

# Bacteria-Based Nanoprobes for Cancer Therapy

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**Abstract:** Surgical removal together with chemotherapy and radiotherapy has used to be the pillars of cancer treatment. Although these traditional methods are still considered as the first-line or standard treatments, non-operative situation, systemic toxicity or resistance severely weakened the therapeutic effect. More recently, synthetic biological nanocarriers elicited substantial interest and exhibited promising potential for combating cancer. In particular, bacteria and their derivatives are omnipotent to realize intrinsic tumor targeting and inhibit tumor growth with anti-cancer agents secreted and immune response. They are frequently employed in synergistic bacteria-mediated anticancer treatments to strengthen the effectiveness of anti-cancer treatment. In this review, we elaborate on the development, mechanism and advantage of bacterial therapy against cancer and then systematically introduce the bacteria-based nanoprobes against cancer and the recent achievements in synergistic treatment strategies and clinical trials. We also discuss the advantages as well as the limitations of these bacteria-based nanoprobes, especially the questions that hinder their application in human, exhibiting this novel anti-cancer endeavor comprehensively.

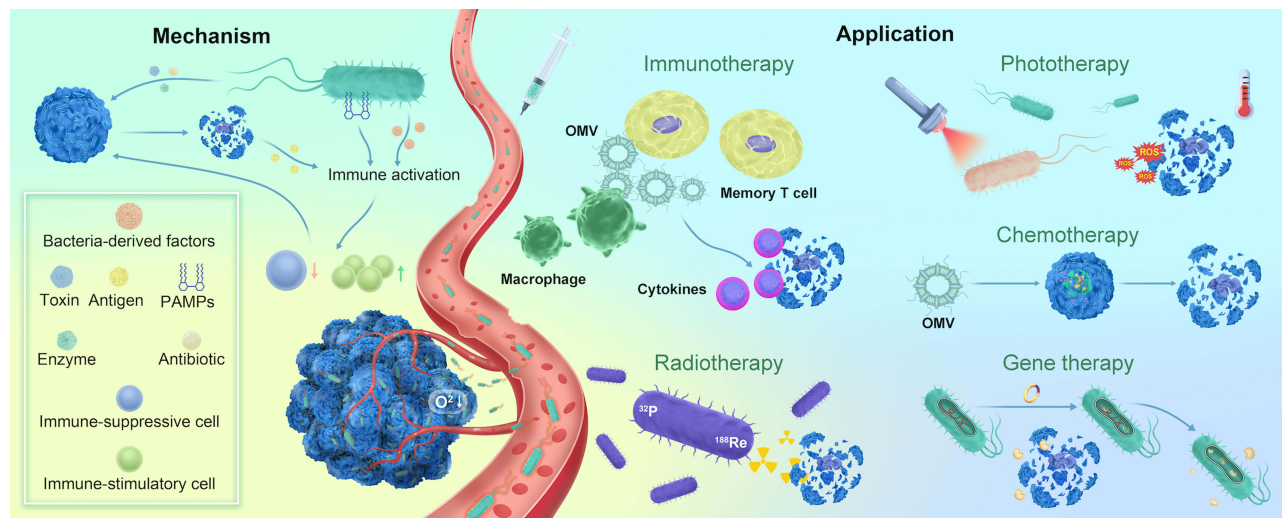
**Keywords:** bacteria-based nanoprobes, bacteria-mediated synergistic cancer therapy, nanomedicine, tumor targeting, tumor immune microenvironment, clinical trial

## Introduction

Nowadays, cancer is considered to rank as the leading cause of death and the single most important barrier to increasing life expectancy in every country of the world in the 21st century.<sup>1</sup> Numerous efforts have been made to conquer cancers. Surgical resection with tumor removal is known as the first-line treatment of many kinds of tumors in their early stage, but for unresectable ones, additional tools are indispensable. Since the late 20th century, new methods, especially chemotherapy and radiotherapy, which utilizes different types of chemotherapeutic drugs or high doses of radiation, are applied to kill tumor cells and shrink tumors. When compared to chemotherapy, the utility of radiotherapy is rather limited since it is only suitable for a tumor confined to a discrete anatomical area.<sup>2</sup> As a systemic therapy, chemotherapy with anti-tumor drugs is capable to treat cancer throughout the whole body even with metastasis.<sup>3</sup> However, the therapeutic effect of chemotherapy is often modest. It was reported that cancer treatment based on a broad use of cytotoxic chemotherapy in unselected cancer patients had reached its therapeutic plateau.<sup>4</sup> Furthermore, the systemic toxicity caused by the lack of selectivity and resistance to chemotherapy and radiotherapy had also severely hampered the effectiveness of these traditional treatments.<sup>5</sup> Therefore, the need to find better treatment alternatives was underscored.

The phenomenon of the selective accumulation of nanometric devices into tumoral tissues discovered first by Matsumura and Maeda in 1986, known as enhanced permeation and retention (EPR) effect, has given nanomedicine an advantage over conventional medicine and started a new chapter of nanomedicine against cancer.<sup>6,7</sup> Since then, hundreds of different nanoparticle (NP)-based drug delivery systems (DDSs) have been developed, from inorganic systems, or organic ones, to hybrid NPs. Theoretically, when administered systemically, NPs leak from the tumor blood vessels into the tumor interstitium through the hyperpermeable tumor vasculatures and are then retained in the tumor due to reduced lymphatic

## Graphical Abstract



drainage which could potentially increase the effectiveness of anti-tumor drugs.<sup>8</sup> The characteristics including passive accumulation within tumor via EPR effect, decoration of various targeting ligands and multiple types of triggers of payload release enable NPs to achieve excellent performance in vitro and in vivo models.<sup>9–13</sup> However, the nano-delivery system mainly based on EPR effect could not always achieve satisfactory therapeutic response in real clinical situations. So far, only a few nano-systems have reached the market and most of them are still associated with adverse effects<sup>14,15</sup> Also, the increase in overall survival of patients with nanomedicine treatment is modest for many cases.<sup>16</sup>

Several reasons might reduce the therapeutic effect of NPs. First, NPs are passively targeting the cancers which would sometimes result in insufficient drug loading-NPs to reach the target sites.<sup>3</sup> Second, the dense extracellular matrix of tumor hampers the particle penetration and limits the distribution of the nanocarrier only on the tumor periphery.<sup>17</sup> Furthermore, the high interstitial pressure presenting in solid tumors makes NP diffusion rather difficult.<sup>18</sup> Despite the less toxicity than conventional therapeutics, the stability, biocompatibility and long-term potential safety of the synthetic nanocarriers are far from ideal for in vivo application.<sup>19</sup> In these cases, biological carriers, especially bacterial-based carriers, exhibit promising properties and gradually attract people's attention.

In comparison to synthetic NPs, bacteria-based NPs exert many benefits in cancer therapy. On the one hand, bacteria and their derivatives are endowed with tumor targeting intrinsically rather than based on EPR effect. In particular, it is the tumor hypoxic and immunosuppressive microenvironment and its special structure like flagellum and chemotaxis that enable bacteria to target, colonize and proliferate.<sup>20</sup> On the other hand, owing to the intrinsic targeting ability of bacteria, the loaded toxic drugs of bacterial NPs play a therapeutic role with spatial and temporal precision and without systemic effects as much as possible.<sup>21,22</sup> Moreover, it is promising to help ensure biocompatibility of NPs by means of choosing a commensal or probiotic bacterial strain, or live attenuated bacteria or derivatives.<sup>23</sup>

This review focuses on bacterial therapy against cancer from the following three aspects: development, mechanism and advantages. Subsequently, the bacteria-based nanoprobe against cancer and the recent achievements in synergistic treatment strategies and clinical trials are introduced. Ultimately, the limitations and future prospects of bacteria-based nanoprobe are anticipated. These will provide scientific guidance and strategic reference for expanding the construction of bacteria-based nanosystems and their significance in cancer therapy.

# Bacteria vs Cancer—History, Mechanism and Advantages

## The History of Bacterial Therapy

The history of bacteria-based anticancer treatment dates back to 1813, when Vautier reported his observation of tumor regression in patients who concurrently suffered from gas gangrene caused by a *Clostridium* infection.<sup>24</sup> Inspired by this, several attempts were made to shrink cancers upon infection of erysipelas. In 1891, W.B. Coley, an American surgeon, reported his experience in treating sarcoma by inoculation of streptococcus erysipelatus.<sup>25</sup> He dedicated his whole career to exploring the anti-cancer effect after the inoculation of the mixed toxin of the streptococcus erysipelas and the *Bacillus Prodigiosus*, known as ‘Coley’s toxin’, whose active agent was later reported to be interleukin-12.<sup>26,27</sup> At that time, with stable replicability in clinical use, Coley’s work was controversial, and gradually the pioneering bacterial therapy gave way to arising chemotherapy and radiotherapy. But science will never stop its pace.

In 1929, Pearl found that cancer survivors had a higher incidence of active or healed tuberculosis (TB) than individuals dying of cancer, implying TB’s protection against cancer.<sup>28</sup> In the second part of 20th century, the anticancer effects of bacilli Calmette-Guérin (BCG), a live attenuated obligate anaerobic strain of *Mycobacterium bovis*, were intensively investigated, during which the underlying theories and substances in bacterial-based immunotherapy were subsequently discovered.<sup>29,30</sup> Efforts were made to treat cancers by BCG vaccine and in 1990, the general use of intravesical BCG for the treatment of noninvasive bladder cancer was officially approved by FDA.<sup>31,32</sup> The breakthrough in synthetic biology, nanotechnology and genetic engineering makes the use of live attenuated bacteria and their derivatives as anti-cancer therapeutics a tangible reality.

## Main Mechanism of Bacterial Therapy

### Intrinsic Tumor Targeting

It is well documented that when administered systemically, therapeutic bacteria disseminate to both tumor and healthy tissues with similar bacterial amount mainly in a passive way. However, several hours or days later, live bacteria could only be detected in solid tumor rather than normal, inflammatory or non-tumoral hypoxic regions. This tumor-targeting phenomenon could be explained by two intrinsic characteristics of the tumor: hypoxia and immunosuppression.

The unlimited proliferation of malignant cells and chaotic neovascularity leads to tumor metabolism environment (TME), which is well summarized as (a) hypoxia, oxygen concentrations  $\leq 10\text{mmHg}$ ; (b) anaerobic metabolism-induced acidity status; and (c) necrosis, contributing to bacterial proliferation in the tumor tissue.<sup>33,34</sup> The hypoxia status of a tumor leads to resistance to cytotoxic agents and radiation, and finally brings poor prognosis. Furthermore, the function of immune cells in vivo was affected by the over-expression of HIF-1 $\alpha$  and VEGF, contributing to the immune privilege of solid tumors.<sup>35,36</sup> It is the hypoxic status and lack of immune clearance in tumor region that provide a suitable environment for live bacteria to colonize and proliferate.

On the bacteria side, live bacteria can move by acquiring energy from the surrounding environment. The flagellum and chemotaxis enable these live creatures penetrate deep into the tumor against the high interstitial fluid pressure (IFP), differentiating these bacteria from other anti-cancer systems that could only diffuse passively.<sup>37</sup> Chemotaxis and tumor necrosis factor (TNF- $\alpha$ ) are also proved to contribute to the initial phase of the penetration of bacteria into tumor tissue from vessels. For example, the chemotactic compounds produced by the dead cancer cells within the tumor attract *Salmonella* to adhere to the vessel walls of tumor. As early as 30 minutes after injection, the concentration of TNF- $\alpha$  increased significantly in parallel to the start of the intra-tumoral colonization of bacteria, and peaked between 30–120 min.<sup>38</sup> The cytokine, TNF- $\alpha$ , leads to vessel disruption, hemorrhage and necrosis, thereafter, creating ideal environment for bacterial germination. The pre-treatment of anti-TNF- $\alpha$  could not inhibit but retard the intra-tumoral colonization of *Salmonella*, which convinced the effect of TNF- $\alpha$  in tumor invasion behavior of bacteria.

Other factors including EPR effect in nano-scale bacteria and possible entrapment of bacteria by occluded/dying vessels also contribute to the intrinsic tumor-targeting ability of bacteria.<sup>39</sup> Some bacteria-derived nanovesicles like minicells can be equipped with the specific antibodies or ligands in order to achieve the precise tumor-targeting.<sup>40</sup>

## Anticancer Agents Secreting and Immune Response Activating

Bacteria have evolved sophisticated mechanisms to inhibit the growth of competitors, and in cancer, the competitors are challengeable cancer cells.<sup>41</sup> Many successful experiments have proved the ability of live bacteria to cause tumor regression. The native therapeutic mechanism of the attenuated live bacteria could be roughly devoted to 2 parts: the secretion of anti-cancer agents and activation of immune response.

The anticancer agents of bacteria mainly include 3 categories: antibiotics, toxins and enzymes.<sup>42,43</sup> Several antibiotics of bacteria origin against tumor were intensively investigated in the past and now are available in market, for instance, Bleomycin and Doxorubicin. Toxins are groups of ribosomal-synthesized bacterial proteins to disrupt the physiological behavior of target cells, and some of them exhibit anticancer activities. For example, colicins, provided by different strains of *Escherichia coli*, represent cytotoxicity towards cancer cells by a series of mechanism including depolarization of the cytoplasmic membrane, DNase and RNase activity, etc, exhibiting anticancer properties toward a variety of human ovarian cell lines.<sup>44,45</sup> Another common group of toxins, including Cytolysin A and Listeriolysin O etc., belongs to pore-forming toxin family, which can damage cell membrane, ablate the chemical gradients needed to keep a cell functioning or allow entrance of bacteria into cytosol, often leading to cell death.<sup>46,47</sup> Some enzymes of bacterial origin display anticancer properties as well, and the representatives of which were the nitrate and nitrite reductases provided by *Salmonella* strains.<sup>48</sup> Under hypoxia circumstances, nitrate and nitrite reductases are capable to convert nitrate and nitrite to the nitric oxide free radical molecule (NO), which has anticancer activity via arresting tumor proliferation.<sup>49</sup>

Besides the anticancer agents mentioned above, bacteria's ability to arouse immune activation is another key to kill tumor. Tumors are known to maintain immune tolerance by resembling normal tissue, but bacteria act as a strong immune stimulator. By secreting bacteria-derived factors, pathogen-associated molecular patterns (PAMPs), for instance, and releasing tumor antigens after disruption of tumor cells, bacteria successfully activate both the innate and adaptive immune systems in the tumor environment, exhaust immune-suppressive cells and increase pro-immunostimulatory lymphocyte and CD8+ T cell infiltrates within the tumor<sup>50-53</sup> Finally, with the immune effects of multiple immune cells, cytokines and chemokines, the immune system is modulated and activated, and cancer cells are recognized and attacked as the consequences.

Due to the inherited cargoes from the donor bacteria, bacteria-derived nanovesicles possess potent immunogenicity in immune response activation. For example, with the same antigens or PAMPs like lipopolysaccharide (LPS), peptidoglycan, and flagellin as the original bacteria, outer membrane vesicles (OMVs) are highly immunogenic nanovesicles that can robustly activate a variety of toll-like receptor (TLR) signaling pathways.<sup>54,55</sup> It was found that intravenous administration of OMVs alone could elicit interferon (IFN)- $\gamma$  production and nonspecific antitumor immune response.<sup>56</sup> Bacterial ghosts (BGs) can not only directly activate immune cells including dendritic cells (DCs), macrophages, and B or T cells, but also activate epithelial cells, fibroblasts, and keratinocytes, all of which can indirectly attract immune cells.<sup>57</sup> Also, BGs can be able to induce higher quality immune responses with the preservation of the epitopes of virulent bacteria strains.

## The Advantages of Bacteria-Based Tumor Treatment

With the tumor-targeting and specific anti-cancer therapeutic effects, bacteria-based tumor treatment has comparative advantages over traditional treatments. Bacteria 1) have the capability of preferentially colonizing the core area of the tumor to achieve accurate drug delivery and therapeutic effects; 2) can effectively activate immune system in the tumor immunosuppressive microenvironment and eliminate tumor cells both directly and indirectly; 3) can be developed as carriers to exert multifunctional anti-tumor efficacy constructed with various nanomaterials; 4) like probiotic strains, live attenuated bacteria or engineered derivatives, can enhance its biocompatibility and biosafety; 5) can be decorated with specific luminescent materials or imaging agents to realize therapeutic visualization *in vitro* and *in vivo*.

More and more attempts have been made to synthesize bacteria and its components to digger the bacterial ability as carriages to deliver medicine, antibodies, cytokines, etc. to trigger more intensive and profound anti-cancer results.<sup>58,59</sup> In the next part, we aim to introduce the most frequently used live attenuated bacteria and their engineered derivatives which have joined the fight against cancers as NPs.

## The Bacteria-Based NPs to Fight Cancer

These bacteria-based nanoprobes against cancer can be further categorized by their structure and the preloaded treating materials.

### Live Attenuated Bacteria

The qualified anti-cancer bacteria need to be attenuated, which means they can infect a host and induce a strong immune response against tumor, but do not cause disease. Historically, the attenuated bacterial vectors were once achieved by chemical mutagenesis or serial passages under laboratory conditions, whose successful examples were illustrated as *Salmonella enterica* serovar Typhi Ty21a and *Mycobacterium bovis* BCG.<sup>60</sup> The modern biotechnology has revolutionized the mechanism of attenuation by deleting or mutating the essential genes involved in virulence regulatory system through recombinant DNA techniques. These mutations attenuate the strain whilst preserving its immunogenicity.<sup>61</sup> Next, we will introduce the most frequently used live attenuated bacteria as NPs to fight cancers.

#### Salmonella

*Salmonella* is a genus of rod-shaped Gram-negative bacteria of the family Enterobacteriaceae. They are facultative anaerobic, intracellular pathogens with cell diameters between about 0.7 and 1.5  $\mu\text{m}$ , lengths from 2 to 5  $\mu\text{m}$ , equipped with peritrichous flagella (all around the cell body). The serotypes of *Salmonella* can be divided into two main groups—typhoidal and nontyphoidal. A member in typhoidal group, *Salmonella enterica* serovar Typhimurium (hereafter *S. Typhimurium*), has been extensively studied as an antitumoral tool. Many well-known engineered *S. Typhimurium* strains are used in anti-tumoral research, including VNP20009 (pur I; -msbB-), A1-R (leu-/arg-), SL7207 (aroA-) and the  $\Delta\text{ppGpp}$  strain SHJ2037 (relA-/spoT-), etc.<sup>62-65</sup> For example, in *S. Typhimurium* strain VNP20009, purI and msbB are deleted chromosomally, resulting in the deficiency of adenine synthesis and modifying the Lipid-A structure. Thus, the virulence of VNP20009 is greatly attenuated. The maximum tolerated dose (MTD) of VNP20009 is significantly reduced by 50,000 times compared to that of the wild-type strain, while the LD50 of it was increased more than 10,000-fold compared with the wild-type strain.<sup>66</sup> *S. Typhimurium* strain A1-R, auxotrophic for leucine and arginine, can suppress the growth of different types of tumors, including prostate, breast and pancreatic cancers and spinal cord glioma, and eradicate cancer metastases.<sup>67-72</sup> Besides virulence attenuation, by placing the essential gene *asd* under a hypoxia-induced promoter, an obligate anaerobic strain called YB1 was created from SL7207 strain. With the characteristics of anaerobiosis, YB1 can only colonize in the hypoxic and necrotic regions of tumor, which enhances the tumor specification.<sup>65,73,74</sup>

*S. Typhimurium* can seek the openings between the disorganized endothelial cells and entered the tumor in both active and passive ways. In the passive aspect, the hemodynamics played an important role in the attachment of *Salmonella* to the tumor vessel wall. In vessels with low flow rates (defined as  $< \sim 1.5 \text{mm/sec}$ ), *S. Typhimurium* were observed to have interaction with vessel wall, but in vessel with higher blood velocity, no bacteria adherence was detected.<sup>35</sup> Except the hemodynamic factors, the motility of *S. Typhimurium* also facilitated its intra-tumoral accumulation. The necessary components for the mobility of *S. Typhimurium* include the proper flagella constructs, signal transduction proteins, active motor function and chemotaxis. Forbes et al proved the chemotaxis, directed by different chemoreceptors, guided *S. Typhimurium* into tumor cylindroids. These chemoreceptors, including the aspartate receptor, the serine receptor and the ribose/galactose receptor played vital roles in the process of chemotaxis initiation, tumor penetration and necrosis colonization.<sup>75,76</sup> In addition, the nutrients and protection provided by hypoxic and necrotic environments further explained the accumulation of *S. Typhimurium* in tumor rather than in normal tissues.<sup>77</sup> After colonization, *S. Typhimurium* was reported to kill cancer cells directly by inducing apoptosis and autophagy through a variety of mechanisms.<sup>68,78</sup> Moreover, *Salmonella* can inhibit angiogenesis and activate the host immune response against tumor tissue to suppress tumor growth.<sup>79-82</sup>

#### Listeria

*Listeria monocytogenes* (hereafter *Lm*) are Gram-positive, facultatively anaerobic, rod-shaped bacteria with the size about 0.5\*2-3 $\mu\text{m}$ .<sup>83</sup> The motility mechanism of *Lm* largely depends on environmental temperature. At 20–25  $^{\circ}\text{C}$ , the

flagella are mainly responsible for its motility, but at 37 °C, Lm can only move within eukaryotic cells by explosive polymerization of actin filaments.<sup>83</sup> It is famous for its ability to infect humans (especially the immunodeficient ones) and produce a variety of symptoms including gastroenteritis, meningitis, and encephalitis.<sup>84</sup> The entrance of Lm into the mammalian cells started from their binding to receptors on host cells and endocytosis thereafter via the guidance of internalin A (InlA) and internalin B (InlB) proteins.<sup>85</sup> Then, Lm are free to move throughout the host cell cytosol via expression of the virulence factor actin assembly-inducing protein (ActA) and finally form protrusions which can be internalized by neighboring cells, leading to dissemination of the infection.<sup>86</sup>

Since wild-type Lm is pathogenic, many efforts have been made to create the attenuated, safe strains for clinical usage. Basically, the attenuation strategies of Lm can be divided into three types: deletion of virulence genes, episomal replacement of virulence or metabolic genes and heat-killing but with active metabolism.<sup>87</sup> In detail, the representative of gene-deleted Lm is Lm  $\Delta actA/\Delta inlB$  strain, which is also known as Live Attenuated Double-Deleted (LADD) (Aduro BioTech Inc., Berkeley, CA, USA). The deletion of both *inlB* and *actA* makes it possible for the strain to selectively infect antigen-presenting cells and reduce toxicity.<sup>88</sup> The deletion of a gene followed by reintroduction of that gene via plasmid can also result in attenuation, whose example is LM  $\Delta dal/\Delta dat$  strain (LMDD). The *dal* and *dat* genes are necessary for Lm to synthesize D-alanine to build peptidoglycan and lipoteichoic acid.<sup>89</sup> After deleting *dal* and *dat* genes, the strain is transformed with a plasmid encoding the *dal* gene under a constitutively active promoter, thus achieving the restoration of virulence.<sup>90–93</sup>

After photochemical treatment, the DNA of Lm was irreversibly destroyed. Nevertheless, the strain is still able to invade host cells and secrete TAA products to elicit antitumor CD8+ T-cell responses. The DNA-destroyed strain is called killed but metabolically active (KBMA) Lm strain, which is not available in clinical trials yet.<sup>94,95</sup>

The common strategy to enhance the anti-tumoral response in Lm is to fuse TAA with native antigens of the strain.<sup>96</sup> The two most common fusion partners with TAAs are modified versions of Listeriolysin O (LLO) and *ActA*. LLO is a famous virulence factor, which helps Lm perforate the phagosome and enter the cytosol. Since LLO is not phagosome-specific, a truncated version of LLO (tLLO) has been engineered and widely used in anti-tumoral vaccines.<sup>97,98</sup> In the explorative study designed by Gunn et al, two recombinant Lm strains, Lm-E7 and Lm-LLO-E7 were injected into mice with tumors secreting the human papilloma virus-16 (HPV-16) E7 protein, which is expressed in HPV-16-associated cervical cancer cells and Lm-LLO-E7, but not Lm-E7, was found to induce the complete regression in 75% E7-expressing tumors. Since then, numerous studies have been carried out to explain the antitumor effects of tLLO and several hypotheses have been testified. First, tLLO contains PEST-like sequences, which can improve vaccine responses towards TAAs.<sup>99,100</sup> Second, tLLO and other pore-forming toxins can signal through pattern recognition receptors.<sup>101,102</sup>

Moreover, tLLO may enhance antitumor responses through changes in Tregs.<sup>103,104</sup> Although not completely explained, tLLO still makes Lm a promising candidate as an anti-tumor vaccine. Another different strategy for Lm to evoke anti-tumor response involves fusing TAAs to *ActA*. The truncated *ActA* sequences vary in lengths.<sup>95,105</sup> Similar to tLLO, fusion of E7 with tActA induces superior antitumor immunity compared to E7 alone.<sup>106</sup> Like tLLO, tActA contains PEST domains, but it may possess PAMP-like attributes as well, leading to the production of pro-inflammatory cytokines.<sup>106,107</sup> However, the precise mechanisms for its adjuvant-like properties are not fully understood yet. After comparison, *ActA-E7* or *tLLO-E7* Lm stains resulted in similar levels of circulating E7-specific CD8+ T cells and yielded similar antitumor effects, while tLLO-E7 seemed to induce higher percentages of tumor-infiltrating E7-specific CD8+ T cells.<sup>99,105</sup> Similar tumor-response level of both strains indicates the anti-tumor potency of Lm.

## Clostridium

As one of the largest prokaryotic genera, Clostridium comprises a phylogenetically heterogeneous group of strictly anaerobic, Gram-positive bacteria. One of their distinct features is the production of bottle-shaped endospores. Cells are in rod shape and motile with flagellum. Well-studied species linked to human diseases are Clostridium tetani, the causative agent of tetanus, and Clostridium botulinum, which is related to food poisoning and causes botulism.<sup>24</sup>

Although Vautier reported the phenomenon of cancer regression in patients suffered from gas gangrene in 1813, it was in 1947 that the first research on the anti-tumor potential of clostridia spores was reported.<sup>108</sup> They found that the subsequent vegetative growth of the organism resulted in significant tumour lysis and extended their survival compared

with tumour-bearing mice that were not treated with clostridial spores. Since then, multiple studies have been carried out to investigate the therapeutic effects of Clostridium against cancer, which involved multiple stains, including *C. histolyticum*, *C. tetani*, *C. oncolyticum* (sporogenes), *C. beijerinckii* (acetobutylicum), etc.<sup>109,110</sup> The most frequently investigated Clostridium strain is Clostridium novyi-NT (hereafter *C. novyi-NT*), which was the  $\alpha$ -toxin-free strain derived from Clostridium novyi. After intravenous injection of  $10^8$  spores, *C. novyi-N* strain colonized and proliferated in the necrotic regions of tumor, causing cytolysis and tumor growth retardation, but unfortunately, without tumor control.<sup>111</sup>

Although unable to eliminate tumor thoroughly, the anti-tumoral mechanism of *C. novyi-NT* is still deeply investigated. A variety of secreted bacterial toxins (eg phospholipases, haemolysins) and enzymes (eg proteases and lipases) destroy the neighboring hypoxic cancer cells and activate the immune response in order to eliminate pathogens.<sup>112,113</sup> Once *C. novyi-NT* spores reach cancer cells, they induce expression of specific antibodies against hypoxia inducing factor-1 (HIF-1) as well as different cytokines such as TNF- $\alpha$ , IL-2 and IL-12 to inhibit the growth of the cancer cells and angiogenesis.<sup>114</sup>

### Escherichia coli

*Escherichia coli*, also known as *E. coli*, is a member of the bacterial family of Enterobacteriaceae. It is a Gram-negative, facultative anaerobe, nonsporulating bacterium with rod shape.<sup>115</sup> The size of it is around 2.0  $\mu\text{m}$  in length and 0.25–1.0  $\mu\text{m}$  in diameter.<sup>116,117</sup> Different from *S. Typhimurium* and *Lm* strains mentioned above, the frequently used tumor-targeting probiotic *E. coli* strains were nonvirulent. Among them, *Escherichia coli* Nissle 1917 (EcN) is the most famous one, which is an intestinal probiotic isolated from a soldier who survived from a severe outbreak of diarrhea during World War I.<sup>118</sup> Since EcN strain does not produce any toxins with great tolerance in various populations, it is safe to administer it orally or through injection.<sup>119</sup> It was found that the tumor/liver ratio of EcN colonization at day 2 after intravenous injection was at least 10,000:1 in 4T1 tumor-bearing BALB/c mice, indicating the characteristics of high tumor-selective replication of EcN.<sup>120</sup> After genetical modification, EcN strain can express therapeutic proteins, aurin and tumstatin for instance, which was proved to trigger the apoptosis of melanoma cells and inhibit angiogenesis.<sup>121,122</sup> Symbioflor-2, another probiotic *E. coli* strain, is also investigated for its antitumoral potency.<sup>123,124</sup> It was proved to be systemically administered and induced an early, albeit lower TNF $\alpha$  response compared with the attenuated *S. Typhimurium* strain SL7207.<sup>124</sup> Altogether, the safety of probiotic *E. coli* strains is guaranteed; however, the therapeutic response is not that prominent.

Other strains of bacteria include *Lactococcus Lactis*,<sup>125,126</sup> *Lactobacillus acidophilus*,<sup>127</sup> etc. have also shown promising anti-cancer efficiency in pre-clinical trials which could be found in other papers.

## Bacteria-Derived Nanovesicles

Given the potential proliferation of bacteria, the nanovesicles synthesized from bacteria components were equivalently useful and much safer in cancer treatment.

### Bacterial Ghost

BG is the envelope of gram-negative bacteria emptied of its cytoplasmic contents through cloned lysis/tunnel formation. Since the cytoplasmic components are removed, BG cannot proliferate but the cell morphology and cell surface structure, including outer membrane proteins, adhesins, LPS and peptidoglycan layer are preserved.<sup>128</sup> BGs derived from different bacteria (eg *E. coli*, *Helicobacter pylori*, *Mannheimia haemolytica*) have been proved to drive efficient maturation and activation of DCs and have the potential to act as adjuvants and/or candidate vaccines through improving maturation and functioning of DCs.<sup>129,130</sup> Besides these, the surface of BGs can be modified to present antigens to elicit specific humoral/cellular responses to target cancer cells while the empty space of BGs can be used to contain different types of payloads like drugs, contrast agents, etc.<sup>131</sup>

### Bacterial Minicells

Bacterial minicells are nano-sized (100–300 nm diameter), non-living, anucleated, non-dividing cells that attributed to the mutation in minC or minD gene.<sup>132</sup> These mutations usually lead to polar septation instead of the mid-cell one, thus

resulting in the subsequent formation of the small spherical minicells lacking chromosomal DNA.<sup>133</sup> Bacterial minicells contain all the components similar to their parent bacterial cells, except DNA. Although cannot proliferate, minicells are still metabolically active, like ATP synthesis, translation of mRNA, transcription and translation of plasmid DNA.<sup>134</sup> The basic bioprocess of minicells in target cells are endocytosis, intracellular degradation and payloads release.<sup>135</sup> Bacterial minicells are proved to minimize the side effects and increase the biocompatibility of cancer therapy and can reduce phagocytosis.<sup>136,137</sup> They are transported across the macrophage membranes and maintained stable for around 6 hours inside the cells.<sup>137</sup> The capability of encapsulating drug molecules of bacterial minicells is prominent as well. One minicell can encapsulate one million molecules of doxorubicin (DOX), whereas only around approximately 10,000 drug molecules are loaded into each liposome.<sup>133,138</sup> Moreover, with the combined characteristics of NPs and bacteria, minicells are more likely to target in the hypoxic regions of cancer and avoid clearance for a long period. But that is not enough. In order to achieve the precise targeting, the minicells are equipped with the bispecific antibodies (bsAbs), in which one arm interacts with the O-polysaccharide component present in LPS on the minicell surface and other arm interacts with the cell surface receptor specific to the target cancer cells.<sup>138</sup> EGFR and Her2/neu are the major targets of minicells modified with a bsAb, and several studies have testified the ability of bacterial minicells as an ideal delivery system for cancer treatment.<sup>139–141</sup>

### Outer Membrane Vesicles

OMVs are nanosized (20–250 nm diameter), spherical proteo-liposomes. They were first discovered by S. Chatterjee as the secretion products from *Vibrio Cholerae*, but currently OMVs are known as originated nanoscale vesicles from the surface of various Gram-negative bacteria by the route of blebbing or endolysin-triggered cell lysis.<sup>142,143</sup> OMVs consist of an outer leaflet of LPS and an inner leaflet of phospholipid. Multiple studies have illustrated that OMVs contain periplasmic and cytosolic proteins, DNA and RNA.<sup>144–147</sup> Phospholipids and LPS, as the main lipid component of OMVs, derivate from the outer membrane and the cell wall of gram-negative bacteria, respectively, representing the same antigens and PAMPs as the original bacteria.<sup>55</sup> These antigen proteins and PAMPs are transmitted to antigen presenting cells (APCs), thereby eliciting a pro-inflammatory host immune response.<sup>148</sup> Equipped with NP-specific EPR effect and a strong immune response induced by a variety of proteins and PAMPs, OMVs exhibit an enormous potential in anti-tumoral therapy.

### Magnetosomes

Magnetotactic bacteria (MTB) are iron-rich particles within intracytoplasmic membrane vesicles, observed and reported by Bellini and Blakemore, respectively, in the 20th century.<sup>149</sup> They are composed of an iron crystal, either magnetite (an iron oxide) or greigite (an iron sulfide), with the size of 20–60 nm surrounded by a proteolipidic membrane.<sup>150</sup> Magnetosomes are produced by MTB in a complex genetically controlled process that leads to the formation of colloiddally stable particles naturally covered by lipids, with high magnetism and a narrow size distribution. The size, morphology and alignment of magnetosomes varied depending on the MTB species and the minerals inside basically belong to 2 types: an iron oxide ( $\text{Fe}^{2+}\text{Fe}^{3+}_2\text{O}_4$ ), also called magnetite crystals, and greigite, an iron sulfide ( $\text{Fe}^{2+}\text{Fe}^{3+}_2\text{S}_4$ ), also called greigite crystals.<sup>151,152</sup> These ferrimagnetic materials consist of one or several magnetic domains, but the mature magnetosomes are stable single domain with the magnetic moments of all their constituting atoms pointing to the same direction.<sup>153</sup> Magnetosomes do not display toxic properties for eukaryotic models, therefore they are low-risk for systemic administration.<sup>154</sup> Due to their magnetic behavior, magnetosomes are currently used as efficient contrast agents for MRI. Compared with chemical contrast agents, magnetosomes do offer better contrast images.<sup>155,156</sup> After surface decoration, the imaging of magnetosomes can be specific to cancer regions.<sup>157</sup> They can also be used for curative purposes. The magnetism of magnetosomes is used to enhance the treatment efficiency in magnetic hyperthermia or photothermia against cancer.<sup>158</sup> Furthermore, after genetically modified, the magnetosomes can load chemotherapeutic drugs or gene therapy agents, target certain cancer cells and exhibit long-term release.<sup>159–161</sup>

### S-Layer of Bacteria

Many strains of archaea and bacteria (both Gram-positive and Gram-negative) contain regular protein arrays as the outermost component of their cell envelopes, which is referred as surface layer, or S-layers.<sup>162</sup> They are highly porous



protein/glycoprotein meshworks with unit cell sizes of 3–30 nm. The key features of S-layer proteins include self-assembly, structural regularity, mechanical and osmotic stability, predictable physicochemical properties, making it the ideal vehicles for drug delivery.<sup>163,164</sup> Moreover, the uniform patterning, unique morphology and the presence of functional groups make them useful biopolymers in biotechnology applications, such as designer matrices, porous ultrafiltration membranes, and substrates for the immobilization of active compounds.<sup>165,166</sup>

## The Treatment Strategy of Bacteria-Based NPs

Since the anti-tumor efficiency of bacteria monotherapy is not prominent, bacteria and their derivatives are frequently employed in hybrid therapies. In this section, we elaborated in detail on the current knowledge of the bacteria-mediated synergistic cancer therapy (BMSCT) and the recent achievements.

### Synergistic Bacteria-Mediated Chemotherapy

Chemotherapy has long been the main treatment for various types of primary cancer and metastasis. However, the delivery efficiency is still impeded by the off-target side effects and the limited intratumoral delivery. Although NPs or other target delivery system have gained some improvements, the chaotic vascular structure and high interstitial fluid pressure can still diminish the drug supply to the tumor center, which severely affect the therapeutic effects of the drugs. Given the specific characteristics of bacteria like active mobility, tumor-targeting and hypoxic-region penetration, bacteria-mediated chemotherapy may overcome the obstacles chemotherapy encountered currently.

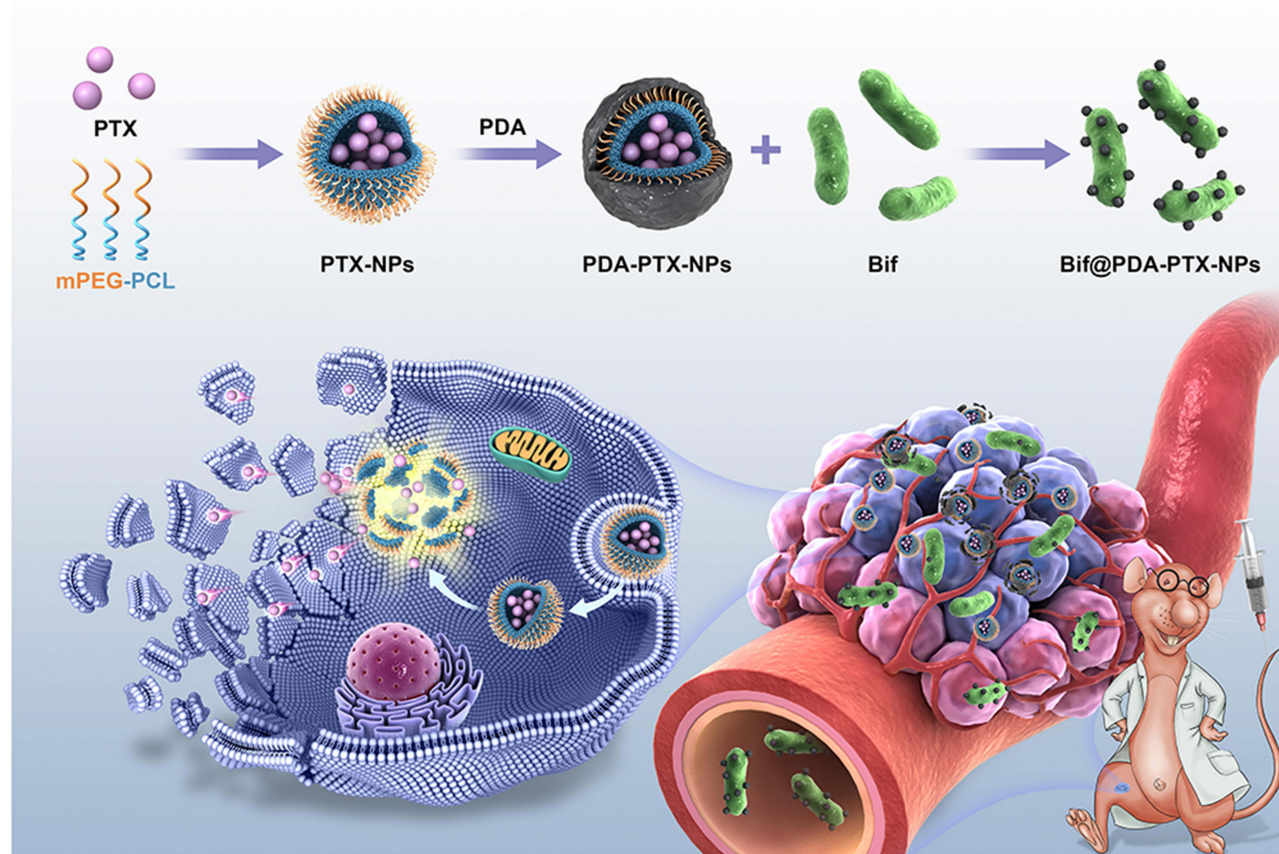
In the bacteria-mediated hybrid chemotherapy, bacteria and their derivatives usually participate as the vectors. Fu S et al successfully developed a series of feasible strategies for the targeted delivery of chemotherapeutic drugs to the interior hypoxic areas of solid tumor tissue in virtue of the inherent-targeting ability of the anaerobic *Bifidobacterium infantis* (*B. infantis*, Bif), thus potentially improving efficacy and alleviate toxic side effects.<sup>167–170</sup> For instance, a Bif-based biohybrid (Bif@PDA-PTX-NPs) was designed to deliver polydopamine (PDA)-coated paclitaxel NPs (PTX-NPs) to tumor tissues, which realized the growth inhibition of A549 xenografts in nude mice, and the survival prolongation of the tumor-bearing mice without any systemic or localized toxicity, providing a new therapeutic strategy for targeted chemotherapy to solid tumors<sup>167</sup> (Figure 1).

With much smaller size (20–250 nm) compared with their parent bacteria, bacterial OMVs and minicells were more frequently used to carry chemotherapeutic drugs to treat colorectal carcinoma, lung cancer, glioma, breast cancer, etc. For example, the OMV-coated, tegafur-loaded (a prodrug of 5-FU) polymeric micelles demonstrated substantial improvement in terms of tumor inhibitory rate and survival extension in B16F10 metastatic mouse models.<sup>171</sup> Kuerban et al developed another doxorubicin-loaded *Klebsiella pneumoniae*-derived OMV system which presented a substantial tumor growth inhibition with favorable tolerability with extensive apoptotic cells and necrosis in A549 tumor tissues in vivo.<sup>172</sup>

For non-pathogenic bacteria, two concerns should be noted. One is the toxicity at the dose required for therapeutic efficacy. A lower dose provides safety but treatment efficacy, which has resulted in the termination of some clinical trials.<sup>173</sup> The other concern is the deterioration of motility of bacteria after loading drugs. Intestinal probiotics EcN, with the loading of DOX via acid-labile linkers of cis-aconitic anhydride, have overcome both challenges. The number of conjugated DOX molecules per one bacterium was around  $4.7 \times 10^5$ , and the average velocities of the conjugated and free EcN were 9.0 and 9.8  $\mu\text{m/s}$ , respectively. The constructed EcN-Dox treatment realized the bacteria-directed accumulation and acid-responsive release of anticancer drugs in tumors, inhibited tumor growth, prolonged animal survivals and induced the apoptosis of tumor cells<sup>141,174</sup> (Figure 2).

### Synergistic Bacteria-Mediated Radiotherapy

Different from external beam radiotherapy, the bacteria-mediated radiotherapy usually refers to targeted radionuclide therapy, in which the bacteria system carries radionuclides to the targeted tumor region and releases radioactive particles, finally destroying the cancerous cells. Claudia G. et al have made some attempts in the treatment of early and advanced pancreatic ductal adenoma by establishing attenuated *Listeria*-based radionuclide delivery platform. They demonstrated in the metastatic pancreatic mouse tumor model that live attenuated *Listeria* coupled with radionuclide <sup>188</sup>Rhenium, could deliver radioactivity to the metastases and less abundantly to primary tumors without affecting normal cells, and



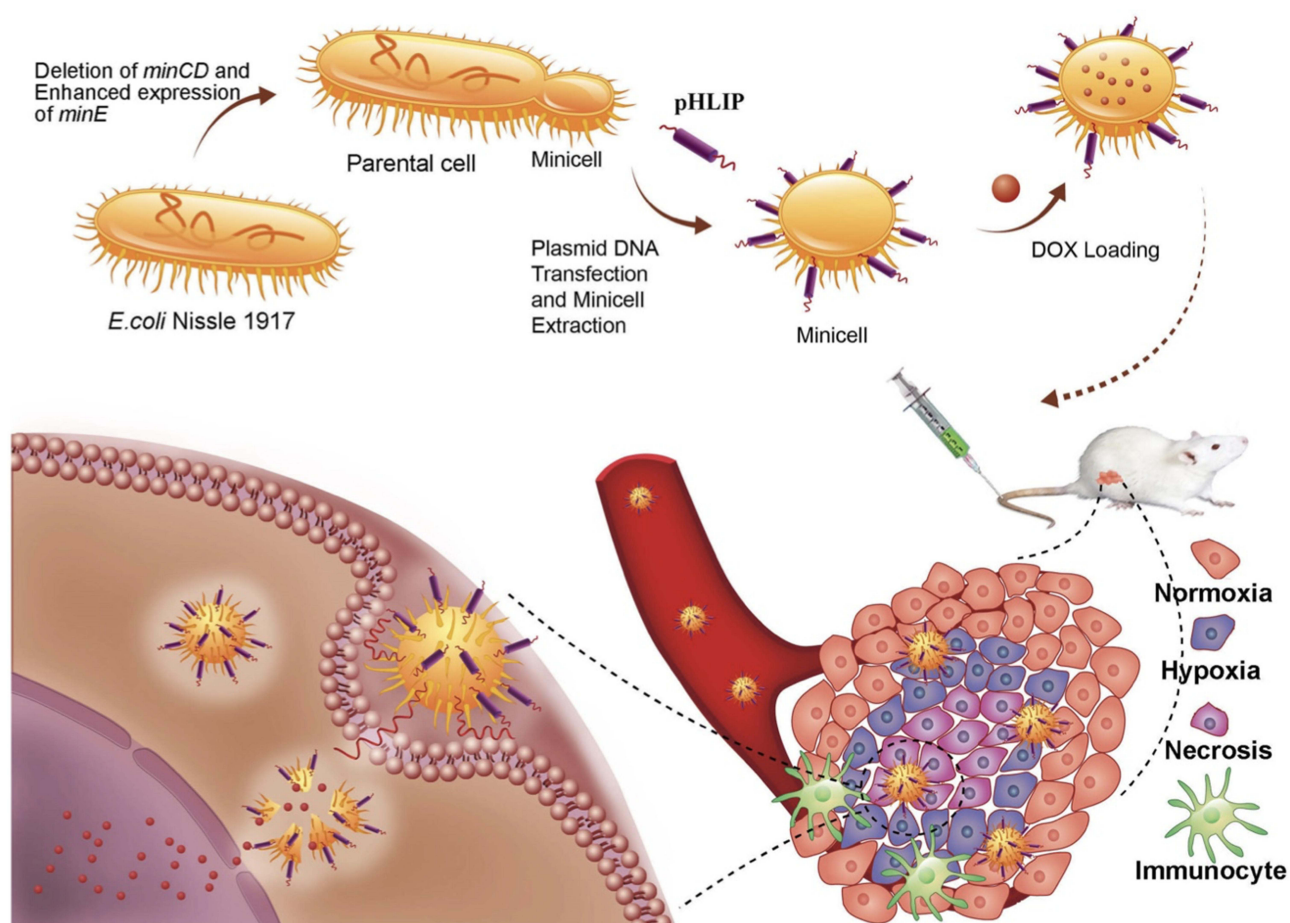
**Figure 1** Schematic diagram shows the construction of the Bif@PDA-PTX-NPs biohybrid and its intelligent responsibility to reductive tumor microenvironment through self-driven targeting to hypoxic regions of tumor.

**Notes:** Adapted with permission from Shi H, Chen L, Liu Y, et al. Bacteria-Driven Tumor Microenvironment-Sensitive Nanoparticles Targeting Hypoxic Regions Enhances the Chemotherapy Outcome of Lung Cancer. *International Journal of Nanomedicine*. 2023;18:1299–1315.<sup>167</sup>

finally killed tumor cells.<sup>175</sup> Four years later, their team developed another *Listeria*-based radionuclide delivery system by incorporating attenuated *Listeria* with <sup>32</sup>P. They found <sup>32</sup>P-*Listeria* predominantly accumulated in tumors and metastases and exhibited a great anti-tumor effect on primary and metastatic tumors in both pancreatic cancer models Panc-02 and KPC at early and advanced stages of pancreatic cancer, while *Listeria* alone did not have significant effect on advanced pancreatic cancer.<sup>176</sup> However, no matter the way they used to connect radionuclide with bacteria, the radio-toxicity, especially the harm induced by free radionuclide shedding from the delivery system should be given enough attention.

### Synergistic Bacteria-Mediated Phototherapy

Phototherapy has long been a method to eradicate tumor via the exposure of patients to light after the administration of photosensitizing agents. Basically, it can be further divided into 2 main approaches: photothermal therapy (PTT) and photodynamic therapy (PDT). In brief, in PTT, photothermal agents are irradiated by light of a specific wavelength, absorbed energy from photons and transformed the energy into heat, resulting in localized thermal damage. Different from PTT, PDT is largely a photochemical process that relies on light interaction with a photosensitizer, generating reactive oxygen species (ROS) and finally inducing cytotoxic effects.<sup>177</sup> However, there are still some challenges, for example, the limited depth of light penetration, the tumoral resistance to PTT and the most important one, the relatively low delivery efficiency of photothermal transduction agents (PTAs). Therefore, the tumor-targeting characteristics of bacteria have inspired new thoughts to the precise target in the field of phototherapy.



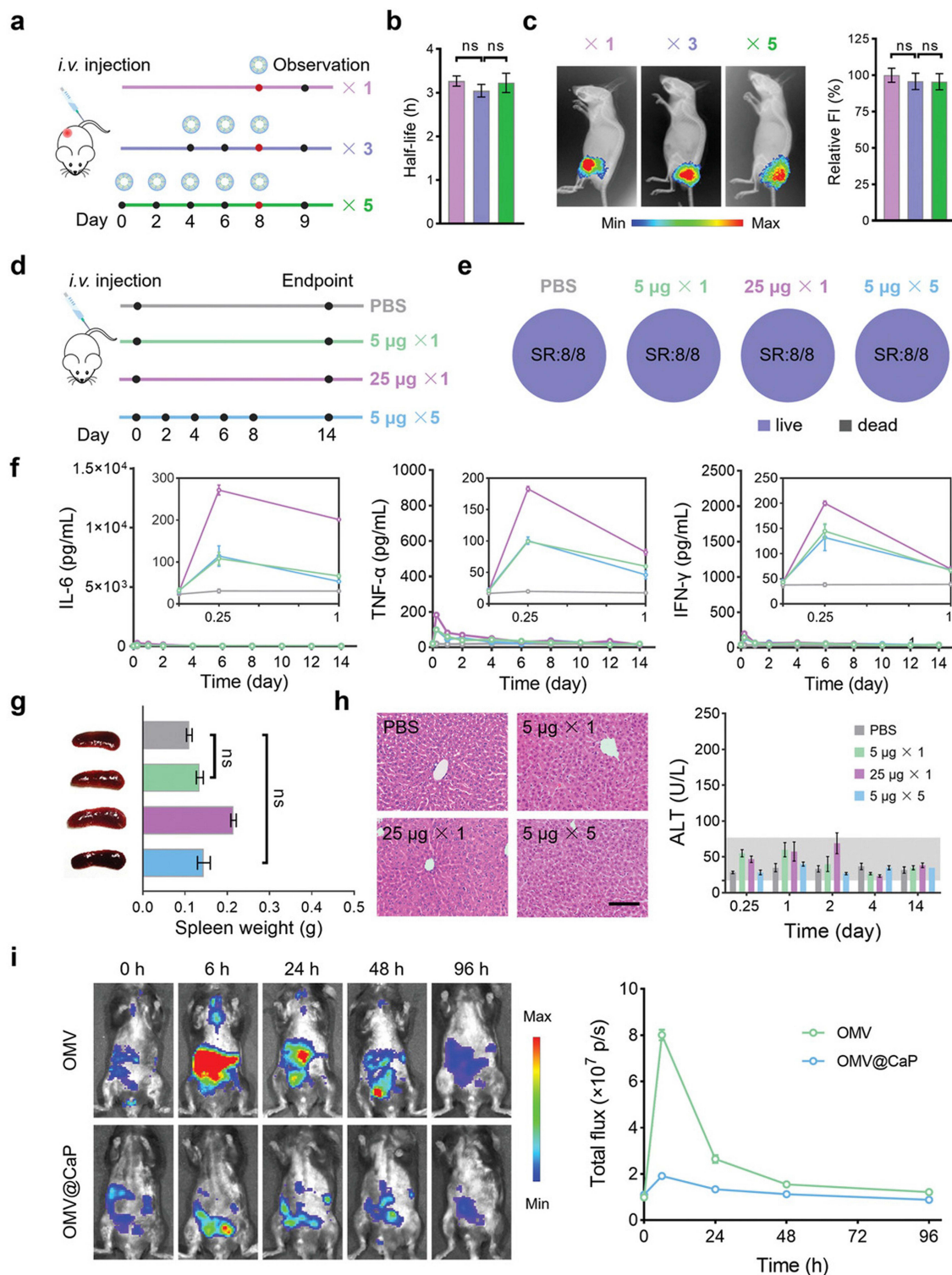
**Figure 2** Schematic illustration depicting the construction of the minicells pHLP for targeted delivery of chemotherapeutic drugs into the hypoxic regions of solid tumors to kill cancer cells.

**Note:** Adapted with permission from Zhang Y, Ji W, He L, et al. *E. coli* Nissle 1917-Derived Minicells for Targeted Delivery of Chemotherapeutic Drug to Hypoxic Regions for Cancer Therapy. *Theranostics*. 2018;8(6):1690–1705.<sup>141</sup>

In the work conducted by Shuang Q. et al, the authors establish a bacteria-related NP platform by employing calcium phosphate (CaP) to cover the surface of *E. coli*-derived OMVs (Figure 3). They selected an FDA-approved photosensitizer, ICG, to integrate with OMV@CaPs. For mice treated with OMV@CaP-ICGs. The tumor temperature rises up to  $\approx 45^{\circ}\text{C}$  after a mild NIR irradiation. Flow cytometry analysis indicated that OMV@CaP-ICGs treatment promoted the DC activation from 2.19% to 40.18% and increased the percentage of  $\text{CD8}^{+}$  T cells in vitro.

Then OMV@CaP- ICGs were intravenously injected into mice with luc-4T1 tumors every other day for 5 times. Twenty-four hours after the first injection, the tumors were irradiated with an 808 nm laser at a low power density ( $0.5 \text{ W}/\text{cm}^2$ ) for 15 min and the temperature of tumor reached around  $45^{\circ}\text{C}$ . It was recorded that after this PTT treatment, the tumor growth was completely suppressed and all mice in OMV@CaP- ICG group stayed alive until the endpoint at day 80.<sup>178</sup>

In terms of good biodegradability and biocompatibility, organic PTAs bring negligible health risk and are favorable in recent bacterial-mediated phototherapy studies. WF. Chen et al developed a polydopamine-mediated photothermal system by coating VNP20009 with polydopamine molecules. Three days after injected via the tail vein with 0.1 mL pDA-VNP, C57BL/6 mice bearing B16F10 tumor (tumor volumes were close to  $80 \text{ mm}^3$ ) were irradiated for 5 min with a near-infrared laser (808 nm,  $1.18 \text{ W}/\text{cm}^2$ ). Then tumor temperature reached up to  $55.0^{\circ}\text{C}$  and all mice treated with pDA-VNP PPT showed a complete tumor regression without apparent tumor relapse or metastasis during the 90-day experiment.<sup>179</sup> Similarly, Vipul Gujrati et al produced OMVs encapsulating biopolymer-melanin ( $\text{OMV}^{\text{Mel}}$ ) using a *E. coli* K12 strain expressing a tyrosinase transgene, which can be further detected by optoacoustic imaging. After intra-tumoral injection of  $\text{OMV}^{\text{Mel}}$  in mice bearing 4T1 subcutaneous tumors, the tumor surface temperature in the laser-



**Figure 3** OMV@CaPs for enabling efficient tumor accumulation and overcoming the severe inflammatory effect.

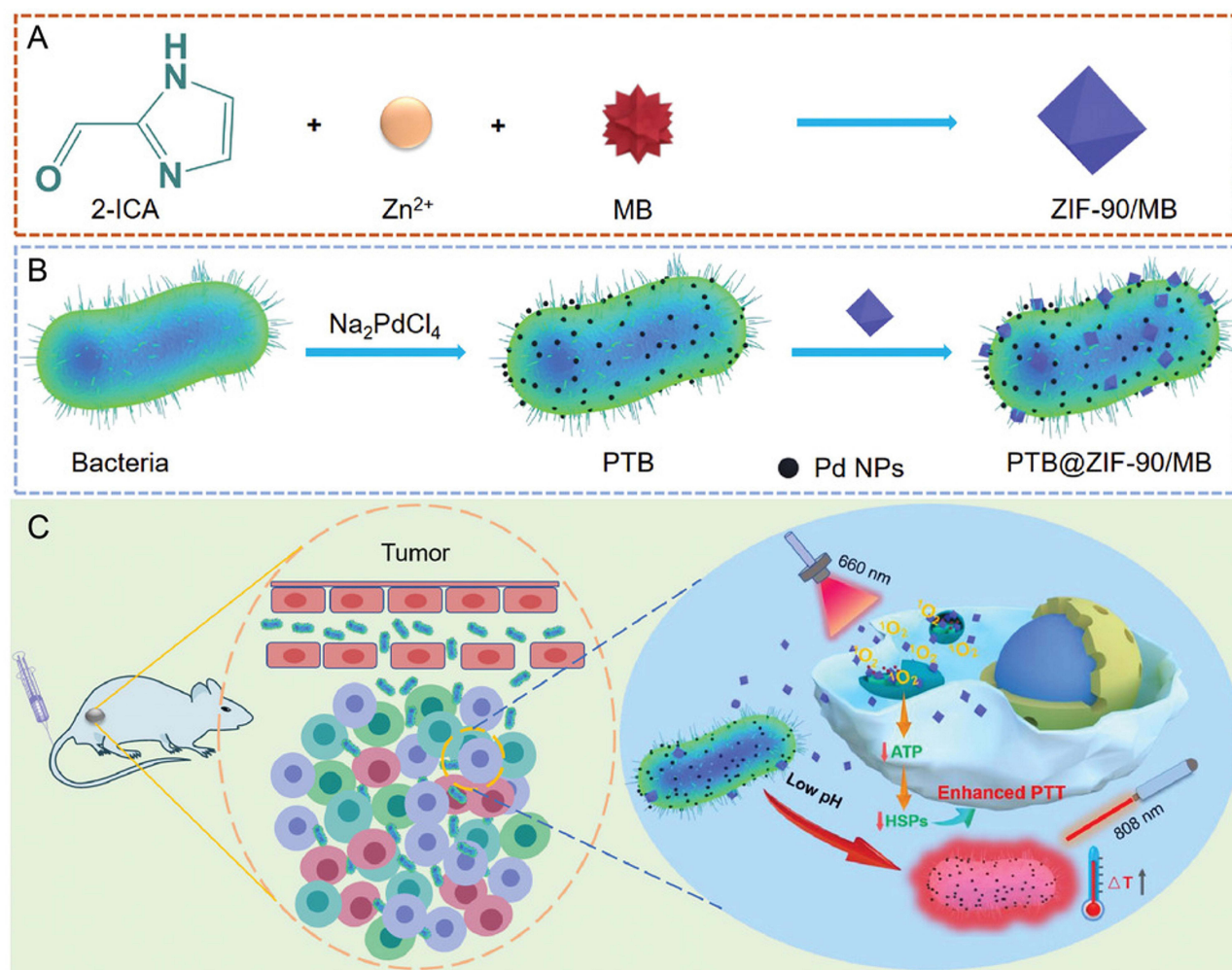
**Notes:** (a) Experimental design for testing tumor accumulation of OMV@CaPs in CT26-tumor-bearing mice with different injection modes (×1, ×3, and ×5). Each injection dose of OMV@CaPs contained 5 μg total protein of OMVs. Red dots mean administration of Cy7-labeled OMVs, while black dots mean unlabeled OMVs. (b) Half-life of OMVs with different injection modes (n = 3). (c) Representative fluorescence images of CT26-tumor-bearing mice 24 h after intravenous injection with Cy7-labeled OMV@CaPs (left) and their relative tumor accumulation efficiencies (n = 3) (right). (d) Experimental design for in vivo toxicity evaluation. (e) SR of each group at the endpoint (n = 8). (f) Systemic cytokines (IL-6, TNF-α, and IFN-γ) release profiles at different time points (n = 3). (g) Weight of spleens in each group and the corresponding representative photographs at the endpoint (n = 3). (h) H&E staining of liver (scale bar: 200 μm) and the serum level of ALT at different time points (n = 3). (i) Representative bioluminescence images of IFN-γ-IRES-Venus-AkaLuc mice after intravenous injection with OMVs and OMV@CaPs (equivalent protein amount) at different time points (left) and their corresponding total flux of luciferase signals (n = 3) (right). Data in (b, c), and (f–i) are presented as the mean ± SEM. Statistical significance was calculated via one-way ANOVA with a Tukey post hoc test. ns, not significant. Reprinted with permission from Qing S, Lyu C, Zhu L, et al. Biomaterialized Bacterial Outer Membrane Vesicles Potentiate Safe and Efficient Tumor Microenvironment Reprogramming for Anticancer Therapy. *Adv Mater.* 2020;32:1–14.<sup>178</sup> © 2020 Wiley-VCH GmbH.

exposed area (800 nm, 1.5 W/cm<sup>2</sup>, 6 min) reached as high as 56 °C and the tumor mass nearly disappeared. Therefore, the combination of bacterial components and organic PTAs could noninvasively be monitored the spatio-temporally, achieved therapeutic effect and resulted in very limited toxicity.<sup>180</sup>

Bacteria-mediated phototherapy system does help in the tumoral accumulation of PTAs, but other challenges still exist, for example, the heat resistance caused by heat shock protein (HSP). In the research performed by QW. Chen et al, they developed a tumor-targeting a photothermal bacterium (PTB) based on *Shewanella oneidensis* MR-1 and hybridize Zeolitic imidazole frameworks-90 (ZIF-90) encapsulating photosensitizer methylene blue (MB) on the surface of the living PTB. Under light illumination, MB could be released at mitochondria and cause mitochondrial dysfunction, which further contributed to adenosine triphosphate (ATP) synthesis inhibition and HSP down-regulated expression, overcoming the obstacle of heat tolerance and achieving the treatment enhancement of PTT<sup>181</sup> (Figure 4). Although the history of bacteria-mediated phototherapy is short, the promising results in relative studies encourage more brave attempts in this field.<sup>182–184</sup>

### Synergistic Bacteria-Mediated Gene Therapy

Beside these traditional treatments, gene therapy provides an alternative to cancer patients, especially those with metastasis. Basically, gene therapy involves the delivery of genetic information to patients to facilitate the production



**Figure 4** Schematic illustration of (A) synthetic procedure of ZIF-90/MB, and (B) synthetic procedure of PTB and PTB@ZIF-90/MB, and (C) mechanism of tumor targeting and photothermal tumor ablation of PTB@ZIF-90/MB upon laser irradiation.

**Notes:** Reprinted with permission from Chen QW, Liu XH, Fan JX, et al. Self-Mineralized Photothermal Bacteria Hybridizing with Mitochondria-Targeted Metal–Organic Frameworks for Augmenting Photothermal Tumor Therapy. *Adv Funct Mater.* 2020;30. <sup>181</sup> © 2020 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

of therapeutic proteins. The main problem of gene therapy is how to deliver these genetic products into target cells precisely. The characteristics of hypoxia region targeting of bacteria makes them ideal carriers for gene delivery.

Cancer cell is the main target of gene therapy, and the induction of cell apoptosis is a direct method to eradicate cancer. The VP3 protein of Chicken anemia virus, named apoptin, is capable of inducing G2/M arrest and apoptosis in several tumor cells. Cecropin B is a small molecule which could potentiate apoptin-induced apoptosis. Therefore, Xi Wang et al combined these two genes and used bacterial magnetic particles (*Magnetospirillum Gryphiswaldense* MRS-1) as carriers to deliver these genes to cancer cells. The constructed delivery system, BMP-pVAX1-VA, was proved to promote apoptosis of HepG2 by enhancing membrane deficiency and caspases upregulation in vitro. Via injection subcutaneously adjacent to tumor, BMP-pVAX1-VA showed ability to inhibit tumor growth in HepG2-bearing mice, which demonstrated that bacterium was an efficient and attractive gene delivery system.<sup>160</sup>

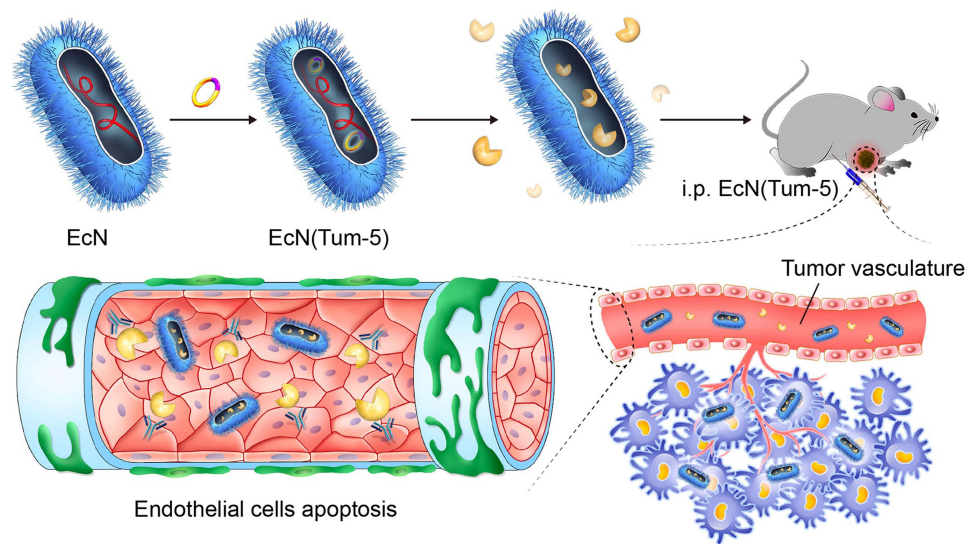
Besides gene transfection, another strategy to induce apoptosis in tumor cells is to take advantages of gene modification of bacteria themselves. Recently, the expression of apoptosis-related proteins by modifying genes in bacteria is popular in anti-cancer treatment. In the work conducted by Huan Wang et al, a eukaryotic expressing VNP20009-AbVec-Igk-AIF (V-A-AIF) strain was established. It was noted that the AIF expressed by the V-A-AIF strain significantly enhanced the apoptosis of B16F10 cells in vitro and decreased the tumor volume, and prolonged the lifespan in a melanoma-bearing mouse model after intravenous injection.<sup>185</sup> Another example is the construction of Bax BH3 peptide-released *Salmonella Typhimurium* SL3261. Inspired by the previous findings from Armando M. et al, they developed a peptide delivery system by applying the live attenuated *Salmonella enterica* serovar Typhimurium strain SL3261 expressing and releasing cell-permeable Bax BH3 peptide through the MisL autotransporter system. In vitro experiments demonstrated that the cell-permeable Bax BH3 peptide released from *S. enterica* promotes apoptosis of Romos cells whereas the in vivo study also proved the existence of antitumor activity of this bacterial-based system in a murine xenograft model of human B NHL.<sup>186</sup> Many molecules could activate apoptosis of cancer cells, which could be further investigated.<sup>187</sup>

Aside from apoptosis induction, disruption of tumor angiogenesis is also promising in retarding tumor growth. Basic fibroblast growth factor (BFGF), as a compensative molecule of VEGF, can cause resistance to anti-VEGF therapy and is regarded as another potential antitumor target. Based on these knowledgements, Huang et al constructed a BFGF-expressing *E. coli* DH5a competent strain and collected the BFGF-OMVs. After 3 times of BFGF-OMVs immunization, TC-1 tumor cells were subcutaneously inoculated into mice. These studies indicated that the growth of both the primary tumor and metastasis was completely suppressed by strong anti-BFGF autoantibody expression, angiogenesis inhibition and the reversion of tumor immune suppression barriers.<sup>188</sup> Similarly, an engineered strain of EcN was established to express Tum-5, the anti-angiogenic active fragment encoding amino acids of Tumstatin. It was proved that EcN (Tum-5) could specifically colonize the tumor and B16 melanoma tumor growth was remarkably restrained compared with pure EcN or Tum-5 deliveries<sup>121</sup> (Figure 5).

### Synergistic Bacteria-Mediated Immunotherapy

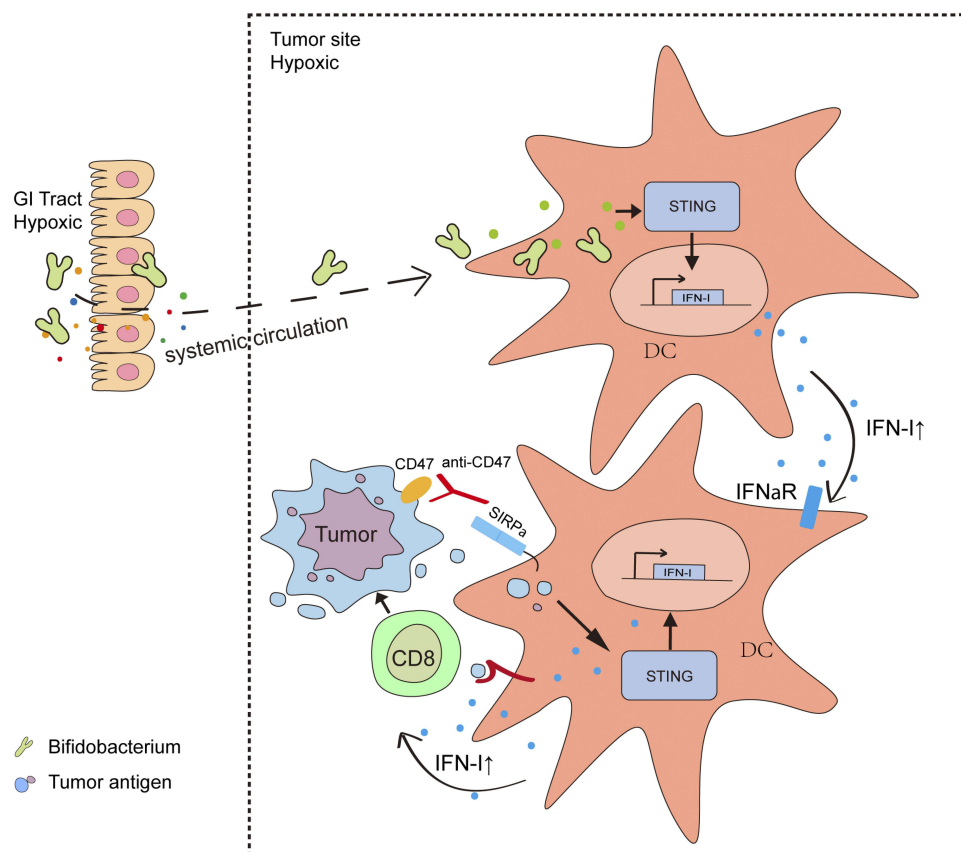
Immunotherapy, as an emerging and promising strategy in cancer treatment, brings hopes for precision cancer treatment. However, hypoxia is considered as one of the key characteristics of TME, which facilitates the immune suppressive tumor environment.<sup>189,190</sup> Bacteria-mediated therapy combined with immunology can enhance the efficacy of immunology through potentiating precise tumor targeting and immune response modulation.<sup>191,192</sup>

Bacteria-based NPs can reduce tumor burden synergistically with other treatment by eliciting an antitumor host immune response through producing immune-stimulating molecules and metabolites. *Bifidobacterium*, an anaerobic commensal bacterium residing in the GI tract with low toxicity, enhanced the antitumor efficacy of CD47-based immunotherapy. Live bacteria could produce secondary metabolites to trigger the STING signaling pathway inside DCs and modulate the immune response elicited by CD47 blockade through the capacity to actively target and colonize in the tumor microenvironment<sup>193</sup> (Figure 6). *Bifidobacterium bifidum* modulated the immunological background and enhanced PD-1 blockade or oxaliplatin treatment efficacy by facilitating the production of interferon- $\gamma$ , probably through the enhanced biosynthesis of immune-stimulating molecules and metabolites.<sup>194</sup>



**Figure 5** Tum-5 protein (yellow) could be solubly expressed in EcN and secreted to the medium. The engineered bacteria (blue) were rapidly specially colonized in mouse tumors. Tum-5 bound to integrin receptors on the surface of vascular endothelial cells to induce endothelial cell apoptosis. This process would cause blood vessels to shrink, then the tumor growth was suppressed.

**Notes:** Adapted with permission from He L, Yang H, Liu F, et al. Escherichia coli Nissle 1917 engineered to express Tum-5 can restrain murine melanoma growth. *Oncotarget*. 2017;8(49):85772–85782.<sup>121</sup>



**Figure 6** Schematic illustration depicts the mechanism by which gut microbiota (Bifidobacterium) preferentially colonize in tumor sites and facilitate immunotherapy via STING signaling.

**Notes:** Adapted with permission of Rockefeller University Press, from Shi Y, Zheng W, Yang K. Intratumoral accumulation of gut microbiota facilitates CD47-based immunotherapy via STING signaling. *J Exp Med*. 2020;217(5):e20192282; permission conveyed through Copyright Clearance Center, Inc.<sup>193</sup>

In addition to the aforementioned utilization of bacteria products as immunostimulants, bacteria can be developed as drug carriers to elicit immunology modulation, such as polarization of M2 macrophages to M1 macrophages. It was proposed that EcN<sub>Z/F</sub>@Au loaded with macrophage phenotype regulator zoledronic acid (ZOL) enhanced valid polarization of tumor-associated macrophages toward the M1 phenotype and an effective production of proinflammatory cytokines, leading to a synergistic efficacy on tumor growth inhibition.<sup>195</sup>

All these new synthesized therapies are all benefiting from the precise tumor-targeting characteristics of bacteria. With editable gene materials and bacteria-induced immune responses, the bacteria-based delivery system is destined to have bright future in human's fight against cancer.

## The Clinical Trials of Bacteria-Based NPs

With great success of various bacteria-based anti-cancer treatments in pre-clinical studies, bacteria and their derivatives are expected to be alternatives of treatments for primary and metastatic tumors ([Supplementary Table 1](#)). However, the very first trial of VNP20009 was claimed to fail with some tumor localization and no tumor retardation effects in the Phase I study, although no safety concerns were detected.<sup>196</sup> In 2020, another live attenuated strain of *S. Typhimurium* called Salmonella-IL2, was used to treat metastatic gastrointestinal cancers in 22 patients. Although no significant toxicities were detected by the doses of up to 10<sup>10</sup> colony forming unit, but still, no survival advantage was observed. Nevertheless, the demonstration of significant increase in circulating NK and NK-T cell suggested the immunological impact of Salmonella-IL2 on human body, which is a progression forward.<sup>197</sup>

Another strain which has been investigated intensely is CRS-207, a live, attenuated LM strain carrying double deletions (LADD) and secreting mesothelin. Mesothelin is a tumor-associated antigen present on normal mesothelial cells and highly expressed by many human tumor types, including mesotheliomas, pancreatic adenocarcinomas, non-small cell lung cancers (NSCLC), and ovarian cancers. Therefore, CRS-207 was investigated in the clinical trials to explore the usefulness and safety in cancer treatment. A phase I study in patients with advanced mesothelioma, non-small-cell lung cancer, ovarian cancer, and pancreatic adenocarcinoma first revealed that CRS-207 was well tolerated at a dose of 10<sup>9</sup> CFU and could induce a robust innate and adaptive mesothelin-specific T-cell response (NCT00327652)<sup>198</sup> Then, an open-label, randomized, controlled Phase II study of 90 patients with previously treated metastatic pancreatic cancer found that the overall survival did improve by sequential administration of low-dose cyclophosphamide (Cy), GVAX (granulocyte-macrophage colony-stimulating factor-secreting allogeneic pancreatic tumor cells) followed by CRS-207 compared with Cy/GVAX alone.<sup>199</sup> In light of the promising results, a phase II b randomized, multicenter study was further conducted to compare Cy/GVAX+CRS-207 with chemotherapy in patients with metastatic pancreatic cancer (NCT02004262). But it turned out that in a larger cohort (213 patients) the combination of Cy/GVAX+CRS-207 did not improve survival over chemotherapy.<sup>200</sup> Except metastatic pancreatic cancer, another CRS-207 clinical trial aimed to investigate the treatment efficiency of malignant pleural mesothelioma (MPM). A total of 60 patients with unresectable MPM were enrolled in this multicenter, open-label phase Ib study. The results showed that administration of CRS-207 was safe and the combination treatment with CRS-207 immunotherapy and chemotherapy led to tumor size reduction, disease control and improved survival.<sup>201</sup> Further clinical trials concerning the treating efficiency of CRS-207 are anticipated.

Bacterial derivatives, like minicells, packaged with chemotherapeutic drugs are also translating from laboratory to clinics. Preclinical studies showed that epidermal growth factor receptor (EGFR)-targeted, paclitaxel-loaded *Salmonella Typhimurium*-derived minicells (<sup>EGFR</sup>minicells<sub>pac</sub>) have antitumor effects in xenograft models. Then, an open-label, multi-center, first-time-in-human Phase I trial was launched. Twenty-eight patients with advanced solid tumors were enrolled and received infusion of <sup>EGFR</sup>minicells<sub>pac</sub> with different concentrations. This study confirmed that the maximum tolerated dose was defined as 10<sup>10</sup> <sup>EGFR</sup>minicells<sub>pac</sub> and nearly 50% patients achieved stable disease as their best response (ACTRN12609000672257).<sup>139</sup>

Recent clinical trials are exploring the treatment safety and possibility of various live attenuated bacteria. For instance, the administration of SGN1, a genetically modified strain of *Salmonella typhimurium* (VNP20009-M) that expresses L-Methioninase, is ready to recruit patients with advanced solid tumors to assess the safety and tolerability (NCT05038150, NCT05103345). Other trials involve genetically modified *Bifidobacterium longum*, *Clostridium novyi-NT* spores, *Yersinia enterocolitica* et al against different advanced solid tumors. Although more and more Phase I/II trials are launched, it still needs a period of time to reveal the real treating efficacy of these mini-robots in human body. Typical clinical trials are summarized in [Table 1](#).



**Table 1** Examples of Anti-Cancer Bacterial Clinical Studies

Biological Agent	Bacterium	NCT Number	Indication	Status	Results	Published Article/Website
VNP20009	Salmonella	NCT00004988	Advanced and/or metastatic, histologically documented solid tumors	Completed in 2002	/	2002, J Clin Oncol <sup>196</sup>
C. novyi-NT	Clostridium	NCT00358397	Treatment-refractory solid tumor	Terminated in 2016	Design problem	<a href="https://clinicaltrials.gov/study/NCT00358397">https://clinicaltrials.gov/study/NCT00358397</a>
CRS 207	Listeria Monocytogenes	NCT00585845	Advanced cancer of the ovary or pancreas, non-small cell lung cancer, or advanced malignant epithelial mesothelioma	Terminated in 2009	No reason provided	2012, Clin Cancer Res <sup>198</sup>
Mixed bacteria vaccine		NCT00623831	NY-ESO-1 expressing cancers	Completed in 2012	/	2012, Clin Cancer Res <sup>202</sup>
ADXS11-001	Listeria Monocytogenes	NCT01598792	HPV-16 +ve Oropharyngeal Carcinoma	Terminated in 2014	Patient 2 suffered DLT post-vaccination. The vaccine manufacturers withdrew support for the study	<a href="https://clinicaltrials.gov/study/NCT01598792">https://clinicaltrials.gov/study/NCT01598792</a>
APS001F	Recombinant Bifidobacterium longum	NCT01562626	Advanced and/or metastatic solid tumors	Suspended in 2021	No reason provided	<a href="https://clinicaltrials.gov/study/NCT01562626">https://clinicaltrials.gov/study/NCT01562626</a>
C. novyi-NT	Clostridium	NCT01924689	Treatment-refractory solid tumor malignancies	Completed in 2017	Single intratumoral injection of C. novyi-NT is feasible. Toxicities can be significant but manageable.	<a href="https://clinicaltrials.gov/study/NCT01924689">https://clinicaltrials.gov/study/NCT01924689</a>
HS-410	Intravesical Bacillus Calmette-Guerin (BCG)	NCT02010203	Bladder Tumor	Terminated	Inability to accrue due to changing treatment landscape	2017, Journal of Clinical Oncology <sup>203</sup>
JNJ-64041809	Live Attenuated Listeria Monocytogenes	NCT02625857	Metastatic castration-resistant prostate cancer	Completed in 2019	No results provided	2022, Prostate Cancer Prostatic Dis <sup>204</sup>
JNJ-64041757	Live Attenuated Listeria Monocytogenes	NCT02592967	Non-small Cell Lung Cancer	Terminated in 2018	Development of JNJ-64041757 discontinued due to lack of efficacy	<a href="https://clinicaltrials.gov/study/NCT02592967">https://clinicaltrials.gov/study/NCT02592967</a>
bacTRL-IL-12	Live, genetically modified Bifidobacterium longum	NCT04025307	Advanced, treatment-refractory solid tumors	Terminated in 2021	Sponsor decision	<a href="https://clinicaltrials.gov/study/NCT04025307">https://clinicaltrials.gov/study/NCT04025307</a>

(Continued)

Table 1 (Continued).

Biological Agent	Bacterium	NCT Number	Indication	Status	Results	Published Article/Website
Pembrolizumab	Clostridium novyi-NT spores	NCT03435952	Advanced solid tumors	Recruiting	/	2021, Clin Cancer Res <sup>205</sup>
/	Ty21a bacteria	NCT03421236	Non-muscle invasive bladder cancer	Recruiting	/	2022, Eur Urol Open Sci <sup>206</sup>
SGNI	Genetically modified strain of Salmonella enterica	NCT05038150	Refractory solid tumors	Not yet recruiting	/	2023, Journal of Clinical Oncology <sup>207</sup>
Pembrolizumab	T3P-Y058-739, a genetically-modified, live attenuated strain of the bacterium Yersinia enterocolitica	NCT05120596	Advanced solid tumors	Recruiting	/	<a href="https://clinicaltrials.gov/study/NCT05120596">https://clinicaltrials.gov/study/NCT05120596</a>
SGNI	Genetically modified strain of Salmonella enterica	NCT05103345	Advanced solid tumors	Not yet recruiting	/	2023, Journal of Clinical Oncology <sup>207</sup>

## Challenges and Future Perspectives

In all the published works, the promising anti-cancer bacteria and their derivatives have the common features including 1) targeting only tumor cells; 2) having tolerable toxicities and side effects and 3) being generically stable, which are the foundation of the optimal microorganism to be applied in human trials. Although many bacteria-based nanoprobe did perform well in animal experiments, we still need to consider the following questions which may hinder their application in human.

The first question is how to guarantee the high local concentration of the bacteria-derived carriers. It is known that bacteria are relatively small compared to mammalian cells, in order to avoid severe side effects, the concentration of injected bacteria or their derivatives should not be very high, which means that the functional proteins they delivered must be effective at a low concentration. Therefore, the tumoral concentration of bacteria-based materials after administration should be investigated carefully which can help to estimate the treatment efficacy of the subsequent molecules.

Second is the how to control the tumor localization and delivery toward efficient bacterial therapy. Bacteria, given their intrinsic mobility, are proved to have tumor targeting capability in both active and passive methods. With deepening insight into bacteria-based treatments, the live attenuated bacteria and their derivatives are modified in their structures and some are designed to carry various functional materials, which may deprive them of their original mobility which in turn may prolong the time they used and reduce the number of bacteria for localization or drug delivery. Moreover, diverse small-sized nanomaterials can accumulate intracellularly rather than outside the cell or on the membrane so as to exert potentially specific mechanisms of antitumor effects toward activation of precise cancer cell death pathways.<sup>208</sup> Hence, it is a novel field in anti-tumor bacterial therapy to investigate more about how to target specific organelles intracellularly.

The third question is how to guarantee the safety of the bacterial therapy. In almost all phase I studies of anti-tumor bacterial treatments, few side effects were reported. However, off-target effects and possible bacterial infection remain to be unavoidable concerns. Since robust intratumoral colonization of bacteria is essential for optimal therapeutic effect, such aggressive infection will inevitably cause infectious symptoms and laboratory changes. More researches are required to investigate how to keep or shift the balance towards an effective therapy with minimal safety concerns.<sup>209</sup> For example, multifunctional synergetic therapy combined with novel cancer therapy with engineered bacteria or extracted individual component, with high therapeutic effects and low toxic and side effects, is developed as one of the novel directions in the realm of anti-tumor bacterial therapy.

Translation from pre-clinical studies to clinical usage is a huge step. Differing from non-viable clinical agents, live therapeutic bacteria cannot be sterilized by the conventional standard. It is an uphill struggle to guarantee the safety, consistency and stability of the products. The mature and standard manufacturing processes of the bacterial-based products, like good manufacturing practices (GMP), need to be well-established in each case from producing, purifying to harvesting live bacteria following strict aseptic protocols with real-time supervision. Furthermore, more regulated issues including unintended spreading among public, the potential infection etc. should also be considered as well.

## Conclusion

Bacteria and bacteria-derived NPs have demonstrated promising potential in anti-cancer treatment. These bacterial-derived organisms are smart to realize intrinsic tumor targeting in both active and passive ways and retard tumor growth with anti-cancer agents secreted and immune response. Based on these advantages, bacteria and their derivatives are further developed into various platforms by delivering additional molecules and drugs in synergistic bacteria-mediated anticancer treatments. These platforms achieved excellent anti-tumor performances *in vivo* and *in vitro* studies and some of them have started clinical trials which brings hope to advanced tumor patients.

Nevertheless, the challenges concerning bacterial therapy still exist. How to guarantee the efficiency and reduce the possible infection after bacteria-derived agents injected? How to successfully translate the bacterial-related NPs from animal experiments to application in human? Current clinical trials are still in the exploration stage of bacteria-inspired medicine. We still have much to learn about the bacteria-based anti-cancer treatment. But with the deepening insights and ever-growing knowledge, these tiny robots may join us as strong weapons in our war against cancer in the near future.

## Abbreviations

EPR, Enhanced permeation and retention; NP, Nanoparticle; DDS, Drug delivery systems; TB, Tuberculosis; BCG, Bacilli Calmette-Guérin; TME, Tumor metabolism environment; IFP, Interstitial fluid pressure; TNF- $\alpha$ , Tumor necrosis factor; NO, nitric oxide; PAMP, Pathogen-associated molecular patterns; LPS, lipopolysaccharide; OMV, Outer membrane vesicles; TLR, toll-like receptor; IFN, interferon; BG, Bacterial ghost; DC, dendritic cells; S. Typhimurium, Salmonella enterica serovar Typhimurium; MTD, Maximum tolerated dose; LM, Listeria monocytogenes; InlA, internalin A; InlB, internalin B; ActA, actin assembly-inducing protein; LADD, Live attenuated double-deleted; LMDD, LM  $\Delta dal/\Delta dat$  strain; KBMA, Killed but metabolically active; LLO, Listeriolysin O; tLLO, truncated version of LLO; HPV-16, Human papilloma virus-16; C. novyi-NT, Clostridium novyi-NT; HIF-1, hypoxia inducing factor-1; EcN, Escherichia coli Nissle 1917; DOX, Doxorubicin; bsAb, bispecific antibodies; APC, antigen presenting cell; MTB, Magnetotactic bacteria; BMSCT, Bacteria-mediated synergistic cancer therapy; Bif, Bifidobacterium infantis; PDA, polydopamine; PTT, Photothermal therapy; PDT, Photodynamic therapy; ROS, Reactive oxygen species; PTA, Photothermal transduction agent; CaP, Calcium phosphate; OMV<sup>MeI</sup>, OMVs encapsulating biopolymer-melanin; HSP, Heat shock protein; PTB, Photothermal bacterium; ZIF-90, Zeolitic imidazole frameworks-90; MB, Methylene blue; ATP, Adenosine triphosphate; V-A-AIF, VNP20009-AbVec-Igk-AIF; BFGF, Basic fibroblast growth factor; ZOL, zoledronic acid; NSCLC, non-small cell lung cancers; Cy, cyclophosphamide; MPM, Malignant pleural mesothelioma; EGFR, Epidermal growth factor receptor; GMP, Good Manufacturing Practices.

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## Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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## Disclosure

The authors declare that they have no competing interests.

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