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Functional characterization of a potent anti-tumor polysaccharide in a mouse model of gastric cancer

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ABSTRACT

Aims: Natural polysaccharides are emerging as a new class of immunomodulatory agents due to their potent immunostimulatory effects and suitable biocompatibility. The aim of this study was to identify potent and selective anticancer activity of a bioactive polysaccharide.

Materials and methods: *In vitro*, viability assay was performed to screen 16 of bioactive polysaccharides in a panel of normal and cancer cell lines. Foci formation, soft agar, BrdU incorporation, cell cycle analyses, and β-galactosidase staining were performed to validate the screening results. *In vivo*, both murine gastric cancer syngeneic and a human gastric tumor xenografts models were applied. Tumor histology, immunohistochemical staining, cytokine array and flow cytometry analyses were assayed.

Key findings: BSP (bamboo shaving polysaccharides) was identified as the most selective polysaccharide for inhibiting the growth of six gastric cancer cell lines while having no toxic effect on normal gastric mucosal cells. Similarly, BSP had more potent killing effect on a subset of human stomach cancer cells than liver or lung cancer cells. *In vivo*, BSP significantly inhibited tumor growth and prolonged the survival of mice bearing a gastric tumor; these effects are mediated by tumor cell apoptosis and remodeling of the tumor microenvironment by boosting both immune cell subpopulations and cytokine production in murine gastric cancer syngeneic model. A significant decrease of F4/80-positive tumor-associated macrophages was also observed.

Significance: The findings of this study suggest that the potent and selective anti-tumor activity of bioactive polysaccharides such as BSP warrants clinical testing for the treatment of gastric cancer.

1. Introduction

Gastric cancer (GC) is the third most common cause of cancer-related death worldwide [1], and systemic chemotherapy remains the principal treatment option for patients with advanced and/or metastatic GC. However, traditional chemotherapeutic agents are cytotoxic and can have severe side effects due to damage caused to normal cells. Recently, HER2-targeted therapy and PD-1–based immunotherapy were shown to provide clinical benefits for GC patients, but to only a limited extent [2]. Therefore, safer and more potent agents for treating GC are urgently needed.

In recent years, a growing number of studies have shown that polysaccharides can have a wide range of biological properties, including anti-virus activity, anti-aging activity, hematopoietic stimulation, immune regulation, and anti-tumor activity [3]. The anticancer properties associated with bioactive polysaccharides derived from plants and other sources underscore the possible role that these molecules may play in cancer therapy [4]. These biologically active polysaccharides are present in a variety of medicinal plants; for example, hetero-β-D-glucans have been isolated from mushroom fruit bodies [5]. Moreover, several polysaccharides have been found to have immunoregulatory activity in both innate and acquired immunity either by increasing the number of immune cells-including monocytes, dendritic cells, macrophages, NK cells, and cytotoxic lymphocytes-or by promoting the production of cytokines production [6].

Polysaccharides present in the shavings of bamboo (*Caulis bambusae in taeniam*) are a water-soluble *O*-acetylated-arabinoxylan extracted from the intermediate layer of bamboo stems using the hot water

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extraction and ethanol precipitation method. Bamboo shaving polysaccharide (BSP) has been shown to have immunoregulatory properties in cyclophosphamide-immunosuppressed mice [7]. Here, we examined the anti-cancer activity of BSP using several cancer cell lines, a syngeneic gastric tumor mouse model and human gastric cancer xenografts in nude mice. Our results suggest that BSP may have high therapeutic potential for treating gastric cancer.

2. Methods

2.1. Cell lines

Five human gastric cancer cell lines (AGS, BGC-823, MGC-803, HGC-27, and MKN-45), three human lung cancer cell lines (NCI-H520, NCI-H1688, and A549), three human liver cancer cell lines (Huh-7, HepG2, and Sk-Hep1), the human normal gastric epithelial cell line GES-1, human lung-bronchi cell line (BEAS-2b), human hepatocyte cell line (HepLL) and the mouse forestomach carcinoma (MFC) cell line were purchased from Cellbank at the Chinese Academy of Sciences (Shanghai, China). All cells were cultured using appropriate conditions.

2.2. In vitro treatment with bioactive polysaccharides

Bioactive polysaccharides extracted from the following plants were purchased from Ci Yuan Biotechnology Co. (Xi'an, China): *Angelica sinensis*, *Astragalus membranaceus*, *Cornus officinalis*, *Cucurbita moschata*, *Epimedium brevicornu*, *Ganoderma lucidum*, *Glycyrrhiza uralensis*, *Hericium erinaceus*, *Lentinus edodes*, *Lycium barbarum*, *Polygonatum sibiricum*, *Polyporus umbellatus*, *Poria cocos*, *Glehnia littoralis*, and *Ziziphus zizyphus*; each extract had a molecular weight (MW) of 10–15 kDa and > 90% purity. BSP was extracted and purified as previously reported [8]. In brief, bamboo shavings were subjected to steam explosion, and the resulting BSPs was purified using hot water extraction and ethanol precipitation. Anion-exchange chromatography with diethylaminoethanol (DEAE)-sepharose fast flow was subsequently performed in order to achieve 90.2% purity (MW: 11.3–12.8 kDa). The polysaccharide powders were dissolved in culture medium at room temperature and then filtered using a 0.22-μm hydrophilic PVDF membrane (Millipore). Lentinan (purity: > 99%, LUYE PHARMA, Nanjing) was purchased from Zhejiang Cancer Hospital. The screening assay was performed at a final concentration of 4 mg/ml.

2.3. Cell viability assay

Cell viability was measured using the CCK-8 assay in accordance with the manufacturer's instructions (Sigma-Aldrich).

2.4. Foci formation assay and soft agar assay

MFC, MKN-45 and BGC-823 cells (500 cells/well) were incubated in 6-well plates at 37 °C for 24 h and then treated with 0, 0.25, 0.5, 1.0, 2.0, or 4.0 mg/ml BSP. When the cells grew to visible colonies, the colonies were washed once with phosphate-buffered saline (PBS) and fixed in 4% paraformaldehyde (Sigma-Aldrich) for 20 min. The cells were then stained with crystal violet, and the number of colonies was counted using light microscopy.

2.5. β-Galactosidase staining assay

MFC, MKN-45 and BGC-823 cells were fixed in 2% paraformaldehyde for 15 min, washed with PBS, and stained using a β-galactosidase staining kit (Beyotime) for 16 h. The cells were then visualized using microscopy (Olympus).

2.6. Syngeneic murine gastric cancer model

Mice from the inbred strain 615 (6–8 weeks of age, 20–25 g) were purchased from the Institute of Hematology, Chinese Academy of Medical Sciences (Tianjin, China). MFC cells in the log phase of growth were suspended in 0.9% saline at 3×10^7 cells/ml, MKN-45 cells were suspended at 1.5×10^8 cells/ml. The mice were lightly anesthetized with isoflurane inhalation (Sigma), and 3×10^6 (MFC) or 1.5×10^7 (MKN-45) cells were injected subcutaneously (*s.c.*) into the back of each mouse in a volume of 100 μl. Tumor size was measured using a digital vernier caliper, and tumor volume (in mm^3) was calculated using the following equation: [Tumor volume = (length × width^2) / 2], with length and width measured in mm. All experimental protocols involving animals were approved by the Institutional Animal Care and Use Committee of the Laboratory Animal Center, Zhejiang University.

2.7. In vivo treatment of BSP and clodrosome

BSP powder was dissolved in 0.9% saline at room temperature and filtered through a 0.22μm hydrophilic PVDF membrane (Millipore). Mice received daily *s.c.* or intraperitoneal (*i.p.*) injections of either 0.9% saline (as a control) or BSP solution (400 or 800 μg/g body weight); at the indicated time points, the animals were sacrificed. For the clodrosome experiment, mice received a daily injection of BSP (800 μg/g body weight, *i.p.*) or saline for 2 weeks; on day 5 and day 11, 100 μl of clodrosome (clodronate encapsulated liposomes, Liposoma) was injected into the tail vein.

2.8. BrdU-incorporation

MFC cells were treated with BSP for 72 h, followed by adding 20 μM 5-bromo-2′-deoxyuridine (BrdU) per well to the culture medium for 30 min and then fixed in 70% ice-cold ethanol. After treatment with 2 M HCl/0.5% Triton X-100 for 30 min at 37 °C, the cells were incubated with anti-BrdU antibody (B44; BD Biosciences) for 1 h, followed by incubation with fluorescein isothiocyanate-conjugated (FITC) anti-mouse antibody (eBioscience) at 37 °C for 30 min. These cells were collected and labeled with PI in calcium buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM CaCl2) at room temperature for 15 min. Flow cytometry was performed on a FACS FC500 ML flow cytometer (BD Biosciences).

2.9. Analysis of cell cycle

MFC cells were treated with 0, 1.0, 2.0, 4.0 mg/ml BSPs for 72 h, then digested and fixed with 70% ice-cold ethanol for 24 h. The cells were then collected and incubated with Propidium iodide (PI) in calcium buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM CaCl2) for 15 min at room temperature. Flow cytometry was performed using a FACS FC500 ML flow cytometer (Beckman Coulter).

2.10. Immunohistochemical staining

Sections of the tumors, including adjacent tissues, were incubated with the following primary antibodies: anti-mouse Ki-67 (5 μg/ml, 14-5698, BD eBioscience), anti-mouse F4/80 (1:100, ab100790, Abcam), anti-CD45 (1:200, ab10558, Abcam), anti-CD8 (1:500, ab203035, Abcam), and anti-Integrin alpha 2 (1:200, ab181548, Abcam). Staining was visualized using the appropriate secondary antibody (Dako Real Envision/HRP, Rabbit/Mouse, K5007) and 3,3′-diaminobenzidine with peroxidase substrate (Dako Real DAB Chromogen, K5007). The sections were counterstained with hematoxylin. The TdT *In Situ* Apoptosis Detection Kit (4810-30-K, R&D) and the SignalStain Apoptosis (Cleaved Caspase-3) IHC Detection Kit (12692, CST) were also used to stain tumor tissues. Semi-quantitative scoring of positive immunostaining was performed by trained pathologists.

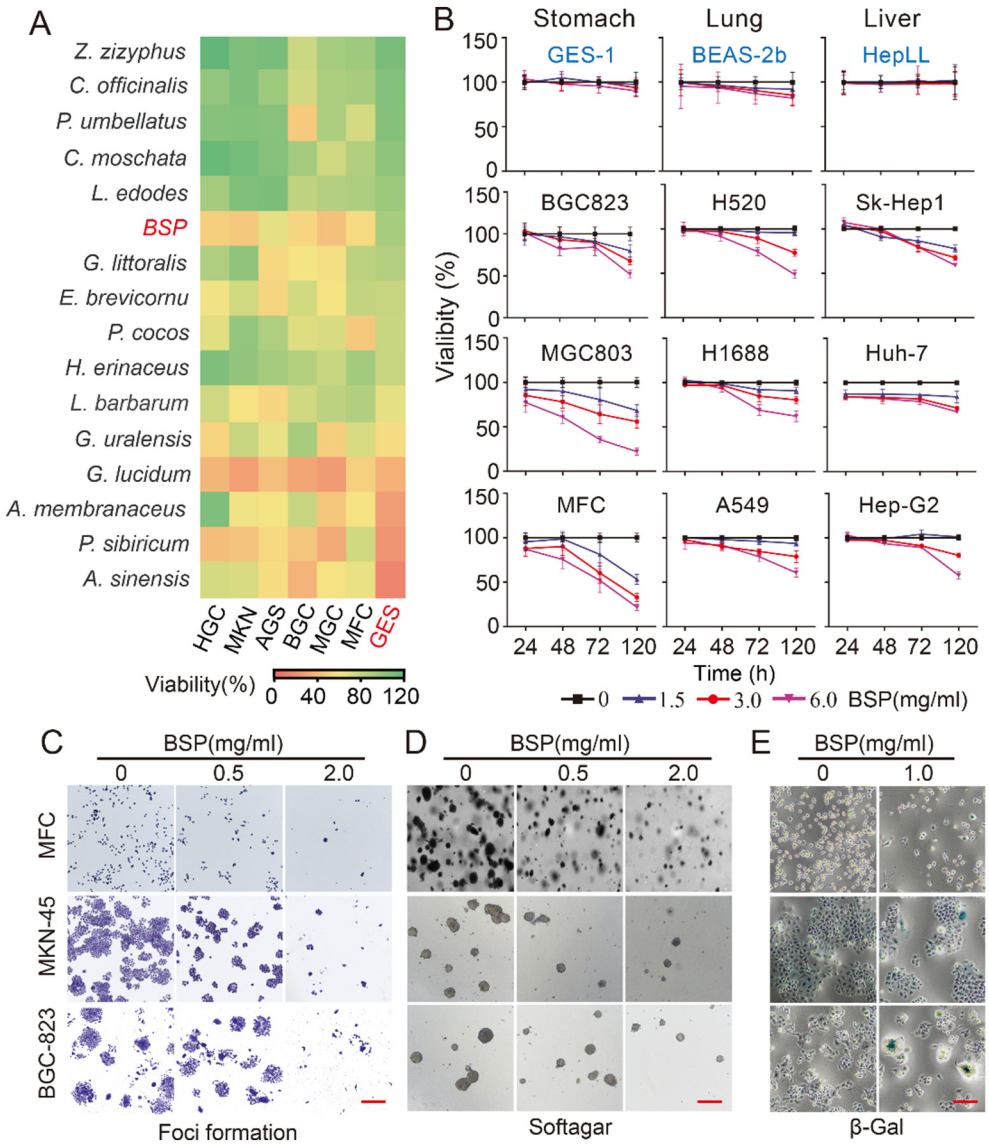

Fig. 1. Summary of the effect of 16 polysaccharides on a panel of gastric cancer cell lines, and the effect of BSP on stomach, lung, and liver cell lines. (A) Heat map showing cell viability of six gastric cancer cell lines and a human mucosal epithelial (GES-1) cell line following treatment with the indicated bioactive polysaccharides. The percent viability was calculated and is represented as a heat map, with green and red representing cell survival and cytotoxicity, respectively. For details, see Table S1. (B) Time course of cell viability measured for the indicated stomach, lung, and liver cancer cell lines treated with the indicated concentration of BSP ($N = 6$/group). (C) Plate clone formation assay and (D) soft agar assay of MFC, MKN-45 and BGC-823 cells treated with 0, 0.5 or 2.0 mg/ml BSP for 12 days. (E) β-galactosidase (β-Gal) staining of MFC, MKN-45 and BGC-823 cells treated with the indicated concentration of BSP for 10 days. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

2.11. Quantitative real-time PCR analysis

Total RNA was isolated using TRIzol reagent (Invitrogen) and reverse-transcribed into cDNA using the PrimeScript RT kit (Takara). For real-time PCR analysis, cDNAs were amplified using a two-step qRT-PCR method (Bio-Rad) with Fast SYBR Green Master Mix (Takara) in accordance with the manufacturer's instructions. The primers used are listed in Table S3. The expression level of each gene was normalized to the housekeeping gene β-actin (*ACTB*).

2.12. Western blot analyses

Extracted proteins were reconstituted in loading buffer and the mixture was boiled for 5 min. Equal amounts (30–100 μg) of the denatured proteins were loaded into each lane and separated on a 12% SDS polyacrylamide gel. Primary antibodies: Caspase-3 (1:2000, CST), cleaved-Caspase-3 (1:1000, CST), Cyclin-A (1:200, Santa Cruz), Cyclin-D1 (1:200, Santa Cruz), Cyclin-E (1:200, Santa Cruz), Cdk-2 (1:200, Santa Cruz), Cdk-4 (1:200, Santa Cruz), Phospho-Rb (1:500, ImmunoWay), Phospho-Cdk2^{T160} (1:500, ImmunoWay), P21^{Cip1} (1:1000, CST),Bcl-2 (1:1000, CST), Bax (1:1000, CST), Bik (1:1000, CST), β-Actin (1:2000, Santa Cruz), overnight at 4 °C. They were then incubated with horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse secondary antibody (1:3000, CST) for 2 h before being visualized with diaminobenzidine solution.

2.13. Flow cytometry analysis

Peripheral blood cells were collected in EDTA-anticoagulant tubes (Falcon), and bone marrow (BM) and spleen cell suspensions were filtered using 100-μm cell strainers (Falcon). Erythrocytes were lysed using ACK lysis buffer containing 8.29 g/l NH_4Cl, 1 g/l $KHCO_3$, and 0.0372 g/l $Na_2EDTA{\cdot}2H_2O$. Cell suspensions were stained with the following fluorochrome-conjugated anti-mouse monoclonal antibodies: APC-CD3e (17-0031-81), PE-CD4 (12-0041-82), PE-CD8b (12-0083-81), APC-CD19 (17-0191-81), FITC-NK1.1 (11-5941-81), APC-CD11b (17-0118-42), FITC-CD11c (11-0116-42), APC-CD14 (17-0191-81), PE-cy7-ly6c (25-5932-82), PE-F4/80 (12-4801-80), or FITC-Gr-1 (11-5931-82) (all from eBioscience). Flow cytometry was performed using a model FC500MCL flow cytometer (Beckman Coulter), and data were analyzed using CXP Analysis Software v3.0 (Beckman Coulter).

2.14. Cytokine measurements

Plasma cytokine levels in BSP-treated and control-treated mice were measured using the C-Series Mouse Cytokine Antibody Array C1 (AAM-

CYT-1, RayBio) in accordance with the manufacturer's instruction. The signal density of each spot was normalized using the following algorithm: [X (Ny) = X (y) * P1 / P (y)], in which P1 is the mean signal density of Positive Control spots on a reference array, P (y) is the mean signal density of Positive Control spots on Array "y", and X (Ny) = X (y) is the normalized signal intensity for spot "X" on Array "y".

2.15. Statistical analyses

Except where indicated otherwise, all summary data presented as the mean ± SD. SPSS statistical software v17.0 (Chicago, IL) and GraphPad Prism v6.0 (La Jolla, CA) were used for statistical analysis. The Student's *t*-test was used to compare two groups, and the log-rank (Mantel-Cox) test was used to analyze the survival curves. Comparisons among multiple groups were performed by one-way ANOVA followed by Tukey's *post hoc* test. p-Value < 0.05 was considered statistically significant.

3. Results

3.1. BSP potently inhibits the growth of cancer cells lines

Among a panel of 16 bioactive polysaccharides, BSP was the most potent at inhibiting the growth of six gastric cancer cell lines (Fig. 1A, Table S1), including HGC-27, MKN-45, BGC-823, and MGC-803 cells, but had little effect on normal gastric epithelial (GES-1) cells (Fig. 1A, Table S1). Moreover, BSP inhibited the growth of nine cancer cell lines in both a time-dependent and dose-dependent manner (Fig. 1B). After 120 h of culture, 6 mg/ml BSP reduced the growth of the gastric cells lines BGC-823, MGC-803, and MFC by 47.76%, 77.94%, and 78.32%, respectively; in contrast, the same treatment reduced the growth of three lung cancer cells by approximately 37–51% and reduced the growth of three liver cancer cell lines by approximately 32–40% (Fig. 1B). In contrast, BSP showed no significantly growth inhibitory effect on human gastric (GES-1, 9.73%), lung (BEAS-2b, 17.94%) and liver (HepLL, 2.67%) normal cell lines at the concentration of 6 mg/ml for 120 h (Fig. 1B). Taken together, these *in vitro* results suggest that gastric cancer cell lines are more sensitive to BSP treatment compared to other cancer cells. A foci formation assay (Fig. 1C) and colony-counting assay (Fig. 1D) revealed significant reduced anchorage-independent growth of BSP-treated MFC,MKN-45 and BGC-823 cells compared to untreated cells. In addition, β-galactosidase staining revealed that BSP-treated cells undergo senescence (Fig. 1E).

3.2. BSP suppressed proliferation and arrested gastric cancer cells at G1 phase

We next analyzed cell proliferation and cell cycle by BrdU incorporation and PI staining. Shown in Fig. 2A, C, the proportion of BrdU positive MFC cells were significantly decreased in a dose-dependent manner. Meantime, we observed the BSP-treated MFC cell cycle was arrested at G1 phase (Fig. 2B, D), even at low dose (1 mg/ml) of BSP threated group, the proportion of cells at G1-phase was increased to 68.24% compared to 54.16% in control group.

To explore a potential molecular mechanism, we measured expression levels of several critical genes involved in G1/S transition with or without BSP treatment. We found both mRNA and protein levels of cyclin E were up-regulated in BSP treated groups (Fig. S2A, B) in a dose-dependent manner. Importantly, two major regulators in G1 phase of cell cycle, phosphorylated p-Cdk2^{T160} and p-Rb, were significantly down-regulated, while p21^{Cip1}, a potent cyclin-dependent kinase2 inhibitor (CKI) was significantly upregulated in BSP treated cells (Fig. 2E). Since p21^{Cip1} is known to regulate both senescence and apoptosis, we next examined cell apoptosis using Annexin V-FITC/PI staining *via* flow cytometry and cleaved Caspase-3 by western blot. As shown in Figs. 2E and S2C, cleaved Caspase-3 as well as Annexin V showed no difference between BSP treated and non-treated control group.

3.3. BSP treatment inhibits tumor growth and prolongs the survival of MFC tumor–bearing mice

To investigate the anti-tumor activity of BSP *in vivo*, we firstly used a syngeneic murine gastric cancer model. Mice were injected with MFC-615 cells and then treated for 3 weeks with BSP (400 or 800 μg/g body weight, *s.c.* injection), Lentinan (50 μg/g body weight) (purity: > 99%, LUYE PHARMA, Nanjing) or saline. Treatment with 800 μg/g BSP significantly reduced tumor growth compared to saline-treated mice (p = 0.0087) (Fig. 3A, B); in contrast, 400 μg/g BSP (p = 0.5459) or Lentinan control (p = 0.7420) had no significant effect on tumor growth (Fig. S1A, B). Consistent with reduced tumor growth, mice that received 800 μg/g BSP had prolonged survival compared to saline-treated animals (p = 0.0011, Log-rank (Mantel-Cox) test) (Fig. 3C). Similarly, tumor inhibitory effect was observed when *i.p.* injected BSP into either MFC syngeneic gastric tumor mouse model (p = 0.0181) (Fig. 3D, E) or nude mice bearing human gastric cancer xenografts (p = 0.0390) (Fig. 3F, G).

3.4. BSP induces cell apoptosis in MFC tumor-bearing mice

Immunohistochemical staining of tumor tissues for the cell proliferation marker Ki-67 revealed a slight increase of Ki-67 positive cells in BSP-treated mice compared to saline-treated mice (p = 0.0156) (Fig. 4A, C); however, cell apoptosis was increased considerably in the BSP-treated tumors compared to saline-treated controls, reflected by a 3.82-fold increase in TUNEL staining and a 2.53-fold increase in cleaved Caspase-3 (Fig. 4B, C). In addition, the mRNA levels and protein levels of the pro-apoptotic family members Bax and Bik were increased in the tumors of BSP-treated mice compared to saline-treated controls, whereas the anti-apoptotic family member Bcl-2 was down-regulated (Fig. 4D, E). Taken together, these results are consistent with increased cell apoptosis in the tumors of BSP-treated mice.

3.5. BSP treatment increases the number of lymphocytes and cytokine production

A variety of immune cells types and cytokines have been implicated in apoptosis of cancer cells [9]. Therefore, to determine whether BSP has immunoregulatory properties *in vivo*, we used FACS analysis to measure the cell populations in the BM (Fig. 5A), peripheral blood (Fig. 5B), and spleen (Fig. 5C) of these tumor-bearing mice. We found that the proportions of CD3+, CD4+, CD8+, and NK1.1+ cells were significantly increased in the peripheral blood, and the proportions of CD19+ and CD11b+ cells were increased in both the peripheral blood and spleen. Immunohistochemical staining of the cells that infiltrated the tumor and non-tumor adjacent tissues revealed that both CD45+ and CD8+ cells were increased in the adjacent tissues but not in the tumor tissue; in contrast, the number of NK cells was unchanged in both the tumor and adjacent tissues (Fig. S4). Surprisingly, the lower BSP dose (*i.e.*, 400 μg/g body weight, *s.c.*), which had no apparent effect on tumor growth (Fig. S1A, B), cell proliferation (Fig. S1D, E), and apoptosis (Fig. S1D, E), significantly increased the number of lymphocytes in both the peripheral blood and the spleen (Fig. S1F, G). Moreover, mice treated with 800 μg/g BSP had significantly higher serum levels of a wide range of cytokines and signaling factors, including GM-CSF, IL-2, IL-6, IL-9, IL-10, IL-13, IFN-γ, MCP-1 (CCL2), SCF, and MCP-5 (Figs. 6, S3 and Table S2). Taken together, these data suggest that treating tumor-bearing mice with BSP can stimulate the immune response.

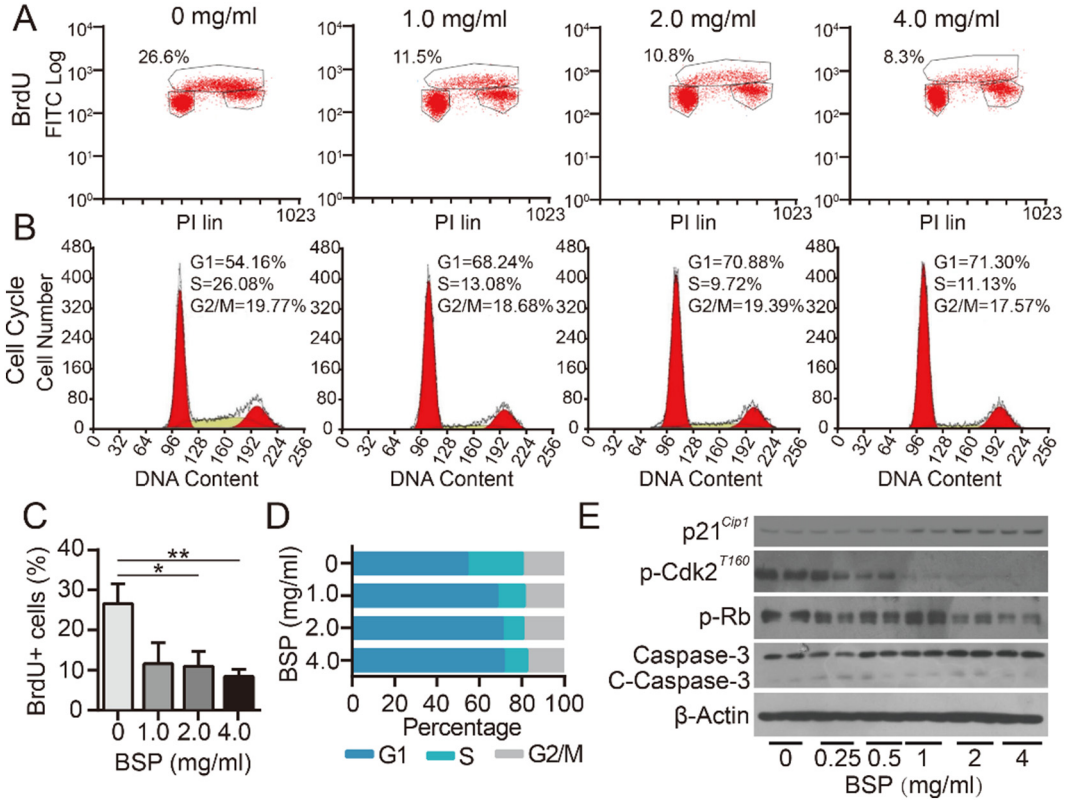

Fig. 2. Effect of BSP on cell proliferation and cell cycle *in vitro*. (A) Proportions of BrdU positive MFC cells treated with 0, 1.0, 2.0, 4.0 mg/ml of BSP for 72 h. (B) Cell cycle analyses. (C) Quantification data of (A). (D) Percentages of MFC cells at G1, S and G2/M phases treated with 0, 1.0, 2.0, 4.0 mg/ml of BSP. (E) Protein levels of p-Cdk2^{T160}, p21^{Cip1}, p-Rb, Caspase-3 and cleaved-Caspase-3 (C-Caspase-3) in BSP-treated MFC cells as indicated. $^{*}p < 0.05$, $^{**}p < 0.01$.

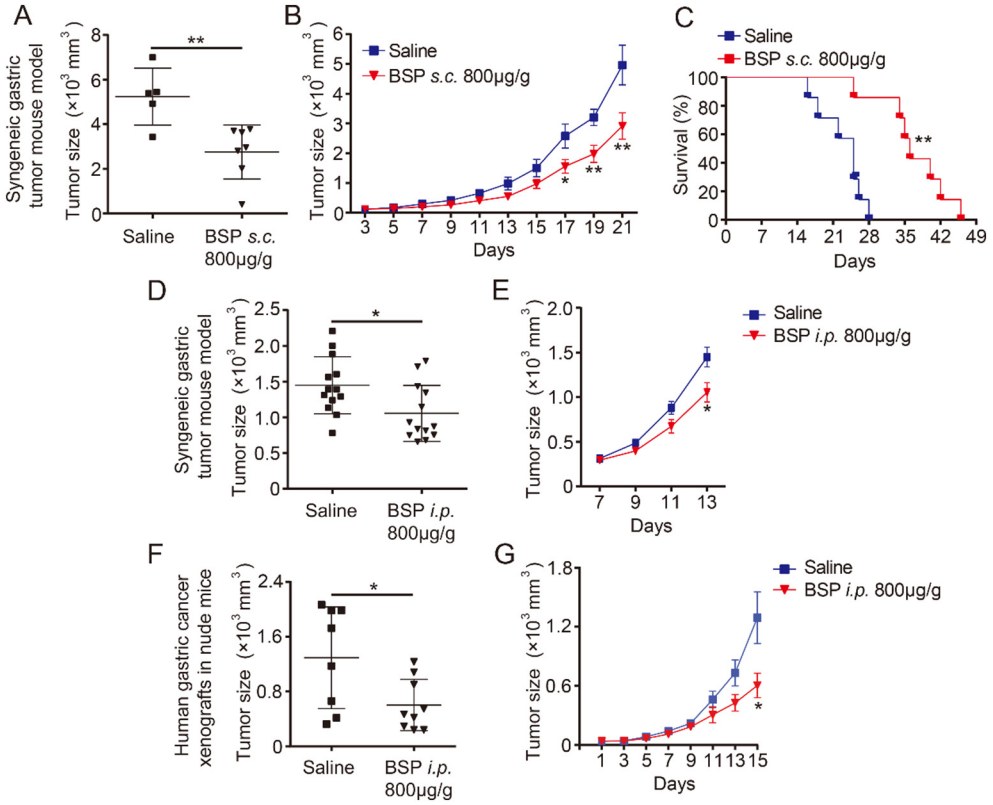

Fig. 3. BSP reduces tumor growth and improves survival in a mouse model of gastric cancer. (A) Tumor size was measured in 615 mice 3 weeks after subcutaneous transplantation of MFC cells (3×10^6) followed by daily *s.c.* injections of BSP (800 μg/g body weight) or saline ($N = 8$ mice/group). (B) Time course of MFC tumor size in mice treated daily with BSP (800 μg/g body weight) or saline for 21 days after transplantation ($N = 7$ mice/group, mean ± sem). (C) Kaplan-Meier survival curves of MFC tumor–bearing mice treated with BSP or saline; the log-rank test was used to analyze the difference in survival between the two groups. (D) Tumor size was measured in 615 mice 13 days after subcutaneous transplantation of MFC cells (3×10^6) followed by daily *i.p.* injections of BSP (800 μg/g body weight) or saline ($N = 13$ mice/group, mean ± sem). (E) Time course of MFC tumor size in 615 mice. (F) Tumor size was measured in BALB/c nude mice subcutaneous transplantation of MKN-45 cells (1.5×10^7) followed by 15 days of daily *i.p.* injections of BSP (800 μg/g body weight) or saline ($N = 8$ or 9 mice/group). (G) Time course of MKN-45 tumor size in BALB/c nude mice daily *i.p.* injections of BSP (800 μg/g body weight) or saline ($N = 9$ mice/group, mean ± sem). $^{*}p < 0.05$, $^{**}p < 0.01$.

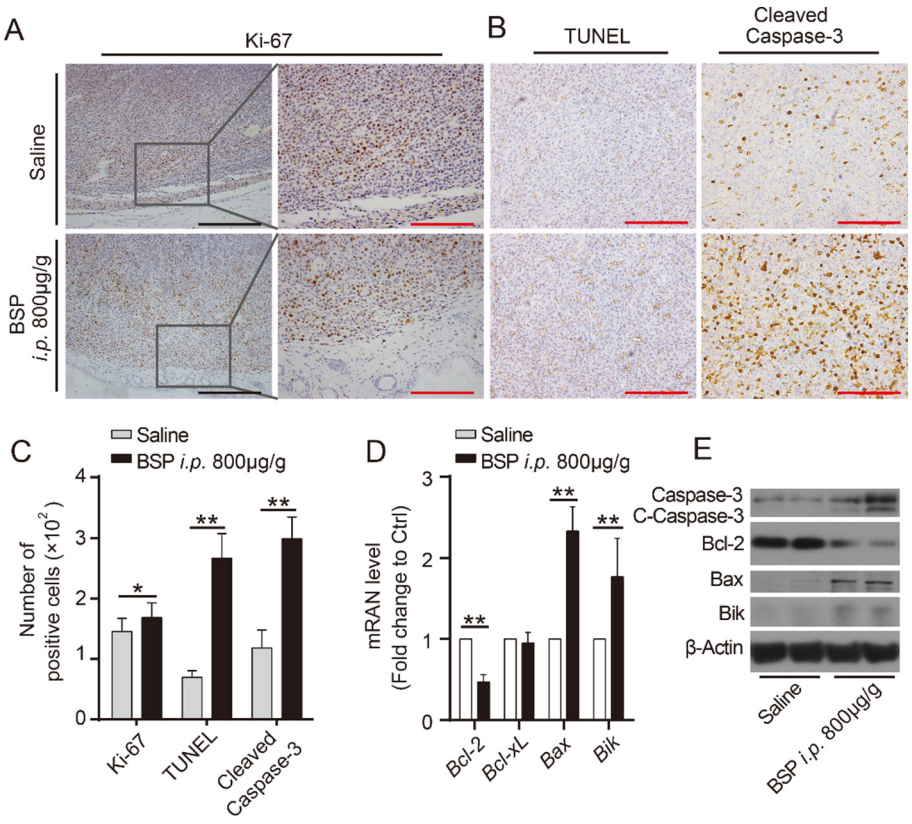

Fig. 4. BSP increases apoptosis of MFC tumor cells in tumor-bearing mice. Tumor-bearing mice were treated daily with *i.p.* injections of BSP (800 μg/g body weight) or saline. Tumor sections were then prepared and stained for (A, C) Ki-67, (B, C) TUNEL, or cleaved Caspase-3 (N = 9-15/group). The black and red scale bars represent 400 μm and 100 μm, respectively. (D) The mRNA levels, and (E) protein levels of the indicated apoptosis-related genes measured in tumor tissues obtained from BSP-treated and saline-treated mice (N = 6/group). $^{*}p < 0.05$, $^{**}p < 0.01$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.6. BSP treatment decreases the percentage of macrophages in the spleen and tumor tissues

Based on FACS analyses, we found that the relative percentages of CD11c+ dendritic cells (DCs) and CD11b + F4/80+ macrophages are reduced in the peripheral blood and spleen of BSP-treated mice; in contrast, CD11b + ly6c monocytes were not affected by BSP treatment (Fig. 7A). As reviewed by Qian and Pollard, several studies found that tumor-associated macrophages (TAMs) are strongly associated with tumor growth, progression, and metastasis [10]. We found that the number of F4/80+ macrophages are reduced in both tumor and adjacent tissues (Fig. 7B, C). To further examine the role of TAMs in tumor growth, we treated MFC tumor-bearing mice with clodrosome (a macrophage-depleting reagent) in the presence or absence of BSP and found significantly fewer CD11b + F4/80+ cells in the spleen (Fig. 7D) and significantly reduced tumor size (Fig. 7E, F).

4. Discussion

Cancer remains the leading cause of human death worldwide. Searching for novel effective anticancer agents of nature origin holds a great promise to improve outcome of cancer patients. Emerging evidence supports the notion that natural plant-derived polysaccharides have unique advantages for treating cancer either combined with chemotherapies or served as an adjuvant treatment [11–13]. However, clinical use of novel bioactive polysaccharides is yet to be developed.

In this study, we report that BSP, a novel polysaccharide isolated from bamboo shavings, has selective *in vitro* cytotoxicity against a variety of human gastric cancer cell lines while sparing non-cancerous cells, indicating that its toxic effects are specific for cancer cells. In a syngeneic mouse model of gastric cancer, we demonstrate that BSP has potent anti-tumor and immunomodulatory properties. We found that BSP potently inhibits tumor growth by inducing apoptosis in tumor cells. This finding is clinically relevant, as treating tumor-bearing mice with BSP significantly extended survival time compared to control

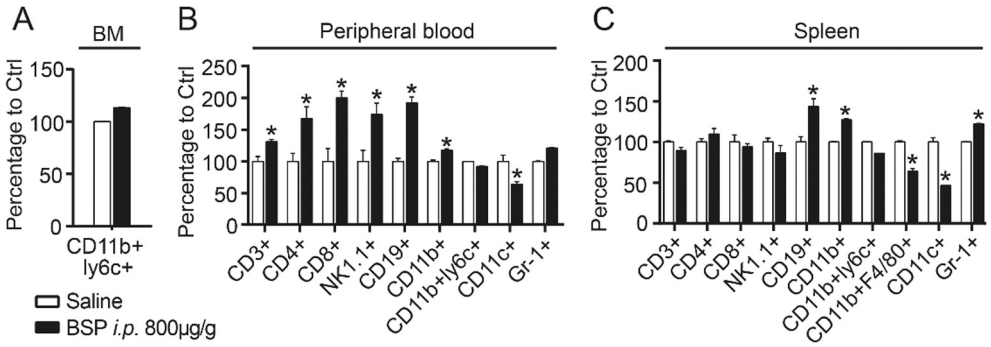

Fig. 5. Summary of the effect of BSP treatment on immune cells in (A) the bone marrow (BM), (B) the peripheral blood, and (C) the spleen. Mice were treated with either BSP (800 μg/g body weight, *i.p.*) or saline (Ctrl), and the number of cells is expressed as a percentage relative to the respective control group (N = 3). $^{*}p < 0.05$.

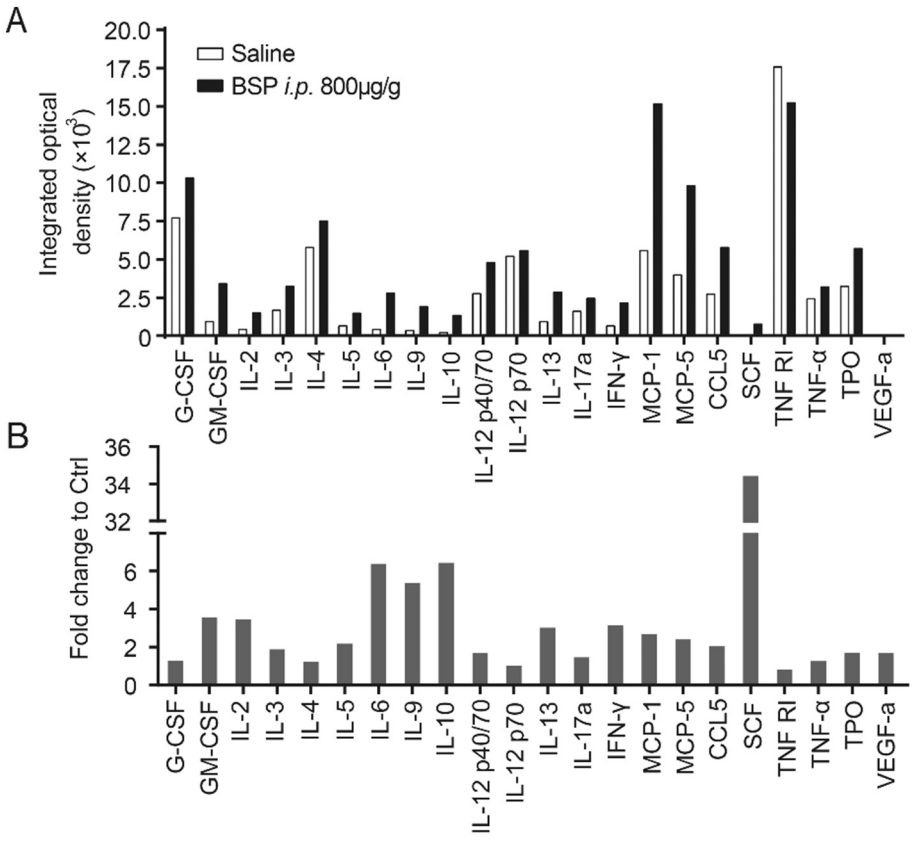

Fig. 6. Summary of the effect of BSP treatment on the serum levels of a panel of cytokines. (A) The indicated cytokines were measured in the serum of mice treated with BSP (800 μg/g body weight, *i.p.*) or saline, and the integrated signal density of each spot was quantified (see Fig. S3 and Table S2 for details). (B) Summary of the fold change in each cytokine in the serum of BSP-treated mice relative to the respective control.

mice. Moreover, we found that BSP can remodel the tumor microenvironment by inducing lymphocytes and increasing inflammatory cytokine levels while reducing macrophage numbers in tumor-bearing mice. Notably, we showed that BSP significantly suppressed growth of human gastric cancer xenografts in nude mice, which highlights the potential clinical implications of BSP in treating gastric cancer.

Interestingly, induction of apoptosis by BSP is observed only *in vivo*. *In vitro*, we found BSP potently inhibited cellular proliferation and arrested gastric cancer cells at G1 phase of cell cycle through down-regulation of p-CDK2^{T160}, p-RB and up-regulation of p21^{Cip1}. In contrast, high dose of BSP (800 μg/g body weight)-treated tumor-bearing mice presented significant apoptosis as evidenced by elevated cleaved-Caspase 3, Bax and Bik levels (Fig. 3), which is consistent with previous reports with respect to the apoptotic effect of bioactive polysaccharides, such as *Ganoderma lucidum* polysaccharides [14], *Cordyceps militaris* polysaccharides [15], and *Grifola frondosa* polysaccharides [16].

To date, anti-cancer polysaccharides are mainly derived from fungi, in which their major bioactive component is β-glucan [17,18]. It is reported that β-glucan containing-polysaccharides are ideal biological response modifiers (BRM) to enhance the immunity [19,20]. In our study, xylan is the bioactive component in BSP [7,8], which antitumor activity has not been investigated. Compared to polysaccharides extracted from edible mushroom, such as *Lentinus edodes* (LNT), BSP displayed a potent immune stimulatory effect through promoting immune cells including T cells, B cells, NK cells, suggesting that BSP might be involved in the early stage of immune cell differentiation. Indeed, our cytokine array analyses revealed that BSP significantly increased the levels of stem cell factor (SCF) (Figs. 6, S3, Table S2), which has been reported to play a role in the differentiation and maturation of immunocytes [21–23]. Whether SCF plays a functional role in the effect of BSP remains to be further explored.

Nevertheless, the immunoregulatory activity of these natural polysaccharides has attracted growing attention in the field of cancer immunotherapy. The cytokine stimulatory effect of BSP, especially the increase of IL-2 and IFN-γ levels in the sera of tumor bearing mice is particular interesting. As both IL-2 and IFN-γ have been proved to be indispensable in T cell-mediated immunotherapy [24,25]. Noteworthy, as the effector cells, macrophages treated with *Ganoderma lucidum* polysaccharides produce large amounts of the cytokines IL-6, TNF-α, and IL-1β [26]. Likewise, *Coriolus versicolor* polysaccharides increase the phagocytotic capacity of macrophages [27], and *Hericium erinaceus* polysaccharides stimulate macrophages to increase their release of NO and induce NF-κB signaling [28]. In addition, polysaccharides extracted from *Agaricus blazei murill* can significantly increase the number of macrophages in the peritoneum of mice carrying S180 sarcoma cell tumors [29].

In conclusion, our findings provide compelling evidence to support the development of bioactive polysaccharides-particularly polysaccharides extracted from bamboo shavings-as an effective treatment of various forms of cancer, including gastric cancer. Future studies should therefore focus on the clinical development of BSP either as an adjuvant therapy or for use in combination with standard care in order to effectively treat gastric cancer.

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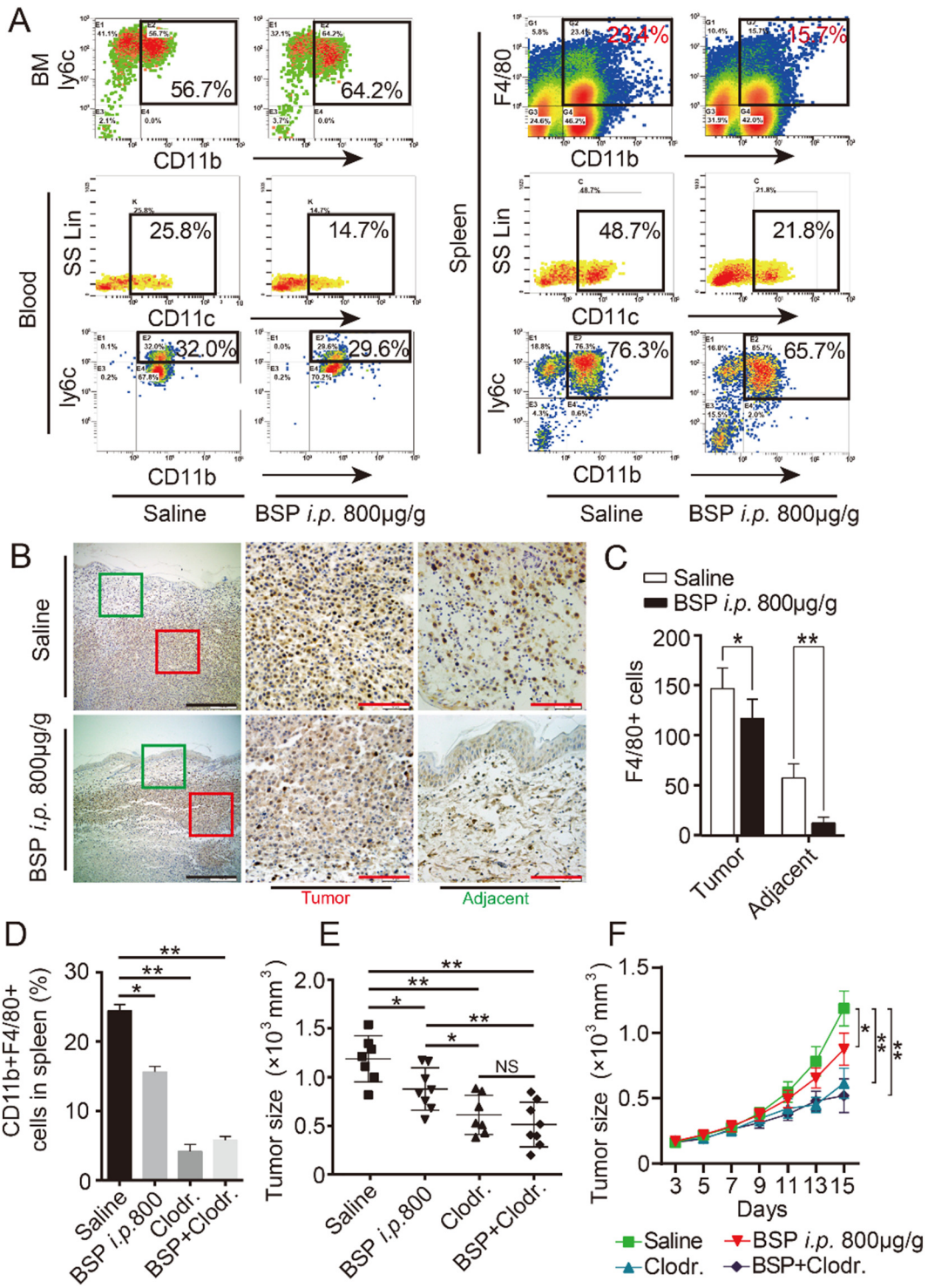

Fig. 7. Macrophage numbers are significantly reduced in tumor tissues following BSP treatment. (A) FACS plots showing the percentage of CD11b + ly6c+ monocytes in the bone marrow (BM), CD11c+ dendritic cells and CD11b + ly6c+ monocytes in the blood, and CD11b + F4/80+ macrophages, CD11c+ dendritic cells, and CD11b + ly6c+ monocytes in the spleen of mice treated with either BSP (800 μg/g body weight, *i.p.*) or saline. (B) Immunohistochemical staining of F4/80 in the tumor (red boxes) and in the adjacent healthy tissue (green boxes). The scale bars represent 400 μm (left column) and 100 μm (middle and right columns). (C) Quantification of F4/80-positive cells as shown in (B) ($N = 6$/group). (D, E and F) Percentage of CD11b + F4/80+ cells in the spleen (D) ($N = 3$/group), tumor size (E) ($N = 8$/group), and tumor growth curve (F) measured in mice treated for 2 weeks with saline or BSP (800 μg/g body weight, *i.p.*) and/or clodrosome (Clodr.). $^{*}p < 0.05$, $^{**}p < 0.01$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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Competing interests

The authors declare that they have no competing interests.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lfs.2019.01.003.

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