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The effect of shark liver oil on the tumor infiltrating lymphocytes and cytokine pattern in mice

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ABSTRACT

Ethnopharmacological relevance: Shark Liver Oil (SLO) is a traditional medicine that has been widely used in Scandinavian folk to augment the immune response in some immune-related diseases, especially as an anti-cancer agent.
Aim of the study: The object of this project was to confirm the anti-cancer effect of SLO and the possible involving mechanisms.
Materials and methods: Using delayed-type hypersensitivity (DTH) response in normal mice, the optimal dose for stimulation of cellular immunity was obtained and injected intraperitoneally to the tumor-bearing mice. Cytokine pattern of splenic MNCs was tested by ELISA. The percentage of CD_4^+ and CD_8^+ lymphocytes in tumor-infiltrating lymphocytes was determined by flow cytometry. Also the rate of increase in tumor volume measured.
Results: Our findings indicated that SLO highly augments delayed-type hypersensitivity response against sheep Red Blood Cell (sRBC) in mice. Furthermore, intraperitoneal injection of SLO to tumor-bearing mice could increase T-cell infiltration into the tumor and lower the increasing rate of tumor's volume. Also, it changes the cytokine pattern of the splenic Mononuclear cells (MNCs) to Th1.
Conclusion: Increase in IFN-γ (resulting in enhanced cellular immunity) and increase in especially CD_8^+ lymphocytes accompanied by a decrease in tumor size are among the signs of its anti-tumor effect. Accordingly, we suppose that SLO is a good candidate for further studies in cancer therapy.
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1. Introduction

Shark liver oil (SLO) has been found to be useful in the treatment of conditions resulted from inadequate immune response, and also in the adjunctive treatment of several types of cancer (Lewkowicz et al., 2006). It has widely been used in Scandinavian countries as an anti-tumor complementary medicine (Krotkiewski et al., 2003). Initial clinical use was for treating leukemia and as a complementary agent in cancer therapy, especially to prevent radiation sickness from cancer X-ray therapy, as well as in the treatment of infectious diseases (Brohult and Holmberg, 1954; Brohult, 1958; Pugliese et al., 1998; Szostak and Szostak-Wegierek, 2006). Also supportive treatments with shark oil components normalize complement level, natural killer cells activity and reactive oxygen intermediates production by peripheral blood leukocytes in rheumatoid arthritis patients (Tchorzewski et al., 2002).

After SLO intake, a set of changes including increased response of neutrophils towards bacteria, increased level of C4 component of complement in blood, rise in total antioxidant status of serum and predominance in the production of Type I cytokines; IFN-γ, TNF-α and IL-2 by peripheral blood mononuclear cells were noticed (Lewkowicz et al., 2005). Moreover, caution has to be taken when SLO is routinely consumed as health supplement, because of its effect on lipid metabolism and hypercholesterolemic effect as reported in hamsters (Zhang et al., 2002; Lewkowicz et al., 2005).

SLO contains great amounts of alkylglycerol and squalene (40% or more), and moderate amount of n-3 polyunsaturated fatty acids (N-3 PUFA) (Skopinska-Rozewska et al., 2003; Lewkowicz et al., 2006; Szostak and Szostak-Wegierek, 2006; Vazquez et al., 2008). N-3 PUFA is of great importance in atherosclerosis prevention (Szostak and Szostak-Wegierek, 2006). It is essential for optimal neonatal development (Mitre et al., 2005). Recently, detailed biochemical analyses have shown that not all fatty acid families possess the same tumor-promoting potential. In general, diets containing high levels of the n-6 polyunsaturated fatty acids have routinely enhanced tumorigenesis in lipid sensitive carcinogen-induced and tumor transplant tumor models, whereas diets with equivalent

Abbreviations: SLO, shark liver oil.

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levels of n-3 polyunsaturated fatty acids have diminished tumorigenesis (Cave, 1991). PUFA induced growth inhibition and cytotoxicity to tumor cells may, at least in part, be due to enhanced free radical generation (Das et al., 1987). Alkylglycerols and squalene are modulators of immunity to infections and cancer (Mitre et al., 2005; Szostak and Szostak-Wegierek, 2006). They have immunostimulating and haematopoietic properties. Alkylglycerols may control immune response possibly through modification of platelet activating factor (PAF) and diacylglycerol (DAG) production (Szostak and Szostak-Wegierek, 2006). PAF can increase the growth of breast cancer in cell cultures (Bussolati et al., 2000) and activates protein kinase C (Shukla, 1992; Roth et al., 1996). Activation of protein kinase C is an essential step in cell proliferation and can be inhibited by alkylglycerols (Pugliese et al., 1998). Glycerol ethers exist in the bone marrow fat of mammals and in the membrane phospholipids. The methoxy-substituted glycerol ethers supplied in the diet may stimulate the bone marrow and/or may be incorporated into membrane lipids, thereby, change the structure and function of the membranes (Boeryd et al., 1978). Alkylglycerols have immunostimulatory action on macrophages and result in increase in leukocyte and thrombocyte counts (Hasle and Rose, 1991; Pugliese et al., 1998). Alkylglycerols have also anti-angiogenic effect and the anti-tumor activity of SLO is likely mediated by the presence of alkylglycerols (Pedrono et al., 2004).

Anti-tumor activity of SLO components is possibly based on different mechanisms, i.e. the induction of apoptosis in neoplastic cells, suppression of signal transduction, inhibition of angiogenesis, promoting of transmembrane transport of cytotoxic agents and shifting of cytokine profile (Szostak and Szostak-Wegierek, 2006). In this work, the effects of SLO on tumor volume and TCD_4^+ and CD_8^+ cells in tumor infiltrating lymphocytes (TILs) and the profile of cytokine production of the splenic mono-nuclear cells (MNCs) in breast cancer-bearing mice were evaluated.

2. Materials and methods

2.1. Mice

Female inbred BALB/c mice (8–10 weeks old, 20–25 g) were purchased from *Pasteur Institute*, Tehran, Iran. They were kept in animal house of Tarbiat Modares University, given sterilized water and autoclaved standard mouse food throughout the study.

2.2. Shark liver oil

Shark liver oil capsules were purchased from Kraftsatim ehf Company (Iceland). Each capsule contains 100% pure natural arctic shark liver oil (350 mg/capsule), extracted from the liver of the Greenland Shark (Somniosus microcephalus). Contains natural vitamin A, D and E (minor amount). Included in this oil is alkylglycerols (35 mg/capsule) and omega-3 polyunsaturated fatty acids (42 mg/capsule). The SLO obtained from the capsules, was diluted using Tyrode's buffer (Ebtekar and Hassan, 1993) to prepare various concentrations.

2.3. Delayed-type hypersensitivity (DTH) test

To evaluate the effects of SLO on DTH response, 35 normal mice were primed subcutaneously in the back with 1×10^8 sRBCs (purchased from Razi Institute, Tehran, Iran) on the day 1. Then, the animals were divided into 7 groups. Five groups of the mice were injected intraperitoneally with serial concentrations (50, 10, 5, 2.5 and 0.1 mg/kg/day) of SLO (0.1 ml) in a 5 day period, daily. The other two groups (as controls) were injected with Tyrode buffer and cooking oil (sunflower) in the same route, volume and time to the shark oil groups. On the day 5, the sensitized animals were challenged with 1×10^8 sRBCs injected subcutaneously on the left hind footpad. The increase in the footpad thickness was measured 24, 48 and 72 h after the booster injection of sRBCs by digimatic caliper (Mitutoyo, Japan) and the results were expressed as the percentage increase in the footpad thickness. The results were calculated according to the following formula by Ebtekar and Hassan (1993):

$$\frac{\text{Left footpad challenged with sRBC} - \text{right footpad}}{\text{right foot pad}} \times 100.$$

2.4. Tumor transplantation and measurement of the tumor volume

Spontaneous mouse mammary tumor (SMMT) spontaneously developed in female BALB/c mice. SMMT is an invasive ductal carcinoma (Hassan et al., 2003).

Tumor tissue was separated from the breast cancer-bearing BALB/c mice and cut into pieces of less than 0.5 cm^3 with forceps and scalpel. Each piece was transplanted subcutaneously to the syngenic female BALB/c mice. After about 2 weeks, when tumor tissue appeared, they were randomly divided into groups of five mice. According to the results obtained from DTH test, the optimum dose of SLO was selected. The first group (test) was inoculated with 10 mg/kg/day SLO intraperitoneally for 20 continuous days. The second and the third groups were inoculated daily with Tyrode and sunflower oil, respectively, with the same procedure. The tumor volume was measured in all the three groups by digimatic caliper (Mitutoyo, Japan), starting from the day 1 up to the 20th day, every 5 days. Tumor size was calculated by the following formula (Singh et al., 1996):

$$V = \frac{1}{2} \times LW^2$$

where V is the volume, L is the length and W is the width.

2.5. Measurement of the intra-tumor T-cell subpopulations

2.5.1. Mononuclear cells separation of tumor

On the day 20, the animals were killed by dislocation of neck and the solid tumors were cut into small pieces and minced with forceps and scalpel. The pieces were rinsed twice with phosphate buffer saline (PBS) and passed through a 150 μm stainless steel mesh. The MNCs of the suspension were isolated by density centrifugation ($700 \times g$, 15 min, and 20 °C) using ficole hypaque (Baharafshan, Iran). Then the layer removed and washed twice with PBS for 10 min in $360 \times g$ and 4 °C. The precipitated cells were resuspended in PBS containing 2% fetal calf serum (Gibco, England) and were stained with tripan-blue. The viability was 90%. After counting, 1×10^5 of the cells was poured into each flowcytometric tube and labeled with monoclonal antibodies.

2.5.2. Immunofluorescent staining

For staining the cells obtained from the tumor tissues, fluorescent anti-CD_4 and anti-CD_8 antibodies (Becton Dickinson Company) were used. We established the reference immunophenotypic pattern using standard procedures. In this study, 100 μl of intra-tumor cell suspension was treated as follows.

We prepared three samples of 1×10^5 MNCs (100 μl) in three flowcytometric tubes and added 1 μl of anti-CD_4 mAbs conjugated with fluorescein isothiocyanate (FITC) to the first tube, anti-CD_8 mAbs conjucated with fluorescein isothiocyanate (FITC) to the second tube and isotype control to the third tube. The samples were then kept at 4 °C and in the dark for 30 min. After washing twice with PBS-FCS 2%, the CD_4^+ or CD_8^+ cell percents in the MNCs of TILs in each sample were determined on a Beckman Coulter flowcytometer (Lal et al., 1988).

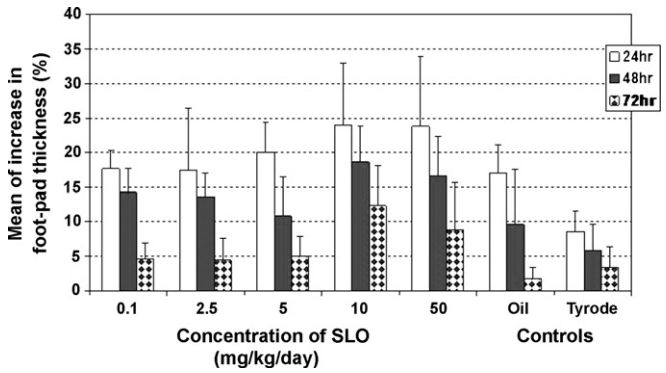

Fig. 1. Effect of various concentrations of SLO on DTH response (mean ± SD). Measurement of foot-pad size after 48 h showed that 10 mg/kg/day of SLO have the most effect on cellular immunity comparing to control groups (oil and tyrode)—SLO: shark liver oil; Oil: sunflower oil (control); Tyrode: diluting buffer (control); SD: standard deviation.

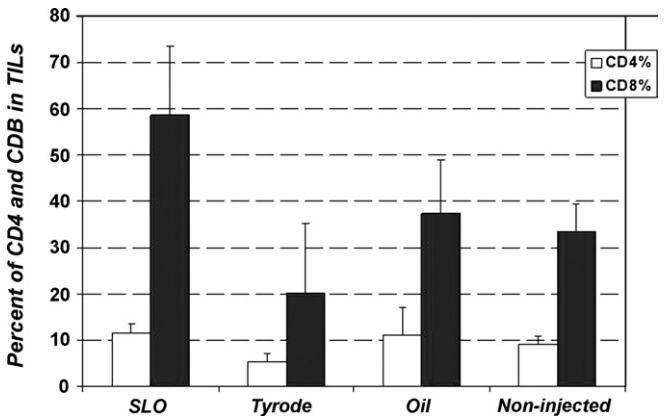

Fig. 2. Percent of CD_4^+ and CD_8^+ T cells in the experimental and control groups (mean ± SD). Flowcytometric results indicated that infiltration of CD_8^+ cells in SLO group was higher than other control groups—SLO: shark liver oil; Oil: sunflower oil (control); Tyrode: diluting buffer (control); SD: standard deviation.

2.6. Splenic MNCs separation and measurement of the cytokines by ELISA

To evaluate the effect of SLO on cytokine production of the splenic MNCs after the treatment and compare the results with the control groups, the MNCs of spleen were separated. Under sterile conditions, spleens were removed and single cell suspensions were prepared in RPMI 1640 (GIBCO, UK). RBCs were osmotically lysed using 0.75% NH_4Cl in Tris buffer (0.02%, pH 7.2). The MNCs were isolated by density centrifugation (700 × *g*, 15 min, and 20 °C) using ficole hypaque (Baharafshan, Iran). Then the layer removed and washed twice with PBS for 10 min in 360 × *g* and 4 °C. The precipitated cells were resuspended in RPMI 1640 (GIBCO, UK) containing 10% fetal calf serum (Gibco, England). After tripan-blue staining and counting, cell viability was more than 90%. 4×10^5 of the cells was poured into each well of the 96-well micro plates and then phytohemagglutinin (PHA) was added for the stimulation of the cells at 5 μg/ml final concentrations. The obtained mixture was incubated for 60 h at 37 °C and 5% CO_2. Then, the supernatants were collected and freezed at −70 °C, till cytokine assay.

ELISA kit (R&D) was used to measure IFN-γ and IL-4. Briefly, after washing the wells with buffer, the standard samples were added to each well, and then biotin conjugates were added. The obtained mixtures were incubated for 2 h. The microplates were washed three times with washing buffer and Stereptoavidin-HRP was added. The plates were incubated for 1 h at 37 °C, and then washed by washing buffer. The TMB substrate solution was dispensed for 15 min, and then stop solution was added. ELISA reader (450 nm filter) was used to read the results.

2.7. Statistical analysis

Non-parametric independent *T*-test, one-way ANOVA and Duncan test were used to calculate the significance of statistical comparisons. In all the analyses, a *p*-value of <0.05 was considered statistically significant.

3. Results

3.1. Effect of SLO on delayed-type hypersensitivity responses

In order to assess the effect of SLO on DTH response, 35 mice were used and the protocol in Fig. 1 was performed. The results obtained by the injection of various concentrations of SLO (50, 10, 5, 2.5 and 0.1 mg/kg/day) indicated that SLO at the doses of 50 ($p = 0.036$) and 10 mg/kg/day ($p = 0.007$) had maximum enhancing effect on the DTH response after 48 h (see Fig. 1). No significant differences were noticed in the footpad thickness between the sunflower oil group and the Tyrode group ($p > 0.05$). In order to assess the anti-tumor effect of SLO, the dose of 10 mg/kg/day was selected for a 28 day period of injection to the tumor-bearing mice.

3.2. Effect of SLO on tumor infiltrating lymphocytes

In order to measure the percent of lymphocyte infiltrated breast tumor, 20 mice were used. The results (see Fig. 2) showed that the percentage of CD_8^+ lymphocytes increased in the mice injected with SLO compared with the Tyrode ($p = 0.001$) or non-treated group ($p < 0.026$), while the sunflower oil group did not show any significant increase in the CD_4^+ or CD_8^+ TILs compared with the Tyrode or non-injected group ($p > 0.05$). The result of CD_4^+/CD_8^+ ratio is shown in Table 1.

3.3. Effect of SLO on tumor volume

In order to evaluate the tumor volume in the tumor-bearing animals, 15 mice were used and the protocol in Fig. 3 was performed. SLO was intraperitoneally injected daily and the tumor size was measured every 5 days. The results indicated that the injected dose of SLO (10 mg/kg/day) decreased the rate of tumor growth compared with the Tyrode group, but it was not statistically significant with the *p*-value of 0.05 ($p = 0.216$). While the sunflower oil group showed no significant ($p = 0.814$) differences comparing with Tyrode group in this regard.

3.4. Effect of SLO on the modulating of cytokine pattern

In order to evaluate the cytokine pattern in the tumor-bearing animals injected with SLO, 25 mice were used and the protocol in

Table 1
According to flowcytometric results, CD_4^+/CD_8^+ in TILs of SLO group is lower than other control groups, which is the outcome of more recruitment of CD_8^+ T cells—SLO: shark liver oil; Oil: sunflower oil (control); Tyrode: diluting buffer (control); SD: standard deviation.

Group	CD4/CD8 ratio
SLO	0.19924
Oil	0.29564
Tyrode	0.36944
Non-injected	0.27104

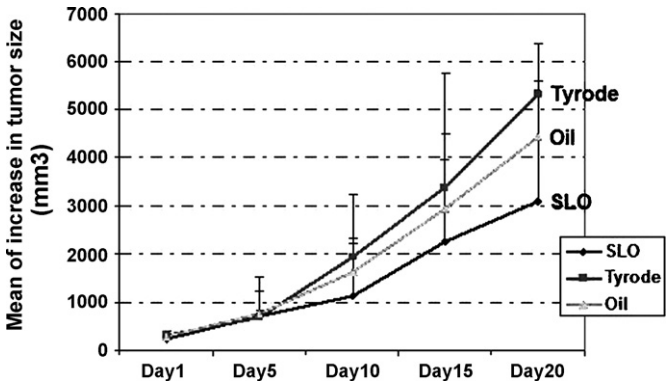

Fig. 3. Rate of increase in tumor volume (mean ± SD) in the experimental and control groups. Increase in tumor volume in SLO group was lower than other groups—SLO: shark liver oil; Oil: sun flower oil (control); Tyrode: diluting buffer (control); SD: standard deviation.

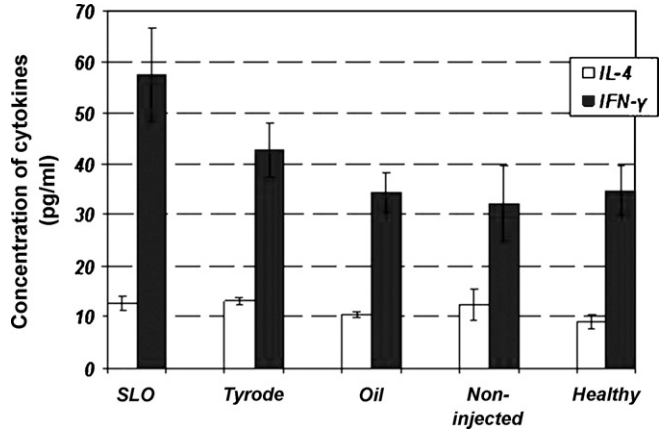

Fig. 4. Concentration of cytokines in experimental and control groups (mean ± SD). SLO could increase in IFN-γ, so shift the immune system to Th1—SLO: shark liver oil; Oil: sun flower oil (control); Tyrode: diluting buffer (control); SD: standard deviation.

Fig. 4 was applied. The results showed that SLO injection caused significant increase in IFN-γ production ($p = 0.007$) comparing to Tyrode, and significant difference comparing with the sunflower oil, non-injected and normal healthy groups of mice ($p = 0.00$). However, no significant differences were noticed in the level of IL-4 among all the groups.

4. Discussion and conclusions

SLO, a medicinal supplement, has attracted considerable attention because of this idea that sharks have been claimed to be resistant to cancer (Skopinska-Rozewska et al., 2003). SLO is an inhibitor of tumor neovascularization (Pedrono et al., 2004). Alkylglycerols, a component of SLO, have been shown to decrease the growth, vascularization and dissemination of Lewis lung carcinoma tumors in mice (Pedrono et al., 2004). SLO suppressed neovascular response in mice grafted with sarcoma L-1 syngeneic cells, human kidney cancer and human urinary bladder cancer cells (Skopinska-Rozewska et al., 1999). The alkylglycerols and their methoxyderivates present in shark liver oil showed a clear apoptotic/necrotic effect on human prostate and mammary carcinoma cell lines (Krotkiewski et al., 2003). After transplantation of syngeneic L-1 sarcoma in BALB/c mice, squalene and polyunsaturated fatty acids significantly diminished cutaneous angiogenesis induced by tumor cells and tumor growth (Skopinska-Rozewska et al., 2003).

In this study, we investigated the effect of SLO on DTH test, tumor volume, percentage of CD_4^+ and CD_8^+ T of tumor infiltrating lymphocytes and cytokine profile in breast cancer-bearing mice.

Since the DTH test is a reliable marker of cellular immune response, we initially performed the DTH test to obtain the most effective dose of SLO. We used this test as primary screening method to obtain a dose of SLO with the strongest stimulatory effect on cellular immune response. Then, based on the results of DTH test, the breast cancer-bearing mice were injected with the same route and dose of SLO. This study indicates that 50 and 10 mg/kg/day doses of SLO have the strongest immunomodulatory properties, augmenting specifically the cellular branch of immune response. For injection of SLO to tumor-bearing mice, we chose the dose of 10 mg/kg/day SLO rather than 50, because of their nearly similar responses. In most of the conditions, a dominant cellular response could provide protection against the disease progression.

It is clear from our results that IP injection of SLO results in significant immunomodulatory and anti-tumor activity in BALB/c mice with a mammary tumor model. Significant changes in immune function and tumor growth following SLO injection with a significant increase in CD_8^+ TILs relative to sunflower oil were seen. We suppose that there is a possible causal link between increased CD_8^+ TILs and decreased tumor growth.

Our findings from the intraperitoneal injection of 10 mg/kg/day SLO showed that the SLO decreased the rate of tumor growth in the breast cancer-bearing mice. It is consistent with the report that alkylglycerols or SLO reduced the tumor growth of Lewis lung carcinoma tumors in mice (Nowicki and Baranska-Rybak, 2007). Also intraperitoneal inoculation of SLO could cause a significant increase in the infiltration of especially CD_8^+ subpopulation of T lymphocytes. It has been shown that T-cell infiltration into the tumor has decreased in tumor progression cases (Muhonen et al., 1994). Adoptive immunotherapy using TILs proliferated in vitro and injected ex vivo indicated that these TILs can help tumor treatment (Devita, 1998), due to their high frequency of either tumor-specific CTL or their precursors (Rosenberg et al., 1986).

In humans, the presence of TILs may be predictive of improved clinical outcomes (Andaloussi and Lesniak, 2006). CD_8^+ TILs are important in the host immune defense against tumor progression and the TIL density has been shown to be correlated with favorable survival in the patients with various cancers like melanoma, colorectal cancers (CRCs) and ovarian cancers (Ropponen et al., 1997; Galon et al., 2006). Intratumoral T cells could modify tumor stroma or tumor cells in a way that attenuates the metastatic potential of tumor cells (Galon et al., 2006).

The infiltration by CD_3^+ CD_4^+ T cells or a subpopulation of CD_4^+ T cells with immunosuppressive properties, so called as regulatory T cells, was reported to counteract the beneficial effect of CD_8^+ T cells. High ratios between CD_8^+ T cells and the other cell types were associated with improved survival (Kondratiev et al., 2004). On the other hand, it seems that cooperation between the infiltrating CD_4^+ and CD_8^+ T cells in tumors might be important.

Activation of CD_4^+ T cells is required for immunization of CD_8^+ T cells against cancer. For activation and maintenance of tumor infiltrating CD_8^+ T cells, CD_4^+ T cells play an important role by secreting cytokines such as interleukin-2, which is required for CD_8^+ T cell growth and proliferation. Further CD_4^+ T cells are necessary for the full anti-tumor activity of CD_8^+ T cells (Hiraoka et al., 2006).

Our data showed that SLO can highly increase infiltration of CD_8^+ T cells. Thus the administration of this substance can be considered as a method for T-cell recruitment into the tumor. As shown in Fig. 2, SLO had a stronger stimulatory effect on CD_8^+ TILs than CD_4^+ TILs which resulted in decreasing of the T (CD_4^+/CD_8^+) ratio (see Table 1).

It has been claimed that CD_4^+ and CD_8^+ expression in human breast cancer was significantly different between the lymph node metastases group and the lymph node negative group. An increased

CD_4^+/CD_8^+ ratio of the TIL isolated from human breast adenocarcinoma may indicate development of metastases (Chin et al., 1992; Macchetti et al., 2006). According to our findings in murine model, SLO-mediated decrease in CD_4^+/CD_8^+ ratio. If similar results are obtained in humans, SLO could prevent the lymph node metastases in breast cancer-bearing patients.

Furthermore, our investigation indicated that the splenic MNCs from the mice-bearing breast carcinoma injected with SLO preferentially produced Th1 cytokines (i.e. IFN-γ) upon activation compared with the production observed in the splenic MNCs of the control groups. Also, Merly et al. showed that shark cartilage, preferentially, induces Th1 type cytokines in human peripheral blood leukocytes (Merly et al., 2007). Th1 responses lead to increased cellular immune responses to tumor as well as increased secretion of tumoricidal IFN-γ and TNF-α (Constant and Bottomly, 1997). Th1 CD_4^+ T-helper cells also enhance antitumor immune responses by secretion of IFN-γ, which in turn induces activation of macrophage cytotoxic activity (Stout and Bottomly, 1989). Th1 responses are thought to be beneficial toward antitumor immunity, whereas Th2 responses may down-regulate cell-mediated antitumor immunity and enhance protumor humoral responses (Johansson et al., 2007).

The SLO-mediated augmentation of DTH response, increased infiltration of CD_8^+ T cell in tumors and altered cytokine profile to Th1 (IFN-γ) are strong indicators of the therapeutic potential of SLO for cancer patients. The controlled manipulation of immune response by natural pharmacological means is a highly sought goal of clinicians due to their favorable therapeutic results and less side effects and toxicity.

Further mechanisms of inhibition of tumor growth needs to be studied more in the future. More research on the effect of SLO on cytokine patterns in TILs, immune cell subtypes and intracellular signaling events in murine models and humans is necessary to shed light on the exact mechanisms and scope of the effect of this compound. Our findings in the present study indicated that this supplement may be administered for prophylaxis and treatment of disease in immunocompromised patients.

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