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# Clostridium sporogenes-mediated intratumoral production of cytokines, combined with radiotherapy, reduces tumor growth and prolongs survival

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# Abstract

Cancer immunotherapy transformed cancer treatment, as reflected by the continued development of immunotherapeutics for multiple cancer types. Despite successes, adverse effects due to systemic exposure remain a major challenge, potentially tackled using tumor-targeted strategies. Most solid cancers contain necrosis, associated with poor prognosis. This can be exploited by anaerobic bacteria, which selectively colonize necrotic regions, thereby eliciting anticancer effects. We “armed” *Clostridium sporogenes-nontoxic (NT)* to express immunotherapeutic genes serving as intratumoral Trojan horse. Genes encoding murine interleukin-2 (mIL-2) or Granulocyte-Macrophage Colony-Stimulating factor (mGM-CSF) were chromosomally inserted. Genetic insertions did not affect germination, growth, or colonization properties of clostridial strains. ELISA results showed successful secretion of mIL-2 and mGM-CSF up to ~3400 and ~560 pg/ml, respectively. Secreted mIL-2 increased T-cell proliferation 6-fold, while secreted mGM-CSF increased NF-κB expression ~2-fold compared to control.*In vivo* combined with radiotherapy (RT), significant tumor growth inhibition was observed with *C. sporogenes-NT* ($p<0.01$) +RT ($p<0.01$), *C. sporogenes-NT-mIL-2* ($p<0.0001$) +RT ($p<0.0001$), and *C. sporogenes-NT-mGM-CSF*+RT ($p<0.01$). Treatment with clostridial strains significantly prolonged survival ($p<0.001$) up to tumor cure upon *C. sporogenes-NT-mIL-2* +RT treatment, without causing toxicity. Our data reports successful secretion of active immunotherapy by *C. sporogenes-mIL-2*and *C. sporogenes-NT-mGM-CSF,* resulting in prolonged survival *in vivo*.


# Introduction

Despite major advances in research, cancer remains a leading cause of death worldwide. The hallmarks of cancer “avoiding immune destruction” and “tumor promoting inflammation” emphasize the role of immunity in tumor progression [1]. During the 18th century, Dr Wiliam Coley reported that intratumoral infection with certain bacteria contributed to cancer regression. His bacterial approach is suggested to be the first anticancer immunotherapeutic strategy, i.e. (re-) activating the host immune system to fight cancer [2]. Over the past decades, immunotherapy has revolutionized anticancer treatment e.g. with the use of cytokines to activate the immune system, however, amongst other challenges, development of immune-related adverse events (irAEs) limits their potential. Currently, only a few cytokines are licensed for clinical use in only a limited number of cancer types, among which interleukin (IL)-2 and granulocyte-monocyte colony-stimulating factor (GM-CSF) [3]. High-dose IL-2 therapy has shown therapeutic effects and significant improvement in disease-free survival in a fraction of patients with metastatic melanoma or metastatic renal cell carcinoma [4, 5]. However, systemic IL-2 exposure has led in a subset of cancer types to a broad spectrum of adverse events, such as pulmonary edema, cardiomyopathy, hepatitis, cholecystopathy, pancreatitis, colitis, thyroiditis, and encephalopathy, potentially caused by increased capillary permeability [6]. GM-CSF is FDA approved in combination with chemotherapy where it functions as an immunomodulatory adjuvant. While systemic administration of GM-CSF is generally well tolerated, adverse events still occur [7, 8]. Furthermore, depending on the cancer type and dosing, GM-CSF can have protumor or antitumor effects resulting in a narrow therapeutic window [9].

Direct drug delivery is hypothesized to overcome irAEs, and several methods have been described for tumor-specific immunotherapy, among which drug-delivery by using anticancer bacteria which specifically colonize tumors. Although no tumor regression was observed, significant higher intratumoral IL-2 levels have been found upon intravenous administration of *Escherichia coli Nissle 1917* engineered to produce murine IL-2 [10]. *Salmonella typhimurium* excreting Flagellin B conjugated to IL-15 showed suppression of tumor growth upon intratumoral colonization *in vivo* explained by activation of innate and adaptive anticancer immune responses [11]. *Clostridium sporogenes-NT* is a strictly anaerobe, spore-forming bacterium which is known to thrive specifically in highly aggressive necrotic tumors and is therefore studied as bacterial-based tumor-targeting therapy. Upon intravenous spore injection, *C. sporogenes-NT* spores migrate in the anoxic/necrotic areas of the tumor microenvironment (TME), germinate into vegetative cells, and colonize the tumor [12]. Genes encoding for murine (m)IL-2 and mGM-CSF were previously stably inserted into the chromosome of *C. sporogenes-NT* using optimized CRISPR-Cas techniques [13]. We hypothesize that intratumoral colonization with *C. sporogenes-NT* recruits immune cells towards the TME, and clostridium-mediated delivery of pro-inflammatory cytokines, such as mIL-2 or mGM-CSF, will trigger these recruited immune cells towards an anticancer response. When combined with radiotherapy (RT), these immune-regulated anticancer effects will be enhanced as RT increases the amount of necrosis, and therefore the levels of intratumoral bacteria, and primes the tumor for immunotherapy [14–17].

Here, we investigate the *in vitro* and *in vivo* therapeutic effects of *C. sporogenes-NT-mIL-2* and *C. sporogenes-NT-mGM-CSF*. We show successful production and secretion of biologically active cytokines *in vitro* and demonstrate that gene insertion did not impair bacterial sporulation, germination, or growth, and intravenous injection of spores in tumor-bearing animals did not lead to systemic toxicity. Intratumoral colonization with immunotherapeutic clostridium strains in syngeneic tumor models resulted in recruitment of immune cells towards the TME, tumor growth inhibition, and prolonged survival. Moreover, when combined with RT, the therapeutic effect of cytokine producing clostridia strains was increased up to complete tumor cure. These data highlight the potential of this Clostridium-Mediated ImmunoTherapy (CMIT) approach, leveraging effective tumor-specific immunotherapy while escaping systemic toxicity.

# Methods

Cell culture

Murine CT26 (kindly provided by Philogen S.p.A.), 4T1 (ATCC), RAW-Blue (Invivogen, kindly provided by Manlio Fusciello and Vincenzo Cerullo, IVT lab, University of Helsinki), and CTLL-2 (ATCC) luciferase (luc)+ cells (generated using protocol described elsewhere [18]) were cultured in DMEM or RPMI (Sigma-Aldrich) supplemented with 10% Fetal Bovine Serum (FBS, Bodinco) at 37°C and 5% $CO_2$. CTLL-2 luc+ cells were supplemented with hIL-2 (200 pg/mL) and Neomycin (50 mg/mL). RAW-Blue cells were supplemented with Normocin (100 µg/mL), Zeocin (200 µg/mL), and cultured with heat-inactivated FBS (30 mins 56°C).

Bacterial cultures

Generation of *C. sporogenes-NT* [12], and chromosomal integration of therapeutic genes resulting in *C. sporogenes-NT-mIL-2*, and *C. sporogenes-NT-mGM-CSF* [13] have been described previously. Bacterial liquid cultures were maintained in Peptone (20 mg/mL, Merck), Yeast (15 mg/mL, Merck), and sodium Thioglycolate (1 mg/mL, Merck) media (PYT). For bacterial solid plate cultures, PYT medium was supplemented with 15 mg/mL Oxoid Agar Technical (Agar No. 2, Thermo Fisher). Growth of the *C. sporogenes* strains was established in an anaerobic cabinet (model MG1000 Mark II, Don Whitley Scientific Ltd.; 80% $N_2$, 10% $CO_2$, 10% $H_2$) at 37°C.

Spheroid cultures

For spheroid generation, 1000 cells/well were seeded in a 96-well Clear Round Bottom Ultra Low Attachment Microplate (Corning). Plates were spun down for 10 minutes at 1000 rpm and were placed in a 5% $CO_2$ incubator at 37°C for at least three days to allow formation of stable spheroids. To induce intra-spheroid colonization, *C. sporogenes* spores were added (1000 spores/spheroid) at day 5 post spheroid seeding. Spheroids colonization was confirmed by determination of the colony forming unit (CFU) per spheroid using the dilution plating assay as described elsewhere [19].

# ELISA

All ELISA assays have been performed according to manufacturers' protocols (Invitrogen Mouse TNF alpha, Mouse IL-2, Mouse GM-CSF Uncoated ELISA Kit). ELISAs were performed with either bacterial culture supernatant or cell culture samples. Bacterial culture samples were obtained by subculturing preferred overnight culture to fresh media. 3, 5 and 7 hours post-subculturing, samples were taken for OD600 measurements, spun down at 10.000g for 10 mins, and supernatant was collected and stored at -20°C. Cellular culture samples were obtained by collecting 100 µL supernatant from either 3D (spheroid) or 2D cell cultures at preferred time points.

CTLL-2 proliferation assay

CTLL-2 luc+ cells were seeded in a 6-well plate ($1.5x10^5$ cells/well) and starved from IL-2 for 24h. The next day, CTLL-2 luc+ cells were seeded in a 96-well plate (20.000 cells/well). Spheroid supernatant samples and positive controls containing IL-2 (200 pg/mL) were added to the CTLL-2 luc+ cells in a 1:1 dilution. After 24h incubation, a luciferase assay (Luciferase Assay System, Promega) was performed according to the manufacturer's protocol to detect number of viable cells. Absorbance was detected using the SpectraMax iD5.

RAW-Blue quantiblue assay

RAW-Blue cells were seeded in a 96-well plate (100.000 cells/well) and spheroid supernatant samples were added to the RAW-Blue cells in a 1:1 dilution. After 24h incubation, NF-kB expression levels were

detected using the QUANTI-Blue assay (Invitrogen) according to the manufacturer's protocol.

## Animals

All animal experiments were in accordance with local institutional guidelines for animal welfare and approved by the Animal Ethical Committee of the University of Maastricht (AVD10700202418254). Female immunocompetent BALB/cOlaHsd mice (Envigo), aged 8 weeks, were injected subcutaneously with $5x10^6$ CT26 or 4T1 cells resuspended in Matrigel (Corning). For toxicity studies, animals were injected intravenously with bacterial spores (dose range between $1x10^8$-$1x10^9$ spores/animal in 100 µL PBS) when tumor reached a size of > 350 $mm^3$. Body weight and toxicity were measured daily, and animals were sacrificed after 48h for downstream analysis. For therapeutic efficacy studies, tumors were locally irradiated (5Gy, 15 MeV, 300 MU/min) using a Varian Truebeam linear accelerator when volume reached 300–350 $mm^3$. Spores ($1x10^8$ spores/animal in 100 µL PBS) were injected intravenously and tumor volume as well as animal body weight were monitored at least 3 times a week. Tumor growth was followed until reaching ethically defined humane endpoint (time to reach 4 times starting volume, T4xSV) or until cured (no tumor for > 60 days). Enhancement ratio (ER) was calculated by dividing mean T4xSV irradiated tumors by T4xSV unirradiated tumors. At endpoint, tumors were excised and dissociated using a gentleMACS Dissociator (Miltenyi) and single cells were stored at -20°C for colonization assays or filtered (EASYstrainer 70µM Greiner) and stored in freezing media (85% FBS, 10% DMSO, 5% of 50% glucose) at -80°C for flow cytometric analysis. Furthermore, blood was collected, spun down (2100 rpm, 10 mins, 4°C) to separate cells from plasma, both stored at -80°C.

## Flow cytometry

Cells were washed once in FACS buffer (0.1% FBS in 1x PBS), diluted to appropriate concentrations (0.25-1.0 $x10^7$ cells/mL), and stained. Antibodies and dilutions are mentioned in Table 1. Samples were flushed through a Falcon 5 ml polystyrene cell-strainer-capped tube (VWR) prior to measurement. Samples were analyzed using BD Bioscience FacsCanto II Flow Cytometer and corresponding FlowJo v10.8.1 software. Samples containing < 2% viable cells were excluded from analysis. The used gating strategy is depicted in supplementary Fig. 1.

Table 1
Antibodies, clones, company, and dilutions used for flow cytometry

| Antibody | Clone | Company | Dilution |
|---|---|---|---|
| FC-block CD16/CD32 | 2.4G2 | BD Biosciences | 1:100 |
| CD45 APC-Cy7 | 30-F11 | BD Pharmingen | 1:200 |
| CD3 FITC | REA641 | Miltenyi | 1:100 |
| CD19 PE | REA749 | Miltenyi | 1:100 |
| NK46 APC | REA815 | Miltenyi | 1:100 |
| CD8 PE-Cy7 | 53 – 6.7 | BD Biosciences | 1:100 |
| Ly6G Percp-Cy5.5 | REA526 | Miltenyi | 1:50 |
| Live/dead aqua | / | Invitrogen | 1:1000 |
| F4/80 PE-Cy7 | BM8 | Invitrogen | 1:200 |

# Statistical analysis

Statistical evaluations were performed with GraphPad Prism 8.0.2 software (San Diego, CA, United States). To assess *in vitro* therapeutic effects between *C. sporogenes-NT-mIL-2* and *C. sporogenes-NT-mGM-CSF* compared to control, an unpaired *t*-test was used per time-point. To assess therapeutic effects comparing two groups, an unpaired *t*-test was used. One-way ANOVA with Dunnett's post-test per time-point was used when comparing > 2 groups. *In vivo* survival was assessed using a Log-rank (Mantel-Cox) test.

# Results

**Genetically modified C. sporogenes-NT secrete cytokines without impairing bacterial growth**

To assess the effect of therapeutic gene insertion on bacterial growth and immunotherapeutic production and secretion, bacteria were grown in an overnight culture. OD600 measurements confirm that bacterial growth is not impaired in genetically modified strains compared to control (Fig. 1a). Next, to assess immunotherapeutic production and secretion, cytokine levels were measured in the culture supernatant. For both *C. sporogenes-NT-mIL-2* (Fig. 1b) and C. *sporogenes-NT-mGM-CSF* (Fig. 1c), elevated cytokine levels were detectable in all samples compared to control samples.

**Figure 1: Genetically modified bacteria grow in liquid culture and produce cytokines.** a: OD600 of *C. sporogenes-NT* (control), *C. sporogenes-NT-mIL-2*, and *C. sporogenes-NT-mGM-CSF* 3, 5, and 7h after start culture. b: mIL-2 production in supernatant of *C. sporogenes-NT-mIL-2* and *C. sporogenes-NT* (control) bacterial cultures. c: mGM-CSF production in supernatant of *C. sporogenes-NT-mGM-CSF* and *C.*

*sporogenes-NT* (control) bacterial cultures. Data represents the mean ± SD of 3 independent bacterial cultures of therapeutic strains.

**Secreted cytokines from genetically modified C. sporogenes-NT are biologically active**

To assess if genetically modified bacteria are still able to form spores and germinate into necrotic areas, growth in previously validated spheroid models [19] was investigated (Fig. 2a). Intra-spheroid colonization with *C. sporogenes-NT-mIL-2* was present within 48 hours after spore addition, peaked after 4 days (day 9), and then stabilized (Fig. 2b). Next, it was investigated whether the bacteria could secrete cytokines upon intra-spheroid colonization. Increasing levels of mIL-2 were observed in media from spheroids colonized with *C. sporogenes-NT-mIL-2*, compared to media from spheroids colonized with *C. sporogenes-NT* ($p < 0.0001$). In line with spheroid colonization, mIL-2 levels peaked 4 days post-spore addition and stabilized (Fig. 2c). Finally, it was determined if the secreted mIL-2 was biologically active using a cytotoxic T cell proliferation assay. T cell proliferation was significantly increased, up to 8-fold ($p < 0.001$), upon addition of *C. sporogenes-NT-mIL-2* treated spheroid medium, compared to control (Fig. 2d).

Similar experiments were performed for *C. sporogenes-NT-mGM-CSF* (Fig. 3a). Upon spore addition, *C. sporogenes-NT-mGM-CSF* colonized CT26 spheroids, with numbers increasing daily (Fig. 3b). Increasing levels, up to 600 pg/mL, of mGM-CSF were observed in the media of spheroids colonized by *C. sporogenes-NT-mGM-CSF*, while absent in spheroid media treated with *C. sporogenes-NT* control (Fig. 3c) ($p < 0.001$). As literature [20] showed that NF-κB is increased upon mGM-CSF treatment in macrophages, NF-κB expression was assessed in RAW-Blue macrophages upon treatment with medium from colonized spheroids. Media from both *C. sporogenes-NT* and *C. sporogenes-NT-mGM-CSF* colonized spheroids increased NF-κB expression compared to control. mGM-CSF enriched media increased NF-κB to significantly higher levels ($p < 0.0001$) compared to *C. sporogenes-NT* control media, also when corrected for CFU/spheroid (Fig. 3d and e), confirming the biological activity of mGM-CSF upon secretion.

NF-κB expression can be increased in both anti- and pro-inflammatory macrophages. Especially tumor-associated anti-inflammatory macrophages are associated with increased NF-κB [21]. To assess the inflammatory state of macrophages treated with spheroid media, secretion of the pro-inflammatory cytokine TNFα was measured. Increased TNFα secretion was observed upon macrophage treatment with bacterial secreted mGM-CSF, which is likewise visible when TNFα levels are corrected for CFU/spheroid (Fig. 3f and g). In line with NF-κB data, TNFα secretion likewise increased upon macrophages treated medium from *C. sporogenes-NT* colonized spheroids, compared to control.

**Genetically modified C. sporogenes-NT colonize tumors in vivo without causing acute toxicity, but cause model-dependent long-term toxicity**

First, the most efficient spore-dose to use *in vivo* was determined, which is defined as a spore-dose resulting in intratumoral colonization upon intravenous spore-injection without causing acute toxicity. Both *C. sporogenes-NT-mIL-2* and *C. sporogenes-NT-mGM-CSF* were able to induce dose-dependent

intratumoral colonization as similar levels of germinated bacteria per spore dose were observed in both CT26 and 4T1, up to ~2x$10^{13}$ bacteria/g tumor (supplementary Fig. 2). No signs of acute toxicity and body weight loss have been observed within the 48h monitoring period.

Next, therapeutic efficacy of *C. sporogenes-NT-mIL-2* combined with RT was assessed in CT26 tumors. In line with earlier results, no acute toxicity within 48h from spore injection was observed. However, within a timespan of 72h to 7 days, all animals treated with bacterial spores (strain- or treatment-independent) demonstrated severe signs of local inflammation with abscess formation, with even a subset of the tumors (~ 8%) partially disappearing, leaving the animals with an ulcerating lesion on the flank. Considering the ethical humane endpoints, the experiment was aborted. Additionally, it was confirmed that these observations were independent from tumor size or spore concentration.

Based on previous observations in CT26, a pilot-study was performed in 4T1 tumor-bearing animals to assess occurrence of long-term toxicity. In contrast to the CT26 tumor-bearing animals, no toxicity was observed > 25 days post spore-injection when experimental endpoint was reached. Hence, for further *in vivo* experiments, 4T1 tumor-bearing animals were used.

**Intratumoral colonization with C. sporogenes results in prolonged survival in vivo**

Long-term therapeutic efficacy of *C. sporogenes-NT*, *C. sporogenes-NT-mIL-2*, and *C. sporogenes-NT-mGM-CSF* has been compared with a vehicle, i.e. PBS without spores, treatment arm in 4T1 tumor-bearing animals. Clostridial spores have been administered as monotherapy or 24h after radiotherapy, given at a tumor-necrosis inducing dose (Fig. 4a). Body weight of animals treated with vehicle, *C. sporogenes-NT*, and *C. sporogenes-NT-mGM-CSF* (regardless of RT treatment) did not change during the experiment (Fig. 4b, d, and h). *C. sporogenes-NT-mIL-2* spore injection caused a drop in body weight (~ 5–10%) within 48h post-spore injection, which restored afterwards (Fig. 4f).

On day 7 post-spore injection, a significant tumor growth inhibition (TGI) was observed for *C. sporogenes-NT* ($p = 0.0007$) and *C. sporogenes-NT-mIL-2* ($p < 0.0001$) compared to vehicle (Table 2). A significant TGI was also observed when combining RT with *C. sporogenes-NT* ($p = 0.0010$), *C. sporogenes-NT-mIL-2* ($p < 0.0001$), and *C. sporogenes-NT-mGM-CSF* ($p = 0.006$) compared to vehicle + RT. Additionally, tumors treated with *C. sporogenes-NT-mIL-2* + RT were significantly smaller ($p < 0.0001$) compared to other treatment arms. Blood samples from day 7 post-spore injection revealed no systemic elevation of mIL-2 and mGM-CSF levels in *C. sporogenes-NT-mIL-2* ($p = 0.5809$) and *C. sporogenes-NT-mGM-CSF* ($p = 0.1119$) treated groups (supplementary Fig. 3).

Treatment with clostridial spores significantly ($p < 0.001$) increased survival for all experimental groups compared to vehicle control (Fig. 4c, e and g). Furthermore, time to reach 4 times starting volume (T4xSV) increased for *C. sporogenes-NT-mIL-2 + RT* ($p = 0.0024$) and *C. sporogenes-NT-mGM-CSF* + RT ($p = 0.0094$) compared to control + RT (Table 2). Individual tumor growth per experimental group is depicted in supplementary Fig. 4. RT did not T4xSV in animals treated with vehicle (median T4xSV RT + PBS: 14.13 days vs median T4xSV PBS: 15.2 days, ER: 0.93, $p = 0.6113$), with *C. sporogenes-NT* (median T4xSV RT +

*C. sporogenes-NT*: 18.26 days vs median T4xSV *C. sporogenes-NT*: 20.94 days, ER: 0.87, p = 0.4992) or *C. sporogenes-NT-mGM-CSF* (median T4xSV RT + *C. sporogenes-NT-mGM-CSF*: 29.54 days vs median T4xSV *C. sporogenes-NT-mGM-CSF*: 28.53 days, ER 1.03, p = 0.8817). RT increased the therapeutic efficacy of *C. sporogenes-NT-mIL-2* (median T4xSV RT + *C. sporogenes-NT-mIL-2*: 31.55 days vs median T4xSV *C. sporogenes-NT-mIL-2* 21.81 days, ER 1.45, p = 0.0874).

Table 2
Time to reach 4x starting volume (T4xSV), enhancement ratio (ER) of radiotherapy (RT) per therapeutic bacterial strain, and percentage tumor growth inhibition (TGI) on day 7 post-spore injection.

| **Experimental group** | **T4xSV** | **ER**<br><br>**RT versus non-RT** | **P-value ER** | **TGI (%)** | **P-value TGI versus vehicle** |
|---|---|---|---|---|---|
| Vehicle control | 15.2 ± 4.06 | 0.93 ± 0.26 | 0.6113 | 1.82 ± 29.87 | - |
| Vehicle control + RT | 14.13 ± 3.91 | | | 5.33 ± 25.72 | 0.9960 |
| *C. sporogenes-NT* | 20.94 ± 7.68 | 0.87 ± 0.38 | 0.4992 | 36.54 ± 23.70 | 0.0007 |
| *C. sporogenes-NT* + RT | 18.26 ± 7.98 | | | 33.31 ± 21.90 | 0.0012 |
| *C. sporogenes-NT-mIL-2* | 21.81 ± 5.79 | 1.45 ± 0.64 | 0.0874 | 45.47 ± 31.42 | < 0.0001 |
| *C. sporogenes-NT-mIL-2* + RT | 31.55 ± 13.98 | | | 73.00 ± 17.69 | < 0.0001 |
| *C. sporogenes-NT-mGM-CSF* | 28.53 ± 12.31 | 1.03 ± 0.42 | 0.8817 | 21.84 ± 33.46 | 0.1132 |
| *C. sporogenes-NT-mGM-CSF* + RT | 29.54 ± 12.01 | | | 34.22 ± 28.35 | 0.0008 |

**Intratumoral colonization with C. sporogenes changed the TME immune cell landscape**

Flow cytometric analysis of tumors obtained 7 days post-spore injection (Fig. 4a) indicated no significant changes in CD45 + recruitment upon 7 days intratumoral colonization with *C. sporogenes*, regardless of strain or RT (Fig. 5a and e). Significant lower levels of F4/80 + cells were observed in *C. sporogenes-NT-mIL-2* (p = 0.0053) and *C. sporogenes-NT-mGM-CSF* (p = 0.0002) colonized tumors compared to *C. sporogenes-NT* (Fig. 5b). This effect disappeared when combining with RT, especially due to the reduced levels of F4/80 + cells for *C. sporogenes-NT* (Fig. 5f). While not significant, a trend towards increased levels of Ly6G+ cells was observed in tumors colonized with *C. sporogenes* when combined with RT (Fig. 5c and g). The majority of these Ly6G+ cells had an intermediate signature (Fig. 5d and h). In contrast to non-RT treated groups, *C. sporogenes-NT* + RT (p = 0.0029) and *C. sporogenes-NT-mGM-CSF* + RT (p = 0.0027) treated tumors contained significantly lower levels NKp46 +

cells compared to vehicle + RT. A similar effect was observed for *C. sporogenes-NT-mIL-2* + RT, albeit not significant (p = 0.0638) (Fig. 5i and m). Expression levels of CD3+, CD8 + and CD19 + cells only marginally changed for the different experimental groups (Fig. 5j-l and n-p).

# Discussion

Bacterial-based anticancer strategies have been investigated for decades, using a wide variety of bacteria, such as *Salmonella*, *E. coli*, *Listeria*, and *Clostridium* [10, 11, 22] and intratumoral bacteria have been described to elicit a wide spectrum of inflammatory effects in the TME, both protumor and antitumor [23]. Among these species, *Clostridium* is a strict anaerobic bacterium, making it highly specific for vegetating in necrotic tumor regions. In context of *Clostridium*, immune-related anticancer effects have been observed previously. For example, *C. novyi* has been reported to modulate the immunological landscape of the TME, as murine and dog studies with *C. novyi-Nontoxic (NT)* reported anticancer effects via tumor cell lysis and host immune system activation [24, 25]. In a phase I clinical trial, germination was observed upon intratumoral *C. novyi-NT* spore injection, resulting in tumor colonization, cancer cell lysis, and increased infiltration of T and myeloid cells [26]. Furthermore, in the 60s-70s, intravenous injection of *C. sporogenes* spores in cancer patients resulted in liquification of the tumor, suggesting oncolysis and inflammation, but tumor regression was not observed [27, 28]. These effects can be attributed to *C. sporogenes* producing cyclic di-AMP which is an agonist for the STING pathway [29]. The results with *C. sporogenes-NT* described in the current study are in line with these findings. *In vitro*, macrophage activation was observed, leading to enhanced NF-κB levels and secretion of the pro-inflammatory cytokine TNFα upon stimulation with medium from *C. sporogenes-NT* treated spheroids. *In vivo*, intratumoral colonization with *C. sporogenes-NT* resulted in prolonged survival and increased F4/80 + cells but did not result in tumor regression. *C. sporogenes* is tolerated by the immune system as this species belongs to the commensal flora of the gut and branched-chain fatty acids and short-chain fatty acids generated by *C. sporogenes* enhance activity of regulatory T cells [30]. This phenomenon is suggested to decrease the infiltration of CD8 + cells in *C. sporogenes-NT* colonized tumors as reflected in the presented data. It is hypothesized that immunotherapeutic strains overcome this by additional activation of the immune system, as observed by *C. sporogenes-NT-mIL-2* + RT treated animals.

While no significant changes in the TME immune landscape have been observed, our data supports the concept of tumor-targeted immunotherapeutic delivery. IL-2 produced within the tumor by *C. sporogenes-NT-mIL-2* combined with RT resulted in prolonged survival and enhanced anticancer effects up to complete tumor cure, as compared with no additive therapeutic effects when combining RT with vehicle or *C. sporogenes-NT*. The synergy between RT and immunotherapy have been extensively described in literature [31]. RT does not only initiate direct killing of cancer cells but also activates immune-related responses via the release of tumor-associated antigens and the enhancement of immune cell infiltration [32]. *In vivo*, intratumoral injection with IL-2 combined with fractionated RT (5x 2Gy) resulted in tumor growth delay and ultimately complete tumor cure compared to monotherapy [33]. Zegers et al. demonstrated that the combination of the tumor-specific immunocytokine L19-IL-2 with RT (10Gy)

resulted in increased tumor growth delay, increased survival, and enhanced infiltration of CD8 + T cells, compared to monotherapy with either RT of L19-IL-2 [34]. In contrast, several clinical trials have not observed an increased therapeutic effect of IL-2 + RT. Safwat et al. found increased NK cell infiltration but no increased therapeutic effect when combining subcutaneous IL-2 with low-dose total body irradiation for treatment of metastatic melanoma [35]. Curti et al. did not observe a significant difference in high-dose intravenous IL-2 combined with stereotactic body radiation therapy compared to IL-2 monotherapy for treating advanced melanoma [36]. However, Jacobs et al. demonstrated a significant improvement in disease-free survival when RT was combined with intratumoral injection of IL-2 in patients with nasopharyngeal carcinoma [37]. Van Limbergen et al. reported in a phase I safety trial 2 patients with long-term progression-free survival after treatment with RT combined with L19-IL-2 [38]. These studies indicate the need for delivering high IL-2 doses in a tumor specific manner. The current study addresses this need by exploiting *C. sporogenes* as an intratumoral delivery system for biologically active IL-2 upon intravenous spore administration, resulting in enhanced therapeutic efficacy, especially when combined with RT as a tumor necrosis inducing agent, while avoiding systemic toxicity.

Similar to *C. sporogenes-NT-mIL-2*, treatment with *C. sporogenes-NT-mGM-CSF* resulted in prolonged survival accompanied with an increased infiltration of CD8 + cells. GM-CSF stimulates production, maturation, and activation of innate immune cells such as neutrophils, macrophages, and dendritic cells, however, in context of cancer the exact effects of this cytokine remain elusive [9]. Yoshimura et al. found that 4T1 cancer cells, as used in the current study, can produce GM-CSF by itself which promotes as such tumor growth by activation of tumor-associated macrophages and neutrophils, however, neutralizing this GM-CSF production had limited effect on tumor progression [39]. Interestingly, Mihalik et al. reported that intratumoral injection of high-dose GM-CSF in 4T1 tumors drove TAMS towards an M1-like phenotype, which is related to an antitumor response [40]. Furthermore, intratumoral injection of oncolytic adenovirus expressing GM-CSF enabled tumor growth inhibition in 4T1 tumors compared to control [41]. These data suggest that the protumor effect of GM-CSF in 4T1 tumors can be converted into an antitumor response using GM-CSF, in line with the current findings with respect to the anticancer effects of *C. sporogenes-NT-mGM-CSF*. Additionally, clinical trials in prostate cancer testing the effects of systemic GM-CSF, reported enhanced recruitment of CD8 + T cells towards the TME, suggesting that systemic GM-CSF can modulate T-cell infiltration, similar as observed in the current study. However, the clinical study additionally points out that systemic exposure to GM-CSF subsequently affects other immune cell subsets, as circulating CD8 + and regulatory T cells increase, while circulating myeloid and plasmacytoid dendritic cells decrease [7]. Effects thereof have not been reported, but systemic changes in immune cell subsets support again the need for tumor-specific delivery.

In the field of bacterial-based anticancer therapy, CT26 is a commonly used murine colon cancer cell line. ClyA expressing *Salmonella* has been injected intraperitoneal into CT26 tumor-bearing animals, resulting in adequate colonization and increased tumoricidal activity [42]. Large (700–900 $mm^3$) CT26 tumors have been treated with *C. novyi*, resulting in robust germination and tumor regression [43]. Previously, our group has injected *C. sporogenes-NT* spores intravenously in CT26 tumor-bearing animals

at a tumor volume of 200 $mm^3$ [12]. Although the extensive research with this tumor model, bacterial-related side effects as observed in the current study have not been described before. Jiang et al. describes the occurrence of black lesions on the skin, similar as observed in the current study, when treating CT26 tumor-bearing animals with engineered *Salmonella*, however, no further details were provided regarding these side effects [44]. Within immune-oncology research, the CT26 tumor model is frequently used in context of immunological “hot” tumors, whereas 4T1 tumor is considered as immunologically “cold”. Hence, as CT26 tumors contain more pro-inflammatory tumor infiltrating lymphocytes, this tumor model is more susceptible towards immunotherapy [45]. We therefore speculate that the immunomodulatory effects as a result of intratumoral colonization with *C. sporogenes-NT* resulted in local inflammation of the tumor causing vasculitis and black lesions specific for CT26 tumors due to the high infiltration of immune cells [46]. More research is necessary to investigate the differential biological effects of intratumoral colonization with *C. sporogenes* between immunological “hot” and “cold” tumors.

There are several limitations to consider within this study. Immune system-related differences between Balb/C and C57/Bl6 mice have been reported. C57/Bl6 animals have more Th1 immune responses, while Balb/C are known to be more biased towards Th2 responses [47]. Hence, it might be interesting to study therapeutic effects of the CMIT approach in a C57/Bl6 model. Additionally, therapeutic effects of bacterial-based mIL-2 and mGM-CSF have not been compared to intravenously administered mIL-2 and mGM-CSF as monotherapy. Cytokine levels are correlated to the number of bacteria (Fig. 1, 2, and 3), and number of bacteria varies per tumor. Therefore, it is difficult to estimate the exact levels of intratumoral cytokines. In addition, high doses of systemically administered IL-2 are necessary to acquire therapeutic efficacy, indicating that only a small fraction of the IL-2 reaches the tumor, resulting in high healthy tissue toxicity [48].

In conclusion, our data demonstrate that intratumoral colonization with *C. sporogenes-NT* strains results in tumor growth delay and increased survival *in vivo* compared to controls. Moreover, the combination of our CMIT strategy with RT, further increases the anticancer effects. Previously, abscopal effects have been observed when combining IL-2 with RT [33, 49], hence this effect could be interesting to study likewise for our *C. sporogenes-NT-mIL-2* + RT strategy. Furthermore, as an immunologically “cold” TME remains a major hurdle for immunotherapy due to resistance to therapy, the combination of our bacterial-based therapy with conventional immunotherapy could result in higher therapeutic efficacy, as the bacteria create a “hot” TME, more favorable for immunotherapy.

## Declarations

# Ethical approval

All animal experiments were in accordance with local institutional guidelines for animal welfare and approved by the Animal Ethical Committee of the University of Maastricht (AVD10700202418254).

**Competing interest**

LJD and JT have, outside of the submitted work, shares in the company Convert Pharmaceuticals. LJD and JT have, in context of the submitted work, shares in the company LivingMed Biotech. The other authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as potential conflict of interest.


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# Author contributions

Experimental design –JL, JT, LJD. Execution of *in vitro* studies – JL, LN. Execution of *in vivo* studies – JL, JB, FV, LS, RB, LN, LJD. Data analysis and discussion – JL, VTCV, JT, LJD. Manuscript writing – JL, JT, LJD. All authors revised and agreed on the content described in the manuscript.


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## Data availability statement

The data analyzed during the current study are available from the corresponding author on reasonable request.

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# Figures

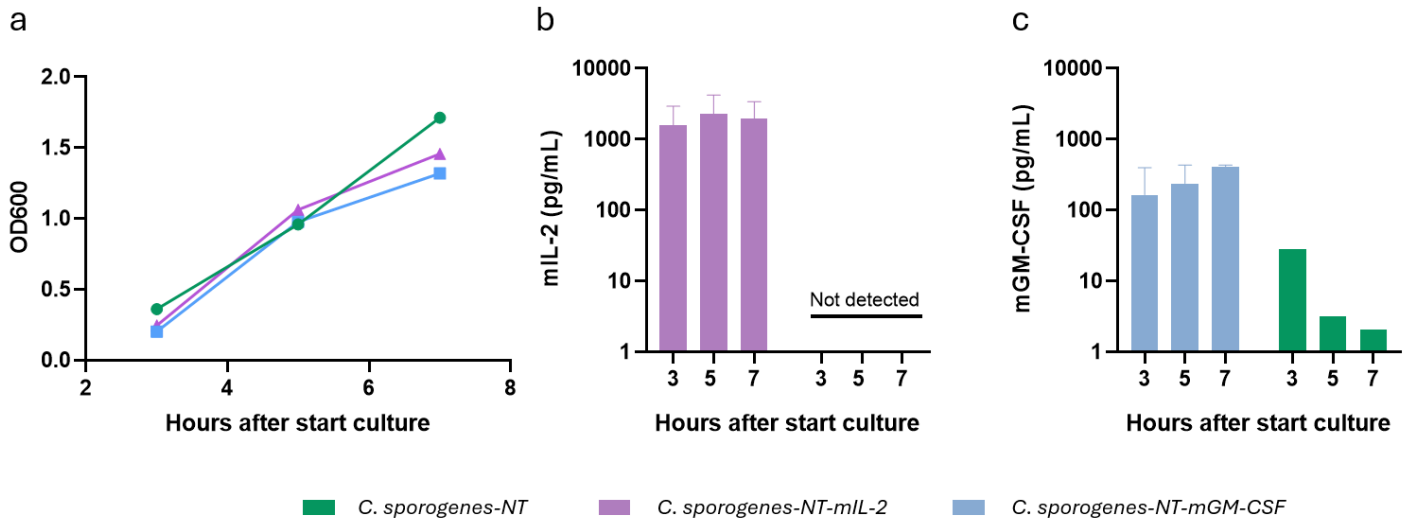



**Figure 1**

**Genetically modified bacteria grow in liquid culture and produce cytokines.** a: OD600 of *C. sporogenes-NT*(control), *C. sporogenes-NT-mIL-2*, and *C. sporogenes-NT-mGM-CSF*3, 5, and 7h after start culture. b: mIL-2 production in supernatant of *C. sporogenes-NT-mIL-2* and *C. sporogenes-NT* (control) bacterial cultures. c: mGM-CSF production in supernatant of *C. sporogenes-NT-mGM-CSF*and *C. sporogenes-NT* (control) bacterial cultures. Data represents the mean ± SD of 3 independent bacterial cultures of therapeutic strains.

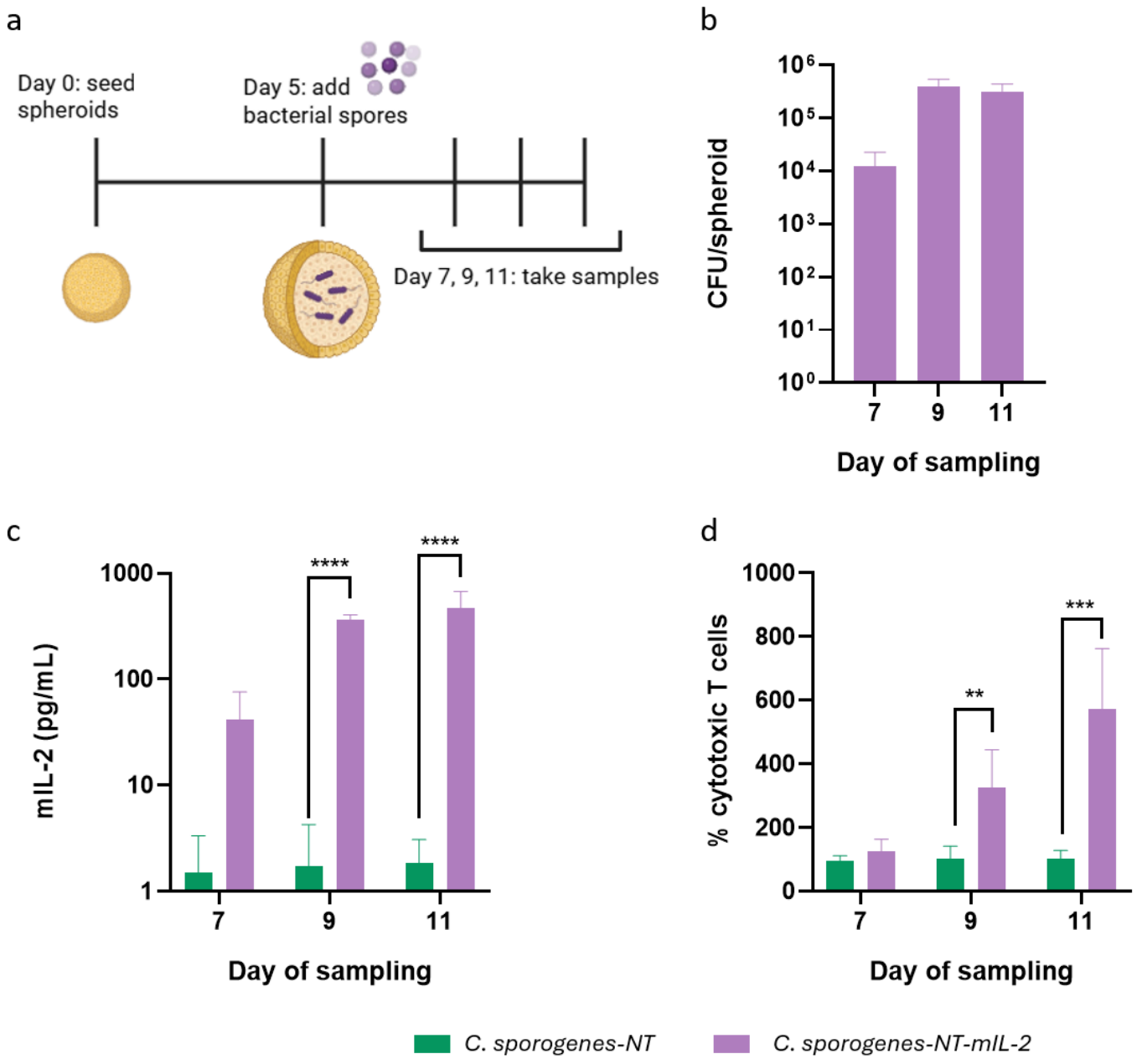
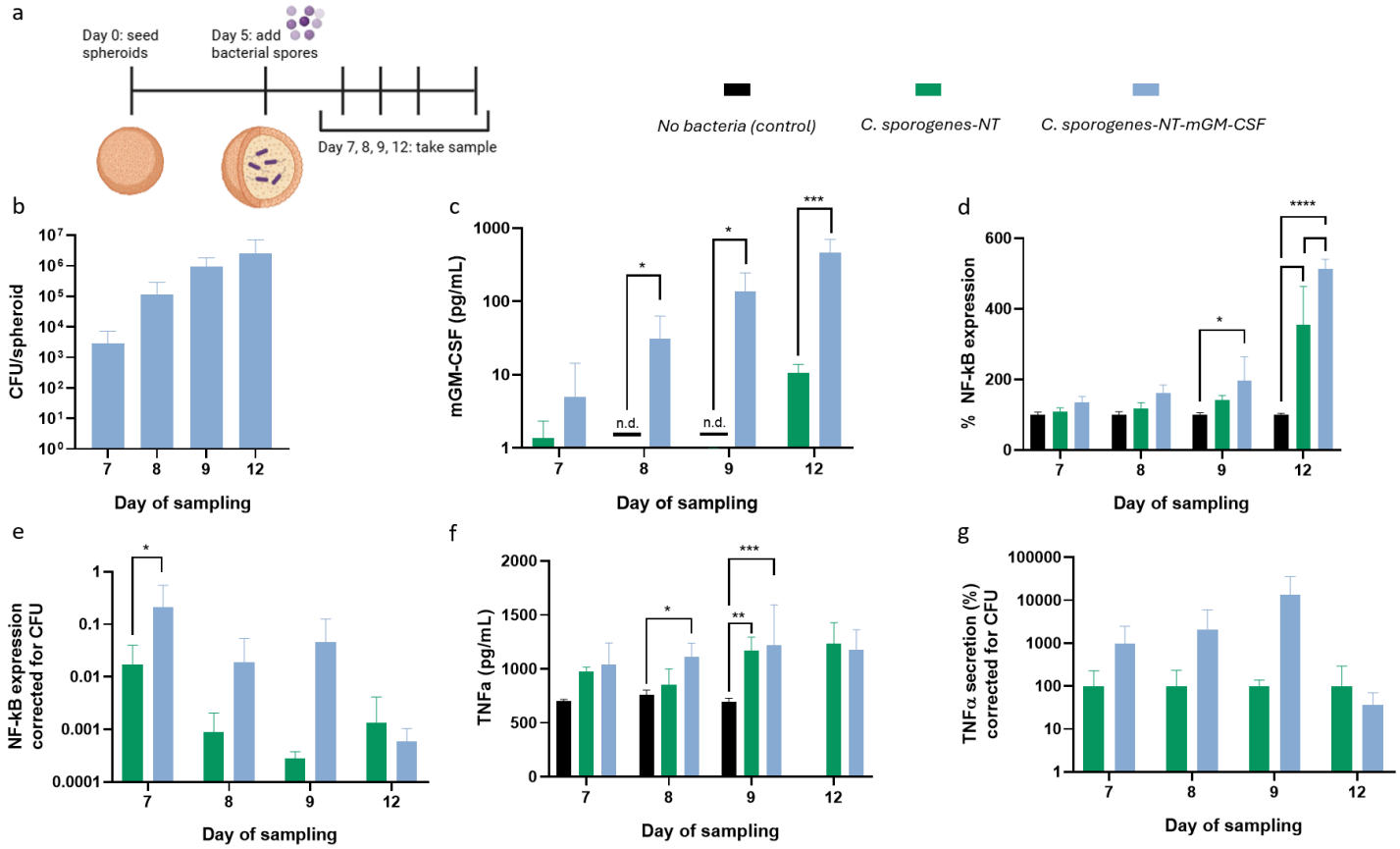


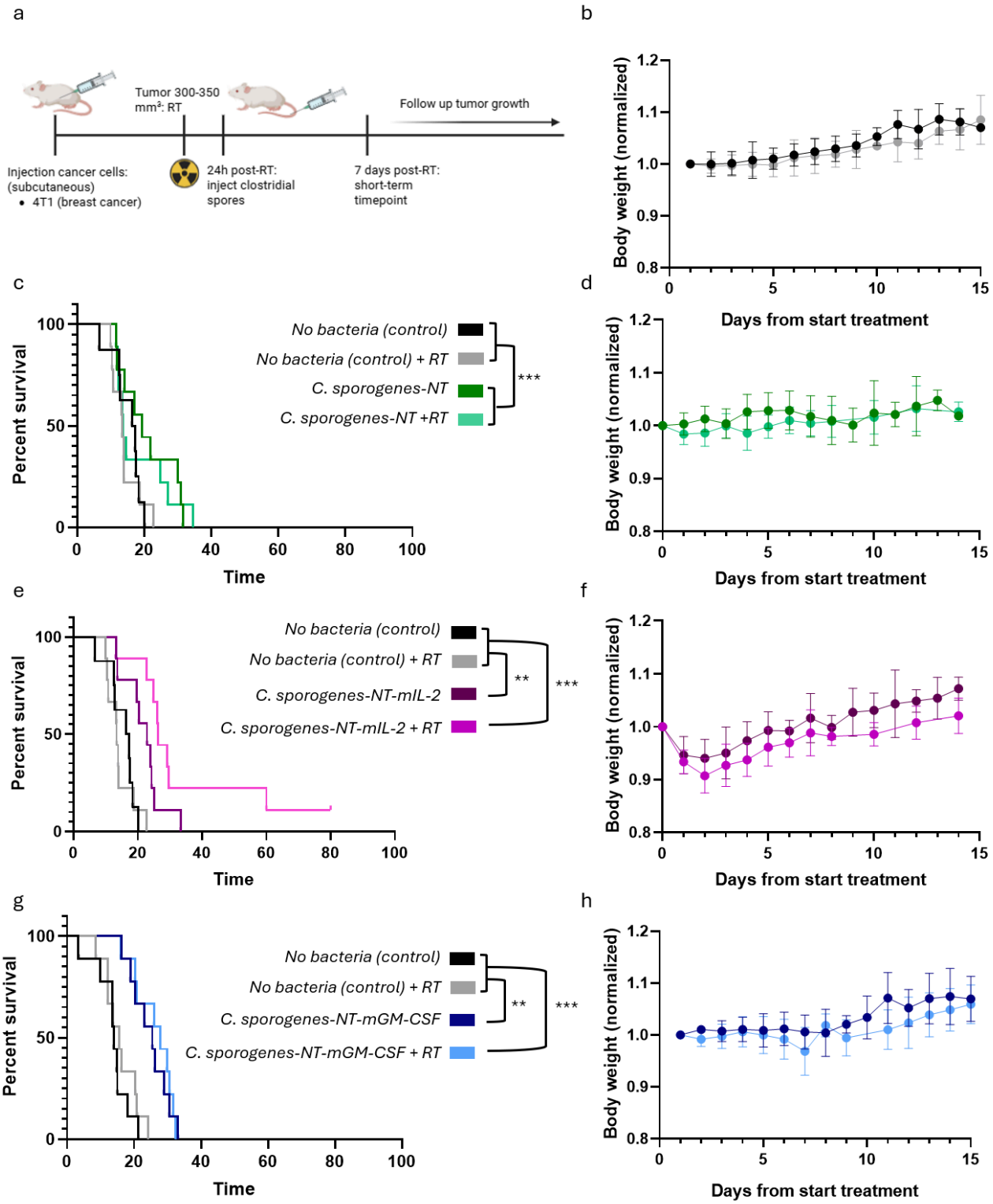

**Figure 2**

***C. sporogenes-NT-mIL-2* produces biologically active mIL-2 upon intra-spheroid colonization**. a: experimental setup. b: *C. sporogenes-NT-mIL-2* CFU/spheroid at day 7, 9, and 11. c: mIL-2 levels in spheroid media and d: percentage increase in cytotoxic T cells upon addition spheroid media from *C. sporogenes-NT-mIL-2 or C. sporogenes-NT treated spheroids*, at day 7, 9, and 11. Data represent the mean ± SD of 6 biological replicates. ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.




## Figure 3

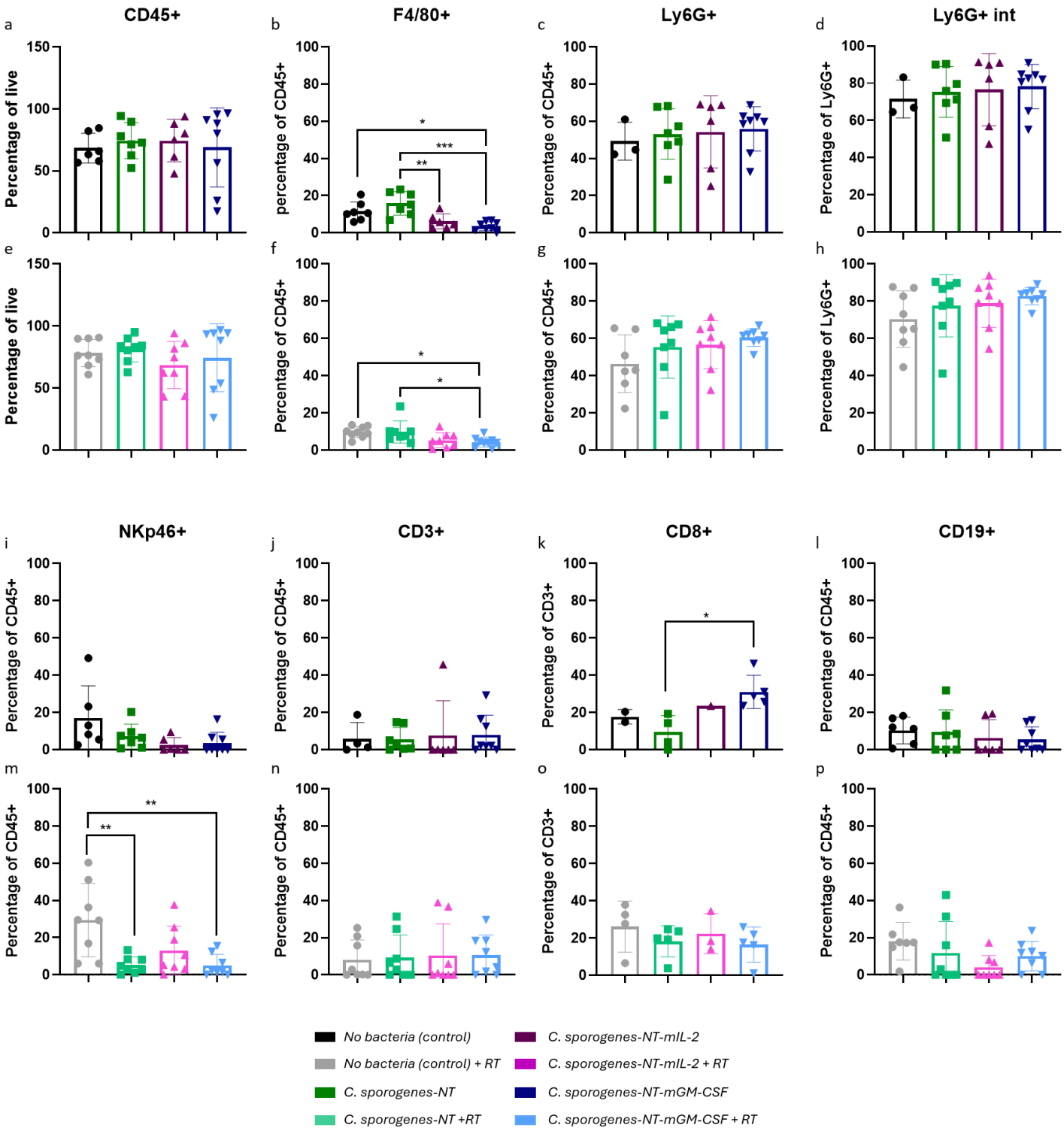
***C. sporogenes-NT-mGM-CSF* produces biologically active mGM-CSF upon intra-spheroid colonization**. a: experimental setup. b: *C. sporogenes-NT-mGM-CSF* CFU/spheroid on day 7, 8, 9, and 12. c: mGM-CSF levels in spheroid media. d: NF-kB assay with spheroid media. Percentage increase in NF-kB expression in RAW-Blue cells treated with media from *C. sporogenes-NT-mGM-CSF or C. sporogenes-NT treated spheroids* per time point. e: NF-kB expression corrected for CFU/spheroid. f: TNFa levels secreted by RAW-Blue cells treated with media from *C. sporogenes-NT-mGM-CSF or C. sporogenes-NT treated spheroids* per time point. g: TNFa secretion corrected for CFU/spheroid. Data represent the mean ± SD of 6 biological replicates. * p<0.05, *** p<0.001, **** p<0.0001.




## Figure 4

**Intratumoral C. sporogenes results in prolonged survival *in vivo***. a: Experimental timeline. Body weight progress of animals treated with vehicle and vehicle + RT (b), *C. sporogenes-NT* and *C. sporogenes-NT* + RT (d), *C. sporogenes-NT-mIL-2* and *C. sporogenes-NT-mIL-2* + RT (f), and *C. sporogenes-mGM-CSF* and *C. sporogenes-NT-mGM-CSF* + RT (h). Survival curves of animals treated with *C. sporogenes-NT* and *C. sporogenes-NT*+ RT (c), *C. sporogenes-mIL-2* and *C. sporogenes-mIL-2* + RT (e), and *C. sporogenes-NT-*

*mGM-CSF* and *C. sporogenes-NT-mGM-CSF* + RT (g), compared to vehicle and vehicle + RT. Data represent the mean ± SD of 9 animals per experimental group. *** p<0.001, **** p<0.0001.




## Figure 5

**Intratumoral colonization with C. sporogenes changes the TME immune landscape.** Flow cytometric analysis of immune cell subsets present in the TME in tumors treated with vehicle, *C. sporogenes-NT*, *C. sporogenes-NT-mIL-2*, and *C. sporogenes-NT-mGM-CSF*. a, b, c, d & i, j, k, l: Nonirradiated treatment groups.

e, f, g, h & m, n, o, p: Irradiated treatment groups. Data represents the mean ± SD of 9 animals per experimental group. * p<0.05, ** p<0.01, *** p<0.001.

## Supplementary Files

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