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Original Article

Comparison of four methods of colon cancer cell lysates preparation for *ex vivo* maturation of dendritic cells

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Abstract

Background and purpose: One of the most effective methods for the development of dendritic cell (DC)-based cancer immunotherapy is *ex vivo* pulsing of DCs with tumor cell lysates (TCLs). However, antitumor immune responses of DCs are significantly influenced by how TCLs were prepared. Here, we compared four strategies of TCL preparation derived from colon cancer cells, HT-29, for *ex vivo* maturation of DCs.
Experimental approach: Peripheral blood monocytes were isolated from healthy volunteers and incubated with granulocyte macrophage colony-stimulating factor and interleukin (IL)-4 to differentiate into DCs in 10 days. Morphological properties, phenotype characteristics (i.e. CD83 and CD86), and cytokine production (i.e. IL-10 and interferon gamma) of DCs loaded with four different TCLs (i.e. freeze-thaw, hypochlorous acid (HOCl), hyperthermia, and UV irradiation) were evaluated.
Findings/Results: HOCl preparations led to the generation of DCs with higher surface expression of maturation biomarkers (particularly CD83), while UV preparations resulted in DCs with lower levels of surface biomarkers compared to freeze-thawed preparations. The supernatant of DCs pulsed with HOCl preparation showed significantly higher levels of interferon gamma and lower levels of IL-10 compared with the other groups.
Conclusion and implications: Our results suggest that pulsing DCs with HOCl preparation may be superior to other TCLs preparation strategies, possibly due to induction of rapid necrotic cell death.

Keywords: Antigen loading; Colorectal cancer; Dendritic cell; Hypochlorous acid, Tumor cell lysate.

INTRODUCTION

Recent advances in the early detection and treatment of colorectal cancer (CRC) have improved the 5-year survival rate of patients, particularly in developed countries. However, CRC is still among the five most common cancers in both genders and the fourth cause of cancer-related deaths worldwide (1-3). Surgical resection increases 5-year survival by 90%, but it only helps patients with disease localized to the mucosa. Non-surgical treatments such as chemotherapy are approved for regionally metastatic colorectal cancer but have modest or no efficacy against distant metastases. Moreover, these treatments have limitations due to their side effects (4). Therefore, more effective treatment strategies for patients with metastatic CRC should be developed.

Cancer immunotherapy is a novel treatment approach that utilizes specific and amplified patients' innate and adaptive immune responses to detect and remove tumor cells; thus, it could be a more efficacious treatment strategy for metastatic cancers (5).

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There are several immunotherapeutic approaches under development and investigation for patients with CRC, such as peptide vaccines, whole tumor cell vaccines, viral vector-based cancer vaccines, cytokine therapy, antibody-based immunotherapies, and dendritic cell-based cancer vaccines (6,7).

Dendritic cells (DCs) have a critical role in presenting tumor immunogenic peptides to T cells and activating them. According to many studies, the DC-based vaccine is one of the safe cancer immunotherapy strategies. Two main types of tumor antigens are used in DC-based vaccines, including defined antigens (*e.g.,* peptide and DNA) and undefined antigens (*e.g.* tumor cell lysate (TCL), intact tumor cells, and total tumor RNA). Unlike the former type of vaccine, which is usually restricted to patients bearing the major histocompatibility complex (MHC) class I restricted tumor-associated antigens that fail to elicit $CD4^+$ T cell immune response, the latter type induces both $CD4^+$ and $CD8^+$ T cell responses as it is not restricted to a certain MHC class of tumor antigens, and as a result diminishes the potential for immune escape due to antigen loss (8).

One of the most effective methods of loading DCs with tumor antigens is pulsing them with TCLs *ex vivo* (4). TCLs are ordinarily prepared by subjecting tumor cells to freeze and thaw cycles, although DCs pulsed with such TCLs have shown modest *in vivo* efficacy and even freeze-thawed TCLs sometimes suppress DC maturation and activation (8). Several studies have attempted to increase the immunogenicity of TCLs by using different TCL preparation strategies, such as subjecting tumor cells to hyperthermia, UV-irradiation (8), and hypochlorous acid (HOCl) to overcome immunosuppressive mechanisms within the tumor microenvironment (9). Hyperthermia is a rapid and simple method of TCL preparation. It was reported that subjecting cancer cells to hyperthermia compared with irradiation was more effective for pulsing DC and provided stronger tumor-specific T cell responses against laryngeal cancer probably through enhancing heat shock protein expression, which might improve tumor-associated antigen delivery to DCs (8). Another research group reported that pulsing DCs with irradiated tumor cells exhibited greater *in vivo* protection against melanoma than other strategies (*i.e.,* freeze-thawed or boiled TCLs), likely due to a different mechanism of tumor cell death (10). They concluded that irritated TCL-loading strategy might have superior efficacy for developing a DC-based vaccine for solid malignancies. Chiang *et al*. reported that HOCl-oxidation improved immunogenicity and uptake of ovarian TCLs by DCs and enhanced antitumor T cell responses, probably *via* providing better exposure of tumor antigens (9). However, the concentration of HOCl must be optimized for each tumor strain to improve the maturation and stimulation of DCs (9). Since different types of tumor cells have various immunogenicities, the optimum TCL preparation strategy must be experimentally determined for each malignancy. There are some reports on the impact of TCL preparation strategy on the maturation and activation of DCs. For instance, Chiang *et al.* compared the effectiveness of three different TCL preparation strategies (*i.e.,* freeze-thaw, HOCl-oxidation, and irradiation) for the maturation of DCs in the induction of *ex vivo* and *in vivo* immune responses against ovarian cancer. They found that pulsing DCs with HOCl-oxidized TCL was more efficient for eliciting antitumor T cell response and *in vivo* tumor suppression (11). However, to the best of our knowledge, the effect of colon cancer cells lysate preparation strategies on DCs differentiation and activation has not been previously evaluated. The aim of this study was to compare four different strategies of TCL preparations derived from colon cancer cells, HT-29, for *ex vivo* maturation of DCs. We monitored the immunogenicity of TCL preparations using morphological changes, level of cytokines secretion, and surface expression of maturation markers of DCs *in vitro*.

MATERIALS AND METHODS

Cancer cell line

HT-29 colon cancer cells were provided by the Pasteur Institute of Iran. Cells were cultured in Dulbecco's modified eagle medium (DMEM) high glucose (Bioidea, Iran) supplemented with 10% fetal bovine serum (FBS, Bioidea, Iran),

100 U/mL penicillin, and 100 µg/mL streptomycin (Bioidea, Iran) and were maintained in a humidified incubator at 37 °C with 5% CO_2.

Preparation of tumor cell lysates

The HT-29 colon cancer cells were harvested, washed, and re-suspended at 2×10^6 cells/mL in phosphate-buffered saline (PBS). These cells were divided into four flasks to be treated with four different strategies, including UV irradiation, HOCl treatment, hyperthermia, and freeze-thaw approach. In the UV irradiation method, HT-29 cells were washed and re-suspended in complete DMEM-high glucose (*i.e.*, supplemented with FBS and antibiotics), the cells were plated in 10 cm Petri dishes and subjected to a 254 nm UVC-irradiation for 10 min to induce apoptosis. The cells were incubated overnight at 37 °C, 5% CO_2, and harvested on the following day for the freeze-thaw procedure (see below) (12). 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 50 µM and was immediately added to HT-29 cells. The cells were maintained in the incubator at 37 °C, 5% CO_2 for 1 h, and were agitated gently every 30 min to induce oxidation-dependent cancer cell death. The HOCl-treated cells were centrifuged at 600 *g* for 6 min and washed twice with PBS and suspended in PBS for the freeze-thaw procedure (see below) (9,13). In the hyperthermia approach, HT-29 cells were heat-treated at 42 °C for 1 h in a thermostatically controlled water bath and then were imposed to freeze-thaw procedure (see below) (8). Freeze-thaw strategy, besides cancer cells that were treated only with the freeze-thaw strategy, all cells treated with mentioned strategies were subjected to 6 cycles of freeze (freezing in liquid nitrogen for 5 min) and thaw (quick thawing at 37 °C) (13).

For all strategies, the lysates were subjected to trypan blue staining to confirm the absence of viable cancer cells under an optical microscope. The percentage of cell viability was calculated by dividing the number of uncolored cells by the total number of cells (0% viability was acceptable) (14). The whole TCLs were centrifuged (3500 *g* for 5 min at 25 °C); cell debris was removed, and the supernatants were collected (15), and the total protein content of the supernatant was determined by Bradford assay (16). The aliquots of supernatants were kept at -80 °C until used.

Ethics statements

Peripheral blood samples were taken from healthy (aged less than 40 years) people who were referred to Isfahan Phlebotomy Center Human after obtaining informed consent. Donors were informed that their blood would be used for research purposes and consented to their blood procurement. All stages of the study were approved by the Ethics Committee of Isfahan University of Medical Sciences (Ethics No. IR.MUI.REC.1396.3.385).

Generation of monocyte-derived dendritic cell

Peripheral blood mononuclear cells (PBMCs) were isolated from peripheral blood of donors by centrifugation with Ficoll density gradient media and were plated at a concentration of 7.7 × 106 cells/mL in T25 flasks containing complete Roswell Park Memorial Institute (RPMI) 1640 medium (Bioidea, Iran). After 2 h of incubation at 37 °C and 5% CO_2, non-adherent cells were gently removed from the flasks (9). The adherent cells (monocytes) were cultured in a complete RPMI medium containing 50 ng/mL of granulocyte macrophage colony-stimulating factor (GM-CSF; R & D, USA) and 20 ng/mL of interleukin (IL)-4 (InvivoGen, USA). The medium was replaced on days 3, 5, and 8, and GM-CSF and IL-4 were added at 25 and 10 ng/mL on mentioned days, respectively. The purity of the adherent cells was assessed using anti-CD14-fluorescein isothiocyanate (FITC) antibodies (Abcam, USA) on the second day.

Pulsing DCs with TCLs

On day 8, immature DCs (3×10^6) were pulsed with TCLs containing 100 µg/mL of protein, which were produced using different strategies, according to the previous study (17). TCL at equivalent protein concentrations was used for DC pulsing to determine if TCLs preparation strategy influences the immunogenicity of the obtained lysate. On day 9,

lipopolysaccharide (LPS; InvitroGen, USA) was added to the flasks (except for negative control) at 2.5 µg/mL.

Characterization of pulsed DCs

The cells were daily observed under the microscope to evaluate their morphological properties. On day 10, DCs were isolated from the medium using trypsin-ethylenediaminetetraacetic acid (EDTA) (0.25%) and then collected by centrifugation (2500 rpm). The phenotype of DCs was determined using anti-CD83-PE (InvitroGen, USA) and anti-CD86-PE/CY5 (Abcam, USA). Appropriate human IgG isotype controls (*i.e.,* IgG2b, kappa (PE/CY5), and IgG1, kappa (PE)) were used to determine the levels of background staining. Approximately 10^4 cells per sample were analyzed using a Becton Dickinson FACS Calibur (USA) flow cytometer. On day 10, the media supernatant of DCs was collected and stored at -20 °C for further analysis. To evaluate the cytokine production by mature DCs, the level of cytokines in the supernatants was assessed by Human IL-10 ELISA development kit (PeproTech, UK) and Human interferon (IFN)-γ ELISA development kit (PeproTech, UK) as described by the manufacturer.

Statistical analysis

Each experiment was repeated at least three times to ensure the reproducibility of the results. Data are expressed as means ± standard deviation (SD). The data were analyzed using Graph Pad Prism 8 (La Jolla, CA, USA). One-way analysis of variance (ANOVA) and post hoc tests were used to make a comparison between DCs pulsed with different TCL preparation strategies. A P value < 0.05 was considered as a statistically significant difference.

RESULTS

Tumor cell lysates preparation

The amount of protein in TCLs was determined by the Bradford assay. No significant differences were observed in terms of protein content between four different TCL preparation strategies (Fig. 1). The protein content of TCL prepared by hypothermia was insignificantly higher than other groups (P > 0.05).

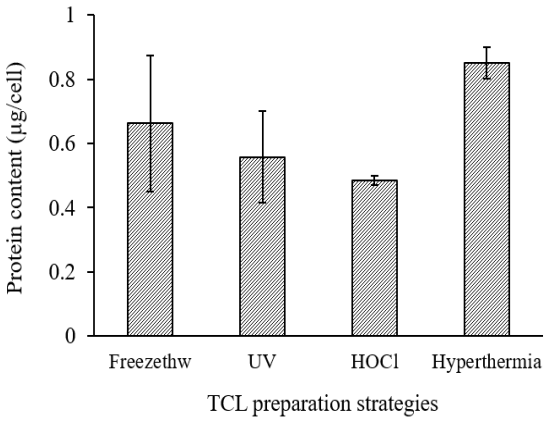

Fig. 1. Protein content of lysate prepared by four different TCL preparations strategies. Data are presented as µg of proteins per tumor cell (mean ± SD, n = 3). TCL, Tumor cell lysates.

Isolation of monocytes and generation of dendritic cells

Our findings showed that upon using the plastic adherence method, 6.2×10^5 cells/mL of adherent cells were obtained from 5.6×10^6 cells/mL of starting PBMCs. To evaluate the purity of the adherent cells (*e.g.*, monocyte), on the second day, cells were harvested, stained, and analyzed by flow cytometry for the expression of CD14. According to flow cytometric analysis, more than 93% of cells were $CD14^+$ positive. The high purity of $CD14^+$ cells suggests that it is possible to isolate high-purity $CD14^+$ cells only by using the adhesive feature of these cells, without using microbeads. DCs generated using 10-day protocol were $(1.5 \pm 0.5) \times 10^5$, representing a yield (%) of 2.7 ± 0.9 for PBMC or 23.1 ± 7 for $CD14^+$ cells.

Pulsing dendritic cells with Tumor cell lysates

Morphological characterization

The morphological properties of the cells were observed under a light microscope during the experiment period. On day 1, cells were round, spherical, and adherent to the surface of the flask (a typical feature of monocytes) (Fig. 2). After 7 days of treatment with GM-CSF and IL-4, the majority of cells formed clusters, which are displayed by typical dendritic cells (Fig. 2).

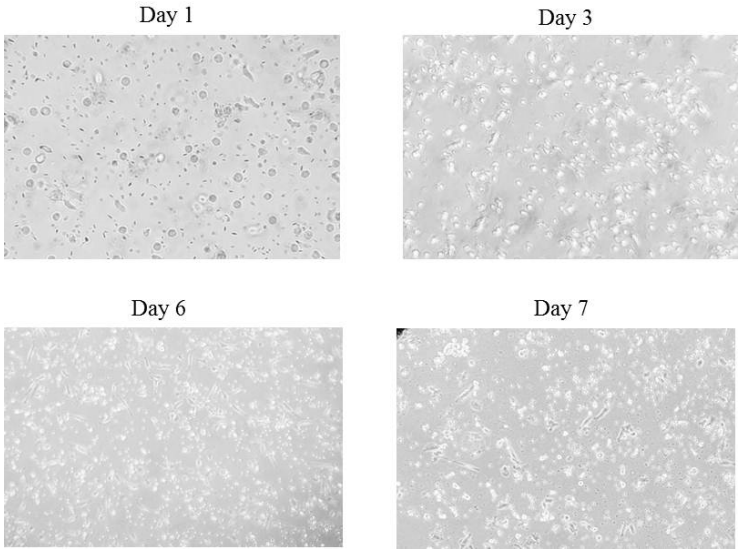

Fig. 2. Morphologic characteristic of the monocyte-derived dendritic cell on days 1, 3, 6, and 7. Cells were isolated by adherence method and co-culture with interleukin 4 and granulocyte macrophage colony-stimulating factor and then analyzed by inverted microscope. Magnification: × 200.

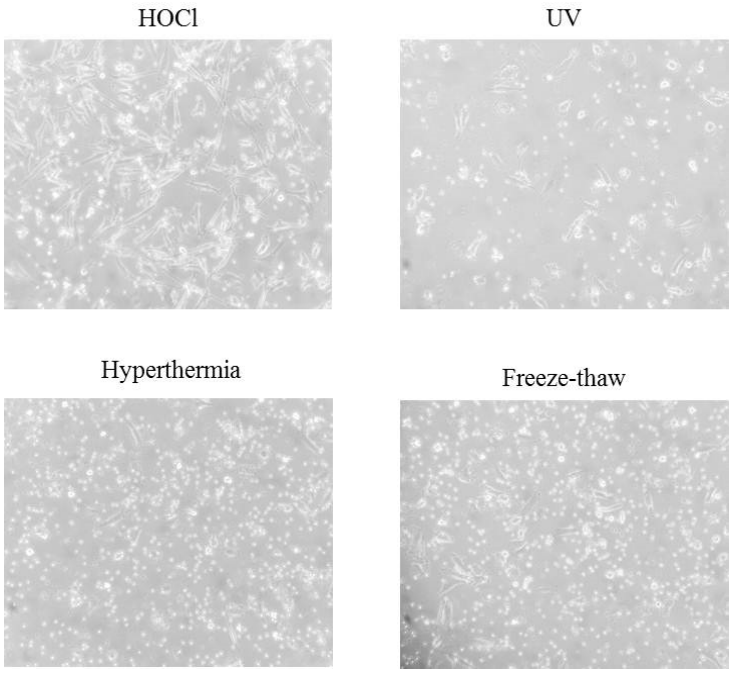

Fig. 3. Morphologic characteristics of dendritic cells pulsed with different tumor cell lysates preparations. Dendritic cells were analyzed by an inverted microscope on day 10. Magnification: × 200.

The effect of TCLs preparation strategies on the DC morphological changes was also evaluated (Fig. 3). On day 10, the majority of DCs pulsed with HOCl-treated TCLs exhibited cytoplasmic projections, many small dendrites, or multiple elongated dendrites, a typical appearance of mature DCs. Additionally, they increased in number and size, which is a characteristic feature of maturation.

Surface markers expression

The evaluation of phenotypic biomarkers of DCs maturation (*i.e.* CD83 and CD86) was performed by flow cytometry. The expression profile of the biomarkers by DCs on day 10 was demonstrated in Fig. 4. Compared with the control group, the expression of CD83 insignificantly increased in DC groups pulsed with HOCl, freeze-thaw, or hyperthermia-treated TCLs. There was no significant difference in the expression of CD86 in DCs groups pulsed with different TCLs preparation strategies. The DCs pulsed with UV-treated TCLs expressed the lowest levels of CD83 and CD86 compared with the control.

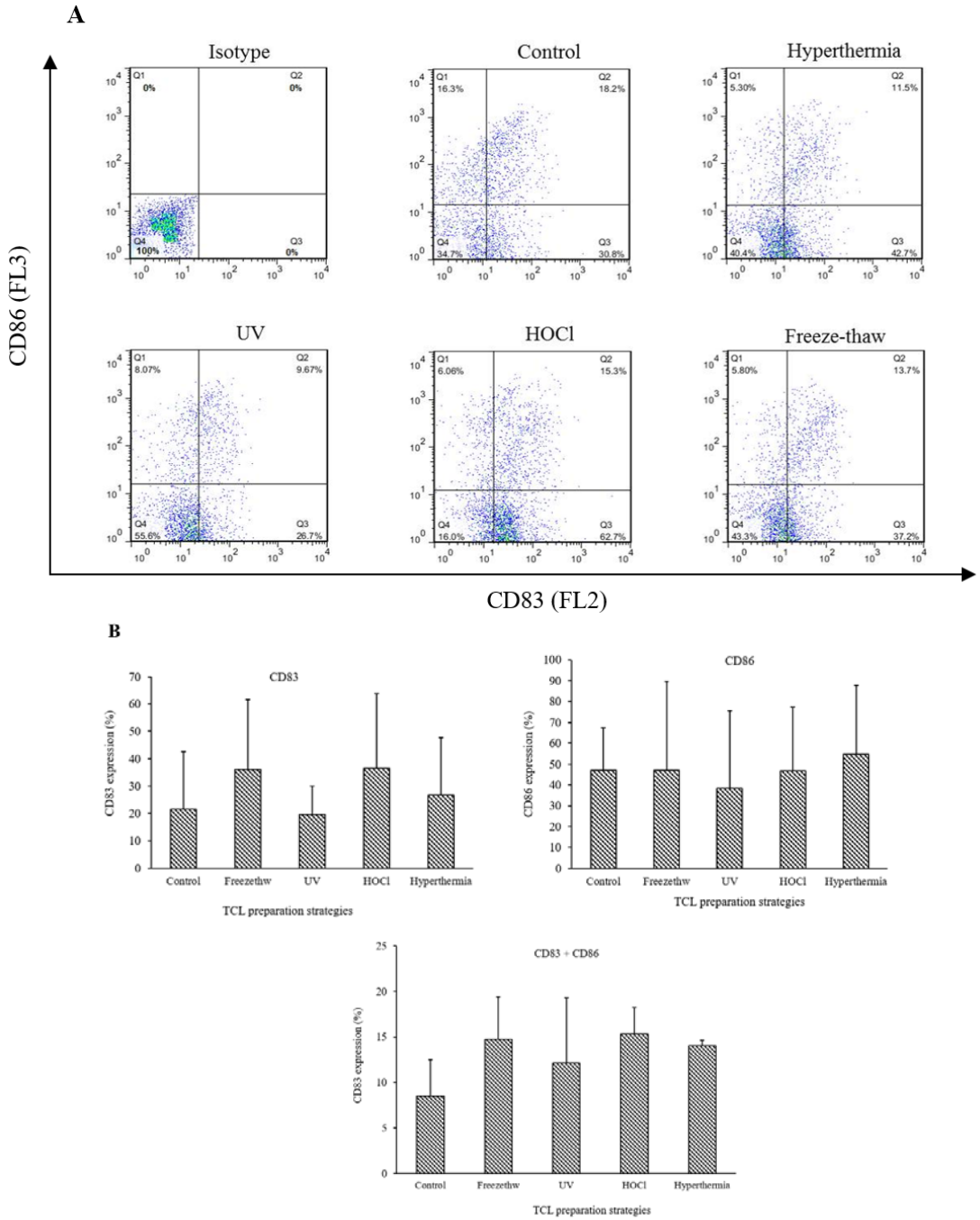

Fig. 4. Surface markers expression. Dendritic cells pulsed with different TCL preparations were harvested, stained for CD83 and CD86, and analyzed by flow cytometry. (A) Representative flow cytometry profile (one of three experiments); (B) the percentage of stained cells with CD83 and CD86 for different TCL preparation strategies. Data are expressed as mean ± SD, n = 3. TCL, Tumor cell lysates.

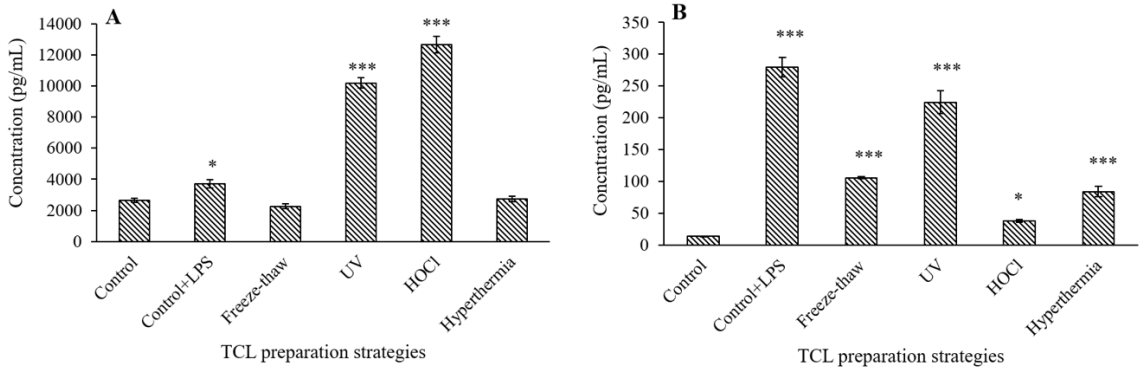

Fig. 5. Secretion of cytokines. The concentration of (A) interferon γ and (B) interleukin 10 were assessed in the supernatants of dendritic cells on day 10 by ELISA methods. Data are expressed as mean ± SD, n = 3. **P* ≤ 0.05 and ****P* ≤ 0.001 indicate significant differences compared with the control group. LPS, Lipopolysaccharide.

Secretion of cytokines

The secretory level of IFN-γ and IL-10 were assessed in the supernatants of DCs on day 10. As the data (Fig. 5) suggest, the levels of IFN-γ (as an activating cytokine) in the supernatant of DCs pulsed with HOCl or UV-treated TCLs were significantly higher than the level of the other groups. The level of IL-10 (as an inhibitory cytokine) was significantly lower in the supernatant of DCs pulsed with HOCl-treated TCLs. However, unlike the HOCl group, DCs pulsed with UV-treated TCLs showed a significantly higher level of IL-10 compared with other groups.

DISCUSSION

Nowadays, targeting different types of advanced and metastatic cancers is possible due to the recent improvements in DC-based vaccines (18). However, further studies are still required to improve the efficacy of DC vaccines and overcome DC processing limitations.

The most well-established DCs generation approach for cancer immunotherapy is a standard 7-day protocol consisting of differentiation of monocytes into immature DCs (*i.e.* culturing monocytes in the presence of GM-CSF and IL-4, which usually takes six days and then pulsing immature DCs with tumor antigens for 18-24 h) (19). In the present study, we extended the length of the DC generation process to 10 days (*i.e.,* monocytes were cultured in the presence of cytokines for 7 days to completely differentiate into immature DCs, then on day 8 loaded with TCLs and on day 9 treated with LPS) and maintained the cells in the same flask during the whole process. We observed remarkably higher CD83 and CD86 expressions in DCs generated based on the 10-day protocol compared to the DCs obtained according to the 7-day protocol (data not shown). In agreement with our findings, Chiang *et al.* reported that DC culture duration could influence expression surface biomarkers and secretion of cytokine by mature DCs (12). Accordingly, the length of DC generation must be experimentally optimized for each case. Furthermore, the manipulation steps of the procedure can affect the viability, yield, and activity of the resulting DCs (20). The ten-day protocol developed in this work involves fewer manipulation steps (*e.g.,* harvesting, centrifugation, and re-suspending) compared with the classic protocol making it less susceptible to cell losses.

Here, we used DC maturation in the presence of LPS. Both TNF-α and LPS are widely used as a single agent for the *ex vivo* maturation of DCs (21). However, DCs maturation with LPS resulted in the generation of polarized type-1 DCs responses, particularly when combined with INF-λ (22). Previous studies showed that type-1-polarization of DCs led to enhanced anti-tumor T cell responses *in vitro* and improved *in vivo* efficacy of DC-based vaccines (22).

Another step of DC processing that profoundly influences the efficacy of the vaccine is loading DC with tumor antigens.

Different antigen-loading strategies have been used for DC-based vaccination, including pulsing DC with TCLs, which provides the patient's library of tumor antigens. Therefore, DC loading with autologous TCLs can be more effectively applied for immunotherapy of heterogeneous malignancies that have few defined tumor-specific antigens, such as colon cancer (23). Furthermore, personalized immunotherapy is becoming more important in the treatment of CRC, which is mostly diagnosed in the advanced stages (*i.e.* III and IV) and may not respond to routine cancer treatment (*e.g.* chemotherapy and surgery). Here, to prepare TCLs, we used the HT-29 cells, which are widely used as an *in vitro* model of colon cancer. HT-29 is a human colorectal adenocarcinoma cell line derived from a primary tumor with metastases to regional lymph nodes (*i.e.*, Ducks' C) and harbors a mutation in p53, BRAF, and PI3K, which can lead to an increase in chemotherapy resistance (24). The approach reported in this study can be applied in TCLs preparation for other cell lines with similar features.

Our finding showed that there was no significant difference in terms of the total amount of protein lysate between four different TCL preparation strategies. In agreement with our results, Callmann *et al.* reported that the total protein of HOCl-oxidized tumor lysate was similar to that of lysate collected from freeze-thawed tumor cells, although larger protein bands appearing in the non-oxidized sample (25). It should be noticed that TCL preparation strategies such as hyperthermia, HOCl-oxidation, or irradiation may impact on the protein population of the lysate by changing expression, stability, and folding of cancer cell proteins; therefore, these strategies can influence antigenicity and immunogenicity of TCLs.

In the present study, we compared the effectiveness of four different strategies of TCL preparation derived from colon cancer in terms of *in vitro* DC maturation. The basic procedure for all four strategies was repetitive freeze-thaw cycles, which is the most common TCLs preparation method and is widely applied for clinical purposes. Some studies reported that freeze-thawed TCLs could suppress *in vitro* maturation and function of DCs and even inhibit *in vivo* antitumor responses (26). Here, HOCl preparations led to the generation of DCs with higher surface expression of maturation biomarkers (particularly CD83), while UV preparations resulted in DCs with a lower level of surface biomarkers compared to freeze-thawed preparations. However, there is no statistically significant difference between groups in terms of the expression of biomarkers. The higher expression of CD83 in DCs pulsed with HOCl preparation compared with other preparations (*e.g.,* UV and freeze-thaw) has also been observed in some other studies. Chiang *et al.* (9) reported that HOCl-oxidized ovarian tumor cells resulted in higher expression of biomarkers (*i.e.* CD83, CD86, and CD40), which was directly dependent on concentrations of HOCl. It was proposed that rapid induction of necrosis by HOCl oxidation leads to enhancement of immunogenicity (*i.e.,* improvement of antigens uptake and processing by DCs), probably through unfolding and exposing tumor antigens and heat shock proteins (27). Expression of costimulatory molecules such as CD83 and CD86 are generally considered as biomarkers for mature DCs, and they are essential for induction of T-cell proliferation and activation by mature DCs. CD80 and CD83 molecules are upregulated in mature DCs, while CD86 expresses before these molecules in the early stage of maturation (28). Therefore, it can be inferred from our results that in HOCl and freeze-thaw preparations, most DCs go through the final stages of maturation. However, assessment of other maturation markers, *e.g.,* CD40, CD80, and HLA-DR, still are needed to confirm our results.

The secretory levels of IFN-γ, as an autocrine mediator for the maturation of DCs (29) and IL-10, as an inhibitory cytokine associated with tolerogenic DCs (*i.e.,* expressing lower of T-cell stimulating molecules), were also evaluated (30). Consistent with the results of phenotype characterization, the supernatant of DCs pulsed with HOCl preparation showed significantly higher levels of IFN-γ and lower levels of IL-10 compared with other groups. DCs pulsed with UV preparations secret higher levels of both IFN-γ and IL-10 compared with the

positive control. The lower immunogenicity of UV-irradiation compared with other strategies can be explained based on the fact that UV-irradiation produces a mixed population of apoptotic and necrotic tumor cells that leads to degradation of important antigens, and as a result, reduces the antigen processing or presentation (31).

CONCLUSIONS

In summary, the developed protocol in the present study is a simple and effective strategy for generating monocyte-derived dendritic cells using the plastic adherence method. Furthermore, *in vitro* comparison of the efficacy of four TCL preparation methods in terms of DCs phenotype and cytokine profile revealed that pulsing DCs with HOCl preparation may be superior to other TCLs preparation strategies, possibly due to induction of rapid necrotic cell death which leads to more efficiently activation of DCs and enhancement of T cell proliferation. These findings indicate the importance of characterization and optimization of TCLs preparations for the development of effective cancer vaccines for clinical applications. Future work will be focused on *in vitro* stimulation of T-cells primed by mature DCs and *in vivo* vaccination with mature DCs loaded with different TCL preparations.

Acknowledgments

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Conflict of interest statement

The authors declared no conflict of interest in this study.

Authors' contribution

V. Akbari was the principal investigator of the project; M. Roufarshbaf and N. Esmaeil planned for the project; M. Roufarshbaf and N. Esmaeil, and V. Akbari performed the required experiments; M. Roufarshbaf wrote the manuscript in consultation with V. Abari and N. Esmaeil. All authors discussed the results and contributed to and approved the final form of the manuscript.

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