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REVIEW ARTICLE

Clostridium novyi-NT in cancer therapy

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Abstract The attenuated anaerobic bacterium *Clostridium novyi*-NT (*C. novyi*-NT) is known for its ability to precisely germinate in and eradicate treatment-resistant hypoxic tumors in various experimental animal models and spontaneously occurring canine sarcomas. In this article, we review the therapeutic and toxicologic aspects of *C. novyi*-NT therapy, key challenges and limitations, and promising strategies to optimize its performance *via* recombinant DNA technology and immunotherapeutic approaches, to establish *C. novyi*-NT as an essential tool in cancer therapy.

Introduction

Cancer is a genetic disease arising from a series of somatic mutations that drive aberrant cellular processes and unchecked cell division.[1,2] As a cancer grows, an *ad hoc* tumor vasculature develops. The result is a heterogeneous tumor, composed of normoxic, hypoxic, and necrotic regions. While somatic mutations and alterations in cellular processes can be targeted with current systemic therapy, resistance almost always develops. First, efficacy of systemic therapy is dependent on tumor vasculature. If a tumor is not well vascularized, as often is the case, systemic therapies do not penetrate all areas of the tumor at levels that are cytotoxic.[3] Second, even if the therapeutic reaches levels that are pharmacologically active, a rare pre-existing resistant clone is often present and expands after treatment.[4] The consequence is a rapid resurgence of a previously conquered foe.[5,6]

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Perturbations of tumor physiology and the resulting regions of hypoxia and necrosis, however, represent an ideal environment for anaerobic bacteria.[7] Specifically, anaerobic bacteria — or their spores — can be injected systemically or directly into the tumor and selectively grow in areas of hypoxia, resulting in tumor destruction. Importantly, therapies based on anaerobic bacteria such as *Clostridium spp.* can potentially overcome many of the disadvantages of systemic therapies and offer a precise way to eradicate tumors that would otherwise be untreatable.[8] In addition, bacterial therapy harnesses the power of the host immune system. It is for these reasons that bacterial anticancer therapies are gaining recognition.

History of bacterial anticancer therapy

The concept of using bacteria to treat cancers has a long history. Over 100 years ago William Coley, a surgeon in New York, conducted the first systematic assessment of bacterial anticancer therapy.[9,10] He intentionally inoculated cancer patients with cultures of *Streptococcus pyogenes*, the causative agent of erysipelas, to induce a local infection.[9] To mitigate the harmful and unpredictable effects of infection, heat inactivated *S. pyogenes*, later coupled with *B. prodigiosis*, was used to treat a large series of patients.[11] Interestingly, Coley observed long-term responses in some patients, although the efficacy was variable. Since then, many genera of bacteria have been shown to preferentially accumulate in tumors to varying degrees of success: *Salmonella typhimurium*, *Escherichia coli*, *Listeria monocytogenes*, *Clostridium spp.*, *Bifidobacterium spp.* and *Mycobacterium bovis*.[7,12–14]

A critical aspect of bacterial anticancer therapy is tumor localization. Bacterial species that localize specifically to the tumor environment while demonstrating an inability to colonize normal tissue are thought to increase targeting specificity and reduce toxicities associated with treatment.[12–15] As such, obligate and facultative anaerobes have been preferentially studied as anticancer agents to take advantage of the physiological differences between neoplastic and normal tissues. In particular, *Clostridium spp.* (*C. butryicum* and *C. novyi*) and *Salmonella typhimurium* have been extensively studied.

The *Clostridia* genus consists of a large and heterogeneous group of gram-positive, spore-forming bacteria that exclusively grow in the absence (or at low levels) of oxygen.[16] Various subtypes have been tested as anti-cancer agents including *C. butyricum*, *C. tetani*, *C. histolyticum*,[17,18] *C. beijerinckii*[19] and *C. acetobutylicum*.[20] The first evidence of the ability of *Clostridium spp.* to colonize tumors came from studies investigating the intravenous injection of *C. tetani* into mice with transplanted and spontaneous tumors.[21] Subsequent work demonstrated that the non-pathogenic M–55 strain of the bacterium *C. butyricum*, that was later on reclassified as *C. sporogenes* ATCC13732, colonized transplanted tumors in mice and produced extensive oncolysis after IV injection.[22] Interestingly, while non-pathogenic strains of *Clostridia* were able to colonize in tumors, oncolysis was less extensive than that observed with *C. butyricum*.[22,23] After these initially encouraging results, five patients with locally advanced tumors were given 1×10^{10} *C. butyricum* spores (M–55 stain) *via* IV injection.[24] Oncolysis was observed in three patients and one patient showed clinical benefit. However, no patient with overt signs of spore germination and oncolysis experienced a complete response. The safety and efficacy of *C. butyricum* was assessed further in 49 patients with glioblastomas, the most aggressive primary brain tumor.[25] Each patient received 1×10^{9} spores injected into the carotid artery. Patients with a minimal clinical response to the first injection of *C. butyricum* spores received a second injection, two weeks after the first. Germination of *C. butyricum* spores and development of an abscess were common, often requiring surgical debridement. Notably, sixteen patients died during treatment and in those who survived, the rate of recurrence was unaffected.[25]

While the preclinical and clinical studies of *C. butyricum* spores demonstrated that treatment resulted in reliable germination confined to neoplastic tissue, complete destruction of a tumor, including its well-oxygenated regions inhospitable to anaerobic bacteria, was a challenge. Thus, the next phase of the development of bacterial therapy based on *Clostridium spp.* involved methods to simultaneously kill both well-oxygenated and hypoxic regions of the tumor. These methods have focused on two approaches to synergistically kill neoplastic cells in well-oxygenated tumor regions: 1) co-treatment with a cytotoxic drug, and 2) genetic modification of *Clostridium spp.* Supportive studies investigated the treatment with IV injected spores of *Clostridium spp.* and chemotherapeutic agents in tumor bearing mice and hamsters. These studies demonstrated an increased oncolysis and responses with cyclophosphamide and 5-Flurorouracil (5-FU), but not other agents.[26,27] Similarly, several *Clostridium spp.* have been genetically engineered to contain cytosine deaminase, an enzyme from *Escherichia coli* that converts 5-fluorocytosine, a non-toxic pro-drug, to 5-FU, a toxic chemotherapeutic agent.[19,28,29] Here, spore germination and tumor colonization result in an increase in tumor concentrations of 5-FU and enhanced anti-tumor responses.[29]

Development of *C. novyi*-NT as a therapeutic agent

With the advancement of recombinant DNA technology in the last few decades, the interest of using bacteria as anticancer agents has re-emerged.[7] At the brink of the 21st century, Vogelstein and colleagues revived the idea of bacterial anti-cancer therapy and screened a panel of 26 anaerobes for their ability to populate and disseminate within a human colorectal cancer xenograft model. *Clostridium novyi* (*C. novyi*, ATCC #19402) was identified as the most superior strain and subsequently rendered nonpathogenic by eliminating a residential phage carrying α-toxin, a major toxin responsible for toxicity.[15] The resulting clone, named *C. novyi*-NT, has been thoroughly investigated since and is currently undergoing clinical evaluation in a human Phase I trial for patients with treatment-refractory tumors (NCT01924689).

C. novyi is ubiquitously encountered in the soil and feces of the environment from where it can infect animals and

humans.[16] Although rare, human infections of wild-type *C. novyi* leading to gas gangrene are often fatal particularly after traumatic wounds or illicit drug use due to the secretion of the lethal α-toxin,[30] which was deleted in the therapeutic strain. *C. novyi*-NT is a highly motile bacterium that is exquisitely sensitive to oxygen; vegetative forms cannot survive in oxygen while bacterial spores can only germinate in hypoxic conditions.[31] Fig. 1 shows micrographs of air-dried *C. novyi*-NT spores, several of which exhibit a shell "tail" at the pole (Fig. 1A, B) and vegetative bacteria, with propeller-like flagella as a means of motility (Fig. 1D). Once *C. novyi*-NT spores are exposed to germination conditions, the spores collapse (Fig. 1C, c1—3) and bacteria outgrow the spores leaving only an empty spore coat behind (Fig. 1C, e1—3).

A variety of syngeneic and xenograft experimental tumors across multiple animal species have been treated with intratumoral (IT) or IV administration of *C. novyi*-NT spores including colon and pancreatic cancers in mice, aggressive squamous cell carcinoma in rabbits, and glioblastomas in rats.[15,32—36] Once administered, *C. novyi*-NT spores germinate locally within tumors and precisely spread throughout the tumor and its microsatellites, causing hemorrhagic necrosis, tumor cell lysis and tumor regression.[15,32,35,36] As such, a single IV dose of *C. novyi*-NT spores into mice and rabbits bearing tumors is sufficient to induce local tumor necrosis and an intense inflammatory response, with complete responses seen in 25—30% of the treated animals.[32] Notably, these responses were durable and animals developed long-term cellular immunity to the original tumors. In the remaining animals with a partial response to *C. novyi*-NT tumors regrew presumably from a well-vascularized tumor rim that is more resistant to *C. novyi*-NT. One problem of the systemic spore administration is the discrepancy between the need for a large injected spore dose and the relatively small fraction of spores that are delivered to tumors,[37] thus limiting the efficacy of the therapy and potentially creating side effects, particularly in large animals and human patients that have relatively large blood volumes. By contrast, IT spore administration can theoretically deliver orders of magnitude more spores directly into the tumor. Indeed, *C. novyi*-NT IT administration appears to be more effective in delivering a therapeutic dose of spores to the tumor, which was most convincingly shown in the orthotopic F98 rat glioblastoma model.[35,36]

Toxicologic evaluation of *C. novyi*-NT spores in mice and rabbits found that spores had only minimal clinical toxicity without an anaerobic space to colonize and were rapidly cleared from the circulation by the reticuloendothelial system.[37] Not surprisingly, *C. novyi*-NT associated toxicity was dependent on germination, the spore dose, route of administration and particularly tumor size, being most pronounced in animals with larger tumors.[37] Clinical signs of toxicities include lethargy, weight loss and abscessation as expected with typical bacterial infections and were predominantly observed in rodents, and to a lesser degree in rabbits. The treatment-related mortality

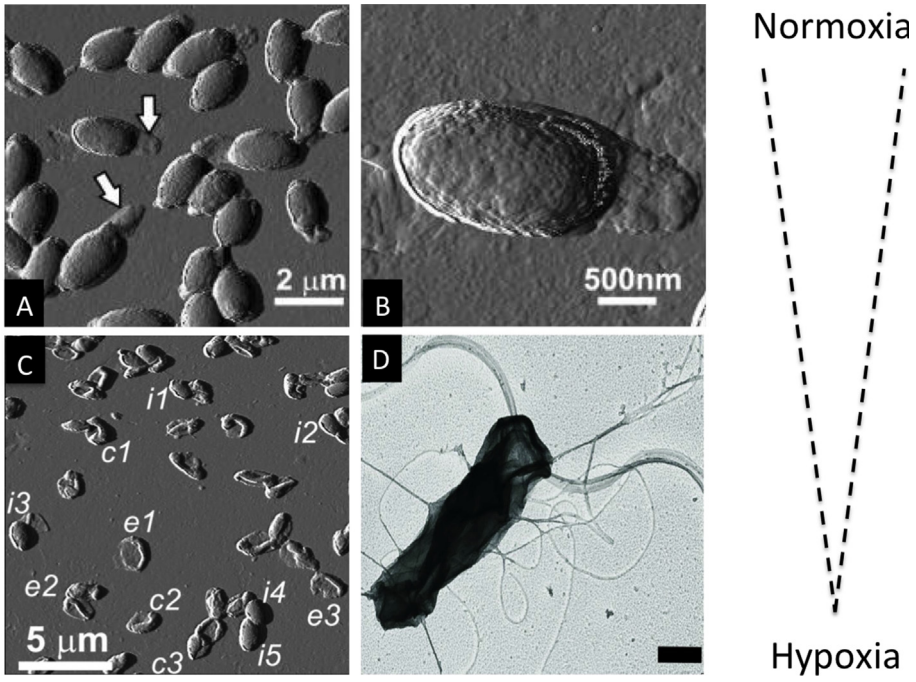

Fig. 1 Micrographs of *C. novyi*-NT spores and outgrowth. (A) Atomic force micrograph of air-dried *C. novyi*-NT spores. Some of the spores have a "tail" corresponding to the amorphous shell (arrow). (B) Magnification of a single *C. novyi*-NT spore highlighting the irregular spore surface and a "tail". (C) Phase-contrast micrograph of spores exposed to germination medium for 24 h. Depicted are spores that are fully intact (i1—i5), collapsed after germination (c1-c3), or fully outgrown only leaving an empty spore coat behind (e1—e3). Fig. 1A—C are reprinted from[41] with permission from the Journal of Bacteriology. (D) Electron micrograph of a vegetative *C. novyi*-NT with numerous flagella. (Bar = 0.5 μm). Reprinted from[38] with permission from PNAS, Copyright (2003) National Academy of Sciences, U.S.A.

varied between animal species and was reported as 10–20% in mice.[37] Toxicity was manageable in rodents by administering antibiotics or by ensuring adequate hydration.[36,37]

Since cures with *C. novyi*-NT alone are relatively rare, *C. novyi*-NT can be combined with a variety of chemotherapeutic agents or radiation, a strategy known as Combination Bacteriolytic Therapy or COBALT.[15,33,38,39] It is based on the rational that *C. novyi*-NT destroys the hypoxic and necrotic parts of the tumors, which are traditionally resistant to radiation and cytotoxic agents, while chemotherapies and radiation attack the tumor cells in the proliferating, non-hypoxic areas of the tumor (Fig. 2). Both modalities — chemotherapeutics, particularly microtubule interacting drugs, and various forms of radiation — resulted in synergistic responses when combined with *C. novyi*-NT with partial or complete regressions in the majority of the various cancer xenograft models.[33,38] However, it should be noted that in combination with radiation and certain cytotoxic agents increases in toxicity were observed.

Genome of *C. novyi*-NT and aspects of bioengineering

The 2.55-Mb *C. novyi*-NT genome is smaller in size and coding capacity than previously sequenced *Clostridium* species.[40] In contrast to its vegetative form, spores of this organism are stable in the presence of oxygen. Transmission electron and atomic force microscopy revealed that *C. novyi*-NT spores are surrounded by an amorphous layer intertwined with honeycomb parasporal layers, which are sequentially dissolved during the germination process.[41] Interestingly, many of the genes encoded by spore mRNA were not expressed in vegetative cells. Most of these spore-specific genes encode either spore coat proteins or proteins with redox activity, which presumably could aid germination by scavenging reactive oxygen species.[40] Spore-specific transcripts were also highly represented in the transcriptomes of *C. novyi*-NT treated tumors, suggesting extensive sporulation as a result of the struggle within a hostile host tumor environment. Expression of the vegetative *C. novyi*-NT-specific genes varied dependent on the growth phase. During the early-log growth phase, several genes involved in energy metabolism and biosynthesis of cofactors and vitamins were frequently expressed.[40] By contrast, genes responsible for the biosynthesis, transport of amino acids and other precursor molecules were upregulated in the mid-log phase, while only 7 genes predominantly involving metabolic enzymes were preferentially expressed in late-log phase.[40] Furthermore, a group of genes encoding enzymes involved in fatty acid and lipid metabolism were detected in transcriptomes of infected tumors, which could mirror the adaptation of *C. novyi*-NT to the membrane-rich environment at the infection site.[40]

Of crucial clinical interest was the identification of genes that may potentially affect the efficacy and toxicity associated with *C. novyi*-NT infections. These included 153 gene products that were predicted to be either cell-surface associated, secreted or had cytolytic properties, such as various protein and lipid degrading enzymes (e.g. phospholipase C), toxins (e.g. two hemolysins and two tetanolysins), as well as proteins involved in lipid biosynthesis, cell motility and chemotaxis (e.g. flagellin).[40] This knowledge has opened the field for genetic manipulation and bioengineering of *C. novyi*-NT.

Genetic modifications will be necessary to design a *C. novyi*-NT strain with the desired therapeutic properties and toxicological profile. The *Clostridia* species, however, has traditionally posed significant challenges because they are not easily transformable using conventional plasmid vectors and electroporation techniques. Recently, a technique utilizing the retrohoming ability of group II introns

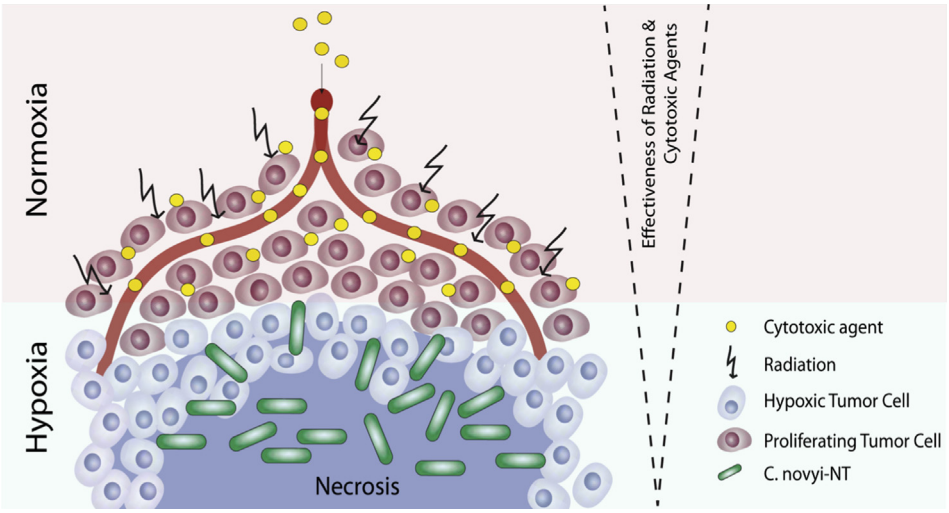

Fig. 2 Schematic overview of COBALT. In tumors, blood vessels are structurally and functionally abnormal, resulting in the development of hypoxic, quiescent tumor areas and well-oxygenated, proliferating tumor regions. In COBALT therapy, *C. novyi*-NT destroys the hypoxic and necrotic parts of the tumors, which frequently give rise to relapse and are traditionally resistant to radiation and cytotoxic agents, while well-oxygenated tumor regions largely inaccessible to *C. novyi*-NT are susceptible to conventional cytotoxic therapies.

(TargeTron) has been validated in *Clostridia*, which will expand the scale of genetic manipulations for this species.[42,43] Potential genetic manipulations to increase effectiveness or decrease toxicity include (a) knock-out of candidate genes that introduce unnecessary toxicity; (b) knock-in of genes that limit toxicity for example through expression of proteins that alter the host immune response; (c) knock-in of genes to increase therapeutic efficacy for example proteins with cytotoxic activities or enzymes metabolizing prodrugs; immune-modulators or cytokines to stimulate the anti-cancer immunity and (d) modifications to endogenous processes that control bacterial proliferation within tumors. Though technically feasible, these processes are very complex and could result in unintended phenotypes, which might interfere with treatment efficacy. On the other hand, the development of genetically optimized strains that are tailored to the patient's cancer could be highly rewarding and finally establish bacteria as an essential treatment tool in cancer.

Tumor targeting and destruction by *C. novyi*-NT

C. novyi has the ability to target tumors with hypoxia, which is observed in almost all solid cancers including glioblastomas (Fig. 3C). Once *C. novyi*-NT spores meet the hypoxic tumor region, they germinate and induce substantial tumor regression. Tumor specificity is demonstrated by the observation that vegetative bacteria cannot spread into normal tissue owing to higher oxygen levels (Fig. 3A–B).[15,33] While larger tumors are well known to develop areas of low oxygen tension and necrosis, we have also found that *C. novyi*-NT is able to localize to small islands of tumor cells, suggesting that in fact microscopic foci of malignant cells could provide sufficient hypoxia to support bacterial germination.[35,36]

The mechanisms underlying bacterial tumor destruction are incompletely understood and may include direct tumor cell destruction as well as activation of the host immune system. The genomic and transcriptome analyses of *C. novyi*-NT allowed the identification of several highly expressed genes encoding extracellular proteins, which could play a key role in this process. Most notable are three lipid-degrading proteins: phospholipase C (NT01CX0979) and two lipases (NT01CX2047 and NT01CX0630), all of which alter the structure of lipid bilayers, change membrane permeability and thereby create direct cytotoxicity.[40] Furthermore, the ability of phospholipases to activate inflammatory responses and induce anti-tumor immunity could have a major effect on the battle between tumor and bacteria.[40]

Apart from direct cytoreduction, *C. novyi*-NT also induces a strong inflammatory response involving pro-inflammatory cytokines such as IL-6, G-CSF, MIP-2, and TIMP-1 that recruit a substantial amount of immune cells

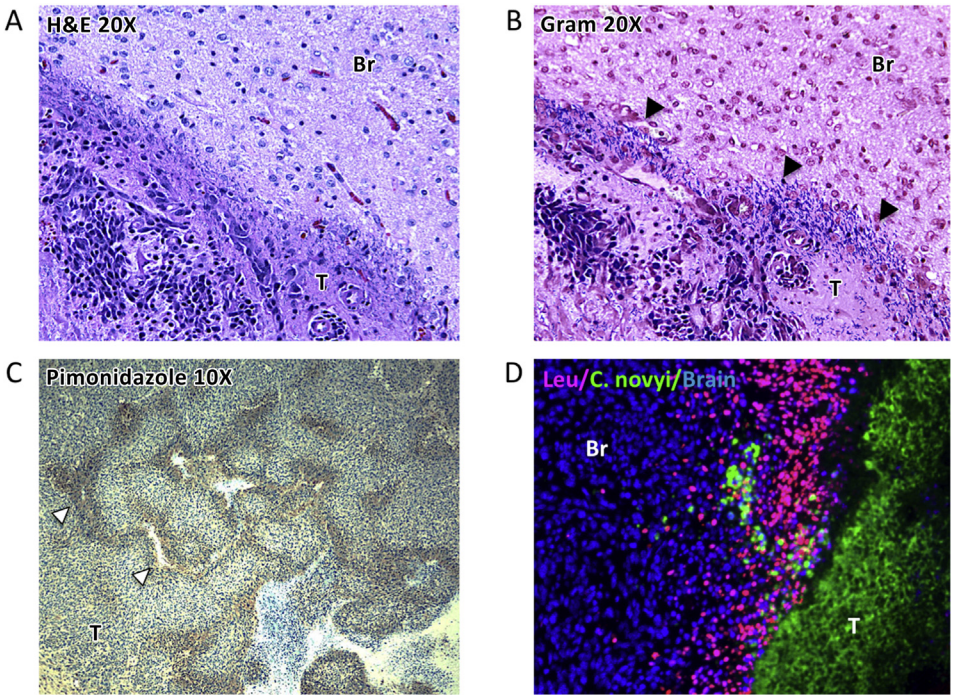

Fig. 3 Histological sections of rodent glioblastomas during *C. novyi*-NT treatment. (A) Histological H&E section of syngeneic F98 glioblastoma treated with *C. novyi*-NT. (B) Gram stained brain sections revealed that vegetative *C. novyi*—NT bacteria are exclusively confined to the tumor (black arrowhead) but not in normal brain parenchyma. (C) Pimonidazole staining of F98 glioblastoma demonstrating intratumoral distribution of hypoxia, shown in brown (white arrowhead). (D) *C. novyi*-NT infection induces a potent local host-inflammatory response in orthotopic rodent glioblastoma. A ring of leukocytes, stained by anti-CD45 (red), surrounded the tumor and restrained the spread of *C. novyi*-NT (green). DAPI is shown in blue. Abbreviations: Br, brain with remaining tumor; T, eradicated tumor.

(Fig. 3D) to the infection site to generate a durable adaptive anti-tumor immunity.[32,36] It is conceivable that immunogenic cell death (ICD), which is generated by tumor associated antigen specific T cells that were triggered by dying tumor cells along with the release of Damage-Associated Molecular Pattern molecules (DAMPs) by dendritic cells,[44–46] may play a substantial role in this process. While exact mechanisms for *C. novyi*-NT related tumor destruction are unknown, the observation that vaccination of BALB/c mice with *Clostridium difficile* (*C. diff.*) toxin B (TcdB)-treated CT26 and B16—F10 melanoma cells resulted in a long-term tumor specific immune response support this hypothesis.[47] Enhancing this effect could further optimize the therapeutic efficacy because the anti-tumor immune response may mediate tumor regression at distant tumor sites in which *C. novyi*-NT infection is not established. Regardless, more studies are needed to further understand how *C. novyi*-NT mediates tumor destruction and the role the immune system plays in this process.

Counteracting immunosuppression

Although cancer cells can be detected and destroyed by the immune system, there is overwhelming evidence that cancer cells paralyze the immune responses. Cancer cells are equipped with an arsenal of escape mechanisms: they can limit their display of antigens because of restricted presentations or improper maturation of Antigen-Presenting Cells (APC), express ligands, e.g. Programmed Death-Ligand 1 (PD-L1), for the receptors that impede the function of immune cells, secrete immune-suppressive cytokines, e.g. TGF-β and IL-10, and induce negative regulatory immune cells such as regulatory T cells (Treg) and Myeloid-Derived Suppressor Cells (MDSC).[48–52]

We believe that a strong immune-activating stimulus, such as a bacterial infection, is able to counteract some of the tumor-induced immune-suppression mechanisms by recruitment of immune cells and local production of cytokines and chemokines. Research on oncolytic viruses has provided support for this hypothesis.[53,54] It is very conceivable that a productive *C. novyi*-NT infection, just like an oncolytic virus, can elicit an adaptive anti-tumor immune response through lysis of tumor cells and release of tumor-associated antigens into the microenvironment. As part of this process, two characteristic bacteria-related signaling pathways, the Pathogen-Associated Molecular Patterns (PAMPs) and DAMPs, become activated and promote maturation of APCs, which, along with various cytokines, stimulate antigen-specific $CD4^+$ and $CD8^+$ T cells to launch an adaptive immune response against cancer cells. Immune-activating adjuvants including curcumin and cyclic diguanylate among others could in principle further enhance this effect.[55,56]

The ability of *C. novyi*-NT to modulate the tumor environment provides a strong rational for the combination with other agents that target additional immune-suppressive mechanisms to optimize the therapeutic success. Various groups including ours have shown that several therapeutic agents can reduce tumor-induced immune suppression. For example, the epigenetic modulator entinostat, anthracycline doxorubicin and anti-metabolites gemcitabine and 5-FU can decrease the number of MDSCs and thus, reverse the immune-suppression.[44,57–60] Furthermore, sunitinib and vemurafenib, a multi-kinase inhibitor and a BRAF inhibitor, respectively, as well as several neutralizing antibodies against GM-CSF receptor, IL-6 receptor, VEGF-A, or stem cell factor (SCF) have been reported to inhibit MDSC expansion and mobilization.[61–64] Likewise, selective depletion of Tregs and MDSCs with low-dose cyclophosphamide has also been practiced in clinical studies.[65]

Perhaps the most promising approach is the combination with T cell checkpoint inhibitors, which have been extensively studied in various cancers. The FDA-approved nivolumab, pembrolizumab and pidilizumab inhibit the interaction of the Programmed Cell Death 1 (PD-1) receptor with its ligand PD-L1, whereas ipilimumab targets Cytotoxic T Lymphocyte Associated Protein 4 (CTLA-4). Several other checkpoint modulators that target PD-L1 (e.g. avelumab), Lymphocyte Activation Gene 3 (LAG-3), T Cell Immunoglobulin and Mucin-3 (TIM-3), IDO or OX40 are currently in clinical development.[48,66–68] These agents could help reverse immune suppression and prove synergistic with *C. novyi*-NT.

Comparative studies with *C. novyi*-NT

The preclinical animal studies demonstrated that the features of *C. novyi*-NT anticancer therapy are attractive to continue development. First, *C. novyi*-NT spores were able to reliably germinate in the hypoxic regions of solid tumors.[15] Second, after germination, growth of the vegetative *C. novyi*-NT led to profound tumor lysis and long-term responses in 25%—30% of tumor bearing mice and rabbits after a single intravenous (IV) dose.[32] Third, *C. novyi*-NT therapy produces durable resistance in mice to re-challenge with the same tumor cell line, indicating stimulation of a long-lasting antitumor immune response.[32] These qualities led to evaluation of the safety and efficacy of *C. novyi*-NT in pet dogs with spontaneously occurring tumors. Increasing evidence suggests that spontaneously occurring tumors in pet dogs more closely resemble human tumors as they[8,69]:

1. Are of host origin,
2. Occur in genetically outbred populations,
3. Occur in hosts with intact immune systems,
4. Share similarities in somatically mutated cancer driver genes,
5. Are of similar size and histological type,
6. Share environmental influences.

To identify the maximum tolerated dose (MTD) of a single IV injection of *C. novyi*-NT spores, 6 dogs with spontaneously occurring solid tumors were treated as part of a standard phase I dose-escalation study.[70] In this study, two dogs were treated at the initial dose of 3×10^8 spores and experienced dose limiting toxicity (DLT), including abscess formation in a metastatic lymph node in one dog. Subsequently, the dose was reduced to 3×10^7 *C. novyi*-NT spores. At the reduced dose, 2 of 4 treated dogs experienced DLT, specifically, abscess formation at the site of the primary tumor of one dog and the spleen of the second dog.

In both cases, *C. novyi* bacteria were confirmed to be present by culture and DNA sequencing. The most common toxicity, experienced by all 6 dogs, was fever. Other common treatment related toxicities included inappetence and hematologic and biochemical abnormalities. Importantly, this study unequivocally demonstrated the ability of *C. novyi*-NT spores to germinate in spontaneously occurring solid tumors and form an abscess after IV injection.

In a second study, 16 dogs with spontaneously occurring solid tumors received 1×10^8 *C. novyi*-NT spores *via* intratumoral (IT) injection weekly for a maximum of four weeks.[35] In this study, the authors reasoned that due to the large blood volume and small tumor sizes seen in pet dogs compared to mice, previous IV injection of spores in pet dogs had resulted in too few spores reaching the target tumor. As such, IT injection was used to deliver an increased number of spores directly into target tumors. Treatment was well tolerated and associated with local infection. Importantly, abscess formation and tumor destruction were common, with 6 dogs experiencing an objective response. Of these dogs, 3 had complete responses and 3 had partial responses.

Based on the safety and efficacy in pet dogs, a Phase I human trial with IT injected *C. novyi*-NT spores was initiated. One patient with advanced leimyosarcoma was treated with 1×10^4 *C. novyi*-NT spores injected directly into a target tumor. The single injection of IT spores in this patient resulted in tumor necrosis and formation of an abscess that required surgical debridement.[35] As reported in dogs, toxicities included those associated with local bacterial infection: an increase in white blood cell count, fever, and pain.

Regulatory concerns

C. novyi-NT differs from standard drugs in several exceptional ways: it is live and vegetative forms self-propagate within tumor tissues. Therefore, like all live therapeutic biologics, *C. novyi*-NT poses unique regulatory and manufacturing challenges. In the United States, the use of biologic and recombinant DNA technology is regulated by two federal agencies: the Food and Drug Administration (FDA), and the Office of Biotechnology Activities (OBA) at the National Institutes of Health, which also organizes the Recombinant DNA Advisory Committee (RAC) and provides recommendations related to the recombinant DNA technology used in these agents. For a detailed overview we would like to refer the reader to an excellent review by Husain[71] and various guidance documents released by the FDA including "Guidance for Industry: Design and Analysis of Shedding Studies for Virus or Bacteria-Based Gene Therapy and Oncolytic Products"[72] and "Guidance for Industry: Determining the Need for and Content of Environmental Assessments for Gene Therapies, Vectored Vaccines, and Related Recombinant Viral or Microbial Products".[73]

Conclusions and future directions

C. novyi-NT holds great promise as a cancer therapeutic but numerous challenges remain to be solved before this therapy can obtain regulatory approval and be applied in clinic. Preclinical and early human experiences have demonstrated *C. novyi*-NT's potential in cancer therapy and yet, such a therapeutic infection carries a risk for a significant toxicity, similar to other bacterial infections. Although the use of the attenuated strain has significantly decreased treatment related toxicity, additional studies to understand the mechanism of toxicity and tumor destruction are necessary to further improve the therapeutic index. Solving these issues *via* synthetic biology could overcome limitations that hamper current cancer therapy and enables genetic fine-tuning of *C. novyi*-NT for individualized cancer therapy. Once perfected, successful *C. novyi*-NT therapy could utilize various treatment strategies dependent on the patient and tumor characteristics: IT spore delivery may maximize treatment effect in locally advanced tumors, however, this approach could be insufficient for metastatic disease, in which case systemic spore administration might be preferred. Finally, determining the optimal combination of *C. novyi*-NT with other cancer therapies will be critical to maximize treatment success. One very exciting opportunity is the combination of bacteriolytic therapy with immunotherapies to augment the host immune response and trigger a robust antitumor immunity.

Conflicts of interest

Under a licensing agreement between BioMed Valley Discoveries Inc. and the Johns Hopkins University, S.Z. is entitled to a share of royalties received by the University on sales of products described in this article. S.Z is also Founding Scientific Advisor of Personal Genome Diagnostics Inc., a company focused on the identification of genetic alterations in human cancer for diagnostic and therapeutic purposes. The terms of these arrangements are under ongoing management by the Johns Hopkins University in accordance with its conflict of interest policies. The other authors declare no conflict of interest in the works described in this article.

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