

Spleen lymphocyte function modulated by a cocoa-enriched diet

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Introduction

Cocoa has recently become a matter of interest as a therapeutic natural product due to its flavonoid content. Because cocoa flavonoids are potent anti-oxidants [1,2], its intake could be valuable in certain pathological and/or physiological states associated with free radical production. In this regard, our previous *in vitro* studies have shown the ability of cocoa flavonoids to modulate lymphocyte activation [3] and down-regulate inflammatory mediators produced by stimulated macrophages [4]. However, these effects cannot be extrapolated directly to humans, as bioavailability and metabolism factors must be taken into account. In this regard, cocoa flavonoids are stable during gastric transit [5], absorbed rapidly and found in plasma after cocoa beverage consumption [6,7]. Recently, *in vivo* studies have demonstrated some beneficial effects of cocoa by inhibiting platelet function in atherosclerosis and enhancing anti-oxidant status [8,9], but its effects on immune function remain unclear.

The spleen is an important organ for immune homeostasis [10]. After activation, T helper (Th) lymphocytes differentiate and proliferate into Th1 and Th2 effector cells [11].

Summary

Previous studies have shown the down-regulating *in vitro* effect of cocoa flavonoids on lymphocyte and macrophage activation. In the present paper, we report the capacity of a long-term rich cocoa diet to modulate macrophage cytokine secretion and lymphocyte function in young rats. Weaned rats received natural cocoa (4% or 10% food intake), containing 32 mg flavonoids/g, for 3 weeks. Spleen immune function was then evaluated through the analysis of lymphocyte composition, their proliferative response and their ability to secrete cytokines and Ig. In addition, the status of activated peritoneal macrophages was established through tumour necrosis factor (TNF)- α secretion. The richest cocoa diet (10%) caused a reduction of TNF- α secretion by peritoneal macrophages showing anti-inflammatory activity. Similarly, although a 10% cocoa diet increased lymphocyte proliferation rate, it down-regulated T helper 2 (Th2)-related cytokines and decreased Ig secretion. These changes were accompanied by an increase in spleen B cell proportion and a decrease in Th cell percentage. In summary, these results demonstrate the functional activity of a cocoa-high dosage in down-regulating the immune response that might be beneficial in hypersensitivity and autoimmunity.

Keywords: cocoa, cytokines, immunoglobulins, lymphocytes, spleen

Th1 cells produce proinflammatory cytokines such as interferon (IFN)- γ , whereas Th2 contributes to B cell proliferation and differentiation by secreting mainly interleukin (IL)-4. However, Th2 hyperresponse may lead to allergic reactions by inducing IgE, up-regulation of its receptor and eosinophil recruitment [11].

Our previous *in vitro* studies, showing immune modulation activity of cocoa flavonoids, prompted us to study the effect of long-term cocoa intake on the systemic immune system in young rats. In this report, we focused on spleen immune function through the analysis of lymphocyte composition and its functional status, including Ig and cytokine secretion ability, lymphocyte activation and proliferation. The functional status of peritoneal macrophages was also evaluated.

Materials and methods

Diets and animals

Natural Forastero cocoa (Nutrexpa, Barcelona, Spain), containing 32 mg polyphenols/g according to the Folin-Ciocalteu method [12], was used. Standard diet

Table 1. Composition of the experimental diets (g/kg); the 10% cocoa-enriched diet was prepared from the AIN-93G control diet removing 72.8 g/kg (16 g/kg corn starch, 11 g/kg soybean oil, 25.5 g/kg cellulose and 22 g/kg casein) and adding natural cocoa.

Components	Control chow (AIN-93G) g/kg	10% cocoa-enriched chow g/kg
Casein	200	178
L-cystine	3	3
Corn starch	397.486	381.486
Maltodextrin	132	132
Sucrose	100	100
Soybean oil	70	59
Cellulose	50	24.5
Mineral mix (TD94046)	35	35
Vitamin mix (TD94047)	10	10
Choline bitartrate	2.5	2.5
TBHQ	0.014	0.014
Natural cocoa powder	–	100
22% protein		
16% carbohydrate		
11% lipid		
25.5% cellulose		
Total calories (kcal/kg diet)	3700	3700

TBHQ: anti-oxidants—tertiary butylhydroquinone.

corresponded to the American Institute of Nutrition (AIN)-93G formulation [13]. Ten per cent cocoa diet was obtained from modified AIN-93G containing 100 g cocoa/kg (Table 1). This chow has the same proportion of carbohydrates, lipids, proteins and calories as the standard diet.

Mothers with 15 day-old Wistar rat litters (50% male, 50% female) were obtained from Harlan (Barcelona, Spain). Rats were housed in controlled conditions of temperature and humidity in a 12 : 12 light : dark cycle. At day 21, pups were weaned and assigned randomly to the following dietary groups:

- **4% cocoa group:** animals received daily 4.8 g cocoa/kg rat by oral gavage. According to the chow intake per day, this dose corresponded to ~4% (g cocoa/100 g chow). Rats had free access to standard chow and water.
- **4% cocoa control diet group:** animals received daily water (cocoa vehicle) by oral gavage. Rats had free access to standard chow and water.
- **10% cocoa group:** animals had free access to water and chow containing 10% (w/w) cocoa (Table 1).
- **10% cocoa control diet group:** animals had free access to water and standard chow.

Six-week-old rats were anaesthetized with ketamine/xylazine [intramuscularly (i.m.)] to obtain peritoneal macrophages, spleens and blood to obtain serum. Studies were performed in accordance with the institutional guidelines for the care and use of laboratory animals. Experimental procedures were approved by the Ethical Committee for Animal Experimentation of the University of Barcelona (ref. 3131).

Isolation and culture of peritoneal macrophages

Ice-cold sterile phosphate-buffered saline (PBS) (40 ml) was injected into the peritoneal cavity. Abdominal massages were performed immediately to induce cell migration. Cell suspension was aspirated, centrifuged (170 g, 5 min, 4°C) and resuspended in cold RPMI-1630 media containing 10% fetal bovine serum (RPMI-FBS) (PAA, Pasing, Austria). Cells were plated at 1.2×10^6 /ml in RPMI-FBS (37°C, 5% CO₂) for 5 h to allow macrophage adhesion. Non-adherent cells were removed by washing three times with warm PBS. The attached macrophages were stimulated by addition of 1 µg/ml lipopolysaccharide (LPS) from *Escherichia coli* O55:B5 (Sigma, Madrid, Spain). After 24 h, cells were harvested to determine cell viability by double staining with acridine orange and ethidium bromide followed by fluorescence light microscopic analysis. Supernatants were collected and stored at –80°C for further tumour necrosis factor (TNF)-α quantification.

Isolation and culture of spleen lymphocytes

Spleen cell suspensions were obtained in sterile conditions by passing the tissue through a steel mesh (Collector™; Bellco, Vertieb, Austria). Cells were then centrifuged (500 g, 5 min, 4°C) and resuspended with 1 ml of 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×. Then, cells were washed and plated at 10⁶/ml in RPMI-FBS containing 0.05 mM 2-mercaptoethanol (ME) (Merck, Darmstadt, Germany) for 3 and 6 days. Supernatants were stored at –20°C until Ig quantification.

Phenotype by immunofluorescence staining and flow cytometry analysis

Lymphocyte phenotype was analysed just after cell isolation by double-staining using fluorochrome-conjugated monoclonal antibodies (mAbs) followed by flow cytometry analysis. Mouse anti-rat mAbs conjugated to fluorescein isothiocyanate (FITC) or phycoerythrin (PE) used here included: anti-T cell receptor (TCR)-αβ (R73), anti-CD4 (OX-35), anti-CD8α (OX-8) (BD Biosciences, Heidelberg, Germany) and anti-CD45RA (OX-33) (Caltag, Burlingame, CA, USA); 2×10^5 cells were labelled with saturating concentrations of FITC- and PE-mAbs in PBS pH 7.2 containing 1% FBS and 0.09% sodium azide (NaN₃) (30 min, 4°C, in darkness). A negative control staining using an isotype-matched mAb was included for each sample. After washing with PBS pH 7.2, cells were fixed with 0.5% p-formaldehyde and stored at 4°C in darkness. Analysis was performed using a Coulter Epics XL2 Corporation cytometer (Miami, FL, USA). Lymphocyte populations were defined as: B (CD45RA⁺ CD4⁺), Th (TCR-αβ⁺ CD4⁺), Tc (TCR-αβ⁺ CD8⁺) and CD8⁺ natural

killer (NK) cells plus $\gamma\delta$ T cells ($CD8^+$ TCR- $\alpha\beta^-$). Results were expressed as percentage of positive cells in the lymphocyte population, selected previously according to their forward (FSC)- and side (SSC)-scatter characteristics.

CD25 expression on spleen lymphocytes after mitogen stimulation

Spleen cells were plated at 1×10^6 /ml in complete media and stimulated with phorbol myristate acetate (PMA) (250 ng/ml) plus ionomycin (Io) (250 ng/ml) (Sigma). After 24 h, cells were harvested to determine cell viability and CD25 surface expression, and supernatants were frozen at -80°C until enzyme-linked immunosorbent assays (ELISA) were performed. Cell viability was determined by double staining with acridine orange and ethidium bromide followed by fluorescence light microscopic analysis. To evaluate CD25 expression, cells were double-stained with PE-anti-rat TCR- $\alpha\beta$ and FITC-anti-rat CD25 mAbs (Biosciences) following the protocol described above.

Results were expressed as percentage of activated T lymphocytes (TCR $\alpha\beta^+$ CD25 $^+$) in a T lymphocyte (TCR- $\alpha\beta^+$) population. CD25 expression was quantified by means of fluorescence intensity (MFI), which is proportional to CD25 surface density, and was expressed as follows:

$$\text{Percentage of CD25 expression} = (C/R) \times 100,$$

where

$$C = [(MFI_{CD25})_{\text{stimulated cells}} - (MFI_{CD25})_{\text{non-stimulated cells}}]_{\text{cocoa diet}}$$

$$R = [(MFI_{CD25})_{\text{stimulated cells}} - (MFI_{CD25})_{\text{non-stimulated cells}}]_{\text{control diet}}$$

Cytokine quantification by ELISA

Levels of IL-2, IL-4, IL-10 and IFN- γ secreted by PMA/Io-stimulated lymphocytes and TNF- α produced by LPS-stimulated macrophages were quantified using rat ELISA sets from Biosource (Nivelles, Belgium) and BD Pharmingen (Erembodegem, Belgium). ELISAs were performed as specified by the manufacturer. Cytokine concentrations from supernatants were calculated from each ELISA plate standard.

Lymphocyte proliferation assay

Spleen lymphocytes were cultured at 1×10^5 /100 μl in 96-well plate and stimulated with PMA (250 ng/ml) plus Io (250 ng/ml) for 48 h at 37°C and 5% CO_2 . Lymphocyte proliferation was determined by ELISA technique using Cell Proliferation Biotrak (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 carried out following the manufacturer's instructions. Absorbance values correlate directly with the amount of DNA synthesized and therefore

to the number of proliferating cells in culture. Results were expressed as follows:

$$\% \text{ Proliferation} = (A/B) \times 100,$$

where

$$A = [(\text{absorbance}_{\text{cells}} - \text{absorbance}_{\text{non-stimulated cells}}) / \text{absorbance}_{\text{non-stimulated cells}}]_{\text{cocoa diet}}$$

$$B = [(\text{absorbance}_{\text{stimulated cells}} - \text{absorbance}_{\text{non-stimulated cells}}) / \text{absorbance}_{\text{non-stimulated cells}}]_{\text{control diet}}$$

IgA-secreting cells quantification by enzyme-linked immunospot assay (ELISPOT)

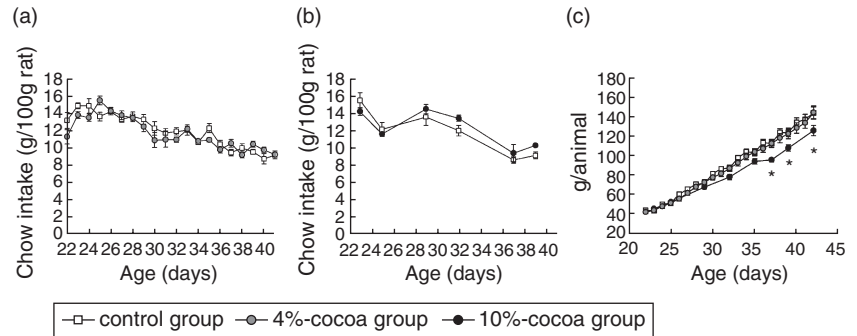
IgA-secreting cells (IgA-SC) from spleen were counted using the ELISPOT technique. In sterile conditions, a 96-well nitrocellulose plate (Multiscreen MAHAN 4510; Millipore, Eschborn, Germany) was coated with anti-rat IgA mAb (BD Biosciences) at 15 $\mu\text{g}/\text{ml}$ in PBS pH 7.2 (overnight, 4°C in a humidified chamber). Unbound antibodies were washed away using PBS and the remaining binding sites were blocked with RPMI-FBS for 1 h at 37°C . Serial dilutions of spleen cell suspensions (2×10^5 , 1×10^5 , 5×10^4 and 2.5×10^4) were then incubated for 20 h at 37°C and 5% CO_2 . To remove cells, the plate was washed 10 \times with PBS containing 0.25% Tween 20 (PBS-Tw) and 1 \times with distilled water. Biotin-conjugated anti-rat IgA mAb (2 $\mu\text{g}/\text{ml}$ in PBS) (BD Biosciences) was added and incubated for 2 h at room temperature (RT). The plate was washed again (5 \times with PBS-Tw) and then incubated with extravidin-peroxidase conjugate (Sigma) at 4 $\mu\text{g}/\text{ml}$ for 1 h at RT. After washing again (5 \times with PBS-Tw), spots (each one corresponds to one IgA-SC) were visualized by the addition of the substrate solution (3-amino-9-ethyl-carbazole [AEC] plus H_2O_2 in 0.1 M acetate solution; Sigma). The reaction was stopped by washing the plate with tap water for 5 min.

Spots were counted automatically by the ELISPOT reader system (AID, Strassberg, Germany), which distinguishes cells with low and high secretory capacity.

Ig quantification in serum and in supernatant from spleen cell culture by ELISA

Levels of IgM and IgG secreted during 3 and 6 days by non-stimulated spleen cells and sera IgA, IgM and IgG were quantified by ELISA. Ninety-six-well polystyrene plates (Nunc Maxisorp, Wiesbaden, Germany) were coated with anti-rat IgA, anti-rat IgM mAbs (BD Biosciences) or anti-serum to rat IgG (Cappel, Aurora, OH, USA) at 2, 2.5 and 20 $\mu\text{g}/\text{ml}$ in PBS, respectively (overnight in a humidified chamber). Thereafter, remaining binding sites were blocked with PBS-1% BSA (1 h, RT). Plates were washed (3 \times with PBS-0.05% Tw and 1 \times with PBS) and then supernatants, sera and standard Ig dilutions (BD Biosciences) in PBS-Tw-1% BSA were added (3 h, RT). Plates were washed again and biotinylated anti-rat IgA or IgM (BD Biosciences) at 0.625 and 1 $\mu\text{g}/\text{ml}$, respectively, were added to the corresponding wells

Fig. 1. Chow intake (g/100 g rat) (a, b) and body weight (c) were monitored for 3 weeks of 4% or 10% cocoa administration. Values are expressed as mean \pm standard error of the mean ($n = 10-18$). *Significant differences ($P < 0.05$) between 10% cocoa group and its corresponding control group.



and incubated for 2 h at RT. After washes, plates were incubated with extravidin–peroxidase conjugate (4 $\mu\text{g/ml}$ in PBS-Tw–1% BSA) for 30 min at RT. A peroxidase-conjugated anti-rat Ig antibody was used for IgG detection (Dakocytomation, Glostrup, Denmark). Immunoglobulins (Igs) were detected by the addition of the substrate solution (o-phenylenediamine dihydrochloride [OPD] plus H_2O_2 in 0.2 M phosphate – 0.1 M citrate buffer pH 5). The enzyme reaction was stopped with 3M H_2SO_4 and absorbance was measured at 492 nm.

Statistical analysis

The software package SPSS 10.0 (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. Conventional one-way analysis of variance (ANOVA) was performed, considering diet group as an independent variable. When cocoa intake had a significant effect on the dependent variable, Bonferroni's test was applied. Significant differences were accepted when $P < 0.05$. After comparing the experimental groups, no statistical differences were seen between both control groups. Therefore, in order to simplify their interpretation, results from the two control groups (gavage and non-gavage) were pooled in the graphs. Significant differences marked in the graphs are due to the comparison between cocoa group and its corresponding control group.

Results

Body weight and chow intake

Body weight and chow intake were monitored daily throughout the study. Food intake was similar between

groups, regardless of whether cocoa was administered by oral gavage (Fig. 1a) or was included in the chow (Fig. 1b). Body weight (Fig. 1c) fitted the same pattern in the 4% cocoa group as in control rats. However, it was reduced in the 10% cocoa group in the last week of treatment ($P < 0.01$).

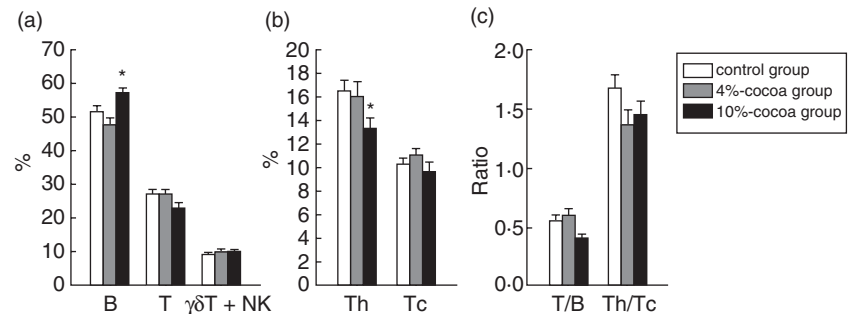
Influence of cocoa diet on spleen lymphocyte composition

The proportion of the main spleen lymphocyte populations and the ratio between them are summarized in Fig. 2. The 4% cocoa diet did not modify spleen lymphocyte composition significantly; however, rats fed with the 10% cocoa diet showed a significantly different composition pattern from that of its corresponding control group. The 10% cocoa diet increased the proportion of B cells ($P < 0.05$) and seemed to reduce the proportion of T cells compared with control animals ($P = 0.056$) (Fig. 2a). T cell percentage reduction in the 10% cocoa group was caused by a 22% decrease in spleen Th lymphocytes ($P < 0.05$) (Fig. 2b). However, T/B and Th/Tc cell ratios did not reflect these changes significantly (Fig. 2c).

Influence of cocoa diet on spleen lymphocyte activation

Activation of spleen lymphocytes was measured by means of CD25 expression, IL-2 secretion and proliferation rate. Moreover, cell viability and IFN- γ , IL-4 and IL-10 secretion were also determined after 24 h of activation.

Fig. 2. Effects of cocoa-enriched diet on spleen lymphocyte composition. Percentage of the main spleen lymphocyte populations (B, T and peripheral CD8^+ natural killer cells and T cell receptor- $\gamma\delta$ T cells) (a), T subsets (Th and Tc) (b) and the ratio between them (T/B, Th/Tc) (c). Each bar represents the mean \pm standard error of the mean ($n = 10-18$). *Significant ($P < 0.05$) differences between 4% or 10% cocoa groups and their respective control groups.



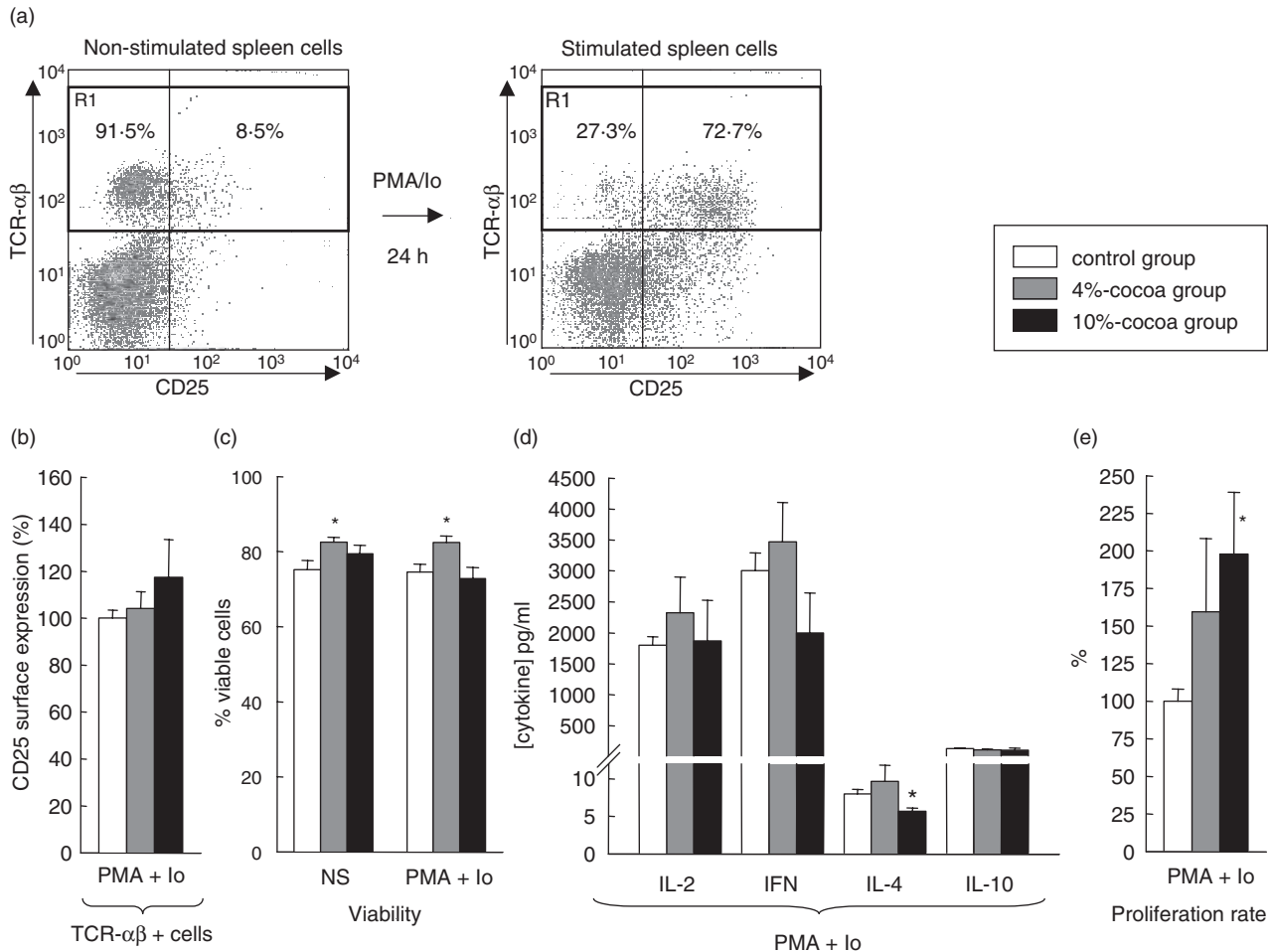


Fig. 3. Effects of cocoa-enriched diet on spleen T lymphocyte activation and proliferation. (a) Representative biparametric cytogram showing the distribution of spleen T lymphocytes according to the expression of T cell receptor- $\alpha\beta$ and CD25 from a representative control rat after 24 h of non-stimulated and phorbol myristate acetate/ionomycin (PMA/Io)-stimulated cell culture. (b) CD25 expression on spleen T lymphocytes after 24 h of PMA/Io stimulation in control group and in 4% or 10% cocoa groups. (c) Viability of non-stimulated and PMA/Io-stimulated lymphocytes from control group and 4% or 10% cocoa groups after 24 h of culture. (d) Levels of interleukin (IL)-2, interferon (IFN)- γ , IL-4 and IL-10 secreted by stimulated PMA/Io lymphocytes from control group and 4% and 10% cocoa groups. (e) Proliferation rate (%) of spleen cells from control group and 4% or 10% cocoa groups after 48 h of PMA/Io stimulation. Each bar represents the mean \pm standard error of the mean ($n = 10-18$).

*Significant differences ($P < 0.05$) between 4% or 10% cocoa groups and their respective control groups.

In non-stimulating conditions, spleen showed $\sim 10\%$ of CD25⁺ T lymphocytes expressing low CD25 surface levels (Fig. 3a). After 24 h of PMA/Io addition, $\sim 70-75\%$ of T lymphocytes became CD25⁺ (Fig. 3a). Neither the percentage of CD25⁺ T cells (data not shown) nor CD25 cell surface expression were modified by cocoa intake (Fig. 3b). Cell viability was not altered after PMA/Io stimulation and rats fed the 4% cocoa diet showed a significant increase ($P < 0.05$) in lymphocyte viability under both non-stimulating and stimulating conditions (Fig. 3c). Analysis of supernatants obtained after 24 h of spleen cell stimulation showed that PMA/Io addition induced IL-2 secretion (~ 1800 pg/ml), which was not modified significantly by cocoa intake (Fig. 3d). Therefore, although spleen Th lymphocyte proportion decreased in rats fed the 10% cocoa diet,

IL-2 levels were similar to control group levels, which suggest an increase in the IL-2 cell rate production by spleen Th cells. Moreover, IFN- γ , IL-4 and IL-10 were produced after PMA/Io stimulation (~ 3000 pg/ml, 8 pg/ml and 130 pg/ml, respectively, in control samples). Activated spleen lymphocytes obtained from 10% cocoa rats secreted lower amounts of IL-4 ($P < 0.05$) than those from control animals (Fig. 3d). In addition, IFN- γ tended to decrease after this diet and IL-10 secretion was not modified by any cocoa diet (Fig. 3d).

Proliferation rate, measured 48 h after PMA/Io activation, increased in spleen cells obtained from rats fed the 10% cocoa diet ($P < 0.05$ compared with control values) (Fig. 3e). In the 4% cocoa group a tendency of proliferation rate increase was observed, although results were not statistically significant ($P = 0.081$).

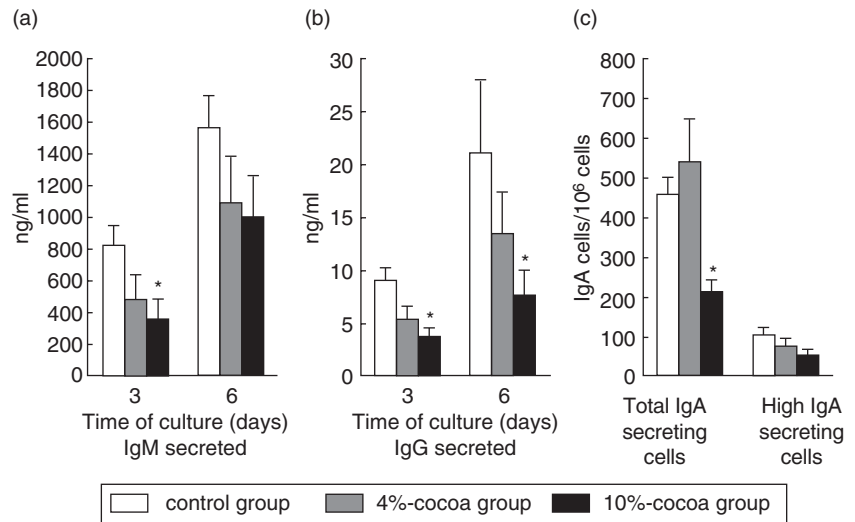


Fig. 4. Effects of cocoa-enriched diet on spleen cell Ig secretion. IgM (a) and IgG (b) production after 3 and 6 days of spleen cell culture. (c) Percentages of total IgA- and high IgA-secreting cells in spleen from control group and 4% or 10% cocoa groups. Each bar represents the mean \pm standard error of the mean ($n = 10-18$). *Significant ($P < 0.05$) differences between 4% or 10% cocoa groups and their respective control groups.

Influence of cocoa diet on spleen antibody secretion ability and sera immunoglobulin levels

Spleen IgM and IgG secreting ability was quantified in supernatants from spleen cell cultures. After 3 days of culture, spleen cells from control groups secreted ~800 ng/ml of IgM, which doubled after 6 days of incubation. IgM secretion was decreased significantly by 10% cocoa diet ($P < 0.05$) (Fig. 4a). Similarly, spleen cells obtained from rats given the 10% cocoa diet secreted less IgG than those from control animals ($P < 0.05$) (Fig. 4b).

Spleen IgA secreting cells were quantified by means of ELISPOT because IgA levels in spleen cell culture supernatants were not detectable by ELISA. This technique enumerates total SC and high-capacity SC according to spot size and intensity. Neither spleen IgA-SC nor high-capacity IgA-SC counts were modified significantly by the 4% cocoa diet. However, the 10% cocoa diet reduced spleen IgA-SC

($P < 0.05$) significantly without affecting high IgA SC numbers (Fig. 4c).

Sera IgG, IgM and IgA were also quantified at the end of this study. These Ig levels were not modified significantly by the 4% cocoa diet, but were lower in rats fed with the 10% cocoa diet, with Ig levels being ~65% lower for both IgA and IgM and ~35% lower for IgG than levels in control animals ($P < 0.05$) (Fig. 5).

Influence of cocoa diet on TNF- α secretion from peritoneal macrophages

Along with spleen lymphocyte function, peritoneal macrophage function was measured in rats fed with cocoa diets. Because plate-attached macrophages were 100% viable before *ex vivo* stimulation, after 24 h LPS induced a 35% viability reduction in peritoneal macrophages from the control and 4% cocoa groups, but viability loss was lower in

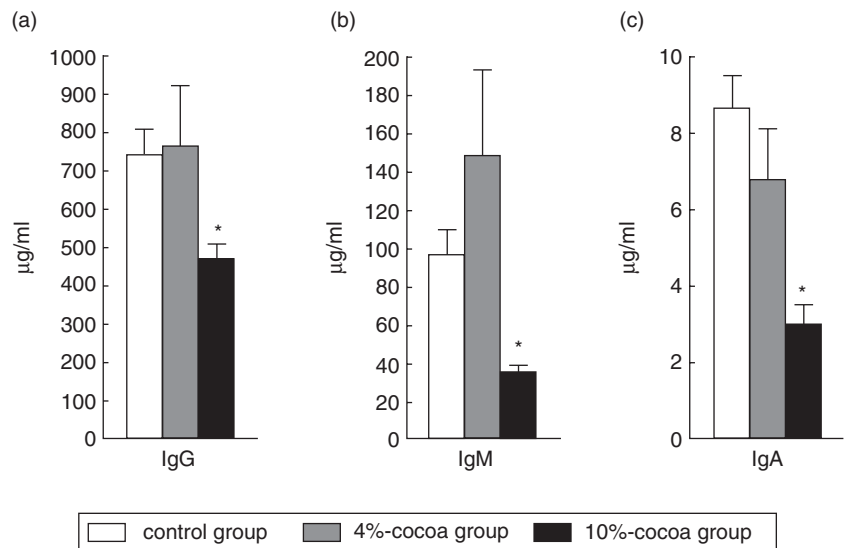


Fig. 5. Effects of cocoa-enriched diet on sera IgG (a), IgM (b) and IgA (c). Each bar represents the mean \pm standard error of the mean ($n = 10-18$). *Significant ($P < 0.05$) differences between 4% or 10% cocoa groups and their respective control groups.

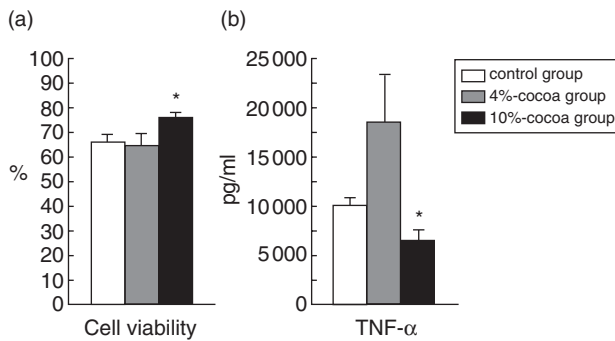


Fig. 6. Effects of cocoa-enriched diet on viability (a) and tumour necrosis factor (TNF)- α production (b) in lipopolysaccharide-stimulated peritoneal macrophages. Each bar represents the mean \pm standard error of the mean ($n = 10-18$). *Significant ($P < 0.05$) differences between 4% or 10% cocoa groups and their respective control groups.

macrophages obtained from rats fed with the 10% cocoa diet ($\sim 24\%$, $P < 0.05$ compared with control group) (Fig. 6a). Although macrophage viability increased 10%, the cocoa diet reduced significantly TNF- α secretion by LPS-stimulated macrophages (Fig. 6b).

Discussion

The present paper reports the effects of long-term cocoa high intake on peritoneal macrophages and on the spleen immune function of young rats. Weaned rats were fed for 3 weeks with cocoa-enriched diets (4% or 10% food intake), a period that corresponds to their infancy.

Macrophages participate in the generation of reactive oxygen and nitrogen species and in the production of proinflammatory cytokines [14]. We have found previously that cocoa flavonoids reduced TNF- α and monocyte chemoattractant protein (MCP)-1 secreted by a LPS-stimulated macrophage cell line [4]. In the present study, we show that long-term cocoa intake also modulates peritoneal macrophage function. The 10% cocoa diet tested here improved cell viability and reduced TNF- α secretion in peritoneal macrophages after LPS stimulation. These results suggest that cocoa, perhaps by means of its flavonoids, also exerts *in vivo* anti-inflammatory activity. This effect is consistent with the *in vivo* anti-inflammatory properties of some other flavonoids in murine models [15,16]. In addition, cell viability improvement may be attributed to a modulation of pathways linked to cell death and survival induced by cocoa.

The spleen was chosen as a representative systemic lymphoid organ to evaluate the immune system status. Rats fed the 4% cocoa diet did not show significant changes in spleen lymphocyte composition and function (antibody and cytokine secretion). However, a tendency to increase proliferation rate and to reduce Ig secretion prompted us to determine whether higher cocoa doses could modulate spleen immune function significantly. The same study design was applied to

weaned rats given higher amounts of cocoa (10% of food intake). In this setting, as the cocoa dose was too high to be administered by a single oral gavage, it was included in the chow. It is important to highlight that cocoa flavonoids were absorbed and metabolized proportionally to cocoa daily intake, regardless of the type of administration (oral gavage *versus* chow), as demonstrated by the amount of flavonoid metabolites excreted in 24-h rat urines of cocoa-fed animals [17].

In this study, although the B cell proportion was increased significantly by the 10% cocoa diet, spleen Ig secretion was down-regulated, sera Ig levels also being reduced. Taken together, these results suggest that cocoa interferes in B lymphocyte differentiation into Ig-secreting cells. Given that B lymphocyte activation and differentiation are linked tightly to T cell function, the analysis of cytokines secreted by T lymphocytes might help to shed light on this issue. Lymphocyte activation response was evaluated after *ex vivo* activation. In response to PMA/Io, T lymphocytes proliferate through the enhancement of the IL-2/IL-2R system [18,19]. In 10% cocoa-fed rats, lymphocyte proliferation was increased substantially over the control group, although neither CD25 expression nor IL-2 secretion were modified. Because 10% cocoa animals showed a reduced Th proportion, the proliferation enhancement could be explained by a higher IL-2 production per Th cell. These results appear to diverge from those of our previous *in vitro* studies in which cocoa flavonoids diminished T cell activation by down-regulating IL-2 secretion and its high-affinity receptor (IL-2R α or CD25) [3]. These apparently contradictory results can be attributed most probably to differences in the type of cocoa compounds that reach cells. In *in vitro* studies, lymphocytes were incubated with a purified cocoa flavonoid extract, while in the present study we administered whole cocoa to rats. Other immunologically active compounds contained in cocoa, such as methylxanthines and fatty acids, may contribute to cocoa *in vivo* effects. In addition, as cocoa is highly metabolized before and after absorption [20,21], the flavonoid profile that reaches the spleen is clearly not the same as in the original product.

To study the effect of cocoa on spleen T cell effector response after activation, apart from IL-2, other cytokines including IFN- γ , IL-4 and IL-10 were quantified. IL-4, produced by Th2 cells, was decreased in PMA/Io-stimulated splenocytes from the 10% cocoa group, whereas IFN- γ and IL-10 were not significantly affected. IL-4 is essential for Th2 development and is associated with humoral type immune responses through the induction of antibody isotype switching and B cell proliferation [22]. Taken together, our results suggest that reduced Ig levels found in 10% cocoa-fed rats could be attributed, at least, to the decrease in Th proportion and especially to the Th2 down-regulatory effect.

The effects reported here are produced by a high cocoa dosage that cannot be included in a normal diet but could be achievable through supplementation. Cocoa compounds that affect the spleen immune system may be flavonoids, but

other compounds must not be excluded. For example, fibre and lipidic compounds may influence the immune system.

Finally, it must be highlighted that although the amount of food intake was similar in all experimental groups, in the third week of treatment a lower body weight increase was observed in rats fed the 10% cocoa diet. This effect could be attributed most probably to a lower adipose tissue synthesis, as described elsewhere, when rats received a 12.5% cocoa diet [23]. It is probable that flavonoids and methylxanthines contained in cocoa are responsible for body weight loss due to their hypolipidaemic and cellular energy stimulating properties [24,25].

In summary, continuous cocoa high intake down-regulates Th2 function and consequently Ig secretion in splenocytes from young rats, suggesting a potential beneficial effect in hypersensitivity and autoimmunity. In addition, cocoa also may exert an anti-inflammatory activity down-regulating macrophage cytokine secretion.

Acknowledgements

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