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Candida Albicans Activated Splenocytes Promote Strong Immune Responses in a Murine Model of Breast Cancer

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ABSTRACT

Background: The potential of Candida *albicans* to modulate antigen-presenting cells maturation has been documented in past studies. Dendritic cells are critical modulators in the orchestration of adaptive immune responses alongside myeloid subtypes, which play an important role in the presentation of antigens to T cells. The aim of this study was to evaluate the efficacy of splenocytes activated with the extract of heated 4T1 cells and the yeast form of C. *albicans* against breast cancer growth *in vivo*.

Methods: 4T1 cells were subcutaneously injected into the left flanks of female BALB/c mice (n=40). At a time when palpable tumors had developed, experimental groups were immunized twice at one-week interim with either activated splenocytes with the extract of heated 4T1 or the killed preparation of yeast form of C. albicans or a combination of the two-One week after the second injection, one-half of animals (n=20) were euthanized to investigate the immune response profile.

Results: Administration of activated splenocytes with the combination protocol caused a favorable survival curve and slower rates of tumor development compared to other tumor-bearing mice. Moreover, combination therapy significantly increased the secretion of IFN-γ, respiratory burst and nitric oxide production and conversely diminished the secretion of IL-4, IL-10 and TGF-β in the splenocyte population.

Conclusion: Since the murine 4T1 cell line is similar to the final stage of human breast carcinoma, we postulate that activated splenocytes with the extract of heated 4T1 cells and yeast form of C. albicans can reduce tumor development in tumor-bearing mice.

Keywords: 4T1, Breast cancer, Candida albicans, Dendritic cell, Macrophage

INTRODUCTION:

Breast cancer is the most commonly diagnosed cancer and the leading cause of death in females around the world[1]. Murine breast carcinoma 4T1 is an easily transplantable and invasive cell line which resembles stage IV human breast cancer with similar kinetics in mice[2]. Similar to human breast cancer, the 4T1 cells thrive and metastasize spontaneously from the primary tumor site in the mammary gland to multiple distant organs such as lymph nodes, blood, liver, lungs, brain, and bones in a very similar way to human breast cancer[2].

The immune system recognizes tumors as malignant cells and attempts to destroy them in the primary stage of development. Nevertheless, solid tumor cells usually evade the pressure of the immune responses and continue to increase and spread throughout the body and eventually contribute to clinically significant masses. The murine spleen contains a diversity of myeloid and dendritic cells (DCs)[3,4]. Since DCs are the most efficient antigen-presenting cells in the immune system, they occupy the leading position in the orchestration of adaptive immune response[5]. These masterminds play a crucial role in the initiation and subsequent direction of cellular and humoral immunity as well as participating in the activation of natural killer cells and natural killer T cells[6]. This has led to DC-based cancer-specific immunotherapy receiving a significant amount of attention in recent years[6,7]. Alongside DCs, distinct subsets of myeloid cells such as macrophages have been previously described in the murine spleen, with each subset differing subtly in phenotype, function and location[4].

Candida albicans, a common member of human gut flora, grows in a variety of morphological forms from unicellular budding yeast to true hyphae with parallel-sided walls[8]. Interestingly, DCs and macrophages can accurately discern yeast and hypha form[9]. Consequently, the interaction of DCs and macrophages with the yeast form of C. albicans leads to the acquisition of Th1 response-promoting function while hypha-pulsed DCs induce Th2 responses[10]. Since Th1 oriented immune response is the primary weapon of the immune system against tumor cells, we hypothesized that the combination of 4T1 cell lysates and killed preparation of C. albicans would boost anti-tumor responses[11,12].

METHODS:

Animals

Forty female inbred BALB/c mice, 6–8 weeks of age, were purchased from the Pasteur Institute of Iran. The animals were housed under controlled environmental conditions (25°C and a 12-hour light/dark cycle) in plastic boxes lined with wood shavings and received food and water ad libitum. All studies conformed to the principles for laboratory animal research outlined by the Animal Welfare Act and were approved by the Urmia University Animal Care and Use Committee (ethical ref code.: IR,Urmia,Record). Treated animals were twice immunized at a one-week interval and control tumor-bearing mice only received PBS on the same schedule. One week after the second immunotherapy, half of the animals in each group (n=5) were euthanized to investigate the immune responses.

Splenocyte Isolation and Activation

Splenocytes were isolated from tissues as described before with minor modifications[13]. In brief, spleens were aseptically collected and a collagenase-digested suspension was made in an RPMI-1640 medium supplemented with 10% fetal calf serum. The red blood cells were omitted using RBC lysis buffer. Next, the cell suspension was centrifuged in dense bovine serum albumin to obtain a fraction with a low buoyant density. Afterward, 4 ml of cell suspension (1×10^7 cells/ml) was pre-incubated in a 60-mm tissue culture dish for 90 min at 37 °C in a moist atmosphere of 5% CO2. The resultant adherent cells are mainly dendritic cells

(~80%), but other cell types such as macrophages and lymphocytes are still present. The non-adherent cells were discarded by twice washing the surface of each dish with 37°C RPMI-1640. Next, each dish was incubated for 30 to 60 min to release additional contaminating lymphocytes from the plastic surface. Finally, the mediums were refreshed and incubated for another 20 hours. During this time, adherent cells would detach gradually. The resultant cells are mostly spleen dendritic cells and macrophages.

The collected cells were pulsed with heated tumor lysate (100 ug/10^6 cells) or yeast preparation or both, and incubated for 20 hours before therapy. After overnight culture, the monolayer was detached by collagenase. The harvested cells were washed twice and viability was evaluated by Trypan blue dye exclusion.

Preparation of C. albicans Extract and Tumor Lysate

C. *albicans* (ATCC:10231) was provided by Pasteur Institute of Iran and inoculated in Sabouraud's Dextrose Agar (SDA) media and incubated at 37°C for 48 hours. The growth appeared in 48 hours as white-colored and smooth colonies. Afterward, yeasts were rinsed twice with PBS (Phosphate buffer saline) and centrifuged (3000 x g, 10 min)[9]. To induce heat shock proteins, isolated yeasts were exposed to non-lethal heat shock (42°C and 30min). Ultimately, the heated yeast was lysed by repeated freeze and thaw cycles. The protein concentration of the prepared lysate was determined by Bradford assay and stored at -70 °C as aliquots before use.

To generate tumor lysate, 4T1 cells were exposed to non-lethal heat shock, 42°C, for 30 min to induce heat shock proteins. Afterward, 4T1 lysate was obtained by repeated freeze and thaw cycles. The supernatant was collected by centrifugation and passed through a 0.2 µm pore filter. The protein concentration of the lysate was calculated by the Bradford method and stored at -70°C as aliquots before use[14].

Tumor Challenge

To generate the tumors, mice were challenged subcutaneously in the left flank with 1×10^4 viable tumor cells in 50 µL of PBS[14]. Tumor growth was assessed every five days by a caliper. Tumor volume in mm^3 was computed using the formula of an ellipsoid (length × width × height × 0.5236).

Immunotherapy

Immunotherapy was initiated when all the animals had developed a palpable tumor mass. Tumor-bearing mice were randomly divided into four groups (n:10); C: control tumor-bearing mice, T1: received candida albicans extract, T2: received tumor cell lysate and T3: received a combination of both candida extract and tumor lysate. Vaccines were administrated by subcutaneous (S.C.) injection in the right flank. All the treatment groups, T1, T2, and T3, were twice immunized at a one-week interval with 10^6 cells/100µL PBS maturated with heated candida extract or tumor cell lysate or both, respectively. Control tumor-bearing mice only received PBS on the same schedule. Ten days after the second immunotherapy, one-half of the animals were euthanized for evaluation of immunological responses.

Cytokines Assay

In brief, spleen cells were aseptically isolated from mice at the bleeding time. Single-cell suspensions of cells were prepared in DMEM medium supplemented with 10% fetal calf serum and red RBCs removed by RBC lysis buffer[15]. Next, cell suspensions (2×10^6 cells/ml) were incubated in 24-well plates and pulsed with antigens derived from tumor cells by freeze and thaw (100µg/ml) and 25µL PHA solution (1mg/ml). The tumor antigen was prepared, as described previously[15]. The culture supernatants were collected after 72 hours. The concentration of IFN-γ, IL-4, IL-10 and TGF-β were measured by ELISA according to the manufactur-

er's instructions.

Assessment of Nitric Oxide in the Splenocyte Population

The nitric oxide production was determined by assaying the nitrite levels of the spleen cells culture supernatants using the Griess reagent[16]. The cell-free supernatants (50 µl) were isolated and mixed with 50 µl Griess reagent (0.1% sulfanilamide, 3% phosphoric acid and 0.1% naphthyl ethylenediamine), then incubated at room temperature for 10 min in the dark. After incubation, absorbance was quantitated at 540 nm on a microplate reader (Dynatech, Denkendorf, Germany). The nitrite concentration was calculated based on a standard curve.

Cytotoxicity Assay

The proliferation potential of lymphocytes in the splenocyte population was checked using the MTT assay. In brief, the splenocytes were plated in 96-well flat-bottomed (1×10^5 cells/100µl/well) and stimulated with 25 µLPHA solution (1 mg/ml) or medium. After 72 hours of incubation, the cultures were pulsed with 20 µl of the MTT solution (5 mg/ml) for 4 hours at 37°C. Then, 150 ml of DMSO was added and shaken vigorously to dissolve the formazan crystals. The optical density (OD) at 550 nm was measured by a microplate reader (Dynatech, Denkendorf, Germany). The experiments were performed in triplicate sets. Data were expressed as the proliferation index according to the ratio of the OD550 of stimulated cells with PHA to the OD550 of non-stimulated cells[15].

Statistical Analysis

Statistical analysis was performed using the Kruskal-Wallis test followed by pair-wise comparisons using the Mann-Whitney U test with Bonferroni adjustment. The Kaplan–Meier estimator was applied to evaluate the survival function from lifetime data. Results were reported as mean ± S.D. Values of $p< 0.05$ were considered statistically significant.

RESULTS:

Following tumor induction, animals were monitored every five days for the first sign of a palpable tumor until day 60 (**Fig.1**). Cell therapy protocols were initiated on day 12 after tumor induction when individual mice developed a palpable tumor mass. Kaplan-Meier curves are illustrated for mice in different groups in **Fig. 2** T3 mice, which received the combination of both tumor lysate and yeast extract showed a more favorable survival curve than other tumor-bearing mice. Noticeably, at least 20% of T3 mice were alive until day 59 after tumor induction. The survivability rate of the mice who were not treated with the combination treatment was inferior so that all animals died at day 38 (T1 mice) and 48 (T2 mice) after the tumor induction (**Fig. 2**).

The growth rates of tumor in T3 mice were slower than those that received only yeast extract or tumor lysates (**Fig. 3**). Precisely, tumor growth rates were hindered in T3 mice from day 27 until the end of the study (**Fig. 3 and 4**). On the other hand, tumor volume alteration was not statistically different between the mice received uncompleted maturation protocol (T1 and T2) and the control tumor-bearing mice (C) (**Fig.3**).

A significant increase in splenocyte proliferation was recorded in the mice received the combination therapy while the difference between other groups was not meaningful (**Fig.5**). Cytokine assay indicated that the combination protocol caused a more prominent secretion of IFN-γ and conversely reduced the secretion of IL-4, IL-10, and TGF-β in the splenocyte population compared to the splenocytes from the control tumor-bearing mice and the other tumor-bearing mice that received different splenocyte protocols (**Fig.6**). According to **Fig.7**, nitric oxide production by splenocytes was also increased in the tumor-bearing mice that received splenocytes matured by combination protocol

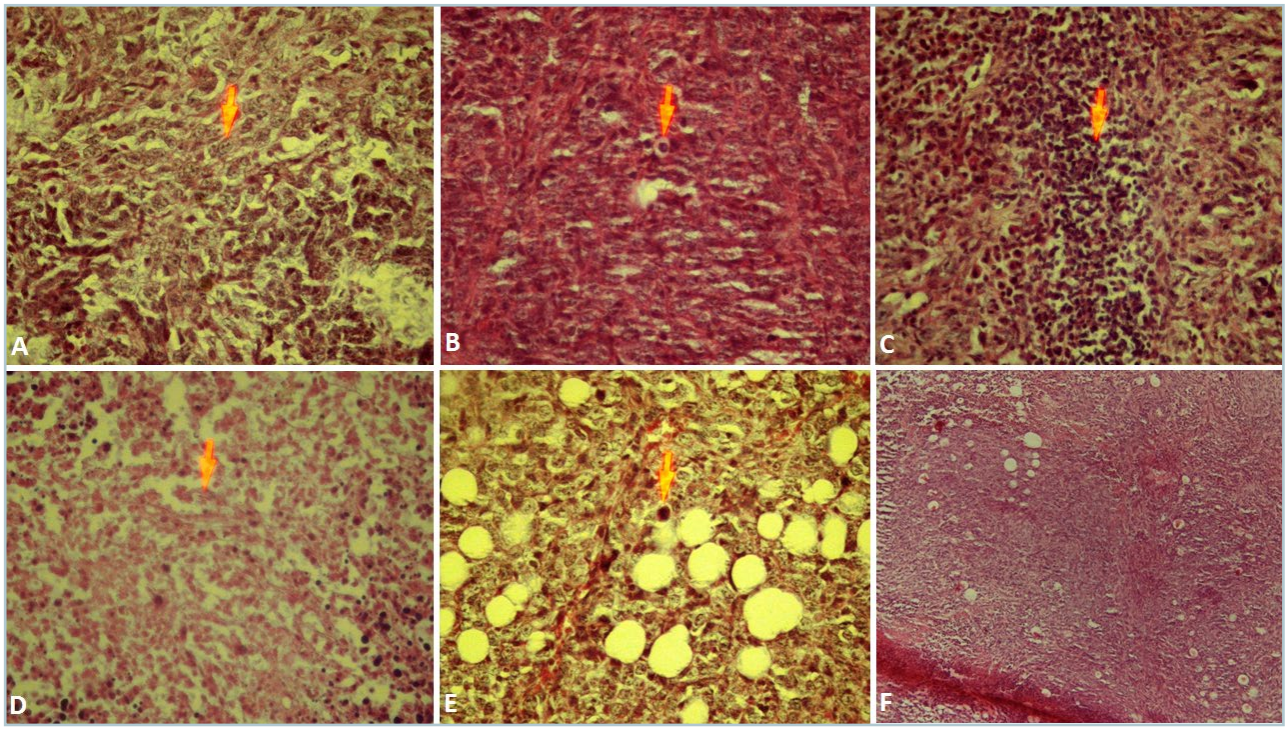

Figure 1. To generate tumor, mice were challenged subcutaneously in the left flanks with 1× 10^4 viable tumor cells. **A**: Mitosis of a tumor cell (Anaphase), **B**: darkly stained tumor cell (hyperchromatic) and an increased nuclear-to cytoplasmic ratio **C:** a chronic inflammatory view (lymphocyte and eosinophil), **D**: necrotic area of tissue section, E: a tumor cell with huge nuclei (bizarre cell) and F: a sheet of the compact tumor cells.

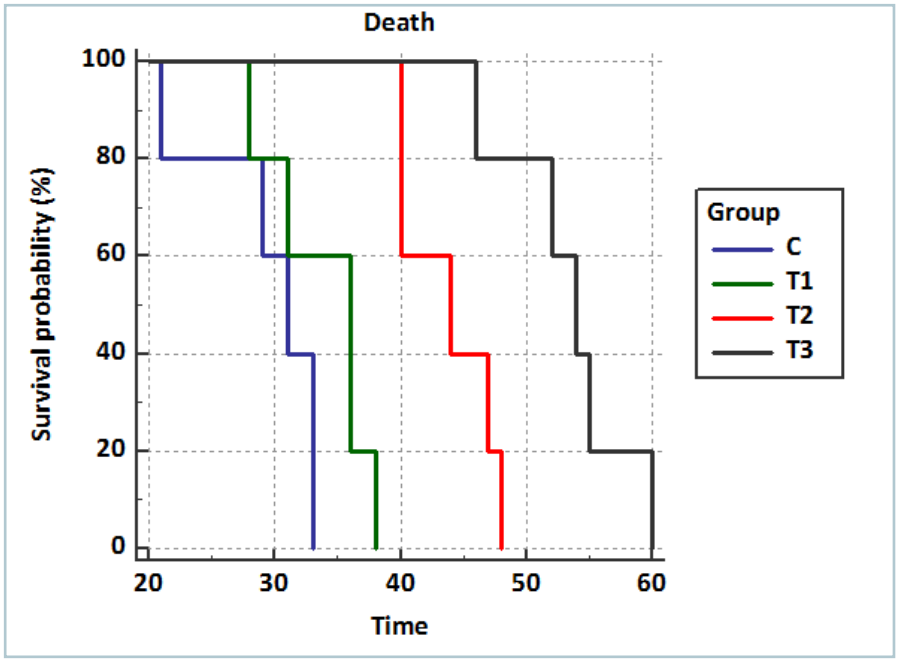

Figure 2. Survival rate. Comparison of Kaplan–Meier Survival Curve of Balb/c mice challenged with 4T1 cells. Cell therapy was initiated when all of the mice showed a palpable tumor. C: tumor bearing mice. T1, T2 and T3: mice were twice immunized at a one-week interval with 10^6 cells /100µL PBS maturated with heated candida extract or tumor cell lysate or both, respectively.

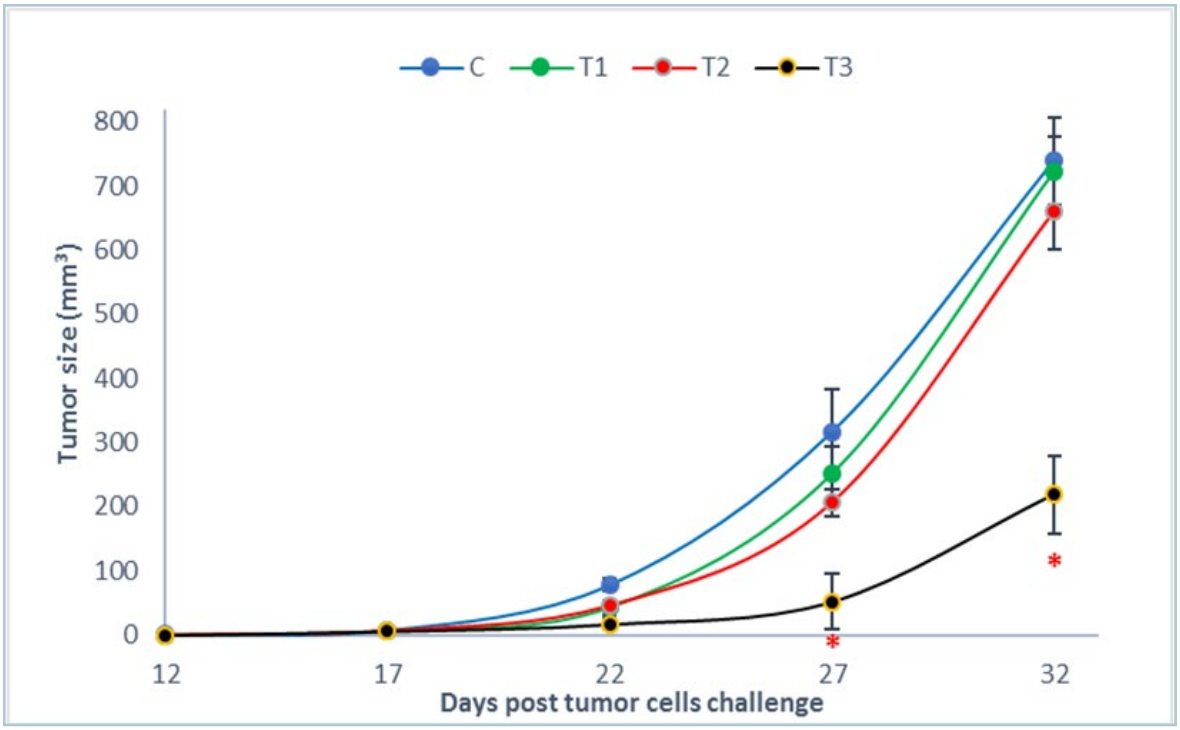

Figure 3. Tumor size. Mammary tumor size after the immunotherapy. The tumor size was computed every 5 days using a caliper. Tumor volume in mm3 was measured by the formula of an ellipsoid (length × width × height × 0.5236). C: tumor bearing mice. T1, T2 and T3: mice were twice immunized at a one-week interval with 10^6 cells /100µL PBS maturated with heated candida extract or tumor cell lysate or both, respectively (* P < 0.01 versus other groups).

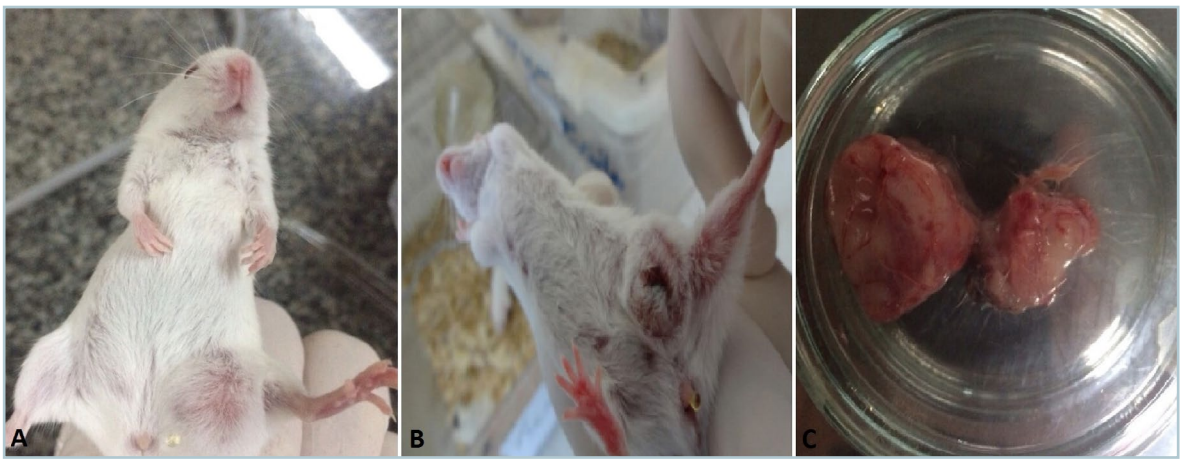

Figure 4. Tumor volume alteration was significant between the mice received the splenocytes pulsed with heated tumor cells and heated yeast form of C. albicans and control tumor-bearing mice. A) Treated mouse at day 60, B) Control tumor-bearing mouse at day 32, C) Dissected tumor masses; the left mass related to the control tumor-bearing mouse and right mass pertains to treated mouse received combined therapy.

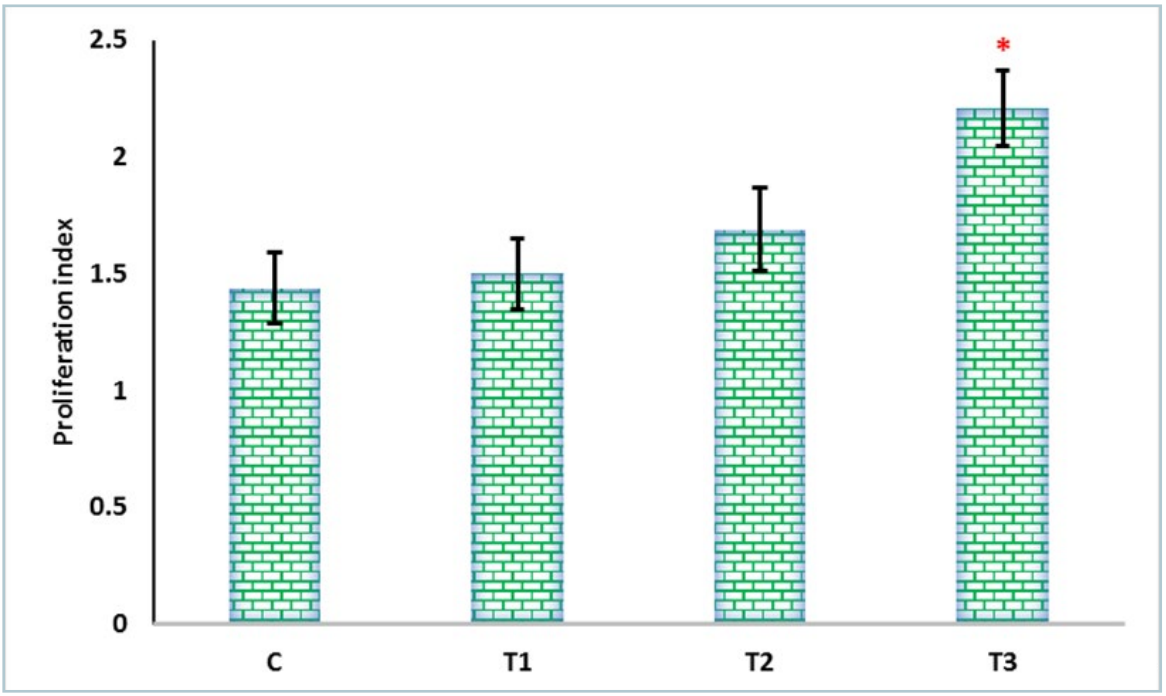

Figure 5. Splenocyte proliferation index. Evaluation of the immunotherapy on the splenocytes proliferation index. One half of the mice in each group were euthanized 10 days after the last therapy and splenocytes were isolated and cultured for 72 under the described materials and methods. C: tumor bearing mice. T1, T2 and T3: mice were twice immunized at a one-week interval with 10^6 cells /100µL PBS maturated with heated candida extract or tumor cell lysate or both, respectively (* P < 0.01 versus other groups).

compared to other tumor-bearing mice. The level of nitric oxide production in tumor-bearing mice that received activate splenocytes by tumor lysate was higher than mice that received yeast extract alone.

DISCUSSION:

The innate immune response is activated when a danger signal or pathogen-associated antigens are recognized by pattern recognition receptors (PRRs)[17,18]. When PRRs recognize these molecules, a series of intracellular signaling events would be initiated which ultimately result in the release of cytokines and up-regulation of co-stimulatory molecules on the surface of antigen-presenting cells[18]. However, the 4T1 cancer cell line has been known as a poorly immunogenic cell line which mostly evades immune surveillance[14]. The immature tissue DCs possess low levels of MHC and co-stimulatory molecules which make them unable to expeditiously activate naive T lymphocytes. Therefore, their maturation and activation are associated with the local microenvironment and the nature of danger signals that PRRs sense[10]. Dendritic cells process and present tumor antigens to CD4+ and CD8+ T cells and play a central role in immune response modulating to determine the type of response. Lately, a new dendritic-like cell which called L-Dc has been reported in murine spleen which is phenotypically and functionally distinct from the

conventional dendritic cell and myeloid subtypes[3]. This cell can activate CD8+ T cells effectively and induce cytotoxic effector cells. That explains why cancer vaccines including DC-based cancer vaccines are considered as promising immunotherapy strategies. Cancer vaccines are developed to promote tumor-specific immune responses against specific tumor antigens. In 2014, Hadjati and et al demonstrated that Listeria monocytogenes activated DC-based vaccine promotes Th1 anti-tumor responses in tumor-bearing mice[19]. Several excellent studies demonstrated that yeast form of C. albicans not only stimulates CD 4+ T cell but also promotes the development of CD8+ cytotoxic T cells via DC cross-presentation. We hypothesized that pre-activation of murine splenocyte, which mostly includes DCs and macrophages, with killed preparation of the yeast form of the fungus C. albicans and tumor lysate can promote CD4+ and CD8+ T-cell responses.

The induction of heat shock proteins (HSPs) is another approach to promote the immunogenicity of tumor cells. Heat shock proteins, especially Hsp70 family, act as classical chaperones which promote tumor antigen uptake by antigen presenting cells[20]. Interestingly, HSP-antigen complexes can be conducted toward the conventional exogenous MHC class II pathway

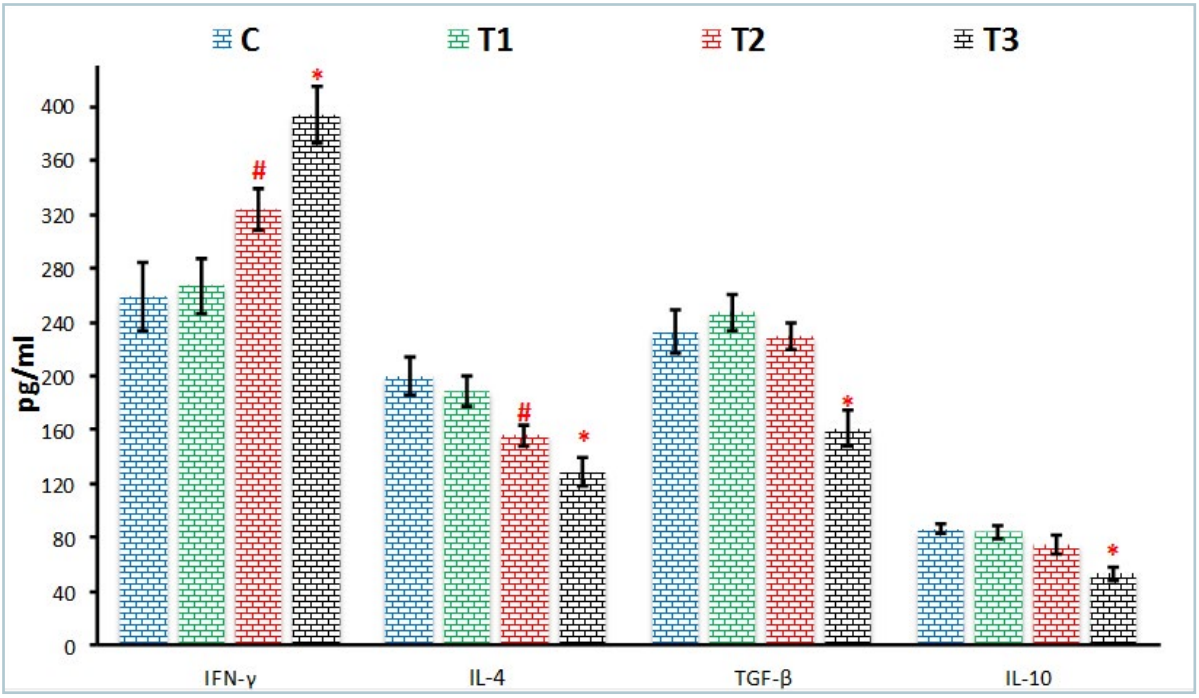

Figure 6. Cytokines changes. Effects of the splenocytes therapy on cytokine production by splenocytes. One half of the mice in each group were euthanized 10 days after the last splenocytes therapy and splenocytes were isolated and cultured for 72 under the described materials and methods. C: tumor bearing mice. T1, T2 and T3: mice were twice immunized at a one-week interval with 10^6 cells /100μL PBS maturated with heated candida extract or tumor cell lysate or both, respectively (* P <0.01versus other tumor bearing mice; # P <0. 01 versus tumor bearing mice received splenocytes maturated by heated candida extract or control tumor bearing mice).

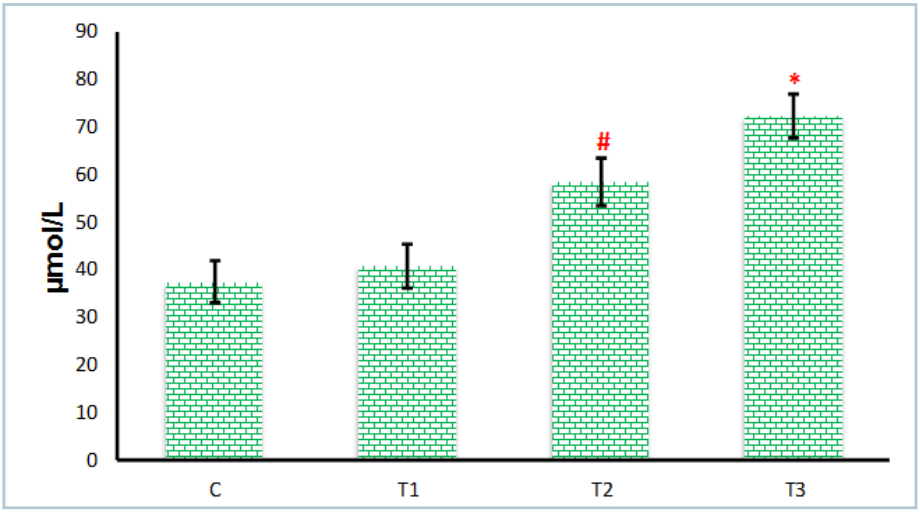

Figure 7. Nitric oxide production. Evaluation of the immunotherapy on the nitric oxide production in the splenocyte population. One half of the mice in each group were euthanized 10 days after the last therapy and splenocytes were isolated and cultured for 72 under the described materials and methods. C: tumor bearing mice. T1, T2 and T3: mice were twice immunized at a one-week interval with 10^6 cells /100µL PBS maturated with heated candida extract or tumor cell lysate or both, respectively (* P <0.001versus other tumor bearing mice; # P <0. 01 versus tumor bearing mice received splenocytes maturated heated candida extract or control tumor bearing mice).

and more importantly may induce cross-presentation of antigens by DCs[21]. Antigen cross-presentation is a key step toward the generation of effector CD8+ T cell responses against tumor cells and viral antigens[14,21]. In this survey, we used a sublethal dose of heat to induce HSP70 in both 4T1 cells and yeasts. Bausero and et all reported that the exposure of 4T1 cells to non-lethal heat shock increases the surface expression of Hsp70 family and suppress the growth and metastatic potential of 4T1 cells in vivo[22]. In addition, HSP70 family of C. albicans is highly immunogenic for both human and mice and would enhance immune response instantly[23]. Our results demonstrated that our cancer vaccine in which cells were matured by the combination of the heated 4T1 cells lysate and killed preparation of yeast form of C. albicans significantly potentiates splenocytes proliferative response. Abtahi et al reported that IFN-γ is not only crucial for 4T1 tumor surveillance but also there is a direct correlation between IFN-γ production and tumor regression[14]. A Previous study described that innate immunity recognizes spores of Bacillus anthracis, which composed of several layers of different sugar polymers including mannans, glucans, chitosan, and dityrosine, via MyD88-dependent receptors and responds by secreting IL-12p40 and IFN-γ cytokines, which may ultimately aid in resisting infection[24,25]. Interestingly, C. albicans cell walls contain similar sugar polymers[26].

The attained data indicate that IFN-γ production by splenocytes after treatment was significantly up-regulated in the tumor-bearing mice which received combined protocol. However, the level of IFN-γ did not show any noticeable difference among other groups. The tumor cells tend to evade immune surveillance subtly by the production of immunosuppressive cytokines such as TGF-β and IL-10. Indeed, these cytokines suppress inflammatory responses by inhibiting lymphocyte proliferation and macrophage activation.

As a result, cell-mediated immunity that is an essential arm of the immune system to control tumor growth is hold back. In addition, both TGF-β and IL-10 promote the development of Treg cells which play a distinct role in a wide variety of malignancies. Many studies have reported that Treg cells limit immune responses to tumors via production of TGF-β, IL-10 and other suppressive molecules[27-29]. Another pro-tumor cytokine is IL-4 which regress Th1-macrophages axis. It has been reported that IL-4 directly potentiate tumor growth in the human breast malignancy[30]. Our results demonstrate that the cell therapy by activated splenocytes diminishes the levels of TGF-β, IL-10, and IL-4 in a significant manner. The previous studies have implicated that yeast form of C. albicans could provoke M1 inflammatory macrophages which producing a high concentration of nitric oxide[10]. Similarly, our findings show that the production of nitric oxide by splenocytes increased more significant in the tumor-bearing mice which received combined protocol than other tumor-bearing mice received each of the immunotherapies alone.

In sum up, higher survival rate and significantly reduced tumor size alongside enhanced cellular response of immune system make us believe that our combined protocol is a successful strategy to control 4T1 cell development in murine model of breast cancer. For the first time, we demonstrated that C. albicans can induce both innate and adaptive immune response in tumor-bearing mice via activation of splenocytes. As murine 4T1 cell line is similar to the final stage of human breast carcinoma, we postulate that this protocol may help us to control tumor development in afflicted patients. However, the current study is a preliminary and further studies should be undertaken.

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DISCLOSURE:

There are no conflicts of interest.

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