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Early Treatment Schedule Optimization of Trained-Immunity Mediated Immunotherapy of Non-Muscle Invasive Bladder Cancer (NMIBC): A Research Protocol

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Abstract

Introduction: Bacillus Calmette-Guérin (BCG) is commonly used as an immunotherapeutic agent following tumour resection in high-risk non-muscle invasive bladder cancer patients. Past studies have shown that BCG confers non-specific innate immune reprogramming, resulting in altered innate immune responses following a secondary antigen challenge. This evidence for innate immune memory has resulted in the identification of a phenomenon known as trained immunity. Recent studies have described the beneficial effects of BCG immunotherapy in terms of trained immunity acquisition and have identified key pro-inflammatory cytokines as trained immunity markers. However, previous studies have not analyzed the impacts of alteration of the standard BCG immunotherapy schedule on acquisition of trained immunity. It is hypothesized that accelerating the BCG treatment schedule will result in greater acquisition of trained immunity, marked by increased levels of IFN-γ and IL-2.

Methods: The proposed protocol will make use of a lab-generated murine NMIBC model. The model will be verified via ultrasonography and intravesical BCG instillations will follow both standard and accelerated immunotherapy schedules. A multiplex assay, targeting 40 pro-inflammatory cytokines, will be employed to measure the levels of cytokines in the plasma, and cytokines produced by PBMCs following secondary stimulation with antigen *in vitro*.

Anticipated Results: It is anticipated that ultrasonography will confirm successful generation of the NMIBC murine model. Furthermore, it is expected that the acceleration of BCG instillations will cause upregulation of pro-inflammatory cytokines, specifically IL-2 and IFN-γ, after week 1 of treatment. Following the BCG treatment, greater induction of trained immunity, marked by the upregulation of IL-1β, IL-2, TNFα, and IFN-γ, is expected relative to untreated controls.

Discussion: The quantitative results of the multiplex cytokine assay will be used to conduct 1-way ANOVA tests to compare several parameters; cytokine expression will be compared between time points, treatment groups, and sampling methodologies.

Conclusion: Results of the proposed study will guide future research on BCG immunotherapy by identifying potential points of optimization for the BCG treatment schedule of bladder cancer. Assessment of non-classical cytokines along with classically defined trained immunity cytokines will aid future studies in tailoring their assays for determining acquisition of trained immunity.

Keywords: immunotherapy; bladder cancer; bacillus calmette-guérin (BCG); trained immunity; NMIBC

Introduction

Global statistics identify cancer as the leading cause of death in most countries throughout the world [1]. GLOBOCAN data for the year 2020 present a total cancer incidence of about 19 million worldwide [1]. Among the 36 cancer types documented by GLOBOCAN, bladder cancer (BC) contributed 3% to the overall cancer incidence in 2020 [1]. In Canada, bladder cancers rank as the 5th most common type of cancer, translating to about 8% of total cancer incidence [2]. Bladder cancers can be broadly classified into two types: non-muscle invasive bladder cancer (NMIBC) or muscle invasive bladder cancer (MIBC) [3]. Of all bladder cancers diagnosed, 4/5 patients present with NMIBC however, nearly a quarter of these patients develop MIBC which is more likely to metastasize aggressively and result in mortality [3]. Furthermore, a maximal 85% chance of recurrence of NMIBC after treatment puts patients at greater risk of developing the more lethal MIBC [4].

Transurethral resection of bladder tumour (TURBT) in combination with intravesical administration of chemotherapeutic or immunotherapeutic agents is the current standard of care for NMIBC patients [3, 5]. Immunotherapies are treatments that target the cells of the immune system, with the goal of modulating their activity to enhance tumour clearance [6, 7]. Immunotherapies typically involve the use of substances that alter the immune microenvironment, polarizing immune signaling which drives immune cell activity [6]. For patients with high-risk NMIBC, intravesical administration of Bacillus Calmette-Guérin (BCG), an immunotherapeutic agent, is the treatment

of choice following TURBT [5]. BCG originated in the early 1920s as a result of hundreds of *Mycobacterium bovis* (*M. bovis*) recultures, conducted by Albert Calmette and Camille Guérin [5]. Originally, BCG was found to have a protective effect against tuberculosis infection in both animals and humans, and, to this day, is the only tuberculosis vaccine that is commercially available [5].

In vertebrates, the innate immune system is the first line of defence against pathogens, which include endogenously derived immune challenges (like tumours). Innate immune cells use cell surface receptors called pattern recognition receptors (PRRs) to recognize pathogen-associated (or danger-associated) molecular patterns (PAMPs or DAMPs respectively) [8]. Recognition of PAMPs or DAMPs by PRRs induces the activation of a network of signalling molecules which activate the innate immune cells including macrophages, dendritic cells (DCs) and neutrophils [8]. For many years, it was thought that immunological memory was solely a characteristic of the adaptive immune system, however current evidence suggests that innate immune cells have some memory capacity [9]. Trained immunity (TI) refers to the long-term, heterologous, modulation of innate immune responses following preliminary challenge with antigen [7, 10, 11]. The immunological memory generated via TI is due to epigenetic changes of genes that regulate pro-inflammatory phenotypes, unlike the somatic recombination that occurs in the adaptive immune system [9]. TI is identified by the increase in pro-inflammatory cytokines upon non-specific secondary stimulation, following a return to baseline cytokine secretion [11].

BCG is a vaccine used for prevention of tuberculosis in humans and evidence shows that it has the capacity to train the innate immune system [12, 13]. Past research has demonstrated that administration of the BCG vaccine also confers non-specific protection from various other pathogens in the patient [12, 13]. In the lens of NMIBC immunotherapy, intravesical administration of BCG into the bladder, after TURBT increases the proportion of patients remaining tumour free after surgery [14]. This has been attributed to BCGs ability to train innate immune cells. BCG-trained macrophages have been found to switch to a pro-inflammatory M1 phenotype after instillation [15]; an increase in pro-inflammatory cytokines IL-1β, IL-2, IL-6, IL-8, IL-10, IFN-γ, and TNFα have also been observed [11, 16]. The standard treatment regimen involves post-operative intravesical BCG instillation on a weekly basis for 6 weeks. If the patient is observed to remain tumour free, a maintenance schedule ensues, involving 6-week instillations of BCG every 1-3 months [5]. When peripheral blood mononuclear cells (PBMCs), from patients who did not have the TI phenotype, were analyzed by Graham et al. before and after the standard BCG treatment schedule, the level of pro-inflammatory cytokines produced by PBMCs upon secondary stimulation with LPS was varied, with no distinct increase identified [11]. Furthermore, analysis of plasma levels of cytokines revealed no difference between pre- and post-BCG treatment [11]. Interestingly, a study conducted by Jackson et al. found that some beneficial cytokines such as IL-2 and IFN-γ do not appear in the urine until later in the BCG treatment schedule [16]. Considering these findings, TI was found to increase rates of complete remission among NMIBC patients [11]. Since innate immune memory is associated with heightened levels of pro-inflammatory cytokines, optimizing the initial BCG treatment schedule to maximize TI-induced upregulation of pro-inflammatory cytokines may be a valid strategy in improving patient outcomes. This proposed protocol aims to test the effectiveness of increasing the number of intravesical BCG instillations in the first week of treatment in a syngeneic NMIBC murine model, via combined *in vitro* and *in vivo* analyses. To establish the NMIBC murine model, female C57BL/6 mice will be catheterized with MB49 murine bladder cancer cells. MB49 cells originated from the C57BL/6 murine strain, making it a suitable tumorigenic agent in C57 mice, with a transformation success rate from 30-100% [17, 18]. The *in vitro* analysis will serve as an assessment of cytokine levels produced by PBMCs from NMIBC mice after secondary stimulation with lipopolysaccharide (LPS). LPS is a bacterial PAMP which is a ligand of PRRs on innate immune cells, like PBMCs [9]. The *in vivo* analysis will assess plasma cytokine levels of NMIBC mice. It is hypothesized that increasing the number of BCG instillations in the first week of treatment will result in a significant increase in pro-inflammatory cytokine production over the standard treatment schedule and will result in the appearance of IL-2 and IFN-γ earlier in the treatment schedule.

Methods

Setup and Verification of the NMIBC Murine Model

NMIBC will be induced in female C57BL/6 mice via catheterization with MB49 murine bladder cancer cells at a concentration of 5×10^5 cells/mL. Prior to catheterization, mice will be deprived of water for 6 hours to prevent urinary leakage after inoculation. The inoculum will be allowed to dwell in the mice for 2 hours and will be subsequently evacuated. Visual confirmation of tumorigenesis will be conducted via high-frequency ultrasonography (US) (Vevo F2, Fujifilm VisualSonics) after 14 days post-inoculation following methodology outlined by Chan et. al. [15]; subsequently TURBT will be performed. A control group will be established of which mice will be inoculated with phosphate-buffered saline (PBS) instead of MB49 cells.

BCG Treatment Schedule and Sampling

Following TURBT, mice will be split into 6 groups with 6 biological replicates per group. Following the methodology of Grossman et al., the first group will be enrolled in a standard BCG treatment schedule involving 8 mg/mL intravesical instillations of BCG, once a week, for 6 weeks following tumour resection [18]. The second group will be placed on an accelerated BCG treatment schedule,

with intravesical administration; 8 mg/mL BCG will be administered on 3 consecutive days in the first week of treatment. Following the first week, mice in group 2 will be returned to the standard BCG treatment schedule. Finally, negative control groups will receive intravesical instillations of PBS instead of BCG; four negative control groups will be established, broadly categorized by whether mice were first inoculated with MB49 cells or PBS. In both categories, mice will be further split to follow either one of the two treatment schedules described above. Therefore, the control groups will be as follows: i) PBS-inoculated mice, catheterized with PBS following the standard schedule, ii) PBS-inoculated mice, catheterized with PBS following the accelerated schedule, iii) MB49-inoculated mice, catheterized with PBS following the standard schedule and, iv) MB49-inoculated mice, catheterized with PBS following the accelerated schedule.

Sampling of PBMCs and blood plasma will be conducted at 4 time points among all groups, relative to the initiation of BCG treatment: i) before initiation (Day 0), ii) after week 1 (Day 7), iii) after week 3 (Day 21), and iv) after week 6 (post-BCG treatment, Day 42). Sufficient blood will be extracted from the saphenous vein to support both plasma and PBMC analyses. PBMCs and plasma will be isolated using a density gradient based separation technique in Lymphoprep™ media (STEMCELL Technologies, Catalog # 07801). Isolated PBMCs will subsequently be cryopreserved in CryoStor® CS10 media (STEMCELL Technologies, Catalog # 07952) instead of a lab-produced dimethyl sulfoxide-fetal bovine serum (DMSO-FBS) solution to limit potential contamination. Isolated plasma will be stored at –20°C following recommendations from the World Health Organization (WHO) [19].

Mouse monocytes will be isolated via negative selection using magnetic bead-based isolation via the EasySep™ separation system (STEMCELL Technologies, Catalog # 19861). Isolated monocytes will be plated on 96-well plates at 1 x 10^5 cells per well and will be stimulated with 10 ng/mL LPS (Sigma Aldrich, Catalog # L5293) following the procedure of Graham et. al. [9].

Sample & Data Analysis

Monocyte culture supernatants and plasma sample proteins will be analyzed using a quantitative, 40-target, pro-inflammatory cytokine multiplex assay (Abcam, catalog # ab197469). The multiplex assay will target the typical cytokines associated with TI including IL-1β, IL-2, IL-6, IL-10, IFN-γ and TNFα; the assay will also cover other pro-inflammatory cytokines that may be affected as a result of inducing TI with BCG therapy. The proposed multiplex assay has a limit of detection (LOD) of 3.33x standard deviations above the mean background, and a limit of quantification of 10x standard deviations above the mean background.

Data will be analyzed using GraphPad Prism software (GraphPad Software, USA); cytokine expression levels will be normalized to a reference multiplex assay for each condition, mean expression among biological replicates will be calculated for each sampling time point, and 1-way ANOVA tests will be performed for each of the TI and other quantified cytokines. Statistical testing will be performed across groups at each time point, within groups across time points.

Figure 1 presents a summary of the key points described in the above methodology, in a graphical format.

Results

Generation of the murine NMIBC model is anticipated to be around 90% successful, following a procedure outlined by Chan et al. It is expected that US images will reveal presence of superficial bladder tumour in mice at the 14-day mark, as previously conducted analyses have identified tumorigenesis via US at the 11-day mark [17].

Raw data from multiplex assays, will be presented in the form of protein expression levels in monocyte culture supernatants and plasma. Based on past literature, it is expected that TI will be induced in groups treated with BCG and, levels of TNF-α and IL-1β will be increased in monocytes and plasma over control groups [12, 16]. That is, greater expression of IL-1β, IL-2, IL-6, IL-10, IFN-γ, and TNFα [11, 16]. A previous study conducted by Graham et. al. in human NMIBC patients, lacking the TI phenotype, discovered no significant changes in pro-inflammatory cytokines released by monocytes pre- and post-BCG treatment [11]. Thus, it is anticipated that monocytes of the BCG treatment groups will have higher expression of pro-inflammatory cytokines relative to their respective negative controls.

It is anticipated that mice receiving BCG treatment on an accelerated schedule will express higher levels of IL-2 and IFN-γ in blood plasma after the first week of treatment, compared to week 1 samples of all other treatment groups; past studies have observed that these beneficial cytokines, that indicate acquirement of TI, appear only later in the standard BCG treatment schedule [16]. Similarly, upon secondary challenge with LPS, monocytes from mice in the accelerated BCG schedule are expected to produce more pro-inflammatory cytokines after week 1 of treatment, relative to the negative control and standard BCG treatment groups. Midway through treatment, it is expected that samples from the accelerated schedule produce similar cytokine expression profiles as day 42 samples from the standard schedule. In other words, it is anticipated that the accelerated treatment schedule will reach maximal pro-inflammatory cytokine expression sooner than the standard treatment schedule.

Finally, it is expected that expression of pro-inflammatory cytokines will either plateau or taper off near the end of the time course. For both treatment schedules, it is anticipated that levels of IL-1β, TNFα and IFNγ approach a limit based on previous studies conducted by Graham et. al. and van Puffelen et. al [11, 12].

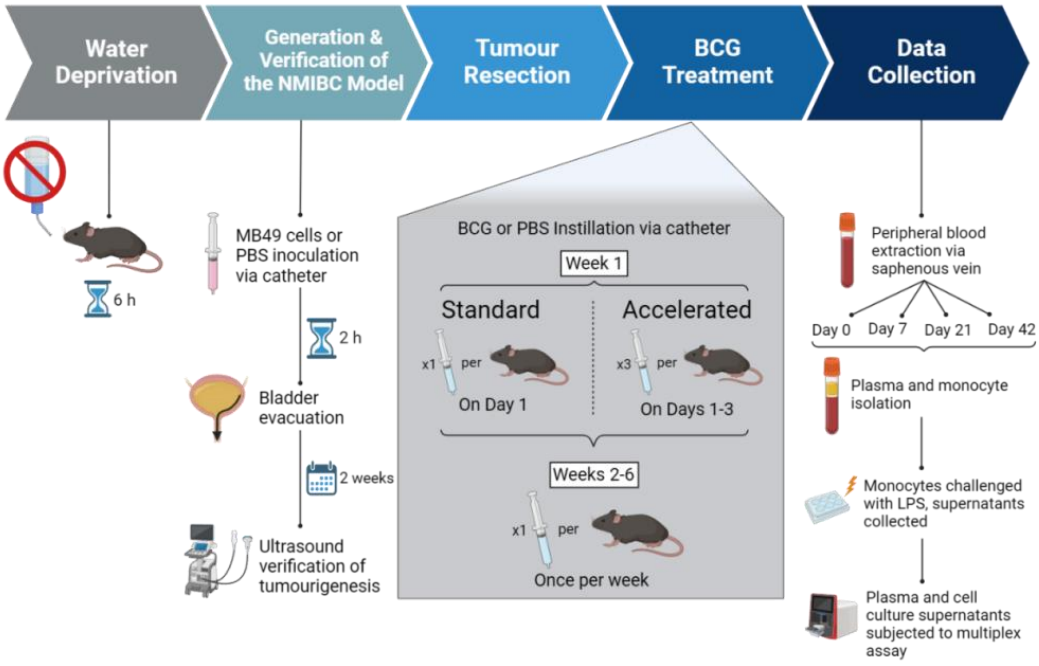

Figure 1. Graphical Summary of the Proposed Methodology. The methodology is split into 5 parts. Before the study begins, mice are subject to water deprivation for 6 hours. After water deprivation, mice will be inoculated with either MB49 cells or PBS and will be allowed to evacuate themselves after a 2 hour dwell time. Two weeks of development will be allowed, and tumorigenesis will be determined via ultrasonography. For mice in the group which received MB49 cells, tumour resection will be performed. Following tumour resection, mice will be enrolled in either standard or accelerated BCG treatment schedules; after week 1 of each schedule, both groups of mice will shift to the standard BCG treatment timeline. Finally, peripheral blood from the saphenous vein will be collected and both monocytes and plasma isolated. Monocytes will be stimulated with LPS in vitro. Cell culture supernatants and previously collected plasma samples will be subjected to a multiplex assay for pro-inflammatory cytokines. NMIBC – non-muscle invasive bladder cancer; BCG – Bacillus Calmette-Guérin; PBS – phosphate buffered saline. Created with BioRender.com.

Discussion

Few studies have conducted a combinatorial *in vivo* and *in vitro* assessment of TI in mice. US imaging will be used to visually assess the appearance of superficial tumour. Following tumorigenesis, the multiplex cytokine assay will allow several key comparisons to be made using 1-way ANOVA tests. Statistical analyses of cytokine expression will allow comparison of sampling methodologies and will serve as an internal validation system for the detection of TI during and after BCG treatment. Since the proposed study focuses on accelerating the BCG treatment schedule as a method to optimize the induction of TI, it inherently involves a time course. Therefore, statistical analyses will be conducted among every time point between, and within each of the groups. The results of the analyses will provide two-fold information on whether accelerated BCG treatment is beneficial to the induction of TI; considering the treatment groups, depending on the extent to which TI is induced at each time point, the results can be interpreted to provide a clear understanding of whether or not accelerated BCG treatments are truly beneficial. Furthermore, the results will provide information on which time point during the treatment schedules will result in the greatest TI phenotype among monocytes and in blood plasma (i.e., greater expression of pro-inflammatory cytokines), and may give secondary insight on BCG treatment scheduling. Similarly, while observation of classically defined TI cytokines via the multiplex assay will be conducted, other pro-inflammatory markers will also be assessed. This will provide information on new biomarkers which may potentially indicate TI acquisition.

Although the proposed study will analyse the extent to which TI is acquired via BCG immunotherapy, it does not include measurements of overall survival (OS), complete remission (CR) rate, and recurrence rate among the samples. In previous studies, BCG has been shown to result in the acquisition of TI and has been observed to reduce recurrence of NMIBC in patients [12, 13, 20]. Thus, the results of the proposed study could be used to imply the effect on recurrence, however future studies should focus on discrete experiments to measure this parameter in relation to TI.

Conclusions

Alteration of the standard BCG treatment schedule is anticipated to provide insight into optimizing the immunotherapy scheduling for NMIBC patients. It is expected that increasing the frequency of intravesical BCG instillations in the first week of immunotherapy, will increase TI acquisition in a murine model. The objective of the proposed research protocol is to build upon the current understanding of the role of TI in mediating the effectiveness of BCG immunotherapy. Furthermore, assessment of both classical and non-classical TI markers may provide a new foundation for which future studies on TI can experiment on. Guided by the current proposed research protocol, future studies should assess TI in relation to CR and OS of patients or samples.

List of Abbreviations

BC: bladder cancer
BCG: bacillus calmette-guérin
CR: complete remission
DMSO: dimethyl sulfoxide
FBS: fetal bovine serum
LOD: limit of detection
LOQ: limit of quantification
LPS: lipopolysaccharide
MIBC: muscle invasive bladder cancer
NMIBC: non-muscle invasive bladder cancer
OS: overall survival
PBMC: peripheral blood mononuclear cell
PBS: phosphate-buffered saline
TI: trained immunity
US: ultrasonography
WHO: world health organization

Conflicts of Interest

The author declares that they have no conflicts of interest.

Ethics Approval and/or Participant Consent

The proposed study will require approval from a qualified Research Ethics Board and will follow the guidelines set by the Canadian Council of Animal Care.

Authors' Contributions

DAS: made substantial contributions to the design and concept of the proposed study. Drafted and critically revised the manuscript and gave final approval of the version to be published.

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