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Research article

Boosting immunotherapy efficacy: Empowering the Potency of Dendritic cells loaded with breast cancer lysates through CTLA-4 suppression

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ABSTRACT

Anticancer immunotherapies with a dendritic Cell (DC) basis are becoming more popular. However, it has been suggested that the tumor's immunosuppressive mechanisms, such as inhibitory immunological checkpoint molecules, reduce the effectiveness of anticancer immunogenicity mediated by DC. Thus, overcoming immune checkpoints and inducing effective antigen-specific T-cell responses uniquely produced with malignant cells represent the key challenges. Among the inhibitory immune checkpoints, DCs' ability to mature and present antigens is decreased by CTLA-4 expression. Consequently, we hypothesized that by expressing CTLA-4 cells on DCs, the T cells' activation against tumor antigens would be suppressed when confronted with these antigens presented by DCs. In this research, by loading cell lysate of breast cancer (BC) on DCs and the other hand by inhibiting the induction of CTLA-4 using small interfering RNA (siRNA), we assessed the functional activities and phenotypes of DCs, and also the responses associated with T-cells following co-culture DC/T cell. Our research has shown that the suppression of CTLA-4 enhanced the stimulating capabilities of DCs. Additionally, CTLA-4-suppressed BC cell lysate-loaded DCs produced more IL-4 and IFN-ϒ and increased T cell induction in contrast to DCs without CTLA-4 suppression. Together, our data point to CTLA-4-suppressed DCs loaded with BC cell lysate as a potentially effective treatment method. However, further research is required before employing this method in therapeutic contexts.

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1. Introduction

According to 2019 WHO estimates, cancer is the leading or second leading cause of death before age 70 in 120 out of 190 countries [1,2]. Breast cancer, the most common cancer among women worldwide, is a genetically and clinically heterogeneous malignancy and a leading cause of death [3–6]. Treatment strategies for BC contain radiation and surgery and systemic approaches such as anti-HER2 therapy, chemotherapy, hormonal therapy with raloxifene or tamoxifen [7], and immunotherapy [8]. However, potential challenges with these treatments include side effects like systemic toxicity, chemotherapy resistance, and potential cancer recurrence [7,9].

Advancements in understanding pathological mechanisms have led to the emergence of immunotherapy as an effective cancer treatment [10]. This approach enhances targeted immune responses against cancer cells by using Dendritic Cells (DCs) [11]. DCs, specific antigen-presenting cells (APCs), bridge adaptive and innate immunity by activating T cells against tumor antigens [12,13]. Current cancer immunotherapy strategies focus on harnessing DCs to tailor immune responses, particularly tumor-specific T-cell reactions [14,15]. In breast cancer, DCs can be primed in the lab with tumor antigens and then re-administered to patients, enhancing immune responses against tumor antigens [16,17]. Studies show that DCs can induce T lymphocyte responses to tumor antigens when pulsed with a tumor cell lysate [18,19]. This activation of T cells by DCs reduces tumor recurrence by increasing immunological memory and decreasing tumor volume [14,15]. Various studies have explored Dendritic Cell (DC) therapy for breast cancer, with several clinical trials currently ongoing, including NCT03387553, NCT04373031, NCT03384914, and NCT02491697.

Dendritic Cells (DCs) are crucial for transferring tumor-associated antigens (TAAs) to T lymphocytes in cancer immunotherapy, but their effectiveness is limited by immune regulatory checkpoint molecules, particularly CTLA-4 [20]. CTLA-4, an inhibitory molecule expressed on DCs and other immune cells, regulates their function and reduces DCs' development and antigen presentation activity [21–24]. It has been shown in a study that CTLA-4 expression on moDCs decreases their maturity and antigen presentation capacity [25]. Also, according to reports, activated moDCs and LPS co-culturing of CTLA-4-positive breast cancer cells can reduce the maturation markers of DCs [26]. Ipilimumab, an anti-CTLA-4-blocking antibody, demonstrated notable therapeutic results and received approval by the FDA in 2011 to treat metastatic melanoma [27]. Furthermore, Son et al. have shown that in irradiated tumors, anti-CTLA-4 antibodies can boost immature DCs-mediated immune responses [28]. Other immune checkpoints like PD-1 are expressed on dendritic cells and can inhibit their function. Thus, its inhibition can improve the dendritic function. It has been reported that the combination of anti-PD-1 antibodies and the DC vaccine can suppress the expression of immunological checkpoints on DCs, keep DCs' capacity to presenting antigens and boost the immune responses of T lymphocytes specific to tumors [29].

Regarding the topics mentioned above, it appears that inhibition of CTLA-4 on DCs along with a tumor cell lysate-loaded in DCs augments activation of T cells more effectively, suggesting the practicable and applicable immunotherapy strategy against BC.

This study is aimed to investigate the impact of inhibiting CTLA-4 on dendritic cells (DCs) loaded with breast tumor lysate, specifically focusing on the enhancement of autologous T lymphocyte activity and cytokine production. Furthermore, the study seeks to ascertain whether inhibiting CTLA-4 on these DCs can boost the effectiveness of DC vaccination in cancer treatment.

2. Methods

2.1. Materials

Complete Medium (CM) inclusive of RPMI 1640 (Gibco, USA, NY), comprises 100 IU/ml penicillin (Sigma-Aldrich, Mannheim, Germany), Fetal Bovine Serum (FBS10 %) (Cayman Chemical, Michigan, USA), 2-mercaptoethanol (2 ME) (Sigma Chemical, Munich, Germany) streptomycin 100 μg/ml (Sigma-Aldrich, Mannheim, Germany), L-glutamine (2 mmol/L) was ordered from Santa Cruz (CA, USA). Lipopolysaccharide (LPS), recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF), and recombinant human interleukin-4 (IL-4) were ordered from Cayman Chemical (Michigan, USA). Ficoll and Carboxyfluorescein succinimidyl ester (CFSE) cell labeling kits were bought from Santa Cruz (CA, USA). The Human Pan T cell isolation Kit and Bradford protein assay kit were ordered from Santa Cruz (CA, USA). Antibodies employed for phenotypic characterization of the cells were anti-CD11c-FITC, anti-CD40-CF blue, and anti-HLA-DR-APC, anti-$CD86^{-}$ PerCP-cy5.5, and anti-CD14-FITC from Gibco (USA, NY).

2.2. Tumor cell lysate preparation

Pasteur Institute (the National Cell Bank of Iran) provided the MCF-7, MDA-MB-231, and MDA-MB-468 human BC cell lines for this study. These cells were transferred to CM and then kept at 37 °C and humidity with CO_2 5 %. After the confluence of cultivate cells gained 85 %, they were harvested utilizing Trypsin, washed with serum (twice), and suspended in sterile PBS buffer (concentration: 1×10^7 cells/mL). Cells underwent 6 rapid freeze-thaw cycles in liquid nitrogen and a 37 °C water bath to produce lysates of tumor cells. The created lysate was next subjected to a 15-s sonication process to generate maximum tumour antigens from cancer cell lysates. The lysates of tumor cells were centrifuged at 1500 rpm to remove cell debris (15 min at 4 °C). A 0.2-mm filter was used to filter the obtained supernatant. The Bradford assay was applied to measure how much protein was present in the lysates. All lysates were refrigerated at −80 °C Until they were used.

2.3. Isolation of peripheral mononuclear cell (PBMC) and production of DCs

Heparin-containing sterile falcons were used to collect fresh peripheral blood (PB) from three healthy volunteers. These samples were then fractionated along Ficoll gradients to extract the PBMCs. Monocytes were separated from PBMCs using the plastic adherence

method. In 6-well plates, PBMCs were cultivated in RPMI-1640 medium (5×10^7 cells/mL concentration) for this purpose. After incubation (2h, 37 °C), suspended cells were eliminated by washing and the adherent cells were cultivated in CM supplemented (50 μM of 2 ME, 20 ng/mL of IL-4, 40 ng/mL of GM-CSF). The cultivated cells were nourished by deleting 0/5 of the medium and adding fresh CM comprising GM-CSF and IL-4 (days 2 and 4). On day 6, immature DCs (iDCs) were collected, and the culture medium was then supplemented with 80 μg/mL of mixed lysate of cell lines of human BC. CM was nourished with 100 ng/mL of LPS after 5 h at 37 °C incubation. Following one-day incubation, mature DCs (mDCs) loaded with tumor cell lysate were generated.

2.4. Phenotypical and morphological specification of DCs

Using the inverted light microscope (Olympus Corporation, Tokyo, Japan), we looked at the morphologic specification of monocytes and DCs and took pictures of the results. To examine the phenotype of mDCs, CTLA-4-blocked mDCs, and iDCs, particular surface markers to these cells, such as CD40 (anti-CD40-CF-blue), CD86 (anti-CD86-PerCP-cy5.5), HLA-DR (anti-HLA-DR-APC), and CD11c (anti-CD11c-FITC) were stained. The cells were assessed by the flow cytometry assay and FlowJo software version 10.5.3 and MACSQuant cytometer (Miltenyi Biotec, Auburn, CA, USA).

2.5. Preparation of siRNA and transferring to DCs

The transfection reagent and CTLA-4-siRNA were purchased from Sigma-Aldrich (Mannheim, Germany). The sorted sequence of CTLA-4-siRNA is indicated in Table 1. The mDCs were collected and then by using Gene Pulser Xcell (Bio-Rad, USA) placed with several pulse voltages (160, 180, and 200 V) in order to find the optimal pulse voltage for CTLA-4-siRNA transfection. With FITC-labeled control siRNA (Cayman Chemical, Michigan, USA), the effectiveness of siRNA transfection at various pulse voltages was assessed. Next, different CTLA-4-siRNA (40, 60, and 80 pmol) concentrations were transfected into mDCs after detecting the suitable voltage (160 V) for transfecting siRNA. Subsequently, mDCs were placed into a 6-well plate comprising a complete medium. After 48 and 72 h of incubation, the level of CTLA-4 was examined utilizing qRT-PCR. The optimal pulse voltage and siRNA dose were acquired for subsequent tests based on the obtained data.

2.6. Separation of $CD3^+$ T-cell and CFSE labeling

By the manufacturer's guide, autologous $CD3^+$ T cells from the same people who produced the DCs were isolated from PBMCs, applying a human Pan T Cell separation Kit employing magnetically activated cell sorting (MACS). After the PBMCs had been extracted, the suspension of cells was centrifuged (400g −15 min). The supernatant was eliminated, and then Pan T cell biotin Ab cocktail (10 μL) and 40 μl of MACS buffer were poured per 1×10^7 total cells. After 5 min of incubation at 2–8 °C, 15 μL of Pan T Cell Micro bead cocktail and 30 μL of MACS buffer were combined per 1×10^7 total cells. Following incubation (2-8°C-10 min), the cells were washed with MACS buffer (3 mL) and re-mixed with it (500 μL). Cell suspension was added to the MACS column and placed in the MACS magnetic field isolator. Unlabeled cells are referred to as negatively selected $CD3^+$ T-cells. Following the manufacturer's recommended procedure, isolated $CD3^+$ T cells were CFSE-labeled.

In summary, pure T cells were suspended again in PBS and exposed to 5 mM CFSE for incubated 5 min in the dark. Through mixing an RPMI-1640 medium (25 % FBS), the continuation of the reaction was prevented. All cells were placed in a warm cell culture medium following the final washing process.

2.7. Investigating the growth of $CD3^+$ T cells

The rate of mDCs and CTLA-4-blocked-mDCs to promote the growth of autologous T cells was assessed by T/DCs co-culture. The mDCs and CTLA-4-blocked-mDCs as stimulator cells and CFSE-labeled autologous $CD3^+$ T cells as responder cells were co-seeded in a

Table 1
List of primer sequences and siRNA.

Gene	Forward/Reverse	Sequence
CTLA-4-siRNA	sense	GUAUCUGAGUUGACUUGACAGAACA
	Antisense	UGUCUGUCAAGUCAACUCAGAUACCA
CTLA-4	F	TCAGTCCTTGGATAGTGAGGTTC
	R	TCAGTCCTTGGATAGTGAGGTTC
T-bet	F	TCTCCTCTCCTACCCAACCAG
	R	CATGCTGACTGCTCGAAACTCA
IL-10	F	AGGAAGAGAAACCAGGGAGC
	R	GAATCCCTCCGAGACACTGG
GATA3	F	GCATCCAGACCAGAAACCGAA
	R	TCGCGTTTAGGCTTCATGATACT
FOXP-3	F	CAGCCAGTCTATGCAAACC
	R	GTCTTGTGTCAGTTTGAGGGTC
18S	F	5′ CTACGTCCCTGCCCTTTGTACA-3′
	R	5′ -ACACTTCACCGGACCATTCAA-3′

96-well plate (1:10 and1:5). As a positive control, Phytohemagglutinin (PHA) (5 %) (Cayman Chemical, Michigan, USA) was employed to stimulate T cells. In contrast, the unstimulated group included co-cultured iDCs and T cells. After incubation under dark circumstances (4 days), the growth of the CFSE-labeled T cells was evaluated via flow cytometry.

2.8. Assessment of cytokines

The cells were seeded at a ratio of 1:5 on a 24-well plate in order to assess the ability of CTLA-4-blocked-mDCs and mDCs to induce release of cytokine from autologous T cells, which is extracted from $CD3^{+}$ T cells. After the co-cultures were stimulated with DCs (48 h), the cells were gathered, and the rate of IFN-ϒ, TGF-β, and IL-4 were determined utilizing ELISA kits (Thermo Fisher Scientific, USA). Using ELISA kits, the amounts of IL-10 and IL-12 in CTLA-4-silenced-mDCs and mDCs' culture supernatants were also measured.

2.9. Isolation of RNA and qRT-PCR

The TRIzol reagent (Tonk Bioscience LLC, USA) was used to isolate total cellular RNA by the manufacturer's instructions. Then, using a spectrophotometer, the amount of RNA was determined. The RNA in this study was kept at −80 °C. Applying RT-PCR (BioFACT 2step 2X) Pre-Mix (Taq) complementary DNA (cDNA) was synthesized, and the level of all genes was examined utilizing the Applied Biosystems StepOnePlusTM RT-PCR System (Life Technologies, USA). After each experiment, the amount of the target and relative mRNAs was normalized using the 2-ΔΔCt technique.

2.10. Statistical analysis

GraphPad Prism v8.1.2 (California, USA) was used to analyze the raw data. Obtained data were compared using Mann–Whitney (the nonparametric version of Student's t-test) and the Kruskal Wallis (the nonparametric version of ANOVA). Data were obtained from biologically independent samples, and each parameter was assessed in triplicate. Each group's data were reported as mean ± SD with the significance cut-off of p-value ≤0.05 (ns: not significant; *: $P \leq 0.05$; **: $P \leq 0.01$; ***: $P \leq 0.001$; and ****: $P \leq 0.0001$), and Each parameter was assessed three times.

3. Results

3.1. CTLA-4 gene was dramatically decreased after transfection of siRNA in mDCs

Various pulse voltages were used to transfect mDCs to determine the optimum voltage for transfection (160, 180, and 200 V). The transfection rate was revealed to be close to 90 % in all of them and did not significantly differ between the specified voltages. Therefore, to reduce the stress on the cells during pulsing, the least voltage (160V) was chosen (Fig. 1A). Furthermore, qRT-PCR was applied to assess the efficiency of siRNA in gene silencing after CTLA-4-siRNA was transfected into mDCs at different concentrations. As a comparison to the control group of untransfected mDCs, CTLA-4-siRNA (60 pmol) lowered expression of CTLA-4 mRNA level in transfected cells more significantly than 40 and 80 pmol (Fig. 1B, P value: 0.0001) and subsequent tests were performed with an ideal CTLA-4-siRNA concentration of 60 pmol and an ideal transfection voltage of 160 V.

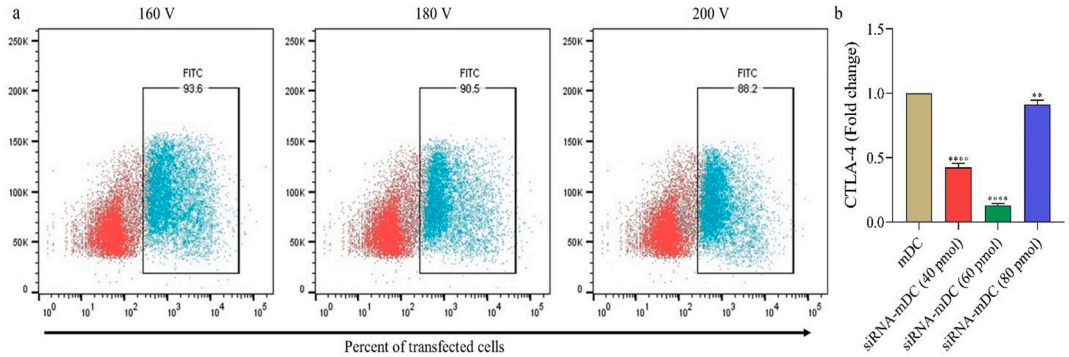

Fig. 1. The CTLA-4 gene was effectively suppressed in mDCs by siRNA transfection. (a) Efficiency of mDCs transfection across different pulse voltages. The optimal voltage was found to be 160 V, with over 93 % of the mDCs successfully transfected using FITC-labeled control siRNA. The Trypan blue exclusion test revealed that all transfected and untransfected DCs had more than 90 % viability. (b) Compared to untransfected mDCs, the expression of CTLA-4 mRNA was inhibited in mDCs that had been transfected with 60 pmol of CTLA-4 siRNA compared to the control group (**$P \leq 0.01$ and ****$P \leq 0.0001$). mDCs, Tumor cell lysate-loaded mature dendritic cells; CTLA-4, Cytotoxic T-lymphocyte-associated protein-4.

3.2. CTLA-4 deletion significantly improved the activation and maturation of DCs

Microscopically examination of adherent monocytes and differentiated DCs in vitro culture indicated morphological changes (Fig. 2A). As explained before, upregulating maturation and antigen presentation evaluated the phenotypic characteristics of different dendritic cells (including CTLA-4-silenced-mDCs, iDCs, and mDCs). According to flow cytometry evaluations, the most critical surface markers expressed by these cells include CD40, CD86, HLA-DR, and CD11c (Fig. 2B). Then, the difference in expression of these markers' surface depending on the median fluorescence intensity (MFI) between these DCs was investigated. Based on the findings of the analysis, CTLA-4 silenced mDCs compared to iDCs, these markers expression levels increased significantly in CD40 ($P \leq 0.0001$), CD86 ($P \leq 0.01$), HLA-DR ($P \leq 0.0001$), and CD11c (P value ≤ 0.01) (Fig. 2C). Furthermore, compared to mDCs, CTLA-4 silencing con siderably enhanced the level of CD40 ($P \leq 0.01$), HLA-DR ($P \leq 0.05$), and CD86 ($P \leq 0.01$), but this increase was not significant in CD11c (Fig. 2C). Moreover, it has been demonstrated that stimulated DCs can generate pro-inflammatory cytokines. The amount of IL-12 and IL-10 were determined by ELISA to describe the impact of CTLA-4 silencing. Also, IL-10 and TNF-α mRNA level was measured by qRT-PCR. Findings revealed that suppression of CTLA-4 decreased the IL-10 expression (Fig. 3A–$P \leq 0.01$), but contrary to expectation, this suppression also decreased the TNF-α level (Fig. 3A–$P \leq 0.0001$). Additionally, after CTLA-4 suppression, IL-12 amounts were enhanced compared to mDCs in the cell culture supernatants (Fig. 3B–$P \leq 0.01$). Also, our data indicated no significant difference in IL-10 mRNA expression in the supernatant of CTLA-4-blocked mDCs compared to mDCs (Fig. 3B).

3.3. CTLA-4 suppression in DCs increases the activity of T cells

In this study, after suppressing CTLA-4 in mDCs, its inhibitory effect on antitumor responses of T cells and cytokine generation by these cells was investigated. For this purpose, a co-culture experiment was performed to assess proliferation utilizing mDCs and CTLA-4-silenced-mDCs as a stimulator and CFSE-labeled autologous $CD3^+$ T cells as a responder in a 1:10 and 1:5 ratio. The findings revealed that all groups showed higher T cell growth at a ratio of 1:5, compared to 1:10 (Fig. 4A and B). Moreover, $CD3^+$ T cell proliferation enhancement was observed following the suppression of CTLA-4 expression in mDCs in a ratio of 1:5 (Fig. 4A and B). However, it is interesting to know that despite the enhanced ability to induce the growth of $CD3^+$ T cells through CTLA-4-blocked-mDCs, in the 1:10 ratio, this increase was not significant. Also, to evaluate the T cells' antitumor activity, the amount of TGF-β, IL-4, and IFN-ϒ in T and DC cells was measured. The obtained results demonstrated that the induction rate of IL-4 and IFN-ϒ in the CTLA-4-silenced mDCs and T cells significantly increased significantly than in T cell/mDC co-culture (Fig. 5A) that was caused by an increment in $CD3^+$ T cell growth. Furthermore, no dramatic difference was observed in the TGF-β level in the co-culture supernatant of CTLA-4-silenced-mDCs and T cells compared to the mDCs and T cells co-culture (Fig. 5A). In contrast to mDCs/T cells co-culture, our result illustrated that T cells obtained from the CTLA4-silenced-mDCs/T cells exhibited enhanced amounts of T-bet, IFN-ϒ, GATA3, and IL-10 mRNA expression (Fig. 5B). In addition, as opposed to mDCs/T cell co-culture, FOXP3 mRNA in T cells isolated from the CTLA-4-blocked-mDCs/T cells was significantly dropped (Fig. 5B).

4. Discussion

Breast cancer is a prevalent and potentially fatal disease affecting women globally. Research and awareness are crucial for early

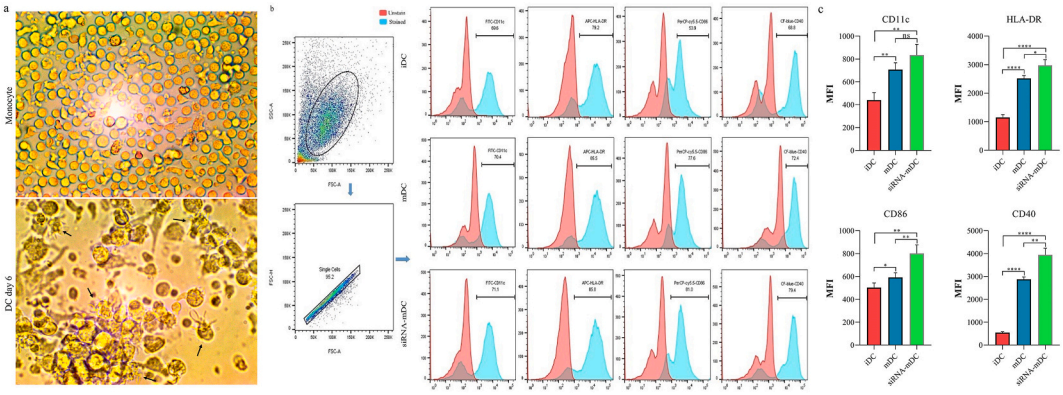

Fig. 2. Characterization of DCs' morphological and phenotypical. (a) Changes in morphology during adherent monocyte and differentiated DC culture in vitro. DCs with normal morphology and sharp dendrites are indicated by arrows. (b) iDC, mDC, and CTLA-4-silenced mDC phenotypic characterization were measured using flow cytometry to express surface markers like CD11c, HLA-DR, CD86, and CD40. The data are presented as a percent of the cells stained with various markers (Figures A and B supplied as representative of all samples). (c) The expression of CD11c, HLA-DR, CD86, and CD40 is displayed as MFI among iDCs, mDCs, and CTLA-4-silenced-mDCs (ns: not significant, *P value ≤ 0.05, **P value ≤ 0.01, and ****P value ≤ 0.0001). DCs, dendritic cells; CTLA-4, Cytotoxic T-lymphocyte-associated protein-4; siRNA-mDCs, CTLA-4-silenced mDCs; mDCs, Tumor cell lysate-loaded mature dendritic cells; iDCs, Immature dendritic cells; HLA-DR, Human leukocyte antigen-DR isotype; MFI, Median fluorescence intensity.

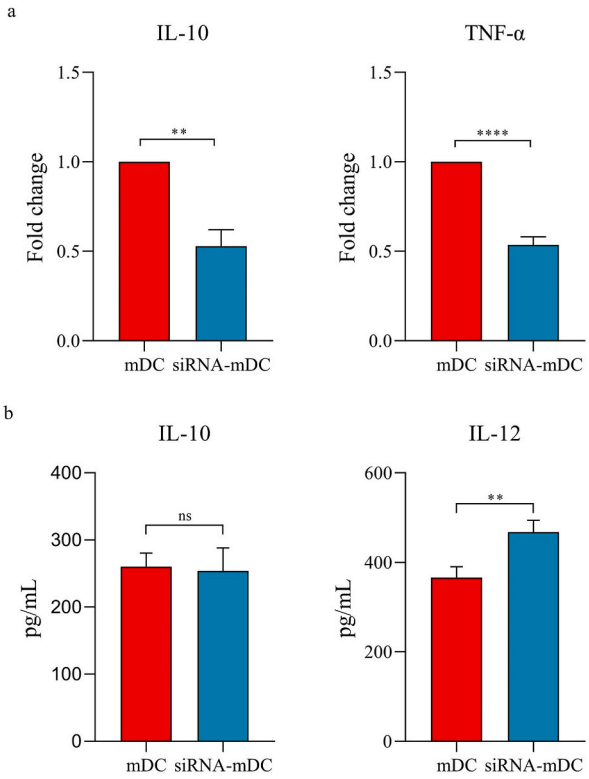

Fig. 3. Cytokine secretion and expression profiles in mDCs and CTLA-4-silenced-mDCs (a) qRT-PCR was used to measure the expression levels of TNF- α and IL-10. (b) Using ELISA in the cell culture supernatants, the amounts of IL-12 and IL-10 were assessed (ns: not significant, **P ≤ 0.01, and ****P ≤ 0.001). qRT-PCR, Quantitative Real-time polymerase chain reaction; mDCs, Tumor cell lysate-loaded mature dendritic cells; CTLA-4, Cytotoxic T-lymphocyte-associated protein-4; siRNA mDCs, CTLA-4-silenced mDCs; ELISA, Enzyme-linked immunosorbent assay.

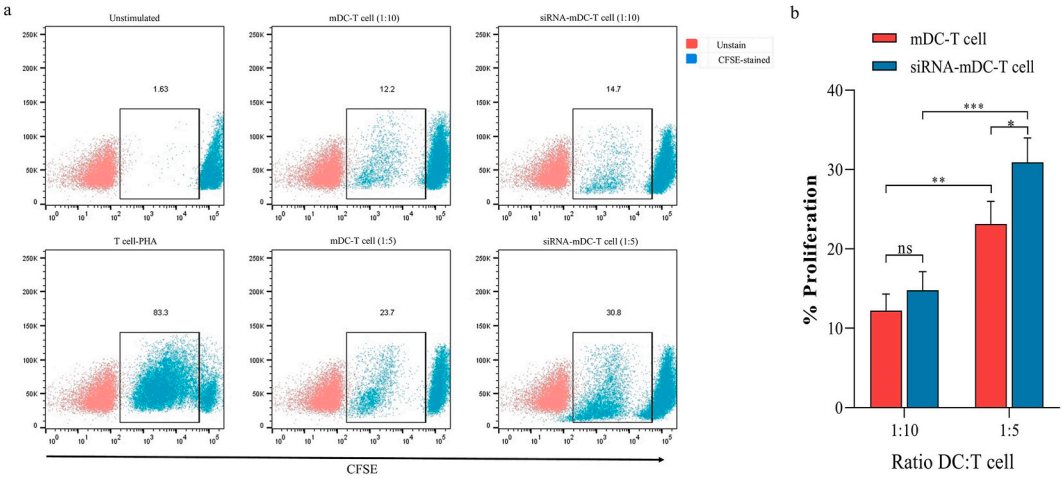

Fig. 4. CTLA-4-silenced-mDCs significantly increase $CD3^+$ T cell proliferation. (a) The percent of CFSE-labeled autologous $CD3^+$ T cells activated by mDCs and CTLA-4-silenced-mDCs at a 1:5 and 1:10 DC/T cell ratio was evaluated by FACS by quantifying the CFSE loss. (b) After CTLA-4 knockdown, the ability of mDC to proliferate T cells was improved (ns: not significant, *P value ≤ 0.05, **P value ≤ 0.01, and ***P value ≤ 0.001). mDCs, Tumor cell lysate-loaded mature dendritic cells; siRNA-mDCs, CTLA-4-silenced mDCs; CTLA-4, Cytotoxic T-lymphocyte-associated protein-4; FACS, Fluorescent activated cell sorting.

detection, improved treatments, and prevention, ultimately reducing its strain on individuals and healthcare systems [30,31]. Among its various subtypes, studying triple-negative (TNBC) and HER2-negative breast cancer holds significant importance due to their unique challenges. The aggressive nature of TNBC, coupled with its propensity to grow and spread rapidly, underscores the urgency for

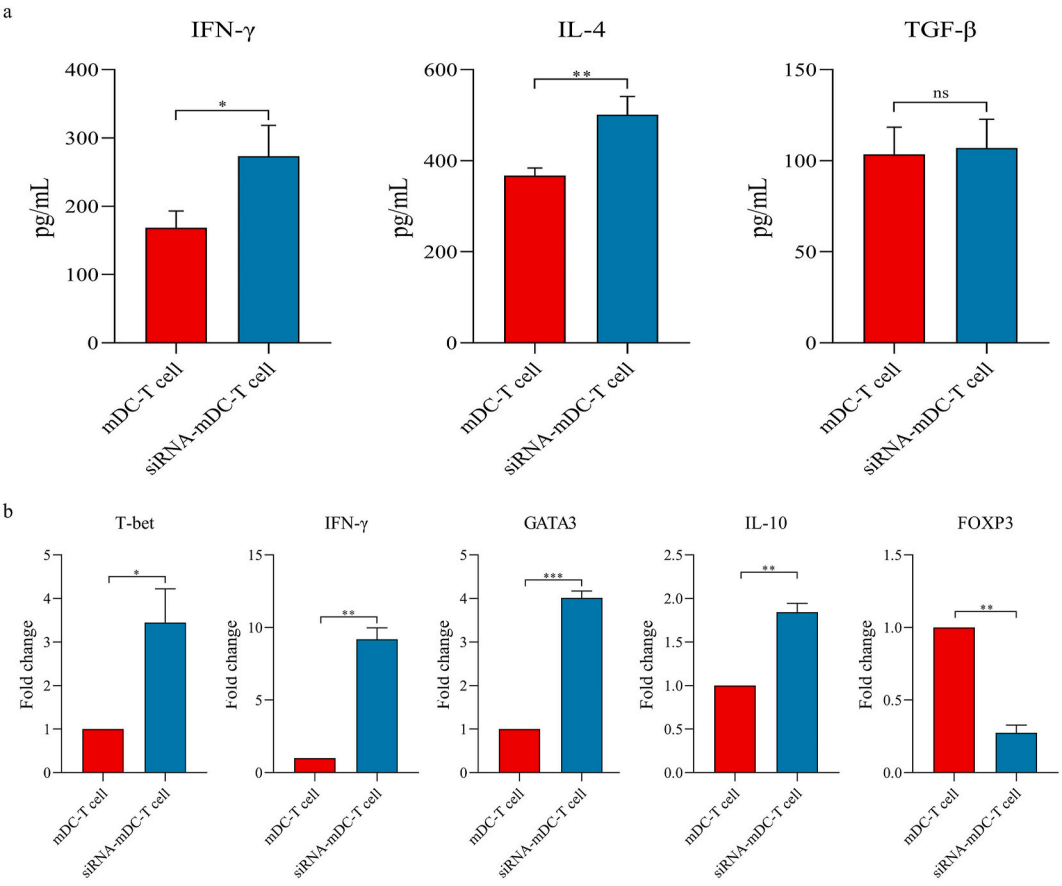

Fig. 5. CTLA-4 silencing improved T cell-mediated effector activities in mDCs. After co-culture with mDCs and CTLA-4-silenced-mDCs, T cells produce cytokines. (a) IFN-ϒ, IL-4, and TGF-β secretion by $CD3^+$ T cells were assessed in supernatants of DC/T cell co-culture by ELISA. (b) qRT-PCR was used to analyze the expression of T cell-mediated transcription factors, including GATA3, T-bet, IFN-ϒ, IL-10, and FOXP3, by $CD3^+$ T cells after co-culture with mDCs and CTLA-4-silenced-mDCs; (*P ≤ 0.05, **P ≤ 0.01, and ***P ≤ 0.001). DC, dendritic cell; mDCs, Tumor cell lysate-loaded mature dendritic cells; siRNA-mDCs, CTLA-4-silenced mDCs; CTLA-4, Cytotoxic T-lymphocyte-associated protein-4; ELISA, Enzyme-linked immunosorbent assay; GATA3, GATA binding protein 3; T-bet, T-box protein expressed in T cells; FOXP3, Forkhead box P3; qRT-PCR, Quantitative reverse transcription polymerase chain reaction.

timely and effective treatments [32]. The primary treatment for TNBC, aggressive chemotherapy, is riddled with side effects, often diminishing the patient's quality of life and may also pose economic challenges for some patients. This highlights the need for research that can pave the way for cost-effective and efficient therapeutic interventions. Thus, treatment approaches based on immunotherapy appear to be the most potent alternatives with fewer side effects [32,33]. In this context, the study of breast cell lines, specifically TNBC is considered. In the present study, we studied MCF-7, MDA-MB-231, and MDA-MB-468 cell lines. MDA-MB-231 and MDA-MB-468 are employed to address the challenges of treating Triple-negative breast cancer (TNBC) [34] and MCF-7 is used as HER2 negative cell line [35]. Each of these cell lines has its distinct genetic and molecular landscape, making them suitable for different types of research studies for the development of immunotherapy.

Dendritic cells can be divided into different types including type 1 conventional DCs (cDC1), type 2 conventional DCs (cDC2), myeloid DCs (mDCs), lymphatic DCs (pDCs), regulatory DCs (regDCs). In humans cDC1 have markers such as CD141, XCR1, CLEC9A, and CADM1 and cDC2s express CD1c, CD11c, and SIRPa [36]. MoDCs are identified in humans using HLA-DR, CD11c, BDCA1, CD1a, FceRI, CD206, CD172a, CD14, and CD11b in addition to M-CSFR and ZBTB46. These cells stimulate the responses of $CD4^+$ and $CD8^+$ T cells and developed under inflammatory conditions are involved in inducing and regulating immune responses [36]. pDCs expresses CD11c, IRF8, and MHC-II molecules. The capacity of this cell to generate a significant quantity of type I interferons is its most notable characteristic [37]. Human regulatory DCs, when compared to their normal counterparts, showed deficient low levels of costimulatory molecules (such as CD40, CD80, and CD86), but high expression levels of MHC [38].

In cancer immunotherapy, using dendritic cells to activate antitumor responses of $CD8^+$ and $CD4^+$ T cells has been considered. Dendritic cells pulsed with lysate are more potent in inducing T-cells; However, tumor cells employ a range of immunoregulation mechanisms that disrupt the performance of these dendritic cells and compromise the efficacy of treatments related to dendritic cells, and allow cancerous cells to evade immune surveillance. Immune checkpoint molecules are pivotal in shaping the tumor microenvironment [39]. CTLA-4 plays a crucial role in blocking antitumor T cells. CTLA-4 can bind to CD86 and CD80, interfering with the

CD28-mediated signaling in T cells and inhibiting T cell induction [40]. The interaction between CD28 and CD80/CD86 is augmented, thereby promoting the immune responses of $CD8^+/CD4^+$ cells [41,42]. CTLA-4 can be expressed in other cells, such as DCs, and have immunoregulatory impacts on these cells [21,24]. The presence of CTLA-4 in these cells decreases their maturation by reducing the expression of CD83, as well as these cells have a low ability to present antigens to T cells [24].

Many studies have tried to use tumor lysate-pulsed DCs [43–45] or prevent immune checkpoints [46,47] in DCs to enhance the effectiveness of treatment based on dendritic cells. Nonetheless, no study has simultaneously evaluated tumor antigen delivery on DCs and the effect of CTLA-4 blockage in cancer therapy. Because of the expression of CTLA-4 on dendritic cells and having a negative impact on their activity, we inhibited the CTLA-4 in BC cell lysate-pulsed DCs through the delivery of siRNA to raise the effectiveness of dendritic cell therapy. To reduce the CTLA-4 level in BC cell lysate-loaded DCs, duplex CTLA-4 siRNA with high specificity, longevity, and stability were used. In our research, following transfection using CTLA-4 siRNA (60 pmol), the level of CTLA-4 genes significantly decreased in transfected mDCs compared to normal mDCs. Following determining the effective and optimal concentration and voltage for the delivery of siRNA, we evaluated the influence of CTLA-4 inhibition on mDCs by measuring cytokine secretion characteristics and surface molecule expression patterns. Thus, despite no significant increase in CD11c expression, CTLA-4 inhibition in these cells could dramatically amplify the CD40, CD86, and HLA-DR expressions, indicating the capability of CTLA-4-blocked mDCs in stimulating T cells. Cytokines released by DCs are another crucial factor for the stimulation of T-cells. Whereas the production of IL-10 is related to tolerogenic DCs, induced DCs generate IL-12 and TNF-α cytokines [48]. We found that after CTLA-4 silencing in mDCs, there was a reduction in the TNF-α and IL-10 expression. Also, by suppressing CTLA-4, the amount of IL-12 secretion dramatically rose, while the level of IL-10 remained unchanged. Furthermore, we evaluated the effectiveness of CTLA-4-blocked mDCs on T-cells, and our data showed that these DCs induced T-cell responses. Inhibition of CTLA-4 and loading of BC cell lysate in DCs caused a dramatic enhancement in the growth of $CD3^+$ T cells by the method of autologous co-culture in comparison to DCs loaded with BC cell lysate. We also determined the secretion of cytokines through T cells due to the suppression of CTLA-4 in DCs. Inhibition of CTLA-4 leads to an enhancement in the generation of IL-4 and IFN-γ, cytokines linked to Th2 and Th1, respectively. Nonetheless, the reduction in TGF-β (a marker of Treg) was not changed. In addition, the level of transcription markers related to responses of T cells was assessed. Our result demonstrated that mRNA expression of T-bet (Th1 marker) and GATA3 (Th2 marker) increased dramatically after treatment with CTLA-4-blocked -mDCs, while FOXP3 mRNA (Treg marker) decreased in these cells in $CD3^+$ T cells.

In DC therapy, various studies have attempted to separately block immune checkpoint molecules or load tumor cell lysates on dendritic cells, including CTLA-4. To support our data, Dea Suk Kim et al. indicated thatDC vaccination utilizing DCs comprising tumor antigens can cause tumor inhibition in melanoma patients [49]. Aerts et al., through producing IFN-γ in a mesothelioma-mouse model, showed that the response of T-cells increased after using DCs loaded with tumor lysate, which is in line with our findings [45]. Consistent with our data, the generation and increase of IFN-γ from T cells increased by stimulating cancer cell-loaded DCs [50]. Consistent with our findings, in patients who suffer from ovarian cancer and melanoma, the use of a type of CTLA-4 blocking antibody (MDX-CTLA4) caused promising tumor regression and antitumor immunity [51]. Van Den Bergh and colleagues demonstrated that PD-L1/L2-blocked dendritic cells enhanced TNF-α generation and T-cell proliferation compared to control DCs, confirming our result [46]. Cedric Menard et al.'s findings revealed that blocking CTLA-4 in melanoma patients increases treatment success by increasing

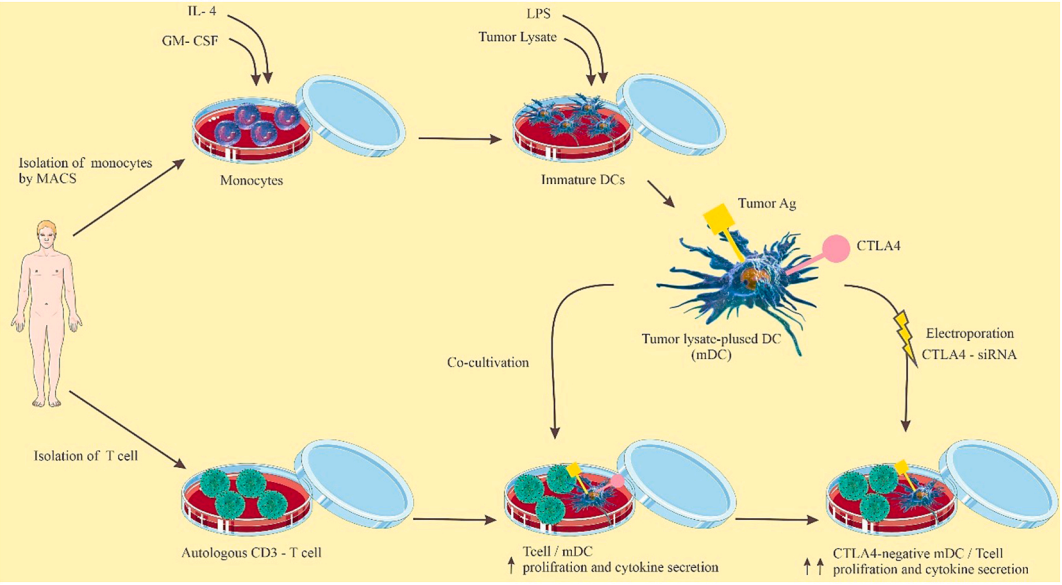

Fig. 6. CTLA-4 molecules suppression in tumor-lysate-pulsed-DCs improves autologous $CD3^+$ T-cell responses. Silencing of CTLA-4 gene in mDCs via using siRNA enhances their stimulatory properties. Thus, Co-culture of CTLA-4-silencing mDCs with autologous $CD3^+$ T cells augments T-cell mediated responses. CTLA-4, Cytotoxic T-lymphocyte-associated protein-4; DC, dendritic cell; mDC: tumor-lysate-pulsed mature dendritic cell; LPS: Lipopolysaccharide; siRNA, small interfering RNA.

memory T cells [52]. Vonderheide RH et al. proved that utilizing antibodies against CTLA-4 (Tremelimumab) increased the activation of $CD8^+$ cells to BC by increasing $ICOS^+$ T cells [53]. Oh, SA and colleagues' findings indicated that inhibition of PD-L1 in dendritic cells improved anticancer responses of $CD8^+$ T-cells [54]. In high stimulatory conditions, such as stimulation with CD3 or exposure to mature autologous DCs, $CD4^+$ T cells obtain inhibitory characteristics, such as expression of FOXP3 genes as well as producing IL-10, IL-4, IL-2, and IFN-γ cytokines [55,56].

Previous studies have attempted to individually load tumor cell lysates onto DCs or silence inhibitory immune checkpoints, such as PD-L1/PD-L2, to develop effective DC-based cell therapy. A study demonstrated that using dendritic cells loaded with human gastric tumor lysates, the expression of dendritic cell surface molecules, including CD80, CD83, CD86, and human leukocyte antigen-DR (HLA -DR) and cytokine production by both DC and DC/T cell cultures (i.e., interleukin IL-12:IL-10 and interferon IFN-γ:IL-4 ratio) increased [57]. Also, in another study on patients with metastatic melanoma, it has been reported that vaccinating these patients using dendritic cells loaded with tumor cell lysis has improved antitumor immunity responses [58]. In addition, GN Shi et al. have shown that using dendritic cells loaded with tumor cell lysates improved antitumor immune responses, increased serum levels of IFN-γ and IL-4, and prevented tumor growth in melanoma mice [59]. Furthermore, in a similar study, it was shown that antigen-loaded dendritic cells stimulated T-cell proliferation and induced the production of high concentrations of IL-12 and IFN-γ but only low levels of IL-10 and cause a strong antitumor immune response in-vivo [60]. Q Peng et al. reported that PD-L1 on dendritic cells limits T-cell responses, and harnessing of PD-L1 in DCs invigorates antitumor immune responses [61]. In another study, in a human breast tumor-bearing SCID model, Ge et al. demonstrated that silencing PD-L1 signaling could enhance DC maturation, proliferation, and IL-12 secretion and potentiate DC primary T-cell responses [62]. In these studies, DCs were loaded individually with tumor lysate or blocked for inhibitory immune checkpoints such as PD-L1/PD-L2; however, there is no study regarding the simultaneous silencing of CTLA-4 and tumor cell lysate loading on DCs to boost the effectiveness of DC-based immunotherapy. In our study, we used the combination of breast tumor lysate and immune checkpoint suppression to increase the efficacy of dendritic cells, and based on our findings, CTLA-4 blockage in DCs loaded with breast cancer cell lysates increases dendritic cell efficacy and autologous T cell activation and cytokine secretion, indicating an appropriate therapeutic technique for the following clinical and preclinical research (Fig. 6).

5. Conclusion

In this research, for the first time, we indicated that blocking CTLA-4 enhanced the stimulatory activity and maturation of DCs loaded with breast cancer cell lysate. Additionally, these modified DCs significantly increased the cytokine secretion and activation of co-cultured T-cells compared to normal DCs. Given these results, this anticancer therapeutic approach is looked into more thoroughly in preclinical studies to support this idea.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The study was reviewed and approved by the ethical committee of Tabriz University of Medical Sciences with the approval code of IR.TBZMED.REC.1399.10.46. The patients/participants provided written informed consent to participate in this study.

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CRediT authorship contribution statement

Mohammad Bakhshivand: Writing – original draft, Methodology, Investigation, Conceptualization. **Javad Masoumi:** Methodology, Investigation. **Farid Ghorbaninezhad:** Methodology, Investigation. **Leili Aghebati-Maleki:** Formal analysis, Conceptualization. **Dariush Shanebandi:** Formal analysis, Conceptualization. **Siamak Sandoghchian Shotorbani:** Conceptualization. **Farhad Jadidi-Niaragh:** Conceptualization. **Amir Baghbanzadeh:** Writing – review & editing. **Nima Hemmat:** Writing – review & editing. **Elham Baghbani:** Visualization. **Amir Ghaffari:** Writing – review & editing. **Behzad Baradaran:** Validation, Supervision, Project administration.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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