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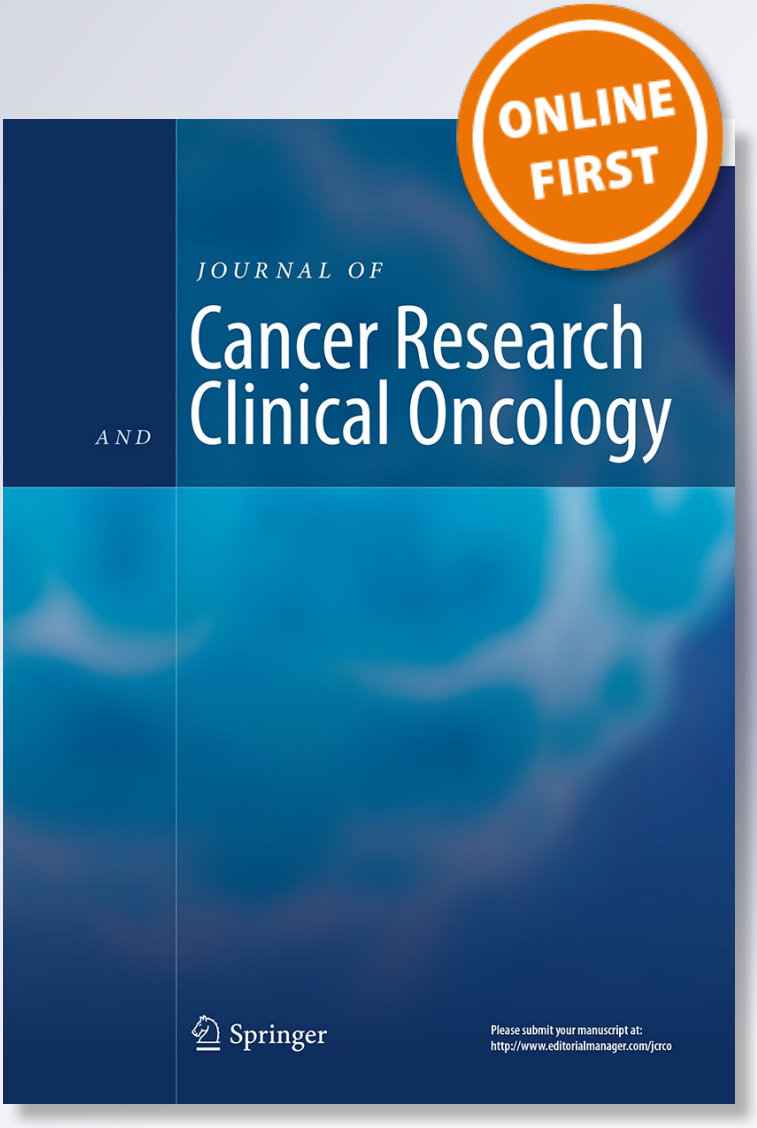

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ORIGINAL ARTICLE – CLINICAL ONCOLOGY

Prospective study of the safety and efficacy of a pancreatic cancer stem cell vaccine

Mao Lin[1] · Yuan-Ying Yuan[1] · Shu-Peng Liu[1] · Juan-Juan Shi[1] · Xin-An Long[1] · Li-Zhi Niu[1] · Ji-Bing Chen[1] · Qiao Li[2] · Ke-Cheng Xu[1]

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Abstract
Introduction In this trial, we isolated and cultured pancreatic cancer stem cells (CSCs) to produce a vaccine and prospectively evaluated its safety and efficacy in low-, medium-, and high-dose groups.
Material and methods Between February and October 2014, we enrolled 90 patients who met the enrollment criteria and assigned them to three groups ($n = 30$). CSC-specific and CSC-non-specific immunity pre- and post-vaccination were compared by Dunnett's multiple comparison test (one-way ANOVA). The data are presented as the mean±standard deviation. Local and systemic adverse events were recorded in the nursing records and compared using the Chi-square test. All statistical analyses were conducted using GraphPad software (GraphPad, San Diego, CA, USA).
Results Throughout the trial, an injection site reaction was the most common reaction (54 %), and fever was least common (9 %). The incidence of these side effects did not vary among the three groups. When the pre- and post-vaccination immunity was compared, we found that both CSC-nonspecific and CSC-specific responses were significantly increased in the high-dose group.
Conclusion This study is the first clinical trial of a pancreatic CSC vaccine and preliminarily proves its safety and efficacy.

Keywords Cancer stem cell · Vaccine · Pancreatic cancer · Safety · Efficacy

Introduction

Pancreatic cancer-related mortality is almost invariably due to metastasis (Siegel et al. 2011), which occurs in more than half of the patients diagnosed with this disease, often years after the primary tumor is diagnosed and removed (Jemal et al. 2010). The therapeutic alternatives for metastatic pancreatic cancer are mainly involve the systemic administration of cytotoxic chemotherapeutic agents (Heinemann et al. 2008; Wolff 2007), and the long-term survival is greatly dependent on the nature of the metastases and physical condition of patients (Moore et al. 2007; Tokh et al. 2012). Many pilot studies have confirmed that cryoablation, primarily in early and late stages of pancreatic cancer, is associated with improved therapeutic efficacy and fewer side effects (Xu et al. 2008a, b). Indeed, cryotherapy or cryoimmunotherapy may prolong the survival of patients with metastasis (Niu et al. 2013), and immunotherapy may have powerful therapeutic effects under low-burden load conditions (Kaneko et al. 2005; Koido et al. 2011).

Traditional DC-CIK immunotherapy supplements the lymphocyte count and function effectively, but has no tumor specificity (Marten and Buchler 2008; Thanendrarajan et al. 2011) and results in poor memory immune responses, which are important for the long-term prevention of tumor recurrence. Currently, accumulating evidence suggests that a tumor cell subpopulation with distinct stem

✉ Ji-Bing Chen
jibingchen398@163.com

✉ Qiao Li
qiaoli@umich.edu

[1] Fuda Cancer Hospital, Jinan University School of Medicine, Guangzhou 510665, China

[2] Comprehensive Cancer Center, University of Michigan, 1520 MSRB-1, Ann Arbor, MI, USA

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cell-like properties (the so-called cancer stem cells, CSCs) is responsible for tumor initiation, invasion, and metastasis in patients with pancreatic cancer (Habib and Saif 2013; Kumar et al. 2013; Xu 2013). In the laboratory, CSCs are identified by the CD44 marker and their ability to form new pancreatic cancer colonies through serial transplantations in immunodeficient hosts, thus reestablishing tumor heterogeneity (Ohara et al. 2013; Palagani et al. 2012; Wood 2014). Furthermore, while pancreatic cancer stem cells can also be identified by other surface markers, the CD44 MicroBead kit is the only commercialized kit that has been validated for pancreatic cancer stem cell identification.

To assess the feasibility of generating CSC vaccines for clinical use, we harvested peripheral blood and tumor specimens from patients with pancreatic adenocarcinoma. Lymphocytes were isolated from peripheral blood mononuclear cells (PBMCs), while CSCs were isolated from the tumor specimens. Cytotoxic T cell- and antibody-mediated CSC-specific and CSC-nonspecific immunity before and after the vaccination, as well as adverse event occurrence were compared.

Materials and methods

Ethics

This clinical trial was registered in the US National Institutes of Health (ID: NCT02074046; Ph1/Ph2) and approved by the Ethics Committee of Guangzhou Fuda Cancer Hospital. In accordance with the Declaration of Helsinki, written informed consent was obtained from each participant in Fuda Cancer Hospital.

Patient selection

This was a prospective study of the therapeutic effects of a CSC vaccine for pancreatic adenocarcinoma patients enrolled between February and October 2014. We enrolled 90 patients who fulfilled the following criteria: (1) had at least one resectable tumor, and other tumors could be well controlled by cryosurgery, brachytherapy, or chemotherapy, (2) the expected survival was >3 months, (3) were between 30 and 75 years of age, (4) the Karnofsky performance status score was >70, and (5) immune parameters fell with the following normal ranges: total T cells, 603–2990/μL; cytotoxic T cells, 125–1312/μL; helper T (Th) cells, 441–2156/μL; platelets $\geq 80 \times 10^9$/L; white blood cells $\geq 3 \times 10^9$/L; neutrophils $\geq 2 \times 10^9$/L; hemoglobin ≥90 g/L; prothrombin time international normalized ratio, 0.8–1.5; adequate hepatic function (bilirubin <20 μM, aminotransferase <60 U/L); and renal function (serum creatinine <130 μM, serum urea <10 mM), (6) absence of level 3 hypertension, severe coronary disease, myelosuppression, respiratory disease, acute or chronic infection, and autoimmune diseases. The contraindications for participation were T cell lymphoma, ongoing organ transplant, or patients presenting within 7 days of systemic chemotherapy. The enrolled patients were allocated to three groups ($n = 30$) with parallel assignments.

Preparation of CSC vaccine

The CSC vaccine contained three components: pancreatic CSC fragments, a multioil fat emulsion ($C_{6–24}$; Fresenius Kabi GmbH, Graz-Puntigam, Austria), and mannatide (Duokang; Chengdu Lier Pharmaceutical, Chengdu, China). The total vaccine volume was 0.5 mL: 0.3 mL CSC/saline solution (10^5, 3×10^5, and 5×10^5 CSCs for the low-, medium-, and high-dose groups, respectively), and 0.1 mL of each of the remaining components.

The CSC preparation process involved seven steps: (1) solid tumor resection (e.g., primary lesions or metastases in pancreas, liver, or lung with a diameter usually >4 cm), (2) preparation of single-cell suspensions using a human Tumor Dissociation Kit (no. 130-095-929; Miltenyi Biotec, Bergisch Gladbach, Germany), (3) CSC separation using a human CD44 MicroBead Kit (no. 130-095-194; clone MC56; Miltenyi Biotec), (4) CSC amplification and culture using a human StemMACS MSC Expansion Media Kit XF (no. 130-104-182; Miltenyi Biotec), (5) detection of cell purity by phycoerythrin (PE) antihuman CD44s (no. 130-095-180; clone DB105; Miltenyi Biotec), (6) cell counting and dilution to different concentrations, and (7) CSC lysis by repeated freezing and thawing five times. All steps were performed in accordance with the corresponding manufacturer instructions.

Vaccinations and tests

We used a vaccination schedule that has been previously established in an animal study from our group (Ning et al. 2012); the vaccination involved two subcutaneous injections on either side of the deltoid muscle, interspersed by a 1-week interval. Peripheral blood (~30 mL) was drawn for in vitro testing before and 1 week after vaccination (Fig. 1).

Peripheral blood (2 mL) was used for detecting CSC-nonspecific immunity by flow cytometry (FACSCanto™ II; BD, Franklin Lakes, NJ, USA). Multitest 6-color TBNK Reagent (no. 644611; BD) was used to detect the number of lymphocyte subsets; Cytometric Bead Array (CBA) Human Th1/Th2 Cytokine Kit II (no. 551809; BD) was used to detect cytokine expression levels. All steps were performed in accordance with the corresponding manufacturer instructions.

Using Ficoll-Hypaque density centrifugation, PBMCs and plasma were harvested from the remaining blood

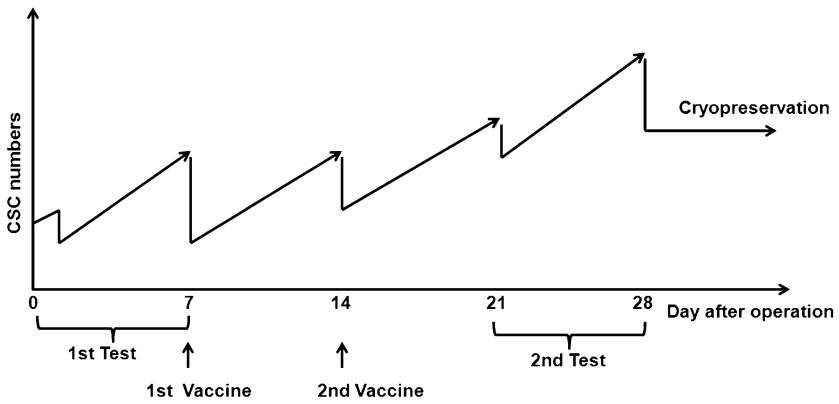

Fig. 1 Vaccination and test schedule, and trend chart of CSC numbers. *Broken lines* represent the gradual amplification of CSCs and single consumption during vaccination or testing

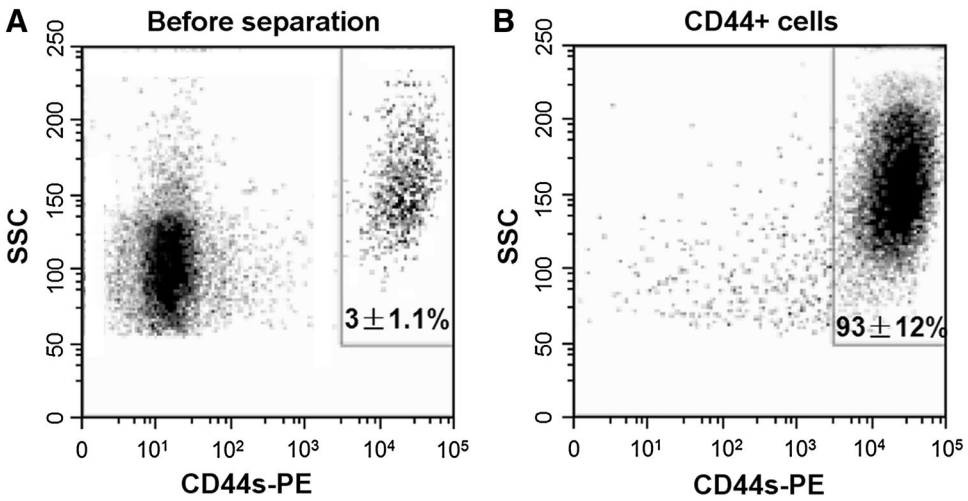

Fig. 2 CSCs in pancreatic cancer mass. **a** CSC purity in tumor cell suspension before separation. **b** CSC purity in separation products obtained using the human CD44 MicroBead Kit

samples, and cytotoxic T cell- and antibody-mediated CSC-specific immunity before and after vaccination were compared. CSC-specific testing included CSC binding by immune plasma, and complement-dependent cytotoxicity (CDC) and cytotoxic T lymphocyte (CTL) tests. Plasma CSC-binding antibodies were detected using fluorescein isothiocyanate antihuman immunoglobulin G (IgG, no. 130-099-229; clone IS11-3B2.2.3; Miltenyi Biotec) using previously described protocols (Ning et al. 2012). T lymphocytes were separated by human CD3 MicroBeads (no. 130-050-101; Miltenyi Biotec), activated by antihuman CD3/28 (no. 300314/302934, BioLegend, San Diego, CA, USA) and expanded using interleukin (IL)-2 (no. 200-02, PeproTech, Rocky Hill, NJ, USA), while DCs were induced and cultured using IL-4 (no. 200-04, PeproTech) and granulocyte–macrophage colony-stimulating factor (no. 300-03, PeproTech), as previously described (Niu et al. 2013).

Evaluation and statistical analysis

Complications were recorded and classified in accordance with the Common Terminology Criteria of Adverse Events version 4.02. Radiographic local tumor control was assessed using image-guided tumor ablation criteria (Goldberg et al. 2005). Patient demographics were compared among the three groups before vaccination by two-way analysis of variance (ANOVA); CSC-specific and CSC-nonspecific immunity pre- and post-vaccination were compared by Dunnett's multiple comparison test (one-way ANOVA), and data are presented as the mean ± standard deviation. Local and systemic adverse events were marked in the nursing records and compared using the Chi-square test. All analyses were conducted using GraphPad software (GraphPad, San Diego, CA, USA).

Results

Identification of CSCs

As per the protocol depicted in Fig. 1, tumors were surgically resected from the patients, and CSCs were then separated in the lab. Before separation, CSC purity was 3 ± 1.1 %; after separation, CSC purity increased up to

Table 1 Patient demographics

Patient characteristics before vaccination	Total ($n = 90$)	Low-dose group ($n = 30$)	Medium-dose group ($n = 30$)	High-dose group ($n = 30$)	P value
Gender (male/female)	43/47	16/14	15/15	12/18	>0.05
Median age (years)	55	58	54	47	>0.05
Differentiated degree of adenocarcinoma					
High	44	16	15	13	>0.05
Moderate	34	10	12	12	>0.05
Poor	12	4	3	5	>0.05
Clinical stage					
II	30	11	7	12	>0.05
III	36	12	14	10	>0.05
IV	24	7	9	8	>0.05
Karnofsky performance status					
80	26	7	9	10	>0.05
90	31	11	11	9	>0.05
100	33	12	10	11	>0.05
Chemotherapy	48	16	17	15	>0.05
Cryosurgery	51	14	18	19	>0.05
Surgery	90	30	30	30	>0.05

Factors of low-, middle-, and high-dose group were compared by two-way ANOVA

Table 2 Comparison of lymphocyte number and function before and after vaccination

Test items of lymphocyte	Testing results			
	Test 1 ($n = 90$)	Test 2 (low-dose group)	Test 2 (medium-dose group)	Test 2 (high-dose group)
Number (cell/μL)				
Total T cell	1392 ± 47	1410 ± 62	1467 ± 74	1790 ± 79**
CD8+T cell	504 ± 12	566 ± 13	606 ± 17*	650 ± 15*
CD4+T cell	64 ± 16	681 ± 28	704 ± 21	739 ± 18*
NK cell	293 ± 17	407 ± 37	437 ± 49	510 ± 58**
B cell	192 ± 14	245 ± 12	348 ± 22	434 ± 30**
Function (pg/mL)				
IL-2	10 ± 3	12 ± 4*	16 ± 3*	21 ± 4**
TNF-β	3 ± 2.4	4 ± 3.5	8 ± 2	16 ± 2.8*
IFN-γ	5 ± 3.1	6 ± 2.9	9 ± 3	15 ± 3.4*
IL-4	12 ± 2	11 ± 2.7	9 ± 3.2	10 ± 3
IL-6	18 ± 3.2	20 ± 3.1	18 ± 4	21 ± 9.2
IL-10	11 ± 2.6	12 ± 3.3	11 ± 2.5	10 ± 3.3

Every cell subset or cytokine were analyzed using Dunnett's multiple comparison test (one-way ANOVA)

NK cell natural killer cell, *IL* interleukin, *TNF* tumor necrosis factor, *IFN* interferon

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

93 ± 12 % (Fig. 2). All CSCs separated from the tumor mass were sufficient for culture, vaccination, and testing, and CD44+ cell purity after 2- and 4-week culture was similar to that immediately following separation (data not shown).

Clinical data

The patients ($n = 90$) enrolled in this study were from China (36/90), Indonesia (25/90), and Malaysia (29/90). Analysis of the pre-treatment data revealed no statistical

differences in patient demographics among the three groups (Table 1). Immune monitoring was carried out both pre-vaccination (Test 1) and post-vaccination (Test 2) (Table 2; Fig. 4).

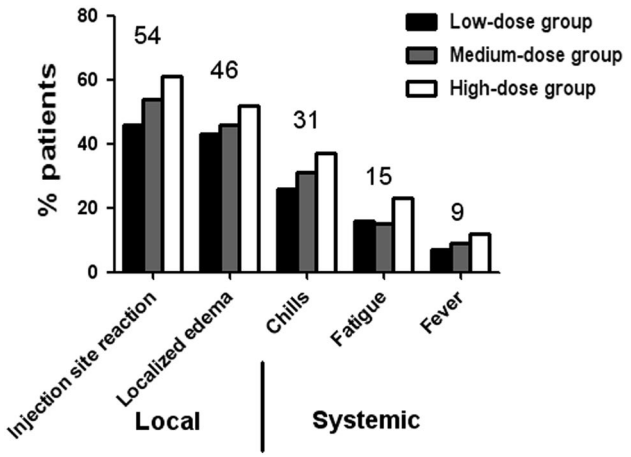

Fig. 3 Local and systemic adverse events. The incidences of each Grade 1 adverse event that occurred in the three groups are depicted in separate columns; figures above the columns indicate the average incidences in all patients

Adverse events

Throughout the trial, the adverse events experienced by the patients were recorded and included local (mainly injection site reaction or localized edema) and systemic (mainly chills, fatigue, or fever) reactions. The most common reaction was an injection site reaction (54 %), while fever was the least common (9 %). Other possible side effects such as blood or bone marrow changes were not detected. All adverse events were Grade 1, and all reactions were resolved within 1 day of treatment of the specific symptoms and did not recur. Adverse event occurrence was compared using the Chi-square test; there was no difference among the three groups ($P = 0.996$, Fig. 3).

CSC-nonspecific and CSC-specific immunity

Lymphocyte count and function before (Test 1) and after (Test 2) vaccination were compared, with patients in all three groups showing an increase in these parameters after vaccination (Table 2). Patient pre-vaccination data were merged and compared with that obtained post-vaccination (Table 2); the cell numbers for all subsets examined were significantly higher after vaccination in the high-dose

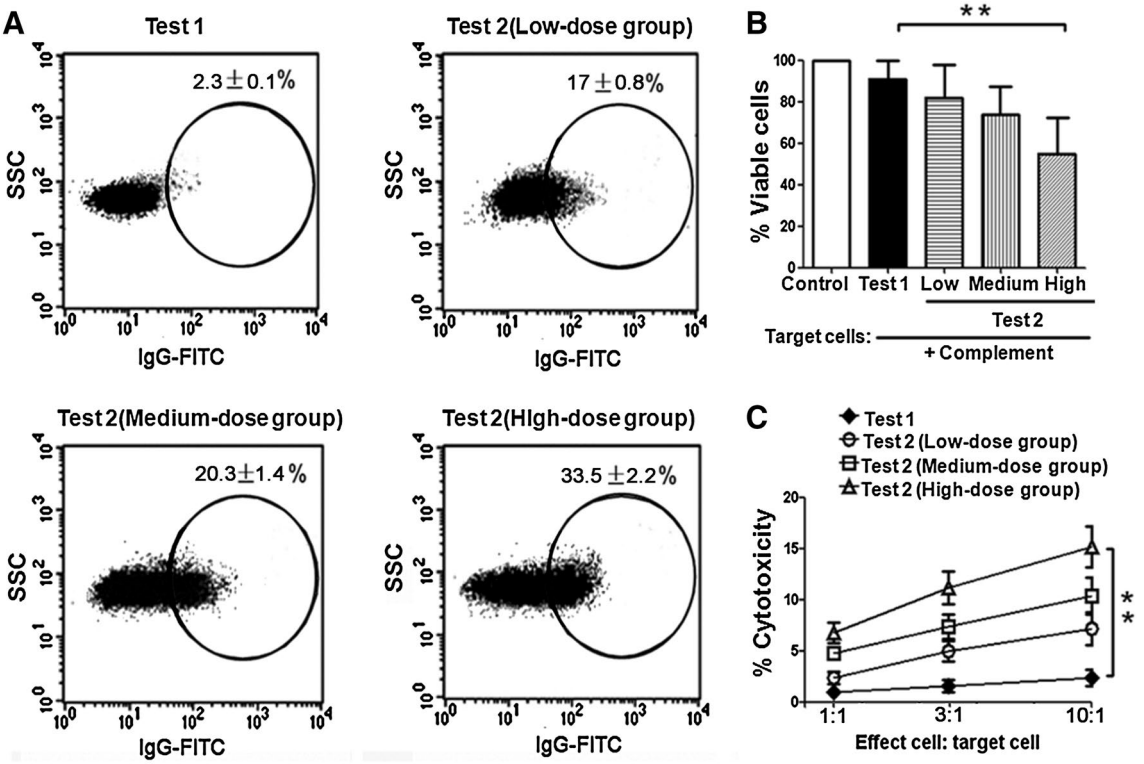

Fig. 4 Comparison of CSC-specific immunity pre- and post-vaccination. **a** Flow cytometry results of CSC-specific antibody levels in immune plasma and IgG levels. **b** CSC targeting by CSC-primed CDC; 10^5 viable CSCs were incubated with immune plasma for 1 h (control group: no immune plasma), then rabbit complement was added and incubated for another 1 h. Viable cells were then counted under a microscope after trypan blue staining. **c** CSC targeting by CSC-primed CTLs; CSC killing was evaluated using a lactate dehydrogenase release assay. A higher percentage of cytotoxicity indicated more cell lysis. *$P < 0.05$

group. Additionally, Th1-type cytokine levels were increased in the medium- and high-dose groups, while Th2-type cytokine levels were essentially unchanged.

CSC-specific immunity was also compared (Fig. 4) by examining CSC binding by immune plasma: The frequency of IgG-positive cells was significantly higher in all the groups post-vaccination ($P < 0.001$, Fig. 4a);

CSC lysis by immune plasma, as determined by the percentage of viable cells after CDC (91 ± 10 %; Test 1), was increased in the high-dose group (55 ± 19 %, $P < 0.01$; Fig. 4b). When CSC destruction by CTLs was examined, the cytotoxicity effects (for three effect/target proportions) were all significantly increased in the high-dose group ($P < 0.01$, Fig. 4c).

Discussion

The subject of some controversy, CSCs are considered capable of symmetric or asymmetric self-renewal, are resistant to standard chemotherapies or radiotherapies, and are less differentiated and tumorigenic, as evidenced by their capacity to establish tumors in immunodeficient mice even when a low number of cells is injected (Dick 2008; Visvader 2011). As the failure of current therapies to control cancer can be attributed to their inability to eliminate CSCs, it is critical to develop strategies that eliminate these stem cell-like tumor cells. Recent data suggest that immune-based approaches may be particularly attractive prospects for targeting CSCs: One strategy is to target CSCs via monoclonal antibodies such as CD47 (Majeti et al. 2009) and CD123 (Jin et al. 2009), which have been shown to eradicate leukemia stem cells in preclinical models. Another approach, which has also produced good effects in animal models, involves harnessing cellular immune components, such **as** antitumor T cells (Sato et al. 2009; Schatton and Frank 2009) or antitumor DCs (Ning et al. 2012; Xu et al. 2009), Currently, commercial CSC separation and amplification kits are widely used, offering technical guarantees for the clinical application of this vaccine. This study represents the first trial of a pancreatic CSC vaccine in clinical use and is a step forward in the exploration of cellular immune responses targeting CSCs. Importantly, no blood or bone marrow changes were detected in any patient, and local or systemic side effects that appeared could be alleviated within the day. From the perspective of efficacy, CSC-specific and CSC-nonspecific lymphocytes were both significantly increased as the number of CSCs in the vaccine was increased. Our findings support that the vaccination resulted in an activation of Th1 immune pathways (as evidenced by increases in IL-2, TNF-α, and IFN-γ levels post-vaccination) and increased the number of NK cells and T cells.

Additionally, our protocol was tailored to maximize the safety and efficacy of the vaccine: (1) all patients underwent cytoreductive surgery, radiotherapy, or cryosurgery to reduce the tumor burden and then received the vaccination. The organ-like structural environment of tumors is very hostile toward immune cells and hence may play a role in local immune tolerance (Plate 2011); a low tumor burden provides the necessary conditions for the in vivo survival and amplification of specific lymphocytes. (2) DC activation plays a central role in pancreatic cancer, where the level of tumor-specific lymphocytes decreases prior to tumor recurrence and increases again with immunization (Kaneko et al. 2005; Koido et al. 2011). Our experience with animal experiments established the foundations of how and when to vaccinate (Ning et al. 2012); although the drawing of extra blood for DC preparation is difficult, the addition of adjuvants (Mannatide Injection) compensated for this drawback and produced a good immune effect. (3) Ongoing CSC cultures ensured antigen activity during vaccination and detection. (4) Given the differences between allogeneic CSCs from different individuals, our approach of generating patient-derived CSC vaccines for each study subject guaranteed a CSC-specific response and appropriate activation of the T cell repertoire.

In summary, this study provides novel and preliminary evidence of the safety and efficacy of the pancreatic CSC vaccine in a clinical trial, but the vaccine requires further refining. At the cellular level, CSC vaccination may modulate immune responses via several mechanisms. For example, CSCs may selectively not express tumor-associated antigens associated with differentiation and may therefore be resistant to immune-mediated rejection or evade host immunosurveillance through the absence or downregulation of major histocompatibility complex class I molecules (Aptsiauri et al. 2007). Therefore, the mechanism by which the pancreatic CSC vaccine strengthens the specific and nonspecific response requires further investigation. From the viewpoint of a long-term curative effect, the benefit of this vaccine on progression-free survival and overall survival also requires further research.

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Conflict of interest We declared that we have no conflict of interest.

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