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Original article

Intestinal immune-potentiation by a purified alkylglycerols compound

Makoto-Kehchs Kantah[1], Hiroshi Wakasugi[1], Archana Kumari[2], Pedro Carrera-Bastos[3], Beniamino Palmieri[4], Yasuhiro Naito[5], Roberto Catanzaro[6], Ryuichi Kobayashi[1], Francesco Marotta[7]

[1] Suheiro Chem-Tech Center, Kumamoto, Japan; [2] Université du Québec, Institut National de la Recherche Scientifique, Centre Eau, Terre and Environnement, Québec, Canada, [3] Center for Primary Health Care Research, Faculty of Medicine, Lund University, Malmö, Sweden; [4] Dept. of Surgery, University of Modena, Italy; [5] Immunology Research Center and Clinics, Nagoya, Japan; [6] Gastroenterology Unit, Dept. of Internal Medicine, University of Catania, Italy; [7] ReGenera Research Group for Aging Intervention, Milano, Italy

Abstract. Alkylglycerols have shown immune stimulant and adjuvant activity in several studies and the aim of the present research was to assess in particular the effect of shark liver-derived alkylglycerols on gut immune system. C57BL/6 mice, fed under specific pathogen free conditions, were randomly divided into two groups: a) fed normal laboratory food or b) added with alkylglycerols (2 mg/day/mouse) for 3 weeks. Intraepithelial lymphocytes (IEL) were retrieved from the small intestine and tested for NK and tumor cytotoxicity. Lymphocytes from liver, spleen and IEL were also assessed as for their counting and phenotypic characterization. Under supplementation with alkylglycerols, the number of lymphocytes yielded by the small intestine increased by to almost 40%. Moreover, the ratio of $CD8\alpha\beta^{+}TCR\alpha\beta^{+}$ cells/$CD8\alpha\alpha^{+}TCR\alpha\beta^{+}$ cells remarkably increased. In parallel with this reshaping in the distribution of lymphocyte subsets, tumor cytotoxicity of IEL against P815 cells and cytokine production from circulating lymphocytes were also enhanced. These data show that phylogenetically developed lymphocytes ($CD8\alpha\beta^{+}$, $TCR\alpha\beta^{+}$) were significantly activated by the oral administration of alkylglycerols. The present results indicate that purified alkylglycerols might have such significant potential via the enhancement of intestinal immunity, especially in the small intestine.(www.actabiomedica.it)

Key words: alkylglycerol, gut immunity, sharek liver

Introduction

Fish oils are better known for their n-3 polyunsaturated fatty acids (PUFA) content. Nevertheless, they contain several other active compounds that modify cell activity and influence various physiological functions. Shark liver oils, despite containing relatively low amounts of n-3 PUFA, are rich in alkylglycerols and squalene. Alkylglycerols may control immune response possibly throw modification of platelet activating factor (PAF) and diacylglycerol production. Alkylglycerols are structurally different from the ester and ether derivatives of lysophospholipids, however they all show ability to activate macrophages and even small doses of these agents can enhance macrophage activity (1). Indeed, the administration of alkyl derivatives to mice significantly enhances ingestion activity of macrophages within 48 hours. Accordingly, in *in vivo* studies, the administration of very small doses (less than 100 ng/mouse) to mice are sufficient to induce a greatly enhanced macrophage ingestion activity (2). Also *in vitro* experiments exposing cultured

peritoneal cells with a very low concentration (50 ng/ml) of dodecylglycerol, demonstrated that this alkylglycerol is one of the most potent macrophage stimulators by inducing a significant activation in 2-3 hours. Such efficient mechanism of ingestion of macrophages involves the participation of non-adherent and adherent cells by a stepwise exchange of signaling factors. Therefore, it is likely that dodecylglycerol treatment of B-cells triggers initiation of development of macrophage ingestion capacity. Since macrophages are antigen-presenting cells, treatment of animals with these agents potentiates host immune systems and have been suggested to possess antitumor activity through different mechanisms, ie., induction of apoptosis of neoplastic cells, suppression of signal transduction, inhibition of angiogenesis and promotion of transmembrane transport of cytotoxic agents (3). On the other hand, they have an extremely safe profile and administration of large doses of alkylglycerols and alkyldiacylglycerols, whatever the route either oral, subcutaneous, intramuscular and intraperitoneal, have shown no adverse effects in animal toxicity studies (4). It is also known that alkylglycerols when orally administered. are rapidly incorporated at different concentrations into several organs and tissues as metabolites, namely alkyldiacylglycerols and alkylphospholipids (5). For instance, the relative concentration of alkylglycerols to the above metabolites is high in the stomach (82%), brain (40%) and intestine (18%). From the metabolic viewpoint, alkylglycerols are phosphorylated by an ATP:alkylglycerol a phosphotransferase to form 1-*O*-alkyl-2-lyso-*sn*-glycerol-3- β phosphate. Via this pathway, the alkylglycerols from dietary intake or resulting from metabolism enter the biosynthetic pathways responsible for the production of structural lipids of cellular membrane bilayers and precursors of lipid mediators including PAF. Such a modulation of PAF synthesis might favour a number of beneficial biological actions such as the stimulation of cells involved in immunological responses (6). Cumulative evidence has revealed that the potentiation of intestinal immunity is extremely important for immunological tolerance, anti-tumor effects, innate immunity against intracellular pathogens. Given that two-third of immune system is located within the gastrointestinal tract, the aim of the present study was to assess the potential benefit of alkylglycerol administration on intestinal immunity.

Materials and Methods

C57BL/6 (B6) mice at the age of 8-10 weeks were used in this study. All mice were fed under specific pathogen free conditions. Animals were individually housed in a temperature- and humidity-controlled room (23±1°C and 55±5%, respectively), under a 12-h light/dark cycle for one week before use in the experiment. Afterwards, they were randomly divided into two groups: a) fed normal laboratory food or b) added with alkylglycerols, as specified below.

Oral administration of alkylglycerols

Alkylglycerols (kindly donated by EuroHealth International) was mixed with standard chow food and animals were supplemented as to assure they would ingest 2 mg of alkylglycerols/day. The supplementation lasted 3 weeks while control mice received unsupplemented standard food.

Intraepithelial lymphocyte preparation. Mice anesthetized with ether were sacrificed by total bleeding from the incised axillary artery and vein. The organs to be used for the experiments were removed and lymphocytes were obtained as follows. Hepatic lymphocytes were isolated as previously described (7). Briefly, the liver was pressed through 200-gauge stainless steel mesh and suspended in Eagle's MEM medium supplemented with 5 mM Hepes and 2% heat-inactivated newborn calf serum. After being washed once with the medium, the cells were fractionated by centrifugation in 15 mL of 35% Percoll solution (Amersham Biosciences, Uppsala, Sweden) for 15 min at 440 X g. The resulting pellet was resuspended in erythrocyte lysing solution (155 mM NH_4C1, 10 mM $KHCO^3$ 1 mM EDTA-Na, and 17 mM Tris, pH 7.3). Splenocytes were obtained by forcing the spleen through 200-gauge stainless steel mesh and were treated with 0.2% NaCL solution to remove erythrocytes.

Intraepithelial lymphocytes (IEL) were retrieved from the small intestine as recently elsewhere de-

scribed (8). Briefly, the small intestine was removed through an abdominal incision and quickly flushed with cold PBS (pH 7.2) to eliminate any luminal contents. The mesentery and Peyer's patches were then resected and collected in Petri dishes containing saline solution. The tissues were then embedded in Jung tissue freezing medium (Leica Microsystem, Nussloch, Germany), immediately frozen in liquid nitrogen and stored at -80°C until use. To block endogenous peroxidise activity, the sections were treated with 9% H_2O_2 in PBS for 1h at RT and washed in PBS containing 0.1% bovine serum albumin. The intestine was sectioned longitudinally and cut into 1-2 cm fragments. These fragments were incubated for 15 min in 20 mL $Ca^{2+}$and Mg^{2}-free Dulbecco's PBS containing 5 mM EDTA, in a 37°C shaking-water bath. The supernatant was then collected. The cell suspensions were collected and centrifuged in a discontinuous 40%/80% Percoll gradient at 830 X g for 25 min. Cells from the 40%/80% interface were collected.

Assessment of Intestinal Immune System Activity

In vivo activity was measured as described previously (9). Suspensions of Peyer's patch cells in RPMI-1640 medium (Gibco, Grand Island, NY) supplemented with 5% fetal bovine serum (RPMI 1640-FBS) were prepared from the small intestine of mice who had been orally administered alkylglycerols or normal food (control). Peyer's patches were digested for 20 min at 37°C in Hank's medium containing 0.150 μg/ml collagenase VIII (Sigma), 5 μg/ml DNase (Sigma), 5% fetal bovine serum and 5 mM EDTA using gentle agitation. Peyer's patches were then mechanically dissociated through a metallic sieve. Aliquots (200 μl) of the cell suspension (1-2 x 10^6 cells/ml in RPMI 1640-FBS) of Peyer's patch cells were cultured in 96-well fiat bottom microtiter plates for 6 days at 37°C in a humidified atmosphere of 5% $C0_2$-95% air.

The fluorescence intensity was measured by Fluoroscan II at an excitation wavelength 544 nm and an emission wavelength of 590 nm. The modulatory activity on the intestinal immune system was expressed as fluorescence intensity compared to that of control. *In vivo* activity was obtained from five experiments in all case.

Phenotypic characterization of lymphocytes by Inmunofluorescence. Before specific staining, nonspecific binding was blocked with 2% human serum in PBS containing 0.5% bovine serum albumin and 0.05% Triton X-100 for 1 h at room temperature. Standard flow cytometric analysis was performed as previously described (10). FITC-conjugated anti-CD3 (145-2C11), anti-CD8α (53-6.7), PE-conjugated anti-NKI.I (PK136), antiIL-2Rβ (TM-β1), anti-CD45R/B220 (RA3-6B2), anti-CD4 (PM4-5), anti-CD8β(53-5.8), biotinconjugated anti-TCRαβ (H57-597), anti-TCRγδ mAbs (GL3) and their isotype controls were obtained from PharMingen (San Diego, CA). Biotin-conjugated reagents were developed using Tricolor conjugated streptavidin.

Cytotoxicity assay. Cytotoxicity assay was performed as elsewhere described (10). YAC-1 and P815 target cells were labeled with sodium [^{51}Cr] chromate for 2 h and washed three times with RPMI-1640 medium supplemented with 10% fetal calf serum (FCS). P815 target cells were preincubated with anti-TCR αβ (H57-597, 1 μg/mL), anti-TCRγδ (GL3, 1 μg/mL), and anti-CD3ε (145-2C11, 1 μg/mL), respectively. Effector cells were serially diluted and mixed with [^{51}Cr]-labeled target cells (1 X 10^4 cells) in a 96-well microculture plate. The plate was centrifuged and incubated for 4 h at 37°C. At the end of the culture, 100 mL of supernatant was extracted and counted in a gamma counter.

Quantification of cytokines production from circulating lymphocytes. Sera obtained from each mouse were used to detect the concentration of interleukin-2 (IL-2) and tumor necrosis factor alpha (TNFα) using Cytometric Bead Array (CBA) kits (BD Biosciences, San Jose, CA) according to the manufacturer's instructions. Briefly, 50 μl of samples was added to a mixture of 50 μl each of capture antibody-bead reagent and detector antibody-phycoerythrin (PE) reagent. The mixture was subsequently incubated for 3 h at room temperature in the dark, and washed to remove unbound detector antibody-PE reagent. Following acquisition of sample data by flow cytometry using a FACScan (FACSCalibur™ Flow cytometer, BD Biosciences, Franklin Lakes, USA), the sample results

were generated in graphical and tabular formats using BD CBA Analysis Software (BD Biosciences). All values were calculated upon the negative control in each assay. The cytokine level was set as 0 pg/ml if the fluorescent signal of a sample was equal to or below the fluorescence of the negative control,

Statistics

All results are expressed as the mean ± S.E. The differences between the control and the treatments in these experiments were tested for statistical significance by Student's t-test. The differences between the treatments were tested by Scheffe test. A value of $P < 0.05$ was considered to indicate statistical significance.

Results

Lymphocyte counting and subset analysis: effect of the administration of alkylglycerols

The number of lymphocytes in the liver and spleen was not affected by the oral administration of alkylglycerols, whereas that one in the small intestine (IEL) increased to almost 40% ($p < 0.01$) (Fig. 1).

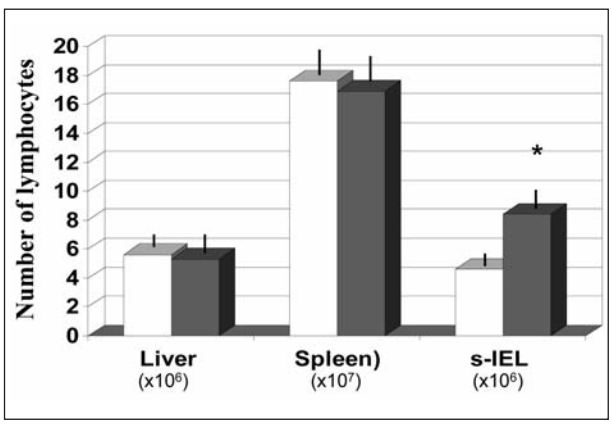

Figure 1. Total lymphocytes yield from different organs: effect of alkylglycerols supplementation.
Legend: Number of total lymphocytes yielded by the liver, spleen and small intestine in mice fed with alkylglycerols. White bars: unsupplemented animals; grey bars: animals supplemented with alkylglycerols. *p<0.05 vs control

In order to study the distribution of lymphocyte subsets in various immune organs, two-color staining for CD3 and IL-2Rβ and for CD3 and NK1.1 were performed (Fig. 2A). Mice fed with alkylglycerols were examined on day 14 after supplementation. In the liver, $CD3^-IL\text{-}2\beta^+$ were NK cells, $CD3^-$ and $IL\text{-}2R\beta^+$ were extrathymic T cells and $CD3^+IL\text{-}2R\beta^-$ were conventional T cells of thymic origin as reported by Watanabe et al. (12). NK cells and extrathymic T cells were abundant in the liver, but these subsets were few in the spleen. The distribution pattern of these lymphocyte subsets in the liver and spleen was not affected by the administration of alkylglycerols. Regarding the small intestine, $CD3^+IL\text{-}2R\beta^-$ (mainly αβT cells) and $CD3^+IL\text{-}2R\beta^+$ (mainly γδT cells) were represented. This pattern was also unaffected by the administration of alkylglycerols. A similar staining pattern without modification by the supplementation was also produced by two-colour staining for CD3 and

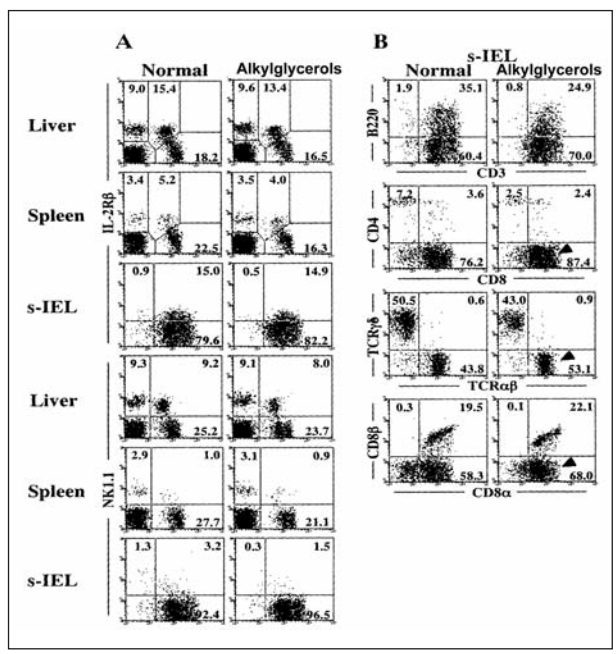

Figure 2. Phenotypic characterization of lymphocytes under alkylglycerol supplementation.
Legend: Phenotypic characterization of lymphocytes by two-colour immunofluorescence tests. A. Two-colour staining for CD3 and IL-2Rβ (or NK1.1), B. Two-colour staining for various combinations. Numbers in the figure represent the percentages of fluorescence-positive cells in corresponding areas, expressed as a mean of three separate experiments

NK1.1 (Fig. 3A bottom). CD3 NK 1.1$^+$ were NK cells and CD3$^-$ NK 1.1$^+$ were NKT cells (approximately 50% of extrathymic T cells were NKT cells).

To further characterize the phenotype of lymphocyte subsets, different stainings were performed with particular interest for the small intestine (Fig. 2B). Among IEL in the small intestine (s-IEL), B220 1T cells were present. Their level decreased after the administration of alkylglycerols (35.1%-24.9%). Two-colour staining for CD4 and CD8 showed that the proportion of CD8T cells increased in alkylglycerols-supplemented animals. This population was found to contain high proportions of TCRαβ$^+$ cells and CD8α$^+$CD8β$^+$ cells (i.e., CD8 αα homodimer cells).

Three-colour staining for CD8α, CD8β and TCRαβ (or TCRγδ) was then carried out to examine the distribution of TCRαβ$^+$ and TCRγδ$^+$ cells among CD8α$^+$β$^+$ cells and CD8α$^+$β$^-$cells in the small intestine (Fig. 3) and the proportion of TCRαβ$^+$ cells and TCRγδ$^+$ cells was estimated by gated analysis. In normal mice, nearly 60% of the CD8αβ cells were TCRαβ$^+$ and 40% of them appeared to be TCRγδ$^+$. On the other hand, approximately 30% of the CD8αα

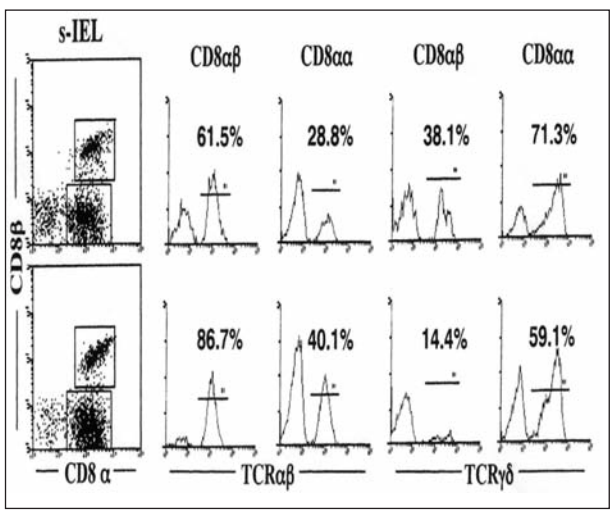

Figure 3. Phenotypic characterization of lymphocyte subsets in the small intestine
Legend: IEL were isolated from the small intestine in mice fed with alkylglycerols. Three-color staining for CD8α, CD8β and TCRαβ (or TCRγδ) was conducted. By gated analysis, the expression of TCRαβ and TCRγδ was estimated in CD8αβ cells and CD8αα cells. Numbers in the figure represent the percentage of fluorescence-positive cells in corresponding areas

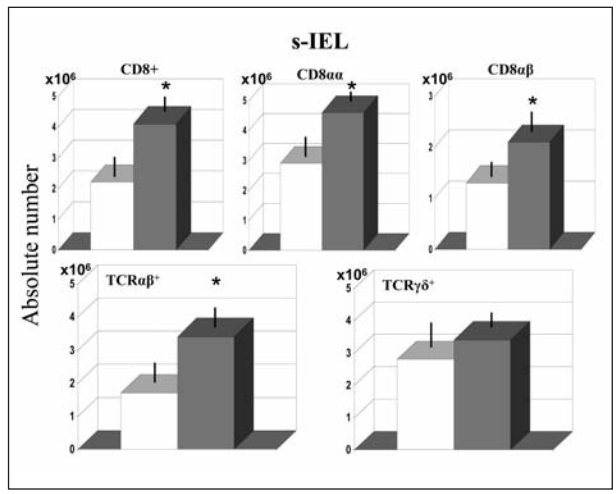

Figure 4. Variation of absolute number of intra-epithelial lymphocyte subsets: effect of alkylglycerols supplementation
Legend: Comparisons of the absolute number of lymphocyte subsets in the small intestine in mice fed with alkylglycerols. White bars: unsupplemented animals; grey bars: animals supplemented with alkylglycerols. *p<0.05 vs control

cells were TCRαβ$^+$ and 70% of them were TCRγδ$^+$. The administration of alkylglycerols changed this distribution pattern, namely, the proportion of TCRαβ$^+$ cells increased and that of TCRγδ$^+$ cells decreased among both CD8αβ cells and CD8αα cells.

These experiments were repeated three times and the absolute numbers of lymphocyte subsets were calculated accordingly (Fig. 5). The number of whole CD8$^+$ cells significantly increased following the administration of alkylglycerols ($p < 0.01$). Among CD8$^+$ cells, the increase in the number of CD8αα cells was far more remarkable than that of CD8αβ cells. The number of TCRαβ$^+$ cells significantly increased ($p<0.05$) whereas that of TCRγδ$^+$ cells showed a non-significant trend increase.

Cytotoxicity of IEL in the small intestine: effect of alkylglycerols

NK cytotoxicity assays against YAC-1 cells and tumor cytotoxicity against P815 cells were carried out. The administration of alkylglycerols did not exert any significant effect on NK cytotoxicity nor changed the pattern of a comparably stronger cytotoxicity from lymphocytes isolated from the liver (data not shown).

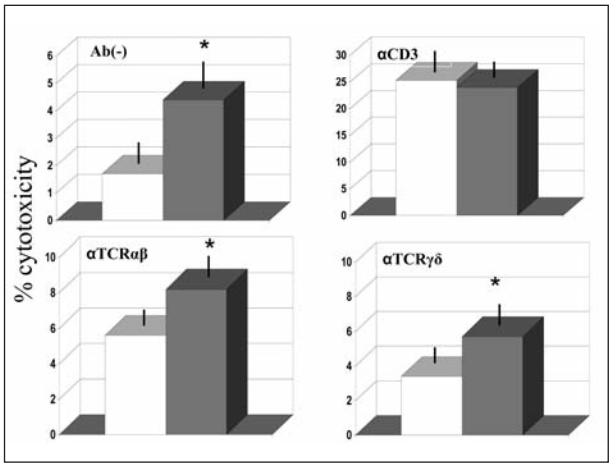

Figure 5. Tumor cytotoxicity of s-IEL against P815 myeloma cells.
Legend: Tumor cytotoxicity experiments tested in IEL from the small intestine and targeting P815 myeloma cells White bars: unsupplemented animals; grey bars: animals supplemented with alkylglycerols. *p<0.05 vs control

On the other hand, tumor cytotoxicity experiments tested in IEL from the small intestine and targeting P815 myeloma cells showed a significant change in mice fed with alkylglycerols (Fig. 5). Infact, apart from anti-CD3 mAb, IEL isolated from supplemented mice showed significantly increased tumor cytotoxicity ($p < 0.05$).

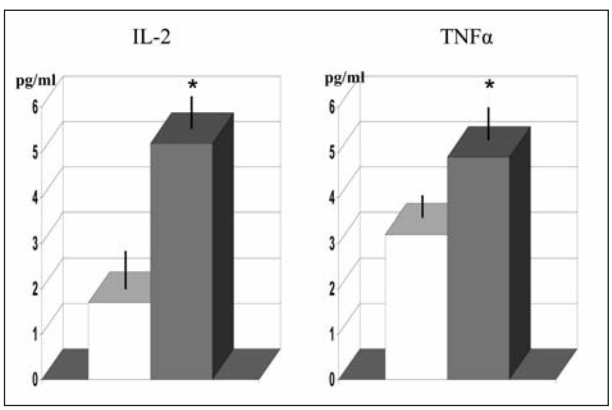

Figure 6. Cytokine production from circulating lymphocytes in mice fed alkylglycerols.
Legends: Lymphocytes were isolated from mice fed with alkylglycerols. Experiments were performed in triplicate and data are expressed as the mean and one SD. White bars: unsupplemented animals; grey bars: animals supplemented with alkylglycerols. *p<0.05 vs control

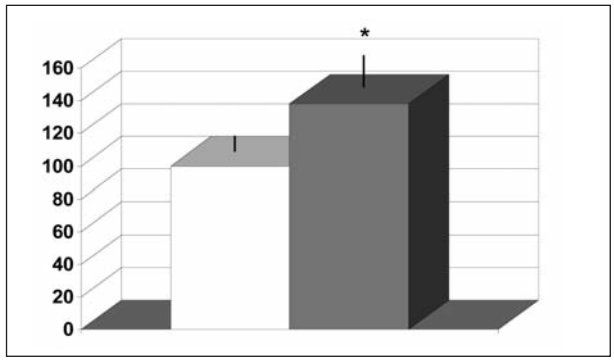

Figure 7. Intestinal Immune System Modulatitng Activity.
Legends: Ex-vivo intestinal immune system modulating activity under alkylglycerols oral administration. Baseline control value, measured in animals fed distilled water, was arbitrarily considered as 100. White bars: unsupplemented animals; grey bars: animals supplemented with alkylglycerols.
*p<0.01 vs control

The levels of IFN-γ, IL-4 and IL-5 were also examined, but were found to be unchanged by the oral administration of alkylglycerols (data not shown). On the other hand, lymphocyte secretion of IL-2 and TNF-a significantly increased in mice fed diet added with alkylglycerols as compared to levels observed in mice without supplementation (fig. 6, $p < 0.05$).

Ex Vivo evaluation of Intestinal Immune System Activity

The admininistration of alkylglycerols showed a significant increase of fluorescence intensity (fig. 7, $p < 0.01$)

Discussion

Fish oil beneficial effects have been investigated in a number of animal disease models as well as human studies. Indeed, fish oils supplementation has been widely used in prevention and treatment of the diseases in humans, although most of the research has been focused on n-3 PUFA. The main effects of shark liver oil are the result of the biological activity of squalene and 1-O-alkylglycerols, which dominate in the composition of the oil quantitatively.

1-*O*-Alkylglycerols, naturally occurring lipids are found in remarkable quantities in hematopoietic or-

gans such as bone marrow and in milk. They are especially abundant in the liver of several species of sharks, whose liver oil may contain as much as 50% alkylglycerols (11, 12). In mammals, alkylglycerols from dietary sources are absorbed without cleavage of their ether bond, and are used as precursors of membrane phospholipids in different tissues (13). Shark liver oil has been traditionally used in Scandinavian medicine against overall frailty and for wound healing. Studies have been performed to confirm and establish the therapeutic properties of these compounds and beneficial effects in cancer treatment, such as preventive action of alkylglycerols on radiotherapy side effects, including leukopenia and thrombocytopenia, have been reported (14-16) although the molecular basis of such effects are still poorly understood. One possible mechanism is the incorporation of alkylglycerols into pools of platelet-activating factor (PAF) precursor and subsequent modification of PAF biosynthesis. Indeed, dietary alkylglycerols are incorporated into 1-*O*-alkyl-2-acyl-*sn*-glycero-3- (RAcylGro*P*Etn) and 1-*O*-alkyl-2-acyl- *sn*-glycero-3-phosphocholine (RAcyl-Gro*P*Cho) in rat intestinal mucosal cells and in other organs (12, 17). This incorporation into 1-alkyl-phospholipids is of particular interest because it represents the pool of precursors for biosynthesis of platelet-activating factor (PAF), which is an important mediator on various cell types and systems. Accordingly, Lewkowicz et al. (18) have demonstrated that by administering shark liver oil supplementation in high doses (3.6 g of squalene, 3.6 g of alkylglycerols and 750 mg of n-3 polyunsaturated fatty acids per day for 4 weeks) in 13 volunteers, it occurred an increased bactericidal response of neutrophils, increased level of C4 complement fraction and the predominance of Type I cytokine IFN-γ, TNF-α and IL-2 production by peripheral blood mononuclear cells.

In our study, the increase in the number of lymphocytes in the small intestine was prominent following the oral administration of alkylglycerols. However, relative enrichment of CD8αβ$^+$ TCRapt cells was rather peculiar. On the other hand, the proportion of CD8αα$^+$ TCRγδ$^+$ cells showed a slight decline (Fig. 4). These results suggest that phylogenetically developed T cells in the intestine can be efficiently activated by alkylglycerols. Interestengly, alkylglycerols have been shown to activate macrophages *in vivo* (19). However, this took place only in the presence of non-adherent B an T cells *in vitro*, thus suggesting multifactorial cell-to-cell interactions (20). Direct stimulating activity of alkylglycerols on calcium signaling in human Jurkat T lymphocytes has also been observed *in vitro* (21). It is known that IEL located in the small intestine exert tumor cytotoxicity properties against P815 myeloma cells and this cytotoxicity is enhanced by anti-CD3, antiTCRαβ and anti- TCRγδ mAbs coated on assay plates (22). In this regard, in our study we have shown that small amounts of alkylglycerols, quite comparable to the ones recommended in humans, were able to potentiate intestinal immunity. In particular, the number of lymphocytes yielded by the small intestine increased and the tumor cytotoxicity against P815 cells was significantly increased by the oral administration of alkylglycerols.

These results are also related to the enhacement of tumor cytotoxicity and the increased production of TNF-a and IL-2. In contrast, CD8aa+ (CD8 homodimer) cells and TCRyb+ cells are known to be the most primitive form of lymphocyte subsets in phylogeny. Overall, it would appear that alkylglycerols may be effective for the potentiation of intestinal immunity and expected to act in a similar manner also in humans although studies are awaited to further elucidate its mechanisms as well the whole potentiality in clinics. Indeed, the human bacterial flora may be a further relavant factor in modulating this action. This is because less that a decade ago, Chorostowska-Wynimko et al. (23) evaluated the effects on murine humoral response of the combined administration of lyophilized combination of three lactic acid bacteria: together with alkylglycerol-rich shark liver oil and observed a significant synergistic stimulatory effect of lactic acid bacteria and alkylglycerols in both post- as well as in pre-immunization treatment schedules with sheep red blood cells.

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Correspondence: Prof. Francesco Marotta, MD, PhD
Piazza Firenze, 12
20154 Milano, Italy
E-mail: fmarchimede@libero.it