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

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Running head: Rat intestinal IgA after cocoa intake

ABSTRACT

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

Keywords: gut immune system, flavonoids, mucosal antibodies

1. Introduction

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

Differentiation of B cells into IgA+ B cells occurs in PPs and, to a lesser extent, in ILF and MLNs [5]. Multiple cytokines such as transforming growth factor-β1 (TGF-β1), interleukin (IL)-5, IL-6 IL-10 and IL-21 are required to IgA class switching and to

promote IgA-committed B cells to proliferate and differentiate into IgA^+ B cells [8-11]. TGF-β1 plus the interaction of CD40 on B cells with CD40 ligand (CD40L) on T cells are crucial to elicit IgA class switching of activated B cells in germinal centers of PPs [12,13]. These IgA^+ B cells migrate from the PPs to the draining MLNs, and home back to the intestinal lamina propria via the thoracic duct and bloodstream to further differentiate into IgA-SCs [7]. This gut-homing system requires the integrin α4β7 on activated gut lymphocytes which binds to its receptor MAdCAM-1 on endothelial cells within the intestinal mucosa[5]**.** Moreover, gut-homing depends on chemokines such as CCL25 and CCL28. In humans and mice, crypt epithelial cells produce CCL25, which interacts with CCR9 on B and also T cells. CCL28 is a mucosal chemokine that assists cell homing in the large and small intestine, interacting with CCR10 [14]. However, this process involving PPs is not the only one for IgA synthesis. Alternatively, IgA+ B cells can be generated within ILFs and lamina propria in a T cell-independent manner. These mechanisms involve toll-like receptors (TLRs) and activated dendritic cells (DCs) producing B-cell activating factor from the TNF family (BAFF) and a proliferation-inducing ligand (APRIL) [12,15]. In any case, mucosal IgA-SCs mainly release dimers and some larger polymers of IgA, which are actively secreted to the apical surface of epithelial cells by the polymeric immunoglobulin receptor (pIgR) expressed on the basolateral surface [2].

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

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

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

2. Material and methods

2.1. Chemicals

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

2.2. Animals and experimental design

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

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

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

2.3. Sample collection

Fecal and sera samples were collected before the diet (week 0), in the middle (week 3.5) and at the end of the study (week 7) and kept at –20ºC for further immunoglobulin quantification. At the end of the dietary intervention, the rats were anesthetized intramuscularly with ketamine/xylazine. MLNs were removed in aseptic conditions for PCR analysis. The small intestine (SI) was excised, divided into two fragments and carefully flushed with sterile 0.9% NaCl solution to remove fecal content. The distal fragment of the SI was opened lengthwise and PPs were excised for PCR analysis, as well as a maximum of 30 mg of tissue corresponding to distal jejunum/proximal ileum without PPs. The remaining distal fragment of the SI was used 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 ºC
170 overnight before storing at -20 ºC.
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172 *2.4. Fecal homogenate obtention*
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174 Fecal samples were dried for 70 min at 37 ºC 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.
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180 *2.5. Immunoglobulin quantification in serum, gut wash and feces by ELISA*
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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 H_2O_2 were added for the detection of bound peroxidase. The reaction was

194 stopped by adding 3M H_2SO_4. 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.
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200 *2.6. Assessment of RNA gene expression by Real Time PCR*
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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 RNAeasy® 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*

(Rn00572010_m1, inventoried (I)), *Il5* (Rn99999143_mH, inventoried), *Il6* (Rn01410330_m1), *Cd40* (Rn01423583_m1,I), *Rara* (Rn00580551_m1, I), *Rarb* (Rn01537835_m1, I), *Ccr9* (Rn00597283_m1, I), *Tlr2* (Rn02133647_s1, I), *Tlr4* (Rn00569848_m1, I), *Tlr7* (Rn01771083_s1, I), *Tlr9* (Rn01640054_m1, I), *Pigr* (Rn00562362_m1, I), *Ccl25* (Rn0143351_m1, I) and *Ccl28* (Rn00586715_m1, I). Quantitative PCR assays were performed in duplicate for each sample using an ABI PRISM®7700 Sequence Detection System (AB). Quantification of the genes of interest was normalized to the housekeeping genes *Hprt1* (Rn01527840_m1, I) and *Gusb* (Rn00566655_m1, I). The amount of target mRNA relative to the endogenous control expression and relative to values from the reference group was calculated using the $2^{-\Delta\Delta Ct}$ method, as previously described [27], where Ct is the cycle number at which the fluorescence signal of the PCR product crosses an arbitrary threshold set within the exponential phase of the PCR and $\Delta\Delta Ct = [(Ct_{\text{target (unknown sample)}} - Ct_{\text{endogenous control (unknown sample)}})] - [(Ct_{\text{target (reference sample)}} - Ct_{\text{endogenous control (reference sample)}})]$. Results are expressed as the mean ± SEM of the percentage of these values for each experimental group compared with its reference age group, which represents 100% gene expression.

2.7. Statistical analysis

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

3. Results

3.1. Effect of cocoa diet on body weight

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

3.2. Effect of cocoa diet on serum immunoglobulins

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

3.3. Effect of cocoa diet on intestinal immunoglobulins

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

3.4. Expression of constitutive genes

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

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

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294 *Iga*, *Tgfb1*, *Il6*, *Il5* and *Cd40* expression was assessed in SI, PPs and MLNs after
295 7 weeks of cocoa intake (Fig. 4*A*, 4*B*, 4*C*). 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.

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

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307 *Rara*, *Rarb* and *Ccr9* were analyzed in PPs, MLNs and SI (Fig. 4*D*, 4*E*, 4*F*).
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.

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

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319 Expression of *Tlr2, Tlr4, Tlr7* and *Tlr9* was assessed in PPs, MLNs and SI (Fig.
320 4*G*, 4*H*, 4*I*). *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

4. Discussion

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

Firstly, we focused on the main pathway that brings about differentiation and maturation of B cells inducing them to become IgA-SCs, the T cell-dependent process that takes place in either PPs or MLNs, inductive sites of the intestinal immune system [3]. This process depends on cytokines such as TGF-β1 and IL-6, among others [7,10]. The results obtained in the present study showed no significant changes in TGF-β1 whereas IL-6 was depleted in the PPs and MLNs of animals that were fed cocoa. As IL-6 is secreted by DCs in PPs [15] we can suggest that some cocoa compounds reaching the intestine could act on these cells and modulate the secretion of IL-6 involved in IgA^+ B cell differentiation. In addition, the interaction between T and B cells through CD40L-CD40 is crucial to elicit IgA class switching on activated B cells in the germinal centers of PPs [12]. The expression of CD40 did not change in either PPs or MLNs after cocoa intake, which is in accordance with previous studies that have

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

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

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

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

In addition to the T-dependent way to secrete IgA referred to so far, IgA+ B cells can be alternatively generated in a T cell-independent manner which involves TLR 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, wich 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.

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

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

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

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

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FIGURE LEGENDS

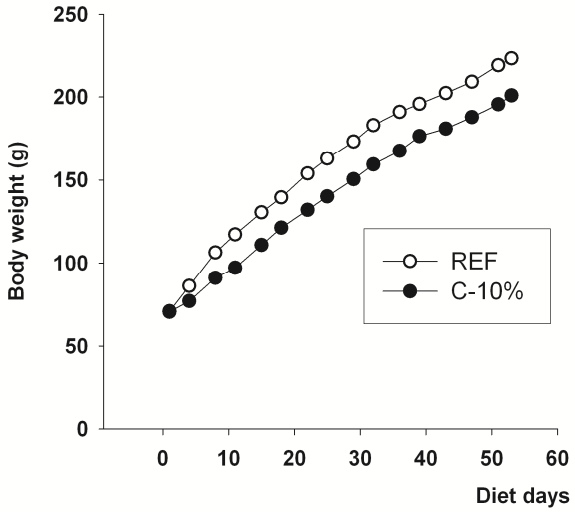
Figure 1. Body weight of female Wistar rats fed a cocoa (●) or standard (○) diet over 7 weeks. Data are means ± SEM (n = 7). Cocoa intake resulted in a lowered growth curve from day 8 and until the end of the study ($P < 0.01$).

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

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

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

649 **Figure 1**



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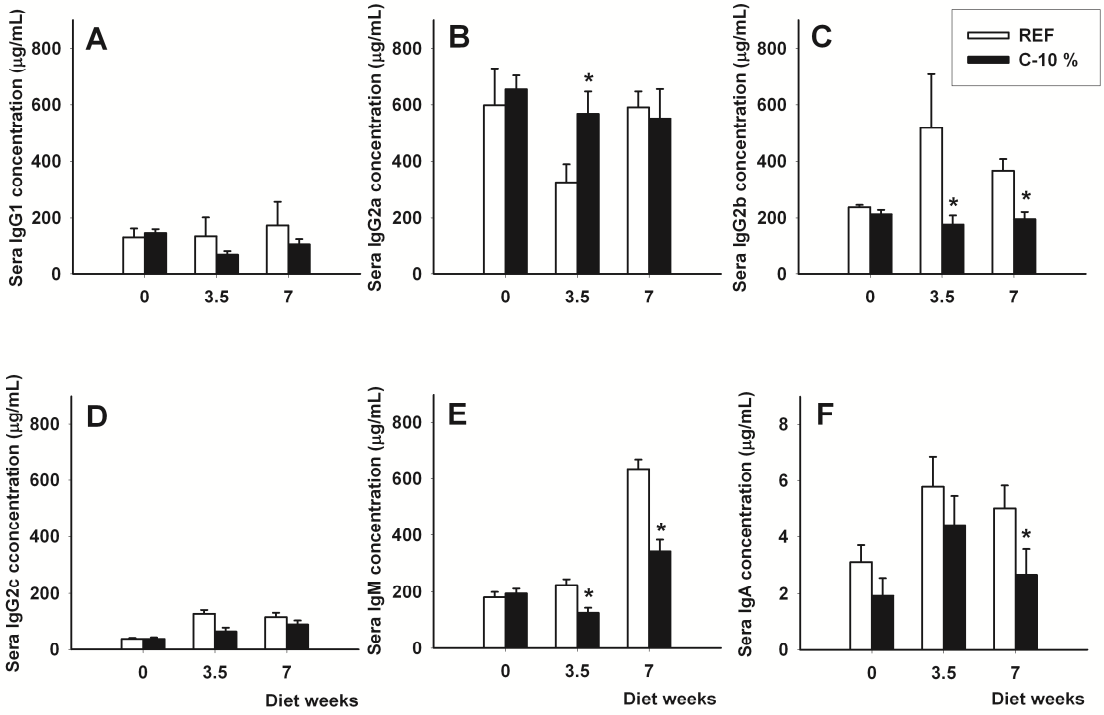
652 **Figure 2**



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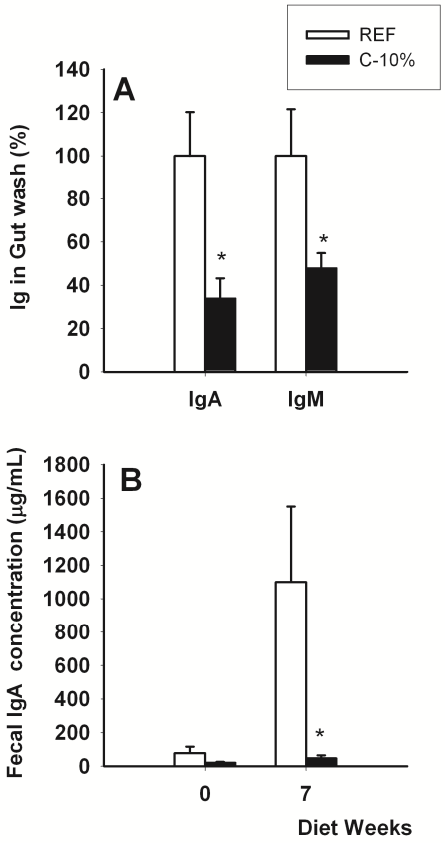
655 **Figure 3**



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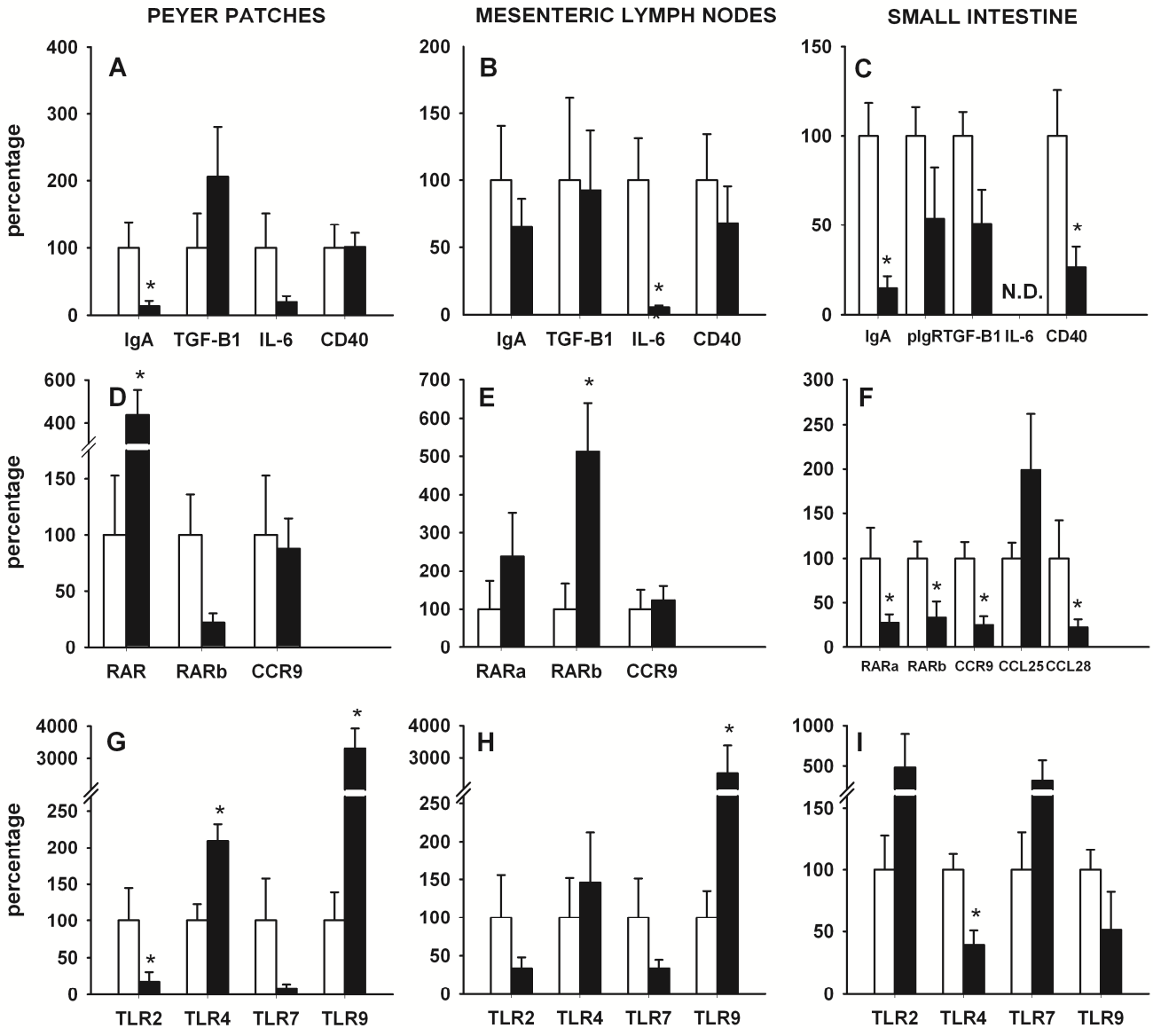
659 **Figure 4**



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