

Overcoming the nutritional immunity by engineering iron scavenging bacteria for cancer therapy

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Abstract

Certain bacteria demonstrate the ability to target and colonize the tumor microenvironment, a characteristic that positions them as innovative carriers for delivering various therapeutic agents in cancer therapy. Nevertheless, our understanding of how bacteria adapt their physiological condition to the tumor microenvironment remains elusive. In this work, we employed liquid chromatography-tandem mass spectrometry to examine the proteome of *E. coli* colonized in murine tumors. Comparing to *E. coli* cultivated in the rich medium, we found that *E. coli* colonized in tumors notably upregulated the processes related to ferric ions, including the enterobactin biosynthesis and iron homeostasis. This finding indicated that the tumor is an iron-deficient environment to *E. coli*. We also found that the colonization of *E. coli* in the tumor led to an increased expression of lipocalin 2 (LCN2), a host's protein that can sequester the enterobactin. We therefore engineered *E. coli* in order to evade the nutritional immunity provided by LCN2. By introducing the *IroA* cluster, the *E. coli* synthesizes the glycosylated enterobactin, which creates steric hindrance to avoid the LCN2 sequestration. The *IroA-E. coli* showed enhanced resistance to LCN2 and significantly improved the anti-tumor activity in mice. Moreover, the mice cured by the *IroA-E. coli* treatment became resistant to the tumor re-challenge, indicating the establishment of immunological memory. Overall, our study underscores the crucial role of bacteria's ability to acquire ferric ions within the tumor microenvironment for effective cancer therapy.

eLife assessment

This **valuable** study combines proteomics and a mouse model to reveal the importance of iron uptake in bacterial therapy for cancer. The evidence presented is **convincing**. Notably, the authors showed upregulation of iron uptake of bacteria significantly inhibits tumor growth in vivo. This paper will be of interest to a broad audience including researchers in cancer biology, cell biology, and microbiology.

Introduction

Bacterial therapy has re-emerged as a promising modality for cancer treatment, building on the pioneering works of William Coley in the late 19th century (Carlson et al. 2020). As a living therapeutic agent, bacteria offer several advantages over traditional cancer treatments, including (1) active tumor targeting (Yamamoto et al. 2016; Kim et al. 2023), (2) tumor colonization (Westphal et al. 2008; Weibel et al. 2008), (3) immune system stimulation (Qiu et al. 2020; Yang et al. 2023), and (4) great engineerability for customized functionalities (Gurbatri et al. 2020; Hu et al. 2022; Canale et al. 2021). The tumor microenvironment (TME) provides unique cues, such as hypoxia, low pH values, and immune suppressors, which facilitate the selective targeting and colonization of certain bacteria, including *E. coli* (Ryan et al. 2009; Flentie et al. 2012). However, the restricted resource in the TME may pose challenges for bacterial survival and growth, potentially limiting their anti-tumor efficacy. While the molecular physiology of *E. coli* has been extensively studied under well-controlled laboratory conditions (Han and Lee 2006; Mateus et al. 2020), it is unclear how *E. coli* adapts to the nutrition-limited and immune-responsive environment in tumors. An in-depth understanding of the intricate relationship between the TME and the adaptation of colonized bacteria may provide hints to unlock the full potential of bacterial therapy against cancers.

It is well-established that the innate and adaptive immunity are sequentially activated upon bacterial infection in humans. However, even before the innate immune response commences, a critical defense mechanism known as “nutritional immunity” serves as the first line of protection to hinder the bacterial infection. Nutritional immunity is employed by the host organism through restricting the availability of essential nutrients to the invading pathogens (Weinberg 1975; Murdoch and Skaar 2022). For example, humans have evolved specialized proteins to chelate trace minerals, such as iron, zinc, and manganese, keeping their free-form concentrations at very low levels within the body (Andrews and Schmidt 2007; Kambe et al. 2015; Roth et al. 2013). In response, pathogens have counter-evolved mechanisms to evade nutritional immunity. For instance, a variety of siderophores are developed by bacteria to acquire ferric ions from the host (Kramer et al. 2020). Intriguingly, humans have further adapted by evolving siderophore-sequestering proteins, such as lipocalin 2 (LCN2), for bacterial inhibition (Singh et al. 2015; Bachman et al. 2009). These co-evolutionary events highlight the importance of nutritional immunity in combating bacteria (Golonka et al. 2019). Previous studies on bacterial cancer therapy have rarely examined the role of nutritional immunity, which may limit bacteria’s therapeutic efficacy.

In this work, we aimed to understand how bacteria modulate their physiological states at the molecular level in response to the tumor microenvironment. We employed liquid chromatography-tandem mass spectrometry (LC-MS/MS) for a quantitative comparison of the proteome between *E. coli* cultured in nutrient-rich medium and *E. coli* colonized in tumors. We found that *E. coli* colonized in tumors dramatically increases the protein expressions involving in the enterobactin biosynthesis and the iron ion homeostasis. This finding suggested that *E. coli* was stressed in an iron-deficient environment. Driven by this discovery, we hypothesized that enhancing the iron acquisition ability of *E. coli* might improve its anti-tumor activity. We engineered *E. coli* with enhanced iron scavenging capacity by introducing LCN2 blockers, including cyclic-di-GMP and glycosylated enterobactin. The engineered bacteria evaded the nutritional immunity and achieved complete tumor emission in mouse models, highlighting strategy for improving anti-tumor bacterial therapy through circumvention of nutritional immunity.

Results

Tumor is an iron-deficient microenvironment for bacterial colonization

While many bacteria are known to colonize and proliferate in the TME, it remains elusive how bacteria adapt to such a nutrition-limited environment as compared to a nutrition-rich one. To this end, we performed quantitative LC-MS/MS experiments to compare the proteome of *E. coli* colonized in the murine tumors or cultured in the rich medium (LB broth) (Fig. S1). Not surprisingly, there are many more proteins enriched in the rich medium than in the tumor condition. The Venn diagram and the volcano blot revealed hundreds of protein IDs enriched in the rich medium condition (Fig. 1a, 1b, Table S1). The Gene Ontology(GO)-term analysis revealed that many of these proteins are associated with the machineries of biosynthetic processes, cell division, and energy production (Fig. 1c), reflecting that the *E. coli* was under a highly proliferative state in the rich medium. Interestingly, there were also 71 proteins that were preferentially expressed in the tumor condition over the rich medium condition. The GO-term analysis and the hierarchical clustering revealed that many of these proteins are involved in the processes of enterobactin synthesis and iron ion homeostasis (Fig. 1d, 1e, and Fig. S2). Fig. 1f showed that the individual proteins in these two processes were markedly up-regulated in the tumor condition. These proteins are known to be tightly controlled by the iron sensing system in *E. coli*, and only become up-regulated under the stress of iron deficiency (Seo et al. 2014). It is worth mentioning that while enterobactin facilitates the uptake of ferric ions into bacteria, the host immune cells can counteract by secreting a protein called LCN2, which possesses a specialized pocket to bind and sequester enterobactin (Fischbach et al. 2006). To investigate this possibility, we employed LC-MS/MS and analyzed the LCN2 expression in the tumors with or without *E. coli* colonization. Indeed, we observed a significant up-regulation of LCN2 expression in the tumors with *E. coli* inoculation (Fig. 1g). Collectively, our data suggest that tumor is an iron-deficient environment for *E. coli* colonization.

Cyclic-di-GMP-producing *E. coli* synergizes with iron chelators for cancer therapy

Based on the proteomic findings, we aimed to focus our therapeutic strategies on modulating the iron competition between bacteria and cancer cells in the tumor. First, we hypothesize that an iron chelator that lowers the effective pool concentration of iron may provide a selection pressure, which disfavors the growth of cancer cells over bacteria. We tested three iron chelators, deferoxamine, ciclopirox, and VLX600, which have been approved for clinical trials or medical applications (Lang et al. 2019; Qi et al. 2020; Fryknas et al. 2016). All three chelators showed high cytotoxicity toward the cancer cells, suggesting the essential role of irons for the survival of mammalian cells (Fig. 2a). On the other hand, *E. coli* can better tolerate these iron chelators at relatively high concentrations. Among them, VLX600 was the most potent drug against the cancer cells (IC₅₀ = 0.33 μM) and provided the largest therapeutic window (280-fold difference) between the cancer cells and *E. coli*. In addition to the iron chelator, we also attempted to find approaches for counteracting the enterobactin sequestering function of LCN2. It has been reported that cyclic di-GMP (CDG) can block the binding between LCN2 and enterobactin, therefore restoring the functionality of enterobactin (Li et al. 2015). In our bacterial culture study, we validated that the addition of CDG can enhance bacteria survival in the presence of LCN2 (Fig. S3), thus prompting our effort to prepare CDG-expressing bacteria. We engineered the *E. coli* by introducing a plasmid that carries the gene of diguanylate cyclase (DGC), an enzyme responsible for catalyzing the biosynthesis of cyclic di-GMP (CDG) (Lv et al. 2019). We showed that the *E. coli* transformed with the DGC plasmid (hereafter referred to DGC-*E. coli*) actively synthesized CDG and secreted it into

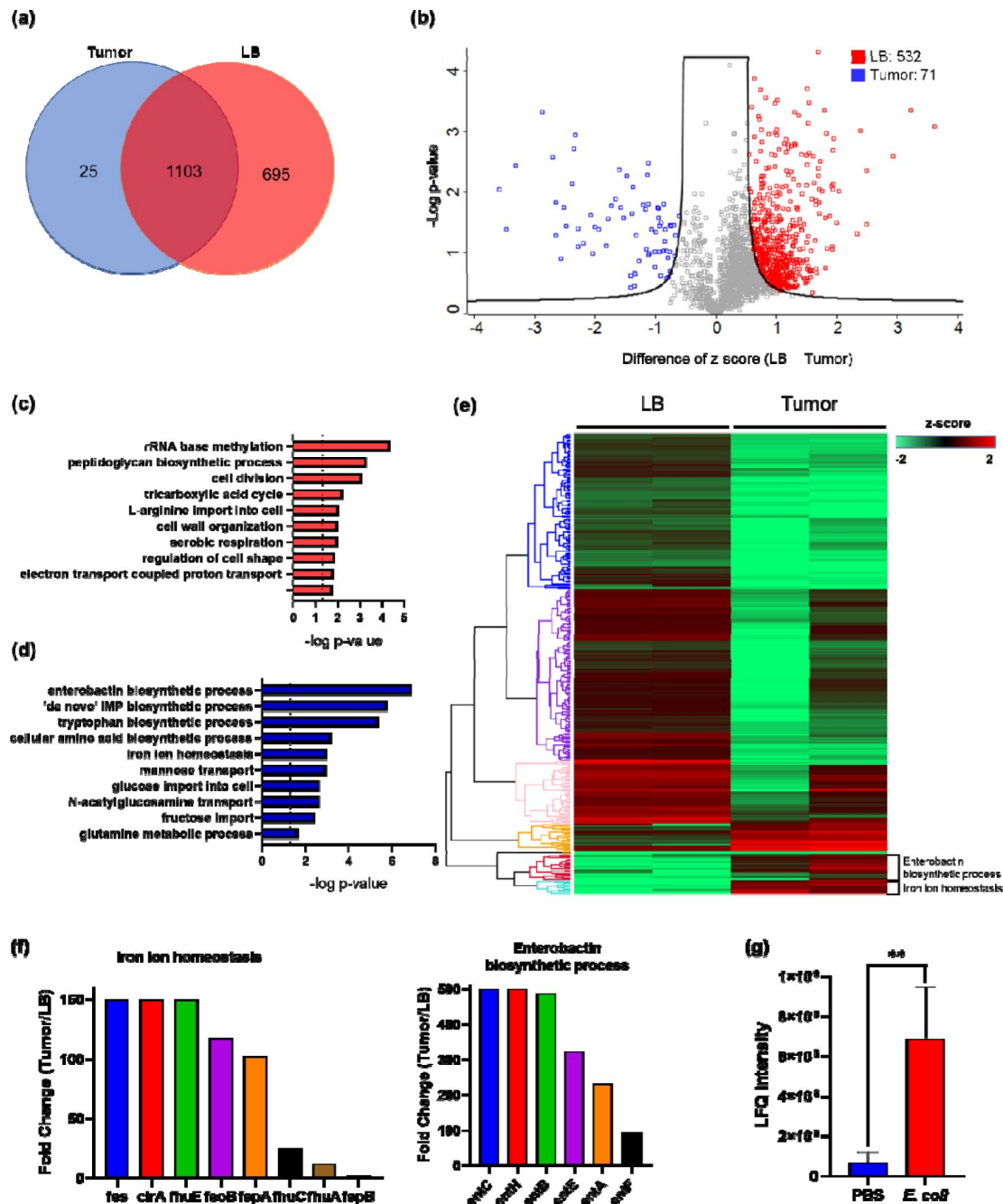


Figure 1.

The quantitative proteomic analysis of *E. coli* in the rich medium and the tumor microenvironment.

(a) The Venn diagram of the *E. coli* protein IDs identified in the rich medium and in the TME. (b) The volcano plot of the *E. coli* protein IDs quantified in the rich medium and in the TME. (c) The GO-term analysis of the protein IDs enriched in the rich medium condition. (d) The GO-term analysis of the protein IDs enriched in the tumor condition. (e) The hierarchical clustering analysis of the protein IDs identified in the rich medium and in the TME. Each column is a biological replicate. (f) Left: the fold changes of individual proteins in the iron ion homeostasis process. These proteins are involved in transporting or processing the iron ions. Right: the fold changes of individual proteins in the enterobactin biosynthesis process. (g) Label-free quantification of LCN2 in the tumors with and without *E. coli* inoculation. The error bars represent mean $\pm$ SD. Statistical analyses were performed by Student's T test (** $p < 0.01$).

the supernatant as detected by the LC/MS-MS mass spectrometry (**Fig. 2b**). It is worth noting that CDG is also a potent ligand for the STING pathway, which can stimulate anti-tumor immunity (Diner et al. 2013; Krasteva and Sondermann 2017; Chattopadhyay et al. 2020). When applying the DGC-*E. coli* supernatant to the macrophages, the macrophages enhanced the IFN- β secretion, indicating the activation of the STING pathway by CDG (**Fig. 2c**).

We evaluated the anti-tumor activity of VLX600 and DGC-*E. coli* in a syngeneic mouse model. The MC38 tumor-bearing mice received VLX600 and/or DGC-*E. coli* as depicted in **Fig. 2d**. The VLX600 monotherapy only marginally suppressed the tumor growth as compared to the PBS control (**Fig. 2e**). The DGC-*E. coli* monotherapy, although inhibited the tumor progression to a certain degree, did not show superior efficacy than the wild-type *E. coli* without the DGC plasmid transformation. Strikingly, the combination of DGC-*E. coli* and VLX600 showed significantly improved efficacy as compared to the individual mono-therapies. The tumor sizes were greatly suppressed, and two out of the four mice achieved complete remission (**Fig. 2e** and **2f**). Of note, the combination of the wild-type *E. coli* and VLX600 did not result in such an improved activity, indicating that the CDG expression contributed to bacteria's synergism with the iron-chelating VLX600. The combination of CDG-expressing bacteria and VLX600 also established robust anticancer adaptive immunity as the mice cured by the combinatorial therapy showed no tumor development in a rechallenge study (**Fig. S4**). These results highlight the benefit of enhancing anticancer bacterial therapy through nutritional immunity manipulation.

Salmochelin-secreting *E. coli* significantly impeded tumor growth

Encouraged by the results shown above, we sought to engineer *E. coli* with a more specific iron scavenging functionality to assess its benefit against nutritional immunity. In nature, bacteria have evolved a strategy to block LCN2 through the expression of glycosylated enterobactin or salmochelin (Fischbach et al. 2006). Some pathogenic *E. coli* strains carry a gene cluster called IroA, which consists 5 genes to perform enterobactin glycosylation and processing. The sugars on the enterobactin create steric hindrance to the LCN2 pocket, thereby abolishing LCN2 binding. To investigate this effect, we cloned the IroA cluster into a plasmid and transformed it into a non-IroA-carrying *E. coli* strain BL21(DE3). A non-enterobactin-expressing Δ entE-*E. coli* strain, which is particularly susceptible to LCN2 binding, was employed for comparison. We incubated the *E. coli* with varying concentrations of LCN2 and measured their viability by colony formation assay. **Fig. 3a** shows that the *E. coli* without the IroA plasmid transformation (referred as WT-*E. coli*) was sensitive to LCN2, whereas the *E. coli* transformed with the IroA plasmid (referred as IroA-*E. coli*) was significantly more resistant to LCN2. The IroA-*E. coli* also showed stronger potency in acquisition of the iron ions than the WT-*E. coli* while the LCN2 was presented in the environment (**Fig. 3b**). In line with this observation, we found that the enterobactin (including the glycosylated form) extracted from the IroA-*E. coli* was more cytotoxic to the cancer cells than that extracted from the WT *E. coli* (**Fig. 3c**).

In mouse models of colon, breast, and melanoma cancers, the IroA-*E. coli* was significantly more efficacious than the WT-*E. coli*. In a MC38 mouse colon cancer model, six out of ten mice treated by IroA-*E. coli* achieved complete remission, whereas none of the mice treated by the WT-*E. coli* experienced a cure (**Fig. 3d–3f**, and **Fig. S5a**). In two other mouse models of E0771 breast cancer and B16F10 melanoma, the IroA-*E. coli* demonstrated improved anti-tumor ability as compared to WT bacteria (**Fig. 3g–3j**, and **Fig. S5b–5c**). It should be pointed out LCN2 expression has been shown to elevate the aggressiveness of breast (Yang et al. 2009), melanoma (Adler et al. 2023), and colon cancers (Chaudhary et al. 2021), suggesting that WT bacteria may show reduced anticancer activity in more aggressive cancer types due to higher iron competition. Overall, we showed that the IroA cluster equips *E. coli* with an effective iron-scavenging capability, exerting a potent anti-tumor effect in the LCN2-rich tumor microenvironment.

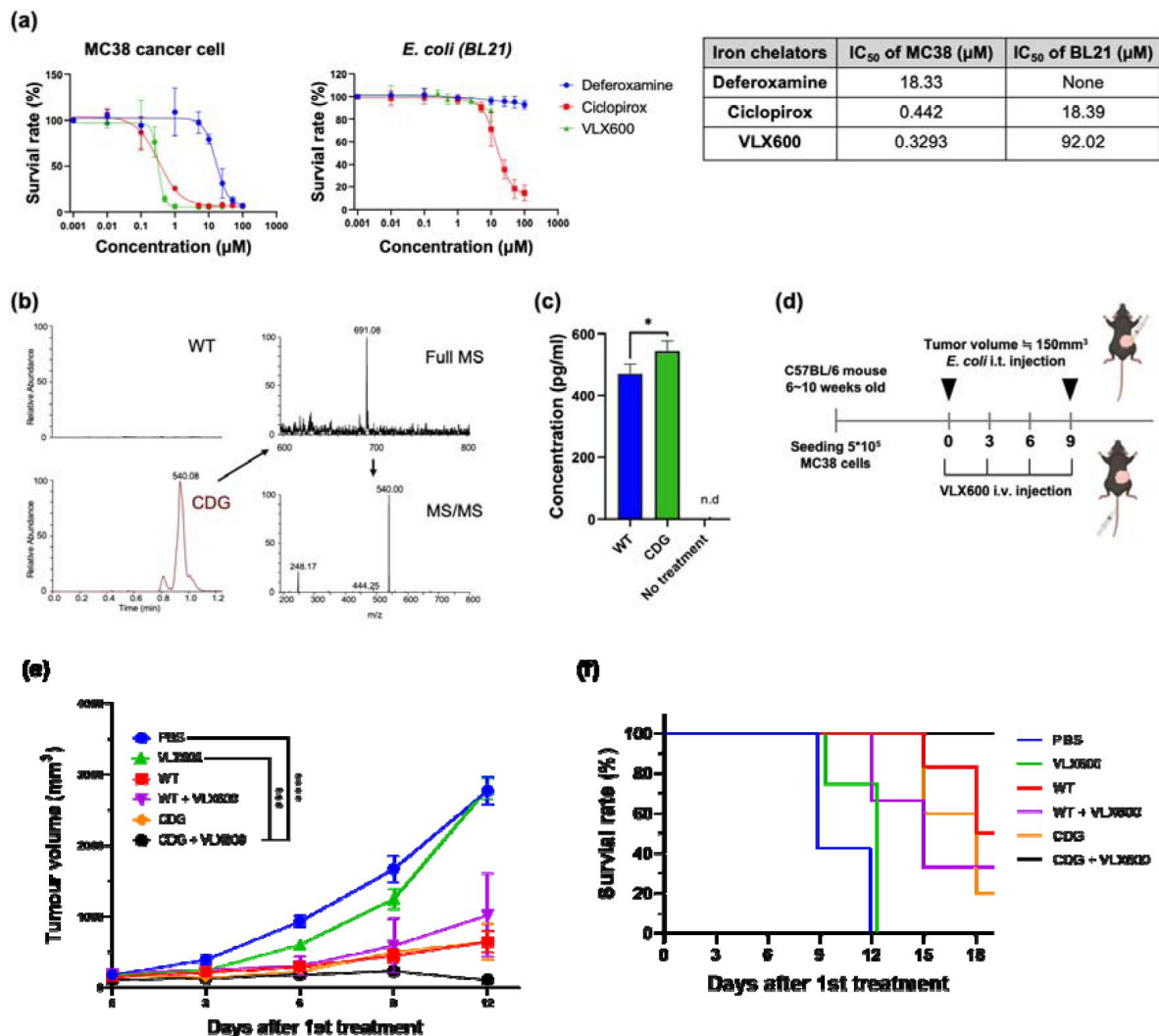


Figure 2.

Combination of iron chelator and DGC-*E. coli* for cancer therapy.

(a) Toxicity profiles of various iron chelators against MC38 cancer cells and *E. coli*. (b) Identification of cyclic-di-GMP secretion from DGC-*E. coli* by LC-MS/MS. The precursor ion and the fragmented product ions correspond to the correct molecule weights of CDG. (c) IFN- β secretion by RAW264.7 cells treated with the supernatants from wild-type *E. coli* or DGC-*E. coli*. (d) Schematic illustration of mouse treatments. The DGC-*E. coli* was intratumorally delivered on Day 0 and Day 9, whereas VLX600 was intravenously administrated every three days from Day 0 to Day 9. (e) Tumor growth curve for various treatment groups. The complete remission was only achieved in the CDG+VLX600 group (CR=2/4). (f) The Kaplan-Meier analysis for different treatment groups. The mouse was considered dead when the tumor volume exceeded $1,500 \text{ mm}^3$. The error bars represent mean $\pm$ SD. Statistical analyses were performed by Student's T test (* $p < 0.05$).

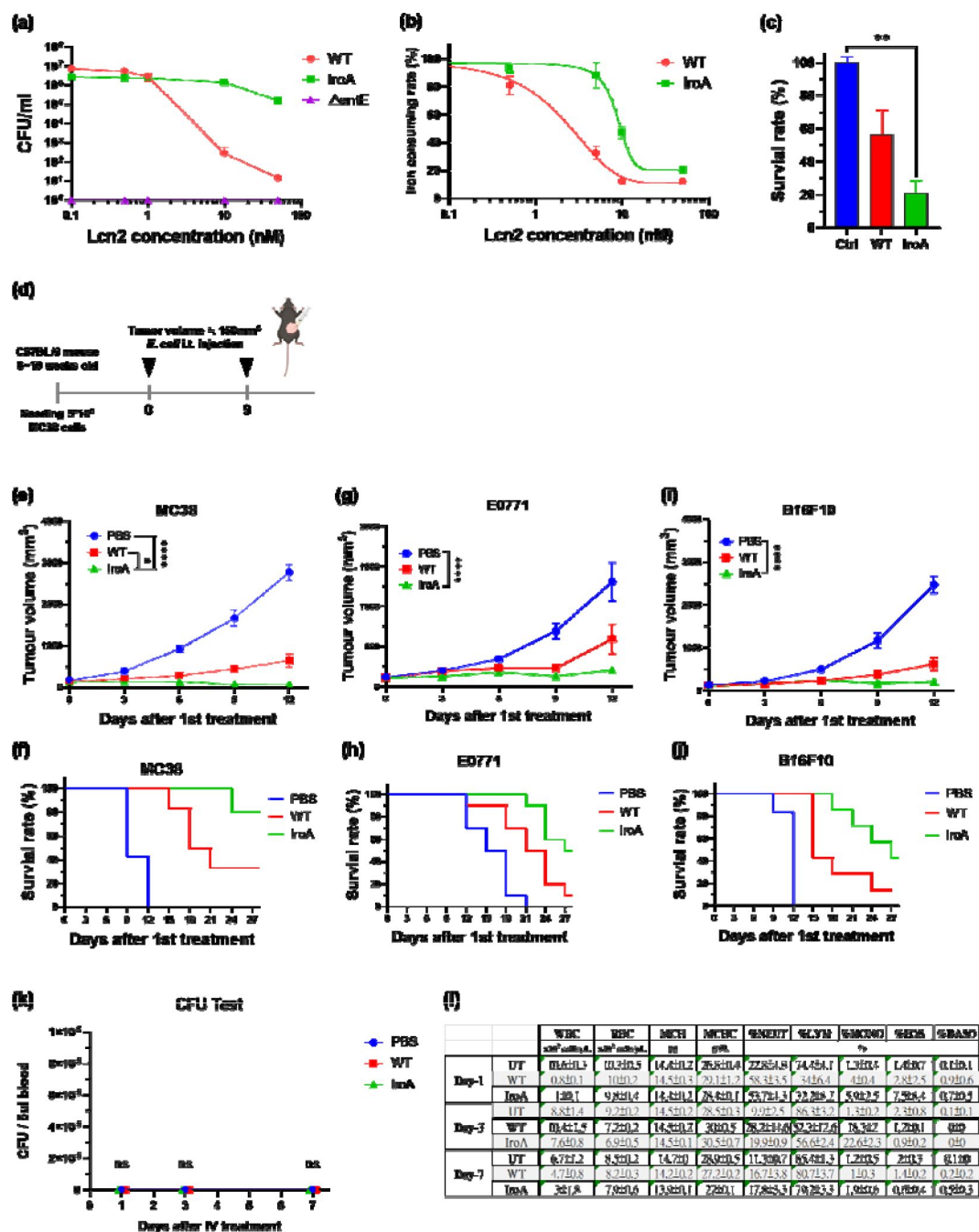


Figure 3.

Characterization of IroA-E. coli for anti-tumor activity

(a) *E. coli* viability in varying concentrations of LCN2 protein. The ΔentE strain, which could not generate enterobactin, was used as a negative control. (b) Iron consuming ability of *E. coli* determined by the CAS assay reagent. (c) Cytotoxicity of enterobactin on the MC38 colon cancer cells. The enterobactin was extracted from an equal supernatant volume of the WT-*E. coli* or the IroA-*E. coli* culture. The extraction buffer (DMSO) was used as a negative control. (d) Treatment schedule of IroA-*E. coli* in tumor-bearing mice. Two intratumoral injections were administered on Day 0 and Day 9. (e) Tumor growth curves across various treatment groups. (f) The Kaplan-Meier analysis for the mice in different treatment groups. (g) E0771 breast tumor growth curves for the different treatment groups. (h) Survival curves for mice in different treatment groups. (i) B16F10 melanoma tumor growth curves for the different treatment treatment groups. (j) Survival curves for mice in the different treatment groups. (k) The bacterial burden from the blood of mice on day 1, 3, and 7 following intravenous administration with different bacteria. (l) Whole blood cell analyses for the different treatment groups. The error bars represent mean ± SD. Statistical analyses were performed by One-way ANOVA (** *p* < 0.01).

To evaluate the safety of IroA-*E. coli* in comparison to WT bacteria, we intravenously administered the bacteria and conducted serial whole blood analyses on days 1, 3, and 7 post-injection to assess bacterial burden and blood cell counts (**Fig 3k** [3l](#) [3m](#)). For both WT-*E. coli* and IroA-*E. coli*, bacterial burden was undetectable in the blood. Additionally, whole blood cell analysis indicated that IroA-*E. coli* did not adversely affect the immune system within the circulatory system compared to WT-*E. coli*. By day 7, all treatment groups had comparable blood cell counts to the untreated (UT) groups. These findings demonstrate that IroA-*E. coli* could enhance anti-tumor treatment without incurring additional risks of bacteremia or sepsis relative to WT-*E. coli*.

IroA-*E. coli* is less iron-deficient than WT-*E. coli* in the tumor microenvironment

Because the salmochelin secreted by IroA-*E. coli* is resistant to the sequestration of LCN2, we speculated that IroA-*E. coli* could have ameliorated the iron deficiency problem in the TME. To verify this speculation, we quantitatively compared the proteome of WT-*E. coli* and IroA-*E. coli* colonized in the tumors (**Fig. 4a** [4b](#) [4c](#)). The GO-term analysis revealed that the many proteins enriched in WT-*E. coli* belong to the enterobactin biosynthesis process and the iron homeostasis. **Fig. 4b** [4c](#) and **Fig. 4c** [4d](#) showed the fold changes of the individual proteins in these two terms. Strikingly, all of these proteins were expressed at a much higher level in WT-*E. coli* than in IroA-*E. coli*. These results suggest that IroA-*E. coli* was less stressed by the iron deficient environment in the TME, which is in accordance with IroA-*E. coli*'s superior iron scavenging ability from the glycosylated enterobactin. Besides the proteome of *E. coli*, we also examined the proteome changes in the host cells. We found that two iron-related proteins in mice, transferrin and transferrin receptor, were elevated in the IroA-*E. coli* treatment as compared to the WT-*E. coli* treatment (**Fig. 4d** [4e](#)). These two proteins are known to be up-regulated when the cells sense a lack of iron in the environment (Ponka and Lok 1999 [4f](#); Theil 1990 [4g](#); Ponka et al. 2015 [4h](#)). Our finding corroborates a competitive scenario between the host cells and *E. coli* where the potent acquisition of ferric iron by IroA-*E. coli* posed an iron-deficient stress to the host cells.

Tumor suppression by iron-scavenging IroA-*E. coli* establishes anticancer adaptive immunity

Given that the IroA-*E. coli* treatment achieved complete tumor remission in multiple tumor models, we sought to investigate whether it also triggered adaptive immune responses against cancers. For the MC38-burdened mice that achieved complete tumor remission upon IroA-*E. coli* treatment, the mice were re-challenged with MC38 cancer cells 6 weeks following tumor eradication. The absence of tumor growth upon rechallenge indicates establishment of robust anticancer adaptive immunity (**Fig. 5a** [5b](#) [5c](#)), which led us to further analyze the tumor-infiltrating lymphocytes (TILs) in the mice treated by PBS, WT-*E. coli*, IroA-*E. coli*. We found that the TILs, especially the CD8⁺ T cells, were elevated in the IroA-*E. coli* group compared to the WT-*E. coli* group (**Fig. 5b** [5c](#)), highlighting the superior capacity of IroA-*E. coli* treatment towards enhancing CD8⁺ T cell infiltration into the tumor microenvironment. The enhanced CD8⁺ T cell infiltration can be attributed to the prolonged intratumoral colonization by the IroA-*E. coli* (**Fig. S6** [S7](#)), which can confer sustained immune stimulation for immune cell recruitment. We further performed a CD8⁺ T-cell depletion study, which showed that the anti-tumor activity of IroA-*E. coli* was partially weakened upon anti-CD8 depletion (**Fig. 5c** [5d](#)). These results demonstrate that the robust tumor suppression by the IroA-*E. coli* therapy can be attributed to in part to the elicitation of tumor-specific cytotoxicity T cells, which can in turn provide durable anticancer immunity following bacterial clearance.

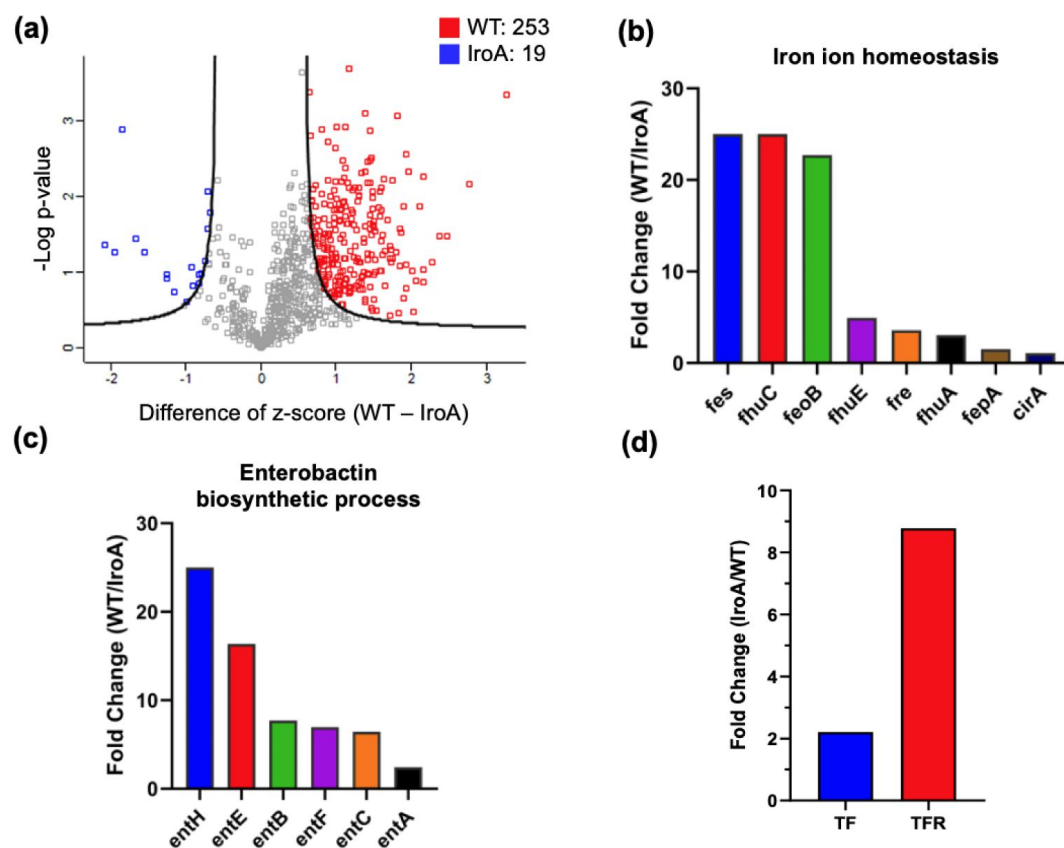


Figure 4.

Quantitative proteomic analysis comparing IroA-*E. coli* and WT-*E. coli* in the TME.

(a) Volcano plot analysis between the proteomes of WT-*E. coli* and IroA-*E. coli* in the mouse tumors. (b) Fold changes of the proteins involved in the iron ion homeostasis. All the fold changes are > 1. (c) Fold changes of the proteins involved in the enterobactin biosynthetic process. All the fold changes are > 1. (d) Fold changes of transferrin and transferrin receptor in the tumor.

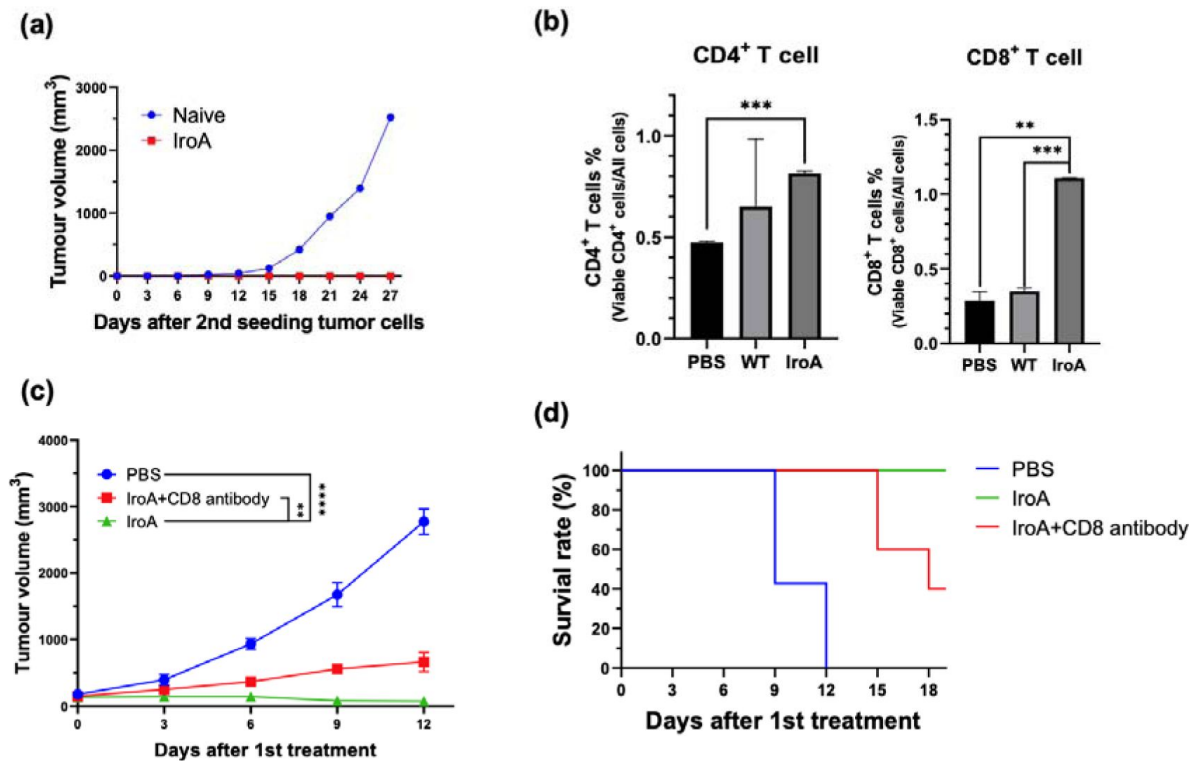


Figure 5.

IroA-*E. coli* treatment stimulated the adaptive immune system for anti-tumor activity.

(a) The mice cured by IroA-*E. coli* were re-challenged with a subcutaneous inoculation of 2.5×10^5 MC38 cells. No tumor formation was observed. The naïve mice were used as controls. (b) The proportions of tumor-infiltrating CD4⁺ and CD8⁺ T cells in different treatment groups. (c) The tumor-bearing mice were treated with IroA-*E. coli* in the presence or absence of the anti-CD8 depletion antibody. (d) Survival curves of mice in different treatment groups. The error bars represent mean $\pm$ SD. Statistical analyses were performed by One-way ANOVA (** $p < 0.01$, *** $p < 0.001$).

Systemic delivery of IroA-*E. coli* and oxaliplatin show synergistic tumor suppression

It has been reported that *E. coli* possesses great tumor targeting ability following intravenous injection in mice due to the hypoxic and immune-suppressive tumor microenvironment. Our previous study has also shown that the combination of *E. coli* and oxaliplatin synergistically suppresses the tumor growth (Lim et al. 2024 [DOI](#)). We therefore attempted to apply IroA-*E. coli* and oxaliplatin for cancer treatment using a systemic delivery approach as depicted in Fig 6a [DOI](#). All mice maintained over 90% of their weights and remained active and healthy during the course of the treatments (Fig. 6b [DOI](#)). Unlike the intratumoral injection, the intravenous injection of *E. coli*, either the wild-type or the IroA transformant, resulted in subdued anti-tumor efficacy (Fig. 6c [DOI](#)–6e [DOI](#)). Also, the monotherapy of oxaliplatin only slightly inhibited the tumor growth. The combination of oxaliplatin with the wild-type *E. coli* delayed the tumor progression but did not achieve complete remission. Remarkably, the combination of oxaliplatin and IroA-*E. coli* significantly suppressed the tumor growth and achieved complete remission in 1 out of 4 mice. Overall, our data revealed that the systemic delivery of IroA-*E. coli* was synergistic with the oxaliplatin chemotherapy in the mouse tumor models.

Discussion

There are accumulating studies that apply bacteria as drug delivery vehicles for cancer therapy with various payloads, including toxins, cytokines, immune checkpoint inhibitors, etc. However, in order to optimize the therapeutic outcomes of these engineering endeavors, it is also very important to understand the bacterial adaptation in the TME. The growth conditions differ vastly between the *in vitro* cultivation and the intratumoral environments. The rich medium provides ample nutrients for the optimal bacterial growth and payload production, whereas tumor is a nutrient-deprived, acidic, and hypoxic environment, which may stress the bacteria and restraint the engineered functionality. Also, unlike the *in vitro* cultivation, bacteria face the competition from the cancer cells as well as the surveillance from the host's immune system. This work provides the first proteomic data comparing *E. coli* cultured in the rich medium and colonized in the mouse tumor. The results show that the TME is an iron-deficient environment for *E. coli*. Moreover, upon the bacterial inoculation, the host cells up-regulate LCN2 to further block the iron uptake of *E. coli* by enterobactin (Singh et al. 2015 [DOI](#); Bachman et al. 2009 [DOI](#)). This observation inspired us to develop *E. coli* that overcame the nutritional immunity from the host. This discovery-driven design, especially the IroA implementation, proves to be highly effective in enhancing the anti-tumor activity of *E. coli*. It is of interest to investigate that if the iron uptake ability is also critical for other types of bacteria when applied in cancer therapy.

In addition to the enterobactin biosynthetic process and the iron ion homeostasis, our proteomic data also revealed other insights into the key nutrients whose scarcity within the TME may impede the bacterial growth. For examples, the proteins involving “the *de novo* synthesis of IMP” are highly enriched in the *E. coli* grown in tumors compared to the rich medium condition. Inosine phosphate (IMP) is the precursor of purine. When the environment is deficient of purine, the bacteria need to synthesize them using *de novo* pathways (Rolfes and Zalkin 1988 [DOI](#); Cho et al. 2011 [DOI](#); Meng et al. 1990 [DOI](#)). A previous report by Samant et al. has shown that the genes associated with the *de novo* purine synthesis are the most critical factors for the growth of *E. coli* or other gram-negative bacteria in human serum (Samant et al. 2008 [DOI](#)). Their data indicate that, similar to iron, humans also control the purine at a very low level in blood as a strategy of nutritional immunity in order to restraint the proliferation of bacteria. In light of these findings, one potential avenue could involve engineering *E. coli* to bolster its ability for *de novo* nucleotide

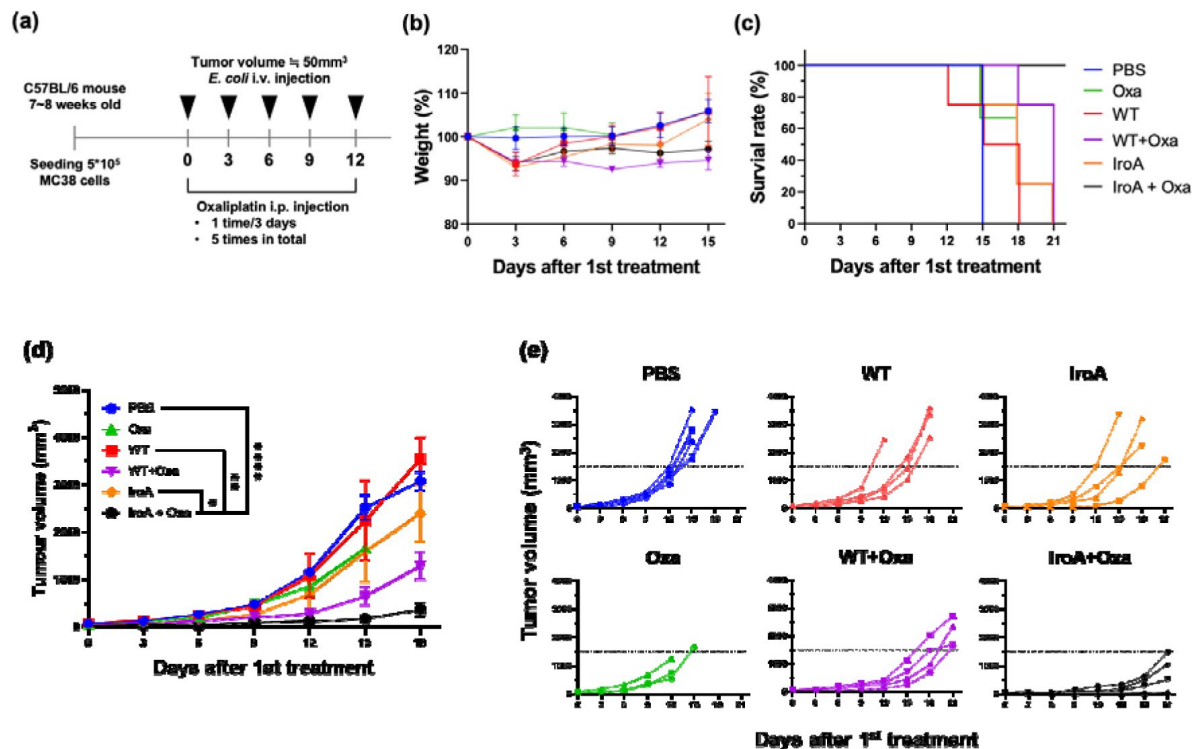


Figure 6.

Synergistic anti-tumor activity of IroA-*E. coli* and oxaliplatin.

(a) The scheme of the systemic delivery of IroA-*E. coli* and oxaliplatin in the tumor-bearing mice. (b) The alteration of mouse weights during the treatment course. (c) Survival curves of the mice in various treatment groups. (d) The average tumor growth curves of different treatment groups. (e) The tumor growth curves of individual mice in (d). The error bars represent mean $\pm$ SD. Statistical analyses were performed by One-way ANOVA (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$).

biosynthesis, therefore facilitating better adaptation to the TME. Given that bacteria and cancer cells vie for growths within the TME, an enhanced bacterial adaptation to the TME may potentially improve the anti-tumor activity.

Overall, our research revealed that the tug of war for iron plays a critical role when applying bacteria for cancer therapy. To aid the bacteria in this war, we have adopted several approaches, including the engineering of *E. coli* to secrete cyclic-di-GMP and salmochelin. These methods have demonstrated effectiveness in treating murine tumors, resulting in a significant portion of complete remission. Notably, the cured mice have also established durable anti-tumor adaptive immunity. Our iron-scavenging strategy opens new avenues by overcoming the nutritional immunity hurdles in the realm of bacteria-based cancer therapy.

Materials and methods

Cancer cell strain and cultivation

The MC38 murine colon cancer cells were cultured in DMEM supplemented with 10%FBS, 1% penicillin-streptomycin solution (100U/ml), 1mM sodium pyruvate, 10mM HEPES, and 1% MEM Non-Essential Amino Acids Solution (100X). RAW264.7 macrophage cells were cultured in DMEM supplemented with 10%FBS, and 1% penicillin-streptomycin solution (100U/ml). All cells lines were maintained in a humidified incubator at 37°C and 5% CO₂.

Mouse experiments

All animal experiments were conducted under specific pathogen-free conditions according to the guidelines approved by the Animal Care and Usage Committee of Academia Sinica. Mice were housed at temperature of 19-23°C with a 12-hour light-dark cycle and a humidity of 50-60%. A maximum of five mice were housed in a single individually ventilated cage with soft wood for nesting. Tumor dimensions were measured using a caliper, and tumor volume was calculated using the following formula: $0.52 \times ((\text{tumor length} + \text{tumor width})/2)^3$. Mice aged 6-10 weeks were subcutaneously injected with either 5×10^5 MC38 murine colon cancer cells, 5×10^5 E0771 breast cancer cells, or 1×10^5 B16F10 melanoma cancer cells suspended in 100 μ L PBS at the right flank. The *Escherichia coli* strain BL21(DE3) was cultured in LB medium at 37°C overnight. The overnight cultures were diluted 100-fold in fresh LB medium and incubated at 37°C for ~3 hours until the log phase. Prior to the intratumoral injection, the bacteria density was determined by measuring the OD600 (1 OD = 4×10^8 CFU/ml). For the proteomic experiment, the tumor-bearing mice were intratumorally injected with 4×10^8 BL21(DE3) in 50 μ L PBS when the tumor volumes reached approximately 150 mm³. The tumors were harvested the following day for the LC-MS/MS analysis. For the intratumor-injection-based therapeutic experiment, the tumor-bearing mice were treated when the tumor volume reached ~150 mm³. The tumor-bearing mice were intratumorally injected with 4×10^8 BL21(DE3) on day 0 and day 9. VLX 600 (4.5 mg/kg) was administered via intravenous injection every three days for a total of four injections. For the intravenous-injection-based therapeutic experiment, the tumor-bearing mice were intravenously injected with 1×10^8 BL21(DE3) in 100 μ L PBS every three days for four times. The Oxaliplatin (5 mg/kg) was administered intraperitoneally every three days for a total of five injections. For the CD8⁺ T cell depletion experiment, the mice received intraperitoneal injections of 100 μ g anti-mouse CD8 α antibodies (BioXCell, Cat# BE0061) on days -6, -2, 2, 6, and 10, along with the intratumoral injections of 4×10^8 BL21(DE3) on days 0 and 9. For tumor re-challenge study, MC38-burdened mice following bacterial therapy were subcutaneously injected with 5×10^5 MC38 murine colon cancer cells for monitoring. Naïve mice were challenged similarly as a control. Mice were considered dead and euthanized when the tumor reached a volume of 1,500 mm³.

Proteomics sample preparation

For the tumor-colonized bacteria, the tumors were excised, and homogenized, and processed to extract intratumoral bacteria cells. Red blood cells were removed using RBC lysis buffer. The mouse cells were removed through low-speed centrifugation at 1,200 g for 2 min three times. The *E. coli* was collected by centrifugation at 4,500 g for 20 min. The samples were lysed using 4% SDS, 100mM Tris-HCL (pH 9), and 1x protease inhibitor cocktail set III. The cell lysates were heated at 95°C for 5 minutes and sonicated for 15 min using a Bioruptor Plus (Diagenode). The supernatant was collected after centrifugation at 18,000 g for 30 min at 4°C. Approximately 50 µL supernatant was mixed with 200 µL methanol, 50 µL chloroform, and 150 µL double-distilled water. The aqueous phase was removed after sitting the sample at room temperature for 10 minutes. Subsequently, the sample was mixed with another 150 µL methanol. The pellet was collected, dried for 20 minutes, and resuspended in 8 M urea and 50 mM triethylammonium bicarbonate buffer. The samples were reduced with 10 mM DTT, alkylated with 50 mM IAA, and digested using LysC and trypsin. Following acidification, the supernatant was loaded onto the SDB-XC StageTips (Rappsilber et al. 2007 [\[1\]](#)) and eluted by 80% ACN containing 0.1%TFA. The sample was lyophilized and stored at -20°C before further LC-MS/MS analysis. For the bacteria grown in the rich medium, the *E. coli* strain BL21(DE3) was cultured in LB medium at 37°C overnight. The overnight culture was diluted 100-fold in fresh LB medium and incubated at 37°C until the log phase (OD=0.6). The bacteria were harvested by centrifugation and treated similarly to the bacteria harvested from tumors.

LC-MS/MS experiments

The sample was loaded onto the trap column (2 cm × 75 µm i.d., Symmetry C18), and then separated on a nanoACQUITY UPLC System (Waters, USA) equipped with a 25 cm × 75 µm i.d. BEH130 C18 column (Waters, USA) using a 5-35% buffer B (buffer A: 0.1% formic acid; buffer B: 0.1% formic acid in acetonitrile) gradient as the separation phase and a flow rate of 300 nl/min. The total running time was 120 min. The mass spectrometric data were collected on a high-resolution Q Exactive HF-X mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) operating in the data-dependent mode. Full MS resolution was set to 60,000 at 200 m/z and the mass range was set to 350–1600. dd-MS2 resolution was set to 15,000 at 200 m/z. Isolation width was set to 1.3 m/z. Normalized collision energy was set to 28%. The LC-MS/MS data were matched with the human SwissProt database using the Mascot search engine v.2.6.1 (Matrix Science, UK) with the following parameters: the mass tolerance of precursor peptide was set to 10 ppm, and the tolerance for MS/MS fragments was 0.02 Da.

Proteomics data processing and statistical analysis

Raw MS data were processed using MaxQuant version 2.0.1. Database search was performed with the Andromeda search engine through the Uniprot database(Cox et al. 2011 [\[2\]](#)). Both protein and peptide level were filtered by a 1% false discovery rate (FDR). The variable modification setting included oxidation(M) and Acetyl (Protein N-term), and the fixed modification setting included carbamidomethyl(C). The “match between runs” was set as 1 minute, and the MaxQuant LFQ algorithm was employed for normalization. The statistical analysis was performed using Perseus version 1.6.15.0 and Prism version 8.0.2(Tyanova et al. 2016 [\[3\]](#)). The proteinGroups output table from MaxQuant was utilized for proteomics analysis. The potential contaminant, reverse, and only-identified-by-site were filtered out. The LFQ-intensity was log2-transformed and filtered for validity. NaN values were imputed, and bacterial cell LFQ intensity was normalized using z-score. The LFQ intensity of bacterial cells was normalized using z-score (n-average/standard deviation). A t-test with an FDR of 0.05 and S0 of 1 was performed to extract significantly different proteins. These proteins were uploaded to the DAVID database for biological interpretation, and the results were visualized in Prism. The raw data of LFQ intensity for significantly different proteins were averaged to calculate the difference in protein expression level.

Enterobactin extraction

BL21(DE3) was cultured in the LB medium at 37°C overnight. The overnight culture was 100-fold diluted to the M9 medium supplemented with 0.2% casamino acids, 0.2% glucose, 1 mM MgSO₄, and 1 mg/mL vitamin B1 and grown for 20 hours. The bacteria were removed by centrifugation, and the supernatant was sterilized using a 0.22 µm filter. The cell-free supernatant was acidified to pH=2 using 10N HCl. An equal volume of ethyl acetate was added to the acidified supernatant and mixed using Intelli Mixer ERM-2L. The organic fraction was collected after 30 min incubation at room temperature and dried using a miVac centrifugal concentrator. All samples were resuspended in DMSO and stored at -20°C for further experiments.

Cytotoxicity assay of enterobactin

1×10^4 MC38 cells in a 96-well plate were treated with the enterobactin extracted from WT-*E. coli* or IroA-*E. coli* for 48 hours. The cell viability was measured using the Cell Counting Kit-8 (CCK-8) according to manufacturer's protocol. A cell-free mixture was used as a background reference, and the untreated cells were used as a control.

Characterization of cyclic-di-GMP (CDG) secreted from DGC-*E. coli*

The DGC plasmid carries the gene of the diguanylate cyclase fragment 82-248 residues from *Thermotoga maritima* with a single mutation of Arg158Ala. The BL21(DE3) bacteria transformed with the DGC plasmid were cultured in LB at 37°C overnight. The overnight cultures were 100-fold diluted to fresh LB supplemented with 0.1mM IPTG and kanamycin (50 µg/ml) and cultured at 37°C for 20 hours. The bacteria were pelleted, and the supernatant was collected and filtered using a 0.22 µm strainer. The supernatant was analyzed by LC-MS/MS to identify the CDG.

Interferon-β quantification

5×10^5 RAW264.7 cells in a 24-well plate were treated with the conditioned medium from DGC-*E. coli* or non-transformed *E. coli* for 18 hours. Subsequently, the cell culture medium was collected for IFN-β quantification. The IFN-β levels were measured using the Mouse IFN-beta ELISA kit (R&D, #P318019) following the manufacturer's protocol.

Iron chelating assay

The iron uptake ability of bacteria was determined using chrome azurol S (CAS) assay (Louden et al. 2011 [\[3\]](#)). The BL21(DE3) cells with or without IroA transformation were cultured in the M9 medium at 37°C overnight. The bacteria were collected by centrifugation and washed twice with PBS. 10^8 bacteria were inoculated into fresh M9 medium with CAS reagent and different concentrations of Lcn2 protein and incubated at 37°C overnight. The A630 of the bacterial cultured medium was measured to quantify the iron-consuming ability of the bacteria.

Lipocalin 2 resistance assay

The IroA-*E. coli* or non-transformed *E. coli* was cultured overnight in the LB medium. Next day, the bacterial culture was diluted to fresh RPMI supplemented with 10% FBS by 100-fold and incubated at 37°C for 5 hours to reach the log phase. 10^5 bacteria were treated with different concentrations of LCN2 and incubated at 37°C for 20 hours. The live bacteria were quantified by serial titration on LB agar plates.

Tumor-infiltrating lymphocyte (TIL) analysis

Tumor tissues were cut into small pieces in the digestion buffer (1mL RPMI supplemented with 10% FBS, 0.33 mg collagenase type IV (Sigma, Cat#C5138), and 66 µg DNase I from bovine pancreas (Cyrusbioscience, Cat#101-9003-98-9)) and transferred into a C tube followed by sample processing

using gentleMACS. The cell suspensions were filtered through a 40 μ m strainer. The RBC lysis buffer was added to the tumor suspension to remove red blood cells. The cells were then blocked using the CD16/32 Fc blocker for 5 minutes on ice. The T cells were stained with eFluor780 viability dye, PE-CD45, APC-CD4, and FITC-CD8a antibodies. The stained T cells were analyzed by flow cytometer (Attune NxT cytometer), and the data were processed using FlowJo software.

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Competing interests

The authors declare no conflict of interest.

Author contributions

S.W.H., K.Y.M., and C.M.H. conceived and designed the experiments. S.W.H., S.K.L., Y.A.Y., Y.C.P., and W.J.L. performed the experiments. S.W.H., K.Y.M., C.Y.M., and C.M.H. discussed the experimental results and wrote the manuscript. All authors provided clarification, guidance, and revision on the manuscript.

Supplementary Information

List of supplementary figures

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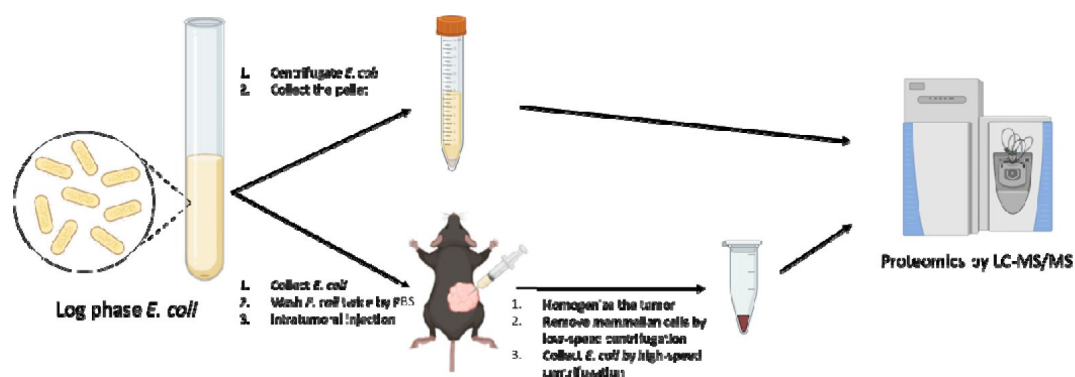


Figure S1.

Workflow of quantitative proteomics for *E. coli* cultured in rich medium and *E. coli* colonized in murine tumors.

The *E. coli* BL21(DE3) were harvested from rich medium and tumors, respectively, and subject to the LC-MS/MS analysis.

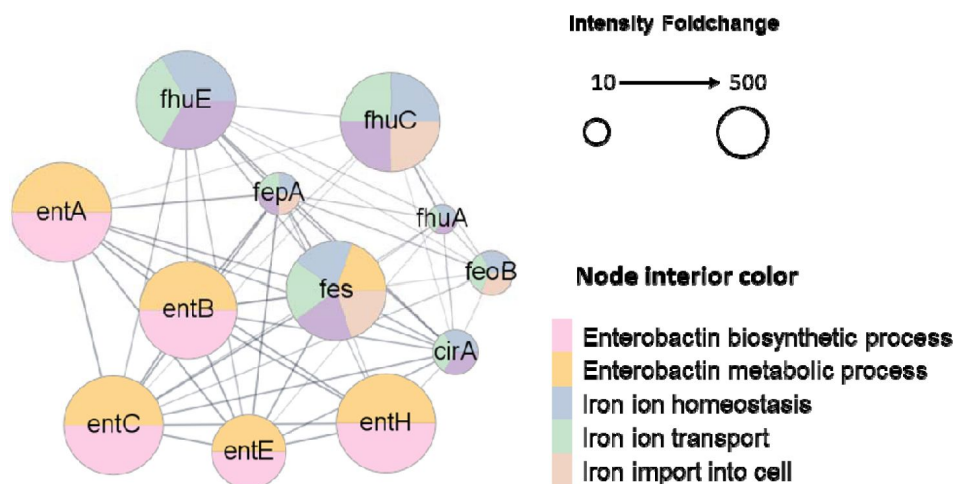


Figure S2.

STRING network analysis.

The proteins involving enterobactin biosynthesis and iron ion homeostasis were enriched in the *E. coli* colonized in tumors. The STRING diagram was drawn for the representative proteins in these two processes. The node size represents the fold change (tumor/rich medium) of the protein determined the label-free quantification method. The different interior colors of the nodes represent various biological processes defined by GO terms. The lines indicate the existence of experimental evidence between the connected nodes.

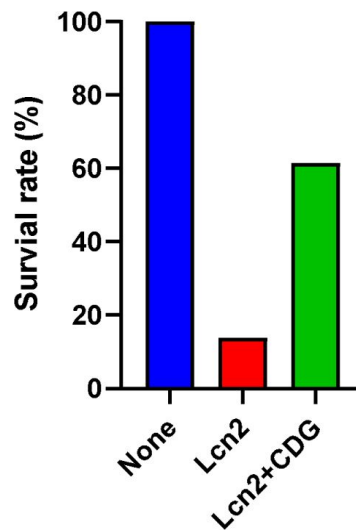


Figure S3.

High concentration of cyclic-di-GMP partially blocks LCN2.

E. coli (1×10^4) were cultured in RPMI medium supplemented with LCN2 at 0.15 μM and Cyclic-di-GMP at 7.5 μM overnight. The number of survival bacteria were determined by plating the overnight culture on the LB agar plates. The survival rate was normalized to the no-treatment group.

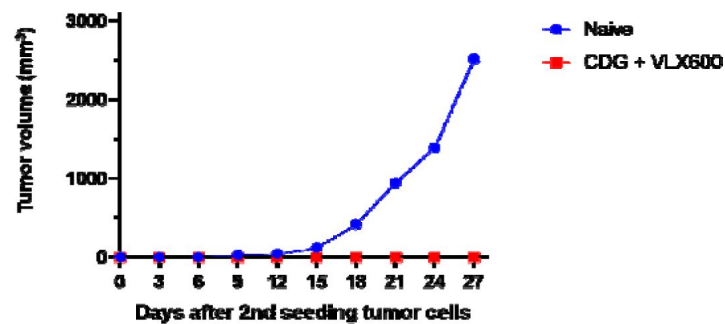


Figure S4.

Tumor re-challenge experiments in mice.

The mice cured by DGC-*E. coli* and VLX600 combination therapy were re-challenged with the same cell line (MC38) subcutaneously at the opposite flank. No tumor growth was observed. The naïve mice were used as controls.

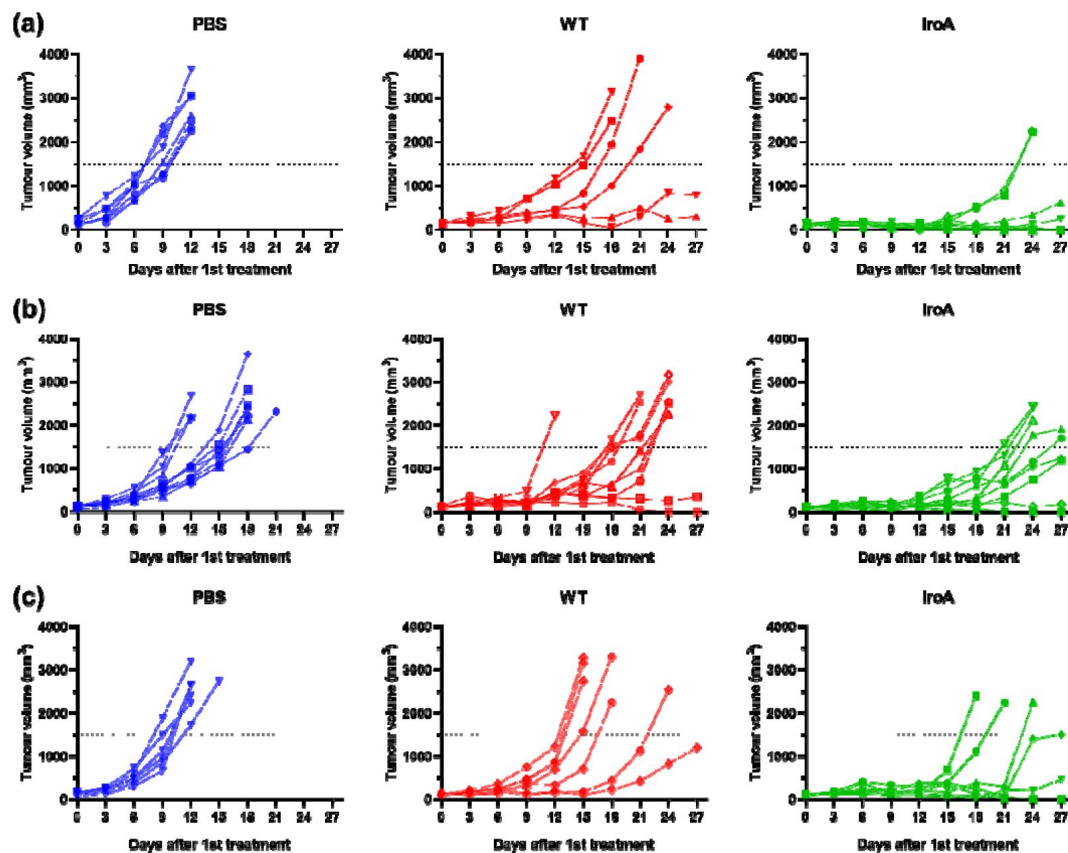


Figure S5.

The tumor growth curves of individual mice in MC38, E0771, B16F10 tumor models

(a) The tumor growth curves of individual mice in MC38 tumor model (b) The tumor growth curves of individual mice in E0771 tumor model (c) The tumor growth curves of individual mice in B16F10 tumor model

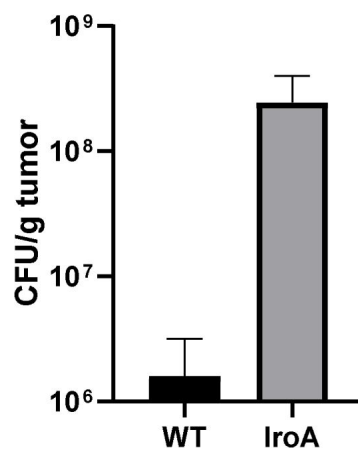


Figure S6.

IroA-*E. coli* treatment resulted in higher bacterial colonization in tumors as compared to WT bacteria.

The bacteria were intratumorally delivered to tumor-bearing mice for fifteen days. The tumors were collected and homogenized for measuring the bacteria number. The number of survival bacteria was determined by plating the overnight culture on the LB agar plates.

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Reviewer #1 (Public Review):

In this manuscript, Huang and colleagues explored the role of iron in bacterial therapy for cancer. Using proteomics, they revealed the upregulation of bacterial genes that uptake iron, and reasoned that such regulation is an adaptation to the iron-deficient tumor microenvironment. Logically, they engineered *E. Coli* strains with enhanced iron-uptake efficiency, and showed that these strains, together with iron scavengers, suppress tumor growth in a mouse model. Lastly, they reported the tumor suppression by IroA-E. *Coli* provides immunological memory via CD8⁺ T cells. In general, I find the findings in the manuscript novel and the evidence convincing.

(1) Although the genetic and proteomic data are convincing, would it be possible to directly quantify the iron concentration in (1) *E. Coli* in different growth environments, and (2) tumor microenvironment? This will provide functional consequence of upregulating genes that import iron into the bacteria.

(2) Related to 1, the experiment to study the synergistic effect of CDG and VLX600 (lines 139-175) is very nice and promising, but one flaw here is a lack of the measurement of iron concentration. Therefore, a possible explanation could be that CDG acts in another manner, unrelated to iron uptake, that synergizes with VLX600's function to deplete iron from cancer cells. Here, a direct measurement of iron concentration will show the effect of CDG on iron uptake, thus complementing the missing link.

(3) Lines 250-268: Although statistically significant, I would recommend the authors characterize the CD8⁺ T cells a little more, as the mechanism now seems quite elusive. What signals or memories do CD8⁺ T cells acquire after IroA-E. *Coli* treatment to confer their long-term immunogenicity?

(4) Perhaps this goes beyond the scope of the current manuscript, but how broadly applicable is the observed iron-transport phenomenon in other tumor models? I would recommend the authors to either experimentally test it in another model, or at least discuss this question.

<https://doi.org/10.7554/eLife.90798.2.sa2>

Reviewer #2 (Public Review):

Summary:

The authors provide strong evidence that bacteria, such as *E. coli*, compete with tumor cells for iron resources and consequently reduce tumor growth. When sequestration between LCN2 and bacterobactin is blocked by upregulating CDG(DGC-*E. coli*) or salmochelin(IroA-*E.coli*), *E. coli* increase iron uptake from the tumor microenvironment (TME) and restrict iron availability for tumor cells. Long-term remission in IroA-E.*coli* treated mice is associated with

enhanced CD8⁺ T cell activity. Additionally, systemic delivery of IroA-E.coli shows a synergistic effect with chemotherapy reagent oxaliplatin to reduce tumor growth.

Strengths:

It is important to identify the iron-related crosstalk between E. coli and TME. Blocking lcn2-bacterobactin sequestration by different strategies consistently reduce tumor growth.

Weaknesses:

As engineered E.coli upregulate their function to uptake iron, they may increase the likelihood of escaping from nutritional immunity (LCN2 becomes insensitive to sequester iron from the bacteria). Would this raise the chance of developing sepsis? Do authors think that it is safe to administrate these engineered bacteria in mice or humans?

<https://doi.org/10.7554/eLife.90798.2.sa1>

Reviewer #3 (Public Review):

Summary:

Based on their observation that tumor has an iron-deficient microenvironment, and the assumption that nutritional immunity is important in bacteria-mediated tumor modulation, the authors postulate that manipulation of iron homeostasis can affect tumor growth. This paper uses straightforward in vitro and in vivo techniques to examine a specific and important question of nutritional immunity in bacteria-mediated tumor therapy. They are successful in showing that manipulation of iron regulation during nutritional immunity does affect the virulence of the bacteria, and in turn the tumor. These findings open future avenues of investigation, including the use of different bacteria, different delivery systems for therapeutics, and different tumor types. The authors were also successful in addressing the reviewer's concerns adequately.

<https://doi.org/10.7554/eLife.90798.2.sa0>

Author response:

The following is the authors' response to the previous reviews.

Reviewer #1 (Public Review):

In this manuscript, Huang and colleagues explored the role of iron in bacterial therapy for cancer. Using proteomics, they revealed the upregulation of bacterial genes that uptake iron, and reasoned that such regulation is an adaptation to the iron-deficient tumor microenvironment. Logically, they engineered E. Coli strains with enhanced iron-uptake efficiency, and showed that these strains, together with iron scavengers, suppress tumor growth in a mouse model. Lastly, they reported the tumor suppression by IroA-E. Coli provides immunological memory via CD8⁺ T cells. In general, I find the findings in the manuscript novel and the evidence convincing.

(1) Although the genetic and proteomic data are convincing, would it be possible to directly quantify the iron concentration in (1) E. Coli in different growth environments, and (2) tumor microenvironment? This will provide the functional consequences of upregulating genes that import iron into the bacteria.

We appreciate the reviewer's comment regarding the precise quantification of iron concentrations. In our study, we attempted various experimental approaches, including Immunohistochemistry utilizing an a Fe³⁺ probe, iron assay kit (ab83366), and Inductively Coupled Plasma Mass Spectrometry (ICP-MS). Despite these attempts, the quantification of oxidized Fe³⁺ concentrations proved challenging due to the inherently low levels of Fe ions and difficulty to distinguish Fe²⁺ and Fe³⁺. We observed measurements below the detection threshold of even the sensitive ICP-MS technique. To circumvent this limitation, we designed an experiment wherein bacteria were cultured in a medium supplemented with Chrome Azurol S (CAS) reagent, which colorimetrically detects siderophore activity. We compared WT bacteria and IroA-expressing bacteria at varying levels of Lcn2 proteins. The outcome, as depicted in the updated Fig. 3b, reveals an enhanced iron acquisition capability in IroA-E. coli under the presence of Lcn2 proteins, in comparison to the wild-type E. coli strains. In addition to the Lcn2 study, the proteomic study in Figure 4 highlights the competitive landscape between cancer cells and bacteria. We observed that IroA-E. coli showed reduced stress responses and exerted elevated iron-associated stress to cancer cells, thus further supporting the IroA-E. coli's iron-scavenging capability against nutritional immunity.

(2) Related to 1, the experiment to study the synergistic effect of CDG and VLX600 (lines 139-175) is very nice and promising, but one flaw here is a lack of the measurement of iron concentration. Therefore, a possible explanation could be that CDG acts in another manner, unrelated to iron uptake, that synergizes with VLX600's function to deplete iron from cancer cells. Here, a direct measurement of iron concentration will show the effect of CDG on iron uptake, thus complementing the missing link.

We appreciate the reviewer's comment and would like to point the reviewer to our results in Figure S3, which shows that the expression of CDG enhances bacteria survival in the presence of LCN2 proteins, which reflects the competitive relationship between CDG and enterobactin for LCN2 proteins as previously shown by Li et al. [Nat Commun 6:8330, 2015]. We regret to inform the reviewer that direct measurement of iron concentration was attempted to no avail due to the limited sensitivity of iron detecting assays. We do acknowledge that CDG may exert different effects in addition to enhancing iron uptake, particularly the potentiation of the STING pathway. We pointed out such effect in Fig 2c that shows enhanced macrophage stimulation by the CDG-expressing bacteria. We would like to accentuate, however, that a primary objective of the experiment is to show that the manipulation of nutritional immunity for promoting anticancer bacterial therapy can be achieved by combining bacteria with iron chelator VLX600. The multifaceted effects of CDG prompted us to focus on IroA-E. coli in subsequent experiments to examine the role of nutritional immunity on bacterial therapy. We have updated the associated text to better convey our experimental design principle.

Lines 250-268: Although statistically significant, I would recommend the authors characterize the CD8+ T cells a little more, as the mechanism now seems quite elusive. What signals or memories do CD8+ T cells acquire after IroA-E. Coli treatment to confer their long-term immunogenicity?

We apologize for the overinterpretation of the immune memory response in our previous manuscript and appreciate the reviewer's recommendation to further characterize CD8+ T cells post-IroA-E. coli treatment. Our findings, which show robust tumor inhibition in rechallenge studies, indicate establishment of anticancer adaptive immune responses. As the scope of the present work is aimed at demonstrating the value of engineered bacteria for overcoming nutritional immunity, expounding on the memory phenotypes of the resulting cellular immunity is beyond the scope of the study. We do acknowledge that our initial writing overextended our claims and have revised the manuscript accordingly. The revised

manuscript highlights induction of anticancer adaptive immunity, attributable to CD8⁺ T cells, following the bacterial therapy.

(3) Perhaps this goes beyond the scope of the current manuscript, but how broadly applicable is the observed iron-transport phenomenon in other tumor models? I would recommend the authors to either experimentally test it in another model or at least discuss this question.

We highly appreciate the reviewer's suggestion regarding the generalizability of the iron-transport phenomenon in diverse tumor models. To address this, we extended our investigations beyond the initial model, employing B16-F10 melanoma and E0771 breast cancer in mouse subcutaneous models. The results, as depicted in Figures 3g to 3j and Figure S5, demonstrate the superiority of IroA-E. coli over WT bacteria in tumor inhibition. These findings support the broad implication of nutritional immunity as well as the potential of iron-scavenging bacteria for different solid tumor treatments.

Reviewer #2 (Public Review):

Summary:

The authors provide strong evidence that bacteria, such as E. coli, compete with tumor cells for iron resources and consequently reduce tumor growth. When sequestration between LCN2 and bacterobactin is blocked by upregulating CDG(DGC-E. coli) or salmochelin(IroA-E.coli), E. coli increase iron uptake from the tumor microenvironment (TME) and restrict iron availability for tumor cells. Long-term remission in IroA-E.coli treated mice is associated with enhanced CD8⁺ T cell activity. Additionally, systemic delivery of IroA-E.coli shows a synergistic effect with chemotherapy reagent oxaliplatin to reduce tumor growth.

Strengths:

It is important to identify the iron-related crosstalk between E. coli and TME. Blocking lcn2-bacterobactin sequestration by different strategies consistently reduces tumor growth.

Weaknesses:

As engineered E.coli upregulate their function to uptake iron, they may increase the likelihood of escaping from nutritional immunity (LCN2 becomes insensitive to sequester iron from the bacteria). Would this raise the chance of developing sepsis? Do authors think that it is safe to administrate these engineered bacteria in mice or humans?

We appreciate the reviewer's comment on the safety evaluation of the iron-scavenging bacteria. To address the concern, we assessed the potential risk of sepsis development by measuring the bacterial burden and performing whole blood cell analyses following intravenous injection of the engineered bacteria. As illustrated in Figures 3k and 3l, our findings indicate that the administration of these engineered bacteria does not elevate the risk of sepsis. The blood cell analysis suggests that mice treated with the bacteria eventually return to baseline levels comparable to untreated mice, supporting the safety of this approach in our experimental models.

Reviewer #3 (Public Review):

Summary:

Based on their observation that tumor has an iron-deficient microenvironment, and the assumption that nutritional immunity is important in bacteria-mediated tumor

modulation, the authors postulate that manipulation of iron homeostasis can affect tumor growth. They show that iron chelation and engineered DGC-E. coli have synergistic effects on tumor growth suppression. Using engineered IroA-E. coli that presumably have more resistance to LCN2, they show improved tumor suppression and survival rate. They also conclude that the IroA-E. coli treated mice develop immunological memory, as they are resistant to repeat tumor injections, and these effects are mediated by CD8+ T cells. Finally, they show synergistic effects of IroA-E. coli and oxaliplatin in tumor suppression, which may have important clinical implications.

Strengths:

This paper uses straightforward in vitro and in vivo techniques to examine a specific and important question of nutritional immunity in bacteria-mediated tumor therapy. They are successful in showing that manipulation of iron regulation during nutritional immunity does affect the virulence of the bacteria, and in turn the tumor. These findings open future avenues of investigation, including the use of different bacteria, different delivery systems for therapeutics, and different tumor types.

Weaknesses:

- *There is no discussion of the cancer type and why this cancer type was chosen. Colon cancer is not one of the more prominently studied cancer types for LCN2 activity. While this is a proof-of-concept paper, there should be some recognition of the potential different effects on different tumor types. For example, this model is dependent on significant LCN production, and different tumors have variable levels of LCN expression. Would the response of the tumor depend on the role of iron in that cancer type? For example, breast cancer aggressiveness has been shown to be influenced by FPN levels and labile iron pools.*

We highly appreciate the reviewer's insightful comment on the varying LCN2 activities across different tumor types. In light of the reviewer's suggestion, we extended our investigations beyond the initial colon cancer model, employing B16-F10 melanoma and E0771 breast cancer in mouse subcutaneous models. The results, as depicted in Figures 3g to 3j and Figure S5, demonstrate that IroA-E. coli consistently outperforms WT bacteria in tumor inhibition. We acknowledge the reviewer's comment regarding LCN2 being more prominently examined in breast cancer and have highlighted this aspect in the revised manuscript. For colon and melanoma cancers, several reports have pointed out the correlation of LCN2 expression and the aggressiveness of these cancers [Int J Cancer. 2021 Oct 1;149(7):1495-1511][Nat Cancer. 2023 Mar;4(3):401-418], albeit to a lesser extent. These findings support the broad implication of nutritional immunity as well as the potential of iron-scavenging bacteria for different solid tumor treatments. The manuscript has been revised to reflect the reviewer's insightful comment.

- *Are the effects on tumor suppression assumed to be from E. coli virulence, i.e. Does the higher number of bacteria result in increased immune-mediated tumor suppression? Or are the effects partially from iron status in the tumor cells and the TME?*

We appreciate the reviewer's question regarding the therapeutic mechanism of IroA-E. coli. Bacterial therapy exerts its anticancer action through several different mechanisms, including bacterial virulence, nutrient and ecological competition, and immune stimulation. Decoupling one mechanism from another would be technically challenging and beyond the scope of the present work. With the objective of demonstrating that an iron-scavenging bacteria can elevate anticancer activity by circumventing nutritional immunity, we highlight

our data in Fig. S6, which shows that IroA-E. coli administration resulted in higher bacterial colonization within solid tumors compared to WT-E. coli on Day 15. This increased bacterial presence supports our iron-scavenging bacteria design, and we highlight a few anticancer mechanisms mediated by the engineered bacteria. Firstly, as shown in Fig. 4d, IroA-E. coli is shown to induce an elevated iron stress response in tumor cells as the treated tumor cells show increased expression of transferrin receptors. Secondly, our experiments involving CD8⁺ T cell depletion indicates that the IroA-E. coli establishes a more robust anticancer CD8⁺ T cell response than WT bacteria. Both immune-mediated responses and alterations in iron status within the tumor microenvironment are demonstrated to contribute to the enhanced anticancer activity of IroA-E. coli in the present study.

- *If the effects are iron-related, could the authors provide some quantification of iron status in tumor cells and/or the TME? Could the proteomic data be queried for this data?*

We appreciate the reviewer's query regarding the quantification of iron concentrations. In our study, we attempted various experimental approaches, including Immunohistochemistry utilizing an a Fe³⁺ probe, iron assay kit (ab83366), and Inductively Coupled Plasma Mass Spectrometry (ICP-MS). Despite these attempts, the quantification of oxidized Fe³⁺ concentrations proved challenging due to the inherently low levels of Fe ions and difficulty to distinguish Fe²⁺ and Fe³⁺. We observed measurements below the detection threshold of even the sensitive ICP-MS technique. Consequently, to circumvent this limitation, we designed an experiment wherein bacteria were cultured in a medium supplemented with Chrome Azurol S (CAS) reagent, which colorimetrically detects siderophore activity. We compared WT bacteria and IroA-expressing bacteria at varying levels of Lcn2 proteins. The outcome, as depicted in the updated Fig. 3b, reveals an enhanced iron acquisition capability in IroA-E. coli under the presence of Lcn2 proteins, in comparison to the wild-type E. coli strains. In addition to the Lcn2 study, the proteomic study in Figure 4 highlights the competitive landscape between cancer cells and bacteria. We observed that IroA-E. coli showed reduced stress responses and exerted elevated iron-associated stress to cancer cells, thus further supporting the IroA-E. coli's iron-scavenging capability against nutritional immunity.

Reviewing Editor:

The authors provide compelling technically sound evidence that bacteria, such as E. coli, can be engineered to sequester iron to potentially compete with tumor cells for iron resources and consequently reduce tumor growth. Long-term remission in IroA-E.coli treated mice is associated with enhanced CD8⁺ T cell activity and a synergistic effect with chemotherapy reagent oxaliplatin is observed to reduce tumor growth. The following additional assessments are needed to fully evaluate the current work for completeness; please see individual reviews for further details.

We appreciate the editor's positive comment.

(1) The premise is one of translation yet the authors have not demonstrated that manipulating bacteria to sequester iron does not provide a potential for sepsis or other evidence that this does not increase the competitiveness of bacteria relative to the host. Only tumor volume was provided rather than animal survival and cause of death, but bacterial virulence is enhanced including the possibility of septic demise. Alternatively, postulated by the authors, that tumor volume is decreased due to iron sequestration but they do not directly quantify the iron concentration in (1) E. Coli in different growth environments, and (2) tumor microenvironment. These important endpoints will provide the functional consequences of upregulating genes that import iron into the bacteria.

We appreciate the editor's comment and have added substantial data to support the translational potential of the iron-scavenging bacteria. In particular, we added evidence that the iron-scavenging bacteria does not increase the risk of sepsis (Fig. 3k, l), evidence of increased bacteria competitiveness and survival in tumor (Fig. S6), and iron-scavenging bacteria's superior anticancer ability and survival benefit across 3 different tumor models (Fig. 3e-j; Fig. S5). While direct measurement of iron concentration in the tumor environment is technically difficult due to the challenge in differentiating Fe²⁺ and Fe³⁺ by available techniques, we added a colorimetric CAS assay to demonstrate the iron-scavenging bacteria can more effectively utilize Fe than WT bacteria in the presence of LCN2 (Fig. 3b). These results substantiate the translational relevance of the engineered bacteria.

(2) There is no discussion of the cancer type and why this cancer type was chosen. If the current tumor modulation system is dependent on LCN2 activity, there would need to be some recognition that different tumors have variable levels of LCN expression. Would the response of the tumor depend on the role of iron in that cancer type?

We appreciate the comment and added relevant text and citations describing clinical relevance of LCN2 expression associated with the tumor types used in the study (breast cancer, melanoma, and colon cancer). Elevated LCN2 has been associated with higher aggressiveness for all three cancer types.

(3) To demonstrate long-term anti-cancer memory was established through enhancement of CD8⁺ T cell activity (Fig 5c), the "2nd seeding tumor cells" experiment may need to be done in CD8 antibody-treated IronA mice since CD8⁺ T cells may play a role in tumor suppression regardless of whether or not iron regulation is being manipulated. It appears that the control group for this experiment is naive mice (and not WT-E. coli treated mice), in which case the immunologic memory could be from having had tumor/E. coli rather than the effect of IroA-E. coli.

We acknowledge that our prior writing may have overstated our claim on immunological memory. Our intention is to show that upon treatment and tumor eradication by iron-scavenging bacteria, adaptive immunity mediated by CD8 T cells can be elicited. We also did not consider a WT-E. coli control as no WT-E. coli treated group achieved complete tumor regression. We have modified our text to reflect our intended message.

Reviewer #1 (Recommendations For The Authors):

All the figures seem to be in low resolution and pixelated. Please upload high-resolution ones.

We have updated figures to high-resolution ones.

Reviewer #2 (Recommendations For The Authors):

Some specific comments towards experiments:

(1) For Fig 2 f/ Fig 3f/ Fig 5d/ Fig 6c, the survival rate is based on the tumor volume (the mouse was considered dead when the tumor volume exceeded 1,500 mm³). Did the mice die from the experiment (how many from each group)? If it only reflects the tumor size, do these figures deliver the same information as the tumor growth figure?

We appreciate the reviewer's comment. The survival rate is indeed based on tumor volume, and we used a cutoff of 1500 mm³. No death event was observed prior to the tumors reaching 1500 mm³. Although the survival figures cover some of the information conveyed by the

tumor volume tracking, the figures offer additional temporal resolution of tumor progression with the survival figures. Having both tumor volume and survival tracking are commonly adopted to depict tumor progression. We have the protocol regarding survival monitoring to the materials and method section.

(2) Fig 3a, not sure if entE is a good negative control for this experiment. Neg. Ctrl should maintain its CFU/ml at a certain level regardless of Lcn2 conc. However, entE conc. is at 100 CUF/ml throughout the experiment suggesting there is no entE in media or if it is supersensitive to Lcn2 that bacteria die at the dose of 0.1nM?

We appreciate the reviewer's comment. The Δ entE-E. coli was indeed observed to be highly sensitive to LCN2. We included the control to highlight the competitive relationship between entE and LCN2 for iron chelation, which is previously reported in literature [Biomaterials 32, 453–467 (2019)].

(3) Fig 4, the authors harvested bacteria from the tumor by centrifuging homogenized samples at different speeds. Internal controls confirming sample purity (positive for bacteria and negative for cells for panels a,b,c; or vice versa for panel d) may be necessary. This comment may also apply to samples from Fig 1.

We acknowledge the reviewer's concern and would like to point out that the proteomic analysis was performed using a highly cited protocol that provides reference and normalization standards for E. coli proteins [Mol Cell Proteomics. 2014 Sep; 13(9): 2513–2526]. The reference is cited in the Materials and Method section associated with the proteomic analysis.

(4) To demonstrate long-term anti-cancer memory was established through enhancement of CD8+ T cell activity, the "2nd seeding tumor cells" experiment may need to be done in CD8 antibody-treated IronA mice.

We have modified our claims to highlight that the tumor eradication by iron scavenging bacteria can establish adaptive anticancer immunity through the elicitation of CD8 T cells. We apologize for overstating our claim in the previous manuscript draft.

Minor suggestions:

(1) Please include the tumor re-challenge experiment in the method section.

The re-challenge experiment has been added to the method section as instructed.

(2) Please cite others' and your previous work. E.g. line 281, 282, line 306-307.

We have added the citations as instructed.

(3) Line 448, BL21 is bacteria, not cells.

We have made the correction accordingly.

Reviewer #3 (Recommendations For The Authors):

- *The authors postulate that IroA-E. coli is more potent than DGC-E. coli in resisting LCN2 activity, and that this potency is the cause of the increased tumor suppression of this engineered strain. If so, Fig 3a should include DGC-E. coli for direct comparison.*

We appreciate the reviewer for the comment and would like to clarify that we intended construct IroA-E. coli as a more specific iron-scavenging strategy, which can aide the discussion of nutritional immunity and minimize compounding factors from the immune-stimulatory effect of CDG. We have modified our text to clarify our stance.

- *The data refers to the effects of WT bacteria-mediated tumor suppression, e.g. Figure 3e shows that even WT bacteria have a significant suppressive effect on tumor growth. Could the authors provide background on what is known about the mechanism of this tumor suppression, outside of tumor targeting and engineerability? They only reference "immune system stimulation."*

We appreciate the reviewer's comment and would like to refer the reviewer to our recently published article [Lim et al., EMBO Molecular Medicine 2024; DOI: 10.1038/s44321-023-00022-w], which shows that in addition to immune system stimulation, WT bacteria can also be perceived as an invading species in the tumor that can exert differential selective pressure against cancer cells. Competition for nutrient is highlighted as a major contribution to contain tumor growth. In fact, the nutrient competition that we observed in the prior article inspired the design of the iron scavenging bacteria towards overcoming nutritional immunity. We have cited this recently published article to the revised manuscript to enrich the background.

- *The authors claim that there is immunologic memory because of tumor resistance in re-challenged mice after IroA-E. coli treatment (Fig 5c). It appears that the control group for this experiment is naive mice (and not WT-E. coli treated mice), in which case the immunologic memory could be from having had tumor/E. coli rather than the effect of IroA-E. coli.*

We have modified our claims to highlight that the tumor eradication by iron scavenging bacteria can establish adaptive anticancer immunity through the elicitation of CD8 T cells. We did not intend to highlight that the adaptive immunity stemmed from IroA-E. coli only, and we intend to build upon current literature that has reported CD8+ T cell elicitation by bacterial therapy. The IroA-E.coli is shown to enhance adaptive immunity. We also did not consider a WT-E. coli control as no WT-E. coli treated group achieved complete tumor regression.

- *The authors claim that CD8+ T cells are mechanistically important in the effects of iron status manipulation in E. coli-mediated tumor suppression (Fig 5). In order to show this, it seems that Fig 5c should include WT-E. coli and WT-E. coli+CD8 ab groups, as it may be that CD8+ T cells play a role in tumor suppression regardless of whether or not iron regulation is being manipulated.*

We apologize for the confusion from our prior writing. We have modified our claims to highlight that the tumor eradication by iron scavenging bacteria can establish adaptive anticancer immunity through the elicitation of CD8 T cells. We did not intend to convey that CD8+ T cells are mechanistically important in the effects of iron status manipulation.