

Utilizing Bacteria-Derived Components for Cancer Immunotherapy

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Abstract

Bacteria-related cancer immunotherapy, because of its mechanisms and useful applications in the induction of anti-tumor immunity, has gained substantial attention in recent decades. Bacteria can enable targeting of tumors, and specifically can colonize the core tumor area. Because they contain many pathogen-associated molecular patterns—which efficiently stimulate immune cells, even within microenvironments that suppress anti-tumor immunity—bacteria boost immunological recognition leading to the destruction of malignant cells. This Editorial highlights various bacteria with immunotherapeutic effects and their by-products used as immunotherapeutics.

Keywords

Immunotherapeutics, immunotherapy, *Listeria*, *Salmonella*, *Lactobacillus*.

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Introduction

The complexity of bacteria, as living organisms, influences the challenges and risks associated with transforming them into anti-tumor weapons. This complexity enables scientists to fine-tune the diverse capabilities of various bacterial strains to elicit anti-tumor effects that are impossible to achieve with other medications [1]. The advantages of using bacteria for tumor targeting lies in their ability to function as vehicles transporting therapeutic agents to tumor tissue as well as to interact with the immune system (Figure 1), thus resulting in recognition and elimination of malignant cells [2]. Intravenous, subcutaneous, and intra-tumoral injections are used to introduce the bacteria to the host's body [3]. Subsequently, the bacteria spread across the noncancerous body parts of the host in addition to solid tumors [4]. The number of bacterial cells in the body that spread through the vasculature and normal tissue markedly decreases within hours or days, because of the oxygen-rich physiology of the human body and immunological elimination. The bacteria are eventually eliminated, thus preventing any possible toxic effects to the host [5]. After the bacteria arrive at a solid tumor, they move to the hypoxic

necrotic core areas of tumors through various processes, including chemotaxis [6]. The hypoxic tumor microenvironment (TME), in addition to the nutrients released by expired tumor cells, facilitates the growth of anaerobic bacteria. The immune system cannot rapidly eliminate the bacteria in the tumor, owing to the immunosuppressive TME. When the bacteria multiply and arrive at the tumor cells, the immune system is stimulated through contact of many immune cells with the tumor [7]. The major categories of bacteria and their functions in cancer immunotherapy are summarized in Table 1. Several bacterial drug-delivery methods for cancer treatment are in clinical trials, despite difficulties and restrictions regarding their production, adverse effects, stability, and mutations. Vion Pharmaceuticals evaluated *Streptococcus typhimurium* VNP20009 in 24 patients with melanoma in phase I. However, no objective tumor shrinkage was observed, although proinflammatory cytokines were elevated [8–10]. *Clostridium novyi*-NT spores have been applied in phase I clinical trials and demonstrated good outcomes after intratumoral injection. However, the *Clostridium* cells cannot completely destroy the tumor cells, thereby resulting in recurrence [11]. Similarly, the safety of bacterial minicells designed to deliver paclitaxel to cancer

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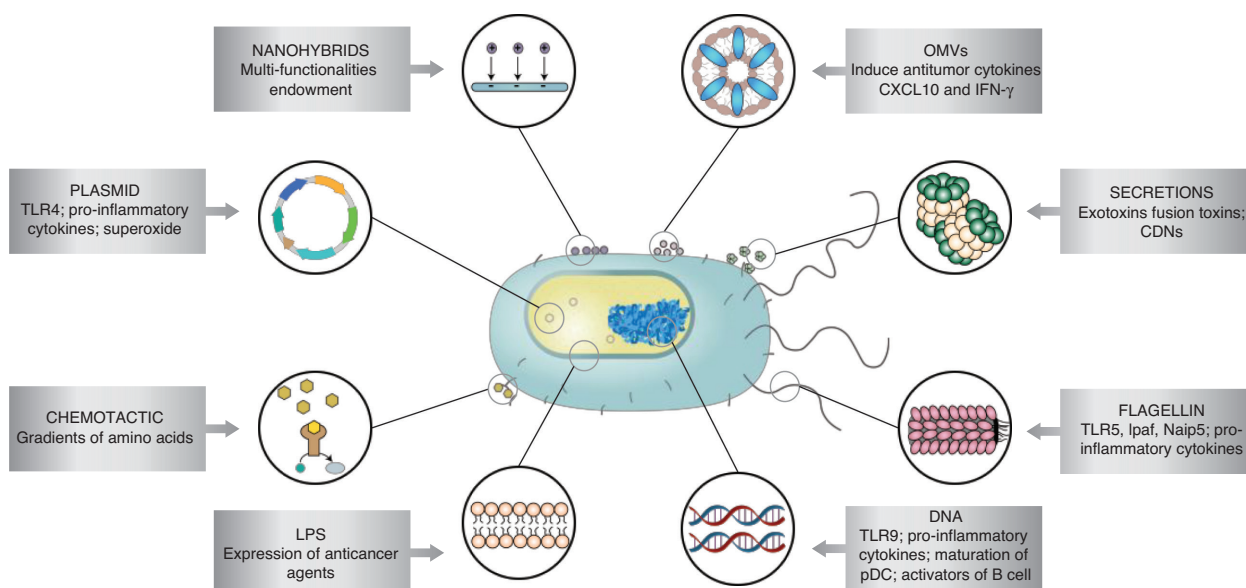


Figure 1 Illustration of the essential characteristics of bacteria and bacterial elements, including those in native and engineered bacteria. The blue box includes descriptions of each bacterial component and the interactions between the immune system and bacterial components.

Table 1 Major Categories of Bacteria

Bacteria	Immunotherapy	Ref
<i>Listeria</i>	<i>Listeria</i> initiate the death of tumor cells through immune-cell activation and stimulate CD8+ T cells to eliminate remaining tumor cells, while additionally preventing metastasis. <i>Listeria</i> contaminate bone-marrow-derived suppressor cells (MDSCs) at tumor sites, thus decreasing MDSCs and subsequently facilitating a shift from immunosuppressive to immunostimulatory status. Moreover, IL-12 can be obtained after the residual infected MDSCs adopt an immunostimulatory phenotype, thus enhancing T cell and NK cell activity.	[13–15]
<i>Clostridium Bacillus</i>	<i>Clostridium</i> activates apoptosis within tumors by facilitating the activity of tumor necrosis factor–associated apoptosis-inducing ligand (TRAIL) from polymorphonuclear neutrophils. Furthermore, the early spread of <i>Clostridium</i> in solid tumors facilitates granulocyte and macrophage filtration of tumor cells, thus increasing adaptive immunity and the engagement of immune cells (e.g., CD8+ T cells) at tumor sites, owing to increased chemokine secretion.	[7, 16–18]
<i>Salmonella</i>	Pathogen-associated molecular patterns such as LPS, <i>Salmonella</i> , and flagellin are identified by antigen-presenting cells (APCs). Flagellin facilitates the development of APCs, and upregulates pro-inflammatory cytokines (such as IL-12) and co-stimulatory molecules such as CD40, through binding and via the activation of Nod-like receptors (NLRs) and TLR5 on APCs. Because of their inflammatory activity, they further stimulate the production of interferon-gamma (IFN- γ) and the T helper type 1 (Th1) cell–mediated immune response. Macrophages and DCs secrete IL-1 β and TNF- α , which are pro-inflammatory, during this process, as LPS-induced TLR4 signaling and tumor cell debris are stimulated. Moreover, the secretion of IFN- γ , after activation of NK cells via a TLR-independent pathway that includes myeloid differentiation factor 88 (MyD88) and IL-18, is facilitated by flagellin. The secreted IFN- γ decreases the frequency of CD4+ CD25+ regulatory T cells within the TME.	[19–24]
<i>Lactobacillus</i>	<i>Lactobacillus</i> prevents colonization by infectious agents, stimulates the immune system, and has been demonstrated to elicit direct cytotoxic outcomes in cancer cells. Some strains of <i>Lactobacillus</i> have demonstrated antimutagenic properties [4–9]. Lactobacilli modify the Th1/Th2 balance, according to several studies. Th1-type responses are beneficial in cancer immunotherapy because they activate cytotoxic T lymphocytes. Th1 cells directly kill tumor cells by secreting cytokines that trigger death receptors. Th1 development is dependent on IL-4 in the absence of IL-12 [10].	[25–32]

cells has been assessed in a human phase I clinical trial on patients with advanced solid tumors. No deaths were reported during the experiment, thus indicating that the bacterial minicells are safe and have modest clinical efficacy [12]. These clinical results have demonstrated several difficulties in clinical application. However, new combinatory strategies with bacterial medication delivery are expected to improve intratumoral bacterial colonization and therapeutic output.

Bacterial components as immunotherapeutics

Bacterial outer-membrane vesicles

Gram-negative bacteria generate nano-sized spherical vesicles called outer-membrane vesicles (OMVs). OMVs are composed primarily of cellular elements of the bacterial periplasm

and the outer membrane, such as proteins, membrane lipids, peptidoglycans (PGs), lipopolysaccharide (LPS), and various virulence factors [33, 34]. OMVs contain several intracellular components, including RNA, DNA, intracellular proteins, metabolites, and ions. [35–38]. The mechanisms underlying the production of OMVs remain unclear. However, three widely acknowledged hypotheses may explain how OMVs are produced. First, OMV production may be due to the accumulation of phospholipids within the external membranes of the bacteria, in addition to regulation by the VacJ/Yrb ATP-binding cassette transport system present in most Gram-negative bacteria [39]. Second, the cross-linking between the bacterial external membrane and the PG layer–lipoprotein crosslinks in the cell walls of Gram-negative bacteria—which comprise PG and the outer membrane, with covalent bonds to preserve the envelope structure—may be involved. When the PG layer decomposes, a portion of the outer layer dissociates from the PG layer and extends outside the cell, thus resulting in formation of OMVs [40]. Finally OMVs are formed because of periplasmic accumulation of misfolded proteins and abnormal envelope components, which decrease the strength of the envelope and consequently divide the PF layer and the outer-membrane layer [41].

OMVs with many microbe-associated molecular patterns (such as LPS, PG, RNA, or DNA) facilitate interaction with host pattern-recognition receptors, thus stimulating the

innate-immunity response (Figure 2). Because of the abundance of natural adjuvant elements in OMVs, the administration of OMVs (packaging small interfering RNAs) obtained from a mutated *E. coli* strain has been found to upregulate the production of the cytokines TNF- α , IL-6, and IFN- γ , as well as the anti-tumor cytokine CXCL10, all of which promote anti-tumor immunity [42, 43]. The most important microbe-associated molecular pattern may be LPS, which comprises a core polysaccharide and lipid A, as well as O-antigen, a polysaccharide on the bacterial outer-membrane surface, and is the manifestation of bacterial cell antigens [44]. Lipid A is strongly inflammatory and regulates the immune response by prompting immune-cell production of antibodies against various antigens; it is central to the biological activity of LPS. Nonetheless, studies have shown that an excess of LPS leads to immunosuppressive reactions: blocking lipid A inhibits the activity of endotoxin, thereby decreasing immunosuppression [45, 46]. OMVs decrease the toxicity of LPS as immune adjuvants, and block lipid A function via inactivating of *msbB* gene; thus resulting in attenuation of immunosuppression, thus resulting in attenuation. Kim et al. have eliminated the *msbB* gene that encodes *E. coli* endotoxin, thus blocking lipid A-mediated immunosuppression [43]. Moreover, their study has indicated that use of G-bacteria inhibits tumor cell growth in a murine colon cancer model. NK cells and T cells, after

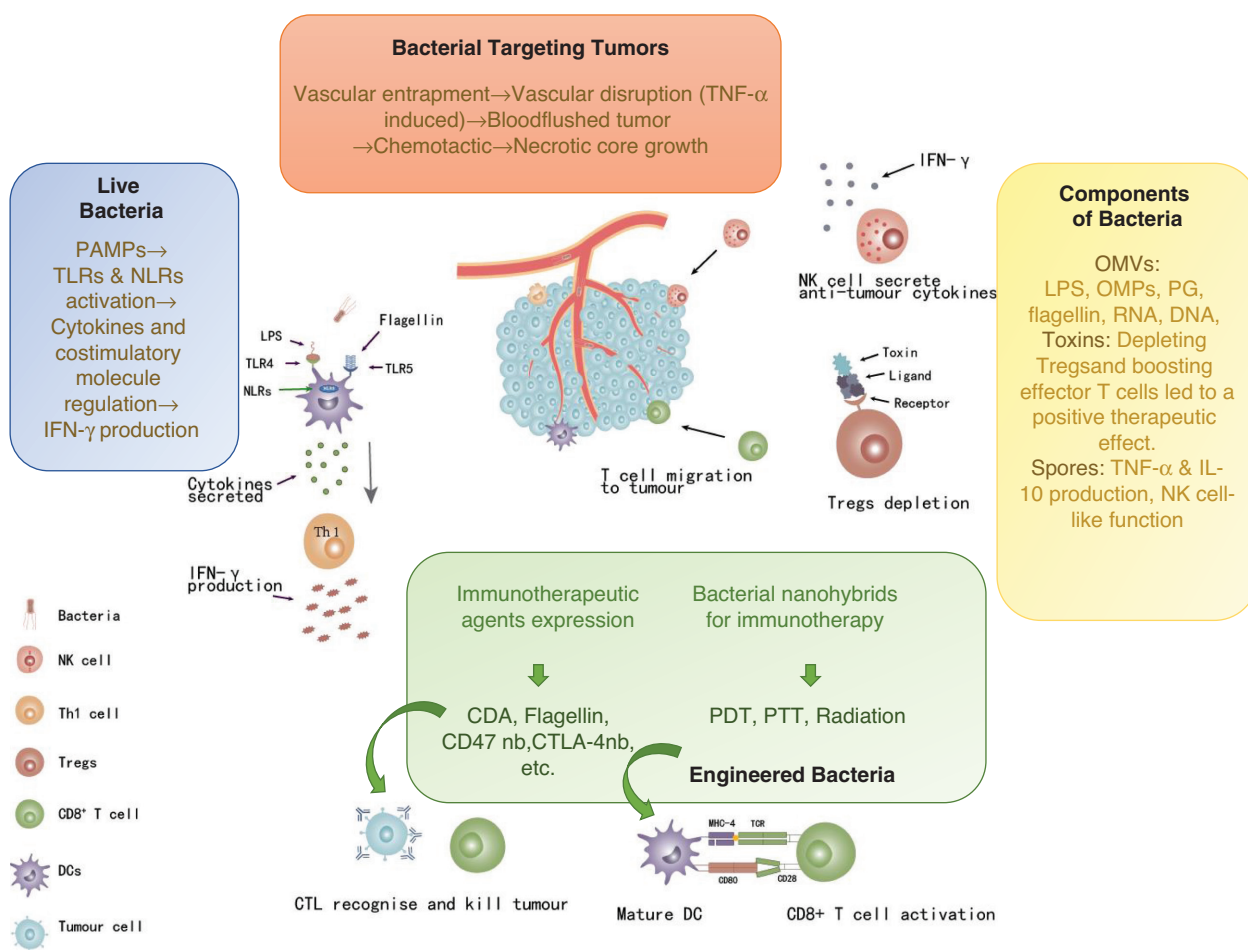


Figure 2 An overview of bacteria-mediated immunotherapy, showing how bacteria target tumors, including how naive live bacteria trigger the immune system, how modified bacteria are used in immunotherapy, and the different bacterial components used in immunotherapy.

stimulation by OMVs, secrete INF- γ , which inhibits tumor growth. Moreover, naturally produced OMVs have been used as carriers for drug delivery. For instance, OMVs have been loaded with immunomodulatory molecules, photosensitizers, and chemotherapy drugs, and used to transport these cargo to targeted tumor cells, thus achieving a combination of immunotherapy and phototherapy. In one study, Chen et al. have coated polymer micelles packed with drugs with DSPE-PEG-RGD-hybridized bacterial OMVs, and tested this novel nanomedicine's efficacy in immunotherapy for cancer and the prevention of metastasis [47]. This study demonstrated that the OMV nanomedicine directly interferes with immune cells, thereby inducing cytotoxicity via activation of the inflammatory response, which in turn activates the host immune response. Moreover, OMV-coated micelles loaded with tegafur have been found to regulate chemotherapy and the immune system, thus preparing melanoma-specific cytotoxic T lymphocytes and additionally suppressing pulmonary metastasis. Thus, to augment OMV functionality and achieve better tumor suppression, two designs have been proposed. The first involves hybridization of lipid polymers (or other biological membranes) with OMVs to attain new functions or for improve efficiency. The second uses the high loading capacity of OMVs to boost the anti-tumor immune response to other treatments and the OMVs themselves by delivering therapeutic agents (such as chemotherapy drugs, immune adjuvants, or photosensitizers) to tumor sites.

Bacterial toxins

Bacterial toxins are exceptionally toxic proteins that are generated and released by bacteria that possess specific functionality such as cell cycle arrest and apoptotic cell death etc. These toxins have been demonstrated to be a potent tool for the treatment of cancer, owing to their considerable toxicity [48]. Anti-tumor bacterial toxins are divided into two categories: those conjugated to the tumor cell antigen surface and those conjugated to ligands. Bacterial toxins that target specific antigens (which are highly expressed on the tumor surface) such as *Clostridium perfringens* enterotoxin, diphtheria toxin (DT), and *Pseudomonas* exotoxin, are used for the targeting and elimination of tumor cells [49–51]. DT is used primarily for the treatment of tumors both in murine models and humans, because it has relatively few anti-tumor effects [52], possibly because of its high cytotoxicity or its simultaneous induction of anti-tumor immunity. Buzzi et al. have engineered cross-reacting non-toxic material 197 (CRM197) for the treatment of a specific group of cancer patients [53]. As a non-toxic variant of DT, CRM197 has immunological functions similar to those of DT. Like DT, CRM197 targets heparin-binding epidermal growth factor, which is commonly overexpressed in tumor cells. Moreover, subcutaneous injection of CRM197 results in inflammatory immunological reactions, thereby activating a biological anti-tumor response. On the basis of these results, the authors have suggested that TNF- α and neutrophils may be associated with the anti-tumor process. Thus, bacterial toxins not only affect tumor cells but also initiate anti-tumor immunity. Fusion proteins comprising targeting antibody

fragments and bacterial toxins are also known as immunotoxins [54]. The targeting antibody fragments act on cancer cells and increase the potency of bacterial toxin fragments in eliminating targeted cells. Bacterial immunotoxins have powerful cytotoxicity through inhibition of protein translation and have been demonstrated to be highly effective in the treatment of several hematological diseases [55, 56]. In an earlier study, Ontak, a fusion protein consisting of anti-IL-2 and DT, has achieved satisfactory outcomes in the treatment of chronic lymphocytic leukemia, because chronic lymphocytic leukemia cells overexpress high-affinity IL-2 receptors [57]. For immunotoxin treatment, repeated administration of the drug is required, as in chemotherapy, to maintain the lethal concentrations necessary for optimal results. However, repeated treatment is restricted by immunogenetics, i.e., the development of anti-drug antibodies. After treatment with immunotoxins, several patients have shown a rapid immune response and the generation of anti-drug antibodies, which neutralize immunotoxins and consequently prohibit multiple administrations. To solve this problem, researchers have attempted to combine immunotoxins with chemotherapy drugs, and to modify bacterial toxins to avoid their identification by the immune system. These approaches have achieved the necessary decrease in immunogenicity. Another direct and widely accepted method is the deletion or mutation of T cell epitopes through the design of recombinant proteins to decrease immunogenicity. Mazor et al. have discovered a novel immunotoxin containing a disulfide-stabilized Fv of the anti-Tac antibody and PE38 bearing nine-point mutations in domains II and III. Furthermore, they have demonstrated that domain II is necessary for CD25-mediated cell destruction—a process distinct from CD22-mediated internalization. The recently engineered immunotoxin LMB-142 has demonstrated greater cytotoxic action in humans *in vitro* and a five-fold lower non-specific toxicity in murine models than LMB-2 (anti-Tac(Fv)-PE38).

Beyond the use of immunotoxins in the treatment of T cell malignancies and other solid tumors, Tregs depleting fusion-protein toxins have great promise in cancer immunotherapy. Tregs consist of T cells, which are considered the “brakes” of the immune response mediated by effector T cells. Moreover, they have major roles in immune tolerance, the prevention of autoimmune disease, and the inhibition of anti-tumor immunity [58, 59]. Tregs, the drivers of the immunosuppressive microenvironment, through processes such as interleukin consumption and immune suppression, promote tumor growth; consequently, their use in many immunotherapies has increased [60–62]. One technique for exhausting Tregs involves moving bacterial toxins to make use of their inherent cytotoxicity to directly eliminate Tregs. This method restores the normal binding of bacterial toxins to the ligands present on the receptors of Tregs. Consequently, cells rich in Treg receptors eliminate the toxins themselves. This process is beneficial, because it alleviates the TME's immunosuppressive nature and minimizes the toxicity of bacterial toxins toward non-targeted cells. Moreover, the high expression of Foxp3 in Tregs increases CD25 expression on the surfaces of Tregs, thus leading to the formation of heterotrimeric high-affinity IL-2 receptors [63, 64]. The abundance of CD25 on Tregs results

in exhaustion of IL-2 within the local microenvironment. However, a decrease in the number of cytokines results in apoptosis of activated effector T cells [65]. Thus, CD25 is an example of a targeted site. Cheung et al. have engineered a next-generation IL-2 receptor-targeted diphtheria fusion toxin with excellent anti-tumor effects in decreasing Tregs [66]. Moreover, this fusion toxin has a beneficial synergistic effect with anti-PD-1 in the treatment of melanoma. However, the clinical applications of denileukin diftotox or Ontak (fusion protein consisting of the bacterial toxin DT and anti-IL-2) are limited by a danger of vascular leakage and production issues related to aggregation and purity. One production approach has used *Corynebacterium diphtheriae* to directly reproduce the fully folded and biologically active s-DAB-IL-2 as a monomer within the culture medium. Moreover, the highly developed fusion protein s-DAB-IL-2(V6A) has been generated through the mutation of a single amino acid (V6A). In comparison to s-DAB-IL-2, V6A has 50 times less vascular leakage *in vitro* with ~3.7 times lower lethality in mice. In a murine model of melanoma, s-DAB-IL-2(V6A) monotherapy as well as combination therapy using anti-PD-1 have been found to increase inhibition of tumor cell growth. The desirable therapeutic effects are associated with a decline in Tregs and the proliferation of effector T cells. Although bacterial toxins are generally considered to have high toxicity toward tumor cells, through fusion with Treg-targeted proteins, they also effectively exhaust Tregs and facilitate an anti-tumor response. More importantly, the association of bacterial toxins with immune-checkpoint blockade enables their application in cancer immunotherapy.

Bacterial spores

Spores are inactive forms of bacteria and are thus extremely resistant. They can live in oxygen-rich cells for extended durations without germinating. After encountering a suitable environment, such as the hypoxic/necrotic area within the tumor core, spores undergo germination and multiplication. Because no critically hypoxic microenvironments are present in normal human tissues, spores do not exhibit toxicity in human organs under physiological conditions. Many researchers have administered a *Clostridium histolyticum* spore suspension into tumor cells and observed effective inhibition of transplanted rat sarcomas without apparent systemic toxicity [52]. In some reports, the mice died from tetanus within 48 hours after the intravascular injection of *Clostridium* spores in tumor-infected murine models, and this effect was not limited to intratumoral injections [67]. The healthy mice receiving the same treatment remained asymptomatic for as long as 40 days, thus establishing that spores demonstrate tumor-specific germination even after vascular administration. *Clostridium novyi* (*C. novyi*) has been widely studied because of its high sensitivity to oxygen and high mobility because of its peritrichous flagella [6]. These two factors contribute to the tumor enrichment of *C. novyi* even when only a small amount of spore germination occurs. The main systemic toxin (α -toxin) gene of *C. novyi* has been isolated and used to generate a novel attenuated *C. novyi*-NT, which has better

application prospects because of its lower systemic toxicity [68]. Agrawal et al. have noted that systemic administration of *C. novyi*-NT spores in fully immune mice with tumor cells results in long-term tumor regression [7]. *C. novyi*-NT spores have been found to spread throughout the body after systemic injection. However, the anaerobic properties of the environment cause them to germinate in only the necrotic core of the tumor, which is relatively hypoxic. The germinated bacteria then eliminate the surrounding tumor cells through local production of lipases, proteases, and other degrading enzymes. Meanwhile, the host responds to this local infection by secreting immunostimulatory cytokines, including MIP-2, IL-6, tissue inhibitor of metalloproteinases 1 (TIMP-1), and granulocyte colony-stimulating factor (G-CSF), thus facilitating the infiltration of tumors by various immune cells. Initially, only a neutrophil response is observed, but monocytes subsequently participate. The spread of bacterial infection is inhibited by the inflammatory response, thus also providing a second layer of control beyond the initial layer supplied by the anaerobic environment. Moreover, the elimination of tumor cells is facilitated by inflammation via generation of reactive oxygen species, and proteases and other enzymes. In addition, inflammation evokes an efficient cellular immune reaction, which continues to eliminate tumor cells remaining to be destroyed by bacteria. Malignant cells have promisingly been found to be eradicated in 30% of mice with tumors. In a later study, the administration of *C. novyi*-NT spores into naturally occurring tumor cells in dogs was found to provoke a powerful immune response [69]. Intratumoral inoculation with *C. novyi*-NT spores enhances phagocytosis as well as the functions of NK-like cells. Moreover, intravenous injection of *C. novyi*-NT spores results in TNF- α production activated by LPS and IL-10 production triggered by LTA, and enhances NK-like-cell function. These findings have demonstrated that the administration of *C. novyi*-NT spores produces long-term alterations in the functions of immune cells. In a different study, Heaps et al. have injected engineered clostridial spores into the blood circulation and successfully suppressed and healed human colon carcinoma in a murine xenograft model [70]. The engineered spores germinated and became activated after reaching the hypoxic necrotic areas of the tumor core, after which they released a prodrug-converting enzyme, which converts non-toxic prodrug molecules to a potent cytotoxic form at the tumor site, thus resulting in the death of tumor cells.

Conclusion

Extensive research conducted on the interactions between tumor cells and the human immune system has indicated that immunotherapy may be one of the most promising approaches to cancer treatment. Because both bacteria and their constituents naturally stimulate the host's immune system, they generate a strong anti-tumor immune response. Although the definitive interactions among tumors, bacteria, and the immune system remain unclear, further research will shed light on how bacteria might be adapted to regulate this interaction to achieve better outcomes. With developments

in synthetic biology, many solutions for these issues are being discovered, and this area of research is expected to be highly worthwhile. Clinicians might be able to control the engineering and release of immunotherapeutic agents by deciding on the number of gene copies, enhancing bacterial

strength and metabolic rate, and adjusting the initial bacteria injection dosage. For patients with various tumors in distinct stages, a personalized immunotherapy plan could be achieved by adjusting the intensity of the therapeutic agents to achieve optimal treatment effects.

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