ORIGINAL ARTICLE
Iran J Allergy Asthma Immunol
February 2024; 23(1):97-106.
DOI: 10.18502/ijaai.v23i1.14957

Exosomes Derived from Heat-shocked Tumor Cells Promote *In vitro* Maturation of Bone Marrow-derived Dendritic Cells

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Received: 9 March 2023; Received in revised form: 8 November 2023; Accepted: 24 November 2023

ABSTRACT

Dendritic cells (DCs), professional antigen-presenting cells that process and deliver antigens using MHC II/I molecules, can be enhanced in numerous ways. Exosomes derived from heat-shocked tumor cells (HS-TEXs) contain high amounts of heat-shock proteins (HSPs). HSPs, as chaperons, can induce DC maturation. This study aimed to investigate whether HS-TEXs can promote DC maturation.

To generate DC, bone marrow-derived cells were treated with Interleukin-4 and GM-CSF. Exosomes were isolated from heat-treated CT-26 cells. The expression level of HSP in exosomes was checked by western blot and the increase in the expression of this protein was observed. Then, HS-TEXs were co-cultured with iDCs to determine DC maturity, and then DCs were co-cultured with lymphocytes to determine DC activity.

Our results showed that DCs treated with HS-TEXs express high levels of molecules involved in DC maturation and function including MHCII, CD40, CD83, and CD86. HS-TEXs caused phenotypic and functional maturation of DCs. In addition, flow cytometric results reflected a higher proliferative response of lymphocytes in the iDC / Tex + HSP group.

HS-TEXs could be used as a strategy to improve DC maturation and activation.

Keywords: Dendritic cell; Exosome; Heat-shock protein; Tumor

INTRODUCTION

Dendritic cells (DCs) are licensed antigen-presenting cells (APCs) and have an unparalleled ability to detect and deliver antigens to other immune cells, especially naive T lymphocytes, and initiate the immune response.[1,2] DCs not only stimulates the proliferation of antigen-specific T cells but also secrete different cytokines for the polarization of T cells into different subgroups.[3] Scientists have used these cells to treat autoimmune diseases and cancers since they can deliver antigens and induce cell polarization.[4]

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Tumor cells-derived exosomes which are called texosomes (TEX), contain innate immune stimulatory molecules including HMGB1, surface antigens, HSPs, and other proteins and also nucleic acids such as RNAs. Also, these nano-sized carriers are suggested to present MHC molecules (especially class I) with antigens. In addition to direct membrane fusion with DCs, the Texosome can deliver its components directly into the cell while leaving the Texosome membrane lipids and proteins on/inside the plasma membrane of the DCs. Texosomes can also dock to DC plasma membranes without apparent internalization. DCs would then be able to arouse T cells by presenting texosome-MHC molecules.[5] Antigens from texosomes can be processed and delivered by MHC I / II molecules, as well as by overexpressing co-stimulatory molecules. Additionally, transferred texosome mRNAs could be translated in DCs to produce and present tumor antigens. Texosomes interact differently with monocytes. T cells may be stimulated more by immature DCs (iDCs) that undergo maturation.[6,7] Calreticulin, various heat shock proteins (HSPs), high mobility group box 1 (HMGB1), and other damage-associated molecular patterns (DAMPs) are deposited on the cell surface or secreted after heat shock, which further stimulates the immune response.[8] Studies have shown physiological challenges like infection, inflammation, injury, or usage of irradiation or exogenous stress-like heat change the proteomic or genetic content of cells and their exosomes.[9-13] Also, according to the study that examined hyperthermia (40-46 degrees C) effects on cancer cells' exosome production, following hyperthermia, exosomes production are increase.[9,14,15] Tumor antigens bind strongly to HSP70 and this combination improves antigen delivery to APCs, as well as provides better cross-presentation of tumor antigens, thereby breaking host immune system tolerance to the tumor.[16-18] These molecules directly transmit immunogenic signals to DCs.[19] When HSPs bind to DC membrane receptors, cargo is processed and presented on MHC; induction of cell signaling results in enhanced MHC expression and co-stimulatory molecules; increased release of modulatory molecules, including cytokines.[20-23] Due to the specific characteristics of texosomes and HSP mentioned above, we are going to investigate the effects of HSP70-enriched texosomes (HS-TEX) on DC maturation and activity.

MATERIALS AND METHODS

Animals

Six to eight-week-old BALB/C mice (18-22 g) were purchased from thePasteur Institute of Iran. Mice were kept under free available food and a standard laboratory chow diet.

Cell Line

Pasteur Institute (Tehran, Iran) provided the murine CT-26 cell line (murine colorectal cancer cell line), which was grown in RPMI 1640 (Biowest, Nuaillé, France) supplemented with 10% fetal bovine serum (FBS, Gibco, Grand Island, NY) at 37ºC with 5% CO2.

Texosome Isolation

CT26 cell line was cultured in serum-free RPMI-1640. At this stage, we had two groups of cells. Group 1 included a cultured CT26 cell line without any manipulation, group 2 included a CT26 cell line, heated to 43 degrees for 30 min, followed by recovery for 1 day at 37ºC.[24] For the removal of cell debris, the collected supernatant from both groups was centrifuged (15 min, 3000 rpm). As directed by the manufacturer, exosomes were extracted using the EXOCIB kit (Cib Biotech, Tehran, Iran). The protein contents of the exosome pellets were measured using the bicinchoninic acid assay (BCA assay) kit (Parstous biotechnology, Mashhad, Iran).

Characterization of Texosome

The exosome size distribution was assayed via dynamic light scattering analysis (DLS) (Zetasizer NANO, Malvern).

For morphological analysis of exosomes by transmission electron microscopy (TEM), exosome fixation was performed with 2% paraformaldehyde, applied onto the formvar-coated grid, and stained with 2% uranyl acetate. Then, an electron microscope (Zeiss-EM10c-100KV) was used to observe and capture images of isolated exosomes.

We used a 3.7% glutaraldehyde solution to fix exosomes for scanning electron microscopy (SEM) analysis. After washing with PBS, samples were dehydrated in alcohol, and then coated with palladium and gold. SEM images were taken with a Mira3 TESCAN (scanning electron microscope).

Expression levels of CD81 and CD63 as exosome markers were evaluated by flow cytometry. The isolated exosomes were fixed on latex microbeads that were

within the detection range of the flow cytometry (3.9 μM). For 15 minutes, 5 μg of exosomes were incubated with 10 μL latex beads. Then exosome and bead mixture was incubated at 4°C overnight. Glycine was added to inactivate any remaining binding sites on the surfaces of the beads. Afterward, the suspension was centrifuged. PBS containing 0.5% bovine serum albumin was used to rinse the pellet three times (BSA). Finally, the pellet was stained with anti-CD63/PE and anti-CD81/FITC.

Dendritic Cell Generation

Female BALB/c mice were euthanized by CO2 asphyxiation. To obtain bone marrow cells, tibias and femurs were removed and flushed. The cell suspension was centrifuged for 8 min at 250 g. The cell pellet was cultured in RPMI 1640 supplemented with 10% FBS, 50 mM β-mercaptoethanol, 20 ng/mL recombinant mouse interleukin-4 (rmIL-4; PeproTech, USA), 20 ng/mL recombinant mouse granulocyte-macrophage colony-stimulating factor (rmGM-CSF; PeproTech, USA), and, 1% Pen/Strep (Biosera, France). A total of 6×10^5 cells/mL were seeded in 24-well plates. After 3 days, the cells were fed with a fresh medium containing 20 ng/mL rmIL-4 and 20 ng/mL rmGM-CSF. The non-adherent or loosely adherent cells were collected on day 6 and then transferred to another well for maturation.[2,25]

Uptake of Labeled Texosome

Exosomes were tagged with PKH67 fluorescent dye (Sigma-Aldrich, Bornem, Belgium) according to the manufacturer´s instructions. Harvested DCs (1×10^5) were cultured with tagged exosomes. Cells were washed and observed with a fluorescent and confocal microscope after 24 hours (Leica TCS SPE, Wetzlar, Germany).

Evaluation of Dendritic Cell Maturation

To assess the impact of texosomes on DCs, iDCs were cultured with 30 μg/ml texosomes for 24 hr. DCs´ surface markers, including CD86, CD11b, CD11c, CD80, CD40, and MHCII, were analyzed in iDCs, iDCs were treated with HS-TEXs, iDCs treated with normal texosomes, iDCs treated with lipopolysaccharide (LPS) (mDC) groups.

Lymphocyte Proliferation Test

To determine DC activation potency, a lymphocyte proliferation test was performed on four groups (iDC, iDC/Tex, iDC/Tex+ HSP, and mDC). For this purpose, spleen lymphocytes of female BALB/C were tagged with CFSE (carboxyfluorescein diacetate succinimidyl ester) (Thermo Fisher Scientific, CA, USA). For CFSE labeling, after extraction of spleen lymphocytes and lysis of RBCs with ammonium-chloride-potassium (ACK) lysing buffer, lymphocytes were counted and their viability was determined using trypan blue. To label lymphocytes with CFSE dye, a cell suspension (20×10^6 cells/mL) was prepared and mixed with 5 μM of CFSE for 10 minutes. The cell suspension was then washed with RPMI enriched with 5% FBS to complete the labeling steps.

To prevent the proliferation of DCs, cells were cultured with 25 μg /mL mitomycin C. To evaluate the activating potency, at a 1:10 ratio, DCs (iDC, iDC/Tex, iDC/Tex+HSP, and mDC) were cocultured with CFSE-labeled lymphocytes. The cells were cultured in a 96- well plate for 48 h. lymphocyte proliferation rate was assessed by flow cytometry (analyzed by FlowJo software).

Western Blotting

Protein concentrations of texosomes were estimated by the BCA protein assay kit (Parstous biotechnology, Mashhad, Iran). Then, an appropriate amount of total protein was loaded onto a 10-15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After that, the proteins in the gel were transferred to the nitrocellulose membrane for 1 hour. The membrane was blocked and incubated at 4˚C overnight with primary antibodies specific to HSP70 or β-actin (R&D Systems, USA). Following three buffer washes, the membrane was incubated for 1 hour with secondary antibodies conjugated to horseradish peroxidase (HRP) (R & D Systems, USA). The enzyme-substrate was then added, revealing protein bands. Protein expression was measured using the ImageJ 1.52v software (National Institute of Health, USA).

Statistical Analysis

The statistical analysis was carried out using the Graph Pad prism (V8.0.1.244). The data were presented as mean ± standard deviation. One-way ANOVA was used to examine the differences between the groups. When the P-value was less than 0.05, the differences were considered significant. $*p<0.05$, $**p<0.01$, $***p<0.001$, $****p<0.0001$.

RESULTS

Characterization of CT-26 Cell-derived Exosomes

The exosome structure was examined microscopically using the TEM technique. The images showed that the

integrity of the exosome membrane was preserved during the separation process and the spherical structure was not damaged (Figure 1A). Using SEM microscopy, small exosomes with spherical morphology were seen in the pictures (Figure 1B). Examination of DLS showed that the isolated exosomes had an average size of 90 nm (Figure 1C). In this study, to confirm the nature of the isolated exosomes, expression levels of CD63 and CD81 surface molecules were evaluated by flow cytometry. Figure 1D shows that the isolated exosomes express both CD63 and CD81 markers.

Effect of Temperature on Expression of HSP70

To explore the temperature influence on HSP70 expression in exosomes, CT26 cell lines were cultured in serum-free RPMI-1640. Upon appropriate confluency, cells were heated to 43 degrees for 30 minutes, followed by recovery at 37°C for one day. Cell culture supernatants were collected for exosome isolation. The expression of HSP70 was determined by Western blot with specific antibodies. β-actin was used as an internal control (n= 4). Figure 1E- F shows that with the increase in temperature, a significant increase in the expression of HSP70 protein in CT26 cell derived-exosomes is observed.

DCs Morphology

Morphology of DCs was characterized using phase-contrast microscopy. Microscopic findings were indicative of the maturation process as evidenced by observation of vacuolated cytoplasm and branched dendrites and macropinosome in iDCs/Tex+ HSP group. Also, in the mDC group, observation of vacuoles in the cytoplasm and macropinosomes revealed the maturation process (Figure 2).

The Uptake of Exosomes by DCs

To examine exosome uptake by DCs, the exosomes were labeled with PKH67 dye. Then, DCs were cocultured with the PKH67-exosomes for 24 hours followed by observation of the cells under confocal fluorescence microscopy. DCs showed fluorescent signals. Blue fluorescence is indicated by red arrows, and depicts the nuclei of cells. Green fluorescence is indicated by yellow arrows and depicts the presence of labeled exosomes in the cytoplasm. Color intensity is directly proportional to the amount of the labeled exosome uptaken. These results indicate that DCs have successfully internalized exosomes (Figure 3).

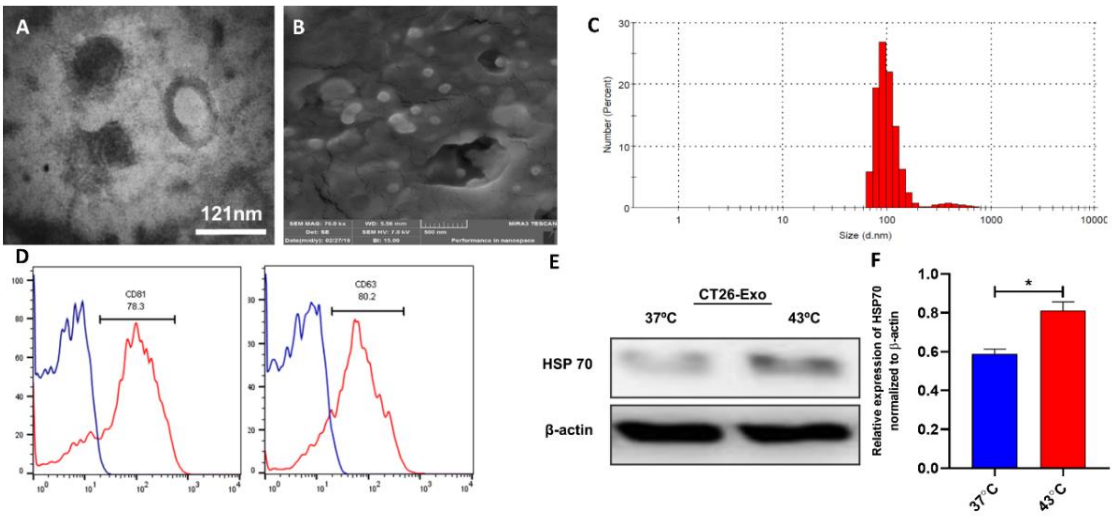

Figure 1. Characterization of CT-26 cell-derived exosomes. (A) The morphology of exosomes was analyzed using transmission electron microscopy (TEM). (B) Scanning electron microscopy (SEM) analysis of exosomes. (C) The size distribution of CT-26 cell-derived exosomes was measured by dynamic light scattering (DLS). (D) the exosomal markers CD63 and CD81 were detected in the isolated exosome by flow cytometry. (E) Release of HSP70 from exosomes following hyperthermia treatment of CT26 cells. Cells were heated to 43 °C for 30 min and recovered for 24 h at 37 °C. After incubation, exosomes were isolated for western blot analysis of the HSP70 protein. β-actin was used as an internal control. (F) HSP70 expression was quantified by densitometric analysis using ImageJ 1.52v software (n= 4). Data are shown as the mean ± SD. Comparisons between groups were analyzed by one-way ANOVA, * p<0.05.

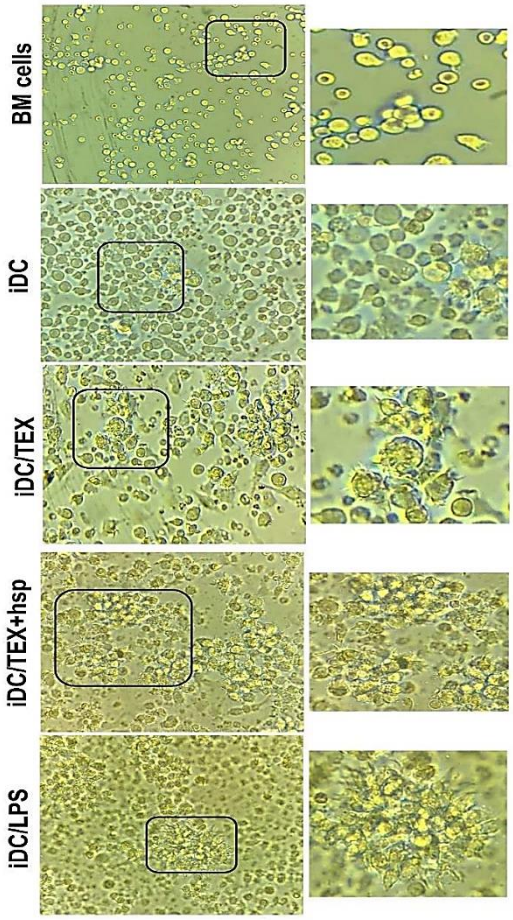

Figure 2. Dendritic Cells (DCs) Morphology. Phase-contrast microscopy images of Bone marrow cells (BM cell), immature DCs (iDC), immature DCs treated with texosome (iDC/Tex), immature DCs treated with exosomes derived from heat-shocked tumor cells (HS-TEXs) (iDC/Tex+HSP), and iDCs treated with lipopolysaccharide (LPS).

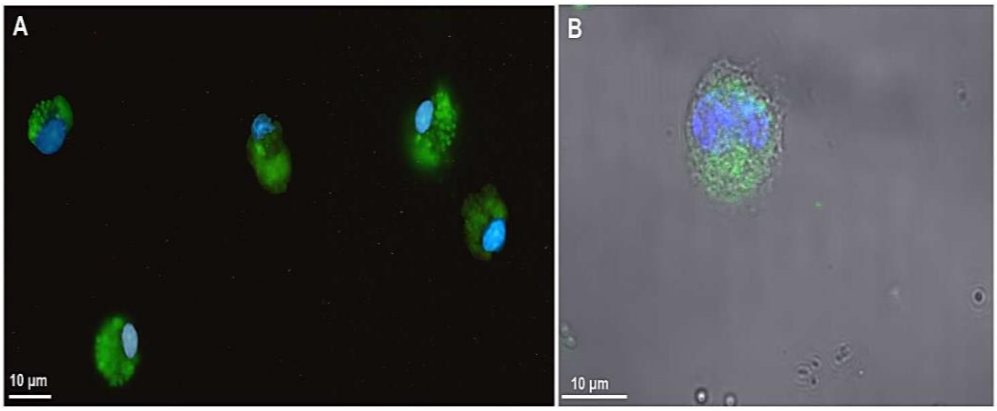

Figure 3. Exosome uptake by Dendritic Cells (DCs). The incorporation of exosomes into DCs was illustrated by fluorescent and confocal microscopy 24 h after the co-culture of DCs with PKH67- labeled exosomes. Results indicated that exosomes (green) up taken by DCs. Images obtained by fluorescent microscopy of PKH67-labeled exosomes with 4',6-diamidino-2-phenylindole (DAPI)-stained nuclei (A), confocal microscopy of localization of labeled exosomes in the cytoplasm (B).

The Effect of Exosomes on The Maturation of DC

To investigate the effects of exosomes on iDCs, different groups were designated: iDC, iDC/Tex, iDC/Tex+ HSP, and mDC. For phenotypic evaluation, the surface markers CD11c, CD86, MHCII, CD83, and CD40 were examined by flow cytometry. Our flow cytometry findings demonstrated increased size uniformity and expression of the markers in the iDC/ Tex+ HSP group compared to the other groups. Analysis of the surface molecules showed that HSP70, similar to LPS, can induce DC maturation. Heat treatment significantly enhanced the frequency (%) and mean fluorescence intensity (MFI) of MHCII (90.27±5 vs. 68.9±4.4 % and 102.12±2.7 vs. 34.37 ± 1.3 MFI unite; p<0.001 and p<0.001, respectively), CD86 (89.2±3.6 vs. 72.5±4.2 % and 66.97±11.1 vs. 27.97±6.2 MFI unite; p<0.001 and p<0.01, respectively), and CD40 (97.2±2.4 vs. 68.15±1.5% and 38±7.3 vs. 22.47±3.2; p<0.001 and p<0.01) and CD83 (89.2±3.6 vs. 68.57±5.3% and 51±7.6 vs. 32±7 MFI unite; p<0.001 and p<0.05, respectively) compared with iDCs (Figures 4 A-B).

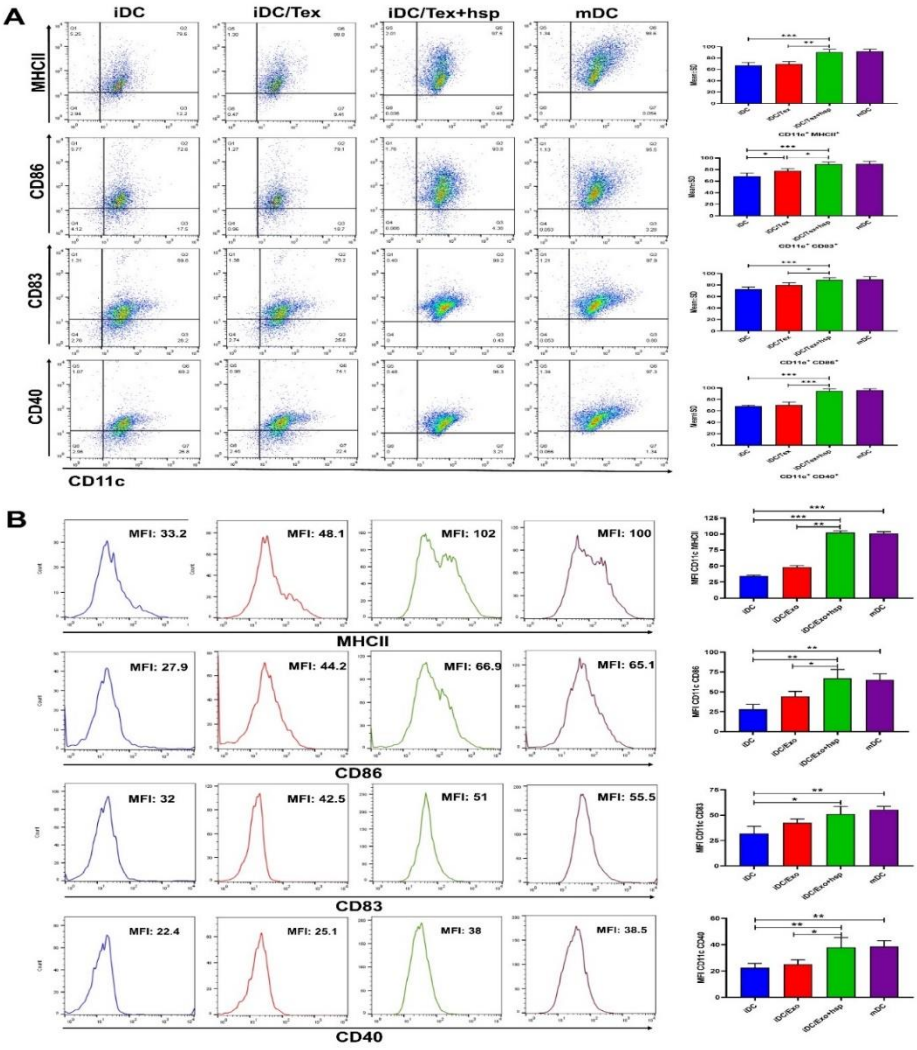

Figure 4. The effects of exosomes derived from heat-shocked tumor cells (HS-TEXs) and/or lipopolysaccharide (LPS) on surface marker expression of immature DCs (iDCs). Dot plot diagrams and the mean ± SD of CD11c, CD86, MHCII, CD83, and CD40 in different groups (A). Representative histogram and mean fluorescence intensity (MFI) (arbitrary units [AU] of fluorescence) ± SD of CD11c, MHCII, CD86, CD83 and CD40 in different groups (B) including iDC, iDCs treated with texosomes (iDC / Tex), iDCs treated with HS-TEXs (iDC / Tex + HSP) and iDCs treated with LPS (mDC) (n= 4). Comparisons between the groups were analyzed by one-way ANOVA (*p<0.001, ** p<0.01* p<0.05).**

T Lymphocyte Proliferation Analysis

To perform this test, DCs in different groups including iDCs without any manipulation (iDC), iDCs treated with texosomes (without manipulation) (iDC / Tex), iDCs treated with HS-TEXs (iDC / Tex+ HSP), and iDCs treated with LPS (mDC) were co-cultured with CFSE- lymphocytes. Analysis of flow cytometric results reflected a higher proliferative response of lymphocytes in the iDC / Tex + HSP group in comparison to the iDC/Tex and iDC groups. The findings also revealed a higher proliferation rate of T lymphocytes cocultured with iDC / Tex compared to the lymphocytes in the iDC group (Figure 5).

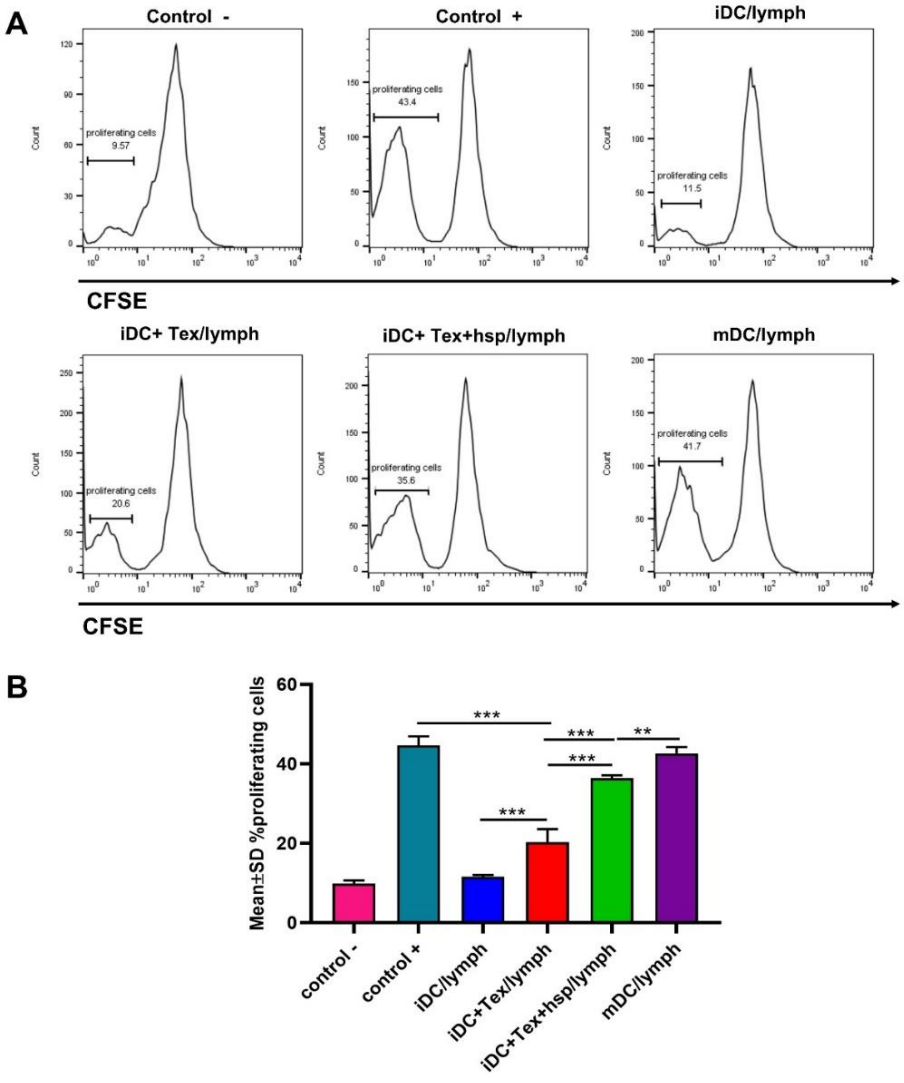

Figure 5. Flow cytometric analysis of lymphocyte proliferation. (A) Histogram and (B) mean±SD value of lymphocyte proliferation in different groups (n=4). DCs in different groups including immature DCs (iDC), iDCs treated with texosomes (without manipulation) (iDC/Tex), iDCs treated with exosomes derived from heat-shocked tumor cells (HS-TEXs) (iDC/Tex+HSP) and iDCs treated with lipopolysaccharide LPS (mDC) were co-cultured with carboxyfluorescein diacetate succinimidyl este (CFSE)- labeled lymphocytes at 1:10 ratio. Negative control: CFSE-labeled responder lymphocytes without any treatment. Positive control: CFSE-labeled responder lymphocytes treated with 2% Phytohaemagglutinin (PHA). Comparisons between the groups were analyzed by one-way ANOVA (*p<0.05, *** p<0.001).

DISCUSSION

Exosomes are intercellular messengers secreted by various cell types.[26,27] Recent evidence has indicated tumor cell-derived exosomes can evoke sufficient antitumor immunity.[28] Promoted anti-tumor responses by texosome might be attributable to their tumor antigen and HSP contents.[29]

Based on our findings, HS-TEXs resulted in enhanced expression by DCs of MHCII and costimulatory molecules compared to normal texosome. In addition, HS-TEXs promoted DCs' immunostimulatory ability in the MLR test. Heat treatment on tumor cells resulted in the secretory of HSP-enriched texosomes. Evidence has shown that HSPs diffused into the extracellular environment exert immunological functions.[18,30] Heat treatment is associated with increased recruitment of HSP into exosomes and promotes immunostimulatory effects of texosomes.[31,32] HSPs are chaperones that facilitate the presentation of antigenic peptides.[33] Exosomes containing high HSP levels could enhance protective immunity.[34] Tumor antigens associated with texosomes can be presented by MHCI and MHCII on mature DCs, where they can play significant roles in promoting T cell responses and skew immune responses toward T helper1 (Th1) type and cytotoxic T lymphocyte (CTL) responses, which highly contribute to tumor eradication.[35,36] Note, however, that here, we only examined the augment expression of HSP70 in the texosomes and showed that the raised expression is involved in DC maturation. Heat shock may also modulate other exosomal factors that influence DC maturation. More investigation is required to confirm the accurate effect of HSP70 in DC maturation, including blocking the HSP70 receptor on DCs or employing texosomes (derived from tumor cells not subjected to heat shock) loaded with HSP70 protein. Therefore, our study has this weakness. These changes in functional and phenotypic properties of iDCs exposed to HSP70 highlight this chaperone as a maturation signal.[33] Studies have demonstrated that HSPs induce the expression of the maturation marker CD83 on iDCs and elevate their co-stimulatory molecules, thereby improving their immunostimulatory functions.[13]

HSP-enriched exosomes released from tumor cells incorporate tumor antigens capable of activating DCs. Therefore, these DCs could trigger specific anti-tumor immune responses that inhibit tumor growth.[34] This finding can be an optimistic point in tumor DC therapy and DC-based cancer vaccine design. However, as this study, which serves as the first step in investigating the synergistic effect of heat and texosomes on enhancing the efficiency of dendritic cells, has been conducted in an invitro setting, the introduction of this strategy into the therapeutic domain necessitates an evaluation of the efficacy of heated tumor cell-derived texosomes in invivo conditions, across various tumor models and at different stages of tumorigenesis. Subsequently, in more comprehensive studies, the mechanism of action of these texosomes and the dendritic cells activated by them will be elucidated.

In conclusion, based on the findings of this study, the concurrent application of heat and taxosomes results in the reinforcement of dendritic cell maturation, activation, and tumor antigen presentation.

STATEMENT OF ETHICS

The study was approved by the Ethics Committee of the Faculty of Medicine (IR.SBMU.MSP.REC.1396.142), Shahid Beheshti University of Medical Sciences, Iran.

FUNDING

This study was sponsored by a grant from Shahid Beheshti University of Medical Sciences (Grant No 10059).

CONFLICT OF INTEREST

The authors declare that there are no conflict of interests.

ACKNOWLEDGEMENTS

Not applicable.

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