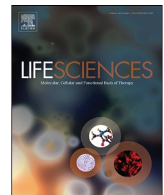
Life Sciences 219 (2019) 136–143

Contents lists available at ScienceDirect

Life Sciences

journal homepage: www.elsevier.com/locate/lifescie



Induction of T cell-mediated immune response by dendritic cells pulsed with mRNA of sphere-forming cells isolated from patients with gastric cancer

Vahid Bagheri[a], Mohammad Reza Abbaszadegan[b,*], Bahram Memar[c], Mohammad Reza Motie[c], Mahdi Asadi[c], Reihaneh Alsadat Mahmoudian[e], Mehran Gholamin[b,d,**]

[a] Cellular and Molecular Research Center, Birjand University of Medical Sciences, Birjand, Iran
[b] Medical Genetics Research Center, Mashhad University of Medical Sciences, Mashhad, Iran
[c] Surgical Oncology Research Center, Imam Reza Hospital, Faculty of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran
[d] Department of Laboratory Sciences, School of Paramedical Sciences, Mashhad University of Medical Sciences, Mashhad, Iran
[e] Immunology Research Center, Mashhad University of Medical Sciences, Mashhad, Iran

ARTICLE INFO

Keywords:
Cancer stem cells
Sphere-forming cells
Dendritic cells
T cells
Cytotoxicity

ABSTRACT

Gastric cancer (GC) as the third most common cause of cancer-associated mortality worldwide is one of the cancers with very high heterogeneity. Cancer stem cells (CSCs) as a small subset of cancer cells in solid tumors with the self-renewal, differentiation and tumorigenic ability are responsible for tumor initiation, progression, recurrence, metastasis, and resistance to current treatments. Therefore, eradication of CSCs is very vital to cure cancer. Here, we first isolated and identified sphere-forming cells in tumor tissue from four GC patients and then analyzed T cell responses induced by monocyte-derived dendritic cells (DCs) loaded with total mRNA of sphere-forming cells in terms of interferon-gamma (*IFN-γ*) gene expression and specific cytotoxicity. Spheroid colonies were formed in serum-free media. Sphere-forming cells dissociated from tumorspheres heterogeneously expressed CD44, CD54, and epithelial cell adhesion molecule (EpCAM) markers and generated one tumor in nude mice. These results demonstrated that gastric CSCs were enriched in tumorspheres. Cytokine-matured DCs loaded with mRNA of sphere-forming cells were able to induce *IFN-γ* gene expression in T-lymphocytes after a 12-day co-culture. mRNA level of *IFN-γ* gene in these lymphocytes was more highly expressed compared to stimulated T-lymphocytes by DCs transfected with normal tissue (6.4–9.39 folds). Cytotoxic activity of primed T-lymphocytes with antigens of sphere-forming cells was significantly higher than normal tissue antigens and mock DCs ($P \leq 0.0001$). Taken together, DCs loaded with mRNA of sphere-forming cells that elicit effectively specific T cell-mediated immune responses *in vitro*, may be considered as a promising therapeutic vaccination in GC patients in future.

1. Introduction

Gastric cancer (GC) is the fifth common cancer worldwide as well as the third cause of cancer-associated mortality. Surgery, chemotherapy, and radiotherapy are used for GC treatment [1]. However, the 5-year relative survival rate due to high relapse rates, metastatic nodule, and resistance to chemotherapeutic drugs is low (20–30%) [2].

Cancer stem cells (CSCs) are rare populations of tumor cells and characterized by self-renewal, differentiative, and tumorigenic properties [3]. The CSC hypothesis suggests that upon eradicating stationary and circulating CSCs, cancer due to differentiation and/or cell death could regress [4,5]. The CSCs due to their role in the tumor onset, progression, metastasis, and recurrence, as well as drug resistance are very much considered for therapeutic targets, especially immunotherapy [6–9] which induces CSC-specific cytotoxic reactions. Dendritic cell (DC)-based immunotherapy is a very promising strategy to prime antitumor-specific T cell responses.

Dendritic Cells (DCs) are professional antigen presenting cells (APCs) and the most effective activator of resting or naive T cells *in vitro* and *in vivo* [10]. DCs in the tumor site and regional lymph nodes mediate antitumor immune responses by activating T cells, in particular $CD8^+$ T lymphocytes [11]. Immature DCs due to high endocytic activity and low activation have high capacity in the antigen uptake. Thus, generation of these cells from peripheral blood monocytes and

* Correspondence to: M.R. Abbaszadegan, Medical Genetics Research Center, Mashhad University of Medical Sciences, Mashhad, P.O. Box 345-91357, Iran.
** Correspondence to: M. Gholamin, Department of Laboratory Sciences, Faculty of Paramedical Sciences, Mashhad University of Medical Sciences, Mashhad, P.O. Box 345-91357, Iran.
E-mail addresses: AbbaszadeganMR@mums.ac.ir (M.R. Abbaszadegan), GholaminM@mums.ac.ir (M. Gholamin).

https://doi.org/10.1016/j.lfs.2019.01.016
Received 10 September 2018; Received in revised form 29 December 2018; Accepted 10 January 2019
Available online 12 January 2019
0024-3205/ © 2019 Elsevier Inc. All rights reserved.

also their incubation with tumor proteins, peptides, and lysates or transfection with DNA and RNA *in vitro* is the basis of cancer immunotherapy [12]. Among DCs loaded with different forms of antigen, use of RNA has several advantages. A key advantage of using RNA is the ability to amplify *in vitro*. Therefore, a limited amount of tumor tissue is sufficient for the production of a large number of DCs pulsed with antigen [13]. Another advantage is that all tumor-associated antigens (TAAs) without the need to identify and know will be processed and presented on DC surface at the same time, resulting in induction and expansion of both $CD4^+$ and $CD8^+$ T cells. RNA, unlike protein and peptide antigens, does not require DCs with the specified HLA allele for the priming of $CD4^+$ and $CD8^+$ T cells. RNA due to easy degradation as well as quick clearance of organism minimizes the chance of causing side effects such as generation of autoantibodies and autoimmune disease [14]. In addition to relatively easy administration of RNAs relative to naked DNA, short-lived RNA constructs, unlike DNA, are not integrated into the DCs genome. Therefore, these constructs do not result in the introduction of oncogenic activity into DCs that returned to the patient [14,15].

It was reported that the characteristics of spheroid (three-dimensional) cultures of CSCs in areas such as gene expression profiles, tumor heterogeneity and morphology resemble original cancer [16–18]. Therefore, formation of spheroid colonies in single cell-based assays could be reliability objectives for development of immunotherapy targeting CSCs. It is also believed that three-dimensional tumorspheres can provide patient-derived CSCs (sphere-forming cells) for evaluation of DC-based approaches, especially DCs pulsed with total mRNA, in order to induction of tumor-specific T cell responses, what defined the main goal of our study.

Identification, characterization, and classification of CSCs remain controversial. None of the known markers for the identification of CSCs in all types of tumors, even a particular type of cancer such as gastric cancer are universal and reliable [3,18–21]. The most commonly used marker of gastric CSCs (GCSCs) is CD44 protein that reproduce a GC tumor in a mouse xenograft model. Other protein such as CD44V8-10, CD133, CD24, CD54, CD90, CD49f, CD71, EpCAM, and ALDH1 are also considered as reliable gastric GCSCs markers [22]. Therefore, in present study, we used sphere-forming cells regardless expression markers for DC-based immunotherapy.

In this study, we first isolated and identified sphere-forming cells from the patients with GC. Then, we utilized autologous DCs transfected with total mRNA of sphere-forming cells as the whole antigen for priming T cell response. Finally, the response of T cells induced by antigens of sphere-forming cells was compared with the response of T cells induced by antigens of gastric normal tissue and mock DCs.

2. Materials and methods

2.1. Isolation and in vitro expansion of sphere-forming cells from gastric tumor specimens

Tumor tissue fragments were obtained from four patients listed in Table 1 after surgical removal. After mechanical and enzymatic dissociation of tumor samples, single cells were cultured in a serum-free DMEM/F12 (Biosera, NUAILLE, France) medium supplemented with 8 mM HEPES (Biosera), 20 ng/ml EGF (Gibco, Carlsbad, California, USA), 10 ng/ml bFGF (Gibco), 4 μg/ml heparin (Sigma-Aldrich, St. Louis, Missouri, USA), 2% B-27 supplement (Gibco), 100 U/ml penicillin, 100 μg/ml streptomycin and 0.4 μg/ml amphotericin B in T-75 low attachment flask (Corning, New York, USA) at 37 °C in a humidified 5% CO_2 incubator for 1–2 months.

Table 1
Clinicopathological characteristics of patients.

Patient	Sex/age	Site	Grade	Stage
1	M/52	None-cardia	Poor	T3N0M0
2	M/59	Cardia	Moderate	T3N1M0
3	M/71	None-cardia	Poor	T4aN3aM0
4	M/62	None-cardia	Moderate	T2N0M0

2.2. Flow cytometry analysis of cell surface markers CD44, CD54 and EpCAM

Cells were dissociated from primary spheres with TrypLE™ Select 1× (Gibco) solution for 60 min at 37 °C and stained using manufacturer-suggested concentrations (Biolegend, San Diego, California, USA) of monoclonal antibodies against CD44 (FITC-conjugated), CD54 (PE-conjugated), and EpCAM (APC-conjugated) for 15–20 min on ice in the dark. After staining and washing, the cells were analyzed by a flow cytometry (BD FACSCalibur, Franklin Lakes, New Jersey, USA). The FlowJo software package (FlowJo, http://www.flowjo.com) was used for processing data. Controls were carried out using FITC-IgG1, PE-IgG1, and APC-IgG1 isotype control antibodies.

2.3. In vivo tumorigenicity assay

Male nude mice (C57BL/6 strain), 4–6-weeks old were used in tumorigenicity experiments. All animal experiments were complied with the ARRIVE guidelines and carried out in accordance the National Institutes of Health guide for the care and use of Laboratory animals as well as were approved by Institutional Animal Care and Use Committee of Mashhad University of Medical Sciences. Sphere-forming cells (1×10^6 cells/ml) suspended in serum-free DMEM/Matrigel (Sigma-Aldrich) (1:1) were subcutaneously injected into the right flank of mice in North Research Center, Pasteur Institute of Iran. The mice were kept for four weeks to form a tumor.

2.4. mRNA isolation and amplification

Total mRNA from sphere-forming cells and gastric normal tissue were isolated using mRNA Isolation Kit (Roche, Basel, Switzerland) according to the manufacturer's instructions. The SMART mRNA Amplification (Clontech, California, USA) and mMESSAGE mMACHINE T7 Transcription (Ambion, California, USA) Kits were used for generation of large amounts of mRNA and *in vitro* transcription, respectively. GFP mRNA as a reporter gene was also transcribed *in vitro*.

2.5. DC generation

Peripheral blood mononuclear cells (PBMCs) were obtained from 30 ml heparinized peripheral blood of patients *via* Ficoll-Hypaque (Biosera). PBMCs were plated in T-25 flasks in RPMI-1640 (Biosera) complete medium (containing HEPES and glutamine) supplemented with 10% heat-inactivated FBS (Gibco) and incubated for 2 h at 37 °C. Unattached cells as peripheral blood lymphocytes (PBLs) were gently washed away and frozen in freezing medium (80% FBS + 10% Glucose + 10% DMSO) for future use. Adherent blood monocytes were then cultured in RPMI-1640 complete medium supplemented with 10% heat-inactivated autologous plasma, 1000 U/ml GM-CSF (Biolegend) and 500 U/ml IL-4 (Biolegend) for 6 days.

2.6. DC transfection

Prior to electroporation, a total of 1×10^6 immature DCs was washed three times in cold OPTI-MEM medium (Gibco), resuspended in 100 μl OPTI-MEM medium containing 5 μg total mRNA and placed in a 5 mm gap cuvette. Electroporation was performed at 300 V for 500 μs using a BTX ECM 830 squarewave electroporator (Genetronics Inc., San Diego, CA, USA). Immature DCs electroporated without mRNA were called mock DCs. For assessing RNA transfection efficiency, immature

DCs were electroporated with GFP mRNA. The electroporated immature DCs were immediately transferred to 6-well plates with 3 ml RPMI-1640 complete media containing 10% heat-inactivated autologous plasma, 1000 U/ml GM-CSF and 500 U/ml IL-4 and incubated for 2–4 h. Maturation agents including 1000 U/ml TNF-α (Gibco), 700 U/ml IL-1β (Gibco), 16,500 U/ml IL-6 (Gibco) and 1 μg/ml PGE2 (Sigma-Aldrich) were then added to culture media. DCs were cultivated for 18–36 h at 37 °C in 5% CO_2.

2.7. Cell surface marker analysis of immature and mature DCs by Flow cytometry

Immunophenotyping of immature and mature DCs was performed by staining cell surface antigens using a panel of monoclonal antibodies (Biolegend) specific for CD14 (monocytes), CD1a (DCs), CD80 (monocytes and DCs), CD83 (DCs), CD86 (monocytes and DCs), CD40 (DCs), HLA-DR, and CCR-7 as well as the appropriate isotype-matched controls.

2.8. Co-culture of total mRNA-transfected DCs with autologous PBLs

DCs transfected with total mRNA of sphere-forming cells and gastric normal tissue as well as mock DCs were separately added to autologous PBLs at a 1:10 (stimulator: responder) ratio in RPMI1640 complete medium supplemented with 10% heat-inactivated autologous plasma in round bottom 96-well plates, and cultured at 37 °C in 5% CO_2 for 12 days. IL-2 (Gibco) at 20 U/ml was added on day 3.

2.9. Interferon-gamma gene expression in T lymphocytes stimulated by mRNA-transfected DCs

Total mRNA was isolated from T lymphocytes stimulated by DCs transfected with total mRNA of sphere-forming cells and gastric normal tissue using mRNA Isolation Kit (Roche) according to the manufacturer's instructions followed by DNase treatment. Concentration and purity of mRNA and cDNA synthesis were determined using spectrophotometer (WPA Biowave II, Great Britain) and Easy cDNA Synthesis Kit (Pars Tous Biotechnology, Mashhad, Iran), respectively. The mRNA level of *IFN*-γ gene was quantitated by real-time PCR. Real-time PCR was done using a Mx3000P QPCR System (Stratagene, La Jolla, CA, USA) in the presence of SYBR-green. The reaction conditions were: 95 °C for 10 min and 40 cycles at 95 °C for 30 s, 56 °C for 30 s, and 72 °C for 30 s. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was amplified as an internal control gene. The relative quantitation of *IFN*-γ gene expression was analyzed using the comparative Ct method (Fold change = $2^{-\Delta\Delta Ct}$) [23]. $2^{-\Delta\Delta Ct}$ = [(Ct *IFN*-γ gene - GAPDH gene)stimulated T-lymphocytes with mRNA-loaded DCs of sphere-forming cells - (Ct IFN-γ gene - GAPDH gene)stimulated T-lymphocytes with mRNA-pulsed DCs of gastric normal tissue]. Primer sequences for *IFN*-γ were 5′-GCTCTGAGACAATGAACGCT-3′ (forward) and 5′-AAAGAGATAATC TGGCTCTGC-3′ (reverse). Primer sequences for GAPDH were also 5′-GGAAGGTGAAGGTCGGAGTCA-3′ (forward) and 5′-GTCATTGATGG CAACAATATCCACT-3′ (reverse).

2.10. Calcein-acetyoxymethyl cytotoxicity assay

The cytotoxic activity of stimulated T-lymphocytes was determined using a calcein-release cytotoxicity assay. Briefly, 1×10^6 sphere-forming cells (target cells) re-suspended in serum-free RPMI1640 complete medium were incubated with 15 μM calcein-AM (Sigma-Aldrich) at 37 °C for 30 min with occasional shaking. After washing three times with PBS, the cells were re-suspended in PBS containing 5% FBS. The calcein-AM labelled target cells (5×10^3 cells/well) were incubated with T lymphocytes stimulated by DCs loaded with total mRNA of sphere-forming cells and gastric normal tissue, as well as mock DCs at various effector:target cell ratios (1:1, 10:1, 20:1) in 96-well U-bottomed microtitre plates (triplicate wells/sample) at 37 °C for 4 h. After centrifugation and transferring 100 μl of the supernatant from each well into black 96-well flat-bottomed plates (SPL Life Sciences, Gyeonggi-do, Korea), the fluorescence of each supernatant was monitored at 490 nm excitation and 520 nm emission wavelengths using Synergy™ H4 Hybrid Multi-Mode Microplate Reader (Biotek, Vermont, USA). The cytotoxicity of the stimulated T-lymphocytes was calculated by the following formula: % Specific Lysis = [(experimental release − spontaneous release) / (maximum release − spontaneous release)]. Maximum release and spontaneous release were obtained from incubation of target cells with and without detergent (2% Triton X-100), respectively.

2.11. Statistical analysis

Statistical calculations were performed using GraphPad Prism version 7. Results were expressed as mean ± SE. Groups were compared using One-Way ANOVA. $P \leq 0.0001$ was considered statistically significant.

3. Results

3.1. Tumorigenic spheroid colonies formed from tumor tissue of patients with GC

CSCs that formed spheroid colonies in culture have extensive similarities to endogenous CSCs in human tumor tissues [21,24]. Therefore, we dissociated and cultivated four GC biopsies under serum-free conditions to induce the formation of spheroid colonies and then examined surface proteins of sphere-forming cells to determine the identity of gastric CSC (GCSC) markers. Primary spheres were seen in culture after four weeks (Fig. 1A). Flow cytometry analysis in accordance with our previous report [18] as well as results of previous studies [19,20] demonstrated that sphere-forming cells dissociated from primary spheres overexpressed CD44, CD54, and EpCAM surface markers (Fig. 1B). Moreover, inoculation of sphere-forming cells into three immunocompromised mice for each patient showed that these cells were able to form a tumor in 100% of the mice after four weeks (Fig. 1C). Thus, these data indicated that sphere-forming cells were of gastric origin, overexpressed CD44, CD54, and EpCAM GCSC markers and generated tumors in nude mice, suggesting that GCSCs are enriched in spheroid colonies.

3.2. Monocytes-derived DCs phenotype

DCs are large irregular cells with typical dendritic morphology and were generated from adherent monocytes isolated from PBMCs (Fig. 2A). DCs staining using various markers of activation and maturation revealed that expression levels of surface molecules CD83, CD40, and HLA-DR (as maturation markers), CD80 and CD86 (as activation markers), and chemokine receptor CCR7 in mature DCs compared to immature DCs increased. Furthermore, downregulation of CD14 expression, as well as upregulation of CD1a expression in both immature and mature DCs indicated that monocytes had fully developed into DCs (Fig. 2B).

3.3. Upregulation of IFN-γ gene confirmed stimulation of T lymphocytes by DCs loaded with total mRNA of sphere-forming cells

To measure *IFN*-γ mRNA expression in stimulated T-lymphocytes by DCs transfected with total mRNA of sphere-forming cells compared with stimulated T-lymphocytes by DCs pulsed with total mRNA of gastric normal tissue, we used comparative real-time PCR. Stimulated T-lymphocytes with mRNA-pulsed DCs of gastric normal tissue were used as control. The results showed that *IFN*-γ gene expression in stimulated T-lymphocytes with antigens of sphere-forming cells isolated

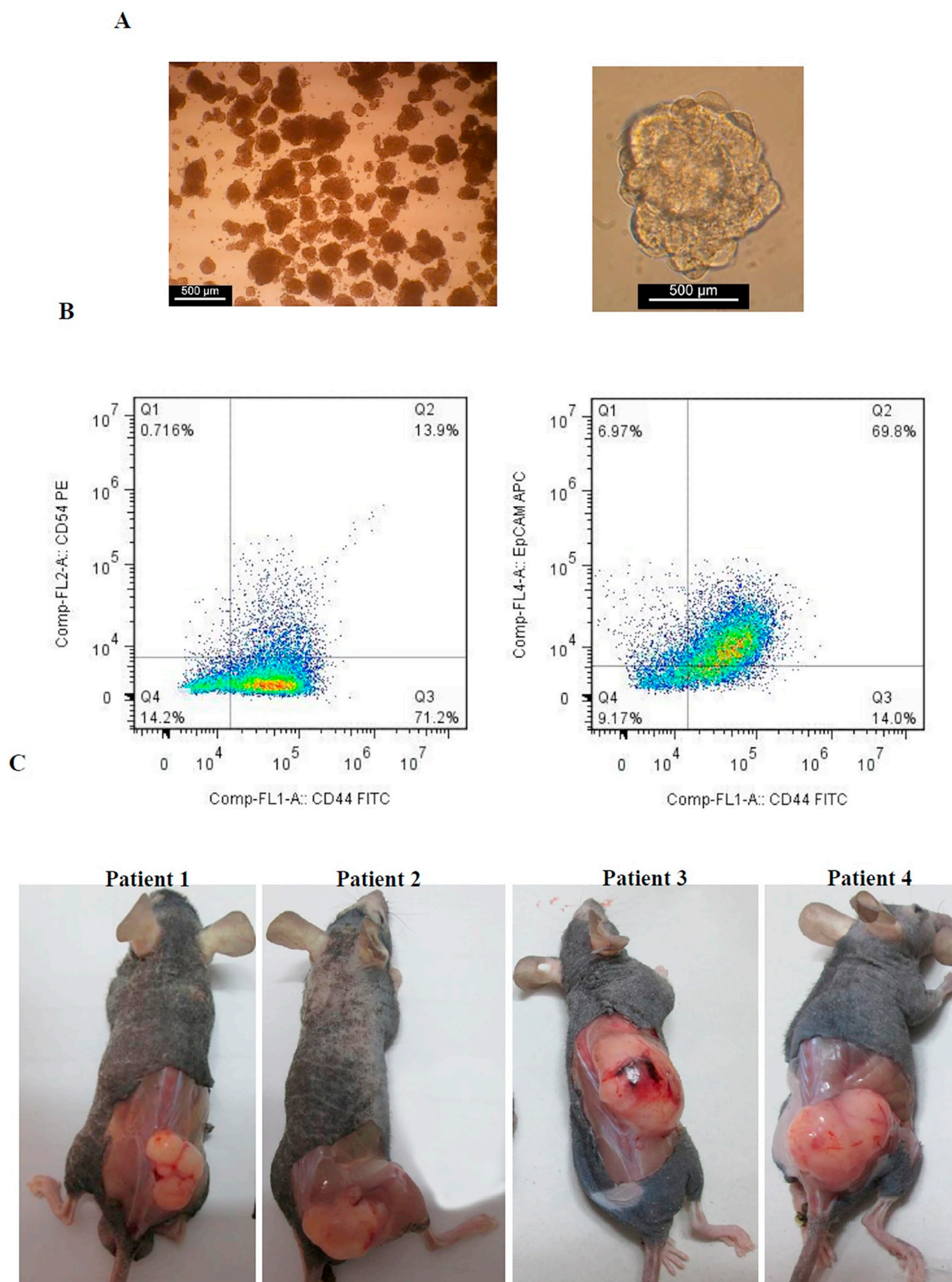


Fig. 1. Tumor spheres derived from human GC specimens overexpressed surface markers of CD44, CD54, and EpCAM as well as exhibited high tumorigenicity *in vivo*. A) Primary spheres were directly generated from tumor cells dissociated from human GC specimen. (Left panel: original magnification 4×; right panel: original magnification 40×). B) Flow cytometry analysis of CD44, CD54 and EpCAM expression in sphere-forming cells. After staining using anti-CD44 FITC, anti-CD54 PE and anti-EpCAM APC monoclonal antibodies, sphere-forming cells showed heterogeneous staining. The frequency of $CD44^{+}$ cells, $CD44^{+}CD54^{+}$ cells and $CD44^{+}EpCAM^{+}$ cells were 84%, 14%, and 69.8%, respectively. C) A tumor formation in nude mice injected with sphere-forming cells. Abbreviations EpCAM, epithelial cell adhesion molecule.

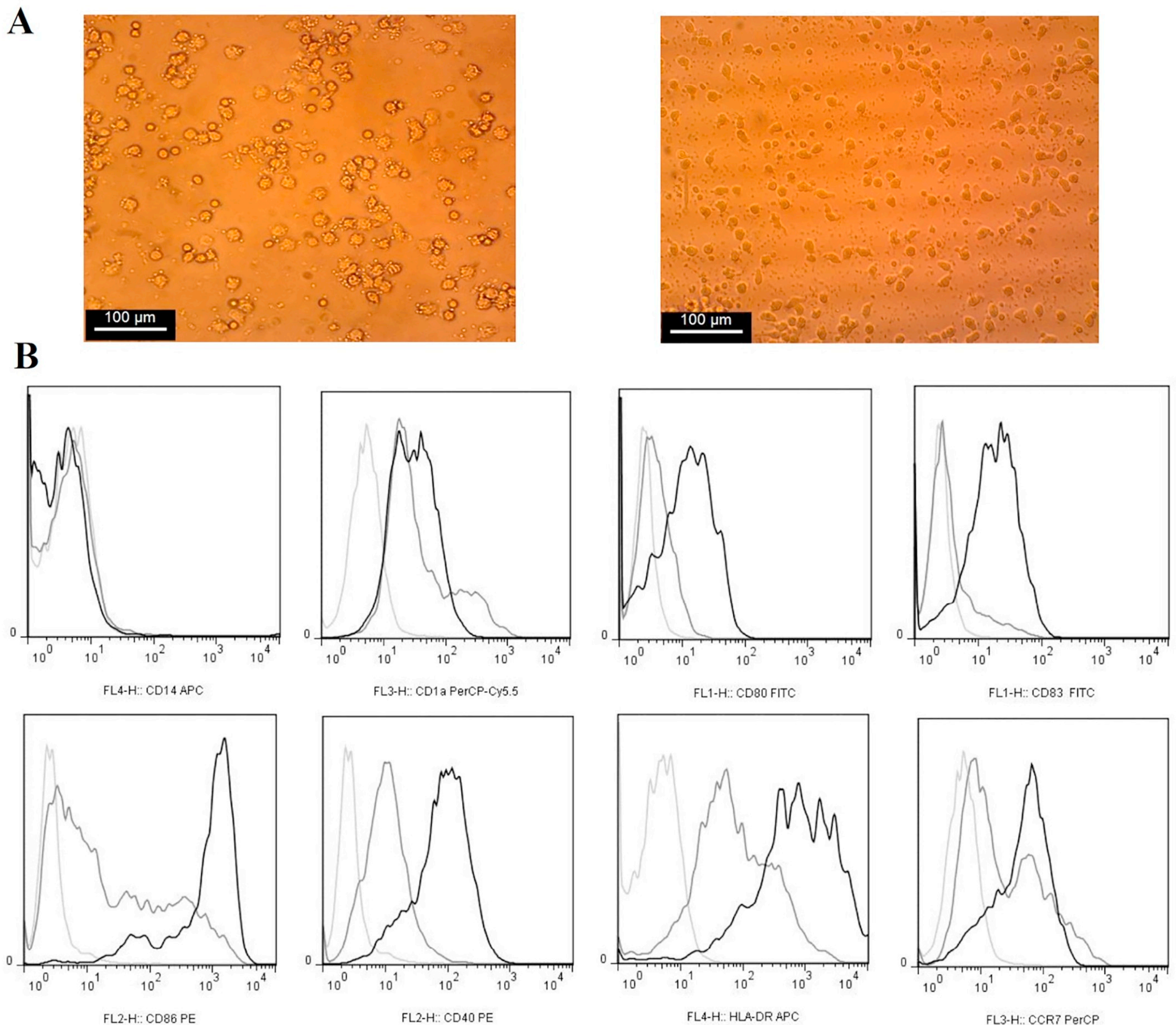


Fig. 2. Monocytes-derived DCs. A) Differentiation of monocytes to mature DCs (Left panel: monocytes, original magnification 4×; right panel: DCs, original magnification 4×). Monocytes were cultivated in the presence of GM-CSF and IL-4 cytokines to differentiate immature DCs. After transfection, DCs maturation was induced in the presence of maturation factors (TNF-α, IL-1β, IL-6, and PGE2). B) Representative histograms of flow cytometric analysis of DCs. Immature and mature DCs were stained using a panel of monoclonal antibodies (anti-CD14, -CD1a, -CD80, -CD83, -CD86, -CD40, -HLA-DR, and -CCR-7). (Bright line - isotype control, Gray line - immature DC, Black line - mature DC. Abbreviations PGE2, prostaglandin E2.

from patients was upregulated in comparison with stimulated T-lymphocytes with antigens of gastric normal tissue (Fig. 3). *IFN*-γ gene expression in stimulated T-lymphocytes with mRNA-loaded DCs of sphere-forming cells in patients 1,2 3, and 4 was 6.4, 7.6, 8.17 and 9.39 fold greater than the control, respectively.

3.4. Stimulated T-lymphocytes with antigens of sphere-forming cells induced specific lysis of sphere-forming cells

To evaluate the cytotoxic activity of stimulated T-lymphocytes by DCs loaded with total mRNA of sphere-forming cells, we used a 4-h calcein release assay. As shown in Fig. 4, cytotoxicity against sphere-forming cells was demonstrated for all four patients at effector:target cell ratios of 1:1 (ranging 6.4% to 11.5%), 10:1 (ranging 19% to 23.6%), 20:1 (ranging 41% to 50.2%). Lysis of sphere-forming cells by stimulated lymphocytes by DCs transfected with total mRNA of gastric normal tissue and mock DCs was also observed at various effector:target cell ratios. However, lysis of sphere-forming cells by stimulated T-lymphocytes with antigens of sphere-forming cells was significantly higher than lysis of sphere-forming cells by stimulated T-lymphocytes with antigens of gastric normal tissue and mock DCs ($P \leq 0.0001$).

4. Discussion

CSCs played the important role in carcinogenesis and recurrence of GC. Eradication of residual GCSCs after surgery due to chemotherapy and radiotherapy resistance, metastasis and relapse is also difficult. Therefore, DC-based immunotherapy aimed at targeting GCSCs would offer an appealing therapeutic strategy for treating GC [2,3]. Enrichment of GCSCs using tumorsphere cultivation technique caused the propagation of stem-like cells from tumors. Tumorspheres expanded in serum-free medium containing several growth factors display stem cell-

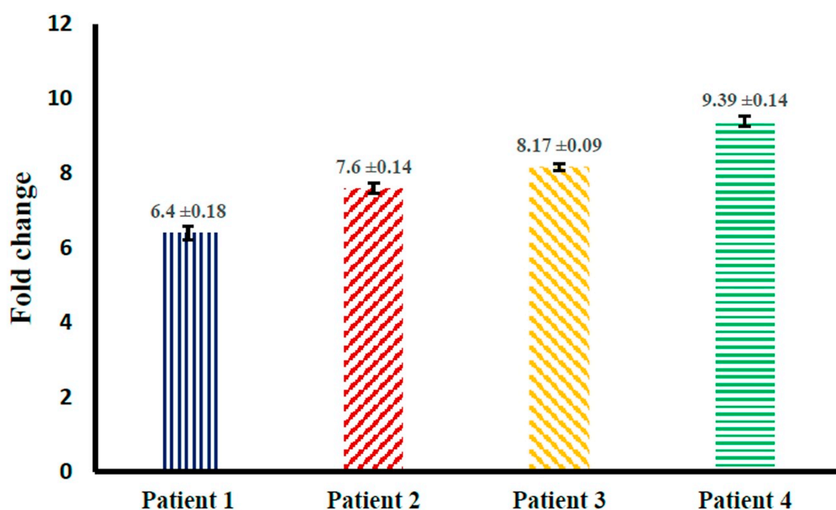


Fig. 3. Gene expression analysis of *IFN*-γ in stimulated T-lymphocytes by DCs loaded with total mRNA of sphere-forming cells. Comparative real-time PCR results showed that mRNA expression of *INF*-γ gene in stimulated T-lymphocytes by DCs pulsed with total mRNA of sphere-forming cells isolated from four patients was upregulated compared with stimulated T-lymphocytes by DCs transfected with total mRNA of gastric normal tissue (6.4–9.39 folds). Stimulated T-lymphocytes with mRNA-loaded DCs of gastric normal tissue were used as control.

like properties and maintain their molecular expression profiles as well as their tumorigenic phenotype [18,25]. Thus, we used the sphere-forming assay for enrichment of stem-like cells. Expression of CD44, CD54 and EpCAM markers as well-known GCSCs surface markers were analyzed in sphere-forming cells. All three markers associated with metastasis of GC [18–20]. Our study showed that CD44, CD54 and EpCAM markers were expressed in sphere-forming cells. However, the percentage of cells expressing these markers in tumorspheres was different. In the other words, spheroid colonies contain various kinds of cells, implying sphere heterogeneity. Thus, we used all sphere-forming cells dissociated from tumorspheres regardless of the type of expression marker for DC-based immunotherapy.

DCs as inducing $CD4^+$ and $CD8^+$ T cell-mediated responses play a unique and important role in antitumor immunity as well as the rejection of tumors [26,27]. DC maturation is vital for effective stimulation of T cell responses. Phenotypic characterization of monocytes-derived DCs from four GC patients indicated that these cells are $CD14^{lo}$, $CD1a^{hi}$, $CD83^{med}$, $CD40^{hi}$, $CD80^{med}$, $CD86^{hi}$, HLA-DR^{hi} and $CCR7^{hi}$. In fact, DCs were able to induce upregulation of CD80, CD86, CD83, CD40, and CCR7 in presence of maturation agents. In DC-based immunotherapy, the choice of antigen and the method of antigen loading are two very important issues. In the current study, we have chosen to use total mRNA of sphere-forming cells. Previous studies have shown that DCs loaded with mRNA offers numerous advantages over other DC-based approaches [13,28]. mRNA advantages are similar to the whole cell lysate, especially induction of both $CD4^+$ and $CD8^+$ T cell responses simultaneously. In this study, consistent with previous experimental investigations [29–31], we demonstrated two methods that DCs loaded with total mRNA of sphere-forming cells were able to stimulate and activate lymphocytes. The first method was characterized by mRNA expression level of *IFN*-γ gene in stimulated T-lymphocytes. *IFN*-γ gene expression levels in stimulated T-lymphocytes by DCs pulsed with total mRNA of sphere-forming cells dissociated from four patients were 6–9 folds more than stimulated T-lymphocytes by DCs loaded with total mRNA of gastric normal tissue. The second method was determined by the cytotoxic activity of stimulated T-lymphocyte. Our results showed that DCs pulsed with antigens of sphere-forming cells induced a lytic activity against sphere-forming cells. Cytotoxic activity of primed T-lymphocytes by DCs loaded with total mRNA of sphere-forming cells at various effector:target cell ratios was significantly higher than DCs pulsed with total mRNA of gastric normal tissue and mock DCs in each patient ($P \leq 0.0001$).

Although in our study, DCs transfected with total mRNA of sphere-forming cells elicited CTLs that were able to recognize and lyse sphere-forming cells, a point that cannot be overlooked is that total mRNA as antigen source contain specific antigens of sphere-forming cells as well as self-antigens, resulting in tolerance break and destruction of gastric normal tissue. However, the benefit of using total mRNA as the whole antigen from tumors for loading DCs have been demonstrated in many patients without induction of autoimmunity [32–34]. Furthermore, subtractive hybridization techniques can be used for enrichment of specific transcripts of sphere-forming cells and removal of common transcripts (auto-antigens) between sphere-forming cells and gastric normal tissue [35,36]. Moreover, our data showed that primed T-lymphocytes by normal gastric antigens of all four patients lysed very few sphere-forming cells at effector:target cell ratios of 1:1 (ranging 0.7% to 1.7%), 10:1 (ranging 2% to 2.7%), 20:1 (ranging 3% to 5.8%). There were no significant differences in cytotoxic activity between primed T-lymphocytes by normal gastric antigens and mock DCs. Thus, DCs loaded with antigens of sphere-forming cells might specifically induce CTLs, leading to lysis of sphere-forming cells.

Some previous studies have demonstrated that tumor cell mRNA-transfected DCs enhance T lymphocyte proliferation and induce tumor Immunity and cytotoxic T lymphocyte responses *in vitro* [8,9,37,38]. However, most antitumor immune responses may be directed against differentiated tumor cells because > 95% and usually < 5% of tumor mass form differentiated tumor cells and CSCs, respectively. Therefore, considering CSC hypothesis as well as CSCs role in cancer development, metastasis, recurrence, and chemoradiotherapy resistance, enrichment of CSCs can provide more effective therapeutic strategies, especially DC-based approaches.

5. Conclusion

The sphere-forming capability is useful for the propagation and enrichment of GCSCs. Our findings demonstrated that DCs loaded with total mRNA of sphere-forming cells could elicit T cell-mediated immune responses in patients with GC *in vitro*. Therefore, considering adverse autoimmune events or other side effects, DCs pulsed with antigens of sphere-forming cells due to the lysis of GCSCs by primed T-lymphocytes *in vitro* can be utilized as a therapeutic vaccination against autologous CSCs in GC patients in the future.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgements

This research was supported in part by grants from Mashhad University of Medical Sciences (930565 and 930607), and by grant REP152 from Presidency of the Islamic Republic of Iran.

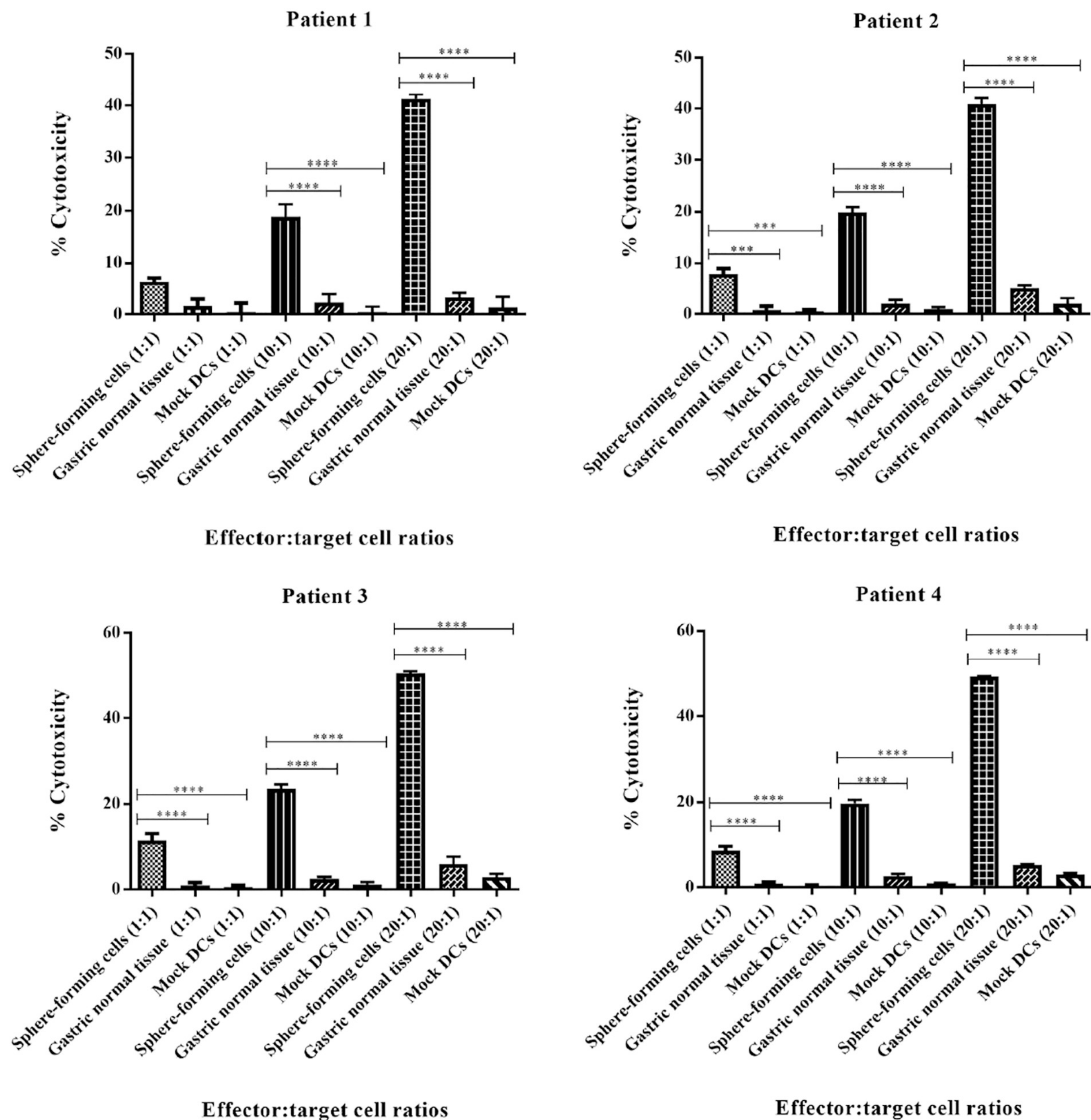


Fig. 4. Cytotoxic activity of stimulated T-lymphocytes by DCs loaded with total mRNA of sphere-forming cells. Calcein-release assays were performed to measure the cytotoxic activity of stimulated T-lymphocytes by DCs transfected with total mRNA of sphere-forming cells and gastric normal tissue as well as mock DCs against sphere-forming cells at various effector:target ratios (1:1, 10:1, 20:1). The results from four patients were expressed as a mean of triplicates (mean ± SE). Stimulated T-lymphocytes with antigens of sphere-forming cells isolated from four patient demonstrated effective lysis of sphere-forming cells but stimulated T-lymphocytes with antigens of gastric normal tissue as well as mock DCs did not induce significant lysis of sphere-forming cells ($P \leq 0.0001$).

References

[1] R. Herrero, J. Parsonnet, E.R. Greenberg, Prevention of gastric cancer, JAMA 312 (2014) 1197–1198, https://doi.org/10.1001/jama.2014.10498.

[2] P. Khosravi Shahi, et al., Management of gastric adenocarcinoma, Clin. Transl. Oncol. 9 (2007) 438–442.

[3] M.R. Abbaszadegan, et al., Isolation, identification, and characterization of cancer stem cells: a review, J. Cell. Physiol. 232 (2017) 2008–2018, https://doi.org/10.1002/jcp.25759.

[4] T. Reya, et al., Stem cells, cancer, and cancer stem cells, Nature 414 (2001) 105–111, https://doi.org/10.1038/35102167.

[5] P.B. Gupta, C.L. Chaffer, R.A. Weinberg, Cancer stem cells: mirage or reality? Nat. Med. 15 (2009) 1010–1012, https://doi.org/10.1038/nm0909-1010.

[6] P.V. Pham, et al., Targeting breast cancer stem cells by dendritic cell vaccination in humanized mice with breast tumor: preliminary results, Onco Targets Ther. 9 (2016) 4441–4451, https://doi.org/10.2147/OTT.S105239.

[7] Q. Chen, et al., Immunotherapy with dendritic cells and cytokine-induced killer cells for MDA-MB-231 breast cancer stem cells in nude mice, Am. J. Transl. Res. 8 (2016) 2947–2955.

[8] J.C. Sun, et al., Dendritic cells-mediated CTLs targeting hepatocellular carcinoma stem cells, Cancer Biol. Ther. 10 (2010) 368–375.

[9] Z.Y. Xiao, et al., An experimental study of dendritic cells transfected with cancer stem-like cells RNA against 9L brain tumors, Cancer Biol. Ther. 11 (2011) 974–980.

[10] S. Adams, D.W. O'Neill, N. Bhardwaj, Recent advances in dendritic cell biology, J. Clin. Immunol. 25 (2005) 87–98, https://doi.org/10.1007/s10875-005-2814-2.

[11] L. Spel, et al., Antitumor immune responses mediated by dendritic cells: how signals derived from dying cancer cells drive antigen cross-presentation, Oncoimmunology 2 (2013) e26403, , https://doi.org/10.4161/onci.26403.

[12] J. Banchereau, A.K. Palucka, Dendritic cells as therapeutic vaccines against cancer, Nat. Rev. Immunol. 5 (2005) 296–306, https://doi.org/10.1038/nri1592.

[13] D. Boczkowski, et al., Induction of tumor immunity and cytotoxic T lymphocyte responses using dendritic cells transfected with messenger RNA amplified from tumor cells, Cancer Res. 60 (2000) 1028–1034.

[14] C.L. Chiang, G. Coukos, L.E. Kandalaft, Whole tumor antigen vaccines: where are we? Vaccines (Basel) 3 (2015) 344–372, https://doi.org/10.3390/vaccines3020344.

[15] P. Parajuli, S. Mathupala, A.E. Sloan, Systematic comparison of dendritic cell-based

immunotherapeutic strategies for malignant gliomas: in vitro induction of cytolytic and natural killer-like T cells, Neurosurgery 55 (2004) 1194–1204.
[16] L.B. Weiswald, D. Bellet, V. Dangles-Marie, Spherical cancer models in, Tumor Biol. 17 (2015) 1–15, https://doi.org/10.1016/j.neo.2014.12.004.
[17] S. Nath, G.R. Devi, Three-dimensional culture systems in cancer research: focus on tumor spheroid model, Pharmacol. Ther. 163 (2016) 94–108, https://doi.org/10.1016/j.pharmthera.2016.03.013.
[18] V. Bagheri, et al., Isolation and identification of chemotherapy-enriched sphere-forming cells from a patient with gastric cancer, J. Cell. Physiol. 233 (2018) 7036–7046, https://doi.org/10.1002/jcp.27578.
[19] T. Chen, et al., Identification and expansion of cancer stem cells in tumor tissues and peripheral blood derived from gastric adenocarcinoma patients, Cell Res. 22 (2012) 248–258, https://doi.org/10.1038/cr.2011.109.
[20] M.E. Han, et al., Cancer spheres from gastric cancer patients provide an ideal model system for cancer stem cell research, Cell. Mol. Life Sci. 68 (2011) 3589–3605, https://doi.org/10.1007/s00018-011-0672-z.
[21] S. Nagrath, et al., Isolation of rare circulating tumour cells in cancer patients by microchip technology, Nature 450 (2007) 1235–1239, https://doi.org/10.1038/nature06385.
[22] D. Brungs, et al., Gastric cancer stem cells: evidence, potential markers, and clinical implications, J. Gastroenterol. 51 (2016) 313–326, https://doi.org/10.1007/s00535-015-1125-5.
[23] T.D. Schmittgen, K.J. Livak, Analyzing real-time PCR data by the comparative C(T) method, Nat. Protoc. 3 (2008) 1101–1108.
[24] C.A. O'Brien, et al., A human colon cancer cell capable of initiating tumour growth in immunodeficient mice, Nature 445 (2007) 106–110, https://doi.org/10.1038/nature05372.
[25] L.P. Deleyrolle, et al., Determination of somatic and cancer stem cell self-renewing symmetric division rate using sphere assays, PLoS One 6 (2011) e15844, , https://doi.org/10.1371/journal.pone.0015844.
[26] A.Y. Huang, et al., Role of bone marrow-derived cells in presenting MHC class I-restricted tumor antigens, Science 264 (1994) 961–965.
[27] S. Ishigami, et al., Prognostic value of HLA-DR expression and dendritic cell infiltration in gastric cancer, Oncology 55 (1998) 65–69.
[28] S.K. Nair, et al., Induction of primary carcinoembryonic antigen (CEA)-specific cytotoxic T lymphocytes in vitro using human dendritic cells transfected with RNA, Nat. Biotechnol. 16 (1998) 364–369, https://doi.org/10.1038/nbt0498-364.
[29] M. Gholamin, et al., Induction of cytotoxic T lymphocytes primed with tumor RNA-loaded dendritic cells in esophageal squamous cell carcinoma: preliminary step for DC vaccine design, BMC Cancer (2010), https://doi.org/10.1186/1471-2407-10-261.
[30] C. Pilon, et al., CD40 engagement strongly induces CD25 expression on porcine dendritic cells and polarizes the T cell immune response toward Th1, Mol. Immunol. 46 (2009) 437–447, https://doi.org/10.1016/j.molimm.2008.10.014.
[31] N. Delirezh, et al., Autologous dendritic cells loaded with apoptotic tumor cells induce T cell-mediated immune responses against breast cancer in vitro, Cell. Immunol. 257 (2009) 23–31, https://doi.org/10.1016/j.cellimm.2009.02.002.
[32] E.O. Vik-Mo, et al., Therapeutic vaccination against autologous cancer stem cells with mRNA-transfected dendritic cells in patients with glioblastoma, Cancer Immunol. Immunother. 62 (2013) 1499–1509, https://doi.org/10.1007/s00262-013-1453-3.
[33] Z. Su, et al., Immunological and clinical responses in metastatic renal cancer patients vaccinated with tumor RNA-transfected dendritic cells, Cancer Res. 63 (2003) 2127–2133.
[34] J.A. Kyte, et al., Phase I/II trial of melanoma therapy with dendritic cells transfected with autologous tumor-mRNA, Cancer Gene Ther. 13 (2006) 905–918, https://doi.org/10.1038/sj.cgt.7700961.
[35] A.N. Houghton, Cancer antigens: immune recognition of self and altered self, J. Exp. Med. 180 (1994) 1–4.
[36] R.A. Mahmoudian, M.R. Abbaszadegan, M. Gholamin, Applying subtractive hybridization technique to enrich and amplify tumor-specific transcripts of esophageal squamous cell carcinoma, Pathol. Oncol. Res. 23 (2017) 271–279, https://doi.org/10.1007/s12253-016-0090-5.
[37] Z. Song, et al., Enhanced antitumor effects of a dendritic cell vaccine transfected with gastric cancer cell total RNA carrying the 4-1BBL gene in vitro, Exp. Ther. Med. 3 (2012) 319–323, https://doi.org/10.3892/etm.2011.394.
[38] B.Y. Liu, et al., Antitumor effects of vaccine consisting of dendritic cells pulsed with tumor RNA from gastric cancer, World J. Gastroenterol. 10 (2004) 630–633.