Theobromine is responsible for the effects of cocoa on the antibody immune status of rats

Mariona Camps-Bossacoma[1], Francisco J. Pérez-Cano[1], Àngels Franch[1], Margarida Castell[1]*

[1] Secció de Fisiologia, Departament de Bioquímica i Fisiologia, Facultat de Farmàcia i Ciències de l'Alimentació, Universitat de Barcelona (UB), Av Joan XXIII 27-31, 08028 Barcelona (Spain); Institut de Recerca en Nutrició i Seguretat Alimentària (INSA-UB), UB

*Corresponding author: Section of Physiology, Department of Biochemistry and Physiology, Faculty of Pharmacy and Food Science, University of Barcelona, 08028 Barcelona, Spain. Tel.: +34 93 402 45 05; Fax: +34 93 403 59 01. E-mail address: margaridacastell@ub.edu (Margarida Castell).

E-mails: marionacamps@ub.edu; franciscoperez@ub.edu; angelsfranch@ub.edu

List of authors: Camps-Bossacoma, Pérez-Cano, Franch, Castell

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Abbreviations

APC: allophycocyanin; CC: cocoa group, fed 10% cocoa diet; ELISA: enzyme-linked immunosorbent assay; FITC: fluorescein isothiocyanate; GALT: gut-associated lymphoid tissues; Ig: immunoglobulin; MLN: mesenteric lymph nodes; PARP-1: poly (ADP-ribose) polymerase 1; PE: phycoerythrin; perCP: peridinin-chlorophyll-protein; RF: reference group, fed standard diet; sIgA: secretory IgA; sIgM: secretory IgM; TB: theobromine group, fed 0.25% theobromine diet; Treg: T regulatory lymphocytes

Conflicts of interest

None of the authors has any conflicts of interest to declare.

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Abstract

Background: A 10% cocoa-enriched diet influences the immune system functionality including the prevention of the antibody response and the induction of lower immunoglobulin concentrations. However, neither cocoa polyphenols nor cocoa fiber can totally explain these immunoregulatory properties.

Objective: This study aimed to establish the influence of cocoa theobromine in systemic and intestinal immunoglobulin concentrations and to determine the effect of cocoa or theobromine feeding on lymphoid tissue lymphocyte composition.

Methods: Three-week-old female Lewis rats received either a standard diet (AIN-93M, RF group), a 10% cocoa diet (CC group) or a 0.25% theobromine diet (the same amount provided by the CC diet, TB group) in 2 separated experiments lasting 19 (Experiment 1) or 8 days (Experiment 2). Serum IgG, IgM, IgA and intestinal secretory IgA (sIgA) concentrations were determined. In addition, at the end of Experiment 2, thymus, mesenteric lymph nodes (MLN) and spleen lymphocyte populations were analyzed.

Results: Both CC and TB groups in Experiments 1 and 2 showed similar serum IgG, IgM and IgA and intestinal sIgA concentrations that were lower than those in the RF group (46-98% lower in Experiment 1 and 23-91% lower in Experiment 2) ($p<0.05$). In addition, in Experiment 2, the CC and TB diets similarly changed the thymocyte composition by increasing CD4-CD8- (+133%) and CD4+CD8- (+53%) proportions ($p<0.01$), MLN composition by decreasing the percentage of Th lymphocytes (-3%) ($p=0.015$) and spleen composition by increasing the proportion of Th lymphocytes (+9%) ($p<0.001$) after 1 week of diet treatment.

Conclusions: The theobromine in cocoa plays an immunoregulatory role that is responsible for cocoa's influence on both systemic and intestinal antibody concentrations and also for modifying lymphoid tissue lymphocyte composition in young healthy Lewis rats. The majority of these changes are observed after a single week of consuming a diet containing 0.25% theobromine.

Keywords: cocoa, immune system, immunoglobulins, lymphoid tissues, mesenteric lymph node, methylxanthine, spleen, theobromine, thymus

1. Introduction

Cocoa, derived from *Theobroma cacao* fermented seeds, was introduced by the Mayan and Aztec civilizations as dietary and medicinal food and was diffused to Europe in the mid-1500s (1). At present, cocoa products are consumed worldwide (2), mainly as a snack due to its pleasurable taste. Furthermore, an increasing number of health properties have been attributed to its consumption, such as promoting cardiovascular health, preventing metabolic and endocrine disorders and improving cognition, mood and behavior (2–7).

In previous studies, we extensively reported that the feeding of a cocoa-enriched diet has an immunoregulatory effect on rats. Specifically, a diet containing 10% (w/w) cocoa in 3- or 6-week-old Wistar, Lewis or Brown-Norway rats influences systemic and intestinal immunoglobulin concentrations and lymphoid tissue composition (8–10). In particular, in the thymus, a cocoa diet increases the proportion of mature single positive CD4+CD8- cells and decreases the immature CD4+CD8+ cells (8). In mesenteric lymph nodes (MLN), cocoa intake increases the proportion of TCRγδ+ cells and Tc cells (9), whereas in the spleen, this nutritional intervention induces a higher percentage of B cells together with a reduction in the Th lymphocyte proportion (10). With regard to immunoglobulins, a diet containing 10% (w/w) cocoa to six-week-old female Wistar rats for three weeks decreases the concentration of serum IgG2a and IgM and secretory IgA (sIgA) and secretory IgM (sIgM) (11). Similar results were observed after two weeks of cocoa diet in eight-week-old male Lewis rats feeding different cocoa flavonoid-enriched diets with a flavonoid content ranging between 0.2 and 0.8% (w/w) (12). In this vein, the immunoregulatory influence of cocoa has been used to prevent the development of both allergy and oral sensitization in rat models, in which it has effectively prevented specific antibody synthesis (13–15).

Cocoa contains carbohydrates, proteins, lipids, fiber, minerals, polyphenols and methylxanthines (16). Among polyphenols, flavonoids are the most important and include procyanidins, epicatechin and catechin. Over the last decades, a great number of cocoa benefits have been described as a result of the antioxidant and anti-inflammatory properties of these polyphenols (2,17–19). However, cocoa is also a source of methylxanthines. These compounds are derived from xanthines and are found in several

vegetal derivatives (20), such as coffee, tea and cocoa, which contain caffeine, theophylline and theobromine as the most relevant methylxanthines, respectively (21). Cocoa contains both theobromine and caffeine, the first being the most abundant (22). Currently, several theobromine health effects have been described. In this context, among other physiological effects, theobromine acts on oral health, suppresses cough, produces bronchodilation in asthma patients, and inhibits acid uric crystallization (22–25). Today, there is increasing evidence regarding the important role played by this methylxanthine in the healthy properties of cocoa (22,23). Theobromine effects can be related to its action in the inhibition of the adenosine receptor, phosphodiesterases and/or poly(ADP-ribose) polymerase-1 (22). As the latter enzyme has many actions on the immune system, such as promoting inflammatory response affecting both innate and adaptive immune response (26), it is plausible that its inhibition could be responsible for cocoa's immunoregulatory effect.

In recent years, interest in the identification of those cocoa compounds that play an immunoregulatory role has grown. For that reason, several experimental designs have been carried out in order to ascertain whether polyphenols or cocoa fiber are responsible for the immunomodulatory properties of cocoa. Nevertheless, in both cases, the results showed that these components only partially explained the impact of cocoa on the immune system and, therefore, other cocoa compounds might contribute to the attenuation of the humoral immune response (12,27,28). In fact, the effect of theobromine on the immune system is not yet known. On the basis of these previous studies, we aimed to establish the influence of cocoa theobromine in systemic and intestinal immunoglobulin concentrations and to determine the effect of one week of cocoa or theobromine feeding on lymphoid tissue lymphocyte composition.

2. Methods

2.1. Animals and experimental nutritional intervention

Two separate experiments, Experiment 1 and Experiment 2, were carried out differing only in length. In both cases, three-week-old female Lewis rats obtained from Janvier Labs (Saint Berthevin Cedex, France) were housed (2–3 rats/cage) under controlled conditions of temperature and humidity in a 12 h

/ 12 h light/dark cycle. In both experiments, the rats were randomly assigned into three dietary groups (n = 6–7 each): the reference (RF) group, fed with the standard diet AIN-93M (Harlan Laboratories Inc., Madison, WI); the cocoa (CC) group, fed a 10% (w/w) cocoa diet; and the theobromine (TB) group fed a standard diet with 0.25% (w/w) theobromine, which was the same amount provided by the 10% cocoa diet (**Supplemental Table 1**). The food containing 10% cocoa was kept isoenergetic by extracting to a basal mix the amount of protein, lipid, carbohydrate, fiber, mineral and vitamins provided by 10% cocoa. Therefore, the addition of cocoa to the basal mix has the same amount of macronutrients and micronutrients as AIN-93M diet, providing about 7.6% of the total energy provided by the diet (3.8 kcal/g). Animals were given free access to water and food. Experiment 1 lasted 19 days and Experiment 2 lasted 8 days. All the experimental procedures were performed according to the Guide for the Care and Use of Laboratory Animals, reviewed and approved by the Ethical Committee for Animal Experimentation of the University of Barcelona (CEEA/UB ref. 5988).

2.2. Sample collection and processing

Blood and fecal samples were collected throughout the Experiment 1 (feces on days 0, 8 and 19 and blood on day 19) and 2 (feces on days 0, 4 and 8 and blood on days 4 and 8). Serum was kept at -20°C until immunoglobulin (IgG, IgG isotypes, IgM and IgA) quantification. Fecal samples were collected and treated as in previous studies in order to obtain fecal homogenates (20 mg/mL), which were kept at -20°C until sIgA quantification (30).

At the end of Experiment 2, on day 8, the animals were euthanized and the thymus, MLN and spleen were carefully removed. Thymus and spleen were immediately weighed. For lymphocyte isolation, tissue samples were passed through a sterile mesh cell strainer (40 µm, ThermoFisher Scientific) and the resultant cell suspensions were used for thymus and MLN samples, whereas spleen samples were submitted to the osmotic lyses of erythrocytes as previously described (10). Cell counting and viability were determined by a Countess™ Automated Cell Counter (Invitrogen™, Thermo Fisher Scientific).

2.3. Immunoglobulin determination

Serum IgG, IgG1, IgG2a, IgG2b, IgG2c, IgM, IgA and intestinal sIgA concentrations were quantified by a sandwich enzyme-linked immunosorbent assay (ELISA) from Bethyl Laboratories (Montgomery, TX, USA), following the manufacturer's instructions. Absorbance was measured at 492 nm by a photometer (Labsystems Multiskan, Helsinki, Finland) and data were interpolated with Multiskan Ascent v.2.6 software (Thermo Fisher Scientific S.L.U, Barcelona, Spain) according to the concentration of the corresponding standards.

2.4. Assessment of lymphocyte composition by flow cytometry analysis

Lymphocytes from thymus, MLN and spleen were stained with the mouse anti-rat CD4, CD8α, CD8β, TCRαβ, TCRγδ, NKR-P1A (BD Biosciences, Oxford, UK) and CD62L (Biolegend, San Diego, CA, USA) monoclonal antibodies conjugated to fluorescein isothiocyanate (FITC), phycoerythrin (PE), peridinin-chlorophyll-protein (PerCP) or allophycocyanin (APC), as previously described (14). All results were assessed by the Flowjo v.10 software (Tree Star Inc., Ashland, OR, USA).

2.5. Statistical analysis

For the statistical analysis, results were evaluated with the software package SPSS 22.0 (IBM Statistical Package for the Social Sciences, version 22.0, Chicago, IL, USA). Data from the 3 groups were compared to one. The Levene test was performed to assess the homogeneity of variance (homoscedasticity) of the results, and the Shapiro–Wilk test to assess their distribution. Homogeneity of variance and normal distributed data were analyzed by the parametric test one-way ANOVA followed by Bonferroni's post hoc test. Body weight values and immunoglobulins concentrations studied at different time points were evaluated by repeated measures ANOVA. Significant differences were established when $p \leq 0.05$.

In contrast, the results having different variance and/or different distribution (IgG isotype concentrations) were evaluated by the Kruskal–Wallis and Mann–Whitney U nonparametric tests. These results were represented using Whisker plots.

3. Results

3.1. Effect of cocoa theobromine on serum immunoglobulins

At the end of Experiment 1, after 19 days of consuming the CC or TB diet, the concentration of IgG, was similar in the CC and TB groups and it was lower than that in the RF group ($p<0.001$) (**Figure 1A**). However, no differences between groups were seen earlier (**Figure 1B**). The lower total IgG concentration on day 19 was mainly due to lower levels of IgG1, IgG2b and IgG2c isotypes ($p\leq0.050$) (Figure 1A–B and **Supplemental Figure 1**).

Total serum IgM and IgA in Experiment 1 were also lower ($p<0.001$) after CC and TB interventions (**Figure 1C–D**). The analysis in the Experiment 2 revealed that IgM and IgA levels in CC and TB group were already lower than those in the RF group at days 4 and 8, respectively ($p\leq0.005$ and $p\leq0.003$, respectively) (**Figure 1E–F**).

3.2. Effect of cocoa theobromine on intestinal sIgA

In order to ascertain the contribution of the theobromine in cocoa in the intestinal compartment, sIgA was also quantified in feces samples throughout both experiments (**Figure 2**). RF group showed an increased pattern in intestinal IgA content associated with age ($p<0.001$). This increase did not occur in the CC and TB groups which had similar concentrations of intestinal sIgA that were lower than those present in RF group ($p<0.001$). The difference was already detectable after 4 days of CC or TB intake ($p\leq0.003$) (**Figure 2B**).

3.3. Effect of cocoa theobromine on thymus, MLN and spleen

In the Experiment 2, the three groups had similar body weight at the beginning of the study (**Table 1**). At the end of the study, after 1 week of diet, the CC and TB groups had higher body weight than that observed at the beginning ($p<0.001$) but the increase was lower than that of the RF group ($p<0.001$) although no differences were detected in the relative food intake between the three studied groups (Table 1). Moreover, the CC and TB groups had lower lymphoid tissues relative weights compared with the RF group after one week of diet ($p\leq0.005$) (Table 1).

The phenotype of lymphocytes from thymus, MLN and spleen was studied after one week of the nutritional intervention (Experiment 2). Lymphocytes from thymus are classified into four subsets according to the expression of CD4 and CD8 molecules (**Figure 3, Supplemental Figure 2**). The most immature population are CD4-CD8- cells (double-negative or DN), then these cells turn into CD4+CD8+ cells (double-positive or DP) to finally become CD4-CD8+ or CD4+CD8-cells (single positive or SP), corresponding to the most mature thymocytes which eventually migrate to the peripheral lymphoid organs (31,32). Both CC and TB groups showed a higher relative amount of CD4-CD8- and CD4+CD8- cells than RF group ($p<0.001$ and $p\leq0.037$, respectively), whereas CD4+CD8+ cell proportion was lower ($p=0.001$) (**Figure 3A**, Supplemental Figure 2). In addition, in the thymocyte maturation, there was a gradual increase in the expression of the antigenic receptor TCRαβ, TCRαβ^{high} being the most mature cells. A lower proportion of CD4-CD8-TCRαβ^{high} was observed in both nutritional interventions with respect to the RF group ($p\leq0.004$) (**Table 2**). Otherwise, in CD4-CD8+ cells, the proportion of TCRαβ^{high} thymocytes in the CC and TB groups was higher than in the RF group ($p\leq0.001$) whereas both that of TCRαβ^{low} and TCRαβ- cells was lower ($p\leq0.034$) (**Table 2**). With regard to the MLN, the proportions of the main lymphocyte subsets, e.g. CD45RA+ (B lymphocytes), TCRαβ+, TCRγδ+ and NK cells, were not modified in CC or TB groups (**Figures 4A**). However, both the CC and TB groups showed a lower proportion of TCRαβ+CD4+ (Th) lymphocytes ($p\leq0.027$) and higher proportion of TCRαβ+CD8+ (Tc) cells ($p\leq0.016$) than RF animals (**Figure 4B**). Consequently, Th/Tc ratio was similarly reduced after the CC or TB diets ($p\leq0.013$) (**Figure 4C**). In the case of TCRγδ+ cells, no significant differences were observed as a result of the diets in either the CD8αα+ or CD8αβ+ subsets (**Supplemental Figure 3**). With regard to the proportion of those Th, Tc and B cells expressing CD62L (L-selectin), a different pattern was found after the cocoa-enriched diet only, which showed a reduced proportion of CD45RA+CD62L+ cells ($p=0.040$) and a higher proportion of CD45RA+CD62L- ($p=0.040$) (**Figure 4D**).

The spleen was also affected after 8 days of either the CC or TB diet. In particular, after both interventions there was a lower proportion of TCRγδ+ and NK cells compared with that of the RF group ($p\leq0.038$ and $p<0.001$, respectively) although no significant differences were found in TCRαβ+

and CD45RA+ lymphocytes (**Figures 5A**). However, studying the TCRαβ+ subsets in depth, in contrast to what happened in the MLN, after both diets there was a higher proportion of Th cells than in the RF group ($p<0.001$), whereas there was a lower percentage of Tc and NKT cells ($p\leq0.043$ and $p<0.001$, respectively) (**Figure 5B**). As a result, the Th/Tc ratio was significantly higher in the CC and TB groups than in the reference group ($p\leq0.013$) (**Figure 5C**). The percentage of TCRγδ+CD8αα+ and TCRγδ+CD8αβ+ cells in the three groups was similar (**Supplemental Figure 4A**). In the case of the CD62L marker on CD4+, CD8+ and CD45RA+ cells, no differences were detected as a result of the nutritional interventions (**Supplemental Figure 4B**).

4. Discussion

Previous studies carried out in rats demonstrated that a 10% (w/w) cocoa diet has the potential to regulate the immune status, attenuating the antibody concentrations both in systemic and intestinal compartments (9–11,33,34), and modifying lymphoid tissue composition mainly in the gut-associated lymphoid tissue (GALT) (8–11). These effects could not be totally attributed to the cocoa polyphenol or fiber content (12,35,36). The present data show, for the first time, the role of theobromine in cocoa's effects on antibody concentrations and on lymphoid tissues in young healthy rats. In addition, this study reveals the effects of cocoa and theobromine on lymphoid tissue after only one week of intervention.

The current results demonstrate that the CC diet influences the circulating concentrations of immunoglobulins. Specifically, the rats fed the CC diet had lower IgG concentration than the RF group after 19 days of diet, in accordance with the previous reported effects of a 10% cocoa diet for 3 weeks on 3-week-old Wistar rats (10,35), whereas it did not match with a study carried out in older animals, in particular in 8-week-old Lewis rats (12). These results reflect the importance of age in cocoa's effects on IgG. The higher sensitivity in young rats could be due to the more immature immune system. Rats are born with an immature immune system that develops during suckling and that even at weaning time is not yet mature (37). Regarding the sensitivity of each IgG isotype (IgG2c>IgG2b>IgG1), previous studies modified them differently (11,27,34), reflecting that, among others, rat strain and environment could also be important in the influence of cocoa on IgG.

Nonetheless, it is worth noting that theobromine on its own produced an identical effect on serum IgG and its isotypes concentrations to that of cocoa.

Likewise, TB and CC groups also showed similar lower concentrations of IgM and IgA with respect to RF group. These results partially or completely agree with those found after 3 or 7 weeks of 10% cocoa diet in 3-week-old or 6-week-old Wistar rats (10,11,34,35), and after 2 weeks of different cocoa-polyphenol-enriched diets in 8-week-old Lewis rats (12). In addition, the current data show that IgM was affected earlier than IgA and both immunoglobulins earlier than IgG. In any case, most importantly, we can conclude that theobromine is responsible for cocoa's influence on systemic immunoglobulin concentrations.

With respect to intestinal antibodies, sIgA content increased in young rats throughout the studied period as previously reported (30), and this increasing pattern was avoided by the cocoa diet as a result of its theobromine content. This down-regulatory effect was already observed after 4 days of the nutritional intervention and corresponded with prior data obtained after one (11,12), two (9,27), three (27,35), six (33) or seven weeks (34) of cocoa feeding using different rat strains. It is worth noting that, physiologically, IgA is the last antibody synthesized (38) and therefore, being the most immature, it could be the most sensitive to the immunoregulatory effect of the diet. On the other hand, changes in intestinal sIgA could be attributed to modifications in intestinal microbiota. In fact, both the CC and TB diets induced changes in the gut bacteria pattern (39,40) but these modifications differ between the two interventions. In consequence, this suggests that the microbiota changes might not be the primary determinant of the effect on sIgA concentrations.

On the other hand, this study also focused on the effect of CC and TB diets on the lymphoid tissues. Previous studies have reported the influence of cocoa diet on MLN after at least 3 or 4 weeks of diet (9,14,41,42), whereas studies on the spleen and the thymus are very limited (8,10). Indeed, in none of the studied lymphoid organs are there any results regarding such a short diet period. First of all, it must be taken into consideration that after only one week of either CC or TB diet, body weight was lower than that in the RF group. This fact could be considered a limitation of the study, although the relative food consumption (calculated per 100 g body weight) did not change during the study as previously

reported (11,14). On the other hand, the relative weight of spleen and thymus from animals fed CC or TB was lower than that in rats fed the standard diet, suggesting that these compounds could, among other changes, inhibit the proliferation of lymphocytes, interfere with lymphocyte trafficking to lymphoid organs or increase the progressive thymus age-related regression (43) that will eventually reduce lymphoid tissue organ weight. Nevertheless, further studies are required to determine the exact impact of TB in such lymphoid organs. In addition, the study of the thymus composition revealed that, in comparison to the RF group, CC and TB intake resulted in a higher proportion of CD4-CD8- cells and CD4+CD8- lymphocytes, whereas there was a lower proportion of CD4+CD8+ cells. Furthermore, both nutritional interventions resulted in a lower expression of TCRαβ on CD4-CD8- cells. The relatively higher amount of the less mature cell type (CD4-CD8- cells) is in line with previous data obtained with a longer diet period (8). Further studies are required to ascertain whether TB either delays T cell maturation, reduces the arrival of cells and/or enhances the number of cells leaving the thymus. Nevertheless, these thymocyte proportion changes after 8 days of diet were not associated with a lower proportion of T cells in either MLN or spleen. However, longer intake studies have demonstrated a decrease in the proportion of TCRαβ+ cells in lymph nodes (14). These results suggest that the main effect of cocoa can be in T cell maturation in the thymus and, later, those modifications will be reflected in the lymph nodes.

In the current study, in spite of the changes in the thymus populations, the balance of the main populations in the MLN and the spleen was not modified by the diet. These results do not agree with those found in the same tissues for a longer timer (10,14). Therefore, we suggest that these previously observed changes would need an extended cocoa intake. However, the current results in MLN showed a lower proportion of Th cells and a higher proportion of Tc cells after one week of CC or TB intake with respect to RF conditions, as found in longer studies (9,14), which could be due to changes in thymocyte maturation. Nevertheless, in the case of the spleen composition, there was a contrary effect, i.e. a higher proportion of Th cells and a lower Tc cell percentage. This could be due to the movement of Th cells in the thymus to the spleen.

In the spleen, the CC and TB diets induced lower proportions of the TCRγδ+ and NK cells that did not agree with the reported effect in a longer study (10). On the other hand, in the current study, TCRγδ+

cell and NK cell proportions in MLN were not modified by the nutritional interventions. However, other studies reported that cocoa-fed animals had a higher proportion of TCRγδ+ (and sometimes NK cells) in MLN (9,14), Peyer's patches and intestinal intraepithelial lymphocytes (44), which could indicate that these cells leave the spleen to go to these peripheral lymphoid organs, although in terms of the MLN, our study was not long enough to demonstrate such an increase.

The comparison of the effects of the CC and TB diets on the composition of both primary and secondary lymphoid tissues revealed that both diets produced the same degree of action in the modified lymphocyte subsets. Overall, these results indicate that theobromine is the main compound responsible for the immunoregulatory effect of cocoa on the lymphoid tissues, in addition to its previously stated effect on the attenuation of systemic and intestinal antibody concentrations. Until now, different effects of theobromine have been published, but, as far as we know, no previous results suggesting the role of theobromine as an immunoregulatory agent have been published. In this context, although the current study was carried out only in young healthy rats, the results suggest that theobromine has an immunoregulatory potential and could be indicated in some disorders, such as in organ transplantation to prevent rejection (45), in autoimmune disorders (multiple sclerosis, rheumatoid arthritis, systemic lupus erythematosus) (46) and in hypersensitivity reactions (allergic disease and asthma) (47). In particular, the previously reported tolerogenic effect of cocoa on food allergy and oral sensitization rat models (13–15,44) could be due to its theobromine content. Nevertheless, further studies must confirm the potential of theobromine as an immunoregulatory agent in pathological conditions.

With regard to theobromine's mechanism of action, it has been described as a potent inhibitor of the poly(ADP-ribose)polymerase-1 (PARP-1) (48). PARP-1 is a nuclear enzyme that has an essential role in DNA repair (48) and relevant immunological functions, including the regulation of gene transcription in dendritic cells, macrophages and lymphocytes (26). In this context, PARP-1 activation has been associated with pathologic conditions such as in inflammatory response in murine asthma models (49,50), and its inhibition prevents airway eosinophilia and suppresses Th2 cytokine production (51,52). It has also been reported that PARP-1 negatively regulates Treg cell function (53,54) and higher numbers of CD4+CD25+FoxP3+ Treg cells appear in thymus, spleen and lymph

293 nodes of PARP-1 knockout mice (55). Altogether, these facts lead us to think that the reported
294 inhibition of PARP-1 by theobromine (48) could have an important implication in the
295 immunoregulatory role described here. Nevertheless, further studies are required to establish the exact
296 mechanisms and to ascertain the minimum dose required of this methylxanthine for it to be able to
297 produce the observed effects. Moreover, clinical studies are needed in order to confirm such effects in
298 humans and also to establish the optimal age and dose needed. Taking into consideration the Reagan-
299 Shaw formula (56), to achieve the immunoregulatory action of cocoa, a human with a body weight of
300 60 kg, would have to eat more than one chocolate bar per day. Nevertheless, the current findings show
301 that cocoa's immunoregulatory effects can be achieved with theobromine alone which avoids such a
302 high chocolate intake. In addition, it is worth noting that the effects of cocoa and theobromine are
303 mainly observed after just one week of intake, which means that the immunoregulatory properties
304 could be achieve with the acute intake of such compounds.

305 In conclusion, a 10% cocoa-enriched diet, due to its theobromine content, is able to decrease systemic
306 and intestinal immunoglobulin concentrations in young healthy Lewis rats and modify thymus,
307 mesenteric lymph nodes and spleen lymphocyte composition after a single week of dietary
308 intervention suggesting for the first time the role of theobromine as an immunosuppressive agent.

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313 **Authors' contributions to the manuscript**

314 F.J.P-C, À.F and M.C. designed research; M.C-B conducted research, analyzed data and wrote the
315 paper. F.J.P-C and M.C. contributed to the critical revision of the manuscript. All authors read and
316 approved the final manuscript.

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FIGURES

FIGURE 1. Serum IgG (A, B), IgM (C, D), and IgA (E, F) concentrations in Lewis rats fed a reference diet alone or including cocoa or theobromine for 19 (A, C, E; Experiment 1) or 8 (B, D, F; Experiment 2) days. Values are means ± SEMs, n=6-7. Labeled means in a panel without a common letter differ (p≤0.05) CC: cocoa group; NS: nonsignificant (P>0.05); RF: reference group; TB: theobromine group.

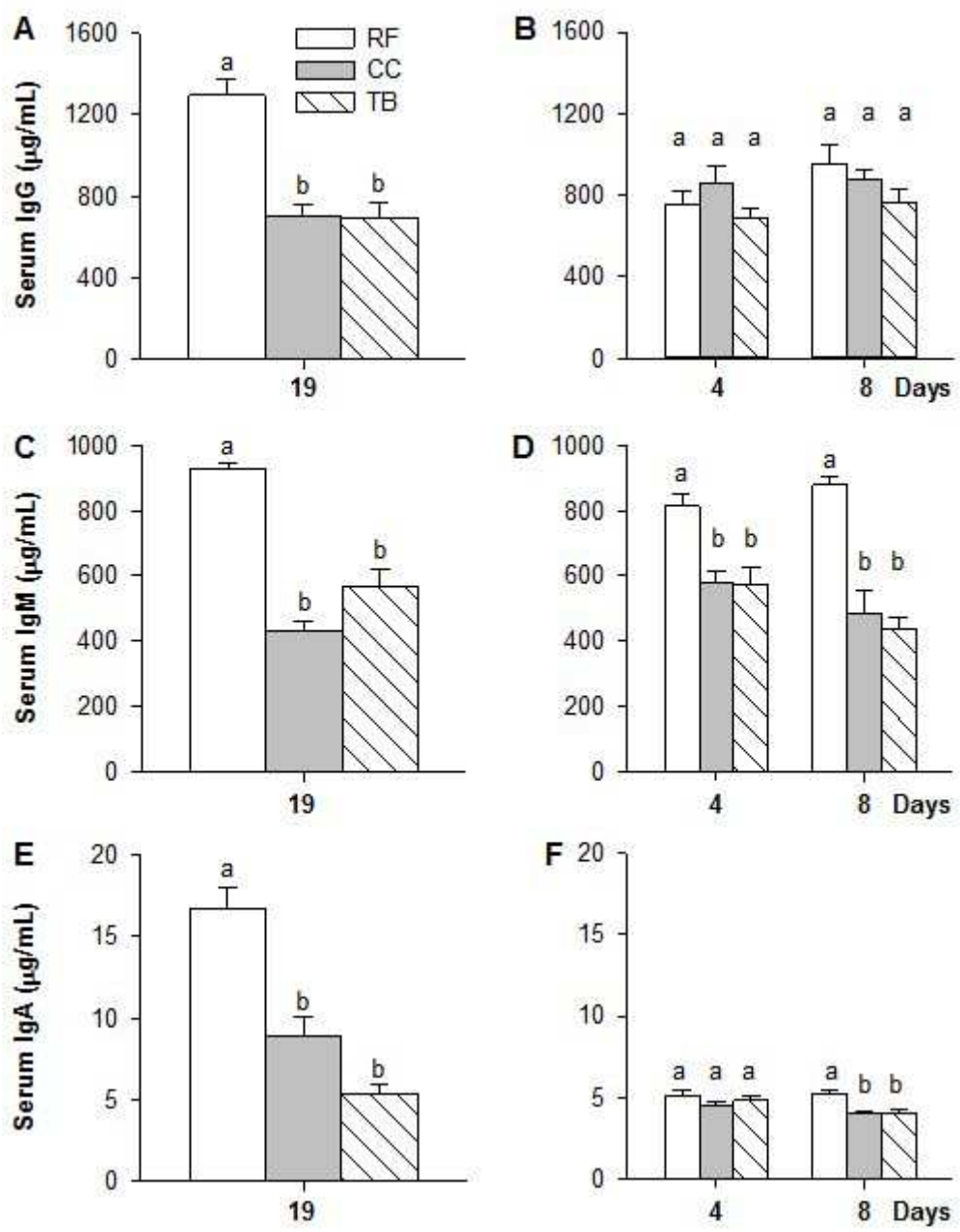


FIGURE 2. Intestinal sIgA concentrations in Lewis rats fed a reference diet alone or including cocoa or theobromine for 19 (A; Experiment 1) or 8 (B; Experiment 2) days. Values are means ± SEMs, n=6-7. Labeled means in a panel without a common letter differ ($p \leq 0.05$). CC: cocoa group; RF: reference group; TB: theobromine group.

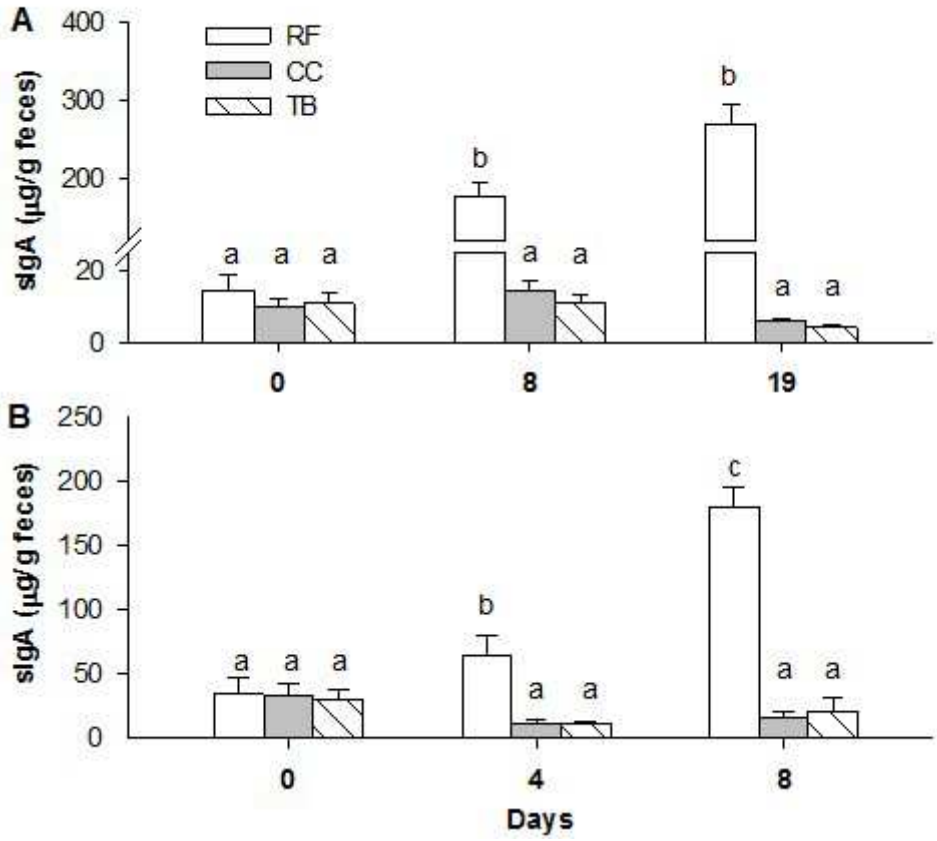


FIGURE 3. Percentage of thymocytes expressing or not CD4 and CD8 molecules in Lewis rats fed a reference diet alone or including cocoa or theobromine for 8 days (Experiment 2). Values are means ± SEMs, n=6. Labeled means in a subset without a common letter differ ($p \leq 0.05$). CC: cocoa group; RF: reference group; TB: theobromine group.

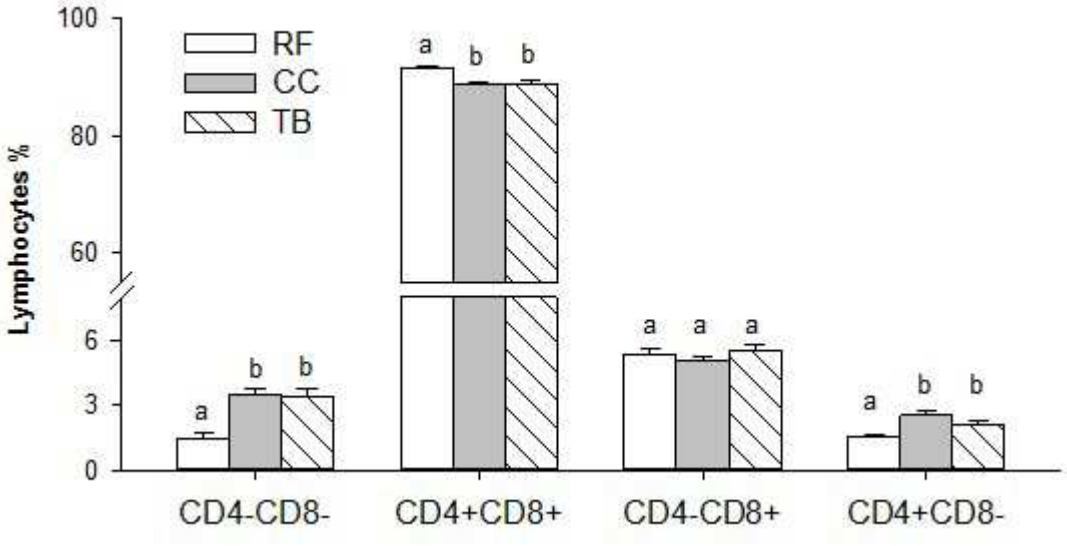


FIGURE 4. Percentage of the main lymphocyte subsets (A) and TCRαβ+ subsets (B), Th/Tc ratio (C), and proportion of CD62L+ and CD62L- cells either in CD4+, CD8+ or CD45RA+ lymphocytes (D) in mesenteric lymph nodes of Lewis rats fed a reference diet alone or including cocoa or theobromine for 8 days (Experiment 2). Values are means ± SEMs, n=6. Labeled means in a subset without a common letter differ ($p \leq 0.05$). CC: cocoa group; RF: reference group; TB: theobromine group.

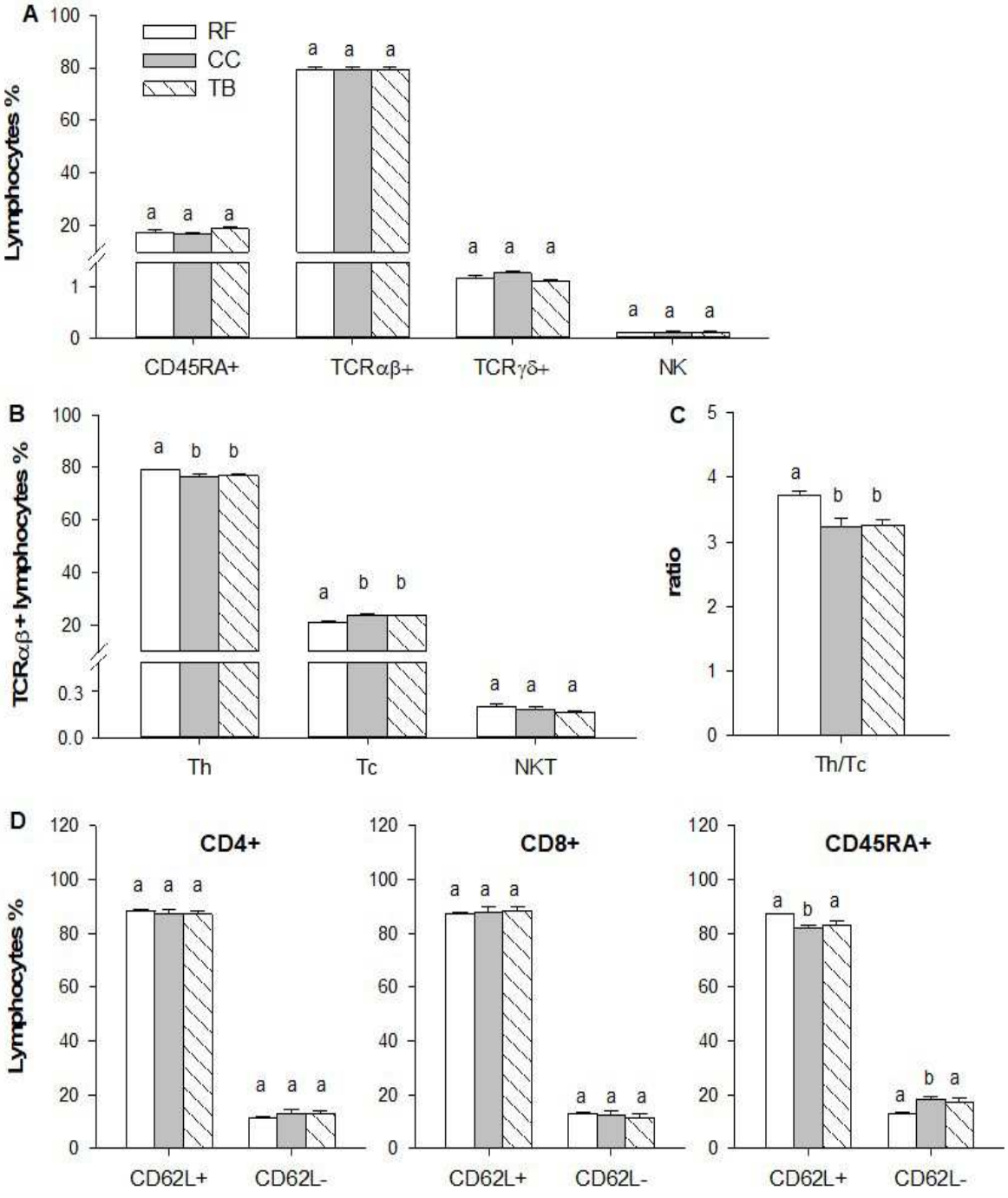
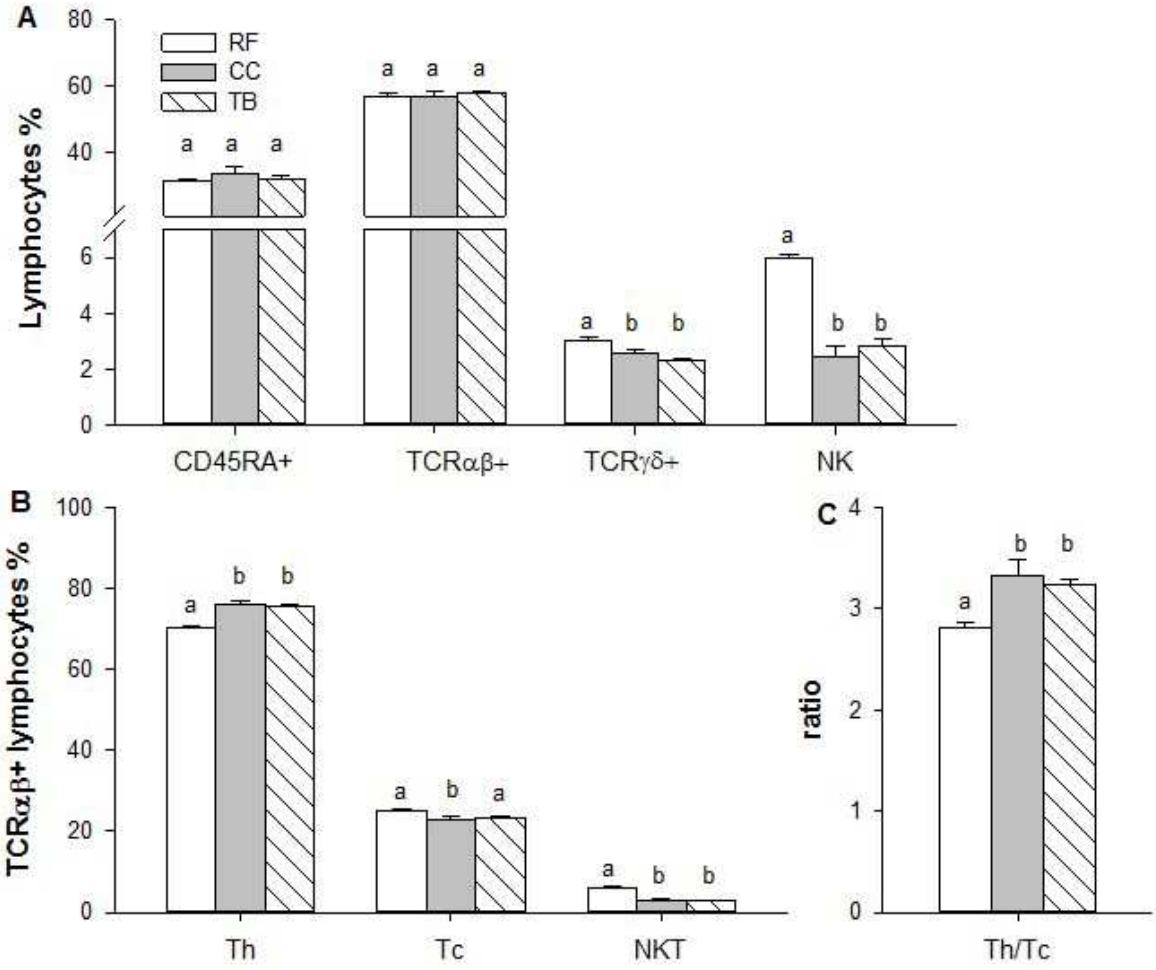


FIGURE 5. Percentage of the main lymphocyte subsets (A) and TCRαβ+ subsets (B), and Th/Tc ratio (C) in spleen of Lewis rats fed a reference diet alone or including cocoa or theobromine for 8 days (Experiment 2).Values are means ± SEMs, n=6. Labeled means in a subset without a common letter differ (p≤0.05). CC: cocoa group; RF: reference group; TB: theobromine group.



TABLES

TABLE 1. Body weight, relative food intake and relative weight of thymus and spleen in Lewis rats fed a reference diet alone or including cocoa or theobromine for 8 days (Experiment 2)[1].

	Group[2]		
	RF	CC	TB
Body weight, g			
Day 0	80.08 ± 2.25	79.98 ± 1.86	80.62 ± 2.17
Day 8	115.38 ± 2.14*[a]	103.42 ± 1.82*[b]	103.65 ± 1.80*[b]
Relative food intake, g/(100 g body x 8d)	60.31 ± 0.27	62.74 ± 0.83	58.32 ± 0.69
Relative tissue weight, g/100 g body			
Thymus	0.39 ± 0.01[a]	0.22 ± 0.01[b]	0.20 ± 0.02[b]
Spleen	0.30 ± 0.01[a]	0.24 ± 0.01[b]	0.25 ± 0.01[b]

1 Values are means ± SEMs, n = 6. Labeled means in a row without a common superscript letter differ, $p \leq 0.05$.

2 CC: cocoa group; RF: reference group; TB: theobromine group.

* Different from day 0, $p < 0.001$

TABLE 2. Percentage of thymocytes according to the expression of TCRαβ (high, low or negative) molecule on each of the four subsets defined by CD4 and CD8 expression in Lewis rats fed a reference diet alone or including cocoa or theobromine for 8 days (Experiment 2)[1].

	Group[2]		
Thymocyte subset	RF	CC	TB
CD4-CD8-, %			
TCRαβ^{low}	29.8 ± 3.7	25.5 ± 2.5	22.1 ± 1.6
TCRαβ^{high}	14.7 ± 1.4^{a}	6.2 ± 1.1^{b}	8.4 ± 1.0^{b}
TCRαβ-	55.5 ± 3.6^{b}	68.2 ± 3.7^{a}	69.5 ± 2.1^{a}
CD4+CD8+, %			
TCRαβ^{low}	81.0 ± 0.7	82.0 ± 0.8	81.0 ± 0.9
TCRαβ^{high}	4.0 ± 0.2	3.9 ± 0.3	4.0 ± 0.2
TCRαβ-	15.0 ± 0.7	14.1 ± 0.9	15.0 ± 1.0
CD4-CD8+, %			
TCRαβ^{low}	24.0 ± 2.5^{a}	10.1 ± 1.5^{b}	12.5 ± 1.5^{b}
TCRαβ^{high}	72.8 ± 2.7^{b}	88.5 ± 1.5^{a}	86.2 ± 1.8^{a}
TCRαβ-	3.0 ± 0.4^{a}	1.5 ± 0.3^{b}	1.4 ± 0.4^{b}
CD4+CD8-, %			
TCRαβ^{low}	30.1 ± 1.8	30.5 ± 2.1	26.0 ± 2.6
TCRαβ^{high}	59.5 ± 1.8	58.3 ± 2.7	63.4 ± 2.7
TCRαβ-	10.3 ± 0.8	11.2 ± 1.1	10.7 ± 1.4

[1] Values are means ± SEMs, n = 6. Labeled means in a row without a common superscript letter differ, $p \leq 0.05$.

[2] CC: cocoa group; RF: reference group; TB: theobromine group.

Online Supporting Material

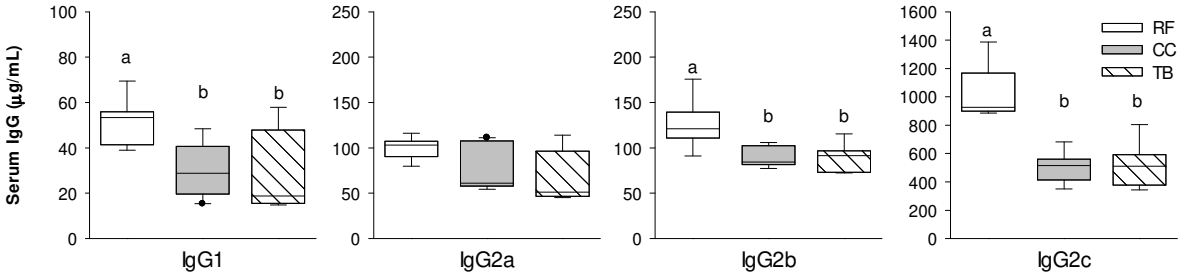
Supplemental Table 1. Composition of the experimental diets fed to Lewis rats for 19-days or 8-days.

Components	Diets [1], g/kg diet			
	RF	**CC**		**TB**
		Basal mix[2]	Cocoa powder[3]	
Carbohydrates	722[4]	696[4]	18	720[4]
Proteins	141[5]	117[5]	23	140[5]
Lipids	38.7[6]	26.0[6]	11.5	38.6[6]
Insoluble fiber	50[7]	24[7]	26	50[7]
Soluble fiber	-	-	8.5	-
Minerals	35.9[8]	27.1[8]	6.1	35.8[8]
Vitamins	10.20[9]	7.92[9]	0.04	10.20[9]
Choline bitartrate	2.5	2.0	-	2.5
Antioxidant (tert-butylhydroquinone)	0.01	0.01	-	0.01
Theobromine	-	-	2.5	2.5
Polyphenols	-	-	4	-
Total	1000	1000		1000

[1] CC: cocoa diet; RF: reference diet (AIN-93M, Harlan Laboratories Inc., Madison, WI); TB: theobromine diet
[2] Harlan Laboratories Inc., Madison, WI
[3] The composition of cocoa has been previously reported (29)
[4] Corn starch, maltodextrin and sucrose
[5] Casein and L-cysteine
[6] Soybean oil
[7] Cellulose
[8] AIN-93-MX (Harlan Laboratories Inc., Madison, WI)
[9] AIN-93-VX (Harlan Laboratories Inc., Madison, WI)

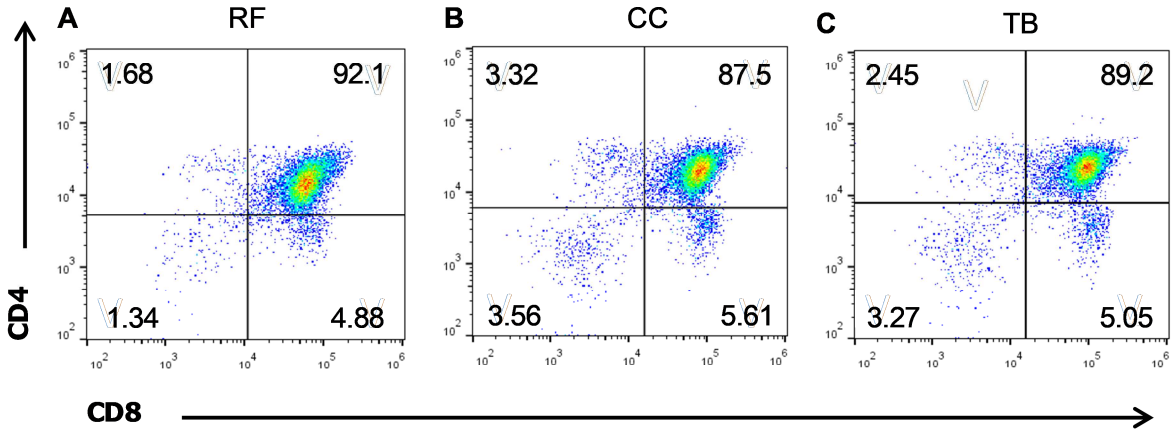
Online Supporting Material

Supplemental Figure 1: Serum IgG isotype concentrations in Lewis rats fed a reference diet alone or including cocoa or theobromine for 19 days (Experiment 1). Values are represented in a Whisker plot, n=6. Labeled means in an isotype without a common letter differ ($p \leq 0.05$). CC: cocoa group, RF: reference group, TB: theobromine group.



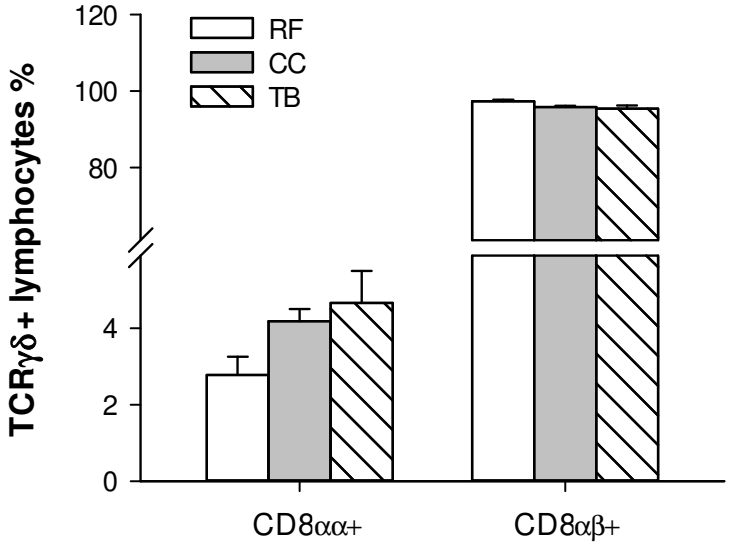
Online Supporting Material

Supplemental Figure 2. Representative flow cytometry histograms of CD4/CD8 expression on thymocytes in Lewis rats fed a reference diet alone or including cocoa or theobromine for 8 days (Experiment 2). CC: cocoa group; RF: reference group; TB: theobromine group.



Online Supporting Material

Supplemental Figure 3. Percentage of TCRγδ+ subsets in mesenteric lymph nodes of Lewis rats fed a reference diet alone or including cocoa or theobromine for 8 days (Experiment 2). Values are means ± SEMs, n=6. No statistically significant results were detected. CC: cocoa group; RF: reference group; TB: theobromine group.



Online Supporting Material

Supplemental Figure 4. Percentage of TCRγδ+ subsets (A), CD62L+ and CD62L- cells either in CD4+ lymphocytes (B), CD8+ lymphocytes (C) or CD45RA+ lymphocytes (D) in Lewis rats fed a reference diet alone or including cocoa or theobromine for 8 days (Experiment 2). Values are means ± SEMs, n=6. No statistically significant results were detected. CC: cocoa group; RF: reference group; TB: theobromine group.