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Effects of kefir fractions on innate immunity

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Abstract

Innate immunity that protects against pathogens in the tissues and circulation is the first line of defense in the immune reaction, where macrophages have a critical role in directing the fate of the infection. We recently demonstrated that kefir modulates the immune response in mice, increasing the number of IgA+ cells in the intestinal and bronchial mucosa and the phagocytic activity of peritoneal and pulmonary macrophages. The aim of this study was to further characterize the immunomodulating capacity of the two fractions of kefir (F1: solids including bacteria and F2: liquid supernatant), by studying the cytokines produced by cells from the innate immune system: peritoneal macrophages and the adherent cells from Peyer's patches. BALB/c mice were fed either kefir solid fraction (F1) or kefir supernatant (F2) for 2, 5 or 7 consecutive days. The number of cytokine (IL-1α, IFNγ, TNFα, IL-6 and IL-10) producing cells was determined on peritoneal macrophages and adherent cells from Peyer's patches. Both kefir fractions (F1 and F2) induced similar cytokine profiles on peritoneal macrophages (only TNFα and IL-6 were up-regulated). All cytokines studied on adherent cells from Peyer's patches were enhanced after F1 and F2 feeding, except for IFNγ after F2 administration. Moreover, the percentage of IL-10+cells induced by fraction F2 on adherent cells from Peyer's patches was significantly higher than the one induced by fraction F1. Different components of kefir have an in vivo role as oral biotherapeutic substances capable of stimulating immune cells of the innate immune system, to down-regulate the Th2 immune phenotype or to promote cell-mediated immune responses against tumours and also against intracellular pathogenic infections.

Keywords: Cytokines; Innate immunity; Immunomodulation; Kefir; Macrophages

Introduction

Innate immunity that protects against pathogens in the tissues and circulation is mediated by a diverse variety of water soluble substances such as cytokines, chemokines, different types of antimicrobial peptides,

Abbreviations: IFN, interferon; IL, interleukin; LAB, lactic acid bacteria; TNF, tumour necrosis factor

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the complement system and a variety of effector cells, mainly phagocytes and natural killer cells (Riera et al., 2003). Considered the first line of defense in the immune reaction, macrophages have a number of important functions, including the ability to phagocytose bacteria, debris and cells, and the capacity to secrete cytokines and reactive metabolites (Jackson and Evans, 2000). Numerous studies have demonstrated the beneficial effects of lactic acid bacteria (LAB) and fermented dairy products in boosting specific or non-specific immune responses (Gill, 1998; Isolauri et al., 2004; Matar et al., 2001; Perdigón et al., 2001; Vinderola et al., 2004). Probiotic microorganisms can exert their beneficial properties mainly through two mechanisms: direct effects of the live microbial cells (probiotics) or indirect effects via metabolites of these cells (biogenics). Biogenics are defined as food components derived from microbial activity which provide health benefits without involving the intestinal microflora (Takano, 2002). The most important biogenics in fermented milks are peptides which are not present prior to fermentation. Kefir is a fermented milk (drink) produced by the action of LAB and yeasts, trapped in a complex matrix of polysaccharides and proteins. Beyond its inherent high nutritional value as a source of proteins and calcium, kefir has a long tradition of being regarded as good for health in countries where it is a staple in the diet (Farnworth, 1999). However, published human or animal feeding trials to substantiate this view or the mechanisms by which kefir exerts its beneficial effects are not numerous. Although the Russian literature contains articles describing the effects of kefir consumption, these studies are not always readily available in western countries (Farnworth and Mainville, 2003). In recent works (Vinderola et al., 2005a; Vinderola et al., 2005b) we demonstrated that kefir, as well as pasteurized kefir, was able to modulate the intestinal mucosa immune response in mice, increasing the number of IgA+ cells in the lamina propria of the small intestine in a dose dependent manner. While kefir induced a typical Th2 response by increasing the number of IgA+, IL-4+, IL-6+ and IL-10+ cells, pasteurized kefir induced simultaneously the production of some pro-inflammatory cytokines (IFNγ and TNFα) but without tissue damage. It was also demonstrated that kefir increased the phagocytic activity of peritoneal and pulmonary macrophages and in addition it was able to enhance mucosal immunity mediated by IgA in distant sites (bronchial tissue). This study was undertaken to further characterize the two main fractions of kefir (solid including bacteria and liquid supernatant) and their contribution to kefir's immunomodulating capacity by studying the cytokines produced by cells from the innate immune system: peritoneal macrophages and the adherent cells derived from Peyer's patches.

Materials and methods

Samples

Kefir (Liberty Company, Brossard, QC) was centrifuged at 2750*g* (Beckman CS 6R centrifuge) for 20 min at 4 °C to produce two fractions: F1 – the solid pellet, and F2 – the supernatant. The two fractions were freeze dried (Freezone 4.5, Labco Co.) and kept refrigerated (4 °C) until tested.

Microbiological screening of kefir fractions

Samples of fresh and freeze dried/rehydrated F1 and F2 were screened for total yeasts, total lactococci and total lactobacilli using potato dextrose agar (PDA) Media (BBL-Becton Dickinson, Cockeysville, MD, USA) with tartaric acid according to the manufacturer's specifications, M17 agar supplemented with 0.5% glucose (Difco Co., Detroit, MI, USA) and lactic acid whey (LAW) agar (www.atcc.org), respectively.

Animals and feeding procedures

A total of 6–8 week-old BALB/c female mice weighing 20–25 g were obtained from Charles River (Montreal, Canada). Each experimental group (sampled at various time) consisted of 5 mice housed together in plastic cages kept in a controlled atmosphere (temperature 22±2 °C; humidity 55±2%) with a 12 h light/dark cycle. Mice were maintained and treated in accordance with the guidelines of the Canadian Council on Animal Care.

Kefir fractions (F1 and F2) were suspended in phosphate buffered saline (PBS, pH 7.4) solution (Sigma–Aldrich, St. Louis, MO, USA) to a concentration of 0.5 mg/ml of protein (Quick Start Bradford Protein Assay, Bio-Rad Laboratories, Hercules, CA, USA). Groups of 5 mice received 200 μl/day of F1 or F2 suspension by gavage for 2, 5 or 7 consecutive days. All groups of mice received simultaneously a conventional balanced diet ad libitum and water. A control group (5 mice) received the same conventional balanced diet and water. Animals were fed in a way that all groups (2, 5 and 7 days and control) were sacrificed on the same day.

Isolation of peritoneal macrophages

The capacity of peritoneal macrophages to adhere to a glass surface was used to isolate them from the peritoneum. After each feeding period, animals were anesthetized and sacrificed by cervical dislocation. The peritoneal cavity was flushed with 5 ml of PBS containing 10 U/ml of heparin (Sigma–Aldrich, St. Louis, MO, USA) and 0.1% bovine serum albumin (BSA) (Jackson ImmunoResearch, West Grove, PA, USA).

The macrophage suspension was washed twice with the PBS buffer, and it was adjusted to a concentration of 10^6 cells/ml. In total, 1 ml of the cell suspension was placed on glass coverslips inside Leighton tubes (5% CO_2 atmosphere) and incubated for 1 h at 37 °C to allow adherence to the glass surface.

Isolation of the adherent population from Peyer's patches

Adherent cells from Peyer's patches were obtained using a modification of a previously described method (Frangakis et al., 1982). After each feeding period, animals were anesthetized and sacrificed by cervical dislocation. Small intestines were removed and placed in Hank's balanced salt solution (HBSS) (Sigma–Aldrich, St. Louis, MO, USA) containing 150 mM HEPES (Sigma–Aldrich), penicillin (100 U/ml, Sigma–Aldrich) and streptomycin (0.1 mg/ml, Sigma–Aldrich). Intestines were flushed 6 times with 10 ml of the same buffer and Peyer's patches were carefully removed and collected in buffer HBSS/HEPES/10% heat-inactivated fetal bovine serum (FBS) containing 50 mM of EDTA (Sigma–Aldrich). Peyer's patches were incubated for 15 min at 37 °C under gentle stirring to allow separation of epithelial cells. Peyer's patches were recovered, washed twice with the buffer and digested in HBSS/HEPES/10% FBS containing 1.5 mg/ml Protease Type XIV (Sigma–Aldrich) and 15 DNase I (Sigma–Aldrich) for 30 min at 37 °C under gentle stirring. The remaining tissues were removed and the supernatant was centrifuged (700*g*) for 10 min at 4 °C. The pellet was washed twice with RPMI 1640 (ATCC, Manassas, VA, USA) and adjusted to a cell concentration of $2–4 \times 10^6$ cells/ml. In total, 1 ml of the cell suspension was placed on glass coverslips inside Leighton tubes (5% CO_2 atmosphere) and incubated for 1 h at 37 °C to allow adherence to the glass surface.

Cytokine determination in adherent cells

Cells adherent to the glass surface, isolated from the peritoneum or Peyer's patches of animals that received F1 or F2, or from control animals, were gently washed with PBS to remove non-adherent cells. Adherent cells were then fixed with formalin (ICC fixation buffer, BD Pharmingen, San Diego, CA, USA) for 15 min at room temperature (RT). Cells were washed twice (5 min, at RT) with PBS, treated with a 1% blocking solution of BSA (Jackson ImmunoResearch, West Grove, PA, USA) and incubated with normal goat serum (diluted 1/20 in PBS containing 1% saponin, Sigma). Subsequent washes were done with PBS-1% saponin for 5 min at RT. The activity of the endogenous peroxidase was blocked with an H_2O_2/methanol solution (peroxidase blocking reagent, DakoCytomation, Glostrup, Denmark). Cells were washed twice and then were incubated for 15 min at RT with avidin and biotin blocking solutions (Avidin/Biotin Blockin Kit, Vector Laboratories Inc., Burlingame, CA, USA) to block the endogenous avidin and biotin; cells were washed twice between these two blocking steps. Cells were then washed twice and incubated for 1 h at RT with rat anti-mouse IL-10, IFNγ or TNFα (BD Pharmingen, San Diego, CA, USA) diluted 1:10 in cytokine ICC diluent buffer (BD Pharmingen, San Diego, CA, USA). For IL-1 alpha and IL-6, a biotinylated rabbit anti-mouse antibody (BD Pharmingen, San Diego, CA, USA) was used (diluted 1:50) for 1 h at RT. For cells treated with IL-10, IFNγ or TNFα antibodies, a biotin conjugated goat anti-rat Ig-specific polyclonal antibody (BD Pharmingen, San Diego, CA, USA) was used as secondary antibody (diluted 1:10) for 30 min at RT. Cells were washed twice and a Vectastain *Elite* ABC Kit (Vector Laboratories, Inc., Burlingame, CA, USA) was used (30 min incubation at RT) to produce the avidin and biotinylated horse-radish peroxidase macromolecular complex. Cells were washed twice and incubated for 20 min at RT with 3,3-diaminobenzidine solution (DAB substrate kit for peroxidase (Vector Laboratories Inc, Burlingame, CA, USA). The development of a brown colour was stopped with a PBS-1% saponin wash. Results were expressed as the percentage of positive cells (those that developed a brown area inside the cytoplasm) counted at $1000 \times$ magnification using a light microscope (Hund Wetzlar H600, Germany).

Statistical analysis

Data were analysed using the one-way ANOVA procedure of SPSS software. The differences among means were detected by the Duncan's Multiple Range Test (SPSS, 1996). Data were considered significantly different when $p < 0.05$.

Results

Mice received daily, by gavage, 100 μg of total protein from the solid fraction (F1) or liquid supernatant (F2) of kefir for 2, 5 or 7 consecutive days. At the end of each feeding period, adherent cells from the peritoneal cavity and Peyer's patches were removed and analysed ex vivo for cytokine production.

Microbiological analyses of F1 and F2 samples after rehydration revealed that the liquid fraction (F2) was devoid of bacteria whereas solid fraction (F1) had the same number of cocci as the fresh product, but a 1 log-order reduction in viable lactobacilli counts were measured in rehydrated F1 compared to the sample

before freeze drying. Yeasts were not able to survive freeze drying and rehydration but remained in F1 as non-viable cells.

Fig. 1 displays the pattern of cytokines induced by the solid fraction (F1) of kefir (containing live bacteria) on peritoneal macrophages. The percentage of IL-1α, IFNγ or IL-10 was not changed compared to control animals, whereas the number of TNFα+ cells significantly increased after 2 and 5 days of kefir solid phase administration and the number of IL-6+ cells significantly increased at all the feeding periods assayed. The adherent cells derived from Peyer's patches showed a greater response to the consumption of kefir solid fraction (F1) than peritoneal macrophages (Fig. 2), since all cytokines studied were up-regulated. IL-6+ and IL-10+ cells increased at all the feeding periods assayed. The percentage of IFNγ+ and TNFα+ producing cells increased after 2 days of F1 administration and IL-1α increased after 2 and 5 days. The oral administration of kefir supernatant (F2) induced a similar pattern of cytokines in peritoneal macrophages (Fig. 3) to the one induced by kefir solid phase (F1). Oral administration of F2 did cause increases in TNFα+ and IL-6+ cells after 2 and 5 days. No effect on the number of IFNγ+ cells was noticed in cells isolated from Peyer's patches of mice that received F2 (Fig. 4). The percentage of TNFα+ cells increased only after 2 days of F2 administration, whereas IL-1α production was enhanced but returned to control values after 7 days of F2 feeding. The percentage of IL-10+ cells increased by the fifth day of F2 administration. The percentage of IL-6+ cells increased for all the days assayed.

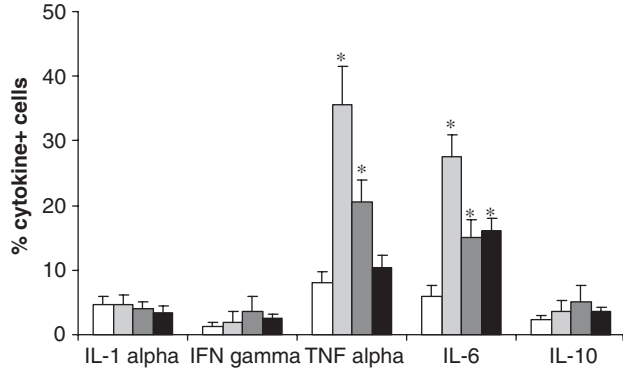

Fig. 1. Effect of the oral administration of the solid phase (F1) of kefir for 2 (▒), 5 (■) or 7 (■) consecutive days on cytokine production by peritoneal macrophages. *Significantly different from control (□) ($p<0.05$). Bars represent mean of 5 animals per time period with standard deviation.

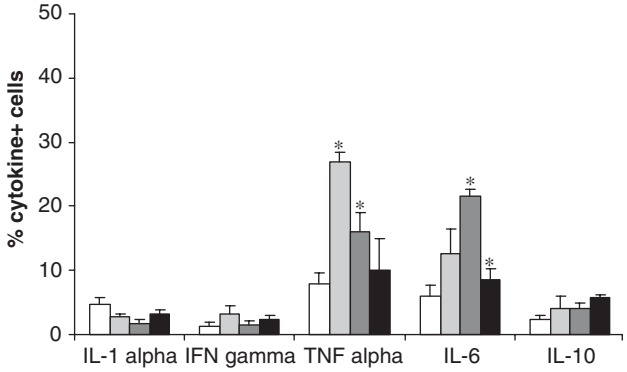

Fig. 3. Effect of the oral administration of the liquid phase (F2) of kefir for 2 (▒), 5 (■) or 7 (■) consecutive days on cytokine production by peritoneal macrophages. *Significantly different from control (□) ($p<0.05$). Bars represent mean of 5 animals per time period with standard deviation.

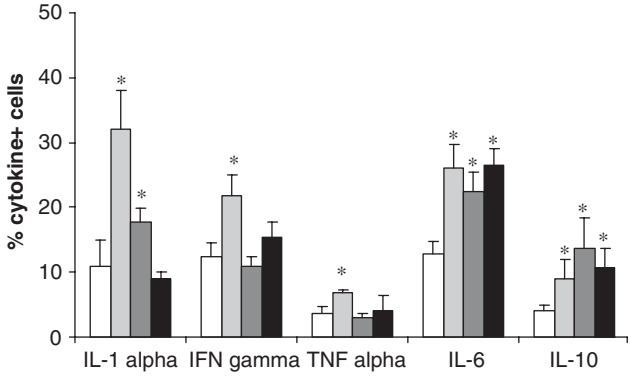

Fig. 2. Effect of the oral administration of the solid phase (F1) of kefir for 2 (▒), 5 (■) or 7 (■) consecutive days on cytokine production by the adherent cells derived from Peyer's patches. *Significantly different from control (□) ($p<0.05$). Bars represent mean of 5 animals per time period with standard deviation.

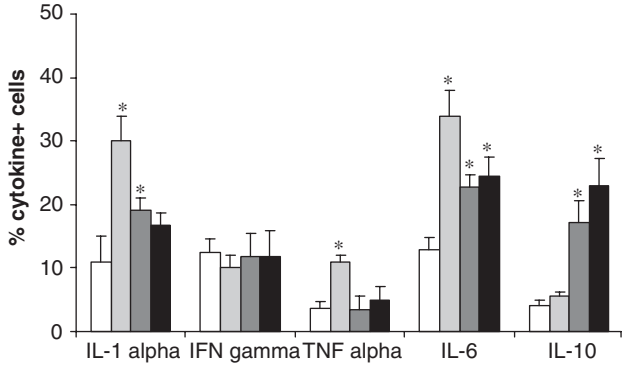

Fig. 4. Effect of the oral administration of the liquid phase (F2) of kefir for 2 (▒), 5 (■) or 7 (■) consecutive days on cytokine production by the adherent cells derived from Peyer's patches. *Significantly different from control (□) ($p<0.05$). Bars represent mean of 5 animals per time period with standard deviation.

When we compared the effects of F1 and F2 on the production of cytokines by the adherent cells of the peritoneal cavity and Peyer's patches, we observed that the levels of TNFα (day 2) and IL-6 (day 2 when compared with day 5) induced by the fraction F1 on peritoneal macrophages were statistically higher ($p<0.05$) than the ones induced by the non-bacterial fraction F2. Fraction F1 also induced statistically higher ($p<0.05$) levels of IFNγ than F2 on adherent cells isolated from Peyer's patches. On the contrary, the fraction F2 induced a higher ($p<0.05$) proliferation of TNFα+ (day 2), IL-6+ (day 2) and IL-10+ cells (day 7) than F1 on cells derived from Peyer's patches. No statistically different results were observed for the percentage of IL-1α+ cells induced by F1 or F2 in peritoneal macrophages or adherent cells from Peyer's patches.

Discussion

The kefir ecosystem is composed of bacteria, yeasts, polysaccharides (kefiran), remaining proteins from milk fermentation and metabolites (peptides for example) produced by the action of live microorganisms on milk proteins during the fermentation process (Farnworth and Mainville, 2003). In this study, fresh kefir was split into two fractions: the solid fraction (F1) and the supernatant fraction (F2). These two fractions were freeze dried and used to study their effect on the release of cytokines by cells that provide innate immunity: peritoneal macrophages and adherent cells derived from Peyer's patches. Some of the bacteria present in the kefir microbiota are encapsulated by a polysaccharide, called kefiran (Farnworth and Mainville, 2003). The fraction F2 was composed of milk sugars and proteins as well as new metabolites produced during milk fermentation such as bioactive peptides (Matar et al., 2000) and also the water-soluble kefiran (Farnworth and Mainville, 2003). A role of the soluble bacterial DNA, possibly present in fraction F2, in the immunomodulation observed must be also acknowledged, since it was reported that bacterial DNA modulated the immune response by a decrease of IL-1β and an increase of IL-10 (Lammers et al., 2003; Jijon et al., 2004). Although most studies of the immunoactive components of Gram (+) bacteria have focused on cell wall components, other factors may be involved. It is well known that peptidic fractions released during milk fermentation are bioactive (Hoerr and Bostwick, 2000; LeBlanc et al., 2002; Korhonen and Pihlanto, 2003). Moreover, water-soluble polysaccharides can be immunostimulatory as has been described in bifidobacteria (Hosono et al., 1997).

In vivo immunomodulating activities of LAB and fermented dairy products containing LABs may be attributed, in part, to altered production of cytokines, which play pivotal roles in coordinating the immune function. In particular, macrophages play their own role in cell-mediated and humoral immunity through the release of different cytokines (Marin et al., 1998). Kefir solids (F1) orally administered to mice induced the production of pro-inflammatory cytokines such as TNFα and IL-6 but not IL-1α, IFNγ or the regulatory IL-10 in peritoneal macrophages. IL-6 is synthesized by macrophages and other cells in response to TNFα among other signals (Abbas et al., 1994). Tejada-Simon and Pestka (1999) reported the in vitro production of TNFα and IL-6 when viable bacteria were put in contact with a cell line of macrophages, whereas the same authors reported simultaneously that only IL-6 was produced by peritoneal macrophages in vivo. Different lactobacilli are able to differentially modulate the immune response (Tejada–Simon and Pestka, 1999; Cross et al., 2004) without enhancement of IFNγ production by macrophages. This agrees with our finding that cytokines are produced by peritoneal macrophages, while other authors have reported an enhanced production of this cytokine followed the administration of LAB (Kitazawa et al., 1994; Miettinen et al., 1999; Cross et al., 2001; Perdigón et al., 2002). The lack of IFNγ production by peritoneal macrophages observed in this study after the oral administration of kefir solids (containing bacteria) might be due to the fact that some encapsulating exopolysaccharides (viz. kefiran) might inhibit some macrophage responses, since neutral sugars present in exopolysaccharides can interfere with the mannose receptor on macrophages (Stout et al., 1992; Pasquier et al., 1997). Moreover, the host response (pro-inflammatory versus regulatory cytokines) to stimuli by LAB is dependent on the strains of bacteria used (Cross, 2002). The production of the pro-inflammatory cytokines IL-1 and IFNγ during the course of pathogenic infections has been reported before (Kaiser et al., 2000; Dornand et al., 2002). IFNγ directs naïve T cells down a Th1 pathway and activates macrophages for enhanced killing of intracellular pathogens (Husband et al., 1999). The absence in this study of enhanced production of IL-1α and IFNγ by peritoneal macrophages could be related to a mild activation of these cells by kefir, since no pathogenic challenge was used in this murine model. TNFα is known to enhance the expression of accessory molecules involved in the adhesion of macrophages to the endothelium, increasing the possibility that other memory cells present in the circulation system are guided to the inflammation site (Roitt and Delves, 2003). In this sense, the enhanced TNFα production by peritoneal macrophages would be better preparing them to access the inflammation site during an infection.

The increase of the pro-inflammatory cytokines IL-1α, IFNγ and TNFα produced by adherent cells derived from Peyer's patches after F1 administration was rapidly

controlled by the increase of the regulatory IL-10, perhaps as a way to avoid tissue damage at the intestinal mucosa. However, IL-6, with its role in the terminal differentiation of B lymphocytes to IgA secreting cells (Goodrich and McGee, 1999), increased and remained higher than control values throughout all the feeding periods assayed. The phospholipid sphingomyelin, present in kefir, has been pointed out as the substance responsible for enhancement of IFNγ production after kefir administration (Osada et al., 1993). After exposure of macrophages to granulocyte-macrophage colony-stimulating factor and IL-4, they can differentiate into functional dendritic cells (Makala et al., 2003). In this sense, the adherent population of Peyer's patches is composed of a complex mixture of cells going from macrophages to dendritic cells at different states of maturation. The production of all cytokines assessed in this study might also be explained by the presence of a highly diverse cell population in the adherent cells isolated from Peyer's patches. These results support the results obtained in a previous study (Vinderola et al., 2005a) and extend the understanding of how immune mechanisms are induced by kefir.

A similar effect to the one produced by the fraction F1 on peritoneal macrophages was observed on this cell population when kefir supernatant (F2) was orally administered to mice. The same pattern of cytokines was up-regulated (TNFα and IL-6) while no effect was observed on IL-1α, IFNγ or IL-10. The absence of IFNγ production by F2 administration might be due to the soluble nature of the antigen (MacMicking, 2004). In spite of their different nature, Gram(+) bacteria (F1) and metabolites present in kefir supernatant (in F2) induced similar biological effects on peritoneal macrophages since the same pattern of cytokines was observed.

In the adherent cells derived from Peyer's patches, the increases of IL-1α and TNFα were also rapidly down-regulated to values similar to those of controls by the increase of IL-10 when F2 was administered. The mode of action of kefir fractions differed in several aspects in relation to the modulation of the innate response. F1 induced a more pro-inflammatory profile than F2, especially on peritoneal macrophages (higher levels of TNFα and IL-6) but also on cells derived from Peyer's patches (higher levels of IFNγ). On the other hand, the fraction F2 induced higher levels of TNFα and IL-6, but especially IL-10, on cells isolated from Peyer's patches than F1. The higher levels of IL-10 found after the administration of kefir supernatant are of potential significance due to the major role of IL-10 in the regulatory network of cytokines controlling mucosal tolerance, and it has been proposed as a potent anti-inflammatory biological therapy in chronic IBD (Braat et al., 2003). These differences in behaviour might also be related to the different nature of the antigens administered: soluble in F2 versus particulate in F1 (Shanahan, 2000). The differences in cytokine responses between peritoneal macrophages and adherent cells from Peyer's patches could be also due to the differential sites of interaction of oral antigens; the immune cells located in the intestinal site could be more activated than peritoneal macrophages due to the differential expression of pattern recognition receptors (Karlsson et al., 2004). Peritoneal macrophages were activated by the cytokines released by the immune cells from the gut and not directly by the antigenic substance administered. This stimulation can increase the phagocytic activity of peritoneal or pulmonary macrophages, as we demonstrated in a previous work (Vinderola et al., 2005a). However, this kind of activation by innocuous antigens is not enough to induce a large activation of peritoneal macrophages with a concomitant enhancement of cytokine release.

Antigen-presenting cells such as monocytes, macrophages and dendritic cells are responsible for detecting microbes and presenting their antigenic structures to T cells, thus eliciting acquired immune responses. In addition, monocytes and macrophages kill microoganisms by phagocytosis and produce pro-inflammatory cytokines (Karlsson et al., 2004). It is important then to keep this immunosurveillant system in an active state. In this work, kefir has been shown to be able to achieve this by acting on the cell populations involved in this task. The induction of pro-inflammatory cytokines by kefir administration without tissue damage, as previously reported (Vinderola et al., 2005a) contributes to maintaining the state of controlled inflammation that takes place under normal conditions observed in the gut mucosa (Monteleone et al., 2002). The present paper begins to identify the different components of kefir which might have multiple in vivo roles as oral biotherapeutic substances capable of stimulating immune cells of the innate immune system such as NK cells. In this way, kefir may be able to down-regulate the Th2 immune phenotype or to promote cell mediated immune responses against tumours and also against intracellular pathogenic infections.

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