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Dietary phytosterols modulate T-helper immune response but do not induce apparent anti-inflammatory effects in a mouse model of acute, aseptic inflammation

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Abstract

Although most studies have focused on the cholesterol-lowering activity of phytosterols, other biological actions have been ascribed to these plant sterol compounds, one of which is a potential immune modulatory effect. To gain insight into this issue, we used a mouse model of acute, aseptic inflammation induced by a single subcutaneous turpentine injection. Hypercholesterolemic apolipoprotein E-deficient (apoE$^{-/-}$) mice, fed with or without a 2% phytosterol supplement, were treated with turpentine or saline and euthanized 48 h later. No differences were observed in spleen lymphocyte subsets between phytosterol- and control-fed apoE$^{-/-}$ mice. However, cultured spleen lymphocytes of apoE$^{-/-}$ mice fed with phytosterols and treated with turpentine showed increased IL-2 and IFN-γ secretion (T-helper type1, Th1 lymphocyte cytokines) compared with turpentine-treated, control-fed animals. In contrast, there was no change in Th2 cytokines IL-4 and IL-10. Phytosterols also inhibit intestinal cholesterol absorption in wild-type C57BL/6J mice but, in this case, without decreasing plasma cholesterol. Spleen lymphocytes of turpentine-treated C57BL/6J mice fed with phytosterols also showed increased IL-2 production, but IFN-γ, IL-4 and IL-10 production was unchanged. The Th1/Th2 ratio was significantly increased both in phytosterol-fed apoE$^{-/-}$ and C57BL/6J mice. We conclude that phytosterols modulate the T-helper immune response in vivo, in part independently of their hypocholesterolemic effect in a setting of acute, aseptic inflammation. Further study of phytosterol effects on immune-based diseases characterized by an exacerbated Th2 response is thus of interest.

Keywords: Phytosterols; T-lymphocytes; Th1: Th2; Cytokines

Introduction

Plant sterol/stanol (phytosterol/phytostanol) consumption is a recommended dietary option to decrease LDL cholesterol (NCEP, 2001) and can be acquired without medical prescription in many countries. Therefore, full understanding of their effects is desirable. Although most studies have focused on the cholesterol-lowering activity of phytosterols, some evidence proposes that these plant sterols may have additional biological actions that include immunomodulatory properties. The use of β-sitosterol and β-sitosterol glucoside in healthy persons or in patients with HIV infection, rheumatoid arthritis and allergic conditions demonstrated enhanced T-cell proliferation and natural killer cell activity with a shift towards a T-helper (Th1) cytokine profile (Bouic, 2001; Bouic et al., 1996; Breytenbach et al., 2001). This is of special interest since such an effect could increase resistance to bacterial and parasitic infections and, therefore, be important in diseases where priming of Th1 helper cells is a targeted goal. However, the dose and composition of the plant sterols used (Bouic, 2001;

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Bouic et al., 1996; Breytenbach et al., 2001) were not those usually used in current food preparations containing plant sterols to decrease plasma cholesterol. We found indirect evidence of potential immunomodulatory activity in intestinal gene expression of a more usual plant sterol mixtures (containing campesterol, stigmasterol and β-sitosterol) and dosis (in mice, 2% of diet) in hypercholesterolemic apolipoprotein (apo) E-deficient ($E^{-/-}$) mice and, partially, in normocholesterolemic C57BL/6J mice (Calpe-Berdiel et al., 2005). Another study found association between the antiatherogenic effects of dietary phytosterols and reduction of proinflammatory cytokine production in $apoE^{-/-}$ mice (Nashed et al., 2005). Phytosterols also decreased prostaglandin release by cultured macrophages (Awad et al., 2004) and modulated leukocyte function in murine models of inflammation (Navarro et al., 2001; Park et al., 2001). Given the clinical potential of these phytosterol actions, the aim of the present study was to assess the effects of dietary phytosterols on immune function in an acute, aseptic, well-controlled inflammation model. As phytosterols decrease plasma cholesterol in hypercholesterolemic $apoE^{-/-}$ mice, but not in normocholesterolemic C57BL/6J, and both hypercholesterolemia and apoE influence immune response, we analyzed both $apoE^{-/-}$ and C57BL/6J mice (Ali et al., 2005; Calpe-Berdiel et al., 2005; Mallat et al., 2003; Nashed et al., 2005; Zhou et al., 1998).

Materials and methods

Animals and diets

All the procedures described were approved by the Ethics Committee of the Agriculture, Livestock and Fishing Department of the Generalitat of Catalonia. The use of $apoE^{-/-}$ mice with a C57BL/6J background was described previously (Escola-Gil et al., 2000). Mice were maintained in a temperature-controlled (20 °C) room with a 12-h light/dark cycle and food and water were provided *ad libitum*. Eight-week-old female mice were randomized in two groups and fed either a control Western-type diet (200 g/kg fat, 0.8 g/kg cholesterol, 170 g/kg protein, 105 g/kg fiber; Mucedola srl, Settimo Milanese, Italy) or a 2% (wt/wt) phytosterol-enriched Western-type diet (wt/wt). The phytosterol product was composed of 20% campesterol, 22% stigmasterol and 41% β-sitosterol (Lipofoods S.L., Gavà, Barcelona, Spain) (Calpe-Berdiel et al., 2005). C57BL/6J mice were obtained from Jackson Laboratories (Bar Harbour, ME) and were fed either a control Western-type diet or a 2% (wt/wt) phytosterol-enriched Western-type diet.

Induction of turpentine-induced acute inflammation and determination of an inflammatory marker

$ApoE^{-/-}$ and C57BL/6J mice were given the control or the phytosterol-supplemented Western-type diet for 4 weeks. Animals were then injected with 100 μL of turpentine oil (Fluka) or 100 μL of saline subcutaneously and euthanized 48 h later by cervical dislocation under anesthesia (Boelen et al., 1996; Kalra et al., 2004; Tous et al., 2005). Blood was then obtained by cardiac puncture. Serum amyloid A (SAA) concentration was measured with a commercially-available enzyme-linked immunosorbent assay (ELISA) kit (BioSource, Camarillo, USA).

Preparation of lymphocyte samples

Spleens were excised and lymphocyte suspension was obtained by passage through stainless steel sieves in RPMI 1640 medium (Innogenetics, Gent, Belgium) supplemented with 10% fetal bovine serum (FBS) (Innogenetics) (Perez-Bosque et al., 2004). Lymphocyte suspension was then centrifuged (355 *g*, 5 min) and the lymphocyte pellet was resuspended in 6 mL RPMI-FBS. This lymphocyte suspension was layered on a 3-mL Ficoll solution (Sigma) and centrifuged at 607 *g* for 30 min. The lymphocyte band at the interface was recovered and resuspended in 4 mL of phosphate-buffered saline (PBS at pH 7.2) containing 10% (v/v) FBS before counting and viability determination. Cell counting and viability were measured by acridine orange and ethidium bromide staining; cell viability was over 75% in all cases.

Immunofluorescence staining and lymphocyte subset characterization

Flow cytometry was used after double staining with a panel of anti-mouse lymphocyte antibodies (Abs) to determine the lymphocyte phenotype subset. The following monoclonal Abs (mAbs) were applied in this study: fluorescein isothiocyanate (FITC)-conjugated anti-mouse CD3 hamster mAb (145–2C11, Becton Dickinson), (FITC)-conjugated anti-mouse CD4 rat mAb (H129.19, Becton Dickinson), phycoerythrin (PE)-conjugated anti-mouse CD8 rat mAb (53–6.7, Becton Dickinson) and (PE)-conjugated anti-mouse NKRP1 rat mAb (PK136, Becton Dickinson). Lymphocytes suspended at 2.5×10^5 cells/100 μL of 10% FBS were labeled by incubating with mAbs 20 min at 4 °C. Stained cells were rinsed twice with PBS containing 10% FBS, centrifuged 5 min at 355 *g*, fixed with 10 g/L paraformaldehyde and counted by flow cytometry (Epics Elite, Coulter). Before the experiments, appropriate dilutions of Abs were established to use saturating concentrations of immunoreagents. A negative control staining was included in each analysis.

Measurement of cytokines

Isolated spleen lymphocytes (2×10^6 cells/mL) from $apoE^{-/-}$ mice fed the control or the phytosterol-enriched diet for 4 weeks and $apoE^{-/-}$ mice fed the same diets and treated with turpentine were cultured in 10% FBS/RPMI 1640 medium for 48 h in the absence or presence of 25 mg/L of the mitogen concanavalin A (ConA). ConA-induced Th1 (IL-2, IFN-γ) and Th2 (and IL-4 and IL-10) cytokine secretion was then measured by ELISA in culture supernatants (eBioscience, San Diego, CA, USA). The same procedure was followed with spleen lymphocytes from C57BL/6J mice. The relative (percentage) Th1/Th2 cytokine

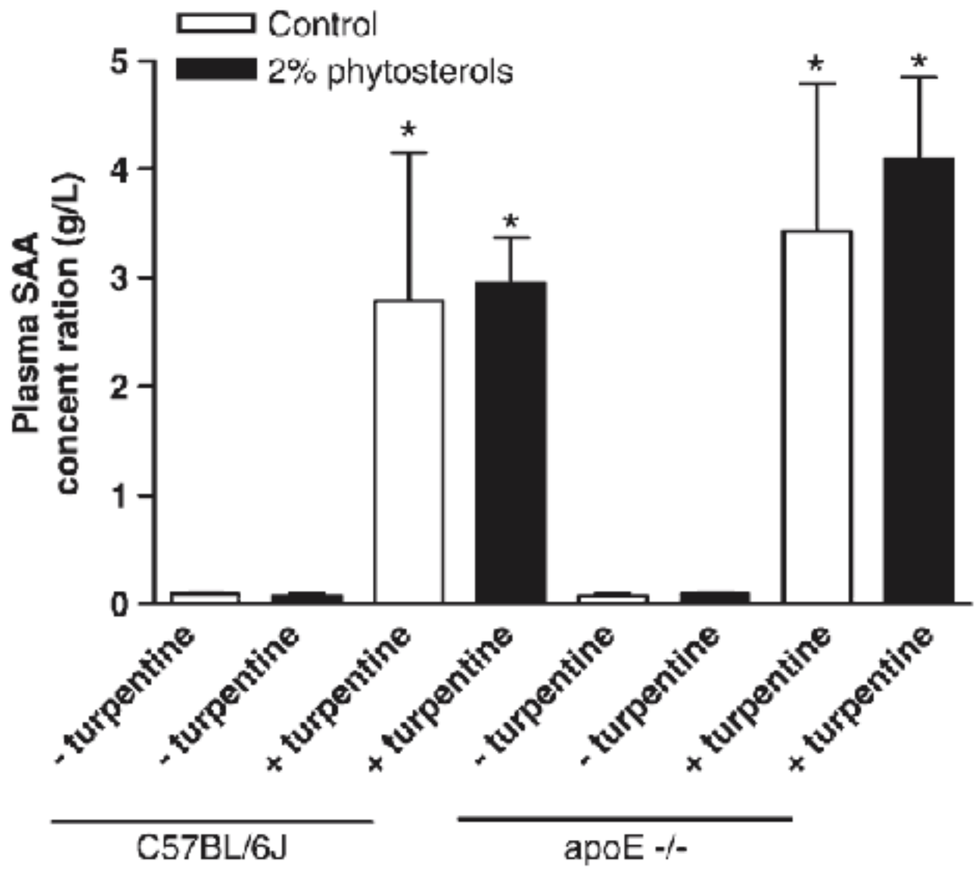

Fig. 1. Mean plasma serum amyloid A (SAA) concentrations from C57BL/6J and apoE$^{-/-}$ mice fed a control or a 2% phytosterol-enriched diet for 4 weeks after 48 h of saline or turpentine treatment. Two independent experiments were performed (3 control and 4 phytosterol-fed animals in each experiment). Results were similar in both experiments and were therefore pooled for analysis (n=6–8). Results are expressed as mean values and SEM of individual animals. *P<0.05 effect of turpentine-mediated inflammation by two-way ANOVA with Bonferroni *post hoc* tests.

ratio was calculated in both types of mice. Taking into account that every cytokine analyzed in the study had a different degree of expression, normalization was done to minimize this fact when calculating the Th1/Th2 ratio. In each animal, each one of the cytokines was normalized and given the same specific weight calculating their expression in relation to a value of 100 arbitrary units (the group mean of each cytokine was set to a rate of 100). The Th1/Th2 ratio was then calculated from these normalized values as the ratio between IL-2+IFN-γ and IL-4+IL-10 in each animal group (6 to 8 values in each). Thus, values of phytosterol-treated animals represent the amount relative to the amount of control mice.

Statistical analysis

Results are expressed as mean values and SEM. Two-way ANOVA with Bonferroni *post hoc* tests was performed using GraphPad Prism version 4.0 for Windows (GraphPad Software, San Diego, CA, USA). Comparison of data for two groups was performed by the U Mann–Whitney test. A value of P<0.05 was considered statistically significant.

Results

Food intake, body weight and plasma SAA inflammatory response

There was no difference between control and phytosterol-treated animals in terms of mobility, behaviour, food intake and weight throughout the study (data not shown). As previously described (Calpe-Berdiel et al., 2005), phytosterols reduced plasma cholesterol concentration in phytosterol-treated apoE$^{-/-}$ mice (11.5±0.04 mM versus 23.0±0.8 in mM control mice), but not in C57BL/6 mice (2.8±0.2 mM versus 3.2±0.3 mM in control mice). Subcutaneous injection of turpentine-induced a similar acute phase response, as measured by plasma SAA concentrations, in both apoE$^{-/-}$ mice and C57BL/6J mice (Fig. 1). No differences in SAA concentration were observed either depending on the supplement of dietary phytosterol or mouse type. SAA concentrations after turpentine injection were similar to those found in models of turpentine-induced chronic inflammation (Tous et al., 2005).

Effects of turpentine and diets on spleen lymphocyte populations

We determined spleen lymphocyte populations in apoE$^{-/-}$ mice fed a control or a 2% phytosterol-enriched diet after 48 h of an injection of saline and, also, in apoE$^{-/-}$ mice fed the same diets after 48 h of a single injection of turpentine to induce acute, aseptic inflammation (Table 1). No differences were found in the subsets evaluated with the exception of a significant rise in NK cells caused by inflammation. The proportion of CD4$^+$/CD8$^+$ T-cells decreased in animals treated with turpentine in comparison to healthy littermates, whereas this ratio remained constant in the two diets (Table 1).

Cytokine secretion of spleen lymphocytes in vitro

We did not detect the selected four cytokines in plasma and their concentration was also below the detection limit when spleen

Table 1
Lymphocyte populations in spleen from apoE$^{-/-}$ mice fed a Western diet or a 2% phytosterol-enriched Western-type diet for 4 weeks and treated or not with turpentine

		CD3$^+$		CD4$^+$		CD8$^+$		NK cells	
		Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Healthy	Control	34.4	4.6	25.6	2.8	7.9	1.8	1.2	0.04
	2% phytosterols	31.4	1.6	23.3	2.0	7.9	1.5	1.6	0.1
Acute inflammation	Control	38.4	11.7	23.5	5.4	14.0	4.4	5.1*	0.9
	2% phytosterols	28.8	4.7	18.9	3.9	9.8	1.5	3.4*	0.4

CD3$^+$ (as T-lymphocytes), CD4$^+$ (as helper lymphocytes), CD8$^+$ (as suppressor/cytotoxic T-lymphocytes) and NK (as natural killer cells) as percentages with respect to the total number of lymphocytes. One experiment was performed in 6 control and 6 phytosterol-fed animals in each experimental situation. Results are expressed as means±SEM. *P<0.05, effect of inflammation by two-way ANOVA. The CD4/CD8 T-cell proportion decreased due to inflammation with turpentine (3.7±0.6 and 3.1±0.4 in control and phytosterol-treated healthy animals vs 1.8±0.2 and 1.7±0.2 in control and phytosterol-treated inflamed-mice) by Two-way ANOVA.

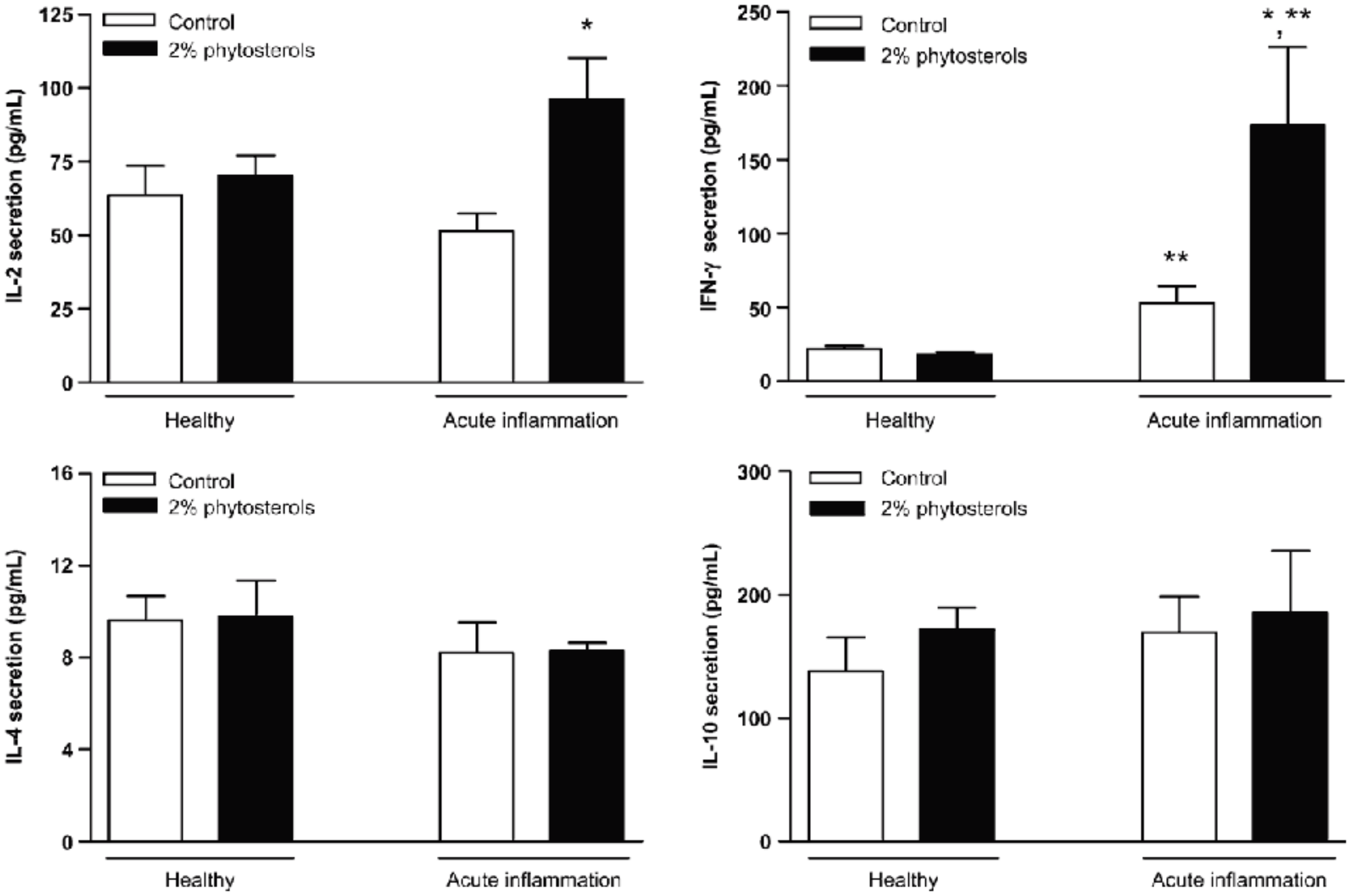

Fig. 2. Cytokine production by spleen lymphocytes from control and turpentine-treated apoE$^{-/-}$ mice fed with or without phytosterols. Spleen lymphocytes isolated from mice, fed with or without a 2% phytosterol-enriched diet (2×10^6 cells/mL), were cultured for 48 h in presence of 25 mg/L of ConA. IL-2, IFN-γ, IL-4 and IL-10 concentrations were determined in the supernatants of the incubation media by ELISA. Values represent mean±SEM, n=6–8 (same animals than Fig. 1). Statistical differences were analyzed by two-way ANOVA with Bonferroni *post hoc* tests. *$P<0.05$, effect of phytosterol-treatment. **$P<0.01$, significant effect on IFN-γ secretion due to acute inflammation revealed by two-way ANOVA.

lymphocytes were not stimulated (data not shown). In contrast, cultured isolated spleen lymphocytes activated with ConA for 48 h showed enhanced cytokine production. Turpentine-induced inflammation was associated with a significant increase in IFN-γ levels (Fig. 2). Dietary phytosterols induced increased Th1 cytokine (IL-2 and IFN-γ) secretion by cultured spleen lymphocytes of apoE$^{-/-}$ mice subjected to turpentine-induced inflammation (1.9 and 3.3-fold, respectively). In contrast, the Th2 cytokines quantified (IL-4 and IL-10) did not significantly change regardless of the presence or absence of inflammation and diet (Fig. 2). To ascertain whether these findings in cytokine levels

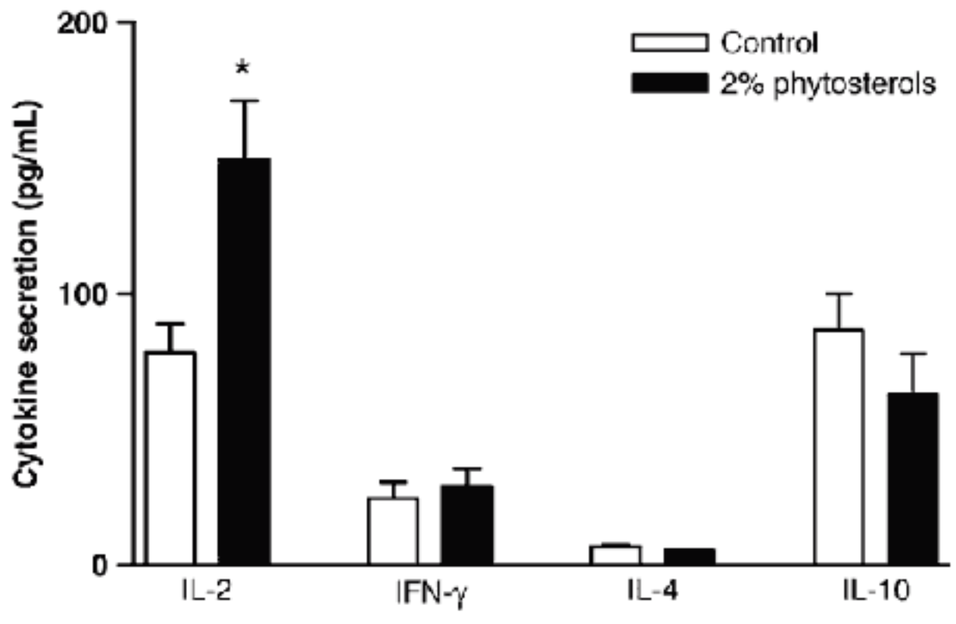

Fig. 3. Cytokine production by spleen lymphocytes from C57BL/6J turpentine-treated mice fed with or without a 2% phytosterol-enriched diet. 2×10^6 cells/mL were cultured for 48 h in presence of 25 mg/L of ConA, and IL-2, IFN-γ, IL-4 and IL-10 concentrations were determined in the supernatants by ELISA. Two independent experiments were performed (3 control and 4 phytosterol-fed animals in each experiment). Results were similar in both experiments and were therefore pooled for analysis (n=6–8). Values represent the mean±SEM. *$P<0.05$ compared to the animals fed the control diet by Mann–Whitney test.

Table 2
Effect of phytosterols on the Th1/Th2 shift of spleen lymphocytes in apoE$^{-/-}$ and C57BL/6J mice with turpentine-induced acute inflammation after 4 weeks of treatment

		Th1/Th2 ratio	
		Mean	SEM
ApoE$^{-/-}$ mice	Control	1.0	0.2
	2% phytosterols	2.9*	0.9
C57BL/6J mice	Control	1.0	0.3
	2% phytosterols	2.1*	0.3

Results are expressed as mean values and SEM of individual animals (data obtained from mice described in Figs. 1 and 2). The Th1/Th2 ratio was calculated, after normalizing each cytokine concentration of control animals to a value of 100 arbitrary units, as the quotient between IL-2+IFN-γ and IL-4+IL-10 averaged for each animal (as described in Materials and methods Section). Values of test animals represent the amount relative to the amount of control mice. *$P<0.05$ compared to the same animals fed the control diet by the U Mann–Whitney test.

were related to the hypocholesterolemic action of phytosterols or absence of apoE, studies in control C57BL/6J mice were performed. In this case, IL-2 secreted levels were nearly 2-fold increased in animals fed the phytosterol-enriched diet whereas IFN-γ did not increase significantly (Fig. 3). The Th1/Th2 ratio was increased by 2.9-fold in turpentine-treated apoE$^{-/-}$ mice and by 2.1-fold in turpentine-treated C57BL/6J mice fed with phytosterols compared with those fed without phytosterol supplementation and treated with turpentine (Table 2).

Discussion

In health, a delicate balance exists between Th1 and Th2 helper cell activity. Th1 response, which is more related to innate immunity, is associated with the clearance of host pathogens using the intracellular milieu to survive. Th2 response is related to B-lymphocyte activation and antibody production. Imbalance of this regulation may favour chronic conditions such as autoimmune disorders, allergies and atherosclerosis (Barnes, 2001; Kero et al., 2001; Romagnani, 1994). To ascertain whether phytosterols influence immune regulation in a highly controlled environment, we used a turpentine-induced sterile model of acute inflammation. The main findings of this experimental study support the concept that phytosterol intake modulates immune equilibrium towards a Th1 response, a concept that was proposed by earlier studies in different types of patients (Bouic, 2001; Bouic et al., 1996; Breytenbach et al., 2001).

However, mice presenting apoE deficiency and fed a phytosterol-enriched diet showed a pronounced release of IFN-γ in response to turpentine compared with phytosterol-fed turpentine-treated C57BL/6J mice (Figs. 2 and 3). ApoE absence increases Th1 response (Ali et al., 2005), whereas lower plasma cholesterol (as observed in apoE$^{-/-}$ mice fed with phytosterols) reverses the Th2 bias induced by hypercholesterolemia (Zhou et al., 1998). Therefore, a combination of the two characteristics – apoE absence and decreased cholesterol mediated by phytosterols – could have produced a stronger, more consistent Th1 shift in apoE$^{-/-}$ mice than in C57BL/6J mice that do have apoE and in which plasma cholesterol does not change with phytosterols.

Another study found elevated production of anti-inflammatory IL-10 cytokine along with reduced proinflammatory cytokine production by LPS-treated spleen cells of phytosterol-fed apoE$^{-/-}$ mice immunized with ovoalbumin (Nashed et al., 2005). From this study one cannot deduce the Th1/Th2 shift in immune response (Nashed et al., 2005) that we have found, whereas the present study did not find evidence of phytosterol-induced anti-inflammatory actions. It is, however, noteworthy that the latter apoE$^{-/-}$ mice were fed a different mixture of phytosterols and were immunized with ovoalbumin (Nashed et al., 2005) whereas ours were subjected to a single turpentine injection. Further, a different inflammatory status could also affect the immune modulatory effect of phytosterols. For instance, we did not find Th1/Th2 changes in mesenteric lymph node lymphocytes in apoE$^{-/-}$ mice fed with phytosterols and treated with dextran sulphate sodium to induce colitis (data not shown) or in apoE$^{-/-}$ mice fed with phytosterols but not subjected to turpentine treatment (Fig. 2).

The substantial changes in T-cell cytokines found in our study could be due to the alteration in spleen subset cell proportion. Cytometric analyses of our study did not reveal differences in spleen lymphocyte subset distribution due to the treatment. However, since absolute cell numbers were not obtained in these experiments, it cannot be ruled out that phytosterols influenced cell number without affecting their relative number.

Phytosterols incorporate into membranes influencing cellular membrane composition and fluidity (Awad et al., 2004) and this may affect eicosanoid, leukotrienes and prostaglandin biosynthesis which are key modulators of signalling pathways involved in immune response (Broughton and Wade, 2002; Moussa et al., 2000; Plat and Mensink, 2005). Phytosterols or their derivatives may also act as LXR ligands (Kaneko et al., 2003; Yang et al., 2004). It is, thus, noteworthy that LXR-dependent gene expression has been shown to play a role in the innate immune response (Joseph et al., 2004) in which the Th1 response is involved. Clearly, the mechanism/s by which the phytosterol intervention leads to altered cytokine production requires more investigation. Also, it will be of special interest to study the effect of dietary phytosterols in inflammatory diseases characterized by a Th2-dominant response such as asthma.

In conclusion, the results of the present study reinforce the evidence for a role of phytosterols in immune modulation. This immunomodulatory action may depend on the existence and type of inflammatory process and may be, in part, independent of apoE and plasma cholesterol level.

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