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RESEARCH ARTICLE

Cocoa-enriched diets modulate intestinal and systemic humoral immune response in young adult rats

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Scope: Previous studies have shown that a highly enriched cocoa diet affects both intestinal and systemic immune function in young rats. The aim of this study was to elucidate whether diets containing lower amounts of cocoa could also influence the systemic and intestinal humoral immune response.

Methods and results: Fecal and serum samples were collected during the study and, at the end, intestinal washes were obtained and mesenteric lymph nodes and small-intestine walls were excised for gene expression assessment. IgA, IgM, IgG1, IgG2a, IgG2b and IgG2c concentrations were quantified in serum whereas S-IgA and S-IgM were determined in feces and intestinal washes. Animals receiving 5 and 10% cocoa for 3 wk showed no age-related increase in serum IgG1 and IgG2a concentrations, and IgG2a values were significantly lower than those in reference animals. Serum IgM was also decreased by the 10% cocoa diet. The 5 and 10% cocoa diets dramatically reduced intestinal S-IgA concentration and modified the expression of several genes involved in IgA synthesis. A diet containing 2% cocoa had no effect on most of the studied variables.

Conclusion: The results demonstrate the downregulatory effect of a 5% or higher cocoa diet on the systemic and intestinal humoral immune response in adult rats.

Keywords:
Cocoa / Gut-associated lymphoid tissue / Humoral immune response / Immunoglobulin

1 Introduction

Cocoa, the dried and fermented seeds derived from *Theobroma cacao*, was being consumed as early as 1100 B.C. by ancient Mesoamerican civilizations [1]. Indeed, the Olmecs, and eventually the Mayans and Aztecs, consumed food and beverages produced from cocoa, which was considered a divine food and was reserved for ritualistic and medicinal purposes [2]. It was not until the 16th century that cocoa was introduced into Europe by Hernán Cortés and, three centuries later, Conrad van Houten developed cocoa powder as we know it today [2, 3].

During the last decade, cocoa has become a subject of great interest mainly due to its high contribution to the total dietary intake of flavonoids, particularly the flavanol monomers (−)-epicatechin and (+)-catechin, and oligomers derived from these monomers, the procyanidins [4, 5]. Flavonoids are a diverse family of naturally occurring polyphenolic compounds with recognized antioxidant potential, which may promote changes in the redox-sensitive signaling pathways involved in diverse gene expression and, in consequence, in several cell functions such as the immune response [6]. In this sense, numerous in vitro studies have highlighted the immunomodulatory properties of cocoa

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Abbreviations: Ab, antibodies; **I,** inventoried; **IgA-SCs,** IgA-secreting cells; **LP,** lamina propria; **MLN,** mesenteric lymph node; **OVA,** ovalbumin; **PP,** Peyer's patch; **RA,** retinoic acid; **RT,** room temperature; **SI,** small instestine; **TGF-β,** transforming growth factor-β; **Th cell,** T-helper cell; **TLR,** toll-like receptor; **Tw,** Tween 20

flavonoids and their metabolites [7–9]. However, very few studies have focused on the in vivo effect of cocoa on the immune system [10–12]. Previous studies in our laboratory demonstrated that cocoa-enriched diets are capable of modifying the lymphocyte subset proportion in the spleen and the concentration of serum immunoglobulins [10]. Moreover, the production of specific antibodies (Ab) and the number of specific IgG-secreting cells in the spleen were reduced by a cocoa diet in ovalbumin (OVA)-sensitized adult rats [12]. Besides affecting the systemic immune function, cocoa also influences the cell composition and functionality of the gut-associated lymphoid tissue. Specifically, a continuous 10% cocoa diet increases the proportion of γδT and B cells in Peyer's Patches (PPs) and reduces the T-helper (Th) cell percentage in young rats [11]. Interestingly, in spite of an increasing B-cell population in PPs, a 10% cocoa diet decreases the number of high-capacity IgA-secreting cells (IgA-SCs) in this compartment [11]. Likewise, a 10% cocoa diet significantly reduces the secretion of IgA into the intestinal lumen of young rats [11]. Similarly, in young rats, a 10% cocoa diet also increases the percentage of γδT and citotoxic T cells and diminishes that of Th cells in mesenteric lymph nodes (MLNs). Additionally, the cocoa dietary intervention down-regulates the secretion of IL-4 by MLN cells, a Th2 cytokine implicated in B-cell differentiation [11].

IgA is the most abundant immunoglobulin in the body and it is estimated that 80% of IgA-SC reside in gut mucosa [13]. IgA constitutes the main humoral mediator of first line mucosal defense and provides non-inflammatory immune protection by neutralizing microbial toxins and pathogens without activating the complement cascade [14]. Moreover, IgA prevents innocuous antigens and/or commensal flora from triggering an inflammatory response and plays a key role in the maintenance of gut microbiota [13, 15]. Prior to producing IgA, B cells have to differentiate into IgA-SCs after antigenic stimulation by means of both T-cell-dependent mechanisms, which are mainly regulated by interaction of CD40 on B cells with CD40 ligand on $CD4^+$ Th cells, and in the absence of cognate B–T interactions (T-cell-independent mechanisms) through toll-like receptor (TLR) interaction among others [15, 16]. Abundant cytokines are involved in IgA class switching and secretion, including IL-5, IL-6, IL-10, IL-21 and the transforming growth factor-β (TGF-β) among others [17–20]. Activated IgA^+ B cells differentiate into IgA plasmablasts and recirculate to the gut lamina propria (LP) directed by the expression of receptors CCR9 and/or CCR10 and the integrin α4β7 [14]. Previous in vivo studies have demonstrated the ability of a highly enriched cocoa diet to decrease the amount of IgA-secreting splenocytes as well as the IgM and IgG synthesis in the spleen of young rats after 3 wk of dietary intervention [10]. Similarly, the cocoa diet down-regulates intestinal S-IgA in weaned rats [11]. However, it is still not known whether the dosage of cocoa was a crucial factor in its immunomodulatory action. The aim of this study was to ascertain whether lower doses of cocoa also had an impact on the systemic and intestinal immune system and, moreover, to study in depth cocoa's effect on intestinal IgA secretion by focusing on some of the intestinal pathways and molecules involved in IgA^+ B-cell homing, and IgA synthesis and secretion into the gut.

2 Materials and methods

2.1 Chemicals

The Natural Forastero cocoa (provided by Nutrexpa SA, Barcelona, Spain) used in this study had a total polyphenol content of 10.62 mg/g with 0.14 mg/g (+)-catechin, 0.83 mg/g (–)-epicatechin and 0.65 mg/g procyanidin B_2.

Ketamine was provided by Merial (Barcelona, Spain) and xylazine by Bayer (Barcelona, Spain). Anti-rat IgA, IgM, IgG2a, IgG2b, IgG2c mAbs; rat IgA, IgM, IgG1, IgG2a, IgG2b and IgG2c recombinant proteins, and biotinylated anti-rat IgA, IgM, IgG1, IgG2a, IgG2b and IgG2c mAbs were purchased from BD Biosciences (Heidelberg, Germany). Anti-rat IgG1 was obtained from BioLegend (San Diego, CA, USA) and peroxidase-conjugated anti-rat Ig mAb was provided by DakoCytomation (Glostrup, Denmark). ExtrAvidin-peroxidase, BSA, *o*-phenylenediamine dihydrochloride, NaCl and 30% hydrogen peroxide were obtained from Sigma-Aldrich (Madrid, Spain). RNAlater® was purchased from Applied Biosystems (Austin, TX, USA).

2.2 Animals and diets

Twenty-five female Wistar rats (6-wk-old) were obtained from Harlan (Barcelona, Spain) and housed in polycarbonate cages (three to four per cage) under controlled conditions of temperature (22 ± 2°C) and humidity (50 ± 10%) in a 12:12 light:dark cycle (light on 8:00–20:00). The animals were given free access to water and chow and were randomly assigned to four dietary groups: the reference group ($n = 6$) was fed with AIN-93 M standard chow (Harlan) and the other three groups received 2% (C-2, $n = 6$), 5% (C-5, $n = 6$) and 10% cocoa (C-10 $n = 7$) in pelleted chow for 3 wk. These diets were produced from a modification of the AIN-93 M formula, by adjusting the amount of carbohydrates, proteins, lipids and fiber provided by the corresponding percentage of cocoa (Table 1). The ingredient mixture was pelletized (1.5 cm diameter) and subsequently dried in a 40°C oven for 36 h at the Drug Products Development Service of the Faculty of Pharmacy at the University of Barcelona. The pelleted diet was thereafter vacuum-packed in order to prevent oxidation and fungi contamination and stored at 4°C until used.

Body weight and food intake were monitored throughout the experiment. Studies were carried out according to the criteria outlined by the Guide for the Care and Use of Laboratory Animals. Experimental procedures were

Table 1. Composition of experimental diets (g/kg diet)

Components	Control chow (AIN-93M; g/kg)	C-2 chow (g/kg)	C-5 chow (g/kg)	C-10 chow (g/kg)
Casein	140	135.6	129	118
L-Cystine	1.8	1.8	1.8	1.8
Corn starch	465.69	462.49	457.69	449.69
Maltodextrin	155.0	155.0	155.0	155.0
Sucrose	100.0	100	100	100
Soybean oil	40.0	37.8	34.5	29
Cellulose	50.0	44.9	37.25	24.5
Mineral mix (TD94049)	35.0	35.0	35.0	35.0
Vitamin mix (TD94047)	10.0	10.0	10.0	10.0
Choline bitartrate	2.5	2.5	2.5	2.5
tert-Butylhydroquinone	0.008	0.008	0.008	0.008
Natural cocoa powder	–	20	50	100
22% Protein		*4.4*	*11*	*22*
16% Carbohydrate		*3.2*	*8.0*	*16*
11% Lipid		*2.2*	*5.5*	*11*
34% Fiber (25.5% cellulose)		*6.8 (5.1)*	*17 (12.75)*	*34 (25.5)*
kcal/g diet	3.6	3.6	3.6	3.6

reviewed and approved by the Ethics Committee for Animal Experimentation of the University of Barcelona (ref. 3131).

2.3 Sample collection

Fecal samples were collected before, and after 1 and 3 wk of the diet beginning and kept at −20°C until analysis. Blood was collected at the same time points and, after centrifugation, serum was separated and kept at −20°C until analysis. Fecal homogenates were obtained as previously described [11] and frozen at −20°C until ELISA IgA quantification.

At the end of the 3-wk nutritional intervention, the rats were anesthetized intramuscularly with ketamine (90 mg/kg) and xylazine (10 mg/kg) in order to obtain blood by cardiac puncture. MLNs and small intestine (SI) were removed in aseptic conditions for further gene expression analysis by real-time PCR. The SI was divided into two fragments and the distal fragment was flushed with sterile 0.9% NaCl solution in order to remove fecal content. Thereafter, this fragment was opened longitudinally and a maximum of 30 mg of tissue corresponding to distal jejunum without PPs was excised for gene expression assessment. MLNs and SI samples for real time PCR were collected in RNAlater® and incubated at 4°C overnight before storing at −20°C. The remaining distal fragment of SI was used to obtain the SI wash by shaking in PBS (37°C, 20 min) and SI washes were conserved at −20°C for IgA and IgM determination.

2.4 ELISA immunoglobulin quantification in serum, SI washes and feces

S-IgA and S-IgM concentrations in SI washes, fecal S-IgA and serum IgA, IgM, IgG1, IgG2a, IgG2b, IgG2c levels were quantified by ELISA. In brief, 96-well polystyrene plates (Nunc Maxisorp, Wiesbaden, Germany) were coated with anti-rat IgA, IgM, IgG1, IgG2a, IgG2b or IgG2c (2 μg/mL in PBS) and incubated in a humidified chamber overnight at room temperature (RT). The remaining binding sites were blocked with PBS containing 1% BSA (PBS-BSA, 1 h, RT). The plate was washed (three times with PBS containing 0.05% Tween 20 (Tw) and once with PBS), and then appropriate diluted samples and standard dilutions in PBS–Tw–BSA were added (3 h, RT). After washing, biotin-conjugated anti-rat IgM, IgG1, IgG2a, IgG2b or IgG2c mAbs were added (1 μg/mL in PBS–Tw–BSA) and incubated for 2 h (RT). Thereafter, peroxidase-conjugated ExtrAvidin (4 μg/mL in PBS–Tw–BSA) was incubated for 30 min (RT). Finally, *o*-phenylenediamine dihydrochloride plus H_2O_2 in an appropriate buffer was added as an enzyme substrate solution for the detection of bound peroxidase. The reaction was stopped by adding 3 M H_2SO_4. Absorbance was measured on a microplate photometer (Labsystems Multiskan, Helsinki, Finland) at 492 nm. Data were interpolated by means of Ascent v.2.6 software (Thermo Fisher Scientific, S.I.U., Barcelona, Spain) into the standard curves, and expressed as μg/mL.

2.5 RNA isolation from SI and MLNs, and assessment of gene expression by real-time PCR

SI and MLN samples were homogenized in a FastPrep® instrument (MP Biomedicals, Illkirch, France) for 40 s. Total RNA was isolated with the RNeasy® mini kit (Qiagen, Madrid, Spain) following the manufacturer's recommendations. The quality of the RNA was assessed by the Agilent 2100 Bioanalyzer with the RNA 6000 LabChip kit (Agilent Technologies, Madrid, Spain) prior to cDNA synthesis. Four

micrograms of total RNA were converted to cDNA using random hexamers (Applied Biosystems (AB), Weiterstadt, Germany). The specific PCR TaqMan® primers and probes (AB) used to measure genes involved in IgA class switching and secretion were: *Iga* (331943, made to order), *Tgfb1* (Rn00572010_m1, inventoried (I)), *Cd40* (Rn01423583_m1, I) and *Pigr* (Rn00562362_m1). The specific PCR TaqMan® primers and probes (AB) used to measure genes involved in gut homing and TLRs were: *Ccr9* (Rn00597283_m1, I), *Rara* (Rn00580551_m1, I), *Rarb* (Rn01537835_m1, I), *Tlr2* (Rn02133647_s1, I), *Tlr4* (Rn00569848_m1, I), *Tlr7* (Rn01771083_s1, I), *Tlr9* (Rn01640054_m1, I), *Ccl25* (Rn0143351_m1, I) and *Ccl28* (Rn00586715_m1, I). Quantification of the genes of interest was normalized to the endogenous control *Gusb* (Rn00566655_m1, I). Real-time PCR assays were performed in duplicate on an ABI PRISM®7700 Sequence Detection System (AB). The amount of target mRNA relative to *Gusb* expression and relative to a calibrator (tissue sample from reference animals) was calculated using the $2^{-\Delta\Delta Ct}$ method, where $\Delta\Delta Ct = [(Ct_{\text{target (unknown sample)}} - Ct_{\text{endogenous control (unknown sample)}}) - (Ct_{\text{target (calibrator sample)}} - Ct_{\text{endogenous control (calibrator sample)}})]$. 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. Results are expressed as the mean ± SEM of the percentage of these values for each experimental group compared with the reference group, which represents 100% gene expression.

2.6 Statistical analysis

The software package SPSS 16.0 (SPSS, Chicago, IL, USA) was used for statistical analysis. The Levene's and the Kolmogorov-Smirnov's tests were applied to assess variance equality and normal distribution, respectively. The one-way analysis of variance followed by the Scheffé's post hoc significance test was used when the assumptions of normality and equal variance were met. In the opposite case, nonparametric tests (Kruskal–Wallis and Mann–Whitney *U* rank-sum test) were used to assess significance. Moreover, the nonparametric Friedman test was used to compare three or more matched groups. Differences were considered statistically significant for p values < 0.05.

3 Results

3.1 Body weight and chow intake

Body weight was monitored throughout the study. Eight days after the start of the dietary intervention and throughout the study, the growth rate of the C-10 group was delayed, as reflected by the lower weight curve, compared to that of the reference group ($p < 0.01$, Fig. 1). The C-2 and C-5 groups showed a body weight time course similar to that observed in the reference group. Chow intake was similar in all groups (data not shown).

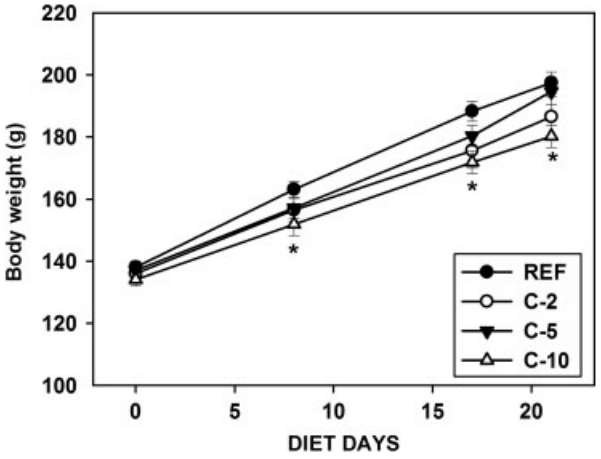

Figure 1. Body weight of female Wistar rats fed a standard (●), 2% cocoa (○), 5% cocoa (▼) or 10% cocoa (△) diet over 3 wk. Data are means ± SEM ($n = 6$–7).

3.2 Serum immunoglobulin concentration

The serum IgG1, IgG2a, IgG2b, IgG2c, IgM and IgA concentrations of all the experimental groups are summarized in Fig. 2. At the beginning of the experiment, the serum of 6-wk-old Wistar rats from all groups contained an IgG1 concentration ranging from 20 to 120 μg/mL with a mean of about 60 μg/mL, IgG2a from 150 to 500 μg/mL with a mean of about 275 μg/mL, IgG2b from 70 to 360 μg/mL with a mean of about 190 μg/mL and IgG2c from 50 to 250 μg/mL with a mean of about 140 μg/mL. In the reference group, the serum concentration of IgG1, IgG2a and IgG2b showed an increasing pattern over the 3-wk studied period (Friedman's test, $p < 0.05$), which means an increase of about 160, 140 and 40%, respectively. The cocoa nutritional intervention tended to avoid the increase in serum IgG1 concentration without achieving statistical significance (C-10 versus REF $p = 0.095$ at wk 3, Fig. 2A), and also resulted in an evident down-regulatory effect on IgG2a concentration, completely preventing the increase produced in the reference group. As a result, at the end of the study the C-5 and C-10 groups showed IgG2a concentrations ~60 and ~70%, respectively, lower than those in the reference animals (Fig. 2B, $p < 0.05$). These reductions were already evident after 1 wk of cocoa intake. On the other hand, serum IgG2b concentration was not significantly modified by any cocoa dosage (Fig. 2C) and that of IgG2c was significantly increased in the C-5 group ($p < 0.05$) and showed a trend to be increased in the C-10 group ($p = 0.07$, Fig. 2D).

The serum IgM concentration in 6-wk-old Wistar rats (before diet beginning) ranged from 80 to 180 μg/mL with a

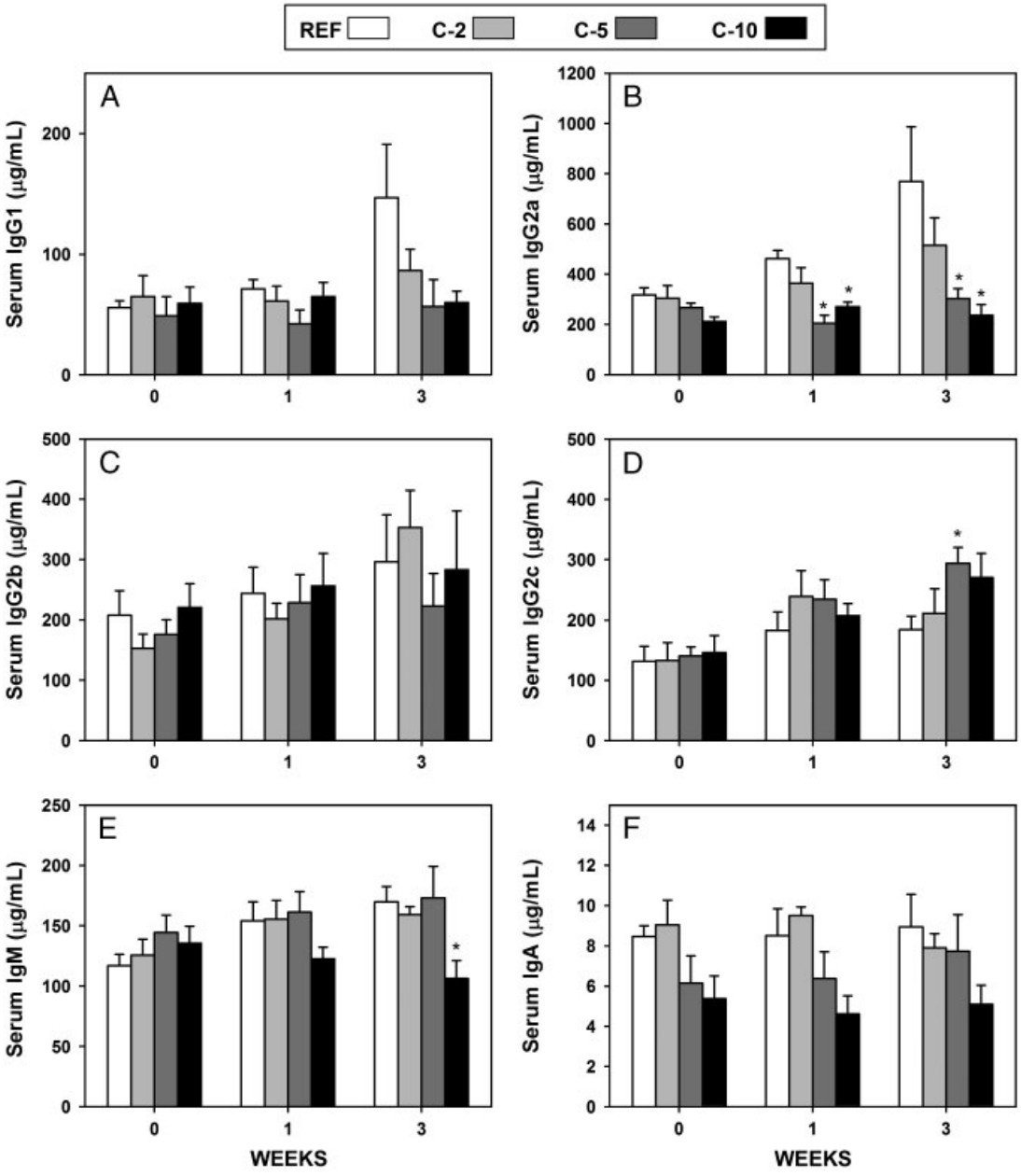

Figure 2. Influence of several cocoa diets on serum IgG1 (A), IgG2a (B), IgG2b (C), IgG2c (D), IgM (E) and IgA (F) concentrations. White bars correspond to the standard diet (reference group), whereas the light-grey, dark-grey and black bars correspond to the 2, 5 and 10% cocoa doses, respectively. Each bar represents the mean ± SEM ($n = 6$–7). $^{*}p < 0.05$ versus REF group.

mean of about 130 μg/mL, and increased by about 45% in reference rats during the study period. In animals fed a 10% cocoa diet this increase was prevented and after 3 wk of cocoa intake the serum IgM concentration was significantly reduced versus reference group (~40%, Fig. 2E, $p < 0.05$). The serum IgA concentration in 6-wk-old Wistar rats from all groups ranged from 2.2 to 13 μg/mL with a mean of about 7.2 μg/mL, and this concentration did not vary in the reference animals over the study. Serum IgA values were not modified by any of the cocoa diets during this period (Fig. 2F).

3.3 Intestinal immunoglobulin concentration

The intestinal S-IgA and S-IgM concentration was quantified in feces before, and after 1 and 3 wk of cocoa diets, and in intestinal washes at the end of the study (Fig. 3). S-IgM was not detected in fecal samples. The fecal S-IgA concentration increased in reference rats over the study ($p = 0.09$). This increase was avoided by the cocoa diets, S-IgA concentration actually decreasing during the study, and this was already significant after 1 wk of the C-5 and C-10 diets.

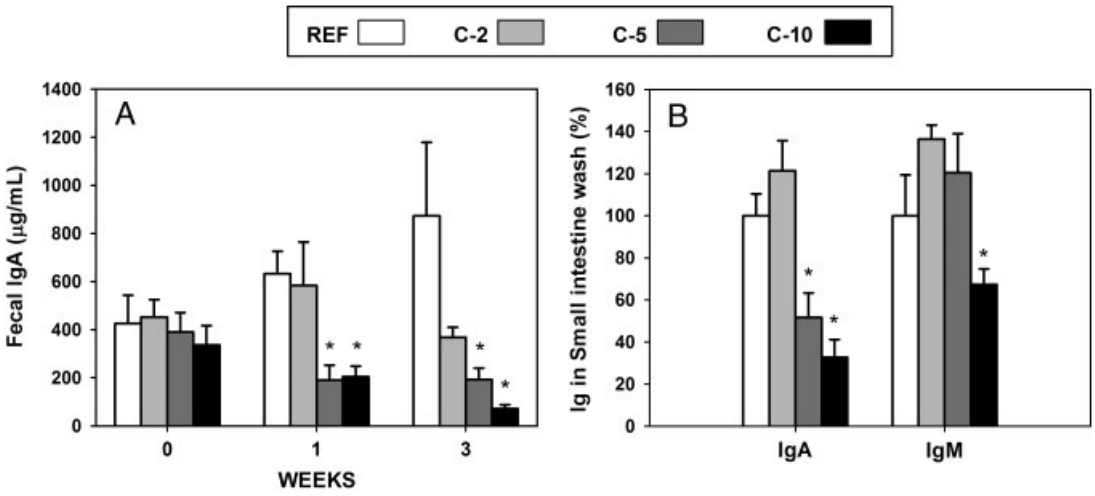

Figure 3. Effect of the cocoa-enriched diet on S-IgA in feces (A) and on S-IgA and S-IgM concentrations in gut wash (B). Gut-wash values were related to those found in the reference group, which is considered to be 100%. White bars correspond to the standard diet whereas the light-grey, dark-grey and black bars correspond to the 2, 5 and 10% cocoa doses, respectively. Each bar represents the mean ± SEM ($n = 6$–7). $^{*}p < 0.05$ versus REF group.

By the end of the study period the cocoa diet had significantly reduced the intestinal S-IgA in a dose-dependent manner (Fig. 3A, C-5 and C-10 groups, $p < 0.05$; C-2 group, $p = 0.14$). The S-IgA concentration obtained in gut washes after 3 wk of the diet followed almost exactly the same pattern (Fig. 3B, C-5 and C-10 groups versus reference group $p < 0.05$). Nevertheless, the S-IgM amounts in the gut washes were not modified in the C-2 and C-5 groups and were reduced by the highest cocoa dose (analysis of variance, $p < 0.05$; Fig. 3B).

3.4 Influence of cocoa diets on IgA class switching and secretion-associated genes

Iga, *Tgfb1*, *Il6* and *Cd40* expression was analyzed in the SI and MLNs after 3 wk of cocoa intake (Figs. 4A and B). The 10% cocoa diet but not the diets with lower amounts of cocoa, significantly reduced the expression of *Iga* and *Tgfb1* in the SI ($p < 0.05$) but did not modify their expression in the MLNs. *Il6* expression was not detected in the SI and the 10% cocoa diet tended to increase its expression in the MLNs (C-10 $p = 0.14$). Cocoa intake did not alter the expression of *Cd40* in any of the studied tissues. Moreover, *Pigr* expression, analyzed in the SI, tended to be slightly reduced by the highest cocoa diet ($p = 0.17$).

3.5 Influence of cocoa diets on genes associated with gut-homing of IgA-SC

Ccr9, Rara and Rarb expression was analyzed in the SI and MLNs and, additionally, *Ccl25* and *Ccl28* genes were assessed in the SI (Figs. 4C and D). Cocoa intake did not modify *Ccr9* expression in either the MLNs or SI. With regards to retinoic acid (RA) receptors, the 10% cocoa diet tended to down-regulate the expression of both *Rara* and *Rarb* ($p = 0.08$) in the SI, but, the same cocoa dose tended to raise the expression of *Rarb* in the MLNs. In the SI, all cocoa diets doubled the expression of the gut-homing *Ccl28* chemokine (C-5 and C-10 groups, $p < 0.05$) whereas *Ccl25* tended to be slightly down-regulated (C-2, $p = 0.11$; C-5, $p = 0.14$; C-10, $p = 0.09$).

3.6 Influence of cocoa diets on TLR expression

The relative expression of *Tlr2*, *Tlr4*, *Tlr7* and *Tlr9* was quantified in the SI and MLNs (Figs. 4E and F). The 10% cocoa diet decreased the *Tlr2* levels in the MLNs by ~50% ($p < 0.05$) and tended to exert the same effect on the SI ($p = 0.11$). *Tlr4* expression tended to be down-regulated by the highest cocoa dose in the SI ($p = 0.10$) but did not in MLNs. Cocoa decreased dose-dependently and significantly the *Tlr7* expression (C-5 and C-10 groups, $p < 0.05$) in the MLNs whereas this gene expression remained unaffected in the SI after cocoa intake. Although the 5% cocoa diet tended to down-regulate *Tlr9* expression in the SI ($p = 0.09$), *Tlr9* was correspondingly up-regulated in the MLNs of all the cocoa groups but this increase was only statistically significant in the C-5 group ($p < 0.05$).

4 Discussion

The present paper assesses the impact of several cocoa-enriched diets on the systemic and intestinal immunoglobulin concentrations after a 3-wk dietary intervention during

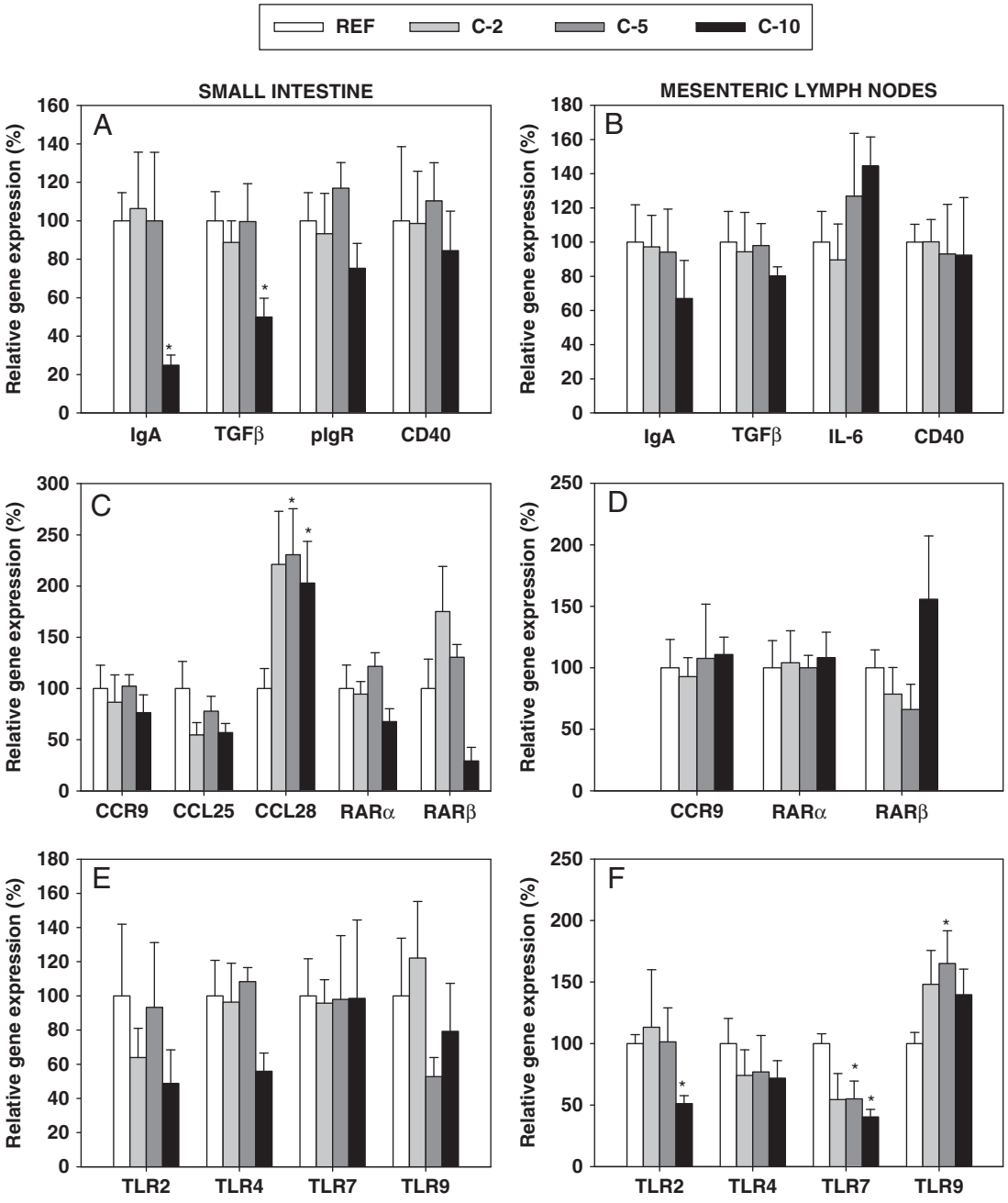

Figure 4. Expression of genes associated with IgA switching, synthesis, secretion, intestinal homing and TLRs in the SI (A, C and E) and MLNs (B, D and F) after cocoa diets. Expression values were normalized using the expression of *Gusb* as the endogenous housekeeping gene. White bars correspond to the standard diet whereas the light-grey, dark-grey and black bars correspond to the 2, 5 and 10% cocoa doses, respectively. Each bar represents the mean ± SEM ($n = 5$–7) of the percentage of the cocoa groups compared with the reference group, which represents 100% gene expression. $^{*}p < 0.05$ versus REF group.

the life of young adult rats. From the three cocoa dosages tested (2, 5 and 10% of food included in the chow), the 5 and 10% cocoa diets attenuated the serum concentration of IgG2a in a dose-dependent manner, whereas the serum concentration of IgG2c and IgM was affected only by 5 and 10% cocoa diets, respectively. Moreover, diets containing 5 and 10% of cocoa dramatically reduced the intestinal IgA concentration, and the 10% cocoa diet modified the expression of several genes involved in IgA synthesis.

Previous studies have shown that serum IgG concentration is reduced in young rats after 3 wk of cocoa intake. In the present study, carried out in older rats, it can be concluded that not all IgG subclasses were affected in the same manner by the cocoa diets. The most affected isotype

was IgG2a, whose serum concentration was diminished as early as 1 wk of cocoa intake. Moreover, cocoa diets tended to avoid the increase in serum IgG1 concentration that was produced by the age. On the other hand, the 5 and 10% cocoa diets increased the serum IgG2c concentration and did not affect that of IgG2b. It should be noted that IgG2a constituted the most abundant IgG in serum and that the isotypes IgG1 and IgG2a exhibited a marked increasing pattern in the reference group during the study. These age-dependent increases in the reference group may reflect the immune maturation of these animals during the study period, i.e. from youth to adult life, rather than environment changes because rats were acclimatized to diet and housing conditions 1 wk before the experiment. The results obtained here concerning the effects of the cocoa diet on IgG isotypes agree with the titers of specific antibodies quantified in OVA-immunized adult rats fed with cocoa. These rats synthesized lower anti-OVA IgG1 and IgG2a titers than OVA-immunized reference animals [12]. In rats, IgG1 and IgG2a isotypes seem to be associated with the Th2 immune response, and IgG2b and IgG2c seem to be dependent on the Th1 response [21–24]. Therefore, it could be suggested that a cocoa diet down-regulates the Th2 antibody response in a dose-dependent manner. Few studies addressing the dietary impact on the systemic humoral response in healthy animals have been reported to date. The effect on Th2 antibodies is in line with that obtained in mice fed with apple polyphenols and the isoflavone genistein, which showed decreased OVA-specific IgG1 antibodies [25, 26]. Moreover, OVA-specific total IgG and IgG1 titers were also reduced in OVA-immunized mice treated with flavonoid glycosides of *Sedum sarmentosum* or *Pollen Typhae* [27, 28]. In addition, some studies on the effect of cocoa diets on cytokine expression indicate that these diets produce a certain imbalance in the Th1/Th2 ratio in favor of Th1 [10, 12], similar to the results obtained with other flavonoids such as apigenin, chrysin, centaurein and centaureidin [29, 30]. All these results may suggest that dietary flavonoids, including those present in cocoa-enriched diets, could produce an attenuating effect on Th2 response and related antibodies. Nevertheless, the mechanisms involved in the differential modulation of such immunoglobulin synthesis are still unknown.

With respect to serum IgM, only the highest cocoa diet decreased IgM concentration at the end of the study as was previously described in younger animals [10]. Conversely, serum IgA concentration was not modified by any of the cocoa doses in young adult rats, which is not in line with previous studies in younger animals [10]. Thus, it seems that a 3-wk high cocoa intake exerts IgM down-regulation regardless of age, although this reduction was more prominent in young rats. Nevertheless, in young adult rats the 10% cocoa diet had no effect on serum IgA concentration. These results may indicate that the earlier the cocoa diet is started, the stronger the modulatory effect that can be achieved on the immune system with a high cocoa dose is. In this sense, an age-dependent effect and the differential roles of several types of dietary fibers on serum IgA concentration when administered to young and aged rats has been reported. Thus, whereas IgA was lower in aged rats fed with guar gum or glucomannan than in those rats receiving cellulose, the opposite was observed with the two diets provided to young rats [31, 32].

The down-modulatory effect of cocoa on immunoglobulin concentration was even more intensive in the intestinal compartment where S-IgA secreted to the intestinal lumen was drastically decreased after 1 wk of the 5 and 10% cocoa diets. These results on S-IgA are not in line with the lack of effect of cocoa on serum IgA concentration. This difference could be due to the functional separation between intestinal and systemic immune systems [33]. The results obtained in the intestinal compartment concur with those obtained in younger rats [11], indicating that S-IgA reduction induced by cocoa seems not to be dependent on age. In contrast to our findings, it has been reported that the consumption of the polyphenol curcumin increases fecal IgA concentration in rats fed a high-fat diet [34]. However, although some probiotics and prebiotics have been shown to increase intestinal S-IgA [35–37], limited information is available about the effects of dietary compounds on intestinal IgA synthesis in healthy animals. In order to detect a possible compensatory mechanism for IgA down-regulation, S-IgM was also analyzed but results showed that S-IgM also tended to decrease in the 10% cocoa group, thus discarding any compensation.

The attenuation of serum or intestinal immunoglobulin synthesis may be the result of a multitude of interferences, but the drastic mucosal IgA reduction observed here after cocoa dietary intervention may involve specific mechanisms located at the intestinal site. In this case, as cocoa reaches the intestine by the oral route, a direct influence on gut-associated lymphoid tissue may be plausible. In the present study the gene expression of some key molecules for IgA synthesis and regulation, mucosal homing and crosstalk with the microbiota through the TLRs were analyzed in the MLNs and SI, and were investigated for possible explanatory mechanisms for the intestinal IgA down-regulation by high cocoa doses. Cocoa intake did not modify the expression of CD40, which is found in activated B cells and interacts with the CD40 ligand on activated Th cells to begin the humoral immune response. Therefore, from our results, it could be suggested that cocoa diet did not exert an effect on this early phase of the antibody synthesis. With regards to the molecular effect of cocoa diets on the IgA switching process, no significant changes were found in the MLNs, considered as an inductor site of intestinal immune response, even in the expression of IL-6, a cytokine involved in the IgA switching process. However, the 10% cocoa diet produced a significant decrease in TGF-β1 from the SI, which might be partly responsible for the down-regulation of the IgA switching process, directly conducted by this cytokine [38]. In fact, the

results show that IgA gene expression was also compromised by the 10% cocoa diet in the SI. However, neither IgA nor TGF-β1 expression was down-regulated by the 5% cocoa diet, which also reduced intestinal IgA, indicating that an additional mechanism may be interfering in the intestinal IgA content.

Once activated, the IgA-committed B cells leave the inductor sites through the thoracic duct and home to the effector gut LP, where differentiation into IgA plasma cells takes place [14]. Intestinal homing is regulated by adhesion molecules and chemokine-mediated interaction including the integrin α4β7, which interacts with MAdCAM-1, and the chemokine receptor CCR9, which binds to CCL25 in the SI [39]. Similarly, the CCL28 chemokine seems to also be important for the recruitment of IgA^+ B cells to the small and large intestine [13]. Here, we found that the cocoa diets did not significantly modify the expression of the CCR9 receptor and CCL25 in the SI. However, the cocoa diets up-regulated the expression of CCL28. This result may indicate a ''rescue mechanism'' to strongly attract the IgA^+ B cells to the gut, in an attempt to compensate for the S-IgA down-regulation. On the other hand, RA produced by DCs from the PPs and MLNs plays a key role in gut-homing imprinting by induction of the CCR9 receptor [40]. As the genomic action of RA is arbitrated by the RA nuclear receptor family RAR [41], we also evaluated the expression of RAR-α as well as RAR-β after the cocoa diets. RAR-α was not modified by the nutritional intervention in any of the studied tissues, which concurs with the fact that CCR9 expression remained invariable, whereas RAR-β showed a tendency to be down-regulated by the 10% cocoa diet in the SI. Since RARα constitutes the most important player in intestinal mucosa [42], the effects of cocoa on RAR-β may not have been sufficient to modify CCR9 expression. In any case, we cannot conclude from these results that the cocoa S-IgA reduction can be attributed to a direct effect on the gut-homing process, but neither can we discount the possibility that other trafficking molecules such as α4β7 or MAdCAM-1, among others, might be affected.

Homing of IgA^+ B cells to the intestinal LP constitutes an efficient mechanism for delivering IgA into the intestinal lumen in association with the transmembrane epithelial protein pIgR [13]. We found that pIgR was not significantly modified by the dietary intervention, thus indicating that cocoa-induced IgA reduction did not occur as a consequence of a decreased transport across the epithelium. The literature describing the effects of nutritional interventions on the arrival of IgA^+ B cells in the LP or IgA secretion into the intestinal lumen is limited and mainly refers to vitamin A deficiency studies [43–45]. On the other hand, several studies point out the pivotal role of TLR signaling in controlling T-cell-dependent and -independent IgA responses both at mucosal and systemic levels [38]. In fact, TLR signaling in intestinal epithelial cells promotes the recruitment of IgM^+ B cells to the LP and enhances their switching to IgA^+ B cells [38, 46]. Specifically, TLR4 signaling in intestinal epithelial cells has been reported to promote the recruitment of B cells to the LP by means of CCL28 and CCL20 chemokines [46] and TLR7 expression is required for IgA responses in respiratory mucosa [47]. Moreover, the addition of TLR2, TLR7 or TLR9 agonists to mononuclear cells cultures highly up-regulates IgA production and secretion [48, 49]. The results in this study showed that the cocoa diets did not bring about significant changes in the SI, but the 5 and 10% cocoa diets modified the expression of some of the studied TLRs in the MLNs. In particular, these diets up-regulated the expression of TLR9 and decreased that of TLR7 in this tissue. The decreased expression of TLR7 could partly explain the lower S-IgA concentration in C-5 and C-10 groups and TLR9 might have been up-regulated as a ''rescue mechanism'' in an attempt to counteract the lowered TLR7 levels. Similarly, the 10% cocoa diet down-regulated the TLR2 levels in the MLNs, which may have also contributed to decrease the intestinal IgA in this group. TLRs are key molecules participating in the recognition of microorganisms and it might be plausible that IgA reduction by a 5 or a 10% cocoa diet might be partly associated with changes in the intestinal microbiota that could somehow be affecting the TLR expression in the inductor site. In fact, the flavanols epicatechin and catechin, which are abundantly present in cocoa and tea, have been reported to conduct changes in the human microbiota and exert prebiotic action [50]. Moreover, resveratrol, a polyphenolic compound highly present in red wine, and the ellagitannins of pomegranate, have also been identified as responsible for changes in the intestinal microbiota in rats with an increase of the *Bifidobacterium* and *Lactobacillus* populations [51, 52]. In any case, further studies should be carried out in order to fully evaluate the effects of cocoa intake on the composition and metabolism of microbiota. In addition to direct effects on microbiota, several studies evidence the TLR-modulating capacity of some polyphenols. In particular, curcumin has been reported to inhibit the dimerization of TLR4, which prevents the activation of downstream signaling pathways [53]. Similarly, epigallocatechin-gallate or luteolin, flavonoids found in green tea and green pepper, respectively, are capable of modulating the MyD88- and TRIF-dependent signaling pathways of TLRs [54, 55]. Thus, we cannot discard a direct effect of cocoa flavonoids on TLR-signaling.

In summary, 3-wk diets containing 5 and 10% cocoa affected the systemic immunoglobulin concentration in adult rats by down-regulating isotypes associated with the Th2 immune antibody response. In addition, the 5 and 10% cocoa diets drastically reduced the intestinal IgA content, which cannot be only attributed to a lower IgA synthesis but also to a number of factors involved in the IgA switching process, and even to the TLR expression that controls the crosstalk between the intestinal microbiota and/or its relationship with gut immune cells. The diet containing 2% cocoa did not show immunomodulatory effects. Further studies should be carried out in order to shed light on the

mechanisms modified by cocoa-enriched diets involved in serum immunoglobulin synthesis, and to study in greater depth the pathways of IgA and IgM gut secretion modulated by cocoa. In any case, a high cocoa intake might play an immunoregulatory role that could favor the oral tolerance acquisition and could be important in diseases involving a dysregulation of intestinal antibody responses such as celiac disease or food allergies.

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