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Research Paper

Allogenic γδ T cell and tumor cell fused vaccine for enhanced immunotherapeutic efficacy of osteosarcoma

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ABSTRACT

Human γδ T cells have displayed their potential in cancer immunity through efficient tumor killing activities. Besides, they are also known for their capacity of antigen presentation. How to improve γδ T cells' immunotherapeutic effect as the cell vaccine is still a great challenge. Herein, we explore the human γδ T cells and tumor cell fused vaccine for enhanced immunotherapeutic efficacy of osteosarcoma. The fusion cell vaccine was prepared by chemical fusion between human γδ T cells and inactive osteosarcoma Saos-2 cells. The fusion process was confirmed by microscopy observation, and flow cytometry analysis further validated the antigen presenting functions of the fusion cells. Moreover, the immunotherapeutic potential of the fusion cells was then verified *via* cytotoxicity assay and cytokine release detection. Our study provided novel immunotherapeutic strategy for patients with osteosarcoma, which merits further practice in the near future.

1. Introduction

Osteosarcoma (OS) is the main primary bone cancer often seen in children and adolescents [1,2]. It is bone-forming tumor characterized by cancer cells producing osteoid matrix and associated with a very complicated tumor microenvironment including stromal cells and immune infiltrates [3,4]. Despite the therapeutic advances over the past decades, the current regimens fall short of expectations for the improvement of patients' prognosis with high relapse rates at nearly 35% [5]. Besides, metastasis still remains the major cause of death in OS patients. As 80%-90% patients are assumed to have metastatic diseases whereas only 20% of them are clinically detectable [6]. Given the current deficiencies in metastasis detection and resulting poor survival rate, it is urgent to develop novel therapeutic strategies that could enhance the host immuno-surveillance for the reduction of such adverse events.

Antigen-presenting cells (APC), such as the dendritic cells (DC) in the mammalian immune system, can process and present the pathogens to launch the immune responses against these 'invaders' [7]. In the past decade, DC-based vaccines have been widely studied for the prevention of tumor metastasis and relapse. However, multiple factors have limited the adoption of this strategy in clinic, accounting for the paucity in the approved cell products [8]. On the other side, γδ T cells, accounting for less than 10% of peripheral blood T cells, are known for their potential to target tumor cells due to their direct recognition of tumor without the restriction of major histocompatibility complex (MHC) molecules [9,10]. Recent studies demonstrated their antigen presentation function and conceivable alternative to DC cells as vaccines in the treatment of cancer [11].

APC and the tumor antigen conjugation hybrids (APC-based vaccines) have long been considered as the ideal candidate for boosting anti-tumor responses [12]. In spite of antigen presentation, the APC-based vaccines can also improve the host immunity and compensate for the immunogenic weaknesses of tumor cells. Up to date, despite the identification of some tumor antigens, few studies have been associated with APC and tumor antigen hybrids [13]. Scientific questions such as complex purification of tumor antigens need to be addressed before the clinical application of such cellular products. In γδ T cells case, their ability of tumor antigen processing is still under investigation [14]. Accordingly, the novel idea of APC fused with tumor cell has been proposed in recent studies [15]. Given the whole repertoire of tumor antigens, this hybrid vaccine could induce a myriad of cytotoxic T

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lymphocyte (CTL) clones and demonstrate potent cytotoxicity against invasive tumor cells [16].

In this study, we isolated the peripheral blood mononuclear cells (PBMC) from healthy donors and expanded the γδ T cells using zoledronic acid. Then, we prepared the allogeneic γδ T cells-based OS vaccine *in vitro*. The results showed that the prepared fusion cells (FCs) demonstrated tumor antigen-specific CTL responses against two human OS cell lines, namely MNNG/HOS and Saos-2. Our results shed a new light on the application of tumor-associated vaccines and provide practicality of immunotherapy with γδ T cells-based vaccine against OS.

2. Materials and methods

2.1. Ethical statement

Research was approved by the Human Research Ethics Committees of the Second Affiliated Hospital, School of Medicine, Zhejiang University (Hangzhou, China). This research was performed in accordance with the Declaration of Helsinki and according to national and international guidelines. Before donating blood, the volunteers had known and confirmed the content that the blood was going to be used for scientific research. We are sure that all the healthy volunteers agreed to participate in the study in verbal informed consent.

2.2. Cell lines and cell culture

The human osteosarcoma lines MNNG/HOS and Saos-2 were obtained from Cell Bank of Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). Their identity was verified by short tandem repeat analysis. All the tumor cell lines were cultured in Dulbecco's modified Eagle's medium (Gibco, Rockville, MD, USA), supplemented with 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA, USA), and 100 μg/mL streptomycin–penicillin. Cells were maintained at 37°C in 5% CO_2.

2.3. Isolation and purification of $CD3^+$ T cells and γδ T cells

Fresh peripheral blood was collected in sodium-heparin vacutainer tubes. PBMCs were isolated by Ficoll density gradient (Sigma Aldrich) centrifugation. $CD3^+$ T cells were isolated and purified from fresh PBMCs of healthy donors by positive selection using human $CD3^+$ T Cell Isolation Kits (Miltenyi Biotech) according to the manufacturer's instructions. The prepared cells were used as responders and reactive cells in the following experiments. For the isolation of γδ T cells, 2.5 μM zoledronate (Zometa; Novartis) and recombinant human IL-2 (200 IU/mL; PeproTech) were added after the seperation of PBMCs. Then cells were supplemented with rIL-2 at the same concentration every two days. Following 14 days culture, the cells were harvested and can be further purified using the human TCR γ/δ^+ T Cell Isolation Kit (Miltenyi Biotech, Bergisch Gladbach, Germany).

2.4. Preparation of FCs by γδ T cells and tumor cell fusion

Methods for fusing the allogeneic γδ T cells and Saos-2 cell line were based on the polyethylene glycol (PEG)-fusion protocol [17]. Before fusion, the Saos-2 cells were treated with 10 mg/mL mitomycin C (MMC) for 4 h, rinsed with PBS (pH 7.4) 3 times to remove the residual of MMC. The inactive Saos-2 cells were then detached for the subsequent application. In a 50 mL centrifuging tube, the freshly-isolated γδ T cells and inactive Saos-2 cells were mixed at a ratio of 5:1 and centrifuged at 800G with brake and acceleration turn off for 10 min. In 90 s, total of 1 mL pre-warmed 50% PEG (MW: 1500, v/v) was added dropwise to the centrifugal sedimentation with continuous and gentle stirring. After 1 min of stewing in 37 °C, RPMI-1640 medium was dripped slowly into the mixture until the overall volume reached 50 mL to end fusion. The solution was centrifuged and the FCs were re-suspended in RPMI-1640 medium, FBS and rIL-2 were added every 2 days. On day 10-12, the FCs were detached and re-suspended in medium spontaneously, that is, the FCs vaccine. The structure of FCs was validated by transmission electron microscopy and laser scanning confocal microscopy.

2.5. Evaluate the fusion efficiency and phenotypes of FCs by flow cytometry

Briefly, cells (1×10^5) were suspended in PBS containing 2% FBS for 10 min to block nonspecifc binding sites and then were incubated at 4°C for 30 min. As to verify the purity of *in vitro* expanded γδ T cells, CD3-Percp/Cy5.5 and TCR-γδ-FITC were used to label the γδ T cells in the sample. As to verify the fusion efficiency of the FCs, the vybrant ® DiD/DiO cell-labeling solution (ThermoFisher Scientific, USA) were used to label the tumor cells and γδ T cells, respectively. As to evaluate the APC-like phenotypes of the fusion cells, a combination of antibodies was used: HLA-DR-PE, CD80-PE, CD86-PE; all were purchased from Biolegend, USA. Low forward scatter elements (debris) were excluded from analysis, and 10,000 events were collected and analyzed by FACSAria cytometer (BD Biosciences).

2.6. Measurement of cytokines by ELISA

T cells were cultured in 24 well plates with complete medium, designated as the effector cells. Fusion cells, γδ T cells alone were plated with effector cells at the ratio of 1:5 (2×10^5 stimulating cells/1×10^6 effector cells) and cultured for 3 days. The supernatants from fusion cells and γδ T cells culture were collected and stored at -80°C until later analysis. Cytokines of IFN-γ, IL-12 concentrations were measured using enzyme-linked immunosorbent assay (R&D systems) according to the manufacturer's instructions.

2.7. The cytotoxic reactions induced by T lymphocytes and FCs in vitro

In order to evaluate the cytotoxic responses of T lymphocytes and FCs, Saos-2 cells were pre-cultured in 96-well microplates for 24 h to attach the bottom wall. After the removal of the culture medium, T lymphocytes and FCs as effector cells were added to Saos-2 cells at ratio of 10:1, 20:1 (effector cell: target cell), the medium and cytokines were used as previously described. The experiment was divided into FCs, $CD3^+$ T cell, FCs + $CD3^+$ T cell and control group respectively. For FCs + $CD3^+$ T cell group, the effector cells were consisted with equal volume of FCs and $CD3^+$ T cell. The Saos-2 cells that cultured in pure medium without effector cells were regarded as the control group. After co-incubated for 48 h, the suspended cells (effector cells and dead target cells) were removed, 10 mL CCK-8 reagents and 100 mL RPMI-1640 media were added to each well and incubated for another 2 h and then, the optical densities (OD) were detected in 450 nm with a microtiter plate (ELISA) reader (Thermo Multiskan MK3, USA). For the evaluation of cytotoxic responses against different target cells, the experiment was divided into FCs, $CD3^+$ T cell, FCs + $CD3^+$ T cell group respectively. E/T ratio was 20:1. The percentage of cytotoxicity (means ± SD of three replications) was calculated with the following equation: killing rate = [(OD value of control well - OD value of reactive well)/OD value of control well] × 100%.

2.8. Statistical analysis

All data are presented as mean ± SD in the legends. Data were treated by SPSS software (version 16.0, SPSS, Chicago, IL, USA). Comparison of means used t test and one-way analysis of variance to determine the statistical difference, when p-value was 0.05 or less, differences were considered statistically significant.

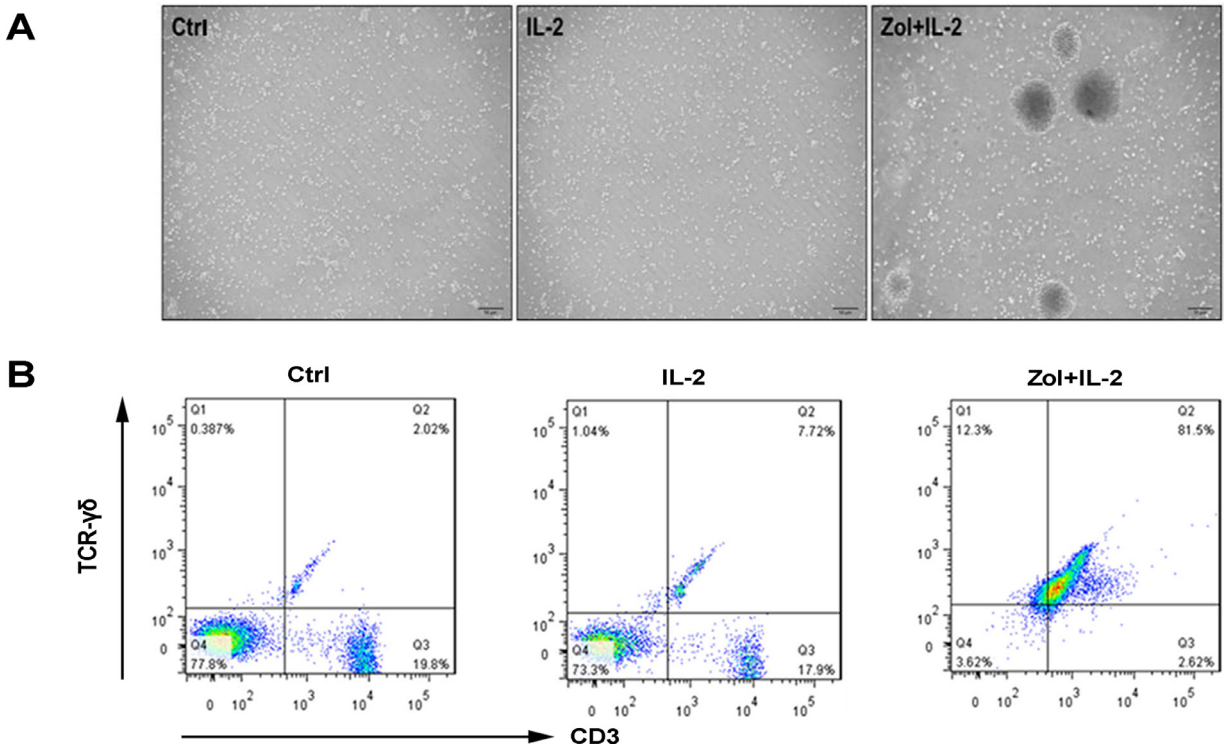

Fig. 1. The expansion of γδ T cells is observed by (A) light microscope and detected by (B) FACS.

3. Results

3.1. The expansion and purification of γδ T cells

Human PBMC were isolated from healthy donors by the application of the Ficoll–Hypaque gradient. Cells were adjusted to 1×10^6 cells/mL and cultured in RPMI 1640 supplemented with 10% FBS and antibiotics. Then, 2.5 μM zoledronate and 200 U/ml rIL-2 were used for the stimulation of γδ T cells with the addition of rIL-2 every two days. After 14 days of expansion, the morphology of γδ T cells were analyzed under light microscopy. As is shown in Fig. 1A, the combination of zoledronate (Zol) and rIL-2 induced the robust proliferation of T cells as compared with the other control groups. It is noticeable that cell aggregates were also observed in the (Zol + rIL-2) treated group, indicating their benign state. As for the expansion efficiency, cell samples were stained with monoclonal antibodies specific for the γδ-TCR receptors and subsequently analyzed via flow cytometry. In spite of the irrelevant αβ T cells and NK cell, 81.5% of the cells in the medium were proved to be γδ T cells (Fig. 1B). Then, the collected γδ T cells were enriched by negative isolation using the human TCR γ/δ^+ T Cell Isolation Kit. As we can see in Fig. 5, 96.6% purity was achieved after the procedure. Cultures with a purity > 95% were enrolled for the following experiments.

3.2. The structures of fusion vaccine

The Day 14 γδ T cells were fused with MMC-pretreated OS cell line Saos-2 via chemical fusion. The structure of fusion cells was validated by TEM. After the fusion process and subsequent cultivation, many γδ T cells combined with the tumor cells. As is shown in Fig. 2A, different stages of fusion process were captured, cytomembrane and cytoplast of the two parent generations had been completely fused. While the karyons of parent cells were clung to each other with an obvious fusion line in this slice (Fig. 2A(c)), the ones in Fig. 2A(a) had already formed the heterokaryons during the chemical fusion processes. Furthermore, DiO-labeled γδ T cells and DiD-labeled Saos-2 cells were used in the confocal microcopy observation. The colocalization of γδ T cells and Saos-2 cells in Fig. 2B also displayed the fusion process. Taken together, these results showed that the prepared fusion cells in this study were composed of the properties from both parent cells.

3.3. Phenotypes of fusion cells

As shown in Fig. 3A, the FACS results showed the labeling efficiency of both involved cells, confirming their viability for the following determination. After the chemical fusion and 7 days of culture, the cells showed with 69.4% double positive of fusion cells. The obtained fusion efficacy is higher than the fusion yields of previous researches in terms of APC-based fusion vaccines [18,19]. The result presented unequivocal evidence that the fusion cells in our research were indeed well prepared. Then, the APC functions of the fusion cells were further assessed by FACS. As is shown in Fig. 3B, FCs had a high expression of CD80, CD86 and HLA-DR molecules, indicating its capacity of antigen presenting in response to stimulators.

3.4. Enhanced T cell activation and tumor specific cytotoxicity effects induced by FCs in vitro

Next, we intended to investigate the immunostimulatory properties of the prepared FCs, the ability to induce the activation of T cells was examined *via* ELISA. Supernatant of the two groups was collected at indicated time points to detect the expression of IFN-γ and IL-12. The results demonstrated that, in contrast to the peripheral-derived γδ T cells, FCs induced significantly higher activation of T cells with the higher expression of IL-12 and IFN-γ, suggesting the effective Th1 immune response, which is favorable for anti-cancer immunity (Fig. 4A). Ever since the FCs had captured and processed a repertoire of antigens during the chemical fusion process, they would be equipped with substantial competence to present the tumor antigens to T cells and elicit the subsequent cytotoxic lysis. In order to investigate whether tumor specific cytotoxicity could be improved by FCs, we evaluated the viability of different target cells via coculturing them with different

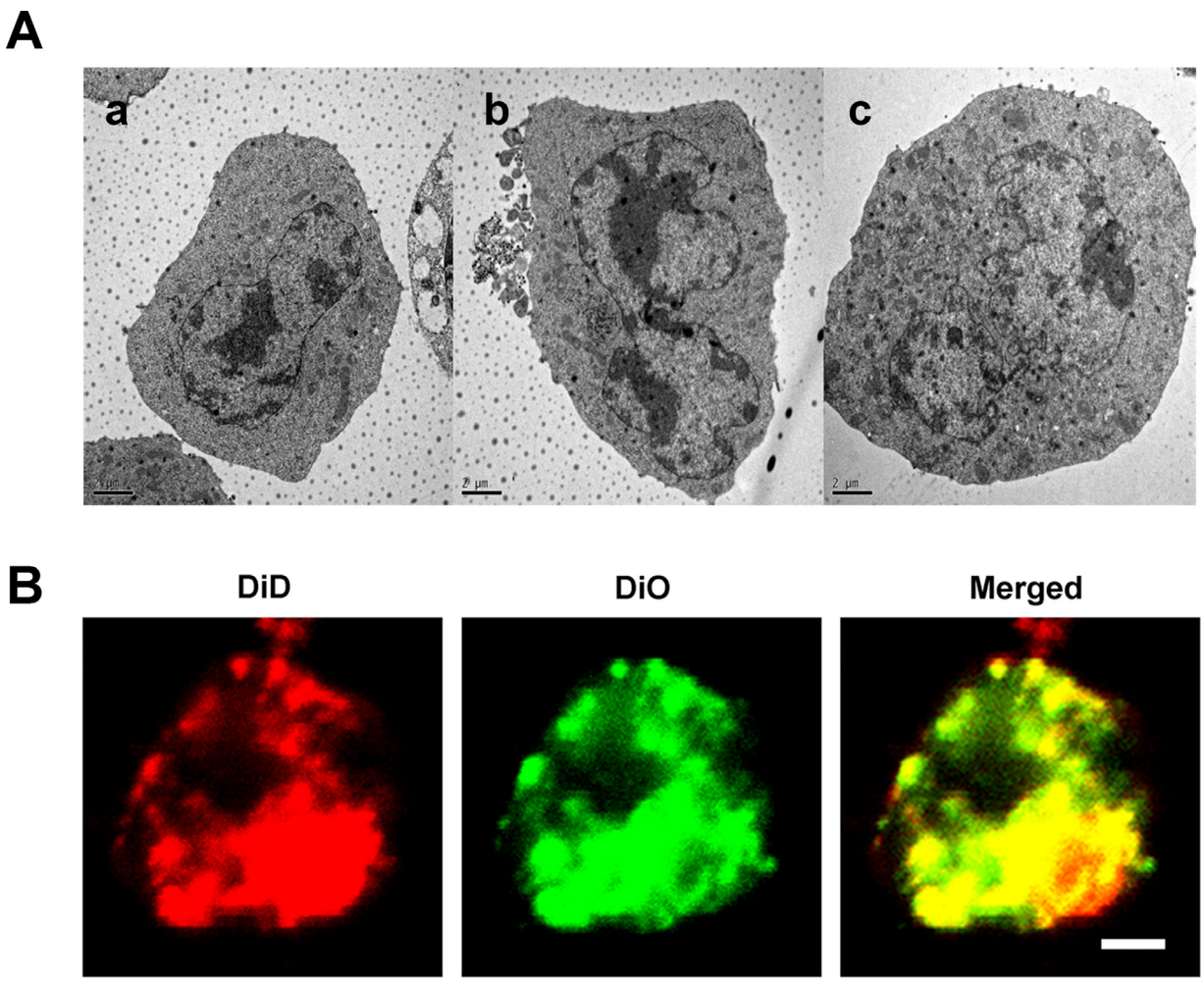

Fig. 2. The structure of fusion cells. (A) The TEM images of fusion cells, displaying different stages of the fusion process, scale bar 2 μm. (B) Confocal micrographs of fusion cells. DiD was used to label Saos-2 cells (red), DiO for γδ T cells (green), Scale bar 10 μm.

groups of effector cells, namely FCs, $CD3^+$ T cells, FCs + $CD3^+$ T cells. The enhanced specific cytotoxicity against Saos-2 cells was observed after 48 h incubation, while more significant cytotoxic effect was witnessed in the (FCs + $CD3^+$ T cells) group when compared with the others (Fig. 4B). Comparatively modest results were achieved from another target cell, namely MNNG/HOS (Fig. 4C). This restricted cytotoxicity can be attributed to the partially shared antigenic components between the two different tumor cells, since the fusion cells were composed of γδ T cells and Saos-2 cells. Collectively, these observations preliminarily confirmed the feasibility of γδ T cell-based fusion vaccine against OS.

4. Discussion

Despite the absence of approved immunotherapeutic agents against sarcomas, it remains an area of active exploration and investigation. To date, multiple modalities to immunotherapy have been evaluated in the treatment of sarcomas, including checkpoint blockade immunotherapy (CBI), adoptive cell therapy (ACT) and cancer vaccines [20]. Recent efforts seek to pursue the success achieved with CBI in other cancers by extending these rationales to sarcoma patients. As clinical experience with single agent CBI has generally been dismal, skeptics insist that relatively lower mutational burden found in sarcomas is inadequate to evoke adoptive immune responses [21,22]. Double agents CBI combined with or without other modalities (such as radiotherapy), however, have provided more promising outcomes [23,24]. On the other hand, highly immunogenic antigens, such as NY-ESO-1 and Her-2, have been adopted in the development of ACT against sarcomas and are demonstrating positive progression in different clinical trials [25,26]. On the contrary, most of the studies on vaccines against sarcomas failed to demonstrate objective responses, which requires much improvement in the preparation of vaccines for clinical application [27,28].

The function of cancer vaccines depends on the activation of APC with the presence of immunogenic antigens. In general, APCs usually take antigens from 'foreign invaders' by phagocytosis and present them to MHC class II/I molecules to evoke the adaptive immune responses for the subsequent defense [29]. Dendritic cells, the notable representative of APC, are involved in many immunotherapeutic strategies, such as the infusion of patients with pre-generated DC-based vaccine. However, *in vitro* culture process can only yield limited number of DCs, thus restraining the development of DC-based vaccines in the clinic [30]. On the other side, γδ T cells were once portrayed as the connector between innate and adaptive immunity, and have been the subject of explosive interest due to their contributions in many types of immune responses [31]. Previous researches demonstrated that human γδ T cells from tonsillar tissues and tumor patients were capable of cross-presenting proteins or antigens to the effector $CD8^+$ αβ T cells, in a manner reminiscent of classic antigen-presenting cells [32]. It is widely accepted that γδ T cells can respond vigorously to phosphoantigens or bisphosphonates, resulting in the large number of expansions during *in vitro* culture. In our study, we adopted zoledronate as well as the delayed addition of IL-2 to achieve nearly 100 times of cell proliferation while

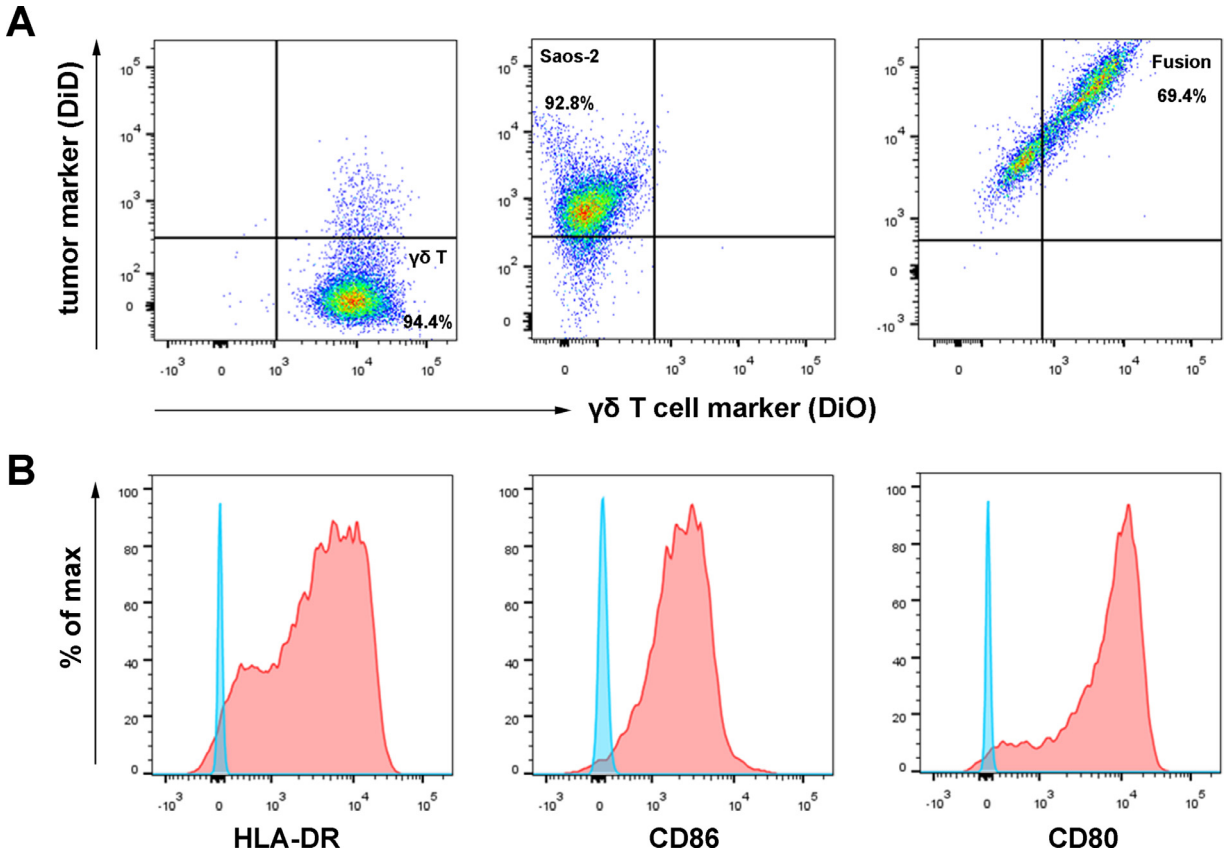

Fig. 3. Characterization of the fusion cells. (A) Labeling efficiency of Saos-2 cells, γδ T cells, and the fusion efficiency. (B) Phenotypes of the fusion cells as the APCs.

preventing the early expansion of irrelevant cells, such as NK cells [33]. Moreover, γδ T cells can be directly activated by the preferentially expressed antigens on tumor cells [34]. These unique capacity makes them advantageous subject in cell-based vaccine over DCs, regardless of the influence from cell frequency and disease stage [35, 36].

Another vital feature of professional APCs lies in their highly efficient antigen uptake and subsequent degradation. Of note, phagocytosis as well as macropinocytosis take place when γδ T cells confront the foreign pathogens, which might contribute to the antigen processing of this new type of APC [37]. Besides, many studies further demonstrated their high efficiency of antigen presentation [11]. Others, on the other hand, proposed numerous strategies to improve the APC-induced immune responses, including electroloading antigens into APCs, fusion of tumor peptides or tumor cells [38–40]. Intriguingly, the fusion cells from tumor cells and APC have been proved to overcome the poor immunogenicity of certain antigens and optimize the antigen presentation process via both class I and II pathways for the more balanced generation of helper and cytotoxic T cell reactions [41,42]. Hence, in the current study, we used polyethylene glycol 2000 to fuse tumor cells with γδ T cells, and a comparatively high fusion rate was achieved while preserving their vitality.

The fusion cells in our study still preserved the APC-like characteristics in terms of phenotype and function: (i) the fusion cells had high expression of HLA-DR and the costimulatory molecule CD80/86. (ii) They induced the secretion of Th 1-oriented cytokines, such as IFN-γ. (iii) More importantly, they triggered the αβ T cells-mediated cytotoxicity against osteosarcoma cells. The above-mentioned typical features were consistent with those found previously with DC-based vaccines [43,44]. Given the whole repertoire of antigens from tumor cells, the immunogenicity triggered by this fusion vaccine is expanded to a comparatively broad range, resulting in robust immune-responses.

Irrespective of these intriguing results of fusion cells-triggered responses *in vitro*, there is still a paucity of evidence for the feasibility of this novel cell vaccines *in vivo*, not to mention the subsequent clinical

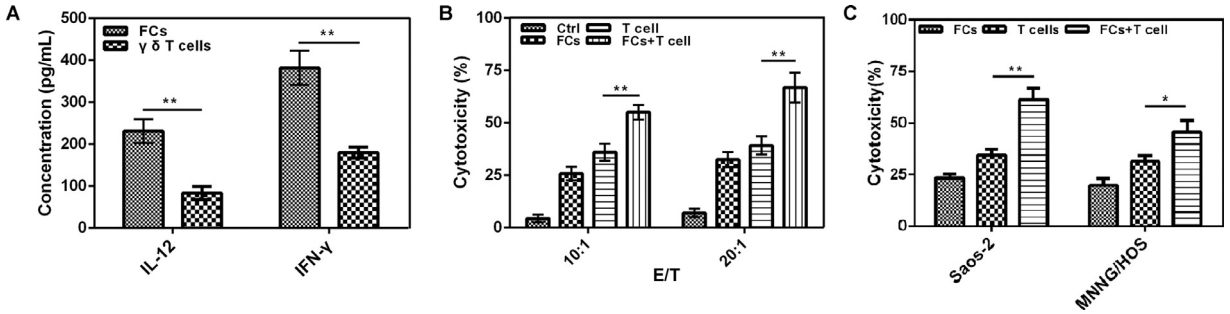

Fig. 4. Enhanced T cell function induced by fusion cells *in vitro*. (A) The cytokines variation of T cells, FCs and FCs + T cells groups. (B) Antigen-specific CTLs by fusion vaccine. The effector cells were harvested and incubated with target cells (Saos-2) at a ratio of 10:1, 20:1 in 96-microwell plates. (C) The cytotoxicity against different target cells (Saos-2, MNNG/HOS) at a E:T ratio of 20:1.

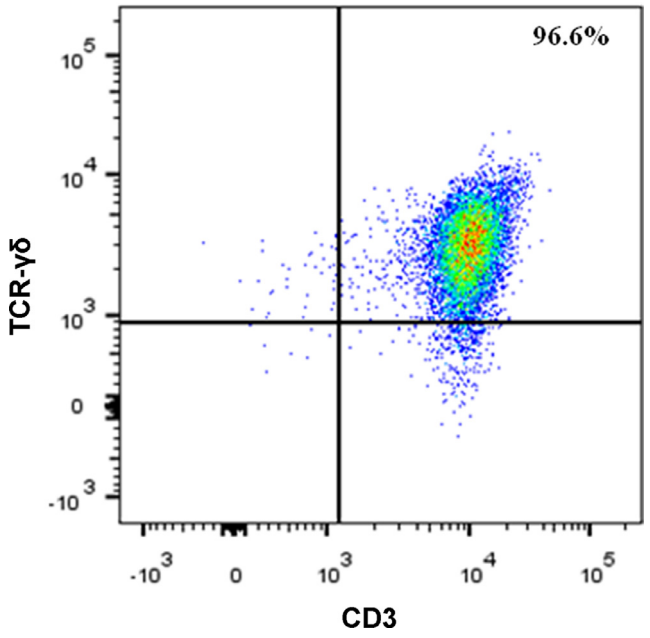

Fig. 5. The isolation efficiency γδ T cells.

trials. Of note, access to the tumor tissues or draining lymph nodes in humans is quite restricted, thus severely compromising the exploration of fusion cells as the novel APCs. On the other side, a substantial amount of evidence has already demonstrated the safety of repetitive infusions of γδ T cells, at least providing the accessibility of further investigation.

5. Conclusion

In summary, we have isolated γδ T cells from human peripheral blood, fused them with osteosarcoma cells. The fusion vaccine has the impressive function of inducing robust cytokine secretion and subsequently inhibiting osteosarcoma cells *in vitro*, making it a potential novel strategy for effective osteosarcoma immunotherapy.

Acknowledgment

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Conflict of interest statement

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, and there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the position presented in, or the review of, the manuscript entitled.

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