Streptococcus bovis Contributes to the Development of Colorectal Cancer via Recruiting CD11b+TLR-4+ Cells

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Data Collection B
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Background: An increasing number of studies have demonstrated that *Streptococcus bovis* and its concomitant inflammatory factors concentrate in the intestine in colorectal cancer (CRC). However, the molecular mechanism of *S. bovis* on colorectal tumorigenesis remains unclear. This study aimed to explore the role of *S. bovis* in carcinogenesis and its potential mechanism in CRC of mice orally pretreated with *S. bovis*.

Material/Methods: The colons of experimental mice were collected and evaluated for the extent of neoplasm. In addition, comparative feces DNA sequencing was adopted to verify the abundance change of *S. bovis* during the progression of CRC in patients.

Results: The results of this study found that *S. bovis* is more likely to be present at higher levels in patients with progressive colorectal carcinoma compared to those adenoma patients and healthy volunteers (*P*<0.05). Pretreatment with *S. bovis* aggravated tumor formation in mice, resulting in more substantial and a higher number of tumor nodes (*P*<0.05). A cytokine expression pattern with increased levels of IL-6, Scyb1, Ptgs2, IL-1β, TNF, and Ccl2 was detected in *S. bovis* pretreated CRC mice (all *P*<0.05). Furthermore, *S. bovis* recruited myeloid cells, especially CD11b+TLR-4+ cells, which could promote pro-tumor immunity in the tumor microenvironment (*P*<0.05).

Conclusions: Collectively, our study indicates that *S. bovis* may induce a suppressive immunity that is conducive to CRC by recruiting tumor-infiltrating CD11b+TLR-4+ cells. In conclusion, *S. bovis* contributes to colorectal tumorigenesis via recruiting CD11b+TLR-4+ cells.

MeSH Keywords: **Colorectal Neoplasms • Myeloid Cells • *Streptococcus bovis***

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Background

Colorectal cancer (CRC) remains the second highest cause of cancer death, according to GLOBOCAN statistics in 2018 [1]. An estimated 376 000 new cases of CRC are reported annually in China, with over 190 000 deaths [2]. While recent treatments restrict the growth of tumors, they also cause postoperative complications and excruciating side effects from radiotherapy and chemotherapy. Therefore, the exploration of a novel therapy for CRC based on a thorough understanding of its pathogenesis has received extensive attention.

Recently, the intestinal microflora has been found to be involved in multi-systemic diseases, including obesity, diabetes, and cancer [3]. Numerous studies suggest that bacterial over-colonization may be involved in the development of CRC [4]. Anaerobic bacteria are widely known to constitute the healthy colorectal microbiota, despite sizeable interpersonal variability [5]. Lumen microbiota usually over-colonize in the intestine, resulting in an increased risk of developing CRC [6]. Moreover, *Fusobacterium nucleatum* can regulate the crosstalk between immune cells and epithelial cells, contributing to the chemotherapy resistance of CRC [7]. Recently, scientists have found that the characteristics of the enriched microflora in colitis-associated CRC are different from those in sporadic CRC [8]. *Streptococcus bovis* is significant among those differences [8]. Epidemiological investigation showed that in CRC patients, the colon is infected with *S. bovis*, even at the initial stage of CRC in the lesion tissues [9]. However, the molecular mechanism behind the involvement of *S. bovis* in CRC remains undefined. This study aimed to explore the role of *S. bovis* in carcinogenesis and its potential mechanism in CRC of mice orally pretreated with *S. bovis*.

Material and Methods

Patients, feces, and tissues

From December 2017 to March 2018, a total of 32 patients with cancer and 30 patients with adenoma underwent radical surgery in the Department of Surgery and Oncology of the Second Affiliated Hospital of Zhejiang University School of Medicine. We obtained the remaining feces from the Department of Clinical Laboratory. All patients had not received adjuvant chemotherapy, immunotherapy, or radiation therapy before surgery. At the same time, another 20 healthy volunteers were enrolled in this study and their corresponding feces were collected. The Ethics Committee of the Second Affiliated Hospital of Zhejiang University Medical College ethically approved the study. The written informed consent obtained from all participants. Feces were then stored in liquid nitrogen at −80°C for RNA examination.

Bacteria and culture

Streptococcus bovis strain (BAA-2069) was bought from American Type Culture Collection (ATCC). The strain was cultured in brain heart infusion broth (Sigma-Aldrich, St. Louis, MO, USA) at 37°C. The strain was grown overnight in broth (3 mL) and then inoculated with fresh broth (1: 100). A spectrophotometer (OD 600 nm) was used to detect bacteria with an absorbance of 0.5 for gavage.

Preparation of RNA and quantitative PCR

According to the manufacturer's instructions, Fecal Genomic DNA Extraction Kit (TianGen, Beijing, China) was used to isolate the DNA in the stool. Total RNA was extracted after elution with Tris-EDTA buffer (pH 8). Total RNA of tissues and cell lines was extracted using RNAiso Plus (TAKARA, Beijing, China) according to the instruction. The extracted RNA was synthesized to cDNA by the PrimeScript ™ RT reagent Kit (TAKARA, Beijing, China). Quantitative polymerase chain reaction (PCR) was done using SYBR® Green Realtime PCR Master Mix (TOYOBO, Shanghai, China) on the Applied Biosystems Veriti Thermal Cycler (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's protocol. The quantitation of the target RNA expression was assessed using the endogenous control by the $2^{-\Delta\Delta Ct}$ method (glyceraldehyde-phosphate dehydrogenase, GAPDH). NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) was used to evaluate the quality of the prepared RNA, and cDNA was measured. All primers used are shown in Supplementary Table 1.

Procedure for mice experiments

Thirty-two C57BL/6 mice (weighted 20 g) were purchased from the Experimental Animal Research Center of Zhejiang University (Hangzhou, Zhejiang, China). All mice were housed at 22°C, 55% humidity, 12 hours circadian rhythm, and in pathogen-free conditions. CRC animal models were performed as described previously [10,11]: one injection of ethoxymethane at day 0 (AOM, 12.5 mg/kg; Sigma-Aldrich, St. Louis, MO, USA); 10 days after AOM injection, 2.5% dextran sulfate sodium sulfate (DSS, MW=36 000–50 000 LLC, MP Biomedicals, USA) drinking for 5 days; 7 days after the previous cycle DSS drinking, next cycle began. The mice were sacrificed on the day the third DSS drinking finished (day 53). For the *S. bovis* group (n=12), each day (10: 00 am), the mice were gavage with *S. bovis* (1.2×10^7 CFU/day per mouse in physiological saline) for 2 weeks. The model group (n=12) was only subjected to AOM/DSS processing. The control group was treated only with drinking water (n=8). At several time points in these 3 groups on 0.5, 1.5, 2.5, 3.5, 4.5, 5.5, 6.5, and 7.5 weeks, the abundance of *S. bovis* was tested to evaluate the efficacy of gavage pretreatment. The weight of the mice was evaluated every 2 weeks

and expressed as a relative weight (body weight change per mouse: test weight/day 0 weight×100%). After the mice were killed on day 53, the intestines were removed for follow-up experiments. The tumor burden of each mouse is shown in Supplementary Table 2. The Animal Ethics Committee has approved all animal experiments following relevant ethical principles and guidelines set out in the Animal Welfare Act and the NIH Guidelines for the Care and Use of Laboratory Animals.

Isolation of immune cells in tumor nodules and flow cytometry analysis

Intertumoral immune cells were isolated as described [12,13] with mild modification. Briefly, the tumor nodes were washed with Dulbecco's phosphate-buffered saline (PBS; Thermo Fisher Scientific, Waltham, MA, USA). Then they were incubated (37°C) in Hank's balanced salt solution containing mixed enzymes (0.1 mg/mL collagenase D, 50 U/mL DNase I, 50 μg/mg dispase, Thermo Fisher Scientific, Waltham, MA, USA) for 30 minutes. The cells mixture was lightly covered with a 70% Percoll solution (Sigma-Aldrich, St. Louis, MO, USA), and then the mixture was lightly covered with 40% Percoll. The solution was centrifuged at 1500 g for 30 minutes at room temperature. The cells between the interfaces were gently aspirated and washed three times with PBS. The single-cell suspension was resuspended, and flow cytometry was performed. After incubation with Fc receptors (BD Biosciences, USA) for a blockade of non-specific signals, single-cell suspensions were incubated with primary antibodies (FITC-anti-CD11b, catalog number 557396, clone M1/70, BD Bioscience, USA) (PE-anti-TLR-4, catalog number 558294, clone MTS510, BD Bioscience, USA) in darkness at 4°C. Samples were measured by Invitrogen Attune NxT Flow Cytometer (Thermo Fisher Scientific, Waltham, MA, USA), and Flowjio was used to Analyze (BD Biosciences, USA) management data.

Statistical analysis

Data are shown as mean±standard error of the mean (SEM) or median of interquartile range. One-way analysis of variance (ANOVA, for example, Figure 1) or Kruskal-Wallis test was used to analyze the comparison of multiple groups. A Mann-Whitney U test (for example, Figures 2A, 2C, 3A, 3C) with a Bonferroni-corrected post hoc test and a Student's *t*-test were used for comparison between the 2 groups. The relationship between the level of *S. bovis* and the clinicopathological characteristics in Table 1 was evaluated by chi-square analysis. $P<0.05$ was considered statistically significant. All statistical analyses were performed using GraphPad 6.0.
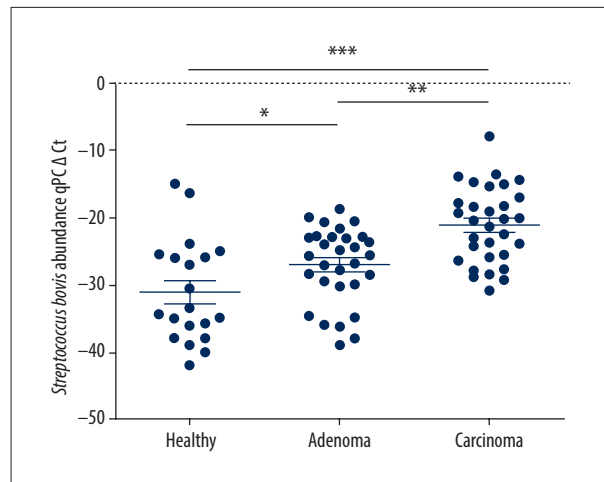

Figure 1. *Streptococcus bovis* are enriched in colorectal carcinoma patients. Abundance of *S. bovis* in feces from healthy subjects (n=20), patients with colorectal adenomas (n=30), and CRC (n=32). *** $P<0.001$.

Results

S. bovis is enriched in colorectal carcinoma patients

The results of this study found that *S. bovis* was more likely to be enriched in cancer patients than in adenoma patients or healthy volunteers at $P<0.01$ (Figure 1). Analyzing the relationship between *S. bovis* abundance and clinicopathological characteristics (Table 1), we found that *S. bovis* was more likely present at patients with larger tumor size ($P=0.028$) and advanced TNM ($P=0.033$). These data indicate that *S. bovis* is related to advance CRC, which indicates that it may promote the development of colorectal cancer (Table 1).

S. bovis accelerates tumor development of CRC *in vivo*

The evaluation of the carcinogenic effect of *S. bovis* on colonic mucosa administered *S. bovis* gavage to mice with AOM/DSS treatment. Nucleic acid assays were used to evaluate the efficiency of gavage pretreatment (Supplementary Figure 1). After the second time of DSS drinking, a more severe weight loss was observed in the *S. bovis* group than in the model group (Figure 2A). As shown in Figure 2B, *S. bovis* pretreatment exacerbated the number of tumor nodules grown in the colorectum at $P<0.01$. Moreover, *S. bovis* pretreatment significantly increased the size of colorectal tumors (the average diameter of the *S. bovis* group was 2.26 mm, the model group was 1.83 mm, $P<0.05$, Figure 2C). In the control group, the colorectal mucosal glands were intact with occasional mild lymphocytic infiltration. In the model group, adenomas and highly differentiated adenocarcinomas could be seen everywhere. In the *S. bovis* group, poorly differentiated adenocarcinoma with disordered arrangement was the most significant change. Compared to
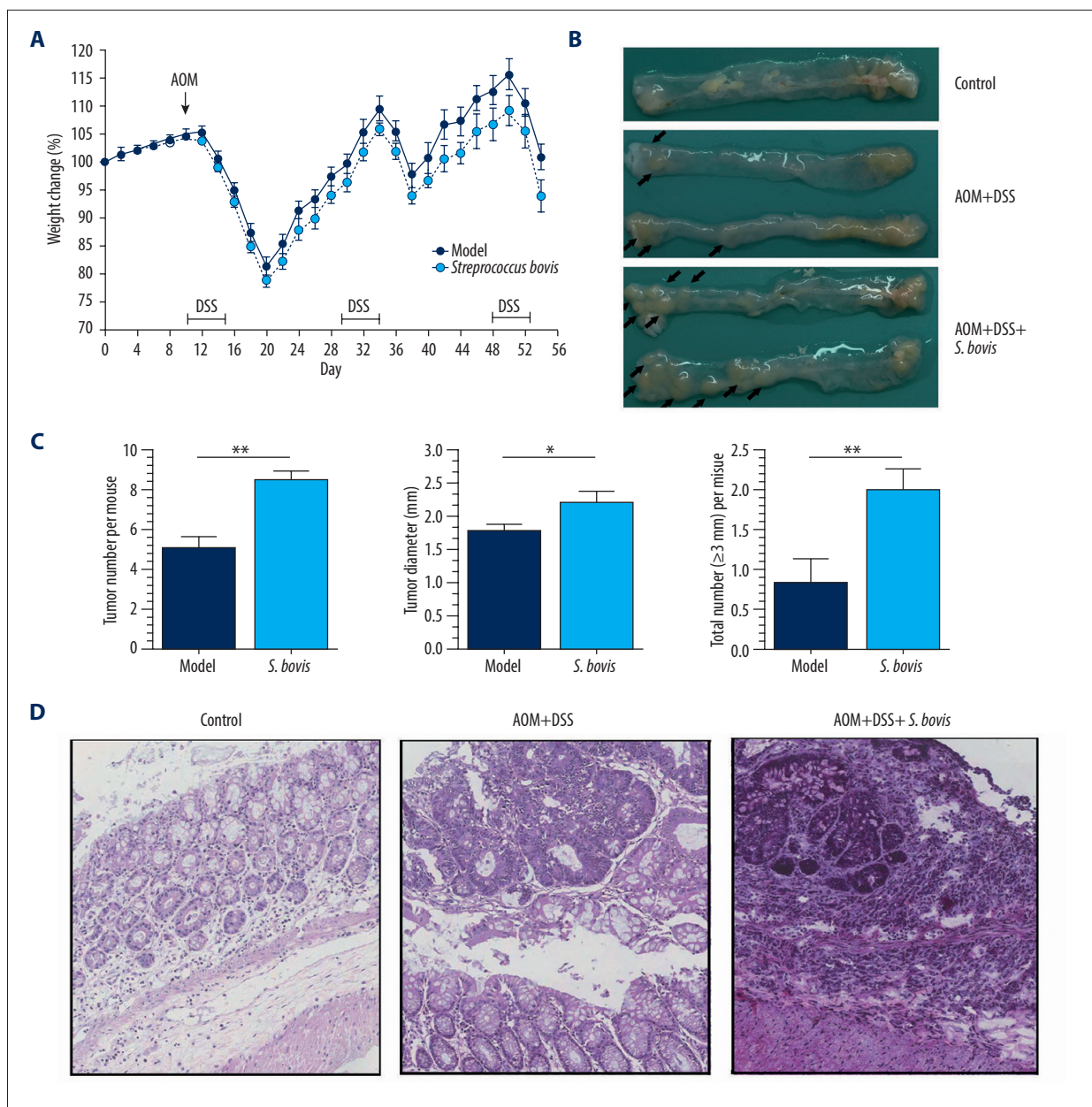
Figure 2. *Streptococcus bovis* accelerates tumor development of CRC *in vivo*. (**A**) Weight changes during the strains pretreatment. (**B**) Colorectal morphology for tumor nodes (black arrows indicate the diameter is larger than 1 mm). (**C**) Comparison between the Model group (n=12) and the *S. bovis* group (n=12). (**D**) Hematoxylin and eosin stained sections of colons in these 3 groups at the 53rd day. * $P<0.05$, ** $P<0.01$.

the model group, the incidence of atypical hyperplasia, crypt abnormalities, and adenocarcinoma was highest in AOM/DSS mice fed with *S. bovis* (Figure 2D). Altogether, these data suggested that *S. bovis* can aggravate tumorigenesis in the colonic mucosa of AOM/DSS mice.

S. bovis contributes to develop CRC by recruiting TLR-4$^+$CD11b$^+$ cells

The evaluation expression pattern of cytokines associated with myeloid cells in the colon microenvironment (Figure 3A). Therefore, the results of this study showed interleukin (IL)-6 at $P<0.01$, Scyb1 (equivalent to human IL-8, $P<0.05$), Ptgs2 (equivalent to human COX-2, $P<0.01$), IL-1β ($P<0.01$), tumor necrosis factor (TNF) ($P<0.05$), and Ccl2 ($P<0.01$) gene expression
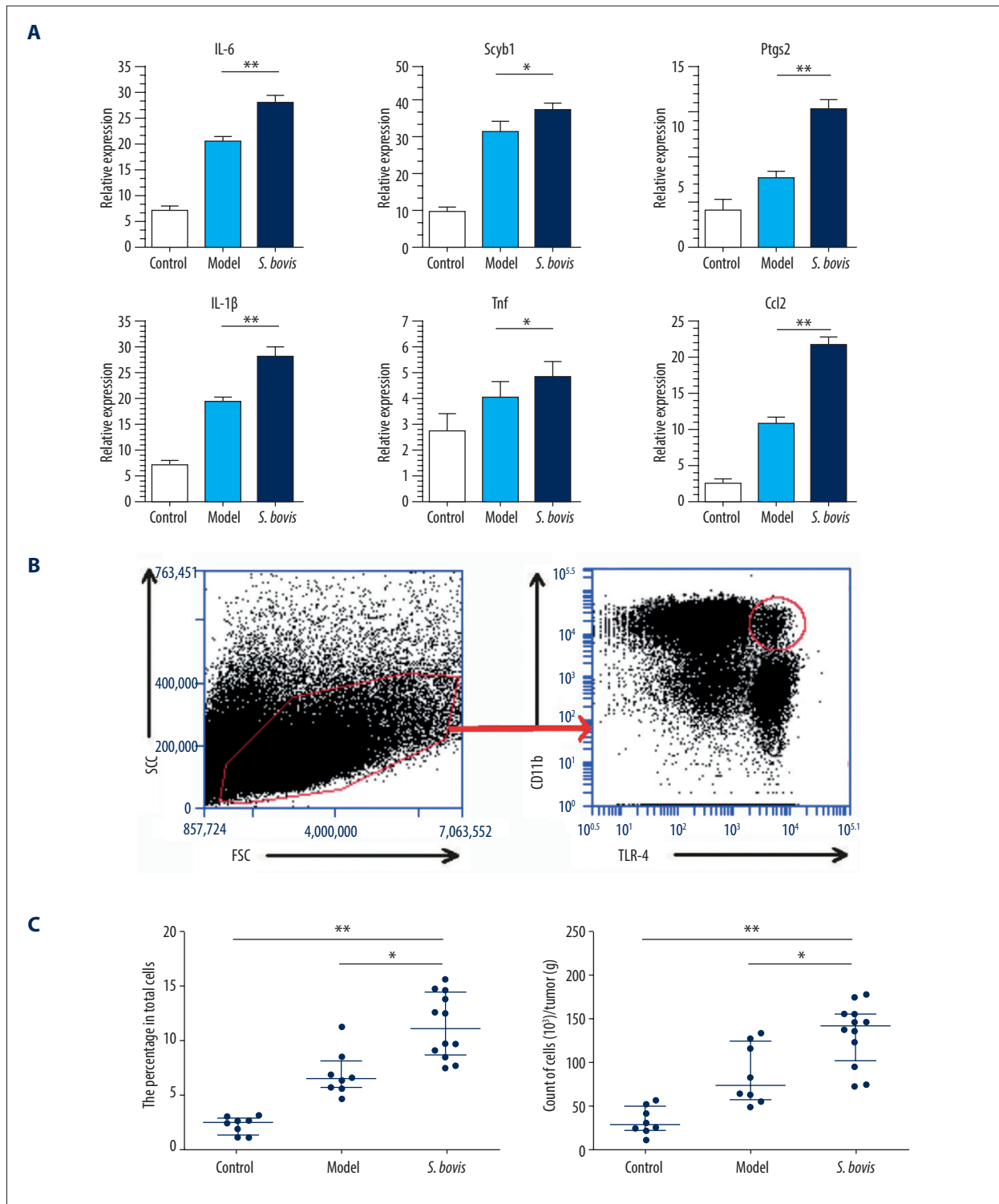
Figure 3. *Streptococcus bovis* contribute to the development of CRC by recruiting TLR-4+CD11b+ cells. (**A**) Relative mRNA expression of IL-6, Scyb, Ptgs2, IL-1β, TNF, and Ccl2 (Model group n=12; *S. bovis* group, n=12). (**B**) The gating methods with density plots for intratumoral myeloid cells. (**C**) Percentage and count of colonic TLR-4+CD11b+ cells (Control group, n=8; Model group, n=12; *S. bovis* group, n=12). * $P<0.05$, ** $P<0.01$.

Table 1. Relationship between abundance of *Streptococcus bovis* and clinicopathological characteristics of CRC patients.

	N	Abundance of *Streptococcus bovis*		P value
		Low (16)	High (16)	
Gender				
Male	18	11	7	0.154
Female	14	5	9	
Age (years)				
≥60	19	8	11	0.280
<60	13	8	5	
Tumor size				
≥4 cm	12	3	9	0.028*
<4 cm	20	13	7	
TNM				
I–II	14	10	4	0.033*
III	18	6	12	
Lymph node metastasis				
No	17	11	6	0.077
Yes	15	5	10	
Tumor site				0.404
Transverse colon	7	5	2	
Descending colon	8	4	4	
Rectum	17	7	10	

Data are analyzed by chi-square test. * *P*<0.05 represents statistical difference.

increased significantly (Figure 3A). To investigate whether tumorigenesis of CRC can be attributed to myeloid cell recruitment caused by *S. bovis*. The measurement of the colon-infiltrated immune cells (Figure 3B) was done. Analysis shows that *S. bovis*-fed mice, tumor nodules TLR-4⁺ CD11b⁺ cells are more abundant than cells in model and control mice (Figure 3C). Supplementary Figure 2 shows a gated control of TLR-4⁺ cells. These results indicate that *S. bovis* may recruit TLR-4⁺ CD11b⁺ cells to promote the development of CRC.

Discussion

Distinct from the other types of cancer, CRC is thought to be caused mainly by colonization and inflammation in the colon [14]. The role of pathogenic bacteria in the development of CRC has gradually attracted scientific attention [15]. Based on the epidemiological investigation of *S. bovis* [15-17], our study explored the response of the colonic microenvironment to high levels of *S. bovis*. An increasing number of reports have addressed the relationship between *S. bovis* and CRC. The production of inflammatory cytokines in response to *S. bovis* such as TNF-α, IL-1β, IL-6, and IL-8 contributes to

the formation of nitric oxide and free radicals, which can promote the tumorigenesis in CRC by modifying cellular DNA [18]. Besides, the production of angiogenic factors such as IL-8 in the colonic mucosa due to *S. bovis* may also be involved in the progression of colon cancer [19,20]. Investigators have found that the wall-extracting antigen of *S. bovis* induces the overexpression of COX2 (Equivalent to mice Ptgs2) *in vitro* [21], which promotes cells proliferation, angiogenesis and inhibits apoptosis through prostaglandins, thereby resulting in CRC [18]. Although previous studies tried to illuminate the association between *S. bovis* and CRC by demonstrating the induction of inflammatory factors by *S. bovis*, the specific molecular mechanism remains unclear. In this study, we analyzed the changes in TLR-4⁺CD11b⁺ cells in the immune microenvironment after pretreatment with *S. bovis* to clarify how *S. bovis* causes the enhancement of these cytokines. As a vital immune role in the development of CRC promoted by *S. bovis* in this study, myeloid cells may be intervened via neutralizing antibodies against TLR-4 or CD11b to mediate the progression of CRC.

CD11b⁺ myeloid cells are heterogeneous in the tumor microenvironment, mainly consisting of macrophages and myeloid-derived suppressor cells (MDSCs). Intratumoral expression of

IL-1β and IL-8, which contribute to tumorigenesis, has been shown to be derived from MDSCs [22]. A previous study reported that long noncoding RNAs could control the immune-suppressive function and differentiation of MDSCs via regulating the expression of Arg-1 and COX2 in MDSCs [23]. Increased TNF recruits MDSCs to the stroma, assisting tumor growth [24]. In addition, CCL2 has been deemed a strong chemokine to macrophages in the development of CRC [25]. Macrophages in the tumor microenvironment cannot only promote the epithelial-mesenchymal transition and stemness of cancer via TNF-α [26] but can also enhance invasion by secreting IL-6 and IL-8 [27]. Although the cytokine expression profile mentioned above was shown to be associated with *S. bovis* introduction in our study (Figure 3), we did not verify the change in each subtype of CD11b+ myeloid cells.

The concentration of intratumoral *S. bovis* in CRC shown in Figure 1 indicates that *S. bovis* may complete a location transformation before or during carcinogenesis. To date, how specific bacteria interact with the tight junctions of the colonic epithelium, cross the mucosal barrier structure, and reach the tumor microenvironment remains unclear. Abnormal expression of claudin proteins, which are responsible for the intestinal mucosal barrier, is modulated in the development of CRC [28]. Additionally, the Muc2 expression declines during the transition from colon adenoma to the malignant tumor [29]. These findings suggest that changes in the surface molecules may make the colon epithelium susceptible to the invasion of specific bacteria, promoting the formation of malignant tumors.

There are some obvious limitations to our study. First, we did not obtain additional kinds of clinical samples (including serum and tumor tissues) apart from feces to investigate changes in *S. bovis* colonized in tumor tissues as well as changes in inflammatory factors (including the baseline levels of those interleukins) in peripheral blood and tumor sites. Second, CD11b+ myeloid cells are so heterogeneous that different types in the tumor microenvironment function in different ways. Although the specific expression profile of cytokines was investigated in the mouse model, changes in specific subtypes of CD11b+ myeloid cells were not verified after *S. bovis* pretreatment. Third, we did not study the intracellular mechanism of the regulation of cytokine secretion via immune cell receptors.

Conclusions

In conclusion, the results of this study show that *S. bovis* cooperates with TLR-4+CD11b+ cells to shape a pro-tumor immunity, accelerating the progression of CRC. Although recent findings reveal the relationship between *S. bovis* and TLR-4+CD11b+ cells in the evolution of CRC. All these provide molecular clues for the diagnosis, treatment, and monitoring of colorectal cancer. Moreover, further investigation may provide information for specific dietary interventions or probiotics that may be helpful in reducing the incidence of colorectal cancer and developing adjuvant therapy.

Supplementary Data

Supplementary Table 1. The primers for genes detected by real-time polymerase chain reaction.

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
Streptococcus bovis	AACGCGAAGAACCTTACCAG	GAGTGCCCAACTGAATGATG
Total bacterial DNA	GCAGGCCTAACACATGCAAGTC	CTGCTGCCTCCCGTAGGAGT
GAPDH	CCCTTCATTGACCTCAACTACA	ATGACAAGCTTCCCGTTCTC

Supplementary Table 2. The tumor burden of each animal in Figure 2.

Streptococcus bovis group (n=12)				Model group (n=8)			
Mouse No.	Tumor number/mouse	Diameter (mm)	Tumor number (≥3 mm)/mouse	Mouse No.	Tumor number/mouse	Diameter (mm)	Tumor number (≥3 mm)/mouse
1	12	3	6	1	7	3	4
2	11	3	5	2	7	3	2
3	10	3	4	3	6	3	1
4	9	3	4	4	5	2	0
5	8	3	3	5	5	1	0
6	8	3	2	6	4	1	0
7	7	3	1	7	3	1	0
8	7	2	0	8	3	1	0
9	7	1	0				
10	6	1	0				
11	6	1	0				
12	6	1	0				

Tumor number/mouse: the count of tumor node in each mouse; Diameter (mm): the size of the largest tumor node in each mouse. Tumor number (≥3 mm)/mouse: the count of tumor node which is larger than 3 mm in each mouse.
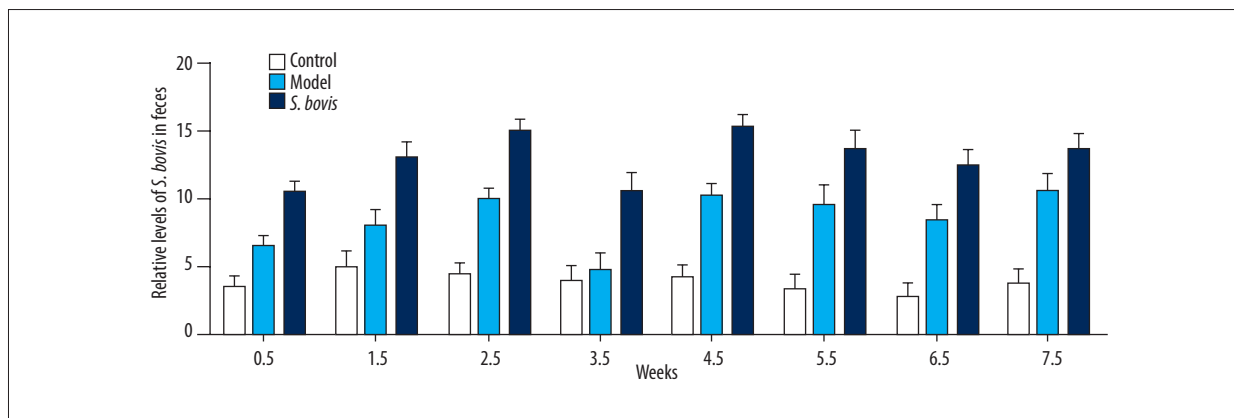
Supplementary Figure 1. The efficacy of *S. bovis* pretreatment showed by abundance of *S. bovis* in feces.
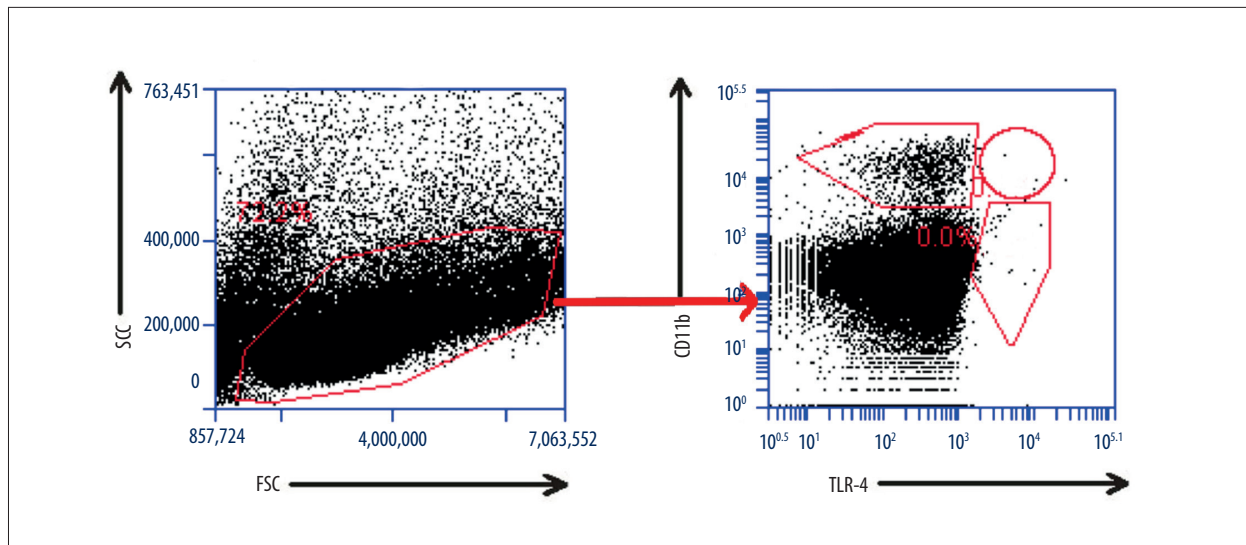
Supplementary Figure 2. The gating control for the TLR-4+ cells.

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