Tumour-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, most late-stage cancers are either insensitive to the therapies to begin with or develop resistance later. Therapy with live tumour-targeting bacteria provides a unique option to meet these challenges. Compared with most other therapeutics, the effectiveness of tumour-targeting bacteria is not directly affected by the 'genetic makeup' of a tumour. Bacteria initiate their direct antitumour effects from deep within the tumour, followed by innate and adaptive antitumour 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 on the basis of clinical needs. Therapeutic approaches using live tumour-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 tumour 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 tumour-targeting bacteria, including a discussion of the regulatory issues.

There are a variety of cytotoxic agents that can kill cancer cells effectively[1]. However, the conventional cytotoxic therapies often eliminate cancer cells at the expense of damaging the normal tissues, resulting in unacceptable toxicities in patients. Therefore, eradication of cancer cells without causing collateral damage is the ultimate goal for all oncologists and cancer researchers. The persistent pursuit of that goal has recently led to two promising clinical 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[2–7]. In spite of its increased targeting precision against tumour cells, targeted therapy is far from perfect[8]. First, targeted therapeutic agents have a spectrum of their own toxicities, some of which are related to the normal functions of the target proteins[9]. Second, the small molecule inhibitors may not be sufficiently specific[10]. 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[11]. Fourth, most tumours do not carry genetic changes currently actionable with established therapies[12].

Immunotherapy can be seen as another targeted therapy, which typically involves T cells reactive to tumour-specific neoantigens or tumour-associated antigens (TAAs)[13]. Recent clinical trials with immune checkpoint blockade have shown remarkable results including durable therapeutic effects on advanced metastatic cancers[14,15]. It is generally believed that sensitivity to immune checkpoint blockade is dependent on the neoantigen burden of the tumours as well as the extent and composition of immune infiltrates in the tumour microenvironment (TME)[16–18]. Unfortunately, most common cancers do not show abundant mutations and infiltrating immune cells and consequently are insensitive to immune checkpoint blockade[16–20]. Major efforts are being made to develop approaches that can sensitize these tumours to immunotherapy[21]. Tumour-targeting bacteria present an alternative approach to overcome the challenges of molecularly targeted therapies and immunotherapy, as they do not rely on the 'genetic makeup' of the tumour and can induce a robust intratumoural inflammatory response (discussed below).

In addition to molecular targets such as oncoproteins and neoantigens, unique pathological alterations at the tissue level can be exploited for tumour targeting. Tumour vasculature is generally irregular and chaotic, leading to insufficient diffusion of oxygen and nutrients into some areas within a solid tumour[22,23]. Cancer cells within these hypoxic areas are dormant but viable[24].

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Obligate anaerobes
Microorganisms that cannot survive in the presence of normal atmospheric concentrations of oxygen.

Facultative anaerobes
Microorganisms that can grow in both the presence and the absence of normal atmospheric concentrations of oxygen.

Germinated *Clostridium* spp.
Vegetative form of clostridia germinated from clostridial spores.

Furthermore, the hypoxic areas can be responsible for clinical relapse after chemotherapy or radiation therapy because they are poorly accessible to systemically delivered therapeutics and oxygen is needed for effective radiation therapy[25]. In addition, low oxygen levels affect the function of immune cells in vivo, contributing to the immune privilege of solid tumours[26]. Nevertheless, it is these same hypoxic and/or necrotic regions that provide a critical niche for bacteria to colonize.

There is a long history of observations that suggest natural bacterial infections can result in antitumour effects against malignant tumours. In 1813, Vautier reported that patients with cancer who developed gas gangrene had regressions of their tumours[27]. 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 *Streptococcus* sp. 'group A' or *Streptococcus pyogenes*) as a means of treating cancers[28–30]. Further pursuit of using bacteria to treat cancers was curtailed later on because the focus of attention was diverted to the then novel radiation therapy. However, the enthusiasm for using live bacteria for cancer treatment was revived in the mid-1990s when the scientific community had a better understanding of the TME and recombinant DNA technology enabled the generation of more potent and less toxic bacterial strains[31].

Many bacterial strains have since been tested in animal models and shown preferential targeting of solid tumours, several of which have advanced to clinical trials[31–37]. One successful example is the use of Bacillus Calmette–Guérin (BCG) in the treatment of bladder cancer[38]. BCG is a live attenuated strain of *Mycobacterium tuberculosis* variant *bovis* originally generated as a vaccine for tuberculosis[39]. 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[40–42]. It is believed that the therapeutic effect of BCG is mainly due to its immunomodulatory activity[43–45]. Nevertheless, the clinical development of live bacteria as therapeutic agents faces substantial hurdles mainly because of potential infection-associated toxicities. In this Review, we discuss the unique aspects of live tumour-targeting bacteria as therapeutic agents, focusing on some of the most investigated strains of *Salmonella* spp., *Clostridium* spp. and *Listeria* spp. as examples. As an increasing number of therapeutic bacterial strains have progressed to the clinical stage, we also highlight issues associated with their clinical translation.

Live tumour-targeting bacteria

Intrinsic tumour targeting

Live bacteria target solid tumours using unique mechanisms. When administered systemically, therapeutic bacteria disseminate to both tumour and healthy tissues. Even though *Salmonella* has been shown to preferentially home to or be retained in the TME enriched in certain metabolites[46], the initial amount of bacteria delivered to the tumour is usually not greater than that delivered to the normal tissues[47–51]. However, bacteria in the circulation and other normal tissues are cleared within hours and days, respectively, while those in the tumour continue to proliferate, often to numbers greatly exceeding the colony-forming units initially administered[47–56]. This selective colonization is likely the result of an immunosuppressive and biochemically unique microenvironment caused by pathological changes associated with solid tumours[46,57–60]. Importantly, anaerobic bacteria do not colonize hypoxic or inflammatory lesions unrelated to neoplasia, as shown in experiments with obligate anaerobes and facultative anaerobes, respectively[48,61,62].

Tumour targeting of *Listeria* spp. involves an additional mechanism. *Listeria* spp. are known to infect not only professional antigen-presenting cells (APCs) such as monocytes or macrophages and dendritic cells but also myeloid-derived suppressor cells (MDSCs) that can deliver the bacteria selectively to the TME, where through a unique mechanism they spread from MDSCs into tumour cells[51,55,63]. *Listeria* spp. inside the tumour-infiltrating immunosuppressive MDSC are protected from immune clearance but are rapidly eliminated from normal tissues that lack immune suppression[51,55]. Obligate anaerobic bacteria such as *Clostridium* spp. are unable to survive in the oxygen-rich environment, thus further reinforcing their tumour-targeting specificity. Interestingly, germinated *Clostridium* spp. have also been observed within micro-invasive lesions where necrosis was not evident as well as in the vicinity of neoplastic vessels in rat glioma models[61,64], raising the possibility that these neoplastic structures provide sufficiently hypoxic, biochemically unique and immunoprivileged microenvironments for bacterial colonization. As discussed in more detail below, facultative anaerobic bacteria can be engineered such that their ability to survive in normal tissues will be further diminished.

Tumour suppression by live bacteria

Localized bacterial infection causes tumour regression through various mechanisms (FIG. 1). Bacteria have intrinsic antitumour activities[65–68], but different strains of bacteria or bacteria in different microenvironments may deploy distinct mechanisms to destroy solid tumours. In addition to the intrinsic antitumour effects, bacterial infection induces innate as well as adaptive immune responses against both tumour-colonizing bacteria and the tumour cells[56–58,61,69–74]. The host immune responses are more critical for the antitumour effects of bacteria such as *Salmonella* spp. that are not sufficiently cytotoxic to tumour cells[69,75]. The dominant mechanism is likely to vary depending on the bacterial species used in the therapy, the types of tumour being treated and even the phases of the bacteria–host interaction. Importantly, bacteria can be genetically engineered to further improve their antitumour activities in a variety of different ways, making them a versatile platform to deliver therapeutic payloads on the basis of clinical needs.

Engineered bacteria

Bacteria can be attenuated for safety reasons or engineered to acquire improved antitumour activities. A large collection of engineered bacterial strains has been generated in laboratories around the world for a

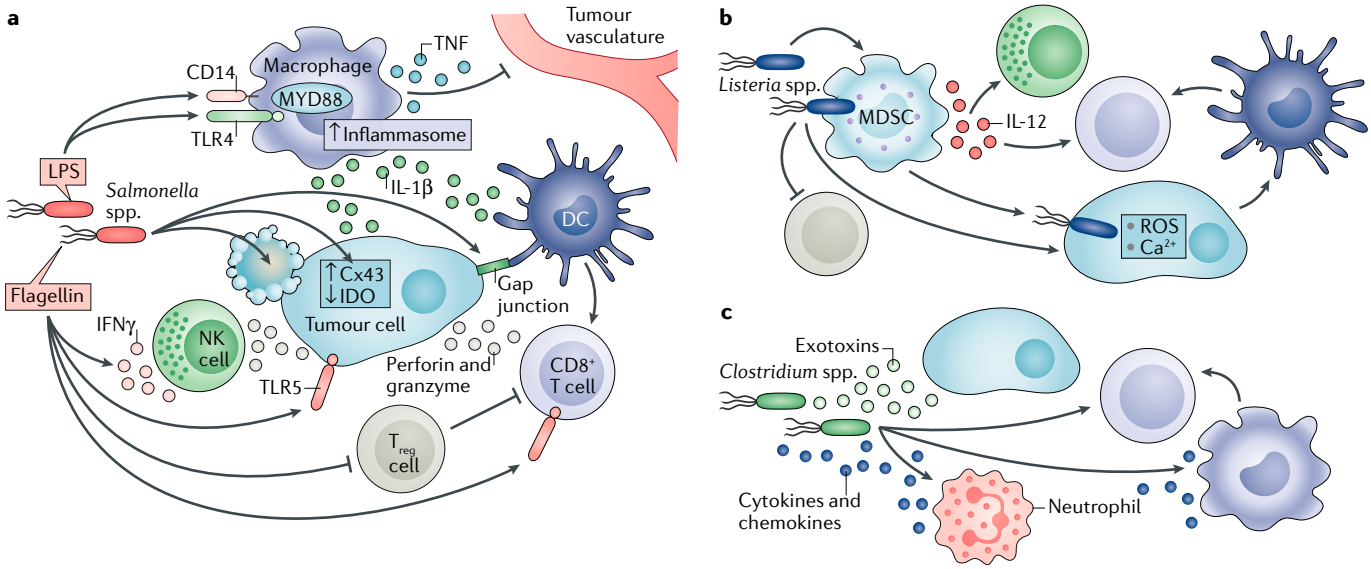

Fig. 1 | **Mechanisms of tumour cell death by tumour-targeting bacteria.** Different bacterial species employ both shared and unique intrinsic mechanisms to eliminate cancer. **a** | Uncontrolled intracellular multiplication of *Salmonella* spp. can lead to bursting of the invaded tumour cells[201]. Alternatively, intracellular bacteria may kill tumour cells by inducing apoptosis or autophagy[201–205]. Macrophages and dendritic cells (DCs) in *Salmonella* spp.-colonized tumours secrete interleukin-1β (IL-1β) that is responsible for the antitumour activity[206]. The elevated IL-1β secretion requires both lipopolysaccharide (LPS)-induced Toll-like receptor 4 (TLR4) signalling and inflammasome activation in macrophages following phagocytosis of *Salmonella* spp.-damaged tumour cells[207]. LPS also elicits tumour necrosis factor (TNF) expression through CD14 (co-receptor for LPS), TLR4 and myeloid differentiation primary response 88 (MYD88)[208,209], leading to disruption of the tumour vasculature[71]. Flagellin, a subunit protein of the bacterial flagellum, improves the $CD8^+$ T cell-dependent antitumour response through activation of TLR5 in a peptide vaccine-based immunotherapeutic setting[210] and decreases the frequency of $CD4^+CD25^+$ regulatory T (T_{reg}) cells[211]. Flagellin can also directly suppress tumour cell proliferation through TLR5 signalling[212]. In addition, an optimized TLR5 agonist derived from *Salmonella* spp. flagellin has been shown to induce a natural killer (NK) cell-mediated antitumour response dependent on perforin[213], and *Salmonella* spp. flagellin can also activate NK cells to produce interferon-γ (IFNγ), a critical cytokine for both innate and adaptive immunity through a TLR-independent pathway involving IL-18 and MYD88 (REF.[214]). *Salmonella* spp. induce upregulation of connexin 43 (Cx43)[215–217], leading to gap junction formation between tumour cells and DCs, which promotes transfer and cross-presentation of processed tumour antigenic peptides[215]. Upregulation of Cx43 in tumour cells also reduces expression of the immunosuppressive enzyme indoleamine 2,3-dioxygenase (IDO)[217]. Both tumour antigen cross-presentation by DCs and decreased IDO further activate $CD8^+$ T cells. **b** | *Listeria* spp. can infect tumour cells directly or with the help of the immunosuppressive myeloid-derived suppressor cells (MDSCs)[55]. Infection of the MDSCs alters a subpopulation of these cells to have an immune-stimulating phenotype characterized by elevated production of IL-12, which is correlated with improved $CD8^+$ T cell and NK cell responses[55]. *Listeria* spp. can directly kill tumour cells through NADPH oxidase-mediated production of reactive oxygen species (ROS) and intracellular calcium mobilization[218]. The immunogenic tumour cell death caused by high levels of ROS activates $CD8^+$ T cells responsible for eliminating both primary tumours and metastases[74,218]. *Listeria* spp. vaccine strains also inhibit MDSCs and T_{reg} cells[219,220]. **c** | *Clostridium* spp. can kill tumour cells through a variety of exotoxins secreted by the colonizing bacteria, some of which (for example, phospholipases, haemolysins and lipases) can damage membrane structures, while others are internalized and interfere with critical cellular functions[65–68]. Similar to infection by other bacterial species, the clostridial infection results in an initial accumulation of granulocytes and macrophages at the infection site[57,69]. This first line of defence prevents the colonizing bacteria from invading into surrounding normal tissues as well as sufficiently perfused and oxygenized tumour regions[58,61]. The cellular response results in elevated cytokines and chemokines that orchestrate a concerted immune response[57,71]. *Clostridium* spp. can also trigger the release of TNF-related apoptosis-inducing ligand (TRAIL) from neutrophils, killing cancer cells through activation of apoptosis[221]. At later time points, adaptive immune cells including $CD8^+$ T lymphocytes are recruited to help eliminate the tumour[57].

Exotoxin
A bacterial toxin secreted into the surroundings.

Gram-negative bacteria
Bacteria including *Salmonella* spp. that are unable to retain the crystal violet stain used in the Gram-staining method for bacterial differentiation owing to only a thin layer of peptidoglycan in their cell walls.

Gram-negative sepsis
A life-threatening complication associated with infection by a Gram-negative bacterium triggering systemic inflammatory responses that can lead to tissue damage and organ failure.

variety of purposes, all of which are 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* was converted to a considerably safer strain (*C. novyi*-NT) by deleting the gene for a lethal exotoxin[76]. Lipopolysaccharide (LPS) in the outer membrane of Gram-negative bacteria is one of the most potent stimulators for the expression of tumour necrosis factor (TNF) and a main inducer of shock in Gram-negative sepsis[77]. Deletion of the *msbB* gene from a *Salmonella* sp. resulted in loss of myristoylation of lipid A, a critical component of LPS, and minimized TNF expression[78]. This modification reduced the toxicity of the *Salmonella* sp. by 10,000-fold. An attenuated strain of *Salmonella enterica* subsp.

Auxotrophic mutation
A mutation that makes an additional nutritional requirement for the growth of the affected bacterium.

Adhesins
Cell surface-exposed bacterial molecules that facilitate adhesion to other cells or surfaces.

Promoter traps
Experimental approaches using reporter activity as the readout to identify particular promoters in a genome by screening libraries constructed with a promoterless reporter gene randomly integrated in the genome or random genomic DNA fragments cloned upstream of a promoterless reporter gene.

enterica serovar Typhimurium named VNP20009 carrying this deletion as well as a purine auxotrophic mutation was isolated and shown to be safe in clinical trials[79,80]. It should be noted that some of the virulence factors may also be responsible for the intrinsic antitumour activity of live bacteria. Whenever possible, attenuation should be achieved without substantially compromising the antitumour activity, unless the bacterial strain is used for the purpose of vaccination only. In this regard, the prototype *msbB*-deficient *Salmonella* strain and the VNP20009 strain retained both tumour-targeting specificity and antitumour activity in the mouse models tested[53,78,81]. *S.* Typhimurium was also made defective in the synthesis of ppGpp, a signalling molecule required for the induced expression of a number of virulence genes[82]. The Δ*p*pGpp 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 while retaining its tumour-targeting capacity[83,84].

Listeria spp. can also serve as tumour-targeting vectors to deliver therapeutic payloads[51,56] but have been used mainly as vaccine strains expressing tumour antigens (reviewed elsewhere[85–87]). *Listeria monocytogenes* has been made safer by deleting *prfA*, the master virulence regulator gene[63]. However, *prfA*-deficient *Listeria* spp. cannot escape from the phagosome into the cytosol of the infected cells, which would prevent the tumour 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 tumour antigens, the *prfA*-deficient strains were engineered to express low levels of PrfA and truncated immunogenic listeriolysin O (LLO) that can be fused with the antigens of choice for improved immunogenicity[87,88]. These strains are referred to as *Lm*-LLO and have been used not only as vaccine strains but also for tumour-targeted delivery of non-vaccine therapeutic payloads[51,56,89]. Attenuation of *L. monocytogenes* can also be achieved by deleting the virulence genes *actA* and *inlB*, which are responsible for bacterial dissemination[90], creating strains known as live attenuated double deleted (LADD)[90,91].

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 and is auxotrophic for leucine and arginine that are likely enriched in the tumour but not in normal tissues[92]. This strain, without further engineering, has shown selective tumour colonization as well as potent antitumour activity in a variety of mouse tumour models[37]. Auxotrophic strains of *L. monocytogenes* have also been generated by insertional inactivation of the *dal* and *dat* genes required for the biosynthesis of D-alanine, a critical component in bacterial cell walls[93]. These attenuated *Listeria* strains can grow in vitro when exogenous D-alanine is provided, but unlike the *Salmonella* A1-R strain, they cannot replicate and spread in vivo. Therefore, they are desirable as vaccine vectors but may not be optimal for tumour-targeted delivery of non-vaccine antitumour payloads.

Increasing tumour targeting

Nonpathogenic or attenuated obligate anaerobes have moderately high tumour specificity, thus resulting in minimal direct cytotoxicity to normal tissues[48,52,94,95]. By contrast, facultative anaerobes such as *Salmonella* spp. and *Listeria* spp. can survive and even proliferate in an oxygenated environment, causing damage to the normal tissues. For facultative anaerobes, improved tumour targeting could reduce their toxicity or increase their efficacy without increasing toxicity. The αvβ3 integrin is overexpressed in multiple cancer types[96]. An *S.* Typhimurium (Δ*p*pGpp) strain displaying an integrin-binding Arg–Gly–Asp (RGD) peptide on its outer membrane protein A (OmpA) showed a >1,000-fold enrichment in αvβ3 integrin-expressing glioma U87MG and melanoma M21 xenografts in mice compared with the control strain and an impressively improved antitumour activity in the αvβ3-positive breast cancer MDA-MB-231 and melanoma MDA-MB-435 xenograft tumour models[97]. Bacteria have been engineered to target TAAs as well. Surface display of antibody fragments against the colorectal cancer-associated carcinoembryonic antigen (CEA) or the lymphoma-associated antigen CD20 made the engineered *S.* Typhimurium strains more effective in suppressing experimental tumours expressing these antigens[98,99]. Importantly, the anti-CD20 strain showed substantially reduced intracellular accumulation in the liver and spleen of the treated mice while maintaining tumour accumulation[99]. Bacteria can also serve as a platform to display modular synthetic adhesins, where different adhesins can be chosen for targeting tumours expressing their specific ligands[100].

Expression systems inducible by tumour-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 tumour-associated factors, novel promoter elements activated in the TME 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 enables 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[101]. Temporally controlled payload expression, once a robust colonization has been established, may be a good approach to address this problem.

Effector systems

Attenuated bacteria alone often cannot eradicate solid tumours. Delivery of therapeutic payloads by tumour-targeting bacteria to augment their efficacy was first described in the mid-1990s[47,102–104]. Various effector systems have since been explored (TABLE 1). Here, we briefly describe different strategies for payload delivery and effector systems categorized on the basis of their antitumour mechanisms.

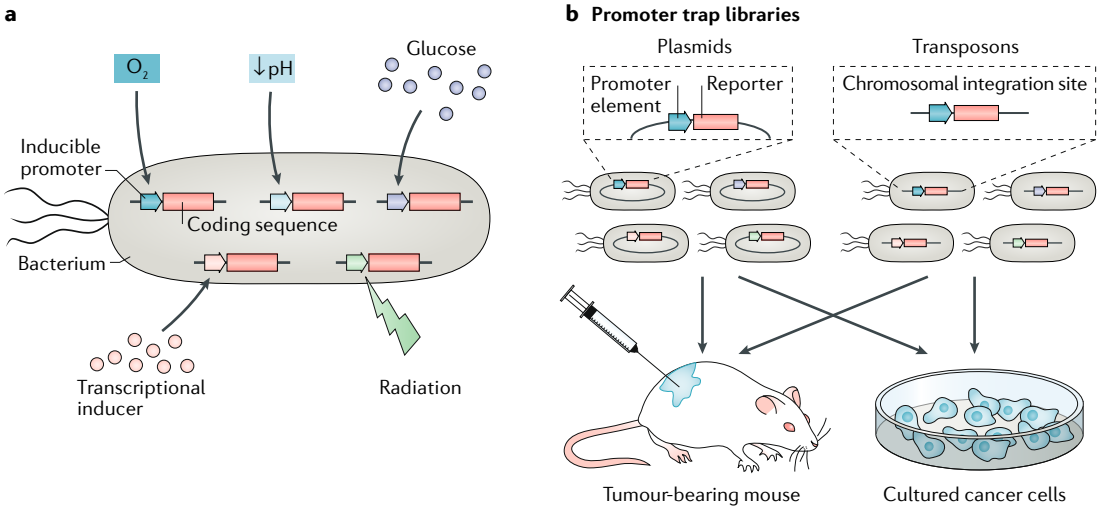

Fig. 2 | **Inducible systems used for targeted colonization and payload expression. a** | Various inducible promoters can be used for either tumour-selective expression or temporally or spatially controlled expression. A *Salmonella* strain has been engineered such that an essential gene is placed under the control of a hypoxia-inducible promoter, while expression of an inhibitory antisense RNA against this gene is activated by an oxygen-inducible promoter to minimize basal expression in oxygenated normal tissues[222]. This strain showed robust tumour colonization and greatly increased clearance from normal tissues, thus resulting in a substantially improved safety profile compared with 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[114]. Promoter elements responsive to low pH were among those identified to be active in the tumour microenvironment (TME) in studies using promoter traps (see below)[223]. A genetic circuit that can be triggered by glucose gradients often present in solid tumours has also been used to engineer bacteria[224], potentially enabling them to express antitumour proteins in metabolically more active tumour regions. Exogenously applied transcriptional inducers such as L-arabinose, acetyl salicylic acid and tetracyclines can tightly regulate the relevant inducible promoters introduced into bacteria[84,107,109,116,117,225–227], providing a means to control colonization or the expression of effector genes in a temporal fashion. Ionic radiation at as low as 2 Gy has also been shown to activate the *recA* promoter on a plasmid transfected into *Clostridium* spp.[228–230], raising the possibility of regulating 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 tumours). **b** | Promoter traps have been employed to identify promoter elements active in the TME[223,231,232]. Promoter trap libraries can be constructed by transforming bacteria with either plasmids containing random genomic DNA fragments cloned upstream of a promoterless reporter gene or transposons containing a promoterless reporter gene that integrate randomly into the bacterial genome. These promoter trap libraries can be either injected into experimental tumours or co-cultured with cancer cells. The bacteria are then recovered and analysed for reporter activities. Clones with high reporter activities are likely to contain promoter elements active in the TME.

Different strategies for payload delivery. The therapeutic payload can be delivered in the form of DNA, RNA or protein depending on its 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 then need to be secreted from the bacteria to achieve their biological effects[105,106]. Alternatively, the vector strains can be engineered such that bacterial lysis is induced for the release of therapeutic payload once a robust tumour colonization has been established[107–109].

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[110–112]. Biological activities of mammalian proteins often depend on correct folding and post-translational 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. In a study using *S.* Typhimurium as a delivery vehicle, β-galactosidase expressed from a eukaryotic cassette induced substantially stronger immune responses than that expressed from a prokaryotic cassette[113]. It should also be noted that proteins produced by intracellular bacteria and those produced by host cells may be targeted to different cellular compartments. A special category of therapeutic bacteria are DNA vaccine strains designed to deliver DNA to APCs[86]. Vaccine strains delivering either DNA or protein are discussed in detail elsewhere[86]. Lastly, short 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 improve the antitumour activity would be to engineer bacterial vectors expressing cytotoxic agents. This strategy requires the bacterial vectors to target tumours with sufficient specificity or the use of inducible promoters for better control of gene expression to avoid toxicity

Table 1 | **Effector systems**

Effector classes	Effectors or targets	Refs
Cytotoxic	Bacterial toxins and immunotoxins[a] (for example, cytolysin A, *Staphylococcus aureus* α-haemolysin, PE, TGFα–PE and TGFα–PE38)	84,114–119,233
	Apoptosis-inducing ligands (for example, TNF, FASL, TRAIL, azurin, Cp53, apoptin and Noxa MTD)	107,109,123–127,234–236
	Agents loaded into or onto bacteria (for example, 188-rhenium, 32-phosphorus, doxorubicin and C_3N_4)	51,56,128,129
Prodrug-converting enzymes	Thymidine kinase, cytosine deaminase, nitroreductase, purine nucleoside phosphorylase, carboxypeptidase G2 and chromate reductase YieF	47,131,132,168,237–242
Immunomodulators	Tumour antigens	85–87,183,243–245
	Cytokines and chemokines (for example, IL-2, IL-4, IL-12, IL-18, IFNγ, GM-CSF, FLT3L, LIGHT and CCL21)	104,137,138,246–253
	Others (for example, heterologous flagellin, α-galactosylceramide and immunodominant recall antigens)	89,139
Tumour stroma targeting	Legumain, VEGFR2, endoglin, thrombospondin 1, TEM8 and PDGFRβ	111,148,150,152–155,254–256
Gene silencing	Silenced targets: IDO, STAT3, BCL-2, MDM2, survivin and MDR1	156,257–264
Synthetic gene circuit	Quorum-sensing gene circuit for controlled payload production	108,265

C_3N_4, carbon nitride; CCL21, C-C motif chemokine ligand 21; endoglin, also known as CD105; FASL, FAS ligand; FLT3L, FMS-related tyrosine kinase 3 ligand; GM-CSF, granulocyte–macrophage colony-stimulating factor; IDO, indoleamine 2,3-dioxygenase; IFNγ, interferon-γ; IL, interleukin; LIGHT, also known as TNFSF14; MDR1, multidrug resistance protein 1; Noxa MTD, Noxa mitochondrial-targeting domain; PDGFRβ, platelet-derived growth factor receptor-β; PE, *Pseudomonas* spp. exotoxin A; PE38, a 38 kDa truncated form of PE; STAT3, signal transducer and activator of transcription 3; TEM8, tumour endothelial marker 8; TGFα, tumour growth factor-α; TNF, tumour necrosis factor; TRAIL, TNF-related apoptosis-inducing ligand; VEGFR2, vascular endothelial growth factor receptor 2. [a]Immunotoxins are toxins linked to an antibody or a ligand that binds specifically to target cells.

to normal tissues. Several bacterial strains have been engineered to express the potent pore-forming bacterial toxin cytolysin A or *Staphylococcus aureus* α-haemolysin under the control of promoters activated by hypoxia[114], L-arabinose[84,115,116] or tetracyclines[117] to ensure safety. An alternative method to increase safety involved expression of chimeric toxic proteins selectively targeted to the tumour cells. For instance, *S.* Typhimurium strains have been engineered to express chimeric proteins comprising tumour growth factor-α (TGFα), an epidermal growth factor receptor (EGFR) ligand and truncated forms of *Pseudomonas* spp. exotoxin A (PE) lacking the native membrane-binding domain[118,119]. Upon release from the bacteria, the chimeric proteins selectively killed EGFR-positive cancer cells and retarded the growth of EGFR-expressing tumours in multiple mouse tumour models[118,119].

Induction of tumour cell apoptosis is an attractive therapeutic approach, but systemic administration of apoptosis-inducing ligands such as TNF, FAS ligand (FASL; also known as TNFSF6) and TNF-related apoptosis-inducing ligand (TRAIL; also known as TNFSF10) is not feasible because of their toxicity or short circulating half-lives[120–122]. To achieve sustained high levels of these proteins in the TME while avoiding systemic toxicity, several groups have engineered bacterial strains for their tumour-targeted delivery[123–127]. An attenuated *S.* Typhimurium strain expressing FASL showed substantial antitumour activities against both subcutaneous and metastatic syngeneic mouse tumour models in a FAS-dependent fashion[125]. In another elegant example, two separate inducible systems were used to drive the expression of the proapoptotic Cp53 peptide derived from the p53 protein and lysis of a *S.* Typhimurium strain to release Cp53 for maximal killing[107].

In addition to genetic engineering for expressing cytotoxic proteins, tumour-targeting bacteria have been used to deliver cytotoxic agents that exert a greater bystander effect on the surrounding uninfected tumour cells[51,56,128,129]. In one study, the high-energy beta emitter 188-rhenium was conjugated to a polyclonal antibody against *Listeria* spp. followed by incubation of the radiolabelled antibody with an attenuated *L. monocytogenes* strain[51]. The resulting radioactive *Listeria* sp. accumulated in metastases after systemic administration and reduced the number of metastases by 90% in a syngeneic Panc02 mouse tumour model. Attenuated *L. monocytogenes* has also been metabolically labelled with 32-phosphorus and shown tumour-suppressive activity in the transgenic KPC mouse model of pancreatic ductal adenocarcinoma (PDAC) (driven by conditional expression of oncogenic *Kras* and mutant *Trp53*)[56]. Another innovative approach capitalized on the ability of some bacteria to generate cytotoxic nitric oxide (NO) from NO_3^- (REF.[129]). Upon photo-irradiation, photoelectrons were excited from the carbon-dot-doped carbon nitride (C_3N_4) loaded onto the surface of *Escherichia coli* and transferred to *E. coli* NO-generating enzymes, resulting in substantially increased production of NO and tumour suppression in mouse syngeneic subcutaneous tumour models. Importantly, the focused photo-irradiation enabled targeted generation of NO.

Prodrug-converting enzymes. Prodrug-converting enzymes were among the first effector systems engineered into tumour-targeting bacteria[47,102,103]. Once expressed by the tumour-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 tumour and across the cell membrane, thus generating a potent bystander effect. Tumour-targeting bacteria have been engineered to express several prodrug-converting enzymes (TABLE 1). Cytosine deaminase converts the non-toxic 5-fluorocytosine (5-FC) to 5-fluorouracil (5-FU), a first-line chemotherapeutic agent for metastatic colorectal cancer[130]. The *S.* Typhimurium VNP20009 strain engineered to express *E. coli* cytosine deaminase showed clearly improved antitumour activity when combined with 5-FC in both mouse syngeneic and human xenograft colorectal tumour models[131].

Similarly, a *Clostridium sporogenes* strain expressing *Haemophilus influenzae* nitroreductase had promising

Bystander effect
In this context, a therapeutic effect on cells that are not infected by bacteria.

antitumour effects as well[132]. Nitroreductase catalyses the conversion of the weak monofunctional DNA-alkylating agent CB1954 into a bifunctional DNA-alkylating derivative that can induce DNA crosslinks and apoptosis[133,134]. Repeated administration of the nitroreductase-expressing strain along with CB1954 achieved sustained tumour control in a subcutaneous human HCT116 colon cancer xenograft model[132]. The efficacy of this effector system depends on robust and sustained tumour colonization by the delivering bacterial vector, which ensures continued high-level expression of the prodrug-converting enzyme[132]. It is worth noting that bacteria also carry endogenous enzymes capable of metabolically activating multiple prodrugs[135,136].

Immunomodulators. To further stimulate antitumour immunity, tumour-targeting bacteria have been engineered to express either tumour antigens or immunoregulatory factors. In addition to vaccination with live bacteria expressing tumour antigens (reviewed elsewhere[85–87]), another approach to augment tumour immunogenicity could involve presenting the immunodominant T cell antigens from tetanus toxoid, poliovirus or measles virus on the surface of tumour cells infected by intracellular tumour-targeting bacteria carrying expression cassettes for these antigens. The immune system in most individuals will have seen these antigens earlier during childhood vaccinations and thus will have generated memory T cells. These T cells can be reactivated upon encountering these antigens again, resulting in killing of the infected tumour cells. Antigen spreading from the dying tumour cells may also take place to induce an immune response against the uninfected tumour cells.

Engineered tumour-targeting bacteria have the ability to bring immunomodulatory proteins to the TME to boost antitumour immunity, and there are several examples of engineered bacterial strains that can achieve this (TABLE 1). For example, an *S.* Typhimurium strain expressing biologically active interleukin-2 (IL-2), generated >20 years ago, reduced hepatic metastases more significantly than a sister strain not expressing IL-2 in a syngeneic intrasplenic tumour model using the MC-38 colon adenocarcinoma cell line[104]. This antitumour activity was shown to depend on natural killer (NK) cells and $CD8^+$ T cells[137]. The IL-2-expressing *Salmonella* strain has also been tested in both canine and human clinical trials (discussed in more detail below). Bacterial strains expressing other cytokines have been generated as well (TABLE 1).

In addition to the classic cytokines and chemokines, other proteins with immunomodulatory activities have been documented to have promising therapeutic effects when delivered by tumour-targeting bacteria. For instance, an attenuated *S.* Typhimurium strain engineered to express LIGHT (also known as TNFSF14), a member of the TNF superfamily, showed considerable antitumour activities in subcutaneous as well as metastatic mouse tumour models[138]. These antitumour activities required both $CD4^+$ and $CD8^+$ T cells. Mobilization of natural killer T cells (NKT cells) could also improve bacterial antitumour activity[89]. In an interesting study with the syngeneic 4T1 breast tumour mouse model, α-galactosylceramide, a glycolipid that can activate NKT cells, was incorporated metabolically into *L. monocytogenes* and shown to help eliminate metastases and improve survival[89]. In a more recent study, heterologous flagellin was employed as a potent immunoregulator[139]; the *S.* Typhimurium ΔppGpp strain was engineered to secrete *Vibrio vulnificus* flagellin B and displayed a markedly improved ability to control tumour growth compared with the parental strain. Further experiments to address the mechanism showed that infection with the *Salmonella* strain activated the Toll-like receptor 4 (TLR4)–myeloid differentiation primary response 88 (MYD88) pathway, presumably through LPS present in the outer membrane of Gram-negative bacteria, resulting in a massive tumour infiltration of macrophages and neutrophils. Secreted heterologous flagellin triggered the TLR5 pathway and further shifted the tumour-infiltrating macrophages towards an M1 phenotype, which was associated with increased levels of tumoricidal mediators including IL-1β, TNF and NO compared with a sister strain not expressing the heterologous flagellin[139].

Recent clinical success with immune checkpoint blockade has prompted a wave of preclinical and clinical studies combining immune checkpoint inhibitory antibodies with therapeutic bacteria or viruses[21,140,141]. These studies tested the hypothesis that intratumoural infection by live microorganisms could establish a more immunogenic microenvironment, thus sensitizing the tumours to immune checkpoint blockade. A more straightforward approach would be to generate bacterial strains secreting immune checkpoint inhibitors such as a programmed cell death protein 1 (PD1)-neutralizing antibody or a soluble PD1 extracellular domain to bind and neutralize the T cell-inhibiting PD1 ligand 1 (PDL1) expressed by tumour cells. This approach is technically possible, as functional single-chain antibodies have been produced from tumour-targeting bacterial strains[142]. These inhibitors can also be expressed by infected tumour cells when intracellular bacteria carrying expression cassettes with mammalian gene promoters and secretory signals are used. As the expression of the immune checkpoint inhibitors is targeted to tumours, this strategy will not activate T cells in normal tissues, thus potentially minimizing toxicity associated with systemic immune checkpoint blockade[143].

Targeting tumour stroma. Tumour cells can evade the immune system by downregulating the expression of tumour antigens as well as proteins involved in antigen processing and cell surface presentation[144]. To circumvent this problem, alternative targeting of the tumour vasculature required for tumour growth may be particularly beneficial for bacterial therapy. As discussed earlier, bacteria preferentially colonize necrotic and/or hypoxic tumour areas. Disruption of tumour vasculature with microtubule-destabilizing agents leads to tumour cell death in the otherwise well-perfused tumour regions and expands bacterial colonization[145–147]. Moreover, bacteria themselves can be engineered to induce disruption of tumour vasculature. Several vaccine strains against critical components of angiogenic tumour vessels have been generated and tested in both

Antigen spreading
Also known as epitope spreading; the expansion of an immune response to antigens that are not the original antigen targeted in the therapy.

Natural killer T cells
(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.

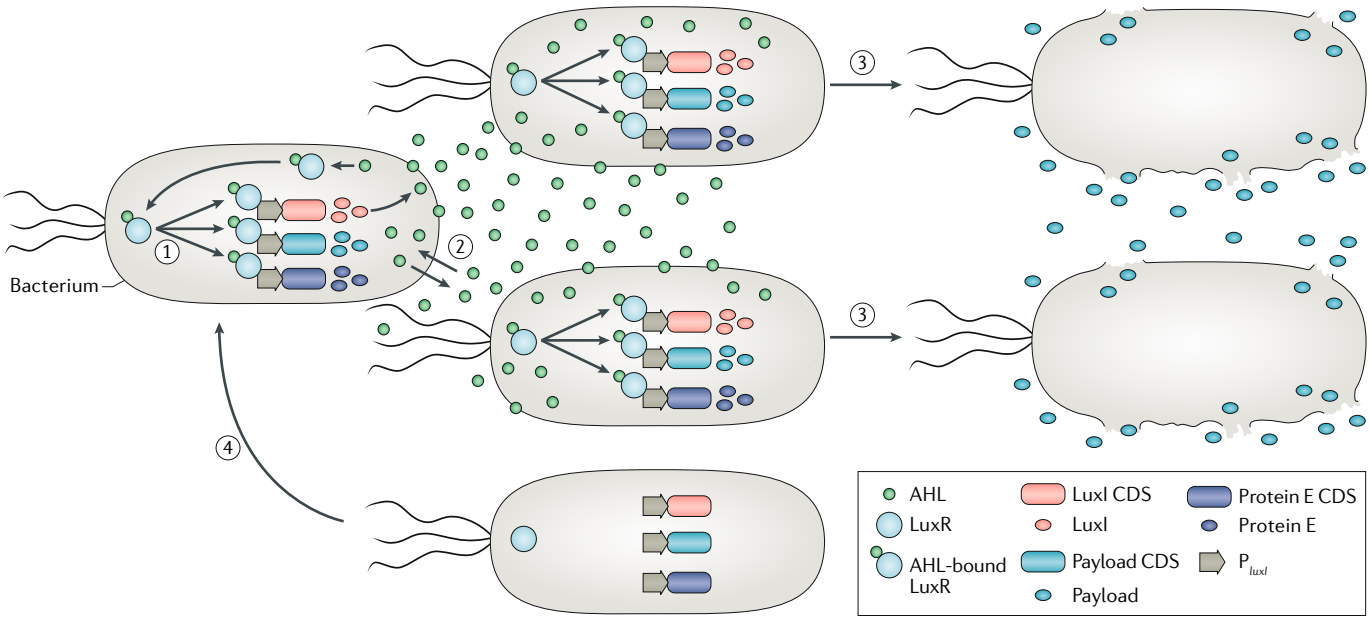

Fig. 3 | **A gene circuit for a transcriptional programme regulating bacterial activities at the population level.** Illustrated is an example of a sophisticated quorum-sensing gene circuit for a transcriptional programme enabling synchronized bacterial population control and therapeutic payload release in repeated cycles[108]. (1) The acyl-homoserine lactone (AHL)-bound transcription factor LuxR interacts with and activates the promoter P_{luxI} that drives the expression of the AHL synthase LuxI to establish a positive feedback loop, the therapeutic payload and the bacteriophage ϕX174 protein E to lyse the bacteria. (2) The AHL signalling molecules can diffuse freely across bacterial cell membranes (indicated by the arrows), enabling synchronization of neighbouring bacteria within the population for a concerted action. At low densities of the bacterial population, AHL molecules diffuse predominately out of bacteria, leaving the gene circuit inactive. An increased bacterial population density enables 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 programme leads to simultaneous lysis of the bacteria within the population by protein E as well as a burst of therapeutic payload release. (4) The few bacteria surviving the lysis repopulate and kick off another cycle of lysis and payload release. CDS, coding sequence.

prophylactic and therapeutic settings[148–155] (TABLE 1). For example, an attenuated *S.* Typhimurium 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-mediated immunity against this self-antigen expressed on proliferating endothelial cells, leading to effective protection against tumour challenge[148]. Another study with an *L. monocytogenes* vaccine strain further suggested that the antitumour activity induced by VEGFR2-based vaccines is dependent on epitope spreading to a tumour antigen[155]. Other stromal components may be targeted as well. For example, intravenous administration of a recombinant hyaluronidase improved colonization and the antitumour effect of an *S.* Typhimurium strain expressing indoleamine 2,3-dioxygenase (IDO)-targeting shRNA[156]. Thus, it is plausible that for some bacterial strains unable to disperse within solid tumours, expression of heterologous enzymes capable of degrading extracellular matrix may help improve their colonization and efficacy. However, this is at present only a hypothesis.

Synthetic gene networks. Both viruses and bacteria can be reprogrammed by genetic engineering, but bacteria can host heterologous DNA of considerably large sizes[157], allowing for more sophisticated reprogramming. 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[158,159]. Therefore, bacteria have been dubbed 'programmable robotic factories' at the microscopic scale[31]. Applying engineering concepts (in particular, those in electrical engineering), investigators have assembled biomolecular modules in bacteria to build genetic networks that can execute logical operations. Typical *cis* (for example, promoters and enhancers) and *trans* (for example, transcription factors and repressors) gene regulatory elements are employed and arranged in unique ways to form feedback and feedforward loops with which the biological equivalents of electronic devices such as toggle switches, oscillators and other sophisticated devices can be fabricated[160–162]. An elegant design using the quorum-sensing elements from *Aliivibrio fischeri* and *Bacillus thuringiensis* arranged to form negative feedback motifs enabled synchronized oscillations of gene expression in a growing population of bacterial cells[163]. In a subsequent study, this quorum-sensing gene circuit was modified to generate synchronized cyclical bacterial population control and anticancer drug delivery as the outputs[108] (FIG. 3). More specifically, once inside the tumour, the tumour-targeting *S.* Typhimurium with this gene circuit underwent repeated cycles of population expansion and

Quorum sensing
A bacterial cell–cell communication process that regulates gene expression in response to fluctuations in population density.

regression by bacterial lysis in response to the density of bacterial cells. The lysis of the cells directly released the anticancer drug made by the bacteria[108]. Thus, this gene circuit provided maximal release of the therapeutic payload through synchronized cell lysis as well as increased safety by maintaining the intratumoural bacterial population at a defined size, consequently minimizing the risk of a potentially lethal systemic inflammatory response. This example illustrates the potential of gene networks to coordinate the behaviour of bacteria at the population level in response to a particular environmental cue for an increased therapeutic index.

Clinical translation

Preclinical animal study is a critical step towards clinical development of tumour-targeting bacteria. The number of published preclinical studies on bacterial cancer therapy has increased exponentially in recent years, and many of these studies have shown promising results in experimental models[31]. Colonization of tumour-targeting bacteria and subsequent antitumour activity can vary substantially among different preclinical models because of the unique TME associated with particular tumour models. In addition to tumour histology, the method used to establish a tumour model can make a substantial difference[164]. For instance, an attenuated *L. monocytogenes* strain was able to colonize both subcutaneously transplanted Panc02 pancreatic tumours and spontaneously occurring tumours in the genetically engineered KPC mouse model of PDAC with comparable efficiencies[56]. However, the 4T1 mammary tumours transplanted subcutaneously into 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 tumours spontaneously developed in transgenic BALB-neuT mice[147]. Interestingly, pretreatment with a vasculature-disrupting agent, which was shown to induce tumour necrosis, drastically improved bacterial colonization of tumours in the autochthonous model. This example underscores the importance of identifying and employing the most appropriate preclinical tumour models for assessment of both efficacy and toxicity that are truly relevant to human patients with cancer. Perhaps a rational and hierarchical approach involving a variety of tumour models will help maximize the chance for the successful clinical development of a tumour-targeting bacterium-based therapeutic product[164].

Despite the rapidly increasing number of published preclinical studies, very few tumour-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 generally 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 requires 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 considerations that need to be addressed before a replication-competent bacterium can be used in humans (BOX 1).

Challenges

Given the unique nature of live engineered bacteria as therapeutic agents, several important issues in clinical translation need to be considered. First, live genetically modified bacteria that carry antibiotic resistance genes or mobile genetic elements such as plasmids that can mediate horizontal gene transfer are generally not appropriate for clinical studies[165]. Chromosomal integration of the expression cassette without antibiotic selection markers provides a safer and more stable way of engineering[166–168]. 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 generating good manufacturing practices (GMP)-grade test articles. In addition, the conventional aerobic and anaerobic culture methods for sterility testing may not be feasible. Thus, performing production and purification in dedicated clean rooms following strict aseptic protocols with frequent in-process monitoring is the most practical way to ensure axenicity. Although the final products cannot be demonstrated to be sterile, they should be assayed to be free from causative agents of diseases or pathological conditions, such as invasive bacterial pathogens listed by the US Centers for Disease Control and Prevention (CDC)[169] and specific pathogens described in the US Pharmacopeia publication (chapter 62)[170], 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 tumour necrosis and/or hypoxia and the abundance of pre-existing tumour-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 and/or necrosis imaging may help define the patient population that would benefit the most from bacterial therapy[171–173]. Additionally, germination and spreading of bacteria may be monitored directly by imaging the replicating bacteria[174–176]. Fourth, when a live biological agent is used in a clinical setting, its potential impact on public health and the environment is always a concern and should be properly addressed.

There is an additional challenge for using oncolytic bacteria. Therapy with oncolytic bacteria is a deliberate attempt to convert a tumour into a localized tumour-destroying infection, which may have serious consequences if not managed properly[48,64,177,178]. As both therapeutic and toxic effects result from a robust infection, a carefully calculated balance is critical. Practically, this is difficult to accomplish, because an antibiotic intervention to prevent or limit the toxicity too early would

Horizontal gene transfer
The transmission of genetic material between different organisms.

Axenicity
The state of a pure culture of microorganisms, entirely free of all other contaminating organisms.

Angiography
A procedure that uses radiography to examine blood vessels.

Box 1 | **Regulatory considerations for clinical investigations of live tumour-targeting bacteria**

Distinct from conventional cancer treatments such as chemotherapies, targeted therapies or monoclonal antibody therapy, live tumour-targeting bacteria have unique regulatory challenges. A detailed description of regulatory considerations and requirements is beyond the scope of this article. Listed below are some points for the sponsors (both academic and commercial) 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
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 and 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 vectors 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 products previously administered to humans, adequate information regarding the manufacturing and characterization of the products 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.

Chemistry manufacturing and controls (CMC)
The process of manufacturing live therapeutic bacteria is vastly more complex than that of small molecules and must take into account several aspects: the most optimal bacterial seed stock and the banking system and the reagents used; the procedures in producing, purifying and harvesting live bacteria; and the type of formulation of the final product and the tests for identity, purity and potency, which face unique challenges for live bacteria as final products (also discussed in the main text).

Pharmacokinetics and dose–response
Live bacterial products do not follow typical patterns of pharmacokinetics and the dose–responses of conventional small molecules, thus posing challenges in determining the optimal starting dose and schedule for administration (also discussed in the main text).

Safety concerns
Safety is the major concern owing 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 relevant infection or sepsis, especially in an immunocompromised host. Administration of antibiotics after 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 trial design would need to consider appropriate plans to mitigate these concerns.

Study population and study design
Discussed in the main text.

Relevant US Food and Drug Administration guidance documents
The guidance document *Recommendations for Microbial Vectors used for Gene Therapy* (September 2016)[199] focuses on the CMC information that investigational new drug application (IND) sponsors should submit in an IND for MVGT 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. Another guidance document, *Preclinical Assessment of Investigational Cellular and Gene Therapy Products* (November 2013)[200], 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.

effectively eliminate the infection before an antitumour effect has been achieved, whereas a late intervention bears the risk of an unpredictable systemic inflammatory response. Successful control 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 intratumoural infection has been established should be inter-disciplinary team decisions.

Study population
In general, for first-in-human 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 in the trials. For clinical trials of live bacterial products, there are additional factors that may influence which patients should be recruited.

The underlying condition of the patients with cancer might make them immunocompromised. They also may need to receive concomitant therapies to control their disease, and some of these therapies (for example, chemotherapy) can be immunosuppressive. To these patients, administering a live bacterial product may pose a substantial risk of infection beyond the target tumour. Thus, in designing a first-in-human trial, the immune status of the patients and their prior treatments or current concomitant therapies need to be considered. Patients who are immunocompromised may be excluded.

Certain patient conditions may particularly predispose patients to developing off-target infections because of the intrinsic properties of the administered live bacterial products. For example, bacteria in general, and anaerobic bacteria, in particular, preferentially proliferate in necrotic tissues. Conditions such as brain abscesses, diverticulitis or recent radiation treatment might promote the unintentional growth of these

Diverticulitis
Inflammation or infection of the small pouches called diverticula formed in the lining of the digestive system.

bacteria in non-target lesions, even though preclinical studies have shown that certain bacterial strains may not be able to colonize the non-malignant lesions[48,61,62]. In addition, some live bacterial products have the potential to colonize foreign bodies such as artificial heart valves, joint replacements or implanted medical devices that may serve as reservoirs for these live products. Therefore, excluding patients with these conditions reduces the risk associated with these products.

Clinical experience

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

Canine studies. Tumours that develop spontaneously in companion dogs serve as an attractive model for human cancers[179,180]. These tumours more closely resemble their human counterparts than preclinical mouse models — originating from cells harbouring naturally occurring mutations in hosts with heterogeneous genetic backgrounds. A few canine studies for tumour-targeting bacteria have been reported (TABLE 2). In one study, the *S.* Typhimurium strain VNP20009 was given by intravenous infusion to 41 client-owned dogs with spontaneous tumours[181]. Complete and partial tumour responses were observed in 15% of the treated animals. Positive bacterial culture was obtained from tumour tissue in 42% of the cases; however, this did not correlate with the administered doses. In another study, intratumoural injection of *C. novyi*-NT spores resulted in objective responses of target lesions in ~38% of 16 evaluable companion dogs[64]. Intriguingly, the objective response rate among the dogs with peripheral nerve sheath tumours was higher, at ~57%. The numbers of dogs used in the trial were likely too small to achieve statistical significance, but these findings should prompt further investigations to identify cancer types particularly sensitive to bacterial therapy.

Tumour-targeting bacteria delivering therapeutic payloads have also been tested in canine patients. The *Salmonella* strain engineered to express IL-2 given in a neoadjuvant and adjuvant setting was combined with amputation and adjuvant doxorubicin to treat canine appendicular osteosarcoma[104,182]. The dogs in this study showed a significantly longer disease-free interval than historical controls treated with amputation and adjuvant doxorubicin alone but not than controls treated with amputation plus carboplatin and doxorubicin.

Human studies. Several *Listeria* vaccine strains have been tested in clinical trials, and some have shown very encouraging results[87,183,184] (Supplementary Table 1). In comparison, human trials with tumour-targeting strains have been scarce. In addition to the historical human studies with live oncolytic bacteria[28–30,177,178], in more recent years, some human clinical trials with tumour-targeting bacteria have been reported, and a few more have been registered with the US federal regulatory authorities[64,79,80,141,167,185–192] (TABLE 2) (a search of the websites EU Clinical Trials Register and UK Clinical Trials Gateway using relevant keywords did not return any result on trials with *Clostridium*, *Salmonella*, *Listeria*, *Bifidobacterium*, *Lactobacillus* or *Escherichia* as cancer therapeutic agents).

Historical studies with the oncolytic M-55 strain of *Clostridium butyricum* (later reclassified as *C. sporogenes* ATCC 13732) have documented robust tumour colonization and tumour lysis in different cancer types[177,178,193]. Similarly, clinical signs of tumour colonization (for example, signs of inflammation at the target tumour including pain, erythema, swelling and spontaneous drainage, systemic signs of infection including fever, and laboratory findings) have been observed in a large fraction of patients treated with either intravenous or intratumoural administration of *C. novyi*-NT spores in more recent phase I trials[187,190,192]. Objective evidence of tumour response has also been shown in these trials. For example, extensive tumour destruction with gas pockets, a signature of infection of the gas-forming *Clostridium* spp., was observed by computed tomography scan in a patient who received direct injection of *C. novyi*-NT spores into a metastatic shoulder lesion of retroperitoneal leiomyosarcoma[64,192]. Biopsies of the lesion revealed extensive tumour necrosis and absence of viable tumour cells. Anaerobic culture of the biopsied material was positive for *C. novyi*-NT, suggesting its involvement in the tumour destruction[64]. However, these treatments with oncolytic bacteria alone failed to eradicate all cancer cells, which inevitably led to progression or relapse[64,177,178,193].

Attenuated *Salmonella* strains and their derivatives engineered to express therapeutic payloads have also been tested in early clinical trials[79,80,167,185,186,188,189] (TABLE 2). Similar to the oncolytic *Clostridium* strains, *Salmonella* strains are reasonably tolerated in patients with cancer. Unexpectedly, the *Salmonella* strains tested so far have yet to show the robust tumour colonization and therapeutic benefit repeatedly observed in preclinical studies. The reason for this discrepancy is unclear, but differences between murine models and patients in terms of tumour accessibility, intratumoural growth, or clearance of bacteria have been proposed as possible reasons[79]. It is worth noting that intratumoural-injected TAPET-CD, an *S.* Typhimurium VNP20009 strain expressing *E. coli* cytosine deaminase, was able to colonize the target tumours and convert 5-FC to 5-FU inside the colonized lesions, resulting in a 3:1 tumour-to-plasma ratio of 5-FU[167] (TABLE 2). This study demonstrated that bacteria colonizing human tumours can express substantial amounts of functional enzymes.

Although small in number, these early human trials have already taught us a few important lessons. First, attenuated tumour-targeting bacteria are reasonably tolerated in human patients. Second, toxicities observed in human patients are very similar to those seen in experimental animals. Third, robust colonization is a prerequisite for significant clinical benefit. Future clinical studies may employ companion diagnostic approaches based on angiography and hypoxia and/or necrosis imaging to define a patient population potentially more sensitive to intratumoural bacterial colonization[171–173].

Table 2 | **Recent and ongoing clinical trials with engineered tumour-targeting bacteria**

Trial species	Bacterial strain	Cancer type	Treatment and outcome	Study status and refs
Canine	*S.* Typhimurium VNP20009	41 patients with soft tissue sarcoma (AUS, FSA, RMS, HPC or MXS), melanoma, carcinomas, osteosarcoma, haemangiosarcoma, lymphoma or mast cell tumour	• i.v. infusion of 1.5×10^5–1×10^8 CFU/kg (dose escalation) with 1–19 doses (mean = 3) • MTD 3×10^7 CFU/kg; tumour colonization observed in 42% cases, with 4 CRs and 2 PRs	Published[181]
	S. Typhimurium SalpIL2 (*S.* Typhimurium χ4550 expressing IL-2)	19 patients with appendicular osteosarcoma	• Neoadjuvant and adjuvant SalpIL2: PO 1×10^5–1×10^9 CFU/patient (dose escalation), with 6 (n = 13), 4 (n = 3), 3 (n = 2) or 1 (n = 1) dose • Amputation • Adjuvant doxorubicin: i.v. 30 mg/m^2 with 5 doses (n = 13) • No dose-limiting toxicity observed; tumour colonization not evident in tumours from 5 patients assayed; and disease-free interval of patients treated with amputation, SalpIL2 and doxorubicin significantly longer than historical comparison group treated with amputation and doxorubicin	Published[182]
	C. novyi-NT	6 patients with haemangiosarcoma, lingual SCC, osteosarcoma, nasal adenocarcinoma or fibrosarcoma	• i.v. infusion of 3×10^8 spores/kg or 3×10^7 spores/kg (1 dose) • Dose-limiting toxicity (abscess formation) observed at 3×10^7 spores/kg; tumour abscess observed in 3 patients; and 4 SD	Published[266]
	C. novyi-NT	16 patients with soft tissue sarcoma (PNST, RMS, FSA or MXS), chondroblastic osteosarcoma, mast cell tumour, melanoma or synovial cell sarcoma	• i.t. injection of 1×10^8 spores/dose with 1–4 doses • Tumour abscess observed in 7 patients, with 3 CRs, 3 PRs and 5 SD	Published[64]
Human	*S.* Typhimurium VNP20009	Phase I; 25 patients with melanoma or RCC	• 30 min i.v. infusion of 1×10^6–1×10^9 CFU/m^2 (dose escalation) (1 dose) • MTD 3×10^8 CFU/m^2; tumour colonization observed in 3 patients in 2 highest dose cohorts; elevated circulating pro-inflammatory cytokines detected; and objective tumour regression not observed	Published[79]
	S. Typhimurium VNP20009	4 patients with melanoma	• 4 h i.v. infusion of 3×10^8 CFU/m^2 (1 dose) • Treatment well tolerated; tumour colonization not evident; and objective tumour response not observed	Published[80]
	S. Typhimurium TAPET-CD (VNP20009 expressing cytosine deaminase)	Phase I; 3 patients with head and neck SCC or oesophageal adenocarcinoma	• TAPET-CD: i.t. injection of 3×10^6, 1×10^7 or 3×10^7 CFU/m^2 (dose escalation) for multiple cycles • 5-FC: PO 100 mg/kg/day divided thrice daily for multiple cycles • Tumour colonization evident in 2 patients; and generation and accumulation of 5-FU observed in the 2 patients with TAPET-CD tumour colonization	Published[167]
	S. Typhimurium VNP20009	Phase I; patients with superficial solid tumours	i.t. injection with dose escalation and up to 3 doses if injected lesions are stable or responding to treatment and non-injected lesions are not progressing	Unpublished[185] and completed
	S. Typhimurium VNP20009	Phase I; patients with unspecified solid tumours	i.v. infusion with dose escalation and up to 12 total doses every 35 days or 2 doses past a CR for patients with SD, PRs or CRs	Unpublished[186] and completed
	S. Typhimurium VNP20009	Phase I; 45 patients with metastatic cancer	i.v. infusion with dose escalation and up to 12 total doses every 35 days for patients with SD, PRs or CRs	Unpublished[188] and completed
	S. Typhimurium SalpIL2 (*S.* Typhimurium χ4550 expressing IL-2)	Phase I; 22 patients with liver metastases of solid tumours	PO with dose escalation, with 1×10^5–1×10^{10} CFU/dose	Unpublished[189] and completed
	C. novyi-NT	Phase I; 2 patients with colorectal cancer	i.v. infusion of 1×10^6 spores/kg (1 dose)	Unpublished[187] and terminated[a]
	C. novyi-NT	Phase I; 5 patients with solid tumour malignancies	i.v. infusion with dose escalation planned and 1×10^5–1×10^7 spores/kg (1 dose)	Unpublished[190] and terminated
	C. novyi-NT	Phase I; 24 patients with solid tumour malignancies	i.t. injection with dose escalation, with 1×10^4–3×10^6 spores/dose (1 dose)	Unpublished[192] and completed
	C. novyi-NT	Phase Ib; patients with treatment-refractory advanced solid tumours	• Anti-PD1 immunotherapy, pembrolizumab: i.v. infusion (200 mg) on day 0 and then every 3 weeks for up to 12 months • *C. novyi*-NT: i.t. injection on day 8 with dose escalation and starting dose at 3×10^4 spores/dose (1 dose)	Unpublished[141], recruiting
	B. longum APS001F (*B. longum* expressing cytosine deaminase)	Phase I/II; patients with advanced and/or metastatic solid tumours	• APS001F ± maltose + 5-FC • APS001F: i.v. infusion on days 1, 2 and 3 and dose escalation • Maltose (10%): i.v. infusion on days 1–5, 8–12 and 15–19 • 5-FC: PO on days 11–15 and 18-22	Unpublished[191] and recruiting

5-FC, 5-fluorocytosine; 5-FU, 5-fluorouracil; AUS, anaplastic and undifferentiated sarcoma; *B. longum*, *Bifidobacterium longum*; *C. novyi*, *Clostridium novyi*; CFU, colony-forming units; CR, complete response; FSA, fibrosarcoma; HPC, haemangiopericytoma; IL-2, interleukin-2; i.t., intratumoural; i.v., intravenous; MTD, maximum tolerated dose; MXS, myxosarcoma; PD1, programmed cell death protein 1; PNST, peripheral nerve sheath tumour; PO, per os (oral administration); PR, partial response; RCC, renal cell carcinoma; RMS, rhabdomyosarcoma; S. Typhimurium, *Salmonella enterica* subsp. *enterica* serovar Typhimurium; SCC, squamous cell carcinoma; SD, stable disease.
[a]Terminated because of a design problem.

Alternatively, engineering bacteria to express proteins targeting tumour vasculature or combining bacteria with microtubule-destabilizing agents may help expand colonization in an otherwise less hypoxic tumour[145–151,153,154].

Conclusions and future perspectives

Tumour-targeting bacteria are ideal vehicles to deliver therapeutic payloads because of their tumour 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 tumour-targeting bacteria, a successful cancer therapy is still likely to require combination approaches in the near future because cancer heterogeneity, at both molecular and histological levels, makes it very difficult to achieve cure with single anticancer agents. Bacteria thrive in necrotic and hypoxic tumour 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 tumour cells in well-perfused tumour areas[25]. Thus, bacteria and cytotoxic therapies should synergize with each other for antitumour activities[76,194–197]. Tumour-targeting bacteria have further been shown to drive the G0–G1 to S–G2–M cell cycle transition of tumour cells, making them more susceptible to chemotherapy[197,198]. Equally, therapies with small molecules targeting tumour vasculature can enlarge the hypoxic niche inside the solid tumour, consequently increasing bacterial colonization[145–147], which is particularly important for tumours without extensive hypoxia. In addition, intratumoural bacterial infection can modulate antitumour immune responses both systemically and in the TME (FIG. 1), making it an attractive possibility to combine live bacteria with other immunotherapeutic approaches such as immune checkpoint blockade. With more rationally designed tumour-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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S.Z., C.G., D.B. and K.L. researched data for the article, wrote the manuscript and reviewed and/or edited the manuscript before submission.

Competing interests
S.Z. is entitled to a share of royalties received by John Hopkins University on sales of products described in this article under a licensing agreement between BioMed Valley Discoveries, Inc. and the university. S.Z. is also a Founding Scientific Adviser of Personal Genome Diagnostics, Inc. and a Founder of PapGene, Inc., which are companies focused on developing genetics-based cancer diagnostics. The terms of these arrangements are under ongoing management by the Johns Hopkins University in accordance with its conflict of interest policies. D.B. has financial interest in Aviex Technologies and Magna Therapeutics and receives royalties from Yale University for technologies based on those described in this article. C.G. has financial interest in Matter Biosciences. K.L. declares no competing interests.

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Supplementary information
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