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Bacteriotherapy: understanding bacterial species specialization based on the established hallmarks of cancer

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Abstract

The prognosis of many cancers has substantially improved through widely accepted therapeutic strategies such as chemotherapy, radiotherapy, and immunotherapy. However, cytotoxicity, reduced accessibility, inability to selectively target tissue, and drug resistance, among other downfalls of traditional modes of treatment make long-term survival and good quality of life difficult to achieve for cancer patients. The complementary or individual administration of bacteriotherapy has been shown to compensate for these deficiencies in traditional treatment. Broadly, qualities such as improved motility, capacity to thrive in aerobic and anaerobic conditions, secretion of toxins, and genetic modifiability allow for a wide range of therapeutic use. The bacterial genera most frequently used in the context of cancer treatment are *Clostridium, Bifidobacterium,* and *Salmonella.* I will discuss these three bacterial genera alongside several others used in cancer bacteriotherapy and highlight their therapeutic advantages based on hallmarks of cancer that they address using the established 'hallmarks of cancer' devised by Hannahan and Weinberg. Ultimately, I argue that species specialization is a promising therapeutic avenue to treat different cancers.

Introduction

By 2040 the world is projected to have at least 27.5 million new cancer cases and 16.3 million cancer-related deaths making cancer an urgent public health concern (*Global Cancer Facts & Figures | American Cancer Society*, n.d.). A key organizational framework reflecting the medical community's evolving understanding of neoplastic diseases is Hanahan and Weinberg's 'hallmarks of cancer.' Along with summarizing ten key biological mechanisms of cancer, this framework also identifies key drug targets leveraged by impactful therapies (Hanahan & Weinberg, 2011). A well-known example is the immunotherapeutic agent, Pembrolizumab which targets the 'avoiding immune destruction' hallmark. By preventing the PD-1 receptor/ligand interaction, the drug enables the immune system to accurately identify cancerous cells. This galvanizes an immune response which leads to the apoptosis of cancerous cells. Other traditional therapies targeting cancer hallmarks include surgery, chemotherapy, and radiotherapy. However, these treatments have numerous limitations including the inability to selectively target neoplastic tissue, cytotoxicity, reduced accessibility to the tumor site, and drug resistance. These detriments can severely compromise the patient's quality of life and/or lead to a relapse of the disease therefore diminishing the chance of long-term survival. Decades of sustained research in the field of bacteriotherapy position it as a mode of treatment that can bridge this therapeutic gap (Patyar et al., 2010; Sedighi et al., 2019).

Bacteriotherapy pertains to the administration of genetically modified bacteria and/or their byproducts. The tremendous strides made in molecular bioengineering alongside the relative ease in genetically manipulating bacteria have enabled scientists to safely administer and closely regulate bacteriotherapy, with significantly positive results. The qualities of bacteria that are useful in cancer treatment include increased motility, accessibility to hypoxic regions, and the production of certain byproducts (spores, toxins, and enzymes). Bacteriotherapy can be administered independently or alongside traditional therapies (Patyar et al., 2010; Sedighi et al., 2019).

The ten most used genera of bacteria in bacteriotherapy are *Clostridium*, *Bifidobacterium, Salmonella*, *Lactobacillus*, *Escherichia, Pseudomonas*, *Caulobacter*, *Listeria*, *Proteus*, and *Streptococcus* (Sedighi et al., 2019). Clinical studies demonstrate unique differences in the biological processes these bacteria target as well as the mechanisms by which they exhibit their antitumor effects. By delineating the mechanism and therapeutic outcome for each bacterial genera using the 'hallmarks of cancer', it becomes apparent that bacterial species specialization is a promising therapeutic avenue.

To demonstrate this, here I specifically review the therapeutic applications for *Clostridium*, *Bifidobacterium,* and *Salmonella*; these three genera are most frequently used as vectors delivering tumoricidal agents and those engineered to express advantageous genes (Sedighi et al., 2019).

Their widespread therapeutic success, variety of cancer hallmarks addressed, and prevalence of these three genera in the literature further support their in-depth exploration. This will be followed by a discussion of cutting-edge applications of the other aforementioned bacterial genera alongside a discourse on how scientific advances have mitigated bacteriotherapy's shortcomings.

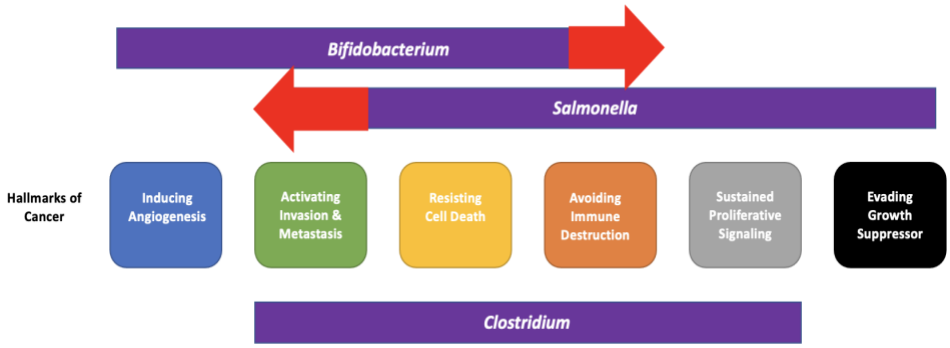

FIGURE 1. Hallmarks of Cancer Targeted by *Clostridium*, *Bifidobacterium* and *Salmonella.* Different Clostridium-based treatments target different cancer hallmarks. Bifidobacterium and Salmonella mainly address the 'avoiding immune destruction' and 'activating invasion & metastasis' hallmarks, respectively as indicated by the red arrowheads. Both bacterial genera in different treatment contexts additionally target other hallmarks as shown. Graphic is adapted from Hanahan & Weinberg, 2011.

Universal Hallmark Addressed by Most Bacteria

One hallmark of cancer consistently targeted by many bacteria is 'reprogramming energy metabolism'. Frequently, tumor cells induce hypoxic conditions to pleiotropically upregulate glycolytic pathways to provide more energy for tumor activity. This further induces cellular proliferation and other hallmarks of cancer (Hanahan & Weinberg, 2011). Many of the bacterial genera discussed exploit this hallmark; having the capacity to function in anaerobic environments allows them to selectively colonize hypoxic tissue allowing for targeted cancer therapy. This helps mitigate damage to surrounding healthy tissue during treatment.

Therapeutic Applications for *Clostridium*

Clostridium primarily addresses the 'reprogramming energy metabolism' hallmark, as well as several others. Indeed, of the three aforementioned genera discussed herein, *Clostridium* addresses the greatest diversity of cancer hallmarks. The following section will discuss how three different *Clostridium*-based treatments address different hallmarks. The administration of recombinant cytotoxin derived from *Clostridium difficile* addresses the 'sustained proliferative signaling' and 'resisting cell death' hallmarks. The use of *Clostridium difficile* based nanoparticles carrying Doxorubicin targets the 'activating invasion & metastasis' hallmark.

Lastly, the administration of *Clostridium novyi-NT* addresses the 'avoiding immune destruction' hallmark.

***Clostridium*-based treatment**	*Clostridium difficile* based recombinant cytotoxin B (rcdtB)	*Clostridium perfringens* enterotoxin based nanoparticles carrying Doxorubicin	*Clostridium novyi-NT* spores
Hallmark(s) addressed	- Sustained proliferative signaling - Resisting cell death	- Activating invasion & metastasis	- Avoiding immune destruction

TABLE 1. Hallmarks of Cancer Targeted by Three *Clostridium*-based treatments.Three different Clostridium-based treatments target four different cancer hallmarks.

Clostridium difficile produces cytotoxin (TcdB) which produces pro-inflammatory cytokines and chemokines that inhibit cell proliferation and promote cell death. Zhang et al. demonstrated that treatment with recombinant cytotoxin B (rcdtB) addresses the 'sustained proliferative signaling' and 'resisting cell death' hallmark. They studied the impact of increasing rcdtB doses on breast cancer progression in BALB/c mice. The tumor volume significantly decreased in the rcdtB treatment group versus the normal treatment group. This was attributed to the significantly down-regulated expression of C-erbB-2 and Cox-2 alongside the significant induction of early and late apoptosis when the 100, 200, and 400 ng/ml doses were administered. C-erbB-2 is an epidermal growth factor receptor (EGFR) tyrosine kinase that stimulates tumor cell proliferation; rcdtB treatment addresses the 'sustained proliferative signaling' hallmark. Subsequent experiments attributed apoptosis induction to the concomitantly decreased expression of Bcl-2, an anti-apoptotic and pro-survival protein; rcdtB treatment also addresses the 'resisting cell death' hallmark (Zhang et al., 2018).

In addition to its toxins, *Clostridium* is also used as a carrier. Shim et al. tested the administration of *Clostridium perfringens* enterotoxin (CPE) based nanoparticles, loaded with Doxorubicin, on the progression of pancreatic cancer. This treatment addresses the 'activating invasion & metastasis' hallmark. CPE was chosen as the carrier since it binds with high affinity to Claudin-4 (CLDN4). CLDN4 is a transmembrane protein that is integral to tight junctions which contribute to governing the metastatic potential of cells by regulating paracellular permeability and cell polarity in epithelial cells (Bhat et al., 2019; Shim et al., 2021). Therefore, this treatment addresses the 'activating invasion & metastasis' hallmark. CPE was additionally conjugated with polysialic acid-based nanoparticles as they accumulate exclusively in tumor tissues and release Doxorubicin (DOX), a chemotherapy agent. The combined entity, DOX-C-SNP, accumulated 2.7 times more in pancreatic tumor tissue versus the control that lacked CPE. Furthermore, the DOX-C-SNP treatment group had smaller tumor volumes versus that of the control groups. This

demonstrates a therapeutic advantage over stand-alone chemotherapy and the therapeutic importance of CPE. Additionally, the bodyweight of the DOX-C-SNP treatment group was significantly greater than that of the DOX group and comparable to that of the saline-treated group. This demonstrates substantially reduced systemic toxicity, which has major implications for improving patient quality of life. All of the factors mentioned above contributed to the significantly prolonged survival of the DOX-C-SNP-treated mice (Shim et al., 2021).

Lastly, one of the most common forms of *Clostridium*-based treatment that has substantially improved disease prognosis is the administration of *Clostridium Novyi-NT* (*C. novyi-NT*) spores, which address the 'avoiding immune destruction' hallmark. *C. novyi-NT* both functions well in hypoxic regions and is also highly motile with its peritrichous flagella making it an appealing bacteriotherapy candidate. When Agarwal et al. administered *C. novyi-NT* spores to mice with CT26 colorectal tumors, over 30% of mice were cured. Morphological analysis revealed a substantial accumulation of inflammatory cells, specifically neutrophils, at the periphery of the tumors. Subsequent experiments confirmed that the administration of *C. novyi-NT* stimulates an immune response, primarily attributed to CD8+ T cells, that resulted in a significantly lesser proportion of mice redeveloping tumors; *C. novyi-NT* addresses the 'avoiding immune destruction' hallmark. Similar results were observed when this treatment was administered in mice with RENCA tumors and rabbits with VX2 tumors. Furthermore, necropsies in a rabbit model showed that *C. novyi-NT* germination and extensive necrosis led to a statistically significant increase in survival. Complete systemic immunity was also observed in rabbits and somewhat in the two mice models (Agrawal et al., 2004). Hence the reproducibility of these favorable results across species and cancer type, along with how *C.novyi-NT* bacteriotherapy alone led to an increase in survival, shows *C.novyi-NT* bacteriotherapy to be a promising therapeutic avenue.

Robert et al. specifically demonstrated *C.novy-NT* bacteriotherapy as a promising therapeutic avenue for humans. After observing a 37.5% objective response rate in their comparative canine trial, the study injected *C.novyi-NT* spores into the metastatic right shoulder tumor of a patient suffering from metastasized retroperitoneal leiomyosarcoma. This intratumoral injection precisely eliminated neoplastic tissue leading to an improvement in survival. Consistent MRIs till day 55 showed ongoing tumor reduction while histopathology showed extensive tumor necrosis with small foci of residual tumor cells. The study last recorded that the patient had a performance status of 1 on the ECOG scale ranging from 0 (patient is active with few restrictions) to 5 (patient is dead) with no signs of infection (*ECOG Performance Status*, n.d.; Roberts et al., 2014). Despite this only being one patient, the rapid and robust local antitumor response coupled with the encouraging comparative canine trial results are cause for potentially replicating this study with more patients.

Therapeutic Applications for *Bifidobacterium*

Unlike *Clostridium*, clinical studies implicate the intrinsic properties of *Bifidobacterium* in mainly addressing the 'avoiding immune destruction' hallmark. *Bifidobacterium* regulates dendritic cell maturation at the host level and influences the production of cytokines and their affiliated components. As shown by Wei et al. that used Tumstatin transformed *Bifidobacterium longum*, *Bifidobacterium* is also an appealing vector that can deliver potent antitumor agents to indirectly address other hallmarks of cancer.

Like *Clostridium*, *Bifidobacterium* is also an anaerobic bacterium that can localize and colonize hypoxic tumor regions. However, unlike *Clostridium* and some of the other genera of interest to this paper, *Bifidobacterium* is typically nonpathogenic ensuring greater safety for the patient. *Bifidobacteria* also contribute to the composition of commensal microbiota and consequently play a role in modulating the patient's response to treatment. There are three mechanisms by which *Bifidobacteria* can help inhibit tumorigenesis. First, by modulating the gut microbiota, *Bifidobacteria* can inhibit the production of carcinogens and activate certain glycolytic pathways that promote a lower intestinal pH reducing exposure of the intestinal epithelium to carcinogens. Second, it can catalyze an immune response involving macrophages, natural killer T-cells, and lymphocytes. Third, *Bifidobacteria* contribute to maintaining host DNA stability and induce apoptosis of tumor cells by, for example, modulating the ratio of Bax/Bcl-2 gene expression (Wei et al., 2016). Consequently, *Bifidobacteria* are capable of addressing the following hallmarks of cancer: 'avoiding immune destruction', 'genome instability & mutation', and 'resisting cell death.' However, according to the literature, in most therapeutic contexts it seems to primarily address the 'avoiding immune destruction' hallmark.

Sivan et al. demonstrated this by showing that a difference in commensal microbiota composition, specifically an increased count of *Bifidobacteria*, conferred antitumor immunity. A significant difference was observed in the growth of subcutaneous B.16.SIY melanoma between two groups of mice with the aforementioned different commensal microbiota compositions. The introduction of commensal bacteria from the better-faring mice to the poorer-faring mice resulted in significantly slower tumor growth and greater immune response. This was analogous to that observed with the systemic administration of PD-L1 antibody therapy. Hence commensal microbiota treatment might be comparable in efficacy to PD-L1 antibody therapy. Combining the two therapies also showed a significant decline in tumor volume versus when PD-L1 antibody therapy was solely administered. The rationale for the success of commensal microbiota treatment, rooted in increased *Bifidobacteria*, is attributed to T-cell responses that take place at the level of the host's dendritic cells (DC). By regulating DC maturation, *Bifidobacterium* indirectly regulates the

improved effector function of tumor-specific CD8+ T cells and greater cytokine production such as that of IFN-Y (Sivan et al., 2015).

Lee et al. provided more insight on the antitumor mechanism of *Bifidobacteria* by testing the impact of *B. adolescentis* isolated from human fecal matter on the RAW 264.7 murine macrophage cell line. The macrophages became "large and rough" in a dose-dependent manner. The morphology was described as similar to that of dendritic cells. There was also a significant increase in TNF-α (tumor necrosis factor-alpha) and NO (Nitric Oxide) production both of which mediate the killing and growth inhibition of tumor cells. TNF-α specifically regulates immune modulation and is cytotoxic to tumor cells (Lee et al., 2008). Hence by regulating DC maturation and stimulating the production of cytokines and antineoplastic agents, *Bifidobacterium* addresses the 'avoiding immune destruction' hallmark.

Bifidobacterium has also been used as a gene delivery vector. Wei et al. created Tumstatin transformed *Bifidobacterium longum* (*BL-Tum*) and administered this treatment to a CT26 mouse model addressing the 'inducing angiogenesis', 'sustained proliferative signaling', and 'resisting cell death' hallmarks. Tumstatin inhibits angiogenesis, the process by which a tumor stimulates the development of new blood vessels to increase access to blood and nutrients facilitating tumor expansion (Hanahan & Weinberg, 2011; Wei et al., 2016). Tumstatin also inhibits cellular proliferation by disrupting protein synthesis and promoting apoptosis, additionally addressing the 'sustained proliferative signaling' and 'resisting cell death' hallmarks. In Wei et al., significant antitumor effects were observed in the *BL-Tum* treatment group versus the control group based on microvessel density, percentage of apoptotic vascular endothelial cells in transplanted tumors, tumor weight, tumor volume, and tumor growth. As such, promising therapeutic success has been demonstrated. Wei et al. also demonstrated that the best mode of administration for this treatment was an injection into the tumor. Therefore, by capitalizing on the 'deregulating cellular energetics' hallmark, *BL-Tum* treatment, administered most effectively through intratumoral injection, mainly addressed the 'inducing angiogenesis' hallmark alongside the 'sustained proliferative signaling' and 'resisting cell death' hallmarks.

Therapeutic Applications for *Salmonella*

In comparison to both *Clostridium* and *Bifidobacterium*, *Salmonella* demonstrates a niche for addressing the 'activating invasion & metastasis' hallmark (Weibel et al., 2008). This is substantiated by how attenuated strains of *S. typhimurium*, one of the most commonly used strains of *Salmonella* in cancer bacteriotherapy, were shown to significantly accumulate and eliminate metastases (Ganai et al., 2011; Hayashi et al., 2009). Additionally, the hallmarks addressed by *Salmonella* depend on

whether they are administered individually, in combination with traditional therapeutics, or as a vector carrying an antitumor agent.

Ganai et al. showed that the attenuated strain of *S. typhimurium*, VNP20003, addresses the 'activating invasion & metastasis' and 'resisting cell death' hallmarks. VNP20003 demonstrated an immediate tropism for subcutaneous tumors. Gradually VNP20003 moved from the edge of the primary tumor to the transition zone and induced apoptosis after sufficiently colonizing a tumor region. The mechanism of apoptosis induction is unclear, however, this preferential movement to the transition zone is explained by how the bacteria experience chemotaxis towards spatial cues produced by the dying cells in the transition zone. The transition zone is the buffer region of dormant cells that border viable and necrotic tumor cells. Both colonization and apoptosis were significantly greater in the transition region versus other tumor regions. This has important therapeutic ramifications since tumor cells in the transition zone are exceptionally resistant to treatment and are associated with tumor aggressiveness and cachexia. VNP20003 also had a significant ability in targeting metastases, especially masses less than 300 micrometers in diameter (Ganai et al., 2011).

Hayashi et al. provided more insight on how *Salmonella* selectively targets metastases. This study administered the *S. typhimurium* A1-R strain to mice infected with metastases by the following cancers taken from human tissue: axillary lymph node metastasis of pancreatic cancer, popliteal lymph node metastasis of fibrosarcoma, and lung metastasis of fibrosarcoma. Post 7-21 days of treatment, all metastases had been eradicated. There was no significant difference in the body weight between the treated and untreated groups indicating minimal treatment toxicity. The treatment success is attributed to two factors. First, *S. typhimurium* can grow in both viable and necrotic/hypoxic regions enabling greater tumor eradication. Second, by injecting the bacteria to target lymphatic metastasis, the study targeted the lymph system which is crucial for bacterial drainage and cancer spread. Lung metastases were eliminated via systemic injection of bacteria (Hayashi et al., 2009). Ultimately, both Ganai et al. and Hayashi et al. showed that attenuated *S. typhimurium* significantly addresses the 'activating invasion & metastasis' hallmark.

When used as a vector to carry antitumor agents and/or when administered alongside traditional cancer therapy, *Salmonella* again shows promising results in regulating tumor growth and addresses different hallmarks. Gao et al. synthesized an oxygen tolerant and highly replicative strain of *S.typhimurium* (KST0652), which was engineered to express the apoptotic protein sATF6 in response to radiation in a dose-dependent manner. This treatment addresses the 'resisting cell death' and 'avoid immune destruction' hallmarks. Before being engineered to express sATF6, KST0650 did not significantly impact tumor size and survival rate. However, when KST0650 was engineered to express sATF6

(KST0652) and administered alongside γ-radiotherapy, a statistically significant synergistic effect on tumor suppression was observed; tumor growth was completely inhibited, and the treatment group was completely protected from death. This is a successful example of combination treatment. The synergistic effect is partially explained by how CHOP, a downstream signaling protein associated with apoptosis and regulated by sATF6, was upregulated. This combination treatment synergistically addresses the 'resisting cell death' hallmark. Both *Salmonella* infection and low-dose radiation can also stimulate a host immune response by inducing the secretion of pro-inflammatory cytokines. They can also enhance antigen presentation leading to the accumulation of cytotoxic T-lymphocytes. Hence this combination therapy could potentially address the 'avoid immune destruction' hallmark (Gao et al., 2020).

Additionally, Yoon et al. showed that *S. typhimurium* can potentially also address either the 'evading growth suppressor' or 'sustaining proliferative signaling' hallmark in CT26 and B16F10 (melanoma) mouse models. They engineered attenuated *S. typhimurium* to carry an expression vector encoding short hairpin RNA targeting INHA (*S. typhimurium*/sh-INHA). The exact function of IHNA is unclear in colon cancer and melanoma; IHNA is said to have tumor suppressor activity in early-stage cancer and oncogenic activity in late-stage cancer. Accordingly, IHNA would be functioning in a manner to suppress cancer in the early stages but would destructively 'sustain proliferative signaling' in late-stage cancer. Significant IHNA expression was observed in tumor tissue versus that of normal adjacent tissue. Administration of *S. typhimurium*/sh-INHA exerted greater cytotoxic effects versus that of the control strains. This treatment also resulted in significant and prolonged tumor growth inhibition leading to significantly prolonged survival; half of the mice in the treatment group were completely cured with no residual cancer cells and no recurrence. Furthermore, no histopathological signs of damage and residual genetically modified *S. typhimurium* were observed post each treatment. This shows that *S. typhimurium* can be administered with few side effects. *S. typhimurium* was also found to reduce the expression of anti-apoptotic proteins (Bcl-2 and Bcl-XL) (Yoon et al., 2018). Hence *S. typhimurium*/sh-INHA treatment addresses the 'resisting cell death' hallmark alongside potentially addressing the 'evading growth suppressor' or 'sustaining proliferative signaling' hallmarks.

Therapeutic Avenues Involving Other Bacterial Genera

Despite the vast success and representation of *Clostridium*, *Bifidobacterium,* and *Salmonella*, other bacterial genera are also used in cancer bacteriotherapy. Here I will focus on how therapies affiliated with other genera reflect the tremendous advantages of cancer bacteriotherapy overall.

As shown thus far bacteriotherapy when combined with traditional therapies can significantly improve treatment outcomes. Jiang et al. further

corroborated this phenomenon using *Escherichia Coli* and radiotherapy (RT). They engineered an *Escherichia Coli* strain, K-12 (a non-pathogenic and commensal strain), to produce cytolysin A (ClyA) and administered this bacterium in primary and metastatic tumor mice models. Hypoxic tumor cells are almost three times more resistant to RT than normoxic cells. *E. Coli* is analogous to *Clostridium* and *Salmonella* in its ability to migrate to and colonize hypoxic tumor regions. However, bacterial colonization of hypoxic regions is always somewhat limited since tumor tissues are rapidly proliferating. Also, while anaerobic bacteria are more efficient in destroying hypoxic cells, RT is more efficient in destroying normoxic cells. Furthermore, cytolysin A is a pore-forming protein that induces macrophage apoptosis and is cytotoxic to mammalian cells. This explains why RT and modified *E. coli* K-12 had a synergistic effect addressing the 'resisting cell death' hallmark. By significantly enhancing the therapeutic effect of radiation, this *E. Coli* based treatment gave rise to the complete suppression of tumor growth in mice. It also significantly reduced metastases development and increased survival time. The success of this combination therapy may warrant the administration of smaller doses of radiotherapy mitigating its side effects (Jiang et al., 2010). Hence bacteriotherapy and traditional therapy compensate for one another's shortcomings leading to improved therapeutic success and potentially reduced side effects.

Another advantage of bacteriotherapy is that both biotechnological innovations and bacteria are highly modifiable. Their malleability allows for endless modifications yielding additional therapeutically advantageous properties and the ability to carry non-conventional tumoricidal agents to address several hallmarks. This has been observed whether it was the use of CPE-based nanoparticles or the delivery of short hairpin RNA to knockdown INHA via *S. typhimurium* (Shim et al., 2021; Yoon et al., 2018). Another example comes from Quispe-Tintaya et al., the first study to report the successful delivery of a radioactive entity via attenuated bacteria. Attenuated *Listeria monocytes* (Listeria^{at}) were coupled to 188-Rhenium producing radioactive Listeria^{at} (RL). This treatment was administered in a highly metastatic pancreatic tumor mouse model. RL treatment decreased the number of metastases by 90% and reduced the primary tumor weight by 64% versus the control groups. The preferential accumulation in metastases is explained by the infected myeloid-derived cells (MDSCs). When Listeria^{at} was injected into the rat tails, it specifically infected myeloid-derived suppressor cells (MDSCs). MDSCs possess immunosuppressive properties and have been implicated in promoting metastases (Trovato et al., 2020). Once infected, the MDSCs then delivered Listeria^{at} to the metastases; this treatment co-opts the 'activating invasion & metastasis' hallmark. The host immune response involving activated T-cells and natural killer cells (NK) in response to the Listeria^{at} infected tumor cells also played a role in treatment success; this treatment also addresses the 'avoiding immune destruction' hallmark.

However, RL's antitumor effect mostly stems from how Listeria^{at} secretes Listeriolysin O (LLO) which induces a high level of reactive oxygen species in the tumor cells of metastases impacting apoptosis and cell survival. This antitumor effect was also heavily influenced by the crossfire effect of 188-Rhenium generated radioactivity. Hence this treatment also circuitously addresses the 'sustained proliferative signaling' and 'resisting cell death' hallmarks. Lastly, this treatment was found to be non-toxic; one week after the last treatment no radioactivity was detected and all Listeria^{at} had been cleared by the immune system (Quispe-Tintaya et al., 2013). The reduced toxicity, targeting of multiple hallmarks, and significant reduction in tumor weight and metastases contribute to the appeal of this treatment and demonstrate how bacteriotherapy can be an effective and holistic course of treatment.

Lastly, bacteriotherapy has demonstrated the potential to improve the objective response and survival rate for cancers with poor prognoses. For certain cancers, the tumor(s) are inoperable and/or existing drugs do not result in a substantial improvement in survival. However, bacteriotherapy can improve the patient survival rate without precipitating a decline in the quality of life. Chang et al. administered PA-MSHA, a derivative strain of *Pseudomonas aeruginosa*, alongside chemotherapy to stage III-IV patients with inoperable non-small cell lung cancer (NSCLC). NSCLC makes up the majority of new lung cancer diagnoses and is known for its poor prognosis. The mechanism of action for PA-MSHA is not entirely clear. However, PA-MSHA possesses rich mannose-sensitive type I fimbriae which can activate the Toll-like receptor 4 (TLR4). TLR4 is widely expressed on innate and adaptive immune cells such as DC and B cells. PA-MSHA also possesses many pathogen-specific molecular patterns (PAMPs) that activate more immune cells by stimulating multiple Toll-like receptors. Tumor cells do not retain these PAMPs required for such stimulation; infection by PA-MSHA addresses the 'avoiding immune destruction' hallmark. The administration of PA-MSHA and chemotherapy resulted in a borderline statistically significant improvement versus the sole administration of chemotherapy. The combination therapy and chemotherapy-treated groups had one year of survival rates of 53.55% and 50.15% respectively. There was also no statistically significant difference in the quality of life between the treatment groups. Therefore, bacteriotherapy can make an albeit small but meaningful difference with regards to the time left for patients with poor prognoses (Chang et al., 2015).

Shortcomings of Bacteriotherapy

The multi-faceted therapeutic appeal of bacteriotherapy is evident. Nonetheless, several drawbacks have precluded the widespread use of bacteriotherapy. Despite many of these bacteria being hailed for their targeting efficacy, certain strains can also reside to a small extent in healthy tissue. For example, in Ganai et al., attenuated *S. typhimurium*

(VNP20003) colonized metastatic tissue by 44% but also colonized the normal liver parenchyma by 0.5%. Since *S. typhimurium* is capable of exerting an antitumor effect on both viable and necrotic/hypoxic tissue, it could compromise the health and safety of the patient (Hayashi et al., 2009). However, Ganai et al. did not consider this a significant concern since the chance of sepsis is reduced when attenuated species are used. Nonetheless, the status of the normal liver parenchyma should have been properly assessed. Both Yoon et al. and Shim et al assessed the status of normal tissue in their studies and observed no significant structural abnormalities or damage. Despite how the deficiencies in the bacteria's targeting efficiency can result in normal tissue retaining a small presence of bacteria, studies have deemed it to be of little concern.

Another considerable drawback is that the bacteria's native toxicity can induce complications in patients (Sedighi et al., 2019). For example, when Robert et al. injected *C. novyi-NT* spores into the human patient with metastasized retroperitoneal leiomyosarcoma, the patient developed a high fever and pain in her right shoulder and scapula that was difficult to control. This was ultimately resolved through antibiotics and hydromorphone and the patient subsequently continued to experience an ongoing reduction in tumor enhancement. This outcome demonstrates that potential complications can be closely monitored and addressed (Roberts et al., 2014).

Additionally, in some cases antibacterial immunity has reduced the efficacy of bacteriotherapy. As demonstrated, many bacteria address the 'avoid immune destruction' hallmark because their presence in the environment indirectly or directly stimulates an aggressive host immune response negatively impacting the tumor. As humans, our environment exposes us to a variety of bacteria. Felgner et al. verified that 20% of the population possess an active antibody titer against *Salmonella*. However, in response to this obstacle, Felgner et al. also demonstrated that by genetically engineering an optimized strain, which includes improving their immune-stimulatory character, a highly immunogenic strain can be synthesized to overcome antibacterial immunity (Felgner et al., 2017). Hence the threat of antibacterial immunity can be addressed through engineering immunostimulatory strains.

However, it is critical to also consider the effectiveness of bacteriotherapy in the long term due to antimicrobial resistance. The risk of exposure and infection from multi-drug resistant (MDR) bacteria varies according to the specific bacterial strain and the patient's profile. General factors that put cancer patients at risk are prolonged hospitalization, medical complications (ex. end-stage disease), and prior exposure to broad spectrum antibiotics (Gudiol & Carratalá, 2014). Such risk factors would be exacerbated in cancer patients receiving bacteriotherapy. Consequently, it is essential for physicians to devise rigorous screening guidelines for cancer bacteriotherapy candidates that account for the aforementioned factors. It is also essential to further develop and employ

the existing antimicrobial stewardship strategies during treatment. This includes accounting for dose optimization of treating bacteria and antibiotics (if necessary) and consistently running clinical cultures and employing rapid techniques to understand and preemptively prevent potential resistance patterns (Gudiol & Carratalá, 2014).

Conclusion

Alongside the aforementioned shortcomings, doubt regarding the choice in bacteria and accompanying traditional therapy has precluded the widespread use of bacteriotherapy. This review utilizes the well-known 'hallmarks of cancer' framework to understand which bacterial genera for what form of bacteriotherapy addresses which hallmark(s) of cancer. In doing so, it is demonstrated that bacterial species specialization based on these hallmarks is a promising therapeutic avenue. Likely, this strategy will help the medical community devise more personalized and effective treatment regimens.

Generally, a wide breadth of bacterial genera seem capable of addressing the 'avoiding immune destruction' hallmark. Otherwise, there is variation across–and sometimes within–each bacterial genus. For each *Clostridium-based* treatment examined, a different cancer hallmark is addressed. The administration of recombinant cytotoxin derived from *Clostridium difficile* addresses the 'sustained proliferative signaling' and 'resisting cell death' hallmarks. Alternatively, the *Clostridium difficile* based nanoparticles carrying Doxorubicin targets the 'activating invasion & metastasis' hallmark. The injection of *Clostridium Novyi-NT* spores addresses the 'avoiding immune destruction' hallmark. When used individually, *Bifidobacterium* mainly addresses the 'avoiding immune destruction' hallmark. However, when used as a vector to deliver an antitumor agent, it can address other hallmarks exclusively relevant to that agent as was observed with *BL-Tum* treatment addressing the 'inducing angiogenesis', 'sustained proliferative signaling', and 'resisting cell death' hallmarks. *Salmonella*, specifically *S. typhimurium, is* known for its heightened ability to target metastasis (Weibel et al., 2008). However, it also addresses the 'resisting cell death' hallmark when used individually and the 'avoiding immune destruction' and 'sustaining proliferative signaling' hallmarks when used to carry an antitumor agent or when used with traditional therapy.

Several avenues of exploration exist to further elaborate upon our understanding of bacteriotherapy. One approach which has garnered significant interest in the medical community is a theranostic approach to treatment that focuses on imaging, monitoring, and concomitantly treating tumors (Sedighi et al., 2019). Jiang et al. which used the *E. coli* K-12 strain & radiotherapy also modified their strain to include the luciferase operon as an imaging marker. Theranostic approaches to bacteriotherapy will allow medical professionals to constantly monitor the bacterial agent

enabling treatment precision and increased ability to avoid complications. Another avenue for exploration is to understand the medical properties of different bacterial genera according to the cancer's tissue of origin since it is a major factor that shapes cancer prognosis. Ultimately cancer bacteriotherapy is a promising therapeutic avenue that warrants greater attention by the medical community.

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