REVIEW

Engineering bacteria as interactive cancer therapies

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With increasing evidence that microbes colonize tumors, synthetic biology tools are being leveraged to repurpose bacteria as tumor-specific delivery systems. These engineered systems can modulate the tumor microenvironment using a combination of their inherent immunogenicity and local payload production. Here, we review genetic circuits that enhance spatial and temporal control of therapeutic bacteria to improve their safety and efficacy. We describe the engineering of interactions among bacteria, tumor cells, and immune cells, and the progression from bacteria as single agents toward their rational combination with other modalities. Together, these efforts are building toward an emerging concept of engineering interactions between programmable medicines using synthetic biology.

Bacteria were first identified as potential cancer treatments in the 19th century, with evidence of tumor regressions observed in patients injected with *Streptococcus pyogenes* and *Serratia marcescens* (*1*). Although it was not known at the time, bacteria are immunostimulatory, directing an immune response toward tumors, and can also preferentially grow in hypoxic and immunosuppressive tumor microenvironments (TMEs). Several genera, such as *Salmonella*, *Escherichia*, *Clostridium*, *Bifidobacterium*, *Proteus*, and *Lactobacillus*, have demonstrated these characteristics, suggesting their utility as tumor-targeting vehicles (*2*, *3*).

Bacteria can be genetically engineered to encode and locally deliver several classes of payloads that might otherwise be toxic if administered systemically, including small molecules, toxins, immunomodulators, pro-drug–converting enzymes, small interfering RNAs, and nanobodies (*3*). These released agents provide a way for bacteria to influence tumor, immune, stromal, microbial, and other cell types within the TME. Furthermore, bacteria and their payloads can interface with external imaging modalities such as magnetic resonance imaging (MRI) and focused ultrasound (FUS) to enable bacterial detection and actuation.

Synthetic biology enables the precise tuning of these bacterial interactions with other cells and technologies to enhance the therapeutic efficacy and safety of bacteria cancer therapy. The development of sense-and-respond genetic circuitry can autonomously control bacterial behavior to regulate where and when they grow and release their payloads (*4*). In this Review, we highlight the progress in engineering *Salmonella typhimurium* and *Escherichia coli* model systems and explore how synthetic gene circuits enable bacteria to more effectively interact with other living and nonliving modalities.

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Engineering the bacteria-tumor interface

Tumor localization

Because of tumor characteristics such as hypoxia and low immune surveillance, administered bacteria can accumulate ~10,000-fold in tumors relative to other tissues. However, some strains can survive or grow in healthy organs, prompting the need for genetic attenuations to reduce inherent virulence and endotoxicity. A notable example is the attenuated *S. typhimurium* VNP20009 strain, which includes chromosomal deletions of *purI* and *msbB*, creating a purine auxotrophic strain and modified lipopolysaccharide (LPS), respectively. Together, these attenuations reduced systemic inflammation, as measured by tumor necrosis factor (TNF-α), in mice (*6*). When clinically evaluated in metastatic melanoma patients, intravenously administered VNP20009 was generally well tolerated, but did not efficiently colonize tumors and provided no therapeutic benefit, demonstrating the challenge of achieving both safety and therapeutic efficacy (*7*).

Strategies to improve the tumor specificity of bacteria include mutagenesis and directed evolution, which select for auxotrophic bacteria that have increased relative growth within tumors or strain variants with increased adherence to cancer cells, respectively (*8*, *9*). Alternatively, bacteria can be engineered to display tumor-targeting moieties such as adhesion peptides and tumor-associated antigens on their membranes (*10*). For example, an attenuated *Salmonella* strain was engineered to display a tumor-homing arginine-glycine-aspartate (RGD) peptide by fusing the peptide to the bacterial outer membrane protein A (OmpA) (*11*). This peptide then bound to $\alpha_v\beta_3$ integrins, which are widely overexpressed on tumor cells, thus increasing the adherence of bacteria to tumor cells compared with healthy cells.

An alternative approach for improved tumor tropism is to leverage genetic circuits that couple bacterial growth to tumor hallmarks such as high concentrations of lactate, low amounts of oxygen, and low pH. In these sensing circuits, the transcription of the essential gene(s) for bacterial growth is controlled by bacterial promoters responding to these environmental cues, thereby limiting bacterial growth to tumors (*12*). Because other tissues may contain one of these signals, combining the sensing circuits through "AND" logic gates further improves tumor specificity and reduces instances of bacterial mutational escape, enabling longer-term biocontainment (*13*).

Once inside the tumor, bacteria can locally deliver high concentrations of a multitude of payloads to specific locations appropriate for the therapeutic targets and type of molecules generated by the bacteria. For example, some small molecules reach their targets by passive diffusion or transport through microbial and mammalian membranes. Other payloads, such as nucleic acids, need to be delivered intracellularly into the cytoplasm or nucleus, and some proteins act on extracellular receptors and require localization to the extracellular space (Fig. 1, A to D).

Targeting the intracellular space

Intracellular delivery has been a long-standing challenge with many orthogonal methods such as virus and nanoparticle delivery platforms developed to access intracellular targets (*14*). As a living medicine, bacteria can demonstrate autonomous control, sensing and responding to the internalization process, and subsequently releasing cargo. Moreover, intracellular delivery is beneficial because it allows for the targeting of proteins and pathways that have been traditionally challenging to pursue.

Bacteria with intracellular life cycles, such as *S. typhimurium*, have been used to secrete an array of cargos, into tumor cells, using a type 3 secretion system (T3SS). T3SS is one of multiple secretion systems found in Gram-negative bacteria in which a needle-like complex enables the direct injection of effector proteins from external bacteria into the cytoplasm of host cells. Studies have improved the efficiency of T3SS-based cytosolic delivery of macromolecules, including synthetic binding proteins such as Designed Ankyrin Repeat Proteins (DARPins) and monobodies (*15*). A generalizable secretion system used for these larger protein families is a pCASP-hyperinvasive locus A (HilA) vector in which expression of the recombinant protein is co-regulated with the *Salmonella* pathogenicity island-1 (SPI-1) operons and tagged with a type III secretion signal sequence and chaperone-binding domain (*15*, *16*). These modifications resulted in the recognition of the heterologous proteins as type III secreted proteins. A regulator of the SPI-1 genes, *hilA*, was also cloned under an arabinose-inducible promoter, allowing for the induction of high levels of protein secretion into target cells. To validate this secretion

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approach, multiple DARPins and monobodies inhibiting the largely undruggable RAS signaling pathway were delivered to the cytosol of human colon cancer cells using this bacterial system, and functional inhibition of RAS signaling was confirmed in vitro.

S. typhimurium can also be phagocytosed and replicate in a *Salmonella*-containing vacuole (SCV) within the host cell. Indeed, the T3SS needle complexes of *Salmonella* can penetrate through the SCV membrane, enabling payload protein secretion into the host cytosol. However, this barrier limits the transport of additional bacterial contents through the SCV once bacteria are internalized by the host tumor cell, prompting investigation into strategies to actively lyse bacteria and break down the SCV. Escape strategies from intracellular species such as *Listeria monocytogenes* and *Shigella flexneri* have been adapted to make *S. typhimurium* more effective at vacuolar escape (*17*). Specifically, the *hlyA* gene encodes for listeriolysin-O (LLO), a pore-forming cytolysin natively found in *L. monocytogenes*, that enables escape of bacterial plasmids and contents. Attenuated *S. typhimurium* engineered to express LLO can deliver plasmids into specific target cells, but the transfer efficiency is low (*18*). For otherwise extracellular bacteria such as *E. coli*, the expression of invasin (encoded by the *inv* gene from *Yersinia pseudotuberculosis*) can enable binding to β1-integrins present on many cell lines and promote bacterial uptake into phagosomes (*19*, *20*). Engineering diaminopimelate auxotrophic *E. coli* strains allows for additional bacterial cell lysis upon entry into mammalian cells because the auxotrophic bacteria are unable to synthesize a cell wall, in turn enabling plasmid DNA delivery to the host cell (*20*). Similar to *S. typhimurium* strains, *E. coli* strains can additionally encode LLO, which, when combined with auxotrophy and invasin expression, enhances the efficiency of nucleic acid transfer to host cells (*18*, *20*, *21*).

Synthetic biology approaches have enabled the integration of spatial and temporal controls to tune bacterial release from the vacuole. For example, inducible circuits have been used to temporally control the transcription of phage-derived lysis genes encoded by internalized *S. typhimurium*, resulting in bacteria cell lysis within the SCV. To enhance the passage of drugs through the SCV membrane, mutations in the *sifA* gene were made to compromise the

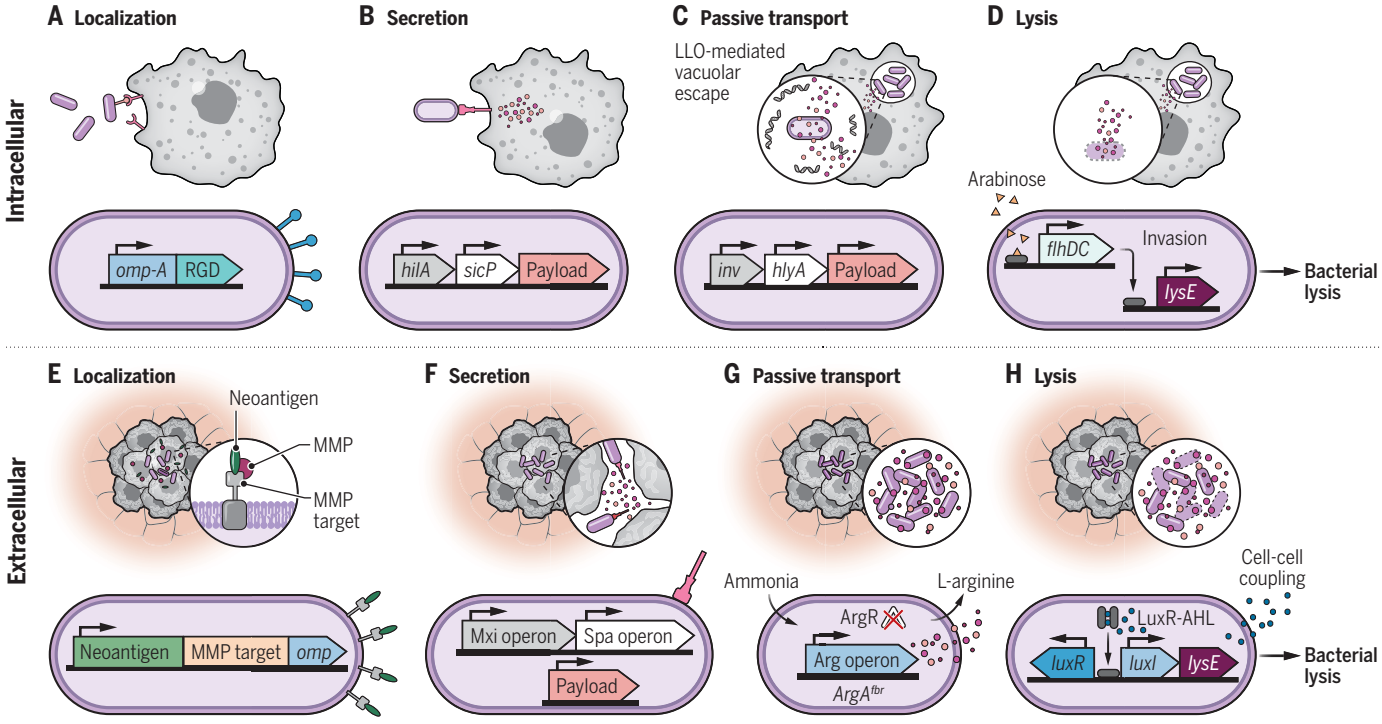

Fig. 1. Engineering the bacteria-tumor interface. Engineered bacteria localize their payloads intracellularly [(A) to (D)] or extracellularly [(E) to (H)] and release them by secretion, diffusion, or lysis mechanisms. (**A**) Bacteria have been engineered to more specifically bind to tumor cells by displaying targeting moieties such as RGD on the external loop of outer membrane proteins such as Omp-A (*11*). (**B**) Modified T3SS secretion using the pCASP-HilA vector enables bacterial delivery of macromolecules. *hilA* and *sicP* are expressed to improve secretion efficiency and the payload is tagged with a type III secretion signal sequence and chaperone binding domain for cytosolic delivery (*15*). (**C**) Expression of invasin, encoded by *inv*, promotes bacterial uptake into phagosomes and increases invasion efficiency of otherwise extracellular bacteria such as noninvasive *E. coli* strains, allowing for protected intravacuolar replication. The addition of LLO, encoded by *hlyA*, increases transfer efficiency of payloads such as plasmids into target cells by forming pores within the vacuolar membrane (*17*). (**D**) Bacteria have also been encoded with lysis circuits to enhance the passage of drugs through the bacterial membrane. An arabinose-inducible circuit regulates expression of the *flhDC* operon, which mediates *Salmonella* motility and invasion. In turn, bacterial lysis, achieved through the expression of by the bacteriophage-derived lysis gene *lysE*, is activated upon invasion (*23*). (**E**) Neoantigens with an MMP target sequence can be expressed with outer membrane proteins (omp) of *S. typhimurium*. Tumor-enriched MMPs can then cleave the MMP target sequence, releasing neoantigens locally within the extracellular tumor space (*41*). (**F**) Extracellular bacteria such as *E. coli* have also been encoded with modified T3SS components, whereby expression of the *mxi* and *spa* operons are necessary for the expression of T3SS structural components. This construct allows the secretion of therapeutic payloads modified with an N-terminal type III secretion signal sequence to be released outside of the tumor cell (*28*). (**G**) Acting as intratumoral bioreactors, *E. coli* have been engineered to metabolize ammonia, a waste product generated by tumors, into L-arginine. Genomic modifications were made to prevent the negative regulation or inhibition of genes in the biosynthesis pathway by deleting ArgR and integrating $ArgA^{fbr}$ (*42*). (**H**) Bacteria have also been engineered with *LuxR*-based QS systems that rely on the diffusion of the autoinducer AHL between cells. In this system, *luxI* produces AHL, which binds to LuxR, engaging the *plux* promoter for positive feedback regulation. Because AHL is also able to diffuse freely between cells, bacteria can sense when they are at a critical density and drive expression of *lysE*, inducing quorum-based lysis and repeated intratumoral drug delivery (*30*).

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integrity of the vacuole, allowing for release of bacterial payload into the host cell cytosol hours after internalization (*22*). In another example, an attenuated *S. typhimurium* strain (Δ*asd* VNP20009) was engineered to self-destruct upon sensing invasion into tumor cells (*23*). Bacterial motility and invasion were tuned by placing the operon, *flhDC*, under an arabinose-inducible circuit. Additionally, bacterial lysis genes were placed under the control of a SPI-2 promoter, which was activated after host cell invasion, further regulating lysis behavior. This "intracellular delivery (ID) *Salmonella*" platform was used to deliver drugs that effectively inhibited intracellular protein phosophatase 1 holoenzymes and mitigated tumor burden in vivo (*23*).

Targeting the extracellular space

Many therapeutic targets, such as tumor cell receptors, necessitate extracellular delivery methods. Generally, targeting extracellular molecules is somewhat simpler in that these delivery strategies depend less on the physical proximity of bacteria to tumor cells. Furthermore, the extracellular space enables bacteria to grow to high densities. As more bacteria grow, more of their payload can be produced and diffuse throughout the tumor space to act upon their intended targets (Fig. 1, E to H).

Extracellular delivery strategies also provide an opportunity to leverage non-invasive bacteria, such as the probiotic strain *E. coli* Nissle 1917 (EcN) (*24*). Because EcN and other inherently extracellular bacteria do not readily secrete most proteins, efforts to translocate recombinant proteins from the cytoplasm have relied on signal peptides and secretion tags (*25*, *26*). For direct delivery of cargo into mammalian cells, *E. coli* can also be engineered to encode a *Shigella*-derived type 3 secretion apparatus (*27*). A modified version of this system, named $PROT_3EcT$ (probiotic type 3 secretion *E. coli* therapeutic) was expressed in EcN and allowed for secretion only within the extracellular space. Specifically, to constrain protein release to outside of the cell, the therapeutic proteins was fused to a sequence lacking the *Ipa* operon necessary for host cell invasion. When evaluated in a preclinical mouse model of colitis, $PROT_3EcT$-secreted anti-TNF-α nanobodies had comparable efficacy to systemically delivered antibodies to TNF-α in reducing inflammation (*28*).

Genetic circuits that use quorum sensing (QS) can enable coordinated bacterial behaviors in the extracellular tumor environment. Because bacteria only reach high densities in the TME, QS effectively acts a tumor-specific signature that can trigger recombinant protein expression (*29*). Additionally, these systems can offer temporal control, where QS parameters control a predetermined bacterial density for when therapeutic expression is activated. Similar to methods for lysing bacteria intracellularly, bacteriophage-derived lysis genes can be cloned under QS control, enabling intratumoral bacterial proliferation, lysis, and therapeutic release of payloads into the extracellular space. For example, a synchronized lysis circuit was designed such that bacteria grow and produce the QS molecule acyl homoserine lactone (AHL). Over time, AHL accumulates to a threshold concentration, triggering a lysis event and releasing bacterial contents upon reaching a critical density. After lysis, a small number of remaining bacteria begin to produce AHL anew, and the process continues in a cyclical fashion, allowing for repeated drug delivery within tumors (*30*). Such circuits have been used to release various toxins and immunotherapeutics, including hemoylysin E (hlyE), cell death domain–RGD integrin peptide (CDD-iRGD), C-C chemokine ligand-21 (CCL-21), and nanobodies targeting Cluster of Differentiation 47 (CD47), Cytotoxic T-lymphocyte associated protein 4 (CTLA-4), and Programmed death-ligand 1 (PD-L1), from both *S. typhimurium* and *E. coli* strains. Furthermore, therapeutic efficacy has been demonstrated in mice bearing colorectal, melanoma, breast, and lymphoma subcutaneous tumors, suggesting its versatility as a release mechanism (*30*–*32*).

QS approaches provide autonomous spatial and temporal control that can confine bacteria to tumors and allow for continuous and sustained therapeutic delivery. Furthermore, these circuits can be tuned such that multiple payloads can be delivered sequentially in accordance with a predetermined dosing regimen. Although this has not been achieved in bacteria, examples of similar recombinase-based circuits resulting in sequential gene expression have been explored in mammalian cells (*33*). Some limitations of QS-based strategies include their reliance on reaching a critical bacteria density, which may not be achieved across all tumor sizes, and the use of bacteriophage-derived lysis genes, which can induce a strong evolutionary pressure for mutation.

Reprogramming the immune system

The introduction of engineered bacteria into a host can prompt a predictable immune response, thereby establishing a bacteria-immune cell interface and an exploitable response timeline. Bacteria are inherently immunogenic by virtue of their overall foreignness through expression of surface and intracellular biomolecules that activate innate immune receptors, secretion of immunostimulatory metabolites, and the ability of certain species to inject effector proteins that permit them to invade tumor and local immune cells (*34*). Immune stimulation by bacteria increases when bacterial lysis products are released within the necrotic tumor core or upon killing and phagocytosis of bacteria by cellular and humoral immune components. Beyond these intrinsic immunostimulatory features, synthetic biology techniques can be used to engineer bacteria that deliver cargo capable of targeting discrete steps in the development of an antitumor immune response, potentially synergizing with the inherent innate immune activation elicited by bacteria to enhance the overall therapeutic efficacy of bacterial cancer therapies across multiple tumor types (Fig. 2).

Programming innate immunity

At the earliest stages of colonization and intratumoral proliferation, the presence of bacterial ligands can act as immune adjuvants to stimulate the recruitment and activation of monocytes, macrophages, and neutrophils. These innate immune cells participate in the lysis and clearance (through phagocytosis) of tumor-colonizing bacteria and produce inflammatory cytokines in response to the detection of liberated surface bacterial components by their expressed repertories of Toll-like receptors (TLRs). An attenuated *S. typhimurium* was engineered to produce and secrete heterologous flagellin, FlaB, which was derived from another bacterial species, *Vibrio vulnificus*, and was more potent than flagellin native to *Salmonella* (*35*). This modified strain of *S. typhimurium* expressing *flaB* stimulates an innate immune response through the cooperative activation of TLR4 and TLR5, where TLR4 recognizes *S. typhimurium* LPS and TLR5 recognizes the secreted FlaB. Recognition of *S. typhimurium* LPS induced the infiltration of monocytes, macrophages, and neutrophils into the TME, which, in conjunction with their recognition of LPS through TLR4, may detect FlaB through TLR5 and support an observed M2 macrophage (protumor) to M1 macrophage (antitumor) shift and increased secretion of the antitumor cytokines interleukin-1β (IL-1β) and TNF-α. Testing of the system in murine and human colon cancer models demonstrated delayed primary tumor growth and inhibition of metastases only when bacteria were engineered to secrete FlaB. The necessity of both components suggests that bacteria can encode payloads to uniquely and effectively alter immune functions (*35*).

As an innate immune response continues, antigen-presenting cells (APCs) will enter the tumor and likely engulf dead tumor cells and intratumoral bacteria, thereby providing further interactions that can be precisely modulated for an enhanced antitumor response. For example, EcN can be engineered to deliver STING agonists such as a cyclic di-AMP (CDA)–producing enzyme and passively release their payload within intratumoral phagocytic APCs (*36*). This strain, SYNB1891, relies on its phagocytic uptake to deliver CDA directly to APCs and induce a type I interferon

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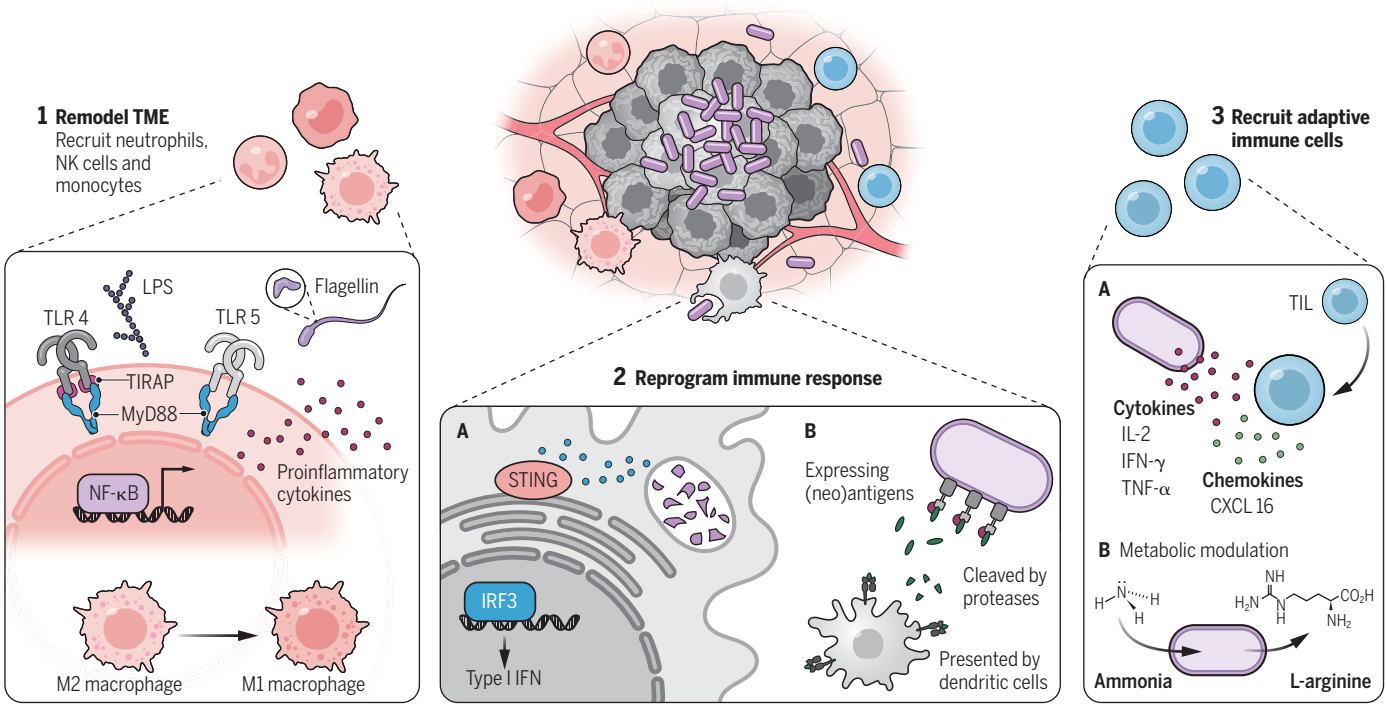

Fig. 2. Engineering the bacteria-immune interface. As single agents, bacteria are immunogenic and can remodel the TME through engagement of TLR-4 and TLR-5, which are stimulated by bacterial LPS and flagella, respectively. Their presence can also lead to an influx of innate immune cells such as neutrophils, natural killer (NK) cells, and monocytes into the tumor and change the phenotype of resident macrophages. As immune cells infiltrate into the tumor, bacteria are phagocytosed, presenting an opportunity to deliver immune cell–specific cargo intracellularly. For example, *E. coli* have been engineered to deliver STING agonists to intratumoral APCs, thereby inducing an IFN-I response (*36*). *S. typhimurium* have also been encoded to express neoantigens on their outer membrane so that once inside the tumor, they can be cleaved, taken up, and presented by surrounding dendritic cells (*41*). Bacteria can also engage the adaptive immune system through the production of immunomodulators such as cytokines and chemokines to recruit TILs into the tumor space. In addition to directly producing immunomodulatory cargo, they have also been engineered to convert tumor metabolic waste products such as ammonia into metabolites such as L-arginine, which has been correlated with increased frequency of TILs, thereby remodeling the TME (*42*).

(IFN-I) response. Combined with the benefit of bacteria-mediated proinflammatory cytokine production, delivery of STING agonists resulted in durable antitumor immunity and tumor regression in multiple murine tumor models. Currently, SYNB1891 is being evaluated in a phase 1 clinical trial of patients with advanced solid tumors and lymphomas (NCT04167137). Similarly, STACT (*S. typhimurium*–attenuated cancer therapy), another strain in clinical development, was engineered to encode a Three Prime Repair Exonuclease 1 (TREX-1) inhibitor, and leverages a similar mechanism, ultimately activating the STING pathway upon uptake by tumor-resident APCs (*37*).

Programming adaptive immunity

As APCs engulf cells, they can present new antigens and stimulate an adaptive immune response. Antigens derived from native intracellular bacteria within the tumor microbiome, specifically patient-derived melanoma tumors, can be presented by melanoma cells and infiltrating APCs, thus activating adaptive immunity (*38*). Additionally, *Listeria*-based approaches have been commonly used to deliver intracellular payloads, including tumor antigens (*39*, *40*). Although *L. monocytogenes* is common for antigen delivery because of its preference for APC invasion, attenuated *S. typhimurium* have been used as an alternative chassis for neoantigen peptide delivery. In one study, multiple neoantigen peptides were tethered to the outer *S. typhimurium* membrane with a matrix metalloproteinase (MMP) target sequence (*41*). Once engineered bacteria home to tumors, neoantigens can be cleaved from bacteria by proteases abundantly found in the tumor and released into the TME for site-specific recruitment and activation of lymphocytes. Treatment of murine colorectal tumors with the engineered neoantigen-producing strain led to an increase in proinflammatory cytokines such as IL-2, TNF-α, and INF-γ, and increased the accumulation of tumor-infiltrating lymphocytes (TILs).

In addition to presenting immunomodulators, intratumoral bacteria can also remodel the TME and indirectly potentiate an adaptive immune response through metabolic modulation. For example, EcN was engineered to convert ammonia, a metabolic waste product in tumors, into L-arginine (*42*). Further modifications were made to the EcN genome to increase L-arginine production, including deleting the arginine repressor (ArgR), thereby preventing the negative regulation of genes in the biosynthesis pathway. Additionally, a feedback-resistant dominant mutant version of ArgA ($ArgA^{fbr}$) was integrated into the bacterial genome to prevent inhibition of the pathway by high levels of L-arginine. When combined with checkpoint blockade, intratumoral injections of this L-arginine–producing strain resulted in increased accumulation of TILs and enhanced overall therapeutic efficacy (*42*).

Bacteria can also be encoded with a multitude of payloads to further program the adaptive antitumor immune response by the activation and recruitment of immune cells through the production of immune checkpoint blockade nanobodies, cytokines, and chemokines (*2*). Tumors lacking infiltrating cytotoxic T cells can become more responsive to immune-based therapies through the production of intratumoral cytokines and chemokines. Examples of cytokines recombined into the bacterial genome include, but are not limited to, IL-2, IL-18, and CCL-21, all of which work to ultimately stimulate immune effector functions against cancer cells. IL-2 has been the most extensively investigated, with examples of IL-2 producing *S. typhimurium*

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strains demonstrating anticancer and prophylactic properties (*2*). EcN can also be encoded to produce C-X-C chemokine ligand 16 (CXCL16) and promote chemotaxis of cytotoxic T cells into tumors (*43*), subsequently supporting tumor regression. Collectively, these studies demonstrate an opportunity to exploit the inherent temporal structure of an immune response to tune bacteria-immune cell interactions. At each stage of the response, bacteria can produce payloads to communicate with specific immune cells, reprogramming a more effective antitumor response.

Engineering microbial interfaces

In addition to interfacing with tumor and immune cells within the TME, bacteria can also work in combination with materials and technologies outside of the tumor. External technologies such as ultrasound and magnetic-based approaches can further manipulate bacterial behavior, allowing for tumor visualization and remote control to precisely tune bacterial location and timing of therapeutic release within the tumor. Moreover, nanoparticles, their cargo, and radiation therapy can remodel the TME and modulate bacterial interaction with the immune system. When combined, these technologies create systems of living and nonliving modalities in which each singular therapy can supplement the limitations of the other, resulting in an overall improved outcome (Fig. 3).

Interfacing with nonliving technologies

Imaging modalities such as positron emission tomography (PET) and MRI have long been used to assist in cancer detection and visualization. These imaging techniques can also be used in combination with native or engineered bacteria enhancing visualization of tumor and bacterial localization in situ (*44*). In addition to imaging-based detection, bacteria can produce molecules recoverable in urine, blood, and stool for additional noninvasive diagnoses (*45*–*47*). Once inside the tumor, engineered bacteria can report on tumor presence, burden, and possibly on microenvironmental conditions when coupled to bacteria-based sensors and circuits.

Moreover, external actuation can be used to interface with microbes in vivo. FUS is one such approach with the ability to penetrate deeply into tissue with high spatial resolution, allowing engineered bacteria to be visualized and manipulated within precise locations. To facilitate bacterial visualization, *E. coli* and *S. typhimurium* can be encoded with acoustic reporter genes derived from intracellular, protein-enclosed gas vesicles native to other microorganisms. Expression of these gene clusters enabled bacterial detection by FUS and, more specifically, visual mapping of their locations in the gastrointestinal tract and TME (*48*). Such bacteria can also be engineered with temperature-actuated genetic switches to release immune checkpoint inhibitors (*49*) or cytokines (*50*) intratumorally in response to applied FUS hyperthermia. Finally, FUS can be used in combination with bacteria as a mechanotherapy in which bacteria encode micrometer-scale cavitating bubbles that unleash strong local mechanical effects to disrupt and kill tissue and cells, further lysing bacteria and releasing therapeutic cargo (*51*).

In parallel, multiple efforts have coupled microbes with magnetic guidance to enhance the penetrance of engineered bacteria strains into hypoxic tumor cores. Several studies have leveraged the magneto-aerotactic property of *Magnetococcus marinus*, demonstrating that external magnetic torque-driven actuation can wirelessly control bacteria bearing liposomal cargo and enhance bacterial accumulation deep within the TME (*52*, *53*). Coating the surface of *E. coli* with both magnetic nanoparticles and chemotherapeutic-encapsulating nanoliposomes allowed the magnetic guidance of bacteria through three-dimensional materials, where they homed to the externally placed magnetic field and released payloads in vitro (*54*).

External therapeutic modalities such as radiation can also combine favorably with bacteria and nanoparticle therapies by promoting bacteria-immune cell interactions. Attenuated *S. typhimurium* (VNP20009) coated with positively charged polyamidoamine dendrimer nanoparticles can bind negatively charged antigens through electrostatic interactions. When immunosuppressive tumors are irradiated, tumor antigens are released and engineered bacteria can then transport the antigens to functional dendritic cells at the tumor periphery, eliciting a strong systemic immune response against the tumor (*55*).

Engineering communities of programmable medicines

In addition to combinations with external technologies and nonliving materials, it is also possible to engineer interactions between bacteria and other living cellular modalities. In the simplest case, bacteria can remodel the TME to be more favorable to other microbial and cellular therapies. For example, nonpathogenic *E. coli* encoded with an IFN-I antagonist, B18R, enhanced the intratumoral replication

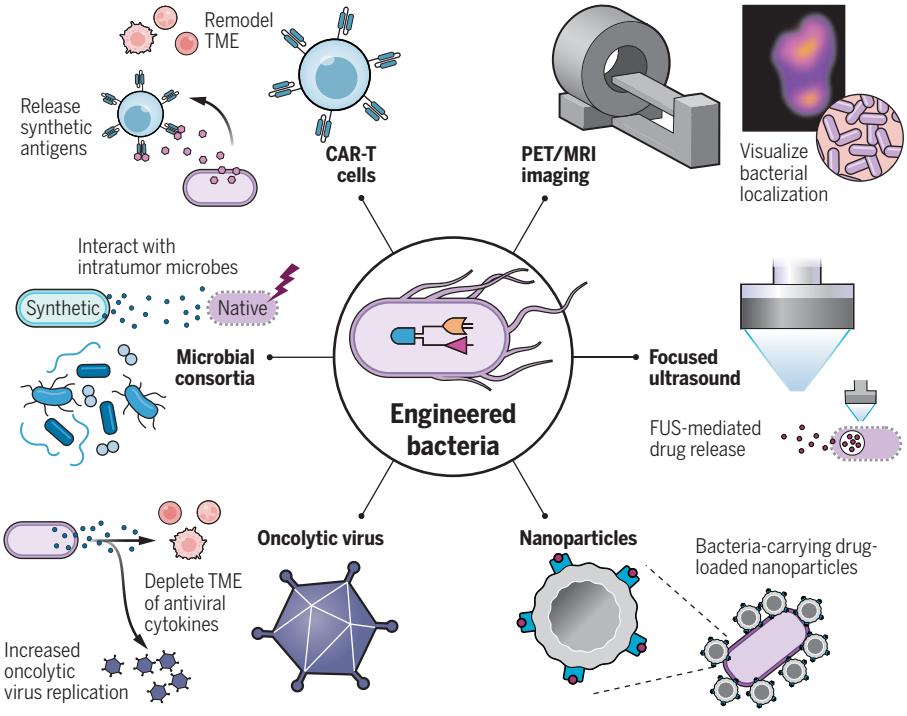

Fig. 3. Engineering bacterial interactions with other modalities. Bacteria can be used together with both living and nonliving technologies for improved diagnostic and therapeutic outcomes. Combinations with imaging techniques such as MRI, PET, and FUS enable tracking and visualization of systemically delivered bacteria. When bacteria are encoded with acoustic reporter genes or thermal switches, FUS can be used to activate therapeutic release. Drug-loaded nanoparticles can be physically bound to bacteria, which can traffic them to tumor depths that they otherwise would be unable to reach. Shifting from nonliving modalities, efforts have focused on engineering interactions between replicating or living modalities. For example, bacteria can remodel the TME, making it more favorable for oncolytic virus therapy. Synthetic consortia of bacteria can also work together to prompt predictable immune responses or limit populations of tumor-promoting bacteria. Finally, CAR-T cells can be activated by bacterial adjuvants and programmed to respond to bacterially released synthetic antigens.

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of vesicular somatitus virus (VSV) by effectively protecting it from immune-mediated clearance (*56*). When used as a monotherapy, neither microbe had any notable antitumor efficacy, but combining the two provided an improved antitumor response and survival outcome in murine cancer models. Underlying this response is the engineering of both microbes such that bacteria depleted the TME of antiviral cytokines, making the environment hospitable to sequentially delivered VSV. In this study, bacteria were repurposed to limit, not provoke, an immune response, and specific bacterial species and immune-effector payloads can be chosen purposefully to complement oncolytic virus therapy. Similarly, bacteria can be favorably combined with chimeric antigen receptor (CAR)–T cell therapies. An attenuated bacterial strain, *Brucella melitenis*, promoted proinflammatory M1 polarization of tumor macrophages and increased the frequency of $CD8^+$ T cells within tumors (*57*). When it was delivered with CAR-T cells to treat a murine model of colon cancer, mice displayed lower tumor burden and their survival significantly improved.

Establishing direct communication between engineered bacteria and cellular therapies, such as CAR T cells, is another strategy to enhance therapeutic outcomes. Probiotic-guided CAR-T cells (ProCARs) were engineered such that bacteria encode a synthetic antigen that can be recognized by the CAR T cells (*58*). In this study, the synthetic antigen served as communication between the engineered bacteria and the ProCARs, allowing for spatiotemporal control of CAR activation within the tumor space after tumor-colonizing bacteria released a synthetic antigen. When evaluated in mice bearing xenografts of human tumors, the ProCAR system significantly delayed tumor growth in multiple models, with an enhanced benefit over a bacteria vehicle and CAR-T cell combination control. Further analysis of the therapeutic response showed increased ProCAR activation from both synthetic antigen presence and stimulation of TLRs by bacteria, demonstrating the potential of engineering communities of programmable medicines (*58*).

In addition to cellular therapies, the human body, and particularly tumors, contains a plethora of microbes that can be manipulated for therapeutic purposes (*59*). Intratumoral bacteria have been linked to chemotherapeutic resistance by metabolizing gemcitabine into an inactive form and other studies have demonstrated that *Fusobacteria* may be associated with cancer progression (*60*). In these cases, engineered bacteria could be designed to sense tumor-promoting microbes and eliminate them by producing antimicrobial payloads. Alternatively, innocuous tumor-resident bacteria could be converted to microbes that assist with antitumor immunity. Another approach is to leverage small microbial consortia as therapeutics. An 11-strain commensal consortium of bacteria was identified within the gut microbiome and shown to mediate cytotoxic T cell immunity and induce $IFN\gamma^+$ $CD8^+$ T cells, potentiating the efficacy of immune checkpoint blockade in inhibiting tumor growth (*61*). Using a synthetic biology approach, designing robust synthetic communities with cooperative or antagonistic symbiosis could be engineered to improve therapeutic outcome (*62*–*64*).

Summary and future outlook

Bacteria are a versatile platform that can be engineered as single agents or in combination with other modalities to improve tumor detection and treatment. Alone, bacteria are immunostimulatory and capable of directing an immune response toward the tumor. They can be further engineered to produce various payloads both extracellularly and intracellularly. By nature of favorable bacterial accumulation within tumor cores, the spatial and temporal drug release profiles differ from conventional systemic therapies, thereby mitigating off-target toxicities and accessing otherwise difficult to reach therapeutic targets (*5*).

Although substantial advances have been made in the progression of bacteria cancer therapies, biocontainment and safety concerns need to be evaluated during clinical translation. Bacillus Calmette-Guerin (BCG), an attenuated *Mycobacterium bovis* strain that is used clinically for bladder cancer treatment, provides a benchmark for a US Food & Drug Administration–approved bacteria cancer therapy. In addition to BCG, attenuated bacterial strains alone or engineered with a payload are also being evaluated in patients (*2*). As systems of bacteria for cancer therapy continue to enter clinical trials, these results will guide future engineering approaches to enhance their effectiveness. Beyond their use as single agents, we envision the use of bacteria therapies in combination with other cellular therapies or external modalities, whereby their interactions with the technologies can be engineered in rationally designed bidirectional systems.

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