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Review

Build-a-bug workshop: Using microbial-host interactions and synthetic biology tools to create cancer therapies

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SUMMARY

Many systemically administered cancer therapies exhibit dose-limiting toxicities that reduce their effectiveness. To increase efficacy, bacterial delivery platforms have been developed that improve safety and prolong treatment. Bacteria are a unique class of therapy that selectively colonizes most solid tumors. As delivery vehicles, bacteria have been genetically modified to express a range of therapies that match multiple cancer indications. In this review, we describe a modular "build-a-bug" method that focuses on five design characteristics: bacterial strain (chassis), therapeutic compound, delivery method, immune-modulating features, and genetic control circuits. We emphasize how fundamental research into gut microbe pathogenesis has created safe bacterial therapies, some of which have entered clinical trials. The genomes of gut microbes are fertile grounds for discovery of components to improve delivery and modulate host immune responses. Future work coupling these delivery vehicles with insights from gut microbes could lead to the next generation of microbial cancer therapy.

INTRODUCTION

In recent years, many bacterial therapies have been developed to treat a wide variety of cancers. Excitement about this class of therapies stems from their potential to provide options to patients with untreatable tumors. Bacterial therapies could overcome the limitations of other therapeutics because they have been shown to (1) colonize primary and metastatic tumors,[1,2] (2) deliver therapeutic molecules directly into tumors and cancer cells,[3,4] (3) directly kill cancer cells,[4,5] and (4) induce antitumor immunogenic responses.[6–8] By specifically focusing delivery to malignant tissue, bacterial therapies have lower systemic toxicities than chemotherapy and hormone therapies and have the potential to treat cancers that are untreatable with surgery and radiation.[9–11] As immunotherapeutic delivery vectors, bacteria avoid the patient specificity of chimeric antigen receptor T cell (CAR-T) and virus-based cancer vaccines.[12]

In this review, we will describe how fundamental discoveries about microbial physiology have been applied to therapeutic bacteria. Although bacterial therapies have shown considerable pre-clinical promise,[4–8,13–19] they have, thus far, not progressed beyond early clinical trials.[20] Success in the clinic has been limited by concerns about safety and efficacy.[21] Four issues that limit bacterial therapies are as follows: (1) toxicity due to cytokine release, (2) weak colonization, (3) mild therapeutic efficacy, and (4) incomplete clearance after treatment.[13,20,22,23] Many of these limitations could be addressed by integrating new concepts from tumor biology and gut pathogenesis. A renewed interest in infectious diseases and the intestinal microbiome has led to discoveries about infection mechanisms and the interactions of microbes with immune cells.[24–27] With appropriate tuning and improved safety and efficacy[3,28,29] bacterial therapies will become a central component of cancer therapy.

THE BUILD-A-BUG WORKSHOP

Here, we introduce the build-a-bug concept (Figure 1). All bacterial therapies contain five fundamental components: (1) a delivered therapeutic, (2) a microbial "chassis," (3) a delivery mechanism, (4) microbial features to control immunogenicity and delivery, and (5) genetic circuits to control gene expression (Figure 1). A generalized procedure for developing a bacterial therapy starts with a therapeutic goal and "builds a bug" to achieve it. In this strategy, therapeutic molecules are selected based on efficacy against specific cell types. Bacterial chassis and other features are selected to best deliver the molecule. In the following sections, we will describe the bacterial genera and their specific infection mechanisms. These mechanisms are the basis of all bacterial therapies. We will then describe the therapies, features, and genetic circuits that have been used in pre-clinical experiments and clinical trials.

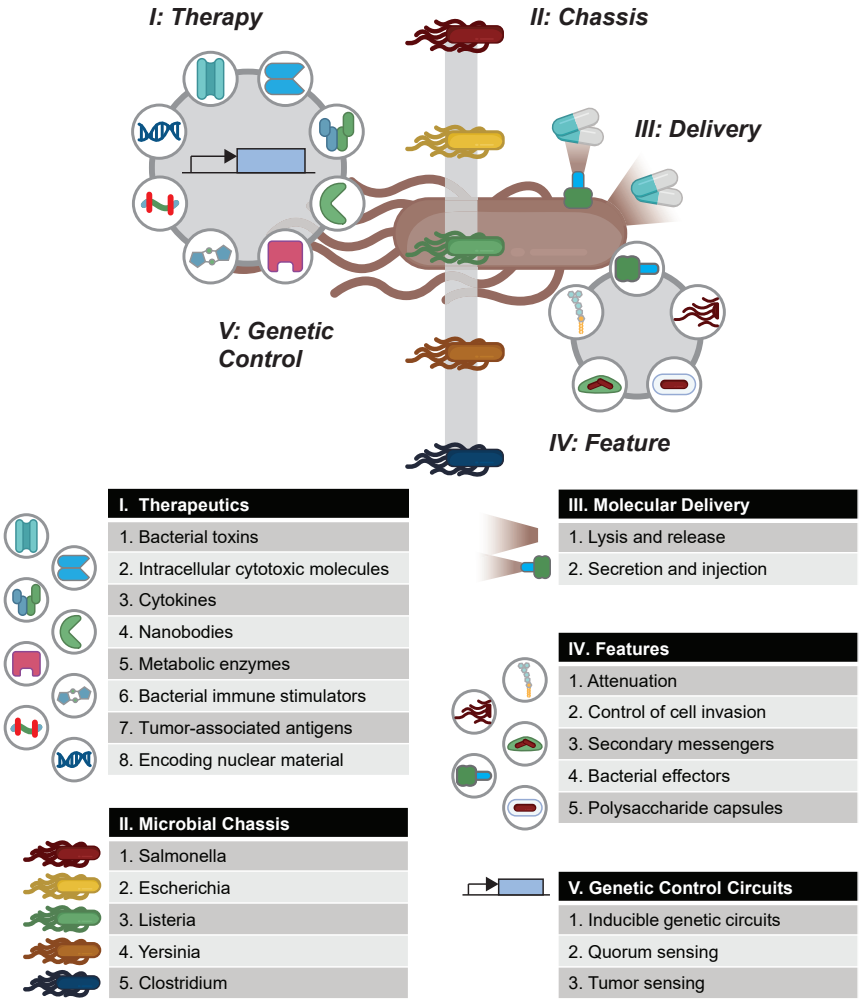

Figure 1. The build-a-bug design strategy
Bacterial therapies contain five components, indicated by I through V: I, a delivered therapeutic; II, a bacterial chassis; III, a molecular delivery mechanism; IV, features to control colonization, delivery, and immunogenicity; and V, genetic control circuits.

BACTERIAL BEHAVIOR IN TUMORS AND THE GUT

Tumors and intestines share characteristics that promote microbial growth (Figure 2). Much has been learned about the behavior of bacteria in tumors by how they interact with epithelial and immune cells in the intestines.[24,25,30–35] Understanding the infection mechanisms of microbial species is essential for creating the next generation of bacterial cancer therapy. The five genera in this review are primarily enteric bacteria, and most knowledge of their behavior has come from a century of infectious disease research.[36] In both tumors and the gut, nutrient gradients affect microbial growth.[2,25,31,37,38] Within the lumen of the ascending colon, fermentable fibers are abundant, and oxygen is virtually absent.[39,40] Tumors contain similar nutrient and oxygen gradients.[41] Viable tumor tissue and functional immune cells reside close to tumor vasculature in regions where nutrient and oxygen concentrations are high.[42] Tumor tissue located far from vasculature is hypoxic and consists mainly of dead cells and debris.[41] Bacteria grow well in these immune-privileged regions because of the absence of functional immune cells.[1,43] Recent discoveries have demonstrated that bacterial species in the intestinal microbiome spontaneously colonize tumor tissue[44] and modulate the efficacy of immune therapies.[45,46]

The five bacterial genera described here (*Salmonella*, *Listeria*, *Escherichia*, *Clostridium*, and *Yersinia*) utilize different mechanisms to promote infection in the intestines (Figure 2, top). Many of these mechanisms are critical for accumulation and therapeutic delivery in tumors (Figure 2, bottom). Because of their unique physiologies, these bacteria target either intracellular or extracellular pathways. Non-pathogenic strains of *Escherichia* and *Clostridium* deliver therapeutics extracellularly.[6,13,19] *Listeria* and *Yersinia* are predominantly intracellular and deliver therapeutics to macrophages and dendritic cells.[16,47,48] Because *Salmonella* is both an intracellular and an extracellular pathogen, it uniquely delivers therapies either to the tumor microenvironment or into cancer cells.[4,8,14,49]

Salmonella

As a therapeutic vector, *Salmonella* (typically *Salmonella enterica* serovar Typhimurium) has selectively delivered active compounds into tumors and reduced volume with minimal toxicity.[4,8,14] *Salmonella* infect both phagocytic and non-phagocytic cells and deliver molecules to both intracellular and extracellular spaces (Figure 2).[4,8,14,50] Because of this breadth, *Salmonella* therapies have targeted epithelial cancer cells,[4] tumor-associated immune cells,[8] and stromal cells.[14]

Natively, *Salmonella* is a facultative anaerobe and a pathogen that infects the large intestine (Figure 2). In the lumen, the microbiome competes with *Salmonella* for available nutrients.[51] *Salmonella* escapes this hostile environment by penetrating the mucosal barrier using flagella-dependent motility.[52,53] Once adjacent to gut epithelial cells, *Salmonella* adhere to the cell membrane with the *Salmonella* pathogenicity island-1 (SPI-1) type-three secretion system (T3SS).[54] Adherent *Salmonella* inject effector proteins into cells, which causes membrane rearrangement and endocytosis into intracellular vacuoles.[55,56]

After invasion into epithelial cells, *Salmonella* modify the intracellular environment to promote survival and prevent clearance.[57,58] Acidification and maturation of *Salmonella*-containing vacuoles (SCVs) activates SPI-2 T3SS and induces injection of SPI-2 effector proteins.[59] These effectors modify the SCV and guard the bacteria from clearance by neutrophils or the complement system.[24] In order to grow and survive within SCVs, the bacteria create *Salmonella*-induced filaments (SIFs). These

filaments connect to the Golgi network of endosomes to harvest critical nutrients.[59] The bacteria continuously modify the SIF network by inserting the SPI-2 T3SS needle into the SCV and injecting effectors into the host cell.[60] Heavy vacuolar modification by reinsertion of SPI-2 T3SS needles causes a small percentage of vacuoles to rupture and release *Salmonella* into the cytoplasm.[61]

Salmonella exploit this escape into the cytoplasm when colonizing the gut.[32–34] The nutrient-dense environment of the cytoplasm promotes hyper-replication.[62] In addition, pathogen-associated molecular patterns (PAMPs), e.g., flagellin and T3SS, activate cytosolic NOD-like receptors (NLRs) and trigger caspase-1-dependent pyroptosis.[63] This form of cell death causes the cell membrane to rupture and release cytosolic *Salmonella* into the extracellular environment.[32–34,61] The extruded bacteria are primed for reinfection by high expression of SPI-1 T3SS and flagella.[33] This reinfection of adjacent epithelial cells perpetuates a cycle that promotes infection and prevents clearance.[32]

Metabolic adaptation within a particular niche is a critical element for survival in the host. For *Salmonella*, the metabolites in the gut do not support proliferation.[64] To circumvent this limitation, *Salmonella* exploits inflammatory reactive oxygen species (ROS) and reactive nitrogen species (RNS) responses.[25,30,65] After traversing the mucosal barrier, *Salmonella* inject effector proteins that recruit neutrophils and stimulates the production of ROS and RNS.[25,30,65] These compounds, e.g., superoxide and nitrate, are generally considered to be antimicrobial.[66] However, *Salmonella* use them as electron acceptors to fuel microbial growth.[25,30,31,65] This manipulation of innate immune responses enables *Salmonella* to survive in the nutrient-limited environment of the intestinal lumen.[25,30,31,65]

Numerous studies have shown that *Salmonella* accumulates in tumors after systemic injection.[1,2] Genetic modification of these bacteria has been shown to controllably produce and deliver therapies that reduce tumor volume.[67,68] Because *Salmonella* localize to two distinct niches, they can be used for both intracellular and extracellular delivery (Figure 2).[4,67,69] We recently showed that, similar to the gut, *Salmonella* invade epithelial cancer cells (carcinoma) in tumors.[4] This intracellular lifestyle makes *Salmonella* particularly useful for targeting internal cellular pathways. Because *Salmonella* also infects phagocytic cells, these bacteria have also been used as delivery systems to tumor-associated immune[8] and stromal cells.[14] Despite invading cells, a significant percentage (~30%) of tumor-colonized *Salmonella* remain outside cells.[4] This localization enable delivery to the local tumor microenvironment and external cell receptors.[4,67,69]

Several strains of *Salmonella* have been investigated as cancer therapies, including VNP20009, Δ*ppGpp*, and A1-R.[70–72] VNP20009 was the first *Salmonella* strain to be investigated in clinical trials.[20,72] This strain contains *msbB* and *purI* deletions that improve tumor selectivity and reduce virulence.[72–74] Clinical trials with VNP20009 demonstrated that the strain is safe and colonizes tumors.[20] In recent studies, it has been shown that VNP20009 can be pre-loaded into macrophages that serve as Trojan horses to deliver functional *Salmonella* into the tumor microenvironment.[50] In pre-clinical models, the Δ*ppGpp* strain has been shown to have reduced virulence, target pancreatic cancer, and safely induce pro-inflammatory immune responses.[8,14,71,75,76] A third therapeutic strain, A1-R, is auxotrophic for branched-chain amino acids (BCAAs).[77] In animal models, this strain colonizes and treats many different tumor types, including soft-tissue sarcoma, follicular-dendritic-cell sarcoma (FDCS), melanoma, pancreatic cancer, ovarian cancer, breast cancer, cervical cancer, and prostate cancer.[70,78]

Escherichia

After birth, commensal *Escherichia coli* (*E. coli*) strains are some of the first organisms to colonize the gastrointestinal tract (Figure 2).[79] When these bacteria initially colonize the intestine, they scavenge oxygen and grow aerobically.[80] Even without oxygen, these facultative anaerobes colonize the large intestine by fermenting soluble fibers, sugars, and other host-produced metabolites.[25,38,40,81] The absence of antimicrobial immune cells in the lumen further enables microbial growth.[82,83] If the bacteria traverse the mucosal barrier, however, they are engulfed by phagocytic cells, and their components are presented by antigen-presenting cells (APCs).[83] The combination of the intestinal microenvironment, metabolic flexibility, and absence of virulence factors promotes colonization in the intestines (Figure 2).[81]

E. coli is well established as a selective delivery vehicle to tumors (Figure 2).[5–7,28,68] Several genetic modifications have been used to enhance its safety and delivery capability. These bacteria have been shown to deliver therapies that target both extracellular ligands[6,7] and targets within phagocytic APCs.[15] Derivatives of the gut commensal strain MG1655 and probiotic Nissle 1917 have been most commonly engineered for cancer treatment, with the latter being used in clinical trials (NCT04167137).[84] A triggered lysis system in Nissle 1917 was developed to deliver therapeutic molecules into the extracellular space.[6,7] Another strain of Nissle 1917 was developed that delivers stimulator of interferon (IFN) genes (STING) agonists after uptake by APCs.[15]

Listeria

Like *Salmonella*, *Listeria monocytogenes* (referred to here by its genus *Listeria*) is an intracellular pathogen that primarily infects the gut and disseminates through the lymphatic system (Figure 2).[85,86] After entering the gut, *Listeria* expresses internalin A or B (*inlA* or *inlB*), which attaches to cadherin receptors on the surface of epithelial cells.[87] Surface attachment accelerates internalization through receptor-mediated endocytosis into vacuoles.[88,89] Shortly after endocytosis, expression of listeriolysin O (LLO) triggers escape into the cytosol,[90] where actin-dependent motility, via ActA, leads to infection of adjacent cells.[91] Entry into APCs within Peyer's patches transports *Listeria* to surrounding lymphoid tissue and disseminates the bacteria.[86]

Genetically modified *Listeria* are well established as vectors for antigen delivery to tumors in mice[16,47,92–94] and in human trials.[21,95,96] Deletion of *actA*, *inlA*, and *inlB* makes these strain stains safe and focuses delivery to APCs by preventing intercellular transport and infection of epithelial cells.[96,97] Inside APCs, *Listeria* expresses LLO, which promotes bacterial escape into the cytosol and delivery of proteins. The *actA*, *inlA*, and *inlB* deletions reduce the virulence of *Listeria* 1,000-fold.[98] Protein delivery into APCs, i.e., dendritic cells and macrophages,[97] induces tumor infiltration and activation of CD8 T cells.[99]

Yersinia

Yersinia is one of the newest genera of bacteria to be explored as a cancer therapy.[48] Like *Salmonella*, *Yersinia enterocolitica* (referred to here as *Yersinia*) produces T3SS injectisomes that have been repurposed to inject therapeutic proteins into the cytoplasm of cancer cells (Figure 2).[48] In its native form, *Yersinia* is a pathogen that causes a mild, self-limiting infection.[100] In the intestine, *Yersinia* activates several mechanisms to prevent clearance by the immune system.[100–102] After ingestion, the temperature and acidity of the gastrointestinal tract induce the expression of virulence factors from the *inv* locus.[100] One of these factors, invasin, adheres the bacteria to the mucus layer and binds B1-integrins on the surface of host epithelial cells.[103] When bound to cells, the T3SS apparatus injects plasmid-encoded *Yersinia* outer proteins (YOPs) that rearrange the cytoskeleton to prevent bacterial phagocytosis and antigen presentation.[101,102,104] Injection of YOPs into the cellular cytoplasm suppresses immune responses and prevents inflammation for 3–4 days.[100] During this phase of silent infection, the bacteria proliferate without clearance.[100] The absence of YOP secretion in plasmid-cured *Yersinia* causes invasin to activate B1-integrin signaling and induces intracellular invasion of the bacteria.[105] With time, the secreted effectors induce pyroptosis and the release of inflammatory cytokines (e.g., interleukin [IL]-1β, tumor necrosis factor alpha [TNF-α], and IL-18).[100] In most cases, these cytokines activate T_h1 CD4 and CD8 T cells, which clear the bacteria and resolve the infection.[100]

To generate a therapeutic strain of *Yersinia enterocolitica*, six YOP effectors, YopH, YopO, YopP, YopE, YopM, and YopT were deleted.[48] This strain utilizes the T3SS apparatus to inject therapeutic proteins into cells.[48] The essential gene for aspartate semialdehyde dehydrogenase (*asd*) was deleted from this strain to improve its safety profile.[48] These organisms were engineered to activate innate immunity (STING and Toll-like receptor [TLR]) in cancer, and their efficacy is currently being tested in a clinical trial for patients with advanced solid tumors (NCT05120596).

Clostridium

Clostridium (most commonly *Clostridium novyi*) is an obligate anaerobe that targets tumors with hypoxia or necrosis (Figure 2).[106,107] As a spore-forming anaerobe, *Clostridium* only germinates in the low-oxygen environment common in the gut lumen or tumor necrosis.[108] In animals, virulent *Clostridium* causes necrotizing hepatitis.[109] These bacteria are opportunistic and require prior organ damage to cause disease.[110] After ingestion, *Clostridium* spores travel through the portal vein to the liver, where they remain dormant.[110] When the liver is damaged by infection or injury, the formation of microscopic hypoxic regions triggers the germination of the spores.[110] After germination, the bacteria expand the area of necrosis by secreting alpha toxins, which have broad activity against *rho* and *ras* GTPases.[111] The alpha toxins cause actin cytoskeleton rearrangement, loss of structural integrity, and cell death.[111]

The efficacy of therapeutic *Clostridium* has been explored in both pre-clinical and clinical settings.[13,18,19] To make *C. novyi* into a safe cancer therapeutic, the alpha toxin was deleted.[13] After germination in tumor necrosis, these bacteria secrete proteases, e.g., collagenase, that destroy the extracellular matrix and other structural components.[112] Although this cytotoxicity is non-specific, the bacteria only germinate in tumor hypoxia, which spares healthy tissue.[13] In the clinic, attenuated (alpha-toxin deleted) *C. novyi* colonize tumors, and toxicities can be managed by administering antibiotics to prevent further germination.[113] In an early clinical trial (NCT01924689), single injections of non-toxic *C. novyi* induced immune responses and demonstrated antitumor activity.[113]

Other gut microbiota

Interest has increased recently in the effect of probiotic bacteria on cancer treatment.[46,114] For example, microbes in the intestines have been shown to produce metabolites that promote tumorigenesis[44] and modulate the efficacy of immune therapies.[46,114] Many tumor sites contain microbiomes that both enhance and repress cancer cell growth.[115] Several probiotic species that have been investigated include *Bifidobacterium infantis*,[116] *Bifidobacterium longum*,[117] *Lactobacillus acidophilus*,[118] *Lactobacillus rhamnosus*,[117] and *Limosilactobacillus fermentum*,[119] just to name a few. Several of these bacteria have been modified to act as single-species therapies.[120] For example, *Bifidobacterium* has been used to deliver viral genes to activate prodrugs[116] or display tumor antigens to sensitize the immune system.[120] When intravenously delivered as spores, *Bifidobacterium*, an obligate anaerobe, specifically colonizes the hypoxic regions of tumors.[116] In addition, many probiotic species have a therapeutic effect in their native forms and are administered as food supplements or as components in fecal transplant.[46] Although beyond the scope of this review, exploration of the complex relationship between microbiota and tumor growth could greatly affect treatment.

THERAPEUTIC MOLECULES

Bacteria have an advantage over other delivery modalities because they continuously and specifically deliver a diverse range of drug types (Figure 3). Bacterial therapies deliver orders of magnitude more therapy to tumors than to healthy tissue because they grow exponentially in malignant tissue.[1,2,121] This selective colonization circumvents the poor targeting of small-molecule therapies that can increase doses and systemic toxicities.[9,11] This specific growth is supported, in part, by the immune-privileged environment in tumors.[43,121] As bacteria grow, they continuously release recombinant therapies into tumor-associated cells or the tumor microenvironment.[4–7,16,48,67,68,93] As a result, dosing with bacterial therapies is different from molecular therapies because the number of bacteria in a tumor is only weakly dependent on the injected dose.[1] For bacterial therapies, efficacy is directly tied to the extent of tumor colonization and the physiologies of the tumors and bacteria.[2,67]

Bacterially delivered therapeutics can be either cytotoxic or immunogenic (Figure 3). Cytotoxic therapeutics reduce malignant tissue by directly killing cancer cells.[4,14,68] Immunogenic therapeutics act by first activating immune cells that in turn eliminate tumors.[7,122] Because they induce immunogenic cell death, cytotoxic drugs often also induce immune responses.[14] Bacterial therapeutics can also be delivered into the extracellular space or directly into cells (Figure 3). Extracellular therapeutics

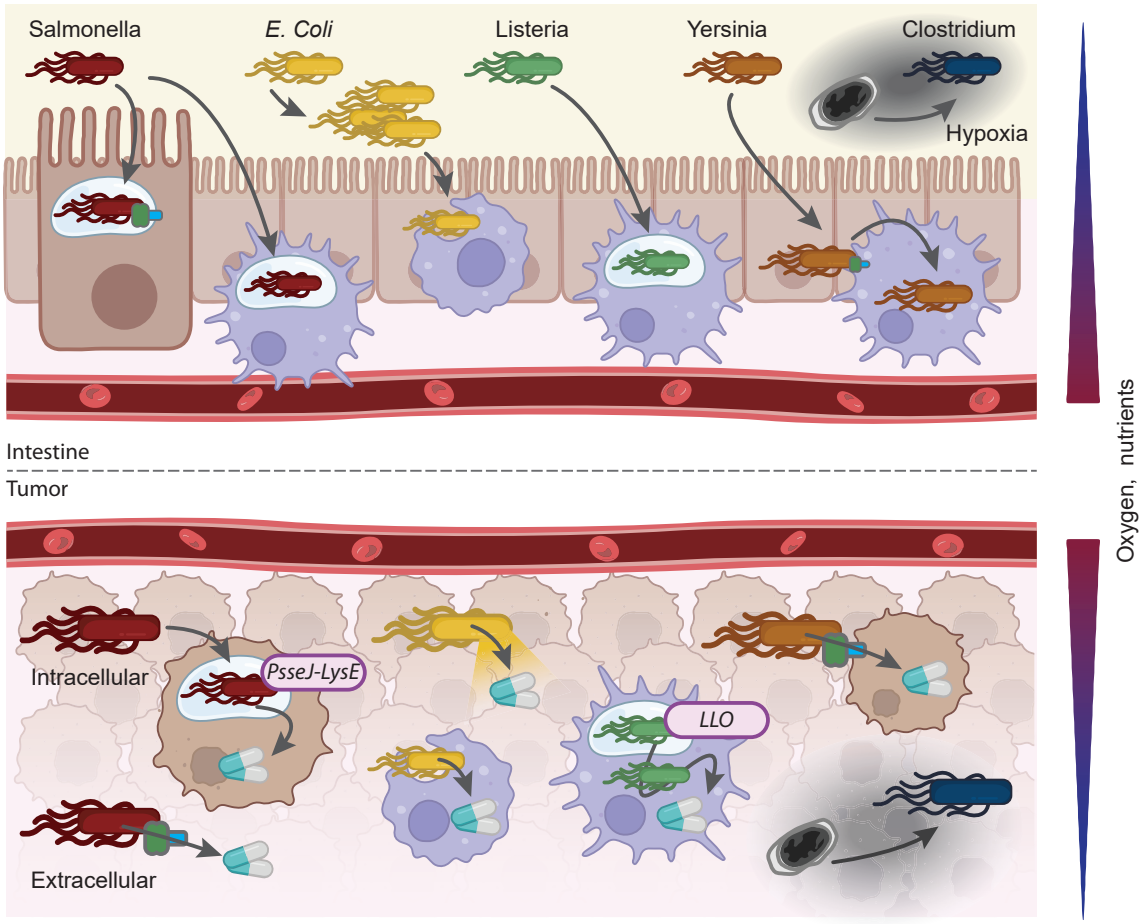

Figure 2. Physiology of five bacterial genera in intestine and tumor environments
In the intestines, *Salmonella* (dark red) invade both epithelial and immune cells. *E. coli* (yellow) proliferates in the intestinal lumen. Like many commensal organisms, if they cross the epithelium barrier, they are consumed by phagocytic immune cells. *Listeria* (green) and *Yersinia* (umber) primarily invade antigen-presenting cells (APCs). *Clostridium* geminates from spores in hypoxic regions of the lumen. In tumors, *Salmonella* deliver molecules into cells (with the *PsseJ-lysE* gene circuit) and into the extracellular space. After triggering lysis, *E. coli* releases molecules into the extracellular space. *E. coli* also deliver molecules into phagocytic antigen-presenting cells (APCs). *Listeria* delivers molecules into APCs after rupturing vacuole membranes with listeriolysin O (LLO). *Yersinia* injects molecules into epithelial cells, and *Clostridium* germinates in tumor hypoxia.

predominantly affect cell membranes, surface proteins, or the tumor microenvironment.[7,123] Intracellular therapeutics target cellular pathways that regulate cell survival or immune induction.[4,122]

Bacterial toxins

Many gut pathogens produce toxins that are effective against tumors (Figure 3A).[3,5,14,68,124,125] For example, delivery of cytolysin A from *Salmonella enterica* (ClyA), alpha toxin from *Staphylococcus aureus* (Hla or SAH), theta toxin from *Clostridium perfringens* (PFO), and hemolysin E from *E. coli* (HlyE) have all reduced tumor growth without eliciting systemic cytotoxicity.[3,5,14,68,124] These toxins form pores in the cellular membrane that disrupt the osmotic balance between the cytoplasm and the surroundings (Figure 3A).[69,125] Disintegration of the membrane quickly leads to necrotic cell death.[68] The number of possible bacterial exo- and endo-toxins is large, but most have not been explored as cancer therapeutics because systemic delivery would be toxic.[69] In addition to directly inducing cell death, many bacterial toxins also compromise barrier integrity by rearranging the actin cytoskeleton.[126] The therapeutic benefits of disrupting epithelial junctions are not completely understood. In the intestine, disruption of the epithelial barrier increases escape of pathogens from the gut into systemic circulation.[127] In the tumor microenvironment, barrier disruption could create channels that improve infiltration of immune cells and enhance antitumor responses. Alternately, these toxins could cause immune dysfunction and hyper-inflammation and impede antitumor efficacy.[128]

Intracellular cytotoxic molecules

There are numerous intrinsic pathways in cancer cells that lead to cell death, including apoptosis, pyroptosis, and autophagy (Figure 3B).[129,130] Intracellular bacterial delivery of key initiating molecules has been shown to trigger these pathways and kill cancer cells.[4] In addition to its direct effect, immunogenic cell death produces antitumor responses by activating immune cells and presentation of tumor antigens.[131] Systemic delivery of cytotoxic molecules has had limited success because of

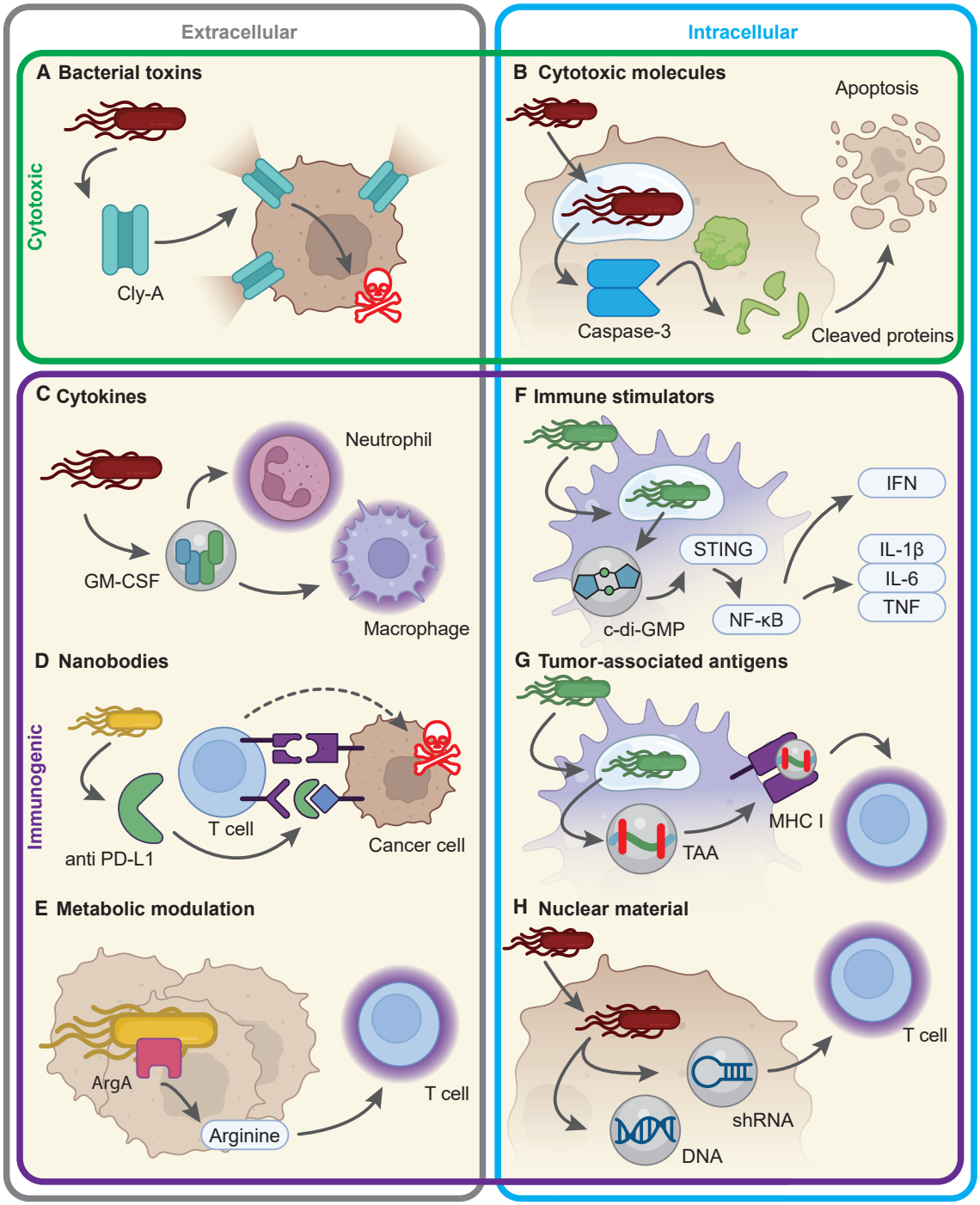

Figure 3. Therapeutic molecules delivered with bacterial vectors

Bacteria have been engineered to deliver molecules that are both cytotoxic (top) and immunogenic (bottom). To effectively interact with their targets, both classes of molecules have been delivered extracellularly (left) and intracellularly (right).

(A) Bacterial toxins (e.g., ClyA, Hla, PFO, or HlyE) kill cells by forming pores in cellular membranes.

(B) Intracellular delivery of cytotoxic molecules (e.g., CT caspase-3 or NIPP1-CD) induces cancer cell death. Delivery of caspase-3 cleaves many protein targets (green fragments), which causes cell blebbing and apoptosis.

(C) Bacterial delivery of cytokines (e.g., GM-CSF, IL-2, or IFNγ) activates immune cells (e.g., neutrophils and macrophages).

(D) Immunological nanobodies (e.g., anti-CD47, PD-L1, or CTLA-4) bind ligands on T cells and cancer cells (here PD-L1) to prevent T cell exhaustion.

(E) Stimulation of metabolic pathways by expression of key enzymes (e.g., ArgA) increases metabolites (e.g., arginine) that limit T cell activation.

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toxicity in healthy tissue.[132] Bacterial vectors prevent this toxicity by directing delivery only to tumors. Two molecules that trigger cell death are constitutively active, two-chain (CT) caspase-3 and the central domain of nuclear inhibitor of protein phosphatase 1 (NIPP1-CD).[4] NIPP1-CD triggers protein phosphatase 1-depenent pathways, independent of its native signaling.[133] When intracellularly delivered to liver cancer cells, NIPP1-CD induces apoptosis.[4] CT caspase-3 is a modified form of the dominant executioner caspase.[134] Once activated, caspase-3 cleaves 500–1,000 protein targets, which causes DNA fragmentation, blebbing of the cell membrane, and death.[135] CT caspase-3 initiates apoptosis independent of cleavage by upstream caspases (caspase-8 and caspase-9) that activate in response to extracellular autocrine signals or detection of DNA damage by p53.[136] When intracellularly delivered to mice with *Salmonella*, CT caspase-3 killed cancer cells, eliminated tumors, and increased survival.[4] Tumor-focused delivery of NIPP1-CD or CT caspase-3 with tumor-colonizing *Salmonella* was not toxic in the blood or healthy organs.[4]

Cytokines

When produced by bacteria, human cytokines activate immune cells and reduce tumor growth (Figure 3C). Three cytokines that have been effective in animal models areIL-2, Granulocyte macrophage-colony stimulating factor (GM-CSF), and IFNγ.[137–139] Sustained production of cytokines is required to activate a pro-inflammatory response against cancer cells within tumors.[140] After colonizing tumors, bacterial therapies continuously produce cytokine molecules, a unique feature of engineered bacteria.[141] In addition, tumor-specific delivery prevents the toxicity associated with systemic delivery.[138] IL-2 is an IL that promotes proliferation and activation of T cells and natural killer (NK) cells.[142] When delivered by attenuated *Salmonella*, IL-2 increases the number of NK cells in the spleen and reduces pulmonary metastases in osteosarcoma.[137] Similarly, GM-CSF is a growth factor that activates neutrophils and macrophages, increases expression of major histocompatibility complex (MHC), and increases recognition of tumor antigens.[143] Delivery of GM-CSF with *Salmonella* prevents the formation of both lung and breast tumors in mice.[139] Like GM-CSF, IFNγ plays a critical role in the body's natural antitumor immune response. IFNγ enhances antigen processing by activating MHC.[144] It also recruits and activates neutrophils, NK cells, and macrophages.[144] When fused to the N-terminal region of SipB, a *Salmonella* translocator protein, delivery of IFNγ induces melanoma cell cytotoxicity and inhibits tumor growth.[138]

Nanobodies

Nanobodies are the antigen-binding domains of camelid heavy-chain antibodies.[145] Unlike humans, camelids have both conventional and heavy-chain-only antibodies.[145] Because of this structure, nanobodies do not require post-translational modification when produced by bacteria.[146] The small size of nanobodies makes them highly soluble and effective at penetrating tumor tissue.[145] Bacterial therapies can produce large quantities of nanobodies to target either intracellular or extracellular antigens.[6,7] When released intracellularly, anti-actin nanobodies specifically bind their target within the cytoplasm.[4] Three examples of immune-stimulatory nanobodies bind extracellular targets: CD47, programmed cell death-ligand 1 (PD-L1), and cytotoxic T lymphocyte-associated protein-4 (CTLA-4).[6,7] Two of these (anti-PD-L1 and anti-CTLA-4) function similar to established immunotherapies and activate immune responses by preventing T cell exhaustion.[7] Similarly, CD47 blockade increases immune responses by inducing phagocytosis of cancer cells.[6] Bacterial delivery of these nanobodies activated infiltrating T cells, increased memory T cells, and regressed tumors.[6,7]

Metabolic modulation

The tumor metabolic environment plays a critical role in the development of antitumor immune responses.[147] Several metabolites prevent T cell exhaustion and promote tumor cell cytotoxicity.[147] More than healthy cells, cancer cells rapidly consume amino acids and sugars within tumors.[129,130] In this depleted environment, infiltrating T cells do not have access to metabolic compounds required for protein synthesis and glycolysis.[147] In the absence of these critical nutrients, T cells become exhausted and lose cytotoxic activity.[147] These metabolic limitations can be overcome by identifying the critical metabolites that limit T cells, e.g., arginine, and manipulating their synthetic pathways in bacteria.[25,38,40,81] The complexity of most metabolic pathways prevents complete reconstruction in recombinant organisms.[148,149] However, controlling expression of key enzymes can increase the production of the essential metabolic compounds within tumors.[123] For example, bacteria with controlled expression of *argR* and *argA* produce arginine, which increases infiltration and activation of T cells.[123]

Bacterial immune stimulators

In addition to toxins and cytokines, bacteria natively produce immunostimulatory molecules (Figure 3F). These include PAMPs, bacterial nucleic acids, and STING agonists.[15] All of these pathways lead to the production of pro-inflammatory cytokines (IL-1β, IL-6, and TNF) or the activation of immune cells.[150] PAMPs are predominantly detected by extracellular TLRs and intracellular NLRs.[150] Two common bacterial PAMPs are flagellin and lipopolysaccharide (LPS).[8,75,151] Flagellin is a TLR5 and NLRC4 agonist that triggers the release of mature IL-1β and induces pyroptosis in macrophages and epithelial cells.[152] LPS is a TLR4 agonist that stimulates the production of TNF and activates antitumor immune cells.[153] Both flagellin and LPS stimulate antitumor immune responses and reduce tumor growth.[8,17] However, because LPS causes septic

(F) Bacterial immune stimulators include PAMPs (e.g., flagellin and LPS), bacterial nucleic acids (e.g., RNA and dsDNA), and STING agonists (e.g., cGAMP, c-di-AMP, and c-di-GMP). As an example, when bacterially delivered c-di-GMP binds the STING protein, it activates NF-κB and induces expression of type 1 IFNs and pro-inflammatory cytokines (IL-1β, IL-6, and TNF).
(G) Bacterially delivered TAAs (e.g., mesothelin and Mage-b) present on MHC class I and activate T cells.
(H) Silencing of PD-1 or STAT3 with bacterial delivered siRNA activates intratumoral CD4 and CD8 T cells. Delivery of the gene for p53 reduces the viability of bladder cancer cells.

shock at low concentrations, it has a narrow therapeutic window.[154]

Bacterial nucleic acids (RNA and DNA) stimulate immune responses through the TLR and STING pathways.[155] Bacterial RNA and double-stranded DNA (dsDNA) are TLR7 and TLR9 agonists that induce tumor cell autophagy.[156] Delivery of these identifiable motifs has reduced the growth of immunologically "hot" tumors in mouse models.[155,156] In the cytoplasm of immune cells, dsDNA from dead bacteria binds the cyclic GMP-AMP synthase (cGAS) sensor, which synthesizes cyclic GMP-AMP (cGAMP).[157,158] Host-synthesized cGAMP binds the STING protein on the endoplasmic reticulum and activates nuclear factor (NF)-κB-dependent expression of type 1 IFNs and pro-inflammatory cytokines (IL-1β, IL-6, and TNF).[157] IFN secretion activates surrounding immune cells to cross-present microbial antigens and generate adaptive immune responses.[159]

STING agonists are another class of immunogenic molecules that have been produced by bacteria (Figure 3F).[15] Many bacterial species secrete cyclic di-nucleotides (cGAMP, c-di-AMP, or c-di-GMP) to regulate virulence and control host immune responses.[158,160,161] When secreted, these secondary messengers bypass cGAS and bind directly to the STING adaptor.[155] Binding STING activates antimicrobial cytokine expression within innate immune cells similar to cGAS synthesized cGAMP.[15] Although STING activation initiates microbial clearance, it also stimulates cross presentation of tumor antigens on MHC class I and activation of antitumor CD8 T cells.[159] When administered into immunologically hot tumors, *E. coli* that synthesizes cyclic AMP reduced tumor growth and eliminated tumors.[15]

Tumor-associated antigens

Several strains of *Listeria* have been engineered to deliver tumor-associated antigens (TAAs) to trigger antitumor immune responses (Figure 3G).[96,97,99,122,162] TAAs are overexpressed in cancers as a result of mutation or upregulated during tumorigenesis.[163] For example, two bacterially delivered TAAs, mesothelin and Mage-b, are overexpressed in many breast, bladder, renal, colorectal, and lung cancers.[163] After systemic injection, *Listeria* selectively colonize tumors and draining lymph nodes.[99] Because of their native physiology, *Listeria* invade and accumulate in APCs (Figure 2).[86] Therapeutic strains have been further engineered to prevent invasion into epithelial cells and focus delivery to these immune cells.[98] In the tumor-draining lymph nodes, bacterially delivered TAAs are processed by APCs (macrophages and dendritic cells) and presented by MHC class I on the cell surface (Figure 3G).[99] This antigen cross-presentation activates CD4 and CD8 T cells and simulates antitumor immune responses.[162,164,165] The activated CD8 T cells infiltrate tumors, produce IFNγ, and convert the microenvironment from immunosuppressive to pro-inflammatory.[99] Upregulation of TLR and NLR agonists with the delivering bacteria increases this T cell response.[164] In humans, delivery of mesothelin is well tolerated and induces production of mesothelin-specific CD8 T cells.[96,97] In animal models, *Listeria* delivery of Mage-b kills mammary tumor cells and reduces metastases.[122,162]

Encoding nuclear material

An alternative to direct delivery of cytotoxic molecules or immunostimulatory ligands is delivery of nuclear material to control gene expression (Figure 3H).[166–168] Vectors have been developed to deliver either encoding DNA or small interfering RNA (siRNA).[163,166,168] Whereas delivered siRNA silences gene expression,[167] bacterial delivery of DNA can restore the function of genes that were altered during transformation.[168] For example, *Salmonella* delivery of the p53 tumor suppressor gene reduces the viability of bladder cancer cells.[168] *Salmonella* vectors have also been developed to silence the expression of programmed death-1 (PD-1)[167] and signal transducer and activator of transcription-3 (STAT3).[169,170] In mice, repressing PD-1 increases the immune response to chloroquine and inhibits the growth of colon cancer metastases.[167] Similarly, *Salmonella* repression of STAT3 activates intratumoral CD4 and CD8 T cells, which inhibited the growth of tumors and metastases.[169,170]

MOLECULAR DELIVERY

Molecular delivery is a critical component of most bacterial therapies.[141] Many delivery systems have been designed to deliver molecules inside cells and into the extracellular environment of tumors.[4–7,14] Introduction of delivery mechanisms has vastly improved efficacy and shifted bacterial therapies from purely immune stimulators to tumor-targeted vectors. There are two predominant systems for extracellular delivery: protein secretion and externally triggered lysis.[5–8,14,67,68] Many bacterial toxins are naturally secreted by a variety of secretory systems.[8,14,68] Alternately, bacteria can be lysed to release proteins into the environment. Lysis can be triggered by promoters that sense the local environment or bacterial density. A quorum-sensing system has been developed that releases therapeutic molecules when the bacteria have colonized tumors and grown to a sufficient density.[5–7,29]

Because bacteria do not naturally release proteins into mammalian cells, an engineered mechanism was created to deliver therapies intracellularly (Figure 2, bottom). To deliver into cancer cells, our group developed a system that repurposed native mechanisms in *Salmonella*.[4,171] We coupled the *Salmonella* promoter, *PsseJ*, with a suicide gene, lysin gene E (*lysE*) from phage phi X174.[4] After invading cells and forming SCVs, detection of the vacuolar environment activates the *PsseJ-lysE* circuit. Subsequent bacterial lysis releases the molecular cargo into the cytoplasm. This lysis system is specific for *Salmonella* because it utilizes a SPI-2 promoter (*PsseJ*) that detects the SCV environment.[4,49] We have shown that this release mechanism can deliver proteins and nanobodies that target pathways in both the cytoplasm and the nucleus.[4,171]

Expression of LLO is another mechanism for intracellular delivery.[85–87,89,172] Both *Listeria* and *Salmonella* naturally invade epithelia cells. When inside cells, expression of LLO forms pores in vacuolar membranes that cause bacteria to translocate to the cytoplasm.[173,174] There, the bacteria lyse and release therapeutic molecules to interact with cytoplasmic targets.[92,94,162,173,174]

FEATURES TO CONTROL SAFETY, COLONIZATION, AND IMMUNE RESPONSES

Genetic modification of the microbial chassis can improve tumor colonization, immune responses, and safety. We will focus on

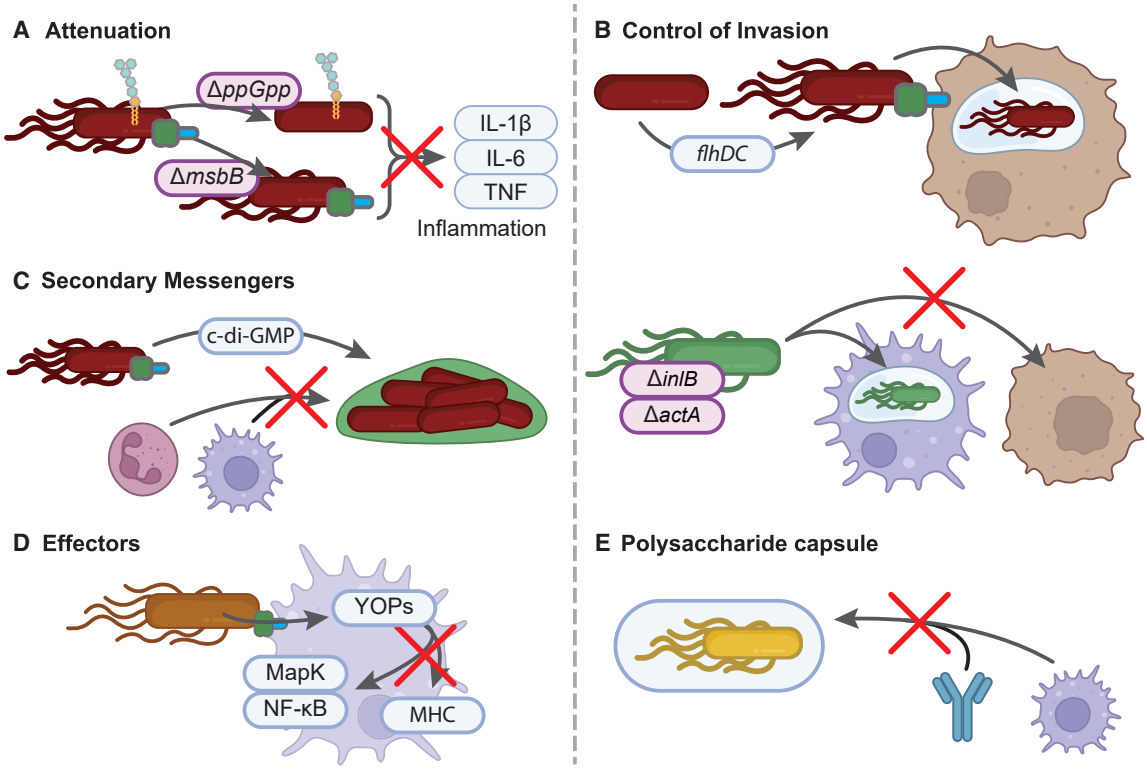

Figure 4. Features to control toxicity, delivery, colonization, and immunogenicity of bacterial therapies

(A) Bacteria can be attenuated by deleting genes (e.g., *ppGpp* or *msbB*) that control production of PAMPs (e.g., flagellin, T3SS, or LPS) and induce production of inflammatory cytokines.

(B) Invasion can be controlled in *Salmonella* by overexpression *flhDC*, which induces formation of structures (flagella and T3SS needles) that drive invasion. In *Listeria*, deletion of critical genes (*inlB* and *actA*) prevents invasion into epithelial cells.

(C) In extracellular bacteria, expression of secondary messengers (e.g., c-di-GMP or c-di-AMP) induces biofilm formation that prevents immune clearance.

(D) Bacterial injection of effectors (e.g., YopE, YopT, YopJ, YopP, or YopH) controls intracellular immunostimulatory pathways.

(E) Formation of a polysaccharide capsule prevents deposition of complement, binding of antibodies, and clearance by phagocytic cells.

five main feature types: (1) attenuation, (2) control of invasion, (3) secondary messengers, (4) bacterial effectors, and (5) polysaccharide capsules (Figure 4).

Attenuation

Two strategies have been used to attenuate therapeutic bacteria: deletion of toxic molecules and metabolic auxotrophy (Figure 4A). With wild-type bacteria, cytokine release syndrome (CRS) is the most common cause of toxicity.[22,23] The production of PAMPs by these bacteria activates the TLR and NLR signaling pathways and induces cytokine release.[151,154] In many engineered strains, modulating ligand expression reduced CRS and improved tumor colonization.[3,73] Two of the methods to attenuate bacteria modify the production of LPS (Δ*msbB*),[72,74,175] or flagellin and T3SS (Δ*ppGpp*).[71,160,176] The deletion of *msbB* prevents the addition of the myristoyl fatty acid moiety to the lipid A component of LPS.[177] Compared with other lipid A mutants, *msbB* does not affect bacterial growth.[177] LPS from Δ*msbB* bacteria does not activate TLR4, reduces TNF production, and diminishes virulence 10,000-fold.[72,74]

Guanine tetraphosphate (*ppGpp*) is an alarmone that activates expression of flagella, SPI-1 T3SS, and SPI-2 T3SS.[160] During starvation, *Salmonella* produces *ppGpp* to promote escape from the nutrient-deprived lumen and invasion of gut epithelial cells.[176] Deletion of two genes (*relA* and *spoT*) that are necessary for its synthesis eliminates production of *ppGpp* and significantly reduces bacterial virulence.[178] Both of these deletions also improve tumor selectivity and enable extracellular delivery to tumors.[72,74] Because Δ*relA* and Δ*spoT* prevent expression of SPI-1 T3SS, Δ*ppGpp* bacteria are exclusively extracellular and cannot deliver intracellularly.[71] As platforms, both Δ*msbB* and Δ*ppGpp* bacteria have safely delivered immunogenic and cytotoxic proteins to tumors.[8,14,69]

Making therapeutic strains auxotrophic for essential nutrients is another form of attenuation that improves bacterial safety.[179] Auxotrophic bacteria have been developed that are unable to synthesize aromatic metabolites, BCAAs, purines, or tryptophan.[77,179–181] The ΔaroA *Salmonella* mutant was developed in the 1980s as a vaccine vector.[180] This deletion prevents the synthesis of aromatic metabolites, e.g., paraaminobenzoic acid and dihydroxybenzoic acid.[180] The A1-R strain of *Salmonella* was developed by selecting for non-toxic BCAA auxotrophs that colonize tumors.[77] The inability to make aromatic compounds or BCAAs limits DNA repair and diminishes cell wall integrity.[182] Because these metabolites are not available in mammals, auxotrophy compromises bacterial fitness in healthy tissue.[179,181] It has less of an effect in tumors, where innate immune cells (e.g., macrophages and neutrophils) are less active.[181] The

purI deletion was developed to improve the safety and tumor colonization of Δ*msbB Salmonella*.[72] A more recent tryptophan mutant was generated by deleting the two genes for tryptophan synthase (*trpA*) and anthranilate synthase (*trpE*).[181] This tryptophan auxotroph is avirulent and effectively colonizes tumors.[181] All of these auxotrophic mutants have improved safety and pharmacokinetic profiles.[70,74,175,179,181]

Control of cell invasion

To increase safety and selectivity, gene circuits that control cell invasion have been coupled intracellular delivery mechanisms (Figure 4B).[4,16,49,92,97] In *Salmonella*, the master regulator *flhDC* controls both motility and cell invasion.[4,49] Combining the inducible *PBAD-flhDC* circuit with the *PsseJ-lysE* delivery circuit controls the timing of cell invasion and protein delivery.[4] After systemic injection, un-induced *Salmonella* grow exponentially in tumors, while clearing from healthy tissue.[4] At maximum colonization, induction of *PBAD-flhDC* triggers cell invasion, which focuses delivery to tumors and increases safety by preventing delivery to healthy tissues.[4] In *Listeria*, deletion of *InlB* and *actA* focuses antigen delivery to APCs.[98] The first deletion, *InlB*, prevents *Listeria* from invading epithelial cancer cells.[98,183] The second deletion, *actA*, prevents intracellular bacteria from spreading to neighboring cells, which confines the bacteria to phagocytic, tumor-associated APCs.[98] In *Salmonella*, deletion of *ppGpp* enhances delivery by preventing cell invasion and focusing delivery to the extracellular space.[8,14]

Secondary messengers

Bacteria secrete secondary messengers to adjust to changing environments and control immune responses after infection (Figure 4C).[160,161] Secondary messengers promote bacterial survival by either stimulating or suppressing immune responses.[160] Two common secondary messengers are *ppGpp* and c-di-GMP.[161] Overexpression of *ppGpp* (or other alarmones) is purely stimulatory because it increases production of PAMPs (e.g., flagellin and T3SS) and triggers TLR-dependent immune responses.[151,160] Cyclic di-nucleotides both stimulate and repress immune response depending on bacterial location.[155] Intracellularly, cyclic di-nucleotides bind the STING adaptor[155] and trigger antimicrobial cytokine expression,[15] as described in the therapeutic section above. In the extracellular space, bacteria use these messengers to signal each other to avoid immune clearance.[161] In *E. coli* and *Salmonella*, c-di-GMP activates cellulose synthase to form biofilms that protect against antimicrobial compounds.[184] Biofilms provide a physical barrier against clearance by neutrophils, phagocytes, or complement.[184] Cyclic di-GMP also binds YcgR, a flagella brake, to switch from a motile to a sessile lifestyle.[185] *Listeria* and other Gram-positive bacteria produce a similar secondary messenger, c-di-AMP, that regulates bacterial growth and virulence.[160] Unlike c-di-GMP, *Listeria* express a single protein (DacA) that synthesizes c-di-AMP.[27] These bacteria also produce biofilms in tumors.[186] Downregulating expression of cyclic di-nucleotides reduces biofilm production and increases bacterial recognition by innate immune cells.[186]

Bacterial effectors

Another feature that controls colonization and immune responses are bacterial effectors (Figure 4D). After cell invasion, facultative bacteria (*Salmonella* and *Yersinia*) secrete T3SS effectors into the cytoplasm to enhance nutrient acquisition and protect against clearance by the immune system.[25,187] Most effectors promote survival by suppressing immune responses.[188] However, several effectors promote survival by stimulating immune cells.[25,31] For example, the T3SS effector SipA, SopE, SopE2, and SopB induce production of inflammatory chemokines that attract neutrophils.[25,187] *Salmonella* without a functional T3SS do attract RNS-producing neutrophils and do not colonize the epithelial niche as well as wild-type *Salmonella*.[25]

Although *Yersinia* is mostly an extracellular pathogen, it also secretes effectors into cells that attenuate and dismantle immune responses (Figure 4D).[100] Effectors YopE and YopT inhibit macrophage phagocytosis by preventing actin cytoskeletal rearrangements within immune cells.[105] Similarly, YopJ and YopP prevent the secretion of pro-inflammatory cytokines by inhibiting MapK and NF-κB signaling.[101,189] Another effector, YopH, targets the antigen presentation pathway, reduces MHC expression, and prevents T cell receptor activation.[102] YopH is a phosphatase that specifically dephosphorylates tyrosine residues.[102] Because tyrosine is an integral amino acid in the T cell receptor and costimulatory CD80/CD86 ligand, dephosphorylation of tyrosine blunts the adaptive immune response.[102] Because of these effectors, *Yersinia* is particularly effective at inhibiting antimicrobial immune responses.[101,102,104,105,189]

Polysaccharide capsules

Most bacteria express polysaccharide capsules on their outer membrane as a physical barrier to inhibit extracellular clearance (Figure 4E).[190] For bacterial cancer therapies, control of capsular polysaccharide (CPS) synthesis reduces immune detection and improves tumor colonization.[3] CPS is composed of sugar molecules and an inner lipid core (lipid A) that is covalently bound to negatively charged, externally facing polysaccharide chains.[190] The negatively charged polysaccharide coat repels the negatively charged membrane of phagocytes and inhibits internalization and subsequent degradation.[190] In the intestines, commensal bacteria express CPS to facilitate a healthy interaction with the host.[191] In this symbiotic environment, the CPS shields bacteria from immune detection and gastrointestinal inflammation.[191] The CPS also shields against antibody and complement deposition on the cellular surface.[190] Because the sugar molecules in the capsid (chondroitin and heparosan) are natively found in humans, the body does not produce strong humoral immune responses to encapsulated bacterial.[190] Encapsulation blunts any activation of complement-mediated bacterial clearance in the blood as well as opsonization-dependent phagocytosis by macrophages in the spleen and liver.[190]

Modulating polysaccharide encapsulation increases bacterial tolerance and tumor colonization.[3] Overexpression of O-antigen and CPS inhibits immune detection and persistence of bacteria *in vivo*.[190,192,193] Controlled expression of CPS in *E. coli* Nissle 1917 increased the tolerance in mice 10-fold.[3] When the CPS system was induced, the engineered *E. coli* disseminated from tumors and colonized uninfected lesions.[3] At the completion of therapy, repression of CPS reversed polysaccharide encapsulation and unmasked the bacteria to the immune system.[3] This control of CPS cleared the bacteria and prevented chronic infection.[3]

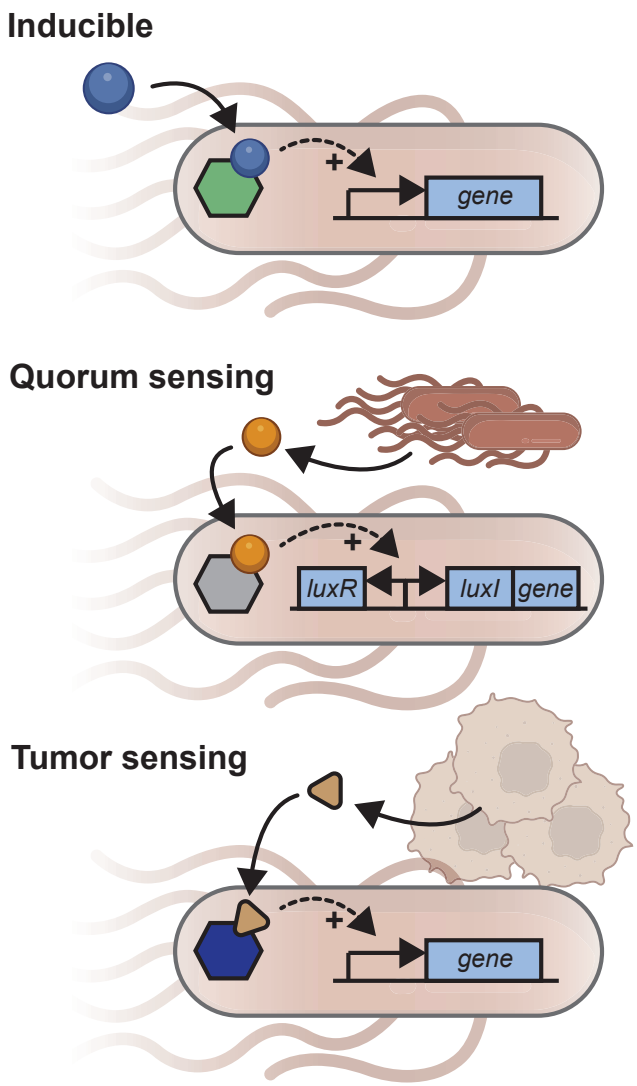

Figure 5. Genetic circuits in bacterial therapies
(A) Inducible circuits trigger gene expression in response to external molecules (e.g., arabinose or IPTG).
(B) Quorum-sensing circuits trigger gene expression in response to bacterial density. The LuxI enzyme produces an autoinducer (orange). Binding of the autoinducer to LuxR leads feedforward amplification and expression of genes.
(C) Tumor-sensing promoters respond to molecules in tumors (e.g., lactate) or the absence of molecules in tumors (e.g., oxygen).

GENE CIRCUITS

Several novel gene circuits have been used to improve the safety and tumor selectivity of bacterial therapies (Figure 5).[5,28,194] Tumor selectivity increases safety by preventing expression in the plasma and in healthy tissue.[194] In turn, selectivity improves efficacy by increasing the concentration of therapeutics at tumor sites.[67,141] Three types of complex genetic circuits (inducible, quorum-sensing, and tumor-sensing) focus gene expression to tumors by (1) controlling the timing of gene expression (Figure 5A), (2) detecting the local bacterial density (Figure 5B), or (3) activating in the tumor microenvironment (Figure 5C).

Another common type of circuit utilizes constitutive promoters. These circuits are a simple way to express features and therapeutic proteins. Unlike more complex promoter systems, genetic circuits built with these promoters do not provide spatial control of expression. Despite this limitation, they are useful in many circumstances because of their simplicity. For example, constitutive promoters can express molecules that (1) are not toxic to healthy tissue, (2) do not escape the bacterial membrane until in neoplastic tissue, (3) are not active until processed in the tumor environment, or (4) are dependent on the expression of partner molecules that are controlled by tumor-selective systems.[4,6,7]

Inducible genetic circuits

Inducible genetic circuits provide a simple method to control the location of gene expression (Figure 5A). These systems control localization by timing gene induction. Systemically injected bacteria clear rapidly from the blood and clearance organs but grow exponentially in tumors.[1,121] Inducing gene expression when the difference in densities is greatest focuses gene expression on tumors.[195] The simplest and most extensively explored systems respond to chemical inducers. For example, the *PBAD* promoter and the *lac* operon induce gene expression in response to the local concentrations of arabinose and isopropyl β-D-1-thiogalactopyranoside (IPTG).[3,4,14] Multiple groups have shown that these chemically inducible promoters focus delivery to tumors and prevent delivery to healthy tissue.[4,8] A limitation of chemically induced promoters is that they require at least one additional injection of the inducer molecule. To be used therapeutically, however, inducible promoters would have to respond to clinically acceptable molecules. For example, arabinose is not appropriate because it is prevalent in many foods, and IPTG has not been approved for use in humans.

Another class of inducible circuit responds to fields and forces that pass through tissue. Examples include the *PrecA* promoter and the PL and PR tandem promoter that respond to radiation and ultrasound.[67,196] Ultrasound waves are highly focused, penetrate deep into tissue, and generate local hyperthermia.[197] Engineered bacteria detect the warmed tissue with thermally inducible genetic circuits.[196,198] Ultrasound induction has effectively delivered IFNγ[196] and immune checkpoint blockade (ICB) nanobodies[196] in tumors. Similarly, ionizing gamma radiation is focused and penetrates tissue.[199] In bacteria, radiation causes dsDNA breaks, which activate the *PrecA* promoter.[67] In mice, this radiation system induced bacterial gene expression and reduced tumor growth.[67]

Quorum sensing

Quorum-sensing systems focus expression on tumors by sensing the local bacterial density (Figure 5B).[5,194] Because the immune system clears bacteria from healthy organs, only tumors have high-density colonies after systemic injection.[194] The most common quorum-sensing system used with bacterial therapeutics is the *LuxI-LuxR* system from *Vibrio fischeri*.[5–7,29] *V. fischeri* uses this circuit to regulate the production of bioluminescent proteins.[200] The system is composed of two genes (*luxI* and *luxR*) and a bidirectional promoter (Figure 5B).[194] In bacteria, the LuxI enzyme constitutively produces a small amount of homoserine lactone autoinducer. At high bacterial

Example	Chassis	Therapy	Delivery	Features	Genetic Circuits
1 	Salmonella	Caspase-3	Intracellular lysis	Δ*msbB* attenuation, Controlled invasion	*PsseJ-lysE, PBAD-flhDC*
2 	Salmonella	ClyA	Extracellular secretion	Δ*ppGpp* attenuation	*PBAD-clyA*
3 	*E coli*	CD47 Nanobody	Extracellular lysis		*PluxR-lysE* (quorum sensing)
4 	Listeria	TAA (Mage-b)	Intracellular	Controlled invasion	Constitutive expression
5 	*E. Coli*	Arginine synthesis	Extracellular		*PfnrS* promoter (hypoxia inducible)

Figure 6. Examples of built bacterial therapies

densities and high autoinducer concentrations, the autoinducer binds LuxR, which induces expression of more LuxI, LuxR, and the therapeutic protein of interest (Figure 5B).[194] As a tool, quorum-sensing circuits can be used in most strains of therapeutic bacteria. Similar density-sensing systems are native to each of these bacteria.[201] In their natural environments, bacteria use quorum sensing to regulate motility, metabolism, and biofilm formation.[202] For cancer therapy, quorum-sensing circuits have been used to focus the expression of both features and therapies (Figure 1). In *Salmonella*, for example, quorum-sensing circuits have been used to deliver the alpha toxin from *Staphylococcus aureus* to tumors, while preventing delivery to healthy tissues (e.g., liver).[194] Quorum-sensing circuits have also been used to control lysis genes in *E. coli* so that therapies are only released in the extracellular space of tumors.[5–7]

Tumor-sensing promoters

Many bacterial species contain promoters that activate gene expression based on the local environment (Figure 5C). To identify promoters that specifically activate in the tumor environment, a *Salmonella* promoter library was created, and lead candidates were injected into tumor-bearing mice.[203] Most promoters identified in this library responded to three tumor characteristics: hypoxia, pH, or lactic acid.[203] Two of the promoters, *PfrdA* and *PglpA*, activated about 1,000 times in hypoxic conditions.[203] As an extension of this discovery, the hypoxia-activated *PfnrS* promoter was used in *E. coli* to drive expression of a STING agonist in tumors without inducing systemic toxicity.[15] Because these promoters are largely conserved,[204] they can be used with several therapeutic strains of bacteria. To amplify tumor specificity, multiple tumor-sensing promoters can be coupled with logic AND gates.[28] To demonstrate this, linking lactate- and hypoxia-sensing promoters with an AND gate increased tumor selectivity by two orders of magnitude.[28] In a clinical setting, this type of logic gate could be used to enhance safety by tailoring gene expression to a patient's specific tumor characteristics.

BUILD-A-BUG EXAMPLES

Several bacterial therapies have been created using multifaceted design strategies similar to the build-a-bug strategy that we have outlined here.[4,6,7,14,122,123,162] Five examples illustrate the modular nature of bacterial design (Figure 6). These examples show how the complexity and plasticity of bacteria enable integration of delivered therapies, immune modulatory features, and genetic control circuits into different bacterial vectors.

Example 1: Intracellular delivery of caspase-3 with *Salmonella*

Engineered *Salmonella* with the *PsseJ-lysE* circuit was used to intracellularly deliver CT caspase-3 (Figure 6).[4] In addition to *PsseJ-lysE*, this strain contained the *PBAD-flhDC* circuit to control cell invasion and the timing of delivery.[4,49] When this therapy was intravenously injected into tumor-bearing mice, the bacteria accumulated in tumors and cleared from healthy tissue.[2] At the apex of this density difference, intraperitoneal injection of arabinose induced *flhDC*, which triggered the production of SPI-1 T3SS and flagella, and initiated cell invasion.[4,49] After invading cancer cells and residing in SCVs, activation of the *PsseJ* promoter drove expression of the *lysE* suicide gene. Bacterial lysis released the protein payload (caspase-3) into the cytoplasm of cancer cells.

This intracellular delivery of active caspase-3 killed cancer cells and reduced tumor volumes.[4] Selective delivery into cancer cells spared healthy tissue and did not affect mouse health.[4] This example demonstrates the modularity of bacterial therapies (Figure 1). It contains two features (intracellular delivery and controlled invasion), has two genetic circuits (*PsseJ-lysE* and *PBAD-flhDC*) that control the location of expression, and delivers a therapeutic that is only functional intracellularly (caspase-3).

Example 2: Extracellular delivery of ClyA with *Salmonella*

Another bacterial therapy used therapeutic *Salmonella* to deliver ClyA (Figure 6).[14] ClyA is an extracellular pore-forming toxin that is natively secreted by *Salmonella*. The engineered strain of *Salmonella* was attenuated by the deletion of *ppGpp* signaling.[71,160] In addition to removing toxicity, this attenuation limited cell invasion by preventing expression of SPI-1 T3SS and flagella.[4,49,71,76] Because ClyA acts on the cell membrane, the strategy required extracellular delivery. The arabinose-inducible promoter PBAD controlled expression of ClyA. After injection of this strain into mice and clearance from healthy tissue, systemic injection of arabinose induced ClyA production and secretion.[14] In mice, this therapy prevented growth of pancreatic tumors and did not affect the health of the mice for the entire course of treatment.[14]

The extracellular and intracellular delivery of cytotoxic molecules (examples 1 and 2), were built on the fundamental infection/invasion mechanisms of *Salmonella*. In both cases, the selected cytotoxic therapy informed which genetically engineered features were necessary for effective therapeutic delivery.[4,14] Because ClyA acts on the cell membrane, it required extracellular delivery. In comparison, caspase-3 is a human

molecule that requires intracellular delivery.[136] Regulating SPI-1 T3SS and flagella expression is a key feature that enables either extracellular or intracellular delivery.

Example 3: Delivery of immunotherapeutic nanobodies with *E. coli*

Engineered *E. coli* Nissle 1917 were used to deliver immune checkpoint and CD47-inhibiting nanobodies and create a focused response against tumors (Figure 6).[6,7] Systemic delivery of ICB can cause severe side effects and result in premature discontinuation of the therapy in cancer patients.[205] Another immunotherapy, magrolimab (anti-CD47), has shown similar promise[206] but also has off-target side effects.[207] To increase tumor-selective delivery, ICB and anti-CD47 nanobodies were delivered with an *E. coli* strain with a quorum-sensing circuit (Figure 6). This circuit controlled bacterial lysis to ensure that nanobodies were only released into tumors with dense bacterial colonies.[5,29] The *E. coli* expressed therapeutic levels of nanobodies without affecting bacterial fitness.[6,7] Because this strain of *E. coli* cannot invade cells, all delivery was extracellular, which was critical for these nanobody therapies. The PD-1, CTLA-4, and CD47 receptors are all located on the outer surface of cells.[208] In animal models, this strategy cleared tumors and generated antitumor immunity but did not produce systemic toxicity, as was seen with systemic delivery.[6,7] In addition, this strain delivered multiple rounds of therapy because the bacteria regrew in the tumors multiple times after a single intravenous injection.[7]

Example 4: Intracellular delivery of tumor antigens with *Listeria*

Live-attenuated *Listeria* were used to deliver TAAs (mesothelin and Mage-b) into cancer-associated APCs (Figure 6).[96,97,99,122,162] The *InlB* and *actA* deletions in the live-attenuated, double-deleted (LADD) strain confined delivery to APCs and prevented invasion of epithelial cancer cells.[98,183] Native expression of LLO causes vacuolar escape, cytoplasmic delivery, and antigen cross presentation on MHC class I.[98,99] Cross presentation of TAAs by APCs is the primary mechanism for activating cognate CD8 T cells and generating adaptive responses against antigens.[99,122,162] When administered to tumor-bearing mice, LADD *Listeria* delivered the TAAs into tumors and generated systemic antitumor immune responses.[122] Systemic delivery reduced the metastatic dissemination of aggressive 4T1 breast cancer.[162] Delivery of TAA into the cytoplasm of APCs was required to induce a CD8 T cell antitumor immune response in immunologically insensitive tumors.[165] This observation demonstrates the unique ability of *Listeria* to sensitize the immune system against both primary and metastatic tumors.

Example 5: Immune-stimulatory arginine synthesis with *E. coli*

E. coli (Nissle 1917) was engineered to revert immunosuppression in tumors by converting ammonia into arginine.[123] Immunosuppressive tumors overexpress arginase, which degrades arginine[209] and impairs T cell survival.[210] An arginine-producing strain was created by deleting the *argR* repressor, which regulates all genes in the arginine synthesis pathway, and incorporating $ArgA^{fbr}$, a mutant enzyme that is not repressed by high levels of arginine.[123] The expression of this feedback-resistant version of *argA*, was restricted to tumors using the hypoxia-inducible, *PfnrS* promoter.[15] Eliminating these two feedback mechanisms significantly increased arginine concentrations in tumors.[123] These engineered bacteria increased the number of TNF-producing T cells, increased the efficacy of ICB, and induced a curative response.[123] This example illustrates an important principle of metabolic manipulation: it is not necessary to encode the entire synthesis pathway. For arginine, this pathway contains eleven genes.[211] Elimination and modification of key regulatory checkpoints (i.e., *argA* and *argR*) can turn natural pathways toward therapeutically useful forms.

NEW MICROBES AND THERAPIES FROM THE GUT

The gut microbiome provides an ever-expanding resource to discover new therapeutics and the bacteria to deliver them. Although model organisms like *E. coli*, *Salmonella*, and *Listeria* have been heavily studied, there are a wide variety of novel microbes that have yet to be characterized. Understanding how these gut organisms activate and suppress tumorigenesis at distant sites[114,212] could identify microbial mechanisms that inhibit tumor growth. Identifying signals (e.g., rho GTPases) that connect the gut microbiome to immune responses in tumors would improve the efficacy of immune therapy.[46,213] Because many organisms of the microbiome are symbiotic with immune cells,[213] identifying their tolerizing mechanisms could improve the safety of bacterial therapies. New attenuation strategies could eliminate the need to remove bacteria after therapy and could reduce safety concerns in clinical trials.[214] Discovering new organisms that colonize tumors[215] without promoting disease progression[44,115] would expand the number of therapeutic strains available for cancer treatment and would accelerate late-stage clinical development.

Conclusions

A resurgent interest in the gut microbiome has paralleled the expansion of bacterial cancer therapies. Incorporation of key discoveries about how microbes affect cells, tumors, and immune responses will propel the development of a diverse set of therapies. Many of these discoveries will be solutions to the fundamental limitations of bacterial therapy. Careful sifting through interactions of microbes with epithelial and immune cells in the intestines could identify powerful new mechanisms. Repurposing overlooked effectors and metabolites in the microbiome would be easier to develop into microbial therapies than traditional molecules. Development of new strains and synthetic circuits will improve pharmacokinetics and safety profiles. Optimized incorporation of immunological and cytotoxic therapeutics will improve treatment efficacy. To assemble these parts in an organized way, we posit the build-a-bug framework. The modularity of bacterial chassis suggests that there is a vast space of unexplored therapies with unique combinations of therapeutic molecules, control circuits, and immune-modulating features. Prior work hints at the breadth of this potential. Microbial strategies have utilized an array of organisms to deliver cytotoxic and immune-stimulatory molecules that are effective against most cancer indications. With enhanced understating of microbial physiology, this potential will expand, and bacterial therapy will become a major pillar of cancer therapy.

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DECLARATION OF INTERESTS

V.R., N.V.D., and N.S.F. are co-founders and shareholders of Ernest Pharmaceuticals, Inc.; V.R. and N.V.D. are employees; and N.S.F. is a member of its Scientific Advisory Board.

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