

The cytokine-mediated network in health and disease with special reference to the gut immune system regulation. Modulation of cytokine functions by nutraceuticals

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RIASSUNTO *Le citochine sono proteine di piccole dimensioni in grado di svolgere molteplici funzioni nell'organismo. Soprattutto a livello intestinale la funzione delle citochine è molto evidente in termini di risposta a stimoli sia esogeni che endogeni (batteri commensali). In particolare, in questa rassegna verrà illustrato il ruolo svolto dalle interleuchine (IL)-17, -23 e -21 in corso di malattie croniche intestinali per una migliore comprensione della patogenesi di queste malattie. Infine, verranno discussi gli effetti sul sistema immunitario di alcune sostanze nutraceutiche quali i polifenoli del vino rosso e relativa vinaccia fermentata nonché del latte di asina e di capra per un loro potenziale impiego in clinica.*

Parole chiave: Citochine; Malattie infiammatorie croniche intestinali; Nutraceutici

ABSTRACT *Cytokines are small size proteins secreted by immune and non-immune cells and endowed with pleiotropic functions in the host. Mostly in the gut mucosa cytokine network function is evident in that it regulates immune responsiveness toward external and internal stimuli, even including commensals. In inflammatory bowel disease, the role of interleukin (IL)-17, IL-23 and IL-21 will be illustrated in order to better understand the pathogenesis of disease. Finally, the effects of some nutraceuticals (polyphenols from red wine, fermented grape marc and donkey's and goat's milk) on the immune system in different experimental settings will be discussed even in terms of potential clinical application.*

Key words: Cytokines; Inflammatory bowel disease; Nutraceuticals

INTRODUCTION

Cytokines are cellular products of small molecular weight (25 kDa about) endowed with pleiotropic regulatory functions, under physiologic and pathologic conditions¹. They act through binding to specific receptors in:

- a) an autocrine way, influencing their own function;
- b) a paracrine way, activating nearby cells;
- c) an endocrine way, reaching distant cellular targets once poured into the circulation¹.

Chemokines are cytokines prevalently released by antigen presenting cells (APCs) and, in particular, macrophages (MΦ) and dendritic cells (DCs) in the early phase of infection². They induce chemotaxis of other immune cells, e.g., polymorphonuclear cells (PMN) as in the case of interleukin (IL)-8, also known as CXCL8².

In general terms, cytokines can be divided into the family of hematopoietins (e.g., growth factors such as colony stimulating factors, IL-7 etc.) and of tumor necrosis factor (TNF)- α , even including several members associated to cellular membranes³. Both families of cytokines are modulators of either innate or adaptive immunity. APCs in response to bacterial challenges secrete the so-called pro-inflammatory cytokines. Among them, IL-8 is a chemoattractant for PMN, while IL-1 β , IL-6 and TNF- α are pyrogenic, also inducing the acute phase response in the liver⁴. APCs also produce IL-12 which induces differentiation of T helper (h)-1 cells⁵.

Th1 and Th2 cytokines are regulators of the adaptive immunity. Th1 cells produce IL-12 and interferon (IFN)- γ

which sustain the cell-mediated immunity⁶. On the other hand, IL-4, IL-5 and IL-6 govern the humoral immunity via synthesis, production and class switch of antibodies (IgG, IgE, and IgA)⁷. Finally, IL-10 and transforming growth factor (TGF)- β play an anti-inflammatory role as products of T regulatory (Treg) cells⁸.

Besides this classical pattern of cytokines, over recent years new ILs have been identified. IL-17 is a product of the newly recognized Th17 cells which mediates a variety of activities ranging from PMN mobilization to allergy and autoimmunity^{9,10}. In the next section, the axis Th17/IL-23 along with the effects mediated by IL-21 on Th17 cells will be elucidated as pathogenetic factors involved in the inflammation bowel disease (IBD) progression. Quite interestingly, IL-22 has been found to be produced by Th17 cells¹¹. The IL-22 receptor is expressed on epithelial cells, thus enabling these cells to protect the host against gram-negative organisms¹¹. Finally, a possible involvement of this cytokine in autoimmunity has been envisaged as in the case of psoriasis¹¹.

In Figure 1, the interplay between pro- and anti-inflammatory cytokines is depicted, as balancing square model.

In this review, a general overview of the cytokine network in the course of IBD will be provided. Thereafter, emphasis will be placed on relevant personal data concerning the *in vitro* and *in vivo* immunomodulating effects of nutraceuticals (red wine polyphenols, fermented grape marc and donkey's and goat's milk). Of note, this review will provide readers with essential details on the methods used according to the aims of this journal.

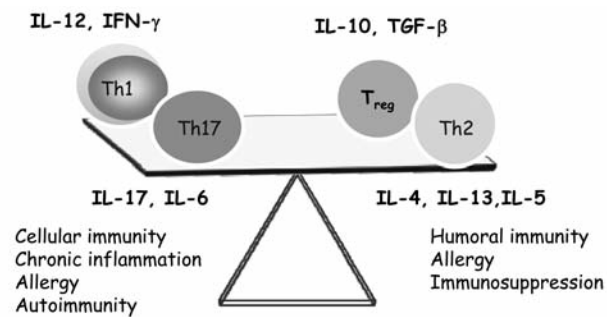


Figure 1
The immune homeostasis maintained by the cytokine network

STRUCTURE AND FUNCTION OF THE GUT

The gastro-intestinal tract (GIT) provides distinct niches for colonization of commensal bacteria, as suggested by differences in the bacterial flora content^{12,13}. Therefore, the anatomical architecture of the GIT also depends on the state of immune activation dictated by microflora¹⁴. Consequentially, not only the epithelial intestinal architecture, but also the density of cells that are closely associated with the innate and adaptive immune response, such as goblet-producing cells, Paneth cells, dendritic cells (DCs), and B and T lymphocytes are quite different, as also evidenced by Peyer's or cecal patches, or isolated lymphoid follicles¹⁴.

The intestinal barrier is composed of a secreted mucus layer, a layer of epithelial cells and the underlying non epithelial mucosal cells (leukocytes) with a broad range of regulatory and effector functions. The epithelial cells represent a structural barrier, generate the majority of components of the secreted barrier, and sense the external danger, thus emanating signals to the innate and adaptive immunity¹⁴. Non epithelial mucosal cells can traverse the epithelial barrier, also generating some components of the secreted barrier, and modulating epithelial cell function as well as the secreted barrier¹⁴. A fundamental function of mucus is to maintain a high concentration of antimicrobial molecules close to the epithelium¹⁵. Among antimicrobial molecules, defensins are secreted in granules produced by Paneth cells into the mucus layer of small intestinal crypts¹⁵. One can speculate that retention of the polycationic defensins in mucus is favored by electrostatic interaction with the polyanionic mucin molecules.

Intestinal epithelial cells are able to phagocytose bacteria, neutralize toxins, sense prokaryotic-associated molecular patterns and interact with underlying innate and adaptive immunity¹⁶. For example, epithelial molecules determine class-switching by B cells in the lamina propria¹⁷. Finally, DCs extend processes between adjacent epithelial cells,^{18,19} and some specialized T cells and Natural Killer (NK) cells are located between epithelial cells²⁰.

Interaction of the Intestinal Barrier with Commensal and Pathogenic Bacteria

The intestinal lumen contains commensals in strict relationship with the host, furnishing nutrients and matu-

ring the immune system^{21,22}. The intestinal secreted and cellular barrier limits contact between bacteria and underlying immune cells. An exception occurs in the dome epithelium above Peyer's patches (PP) in the small intestine which lacks goblet cells and mucus barrier. This allows bacteria to bind to M cells that phagocytose and transport them to underlying DCs for antigen presentation^{23,24}. In healthy individuals, the gut mucosa is composed by a large number of lymphocytes in a status of inflammatory equilibrium between the mucosal immune system, and dietary Ags and intestinal microbiota. A breakdown in this balance results in the development of Crohn's disease (CD) and ulcerative colitis (UC), the two major forms of IBD²⁵.

Activation of the intestinal immune system by intestinal microflora results in gut inflammation, characterized by cross talk between immune and non-immune system *via* cytokines and membrane-bound receptors. The role of cytokines in intestinal inflammation has been demonstrated by the tumor necrosis factor- α (TNF)- α and interferon (IFN)- γ , IL-6, and IL-12 blockade which is of therapeutic benefit in randomized controlled trials²⁶⁻²⁸.

ROLE OF CYTOKINES IN IBD

Cytokines are fundamental signals in the intestinal immune system, able to subvert the physiological status of inflammation in the gut^{29,30}. In IBD, activated DCs and M Φ secrete several cytokines which trigger and differentiate many T cells. In this regard, CD patients produce a Th1-type cytokine profile, whereas UC patients release Th2 cytokines, such as IL-5 and IL-13³¹⁻³³. However, IL-4 levels are decreased in both UC and CD. In addition, TNF- α is produced by both Th1 and Th2 cells as well as by M Φ . Moreover, Th3 and regulatory CD25+CD4+ (Treg) cells exist in the normal gut that produce TGF- β and IL-10, respectively^{34,35}. APCs, Th1, Th2, Treg cells and the most recently characterized Th17 and their derived-cytokines play an important role in IBD³⁶. Therefore, cellular interactions are driven by both classical (such as TNF- α , IFN- γ , IL-1, IL-6, IL-4, IL-5, IL-10, TGF- β) and newly characterized cytokines (like IL-13, IL-12, IL-18, IL-23)³⁷.

CD, as previously mentioned, is associated with a Th1 cell response, leading to an enhanced production of IFN- γ and TNF- α . IL-12 and IL-23 regulate the Th1 differentiation which along with IL-15, IL-18 and IL-21 results in the polarization of Th1³¹. On the other hand, in UC, the local immu-

ne response is rather characterized by CD1 reactive NK T cell generation of Th2 cytokine production³³.

Here, a detailed description of newly identified cytokines will be presented for a better understanding of IBD pathogenesis. Th17 cells are characterized by the production of IL-17, which is an important player in inflammatory responses³⁸. Sequencing the human genome led to the discovery of additional five members of the IL-17 family from IL-17B to IL-17F. IL-17A is exclusively generated by Th17 cells³⁹ via STAT3 activation triggered by IL-23⁴⁰. IL-17 recruits immune cells to peripheral tissues via NF- κ B activation following IL-17 receptor engagement^{41,42}. IL-17 also induces many pro-inflammatory cytokines, even including TNF- α , IL-6, and IL-1 β ⁴³⁻⁴⁵.

Furthermore, TNF- α and IL-6, both products of Th17 cells, not only support Th17 cell development but also cooperate with IL-17 to augment the production of pro-inflammatory mediators⁴⁵.

In mice, TGF- β and IL-6 synergically induce differentiation of naïve T cells into Th17 cells but in humans TGF- β is not necessary like in mice⁴⁶⁻⁵⁰. Evidence has been reported that Treg cells augment the induction of Th17-producing cells by spontaneous release of TGF- β and/or DC-induced generation of TGF- β . In fact, during the course of colitis Treg cells are located in close proximity of CD11c+ cells and pathogenic cells⁵¹.

In this respect, current data indicate⁵² that mice develop a more severe 2,4,6-trinitrobenzenesulfonic acid colitis characterized by increased IL-17 production when they have been administered with FoxP3+ (GFP+) Treg cells during the development of colitis. This evidence suggests that Treg cells contribute to Th17 differentiation *in vivo* under inflammatory conditions.

In humans, Th17 differentiation is induced by IL-1 β and IL-23, and enhanced by IL-6 but is inhibited by TGF- β and IL-12^{53,54}. Finally, Treg cells not only contribute to the differentiation of Th17 cells but also produce IL-17 and IL-22 for the maintenance of the mucosal barrier function³⁸. Quite interestingly, IL-17 as well as Th17 cells are elevated in serum and intestinal tissue of IBD patients but are not detected in inactive of IBD patients tissue as well as other types of colitis^{55,56}.

Recently, it has been demonstrated that p40 is a component not only of IL-12 (p40/p35) but also of the Th1-inducing cytokine IL-23 (p40/p19)⁵⁷⁻⁵⁹. Of note, bacteria-induced DC activation results in IL-23 as opposed to IL-12 production and this fact raises the possibility that IL-23 is also an important mediator in intestinal inflammation⁶⁰. IL-23 may also maintain Th1 cell memory responses by acting directly on CD4+ memory lymphocytes⁵⁹ and activate DCs and M Φ via autocrine mechanism⁶¹.

According to Becker et al.⁶², the augmentation of IL-23 expression in the ileum seems to reflect an increased susceptibility to inflammation in the terminal ileum, a specific site in CD patients⁵⁷.

Indeed, Uhlig et al.⁶³, have reported that IL-23 initiates T-cell-independent gut inflammation and for its effects on Th17 cells⁶⁴ may directly activate M Φ to produce proinflammatory cytokines. Therefore, IL-23 seems to be a pleiotropic cytokine exerting direct effects on both T cells and APCs in the gut, as also underlined by the observation that anti-IL-12/IL-23 p40 antibody (Ab) demonstrated clinical

efficacy in a pilot trial in active CD⁶⁵.

Quite interestingly, a IL-23-Th17 cell axis has also been reported in a murine asthma model in terms of neutrophilic and Th2-mediated eosinophilic airway inflammation⁶⁶.

IL-21 receptors (IL-21) R is a novel orphan Th1 cytokine receptor⁶⁷ with a significant structural homology with the IL-2R β chain. Functionally, IL-21 increases the proliferation and cytokine profile of CD4+ and CD8+ cells⁶⁸ while inhibiting IFN- γ expression in naïve T cells without influencing the expression level of either Th1-related cytokines or T-bet⁶⁹. IL-21 is involved in NK T cell activation and, in particular, augments their cytotoxic response⁷⁰. Furthermore, it promotes CD4+ cells to differentiate into Th17 cells⁷¹. IL-21 is overproduced in the inflamed intestine of patients with CD compared to patients with UC and healthy controls. In addition, exogenous IL-12 further augments IL-21 reduced STAT-4 phosphorylation, T-bet expression, and IFN- γ production. These data suggest that, in CD, IL-21 may expand and maintain the ongoing Th1 response⁷².

Intestinal epithelial cells constitutively express the IL-21R complex which is further enhanced in the inflamed tissue of UC and CD patients, thus suggesting that IL-21 might directly target intestinal epithelial cells during inflammation. *In vitro* IL-21 increases secretion of the M Φ inflammatory protein (MIP)-3 α (CCL20)⁷³. In both CD and UC mucosa, where IL-21 is expressed at high levels, MIP-3 α is upregulated, thus contributing to the recruitment of gut-homing α 4- β 7-expressing T cells.

UC is characterized by a Th2 immune response in which IL-13, produced by NK T cells³², may impair epithelial barrier function by acting upon epithelial apoptosis, tight junctions, and restitution velocity⁷⁴. In addition, it was found that lamina propria mononuclear cells from UC patients secreted high amounts of IL-13 and IL-5^{32,74}. The IL-13+ and IL-5+ cells bear NK specific markers such as CD161 and recognize CD1d, indicating that they are NK T-cells³².

Conclusively, data suggest that the modifications in barrier function in human UC may not only depend on the cytolytic destruction of the epithelial layer by NK T cells but also on the effects of IL-13. Thus, this cytokine may play a dual pathogenic role either affecting epithelial cells or stimulating NKT cells.

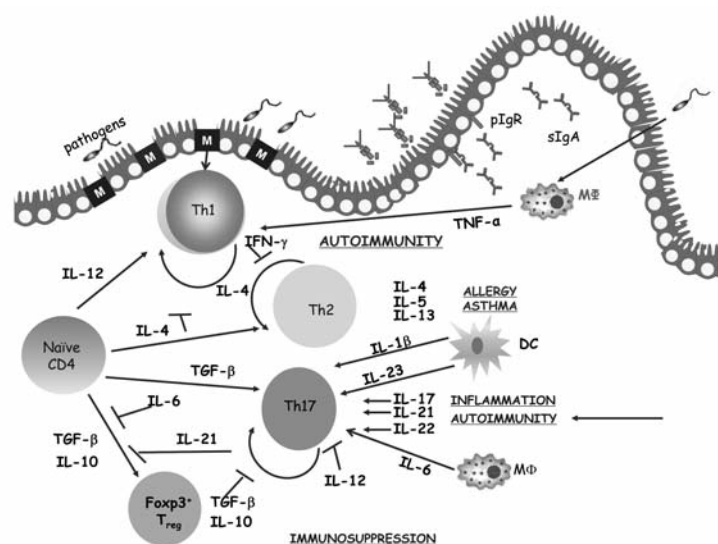
Table 1 and Figure 2 summarize the effects of the major cytokines involved in the pathogenesis of IBD.

NUTRACEUTICALS PRODUCTS AND CYTOKINE RELEASE

In relation to the above cited inflammatory effects of cytokines in the gut, we have focused our attention on the potential anti-inflammatory properties of nutraceutical products such as red wine polyphenols, fermented grape marc (FGM), donkey's and goat's milk, respectively.

Effects of an Italian Red Wine (Negroamaro) on Cytokine Release from Human Peripheral Blood Mononuclear Cells (PMCs)⁷⁵

PBMCs isolated from healthy donors were diluted and layered over Ficoll-Paque (1.077 density) (Sigma-Aldrich,

**Figure 2**

Expression of major cytokines in the human gut inflamed mucosa.

(Th17 differentiation is induced by IL-1 β , IL-21 and IL-23 and enhanced by IL-6, TGF- β and IL-12 inhibit Th17 differentiation.

→ differentiation or enhancement; —| inhibition).

Table 1

Newly identified cytokines involved in IBD development

Cytokine	Cellular source	Function
IL-17	Th17 cells	Inflammation
	Treg cells	Allergy
	$\gamma\delta$ T-cells	Autoimmunity
IL-21	Th17 cells	Differentiation of Th17 cells from CD4+ cells
	Natural killer T (NK) cells	Increased proliferation and cytokine profile of CD4+ and CD8+ cells
	CXCR5+ T follicular h cells	Increased NKT cell activation and cytotoxic responses
IL-23	Dendritic cells	T-cell independent gut inflammation
		Induction of Th17 cells
		Activation of M Φ for pro-inflammatory cytokine release

St. Louis, MO., USA) to obtain cell suspensions that were added to complete medium RPMI-1640 (Milteny Biotec, Bergisch Gladbach, Germany), supplemented with 1 mM sodium pyruvate (Sigma-Aldrich), 1 M HEPES buffer (Sigma-Aldrich), 0.05 mM 2-mercaptoethanol (Sigma-Aldrich), 30% FBS, streptomycin [10 ng/mL]-penicillin [100 UI/mL] (Biowhittaker, Walkersville, USA). Three different samples of Negroamaro were used: as a whole, with polyphenols alone and with alcohol alone at two different dilutions (1:5 and 1:10), respectively.

Controls were represented by samples deprived of polyphenols, and ethanol and by unstimulated cells. The positive control was represented by *E. coli* lipopolysaccharide (LPS) [10 μ g/mL] (O55:B5; Sigma-Aldrich).

For ELISPOT assay, 100 μ l of each wine sample diluted 1:5 and 1:10, respectively, were added to the cells at a concentration of 7×10^7 /ml. Thus, cells were added with 10 μ l of Biotin antibody cocktail (Milteny Biotec), allowing an incubation for 10 min. at 4°C. Next, 20 μ l of CD19 human anti-Microbeads (Milteny Biotec) were supplemented.

After incubation for 15 min, cell-supernatants were pipetted off, and cell pellets were resuspended in running buffer. Cells were isolated using VarioMACS (Milteny Biotec) proceeding by a depletion selection. CD19- cells resuspended in complete medium, were stimulated with phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich) plus ionomycin (Sigma-Aldrich), whole wine and ethanol alone and controls, respectively, and were kept in a plate to let monocytes to adhere at 37°C, 5%CO₂. Next, supernatants containing T lymphocytes alone were used for detection of IFN- γ by ELISPOT⁷⁶.

The effects of red wine polyphenols on other cytokine (IL-1 β , IL-6, IL-10 and IL-12) release were assessed in supernatants by a cytofluorimetric method (Cytometric Bead Array, CBA Kit, Becton Dickinson, San Jose, CA). The kit uses a set of beads coated with cytokine specific antibodies (Abs)⁷⁷, which serves as a capture surface and to each cytokine-specific set of beads was assigned a discrete fluorescence intensity. The data acquired for the negative control (unstimulated cells) were subtracted from

those obtained with stimulated cells. As far as production of cytokines is concerned, the following results were obtained.

IFN- γ release augmented in presence of whole wine at both dilutions (1:5 and 1:10), even if at 1:10 dilution it was less evident. IL-12 production augmented in samples treated with whole wine alone or in presence of LPS. IL-6 release by PBMCs stimulated with whole wine at 1:5 dilution was higher when compared to cells treated with LPS alone, whereas in cells treated with whole wine at 1:10 dilution IL-6 was completely absent. Enhancement of IL-1 β release was observed in whole wine-treated cells at 1:5 dilution and in LPS-treated cells. Finally, IL-10 in PBMCs-stimulated with whole wine at 1:5 dilution was slightly augmented in comparison with LPS-treated cells. On the other hand, release of all cytokines tested were absent in ethanol-stimulated cells, as well as in polyphenols-deprived samples.

In sum, release of cytokines from PBMC stimulated with Negroamaro seems to switch the immune response toward the Th1 type by effect of IL-12, as evidenced by IFN- γ release⁷⁵. On the other hand, production of IL-1 β and IL-6 was present, thus, suggesting a switch toward the inflammatory pathway. However, production of the anti-inflammatory cytokine IL-10 was also induced by Negroamaro, thus indicating that the release of IL-6 and IL-1 β was under the control of IL-10⁷⁸. In turn, IL-6 stimulates IL-10 production causing a local feedback loop limiting the proinflammatory pathway⁷⁹.

All these evidences may account for the maintenance of the homeostasis in the inflamed gut thanks to the activity of Negroamaro polyphenols able to keep in equilibrium the IL-12/IL-10 ratio. Besides the above mentioned property, moderate red wine intake seems to afford protection to the host against invading pathogens *via* cellular and humoral immune responses^{80,81}, even including the production of nitric oxide by monocytes⁷⁵

Fermented Grape Marc and Cytokine Release

In another series of experiments FGM was obtained from skin and seeds of *vitis vinifera* Negroamaro and Koshu, respectively, according to the procedure described in a previous report⁸².

Two models were used for evaluating the anti-inflammatory effects of FGMs (unpublished data).

BALB/c mice were treated with 5% of dextran sodium sulfate (DSS) to induce colitis. In this experiment three groups of BALB/c mice were used:

- Normal group: BALB/c mice untreated with DSS;
- Control group: BALB/c mice treated with DSS;
- FGM-treated group: BALB/c mice treated with DSS plus FGMs deriving from Negroamaro and Koshu, respectively. In this case, FGMs were administered for 6 days with 10, 30 or 100 mg/kg in water.

At 7 days BALB/c mice were sacrificed and colon was homogenized to measure the total TNF- α and IL-1 β content by ELISA. Koshu FGM could significantly reduced both cytokines at 30 and 100 mg/kg, respectively. No effects were observed with Negroamaro FGM. Attenuation of colitis induced by Koshu FGM in comparison to the mice treated with DSS only was macroscopically observed in Koshu treated animals measuring length of colon (normal length as in untreated mice, while colon length was reduced

in DSS mice). In synthesis, Koshu FGM-induced reduction of TNF- α and IL-1 β may account for the mitigation of colitis in FGM-treated animals.

Another experiment was conducted using PP cells of BALB/c mice that were treated with Koshu- and Negroamaro-FGMs added of water or ethanol, respectively, in presence of PMA, ionomycin and brefeldin A. After stimulation for 6 hrs, the cytokine-producing cells were analyzed with a CBA kit.

Negroamaro-FGM suspended in ethanol was able to induce production of IL-10 and IL-12 from PP cells. On the other hand, Koshu-FGM suspended in water suppressed TNF- α and IL-1 β release from PP, while inducing production of IL-10 but not of IL-12. Taken together, these results point out the *in vitro* anti-inflammatory activity of both FGMs.

Effects of donkey's milk on cytokine production from human healthy PBMCs^{83,84}

PBMCs were stimulated with donkey's *colostrum* and milk that were collected from lactating mares (Martina Franca breed) at different time points; *colostrum*: 12, 24, 36, 48, 84, 60, 72, 84, 108 hrs, respectively, and milk: 45, 60, 70, 90, 101, 190, 204, 211, 213, 215, 225 days, respectively. PBMC cultures were performed according to the procedure previously described in this review. CBA kit was used for cytokine determination in supernatants.

In milk, IL-12 release by PBMCs was higher than that of *colostrum*, peaking at 60, 70, and 190 days, followed by a remarkable decreased of activity. *Colostrum*-induced TNF- α activity was undetectable, whereas in milk TNF- α peaked at 10 days with negligible values after this time. On the other hand, milk-induced release of IL-1 β was measured at each time point with a peak at 190 days, whereas *colostrum*-induced IL-1 β production was negligible. Same pattern of responses was observed with IL-10, whose production was stimulated by milk only.

A number of studies have clearly pointed out that donkey's milk for its chemical properties represents the best substitute of human milk^{85,86}. In particular, lactose is the most representative constituent of donkey's milk, being its percentage equal to 6.8 as well as in the human milk⁸⁵. It represents an optimal substrate for the intestinal flora growth in humans and, technologically, can be utilized in the preparation of probiotic solution for human use (e.g., IBD)⁸⁷. Therefore, the *in vitro* effect of milk on human PBMCs may well reflect the *in vivo* situation. Release of IL-1 β and TNF- α , counterbalanced by IL-10 production, might ensure advantageous effects both locally or systemically in the case of intestinal pathogen access into the host.

Role of Goat's milk on cytokine production from human healthy PBMCs⁸⁸

In another set of *in vitro* experiments, 1x10⁶ PBMCs/ml, isolated as previously described, were stimulated with 10 μ l (the optimal dilution observed) of goat's, [Saanen (Switzerland), Ionica (Apulia, South Italy), and a Italian commercial product]] or bovine or donkey's milk, respectively for 24 hrs at 37° C-5% CO₂. Controls were

Table 2
Anti-inflammatory effects of nutraceuticals

Nutraceuticals	Test System	Anti-inflammatory activity
Polyphenols from red wine	Normal human PBMCs <i>in vitro</i>	Production of IL-10 also enhanced by IL-6 release, thus limiting the inflammatory pathway triggered by IL-12 and pro-inflammatory cytokines
Fermented grape marc (FGM) from Negroamaro and Koshu	DDS induced colitis in mice.	Reduction of IL-1 β and TNF- α release <i>ex vivo</i> .
	<i>In vitro</i> experiments with PP cells	Production of IL-10 and reduction of TNF- α and IL-1 β release <i>in vitro</i>
Donkey's and goat's milk	Normal human PBMCs <i>in vitro</i>	Production of IL-10 and contribution to the anti-inflammatory pathway

represented by unstimulated cells. The positive control was represented by *E. coli* LPS (Sigma-Aldrich) [10 μ g/mL].

In supernatants the following cytokines were evaluated: IL-4, IL-5, IL-6, IL-10, IL-12, IL-13, IL-17, TNF- α , TGF- β and granulocyte/colony stimulating factor (G/CSF), using the multi-analyte ELISArray (SuperArray Bioscience Corporation, D.B.A. Italia, Milan, Italy), according to manufacturers' instructions. Although a broad array of cytokines was screened (IL-4, IL-5, IL-6, IL-10, IL-12, IL-13, IL-17, TGF- β and G/CSF) only three of them were measurable in PBMC-treated supernatants: IL-10, TNF- α , and IL-6, respectively. IL-10 production was maximally induced by Saanen milk, whereas Jonica, Donkey, Bovine milk and a commercial preparation were gradually less effective. As far as TNF- α production from PBMCs is concerned the goat commercial milk was the most effective of all when compared to the other milks tested.

The degree of effectiveness in the release of IL-6 progressively decreased from Bovine, Jonica, Donkey, Saanen and, finally, to commercial milk⁸⁸.

In comparison to bovine milk, goat's milk is more easily digested for its higher content in essential fatty acids and higher ratio in short and medium chain fatty acids⁸⁹ and for this reason goat's milk was introduced as a dietary integrator in Southern Europe. In addition, as a consequence of a lower content in lactose goat's milk can be helpful to subjects with intolerance to the bovine counterpart. In sum, these experiments represent the first demonstration of the capacity of various goat's milks to modulate the *in vitro* human immune response and, it is evident that goat's milk is able to promote both innate and adaptive immunity via cytokine production.

As far as cytokine release is concerned, goat's milk seems to be a poor trigger of these mediators. In fact, among a broad array of cytokines screened only TNF- α , IL-6 and mostly IL-10 were detectable. It is likely that in this experimental set-up the ELISA assay used may have represented a limitation in cytokine detection because in a previous study⁸³ using a CBA method donkey's milk could stimulate a broad spectrum of human cytokines and in higher quantities than those revealed in the present study.

On the bases of the data obtained one can speculate that TNF- α , as a proinflammatory cytokine⁹⁰, IL-6, as an acute phase reactant⁹¹ and a growth factor for B cells⁹¹, and IL-10, as an anti-inflammatory cytokine⁹², are produced in concert by effect of goat's milk thus suggesting the

maintenance of the immune homeostasis. In this respect, two recent reports have pointed out that goat's milk is able to attenuate both hapten- and DDS colitis in rats by virtue of the anti-inflammatory activities of oligosaccharides^{93,94}. Therefore, goat's milk might be introduced in the diet of patients with IBD.

The major effects of polyphenol, FGM and milks are illustrated in Table 2.

CONCLUSION

Cytokines intervene in many pathological conditions, even including IBD and food- as well as age-related diseases, such as atherosclerosis⁹⁵. Therefore, cytokines represent important drug targets to prevent and mitigate the development of a low inflammatory status, a common denominator of various pathologies⁹⁵. Quite importantly, IL-27, a product of APCs, plays a modulating role on the Th1, Th2 and Th17 responses in the course of acute or chronic inflammation⁹⁶. Recently, it has been reported the IL-27 p28 subunit requires IL-6 receptor for signaling, and blockade of this receptor with a humanized monoclonal Ab attenuates rheumatic pathologies⁹⁷.

Nowadays, the nutritional approach to prevent inflammatory, metabolic, neoplastic and aged related-diseases is one of the major strategy worldwide in favor of human health and longevity⁹⁸.

Nutraceuticals represent dietary components which may act on the cytokine network and, among them, polyphenols, dairy products as well as probiotics are innovative therapies for correcting the imbalanced immune system.

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