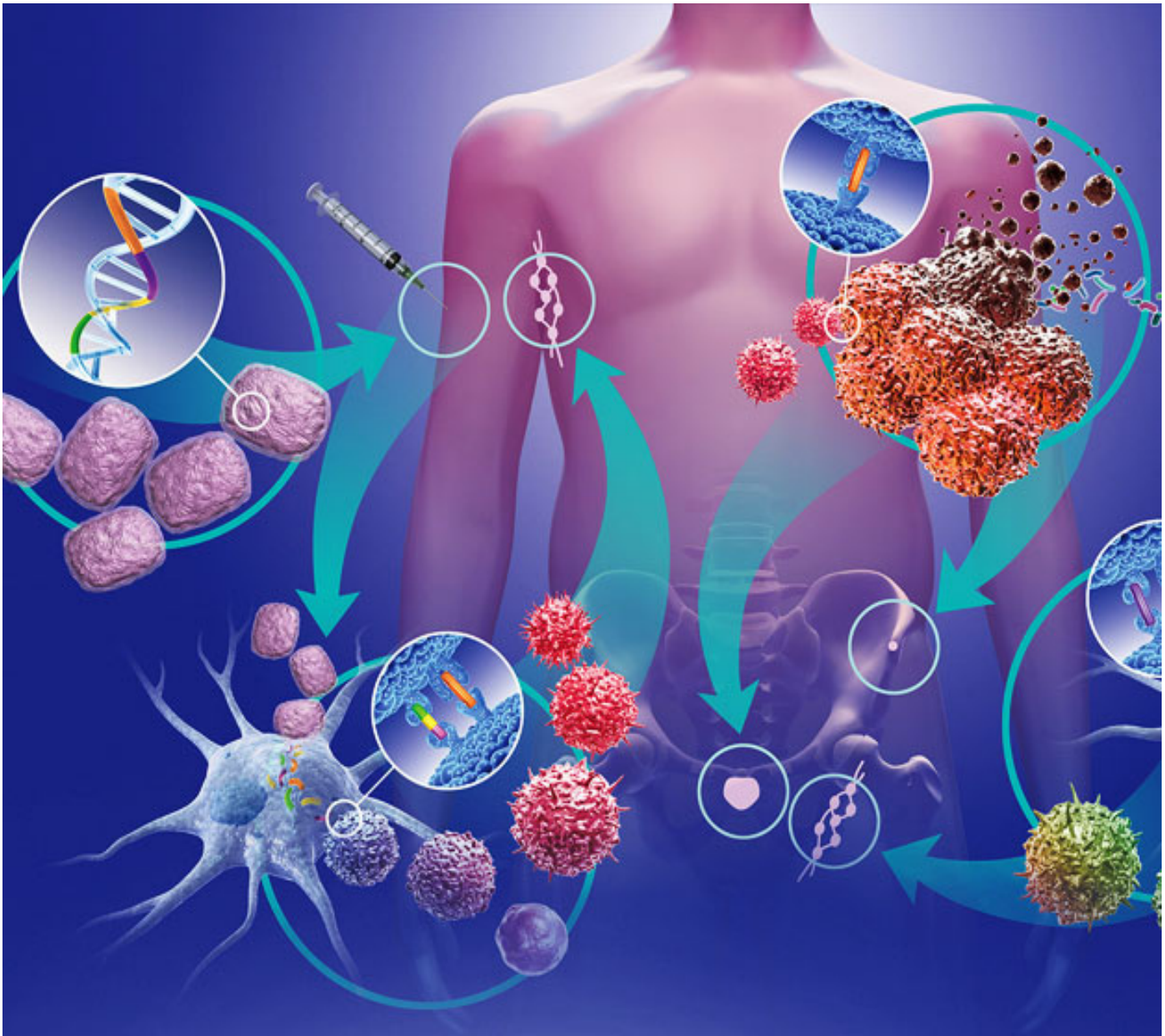

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Microbiological approaches to Treating Cancer

Ender Volkan*; Ovgu Isbilen

Department of Pharmacy, Cyprus International University, Northern Cyprus via Mersin 10 Turkey

Corresponding Author: Ender Volkan,

Faculty of Pharmacy, Biotechnology Research Center, Cyprus International University, Nicosia, Northern Cyprus, 99258, via Mersin 10 Turkey

Email: evolkan@ciu.edu.tr

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Abstract

For over a century, microbes have been utilized in cancer therapy, mainly by means of stimulating the immune system. In recent decades, by the advent of biotechnology and advanced recombinant DNA technology, genetically engineered bacteria and bacterial products have gained popularity as novel approaches for treatment of cancer. These modified microorganisms can directly deliver anti-cancer products or carry out direct homing to tumor sites damaging the cancerous cells. In conjunction with direct tumor damage by microbes, immunotherapy, stimulation of the immune system to attack tumors is also a blooming field along with the emerging data on the significance of microbiome composition on both cancer occurrence and therapy responses. This chapter focuses on a historical perspective and the recent advances on using bacteria against cancer as well as the significance of microbiome on cancer treatment.

Introduction

The interactions of humans with bacterial species have been like a "love/hate relationship" throughout history. Bacteria are often considered as pathogenic enemies of human health however we rely on several bacterial species for homeostasis, immune modulatory mechanisms and the production of foods we consume daily (yogurt, cheese, sauerkraut etc). While bacterial infections are a major threat and have been posing significant danger to human kind throughout history, several "kinder" species of microbes are required to sustain health and prevent dangerous infectious diseases [1]. In fact we carry more bacterial cells in and on our bodies than our own eukaryotic cells: $3.8x10^{13}$ bacterial cells in a 70kg man compared to $3.01x10^{13}$ human cells [2]. These bacteria, which make up our microbiota, are involved in a variety of factors ranging from induction of intestinal vasculature remodeling [3] to prevention of obesity [4], to modulation of autoimmune responses [5-7]. It is clear that this symbiotic relationship is crucial for well-being.

Our body is constantly at an arms race keeping the perfect balance between our immune system, our resident microbiota and prevention of bacterial infections. While we want to avoid bacterial infections, we also want to retain the stability of our microbiota and perhaps, take advantage of easily manipulated bacteria for treatment of other diseases.

A crucial fight that human race as a whole has been facing is the threat of cancer, a modern day nemesis of human health. Cancer, which tends to begin as uncontrolled division of cells, is mediated by complex epigenetic modulation of hundreds of genes. Considering the unique multifaceted nature of cancers and the fact that current treatments tend to inflict serious collateral damage, new therapeutic cancer treatments are under consideration. Some of these promising new cancer treatment methods that benefit from microorganisms include immunotherapy, use of bacterial products and super bugs. This chapter will outline these advents mostly focusing on the strategies of taking advantage of bacteria or bacterial products in cancer treatment and significance of microbiome composition in cancer and its treatment.

First examples of using bacteria in tumor treatment

Bacteria have been considered as therapeutic agents for treatment of tumors for centuries. In early 1700s, Deidier noted that patients with syphilis had lower incidence of malignant tumor development [8]. About 100 years later, in 1813, Vaultier reported tumor regression in his patients who suffered from gas gangrene, which is caused by *Clostridia* species [9,10]. Busch

(1868) and Fehleisen (1882) were the first to treat cancer patients with inoperable tumors using inoculations of *Streptococcus pyogenes*. Similarly, Dr. William Coley in 1891, as a bone sarcoma surgeon at the New York Memorial Hospital observed that sarcoma patients that had *Streptococcus pyogenes* infections at the surgical site after tumor removal had a higher chance of survival. He took advantage of this observation to use bacterial cultures for cancer treatment where he later developed a mixture of bacterial cells and toxins called "Coley's toxins". His mixture included heat killed Gram-positive *Streptococcus* spp. and heat killed Gram-negative bacteria *Serratia marcescens*. He used injections of this mixture to stimulate the immune system of over 1000 sarcoma patients with some success, which represent the first examples of immunotherapy [11]. This method was reportedly used for treatment of sarcomas until 1960s and demonstrates one of the first examples of immunotherapy, where microorganisms and their products were used to stimulate the immune system. Despite some German companies have continued production of Coley's Toxin under the brand name Vaccineurin (Kleef and Hager), it is no longer available as it was not supported by the German Federal Institute of Drugs and Medical Devices. Similarly, Cancer Research UK reports that using Coley's Toxin against cancer may pose significant health risks to patients [12] so is not being recommended as a method of treatment today.

Bacteria in cancer ımmunotherapy

Our immune system is equipped with magnificent arsenal to fight against pathogens like viruses and bacteria as well as abnormal growth of our own cells into tumors. Increasing the efficiency of our armory to act against these threats by stimulating our immune system is intuitive. Interestingly, stimulating the immune system by a pathogen, which incites white blood cells to attack the pathogen may be effective against targeting malignant cells also. As noted earlier, throughout history, instances of cured cancers upon contracting a pathogen have been observed. Similar observations were also made in recent history. In 1999, Bowles and Perkins reported four case studies where regression of malignant brain tumors co-occurred with infection of the surgical tissue with *Enterobacter aerogenes* [13].

Likewise, animal models were used to demonstrate the germination of *Clostridium novyi*-NT spores in hypoxic cancer cells, which ultimately caused destruction of hypoxic regions of cancers. This led to curing of 30% of mice in an immune mediated manner [14]. Glioblastoma, brain tumors notorious for thriving in hypoxic conditions have been targeted by intra-venous *Clostridium novyi*-NT spores in rat models where spore germination was tumor specific and spared normal brain parenchyma and increased rodent survival [15].

Bacille Calmette–Guérin (BCG) vaccine, based on the original development in 1921 as an attenuated strain of *Mycobacterium bovis* for tuberculosis vaccination has been used for superficial bladder cancer treatment since 1976 as a successful immunotherapy agent [16-19]. The cell wall of *M. bovis* is an important immune stimulating component such that it may help in IFNγ secretion and stimulation of Langerhans cells to convert to DCs likely playing a role in treatment of superficial bladder tumors [20,21]. Despite being frequently used as a first line treatment for superficial transitional bladder cell carcinoma, instances of disseminated BCG infection following intravesical instillation has been observed [22].

Other species such as *Pseudomonas aeruginosa* and their adhesive properties have also been effectively utilized in cancer research. A vaccine derived from mannose sensitive hemagglutination pilus strain of *P. aeruginosa* demonstrated immune modulatory effects in gastric cancer-mediated peritoneal dissemination. Via a toll-like receptor4/9-dependent mechanism, the levels of inflammatory cytokines such as IL-12 and other co-stimulatory and antigen-presenting molecules were increased which in turn resulted in reduced peritoneal dissemination in mice treated with the vaccine [23]. Anti-tumor effects of the vaccine obtained from mannose sensitive hemagglutination pilus strain of *P. aeruginosa* were also shown in in vitro settings [23]. *Pseudomonas aeruginosa*-mannose-sensitive hemagglutinin was also demonstrated to have anti-proliferative effects specifically against pancreatic cancer cells without disrupting normal pancreatic duct epithelial cells [24].

Although these observations are mostly due to natural or engineered immune-stimulatory functions of bacteria, potential direct targeting of tumors by bacteria and/or bacterial factors is another possibility. With the advent of bacterial genetics blossoming, it is now possible to use both the immune-stimulatory functions of bacteria as well as taking advantage of their easily manipulated genome to create "super bugs" to our advantage. These bacteria can be engineered to express anti-tumor factors, small RNAs or toxins to directly target cancerous tissues. While engineered or naturallly found bacteria can demonstrate specific actions, directly or indirectly on tumors, it is important to consider the trillions of bacterial cells that inhabit our bodies. Human microbiome and its bacterial composition have been shown to play important role on cancer and effectiveness of cancer therapies.

Role of the resident microbiota in cancer treatment

One approach to limit side effects that may arise from bacterial administration is to use the harmless members of our own microbiota for therapy. While immunotherapy is gaining foothold in the field of cancer treatment, the role host microbiota plays in the treatment of cancer by immunotherapy is becoming more evident. Recent studies suggest that intestinal microbiota may already play a significant role in determining cancer treatment outcome especially that of immunotherapy [25]. For instance the diversity of the gut microbiome has a direct role in determining the efficacy of cancer treatment such that patients with the most diverse microbiome respond best to immunotherapy [26]. Furthermore, emerging research revealed the relationship between antibiotic treatment and immunotherapy. Patients who have received antibiotic treatment before starting immunotherapy did not respond well to therapy that aimed PD-1 (programmed cell death protein 1 ligand 1) blockage [27]. Similarly, the importance of gut microbial composition specifically for advanced melanoma, non-small cell lung carcinoma and renal cell carcinoma immunotherapy was determined when patients who went through antibiotic treatment before or after imnmunotherpy did not respond well to anti-PD-1 treatment. Further investigation revaled that *Akkermansia muciniphila* bacteria was in lower abundance in non-responders such that fecal transplantation of fecal matter from responders to antibiotic treated mice revealed a better prognosis to immunotherapy via T-cell recruitment to the tumor site [27]. Furthermore commensal *Bifidobacterium*, a resident species of the gut microbiome, was demonstrated to play a role in efficiency of immunotherapy by increasing the anti-tumor responses exerted by drugs that target PD-L1 [28]. It is further possible to envision that the side effects associated with mental health deterioration during can-

cer treatment can be addressed by gut microbiome manipulation as evidence points to the mood enhancing properties associated with the gut microbiota, *Lactobacillus helveticus* and *Bifidobacterium longum* [29].Further, gut microbiome also has a mediatory role between tumorigenesis and the initiation of immune responses, which may influence the clinical outcome of colorectal cancer [30]. Previously, Bifidobacterium a commensal, nonpathogenic bacterium was reported to inhibit colon cancer by modulating biomarkers of colon carcinogenesis [31]. The presence of Bifidobacterium gave rise to increased T-cell response at the tumor microenvironment, enhancing host immune response against melanoma, such that the tumor regression was similar to PD-L1-specific antibody therapy where dual therapy of PD-L1 and Bifidobacterium almost eradicated the tumor [28].

In 2010, *Bifidobacterium breve* UCC 2003, a commensal, nonpathogenic bacterium was shown to home to tumors and upregulate interferon-γ production in rodent models [32]. Despite IV administration is a popular method, oral administration of *Bifidobacterium* was successful at translocating from the gastro intestinal tract to the tumor and it did not increase systemic levels of other commensal bacteria. Furthermore, as *Bifidobacterium* was engineered to express the *lux* operon, its tumor targeting abilities was conveniently observed by live whole-body imaging [32]. Similarly, another *Bifidobacterium* species, *Bifidobacterium longum* was shown to localize to and proliferate in solid tumors after systemic application [33,34]. The significance of gut microbiome in cancer treatment became evident in recent studies which revealed that melanoma patients with gut microbial compositon of *Bifidobacterium longum*, *Collinsella aerofaciens*, and *Enterococcus faecium* responded better to anti-PD based immunotherapy methods [35]. Probiotic microbes including *Bifidobacterium* and *Akkermansia* spp. can be a potential safe option to not only target tumors for bacterial gene or antigen delivery but also a potential tumor detection method via ingestion of nonpathogenic bacteria.

Super-bugs

In the past decades the significant knowledge generated in the fields of cancer research and bacterial genetics have posed novel methods of therapy for the tedious disease of cancer by using genetically engineered microorganisms. These bugs can be used as factories to generate cancer specific factors to directly target abnormal tissues and cells. Particularly the attenuated versions of Gram (-) bacteria such as *Escherichia* or *Salmonellae* spp. are great options for cancer therapy due to ease of their genetic manipulation. Similarly oncolytic properties of *Clostridium* spp. and facultative intracellular aspects of *Listeria* spp. are useful tools to achieve efficient delivery of anti-cancer compounds and vaccines. Members of our own microbiota, probiotic bacteria like Bifidobacterium spp. were similarly shown to have a significant impact on tumors. Below, the advances carried out on these microorganisms for cancer treatment are detailed.

***Salmonella* spp. in cancer treatment**

For over 50 years, it has been known that some anaerobic bacteria tend to preferably grow in tumors, suggesting that bacterial delivery of anti-cancer therapeutics by these bacteria is a viable option [36]. Particularly the necrotic and hypoxic conditions that are present within cancerous tissues are suitable conditions for anaerobic bacterial survival. For instance, *Salmonella typhimurium*, a Gram (-) enteric pathogen, was shown to be attracted to tumors via the compounds released by necrotic cells [37]. Furthermore, upon deep penetration into tumorous tissue, the bacterial cells can evade immune reactions such that, if engineered to do so, deliverance of anti-cancer compounds can be effectively carried out by these microogranisms. *Salmonella* species have previously been engineered to increase the efficiency of bacterial targeting strictly to tumors. Specifically, in an attenuated *Salmonella* strain, several metabolic genes have been knocked out which rendered the bacteria auxotrophic for certain nutrients (purines) that are present in high concentrations in tumor sites [38,39]. This allowed targeting of these recombinant microorganisms to cancerous tissues without harming normal tissues [39]. In addition to deletion of metabolic genes, nutrient receptors have been identified on the outer membrane of *Salmonella*, which further allow manipulation of tissue targeting. For instance TAR receptor was shown to detect aspartate, which is secreted by cancerous tissues and TRG receptor helps in migration to necrotic tissues [40]. TRG deficient version of *Salmonella* demonstrated ability to invade quiescent parts of tumor relying solely on its proliferation and penetration capabilities [41]. Deletion or over expression of these genes may aid in further direct targeting of bacterial cells to the tissue of interest.

These methods of engineering *Salmonella* to deliver anti-cancer compounds to the tumor site may be a method to counteract the cancerous tissue from within, similar to a “Trojan horse” mechanism. In another study, *Salmonella typhimurium* was genetically modified to create an attenuated strain such that the strain will encode its infamous Type 3 Secretion System (T3SS) but lacks several of the secreted effectors necessary for its replication within the macrophages and pathogenicity. Hence, this attenuated strain is capable of utilizing its T3SS for delivery of specific antigens (potentially targeting tumors) that can cause T-cell stimulation, while lacking the ability of replicating within the macrophages and causing gut infection [42]. *Salmonella typhimurium* engineered to release angiogenic inhibitors via its T3SS (Endostatin conjugated SopE, an effector protein) was demonstrated to shrink colon tumors in in vivo mouse models [43] making it a hopeful candidate for T3SS mediated delivery system for tumors. T3SS of Salmonella typhimurium has also efficiently been utilized to secrete angiogenic inhibitors inhibiting tumors upon systemic injection in immunocompromised mouse models with colon cancer [43]. This finding is especially intriguing as microbiological therapy of immunocompromised cancer cases is often risky due to the potential of bacterial dissemination.

When talking about presence of potentially pathogenic bacteria in the body, infection or adverse inflammatory reaction is always a concern. Therefore, it is important to employ microorganisms with sensitivity to commercially available antibiotics to keep the bacteria under control. To further address this issue and avoid toxic shock, Lipid A molecule of Gram (-) bacteria like *Salmonella* spp. can be truncated that renders bacteria less immunogenic [38]. In fact, the clinical safety of *Salmonella typhimurium* strain that is unable to produce lipid A, VNP20009, was demonstrated in Phase 1 trials [44]. It is interesting that Lipid A administration into VNP2009 exposed tumors in trans enabled a more robust delivery of anticancer agents [45]. While *Salmonella* spp. can stimulate humoral and cytotoxic responses (which in one hand may be useful for immunotherapy stand point) safety attenuated strains of *Salmonella* spp. are also widely available for use in cancer treatments [46]. A counteracting point of view regarding VNP2009 has emerged in the case of more aggres-

sive types of tumors in immunocompetent cancer models. 4T1 mouse mammary cancer progression was not found to be interfered with by VNP2009 or VPN2009 with restored chemotaxis [47]. Mice exposed to VNP2009 further demonstrated higher rates of morbidity arising from liver disease which indicate the necessity of further comprehensive research on these engineered *Salmonella* species to ensure safety and efficacy on different cancer models [47].

To avoid any attacks by the immune system, tumor cells tend to secrete several immune suppressive cytokines, which prevent proliferation of cytotoxic lymphocytes leading to immune dampening [48]. Reversing this phenomenon with the help of genetically engineered bacteria may be an effective way of directing the immune system towards the tumor, as an "engineered immunotherapy" method. To address this, *Salmonella typhimurium* has been genetically engineered to express immune stimulating molecules like IL-18, CCL21 [49,50]. Studies carried out on murine models using genetically engineered S. typhimurium revealed significant tumor reduction. Similarly, a recombinant strain of *S. typhimurium* expressing a gene encoding LIGHT, a cytokine known to promote tumor rejection, was shown to interfere with primary tumor growth and dissemination of pulmonary metastases, in several mouse tumor models suggesting that immunotherapy carried out using recombinant bacterial techniques is a viable option [51]. The most recent advent on engineered *Salmonella* species utilized an engineered version of *Salmonella typhimurium* that expresses flagellin B protein from *Vibrio vulnificus* such that the engineered bacteria exerted effective antitumor response via immune system activation without significant toxicitiy [52].

Clostridia species in cancer treatment

Historically, *Clostridia* species (Gram-positive anaerobic bacilli) have been among the most widely applied bacterial tumor therapy methods for targeted tumor killing in clinical trial stage [53-55]. Clinical and preclinical studies demonstrated that *Clostridium* spores germinate within tumors causing tumor lysis as they replicate following intravenous delivery [10,56,57]. However, *Clostridium* spores preferably germinate within hypoxic regions of tumors [58]. While this is advantageous, it may potentially allow for eventual regrowth of tumors [54,59]. Also, Clostridium spores seem to require a tumor size of $3cm^3$ to exert their oncolytic effects [53,55]. Therefore, this therapy method is not effective against small metastases but target large tumors. However, in conjunction with other relevant cancer therapies that are already in use, *Clostridium* spores may be evaluated to treat cancer by targeted tumor killing of large tumors.

Listeria in cancer treatment

Listeria monocytogenes is a facultative intracellular pathogen, the causative agent of listeriosis; a form of food poisoning that mainly affects older individuals, pregnant women and newborns [60]. Interestingly, the facultative intracellular aspect of *L. monocytogenes* may be an advantage for cancer therapy as it can potentiate the delivery of cancer antigens directly into the cytosol for quick and efficient presentation by MHC Class I leading to fast T cell responses. This feature of Listeria has been employed such that live attenuated *L. monocytogenes* was engineered to express a variety of tumor antigens like PSA (prostate specific antigen) [61,62] and MAGE (melanoma associated antigen) [63]. Moreover, an attenuated strain of *L. monocytogenes* expressing Human Pappiloma Virus E7 protein was safely used in Phase 1 clinical trials for metastatic cervical cancer [64]. Presentation of these antigens is enhanced by Listeria as it directly delivers them to the host cell cytosol accelerating antigen presentation.

Another attenuated *Listeria monocytogenes* (LM)-based vaccine expressing truncated Listeriolysin O (LLO) has demonstrated the eradication of metastases and the primary tumor in an aggressive mouse breast tumor model [65]. It was also shown that this is due to the combined result of direct killing by Listeria infecting the tumor cells and by cytotoxic T lymphocyte responses against *Listeria* antigens model [65]. A replication deficient *Listeria monocytogenes* with the ability to secrete CD24 (a hepatic cancer cell biomarker associated with apoptosis and metastatis) was intravenously introduced into mice such that upon treatment mice survival and tumor size was significantly reduced. This reduction was revealed to be due to a reduction in regulatory T-cell numbers and an increase in CD8+ T-cell activity at the tumor site [66]. Collectively, these data suggest the ability of engineered *Listeria* to both effectively illicit an immune response and directly interfere with tumors helping clearance of the cancer. *Listeria* is a good example of using bacteria for the dual role of immunotherapy and bacterial delivery of anticancer factors.

Conclusion

Microorganism mediated immunotherapy or different bacterial delivery systems have great potential in adding, improving or revolutionizing the current treatments being used in cancer. However it is clear that further work is needed to both optimize the techniques used and limit the potentially adverse side effects these approaches can elicit. Furthermore, it may be of use to investigate both traditionally used therapies like irradiation or chemotherapy in conjunction with bacterial therapies to investigate any useful, synergistic effects.

Use of recombinant bacteria in humans requires great care in "bio-containtment" approaches to ensure either environmental spread of the used bacteria or lateral gene transfer with environmental bacteria does not occur. Similarly, it is important to restrict usage of bacterial vectors to those sensitive to clinically available antibiotics in order to be able to keep the bacteria under control post-administration. The feature of controlling therapy by simple antibiotic administration is an invaluable aspect of using bacteria for immune-stimulatory or delivery functions.

The potential utilization of bacterial species is not limited for cancer treatment purposes. Treatment of other diseases using microorganisms is also blossoming as *Lactobacillus lactis* is being used by Actogenix (Ghent, Belgium) to deliver anti-inflammatory factors for treatment of oral mucositis that cancer patients tend to encounter due to chemotherapy side effects [67].

Finally in an economical and industrial aspect, usage of bacteria for therapy is both easy and low cost. Most bacteria can easily be manipulated by standard recombinant techniques where the large-scale growth of culturable bacteria is achievable in laboratory settings or biotechnology industries.

Therefore, the major obstacle that needs to be overcome is the avoidance of potential adverse effects and the efficacy of microbiological delivery/ immune-stimulation to eradicate cancerous tissues. Broad range studies are still needed to understand the promising role of microorganisms or bacterial products/ delivery in treatment of cancers and the relationships of their immunostimulatory aspects in cancer to develop novel and safe

treatment options in conjunction with classical therapies. These studies would eventually help improve approaches to manipulate our immune system or manipulate our 'bugs' (microbiota) to efficiently target cancerous tissues or improve therapies.

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