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Original Research

Bacillus Calmette Guérin (BCG) activates lymphocyte to promote autophagy and apoptosis of gastric cancer MGC-803 cell

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Abstract: Bacillus Calmette Guérin (BCG) has a potential anti-tumor effect on gastric cancer. However, the mechanism is still unclear. In this study, we investigated the effect of BCG on gastric cancer cell line MGC-803 and studied the potential cooperation of BCG and lymphocyte in determining the final fate of cancer cells. After treatment with BCG, the cell viability was significantly inhibited in a dosage-dependent manner. Flow cytometry assay showed the apoptosis rates were significantly increased by BCG. Using western blot assay, results showed that BCG increased cleaved-caspase-3, LC-3BII and Atg-3. After cocultured with BCG and lymphocyte, the apoptosis rates, the levels of cleaved-caspase-3, and the protein levels of LC-3BII and Atg-3 were significantly increased compared with BCG or lymphocytes alone groups. ELISA detection found that BCG induced secretion of interferon gamma (IFNg) from lymphocytes. BCG with IFNg also increased levels of cleaved-caspase-3, LC-3BII and Atg-3. Taken together, BCG promotes lymphocyte immunocompetence to induce cell apoptosis and autophagy in MGC-803 cells, might through inducing release of IFNg from peripheral blood lymphocytes.

Key words: Bacillus Calmette Guérin; Lymphocyte; IFNg; Gastric cancer; Autophagy.

Introduction

Bacillus Calmette Guérin (BCG) is an attenuated strain of *Mycobacterium bovis* that has lost its virulence for human (1, 2). Since 1921 when Calmette and Guerin isolated the mycobacterium tuberculosis, BCG has been employed as a vaccine against tuberculosis (TB) and played a significant role in the prevention and treatment of tuberculosis (1). In 1929, Perle et al. discovered a low cancer incidence in TB population, indicating that BCG had a potential anti-tumor function (1, 3). BCG was firstly applied to treat patients with cancer in 1935. However, it was not until the 1950's and 1960's of that the theoretical and clinical research on BCG function on cancer treatment was initiated (4).

Numerous studies have confirmed the effectiveness of BCG as a nonspecific enhancer that can increase phagocytic activity of macrophage, activate lymphocyte, and boost the level of cellular and humoral immunity (5-8). For many years, a great number of researches have focused on BCG application for the prevention and treatment of tumor (9, 10).

Nowadays, BCG is primarily applied as an adjuvant treatment for bladder and malignant tumor, which achieve relatively good effect (11, 12). As an effective anti-tumor modulator, the potential application of BCG for other tumors prospect of BCG is appreciable (13). The anti-tumor mechanism of BCG is hitherto ambiguous.

Although anti-tumor effect of BCG administration was studied by both animal experimental and clinical trials, the exact mechanism of BCG in the treatment of tumors is not fully understood (14, 15). It has been proved that BCG plays an important role in local tumor (16). However, studies have also shown that the complete immune system is indispensable for the function of BCG (17). Ratliff et al found that BCG did not play an anti-tumor role in rats with thymus removed, so BCG is considered to have an anti-tumor effect by activating the body's immune function (17).

In the current research, we identified an anti-tumor effect of BCG on gastric cancer cell line MGC-803. We also studied the potential cooperation of BCG and lymphocyte in determining the final fate of human cancer cells.

Materials and Methods

Cell culture

Human gastric cancer cell line MGC-803 was commercially obtained from Sun Yat-sen University Cancer Center, Guangzhou, China. The cells were cultured in RPMI-1640 medium (Gibco, USA) containing 10% fetal bovine serum (Gibco, USA) and 100μg/ml penicillin/streptomycin, and incubated in standard incubator at 37℃ in saturating humidity of 5% CO_2/95% air. ImmuCyst BCG was made by Sanofi-Pasteur, USA (one vial contained 81 mg BCG with 5% monosodium glutamate).

Lymphocytes were isolated from human peripheral blood using Lympholyte®-H Cell Separation Media (Cedarlane, Canada) according to manufacturer's instruction. All healthy subjects were signed informed consensus form and this study was approved by Ethics Committee of Center for Tuberculosis Control of Guangdong Province, China.

The Recombinant Human IFN-gamma (rhIFNg) Protein was produced by R&D system. (Cat# CAA31639, R&D system, USA). The effect of BCG on lymphocyte or rhIFNg regulated apoptosis and autophagy of gastric cancer cells were performed by Transwell assay.

Cell viability

Cell viability was evaluated by MTT assay as described previously (18). Briefly, cells were seeded in 100 μl RPMI-1640 medium in a 96-well plate with a cell density of 1×10^4 cells/well. There were three parallel wells for each treatment. After adhering to the wells for 8 h, cells were incubated with BCG at different dosages (1 mg/mL, 0.5 mg/mL, 0.25 mg/mL, 0.125 mg/mL, 0.063 mg/mL or 0 mg/mL BCG in phosphate-buffered saline) (19). Then, the cells were cultured in standard incubator at 37℃ in saturating humidity of 5% CO_2/95% air for 24 h or 48 h, washed by phosphate-buffered saline for three times, and incubated with 10μl MTT (5mg/mL) in 100 μl culture solution for another 4 h. The formazan crystals in the living cells were dissolved in 100 μl of DMSO in each well. The absorbance at 490nm (A490) was measured by an enzyme-labeling measuring instrument (iMark; Bio-red, USA).

Cell apoptosis assay

Cell apoptosis was performed by Annexin V/PI assays. Cells were seeded in 1 ml RPMI-1640 medium in 6-well plate with the density of 1.5×10^5 cells/well, and treated with BCG (1 mg/mL, 0.5 mg/mL, 0.25 mg/mL, 0.125 mg/mL, 0.063 mg/mL or 0 mg/mL BCG). After 24 h, the cells were washed by 4℃ PBS twice, and stained with 10 μl Annexin V-FITC and 10 μl PI (5 μg/ml) for 30 min in dark at room temperature. Then, cells were incubated with 300 μl binding buffer (10 mM HEPES, pH 7.4, 140 mM NaOH, 2.5 mM $CaCl_2$). The apoptotic cells were evaluated using flow cytometry (FACS Calibur; BD Biosciences, USA). The apoptosis rates were evaluated.

Transwell assay

In order to explore whether the effect of BCG on lymphocyte or rhIFNg regulated apoptosis and autophagy of gastric cancer cells, we co-cultured the BCG (0.25 mg/mL) and lymphocyte (8.8×10^5 cells/well) or rhIFNg (50 or 100 pg/ml) with 100 μl fetal bovine serum in upper chamber of Transwell with 0.1 μm pore size, and cultured the MGC-803 cells in a normal 6-well plate with a cell density of 4×10^5 cells/well in serum-free medium. After 24 h, the apoptosis and autophagy in MGC-803 cells were performed, and levels of Interferon gamma (IFNg) in medium were detected. The levels of IFNg were performed by commercially ELISA kit (Cat# DIF50, R&D system, USA) according to manufacturer's instruction.

Western Blot assay

Cells were washed by PBS twice, lysed with 80 μl ice-cold lysis buffer (50 mM Tris/HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.25% sodium deoxycholate and a protease inhibitor cocktail) on ice for 15 min, and centrifuged at 12,000 rpm for 15 min. Protein concentration was determined by bicinchoninic acid (BCA) protein assay (Thermo Fisher, USA). Equal amount of proteins from each sample were separated with SDS/PAGE (10-12% gels) and then transferred to PVDF membranes. After blocking with 1%bovin serum albumin, the membranes were incubated in rabbit anti LC-3B (1:1000; Abcam, USA), anti-Atg-3 (1:1000; Abcam, USA), anti pro caspase-3 (1:1000; ab32150, Abcam, USA) or cleaved-caspase-3 (1:500; #9662, Cell signaling technology, USA) antibodies at 4℃ overnight. As a loading control, β-actin was detected with 10μg of anti-β-actin monoclonal antibody. After incubation in horseradish peroxidase (HRP) conjugated secondary antibodies (BOSTER Ltd. China) for 1 h, images were acquired with enhanced chemiluminescence (ECL) western blotting detection reagents (Thermo Fisher, USA). The Quantity One 8 software (Bio-Rad, USA) was used to quantify the band intensities. The gray scale value of the positive band was normalized to the β-actin.

Statistical analysis

All the data were reported in a format of mean ± standard deviation (SD) at least three independent experiments and analyses were performed using SPSS 17.0 software (SPSS, USA). The results were analyzed using the Student's t-test for comparison of two groups, and the one-way ANOVA with Turkey post hoc test for comparison of multiple group. Differences were considered statistically significant at $p<0.05$.

Results

BCG inhibited cell viability of MGC-803 cells in a dose-dependent manner

MGC-803 cells were treated with different dosages of BCG (1 mg/mL, 0.5 mg/mL, 0.25 mg/mL, 0.125 mg/mL, 0.063 mg/mL or 0 mg/mL) for 24 h or 48 h. The cell viability was significantly inhibited in a dosage-dependent manner (Figure 1).

BCG promoted cell apoptosis in MGC-803 cells

MGC-803 cells were treated with different concentrations (1 mg/mL, 0.5 mg/mL, 0.25 mg/mL, 0.125 mg/mL, 0.063 mg/mL or 0 mg/mL BCG) of BCG for 24 h, PI and Annexin V staining and flow cytometry assay were performed to investigate whether BCG promotes

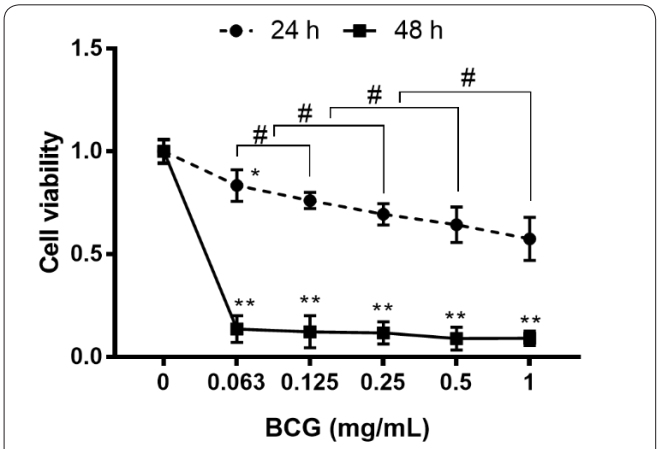

Figure 1. The effect of BCG on cell viability in gastric cancer cell line MGC-803cells. MGC-803 cells were treated with BCG at 1 mg/mL, 0.5 mg/mL, 0.25 mg/mL, 0.125 mg/mL, 0.063 mg/mL or 0 mg/mL for 24 h or 48 h. Control: cells without BCG treatment. *P<0.05, **P<0.01 vs. control; #P<0.05.

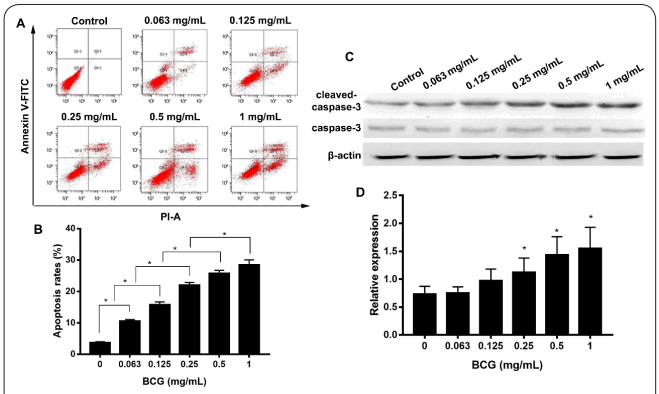

Figure 2. The effect of BCG on cell apoptosis of MGC-803 cells. MGC-803 cells were treated with BCG at 1 mg/mL, 0.5 mg/mL, 0.25 mg/mL, 0.125 mg/mL, 0.063 mg/mL or 0 mg/mL for 24 h. Then, (A) cell apoptosis was performed by Flow cytometry and (B) apoptosis rates were quantified. *P<0.05. (C) The levels of cleaved- and pro-caspase-3 were also detected by Western blot and (D) quantified. Control: cells without treatment. *P<0.05 vs. control.

cell apoptosis (Figure 2A and B). After treatment with BCG for 24 h, the apoptosis rates were significantly increased compared with the control group (Figure 2A and B). Moreover, the percentages of apoptotic cells were significantly increased in a BCG dosage-dependent manner (Figure 2A and B). Using western blot assay, the expression of cleaved-caspase-3 in BCG treated cells were performed. Results showed that BCG increased cleaved-caspase-3 (Figure 2C and D). Thus, BCG increased apoptosis of MGC-803 cells.

BCG promoted autophagy in MGC-803 cells

The autophagy of MGC-803cells was detected by western blot (Figure 3). After treatment with 1 mg/mL, 0.5 mg/mL, 0.25 mg/mL, 0.125 mg/mL, 0.063 mg/mL or 0 mg/mL BCG for 24 h, the protein levels of LC-3BII and Atg-3 were significantly increased in the presence of BCG in a dosage-dependent manner compare with the control group, suggesting BCG treatment was able to trigger autophagy in MGC-803 cells in a dosage-dependent manner.

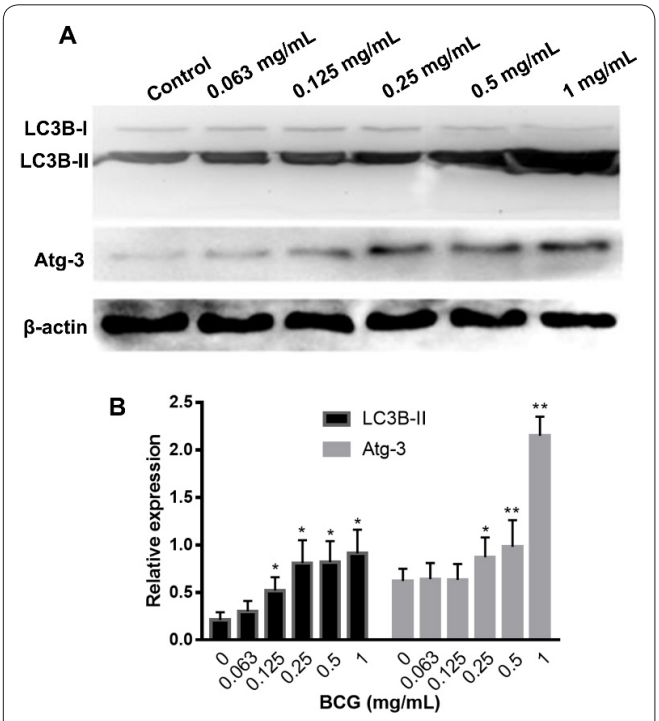

Figure 3. The effect of BCG on cell autophagy of MGC-803 cells. MGC-803 cells were treated with BCG at 1 mg/mL, 0.5 mg/mL, 0.25 mg/mL, 0.125 mg/mL, 0.063 mg/mL or 0 mg/mL for 24 h. Then, (A) the levels of LC3BII and Atg-3 were detected by Western blot and (B) quantified. Control: cells without treatment. *P<0.05, **P<0.01 vs. 0 mg/mL (control).

Coculture of BCG with lymphocytes promoted cell apoptosis in MGC-803 cells

In order to explore whether the effect of BCG on lymphocyte regulated apoptosis in MGC-803 cells, BCG was cocultured with lymphocytes in the upper chamber of Transwell insert, and MGC-803 cells were cultured in the lower chamber. After 24 h, the cell apoptosis rates in MGC-803 cells were significantly increased in coculture group, compared with BCG or lymphocytes alone groups (Figure 4A and B). The BCG in upper chamber induced cell apoptosis compared with control groups (Figure 4A and B), which was similar to the results of directly treating cells with BCG as shown in Figure 2A and B. The levels of cleaved-caspase-3 were also increased in coculture group, compared with BCG or lymphocytes alone groups (Figure 4C and D). Thus, BCG coculutred with lymphocytes induced cell apoptosis.

Coculture of BCG with lymphocytes promoted autophagy in MGC-803 cells

Whether the effect of BCG on lymphocyte regulated autophagy in MGC-803 cells was also performed (Figure 5). BCG was cocultured with lymphocytes in the upper chamber of Transwell insert, and MGC-803 cells were cultured in the lower chamber. After 24 h, the protein levels of LC-3BII and Atg-3 were significantly increased in coculture group, compared with BCG or lymphocytes alone groups (Figure 5A and B). The BCG in upper chamber significantly increased levels of LC-3BII and Atg-3 compared with control groups (Figure 5A and B), which was similar to the results of directly treating cells with BCG as shown in Figure 3. Thus, BCG coculutred with lymphocytes induced autophagy

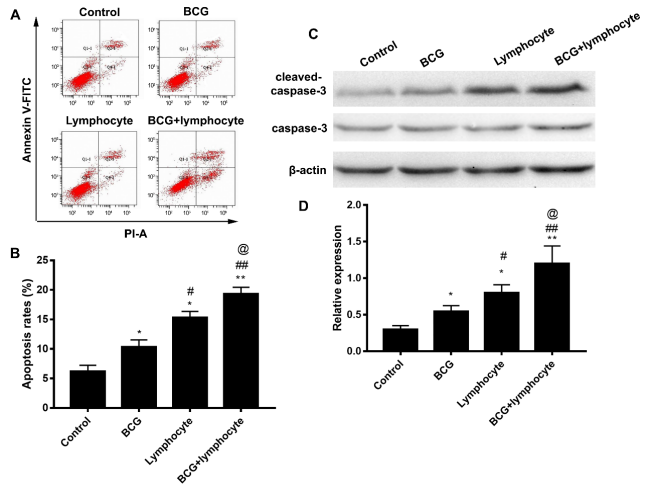

Figure 4. Effect of coculture of BCG with lymphocytes on cell apoptosis in MGC-803 gastric cancer cells. BCG was cocultured with lymphocytes in the upper chamber of Transwell insert, and MGC-803 cells were cultured in the lower chamber. After 24 h, the cell apoptosis of MGC-803 cells were detected by (A) flow cytometry, and (B) apoptosis rates were analyzed. (C) The levels of cleaved- and pro-caspase-3 were performed by Western blot and (D) quantified. Control: cells without treatment. *P<0.05, **P<0.01 vs. control; #P<0.05, ##P<0.01 vs. BCG; @P<0.01 vs. lymphocyte.

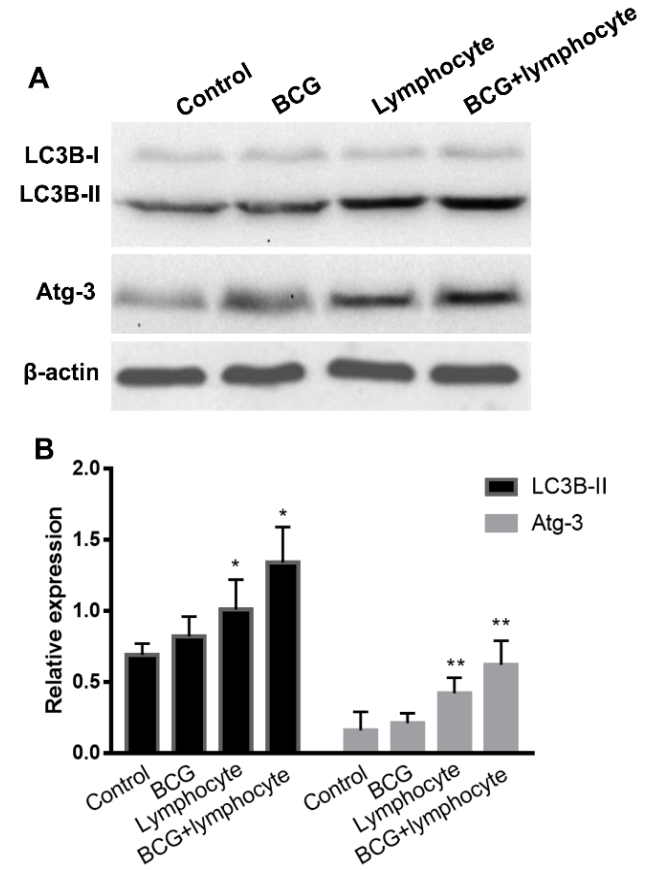

Figure 5. Effect of coculture of BCG with lymphocytes on cell autophagy in MGC-803 gastric cancer cells. BCG was cocultured with lymphocytes in the upper chamber of Transwell insert, and MGC-803 cells were cultured in the lower chamber. After 24 h, (A) Western blot and (B) quantification of LC3BII and Atg-3 levels were performed. Control: cells without treatment. *P<0.05, **P<0.01 vs. control.

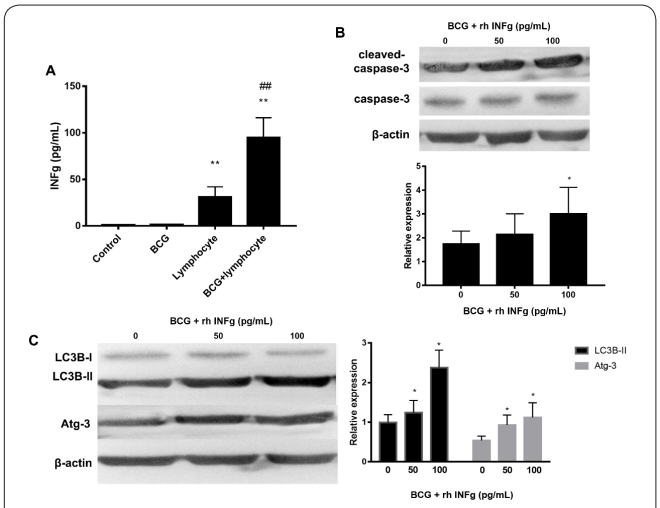

Figure 6. Effects of BCG and IFNg on apoptosis and autophagy in MGC-803 gastric cancer cells . (A) After cocultured the MGC-803 cells with BCG and lymphocytes for 24h, IFNg level in medium was detected by ELISA. **P<0.01 vs. control; ## P<0.01 vs. lymphocyte. After cocultured the MGC-803 cells with BCG and rhINFg for 24 h, (B) levels of cleaved- and pro- caspase-3, and (C) LC3BII and Atg-3 were performed. *P<0.05 vs. BCG+0 pg/mL rhINFg.

in MGC-803 cells.

BCG induced secretion of IFNg from lymphocyte

To explore the effect of BCG on lymphocyte, IFNg level was detected by ELISA (Figure 6A). There were a significantly increased in IFNg level in medium in BCG cocultured with lymphocyte compared with lymphocyte control. Moreover, 100 pg/ml rhIFNg with BCG induced cell apoptosis and autophagy in MGC-803 cells (Figure 6B and C). Thus, BCG promotes lymphocyte immunocompetence to induce cell apoptosis and autophagy in MGC-803 cells.

Discussion

The treatment and molecular mechanism of various cancers are still unclear (20, 21). There have been numerous reports of BCG inhibited development of bladder cancer and intestinal carcinoma (22, 23). BCG administration would be an effective local adjuvant therapy following radical surgery to treat patients with gastrointestinal cancer, but its effect on gastric cancer and the mechanism are still not clear (14, 24, 25). In this study, we found that BCG inhibited cell viability, and induced apoptosis and autophagy in gastric cancer MGC-803 cells. Moreover, BCG promoted IFNg secretion from lymphocyte. Cooperation of BCG and lymphocyte in determining the final fate of human cancer cells was also observed.

BCG can not only induce cancer cells to express antigens that can stimulate specific immune responses, including tumor-associated antigens and cross-reactive antigens such as heat shock proteins, but also induce cancer cells to express adhesion molecules (such as ICAM-1) and Fas receptors, thereby enhancing the binding of immunocompetent effector cells to cancer cells, and inducing cancer cell apoptosis through Fas-fasl and perforin (23). After intravesical infusion of BCG, cancer cells can express major histocompatibility com-

plex-II molecules (MHC-II) (26). MHC-II can activate CD 4+ T through binding to macrophages. BCG can promote the expression of HLA-DR antigens in cancer cells, thereby enhancing the immunogenicity of cancer cells, increasing the susceptibility of cancer cells to immune cells, and the identification and killing activity of immune cells (26). Ikeda et al reported that BCG can induce the expression of surface antigen MHC-II, CD 1, CD 80 and ICAM-1 in mouse bladder cancer cell line MBT-2 and human bladder cancer cell lines T24 and T28 (27). MBT-2 cells treated with BCG can stimulate the production of IL-2 and IFN-γ by BCG-activated lymphocytes (27). Intravesical BCG can induce cancer cells to produce cytokines such as IL-1, IL-6, IL-8, IL-2, IL-12, and INF-γ, and play a direct anti-tumor effect, immune cell network regulation effect and magnify the anti-tumor activity of immune cells. Monocytes of some patients with gastric cancer receiving BCG had an enhanced ability to produce TNF following stimulation with tumor cells (28). In this study, we found BCG not only induced cell apoptosis and autophagy of gastric cancer cells, but also activated lymphocyte to enhance the anti-tumor activity of immune cells. For apoptosis detection, the levels of cleaved-caspase-3 and pro-caspase-3 were performed with different loading quantity of proteins. We used 50 μg for cleaved-caspase-3, and 15 μg for pro-caspase-3. The changes in pro-caspase-3 were not detectable in our study.

The anti-tumor immunity is based on cellular immunity, and BCG is mainly activated by CD4+ and CD8+ T cells (29, 30). The specific immune response of cancer cells to BCG induces the transformation of Th0 cells to Th1 cells, inducing T cell activation and proliferation and mediating cellular immune responses (29, 30). BCG can directly activate NK cells through TLRs, or induce activation of NK cells by activating mononuclear cells to release cytokines IL-12 and IFN-α (31), then killing cancer cells. In addition, it was believed that BCG can overcome the immune escape of bladder tumors. Like a common used positive control lipopolysaccharides in activation of immune system (32), *mycobacterium bovis* BCG augments macrophage activating-factor activity release from peripheral blood lymphocytes by inducing IFNg (33). IFNg and autophagy plays critical roles in controlling bacterial infections (34). IFNg-induced Irgm1 promotes tumorigenesis of melanoma via dual regulation of apoptosis and Bif-1-dependent autophagy (35). In the present study, we found BCG induced IFNg production in lymphocytes, which might help to enhance the anti-tumor activity of immune cells.

Taken together, this study demonstrated that BCG has an anti-tumor effect by directly attacking gastric cancer cells and by inducing immunity. However, there are still several drawbacks need to be further studied. For example, it is not observed whether BCG directly enters into cancer cells. It is still unknown what substances produced by BCG in lymphocytes are responsible for anti-tumor effects in gastric tumor. The association between autophagy and apoptosis in BCG treated gastric cells is still unclear. In addition, we might study the cancer stem cell marker in various gastric cell lines in the future.

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