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Isfahan University of Medical Sciences

Original Article

DCs pulsed with hypochlorous acid-treated tumor cell lysates present antigens efficiently and induce CD8$^+$ T cell activation through cross-presentation

Maryam Abbaspour[1], Nafiseh Esmaeil[2], Mahboubeh Rezazadeh[3,4], Mohsen Minaiyan[5], and Vajihe Akbari[1,*]

[1]*Department of Pharmaceutical Biotechnology, School of Pharmacy and Pharmaceutical Sciences, Isfahan University of Medical Sciences, Isfahan, I.R. Iran.*
[2]*Department of Immunology, School of Medicine, Isfahan University of Medical Sciences, Isfahan, I.R. Iran.*
[3]*Department of Pharmaceutics, School of Pharmacy and Pharmaceutical Sciences, Isfahan University of Medical Sciences, Isfahan, I.R. Iran.*
[4]*Novel Drug Delivery System Research Center, School of Pharmacy and Pharmaceutical Sciences, Isfahan University of Medical Sciences, Isfahan, I.R. Iran.*
[5]*Department of Pharmacology and Toxicology, School of Pharmacy and Pharmaceutical Sciences, Isfahan University of Medical Sciences, Isfahan, I.R. Iran.*

Abstract

Background and purpose: In initiating and regulating immune responses, dendritic cells (DCs) play an important role as antigen-presenting cells. When DCs are exposed to tumor cell lysates, they can stimulate T cells to recognize tumor-associated and tumor-specific antigens and generate an immune response against cancer. The purpose of this study was to compare 4 different approaches for preparing breast tumor cell lysates for pulsing DCs.

Experimental approach: To prepare tumor cell lysates from 4T1 cells, 4 different methods were used, including freeze-thaw, hypochlorous acid (HOCl), hyperthermia, and ultraviolet-B irradiation. The effects of the tumor lysates were assessed on the maturation of DCs and the secretion of cytokines using flow cytometry and ELISA. Furthermore, DCs pulsed with different lysates were also evaluated for their ability to promote CD8+ T cell proliferation and release cytokines.

Findings/Results: The results demonstrated that DCs pulsed with lysate prepared by HOCl exhibited more maturation surface biomarker expression (CD86) than DCs pulsed with freeze-thawed cells or unloaded DCs (control). Furthermore, activated DCs were also found to promote CD8+ T cell proliferation and induce the responses of T cells by producing high levels of IFN-γ, while inhibiting IL-10.

Conclusion and implications: HOCl is capable of releasing tumor antigens while maintaining its ability to stimulate an immune response. DCs-based therapies may be designed based on the findings presented here, demonstrating a cross-presentation of antigens and specific activation of the immune system against breast cancer.

Keywords: Breast cancer; Dendritic cells; Hypochlorous acid; Tumor cell lysate.

INTRODUCTION

A significant number of women worldwide are affected by breast cancer, one of the most common forms of cancer. Traditional treatment options, such as surgery, chemotherapy, and radiation have limited success in eliminating cancer cells. Therefore, it is crucial to develop novel and effective therapies. Cancer immunotherapy aims to establish long-lasting immunity to prevent tumor recurrence by specifically amplifying and targeting the adaptive immune response (1).

*Corresponding author: V. Akbari
Tel: +98-3137927060, Fax: +98-3136680011
Email: v_akbari@pharm.mui.ac.ir

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Dendritic cells (DCs)-based immunotherapy has been extensively investigated in the past two decades, and represents a promising therapeutic approach to treating cancer. DCs, the most potent antigen-presenting cells (APCs), function as a link between innate and adaptive immune systems by processing and presenting tumor-associated antigens (TAAs) to naive T cells *via* major histocompatibility complex (MHC) classes I and II. This process activates and expands $CD4^+$ T helper (TH) lymphocytes and $CD8^+$ cytotoxic T lymphocytes (CTLs) (2,3). Whole tumor antigen consists of epitopes for both $CD8^+$ CTLs and $CD4^+$ TH cells, generating potent and enduring immune responses against tumors (4).

For activating and targeting DCs, they can be loaded, either *in vivo* or *ex vivo,* with various forms of whole tumor antigen including whole tumor cell lysates, exosomes, and tumor-associated peptides (5). Although tumor vaccination with DCs pulsed with whole tumor cells or derivatives has shown promise, it is challenging to induce the robust responses of CTLs with therapeutic efficacy using tumor cell lysates (6,7).

This study compared the efficacy of 4 different strategies including repeated freeze-thawing (FT), lysis by hypochlorous acid (HOCl), ultraviolet B (UVB) ray-irradiation, and lysis by hyperthermia (HSP) for preparing breast tumor cell lysate and evaluated their ability to induce the maturation of DCs and robust T-cell responses *in vitro*.

MATERIALS AND METHODS

Cell culture

4T1 cell line, mouse breast carcinoma cells, was provided by the Pasteur Institute of Tehran, Iran. Cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, and 1% penicillin/streptomycin at 37 °C in a humidified incubator with 5% CO_2.

Preparation of tumor cell lysate

4T1 cells grown to 70-80% confluency were detached with 0.25% trypsin-ethylenediaminetetraacetic acid (EDTA) (Gibco, Scotland) and washed 2 times with phosphate-buffered saline (PBS) and resuspended at 2×10^6 cells/mL in PBS. Various methods, including HOCl, HSP, UV irradiation, and FT were used to treat the cells.

In HOCl treatment, NaOCl (Sigma-Aldrich, Germany) reagent was diluted in Hank's balanced salt solution (Baharafshan, Iran) to prepare the HOCl solution at the 60 µM concentration and immediately added to 4T1 cells. The cells were maintained in the incubator at 37 °C and 5% CO_2 for 1 h, and agitated gently every 30 min to induce oxidation-dependent cancer cell death. The HOCl-treated cells were centrifuged at 600 *g* for 6 min, washed twice with PBS, and suspended in PBS for the freeze-thaw procedure (8,9).

In the hyperthermia approach, 4T1 tumor cells were placed in a water bath at 42 °C for 60 min and then at 37 °C for 12 h. Then, the tumor cells were washed in PBS and subjected to 6 cycles of freeze (in liquid nitrogen) and thaw (at 37 °C in the water bath) to obtain cell lysates.

In the UV irradiation method, 2×10^6 4T1 cells/mL were washed and resuspended in a complete RPMI medium (*i.e.*, supplemented with FBS and antibiotics). Next, the cells were plated in 10-cm Petri dishes and subjected to a 254 nm UVB irradiation for 10 min to induce apoptosis. The cells were incubated overnight at 37 °C and 5% CO_2 and harvested the next day for the freeze-thaw procedure (10).

To implement the FT approach, the cells were exposed to freeze-thaw cycles. Each cycle consisted of 10 min in liquid nitrogen followed by 10 min in a water bath set at 37 °C. This process was conducted 6 times in total. A low-speed centrifugation step was then carried out using $100 \times g$ for 10 min to eliminate large debris and create a freeze-thaw lysate from the supernatant.

All lysates underwent trypan blue staining. After examining under an optical microscope, cell viability percentage was determined by dividing the number of uncolored cells by the total number of cells. The supernatant protein concentration was measured using the Bradford method (11). All lysates were stored at -80 °C until further use.

DCs

DCs derived from female Balb/c mouse (6 to 8 weeks old) bone marrow were generated as described previously (12). Briefly, bone marrow-derived mononuclear cells (MNCs) were collected by flushing the femurs and tibias with RPMI 1640 medium. The MNCs were then centrifuged and resuspended in complete RPMI 1640 medium supplemented with 10% FBS, 1% L-glutamine, 1% penicillin/streptomycin, 20 ng/mL murine recombinant granulocyte-macrophage colony-stimulating factor (GM-CSF; R & D, USA), and 10 ng/mL recombinant murine IL-4 (BioLegend, USA). After 3 days, half of the medium was collected and centrifuged. Then, 5 mL of the new culture medium was added. After 6 days, non-adherent and loosely adherent cells were collected, washed, and used to evaluate the phenotype of immature DCs in experiments.

DCs pulsed with tumor lysate

On the sixth day, immature DCs (2×10^6) were exposed to tumor cell lysates (TCLs) containing 8 µg/mL of protein. The TCLs were produced using different strategies, as described in a previous study (16). To assess the preparation strategy impact of TCLs on the immunogenicity of the lysates, an equivalent amount of protein was used for pulsing DCs. Additionally, lipopolysaccharide (LPS; Sigma-Aldrich, Germany) at a concentration of 1X (10 ng/mL) was added to all the stimulated (activated) flasks, except for the negative control.

Flow cytometry

In this experiment, 0.25×10^6 DCs were treated with antibodies and analyzed using flow cytometry. The DCs were resuspended in PBS and stained with mouse CD11c PE-conjugated antibody and FITC-conjugated anti-mouse CD86 antibody (Biolegend, San Diego, CA, USA).

The cells were gently mixed on a vortex machine and then placed at room temperature in the dark for 30 min. They were rinsed with PBS twice and centrifuged at 300 × *g* for 5 min. Flow cytometry analysis was performed using a FACS Calibur flow cytometer (Becton-Dickinson, Mountain View, CA, USA) and the CellQuest software package (BD Biosciences, USA). Necrotic cells and cellular debris were excluded from the analysis based on their forward scatter/side scatter profiles. For each analysis, 20,000 events were acquired and gated based on CD11c expression and side scatter properties.

Generation of lymphocyte suspensions from the spleen

Six- to 8-week-old Balb/c female mice with weighing 25 ± 2 g were provided from Rouyan institute (Isfahan, Iran) to obtain $CD8^+$ T cells from spleens using aseptic techniques. All experimental procedures were performed according to national and international standards for the care and use of laboratory animals, with approval from the Ethics Committee of Animal Use of Isfahan University of Medical Sciences (ethical code: IR.MUI.RESEARCH.REC.1398.335). The spleens were individually homogenized in 5 mL of RPMI 1640 medium to release splenocytes. The resulting cell suspension was centrifuged for 5 min at 400 *g* and room temperature. After centrifugation, the supernatant was removed, and the cells were resuspended in the remaining volume, approximately 100 µL. Erythrocytes were lysed using red blood cell lysis buffer (0.15 M NH_4Cl, 1 mM $KHCO_3$, and 0.1 mM Na_2EDTA; pH 7.4) per 10^8 cells. The lysis reaction was incubated at room temperature for 3-4 min. To restore iso-osmolarity, 10 mL of PBS containing 2% FBS was added. The single-cell suspensions from individual spleens were pooled, filtered through a 70-µm cell strainer (Fisher, USA), and counted. $CD8^+$ T cells were isolated using magnetic cell sorting *via* negative selection by MojoSort Mouse CD8 T Cell Isolation kit (BioLegend, USA) according to the manufacturer's instructions. Cell samples were taken before and post-magnetic selections and then labeled with a viability dye, carboxyfluorescein succinimidyl ester (CFSE; Thermo Fisher Scientific, USA). To check sample purity, anti-CD4-FITC (clone RM4-4, BioLegend,USA) and anti-CD8-FITC (clone 53-6.7, BioLegend, USA) were used. The morphology of the cells was also assessed under a microscope, with T cells showing a round or oval shape, B cells appearing larger

and more irregular, and natural killer cells having a large granular cytoplasmic appearance.

In vitro T cell expansion

To assess the impact of different methods of tumor lysate protein production on the expansion of antigen-specific $CD8^+$ T cells, 50,000 CD11c bone marrow cells were seeded onto 96-well plates after being isolated as described above. The cells were then incubated for 24 h with 8 µg/mL of tumor lysate protein generated through various methods, along with LPS. CD11c cells untreated with tumor lysate and LPS-free were included as a negative control.

$CD8^+$ T cells were isolated from the spleen using a negative selection kit (MojoSort Mouse CD8 T Cell Isolation Kit). The T cells were then labeled with CFSE (at a concentration of 1 µM, 2 million cells/mL) for 10 min in RPMI medium in a 100-µL total volume.

T cells with $CD11c^{+/-}$ cells treated or untreated were co-cultured on 96-well round-bottom plates at a ratio of 1:5 (DCs:T cells) and maintained at 37 °C and 5% CO_2 in complete RPMI medium (containing 10% FBS). Supernatants were collected at 72 h for future cytokine analysis. The cells were washed twice using PBS and analyzed immediately by flow cytometry. FlowJo Software (Treestar, USA) was used for analyzing T cell proliferation.

Cytokine release

Interferon-gamma (IFN-γ) and interleukin-10 (IL-10) cytokines in the supernatants were determined using enzyme-linked immunosorbent (ELISA) kits (R MAX™ Standard Set, BioLegend, USA) according to the manufacturer's instructions. The concentrations were calculated from the standard curves.

Statistical analysis

Each experiment was repeated 3 times. Data were presented as mean ± standard deviation (SD). Statistical analysis was performed using GraphPad Prism Software 8 (La Jolla, CA, USA), and data were analyzed using ANOVA followed by least-significant difference (LSD). The P-value < 0.05 was considered statistically significant.

RESULTS

Maturation of DCs in response to antigen loading

To induce activation and maturation of DCs, cells were pulsed with whole TCLs prepared by 4 strategies for 24 h. The expression of phenotypic biomarkers such as CD86, indicating the maturation of DCs, was assessed using flow cytometry (Fig. 1A). This biomarker was expressed at a low level before treatment with TCLs. The expression profile of the biomarkers by DCs on day 7 was demonstrated in Fig. 1B. Overall, there was no significant difference in CD86 expression among the DCs exposed to different preparation strategies of TCLs. However, DCs exposed to HOCl showed a significantly higher level of CD86 expression than the control group (Fig.1A).

The proliferation of T cells with tumor lysates prepared by different methods

To measure the proliferation of CFSE-labeled naive T cells, duplicate assays were conducted (Fig. 2A). After dividing a cell, the level of CFSE in the daughter cells was reduced, and a small population of divided cells could be detected to the left of the undivided population (Fig. 2B). To evaluate the impact of different properties of formulations of DCs pulsed by tumor lysate on *in vitro* T cell activation, T cell proliferation was assessed by CFSE dilution using flow cytometry in 96-well tissue culture plates over 72 h. When DCs were exposed to tumor lysate antigens, they stimulated T-cell proliferation (Fig. 2A).

T cell proliferation in different treatment groups demonstrated that co-culturing T lymphocytes with antigen-loaded DCs led to increased cell division compared to unloaded control DCs. Notably, DCs exposed to UV-TCL showed a significant increase in T cell proliferation compared to unloaded control DCs (Fig. 2A). Furthermore, LPS-treated DCs exhibited a higher ability to activate T cells than DCs without LPS (Fig. 2A).

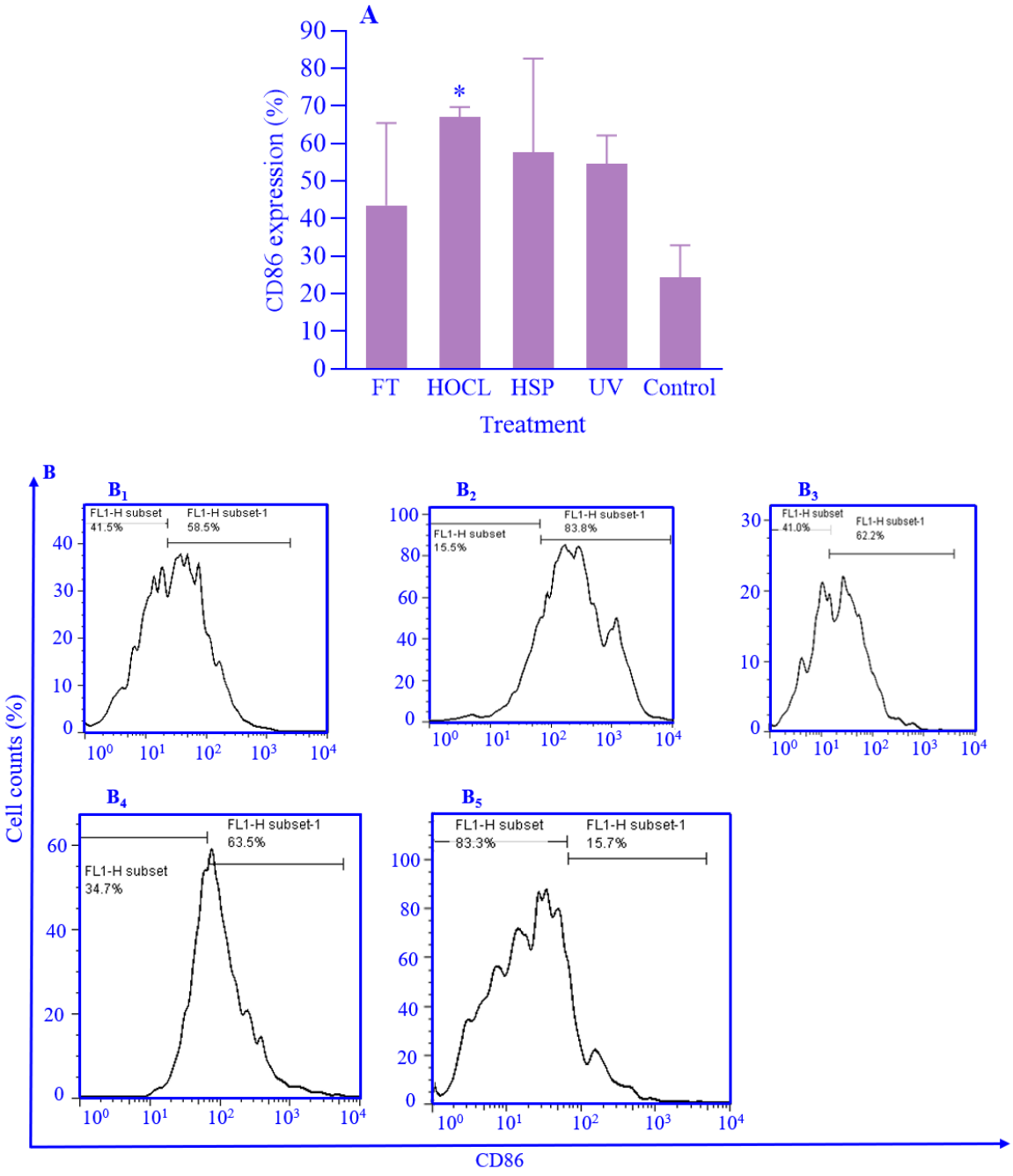

Fig. 1. The maturation of DCs in response to antigen loading. DCs were pulsed with tumor lysates prepared by 4 strategies for 24 h, and then the expression of CD86 was analyzed by flow cytometry. (A) The maturation percentage of DCs plus different tumor cell lysates; (B) Histogram curves demonstrating the maturation of DCs, (B_1) FT; (B_2) HOCL; (B_3) UV; (B_4) HSP; (B_5) control. The control group was considered antigen-unloaded DCs. Data were expressed as mean ± SD, n = 3. $^*P < 0.05$ demonstrates a significant difference compared with control. FT, Freeze-thaw; HOCL, hypochlorous acid; UV, ultraviolet; HSP, hyperthermia; DCs, dendritic cells.

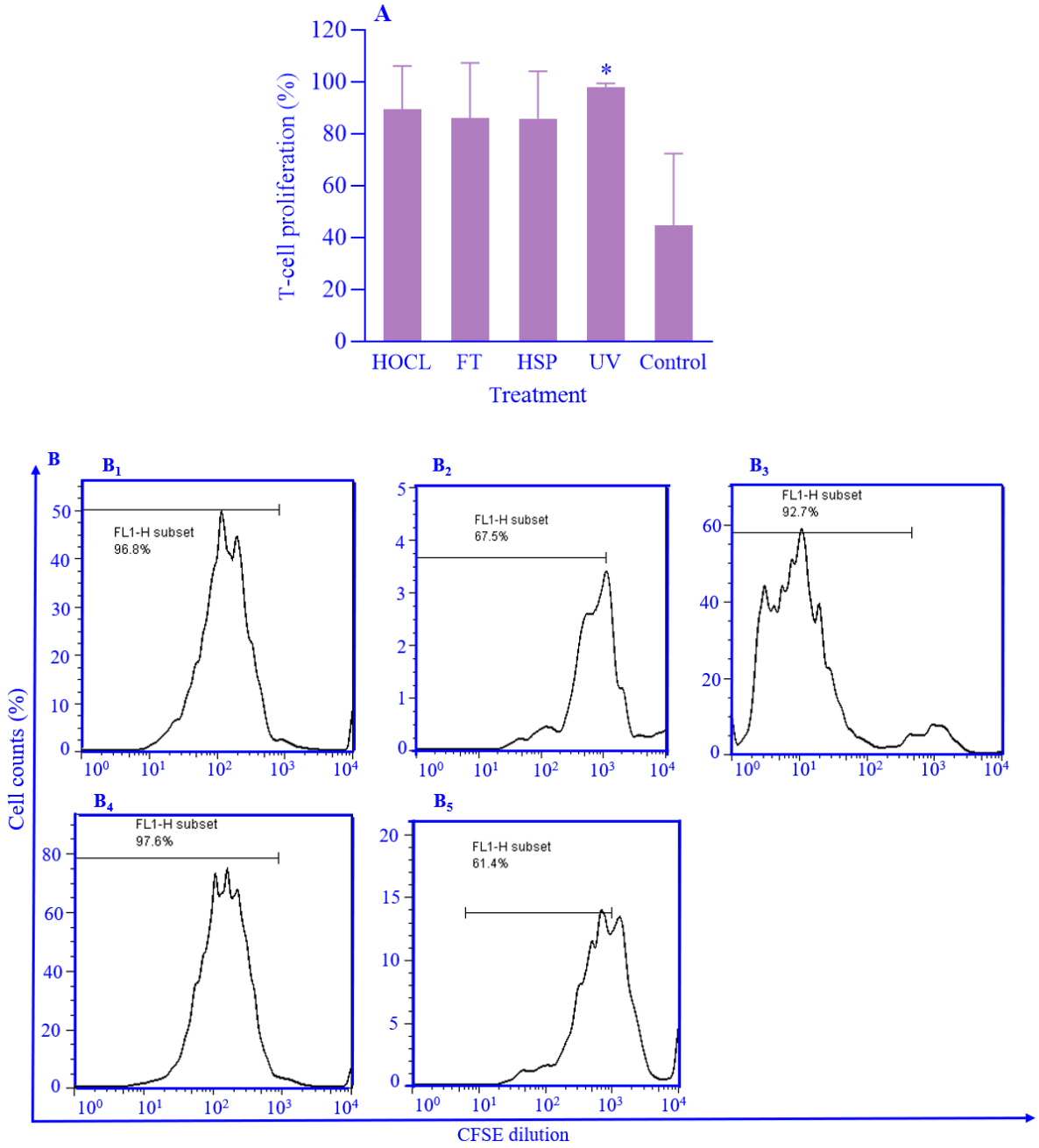

Fig. 2. Proliferation of antigen-specific $CD8^+$ T cells. (A) The percentage of cell proliferation of CFSE-labeled T cells co-cultured with DCs plus different tumor cell lysates; (B) representative FACS histograms demonstrating CFSE dilution within proliferating T cells. (B1) FT; (B2) HOCL; (B3) UV; (B4) HSP; (B5) control. The control group was considered antigen-unloaded DCs. Data were expressed as mean ± SD, n = 3. $^*P < 0.05$ demonstrates a significant difference compared with control. CFSE, Carboxyfluorescein succinimidyl ester; FT, freeze-thaw; HOCL, hypochlorous acid; UV, ultraviolet; HSP, hyperthermia; DCs, dendritic cells.

In vitro cytokine production

The levels of IFN-γ and IL-10 were measured in supernatants collected from DCs pulsed with TCLs prepared by different methods, as well as from T cells co-cultured with DCs loaded with TCLs.

The level of IFN-γ in the supernatant of DCs treated with HOCL was significantly higher than that in the control and FT groups (Fig.3A). A decrease in IL-10 production was also observed in the supernatant of DCs treated with HOCL compared to the control group (Fig.3B).

As depicted in Fig 4A, IFN-γ secretion was significantly increased in T-cells co-cultured with DCs pulsed with HOCL compared to control and DCs pulsed with UV. In contrast, IL-10 was significantly decreased in DCs pulsed with HOCL following tumor lysate engulfment (Fig 4B). The interaction between DCs and T cells resulted in a significant decrease in IL-10 production in the supernatant of DCs treated with HOCL compared to the control group (Fig. 4B).

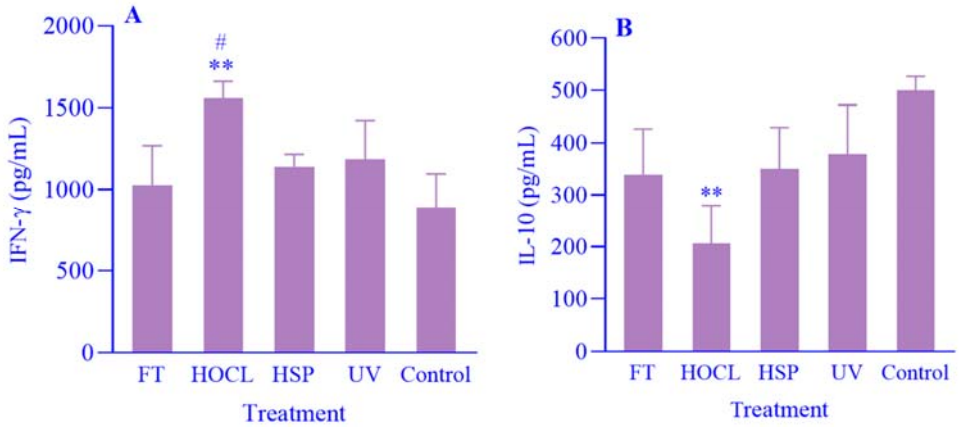

Fig. 3. *In vitro* cytokine production in the supernatant of DCs pulsed with TCLs prepared by different methods. (A) IFN-γ and (B) IL-10 concentrations in the supernatant of DCs were measured by ELISA. The control group was considered antigen-unloaded DCs. Data were expressed as mean ± SD, n = 3. $^{**}P < 0.01$ demonstrates a significant difference compared with the control; $^{\#}P < 0.05$ versus FT. FT, Freeze-thaw; HOCL, hypochlorous acid; UV, ultraviolet; HSP, hyperthermia; DCs, dendritic cells; TCLs, tumor cell lysates; IFN-γ, interferon-gamma; IL-10, interleukin-10.

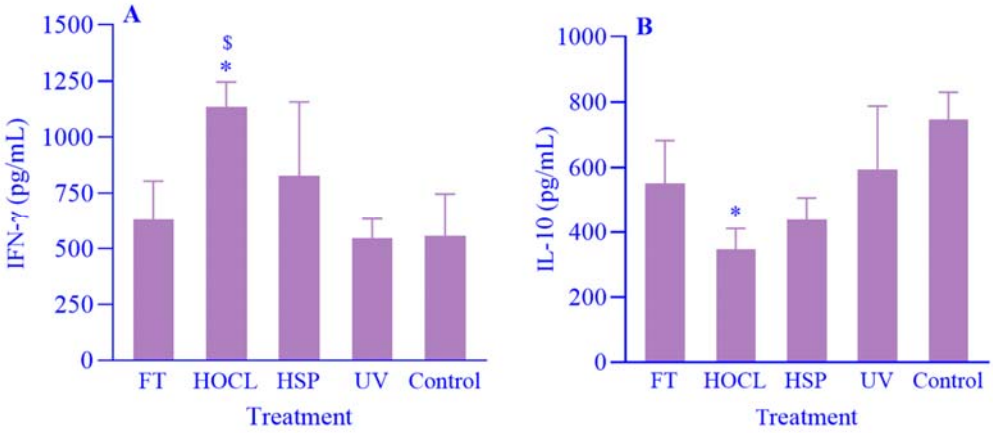

Fig. 4. *In vitro* cytokine production in the supernatant of syngeneic naive T-cells co-cultured with DCs pulsed with tumor cell lysates prepared by different methods. (A) IFN-γ and (B) IL-10 concentrations in the supernatant of T-cell/DCs were measured by ELISA. The control group was considered antigen-unloaded DCs. Data were expressed as mean ± SD, n = 3. $^{*}P < 0.05$ demonstrates a significant difference compared with control; $^{\$}P < 0.05$ versus UV. FT, Freeze-thaw; HOCL, hypochlorous acid; UV, ultraviolet; HSP, hyperthermia; DCs, dendritic cells; TCLs, tumor cell lysates; IFN-γ, interferon-gamma; IL-10, interleukin-10.

DISCUSSION

DCs are the most powerful and professional APCs that stimulate the immune response. In this study, DCs were successfully generated from bone marrow-derived MNCs *ex vivo*. To completely mature the DCs, the loaded cells were subsequently stimulated with TCLs and LPS.

As part of the main objective, the different preparation methods of TCLs were used to induce cell death in 4T1 mouse breast cancer cell lines and create a collection of TAAs capable of activating DCs. This study evaluated whether this priming process would enhance antigen presentation, processing, and cellular immune response. The novelty of the study lied in its comprehensive comparison of different preparation methods of TCLs for breast cancer cells (4T1 as a model of metastatic breast cancer) and their effects on the maturation of DCs and antigen presentation. The current study focused on evaluating the levels of MHC molecules, co-stimulatory molecules, and the levels of IFN-γ and IL-10 secretion for understanding the balance between pro-inflammatory and anti-inflammatory responses in the immune system. The findings demonstrated that DCs pulsed with HOCl-treated tumor cell lysates induced a pro-inflammatory response and enhanced the activation of $CD8^+$ T cells and TH type 1 cells while reducing the anti-inflammatory response compared to other methods.

The maturation of DCs is characterized by their high expression levels of MHC molecules and co-stimulatory molecules, including CD80, CD83, and CD86. These molecules are essential for stabilizing the interaction between DCs and naive T lymphocytes (13). Additionally, mature DCs release pro-inflammatory cytokines such as IFN-γ and IL-12, which activate the Th1 response and initiate CTL activation (14). In the present study, CD86 expression increased in DCs pulsed with tumor lysates prepared by HOCl in comparison to FT and control groups as a result of enhanced antigen presentation, activation of pattern recognition receptors, release of immunostimulatory factors, and oxidative stress (15). In response to oxidative stress generated by HOCl, tumor antigens may be better released and exposed, improving antigen presentation and increasing CD86 expression.

The oxidative stress produced by HOCl can lead to increased necrotic tumor cell death, which can enhance the release and exposure of tumor antigens, leading to improved antigen presentation and CD86 expression. While, irradiated tumor cells that showed approximately 54% CD86 expression, may inhibit the maturation of DCs by downregulation of MHC II and impairing the integrity and function of proteins in the lysate (16).

A study by Roufarshbaf *et al*. compared 4 preparation methods of TCLs and found that HOCl preparation resulted in DCs with higher surface expression of maturation biomarkers and increased levels of IFN-γ production (11). Consistent with these findings, other studies have shown that the co-culture of T cells with APCs loaded with HOCl-treated model antigens increased the activation of T cells and the recognition of antigens (17-20). However, the effect of different preparation methods of TCLs on T cell activation (*i.e.,* proliferation and cytokine release) was not evaluated. The present study evaluated the preparation effect of TCLs on DCs and $CD8^+$ T cells. However, the effects of other immune cells, such as macrophages and regulatory T cells on the adaptive immune response could be further explored to provide a complete understanding of the immune response. In this regard, Zhou *et al*. demonstrated that the maturation of DCs was significantly enhanced by cell-derived secretions from HOCl-treated tumors, and macrophages were polarized towards an M1 phenotype as a result (21). Chiang *et al*. reported that HOCl oxidation increased the expression of biomarkers (*i.e.* CD83, CD86, and CD40), improved the immunogenicity of the ovarian TCLs by DCs, and enhanced T cell responses against the tumor (22). Co-stimulatory molecules, such as CD86 generally considered to be biomarkers of mature DCs are crucial for T cell proliferation (8).

In the present study, DCs-UV exhibited higher levels of T-cell proliferation after 72 h than T cells co-cultured with DCs alone (no antigen), as a control group. In contrast,

DCs-FT reduced the T cell proliferation capacity of loaded DCs followed by the engulfment of tumor lysate. Previous studies reported that soluble factors produced by the tumor cells may participate in the attenuation of the allostimulatory function of DCs. These factors include transforming growth factor β and other tumor-derived lipids without affecting the MHC II phenotypic expression in DCs (23). DCs exposed to LPS and TCLs had higher levels of T cell proliferation compared to the control group. LPS-treated DCs can activate T cells to a broader extent than DCs (no LPS), because LPS enhances antigen presentation, boosts pro-inflammatory cytokine production, and improves immune stimulation, antigen uptake, and processing (24).

IFN-γ and IL-10 are cytokines that play critical roles in the immune system, particularly in the functioning of DCs and T-cells. The balance between IFN-γ and IL-10 signaling in the co-culture of mature DCs and naive T-cells plays a crucial role in shaping the adaptive immune response (25). In this study, the secretion levels of IFN-γ, a pro-inflammatory cytokine important in $CD8^+$ T cells and $CD4^+$ TH type 1, and IL-10, an anti-inflammatory cytokine, were evaluated. Consistent with phenotype characterization, the supernatant of DCs pulsed with HOCl co-cultured with $CD8^+$ T cells showed lower IL-10 and higher IFN- γ levels compared to other groups. DCs pulsed with FT showed higher levels of IL-10 and lower levels of IFN-γ compared to other treatments. The low immunogenicity of the FT method can be explained by its ability to preserve native conformation, minimize denaturation and aggregation, and reduce stress-induced protein modifications, making it less likely to expose epitopes and prevent these antigens from being processed and presented (26). In agreement with Chiang's findings, the current results indicated that DCs pulsed with HOCl tumor lysate expressed cross-present immunodominant epitopes when exposed to a mixture of whole tumor lysate and activated $CD8^+$ T cells (27).

In this study, the method of hyperthermia and UV, which increased the expression of MHC I, and as a result, improved recognition by $CD8^+$ lymphocytes, had an acceptable and appropriate function.

The choice of cell lysate formulation for loading DCs with antigens for cross-presentation depends on the specific experimental or clinical context. Factors such as antigen stability, processing pathways, and the desired immune response need to be considered. Optimization of these formulations can lead to improved cross-presentation efficiency, resulting in enhanced T-cell activation and better immune responses.

This research provided valuable insights into the potential use of HOCl-treated tumor cell lysates as a novel method for enhancing the anti-tumor immune response in breast cancer therapy. By modulating the balance between pro-inflammatory and anti-inflammatory responses in the immune system, this approach has the potential to improve the efficacy of immunotherapy for metastatic breast cancer. The findings of the study had significant implications for the development of new strategies for cancer treatment, highlighting the importance of considering the effects of different tumor cell lysate preparation methods on the maturation of DCs and antigen presentation. Overall, comparing different preparation methods of TCLs and evaluating their effects on the maturation of DCs and immune responses provided valuable insights into enhancing anti-tumor immunity. By further elucidating the mechanisms underlying these effects and addressing the weaknesses mentioned, the study had the potential to make a significant contribution to the field of cancer immunotherapy.

CONCLUSION

Overall, it appears that HOCl could effectively release tumor antigens while maintaining immunogenicity. Additionally, HOCl treatment enhanced the maturation of DCs, which are responsible for activating tumor-specific immune responses. The fact that the DCs promoted TH type 1 immune responses and the release of cytokines like IFN-γ suggests that they may contribute to the stimulation of a robust anti-tumor immune response. Furthermore, the low levels of IL-10 produced by the DCs suggest that they may not contribute to tumor growth or immunosuppression. This is a crucial consideration when developing

effective cancer immunotherapies. The strengths of the study lie in its comprehensive comparison of different preparation methods of TCLs and their effects on the maturation of DCs and antigen presentation. However, the effects of other immune cells, such as macrophages and regulatory T cells on the adaptive immune response could be further explored to provide a complete understanding of the immune response. However, further research is needed to optimize the preparation methods and evaluate the clinical effectiveness of DCs-based immunotherapies using HOCl. Future studies should focus on investigating the impact of activated T cells on breast cancer therapy, both *in vitro* and *in vivo*. Animal models and clinical trials should be utilized to assess the safety and efficacy of this approach in human patients. Additionally, understanding the specific mechanisms underlying T cell-mediated antitumor effects will be crucial for optimizing treatment strategies and improving patient outcomes.

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Conflict of interest statement

The authors declared no conflict of interest in this work.

Authors' contributions

V. Akabri, N. Esmaeil, M. Abbaspour, M. Rezazadeh, and M. Minaiyan contributed to the conceptualization, methodology and investigation; M. Abbaspour wrote the first draft of the manuscript; V. Akbari was responsible for the resources. All authors revised the manuscript and approved the final manuscript as submitted and agree to be accountable for all aspects of the work.

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