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SHORT COMMUNICATION

Sodium butyrate modulates gut microbiota and immune response in colorectal cancer liver metastatic mice

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Abstract Colorectal cancer (CRC) liver metastasis (CLM) is the leading death cause of CRC patients, but there is no satisfied approach to treat CLM. Gut microbiota plays a pivotal role in CRC initiation and development. Targeting dysbiosis of the gut microbiota might open up new opportunities for CLM treatment. Here, we investigated the efficacy of sodium butyrate (NaB), a major product of gut microbial fermentation, in modulating gut microbiota in CLM mice. NaB supplement decreased mouse colon cancer CT26 cell liver metastasis in intrasplenic tumor injection model of BALB/c mice. Using 16S rRNA gene sequencing, we found altered microbiota composition in CLM mice, characterized by increases of Firmicutes and Proteobacteria. NaB beneficially changed dysbiosis in CLM mice. Functional analysis of the KEGG pathways showed that NaB changed pathways related to immune system diseases and primary immunodeficiency in CLM mice. In addition, NaB decreased T regulatory cells and increased natural killer T cells and T helper 17 cells, accordingly decreased IL-10 and increased IL-17 secretion in CLM mice liver. In conclusion, NaB beneficially modulated gut microbiota and improved host immune response in CLM mice. These findings demonstrate the therapeutic potential of NaB in CLM treatment.

Keywords Sodium butyrate · Colorectal cancer · Liver metastasis · Gut microbiota · Immunity

Abbreviations

NaB	Sodium butyrate
CRC	Colorectal cancer
CLM	Colorectal liver metastasis
SCFAs	Short-chain fatty acids
NK	Natural killer

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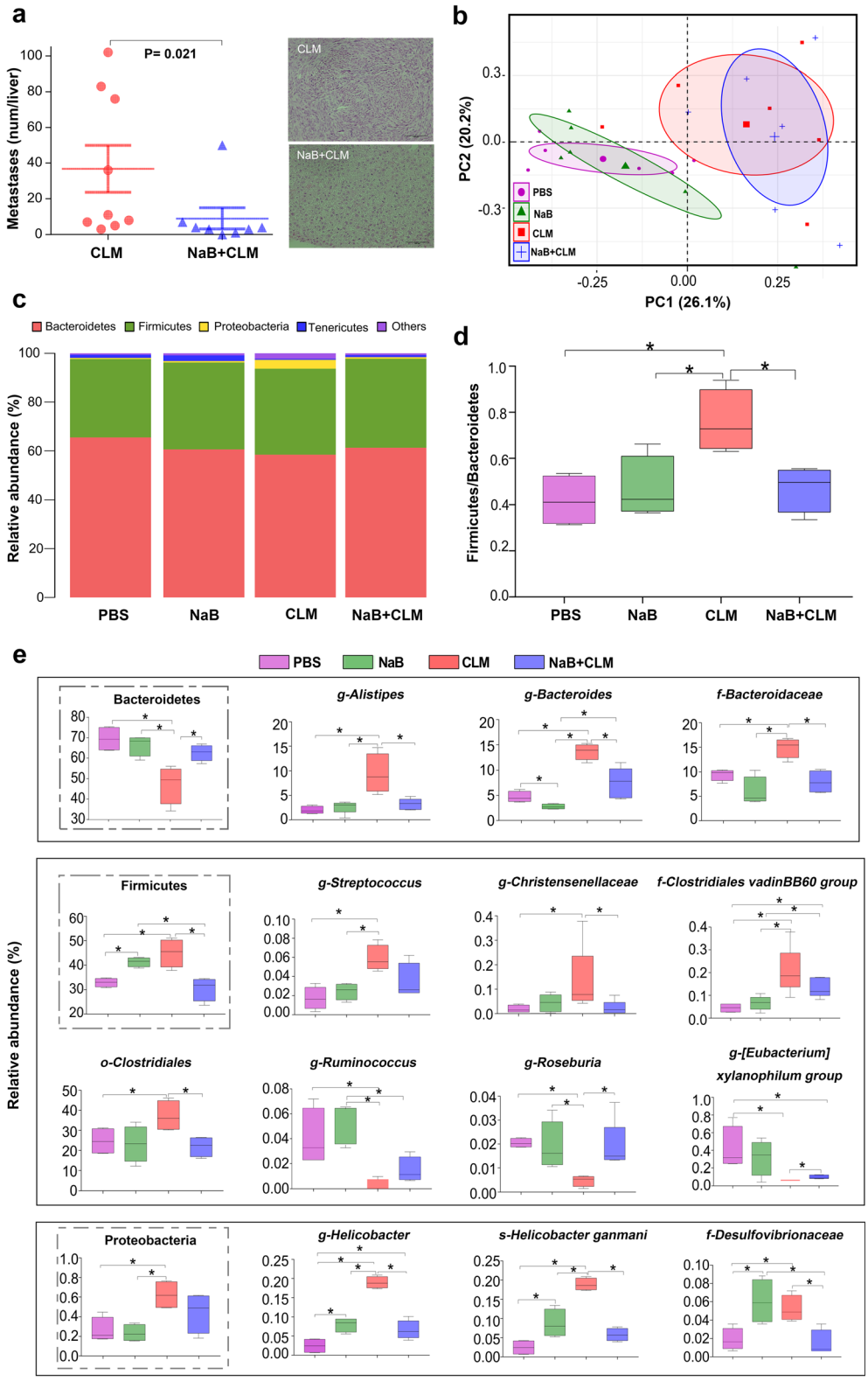

a
P= 0.021
Metastases (num/liver)
CLM
NaB+CLM
b
PC2 (20.2%)
PC1 (26.1%)
PBS
NaB
CLM
NaB+CLM
c
Bacteroidetes
Firmicutes
Proteobacteria
Tenericutes
Others
Relative abundance (%)
d
Firmicutes/Bacteroidetes
e
Bacteroidetes
g-Alistipes
g-Bacteroides
f-Bacteroidaceae
Firmicutes
g-Streptococcus
g-Christensenellaceae
f-Clostridiales vadinBB60 group
o-Clostridiales
g-Ruminococcus
g-Roseburia
g-[Eubacterium] xylanophilum group
Proteobacteria
g-Helicobacter
s-Helicobacter ganmani
f-Desulfovibrionaceae

◀ **Fig. 1** NaB regulates gut microbiota in CLM mice. **a** Analysis of CLM in mice intrasplenically transplanted of CT26 cells without ($n = 9$) or with ($n = 8$) NaB supplement. Left: NaB decreased the number of metastases in liver. Right: HE staining of liver sections isolated from CLM mice with/without NaB supplement. Scale bar, 100 μm. **b** Principal coordinate analysis (PCoA) of gut microbiota. Each symbol represents one mouse ($n = 6$ each group). **c** Differential bacterial abundance analysis at the phylum level. **d** Relative abundance of Firmicutes and Bacteroidetes were measured by qPCR. The Firmicutes-to-Bacteroidetes ratio in each mouse was calculated ($n = 6$). *$P < 0.05$. **e** Mice were treated with PBS or NaB in mice with/without CLM ($n = 6$). Relative abundance of gut bacterial organisms in phylum Bacteroidetes, Firmicutes, and Proteobacteria were measured by qPCR. The line demarks the mean. *$P < 0.05$. In the figure, *g* means genus, *f* means family, *o* means order

Treg	T regulatory
Th17	T helper 17

Liver metastasis is the leading cause of colorectal cancer (CRC)-related death. More than 50% of CRC patients develop colorectal liver metastasis (CLM) throughout their life. Though CRC incidence and mortality rates declined over the past decade, the 5-year survival rate for metastasized CRC patients is less than 10% (Nordlinger et al. 2013). Thus, there is a pressing need for new approaches to treating patients with CLM.

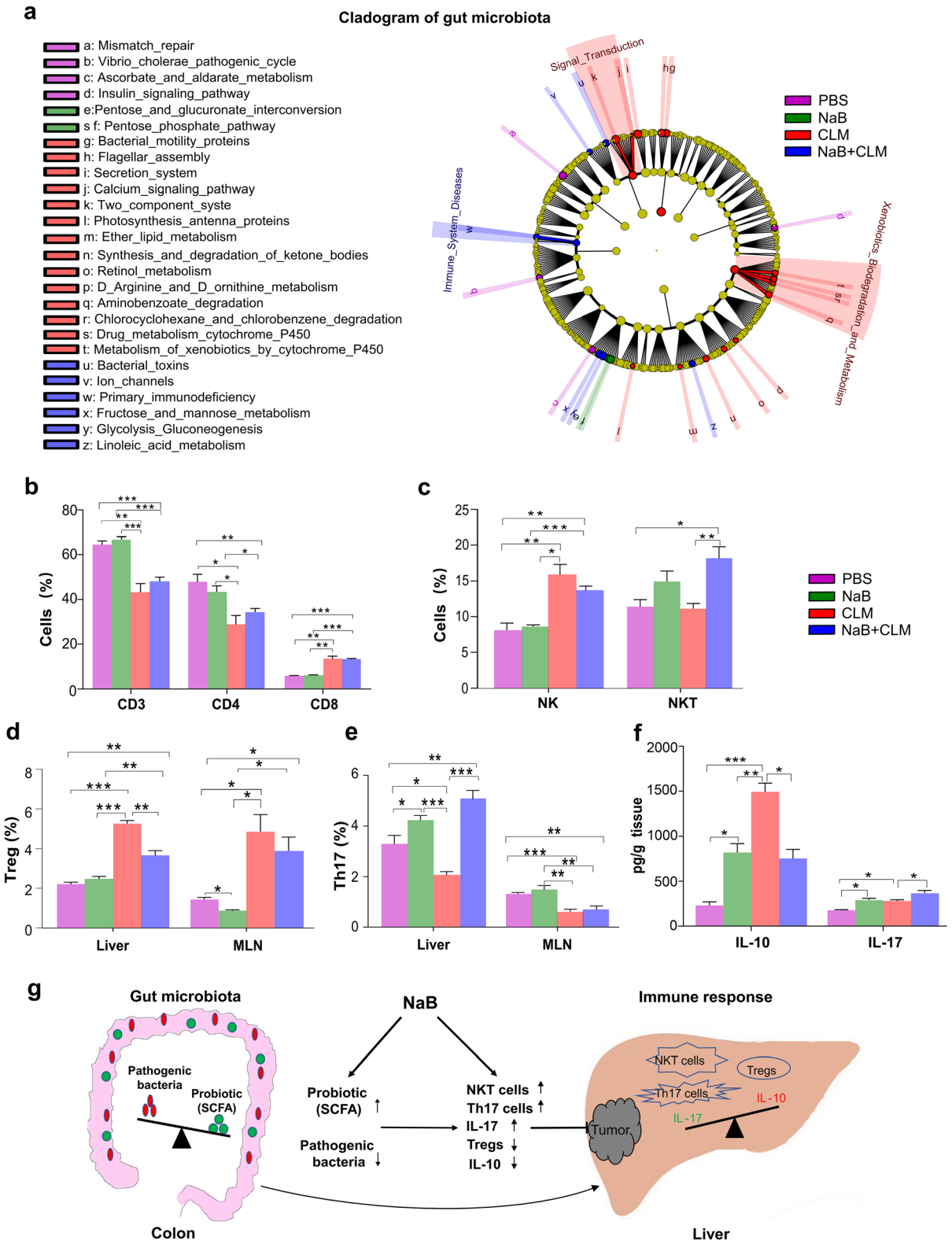
Gut microbiota regulates health and diseases. Increasing evidences suggest that gut microbiota plays a pivotal role in CRC initiation and development. The fecal microbiota from CRC patients promotes tumorigenesis in mice, indicating that altered microbiota promotes CRC formation (Wong et al. 2017). Gut microbiota-stimulated cathepsin K secretion promotes CRC metastasis (Li et al. 2019). Targeting dysbiosis of the gut microbiota might open up new opportunities for CLM treatment.

Diet influences the composition of the host gut microbiome. Gut microbiota ferments dietary fiber to produce short-chain fatty acids (SCFAs), such as butyrate. Sodium butyrate (NaB) administration beneficially changes gut microbiota composition and improves intestinal barrier in high-fat diet mice (Fang et al. 2019). NaB also influences the composition of gut microbiota and host colonic tumorigenesis (Zhang et al. 2018). CRC patients have significant reduction in the number of bacteria that produce SCFAs, including butyrate (Wu et al. 2016). Therefore, NaB may modulate the gut microbiota to attenuate the development of CRC. Here, we found that NaB rebalanced gut microbiota and modulated host immune response in CLM mice. Our findings provide insights into the therapeutic utility of NaB in CLM.

Five-week-old male BALB/c mice were randomly divided into four groups: PBS group (PBS), NaB group (NaB 350 mg/kg/day), CLM group (intrasplenic injection of 2×10^5 CT26 cells), and NaB + CLM group (NaB 350 mg/kg/day, intrasplenic injection of CT26 cells). Mice were sacrificed 10 days after inoculation. Fecal samples were collected for gut microbiota analysis. Liver and mesenteric lymph nodes (MLN) were collected for immune analysis (Supplementary material and methods). Results were presented as mean ± SEM. One-way ANOVA or *t* test was utilized for comparisons among groups. $P < 0.05$ was considered statistically significant.

Our results indicate that NaB had no effect on body weight gain and spleen tumor formation in mice (Fig. S1), but NaB dramatically repressed CLM (Fig. 1a). Clinic studies have demonstrated gut dysbiosis in CRC patients, such as higher level of pathogenic bacteria and lower level of SCFA-producing bacteria (Wirbel et al. 2019; Flemer et al. 2017). Gut dysbiosis has also been observed in mice model of colitis and colitis-associated CRC (Ibrahim et al. 2019), even in mice subcutaneously inoculated with CT26 cells (Wang et al. 2018). PCoA showed that the bacterial flora was changed in CLM mice (Fig. 1b). Taxonomic composition analysis revealed that severe dysbiosis in CLM mice with elevated proportion of Proteobacteria and Firmicutes, and decreased proportion of Bacteroidetes (Fig. 1c). The Firmicutes-to-Bacteroidetes ratio was significantly increased in CLM mice (Fig. 1d). Altered relative abundances of Bacteroidetes and Firmicutes are associated with the risk of CRC (Wirbel et al. 2019). NaB increased proportion of Bacteroidetes and decreased Firmicutes, accordingly reducing the Firmicutes-to-Bacteroidetes ratio in CLM mice (Fig. 1d).

In CLM mice (Fig. 1e), three organisms (*Alistipes*, *Bacteroides*, and *Bacteroidaceae*) related to CRC development in the phylum Bacteroidetes were increased compared with control group mice. Four organisms (*streptococcus*, *Christensenellaceae*, *Clostridiales vadinBB60 group*, and *Clostridiales*) were increased, and three organisms (*Ruminococcus*, *Roseburia*, and *[Eubacterium] xylanophilum group*) in the phylum Firmicutes were decreased compared with control mice. Three organisms (*Helicobacter*, *Helicobacter ganmani*,


a
Cladogram of gut microbiota
a: Mismatch_repair
b: Vibrio_cholerae_pathogenic_cycle
c: Ascorbate_and_aldarate_metabolism
d: Insulin_signaling_pathway
e:Pentose_and_glucuronate_interconversion
s f: Pentose_phosphate_pathway
g: Bacterial_motility_proteins
h: Flagellar_assembly
i: Secretion_system
j: Calcium_signaling_pathway
k: Two_component_syste
l: Photosynthesis_antenna_proteins
m: Ether_lipid_metabolism
n: Synthesis_and_degradation_of_ketone_bodies
o: Retinol_metabolism
p: D_Arginine_and_D_ornithine_metabolism
q: Aminobenzoate_degradation
r: Chlorocyclohexane_and_chlorobenzene_degradation
s: Drug_metabolism_cytochrome_P450
t: Metabolism_of_xenobiotics_by_cytochrome_P450
u: Bacterial_toxins
v: Ion_channels
w: Primary_immunodeficiency
x: Fructose_and_mannose_metabolism
y: Glycolysis_Gluconeogenesis
z: Linoleic_acid_metabolism
Signal_Transduction
Immune_System_Diseases
Xenobiotics_Biodegradation_and_Metabolism
PBS
NaB
CLM
NaB+CLM
b
Cells (%)
CD3
CD4
CD8
c
Cells (%)
NK
NKT
PBS
NaB
CLM
NaB+CLM
d
Treg (%)
Liver
MLN
e
Th17 (%)
Liver
MLN
f
pg/g tissue
IL-10
IL-17
g
Gut microbiota
Pathogenic bacteria
Probiotic (SCFA)
Colon
NaB
Probiotic (SCFA) ↑
Pathogenic bacteria ↓
NKT cells ↑
Th17 cells ↑
IL-17 ↑
Tregs ↓
IL-10 ↓
Immune response
NKT cells
Tregs
Th17 cells
IL-10
IL-17
Tumor
Liver

◀ **Fig. 2** NaB modulates enriched pathways and regulates immune responses in CLM mice. Mice were treated with PBS or NaB in mice with/without CLM ($n = 6$). **a** PICRUSt was used to predict the metagenome functional content of mice. The significant differential abundances in different groups of mice were identified with LEfSe (P value cut-off of 0.05 and LDA score cut-off of 2). Circular dendrogram represented the KEGG functional hierarchy. **b** The frequency of T cells ($CD3^+$, $CD3^+CD4^+$, $CD3^+CD8^+$) in the liver was determined by flow cytometry and analyzed by gating on lymphocytes and $CD3^+$ cells. NaB supplement had no effect on T cells in CLM mice. **c** The frequency of NK cells ($CD49b^+$) and NKT cells (TCR-β^+) in the liver was determined by flow cytometry. NaB increased NKT cells in CLM mice. **d** The frequency of $CD4^+CD25^+$ Tregs in the liver and MLN was determined by flow cytometry and analyzed by gating on $CD127^{Low}$ cells. NaB decreased Tregs in CLM mice liver. **e** The frequency of IL-$17A^+$ Th17 cells in the liver and MLN was determined by flow cytometry and analyzed by gating on $CD4^+$ cells. NaB increased Th17 cells in CLM mice liver. **f** The protein levels of IL-10 and IL-17 in hepatic tissue were determined by ELISA. Bars represent positive percent cells (**b**–**e** mean ± SEM) in different groups of mice. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. **g** Schematic representation of the major molecular mechanism by which NaB suppresses CLM. Dysbiosis of gut microbiota affects host immune response in CLM mice. NaB decreased the relative abundance of pathogenic bacteria and increased the relative abundance of probiotic, such as SCFA-producing bacteria. The changes of gut microbiota by NaB decreased Tregs and increased NKT and Th17 cells, accordingly decreased IL-10 and increased IL-17 secretion, which lead to improved host anti-tumor immune response in CLM liver and suppression of CLM

and *Desulfovibrionaceae*) in the phylum Proteobacteria were increased compared with control. *Alistipes* promotes CRC development via activation of IL-6/STAT3 signaling (Yang and Jobin 2017). *Bacteroides* overrepresented in CRC produces enterotoxin (Snezhkina et al. 2016). *Clostridiales* induces amino acid degradation in CRC (Wirbel et al. 2019). *Helicobacter* is involved in chronic inflammation (Abdulamir et al. 2011). *Desulfovibrionaceae* interferes with epithelial cell oxidative metabolism, which leads to cell damage (Balamurugan et al. 2008). Interestingly, the relative abundances of *Alistipes*, *Bacteroides*, *Clostridiales*, *Helicobacter*, and *Desulfovibrionaceae* were decreased in NaB-treated CLM mice. Thus, NaB could decrease the harmful pathogenic bacteria in CLM mice.

The energy source of colon cells mainly relies on SCFAs, especially butyrate. The gut microbiome of CRC promotes dysregulated glucose metabolism of SCFAs (Wang et al. 2019). The gut dysbiosis in mice with colitis and colitis-associated CRC is characterized by a decrease in the relative abundance of SCFA-producing bacteria, and the concentration of SCFAs accordingly decreased significantly in mice fecal samples (Ibrahim et al. 2019). High-fiber diet alters the microbiota composition and promotes the production of SCFAs in polyposis mice (Bishehsari et al. 2018). NaB significantly increased the relative abundance of *Roseburia* and *[Eubacterium] xylanophilum group* related to SCFA-producing in CLM mice. Thus, NaB could increase probiotic, such as SCFA-producing bacteria in CLM mice.

Furthermore, LEfSe showed that CLM resulted in marked metabolic profile changes (Fig. 2a, S2). Pathways related to signaling transduction, xenobiotics biodegradation, and metabolism were enriched in CLM mice. NaB changed functional pathways of immune system diseases in CLM mice. Gut microbiota plays an important role in shaping immune cell responses (Fessler et al. 2019). The liver is an essential immunological organ with a large number of immune cells. Compared with mice receiving PBS or NaB, CLM mice had decreased $CD3^+$ and $CD4^+$ T cells and increased $CD8^+$ T and NK cells in the liver. NaB had no effect on $CD3^+$, $CD4^+$, $CD8^+$ T, and NK cells, but markedly increased the frequency of natural killer T cells (NKT) cells in CLM mice liver (Fig. 2b, c, S3a). *Bacteroides fragilis* regulates the homeostasis of host invariant NKT cells (An et al. 2014). Pathogenic bacteria *Streptococcus* leads to a rapid reduction of NKT cell numbers (Chen et al. 2014). Study found that bile acid–controlled NKT cell accumulation mediates liver cancer inhibition (Ma et al. 2018). Thus, NaB suppresses CLM partially through inhibiting pathogenic bacteria-induced NKT cell downregulation in the liver.

T helper 17 (Th17)/T regulatory cells (Tregs) ratio and cytokine imbalance are important for CRC development (Amicarella et al. 2017). CRC patients have a higher proportion of Tregs that suppress tumor immune responses (Ling et al. 2007). CLM mice had increased $CD4^+CD25^+CD127^{Low}$ Tregs (Fig. 2d, S3b) and decreased $CD4^+$IL-$17A^+$ Th17 cells (Fig. 2e, S3c) both in the liver and MLN. NaB decreased the frequency of Tregs and increased Th17 cells in the liver but not in MLN. Accordingly, NaB decreased IL-10 protein while increasing IL-17 in CLM mice liver (Fig. 2f). These results demonstrated that the antitumor effect of NaB maybe related to regulating Tregs/Th17 cells and secretion of IL-10/IL-17 in the liver.

Our study revealed the dysbiosis of gut microbiota in CLM mice. Firmicutes to Bacteroidetes ratio significantly increased in CLM mice, but this ratio could be reduced by NaB. NaB decreased the relative abundance of pathogenic bacteria and increased the relative abundance of probiotic, such as SCFA-producing bacteria. Thus, NaB could decrease toxic bacterial products and increase beneficial bacterial metabolites. Moreover, the changes of gut microbiota by NaB increased NKT cells and Th17 cells and decreased Tregs, which lead to improved host anti-tumor immune response in CLM liver (Fig. 2g). This study highlights the therapeutic potential of NaB in CLM treatment.

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Author contributions G. W., Q. D., and H. D. participated in the design of the study. X. M., Z. Z., Z. X., M. F., and Y. H. performed the experiments. Q. D., G. W., X. M., Z. Z., Y. F., and H. D. contributed to data interpretation and wrote the manuscript. All authors read and approved the final manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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