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Intratumoral injection of *Clostridium novyi*-NT spores induces antitumor responses

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Abstract

Species of *Clostridium* bacteria are notable for their ability to lyse tumor cells growing in hypoxic environments. We show that an attenuated strain of *Clostridium novyi* (*C. novyi*-NT) induces a microscopically precise, tumor-localized response in a rat orthotopic brain tumor model after intratumoral injection. It is well known, however, that experimental models often do not reliably predict the responses of human patients to therapeutic agents. We therefore used naturally occurring canine tumors as a translational bridge to human trials. Canine tumors are more like those of humans because they occur in animals with heterogeneous genetic backgrounds, are of host origin, and are due to spontaneous rather than engineered mutations. We found that

intratumoral injection of *C. novyi*-NT spores was well tolerated in companion dogs bearing spontaneous solid tumors, with the most common toxicities being the expected symptoms associated with bacterial infections. Objective responses were observed in 6 of 16 dogs (37.5%), with three complete and three partial responses. On the basis of these encouraging results, we treated a human patient who had an advanced leiomyosarcoma with an intratumoral injection of *C. novyi*-NT spores. This treatment reduced the tumor within and surrounding the bone. Together, these results show that *C. novyi*-NT can precisely eradicate neoplastic tissues and suggest that further clinical trials of this agent in selected patients are warranted.

INTRODUCTION

Therapies that specifically target and destroy cancers must recognize differences between normal and malignant tissues (1–4). These differences include genetic alterations and pathophysiological changes that lead to heterogeneous masses with areas of hypoxia and necrosis (4–8). Systemically delivered anticancer agents rely on tumor vasculature for delivery and, as such, are less effective in poorly vascularized, hypoxic tumor regions (4). Additionally, radiotherapy fails to kill hypoxic cells because oxygen is a required effector of radiation-induced cell death (9). For these key reasons, nonresectable, locally advanced tumors are particularly difficult to manage with conventional therapies.

In contrast, the hypoxic areas of tumors offer a perfect niche for the growth of anaerobic bacteria. In principle, this offers an opportunity for eradication of advanced local tumors in a precise manner, sparing surrounding well-vascularized, normoxic tissue. Since Coley's original work treating cancer patients with *Streptococcus pyogenes* over 100 years ago, a variety of anaerobic bacteria have been considered for this purpose (10–12). This early work failed to produce a viable anticancer agent, in part because of poor reproducibility and unacceptable toxicity. More recent work involved attenuated strains of *Salmonella typhimurium* and others (13, 14). However, whereas phase 1 clinical trials of *S. typhimurium* in both dogs and human patients demonstrated that the bacterium could be safely administered and targeted to tumor, limited efficacy was observed (15, 16). In an effort to augment efficacy with *S. typhimurium* therapy, genetically modified strains incorporating cytosine deaminase, which converts systemically administered 5-flurocytosine to 5-flurouracil, have been developed and evaluated in patients (17).

One particularly promising bacterium, however, is *Clostridium novyi* (18). *C. novyi* is a highly mobile, spore-forming bacterium that is exquisitely sensitive to oxygen. A derivative of the wild-type strain, called *C. novyi*-NT, was generated through removal of the α-toxin gene (18–20). A single dose of intravenously injected *C. novyi*-NT spores into mice and rabbits bearing transplanted syngeneic tumors led to localized tumor necrosis, intense inflammatory responses, and complete responses (CRs) in 25 to 30% of the treated animals (20). On the basis of these data, intravenously injected *C. novyi*-NT spores were evaluated in spontaneously occurring canine tumors (21). However, at doses that exhibited acceptable toxicity, no CRs were observed.

Given the ability of intravenously injected *C. novyi*-NT spores to localize, germinate within, and destroy murine tumors while leaving surrounding normal tissues intact, we hypothesized

that direct intratumoral injection of spores into solid tumors might have advantages over administration via the intravenous route. One problem encountered with systemic injection of spores is the small proportion of spores that actually are delivered to tumors (22). This problem is compounded in large animals and human patients, which have relatively large blood volumes and relatively small tumors compared to mice. With intratumoral injection, orders of magnitude more spores can be directly deposited within the target tumor to overcome this problem. Additionally, intratumoral injection of spores may also have advantages over other conventional forms of local therapy, such as surgery and radiotherapy. Theoretically, *C. novyi*-NT therapy could result in the precise, microscopic excision of neoplastic cells from tumors without the need to excise a margin of normal tissue. Intratumoral injection of *C. novyi*-NT spores could also elicit a potent localized inflammatory response as well as an adaptive immune response against tumor cells (20). On the basis of this reasoning, we investigated the safety and efficacy of intratumorally injected *C. novyi*-NT spores in a preclinical animal model as well as in a comparative study of dogs with spontaneously occurring cancers. We also report the first-in-human data from a patient treated with intratumorally injected *C. novyi*-NT spores.

RESULTS

Precise tumor excision and prolonged survival in rats

High-grade gliomas exhibit notable histopathological variability, with extensive regions of hypoxia and necrosis. Although this tumor type generally does not metastasize, its complexity along with the sheltered location within the central nervous system has made this cancer one of the most difficult to treat. Complete surgical excision is nearly always impossible because of anatomical restrictions and the infiltrative growth pattern leading inexorably to tumor recurrences. Gliomas, therefore, seemed to represent a tumor type for which local injection of *C. novyi*-NT spores could be therapeutically useful. To evaluate this possibility, we orthotopically implanted F98 rat glioma cells engineered to express luciferase into 6-week-old F344 Fisher rats, resulting in locally invasive tumors that were rapidly fatal (fig. S1A). Stereotactic intratumoral injection of *C. novyi*-NT spores into the tumors of these rats resulted in their germination within 24 hours and a rapid fall in luciferase activity, an indicator of tumor burden, within 48 hours (fig. S1, B and C). *C. novyi*-NT germination was demonstrated by the appearance of vegetative forms of the bacterium. Strikingly, *C. novyi*-NT precisely localized to the tumor, sparing adjacent normal cells only a few microns away (Fig. 1, A and B). Moreover, these vegetative bacteria could be seen to specifically grow within and concomitantly destroy islands of microinvasive tumor cells buried within the normal brain parenchyma (Fig. 1, C and D). This bacterial treatment led to a significant survival advantage in this extremely aggressive rat model (fig. S1A; $P < 0.0001$). Brain edema as a result of *C. novyi*-NT germination was common and medically managed. Abscess formation in the brain was not observed in the syngeneic rat model with appropriate use of antibiotics. Abscess formation, however, is a potential side effect of the therapy, which could develop in human patients and would necessitate neurosurgical abscess excision and drainage, a routine clinical procedure. Regardless, given the dismal prognosis of high-grade gliomas, the benefits of *C. novyi*-NT treatment might outweigh associated potential risks.

Canine soft tissue sarcomas resemble human tumors

Preclinical animal studies of anticancer agents often do not recapitulate the observed effects in people. In companion dogs, however, clinically used therapeutic agents induce similar toxicities and effects as found in people (23). Studies of investigational therapies in companion dogs can represent a crucial bridge between preclinical animal studies and human clinical studies. In particular, canine soft tissue sarcomas are an excellent model because they are common in many breeds of dogs and have clinical and histopathological features remarkably similar to those of human soft tissue sarcomas (23, 24). In addition, the superficial location of many soft tissue sarcomas allows for rapid assessment and management of therapy-related abscess formation.

Recent advances in genomics have expanded our knowledge of cancer genetics in people and led to recent evidence of a link between mutational burden, tumor immunogenicity, and response to immunotherapies such as antibodies to programmed cell death 1 (PD-1) and programmed cell death ligand 1 (PD-L1) (25). However, comparatively little is known about the genetic landscape of canine cancers. Because *C. novyi*-NT has been shown to induce a potent antitumor immune response (20), we sought to determine whether canine soft tumor sarcomas were genetically similar to those of humans and, as such, would be a suitable comparative model. Therefore, we sequenced the exome of tumor and matched normal DNA from 10 dogs with soft tissue sarcomas (seven peripheral nerve sheath tumors, one fibrosarcoma, one myxosarcoma, and one synovial cell sarcoma) participating in the comparative study (table S1). This analysis involved the interrogation of 30,194 nominal genes comprising 32.9 Mb of DNA. On average, 16.2 gigabases (Gb) (range, 8.1 to 23.3 Gb) of generated sequence were mapped to the genome, and 92.2% of bases in the targeted regions were covered by at least 10 unique reads in the tumor DNA. Similarly, an average of 16.2 Gb (range, 14.6 to 19.7 Gb) of sequence was mapped to the genome in normal DNA, with 93.6% of targeted bases covered by at least 10 unique reads. Average coverage for each targeted base in the tumor was 158-fold (range, 73 to 227-fold) and 151-fold in the matched normal samples (range, 130- to 178-fold).

Using stringent analysis criteria, we identified 156 somatic mutations and 28 somatic copy number alterations among the 10 soft tissue sarcomas (tables S2 and S3). The range of somatic mutations was 0 to 95 with a mean of 16 per tumor. Mutation prevalence in the soft tissue sarcomas was low, averaging 0.47/Mb (range, 0.00 to 2.89/Mb). Excluding one sample outlier, with 95 somatic alterations, there was a mean prevalence of 0.21 mutations/Mb (range, 0.00 to 0.61/Mb) (table S1), similar to estimates of the mutation rate in human pediatric rhabdoid tumors (26) and other soft tissue sarcomas (27). The most common type of somatic alteration was a missense mutation, with a preponderance of C to T (45.5%) and G to A transitions (34.0%; tables S4 and S5). Amplifications and deletions were less common, with an average of three per tumor (range, of 0 to 17) (table S1). Seven of the 10 canine soft tissue sarcomas harbored no amplifications or deletions.

Single-base substitutions were identified in three tumor suppressor genes that are frequently mutated in human tumors (*NF1*, *MLL3*, and *PTCH1*). Additionally, *MDM4*, an oncogene that has been shown to be amplified but not point-mutated in human cancers, was found to

be amplified (but not point-mutated) in one canine tumor (26, 28–30). The only genes mutated in more than one tumor were *ATP7B* (missense mutations in two tumors) and *AIG1* (amplified in two tumors). Mutations in ATP7B were also found in a human liposarcoma (27). Twenty-two of the 184 somatic alterations in canine tumors occurred in genes previously shown to be mutated in human soft tissue sarcomas (table S6). As our analyses encompassed a number of soft tissue sarcoma histiotypes, larger studies of soft tissue sarcomas in both species will be required to determine whether these represent driver mutations that signify important, conserved tumorigenic pathways. Regardless, the genetic landscapes of canine tumors were similar to those of humans in terms of the numbers of genetic alterations and spectrum of mutations. Specifically, they exclude the possibility that the canine tumors have a very large number of mutations that might make them more likely to mount an immune response than analogous tumor types in humans.

Robust and reproducible responses in spontaneous canine tumors

To investigate the safety and efficacy of intratumoral injection of *C. novyi*-NT spores, we performed a comparative study in 16 dogs with spontaneously occurring solid tumors (Table 1). Each dog received at least one cycle of *C. novyi*-NT spore treatment, defined as a single intratumoral injection of 1×10^8 *C. novyi*-NT spores into one target tumor. Dogs received up to four cycles of treatment with a 1-week interval between cycles. Treated dogs were followed for at least 90 days after the first intratumoral injection.

Nine neutered males, six neutered females, and one intact male were enrolled in the study (table S7). The mean weight of dogs was 29.4 kg (range, 8.1 to 44.3 kg), and their mean age was 10.9 years (range, 7.2 to 14.3 years). Thirteen dogs had a histomorphic diagnosis of soft tissue sarcoma (eight peripheral nerve sheath tumors, one fibrosarcoma, one myxosarcoma, one rhabdomyosarcoma, and one synovial cell sarcoma), and one each had a diagnosis of osteosarcoma, malignant melanoma, and mast cell tumor. Of the 13 soft tissue sarcomas, six peripheral nerve sheath tumors were available for immunohistochemistry. All six were positive for S100 and negative for smooth muscle actin, confirming the histiomorphic diagnosis. Seven of the tumors were grade I, five were grade II, and four were grade III. Eight dogs had previous surgical therapy for their cancers.

All dogs received at least one cycle of treatment, with 53 cycles given of a maximum of 64 planned. Most dogs, 10 of 16, received the intended four cycles. For dogs showing early tumor responses, toxicity, or PD after the first cycle, subsequent cycles were stopped (Table 1). In general, adverse events were mild in severity (>90% grade I or II) and were consistent with local infection at the *C. novyi*-NT spore injection site, including fever (17 incidents), tumor inflammation (12 incidents), tumor abscess (10 incidents), anorexia (9 incidents), and lethargy (6 incidents) (table S8). Clinical signs of an inflammatory response at the injected target lesion site were observed in 14 of 16 dogs (87.5%), including tumor inflammation (12 of 14), tumor abscess (7of 14), tumor pain (5 of 14), and tumor discharge (4 of 14) (Table 2).

Dogs were evaluated for best response on or after day 21 of the study. Two of 16 dogs, 04-R04 and 04-R08, could not be evaluated for responses because the injected tumors were surgically resected before day 21. Dog 04-R04 had a humeral osteosarcoma that experienced

robust germination 2 days after the first intratumoral injection of *C. novyi*-NT, and because of the deep location of the tumor, amputation was performed on day 21 for abscess management. Dog 04-R08 had a peripheral nerve sheath tumor of the medial aspect of the hind paw and received three cycles of treatment before amputation on day 15 for management of PD. Fourteen of 16 dogs were evaluated for responses to treatment. Three had CRs to therapy, three had partial responses (PRs), five had stable disease (SD), and three had PD. The objective response rate for treatment was 37.5% (6 of 16 dogs; 95% confidence interval, 15.2 to 64.6%). Tumor abscesses and responses occurred after one to four cycles of treatment. Dog 11-R01 experienced a PR after a single cycle, 04-R03 had a CR after three cycles, and dogs 04-R02 and 04-R05 had PRs after four cycles, whereas 04-R01 and 04-R06 had CRs after four cycles. Figures 2 and 3 show representative changes in dogs with PR (11-R01) and CR (04-R03), respectively. Resolution of abscesses occurred with surgical management in three of six dogs experiencing an objective response. In these cases, debridement occurred an average of 22 days after the first cycle of treatment. In dog 04-R02, tumor response was assessed before an owner elected amputation for wound management. In dogs 04-R03 and 11-R02, tumor response was assessed after wound debridement. Debrided tissue was available for histopathological analysis in dogs 04-R02 and 04-R03, which demonstrated extensive necrosis and inflammation of the tumor, with numerous Gram-positive bacilli morphologically consistent with *Clostridium* spp. In dog 04-R02, no viable tumor cells were present at the tumor margin. In dog 04-R03, rare scattered tumor cells were observed. However, given the active nature of *C. novyi*-NT–related abscess formation and subsequent immune infiltration and wound healing, it is difficult to speculate on their eventual fate if debridement had not occurred. Regardless of debridement, wound healing was uneventful and complete after 2 to 4 weeks. In addition to surgical management, three of six dogs that had an objective response received antibiotics (ampicillin, amoxicillin, and metronidazole) and analgesics (opioids, tramadol, and nonsteroidal anti-inflammatory drugs) during the course of the study. Overt abscess formation, however, was not always observed before an objective response. Dogs 04-R01 and 04-R06 received four cycles of treatment, with tumor inflammation, but not abscess formation, observed at the day 21 study visit. CRs were noted on day 42 (unscheduled visit) and day 60 study visits in these two dogs, respectively. Three of the six dogs that experienced either CR or PR had a long-term response (fig. S2). In the remaining three dogs, mean time to progression was 106 days (range, 60 to 169 days).

Rapid local tumor destruction in the first human patient

The promising outcomes and favorable risk/benefit profile of *C. novyi*-NT treatment in the comparative canine trial, in conjunction with the results observed in rats, provided a rationale for attempting this treatment in humans. Accordingly, a phase 1 investigational study in human patients with solid tumors that were either refractory to standard therapy or without an available standard therapy was initiated (NCT01924689). The first patient enrolled in this trial is reported herein: a 53-year-old female diagnosed with a retroperitoneal leiomyosarcoma in August 2006. The patient had undergone several surgical resections and received multiple chemotherapy and radiotherapy treatments. However, her disease progressed, with metastatic lesions present in her liver, lungs, peritoneum, and soft tissue in the right shoulder and adjacent right humerus.

Treatment was performed with the planned starting dose of 1×10^4 *C. novyi*-NT spores injected into the patient's metastatic right shoulder tumor with an 18-gauge multipronged needle (day 0). On day 1, the patient experienced mild right-shoulder pain extending to the scapula, which responded to tramadol and acetaminophen. On day 2, her pain required intravenous patient-controlled analgesia with hydromorphone, her leukocyte count increased to 18,300/μl, and she developed fever with a maximum temperature of 39.2°C. On day 3, the pain in the patient's right shoulder and scapula was difficult to control. Her maximum temperature was 37.8°C. The CT scan of the right upper extremity demonstrated extensive tumor destruction with gas in the soft tissue and bony component of the tumor (Fig. 4A). The permeative pattern of gas was consistent with extensive necrosis of the proximal humerus. A CT-guided aspirate of her tumor revealed *C. novyi*-NT growth under anaerobic culture conditions. The patient was then started on antibiotics (piperacillin/tazobactam, metronidazole, and vancomycin), and her fever abated shortly thereafter. On day 4, magnetic resonance imaging (MRI) of the right upper extremity demonstrated markedly diminished enhancement confined to the tumor mass compared to baseline (Fig. 4, B and C). Biopsies from the tumor showed many Gram-positive bacteria and an absence of viable tumor cells (Fig. 5). At the time of the biopsies, a percutaneous drain was placed within the tumor abscess to drain fluid and debris. The patient remained afebrile, and her leukocyte count gradually normalized. She continued on antibiotics and was kept in the hospital for intravenous analgesia until day 20 when she was transitioned to oral analgesics. She was discharged on orally administered metronidazole and doxycycline per protocol. On day 29, a follow-up MRI demonstrated an ongoing reduction in tumor enhancement (Fig. 4D). On day 55, the patient presented with localized pain as a result of an effort–induced pathological fracture of the necrotic right proximal humerus. Subsequent partial resection of the humerus, debridement, and internal fixation with an intramedullary nail and cement spacer resulted in significant improvement in pain and an increase in range of motion. Intraoperative cultures revealed *C. novyi*-NT growth under anaerobic culture conditions. Histopathology demonstrated extensive tumor necrosis with small foci of residual tumor cells. The patient continues to be monitored and currently has a performance status of 1 on the Eastern Cooperative Oncology Group (ECOG) scale with no clinical signs of infection.

DISCUSSION

Most conventional anticancer therapies target the well-vascularized component of tumors. Yet to cure the disease, every neoplastic cell must be destroyed; any remaining cancer cells can regenerate the tumor. This principle has been dramatically illustrated in recent studies with targeted anticancer agents. Although striking remissions can be induced, the tumors nearly always recur within several months because of a tiny fraction (<0.0001%) of cells that harbor resistance mutations before therapy (31–33).

Treatment with intratumorally injected *C. novyi*-NT spores, in principle, offers a way to eradicate neoplastic cells with precision, independent of tumor-specific genetic alterations. In addition to directly killing tumor cells in their hypoxic environments, *C. novyi*-NT has been shown to induce a potent antitumor immune response, both innate and acquired, in preclinical models (20). Although there was no clear evidence to demonstrate an acquired antitumor immune response in the human patient or companion dogs, the striking

inflammatory response that was induced by intratumoral injection of *C. novyi*-NT spores provides unequivocal evidence of an innate immune response. Because *C. novyi*-NT is exquisitely sensitive to oxygen and has never been shown to germinate in normoxic areas of tumors, it is plausible that immunity (either innate or acquired) played a role in those dogs in which durable CRs were obtained. Furthermore, the first human experience with intratumorally injected *C. novyi*-NT spores resulted in a rapid and robust local antitumor response. In this case, proximity of underlying bone may have contributed to a pathological fracture that ultimately required surgery. Patient selection, however, may minimize the risk of similar complications in the future. It is important to point out that this result was produced by only 10,000 spores—a small fraction of the dose used to treat dogs or rats. As the phase 1 trial progresses, it will be interesting to see whether higher doses affect distant metastases, either directly through the spread of spores released from the local site into the circulation or through host-mediated immunity.

Comparative studies in dogs with spontaneous tumors should be incorporated into the debate about the translatability of studies in experimental animal models of cancer (24). In our view, the demonstration of therapeutic effects in spontaneous tumors of dogs can powerfully complement studies of transplanted or genetically induced tumors in preclinical animal models. This complementarity is reinforced by the genetic similarities between human and canine tumors described herein. Together, they can provide a compelling rationale for guiding studies in humans, which is particularly germane for new forms of therapy associated with significant potential toxicity, such as those with *C. novyi*-NT and other biological agents.

Although we have demonstrated that intratumorally injected *C. novyi*-NT spores produce robust, reproducible antitumor responses across several species, there are limitations to our study. Specifically, more human patients with a diverse range of tumor types will need to be treated to determine the maximum tolerated dose and tolerability of treatment, tumor-specific responses, and objective response rate. In addition, our study did not assess a host immune response and whether intratumoral injection of *C. novyi*-NT spores induced an adaptive immune response against tumor cells.

The next steps in this line of research are clear. First, it will be important to further characterize the safety and efficacy of intratumoral *C. novyi*-NT spore treatment. Second, single-agent treatments against diseases like cancer are never optimal. The effects of *C. novyi*-NT spores, at least when administered systemically, are markedly enhanced by combination with carefully chosen chemotherapeutic agents or radiation therapy (19, 34, 35). Because the mechanisms through which *C. novyi*-NT kills tumor cells do not overlap with the mechanisms of action of other forms of therapy, multimodel approaches seem particularly attractive (19). Finally, it will also be of great interest to determine whether immune checkpoint blockade can enhance the antitumor immunity expected from intratumoral *C. novyi*-NT spore treatment (20).

MATERIALS AND METHODS

Study design

The preclinical proof-of-concept study was conducted using the rat orthotopic F98 glioma model to demonstrate *C. novyi*-NT–induced infection specifically and precisely localized in the tumor lesions. Luciferase activity and Kaplan-Meier survival curves were used to assess therapeutic benefit. A comparative study in companion dogs with spontaneous solid tumors was used to bridge translation between preclinical and human studies. The experimental unit was one study dog, and each dog received up to four cycles of treatment. Placebo control, blinding, or randomization was not used in the study. Formal a priori statistical hypotheses were not planned for this comparative study. Descriptive summary statistics and analysis were provided post hoc. The human clinical trial is an ongoing open-label, nonrandomized, multicenter phase 1 study with a standard "3 + 3" dose escalation. The study was designed to (i) determine the safety profile, dose-limiting toxicities, and maximum tolerated dose of *C. novyi*-NT spores in humans with treatment-refractory solid tumor malignancies when administered as a single intratumoral injection; (ii) document preliminary antitumor activity of both the injected tumor and overall response; (iii) study the disposition of circulating *C. novyi*-NT spores; and (iv) measure the host immune and inflammatory responses associated with *C. novyi*-NT treatment.

Cell lines and tissue culture

Rat F98 glioma cell line transfected with luciferase construct via lentivirus was maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin and streptomycin.

Rat orthotopic brain tumor model

All animal experiments involving rats were approved by the Johns Hopkins University Institutional Animal Care and Use Committee. Six-week-old female F344 Fisher rats (weight, 100 to 150 g) were purchased from the National Cancer Institute. For the implantation procedure, female F344 Fisher rats were anesthetized via intraperitoneal injection of ketamine hydrochloride (75 mg/kg; 100 mg/ml ketamine HCl; Abbott Laboratories), xylazine (7.5 mg/kg; 100 mg/ml Xyla-ject; Phoenix Pharmaceutical), and ethanol (14.25%) in a sterile NaCl (0.9%) solution. F98 glioma cells (2×10^4) transfected with a luciferase construct via lentivirus were stereotactically implanted through a burr hole into the right frontal lobe located 3 mm lateral and 2 mm anterior to the bregma, as described before (36).

Tumor size was assessed via a Xenogen instrument with intraperitoneal injection of 8 mg of D-luciferin potassium salt per rat at day 12 after implantation of the tumor cells. Subsequently, 3 million *C. novyi*-NT spores, produced as previously described (18, 37), were stereotactically injected into the intracranial tumor using the same coordinates as described above. The rats were treated with intraperitoneal dexamethasone (10 mg/kg per day) for the first 2 days to minimize the risk of postoperative edema; this closely mimics the standard clinical protocol used in human patients after brain tumor surgery and biopsy. Control rats were stereotactically injected with the same volume of phosphate-buffered

saline (PBS) and treated with intraperitoneal dexamethasone (10 mg/kg per day) for the first 2 days. Animals were observed daily for any signs of deterioration, lethargy, neurotoxicity, or pain in accordance with the Johns Hopkins Animal Care and Use Guidelines. If symptoms of distress were present, supportive therapy with hydration and doxycycline (loading dose of 15 mg/kg intraperitoneally, followed by 10 mg/kg every 12 hours as maintenance) was initiated and continued for a 7-day period. If symptoms persisted and/or resulted in debilitation, moribund animals were euthanized. The effectiveness of intratumorally injected *C. novyi*-NT spores was evaluated by Kaplan-Meier survival curves, as well as remaining tumor burden on brain sections. For the latter, brains were collected postmortem, placed in formaldehyde, and embedded in paraffin for additional pathological studies. Gram-stained slides, counterstained with safranin, and hematoxylin and eosin (H&E) slides were obtained according to standard procedure guidelines.

Statistical analyses

Kaplan-Meier survival curves and luciferase count graphs were created and analyzed with Mantel-Cox and Mann-Whitney tests, respectively, using GraphPad Prism v.5.00 (GraphPad Software).

Genomic DNA isolation for sequencing

Genomic DNA from dogs participating in the comparative study of intratumorally injected *C. novyi*-NT spores was extracted from peripheral blood lymphocytes and formalin-fixed, paraffin-embedded tumor tissue using the QIAamp DNA Mini Kit (Qiagen) according to the manufacturer's protocol.

Sequencing and bioinformatic analysis

Genomic purification, library construction, exome capture, next-generation sequencing, and bioinformatic analyses of tumor and normal samples were performed at Personal Genome Diagnostics (PGDx). In brief, genomic DNA from tumor and normal samples were fragmented and used for Illumina TruSeq library construction (Illumina). The exomic regions were captured in solution using the Agilent Canine All Exon kit according to the manufacturer's instructions (Agilent). Paired-end sequencing, resulting in 100 bases from each end of the fragments, was performed using a HiSeq 2000 Genome Analyzer (Illumina). The tags were aligned to the canine reference sequence (CanFam2.0) using the ELAND algorithm of CASAVA 1.7 software (Illumina). The chastity filter of the BaseCall software of Illumina was used to select sequence reads for subsequent analysis. The ELAND algorithm of CASAVA 1.7 software (Illumina) was then applied to identify point mutations and small insertions and deletions. Known polymorphisms recorded in dbSNP131 (Single Nucleotide Polymorphism database, build 131) (CanFam2.0) were removed from the analysis. Potential somatic mutations were filtered and visually inspected as described previously (38).

Preparation and intratumoral injection of *C. novyi*-NT spores in spontaneous canine tumors

C. novyi-NT spores for use in the comparative canine study were produced as previously described (18, 37). In brief, bacteria were cultured in sporulation medium for at least 2 weeks to ensure maximum yield of mature spores. Mature spores were purified through two consecutive, continuous Percoll gradients followed by four washes and resuspensions in PBS. Sterility testing of the final product was performed by culturing product in Soybean-Casein Digest Medium and Thioglycollate Medium in accordance with Food and Drug Administration (FDA) 21CFR610.12 guidelines (Nelson Laboratories). Germination efficiency assays were performed under anaerobic conditions on Brucella agar with 5% horse blood to ensure that the spores meet preset viability criteria. Spores were packaged in sterile 1.8-ml cryovials with O-ring sealed screw caps (Simport) at a volume of 1000 μl and a concentration of 1×10^9 spores/ml. *C. novyi*-NT cryovials were stored at 2°C to 8°C. For dosing, a 0.4-ml aliquot of the stock spore solution was packaged into 0.5-ml cryovials. After dosing, the cryovials and unused *C. novyi*-NT spores were discarded according to applicable regulations for disposal of Biosafety Level 2 material.

Before intratumoral injection, spores were resuspended with a vortex mixer, mixing at maximum speed for 10 s for a total of three times before being withdrawn into a 1-ml syringe. The injection site was aseptically prepared. If available, ultrasound or CT was used to identify a necrotic region of the tumor. If a necrotic region was not identified, the injection was directed to the center of the tumor. The needle was inserted once into the predefined region, and 100 μl of spore suspension (1×10^8 *C. novyi*-NT spores) was dispensed with even pressure. The injection needle was removed slowly, and the injection site was sterilized.

Design and conduct of the comparative canine study

All animal research involving dogs was performed in compliance with applicable local, state, national, and international animal welfare regulations and adhered to the highest standards of animal care and use. Written informed consent was obtained from the owner before enrollment of each dog. The study protocol and informed consent were approved by the Animal Clinical Investigation (ACI) Animal Care and Use Committee to ensure the ethical care of dogs enrolled in the study.

Client-owned dogs with spontaneous tumors received up to four cycles of intratumoral *C. novyi*-NT spores. A cycle consisted of one intratumoral injection of 1×10^8 *C. novyi*-NT spores (in 100 μl of PBS) into one target tumor. Cycles of intratumoral *C. novyi*-NT spores were typically 1 week apart. No placebo control or masking was used. Dogs were followed for 90 days, and extended follow-up for disease progression and survival was warranted when available. Early withdrawal from the study was allowed for toxicity or PD.

Dogs were enrolled at multiple sites participating in the ACI oncology network. Treatment, management, and study evaluations were overseen by board-certified veterinary oncologists. Enrollment was offered to client-owned dogs with spontaneous solid tumors, with a preference for soft tissue sarcomas that had failed standard therapy or whose owner(s) had

declined such therapy. Participation was restricted to tumor-bearing dogs with a target lesion having a longest diameter between 1 and 7 cm. Dogs with tumors located in areas where abscess development would be catastrophic (for example, nasal tumors that extended into the brain or significant pulmonary metastatic disease) were excluded from the study. Dogs with evidence of an active bacterial infection requiring systemic antibiotic therapy within 7 days or cancer therapy (chemotherapy, radiation therapy, and immunotherapy) within 21 days of *C. novyi*-NT spore treatment were ineligible. Dogs were required to have a performance score of 0 or 1 (table S9) and to be available for the full duration of the study for enrollment. Concurrent use of anticancer agents and participation in other clinical trials were prohibited.

Dogs were hospitalized for 4 days after the first intratumoral injection of *C. novyi*-NT and for 24 to 48 hours after subsequent intratumoral injections for observation at the discretion of the investigator. Intravenous fluid therapy was administered after each intratumoral injection of *C. novyi*-NT spores for 2 hours at a rate of 4 ml/kg per hour. Subcutaneous fluid therapy was administered for 4 days after each intratumoral injection of *C. novyi*-NT spores at a rate of 20 ml/kg per day. Dogs were closely monitored for 6 hours after each intratumoral injection of *C. novyi*-NT spores.

Study evaluations were undertaken as described in table S7. Prescreening evaluations were conducted 1 to 14 days before the first cycle of intratumoral *C. novyi*-NT spore treatment. Dogs were monitored periodically on both an inpatient and outpatient basis during the study. Laboratory samples were taken as defined in table S7 and included a complete blood count, serum biochemistry, prothrombin time, partial thromboplastin time, and urinalysis. Imaging was performed at screening and included regional CT, thoracic radiography, and abdominal ultrasonography. Additional imaging was conducted during the study at the investigator's discretion.

Adverse events were evaluated, where possible, using the Veterinary Co-operative Oncology Group—Common Terminology Criteria for Adverse Events (VCOG-CTCAE) v1.0 (39), with terminology from the Veterinary Dictionary for Drug Related Affairs (VeDDRA) rev.4 (40). Terminologies for adverse events related to *C. novyi*-NT germination (target lesion reactions) are defined in table S10. Clinical observations without appropriate VeDDRA or target lesion reaction terminology were classified separately as uncoded signs (table S11). Relationship to *C. novyi*-NT therapy was determined by the reporting investigator.

Longest diameter tumor measurements of the target (injected) lesion were made on days 0, 7, 14, 21, 60, and 90 after treatment (table S7). Nontarget and new lesions were recorded but not measured. The best overall target response was evaluated on or after the day 21 study visit: CR was defined as the complete disappearance of the target lesion; PR was defined as at least a 30% decrease in the longest diameter of the target lesion; and PD was defined as at least a 20% increase in the longest diameter of the target lesion or the appearance of new nontarget lesions. SD was defined as insufficient decrease or increase in the longest diameter of the target lesion to qualify as CR, PR, or PD. In the case of *C. novyi*-NT–related

abscesses, medical or surgical debridement of necrotic tissue was at the discretion of the investigator.

Evaluation of surgical samples and necropsies was conducted by board-certified veterinary pathologists. Tissue specimens were fixed in 10% neutral buffered formalin and embedded in paraffin. Slides stained with H&E and or Gram-stained slides were prepared for evaluation according to standard procedure guidelines. For immunohistochemistry, formalin-fixed, paraffin-embedded tumor tissue was sectioned at 5 μm, deparaffinized in xylene, and rehydrated through graded alcohols. Antigen retrieval was done by heating slides in unmasking solution for 10 min (catalog no. H-3300, Vector Laboratories). All slides were then incubated in 10% blocking serum from the animal species from which the secondary antibody was made, in PBS for 10 min at room temperature. Primary antibodies S100 (catalog no. Z0311, Dako) and anti–smooth muscle actin (catalog no. M0851, Dako) were used at 1:100 for 60 min at room temperature (41, 42). Secondary antibodies (catalog nos. BA-1000 and BA-2000, Vector Laboratories) labeled with 3,3′-diaminobenzidine were used at 1:500 for 30 min at room temperature. Sections were incubated with ABC reagent (Vector Laboratories) and counterstained with hematoxylin. Tumor grade was assigned based on published criteria (43–46).

Phase 1 human clinical trial of intratumorally injected *C. novyi*-NT spores

An open-label, nonrandomized, multicenter phase 1 safety study of a single intratumoral injection of *C. novyi*-NT spores is currently ongoing in patients with treatment-refractory solid tumors. The clinical study protocol was reviewed and approved by the Institutional Review Board of each participating institution, and all regulatory steps were performed under the guidance of the FDA (http://www.clinicaltrials.gov; NCT01924689). All patients were required to sign a written informed consent form before inclusion in the study.

The primary objective of this phase 1 study is to determine the safety profile, dose-limiting toxicities, and maximum tolerated dose of intratumorally injected *C. novyi*-NT. In addition, the antitumor activity of intratumoral *C. novyi*-NT was explored.

Preparation and intratumoral injection of *C. novyi*-NT spores in the phase 1 study

C. novyi-NT spores were manufactured and formulated by Omnia Biologics Inc. The clinical supply of *C. novyi*-NT spores was packaged in a single-use 2-ml sterile and pyrogen-free, type I borosilicate glass vial with a rubber stopper and aluminum seal with a tamper-resistant cap at a concentration of 8.52×10^8 spores/ml suspended in 1.0 ml of sterile PBS. Vials were stored between 2° to 8°C in a controlled temperature environment under constant temperature monitoring.

After a patient was enrolled in the trial, one vial was shipped to the study site. Further preparation of *C. novyi*-NT was required and occurred on the same day of the intratumoral injection. Dilution of the concentrated spore suspension was performed in a designated biological safety cabinet using sterile saline (0.9%) infusion bags of appropriate size to achieve the required dose based on the assigned cohort. The injection volume (3 ml) was

then withdrawn from the saline bag and injected under radiographic guidance. *C. novyi*-NT spores were injected with an 18-gauge multipronged needle (Quadra-Fuse, Rex Medical).

Design and conduct of human clinical trial

The study was conducted with a standard 3 + 3 dose-escalation design. To enroll on the study, patients must have been diagnosed with an advanced solid tumor malignancy, with a target tumor that was palpable and clearly identifiable under ultrasound or radiographic guidance. In addition, the target lesion must have had a longest diameter ≥1 cm, have been measurable as defined by RECIST (Response Evaluation Criteria In Solid Tumors) 1.1 criteria, and have been amenable to percutaneous injection of *C. novyi*-NT spores.

The eligibility criteria included a history of a treatment-refractory solid tumor malignancy, at least 18 years of age, an ECOG performance status of ≤2, an ability to stay within 45 min of an emergency room, and having a caregiver for 28 days after intratumoral injection. The exclusion criteria included pregnancy; a primary brain malignancy or brain metastases; clinically significant ascites or clinical evidence or history of portosystemic hypertension or cirrhosis; a Glasgow Coma Score <15; a serum creatinine level >1.5× the upper limit of normal, chronic renal failure that required hemodialysis or peritoneal dialysis; an oxygen saturation (Sp02) <95% (room air); a mean arterial blood pressure <70 mmHg; a platelet count ≤100,000/mm^3; a hemoglobin <9.0 g/dl; an absolute neutrophil count <1000 /mm^3; clinically significant pleural effusion, pericardial effusion, circumferential pericardial effusion, or any effusion that was greater than 1.0 cm at any location around the heart; a need for ongoing treatment with an immunosuppressive agent; a history of solid organ transplantation; and systemic or localized infection.

Eligible patients were admitted and enrolled into a dose cohort. Patients remained hospitalized after *C. novyi*-NT spore injection and were observed for 8 days. Patients returned to the clinical site for routinely scheduled follow-up visits, during which time assessments of safety and efficacy were performed.

Clinical response and progression was evaluated using RECIST version 1.1. Objective responses were measured by serial CT or MRI scans of the injected tumor, as well as distant metastases (up to five lesions).

Public health implications of *C. novyi*-NT therapy

C. novyi is a spore-forming, Gram-positive, obligate anaerobe commonly found in soil (47). *C. novyi*-NT was derived from a strain of *C. novyi* by deleting a toxin gene necessary for systemic pathogenicity (18). Extensive preclinical evaluation of *C. novyi*-NT has failed to demonstrate germination of *C. novyi*-NT spores in nontumor tissue (22). In addition, whereas *C. novyi*-NT spores are resistant to oxygen, vegetative *C. novyi*-NT is highly sensitive to oxygen (22). As such, vegetative *C. novyi*-NT is not viable outside the hypoxic tumor micro-environment. Although the risk to health of the public with *C. novyi*-NT therapy is thought to be minimal, precautions for the handling of *C. novyi*-NT and disposal of *C. novyi*-NT–contaminated material were instigated. For the canine comparative study, protective gloves were worn when handling feces, urine, saliva, or tumor discharge from

treated dogs; stool was placed into a sealed plastic bag and disposed with general household waste; items soiled with urine, stool, or tumor discharge were washed separately from other laundry. For the human clinical study, standard protective gowns and gloves were required for healthcare providers.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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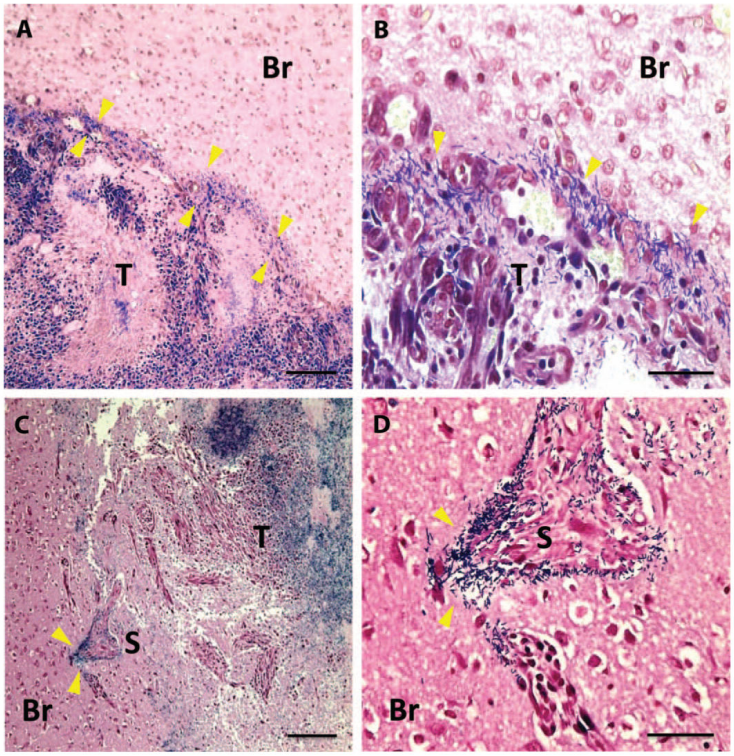

Fig. 1. Germinated *C. novyi*–NT bacteria within microscopic rat brain tumor lesions

Gram stain showed vegetative *C. novyi*–NT bacteria (yellow arrowheads) localized in tumor (T) and stellate microinvasion (S) but not in normal brain tissue (Br) from F344 Fisher rats. (**A**) Interface of tumor and normal brain; scale bar, 30 μm. (**B**) Interface of tumor and normal brain; scale bar, 10 μm. (**C**) Interface of normal brain, tumor, and stellate microinvasion of neoplastic tissue; scale bar, 30 μm. (**D**) *C. novyi*–NT germination evident in stellate microinvasive lesion; scale bar, 10 μm.

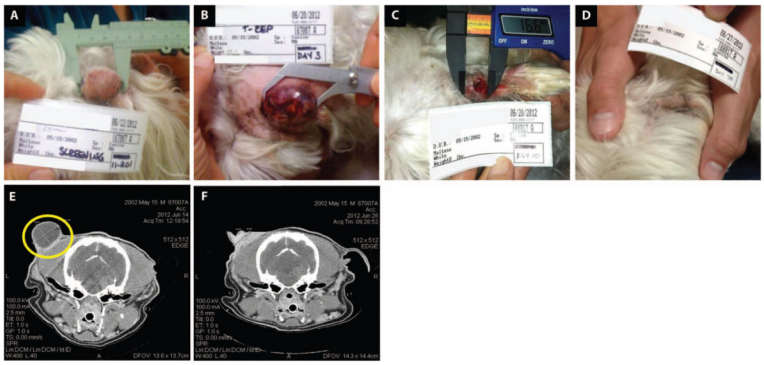

Fig. 2. Photographic and computed tomography images from dog 11-R01 showing a PR to *C. novyi*-NT therapy

Images span pretreatment to day 70 after first intratumoral dose of *C. novyi*-NT spores. (**A**) Pretreatment image of the peripheral nerve sheath tumor. (**B**) Abscess formation on day 3 of the study, with extent confined to tumor. (**C**) Medical debridement after spontaneous abscess rupture and discharge of necrotic and purulent material allowed healing by second intention. (**D**) The wound had healed completely by day 70 of the study, and 77.6% reduction in the largest diameter of the tumor was noted. (**E**) Pretreatment computed tomography (CT) image, taken 4 days before first treatment showed extent of tumor (yellow circle) at the intersection of the pinna and cranium. (**F**) Posttreatment CT image on day 10 of the study showed almost complete debulking of tumor.

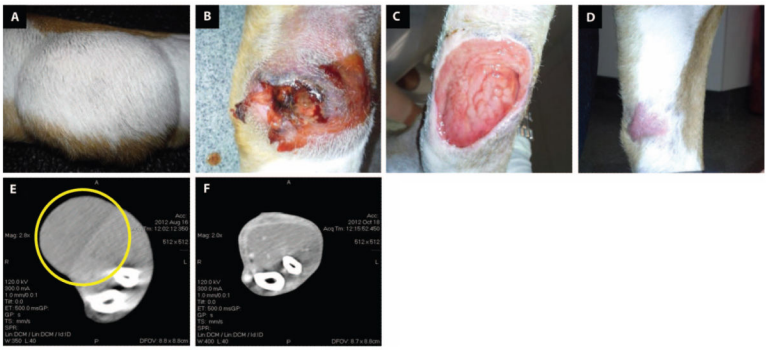

Fig. 3. Photographic and CT images from dog 04-R03 showing a CR to *C. novyi*-NT therapy

Images span pretreatment to day 60 after first intratumoral dose of *C. novyi*–NT spores. (**A**) Pretreatment image of the soft tissue sarcoma. (**B**) Tumor localized abscess formed on day 15 of the study, 1 day after a third dose of *C. novyi*–NT spores. (**C**) Tumor debulking was complete by day 27 of the study, and healthy granulation tissue had formed. (**D**) The wound had healed completely by day 60 of the study, and no residual tumor was noted (CR). (**E**) Pretreatment CT image, taken 5 days before first treatment, showing extent of tumor (yellow circle) on antebrachium. (**F**) Posttreatment CT image on day 62 of the study showing complete loss of tumor mass.

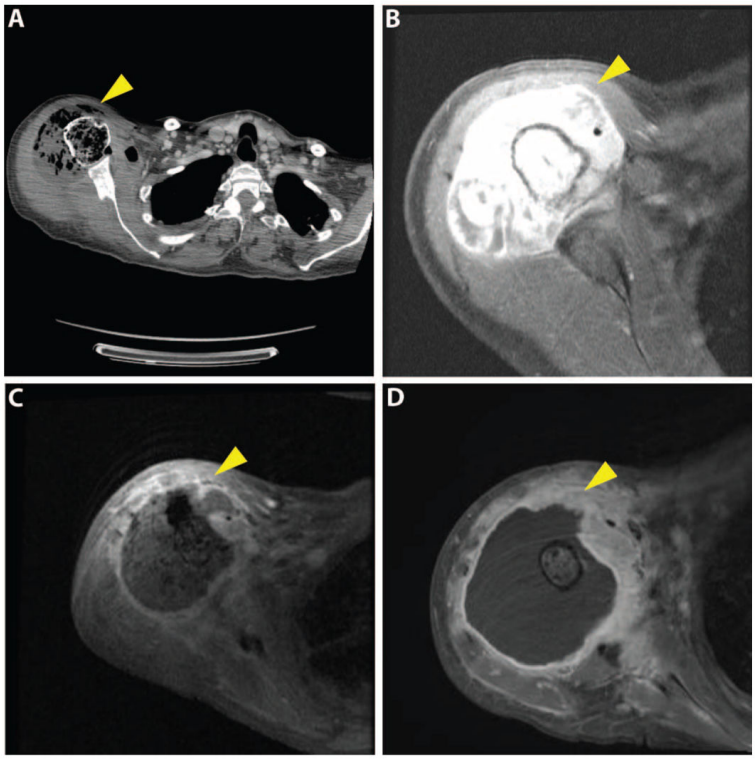

Fig. 4. CT and MRI images from the human patient

(**A**) Posttreatment CT with contrast on day 3 demonstrating evidence of intra- and extramedullary air collection. (**B**) Pretreatment MRI (T_1 with gadolinium contrast) of the right upper humerus showing a contrast enhancing mass involving the soft tissue and possibly adjacent bone. (**C**) Posttreatment MRI on day 4 demonstrating diminished contrast enhancement in the tumor mass compared to baseline. (**D**) Posttreatment MRI on day 29 showing a homogeneous non-enhancing mass consistent with ongoing necrosis. Tumor is highlighted with yellow arrowheads.

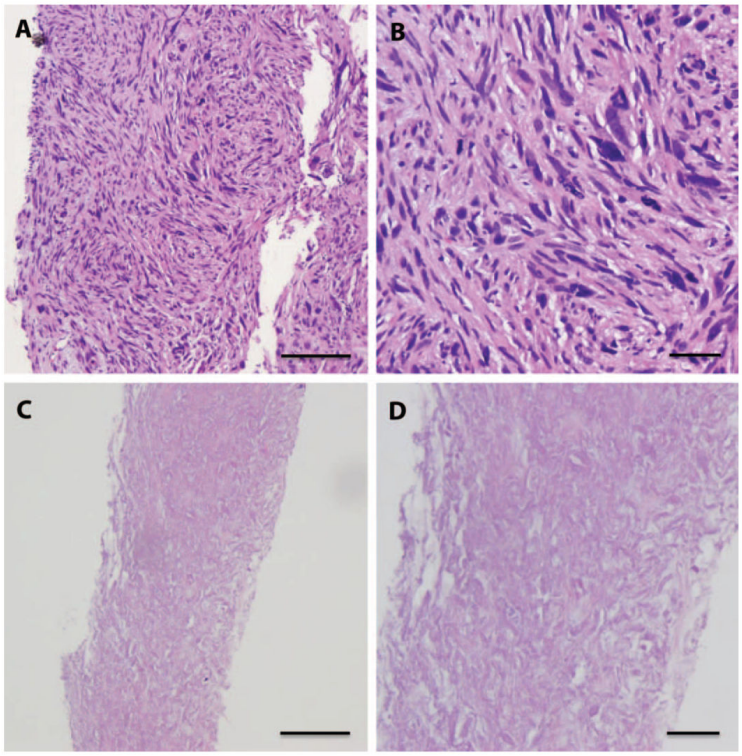

Fig. 5. Extensive tumor necrosis in the human patient treated with *C. novyi*-NT spores

(**A** and **B**) Pretreatment tumor biopsy showing viable tumor (leiomyosarcoma) cells; scale bars, 100 and 30 μm, respectively. (**C** and **D**) Posttreatment tumor biopsy, 4 days after intratumoral injection of *C. novyi*-NT spores, showing extensive necrosis of tumor cells; scale bars, 100 and 30 μm, respectively.

Table 1

Characteristics of the dogs in the comparative canine study.

Case ID	Sex*	Breed	Age (years)	Body weight (kg)	Tumor type†	Grade‡	Location	Longest diameter§ (mm)	Previous treatment‖	Number of *C. novyi*-NT treatment cycles¶
01-R02	FN	Border Collie	14.3	21.7	STS—PNST	II	Left flank	43	None	4
04-R01	MN	Golden Retriever	7.9	34.0	STS—PNST	II	Right maxilla	15	Surgical	4
04-R02	MI	Golden Retriever	12.0	38.8	STS—PNST	I	Right lateral metacarpus	46	Surgical	4
04-R03	MN	Boxer	9.6	29.4	STS—PNST	I	Left medial antebrachium	56	None	3^{TR}
04-R04	FN	St. Bernard	11.7	31.0	OSA_c	III	Right proximal humerus	ND	Surgical	1^{AE}
04-R05	MN	Shetland Sheepdog	14.0	13.4	STS—RMS	III	Right cranial antebrachium	45	Surgical and *C. novyi*-NT spores IV	4
04-R06	FN	Labrador Retriever	11.6	24.3	MCT	III	Right hindlimb digit	23	None	4
04-R08	FN	Shepherd	7.2	28.9	STS—PNST	I	Right medial hindlimb paw	65	Surgical	3^{PD}
10-R01	MN	Golden Retriever	13.7	33.6	OMM	III	Left mandible	27	Surgical	2^{AE}
10-R02	MN	Pit Bull Terrier	10.0	43.6	STS—PNST	I	Right flank	53	Surgical	4
11-R01	MN	Maltese	11.1	8.1	STS—PNST	II	Left pinna	28	Surgical	1^{TR}
11-R02	FN	Labrador Retriever	12.2	30.3	STS—PNST	II	Left stifle	43	None	3^{IV}
11-R04	MN	Husky	10.3	44.3	STS—FBS	I	Right forelimb paw	29	None	4
16-R02	MN	Labrador Retriever	9.8	36.8	STS—MXS	I	Left lateral thigh	91	Surgical	4
16-R03	FN	Shepherd	10.8	20.8	STS—SCS	I	Left forelimb paw	53	Surgical	4
26-R01	MN	Labrador Retriever	7.9	30.8	STS—RMS	II	Right forelimb paw	24	None	4

*FN, female neutered; MN, male neutered; MI, male intact.

†STS, soft tissue sarcoma; PNST, peripheral nerve sheath tumor; OSAc, chondroblastic osteosarcoma; RMS, rhabdomyosarcoma; MCT, mast cell tumor; OMM, oral malignant melamona; FBS, fibrosarcoma; MXS, myxosarcoma; SCS, synovial cell sarcoma.

[‡] Grading based on published criteria (*43*–*46*): I, low grade; II, intermediate grade; III, high grade; NA, not assessed.

[§] Longest diameter at time of first *C. novyi*-NT injection (day 0). ND, unmeasurable because of location.

[//] 04-R05, previous *C. novyi*-NT therapy with a single intravenous injection of 1×10^7 spores/m^2 437 days before the first intratumoral injection of *C. novyi*-NT spores.

[¶] A treatment cycle consisted of one intratumoral injection of 1×10^8 *C. novyi*-NT spores. Dogs received up to four cycles, typically 1 week apart. Reason for receiving fewer than four treatment cycles given in superscript: TR, tumor response; AE, adverse event; PD, progressive disease; IV, fourth dose given intravenously.

Table 2

Summary of clinical responses to intratumoral *C. novyi*-NT therapy.

Case ID	Clinical evidence of germination*	Clinical response†
01-R02	Tumor inflammation, skin disorder, and discharge	PD
04-R01	Tumor inflammation and pain	CR
04-R02	Tumor inflammation and abscess	PR
04-R03	Tumor inflammation, consistency change, discharge, and tumor pain	CR
04-R04	Tumor inflammation and pain	NE
04-R05	Tumor inflammation, consistency change, skin disorder, and pain	PR
04-R06	Tumor inflammation, abscess, and discharge	CR
04-R08	Tumor abscess and discharge	NE
10-R01	—	PD
10-R02	Tumor inflammation, abscess, and pain	SD
11-R01	Tumor inflammation and abscess	PR
11-R02	Tumor inflammation	SD
11-R04	Tumor abscess and consistency change	SD
16-R02	Tumor inflammation	PD
16-R03	Tumor inflammation and abscess	SD
26-R01	—	SD

*Clinical evidence of *C. novyi*-NT germination on or after day 0 of the study includes target lesion reactions (table S10).

†Best response of the target lesion, as defined by the study protocol, after day 21 of the study. NE, not evaluated for response on or after day 21 of the study.