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Design, expression, and characterization of a novel dendritic cell-targeted proteins

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ABSTRACT

In vivo approaches to inducing an effective immune response focus on targeted antigen (Ag) delivery to dendritic cells (DCs). In this study, we developed a new method of targeting plasmid DNA and/or the antigen (Ag)–antibody (Ab) complex to DCs via the DC receptor DEC-205, also known as cluster of differentiation CD205. We cloned and expressed a recombinant protein composed of mouse DEC-205-specific single-chain fragment variable region (mDEC-205-scFv), the streptococcal protein G (SPG) IgG-binding domain and cationic peptide (CP), which named mDEC205-scFv-SPG-CP (msSC). In vitro, the recombinant protein msSC can specifically bind to DCs through the section of mDEC-205-scFv, and bound the Ag–Ab complex via SPG as well as plasmid DNA through electrostatic bonding with CP in vitro. In addition, msSC functioned in a manner similar to anti-DEC-205 monoclonal Ab and bound to mouse bone marrow-derived DCs. It was demonstrated in vivo that msSC can target plasmid DNA to DCs, resulting in efficient uptake and expression. Moreover, msSC can form a complex with pGL3-CMV and transport it to draining lymph nodes when injected in vivo. These results indicate that msSC can be used as a carrier protein for vaccine delivery to DCs via formation of plasmid DNA-Ag–Ab ternary complexes.

1. Introduction

Various methods are used to deliver known antigen (Ag) to a site of infection or tumor formation [1], including electroporation of plasmid DNA injected into muscle, Ag–antibody (Ab) coupling, and loading dendritic cells (DCs) with tumor-associated Ags or whole tumor cells in vitro [2–4]. However, these techniques have several drawbacks such as non-specific Ag targeting and dilution or degradation of the Ag as well as high cost, and are often complex procedures that require special equipment [1]. Recent strategies for inducing an effective immune response in vivo have focused on targeted delivery of Ags to cluster of differentiation CD40 [5] or the DC-specific intercellular adhesion molecule 3-grabbing non-integrin in the surface of DCs [6]. However, these molecules lack specificity as they are expressed by monocytes, eosinophils, and fibroblasts. Nonetheless, CD205 can be used to specifically target the DC receptor DEC-205, which is an endocytic receptor belonging to the mannose receptor family that is highly expressed in DC cells [7,8]. Ag coupled to an anti-DEC205 monoclonal Ab induced an Ag-specific immune response that eliminated metastatic melanoma in a mouse model [4]. While effective, this method depends on consistent and efficient coupling between Ag and Ab [1].

In the present study, the DC-targeted recombinant protein msSC was constructed to improve the efficiency of vaccine uptake by DCs and hence enhance the immune response. msSC consists of mouse DEC-205-specific single-chain fragment variable region (mDEC-205-scFv) [1,9], streptococcal protein G (SPG) immunoglobulin (Ig) G-binding domain [10,11], and cationic peptide (CP) [12,13]. The mDEC-205-scFv domain can specifically bind the DC surface molecule DEC-205, thereby targeting the Ag to DCs. mSC contains an SPG domain that binds IgG to enable efficient binding to Ag–Ab complexes [14–16], as well as a cationic peptide that is capable of binding negatively charged plasmid DNA. This fusion protein has several advantages. First, it can decrease non-specific binding to receptors or cells and improve DNA uptake and Ag-presenting capacity. Secondly, it can bind plasmid DNA and Ags simultaneously to synergistically induce an immune response, thereby promoting cellular and humoral immunity.

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We demonstrate that msSC can bind Ag–Ab complexes through the SPG domain and plasmid DNA through electrostatic coupling. In addition, msSC functioned in a manner similar to anti-DEC-205 monoclonal Ab bound to mDEC-205, effectively binding to mouse bone marrow-derived (mBM)DCs, and enhanced the uptake of plasmid DNA by DCs and the transport of plasmid DNA to draining lymph nodes when injected in vivo. These results indicate that msSC can be used for targeted delivery of vaccines to DCs to eliminate infections or tumors.

2. Materials and methods

2.1. Ethics statement

Pathogen-free female BALB/c mice were obtained from the Experimental Animal Center of Military Medical Sciences. Animals used in this study were 4–6 weeks old and maintained in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health publication no. 85-23, Revised 1996). Experimental procedures conformed to international guidelines for the care and use of laboratory animals and were approved by the Animal Ethics Committee of Beijing Institute of Basic Medical Sciences.

2.2. Plasmids and reagents

pUC57-Amp was obtained from Genewiz, Inc. (South Plainfield, NJ, USA). pET-28a was from Novagen (Billerica, MA, USA). pGL3-CMV containing the luciferase reporter gene for in vivo DC targeting of msSC was purchased from Promega (Beijing, China). pSVK-HBVA (16-kb DNA with hepatitis B virus [HBV] fusion Ag) for the gel retardation assays was constructed by our laboratory. *Escherichia coli* DH5α was used for cloning and transformation of recombinant plasmids. *E. coli* BL21 (DE3) pLysS from a laboratory stock was used for protein expression. Monoclonal rat anti-mouse DEC205 Ab was purchased from eBioscience (San Diego, CA, USA). Mouse anti-His tag antibody, horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG, and HRP-conjugated human and mouse IgG were purchased from Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd. (Beijing, China). Recombinant HB surface Ag (HBsAg) was obtained from Beijing Wantai Bio-Pharmaceutical Co., Ltd. (Beijing, China). Human HB immunoglobulin (HBIg) was obtained from the Chinese Biotechnology Company.

2.3. Media and growth conditions

DH5α cells transformed with pUC57-Amp were incubated in Luria broth (LB) with 100 μg/ml ampicillin at 37 °C. BL21 (DE3) pLysS cells transformed with pET-28a (+) were incubated in LB with 50 μg/ml kanamycin at 37 °C. Protein expression was induced by addition of isopropyl-β-d-thiogalactoside (IPTG) at a final concentration of 1 mM.

2.4. Expression plasmid construction

Recombinant mDEC205-scFv-SPG-CP (msSC) encodes three proteins, including mDEC-205-scFv [17], SPG containing the IgG-binding domain (GenBank accession no.: CAA27638.1), and CP (RSQSRSRYYRQRQRSRRRRRRS) (Fig. 1). mDEC-205-scFv was fused to SPG-CP with the linker (GGGGS)3 to generate msSC. BamHI and SacI restriction sites were introduced at the 5′ and 3′ ends, respectively, of the fusion gene. Following *E. coli* codon optimization, the fused gene was synthesized by Genewiz Inc. (South Plainfield, NJ, USA) and cloned into pUC57-Amp vector. Correct insertion of the DNA fragment was confirmed by sequencing. The fragment with BamHI and SacI sites in pUC57-Amp was subcloned into the pET-28a expression vector to generate pET-28a-msSC.

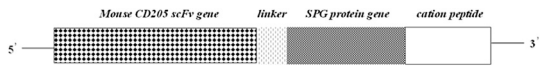

Fig. 1. Schematic illustration of the protocol used to generate msSC fusion protein.

2.5. Expression and purification of mDEC205-scFv-SPG-CP (msSC) protein

pET-28a-msSC was transformed into *E. coli* BL21(DE3) pLysS cells to generate the expression strain BL-pET-28a-msSC. A single colony was inoculated into 2 ml LB containing 50 μg/ml kanamycin at 37 °C for 12 h. Cells were then inoculated into 100 ml fresh LB with kanamycin until mid-log phase (absorbance at 600 nm of about 0.6–1.0) and induced by addition of IPTG. The cells were cultured for an additional 4–6 h and harvested by centrifugation at 8000 × g for 10 min. *E. coli* cells were re-suspended in 10 mM phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, and 2 mM KH2PO4, pH 7.4) and lysed by sonication for 30 min, followed by centrifugation at 8000 × g for 10 min at 4 °C. The cell pellet was washed with PBS containing 2 M urea followed by one wash in 4 M urea. Cell debris was dissolved in 8 M urea and centrifuged at 12,000 × g for 3 min at 4 °C. The protein in the supernatant was dialyzed sequentially against 10 mM PBS containing 6, 4, 2, and 0 M urea at 4 °C. Optimal dilutions were determined to prevent aggregation during refolding. Protein concentration was determined by UV absorption at 280 and 260 nm (reference: 320 nm) on a spectrophotometer (2800 UV/VIS, Shanghai Sunny Hengping Scientific Instrument Co., Ltd., Shanghai, China). msSC was purified from BL-pET-28a-msSC cell inclusion bodies and detected by western blotting using a monoclonal Ab against His and visualized with HRP-conjugated goat anti-mouse secondary Ab and an enhanced chemiluminescence detection system.

2.6. Cytotoxicity assay

Cell viability was assessed using Cell Counting Kit-8 (CCK-8; Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer's instructions. HepG2 cells (5 × 103 per well) were seeded on 96-well plates with 100 μl media one day prior to adding mSC protein. The next day, cells were incubated with 15, 30, 60, or 120 μg/ml msSC protein, for 3 h at 37 °C and 5% CO_2. Water-soluble Tetrazolium Salt 8 reagent was then added, followed by a 3-h incubation. The number of viable cells was measured with a microplate reader (Bio-Rad, Hercules, CA, USA) at optical density (OD) 450. Data are expressed as mean ± standard deviation (SD) of three measurements.

2.7. Determination of msSC binding to human and mouse IgG by enzyme-linked immunosorbent assay (ELISA)

A microplate was coated with 100 μl of 10 mM PBS containing 10 μg/ml msSC fusion protein and incubated overnight at 4 °C. After blocking with 5% not-fat milk powder for 2 h at room temperature, serial dilutions of HRP-conjugated human or mouse IgG (1:10–1:5120) were added to the microplate, followed by a 1-h incubation at 37 °C. The solution was reacted by adding o-phenylenediamine dihydrochloride substrate (Sigma–Aldrich, St. Louis, MO, USA), and msSC binding activity was measured at OD_{450} with a

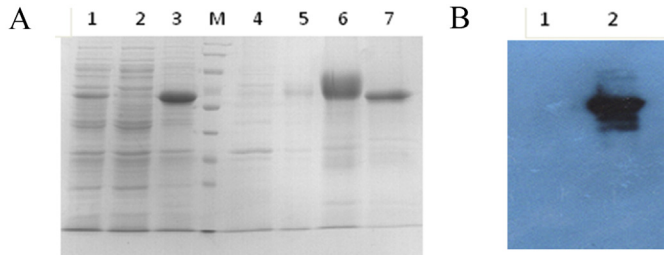

Fig. 2. Expression and purification of recombinant msSC fusion protein. (A) SDS-PAGE analysis of recombinant msSC. Lane 1, whole cell lysate; lane 2, supernatant; lane 3, pellet; lanes 4–6, supernatant of inclusion body fraction washed with 2 M (lane 4), 4 M (lane 5), and 8 M (lane 6) urea; lane 7, msSC after gradient dialysis; M, protein marker. (B) Western blot analysis of purified recombinant msSC. Lane 1, pET-28a empty sample; lane 2, purified recombinant msSC.

microplate reader. An amine:phosphate ratio (N/P) $\geq$ 2.1 was taken as positive.

2.8. Detection of msSC-Ag–Ab ternary complex formation by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

The formation of msSC-Ag–Ab ternar complexes was detected as previously described [14–16]. Briefly, a tube containing 25 μl of HBsAg and HBIg (10 IU) was incubated for 2 h at 37 °C and then overnight at 4 °C. The next day, the Ag–Ab complex was added to 16 μl of msSC protein. After incubating for 2 h at 37 °C with shaking at 20 rpm, the tube was incubated overnight at 4 °C, then centrifuged at 12,000 rpm for 50 min; 60 μl of supernatant was transferred to a new tube containing 30 μl loading buffer. The pellet was resuspended in 60 μl PBS, to which 30 μl of loading buffer were added. The samples were boiled for 5 min and resolved by SDS-PAGE. Negative control samples, Ag, Ab, and msSC protein, were detected by the same procedure.

2.9. Gel retardation assay

To evaluate the ability of msSC to bind plasmid DNA, the DNA and protein were mixed together at various N/P ratios. After incubating at room temperature for 30 min, the mixture was separated by 1.0% agarose gel electrophoresis. The gel was stained with ethidium bromide and visualized using an ultraviolet imaging system.

2.10. Determination of DC binding by msSC

To determine the capacity of msSC protein to bind DCs in vitro, mBMDCs were prepared as described [18]. Briefly, DCs were isolated and cultured in 5-ml Eppendorf tubes after dilution with PBS to the indicated concentration along with msSC protein at room temperature for 1 h. Cells were washed two or three times with PBS, and then stained with P-phycoerythrin (PE)-conjugated goat anti-mouse IgG for 30 min at room temperature followed by two or three washes with PBS and analysis by flow cytometry. DCs incubated with rat IgG and rat anti-mouse DEC205 monoclonal Ab (14-2051; eBioscience) served as negative and positive controls, respectively. PE-labeled goat anti-rat IgG was used as a secondary Ab.

2.11. Targeting msSC protein to DCs in vivo

msSC protein containing mDEC-205-scFv can bind CD205 on the DC surface, but also contains a cationic peptide that can bind plasmid DNA. DEC-205 is highly expressed on Langerhans cells of the skin [19]; as such, anti-DEC-205 monoclonal Ab can be delivered to draining lymph nodes. This was evaluated in vivo by

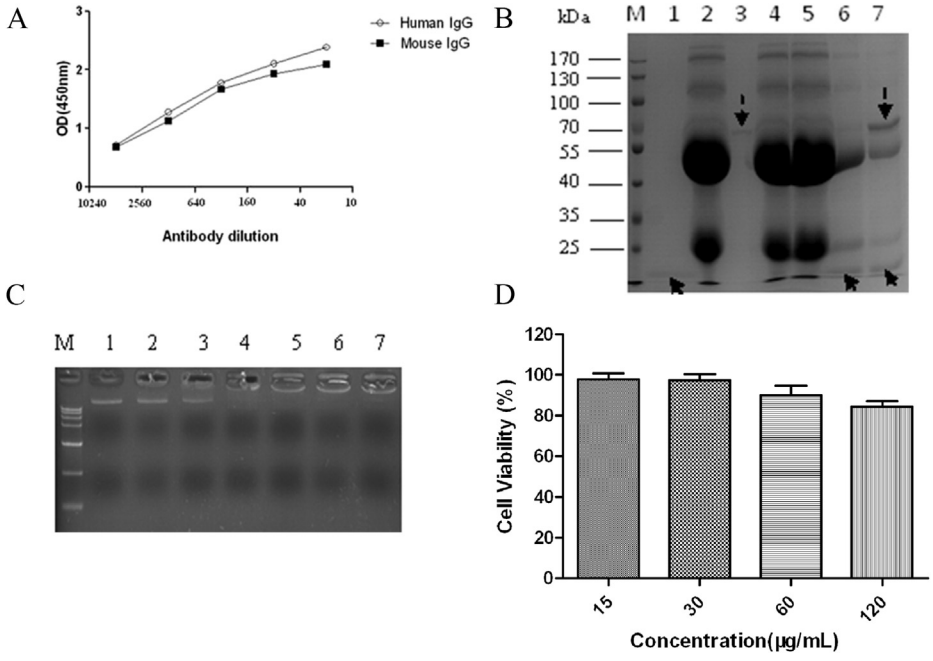

Fig. 3. Analysis the Ab-binding activity, toxicity of msSC protein and protein/DNA complex formation. (A) ELISA analysis of msSC–Ab binding. (B) SDS-PAGE analysis of msSC–Ag–Ab complex formation. Lane 1, Ag (HBsAg); lane 2, Ab (HBIg); lane 3, purified msSC protein; lane 4, supernatant from Ag–Ab complex precipitation; lane 5, supernatant from msSC–Ag–Ab complex precipitation (note the absence of soluble Ag, indicated by an arrow); lane 6, pellet from Ag–Ab complex precipitation (note the absence of soluble Ag and msSC, indicated by arrows); lane 7, supernatant from msSC–Ag–Ab complex precipitation; M, protein marker. (C) Gel retardation assay of msSC and DNA binding was carried out at different N/P ratios. Lane 1, N/P = 0; lane 2, N/P = 5; lane 3, N/P = 10; lane 4, N/P = 20; lane 5, N/P = 40; lanes 6 and 7, N/P = 80; M, DNA marker. (D) The cytotoxicity of msSC protein was evaluated by the CCK-8 assay.

incubating 40 μg of msSC protein with 20 μg of pGL3-CMV plasmid containing the luciferase reporter gene. The protein/DNA complex mixed with 50 μg of poly (I: C) was injected subcutaneously into the footplate of 4–6-week-old female BALB/c mice; 24 h later, luciferase expression was detected by imaging. pGL3-CMV plasmid alone or with an irrelevant protein delivered to the footplate by subcutaneous injection served as a control.

3. Results and discussion

3.1. Construction, expression and purification of recombinant msSC fusion protein

The msSC fusion gene was synthesized and cloned into the pUC57-Amp vector. The msSC gene fragment was isolated from pUC57-Amp-msSC by BamHI/SacI digestion and inserted at the corresponding sites of pET-28a (+). The insertion was confirmed by digesting the recombinant plasmid pET-28a (+)–msSC with BamHI and SacI and by DNA sequencing. pET-28a(+)-msSC was transformed into BL21 (DE3)pLysS cells to generate BL-pET-28a-msSC cells, which were cultured and induced with 1 mM IPTG for 4–6 h. Cell lysates were resolved by SDS-PAGE and the gel was stained with Coomassie Brilliant Blue, which confirmed that msSC was expressed as a fusion protein with an N-terminal His tag (Fig. 2A, lane 1), mainly within inclusion bodies (Fig. 2A, lane 3). The molecular weight of msSC was close to the theoretical mass of about 60 kDa.

Since inclusion bodies contained a high proportion of msSC fusion protein and minimal amounts of contaminating protein, these were dissolved in 8 M urea and refolded by gradient dialysis

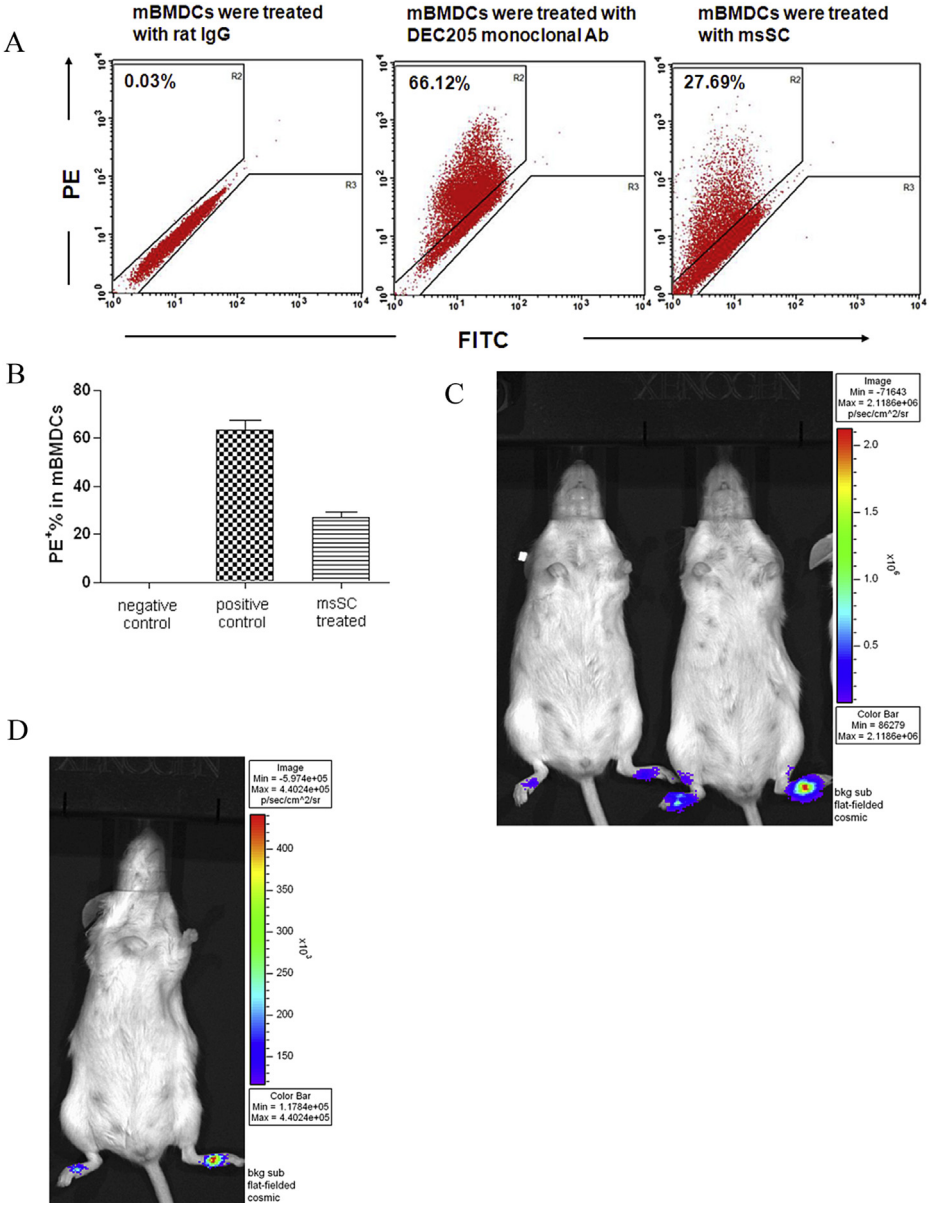

Fig. 4. In vitro and vivo binding capacity of msSC with DCs. (A) and (B) In vitro binding capacity of msSC with mBMDC by flow cytometry. Left: mBMDC were treated with rat IgG; Middle: mBMDC were treated with rat anti-mouse DEC205 monoclonal Ab; Right: mBMDC were treated with msSC protein. (C) In vivo detection of msSC-DNA binding by the luciferase reporter assay. Left: 20 μg of pGL3-CMV + 50 μg of poly (I:C) (subcutaneous footplate injection), Right: 20 μg of pGL3-CMV + 50 μg of poly (I:C) + msSC protein (subcutaneous footplate injection). (D) Fluorescence intensity in mice injected with pGL3-CMV/irrelevant protein compound was detected. (20 μg of pGL3-CMV + 50 μg of poly (I:C) + irrelevant protein (subcutaneous footplate injection).

renaturation, after which the purity of msSC was >90% by Bradford assay (Fig. 2A, lane 7). A western blot analysis using a primary Ab against His (Zhongshan Golden Bridge Biotechnology Co. Ltd., Beijing, China) confirmed of the expression of His-tagged msSC (Fig. 2B, lane 2).

3.2. The Ab-binding activity, toxicity of msSC protein and protein/DNA complex formation

The msSC fusion protein containing the IgG-binding domain of SPG is theoretically capable of binding an IgG Fc segment. The ability of msSC to bind human and mouse IgG was confirmed by ELISA. As shown in Fig. 3A, the msSC protein could bind human and mouse IgG in a various dilution.

A precipitate was observed when the Ag and Ab were mixed with msSC protein at specific ratios 20 rpm, indicating that msSC may form ternary complexes with Ag and Ab. To verify whether this occurs through binding of msSC to the Ab, the precipitate was resolved by SDS-PAGE (Fig. 3B). There was no soluble Ag (lane 4) in the supernatant after centrifugation of the Ag–Ab complex nor any soluble Ag or msSC in the supernatant after centrifugation of the msSC–Ag–Ab complex (lane 5). However, after centrifuging both Ag–Ab and msSC–Ag–Ab complexes, Ag alone (lane 6) or with msSC (lane 7), respectively, were detected in the precipitate. These results indicate that msSC can bind with the Ab to form an msSC–Ag–Ab ternary complex. Given that msSC contains a DC-targeting domain, it was conjectured that msSC can transport Ag–Ab complexes to DCs via complex formation.

The ideal gene delivery vector encodes a protein with DNA-binding capacity [19]. To determine whether msSC can bind DNA in vitro, protein/DNA complex formation at different N/P ratios was examined by Gel retardation assay. The mobility of plasmid DNA decreased at higher N/P ratios, and mobility was completely abolished at N/P = 20 (Fig. 3C, lane 4). These results indicate that msSC can bind negatively charged plasmid DNA, and that in vivo applications of DC-targeted msSC will be determined by an N/P ratio of 20.

The toxicity of the mSC protein was evaluated by the CCK-8 assay. The protein was not toxic to cells even at high concentrations: between 60 and 120 μg/ml, cell viability was decreased slightly but remained at 90% (Fig. 3D). This indicates that msSC protein can be a non-toxic vector for in vivo gene delivery.

3.3. In vitro and vivo binding capacity of msSC with DCs

Given that the fusion protein contains mouse DEC-205 scFv domain, the binding of msSC to mBMDCs was examined by flow cytometry. mBMDCs were treated with rat IgG and rat anti-mouse DEC205 monoclonal Ab as negative and positive controls, respectively, and PE-labeled goat anti-rat IgG was used as the secondary Ab. Rat IgG did not bind mBMDC (Fig. 4A and B, left), while strong binding was observed with rat anti-mouse DEC205 monoclonal Ab (Fig. 4A and B, middle), with the gate of the R2 region reaching 66.12%. The msSC protein bound mBMDCs and the gate of the R2 region reached 27.69% (Fig. 4A and B, right). These results indicate that the msSC fusion protein can directly interact with DCs in vitro.

To assess the ability of msSC to bind DCs in vivo, msSC/pGL3-CMV was injected into the footplate of 4–6-week-old mice, and luciferase activity was examined 24 h later. The entire footplate of injected mice showed a strong fluorescence (Fig. 4C, right), whereas mice injected with pGL3-CMV alone (Fig. 4C, left) or pGL3-CMV/irrelevant protein compounds (Fig. 4D) showed only weak fluorescence. These results suggest that pGL3-CMV was targeted to DCs in the footplate through complex formation with msSC, resulting in enhanced uptake of pGL3-CMV and hence higher luciferase expression.

A low-intensity fluorescent signal was detected in the draining lymph nodes of mouse legs injected with msSC/pGL3-CMV (Fig. 4C, right), consistent with previous reports [19]. The high DEC-205 expression in Langerhans cells of the skin indicates that anti-DEC-205 monoclonal Ab can be delivered to draining lymph nodes. The msSC fusion protein containing mDEC-205-scFv and a cationic peptide can bind to CD205 on the DC surface and plasmid DNA, respectively, enabling the msSC/pGL3-CMV complex to enter the draining lymph nodes. Thus, the immunogenicity of a DNA vaccine could potentially be enhanced through binding to msSC.

4. Conclusions

msSC can bind to DCs via the surface molecule DEC-205; the SPG and CP components can bind to IgG and negatively charged plasmid DNA, respectively. In addition, an msSC–Ag–Ab ternary complex was formed. The msSC fusion protein bound mBMDCs with high efficiency, while plasmid DNA bound to msSC was taken up DCs. These findings indicate that msSC can be used as a safe carrier for the delivery of DNA vaccines directly to DCs.

Conflict of interest

The authors declare that they have no competing interests.

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