Harnessing T-cell activity against prostate cancer: A therapeutic microparticulate oral cancer vaccine

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A B S T R A C T

Prostate Cancer specific immunotherapy in combination with immune stimulating adjuvants may serve as a viable strategy for facilitating tumor regression and preventing recurrence. In this study, an oral microparticulate vaccine encapsulating tumor associated antigens (TAA) extracted from a murine prostate cancer cell line, TRAMP-C2, was formulated with the help of a spray dryer. Microparticles were characterized in vitro to determine their physicochemical properties and antigenicity. Formulated microparticles had an average size of 4.92 ± 0.5 μm with a zeta potential of 7.92 ± 1.2 mV. In order to test our formulation for its ability to demonstrate adequate antigen presentation and co-stimulation, microparticles were tested in vitro on murine dendritic cells. In vitro biological characterization demonstrated the activation of specific immune system markers such as CD80/86, CD40, MHC-I and MHC-II. Following in vitro characterization, in vivo anti-tumor efficacy of the oral microparticulate vaccine was evaluated in C57BL/6 male mice. Combination therapy of vaccine microparticles with cyclophosphamide and granulocyte macrophage-colony stimulating factor (GM-CSF) demonstrated a five-fold reduction in tumor volume as compared to non-vaccinated mice. At the cellular level, cyclophosphamide and GM-CSF augmented the vaccine response as indicated by the reduced tumor volume and significant elevation of cytotoxic T-cell (CTL) CD8+ and (T-helper) CD4+ T-cells compared to mice receiving vaccine microparticles alone. Furthermore, our studies indicate a significant reduction in T-regulatory cells (T-regs) in mice receiving vaccine along with GM-CSF and cyclophosphamide, one of the immune escape mechanisms linked to tumor growth and progression. Thus, oral microparticulate vaccines have the potential to trigger a robust anti-tumor cellular response, and in combination with clinically relevant agents, significantly resist tumor growth and progression.

1. Introduction

Prostate cancer is currently the second leading cause of cancer-related death among men in the United States. According to the American Cancer Society, in 2019 alone, an estimated 174,650 patients will be diagnosed with prostate cancer, and approximately 31,620 patients will succumb to the disease. Metastatic castration-resistant prostate cancer is an advanced stage of prostate cancer observed in patients who have undergone surgery for removal of primary tumor. Currently, there are 6 new agents approved in the United States against prostate cancer which include Sipuleucel-T, apalutamide, cabazitaxel, abiraterone acetate, enzalutamide and radium-223. The introduction of these agents into the clinic is a significant achievement in medicine; however, resistance to chemotherapeutic drugs is still a significant challenge which limits the treatment options available to patients [1,2]. Moreover, the median survival rate in therapy with these agents for patients with castration resistant prostate cancer is in the range of 3–5 months [3].

Cancer immunotherapy is gaining steady momentum following the introduction of Chimeric Antigen Receptor T-cell (CAR-T) therapy and antibodies against immune-suppressive mechanism such as anti-PD1 and anti-CTLA4 [4]. With appropriate clinical guidance and therapy, these medications may greatly enhance the chances of combating the progression of cancer. However, the development of cell-based therapies often involves cumbersome ex vivo culture, stimulation and expansion followed by reintroduction into

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patients (4). TAAs extracted from whole tumor cells possess advantages such as, presenting a pool of antigens from the patient's tumor and activating both CD4^{+} and CD8^{+} T-cells [5]. GVAX-PCa is an example of whole cell tumor vaccine comprising of two allogeneic cell lines LNCaP and PC-3 secreting GM-CSF. GVAX-PCa presents a pool of tumor antigens to the host immune system to recognize and initiate anti-tumor activity. Phase I/II studies on GVAX-PCa proved excellent safety with no incidence of auto-immune responses [6].

Successful cancer immunotherapy hinges on the ability of the patient's immune system to activate macrophages/dendritic cells against the tumor, process tumor antigens, initiate a T-cell response and overcome immune tolerance mechanisms exhibited by tumor cells [7]. Delivery of protein antigens possess several challenges such as protein degradation, inability to be sampled for antigen presentation and inefficient prime-boost responses as pure solutions [8]. Therefore, adjuvants have been utilized along with several protein antigens to boost immune responses. Particulate delivery systems can activate the immune system at the target site, present antigens to antigen presenting cells and protect the antigen from degradation mechanism *in vivo* [9]. The surface chemistry of polymer microparticles/nanoparticles can also be modified to have specific targeting ligands and release the antigens slowly at the intended target site for improving the probability of antigen sampling and immune activation [9]. Therefore, particulate delivery systems not only act as efficient carriers of antigens but may also overcome the requirement of adjuvants currently used in vaccines [10]. Once formulated into delivery systems such as nano/mircoparticles with specific surface chemistries, particulate vaccines can be administered by several non-invasive routes such as inhalation through the lungs and the oral route [10,11].

Oral vaccines for polio [12] and cholera [13] have been administered globally and are a promising strategy to overcome the challenges associated with parenteral vaccinations. Antigen delivery systems for oral administration must overcome challenges such as harsh gastro-intestinal pH and enzymatic conditions [14], efficient uptake by M-cells for antigen sampling, activation of the mucosal, humoral and cellular immune responses and more importantly development of oral tolerance [15]. Particulate delivery systems have the potential to overcome these abovementioned challenges and deliver antigens to the Peyer's patch in the intestine [16,17,18].

The inclusion of immune stimulators such as GM-CSF along with a cancer vaccine platform GVAX® is currently under investigation for the treatment of prostate [19] and pancreatic [20] cancer. GM-CSF is a hematopoietic growth factor and an immune system modulator which has profound effects on functional activities of several circulating leukocytes such as monocytes, lymphocytes and neutrophils to potentiate host defense. GM-CSF also acts as an immune adjuvant by enhancing macrophage activity and increasing dendritic cell maturation/function [21].

Cancer biology is extremely complex; however, researchers have identified a subset of cells that are immuno-suppressive called T-regulatory cells (T-regs). They are implicated in enhancing tumor escape mechanism among other mechanisms such as loss or down-regulation of MHC-I and release of immunosuppressive cytokines [31]. Cyclophosphamide (Cy) is an alkylating agent which has been studied for its immune modulatory properties at low doses. In normal individuals, T-regs play an important role of maintaining self-tolerance and avoiding auto-immunity, however, in case of malignancies, T-regs are recruited by tumors for immune evasion. This renders the patient's immune system incapable of fighting the tumors. Hence, depletion of T-regs may lead to an enhanced anti-tumor immune response [22].

This study addresses several challenges associated with current cancer therapies such as resistance to hormone therapy,

chemotherapy and cross resistance between hormone and chemotherapy for prostate cancer. The paper describes the design and evaluation of an oral microparticulate vaccine against prostate cancer. *In vitro* studies were performed to characterize the vaccine formulation while *in vivo* studies were performed on TRAMP-C2 (TC-2) murine prostate cancer model to delineate the mechanisms at the cellular level. In addition to evaluation of the particulate delivery system we have discussed the effect of two immune-potentiating agents (Cy and GM-CSF) which are used in the clinic as therapies for treatment of malignancies. Hence, our approach not only presents a novel therapeutic vaccine option but also evaluates the combination effect of two therapeutic agents used for oncology [23]. Henceforth, the term 'vaccine microparticle' indicates the use of tumor-associated antigens (TAA) extracted from TC-2 murine prostate cancer cells encapsulated in microparticles.

2. Materials and methods

2.1. Materials

TC-2 cell line was obtained from ATCC, (Manassas, VA) and grown in Dulbecco's Modified Eagle Medium (DMEM), Sigma Aldrich, St. Louis, MO. Fetal bovine serum was purchased from Atlanta biologicals, Atlanta, GA. 1% Penicillin-Streptomycin was purchased from Cellgro, Manassas VA. Four to six weeks old C57BL/6 mice were purchased from Charles River Laboratories, Wilmington, MA. GM-CSF was purchased from Reprokine, Valley cottage, NY. Hydroxypropylmethyl cellulose (HPMCAS AQOAT) and Ethyl cellulose (Aquacoat ECD) was purchased from FMC Biopolymers, Philadelphia, PA. Aleuria aurantia lectin (AAL) was obtained from Vector Labs Inc., Burlingame, CA. Recombinant murine interleukin, IL-2 (5×10^6 units/mg) was purchased from eBiosciences Inc., San Diego, CA. All flow cytometry markers (murine IgG targeted against MHC-I, MHC-II, CD86, CD40, CD80, CD4^{+}T-cells and CD8^{+}T-cells) were purchased from eBiosciences Inc, San Diego, CA. Cy was purchased from Sigma Aldrich, St. Louis, MO. Bradford protein assay kit was obtained from Life Technologies, Grand Island, NY. Micro-BCA protein assay kit was obtained from Thermo Scientific, Rockford, IL. Recombinant murine IL-2 was obtained from PeproTech, Rockhill, NJ. DC 2.4 cell line was obtained from Dr. Kenneth L Rock at the Dana-Faber Cancer Institute, Boston, MA. All other materials used were of analytical grade.

2.2. Preparation of TAA extracted from TC-2 cells

Murine prostate cancer TC-2 cells were cultured in Dulbecco's modified eagle's medium (DMEM) with 4 mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate and 4.5 g/L glucose supplemented with 0.005 mg/ml bovine insulin and 10 nM dehydroisoandrosterone, 90%; fetal bovine serum, 5%; Nu-Serum IV, 5%. Cells were treated with hypotonic buffer (10 mM Tris and 10 mM NaCl) and subjected to five 15 min freeze–thaw cycles at temperatures of −80 °C and 37 °C respectively to extract the tumor associated antigens (TAA) from TC-2 cells (21). To confirm cell lysis and extraction of TAAs from the TC-2 cells, trypan blue exclusion assay was performed. The extracted TAAs were stored in −80 °C for future use.

2.3. Formulation of vaccine microparticles

Spray drying technique was utilized to prepare vaccine microparticles as described elsewhere [24,25]. Briefly, 100 mg of ethyl cellulose was suspended in water and along with 15 mg HPMCAS. 4 mg chitosan glycol was added to the mixture along with 10 mg of trehalose. 0.25%AAL and 0.1% tween-20 was added

to the formulation. Distilled deionized water was added to obtain a final volume of 100 ml. Vaccine microparticles were formulated by incorporating 10%w/w of the TAA. The final aqueous solution was adjusted to pH 7.4 and spray dried using Buchi B-290 spray dryer. The inlet temperature was maintained at 120 °C while the outlet temperature was maintained at 75 °C. The feed rate was set at 20 ml/h while the nozzle diameter was 0.7 mm. The nozzle was cooled at −5 °C using circulating cooler and the resulting microparticles were stored in −20 °C for future use. Blank microparticles were prepared using polymers and excipients alone without any antigens.

2.4. Characterization of vaccine microparticles

Microparticles were imaged with the Phenom® Desktop scanning electron microscope. Briefly, spray dried microparticles were placed on carbon sheets and imaged at 2300×, 5 kV. Malvern Zetasizer ® was used to characterize size and zeta potential of the microparticles. Entrapment efficiency was calculated using the Bradford protein assay following extraction of the TAAs in PBS. Release study of TAAs from microparticles was performed in simulated murine gastric pH (pH3) and intestinal pH (pH5) conditions for 7 h which is greater than the gastro-intestinal transit time in mice. Release samples were analyzed using Micro BCA assay. MTS assay was performed on murine macrophage cell line RAW 264.7, 1% Triton X-100-media mixture was used as positive control.

2.5. In vitro antigenicity of vaccine microparticles on murine dendritic cells

Murine dendritic cells (DC 2.4) were grown and maintained in DMEM with glucose, L-glutamine, and 10% FBS. DC 2.4 cells were passaged at around 70% confluence and seeded in 12 well plates and incubated at 37 °C overnight in 5% carbon dioxide in an incubator. Briefly, 400,000 murine dendritic cells were incubated with 0.3 mg of blank microparticles, vaccine solution and vaccine microparticles for 24 h. Following incubation, the supernatant was collected for nitric oxide assay the cells were washed three times with cold PBS and analyzed for expression of CD40, CD80/86, MHC-I and MHC II using anti-mouse IgG-FITC or PE markers by flow cytometry analysis as per manufacturer's protocol.

2.6. Prostate cancer induction in C57BL/6 mice

In vivo studies were performed on 4–6-week-old male C57BL/6 mice, obtained from Charles River Laboratories, Wilmington, MA. All animal experiments were carried out as per protocols approved by Mercer University's institutional committee for the care and use of laboratory animals. In brief, 5×10^6 live TC-2 cells suspended in media without fetal bovine serum and injected subcutaneously on the back. Tumors were grown until palpable following which tumor volumes were noted using Vernier calipers. Mice were monitored weekly for signs of discomfort and were euthanized at the end of 8 weeks or when tumors were ulcerated. Tumor volume was measured by the following formula, V = 1/2 (length) (width)².

2.7. Oral microparticulate vaccine immunization protocol

The anti-tumor efficacy of vaccine microparticles were evaluated in the C57BL/6 male mice model. Animals (n = 6) were administered with blank or vaccine microparticles using an oral gavage. The animals were administered one prime dose of vaccine microparticles (equivalent to 500 μg of antigens) followed by 3 booster doses bi-weekly. In brief, vaccine microparticles were

suspended in 10 mM citrate buffer pH 4 and administered to the mice using an oral gavage. 50 mg/kg Cy was administered intraperitoneally through a 27G × 1/2 needle (0.4 mm × 13 mm) 24 h prior to oral vaccination. 5 μg GM-CSF was administered through a 27G × 1/2 needle (0.4 mm × 13 mm) needle subcutaneously prior to oral dosing. The untreated group (n = 6) was only injected with TC-2 tumor cells without any treatment, this served as one of our control groups for analysis of T-regulatory (T-reg) cell populations only.

2.8. Determination of T-cell populations in C57BL/6 mice following oral immunization

Mice were euthanized at the end of the study and immune organs were collected as per the IACUC protocol approved by Mercer University. Spleen and lymph nodes of mice were harvested aseptically and pooled in groups of two. Secondary immune organs were gently passed through a cell strainer resulting in a single cell suspension. Erythrocytes were lysed from the cell suspension with the help of ammonium chloride, potassium bicarbonate and EDTA buffer. The cells were then stimulated in 10U/ml recombinant murine IL-2 overnight in complete RPMI. The following day, cells were labelled with murine IgG anti-CD4+-FITC, anti-CD8+-FITC or anti-CD4+-CD25+-APC markers to stain specific cell populations. In case of CD4+-CD25+Foxp3+T-cell, manufacturer's staining protocol was followed. Flow Cytometry analysis was performed on BD Accuri ™ C6 by BD Biosciences, San Jose, CA. All reagents and standards were purchased for flow cytometry was purchased from BD Biosciences and utilized as per manufacturer's protocol.

2.9. Statistical analysis

All statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test or student's t-test. GraphPad Prism 6 (trial version 6.0, GraphPad software, La Jolla, CA) was utilized to perform all statistical analysis. $*p < 0.05$, $**p < 0.001$, $***p < 0.0001$.

3. Results

3.1. Preparation of TAA extracted from TC-2 cells

TC-2 cells were subjected to five freeze- thaw cycles, total TAA concentration was determined to be 2 mg/ml. This concentration was considered for formulation of 10% w/w of antigen loading in microparticles.

3.2. Characterization of vaccine microparticles

Microparticle yield from the spray drying process for the vaccine formulation was observed to be 80% w/w. The average size of the microparticles was 4.92 ± 0.5 μm, however the size distribution of the microparticles ranged from 1 to 5 μm. Vaccine microparticles had a net positive charge of $+7.92 \pm 1.2$ mV. SEM image (Fig. 1) of the microparticles were observed to be irregular in shape with surface indentations. Entrapment efficiency of the TAA antigen was approximately $78 \pm 2.6\%$. Release of TAAs from microparticles was delayed at acidic pH which would help deliver the vaccine in the intestinal segment of the GI tract. The half-life of release was approximately 3 h (Fig. S1) (supplemental). Microparticles did not exhibit any cytotoxicity at concentrations as high as 2.5 mg/ml when incubated with macrophages for 24 h (Fig. 2).
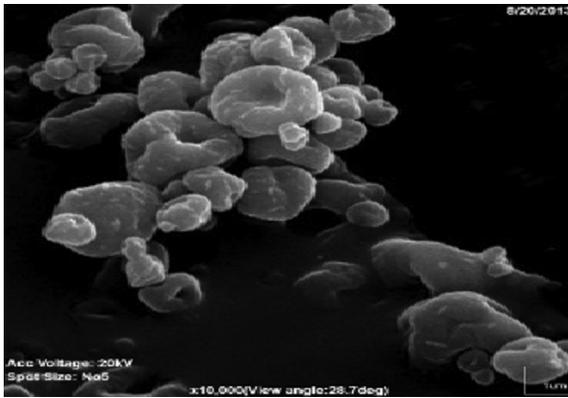
Fig. 1. SEM image of vaccine microparticles. (5 kV, Zoom: 2300×). Microparticles exhibit irregular doughnut shape and a particle size between 1 and 5 μm. Irregular particles result in better uptake into antigen resenting cells.
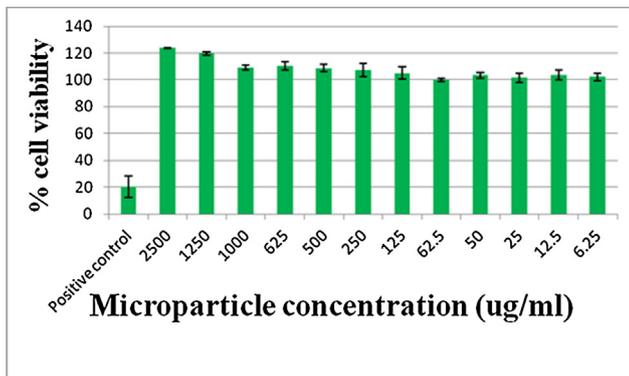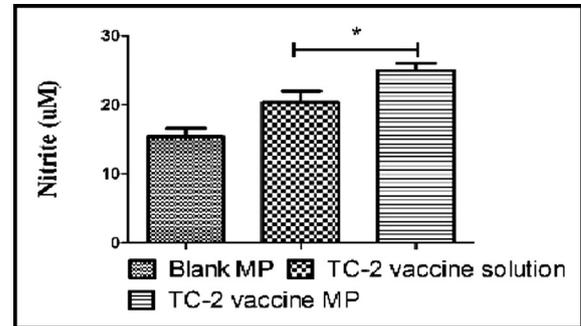

Fig. 3. Release of nitric oxide from DC 2.4 cell line following incubation of Blank microparticles (MP), TC-2 vaccine solution and TC-2 vaccine MP. Vaccine MP significantly increased the release of nitric oxide compared to vaccine solution (*p < 0.05). Blank microparticles did not elicit a significant increase in nitric oxide compared to solution group.

Fig. 2. Cytotoxicity of vaccine loaded microparticles on murine macrophage cell line RAW 264.7. 20,000 RAW 264.7 cells were plated overnight and treated with varying concentrations of vaccine microparticles for 24 h and percent live cells were calculated using the MTS assay. Vaccine microparticles were non-toxic at concentrations of 2.5 mg/ml. In the positive control group, the cell were treated with 1% Triton-X100 in media.
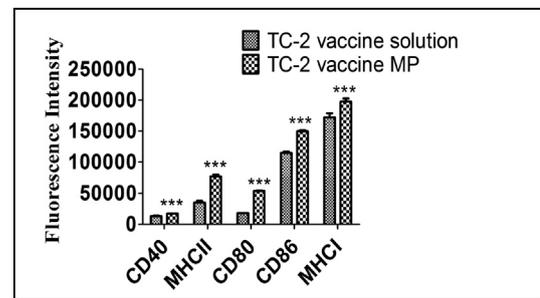

Fig. 4. Expression of CD86, CD40 and Major Histocompatibility Complex (MHC)-II, MHC-I and CD80 on DC 2.4 cell line following incubation with vaccine solution and TC-2 vaccine microparticle (MP) (***p < 0.0001). DC 2.4 cells were incubated with 500 μg vaccine microparticles for 24 h. Vaccine solution (50 μL of 1 mg/mL) was used as control groups. Vaccine MP significantly increased the expression of CD86, CD80, MHC-I and MHC-II and CD40 (p < 0.0001). Blank microparticles when tested did not give any significant increase in expression of antigen presentation or co-stimulatory markers compared to vaccine solution alone indicating minimal effect on the immune response (data not shown).

3.3. In vitro antigenicity of vaccine microparticles on murine dendritic cells

Vaccine microparticles significantly increased (p < 0.05) the release of nitric oxide from murine dendritic cells as compared to vaccine solution and blank microparticles (Fig. 3). Since the release of nitric oxide from dendritic cells was not observed in the blank microparticle group, further in vitro studies with this group were not carried out. Antigen presentation (MHC-I and MHC-II) and co-stimulatory molecule expression (CD86, CD40 and CD80) was (Fig. 4) significantly elevated (p < 0.0001) in TAA encapsulated microparticles as compared to the TAAs in solution (Fig. 4). Thus, TC-2 microparticles may help in activating both CD4+T-cells and CD8+T-cells when administered to mice in vivo.

3.4. Microparticulate vaccine reduces tumor volume in murine prostate cancer model

C57BL/6 mice administered with oral vaccine microparticles demonstrated significant (p < 0.05) reduction in tumor volume as compared to blank microparticle treated group (Fig. 5). Moreover, when mice were administered with a combination of GM-CSF and Cy along with vaccine microparticles, the tumor volume was approximately 5-fold lower as compared to the group treated with vaccine microparticles alone (week 8). Furthermore, at week 8, the
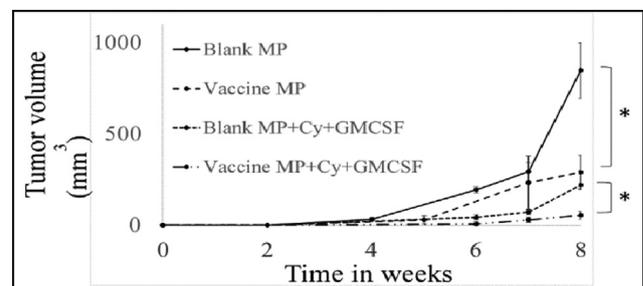

Fig. 5. Tumor volume data of blank microparticles (Blank MP), Cyclophosphamide (Cy) (50 mg/kg), Oral vaccine microparticles (Vaccine MP) and combination of vaccine with cyclophosphamide (Cy) and GM-CSF. Vaccinated mice demonstrate significant (p < 0.05) reduction in tumor volume compared to their respective control groups. Combination treatment of Cy and GM-CSF along with vaccine MP significantly reduced tumor growth compared to vaccine MP and other control groups (*p < 0.05) (n = 6).

tumor volume for mice treated with vaccine microparticles along with GM-CSF and Cy was approximately two times lower as compared to the group treated with blank microparticles in combination with GM-CSF and Cy (control). In fact, two out of six mice in the vaccine microparticle with Cy and GM-CSF had negligible tumor volumes compared to any other group at week 8.

3.5. Vaccine microparticles in conjunction with GM-CSF and Cy augment anti-tumor immune responses

Following tumor volume measurement for 8 weeks, animals were euthanized for analysis of T-cell populations in secondary lymphoid organs. CD4$^+$ and CD8$^+$ T-cells from spleen and lymph nodes were found to be elevated in mice treated with vaccine microparticles (with/without GM-CSF and Cy) compared to their respective control groups. Cells extracted from lymph nodes demonstrate elevated counts of CD4$^+$ and CD8$^+$ T-cells in mice treated with vaccine microparticle alone and vaccine microparticle administered with GM-CSF and Cy (Figs. 7 and 9). However, CD4$^+$ and CD8$^+$ T-cell counts in splenocytes were significantly ($p < 0.001$) elevated in mice treated with vaccine microparticle in conjunction with GM-CSF and Cy as compared to vaccine microparticles alone (Figs. 6 and 8). CD4$^+$CD25$^+$Foxp3$^+$ T-cells (T-regs) were analyzed in mice treated with blank microparticles, vaccine microparticles and vaccine microparticles administered with GM-CSF and Cy. T-regs were found to be significantly ($p < 0.0001$) reduced in mice receiving vaccine microparticles in conjunction with GM-CSF and Cy (Fig.S2). The reduction of T-regs in the GM-CSF and Cy treated groups along with elevation of CD4$^+$, CD8$^+$ T-cells with superior anti-tumor efficacy confirms the role of immune suppressor cells and the importance of inhibiting such immune suppression mechanisms to elicit a robust anti-tumor response.

4. Discussion

TAAs extracted from whole tumor cells elicit a strong anti-tumor immune response primarily owing to the presence of several uncharacterized tumor-associated antigens which can activate the innate and adaptive immune system against tumors [14,24,25]. Therefore, for successful oral vaccination of the extracted antigens, formulation of a delivery system is crucial. Intestinal uptake of microparticles following oral vaccination has been well documented to be effective in activating the immune system [17,25,26]. The microparticulate formulation in this study had a particle size of $4.92 \pm 0.5\ \mu m$ with a net positive change on the microparticle. Studies from De Jesus et al. demonstrated glucan microparticles size less than $5\ \mu m$, on oral administration, were sampled by CD11+ dendritic cells in the Peyer's Patch [27]. Particle size of the delivery system plays a crucial role in delivery of the antigen payload to the M-cells in the intestine. Studies have shown that microparticles greater than $10\ \mu m$ have significantly reduced uptake following oral administration while microparticles less than $5\ \mu m$ were found to redistribute into the spleen. Moreover, $5\ \mu m$ sized particles were found to have the highest antigen payload [28]. For oral delivery of vaccines, particulate delivery systems are essential as they help protect the antigens from harsh pH and enzymatic environments. The TC-2 vaccine microparticulate delivery system was successful in protecting the antigen payload from the harsh acidic pH environment and was optimized to
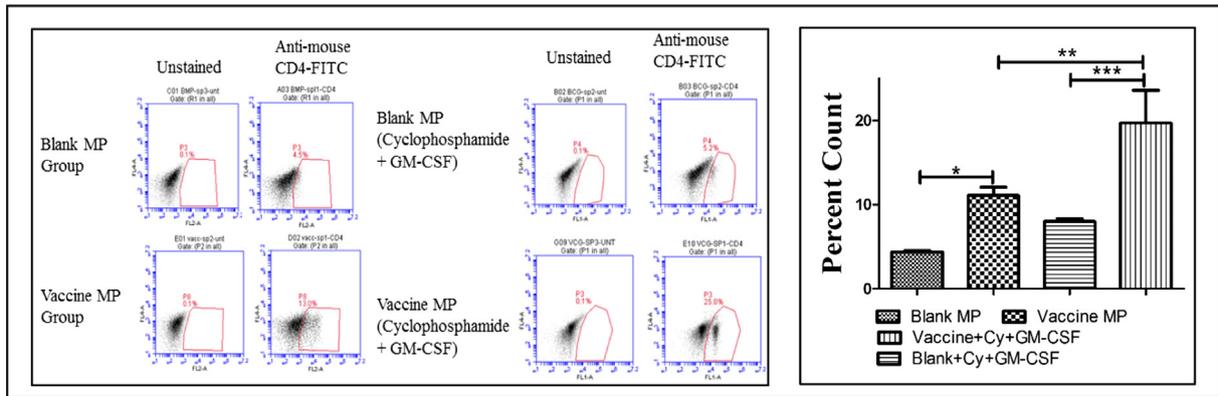
Fig. 6. Percent CD4$^+$ T-cell counts from isolated spleen in mice treated with blank microparticle (MP) and vaccine MP groups (n = 6) (with and without cyclophosphamide and GM-CSF therapy). Vaccine MP treated groups significantly increased CD4$^+$ T-cell counts as compared to blank microparticles. Furthermore, vaccine MP group treated with Cy and GM-CSF demonstrated significant increase in CD4$^+$T-cell counts as compared to vaccine MP alone and their respective controls. (Cy: Cyclophosphamide) (*$p < 0.05$, **$p < 0.001$, ***$p < 0.0001$).
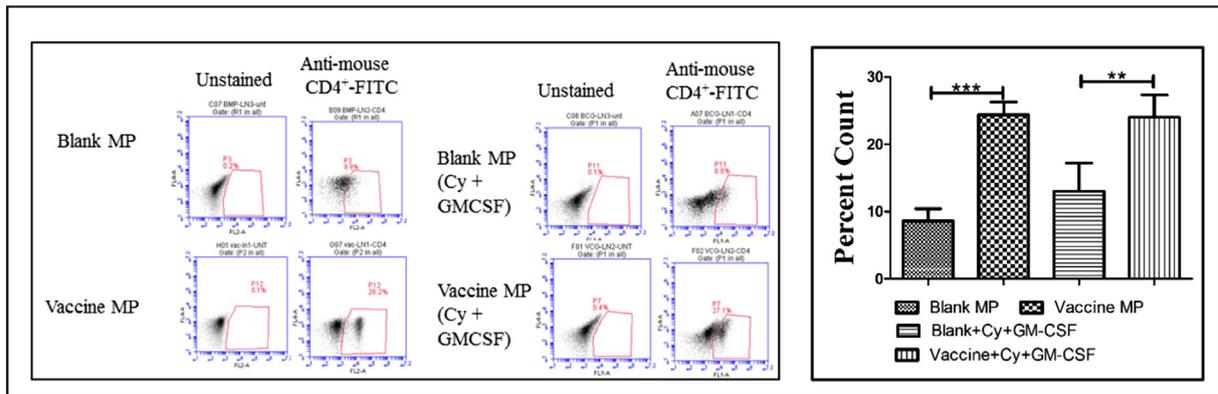
Fig. 7. Flow cytometry graphs and percent CD4$^+$ T-cell counts from isolated lymph nodes in mice treated with blank microparticle (MP) and vaccine MP groups (n = 6) (treated with and without cyclophosphamide and GMCSF therapy). Vaccine MP treated groups (with and without cyclophosphamide and GMCSF therapy) significantly increased helper T-cell counts (Cy: Cyclophosphamide) (*$p < 0.05$, **$p < 0.001$, ***$p < 0.0001$).
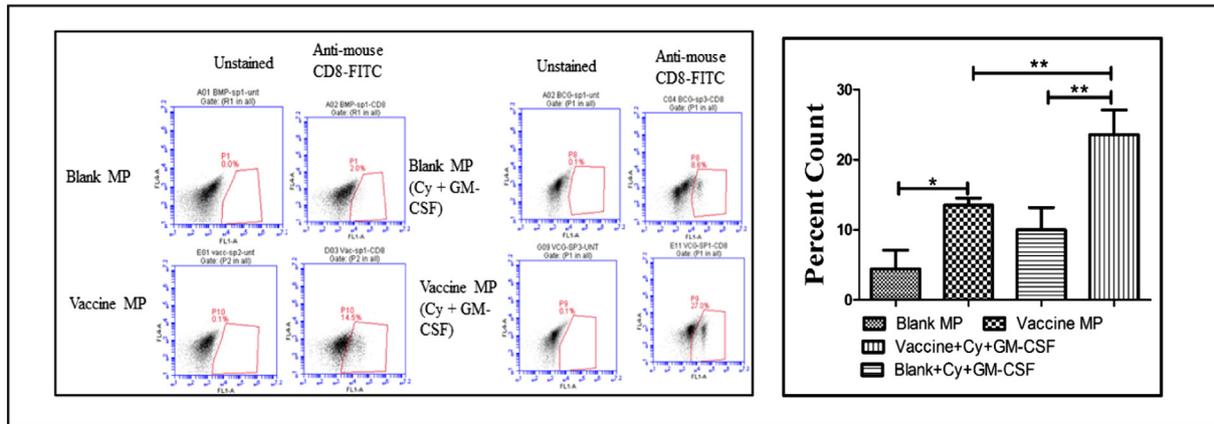
Fig. 8. Flow cytometry graphs and percent CD8$^+$ T-cell counts from isolated spleen in mice treated with blank microparticle (MP) and vaccine MP groups (n = 6) (with and without cyclophosphamide and GM-CSF therapy). Vaccine MP treated groups significantly increased CD8$^+$ T-cell counts as compared to blank microparticles. Furthermore, vaccine MP group treated with Cy and GM-CSF demonstrated significant increase in CD8$^+$T-cell counts as compared to vaccine MP alone and their respective controls. (Cy: Cyclophosphamide) (*p < 0.05, **p < 0.001, ***p < 0.0001).
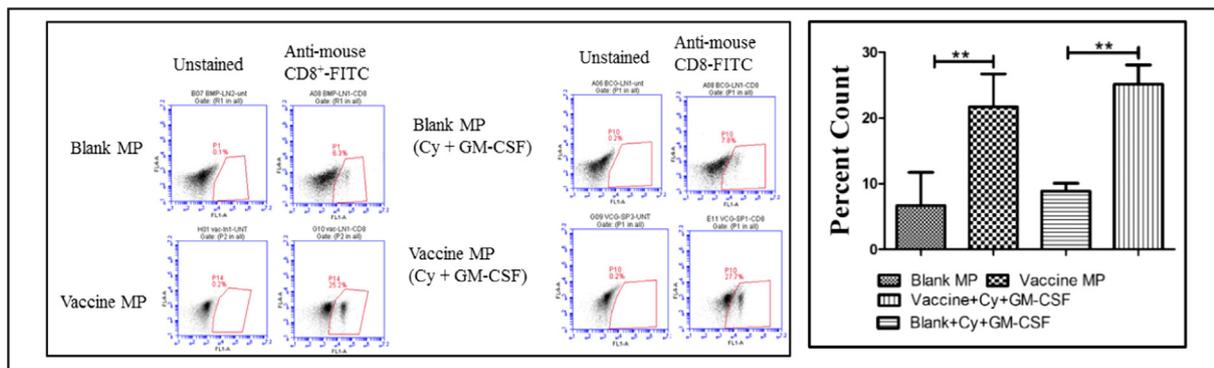

Fig. 9. Flow cytometry graphs and percent CD8$^+$ T-cell counts from isolated lymph nodes in mice treated with blank microparticle (MP) and vaccine MP groups (n = 6) (treated without and with cyclophosphamide and GM-CSF therapy). Vaccine MP treated groups (without and with cyclophosphamide and GM-CSF therapy) significantly increased CD8$^+$ T-cell counts. (Cy: Cyclophosphamide) (*p < 0.05, **p < 0.001, ***p < 0.0001).

deliver the payload in the intestine for activation of the immune system.

Following physicochemical characterization of the microparticles, we performed extensive *in vitro* characterization to test the antigenicity of the delivery system. Vaccine microparticles were efficient in up-regulating the expression of several important immune system markers. Elevated levels of nitric oxide were observed in vaccine treated groups thus signifying activation of the innate immune system which could further lead to adaptive immunity against cancer antigens [46]. Another critical aspect of initiating a sustained and tumor-specific immune response is the process of antigen presentation and expression of co-stimulatory molecules. Specifically, TC-2 TAA loaded microparticles significantly (p < 0.0001) up-regulated the expression of CD80/86, CD40, MHC-I and MHC-II. CD 80/86 up-regulation is associated with activation of T-cells. The activation of both CD80 and CD86 leads to release of IL-4, IL-5 and IFN-γ which can modulate the immune system to act via the Th1 and Th2 pathway [29]. Enhanced expression of MHC-I and CD80 in the vaccine microparticle group suggest that subsequent CD8$^+$ T-cell activation (*in vivo*) may enable initiation of a tumor specific immune response. Researchers have documented the importance of CD8$^+$ T-cell activation and their ability to directly lyse tumor cells [25,30]. However, activation of CD4$^+$ and CD8$^+$ T-cells have been confirmed to have sustained anti-tumor effects [31]. The expression of CD40 and MHC-II is associated with activation of CD4$^+$ T-cells which in-turn is essential in

cytotoxic T-cell (CTL) priming. Studies have demonstrated that the expression of CD40 on APCs can directly activate cytotoxic T-cells without the need for T-helper cell activation. Moreover, when CD40L was blocked, it leads to inhibition of CTL priming [32]. Co-stimulatory molecule expression in cancer therapy is extremely crucial, inefficient interaction of CD40, CD80/86 on dendritic cells with CD40L and CD28 on T-cells respectively, leads to T-cell anergy and production of IL-10 which is associated with complete suppression of T-cell activity [33].

Following *in vitro* studies of vaccine microparticles, *in vivo* studies were conducted on a murine prostate cancer model. *In vivo* observations were consistent with other publications in which the response time following oral vaccination was observed on or after 5–6 weeks [14,16,18,34]. Mice receiving blank microparticles demonstrated steady increase in tumor volume, however, vaccinated mice were observed to have significant (p < 0.05) resistance to tumor growth. An important and noteworthy observation was the reduced tumor volume observed in mice treated with blank microparticles along with Cy and GM-CSF. It is likely that blank microparticles with Cy and GM-CSF non-specifically act to boost the immune system, however, as observed in Fig. 5, there was a steady increase in tumor volume in the blank microparticle group treated with Cy and GM-CSF over 8 weeks. It is likely that the tumor overcomes this non-specific effect of Cy and GMCSF (with blank microparticles) thereby leading to increased tumor volumes and poor remission of the malignancy. Vaccine microparticles

when administered with Cy and GM-CSF demonstrated robust and significant tumor remission. This is an important observation and evidence that suppression of certain immune-suppressive mechanism (T-regs) in cancer is important and that the use of chemotherapeutic drugs and "immune potentiators" alone may not be adequate to combat the tumor.

Specific T-cell activation is crucial in developing effective immuno-oncology therapies and current therapies such as CAR-T therapy specifically activate the T-cells against tumor antigen for recognition and lysis [4]. Elevated cellular levels of CD8$^+$T-cells and CD4$^+$T-cells correlate well to the difference in tumor volume between the groups. CD8$^+$T-cells have been directly linked to anti-tumor activity due to their ability to kill tumor [35,36]. Similarly, CD4$^+$T-cells have been documented to help CTL activation against tumors [31]. In several *ex vivo* studies, human DC were exposed to polymer microparticles loaded with autologous tumor lysates from patients, which demonstrated significant IFN-γ, IL-6 and IL-1β production and low levels of IL-10. These cytokines skew the immune response to a Th1 response [37]. Furthermore, tolerance induction was observed in certain cases where solution tumor lysates were utilized instead of micro/nanoparticles [37]. Therefore, it is likely that encapsulated antigens can overcome tolerance induction, which is a significant concern in cancer therapy [10,37]. One of the most important considerations in single antigen (subunit) cancer vaccines is the possibility of mutations in the tumor and loss of specificity over the duration of the disease, autologous tumor cell lysates overcome this challenge by providing a pool of antigen to the immune system thereby increasing the T-cell repertoire for activating against the cancer tissue [10].

T-cell subsets such as CD4$^+$CD25$^+$Foxp3$^+$ T-cells (T-regs) are responsible for maintaining homeostasis in a healthy individual; however, this subset of T-cells are recruited by tumor cells, thereby leading to immune evasion and subsequent tumor growth. T-regs are also implicated in the down regulation of immune responses to both natural and induced immune system activation [38]. Zhao and colleagues proved that selectivity of a low dose Cy to T-regs primarily stems from reduced intracellular levels of ATP compared to conventional T-cells. T-regs were shown to be sensitive to Cy at concentrations which were non-toxic to splenocytes or CD4$^+$-CD25$^+$Foxp3$^-$ T-cells [39]. Since there are a host of mechanisms involved in cancer progression, combination therapy is often preferred over monotherapy. Scientific studies have proven the effectiveness of combination immunotherapy in several cancers such as prostate, breast and ovarian [40–43]. Studies in patients have demonstrated that combining T-reg inhibition and GM-CSF secreting vaccines leads to proliferation of high-avidity T-cells, associated with prolonged progression-free survival [44].

Resistance to tumor growth, increase in CD4$^+$T-cell and CD8$^+$ T-cell counts and a reduction in T-reg counts, proved the efficacy of the microparticulate vaccine with Cy and GM-CSF therapy to stimulate the immune system specifically against prostate cancer. The utilization of a microparticulate vaccine which can protect the tumor antigens from degradation on oral administration and activate the immune system specifically to combat the malignancy (with/without GM-CSF and Cy) may present a unique way to treat prostate cancer. Wada et al. and colleagues reported a significant tumor reduction when mice were treated with T-GVAX and Cy. The mechanistic findings from this study corroborate the observed findings and further support the need for a combination therapy against prostate cancer [45]. Inclusion of anti-PD1 and anti-CTLA4 therapies in combination with cancer vaccines could be the next step in cancer therapy and our study demonstrates the potential for development of prostate cancer specific anti-tumor efficacy in combination with tumor-suppressive agents.

5. Conclusion

In this study, we have demonstrated the anti-tumor efficacy of an oral microparticulate vaccine containing TAAs extracted from TC-2 in a therapeutic murine prostate cancer model. The particulate delivery system was effective in preventing degradation of the antigen and presenting the antigens to immune cells for anti-tumor activity. Furthermore, we demonstrated the anti-tumor efficacy of two clinically relevant agents (Cy and GM-CSF) in combination with the oral microparticulate vaccine. This approach also addresses the contribution of tumor immune suppression mechanisms and strategies to overcome immune evasion. Thus, combating cancer in a therapeutic setting has many facets that play a crucial role in successful therapy. This study provides an opportunity to further explore oral vaccination with specific cancer antigens that may be utilized for immunotherapy.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgement

Funding

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2019.08.033.

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