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Bacteria-mediated cancer therapy: A versatile bio-sapper with translational potential

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Bacteria are important symbionts for humans, which sustain substantial influences on our health. Interestingly, some bastrains have been identified to have therapeutic applications, notably for antitumor activity. Thereby, oncologists have developed various therapeutic models and investigated the potential antitumor mechanisms for bacteria-mediated cancer therapy (BCT). Even though BCT has a long history and exhibits remarkable therapeutic efficacy in pre-clinical animal models, its clinical translation still lags and requires further breakthroughs. This review aims to focus on the established strains of therapeutic bacteria and their antitumor mechanisms, including the stimulation of host immune responses, direct cytotoxicity, the interference on cellular signal transduction, extracellular matrix remodeling, neoangiogenesis, and metabolism, as well as vehicles for drug delivery and gene therapy. Moreover, a brief discussion is proposed regarding the important future directions for this fantastic research field of BCT at the end of this review.

KEYWORDS

bacteria-mediated cancer therapy, pathogen-associated molecular pattern (PAMP), drug delivery, cancer immunotherapy, bacterial cytotoxicity

Therapeutic bacteria for cancer treatment

Humans and bacteria have a symbiotic relationship. In average, one human being contains approximately 30 trillion human cells and 39 trillion bacteria (1). Diverse microbial community flourish in our epidermal tissues, mucosal tissues and digestive system, regulating our physiological behaviors in sophisticated ways. For example, the notorious *Helicobacter pylori* has been proved to increase the risk of developing gastric cancer. With the advances in detection methods based on genome sequencing, residential bacteria are also identified in tissues which were conventionally considered “sterile”,

including brains, placentas, kidneys and cancerous tissues of breast (2). Even though our knowledge about their roles in human health is still superficial, pioneering scientists and physicians have made great effort to utilize these microorganisms to fight against human cancer.

The modern concept of bacteria-mediated cancer therapy (BCT) originated from more than a century ago. A surgical oncologist, William Coley, tentatively treated cancer patients with a mixture of inactivated *Streptococcus pyogenes* and *Serratia marcescens* (Coley's Toxins) in 1883. From that attempt, isolated bacterial strains replaced deliberate infections by pathogenic bacteria in unsterile conditions during the mysterious natural therapies aiming at suppressing cancerous cell growth with infectious bacteria before the emergence of chemotherapies and radiotherapies. Even though BCT has never been a mainstream clinical treatment option for cancer, mechanistic and translational studies have been continuously devoted to elucidate the mechanisms by which bacteria could influence the growth of solid cancer. More bacteria strains have been chosen with rational consideration of their unique biological properties, and further engineered with modern genetic tools to achieve better therapeutic efficacy and safety profile. Here, we listed those strains which have gone through or in the middle of clinical trials registered under the guideline of U.S. Food and Drug Administration (Table 1).

This review would mainly discuss about the versatility of BCT, the underlying biological mechanisms in the triangular relationship among bacteria, cancer cells and host immune system, as well as problems urgently requiring thorough investigations in this field (Figure 1).

The mechanisms of BCT

Therapeutic bacteria function as immune stimulators

Many bacterial components, such as lipopolysaccharide (LPS), flagella, and bacterial DNA, exhibits substantial and specific influences on the immunostimulatory responses induced by the administration of either live or inactivated bacteriological preparations (16). These pathogen-associated molecular patterns (PAMPs) immediately initiate innate immune responses, marked by the accumulation of granulocytes and macrophages into the sites of infection, as well as a coordinated elevation of proinflammatory cytokines and chemokines (17–19). During BCT in which aerobic or facultative anaerobic bacteria are used, a large titer of bacteria penetrates and colonizes in the hypoxic tumor microenvironment (TME) simultaneously. Such an intense infection, as well as consequent innate immune responses, concomitantly lead to the lysis of neighboring tumor cells and releasement of cellular content, including tumor-associated antigens (TAAs) and tumor-specific antigens (TSAs) (20).

Antigen-presenting cells (APCs) engulf both bacteria and debris of cells infected by bacteria, and process them into antigens coupled with major histocompatibility complex (MHC) (21, 22). Eventually, antigen-specific T cells in draining lymph nodes are activated by APCs, marking the initiation of acquired immune responses. During the phase of acquired immune responses, the presence of bacterial PAMPs in TME is still beneficial to maintain the proinflammatory status of T cells and macrophages (23, 24). Therefore, bacterial PAMPs play key functions for the antitumor effects of therapeutic bacteria (Figure 2).

LPS is an important structural component of the outer wall of Gram-negative bacteria. Structure of LPS consists of lipid A, the core oligosaccharide and the O-antigen. LPS forms a dense network of hydrophobic compounds through ionic and covalent bonds, providing barrier protection for bacterial outer membrane and protecting bacteria from the lethal effects of the complement system and macrophages (25). In addition to its role in bacterial membrane, LPS, especially its lipid A portion, is also an important signaling molecule for the innate immune system (26). LPS specifically activates Toll-like receptor 4 (TLR4) and CD14, which are widely expressed by CD8⁺ T cells, macrophages, natural killer (NK) cells and Dendritic cells (DCs), and initiates tumor-suppressive downstream signaling cascades mainly through myeloid differentiation primary response 88 (MYD88), such as the secretion of proinflammatory cytokines and cytotoxic factors, as well as the formation of inflammasome. For example, bacterial lipopolysaccharide (LPS) enhances prostate cancer metastasis potentially through NF- κ B activation (27). *Salmonella* LPS could induce tumor-specific CD8⁺ T cell responses and the elevation of tumor necrosis factor- α (TNF α) in both TME and peripheral blood (28, 29). Moreover, Thuy Xuan Phan et al. demonstrated that *Salmonella typhimurium* enhanced inflammasome activation in tumor-infiltrating macrophages following their phagocytosis of *Salmonella*-infected tumor cells in a TLR4-dependent manner, and thus increased their level of interleukin-1 β (IL-1 β) secretion (30–32).

Flagellin is a subunit protein at the tip of bacterial flagella, the locomotive organ of bacteria, and it is recognized by TLR5 as a PAMP (33). Flagellin has been shown to induce perforin-dependent NK cell-mediated antitumor responses (34). Flagellin also activates NK cells through a TLR-independent pathway involving IL-18 and MYD88 to produce interferon- γ (IFN γ), a key cytokine in innate and adaptive immunity. Moreover, Francesc Coll et al. demonstrated that flagellin could significantly suppress tumor cell proliferation by decreasing the frequency of CD4⁺CD25⁺ regulatory T (Treg) cells (35). Chung Truong Nguyen *et al.* showed that flagellin also enhances a CD8⁺ T cell-dependent antitumor response in a peptide vaccine-based immunotherapeutic procedure (36).

Bacterial DNA is the genetic material shared by Gram-positive and Gram-negative bacteria, and it has broad-spectrum immune-stimulating effects. CpG motifs are the

TABLE 1 The therapeutic bacteria strains finish or undergo clinical evaluation.

Category	Strain/gene	Type of cancer	No. of patients treated	Clinical phase	NCT identification	Reference/Recruitment Status
<i>Salmonella typhimurium</i>	<i>Salmonella typhimurium</i> VNP20009	Melanoma, renal cell carcinoma	25	Phase I		Published (3)
		Melanoma	4	Phase I		Published (4)
		Refractory, superficial solid tumors	12-40	Phase I	NCT00004216	Unpublished, completed
	<i>Salmonella typhimurium</i> TAPET-CD (<i>VNP20009</i> expressing CD)	Head & neck squamous cell carcinoma, esophageal adenocarcinoma	3			Published (5)
	<i>SalpIL2</i> (<i>Salmonella</i> χ 4550 expressing IL-2)	Liver metastases of solid tumors	22	Phase I	NCT01099631	Unpublished, completed
<i>Salmonella</i> spp.	<i>S. typhi</i> Ty21/Anti-VEGFR-2 (<i>VXM01</i>)	Pancreatic cancer	30	Phase I		Published (6)
	<i>TXSVN</i> vaccine derived from <i>Salmonella</i> sp. (<i>CVD908ssb</i>)	Multiple Myeloma	24		NCT03762291	Unpublished, Recruiting
<i>Clostridium novyi</i>	<i>Clostridium novyi</i> -NT	Colorectal cancer	2	Phase I	NCT00358397.	Unpublished, terminated
		Solid tumor malignancies	5	Phase I	NCT01118819	Unpublished, terminated
		Solid tumor malignancies retroperitoneal leiomyosarcoma	24	Phase I	NCT01924689	Published (7)
<i>Listeria monocytogenes</i>	ANZ-100/CRS-100 (LADD)	Pancreatic cancer, colorectal cancer, and melanoma all with liver metastases	9	Phase I	NCT00327652.	Published (9)
		CRS-207 (LADD)	Pancreatic cancer, mesothelioma, ovarian cancer, non-small-cell lung cancer	17	Phase I	NCT00585845.
	ADXS11-001(<i>Lm-LLO</i>)	Pancreatic cancer	90	Phase II	NCT01417000.	Published (10)
		Mesothelioma	60	Phase I	NCT01675765.	Published (11)
		Pancreatic cancer	303	Phase II	NCT02004262	Published (12)
		Ovarian, fallopian or peritoneal cancer	35	Phase I/II	NCT02575807.	Unpublished; terminated
		Mesothelioma	10	Phase II	NCT03175172.	Unpublished; terminated
		Gastric, gastroesophageal junction, or esophageal cancer	5	Phase II	NCT03122548.	Unpublished; terminated
		Cervical Intraepithelial Neoplasia	81	Phase II	NCT01116245.	Unpublished; terminated
		Cervical cancer	54	Phase II	NCT01266460.	Published (13)
		HPV-16+, p16+OPSCC		Phase I	NCT01598792/ISRCTN47069182	Unpublished; terminated
		HPV positive oropharyngeal squamous cell carcinoma	15	Phase II	NCT02002182.	Unpublished; active, not recruiting
		Anal cancer	11	Phase I/II	NCT01671488.	Unpublished; terminated
		HPV+ Cervical cancer	25	Phase I/II	NCT02164461.	Unpublished; completed
		Cervical or HPV+ Head & neck cancer	66	Phase I/II	NCT02291055.	Unpublished; unknown
Anal or rectal cancer	51	Phase II	NCT02399813.	Unpublished; completed		
Cervical cancer	450	Phase III	NCT02853604.			

(Continued)

TABLE 1 Continued

Category	Strain/gene	Type of cancer	No. of patients treated	Clinical phase	NCT identification	Reference/Recruitment Status
		HPV+ Non-small cell lung carcinoma	124	Phase II	NCT02531854.	Unpublished; active, not recruiting
	<i>Lm -LLO-E7 (Lm -LLO)</i>	Cervical cancer	15	Phase I		Unpublished; unknown
	<i>ADXS-NEO (Lm-LLO)</i>	HER2 expressing solid tumors	12	Phase I/II	NCT02386501.	Published (14)
		Melanoma, Colon cancer, head and neck cancer, non-small cell lung cancer, urothelial carcinoma	5	Phase I	NCT03265080.	Unpublished; active, not recruiting
	<i>ADXS31-142</i>	prostate cancer	51	Phase I/II	NCT02325557	Unpublished; unknown
	<i>ADU-623 (LADD)</i>	Astrocytic tumors	11	Phase I	NCT01967758	Unpublished; completed
	<i>JNJ-64041809</i>	Prostate Cancer	26	Phase I	NCT02625857	Unpublished; completed
	<i>JNJ-64041757</i>	Non-small cell lung cancer,	18	Phase I	NCT02592967	Unpublished; terminated
	<i>pLADD (LADD)</i>	Colorectal neoplasms	28	Phase I	NCT03189030.	Unpublished; terminated
<i>Enterococcus gallinarum</i>	<i>MRx0518</i>	Pancreatic Cancer	15	Phase I	NCT04193904	Unpublished; Recruiting
<i>Bifidobacterium longum</i>	<i>APS001F (B. longum expressing CD)</i>	Advanced and/or Metastatic Solid Tumors	75	Phase I/II	NCT01562626	Unpublished, suspended
	<i>bacTRL-IL-12</i>	Solid Tumours	5	Phase I	NCT04025307	Unpublished, suspended
<i>Clostridium butyricum</i>	<i>CBM 588 Probiotic Strain</i>	Kidney Cancer	30	Phase I	NCT03829111	Published (15)
		Hematopoietic and Lymphoid Cell Neoplasm	36	Phase I	NCT03922035	Unpublished, active, not recruiting
		Kidney Cancer	30	Phase I	NCT05122546	Unpublished, recruiting

structural basis for its immune activity, which are recognized by TLR9 (37–40). Bacterial DNA directly activates mouse macrophages, B cells, and DCs in a TLR9-dependent manner, upregulates the expression of immunostimulatory molecules, modulates immune responses, and induces the secretion of various cytokines, including IL-12, IL-1, and TNF α (39, 41–44). Moreover, bacterial DNA indirectly activates Th1 and CTL through cytokines, DCs, and other monocytic cells (43–45). For example, KJ Stacey et al. showed that bacterial DNA directly activated APCs and upregulated the expression of MHC-class II molecules and costimulatory molecules such as CD86 and CD40, thus activating antigen-specific T cells to stimulate acquired immune responses (46, 47).

Apart from PAMPs, the production of TAAs/TSAs (tumor-associated antigens/tumor-specific antigens) is the key step for the successful shift from innate immune to acquired immune

responses, which have the merit of high specificity and long-lasting memory (48). However, the efficacy of the concomitant lysis of tumor cells by bacteria-induced immune response is largely affected by the health conditions of recipients, such as the competency of immune system and the vasculature status of TME. Therefore, bacteria-mediated antigen delivery system is developed to bypass these limitations, in which bacteria are modified to carry TAA/TSAs by themselves (49, 50). This strategy of delivering TAAs/TSAs could effectively shape the host antitumor immune response and significantly suppress the growth of tumor. For example, Yu Mei et al. utilized the attenuated *Salmonella typhimurium* SL7207 to deliver a murine melanoma vaccine via the transformation of prokaryotic expression plasmids expressing the AIDA-1 autotransporter and DNA vaccine elements (51). Similarly, Xiong et al. constructed a novel SPI2-based oral *Salmonella* vaccine expressing mutant human *Survivin* in

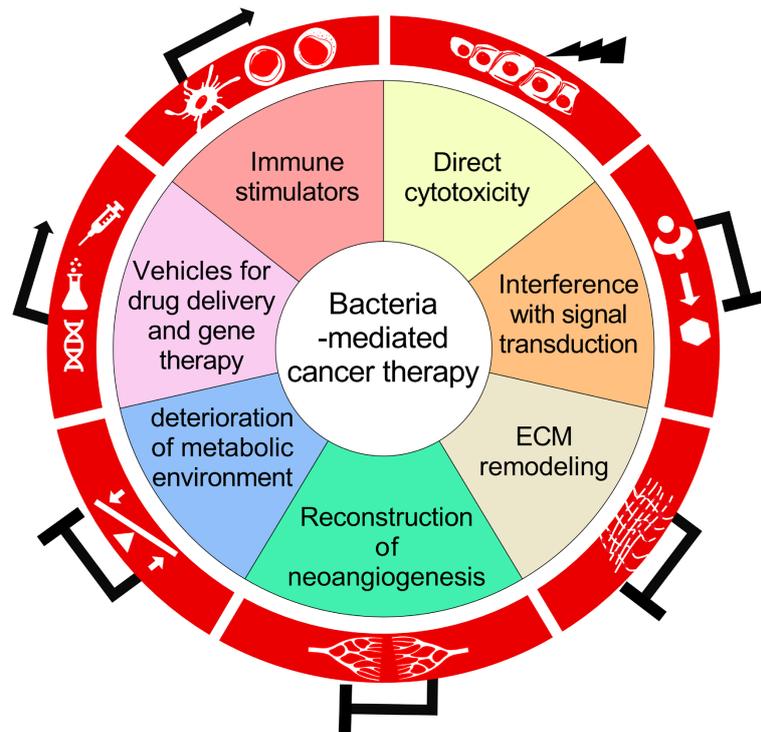


FIGURE 1

A schematic diagram summarizing the biological mechanisms for the antitumor activity of bacteria-mediated therapy.

combination with NKT ligands, and achieved significantly improved generation of antigen-specific effector-memory $CD8^+$ and $CD4^+$ T cells, as well as increased antitumor efficacy, in murine models of colon cancer and glioblastoma (52). Apart from live bacteria-mediated antigen delivery system, bacteria components such as bacterial membrane could be directly used as vaccine adjuvant. For example, Ravi B Patel et al. used bacterial membrane-coated nanoparticles composed of immune activating PC7A/CpG polyplex core to *in situ* capture cancer neoantigens following radiation therapy, which facilitated their up-taken by DC cells to stimulate a strong antitumor T cell response (53).

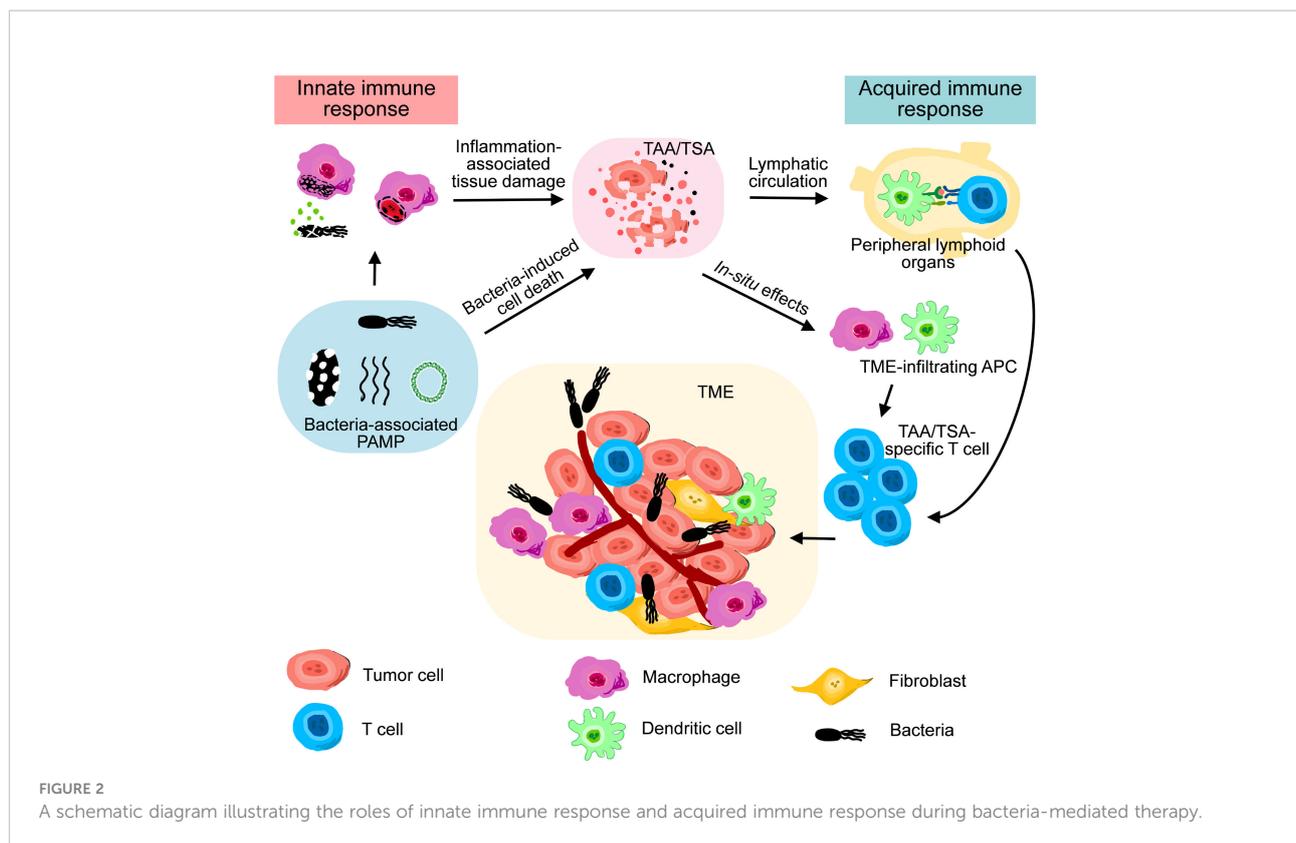
Therapeutic effects of BCT independent of immune responses

In addition to the induction of host immune responses, therapeutic bacteria exhibit direct cytotoxicity to cancer cells. An *in vitro* cell killing experiment showed that *Salmonella typhimurium* A1-R, an attenuated *Salmonella* strain, could cause cell death for prostate cancer cell lines *via* mixed cytotoxic mechanisms independent of immune responses (54). For example, *Salmonella* invasion induced rapid necrosis due to cytoplasmic swelling and membrane disruption (54). Extensive intracellular *Salmonella* duplication also led to the bursting of invaded cells (54).

Moreover, *Clostridium novyi-NT* could secrete phospholipases, hemolysins and lipases to disrupt the structure of lipid bilayers of cancer cells, thereby causing direct cytotoxicity (55, 56).

Apart from direct cytotoxicity, bacterial infection could suppress the growth of cancer cells through interfering with intracellular signal transduction. For example, *Salmonella typhimurium* flagellin could suppress the proliferation of breast cancer cells by activating the membrane-anchored TLR5 of cancer cells (57). *Salmonella* infection would also suppress AKT/mTOR pathway, thus leading to the induction of apoptotic and autophagic pathways (58). *Listeria monocytogenes* could kill tumor cells by enhancing cytosolic reactive oxygen species (ROS) levels through the activation of nicotinamide adenine dinucleotide phosphate oxidase and intracellular calcium mobilization, and such immunogenic tumor cell death would further activate $CD8^+$ T cells to eliminate both primary tumors and metastases (59). Other than the proliferation and death of cancer cells, other cellular behaviors such as drug sensitivity would be influenced by therapeutic bacteria. For example, *Salmonella choleraesuis* induced a significant increase in the protein level of connexin 43 which mediated gap intercellular communication between cancer cells, thus sensitizing them to cisplatin (60).

The extracellular matrix (ECM) of tumor tissues would also be changed after the infection of therapeutic bacteria. ECM is an important component that maintains tissue integrity, and regulates



cell behaviors through transmembrane signaling transduction (61). Solid tumors generally contain a high abundance of collagens and hyaluronic acids (HA), making them stiffer than normal tissues (62, 63). Malignant cells proliferate and migrate faster in such mechanical environment (64, 65). Meanwhile, accumulated ECM also creates structural obstacles physically for various cancer therapies, including chemotherapies and immunotherapies (66, 67). For example, collagen-laminin network forms a firm barrier for the diffusion of large molecules, while excessive HA dampens the passive release of drugs from blood vessels *via* increased osmotic pressure and viscosity of tissue fluid (68). Therapeutic bacteria could reduce the stiffness of ECM by multiple mechanisms. For example, *Salmonella typhimurium* could convert plasminogen to plasmin, a broad-spectrum serine protease, by inactivating Plasminogen activator inhibitor 1 (PAI-1), thus leading to the degradation of laminin as well as the activation of matrix metalloproteinases (MMPs) precursors to degrade collagens (69). *Listeria monocytogenes* are also capable to secrete MMPs (70). Some Gram-positive bacteria such as *Clostridium perfringens* (Mu toxin), *Clostridium difficile*, *Clostridium septicum* (γ toxin) and *Clostridium chauvoei* produces hyaluronidase that degrades HA (71–73). Other mechanisms by which bacteria lead to ECM disruption have been systematically reviewed by Lennert Steukers et al. (74). Taking advantages of these bacterial enzymes targeting intertumoral ECM, therapeutic bacteria have been utilized to combat solid tumors which are difficult to target by conventional chemotherapies. For

example, Nancy D. Ebelt et al. recently reported that genetically modified attenuated *Salmonella typhimurium* expressing exogenously bacterial hyaluronidase could invaded into the desmoplastic tumors and degraded HA with a significantly enhanced efficacy in the orthotopic human pancreatic ductal adenocarcinoma mice models (75).

Besides cytotoxicity, signal transduction interference and ECM remodeling, the reconstruction of neoangiogenesis and metabolism landscape are also important features for bacteria-mediated tumor therapy. For example, *Salmonella typhimurium* VNP20009 treatment could reduce the formation of neovascularization, therefore leading to retarded diffusion of nutrients in TME (76, 77). *Salmonella choleraesuis* has been demonstrated as both tumoricidal and antiangiogenic for the treatment of hepatocellular carcinoma (78). Moreover, both *Salmonella typhimurium* and *Listeria monocytogenes* are glucose-consumers, which would increase nutritional stress for tumor cells and deteriorate the metabolic microenvironment in TME (79, 80).

Therapeutic bacteria as tumor-targeting vehicles for drug delivery

Bacteria own several merits as pharmaceutical carriers for cancer therapies. First, anaerobic and facultative anaerobic

bacteria have a natural tendency of penetrating and colonizing the hypoxic areas in tumor due to intrinsic locomotion, which exceeds the limits of vascularization. Second, bacteria own high surface-to-volume ratio, which provides suitable interface for particle absorption. Third, bacterial surface molecules such as polysaccharides and glycoproteins are potential subjects for chemical modifications. Last but not least, the cytotoxic effects of the delivered compounds and bacteria-induced immune activation might achieve synergistic effects during cancer therapy.

Therapeutic bacteria can be used as live vehicles (similar to “mules”). For example, Shuangqian Yan et al. reported a bacteria@MOFs system in which the flagellum of *Escherichia coli* MG1655 were modified with metal-organic framework (MOF) encapsulating chemical drugs through a one-step *in situ* method, could maintain viability and tumor selectivity to achieve effective delivery of chemical drugs to the poorly-vascularized areas inside the tumors (81). Moreover, additional guiding motifs could be incorporated. For example, Byung-Wook Park et al. linked *Escherichia coli* with drug-loaded polyelectrolyte multilayer microparticles with embedded magnetic nanoparticles at an approximately 1:1 ratio, to which system the authors referred as “microswimmers”, and such drug carriers with a diameter of 1 μm could directionally move at a mean speed of up to 22.5 $\mu\text{m}/\text{s}$ under the orientation of an electromagnetic field, which would benefit the bacteria-mediated therapies for those highly vascularized tumors showing weak hypoxia (82). Packaging materials of different sizes and functions have been applied to decorate therapeutic bacteria (83–86), including polymeric particles (82, 87), polymer tubes (88), red blood cells (89–91), liposomes (92, 93), and nanoparticles (94–97). For example, Mukrime Birgul Akolpoglu et al. reported a biohybrid microbotic platform by combining nanoliposomes and *E. coli* MG1655 (98). Recently, Fenton-like reactions which eliminate cancer cells by generating toxic ROS have developed rapidly as chemodynamic therapies, and therapeutic bacteria improve such therapies by biocatalytically producing H_2O_2 , a key reactant for Fenton-like reactions. For example, magnetic Fe_3O_4 nanoparticle-decorated *Escherichia coli* MG1655 overexpressing respiratory chain enzyme II (NDH-2) could support Fenton-like reaction by continuously producing H_2O_2 , which triggered abundant tumor cell apoptosis by the excessive cytotoxic hydroxyl radicals (94).

However, frequent and repetitive administration is not suitable for live bacteria-mediated therapy, since its slow whole-body clearance might lead to accumulated systemic toxicity. Thus, drugs to be delivered by live bacteria should be chosen with great caution. Specifically, the ideal drugs to be delivered should be able to achieve sustained effects with one single dose.

Vesicles made with bacterial membranes, instead of live bacteria, are promising strategies to improve the safety profile of bacteria-mediated drug delivery. Small bacterial outer membrane vesicles (< 200 nm) are naturally released from

Gram-negative bacteria during proliferation (99), and particles of such size often exhibit strong accumulation in TME through enhanced permeability and retention (EPR) effect during blood circulation. Recently Qi Chen et al. used DSPE-PEG-RGD-decorated bacterial outer membrane vesicles to encapsulate F127 mesoporous nanoparticles containing cytotoxic Tegafur. The obtained nanodrug could be preferentially accumulated at the site of tumors, and simultaneously induced the killing of tumor cells and the activation of immune cells such as macrophages and T cells. Eventually, the tumor antigens released by dead tumor cells initiated potent systemic immune responses eliminating both primary tumors and metastatic lesions (20).

Larger membrane vesicles maintaining the entire bacterial morphology can be produced *via* membrane perforation mediated by lysis gene *E* from bacteriophage ϕX174 (100), and they are often referred to as “bacterial ghost (BG)” by many researchers. BGs can be used immune adjuvants (101), and they have good structure integrity upon lyophilization, which is beneficial for massive production (102). Water-soluble drugs can be loaded into BGs through membrane pores (103), and these pores can be sealed by fusion with small membrane vesicles in the presence of Ca^{2+} ions to prevent drug leakage (104). *In vitro* evaluation showed that such drug-loaded bacterial ghosts could be up-taken by both macrophages and cancer cells, with a preference for macrophages (104). Considering the size of entire bacteria has exceeded the range for strong EPR effect, BGs might not be the best choice for targeting tumor cells, but they are highly efficient for targeting APCs which preferentially engulf bacteria. For example, N.Dobrovolskienė et al. prepared BGs filled with tumor lysate containing numerous tumor antigens, and such antigen-loaded BGs induced a stronger dendritic cell activation compared to LPS (105).

The combination of BCT and photothermal/photodynamic therapy

Photothermal therapy (PTT) and photodynamic therapy (PDT) are new strategies for cancer therapy. PTT uses photosensitizers (PSs) with specific light absorption to convert light energy into heat energy to eliminate cancer cells, while PDT uses PSs to produce excessive ROS in the presence of specific wavelengths of light. PTT, as a non-invasive cancer treatment, can cause strong tumor ablation and simultaneously induce heat shock proteins produced by tumor cells, which is a family of proteins with moderate immunostimulant function (106). In principle, PTT could also provide cancer treatment that causes relatively little damage to surrounding healthy tissue, since thermal effects only occur when near-infrared light is applied and only in the presence of PTT reagents (107). PDT takes

advantage of the active metabolism of tumor tissue; whereby non-toxic photosensitizers accumulate in tumor tissue after injection. When the tumor tissue is irradiated with harmless visible light, the activated photosensitizer transfers its energy to surrounding intracellular oxygen that forms reactive oxygen species (ROS), which specifically destroy the tumor cells and neovascularization (108). PSs for PTT/PDT include organic dye molecules, organic nanoparticles, noble metal materials, carbon-based materials, quantum dots, and metal oxide nanoparticles (109–111). The *in vivo* distribution pattern and local drug concentration of PSs are critical factors determining the antitumor efficacy and systemic toxicity for PTT/PDT.

In bacteria-mediated PTT/PDT therapy, PSs are attached to the surface of bacteria to construct a bacteria-driven PSs delivery system, which significantly improves the tumor-targeting efficacy of PSs. For example, Chen et al. attached the INPs (PSs-containing indocyanine green (ICG)) to the surface of YB1 (an attenuated therapeutic *Salmonella Typhimurium* strain) *via* amide bonds, and the YB1-INPs complex achieved effective targeting hypoxic areas in tumor, thus eliminating bulk tumor tissues in mice after NIR laser irradiation. Meanwhile, YB1-INPs also exhibited satisfying fluorescence (FL) imaging ability (112). Moreover, many bacteria could immobilize environmental metal ions on cell surface through biomineralization process (113). Inspired by this phenomenon, Zhang's group conjugated palladium nanoparticles (Pd NPs) on the surface of the facultative anaerobic bacterium *Shewanella oneidensis* MR-1 by biomineralization, and such bacteria-based photothermal therapeutical platform significantly increased photothermal capacity under near-infrared (NIR) laser irradiation (114). In addition to biomineralization, Cheng-Hung Luo et al. utilized cargo-carrying method and antibody-directed method to decorate anaerobic *Bifidobacterium breve* and *Clostridium difficile* with upconversion nanorods for tumor imaging and Au nanorods for photothermal ablation upon NIR excitation (115).

The combination of BCT and gene therapy

In addition to chemotherapies, bacteria are effective carriers for gene therapies in the form of DNA and RNA. Plasmid is frequently used for bacteria-mediated gene therapies, due to its versatility for the engineering of genetic components, accessibility for massive production, high stability in physiology environment, as well as chemical activeness for further modifications. The gene products delivered by plasmids could be peptides, proteins, short hairpin RNAs and long non-coding RNAs. Both prokaryotic plasmids and eukaryotic plasmids can be delivered by bacteria, but their applications are different for cancer therapies.

Prokaryotic plasmids produce recombinant peptides and proteins within the bacterial protoplasm, and then these products are allocated to cytoplasm, membrane or extracellular

space according to their sequence signature. Generally, the recombinant proteins produced by prokaryotic plasmids are released to TME either by secretion, bacterial lysis or endocytosis by multiple types of cells such as cancer cells, macrophages and granule cells (116, 117). For example, Loeffler et al. introduced the gene LIGHT, which encoded a human cytokine mediating tissue rejection, into attenuated *Salmonella typhimurium* by plasmid pGEN206, and the transformed *Salmonella* exhibited significantly improved antitumor activity without additional *in vitro* toxicity in murine carcinoma cell lines (118). Additionally, *Escherichia coli* stably transformed with a plasmid constitutively expressing nanobody antagonist against CD47 could stimulate systemic tumor antigen-specific immune responses, and induced durable tumor regression and long-term survival in a syngeneic tumor model (119). Such constitutive expressing systems are more suitable for the delivery of immune modulators compared to cytotoxin, since the short time window of uncontrolled bacterial distribution before they are restricted in TME could augment immune responses in favor of tumor elimination, while unintended delivery of cytotoxin to healthy organs would lead to systemic toxicity.

The incorporation of promoters in response to environmental signals, such as hypoxia, low pH and exogenous compounds, allows spatial and temporal regulation on the production of therapeutic molecules in TME. For example, hypoxia-inducible promoters, such as HIP-1 and NirB, are utilized to restrict gene expression within hypoxic TME, thus reducing toxicity to normal tissues (120–124). Meanwhile, chemical-inducible promoters (e.g., pBAD, pTet, and Pm) can activate gene expression through systemic administration of transcriptional inducers (e.g., L-arabinose, tetracyclines, and acetyl salicylic acid, respectively) *in vivo* (125–129).

In most cases, therapeutic proteins produced by bacteria need to be released into TME to convey biological effects (116). Therefore, the bacteria strains can be engineered to induce autolysis within TME, thus releasing therapeutic payloads in a controlled manner. For example, Eva María Camacho et al. developed an inducible autolysis system in response to anhydrotetracycline, in combination with a salicylate cascade system that allowed efficient production of therapeutic molecules in response to aspirin and a *sifA* mutation that liberated bacteria from the vacuoles to a cytosolic location for therapeutic *Salmonella* (130). Moreover, M. Omar Din et al. engineered *Salmonella Typhimurium* to lyse synchronously at a threshold population density and to release genetically encoded cargo in a pulsatile manner (131). Such engineering strategies may inspire development of therapeutic bio-communities within *in vivo* environments, where population dynamics are driven by interacting viruses, bacteria, and host immune cells (132).

Eukaryotic plasmids utilize the transcription and translation machinery of mammalian cells. Compared to prokaryotic

systems, eukaryotic systems have the following advantages: firstly, protein products of eukaryotic systems are more biologically active due to the integrity of post-transcriptional modifications and higher structure, especially for those proteins with large molecular weight (133–135). In a study using *Salmonella Typhimurium* as a delivery vehicle, β -galactosidase expressed from a eukaryotic cassette induced substantially stronger immune responses than that expressed from a prokaryotic cassette (136). Secondly, the choice of therapeutic genes is more flexible, regardless of their subcellular locations of action. For example, RNA-based gene therapies often adopt eukaryotic plasmids to directly produce functional RNAs within target cells, thus bypassing the restrictions of RNA uptake efficacy *in vivo*. Huijie Jia et al. reported that attenuated *Salmonella Typhimurium* harboring eukaryotic plasmids expressing endostatin and Stat3-specific small interfering RNA (siRNA) conferred significant tumor-suppression effects in mouse tumor models (137). Moreover, therapeutic bacteria carrying eukaryotic plasmids encoding cytokines such as IL-4 and IL-18, and angiogenesis inhibitors such as endostatin and thrombospondin all resulted in retardation of tumor growth and prolonged survival of tumor-bearing mice (134, 138–140). Last but not least, eukaryotic plasmids could be loaded either within the protoplasm or on the surface of bacteria. Therefore, a considerable amount of plasmids can be loaded to a single bacterium due to surface-to-volume ratio (127).

However, the efficacy of plasmid endocytosis is the bottleneck for the eukaryotic plasmid-based therapies. Macrophages, the most important phagocytes in TME, are the ideal target for bacteria-mediated gene therapies. G Dietrich et al. reported that eukaryotic expression vectors can be delivered to macrophages using attenuated self-destructing *Listeria monocytogenes* (141). Similarly, Igentschev et al. also developed a plasmid-vector system targeting APCs with *Listeria monocytogenes*, which expressed the heterologous antigens under the control of an eukaryotic promoter in a similar fashion as in plasmids commonly used for vaccination with naked DNA (142). In addition to live bacteria, inactivated bacteria are also capable as vehicles of gene therapies targeting macrophages. For example, M.R.Miri et al. showed that BGs loaded with pEGFP-C1 plasmids were efficiently captured by murine macrophages (102, 143).

There are also some non-plasmid-dependent gene delivery methods. For example, Shuya Lu et al. reported that Chloroquine combined with PD-1 siRNA delivered with attenuated *Salmonella* could significantly enhance the tumor growth inhibition through upregulation of the number and activity of immune cells in tumor tissues (144). Qin Guo et al. developed a system in which bacterial outer membrane vesicles were co-loaded with PTX and Redd1-siRNA (siRNA@M-/PTX-CA-OMVs) to regulate the tumor metabolic microenvironment and suppress tumor growth. Upon reaching the tumor site, the system was first triggered by tumor pH (pH 6.8) to release

PTX. Then, the rest of the system would be taken up by M2 macrophages to increase their level of glycolysis (145).

Bacteria-mediated gene delivery is not only a therapeutic strategy, but also a useful diagnostic method. Genomically engineered bacterial can be detected non-invasively by fluoroscopy (146), magnetic resonance imaging (MRI) (147) and positron emission tomography (PET) (148) scan, which could be used to show the location of tumors. For example, *Escherichia coli* carrying pMW211-dsred plasmid could mark cancerous cells in their exact localization by luminescent signals (149). Studies have shown that there are many microorganisms differentially enriched in healthy population and cancer patients, such as gastric cancer, colorectal cancer, prostate cancer and oral cancer. Among them, many studies are focused on colorectal cancer and intestinal microorganisms, such as *Fusobacterium nucleatum*, *Peptostreptococcus stomatis* and *Streptococcus salivarius*, etc. All of these mentioned bacterial species are positively correlated with the occurrence of colorectal cancer and are expected to be potential diagnostic markers (150–152).

Conclusion and perspective

In contrast with traditional cancer therapies such as chemotherapy and radiotherapy (153–156), which are less effective for the hypoxic tumors, therapeutic bacteria preferentially penetrate and colonize in hypoxic areas in the tumors. Therefore, it is beneficial to combine BCT with traditional cancer therapies. For example, the combination of *Salmonella typhimurium* with 5-fluorouracil inhibited MC26-LucF tumor growth and prolonged survival in mice (131). The combination of *Salmonella typhimurium* VNP20009 and Triptolide, a traditional Chinese medicine, showed significantly enhanced antitumor activity by modulating tumor angiogenesis and host immune response (77). Moreover, preclinical studies demonstrated that combined administration of Cytolysin A-expressing *Salmonella typhimurium* and radiation therapy could reduce tumor growth to a greater extent than bacterial monotherapy (157).

Apart from chemotherapy, radiation therapy is also commonly used for the treatment of solid tumors. While radiation therapy shows good efficacy for many types of cancer, its damage to surrounding normal tissues remains a difficult problem. A few studies regarding *E. coli*, *Salmonella Typhimurium*, *Salmonella*, *Clostridium* and other strains (158–161) have demonstrated that bacterial therapy combined with radiotherapy can reduce radiation-associated damage, improve the therapeutic effect in radiotherapy, and reduce metastasis (162). However, the mechanistic study on the combination of bacteria-mediated cancer therapy and radiotherapy is still superficial, and the behavior of cancer cells, bacteria, and especially immune cells during radiotherapy required more in-depth investigation.

However, the following questions remains to be solved for the research field of bacteria-mediated cancer therapy:

The choice and optimization of bacterial strains

The clinical development of BCT faces substantial obstacles, mainly due to potential infection-related toxicity. For example, the Phase II clinical trial of ADXS11-001, an attenuated *Listeria monocytogenes* therapeutic vaccine, in patients with oropharyngeal cancer (NCT01598792) were suspended after a patient developed systemic listeriosis following vaccination (163).

The ideal strain should meet the following criteria: ① High tumor specificity. For example, a *Salmonella* strain displaying an integrin-binding RGD peptide on its outer membrane protein A (OmpA) showed a >1000-fold enrichment in the $\alpha v\beta 3$ integrin-expressing U87MG and M21 xenografts compared to the control strain and an impressively enhanced antitumor activity in the MDA-MB-231 and MDA-MB-435 xenograft tumor models (164). ② High tumor-killing effect. Bacteria can be engineered to obtain enhanced anti-tumor activity by means such as gene elements coding for cytotoxic agents, as discuss in previous chapters in this review. ③ No/low systemic toxicity. LPS is one of the most potent TNF α stimulators and thus responsible for Gram-negative sepsis (165). Deletion of the *msbB* gene from *Salmonella* results in loss of myristoylation of lipid A, a critical component of LPS, and reduces its toxicity of by 10,000-fold. For example, VNP20009 with deletions of *purI* and *msbB*, two genes necessary for adenine and lipid A synthesis, respectively, have been safely administered to patients with metastatic melanoma and renal cell carcinoma in a phase I clinical study (3, 4). However, no antitumor effect was observed in patients treated with VNP20009, which might due be to the over-attenuation of bacteria (3, 166). Therefore, it should be noted that some of the virulence factors may also be responsible for the intrinsic antitumor activity of live bacteria. Whenever possible, attenuation should be achieved without substantially compromising the antitumor activity, unless the bacterial strain is used for the purpose of vaccination only.

The choice of patients suitable for BCT

The standards for participant recruitments may greatly affect the outcomes of clinical trials (23). The risks and potential benefits must be considered carefully for first-in-human (FIH) trials. In general, only patients without any response to conventional therapies should be enrolled in clinical trials. Even though the

bacteria are highly attenuated, the administration of live bacteria still poses a serious risk of infection. Therefore, the immune status and prior/concomitant therapies of patients should be evaluated sufficiently during the design of clinical trials (167). For example, immunocompromised patients who receive other immunotherapies simultaneously should be excluded (168). In addition, patients with foreign transplants such as artificial heart valves should be excluded, since foreign transplants may provide refuges for oncolytic bacteria to escape immune clearance and cause serious adverse reactions (168).

The interaction between therapeutic bacteria and host bacterial flora

The long-term interaction of bacterial therapy on the normal flora of patients have not been investigated. It is not known yet whether therapeutic bacteria would hibernate in the locations of immune exemption, which might lead to unexpected health crisis in the long term. Moreover, the influence of host bacterial flora, as well as the antibiotic usage habits might substantially influence the effect of bacteria-mediated cancer therapies, which have not been studied comprehensively. Therefore, successful bacteria-mediated cancer therapies require interdisciplinary expertise, including oncologists, infectious disease specialists, immunologists and microbiologists.

Although many published studies on bacteria-based biotherapies have shown promising therapeutic effect in experimental models, its drawbacks are equally evident. Firstly, safety is the major concern due to the infectious nature of the bacteria. Secondly, limited drug loading efficiency is another challenge dampening the anticancer effect of bacteria. Thirdly, the manufacturing process of live bacteria is more complex than that of the small molecule anticancer drugs. Last but not least, when live bacteria could be used in a clinical settings, the potential impact on the environment would be also a concern that should be properly addressed (169, 170).

In conclusion, BCT is an emerging category of experimental cancer treatment, and what we've discovered might be the tip of an iceberg. From the first attempt of Coley's strategy until today, great progress has been achieved. Thus, with more understanding of its mechanism, the bacteria, as well as bacteria-related therapeutics would become powerful weapons in the battle against cancers in the near future.

Author contributions

ML, XC, HG and FY are responsible for the collection, collation, and writing of the manuscript. JC and YQ are responsible for the concept development, revision, and review

of the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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