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Switching Macrophages to Cancer-Killing Machines using Recombinant Type-VI Secretion System in "Tumor-Seeking" Bacteria: A Novel Delivery System for Cancer Treatment

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Abstract

Introduction: Cancer is a complex, heterogeneous disease that is challenging to treat. Immunotherapy, which involves activating or suppressing the immune system, is emerging as a option for many resistant cancers. However, one persistent obstacle is ensuring that the drug reaches the appropriate immune cells as to minimize off-target cellular cytotoxicity. Recent studies have demonstrated the efficacy of some anaerobe bacteria to specifically "seek" the hypoxic tumor microenvironment and are thus valuable vessels to carry anti-tumor drugs.

CD206+ tumor associated macrophages (TAMs) are characteristically anti-inflammatory, innate immune cells that foster a supportive microenvironment to support tumorigenesis. The cGAS-STING pathway can be activated within macrophages, allowing a switch to a pro-inflammatory phenotype. We propose that tumor-seeking bacteria can be effectively manipulated to deliver cGAS to TAMs, resulting in decreased tumor load and minimum toxicity.

Methods: We propose to design a plasmid carrying a constitutively active cGAS, inserted into *E.Coli*. *E.Coli* will contain an injection system, modified with nanobodies specific to CD206 receptors on TAMs. To deliver this drug into the intracellular compartment of the macrophage, here we propose to incorporate a recombinant Type-VI injection system, expressed by these tumor-seeking bacteria, to deliver constitutively active cGAS-STING to the TAMs. In vitro cell culture experiments using mouse and human macrophages will be used to initially test the efficacy of binding and insertion of cGAS sting, followed by intra-tumor or systemic delivery in an in vivo melanoma mouse tumor model.

Results: We anticipate that the *E.Coli* would specifically target CD206+ macrophages both in vitro and within the tumor. The Type-VI injection system would effectively deliver the cGAS into the macrophages, resulting in the activation of the cGAS-STING pathway and the expression of pro-inflammatory cytokine genes, as measured in a cytokine assay. The frequencies and absolute numbers of pro-inflammatory macrophages will be increased over time. We expect that there will be no toxic, or excessive inflammatory effects on the mice administered with the recombinant tumor-seeking bacteria, and the tumor size will be reduced at endpoint.

Conclusion: This proposal explores a novel cancer immunotherapy product exploiting the potential of tumor-seeking recombinant bacteria as a drug carrier.

Keywords: cancer immunotherapy; tumor-associated macrophages; intracellular delivery; cGAS-STING, synthetic biology

Introduction

Cancer is a complex disease characterized by the uncontrolled growth and the spread of abnormal cells. The immune system has adapted to recognize and eliminate foreign cells and pathogens [1], but cancer cells have adapted mechanisms to evade this response. One such process includes the modulation of the tumor microenvironment (TME), which is defined as the cellular environment within a tumor, to become immune suppressive [2]. This is mediated by the secretion of cytokines, such as interleukin-4 (IL-4) and IL-10 by cancer cells, which allows them to suppress the pro-inflammatory anti-tumor mechanisms of nearby immune cells [3].

Macrophages are now known to consist of a heterogeneous group of cells that exist along a spectrum between two major polarized forms of differentiation: M1 and M2. M1 macrophages are pro-inflammatory cells that damage tissues and inhibit proliferation while M2 macrophages tend to promote proliferation and tissue repair. Previous studies described tumor-associated macrophages (TAMs) as M2-leaning macrophages; this aids tumor proliferation through the secretion of cytokines, like IL-4 and IL-13, that bolster an immunosuppressive tumor microenvironment [4]. Additionally, these TAMs produce C-C chemokine ligand 2 (CCL) which recruits monocytes for differentiation into TAMs, feeding a cycle of

tumorigenesis. As such, converting these TAMs to a tumor non-supportive, or M1-leaning, phenotype presents a promising avenue treating cancer [4-6], as these M1 macrophages tend to bolster the antitumor effects of T-cells and other immune cells in the TME [4].

In recent years, immunotherapies have been demonstrated to be powerful tools to reinvigorate the immune system in combatting various types of cancers [5]. Immune checkpoint inhibitors like anti-PDL1(Programmed Death-Ligand 1) or anti-CTLA4(Cytotoxic T-lymphocyte Associated Protein 4), and immunomodulatory therapies have been shown to be effective against tumors [5]. An emerging avenue of immunotherapy is to convert M2-like TAMs from a tumor-supportive phenotype to a tumor non-supportive phenotype [4, 5]. However, one caveat is that many immunotherapies for these TAMs are delivered systemically, which can lead to detrimental immune-related adverse effects (iRAEs) such as myocarditis, pneumonitis, and psoriasis in non-cancerous tissues [7-9].

To decrease off-target cellular cytotoxicity, live bacteria have been investigated as a potential drug-delivery vessel to localize to the tumor microenvironment [10-13]. "tumor-seeking bacteria", such as *Escherichia coli* (*E.Coli*) and *Clostridium novyi* are facultative and obligate anaerobes, respectively, that preferentially localize to tumors due to their hypoxic microenvironment. Moreover, the immunosuppressive tumor niche may allow bacteria to proliferate without being targeted by the immune system [10]. Preclinical, proof-of-concept studies, such as reported by Gurbatri et al, showed that tumor-seeking bacteria can be modified to secrete immunotherapeutics, such as anti-PD-L1 or anti-CTLA-4 nanobodies, within TME [11-14]. Furthermore, they showed that tumor-seeking bacteria increase the efficiency of immunotherapies while avoiding off-tumor iRAEs in mice.

It has been shown by Nguyen et al. that tumor-seeking bacteria provide a way to recruit M1-leaning macrophages in the TME by secreting macrophage-recruiting chemokines [12]. However, it is unknown whether tumor-seeking bacteria can be used to target intracellular pathways in TAMs to convert M2 to an M1 phenotype [15, 16]. Here, we consider targeting the cyclic GMP-AMP synthetase (cGAS)-stimulator of interferon genes (STING) pathway with recombinant tumor-seeking bacteria.

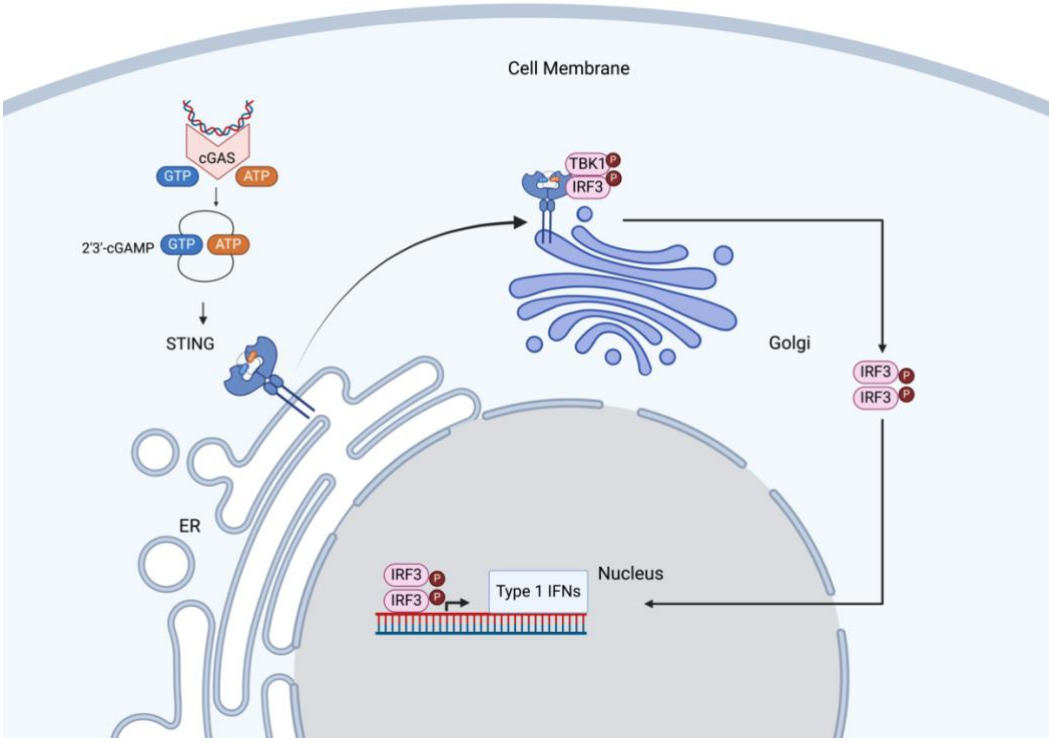


Figure 1. Schematic of the cGAS-STING Pathway. cGAS detects foreign dsDNA and converts 1 GTP and 1 ATP to 2'3'-cyclicGAMP. This compound mediates the dimerization of STING in the membrane of the rough ER. Dimerized STING recruits and phosphorylates TBK1 and IRF3 which leads to IRF3 dimerization and the upregulation of type 1 interferons, via its transcription factor-like activity. Figure created using Biorender.

The cGAS-STING pathway (Figure 1) responds to dsDNA in the cytoplasm; cyclic GMP–AMP synthase (cGAS) converts GTP and ATP into 2'3'-cGAMP which mediates the dimerization of STING. This complex then recruits and activates TANK-binding kinase (TBK-1), which in turn recruits and activates interferon regulatory factor-3 (IRF-3). This will activate downstream signaling pathways, upregulating various type 1 interferons [16, 17]. These pathways can be hijacked for the conversion of TAMs to a tumor non-supportive phenotype [17].

A limitation of these engineered bacteria is that it can be difficult for them to target the cGAS-STING pathway efficiently, due to its intracellular nature. However, it may be possible to circumvent this issue by using an extracellular Contractile Injection System (eCIS). The bacterial genus *Photorhabdus* relies on a type VI secretion system, known as the *Photorhabdus* virulence cassette (PVC), that works by targeting eukaryotic cells, via a weakly attractive phage tail, to inject them with a toxic protein payload known as Pdp1 [18, 19]. The weak attraction of this phage tail limits its ability to deliver proteins to eukaryotic cells.

However, Krietz et al has shown the PVC can be manipulated to inject numerous cell-types, such as macrophages or hippocampal neurons, with a customized protein-payload via the addition of a nanobody motif to the phage tail [19] (Figure 2A). In order to be utilized for injection, the protein-payload must contain a highly disordered region on the N terminus; this "packaging-region" has been shown to be necessary for non-native protein delivery [19]. Therefore, by adding this "packaging-region" domain to an exogenous protein (Figure 2A), bacteria can be engineered to intracellularly deliver proteins to any cell-type.

Here, we propose the utility of a constitutively active cGAS payload in converting macrophages into an M1-leaning phenotype. The cGAS-STING pathway has been difficult to target with high efficiency due to its intracellular nature; therefore, we propose utilizing PVC-expressing *E. Coli* in order to increase this efficiency. PVCs will be engineered to target cluster of differentiation 206 (CD206), a well-known M2 macrophage marker, to deliver a cGAS-STING payload to TAMs [15]. We consider delivering constitutively active cGAS to the macrophage via the PVC system to convert them from an M2-leaning phenotype to an M1-leaning phenotype. We hypothesize that this novel method may be more effective in targeting this pathway than previous methods.

In summary, we propose utilizing the facultative anaerobe *E. Coli* as a "tumor-seeking" vessel to deliver intracellularly cGAS to activate the pro-inflammatory M1 phenotype in TAMs [4, 10-14]. In order to achieve this, we will exploit the type-VI bacterial secretion system to engineered to be expressed in the recombinant *E.Coli*. Upon administration to melanoma-bearing mice, we expect that these M1 macrophages will become increasingly detectable within tumor samples, post-treatment. We further expect that tumor weight may decrease alongside an increase in serum IFN levels. Finally, we will monitor the survival rates of these treated mice in order to validate this treatment.

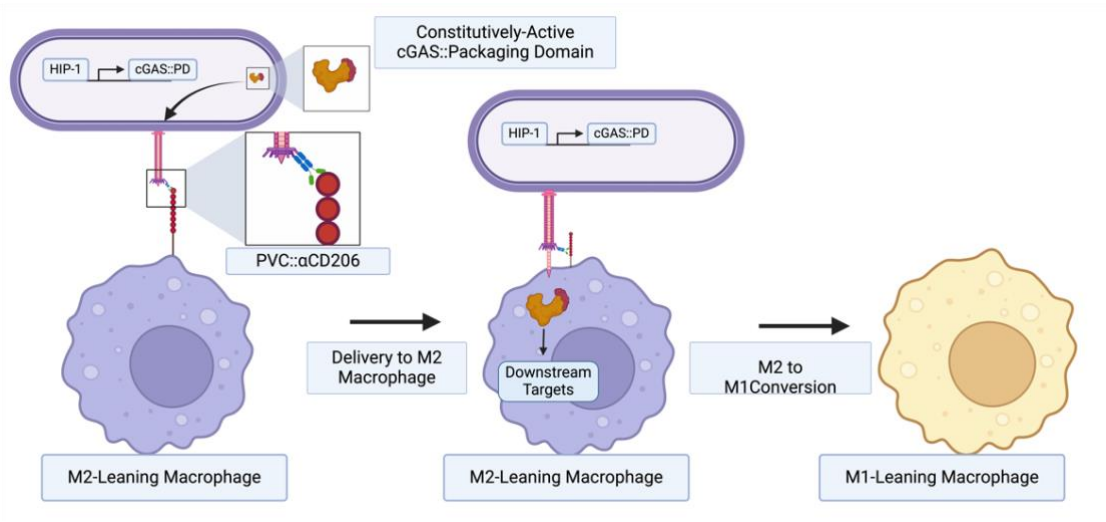
Methods

Bacterial Engineering

The PVC and protein payload will be generated using separate plasmids, as described previously [18, 19]. These plasmids can be generated separately using Cas-9 assisted targeting of chromosome segments (CATCH) method [21] to clone the large PVC gene cluster in one step, as was done previously [18, 19]. This method uses Cas9 to excise a large genomic segment from a bacterial genome before recombining it with a cloning vector; this recombination may be mediated by an overlapping 30bp segment, created by overlap extension PCR, in a Gibson reaction. *Photorhabdus* bacteria will be embedded into agarose gel plugs and spCas9 will be used to excise the PVC fragment, which will be isolated and used to generate plasmids. Payload plasmids will be modified, using CATCH and Gibson/Golden gate assembly to express a GFP::'packaging region' protein construct.

PVC structural, accessory, and regulatory regions will be synthesized de novo as previously described [19]. To target TAMs, PVCs with an anti-macrophage (anti-CD206) nanobodies will be attached to their phage tails (PVC::αCD206) (Figure 1). GFP::'packaging region' constructs will be generated chimerically using recombination. Expression would be driven under the hypoxia inducible-promotor *HIF-1* to prevent premature activation of the injection system [11]. In the context of in-vivo experiments, we consider using a Focused ultrasound (FUS) cassette to prevent macrophage activation within intrinsically hypoxic regions of the body (Figure 2B) [22].

A



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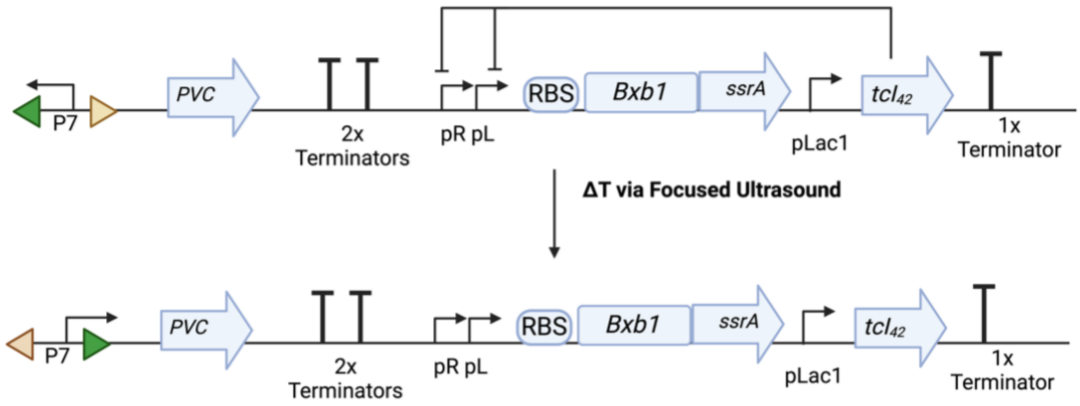

Figure 2. Schematic of Bacterial Administration to Macrophage. (A) The hypoxic TME drives the expression of cGAS::Packaging-Region, through HIP-1. PVC:: αCD206 binds CD206 and facilitates injection of constitutively active cGAS::Packaging-Region construct into the macrophage. Delivery of constitutively active cGAS mediates expression of genes characteristic of M1-like phenotype. (B). We utilize the focused ultrasound (FUS) cassette previously described to upregulate PVC in the TME only [21]. tcI inhibits Bxb1 expression until FUS removes this inhibition. Bxb1 expression then recombines the constitutively active P7 driver in order to upregulate PVC structural and accessory proteins. We may consider using multiple P7 promotors to drive the large 19Kb PVC cassette. Terminators prevent unwanted expression. Figures created with Biorender.

In Vitro Macrophage

To determine whether proteins are delivered intracellularly into macrophages, we will utilize an in vitro assay whereby an immortal murine CD206+ macrophage cell line (BMA3.1A7) will be exposed to 50uL of PVC and GFP::Packaging-Region expressing engineered-bacteria under low pH conditions (pH=6.0); this low pH will activate *HIF-1* promotor. We will seed, fix, and permeabilize 3.5×10^5 BMA3.1A7 in 0.5mL of corresponding media before blocking them using bovine serum albumin to prepare for immunohistochemistry. Engineered bacteria will be administered to some wells as a negative control, while others will receive GFP-expressing bacteria.

E.Coli successfully expressing the recombinant plasmid will be detected by Dylight Chicken anti-GFP (600-901-215) and macrophages will be detected via rabbit anti-mouse CD68 antibodies (28058-1-AP). The signal will be further amplified using the secondary antibodies specific to the species of the primary antibodies, Dylight Donkey anti-chicken Alexa Fluor 488(A78948) and Goat anti-rabbit Alexa Fluor 555(A-21428) secondary antibodies, respectively.

Following incubation, macrophages will be washed and treated with antiseptic solution to reduce excess bacterial presence. They will be imaged within the wells using confocal microscopy (LSM-800) at 400X magnification, with 30um along the Z-stack. Images will be analyzed using Fiji software. The average baseline GFP intensity will be calculated from the imaging background and subtracted from the intensity of the stained sample. Median GFP intensity will be compared across samples.

Delivery of cGAS in Vitro

We will perform an assay on TAMs which we will generate from human tissue samples [23]. CD14+CD16+ monocytes will be isolated from the blood of 4 healthy human donors using a monocyte isolation kit (Stem Cell Technologies). Monocytes will be differentiated into M2-leaning macrophages using IL-4 as previously described [23].

We will use 2 controls: no bacteria and *E. Coli* K-12 MG1655 (ATCC47076) prepared by inoculating a single colony in 30mL of LB medium. Our wells will contain differentiated macrophages. We will treat these samples with bacteria for 2 hours in an anaerobic chamber; we will then identify the type of macrophages in these wells using flow cytometry.

Macrophage Phenotyping using Flow Cytometry

Flow cytometry will be used to assess the frequency and absolute counts of M1 macrophages (CD68+ iNOS+) to M2 macrophages (CD206+ CD163+) across treatment conditions [24]. Antibodies will be sourced from Arigo biolaboratories M1/M2/TAM Antibody panel kit (ARG 30333) FlowJo software will be used to draw gates to define macrophages and gain percentage and absolute values.

Delivery of cGAS in Vivo

7-week-old male and female mice are provided by Jackson Laboratory and housed in the animal facilities in a temperature-controlled room with a normal diet. B16.F10 melanoma cell line (CRL-6475) will be administered to these mice and tumors will be allowed to grow for 22 days before being euthanized alongside a final blood collection via cardiac puncture. Mice will then be randomized into the following treatment groups: *E. coli* expressing cGAS and PVC components driven by the FUS promotor, native *E. coli* as a negative control, smSTING agonist as a positive control, or saline as a negative control. These treatments will be delivered to B16.F10 melanoma-bearing mice both intratumorally, and systemically, via the tail vein. Furthermore, we will administer 50uL of *E. coli* expressing cGAS and PVC components to non-tumor bearing mice without applying FUS.

A signal generator (B&K #4054B) generates the ultrasound signal, which is then amplified (AR #100 A250B) and transmitted to the transducer. The water bath acts as the coupling medium for the ultrasound wave. A Velmex BiSlide motorized positioning system submerges and positions a 0.67 MHz FUS transducer (Precision Acoustics PA717) such that its focal point lies within the tumor [22]. Signal will be administered for a 2-hour period by alternating the heat between 37°C and 47°C at 5-minute intervals, this will be done 3 hours after treatment with bacteria [22].

We will monitor mice weight over time, end-point survival, as well as tumor weight at the end of the treatment period to quantify its efficacy. We will measure the frequency and absolute counts of macrophages via flow cytometry.

Between our treatment groups, we will measure changes in serology markers using a cytokine detection kit (Milliplex). We will specifically assess pro-inflammatory M1 cytokines, such as interferon beta (IFN-β), IL-1β, and IL-12 and anti-inflammatory canonical M2 markers, such as IL-10 and transforming growth factor-beta (TGF-β). Raw data will be analyzed with Luminex.

Statistics

Statistical analysis will be conducted using Graphpad Prism 9 software. Data will be subjected to Shapiro-wilk tests to identify normality. Comparisons between groups will be conducted using paired or unpaired Student's t tests when data are normally distributed or Mann-Whitney test when non-parametric. Comparisons between multiple groups will be performed using a one-way analysis of variance (ANOVA) with Tukey's multiple comparisons test in parametric data. Kruskal-Wallis one-way ANOVA with Dunn's pairwise comparison test will be used with non-parametric data.

Results

Delivery of GFP to Macrophages in Vitro

As previously published, PVCs are anticipated to be effective at delivering protein payloads to the macrophages [19]. In our proposal, the PVC is modified by adding αCD206 motif on the phage tail to enable delivery of GFP payload to CD206+ expressing macrophages [4, 15]. Initial testing with immortalized CD206+ macrophage co-culture system with *E.Coli* containing the recombinant plasmid with GFP or empty vector, is expected to deliver the GFP signal into the macrophages, as detectable by immunohistochemistry. Macrophages exposed to antibodies blocking CD206 while incubated with recombinant *E.Coli* is expected to have a diminished intracellular GFP signal. We expect that some intracellular GFP may be due to the phagocytosis of GFP expressing bacteria independent of the Type-IV secretion pathway. Therefore, we will use GFP-expressing plasmid without Type VI secretion as a baseline, relative to treatment.

We also anticipate that some bacteria may be attached to the surface of the macrophage following incubation, potentially leading to a false positive result. We opted to

use confocal microscopy to discriminate the GFP signal within and outside of the macrophage membrane, using the z-stacks.

Delivery of cGAS in Vitro

While proteins of various sizes, from the 38kDa Cre to the 160kDA spCas9, have been shown to be deliverable [18], we will confirm whether the ~59kDA cGAS protein is capable of being delivered to TAMs. We expect that *E.Coli* expressing constitutively active cGAS with a packaging domain (Figure 2) can deliver cGAS to TAMs. We further hypothesize that this will alter their immunological programming and causing the upregulation of M1-associated genes, similar to other cGAS agonists [14, 16].

We will characterize this change by observing a decrease in the expression of M2 markers (CD206+ CD163+), alongside an increase in the expression of M1 markers (CD68+ iNOS+) using both flow cytometry and cytokine profiling. We hypothesize that the frequency and absolute numbers of M1 macrophages (CD68+ iNOS+) will increase while the frequency and absolute numbers of M2 macrophages (CD206+ CD163+)) will decrease relative to the total macrophage population, after treatment with the recombinant *E.Coli* expressing cGAS. We expect to see no changes in the frequencies and absolute numbers of tumor carrying mice that are untreated compared to those that are treated with empty vector *E. coli*.

Efficacy of recombinant *E.Coli* in melanoma mouse model

Tumor-seeking bacteria may be described as "programmable factories" that can deliver therapeutics into TME. The TME is an attractive ecological niche for these anaerobic bacteria due to the hypoxic conditions. Upon reaching the target destination, the hypoxic environment will activate the *HIF-1* promotor, driving the expression and production of cGAS, and the PVC components. However, we cannot exclude that the *E.Coli* may migrate to other hypoxic regions of the body, like the bone marrow and cartilage.

To attempt to circumvent this issue for off-target effects, we further consider using an ultrasound promotor (Figure 2B) to drive the structural and accessory components of the PVC [18, 22]. This promotor works by taking advantage of the heat generated by FUS to knock TCL42 off the PR and PL promotors, thereby allowing the expression of the BXB1 integrase which inverts the P7 promotor, mediating PVC expression.

We expect that the PVC expressing *E. Coli* will lead to the greatest decrease in tumor size, while also resulting in less serum IFNs than a systemically delivered smSTING agonist. We will further administer 50uL of PVC-expressing *E. coli* solution to a cohort of normal mice without applying FUS in order to identify whether any iRAEs occur independent of FUS activation. We expect to see no change in the severity of these iRAEs; highlighting the specificity of FUS activated immunotherapy.

The tumor weight will be monitored and compared across samples using a one-way ANOVA with Tukey's multiple comparison test on day 20; we expect tumor weight will be significantly lower in the FUS group than in all other treatments. survival rate over 90 days will also be compared across samples using a censured mantel-cox log ranked comparison, with the expectation that the FUS group will have the longest survival rate. Additionally, we will find the M1/M2 ratio in the tumor samples and will compare between groups using a one-way ANOVA with Tukey's multiple comparison test, anticipating that this ratio will be the highest in the FUS group. Finally, we will monitor serum levels of IFNs and compare them across samples using a one-way ANOVA, we hypothesize that this will be increased in the FUS group. We expect that this treatment will impede the growth of cancer, with minimal off target iRAEs, by directly programming the TAMs of the tumor microenvironment to an anti-tumor phenotype.

Discussion

The use of tumor seeking bacteria in immunotherapy is an attractive, emerging new cancer treatment option worth further exploration. Recent studies have shown their utility in locally delivering anti-PDL1 and anti-CTLA4 nanobodies to impede tumor growth [10]. Similarly, there are methods to target cGAS-STING for potent tumor-specific immunomodulation [14]. Here we consider a novel way to activate macrophage cGAS using a PVC which has the potential to increase the efficiency of cGAS-STING targeting, as well as provide a method by which intracellular protein-therapeutic delivery can occur within tumors.

The tumor-specific delivery of constitutively active cGAS is predicted to be able to convert macrophages from a tumor-supportive, M2, phenotype to a tumor non-supportive M1 phenotype [15, 16, 17]. This precise mechanism behind this shift is still being investigated, but it's clear that an increase in cGAS upregulates M1 markers, like CD86, while downregulating M2 markers, like CD163, resulting in an M1-leaning phenotype that is known to create an unfavorable environment for cancer cell growth [17, 25].

A limitation of this treatment is the capacity of FUS to upregulate the PVC components due to impartial heating of the tumor; however, this can be overcome with clinical FUS systems [26]. Alternatively, *HIF-1* can be used to drive the expression of the PVC components; however, this runs the risk of cGAS delivery to macrophages in hypoxic areas, like the bone marrow or cartilage. Off-target effects are a limitation of these bacterial therapeutics, and it is uncertain whether using this recombinant synthetic biology technology would work in other cancers. Extensive preclinical testing is necessary to identify and limit the

extent of these effects. Furthermore, cancer patient heterogeneity may lead to not all patients expressing high

levels of CD206+ macrophages to be targeted. Alternative receptors, such as CD163 may be considered.

cGAS is one of many targets that can modulate the TAMs. Cancer is ever evolving; as such, a wide toolkit of treatment options is necessary to tackle it effectively. Here, we design a commensal therapeutic with the potential to deliver therapeutic proteins to specialized cells in localized anatomical regions. This establishes a pathway that future studies may follow to design commensals that can intracellularly deliver therapeutic proteins to specific cells within the TME.

Conclusion

This proposal explores a novel cancer immunotherapy product exploiting the potential of tumor-seeking bacteria to drive pro-inflammatory M1 macrophage phenotype by intracellularly targeting the cGAS-STING pathway using a naturally prevalent bacterial secretion system. This biotechnology has the potential to deliver a plethora of proteins specifically to cells with diminished off-target cytotoxicity, and establishes a novel mechanism to intracellularly deliver therapeutics to cells within the TME [9]. Ultimately, we hope that this foundational technology would expand the therapeutic toolbox available for fighting cancer in an easily reproducible manner.

List of Abbreviations

CD: cluster of differentiation
cGAMP: cyclic GMP-AMP
cGAS: cyclic GMP-AMP synthetase
eCIS: extracellular contractile injection system
FUS: focused ultrasound
IFN: interferon
IL: interleukin
IRF: interferon regulatory factor
PVC: *photorhabdus* virulence cassette
STING: stimulator of interferon genes
TBK-1: TANK binding kinase-1
TAMs: tumor associated macrophages
TGF: transforming growth factor
TME: tumor microenvironment

Conflicts of Interest

The author declares that he has no conflict of interests.

Ethics Approval and/or Participant Consent

All experimentation will be conducted in accordance with the Canadian Council for Animal Care.

Authors' Contributions

MJ: contributed to study design and planning, drafted the manuscript, and gave final approval of the version to be published.

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