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Research Article

# Modulation of cytokine production by plant sterols in stimulated human Jurkat T cells

**S. Aisling Aherne and Nora M. O'Brien**

Department of Food and Nutritional Sciences, University College Cork, Cork, Republic of Ireland

The plant sterols campesterol, β-sitosterol and β-sitostanol were investigated for potential immunomodulatory effects in Jurkat T cells. Treatments involved supplementing cells with or without concanavalin A (ConA) or phorbol-12-myristate-13-acetate plus ionomycin (PMA+IoM) in the presence or absence of increasing concentrations (10 – 100 μM) of each plant sterol for 24 h. None of the plant sterols significantly affected mitogen-stimulated IL-4, IL-10 or IFN-γ production. However, campesterol, β-sitosterol and β-sitostanol significantly suppressed mitogen-induced IL-2 production in a dose-dependent manner. Both bisindolylmaleimide-I (BIM-I), a specific protein kinase C (PKC) inhibitor, and the immunosuppressant drug known as Tacrolimus (FK506), an IL-2 inhibitor, prevented mitogen-stimulated IL-2 production in Jurkat cells. Treatment with PMA+IoM alone significantly increased PKC activity and the presence of BIM-I prevented PKC activation by PMA+IoM. Following 24 h treatments, the plant sterols did not affect PMA+IoM-enhanced PKC activity, cellular calcium content or calcineurin activity. Intracellular cyclic 3′,5′-adenosine monophosphate (cAMP) levels were significantly reduced by PMA+IoM. The presence of FK506 prevented a PMA+IoM-induced reduction of intracellular cAMP. Likewise the plant sterols behaved in a similar manner as FK506. Our findings suggest that the suppression of IL-2 by the plant sterols was not mediated *via* PKC inhibition and that their effects occurred possibly *via* cAMP modulation and/or a calcium/calcineurin-independent pathway.

**Keywords:** cAMP / Cytokine production / Jurkat cells / Phytosterol / Protein kinase C

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## 1 Introduction

Plant sterols are specific phytochemicals that resemble cholesterol in structure and are found in all plants and in products containing plant-based raw materials [1, 2]. In the human diet, the three most common plant sterols are β-sitosterol, campesterol and stigmasterol [3 – 5] whereas the two most common saturated plant sterols (plant stanols) are β-sitostanol and campestanol. Rich natural food sources include nuts, seeds and legumes [6, 7] and to a lesser extent fruits, cereals and cereal products [7]. Plant sterols have been repeatedly investigated for their cholesterol-lowering effects and their anticancer properties however only limited research has been carried out on other potential bioactive properties such as immune modulation.

The few studies that exist in the literature suggest that plant sterols exhibit immunomodulatory properties both *in vitro* and *in vivo* [8, 9]. The reporting of dietary plant sterols as immunomodulatory compounds started from the initial observations that a plant sterol/sterolin mixture enhanced the cellular responsiveness of T lymphocytes [2, 10]. Since then, Bouic and coworkers have investigated the use of this plant sterol/sterolin mixture on the management of pulmonary tuberculosis in patients [11], the inhibition of immune stress in marathon runners [12] and the management of HIV-infected patients [13].

Cytokines are not only participants in inflammation, immunity and the host response to infectious agents but are also involved in the regulation of many biological systems, including the immune system [14]. They are low-molecular-mass glycoproteins produced by immune cells and many other cell types in response to various activation stim-

**Correspondence:** Dr. S. Aisling Aherne, Department of Food and Nutritional Sciences, University College Cork, Cork, Republic of Ireland
**E-mail:** a.aherne@ucc.ie
**Fax:** +353-21-4270244

**Abbreviations: BIM-I,** bisindolylmaleimide-I; **cAMP,** cyclic 3′,5′-adenosine monophosphate; **ConA,** concanavalin A; **FK506,** immunosuppressant drug also known as Tacrolimus; **IoM,** ionomycin; **IP3,** inositol-1,4,5-triphosphate; **PKC,** protein kinase C; **PMA,** phorbol-12-myristate-13-acetate; **TCR,** T-cell receptor

uli [15, 16]. Jurkat cells have long served as a human T cell model for different immunological studies and the use of mitogens such as concanavalin A (ConA) and phorbol-12-myristate-13-acetate plus ionomycin (PMA+IoM) have been employed to induce cytokine production in this cell model [17–22]. PMA+IoM has been reported to activate IL-2 gene expression by a mechanism different from that of ConA [23]. ConA works *via* the T-cell receptor (TCR)–CD3 complex resulting in subsequent (indirect) activation of both calcineurin and protein kinase C (PKC) in Jurkat cells [21]. In contrast, PMA+IoM act synergistically to induce IL-2 production [24]. PMA is an agonist that mimics diacylglycerol (DAG) and thus can activate T cells directly *via* the PKC pathway [21, 25]. IoM is a calcium ionophore which can activate calcineurin directly [21, 23–25].

To date, there is no information in the literature regarding the effects of individual plant sterols on cytokine production in human Jurkat T cells. Therefore, in this study we investigated if the plant sterols campesterol, β-sitosterol or β-sitostanol, at concentrations of 10, 50 and 100 μM could affect cytokine production in stimulated Jurkat T cells. We also analysed the effects of two inhibitors, namely bisindolylmaleimide-I (BIM-I) and the immunosuppressant drug known as Tacrolimus (FK506). BIM-I is a potent and selective inhibitor of PKC which, in turn, prevents IL-2 production in T cells [26]. The immunosuppressant drug FK506 directly inhibits calcineurin activity thus preventing both T-cell signal transduction and IL-2 transcription [27, 28]. To elucidate our findings we determined cellular calcium content, calcineurin activity, cyclic 3′,5′-adenosine monophosphate (cAMP) levels and PKC activity.

## 2 Materials and methods

### 2.1 Materials

All chemicals and cell culture reagents including β-sitosterol (≥97% purity), β-sitostanol (≥95% purity), PMA, IoM and ConA were purchased from Sigma Chemical (Dublin, Ireland) unless otherwise stated. Campesterol (70% purity) was purchased from Steraloids (London, UK). Bisindolylmaleimide I (also known as GF 109203X) was purchased from Calbiochem (Merck Chemicals, Nottingham, UK). Foetal bovine serum was purchased from Invitrogen (Paisley, Scotland).

### 2.2 Cell culture

Human Jurkat E6.1 T cells were purchased from the European Collection of Animal Cell Cultures (Salisbury, UK). Cells were cultured in an atmosphere of $CO_2$/air (5:95 v/v) at 37°C and were maintained in the absence of antibiotics. Jurkat cells were grown in RPMI-1640 medium supplemented with 10% v/v foetal bovine serum (FBS). According to the literature and/or supplier's instructions, the plant sterols and FK506 were dissolved in EtOH. PMA, IoM and BIM-I were dissolved in DMSO whereas ConA was dissolved in PBS. Control cultures were exposed to the equivalent concentration of carrier (DMSO 0.5%, PBS 0.5% and/or EtOH 0.1%) and were found not to significantly differ from cells grown in media with or without other appropriate carriers. Consequently, control data presented throughout this paper will refer to carrier control cultures, unless stated otherwise. Each experiment was repeated four times and the number of replicates for each condition was 3–4 in every experiment.

### 2.3 Evaluation of cell viability and growth

Jurkat cells ($2 \times 10^5$/mL; $4 \times 10^4$/well) were seeded in 96-well plates with PMA+IoM (10 ng/mL PMA plus 350 ng/mL IoM) or ConA (25 μg/mL) in the presence or absence of campesterol, β-sitosterol or β-sitostanol (10–100 μM) for 24 h. Cell viability and growth were assessed using the MTT assay (Cell Proliferation Kit I MTT, Roche Diagnostics, West Sussex, UK). The MTT assay is designed and repeatedly used for the quantification of cell viability and proliferation. This assay is based on the cleavage of the yellow tetrazolium salt MTT to purple formazan crystals by metabolically active cells. The formazan crystals formed are solubilised and the resulting coloured solution is quantified using a microplate reader at an absorbance of 550 nm with a reference wavelength of 690 nm. Cell viability was expressed as a percentage of carrier control cultures. Cell proliferation was expressed as MTT-reduction index, *i.e.* the amount of dye produced is proportional to the number of metabolically active cells present.

### 2.4 Determination of cytokine production

Initially, a time- and concentration-profile on the cytokine response of human Jurkat T cells to ConA and PMA+IoM were determined over a 72 h time period (data not shown). From the data obtained, one dose of each treatment was selected for subsequent assays.

Jurkat cells ($2 \times 10^5$/mL) were seeded in 96-well plates in the presence or absence of PMA+IoM (10 ng/mL PMA plus 350 ng/mL IoM) or ConA (25 μg/mL) and treated with or without campesterol, β-sitosterol and β-sitostanol (10–100 μM) for 24 h. The release of the cytokines IL-2, IL-4, IL-10 and IFN-γ was determined by ELISA kits from eBioscience (Insight Biotechnology, Wembley, UK). The eBioscience Human Th1/Th2 ELISA Ready-SET-Go kit contains four pairs of antibodies that can be used to discriminate Th1/Th2 subsets based on their expression of IFN-γ, IL-2, IL-4 and IL-10. Absorbance was read at 450 nm with a reference wavelength of 570 nm. Jurkat cells ($2 \times 10^5$/mL) were seeded in 96-well plates in the presence or absence of PMA+IoM (10 ng/mL PMA plus 350 ng/mL IoM) or ConA (25 μg/mL) and treated with or without

 

FK506 or BIM-I (0.1 – 10 μM) for 24 h. IL-2 production was determined by ELISA (as stated previously). The concentration range selected for the inhibitors (0.1 – 10 μM) was based on doses that have been reported in the literature [21, 29 – 32].

### 2.5 Measurements of cellular calcium content and calcineurin activity

Jurkat cells ($2 \times 10^5$/mL) were seeded into 6-well plates in the presence or absence of PMA+IoM (10 ng/mL PMA plus 350 ng/mL IoM) with or without 10 – 100 μM campesterol, β-sitosterol, β-sitostanol or 1 μM FK506 for 24 h. Calcium content was determined by the Quantichrom Calcium Assay kit (BioAssay Systems, Cambridge, UK). A phenolsulphonephthalein dye in the kit forms a very stable blue coloured complex specifically with free calcium. The intensity of the colour measured at 612 nm is directly proportional to the calcium concentration in the sample. Calcium content is expressed relative to the protein content, as determined by the bicinchoninic acid (BCA) method [33]. Calcineurin activity was determined by a cellular calcineurin activity kit (Calbiochem, Merck Biosciences, Nottingham, UK) which is a complete colourimetric assay kit for measuring cellular calcineurin activity and involves the use of RII phosphopeptide, the most well-known substrate for calcineurin.

### 2.6 Assessment of PKC activity

Jurkat cells ($2 \times 10^5$/mL) were seeded in 6-well plates in the presence or absence of PMA+IoM (10 ng/mL PMA plus 350 ng/mL IoM) with or without 10 – 100 μM campesterol, β-sitosterol, β-sitostanol or 1 μM BIM-I for 24 h. PKC activity was determined by an ELISA kit (Stressgen, Cambridge Bioscience, Cambridge, UK) which is based on a solid phase ELISA that uses a specific synthetic peptide as a substrate for PKC and a polyclonal antibody that recognises the phosphorylated form of the substrate. The assay is developed with TMB and a colour develops in proportion to PKC phosphotransferase activity. Colour intensity is measured at 450 nm. PKC activity is expressed relative to the protein content, as determined by the BCA method [33].

### 2.7 Measurement of intracellular cAMP levels

Jurkat cells ($2 \times 10^5$/mL) were seeded in 6-well plates in the presence or absence of PMA+IoM (10 ng/mL PMA plus 350 ng/mL IoM) with or without 10 – 100 μM campesterol, β-sitosterol, β-sitostanol, or 1 μM BIM-I, or 1 μM FK506 for 24 h. The levels of intracellular cAMP were determined by a BioVision cAMP Direct Immunoassay kit (Cambridge Bioscience) which provides a direct competitive immunoassay for sensitive and quantitative determination of cAMP levels in biological samples. The kit provides a new acetylation procedure that significantly improves the detection signal. The intensity of absorbance at 450 nm is inversely proportional to the concentration of cAMP.

### 2.8 Statistical analysis

Results are presented as the mean and SEM. Data were analysed, where appropriate, by one-way analysis of variance (ANOVA) followed by the Dunnett's test (Prism 4, GraphPad, CA, USA). The level of statistical significance is taken as $p < 0.05$ unless stated otherwise.

## 3 Results

### 3.1 Cell viability and growth

Jurkat cells were treated with PMA+IoM (10 ng/mL PMA plus 350 ng/mL IoM) or ConA (25 μg/mL) and incubated with increasing concentrations of campesterol, β-sitosterol or β-sitostanol (10 – 100 μM) for 24 h. The plant sterols had no significant effect on the viability of mitogen-treated Jurkat cells (data not shown). Supplementation with campesterol, β-sitosterol or β-sitostanol (10 – 100 μM) significantly reduced cell growth compared with control cultures ($p < 0.01$; Table 1).

### 3.2 Cytokine production

#### 3.2.1 IL-4, IL-10 and IFN-γ

Treatment of Jurkat cells with the plant sterols alone (10 – 100 μM) had no effect on IL-4, IL-10 or IFN-γ release (data not shown). At all concentrations tested, campesterol,

**Table 1.** Growth of Jurkat cells following supplementation with plant sterols[a)]

| | Cell growth (MTT-reduction index)[b)] | |
|---|---|---|
| | ConA | PMA+IoM |
| Campesterol 10 μM | 0.246 ± 0.059* | 0.290 ± 0.018* |
| Campesterol 50 μM | 0.269 ± 0.050* | 0.272 ± 0.018* |
| Campesterol 100 μM | 0.253 ± 0.060* | 0.250 ± 0.022* |
| β-Sitosterol 10 μM | 0.263 ± 0.058* | 0.297 ± 0.027* |
| β-Sitosterol 50 μM | 0.214 ± 0.060* | 0.234 ± 0.021* |
| β-Sitosterol 100 μM | 0.206 ± 0.056* | 0.212 ± 0.018* |
| β-Sitostanol 10 μM | 0.223 ± 0.047* | 0.283 ± 0.024* |
| β-Sitostanol 50 μM | 0.222 ± 0.054* | 0.273 ± 0.018* |
| β-Sitostanol 100 μM | 0.229 ± 0.053* | 0.257 ± 0.018* |

a) Jurkat cells ($2 \times 10^5$/mL) were supplemented with ConA (25 μg/mL) or PMA (10 ng/mL) plus IoM (350 ng/mL) in the presence or absence of campesterol, β-sitosterol or β-sitostanol (10 – 100 μM) for 24 h.

b) Cell proliferation was assessed using the MTT assay. Data are mean and standard error for four independent experiments.

* $p < 0.01$ from control values (0.423 – 0.490): one-way ANOVA followed by Dunnett's test.

 

**Table 2.** Effects of plant sterols on cytokine release from ConA-treated Jurkat cells[a)]

| | Cytokine[b)] (% ConA treated cells) | | |
|---|---|---|---|
| | IL-4 | IL-10 | IFN-γ |
| ConA | 100.0 ± 7.1[c)] | 100.0 ± 10.3[d)] | 100.0 ± 11.1[e)] |
| ConA + campesterol 10 μM | 98.7 ± 2.3 | 100.0 ± 2.5 | 98.6 ± 8.5 |
| ConA + campesterol 50 μM | 112.9 ± 15.1 | 93.5 ± 4.3 | 83.5 ± 7.8 |
| ConA + campesterol 100 μM | 100.9 ± 11.2 | 96.9 ± 4.7 | 86.7 ± 7.7 |
| ConA + β-sitosterol 10 μM | 70.0 ± 19.1 | 107.1 ± 6.0 | 103.2 ± 9.7 |
| ConA + β-sitosterol 50 M | 79.7 ± 19.5 | 90.9 ± 4.5 | 87.3 ± 6.6 |
| ConA + β-sitosterol 100 μM | 82.8 ± 13.8 | 101.6 ± 6.9 | 91.2 ± 8.2 |
| ConA + β-sitostanol 10 μM | 94.3 ± 22.9 | 92.3 ± 3.9 | 90.0 ± 7.7 |
| ConA + β-sitostanol 50 μM | 81.6 ± 17.0 | 97.0 ± 7.7 | 84.1 ± 7.4 |
| ConA + β-sitostanol 100 μM | 118.6 ± 17.7 | 102.9 ± 7.2 | 94.2 ± 7.3 |
| SA[f)] | NS[g)] | NS | NS |

a) Jurkat cells ($2 \times 10^5$/mL) were supplemented with ConA (25 μg/mL) in the absence or presence of campesterol, β-sitosterol or β-sitostanol (10 – 100 μM) for 24 h.
b) Cytokine production is expressed as a percentage relative to cells treated with ConA. Data are mean and standard error for four independent experiments.
c) 100% represents 3.1 pg/mL whereas basal levels in control cultures ranged from 0.5 to 0.6 pg/mL.
d) 100% represents 23.8 pg/mL whereas basal levels in control cultures ranged from 2.4 to 2.6 pg/mL.
e) 100% represents 82.7 pg/mL whereas basal levels in control cultures ranged from 10.2 to 11.5 pg/mL.
f) SA, statistical analysis by one-way ANOVA.
g) NS, not significantly different from ConA-treated cells.

**Table 3.** Effects of phytosterols on cytokine release from PMA+IoM-treated Jurkat cells[a)]

| | Cytokine[b)] (% PMA+IoM-treated cells) | | |
|---|---|---|---|
| | IL-4 | IL-10 | IFN-γ |
| PMA+IoM | 100.0 ± 4.0[c)] | 100.0 ± 6.5[d)] | 100.0 ± 10.8[e)] |
| PMA+IoM + campesterol 10 μM | 105.3 ± 7.3 | 100.6 ± 2.2 | 104.7 ± 1.6 |
| PMA+IoM + campesterol 50 μM | 86.8 ± 5.3 | 111.9 ± 6.3 | 98.3 ± 6.5 |
| PMA+IoM + campesterol 100 μM | 101.8 ± 7.5 | 102.6 ± 2.6 | 96.7 ± 7.9 |
| PMA+IoM + β-sitosterol 10 μM | 120.4 ± 8.3 | 109.5 ± 6.4 | 108.6 ± 2.9 |
| PMA+IoM + β-sitosterol 50 μM | 99.4 ± 15.5 | 100.8 ± 1.0 | 107.2 ± 3.8 |
| PMA+IoM + β-sitosterol 100 μM | 61.3 ± 15.8 | 109.7 ± 4.7 | 100.6 ± 5.7 |
| PMA+IoM + β-sitostanol 10 μM | 67.3 ± 11.3 | 107.2 ± 3.6 | 100.4 ± 3.7 |
| PMA+IoM + β-sitostanol 50 μM | 128.4 ± 13.0 | 101.7 ± 1.3 | 101.0 ± 1.2 |
| PMA+IoM + β-sitostanol 100 μM | 69.4 ± 14.4 | 101.9 ± 1.3 | 94.7 ± 4.8 |
| SA[f)] | NS[g)] | NS | NS |

a) Jurkat cells ($2 \times 10^5$/mL) were supplemented with PMA (10 ng/mL) plus IoM (350 ng/mL) in the absence or presence of campesterol, β-sitosterol or β-sitostanol (10 – 100 μM) for 24 h.
b) Cytokine release expressed as a percentage relative to cells treated with PMA+IoM. Data are mean and standard error for four independent experiments.
c) 100% represents 1.6 pg/mL whereas basal levels in control cultures ranged from 0.6 to 0.8 pg/mL.
d) 100% represents 7.1 pg/mL whereas basal levels in control cultures ranged from 2.1 to 2.7 pg/mL.
e) 100% represents 169.5 pg/mL whereas basal levels in control cultures ranged from 12.3 to 12.9 pg/mL.
f) SA, statistical analysis by one-way ANOVA.
g) NS, not significantly different from PMA+IoM-treated cells.

β-sitosterol and β-sitostanol did not significantly affect ConA- (25 μg/mL) or PMA+IoM- (10 ng/mL PMA plus 350 ng/mL IoM) enhanced production of IL-4, IL-10 or IFN-γ in Jurkat cells (Tables 2 and 3, respectively).

### 3.2.2 IL-2

Campesterol, β-sitosterol and β-sitostanol significantly reduced PMA+IoM-stimulated IL-2 release from the cells in a dose-dependent manner ($p < 0.01$; Fig. 1). Similarly, campesterol, at concentrations of 50 or 100 μM, significantly suppressed ConA-induced IL-2 production ($p < 0.01$; Fig. 2). Only the highest concentrations of β-sitosterol or β-sitostanol (100 μM) significantly inhibited IL-2 activation by ConA ($p < 0.01$; Fig. 2). For suppression of PMA+IoM stimulated IL-2 production, campesterol was most effective (249 pg/mL), followed by β-sitosterol (384 pg/mL) and β-sitostanol (476 pg/mL). Similarly with ConA stimulation campesterol was the most effective plant

 

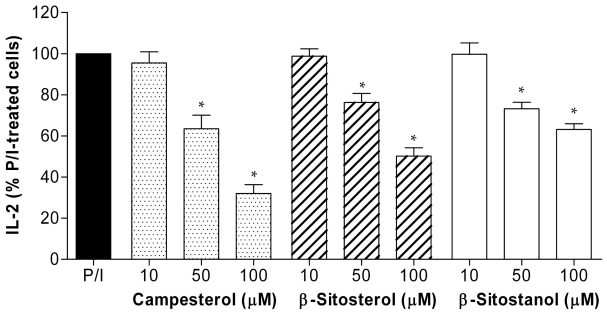


**Figure 1.** IL-2 production. Jurkat cells ($2 \times 10^5$/mL) were supplemented with PMA (10 ng/mL) plus IoM (350 ng/mL) (P/I, black column) in the absence or presence of campesterol (dotted columns), β-sitosterol (striped columns) or β-sitostanol (clear columns) (10–100 μM) for 24 h. IL-2 production is expressed as a percentage relative to cells treated with mitogen alone (P/I). 100% represents 649 pg/mL whereas basal levels of IL-2 were 6–7 pg/mL in control cultures. Data are mean and standard error for four independent experiments. *$p$ < 0.01 compared with P/I, one-way ANOVA followed by Dunnett's test.

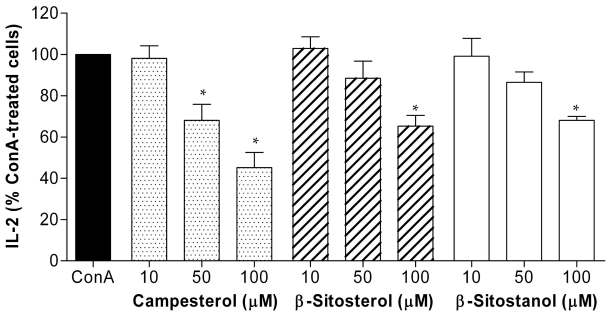


**Figure 2.** IL-2 production. Jurkat cells ($2 \times 10^5$/mL) were supplemented with ConA (25 μg/mL, solid bar) in the absence or presence of campesterol (dotted column), β-sitosterol (striped column) or β-sitostanol (clear column) (10–100 μM) for 24 h. IL-2 production is expressed as a percentage relative to cells treated with mitogen alone (ConA). 100% represents 197 pg/mL whereas basal levels of IL-2 were 6–7 pg/mL in control cultures. Data are mean and standard error for four independent experiments. *$p$ < 0.01 compared with ConA, one-way ANOVA followed by Dunnett's test.

sterol in reducing IL-2 production (91 pg/mL), followed by β-sitosterol (131 pg/mL) and β-sitostanol (147 pg/mL).

IL-2 was activated to a much greater extent in cells treated with PMA+IoM compared with ConA, 649 pg/mL and 197 pg/mL, respectively. The presence of either FK506 or BIM-I (0.1–10 μM) significantly inhibited IL-2 production in Jurkat cells treated with either PMA+IoM (10 ng/mL PMA plus 350 ng/mL IoM) or ConA (25 μg/mL) ($p$ < 0.01; Table 4). At concentrations of 0.1 and 1 μM, FK506 exerted greater inhibitory effects on IL-2 production than BIM-I. However, 10 μM BIM-I or FK506 showed similar levels of IL-2 suppression (Table 4). PMA+IoM-enhanced IL-2 production was more sensitive to inhibition by FK506 and BIM-I compared with ConA.

**Table 4.** Effects of inhibitors on IL-2 production in mitogen-treated Jurkat cells[a)]

| | IL-2 production[b)] (% control) | |
|---|---|---|
| | FK506 | BIM-I |
| ConA | 100.00 ± 6.81 | 100.00 ± 1.54 |
| ConA + 0.1 μM inhibitor | 14.12 ± 0.61* | 63.01[c)] |
| ConA + 1 μM inhibitor | 13.71 ± 0.70* | 56.73 ± 5.41* |
| ConA + 10 μM inhibitor | 12.59 ± 0.21* | 9.62 ± 1.56* |
| PMA+IoM | 100.00 ± 1.54 | 100.00 ± 6.81 |
| PMA+IoM + 0.1 μM inhibitor | 1.00 ± 0.07* | 33.98[c)] |
| PMA+IoM + 1 μM inhibitor | 1.42 ± 0.15* | 1.23 ± 0.27* |
| PMA+IoM + 10 μM inhibitor | 0.73 ± 0.09* | 0.63 ± 0.28* |

a) Jurkat cells ($2 \times 10^5$/mL) were supplemented with or without ConA (25 μg/mL) or PMA (10 ng/mL) plus IoM (350 ng/mL) in the absence or presence of FK506 (0.1–10 μM) or BIM-I (0.1–10 μM) for 24 h.
b) IL-2 production expressed as a percentage relative to cells treated with mitogen alone. Data are mean and standard error for four independent experiments.
c) $n$1 independent experiment.
* $p$ < 0.01; significantly different from cells treated with appropriate mitogen only, one-way ANOVA followed by Dunnett's test.

### 3.3 Cellular calcium content and calcineurin activity

Calcium content and calcineurin activity were measured in Jurkat cells supplemented with PMA+IoM in the presence or absence of 10–100 μM campesterol, β-sitosterol, β-sitostanol or 1 μM FK506 for 24 h. None of the treatments significantly affected cellular calcium content (Table 5) or calcineurin activity (data not shown) after 24 h.

### 3.4 PKC activity

The presence of PMA+IoM for 24 h significantly increased PKC activity in Jurkat cells compared with control cultures ($p$ < 0.05; Fig. 3). Supplementation with BIM-I (1 μM) significantly inhibited PMA+IoM-enhanced PKC activity ($p$ < 0.05). However treatments with campesterol, β-sitosterol or β-sitostanol (10–100 μM) did not alter PKC activation by PMA+IoM (Fig. 3).

### 3.5 cAMP levels

PMA+IoM significantly lowered cAMP levels in Jurkat cells when compared with control cells (Fig. 4). Campesterol, at concentrations of 50 or 100 μM, significantly increased intracellular cAMP concentrations ($p$ < 0.01) compared with cells treated with mitogen (Fig. 4). The presence of 50 μM β-sitosterol or β-sitostanol significantly enhanced PMA+IoM-modulated cAMP levels in the Jurkat cells ($p$ < 0.01 and < 0.05, respectively). Intracellular cAMP concentrations were significantly enhanced by 1 μM

 

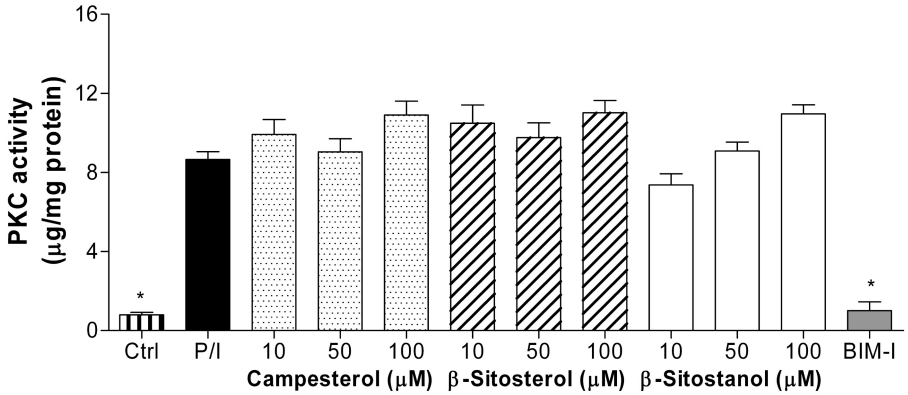


**Figure 3.** PKC activity. Jurkat cells ($2 \times 10^5$ cells/mL) were supplemented with or without PMA (10 ng/mL) plus IoM (350 ng/mL) (P/I) in the absence or presence of 10 – 100 μM campesterol (dotted columns), β-sitosterol (striped columns), β-sitostanol (clear columns) or 1 μM bisindolylmaleimide-I (BIM-I, dark grey column) for 24 h. Data represent mean and standard errors of four independent experiments. *$p < 0.01$: significantly different from P/I, one-way ANOVA, Dunnett's test.

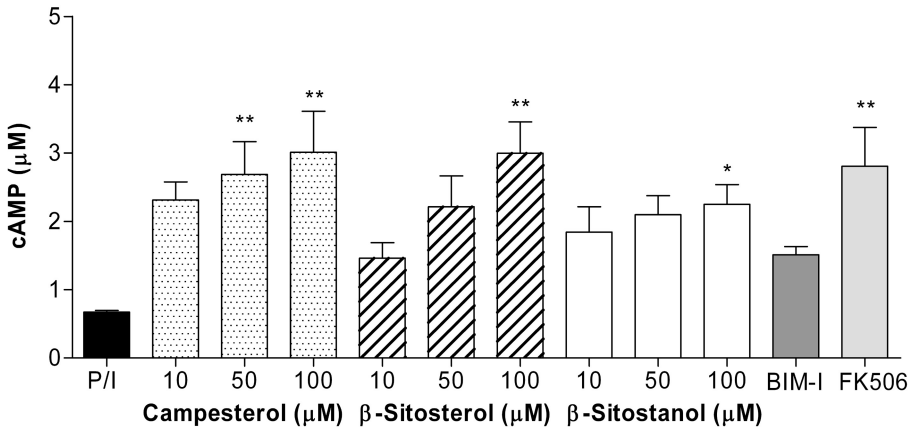


**Figure 4.** Intracellular cAMP concentration. Jurkat cells ($2 \times 10^5$ cells/mL) were supplemented with or without PMA (10 ng/mL) plus IoM (350 ng/mL) (P/I) in the absence or presence of 10 – 100 μM campesterol (dotted columns), β-sitosterol (striped columns), β-sitostanol (clear columns) or 1 μM BIM-I (dark grey column) or 1 μM FK506 (light grey column) for 24 h. Data represent mean and standard errors of four independent experiments. Basal levels of cAMP were 1.7 ± 0.7 μM. *$p < 0.05$, **$p < 0.01$: significantly different from P/I-treated cells, one-way ANOVA, Dunnett's test.

FK506 compared with mitogen-treated cells however supplementation with BIM-I had no significant effect (Fig. 4).

## 4 Discussion

The cholesterol-lowering effect of plant sterols has led to the rapid development of plant sterol-enriched foods and, as a result, increased dietary consumption. However, limited research has been carried out in relation to other potential bioactive properties that plant sterols may possess such as immune modulation. We investigated the effects of three commonly consumed plant sterols on cytokine production in stimulated Jurkat T cells.

Plasma concentrations of plant sterols vary largely between and within different population groups [34]. From a review of 45 studies, campesterol levels in the plasma of normal individuals were shown to range from 6.9 to 27.9 μM whereas baseline plasma β-sitosterol concentrations were shown to range between 2.8 and 16.0 μM [34]. Much lower levels of β-sitostanol have been detected in human plasma with values of 0.14 μM reported [35]. Plasma levels of plant sterols in patients with sitosterolaemia are much higher with typical ranges for β-sitosterol reported between 300 and 1600 μM and for campesterol 200 and 500 μM [36]. Based on recent scientific understandings, there is now concern about the possible unwanted effects of elevated concentrations of plant sterols in serum [35]. Therefore, we included a range of concentrations of each plant sterol to reflect approximate physiological (~10 μM), supplemental (~50 μM) and pharmacological/sitosterolaemia (100 μM) levels.

 

**Table 5.** Calcium content of Jurkat cells treated with mitogens and plant sterols or FK506[a)]

| | Cellular calcium[b)] (μg/mg protein) |
|---|---|
| Control | 64.1 ± 2.9 |
| PMA+IoM | 74.4 ± 3.5 |
| PMA+IoM + campesterol 10 μM | 70.1 ± 2.0 |
| PMA+IoM + campesterol 50 μM | 72.9 ± 1.5 |
| PMA+IoM + campesterol 100 μM | 70.3 ± 1.2 |
| PMA+IoM + β-sitosterol 10 μM | 72.5 ± 2.6 |
| PMA+IoM + β-sitosterol 50 μM | 73.0 ± 4.0 |
| PMA+IoM + β-sitosterol 100 μM | 71.9 ± 2.4 |
| PMA+IoM + β-sitostanol 10 μM | 66.7 ± 2.9 |
| PMA+IoM + β-sitostanol 50 μM | 67.8 ± 1.4 |
| PMA+IoM + β-sitostanol 100 μM | 71.5 ± 1.1 |
| PMA+IoM + FK506 1 μM | 68.6 ± 2.6 |
| SA[c)] | NS[d)] |

a) Jurkat cells ($2 \times 10^5$/mL) were supplemented with PMA (10 ng/mL) plus IoM (350 ng/mL) in the absence or presence of campesterol, β-sitosterol or β-sitostanol (10–100 μM) for 24 h.
b) Data are mean and standard error for four independent experiments.
c) SA, statistical analysis by one-way ANOVA.
d) NS, not significantly different from cells treated with PMA+IoM only.

Campesterol, β-sitosterol or β-sitostanol did not significantly affect the viability of mitogen-treated Jurkat cells whereas their presence significantly reduced cell growth compared with control cultures. This suggests that the cells are not dead but that growth is affected. Similarly, other *in vitro* studies have shown inhibitory effects of β-sitosterol on the growth of various human tumour cell lines including Caco-2 cells [37], HT29 cells [38], HCT116 colon cells [39], LNCaP cells [40] and MDA-MB-231 cells [41, 42]. These antiproliferative effects have been associated with their reported apoptotic and "anticancer" activities such as those which have an effect on membrane structure and function of tumour and host tissue, signal transduction pathways that regulate tumour growth and apoptosis, and immune function of the host [2, 43, 44].

Bouic *et al.* [12] investigated the effects of ingesting a β-sitosterol and β-sitosterol glucoside mixture on IL-6 levels in runners pre- and postmarathon. Postmarathon, the placebo group had higher IL-6 levels than those ingesting BSS:BSSG supplements, however, premarathon runners on the BSS:BSSG diet had much higher levels of IL-6 in their plasma compared to the placebo group. Nashed *et al.* [45] reported that, upon activation with LPS, spleen cells from plant sterol-treated mice had reduced IL-6 and TNF-α levels. In addition, they showed that IL-5, IL-10, IL-13 and IFN-γ production were increased in ovalbumin-supplemented spleen cells isolated from mice fed the plant sterol mix. More recently, Calpe-Berdiel *et al.* [9] fed mice a diet containing a mix of plant sterols for 4 wk followed by treatment with or without turpentine (to simulate acute inflammation). Spleen lymphocytes were isolated and cultured in the presence of ConA for 48 h. Similar to our findings, Calpe-Berdiel *et al.* [9] showed that the plant sterols had no effect on IL-4, IL-10 and IFN-γ production in spleen lymphocytes treated with ConA. In the present study, supplementation with 10 μM campesterol, β-sitosterol or β-sitostanol did not alter mitogen-enhanced IL-2 production in our Jurkat cell model. Plant sterol concentrations of 50 μM and/or 100 μM significantly suppressed IL-2 production stimulated by ConA or PMA+IoM.

Activation of PKC and elevation of calcium/calcineurin pathways are involved in a variety of signalling responses including IL-2 production in T cells [46, 47]. We used two different mitogens, and hence mechanisms, to activate cytokine production in the Jurkat cells. By including ConA and PMA+IoM we were looking at both TCR-dependent and TCR-independent pathways for IL-2 transcription and production [21, 23]. IL-2 suppression by the plant sterols occurred in cells stimulated with PMA+IoM or ConA. This suggests that the plant sterol-induced IL-2 suppressive effects are not dependent on TCR activation. In our study, both FK506 and BIM-I prevented mitogen-stimulated IL-2 production. PMA+IoM significantly increased PKC activity and the addition of BIM-I prohibited PMA+IoM activation of PKC in our cell model. These findings strongly suggest that PKC, and possibly calcium/calcineurin, pathways are involved in ConA- and PMA+IoM-enhanced IL-2 production.

T-cell activation has been divided into early and late events [48]. PKC activation is more prolonged and may mediate, in part, some of the more sustained signalling responses [47]. PKC activity was enhanced by the presence of PMA+IoM in the Jurkat cells; however none of the plant sterols affected mitogen-activated PKC. Similarly, *in vitro* studies on HT-29 cells have reported on the lack of effect of β-sitosterol on phospholipase C, a key enzyme in the PKC pathway, which catalyses the generation of the two second messengers inositol-1,4,5-triphosphate (IP3) and DAG [49]. Moreover, *in vivo* work has demonstrated that β-sitosterol exerts no effect on PKC activity in rat mucosa [50].

FK506 and cyclosporine A (CsA) are potent immunosuppressive drugs widely used in reducing the incidence and severity of allograft rejection after organ transplantation [51]. Not only are the immunosuppressive effects of FK506 and CsA due to the interruption of the calcineurin/NF-AT pathway but apparently the drugs can also work independent of calcium signalling [51, 52]. For instance, FK506 can affect other pathways of T cell activation including the blockade of cytokine receptor expression and cytokine effects on target cells [51, 52]. Despite differences in their structure, both drugs were thought to have identical cellular and molecular effects, *i. e.* the blocking of T cell activation by inhibiting IL-2 production. However, recent research has shown their immunosuppressive mechanisms are not the

 

same [52, 53] and that their molecular effects on T cells are more complex than initially thought. Differences between FK506 and CsA immunosuppressive effects were highlighted by the finding that FK506, not CsA, inhibited (i) IL-2 induced IL-5 production by human T cells and (ii) T cell proliferation stimulated by IL-2 and IL-7 [51]. CsA, while sharing with FK506 the capacity to inhibit cytokine production, had negligible (if any) effect on the cascade alterations within the cytokine network [51]. In the present study, the plant sterols suppressed IL-2 production however no effects were seen on the other cytokines especially IFN-γ. Therefore, it is possible that the plant sterols may have exerted their effects in a similar manner to CsA. Moghadasian [54] studied the interactions between dietary phytosterols and CsA in regard to blood CsA concentration, lipoprotein profiles and histological and morphometrical features of atherosclerotic lesions in ApoE-KO mice. The author suggested that simultaneous consumption of dietary plant sterols and CsA may attenuate post-transplant CsA-related hypercholesterolemia. Moghadasian [54] concluded that additional studies are required to understand the mechanisms by which plant sterols reduce CsA-induced hypercholesterolemia. In our study, FK506 and the plant sterols were not added to the Jurkat cells simultaneously.

After 24 h, cellular calcium content and calcineurin activity was not altered by PMA+IoM, FK506 or the plant sterols in our cell model. Tanaka *et al.* [21] reported that zinc ions significantly lowered IL-2 production stimulated by ConA and PMA+IoM in Jurkat cells. They suggested that because zinc ions suppressed PMA+IoM-enhanced IL-2, in a way similar to FK506, that the action of the zinc ions should reside at least in the downstream of intracellular $Ca^{2+}$ increase [21]. Therefore there are a few possibilities (i) calcium content and calcineurin activity in the Jurkat cells returned to basal levels seeing as that they are early events in T cell activation [47] or (ii) IL-2 suppression may have occurred *via* a calcium/calcineurin-independent pathway [51 – 53].

The first description of a physiological role of cAMP in 1957 gave rise to a concept of cAMP being a second messenger that mediates the effects of a variety of different biologically active agents [55, 56]. A multitude of studies involving the use of cAMP-elevating agents, such as $PGE_2$ and forskolin, have demonstrated significant inhibition in IL-2 production [57 – 60]. Although IL-2 has been reported to inhibit basal as well as $PGE_2$-, isoproterenol- and forskolin-stimulated cAMP production in human T lymphocytes, this inhibitory effect was observed only if cells were pretreated with IL-2 and the membranes activated with $Ca^{2+}$ and ATP [61]. The addition of PMA+IoM resulted in enhanced IL-2 production and reduced cAMP levels compared with control. Conversely, FK506 prevented IL-2 production and significantly enhanced cAMP levels. Campesterol, β-sitosterol and β-sitostanol behaved in a similar manner as FK506 seeing as that the concentrations which significantly suppressed IL-2 production almost correspond exactly to those that elevated cAMP levels.

The clinical implication of high serum plant sterol concentrations, if any, is still unclear and under active investigation [62]. Experimental evidence, including the present findings, suggests that plant sterols may have additional biological effects. This type of specific interaction with IL-2 might prove useful in the treatment of various inflammatory processes, especially in patients requiring immunosuppressive effects [63]. On the other hand, novel agents that influence cAMP and IL-2 levels, which have been developed primarily for targeting other pathophysiological states (*i. e.* lowering plasma cholesterol levels), should be carefully monitored for possible unwanted immune involvement [63]. It is important to bear in mind that in the present study the plant sterols had no effect on IFN-γ, IL-4 and IL-10 levels. Further research is warranted in this area to elucidate the effects of plant sterols on IL-2 production in various research models (both *in vitro* and *in vivo*) and to provide more information on their possible mechanisms of action.

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## 5 References

[1] Piironen, V., Lampi, A. M., Occurrence and levels of phytosterols in foods, in: Dutta, P. C. (Ed.), *Phytosterols as Functional Food Component and Nutraceuticals*, Marcel Dekker, Inc., New York 2004, pp. 1 – 32.

[2] Bradford, P. G., Awad, A. B., Phytosterols as anticancer compounds, *Mol. Nutr. Food Res.* 2007, *51*, 161 – 170.

[3] Heinemann, T., Axtmann, G., Von Bergmann, K., Comparison of intestinal absorption of cholesterol with different plant sterols in man, *Eur. J. Clin. Invest.* 1993, *23*, 827 – 831.

[4] Moreau, R. A., Whitaker, B. D., Hicks, K. B., Phytosterols, phytostanols, and their conjugates in foods: Structural diversity, quantitative analysis, and health-promoting uses, *Prog. Lipid Res.* 2002, *41*, 457 – 500.

[5] Duchateau, D. G., Janssen, H. G., Louter, A. J., Plant sterol analysis in relation to functional foods, in: Dutta, P. C. (Ed.), Phytosterols as functional food components and nutraceuticals, Marcel Dekker, Inc., New York 2004, pp. 75 – 132.

[6] Ryan, E., Galvin, K., O'Connor, T. P., Maguire, A. R., *et al.*, Fatty acid profile, tocopherol, squalene and phytosterol content of brazil, pecan, pine, pistachio and cashew nuts, *Int. J. Food Sci. Nutr.* 2006, *57*, 219 – 228.

[7] Ryan, E., Galvin, K., O'Connor, T. P., Maguire, A. R., *et al.*, Phytosterol, squalene, tocopherol content and fatty acid profile of selected seeds, grains, and legumes, *Plant Foods Hum. Nutr.* 2007, *62*, 85 – 91.

[8] Bouic, P. J., The role of phytosterols and phytosterolins in immune modulation: A review of the past 10 yrs, *Curr. Opin. Clin. Nutr. Metab. Care* 2001, *4*, 471 – 475.

[9] Calpe-Berdiel, L., Escola-Gil, J. C., Benitez, S., Bancells, C. *et al.*, Dietary phytosterols modulate T-helper immune response but do not induce apparent anti-inflammatory effects in a mouse model of acute, aseptic inflammation, *Life Sci*. 2007, *80*, 1951 – 1956.

[10] Bouic, P. J., Etsebeth, S., Liebenberg, R. W., Albrecht, C. F., *et al.*, beta-Sitosterol and beta-sitosterol glucoside stimulate human peripheral blood lymphocyte proliferation: Implications for their use as an immunomodulatory vitamin combination, *Int. J. Immunopharmacol*. 1996, *18*, 693 – 700.

[11] Donald, P. R., Lamprecht, J. H., Freestone, M., Albrecht, C. F., *et al.*, A randomised placebo-controlled trial of the efficacy of beta-sitosterol and its glucoside as adjuvants in the treatment of pulmonary tuberculosis, *Int. J. Tuberc. Lung Dis*. 1997, *1*, 518 – 522.

[12] Bouic, P. J., Clark, A., Lamprecht, J., Freestone, M., *et al.*, The effects of β-sitosterol (BSS) and β-sitosterol glucoside (BSSG) mixture on selected immune parameters of marathon runners: Inhibition of post marathon immune suppression and inflammation, *Int. J. Sports Med*. 1999, *20*, 258 – 262.

[13] Bouic, P. J., Clark, A., Brittle, W., Lamprecht, J. H., *et al.*, Plant sterol/sterolin supplement use in a cohort of South African HIV-infected patients – effects on immunological and virological surrogate markers, *S. Afr. Med. J.* 2001, *91*, 848 – 850.

[14] Friberg, D., Bryant, J., Shannon, W., Whiteside, T. L., In vitro cytokine production by normal human peripheral blood mononuclear cells as a measure of immunocompetence or the state of activation, *Clin. Diagn. Lab. Immunol.* 1994, *1*, 261 – 268.

[15] Akira, S., Hirano, T., Taga, T., Kishimoto, T., Biology of multifunctional cytokines: IL 6 and related molecules (IL 1 and TNF), *FASEB J.* 1990, *4*, 2860 – 2867.

[16] Balkwill, F. R., Burke, F., The cytokine network, *Immunol. Today* 1989, *10*, 299 – 304.

[17] Luongo, D., Bergamo, P., Rossi, M., Effects of conjugated linoleic acid on growth and cytokine expression in Jurkat T cells, *Immunol. Lett*. 2003, *90*, 195 – 201.

[18] Luongo, D., Severino, L., Bergamo, P., De Luna, R., *et al.*, Interactive effects of fumonisin B1 and alpha-zearalenol on proliferation and cytokine expression in Jurkat T cells, *Toxicol. In Vitro* 2006, *20*, 1403 – 1410.

[19] Verlengia, R., Gorjao, R., Kanunfre, C. C., Bordin, S., *et al.*, Comparative effects of eicosapentaenoic acid and docosahexaenoic acid on proliferation, cytokine production, and pleiotropic gene expression in Jurkat cells, *J. Nutr. Biochem*. 2004, *15*, 657 – 665.

[20] Durrieu, C., Degraeve, P., Carnet-Pantiez, A., Martial, A., Assessment of the immunomodulatory activity of cheese extracts by a complete and easy to handle in vitro screening methodology, *Biotechnol. Lett*. 2005, *27*, 969 – 975.

[21] Tanaka, S., Akaishi, E., Hosaka, K., Okamura, S., *et al.*, Zinc ions suppress mitogen-activated interleukin-2 production in Jurkat cells, *Biochem. Biophys. Res. Commun*. 2005, *335*, 162 – 167.

[22] Durrieu, C., Degravaeve, P., Chappaz, S., Martial-Gros, A., Immunomodulating effects of water-soluble extracts of traditional French Alps cheeses on a human T-lymphocyte cell line, *Int. Dairy J.* 2006, *16*, 1505 – 1514.

[23] Gao, J., Caruso, A., De Francesco, M., Canaris, A. D., *et al.*, Heat-killed Bacillus subtilis inhibits T-cell proliferative response to mitogens and recall antigens, *Int. J. Immunopharmacol*. 1996, *18*, 701 – 706.

[24] Michell, B., $Ca^{2+}$ and protein kinase C: Two synergistic cellular signals, *Trends Biochem. Sci.* 1983, *8*, 263.

[25] Arrington, J. L., Mcmurray, D. N., Switzer, K. C., Fan, Y. Y., *et al.*, Docosahexaenoic acid suppresses function of the CD28 costimulatory membrane receptor in primary murine and Jurkat T cells, *J. Nutr.* 2001, *131*, 1147 – 1153.

[26] Toullec, D., Pianetti, P., Coste, H., Bellevergue, P., *et al.*, The bisindolylmaleimide GF 109203X is a potent and selective inhibitor of protein kinase C, *J. Biol. Chem*. 1991, *266*, 15771 – 15781.

[27] Ho, S., Clipstone, N., Timmermann, L., Northrop, J., *et al.*, The mechanism of action of cyclosporin A and FK506, *Clin. Immunol. Immunopathol.* 1996, *80*, S40 – S45.

[28] Lin, C. S., Boltz, R. C., Siekierka, J. J., Sigal, N. H., FK-506 and cyclosporin A inhibit highly similar signal transduction pathways in human T lymphocytes, *Cell. Immunol.* 1991, *133*, 269 – 284.

[29] Minguillon, J., Morancho, B., Kim, S. J., Lopez-Botet, M. *et al*., Concentrations of cyclosporin A and FK506 that inhibit IL-2 induction in human T cells do not affect TGF-beta1 biosynthesis, whereas higher doses of cyclosporin A trigger apoptosis and release of preformed TGF-beta1, *J. Leukoc. Biol*. 2005, *77*, 748 – 758.

[30] Ryu, S., Huppmann, A. R., Sambangi, N., Takacs, P., *et al.*, Increased leukocyte adhesion to vascular endothelium in preeclampsia is inhibited by antioxidants, *Am. J. Obstet. Gynecol.* 2007, *196*, 400e1 – 400e7.

[31] Nunes, J., Klasen, S., Franco, M. D., Lipcey, C., *et al.*, Signalling through CD28 T-cell activation pathway involves an inositol phospholipid-specific phospholipase C activity, *Biochem. J.* 1993, *293*, 835 – 842.

[32] Puente, L. G., Stone, J. C., Ostergaard, H. L., Evidence for protein kinase C-dependent and -independent activation of mitogen-activated protein kinase in T cells: Potential role of additional diacylglycerol binding proteins, *J. Immunol.* 2000, *165*, 6865 – 6871.

[33] Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., *et al.*, Measurement of protein using bicinchoninic acid, *Anal. Biochem*. 1985, *150*, 76 – 85.

[34] Chan, Y. M., Varady, K. A., Lin, Y., Trautwein, E., *et al*., Plasma concentrations of plant sterols: Physiology and relationship with coronary heart disease, *Nutr. Rev.* 2006, *64*, 385 – 402.

[35] Fransen, H. P., De Jong, N., Wolfs, M., Verhagen, H., *et al*., Customary use of plant sterol and plant stanol enriched margarine is associated with changes in serum plant sterol and stanol concentrations in humans, *J. Nutr.* 2007, *137*, 1301 – 1306.

[36] Kritchevsky, D., Chen, S. C., Phytosterols – health benefits and potential concerns: A review, *Nutr. Res.* 2005, *25*, 413 – 428.

[37] Daly, T., Aherne, S. A., O'Connor, T., O'Brien, N. M., The effects of plant sterols on the viability and proliferation of Caco-2 cells, *Proc. Nutr. Soc*. 2007, *66* (Suppl.), 80A.

[38] Awad, A. B., Chen, Y. C., Fink, C. S., Hennessey, T., beta-Sitosterol inhibits HT-29 human colon cancer cell growth and alters membrane lipids, *Anticancer Res.* 1996, *16*, 2797 – 2804.

 

[39] Choi, Y. H., Kong, K. R., Kim, Y. A., Jung, K. O., *et al.*, Induction of Bax and activation of caspases during beta-sitosterol-mediated apoptosis in human colon cancer cells, *Int. J. Oncol.* 2003, *23*, 1657 – 1662.

[40] von Holtz, R. L., Fink, C. S., Awad, A. B., beta-Sitosterol activates the sphingomyelin cycle and induces apoptosis in LNCaP human prostate cancer cells, *Nutr. Cancer* 1998, *32*, 8 – 12.

[41] Awad, A. B., Downie, A. C., Fink, C. S., Inhibition of growth and stimulation of apoptosis by beta-sitosterol treatment of MDA-MB-231 human breast cancer cells in culture, *Int. J. Mol. Med.* 2000, *5*, 541 – 545.

[42] Awad, A. B., Roy, R., Fink, C. S., Beta-sitosterol, a plant sterol, induces apoptosis and activates key caspases in MDA-MB-231 human breast cancer cells, *Oncol. Rep.* 2003, *10*, 497 – 500.

[43] Awad, A. B., Chinnam, M., Fink, C. S., Bradford, P. G., beta-Sitosterol activates Fas signaling in human breast cancer cells, *Phytomedicine* 2007, *14*, 747 – 754.

[44] Awad, A. B., Fink, C. S., Phytosterols as anticancer dietary components: Evidence and mechanism of action, *J. Nutr.* 2000, *130*, 2127 – 2130.

[45] Nashed, B., Yeganeh, B., Hayglass, K. T., Moghadasian, M. H., Antiatherogenic effects of dietary plant sterols are associated with inhibition of proinflammatory cytokine production in Apo E-KO mice, *J. Nutr.* 2005, *135*, 2438 – 2444.

[46] Clapham, D. E., Calcium signaling, *Cell* 1995, *80*, 259 – 268.

[47] Werlen, G., Jacinto, E., Xia, Y., Karin, M., Calcineurin preferentially synergizes with PKC-theta to activate JNK and IL-2 promoter in T lymphocytes, *EMBO J.* 1998, *17*, 3101 – 3111.

[48] Denys, A., Hichami, A., Khan, N. A., n-3 PUFAs modulate T-cell activation via protein kinase C-alpha and -epsilon and the NF-kappaB signaling pathway, *J. Lipid Res.* 2005, *46*, 752 – 758.

[49] Awad, A. B., von Holtz, R. L., Cone, J. P., Fink, C. S., Chen, Y. C., beta-Sitosterol inhibits growth of HT-29 human colon cancer cells by activating the sphingomyelin cycle, *Anticancer Res.* 1998, *18*, 471 – 473.

[50] Awad, A. B., Hernandez, A. Y., Fink, C. S., Mendel, S. L., Effect of dietary phytosterols on cell proliferation and protein kinase C activity in rat colonic mucosa, *Nutr. Cancer* 1997, *27*, 210 – 215.

[51] Almawi, W. Y., Melemedjian, O. K., Clinical and mechanistic differences between FK506 (tacrolimus) and cyclosporin A, *Nephrol. Dial. Transplant.* 2000, *15*, 1916 – 1918.

[52] Miller, J. L., Ericson, S. G., Cyclosporin A and tacrolimus (FK506) differentially alter T-cell receptor expression in vivo, *Immunopharmacol. Immunotoxicol*. 2007, *29*, 105 – 118.

[53] Hamawy, M. M., Knechtle, S. J., An overview of the actions of cyclosporine and FK506, *Transplant. Rev.* 2003, *17*, 165 – 171.

[54] Moghadasian, M. H., Dietary phytosterols reduce cyclosporine-induced hypercholesterolemia in apolipoprotein E-knockout mice, *Transplantation* 2006, *81*, 207 – 213.

[55] Bopp, T., Becker, C., Klein, M., Klein-Hessling, S., *et al.*, Cyclic adenosine monophosphate is a key component of regulatory T cell-mediated suppression, *J. Exp. Med.* 2007, *204*, 1303 – 1310.

[56] Robison, G. A., Butcher, R. W., Sutherland, E. W., Cyclic AMP, *Ann. Rev. Biochem.* 1968, *37*, 149 – 174.

[57] Paliogianni, F., Kincaid, R. L., Boumpas, D. T., Prostaglandin E2 and other cyclic AMP elevating agents inhibit interleukin 2 gene transcription by counteracting calcineurin-dependent pathways, *J. Exp. Med.* 1993, *178*, 1813 – 1817.

[58] Kaminuma, O., Mori, A., Wada, K., Kikkawa, H., *et al.*, A selective type 4 phosphodiesterase inhibitor, T-440, modulates intracellular cyclic AMP level and interleukin-2 production of Jurkat cells, *Immunopharmacology* 1998, *38*, 247 – 252.

[59] Mary, D., Aussel, C., Ferrua, B., Fehlmann, M., Regulation of interleukin 2 synthesis by cAMP in human T cells, *J. Immunol.* 1987, *139*, 1179 – 1184.

[60] Novogrodsky, A., Patya, M., Rubin, A. L., Stenzel, K. H., Agents that increase cellular cAMP inhibit production of interleukin-2, but not its activity, *Biochem. Biophys. Res. Commun.* 1983, *114*, 93 – 98.

[61] Beckner, S. K., Farrar, W. L., Inhibition of adenylate cyclase by IL 2 in human T lymphocytes is mediated by protein kinase C, *Biochem. Biophys. Res. Commun.* 1987, *145*, 176 – 182.

[62] Patel, M. D., Thompson, P. D., Phytosterols and vascular disease, *Atherosclerosis* 2006, *186*, 12 – 19.

[63] Zidek, Z., Adenosine-cyclic AMP pathways and cytokine expression, *Eur. Cytokine Netw.* 1999, *10*, 319 – 328.