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Tumor-targeting bacteria engineered to fight cancer

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Abstract

Recent advances in targeted therapy and immunotherapy have once again raised the hope that a cure might be within reach for many cancer types. Yet, the majority of cancers are either insensitive to the therapies to begin with or develop resistance later on. Therapy with live tumor-targeting bacteria provides a unique option to meet these challenges. Compared to most other therapeutics, the effectiveness of tumor-targeting bacteria is not directly affected by the genetic makeup of a tumor. Bacteria initiate their direct antitumor effects from deep within the tumor, followed by innate and adaptive antitumor immune responses. As microscopic “robotic factories”, bacterial vectors can be reprogrammed following simple genetic rules or sophisticated synthetic bioengineering principles to produce and deliver anticancer agents based on clinical needs. Therapeutic approaches using live tumor-targeting bacteria can either be applied as a monotherapy or complement other anticancer therapies to achieve better clinical outcomes. In this Review, we summarize the potential benefits and challenges of this approach. We discuss how live bacteria selectively induce tumor regression and provide examples to illustrate different ways to engineer bacteria for improved safety and efficacy. Finally, we share our experience and insights on oncology clinical trials with tumor-targeting bacteria, including a discussion on regulatory issues.

There are a variety of cytotoxic agents that can kill cancer cells effectively. However, the conventional cytotoxic therapies often eliminate cancer cells at the expense of damaging the normal tissues, resulting in unacceptable toxicities. Therefore, eradication of cancer cells without causing collateral damage is the ultimate goal to all oncologists and cancer researchers. The persistent pursuit of that goal has recently led to two promising clinical

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advances – molecularly targeted therapy and immunotherapy. Molecularly targeted therapy aims at genes with specific genetic or epigenetic alterations in cancer cells, thus potentially minimizing side effects seen in patients treated with traditional chemotherapeutic agents[1-6]. In spite of its increased targeting precision against tumor cells, targeted therapy is far from perfect[7]. First, targeted therapeutic agents have a spectrum of their own toxicities, some of which are related to the normal functions of the target proteins[8]. Second, the small molecule inhibitors may not be sufficiently specific[9]. Third, resistance or relapse is often observed in patients treated with targeted therapy, resulting from intrinsic resistant genetic changes or selection for a subset of cancer cells with those changes[10]. Fourth, the majority of tumors do not carry currently actionable genetic changes. Immunotherapy can be seen as another "targeted" therapy which involves T cells reactive to tumor-specific neoantigens or tumor-associated antigens (TAAs). Recent clinical trials with immune checkpoint blockade have shown remarkable results including durable therapeutic effects on advanced metastatic cancers[11,12]. It is generally believed that sensitivity to checkpoint blockade is dependent on the neoantigen burdens of the tumor cells and immune infiltrates in the tumor microenvironment[13]. Unfortunately, the majority of the common cancers do not show abundant mutations and infiltrating immune cells, and consequently are insensitive to checkpoint blockade. Major efforts are being made to develop approaches that can sensitize these tumors to immunotherapy. In addition to molecular targets such as oncoproteins and neoantigens, unique pathological alterations at the tissue level can be exploited for tumor targeting. Tumor vasculature is generally irregularly-developed and chaotic, leading to insufficient infusion of oxygen and nutrients in areas within a solid tumor[14,15]. Cancer cells in these areas are dormant but viable[16]. They can be responsible for clinical relapse after chemo or radiation therapy, because the hypoxic areas are poorly accessible to systemically delivered therapeutics and oxygen is needed for effective radiation therapy. In addition, low oxygen levels affect the function of immune cells, contributing to immune privilege of solid tumors. Nevertheless, the necrotic/hypoxic regions provide a critical niche for bacteria to colonize.

There is a long history of observations that suggest natural bacterial infections can result in antitumor effects against malignant tumors. In *1813*, Vautier reported that cancer patients who developed gas gangrene had tumor regressions[17]. Other historical accounts include observations by Busch (1866) that led Fehleisen (1883) and subsequently William B. Coley to experiment with the live infectious agent of erysipelas (later termed Group A *Streptococcus* or *S. pyogenes*) as a means of treating cancers[18-20]. Further pursuit of using bacteria to treat cancers was curtailed later on because of the focus of attention on the then novel chemo and radiation therapies. The enthusiasm for using live bacteria for cancer treatment has revived since the mid-1990s when the scientific community had a better understanding about the tumor microenvironment and recombinant DNA technology allowed generation of more potent and less toxic bacterial strains [21]. Many bacterial strains have since been tested in animal models and shown preferential targeting of solid tumors, several of which have advanced to clinical trials[21-27]. The clinical development of live bacteria as therapeutic agents faces substantial hurdles mainly because of potential infection-associated toxicities. One successful example is the use of Bacillus Calmette-Guérin (BCG) in the treatment of bladder cancer[28]. BCG is a live attenuated strain of *Mycobacterium bovis*

originally generated as a vaccine for tuberculosis. BCG therapy by intravesical administration was first documented in the 1970s and has since become an important treatment option for transitional-cell carcinoma *in situ* of the bladder[29-31]. It is believed that BCG's therapeutic effect is mainly due to its immunomodulatory activity[32-34]. In this Review, we discuss the unique aspects of live tumor-targeting bacteria as therapeutic agents, focusing on some of the most investigated strains of *Salmonella*, *Clostridium*, and *Listeria* as examples. As an increasing number of therapeutic bacterial strains have advanced to the clinical stage, we also highlight issues associated with their clinical translation.

Live tumor-targeting bacteria

Intrinsic tumor-targeting.

Live bacteria "target" solid tumors using unique mechanisms. When administered systemically, therapeutic bacteria disseminate to both tumor and healthy tissues. Even though Salmonella has been shown to preferentially home to or are retained in the tumor microenvironment enriched in certain metabolites[35], the initial amount of bacteria delivered to the tumor is usually not greater than that to the normal tissues[36-38]. However, bacteria in the circulation and other normal tissues are cleared within hours and days, respectively, while those in the tumor continue to proliferate, often to numbers greatly exceeding the colony forming units initially administered[36,38-46]. This selective colonization is likely the result of an immunosuppressive and biochemically unique microenvironment caused by pathologic changes associated with solid tumors[35,47-51]. Importantly, anaerobic bacteria do not colonize hypoxic or inflammatory lesions unrelated to neoplasia, as shown in experiments with obligate and facultative anaerobes, respectively[38,52,53]. Tumor-targeting of *Listeria* involves a different mechanism. *Listeria* is known to infect not only professional antigen-presenting cells (APCs) such as monocytes/macrophages and dendritic cells, but also myeloid-derived suppressor cells (MDSCs) that can deliver the bacteria selectively to tumor[44,45]. *Listeria* inside the tumor-infiltrating immunosuppressive MDSC is protected from immune clearance, but is rapidly eliminated from normal tissues that lack immune suppression. Obligate anaerobic bacteria such as clostridia are unable to survive in the oxygen-rich environment, thus further reinforcing their tumor-targeting specificity. Interestingly, germinated clostridia have also been observed within micro invasive lesions where necrosis was not evident as well as in the vicinity of neoplastic vessels in glioma models[52,54], raising the possibility that these neoplastic structures provide sufficiently hypoxic, biochemically unique, and immunoprivileged microenvironment for bacterial colonization. As discussed in more detail below, facultatively anaerobic bacteria can be engineered such that their ability to survive in the normal tissues will be further diminished.

Tumor destruction by live bacteria.

Localized bacterial infection causes tumor regression through various mechanisms (FIG. 1). Bacteria have intrinsic antitumor activities, but different strains of bacteria or bacteria in different microenvironments may deploy distinct mechanisms to destroy solid tumors. In addition to the intrinsic antitumor effects, bacterial infection induces innate as well as adaptive immune responses against both tumor-colonizing bacteria and the tumor cells[46-48,52,55-59]. The host immune responses are more critical for the antitumor effects of

bacteria such as *Salmonella* that are not sufficiently cytotoxic to tumor cells[55,60]. Numerous studies have suggested that both bacterium-intrinsic and host immune mechanisms are involved in tumor destruction (FIG. 1). The dominant mechanism is likely to vary depending on the bacterial species used in the therapy, the types of tumor being treated, and even the phases of the bacteria-host interaction. Importantly, bacteria can be genetically engineered to further enhance their antitumor activities in a variety of different ways, making them a versatile platform to deliver therapeutic payloads based on clinical needs.

Engineered bacteria

Bacteria can be attenuated for safety reasons or engineered to acquire enhanced antitumor activities. As discussed below, a large collection of engineered bacterial strains have been generated in laboratories around the world for a variety of purposes, all aimed at improving the therapeutic index when bacteria are used either alone or in combination with other cancer therapeutic approaches.

Improving safety.

The safety profile of a therapeutic bacterium can be improved by different approaches. For the known human pathogens, deletion of major virulence genes is often required to minimize their pathogenicity. An exceedingly toxic strain of *Clostridium novyi* (*C. novyi*) was converted to a considerably safer strain (*C. novyi*-NT) by deleting the gene for a lethal exotoxin[61]. Lipopolysaccharide (LPS) in the outer membrane of Gram-negative bacteria is one of the most potent TNFα stimulators and thus responsible for Gram-negative sepsis[62]. Deletion of the *msbB* gene from *Salmonella* resulted in loss of myristoylation of lipid A, a critical component of LPS, and minimized TNFα expression[51]. This modification reduced the toxicity of *Salmonella* by 10,000-fold. An attenuated strain of *Salmonella* named VNP20009 carrying this deletion was isolated and shown to be safe in clinical trials[63,64]. 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. In this regard, the *msbB*-deficient *Salmonella* strain retained both tumor-targeting specificity and antitumor activity in the mouse B16F10 melanoma model[51]. *Salmonella* was also made defective in the synthesis of ppGpp (thus named ΔppGpp), a signaling molecule required for the induced expression of a number of virulence genes[65]. The ΔppGpp strain has a drastically improved safety profile. Interestingly, this strain is also defective in its ability to enter and replicate in the host cells, effectively turning it into an extracellular bacterium[66].

Another way to improve safety is to generate auxotrophic mutants that cannot replicate efficiently in an environment where a particular nutrient required by the mutant strain is scarce. *Salmonella* A1-R represents such a strain, which is auxotrophic for leucine and arginine likely enriched in the tumor but not in normal tissues[67]. This strain, without further engineering, has shown selective tumor colonization as well as potent antitumor activity in a variety of mouse tumor models[27,68]. *Listeria* can be made safer by deleting *prfA*, the master virulence regulator gene[69]. However, *prfA*-deficient *Listeria* cannot escape into the cytosol

of the infected cells, which would prevent the tumor antigens expressed by the vaccine strains from accessing the cytosol for processing and cell surface presentation. To maintain a sufficiently attenuated state while allowing cytosolic delivery of the tumor antigens, the *prfA*-deficient strains were engineered to express low levels of *prfA* and truncated Listeriolysin O (LLO) that can be fused with the antigens of choice for enhanced immunogenicity[70,71]. These strains are referred to as *Lm*-LLO, which have been used not only as vaccine strains, but also for tumor-targeted delivery of non-vaccine therapeutic payloads[44,46,72]. Attenuation of *Listeria* can also be achieved by deleting the virulence genes *actA* and *inlB* responsible for bacterial dissemination, creating strains known as LADD for Live Attenuated Double-Deleted[73,74]. Another method to generate attenuated *Listeria* strains involved insertional inactivation of the *dal* and *dat* genes required for the synthesis of bacterial cell wall[75]. The attenuated strains with these modifications are incapable of replication or spreading *in vivo*. Therefore, they are desired vaccine vectors, but not optimal for tumor-targeted delivery of non-vaccine antitumor payloads.

Increasing tumor targeting.

Obligate anaerobes have relatively high tumor specificity, thus resulting in minimal direct cytotoxicity to normal tissues[38,39,76,77]. In contrast, facultative anaerobes such as *Salmonella* and *Listeria* can survive and even proliferate in an oxygenated environment, causing direct damage to the normal tissues. For facultative anaerobes, improved tumor targeting could reduce their toxicity or enhance their efficacy without increasing toxicity. The αvβ3 integrin is overexpressed on activated endothelial cells and some cancer cells. A *Salmonella* strain displaying an integrin-binding RGD peptide on its outer membrane protein A (OmpA) showed a >1000-fold enrichment in the αvβ3 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[78]. Bacteria have been engineered to target TAAs as well. Surface display of antibody fragments against the colorectal cancer-associated carcinoembryonic antigen (CEA) or lymphoma-associated antigen CD20 made the engineered *Salmonella* strains more effective in suppressing experimental tumors expressing these antigens[79,80]. Importantly, the anti-CD20 strain showed substantially reduced intracellular accumulation in the liver and spleen of the treated mice, while maintaining tumor accumulation[80]. Bacteria can also serve as a platform to display modular synthetic adhesins, where different adhesins can be chosen for targeting tumors expressing their specific ligands[81].

Gene promoters responsive to tumor-associated signals such as hypoxia have also been exploited for both targeted colonization and payload expression (FIG. 2a). In addition to promoters known to be induced by the tumor-associated factors, novel promoter elements activated in tumor microenvironment can be identified using unbiased large-scale screening methods such as those employing “promoter traps” (FIG. 2b). Promoters tightly regulated by exogenously applied chemical transcriptional triggers or by ionic radiation represent another means to control the expression of effector genes (FIG. 2a). While systemic administration of chemical triggers allows a temporal control, focused radiation can provide both temporal and spatial controls. It should also be noted that high-level constitutive expression of heterologous proteins can be a metabolic burden to the bacterial vector, resulting in

decreased fitness and inefficient colonization[82]. Temporally controlled payload expression after a robust colonization has been established may be a good approach to address this problem.

Effector systems.

Attenuated bacteria alone often cannot eradicate solid tumors. Delivery of therapeutic payloads by tumor-targeted bacteria to augment their efficacy was first described in the mid-1990s[83-87]. Various effector systems have since been explored (TABLE 1). Here we briefly describe different strategies for payload delivery and effector systems categorized based on their antitumor mechanisms.

Different strategies for payload delivery.—The therapeutic payload can be delivered in the form of DNA, RNA or protein depending on their intended use and the type of delivery bacteria. In the majority of cases, bacteria are transformed with plasmids carrying gene expression cassettes that direct the expression of therapeutic proteins in the bacteria. The proteins need to be secreted from the bacteria to achieve their biological effects[88]. Alternatively, the vector strains can be engineered such that autolysis is induced for the release of therapeutic payload once a robust tumor colonization has been established[89,90].

In addition to therapeutic proteins, DNA and RNA molecules can also be delivered to targeted cells. Intracellular bacteria can be engineered with DNA cassettes expressing therapeutic proteins under the control of mammalian promoters[91-93]. Biological activities of mammalian proteins often depend on correct folding and posttranslational modifications that may be absent in proteins produced in bacteria. Thus, one advantage of delivering DNA is to produce optimally active proteins by host cells. It should also be noted that proteins produced by intracellular bacteria and those produced by host cells may be targeted to different cellular compartments. In a study using *Salmonella* as a delivery vehicle, β-galactosidase expressed from a eukaryotic cassette induced substantially stronger immune responses than that expressed from a prokaryotic cassette[94]. A special category of therapeutic bacteria are DNA vaccine strains designed to deliver DNA to APCs. Vaccine strains delivering either DNA or protein are discussed in detail elsewhere[95]. Small hairpin RNA (shRNA) and small interfering RNA (siRNA) are popular forms of RNA used for gene silencing and their delivery by intracellular bacteria has been explored in multiple studies (TABLE 1).

Cytotoxic agents.—The most straightforward approach to enhance the antitumor activity would be to engineer bacterial vectors expressing cytotoxic agents. This approach requires the bacterial vectors to target tumors with sufficient specificity or the use of inducible promoters for a better control of gene expression to avoid toxicity to normal tissues. Several bacterial strains have been engineered to express the potent pore-forming bacterial toxin cytolysin A or *S. aureus* α-hemolysin under the control of promoters activated by hypoxia[96], L-arabinose[97-99], or doxycycline[100], to ensure safety. An alternative method to increase safety involved expressing a chimeric protein with tumor growth factor alpha (TGFα), an epidermal growth factor receptor (EGFR) ligand, that targeted the *Pseudomonas* exotoxin A (ToxA, also referred to as PE) to EGFR overexpressed in many cancer types[101,102]. The

chimeric protein was shown to selectively kill EGFR-positive cancer cells and retarded tumor growth in multiple mouse tumor models expressing EGFR.

Induction of tumor cell apoptosis is an attractive therapeutic approach, but systemic administration of apoptosis-inducing ligands such as TNF-α, Fas ligand (FasL), and TNF-related apoptosis-inducing ligand (TRAIL) is not feasible because of their toxicity or short circulating half-life[103-105]. To achieve sustained high levels of these proteins in the tumor microenvironment while avoiding systemic toxicity, several groups have engineered bacterial strains for their tumor-targeted delivery[106-110]. An attenuated *Salmonella* strain expressing FasL showed significant antitumor activities against both primary and metastatic mouse tumors in a Fas-dependent fashion[108]. In another elegant example, two separate inducible systems were used to drive the expression of the cytotoxic Cp53 peptide derived from the p53 protein and autolysis of the bacteria to release Cp53 for maximal killing[89].

In addition to genetic engineering for expressing cytotoxic proteins, tumor-targeting bacteria have been used to deliver cytotoxic agents that can exert greater bystander effect on the surrounding uninfected tumor cells[44,46,111,112]. In one study, the high-energy beta emitter 188-Rhenium (^{188}Re) was conjugated to a polyclonal antibody against *Listeria* followed by incubation of the radiolabeled antibody with an attenuated *Listeria* strain[44]. The resulting radioactive *Listeria* were accumulated in metastases after systemic administration and reduced the number of metastases by 90% in the Panc-02 metastatic mouse tumor model. Another innovative approach capitalized on the ability of some bacteria to generate cytotoxic NO from NO_3^-[112]. Upon photo-irradiation, photoelectrons were excited from the carbon-dot doped carbon nitride (C_3N_4) loaded onto the surface of *Escherichia coli* (*E. coli*) and transferred to *E. coli* NO-generating enzymes, resulting in substantially enhanced production of NO and tumor suppression. The focused photo-irradiation enabled targeted generation of NO.

Prodrug-converting enzymes.—Prodrug-converting enzymes were among the first effector systems engineered into tumor-targeting bacteria. Once expressed by the tumor-localized bacteria, these enzymes can metabolize their systemically administered innocuous substrates (prodrugs) and convert them into cytotoxic products. The major advantage for using prodrug-converting enzymes is that the cytotoxic products are small molecules able to diffuse farther inside the solid tumor and across the cell membrane, thus generating potent bystander effect. Tumor-targeting bacteria have been engineered to express several prodrug-converting enzymes (TABLE 1). Cytosine deaminase (CD) converts the non-toxic 5-fluorocytosine (5-FC) to 5-fluorouracil (5-FU), a first-line chemotherapeutic agent for metastatic colorectal cancer[113]. The VNP20009 *Salmonella* strain engineered to express *E. coli* CD showed clearly enhanced antitumor activity when combined with 5-FC in both mouse syngeneic and human xenograft colorectal tumor models[114]. Similarly, a *C. sporogenes* strain expressing *H. influenza* nitroreductase (NTR) had promising antitumor effect as well[115]. Bacterial NTR catalyzes conversion of the weak monofunctional DNA-alkylating agent CB1954 into a bifunctional DNA-alkylating derivative that can induce DNA crosslinks and apoptosis. Repeated administration of the NTR-expressing strain along with CB1954 achieved sustained tumor control in a mouse xenograft tumor model[115]. The efficacy of this effector system depends on robust and sustained tumor colonization by the

delivering bacterial vector, which ensures continued high-level expression of the prodrug-converting enzyme[115,116]. It is worth noting that bacteria also carry endogenous enzymes capable of metabolically activating multiple prodrugs[117,118].

Immunomodulators.—To further stimulate antitumor immunity, tumor-targeting bacteria have been engineered to express either tumor antigens or immunoregulatory factors. Live bacteria as vectors for tumor vaccination have been reviewed elsewhere[71,95,119]. In addition to vaccination with bacteria expressing tumor antigens, another approach to augment tumor immunogenicity could involve presenting the immunodominant T cell antigens from tetanus toxoid, poliovirus, or measles virus on the surface of tumor cells infected by intracellular tumor-targeting bacteria carrying expression cassettes for these antigens. The immune system in most individuals has seen these antigens earlier during childhood vaccinations, and thus has generated memory T cells. These T cells can be reactivated when seeing these antigens again, resulting in destruction of the infected tumor cells. Antigen spreading from the destructed tumor cells may also take place to induce an immune response against the uninfected tumor cells.

Engineered tumor-targeting bacteria have the ability to bring immunomodulatory proteins to the tumor microenvironment. A number of bacterial strains have been engineered to express immunoregulatory factors in the tumor microenvironment to boost antitumor immunity (TABLE 1). For example, a *Salmonella* strain expressing biologically active IL-2 was generated more than 20 years ago and showed enhanced antitumor activities dependent on natural killer (NK) cells and $CD8^+$ T cells in the mouse MCA-38 hepatic metastasis model[86,120]. The IL-2-expressing *Salmonella* has also been tested in both canine and human clinical trials (discussed below under Clinical translation). Different bacterial species expressing other cytokines have been generated as well (TABLE 1). In addition to the classic cytokines and chemokines, other proteins with immunomodulatory activities have also been documented to have promising therapeutic effects when delivered by tumor-targeting bacteria. For instance, an attenuated *Salmonella* strain engineered to express LIGHT, a member of the TNF superfamily, showed considerable antitumor activities in subcutaneous as well as metastatic mouse tumor models[121]. These antitumor activities required both $CD4^+$ and $CD8^+$ T cells. Mobilization of natural killer T (NKT) cells could also enhance bacterial antitumor activity. In an interesting study with the 4T1 syngeneic mouse tumor model, α-galactosylceramide, a glycolipid that can activate NKT cells, was incorporated metabolically into *Listeria* and shown to help eliminate metastases and improve survival[72]. A recent study employed heterologous flagellin as a potent immunoregulator[122]. In this study, the *Salmonella* ΔppGpp strain engineered to secrete *Vibrio vulnificus* flagellin B displayed markedly improved ability for tumor control compared to the parental strain. Mechanistic studies showed that infection with the Gram-negative *Salmonella* activated the TLR4/MyD88 pathway, presumably by LPS present in the outer membrane of Gram-negative bacteria, resulting in a massive tumor infiltration of macrophages and neutrophils. Secreted heterologous flagellin triggered the TLR5 pathway and further shifted the tumor-infiltrating macrophages toward an M1 phenotype, which was associated with increased levels of tumoricidal mediators including interleukin 1 beta (IL-1β), TNF-α, and nitric oxide (NO).

Recent clinical success with immune checkpoint blockade has prompt a wave of preclinical and clinical studies combining checkpoint inhibitory antibodies with therapeutic bacteria or viruses[123-125]. These studies tested the hypothesis that intratumoral infection by the infectious agents could establish a more immunogenic microenvironment, thus sensitizing the tumors to checkpoint blockade. A more straightforward approach would be to generate bacterial strains secreting checkpoint inhibitors such as an anti-PD-1 antibody or a soluble PD-1 extracellular domain to bind and neutralize the T cell-inhibitory PD-L1 expressed by tumor cells. This approach is technically possible as functional single-chain antibodies have been produced from tumor-targeting bacterial strains[126]. These inhibitors can also be expressed by infected tumor cells when intracellular bacteria carrying expression cassettes with mammalian gene promoters and secretory signals are used. As the expression of the checkpoint inhibitors are targeted to tumors, this approach will not activate T cells in normal tissues, thus potentially minimizing toxicity associated with systemic checkpoint blockade[127].

Targeting tumor stroma.—Tumor cells can evade the immune system by downregulating the expression of tumor antigens as well as proteins involved in antigen processing and cell surface presentation[128]. Targeting tumor vasculature required for tumor growth circumvents this problem and may be particularly beneficial for bacterial therapy. As discussed earlier, bacteria preferentially colonize necrotic/hypoxic tumor areas. Disruption of tumor vasculature with microtubule-destabilizing agents leads to destruction of the well-perfused tumor regions and expands bacterial colonization[129-131]. Bacteria themselves can be engineered to induce destruction of tumor vasculature. Several vaccine strains against critical components of the angiogenic tumor vessels have been constructed and tested in both prophylactic and therapeutic settings(TABLE 1)[132-139]. For example, a *Salmonella* DNA vaccine strain targeting vascular-endothelial growth factor receptor 2 (VEGFR2, also known as FLK-1) was able to break peripheral immune tolerance and elicit cytotoxic T cell (CTL)-mediated immunity against this self antigen expressed on proliferating endothelial cells, leading to effective protection against tumor challenges[132]. Another study with a *Listeria* vaccine strain further suggested that the antitumor activity induced by VEGFR2 vaccines is dependent on epitope spreading to a tumor antigen[139]. Other stromal components may be targeted as well.

Synthetic gene networks.—Both viruses and bacteria can be reprogrammed by genetic engineering, but bacteria can host heterologous DNA of considerably large sizes[140], allowing for more sophisticated reprograming. The powerful recombinant DNA and synthetic biology technologies have even enabled recreation of viable bacterial cells by transplanting entire chemically synthesized genomes into recipient cells[141,142]. Therefore, bacteria have been dubbed “programmable robotic factories” at the microscopic scale[21]. Applying engineering (electrical engineering in particular) concepts, investigators have assembled biomolecular modules in bacteria to build genetic networks that can execute logical operations. Typical cis (e.g. promoters, enhancers) and trans (e.g. transcription factors, repressors) gene regulatory elements are employed and arranged in unique ways to form feedback and feedforward loops, called network motifs, with which the biological equivalents of electronic devices such as toggle switches, oscillators, and other sophisticated

devices can be fabricated[143-145]. An elegant design using the quorum-sensing elements from *Vibrio fischeri* and *Bacillus Thurigensis* arranged to form negative feedback motifs enabled synchronized oscillations of gene expression in a population of bacterial cells[146]. In a subsequent study, this quorum-sensing gene circuit was modified to generate synchronized cyclical population self-control and anticancer drug delivery as the output[90](FIG. 3). Once inside the tumor, the tumor-targeting *Salmonella* with this gene circuit underwent repeated cycles of population expansion and regression by autolysis in response to the density of bacterial cells. The lysis of the cells directly released the anticancer drug made by the bacteria. Thus, this gene circuit provided maximal release of the therapeutic payload through synchronized cell lysis and increased safety by maintaining the intratumoral bacterial population at a defined size, consequently minimizing the risk of a devastating systemic inflammatory response. This example illustrates the potential of gene networks to coordinate the behavior of bacteria at the population level in response to a particular environmental cue for an increased therapeutic index.

Experimental tumor models.—Preclinical animal study is a critical step toward clinical development of tumor-targeting bacteria. Colonization of tumor-targeting bacteria and subsequent antitumor activity can vary substantially among different preclinical models, because of the unique tumor microenvironment associated with particular tumor models. In addition to tumor histology, the method used to establish a tumor model can make a significant difference[147]. For instance, *Listeria* strain colonized the subcutaneously transplanted Panc02 pancreatic tumors and tumors spontaneously occurring in the genetically engineered KPC mice with comparable efficiencies[46]. However, the 4T1 mammary tumors transplanted subcutaneously to BALB/c mice were shown to support the colonization of two different attenuated *Salmonella* strains 10,000-fold more efficiently than the size-matched autochthonous mammary tumors spontaneously developed in transgenic BALB-neuT mice[131]. Interestingly, pretreatment with a vasculature-disrupting agent, shown to induce tumor necrosis, drastically improved tumor colonization in the autochthonous model. This example underscores the importance of identifying and employing the right tumor models for the assessment of both efficacy and toxicity that are truly relevant to human cancer patients. Perhaps a rational and hierarchical approach involving a variety of tumor models will help maximize the chance for the successful clinical development of a tumor-targeting bacterium-based therapeutic product [147].

Clinical translation

The number of published studies on bacterial cancer therapy have increased exponentially in recent years, many of which have shown promising results in experimental models[21]. Nevertheless, very few tumor-targeting bacteria have advanced to clinical stages. Model organisms share many genetic elements and biological pathways with humans, and yet fundamental differences exist. In addition, disease models lack the heterogeneity always seen in the patient population. Consequently, all experimental therapeutic approaches must pass the test in a patient population to show their clinical safety and utility. Translation of any novel therapeutic agent from the laboratory bench to the bedside would require enormous efforts, but is particularly challenging for live bacteria. Use of replication-

competent bacteria in cancer therapy poses major challenges to both investigators and the regulatory authorities. Regulatory issues are among the most important issues that need to be addressed before a replication-competent bacterium can be applied to humans (Box 1).

Challenges.

Given the unique nature of live engineered bacteria as therapeutic agents, a number of challenges should be considered. First, live genetically modified bacteria that carry antibiotic resistance genes or mobile genetic elements that can mediate horizontal gene transfer are generally not appropriate for clinical studies[148]. Chromosomal integration of the expression cassette without antibiotic selection markers provides a safer and more stable way for engineering[149,150]. Second, unlike small molecules or other non-viable clinical agents, live bacteria or bacterial spores cannot be sterilized either by heating or by filtering, which presents a major challenge for manufacturing Good Manufacturing Practices (GMP)-grade test articles. In addition, the conventional regulatory standard for sterility testing would not be feasible. Thus, production and purification in dedicated clean rooms following strict aseptic protocols with frequent in-process monitoring is the most practical way to ensure "sterility" (meaning no contamination from other live microorganisms). Although the final products cannot be demonstrated to be sterile, they should be assayed to be free from causative agents of other diseases or conditions, such as invasive bacterial pathogens listed by the Centers for Disease Control and Prevention (CDC)[151] and specific pathogens described in the United States Pharmacopeia, Chapter 62 [152], as appropriate. Third, live bacteria are proliferative in the target tissue and therefore, the effective (whether therapeutic or toxic) dose is not necessarily correlated with the administered dose. The effective dose depends more on the "quality" of the target tissue, which is defined by the accessibility, the extent of tumor necrosis/hypoxia, and the abundance of preexisting tumor-infiltrating inflammatory cells. These factors determine how easily the systemically administered bacteria can enter their target tissue and whether the target tissue can support a robust bacterial proliferation and spreading of the infection. The development of companion diagnostic approaches such as those based on angiography and hypoxia/necrosis imaging may help define the patient population that would benefit the most from bacterial therapy[153-155]. Additionally, germination and spreading of bacteria may be monitored directly by imaging the replicating bacteria[156-158]. It should also be noted that when low doses of bacteria are given, especially when administered systemically, the successful establishment of an infection in the target tissue is less predictable and may take much longer time to occur. This could pose a greater risk to the patients, because they are more likely to become less vigilant over time. Fourth, oncolytic bacterial therapy is a deliberate attempt to convert a tumor into a localized tumor-destructing infection, which may have serious consequences if not managed properly. The severity of the infection-associated toxicity generally correlates positively with the tumor size and the extent of necrosis/hypoxia inside the tumor that are important determinants for the robustness of the infection. As both therapeutic and toxic effects result from a robust infection, a carefully calculated balance is critical. Practically, this is difficult to achieve, because an antibiotic intervention too early would effectively eliminate the infection before an antitumor effect has been achieved, whereas a late intervention bears the risk of an unpredictable systemic inflammatory response. Effective management of the therapeutic infection requires experts

across disciplines including oncologists, infectious disease specialists, and interventional radiologists or surgeons for managing abscess or non-abscess-forming infections that need invasive management. Therefore, when and how to intervene after an intratumoral infection has been established should be a team decision. Fifth, when a live biological agent is used in a clinical setting, its potential impact on public health and environment is always a concern and should be properly addressed.

Study population.

In general, for first-in-human (FIH) trials, risk and potential benefit need to be considered in the selection of the study subjects. Usually, subjects whose diseases are unresponsive or refractory to standard therapies are enrolled to the trials. For live bacterial products, additional considerations include intrinsic properties of the product and the concomitant therapies.

The underlying condition of the cancer patients might make them immunocompromised. They also may need to receive concomitant therapies to control their disease and some of these therapies (e.g., chemotherapy) can be immunosuppressive. To these patients, administering a live bacterial product may pose a significant risk of infection. Thus, in designing a FIH trial, the immune status of the patients and their prior / concomitant therapies need to be considered. Patients who are immunocompromised or receive concomitant immunotherapeutics may be excluded.

Certain patient conditions may particularly predispose patients to developing infections associated with administered live bacterial products because of their intrinsic properties. The following are examples to consider. (a) Bacteria in general, and anaerobic bacteria in particular, preferentially proliferate in necrotic tissues. Conditions such as brain abscess, diverticulitis or recent radiation treatment might promote the unintentional growth of these bacteria in non-target lesions, even though preclinical studies have shown that certain bacterial strains may not be able to gain access to the non-malignant lesions[38,52,53]. (b) Some live bacterial products have the potential to colonize foreign bodies such as artificial heart valves, joint replacement or implanted medical devices that may serve as reservoirs for these live products. Excluding patients with these conditions reduces the risk with these products.

Clinical Experience.

Several historical clinical observations with live antitumor bacteria have been documented as mentioned earlier. In recent years, carefully designed clinical trials for tumor-targeting bacteria have been conducted in both human patients and companion dogs with spontaneous tumors.

Canine studies.—Tumors developed spontaneously in companion dogs serve as an attractive model for human cancers[159,160]. These tumors resemble their human counterparts – originating from cells harboring naturally occurring mutations in hosts with heterogeneous genetic backgrounds. A few canine studies for tumor-targeting bacteria have been reported (TABLE 2). In one study, the *Salmonella* strain VNP20009 was given by intravenous (IV)

infusion to 41 client-owned dogs with spontaneous tumors[161]. Complete and partial tumor responses were observed in 15% of the treated animals. Positive bacterial culture was obtained from tumor tissue in 42% of the cases; however this was not correlated with the administered doses. In another study, intratumoral injection of *C. novyi*-NT spores resulted in objective responses of target lesions in ~38% of 16 evaluable companion dogs[54]. Intriguingly, the objective response rate among the dogs with peripheral nerve sheath tumors was higher at ~57%. The numbers of dogs used in the trial were likely too small to achieve statistical significance, but should prompt further investigations to identify cancer types particularly sensitive to bacterial therapy. Tumor-targeting bacteria delivering therapeutic payloads have also been tested in canine patients. The *Salmonella* strain engineered to express IL-2 given at a neoadjuvant/adjuvant setting was combined with amputation and adjuvant doxorubicin to treat canine appendicular osteosarcoma[86,162]. The dogs in this study showed a significantly longer disease-free interval (DFI) when compared to historical controls treated with amputation and adjuvant doxorubicin, but not to those treated with amputation plus carboplatin and doxorubicin.

Human studies.—A number of *Listeria* vaccine strains have been tested in clinical trials and some showed very encouraging results (TABLE 3)[71,163,164]. In comparison, human trials with tumor-targeting strains have been scarce. In addition to the historical human studies with live oncolytic bacteria[18-20,165,166], a handful of human clinical trials have been reported and a few more registered with the federal regulatory authorities in more recent years (TABLE 2)[54,63,64,167-175] (a search at EU Clinical Trials Register and UK Clinical Trials Gateway websites using relevant keywords did not return any result on trials with *Clostridium*, *Salmonella*, *Listeria*, Bidobacterium, *Lactobacilli*, or *Escherichia*).

Historical studies with an oncolytic *Clostridium* strain have documented robust tumor colonization and tumor lysis in different cancer types[165,166,176]. Similarly, clinical signs of colonization have been observed in a large fraction of patients treated with either IV or intratumoral administration of *C. novyi*-NT spores in more recent Phase I trials[170,173,175]. Objective evidence of tumor response has also been shown in these trials. For example, extensive tumor destruction along with gas pockets, a signature sign for infection of the gas-forming clostridia, was observed by CT scan in a patient who received direct injection of *C. novyi*-NT spores into a metastatic shoulder lesion[54,175]. Biopsies of the lesion revealed extensive tumor necrosis and absence of viable tumor cells. Anaerobic culture of the biopsied material was positive for *C. novyi*-NT, suggesting its involvement in tumor destruction. However, these treatments with oncolytic bacteria alone failed to eradicate all cancer cells, which inevitably led to progression or relapse.

Attenuated *Salmonella* strains and their derivatives engineered to express therapeutic payloads have also been tested in early clinical trials[63,64,167-169,171,172]. Similar to the oncolytic *Clostridium* strains, *Salmonella* strains are reasonably tolerated in cancer patients. Unexpectedly, the *Salmonella* strains tested so far have yet to show the robust colonization and therapeutic benefit repeatedly observed in preclinical studies. The reason for this discrepancy is unclear, but over-attenuation has been proposed as a reason. It is worth noting that intratumoral-injected *Salmonella* expressing *E. coli* cytosine deaminase was able to colonize the target tumors and convert 5-FC to 5-FU inside the colonized lesions, resulting

in a 3:1 tumor-to-plasma ratio of 5-FU[167]. This study demonstrated that bacteria colonizing human tumors can express significant amounts of functional enzymes.

Although small in number, these early human trials have already taught us a few important lessons. (1) The attenuated tumor-targeting bacteria are reasonably tolerated in human patients and toxicities observed are very similar to those seen in experimental animals. (2) Robust colonization is a prerequisite for significant clinical benefit. Future clinical studies may employ companion diagnostic approaches based on angiography and hypoxia/necrosis imaging to define a patient population potentially more sensitive to intratumoral bacterial colonization[153-155]. Alternatively, engineering bacteria to express proteins targeting tumor vasculature or combining bacteria with microtubule-destabilizing agents may help expand colonization in an otherwise less hypoxic tumor[129,130,132-139].

Conclusions and future perspectives

Tumor-targeting bacteria are ideal vehicles to deliver therapeutic payloads because of their tumor selectivity and vast gene packaging capacity. This essentially unlimited gene packaging capacity would allow not only expression of large and multiple therapeutic proteins, but also engineering of bacteria with gene networks, enabling them to perform more sophisticated tasks in the fight against cancer. Despite the great therapeutic potential of engineered tumor-targeting bacteria, a successful cancer therapy is still likely to require combination approaches in the near future, because cancer heterogeneity, at both molecular and histologic levels, makes it very difficult to achieve cure with single anticancer agents. Bacteria thrive in necrotic and hypoxic tumor regions, but not in the highly perfused areas. The contrary is true for cytotoxic therapies, such as chemotherapeutic agents or radiation, which are often more effective against tumor cells in well perfused tumor areas[177]. Thus, bacteria and the cytotoxic therapies should synergize with each other for antitumor activities[61,178-180]. Tumor-targeting bacteria have further been shown to drive the G0/G1 to S/G2/M cell cycle transition of tumor cells, making them more susceptible to chemotherapy[180,181]. Conversely, therapies with small molecules targeting tumor vasculature can enlarge the hypoxic niche inside the solid tumor, consequently increasing bacterial colonization[129-131], which is particularly important for tumors without extensive hypoxia. In addition, intratumoral bacterial infection can modulate antitumor immune response both systemically and in the tumor microenvironment (FIG. 1), making it attractive to combine live bacteria with other systemic immunotherapeutic approaches such as immune checkpoint blockade. With more rationally designed tumor-targeting bacteria entering clinical studies, therapy with these bacteria will hopefully become another powerful weapon in the arsenal for our fight against cancer in the near future.

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Glossary list

Germinated *Clostridia*
actively growing *Clostridia* germinated from clostridial spores

Exotoxin
bacterial toxin secreted into the surroundings

Gram-negative bacteria
bacteria including *Salmonella* unable to retain the crystal violet stain used in the Gram-staining method for bacterial differentiation

Sepsis
a life-threatening complication associated with an infection triggering systemic inflammatory responses that can lead to tissue damage and organ failure

Auxotrophic mutant
a mutant bacterial strain that has an additional nutritional requirement for growth compared to its parental strain

Promoter traps
experimental approaches to identify particular promoters in a genome by using a promoterless reporter gene

Autolysis
destruction of a cell through a mechanism present within the cell

Bystander effect
referring in this article specifically to therapeutic effect on cells that are not infected by the bacteria

Antigen spreading (or “epitope spreading”)
the expansion of an immune response to antigens that are not the original antigen targeted in the therapy

Natural killer T (NKT) cells
a heterogeneous population of T cells that express an invariant αβ T-cell receptor and a number of cell surface molecules typically associated with natural killer cells

Quorum-sensing
a bacterial cell-cell communication process that regulates gene expression in response to fluctuations in cell-population density

Autochthonous tumors
tumors developed spontaneously, including those developed in genetically modified models or induced by chemical, viral, or physical carcinogens, as opposed to transplanted tumors

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Box 1.

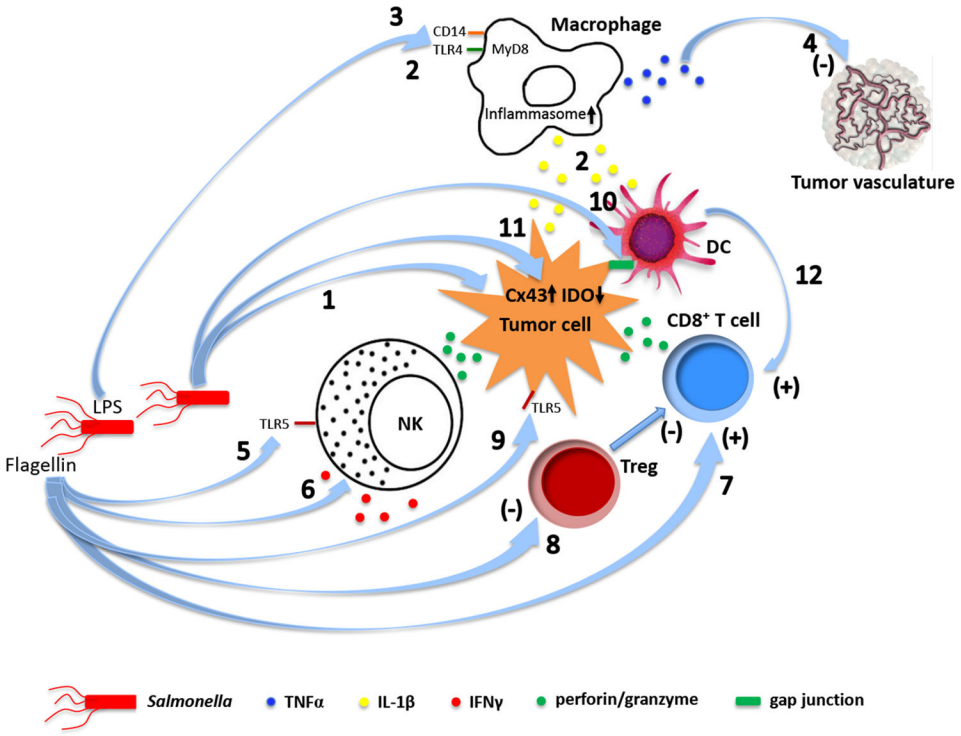
Regulatory considerations for clinical investigations of live tumor-targeting bacteria.

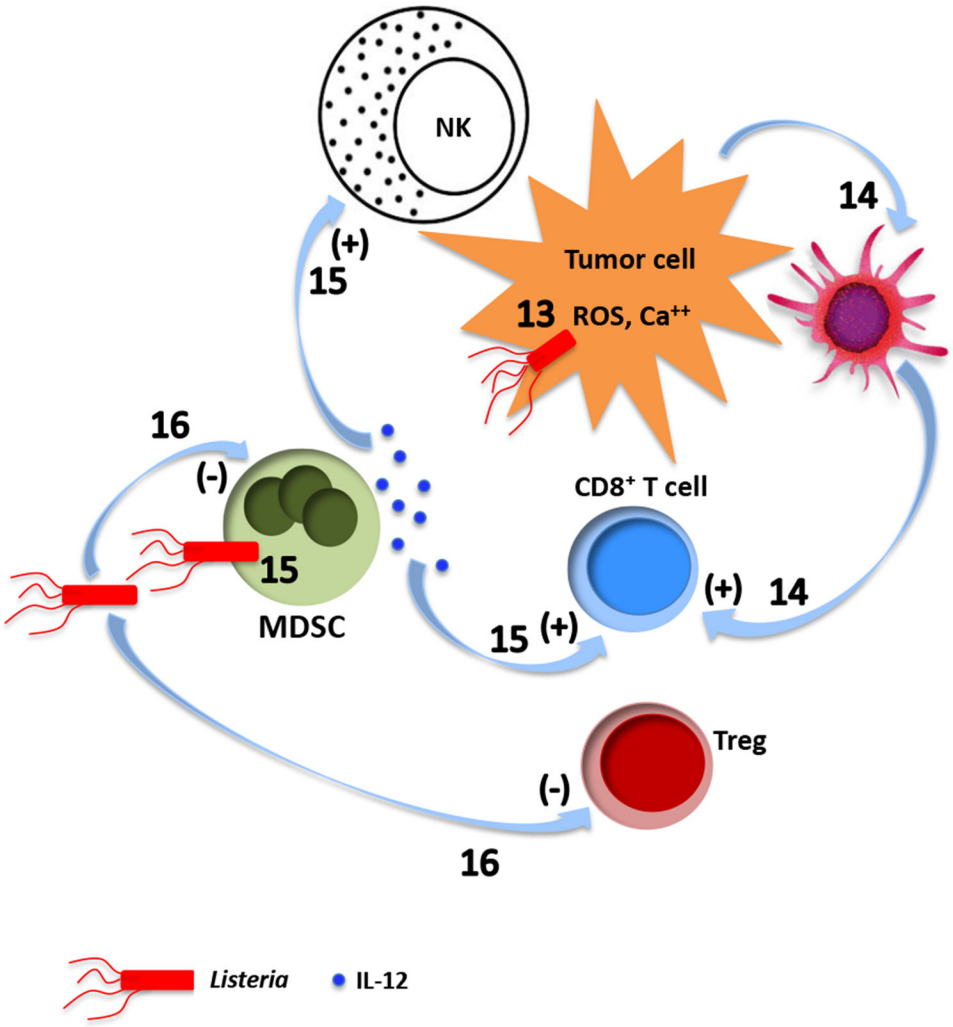
Distinct from conventional cancer treatment such as chemotherapies, targeted therapies or monoclonal antibody therapy, live tumor-targeting bacteria have its unique regulatory challenges. Detailed description of regulatory consideration and requirement is beyond the scope of this section. Listed below are some points for the sponsors to consider in initiation of clinical investigations using live bacteria for cancer treatment. The government regulatory agencies generally encourage the sponsors to consult the published guidance documents and engage with the regulatory agencies early in the development of live bacterium-based products.

- Preclinical study considerations.
  - Preclinical proof-of-concept and safety studies are critically important for several reasons. They support the scientific rationale for proposed clinical studies, guide the selection of the initial clinical dose level, dose-escalation scheme, dosing schedule, and provide adequate safety information for the regulatory authorities to determine whether it is reasonably safe to conduct the proposed clinical trial.
  - If previous human safety and activity data are available for a microbial vector used for gene therapy (MVGT) product including the live bacterium, additional extensive preclinical studies may not be necessary. However, to assess the relevance of the available data to specific product(s) previously administered to humans, adequate information regarding the manufacturing and characterization of the product(s) is required. In addition, sponsors should provide comprehensive activity and safety data from the previous human experience to support the safety of the proposed dosing of the MVGT product.
  - Sponsors are encouraged to actively engage with the regulatory authorities early in product development to discuss above issues.
- **Chemistry Manufacturing and Controls (CMC) considerations.** The process of manufacturing live therapeutic bacteria is vastly more complex than that of the small molecules. Sponsors need to consider the following.
  - The most optimal bacterial seed stock, banking system and the reagents used.
  - The procedures in producing, purifying and harvesting live bacteria.
  - The type of formulation of the final product.

  - The tests for identity, purity and potency which face unique challenges for live bacteria as final products (also discussed in main text).

- **Pharmacokinetics and dose-response considerations.** Live bacterial products do not follow typical patterns of pharmacokinetics and the dose-responses of conventional small-molecule drugs and proteins, thus posing challenges in determining the optimal starting dose and schedule for administration (also discussed in main text).
- **Safety concerns.** Safety is the major concern due to the infectious nature of the products, along with the concomitant medications and procedures for administering these products.
  - Live bacterial products carry the risk of clinically significant infection/sepsis, especially in immunocompromised host. Administration of antibiotics post treatment, and in some cases, prolonged antibiotic administration may be needed to decrease this risk.
  - For some products, it may be necessary that certain procedures are followed to administer these products. There are risks associated with these procedures. Thus, early clinical trials design would need to consider appropriate plans to mitigate these concerns.
- **Study population and study design.** Discussed in main text.
- Relevant U.S. FDA guidance documents.
  - "*Recommendations for Microbial Vectors used for Gene Therapy*" (September, 2016)[182]. This guidance focuses on the chemistry, manufacturing, and control (CMC) information that investigational new drug application (IND) sponsors should submit in an IND for MVGTs and provides an overview of preclinical and clinical considerations for these products. Many principles described in this guidance apply to microbial-based cancer therapies that are not genetically modified as well.
  - "*Preclinical Assessment of Investigational Cellular and Gene Therapy Products*" (November, 2013)[183]. This guidance provides comprehensive recommendations regarding the selection of appropriate animal species and animal models of disease, as well as the overall design of preclinical proof-of-concept and toxicology studies for investigational products, including live bacterial products.





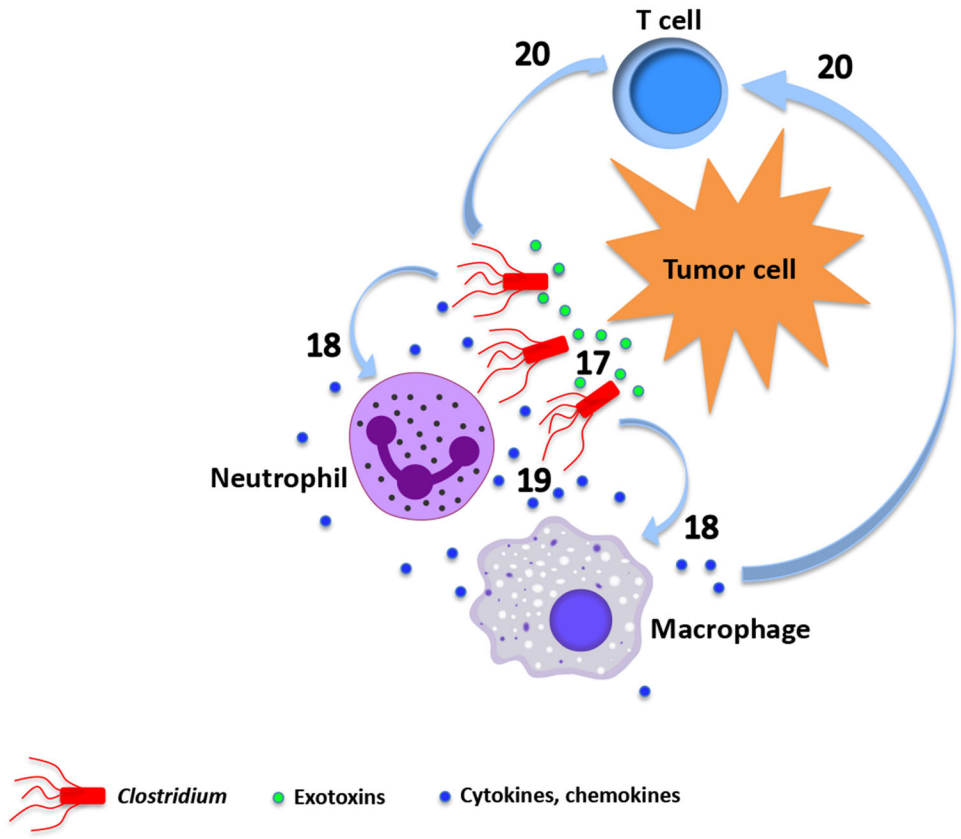


Fig. 1. Mechanisms of tumor destruction by live tumor-targeting bacteria.

Different bacterial species employ both shared and unique intrinsic mechanisms to destroy cancer. **a**. *Salmonella*. (**1**) Uncontrolled bacterial multiplication can lead to bursting of the invaded tumor cells[184]. Intracellular bacteria may also kill tumor cells by inducing apoptosis or autophagy[184-188]. (**2**) Macrophages and dendritic cells in *Salmonella*-colonized tumors secrete IL-1β responsible for the antitumor activity[189]. The elevated IL-1β secretion requires both LPS-induced TLR4 signaling and inflammasome activation in macrophage following phagocytosis of *Salmonella*-damaged tumor cells[190]. (**3**) LPS elicits TNFα expression through CD14, TLR4 and MyD88[191,192], (**4**) leading to disruption of the tumor vasculature[57]. (**5**) Flagellin induces an NK cell-mediated antitumor response dependent on perforin[193], as well as (**6**) release of IFNγ, a critical cytokine for both innate and adaptive immunity, from NK cells through a TLR-independent pathway involving IL-18 and Myd88[194]. (**7**) Flagellin also enhances a TLR5 and $CD8^+$ T cell-dependent antitumor response in a peptide vaccine-based immunotherapeutic setting[195], and (**8**) decreases frequency of $CD4^+CD25^+$ regulatory T cells (Treg)[196]. (**9**) In addition, flagellin can directly suppress tumor cell proliferation through TLR5 signaling[197]. (**10**) *Salmonella* induces upregulation of connexin 43 (Cx43)[198,199,200] , leading to gap junction formation between tumor cells and dendritic cells (DC), which promotes transfer and cross-presentation of processed tumor antigenic peptides[198]. (**11**) Upregulation of Cx43 in tumor cells also reduces expression of the immunosuppressive indoleamine 2,3-dioxygenase (IDO)[200]. (**12**) Both tumor antigen cross-presentation by DC and decreased IDO further activate $CD8^+$ T cells. **b**. *Listeria*. (**13**) *Listeria* can directly kill tumor cells through the nicotinamide adenine

dinucleotide phosphate oxidase (NADPH oxidase)-mediated production of reactive oxygen species (ROS) and intracellular calcium mobilization[201]. (**14**) The immunogenic tumor cell death caused by high levels of ROS activates $CD8^+$ T cells responsible for eliminating both primary tumors and metastases[201,202]. (**15**) *Listeria* infects the immunosuppressive MDSC and alters a subpopulation of these cells into an immune-stimulating phenotype characterized by elevated production of IL-12, which is correlated with improved $CD8^+$ T cell and NK cell responses[45]. (**16**) *Listeria* vaccine strains also inhibits MDSC and Treg[124,203]. **c**. *Clostridia*. (**17**) Direct tumor destruction is caused by a variety of exotoxins secreted by the colonizing clostridia, some of which (e.g. phospholipases, haemolysins, lipases) can damage membrane structures while others are internalized to interfere with critical cellular functions[204-207]. (**18**) Similar to infection by other bacterial species, the clostridial infection results in an initial accumulation of granulocytes and macrophages at the infection site [47,55]. This first line of defense prevents the colonizing bacteria from invading into surrounding normal tissues as well as sufficiently perfused and oxygenized tumor regions[48,52]. (**19**) The cellular response results in elevated cytokines and chemokines that orchestrate a concerted immune response[47,57]. *Clostridia* can also trigger the release of TRAIL from neutrophils, killing cancer cells through activation of apoptosis[208]. (**20**) At later time points, adaptive immune cells including $CD8^+$ T lymphocytes are recruited to fight cancer[47].

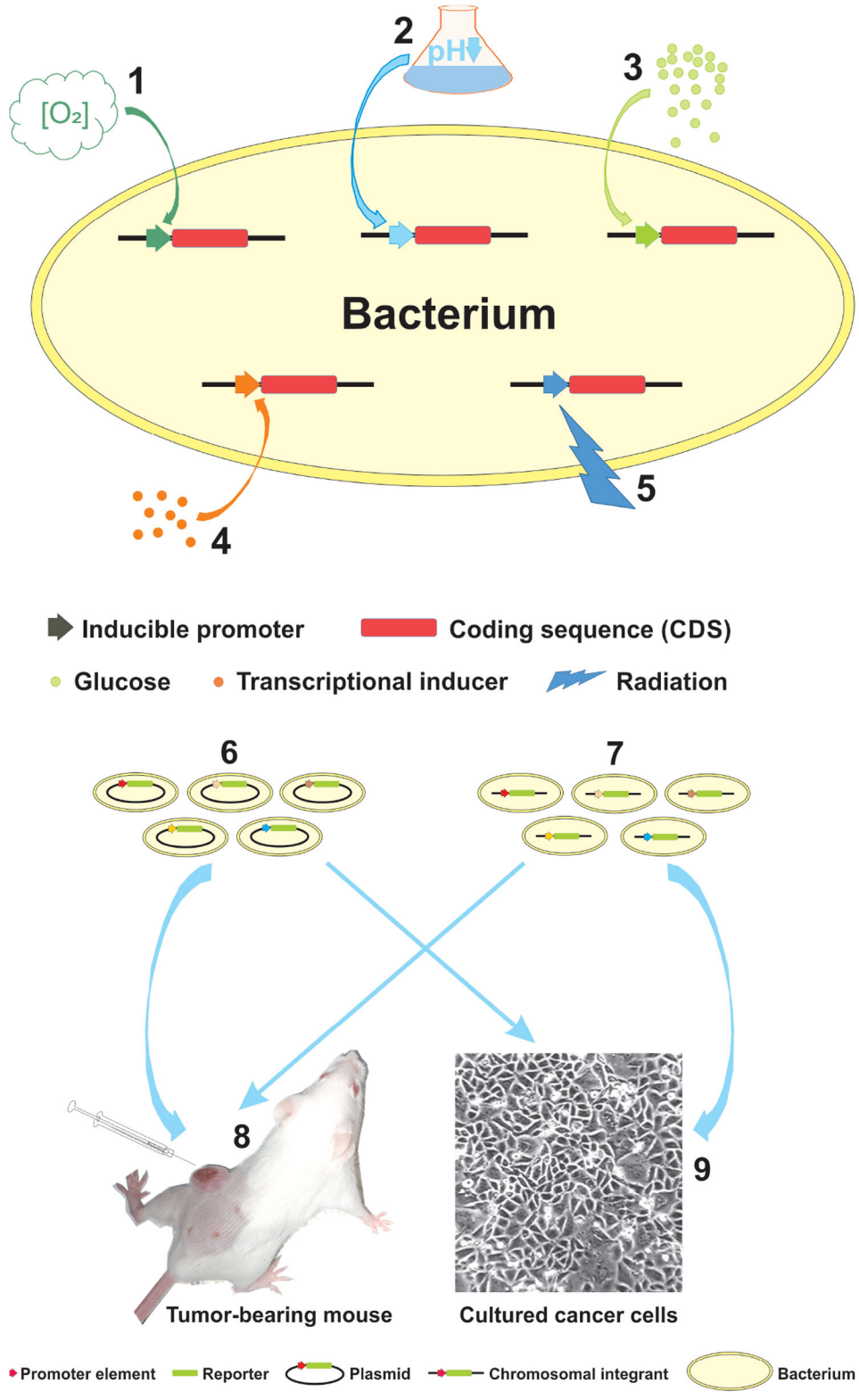


Fig. 2. Inducible promoters used for targeted colonization and payload expression.

a. Various inducible promoters can be used for either tumor-selective expression or temporally or spatially controlled expression. (**1**) A *Salmonella* strain was engineered such that an essential gene was placed under the control of a hypoxia-inducible promoter, while expression of an inhibitory antisense RNA for this gene was activated by an oxygen-inducible promoter to minimize basal level expression in oxygenated normal tissues[209]. This strain showed a robust tumor colonization and greatly enhanced clearance from normal tissues, thus resulting in a substantially improved safety profile compared to the parental strain. Hypoxia-inducible promoters have also been used to direct the expression of effector genes such as those encoding cytotoxic proteins, which requires tighter control for safety reasons[96]. (**2**) Promoter elements responsive to low pH were among the ones identified to be active in tumor microenvironment in studies using "promoter traps" (see below)[210]. (**3**) A genetic circuit that can be triggered by glucose gradients often present in solid tumors has also been used to engineer bacteria[211], enabling them to express antitumor proteins in metabolically more active tumor regions. (**4**) Exogenously applied transcriptional inducers such as L-arabinose, acetyl salicylic acid and doxycycline can tightly regulate the relevant inducible promoters introduced into bacteria[97,99,100,212-215], providing a means to control the expression of effector genes in a temporal fashion. (**5**) Ionic radiation at as low as 2 Gy has also been shown to activate the recA promoter on a plasmid transfected into *Clostridium*[216-218], raising the possibility to regulate effector gene expression with focused radiation treatment at clinically relevant doses (2 Gy is similar to a typical fractionated dose used in radiation therapy in an adjuvant setting for solid tumors). **b.** "Promoter traps" have been employed to identify promoter elements active in the tumor microenvironment[210,219,220]. "Promoter trap" libraries can be constructed by transforming bacteria with either (**6**) plasmids containing random genomic DNA fragments cloned upstream of a promoterless reporter gene, or (**7**) transposons containing a promoterless reporter gene which integrate randomly into the bacterial genome. These "promoter trap" libraries can be either (**8**) injected into experimental tumors or (**9**) co-cultured with tumor cells. Bacteria are then recovered and analyzed for reporter activities. Clones with high reporter activities are likely to contain promoter elements active in the tumor microenvironment.

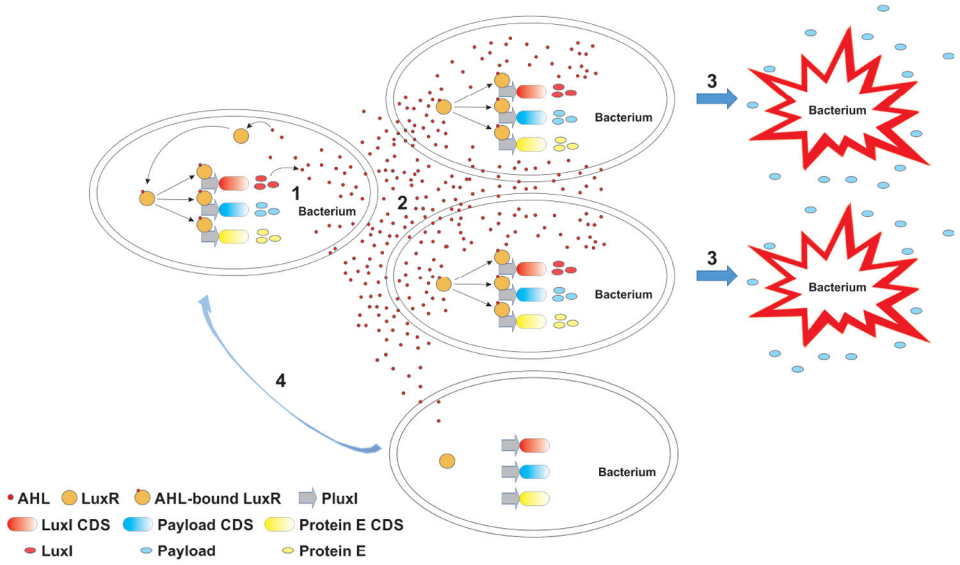


Fig. 3. Gene circuit for a transcriptional program regulating bacterial activities at the population level.

Illustrated is an example of a sophisticated gene circuit for a transcriptional program enabling synchronized population control and therapeutic payload release in repeated cycles. (**1**) The AHL-bound transcription factor LuxR interacts with and activates promoter PluxI that drives the expression of (from top to bottom) the AHL synthase LuxI to establish a positive feedback loop, the therapeutic payload, and the bacteriophage φX174 protein E to lyse the bacteria. CDS, coding sequence. (**2**) The AHL signaling molecules diffuse freely across the cell membranes, enabling synchronization of neighboring bacterial cells in the population for a concerted action. At low densities of the bacterial population, AHL molecules diffuse predominately out of bacterial cells, leaving the gene circuit inactive. Increased population density allows AHL molecules inside the majority of the bacterial cells to accumulate and reach a threshold concentration required to activate the gene circuit. (**3**) Synchronized activation of the transcriptional program leads to simultaneous lysis of bacterial cells in the population by protein E as well as a burst of therapeutic payload release. (**4**) The small number of bacteria surviving the lysis repopulate and kick off another cycle of lysis and payload release.

Table 1.

Effector systems

Effector classes	Effectors or targets	Exemplary studies
Cytotoxic	Bacterial toxins, immunotoxins (e.g. Cytolysin A, *S. aureus* α-hemolysin, *Pseudomonas* exotoxin A, TGFα-PE38)	96-102,221
	Apoptosis-inducing ligands (e.g. TNF-α, FasL, TRAIL, Azurin, Cp53, apoptin, Noxa BTD)	89,106-110,214,222-224
	Agents loaded into/onto bacteria (e.g. ^{188}Re, ^{32}P, doxorubicin, C_3N_4)	44,46,111,112
Prodrug-converting enzymes	Thymidine kinase (TK), Cytosine deaminase (CD), Nitroreductase (NTR), Purine nucleoside Phosphorylase (PNP), Carboxypeptidase G2, YieF	36,114-116,150,225-229
Immunomodulators	Tumor antigens	71,95,119,163,230-232
	Cytokines and chemokines (e.g. IL-2, IL-4, IL-12, IL-18, IFNγ, GM-CSF, Flt3L, LIGHT, CCL21)	86,120,121,233-240
	Others (e.g. heterologous flagellin, α-galactosylceramide, Immunodominant recall antigens)	72,122
Targeting tumor stroma	Legumain, VEGFR2 (FLK-1), Endoglin (CD105), Thrombospondin-1, TEM8, PDGFRβ	92,132,134,136-139,241-243
Gene silencing	Silenced targets: IDO, STAT3, Bcl-2, MDM2, Survivin, MDR	244-252
Synthetic gene circuit	Quorum-sensing gene circuit for controlled payload production	90,253

Table 2.

Clinical trials with engineered tumor-targeting bacteria

Trial	Bacterial strain	No. patients treated/Cancer type	Treatment/Outcome	Reference/ Recruitment Status
Canine trials with tumor-targeting strains	VNP20009	41 patients STS (AUS, FBS, RMS, HPC, MXS), melanoma, carcinomas, OSA, HSA, lymphoma, MCT	IV infusion, 1.5×10^5 - 1×10^8 CFU/kg (dose escalation), 1 - 19 doses (mean=3) MTD 3×10^7 CFU/kg; tumor colonization observed in 42% cases; 4 CR, 2 PR	Published[161]
	C. novyi-NT	6 patients HSA, lingual SCC, OSA, nasal ACA, FBS	IV infusion, 3×10^8 spores/kg, 3×10^7 spores/kg, 1 dose DLT (abscess formation) observed at 3×10^7 spores/kg; tumor abscess observed in 3 patients; 4 SD	Published[254]
	C. novyi-NT	16 patients STS (PNST, RMS, FBS, MXS), OSAc, MCT, melanoma, SCS	IT injection, 1×10^8 spores/dose, 1 - 4 doses Tumor abscess observed in 7 patients; 3 CR, 3 PR, 5 SD	Published[54]
	SalpIL2 (*Salmonella* χ4550 expressing IL-2)	19 patients Appendicular OSA	Neoadjuvant/adjuvant SalpIL2: PO, 1×10^5 - 1×10^9 CFU/patient (dose escalation), 6 (n=13), 4 (n=3), 3 (n=2), 1 (n=1) doses Amputation Adjuvant doxorubicin: IV, 30 mg/m^2, 5 doses (n=13) No DLT observed; tumor colonization not evident in tumors from 5 patients assayed; DFI of patients treated with SalpIL2/doxorubicin significantly longer than comparison group treated with doxorubicin	Published[162]
Human trials with tumor-targeting strains	VNP20009	Phase 1 25 patients Melanoma, RCC	30 min IV infusion, 1×10^6 - 1×10^9 CFU/m^2 (dose escalation), 1 dose MTD 3×10^8 CFU/m^2; tumor colonization observed in 3 patients in 2 highest dose cohorts; elevated circulating proinflammatory cytokines detected; objective tumor regression not observed	Published[63]
	VNP20009	4 patients Melanoma	4 hr IV infusion, 3×10^8 CFU/m^2, 1 dose Treatment well tolerated; tumor colonization not evident; objective tumor response not observed	Published[64]
	TAPET-CD (VNP20009 expressing CD)	3 patients Head & neck SCC, esophageal ACA	TAPET-CD: IT injection, 3×10^6 CFU/m^2, 1×10^7 CFU/m^2, 3×10^7 CFU/m^2 (dose escalation), multiple doses/cycles 5-FC: PO, 100 mg/kg/day divided thrice daily, multiple doses/cycles Tumor colonization evident in 2 patients; generation and accumulation of 5-FU observed in the 2 patients with TAPET-CD tumor colonization	Published[167]
	VNP20009	Phase 1 Refractory, superficial solid tumors	IT injection, dose escalation planned	Unpublished[168], completed
	C. novyi-NT	Phase 1 2 patients Colorectal cancer	IV infusion, 1×10^6 spores/kg, 1 dose	Unpublished[170], terminated
	SalpIL2 (Salmonella χ4550 expressing IL-2)	Phase 1 22 patients Liver metastases of solid tumors	PO, 1×10^5 - 1×10^{10} CFU/dose (dose escalation planned)	Unpublished[172], completed
	C. novyi-NT	Phase 1 5 patients	IV infusion, 1×10^5 - 1×10^7 spores/kg (dose escalation planned), 1 dose	Unpublished[173], terminated

Trial	Bacterial strain	No. patients treated/Cancer type	Treatment/Outcome	Reference/ Recruitment Status
		Solid tumor malignancies		
	APS001F (B. longum expressing CD)	Phase 1/2 75 patients (estimated) Advanced and/or Metastatic Solid Tumors	APS001F ± maltose IV infusion/5-FC PO	Unpublished[174], recruiting
	C. novyi-NT	Phase 1 24 patients Solid tumor malignancies	IT injection, 1×10^4 - 3×10^6 spores/dose (dose escalation), 1 dose	Unpublished[175], completed

STS, soft tissue sarcoma; AUS, anaplastic/undifferentiated sarcoma; FBS, fibrosarcoma; RMS, rhabdomyosarcoma; HPC, hemangiopericytoma; MXS, myxosarcoma; OSA, osteosarcoma; HSA, hemangiosarcoma; MCT, mast cell tumor; SCC, squamous cell carcinoma; ACA, adenocarcinoma; PNST, peripheral nerve sheath tumor; OSAc, chondroblastic osteosarcoma; SCS, synovial cell sarcoma; RCC, renal cell carcinoma; PO, oral administration; CFU, colony-forming unit; MTD, maximum tolerated dose; DLT, dose-limiting toxicity; SAE, serious adverse event; CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease; DFI, disease-free interval; OS, overall survival

Table 3.

Clinical trials with live attenuated *Listeria monocytogenes (Lm)*-based vaccines

Vaccine name (attenuated strain) Combination agent	Peptide antigen expressed	Trial type/No. patients treated/Cancer type	Treatment/Outcome	Reference/ Recruitment Status
Lm-LLO-E7 (*Lm*-LLO)	HPV-16 E7 fused to LLO	Phase 1 15 patients (13 evaluable) Cervical cancer	IV infusion, 1×10^9 CFU, 3.3×10^9 CFU or 1×10^{10} CFU/dose (dose escalation) ×2 All experienced flu-like syndrome, grade 3 AEs reported in 6 patients (40%), DLT (grade 2 diastolic hypotension) reported in 3 patients in the 1×10^{10} CFU group; *Lm*-LLO-E7 rapidly cleared from blood; Positive T cell response (IFNγ-ELISpot) specific to HPV-16 E7 peptides observed after the second dose on day 26 in one patient; 7 (53.8%) SD, 5 (38.5%) PD; 1 (7.7%) PR	Published[255]
ADXS11-001 (*Lm*-LLO) Cisplatin	HPV-16 E7 fused to LLO	Phase 2 109 patients (69 evaluable) Cervical cancer	ADXS11-001 only group (n=55): IV infusion of ADXS11-001 (1×10^9 CFU) on days 1, 29, 57 ADXS11-001 + cisplatin group (n=54): IV infusion of ADXS11-001 (1×10^9 CFU) on days 1, 85, 113, 141 and IV infusion of cisplatin (40 mg/m^2) on days 29, 36, 43, 50, 57 Median OS comprable between the two groups, OS (two groups combined) 34.9% at 12 months and 24.8% at 18 months; most AEs mild to moderate, SAEs reported in two patients (CRS with one and bacterial peritonitis with septicemia in another) treated with ADXS11-001 monotherapy.	Published[256]
ANZ-100/CRS-100 (LADD)	None	Phase 1 9 patients Pancreatic cancer, colorectoal cancer, and melanoma all with liver metastases	A single-dose IV infusion, 1×10^6, 3×10^7, 3×10^8 CFU/dose (dose escalation) Most frequent AEs: transient lymphopenia, hyperglycemia, hypophosphatemia, fever; MTD not reached; CD38 on NK cells increased after treatment; serum MCP-1 and MIP-1β elevated in patients after treatment at highest dose level, elevated IFN-γ and IL-12p70 also observed; ANZ-100 not detected in blood, stool, urine, or sputum at any time point	Published[257,258]
CRS-207 (LADD)	Mesothelin	Phase 1 17 patients Pancreatic cancer, mesothelioma, ovarian cancer, NSCLC	IV infusion, 1×10^8, 3×10^8, 1×10^9, and 1×10^{10} CFU/dose x1-4 every 3 weeks Most frequent AEs: transient lymphopenia, hypophosphatemia, transaminitis, fever, chills/rigors, nausea, fatigue, hypotension; grade 2 CRS reported in 1 patient dosed at 1×10^{10} CFU; MTD 1×10^9; elevated MCP-1, MIP-1β and IP-10 for all dose levels observed; LLO-specific and mesothelin-specific T cell responses (IFNγ-ELISpot) detected; 37% of patients survived for 15 months; CRS-207 detected in blood cultures of 1 patient receiving 1×10^9 CFU at day 4 after 2nd IV infusion, CRS-207 not detected in all remaining blood, stool, or urine samples at any time point	Published[257,259]
CRS-207 (LADD) GVAX pancreas vaccine Cy	Mesothelin	Phase 2 90 patients Pancreatic cancer	Arm A (n=61): IV infusion of Cy (200 mg/m^2) on day 1 of and ID injections of GVAX (5×10^8 cells) on day 2 of weeks 1 and 4; IV infusion of CRS-207 (10^9 CFU) on day 1 of weeks 7, 10, 13 and 16 Arm B (n=29): IV infusion of Cy (200 mg/m^2) on day 1 of and ID injections of GVAX (5×10^8 cells) on day 2 of weeks 1, 4, 7, 10, 13 and 16 OS 6.1 months in arm A vs 3.9 months in arm B (hazard ratio 0.59; P=0.02); stabilized or reduced CA19-9 levels observed in 27% of patients in arm A and 9% of patients in arm B (two-sided P=0.08); An increase (P=0.042) in mesothelin-specific CD8$^+$ T cells over baseline documented at week 20 in arm A only; Most frequent AEs in arm A: injection site reactions, nausea, vomiting, chills, fever, fatigue; grade 3 to 4 lymphopenia and transaminitis observed after CRS-207 infusion	Published[163,260]
ADXS11-001(*Lm*-LLO)	HPV-16 E7 fused to LLO	Phase 2 81 patients	IV infusion, 5×10^7, 3.3×10^8, or 1×10^9 CFU/dose x3 at 28 day intervals; placebo control arm: normal saline x3 at 28 day intervals	Unpublished[261]; terminated

Vaccine name (attenuated strain) Combination agent	Peptide antigen expressed	Trial type/No. patients treated/Cancer type	Treatment/Outcome	Reference/ Recruitment Status
		Cervical Intraepithelial Neoplasia		
ADXS11-001(*Lm*-LLO)	HPV-16 E7 fused to LLO	Phase 2 67 patients (estimated) Cervical cancer	IV infusion on day 1, repeated every 28 days in the absence of disease progression or unacceptable toxicity.	Unpublished[262]; active, not recruiting
ADXS11-001(*Lm*-LLO)	HPV-16 E7 fused to LLO	Phase 1 2 patients HPV-16+, p16+OPSCC	IV infusion, 3.3×10^8, 1×10^9, or 3.3×10^9 CFU/dose (dose escalation) x3 at 28 day intervals	Unpublished[263,264]; terminated
ADXS11-001(*Lm*-LLO) Ablative transoral robotic surgery	HPV-16 E7 fused to LLO	Phase 2 30 patients (estimated) HPV+OPSCC	IV infusion, 1×10^9 CFU/dose x2, administered ~33 and ~14 days before ablative transoral robotic surgery; control arm: surgery only	Unpublished[265]; recruiting
ADXS11-001(*Lm*-LLO) Chemoradiation (mitomycin, 5-FU and radiation)	HPV-16 E7 fused to LLO	Phase 1/2 11 patients Anal cancer	IV infusion, 1×10^9 CFU/dose x4, 1st dose administered 10-14 day before initiation of chemoradiation, 2nd dose administered at least 10 days after completion of chemoradiation; 2nd, 3rd and 4th doses administered at 28 day intervals	Unpublished[266]; terminated
ADXS11-001(*Lm*-LLO)	HPV-16 E7 fused to LLO	Phase 1/2 25 patients (estimated) HPV+ Cervical cancer	1×10^{10} CFU/dose x3 at 28 day intervals	Unpublished[267]; active, not recruiting
ADXS11-001(*Lm*-LLO) MEDI4736 (durvalumab)	HPV-16 E7 fused to LLO	Phase 1/2 66 patients (estimated) HPV+ Head & neck SCC or cervical cancer	IV Infusion, ADXS11-001 plus MEDI4736 or MEDI4736 alone	Unpublished[268]; suspended
ADXS11-001(*Lm*-LLO)	HPV-16 E7 fused to LLO	Phase 2 55 patients (estimated) Anal or rectal cancer	No information provided	Unpublished[269]; active, not recruiting
ADXS11-001(*Lm*-LLO)	HPV-16 E7 fused to LLO	Phase 3 450 patients (estimated) Cervical cancer	Double-blind, placebo-controlled randomized study of ADXS11-001 administered in adjuvant setting after completion of cisplatin-based concurrent chemoradiotherapy; IV infusion administered every 3 weeks for 3 doses for the first 3 months, followed by treatment every 8 weeks for a total of 5 doses or until disease recurrence	Unpublished[270]; recruiting
ADXS11-001(*Lm*-LLO) Pemetrexed	HPV-16 E7 fused to LLO	Phase 2 124 patients (estimated) HPV+ NSCLC	Treatment arm: pemetrexed plus ADXS11-001 immunotherapy Control arm: pemetrexed only	Unpublished[271]; not yet recruiting
ADXS31-164 (*Lm*-LLO)	HER2/neu fused to LLO	Phase 1/2 12 patients HER2/neu+solid tumors	Dose escalation	Unpublished[272]; active, not recruiting
ADXS-NEO (*Lm*-LLO)	Personalized neoepitopes fused to LLO	Phase 1 48 patients (estimated) Colorecal cancer, head & neck SCC, or NSCLC	Dose escalation/de-escalation study with a standard 3 + 3 design; 3 dose levels planned: 1×10^9, 2×10^9, 4×10^9 CFU	Unpublished[273]; recruiting
CRS-207 (LADD) Pemetrexed and cisplatin	Mesothelin	Phase 1b 60 patients Mesothelioma	IV infusion of CRS-207 (1×10^9 CFU) with or without Cy (200 mg/m^2) at weeks 1, 3, 23 and 26; maintenance CRS-207 vaccinations every 8 weeks starting at week 34 until	Unpublished[274]; active, not recruiting

Vaccine name (attenuated strain) Combination agent	Peptide antigen expressed	Trial type/No. patients treated/Cancer type	Treatment/Outcome	Reference/ Recruitment Status
			disease progression; pemetrexed (500 mg/m^2) and cisplatin (75 mg/m^2) at weeks 5, 8, 11, 14, 17 and 20	
CRS-207 (LADD) GVAX pancreas vaccine Cy	Mesothelin	Phase 2b 303 patients Pancreatic cancer	Cy/GVAX + CRS-207 arm: IV infusion of Cy (200 mg/m^2) on day 1 of and ID injection of GVAX (5×10^8 cells) on day 2 of weeks 1 and 4; IV infusion of CRS-207 (1×10^9 CFU) on day 1 of weeks 7, 10, 13 and 16 CRS-207 arm: IV infusion of CRS-207 (1×10^9 CFU) on day 1 of weeks 1, 4, 7, 10, 13 and 16	Unpublished[275]; completed
CRS-207 (LADD) Epacadostat and pembrolizumab	Mesothelin	Phase 1/2 126 patients (estimated) Ovarian, fallopian or peritoneal cancer	Phase 1/arm 1: IV infusion of CRS-207 (1×10^9 CFU), oral administration of epacadostat (100 or 300 mg, twice a day) Phase 1/arm 2: IV infusion of CRS-207 (1×10^9 CFU) only Phase 2/arm 1: IV infusion of pembrolizumab (200 mg), IV infusion of CRS-207 (1×10^9 CFU) Phase 2/arm 2: IV infusion of pembrolizumab (200 mg), IV infusion of CRS-207 (1×10^9 CFU), oral administration of epacadostat (300 mg, twice a day)	Unpublished[276]; active, not recruiting
CRS-207 (LADD) Pembrolizumab	Mesothelin	Phase 2 35 patients (estimated) Mesothelioma	IV infusion of pembrolizumab (200 mg) and CRS-207 (1×10^9 CFU) for 4 × 3-week cycles, followed by pembrolizumab treatment at 3 week intervals and CRS-207 treatment at 6 week intervals up to 24 months	Unpublished[277]; active, not recruiting
CRS-207 (LADD) Pembrolizumab	Mesothelin	Phase 2 79 patients (estimated) Gastric, gastroesophageal junction, or esophageal cancer	IV infusion of pembrolizumab (200 mg) and CRS-207 (1×10^9 CFU) for 4 × 3-week cycles, followed by pembrolizumab treatment at 3 week intervals and CRS-207 treatment at 6 week intervals up to 24 months	Unpublished[278]; active, not recruiting
ADU-623 (LADD)	EGFRvIII and NY-ESO-1	Phase 1 11 patients Astrocytomas	IV infusion, 1×10^8 or 1×10^9 CFU/dose (dose escalation) x4 at 21 day intervals	Unpublished[279]; active, not recuiting
ADU-214/ JNJ-64041757 (LADD)	EGFRvIII and Mesothelin	Phase 1 42 patients (estimated) NSCLC	IV infusion, 1×10^8 or 1×10^9 CFU/dose (dose escalation) at 21 day intervals	Unpublished[280]; recuiting
ADU-741/ JNJ-64041809 (LADD)	Prostate-specific antigens	Phase 1 26 patients Prostate cancer	IV infusion, 1×10^8 or 1×10^9 CFU/dose (dose escalation) at 21 day intervals	Unpublished[281]; active, not recuiting
pLADD (LADD)	Personalized neoepitopes	Phase 1 15 patients (estimated) Colorectal cancer	IV infusion, 1×10^8 or 1×10^9 CFU/dose (dose escalation) at 21 day intervals	Unpublished[282]; recruiting

AEs, adverse events; CRS, cytokine release syndrom; GVAX pancreas, granulocyte-macrophage colony-stimulating factor (GM-CSF)–secreting allogeneic pancreatic tumor cells; Cy, cyclophosphamide; ID, intradermal; HPV, human papillomavirus; OPSCC, oropharyngeal squamous cell carcinoma; NSCLC, non-small-cell lung carcinoma; 5-FU, fluorouracil