微生物学报
Acta Microbiologica Sinica
2024, 64(9): 3489–3505
http://journals.im.ac.cn/actamicrocn
DOI: 10.13343/j.cnki.wsxb.20240157

Research Article 研究报告

地塞米松通过调节中性粒细胞和巨噬细胞向肿瘤的浸润增强减毒鼠伤寒沙门菌介导的肿瘤免疫治疗的抗肿瘤效果

王珍[1]，朴伶华[2]，申铉日[3*]，柳贤德[1*]

1 海南大学 生命科学学院，海南 海口 570228
2 海南医学院 生理学系，海南 海口 571199
3 海南热带海洋学院 食品科学与工程学院，海南 三亚 572022

王珍, 朴伶华, 申铉日, 柳贤德. 地塞米松通过调节中性粒细胞和巨噬细胞向肿瘤的浸润增强减毒鼠伤寒沙门菌介导的肿瘤免疫治疗的抗肿瘤效果[J]. 微生物学报, 2024, 64(9): 3489-3505.

WANG Zhen, PIAO Linghua, SHEN Xuanri, LIU Xiande. Dexamethasone augments the efficacy of attenuated *Salmonella typhimurium*-based cancer immunotherapy by modulating the infiltration of neutrophils and macrophages in the tumor microenvironment[J]. Acta Microbiologica Sinica, 2024, 64(9): 3489-3505.

摘　要：细菌介导的肿瘤免疫治疗(bacterium-mediated cancer immunotherapy, BCI)在癌症治疗中具有诸多优势，但地塞米松(dexamethasone, DEX)联合 BCI 治疗肿瘤的免疫反应机制仍不清晰。【目的】探究 DEX 联合减毒鼠伤寒沙门氏菌 St.ΔppGpp 介导的 BCI 肿瘤治疗效果及机制。【方法】通过鼠源结直肠癌小鼠模型，测定 St.ΔppGpp+DEX 联合治疗的肿瘤抑制效果。使用活体成像测定 St.ΔppGpp 的肿瘤靶向性与定殖时间。通过伊红(hematoxylin and eosin, H&E)染色，测试 St.ΔppGpp+DEX 联合治疗的器官毒性。基于流式细胞术和免疫荧光切片，测定巨噬细胞极化、中性粒细胞的募集和 T 细胞反应。通过 qRT-PCR，检测肿瘤微环境中的炎症因子变化。通过人源结直肠癌症小鼠模型，验证 T 细胞缺失对 St.ΔppGpp+DEX 联合治疗的影响。【结果】St.ΔppGpp+DEX 联合治疗可显著降低肿瘤大小，并提高小鼠生存率。DEX 可延长 St.ΔppGpp 在肿瘤细胞的定殖。St.ΔppGpp+DEX 联合治疗不会损伤重要免疫器官，并可促进 M2 向 M1 型巨噬细胞极化，同时抑制中性粒细胞的募集。T 细

资助项目：国家自然科学基金(32260854)；海南省科技专项(ZDYF2022SHFZ041)；海南省自然科学基金(821RC529, 821RC584)；海南热带海洋大学科研基金(RHDRC202314)

This work was supported by the National Natural Science Foundation of China (32260854), the Science and Technology Special Fund of Hainan Province (ZDYF2022SHFZ041), the Natural Science Foundation of Hainan Province (821RC529, 821RC584), and the Scientific Research Foundation of Hainan Tropical Ocean University (RHDRC202314).

*Corresponding authors. E-mail: SHEN Xuanri, shenxuanri2009@163.com; LIU Xiande, xiande_liu79@hainu.edu.cn

Received: 2024-03-12; Accepted: 2024-05-27; Published online: 2024-05-29

胞缺失不会影响 St.ΔppGpp+DEX 联合治疗效果。【结论】DEX 通过抑制中性粒细胞募集，增加肿瘤微环境中的 M1 型巨噬细胞比例，进而提高减毒鼠伤寒沙门氏菌 St.ΔppGpp 的抗肿瘤疗效。

关键词：细菌介导的肿瘤免疫治疗；鼠伤寒沙门氏菌；地塞米松；肿瘤微环境；细胞免疫反应

Dexamethasone augments the efficacy of attenuated *Salmonella typhimurium*-based cancer immunotherapy by modulating the infiltration of neutrophils and macrophages in the tumor microenvironment

WANG Zhen[1], PIAO Linghua[2], SHEN Xuanri[3*], LIU Xiande[1*]

1 School of Life Sciences, Hainan University, Haikou 570228, Hainan, China
2 Department of Physiology, Hainan Medical University, Haikou 571199, Hainan, China
3 College of Food Science and Engineering, Hainan Tropical Ocean University, Sanya 572022, Hainan, China

Abstract: Bacterium-mediated cancer immunotherapy (BCI) presents numerous advantages in cancer treatment, while the immune response mechanism of dexamethasone (DEX) combined with BCI for tumor treatment remains unclear. **[Objective]** To investigate the therapeutic efficacy and mechanism of dexamethasone in combination with attenuated *Salmonella typhimurium* St.ΔppGpp-mediated BCI. **[Methods]** The inhibitory effects of St.ΔppGpp+DEX on cancer were evaluated in a murine model of colorectal cancer. *In vivo* imaging was utilized to determine the tumor targeting and colonization duration of St.ΔppGpp. Organ toxicity resulted from St.ΔppGpp+DEX treatment was assessed by hematoxylin and eosin (H&E) staining. Macrophage polarization, neutrophil recruitment, and T-cell responses were analyzed by flow cytometry and immunofluorescence assay of sections. The changes in inflammatory cytokines in the tumor microenvironment were examined *via* qRT-PCR. A mouse model transplanted with human colorectal cancer was employed to confirm the effect of T cell depletion on the therapeutic efficacy of St.ΔppGpp+DEX. **[Results]** The combined treatment St.ΔppGpp+DEX significantly decreased tumor size and enhanced the survival rate of mice. DEX extended the colonization of St.ΔppGpp in tumor cells. Furthermore, St.ΔppGpp+DEX did not induce damage to vital immune organs, and it facilitated the polarization of macrophages from M2 to M1 phenotype while suppressing neutrophil recruitment. T cell depletion did not influence the efficacy of St.ΔppGpp+DEX. **[Conclusion]** DEX can enhance the anti-tumor effects of St.ΔppGpp by inhibiting neutrophil recruitment and increasing the proportion of M1 macrophages in the tumor microenvironment.

Keywords: bacterium-mediated cancer immunotherapy; *Salmonella typhimurium*; dexamethasone; tumor microenvironment; cellular immune response

Cancer immunotherapy is considered a valuable treatment approach for various types of cancer[1]. The immune checkpoint blockade (ICB) has exhibited remarkable clinical efficacy in patients with melanoma, non-small cell lung cancer, renal cell carcinoma, bladder cancer, or Hodgkin's lymphoma among the various forms of cancer immunotherapy[2]. ICB works by alleviating the T cell suppression and thereby increasing their tumor cell killing potential[3]. Despite the overall success of immune checkpoint blockade (ICB), its efficacy has been limited in patients with pancreatic and ovarian malignancies, which are characterized by a low frequency of tumor antigen mutations and a lack of T cell infiltration into the tumor[4]. Moreover, the interaction between programmed cell death 1 receptor (PD-1) and its ligand PD-L1 has been found to be associated with a range of immune-related adverse effects and cardiotoxicity[5].

The use of dexamethasone as a first-line prophylactic medication is recommended to effectively mitigate nausea and vomiting in patients undergoing ICB therapy[6]. In addition, it serves as an immunosuppressant, mitigating the deleterious effects of chemotherapy[6-8] and ICB treatment regimens[8-9]. Consequently, the use of dexamethasone in conjunction with other cancer treatments can have a substantial impact on a patient's quality of life and medical expenses. Moreover, dexamethasone has demonstrated potent anti-tumor and anti-angiogenic properties by effectively inhibiting the expression of HIF-1 and vascular endothelial growth factor (VEGF)[10-11].

The bacteria-mediated cancer immunotherapy (BCI) offers several advantages over ICB, ranging from precise tumor targeting to enhanced tumor penetration[12-16]. The BCI recruits various immune cells, such as neutrophils, macrophages, and $CD4^+$/$CD8^+$ T cells, to the tumor microenvironment[16-17]. In addition, BCI can directly eradicate tumor cells *via* bacterial invasion[18-20]. The genetically modified strain of *Salmonella typhimurium* (St.ΔppGpp) exhibits a remarkable capacity for proliferation and persistence within tumor tissue, inducing a robust immune response that leads to rapid regression of the tumor[14,21-22]. The St.ΔppGpp variant has a 10 000 to 1 000 000 times greater 50% lethal dose than the wild-type *S. typhimurium* strain[23].

Because *Salmonella*-mediated cancer therapy cannot completely eradicate tumors alone, it is important to devise an optimal way of combining it with other therapeutic modalities such as chemotherapy or radiotherapy[24-26]. While *Salmonella*-mediated cancer therapy and dexamethasone have demonstrated encouraging outcomes independently, the potential anti-tumor efficacy of their combination remains largely unexplored. In addition, whether dexamethasone can modulate the immune response to St.ΔppGpp is uncertain. Therefore, the objective of this study was to investigate the impact of combined treatment using St.ΔppGpp and dexamethasone on both anti-tumor and immune responses in a xenograft mouse model.

1 Materials and Methods

1.1 Mouse colon cancer model and bacterial injection

To establish the colon cancer xenografts, CT26 cells (1×10^6/mL; American Type Culture Collection [ATCC]) and HT29 cells (1×10^6/mL; ATCC) were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco) supplemented with 10% fetal bovine serum (Gibco), and implanted subcutaneously into the right flank of male SPF BALB/c or BALB/c-nu/nu mice (5–6 weeks old; weighing 18–25 g) obtained from Guangdong Medical Laboratory Animal Center (Foshan, China). The tumor growth was monitored on a daily basis by measuring their dimensions with a caliper, and the tumor volume was calculated using the formula (length×height×width)/2. Once the tumor volume reached 120–160 mm^3, the mice were randomly divided into four groups (n=9/group): phosphate buffered saline (PBS; vehicle control), dexamethasone (DEX), St.ΔppGpp, or St.ΔppGpp+DEX. The DEX groups received a

daily intravenous injection of 0.2 mg/kg dexamethasone, while the St.ΔppGpp groups were administered 3×10^7 colony forming units (CFU) of St.ΔppGpp bacteria *via* the tail vein. The mice were euthanized when the tumor volume reached ≥1 500 mm^3. All experimental procedures involving mice were approved by the Animal Science and Technology Ethics Committee of Hainan University (No. HNUAUCC-2020-00013) and the mice were anesthetized using either 2% isoflurane or ketamine (200 mg/kg).The specific details are shown in the "Approval Document for Experimental Animal Welfare Ethics Related Projects of Hainan University".

The St.ΔppGpp-Lux, a St.ΔppGpp variant engineered to express the luciferase gene, acquired from Zheng Jinhai at Hunan University. The strain was cultivated in Luria Bertani medium supplemented with kanamycin (Sigma-Aldrich) and preserved as 25% glycerol stocks at a temperature of −80 ℃.

1.2 Optical bioluminescence imaging

When the tumor volume reached 120–180 mm^3, the mice underwent intravenous injection of 3×10^7 CFU of St.ΔppGpp-Lux, followed by bacterial bioluminescence imaging. Subsequently, the mice were randomly divided between two groups: St.ΔppGpp-Lux and St.ΔppGpp-Lux+DEX, with six mice per group. The DEX group received a daily intravenous dose of 0.2 mg/kg dexamethasone. Bioluminescence imaging was performed for 9 consecutive days using an IVIS 100 *in vivo* imaging system (Caliper).

1.3 H&E staining

The liver, spleen, kidney, and lung tissues were harvested from euthanized tumor-bearing mice and fixed in 4% paraformaldehyde (PFA) solution to evaluate the toxicity of the St.ΔppGpp+DEX combination therapy. Paraffin-embedded tissue sections (3 μm thick) were prepared and subjected to H&E staining using a commercial staining kit (C0105, Beyotime) according to the manufacturer's instructions.

1.4 Quantitative real-time (qRT)-PCR analysis

TRIzol reagent (Beyotime) was used to extract total RNA from tumors. The extracted RNA was reverse transcribed using the SuperScript II cDNA Synthesis Kit (TaKaRa) and subjected to qRT-PCR using the SuperReal PreMix Plus Reagent (TIANGEN, China). The primer sequences used in this study are provided in Table 1.

1.5 Flow cytometry

Tumor tissue was immersed in a solution of collagenase I (Wuhan Servicebio Technology, Co., Ltd.) and incubated for 45 min at 37 ℃ to extract the cells. Subsequently, the tissue was passed through a 70 μm cell strainer. The cells were then stained with fluorochrome-labeled antibodies targeting CD86, CD206, F4/80, LY6G, CD3, CD4 and CD8 (all from Elabscience Biotechnology, Co., Ltd.) at 4 ℃ for 30 min. A minimum of 10 000 events were recorded on a FACSCalibur flow cytometer (Beckman Coulter CytoFlEX) and the data were analyzed using FlowJo software (Tree Star).

1.6 Zebrafish injury model

The recruitment of neutrophils expressing enhanced green fluorescent protein (eGFP) to the

Table 1 Primers used to amplify cytokine-encoding and housekeeping genes

Gene name	Forward primers (5′→3′)	Reverse primers (5′→3′)
IL-1β	GCAACTGTTCCTGAACTCAACT	ATCTTTTGGGGTCCGTCAACT
TNF-α	CATCTTCTCAAAATTCGAGTGACAA	TGGGAGTAGACAAGGTACAACCC
IL-6	CCTTCCTACCCCAATTTCCAAT	AACGCACTAGGTTTGCCGAGTA
TGF-β	GAAGGCAGAGTTCAGGGTCTT	GGTTCCTGTCTTTGTGGTGAA
G-CSF	CTCAACTTTCTGCCCAGAGG	AGCTGGCTTAGGCACTGTGT
GM-CSF	GCCATCAAAGAAGCCCTGAA	GTGAAATTGCCCCGTAGACC
HPRT	TTATGGACAGGACTGAAAGAC	GCTTTAATGTAATCCAGCAGGT

site of injury was visualized in a transgenic (Tg) (mpx:eGFP) zebrafish model (obtained from the China Zebrafish Resource Center, Wuhan, China) using live imaging. To induce the injury, the notochord tip was transected using a sterile scalpel at 72 hour-post-fertilization (hpf), and the number of neutrophils within 0.2 mm of the incision edge was quantified. Damaged tails were treated with dexamethasone (500 μmol/L, Aladdin, China), and the images were captured on an Olympus fluorescence microscope at 4 and 24 hour-post-injury (hpi). The images were analyzed using Image-Pro Plus software.

1.7 Statistical analysis

The statistical analysis was carried out using SPSS 21.0 software. A *P*-value of less than 0.05 was considered as a measure of statistical significance. The Kaplan-Meier method and log-rank test were used to perform survival analysis. The data were presented as the mean ± standard error of the mean (SEM).

2 Results

2.1 Dexamethasone enhances the anti-cancer activity of St.ΔppGpp-mediated cancer immunotherapy

Although the efficacy of *Salmonella*-mediated BCI and dexamethasone was promising, neither monotherapy could fully mitigate tumor development and metastasis in patients[19-20,27]. Thus, to explore whether dexamethasone could enhance the anti-tumor effect of BCI by regulating the anti-bacterial immune response and remodeling the tumor environment (TME), we examined the potential synergistic effect of the dexamethasone and St.ΔppGpp combination. To this end, mice were intravenously injected with St.ΔppGpp (3×10^7 CFU) on Day 1, and then treated intraperitoneally with dexamethasone (0.2 mg/kg) from Days 2 to 27 (Figure 1A). The results demonstrated that St.ΔppGpp and dexamethasone exhibited a synergistic anti-tumor effect (versus St.ΔppGpp or dexamethasone alone), which was evidenced by tumor regression (Figure 1B and 1C) and the improved survival rates of model mice (Figure 1D). These results indicate that dexamethasone positively regulates St.ΔppGpp-mediated cancer immunotherapy and that combining St.ΔppGpp with dexamethasone may be a promising anti-cancer strategy.

2.2 Dexamethasone promotes the survival of tumor-colonizing St.ΔppGpp

The success of BCI relies on the ability of therapeutic bacteria to efficiently colonize tumors and induce tumor cell apoptosis and endogenous immune responses[16,28]. To investigate whether the synergistic antitumor effect of the combination of St.ΔppGpp and dexamethasone is associated with improved survival of tumor-colonized bacteria, this study evaluated the effect of dexamethasone on survival of bioluminescent tumor-colonized St.ΔppGpp-Lux strains. The data of Caliper IVIS Lumina II showed that the administration of dexamethasone increased the retention time of St.ΔppGpp-Lux-derived bioluminescence from 10 d (for St.ΔppGpp-Lux alone) to 14 d (Figure 2A). These findings indicate a potential correlation between the capacity of dexamethasone when combined with St.ΔppGpp to augment the antitumor efficacy and the increased survival rates of patients with tumors following treatment involving dexamethasone with St.ΔppGpp.

After observing the retention of St.ΔppGpp in tumors, we assessed the potential impact of combining St.ΔppGpp with dexamethasone on lung, liver, spleen, and kidney tissues in tumor-bearing mice. The data showed that the toxicity of the St.ΔppGpp and dexamethasone combination was similar to that of each therapeutic agent alone. Moreover, the combination therapy did not induce abnormalities, such as steatosis, inflammatory infiltrates, or fibrosis (Figure 2B). This suggests that dexamethasone positively regulates the bacterial colonization of tumors and not that of other organs.

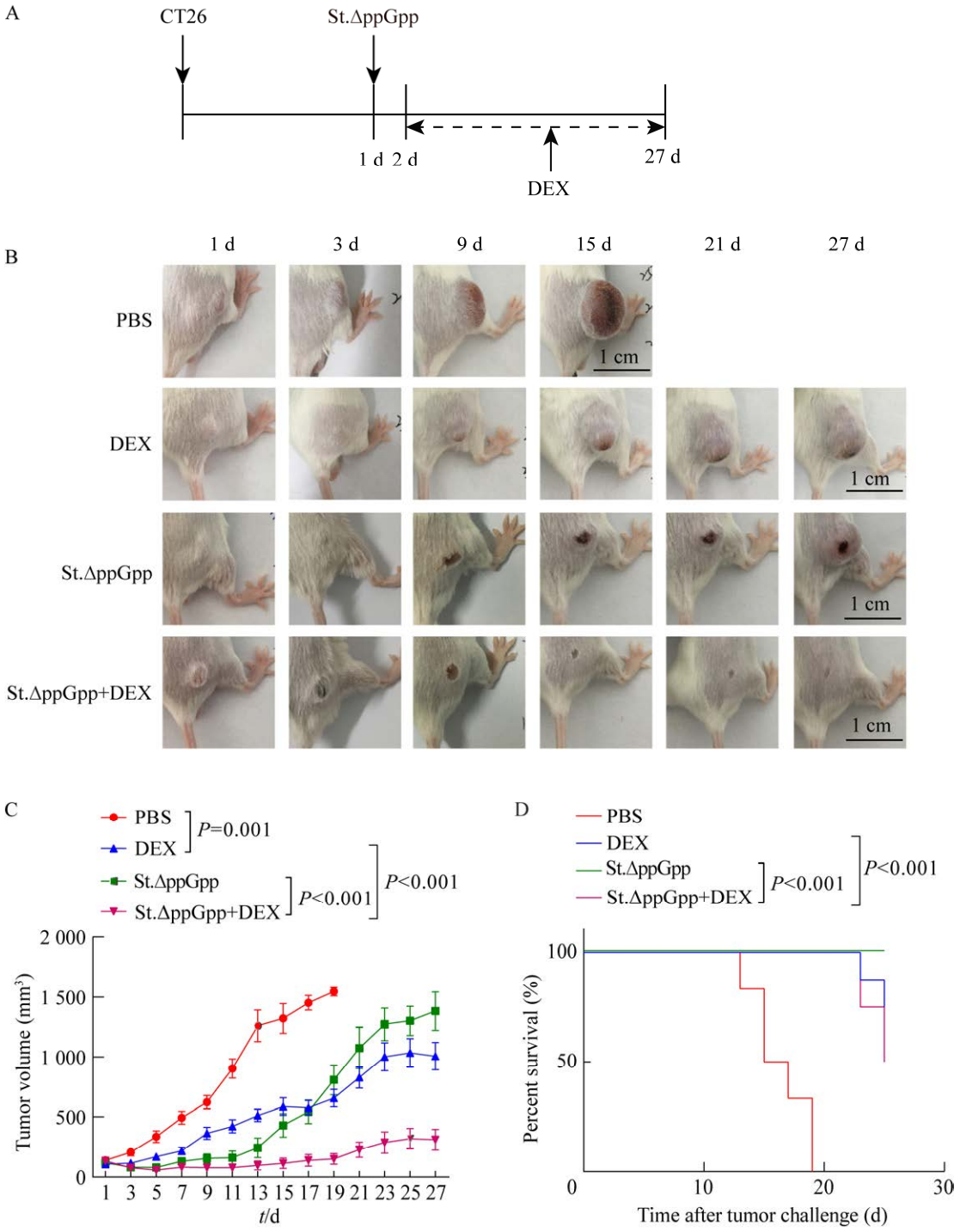

Figure 1 The St.ΔppGpp and dexamethasone combination induces tumor regression in CT26-tumor-bearing mice. A: Schematic diagram showing the treatment timeline used for all the mouse experiments. B: Images of tumors from representative mice from each group. C: Graph depicting changes in CT26 tumor size (n=6). D: Kaplan-Meier survival curves for CT26 tumor-bearing mice (n=6). P≤0.001 indicates significant difference versus the PBS and St.ΔppGpp groups.

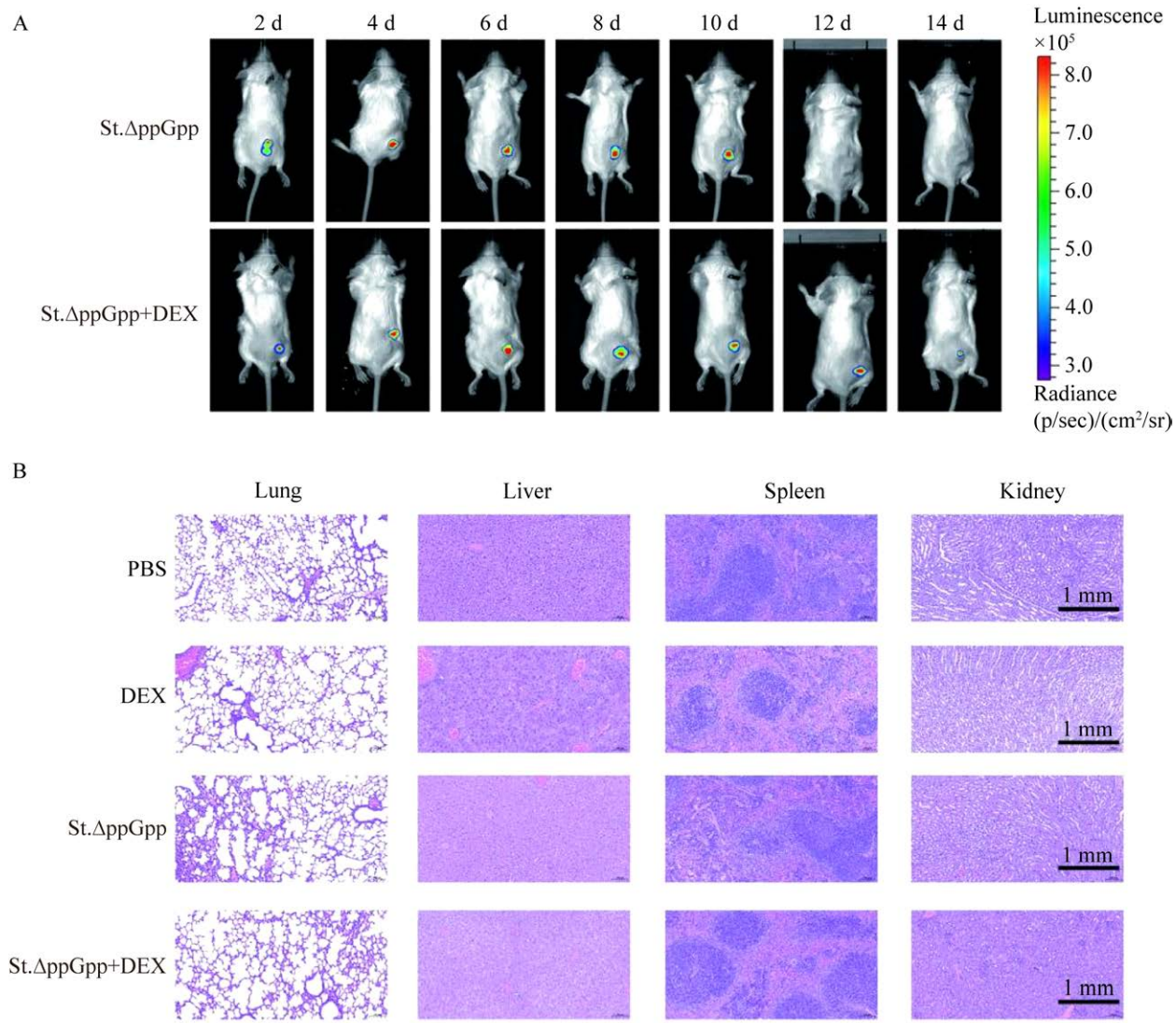

Figure 2 The St.ΔppGpp and dexamethasone combination promotes tumor colonization by *Salmonella* without adverse effects. A: Non-invasive monitoring of bacterial bioluminescence for 14 d (n=8). (p/sec)/(cm^2/sr): Luminous flux particles irradiated per unit area. B: H&E staining of organs isolated from CT26-tumor-bearing mice treated with PBS, dexamethasone (DEX), St.ΔppGpp, or St.ΔppGpp+DEX.

2.3 The effect of dexamethasone on St.ΔppGpp-mediated cellular immune response in the TME

The accumulation of *Salmonella* in tumor tissue can induce a shift in the tumor microenvironment (TME) from an immunosuppressive state to an immunogenic state by recruiting immune cells, including neutrophils, macrophages, and T cells, into the tumor tissue[12,21,29]. The immunogenicity of infected tumors is increased *via* their apoptosis or through the increased presentation of tumor antigens. This increased tumor antigen release and/or presentation stimulates T cells to kill tumor cells[30-32]. To assess the impact of dexamethasone on *Salmonella*-induced anti-tumor cellular immune responses, we employed flow cytometry to quantify the proportions of distinct immune cell populations (specifically neutrophils, M1 and M2 macrophages, as well as $CD4^+$/$CD8^+$ T cells) within the tumor microenvironment.

Hence, the research stained tumor tissues isolated from CT26-tumor-bearing mice with antibodies against F4/80 and CD86 to detect M1-type macrophages; against F4/80 and CD206 to detect M2-type macrophages; against Ly6G to detect neutrophils; against CD3 and CD4 to detect $CD4^+$ T cells; and against CD3 and CD8a to detect $CD8^+$ T cells. Flow cytometry analysis revealed an increase in the proportion of $F4/80^+CD86^+$ M1-type macrophages (Figure 3A), a decrease in the proportion of $F4/80^+CD206^+$ M2-type macrophages (Figure 3B) and $Ly6G^+$ neutrophils (Figure 3C), and no change in the proportions of $CD4^+$ T cells (Figure 3D) and $CD8^+$ T cells (Figure 3E). The findings suggest that dexamethasone increased *Salmonella*-mediated anti-cancer activity by decreasing the infiltration of neutrophils and M2-type macrophages and increasing the infiltration of M1-type macrophages into the tumor.

2.4 Dexamethasone decreases St.ΔppGpp-mediated neutrophil infiltration

Being facultative anaerobic bacteria, *Salmonella* possess a distinctive capability to deeply infiltrate tumor tissue and selectively accumulate in necrotic zones characterized by inadequate vasculature and hypoxia[33]. However, neutrophils can form a barrier, which restricts the entry of *Salmonella* into viable cancer cell zones within the tumor. As such, they negatively regulate the ability of these bacteria to effectively colonize tumors, which ultimately reduces the anti-cancer efficacy of BCI[28]. To investigate the link between dexamethasone, *Salmonella* retention in tumors, and neutrophil tumor infiltration, immunofluorescence staining was performed on tumor tissue collected from CT26-bearing mice. The results demonstrated that dexamethasone significantly decreased neutrophil infiltration into tumors (Figure 4A and 4B), which was consistent with the findings of the flow cytometry analysis. To further verify the effect of dexamethasone on neutrophils, Tg (mpx:eGFP) zebrafish was used as an inflammatory model[34-36]. After injuring the zebrafish tail, the recruitment of neutrophils to the injury site was observed by live imaging, and it was found that dexamethasone significantly inhibited the migration of neutrophils to the injury site (Figure 4C). This finding indicates that dexamethasone blocks *Salmonella*-mediated neutrophil recruitment to the tumor, supporting the flow cytometry and immunofluorescence staining results generated from tumor-bearing mice.

2.5 Dexamethasone increases M1 and decreases M2 macrophage infiltration into St.ΔppGpp-colonized tumors

Tumor-infiltrating macrophages can be categorized into two types: M1, which have anti-tumorigenic activity, and M2, which are pro-tumorigenic. Polarizing M2 macrophages into M1 macrophages represents a promising cancer therapeutic strategy, as it can lead to the transformation of a highly immunosuppressive TME into an immunogenic one[37-39]. To further characterize *Salmonella*-mediated macrophage infiltration, immunofluorescence staining was performed on tumor tissues collected from CT26-tumor-bearing mice. The results of the immunofluorescence staining were consistent with those of the flow cytometry analysis, and showed that dexamethasone increased the *Salmonella*-mediated infiltration of M1 macrophages (Figure 5A and 5B) while decreasing that of M2 macrophages (Figure 5A and 5C) into the tumor, suggesting that the synergistic anti-cancer efficacy of the St.ΔppGpp and dexamethasone combination was associated with increased St.ΔppGpp-mediated M1 macrophage infiltration into the tumor.

2.6 Effect of St.ΔppGpp and dexamethasone on inflammatory cytokines in the TME

Salmonella infection in the TME induces the production of pro-inflammatory cytokines by tumor-infiltrating immune cells, which is associated with *Salmonella*-mediated anti-tumor efficacy[18,40]. The expression of various cytokines within the TME was measured by qRT-PCR and it

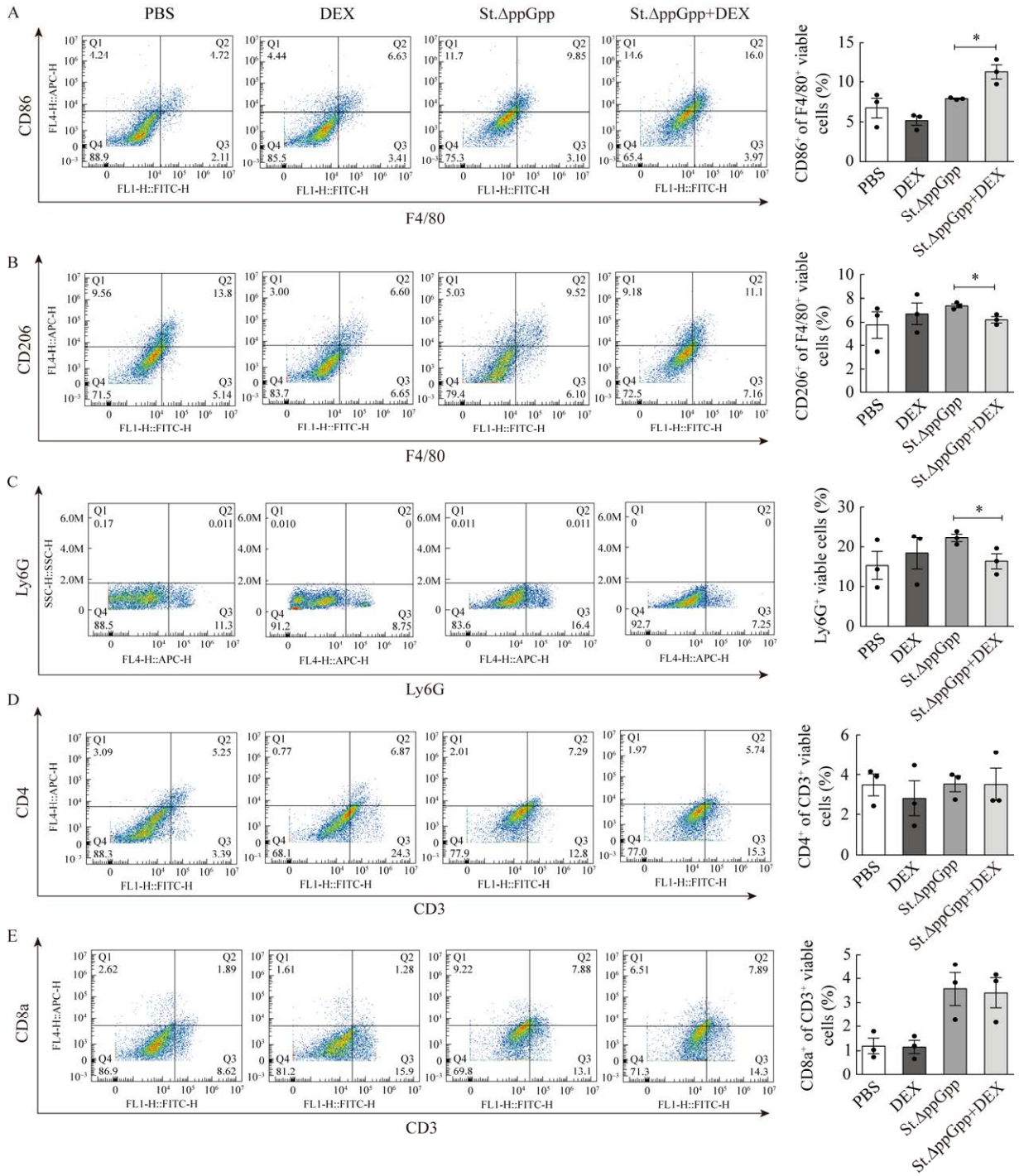

Figure 3 Effect of St.ΔppGpp and dexamethasone combination therapy on the infiltration of immune cells into tumors. Tumors were isolated from CT26-tumor-bearing mice treated with PBS, dexamethasone (DEX), St.ΔppGpp, or St.ΔppGpp+DEX on Day 2 post bacterial injection (n=5). Samples were double stained with fluorescently-labeled antibodies against F4/80 (macrophage marker) and CD86 (M1-type macrophage marker) (A) or CD206 (M2-type macrophage marker) (B), Ly6G (neutrophil marker) (C), CD3 (T cell marker) and CD4 ($CD4^+$ T cell marker) (D), or CD8a ($CD8^+$ T cell marker) (E), and then analyzed by flow cytometry. *: P<0.05, versus the PBS, DEX, and St.ΔppGpp groups.

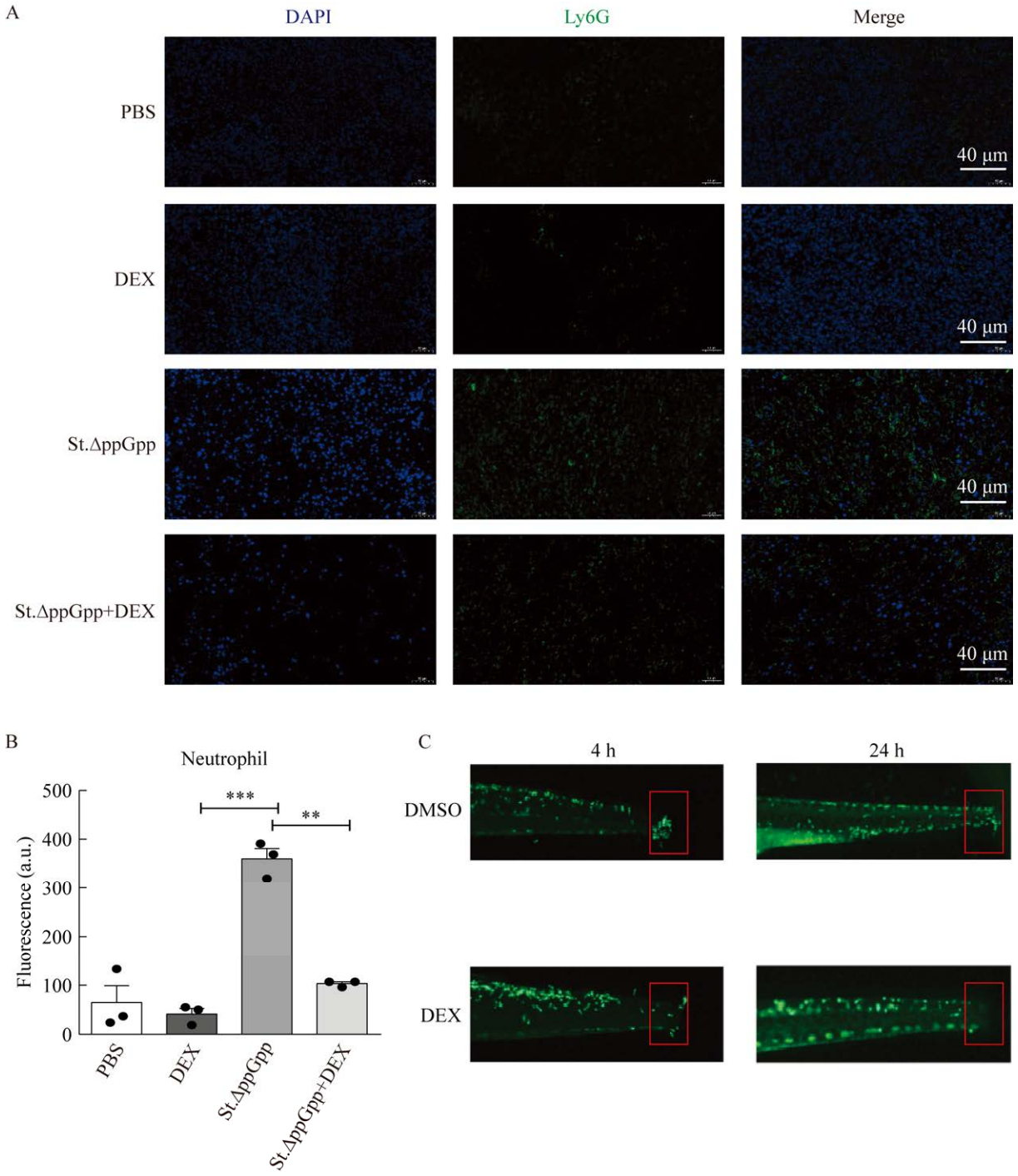

Figure 4 Dexamethasone inhibits St.ΔppGpp-mediated neutrophil infiltration into tumors. A: Representative images of immunofluorescence staining of neutrophils ($Ly6G^+$) in tumors from CT26-tumor-bearing mice treated with PBS, DEX, St.ΔppGpp, or St.ΔppGpp+DEX. B: Quantification of neutrophil-derived immunofluorescence intensity ($Ly6G^+$, green). C: Representative fluorescence images of zebrafish embryos obtained 4 h and 24 h post-treatment with DEX (200 μmol/L). ***: $P<0.001$ and **: $P<0.01$, versus the DEX or St.ΔppGpp groups, respectively.

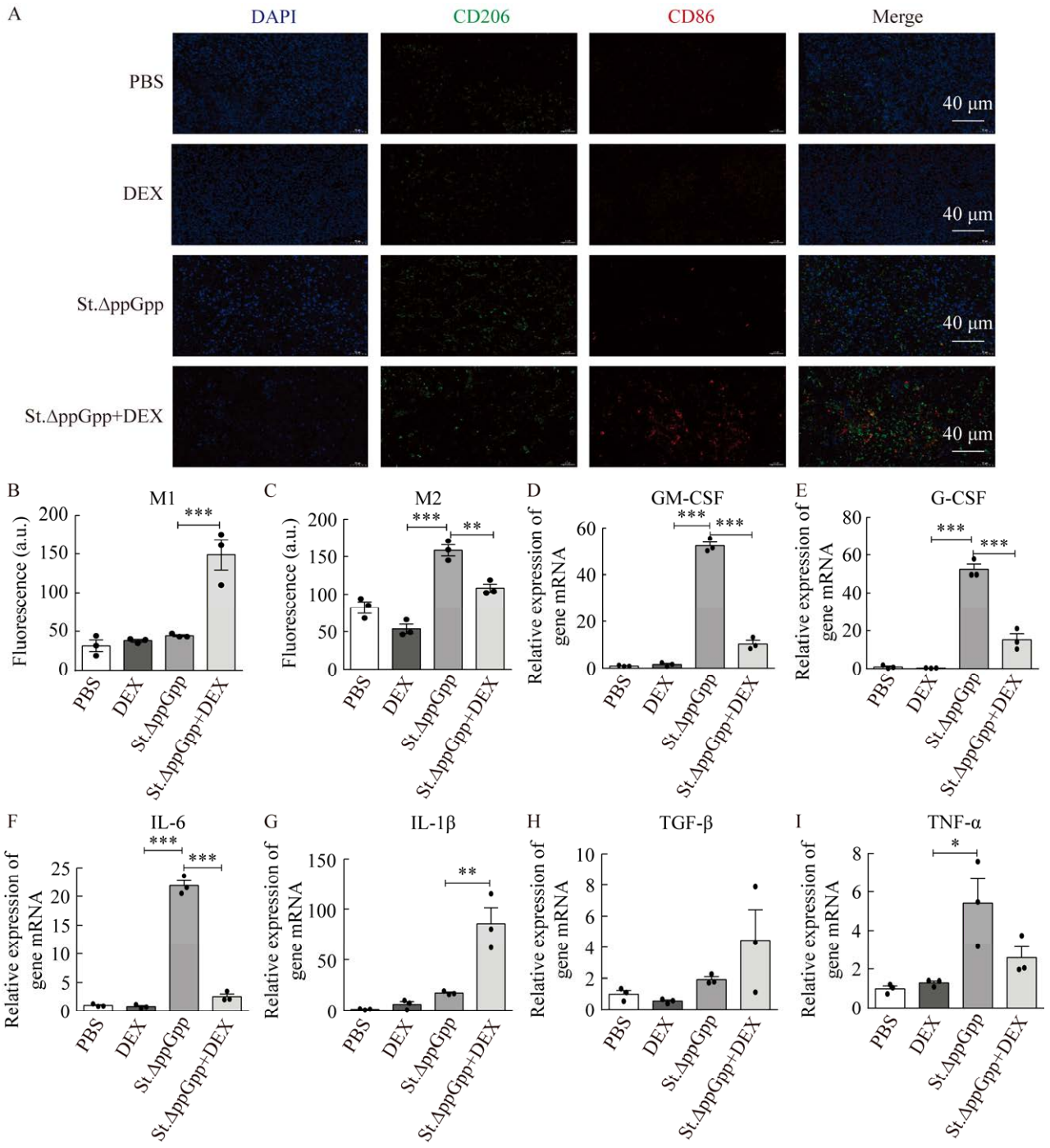

Figure 5 Effects of St.ΔppGpp in combination with dexamethasone on macrophage tumor infiltration and inflammatory cytokines. A: Representative images of immunofluorescence staining for M1-type ($CD86^+$, red) and M2-type ($CD206^+$, green) macrophages in tumors from CT26-tumor-bearing mice treated with PBS, DEX, St.ΔppGpp or St.ΔppGpp+DEX. Quantification of immunofluorescence intensity for B: M1-type ($CD86^+$, red) and C: M2-type ($CD206^+$, green) macrophages. Tumor tissue was isolated from CT26 tumor-bearing mice at day 2 post-bacterial injection (n=3), and mRNA expression of inflammatory cytokines, including D: GM-CSF, E: G-CSF, F: IL-6, G: IL-1β, H: TGF-β, and I: TNF-α, was measured by qRT-PCR. ***: P<0.001 and **: P<0.01, versus the DEX or St.ΔppGpp groups, respectively.

was found that the St.ΔppGpp and dexamethasone combination (versus St.ΔppGpp alone) reduced the expression of GM-CSF, G-CSF, and interleukin (IL)-6 (Figure 5D−5F), while increasing that of IL-1β; the levels of TGF-β and TNF-α were unaltered (Figure 5G−5I). These results suggest a possible link between decreased neutrophil infiltration and the downregulation of genes encoding G-CSF, GM-CSF, and IL-6, as well as between increased M1 macrophage infiltration and the increase in IL-1β expression.

2.7 Dexamethasone enhances the *Salmonella*-mediated anti-cancer response in a T-cell-independent manner

Because our earlier flow cytometry results showed that dexamethasone did not alter the *Salmonella*-mediated infiltration of T cells into tumors, we wanted to eliminate the influence of T cells in further experiments. The combination of St.ΔppGpp and dexamethasone was employed in this study to treat HT29-tumor-bearing mice derived from T cell-deficient BALB/c-nu/nu mice (Figure 6A−6C). Similarly, the combined administration of St.ΔppGpp and dexamethasone synergistically enhanced the antitumor efficacy compared to their individual effects. A previous study had indicated that inhibition of the AKT signaling pathway could impede neutrophil recruitment to inflammation sites. To validate the correlation between inhibition of neutrophil recruitment and the *Salmonella*-mediated anti-tumor effect, we utilized esculetin, an AKT inhibitor, to further suppress neutrophil recruitment to the tumors of T-cell-deficient HT29-tumor-bearing mice. The data showed that the triple combination of St.ΔppGpp, dexamethasone, and esculetin exhibited a significantly enhance anti-tumor effect compared to St.ΔppGpp and either dexamethasone or esculetin alone (Figure 6D and 6E). The findings suggest that targeting the AKT pathway to reduce neutrophil infiltration could be a promising strategy for augmenting the effectiveness of *Salmonella*-mediated anti-tumor therapy.

3 Discussion

Salmonella-mediated cancer immunotherapy has certain advantages over traditional tumor immunotherapy, such as effective targeting, tumor penetration, and immune stimulation, coupled with low toxicity and cost[16]; however, *Salmonella* alone cannot completely eradicate tumors or inhibit tumor metastasis. Therefore, it is increasingly being used as a combined therapy with radiotherapy and chemotherapy in clinical studies. Combination therapy with conventional drugs, which are already in clinical use and well tolerated by patients, is easier to implement than developing new gene therapies or drugs. In this study, the data showed that dexamethasone significantly enhanced the anti-tumor effect of attenuated *Salmonella* BCI and significantly prolonged the survival of these bacteria in a tumor-bearing mouse model. Similar results were obtained in another study, which showed that dexamethasone enhanced the anti-tumor efficacy of gemcitabine by promoting the apoptosis and chemosensitivity of tumor cells[41].

Salmonella exerts three main effects on tumors: (1) it induces apoptosis by infecting tumor cells; (2) its infection of tumor cells leads to the recruitment of more immune cells into the TME; and (3) it increases the production of tumor antigens and the recruitment to antigen-presenting cells such as neutrophils, macrophages, and dendritic cells into the tumor, which ultimately activates T cells to kill tumor cells[17,21,42]. Lee et al. reported that the anti-tumor effect of attenuated *Salmonella* was significantly greater in wild-type mice than in mice lacking $CD4^+$ and $CD8^+$ T cells, indicating that the therapeutic effect of *Salmonella*-mediated cancer immunotherapy was directly related to T cell activation[21,30-31]. The glucocorticoid dexamethasone is frequently employed in clinical practice alongside chemotherapy and radiotherapy to mitigate the adverse effects of these therapeutic modalities through its anti-inflammatory and immunosuppressive mechanisms[6]; however, dexamethasone itself also exerts an

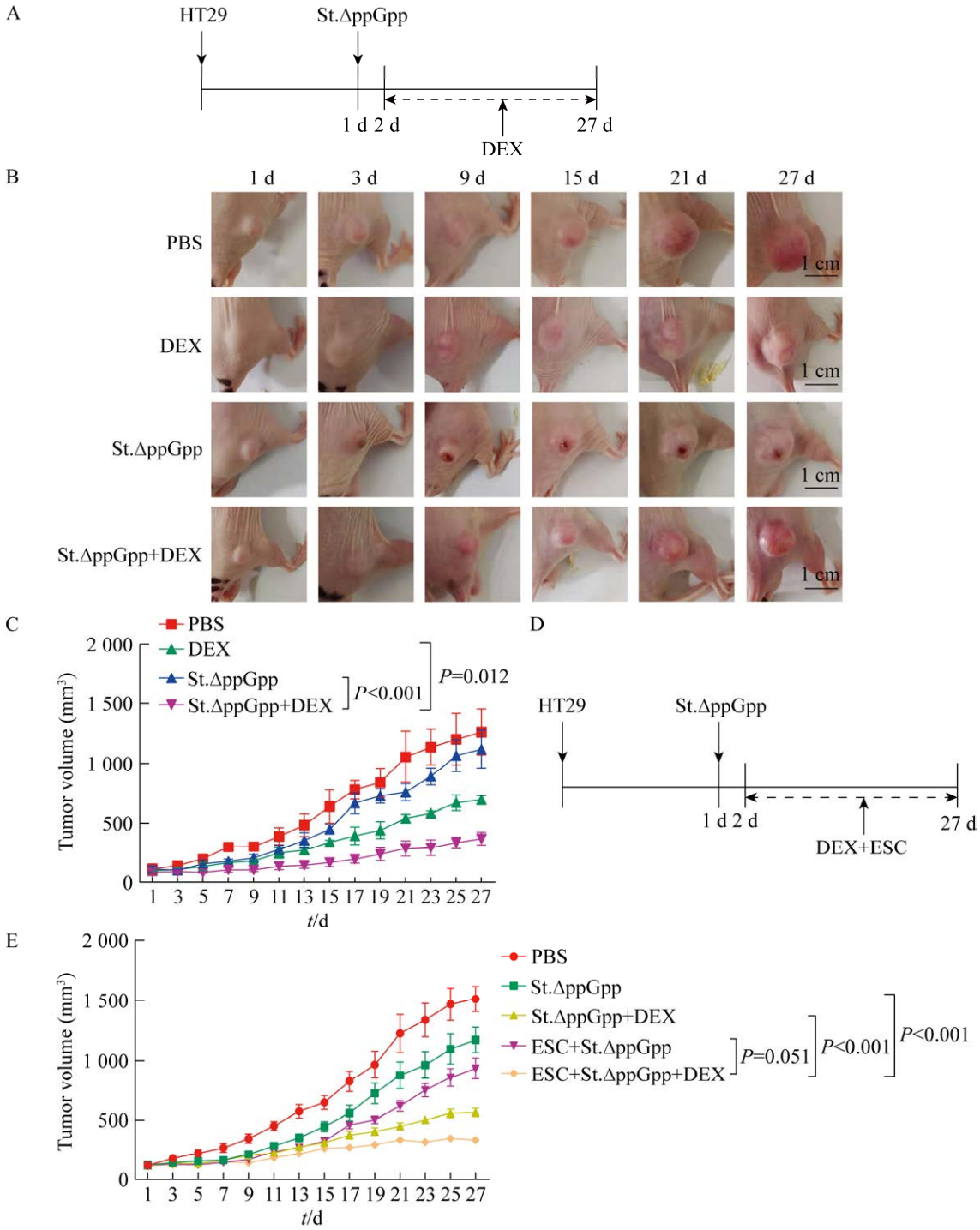

Figure 6 The St.ΔppGpp and dexamethasone combination induces tumor regression in HT29-tumor-bearing nude mice. A: Schematic diagram showing the treatment timeline used for the mouse experiments. B: Images of tumors from representative mice from each group. C: Graph depicting changes in HT29 tumor size (n=6). D: Schematic diagram showing the treatment timeline used for the mouse experiments. E: Graph depicting changes in HT29 tumor size (n=6). P≤0.001 indicates significant difference versus the PBS and St.ΔDppGpp groups. 0.001<P≤0.01 values with different uppercase letters are significantly different versus the PBS and St.ΔppGpp groups.

anti-tumor effects by inhibiting tumor angiogenesis[10-11]. The flow cytometry results generated in the present study showed that dexamethasone had no effect on $CD4^+$ and $CD8^+$ T cells when used in combination with St.ΔppGpp, and instead increased M1 macrophage and a decreased neutrophil and M2 macrophage infiltration into tumors. The anti-inflammatory role of dexamethasone makes it challenging to provide an explanation for this finding.

Westphal et al. reported that using antibody-mediated systemic inhibition of neutrophil recruitment improved the anti-tumor effect of *Salmonella*[28]. The duration of *Salmonella* retention within tumors has an important impact on tumor apoptosis, immune cell infiltration, and anti-tumor effects. Previous studies have shown that natural products such as chlorogenic acid[24] and lovastatin[25] significantly inhibited tumor growth by prolonging the duration of tumor colonization by *Salmonella*. The data demonstrated that dexamethasone effectively suppressed the recruitment of neutrophils to the injury site in a zebrafish model, which was further validated by flow cytometry and fluorescence immunostaining in tumor-bearing mice treated with St.ΔppGpp. The qRT-PCR results also indicated that G-CSF and GM-CSF production was significantly lower in the tumors of mice treated with St.ΔppGpp and dexamethasone than in those treated with St.ΔppGpp alone; this was associated with the dexamethasone-mediated inhibition of neutrophil recruitment into the tumor. Therefore, it was believed that, by inhibiting neutrophil recruitment, dexamethasone prolongs the retention of St.ΔppGpp within tumors and improves the anti-tumor efficacy of this BCI.

Tumor-associated macrophages (TAM) are divided into the M1 and M2 types: M1 macrophages suppress tumor growth by phagocytosis and cytotoxicity, while M2 macrophages promote tumor cell proliferation and tissue invasion through increased angiogenesis[43]. These two types of TAMs can be polarized and converted into one another. A study has shown that attenuated St.ΔppGpp carrying flagellin protein B (FlaB) can polarize M2 to M1 macrophages *via* Toll-like receptor (TLR) 4 and 5 signaling pathways and therefore enhance the anti-tumor activity of *Salmonella*[7]. The utilization of flow cytometry and immunofluorescence staining revealed a significant increase in M1 macrophage infiltration and a decrease in M2 macrophage infiltration within the tumors of St.ΔppGpp-treated mice upon administration of dexamethasone. This finding was supported by another study, which showed that dexamethasone influenced the polarization of TAMs from the pro-tumor M2 to the anti-tumor M1 type[44]. Although the polarization of macrophages was not examined in the present study, we did confirm a direct correlation between the increase in infiltration of M1 macrophages and the synergistic anti-tumor effect observed with the combination of St.ΔppGpp and dexamethasone. The increase in M1 macrophage infiltration can also explain the high expression of pro-inflammatory cytokines (e.g., IL-1β) in the tumors of mice treated with St.ΔppGpp and dexamethasone. Other literature reports also indicated that the IL-1β and TNF-α in tumor infected with therapeutic bacteria were mainly secreted by dendritic cells and macrophages and were closely related to the anti-tumor effect of BCI. Moreover, the restoration of IL-1β and TNF-α levels to pre-treatment levels was associated with tumor recurrence[40]. Therefore, it was posited that the observed elevation in IL-1β levels in this study can be attributed directly to the infiltration of specific immune cells into the tumor.

To eliminate the influence of T cells on tumor therapy, the utilization of thymectomized nude mice for establishing a tumor model facilitates a more comprehensive investigation into the role played by other types of immune cells. Previous research has demonstrated a close association between AKT phosphorylation and neutrophil migration as well as recruitment[24-25]. In the HT29 nude mouse model, the combination of dexamethasone and esculetin (an AKT inhibitor)

can significantly improved the anti-tumor efficacy of St.ΔppGpp. This result also indirectly demonstrated that the inhibition of neutrophil recruitment to the tumor and the activation of M1 macrophages significantly increased the anti-tumor effect of St.ΔppGpp therapy. Further studies will be needed to explore whether there is a connection between the inhibition of neutrophil recruitment and the activation of M1 macrophages in the TME following St.ΔppGpp treatment.

Overall, the effect of dexamethasone on *Salmonella*-mediated BCI can be summarized as follows: (1) it exerts anti-tumor effects by inhibiting angiogenesis; (2) it inhibits neutrophil recruitment, thereby promoting tumor colonization by *Salmonella*; and (3) it directly or indirectly activates M1 macrophages in the TME. Our findings suggest that the combination of dexamethasone and attenuated *Salmonella* has significant implications for the treatment of cancer patients who lack tumor-infiltrating T cells or are unable to use ICB agents, such as those targeting PD-1 or PD-L1.

References

[1] CIARDIELLO D, VITIELLO PP, CARDONE C, MARTINI G, TROIANI T, MARTINELLI E, CIARDIELLO F. Immunotherapy of colorectal cancer: challenges for therapeutic efficacy[J]. Cancer Treatment Reviews, 2019, 76: 22-32.

[2] PAYANDEH Z, KHALILI S, SOMI MH, MARD-SOLTANI M, BAGHBANZADEH A, HAJIASGHARZADEH K, SAMADI N, BARADARAN B. PD-1/PD-L1-dependent immune response in colorectal cancer[J]. Journal of Cellular Physiology, 2020, 235(7/8): 5461-5475.

[3] BOUSSIOTIS VA. Molecular and biochemical aspects of the PD-1 checkpoint pathway[J]. The New England Journal of Medicine, 2016, 375(18): 1767-1778.

[4] DELANOY N, MICHOT JM, COMONT T, KRAMKIMEL N, LAZAROVICI J, DUPONT R, CHAMPIAT S, CHAHINE C, ROBERT C, HERBAUX C, BESSE B, GUILLEMIN A, MATEUS C, PAUTIER P, SAÏAG P, MADONNA E, MAEREVOET M, BOUT JC, LEDUC C, BISCAY P, et al. Haematological immune-related adverse events induced by anti-PD-1 or anti-PD-L1 immunotherapy: a descriptive observational study[J]. The Lancet Haematology, 2019, 6(1): e48-e57.

[5] WANG XD, YANG XH, ZHANG C, WANG Y, CHENG TY, DUAN LQ, TONG Z, TAN SG, ZHANG HJ, SAW PE, GU YM, WANG JH, ZHANG YB, SHANG LN, LIU YJ, JIANG SY, YAN BX, LI R, YANG Y, YU J, et al. Tumor cell-intrinsic PD-1 receptor is a tumor suppressor and mediates resistance to PD-1 blockade therapy[J]. Proceedings of the National Academy of Sciences of the United States of America, 2020, 117(12): 6640-6650.

[6] JANOWITZ T, KLEEMAN S, VONDERHEIDE RH. Reconsidering dexamethasone for antiemesis when combining chemotherapy and immunotherapy[J]. The Oncologist, 2021, 26(4): 269-273.

[7] ROILA F, MOLASSIOTIS A, HERRSTEDT J, AAPRO M, GRALLA RJ, BRUERA E, CLARK-SNOW RA, DUPUIS LL, EINHORN LH, FEYER P, HESKETH PJ, JORDAN K, OLVER I, RAPOPORT BL, ROSCOE J, RUHLMANN CH, WALSH D, WARR D, van der WETERING M.2016 MASCC and ESMO guideline update for the prevention of chemotherapy- and radiotherapy-induced nausea and vomiting and of nausea and vomiting in advanced cancer patients[J]. Annals of Oncology: Official Journal of the European Society for Medical Oncology, 2016, 27(suppl 5): v119-v133.

[8] HESKETH PJ, KRIS MG, BASCH E, BOHLKE K, BARBOUR SY, CLARK-SNOW RA, DANSO MA, DENNIS K, DUPUIS LL, DUSETZINA SB, ENG C, FEYER PC, JORDAN K, NOONAN K, SPARACIO D, LYMAN GH. Antiemetics: ASCO guideline update[J]. Journal of Clinical Oncology, 2020, 38(24): 2782-2797.

[9] SCHMID P, ADAMS S, RUGO HS, SCHNEEWEISS A, BARRIOS CH, IWATA H, DIÉRAS V, HEGG R, IM SA, WRIGHT GS, HENSCHEL V, MOLINERO L, CHUI SY, FUNKE R, HUSAIN A, WINER EP, LOI S, EMENS LA, INVESTIGATORS IT. Atezolizumab and nab-paclitaxel in advanced triple-negative breast cancer[J]. The New England Journal of Medicine, 2018, 379(22): 2108-2121.

[10] SUN NB, JI HJ, WANG W, ZHU Q, CAO M, ZANG Q. Inhibitory effect of dexamethasone on residual Lewis lung cancer cells in mice following palliative surgery[J]. Oncology Letters, 2017, 13(1): 356-362.

[11] GENG Y, WANG J, JING H, WANG HW, BAO YX.

Inhibitory effect of dexamethasone on Lewis mice lung cancer cells[J]. Genetics and Molecular Research: GMR, 2014, 13(3): 6827-6836.

[12] GUO YX, CHEN Y, LIU XQ, MIN JJ, TAN WZ, ZHENG JH. Targeted cancer immunotherapy with genetically engineered oncolytic *Salmonella typhimurium*[J]. Cancer Letters, 2020, 469: 102-110.

[13] ZHOU SB, GRAVEKAMP C, BERMUDES D, LIU K. Tumour-targeting bacteria engineered to fight cancer[J]. Nature Reviews Cancer, 2018, 18: 727-743.

[14] TAN WZ, DUONG MTQ, ZUO CH, QIN YS, ZHANG Y, GUO YX, HONG Y, ZHENG JH, MIN JJ. Targeting of pancreatic cancer cells and stromal cells using engineered oncolytic *Salmonella typhimurium*[J]. Molecular Therapy, 2022, 30(2): 662-671.

[15] CANALE FP, BASSO C, ANTONINI G, PEROTTI M, LI N, SOKOLOVSKA A, NEUMANN J, JAMES MJ, GEIGER S, JIN WJ, THEURILLAT JP, WEST KA, LEVENTHAL DS, LORA JM, SALLUSTO F, GEIGER R. Metabolic modulation of tumours with engineered bacteria for immunotherapy[J]. Nature, 2021, 598: 662-666.

[16] CHEN Y, LIU XQ, GUO YX, WANG JC, ZHANG DX, MEI Y, SHI JF, TAN WZ, ZHENG JH. Genetically engineered oncolytic bacteria as drug delivery systems for targeted cancer theranostics[J]. Acta Biomaterialia, 2021, 124: 72-87.

[17] GRILLE S, MORENO M, BASCUAS T, MARQUÉS JM, MUÑOZ N, LENS D, CHABALGOITY JA. *Salmonella enterica* serovar Typhimurium immunotherapy for B-cell lymphoma induces broad anti-tumour immunity with therapeutic effect[J]. Immunology, 2014, 143(3): 428-437.

[18] BARAK Y, SCHREIBER F, THORNE SH, CONTAG CH, DEBEER D, MATIN A. Role of nitric oxide in *Salmonella typhimurium*-mediated cancer cell killing[J]. BMC Cancer, 2010, 10: 146.

[19] HIROSHIMA Y, ZHAO M, MAAWY A, ZHANG Y, KATZ MHG, FLEMING JB, UEHARA F, MIWA S, YANO S, MOMIYAMA M, SUETSUGU A, CHISHIMA T, TANAKA K, BOUVET M, ENDO I, HOFFMAN RM. Efficacy of *Salmonella typhimurium* A1-R versus chemotherapy on a pancreatic cancer patient-derived orthotopic xenograft (PDOX)[J]. Journal of Cellular Biochemistry, 2014, 115(7): 1254-1261.

[20] IGARASHI K, KAWAGUCHI K, KIYUNA T, MIYAKE K, MIYAKE M, LI SK, HAN QH, TAN YY, ZHAO M, LI YF, NELSON SD, DRY SM, SINGH AS, ELLIOTT IA, RUSSELL TA, ECKARDT MA, YAMAMOTO N, HAYASHI K, KIMURA H, MIWA S, et al. Tumor-targeting *Salmonella typhimurium* A1-R combined with recombinant methioninase and cisplatinum eradicates an osteosarcoma cisplatinum-resistant lung metastasis in a patient-derived orthotopic xenograft (PDOX) mouse model: decoy, trap and kill chemotherapy moves toward the clinic[J]. Cell Cycle, 2018, 17(6): 801-809.

[21] LIANG K, LIU Q, LI P, LUO HY, WANG HJ, KONG QK. Genetically engineered *Salmonella* Typhimurium: recent advances in cancer therapy[J]. Cancer Letters, 2019, 448: 168-181.

[22] ZHENG JH, NGUYEN VH, JIANG SN, PARK SH, TAN WZ, HONG SH, SHIN MG, CHUNG IJ, HONG Y, BOM HS, CHOY HE, LEE SE, RHEE JH, MIN JJ. Two-step enhanced cancer immunotherapy with engineered *Salmonella typhimurium* secreting heterologous flagellin[J]. Science Translational Medicine, 2017, 9(376): eaak9537.

[23] NA HS, KIM HJ, LEE HC, HONG Y, RHEE JH, CHOY HE. Immune response induced by *Salmonella typhimurium* defective in ppGpp synthesis[J]. Vaccine, 2006, 24(12): 2027-2034.

[24] ZHANG WH, PIAO LH, LIU XD. Chlorogenic acid suppresses neutrophil recruitment to tumors by inducing apoptosis and reverse migration[J]. Journal of Functional Foods, 2020, 75: 104216.

[25] ZHANG H, SANG ST, XU HM, PIAO LH, LIU XD. Lovastatin suppresses bacterial therapy-induced neutrophil recruitment to the tumor by promoting neutrophil apoptosis[J]. Journal of Functional Foods, 2021, 86: 104693.

[26] LIU XD, JIANG SN, PIAO LH, YUAN F. Radiotherapy combined with an engineered *Salmonella typhimurium* inhibits tumor growth in a mouse model of colon cancer[J]. Experimental Animals, 2016, 65(4): 413-418.

[27] IGARASHI K, KAWAGUCHI K, KIYUNA T, MIYAKE K, MIYAKE M, SINGH AS, ECKARDT MA, NELSON SD, RUSSELL TA, DRY SM, LI YF, YAMAMOTO N, HAYASHI K, KIMURA H, MIWA S, TSUCHIYA H, SINGH SR, EILBER FC, HOFFMAN RM. Tumor-targeting *Salmonella typhimurium* A1-R is a highly effective general therapeutic for undifferentiated soft tissue sarcoma patient-derived orthotopic xenograft nude-mouse

models[J]. Biochemical and Biophysical Research Communications, 2018, 497(4): 1055-1061.

[28] WESTPHAL K, LESCHNER S, JABLONSKA J, LOESSNER H, WEISS S. Containment of tumor-colonizing bacteria by host neutrophils[J]. Cancer Research, 2008, 68(8): 2952-2960.

[29] KOCIJANCIC D, LESCHNER S, FELGNER S, KOMOLL RM, FRAHM M, PAWAR V, WEISS S. Therapeutic benefit of *Salmonella* attributed to LPS and TNF-α is exhaustible and dictated by tumor susceptibility[J]. Oncotarget, 2017, 8(22): 36492-36508.

[30] LEE CH, HSIEH JL, WU CL, HSU PY, SHIAU AL. T cell augments the antitumor activity of tumor-targeting *Salmonella*[J]. Applied Microbiology and Biotechnology, 2011, 90(4): 1381-1388.

[31] KAIMALA S, MOHAMED YA, NADER N, ISSAC J, ELKORD E, CHOUAIB S, FERNANDEZ-CABEZUDO MJ, AL-RAMADI BK. *Salmonella*-mediated tumor regression involves targeting of tumor myeloid suppressor cells causing a shift to M1-like phenotype and reduction in suppressive capacity[J]. Cancer Immunology, Immunotherapy: CII, 2014, 63(6): 587-599.

[32] SACCHERI F, POZZI C, AVOGADRI F, BAROZZI S, FARETTA M, FUSI PL, RESCIGNO M. Bacteria-induced gap junctions in tumors favor antigen cross-presentation and antitumor immunity[J]. Science Translational Medicine, 2010, 2(44): e3000739.

[33] KANG SR, NGUYEN DH, YOO SW, MIN JJ. Bacteria and bacterial derivatives as delivery carriers for immunotherapy[J]. Advanced Drug Delivery Reviews, 2022, 181: 114085.

[34] STARNES TW, HUTTENLOCHER A. Neutrophil reverse migration becomes transparent with zebrafish[J]. Advances in Hematology, 2012, 2012: 398640.

[35] JONES HR, ROBB CT, PERRETTI M, ROSSI AG. The role of neutrophils in inflammation resolution[J]. Seminars in Immunology, 2016, 28(2): 137-145.

[36] HENRY KM, LOYNES CA, WHYTE MKB, RENSHAW SA. Zebrafish as a model for the study of neutrophil biology[J]. Journal of Leukocyte Biology, 2013, 94(4): 633-642.

[37] GARRIDO-MARTIN EM, MELLOWS TWP, CLARKE J, GANESAN AP, WOOD O, CAZALY A, SEUMOIS G, CHEE SJ, ALZETANI A, KING EV, HEDRICK CC, THOMAS G, FRIEDMANN PS, OTTENSMEIER CH, VIJAYANAND P, SANCHEZ-ELSNER T. $M1^{hot}$ tumor-associated macrophages boost tissue-resident memory T cells infiltration and survival in human lung cancer[J]. Journal for Immunotherapy of Cancer, 2020, 8(2): e000778.

[38] PAN YY, YU YD, WANG XJ, ZHANG T. Tumor-associated macrophages in tumor immunity[J]. Frontiers in Immunology, 2020, 11: 583084.

[39] WANG YC, WANG X, YU JJ, MA FY, LI Z, ZHOU Y, ZENG S, MA XY, LI YR, NEAL A, HUANG J, TO A, CLARKE N, MEMARZADEH S, PELLEGRINI M, YANG LL. Targeting monoamine oxidase A-regulated tumor-associated macrophage polarization for cancer immunotherapy[J]. Nature Communications, 2021, 12(1): 3530.

[40] KIM JE, PHAN TX, NGUYEN VH, DINH-VU HV, ZHENG JH, YUN MS, PARK SG, HONG Y, CHOY HE, SZARDENINGS M, HWANG W, PARK JA, PARK S, IM SH, MIN JJ. *Salmonella typhimurium* suppresses tumor growth *via* the pro-inflammatory cytokine interleukin-1β[J]. Theranostics, 2015, 5(12): 1328-1342.

[41] GONG JH, ZHENG YB, ZHANG MR, WANG YX, YANG SQ, WANG RH, MIAO QF, LIU XJ, ZHEN YS. Dexamethasone enhances the antitumor efficacy of Gemcitabine by glucocorticoid receptor signaling[J]. Cancer Biology & Therapy, 2020, 21(4): 332-343.

[42] LEE CH, HSIEH JL, WU CL, HSU HC, SHIAU AL. B cells are required for tumor-targeting *Salmonella* in host[J]. Applied Microbiology and Biotechnology, 2011, 92(6): 1251-1260.

[43] JACKAMAN C, TOMAY F, DUONG L, ABDOL RAZAK NB, PIXLEY FJ, METHAROM P, NELSON DJ. Aging and cancer: the role of macrophages and neutrophils[J]. Ageing Research Reviews, 2017, 36: 105-116.

[44] XU L, XIA H, NI DS, HU YX, LIU JN, QIN Y, ZHOU Q, YI QY, XIE YJ. High-dose dexamethasone manipulates the tumor microenvironment and internal metabolic pathways in anti-tumor progression[J]. International Journal of Molecular Sciences, 2020, 21(5): 1846.