

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

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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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1 Abstract

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

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

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

15 **Results:** Both CC and TB groups in Experiments 1 and 2 showed similar serum IgG, IgM and IgA and
16 intestinal sIgA concentrations that were lower than those in the RF group (46-98% lower in
17 Experiment 1 and 23-91% lower in Experiment 2) ($p < 0.05$). In addition, in Experiment 2, the CC and
18 TB diets similarly changed the thymocyte composition by increasing CD4-CD8- (+133%) and
19 CD4+CD8- (+53%) proportions ($p < 0.01$), MLN composition by decreasing the percentage of Th
20 lymphocytes (-3%) ($p = 0.015$) and spleen composition by increasing the proportion of Th lymphocytes
21 (+9%) ($p < 0.001$) after 1 week of diet treatment.

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

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

28 1. Introduction

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

35 In previous studies, we extensively reported that the feeding of a cocoa-enriched diet has an
36 immunoregulatory effect on rats. Specifically, a diet containing 10% (w/w) cocoa in 3- or 6-week-old
37 Wistar, Lewis or Brown-Norway rats influences systemic and intestinal immunoglobulin
38 concentrations and lymphoid tissue composition (8–10). In particular, in the thymus, a cocoa diet
39 increases the proportion of mature single positive CD4+CD8- cells and decreases the immature
40 CD4+CD8+ cells (8). In mesenteric lymph nodes (MLN), cocoa intake increases the proportion of
41 TCR $\gamma\delta$ + cells and Tc cells (9), whereas in the spleen, this nutritional intervention induces a higher
42 percentage of B cells together with a reduction in the Th lymphocyte proportion (10). With regard to
43 immunoglobulins, a diet containing 10% (w/w) cocoa to six-week-old female Wistar rats for three
44 weeks decreases the concentration of serum IgG2a and IgM and secretory IgA (sIgA) and secretory
45 IgM (sIgM) (11). Similar results were observed after two weeks of cocoa diet in eight-week-old male
46 Lewis rats feeding different cocoa flavonoid-enriched diets with a flavonoid content ranging between
47 0.2 and 0.8% (w/w) (12). In this vein, the immunoregulatory influence of cocoa has been used to
48 prevent the development of both allergy and oral sensitization in rat models, in which it has effectively
49 prevented specific antibody synthesis (13–15).

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

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

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

76 **2. Methods**

77 *2.1. Animals and experimental nutritional intervention*

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

81 / 12 h light/dark cycle. In both experiments, the rats were randomly assigned into three dietary groups
82 (n = 6–7 each): the reference (RF) group, fed with the standard diet AIN-93M (Harlan Laboratories
83 Inc., Madison, WI); the cocoa (CC) group, fed a 10% (w/w) cocoa diet; and the theobromine (TB)
84 group fed a standard diet with 0.25% (w/w) theobromine, which was the same amount provided by the
85 10% cocoa diet (**Supplemental Table 1**). The food containing 10% cocoa was kept isoenergetic by
86 extracting to a basal mix the amount of protein, lipid, carbohydrate, fiber, mineral and vitamins
87 provided by 10% cocoa. Therefore, the addition of cocoa to the basal mix has the same amount of
88 macronutrients and micronutrients as AIN-93M diet, providing about 7.6% of the total energy
89 provided by the diet (3.8 kcal/g). Animals were given free access to water and food. Experiment 1
90 lasted 19 days and Experiment 2 lasted 8 days. All the experimental procedures were performed
91 according to the Guide for the Care and Use of Laboratory Animals, reviewed and approved by the
92 Ethical Committee for Animal Experimentation of the University of Barcelona (CEEA/UB ref. 5988).

93 *2.2. Sample collection and processing*

94 Blood and fecal samples were collected throughout the Experiment 1 (feces on days 0, 8 and 19 and
95 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
96 until immunoglobulin (IgG, IgG isotypes, IgM and IgA) quantification. Fecal samples were collected
97 and treated as in previous studies in order to obtain fecal homogenates (20 mg/mL), which were kept
98 at -20°C until sIgA quantification (30).

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

105 *2.3. Immunoglobulin determination*

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

112 *2.4. Assessment of lymphocyte composition by flow cytometry analysis*

113 Lymphocytes from thymus, MLN and spleen were stained with the mouse anti-rat CD4, CD8 α , CD8 β ,
114 TCR $\alpha\beta$, TCR $\gamma\delta$, NKR-P1A (BD Biosciences, Oxford, UK) and CD62L (Biolegend, San Diego, CA,
115 USA) monoclonal antibodies conjugated to fluorescein isothiocyanate (FITC), phycoerythrin (PE),
116 peridinin-chlorophyll-protein (PerCP) or allophycocyanin (APC), as previously described (14). All
117 results were assessed by the Flowjo v.10 software (Tree Star Inc., Ashland, OR, USA).

118 *2.5. Statistical analysis*

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

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

130

131 3. Results

132 3.1. Effect of cocoa theobromine on serum immunoglobulins

133 At the end of Experiment 1, after 19 days of consuming the CC or TB diet, the concentration of IgG,
134 was similar in the CC and TB groups and it was lower than that in the RF group ($p < 0.001$) (**Figure**
135 **1A**). However, no differences between groups were seen earlier (**Figure 1B**). The lower total IgG
136 concentration on day 19 was mainly due to lower levels of IgG1, IgG2b and IgG2c isotypes ($p \leq 0.050$)
137 (Figure 1A–B and **Supplemental Figure 1**).

138 Total serum IgM and IgA in Experiment 1 were also lower ($p < 0.001$) after CC and TB interventions
139 (**Figure 1C–D**). The analysis in the Experiment 2 revealed that IgM and IgA levels in CC and TB
140 group were already lower than those in the RF group at days 4 and 8, respectively ($p \leq 0.005$ and
141 $p \leq 0.003$, respectively) (**Figure 1E–F**).

142 3.2. Effect of cocoa theobromine on intestinal sIgA

143 In order to ascertain the contribution of the theobromine in cocoa in the intestinal compartment, sIgA
144 was also quantified in feces samples throughout both experiments (**Figure 2**). RF group showed an
145 increased pattern in intestinal IgA content associated with age ($p < 0.001$). This increase did not occur
146 in the CC and TB groups which had similar concentrations of intestinal sIgA that were lower than
147 those present in RF group ($p < 0.001$). The difference was already detectable after 4 days of CC or TB
148 intake ($p \leq 0.003$) (**Figure 2B**).

149 3.3. Effect of cocoa theobromine on thymus, MLN and spleen

150 In the Experiment 2, the three groups had similar body weight at the beginning of the study (**Table 1**).
151 At the end of the study, after 1 week of diet, the CC and TB groups had higher body weight than that
152 observed at the beginning ($p < 0.001$) but the increase was lower than that of the RF group ($p < 0.001$)
153 although no differences were detected in the relative food intake between the three studied groups
154 (Table 1). Moreover, the CC and TB groups had lower lymphoid tissues relative weights compared
155 with the RF group after one week of diet ($p \leq 0.005$) (Table 1).

156 The phenotype of lymphocytes from thymus, MLN and spleen was studied after one week of the
157 nutritional intervention (Experiment 2). Lymphocytes from thymus are classified into four subsets
158 according to the expression of CD4 and CD8 molecules (**Figure 3, Supplemental Figure 2**). The
159 most immature population are CD4-CD8- cells (double-negative or DN), then these cells turn into
160 CD4+CD8+ cells (double-positive or DP) to finally become CD4-CD8+ or CD4+CD8-cells (single
161 positive or SP), corresponding to the most mature thymocytes which eventually migrate to the
162 peripheral lymphoid organs (31,32). Both CC and TB groups showed a higher relative amount of
163 CD4-CD8- and CD4+CD8- cells than RF group ($p < 0.001$ and $p \leq 0.037$, respectively), whereas
164 CD4+CD8+ cell proportion was lower ($p = 0.001$) (**Figure 3A**, Supplemental Figure 2). In addition, in
165 the thymocyte maturation, there was a gradual increase in the expression of the antigenic receptor
166 TCR $\alpha\beta$, TCR $\alpha\beta^{\text{high}}$ being the most mature cells. A lower proportion of CD4-CD8-TCR $\alpha\beta^{\text{high}}$ was
167 observed in both nutritional interventions with respect to the RF group ($p \leq 0.004$) (**Table 2**).
168 Otherwise, in CD4-CD8+ cells, the proportion of TCR $\alpha\beta^{\text{high}}$ thymocytes in the CC and TB groups was
169 higher than in the RF group ($p \leq 0.001$) whereas both that of TCR $\alpha\beta^{\text{low}}$ and TCR $\alpha\beta^-$ cells was lower
170 ($p \leq 0.034$) (**Table 2**). With regard to the MLN, the proportions of the main lymphocyte subsets, e.g.
171 CD45RA+ (B lymphocytes), TCR $\alpha\beta^+$, TCR $\gamma\delta^+$ and NK cells, were not modified in CC or TB groups
172 (**Figures 4A**). However, both the CC and TB groups showed a lower proportion of TCR $\alpha\beta^+$ CD4+
173 (Th) lymphocytes ($p \leq 0.027$) and higher proportion of TCR $\alpha\beta^+$ CD8+ (Tc) cells ($p \leq 0.016$) than RF
174 animals (**Figure 4B**). Consequently, Th/Tc ratio was similarly reduced after the CC or TB diets
175 ($p \leq 0.013$) (**Figure 4C**). In the case of TCR $\gamma\delta^+$ cells, no significant differences were observed as a
176 result of the diets in either the CD8 $\alpha\alpha^+$ or CD8 $\alpha\beta^+$ subsets (**Supplemental Figure 3**). With regard to
177 the proportion of those Th, Tc and B cells expressing CD62L (L-selectin), a different pattern was
178 found after the cocoa-enriched diet only, which showed a reduced proportion of CD45RA+CD62L+
179 cells ($p = 0.040$) and a higher proportion of CD45RA+CD62L- ($p = 0.040$) (**Figure 4D**).

180 The spleen was also affected after 8 days of either the CC or TB diet. In particular, after both
181 interventions there was a lower proportion of TCR $\gamma\delta^+$ and NK cells compared with that of the RF
182 group ($p \leq 0.038$ and $p < 0.001$, respectively) although no significant differences were found in TCR $\alpha\beta^+$

183 and CD45RA+ lymphocytes (**Figures 5A**). However, studying the TCR $\alpha\beta$ + subsets in depth, in
184 contrast to what happened in the MLN, after both diets there was a higher proportion of Th cells than
185 in the RF group ($p < 0.001$), whereas there was a lower percentage of Tc and NKT cells ($p \leq 0.043$ and
186 $p < 0.001$, respectively) (**Figure 5B**). As a result, the Th/Tc ratio was significantly higher in the CC and
187 TB groups than in the reference group ($p \leq 0.013$) (**Figure 5C**). The percentage of TCR $\gamma\delta$ +CD8 $\alpha\alpha$ +
188 and TCR $\gamma\delta$ +CD8 $\alpha\beta$ + cells in the three groups was similar (**Supplemental Figure 4A**). In the case of
189 the CD62L marker on CD4+, CD8+ and CD45RA+ cells, no differences were detected as a result of
190 the nutritional interventions (**Supplemental Figure 4B**).

191 **4. Discussion**

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

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

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

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

219 With respect to intestinal antibodies, sIgA content increased in young rats throughout the studied
220 period as previously reported (30), and this increasing pattern was avoided by the cocoa diet as a result
221 of its theobromine content. This down-regulatory effect was already observed after 4 days of the
222 nutritional intervention and corresponded with prior data obtained after one (11,12), two (9,27), three
223 (27,35), six (33) or seven weeks (34) of cocoa feeding using different rat strains. It is worth noting
224 that, physiologically, IgA is the last antibody synthesized (38) and therefore, being the most immature,
225 it could be the most sensitive to the immunoregulatory effect of the diet. On the other hand, changes in
226 intestinal sIgA could be attributed to modifications in intestinal microbiota. In fact, both the CC and
227 TB diets induced changes in the gut bacteria pattern (39,40) but these modifications differ between the
228 two interventions. In consequence, this suggests that the microbiota changes might not be the primary
229 determinant of the effect on sIgA concentrations.

230 On the other hand, this study also focused on the effect of CC and TB diets on the lymphoid tissues.
231 Previous studies have reported the influence of cocoa diet on MLN after at least 3 or 4 weeks of diet
232 (9,14,41,42), whereas studies on the spleen and the thymus are very limited (8,10). Indeed, in none of
233 the studied lymphoid organs are there any results regarding such a short diet period. First of all, it must
234 be taken into consideration that after only one week of either CC or TB diet, body weight was lower
235 than that in the RF group. This fact could be considered a limitation of the study, although the relative
236 food consumption (calculated per 100 g body weight) did not change during the study as previously

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

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

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

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

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

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

309

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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 \pm SEMs, n=6-7. Labeled means in a panel without a common letter differ ($p \leq 0.05$) CC: cocoa group; NS: nonsignificant ($P > 0.05$); RF: reference group; TB: theobromine group.

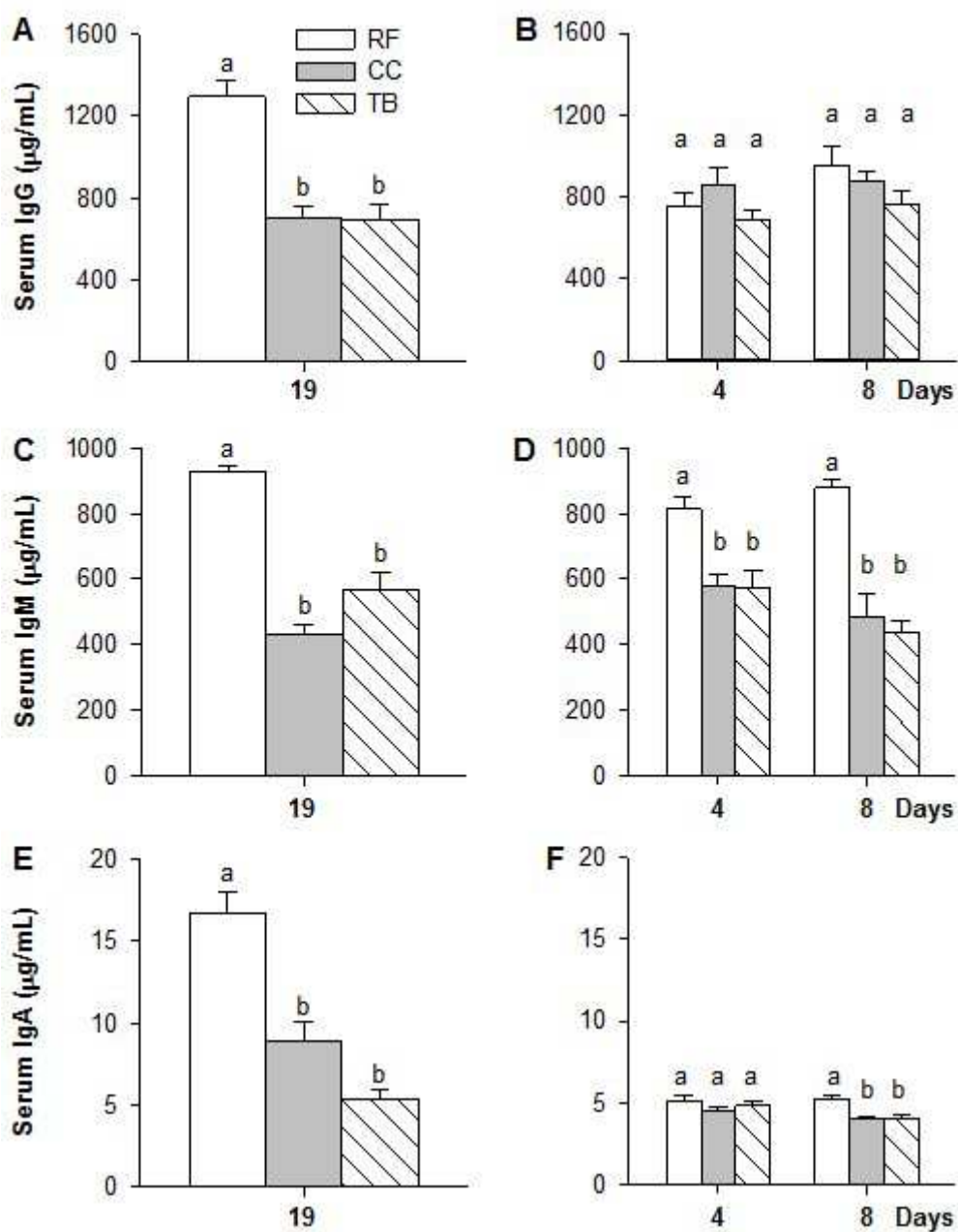


FIGURE 2. Intestinal sIgA concentrations in Lewis rats fed a reference diet alone or including cocoa or theobromine for 19 (A; Experiment 1) or 8 (B; Experiment 2) days. Values are means \pm SEMs, n=6-7. Labeled means in a panel without a common letter differ ($p \leq 0.05$). CC: cocoa group; RF: reference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

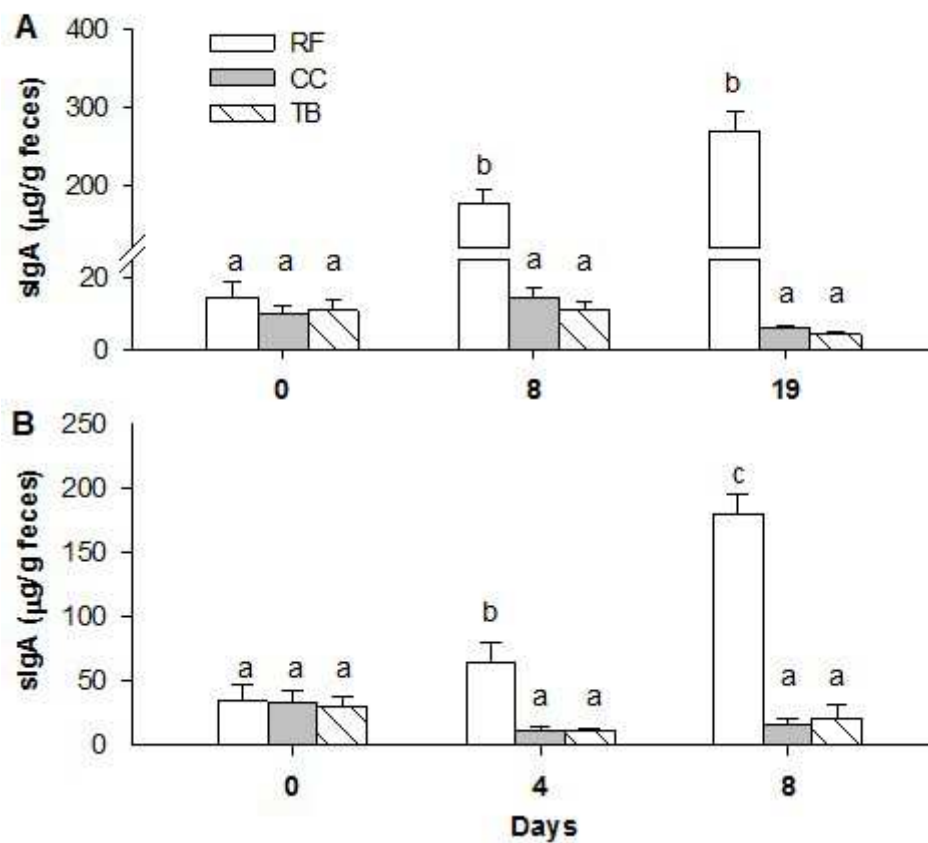


FIGURE 3. Percentage of thymocytes expressing or not CD4 and CD8 molecules in Lewis rats fed a reference diet alone or including cocoa or theobromine for 8 days (Experiment 2). Values are means \pm SEMs, n=6. Labeled means in a subset without a common letter differ ($p \leq 0.05$). CC: cocoa group; RF: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

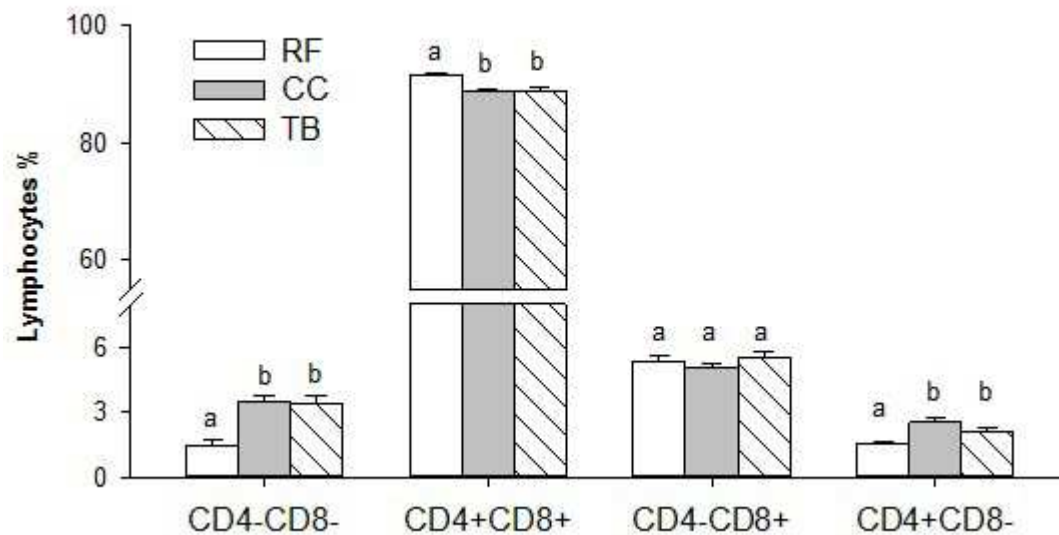


FIGURE 4. Percentage of the main lymphocyte subsets (A) and TCR $\alpha\beta$ + subsets (B), Th/Tc ratio (C), and proportion of CD62L+ and CD62L- cells either in CD4+, CD8+ or CD45RA+ lymphocytes (D) in mesenteric lymph nodes of Lewis rats fed a reference diet alone or including cocoa or theobromine for 8 days (Experiment 2). Values are means \pm 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.

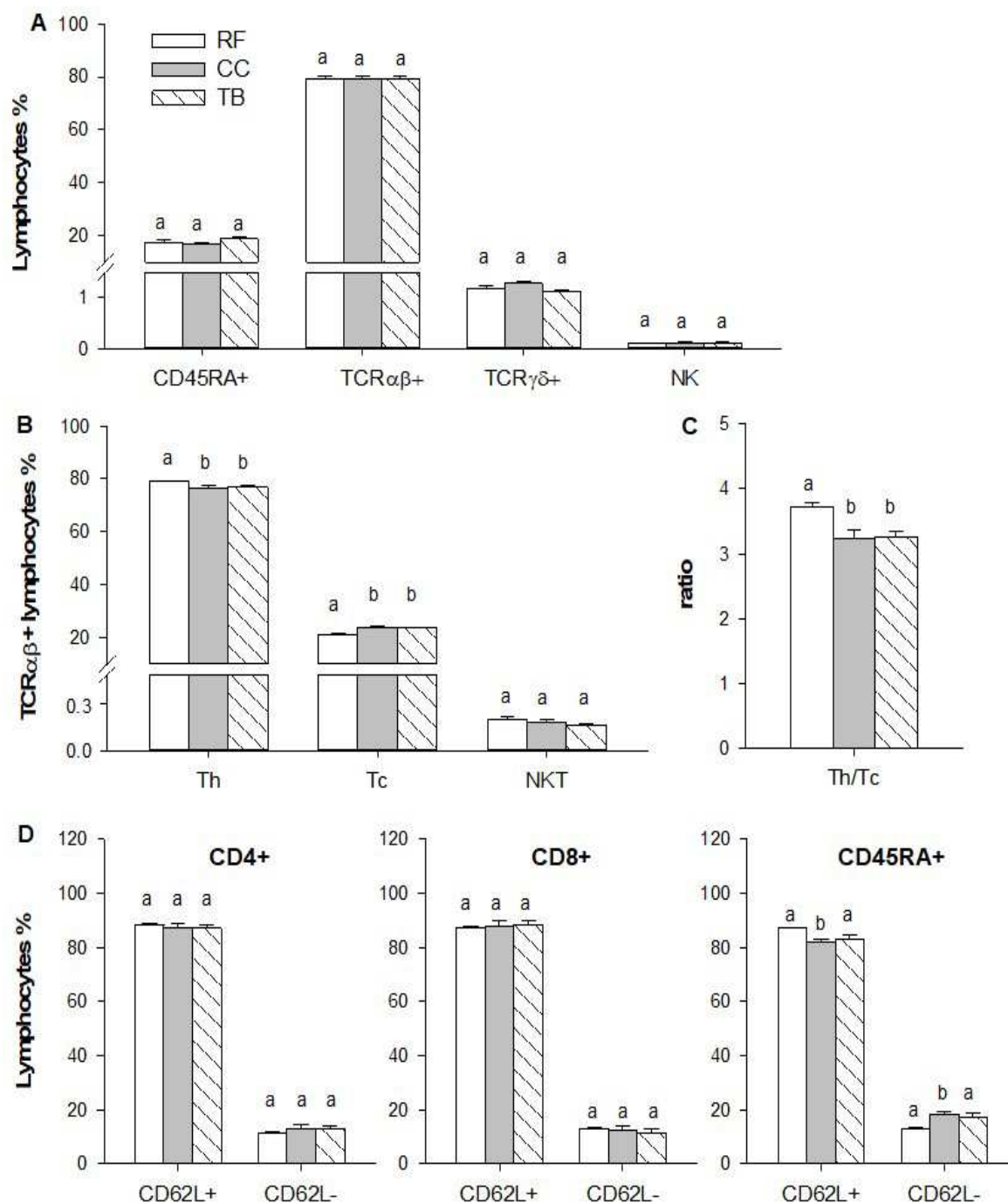
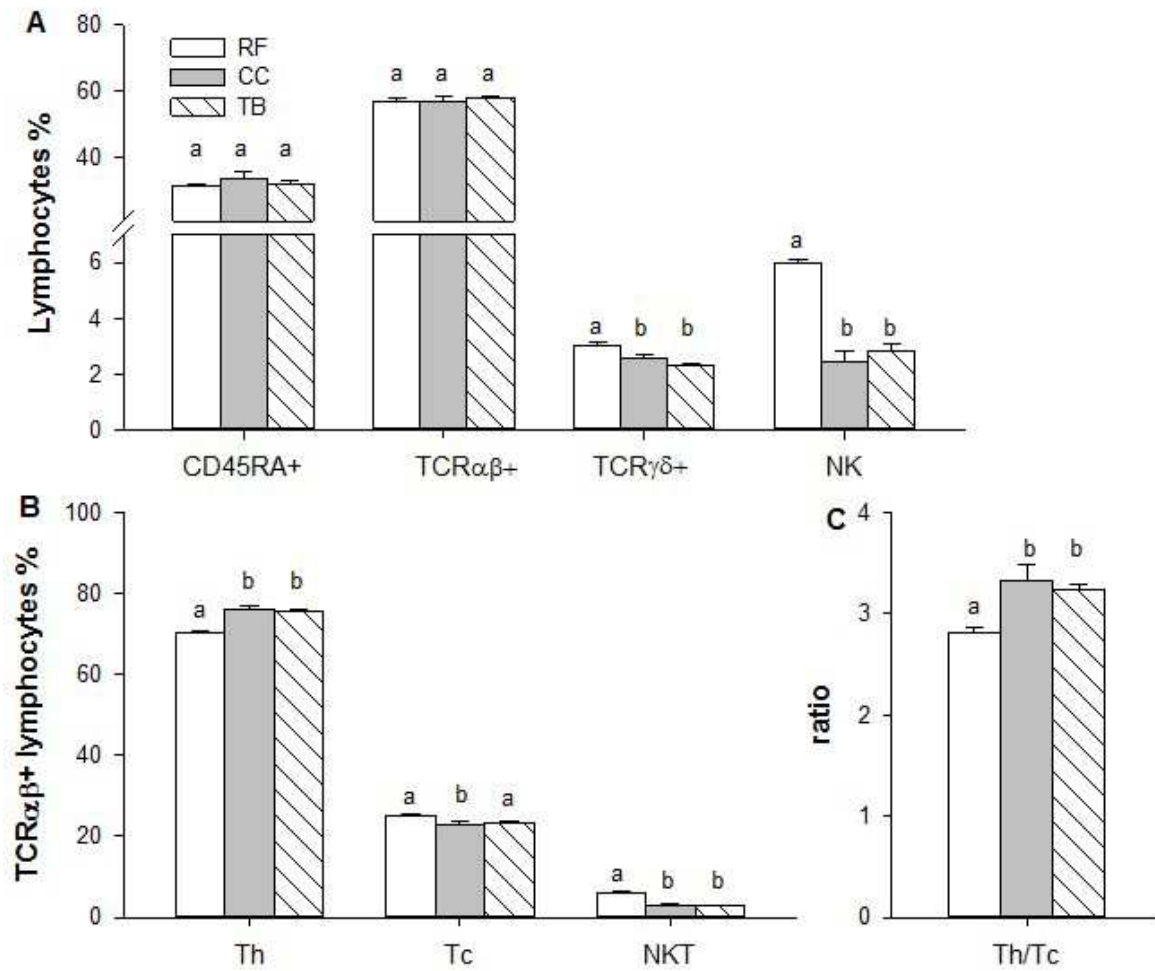


FIGURE 5. Percentage of the main lymphocyte subsets (A) and TCR $\alpha\beta$ + subsets (B), and Th/Tc ratio (C) in spleen of Lewis rats fed a reference diet alone or including cocoa or theobromine for 8 days (Experiment 2). Values are means \pm SEMs, n=6. Labeled means in a subset without a common letter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



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 ≤ 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 TCR $\alpha\beta$ (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.

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

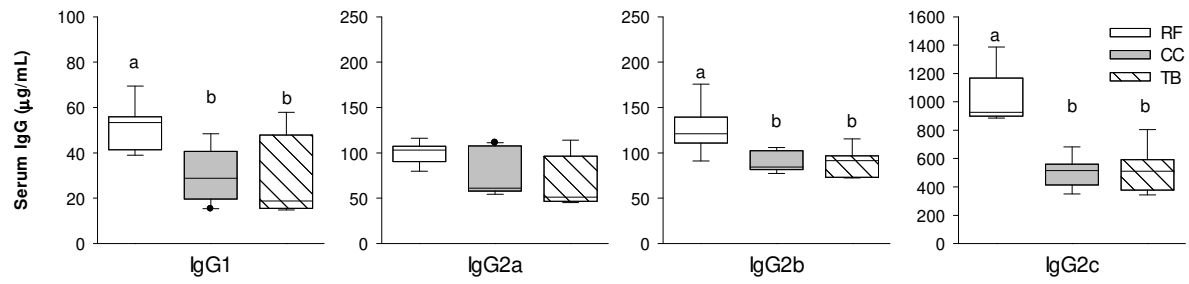
⁷ Cellulose

⁸ AIN-93-MX (Harlan Laboratories Inc., Madison, WI)

⁹ AIN-93-VX (Harlan Laboratories Inc., Madison, WI)

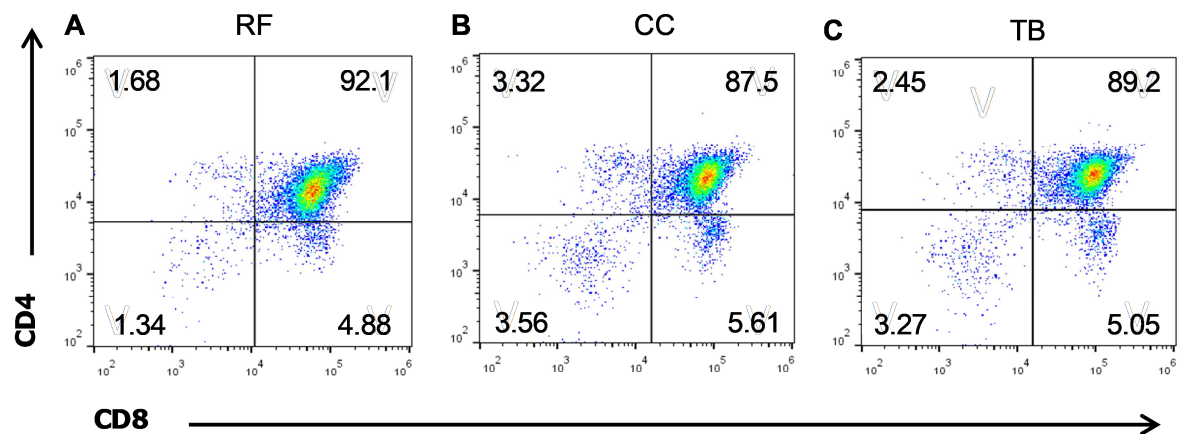
Online Supporting Material

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.



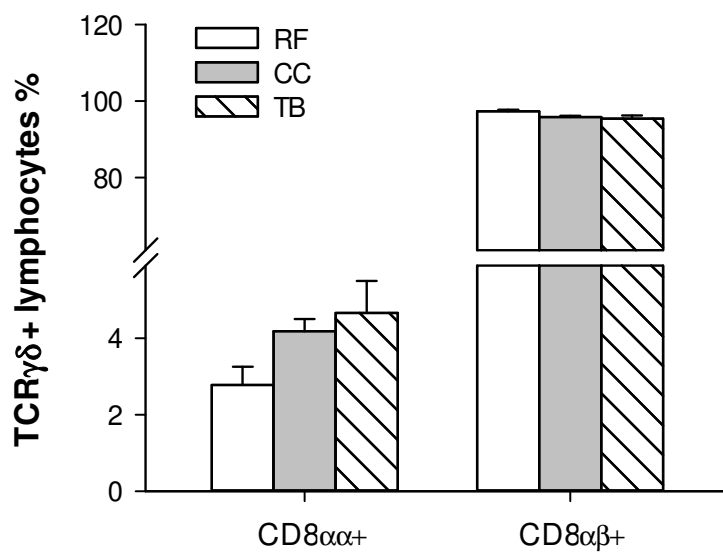
Online Supporting Material

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 TCR $\gamma\delta$ ⁺ 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 \pm 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 TCR $\gamma\delta$ + 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 \pm SEMs, n=6. No statistically significant results were detected. CC: cocoa group; RF: reference group; TB: theobromine group.

