Immunotherapy of Bacillus Calmette-Guérin by targeting macrophages against bladder cancer in a NOD/scid [IL2Rg-/-] mouse model

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Abstract. Bacillus Calmette-Guérin (BCG) is considered to be a successful biotherapy for treating bladder cancer (BCa). However, the underlying mechanisms of BCG have not been completely clarified, to date. The role of macrophages in BCG therapy for BCa has still not been determined *in vivo*. In the present study, the role and potential mechanism of BCG (0.25, 1.25 and 6.25 μg/mouse; intravenous) immunotherapy for BCa was investigated in a NOD/scid [IL2Rg-/-] (NSI) mouse model by targeting macrophages *in vivo*. Notably, it was observed that NSI mice with T24 BCa cells displayed high levels of the macrophage marker CD11b^{+} F4/80^{+} after injection via the tail vein of live BCG, as well as a significant reduction in tumor volume. The levels of the inflammatory and macrophage maturation cytokines, such as tumor necrosis factor-α, interleukin (IL)-1β, IL-6, IL-12P70, TNF superfamily member 11 and monocyte chemotactic protein 1, were significantly increased in the serum and the tumor supernatant compared to that in normal control subjects. Furthermore, BCG promoted the expression of the pro-differential genes Spi-1 proto-oncogene, early growth response protein 1, nuclear factor (NF)-κB and proto-oncogene c-Fos in bone marrow. In conclusion, these observations indicate that the injection of live BCG can target macrophages against bladder tumor growth *in vivo*. The mechanism is likely related to the promotion of macrophage maturation, immune activation and increased numbers of macrophages infiltrating the bladder tumor.

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Introduction

Bacillus Calmette-Guérin (BCG) has been used to treat non-muscle-invasive bladder cancer (BCa) for nearly 40 years (1). It is one of the most successful biotherapies for BCa currently in use (2,3). Despite extensive clinical experience with BCG, the mechanism by which it achieves its therapeutic effect remains a matter for investigation. Abundant evidence indicates that BCG therapy for BCa results in extensive activation of the immune system (4). The requirements for effective live BCG therapy include an intact immune system and close contact of BCG with BCa cells (5). Important constituents of the cellular inflammatory response to BCG include CD4^{+} and CD8^{+} lymphocytes, natural killer cells and granulocytes (6-8). Important elements of the humoral immune response to BCG include tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), interleukin (IL)-2, IL-8, IL-18, IL-12, interferon (IFN)-γ and tumor necrosis factor (TNF)-α (9). In addition, the macrophage response to BCG therapy is evident in the bladder wall of patients as well as in their urine *in vitro* (6,10). Another indirect piece of evidence of the response of macrophages to BCG is the variety of cytokines found in the urine of patients treated with BCG, including IL-6, IL-12, and TNF-α, which are known to be secreted by macrophages exposed to BCG (9,11,12). However, there are currently no published reports that confirm the role of macrophages in BCG therapy for BCa *in vivo*, to the best of our knowledge.

In the present study, with the aim of examining the potential functions of BCG in activating macrophages *in vivo*, functional macrophages were observed in a T24 carcinoma tumor-bearing NOD/scid [IL2Rg-/-] (NSI) BCa mouse model after injection via the tail vein of live BCG. The results showed macrophage recruitment in the blood and maturation of macrophages in the bone marrow. Moreover, macrophages

were expressed at high levels in the tumor environment. Due to the relationship between macrophages and tumor cells, the present study attempted to clarify the mechanism of BCG treatment in BCa. Independent *in vitro* experimental results demonstrated BCG 'targeting' of macrophages. The results demonstrated that BCG was the basis for successful cancer treatment and that one of the mechanisms was the targeting of macrophages could be relevant to the future treatment of BCa.

Materials and methods

BCa in the NSI mouse model. The human BCa cell line T24 was obtained from the American Type Culture Collection. Cells were incubated in culture media consisting of RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) and a 1% penicillin-streptomycin solution (Gibco; Thermo Fisher Scientific, Inc.). Cells were cultured in 5% CO_2 and a normal level of oxygen at 37˚C.

NSI mice (N=24) were obtained from Guangzhou Institutes of Biomedicine and Health (GIBH); this is a novel strain of immunodeficient mice with the absence of T, B and NK cells. Animal experiments were performed in the Laboratory Animal Center of GIBH, and all animal procedures were approved by the Animal Welfare and Ethical Committee of GIBH (approval no. N2014050). Mice were maintained in a barrier facility by sister-brother mating and were specific pathogen-free, according to the current Federation for Laboratory Animal Associations guidelines, which include pathogens such as parvovirus, Sendai virus, Hantan virus, coronavirus, reovirus, cytomegalovirus, Pasteurellaceae and *Mycoplasma pulmonis*. All of the mice were kept in a climate-controlled environment (temperature: 22-26˚C; humidity: 40-50%) with a 14 h light/10 h dark cycle, and were provided with autoclaved food and water *ad libitum*. The establishment of a mouse T24 carcinoma tumor-bearing model for study has been reported in other studies (13,14). In brief, 24 male NSI mice (18-20 g) were randomized into 4 groups (n=6) and inoculated with $5x10^6$ T24 cells/mouse. After 3 weeks, each mouse received a single tail-vein injection of BCG at 0.25, 1.25 or 6.25 μg/mouse; one dose was administered each week. The control group was treated with PBS. Analysis was performed during the following week after the administration of the last dose of BCG. Animals were anesthetized with Avertin (2,2,2-tribromoethanol) at a dose of 240 mg/kg body weight, and sacrificed by cervical dislocation. Samples including blood, spleen and tumor tissue were collected for further analysis. The formula for calculating the tumor volume was $V=1/2 x a x b^2$. The weight of each group's spleen was weighed by electronic balance (WANT Balance Instrument Co., Ltd).

Flow cytometry analysis. For the analysis of macrophage surface antigen expression, allophycocyanin-, phycoery-thrin-, Precp- and FITC-conjugated anti-mouse CD11b (cat. no. 17-0112-81), F4/80 (cat. no. 17-4801-82), CD206 (cat. no. 85-12-2061-82) and Ly-6G (Gr-1; cat. no. 11-5931-82) antibodies were used, diluted 1:50. Mouse isotype controls were also used. The cells were washed and stained for 30 min

at 4˚C. All of the antibodies were obtained from eBioscience unless otherwise stated. Flow cytometric analysis was performed using ACEA NovoCyte™ II (ACEA Biosciences, Inc.). Data analysis was performed using FlowJo 7.6.1 software (FlowJo LLC).

Immunohistochemistry analyses. Standard protocols were used for immunohistochemistry. Tumor tissue was pre-treated with 4% paraformaldehyde for 1 h at room temperature, and the tissue was collected and sequentially cut into 4-μm thick sections. The sections were heated in a tissue-drying oven for 120 min at 65˚C and deparaffinized, followed by rehydration through a gradient ethanol series (100, 95 and 70%). Antigen retrieval procedures were necessary for the exposure of all epitopes, which was achieved by microwaving the sections in 0.01 M citrate buffer (pH 6.0) on full power for 3x5 min. The sections were incubated for 30 min at 37˚C with 5% BSA (cat. no. A8806-1G; Sigma-Aldrich; Merck KGaA). The sections were covered with the F4/80 antibody, which was diluted in diluent buffer to 1:100, overnight at 4˚C. The sections were then washed three times with PBS for 5 min each. The sections were covered with the peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody (cat. no. ab6728; 1:2,000; Abcam) for 30 min at 37˚C, then washed three times with PBS for 5 min each. The sections were stained with DAB for 10 min, and counterstained with hematoxylin for 1 min at room temperature after being rinsed twice in PBS. Photomicrographs were captured using a confocal system (Olympus FV500-IX81; Olympus Corporation) and analyzed with Image-Pro Plus 6 (Media Cybernetics, Inc.).

Measurement of cytokine and growth factor production. Parallel 96-well plates were prepared for multiplex assay analysis to measure TNF-α, IL-1β, IL-6, IL-12P70, vascular endothelial growth factor (VEGF), tumor necrosis factor ligand superfamily member 11 (RANKL), macrophage colony stimulating factor (M-CSF) and monocyte chemotactic protein 1 (MCP-1). The multiplex kits were custom designed in-house and manufactured by R&D Systems (cat. no. LXSAMSM-09; lot no. L121798) according to the manufacturer's instructions. Briefly, the medium was collected from triplicate wells and stored at -80˚C until analysis. Each 96-well filter plate (EMD Millipore) was blocked with 100 μl blocking buffer for 30 min and vacuum-filtered at 2 psi. The 25x multiplex bead mix was mixed using a vortex mixer for 1 min and then mixed for 30 sec before diluting. The bead mix was diluted in wash buffer, and 50 μl was immediately added to each well. Different samples were then added to the assay well and mixed at room temperature for 2 h. After washing, 50 μl of detection antibody was added to each well, and the plates were incubated on a shaker at room temperature for 1 h in the dark. After a final washing step, the beads were then resuspended in 150 μl of wash buffer for analysis. The fluorescence intensity of the detection antibody was determined using the Luminex 200 System (Luminex Corporation). Fluorescence intensity readings of 50 beads/cytokine were collected for each standard and sample dilution.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was determined using RT-qPCR and extracted with
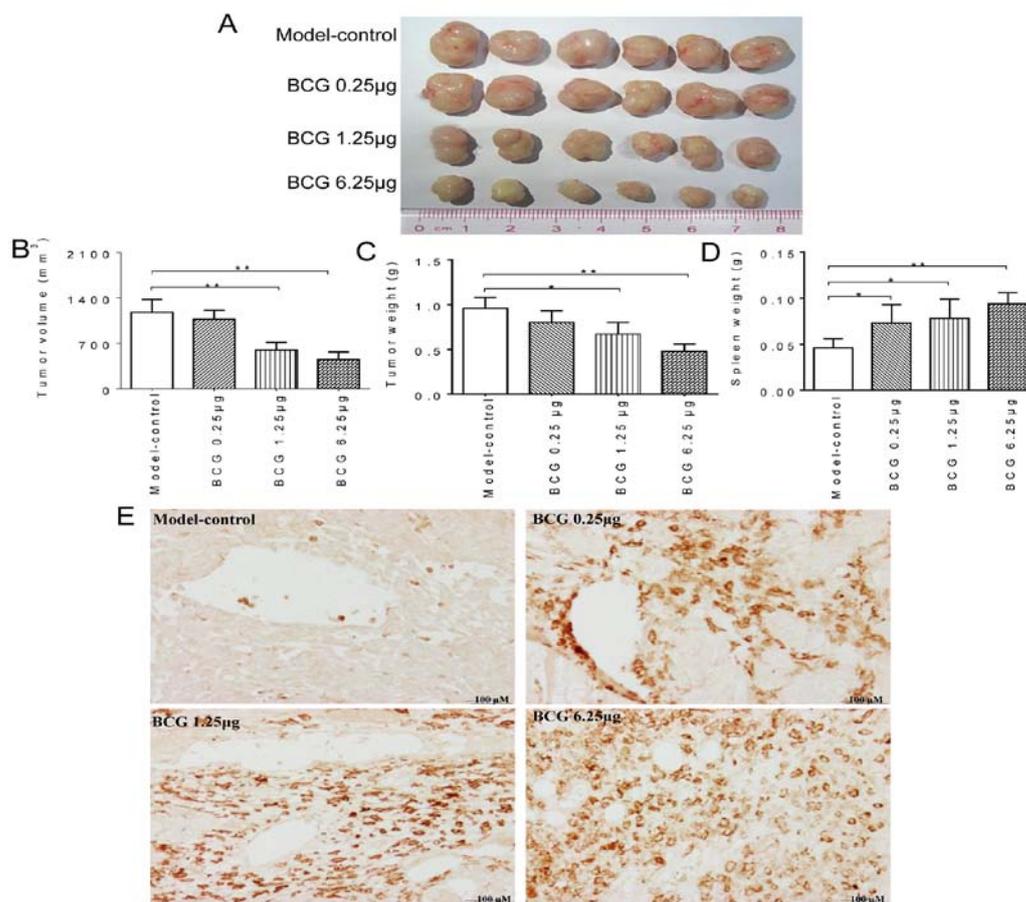
Figure 1. Inhibitory effect of BCG in the T24 tumor-bearing NSI bladder cancer mouse model and immunohistochemistry of F4/80 (macrophages) in the tumor core microenvironment. (A) Morphology of the tumors in the control and different BCG administration groups. The (B) tumor volume and (C) weight results were evaluated for each group. (D) The spleen weight results were evaluated for each group. (E) Immunohistochemistry of the macrophages (detection of F4/80) in the tumor core microenvironment. The results are presented as the mean ± SD of six NSI mice. $^*P<0.05$ and $^{**}P<0.01$. NSI, NOD/scid $^{IL2Rg-/-}$; BCG, Bacillus Calmette-Guérin.

TRIzol® (GenStar Technologies). The EasyScript First-Strand cDNA Synthesis SuperMix kit (TransGen Biotech Co., Ltd.) was used to prepare cDNA from 1,000 ng of RNA under the following conditions: 25˚C for 10 min, 37˚C for 60 min and 70˚C for 10 min. cDNA was then amplified with a TransStart Green qPCR SuperMix kit (TransGen Biotech Co., Ltd.) in a Bio-Rad CFX96 Real-Time PCR System (Bio-Rad Laboratories Inc.). The primers were from Shanghai Jierui Biotechnology Co., Ltd., with the following sequences: 5'-GCTGTCTTGGGT GCATTGGA-3' (forward) and 5'-AAGGGACTTCCTGTA ACAATGCA-3' (reverse) for β-actin; 5'-CGGGGCTGATCT GGGAAAAT-3' (forward) and 5'-CACAGCGTAACCTCG TCTTC-3' (reverse) for interferon regulatory factor 8 (IRF-8); 5'-CAAGCGCATGACGTATCAGAA-3' (forward) and 5'-GCTGTCAAACTGGTAGGTGAG-3' (reverse) for Spi-1 proto-oncogene (PU.1); 5'-GGACGGTGTTGCAGCAGAT-3' (forward) and 5'-GCAGTCTGAGTTCCAGTGGTA-3' (reverse) for RANK; 5'-TCCCCACAGTTGCCTTCAC-3' (forward) and 5'-GAGCGGCGTCTTGCCTTTA-3' (reverse) for NF-κB; 5'-ACTTTGCGCCTACAATTCAGG-3' (forward) and 5'-AACTTGCCAGGGAATGGAACT-3' (reverse) for EGR-1; 5'-TGTCATCGAGCCTAGTGGC-3' (forward) and 5'-CGGGAGATTCAGGGTCCAAG-3' (reverse) for colony stimulating factor 1 receptor (C-FMS); 5'-TTGAGCGAT CATCCCGGTC-3' (forward) and 5'-GCGTGAGTCCATACT GGCAAG-3' (reverse) for proto-oncogene c-Fos (c-Fos). The following thermal profile was used for all qPCR experiments: 50˚C for 2 min; 95˚C for 10 min; and 40 cycles of 95˚C for 15 sec and 60˚C for 1 min. The fit point algorithm of the Light Cycler software was used to calculate Cq (15) values. For the normalization of RNA expression, the levels of β-actin mRNA were used as the internal control, and the gene-specific mRNA expression was normalized against β-actin expression. All samples were prepared and tested in triplicate.

Statistical analysis. Data are expressed as the mean ± SD. Significant differences between the control and treatment group were analyzed by one-way ANOVA followed by Tukey's post-hoc test, or by two-tailed unpaired Student's t-test. Statistical analysis was performed using GraphPad Prism Software version 5.0 (GraphPad Software, Inc.). FlowJo 7.6.1 software was used to analyze the flow cytometry data. P<0.05 was considered to indicate a statistically significant difference.

Results

BCG enhances the macrophage-mediated inhibition of BCa growth in vivo. To assess the effects on macrophage of live
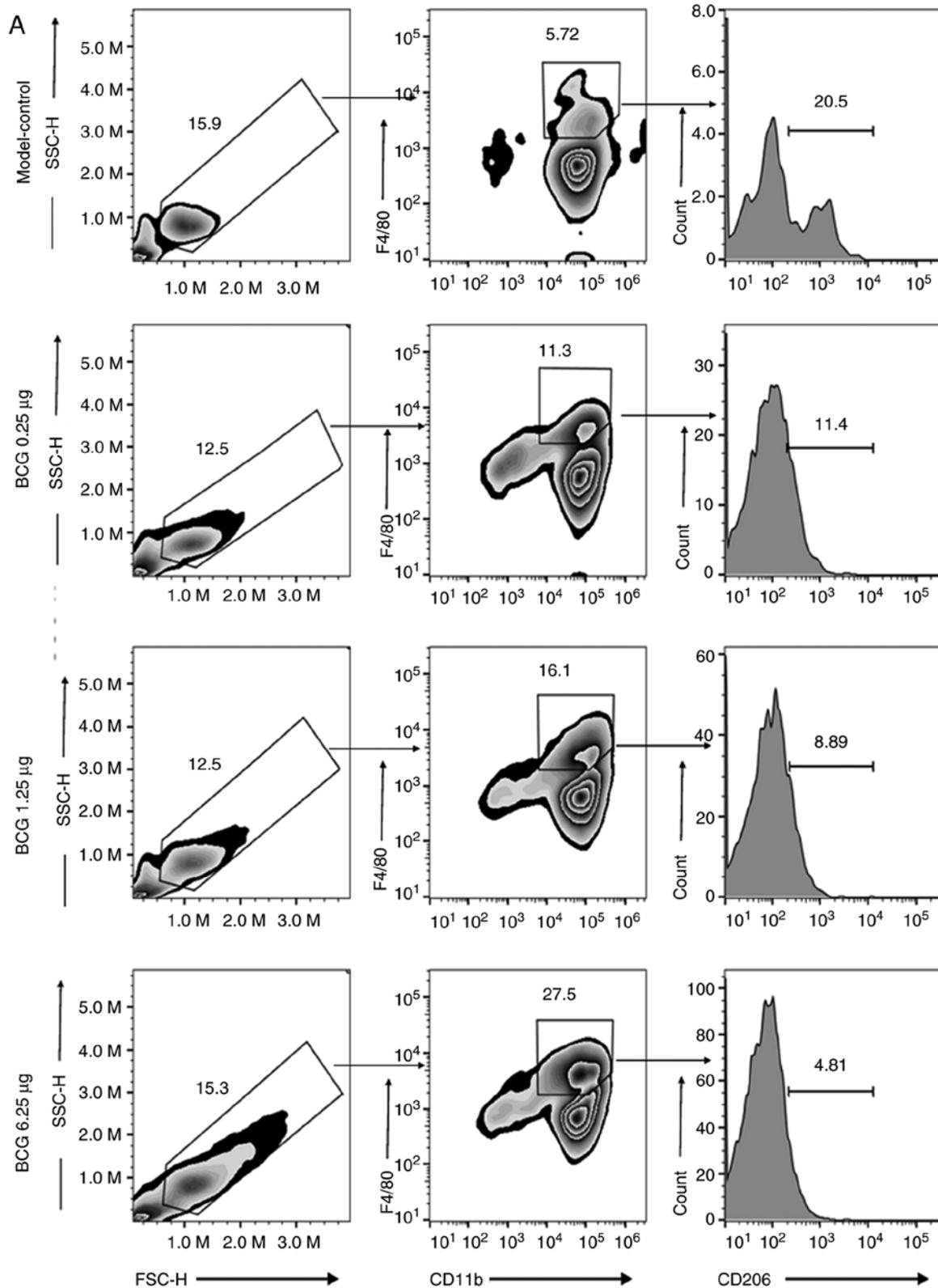
Figure 2. Effect of BCG on the expression of macrophages analyzed using flow cytometry for blood, spleen and bone marrow in NSI mice. (A) CD11b, F4/80 and CD206 were analyzed in the blood.

BCG during BCa growth *in vivo*, NSI mice were inoculated with human BCa T24 cells. Although every mouse in the model-control group and BCG-treatment groups developed tumors (100% tumor incidence) after a 3-week latency, the treatment effect of live BCG (delivered intravenously to the tail) was assessed for 4 weeks. In the present study, no mouse presented with multiple. The largest subcutaneous tumor had a diameter of 1.18 cm, and the largest tumor as a percentage of body weight was 5.1%. It was also observed that no mice were found to have succumbed during this study, and no
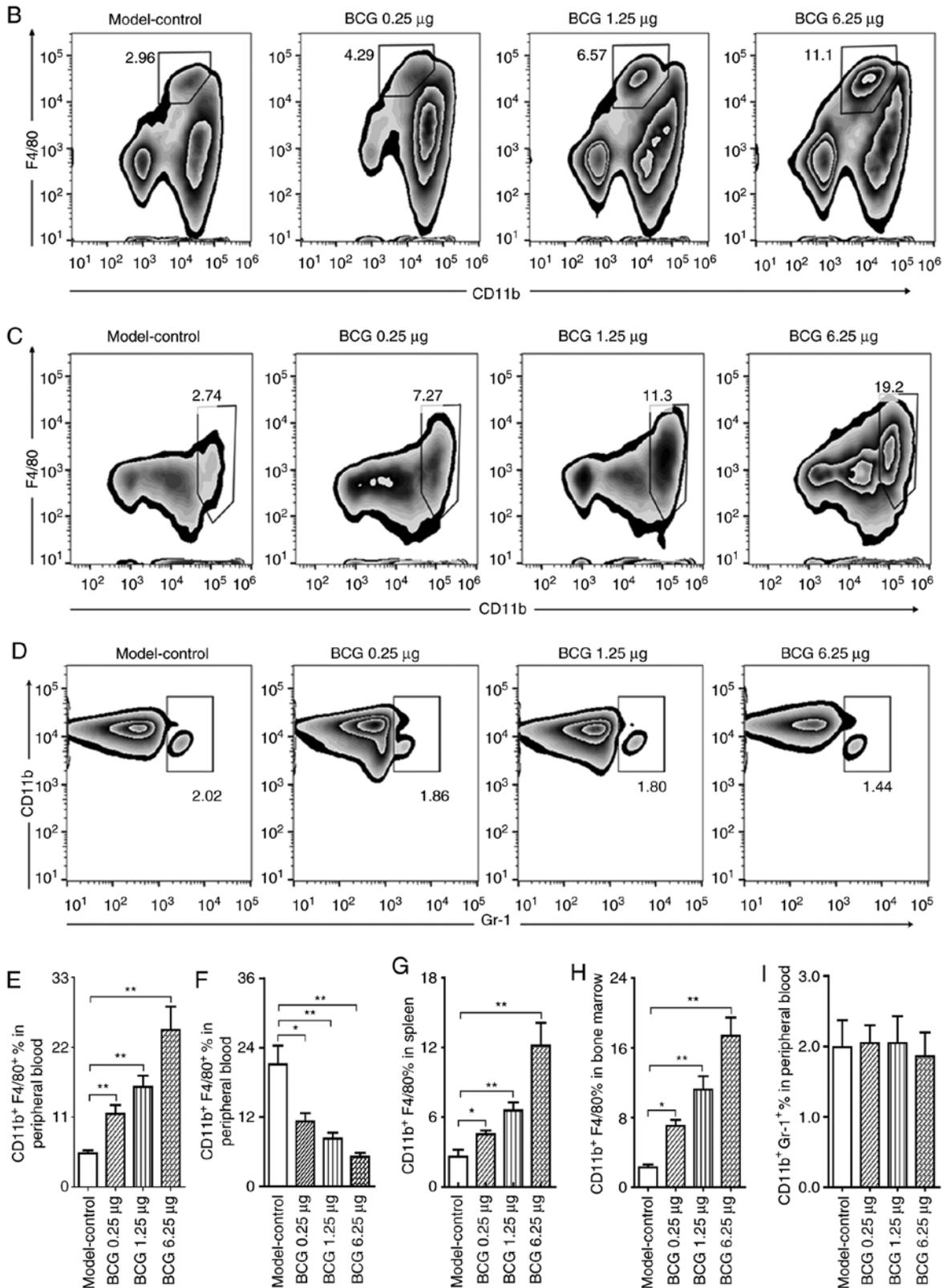
Figure 2. Continued. Effect of BCG on the expression of macrophages analyzed using flow cytometry for blood, spleen and bone marrow in NSI mice. (B) CD11b and F4/80 were analyzed in the spleen. (C) CD11b and F4/80 were analyzed in the bone marrow. (D) CD11b and Gr-1 were analyzed in the blood. (E) CD11b⁺F4/80⁺ cells in the blood. (F) CD11b⁺F4/80⁺CD206⁺ cells in the blood. (G) CD11b⁺F4/80⁺ cells in the spleen. (H) CD11b⁺F4/80⁺ cells in the bone marrow. (I) CD11b⁺F4/80⁺ cells in the bone marrow. $^*P<0.05$ and $^{**}P<0.01$. NSI, NOD/scid $^{IL2Rg-/-}$; BCG, Bacillus Calmette-Guérin.

significant abnormalities were found between the treatment group and the control group. Interestingly, during the treatment period, a significant reduction in tumor growth was observed. Tumor burden, as assessed by tumor size, weight and volume (Fig. 1A-C), in the BCG treated groups (1.25 and 6.25 µg/mouse) were significantly lower (P<0.05) than in
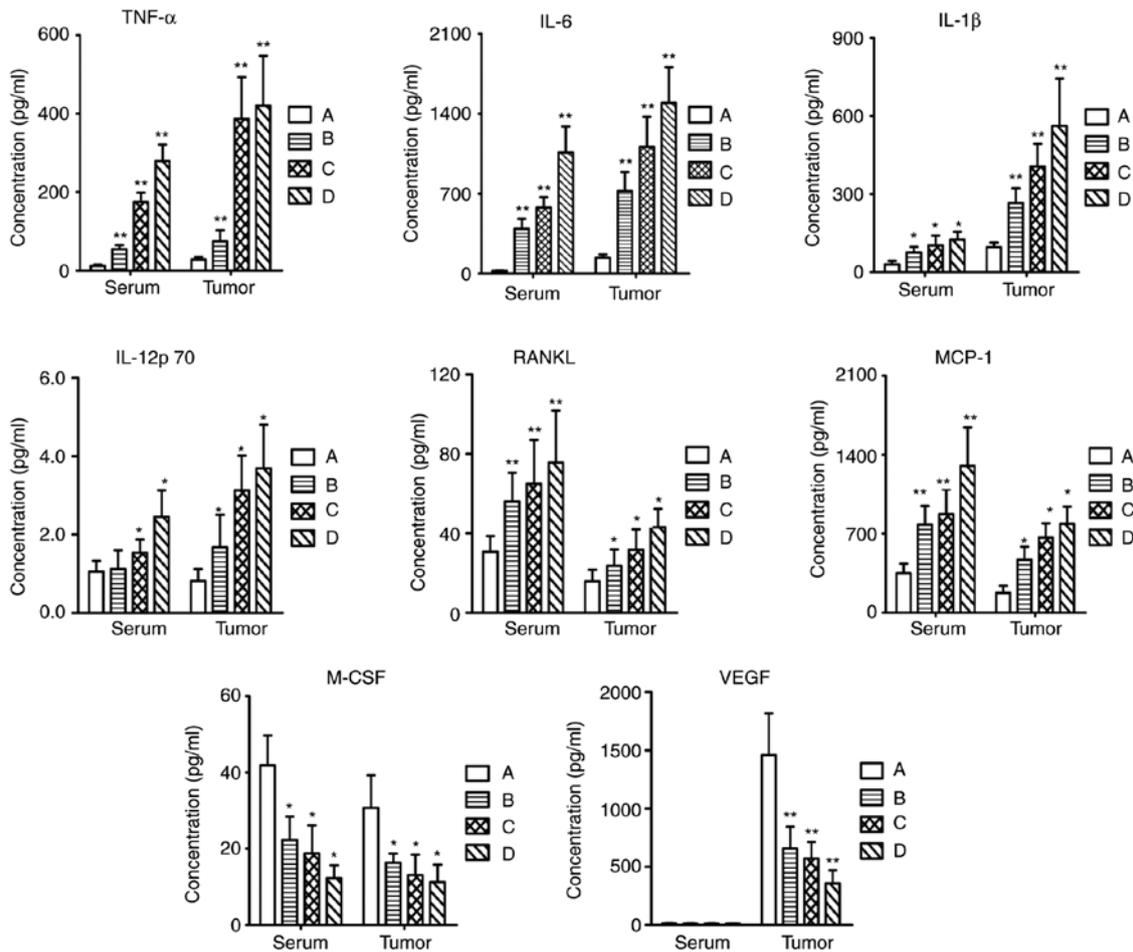
Figure 3. Serum and tumor supernatants were analyzed using a multiplex assay for the production of TNF-α, IL-6, IL-1β, IL-12P70, RANKL, MCP-1, M-CSF and VEGF after treatment of NOD/scid $^{IL2Rg-/-}$ mice with BCG. Each value is expressed as the mean ± SD of three separate experiments. *P<0.05 and **P<0.01 vs. respective control. A, control group; B, BCG 0.25 µg group; C, BCG 1.25 µg group; D, BCG 6.25 µg group; BCG, Bacillus Calmette-Guérin; IL, interleukin; TNF-α, tumor necrosis factor-α; VEGF, vascular endothelial growth factor; RANKL, tumor necrosis factor ligand superfamily member 11; M-CSF, macrophage colony stimulating factor; MCP-1, monocyte chemotactic protein 1.

the control group. In addition, the weight of the spleen in the BCG-treated groups increased compared to that in the control group (Fig. 1D). Similarly, the infiltration of macrophages (detected with anti-F4/80) was markedly increased in NSI mice compared to mice in the control group (Fig. 1E). Based on these results, macrophages were selected for further experimentation and analyses.

BCG enhances macrophage recruitment and differentiation/transformation in the immune system. After observing increased numbers of macrophages in BCa tumors using F4/80 staining, the blood, spleen and bone marrow isolated from the studied NSI mouse model were examined to determine whether immune system parameters were consistent with the results generated using tumor samples. After the mice had been sacrificed, blood-derived macrophages were analyzed using flow cytometry staining with anti-CD11b and F4/80. The M2 phenotype was also extensively analyzed using the marker CD206 (Fig. 2A, E and F). It was observed that BCG caused a marked increase in the number of macrophages and that the proportion of M2 decreased; however, CD11b$^+$/Gr-1$^+$ myeloid-derived suppressor cells (MDSCs) did not significantly change in the blood (Fig. 2D and I). To determine a more
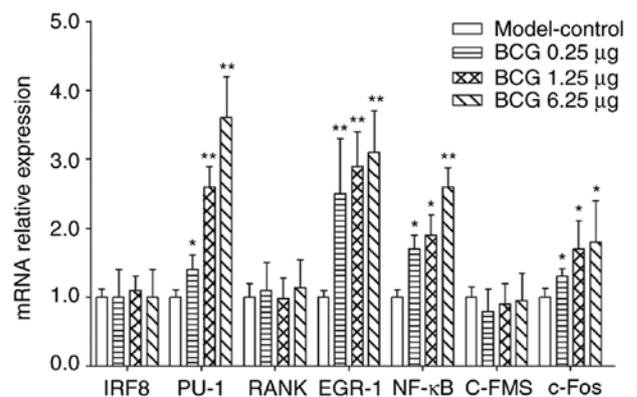
Figure 4. mRNA levels of IRF8, PU-1, RANK, EGR-1, NF-κB, C-FMS and c-Fos in BCG-treated mice were detected using reverse transcription-quantitative PCR in bone marrow. *P<0.05 and **P<0.01 vs. respective model-control group. The results represent the mean ± SD of three separate experiments. BCG, Bacillus Calmette-Guérin; IRF-8, interferon regulatory factor-8; PU.1, Spi-1 proto-oncogene; NF-κB, nuclear factor-κB; C-FMS, colony stimulating factor 1 receptor; c-Fos, c-FOS proto-oncogene.

direct mechanism, further analysis of dissociated spleen cells using flow cytometry confirmed that the expression mainly
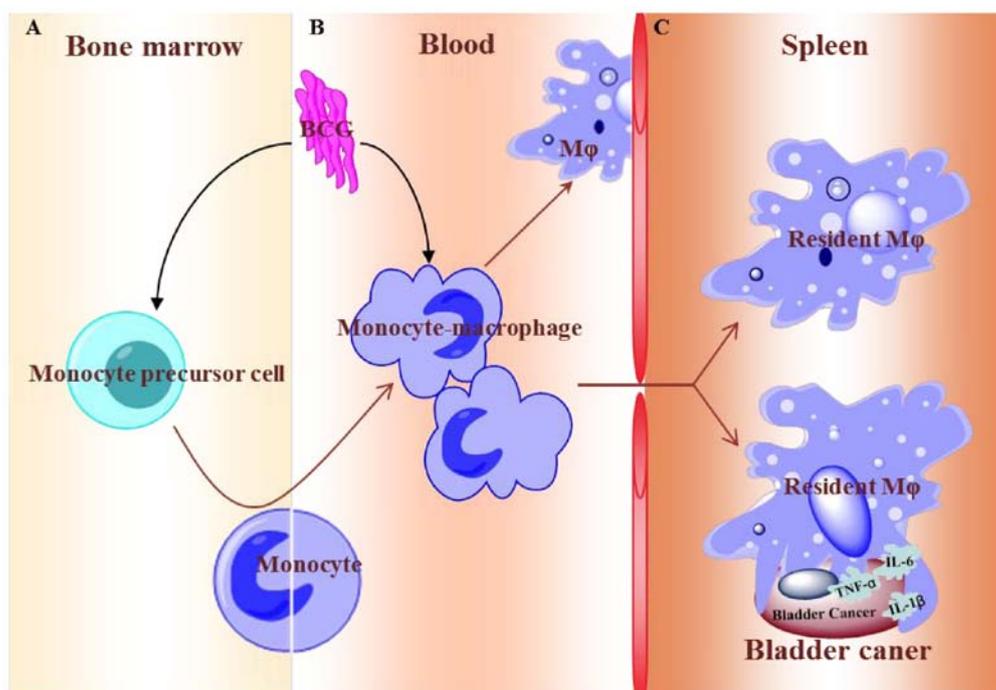
Figure 5. A schematic diagram of the proposed mechanisms of BCG in the promotion of macrophage recruitment and immune activation to inhibit BCa *in vivo*. (A) BCG promotes the transformation of bone marrow precursor monocytes into monocytes and upregulates a series of macrophage-related transcriptional genes. (B) In addition, BCG in the blood circulation induces monocytes and monocyte-macrophages to differentiate in the direction of macrophages and release a large number of cytokines (chemokines) to promote macrophage maturation and immune activation. (C) Increased infiltration of macrophages inhibits BCa growth. BCG, Bacillus Calmette-Guérin; TNF-α, tumor necrosis factor-α; IL, interleukin; BCa, bladder cancer.

consisted of CD11b⁺/F4/80⁺ macrophages, with a six-fold increase in active macrophages compared to the model control (Fig. 2B and G). To determine whether macrophage precursor cells were detected by flow cytometry with anti-CD11b⁺/F4/80⁺, macrophage differentiation/transformation was evaluated using the anti-CD11b⁺/F4/80⁺ macrophage marker in bone marrow (Fig. 2C and H). Notably, it was found that this difference was consistent with the results from the spleen and blood. These data indicated that BCG-induced differentiation/transformation of macrophages began in the bone marrow and affected the entire immune system in a sweeping manner.

BCG increases the expression of TNF-α, IL-1β, IL-6, IL-12P70, VEGF, RANKL, M-CSF and MCP-1, associated with macrophages. The present study suggested that BCG may function by recruiting macrophages to suppress BCa via an increased quantity of macrophage-released soluble cytotoxic factors. The mouse magnetic Luminex assay was performed to evaluate the changes in the expression levels of cytokines; serum and tumor supernatants were collected for this test (Fig. 3). Treatment with BCG markedly increased the recruitment of macrophages, resulting in the upregulation of pro-inflammatory cytokines such as TNF-α, IL-1β, IL-6 and IL-12P70, in a dose-dependent manner (0.25-6.25 μg BCG/mice). Chemokines enhance chemotaxis and the activating factors of macrophages, thereby accelerating the generation of macrophages. In our study, the expression level of MCP-1 was also upregulated after treatment with BCG. In addition, the recruitment of macrophages led to the secretion of RANKL, which strengthened the immune response to BCG. Otherwise, the levels of vascular VEGF and M-CSF in

the serum and tumor supernatants were decreased compared to those in the normal group.

Effect of BCG on IRF-8, PU.1, RANK, EGR-1, NF-κB, C-FMS and c-Fos mRNA expression in bone marrow. Growing evidence demonstrates that IRF-8, PU.1, RANK, EGR-1, NF-κB, C-FMS and c-Fos may be linked to mature macrophage differentiation from monocyte-macrophage precursor cells (16-18). Thus, the present study further investigated the effects of BCG on the gene expression levels in bone marrow tissue from a BCa model. RT-qPCR analysis showed that the expression levels of four genes in bone marrow treated with BCG were significantly overexpressed compared to the control group. As shown in Fig. 4, different doses of BCG in treated mice exhibited 1.4-3.6-fold, 2.5-3.1-fold, 1.7-2.6-fold, and 1.3-1.8-fold up-regulation of the PU-1, EGR-1, NF-κB and c-Fos mRNA levels (P<0.05), respectively.

Discussion

In 1891, the use of microbial products for the treatment of cancer was pioneered by Dr. William B. Coley, who treated cancer patients with intratumoural injections of live *Streptococcus pyogenes* (19). The biologist Pearl (1928) (1), who performed an autopsy study and observed that cancer was less common in patients who had lesions of active tuberculosis, first suggested the idea that mycobacteria might be useful as a therapy for cancer. A study conducted at the Sloan-Kettering Institute showed that mice infected intravenously with BCG were more resistant to the transplantation of tumors (20), leading to the discovery of TNF-α in the serum of BCG-infected

mice. Since BCG was first used by Morales *et al* (21) in 1976, the efficacy of BCG therapy for BCa has been confirmed. Intravesical BCG has been an effective immunotherapy that represents the current standard treatment for patients with high risk non-muscle-invasive BCa (22). However, several gaps in the knowledge still exist, which should be addressed in future efforts to understand this biotherapy for cancer. No published reports have confirmed the role of macrophages in BCG therapy for BCa *in vivo*, to the best of our knowledge. This study comprised innovative exploration of BCG injection in NSI mice; the treatment was administered twice a week for 1 month, relatively close to the duration of clinical treatment BCG in patients with BCa, typically once a week for 6 weeks (23). The upper limit of tumor size was set at 2 cm^3, which was defined as the experimental ethical endpoint. Animals exhibiting signs of this humane endpoint would be sacrificed immediately, although this did not occur in the present study.

The NSI strain does not harbor T, B or NK cells. However, the mice exhibit normal macrophages and other monocytes, and the strain can be adapted to study the physiological roles and mechanisms of macrophages in tumor models (24). The present study showed that BCG directs monocyte precursor cells to differentiate into functional mature macrophages. Upon BCG treatment, monocyte progenitor cells induce the macrophage-specific marker F4/80 and enhance CD11b expression *in vivo*. These cells have overexpressed gene levels of PU-1, EGR-1, NF-κB and c-Fos, which may be linked to the differentiation of macrophages to a mature form. The secretion of cytokines, such as TNF-α, IL-1β, IL-6 and IL-12P70, is also a hallmark of mature macrophage function. Moreover, it was identified that BCG induced a high level of MCP-1, which macrophages require to stimulate differentiation responses. Another observation that supports the role of BCG in stimulating authentic macrophage differentiation is that the proportion of macrophages increased in the blood in the present study.

In the present study, the immunotherapy effect of BCG depended on macrophage activation and tissue infiltration, the increase in the number of macrophages in the bladder tumor, and the secretion of tumor-killing cytokines. Notably, the therapeutic effect of live BCG treatment in the NSI BCa mouse model was significant inhibition of tumor growth, evidence of the important role of macrophages in tumor immunotherapy. The proportion of CD11b$^+$/Gr-1$^+$ MDSCs in the blood did not significantly change, meaning that the macrophages were immune-activated. In the present investigation, based on knowledge of macrophage maturation and immune activation, administration of BCG led to a high state of macrophage immunotherapy, which allowed tumoricidal activity to continue for 1 month.

In summary, the present study may provide an additional theoretical and experimental basis for the use of BCG for tumor immunotherapy (Fig. 5). BCG stimulated the differentiation and maturation of macrophages, thereby increasing the proportion of macrophages in the blood. Moreover, macrophages were activated and the tumoricidal effects were maintained. Relieving the immunosuppressive effects of macrophages, such as tumor progression, vascularization, invasion and metastasis in the tumor microenvironment, is also beneficial to

the enhancement of other immunotherapies. The present study is the first, to the best of our knowledge, conducted to examine the mechanism of BCG in targeting macrophages against BCa in an NSI mouse model. Since a mouse model with a healthy immune system was not used, there can be no clear conclusions about the *in vivo* effects of BCG on lymphocytes. Further study is necessary to determine the effects of BCG as an immunotherapeutic agent for cancer *in vivo*.

Availability of data and materials

The datasets used or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

XZe, QLT and CYZ designed the research study and wrote the paper. QLT, CYZ, LC, CPL, ML, WXX and XZh performed the research study. XZe, XZh and ML revised the manuscript. XZh helped with revising the language of the manuscript.

Ethics approval and consent to participate

The present study was approved by the Animal Welfare and Ethical Committee of Guangzhou Institutes of Biomedicine and Health (approval no. 2014050).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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