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Research Paper

Evaluation of Cytotoxic T-lymphocyte Antigen-4 (CTLA-4) Level in Lymphocytes Exposed to Breast Tumor Cell Lysate

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ABSTRACT

Malignant tumors and cancers are leading causes of death in human societies. T lymphocytes are the main cells in antitumor immunity. One of the best-known inhibitory receptors of T lymphocytes is cytotoxic T lymphocyte antigen-4 (CTLA-4), which plays a crucial role in immunological tolerance. This study aimed to examine the CTLA-4 expression on lymphocytes following exposure to breast tumor cell lysate. We used the 4T1 mouse model of triple-negative breast carcinoma in vitro to evaluate the effect of tumor cell lysate on CTLA-4 expression on T lymphocytes. Tumor cell lysate was prepared from cultured 4T1 tumor cells. Leukocytes were isolated from Balb/c mice and cultured in the presence of tumor cell lysate (tumor cell lysate-treated group) or in the absence of tumor cell lysate (control group). After 4 days of incubation, the leukocytes from both groups were stained with fluorophore-conjugated anti-CD3 and anti-CTLA-4 monoclonal antibodies and analyzed by flow cytometry. Analysis of flow cytometry data showed that the frequency of T lymphocytes was not altered in the tumor cell lysate-treated group compared to the control group ($P > 0.05$). However, the frequency of CTLA-4+ T lymphocytes in the tumor cell lysate-treated group was higher than that in the control group (1.68% vs. 0.97%, $P = 0.05$). These results indicate that tumor cell antigens or other tumor cell components may increase CTLA-4 expression on T lymphocytes, thereby dampening the antitumor immune responses. The observed upregulation of CTLA-4+ T lymphocytes in the presence of breast tumor cell lysate suggests that targeting this molecule with monoclonal antibodies may enhance the efficacy of anticancer therapy in patients with breast cancer.

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Introduction

The process by which normal cells progressively transform into malignancies requires the sequential acquisition of mutations in genes involved in normal homeostatic mechanisms that control proliferation and cell death. These mutations result in the activation of genes that stimulate proliferation or protect against cell death (oncogenes) and the inactivation of genes that normally inhibit proliferation (tumor suppressor genes). These malignant cells proliferate to form a new population of genetically distinct cells, the tumor [1].

The immune system plays a crucial role in recognizing and eliminating malignant cells, with cytotoxic T lymphocytes (CTLs) pivotal in this process [2, 3]. However, several immune checkpoints regulate lymphocyte immune responses [4, 5]. Immune checkpoint pathways are crucial for self-tolerance, which prevents the immune system from attacking the body's cells [4, 5]. Some tumor cells can protect themselves from lymphocyte attack by stimulating immune checkpoint targets [6-9]. Cytotoxic T lymphocyte antigen-4 (CTLA-4), also known as CD152, is the most important immune checkpoint that downregulates immune responses. This protein is upregulated in activated conventional T cells. It is also constitutively expressed on regulatory T cells (Tregs). Its primary function is to prevent excessive tissue damage during immune activation [10, 11].

Breast cancer is one of the most prevalent malignancies among women worldwide [12, 13]. Breast cancer presents unique challenges in immunotherapy, and developing more effective immunotherapeutic strategies is necessary [14]. Tumor-associated antigens (TAAs) specific to breast cancer can stimulate CTL responses [15], and tumor-infiltrating lymphocytes in breast cancer predict the response to chemotherapy and survival outcomes [16]. However, the effectiveness of these cells is often hindered in tumor tissues. Upregulation of CTLA-4 is reported in several types of cancer [17]. Recently, CTLA-4 expression and its clinical significance have been reported in breast cancer patients [18].

Understanding the dynamics of CTLA-4 expression in lymphocytes when exposed to breast tumor antigens is essential for developing more effective immunotherapeutic strategies. Elevated CTLA-4 expression is associated with T lymphocyte exhaustion, a state in which T-lymphocytes lose their effector functions after prolonged antigen exposure. This phenomenon can be particularly relevant in the tumor tissues, where persistent exposure to TAAs may lead to a diminished immune response, ultimately facilitating tumor progression. Therefore, evaluating CTLA-4 levels in lymphocytes provides insights into the immune landscape of immune-related diseases.

This study aimed to evaluate CTLA-4 levels in lymphocytes exposed to breast tumor antigens, providing a comprehensive understanding of the interplay between immune regulation and tumor immunogenicity. Our findings could pave the way for novel immunotherapeutic strategies that harness the immune system to effectively combat breast cancer.

Materials and Methods

Tumor Cell Line and Reagents

Mouse 4T1 breast cancer cell line was obtained from the Pasteur Institute of Iran (Tehran, Iran). 4T1 cells were examined for contaminants with the molecular testing (polymerase chain reaction) and cell culture. The morphology and in vitro growth rate were routinely monitored.

RPMI 1640 culture medium, fetal bovine serum (FBS), and L-glutamine were purchased from Gibco-Invitrogen (UK). Trypsin, sodium pyruvate, penicillin, and streptomycin were purchased from Sigma-Aldrich (USA). Red blood cell lysis buffer, flow cytometry buffer, anti-CD_3 monoclonal antibody conjugated with fluorescein isothiocyanate (FITC), and anti-CTLA-4 monoclonal antibody conjugated with phycoerythrin (PE) were purchased from Biolegend (USA).

Preparation of Tumor Cell Lysate

4T1 cells were cultured in RPMI 1640 supplemented with 10% FBS, 2mM L-glutamine, 100 IU/mL penicillin, and 100 µL/mL streptomycin. Cell incubation was performed at 37˚C in an incubator with an atmosphere containing 5% carbon dioxide (CO_2) and 95% humidity. After the proliferation of tumor cells, cells were detached with trypsin and washed twice with RPMI1640 culture medium. The cells were poured into a cryovial and placed in liquid nitrogen (temperature -196˚C). Then, the cryovial was removed from liquid nitrogen and placed in a water bath at 37˚C for 5 minutes. This process was repeated three times. Subsequently, the contents of the cryovial were filtered through a 0.45 µm pore-diameter filter and placed in a freezer at -70˚C.

Isolation and Culture of Leukocytes in the Presence of Tumor Lysate

To isolate leukocytes, healthy Balb/c mice (8-12-week-old, female) were reared in an animal facility at the Immunology Department of Tehran University of Medical Sciences (Tehran, Iran), euthanized by cervical dislocation, and their spleens were removed. After tearing the splenic capsule, the spleen cells were collected on a plate. Red blood cell lysis buffer containing ammonium chloride (Biolegend, USA) was used to destroy red blood cells. After red blood cell lysis, the cells were washed with

RPMI1640 and passed through an 80 µL pore-diameter cell filter to remove tissue remnants and cell aggregates. Then, leukocytes were cultured in RPMI 1640 supplemented with 10% FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, and 100 IU/mL penicillin and 100 µg/mL streptomycin. Tumor lysates in concentrations of 1 µg/mL and 10 µg/mL were added to the leukocytes. Only the culture medium was added to the cells of the control group. The cells were incubated at 37°C in a 5% CO_2 atmosphere with 95% humidity, and the medium was changed the day after seeding. The cells were examined under an inverted microscope daily.

Measurement of Cytotoxic T-lymphocyte Antigen-4 (CTLA-4) on the Surface of Cultured Lymphocytes

After 4 days of incubation, the cultured cells were collected from the cell culture plates using a Pasteur pipette and transferred to BD flow cytometry tubes. Then, FITC-conjugated anti-CD_3 monoclonal antibody and PE-conjugated anti-CTLA-4 monoclonal antibody were added to the cells. To stain the cells with the isotype control, the isotype antibody prepared in rat was added to the cells in another set of BD flow cytometry tubes. Cells were incubated in the dark for 45 minutes. The cells were washed with 2 mL of flow cytometer buffer and centrifuged for 5 minutes at 2000 rpm. The supernatant was discarded, 500 µL of flow cytometer buffer was added, and the cells were analyzed with a FACSCalibur flow cytometer instrument (Becton Dickinson, USA). For this purpose, the cells were passed through the laser (488) nm as a narrow strip in flow cytometry buffer. Lights with wavelengths of 565-605 nm and 576 nm emitted from each cell were captured by the device's special filters. Data were analyzed using the FlowJo (version 10) CL software.

Statistical Analysis

To test the effect of tumor lysate on the frequency of cultured CD3 lymphocytes and on the expression levels of CTLA-4 on $CD3^+$ lymphocytes, the student's t-test was used. Differences were considered significant at a *P* value ≤ 0.05.

Results

To investigate the effect of breast tumor cell antigens/components on the frequency of T lymphocytes and the expression of the CTLA-4 molecule on T lymphocytes in the presence of tumor lysate and control lymphocytes, only leukocytes cultured in the presence of 1 µg/mL of tumor lysate were analyzed by flow cytometry because the viability of leukocytes cultured in the presence 10 µg/mL of tumor lysate was not sufficient. Lymphocytes were gated based on forward scatter (FSC) and side scatter (SSC) parameters (Figure 1). Then, $CD3^+$ cells were gated on the FITC signal, and $CTLA\text{-}4^+$ cells were gated on the concomitant PE signal (Figure 2). Unstained cells and cells stained with isotype control antibodies were used to detect autofluorescence or background staining to justify FLPMT voltages and to identify negative gates.

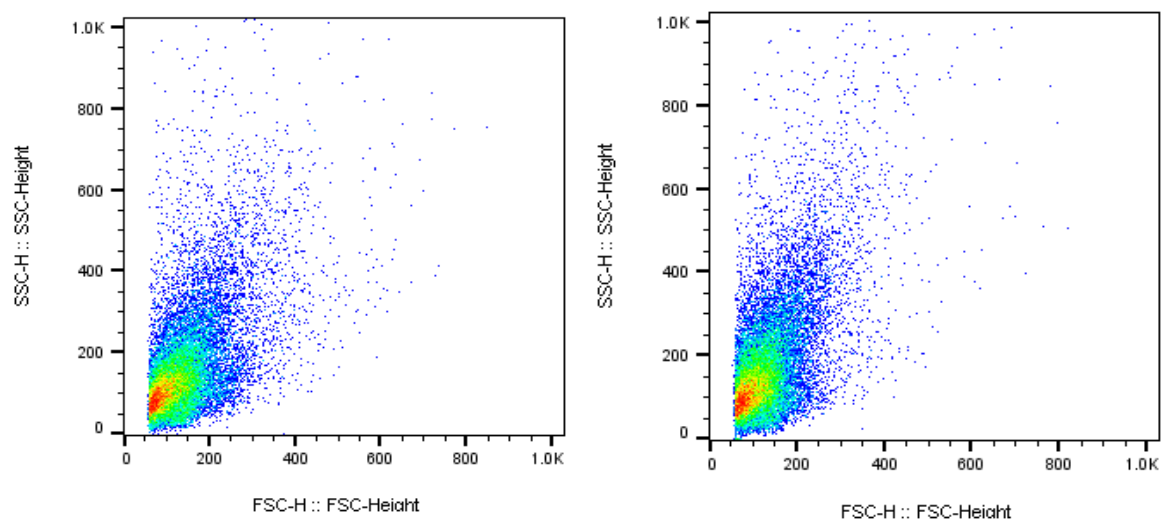

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Figure 1. Forward Scatter/Side Scatter (FSC/SSC) Dot Blot Graphs of Leukocytes in Flow Cytometry Analysis, a. FSC/SSC Dot Blot Graph of Control Leukocytes, b. FSC/SSC Dot Blot Graph of Tumor Cell Lysate-Treated Leukocytes

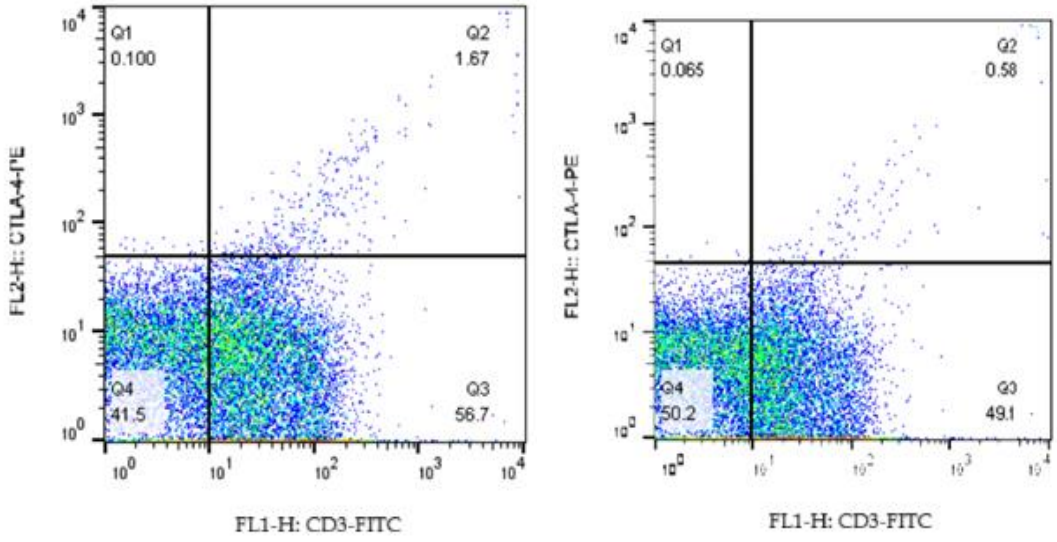

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Figure 2. Dot Plot Graph of $CD3^+$ Cytotoxic T-lymphocyte Antigen-4 (CTLA-4) $^+$Lymphocytes in Flow Cytometry Analysis, a. Dot Plot Graph of $CD3^+$ $CTLA\text{-}4^+$ Lymphocytes in Control Leukocytes, b. Dot Plot Graph of $CD3^+$ $CTLA\text{-}4^+$ Lymphocytes in Tumor Cell Lysate-Treated Leukocytes

By analyzing the obtained data, the percentages of $CD3^+$ lymphocytes and CD3+ lymphocytes expressing CTLA-4 in the control and cultured groups in the presence of tumor lysate were determined. No significant difference in the percentages of $CD3^+$ T lymphocytes was observed between the two groups ($P > 0.05$). The percentage of $CD3^+$ lymphocytes expressing the CTLA-4 molecule was 0.97 ± 0.40 in the control group leukocytes and 1.68 ± 0.28 in the leukocytes cultured in the presence of tumor lysate. The difference between these two groups was statistically significant ($P = 0.05$).

Discussion

The findings of this study provide valuable insights into the interactions between breast tumor cell antigens or other tumor cell components and the expression of CTLA-4 in T lymphocytes. The analysis revealed no significant difference in the percentage of T lymphocytes in the two groups. Nevertheless, the percentage of $CD3^+$ T lymphocytes expressing CTLA-4 was significantly higher in cultured leukocytes exposed to tumor lysate (1.68%) than in control group leukocytes (0.97%). This suggests that the presence of tumor antigens or tumor cell components can enhance CTLA-4 expression on T cells. The increase in CTLA-4 expression in response to tumor lysate indicates a potential mechanism by which tumors may evade immune surveillance, reinforcing the concept that tumor microenvironments can modulate immune responses.

While the overall percentage of $CD3^+$ T lymphocytes did not differ significantly between the control and tumor lysate-treated groups, the differential expression of CTLA-4 highlights the complexity of T cell responses in the tumor microenvironment. This finding is consistent with previous studies showing that tumor cell-derived factors can selectively upregulate inhibitory receptors on T cells, thereby dampening their antitumor function [19, 20]. Mao et al. (2010) detected CTLA-4 expression in breast tissue by immunohistochemical staining and reverse transcription polymerase chain reaction in 60 patients with breast cancer and 30 normal controls. The levels of CTLA-4 on T lymphocytes were determined by flow cytometry in 33 patients and 27 controls. Patients showed strong CTLA-4 expression in all tumor tissue samples at both the protein and *messenger ribonucleic acid* (mRNA) levels, but only weakly positive or negative expression in normal breast tissues. Patients with higher mRNA levels of CTLA-4 had obvious axillary lymph nodes metastases and higher clinical stage [21], highlighting the importance of CTLA4 in the development and progression of breast cancer. In the study by Kassardjian et al. (2018), CTLA-4 was over-expressed in 49 of 93 (52.7%) breast tumors, three of eight (37.5%) ductal carcinoma in situ, 40 of 73 (55%) of invasive ductal carcinomas, four of 10 (40%) of invasive lobular carcinomas and two of two (100%) of invasive tubular carcinomas. All 6 normal breast tissues were interpreted as negative for CTLA-4 staining [22]. Cabioglu et al. (2021) investigated immunohistochemically the expression of CTLA-4 on tumor-infiltrating lymphocytes in 61 patients with locally advanced triple-negative breast cancer after neoadjuvant chemotherapy. They reported that 82% of examined surgical specimens were positive for CTLA-4 expression [23]. Mehrez et al. (2025), in a retrospective cohort study, analyzed 100 primary triple-negative breast cancer cases that had surgical resection. Immunohistochemistry results showed that CTLA-4 was expressed in 45% of triple-negative breast cancer. CTLA-4 expression was significantly associated with lymph node metastasis ($P = 0.009$), distant metastasis ($P = 0.001$), and advanced TNM stage ($P = 0.001$). Multivariate analysis

identified CTLA-4 expression as an independent prognostic predictor for both disease-free survival (P = 0.002) and overall survival (P = 0.003) [24].

CTLA-4 is highly expressed on Tregs. Tregs suppress antitumor immune responses, and a high frequency of Tregs has been associated with worse clinical outcomes in most cancer patients [25]. In this study, we found that breast tumor lysates increased the surface expression of this molecule on T-lymphocytes. These results, along with the results of previous studies, which have shown that the expression of CTLA-4 on the cell surface of lymphocytes suppresses their immune responses [25-27], indicate that exposure of lymphocytes to breast tumor lysate can reduce the immune responses of lymphocytes by upregulation of CTLA-4 on T cells and induction of differentiation of T cells into Tregs in the tumor tissues.

The significance of these results lies in their implications for cancer immunotherapy strategies. In the recent decade, improvements in antitumor immunity in cancer patients and the therapeutic efficacy of cancer immunotherapies using monoclonal antibodies have been reported [28]. The presence of tumor-infiltrating lymphocytes [29] and Tregs [25] in breast cancer provides the rationale for immunotherapy as a potential treatment approach, particularly for triple-negative breast cancer, which currently lacks targeted treatment and is not responsive to standard therapies for a large proportion of patients [30]. Understanding how tumor cell-induced CTLA-4 expression on T cells affects antitumor immunity in patients with breast cancer is helpful in designing immunotherapies aimed at blocking this pathway. In this regard, evaluating CTLA-4 levels in tumor tissues of patients with breast cancer is crucial for successful immunotherapies targeting CTLA-4 with monoclonal antibodies to enhance T cell activation and improve clinical outcomes in various cancers.

Conclusion

In conclusion, this study underscores the importance of investigating the interactions between tumor cell antigens/components and the regulatory mechanisms of T lymphocytes to identify potential biomarkers of immune evasion and treatment resistance in breast cancer. The results showed that breast tumor cells can suppress T cell immune responses by inducing CTLA-4 expression. Further research is warranted to explore the underlying pathways that induce CTLA-4 expression on T lymphocytes exposed to tumor cell components and the therapeutic potential of targeting these pathways in breast cancer patients.

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Ethical Considerations

Compliance with ethical guidelines

This study was approved by the Ethics Committee of Tehran University of Medical Sciences, Tehran, Iran (IR.TUMS.MEDICINE.REC.1395.470).

Conflicts of interest

The authors declared no conflicts of interest.

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