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RESEARCH ARTICLE

Shark cartilage 14 kDa protein as a dendritic cells activator

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Abstract

Low molecular weight components of shark cartilage are reported to have anti-tumor as well as immuno-stimulating effects. Dendritic cells (DCs) are potent antigen-presenting cells (APCs) that have a key role in establishment of anti-cancer immune response. In this study, the effect of 14 kDa protein from shark cartilage was investigated on stimulation and maturation of dendritic cells. The isolated 14 kDa protein from shark cartilage extract was added to DCs medium during overnight culture and their maturation and T cells stimulation potential was investigated. The majority of shark-cartilage-treated DCs expressed higher levels of maturation markers and were more effective in stimulation of allogenic T cells compared with non-treated DCs ($p < 0.05$). Our results showed that shark cartilage 14 kDa protein can potentially be used in DC-mediated T-cells stimulation and induction of desirable immune responses in clinical trials such as cancer immunotherapy. However, further studies are required to examine this proposal.

Keywords

Allogenic MLR, dendritic cell, shark cartilage, T cell stimulation, tumor

History

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Introduction

Shark cartilage has conventionally been qualified with a number of medical benefits; but it is primarily marketed for its anticancer effects[1–5]. Shark cartilage extract has shown anti-angiogenic and anti-tumor activities in animals and humans[6–8]. However, thus far, its efficacy is controversial[9–14]. Also there are some scientific evidences that shark cartilage modulate the cellular and humoral components of the immune system and this activity may make it effective against tumor growth, as well as bacterial, viral, and fungal infections.

We showed previously that shark cartilage protein fraction with the most immuno-stimulatory effects is composed of two proteins with molecular weights of about 14 and 15 kDa[15]. Other investigators have also shown that the low molecular weight component from shark cartilage is a protein with neutral PI and sequence similarity to parvalbumin[16]. Besides Kralovec et al.[17] have shown that although the shark cartilage extract contains lots of proteoglycans, the immunostimulatory properties are mostly related to its protein content[17]. This fraction could augment cellular immune response and T-cell infiltration into the tumor[18]. However, no study has reported shark cartilage effects on development of dendritic cells (DCs) as the most potent antigen-presenting cell (APC) for naïve T-cells. DCs are a group of multifunctional APCs which are present as infrequent leukocytes throughout lymphoid and non-lymphoid tissues[19]. These cells are completely heterogeneous regarding to their hemopoietic lineage, differential morphology, phenotype, and function; however, the ability to stimulate and activate naïve T-cells appears to be shared among various DC subsets. DCs are derived from bone marrow progenitors and lodge in different tissues as immature precursors prior to migration into regional lymph nodes. Following antigen up take and appropriate stimulation, DCs undergo further maturation and migrate to secondary lymphoid tissues where they present Ag to T-cells and induce the immune response. DCs are receiving increasing scientific and clinical interest due to their key role in induction of desirable immune responses and their potential use as biological adjuvant in tumor vaccines, as well as their involvement in the immunobiology of tolerance and autoimmunity[20].

Because of the reported immune potentiating effect of shark cartilage 14 kDa protein on tumor therapy and cell-mediated immunity and considering the chief role of cellular immunity in efficient anti-tumor response, there may be a correlation between shark cartilage and immune cells activation by DCs. Hence, the present study aims to investigate the effect of shark cartilage 14 kDa protein on stimulation and maturation of dendritic cells.

Materials and methods

Mice

Eight- to 10-weeks-old inbred BALB/c ($n = 18$) and C57BL/6 ($n = 5$) mice were obtained from Pasteur Institute of Iran. The mice were housed under standard conditions of hygiene, temperature and humidity with 12-h intervals of light/dark and given free access to food and water. All the experiments were carried out according to the Animal Care and Use Protocol of Tarbiat Modares University (Tehran, Iran).

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Preparation of crude shark cartilage extract

The shark cartilage extract was prepared as we mentioned elsewhere[18]. Simply, cartilage was prepared from notochord of dogfish shark (Persian Gulf, Iran). The notochords of dogfishes were prepared freshly from Iran Fisheries Organization, kept refrigerated during their carriage (maximum 30 h) to our laboratory where the attached residual tissues were removed from it by washing and scrubbing under tap water. The cleaned cartilage was then rinsed with distilled water, cut into small pieces, lyophilized, and pulverized. Ten grams of the cartilage powder of dogfish was afterwards extracted in 100 ml of 0.1 M phosphate buffer containing 4 M guanidine HCl and a protease inhibitor (phenylmethanesulfonyl fluoride (PMSF): 1 mM, Sigma, St. Louis, MO) at pH 5.8 for 48 h with slight stirring at 2–8 °C[7,21,22]. The obtained extract was ultracentrifuged at 100 000*g* for 45 min. The supernatant was isolated and its high molecular weight components were precipitated in 20% PEG (Pharmacia, Uppsala, Sweden).

Isolation and purification of shark cartilage proteins

In order to isolate the 14 kDa protein from the crude extract of cartilage, the extract was first filtered against two Amicon ultrafiltration membranes (Millipore, Billerica, MA) with 30 and 10 kDa cutoff respectively. Column chromatography was also used for further purification of low molecular weight proteins[23]. Simply, sufficient amount of the dried gel (Sephadex G-50, Pharmacia, Uppsala, Sweden) was incubated in distilled water for 72 h at 4 °C and then loaded onto the column ($12 \times 1\ cm^2$). The column was equilibrated with 5 M urea buffer. The residue of 10 kDa amicon membrane was then applied to the column at a flow rate of 0.1 ml/min and the eluted fractions were collected as 1 ml samples. Light absorbance of the elution tubes was measured through spectroscopy at a wavelength of 280 nm. The collected fractions were then dialyzed against PBS buffer with pH 7.4 and sterilized by passing through 0.02 μm Millipore filters.

SDS-PAGE

Polyacrylamide gels were used to examine the purity of proteins and estimate their molecular mass in comparison with standard marker proteins (low MW marker, Pharmacia, Uppsala, Sweden). After electrophoresis, the gels were fixed with methanol and acetic acid formaldehyde for 60 min and stained with coomassie blue (Sigma, St. Louis, MO)[24].

Isolation of splenic dendritic cells and their treatment with shark cartilage 14 kDa protein

DCs from mouse spleen were prepared as we described elsewhere[25,26]. Briefly, BALB/c mice spleens were removed under sterile condition and each spleen was then injected and digested with a cocktail comprising of 0.5 mg/ml collagenase D (Roche Diagnostics, Mannheim, Germany) and 0.02 mg/ml DNase I (Roche Diagnostics, Mannheim, Germany). The obtained cell suspension was washed twice with PBS containing 5 mM EDTA (10 min at 4 °C, 280*g*), overlaid on 12% Nycodenz (Axis-Shield, Oslo, Norway) and centrifuged (600*g*) at room temperature for 15 min. Low density cells were removed from the interface and the cells were washed with cold PBS-EDTA. Cells were then resuspended in complete RPMI-1640 containing 0.5% mouse serum and cultured at 37 °C in 15×60 mm Petri dishes (Falcon, Rochester, NY) for 2 h. Non-adherent cells were afterwards discarded by washings with warm (37 °C) RPMI-1640 containing 0.5% mouse serum in two steps with 30 min intervals. Warm RPMI containing 0.5% mouse serum was then added to the adherent cells and plates were incubated overnight at 37 °C, 5% CO_2, after which the floating DCs were removed and washed twice with cold PBS at 4 °C. Purity of the obtained DCs was determined as percent of $CD11c^+$ cells through flow cytometric analysis. The DCs separation experiments were done five times (three BALB\c mice spleen for each experiment).

In some wells, 25 μg/ml shark cartilage 14 kDa purified protein was added to culture medium during the overnight culture. The appropriate amount of shark protein was determined through pilot studies with serial concentrations of it (data were not presented here).

Flow-cytometric analysis

Dendritic cells (10^5) were suspended in 100 μl PBS, containing 1% normal hamster and normal rat sera. 1 μg of PE-conjugated hamster anti-mouse CD11c and one of the various directly conjugated monoclonal antibodies including FITC-conjugated rat anti-mouse $I\text{-}A^d$, FITC-conjugated rat anti-mouse CD86 and FITC-conjugated rat anti-mouse CD40 or appropriate isotype controls were added to each tube (all antibodies were prepared from BD Biosciences, San Jose, CA). After 30 min of incubation on ice, cells were washed and examined by flow-cytometer (Partec, Münster, Germany). The results were analyzed as two colors dot plots and the negative regions were determined according to isotype controls. For determination of DCs, purity one color staining by anti-CD11c was used. All immunophenotypic experiments were repeated at least five times.

Mixed lymphocyte reaction

Mixed lymphocyte reaction (MLR) test was used for functional analysis of shake cartilage treated and untreated DCs. Allogenic T lymphocytes were isolated from lymph nodes of C57BL/6 mice by two consecutive passages of lymph node cells through a nylon wool column (Polyscience Ltd., Eppelheim, Germany). The purity of isolated T cells was measured by flow-cytometric analysis as $CD3^+$ cells and was always more than 90%. Shark cartilage 14 kDa protein-treated and -untreated DCs were used as stimulator cells after 3000 rad irradiation. 100 μl of the responder T cell suspension (1×10^6 cells/ml) were co-cultured with 100 μl of stimulator DCs (1×10^5 cells/ml) in a U-bottom 96-well plate (NUNC, Roskilde, Denmark) for 3 days in RPMI-1640 containing 50 mm HEPES (Gibco, Berlin, Germany), 110 mg/ml Na-pyruvate (Merck, Darmstadt, Germany), 10% FCS (Gibco, Waltham, MA) and antibiotics. T-cell proliferation was measured via MTT assay. About 15 μl/well of tetrazolium salt solution (5 g/l MTT, Sigma, St. Louis, MO) was added to the cells culture medium 4 h before the ending of culture and tissue culture medium was gently removed following

centrifugation of tissue culture plates. 150 µl DMSO was added into the wells to dissolve the generated insoluble formazan crystals. Optical density (OD) of the wells was measured by micro-plate reader (Molecular Devices, Sunnyvale, CA) at a wavelength of 570 nm. The MLR assay was repeated five times with separate DCs and T cells and each test was also done in triplicate. T cells alone and DCs alone were used as negative controls.

Statistical analysis

The statistical significance of differences between the groups was determined by the non-parametric Mann–Whitney *U*-test and p values less than 0.05 were considered significant. All experiments were repeated at least five times and the results are presented here as mean ± SD of five separate experiments.

Results

Purification of immuno-modulator fraction from shark cartilage

Using guanidine extraction, Amicon ultra-filtration, and gel filtration chromatography, two fractions were purified from shark cartilage. The first fraction was composed of one major band in SDS-PAGE which its molecular weight was estimated by SDS-PAGE to be approximately 14 kDa as compared with standard markers (Figure 1). The mobility of major band was the same in the presence or absence of 2-mercaptoethanol (2ME).

Effect of shark cartilage fraction on maturation markers of DC

Shark cartilage 14 kDa fraction was able to induce maturation of DCs as demonstrated by the increased expressions of MHC II, CD40, and CD86. DC maturation is characterized by the up-regulation of co-stimulatory and Ag-presenting molecules. To evaluate the direct effect of shark cartilage fractions on the DC phenotype, the surface expression of several maturation markers (CD86, CD40, and I-Ad) was analyzed after 16 h of treatment of immature DCs with shark cartilage fractions. The achieved results showed that shark-cartilage-treated DCs ($CD11c^+$) expressed high levels of CD40, CD86, and MHC-II (maturation marker of DCs) compared with non-treated DCs ($p < 0.05$). Thus, treatment of DCs with shark cartilage 14 kDa fraction markedly induced the expression of MHC and co-stimulatory molecules (Figure 2).

Functional activity of shark-cartilage-treated DCs assessed by MLR

DCs treated with 14 kDa fraction of shark cartilage were tested for their ability to stimulate allogeneic T-cells from C57BL/6 mice. Day 3 cultures were assessed for responder T-cell proliferation by MTT assay. The obtained results showed that DCs treated with 14 kDa fraction of shark cartilage induced a significant ($p < 0.05$) increase in the allogeneic responses in comparison with the control DCS (Figure 3A). Therefore, results of MLR exhibited high absorbance and stimulation index (SI) in those T-cells which were stimulated by shark-cartilage-treated DCs compared to non-treated DCs (Figure 3).

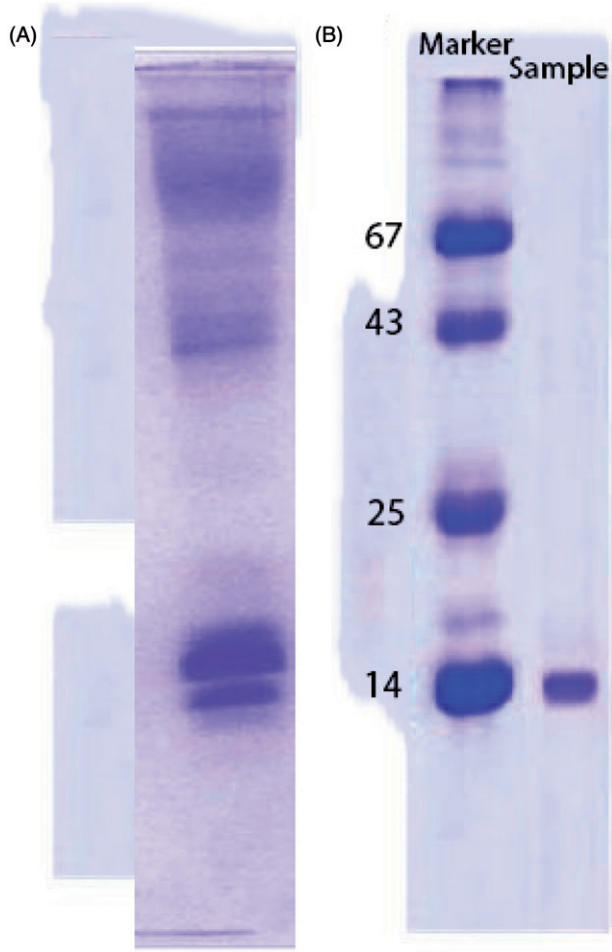

Figure 1. The purification of shark cartilage 14 kDa protein. SDS-PAGE electrophoresis of shake cartilage extract before purification (A) and purified 14 kDa protein after Amicon ultra-filtration and Sephadex G-50 chromatography (B). The second lane (marker) shows the protein weight markers.

Discussion

Following finding that sharks rarely gets cancer[27], some investigations have been made to identify the various pharmaceutical compounds present in its cartilage. However, the cancer in bony fishes is not indeed uncommon[28,29]. Over the recent years, it has been well recognized that some immuno-stimulatory and anti-angiogenic compounds exist in shark cartilage which makes shark much resistant to tumors[7]. A limited number of clinical trials have also been done to examine the anti-tumor properties of shark cartilage and it has been shown that oral administration of shark cartilage results in tumor size reduction and improvement of the life quality[7]. Certain effects of shark cartilage on some cell populations involved in immune responses like T cell, B cells, NK cells, and macrophages have been investigated by our group and others[30]. The current research was done to elucidate the shark cartilage effects on dendritic cells as the most important antigen-presenting cells of the immune system whose use in immunotherapy of cancers has opened a new insight of hope and success.

In previous studies, our group showed that low molecular weight components of shark cartilage especially 14 kDa

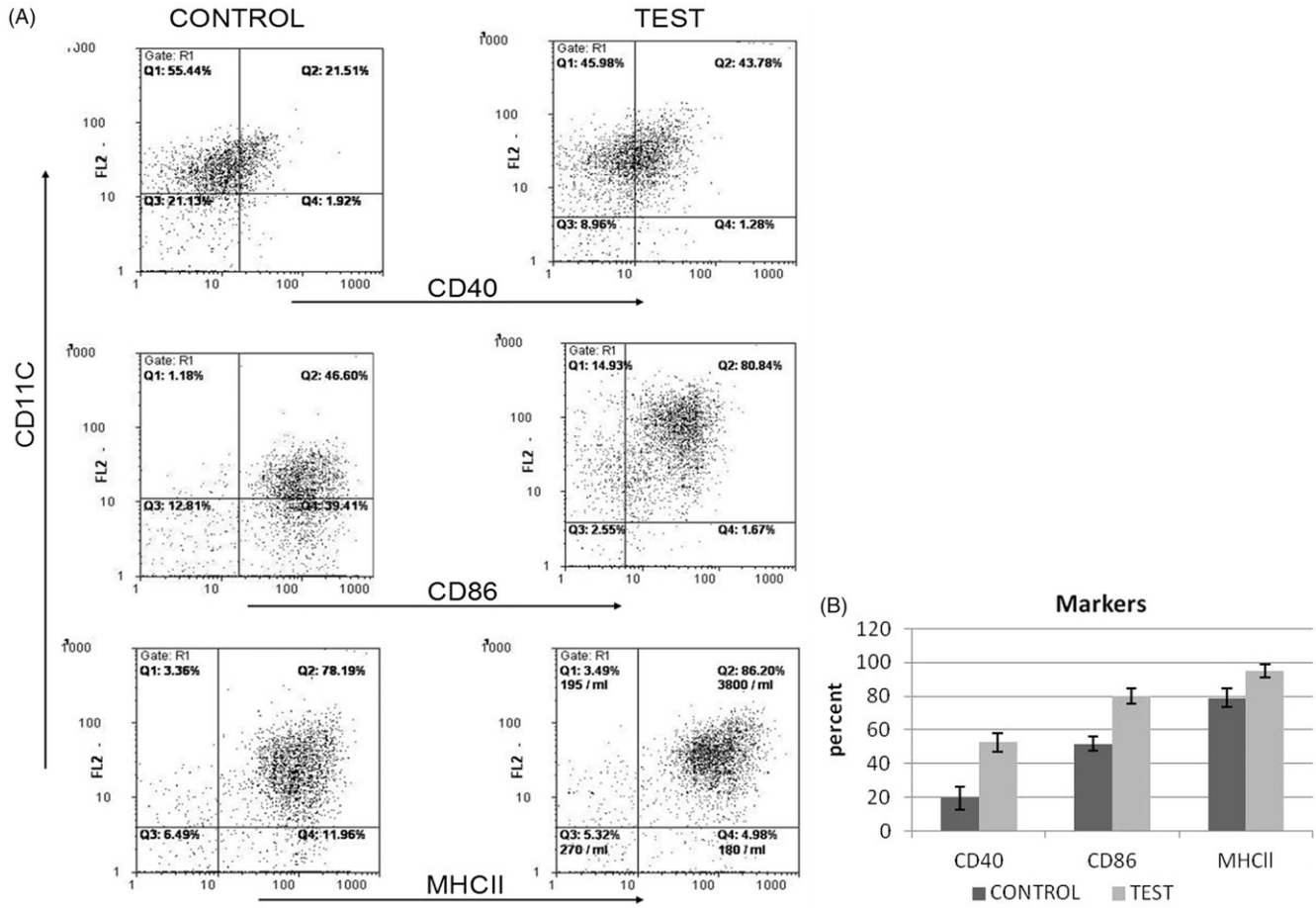

Figure 2. Treatment of DCs with 14 kDa protein of shark cartilage significantly ($p < 0.05$) up-regulate the expression of co-stimulatory molecules on the cell surface. (A) Dot plot flow cytometric analysis of CD40, CD86, and MHC-II expression on shark cartilage treated (TEST) and untreated (CONTROL) DCs (CD11c + cells). The figure is a representative of five separate experiments. (B) Mean ± SD of CD40, CD86, and MHC-II positive cell percents in five separate experiments.

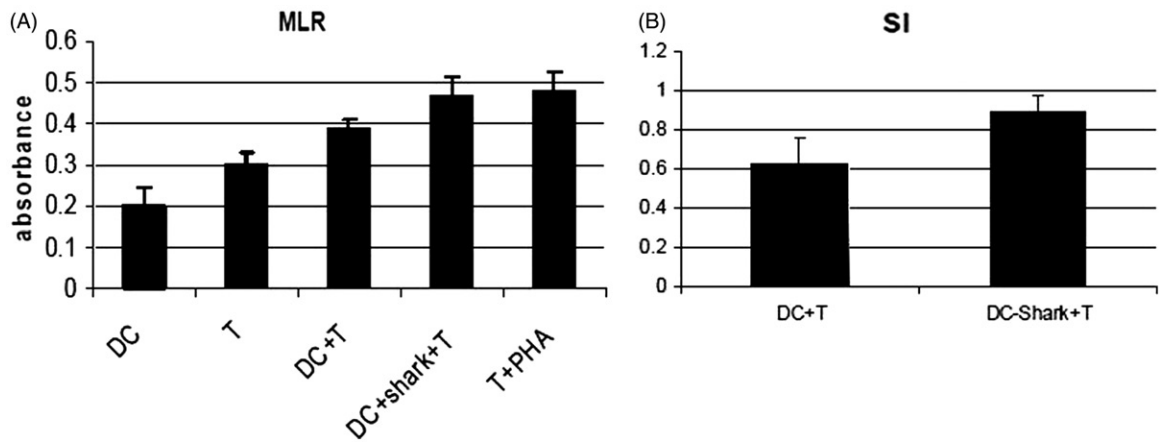

Figure 3. Treatment of DCs with 14 kDa protein of shark cartilage increased the T cell stimulatory potential of DCs (DC + shark + T) compared with non-treated cells (DC+T). (A) Mean ± SD of an absorbance of 570 nm in five separate MTT assays. We used three culture wells for each separate experiment (Triplicate). PHA was used as positive control (PHA+T) and T cells alone (T) and DCs alone (DC) were used as negative controls. (B) Mean ± SD of stimulation index of MLR assay in five separate experiments.

protein exhibit the most potent immunostimulatory effects[18]. So, we decided to examine the effect of the same component on maturation and functions of DCs. The extraction of 14 kDa protein was done in the presence of high concentration of guanidine hydrochloride. Cartilage matrix is made of several types of collagen proteins and several types of proteoglycans[31,32]. Guanidine hydrochloride is the most popular chemical for extraction of proteoglycans and can dissociate frequent hydrophobic interactions between proteoglycan aggregates in cartilage matrix and helps to dissolve its

components[33]. 20% PEG was also used to precipitate high molecular weight proteins in cartilage extract and derivation of low molecular weight proteins[18].

Our results showed that DCs ($CD11c^+$) treated by 14 kDa shark cartilage fraction express higher levels of co-stimulatory molecules CD40, CD86, and MHC-II (a maturation marker of DCs) compared with non-treated DCs.

Naive T cells stimulation and their differentiation into effector cells require recognition of antigens presented on MHC-I and MHC-II molecules at the surface of APCs, especially dendritic cells along with additional signals provided by co-stimulator molecules expressed on APCs. The best-defined costimulatory pathways are the B7:CD28 pathway and the CD40:CD40 ligand (CD40L) pathway[19]. The two known members of the B7 family, B7-1 (CD80) and B7-2 (CD86), are expressed on professional APCs. Recognition of B7 by CD28, which is constitutively expressed by majority of T lymphocytes, enhances Ag-stimulated clonal expansion of T cells, cytokine production, and their differentiation into effector cells[19]. The interaction between CD40L and CD40 also enhances T cell responses. Although the mechanism of this effect is unclear, it is currently believed that CD40:CD40L interactions serve to up-regulate the expression of B7 on APCs and to induce the production of cytokines, such as IL-12, which promote T cell differentiation[19].

In the analysis of functional properties of shake cartilage treated DCs, our results showed an enhanced potency of allogenic T cell stimulation in shake cartilage treated DCs compared with non-treated cells. Regarding the higher expression of co-stimulatory and maturation markers, especially MHC-II molecules on cartilage treated cells, this result is completely expectable. The capacity of DCs to initiate primary immune responses is due to their ability to deliver specific co-stimulatory signals which are essential for T-cell activation from the resting or naïve state into distinct classes of effecter cells[19].

It has been shown that following activation with different stimuli, DCs achieve maturation, where they express high levels of several molecules on the cell surface such as MHC classes I and II, as well as the accessory molecules CD40, CD80, and CD86[19]. In this study, we showed that shark cartilage 14 kDa protein possesses the ability to induce DCs maturation; however, its mechanism of action remains to be elucidated. Merly et al.[30] have examined shark cartilage extracts for induction of cytokines and chemokines in human peripheral blood leukocytes and showed that shark cartilage acid extracts induced high levels of TNFα and IFNγ. These cytokines are well-known DCs maturation inducers[19]. Therefore, it could be assumed that maturation induction property of shark cartilage is due to production of cytokines like TNF-α by DCs themselves or other contaminated leukocytes.

To our best knowledge, this the first report of shark cartilage effects on DCs as the most important antigen presenting cells of the immune system. Considering the growing interest to use of DCs in cancer immunotherapy and having in mind the importance of maturation state in induction of efficient immune response by DCs, and the results of this study which confirmed the high potency of 14 kDa protein of shake cartilage to induce DCs maturation, we suggest this component of shark cartilage as a dominant inducer of DCs maturation for using in DCs-based immunotherapy of cancer. Besides this property, other beneficial antitumor effects have been reported for shark cartilage which could be operative at the same time. In a previous study by our group, we showed that intra-peritoneal injection of shark cartilage 14 kDa fraction to tumor-bearing mice could increase T-cell infiltration into the tumor[18]. Also, there was a significant increase in the CD4/CD8 ratio in tumor infiltrating lymphocytes, while no such changes were found in the peripheral blood lymphocytes[18]. We also showed that this fraction of shark cartilage highly increased DTH response against sRBC in mice[18]. Other studies reported that shark cartilage extract preferentially induces Th1-type inflammatory cytokines while treatment with digestive proteases (trypsin and chymotrypsin) reduced the cytokine induction response by 80%, suggesting that the active component(s) in cartilage extracts is proteinaceous[30]. In addition, purified mouse macrophages cultured with shark cartilage preparations produced significantly higher levels of nitric oxide[17]. Additionally most of the current evidence shows the efficacy of shark cartilage to prevent angiogenesis[34,35]. AE-941 (Neovastat®), a derivative of shark cartilage, which has been of particular research interest in the treatment of some cancers, was granted orphan drug status in 2002 by the food and drug administration (FDA)[36]. In addition to reports of anti-angiogenic properties[37], AE-941 has been noted to exhibit vascular endothelial growth factor inhibition and pro-apoptotic properties[38]. Pre-clinical researches and initial human researches reported good tolerance and potential benefits in renal cell carcinoma[39], prostate[40], non-small cell lung cancer[41], multiple myeloma[42], and bony metastases[43].

All these properties together candidate shark cartilage derivatives as suitable DCs maturation and stimulation factors for using in DCs-based immunotherapy of cancers. However, further studies are necessary to investigate the effects of other shark cartilage fractions on stimulation and maturation of dendritic cells.

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Declaration of interest

All authors have approved the manuscript and agree with its submission to Immunophamacology and Immunotoxicology. The authors also declare that there is no conflict of interest that would prejudice the impartiality of this scientific work. This work was financed and organized by Tarbiat Modares University.

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