Eur J Nutr (2011) 50:563–573
DOI 10.1007/s00394-010-0163-2

ORIGINAL CONTRIBUTION

Improvement of leucocyte functions in mature and old mice after 15 and 30 weeks of diet supplementation with polyphenol-rich biscuits

Mónica De la Fuente · Sonia Medina · Isabel Baeza · Liliana Jiménez

Received: 31 August 2010 / Accepted: 21 December 2010 / Published online: 8 January 2011

Abstract
Purpose To study the effect of diet supplementation with polyphenols on several functions suffering age-related changes, in peritoneal leucocytes from mature and old mice.
Methods Five groups of female ICR mice were used. Four groups received a supplementation (20% wt/wt) of biscuits with different cereal fractions naturally rich in polyphenols (named CO49, CO50, CO52, CO53), containing different amounts of catechin, p-hydroxybenzoic acid, vanillic acid, p-coumaric acid, sinapic acid, ferulic acid, rutin and oryzanol. The control group received only standard maintenance diet. Peritoneal suspensions were obtained after 15 and 30 weeks of diet supplementation, when the age of the animals was 49 ± 2 (mature mice) and 64 ± 2 weeks (old mice), respectively. The functions analysed were: chemotaxis of macrophages and lymphocytes, phagocytosis of particles by macrophages, intracellular superoxide anion levels, lymphoproliferative response to mitogens (concanavalin A and lipopolysaccharide), interleukin-2 secretion and natural killer (NK) activity, as functions that decrease with age, and adherence of macrophages and lymphocytes and tumour necrosis factor-α secretion as functions with age-related increase.
Results The supplementation, in general, increased the functions that decrease with age and decreased those that increase with age. There were differences in the effects shown by the four kinds of biscuits depending on the function studied and the number of weeks of supplementation.
Conclusion Since the immune system has been proposed as a good marker of health and predictor of longevity, diet supplementation with cereals naturally rich in polyphenols could be an important way for health preservation with age and reaching high longevity.

Keywords Leucocyte functions · Polyphenols · Ageing · Mice

M. De la Fuente (✉) · S. Medina · I. Baeza
Department of Physiology (Animal Physiology II), Faculty of Biological Sciences, Complutense University, c/Jose Antonio Novais no. 2, 28040 Madrid, Spain
e-mail: mondelaf@bio.ucm.es

L. Jiménez
Danone Research, Centre de Recherche Daniel Carasso, RD 128, 91767 Palaiseau Cedex, France

Introduction

The immune system, like other physiological systems, suffers age-related changes, which are denominated immunosenescence, with the most pronounced alterations being found in T lymphocytes, although other immune cells such as natural killer (NK) cells, macrophages and B lymphocytes also suffer important changes in their functions with ageing [1, 2]. The main causes of the ageing process seem to be related to oxygen free radicals that injure different biomolecules because of their high reactivity [3, 4]. Moreover, immune cells are especially involved in free radical generation in order to carry out their function, but these free radicals can produce oxidative damage in the immune cells and in the organism if they surpass their antioxidant defence capacity. Thus, an oxidant-antioxidant balance is needed to maintain a correct immune function and the unbalance with more oxidant than antioxidant defences, i.e., the oxidative stress, as occurs in ageing, is the basis of immunosenescence [2, 5]. In addition, it is also known that the immune cell function is a health biomarker and longevity predictor [2, 6, 7]. Recently, it has been suggested that the immune cells can

be involved in the age-related oxi-inflamm-ageing situation of the organism, increasing, if these cells suffer a premature immunosenescence, the rate of ageing of the individual [2]. Thus, the ingestion of diets rich in antioxidant compounds has been proposed as a strategy to slow down the immunosenescence and consequently the rate of ageing [2, 8]. Although there is a great deal of information on the effects of antioxidant compounds on the immune function [2, 8, 9], there are several controversial results with respect to positive effects of antioxidant supplementations, which are mainly due to the type of antioxidants, the time of supplementation and especially the doses used and the age of the subjects [2, 8–11].

Polyphenols are the most abundant antioxidants in our diet, but there are few studies on the effects of foods naturally rich in phenolic compounds, such as those present in cereals, on the immune functions, and these effects are unknown in the aged subjects. Polyphenols, both flavonoids and phenolic acids, show free radical scavenging activities [12–14], but also have other important biological effects including immunomodulatory, antibacterial, antigenotoxic and anti-inflammatory activities [14–20]. Moreover, dietary polyphenol intake seems to prevent different diseases such as degenerative and cardiovascular diseases as well as their risk factors [20–24]. Although there are several studies on the effect of polyphenol compounds on the immune function, which show positive effects on immune cell activities [25–27], research on the physiological effects of diets providing nutritional doses of polyphenols as components of regular foods is still scarce [28, 29]. In a previous study, we showed an improvement of function and redox parameters in leucocytes from adult mice after receiving a diet supplementation for 5 weeks with polyphenol-rich cereals [30]. Although this supplementation also improved immune functions in adult but prematurely ageing mice [31], the effects on aged animals and with a long period of supplementation have not been studied. Thus, the main aim of the present work was to evaluate whether, in mature and old mice, a long-term exposure to diet supplementation during 15 and 30 weeks with biscuits (made with natural fractions of different cereals in order to increase the diversity and quantity of polyphenols naturally present in them but in physiological doses) affects several immune functions, which have been demonstrated by us and other authors to suffer age-related changes and be possible biomarkers of longevity [1, 2, 6, 32, 33].

Materials and methods

Animals and maintenance

Female ICR (CD-1) mice (*Mus musculus*) (Harlan Ibérica, Barcelona, Spain), 32 ± 2 weeks old were used. The mice were specific pathogen-free, as tested by Harlan according to FELASA recommendations, and did not show any sign of malignancy or other pathological processes. Fifty animals were randomly divided into five groups and maintained for 2 weeks for their adaptation to their new location. After this time, four of these groups received a diet with natural polyphenol-rich biscuits and one received standard diet (control group). The animals were housed in polyurethane boxes (ten mice/box), at a constant temperature (22 ± 2 °C), in sterile conditions inside an aseptic air negative-pressure environmental cabinet (Flufrance, Cachan, France), on a 12/12 h reversed light/dark cycle. All mice were treated according to the guidelines of the European Community Council Directives 86/6091 EEC. The study was approved by the Ethical Committee on Animal Research of the Complutense University of Madrid (Spain).

Experimental groups

The control group received a 100% standard maintenance diet (AO4 diet from Panlab L.S. Barcelona, Spain), which does not contain polyphenols and water ad libitum. The diet was in accordance with the recommendations of the American Institute of Nutrition for laboratory animals. The four treated groups received 80% of control diet plus 20% of biscuits elaborated by Danone Vitapole (France) with cereal fractions naturally rich in polyphenols namely CO49, CO50, CO52, CO53 (composition reference included in Table 1). These naturally polyphenol-rich diets were kept in darkness at 4 °C for less than 3 weeks in order to prevent oxidation. Peritoneal suspensions were obtained to evaluate different immune parameters after 15 and 30 weeks of diet supplementation when the age of the animals was 49 ± 2 (mature mice) and 64 ± 2 weeks (old mice), respectively. Animals were the same in both periods of supplementation (they were supplemented for 15 weeks, and after collection of peritoneal suspensions the supplementation was maintained 15 weeks more).

Collection of peritoneal leucocytes

Peritoneal cellular suspensions were obtained between 8:00 and 10:00 h, without killing of the animals (mice were selected at random). The abdomen was cleansed with 70% ethanol, and 3 ml of sterile Hank′s was injected intraperitoneally. After massaging the abdomen, 80% of the injected volume was recovered. Then, macrophage and lymphocyte functions were evaluated, with the macrophages being identified by their morphology and non-specific esterase staining, adjusted to 5.10^5 macrophages/ml and 1.10^6 lymphocytes/ml. Cellular viability, determined in

Table 1 Nutritional composition of biscuits tested

Biscuit	Cereal fraction composition (in % of total flour)	Energy (Kcal)	Proteins (g/100 g)	Lipids (g/100 g)	Carbohydrates (g/100 g)	Catechins (mg/100 g)	P-OH benzoic acid (mg/100 g)	Vanillic acid (mg/100 g)	P-Coumaric acid (mg/100 g)	Sinapic acid (mg/100 g)	Ferulic acid (mg/100 g)	Rutin (mg/100 g)	Oryzanol (mg/100 g)	Total polyphenols content (eq mg acid gallic/100 g)	Total antioxidant capacity (μmol TE/100 g)
CO49	20% of buckwheat, 10% of rice bran	453	7.7	18.24	64.5	0.60	2.30	3.60	1.70	4.80	8.40	0.47	0.24	1,700	510
CO50	20% or rice brand, 10% of buckwheat	448	7.75	19.32	60.7	0	0	0	3.10	14.10	10.10	0.24	0.48	2,000	550
CO52	20% of rice brand, 10% wheat middling	440	7.9	19.55	58.1	0.60	3.00	1.00	3.20	13.90	16.10	0	0.48	1,900	530
CO53	10% of buckwheat, 10% of rice bran, 10% of cold pressed wheat germ	447	9.0	18.69	60.6	0	0	0.40	1.50	12.60	10.00	0.95	0.24	1,900	510

each experiment using the trypan-blue exclusion test, was in all cases higher than 95%.

Assay of macrophage functions

The different steps of the phagocytic process, namely adherence, chemotaxis, phagocytosis and digestion (through the measure of superoxide anion levels) were evaluated.

Adherence capacity assay was performed following a method previously described [10]. Aliquots of 200 μl from the adjusted peritoneal suspension were placed in Eppendorf tubes and incubated 10 min at 37 °C. Adherence index (AI) was calculated according to the following expression: AI = (1−macrophage/ml supernatant after 10 min of incubation/macrophages/ml initial sample (time 0)) × 100.

Chemotaxis assay was performed according to a modification of the original technique described by Boyden [10], which consists in the use of chambers with two compartments separated by a filter with a pore diameter of 3 μm (Millipore, Bedford, MA). Aliquots of 300 μl of the adjusted peritoneal suspension were deposited in the upper compartment, and FMLP (formyl-met-leu-phe) at 10^{-8} M (Sigma, St. Louis, MO) was placed in the lower compartment as chemoattractant agent. Chambers were incubated 3 h at 37 °C and 5% CO_2 and then the filters were fixed and stained. Chemotaxis index was determined by counting the total number of macrophages or lymphocytes on one-third of the lower face of the filters, corresponding to four scans of 5 mm, using an optical microscope (×100 magnification).

Phagocytosis of inert particles (latex beads 1.09 μm diluted to 1% in PBS; Sigma St Louis, MO) was carried out following the method previously described [10]. Aliquots of 200 μl of the peritoneal suspension were placed in MIF (migratory inhibitory factor) plates for 30 min. The adherence monolayer was washed with PBS at 37 °C and then, this monolayer was resuspended in 200 μl of Hank′s solution and incubated with 20 μl of latex. After 30 min of incubation, the plates were washed with PBS, fixed and stained, and the number of latex beads ingested by 100 macrophages was determined by optical microscopy.

Superoxide anion levels were evaluated following the method described by De la Fuente et al. [10], based on the nitroblue tetrazolium (NBT) reduction test in an equimolecular reaction with superoxide anion. Briefly, aliquots of 250 μl of peritoneal suspension were mixed with 250 μl of NBT solution (1 mg/ml; Sigma St Louis, MO). Aliquots of 50 μl of latex beads were added to the stimulated samples and 50 μl of Hank′s to the non-stimulated samples. After 60 min of incubation at 37 °C, the reaction was stopped and, following centrifugation,

the supernatants were discarded and the reduced NBT was extracted with dioxin (Sigma St Louis, MO). Supernatant absorbances were measured at 525 nm. The data obtained were expressed as nmol of NBT reduced per 10^6 macrophages by extrapolating from a standard curve of NBT reduced with 1,4-dithioerythritol (Sigma St Louis, MO).

Assay of lymphocyte functions

Adherence and c*hemotaxis* assay of lymphocytes was performed as previously described for macrophages [10].

Proliferation of lymphocytes was quantified in total peritoneal cellular suspensions adjusted to a final concentration of 5.10^5 leucocytes/ml in complete medium (RPMI-1640; PAA, Austria, plus 10% foetal calf serum, Life Technologies; plus 1% gentamicin, PAA, Austria), following a method previously described [32]. Aliquots of 200 µl were dispensed in 96-well flat-bottomed plates (Nunc, Roskilde Denmark), and 20 µl of concanavalin A (ConA 1 µg/ml) or lipopolysaccharide (LPS, *E. coli*, 055:B5 1 µg/ml; Sigma, St Louis, MO) was added. After 48 h of incubation, Con A and LPS-stimulated culture supernatants were collected to measure the levels of interleukin-2 (IL-2) and TNF-α, respectively. Then, 0.5 µ Ci of ^{3}H-thymidine were added and after 8 h cells were harvested and the thymidine uptake was measured in a beta counter. The results were expressed as percentage of stimulation, 100% being the cpm thymidine uptake at basal condition.

IL-2 and TNF-α levels in the supernatants of leucocyte cultures, after 48 h of incubation with Con A and LPS, respectively, were measured by ELISA kits (R&D System, Minneapolis, USA) and the results expressed as pg/ml.

Natural killer (NK) activity was evaluated using an enzymatic colorimetric assay for cytolytic measurements of target cells (Cytotox 96 TM Promega, Madison, WI, USA) based on the determination of lactate dehydrogenase (LDH) activity using a tetrazolium salt [32]. Cells YAC-1 from a murine lymphoma were used as targets and peritoneal leucocytes as effector cells (with an effector/target rate 10:1) in the NK assay. After 4 h of incubation, LDH activity was measured in the supernatants by addition of the enzyme substrate at absorbance of 490 nm. To determine the percentage of lysis of target cells, the following equation was used: % lysis = $(E-\mathrm{ES}-\mathrm{TS}/M-\mathrm{ES}-\mathrm{TS}) \times 100$, being E the mean of absorbances in the presence of effector cells; ES the mean of absorbances of effector cells incubated alone; TS the mean of absorbances in target cells incubated with medium alone and M the mean of maximum absorbances after incubating target cells with lysis solution.

Statistical analysis

The data were expressed as the mean ± SD of the values. The normality of the samples was tested by the Kolmogorov–Smirnov test. The data were statistically evaluated by the two-way analysis of variance (ANOVA) and Tukey t test for comparisons of parametric samples. $p < 0.05$ was taken as the minimum significance level.

Results

The results concerning the several functions studied in macrophages (that represent different steps of the phagocytic process) are indicated in Table 2 and Fig. 1. The adherence capacity, the first step of the phagocytic process (Table 2), increases significantly with animal age (as already mentioned above, the mice after 15 weeks of treatment are mature and after 30 weeks are old; $p < 0.05$ comparing cells from mature mice with those of the aged). All biscuit treatments resulted in significantly diminished values of the adherence indices with respect to those of the controls in both periods of supplementation, showing during the last period (30 weeks) the most effective decrease ($p < 0.001$) for all biscuits. Following adherence, the macrophages migrate to the infection focus. After diet supplementation, only the CO50 and CO53 biscuits caused an increase in this immune parameter at 30 weeks ($p < 0.05$ and $p < 0.001$, respectively). Regarding phagocytosis capacity, the phagocytosis indices (Fig. 1) show a decrease in old mice ($p < 0.001$) when compared to mature animals. After 15 weeks of supplementation, the CO49 ($p < 0.001$), CO52 ($p < 0.001$) and CO53 ($p < 0.01$) biscuits significantly increased phagocytosis. After 30 weeks, CO50 significantly decreased the phagocytosis index ($p < 0.05$). As regards to superoxide anion levels, the first free radical that macrophages produce by digestion of the ingested material, it could be observed, in control samples, a diminution of superoxide levels in basal as well as stimulated samples ($p < 0.001$) in old animals with respect to mature mice. After 30 weeks of supplementation with CO52 biscuit, the levels of superoxide anion were increased in stimulated samples ($p < 0.001$).

With respect to the effects of the different treatments on lymphocytes, Fig. 2 shows the results obtained for adherence and chemotaxis. As regards to adherence (Fig. 2a), no significant differences were found at the control level between cells from mature and old animals. All the biscuits were able to decrease lymphocyte adherence indices in both periods of supplementation ($p < 0.001$ in all cases with exception of CO49 and CO53, which showed the lowest ($p < 0.05$) after 30-week treatments). Regarding chemotaxis (Fig. 2b), a decrease in this immune parameter

Table 2 Adherence indices, chemotaxis indices and superoxide anion levels (basal and stimulated levels) of peritoneal macrophages from mice after 15 (mature animals) and 30 (old animals) weeks of the different diet supplementations

	Adherence indices	Chemotaxis indices	Superoxide anion (Basal levels)	Superoxide anion (Stimulated levels)
15 weeks of diet supplementation (mature mice)				
Control	45 ± 8	196 ± 50	43 ± 6	64 ± 9
CO49	33 ± 3^{a}	246 ± 45	34 ± 7	72 ± 13
CO50	34 ± 5^{a}	249 ± 51	34 ± 9	51 ± 11
CO52	33 ± 6^{a}	193 ± 36	35 ± 7	64 ± 14
CO53	34 ± 5^{a}	218 ± 41	40 ± 7	72 ± 10
30 weeks of diet supplementation (old mice)				
Control	57 ± 8^{b}	139 ± 26	21 ± 4^{bbb}	34 ± 6^{bbb}
CO49	39 ± 8^{aaa}	186 ± 26	14 ± 3	45 ± 8
CO50	30 ± 6^{aaa}	209 ± 42^{a}	17 ± 4	31 ± 7
CO52	33 ± 9^{aaa}	110 ± 26	27 ± 8	56 ± 13^{aaa}
CO53	36 ± 8^{aaa}	237 ± 63^{aaa}	25 ± 7	47 ± 7

The results are the mean ± SD of eight values corresponding to the same number of mice, each value being the mean of duplicated assays. ^{a} $p < 0.05$, ^{aaa} $p < 0.001$ compared to the control of each period of supplementation. ^{b} $p < 0.05$, ^{bbb} $p < 0.001$ with respect to the corresponding value in 15-week supplementation group

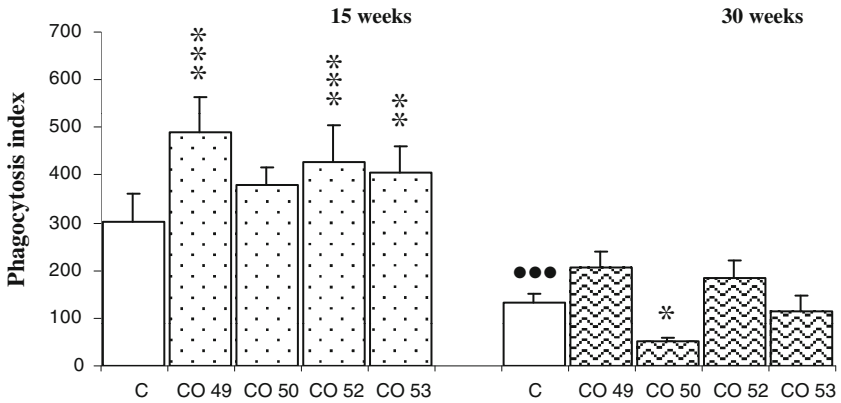

Fig. 1 Phagocytosis indexes (number of latex beads ingested by 100 macrophages) of peritoneal macrophages from mice after 15 (mature animals) and 30 (old animals) weeks of diet supplementation with different polyphenol-rich biscuits. The results are the mean ± SD of eight values corresponding to the same number of animals, each value being the mean of duplicated assays. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ compared to the control of each period of supplementation. •••$p < 0.001$ with respect to the corresponding value in 15-week supplementation group

with age in control samples was observed ($p < 0.05$). The supplementation with CO49, CO50 and CO53 biscuits increased the chemotaxis indices of lymphocytes ($p < 0.05$, 0.001 and 0.01, respectively) after 15 weeks. The same stimulatory effects was observed at 30 weeks, with all biscuits causing an increase in lymphocyte mobility, CO49, CO50 and CO53 showing the highest index values ($p < 0.001$) and CO52 the lowest ($p < 0.05$).

Figure 3 represents proliferation percentages in response to concanavalin A (Con A) and lipopolysaccharide (LPS). A significant decrease ($p < 0.001$) in proliferation at control levels can be observed in cells from old animals with respect to those from mature mice, in response to both Con A and LPS. The biscuits studied are able to increase lymphocyte proliferation in response to Con A (Fig. 3a) after 15 weeks of treatment. However, this stimulatory effect can only be observed with CO52 ($p < 0.01$) and CO53 ($p < 0.001$) biscuits after 30 weeks of treatment. The percentages of proliferation in response to LPS (Fig. 3b) are increased with CO50 and CO52 biscuits at 15 weeks ($p < 0.01$ and 0.001, respectively). After 30 weeks of supplementation, all treatments, except CO49, increase proliferation ($p < 0.001$ for CO50 and CO53, and $p < 0.01$ for CO52) in response to LPS.

With regards to natural killer activity, represented as lysis percentage of tumour cells, and interleukin-2 (IL-2)

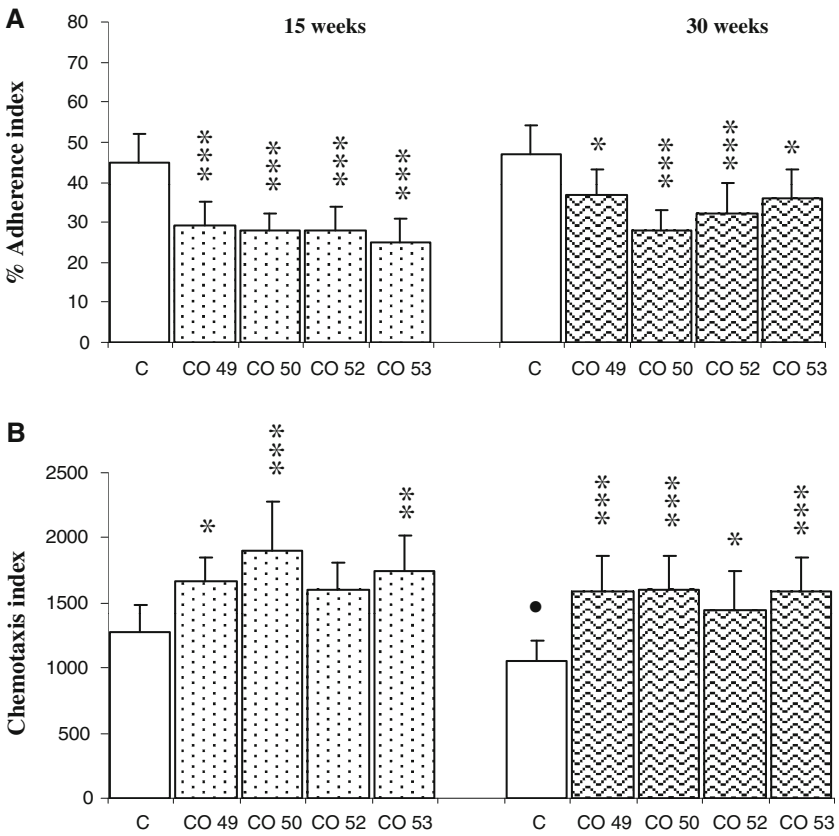

Fig. 2 Adherence (**a**) and chemotaxis indexes (**b**) of peritoneal lymphocytes from mice after 15 (mature animals) and 30 (old animals) weeks of diet supplementation with different polyphenol-rich biscuits. The results are the mean ± SD of eight values corresponding to the same number of animals, each value being the mean of duplicated assays. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$ compared to the control of each period of supplementation. $\bullet p < 0.05$ with respect to the corresponding value in 15-week supplementation group

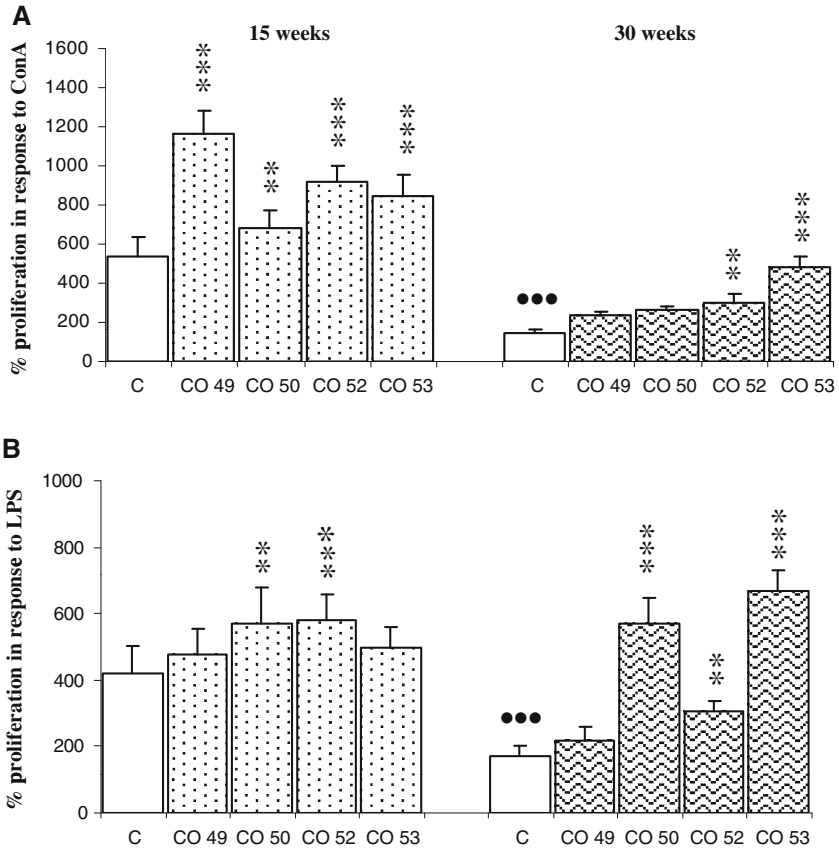

Fig. 3 Proliferation, in response to the mitogens concanavalin A (Con A) (**a**) and lipopolysaccharide (LPS) (**b**), of peritoneal lymphocytes from mice after 15 (mature animals) and 30 (old animals) weeks of diet supplementation with different polyphenol-rich biscuits. The results are the mean ± SD of eight values corresponding to the same number of animals, each value being the mean of duplicated assays. $**p < 0.01$; $***p < 0.001$ compared to the control of each period of supplementation. $\bullet\bullet\bullet p < 0.001$ with respect to the corresponding value in 15-week supplementation group

Table 3 Natural killer activity and interleukin 2 levels of peritoneal leucocytes from mice after 15 (mature animals) and 30 (old animals) weeks of the different diet supplementations

	Natural killer activity (% lysis)	Interleukin-2 levels (pg/ml)
15 weeks of supplementation (mature mice)		
Control	26 ± 5	243 ± 24
CO49	33 ± 6	341 ± 53^{aa}
CO50	37 ± 5^{aa}	380 ± 65^{aaa}
CO52	36 ± 5^{aa}	314 ± 83
CO53	45 ± 7^{aaa}	341 ± 48^{aa}
30 weeks of supplementation (old mice)		
Control	23 ± 2	159 ± 30^{b}
CO49	35 ± 5^{aaa}	226 ± 38
CO50	32 ± 3^{a}	182 ± 42
CO52	37 ± 8^{aaa}	235 ± 55
CO53	39 ± 6^{aaa}	239 ± 39

The results are the mean ± SD of eight values corresponding to the same number of animals, each value being the mean of duplicated assays. ^{a} $p < 0.05$, ^{aa} $p < 0.01$, ^{aaa} $p < 0.001$ compared to the control of each period of supplementation. ^{b} $p < 0.05$ with respect to the corresponding value in 15-week supplementation group

levels, the results are shown in Table 3. No significant differences with age were observed on natural killer activity between control samples. After 15 weeks, CO50 and CO52 increase lysis percentages ($p < 0.01$), as well as CO53 ($p < 0.001$). All treatments significantly increased natural killer activity after a 30-week period (CO49, CO52 and CO53 $p < 0.001$; and CO50 $p < 0.05$). As regards the IL-2 levels, a decrease with age in control samples was observed ($p < 0.05$). The supplementation with CO49 ($p < 0.01$), CO50 ($p < 0.001$) and CO53 ($p < 0.01$) increased IL-2 levels after 15 weeks, and no significant effects were observed after 30 weeks.

Besides, alpha-tumour necrosis factor levels (TNF-α) were measured. In reference to control samples, increased levels of this cytokine ($p < 0.05$) were shown in old mice (246 ± 28 pg/ml), in comparison with mature animals (202 ± 36 pg/ml). No effects on TNF-α levels were observed with the different treatments after 15 weeks, whereas after 30 weeks of supplementation with CO52 a significant decrease in TNF-α levels (191 ± 36, $p < 0.05$) was shown.

Discussion

In the present work, we have shown the favourable effects of long-term intake (15 and 30 weeks) of different biscuits containing nutritional concentrations of various combinations of cereal fractions naturally rich in polyphenols improving several relevant function parameters in immune cells from mature and old mice, which suffer age-related changes. The differences observed at control level between both ages in immune parameters are similar to those shown by several authors to occur with ageing [1, 2, 32–34]. Thus, the immune cells from the mice used in the present study show, in general, the previously observed age-related changes.

The adherence, the first step in the immune response of immune cells increases with age in macrophages. Adherence is a process related to oxidative stress, which implies an increased expression of adhesion molecules and other oxidant and inflammatory factors [34], and since ageing is associated with high levels of free radicals, an increase with ageing of the adherence function has been observed [2, 34]. An adequate adherence is needed for cell migration towards inflammatory foci, but an excessive adherence could represent a drawback for the cells to reach the infectious focus. Treatments were able to decrease the adherence indices of macrophages and lymphocytes after both periods of supplementation. Polyphenols, through their antioxidant role could reduce oxidative stress and the expression of adhesion molecules. In general, the described effects of polyphenols have demonstrated a clear inhibitory action on adhesion. Thus, certain types of polyphenols such as ferulic acid [35] and catechins [36] may decrease the expression of adhesion molecules by endothelial cells, and also may reduce monocyte adhesion. Moreover, epigallocatechin gallate (EGCG), one of the main phenolic components of green tea, inhibits adhesion of human neutrophils [37] and of the human monocyte cell line [38]. These two kinds of polyphenols, and especially the ferulic acid, present in all the biscuits studied, could be responsible of the decrease in the adherence of leucocytes found after supplementation.

With ageing, a decreased chemotaxis in macrophages and lymphocytes has also been previously described [2, 34]. The different biscuits did not show any differences after 15 weeks of supplementation with respect to macrophage control values of this immune parameter. Nevertheless, after the longest period of polyphenol-rich biscuit supplementation (30 weeks), CO50 and CO53 biscuits were able to increase macrophage chemotaxis. With respect to lymphocytes, in general, it can be observed an enhancement of chemotaxis in both periods of supplementation. There are few studies on the effect of polyphenols on cell migration or chemotaxis to sites of inflammation. Similar results to those shown in the present work have been found with several polyphenols, which enhance both f-MetLeuPhe directed and random migration of murine neutrophils in vitro [39]. However, other research shows controversial results. Thus, after in vitro and in vivo supplementation with the polyphenol rutin, an

inhibition of chemotaxis of rat neutrophils has been reported [40]. Nevertheless, in prematurely ageing mice, the supplementation for 5 weeks with a cereal rich in rutin increased the peritoneal macrophage chemotaxis [31]. Moreover, in adult mice a diet supplementation for that time with different polyphenol-rich cereals improves chemotaxis of peritoneal lymphocytes and macrophages [30].

The phagocytosis activity, that is the main macrophage function, decreases in old mice at control levels when compared to the values of mature animals, in agreement with previous results [2, 34]. In general, the different treatments increased this function after 15 weeks of treatment. The effects shown by biscuits change depending on the period studied. Thus, CO50 did not exert any effect after 15 weeks but inhibited significantly phagocytosis after 30 weeks. Besides, the stimulatory effect exerted by CO49, CO52 and CO53 after 15 weeks, disappears after 30 weeks of polyphenol-biscuits intake. It is possible that the different amounts and composition of polyphenols present in the different biscuits may be responsible for these effects. It has been demonstrated that a synthetic lipophilic derivate, 3-palmitoyl-(+)-catechin, enhances the phagocytic activity of guinea pig kupffer cells in vivo according to Piazza et al. [41]. Therefore, the presence of catechin in CO49 and CO52 biscuits could explain their enhancement of phagocytosis after 15 and 30 weeks of supplementation. In a previous study, diet supplementation for 5 weeks with polyphenol-rich cereals increased the phagocytic capacity of peritoneal macrophages from prematurely ageing mice [31].

The digestion of the phagocytized particles by macrophages takes place through the respiratory burst in which NADPH oxidase is activated catalyzing a reaction that produces superoxide anion. Without phagocytic stimulus these cells can also produce superoxide anion increasing the NADPH synthesis. In the present study, peritoneal macrophages from old mice show lower levels of intracellular superoxide anion, both at basal levels and with phagocytic stimulus, than those from mature animals. This confirms the lower effectiveness of foreign material destruction at this age and this fact is in agreement with previous results showing an age-related decrease in the intracellular levels of superoxide anion in peritoneal leucocyte of mice and an increase in extracellular superoxide levels, which could produce serious host tissue damage [2, 34]. The different kinds of biscuits do not show, in general, any effect at basal level on this free radical in both periods of supplementation. However, in stimulated samples, CO52 increases the levels of this anion after 30 weeks of treatment. In most cases the studies with polyphenols have been performed measuring extracellular superoxide anion levels, and the scavenging properties of polyphenols on this free radical showed these levels decreased [42, 43]. Nevertheless, several antioxidant compounds such as vitamin C, vitamin E, gluthation, tioproline or N-acetylcysteine, have shown in vitro and in vivo an increase in the levels of intracellular superoxide anion [2, 10, 44]. Moreover, a diet supplementation with polyphenol-rich cereals also increased the intracellular superoxide anion levels in peritoneal leucocyte from adult mice [30] and from prematurely ageing mice [31].

The most pronounced alterations with ageing seem to be found in T lymphocyte functions, with a decrease in their proliferative response and IL-2 production [1, 2]. The results of the present work confirm these previous studies since a significant reduction of the proliferation of lymphocytes and also of IL-2 concentrations have been found at control level in old mice with respect to the mature animals. In general, the naturally polyphenol-rich biscuits studied are able to improve proliferation in response to mitogens after both intake periods, as well as to enhance the levels of IL-2 after 15 weeks of treatment. Other authors have found that 2 weeks after polyphenol-fruit juice consumption, IL-2 levels increased significantly, as well as human lymphocyte responsiveness to mitogens [25]. In this context, an in vitro study on humans has demonstrated that polyphenols such as ferulic, p-coumaric and vanillic acids enhance the activity of human lymphocyte proliferation as well as the secretion of interferon-gamma [45]. Ferulic and p-coumaric acids are the main polyphenols in cereals, and the biscuits tested have a high content of these molecules, which could be responsible of the increased lymphocyte proliferation observed in our study. In a previous work, the diet supplementation with polyphenol-rich cereals showed an increase in IL-2 release and proliferation of peritoneal T lymphocytes in both adult and prematurely ageing mice, with the p-coumaric acid being an important candidate to mediate this effect [30, 31]. Nevertheless, controversial results have been reported regarding lymphocyte proliferation and IL-2 release depending on the polyphenol studied. Thus, the cacao liquor polyphenols, in vitro, inhibit human lymphocyte proliferation in response to mitogens [46] and no effect of five polyphenols in an in vitro study on IL-2 concentrations have been reported by Miles et al. [47]. Moreover, epigallocatechin-3-gallate inhibited the spleen T cell proliferation in mice and its supplementation resulted in a lower IL-2 receptor expression [48].

With respect to the antitumoural activity of NK cells, no differences were found at control levels between the ages studied. Controversial results with respect to NK activity with ageing have been observed, thus no change or a decline of this immune function with age has been reported [2, 49]. Supplementation with biscuits rich in polyphenols was able to improve this function after 15 weeks (with the exception of CO49), and 30 weeks of supplementation.

In agreement with our results, a study performed in adult humans consuming polyphenol-fruit juice, in a total period of 10 weeks, showed an improvement of the natural killer activity [25]. In vitro quercetin showed a decrease in lytic activity of NK cells, whereas catechin increased this function [50]. Moreover, green tea catechins maintained better NK activity in senescence-accelerated mice prone and decreased their tumour metastasis [51], and a diet supplementation with polyphenol-rich cereals for 5 weeks increased the NK activity of peritoneal leucocytes from prematurely ageing mice [31]. However, in an in vivo study performed in humans, the NK activity was not affected by polyphenols present in red wine [52].

With ageing also increases the release of proinflammatory cytokines such as TNF-α [2, 34] in agreement with the results obtained in the present work. The effect of CO52 biscuits decreasing the levels of TNF-α after 30 weeks of ingestion could show a useful anti-inflammatory action at this old age. The majority of the studies about the effect of polyphenols on the secretion of TNF-α are controversial. A recent study shows that a phenolic synthetic compound blocks this inflammatory cytokine production in macrophages [53]. Catechins have been particularly well studied on TNF-α secretion and showed an inhibitory effect in mouse macrophages [54, 55], but no significant effect in whole blood human cultures [56] and in a mouse monocyte/macrophage cell line (RAW 264.7) [57]. The diet supplementation for 5 weeks with several polyphenol-rich cereals decreased the release of TNF-α in peritoneal leucocyte of adult mice, with exception of one of the cereal studies, that without p-coumaric acid [30]. Nevertheless, other authors did not observe any effect on TNF-α production in whole blood cultures in the presence of p-coumaric acid [58]. The immunomodulatory role of the polyphenols studied in the present work, lowering the age-overactivated functions of leucocytes such as adherence and TNF-α release and, at the same time, stimulating the impaired ones such as the other function studied here, to reach the best physiological levels in each immune function, has already been shown with other antioxidants [2].

The present work demonstrates that age-associated impairment of the immune system may be attenuated by ingestion of nutrients present in the biscuits studied. Probably, the polyphenol content of these biscuits could be the main compound responsible for the improvement observed on several immune cell functions. Nevertheless, we cannot assume that the changes found in immune status are due to one individual polyphenol, and we must also consider that there are other components of the biscuits (such as lipids) that also influence immune status, and thus, additional studies in this area would be needed. Presently, all that we can say is that these effects are possibly a consequence of the concrete combination of the different types of polyphenols as well as the physiological amounts of them present in the biscuits. The use of combinations of optimal doses of antioxidant and anti-inflammatory phytochemicals for dietary intake, better than an antioxidant alone [59], could result in an appropriate way to slow down the progressive tendency to decline of the immune responses with advancing age, as many studies have shown [2, 8–10, 44]. Moreover, the potential antioxidant and anti-inflammatory action of polyphenols [12–17] could not always explain their pivotal physiological role, since, polyphenols, like other antioxidants, not only can be considered as merely scavenging radicals but also as modulators and regulators of several physiological functions including immune responses [2, 18, 19, 60, 61]. In addition, the role that show several nutrients increasing antioxidant defences by up-regulating the expression and activity of antioxidant enzymes normally present in the cell, such as it has been observed in some polyphenol compounds, seems the best way to improve longevity [11].

Since the immune system declines with age, increasing morbidity and mortality [62], naturally rich polyphenol food may be an important way to preserve health with age. In fact, the few studies on the effects of polyphenol supplementation in the immune functions of chronologically old subjects or with premature or accelerated ageing have shown positive effects [26, 27, 31, 51, 63], and in several cases this supplementation improved the immune functions restoring the values to those in adults. For such reason, further efforts have to be focused to understand the mechanisms by which these compounds influence immunity during ageing. In conclusion, the present work reveals that the ingestion of diets enriched in cereal polyphenols through ageing is useful against the age-related immunological decline and thus, since immune function has been shown to be a health and longevity predictor, increase healthy longevity.

Acknowledgments This work has been supported by grants from DANONE-VITAPOLE, and the Ministry of Science and Innovation (MICINN, BFU 2008-04336), the UCM Research Group (910379ENEROINN) grants and RETICEF (RD06/0013/0003) from ISCIII, Spain.

References

1. Pawelec G, Barnett Y, Forsey R et al (2002) T cells and aging. Front Biosci 7:D1056–D1183
2. De la Fuente M, Miquel J (2009) An update of the oxidation-inflammation theory of aging. The involvement of the immune system in oxi-inflamm-aging. Curr Pharm Des 15:3003–3026
3. Harman D (1956) Aging: a theory based on free radicals and radiation chemistry. J Gerontol 11:298–300
4. Miquel J (1998) An update on the oxygen stress-mitochondrial mutation theory of aging: genetic and evolutionary implications. Exp Gerontol 33:113–126

5. Knight JA (2000) Review: free radicals, antioxidants, and the immune system. Ann Clin Lab Sci 30:145–157
6. Wayne SJ, Rhyme RL, Garry PJ, Goodwin JS (1990) Cell-mediated immunity as a predictor of morbidity and mortality in subjects over 60. J Gerontol 45:M45–M48
7. Arranz L, Caamano J, Lord JM, De la Fuente M (2010) Preserved immune functions and controlled leukocyte oxidative stress in naturally long-lived mice: possible role of nuclear factor-kappaB. J Gerontol A Biol Sci Med Sci 65:941–950
8. De la Fuente M (2010) Murine models of premature ageing for the study of diet-induced immune changes. Improvement of leukocyte functions in two strains of old prematurely ageing mice by dietary supplementation with sulphur-containing antioxidants. Proc Nutr Soc 69:651–659
9. Chandra RK (2004) Impact of nutritional status and nutrient supplements on immune responses and incidence of infection in older individuals. Ageing Res Rev 3:91–104
10. De la Fuente M, Miquel J, Catalan MP, Victor VM, Guayerbas N (2002) The amount of thiolic antioxidant ingestion needed to improve several immune functions is higher in aged than in adult mice. Free Radic Res 36:119–126
11. Viña J, Gomez-Cabrera MC, Borras C (2007) Fostering antioxidant defences: up-regulation of antioxidant genes or antioxidant supplementation? Br J Nutr 98:S36–S40
12. Rice-Evans CA, Miller NJ (1996) Antioxidant activities of flavonoids as bioactive components of food. Biochem Soc Trans 24:790–795
13. RiceEvans CA, Miller NJ, Paganga G (1996) Structure-antioxidant activity relationships of flavonoids and phenolic acids. Free Radical Biol Med 20:933–956
14. Manach C, Scalbert A, Morand C, Remesy C, Jimenez L (2004) Polyphenols: food sources and bioavailability. Am J Clin Nutr 79:727–747
15. Middleton EJ (1998) Effect of plant flavonoids on immune and inflammatory function. Adv Exp Med Biol 439:175–182
16. Kim HP, Son KH, Chang HW, Kang SS (2004) Anti-inflammatory plant flavonoids and cellular action mechanisms. J Pharmacol Sci 96:229–245
17. Hughes DA (2005) Plant polyphenols: modifiers of immune function and risk of cardiovascular disease. Nutrition 21:422–423
18. Ramiro-Puig E, Castell M (2009) Cocoa: antioxidant and immunomodulator. Br J Nutr 101(7):931–940
19. Magrone T, Jirillo E (2010) Polyphenols from red wine are potent modulators of innate and adaptive immune responsiveness. Proc Nutr Soc 69(3):279–285
20. Sies H (2010) Polyphenols and health: update and perspectives. Arch Biochem Biophys 501:2–5
21. Giugliano D (2000) Dietary antioxidants for cardiovascular prevention. Nutr Metab Cardiovasc Dis 10:38–44
22. Tapiero H, Tew KD, Ba GN, Mathe G (2002) Polyphenols: do they play a role in the prevention of human pathologies? Biomed Pharmacother 56:200–207
23. Passamonti S, Terdoslavich M, Franca R, Vanzo A, Tramer F, Braidor E, Petrussa E, Vianello A (2009) Bioavailability of flavonoids: a review of their membrane transport and the function of bilitranslocase in animal and plant organisms. Curr Drug Metab 10:369–394
24. Uto-Kondo H, Ayaori M, Ogura M et al (2010) Coffee consumption enhances high-density lipoprotein-mediated cholesterol efflux in macrophages. Circ Res 106:779–787
25. Bub A, Watzl B, Blockhaus M, Brivida K, Liegibel U, Müller H, Pool-Zobel BL, Rechkemmer G (2003) Fruit juice consumption modulates antioxidative status, immune status and DNA damage. J Nutr Biochem 14:90–98
26. Baeza I, De Castro NM, Alvarado C, Alvarez P, Arranz L, Bayon J, De la Fuente M (2007) Improvement of immune cell functions in aged mice treated for 5 weeks with soybean isoflavones. Ann NY Acad Sci 1100:497–504
27. Baeza I, De Castro NM, Arranz L, De la Fuente M (2010) Soybean and green tea polyphenols improve immune function and redox status in very old ovariectomized mice. Rejuvenation Res doi:10.1089/rej.2010.1049
28. Boyle SP, Dobson VI, Duthie SJ, Kyle JA, Collins AR (2000) Absorption and DNA protective effects of flavonoid glycosides from an onion meal. Eur J Nutr 39:213–223
29. Pedersen CB, Kyle J, Jenkinson AM, Gardner PT, McPhail DB, Duthie GG (2000) Effects of blueberry and cranberry juice consumption on the plasma antioxidant capacity of healthy female volunteers. Eur J Clin Nutr 54:405–408
30. Alvarez P, Alvarado C, Mathieu F, Jimenez L, De la Fuene M (2006) Diet supplementation for 5 weeks with polyphenol-rich cereals improves several functions and the redox state of mouse leucocytes. Eur J Nutr 45:428–438
31. Alvarez P, Alvarado C, Puerto M, Schlumberger A, Jimenez L, De la Fuente M (2006) Improvement of leukocyte functions in prematurely aging mice after five weeks of diet supplementation with polyphenol-rich cereals. Nutrition 22:913–921
32. Guayerbas N, Puerto M, Victor VM, Miquel J, De la Fuente M (2002) Leukocyte function and life span in a murine model of premature immunosenescence. Exp Gerontol 37:249–256
33. Guayerbas N, De la Fuente M (2003) An impairment of phagocytic function is linked to a shorter life span in two strains of prematurely-ageing mice. Develop Comp Immunol 27:339–350
34. De la Fuente M, Hernanz A, Guayerbas N, Alvarez P, Alvarado C (2004) Changes with age in peritoneal macrophage functions. Implication of leukocytes in the oxidative stress of senescence. Cell Mol Biol 50:OL683–OL690
35. Ma ZC, Hong Q, Wang YG et al (2010) Ferulic acid attenuates adhesion molecule expression in gamma-radiated human umbilical vascular endothelial cells. Biol Pharm Bull 33:752–758
36. Koga T, Meydani M (2001) Effect of plasma metabolites of (+)-catechin and quercetin on monocyte adhesion to human aortic endothelial cells. Am J Clin Nutr 73:74174–74178
37. Hofbauer R, Frass M, Gmeiner B, Handler S, Speiser W, Kapiotis S (1999) The green tea extract epigallocatechin gallate is able to reduce neutrophil transmigration through monolayer of endothelial cells. Wien Klin Wochensch 111:278–282
38. Melgarejo E, Medina MA, Sánchez-Jimenez F, Urdiales JL (2009) Epigallocatechin gallate reduces human monocyte mobility and adhesion in vitro. Br J Pharmacol 158(7):1705–1712
39. Kenny MT, Balistrery FJ, Torney HL (1990) Flavonoid modulation of murine neutrophil cytokinesis. Immunopharmacol Immunotoxicol 12:527–541
40. Selloum L, Bouriche H, Tigrine C, Boudoukha C (2003) Anti-inflammatory effect of rutin on rat paw oedema, and on neutrophils chemotaxis and degranulation. Exp Toxicol Pathol 54:313–318
41. Piazza M, Borgia G, Crowell J, Nappa S, Lambrase A, Perrissoud D, Schreil W (1985) The effect of 3-palmitoyl-(+)-catechin on kupffer cells in guinea pig liver. Hepatology 5:867–869
42. Martínez J, Moreno JJ (2000) Effect of resveratrol, a natural polyphenolic compound, on reactive oxygen species and prostaglandin production. Biochem Pharmacol 59:865–870
43. O′Dowd Y, Driss F, Dang PMC, Elbim C, Gougerot-Pocidalo MA, Pasquier C, El-Benna J (2004) Antioxidant effect of hydroxytyrosol, a polyphenol from olive oil: scavenging of hydrogen peroxide but not superoxide anion produced by human neutrophils. Biochem Pharmacol 68:2003–2008
44. Alvarado C, Álvarez P, Puerto M, Gausseres N, Jiménez L, De la Fuente M (2006) Dietary supplementation with antioxidants improves functions and decreases oxidative stress of leukocytes from prematurely ageing mice. Nutrition 22:767–777

45. Chiang LC, Ng LT, Chiang W, Chang MY, Lin CC (2003) Immunomodulatory activities of flavonoids, monoterpenoids, triterpenoids, iridoid glycosides and phenolic compounds of Plantago species. Planta Med 69:600–604
46. Sanbongi C, Suzuki N, Sakane T (1997) Polyphenols in chocolate, which have antioxidant activity, modulate immune functions in human in vitro. Cell Immunol 177:129–136
47. Miles EA, Zoubouli P, Calder PC (2005) Effects of polyphenols on human Th1 and Th2 cytokine production. Clin Nutr 24:780–784
48. Wu D, Guo Z, Ren Z, Guo W, Meydani SN (2009) Green tea EGCG suppresses T cell proliferation through impairment of IL-2/IL-2 receptor signalling. Free Radic Biol Med 47(5):636–643
49. Ferrandez MD, Correa R, Del Rio M, De la Fuente M (1999) Effects in vitro of several antioxidants on the natural killer function of aging mice. Exp Gerontol 34:675–685
50. Exon JH, Magnuson BA, South EH, Hendirx K (1998) Dietary quercetin, immune functions and colonic carcinogenesis in rats. Immunopharmacol Immunotoxicol 20:173–190
51. Shimizu K, Shimizu NK, Hakamata W, Unno K, Asai T, Oku N (2010) Preventive effect of green tea catechins on experimental tumor metastasis in senescence-accelerated mice. Biol Pharm Bull 33:117–121
52. Watzl B, Bub A, Pretzer G, Roser S, Barth SW, Rechkemmer G (2004) Daily moderate amounts of red wine or alcohol have no effect on the immune system of healthy men. Eur J Clin Nutr 58:40–45
53. Hwang JM, Yu JY, Jang YO, Kim BT, Hwang KJ, Jeon YM, Lee JC (2010) A phenolic acid phenethyl urea compound inhibits lipopolysaccharide-induced production of nitric oxide and pro-inflammatory cytokines in cell culture. Int Immunopharmacol 10:526–532
54. Chen CC, Chow MP, Huang WC, Lin YC, Chang YJ (2004) Flavonoids inhibit tumor necrosis factor-alpha-induced up-regulation of intercellular adhesion molecule-1 (ICAM-1) in respiratory epithelial cells through activator protein-1 and nuclear factor-kappa B: structure, activity relationships. Mol Pharmacol 66:683–693
55. Ichikawa D, Matsui A, Imai M, Sonoda Y, Kasahara T (2004) Effect of various catechins on the IL-12p40 production by murine peritoneal macrophages and a macrophage cell line, J774.1. Biol Pharm Bull 27:1353–1358
56. Crouvezier S, Powell B, Keir D, Yaqoob P (2001) The effects of phenolic components of tea on the production of pro- and anti-inflammatory cytokines by human leukocytes in vitro. Cytokine 13:280–286
57. Wang J, Mazza G (2002) Effects of anthocyanins and other phenolic compounds on the prodcution of tumor necrosis factor alpha in LPS/IFN-gamma-activated RAW 264.7 macrophages. J Agric Food Chem 50:4183–4189
58. Miles EA, Zoubouli P, Calder PC (2005) Differential anti-inflammatory effects of phenolic compounds from extra virgin olive oil identified in human whole blood cultures. Nutrition 21:389–394
59. Liu RH (2004) Potential synergy of phytochemicals in cancer prevention: mechanism of action. J Nutr 134:3479S–3485S
60. Brigelius-Flohé R (2005) Introductory remarks to the new series of reviews: from dietary antioxidants to regulators in cellular signalling and gene expression. Free Rad Res 39:351
61. Queen BL, Tollefsbol TO (2010) Polyphenols and aging. Curr Aging Sci 3:34–42
62. Dorshkind K, Swain S (2009) Age-associated declines in immune system development and function: causes, consequences, and reversal. Curr Opin Immunol 21:404–407
63. Alvarez P, Alvarado C, Jimenez L, De la Fuente M (2008) Effects of a diet with polyphenol-rich cereal supplementation on the function and redox state of peritienal leukocytes from mice: differences between a short (5 weeks) and long (20 weeks) period of supplementation. Proc Nutr Soc 67 (OCE):E23