

A novel immunotherapy for superficial bladder cancer by intravesical immobilization of GM-CSF

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Abstract

In situ gene therapy with granulocyte-macrophage colony-stimulating factor (GM-CSF) was demonstrated to successfully inhibit tumour cell growth in a mouse orthotopic bladder cancer model, but suffered from several disadvantages, such as limited efficiency for gene delivery, low expression efficiency of the transgene and the safety concern resulting from viral vector. In order to address the limits, a novel immunotherapy was developed attentively through immobilization of streptavidin-tagged bioactive GM-CSF on the biotinylated mucosal surface of bladder wall on the basis of both the unique property of streptavidin (SA) to bind rapidly and almost irreversibly to any biotin-linked molecule and the outstanding ability of biotin to be incorporated easily into the proteins on the cell surface. The mouse orthotopic model of MB49 bladder cancer was used to evaluate the feasibility and efficacy of the novel immunotherapy performed twice a week for 3 weeks. Briefly, 1 day after intravesical implantation of 1×10^6 MB49 tumour cells in C57BL/6 mouse, 100 μ l of 1 mg/ml NHS-PEO4-biotin was instilled and allowed to incubate in the bladder for 30 min., followed by intravesical instillation of 100 μ l of 0.15 mg/ml SA-GM-CSF bifunctional fusion protein and incubation for 1 hr. SA-GM-CSF fusion protein was shown to be immobilized efficiently and durably on the biotinylated mucosal surface of bladder wall. The bladder cancer incidence was dramatically decreased from 100% in the control group to 37.5% in the SA-GM-CSF group. Importantly, 70% of the SA-GM-CSF-cured mice were protected against a second intravesical wild-type MB49 tumour challenge, indicating that an effective anti-tumour immunity was generated against MB49 bladder cancer. Thus, the novel immunotherapy may be an attractive therapeutic alternative and should be evaluated in bladder cancer patients.

Keywords: biotinylation • fusion protein • GM-CSF • immunotherapy • streptavidin • superficial bladder cancer

Introduction

Intravesical administration of Bacillus Calmette–GuÈrin (BCG) after transurethral resection is by far the most effective local therapy for superficial bladder cancer, the fifth most common cancer in the world. However, relapses are frequent and some patients develop resistance to BCG therapy [1, 2].

Granulocyte-macrophage colony-stimulating factor (GM-CSF) is secreted by many cell types, including B and T cells, macrophages, mast cells, endothelial cells and fibroblasts. It functions as a white blood cell growth factor to stimulate stem cells to

produce granulocytes (neutrophils, eosinophils and basophils) and monocytes including specialized antigen-presenting cells. A comparative analysis of vaccination with irradiated, murine tumour cells engineered to express a large number of immunostimulatory molecules has established the superior ability of GM-CSF to evoke potent, specific and long-lasting anti-tumour immunity. The tumour vaccination involves enhanced tumour antigen presentation by recruited dendritic cells and macrophages; the coordinated functions of CD4+ and CD8+ T cells, CD1d-restricted NKT cells and antibodies mediate protective immunity [3]. *In situ* gene therapy of GM-CSF was demonstrated to successfully inhibit tumour cell growth by decreasing the tumour incidence from 76.9% in the control group to 15.4–30.8% in the treatment group in the mouse orthotopic model of MB49 bladder cancer [4, 5]. However, a series of difficulties should be overcome before the full potential of gene transfer-based immunotherapy is realized clinically. These difficulties include the low gene transfer efficiency, the low transgene expression level and biosafety

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concern arising from the introduction of foreign genetic material into the patient [6–8]. Therefore, a novel alternative method that allows the efficient and durable display of exogenous immunostimulators such as GM-CSF on the bladder mucosal surface may have important therapeutic implications for superficial bladder cancer.

Streptavidin (SA) is a *Streptomyces avidinii*-derived, nonglycosylated homo-tetrameric protein, which can bind up to four D-biotin molecules. SA binds to biotin in a noncovalent fashion with an extremely high affinity with the K_D value of 10^{-15} M, 10^3 – 10^6 folds higher than that for typical antigen–antibody interaction. Due to both the property of SA to bind rapidly and almost irreversibly to any biotin-linked molecule and the ability of biotin to be incorporated easily into various biological materials, SA-biotin system has been widely used in many biomedical fields [9, 10]. Based on the specific and tight interaction between SA and biotin, we developed a novel intravesical immunotherapy for superficial bladder cancer, which included preparation of SA-tagged GM-CSF (SA-GM-CSF) bifunctional fusion protein, biotinylation of the bladder mucosal surface and immobilization of SA-GM-CSF fusion protein on the biotinylated bladder mucosal surface. Using the mouse orthotopic model of MB49 bladder cancer, we demonstrated that the efficient and durable display of SA-GM-CSF fusion protein was achieved on the biotinylated bladder mucosal surface indicating that this approach could effectively suppress bladder tumour growth and remarkably induce strong and long-lasting immunity against the wild-type MB49 tumour.

Materials and methods

Cell culture and animals

The MB49 cell line (gift from Dr. I.C. Summerhayes in Lahey Clinic, Burlington, MA, USA) is a carcinogen-induced transitional cell carcinoma derived from C57BL/6 male mice. The MB49 cells were cultured in DMEM supplemented with 10% foetal bovine serum, 1% penicillin/streptomycin and 0.1% sodium pyruvate (Invitrogen, Carlsbad, CA, USA). C57BL/6 mice were purchased from the certified animal facility of Southern Medical University (Guangzhou, China).

Construction of SA-GM-CSF-expressing plasmid

The genomic DNA extracted from *S. avidinii* (ATCC, Manassas, VA, USA) with the DNase kit (Qiagen, Valencia, CA, USA) was used as a template to PCR-amplify the cDNA encoding mature SA with Platinum *Pfx* DNA Polymerase system (Invitrogen) and the following PCR primer pair containing a NdeI site and one 6xHis tag at the up-stream primer and an EcoRI site and a glycine/serine-rich flexible linker at the down-stream primer: 5' GGAATTCATATGCATCATCACCATCACCATGAGGCCGGCATACCCGGCACCCTGG 3' (55nt) and 5' GGAATTCGCCGATCCGCCCGCCGCTGCCTCCGCCCGCTGCCCGCTGCTGCTGAACGGCGTGCAGCGGGTTGCC 3' (82nt). Murine total RNA, extracted from PHA-activated murine peripheral blood mononuclear cells with Trizol reagent, was used to clone mouse

GM-CSF cDNA by RT-PCR with the Superscript II transcriptase (Invitrogen) and the following PCR primer pair containing an EcoRI site at the up-stream primer and a HindIII site at the down-stream primer: 5' GGAATTCGCACCCACCCGCTCACCATCAC 3' (30nt) and 5' CCCAAGCTTTCATTTTGGCCTGGTTTTTTCG 3' (32nt). The desired restriction enzyme cloning sites were incorporated into the PCR primers, and the SA-GM-CSF fusion protein was designed to contain single 6xHis tag at its N-terminal. In addition, the glycine/serine-rich flexible linker of 18-mer (L) was inserted between SA and GM-CSF with the following DNA sequence: ACG AGC GGG GGC AGC GGG GGC GGA GGC AGC GGC GGG GGC GGA TCC GGC GAA TTC. The resultant fusion gene SA-GM-CSF was cloned into pET24a vector (Novagen, Madison, CA, USA) to get the expression plasmid 6His-SA-L-GM-CSF-pET24, and then verified by DNA sequencing from both directions with T7 promoter and T7 terminator primers.

Expression of SA-GM-CSF fusion protein

The resultant expression plasmid 6His-SA-L-GM-CSF-pET24 was transformed into BL21 (DE3) pLysS strain of *E. coli* (Novagen) to produce GM-CSF fusion protein. The bacterial cells were grown in a shaking flask containing 100 ml LB medium with kanamycin (50 mg/ml) at 37°C. When the optical density at 600 nm (OD_{600}) of the culture was around 0.3, the expression of SA-GM-CS fusion protein was induced with isopropyl- β -D-thiogalactoside (IPTG) at the final concentration of 1 mM. When the OD_{600} reached about 2.0, aliquots from the harvested cells were taken and analysed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie Brilliant Blue R-250 staining.

Purification and refolding of SA-GM-CSF fusion protein

SA-GM-CSF fusion protein was collected as inclusion body after cell lysis by sonication, and washed consecutively for 5 min. on a rotating platform with each solution: A (20 mM Tris-HCl, 2 mM EDTA, pH 8.0), B (20 mM Tris-HCl, 10 mM EDTA, 2 mM mercaptoethanol, 0.1% Triton X-100, pH 8.0), C (20 mM Tris-HCl, 2 M Urea, 2 mM EDTA, pH 8.0), D (20 mM Tris-HCl, 50% isopropanol, 2 mM EDTA, pH8.0) and E (20 mM Tris-HCl, pH8.0). After washing, the inclusion body was dissolved in 8M urea buffer (containing 50 mM sodium phosphate, 10 mM Tris-HCl, 1 mM EDTA, 100 mM mercaptoethanol, pH 8.0), and purified on the Ni-NTA column (Qiagen, Valencia, CA, USA) according to the manufacture's protocol. The purified SA-GM-CSF fusion protein was adjusted to 0.2 at OD_{280} , dialysed against the refolding buffer (100 mM NaHCO_3 , 1.0 M urea, pH 9.0, GSH 1 mM, GSSG 0.2 mM at 4°C overnight with gentle agitation, and re-dialysed in the second buffer [50 mM $(\text{NH}_4)_2\text{CO}_3$, 500 mM NaCl, pH 11] at 4°C for additional 6 hrs. After centrifugation, the refolded SA-GM-CSF fusion protein was further purified through the 2-aminobiotin agarose column (Sigma, St. Louis, MO, USA). The bound SA-GM-CSF fusion protein was eluted with 50 mM NaAc, 150 mM NaCl, pH 4.0, adjusted to pH 8.0 with 500 mM $(\text{NH}_4)_2\text{CO}_3$, 5.0 M NaCl, pH 11, and then passed through Detoxi-Gel Endotoxin Removing Gel (Pierce, Rockford, IL, USA) to remove bacterial endotoxin contaminants. The amount of the SA-GM-CSF fusion protein in the preparations was quantitatively measured by use of mouse GM-CSF Single Analyte ELISArray Kit (SABiosciences, Frederick, MD, USA).

Similarly, SA-tagged green fluorescence protein named SA-GFP fusion protein was expressed, purified and refolded.

Western blotting

The purified and refolded SA-GM-CSF fusion protein under both denaturing-reducing and native conditions was separated by SDS-PAGE and transferred onto a polyvinylidene difluoride (PVDF) membrane with a semi-dry transfer apparatus (Bio-Rad, Hercules, CA, USA). The PVDF membrane was incubated consecutively at 37°C for 1 hr with each solution: the blocking buffer containing 5% dry milk and 0.5% Tween-20 in 1 × phosphate buffered saline (PBS), rat anti-mouse GM-CSF polyclonal antibody (BD Biosciences, San Diego, CA, USA) at 1:500 dilution in the blocking buffer, and horseradish peroxidase (HRP)-conjugated goat anti-rat antibody (1:5000) in the washing buffer (1% BSA, 0.2% Tween-20, 1 × PBS). After extensive washing of the membrane, the blot was developed with 3, 3'-diaminobenzidine (DAB) display liquid kit (Boshide Corporation, Wuhan, China).

Bioactivity assays of free and cell membrane-bound SA-GM-CSF bifunctional fusion proteins

Flow cytometric analysis and bone marrow cell proliferation assays were performed to test the bifunctionality of SA-GM-CSF fusion protein, *i.e.* both biotin-binding property and GM-CSF bioactivity.

To examine the biotin-binding property of SA-GM-CSF fusion protein, 5×10^7 MB49 cells were incubated in 1 × PBS containing 1.0 mg/ml EZ-Link NHS-PEO4-Biotin (Pierce) for 1 hr at room temperature. The biotinylated cells (10^6 /ml) were washed extensively with 1 × PBS and then modified with 0.1 mg/ml SA-GM-CSF fusion protein in 1 × PBS for 1 hr at room temperature. After extensive washing with 1 × PBS, the modified cells were stained with phycoerythrin-conjugated anti-GM-CSF monoclonal antibody (BD Biosciences) for 1 hr at 37°C, and then analysed to determine SA-GM-CSF-cell-surface modification efficiency with a flow cytometer (Becton Dickinson, San Jose, CA, USA).

GM-CSF bioactivity of SA-GM-CSF fusion protein was assessed by bone marrow cell proliferation. Briefly, bone marrow cells were prepared from femurs and tibias of C57BL/6 mice by flushing the marrow cavity. The cells were plated at 1×10^4 cells each well, left unstimulated or stimulated with indicated concentrations of either SA-GM-CSF fusion protein or recombinant mouse GM-CSF as the positive control (R & D systems, Minneapolis, MN, USA), and cultured for 3 days at 37°C in a 5% CO₂ humidified incubator. Twenty μ l of 5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution was added to each well and incubated for 4 hrs. The reaction was stopped by adding 200 μ l of dimethyl sulphoxide to each well, and the absorbance was measured at 570 nm with a microplate reader.

To determine whether the cell membrane-bound SA-GM-CSF fusion protein still retained the bioactivity of its GM-CSF moiety, 1×10^5 SA-GM-CSF-modified MB49 cells were collected, suspended in 0.5 ml 1 × PBS and then lysed by three cycles of freeze and thaw. Membrane fractions were collected by centrifugation at 15,000 *g* at 4°C for 5 min. and suspended in 0.5 ml of complete medium. Aliquots of cell membrane fractions as indicated were added to 1×10^4 bone marrow cells each well. With cell membrane fractions from SA-GFP-modified or recombinant mouse GM-CSF-treated MB49 cells as negative control, GM-CSF-dependent cell proliferation was assessed by MTT assay as described above.

Intravesical immobilization of SA-GM-CSF fusion protein for immunotherapy of superficial bladder cancer

The mouse orthotopic model of MB49 bladder cancer was used to evaluate the feasibility and efficacy of the novel immunotherapy through intravesical immobilization of SA-GM-CSF bifunctional fusion protein on the biotinylated mucosal surface of bladder wall. Briefly, 8- to 10-week-old female C57BL/6 mice, weighing 15–20 grams, were anaesthetized with intraperitoneal sodium pentobarbital (68 mg/kg body weight). Subsequently, a 24-gauge Insyte IV catheter (BD Biosciences) was inserted through the urethra into the bladder using mineral oil as a lubricant. In order to prepare the bladder for tumour implantation, the pretreatment was performed, *i.e.* a brief acid (100 μ l of 0.1 M HCl) exposure (15 sec.), followed by alkaline (100 μ l of 0.1 M NaOH) neutralization (5 sec.), which caused chemical lesions on the bladder mucosal surface. The content was washed out by transurethral infusion of 1 × PBS. After the pretreatment, 100 μ l of MB49 cells (1×10^6) were instilled and allowed to incubate in the bladder for 1 hr. One day after tumour implantation, 100 μ l of 1 mg/ml NHS-PEO4-biotin was instilled and allowed to incubate in the bladder for 30 min., followed by intravesical instillation of 100 μ l of 1 × PBS, GM-CSF (0.15 mg/ml), SA-GFP (0.15 mg/ml), or SA-GM-CSF (0.15 mg/ml) and incubation for 1 hr. The SA-GM-CSF group had 16 mice and other groups (1 × PBS, GM-CSF and SA-GFP) had 10 mice individually. The treatment was performed twice a week for 3 weeks. Mice were carefully monitored for health status (body temperature, body weight and food consumption), haematuria and palpable tumour.

On day 60 after MB49 the implantation, the SA-GM-CSF-cured mice, which were found to have no haematuria and palpable tumour at the indicated time, were challenged with 100 μ l of wild-type MB49 cells (1×10^6) implanted into the pretreated bladder and monitored for survival.

Histological and immunohistochemical analyses in the mouse orthotopic model of MB49 bladder cancer

Mice were sacrificed at the indicated time-points after implantation or intravesical treatment. Bladders were then removed, waxed with formalin and embedded in paraffin. Haematoxylin and eosin staining was performed on 4- μ m sections. Sections were inspected for tumour incidence and histological stage based on tumour size and localization. In addition, the snap-frozen bladder specimens from the SA-GM-CSF-treated mice were obtained for cryosection on days 1, 4, 7 and 10 after intravesical treatment, and then immunohistochemical analysis was performed to assess the persistence of the SA-GM-CSF fusion protein immobilized on the biotinylated bladder mucosal surface with biotin-conjugated HRP and DAB display liquid kit (Boshide Corporation).

Tumour-specific cytotoxicity assay

The cytotoxic T lymphocyte (CTL) cytotoxicity was assessed with lactate dehydrogenase (LDH) cytotoxicity detection kit (Clontech, Mountain View, CA, USA). Briefly, spleen cells were pooled from three naive C57BL/6 mice, three tumour-cured and three tumour-bearing mice in the SA-GM-CSF-treated group on day 40 after the last intravesical therapy, and then were stimulated *in vitro* with 20 U/ml of recombinant human IL-2 (R & D

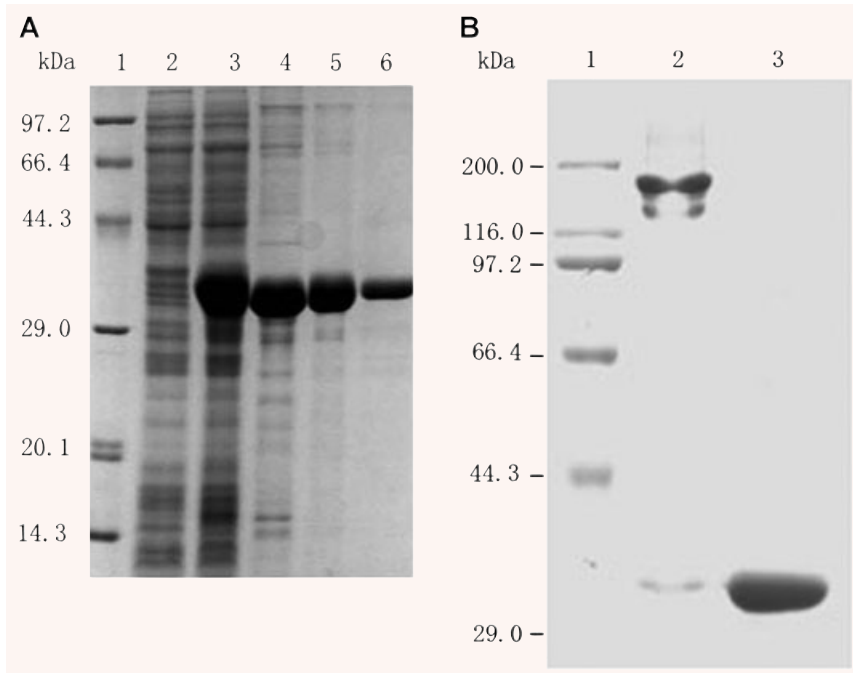


Fig. 1 Analysis of expression, purification and refolding of SA-GM-CSF fusion protein. **(A)** Analysis of expression and purification of SA-GM-CSF fusion protein by 12% SDS-PAGE. Lane 1: Protein markers; Lane 2: Total proteins of bacteria not induced by IPTG; Lane 3: Total proteins of bacteria induced by IPTG; Lane 4: Inclusion body of SA-GM-CSF; Lane 5: SA-GM-CSF purified on the Ni-NTA column; Lane 6: SA-GM-CSF purified through the 2-iminobiotin agarose column. The experiments were repeated three times. **(B)** Characterization of the refolded SA-GM-CSF fusion protein by Western blotting. Lane 1: Protein markers; Lane 2: Under native condition; Lane 3: Under reducing/denaturing condition. The experiments were repeated three times.

systems) plus irradiated MB49 tumour cells for 5 days. Effector cells were then harvested, washed and adjusted to the concentration of 1×10^6 cells/ml. A varying number of effector cells were added in triplicate to the target MB49 cells (1×10^4 cells/well) at the desired E:T ratios. After the cell mixture was incubated for 6 hrs, 100 μ l of culture supernatant was collected to measure LDH activity. The mean percentage specific lysis was calculated as the following: Specific Lysis (%) = [(Effector:Test Cell Mix – Effector Cell Control) – Low Control] \times 100/(High Control– Low Control).

Statistical analysis

The results shown were representative of at least two independent experiments. The results other than those of survival were statistically analysed with an unpaired t-test. Survival curve was plotted using the Kaplan–Meier method and difference in survival between the groups was compared by the log-rank test. $P < 0.05$ was considered statistically significant. Survival comparison and unpaired Student's t-test were performed with GraphPad Prism 3.0 software (GraphPad Software, San Diego, CA, USA).

Results

Generation and characterization of SA-GM-CSF bifunctional fusion protein

The recombinant SA-GM-CSF fusion protein was designed to contain a single $6 \times$ His tag at its N-terminus, which is convenient for Ni-NTA affinity purification, and an 18-mer glycine/serine-rich

flexible linker between SA and GM-CSF, which allows these two moieties of SA-GM-CSF fusion protein to refold as independently as possible. It was expressed at about 25% of the total cellular proteins as inclusion body in *E. coli*. The purity of SA-GM-CSF fusion protein was 60% after extensive washing of the inclusion body, about 90% through the Ni-NTA column, and up to 95% after pass-through the 2-iminobiotin agarose column. The recovery efficiency of SA-GM-CSF fusion protein was about 70% (Fig. 1A).

The refolded SA-GM-CSF fusion protein formed tetramers with the molecular weight of 130 kD and higher order structures under native condition. However, dissociation of multimerized SA-GM-CSF fusion protein into the monomer with the molecular weight of 34 kD occurred under denaturing-reducing condition when the sample was heated at 100°C (Fig. 1B).

The refolded SA-GM-CSF fusion protein exhibited full biotin-binding property and GM-CSF bioactivity. As flow cytometric analysis showed, SA-GM-CSF fusion protein was displayed efficiently on the biotinylated surface of MB49 cells through the specific and tight interaction between SA and biotin (Fig. 2A). The ability of SA-GM-CSF fusion protein to promote the proliferation of bone marrow cells from C57BL/6 mice was studied *in vitro*. SA-GM-CSF fusion protein stimulated the bone marrow cells in a dose-dependent manner with recombinant mouse GM-CSF as the positive control. Its median effective dose (ED₅₀) was typically 0.1–0.2 ng/ml while ED₅₀ of recombinant mouse GM-CSF was 0.05–0.1 ng/ml.

To investigate GM-CSF bioactivity of SA-GM-CSF immobilized on the biotinylated surface of MB49 cells, we evaluated whether the membrane-bound component was able to stimulate syngeneic bone marrow cells. Cell membrane fractions harvested from the SA-GM-CSF-immobilized MB49 cells were able to stimulate the

proliferation of bone marrow cells in a dose-dependent manner, indicating that SA-GM-CSF fusion protein immobilized on the biotinylated surface of MB49 cells still retained the bioactivity of its GM-CSF moiety (Fig. 2B).

The feasibility and efficacy of the novel immunotherapy for superficial bladder cancer

The mouse orthotopic model of MB49 bladder cancer was used to evaluate the feasibility and efficacy of the novel immunotherapy through intravesical immobilization of SA-GM-CSF bifunctional fusion protein on the biotinylated mucosal surface of mouse bladder wall (Fig. 3). First, we determined the desired concentration, 1 mg/ml of the biotinylating reagent NHS-PEO4-biotin, for efficient biotinylation of the bladder mucosal surface by titration of 0.1, 0.2, 0.5, 1.0 and 2.0 mg/ml. Secondly, we found that about 3.0 μ g of SA-GM-CSF fusion protein was immobilized each treatment by comparing the original SA-GM-CSF concentration in the administered solution with that in the washed-out solution after instillation. Thus, we decided to instill 100 μ l of 0.15 mg/ml SA-GM-CSF fusion protein (*i.e.* 15 μ g), which is 5 times as much as the saturating amount of 3.0 μ g each treatment. After intravesical immobilization, the persistence of SA-GM-CSF fusion protein on the bladder mucosal surface was visualized with biotin-conjugated HRP and DAB display liquid kit through the specific interaction of biotin-conjugated HRP with the SA moiety of the SA-GM-CSF fusion protein. SA-GM-CSF fusion protein was displayed efficiently and durably on the biotinylated mucosal surface of mouse bladder wall for up to 7 days (Fig. 4B–E). In contrast, no SA-GM-CSF fusion protein was detected on the un-biotinylated bladder mucosal surface (Fig. 4A).

On day 60 after the MB49 implantation, the SA-GFP-treated group and the soluble GM-CSF-treated group had 2 out of 10 (20%) and 1 out of 10 (10%) survival with palpable tumour, respectively. In contrast, the SA-GM-CSF-treated group had 10 out of 16 (62.5%) survival, and 6 out of the 10 (60%) surviving mice were cured (*i.e.* without haematuria and palpable tumour) (Fig. 5A). The survival curves were significantly different between the SA-GM-CSF and the SA-GFP groups ($P < 0.05$), and between the SA-GM-CSF and the soluble GM-CSF groups ($P < 0.01$). Importantly, 70% of the SA-GM-CSF-cured mice were protected against a second intravesical wild-type MB49 challenge whereas only 10% of naïve mice survived the challenge with palpable tumour ($P < 0.01$) (Fig. 5B). In addition, we found that the survival rate was decreased to 6 out of 16 (37.5%) when the treatment was given once a week instead twice a week, indicating that the sustainable amount of GM-CSF in the tumour site is critical for the immunotherapy of superficial bladder cancer.

During the treatment, there were no significant treatment-related toxicities, such as fever, weight loss and reduction of food consumption. In line with the observations, the histological examination confirmed that no obvious pathological changes were found in the bladder walls of the SA-GM-CSF-cured mice, which were randomly selected on days 10, 20 and 40 after the last

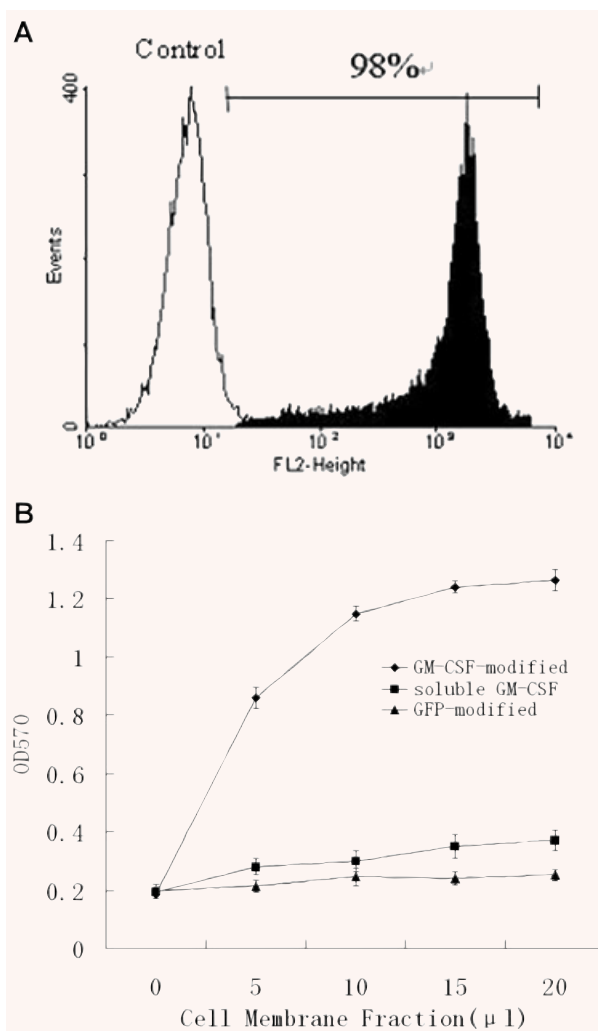


Fig. 2 Bioactive assays of free and membrane-bound SA-GM-CSF bifunctional fusion proteins. **(A)** Efficient display of SA-GM-CSF bifunctional protein on the biotinylated surface of MB49 cells. MB49 cells were biotinylated in freshly prepared EZ-Link NHS-PEO4-Biotin in $1 \times$ PBS for 1 hr at room temperature. 10^6 /ml cells were then washed extensively and incubated with 100 ng of SA-GM-CSF bifunctional protein in $1 \times$ PBS for 1 hr. The presence of SA-GM-CSF was assessed using phycoerythrin-conjugated anti-GM-CSF monoclonal antibody for flow cytometric analysis with un-biotinylated MB49 cells as control. The result was represented as one of the experiments repeated three times. **(B)** The intact GM-CSF bioactivity of SA-GM-CSF fusion protein immobilized on the biotinylated surface of MB49 cells. Suspension of 1×10^5 MB49 cells modified by SA-GM-CSF, SA-GFP or GM-CSF were collected and suspended in 0.5 ml of $1 \times$ PBS. Cells were lysed by three cycles of freeze and thaw. Membrane fractions were collected and suspended in 0.5 ml of complete medium. Aliquots of cell membrane fractions as indicated were added to 1×10^4 C57BL/6 bone marrow cells each well and incubated for 3 days. The cell proliferation was assessed by MTT assay. Experiments were done in triplicate, and repeated three times.

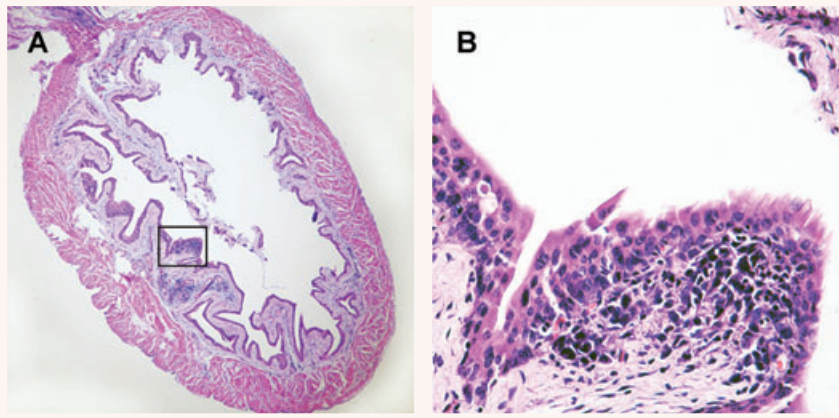


Fig. 3 Establishment of the mouse orthotopic model of MB49 superficial bladder cancer. Eight to 10-week-old female C57BL/6 mice, weighing 15–20 grams, were anaesthetized with intraperitoneal sodium pentobarbital (68 mg/kg body weight). Subsequently, a 24-gauge Insyte IV catheter was inserted through the urethra into the bladder using mineral oil as a lubricant. In order to prepare the bladder for tumour implantation, a brief acid (0.1 M HCl, 100 μ l) exposure (15 sec.), followed by alkaline (0.1 M NaOH, 100 μ l) neutralization, caused chemical lesions on the bladder wall. After 15 sec., the content was washed out by transurethral infusion of 1 \times PBS. After the pretreatment, 100 μ l

of MB49 cells (1×10^6) were instilled and allowed to incubate in the bladder for 1 hr. On day 7 after intravesical implantation of MB49 cells, bladder sections were prepared and stained with haematoxylin and eosin. **(A)** Original magnification $\times 40$. **(B)** Original magnification $\times 400$.

intravesical treatment. In addition, we found that biotinylation and SA-GM-CSF immobilization had no significant effect on the cell viability *in vitro* (Figs S1 and S3).

To determine whether the SA-GM-CSF treatment affected CTL function, splenocytes were pooled from naïve mice, tumour-cured or tumour-bearing mice in the SA-GM-CSF-treated group, and stimulated with irradiated MB49 cells in the presence of IL-2 for 5 days to prepare effector cells. The percentages of specific killing of the effector cells pooled from naïve mice, the tumour-bearing and the tumour-cured mice in the SA-GM-CSF-treated group were determined at the effector as target ratios of 1:1, 25:1 and 50:1, respectively (Fig. 6). There were significant differences between the cytotoxic activities of the tumour-cured and the tumour-bearing mice in the SA-GM-CSF-treated group ($P < 0.01$), and of the naïve and the tumour-bearing mice in the SA-GM-CSF-treated group ($P < 0.05$).

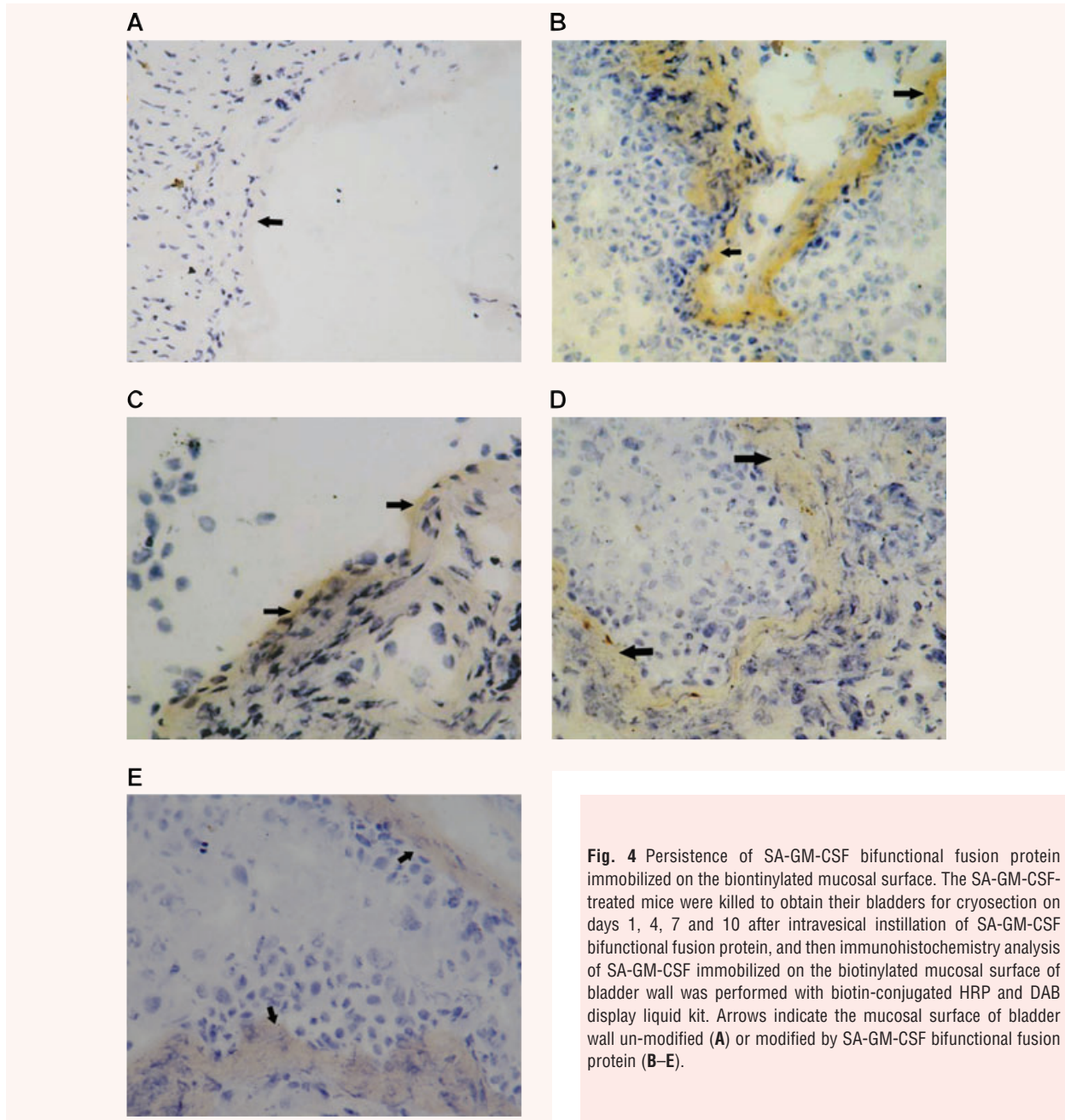
Therefore, these observations indicated that the immunotherapy for MB49 superficial bladder cancer induced strong and long-lasting immunity against the wild-type MB49 tumour challenge by intravesical immobilization of SA-GM-CSF fusion protein on the biotinylated bladder mucosal surface.

Discussion

The majority of bladder cancers are superficial at the time of diagnosis and most of them (60–70%) have a propensity for recurrence after initial transurethral resection of bladder tumour. Some (15–25%) of patients are at high risk for progression to invasive bladder cancer. Intravesical administration of BCG after transurethral resection has remained the most effective local therapy for superficial bladder cancer. However, approximately one-third of the patients fail to respond and most patients eventually

relapse. In addition, there are pronounced side effects of BCG therapy such as BCG sepsis and the high frequency of BCG-induced cystitis [1, 2]. Based on the fact that BCG induces cytokines and thus activates the local immune response, intravesical administration of recombinant cytokines such as IFN α , TNF α , GM-CSF or IL-2 has been used in clinical trials with encouraging results [11–14]. However, due to the instability and short half-life in urine of recombinant cytokines, the intravesical treatment requires repeated large dose applications. One solution to these limitations lies in the intravesical *in situ* gene transfer with cytokine genes. This would allow a sustained and prolonged local release of cytokines at the tumour site, since high cytokine level in the vicinity of tumour cells is crucial for the cancer immunotherapy [3–8]. In order to circumvent the weaknesses of genetic modification such as low transfer efficiency, low transgene expression level, and biosafety concern associated with viral vector-mediated gene transfer, we have developed a novel platform for tumour vaccination to allow rapid, efficient and durable display of multiple synergistic immunostimulators such as cytokines and costimulators on the biotinylated surface of tumour cells through the specific and tight interaction between SA and biotin [15].

In this study, we reported the high-level expression in *E. coli*, purification and refolding of SA-GM-CSF fusion protein. We showed that the refolded SA-GM-CSF fusion protein exhibited bifunctionality, *i.e.* full biotin-binding property and GM-CSF bioactivity. Furthermore, we demonstrated that the bifunctional fusion protein SA-GM-CSF was displayed efficiently and durably on the biotinylated bladder mucosal surface. Our data indicated that the novel immunotherapy was able to induce strong and long-lasting anti-tumour immunity against the implanted MB49 tumour, *i.e.* effective suppression of the tumour growth in the mouse orthotopic model of MB49 bladder cancer and protection of most of the SA-GM-CSF-cured mice from a second intravesical wild-type MB49 tumour challenge.



It is worth noting that the novel immunotherapy achieved only 67.5% cure rate in the mouse orthotopic model of MB49 bladder cancer that delayed treatment initiation by only 1 day following instillation of the bladder tumour. Our preliminary data indicated that it was mainly due to the microenvironment of MB49 tumour, which included infiltration of T regulatory cell, up-regulation of IL-10, TGF- β or/and the immunosuppressive enzyme, indoleamine-pyrrole 2,3-dioxygenase (IDO). Thus, the efficacy of SA-tagged

bioactive GM-CSF alone may theoretically be improved remarkably in combination with an IDO inhibitor or/and other SA-tagged synergistic immunostimulators such as TNF α or/and CD40L, which is currently under our investigation [16–18].

Interestingly, our results showed that the SA-GFP treatment also increased the animal survival. This may be due to the strong immunogenicity of SA in mouse. SA is a bacterial protein, which could induce a humoral immune response, with minimal

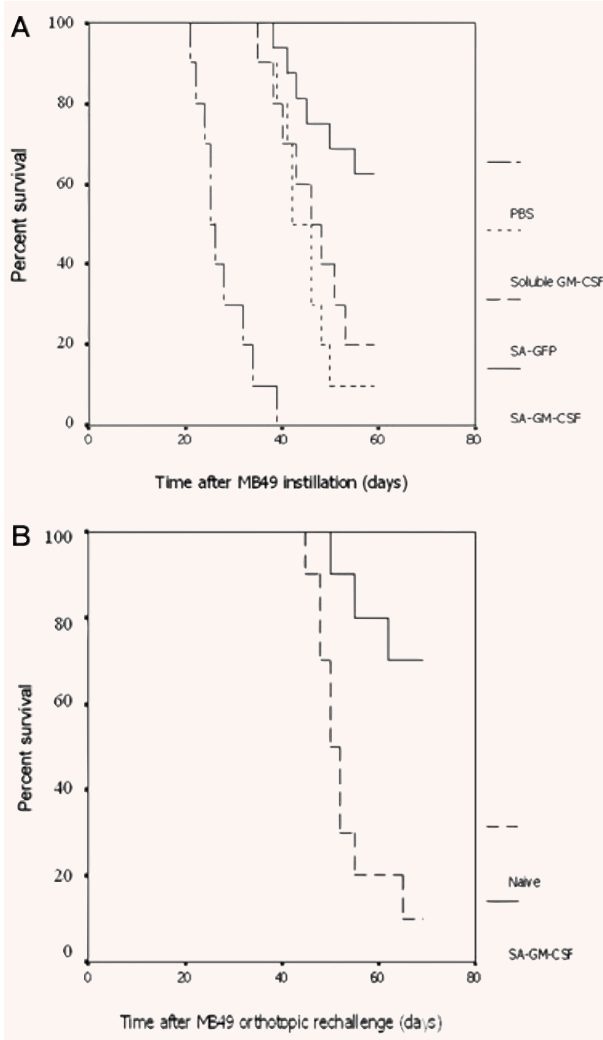


Fig. 5 Intravesical immobilization of SA-GM-CSF bifunctional fusion protein for immunotherapy of superficial bladder cancer. **(A)** Therapeutic efficacy of SA-GM-CSF bifunctional fusion protein in the orthotopic mouse model of MB49 bladder cancer. Mice implanted with orthotopic MB49 tumour were instilled with 1×10^6 PBS (10 mice), soluble GM-CSF (10 mice), SA-GFP (10 mice) and SA-GM-CSF (16 mice), respectively. The survival curves were significantly different between the SA-GM-CSF-treated and the SA-GFP-treated groups ($P < 0.05$), and between the SA-GM-CSF-treated and the soluble GM-CSF-treated groups ($P < 0.01$). The result was represented as one of the experiments repeated three times. **(B)** Protective immunity against a second intravesical MB49 challenge in SA-GM-CSF-cured mice. Mice undergoing complete tumour regression were re-challenged with the intravesical implantation of 1×10^6 wild-type MB49 cells and monitored for survival. Seven out of 10 SA-GM-CSF-cured mice were protected against the second intravesical MB49 challenge whereas only one of 10 naïve mice survived the challenge with palpable tumour ($P < 0.01$). The result was represented as one of the experiments repeated three times.

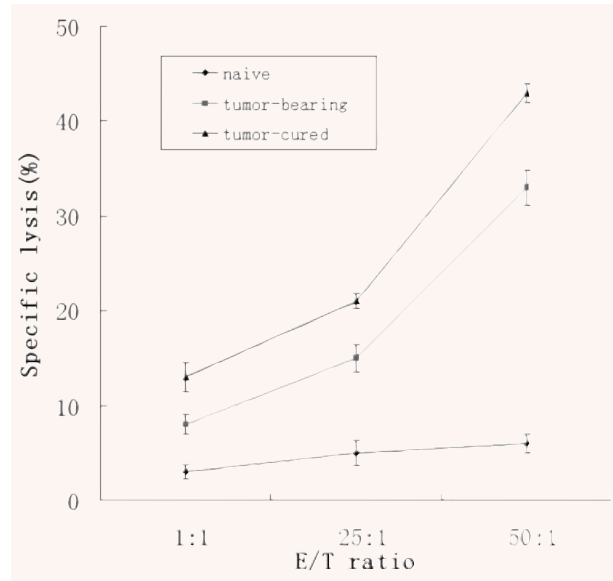


Fig. 6 The SA-GM-CSF treatment could induce and enhance CTL function. Splenocytes were pooled from naïve mice, tumour-cured or tumour-bearing mice in the SA-GM-CSF-treated group, and stimulated with irradiated MB49 cells in the presence of IL-2 for 5 days to prepare effector cells. The CTL cytotoxicities were determined with LDH cytotoxicity detection kit at the effector to target ratios of 1:1, 25:1 and 50:1, respectively. The cytotoxicities had significant differences between the tumour-cured and the tumour-bearing mice in the SA-GM-CSF-treated group ($P < 0.01$), and between naïve and the tumour-bearing mice in the SA-GM-CSF-treated group ($P < 0.05$).

immunological side effects as shown in the clinical trials of pretargeted radioimmunotherapy [19, 20]. GFP is also an exogenous protein for mouse. Thus, SA-GFP, as an exogenous fusion protein, could stimulate immune response in mice and thus enhance their immunocompetence. However, a significant improvement of the immunotherapy was observed in the SA-GM-CSF test group in comparison with the SA-GFP control group, showing that the majority of the protective effect was clearly due to immunostimulation with GM-CSF.

In summary, we have developed a novel immunotherapy for superficial bladder cancer without significant treatment-related toxicity by intravesically immobilizing SA-tagged bioactive GM-CSF on the biotinylated bladder mucosal surface under physiological condition. This immunotherapy may be an attractive therapeutic alternative and should be evaluated in bladder cancer patients.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1 Histological effect of the SA-GM-CSF immobilization on the mouse bladder wall. Bladder sections were prepared from the SA-GM-CSF-cured at days 10 (**A**) and 40 (**B**) after the last intravesical treatment, and stained with haematoxylin and eosin (original magnification $\times 400$).

Fig. S2 Identification of the cellular infiltrate in the MB49 superficial bladder cancer of SA-GM-CSF-treated and SA-GFP-treated mice by the ribonuclease protection assay. To determine the composition of the cellular infiltrate, we analysed the gene expression of cell surface markers of T cells (TCR δ , TCR α , CD3 ϵ , CD4, CD8 α ,

CD8 β), B cells (CD19), macrophages (F4/80) and leucocytes (CD45) with housekeeping genes L32 and GAPDH as internal controls in the MB49 superficial bladder cancer of SA-GM-CSF-treated and SA-GFP-treated mice on day 12 after MB49 implantation by use of the RiboQuant Multi-Probe RNase Protection Assay System (Pharmingen, San Diego, CA, USA).

Fig. S3 Effect of biotinylation and SA-GM-CSF immobilization on the viability of MB49 cells. 5×10^7 MB49 cells were incubated in $1 \times$ PBS containing 1.0 mg/ml EZ-Link NHS-PEO4-Biotin for 1 hr at room temperature. The biotinylated cells (10^6 /ml) were washed extensively with $1 \times$ PBS and then modified with 0.15 mg/ml SA-GM-CSF fusion protein in $1 \times$ PBS for 1 hr at room temperature. After extensive washing with $1 \times$ PBS, the modified cells were stained with $1 \mu\text{g/ml}$ propidium iodide for 10 min. or with FITC-labelled anti-GM-CSF monoclonal antibody for 1 hr at 37°C , and then analysed to determine the cell viability (**B**, **D**) and SA-GM-CSF-cell-surface modification efficiency (**A**, **C**) by flow cytometry.

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