Engineering Cell-Based Systems for Smart Cancer Therapy

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Due to the difficulty of targeting systemically delivered therapeutics for cancer, interest has grown in exploiting biological agents to enhance tumor accumulation and mediate localized drug delivery. Equipped with onboard sensing and active motility, some cells respond to specific cues of the tumor microenvironment, making them ideal candidates for smart cancer therapy. Herein, recent progress and developments are presented on the use of four of the most promising cell-based systems for tumor targeting and drug delivery—immune cells, stem cells, platelets, and bacteria. Strategies to further enhance specificity at the tissue and cell level are discussed, including genetic engineering, chemical cell surface modification, and the use of external physical stimuli. With crucial ongoing efforts addressing the safety and efficacy of living intelligent therapeutics, a new era of cancer medicine is on the horizon.

1. Introduction

Functional nanomaterials continue to hold great promise in cancer therapy for overcoming delivery challenges posed by the administration of conventional drugs. They have been designed to surmount physiological barriers at several scales, improving biodistribution, enhancing accumulation in the tumor microenvironment (TME), targeting specific cells, and influencing intracellular trafficking.[1,2] Nevertheless, the expectations surrounding nanoparticle (NP) drug carriers have remained largely unfulfilled due to heterogeneity among cancer types and diffusion-limited transport in tumors.[3–5] Viable quiescent cells in deeper parts of the tumor, which significantly contribute to drug resistance and clinical relapse, are particularly hard to reach by systemically administered therapeutics, and external radiotherapy is also less effective in the poorly oxygenated tumor core.[6,7]

Due to the difficulties faced in drug delivery, interest has grown in exploiting biological agents able to actively migrate to tumors.[8,9] Some cell types are naturally equipped with encoded onboard sensing that allows them to autonomously follow cues of the cancer environment, and intercellular communication can further enhance their tumor homing.[10,11] Once a tumor is reached, innate toxicity, codelivered drugs, or toxins secreted on-site can initiate a therapeutic response. These features allow cells to be recast as programmable living vehicles with the potential to contribute toward the ultimate goal of cancer treatment—the selective and complete eradication of cancer cells with minimal off-target effects.[12]

Several types of prokaryotic and eukaryotic cells have been identified as candidates for cell-based cancer therapeutics, with a few strains of bacteria and immune cells commonly favored by investigators.[13] The idea to use bacteria to treat cancer dates back to the late 19th century, when the bone surgeon William Coley observed tumor regression in cancer patients with skin infections.[14] At the tissue level, bacteria are capable of deep penetration into the tumor, relying on their own flagellar propulsion for motility.[15] Today, tools offered by synthetic biology have made it possible to engineer bacteria for cancer therapy with safe and specific functionality.[16] Coley’s experiment provided another lesson that led to his moniker as the “father of immunotherapy.” Immune cells can be exploited in the fight against cancer, as they have evolved as key players in virtually any battlefield throughout the body. Their capability to actively migrate to tumors and orchestrate complex responses renders them ideal candidates as another class of anticancer agents.[6,17] Similarly, stem cells, with their substantial contribution to tissue regeneration, are also capable of homing to sites of inflammation such as tumors.[18] Low-immunogenicity further facilitates exploitation of these cells for drug delivery applications. Mesenchymal stem cells (MSCs) also modulate immune response in damaged tissue, motivating the reengineering of these multipotent cells.[13,19]

Lastly, platelets are another cell type that can function as an intelligent cell-based delivery system. These anucleate cells circulating in blood do not possess the biological machinery for reproduction and active migration, but their close interactions with cancer cells, whether in primary tumors, circulating tumor cells (CTCs), or in metastases, have made them attractive candidates for drug delivery.[20–22] Their role in vascular integrity and immunomodulation is linked with their selective activation in the presence of cancer and their blood-borne nature provides improved drug pharmacokinetics with minimal immunogenicity.[23]

In this review, we survey cell-based agents capable of active, intrinsic homing to tumor sites. We summarize the

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fundamentals of tumor tropism in each of these cell types, focusing on the involved biological pathways. We highlight the limitations of relying on intrinsic homing in cell-based therapeutics, and discuss technological advances and strategies to improve homing at the tissue scale and enhance targeting at the cellular scale. Following successful homing and recognition of the target, we summarize different forms of therapeutic action that can be exerted by these cell-based platforms in cancer therapy. Focus is placed on covering studies that showed augmented therapeutic effects when cells are engineered to carry drug cargo or genetically modified to produce therapeutic substances. Finally, the review discusses current limitations and outlook for cell-based delivery systems.

2. Tumor Homing

Effective means to transport drug cargo to deep seated tumor sites constitutes the first crucial step in cancer therapy. Certain living cells equipped with onboard sensing and motility may serve as intelligent anticancer agents as they are capable of migrating to and penetrating tumors. The precise sensing mechanism and mode of motion depends on the cell type and is heavily affected by their physiology and behavior in the presence of cancer cells, which is described in Section 2.1 according to cell type (Figure 1). In vivo, however, this intrinsic homing capacity is often hindered by physiological barriers at the tissue level, and in addition suffers from limited anticancer specificity at the cellular level. As such, various strategies have been developed, including the use of external cues for guidance (Section 2.2) and leveraging synthetic biology for enhanced specificity (Section 2.3), to overcome these challenges.

2.1. Intrinsic Tumor Homing Mechanisms

2.1.1. Immune Cells

A variety of innate and adaptive immune cell subpopulations are known to actively migrate toward the tumor niche, making them attractive vectors for the delivery of therapeutic agents. Extravasation and entry into peripheral tissues unfolds along an orchestrated sequence of events consisting of four steps: rolling, activation, arrest, and transmigration. Once in the tumor, they exhibit two distinct modes of motion: 1) mesenchymal migration which is an adhesion-mediated strategy where cell edges protrude, adhere, and detach alongside proteolytic degradation of the extracellular matrix (ECM), and 2) amoeboid migration which is faster and mainly independent of adhesion and proteolysis of the ECM.[24]

Neutrophils are among the first immune cells recruited to sites of inflammation, where they phagocytose pathogens and release cytokines to recruit other leukocytes.[25,26] Neutrophils extravasate and then move toward tumors in response to inflammatory chemokine signals secreted by cancer and stromal cells. First, neutrophils undergo rolling by transiently attaching and detaching to the endothelium through interactions between P-selectin glycoprotein ligand 1 (PSGL-1), CD44, E-selectin ligand-1 (ESL-1), and L-selectin expressed by neutrophils and endothelial P- and E-selectins.[27,28] Chemokines induce a change in conformation of integrins on the neutrophil cell surface.
shifting from low to high affinity which facilitates cell arrest.[29] Next, cell arrest is mediated by interactions between α4β7, αβ1, and αβ2 integrins on the neutrophil and endothelial intercellular adhesion molecule 1/2 (ICAM-1/2), vascular cell adhesion molecule 1 (VCAM-1), and mucosal vascular addressin cell adhesion molecule 1 (MadCAM-1).[30] Transmigration is then facilitated by interactions between platelet endothelial cell adhesion molecule (PECAM-1), αβ1 integrin, and αβ2 integrin on the neutrophil, and endothelial PECAM-1, ICAM-1, and VCAM-1.[30] The migration of neutrophils toward the TME is mediated by chemokine signals, such as CXCL1, CXCL2, and CXCL5, which are ligands for the CXCR2 receptor.[31,32] While high levels of tumor-associated neutrophils (TANs) have been associated with protumoral effects and a poor prognosis, TANs have also been shown to display functional heterogeneity which can also result in antitumoral responses.[33]

Unlike neutrophils, macrophages are largely tissue resident. They differentiate from circulating monocytes after extravasation, and patrol peripheral tissues for invading pathogens, cell debris, and cancerous cells.[34,35] Macrophages home to tumors in response to a variety of chemoattractant signals, including colony-stimulating factor 1 (CSF1),[36] vascular endothelial growth factor (VEGF),[37] chemokine ligand 5 (CCL5),[38] and possibly chemokine ligand 2 (CCL2), although its role in cancer is under debate.[39-44] Macrophages can be categorized as either proinflammatory (M1 polarization), playing a role in the elimination of immunogenic cancer cells during carcinogenesis, or anti-inflammatory (M2 polarization) where their role is protumorigenic.[45,46] While the M1/M2 model has proved useful, transcriptomic analysis indicates that this paradigm is oversimplified and suggests, instead, that a spectrum of tumor-associated macrophages exists.[47]

Dendritic cells (DCs), like macrophages, are located in almost every tissue in the body and survey peripheral tissue for pathogen-specific signals.[48] DCs are specialized antigen-presenting cells (APCs) that play a crucial role in the priming and establishment of the antitumor adaptive immune response. Clinical trials have studied DC-based cancer vaccines using autologous DCs stimulated with tumor antigens to elicit an antitumor T cell response.[49-51] These studies have yielded promising results in melanoma cancer, and Sipuleucel-T is one example of a cell-based immunostimulant that has been approved by the U.S. Food and Drug Administration for use in the treatment of patients with prostate cancer.[52] During tumorigenesis, activated DCs migrate to lymph nodes where they prime CD4+ and CD8+ T cells through antigen presentation and appropriate costimulation. Activated T cells enter circulation from the lymphatic system, extravasate, and home to the tumor site, where they exert an effector immune response to eliminate immunogenic cancer cells. CD8+ T cells differentiate into cytotoxic T lymphocytes (CTLs) that exert an antitumoral response through the production of granules containing perforin and granzyme.[53,54] CD4+ T helper 1 (Th-1) cells orchestrate an antitumoral response through the secretion of proinflammatory cytokines such as interleukin-2 (IL-2), interferon gamma (IFN-γ), and tumor necrosis factor alpha (TNF-α), which promotes tumor antigen presentation, CTL cytotoxicity, and an antitumor response in macrophages.[55]

These intrinsic tumor homing capabilities have prompted the use of leukocytes as a new class of living vehicles for targeted payload delivery. Yet, the success of these approaches, particularly in the case of solid tumors, is highly dependent on the immunogenicity of cancer cells and the ability of the immune cells to infiltrate tumors. Tumors can be classified as “hot” or “cold” according to the degree of T cell inflammation.[56] Hot tumors have a high level of tumor-infiltrating lymphocytes (TILs), while cold tumors are defined by a failure of T cell penetration or a complete lack of T cell recruitment. As immunotherapy in its various forms, including checkpoint blockers, vaccines, and chimeric antigen receptor (CAR) T cell therapies, are aimed at enhancing the body’s innate antitumor immune response, the inability of T cells to infiltrate the tumor results in partial response rates or renders the therapy ineffective. As such, there have been efforts to develop strategies for improved T cell infiltration through the administration of antiangiogenic therapy,[57] oncogenic pathway inhibitors,[58,59] and transforming growth factor beta (TGF-β) inhibitors.[60,61]

2.1.2. Stem Cells

Mesenchymal stem cells (MSCs), also termed mesenchymal stromal cells, are a heterogeneous population of adult progenitor cells characterized by multilineage differentiation capacity and self-renewal capability.[62] They were first discovered in the bone marrow,[63] but have since been isolated from a vast range of tissues,[64] including dental pulp,[65] adipose tissue,[66] connective tissue,[67] and the umbilical cord.[68] Although MSCs may exhibit considerably different transcriptomic profiles depending on their tissue of origin,[69,70] they can be distinguished from other progenitor cells by the expression of a specified set of surface antigens and a capacity for trilineage differentiation toward adipocytes, chondrocytes, and osteoblasts.[71]

MSCs inherently home to damaged tissue where they play a key role in tissue repair and regeneration.[72] Like damaged tissues, tumors release chemoattractant signals that recruit MSCs to the tumor site. Once released into circulation, MSCs undergo a multistep process to migrate to the tumor. In the first step, selectins expressed by endothelial cells facilitate the attachment and rolling of MSCs along the vasculature wall. While the specific selectin used in this process is still under debate, P-selectin has been identified as a probable candidate.[73] MSC adhesion and rolling was induced on immobilized P-selectin and was suppressed after pretreatment of an endothelial mono-layer with an anti-P-selectin antibody.[73] However, as MSCs do not express PSGL-1,[73,74] the primary high affinity counterreceptor for P-selectin, other ligands have been investigated for their possible role in this interaction. CD24, a ligand for P-selectin-mediated rolling of myeloid cells and metastatic cancer cells,[75-77] was suggested as a possible ligand in adipose-derived stromal cells.[78] In another study, galectin-1 was identified as a possible mediator of the interaction of MSCs with P-selectin.[79]

Chemoattractant signals play a significant role in MSC activation and the CXCL12/CXCR4 axis has been widely studied as a mediator of MSC migration toward the tumor niche.[80-84] However, as not all MSCs express CXCR4,[85] it has been shown that a subpopulation of MSCs express CXCR7 which is another receptor for CXCL12.[86-88] Stimulation with CXCL12 triggers binding of cytoskeletal proteins, such as talin, to the integrin
β subunit cytoplasmic domain, inducing a shift from low to high affinity conformation.[89] Once activated, integrins, such as integrin α4β1, facilitate cell arrest by binding to VCAM-1 on the endothelial cell surface. MSCs then transmigrate between endothelial cells and secrete matrix metalloproteinases (MMPs), including MMP2[90] and MMP 14,[91] to break down the endothelial basement membrane.[92] Finally, MSC migration through the interstitium is mediated by ECM degrading enzymes such as MMP-2[93] and is guided by chemotactic signals from cytokines, such as interleukin-8 (IL-8),[94] and growth factors, such as TGF-β1 and platelet-derived growth factor (PDGF).[94,95] Within the TME, the paracrine function of MSCs affects tumor development by influencing angiogenesis, cell proliferation, cell viability, and immunomodulation. While some studies indicate that cross-talk between MSCs and tumor cells contributes to cancer pathogenesis,[96–101] evidence also supports the tumor suppressive effects of MSCs.[102–107] For instance, MSCs have been found to induce cell cycle arrest by inhibiting the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT) signaling pathway.[108,109] The discrepancies in these findings may be due to differences in isolation and culture methods, tissue sources, and experimental models and conditions.[110]

Despite their vast array of paracrine functions, the feature of MSCs that has been widely exploited in a therapeutic context is their innate ability to migrate to the tumor niche. While these efforts have illustrated the potential of MSC-based therapies, efficient delivery of MSCs remains a challenge. Successful MSC transductions require high doses to achieve sufficient amounts at the target site, as the bulk of intravenously administered MSCs are cleared from circulation or become trapped in lung capillaries.[111–114] As such, a variety of approaches have been explored to increase MSC accumulation, including guidance by external stimuli.

2.1.3. Platelets

Although platelets are small anucleate blood-borne cells lacking the active motility exhibited by mammalian cells and bacteria, their unique features make them an attractive candidate for another type of cell-based therapeutic platform.[17,115] These discoid cells are widely studied because of their critical role in hemostasis and wound healing.[116,117] Nature has evolved on/off switches in the physiology of platelets enabling them to respond only when activated. Von Willebrand factor (vWF), collagen, thrombin, and adenosine diphosphate (ADP) are considered as the main triggers for initiation and stabilization of the activation.[118,119] This switch and the specificity associated with it can serve as a homing mechanism when the goal is selectively targeting cells or tissues that trigger this activation. This paradigm, together with their role in vascular integrity, has led to the introduction of platelet-based drug delivery systems in wound healing and antithrombosis applications.[120,121]

Aside from the role of platelets in normal physiology, they can be “deceived” to function in favor of pathologies, with cancer being the most widely known.[122] This alliance was first reported by Armand Trouseau (1865) following observations of thrombocytosis and hypercoagulability in cancer patients.[20,123] This phenomenon is shown to be mainly driven by tumor-derived cytokines, interleukin-6 (IL-6) being the most important one, which trigger the growth of megakaryocytes, either directly or through thrombopoietins produced by the liver.[124,125] The resulting increased platelet count is linked with poor prognosis in cancer patients.[21] Platelets contribute to the inflammatory conditions in cancer, which also correlates to a poor prognosis. Sustained release of active TGF-β from platelets, driven by the catalytic role of thrombin, assists tumor immune evasion by modulating the functions of immune cells.[126–129] This process thereby supports the chronicity of the inflammatory environment in the tumor niche through immunosuppressive functions.[130,131]

Different mechanisms are proposed for the interaction of cancer cells with platelets in the context of tumor cell-induced platelet aggregation (TCIPA).[131–133] Platelet activation occurs as a result of the secretion of thrombin which binds to PAR receptors, the release of ADP which interacts with P2Y1 and P2Y12 receptors, and the expression of podoplanin which binds to CLEC-2 expressed on the surface of the platelets, among others.[138,139] In addition, cancer cell expression of tissue factor (TF), the major activator of the coagulation cascade, and its elevated serum level further contributes to TCIPA. The role of thrombospondin A[134,135] and MMPs, such as MMP-2,[136] in this process was also reported.[134] All these interactions provide opportunities to leverage selective activation of platelets in cancer to target not only primary tumors and metastases, but also CTCs which evade the immune system and colonize the metastatic niche.[137]

Thrombocytosis supports the progression of cancer which together with tumor-derived thrombopoiesis create a vicious cycle.[21] Activated platelets release growth factors, such as PDGF, TGF-β, and VEGF, that are stored in their α-granules and promote tumor growth.[118] In the thrombogenic TME, secretion of proangiogenic factors from α-granules dominate the antiangiogenic factors, which further helps the tumor with neovascularization.[137,138] Interestingly, some studies have elucidated the role of platelets in the formation of a premetastatic niche through the recruitment of granulocytes using CXCL5/7 chemokines,[139] or releasing TGF-β and MMP-1.[140] Their role in metastasis, however, is not limited to the premetastatic niche. By forming platelet tumor cell aggregates in circulation, they are able to shield CTCs from shear stress and immune surveillance.[23,118] TGF-β secreted by platelets weakens the activity of natural killer (NK) cells and promotes epithelial-to-mesenchymal transition (EMT) of cancer cells.[123,141] TNF-α-mediated cell death is also reduced in the presence of platelets.[142] Evidence of the transfer of platelet-derived major histocompatibility class I (MHCI) molecules to cancer cells further highlights the protective role of platelets.[143] Finally, extravasation of cancer cells across endothelial monolayers is facilitated by ADP and ATP-induced permeability, resulting from interaction with endothelial P2Y receptors,[144,145] as well as P-selectin and integrin-mediated firm adhesion.[136,146]

Despite selective activation by cancer cells, homing of platelets into the TME is yet to be fully understood. There is growing evidence that platelets are passively and actively recruited to promote tumor growth and metastasis.[22,115] Leaky and abnormal vasculature in the tumor exposes platelets to pathologic blood
flow and subendothelial components.\textsuperscript{[21,147]} Secretion of platelet binding or activating agents by tumor cells and defective endothelial cells contributes to this homing.\textsuperscript{[21,138]} Regulation of tumor infiltration by focal adhesion kinase (FAK) for platelets has also been reported where tumor growth was reduced as a result of FAK-deficient platelets.\textsuperscript{[148]} An analogous effect was observed for P-selectin, suggesting that these two proteins play a critical role in the migration of platelets into the TME.\textsuperscript{[149]} Chen et al. investigated the homing ability of platelets in different tumor models.\textsuperscript{[150]} Using P-selectin targeted peptide (PSN) which targets activated platelets, they noted that while activated platelets were present in the breast tumor site, there was no detectable signal from PSN in pancreatic tumors. This effect was attributed to differences in the TME, where pancreatic tumors have higher interstitial pressure and lower blood perfusion compared with breast cancer. Furthermore, intrinsic homing of platelets to wound sites could be exploited in the context of cancer therapy for postsurgical applications.\textsuperscript{[9]}

2.1.4. Bacteria

A unique trait of bacteria that distinguishes them from other living cell-based therapeutics is their ability to swim in complex biological fluids, facilitated by the combination of onboard sensing and flagellar activity.\textsuperscript{[15,151]} This form of locomotion, characterized by efficient self-propulsion in response to changes in the surrounding environment, renders bacteria autonomous living systems capable of finding their optimal growth conditions,\textsuperscript{[152,153]} potentially within the TME. Targeted swimming toward environments that favor bacterial growth, known as taxis, requires highly organized coordination between the sensory system and flagellar motor.\textsuperscript{[152,154]} This is achieved through a two-component regulatory system (TCRS) composed of sensors and response regulators.\textsuperscript{[155,156]} Signal transduction linking these two components only leads to a beneficial behavioral response when it is able to sense spatial variations of relevant compounds. Due to the small size of a single bacterium (on the order of 1 μm), random swimming over large distances is necessary for sampling the environment and detecting gradients.\textsuperscript{[154,156]} This continuous explorative cycle of sense and move is manifested in different forms such as the run-and-tumble motion in Escherichia coli\textsuperscript{[157]} or the forward, reverse, and flick pattern in Vibrio alginolyticus.\textsuperscript{[158,159]}

The taxis behavior of bacteria can be grouped in two general categories: classical chemotaxis, where the response only depends on the presence of chemical stimuli, and energy-taxis, such as aerotaxis and phototaxis, in which the cellular metabolism of the stimuli and the energy level regulate the swimming behavior.\textsuperscript{[160]} While membrane-spanning chemoreceptors, known as methyl-accepting chemotaxis proteins (MCPs), bind to specific attractant or repellent chemoeffectors, energy transducer proteins interact with the electron transport system to sense the energy level. The transduction pathways of bacterial taxes converge further down the signaling network where, for example, in E. coli the phosphorylated CheY protein interacts with the flagellar motor, which switches the direction of rotation of the motor.\textsuperscript{[156,160]} Although a general understanding of the signal transduction pathway has been elucidated from studying E. coli and Salmonella, sensory systems with higher complexity featuring more diverse receptors have been identified in other genera.\textsuperscript{[152,154]}

Due to the viscous-dominated low Reynolds flow regime experienced by these microorganisms, they have evolved to rely on molecularly driven rotation of flagellar flagella for propulsion.\textsuperscript{[161,162]} These slender organelles are efficient at converting rotational motion into translational displacement by generating drag-induced thrust.\textsuperscript{[163,164]} Molecular motors are powered by electrochemical gradients of protons or sodium ions to rotate the flagella at frequencies of up to 300 Hz (for H\textsuperscript{+}) and 1300 Hz (for Na\textsuperscript{+}).\textsuperscript{[165]} Leading to swimming velocities in the range of tens and hundreds of micrometer per second, respectively. Depending on the species and configuration of flagella, different strategies have been observed for changing direction in fluids and more complex media such as mucus and biofilms.\textsuperscript{[153,154]}

Taxis-guided self-propulsion has motivated the use of these microorganisms in the treatment of hard-to-access tissues, such as solid tumors. Despite observations of tumor regression in patients with bacterial infection in the early 19th century, as well as later experiments with live bacteria for cancer treatment, tumor homing of bacteria was first studied in 1955 by Malmgren and Flanigan.\textsuperscript{[166]} Their study showed exclusive localization of Clostridium in tumors following intravenous injection. Tumor colonization of this anaerobe was also observed in rat models where normal tissues only contained bacterial spores without any germination.\textsuperscript{[167]} Similarly, Bifidobacterium, another anaerobic bacteria, demonstrated selective tumor localization following systemic administration into mouse models.\textsuperscript{[168]}

Most solid tumors are characterized by the presence of a necrotic core and hypoxic regions in areas distant from the closest immature blood vessel.\textsuperscript{[4,169]} Therefore, low oxygen levels are considered the main driver for aerotactic migration, followed by sustained germination and colonization of obligate anaerobes, which exhibit low oxygen tolerance.\textsuperscript{[170]} Interestingly, when the obligate anaerobe C. novyi-NT was injected into mice bearing poorly vascularized non-neoplastic tissues characterized by hypoxia, no bacterial colonization occurred. This was consistent with a later finding in a rat ischemic brain model.\textsuperscript{[171]} These studies highlight the role of unique biochemical parameters of the TME in the infiltration of the solid tumor by strict anaerobes.\textsuperscript{[7]}

Other mechanisms have been proposed for the tumor homing of facultative anaerobes that can also survive and grow under the normal oxygen concentrations present in healthy tissues.\textsuperscript{[12]} Studying gene knockouts indicated the role of aspartate receptors in the chemotaxis-driven tumor targeting of S. typhimurium.\textsuperscript{[172]} Serine receptors played a role at the start of infiltration, and directed penetration toward a necrotic core was achieved through ribose/galactose receptors. Signal transduction mutants were shown to lack migration toward tumor tissues.\textsuperscript{[173]} Moreover, the correlation between both homing and intratumoral growth of bacteria with the size of the tumor tissue illustrated the key role of hypoxic viable cells in chemotaxis.\textsuperscript{[174]} Interestingly, Listeria uses an alternative strategy by targeting the immunoprivileged tumor tissue through infection of myeloid-derived suppressor cells (MDSCs) that infiltrate the tumor.\textsuperscript{[175]}

While the aforementioned anaerobes display a range of responses to oxygen levels, other unique features of the TME
facilitate the homing of all classes of bacteria in a similar manner. The role of the immune system in the homing of both obligate and facultative anaerobic strains is complex. All tumor-targeting bacteria differentially take advantage of the immunosuppressive nature of the TME, particularly in the necrotic core. It also contributes to the dissemination pattern of facultative anaerobes in tumors, which is analogous to obligate anaerobes despite differences in physiology. Targeting and replication in tumors by aerobic strains further underpins the argument in favor of the critical role of defective immunosurveillance compared with the influence of hypoxia. While accumulation of bacteria at the location of quiescent viable cells inside tumors would lead to the highest treatment efficacy, immune cells limit the presence of tumor-infiltrating bacteria to necrotic regions. However, in vivo, the well-perfused rim of the tumor, which is not colonized by bacteria, was shown to be eradicable through immune-mediated responses in immunocompetent mice. Overall, hypoxia development, release of bacterial nutrients, and the immune-suppressive environment in solid tumors are all intertwined with irregular leaky tumor vasculature. Consequently, passive entrapment of bacteria in tumor blood vessels and infiltration through endothelial gaps, which could be accelerated by TNF-α has been considered as another contributing factor in the tumor tropism of bacteria.

Targeted colonization of the tumor is the basis of almost all bacterial cancer therapies, but this process is contingent upon the homing and achievement of adequate accumulation. Accumulation is reliant on administration of a minimum amount of bacteria and the presence of a necrotic core, which is correlated with the developmental stage of the tumor. Both Gram-negative and Gram-positive bacteria, regardless of their oxygen requirements and pathogenicity, were able to colonize syngeneic and xenograft tumors in immunocompromised and immunocompetent mice. A comprehensive understanding of the necessary conditions for colonization has been the subject of various studies.

2.2. Externally Enhanced Tumor Specificity at a Tissue Level

As originally demonstrated for nonliving NP-based drug formulations, external stimuli can also serve to improve selective targeting of cell-based therapeutics (Figure 2). Responsiveness to internal stimuli, mainly in the form of chemotaxis and aerotaxis, enables cell-based therapeutics to act autonomously, but at the cost of a relatively slow response and slightly random behavior. Conversely, interactions with external cues enable faster control, which is accompanied by design complexities and potentially the need for closed-loop control. Additional modification of the cells is also necessary when responsiveness is not intrinsic to the cell type. External control confers dual-targeting functionality to cellular agents that are already equipped with intrinsic homing mechanisms. This added specificity leads to higher safety levels because the same efficacy can be achieved at lower dosages.

Among all physical stimuli, the use of magnetic energy has gained the most interest (Figure 2A). The human body is fully penetrable by magnetic fields which allows magnetically responsive agents to target deep-seated organs. Magnetotactic bacteria (MTB), a group of bacteria that biomineralize membrane-bound iron-rich nanocrystals called magnetosomes, have strongly contributed to this interest. Chains of magnetosomes serve as compass needles for MTB, helping them navigate along the Earth’s magnetic field while swimming toward the oxic-anoxic interface (OA1), a behavior known as magnetooaerotaxis. The idea of using MTB as trackable medical micro-robots was proposed over a decade ago and since then, different strains of MTB such as Magnetococcus marinus MC-1 and Magnetospirillum magneticum AMB-1 and Magnetospirillum gryphiswalense MSR-1 have been studied in vivo for drug delivery into tumors. In terms of navigation strategies, most studies focused on force-based field gradients or directing magnetic fields. Following peritumoral injection in mice bearing flank tumors, approximately 55% of the MC-1 exposed to directing magnetic field accumulated in hypoxic region of the tumor and almost twofold higher tumor infiltration was shown for magnetically assisted AMB-1. Furthermore, the ability of bacteria to act as magnetic resonance imaging (MRI) negative contrast agents and the correlation with concentration has been demonstrated. Recently, using rotating magnetic field as a means of actuation of MTB to convectively enhance the transport of codelivered NPs was proposed. Although more sophisticated control schemes, such as point-to-point closed-loop control or the use of a virtual magnetic monopole, have been proposed for MTB guidance, none have been implemented in vivo to date.

Magnetically enhanced tumor targeting can also be applied to nonmagnetic living cells (Figure 2A). This strategy requires the integration of magnetic nano/microparticles into the living system. Magnetic particles conjugated onto the surface of bacteria such as E. coli and Serratia marcescens conferred the ability to align with magnetic fields, with swimming velocities lower than that of sole bacteria. Microswimmers created by attaching several E. coli to multilayer microparticles loaded with doxorubicin and magnetite were demonstrated to have mean swimming speeds of up to 22.5 μm s⁻¹ and to deliver doxorubicin in 4T1 breast cancer cells in vitro under a chemoattractant gradient and a magnetic field. Similar microswimmers composed of multiple S. marcescens bacteria attached to a superparamagnetic bead were shown to have mean swim speeds of up to 7.3 μm s⁻¹ and were able to be steered along 2D trajectories using low magnetic field amplitudes (<10 mT). Through genetically engineered expression of iron-storage ferritin and magnetosomes, E. coli and MSCs, respectively, have been rendered responsive to magnetic fields.

Innate phagocytosis makes macrophages and neutrophils readily useable candidates as magnetic carriers through the internalization of magnetic particles (Figure 2A). Pulsed field gradients were applied to oncolytic virus-carrying macrophages and MRI scanners were used to concurrently track and steer the magnetic systems. Enhanced motility under magnetic fields was reported for macrophages carrying paclitaxel-loaded magnetic liposomes and poly(lactic-co-glycolic acid) (PLGA) particles containing docetaxel and magnetic nanoparticles (MNPs). Recently, enhanced tumor accumulation of macrophages loaded with MNPs and thermosensitive liposomes was...
demonstrated for mice with magnets attached to their tumors. In another study, neutrophils phagocytosed drug-containing magnetic nanogels which enabled their guidance by rotating magnetic fields toward the mouse brain. Following long-range control with magnetic fields, the cells crossed the blood–brain barrier mostly relying on chemotaxis in a postoperative glioma model.

Static fields alone are unable to focus magnetic agents at an interior point to create stable traps, as demonstrated by Earnshaw’s theorem. This, together with the rapid decay of magnetic forces resulting from field gradients, limits application of static fields to shallow targets. As such, while promising in small animal models, very high fields are required to achieve the same level of forces at larger scales. This scalability issue can partly be circumvented through the use of relatively weak directional static fields to align the motile agent in the specified direction. However, success of this guiding strategy is bounded by self-propulsion forces which may not be sufficient to overcome robust physiological barriers. Rotating magnetic fields, that also bypass the high field requirement, can serve as an attractive option in the absence of self-propulsion or when propulsive forces are not sufficiently strong.

Another natural taxis with respect to external stimuli is phototaxis, which is active migration in response to light intensity (Figure 2A). Photosynthetic bacteria (PSB) *Rhodobacter johnii* showed higher tumor accumulation in NIR irradiated tumors. Coculture with multicellular MCF-7 spheroids showed enhanced infiltration of the tumor core when subjected to NIR irradiation. Following peritumoral injection of PSB into tumor-bearing mice, PSB accumulation in hypoxic regions of the tumor was increased by exposure to NIR light. Although spatial control was achieved by light-induced migration, the application of this approach is restricted to superficial tumors given the limited penetration depth of light in the body.

In addition to taxis behaviors controlled by external stimuli, internal taxis mechanisms, such as chemotaxis, can be triggered by artificially producing a diseased state through the application of external stimuli (Figure 2B). Cell types responding to acute inflammation and “injuries”, namely neutrophils as the first line of defense and platelets as the guards of vascular integrity, are recruited rapidly to the targeted site. Such externally induced chemotaxis was exploited to deliver checkpoint inhibitors by platelets after photothermal ablation, photodynamic therapy, application of focused ultrasound, and radiotherapy. In another study, photosensitized tumors were infiltrated by neutrophils carrying photothermal agents, which subsequently increased the efficacy of thermal ablation. Magnetic hyperthermia, photodynamic therapy, and high-intensity focused ultrasound have also been used for improved T cell priming. Through induction of immunogenic cell death and release of tumor-specific...
antigens, which are presented to cytotoxic T cells by DCs, amplified T cell infiltration can be achieved.

2.3. Engineering-Enhanced Tumor Specificity at the Cellular Level

Engineered cell-based therapies are transforming cancer therapy by exploiting living cells capable of intelligent sensing and sophisticated responses for improved targeting and specificity of therapeutic effects. The unprecedented response rates of CD19-targeted CAR-T cell therapies have provided a clear demonstration of the value of cell engineering in cancer therapy (Figure 3).[233] CARs are synthetic receptors that allow major histocompatibility complex (MHC)-independent retargeting of T cells for the recognition and elimination of cells expressing user-defined antigens.[227–229] While CAR-T therapy has been transformative, there are still challenges with the development of cytokine release syndrome, resistance caused by antigen escape, as well as concerns about on-target off-tumor effects[230,231] that can result in profound toxicities, particularly in the case of solid tumors.[232]

Control switches that enable external regulation of T cell function have been developed with the aim of increasing the safety of CAR-T therapy. One approach involves the use of bifunctional adaptors composed of a tumor antigen-specific antibody fragment fused to a peptide neoepitope (Figure 3A).[233] This adaptor or “switch” mediates the temporally controlled formation of immunological synapses in which the target cell and CAR-T cell interact in a structurally defined manner. Switchable CAR-T cells exhibit potent, dose-dependent antitumor activity in vivo with lower cytokine levels than conventional CAR-T cells.[233,234] In addition, the CAR-T cells could be retroactively by exchanging the antibody on the adaptor, eliminating the need to develop a new CAR for each target. This strategy has even shown efficacy in solid tumors.[233] Switchable CAR-T cells targeting human epidermal growth factor receptor 2 (HER2) induced complete remission in aggressive, patient-derived late-stage pancreatic tumor models following administration of the adaptor molecule.

Synthetic biology has also been harnessed to create sophisticated multigene circuits that produce programmed outcomes with the aim of enhancing specific targeting of cancer cells, without affecting normal cells. Several strategies have been developed to engineer CAR-T cells that execute Boolean logic, where a combination of inputs results in a programmed output. To address the issue of antigen escape, OR-gate CARs composed of independent antigen recognition domains that can be activated by two different antigen ligands have been developed (Figure 3B). CARs targeting both CD19 and HER2 displayed effector functions upon encounter with either antigen alone and exhibited enhanced potency when both receptors were simultaneously engaged.[236] Zah et al. developed bispecific CARs targeting the B cell specific antigens CD19 and CD20 that exhibited cytotoxicity against target cells expressing CD19 and CD19’ mutants, with comparable efficiency in vivo.[237]

To enhance on-target activity, AND NOT-gate circuits have been engineered by combining a CAR for one antigen with an inhibitory CAR (iCAR) for another antigen (Figure 3C). iCARs with an intracellular signaling domain of the immunoinhibitory receptors PD-1 or CTLA-4 reversibly inhibit T cell function upon antigen recognition, enabling discrimination between target and off-target cells.[238] Precise tumor recognition can also be accomplished by CAR AND-gates that require a specific combination of antigens for activation through the expression of two separate CARs (Figure 3D).[239,240] Kloss et al. transduced T cells with a CAR that provided suboptimal activation upon binding of prostate stem cell antigen (PSCA) as well as a chimeric costimulatory receptor (CCR) that recognizes prostate-specific membrane antigen (PSMA).[241] The dual-targeting T cells selectively eradicated double-positive tumors but did not affect single-antigen positive tumors. Another similarly effective strategy for engineering dual-antigen recognition utilizes a circuit in which activation by an antigen for a synthetic Notch (synNotch) receptor induces the expression of a CAR for a second antigen.[242] Engagement of the synNotch receptor ligand triggers proteolytic cleavage of the intracellular domain that contains a transcriptional regulator which activates the transcription of

![Figure 3](https://www.advancedsciencenews.com/doi/figure/10.1002/adv.21001348)
3. Strategies for Therapeutic Action at the Tumor Site

The therapeutic properties of immune cells, stem cells, platelets, and bacteria have been augmented by the addition or induced production of therapeutic payloads. Combined with onboard sensing and autonomous navigation, these biohybrid delivery vectors offer multiple therapeutic advantages over conventional cancer therapies. The improved therapeutic outcomes accomplished using cell-based cancer treatments can generally be attributed to well-controlled drug release that is achieved through such systems.

3.1. Immune Cells

Phagocytes that innately home to hard-to-reach tumor regions have been leveraged to create therapeutic “Trojan horses.” Payloads are phagocytosed by immune cells ex vivo and this intracellular cargo is shuttled into tumors for targeted delivery with decreased systemic toxicity of the cargo. Fu et al. loaded therapeutic concentrations of doxorubicin (DOX) into the macrophage-like cell line RAW264.7 without significantly compromising cell viability. However, cell enlargement and a decrease in the migration of DOX-loaded macrophages were observed. Loading macrophages with DOX encapsulated in PLGA NPs or liposomes has been shown to reduce toxicity compared with free DOX. Zhang et al. achieved a high loading efficiency (16.6 pg DOX per cell) without disrupting cell functions using silica-based nanocapsules. The silica casing was shown to be resistant to enzymatic degradation in phagolysosomes, resulting in more sustained drug release. Comparable accumulation of untreated and DOX-loaded macrophages was observed in a U87MG xenograft model, and histological analysis showed apoptotic cells in both the periphery and core of DOX-macrophage treated samples. Macrophage Trojan horses have also been used for the horizontal transfer of siRNA. Transfer of calcium integrin binding protein-1 (CIB1)-siRNA reduced the growth of tumorspheres composed of MDA-MB-468 cells and loaded macrophages.

Other phagocytic cells have also successfully been used as agents for targeted payload delivery. Ly6C<sup>hi</sup> monocytes were used as carriers of pH-sensitive micelles loaded with the anticancer drug paclitaxel (PTX) (Figure 4A). PTX-micelles were transferred to cancer cells through endocytosis and PTX was released when exposed to the acidic environment within the endo/lysosomes. Monocytes homed to tumors following intravenous administration and suppressed lung metastasis and tumor growth. Xue et al. loaded neutrophils with liposomes containing the anticancer agent PTX. PTX-neutrophils were able to cross the blood–brain barrier and home to residues of resected glioma in mice. The neutrophils released the intracellular payload in response to inflammatory cues which resulted in the inhibited recurrence of tumors. Zhang et al. fabricated magnetically responsive “neutrobots” through the phagocytosis of gelatin hydrogels containing PTX and iron-oxide MNPs enveloped in an E. coli cell membrane (Figure 4B). Magnetic actuation using rotating magnetic fields augmented neutrobot tumor accumulation in a model of postoperative glioma, and release of PXT in response to inflammatory signals resulted in inhibited tumor growth and increased survival rates.

Cellular Trojan horses have also been used as mediators of photothermal therapy and magnetic hyperthermia. Neutrophils loaded with gold nanorods and macrophages loaded with gold NPs or tungsten oxide-loaded NPs efficiently meditated photothermal therapy, resulting in ablation when tumors were irradiated with NIR. In an in vitro 3D tumor model consisting of cancer cells and loaded macrophages, exposure to alternating magnetic field (AMF) led to release of maytansinoid, a cytotoxic agent, that was covalently linked to the particles via a thermosensitive linker which resulted in a significant reduction in cell metabolic activity.

Concurrent photothermal–chemotherapy has also been achieved with such cellular Trojan horses through the addition of DOX to intracellular cargo (Figure 4C). Dual-purpose macrophages have been used for the treatment of tumor hypoxia through NIR-triggered release of nitric oxide (NO). A photoactivatable NO-releasing manganese–nitosyl complex and Nd<sup>3+</sup>-doped NPs were encapsulated in a biodegradable polymer and loaded into bone marrow-derived murine macrophages. These macrophages penetrated 3D tumor spheroids and released detectable amounts of NO following NIR irradiation leading to a reduction of hypoxia inducible factor 1 alpha (HIF-1α) levels in the tumor cells.

T cells and NK cells have been used as carriers for cell-mediated delivery of nanotherapeutics in tumors sites (Figure 4D). Autologous polyclonal T cells were primed ex vivo and functionalized with multilamellar lipid nanocapsules containing the antineoplastic drug SN-38. Following adoptive
transfer, functionalized T cells delivered tenfold higher doses of SN-38 into lymphoid tumors in vivo.\(^{268}\) Tang et al. developed interleukin-15 (IL-15) super-agonist nanogels that respond to local changes in redox activity as a result of T cell receptor activation.\(^{269}\) Localized drug delivery following antigen recognition resulted in eightfold higher cytokine doses and intratumoral T cell expansion, which led to increased tumor clearance in vivo.

Triggered release of therapeutic cargo from T cells has been achieved through the use of genetic engineering. synNotch receptors were used to induce local, antigen-dependent production of a range of payloads, including antibodies, adjuvants, and cytokines from programmed T cells.\(^{270}\) External cues, such as heat, have also been used to trigger payload release from engineered T cells. Heat shock promoters were used to mediate the activation of gene circuits in primary T cells.\(^{271}\) Thermal actuation enabled controllable expression of interleukin-21 (IL-21) as well as the expression of CARs. It was demonstrated that these heat-responsive cells could reduce the viability of target tumor cells. Miller et al. engineered Jurkat T cells with gene switches constructed from the heat shock protein HSP70B’ (HSPA6) promoter which were activated by photothermal heating within a temperature range of 40–45°C.\(^{272}\) Subcutaneous matrigel implants containing engineered Jurkat T cells and gold nanorods exhibited high thermal tolerability and long-term control of the cells for up to 2 weeks.

### 3.2. Stem Cells

Stem cells may also serve as promising Trojan horses given their tumor homing properties. MSC-based Trojan horses have been developed through the internalization of the drug-loaded NPs via both passive uptake and active endocytosis. Payload internalization is dependent on several factors, including the size of the cargo, the surface properties, and the incubation time.\(^{273}\) Notably, the viability and migratory properties of the MSCs must be preserved. Zhao et al. developed a cell-based transport strategy using DOX-loaded PLGA NPs internalized by MSCs, with a loading of 21 pg/cell.\(^{274}\) Tumor infiltration of intravenously administered MSCs significantly reduced the number of metastatic nodules compared with controls in a murine metastatic melanoma model.

The intrinsic tumor tropism of MSCs has been combined with anticancer drug carriers to create potent, living therapeutic systems. DOX-loaded silica nanorattles were coupled to the
MSC cell membrane through antibody–antigen binding.\textsuperscript{[275]} Intratumoral injection of these MSCs resulted in a uniform distribution and increased retention of DOX in the tumor compared with controls. Mooney et al. conjugated pH-responsive NPs loaded with the anticancer agent docetaxel (DTX) to neural stem cells (NSCs).\textsuperscript{[276]} The NPs disassembled below pH 6.3 and intratumoral injection of NSC-NP conjugates reduced tumor vasculature and cell proliferation in vivo.

Engineered expression of antitumor proteins has also been implemented using MSCs. A pioneering study by Nakamizo et al. demonstrated the ability of engineered stem cells to be used as targeted delivery vehicles.\textsuperscript{[95]} MSCs engineered to secrete IFN-β were injected intratumorally into U87 intracranial glioma tumor-bearing mice which enhanced survival compared with controls. Human adipose-derived MSCs (hAMSCs) have been engineered to secrete bone morphogenetic protein 4 (BMP4), a key regulator of cell proliferation, which is known to have antitumor effects on brain tumor-initiating cells. Mangraviti et al. transfected hAMSCs using an engineered polymeric NP and transfected cells did not exhibit any change in cell surface markers.\textsuperscript{[277]} Engineered hAMSCs homed to and penetrated tumors in a human glioblastoma rat model following both intravenous and intranasal administration, which led to improved survival compared with those treated with control cells. Intracardiac injection of engineered hAMCSs reduced proliferation and migration of cancer cells in an intracranial glioblastoma murine model.\textsuperscript{[278]}

3.3. Platelets

Platelet-based drug delivery offers multiple benefits in addition to their tumour homing which is associated with TCIPA. As biological cells abundant in the body, they reduce potential production costs and immunogenicity associated with many synthetic drug carriers.\textsuperscript{[6,9,10]} Their high encapsulation efficiency, which is linked to their ability to actively sequester molecules, along with their lifespan, enhances the pharmacokinetics of drugs.\textsuperscript{[17,23,279,280]}

Treatment of primary tumors using platelets as carriers has proved to be an effective strategy. Sarkar et al. exploited platelets to transport the anticancer agent DOX and release it at the tumor site.\textsuperscript{[281]} Growth suppression of Ehrlich ascites carcinoma (EAC) cells in the peritoneal cavity of mice injected intraperitoneally with DOX-platelets was confirmed through measurement of accumulated ascites fluid. In addition, collected samples revealed apoptosis of EAC cells. Xu et al. loaded platelets with DOX without experiencing compromised structural integrity and functions.\textsuperscript{[282]} The DOX release profile was shown to be pH-dependent, with highest release in acidic environments. In addition, low cardiotoxicity of this formulation was confirmed by assessing the viability of myocardial cells incubated with DOX-platelets. Comparison with well-studied liposomal DOX demonstrated less uptake by macrophages for DOX-platelets. Circulation half-life of DOX increased from 1.9 to 29.2 h as a result of encapsulation in platelets which also led to higher accumulation in the tumor and lower concentrations in the heart. Adding anti-CD22 monoclonal antibodies to this system further enhanced specific internalization of DOX by cancer cells and resulted in even higher tumor growth inhibition.\textsuperscript{[283]}

To prevent recurrence of cancer following surgical operation, Wang et al. leveraged the targeting ability of platelets for wound sites.\textsuperscript{[284]} An antibody against programmed-death ligand 1 (aPDL1) was attached covalently to the surface of platelets. In vitro stimulation revealed release of aPDL1 upon activation and its binding to cancer cells. Mouse melanoma models with incomplete tumor removal showed reduced relapse and prolonged survival when treated with aPDL1-platelets. Both CD8+ and CD4+ T cells infiltrated tumors of this group, reflecting a T cell-mediated antitumor immune response. In a mouse metastasis model, free aPDL1 led to lowered metastatic foci in the lungs, yet it failed to prevent tumor relapse, while the aPDL1-platelets treated group demonstrated a significant decrease in metastatic sites and tumor recurrence.

Like surgeries, other physical therapies of tumors using heat, ultrasound, and irradiation, among others, are also associated with recurrence and metastasis.\textsuperscript{[215]} Han et al. engineered platelets to carry aPDL1 to thermally ablated tumors which were treated with PLGA-coated indocyanine green (PLGA-ICG) as a photothermal agent. aPDL1-platelet treatment led to efficient targeting and, consequently, suppressed tumor growth and improved survival. Targeting ability of this biohybrid platform was also confirmed in the case of photodynamic therapy, high intensity focused ultrasound, and radiotherapy.

A recent study took advantage of the synergy between platelet tumor tropism and damage targeting.\textsuperscript{[285]} Naphthalene diimide–bithiophene block copolymer, a photothermal material, along with the immunostimulator R837 hydrochloride were loaded into platelets and this system was tested in murine models. Upon intravenous injection, platelets enriched tumors through defective vascular endothelial cells present in the TME. Subsequently, tumors were irradiated with NIR, reinforcing aggregation of platelets based on their response to tissue damage. This photothermally enhanced aggregation led to enhanced delivery of the immunostimulator. As a result, tumor-associated antigens released from ablated tumors induced a stronger immune response to metastatic and recurrent tumors.

Selective targeting of platelets in vivo to deliver active agents to tumors constitutes another treatment strategy. Chen et al. used P-selectin targeted peptide (PSN) which binds to activated platelets.\textsuperscript{[150]} Following photothermal therapy of mice with breast and pancreatic cancer models, efficient treatment was evident only in the former group. This selective effect was attributed to the higher interstitial pressure and lower blood perfusion in the TME of pancreatic versus breast cancer.

Platelets as anucleated cells cannot be directly genetically engineered. However, modifying corresponding progenitor cells provides an opportunity of producing platelets with desired functionalities. In a study by Li et al., genetically engineered hematopoietic stem and progenitor cells were shown to produce platelets expressing tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) on the cell surface.\textsuperscript{[286]} Following bone marrow transplantation of the modified stem cells, 40% of circulating platelets were TRAIL-expressing and a significant reduction in prostate cancer metastases was shown in vivo. In a
similar study, platelets expressing the programmed cell death protein 1 (PD-1) were produced from genetically engineered megakaryocyte progenitor cells.\textsuperscript{[287]} Intravenously injected PD-1-expressing platelets accumulated at postsurgical sites following incomplete tumor removal and reverted exhausted CD8\(^+\) T cells, slowing down tumor growth. In addition, loading PD-1 platelets with cyclophosphamide resulted in depletion of regulatory T cells, higher frequency of reinvigorated CD8\(^+\) T cells, and prevention of tumor relapse.

### 3.4. Bacteria

The innate tumor homing of bacteria, although a critical prerequisite, is not the sole motivation to use these microorganisms in cancer treatment. Different forms of antitumor activities provided by bacteria have fueled studies on these living therapeutic agents.\textsuperscript{[12]} Their antitumor effects stem from either innate toxicity, delivery of cargo, or on-site production and release of drugs by genetically engineered bacteria (Figure 5).\textsuperscript{[7,11,16]} In addition to these cytotoxic mechanisms, bacteria trigger immune-mediated antitumor responses.\textsuperscript{[7,180]} Relative contributions of these two pathways in cancer cell death depends on the species, specific TME, and time of intervention.\textsuperscript{[12,16]}

Native cytotoxicity of different species has been studied and various mechanisms were proposed. \textit{Salmonella} as intracellular pathogens were shown to induce cell death through apoptosis\textsuperscript{[288]} and autophagy\textsuperscript{[289]} by production of toxins or uncontrolled replication inside the cell. They also compete for nutrients within the TME. \textit{Listeria} give rise to elevated intracellular reactive oxygen species (ROS)\textsuperscript{[290]} which thereby kills cancer cells. \textit{Clostridium} secrete many exotoxins such as phospholipases and hemolysins which disrupt the cell membrane and interfere with cellular functions.\textsuperscript{[291,292]}

These direct anticancer effects are complemented by immune-mediated cell death. Tumors colonized with \textit{Salmonella} demonstrated secretion of interleukin-1\(\beta\) (IL-1\(\beta\)) from macrophages and DCs induced by LPS\textsuperscript{[293]} or phagocytosis of infected cells leading to pyroptosis\textsuperscript{[180,294]} Enhanced cross-presentation of antigens to DCs through gap junctions\textsuperscript{[295]} and flagellin-mediated activation of CD8\(^+\) T cells and NK cells resulting in production of IFN-\(\gamma\)\textsuperscript{[296]} have also been reported for this genus.\textsuperscript{[180]} Phenotypic alteration in MDSCs infected by \textit{Listeria} boosts IL-12 levels, which is accompanied by a stronger antitumor response from NK and CD8\(^+\) T cells.\textsuperscript{[175,182,297]} In the case of \textit{Clostridium}, secretion of IL-6, granulocyte-colony-stimulating factor, and macrophage inflammatory protein 2 is considered responsible for recruiting

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**Figure 5.** Engineered bacteria for carrying therapeutic payloads and controlled release. A) Conjugation of nanoparticles to the surface of \textit{Salmonella} forming biohybrid cargo-carrying microrobots with autonomous targeting. Reproduced with permission.\textsuperscript{[305]} Copyright 2018, John Wiley and Sons. B) Magnetic guidance of cargo-carrying magnetotactic bacteria strain AMB-1 for photothermal therapy. Reproduced with permission.\textsuperscript{[196]} Copyright 2020, John Wiley and Sons. C) Photothermal therapy mediated by enhanced accumulation of photosynthetic bacteria \textit{Rhodobacter johrii} under NIR irradiation. Reproduced with permission.\textsuperscript{[218]} Copyright 2021, American Chemical Society. D) Genetically engineered \textit{E. coli} for controlled production and release of the nanobodies through intratumoral quorum lysis. Reproduced with permission.\textsuperscript{[320]} Copyright 2019, Springer Nature.
immune cells\cite{298} and triggering the release of TRAIL from neutrophils.\cite{299} Modification of the cell membrane with targeting moieties can further boost therapeutic effects by enhancing tumor accumulation. In vivo studies using an engineered attenuated RGD-displaying \textit{Salmonella} revealed prolonged survival of mice, with 80\% enduring up to 160 days after tumor inoculation. Similarly, attenuated \textit{Salmonella} with a CEA-specific antibody resulted in inhibited growth of tumors as well as local accumulation of CD3+ T cells and CD11b+ macrophages.\cite{247}

Analogous engineering routes are also used to attach drug payloads onto bacterial membranes.\cite{100,101} Nguyen et al. developed a cargo-carrying system featuring tumor targeting bacteria \textit{S. typhimurium} and PTX-loaded microliposomes.\cite{102} The average velocity of this kind of “bacteriobot” was reduced to 3 μm s \(^{-1}\). In their final study, these bacteriobots selectively reduced the viability of cancer cells in vitro. Another example was developed by Park et al. who attached polystyrene carboxylated microbeads onto attenuated \textit{S. typhimurium}.\cite{103} Chemotaxis-driven migration speeds of approximately 30 μm min \(^{-1}\) were observed for the bacteriobots when exposed to lysates or cancer cell spheroids while their velocity reached approximately 5 μm min \(^{-1}\) in the presence of normal cells. In another study, the radioisotope \(^{188}\text{Rhenium}\) was conjugated to attenuated live \textit{Listeria monocytogenes} via strain-specific antibodies.\cite{304} Mice with metastatic pancreatic tumors were injected intraperitoneally and bacterial proliferation was found in metastatic sites, while healthy tissues were spared. Daily low dose injections for sustained delivery of radioactive agents resulted in a 90\% reduction of the number of metastases compared with 50\% for \textit{Listeria} alone. The measured radioactivity level in the liver and kidneys was not detectable by day 7. Suh et al. developed a biohybrid autonomous drug delivery platform composed of PLGA NPs conjugated to an attenuated auxotrophic mutant of \textit{S. typhimurium} (Figure 5A).\cite{105} This chemotaxis-defective strain demonstrated significantly better tumor penetration in vitro compared with NPs alone, and their conjugation resulted only in a slight decrease in this ability. Intercellular transmigration was determined as the dominant mode of translocation inside tumor masses highlighting the role of TME in tumor penetration of bacteria. Invasion assays also revealed a decreased number of bacteria invading the cells when coated with polyethylene glycol (PEG) or carrying conjugated NPs.

Therapeutic cargos have been integrated with bacteria that are innately responsive to external physical cues for enhanced delivery at the tissue scale. A similar strategy has been adopted for nonresponsive bacteria where they are modified with nanomaterials to allow for external control. Taherkhani et al. investigated the potential of the MTB strain MC-1, as a drug carrier.\cite{106} MTB-liposome conjugates were synthesized by utilizing the amine groups on the surface of the Gram-negative bacteria. Reduced motility was correlated with the number of attached liposomes and residence time in buffer. The same system was used in vivo to target the hypoxic core of tumors under directing magnetic fields.\cite{195} Another strain of MTB, AMB-1, was also used as a magnetically controllable motile carrier. Here, integrated indocyanine green nanoparticles (INPs) rendered this biohybrid platform capable of imaging and photothermal therapy (Figure 5B).\cite{196} Average mean velocity of this biohybrid microbot was reported to be 13.3 μm s \(^{-1}\). They were peritumorally injected and guided using magnetic field gradients. Photothermal therapy using NIR irradiation inhibited tumor growth, and no significant changes in biochemical parameters associated with liver and kidney function implied acceptable safety levels. In addition to chemically conjugated payloads, DOX-internalized MTB was also recently presented, which was motivated by chelation of DOX with Fe\cite{197,198}. Drug-internalized MSR-1 maintained their motility for 3 h. In vivo, this motile platform resulted in better targeting and higher tumor growth suppression compared with inactivated bacteria and free DOX. The same strain has also been tested for applications in magnetic hyperthermia.\cite{107} Similarly, external control has been presented for bacteria that are either coupled to or engineered to synthesize magnetic particles.\cite{206,208,209}

While most studies have focused on the application of magnetic fields, the responsiveness of bacteria has also been exploited for other types of stimuli. Zheng et al. proposed living PSB as an ideal carrier possessing both hypoxia targeting and NIR phototaxis which could be leveraged for photothermal therapy (Figure 5C). While PSB alone suppressed tumor growth after 10 days, which was attributed to nutrient competition, irradiated PSB resulted in much smaller tumor sizes.

A third group of bacteria-based therapeutics comprises genetically engineered bacteria with encoded functionalities to enhance targeting and therapeutic efficiency. These genetic modifications were implemented in both probiotic and pathogenic strains. In a pioneering work by Pawelek et al., different auxotrophs of engineered \textit{Salmonella} were explored as anticancer vectors.\cite{108} Engineered auxotrophs expressed the herpes simplex virus thymidine kinase (HSV-TK) gene which led to activation of the produg ganciclovir and suppressed tumor growth. Similarly, attenuated \textit{S. typhimurium} was genetically engineered to produce IL-2 by cloning the human gene for IL-2 into a plasmid.\cite{109} Mice bearing metastases in the liver demonstrated significantly lower number of metastases when gavaged with IL-2 expressing bacteria in contrast to an attenuated strain without IL-2. These bacteriobots were able to colonize the tumor for at least 4 weeks. More recently, Leventhal et al. developed a therapeutic platform based on nonpathogenic \textit{E. coli} Nissle (EcN) that activated the STING pathway.\cite{110} Reduced tumor growth was observed in melanoma-bearing mice treated with intratumoral injections of EcN. Triggering localized inflammation by intratumoral production of the STING-agonist cyclic diAMP resulted in better tumor regression compared with control EcN.

Further genetic circuits have been introduced to control production and release of the therapeutic agents. Camacho et al. engineered \textit{Salmonella} with an inducible autolysis system leading to lysis of bacteria triggered by anhydrotetracycline.\cite{311} This system was complemented with aspirin-dependent production of the therapeutic agent and a siRNA mutation which allowed the bacteria to escape the vacuoles when internalized by the cells. Infection of cancer cells with engineered bacteria producing Cp53 confirmed proliferation of this strain inside cells and Cp53-induced cell death. In a microfluidic model of perfused tumor tissue, \textit{E. coli} expressing Trz1, a glucose-sensing receptor,\cite{312} was shown to detect glucose gradients and express green fluorescent protein (GFP) in response.\cite{313} Based on this data, mathematical models predicted treatment of 99.2\% of cells within a tumor using Trz1-activated drug delivery compared with 70.8\% with similar systemically delivered drugs.
Incorporating inducible elements sensitive to oxygen levels is another attractive strategy due to its potential in localizing the antitumor effects. In a study by Anderson et al., environmental cues, including hypoxia, cell density, and inducible inputs, were used to control the internalization of E. coli. This response was achieved by placing the expression of the invasin gene from Yersinia pseudotuberculosis under the regulation of the quorum sensing lux operon, a hypoxia-induced fdhF promoter, or an arabinose-inducible araBAD promoter. This resulted in 8% of added bacteria being recovered following lysis of cancer cells expressing β1-integrins, while levels of bacteria without invasin expression were undetectable. Similarly, S. typhimurium with a gene encoding the production of Hlye, a cytotoxic protein, under the control of a hypoxia-inducible promoter (FF + 20β) colonized hypoxic tumor regions after systemic administration, leading to a reduction in tumor growth and an increase in tumor necrosis. S. typhimurium was also programmed in another study to solely survive in anaerobic conditions by placing an essential gene, aspartate-semialdehyde dehydrogenase (asad), under the regulation of a hypoxia-conditioned promoter. In vivo, the intravenously administered engineered strain successfully colonized tumors and repressed growth while undergoing lysis and clearance in normal tissues. Mengesha et al. developed a hypoxia-inducible promoter (HIP-1) derived from the Salmonella pepT promoter. Following systemic administration of the bacteria, the reporter gene expression for GFP and RFP was restricted to the hypoxic tumors and was approximately 15-fold higher compared with a constitutive promoter.

Another intriguing opportunity is engineering the therapeutic response as a function of quorum sensing—the ability of some bacteria to regulate gene expression in response to density of the cell population. The bacterial quorum sensing autoinducer-2 (AI-2) signaling pathway of E. coli was rewired to enable programmed motility, sensing, and payload delivery based on the density of epidermal growth factor receptor (EGFR). In vitro, the engineered bacteria had the ability to survey surfaces to distinguish between diseased and healthy cells, and initiate DsRed gene expression in response. ECT was engineered to produce and release nanobodies targeting programmed cell death-ligand 1 (PD-L1) and cytotoxic T lymphocyte-associated protein-4 (CTLA-4) intratumorally once a quorum was reached. Compared with clinically relevant antibodies, a single dose of intratumorally injected engineered bacteria resulted in an increase in activated T cells, tumor regression, as well as an abscopal effect. Using similar intratumoral AI-2 signaling, a nonpathogenic E. coli strain was engineered to lyse and release a nanobody antagonist of CD47, a receptor that promotes tumor cell survival.

4. Conclusion

Cell-based therapies are ushering in a new era of drug delivery in which the unique attributes of immune cells, stem cells, platelets, and bacteria are leveraged to overcome current limitations in cancer treatment. Using onboard sensing, autonomous motion, and intercellular communication, these cells can home to tumors. In addition, their innate features are amenable to modification and augmentation via genetic reprogramming. When used as drug carriers, they are capable of acting as controlled delivery depots and improving the pharmacokinetic profiles of their payload. Although several examples are already clinically used or are being tested in clinical trials, limitations still must be overcome to unleash the full potential of cell-based therapies. Inadequate infiltration resulting mostly from heterogeneity of tumors at both cellular and tissue level leads to incomplete eradication of cancer cells. Safety concerns, particularly in the case of CAR-T cells and bacteria, also necessitate further study of their tolerance in vivo. Developing effective means to upscale production of cells using good manufacturing practice (GMP) principles is another critical aspect in cell-based therapies.

Several of these limitations are already being addressed. External cues including magnetic fields and light have been used to boost tumor homing and therapeutic efficacy by assisting chemotaxis and guiding cells to tumor sites. Higher specificity in the TME toward cancer cells has been achieved by cell membrane modifications. Synthetic biology provides a powerful toolkit to tweak cells and provide therapeutic vectors capable of multifaceted responses to a vast array of disease signatures. Specifically, synthetic biology has offered the means to increase specificity and safety by producing attenuated strains, allowing spatiotemporally controlled release, and integrating further sensing or targeting functionalities. Current efforts, particularly for CAR-T cells, are focused on the development of autonomous, feedback circuits that trigger downregulation in response to indications of toxicity. For bacteria, the use of attenuated auxotrophic mutants has shown promise in mitigating unintended immunogenicity. Such prevention-based strategies could improve safety and increase the therapeutic potential of these living systems.

Considering the unique attributes of each cell-based system, their complementary therapeutic actions could be envisioned in the context of combination therapy. For instance, bacteria tend to colonize the hypoxic region of the tumor, whereas immune cells infiltrate the well-oxygenated rim. On the contrary, platelets exhibit unique targeting to CTCs. Combining engineered bacteria for local release and CAR-T cells with enhanced specificity could potentially provide a successful treatment of primary tumors, complemented by platelet-mediated recognition and eradication of intravasated cancer cells. Such synergistic effects are already studied in platelet or bacteria-based delivery of immune checkpoint inhibitors. Potency of these treatment strategies improves when physical therapies join forces with living therapeutics to combat cancer. Micro- or nanoagents that mediate application of external stimuli as a part of the therapy can be included in the delivered payload in this scenario. Making the best use of all available weapons in the realm of cell-based therapeutics, great advancements in cancer treatment seem to be on the horizon.
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Conflict of Interest

The authors declare no conflict of interest.

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