Original Article

Immunization with Hydatid Cyst Wall Antigens Can Inhibit Breast Cancer through Changes in Serum Levels of Th1/Th2 Cytokines

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Abstract

Background: Hydatid cysts are the larval stage of *Echinococcus granulosus*, which lead to humoral and cellular immune responses in hosts. Such immune responses play a key role in the inhibition of tumor growth and cancers. To test this hypothesis, it was attempted not only to examine the changes in serum level of some Th1 and Th2 cytokines but also to find relationships between the cytokines and cancer in 4T1 breast cancer-bearing mice immunized with hydatid cyst wall (HCW) antigens. **Methods:** Six to eight-week-old Balb/c female mice were immunized with alum, PBS and HCW antigens, including crude extract of HCW (laminated layer) 28 and 27 kDa protein bands (upper and lower bands) and then challenged with 4T1 breast cancer cells. The amounts of IL2, TNF-α, IFN-γ (Th1 cytokines), and IL4 (Th2 cytokine) were estimated using ELISA. Correlations between these cytokines and cancer parameters (tumor growth, metastasis, and survival) were determined by Pearson's correlation coefficients. **Results:** Overall, HCW antigens increased the amounts of IL2, TNF-α, IFN-γ, and IL4. Pearson's correlation coefficients indicated reverse relationships between changes in amounts of these cytokines and tumor growth/metastasis. However, except for IL-4, all cytokines had a direct relationship with mouse survival. **Conclusions:** The results of this study indicated that the inhibition of breast tumor growth and metastasis and improvement of survival in 4T1 mice immunized with HCW antigens, especially laminated layer and 27 kDa protein band can occur through a rise in the levels of cytokines.

Keywords: *Breast neoplasms, cytokines, hydatid cyst, immunization*

Introduction

According to the World Health Organization (WHO) report, breast cancer is the most common cancer among women around the world, which influences 2.1 million women each year.[1] There are various treatments depending on the type of breast cancer, the stage of cancer, sensitivity to hormones, the patient's age, overall health, and preferences. The treatments include surgery, chemotherapy, radiation therapy, hormone therapy, and targeted drug therapy. However, all the mentioned treatments have adverse effects that are inescapable.[2-4] To find new ways for cancer treatment with minimal side effects, introducing foreign antigens similar to cancer antigens can be a promising approach to overcome tumor growth.[5-8] In fact, these antigens will probably stimulate the patient's immune system to remove tumor cells producing antigens similar to foreign antigens.[8] Previous studies showed that antigens extracted from human parasites could play a key role in cancer therapy in animal models.[9-13] Hydatid cyst is one of these parasites that have the potential to be used for the cure.[14-16]

Hydatid cyst with cosmopolitan distribution is the larval stage of the tapeworm, *Echinococcus granulosus*, which is found in human and animals' viscera. This parasite is now classified into different species and strains.[17] Humans are infected following ingestion of parasite eggs which are excreted in dogs' feces and lead to contamination of foods. Hydatid cyst wall (HCW) outwardly composed of laminated layer and germinal layer. The latter layer produces brood capsules and protoscolices. The cyst wall also is enclosed by a fibrous layer (precyst) which consists of fibrous elements and host immune cells.[18,19] Antigens of HCW can play a key role in the modification of host immune responses.[20,21]

Parasitic infections activate both innate and acquired immune responses, which

How to cite this article: Shakibapour M, Shojaie B, Yousofi Darani H. Immunization with hydatid cyst wall antigens can inhibit breast cancer through changes in serum levels of Th1/Th2 cytokines. Int J Prev Med 2020;11:189.

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Access this article online

Website: www.ijpvmjournal.net/www.ijpm.ir

DOI: 10.4103/ijpvm.IJPVM_311_19

Quick Response Code:

the latter consists of cellular and humoral reactions.[22] As part of cellular responses, cytokines play a main role in cell signalling during parasitic infections.[23] Two distinct cytokine patterns, including Th1 and Th2, have been identified in response to the hydatid cyst. Type one T helper (Th1) cells produce interleukin 2 (IL2), interferon gamma (IFN-γ) and tumor necrosis factor-alfa/beta (TNFα/β), while type two T helper (Th2) cells secrete IL4, IL5, IL6, and IL10.[24] In the early stage of hydatid cyst infection, the host's reaction is related to Th1 cytokine, which can inhibit the development of hydatid cyst. This reaction shifts to a Th2 cytokine response in the later chronic stage. This Th2 response may be in favor of the parasite establishment.[25]

Hydatid cyst usually located in the host tissues, and this direct contact of the cyst wall with host tissues plays an important role in the stimulation and regulation of cellular and humoral immune responses to establish the hydatid cyst. In a previous investigation, it has been shown that some antigens of HCW, especially 27 kDa protein band, have an immunological cross-reaction with sera of patients with breast cancer.[26] The objective of the present study was to examine the immune responses of 4T1 breast cancer-bearing mice immunized with HCW antigens by determining the serum level of some cytokines, such as IL2, TNF-α, IFN-γ, and IL4. Moreover, in this research, it was attempted to find relationships between cytokines (IL2, TNF-α, IFN-γ, and IL4) and cancer parameters (tumor growth, metastasis, and survival).

Methods

Preparation of HCW antigens

Sheep livers with hydatid cysts were collected from Khomeinishahr slaughterhouse in Isfahan, Iran. Hydatid cyst fluid was aspirated from cysts and examined for observation of protoscolices. Cysts containing protoscolices were used for this study. To prepare cyst wall antigens, cyst walls were detached from the surrounding tissues and collected in a test tube containing the physiological saline solution and washed three times with normal saline solution and kept at -20°C. The frozen cyst walls were then minced by a pounder. The minced mixture was sonicated for 5 min and then centrifuged at 5000 rpm for 5 min. The supernatants in the form of the crude extract of HCW were used as crude cyst wall antigens (laminated layer antigen). In addition, a part of this crude extract was subjected to SDS-PAGE and following staining two protein bands with molecular weight about 27 and 28 kDa (the lower and the upper bands) were detected on the gel, which for simplifying they were named upper (28 kDa) and lower (27 kDa). According to primary experiments (data not shown), the interaction of sera of patients suffering breast cancer with protein bands obtained from SDS-PAGE of crude extract of HCW was the basis for the selection of these two protein bands (the lower and the upper bands). These two protein bands were cut out of the gel separately by a razor blade and purified from the gel using electroelution method.[27] The purified proteins were used as 27/28 kDa antigens (lower and the upper antigens).

Mouse mammary carcinoma cells culture

4T1 a (C604) was purchased from the Pasteur Institute of Tehran, Iran. The cells were cultured in RPMI 1640 medium (Sigma-Aldrich, Germany) containing 10% fetal bovine serum (FBS) (Sigma-Aldrich, Germany) and 1% of antibiotics mixture containing penicillin (Sigma-Aldrich, Germany) and streptomycin (Sigma-Aldrich, Germany). The cells were incubated in a humidified incubator at 37°C in 5% of CO2 atmosphere.

Mouse immunization with HCW antigens and induction of breast cancer in mice

Six-to-eight-week-old BALB/c female mice were studied in the present research. The mice were purchased from the Pasteur Institute of Tehran, Iran. They were maintained and examined according to the Institutional Animal Care guidelines and Ethical Committee of Isfahan University of Medical Sciences. The mice were kept in animal research house in groups of six mice per cage and fed with appropriate food and clean water. Groups of one, two, and three were injected subcutaneously with 125 μL of each HCW antigens, including crude extract of the cyst wall (laminated layer), the purified upper and the lower bands obtained from SDS-PAGE of the cyst wall absorbed on 125 μL alum as an adjuvant, respectively. Groups of four and five were injected with 125 μL alum alone and in combination with 125 μL PBS as the control groups, respectively. Mice were given boosters every 5 days up to five injections. At each immunization, 50 μg of each antigen was injected. Five days after the last antigen injection, all the mice were injected with 4T1 breast cancer cells (one million cells per mice) at the mammary fat pad. About 24 days after 4T1 injection, blood samples were taken from each mouse, and their sera were kept at -20°C until they were used. The size of the developed tumors was also measured. For this purpose, two dimensions of each tumor were measured using a digital caliper, and the tumor volumes were calculated according to the formula;

$$\text{Tumor volume } (mm^3) = \text{Length } (mm) \times \text{Width } (mm)^2 \times 0.52$$

These mice were followed up for 2 months to evaluate their survival time and metastasis of breast cancer to the lung. At the half time of an 80-day experiment period, the number of the live 4T1 mice that had received different treatments of HCW antigens was counted. To determine metastasis status, the number of nodules formed in the lung of mice was also counted under a stereomicroscope after mouse death.

Assessment of mouse serum cytokines by ELISA

ELISA tests were performed according to the instructions of Cytokine ELISA Kits (eBioscience, USA) to measure IL-2, IL-4, TNF-α, and IFN-γ. Briefly, different anticytokine antibodies (anti: IFN-γ, IL-2, IL-4, and TNF-α) were coated on ELISA plate wells and the plates were incubated overnight at 2–8°C. Afterwards, the plates were washed and blocked with ELISA/ELISAPOT (1% BSA in TBS) for 1 h. The plates were then incubated with the mouse sera and kept for 2 h at room temperature. After washing, detecting antibody (Antimouse IL2/IL4/TNF-α/IFN-γ) was added to each well and incubated for 1 h at room temperature. The plates were then washed and incubated with secondary antibody at room temperature for 30 min. In the next step, following washing, the substrate solution (TMB) was added to each well and incubated at room temperature for 15 min. Finally, stop solution was added and optical densities (OD) of wells were measured (at 450 nm) using an ELISA reader. The amount of each cytokine in sera was estimated according to the instructions of each kit. ELISA test was performed in triplicate for each sample.

Statistical analysis

All experiments were performed in a completely random design with three replications for each analysis. One-way ANOVA and Duncan's multiple range tests were used to compare the mean values. All graphics were drawn by Microsoft Excel 2010. Moreover, Pearson's correlation coefficients (r) were calculated separately by data of cytokines (IL2, TNF-α, IFN-γ, and IL4) and cancer parameters (tumor growth, metastasis and survival) for 4T1 breast cancer-bearing mice immunized with HCW antigens, alum and PBS by SPSS21 software (IBM SPSS Statistics for Windows, Version 21.0. Armonk, NY: IBM Corp, 2012).

Results

The amounts of cytokines in the mice immunized with HCW antigens

In the present study, the changes in level of cytokines which are indicators for Th1 such as IL2, TNF-α, and IFN-γ and for Th2 including IL4, were evaluated in 4T1 Balb/c mice (breast cancer) that had been immunized with HCW antigens (laminated layer, 28 and 27 kDa protein bands). Overall, results showed that antigens increased the amounts of some Th1 and Th2 cytokines in Balb/c mice [Figure 1a-d].

The level of IL2 was significantly higher in the mice injected with HCW antigens (laminated layer, upper (28 kDa), and lower (27 kDa) protein bands) than the control groups (Alum alone or PBS). Though the amount of IL2 in mice treated with laminated layer was significantly greater than the group injected with 28 kDa antigen (upper band), there was no remarkable difference between mice that received antigens of laminated layer and 27 kDa

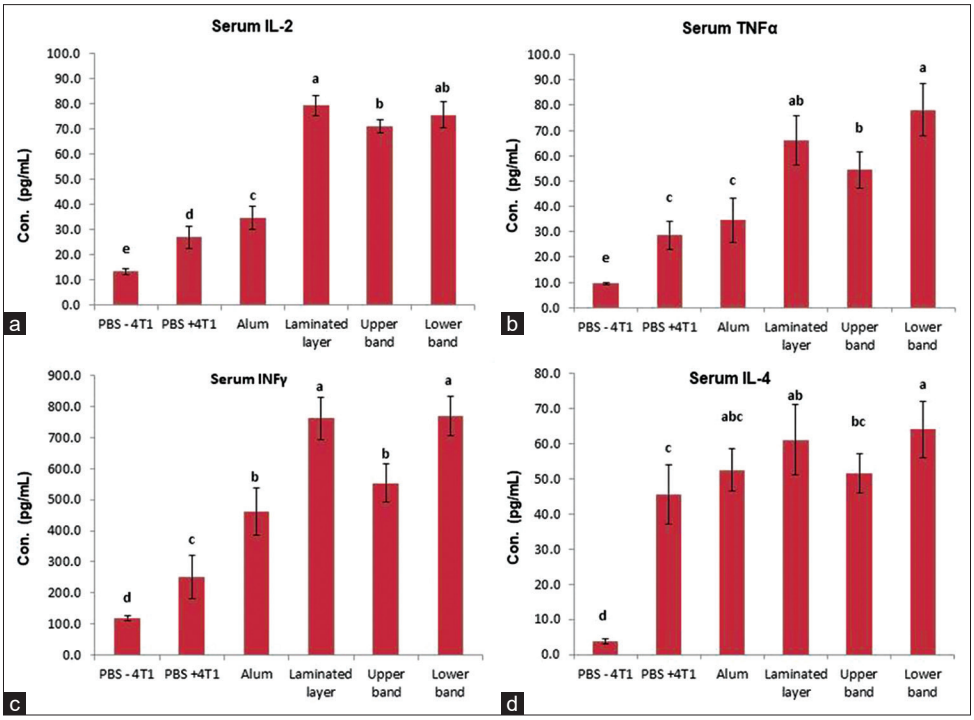

Figure 1: The serum level of Th1 and Th2 cytokines, including a) IL-2, b) TNF-α, c) IFN- γ, and d) IL- 4 in normal mice and 4T1 mice immunized with alum, PBS and HCW antigens, including crude extract of HCW (laminated layer) 28 kDa (upper band) and 27 kDa (lower band) proteins. Mouse immunization was performed before injection of 4T1 breast cancer cells. Values are mean ± SD (*n* = 3). One-way ANOVA and Duncan's multiple range tests were used to compare the mean values. The dissimilar lowercase letters on the columns represent a statistically significant difference among different treatments (*P* < 0.05)

protein band (lower band). Moreover, no notable difference was observed in mice treated with 27 and 28 kDa protein bands. The highest and lowest level of IL2 was related to cancerous mice (Balb/c + T41) immunized with laminated layer (approximately 80 pg/mL), and normal mice treated with PBS (about 13 pg/mL), respectively [Figure 1a].

The amount of TNF-α was significantly greater in groups treated with cyst wall antigens (crude extract HCW, 27 and 28 kDa protein bands) in comparison with the control groups (alum alone or PBS). Unlike IL2 results, a significant difference was observed in amounts of TNF-α between mice immunized with antigens of 28 (upper band) and 27 kDa (lower band). However, the difference between groups treated with antigens of the laminated layer and the upper band was not noticeable. 4T1 mice injected with 27 kDa antigen had the maximum level of TNF-α (about 80 pg/mL) while normal mice under the control condition (PBS injection) showed the lowest level of TNF-α, about 10 pg/mL [Figure 1b].

The amounts of IFN-γ increased remarkably in the mice immunized with laminated layer and 27 kDa antigens in comparison with the control groups (alum alone or PBS) and the group treated with 28 kDa protein band (upper band) as well. There was no significant difference between groups injected with 28 kDa protein band and Alum. The highest level of IFN-γ about 770 pg/mL was related to 4T1 mice immunized with 27 kDa antigen, whereas the minimum amount of IFN-γ (about 119 pg/mL) was observed in normal mice treated with PBS [Figure 1c].

The amounts of IL4 in groups immunized with laminated layer and 27 kDa (lower band) antigens was dramatically more than mice treated with 28 kDa protein band (upper band) and the control mice (PBS). No significant difference was observed among mice injected with laminated layer, upper protein band and alum. The mice treated with 27 kDa protein band (lower band) and PBS had the greatest and the lowest amount of IL4 (about 64 and 4 pg/mL), respectively [Figure 1d].

Analysis of breast cancer status in the mice treated with HCW antigens

The changes in some parameters indicating breast cancer progress including tumor growth, metastasis, and survival [Figures 2a-c] were presented in 4T1 mice that had been immunized with HCW antigens (crude extract of HCW (laminated layer) 28 kDa (upper band), and 27 kDa (lower band) proteins).

The results of tumor volume as an indicator for tumor growth showed that tumor sizes in mice injected with laminated layer and 27 kDa (lower band) antigens were significantly smaller than tumor sizes in mice immunized with 28 kDa (upper band) antigen and the control mice (injected with alum alone or PBS). In addition, no

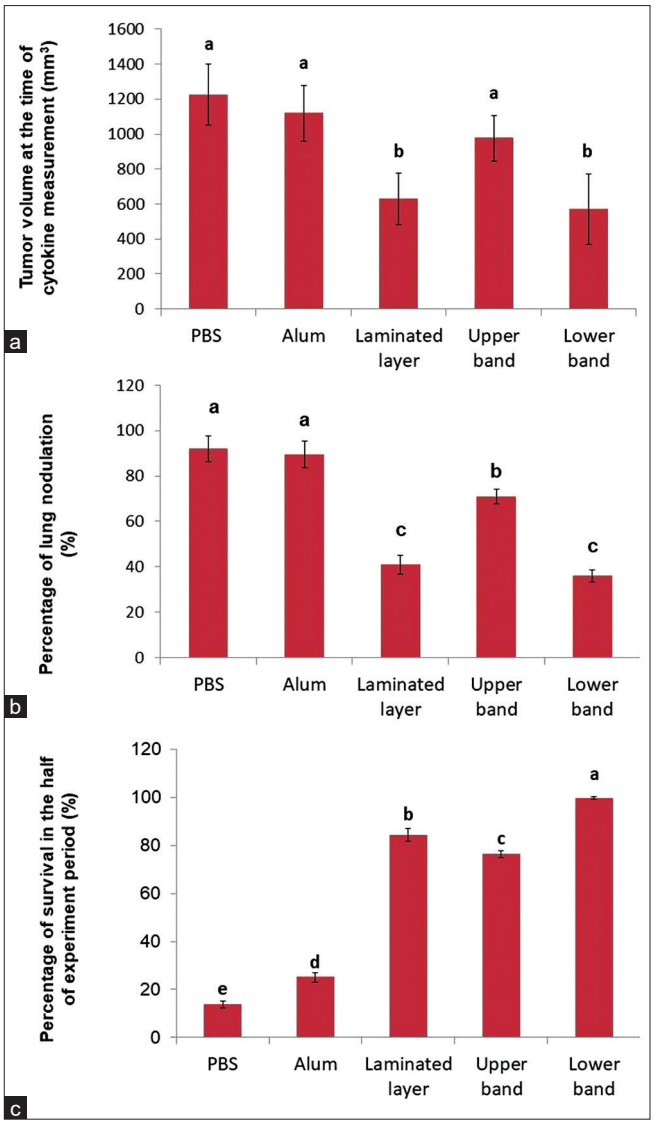

Figure 2: Changes in parameters indicating breast cancer progress, including a) tumor volume (mm³), b) percentage of lung nodulation, and c) percentage of survival at the half time of an 80-day experiment period in 4T1 mice immunized with hydatid cyst wall antigens, including crude extract of hydatid cyst wall (laminated layer) 28 kDa (upper band) and 27 kDa (lower band) protein bands, alum and PBS. Mouse immunization was performed before injection of 4T1 breast cancer cells. Values are mean ± SD (n = 3). One-way ANOVA and Duncan's multiple range tests were used to compare the mean values. The dissimilar lowercase letters on the columns represent statistically significant difference among different treatments ($P < 0.05$)

remarkable difference was observed between mice injected with 28 kDa (upper band) protein band and the control mice treated with alum alone or PBS [Figure 2a].

The formation of nodules in the lung or nodulation in mice immunized with HCW antigens indicated the metastasis status of breast cancer to the lung. The percentage of lung nodulation in mice injected with laminated layer and 27 kDa (lower band) antigens was noticeably lower than mice immunized with 28 kDa (upper band) antigen and the control mice injected with alum alone or PBS (about 40% vs. 70–90%). Moreover, there was a significant difference

in the percentage of lung nodulation between mice injected with 28 kDa (upper band) antigen and the control mice treated with alum alone and PBS [Figure 2b].

The results of the survival percentage of mice treated with HCW antigens, alum alone and PBS at the half time of an 80-day experiment period [Figure 2c] showed that 100% of mice injected with 27 kDa protein band (lower band) were alive. However, a reduction in survival percentage was observed in groups injected with laminated layer, 28 kDa antigen (upper band), alum alone and PBS, respectively. The lowest survival percentage (about 14%) was related to mice treated with PBS.

Correlations between cytokines and tumor volume, metastasis, and survival

Pearson's correlation coefficients (r) used to evaluate changes in IL2, TNF-α, IFN-γ, and IL4 relative to tumor volume metastasis and survival indicated significant and negative correlations between all cytokines and tumor volume. Significant and negative correlations were also observed between all cytokines and metastasis. However, with exception of IL4, there were significant and positive correlations between cytokines and survival [Table 1]. The Pearson's correlation coefficient (r) close to 1 or -1 shows that there is a strong positive or negative relationship between parameters of interest, respectively, whereas Pearson's correlation coefficients (r) close to zero indicates a weak relationship between them (SPSS Tutorials: Pearson Correlation. http://libguides.library.kent.edu/SPSS/Pearson Corr).

In terms of the correlation between parameters indicating breast cancer progress (tumor volume, metastasis and survival), the results of Pearson's correlation coefficients showed that there was a significant and positive correlation between tumor volume and metastasis, while both parameters had a significant and negative correlation with survival.

Discussion

Changes in amounts of cytokines in response to HCW antigens and breast cancer

In this study, it has been shown that concentrations of IL2, TNF-α, IFN-γ, and IL4 significantly increased in mice injected with antigens of HCW (laminated layer, 28 and 27 kDa protein bands) in comparison with those injected with alum alone or PBS. An increase in some cytokines (IL2, IL4, and IFN-γ) in mice inoculated with *Echinococcus granulosus* protoscolices have already been detected.[28] Moreover, a rise in levels of cytokines, including IFN-γ, IL-12, IL-16, IL-18, IL-4, IL-5, IL-10, and IL-13 in sera of patients infected with hydatid cyst was reported by Mezioug and Touil-Boukoffa (2009). They showed that both Th1 and Th2 cell subsets are active in human hydatidosis.[29] Our results showed amounts of IL2, TNF-α, IFN-γ, and IL4 increased in mice immunized with HCW antigens and challenged with breast cancer cells. Walker II and colleagues (2017) represented an increased production of such cytokines in response to breast cancer in Balb/c mice treated with 4T1 cell line (inducing mammary tumors).[30] However, for the first time, we reported the cytokine changes in cancerous mice immunized with HCW antigens, which implies the induction of cytokine production by both HCW antigens and breast tumors. Higher levels of IL2, TNF-α, IFN-γ and IL4 in mice injected with antigens of 27 kDa protein band (lower band) and laminated layer could be a result of stronger immune responses to these antigens in comparison to antigens of 28 kDa (upper band). Such immune responses indicate that antigens of 27 kDa protein band (lower band) and laminated layer were probably more incompatible than antigens of 28 kDa protein band (upper band) to mice immune system, and by producing large amounts of cytokines, mice probably have been able to adapt to these external antigens. In support of this idea, the elevation of cytokine levels has been reported as a mechanism for host adaptation to foreign invaders and external antigens.[31,32]

Tumor growth, metastasis and survival in response to HCW antigens

In our results, the significantly smaller size of breast tumors in mice immunized with antigens of 27 kDa protein band (lower band) and laminated layer in comparison with other treatment groups represented the positive effect of these antigens on inhibition of breast tumor growth in mice. Other studies have shown antitumor effects of hydatid cyst on breast cancer. For instance, hydatid cyst fluid promoted apoptosis in breast cancer cells.[14] The prevention of tumorigenesis by DMBA in mammary tissues of rats with hydatid disease is also another example.[33] Moreover, injection of human hydatid cyst fluid (HCF) to mice (before

Table 1: Pearson's correlation coefficients (r). Correlation coefficients between cytokines (IL2, TNF-α, IFN-γ, and IL4) and cancer parameters (tumor growth (tumor volume), metastasis, and survival) in 4T1 breast cancer-bearing mice immunized with HCW antigens, including crude extract of HCW (laminated layer) 28 kDa (upper band) and 27 kDa (lower band) protein bands, alum and PBS

	Pearson's Correlation coefficients						
	IL2	**TNFa**	**INFy**	**IL4**	**Tumor volume**	**Metastasis**	**Survival**
Tumor volume	-0.894*	-0.972**	-0.968**	-0.968**	1	0.995**	-0.926*
Metastasis	-0.904*	-0.974**	-0.947*	-0.939*	0.995**	1	-0.934*
Survival	0.978**	0.981**	0.930*	0.852	-0.926*	-0.934*	1

*Correlation is significant at the 0.05 level (2-tailed). **Correlation is significant at the 0.01 level (2-tailed)

tumor cell challenge) protected 40% of them against colon tumor growth.[34] In another study, the treatment of mice bearing melanoma with hydatid cyst antigens led to tumor growth inhibition.[16]

In our study, a lower percentage of lung nodulation following immunization of mice with antigens of 27 kDa protein band (lower band) and laminated layer of HCW indicated that these antigens could decrease the rate and intensity of cancer metastasis though they were not able to prevent the immigration of cancer cells from the breast to the lung tissues. However, an increase in cancer metastasis from mammary tissue to the liver has been reported in mice treated with both protoscoleces of *Echinococcus granulosus* and 4T1 breast tumor cells, which indicated the pathogenic role of this parasite in exacerbating cancer.[35]

The high percentages of survival (75–100%) which was observed in mice treated with antigens of HCW (laminated layer, 28 and 27 kDa protein bands) demonstrated that such treatments might delay the cancer-related death. In agreement with our result, it has been reported that malaria infection could prolong the survival of mice with Lewis lung cancer.[36] Berriel and colleagues (2013) also showed that vaccination of mice with human hydatic cyst fluid (HCF) resulted in the survival of 40% of mice injected with CT26 colon cancer cells by the end of the experiment period (100 days after tumor challenge).[34]

Relationships between cytokines (IL2, TNF-α, IFN-γ and IL4) and cancer parameters (tumor growth, metastasis and survival) in 4T1 mice immunized with HCW antigens

Previous research showed that cytokines play an important role in the function of the immune system in breast cancer.[37] Significant and negative correlations between cytokines (IL2, TNF-α, IFN-γ, and IL-4) and tumor volume confirmed reverse relationships between changes in the amount of these cytokines and tumor growth, which means any increase in the amount of these cytokines can lead to prevention of tumor growth, while low concentration of these cytokines may promote tumor growth. It has been shown that the administration of IL-2 led to inhibition of breast cancer in patients.[37] The ability of IFN-γ to inhibit the growth of breast cancer cells has previously been demonstrated.[38] Although there are some reports that TNF-α inhibits tumor growth, some other pre-clinical findings suggest that TNF-α may promote cancer development and progression.[39] IL-4 has been known for its anti-tumor activity against various cancers, such as breast cancer.[40] In addition, our results showed reverse relationships between changes in amounts of IL-2, TNF-α, IFN-γ, and IL-4 and metastasis. As a matter of fact, the low amounts of these cytokines might increase metastasis, whereas a rise in the levels of IL-2, TNF-α, IFN-γ, and IL-4 probably will decrease the occurrence of metastasis. Ma *et al*., (2017) showed that higher level of TNF-α in stage III breast cancer were associated with lymph node metastasis.[41] In breast cancer patients with skin metastasis, topical injection of IFN-γ resulted in complete or partial regression of the skin lesions.[38] It has been reported that blocking IL4 signalling mediates breast cancer cell proliferation, invasion, and tumor growth by down-regulation of MAPK activity.[42] However, except for IL-4, all cytokines had a direct relationship with mice survival. Therefore, immunization with the HCW antigens that elevated serum levels of IL2, TNF-α, and IFN-γ remarkably in tumor-bearing mice resulted in an increase in survival, suggesting that these cytokines may contribute to anti-cancer effects of HCW antigens. However, according to our results, the percentage of survival depends on the levels of these cytokines.

Conclusions

The results of the present study showed that immunization of mice with HCW antigens, especially with laminated layer and 27 kDa protein band increased the amounts of cytokines, including IL2, TNF-α, IFN-γ, and IL4. These results indicated that a rise in the level of such cytokines in response to immunization with HCW antigens can inhibit breast tumor growth and metastasis and improve survival in 4T1 mice. Therefore, it is suggested that the usage of HCW antigens could be a promising approach in cancer immune therapy.

Acknowledgments

Thanks to the Isfahan University of Medical Sciences for financial support and for providing space and facilities.

Financial support and sponsorship

This work was supported by a research grant (number: 395309) from Isfahan University of medical sciences.

Conflicts of interest

There are no conflicts of interest.

Received: 29 Aug 19 **Accepted:** 31 Oct 19
Published: 11 Dec 2020

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