PostDoc Journal
Vol. 4, No. 11, November 2016

Journal of Postdoctoral Research
www.postdocjournal.com

Shaping cancerous landscapes with microbial communities

Aditya Kulkarni, Ph.D.
Department of Biochemistry, University of Texas Southwestern Medical Center,
Dallas TX 75390
Email: aditya.kulkarni@utsouthwestern.edu

Abstract

The dynamic interplay between resident microbiota, host immunity and anti-cancer therapy has generated a captivating enigma underlying the assignment of cause-effect relationships among these factors. The diverse effects of microbes on carcinogenesis, ranging from preventing or promoting cancer to dictating therapeutic outcomes, complicates the understanding of the relationship between the microbiota and the host. Understanding how host-microbe interactions are influenced by genes and environment in carcinogenesis, and applying that knowledge for cancer detection and treatment are gathering prime interest. This review scrutinizes the host-microbe relationship in the context of cancer by discussing the latest findings involving the host-microbe-drug interaction axes.

Keywords: microbiome, anti-cancer therapy, immune system, bacteria mediated tumor therapy

I. Introduction

There are several microscopic communities living on or inside our bodies. Together they orchestrate a carefully balanced symbiotic relationship. The human body provides a place for them to survive and thrive. Microorganisms maintain the body's homeostasis by modulating the epithelial tissue expression of genes involved in nutrient uptake and metabolism, mucosal barrier function, enteric nervous system and motility, hormonal responses, angiogenesis, cytoskeleton and extracellular matrix, signal transduction, and general cellular functions [1]. It is an important relationship, with any disequilibrium of these unique communities being linked to a number of diseases, including cancer.

Investigations in recent years delving into the interplay between the commensal human microbiota, cancer progression and accompanying therapy are leading to the definition of a new terminology called 'oncomicrobiome'. This emerging concept in cancer biology implicates the microbiota as a powerful environmental factor modulating the carcinogenic process. Some of the ways in which the microbial community can enhance or diminish a host's risk of developing cancer and/ or ability to respond to anti-cancer therapy include: 1) altering the balance of host cell proliferation and death through induction of pro- or anti-inflammatory programs, 2) guiding immune system function, 3) influencing metabolism (re-activation or detoxification) of host-produced factors, dietary components and xenobiotics/ pharmaceuticals, 4) probiotic- or antibiotic-driven changes in abundance and/ or localization of specific microbes, 5) inducing genotoxic stress in healthy or tumor cells and 6) loss of symbiosis-permissive mucosal barriers between host and microbe. Carcinogenesis has been hypothesized to be related to microbial dysbiosis under context-specific conditions, and has been relatively well documented in case of colorectal cancer [2]. Loss of recognition of microbial species by host [3], bacteria-induced mutations in host DNA [4], epigenetic alterations in host gene expression driven by bacterial metabolites that act as cofactors, modifiers or allosteric regulators [5], infection-associated chronic inflammation [6, 7], indirect microbial effects on energy uptake and

metabolism [2] are few mechanisms that have been suggested to regulate host cell apoptosis, proliferation and migration directly or via cytokines or hormones. In this review, I specifically evaluate 1) how microbes may contribute to responsiveness to various kinds of anti-cancer therapy, 2) how microbes may be genetically modified to improve anti-cancer therapeutic efficacy and limit toxicity, and 3) the opportunity for microbes to be developed as biomarkers for cancer diagnosis.

II. Contribution of microbes to responsiveness to anti-cancer therapy

Depending upon the type and stage of cancer, various kinds of anti-cancer approaches have clinically been approved as standard of care chemotherapy or are being included as concurrent or combination adjuvant therapy. The impact of the diverse microbiota on response to such treatments is only recently becoming more evident, as discussed further in this section. Reports from diverse model systems, have displayed the direct involvement of resident microbes in mediating an anti-neoplastic response.

(i) Oxaliplatin

The platinum compound oxaliplatin has been used to treat various gastrointestinal (GI) malignancies, particularly advanced colorectal cancer. Oxaliplatin initiates tumor cytotoxicity by forming platinum-DNA adducts and intra-strand cross-links eventually leading to irreparable DNA damage and apoptotic death of the cancer cell [8]. Oxaliplatin chemotherapy has been demonstrated to work in part by boosting inflammation. Oxaliplatin, that was initially not linked to work via activation of the body's immune system, also surprisingly relied on the gut microbiota for successful eradication of tumors in animal studies [9]. Antibiotic-treated and germ-free mice bearing tumors had reduced tumor regression and survival compared with control mice receiving platinum therapy, with the antibiotic-treated mice exhibiting reduced production of ROS (Reactive Oxygen Species) and reduced cytotoxic effects. The production of ROS required for oxaliplatin genotoxicity *in vivo* were shown to be mostly derived from tumor-associated inflammatory cells. Gene-expression analysis showed that induction of pro-inflammatory genes was decreased in the absence of microbiota after oxaliplatin treatment, indicating that inflammation was essential to the anti-tumor effect of the drug [9]. If the microbiome is altered in such a way that inflammation is reduced, these therapeutic agents are less effective. Upon depletion of myeloid cells, the ability of oxaliplatin to induce tumor regression and to increase survival was impaired. This suggests that the reduced effect of oxaliplatin in antibiotic-treated or germ-free mice is partially due to reduced myeloid-cell ROS production. The commensal effect on Oxaliplatin's antitumor cytotoxicity was proposed to be related to microbial product sensing. Besides platinum complexes, drugs such as anthracyclines, alkylating agents, podophyllotoxins, and Camptothecin induce ROS as part of their anticancer activity exhibiting a similar manner of regulation. The gut microbiota can be said to prime myeloid cells for increased Reactive Oxygen Species (ROS) production in the tumor microenvironment. The resultant intratumoral oxidative stress augments the Oxaliplatin-associated DNA damage. The microbiota and in turn the immune system cooperate to potentiate the efficacy of Oxaliplatin.

(ii) Irinotecan

Irinotecan is a semisynthetic analogue of the natural alkaloid Camptothecin. Its main use is in colon cancer, in particular, in combination with other chemotherapy agents. It has also been used against lung and brain tumors as well as refractory forms of leukemia and lymphoma. Irinotecan, a prodrug, is hydrolyzed by carboxylesterases to its active metabolite, SN-38, an inhibitor of Topoisomerase I [10]. The poisoning of the catalytic cycle of Topoisomerase I by SN-38 eventually leads to

inhibition of both DNA replication and transcription, especially in rapidly dividing cells, which would be ideal for targeting cancer cells. In clinical trials, however, it was observed that the dose-limiting side effect of Campthothecin and its derivatives including Irinotecan is severe diarrhea [11]. As part of routine hepatic biotransformation, active SN-38 is inactivated and detoxified to SN-38G by glucuronidation by uridine diphosphate glucuronosyltransferase 1A1 (UGT1A1). SN-38G is excreted via biliary ducts into the GI tract. Once in the intestines, though, SN-38G serves as a substrate for bacterial β-glucuronidase enzymes of the symbiotic commensal microbiota that scavenge for and remove the glucuronide group as a carbon source, producing reactivated SN-38 [12]. This new toxic form of SN-38 destroys rapidly dividing intestinal epithelial cells and causes severe GI distress including diarrhea. High SN-38 levels in the intestinal lumen prevent dose intensification and efficacy achievement in up to 40% of treated patients [12].

Employing antibiotics to reduce GI bacteria levels prior to Irinotecan treatment does not represent a preferred treatment option because the indiscriminate killing of bacteria can be deleterious. Intestinal microbiota plays essential roles in carbohydrate metabolism, vitamin production, and the processing of bile acids, sterols, and xenobiotics [13]. Thus, the removal of GI bacteria is not recommended for patients already challenged by neoplastic growths and chemotherapy. In addition, elimination of symbiotic GI flora increases the chances of infections by pathogenic bacteria including enterohemorrhagic *Escherichia coli* and *Clostridium difficile*. Through structural and chemical biology advances, potent and selective pharmacological inhibitors of β-glucronidases from various bacterial species, but not the mammalian version, are being developed to eliminate the GI toxicity of Irinotecan without killing the bacterial symbiotes required for intestinal health [12]. During the screening of potential β-glucuronidase blockers to be combined with Irinotecan therapy, along with successful reduction in diarrhea, it is important to note that the drugs should not alter the levels of active SN-38 in the bloodstream. This strategy can thereby widen the therapeutic window of Irinotecan. Oral administration of these inhibitors has been effective at alleviating the GI toxicity of Irinotecan in mouse models [12], and this approach may allow the dose or duration of Irinotecan-based chemotherapy to be ramped up in patients. One might expect these inhibitors to be effective in combination with other chemotherapeutics that are glucuronidated in the liver and reactivated by bacterial β-glucuronidases in the gut.

(iii) Cyclophosphamide

Anti-cancer chemotherapeutics often cause mucositis (a debilitating mucosal barrier injury associated with bacterial translocation) and neutropenia (an abnormally low concentration of neutrophils), two complications that require treatment with antibiotics, which in turn can result in dysbiosis. Some anti-neoplastic agents mediate part of their anti-cancer activity by stimulating anti-cancer immune responses. Cyclophosphamide is a prominent DNA alkylating chemotherapy agent used in hematologic malignancies and solid tumors. It has been known to disrupt the intestinal epithelial barrier, affecting mucosal integrity and causing the gut to leak certain bacteria. Cyclophosphamide treatment induces mucosa-associated microbial dysbiosis and provokes selective translocation of distinct Gram-positive bacterial species (*Lactobacillus johnsonii, Lactobacillus murinus and Enterococcus hirae*) in secondary lymphoid organs such as the lymph nodes and the spleen [14]. Bacteria now accumulated in lymphoid tissue outside the gut stimulate the generation of memory T helper 1 (Th1) cells and a specific subset of “pathogenic” T helper 17 (pTh17) effector cells that subsequently migrate to the tumor and kill it. Tumor-bearing mice that were germ-free or that had been treated with antibiotics like

Vancomycin to kill Gram-positive bacteria showed a reduction in pTh17-mediated immune response and their tumors were resistant to Cyclophosphamide. Adoptive transfer of pTh17 cells partially restored the anti-tumor efficacy of Cyclophosphamide [14]. These results suggest that the composition of the gut microbiota helps shape the anticancer immune response.

(iv) Immune checkpoint blockade therapy

Multiple mechanisms underlying inherent prevention of an effective anti-cancer immune response have been described, including signaling via immunosuppressive and anti-inflammatory factors, such as nitric oxide, arginase, Transforming Growth Factor-β (TGF-β) and IL-10 that are produced by both classically and alternatively activated macrophages, other myeloid cell subsets, and regulatory T cells. In addition to malignant tumor cells, stromal cells and hematopoietic cells also express ligands such as the B7 family molecules and PD-L1/2 that trigger the immune checkpoint T cell receptors, CTLA-4 and PD-1 respectively. They cause blunting of T cell-mediated antitumor activity [15]. Thus, inflammation and immunity should be considered inherent characteristics of cancer, and “local chronic inflammation” and “evasion of the immune system” are now included among the hallmarks of cancer.

In the past few years there has been very promising progress in the therapy of melanoma, kidney and lung cancers with respect to boosting the patient's immune response against the tumor using immune checkpoint inhibitors such as antibodies blocking the CTLA-4 or PD-1 receptors or PD-L1 ligand. Many recent studies reporting the role of the commensal microbiota in modulating the response to cancer immunotherapy, immunogenic chemotherapy, and adoptive T cell transfer have raised the possibility that the microbiota may also modulate the clinical efficacy of this new class of anticancer drugs. Two path breaking studies have addressed this question by identifying specific gut-resident bacteria as drivers of checkpoint blockade immunotherapy in preclinical tumor models [16, 17].

Antibodies that inhibit either CTLA-4 or PD-L1 have shown particular promise by triggering a checkpoint blockade that unleashes a robust immune response against cancer cells. Although killer T cell infiltration of solid tumors has been associated with favorable patient outcomes, the mechanisms responsible for variable immune responses between individuals are not completely understood. Interestingly, the efficacy of anti-CTLA4 and anti-PD-L1 treatments was found to be dependent on the composition of the patient gut microbiota and its ability to induce the maturation of dendritic cells. This involves a vigorous mobilization of tumor infiltrating cytotoxic T lymphocytes. Distinct bacterial species were associated with augmented dendritic cell function leading to enhanced and microbe-specific killer T cell priming and accumulation in the tumor microenvironment. Presence of *Bifidobacterium* species promoted anti-PD-L1 efficacy [16] whereas *Bacteroides* promoted anti-CTLA4 efficacy [17]. Fecal microbial transplantation from humans to mice confirmed that treatment of melanoma patients with antibodies against CTLA-4 favored the outgrowth of *Bacteroides fragilis* with anti-cancer properties [16]. Additionally, therapeutic feeding of these particular immunostimulatory bacteria improved the efficacy of immunotherapy in mouse models lacking those bacteria. Dendritic cells from microbe-fed mice in these studies showed elevated expression of genes associated with antitumor immunity and heightened capability for T cell activation [16, 17].

All the pieces of evidence described above demonstrate an unsuspected role for commensal microbiota in regulating various anti-cancer therapeutic strategies either positively through microbial shifts enhancing anti-tumor immunity or negatively through influencing drug metabolism.

III. Manipulating microbes for anti-cancer therapy

Any immunotherapy essentially works by hacking your immune system. The therapeutic moiety teaches the immune system how to recognize and attack the previously hidden cancer cells that it would otherwise ignore. Origins of one of the most promising areas of cancer immunotherapy can be traced back to about a century ago with the rather serendipitous introduction of Coley's toxins: controlled bacteria that might be the most powerful tool yet to turn the immune system into a cancer-fighting machinery [18]. Appreciating the relevance of microbes as key modulators of benefits and adversities associated with anti-cancer therapy, scientists have been attempting to engineer microbes and their derivatives in different ways that can improve the balance between therapy-linked efficacy and toxicity. This is leading to a wave of Bacteria-Mediated Tumor Therapy (BMTT) trials. BMTT differs from conventional immunotherapy in the sense that a bacterial infection itself can exert toxic effects on individual cancer cells, rather than recruiting the immune system. The battle in this approach is to fine-tune the bacterium's ability to reach a solid tumor, thrive in the local environment and execute its engineered function or manifest its virulence in a manner that ultimately kills most of the tumor cells while preventing damage to healthy tissues.

(i) Optimizing microbial strain design

Exploiting the uniqueness of the tumor biology and understanding the appropriate microbial features for optimizing strain design will be critical factors governing the success of the BMTT strategy. *Salmonella, Clostridium* and *Listeria* species have been among the few microorganisms that have exhibited potential in entering, colonizing and destroying cancer cells. They have been modified in various ways to make them more suitable as BMTT agents. The intrinsic anti-tumor response generated by bacteria is most likely connected to their expression of prominent Microbial Associated Molecular Patterns (MAMPs) such as Lipopolysaccharides (LPS, the immunogenic element in bacterial cell membranes) and flagella (regardless of their functionality in motility or chemotaxis) [19]. Interference with these virulence factors or restricting the *in vivo* survival of bacteria by metabolic auxotrophies can serve as amenable strategies for modification. However, such modifications can easily lead to over-attenuation of strains that might increase their safety but at the same time compromise their ability to mount an adequate anti-tumor response.

One of the frequently distinguishing characteristics of solid tumors is the formation of a necrotic, hypoxic zone with low partial pressure of oxygen in the interior of the tumor. Obligate anaerobic bacteria like *Clostridia* were preferentially chosen for anti-cancer therapy since their spores germinate only in the absence of oxygen. Although germination of *Clostridia* was confined to hypoxic regions, the related toxicity led to high mortality rates using this type of bacteria. To increase the safety of *Clostridia*, virulence factors like the lethal α-toxin were deleted from potential therapeutic strains. Besides experimental studies in mice, *Clostridium novyi-NT* (non-toxic) has already been tested in preclinical and clinical trials using dogs as well as human patients [20]. A human patient with advanced leiomyosarcoma was chosen for treatment with an intratumoral injection of *C. novyi-NT* spores. This treatment resulted in regression of the tumor within and surrounding the bone [20]. *C. novyi-NT* was thereafter suggested to precisely eradicate neoplastic tissues, warranting further clinical trials of this agent in selected patients. *C. novyi-NT* is unique because it thrives in a low-oxygen environment where it begins to divide and grow, and in the process kills cancer cells. The bacteria then stop growing at the tumor boundary, where there is more oxygen, preventing them from intruding any further into healthy cells. Furthermore, orthotopic

glioblastomas were successfully targeted with *C. novyi* spores upon intravenous infection in a rat model, in turn indicating that the spores are able to pass the blood brain barrier under certain conditions [21]. The mechanism of the antitumor effect by *Clostridia* is poorly understood. Although these bacteria are able to successfully target neoplastic tissue without seriously harming the host, their application is limited to large solid tumors with hypoxic centers.

To overcome the limitation of confinement to hypoxic regions and to address the problem that tumors grow out from viable oxygenated tissue, facultative anaerobic bacteria like *Salmonella typhimurium* became the focus of initial BMTT experiments. However, as *Salmonella* can grow under aerobic conditions, they are not restricted to merely colonizing tumors but are also able to disseminate to healthy organs like spleen and liver. Therefore, to ensure safe application, *Salmonella* needs to be adapted. The prominent *Salmonella* strains VNP20009 (*in vitro*) and A1-R (*in vitro* and *in vivo*) were created by passaging bacteria from tumor to tumor either in cell culture or in mice, so as to develop a tumor-adapted phenotype concomitantly exhibiting high tumor specificity [22]. Auxotrophy for purines or Arginine and Leucine, respectively, rendered these *Salmonella* variants metabolically deficient and highly tumor-specific [23]. However, the isolation of spontaneously appearing mutant bacterial clones through selective pressure represents a challenge to appropriately tailor bacterial strains. Due to the uncertainty associated with such a non-specific method of attenuation, targeted gene editing would be a better choice for customizing bacteria for anti-cancer therapy.

The immune recognition of *Salmonella* and induction of an immune response are factors that directly correlate with the presence of various MAMPs. To survive in a hostile environment *Salmonella* may either modify the structure of LPS or downregulate the expression of flagella. To counteract such mechanisms, a promising recombinant strategy would be to reinstate the immunogenicity of *Salmonella* via modification of immunogenic targets or MAMPs. For example, a hexa-acylated Lipid A structure was shown to be highly efficient at immune stimulation, whereas tetra-acylated Lipid A acted antagonistically [24]. In addition, it was shown that *Salmonella* variants bearing both flagella proteins FliC and FljB trigger an increased host immune response upon oral administration [25]. These examples demonstrate that the immunogenicity of attenuated bacteria can be enhanced when the MAMPs are modified in such a way that host pattern recognition receptors are more efficiently stimulated.

Modifying the expression or activity of certain MAMPs could have pleiotropic effects, some detrimental, that may affect the gene regulatory circuits of bacteria in a more general way. Therefore, a wild-type like phenotype of bacteria that is only conditionally modified may be the next step in strain design. Currently, two concepts are being evaluated using this rationale, namely, delayed attenuation and delayed lysis. Genetically modified mutants generated in these two approaches exhibit a wild-type like phenotype upon *in vivo* administration whereas manifestation of the intended ultimate phenotype usually driven by loss of an inducer kicks in later. For instance, auxotrophic or attenuated bacteria may maintain viability through gene complementation by expressing a gene product under an inducible promoter like P_{BAD} or P_{tet} in the presence of arabinose or anhydrotetracycline, respectively [26]. Such bacteria can be stably induced and complemented in culture. *In vivo*, the inducers are diluted out and are no longer available. As a consequence, the bacteria will lose their wild-type phenotype and become attenuated after a few rounds of replication. This delayed attenuation system was recently deployed for

Salmonella to modify the LPS structure under the control of P_{BAD}. The effect was evaluated in a murine tumor model [26]. Compared to the bacteria harboring a complete gene deletion and affecting LPS expression immediately upon inoculation into the mice, the initial wild-type like phenotype of the delayed attenuation strain induced a stronger immune response that significantly enhanced its anti-tumor activity [26]. None of the mice succumbed to the infection and the health status of the mice was only transiently affected after bacterial administration.

Similarly, in a delayed lysis *Salmonella* system, cell wall synthesis is abrogated in the absence of arabinose *in vivo* [27]. The bacteria are thus not able to establish a systemic infection. However, the sudden microbial death *in vivo* might cause complications like septic shock in the host due to release of large amounts of bacterial endotoxins. Nevertheless, the system was successfully tested to vaccinate mice against influenza viruses, by a targeted release of intracellular virus-specific antigens by the bacteria [28]. This system may show comparable utility in a cancer model and should thus be explored. These studies demonstrate that modern strategies are more widely effective when both attenuation and optimization are accommodated in the same therapeutic strain.

(ii) Microbes as delivery vehicles for therapeutic molecules

Bacteria could further be exploited as opportunistic infectious agents designed to shuttle therapeutic agents directly into cancerous tissue. This should maximize their intended effect while reducing systemic side effects. Various preclinical trials have shown the ability of different bacterial strains to migrate to tumor sites, locally produce therapeutic agents, and mediate highly effective and specific therapeutic responses [29, 30]. Exploiting bacteria as live vector systems could represent the next generation of strain design. However, this promising idea is beset with its own set of challenges. At least two components including (i) a tumor-specific microbe-based platform and (ii) a cytotoxic compound or payload that can be synthesized and actively secreted or delivered by the microbes, are required.

In this context, a few concepts are currently under investigation. The first one employs prodrug converting enzymes produced by bacteria. This strategy relies on enzymes that are capable of converting a systemically administered inactive prodrug into an active cytotoxic drug. As the enzyme would be present primarily in vicinity of the bacteria and facilitate local conversion at only this site, this method provides good tumor specificity. The therapeutic benefit of enzymes like cytosine deaminase and nitroreductase expressed by either *Clostridia* or *Listeria* has been tested [31, 32]. However, while they showed promising activity *in vitro*, no significant improvement of therapeutic effects was observed *in vivo*. This could most likely be attributed to low enzyme expression levels, low enzyme secretion efficiency or low prodrug conversion inside the cancerous environment and needs to be optimized for further attempts. However, attenuated *Salmonella* strains as carriers of enzymes like cytosine deaminase [33] (which was effective in treating subcutaneously implanted colon tumors in mice by converting 5-Fluorocytosine to 5-Fluorouracil), thymidine kinase [34] (which caused dose-dependent suppression of tumor growth and prolonged survival in melanoma-bearing mice, in addition to that seen with the bacteria alone, by phosphorylation of Ganciclovir to its active form) or carboxypeptidase G2 [35] (which showed tumor growth reduction in cases of mouse melanoma, and human breast and colon carcinomas by converting a range of mustard prodrugs to active DNA cross-linking agents) have been successfully used in preclinical and clinical setups. Nevertheless, the patient cohort needs to be enlarged to significantly validate any promising results.

The second approach concerns production and subsequent secretion of therapeutically active compounds by the bacteria themselves during tumor colonization. Therapeutic molecules include bacterial toxins like α-Hemolysin or Azurin [36, 37], recombinant effector proteins such as TNF-α and IL-2 [38], or small hairpin RNAs [39] (shRNAs) targeting, for instance, STAT3 (in case of Hepatocellular Carcinoma) [40] or IDO (in case of melanoma) [41]. The transport of these molecules across bacterial membranes to the extracellular environment around or within the tumor is critical. The basic idea is to fuse therapeutic agents to signaling molecules that determine release via a particular bacterial secretory pathway, ensuring continuous secretion. However, the fusion to a signaling peptide could be a limiting factor in this strategy. The agent may lose its activity due to, for example, conformational alteration or non-native refolding of the fusion complex upon secretion. However, proof of principle has been successfully demonstrated by Singer et al., where recombinant neuroactive peptides were delivered via the flagellar type 3 secretion system (fT3SS) [42]. Furthermore, the efficacy of fT3SS for delivery was assessed in a cancer vaccine, where the codon-optimized human tumor-associated antigen Survivin, an oncoprotein overexpressed in most human cancers, was genetically fused to the *Salmonella* secreted effector protein SseJ, and delivered in the cytosol of antigen-presenting cells. As a result, complete tumor regression in lymphoma-bearing mice was observed [43]. Thus, delivery via the fT3SS of *Salmonella* may represent a promising foundation for active delivery. Complex constructs with multiple domains for direct or indirect recognition of, binding to and killing of cancer cells could in principle be engineered in various microbial strains.

In another path breaking effort, scientists have created a nanoporous biosilica created from the diatom microalga *Thalassiosira pseudonana* [44]. This diatom was engineered in a two-step process: (i) genetic alteration to display GB1, an immunoglobulin G (IgG)-binding domain of protein G on the biosilica surface, enabling attachment of the tumor cell-targeting antibody specific for the p75 neurotrophin receptor (p75NTR) which specifically and readily attached to neuroblastoma cells but not to fibroblasts, and (ii) incorporation of the chemotherapeutic agent Camptothecin or SN-38 into the silica-binding carriers using an established method to encapsulate hydrophobic drug molecules into cationic micelles and liposomes, in turn minimizing the off-target toxicity. The algae-based drug-delivery vehicle is biodegradable and completely harmless to healthy cells. Such chemotherapy delivered by microalgae led to appreciable tumor regression in the mouse neuroblastoma model employed in this study. This system served as a good foundation for subsequent studies demonstrating how engineered microbial sources may be used as versatile vehicles for the targeted delivery of anticancer drugs to tumor sites. For instance, in a recent report by Felfoul et al. [45] immunodeficient mice were injected with *Magnetococcus marinus,* the microorganism employed to transport nanoliposomes encapsulating SN-38 into tumor hypoxic regions, coupled with navigation via an external energy source. The authors suggested that harnessing swarms of bacteria exhibiting magneto-aerotactic migration behavior and very low immunogenicity can dramatically improve the therapeutic index of drug-loaded nanocarriers, while reducing systemic toxicity and ensuring safety.

For the most part, BMTT has incorporated laboratory-made strains, and while results in murine models have been impressive, outcomes in patients have been inconsistent, with the inherent pathogenicity and immunogenicity of the bacteria employed outweighing therapeutic responses in patients [13]. Still, the development of microbial vectors as delivery vehicles for therapeutic agents is an

exciting area of research that is gaining acceptance by clinicians and regulatory authorities for its potential to deliver positive clinical outcomes.

Use of Clostridial species for targeted tumor killing and attenuated *Salmonella* or *Listeria* vectors for oral vaccination or tumor gene delivery, represent the most widely applied bacterial vectors at the clinical trial level [46, 47, 48, 49, 50]. In a clinical trial performed on metastatic pancreatic ductal adenocarcinoma (PDA) patients, administration of CRS-207, a live-attenuated *Listeria monocytogenes* expressing the cancer antigen mesothelin, along with low dose Cyclophosphamide and GVAX (another vaccine evaluated in PDA) significantly extended overall survival with minimal toxicity [51].

Similarly, a number of live attenuated strains of Listeria have been developed expressing a broad range of tumor antigens, such as Her-2/neu [52, 53] (an oncoprotein associated with a wide variety of cancers), Melanoma Associated Antigen (MAGE) [54] and prostate specific antigen (PSA) [55, 56], and HPV16 E7 [57]. The cytoplasmic location of L. monocytogenes is crucial as this potentiates entry of the antigen into the Class I MHC antigen-processing pathway leading to priming of specific CD8+ T-cell responses. Intravenously administered attenuated L. monocytogenes expressing HPV16 E7 was recently used in phase I clinical trial on patients with metastatic cervical cancer [57]. Apart from some flu-like symptoms and fever-related hypertension in some patients, the vector was well tolerated. In addition, 30% tumor reduction was noted with an increase in overall survival, indicating the safety and efficacy of listerial vectors in patients and paving the way for clinical development of this vector strategy.

Persistent infection after chemotherapy, genetic instability of engineered strains and determining the correct combination therapies are additional challenges that remain to be addressed before optimized bacteria can be implemented in the clinic for anti-cancer therapy.

Immunosuppression in cancer patients can occur via neutropenia, disruption in the barriers to infection and shifts in the microbial flora, caused by the malignancy itself, treatment procedures (surgery, radiation, chemotherapy) or reduced utilization of nutrition. These constitute some of the risk factors generally making a cancer patient more prone to primary, secondary or nosocomial infections. Due to low white blood cell count, patients may not have the usual signs and symptoms when developing an infection such as redness, swelling, pus formation, cough, nasal drainage etc. Making the distinction between patients at low and high risk depending upon their bone marrow function is critical in determining clinical success. Applying BMTT in such situations raises even higher safety concerns regarding the pathogenicity and immunogenicity of microbial species supposed to mediate therapy, as they are known to cause life-threatening infections in clinical practice. Through BMTT related bacterial translocation, endogenous microorganisms can move into the bloodstream, resulting in bacteremia [58]. Although the mortality attributed to such infections has decreased over the years, due to the development of beta-lactam antibiotics and fluoroquinolones, the types of infections have changed as new resistant and opportunistic microorganisms emerge [58]. Efforts to refine the process have involved prophylactic antibiotic regimens [59], developing more specific antibiotics targeting the desired microbial class [59], and genetic engineering of strains to enable encoding of additional heterologous genes [60, 61, 62] Both *Clostridium* and *Salmonella* have been shown to be non-pathogenic in multiple animal species [63, 64] and in human trials [50, 65, 66], but any retained virulence could be problematic for immunocompromised late-stage cancer patients.

Genetic instability of attenuated strains is a potential problem because mutations could create ineffective (loss of function) or harmful (gain of function) phenotypes, leading to failure of therapy and/ or exaggerated infection. The rate of mutation will need to be estimated for each strain to be able to specify the maximum permissible time limit that bacterial colonies could remain in tumors before being eliminated using specific antibiotics. Genetic stability could be enhanced by creating clean deletions in virulence factors, identifying and modifying multiple virulence factors to reduce the probability of reversion, or by incorporating engineered genes on the bacterial chromosome thereby limiting homologous recombination and horizontal gene transfer [67].

The ultimate BMTT can be thought to consist of a collection of strains designed for specialized purposes rather than a single perfect strain. Successful treatment could utilize these strains cooperatively and in combination with chemotherapy by means of a detectable facultative anaerobe for diagnosis; an engineered immunogenic stain to sensitize the immune system; an obligate anaerobe to treat inoperable primary tumors; and a motile strain controllably producing a cytotoxic agent to treat diffuse tumors and metastatic disease [67]. The genetic flexibility of bacterial strains can be exploited to tune them for individualized therapy, targeting to multiple tumor sites and precise control of cytotoxicity. Once perfected, anti-cancer bacteria are expected to be an essential clinical tool, able to perform functions unachievable by other therapies, such as detect, prevent, and treat tumors and metastases.

IV. Microbes as biomarkers for cancer diagnosis

Rapid advances in the engineering of genetic circuitry in living cells has positioned synthetic biology as a remarkable tool to address numerous biomedical problems, including disease diagnosis. One challenge in exploiting synthetic biology for translational applications is to engineer microbes that are well tolerated by patients and seamlessly integrate with existing clinical methods. The host strain *Escherichia coli* Nissle 1917 (EcN) has an established safety record in clinical trials for oral delivery to GI disorders and has therefore been used to develop an orally administered diagnostic agent. This agent functions non-invasively and indicates the presence of primary or metastatic liver cancer by producing visibly detectable signals in urine. Motivated by the need for an accessible, highly sensitive and specific tool for detection of micrometastases that are beyond the reach of existing diagnostic tools, Danino et al. [68] engineered EcN, with a series of expression cassettes. The corresponding diagnostic platform called PROP-Z (programmable probiotics with lacZ) is made up of probiotic EcN bacteria transformed with a dual-stabilized, high-expression lacZ vector as well as a genomically integrated luxCDABE cassette that allows for luminescent visualization without providing exogenous luciferin. Upon oral delivery, these probiotics rapidly (within 24 hours) translocate across the GI tract and selectively expand within tumor cells present in the liver. The natural reticuloendothelial filtration of gut-derived venous outflow to the liver maximizes liver exposure of gut bacteria, and thus, orally delivered microbes selectively colonize liver tumors. EcN robustly colonized tumor tissue in rodent models of liver metastasis after oral delivery but did not colonize healthy organs or fibrotic liver tissue. No deleterious health effects were observed on the mice for more than 12 months after oral delivery [68]. PROP-Z expresses high levels of the enzyme β-galactosidase which can cleave systematically injected, cleavable substrates. Cleavage products of the substrates filter through the renal system to generate a high-contrast urine signal for detection. Probiotics can thus be programmed to safely and selectively deliver synthetic gene circuits to diseased tissue microenvironments *in vivo*.

Liver cancers frequently metastasize to the colon, lungs, ovaries or pancreas before they are detected making timely detection of liver metastasis a pressing clinical need. Liver cancer is a difficult malignancy to detect with conventional imaging because of poor tumor-to-organ contrast. The PROP-Z technology may be useful for detection of primary hepatocellular carcinoma in patients with known risk factors for malignant transformation (for example, obesity, chronic viral hepatitis infections and prior treatment for primary liver, colorectal, breast or pancreatic cancers). The PROP-Z platform architecture is highly modular and could be repurposed for various applications. As genetic signatures of certain cancers become more consolidated, bacteria could, for instance, be programmed to recognize those signatures, aiding in providing early stage diagnoses, monitoring a patient's response to treatment, and delivering appropriate treatment. Tumors in other organs that are exposed to high bacterial concentrations from the GI tract, such as colorectal cancers, may also be amenable to detection with this system. To be able to treat tumors outside the gut or liver with this strategy, a higher dose, direct injection into the tumor, or alternative homing strategies can be applied. One advantage in using bacterial diagnostics is their susceptibility to antibiotics, which can be administered to eliminate the agent.

Moving forward, there are many issues that must be addressed while considering the clinical translation of the PROP-Z or similar platforms. For example, the selective trafficking of oral PROP across the gut wall and colonizing liver lesions relative to the pre-existing gut microbiome must be investigated in humans because of the many species-specific differences between rodents and humans. Special attention must also be paid to the fate of PROP in patients who are already undergoing therapy that may have immunomodulatory effects (e.g. radiation, cytotoxic chemotherapy, and immunotherapy). Another potential concern is interference of PROP or the resultant inflammatory response with radiographic imaging or positron emission tomography surveillance studies. Lastly, any approach using engineered bacterial species in patients will require regulatory approval before becoming a clinical reality. In this regard the regulatory landscape that is being established for fecal microbial transplantation will be beneficial.

Similarly, Zackular et al. [69] have characterized the gut microbiome in patients from three clinical groups representing the stages of colorectal cancer progression: health, adenoma and carcinoma. Analysis of the stool-derived gut microbiome in terms of sequence comparisons of the 16S rRNA coding gene from each sample revealed both enrichment and depletion of several bacterial populations associated with adenomas and carcinomas. Combined with known clinical risk factors of colorectal cancer (such as BMI, age, race), data from the gut microbiome significantly improved the ability to differentiate between healthy, adenoma, and carcinoma clinical groups relative to risk factors alone [69]. This demonstrates the feasibility of using the composition of the gut microbiome to detect the presence of precancerous and cancerous lesions. These results warrant further studies with diverse populations and linkage to other stool markers, dietary data, and personal health information.

V. Conclusion

The unexpected influence of commensal intestinal bacteria on the outcome of cancer treatment [70, 71, 72] and the function of anti-cancer immunity poses new questions from a preclinical and clinical standpoint in the oncology field. Delineating the complex roles of microbiota, not only from the gut but also the skin and oral cavity, in response to chemotherapy in a range of model systems and undertaking epidemiologic studies with microbiome analysis in patients with and at risk of cancer will be critical for establishing the

microbiota as potent combination therapy agents that enhance efficacy and/ or diminish toxicity of existing approved anti-cancer treatments.

According to the various reports discussed in this review, the commensal microbiota is seen to differentially affect the type of inflammatory tone required for response to different therapeutic protocols. This unveils new risks associated with antibiotic medication during cancer treatments as well as the opportunities to improve cancer treatment by manipulating the human gut microbiota. Further investigations are needed to determine whether a potential molecular mimicry between distinct microbes and tumor neoantigens could account for the toxicity and/or efficacy of immune checkpoint blockers, currently in the vanguard of anticancer therapy. Efforts to profile the gut microbiome of patients undergoing checkpoint blockade could yield both strategies to maximize the clinical benefit of cancer immunotherapy and biomarkers for predicting therapeutic response.

It is debatable whether specific alterations in the gut microbiota are instrumental or detrimental to the efficacy of anticancer chemotherapy. On one hand, local or systemic bacterial infections can complicate cancer therapy through reducing anti-tumor efficacy or increasing off-target toxicity. On the contrary, for drugs whose cytotoxicity is controlled by bacteria, deliberate modification of the bacterial content of cancer patients or introduction of microbes as delivery vehicles of drug cargo can serve to improve their therapeutic index. It is tempting to speculate that the clinical profile of at least some chemotherapeutics can be improved by combinatorial interventions involving one or more antibiotics, probiotics, prebiotics or postbiotics.

Joining a list of recent next-generation cancer diagnostic applications (including genetic, epigenetic and proteomic analyses, circulating tumor cell assays, biomarker profiling and monitoring), programmable probiotics can aid in early identification of micrometastatic disease that may result in improved patient outcomes. With a growing population of patients at risk of developing cancer, a highly sensitive, specific, nonsurgical, nonradioactive method for repeated monitoring such as the PROP-Z may be clinically highly adopted. Probiotics may be further engineered to allow a) urinalysis by low-cost paper tests, b) addition of newer substrates for biochemical colorimetric or imaging-based diagnosis, and c) integration with other biomarkers for cancer.

With promising associations, but not necessarily confirmed causative links, emerging between the microbiome and evolution of cancer driven by both genes and environment, it can be contemplated that the screening, treatment and surveillance of cancer patients will one day incorporate microbiome sequencing in addition to sequencing one's genome, making personalized medicine even more advanced. Manipulating the composition of the gut microbiota as a methodology to optimize responses to therapy in the clinic is a relatively new concept, and additional studies are required to understand the clinical value of such an approach. In this context, the broad spectrum of most conventional antibiotics and the intersubject heterogeneity of the gut microbiota constitute major obstacles. Highly specific antimicrobials such as bacteriocins [73] that may also serve as anticancer agents, along with the development of new technologies allowing rapid characterization of the gut microbiota on a personalized basis may, in part, circumvent these issues. Carefully tailored modulation of the human microbiota may, therefore, constitute a viable strategy for improving the clinical efficacy of anti-cancer chemo-, radio- and immunotherapy.

References

[1] Leser, Thomas D., and Lars Mølbak. "Better Living through Microbial Action: The Benefits of the Mammalian Gastrointestinal Microbiota on the Host." *Environmental Microbiology* 11, no. 9 (2009):2194-206. https://dx.doi.org/10.1111/j.1462-2920.2009.01941.x PMid:19737302

[2] Sobhani, I., A. Amiot, Y. Le Baleur, M. Levy, M.-L. Auriault, J. T. Van Nhieu, and J. C. Delchier. "Microbial Dysbiosis and Colon Carcinogenesis: Could Colon Cancer Be Considered a Bacteria-related Disease?" *Therapeutic Advances in Gastroenterology* 6, no. 3 (2013): 215-29. https://dx.doi.org/10.1177/1756283X12473674 PMid:23634186 PMCid:PMC3625019

[3] Arthur, J. C., E. Perez-Chanona, M. Muhlbauer, S. Tomkovich, J. M. Uronis, T.-J. Fan, B. J. Campbell, T. Abujamel, B. Dogan, A. B. Rogers, J. M. Rhodes, A. Stintzi, K. W. Simpson, J. J. Hansen, T. O. Keku, A. A. Fodor, and C. Jobin. "Intestinal Inflammation Targets Cancer-Inducing Activity of the Microbiota." *Science* 338, no. 6103 (2012): 120-23. https://dx.doi.org/10.1126/science.1224820 PMid:22903521 PMCid:PMC3645302

[4] Cuevas-Ramos, G., C. R. Petit, I. Marcq, M. Boury, E. Oswald, and J.-P. Nougayrede. "Escherichia Coli Induces DNA Damage in Vivo and Triggers Genomic Instability in Mammalian Cells." *Proceedings of the National Academy of Sciences* 107, no. 25 (2010): 11537-1542. https://dx.doi.org/10.1073/pnas.1001261107 PMid:20534522 PMCid:PMC2895108

[5] Paul, Bidisha, Stephen Barnes, Wendy Demark-Wahnefried, Casey Morrow, Carolina Salvador, Christine Skibola, and Trygve O. Tollefsbol. "Influences of Diet and the Gut Microbiome on Epigenetic Modulation in Cancer and Other Diseases." *Clinical Epigenetics Clin Epigenet* 7, no. 1 (2015). https://dx.doi.org/10.1186/s13148-015-0144-7 PMid:26478753 PMCid:PMC4609101

[6] Balkwill, F., and A. Mantovani. "Cancer and Inflammation: Implications for Pharmacology and Therapeutics." *Clin Pharmacol Ther Clinical Pharmacology & Therapeutics* 87, no. 4 (2010): 401-06. https://dx.doi.org/10.1038/clpt.2009.312 PMid:20200512

[7] Belkaid, Yasmine, and Timothy W. Hand. "Role of the Microbiota in Immunity and Inflammation." *Cell* 157, no. 1 (2014): 121-41. https://dx.doi.org/10.1016/j.cell.2014.03.011 PMid:24679531 PMCid:PMC4056765

[8] Raymond E, S. Faivre, JM Woynarowski, and SG Chaney. "Oxaliplatin: mechanism of action and antineoplastic activity." Seminal Oncology 25.2 (1998):4-12 PMid:9609103

[9] Iida, N., A. Dzutsev, C. A. Stewart, L. Smith, N. Bouladoux, R. A. Weingarten, D. A. Molina, R. Salcedo, T. Back, S. Cramer, R.-M. Dai, H. Kiu, M. Cardone, S. Naik, A. K. Patri, E. Wang, F. M. Marincola, K. M. Frank, Y. Belkaid, G. Trinchieri, and R. S. Goldszmid. "Commensal Bacteria Control Cancer Response to Therapy by Modulating the Tumor Microenvironment." Science 342.6161 (2013): 967-70. http://dx.doi.org/10.1126/science.1240527 PMid:24264989

[10] Pommier, Yves, Elisabetta Leo, Hongliang Zhang, and Christophe Marchand. "DNA Topoisomerases and Their Poisoning by Anticancer and Antibacterial Drugs." Chemistry & Biology 17.5 (2010):421-33 http://dx.doi.org/10.1016/j.chembiol.2010.04.012 PMid:20534341

[11] Abigerges D, GG Chabot, JP Armand, P Hérait, A Gouyette, and D Gandia. "Phase I and pharmacologic studies of the camptothecin

analog irinotecan administered every 3 weeks in cancer patients." Journal of Clinical Oncology 13.1 (1995):210-21. http://dx.doi.org/10.1200/jco.1995.13.1.210 PMid:7799022

[12] Wallace, B. D., H. Wang, K. T. Lane, J. E. Scott, J. Orans, J. S. Koo, M. Venkatesh, C. Jobin, L.-A. Yeh, S. Mani, and M. R. Redinbo. "Alleviating Cancer Drug Toxicity by Inhibiting a Bacterial Enzyme." Science 330.6005 (2010): 831-35. http://dx.doi.org/10.1126/science.1191175 PMid:21051639 PMCid:PMC3110694

[13] Nicholson, J. K., E. Holmes, J. Kinross, R. Burcelin, G. Gibson, W. Jia, and S. Pettersson. "Host-Gut Microbiota Metabolic Interactions." Science 336.6086 (2012): 1262-267. http://dx.doi.org/10.1126/science.1223813 PMid:22674330

[14] Viaud, S., F. Saccheri, G. Mignot, T. Yamazaki, R. Daillere, D. Hannani, D. P. Enot, C. Pfirschke, C. Engblom, M. J. Pittet, A. Schlitzer, F. Ginhoux, L. Apetoh, E. Chachaty, P.-L. Woerther, G. Eberl, M. Berard, C. Ecobichon, D. Clermont, C. Bizet, V. Gaboriau-Routhiau, N. Cerf-Bensussan, P. Opolon, N. Yessaad, E. Vivier, B. Ryffel, C. O. Elson, J. Dore, G. Kroemer, P. Lepage, I. G. Boneca, F. Ghiringhelli, and L. Zitvogel. "The Intestinal Microbiota Modulates the Anticancer Immune Effects of Cyclophosphamide." Science 342.6161 (2013): 971-76. http://dx.doi.org/10.1126/science.1240537 PMid:24264990 PMCid:PMC4048947

[15] Dzutsev, Amiran, Romina S. Goldszmid, Sophie Viaud, Laurence Zitvogel, and Giorgio Trinchieri. "The Role of the Microbiota in Inflammation, Carcinogenesis, and Cancer Therapy." European Journal of Immunology 45.1 (2014): 17-31. http://dx.doi.org/10.1002/eji.201444972 PMid:25328099

[16] Sivan, A., L. Corrales, N. Hubert, J. B. Williams, K. Aquino-Michaels, Z. M. Earley, F. W. Benyamin, Y. Man Lei, B. Jabri, M.-L. Alegre, E. B. Chang, and T. F. Gajewski. "Commensal Bifidobacterium Promotes Antitumor Immunity and Facilitates Anti-PD-L1 Efficacy." Science 350.6264 (2015): 1084-089. http://dx.doi.org/10.1126/science.aac4255 PMid:26541606 PMCid:PMC4873287

[17] Vetizou, M., J. M. Pitt, R. Daillere, P. Lepage, N. Waldschmitt, C. Flament, S. Rusakiewicz, B. Routy, M. P. Roberti, C. P. M. Duong, V. Poirier-Colame, A. Roux, S. Becharef, S. Formenti, E. Golden, S. Cording, G. Eberl, A. Schlitzer, F. Ginhoux, S. Mani, T. Yamazaki, N. Jacquelot, D. P. Enot, M. Berard, J. Nigou, P. Opolon, A. Eggermont, P.-L. Woerther, E. Chachaty, N. Chaput, C. Robert, C. Mateus, G., Kroemer, D. Raoult, I. G. Boneca, F. Carbonnel, M. Chamaillard, and L. Zitvogel. "Anticancer Immunotherapy by CTLA-4 Blockade Relies on the Gut Microbiota." Science 350.6264 (2015): 1079-084. http://dx.doi.org/10.1126/science.aad1329 PMid:26541610 PMCid:PMC4721659

[18] Coley, William B. "The Treatment Of Malignant Tumors By Repeated Inoculations Of Erysipelas." The American Journal of the Medical Sciences 105.5 (1893): 487-510. http://dx.doi.org/10.1097/00000441-189305000-00001

[19] Felgner, Sebastian, Dino Kocijancic, Michael Frahm, and Siegfried Weiss. "Bacteria in Cancer Therapy: Renaissance of an Old Concept." International Journal of Microbiology 2016 (2016):1-14 http://dx.doi.org/10.1155/2016/8451728 PMid:27051423 PMCid:PMC4802035

[20] Roberts, N. J., L. Zhang, F. Janku, A. Collins, R. Y. Bai, V. Staedtke, A. W. Rusk, D. Tung, M. Miller, J. Roix, K. V. Khanna, R. Murthy, R. S. Benjamin, T. Helgason, A. D. Szvalb, J. E. Bird,

S. Roy-Chowdhuri, H. H. Zhang, Y. Qiao, B. Karim, J. Mcdaniel, A. Elpiner, A. Sahora, J. Lachowicz, B. Phillips, A. Turner, M. K. Klein, G. Post, L. A. Diaz, G. J. Riggins, N. Papadopoulos, K. W. Kinzler, B. Vogelstein, C. Bettegowda, D. L. Huso, M. Varterasian, S. Saha, and S. Zhou. "Intratumoral Injection of Clostridium Novyi-NT Spores Induces Antitumor Responses." Science Translational Medicine 6.249 (2014) http://dx.doi.org/10.1126/scitranslmed.3008982 PMid:25122639 PMCid:PMC4399712

[21] Staedtke, Verena, Ren-Yuan Bai, Weiyun Sun, Judy Huang, Kathleen Kazuko Kibler, Betty M. Tyler, Gary L. Gallia, Kenneth Kinzler, Bert Vogelstein, Shibin Zhou, and Gregory J. Riggins. "Clostridium Novyi-NT Can Cause Regression of Orthotopically Implanted Glioblastomas in Rats." Oncotarget 6.8 (2015): 5536-546. http://dx.doi.org/10.18632/oncotarget.3627 PMid:25849940 PMCid:PMC4467385

[22] Low, Kenneth Brooks, Martina Ittensohn, Xiang Luo, Li-Mou Zheng, Ivan King, John M. Pawelek, and David Bermudes. "Construction of VNP20009: A Novel, Genetically Stable Antibiotic-Sensitive Strain of Tumor-Targeting Salmonella for Parenteral Administration in Humans." Suicide Gene Therapy: 47-60. PMid:14657558

[23] Hoffman, Robert M. "Tumor-seeking Salmonella Amino Acid Auxotrophs." Current Opinion in Biotechnology 22.6 (2011): 917-23. http://dx.doi.org/10.1016/j.copbio.2011.03.009 PMid:21498066

[24] Saitoh, S.-I. "Lipid A Antagonist, Lipid IVa, Is Distinct from Lipid A in Interaction with Toll-like Receptor 4 (TLR4)-MD-2 and Ligand-induced TLR4 Oligomerization." International Immunology 16.7 (2004): 961-69. http://dx.doi.org/10.1093/intimm/dxh097 PMid:15184344

[25] Eom, Jeong Seon, Jin Seok Kim, Jung Im Jang, Bae-Hoon Kim, So Young Yoo, Ji Hyeon Choi, Iel-Soo Bang, In Soo Lee, and Yong Keun Park. "Enhancement of Host Immune Responses by Oral Vaccination to Salmonella Enterica Serovar Typhimurium Harboring Both FliC and FljB Flagella." PLoS ONE 8.9 (2013). http://dx.doi.org/10.1371/journal.pone.0074850 PMid:24069357 PMCid:PMC3775770

[26] Frahm, Michael, Sebastian Felgner, Dino Kocijancic, Manfred Rohde, Michael Hensel, Roy Curtiss, Marc Erhardt, and Siegfried Weiss. "Efficiency of Conditionally Attenuated Salmonella Enterica Serovar Typhimurium in Bacterium-Mediated Tumor Therapy."Molecular Biology 6.2 (2015) PMid:25873375 PMCid:PMC4453544 http://dx.doi.org/10.1128/mBio.00254-15

[27] Kong, W., S.-Y. Wanda, X. Zhang, W. Bollen, S. A. Tinge, K. L. Roland, and R. Curtiss. "Regulated Programmed Lysis of Recombinant Salmonella in Host Tissues to Release Protective Antigens and Confer Biological Containment." Proceedings of the National Academy of Sciences 105.27 (2008): 9361-366. http://dx.doi.org/10.1073/pnas.0803801105 PMid:18607005 PMCid:PMC2453710

[28] Ashraf, Shamaila, Wei Kong, Shifeng Wang, Jiseon Yang, and Roy Curtiss. "Protective Cellular Responses Elicited by Vaccination with Influenza Nucleoprotein Delivered by a Live Recombinant Attenuated Salmonella Vaccine." Vaccine 29.23 (2011): 3990-4002. http://dx.doi.org/10.1016/j.vaccine.2011.03.066 PMid:21466806 PMCid:PMC3092860

[29] Baban, Chwanrow K., Michelle Cronin, Deirdre O' Hanlon, Gerald C. O'Sullivan, and Mark Tangney. "Bacteria as Vectors for Gene Therapy of Cancer." Bioengineered Bugs 1.6 (2010): 385-94. http://dx.doi.org/10.4161/bbug.1.6.13146 PMid:21468205 PMCid:PMC3056088

[30] Cummins, Joanne, and Mark Tangney.

"Bacteria and Tumors: Causative Agents or Opportunistic Inhabitants?" Infectious Agents and Cancer Infect Agents Cancer 8.1 (2013) PMid:23537317 PMCid:PMC3668256 http://dx.doi.org/10.1186/1750-9378-8-11

[31] Theys, Jan, Willy Landuyt, Sandra Nuyts, Lieve Van Mellaert, Allan Van Oosterom, Philippe Lambin, and Jozef Anné. "Specific Targeting of Cytosine Deaminase to Solid Tumors by Engineered Clostridium Acetobutylicum." Cancer Gene Therapy 8.4 (2001): 294-97. http://dx.doi.org/10.1038/sj.cgt.7700303 PMid:11393282

[32] Stritzker, Jochen, Sabine Pilgrim, Aladar A. Szalay, and Werner Goebel. "Prodrug Converting Enzyme Gene Delivery by L. Monocytogenes." BMC Cancer 8.1 (2008). PMid:18402662 PMCid:PMC2329648 http://dx.doi.org/10.1186/1471-2407-8-94

[33] King, Ivan, David Bermudes, Stanley Lin, Michael Belcourt, Jeremy Pike, Kimberly Troy, Trung Le, Martina Ittensohn, John Mao, Wenshang Lang, Jacob D. Runyan, Xiang Luo, Zujin Li, and Li-Mou Zheng. "Tumor-Targeted Salmonella Expressing Cytosine Deaminase as an Anticancer Agent." Human Gene Therapy 13.10 (2002): 1225-233. http://dx.doi.org/10.1089/10430340232013900 05 PMid:12133275

[34] Pawelek, J. M., K. B. Low, and D. Bermudes. "Tumor-targeted Salmonella as a Novel Anti-melanoma Vector." Melanoma Research 7, Supplement 1 (1997). PMid:9377566

[35] Friedlos, F., P. Lehouritis, L. Ogilvie, D. Hedley, L. Davies, D. Bermudes, I. King, J. Martin, R. Marais, and C. J. Springer. "Attenuated Salmonella Targets Prodrug Activating Enzyme Carboxypeptidase G2 to Mouse Melanoma and Human Breast and Colon Carcinomas for Effective Suicide Gene Therapy." Clinical Cancer Research 14.13 (2008): 4259-266. http://dx.doi.org/10.1158/1078-0432.CCR-07-4800 PMid:18594008

[36] Swofford, Charles A., Adam T. St. Jean, Jan T. Panteli, Zachary J. Brentzel, and Neil S. Forbes. "Identification of Staphylococcus Aureus α-hemolysin as a Protein Drug That Is Secreted by Anticancer Bacteria and Rapidly Kills Cancer Cells." Biotechnology and Bioengineering 111.6 (2014): 1233-245. http://dx.doi.org/10.1002/bit.25184 PMid:24415346

[37] Jean, Adam T. St., Charles A. Swofford, Jan T. Panteli, Zachary J. Brentzel, and Neil S. Forbes. "Bacterial Delivery of Staphylococcus Aureus Alpha-Hemolysin Causes Tumor Regression and Necrosis in Murine Tumors." Molecular Therapy, 2014. http://dx.doi.org/10.1038/mt.2014.36 PMid:24590046 PMCid:PMC4089002

[38] Barbe, Sofie, Lieve Mellaert, Jan Theys, Nick Geukens, Elke Lammertyn, Philippe Lambin, and Jozef Anna. "Secretory Production of Biologically Active Rat Interleukin-2 by Clostridium Acetobutylicum DSM792 as a Tool for Anti-tumor Treatment."FEMS Microbiology Letters 246.1 (2005): 67-73. http://dx.doi.org/10.1016/j.femsle.2005.03.037 PMid:15869963

[39] Yang, Nan, Xiangying Zhu, Lishan Chen, Shenghua Li, and Daming Ren. "Oral Administration of Attenuated S. Typhimurium Carrying ShRNA-expressing Vectors as a Cancer Therapeutic." Cancer Biology & Therapy 7.1(2008):145-51. http://dx.doi.org/10.4161/cbt.7.1.5195 PMid:18059172

[40] Y. Tian, Y., B. Guo, H. Jia, K. Ji, Y. Sun, Y. Li, T. Zhao, L. Gao, Y. Meng, D. V. Kalvakolanu, D. J. Kopecko, X. Zhao, L. Zhang, and D. Xu.

"Targeted Therapy via Oral Administration of Attenuated Salmonella Expression Plasmid-vectored Stat3-shRNA Cures Orthotopically Transplanted Mouse HCC." Cancer Gene Therapy 19.6 (2012): 393-401. http://dx.doi.org/10.1038/cgt.2012.12 PMid:22555509 PMCid:PMC3891655

[41] Blache, C. A., E. R. Manuel, T. I. Kaltcheva, A. N. Wong, J. D. I. Ellenhorn, B. R. Blazar, and D. J. Diamond. "Systemic Delivery of Salmonella Typhimurium Transformed with IDO ShRNA Enhances Intratumoral Vector Colonization and Suppresses Tumor Growth." Cancer Research 72.24 (2012): 6447-456. http://dx.doi.org/10.1158/0008-5472.CAN-12-0193 PMid:23090116 PMCid:PMC3525777

[42] Singer, H. M., M. Erhardt, A. M. Steiner, M.-M. Zhang, D. Yoshikami, G. Bulaj, B. M. Olivera, and K. T. Hughes. "Selective Purification of Recombinant Neuroactive Peptides Using the Flagellar Type III Secretion System." Molecular Biology 3.3 (2012). PMid:22647788 PMCid:PMC3372961 http://dx.doi.org/10.1128/mBio.00115-12

[43] Xu, X., W. A. H. Hegazy, L. Guo, X. Gao, A. N. Courtney, S. Kurbanov, D. Liu, G. Tian, E. R. Manuel, D. J. Diamond, M. Hensel, and L. S. Metelitsa. "Effective Cancer Vaccine Platform Based on Attenuated Salmonella and a Type III Secretion System." Cancer Research 74.21 (2014):6260-270. http://dx.doi.org/10.1158/0008-5472.CAN-14-1169 PMid:25213323 PMCid:PMC4216746

[44] Delalat, Bahman, Vonda C. Sheppard, Soraya Rasi Ghaemi, Shasha Rao, Clive A. Prestidge, Gordon Mcphee, Mary-Louise Rogers, Jacqueline F. Donoghue, Vinochani Pillay, Terrance G. Johns, Nils Kröger, and Nicolas H. Voelcker. "Targeted Drug Delivery Using Genetically Engineered Diatom Biosilica." Nature Communications 6 (2015): 8791. http://dx.doi.org/10.1038/ncomms9791 PMid:26556723

[45] Felfoul, Ouajdi, Mahmood Mohammadi, Samira Taherkhani, Dominic De Lanauze, Yong Zhong Xu, Dumitru Loghin, Sherief Essa, Sylwia Jancik, Daniel Houle, Michel Lafleur, Louis Gaboury, Maryam Tabrizian, Neila Kaou, Michael Atkin, Té Vuong, Gerald Batist, Nicole Beauchemin, Danuta Radzioch, and Sylvain Martel. "Magneto-aerotactic Bacteria Deliver Drug-containing Nanoliposomes to Tumour Hypoxic Regions." *Nature Nanotechnology* 11, no. 11 (2016): 941-47. http://dx.doi.org/10.1038/nnano.2016.137 PMid:27525475

[46] Nemunaitis, John, Casey Cunningham, Neil Senzer, Joseph Kuhn, Jennifer Cramm, Craig Litz, Robert Cavagnolo, Ann Cahill, Caroline Clairmont, and Mario Sznol. "Pilot Trial of Genetically Modified, Attenuated Salmonella Expressing the E. Coli Cytosine Deaminase Gene in Refractory Cancer Patients." *Cancer Gene Therapy* 10, no. 10 (2003): 737-44. http://dx.doi.org/10.1038/sj.cgt.7700634 PMid:14502226

[47] King, Ivan, Martina Itterson, and David Bermudes. "Tumor-Targeted Salmonella Typhimurium Overexpressing Cytosine Deaminase: A Novel, Tumor-Selective Therapy." *Gene Therapy of Cancer Methods in Molecular Biology™*,(2009):649-59. http://dx.doi.org/10.1007/978-1-59745-561-9_33 PMid:19565926

[48] Heppner, F., and J. R. Moese. "The Liquefaction (oncolysis) of Malignant Gliomas by a Non Pathogenic Clostridium." *Acta Neurochirurgica* 42, no. 1-2 (1978): 123-25. http://dx.doi.org/10.1007/bf01406639 PMid:696441

[49] Tangney, Mark, and Cormac Gahan. "Editorial Hot Topic: Bacterial Vectors for Gene & Cell Therapy." *CGT Current Gene Therapy* 10, no. 1 (2010): 1-2.

http://dx.doi.org/10.2174/156652310790945584 PMid:20230364

[50] Toso, J. F., V. J. Gill, P. Hwu, F. M. Marincola, N. P. Restifo, D. J. Schwartzentruber, "Phase I Study of the Intravenous Administration of Attenuated Salmonella Typhimurium to Patients With Metastatic Melanoma." *Journal of Clinical Oncology* 20, no. 1(2002):142-52. http://dx.doi.org/10.1200/jco.20.1.142 PMid:11773163 PMCid:PMC2064865

[51] Le, D. T., A. Wang-Gillam, V. Picozzi, T. F. Greten, T. Crocenzi, G. Springett, M. Morse, H. Zeh, D. Cohen, R. L. Fine, B. Onners, J. N. Uram, D. A. Laheru, E. R. Lutz, S. Solt, A. L. Murphy, J. Skoble, E. Lemmens, J. Grous, T. Dubensky, D. G. Brockstedt, and E. M. Jaffee. "Safety and Survival With GVAX Pancreas Prime and Listeria Monocytogenes-Expressing Mesothelin (CRS-207) Boost Vaccines for Metastatic Pancreatic Cancer." *Journal of Clinical Oncology* 33, no. 12 (2015):1325-333. http://dx.doi.org/10.1200/jco.2014.57.4244 PMid:25584002 PMCid:PMC4397277

[52] Seavey, M. M., Z.-K. Pan, P. C. Maciag, A. Wallecha, S. Rivera, Y. Paterson, and V. Shahabi. "A Novel Human Her-2/neu Chimeric Molecule Expressed by Listeria Monocytogenes Can Elicit Potent HLA-A2 Restricted CD8-positive T Cell Responses and Impact the Growth and Spread of Her-2/neu-positive Breast Tumors." *Clinical Cancer Research* 15, no. 3 (2009): 924-32. http://dx.doi.org/10.1158/1078-0432.ccr-08-2283 PMid:19188163

[53] Singh, R., M. E. Dominiecki, E. M. Jaffee, and Y. Paterson. "Fusion to Listeriolysin O and Delivery by Listeria Monocytogenes Enhances the Immunogenicity of HER-2/neu and Reveals Subdominant Epitopes in the FVB/N Mouse." *The Journal of Immunology* 175, no. 6 (2005): 3663-673. http://dx.doi.org/10.4049/jimmunol.175.6.3663 PMid:16148111

[54] Kim, S. H., F. Castro, D. Gonzalez, P. C. Maciag, Y. Paterson, and C. Gravekamp. "Mage-b Vaccine Delivered by Recombinant Listeria Monocytogenes Is Highly Effective against Breast Cancer Metastases." *British Journal of Cancer* 99, no. 5 (2008): 741-49. http://dx.doi.org/10.1038/sj.bjc.6604526 PMid:18728665 PMCid:PMC2528142

[55] Shahabi, Vafa, Mariela Reyes-Reyes, Anu Wallecha, Sandra Rivera, Yvonne Paterson, and Paulo Maciag. "Development of a Listeria Monocytogenes Based Vaccine against Prostate Cancer." *Cancer Immunology, Immunotherapy* 57, no. 9 (2008): 1301-313. http://dx.doi.org/10.1007/s00262-008-0463-z PMid:18273616

[56] Wallecha, A., P. C. Maciag, S. Rivera, Y. Paterson, and V. Shahabi. "Construction and Characterization of an Attenuated Listeria Monocytogenes Strain for Clinical Use in Cancer Immunotherapy." *Clinical and Vaccine Immunology* 16, no. 1 (2008): 96-103. http://dx.doi.org/10.1128/cvi.00274-08 PMid:19020110 PMCid:PMC2620657

[57] Maciag, Paulo Cesar, Siniša Radulovic, and John Rothman. "The First Clinical Use of a Live-attenuated Listeria Monocytogenes Vaccine: A Phase I Safety Study of Lm-LLO-E7 in Patients with Advanced Carcinoma of the Cervix." *Vaccine* 27, no. 30 (2009): 3975-983. http://dx.doi.org/10.1016/j.vaccine.2009.04.041 PMid:19389451

[58] Oncology (Williston Park). 1997 Jun;11(6):772, 775-6. Bacterial infection in patients with cancer: focus on prevention. PMid:9244471

[59] Gafter-Gvili, A., P. Mical, M. Van Der Wetering, L. Kremer, A. Fraser, and L. Leibovici. "Antibiotic Prophylaxis for Bacterial Infections in Afebrile Neutropenic Patients following Chemotherapy." *Protocols The Cochrane Database of Systematic Reviews*, 2003.

http://dx.doi.org/10.1002/14651858.cd004386 PMid:16235360

[60] Liu, S-C, Np Minton, Aj Giaccia, and Jm Brown. "Anticancer Efficacy of Systemically Delivered Anaerobic Bacteria as Gene Therapy Vectors Targeting Tumor Hypoxia/necrosis." *Gene Therapy* 9, no. 4 (2002): 291-96. http://dx.doi.org/10.1038/sj.gt.3301659 PMid:11896468

[61] Theys, J., O. Pennington, L. Dubois, G. Anlezark, T. Vaughan, A. Mengesha, W. Landuyt, J. Anné, P. J. Burke, P. Dûrre, B. G. Wouters, N. P. Minton, and P. Lambin. "Repeated Cycles of Clostridium-directed Enzyme Prodrug Therapy Result in Sustained Antitumour Effects in Vivo." *British Journal of Cancer* 95, no. 9 (2006): 1212-219. http://dx.doi.org/10.1038/sj.bjc.6603367 PMid:17024128 PMCid:PMC2360559

[62] Theys, Jan, Willy Landuyt, Sandra Nuyts, Lieve Van Mellaert, Allan Van Oosterom, Philippe Lambin, and Jozef Anné. "Specific Targeting of Cytosine Deaminase to Solid Tumors by Engineered Clostridium Acetobutylicum." *Cancer Gene Therapy* 8, no. 4 (2001): 294-97. http://dx.doi.org/10.1038/sj.cgt.7700303 PMid:11393282

[63] Low KB, et al. "Lipid A mutant Salmonella with suppressed virulence and TNFalpha induction retain tumor-targeting in vivo." Nature Biotechnology (1999); 17:37–41. http://dx.doi.org/10.1038/5205 PMid: 9920266

[64] Thamm, D. H. "Systemic Administration of an Attenuated, Tumor-Targeting Salmonella Typhimurium to Dogs with Spontaneous Neoplasia: Phase I Evaluation." *Clinical Cancer Research* 11, no. 13 (2005): 4827-834. http://dx.doi.org/10.1158/1078-0432.ccr-04-2510 PMid:16000580

[65] Heimann, David M., and Steven A. Rosenberg. "Continuous Intravenous Administration of Live Genetically Modified Salmonella Typhimurium in Patients With Metastatic Melanoma." *Journal of Immunotherapy* 26, no. 2 (2003): 179-80. http://dx.doi.org/10.1097/00002371-200303000-00011 PMid:12616110 PMCid:PMC2656370

[66] Nemunaitis, John, Casey Cunningham, Neil Senzer, Joseph Kuhn, Jennifer Cramm, Craig Litz, Robert Cavagnolo, Ann Cahill, Caroline Clairmont, and Mario Sznol. "Pilot Trial of Genetically Modified, Attenuated Salmonella Expressing the E. Coli Cytosine Deaminase Gene in Refractory Cancer Patients." *Cancer Gene Therapy* 10, no. 10 (2003): 737-44. http://dx.doi.org/10.1038/sj.cgt.7700634 PMid:14502226

[67] Forbes, Neil S. "Engineering the Perfect (bacterial) Cancer Therapy." *Nature Reviews Cancer* 10, no. 11 (2010): 785-94. http://dx.doi.org/10.1038/nrc2934 PMid:20944664 PMCid:PMC3756932

[68] Danino, T., A. Prindle, G. A. Kwong, M. Skalak, H. Li, K. Allen, J. Hasty, and S. N. Bhatia. "Programmable Probiotics for Detection of Cancer in Urine." Science Translational Medicine 7.289 (2015). http://dx.doi.org/10.1126/scitranslmed.aaa3519 PMid:26019220 PMCid:PMC4511399

[69] Zackular, J. P., M. A. M. Rogers, M. T. Ruffin, and P. D. Schloss. "The Human Gut Microbiome as a Screening Tool for Colorectal Cancer." Cancer Prevention Research 7.11 (2014): 1112-121. http://dx.doi.org/10.1158/1940-6207.CAPR-14-0129 PMid:25104642 PMCid:PMC4221363

[70] Zitvogel, L., L. Galluzzi, S. Viaud, M. Vetizou, R. Daillere, M. Merad, and G. Kroemer. "Cancer and the Gut Microbiota: An Unexpected Link." Science Translational Medicine 7.271 (2015).

http://dx.doi.org/10.1126/scitranslmed.3010473 PMid:25609166 PMCid:PMC4690201

[71] Schwabe, Robert F., and Christian Jobin. "The Microbiome and Cancer." Nature Reviews Cancer 13.11 (2013): 800-12. http://dx.doi.org/10.1038/nrc3610 PMid:24132111 PMCid:PMC3986062

[72] Garrett, W. S. "Cancer and the Microbiota." Science 348.6230 (2015): 80-86. http://dx.doi.org/10.1126/science.aaa4972 PMid:25838377

[73] Yang, Shih-Chun, Chih-Hung Lin, Calvin T. Sung, and Jia-You Fang. "Antibacterial Activities of Bacteriocins: Application in Foods and Pharmaceuticals." Front. Microbiol. Frontiers in Microbiology 5 (2014). http://dx.doi.org/10.3389/fmicb.2014.00241 PMid:24904554