Available online at www.sciencedirect.com
ScienceDirect
Journal of Nutritional Biochemistry 19 (2008) 555 – 565
Journal of Nutritional Biochemistry

Intestinal immune system of young rats influenced by cocoa-enriched diet

Emma Ramiro-Puig[a], Francisco J. Pérez-Cano[a], Sara Ramos-Romero[a], Teresa Pérez-Berezo[a], Cristina Castellote[a], Joan Permanyer[b], Àngels Franch[a], Maria Izquierdo-Pulido[b], Margarida Castell[a,*]

[a]Department of Physiology, Faculty of Pharmacy, University of Barcelona, 08028 Barcelona, Spain
[b]Department of Nutrition and Food Science, Faculty of Pharmacy, University of Barcelona, 08028 Barcelona, Spain

Received 14 March 2007; received in revised form 28 June 2007; accepted 5 July 2007

Abstract

Gut-associated lymphoid tissue (GALT) maintains mucosal homeostasis by counteracting pathogens and inducing a state of nonresponsiveness when it receives signals from food antigens and commensal bacteria. We report for the first time the influence of continuous cocoa consumption on GALT function in rats postweaning. Weaned Wistar rats were fed cocoa-enriched diets (4% or 10% food intake) for 3 weeks. The function of the primary inductive sites of GALT, such as Peyer's patches (PP) and mesenteric lymph nodes (MLN), was evaluated through an analysis of IgA-secretory ability and lymphocyte composition (T, B and natural killer cells), activation (IL-2 secretion and IL-2 receptor α expression) and proliferation. T-helper effector cell balance was also established based on cytokine profile (interferon γ, IL-4 and IL-10) after mitogen activation. A 10% cocoa intake induced significant changes in PP and MLN lymphocyte composition and function, whereas a 4% cocoa diet did not cause significant modifications in either tissues. Cocoa diet strongly reduced secretory IgA (S-IgA) in the intestinal lumen, although IgA's secretory ability was only slightly decreased in PP. In addition, the 10% cocoa diet increased T-cell-antigen receptor γδ cell proportion in both lymphoid tissues. Thus, cocoa intake modulates intestinal immune responses in young rats, influencing γδ T-cells and S-IgA production.

Keywords: Cocoa; Rat; Lymphocytes; γδ T cells; IgA

1. Introduction

Extensive research demonstrates that diet is crucial to maintaining an optimal immune function [1,2]. During food digestion and nutrient absorption, the small intestine is exposed to a large variety of environmental antigens [3]. In this compartment, gut-associated lymphoid tissue (GALT) fences off potentially harmful intestinal antigens and induces tolerance to innocuous luminal food antigens.

Anatomically, GALT is composed of organized lymphoid structures and diffusely distributed cell populations (intraepithelial lymphocytes and lamina propria lymphocytes). Organized GALT comprises Peyer's patches (PP), isolated follicles and mesenteric lymph nodes (MLN), which are considered primary mucosal immune-inductive sites and sources of IgA-secreting cells (IgA-SC). PP are highly specialized lymphoid follicles in the small intestine that mainly contain B-lymphocyte aggregates, follicular dendritic cells and T-cell-rich areas. MLN are also composed of defined T- and B-cell-dependent compartments and constitute the second line of defense by filtering mesenteric lymph vessels. Antigen presentation to immune effector cells is concentrated at these organized lymphoid tissues [4]. Thus, luminal antigens are taken up, transported into PP and presented to naïve T lymphocytes by antigen-presenting cells (APC), including follicular dendritic cells and macrophages. Immature T and B cells migrate to MLN, where maturation and clonal expansion take place. Then mature cells leave MLN and enter the bloodstream through the thoracic duct. After recirculation, they home to effector sites (i.e., mucosal diffused GALT) where differentiation into mature effector cells is completed [5].

Specific recognition of antigenic peptides by T-cell receptors triggers an intracellular signaling cascade that

* Corresponding author. Tel.: +34 93 402 45 05; fax: +34 93 403 59 01.
E-mail address: margaridacastell@ub.edu (M. Castell).

0955-2863/$ – see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.jnutbio.2007.07.002

Table 1
Composition of experimental diets (g/kg)

Components	Control chow (AIN-93G; g/kg)	10% cocoa-enriched chow (g/kg)
Casein	200	178
L-Cystine	3	3
Cornstarch	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
tert-Butylhydroquinone	0.014	0.014
Natural cocoa powder 22% protein 16% carbohydrate 11% lipid 25.5% cellulose	–	100
Total calories (kcal/kg diet)	3700	3700

The 10% cocoa-enriched diet was prepared from the AIN-93G control diet, removing 72.8 g/kg (16 g/kg cornstarch, 11 g/kg soybean oil, 25.5 g/kg cellulose and 22g/kg casein) and adding natural cocoa.

involves the induction of IL-2 and IL-2 receptor subunit α (IL-2Rα or CD25) [6]. IL-2 binds to its high-affinity receptor, formed by the assembly of three subunits (α, β and γ), and stimulates transduction pathways that result in T-helper (Th)-cell proliferation and B-cell differentiation [6]. Activated Th lymphocytes in GALT are classified into the following subsets of effector cells: Th1, Th2 and T regulatory (Treg: Tr1 and Th3). Th1 cells produce proinflammatory cytokines such as tumor necrosis factor α and interferon γ (IFNγ), whereas Th2 and Treg cells counteract Th1 function by secreting IL-4 and IL-10 or transforming growth factor (TGF) β, respectively [7]. Th2 and Th3 cells also favor the differentiation of antigen-specific B cells into predominantly IgA plasma cells. However, IL-4 hypersecretion may lead to allergic response by increasing intestinal permeability, inducing IgE synthesis and up-regulating its receptor, as well as by enhancing eosinophil recruitment [7,8]. Cytokines secreted by Treg cells (IL-10 and TGF-β) contribute to the maintenance of oral tolerance to innocuous antigens [7]. Secretory IgA (S-IgA) is the principal immunoglobulin in mucosal surfaces and exerts a protective role against the invasion of harmful microorganisms and toxins [9].

In many countries, cocoa is commonly consumed, especially by children and young people [10,11]. To date, the consumption of cocoa products in the European Union and United States is relatively high, estimated at ~2.6 and 2.35 g/day cocoa powder per capita, respectively [11]. However, the influence of cocoa intake on health and disease remains to be explored in depth.

Since cocoa is a rich source of potent antioxidants, mainly flavonoids [12], its intake could be beneficial in preventing or ameliorating certain pathological and physiological states that are linked to a high production of free radicals and other reactive oxygen species. In this regard, recent in vivo studies have shown that cocoa reduces some oxidative markers and enhances antioxidant status [13], but little is known about its possible influence on immune function. Our previous in vitro studies have shown the ability of cocoa (through its flavonoids) to down-regulate inflammatory mediators, such as cytokines and nitric oxide, produced by stimulated macrophages and to modulate lymphocyte activation [14,15]. These promising results prompted us to study the influence of continuous cocoa consumption on GALT of weaned rats when the mucosal immune system is still in maturation [16]. In this article, we focused on the functions of lymphocytes from PP and MLN (i.e., phenotype composition, S-IgA production, and lymphocyte activation and proliferation abilities).

2. Materials and methods

2.1. Reagents and diets

RPMI 1640 medium, glutamine, penicillin–streptomycin, fetal bovine serum (FBS) and bovine serum albumin (BSA) fraction V were purchased from PAA (Pashing, Austria). Dithiothreitol (DTT), phorbol 12-myristate 13-acetate (PMA), ionomycin calcium salt (Io), acridine orange (AO), ethidium bromide (EB), 30% (vol/vol) hydrogen peroxide, 3-amino-ethyl carbazole (AEC), *o*-phenylenediamine dihydrochloride (OPD), extravidin–peroxidase, paraffin, hematoxylin, eosin and Masson trichrome stain were obtained from Sigma-Aldrich (Madrid, Spain). Sodium azide, 2-mercaptoethanol (ME), *p*-formaldehyde, xylol and ethanol were provided by Merck (Darmstadt, Germany).

Fluorescein isothiocyanate (FITC)-conjugated anti-rat T-cell-antigen receptor (TCR) αβ (R73) and CD25 (IL-2Rα chain, p55, OX-39) monoclonal antibodies (MAb); phycoerythrin (PE)-conjugated anti-rat TCRαβ (R73), CD4 (OX-35) and NKR-P1A (10/78) MAb; and peridinin–chlorophyll *a* protein (PerCP)-conjugated anti-rat CD8α (OX-8), anti-rat IgA (A93-3) and anti-rat IgM (G53-238) MAb; rat IgA standard, rat IgM standard and biotinylated anti-rat IgA (A93-2) and IgM (G53-338) MAb were purchased from BD Biosciences (Heidelberg, Germany). PE–anti-rat CD45RA (OX-33) MAb were obtained from Caltag (Burligame, CA, USA).

Natural Forastero cocoa (Nutrexpa SA, Barcelona, Spain) containing 32 mg/g polyphenols was used for this study. AIN-93G formulation [17], which provides the nutrients required for optimal rat growth, was used as the control diet. A 10% cocoa diet, containing 100 g/kg cocoa, was produced from modified AIN-93G. Diet compositions are detailed in Table 1.

2.2. Animals and experimental design

Dams with 15-day-old Wistar rat litters (50% male, 50% female) were obtained from Harlan (Barcelona, Spain). Rats were housed in cages (10 pups per lactating

mother) under temperature- and humidity-controlled conditions and a 12:12 light/dark cycle. On Day 21, pups were weaned and randomly assigned to the following dietary groups:

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

Body weight and food intake were monitored throughout the study. Studies were performed in accordance with the institutional guidelines for the care and use of laboratory animals, and experimental procedures were approved by the Ethical Committee for Animal Experimentation of the University of Barcelona (ref. 3131).

2.3. Sample collection

Fecal samples were collected at 2 or 3 weeks of cocoa intake by abdominal massage and immediately frozen at −20°C for further IgA quantification. After 3 weeks of cocoa or control diet, rats were anesthetized intramuscularly with ketamine/xylazine, and MLN and the small intestine were collected. The small intestine was divided into four fragments and carefully flushed with 0.9% NaCl solution to remove fecal content. A 0.5-cm segment of the distal jejunum was separated for histological analysis. The intestine was opened lengthwise, and visible PP were excised for immediate lymphocyte isolation. The intestinal tissue without PP was cut into 5-mm pieces, weighed and incubated with phosphate-buffered solution (PBS) for 10 min at 37°C in a shaker. The suspension obtained was then centrifuged (500×*g*, 10 min, 4°C), and supernatants (gut wash) were stored at −20°C until S-Ig quantification by enzyme-linked immunosorbent assay (ELISA) technique.

2.4. PP lymphocyte isolation

The excised PP were incubated in sterile conditions with RPMI medium containing 1 mM DTT (5 min, 37°C). Thereafter, PP were washed with RPMI medium and passed through a steel mesh. The obtained cell suspension was purified by filtration in sterile cotton gauze and then centrifuged (500×*g*, 10 min, 4°C) and resuspended in RPMI containing 10% FBS and 0.05 mM ME (complete medium). Cell counting and viability were determined by double staining with AO/EB, followed by fluorescence light microscopical analysis.

2.5. MLN lymphocyte isolation

MLN lymphocytes were obtained in sterile conditions by passing the tissue through a sterile steel mesh. Cell suspension was incubated on ice to remove tissue debris by sedimentation for 10 min. Then cells were centrifuged (500×*g*, 5 min, 4°C) and resuspended with complete RPMI medium. Cell counting and viability were determined by double staining with AO/EB, followed by fluorescence light microscopical analysis.

2.6. Phenotype by immunofluorescence staining and flow cytometry analysis

PP and MLN lymphocytes were stained with anti-rat MAb conjugated to FITC, PE or PerCP: anti-TCRαβ (R73), anti-CD4 (OX-35), anti-CD8α (OX-8), anti-CD45RA (OX-33) and anti-NKR-P1A (10/78). Cells (2×10^5) were labeled with saturating concentrations of FITC MAb, PE MAb and PerCP MAb in PBS (pH 7.2) containing 1% FBS and 0.09% NaN_3 (30 min, 4°C, in darkness). Negative control staining using isotype-matched MAb was included for each sample. After the cells had been washed with PBS (pH 7.2), they were fixed with 0.5% *p*-formaldehyde and stored at 4°C in darkness. Analyses were performed using a Coulter Epics XL2 Corporation cytometer (Coulter, Miami, FL, USA), and data were assessed by the cytometer software Summit V3.1 (Cytomation, Inc.). Lymphocyte populations were defined as: B ($CD45RA^+CD4^-$), T [$TCR\alpha\beta^+$ and $TCR\gamma\delta^+$ ($TCR\alpha\beta^-CD8^+NKR\text{-}P1A^-$)], T-subset [Th ($TCR\alpha\beta^+CD4^+$) and Tc ($TCR\alpha\beta^+CD8^+$)] and natural killer (NK; $NKR\text{-}P1A^+$) cells. Results were expressed as percentages of positive cells in the lymphocyte population previously selected according to their forward scatter (FSC) and side scatter (SSC) characteristics.

2.7. CD25 expression in MLN lymphocytes after PMA/Io stimulation

MLN lymphocytes were plated at 1×10^6 ml^{-1} in complete RPMI medium and stimulated with PMA (250 ng/ml) plus Io (250 ng/ml). After 24 h, cells were harvested to determine cell viability and CD25 surface expression, and supernatants were frozen at −80°C until cytokine ELISA assays. Cells were double stained with PE–anti-rat TCRαβ and FITC–anti-rat CD25 MAb following the same protocol described above. Results were expressed as percentages of activated T lymphocytes ($TCR\alpha\beta^+CD25^+$) in a T-lymphocyte ($TCR\alpha\beta^+$) population that was gated according to FSC/SSC lymphocyte characteristics. CD25 expression was quantified through mean fluorescence intensity (MFI), which is proportional to CD25 surface density and is expressed as follows:

$$\%\ \text{CD 25 expression} = (C/R) \times 100$$

where

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

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

2.8. Lymphocyte proliferation assay

MLN lymphocytes were plated at 1×10^6 ml^{-1} on a 96-well plate and stimulated with PMA (250 ng/ml) plus Io (250 ng/ml) at 37°C in a 5% CO_2 atmosphere. After 48 h of incubation, lymphocyte proliferation was determined by a modified ELISA technique using Cell Proliferation Biotrak (Amersham Biosciences, Munich, Germany). This assay was based on the measurement of 5-bromo-2′-deoxyuridine incorporation into proliferating cells during DNA synthesis and was carried out as specified by the manufacturer. Absorbance (Ab) 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

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

$$B = [Ab_{\text{stimulated cells}} - Ab_{\text{non-stimulated cells}})/Ab_{\text{non-stimulated cells}}]_{\text{reference diet}}$$

2.9. IgA secretion ability determination by ELISPOT

IgA-SC from MLN and PP were quantified 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 (A93-3) at 15 μg/ml in PBS (pH 7.2; overnight at 4°C in a humidified chamber). Unbound antibodies were washed away using PBS, and the remaining binding sites were blocked with RPMI–10% FBS for 1 h at 37°C. Then serial dilutions of cell suspension (2×10^5, 1×10^5, 5×10^4 and 2.5×10^4) were incubated for 20 h at 37°C in a 5% CO_2 atmosphere. Then the plate was washed 10× with PBS–0.25% Tween-20 (Tw) and once with distilled water to remove cells. Biotin-conjugated anti-rat IgA MAb (A93-2) (2 μg/ml in PBS) was added and incubated for 2 h at room temperature (RT). The plate was washed again (5× with PBS-Tw) and incubated with extravidin–peroxidase conjugate at 4 μg/ml for 1 h at RT. After repeated washes (5× with PBS-Tw), spots (each one corresponding to one IgA-SC) were detected by the addition of a substrate solution (AEC plus hydrogen peroxide in 0.1 M acetate solution). The reaction was stopped by washing the plate with tap water for 5 min. Spots were quantified automatically by the ELISPOT reader system (AID, Strassberg, Germany). We distinguished between cells with low secreting ability and cells with high secreting ability according to spot size and intensity.

2.10. Cytokine secretion by ELISA

Levels of IL-2, IL-4, IL-10 and IFNγ secreted by PMA/Io-stimulated lymphocytes from MLN were quantified using rat OptEIA sets from BD Pharmingen (Madrid, Spain). ELISA was performed as specified by the manufacturer. Cytokine concentrations in supernatants were calculated by interpolating absorbance values into corresponding standard curves.

2.11. Fecal homogenate preparation

Fecal samples were thawed at RT and dried for 70 min at 37°C, followed by 30 min at RT before weighing. Then fecal samples were diluted in PBS (20 mg/ml) and homogenized using Polytron (Kinematica, Switzerland). Homogenates were centrifuged (500×*g*, 15 min, RT), and supernatants were frozen at −20°C until ELISA IgA quantification.

2.12. ELISA Ig quantification in gut wash and feces

S-IgA levels in gut wash (supernatants obtained after the incubation and centrifugation of intestinal tissue) and feces were quantified by ELISA technique. Ninety-six-well polystyrene plates (Nunc Maxisorp, Wiesbaden, Germany) were coated with anti-rat IgA (2 μg/ml in PBS) in a humidified chamber overnight. Thereafter, the remaining binding sites were blocked with PBS–1% BSA (1 h, RT).

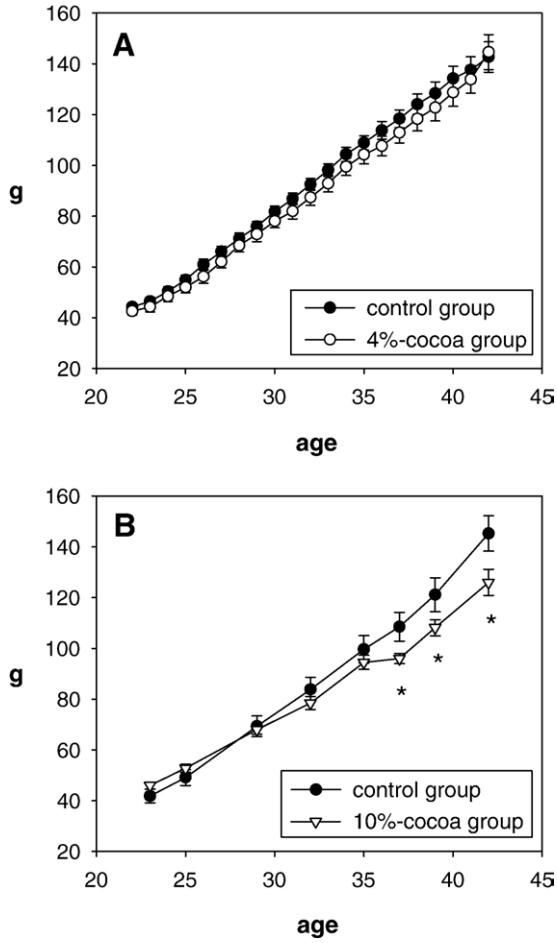

Fig. 1. Body weight throughout 3 weeks of 4% cocoa (A) or 10% cocoa (B) administration. Values are expressed as mean±S.E.M. (n=10–18). *Significant differences (P<.05) between the 10% cocoa group and its corresponding control group.

The plate was washed (thrice with PBS–0.05% Tw and once with PBS), and then appropriate diluted samples (gut wash and fecal extract) and standard IgA dilutions in PBS–Tw–1% BSA were added (3 h, RT). After repeat washing, biotinylated anti-rat IgA (0.0625 μg/ml in PBS–Tw–1% BSA) was added and incubated for 2 h at RT. Then the plate was incubated with extravidin–peroxidase conjugate (4 μg/ml in PBS–Tw–1% BSA) for 30 min at RT. Captured S-IgA was detected by the addition of enzyme substrate solution (OPD plus hydrogen peroxide in 0.2 M phosphate–0.1 M citrate buffer, pH 5). Enzyme reaction was stopped with 3 M H_2SO_4, and absorbance was measured at 492 nm. Data were interpolated into the IgA standard curve, and S-IgA levels were expressed as nanograms per milliliter in gut wash or as nanograms per gram in fecal sample. After the obtainment of S-IgA results, secretory IgM (S-IgM) levels were also determined in gut wash by a similar ELISA technique.

2.13. Histological samples

Intestinal segments were placed in cassettes and fixed in 10% buffered formaldehyde. Tissue samples were washed with tap water, dehydrated in increasing concentrations of ethanol [70% (1 h), 96% (3×, 1 h)] and embedded in paraffin. Tissue sections were cut at 4 μm on a rotatory microtome (Leitz, Wetzlar, Germany), mounted on glass slides and dried overnight at 37°C. Tissue sections were then cleared and hydrated by immersion in xylol and ethanol solutions [xylol (1×5 min, 1×10 min), absolute ethanol (2×5 min) and distilled water (1×5 min)]. Hydrated sections were stained with hematoxylin–eosin and Masson trichrome stain. Histological analysis was blindly performed by an experienced pathologist.

2.14. Statistical analysis

Results were expressed as mean±S.E.M. The software package SPSS 10.0 (SPSS, Inc., Chicago, IL, USA) was used for statistical analysis. Conventional analysis of variance (ANOVA) was performed, with diet group as an independent variable. When cocoa intake was found to have a significant effect on the dependent variable, Bonferroni's test was applied. Significant differences were accepted when $P<.05$. Upon a comparison of experimental groups, no statistical differences were seen between both control groups. Therefore, in order to simplify their interpretation, the results from the two control groups (gavage and nongavage) were pooled on graphs. The standard error of the mean of the control groups on the graphs indicates low dispersion for these groups. Significant differences marked on the graphs are due to the comparison between the treatment group and its corresponding control group.

3. Results

3.1. Effect of cocoa diet on body weight and small intestine structure

The body weight of animals in the 4% cocoa group did not differ from the control group (Fig. 1A) but was significantly reduced in the 10% cocoa group (Fig. 1B) ($P<.05$). This effect was not related to a lower chow intake, as chow intake in the 4% cocoa group, in the 10% cocoa group and in both control

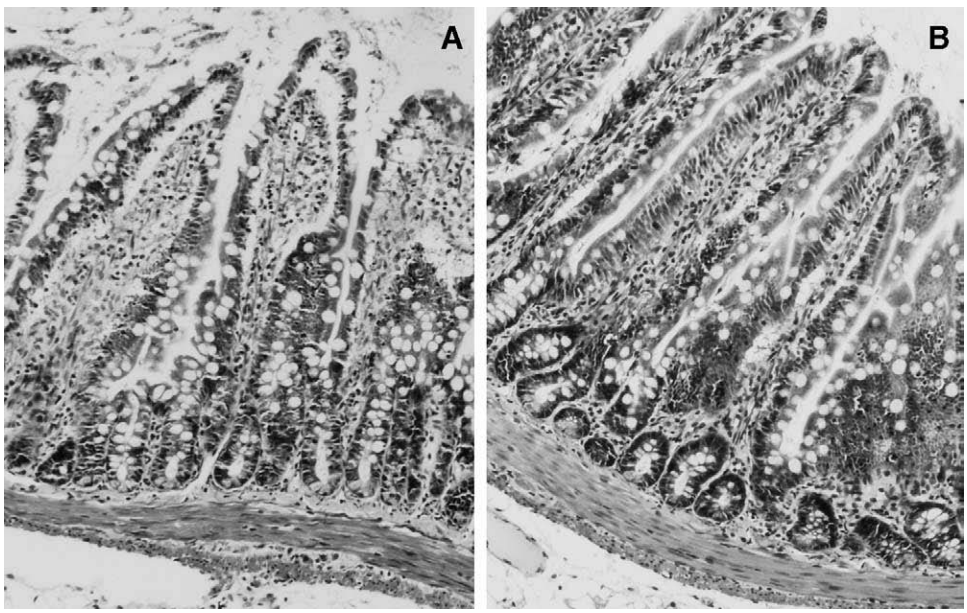

Fig. 2. Sections of the distal jejunum stained with Masson trichrome stain from a representative control rat (A) and a rat fed a 10% cocoa diet (B) (original magnification, ×10).

groups ranged from 9 to 15 g/100 g/day, with no statistical differences among the four groups.

Macroscopical analysis of the small intestine showed no significant changes in weight, length and PP number (data not shown). In addition, a histological study that searched for epithelial lesions or/and cell detachment, mucosal hyperplasia, inflammation and morphological changes in intestinal layers revealed no intestinal lesions in rats fed cocoa-enriched diets (Fig. 2).

3.2. Effect of cocoa diet on PP and MLN lymphocyte composition

The percentages of B, TCRαβ$^+$ (Th and Tc), γδ T and NK cells in PP and MLN and the ratio among them are summarized in Fig. 3. The 4% cocoa diet did not modify PP and MLN lymphocyte composition; however, rats fed the 10% cocoa diet showed a composition pattern different from that of control animals (Fig. 3). In PP, the 10% cocoa diet

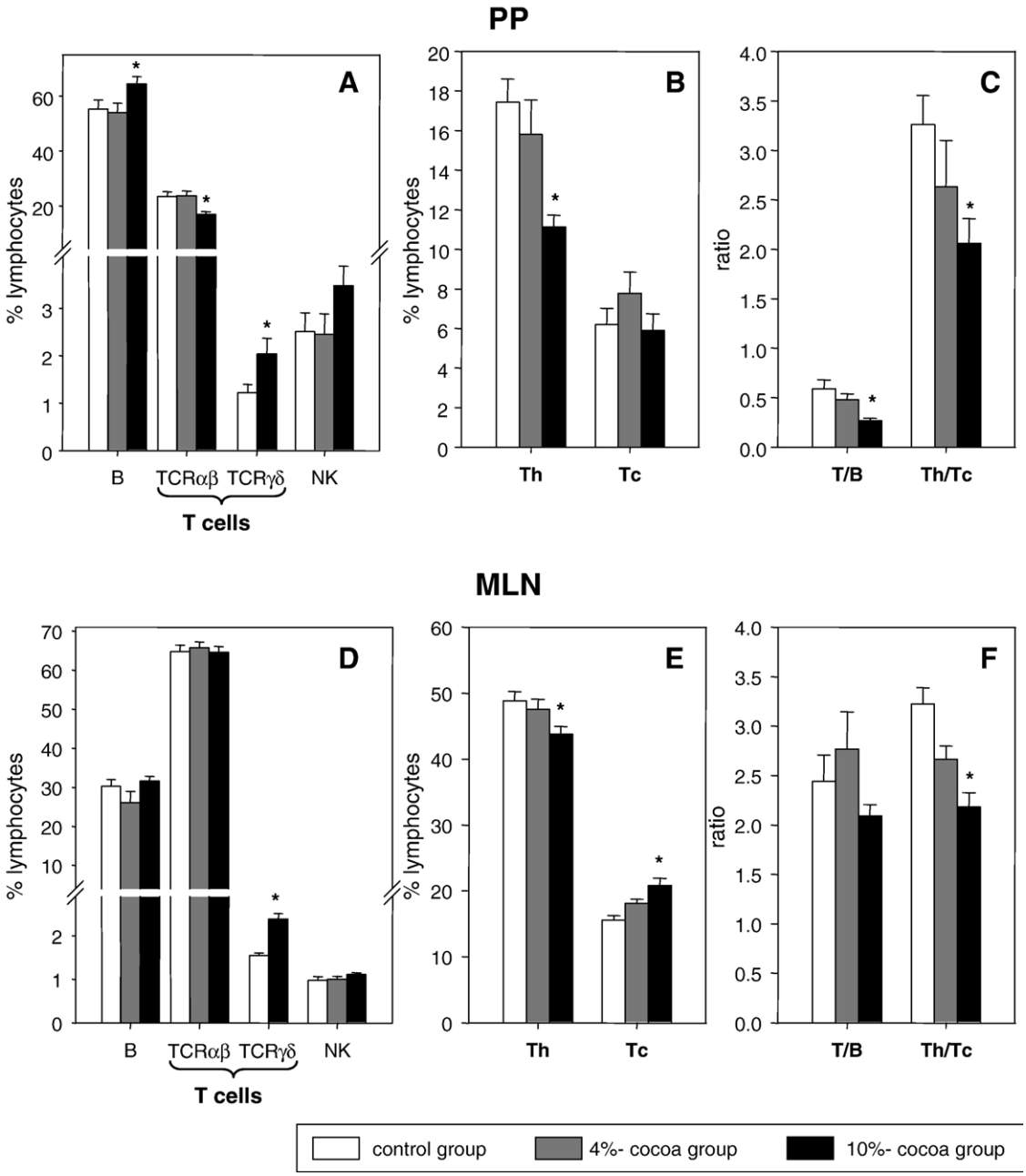

Fig. 3. Effect of cocoa-enriched diet on PP (A–C) and MLN (D–F) lymphocyte composition in young rats. Percentages of the main lymphocyte populations (B, T and NK cells) (A and D), T subsets (Th and Tc) (B and E) and the ratios between them (C and F). Each bar represents the mean±S.E.M. (n=10–18). *P<.05, 4% or 10% cocoa group versus their respective control groups (ANOVA followed by Bonferroni's test).

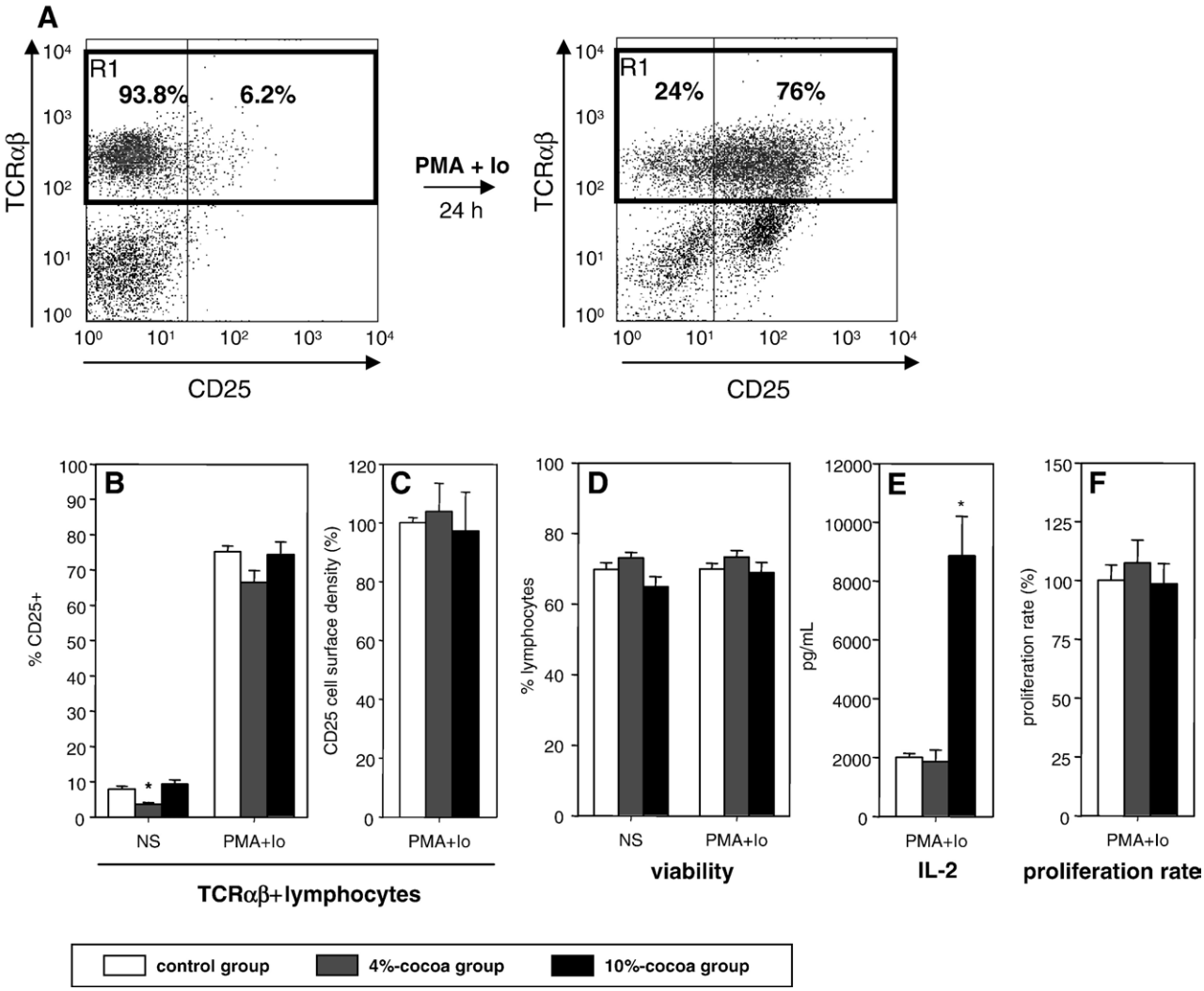

Fig. 4. Effect of cocoa-enriched diet on MLN T-lymphocyte activation and proliferation in young rats. (A) Representative biparametric cytogram showing the distribution of MLN T lymphocytes according to the expression of TCRαβ and CD25 from a representative control rat after 24 h of nonstimulated and PMA/Io-stimulated cell culture. (B) Percentage of $CD25^+$ lymphocytes in $TCR\alpha\beta^+$ cells after 24 h of PMA/Io stimulation in the control group and in the 4% or 10% cocoa group. (C) CD25 surface density on MLN T lymphocytes after 24 h of PMA/Io stimulation in the control group and in the 4% or 10% cocoa group. (D) Viability of nonstimulated and PMA/Io-stimulated lymphocytes from the control group and the 4% or 10% cocoa group after 24 h of culture. (E) IL-2 secretion (pg/ml) of stimulated PMA/Io lymphocytes from the control group and the 4% or 10% cocoa group. (F) Proliferation rate (%) of MLN cells from the control group and the 4% or 10% cocoa group after 48 h of PMA/Io stimulation. Each bar represents the mean±S.E.M. (n=10–18). *P<.05, 4% or 10% cocoa group versus their respective control groups (ANOVA followed by Bonferroni's test).

reduced $TCR\alpha\beta^+$ T-cell proportion by up to ~28% and increased B- and γδ T-cell percentages, being ~16% and ~67% higher than those of the control group, respectively (P<.05) (Fig. 3A). PP T-cell reduction corresponded to a decrease in Th-subset proportion (P<.05) (Fig. 3B). Both T/B and Th/Tc ratios were reduced by the 10% cocoa group with respect to its control group (P<.05) (Fig. 3C).

In MLN, the 10% cocoa diet increased γδ T-cell proportion by ~55% (P<.05) (Fig. 3D). Although $TCR\alpha\beta^+$ T-cell percentage was not modified in the 10% cocoa group, the ratio among their subsets was strongly reduced (P<.05) (Fig. 3F). Thus, Tc proportion increased (~33% higher than that in control animals) (P<.05), whereas Th percentage diminished (~10% lower than that in control animals) (P<.05) (Fig. 3E).

3.3. Effect of cocoa diet on MLN lymphocyte activation

MLN lymphocyte activation was determined by measuring CD25 expression and IL-2 secretion (markers of early activation) after 24 h of PMA/Io addition and their proliferative ability rate after 48 h of incubation. Cell viability and IFNγ, IL-4 and IL-10 production were also quantified after 24 h of mitogen activation.

In nonstimulating conditions, MLN showed ~10% of activated T cells ($CD25^+TCR\alpha\beta^+$) with low CD25 surface

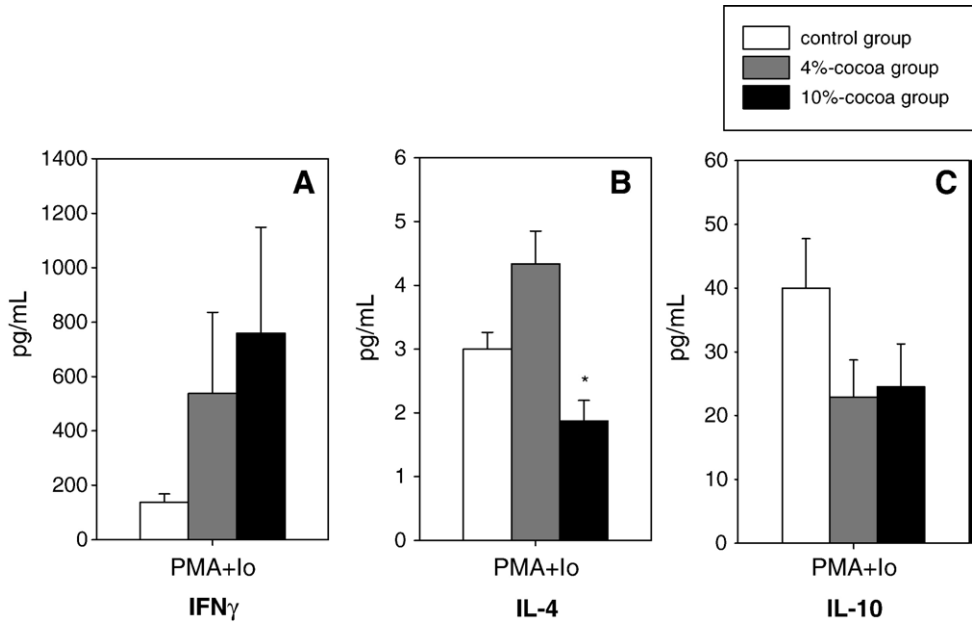

Fig. 5. IFNγ (A), IL-4 (B) and IL-10 (C) secretion of PMA/Io-stimulated MLN cells from rats fed the control diet and the 4% or 10% cocoa-enriched diet. Each bar represents the mean±S.E.M. (n=10–18). *P<.05, 4% or 10% cocoa group versus their respective control groups (ANOVA followed by Bonferroni's test).

expression, whereas after 24 h of PMA/Io stimulation, $CD25^+$ cell proportion in T lymphocytes increased by up to ~75% (Fig. 4A and B). The 4% cocoa diet reduced the proportion of $CD25^+$ T lymphocytes in nonstimulating conditions (P<.05); however, when cells were mitogen stimulated, no significant differences in $CD25^+$ T-cell percentage between groups were found (Fig. 4B). Moreover, after mitogen stimulation, CD25 surface density was not modified by any cocoa diet tested (Fig. 4C). Cell viability in all diet groups was ~75% after 24 h of cell culture and was not altered by PMA/Io addition (Fig. 4D). ELISA analysis of supernatants obtained after 24 h of MLN cell stimulation showed that PMA/Io addition induced IL-2 secretion (~2000 pg/ml), which was strongly enhanced by the 10% cocoa diet (P<.05) (Fig. 4E). However, no increase in proliferation rate was observed after 48 h of cell culture (Fig. 4F).

3.4. Effect of cocoa diet on Th-effector cytokines

In order to study whether cocoa-enriched diet modulates Th-effector cytokines in MLN, apart from IL-2, other cytokines, including IFNγ, IL-4 and IL-10, were quantified

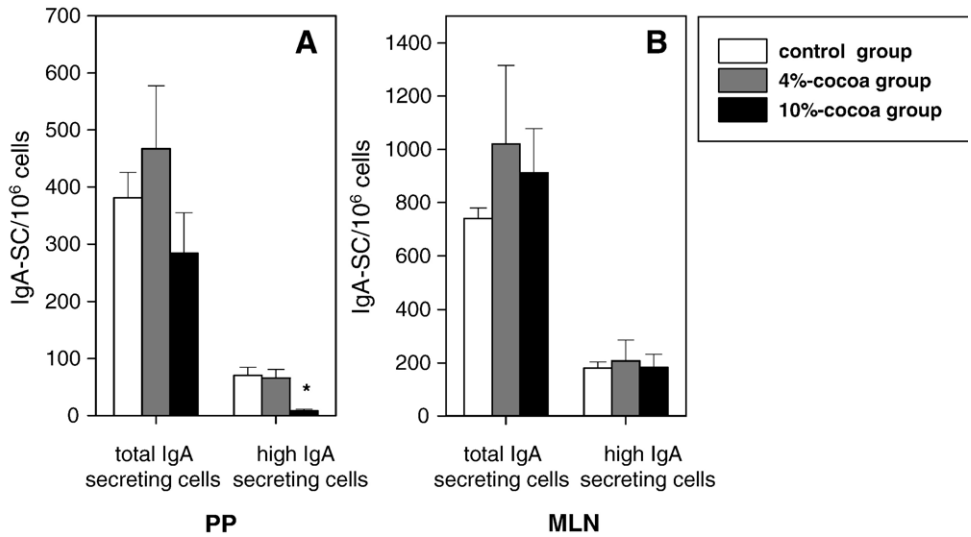

Fig. 6. Number of total and high-capacity IgA-SC in PP (A) and MLN (B) from the control diet group and the 4% or 10% cocoa-enriched diet group. Each bar represents the mean±S.E.M. (n=10–18). *P<.05, 4% or 10% cocoa group versus their respective control groups (ANOVA followed by Bonferroni's test).

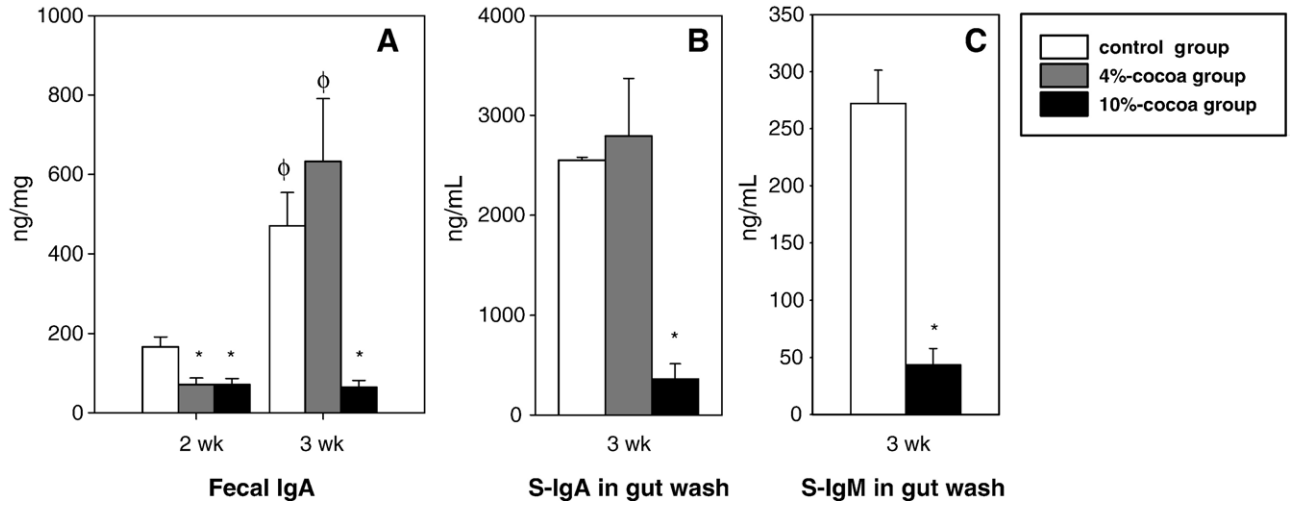

Fig. 7. Effect of cocoa-enriched diet on S-IgA in feces (A), and in gut wash (B), and on S-IgM in gut wash (C). Each bar represents the mean±S.E.M. (n=10–18). *P<.05, cocoa groups versus their respective control groups (of the same animal age). $^{\Phi}P$<.05, between days from the same animal group (ANOVA followed by Bonferroni's test).

in cell supernatants after 24 h of PMA/Io stimulation. The IFNγ, IL-4 and IL-10 secreted by MLN cells from reference animals reached ~138, ~3 and ~40 pg/ml, respectively, after PMA/Io stimulation. The 10% cocoa diet decreased IL-4 (P<.05) and IL-10 secretion by ~40% (statistically nonsignificant), whereas IFNγ production tended to increase (also statistically nonsignificant). These results suggest that the 10% cocoa diet down-regulates Th2 responses. The 4% cocoa intake did not produce any significant modification; however, a certain increase in IFNγ secretion and a decrease in IL-10 were also observed (Fig. 5).

3.5. Effect of cocoa diet on the number of IgA-SC in PP and MLN

IgA-SC in MLN and PP were enumerated by means of ELISPOT. Total and high-capacity SC were distinguished based on spot characteristics (size and intensity). In both tissues, the numbers of total and high-capacity IgA-SC were not significantly modified by the 4% cocoa diet (Fig. 6). However, PP cells from rats fed the 10% cocoa diet showed a depletion of high-capacity IgA-SC count (P<.05) (Fig. 6A).

3.6. S-Ig in gut wash and fecal samples

S-IgA was quantified in feces after 2 and 3 weeks of cocoa intake (Fig. 7A), and S-IgA in the small intestine wash was also determined at the end of the study (Fig. 7B). In control animals, fecal S-IgA levels increased according to age (P<.05). The 4% cocoa diet produced a delay in gut S-IgA secretion: Fecal S-IgA levels were low on the second week of diet, but a week later were similar to those found in the fecal samples and gut wash of control animals. As is observed at 2 and 3 weeks of treatment, the 10% cocoa diet caused less S-IgA secretion in the gut than the control diet (P<.05). These results prompted us to quantify S-IgM in gut wash, and a significant decrease in this immunoglobulin isotype was also found in the 10% cocoa group (Fig. 7C) (P<.05).

4. Discussion

Cocoa is a rich source of flavonoids with antioxidant properties [11]. In previous in vitro studies, our group and others have demonstrated the immunomodulatory properties of cocoa flavonoids [14,15,18]. These results have prompted us to study whether cocoa influences immune function in vivo. We have recently found that cocoa intake modulates spleen immune function in young rats by down-regulating Th2-related cytokines and Ig secretion [19]. Moreover, we report here for the first time the effects of continuous cocoa intake on GALT function (PP and MLN) in weaned rats.

Weaned rats were fed cocoa-enriched diets (4% and 10% food intake) for 3 weeks corresponding to their infancy. Firstly, cocoa was administered by oral gavage (0.48 g cocoa/kg animal, corresponding to ~4% food intake). Given the few observed effects on immune function, a higher cocoa dose was subsequently tested by applying the same study design. Since the physical properties of cocoa powder did not allow the administration of >0.48 g cocoa/kg animal by a single oral gavage, cocoa was then included in rat chow (10% wt/wt). Cocoa flavonoids were efficiently absorbed and dose dependently metabolized by rats regardless of the administration protocol (gavage vs. chow) [20]. During the study period, rats fed both cocoa diets had food intakes similar to their corresponding control animals. However, the 10% cocoa diet reduced body weight on the third week of the study. This effect may be attributed to lower adipose tissue synthesis, as described elsewhere, when rats consumed a 12.5% cocoa diet [21]. Further studies will be needed to

establish the cocoa compounds responsible for this effect and the mechanism involved in reducing body weight increase.

Cocoa diet did not morphologically affect the intestinal structure, but high intakes (10% cocoa) influenced some important aspects of GALT function. Profound changes in PP and MLN lymphocyte composition were found in rats fed the 10% cocoa diet. γδ T-cell proportion significantly increased in tissues from the 10% cocoa group. Intestinal γδ T lymphocytes are mainly involved in innate immunity and help to maintain mucosal homeostasis by participating in oral tolerance to food antigens and intestinal flora, in mucosal tissue repair and in immunity to viral antigens and tumor cells [22–24]. Moreover, a recent study has shown that activated human γδ T cells can also serve as APC and induce both Th- and Tc-cell-mediated responses [25]. Our results agree with those of Akiyama et al. [26], who found an increase in intestinal γδ T-cell percentage with apple polyphenol intake in healthy mice. In murine models of food allergy, apple polyphenols prevented the development of oral sensitization, and this inhibition correlated with a rise in intestinal γδ T-cell population [26]. Taken together, these results suggest that certain diets rich in flavonoids may be capable of increasing γδ T-cell proportion in GALT, and this may contribute to the prevention of food allergies, which are very common during infancy. However, cocoa's effects on other immune cells must be also taken into account.

In our study, B-cell percentage increased in PP from the 10% cocoa group. Intestinal B lymphocytes differentiate mainly into IgA plasma cells. From lamina propria (effector site), IgA is released to the intestinal lumen by binding to poly-Ig receptors (p-IgR) on epithelial cells. S-IgA is considered to be ideal for protecting the mucosal surface since it mediates immune exclusion of pathogens without triggering an inflammatory response [27]. In the present study, cocoa intake decreased S-IgA secretion to the intestinal lumen. The 4% cocoa diet only delayed this secretion since S-IgA levels in feces were restored 3 weeks after the cocoa administration period. S-IgA secretion was reduced longer by the 10% cocoa diet; from our results, we cannot determine whether S-IgA levels can be restored later. S-IgM constitutes minor Ig secreted to the gut, but it can be up-regulated as a compensatory mechanism in certain states of S-IgA deficiency [28]. Given the low S-IgA levels found in the 10% cocoa group, S-IgM in gut wash was subsequently quantified, and a decrease in intestinal S-IgM levels was also observed, showing a lack of S-IgM compensatory mechanism. PP and lamina propria constitute the major sources of Ig-SC in the intestinal mucosa. In our study, total PP and MLN IgA-SC were not modified by any of the cocoa diets tested. However, rats fed the 10% cocoa diet showed depletion of PP IgA-SC with high functionality. The decrease of high-capacity IgA-SC in PP could partly explain the lower S-IgA levels found in the 10% cocoa group. However, other factors, such as decrease in lamina propria IgA-SC or/and p-IgR down-regulation, may also contribute to luminal IgA reduction. On the other hand, IgA secretion impairment in PP from animals in the 10% cocoa group could be attributed to reduction in Th cells, which are essential for B-lymphocyte activation and differentiation into Ig-SC [29]. Few studies addressing dietary effects on GALT function in healthy animals have been reported to date. In contrast to our findings, some dietary compounds, especially probiotics and fiber, have been shown to enhance intestinal S-IgA production in healthy animals [30–32]. However, the physiological consequences of S-IgA modulation on healthy individuals remain to be explored in depth.

Evaluation of T-lymphocyte function may help to determine B-cell behavior in GALT. T-lymphocyte proportion in MLN was not affected, but the Th/Tc ratio was shifted to the cytotoxic subset. Tc lymphocytes play an important role in protection against mucosal viral and bacterial infections [27]. MLN lymphocyte activation was evaluated after PMA/Io addition. PMA is a protein kinase activator that induces T-lymphocyte activation through a pathway similar to antigen binding to cell receptors [33]. Ionomycin, a calcium ionophor salt, synergizes with PMA [34]. In response to PMA plus ionomycin, T lymphocytes are activated and proliferate through overexpression of the IL-2/IL-2R system. In our study, MLN lymphocyte proliferation was not modified by cocoa intake, although IL-2 secretion was strongly enhanced in animals fed the 10% cocoa diet. It is important to highlight that the 10% cocoa diet did not modify IL-2R (or CD25) surface expression, and this may partly explain why proliferation rate remained unchanged. However, we cannot discard the involvement of other mechanisms.

To ascertain whether cocoa modified GALT Th-effector cytokines after mitogen activation, we quantified IFNγ, IL-4 and IL-10. Neither the secretion of IFNγ (produced mainly by Th1 subset) nor the secretion of IL-10 (produced by Tr1 cells) achieved statistically significant differences with any of the cocoa-enriched diets tested with respect to their corresponding control groups. IL-4, a Th2 cytokine involved in B-cell differentiation, was down-regulated by high cocoa intake. Although IL-4 secretion was reduced in stimulated MLN cells, the number of IgA-SC was not modified in this lymphoid tissue. Taken together, these results suggest that cocoa modified other factors (apart from IL-4 secretion) involved in B-cell maturation. IL-4 also induces IgE up-regulation and increases intestinal permeability [8]; therefore, IL-4 down-regulation, along with the γδ T-cell increase induced by cocoa diet, may be beneficial in reducing certain states of hypersensitivity, such as food allergy.

In conclusion, high cocoa intake induced important changes in PP and MLN lymphocyte function. Cocoa diet strongly reduced S-IgA levels in the intestinal lumen, which is partly attributed to the down-regulation of Th2 effect on B cells in PP. In addition, cocoa increased γδ T cells in both lymphoid tissues and Tc cells in MLN. These effects may be useful in preventing viral and bacterial infections and in reducing the development of food allergies in infancy by inducing oral tolerance. However, further studies must be

performed to evaluate these potential therapeutic effects and their safety.

Acknowledgments

The authors thank Dr. Montserrat Plana and colleagues (Idibaps, Hospital Clinic, Barcelona) for the loan of the ELISPOT reader and for their technical assistance. We also thank Carolina Ramírez for her help with laboratory work and Josep Mis for histological technical assistance.

This study was supported by Nutrexpa SA and by grants from the Spanish Ministry of Education and Science (CDTI 050618 and AGL2005-002823). E.R.P. is the recipient of a fellowship from the Generalitat de Catalunya (2003FI 00578).

References

[1] Calder PC, Kew S. The immune system: a target for functional foods? Br J Nutr 2002;88(Suppl 2):S165–77.
[2] Meydani A, Ahmed T, Meydani SN. Aging, nutritional status, and infection in the developing world. Nutr Rev 2005;63(7):233–46.
[3] Menezes Jda S, Mucida DS, Cara DC, Alvarez-Leite JI, Russo M, Vaz NM, et al. Stimulation by food proteins plays a critical role in the maturation of the immune system. Int Immunol 2003;15(3):447–55.
[4] Spahn TW, Kucharzik T. Modulating the intestinal immune system: the role of lymphotoxin and GALT organs. Gut 2004;53(3):456–65.
[5] Mowat AM. Anatomical basis of tolerance and immunity to intestinal antigens. Nat Rev Immunol 2003;3(4):331–41.
[6] Nelson BH, Willerford DM. Biology of the interleukin-2 receptor. Adv Immunol 1998;70:1–81.
[7] Forchielli ML, Walker A. The role of gut-associated lymphoid tissues and mucosal defence. Br J Nutr 2005;93(S1):41–8.
[8] Colgan SP, Resnick MB, Parkos CA, Delp-Archer C, McGuirk D, Bacarra AE, et al. IL-4 directly modulates function of a model human intestinal epithelium. J Immunol 1994;153(5):2122–9.
[9] Russell MW, Kilian M. Biological activities of IgA. In: Mestecky J, Lamm ME, Strober W, Bienenstock J, McGhee JR, Mayer L, editors. Mucosal immunology. San Diego: Elsevier Press; 2005. p. 267–91.
[10] Spanish Department of Agriculture Fishery and Food. La alimentación en España. Ministerio de Agricultura, Pesca y Alimentación. Madrid (España); 2002.
[11] Vinson JA, Proch J, Bose P, Muchler S, Taffera P, Shutta D, et al. Chocolate is a powerful ex vivo and in vitro antioxidant, antiatherosclerotic agent in an animal model, and a significant contributor to antioxidants in the European and American diets. J Agric Food Chem 2006;54(21):8071–6.
[12] Pietta PG. Flavonoids as antioxidants. J Nat Prod 2000;63(7):1035–42.
[13] Mateos R, Lecumberri E, Ramos S, Goya L, Bravo L. Determination of malondialdehyde (MDA) by high-performance liquid chromatography in serum and liver as a biomarker for oxidative stress. Application to a rat model for hypercholesterolemia and evaluation of the effect of diets rich in phenolic antioxidants from fruits. J Chromatogr B Analyt Technol Biomed Life Sci 2005;827(1):76–82.
[14] Ramiro E, Franch A, Castellote C, Andres-Lacueva C, Izquierdo-Pulido M, Castell M. Effect of *Theobroma cacao* flavonoids on immune activation of a lymphoid cell line. Br J Nutr 2005;93(6):859–66.
[15] Ramiro E, Franch A, Castellote C, Perez-Cano F, Permanyer J, Izquierdo-Pulido M, et al. Flavonoids from *Theobroma cacao* down-regulate inflammatory mediators. J Agric Food Chem 2005;53(22): 8506–11.
[16] Perez-Cano FJ, Castellote C, Marin-Gallen S, Franch A, Castell M. Neonatal immunoglobulin secretion and lymphocyte phenotype in rat small intestine lamina propria. Pediatr Res 2005;58(1):164–9.
[17] Reeves PG, Nielsen FH, Fahey Jr GC. AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet. J Nutr 1993;123(11):1939–51.
[18] Sies H, Schewe T, Heiss C, Kelm M. Cocoa polyphenols and inflammatory mediators. Am J Clin Nutr 2005;81(Suppl 1):304S–12S.
[19] Ramiro-Puig E, Pérez-Cano FJ, Ramírez-Santana C, Castellote C, Izquierdo-Pulido M, Permanyer J, et al. Spleen lymphocyte function modulated by a cocoa-enriched diet. Clin and Exp Immunol 2007;149: 535–42.
[20] Ramiro-Puig E, Urpí-Sardà M, Pérez-Cano FJ, Franch A, Castellote C, Andrés-Lacueva C, et al. Cocoa-enriched diet enhances antioxidant enzyme activity and modulates lymphocyte composition in thymus from young rats. J Agric Food Chem 2007;55:6431–8.
[21] Matsui N, Ito R, Nishimura E, Yoshikawa M, Kato M, Kamei M, et al. Ingested cocoa can prevent high-fat diet-induced obesity by regulating the expression of genes for fatty acid metabolism. Nutrition 2005;21 (5):594–601.
[22] Boismenu R. Function of intestinal gammadelta T cells. Immunol Res 2000;21:123–7.
[23] Born WK, Reardon CL, O'Brien RL. The function of gammadelta T cells in innate immunity. Curr Opin Immunol 2006;18(1):31–8.
[24] Hanninen A, Harrison LC. Gamma delta T cells as mediators of mucosal tolerance: the autoimmune diabetes model. Immunol Rev 2000;173:109–19.
[25] Brandes M, Willimann K, Moser B. Professional antigen-presentation function by human gammadelta T cells. Science 2005;309:264–8.
[26] Akiyama H, Sato Y, Watanabe T, Nagaoka MH, Yoshioka Y, Shoji T, et al. Dietary unripe apple polyphenol inhibits the development of food allergies in murine models. FEBS Lett 2005;579(20):4485–91.
[27] Kunisawa J, Kiyono H. A marvel of mucosal T cells and secretory antibodies for the creation of first lines of defense. Cell Mol Life Sci 2005;62(12):1308–21.
[28] Brandtzaeg P, Nilssen DE. Mucosal aspects of primary B-cell deficiency and gastrointestinal infections. Curr Opin Gastroenterol 1995;11:532–40.
[29] Biewenga J, van Rees EP, Sminia T. Induction and regulation of IgA responses in the microenvironment of the gut. Clin Immunol Immunopathol 1993;67(1):1–7.
[30] Hosono A, Ozawa A, Kato R, Ohnishi Y, Nakanishi Y, Kimura T, et al. Dietary fructooligosaccharides induce immunoregulation of intestinal IgA secretion by murine Peyer's patch cells. Biosci Biotechnol Biochem 2003;67(4):758–64.
[31] Lim BO, Yamada K, Nonaka M, Kuramoto Y, Hung P, Sugano M. Dietary fibers modulate indices of intestinal immune function in rats. J Nutr 1997;127(5):663–7.
[32] Roller M, Rechkemmer G, Watzl B. Prebiotic inulin enriched with oligofructose in combination with the probiotics *Lactobacillus rhamnosus* and *Bifidobacterium lactis* modulates intestinal immune functions in rats. J Nutr 2004;134(1):153–6.
[33] Dornand J, Bouaboula M, d'Angeac AD, Favero J, Shire D, Casellas P. Contrasting effects of the protein kinase C inhibitor staurosporine on the interleukin-1 and phorbol ester activation pathways in the EL4-6.1 thymoma cell line. J Cell Physiol 1992;151(1):71–80.
[34] Truneh A, Albert F, Golstein P, Schmitt-Verhulst AM. Calcium ionophore plus phorbol ester can substitute for antigen in the induction of cytolytic T lymphocytes from specifically primed precursors. J Immunol 1985;135(4):2262–7.