolysates, it is expected that they would exert multiple effects through
386 different mechanisms.[36] As compared to the hydrolysates produced by pepsin and
387 Neutrase, those produced by alcalase did not show distinct features regarding their RP-



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388 HPLC profile, albeit they had a smaller molecular mass distribution. Further studies are
389 being conducted to correlate particular physicochemical characteristics with the
390 immunomodulatory properties of EW-derived peptides, as well as to further validate
391 these results in more sophisticated *in vitro* and animal models.

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509 **Figure captions**

510

511 **Fig. 1.** Effects of different concentrations (20, 100 and 200 μg/ml) of ovalbumin (O),
512 lysozyme (L), ovomucoid (M), egg white (E) and their hydrolysates produced with
513 pepsin (P), Neutrase (N), and alcalase (A) on concanavalin A (ConA)-induced secretion
514 of IFN-γ **(a)**, TNF-α **(b)**, IL-13 **(c)**, and IL-10 **(d)** by mouse splenocytes. Data are
515 means ± standard deviation of triplicates. * $P<0.05$ compared to the ConA-stimulated
516 control.

517

518 **Fig. 2.** Effect of ovalbumin (O), lysozyme (L), ovomucoid (M), egg white (E) and their
519 hydrolysates produced with pepsin (P), Neutrase (N), and alcalase (A) (200 μg/ml) on
520 lipopolysaccharide (LPS)-induced secretion of IgG1 by mouse splenocytes **(a)**, and
521 mesenteric lymph node cells **(b)**. Data are means ± standard deviation of triplicates. *
522 $P<0.05$ compared to the LPS-stimulated control. • $P< 0.05$ compared to the respective
523 intact protein.

524

525 **Fig. 3.** Effect of ovalbumin (O), lysozyme (L), ovomucoid (M), egg white (E) and their
526 hydrolysates produced with pepsin (P), Neutrase (N) and alcalase (A) (200 μg/ml) on
527 *tert*-butyl hydroperoxide-induced intracellular ROS generation by lipopolysaccharide
528 (LPS)-stimulated mouse splenocytes **(a)**, and mesenteric lymph node cells **(b)**. Data are
529 expressed as percentage of the LPS-stimulated control ± standard deviation of
530 triplicates. * P<0.05 compared to the LPS-stimulated control. • $P< 0.05$ compared to the
531 respective intact protein.

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**Table 1.** Peptide mass distribution in the hydrolysates of ovalbumin (O), lysozyme (L), ovomucoid (M) and egg white (E) with pepsin (P), Neutrase (N) and alcalase (A). The masses included in each mass range are expressed as percentage of all the masses detected by MALDI-TOF analyses.

| Mass Range (Da) | Frequency (%) | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **OP** | **ON** | **OA** | **LP** | **LN** | **LA** | **MP** | **MN** | **MA** | **EP** | **EN** | **EA** |
| ≤ 600 | 7.72 | 6.97 | 15.52 | 5.29 | 4.79 | 13.33 | 3.41 | 4.23 | 8.17 | 4.86 | 3.42 | 5.96 |
| 601-900 | 37.40 | 30.31 | 55.75 | 32.60 | 35.21 | 50.67 | 30.30 | 23.78 | 49.03 | 35.07 | 28.57 | 50.47 |
| 901-1200 | 24.39 | 20.91 | 22.99 | 17.18 | 22.25 | 19.11 | 17.80 | 14.98 | 21.40 | 25.00 | 19.88 | 19.44 |
| 1201-1500 | 6.50 | 11.50 | 2.87 | 14.54 | 18.03 | 10.67 | 9.85 | 12.05 | 12.06 | 15.63 | 11.49 | 9.72 |
| 1501-3000 | 10.98 | 21.25 | 0.57 | 25.99 | 14.37 | 4.00 | 18.18 | 15.31 | 7.00 | 6.25 | 22.36 | 6.90 |
| ≥ 3001 | 13.01 | 9.06 | 2.30 | 4.41 | 5.35 | 2.22 | 20.45 | 29.64 | 2.33 | 13.19 | 14.29 | 7.52 |



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**Table 2.** Effects of ovalbumin (O), lysozyme (L), ovomucoid (M), egg white (E) and their hydrolysates with pepsin (P), Neutrase (N) and alcalase (A) on proliferation and viability of splenocytes. Proliferation of concanavalin A (ConA)- and lipopolysaccharide (LPS)-stimulated splenocytes cultured with 200 µg/mL of the intact proteins and their hydrolysates was assessed by CFSE labelling and the results expressed as the percentage of cells in the final population that divided at least once. Viability was measured by the MTT assay either on non-stimulated (RPMI), ConA- or LPS- stimulated splenocytes (taken as 100%) and the corresponding percentage was calculated in the cultures treated with 200 µg/mL of the proteins and their hydrolysates.

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| | Proliferation ConA-stimulated cells | Proliferation LPS-stimulated cells | Viability RPMI | Viability ConA-stimulated cells | Viability LPS-stimulated cells |
|---|---|---|---|---|---|
| **RPMI** | 4.27 ± 0.10 * | 12.95 ± 0.78 * | 100.00 ± 0.00 | - | - |
| **ConA** | 74.25 ± 0.49 | - | - | 100.00 ± 0.00 | - |
| **LPS** | - | 29.30 ± 0.28 | - | - | 100.00 ± 0.00 |
| **O** | 75.95 ± 0.92 | 25.60 ± 0.99 | 152.12 ± 16.35 | 106.30 ± 1.35 | 79.91 ± 4.31 * |
| **OP** | 72.00 ±1.13 | 22.95 ± 0.64 * | 160.25 ± 17.12 | 107.12 ± 2.68 | 92.34 ± 1.22 |
| **ON** | 65.25 ± 0.92 * • | 27.20 ± 1.27 | 170.63 ± 21.13 | 110.06 ± 2.67 | 82.06 ± 2.46 |
| **OA** | 66.75 ± 0.78 * • | 22.65 ± 0.21 * | 144.10 ± 11.32 | 105.81 ± 4.25 | 78.11 ± 6.10 * |
| **L** | 73.25 ± 0.92 | 25.65 ± 1.06 | 93.98 ± 4.02 | 109.32 ± 3.60 | 83.14 ± 4.02 |
| **LP** | 70.45 ± 1.20 | 25.20 ± 0.42 * | 127.03 ± 1.35 | 104.50 ± 1.24 | 88.63 ± 1.87 |
| **LN** | 64.55 ± 0.92 * • | 26.25 ± 1.06 | 145.00 ± 1.37 | 106.21 ± 4.56 | 86.78 ± 1.69 |
| **LA** | 67.75 ± 0.07 * • | 24.70 ± 0.85 * | 123.21 ± 10.11 | 110.00 ± 3.26 | 89.08 ± 1.30 |
| **M** | 72.00 ± 1.27 | 29.05 ± 1.20 | 121.25 ± 7.32 | 100.36 ± 4.11 | 81.41 ± 3.32 * |
| **MP** | 69.80 ± 1.13 | 26.75 ± 0.49 | 138.31 ± 12.35 | 111.21 ± 1.96 | 87.22 ± 9.03 |
| **MN** | 71.10 ± 1.13 | 25.45 ± 0.78 * • | 171.10 ± 29.64 | 109.45 ± 3.97 | 83.86 ± 7.31 |
| **MA** | 70.40 ± 0.57 | 23.60 ± 0.85 * • | 117.45 ± 23.96 | 113.13 ± 9.13 | 89.64 ± 1.65 |
| **E** | 74.70 ± 1.41 | 28.30 ± 0.99 | 133.08 ± 39.45 | 102.35 ± 1.25 | 90.49 ± 4.06 |
| **EP** | 71.15 ± 1.06 | 31.10 ± 0.57 | 119.20 ± 33.56 | 106.01 ± 4.78 | 94.12 ± 3.98 |
| **EN** | 65.40 ± 0.57 * • | 33.45 ± 0.21 * • | 141.20 ± 27.12 | 105.60 ± 1.95 | 93.30 ± 5.15 |
| **EA** | 69.15 ± 1.34 * • | 31.15 ± 0.32 | 126.00 ± 25.32 | 107.19 ± 4.36 | 87.49 ± 8.23 |

* $P< 0.05$ compared to the respective control within columns.

• $P< 0.05$ compared to the respective intact protein within columns.



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Fig. 1

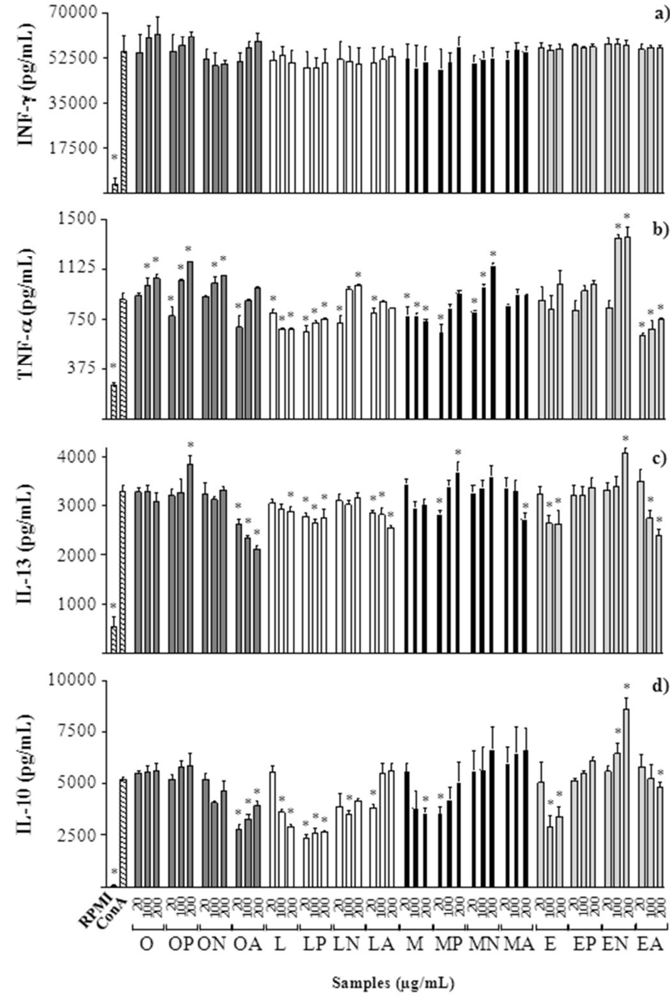


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Fig. 2

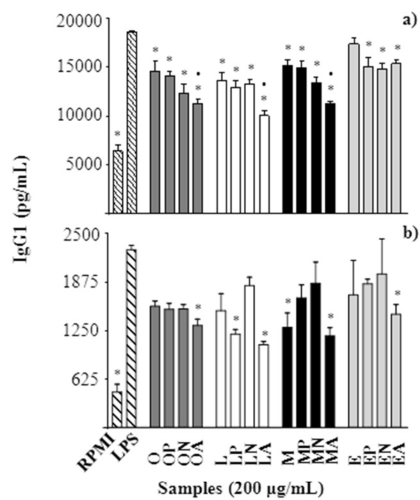


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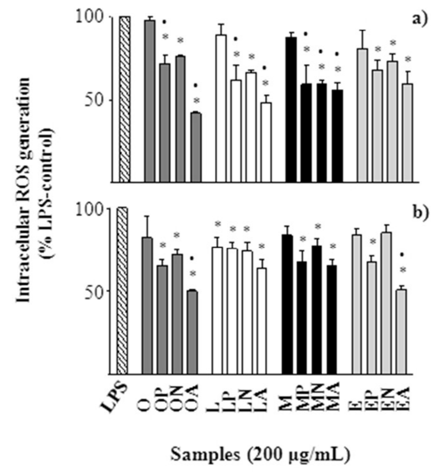
Fig. 3

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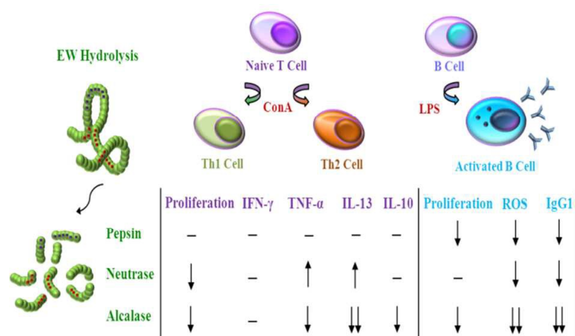
Egg white proteins hydrolysed with different enzymes exert immunomodulating effects and can be used as Th1- or Th2-skewing mediators

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