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Engineering *Proteus mirabilis* improves antitumor efficacy via enhancing cytotoxic T cell responses

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Cancer immunotherapy based on bioengineering of bacteria can effectively increase anticancer immune responses. However, few studies have investigated the antitumor potential of engineering *Proteus mirabilis*. Here, we genetically engineered *P. mirabilis* to overexpress *Vibrio vulnificus* flagellin B (FlaB) protein in a murine CT26 tumor model. We found that a large number of FlaB-expressing *P. mirabilis* colonized tumor tissues, enhanced T cell infiltration and secretion of cytokines and cytotoxic proteins in tumors, and significantly restrained tumor growth. Our results also showed that programmed death ligand 1 (PD-L1) expression in tumor-infiltrating immune cells was elevated after treatment with FlaB-expressing *P. mirabilis*. In addition, combination therapy with FlaB-expressing *P. mirabilis* and PD-L1 blockade synergistically improved antitumor efficacy by enhancing infiltration of $CD8^+$ cells. Furthermore, serum liver biochemical indices of mice increased in the short term in both the *P. mirabilis* and the FlaB-expressing *P. mirabilis* treatment groups but gradually recovered in the later stage of treatment so that FlaB protein expression did not increase the toxicity of *P. mirabilis in vivo*. Taken together, our results suggest that *P. mirabilis* could serve as an engineered bacterium for bacterium-based cancer immunotherapy.

INTRODUCTION

Colorectal cancer (CRC) is a malignant solid tumor with increasing incidence and mortality rates.[1] Standard therapies for CRC are surgery, chemotherapy, and radiotherapy; however, chemotherapy and radiotherapy in particular have adverse effects because of their nonspecific cytotoxicity to normal cells.[2] Cancer immunotherapy is a new alternative that can trace its origins to 1866, when marked tumor inhibition was observed in cancer patients with *Streptococcus pyogenes* infection.[2] Later, William Coley, known as the "Father of Immunotherapy," developed Coley's toxins immunotherapy; however, this was opposed by health practitioners because of its non-reproducible outcomes.[3] In recent years, there has been renewed interest in the use of bacteria in cancer immunotherapies, although many problems remain to be solved before such therapies are suitable for clinical applications.

Our preliminary results show that *Proteus mirabilis*, a Gram-positive, extracellular, opportunistic pathogen, can colonize the tumor site and inhibit tumor growth and spontaneous lung metastasis in a mouse model,[4] although wild-type *P. mirabilis* does not show a strong antitumor effect for therapeutic use. However, bacteria can be bioengineered to express antitumor immunomodulators to enable them to exert stronger antitumor effects;[5–7] moreover, tumor-targeting bacteria are perfect versatile platforms for delivery of therapeutic payloads owing to their tumor-targeting ability and enormous gene packaging capacity.[8] The expression of NKp46 and CD11c is increased after *P. mirabilis* treatment, but the antitumor immune responses induced by *P. mirabilis* expressing endogenous flagellin are not strong enough.[4] FlaB, which is not expressed in either swimmer or swarmer *P. mirabilis*,[9,10] is a good anticancer immunotherapy adjuvant that can activate innate immunity, enhance M1 macrophage polarization, and enhance T cell response.[11,12]

Immune checkpoint blockade therapy promotes T cell activation to stimulate antitumor responses and produce sustained cancer inhibition.[13] The most frequently used immune checkpoint inhibitors include programmed cell death protein 1 (PD-1) and programmed death ligand 1 (PD-L1), which have proven extremely effective in patients with metastatic melanoma, non-small-cell lung cancer, and bladder cancer.[14–16] However, anti-PD-1/PD-L1 therapy does not seem to be beneficial for most CRC patients.[2] Therefore, improving the effectiveness of PD-1/PD-L1 blockade remains a challenge. Notably, several studies have indicated that bacterial therapy combined with PD-L1 antibodies can improve the effectiveness of immune checkpoint therapy.[17,18]

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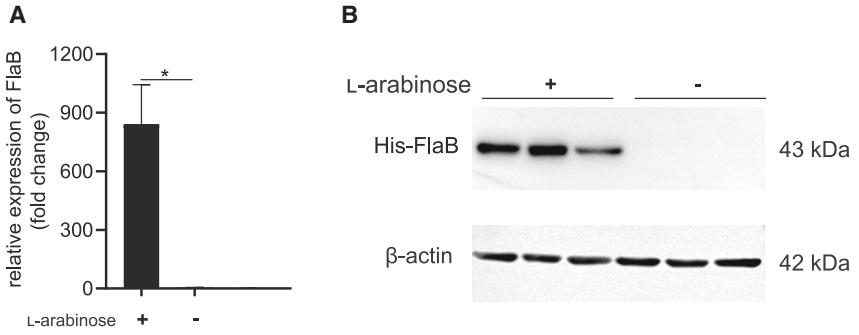

Figure 1. Introduction of the *FlaB* gene into *P. mirabilis* and expression in the murine CRC model

(A) Total RNA (n = 3) was isolated from tumor tissues infected with FlaB-expressing *P. mirabilis* (*P. mirabilis* FlaB) with (+) or without (–) L-arabinose induction (24 h 3 days post induction). FlaB mRNA expression was quantified by RT-qPCR and normalized to the 16S housekeeping gene. (B) Expressions levels of FlaB protein were detected by western blot analysis. Samples were prepared with (+) or without (–) 0.2% L-arabinose.

The purpose of the present study was to enhance the antitumor effect of *P. mirabilis* by expressing the FlaB protein and, further, to show that the therapeutic efficacy of immune checkpoint inhibitors could be improved by combining engineered *P. mirabilis* with PD-L1 blockade in CRC treatment. The results of this study may contribute to improving the effectiveness of both bacterium-based cancer immunotherapy and immune checkpoint therapy for CRC.

RESULTS

Engineering *P. mirabilis* to express FlaB

To confirm FlaB expression in tumor tissues of mice after intravenous injection of engineered *P. mirabilis*, we performed quantitative reverse-transcription PCR (RT-qPCR) and western blot analysis. The results demonstrated that the mRNA expression of FlaB in tumor tissues of mice injected with L-arabinose was much higher than that of those without L-arabinose induction (Figure 1A). In addition, FlaB protein expression in tumor tissues of mice with L-arabinose induction was higher than that in the absence of L-arabinose (Figure 1B). Together, these results showed that the FlaB gene had been successfully introduced into *P. mirabilis* and was expressed in our murine CRC model.

Engineered *P. mirabilis* distributes to tumor lesions

To investigate the biodistribution of *P. mirabilis* transferred with or without the FlaB plasmid in mice, we counted the numbers of live *P. mirabilis* in the tumors and viscera of tumor-bearing mice after they had been intravenously injected with bacteria. We found no statistically significant difference in biodistribution of *P. mirabilis* between the FlaB-expressing *P. mirabilis* injection and *P. mirabilis* injection groups at different time points, and large numbers of engineered bacteria remained in tumor tissue for more than 5 days (Figure 2). Together, these results show that engineered *P. mirabilis* retains its ability to target tumors in a murine cancer model.

Anti-tumor effects of FlaB-expressing *P. mirabilis*

To observe the antitumor ability of engineered *P. mirabilis*, *P. mirabilis* or FlaB-expressing *P. mirabilis* was intravenously injected into tumor-bearing mice, and FlaB expression was induced with L-arabinose injection (Figure 3A). The tumor volumes of mice in the FlaB-expressing *P. mirabilis* treatment group were significantly smaller than those in the control group and the *P. mirabilis* treatment group (Figures 3B and 3C). The survival rate of mice was 16.6% higher in the FlaB-expressing *P. mirabilis* injection group compared with the *P. mirabilis* treatment group (Figure 3D). Together, these results indicate that FlaB overexpression enhances the capacity of *P. mirabilis* to fight against cancer.

Effects of FlaB-expressing *P. mirabilis* on tumor-infiltrating T cells

To explore the effects of FlaB-expressing *P. mirabilis* on tumor-infiltrating T cells, we collected tumor tissues from mice 24 h after L-arabinose injection. Immunohistochemistry (IHC) staining showed that there was more infiltration of $CD8^+$ and $CD4^+$ T cells in the FlaB-expressing *P. mirabilis* treatment group compared with the control and *P. mirabilis* treatment groups (Figures 4A and 4B). Flow cytometry results confirmed that there were higher numbers of $CD3^+$ $CD8^+$ and $CD3^+$ $CD4^+$ T cells in tumor tissues of mice in the FlaB-expressing *P. mirabilis* treatment group compared with the *P. mirabilis* injection and control groups (Figure 4C). Subsequently, we detected the cytokines interferon γ (IFN-γ) and tumor necrosis factor alpha (TNF-α) and cytotoxic granule-associated protein granzyme B and perforin, which are primarily produced by cytotoxic $CD8^+$ T effector cells. Enzyme-linked immunosorbent assay (ELISA) results indicated that FlaB-expressing *P. mirabilis* injection induced higher concentrations of TNF-α, IFN-γ, granzyme B, and perforin in the tumor microenvironment (TME) in the murine CT26 tumor model compared with treatment with wild-type *P. mirabilis* (Figure 4D). Together, these results show that FlaB-expressing *P. mirabilis* treatment can increase cytotoxic T cell infiltration and cytotoxic granule-associated protein secretion in the TME.

FlaB-expressing *P. mirabilis* induces higher expression of PD-L1

To elucidate the effects of the engineered *P. mirabilis* on PD-L1 expression in tumor cells and immune cells in the TME, we conducted flow cytometry and IHC staining analyses. The flow cytometry results showed that the expression level of PD-L1 in tumor tissues was higher in the FlaB-expressing *P. mirabilis* treatment group than in the control or *P. mirabilis* group (Figure 5A). Further, IHC staining indicated that expression of PD-L1 on immune cells in the FlaB-expressing *P. mirabilis* treatment group was significantly increased compared with that in the *P. mirabilis* treatment and the control groups (Figures 5B and 5C). However, the percentage of $PD\text{-}L1^+$ tumor cells

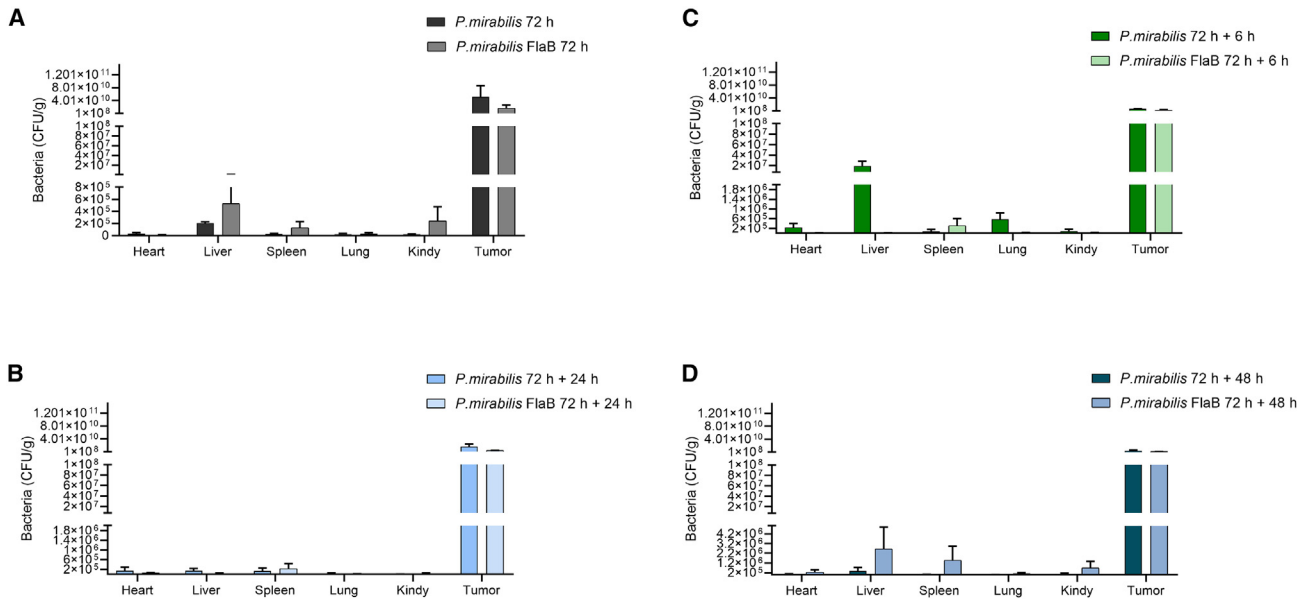

Figure 2. *P. mirabilis* FlaB retains its ability to target tumors

Tumor-bearing mice were injected intravenously with *P. mirabilis* and *P. mirabilis* FlaB; then, FlaB expression was induced 3 days after bacterial treatment via intraperitoneal injection of L-arabinose. Numbers of colony-forming units (CFUs) of bacteria in tumors and organs (heart, liver, spleen, lungs, and kidneys) were calculated at 0 h (A), 6 h (B), 24 h (C), and 48 h (D) with (+) or without (−) L-arabinose induction. Data are presented as the mean ± SEM. *$p < 0.05$, **$p < 0.01$.

was not statistically significantly different among the three groups (Figures 5B and 5C). Together, these results show that FlaB-expressing *P. mirabilis* increases expression of PD-L1 on tumor-infiltrating immune cells in a murine tumor model.

Combination of FlaB-expressing *P. mirabilis* and PD-L1 blockade enhances tumor regression

Expression levels of PD-L1 in tumor-infiltrating immune cells significantly increased after treatment with FlaB-expressing *P. mirabilis*; thus, we hypothesized that co-treatment with FlaB-expressing *P. mirabilis* and PD-L1 blockade might result in a synergistic antitumor effect. Hence, FlaB-expressing *P. mirabilis* therapy was combined with PD-L1 blockade in our murine CT26 tumor model (Figure 6A). Tumor volumes of mice were statistically significantly smaller in the co-treatment group compared with the groups treated with FlaB-expressing *P. mirabilis* alone or PD-L1 blockade only (Figures 6B and 6C). Further, IHC staining showed that PD-L1 expression in the mouse tumors was significantly blocked in the co-treatment group compared with the FlaB-expressing *P. mirabilis*-alone treatment group (Figure 6D). Moreover, we found that the combination treatment significantly strengthened the infiltration of $CD8^+$ T cells in the TME compared with other treatments (Figure 6D). Together, these results suggest that co-treatment with FlaB-expressing *P. mirabilis* and PD-L1 blockade confers synergistic antitumor activity.

Safety of FlaB-expressing *P. mirabilis* treatment

To evaluate the safety of FlaB-expressing *P. mirabilis* therapy, we examined liver and kidney histopathology and serum biochemical indices of liver injury. The livers and kidneys of mice treated with *P. mirabilis* and engineered *P. mirabilis* showed no significant histological changes compared with those in the control group (Figure S1). Concentrations of serum alanine transaminase (ALT) and aspartate transaminase (AST) in mice increased beyond the normal range 24 h after L-arabinose induction in both the *P. mirabilis* and engineered *P. mirabilis* treatment groups (Table 1). However, they had returned to normal levels by the end of treatment, and there was no statistically significant difference among the three groups (Table 2). Serum alkaline phosphatase (ALP) expression was not statistically significantly different among the three groups (Tables 1 and 2). Together, those results indicate that FlaB-expressing *P. mirabilis* does not increase the *in vivo* toxicity of *P. mirabilis*.

DISCUSSION

Tumor-colonizing bacteria can attract T lymphoma cells to the tumor site and effectively motivate innate and adaptive immune systems to boost the immunogenicity of "cold tumors," which are characterized by a lack of cytotoxic T cell infiltration.[19] In the present study, we genetically engineered *P. mirabilis* to overexpress FlaB protein. We found that a large number of FlaB-expressing *P. mirabilis* colonized in tumors, enhanced $CD8^+$ T cell infiltration and secretion of cytotoxic proteins in the TME, and significantly inhibited tumor growth in a mouse tumor model. Our results also showed that PD-L1 expression in tumor-infiltrating immune cells was enhanced after treatment with FlaB-expressing *P. mirabilis*. In addition, combination therapy with FlaB-expressing *P. mirabilis* and PD-L1 blockade improved therapeutic efficacy by enhancing the infiltration of $CD8^+$ cells. Finally, FlaB-expressing *P. mirabilis* did not generally increase the *in vivo*

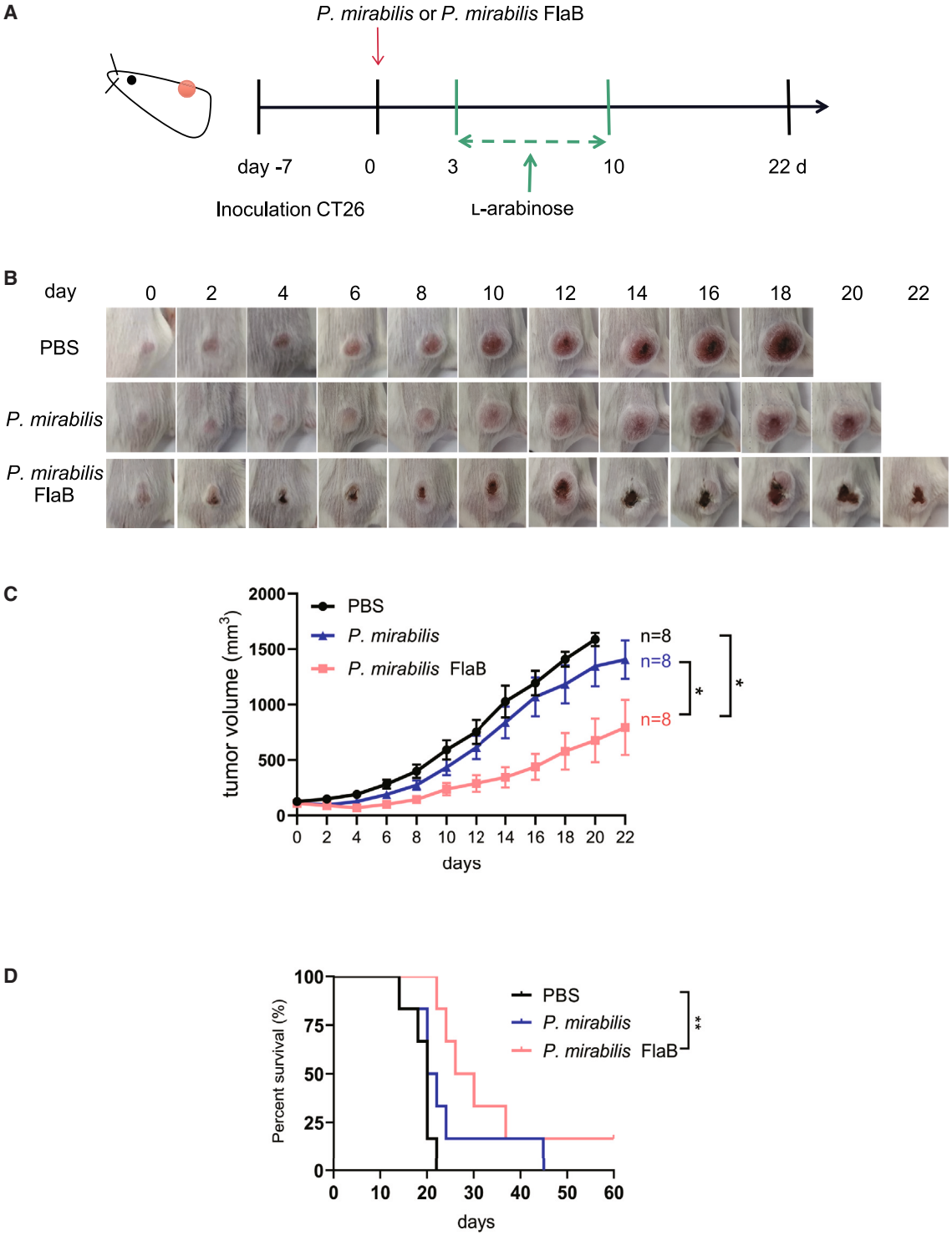

Figure 3. FlaB overexpression enhances the anticancer effect of *P. mirabilis*

(A) Study protocol. BALB/C mice (n = 8 per group) were subcutaneously injected with CT26 cells (1×10^6 cells). When tumor volumes reached about 150 mm^3, mice were divided into three treatment groups: PBS alone, *P. mirabilis*, and *P. mirabilis* FlaB. Mice in the *P. mirabilis* and *P. mirabilis* FlaB groups received intravenous injections of 6×10^7 CFU bacteria, followed by 0.12 g of L-arabinose daily from 3 days after bacterial injection. (B) Images of tumors from representative mice from each group. (C) Tumor volumes in each group were monitored every other day. (D) Kaplan-Meier survival curves for CT26 tumor-bearing mice. *$p < 0.05$, **$p < 0.01$.

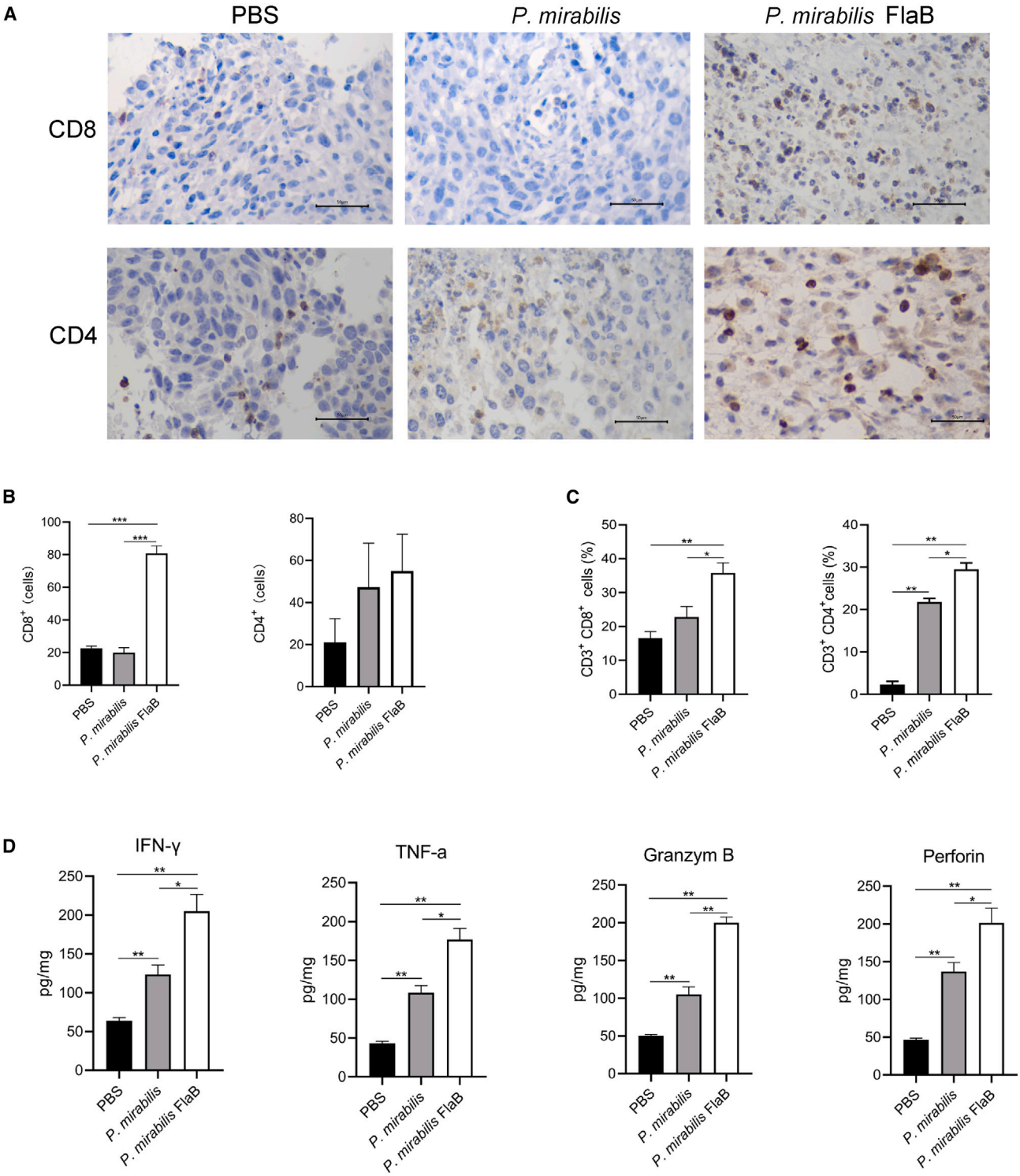

Figure 4. FlaB-expressing *P. mirabilis* treatment can increase cytotoxic T cell infiltration and cytotoxic granule-associated protein secretion in TME

(A) Representative figures showing IHC staining of $CD8^+$ and $CD4^+$ T cells in tumor tissues. Scale bar, 50 μm. (B) Numbers of $CD8^+$ and $CD4^+$ T cells were statistically assessed based on five high-power fields (n = 3). (C) Samples were double-stained with CD3 and CD4 or CD8 antibodies and then analyzed by flow cytometry (n = 3). (D) TNF-α, IFN-γ, granzyme B, and perforin concentrations were measured in tumor suspensions 24 h after L-arabinose induction in the murine CT26 tumor model (n = 3).

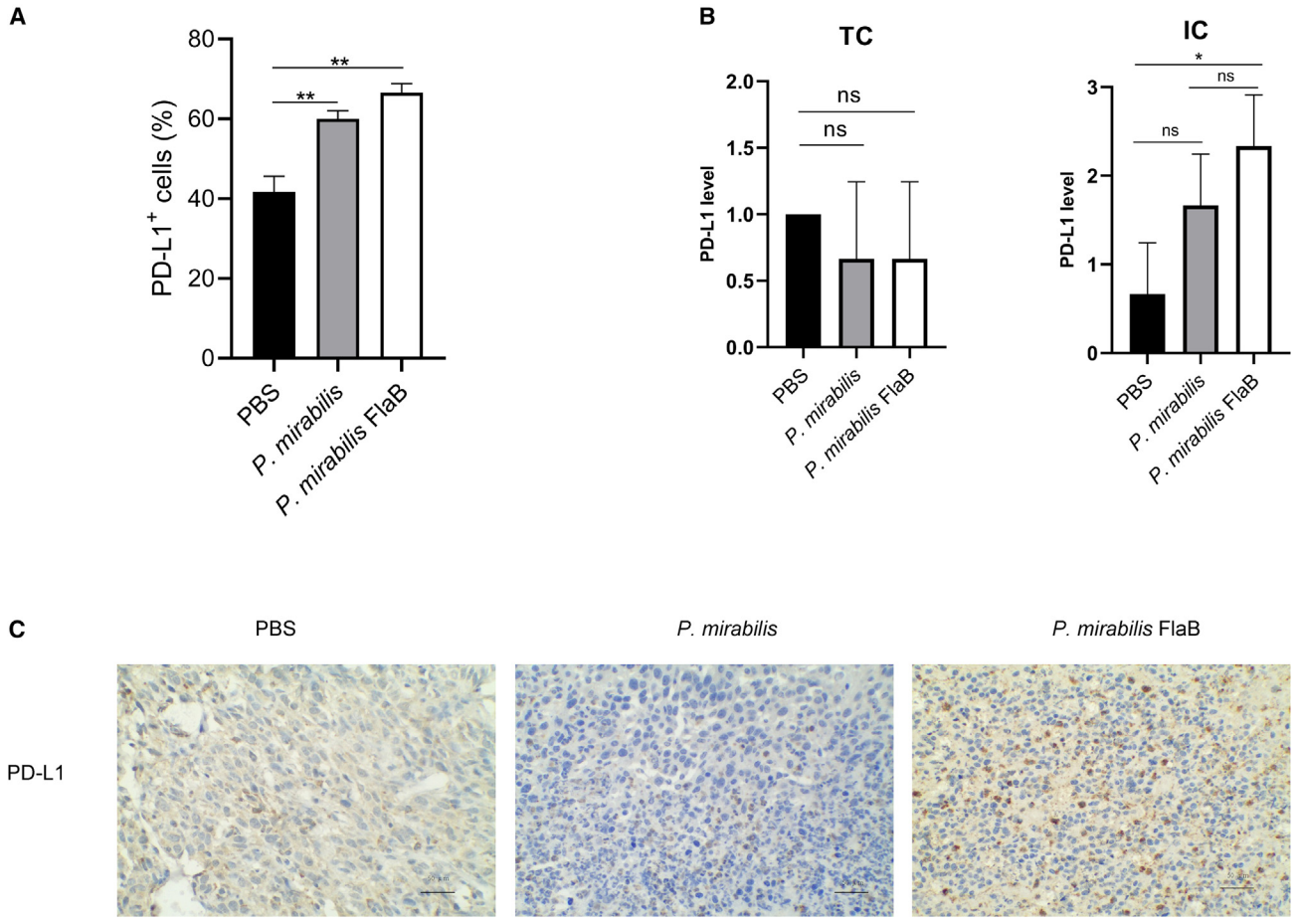

Figure 5. *P. mirabilis* FlaB increases expression of PD-L1 on tumor-infiltrating immune cells in the murine tumor model

(A) Mouse tumors were stained with the PD-L1 antibody and analyzed by flow cytometry 24 h after intraperitoneal injection of with L-arabinose (n = 3). (B) Assessment of expression of PD-L1 in immune cells (ICs) and tumor cells (TCs) from five high-power fields (n = 3). *$p < 0.05$, **$p < 0.01$. (C) Representative IHC results of samples stained with PD-L1; scale bar, 50 μm.

toxicity of *P. mirabilis*. Taken together, our findings may contribute to improving the efficacy of bacterium-based cancer immunotherapy and immune checkpoint therapy for CRC.

Several facultative and obligate anaerobic bacteria, including *Clostridium*, *Bifidobacterium*, *Salmonella*, *Escherichia*, and *Lactobacillus*, can specifically target tumors and inhibit their growth.[20] These bacteria can be induced via genetic engineering techniques to produce therapeutic payloads, including cytotoxic agents, immunomodulators, cytokines, and nanobodies, to increase their antitumor activity.[21] Notably, *Salmonella enterica* Typhimurium has exhibited robust inhibitory effects on tumor growth and metastasis in animal models, leading to the emergence of attenuated *S. enterica* Typhimurium strains. However, despite the safe administration of these *Salmonella* strains, human clinical trials did not show significant tumor-inhibitory effects comparable with those observed in numerous animal experiments.[22] Our work focuses on discovering bacteria with more antitumor activity and evaluating their potential value for entering clinical trials. However, no genetically modified *P. mirabilis* has yet been reported. In the present study, we introduced a plasmid carried FlaB into *P. mirabilis* by electroporation and verified that the FlaB protein was overexpressed. The mRNA and protein expression of FlaB in wild-type *P. mirabilis* were undetectable in our present study. Similarly, a study indicates that several FlaB-specific primers fail to yield a product in *P. mirabilis* by northern blot analysis.[23] We also confirmed that the biodistribution of *P. mirabilis* in tumor-bearing mice was not affected by the introduction of FlaB. However, the defect of our study was that the accumulation of *P. mirabilis* in the gut, which may also be a suitable viscera for the colonization,[24,25] was not explored. There are three potential mechanisms to elucidate the tumor-targeting properties of bacteria: (1) hypoxia, a well-characterized feature of most solid tumors, facilitates the tumor-specific accumulation of anaerobes;[26,27] (2) low immune surveillance;[27] and (3) motile bacteria can move through chemotaxis toward tumor tissue by sensing specific chemicals secreted by the TME.[28] As a result, we hypothesize that *P. mirabilis*

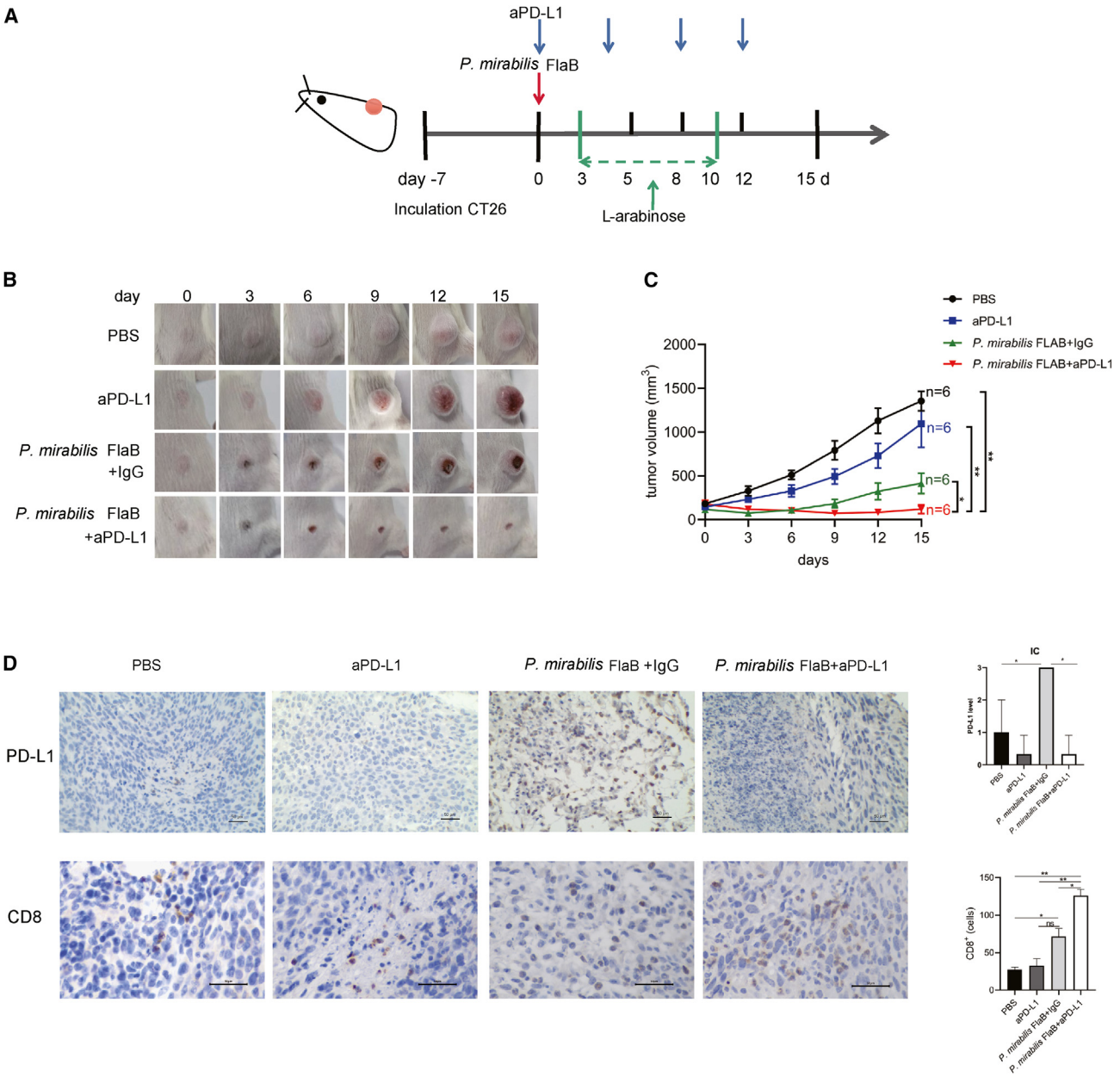

Figure 6. Co-treatment with *P. mirabilis* FlaB and PD-L1 blockade confers synergistic antitumor activity by increasing levels of tumor-infiltrating CD8⁺ T cells

(A) Study protocol. Mice were intravenously injected with *P. mirabilis* FlaB or PBS when the tumor had grown to ~150 mm^3 in the murine CT26 tumor model. The PD-L1 antibody (aPD-L1) or IgG isotype control antibody was intraperitoneally administered on days 0, 4, 8, and 12 after bacterial injection. Three days after bacterial injection, the mice in the *P. mirabilis* FlaB group received daily intraperitoneal injection of L-arabinose for 8 consecutive days. (B) Images of tumors from representative mice from each group (n = 6). (C) Tumor volumes in each group were monitored every other day. (D) Representative plot of IHC staining of CD8 and PD-L1 in tumor tissue 24 h after PD-L1 blockade treatment. Numbers of $CD8^+$ T cells and $PD\text{-}L1^+$ ICs were statistically assessed from five high-power fields. *p < 0.05, **p < 0.01.

preferentially accumulates in tumors rather than normal tissues, such as the gastrointestinal lumen, even though the oxygen concentration in the gastrointestinal lumen is close to 0%.[24,25] Our findings will provide a reference for future genetic modification of *P. mirabilis*.

FlaB is a major functional protein in the adhesion and cytotoxicity of *Vibrio vulnificus*,[29] as well as being an immune adjuvant that enhances T cell responses *in vivo*. FlaB combined with infrared light can synergistically restrain tumor growth by activating antitumor immune responses.[29] The P domain (Pd) vaccine itself cannot induce

Table 1. Serum liver biochemical indices of mice on day 4 after treatment with bacteria

Group	ALT (U/L)	AST (U/L)	ALP (U/L)
PBS	37 ± 5.39[a]	190.73 ± 142.66[a]	51.83 ± 62.83[a]
P. mirabilis	432.6 ± 452.09[b]	276.13 ± 124.99[b]	45.47 ± 40.57[a]
P. mirabilis FlaB	355.57 ± 311.68[b]	350.2 ± 155.18[b]	26.83 ± 36.69[a]

ALT, alanine transaminase; AST, aspartate transaminase; ALP, alkaline phosphatase. Data are presented as the mean ± SEM. Within each column, different superscript letters (a, b) indicate significant differences ($p < 0.05$).

cell-mediated immune responses, but a combination of the Pd vaccine and FlaB has been found to stimulate strong $CD4^+$ IFN-γ^+ and $CD8^+$ IFN-γ^+ T cell responses dependent on Toll-like receptor 5.[30] FlaB also enhances the $CD8^+$ T cell response of epidermal growth factor receptor variant III in a mouse brain glioblastoma model.[31] Cytotoxic $CD8^+$ T effector cells mainly produce IFN-γ and TNF-α and have a powerful capacity to target antigen-bearing cells by releasing perforin and granzyme B.[32–34] According to our observation, there were 50% (4 of 8), 62.5% (5 of 8), and 83.3% (5 of 6) of mice with non-spontaneous ulcerated tumors within 6–24 h after bacterium injection in the *P. mirabilis* group, the FlaB-expressing *P. mirabilis* (*P. mirabilis* FlaB) group, and the *P. mirabilis* FlaB combined with a-PD-L1 group, respectively, suggesting an immune response. The destruction of non-spontaneous necrosis is a hallmark response in colonized tumors that may also be a result of host defense mechanisms against invading bacteria due to indirect tissue destruction by specific immune cells.[35,36] Weibel et al.[37] also describe large areas of necrosis in colonized tumors that are presumably triggered by bacterially activated TNF-α-producing M1 macrophages. Our analysis revealed that FlaB-overexpressing *P. mirabilis* treatment enhanced $CD8^+$ T lymphoma cell infiltration in the tumor tissues of mice compared with treatment with *P. mirabilis* alone. Secretion of IFN-γ, TNF-α, perforin, and granzyme B was also augmented in the TME after *P. mirabilis* FlaB injection. We deduced that *P. mirabilis* FlaB may exert its antitumor effect mainly via enhancing T cell responses. The possible mechanism may be that FlaB enhances $CD8^+$ T cell anti-tumor immune responses via stimulating Toll-like receptors (TLRs) 5.[11,12,30] A recent study reported that $CD4^+$ effector cells could indirectly contribute to antitumor immunity by interacting with $CD11c^+$ major histocompatibility complex (MHC) class II^+ immune cells at invasive margins.[38] In the present study, we also observed an increase in $CD4^+$ cells in the TME after treatment with *P. mirabilis* FlaB; we thus speculated that a small percentage of these cells might be able to eliminate tumors independent of $CD8^+$ T cells. We did not further consider this possibility here, but it will be worth investigating in future research.

Table 2. Serum liver biochemical indices of mice on day 21 after treatment with bacteria

Group	ALT (U/L)	AST (U/L)	ALP (U/L)
PBS	50.03 ± 15.54[a]	279.37 ± 109.09[a]	64.1 ± 70.33[a]
P. mirabilis	45.07 ± 5.6[a]	175.8 ± 144.75[a]	30.1 ± 35.37[a]
P. mirabilis FlaB	33.47 ± 11.46[a]	171.33 ± 24.01[a]	24.33 ± 23.47[a]

ALT, alanine transaminase; AST, aspartate transaminase; ALP, alkaline phosphatase. Data are presented as the mean ± SEM. Within each column, letter a indicates no significant differences ($p > 0.05$).

Following remarkable success in clinical trials, several PD-1 and PD-L1 antibodies have been approved for treating various cancers.[39] PD-1 and the PD-L1 axis are responsible for T cell activation, proliferation, and cytotoxic secretion, enabling escape of antitumor immunity in cancer.[40] Expression of PD-L1 can be detected in lymphocytes, macrophages, dendritic cells, and mast cells as well as in tumor cells and tumor stroma.[39] A study reported that PD-L1 expression is significantly higher in macrophages than in other immune cells and that it is associated with longer survival of patients and may predict treatment outcomes.[41] Another study demonstrated that PD-L1 expressed in both cancer cells and immune cells could functionally modulate cytotoxic T lymphocytes in the TME.[42] For patients with non-inflammatory tumor types, α-PD-1/PD-L1 monotherapy shows little clinical antitumor effect; however, some α-PD-1/PD-L1 combination therapies have shown clinical benefits and improved response rates.[43] Coincidentally, bacterial therapy substantially promotes transformation of non-inflammatory tumors to an inflammatory type; this may be a means of overcoming low immunogenicity-mediated immune responses to α-PD-1/PD-L1 monotherapy. In the present study, we found that *P. mirabilis* FlaB injection increased PD-L1 expression in immune cells in the TME and enhanced the antitumor efficacy of PD-L1 blockade in a murine CRC model. Similarly, an engineered probiotic Nissle1917 strain has shown significant synergistic effects on tumor clearance when combined with PD-L1 antibodies by increasing levels of tumor-infiltrating T lymphocytes.[15] Gao et al.[18] found that *Fusobacterium nucleatum* induced PD-L1 expression and increased infiltration of cytotoxic $CD8^+$ lymphocytes during PD-L1 blockade treatment. A combined treatment consisting of FlaB-expressing *Salmonella* with interleukin-15 (IL-15) and PD-L1 blockade showed an enhanced therapeutic effect in murine 4T1 and B16F10 models.[44] Although *P. mirabilis* FlaB showed minor toxicity to mice at the beginning of treatment, generally it had relatively low toxicity. We also carefully observed the urine of the mice and found no mice with urinary tract infections (UTIs), kidney stones, or other obvious abnormalities in the 60-day survival observation experiment. Moreover, other strains of *P. mirabilis* have been reported to have little toxicity to mice.[45,46] In addition, *P. mirabilis* displays greater sensitivity to many antibiotics than other bacteria,[47] which is an advantageous characteristic. Therefore, we think it worth further exploration. However, attenuated *P. mirabilis* strains will be more suitable for clinical use, and future studies may consider modifying bacteria to reduce adverse effects. The efficiency strategies for reducing toxicity of *P. mirabilis* without altering its tumor-targeting ability may include (1) genetically carrying an anti-tumor agent by *P. mirabilis* to reduce the bacterial dose, (2) genetically deleting the virulence gene of it to attenuate the toxicities, and (3) selecting the nutrient-deficient strains by gamma irradiation or a chemical mutagen to make it specificity survive in the TME. Nevertheless,

engineered *P. mirabilis*-based therapy appears to be a potential treatment strategy for use in combination with PD-L1 blockade.

In conclusion, in this study, *P. mirabilis* was engineered to induce the expression of FlaB protein, and this modification was shown to improve the antitumor activity of *P. mirabilis*. In addition, *P. mirabilis* FlaB treatment increased levels of tumor-infiltrating $CD8^+$ T lymphocytes, enhanced secretion of cytotoxic cytokines, and synergistically improved the tumoricidal effect of PD-L1 blockade. The results of this study may contribute to the clinical transformation of bacterium-mediated antitumor therapy.

MATERIALS AND METHODS

Cell line, plasmid, and bacterial strain

CT26 cells were obtained from the American Type Culture Collection (ATCC; VA, USA) and cultured in Dulbecco's modified Eagle's medium (Gibco, NY, USA) with 10% fetal bovine serum (Newzerum, Christchurch, New Zealand). *P. mirabilis* Hauser (ATCC 35659) was purchased from the ATCC and grown in lysogeny broth (LB) at 37°C. The plasmid pBAD-pelB-FlaB (pFlaB) and pEmpty (carrying an ampicillin resistance gene) were gifted by J.H. Zheng (Hunan University, China).[11] The *flaB* gene was appended with a His_6 tag at the C terminus and synthesized by Gene Grate (Wuhan, China). The pBAD plasmid vector was cloned into the *flaB-his* gene by digestion with NcoI and PmeI and ultimately contained a His_6 tag (pFlaB-His). Then, 100 ng of pFlaB-His was mixed with *P. mirabilis* and electroporated at 2.0 kV in an electroporation cuvette. The transferred strains were preserved in LB medium with ampicillin and kept at −80°C with a final concentration of 25% glycerin.

Mouse experiments

All mouse experiments were approved and oversighted by the Hainan University Institutional Animal Use and Care Committee (HNUAUCC-2022-00070). Male BALB/c mice (5–6 weeks old) were purchased from the Guangdong Medical Laboratory Animal Center (Guangzhou, China). CT26 cells (1×10^6 cells) were implanted into the right flanks of mice by subcutaneous injection to produce tumor-bearing mouse xenografts. Tumor volumes were calculated using the formula (length × height × width)/2. Tumors were measured every other day.

When tumor volumes reached about 150 mm^3, mice were treated with phosphate-buffered saline (PBS) alone or with *P. mirabilis* or *P. mirabilis* FlaB at a dose of 6×10^7 colony-forming units (CFUs) in 100 μL PBS, administered into the tail vein. The mice were also treated daily with 0.12 g of L-arabinose (V900920, Sigma, MO, USA) by intraperitoneal injection 72 h after bacterial injection.[9] For studies in combination with thePD-L1 antibody, mice were divided randomly into the following groups: control group, PD-L1 blockade group, *P. mirabilis* FlaB + isotype control antibody (*P. mirabilis* FlaB + immunoglobulin G [IgG]) group, and (*P. mirabilis* FlaB+aPD-L1 group. The PD-L1 antibody (BE0101, Bio X Cell, NH, USA) or an isotype control antibody was injected intraperitoneally at 5 mg/kg on days 1, 5, 8, and 11. When tumor volumes were greater than or equal to 1,500 mm^3 or on day 22 post treatment, mice were euthanized, and tumors, visceral organs, and serum were collected.

RT-qPCR analysis

To confirm the expression of FlaB mRNA *in vivo*, tumor tissues of mice were collected 24 h after L-arabinose injection and treated with RNAwait (Biosharp, Hefei, China). We extracted total RNA from the tumors using TRIzol reagent (Tiangen, Beijing, China) and reverse transcribed it into cDNA using a SuperScript II cDNA Synthesis Kit (Takara Biotechnology, Beijing, China). The qPCR was carried out with SYBR Green (Takara Biotechnology). The 16S gene of *P. mirabilis* was used as a housekeeping gene.[48] The primers used are listed in Table S1.

Western blotting analysis

To confirm the expression of FlaB protein *in vivo*, mouse tumor tissues were collected 24 h after L-arabinose injection, and total proteins were obtained from those tissues by adding ice-cold RIPA (radioimmunoprecipitation assay) lysis buffer (Servicebio, Wuhan, China). Equivalent amounts of protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred to a polyvinylidene fluoride membrane. The membrane was blocked with 5% skim milk for 1 h at room temperature and then incubated with anti-His_6 antibody (9F2) (1:4,000, Fujifilm Wako Pure Chemicals Industries, VA, USA) at −4°C overnight. Anti-β-actin (bs-0061r, Bioss Antibodies, Beijing, China, 1:5,000) was used as a housekeeping marker. The bands on the membrane were developed with a Super ECL Plus Kit (Boster Biological Technology, Wuhan, China) and recorded via Image Lab software (4600SF, Tanon Science & Technology, Xinjiang, China).

Biodistribution of *P. mirabilis in vivo*

To investigate the biodistribution of *P. mirabilis* FlaB *in vivo*, tumor-bearing mice were injected with *P. mirabilis* or *P. mirabilis* FlaB at a dose of 6×10^7 CFUs per mouse via the tail vein when the tumor volume was about 150 mm^3 L-arabinose was administered by intraperitoneal injection 72 h after bacterium injection.[11] 6 h, 24 h, and 48 h after L-arabinose injection, mice (n = 3) were euthanized, and their tumors and visceral organs were collected. These collected tissues were sterilely weighed; then, a 5-fold volume of cold PBS was added, and the tissues were homogenized. We next performed a 10-fold serial dilution of the tissue suspensions and coated them in LB agar medium containing ampicillin. After 24 h of culture in incubators at 37°C, we counted and calculated the CFUs of bacteria.

Histopathological observation and IHC analysis

Tissues were fixed in 4% paraformaldehyde for 48 h and embedded with paraffin. Then, sections were stained with hematoxylin and eosin. For IHC staining, sections were rehydrated by immersion in a alcohol series gradient (100%, 95%, 75%, and 50%) for 5 min each. Then, sodium citrate buffer (pH 6.0) was used for antigen retrieval, with heating in a pressure cooker. Endogenous peroxidase activity of sections was blocked by incubation with 3% H_2O_2 for 20 min. Next, sections were blocked with 5% bovine serum albumin

for 30 min at room temperature and incubated with primary antibodies overnight at 4°C, followed by further incubation with peroxidase-linked secondary antibody for 50 min at room temperature. The primary antibodies were CD8 (bs-0648R, Bioss Antibodies, 1:200), CD4 (bs-0647R, Bioss Antibodies, 1:200), and PD-L1 (bs-1103R, Bioss Antibodies, 1:200). Finally, the sections were developed using 3,3′diaminobenzidine (DAB; Boster, Wuhan, China).

CD8, CD4, and PD-L1 positivity were defined based on membrane staining of tumor cells. Numbers of cells expressing CD8 and CD4 were determined from five random high-power fields. PD-L1 expression levels were scored based on IHC staining. In tumor cells, expression scores were defined based on the percentage of PD-L1$^+$ tumor cells as follows: level 3, 50% or greater; level 2, 5% or greater and less than 50%; level 1, 1% or greater and less than 5%; and level 0, less than 1%. In immune cells, scores were defined based on the percentage of tumor area as follows: level 3, 10% or greater; level 2, 5% or greater and less than10%; level 1, 1% or greater and less than 5%; and level 0, less than 1%.[49]

Flow cytometry

Tumors were disaggregated mechanically and enzymatically with 2 mg/mL collagenase type 2 (Gibco). Then, single-cell suspensions were fixed with fixation buffer for 20 min at 4°C and stained with fluorochrome-conjugated antibody cocktails for 15 min at 4°C. The following antibodies were used: CD3- fluorescein isothiocyanate (FITC; F2100301, MultiSciences, Hangzhou, China), CD8α-PE (F2100802, MultiSciences), CD4-APC 1/20 (F2100403, MultiSciences), and PD-L1-APC (F2127403, MultiSciences). Flow cytometry data were acquired on a Guava easyCyte flow cytometer (Millipore, MA, USA) and analyzed using FlowJo software (Becton Dickinson, NJ, USA).

Cytokine assays

To explore the regulatory effects of *P. mirabilis* FlaB on expression of cytokines related to T cell activation, we detected the expression of IFN-γ, TNF-α, granzyme B, and perforin in the TME. 24 h after L-arabinose injection, tumor tissues were collected and homogenized in ice-cold PBS by 20 cycles of homogenization for 5 s at 60 Hz at intervals of 5 s. Total protein concentration of the supernatant was determined using a bicinchoninic acid (BCA) assay kit (AR0146, Boster). Secretion of IFN-γ, TNF-α, granzyme B, and perforin in the supernatant was measured with a mouse ELISA kit (Meimian, Yancheng, China).

Statistical analysis

All statistical analyses were performed using GraphPad Prism software v.8.0 and SPSS v.25.0. Statistical significance of the experimental results was evaluated using unpaired two-tailed Student's t test. Survival times were evaluated by Kaplan-Meier analyses, and comparisons were performed using log rank tests. Each experiment included three replicates per condition. All data are expressed as the mean ± SEM. p values of less than 0.05 were considered to indicate statistical significance.

DATA AND CODE AVAILABILITY

The data that support the findings of this study are included in the supplemental information or available from the corresponding authors upon request.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.omton.2024.200770.

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AUTHOR CONTRIBUTIONS

H.Z. designed the study, analyzed data, and wrote the article. Y.L. performed the experiments and analyzed data. X.Z. performed the experiments. X.L. supervised the project and wrote the article. All of the authors critically read and approved the manuscript text.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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