Research Article

Influence of a cocoa-enriched diet on specific immune response in ovalbumin-sensitized rats

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Previous studies in young rats have reported the impact of 3 weeks of high cocoa intake on healthy immune status. The present article describes the effects of a longer-term cocoa-enriched diet (9 weeks) on the specific immune response to ovalbumin (OVA) in adult Wistar rats. At 4 weeks after immunization, control rats produced anti-OVA antibodies, which, according their amount and isotype, were arranged as follows: IgG1 > IgG2a > IgM > IgG2b > IgG2c. Both cocoa diets studied (4% and 10%) down-modulated OVA-specific antibody levels of IgG1 (main subclass associated with the Th2 immune response in rats), IgG2a, IgG2c and IgM isotypes. Conversely, cocoa-fed rats presented equal or higher levels of anti-OVA IgG2b antibodies (subclass linked to the Th1 response). Spleen and lymph node cells from OVA-immunized control and cocoa-fed animals proliferated similarly under OVA stimulation. However, spleen cells from cocoa-fed animals showed decreased interleukin-4 secretion (main Th2 cytokine), and lymph node cells from the same rats displayed higher interferon-γ secretion (main Th1 cytokine). These changes were accompanied by a reduction in the number of anti-OVA IgG-secreting cells in spleen. In conclusion, cocoa diets induced attenuation of antibody synthesis that may be attributable to specific down-regulation of the Th2 immune response.

Keywords: Antibody subclass / Cocoa / Immunization / Th1/Th2 balance

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1 Introduction

The Mayans and Aztecs considered cocoa a divine beverage with medicinal properties. Now, the health benefits of cocoa known by these ancient civilizations are again emerging, and cocoa has been recognized as a rich source of polyphenol antioxidants, particularly flavonoids. Cocoa powder mainly contains (−)-epicatechin and (+)-catechin, as well as procyanidins, the polymers derived from these monomers [1, 2]. Several studies have demonstrated the antioxidant capacity of cocoa flavonoids and their metabolites [3–5]. Epicatechin and catechin are very effective for neutralizing several types of reactive radicals [5–8]. In fact, cocoa intake increases the total antioxidant capacity and has been found to decrease lipid oxidation products in murine plasma and human plasma from healthy subjects [9–11].

In addition to its antioxidant properties, cocoa has immunoregulatory effects on innate and acquired immunity. In the former case, cocoa extract decreases the release of inflammatory mediators from macrophages, such as tumor necrosis factor (TNF)-α, monocyte chemoattractant protein 1, and nitric oxide *in vitro* [12, 13]. Cocoa also influences acquired immunity [14]. The acquired immune response begins when naïve T helper (Th) cells with a specific antigen receptor are activated by interactions with antigen-presenting cells [15]. To give rise to effector cells, Th cells are exposed to activation signals from complexes of antigenic peptide bound to MHC and costimulatory molecules [16]. Furthermore, Th cells are influenced by cytokine signals from the environment [17]. Th cell activation triggers production of IL-2, a cytokine with multiple functions, includ-

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Abbreviations: **Ab**, antibody; **AEC**, 3-amino-9-ethyl-carbazole; **ANOVA**, analysis of variance; **FBS**, fetal bovine serum; **GALT**, gut-associated lymphoid tissue; **LN**, lymph node; **OPD**, *o*-phenylenediamine; **OVA**, ovalbumin; **PMA**, phorbol myristate acetate; **SC**, secreting cells; **Th**, T helper

ing promotion of lymphocyte proliferation [18]. Th effector cells can be divided into two distinct subsets on the basis of their functional capabilities and the profile of cytokines they produce [19]: the Th1 subset secretes cytokines that are usually associated with cell-mediated immune responses, such as IFNγ, IL-2, and TNFα, and the Th2 subset produces cytokines linked to the humoral-type immune response, such as IL-4, IL-5 and IL-13, which help B cells to proliferate and differentiate. IFNγ and IL-12 are thought to be the major cytokines that induce Th1 differentiation and, moreover, IFNγ prevents the outgrowth of Th2 cells [20]. IL-4 has the greatest influence in driving Th2 differentiation [21, 22].

In the earliest phases of the acquired immune response, cocoa extract reduces *in vitro* T lymphocyte proliferation by down-regulating IL-2 and IL-2R expression [23 – 25]. Cocoa procyanidins also decrease IL-2 produced by stimulated peripheral blood mononuclear cells [24]. Moreover, cocoa extract slightly increases *in vitro* IL-4 secretion, while epicatechin produces a greater stimulatory effect [25]. In addition, short-chain cocoa procyanidins enhance IL-4 and IL-5 release, whereas long-chain procyanidins reduce both Th2 cytokines [26, 27].

Cocoa immune regulation has been also tested *in vivo*. In a previous study, we investigated the impact of cocoa-rich diets on lymphoid tissue of healthy young rats. Three-week cocoa intake promoted the progress of immature thymocytes toward more mature T cell stages [28]. Moreover, the cocoa diet increased the proportion of thymus $CD4^-CD8^-$ cells [28], which possess multi-lineage potential, including B and T cells, myeloid cells, natural killer cells and dendritic cells [29]. Cocoa intake not only restrains T cell maturation, but also affects lymphocyte composition and function in secondary lymphoid tissues. Continuous, high cocoa intake in young rats increased the proportion of B cells and decreased the proportion of Th cells present in the spleen [14]. In addition, in gut-associated lymphoid tissue (GALT), cocoa intake reduced Th lymphocyte percentage and increased B and γδ T cell percentages in Peyer's patches, and increased γδ T cell percentage and reduced Th percentage in mesenteric lymph nodes (LNs) [15]. Although cocoa resulted in a B cell increase in spleen and Peyer's patches, it is noteworthy that the ability of these cells to secret immunoglobulins was down-regulated. This fact was reflected by lower plasma IgG, IgM and IgA levels and reduced S-IgA and S-IgM concentration in the intestinal lumen [14, 30].

In the present article, we further analyze the *in vivo* impact of continuous cocoa diets on the immune system. The aim of the present study was to investigate whether two cocoa-enriched diets affect T cell function and the development of the humoral immune response to ovalbumin (OVA). We report that high cocoa intake produces a marked attenuation of antigen-specific antibody (Ab) production in OVA-sensitized rats.

2 Materials and methods

2.1 Chemicals

The Natural Forastero cocoa (Nutrexpa, Barcelona, Spain) used in this study presented a total polyphenol content of about 22 mg/g with 2.2 mg/g (−)-epicatechin, 0.74 mg/g (+)-catechin and 1.7 mg/g procyanidin B2 [28].

OVA (grade V), casein, phorbol myristate acetate (PMA), ionomycin, peroxidase-conjugated extravidin, *o*-phenylenediamine (OPD), 3-amino-9-ethyl-carbazole (AEC), L-glutamine, streptomycin and penicillin were from Sigma-Aldrich Chemie (Steinheim, Germany). Inject alum was obtained from Pierce (Rockford, IL, USA). Biotin-conjugated anti-rat IgM, IgG, IgG1, IgG2a, IgG2b and IgG2c mAb were purchased from BD Pharmingen (Madrid, Spain), and peroxidase-conjugated anti-rat Ig Ab from Dako cytomation (Glostrup, Denmark).

RPMI 1640 medium and fetal bovine serum (FBS) were obtained from BioWhittaker (Verviers, Belgium), and 2-mercaptoethanol was provided from Merck (Darmstadt, Germany).

2.2 Animals and diets

Wistar rats (21 days old, 50% male, 50% female) were obtained from Harlan (Barcelona, Spain). Rats were housed under conditions of controlled temperature and humidity in a 12:12 light:dark cycle. Rats were assigned to one of the following three dietary groups: (i) 4% cocoa diet group: animals fed chow containing 4% cocoa starting at their arrival (3 weeks old) and continuing to the end of the study (12 weeks old); (ii) 10% cocoa diet group: animals fed chow containing 10% cocoa starting at their arrival (3 weeks old) and continuing to the end of the study (12 weeks old); or (iii) control group: animals fed control chow.

The AIN-93G formulation (Harlan) was used as the control diet. Previous studies have demonstrated that this diet does not contain detectable amounts of polyphenols [28]. The 4% and 10% cocoa diets were produced with a modification of the AIN-93G formula, consisting of subtracting the amount of carbohydrates, proteins, lipids and fiber provided by the corresponding percentage of cocoa.

Studies were 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 (ref. 3129).

2.3 OVA immunization

OVA immunization was carried out when rats were 8 weeks old. All animals received 10 mg/kg OVA emulsified with alum adjuvant by intraperitoneal injection. At 4 weeks after immunization, rats were anesthetized to obtain blood by

cardiac puncture, and spleen and mesenteric LNs were excised. Serum was kept at –20°C for further use.

2.4 Determination of serum anti-OVA Ab levels

Indirect ELISA assays were performed to determine specific serum anti-OVA Ab concentrations, particularly Ab belonging to the IgG1, IgG2a, IgG2b, IgG2c and IgM isotypes. In brief, ELISA plates (Nunc Maxisorp, Wiesbaden, Germany) were coated with 100 µL OVA solution (10 µg/mL OVA in PBS), left overnight and blocked with 0.5% gelatin in PBS for 1 h. After washing three times with PBS containing 0.05% Tween 20 and once with PBS alone, appropriately diluted serum samples were added and incubated for 3 h. After washing, biotin-conjugated anti-rat IgM, IgG1, IgG2a, IgG2b or IgG2c Ab were added and incubated for 2 h. Thereafter, peroxidase-conjugated extravidin was incubated for 30 min. Lastly, an OPD solution was added for detection of bound peroxidase; 3 N H_2SO_4 was added to stop the reaction. OD was measured on a microtiter plate photometer (Labsystems Multiskan, Helsinki, Finland) at 492 nm. The Ab units for each isotype were calculated by comparison with the arbitrary units assigned to a pool of OVA-immunized rat sera diluted adequately to match the tested samples.

2.5 Preparation and culture of spleen and LN lymphocytes

Spleen and mesenteric LN lymphocytes were isolated immediately after organ excision. Spleen cell suspensions were obtained by passing the tissue through a steel mesh in cold sterile conditions. Cells were then centrifuged ($600 \times g$, 5 min, 4°C) and resuspended in PBS (pH 7.2). Erythrocytes were lysed by adding distilled water to the cell suspension for 5 s. Tonicity was restored by adding PBS ($10 \times$). Cells were then washed and resuspended with RPMI 1640 medium supplemented with 10% FBS, 100 IU/mL streptomycin-penicillin, 2 mM L-glutamine, and 0.05 mM 2-mercaptoethanol (complete culture media).

LN cell suspensions were obtained in sterile conditions by passing the tissue through a steel mesh. The cell suspension was left on ice for 10 min to remove tissue debris by sedimentation. Cells were then centrifuged ($600 \times g$, 5 min, 4°C) and resuspended in complete culture media.

2.6 Quantification of anti-OVA Ab-secreting cells by ELISPOT

Anti-OVA IgM- and IgG-secreting cells (SC) from spleen and LN were counted by the ELISPOT technique. A 96-well nitrocellulose plate (Multiscreen MAHAN 4510, Millipore, Eschborn, Germany) was coated with OVA diluted in PBS (20 µg/mL) in sterile conditions (overnight, 4°C). The plate was then blocked with complete culture media for 1 h at 37°C. Freshly isolated spleen and LN cells were plated at serial dilutions (2.5×10^5, 1.25×10^5 and 0.625×10^5 cells/well) and were incubated for 18 h (37°C, 5% CO_2). Cells were then removed by washing with PBS containing 0.25% Tween 20 ($\times 10$), and with distilled water ($\times 1$). Biotin-conjugated anti-rat IgM or IgG Ab (2 µg/mL in PBS) was added and incubated for 2 h at room temperature. The plate was washed again, and then incubated with extravidin-peroxidase conjugate (4 µg/mL) for 1 h at room temperature. Spots (each one corresponding to one anti-OVA Ab-SC) were visualized by addition of the substrate solution (AEC plus H_2O_2 in 0.1 M acetate solution). The reaction was stopped by rinsing with tap water for 5 min. Spots were counted automatically by the computer-assisted ELISPOT image analysis (ELISPOT reader system, AID, Strassberg, Germany).

2.7 Determination of anti-OVA Ab produced by LN and spleen cell culture

Levels of total anti-OVA Ab secreted over 4 days by non-stimulated spleen and LN cells (10^6 cells/mL) were quantified by an ELISA technique similar to the one described above. In this case total anti-OVA Ab levels were detected directly using peroxidase-conjugated anti-rat Ig (1.3 µg/mL).

2.8 Quantitation of LN and spleen cell proliferation

LN and spleen cells were cultured at 3.5×10^5 cells in 200 µL and stimulated with OVA (10 µg/mL) or casein (negative control protein, 10 µg/mL), or non-stimulated, for 96 h (37°C, 5% CO_2). Cell proliferation was determined by an ELISA method using Cell Proliferation Biotrak from Amersham Biosciences (Munich, Germany). This assay is based on the measurement of 5-bromo-2′deoxyuridine incorporation during DNA synthesis in proliferating cells, and was performed following the manufacturer's instructions.

2.9 Cytokine quantification after LN and spleen activation

LN and spleen cells were cultured at 10^6 cells/mL in 12-well plates and stimulated with PMA (250 ng/mL) plus ionomycin (250 ng/mL) for 24 h. Levels of IL-2, IL-4 and IFN-γ from supernatants were quantified using rat ELISA kits from Biosource (Nivelles, Belgium) and BD Pharmingen (Erembodegem, Belgium). ELISAs were performed according to the manufacturers' recommendations.

2.10 Statistical analysis

A one-way analysis of variance (ANOVA) followed by the bilateral Dunnet *post hoc* test was used to compare the cocoa diet groups with the control diet. Differences were considered statistically significant at p values of <0.05.

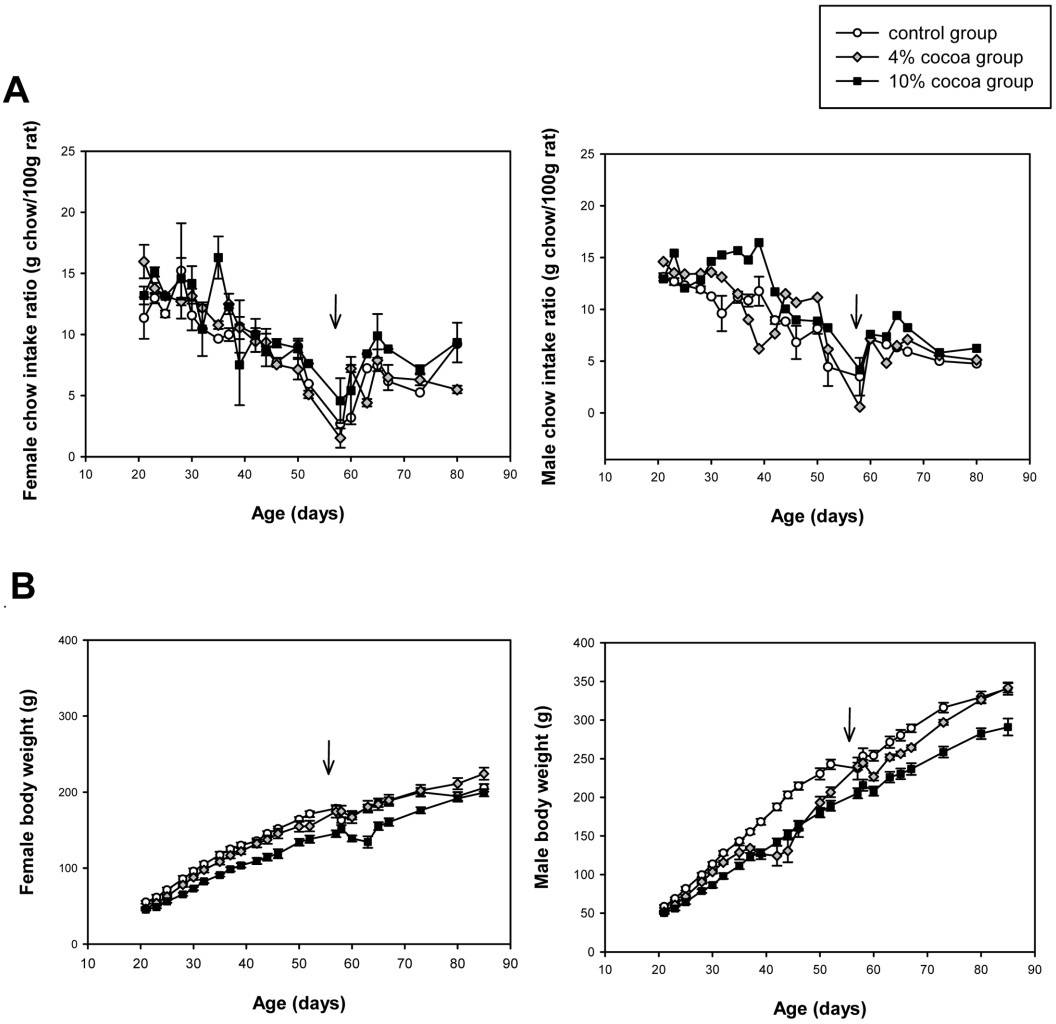

Figure 1. (A) Chow intake ratio (g/100 g rat) and (B) body weight monitored over the cocoa diets according to the rat sex. On day 58 (arrow), rats were immunized with OVA. Values are expressed as mean ± SEM (n=10 – 13).

3 Results

3.1 Chow intake and body weight

Body weight and chow intake were monitored throughout the study. Chow intake was referred to body weight (g chow/100 g animal) and this ratio was influenced by age (younger animal, higher intake) and the stress caused by immunization. Food intake was similar among females and males, regardless of whether the chow contained cocoa (Fig. 1A). The body weight time course in both females and males from the 10% cocoa group showed a lower slope than that of the control rats ($P < 0.01$) (Fig. 1B). The effect of cocoa diets on body weight has been reported in previous studies [14, 31].

3.2 Serum OVA-specific Abs

Serum concentrations of specific OVA Ab belonging to the IgM, IgG1, IgG2a, IgG2b and IgG2c isotypes from all the experimental groups are summarized in Fig. 2. At 4 weeks after immunization, rats produced a high amount of anti-OVA Abs. The control animals produced anti-OVA titers arranged as: IgG1 > IgG2a > IgM > IgG2b > IgG2c. Both cocoa diets produced a decrease in the main serum anti-OVA Ab levels. Thus, animals fed 4% cocoa showed reductions up to ~15% in anti-OVA IgG1 (p <0.01); up to ~65% in anti-OVA IgM ($p < 0.05$), and up to ~20% in anti-OVA IgG2c ($p < 0.01$). The impact of the 10% cocoa diet was more pronounced with decreases of up to ~4% in anti-OVA IgG1 levels ($p < 0.01$); up to ~45% in anti-OVA IgM ($p < 0.05$); up to ~14% in anti-OVA IgG2a ($p < 0.05$) and up to ~13% in anti-OVA IgG2c ($p < 0.01$). In addition, the 10% cocoa group presented increased levels of anti-OVA IgG2b ($p < 0.05$), whereas the 4% group showed no changes in this parameter.

The level of anti-OVA Ab secreted by LN and spleen cell cultures over 4 days was also measured (Fig. 3). The reduction in anti-OVA Ab secretion in the cocoa groups was also

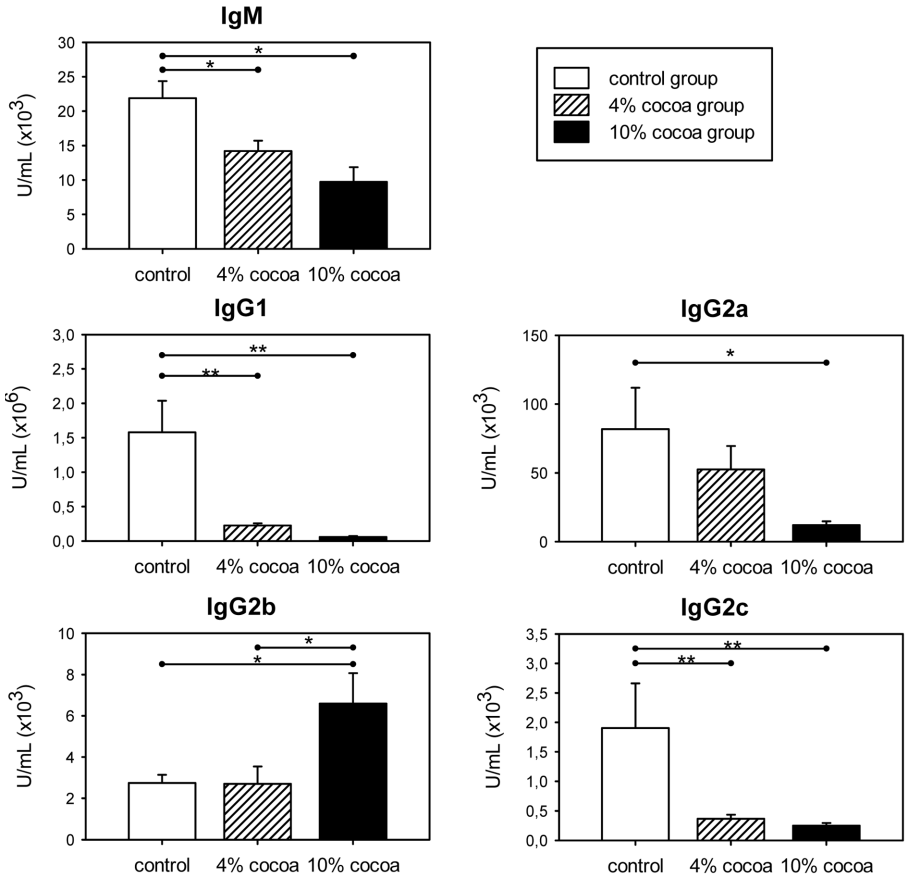

Figure 2. Effects of cocoa-enriched diets on serum anti-OVA Abs belonging to IgM, IgG1, IgG2a, IgG2b and IgG2c isotypes. Each bar represents the mean ± SEM (n=10–13). * $p < 0.05$ ** $p < 0.01$.

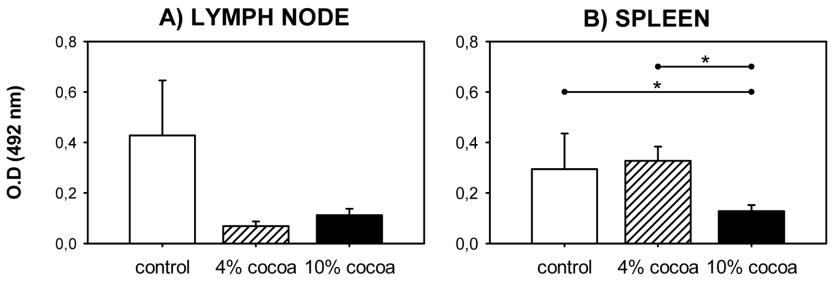

Figure 3. Effects of cocoa-enriched diets on the (A) LN and (B) spleen cell ability to secret anti-OVA Ab *in vitro*. Each bar represents the mean ± SEM (n = 7–11). * $p < 0.05$.

patent in these cell models, but only the spleen cells from animals in the 10% cocoa group showed statistically significant differences ($p < 0.05$).

3.3 Anti-OVA Ab-SCs

LN and spleen anti-OVA Ab-SC were quantified by ELISPOT (Fig. 4). Neither LN nor spleen anti-OVA IgM-SC counts were significantly modified by either cocoa diet. Nonetheless, the number of OVA-specific IgG-SC in both tissues were significantly decreased in the cocoa groups ($p < 0.05$).

3.4 LN and spleen cell proliferative response

Cell suspensions from LN and spleen were OVA-stimulated *in vitro* and the proliferative response was measured 96 h later. As shown in Fig. 5, LN cells proliferated under OVA stimulation compared to casein or medium ($p < 0.05$). There was no difference between control cells and those obtained from cocoa-diets, *i.e.* the ability of cells to proliferate upon specific antigen stimulation was not modified by either cocoa diet, in contrast to cocoa effect on Ab production. Similar results were obtained for spleen lymphocytes, although their OVA-specific proliferative response was lower than that in LN cells.

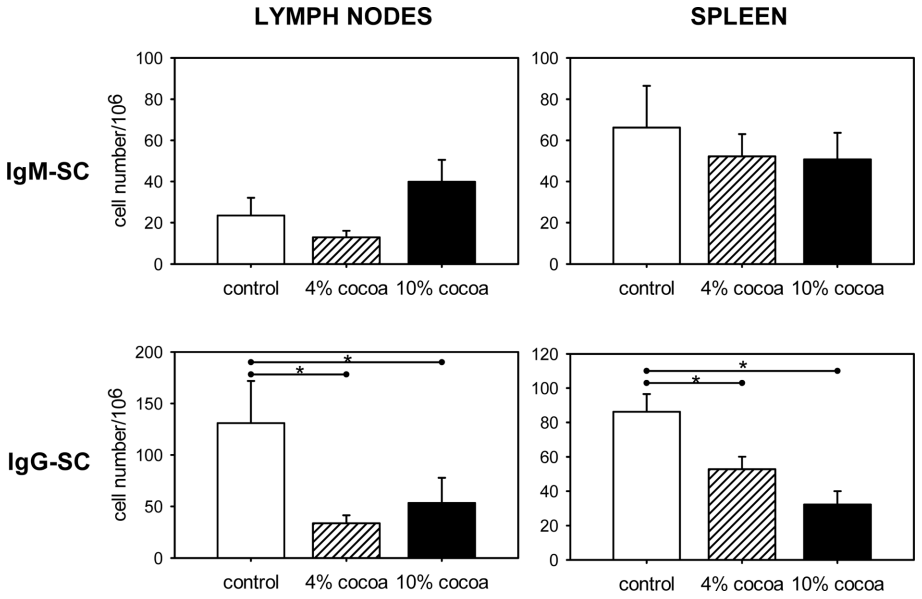

Figure 4. Effect of cocoa-enriched diets on the number of anti-OVA-SCs from LNs and spleen. Each bar represents the mean ± SEM (n=10 – 13). * $p < 0.05$.

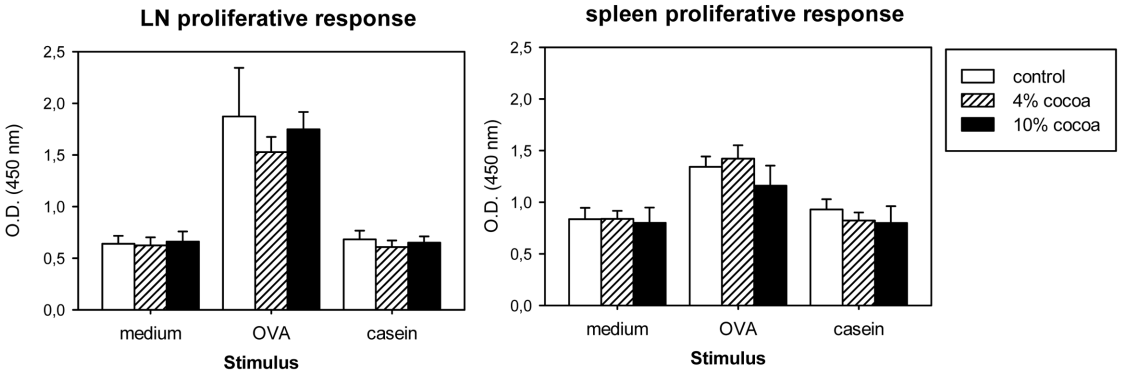

Figure 5. Effect of cocoa-enriched diets on proliferative response of LN and spleen lymphocytes. Cells were stimulated by medium (background), OVA or casein (negative control). Each bar represents the mean ± SEM (n=8 – 11).

3.5 Cytokine secretion after PMA/ionomycin cell stimulation

IL-2, IL-4 and IFNγ were quantified in LN and spleen cell supernatants obtained 24 h after PMA/ionomycin activation (Fig. 6). IL-2 levels secreted by both lymphoid tissues from animals fed cocoa did not significantly differ from those of control animals. Results corresponding to IL-4 secretion in LN were similar in all groups, but a decrease in IL-4 concentration was detected in spleen cells from animals fed 4% ($p < 0.05$) and 10% cocoa ($p = 0.087$). On the other hand, cocoa diets increased IFNγ secretion by LN cells (p=0.111 and $p < 0.05$ in 4% and 10% cocoa groups, respectively).

4 Discussion

Previous studies have shown that cocoa flavonoids are dose-dependently absorbed by rats and excreted in urine [28]. Cocoa intake exerted some effects on the healthy immune status of young animals fed cocoa for 3 weeks [14]. The present article reports the impact of longer-term (9 weeks) high cocoa intake on the specific immune response to OVA.

In a previous study, we observed that young rats fed a cocoa-enriched diet showed significant changes in spleen and mesenteric LN lymphocyte composition and function (Ig and cytokine secretion) [14, 30]. The B cell proportion was significantly increased in spleen, but Ig secretion from spleen and mesenteric LN was down-regulated, resulting in reduced serum Ig levels. In the present study, levels of OVA-specific Ab were also found to be attenuated in adult animals fed 4% or 10% cocoa diets. This immunomodulator effect on OVA-specific Ab production was higher for the 10% cocoa diet group than for the 4% cocoa diet group. *i.e.*, there was a dose-dependent effect. An interesting finding from this study is that the impact on humoral response

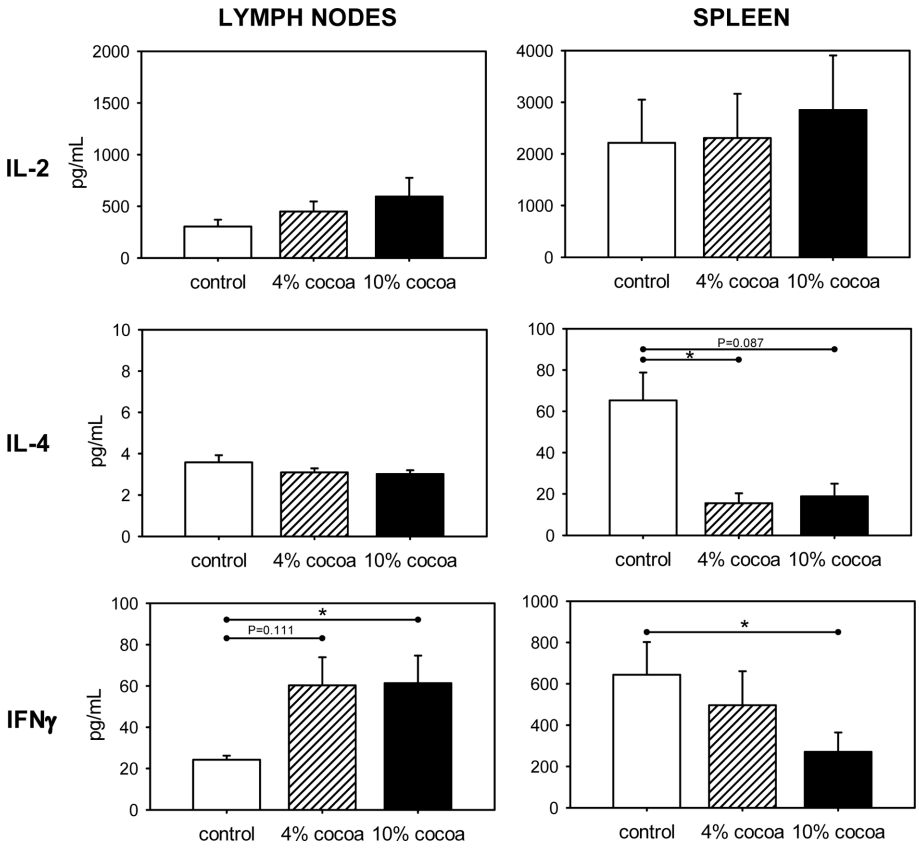

Figure 6. Effect of cocoa-enriched diets on cytokine release from LN and spleen cells PMA/ionomycin-stimulated. Each bar represents the mean ± SEM (n=7–9). * $p < 0.05$.

did not affect all Ab isotypes in a similar manner: anti-OVA IgM, IgG1, IgG2a and IgG2c levels were decreased, whereas anti-OVA IgG2b levels held steady or increased with the 10% cocoa diet. It is noteworthy that IgG isotypes can be associated with type Th1 or Th2 immune responses. In the rat, IgG1 and IgG2a are associated with the Th2 response, while IgG2b depends on the Th1 response [32–35]. Therefore, based on our results, we suggest that cocoa diet mainly down-regulates the Th2 Ab response, whereas it maintains Th1 Ab formation.

There are few studies investigating the impact of flavonoids on the humoral immune response. Apple polyphenols administered to mice decreased OVA-specific IgG1 Abs [36]. Similarly, a soybean isoflavone, genistein, produced a reduction in OVA-specific IgG1 in mice [37]. In addition, mice fed with two flavones, chrysin and apigenin, showed decreased levels of total IgE, another Th2-induced Ab isotype [38].

Modulation of OVA-specific Ab synthesis can result from interference at several levels during development of the humoral immune response. In the early phases, proliferation of specific Th cells occurs under antigen stimulation, which is promoted by IL-2 [18]. Our results indicate that LN and spleen lymphocytes from OVA-immunized cocoa-fed animals were able to proliferate under OVA stimulation in a manner similar to cells obtained from OVA-immunized control rats; IL-2 levels secreted from these cells after non-specific stimulation were also unmodified. Therefore, it is possible that cocoa did not interfere with these initial immune events. In contrast to our results, Kogiso *et al.* [37] showed that the decrease in anti-OVA IgG1 levels in genistein-fed mice was accompanied by an inhibitory effect on OVA-specific lymphocyte proliferation. The differences could be related to a particular mechanism of action for each flavonoid and/or to the effect of other compounds present in cocoa.

Following activation and proliferation of Th cells, specific lymphocytes polarize into Th1 or Th2 responses according to the environment. Our results indicate that spleen and LN cells from animals fed cocoa presented a certain imbalance in their ability to secrete IFNγ and IL-4. This imbalance was reflected by a decrease in IL-4 secretion in spleen and an increase in IFNγ release from LN. These results agree with those obtained in young rats undergoing 3 weeks of cocoa consumption [14], and those obtained in OVA-immunized mice after chrysin and apigenin intake [38]. The stimulatory effect of IFNγ has also been reported for other flavonoids, such as centaurein and centaureidin [39]. Since IL-4 drives Th2 differentiation [21, 22] and IFNγ prevents the growth of these cells [20], we

can postulate that some flavonoid-rich diets, such as cocoa diets, may down-regulate Th2 immune responses by modulating cytokine production during Th polarization.

Eventually in the humoral immune response, antigen-specific Th cells cause specific B cells to undergo proliferation, affinity maturation, and isotype switch recombination [40]. This reaction ultimately produces plasma cells that secrete copious amounts of Ab. Cytokines, such as IL-4, IL-10 and IL-6, influence the development of Ab SCs [41]; specifically, IL-4 controls the specificity of immunoglobulin class switching [42]. We also determined the number of OVA-specific IgG- and IgM-SC from cocoa-fed animals. The results showed that IgG-SC were significantly reduced in spleen and LN in the cocoa groups. Therefore, a diet with high cocoa content must interfere with the humoral immune response to OVA in a way that is reflected in a lower number of specific Ab-SC. This effect could be partially attributed to the decrease in IL-4 secretion and the increase of IFNγ production.

In summary, highly cocoa-enriched diets produce an attenuating effect on Ab synthesis. The mechanism involved in this action perhaps is partially attributable to an imbalance between cytokines belonging to Th1 and Th2 effector immune responses. Further studies must be orientated to elucidating the exact mechanism of cocoa compounds in modulating the humoral immune response. In any case, high cocoa intake might play an immunoregulatory role that could be important in diseases involving excessive Th2 immune responses, such as allergies.

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The authors have declared no conflict of interest.

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