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Immunotherapy of Metastatic Mouse Breast Cancer by Adherent Splenocytes Pulsed With Extracts of Heated Tumor Cells and *Lactobacillus Casei*

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Abstract

Introduction: Flask-adherent Splenocytes (SACs) fulfill antigen-presenting cell requirements of acquired immune responses. This study was done to evaluate the efficacy of new immunotherapy against breast cancer made by SACs pulsed with the extract of heated 4T1 cells and *Lactobacillus casei*, as a probiotic.

Materials and Methods: Mammary carcinoma was induced by injection of 4T1 cell line in the flank of female Balb/c mice. The first SACs therapy was started on day 11 after tumor induction when all animals had developed a palpable tumor. SACs therapy was done twice at a 10-day interval.

Results: Mice with mammary tumors received SACs pulsed with combined heated 4T1 cells and *L. casei* determined a more desirable survival curve and a slower rate of tumor development compared to the other groups. At least 20% of the group receiving combined immunotherapy were alive by day 58. Those mice receiving SACs pulsed with the Lysate of heated tumor cells died by day 45.The maximum survival of other mice was up to 38 days after tumor induction. Moreover, SAC pulsed with combined agents significantly amplified the secretion of Interferon-γ (IFN-γ), and conversely reduced the secretion of Transforming growth factor-β (TGF-β) and Interleukin 4 (IL-4) in the splenocyte population compared to splenocytes from other groups. Combined immunotherapy increased the expression of p53 and caspase 3 genes and reduced the exertion of BCL2 more than other immunotherapy protocols.

Conclusion: Immunotherapy with SACs pulsed with heated 4T1 cells and *L. casei* promotes beneficial outcomes in the mouse model of breast cancer.

Keywords: Breast cancer, Immunotherapy, Apoptosis, *Lactobacillus casei*, Antigen-Presenting Cell, Spleen

1. Introduction

Nowadays, malignancy is one of the leading causes of mortality in the world. Breast cancer is one of the most dangerous ones in the female population, accounting for 25% of malignancies in women worldwide [1, 2]. Breast cancer is very invasive and spreads rapidly in the body [2-4]. The spontaneously generated 4T1 cell line was derived in BALB/c mice. These cells can metastasize to various organs similar to the tissues involved in metastatic breast cancer in humans [5, 6].

Low immunogenic tumor cells such as 4T1 and human adenocarcinoma mammary tumor can grow rapidly and evade immune responses [6]. Therefore, strategies that can change the type of polarization are considered appropriate. Therefore, it is necessary to use mechanisms that activate immune cells against these tumor cells.

Dendritic cells are professional antigen-presenting cells that act as initiator and regulators of specific immune responses against various antigens, such as tumor antigens. Dendritic Cells (DCs) can be used in cancer immunotherapy. Tumor antigen-loaded DCs can enhance the cytotoxic T cells or T helper 1 (Th1) responses that are needed to eradicate most cancers [7]. It is possible to prepare dendritic cells for immunotherapy from various sources. Flask-adherent Splenocytes (SACs) are one of the main sources of dendritic cell production in mice [8].

Previous studies have shown the effect of bacterial components on the maturation of dendritic cells. For example, it has been shown that the use of Listeria monocytogenes antigens in combination with tumor antigens priming of dendritic cells has enhanced immunotherapy against the fibrosarcoma mouse model [9]. In recent years, the role of lactic acid bacteria (Lactobacilli) in preserving human health and stimulating the immune system has been significantly considered [10-12] These agents are also able to stimulate immune responses such as the secretion of cytokines by lymphocytes [13, 14]. In addition, oral consumption of Lactobacillus has anti-breast cancer activity and prevents tumor metastasis [15]. However, there have been no studies on the use of *L. casei* as a factor in the maturation of dendritic cells pulsed with tumor antigens. This study set out to investigate the efficacy of new immunotherapy against breast cancer made by SACs pulsed with the extract of heated 4T1 cells and *L. casei*.

2. Materials and Methods

This study was an experimental study that was performed as a case-control study.

Reagents

Fetal Calf Serum (FCS) and Dulbecco's Modified Eagle Medium (DMEM) were obtained from GIBCO/Life Technologies Inc. (Gaithersburg, MD). Enzyme-Linked Immunosorbent Assay (ELISA) kits were purchased from Qiagen (Hilden, Germany). Other reagents were procured from Sigma-Aldrich (St. Louis, MO).

Isolation of flask-adherent splenocytes

Flask-adherent Splenocytes (SACs) were isolated as described previously with some modifications [16, 17]. After euthanizing, the spleens of Balb/c mice were removed under sterile conditions and a single cell suspension was prepared. The pellet was suspended in 2 ml of RPM medium and gently transferred to 2-3 ml of Nicodense gradient medium and centrifuged for 10 minutes at 900 g at 4°C. The low-density cells were carefully removed, washed, and centrifuged twice as in the previous step. To remove non-adherent cells (lymphocytes), cell suspensions were incubated in the culture flask for 90 minutes. Afterward, non-adherent cells were removed by gently washing the plate. Cells adhering to the plate were cultured for 18 hours in a complete culture medium at 37°C and 5% CO_2. During this time, cells with similar functions to dendritic cells are separated from the bottom of the plate after maturation.

During the 18-hour culture and depending on the type of treatment group that these cells are to be used, these cells were pulsed with Lysate of heated tumor cells (100µg/ml), killed preparation of *L. casei* (100µg/ml), or a combination of both. To remove unused peptides, cells were washed three times with saline phosphate buffer.

Culture and proliferation of mouse adenocarcinoma cell line (4T1)

4T1 is an adherent murine mammary carcinoma cell line from a BALB/cfC3H mouse. The 4T1 cell line was obtained from the Pasteur Institute of Iran. Cells were cultured in DMEM medium containing 10% FBS, 1 mM L-glutamine, and penicillin/streptomycin in an incubator containing 5% CO_2 at 37°C. When 80% of the surface of the flask was covered by the cells, cell passage was performed.

Induction of heat shock in cells and preparation of tumor cell extracts

In order to increase the stress level of proteins in tumor models, they were subjected to sub-lethal shock. The cells in the flask were heated at 42°C for 60 min using a Ben Marie, and were then placed at 3°C for 12 h;this time was selected as the best time using preliminary studies. Tumor cells underwent necrosis and lysis by freezing in liquid nitrogen and re-melting using a 37-degree Celsius bin (four times). The cell membranes were precipitated using a centrifuge at 4000 rpm. The supernatant, which is the cell extract, was separated and the extract was passed through a 0.2-micron filter. The

amount of protein resulting from the lysis of the extract from one million 4T1 cells per milliliter was measured by the Bradford method.

Bacterial antigen preparation

L. casei (ATCC: 393) was procured from Pasteur Institute of Iran and cultivated in a Rogosa's medium for 24 h at 37°C. Afterward, bacteria were washed twice with PBS and heated at 60°C for 60 minutes. A sonicator was used to prepare bacterial lysates (3 times for 2 minutes with a power of 5 and a cycle of 50).

Tumor induction, evaluation and treatment

The study population included BALB/c mice, within the age range of 6 to 8 weeks, obtained from the Pasteur Institute of Iran. All animal studies were performed following the regulations of the Ministry of Health of the Islamic Republic of Iran and were approved by the Ethics Committee for Animal Experiments of Urmia University (IR-UU-AEC-1644).

Mice were kept at an appropriate temperature (in the range of 23°C) and 12-hour light cycles. Animals had free access to food and water. In order to induce the mammary carcinoma, 1×10^4 live tumor cells in a volume of 30 µl of Phosphate-buffered saline (PBS) were injected subcutaneously in the right flanks of each mouse. Tumor growth was assessed every 5 days by using a digital caliper. Tumor volume was calculated using the ellipsoid formula (length × width × height 0.5236).

Immunotherapy protocol was initiated once all the mice had developed a palpable breast tumor. If the tumor reached a size of 800 mm, the animal was euthanized and excluded from the study. At this time, the tumor-bearing mice were randomly divided into 5 equal groups (n=10): mice, SACs, SACs pulsed with the Lysate of heated tumor cells, SACs pulsed with the killed preparation of *L. casei*, SACs pulsed with combined the Lysate of heated tumor cells and the killed preparation of *L. casei*. Mice were twice vaccinated with ten-day intervals in the right flank with 10^6 SACs. All injections were administrated via subcutaneous injections in the left flank. Saline-treated tumor-bearing mice received 200 µl saline solution at the same schedule.

Ten days after the last immunotherapy, half of the animals were euthanized for immunological evaluation in the splenocytes population. The other half of the mice were kept until death to draw a survival curve.

Half of the mice were vaccinated ten days after the last vaccination. The spleen of the mice was separated and homogenized in a homogenizer to separate the cells. It was then centrifuged at 1700 rpm for 5 minutes. The erythrocytes were omitted by ACK buffer. Afterward, a cell suspension (2×10^6 cells/mL) was cultured in 24-well plates and primed with tumor antigens derived from the 4T1cells by freeze and thaw (50 µg/mL). After 72 h, the culture supernatants were isolated, and the secretion of Transforming growth factor-β (TGF-β), Interferon gamma (IFN-γ),Interleukin 10 (IL-10), and Interleukin 4 (IL-4) was monitored via the ELISA method according to the manufacturer's instructions.

Cytotoxicity potential of Natural killer (NK) assay

Cytotoxic activity of the NK population of splenocytes was monitored by a Lactate Dehydrogenase (LDH) cytotoxicity detection kit (Takara Bio USA, Inc.). In this assay, the splenocytes were effector cells, and the 4T1 cells were target cells. Splenocytes were isolated as described above. The effector and target cells were rinsed with DEMEM containing 1% BSA. The effector and target cells co-cultured at a ratio of 50/1 at 37°C for 6 hours. After centrifugation, the supernatants were transferred to 96-well flat-bottomed, and the LDH level was detected accrediting to the kit's manufacture guideline. The percentage of cell-mediated cytotoxicity was recorded by the following formula: cytotoxicity (%) = (experimental release–spontaneous target release–spontaneous effector release) / (maximal target release– spontaneous target release) × 100%.

Evaluation of apoptosis in tumor masses

In brief, YTzol pure RNA Kit (YTA, Iran) was used to extract the total RNA from frozen tumors according to the manufacturer's instructions. Complementary DNAs (cDNAs) were prepared by a cDNA Synthesis Kit (YTA, Iran) according to the manufacturer's guidelines. The real-time PCR amplification was done in triplicate by an SYBR Green kit according to the manufacturer's instruction. In previous experiences performed by our group, to select the appropriate reference gene in tumors induced by 4T1 cells, the expression stability of two well-known genes *β-actin* and Glyceraldehyde-3-Phosphate Dehydrogenase (*GAPDH*) were evaluated using NortFindler and BestKeeper software, and values of ΔCt were compared. The results showed that the *GAPDH* gene is the most suitable reference gene for normalizing the expression of 4T1 induced tumor genes (Data not shown). The mRNA quantity was defined from the standard curve and normalized relative to the amount of

mRNA in the reference gene. The sequences of the specific forward and reverse primers are shown in Table 1.

Statistical analysis

Data analysis was performed using the Kruskal–Wallis test, followed by pairwise comparisons, using the Mann–Whitney U-test with the Bonferroni adjustment at a 95% confidence level. To evaluate the survivability of tumor-bearing animals, The Kaplan–Meier estimator was performed. Data were displayed as mean and standard deviations.

3. Results

Clinical appearance

After tumor induction, mice were monitored every five days for the first sign of a palpable tumor. All immunization protocols were started on day 11 after tumor induction, when a palpable tumor was observed in all mice, and continued until day 59, when mice in all groups died.

As shown in Figure 1 A, mice that received SACs pulsed with both the Lysate of heated tumor cells and the killed preparation of *L. casei* showed a more favorable survival curve than mice in other groups. At least 20% of mice in this group were alive by day 58 after tumor injection. However, all mice receiving SACs pulsed with the Lysate of heated tumor cells died by day 45 after tumor induction. The survival rate of mice in other groups was very low, so that the maximum survival of other mice was up to 38 days after tumor induction (Figure 1 A).

According to Figure 1 B, a comparison of tumor growth changes between different groups showed a significant decrease in tumor growth intensity in immunized mice receiving combined immunotherapy from the 27th day after induction until the end of the study compared to tumor-bearing mice receiving saline or SACs. This time started in the immunized mice vaccinated with SACs pulsed with the Lysate of heated tumor cells from day 32 after the induction compared to tumor-bearing mice receiving saline or SACs. The change in tumor volume in the tumor-bearing mice of the other groups did not show a significant difference compared to the tumor-bearing mice which received saline (Figure 1 B).

Immunological evaluation

According to Figure 2, only IFN-γ increased significantly in the supernatant of splenocytes isolated from tumor-bearing mice which received combined immunotherapy compared to other groups. Measurement of IL-4, TGF-β and IL-10 levels in the supernatant of splenocytes isolated from tumor-bearing mice showed that the level of these cytokines in the groups vaccinated with SACs pulsed with combined Lysate of heated tumor cells and the killed preparation of *L. casei* or SACs pulsed with of heated tumor cells significantly reduced compared to the control group. However, the decrease of IL-4 and TGF-β levels in the group that received combined immunotherapy was more significant than the level of these cytokines in tumor mice vaccinated with SACs pulsed with heated tumor cells (Figure 2).

The assessment of LDH released from tumor cells is a fast and straightforward colorimetric assay to quantify the cytotoxicity potential of the NK cell population. The findings indicated that NK cytotoxicity was only significantly mounted in the tumor-bearing mice obtaining the combined immunotherapy compared to the other animals (Figure 3).

Assessment of mRNA expressions of apoptosis-related gens

Attained results showed that the mRNA expression of p53 significantly increased in tumor-bearing mice receiving SACs pulsed with combined the Lysate of heated tumor cells and the killed preparation of *L. casei* or SACs pulsed with the Lysate of heated tumor cells compared to the tumor-bearing mice receiving SACs alone or SACs pulsed with the killed preparation of *L. casei*. Albith, combined immunotherapy increased the expression ratio of p53 more profoundly than other im-

Table 1. Primer sequences and sizes of RT-PCR products

Gene	Forward Primer	Reverse Primer
GAPDH	5'TCAACAGCAACTCCCACTCTTCCA 3'	5'ACCCTGTTGCTGTAGCCGTATTCA3'
P53	5'-GCCATGGCCATCTACAAGAA-3'	5'-CTCGGGTGGCTCATAAGGTA-3'
bcL2	5'-CTTTGAGTTCGGTGGGGTCA-3'	5'-AGTTCCACAAAGGCATCCCA-3'
Caspase 3	5'-AAGATACC GGTGGAGGCTGA-3'	5'-AAGGGACTGGATGAACCACG-3'

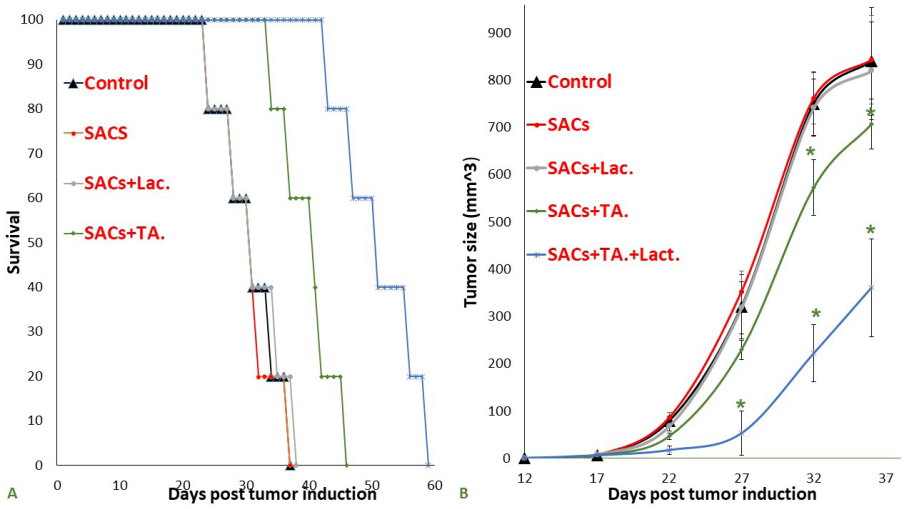

Figure 1. Clinical evaluation of tumor-bearing rats. Immunotherapy was started on day 11 after induction and repeated 10 days later. A) Evaluation of rat survival rate. B) Assessment of the mammary tumor size. Tumor size is measured with a caliper every 5 days. Overall, the use of SACs pulsed with both the Lysate of heated tumor cells and the killed preparation of *L. casei* led to better results. (*P<0.05 vs. the control tumor-bearing mice and/or the tumor-bearing mice receiving other treatment). SACs: Flask-adherent splenocytes; SACs+LAC.: Flask-adherent splenocytes pulsed with killed preparation of *L. casei*; SACs+TA.: Flask-adherent splenocytes pulsed with the Lysate of heated tumor cells; SACs+LAC. +TA.: Flask-adherent splenocytes pulsed with combined killed preparation of *L. casei* and the Lysate of heated tumor cells.

munotherapy protocol (Figure 4). Moreover, the lowest caspase-3 mRNA expression and conversely the highest BCL-2 and p53 mRNA expression were significantly detected in tumor-bearing mice immunized with SACs pulsed with the killed body of *L. casei* and tumor antigens compared to the other groups (Figure 4).

4. Discussion

The present investigation demonstrated that immunotherapy with SACs pulsed with heated 4T1 cells and *L. casei* promotes beneficial outcomes in the mouse model of breast cancer by intensifying anti-tumor immune responses and promoting apoptosis in tumor cells. Among the immune responses, the cellular immune arm and the Th1 responses play an essential role against tumor cells [18]. Adjuvants are beneficial substances that can lead the

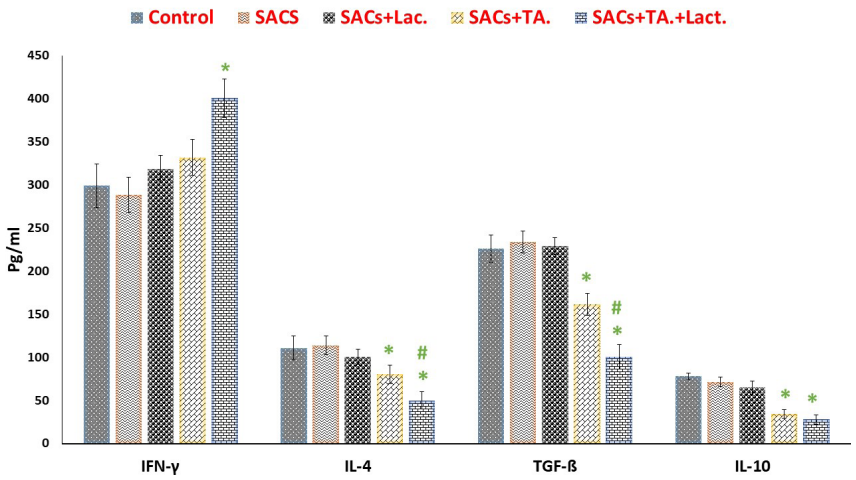

Figure 2. Effects of the immunotherapy on cytokine production in the splenocytes. One half of the tumor-bearing mice were euthanized one week after the last immunotherapy, and the splenocytes were cultured for 72 hours. (*P<0.05 vs. the control tumor-bearing mice and/or the tumor-bearing mice receiving other treatment, # P<0.05 vs. the tumor-bearing mice receiving flask-adherent splenocytes pulsed with the Lysate of heated tumor cells). SACs: Flask-adherent splenocytes; SACs+LAC.: Flask-adherent splenocytes pulsed with killed preparation of *L. casei*; SACs+TA.: Flask-adherent splenocytes pulsed with the Lysate of heated tumor cells; SACs+LAC. +TA.: Flask-adherent splenocytes pulsed with combined killed preparation of *L. casei* and the Lysate of heated tumor cells.

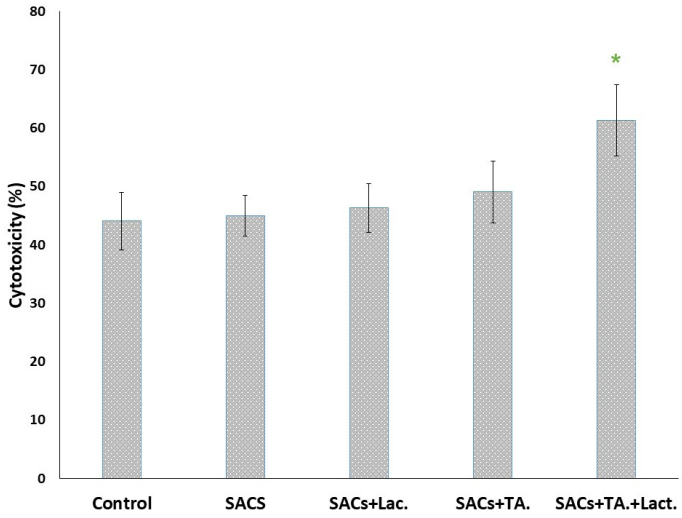

Figure 3. Effects of the immunotherapy on the tumor cytotoxicity of the splenocyte population. Cytotoxic activity of the NK population of splenocytes was monitored by a Lactate Dehydrogenase (LDH) cytotoxicity detection kit. (*P<0.05 vs. the control tumor-bearing mice and/or the tumor-bearing mice receiving other treatment). SACs: Flask-adherent splenocytes; SACs+LAC.: Flask-adherent splenocytes pulsed with killed preparation of *L. casei*; SACs+TA.: Flask-adherent splenocytes pulsed with the Lysate of heated tumor cells; SACs+LAC. +TA.: Flask-adherent splenocytes pulsed with combined killed preparation of *L. casei* and the Lysate of heated tumor cells.

immune response to the desired arm of the immune system [6]. However, the only approved adjuvant in humans is the alum adjuvant, which is only capable of generating Th2 responses [19]. Another approach to create an appropriate and targeted anti-tumor response is to use dendritic cells. These cells are professional antigen presenting cells that act as powerful primers and regulators of specific immune responses against various antigens [7, 19, 20].

As mentioned earlier, the majority of SACs isolated in this study were dendritic cell populations [17]. However, proving the existence of dendritic cells requires flow-cy-

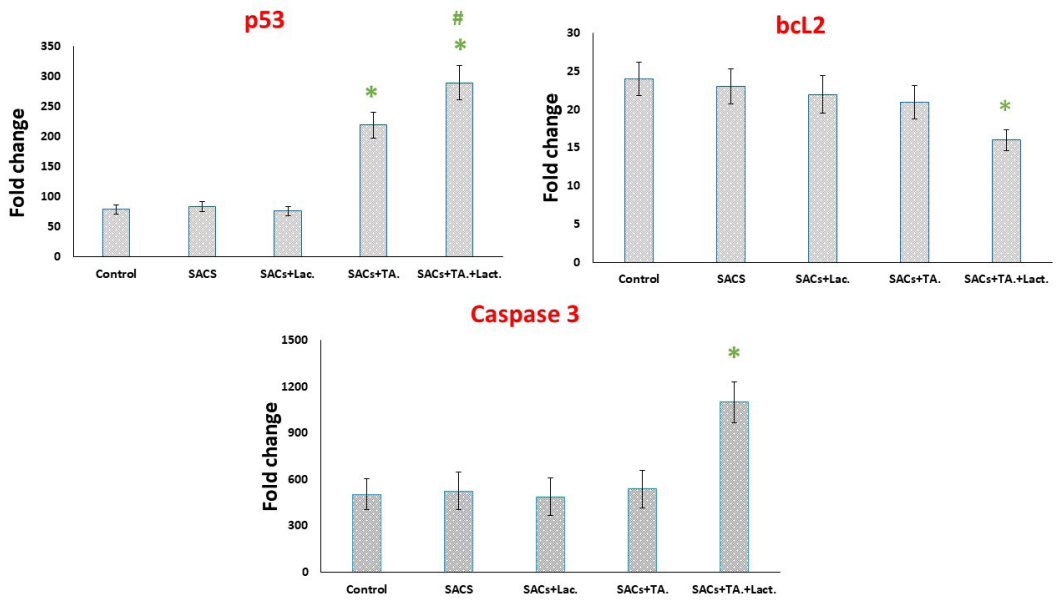

Figure 4. The mRNA expression of P53, bcL2, and Caspase 3 in different experimental groups. (*P<0.05 vs. the control tumor-bearing mice and/or the tumor-bearing mice receiving other treatment, # P<0.05 vs. the tumor-bearing mice receiving flask-adherent splenocytes pulsed with the Lysate of heated tumor cells). SACs: Flask-adherent splenocytes; SACs+LAC.: Flask-adherent splenocytes pulsed with killed preparation of *L. casei*; SACs+TA.: Flask-adherent splenocytes pulsed with the Lysate of heated tumor cells; SACs+LAC. +TA.: Flask-adherent splenocytes pulsed with combined killed preparation of *L. casei* and the Lysate of heated tumor cells.

tometric tests, which were not performed due to financial constraints in the current survey. Therefore, in the present investigation, instead of using the term dendritic cell, the term SACs was used.

Heat Shock Proteins (HSPs) are a group of highly conserved proteins that occur in many organisms. Expression of these molecules by a wide range of stimuli such as heat shock [21]. Interestingly, some members of the heat shock protein family, such as the HSP70 family, can bind to antigens. It leads to increased uptake of these antigens via antigen presenting cells by CD94 receptors [22, 23]. It should be noted that the removal of tumor antigens with HSP70 molecules promotes cross presentation through MHC class 1 molecule and triggers cellular immune responses [22]. Accordingly, in this study, to adequately supply tumor antigens to spleen cells adhering to 4T1 cell extract previously exposed to non-lethal heat shock to induces the expression of the HSP70 molecules, and increases the final polarization toward Th1 responses.

The micro-environment in which the dendritic cells are located has led to the polarization of these cells into a specific subgroup, which ultimately plays a role in the direction of immune responses. For example, microbial products can increase the incidence of co-stimulatory molecules (CD80 and CD86) and MHC molecules at the cell surface by binding to pattern-recognition receptors present on the surface of dendritic cells [24]. In the meantime, like *L. casei* some microbial products may enhance immune responses toward Th1 [25, 26]. In this regard, we use the killed preparation of *L. casei* to activate pattern recognition receptors of SACs. Previous findings suggest that *L. casei* increases the expression of MHC molecules and the expression of CD86 co-stimulatory molecules on dendritic cells, as well as inducing IL-12 production through these cells [26]. IL-12 produced by dendritic cells is one of the most critical factors in polarizing the response of lymphocytes to Th1 [6, 25]. Our findings suggest that the use of SACs alone or SACs pulsed with the killed preparation of *L. casei* did not have any selective advantage in increasing the survival of bearing mice or reducing the mammary tumor size. However, the use of SACs pulsed with the Lysate of heated tumor cells significantly increased the survival rate of mice and decreased the growth rate of tumors compared with the previous two groups of mice. More importantly, combined immunotherapy with killed preparation of *L. casei* and the Lysate of heated tumor cells had synergistic effects, promoting a more favorable survival curve and slower malignancy growth when compared to other tumor-bearing mice.

Other experiments in this study were designed to find some of the mechanisms involved int these results. Tumors are able to escape immune responses by producing certain mediators such as IL-4, TGF-β, and IL-10. These cytokines are able to inhibit the main arm of immune responses against tumors, namely inflammatory macrophages (M1) and Th1 cells [27]. IL-4 may directly increase the growth of breast cancer tumor cells in humans [28]. TGF-β and IL-10 inhibit the effective activation of classical lymphocytes and macrophages [27]. Both recent classes of cytokines have led to the induction of regulatory T cells, which also play a role in inhibiting antitumor responses [6, 29]. On the other hand, regulatory T cells themselves are able to produce these cytokines [12, 29]. Our findings show that SACs primed with heated tumor antigens and heated preparation of *L. casei* and SACs primed with heated tumor antigens significantly reduced the levels of TGF-β and IL-4 cytokines more pronounced than other groups.

Previous experience has shown that the level of INF-γ as a major cytokine of Th1 responses is directly related to antitumor responses [6]. Based on our findings, the INF-γ response significantly increased only in the group that received SACs primed with the killed preparation of *L. casei* and heated tumor antigens.

In addition to the overgrowth of cancer cells, a decrease in the apoptosis rate is also one of the factors in the development of tumors. Numerous investigations have reported the roles of *p53*, *Bax*, *Bcl-2*, and *caspase-3* genes in the induction of apoptosis and cancer treatment [22, 30, 31]. Caspase cascades are involved in the development and progression of apoptosis. In the caspase family, the most important regulator of apoptosis is caspase-3 [32]. The anti-apoptotic protein Bcl-2 prevents apoptosis through the mitochondrial pathway and high expression of this protein is essential for the survival of tumor cells [33]. P53 is a multifunctional tumor suppressor protein that possesses an important function to modulate the apoptosis response, *Bcl-2* and *Bax* gene expression, and DNA repair mechanisms for protecting genomic stability [34]. Our study detected that the lowest caspase-3 mRNA expression and conversely the highest BCL-2 and p53 mRNA expression significantly occurred in tumor-bearing mice vaccinated with SACs pulsed with a killed body of *L. casei* and tumor antigens compared to the other groups.

Natural Killer (NK) cells are indispensable innate cells the participated in the destruction of tumor cells. NK cells destruct malignant cells directly by promoting of apoptosis and the production of cytokines like IFN-γ

[35]. Attained data in the current study suggested that cytotoxicity of NK cells was significantly elevated only in tumor-bearing mice vaccinated with killed preparation of *L. casei* and heated tumor antigens.

5. Conclusion

Collectively, it seems that SACs pulsed with the killed body of *L. casei* and tumor antigens lead to a significant increase in the survival rate of infected tumor-bearing mice, and a decrease in the growth rate of tumors compared to other groups. It seems that the main advantage of this treatment compared to other groups is a significant increase in INF-γ, nitric oxide production, and NK cytotoxicity and a decrease in the levels of TGF-β, IL-4, and IL-10 cytokines by the immune system of treated mice. Combined immunotherapy also increased the expression of *p53* and *caspase 3* genes and reduced the exertion of BCL2 more than other immunotherapy protocol. This study is preliminary and more detailed and in-depth studies are needed. The most important limitation of this study is the measurement of immune responses in the population of splenocytes and not in the internal environment of the tumor.

Ethical Considerations

Compliance with ethical guidelines

This study was approved by the Ethics Committee of the Veterinary Faculty of Urmia University, Urmia,Iran (IR-UU-AEC-1464/AD/3) .

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Author's contributions

Conceptualization and Supervision: Seyyed Meysam Abtahi Froushani and Amir Tukmachi; Methodology: Seyyed Meysam Abtahi Froushani ; Investigation, Writing – original draft, and Writing – review & editing: All authors; Data collection: Kave Golpasandi; Data analysis: Kave Golpasandi and Seyyed Meysam Abtahi Froushani; Funding acquisition and Resources: Seyyed Meysam Abtahi Froushani and Amir Tukmachi.

Conflict of interest

The authors declared no conflict of interest.

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