

1 **MECHANISMS INVOLVED IN DOWN-REGULATION OF INTESTINAL IgA**
2 **IN RATS BY HIGH COCOA INTAKE**

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23 **Running head:** Rat intestinal IgA after cocoa intake

24

25 **ABSTRACT**

26 Previous studies have shown that rat intestinal IgA concentration and lymphocyte
27 composition of the intestinal immune system were influenced by a highly enriched
28 cocoa diet. The aim of this study was to dissect the mechanisms by which a long-term
29 high cocoa intake was capable of modifying gut secretory IgA (S-IgA) in Wistar rats.
30 After seven weeks of nutritional intervention, Peyer's Patches (PPs), mesenteric lymph
31 nodes (MLNs) and the small intestine (SI) were excised for gene expression assessment
32 of IgA, TGF- β , CCR9, IL-6, CD40, retinoic acid receptors (RAR α and RAR β), CCL25
33 and CCL28 chemokines, pIgR and toll-like receptors (TLR) expression by real time
34 PCR. As in previous studies, S-IgA concentration decreased in intestinal wash and fecal
35 samples after cocoa intake. Results from the gene expression showed that cocoa intake
36 reduced IgA and IL-6 in PPs and MLNs, whereas in SI cocoa decreased IgA, CCR9,
37 CCL28, RAR α and RAR β . Moreover, cocoa-fed animals presented an altered TLR
38 expression pattern in the three compartments studied. In conclusion, a high cocoa diet
39 down-regulated cytokines such as IL-6, which is required for the activation of B cells to
40 become IgA-secreting cells (IgA-SCs), chemokines and chemokine receptors, such as
41 CCL28 and CCR9 together with RAR α and RAR β , which are involved in the gut-
42 homing of IgA-SCs. Moreover, cocoa modified the cross-talk between microbiota and
43 intestinal cells as was detected by an altered TLR pattern. These overall effects in the
44 intestine may explain the intestinal IgA down-regulatory effect after the consumption of
45 a long-term cocoa-enriched diet.

46

47 **Keywords:** gut immune system, flavonoids, mucosal antibodies

48

49 **1. Introduction**

50

51 The gut associated lymphoid tissue (GALT) constitutes the most extensive and
52 complex part of the immune system in the body. Every day it receives a huge antigenic
53 load and it is able to distinguish between invasive pathogens and innocuous antigens
54 from food and commensal bacteria. Structurally, the GALT is divided into organized
55 and diffuse compartments. Organized GALT is formed by isolated lymphoid follicles
56 (ILF) and associated lymphoid follicles or Peyer's patches (PPs). Diffuse or effector
57 GALT is formed by lymphocyte populations scattered across the epithelial cells
58 (intraepithelial lymphocytes, IELs), or in the intestinal lamina propria (lamina propria
59 lymphocytes, LPLs). Moreover, the mesenteric lymph nodes (MLNs) are part of the
60 intestinal immune system although they are not referred to as GALT as they do not
61 sample antigens directly [1]. M cells from PPs are specialized in luminal antigen uptake
62 and transport towards antigen-presenting cells (APCs), which interact with
63 interfollicular T lymphocytes or migrate towards MLNs [2]. This T cell-dependent
64 process brings about differentiation and maturation of B cells, inducing them to become
65 IgA⁺ cells and later IgA-secreting cells (IgA-SCs) [3]. Secretory-IgA (S-IgA) is the
66 main humoral mediator in the intestine (80-90%) [4,5] and provides a first line of non-
67 inflammatory immune protection at mucosal surfaces by neutralizing microbial
68 pathogens and exotoxins and by processing innocuous dietary antigens and commensal
69 microbes [6,7]. S-IgA plays a key role in the maintenance of gut homeostasis and oral
70 tolerance and its function and production is tightly regulated [2].

71 Differentiation of B cells into IgA⁺ B cells occurs in PPs and, to a lesser extent,
72 in ILF and MLNs [5]. Multiple cytokines such as transforming growth factor- β 1 (TGF-
73 β 1), interleukin (IL)-5, IL-6 IL-10 and IL-21 are required to IgA class switching and to

74 promote IgA-committed B cells to proliferate and differentiate into IgA⁺ B cells [8-11].
75 TGF- β 1 plus the interaction of CD40 on B cells with CD40 ligand (CD40L) on T cells
76 are crucial to elicit IgA class switching of activated B cells in germinal centers of PPs
77 [12,13]. These IgA⁺ B cells migrate from the PPs to the draining MLNs, and home back
78 to the intestinal lamina propria via the thoracic duct and bloodstream to further
79 differentiate into IgA-SCs [7]. This gut-homing system requires the integrin α 4 β 7 on
80 activated gut lymphocytes which binds to its receptor MAdCAM-1 on endothelial cells
81 within the intestinal mucosa[5]. Moreover, gut-homing depends on chemokines such as
82 CCL25 and CCL28. In humans and mice, crypt epithelial cells produce CCL25, which
83 interacts with CCR9 on B and also T cells. CCL28 is a mucosal chemokine that assists
84 cell homing in the large and small intestine, interacting with CCR10 [14]. However, this
85 process involving PPs is not the only one for IgA synthesis. Alternatively, IgA⁺ B cells
86 can be generated within ILFs and lamina propria in a T cell-independent manner. These
87 mechanisms involve toll-like receptors (TLRs) and activated dendritic cells (DCs)
88 producing B-cell activating factor from the TNF family (BAFF) and a proliferation-
89 inducing ligand (APRIL) [12,15]. In any case, mucosal IgA-SCs mainly release dimers
90 and some larger polymers of IgA, which are actively secreted to the apical surface of
91 epithelial cells by the polymeric immunoglobulin receptor (pIgR) expressed on the
92 basolateral surface [2].

93 Over the last decade, an increasing interest has been focused on the
94 identification of natural biologically active nutrients with the potential to modulate the
95 activity of the immune system. In this regard, a vast number of studies have highlighted
96 the health benefits of polyphenolic compounds, particularly flavonoids, due to their
97 antioxidant properties [16]. Cocoa and cocoa-based products such as chocolate
98 represent some of the main natural sources of dietary flavonoids, including

99 (-)-epicatechin, (+)-catechin and their oligomers, the procyanidins [17,18]. Although the
100 antioxidant and immunomodulatory capacities of cocoa flavonoids have been
101 investigated mainly *in vitro* [19-21], less is known about the *in vivo* effect of cocoa on
102 the immune system [22]. Previous studies in our laboratory have demonstrated that a
103 dietary intervention with cocoa is capable of modifying the composition and
104 functionality of several lymphoid tissues in young rats, including the GALT [23,24]. In
105 particular, a continuous cocoa intake increases the percentage of $\gamma\delta$ T cells and reduces
106 the proportion of Th cells in both PPs and MLNs. Cocoa intake in rats also augments
107 B-cell proportion in PPs but depletes cells with a high capacity to secrete IgA [24]. In
108 fact, a 10% cocoa diet decreases S-IgA concentration in the intestinal lumen of young
109 rats, as is reflected by the lower S-IgA content in fecal samples and small-intestine wash
110 [24].

111 Based on the complex mechanisms of IgA regulation and the down-regulation of
112 S-IgA after a cocoa diet, the aim of the present study was to dissect some of the
113 mechanisms by which a long-term cocoa intake may affect IgA production. We focused
114 on intestinal pathways and molecules involved in IgA+ B cell homing and IgA synthesis
115 in three different compartments of the intestinal immune system PPs, MLNs and small-
116 intestine wall containing lamina propria, as representative tissues of the inductor and
117 effector sites.

118

119 **2. Material and methods**

120

121 *2.1. Chemicals*

122

123 The Natural Forastero cocoa (provided by Nutrexp SA, Barcelona, Spain) used
124 in this study contained a total polyphenol content of 10.62 mg/g with 0.83 mg/g (-)-
125 epicatechin, 0.14 mg/g (+)-catechin and 0.65 mg/g procyanidin B2. ExtrAvidin-
126 peroxidase, o-phenylenediamine dihydrochloride (OPD), bovine serum albumin (BSA)
127 and 30% hydrogen peroxide were obtained from Sigma-Aldrich (Madrid,
128 Spain). Mouse anti-rat IgA (A93-3), IgM (G53-238), IgG2a (B46-7), IgG2b (G15-337),
129 IgG2c (A92-3) MAb, rat IgA, IgM, IgG1, IgG2a, IgG2b and IgG2c recombinant
130 proteins, and biotinylated anti-rat IgA (A93-2), IgM (G53-238), IgG2a (R19-15), IgG2b
131 (G15-337) and IgG2c (A92-1) MAb were purchased from BD Biosciences (Heidelberg,
132 Germany). Anti-rat IgG1 (MRG1-58) was obtained from BioLegend (San Diego, CA)
133 and peroxidase-conjugated anti-rat Ig MAb was provided by DakoCytomation
134 (Glostrup, Denmark). RNAlater® was purchased from Ambion (Applied Biosystems,
135 Austin, TX).

136

137 *2.2. Animals and experimental design*

138

139 Three-week-old female Wistar rats were obtained from Harlan (Barcelona,
140 Spain) and housed in cages under conditions of controlled temperature and humidity in
141 a 12:12 light-dark cycle. After 6 days of acclimatization, the rats weighing 66-74 g were
142 randomly assigned to two dietary groups (n=7 each group): the reference group which

143 was fed with a standard diet, and the cocoa group which received chow containing 10%
144 (w/w) cocoa for 7 weeks.

145
146 The AIN-93G formulation [25] (Harlan) was used as the control standard diet. The
147 cocoa diet was produced from a modification of the AIN-93G formula, as previously
148 described [24]. In brief, we used a basal mix (Harlan) in which the proportion of
149 proteins, carbohydrates and lipids had been modified in such a way that the addition of
150 10% cocoa (100 g/kg) resulted in a final isoenergetic diet with the same macronutrient
151 composition as the AIN-93G diet. Animals were given free access to water and chow *ad*
152 *libitum*, and body weight and food intake were monitored throughout the experiment.
153 The study was performed according to the criteria outlined by the Guide for the Care
154 and Use of Laboratory Animals. Experimental procedures were reviewed and approved
155 by the Ethical Committee for Animal Experimentation of the University of Barcelona.

156

157 2.3. Sample collection

158

159 Fecal and sera samples were collected before the diet (week 0), in the middle
160 (week 3.5) and at the end of the study (week 7) and kept at -20°C for further
161 immunoglobulin quantification. At the end of the dietary intervention, the rats were
162 anesthetized intramuscularly with ketamine/xylazine. MLNs were removed in aseptic
163 conditions for PCR analysis. The small intestine (SI) was excised, divided into two
164 fragments and carefully flushed with sterile 0.9% NaCl solution to remove fecal
165 content. The distal fragment of the SI was opened lengthwise and PPs were excised for
166 PCR analysis, as well as a maximum of 30 mg of tissue corresponding to distal
167 jejunum/proximal ileum without PPs. The remaining distal fragment of the SI was used
168 to obtain the gut wash for IgA determination as previously described [24]. All tissue

169 samples for PCR were immediately immersed in RNAlater® and incubated at 4 °C
170 overnight before storing at -20 °C.

171

172 *2.4. Fecal homogenate obtention*

173

174 Fecal samples were dried for 70 min at 37 °C in a thermostatically controlled
175 incubator and for 30 min at room temperature (RT) before being weighed. Thereafter,
176 fecal samples were diluted in PBS (20 mg/ml) and homogenized using a Polytron®
177 (Kinematica, Lucerne, Switzerland). Homogenates obtained were then centrifuged (500
178 g, 15 min, RT), and supernatants were frozen at -20 °C until ELISA IgA quantification.

179

180 *2.5. Immunoglobulin quantification in serum, gut wash and feces by ELISA*

181

182 S-IgA and S-IgM levels in gut wash and feces, and serum IgA, IgM, IgG1,
183 IgG2a, IgG2b, IgG2c concentrations were quantified by ELISA. Ninety-six-well
184 polystyrene plates (Nunc MaxiSorp, Wiesbaden, Germany) were coated with mouse
185 anti-rat IgA, IgM, IgG1, IgG2a, IgG2b or IgG2c MAb (2 µg/ml in PBS) and incubated
186 in a humidified chamber overnight. Thereafter, the remaining binding sites were
187 blocked with PBS containing 1% BSA (PBS-BSA, 1 h, RT). The plate was washed
188 three times with PBS containing 0.05% Tween 20 (PBS-Tw) and once with PBS; then
189 appropriate diluted samples and standards in PBS-Tw-BSA were added (3 h, RT).
190 After washing, biotin-conjugated mouse anti-rat IgA, IgM, IgG1, IgG2a, IgG2b or
191 IgG2c MAb were added (1 µg/ml in PBS-Tw-BSA, 2 h, RT). Thereafter, peroxidase-
192 conjugated ExtrAvidin (4 µg/ml in PBS-Tw-BSA) was incubated for 30 min. Lastly,
193 OPD and H₂O₂ were added for the detection of bound peroxidase. The reaction was

194 stopped by adding 3M H₂SO₄. Absorbance was measured on a microtiter plate
195 photometer (Labsystems, Helsinki, Finland) at 492 nm. Data were interpolated by
196 means of Multiskan *Ascent* v.2.6 software (Thermo Fisher Scientific S.L.U, Barcelona,
197 Spain) into the standard curves, and expressed as µg/mL in sera, gut washes and fecal
198 samples.

199

200 2.6. Assessment of RNA gene expression by Real Time PCR

201

202 For RNA isolation, tissue samples in RNA later® were transferred into lysing
203 matrix tubes (MP Biomedicals, Illkirch, France) containing an appropriate buffer and
204 homogenized in a FastPrep®-24 instrument (MP Biomedicals) for 40 s. Lysates were
205 centrifuged for 3 min at 12000 g to eliminate excess tissue debris in and transferred into
206 new tubes. RNA was isolated by the RNeasy® mini kit (Qiagen, Madrid, Spain)
207 following manufacturer's recommendations. RNA was quantified with a NanoDrop
208 spectrophotometer and NanoDrop IVD-1000 v.3.1.2 software (NanoDrop Technologies,
209 Wilmington, DE). The Agilent 2100 Bioanalyzer with the RNA 6000 LabChip 1 kit
210 (Agilent Technologies, Madrid, Spain) was used to provide an RNA integrity number
211 (RIN) for each sample.

212 Four µg of total RNA were reverse-transcribed in a thermal cycler PTC-100
213 using random hexamers and TaqMan® Reverse Transcription Reagents (Applied
214 Biosystems, AB, Weiterstadt, Germany). A final volume of 1 µL was used to confirm
215 the reaction of each sample by conventional PCR using rat β-actin primers and
216 conditions previously established in our laboratory [26].

217 Specific PCR TaqMan® primers and probes (Applied Biosystems, AB,
218 Weiterstadt, Germany) were used to measure *Iga* (331943, made to order), *Tgfb1*

219 (Rn00572010_m1, inventoried (I)), *Il5* (Rn99999143_mH, inventoried), *Il6*
220 (Rn01410330_m1), *Cd40* (Rn01423583_m1,I), *Rara* (Rn00580551_m1, I), *Rarb*
221 (Rn01537835_m1, I), *Ccr9* (Rn00597283_m1, I), *Tlr2* (Rn02133647_s1, I), *Tlr4*
222 (Rn00569848_m1, I), *Tlr7* (Rn01771083_s1, I), *Tlr9* (Rn01640054_m1, I), *Pigr*
223 (Rn00562362_m1, I), *Ccl25* (Rn0143351_m1, I) and *Ccl28* (Rn00586715_m1, I).

224 Quantitative PCR assays were performed in duplicate for each sample using an ABI
225 PRISM®7700 Sequence Detection System (AB). Quantification of the genes of interest
226 was normalized to the housekeeping genes *Hprt1* (Rn01527840_m1, I) and *Gusb*
227 (Rn00566655_m1, I). The amount of target mRNA relative to the endogenous control
228 expression and relative to values from the reference group was calculated using the
229 $2^{-\Delta\Delta C_t}$ method, as previously described [27], where C_t is the cycle number at which the
230 fluorescence signal of the PCR product crosses an arbitrary threshold set within the
231 exponential phase of the PCR and $\Delta\Delta C_t = [(C_{t_{\text{target}}(\text{unknown sample})} - C_{t_{\text{endogenous control}}(\text{unknown sample})}) - [(C_{t_{\text{target}}(\text{reference sample})} - C_{t_{\text{endogenous control}}(\text{reference sample})})]$. Results are expressed as
232 the mean \pm SEM of the percentage of these values for each experimental group
233 compared with its reference age group, which represents 100% gene expression.

235

236 2.7. Statistical analysis

237

238 The software package SPSS 16.0 (SPSS, Inc., Chicago, IL) was used for
239 statistical analysis. The data were analyzed by the Mann–Whitney U test. A P value of
240 < 0.05 was considered statistically significant.

241

242 **3. Results**

243

244 *3.1. Effect of cocoa diet on body weight*

245

246 Body weight and chow intake were monitored throughout the study (3 times per
247 week). The growth of animals with the cocoa diet was slower than that of the reference
248 animals ($P < 0.01$, Figure 1). This effect was not associated with a lower chow intake
249 because food intake was similar between both groups (data not shown) as reported
250 previously [23,24,28].

251

252 *3.2. Effect of cocoa diet on serum immunoglobulins*

253

254 Concentrations of IgG1, IgG2a, IgG2b, IgG2c, IgM and IgA were quantified in
255 serum before, in the middle and at the end of dietary intervention. Data from the
256 experimental groups are summarized in Figure 2. At the end of the study, the
257 predominant IgG isotype present in the reference animals' serum was IgG2a (~600
258 $\mu\text{g/mL}$), followed by IgG2b (~370 $\mu\text{g/mL}$), IgG1 (~170 $\mu\text{g/mL}$) and IgG2c (~115
259 $\mu\text{g/mL}$). Serum IgM and IgA concentrations were about 635 $\mu\text{g/mL}$ and 5 $\mu\text{g/mL}$,
260 respectively. A long-term cocoa diet significantly modified serum immunoglobulin
261 concentrations: IgG2b, IgM and IgA concentrations being ~50% lower than values in
262 the reference animals at the end of the study (Fig. 2C, 2E, 2F, $P < 0.05$). This reduction
263 was already evident for IgG2b and IgM after 3.5 weeks of cocoa intake. Moreover,
264 cocoa intake tended to decrease serum IgG1 and IgG2c concentrations (Fig. 2A and
265 2D). The cocoa diet did not modify IgG2a concentration after a 7-week intake but we
266 found an increase of this isotype in the middle of the study (Fig. 2B, $P < 0.05$).

267

268 *3.3. Effect of cocoa diet on intestinal immunoglobulins*

269

270 Intestinal IgM and IgA production was quantified by means of evaluation in
271 feces before and after 7 weeks of cocoa intake, and in intestinal wash at the end of the
272 study (Figure 3). The cocoa diet produced a decrease of S-IgA and S-IgM in intestinal
273 wash (Fig. 3A, $P < 0.05$). Fecal IgA concentration increased according to age in the
274 reference group, and that increase was inhibited by the dietary intervention with cocoa
275 (Fig. 3B, $P < 0.05$). S-IgM was not detected in fecal samples.

276

277 *3.4. Expression of constitutive genes*

278

279 Expression levels of two commonly used housekeeping genes, *Gusb* and *Hprt1*,
280 were analyzed in all tissues of both groups. Ideally, all cell types and tissues should
281 constitutively express housekeeping genes independently of experimental conditions
282 and they should not be affected by interventional, environmental or regulative factors.
283 We found that *Gusb* expression was relatively homogenous among samples whereas
284 *Hprt1* expression fluctuated regardless of the diet (data not shown). This result
285 prompted us to discard *Hprt1* as a normalizing gene and results were referred to *Gusb*
286 expression (Figure 4). The coefficients of variation for both *inter*- and *intrassay*
287 determinations were < 2 and 1% respectively, indicating the high reproducibility of the
288 assay and that dispersion among samples within the same group was due to animal
289 physiological variations.

290

291

292 3.5. *Effect of cocoa diet on genes related to IgA class switching and secretion*

293

294 *Iga*, *Tgfb1*, *Il6*, *Il5* and *Cd40* expression was assessed in SI, PPs and MLNs after
295 7 weeks of cocoa intake (Fig. 4A, 4B, 4C). Additionally, *Pigr* expression was also
296 analyzed in SI. In cocoa-fed animals, *IgA* was down-regulated in both PPs and SI ($P <$
297 0.05) and tended to decrease in MLNs. The cocoa diet did not significantly modify
298 *Tgfb1* expression in any of the tissues considered; however, *Il6* was reduced ~95% in
299 MLNs ($P < 0.05$) and tended to decrease in the PPs of the cocoa group animals whereas
300 *Il6* expression was not detected in the SI. *Il5* expression was also too low to be detected
301 in the analyzed tissue samples. Cocoa intake reduced *Cd40* expression in SI ($P < 0.05$)
302 but *Cd40* was not modified, either in PPs or in MLNs. *Pigr* tended to be reduced in the
303 SI from cocoa-fed rats.

304

305 3.6. *Effect of cocoa diet on genes associated with IgA-secreting cell homing*

306

307 *Rara*, *Rarb* and *Ccr9* were analyzed in PPs, MLNs and SI (Fig. 4D, 4E, 4F).
308 Moreover, *Ccl25* and *Ccl28* expression was also studied in SI. The cocoa diet produced
309 that *Rara* was up-regulated 5-fold in PPs ($P < 0.05$) and tended to be augmented in
310 MLNs; however, this gene was decreased ~70% in the SI of cocoa-fed animals ($P <$
311 0.05). With regards to *Rarb*, the cocoa group showed a 5-fold expression increase in
312 MLNs ($P < 0.05$) whereas it decreased in PPs and SI ($P < 0.05$ in SI). Cocoa intake did
313 not alter *Ccr9* expression in PPs or MLNs but reduced its expression in SI ($P < 0.05$).
314 With regards to chemokine gene expression in SI, the cocoa diet significantly reduced
315 *Ccl28* expression (75%, $P < 0.05$) but did not significantly modify *Ccl25* expression.

316

317 3.7. *Effect of cocoa on TLR gene expression.*

318

319 Expression of *Tlr2*, *Tlr4*, *Tlr7* and *Tlr9* was assessed in PPs, MLNs and SI (Fig.
320 4G, 4H, 4I). *Tlr2* was decreased up to ~ 80% in the PPs of cocoa-fed animals ($P < 0.05$)
321 as well as in MLNs, but increased in SI. A similar pattern was also found for *Tlr7*
322 expression. *Tlr4* expression augmented ~ 2-fold in PPs ($P < 0.05$), was not modified in
323 MLNs whereas it was reduced in the SI of cocoa group rats ($P < 0.05$). Cocoa intake
324 drastically increased *Tlr9* expression in both PPs and MLNs ($P < 0.05$).

325

326 4. Discussion

327

328 In previous studies, we reported that cocoa flavonoids possess *in vitro* and *in*
329 *vivo* modulator effects on the immune system [24,29-31]. Specifically, we found that a
330 cocoa-enriched diet in young rats over 3 weeks produced a down-modulator effect on
331 intestinal IgA content [24]. Similarly, here we have found that a high and continuous
332 cocoa intake reduced intestinal IgA concentration. The intestinal IgA drop as a result of
333 cocoa intake suggests that a cocoa diet could influence specific mechanisms involved in
334 IgA production located at the intestinal site. The present study is focused on the gene
335 expression of some molecules related to the IgA synthesis, IgA-SCs, and gut cell
336 homing and lumen secretion in order to achieve an understanding of some pathways
337 within the complex intestinal immune system that could be involved in intestinal IgA
338 modulation by cocoa.

339 Firstly, we focused on the main pathway that brings about differentiation and
340 maturation of B cells inducing them to become IgA-SCs, the T cell-dependent process
341 that takes place in either PPs or MLNs, inductive sites of the intestinal immune system
342 [3]. This process depends on cytokines such as TGF- β 1 and IL-6, among others [7,10].
343 The results obtained in the present study showed no significant changes in TGF- β 1
344 whereas IL-6 was depleted in the PPs and MLNs of animals that were fed cocoa. As
345 IL-6 is secreted by DCs in PPs [15] we can suggest that some cocoa compounds
346 reaching the intestine could act on these cells and modulate the secretion of IL-6
347 involved in IgA⁺ B cell differentiation. In addition, the interaction between T and B
348 cells through CD40L-CD40 is crucial to elicit IgA class switching on activated B cells
349 in the germinal centers of PPs [12]. The expression of CD40 did not change in either
350 PPs or MLNs after cocoa intake, which is in accordance with previous studies that have

351 shown that a cocoa diet increases the proportion of B cells in PPs [24]. Therefore,
352 although the cocoa diet decreased soluble factors, such as IL-6, which promote IgA+ B
353 cells, it seems that it had no influence on the direct interaction between T and B cells.
354 However, lower IgA gene expression was found in PPs and MLNs, which could
355 eventually mean that there would be less differentiation in IgA+ B cells, and/or lower
356 IgA synthesis ability in these compartments. This suggestion agrees with previous
357 findings that have shown that, after a cocoa diet, although the total number of IgA-SCs
358 does not change, the number of high-capacity IgA-SCs in PPs decreases [24].

359 Physiologically, after the maturation process in the inductive sites, i.e. PPs and
360 MLNs, activated IgA+ B cells migrate from there and home back to the intestinal
361 lamina propria, as the effector site, to further differentiate into IgA-SCs [7]. This
362 gut-homing system requires the expression of the chemokine receptor CCR9 on IgA+ B
363 cells which binds to its ligand CCL25, thus promoting cell recruitment to the intestinal
364 lamina propria [14]. We found that the cocoa diet did not modify CCR9 expression in
365 either the PPs or MLNs but reduced it in the SI. Interestingly, CCL25 gene expression
366 was augmented in this last tissue in cocoa-fed animals. These results suggest that
367 molecules involved in the gut homing of IgA-SCs would be modified not in the
368 inductive sites but in the effector tissues, and it seems that small intestine lamina propria
369 increased CCL25 in an attempt to strongly attract the reduced number of CCR9-
370 expressing cells. On the other hand, DCs from PPs and intestinal lamina propria have
371 been shown to induce CCR9 expression on IgA+ B cells by means of retinoic acid (RA)
372 production [32]. The action of RA is mediated by its ligation to RA nuclear receptors
373 such as RAR α and RAR β [33]. Here we found that the cocoa diet up-modulated RAR α
374 and RAR β gene expression in PPs and MLNs, respectively, but both were reduced in SI.
375 This last result agrees with the decreased CCR9 gene expression found in SI, and it

376 allows us to postulate that lower CCR9 values in intestinal lamina propria could be a
377 consequence of a decrease in the expression of RAR α and RAR β in B cells present in
378 this compartment. The meaning of the up-regulation of RAR α and RAR β in the
379 inductive sites, where CCR9 expression was maintained after diet intervention, remains
380 to be elucidated. On the other hand, we have found that SI from cocoa-fed rats had
381 reduced values of CCL28 gene expression, a chemokine produced by epithelial cells
382 which selectively attracts IgA⁺ B cells [14]. Taking together all these results, we suggest
383 that a high cocoa diet induces a lower number of IgA⁺ B cells reaching the intestinal
384 lamina propria by the down-modulation of chemokines (such as CCL28, involved in
385 homing both to the small and the large intestine) or chemokine receptors (such as
386 CCR9, mediated in part by the down-regulation of RAR), although some mechanisms in
387 the own gut lamina propria work efficiently (CCL25 synthesis).

388 After the homing and differentiation processes, IgA-SCs of intestinal lamina
389 propria release dimmers or larger polymers of IgA which are actively secreted to the
390 apical surface of epithelial cells by the polymeric immunoglobulin receptor (pIgR)
391 expressed on the basolateral surface [2]. Cocoa-fed animals showed a lower expression
392 of IgA and CD40 in SI, and a variable expression of pIgR. These results allow us to
393 hypothesize that after cocoa intake intestinal lamina propria contained lower numbers of
394 activated B cells (CD40+) and IgA-SCs, which agrees with the reduction of homing and
395 activation mechanisms presented above. Nevertheless, the way IgA was transported
396 across the epithelial layer mediated by the pIgR was not significantly affected by this
397 dietary intervention.

398 In addition to the T-dependent way to secrete IgA referred to so far, IgA+ B
399 cells can be alternatively generated in a T cell-independent manner which involves TLR
400 signaling, among others ways [15]. Cocoa-fed animals showed changes in the

401 expression of at least all the considered TLRs: TLR2, which recognizes components
402 from gram-positive bacteria; TLR4, which recognizes LPS or gram-negative bacteria;
403 TLR7, which is found in endosomes and recognizes single-stranded RNA from viruses,
404 and TLR9, which is also found in endosomes and acts as a receptor for CpG in bacterial
405 and viral DNA [34]. A high and continuous cocoa diet produced an up-regulation of
406 TLR4 and TLR9 and a down-regulation of TLR2 and TLR7 in PPs and MLNs.
407 Conversely, in SI, cocoa-fed animals showed lower values for TLR4 and TLR9 and a
408 higher expression of TLR2 and TLR7. TLRs are expressed preferentially in tissues that
409 are in constant contact with microorganisms [34,35] and changes in the TLR expression
410 could reflect changes in the intestinal microbiota and/or its relation with intestinal
411 immune cells [36]. Therefore, the overall change in the TLR expression found here,
412 whatever the meaning of contradictions between the inductor and effector sites, could
413 be a consequence of changes in intestinal microbiota induced by the cocoa-enriched
414 diet. In fact, a recent study has shown in humans that the daily consumption of a cocoa
415 beverage rich in flavanols significantly increased the growth of *Lactobacillus spp.* and
416 *Bifidobacterium spp.* and decreased that of *Clostridium histolyticum* group [37].
417 Similarly, ~~Thus~~ wine-treated rats show gut prevalence of *Bacteroides*, *Lactobacillus* and
418 *Bifidobacterium* [38] and pigs administered with tea polyphenols increase intestinal
419 *Lactobacillus* [39]. Moreover, berries and their phenolics selectively inhibit the growth
420 of pathogenic bacteria in humans [40,41]. In consequence, it seems that the
421 consumption of flavanol-rich food seems to exert prebiotic actions [37, 42]. In any case,
422 further studies must determine the microbiota composition of rats fed a cocoa diet. On
423 the other hand, it would be interesting to know the relation, if any, between changes in
424 the TLRs of the three tissues and the IgA-secreting function of the intestinal immune
425 system. It has been reported that TLR4 signaling in the intestinal epithelial cells

426 promotes the recruitment of B cells to the lamina propria by means of CCL28 and
427 CCL20 chemokines [43]. Moreover, TLR4 expression has been directly correlated to a
428 higher number of IgA-SCs in the lamina propria and increased IgA in the feces of
429 transgenic mice that express a constitutively active form of TLR4 on intestinal epithelial
430 cells [34,43]. As we found that a high cocoa diet produced a down-regulation of TLR4
431 and also CCL28 in SI, it could be suggested that TLR alterations could be involved in
432 the lower recruitment of IgA-secreting cells to the intestinal lamina propria. Other
433 studies have shown the effect of polyphenols on TLR expression. Thus,
434 epigallocatechin-gallate (EGCG) reduces TLR4 gene expression on macrophages *in*
435 *vitro* [44], and an interventional study with orange juice with hesperidin and naringenin
436 produced a reduction in TLR2 and TLR4 mRNA and protein expression in PBMC [45].
437 Moreover, EGCG and curcumin have been shown to be able to block TLR4
438 glycosilation and homodimerization [46,47], in both cases inhibiting the activated
439 downstream molecules. In addition, two downstream signaling adaptors of TLRs,
440 MyD88 and TRIF proteins, have been specifically inhibited by resveratrol [48], luteolin
441 [49], EGCG [50] or curcumin [47]. Thus, we cannot disregard the effect of cocoa
442 flavonoids on TLR-related pathways, which could contribute to their down-regulatory
443 role on IgA. The potential bioactivity of flavonoids depend on their bioavailability and,
444 while monomeric flavonoids are rapidly absorbed in the small intestine, the polymeric
445 forms -present in high proportion in cocoa- reach intact into the colon where they are
446 metabolized by the intestinal microbiota into various phenolic acids [51]. In this sense,
447 several dietary interventions have evidenced the accessibility of cocoa flavonoids in the
448 large intestine [52,53], where they or their metabolites might exert a modulatory effect
449 on microbiota and, consequently, on the TLR expression.

450 On the other hand and in addition to the effects on the gut, the high cocoa diet
451 reduced IgG, IgM and IgA serum concentrations in Wistar rats, in agreement with
452 previous studies [31]. Furthermore, the influence of the cocoa intake on IgG depended
453 on the isotype, IgG2b being the most reduced by the diet. As IgG2b isotype is
454 associated with Th1 immune response in rats [54-56], it seems that the cocoa diet
455 tended to reduce Th1 immune responses. This suggestion agrees with the anti-
456 inflammatory properties shown in cocoa intake [57]. Nevertheless, it still remains to be
457 seen how a cocoa diet acts on pathways involved in the production of each IgG isotype.
458 On the other hand, there are also unknown mechanisms that decrease serum IgM and
459 IgA, although these reductions could partially reflect the reduction of mucosal
460 immunoglobulin synthesis.

461 Finally, the 10% cocoa diet produced an attenuating effect on the body weight
462 increase of the animals, despite the food consumption was similar in all experimental
463 groups. This effect has been reported in previous studies [23,24] and could be attributed
464 to the gene regulation of mechanisms implicated in the adipose tissue synthesis, as
465 described elsewhere [28].

466 In summary, we have demonstrated by using a continuous and high cocoa-
467 enriched diet in Wistar rats that compounds present in cocoa interact with mechanisms
468 involved in intestinal IgA production, leading to a lower IgA secretion. These
469 mechanisms comprise cytokines produced by DCs, such as IL-6, required in the
470 induction site (PPs and MLNs) and chemokines and their receptors, such as CCL28 and
471 CCR9 together with RAR α and RAR β , needed for gut homing. Moreover, a high cocoa
472 diet also modified the cross-talk between microbiota and the intestinal cells, as shown
473 by an altered TLR pattern. Finally, all these changes seem to produce a lower number of
474 IgA-SCs and/or a lower ability to synthesize this antibody in the small intestine. Further

475 studies must be considered to explore the precise compounds and amount of cocoa
476 responsible for this action.

477

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479

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622

623 **FIGURE LEGENDS**

624

625 **Figure 1.** Body weight of female Wistar rats fed a cocoa (●) or standard (○) diet over 7
626 weeks. Data are means \pm SEM ($n = 7$). Cocoa intake resulted in a lowered growth curve
627 from day 8 and until the end of the study ($P < 0.01$).

628

629 **Figure 2.** Effect of a cocoa enriched diet on serum IgG1(A), IgG2a (B), IgG2b (C),
630 IgG2c (D), IgM (E) and IgA (F) isotypes. Black bars correspond to the cocoa diet and
631 white bars correspond to the standard diet. Each bar represents the mean \pm SEM ($n = 5$ –
632 7). * $P < 0.05$

633

634 **Figure 3.** Effect of the cocoa-enriched diet on S-IgA and S-IgM in gut wash (A), and on
635 S-IgA in feces (B). Gut wash values are related to those found in the reference group
636 which are considered as 100%. Black bars correspond to the cocoa diet and white bars
637 correspond to the standard diet. Each bar represents the mean \pm SEM ($n = 6$ –7).
638 * $P < 0.05$

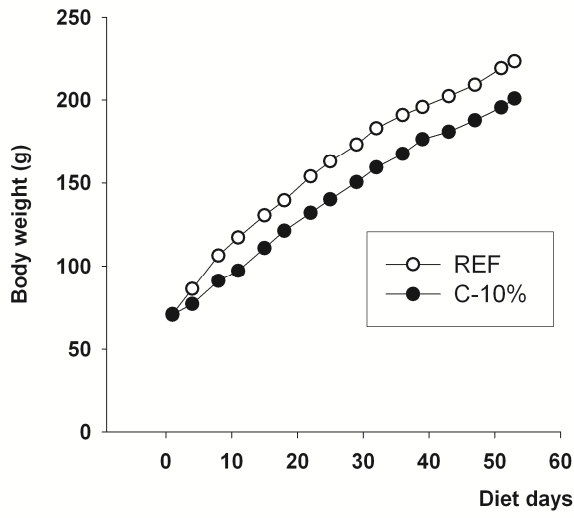
639

640 **Figure 4.** Expression of genes associated with IgA synthesis, secretion, switching,
641 intestinal homing and TLRs in PPs (A, D, G), MLNs (B, E, H) and SI (C, F, I) after the
642 cocoa diet. Expression levels were normalized using the expression of *Gusb* as the
643 endogenous housekeeping gene. Black bars correspond to the cocoa diet and white bars
644 correspond to the standard diet. Each bar represents the mean \pm SEM ($n = 5$ –7) of the
645 percentage of the cocoa group compared with the reference group, which represents
646 100% gene expression. * $P < 0.05$.

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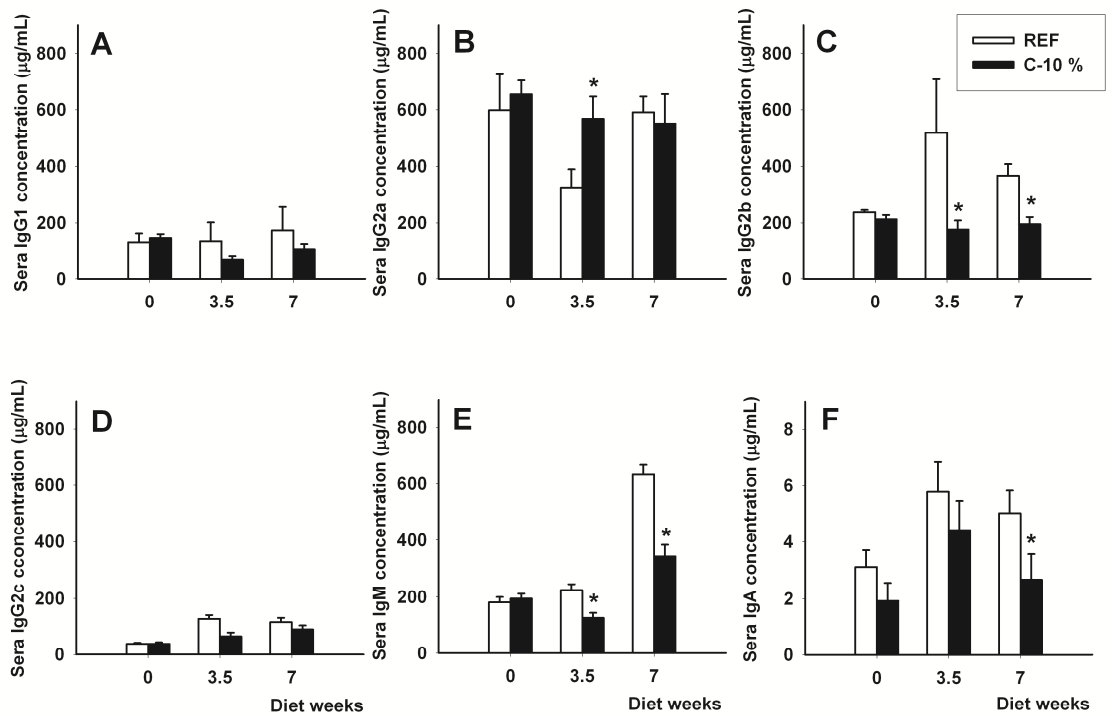
649 **Figure 1**



650

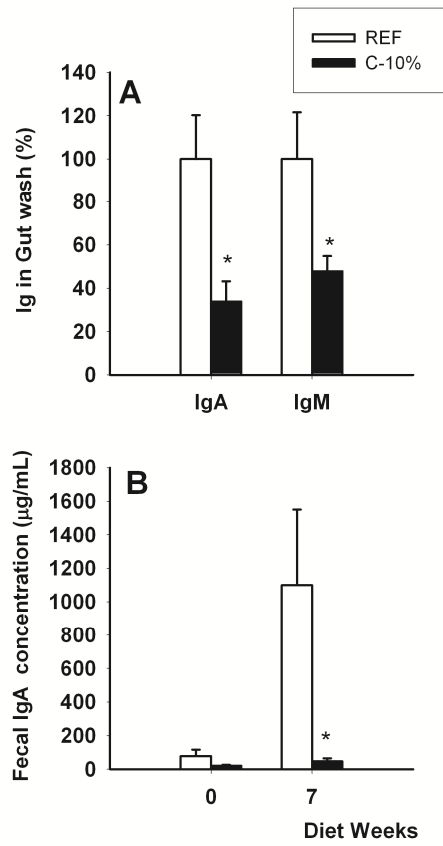
651

652 **Figure 2**



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654

655 **Figure 3**

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659 **Figure 4**

