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TITLE: Investigation of Genetic and Immune Mechanisms of Response to BCG for Non-Muscle Invasive Bladder Cancer: A Translational Study of S1602

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13. SUPPLEMENTARY NOTES

14. ABSTRACT

We laid significant groundwork in year 1. Multiple IRB and HRPOs were filed and we are nearly completely ready to begin the expression and genomic profiling. We have identified a critical role of gamma-delta T cells, that will allow us to shift experiments from Aim 3 to result in future success of this project.

15. SUBJECT TERMS
Bladder cancer, immunotherapy, tumor response

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
Unclassified	Unclassified	Unclassified	Unclassified	10	19b. TELEPHONE NUMBER *(include area code)*

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1. **INTRODUCTION:** In Year 1 of our team translational science project, we laid the groundwork to achieve success in years 2 and 3. Our collaborative met every other week to discuss research challenges and progress. We successfully developed contracts and IRB submissions that will allow for the transfer of guarded patient related genetic data and specimens for Aims 1-3. Critically, we developed Institutional Regulatory Board (IRB) protocols to send slides for RNA and DNA extraction from Northwestern University (NU) to John's Hopkins (JH) for Aim 2. JH is near completion of their IRB approval to analyze the RNA from these slides. Based on the findings of related work on the topic of BCG response, we identified weaknesses in our approach to Aim 1 and Aim 3 that will be updated in years 2 and 3 to focus on our research on innate and acquired immune activation in response to BCG stimulation

2. **KEYWORDS:** BCG, immune response, transcriptome profiling

3. **ACCOMPLISHMENTS:**

What were the major goals of the project?

1. HRPO submission, Review, and Approval (4 mo) -***80% complete***
2. Assembly, extraction and characterization of retrospective cohort (12 mo) ***100% complete***
3. Identification of S1602 CIS tumors for the Validation Cohort (36 mo). ***0% complete***
4. Variant Analysis of DNA Damage Response Genes (retrospective and validation cohort) ***0% complete***
5. Calculation of Total Mutation Burden (36 mo) **0% complete**
6. Integration of Aim 1 findings with Aim 2 and Aim 3 (36 mo) ***0% complete***
7. Library Creation and Next Generation Sequencing for RNA-Seq- with bioinformatic Discovery (36 mo) ***0% complete***
8. Identification of Tumor sub-types (12-36 mo) ***0% complete***
9. Identification of Immune Signatures (12-36 mo) ***0% complete***
10. Determine predictive value of immune monitoring during BCG therapy. (4-36 mo) **25% complete**
11. Characterize local immune responses across molecular subtypes and validate a predictive signature using BCG-induced cytokines. (4-36 mo**) 25% complete**
12. Test hypothesis that bladder immune cells are influenced by tumor subtype and predict response to BCG. (4-36 mo) ***25% complete***

What was accomplished under these goals?

A. Major Activities

Material Transfer Agreements (MTAs)- MTAs have been established to share tissue between Johns Hopkins (JH), the University of Texas Health San Antonio (UTHSA) and Northwestern University (NU). These documents will allow sharing of materials, including tissue specimens with clinical annotation. These materials will be used for downstream RNA and DNA assays to perform the proposed research.

Protocols written and approved by local Institutional Review Board (IRB) and Human Research Protection Office (HRPO). Eight HRPO documents have been generated or are in the process of approval. These include the creation of HRPO protocols for retrospective and prospective collection of human tissues necessary to perform this research. Except for 1 HRPO, all IRBs are HRPOs are approved at five sites (NU, UTSA, JH, U Colorado and U Washington).

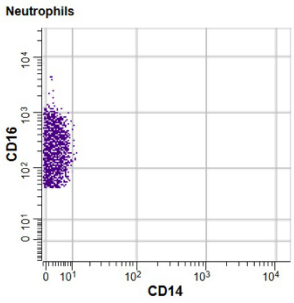
E00220.1a (NU). IRB approved, HRPO approved
E00220.2a (NU) IRB approved, HRPO approved
E00219.1 (UTSA) IRB approved, HRPO approved
E00220.2b (UTHSCSA Site) and E00220.2d (STVHS Site) IRB approved and HRPO approved
Determinants of BCG sensitivity and resistance in bladder cancer. (JH) IRB in revisions
E00220.2c (Fred Hutchinson) IRB approved and HRPO approved
E00220.3a (University of Colorado) IRB approved and HRPO approved
E00220.4a (NU) IRB approved and HRPO approved.

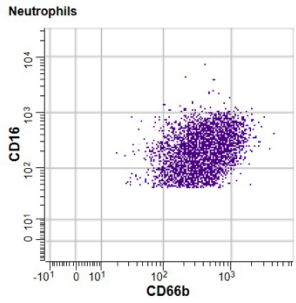
Assembly, extraction and characterization of a retrospective cohort.

Aim 1 and 2 of our proposal focused on development of a retrospective cohort and evaluation by RNA and DNA analysis. As planned, n=50 patients with non-muscle invasive bladder cancer who underwent intravesical BCG treatment were identified at UTHSA. Thus far, n=25 specimens with clinical annotation were sent to JH. An additional n=25 have been clinically annotated and we expect to ship these within 2 weeks. 100 patients treated with BCG were identified at Northwestern and are ready to dispatch to JH once IRB approval is achieved. Because of the complexity of the primary objective- clinical and pathologic response to BCG- we decided to increase the total cohort by adding patients from NU. This was approved by the IRB and HRPO

Significant results or key outcomes

a. **Evaluation of post-BCG urinary immune cells using CyTOF**. We initiated characterization of immune cells of the urine using mass cytometry. We conducted analysis of post-BCG urine in n=6 samples of urine. We expected to find a high frequency T lymphocytes (T cells) but instead we found a large population of granulocytes, including CD14-, CD66b dim/+, CD16+, HLA-DR- neutrophils and relatively few lymphocytes. The CD3 T cell population represented 0.67% of the live cells. CD4 and CD8 were detected in very few numbers and γδ T cells (i.e., γδ+, CD4-, CD8-) were not detected (**Table**). Because of the additional steps in preparation of samples for CyTOF, we suspected that some immune cells, including T cells could have been lost or undetected in CyTOF analyses. Therefore, we repeated the analysis using traditional flow and we used two separate antibody clones to detect γδ T cells. Using flow cytometry, we identified T cells in the urine during BCG instillation (Figure). We also compared the urine immune cells to peripheral blood from matched patients. Remarkably, we found that γδ T cells – although extremely low in peripheral blood, had a higher proportion in urine compared to CD8 + T cells. This was a promising finding because it aligns with our other data that implicates γδ T cells in BCG's activity.

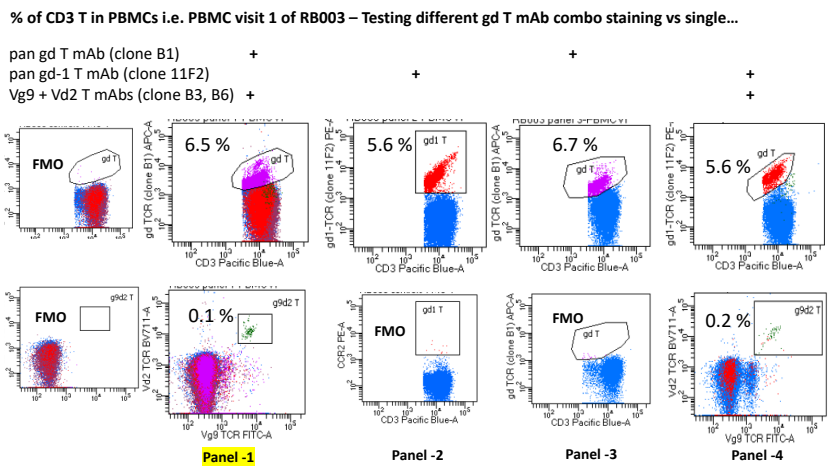




	Count	% Intact Live	Parent	% Parent
Intact Live Cells	19039	100.00	All events	94.03
Lymphocytes	128.00	0.67	Intact Live	0.67
CD3 T Cells	112.00	0.59	Lymphocytes	87.50
CD8 T Cells	23	0.12	CD3 T Cells	20.54
Naive	0.00	0.00	CD8 T Cells	0.00
Central memory	0.00	0.00	CD8 T Cells	0.00
Effector memory	17	0.09	CD8 T Cells	73.91
Terminal effector	6	0.03	CD8 T Cells	26.09
CD4 T Cells	69	0.36	CD3 T Cells	61.61
Naive	1	0.01	CD4 T Cells	1.45
Central memory	4	0.02	CD4 T Cells	5.80
Effector memory	20	0.11	CD4 T Cells	28.99
Terminal effector	44	0.23	CD4 T Cells	63.77
GD T Cells	0	0.00	CD3 T Cells	0.00
MAIT NKT Cells	20	0.11	CD3 T Cells	17.86
B Cells	8.00	0.04	Lymphocytes	6.25
Naive B	2	0.01	B Cells	25.00
Memory B	6	0.03	B Cells	75.00
Trans B	0.00	0.00	B Cells	0.00
Plasmablasts	0.00	0.00	B Cells	0.00
NK Cells	8	0.04	Lymphocytes	6.25
Monocytes	1358	7.13	Intact Live	7.13
Classical	1201	6.31	Monocytes	88.44
Transitional	137	0.72	Monocytes	10.09
Non-classical	20	0.11	Monocytes	1.47
Dendritic Cells	21.00	0.11	Intact Live	0.11
pDC	20	0.11	Dendritic Cells	95.24
mDC	1	0.01	Dendritic Cells	4.76
Granulocytes	16395.00	86.11	Intact Live	86.11
Neutrophils	15613	82.01	Granulocytes	95.23
Basophils	17	0.09	Granulocytes	0.10
Eosinophils	547	2.87	Granulocytes	3.34
CD66b- Neutrophils	218	1.15	Granulocytes	1.33
Other	1137	N/A	All events	5.97

marker	pos cell	neg cell	bet	grade	pos mediar	neg mediar
CD3	CD4	B	3.7	Very Good	382.91	1.83
CD19	B	CD4	7.5	Extremely Good	468.88	1.78
CD14	Mono Class	CD4	3.7	Very Good	51.98	1.72
CD28	CD8 Nv+CM	CD8 TE		Good		
CD27	CD8 Nv+CM	CD8 TE				
CD16	NK CD16+	CD8 Nv+CM				
CD45RA	CD4 Nv	CD4 EM	10.1	Extremely Good	57.43	9.85
CD45RC	CD4 EM	CD4 Nv	9.4	Extremely Good	220.70	10.86
CXCR3	CD4 EM	Mono	0.0	Extremely Weak	18.02	18.54
CXCR5	B	Mono	0.2	Extremely Weak	12.39	12.85
CCR6	B	CD4 Nv	0.9	Fairly Weak	14.81	8.58
CCR4	CD4 CM Treg	Mono	1.5	Moderate	19.80	7.34
TCR gd	T gd	CD8 Nv+CM				
CD26	MAIT NKT	NK	2.1	Good	143.84	13.70
CD127	CD4 TE	Mono	0.0	Extremely Weak	19.43	21.41
CD56	NK	B	1.3	Moderate	129.22	15.41
CD161	MAIT NKT	Mono	6.9	Extremely Good	469.43	11.40
CD8	CD8	CD4	5.9	Extremely Good	1107.24	10.43
CD4	CD4	CD8	8.9	Extremely Good	638.79	12.45
CCR7	CD8 Nv+CM	CD8 TE				
CD25	CD4 CM Treg	B	0.8	Fairly Weak	98.70	17.15
HLADR	B	CD4 Nv	7.0	Extremely Good	156.58	11.34
CD38	Mono Class	CD8 TE	0.4	Very Weak	167.16	260.75
CD20	B	CD4	2.8	Good	51.84	5.32
IgD	B Nv	CD4	0.1	Extremely Weak	1.40	1.13
CD66b	Neutrophils	CD8	7.0	Extremely Good	598.49	11.60
CD45	CD4	Neutrophils	2.3	Good	860.73	156.38
CD123	pDC	mDC	0.2	Extremely Weak	12.92	13.45
CD11c	mDC	pDC	30.0	Extremely Good	353.90	1.84

b. **Flow cytometry to detect γδ T cells in urine**. As we were unable to detect γδ T cells in urine using mass cytometry, we worked to identify these cells using flow cytometry. We experimented with 3 different antibody clones for detection including a pan γδ T cell monoclonal antibody clone (clone B1), a pan- gd-1 mAb (clone 11F2), and a Vg9 + Vd2 mAb clone (clone B3, B6). We used flow minus one (FMO) gating to identify the population. As shown in the figure, we were able to detect populations of γδ T cells in urine with variable numbers based on the mAb clone used. The pan γδ T cell B1 clone detected the most γδ T cells (Figure) and was reliable on repeated examinations (not shown).



What opportunities for training and professional development has the project provided?

Nothing to Report.

How were the results disseminated to communities of interest?
Nothing to Report

What do you plan to do during the next reporting period to accomplish the goals?

During the next reporting period, we have revised plans as outlined below under "Changes/Problems" section. Specifically

1) will collect germline DNA from patients in retrospective cohort to perform accurate whole exome sequencing
2) will add single-cell RNA seq to Aim 2 to determine the change in immune cell transcription before and after BCG
3) will change the TruCulture to an alternate assay to determine BCG-specific response

4. **IMPACT:**

What was the impact on the development of the principal discipline(s) of the project?
The major findings that affected the direction of this project was work from Dr. Svatek's laboratory identifying the important role of γδ T cells

What was the impact on other disciplines?
We found that TruCulture has limitations with regards to use for characterization of BCG-specific immunity. Despite prior published literature suggesting its value in this area, it clearly has important limitations that preclude it from being used to assess immunity to live BCG organisms. Alternative uses of TruCulture could be to utilize specific BCG proteins of interest or purified protein derivative, but this will not provide a representation of the complex and multifactorial response to the mulititude of BCG proteins on live BCG organisms.

What was the impact on technology transfer?
Nothing to report

What was the impact on society beyond science and technology?
Nothing to report

5. **CHANGES/PROBLEMS:**

Changes in approach and reasons for change

Aim 1, Major Task 2. We have found that using germline DNA is critical to performing whole exome sequencing. Thus, we will begin collecting DNA from patients from our retrospective cohort. This will greatly improve the accuracy of sequencing results. We inititally identified this problem when performing WES and while this can be approximated, having germline DNA is much "cleaner" from a bioinformatics perspective.

Aim 2. We will add a Major Task to perform single-cell RNA-Seq of bladder tumor tissue before and after BCG treatment to characterize the immune cell function and tumor/epithelial cell expression after BCG treatment. This will facilitate Aim 3.

Aim 3, Major Task 10, was to "Determine predictive value of TruCulture immune monitoring during BCG therapy". We had plan to prospectively enroll patients receiving BCG to evaluate their BCG-specific immune response using TruCulture and to correlate the immune response with the PPD test, and urine cytokine response and clinical outcomes. The TruCulture device is made of a small collection container that contains the antigen or protein of interest and is mixed with whole blood in this chamber. Then, the chamber is incubated in a heat block and subsequently, the blood is analyzed for a panel of cytokines. In this way, immune cells release cytokines in response to the antigen/protein of interest and this response can be measured by quantifying the amount of cytokines secreted. Our objective (3.1) was to compare the BCG-specific immune response measured by TruCulture to the more traditional PPD response. We had planned to do this using patients enrolled on to S1602 at NU and UTHSA. We obtained IRB and HRPO approval at both sites early on during our award period. However, the company (Myriad-RBM) was slow in communication with us regarding developing the tubes needed for the protocol. We were finally able to submit a purchase order (PO) for n=50 tubes in October 2018, which would have enabled us to start enrolling patients and performing the assay as planned. In January 2019, we received a call from the company that Dr. Manfred Schmolz, the inventor of TruCulture, wanted to speak with us because they had a problem filling our order. They stated that although they had previously used BCG for the TruCulture device, they were no longer able to use BCG in the container for a variety of reasons. They offered to use the purified protein derivative (PPD) protein placed into the TruCulture container as an alternative. Our study team discussed this option. We decided that this approach would not provide substantial improvement in science to be worth the expense and time. Thus, in the early part of 2019, our team made the decision not to pursue the TruCulture experiment for Aim 3.

Revised approach. In order to fulfill the mission of Major Task 10, we propose to use an alternative method for assessing BCG-specific immunity that we recently developed. In this assay, patients' whole blood is labeled with CFSE and then mixed with live BCG, allowed to incubate for 7 days, and then cytokine (IFN-gamma) and proliferation (CFSE dilution) is measured by flow cytometry for CD4, CD8, and $\gamma\delta$ T cells. Thus, this assay captures similar data to TruCulture. In addition, we will be able to determine specific T cell subset responses which was not possible with the TruCulture device. We have already made progress in establishing the feasibility and reliability of this approach (Figure). Because we are now into the 2nd year of our award period, we have limited amount of time sufficient patients in order to fulfill Major Task 10. Therefore, we plan to seek HRPO approval to utilize existing specimens available to us from a trial that recently completed accrual (NCT02753309) to answer the questions posed by Major Task 10. This includes correlating the BCG-specific immune response with PPD, urinary cytokines and clinical data, all of which were collected on approximately n=30 patients receiving intravesical BCG.

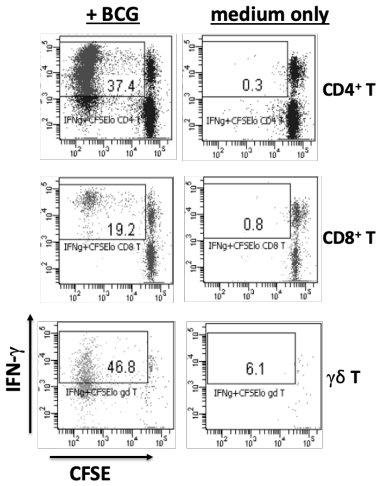


Figure. Measuring BCG-specific proliferation and effector function of circulating CD4$^+$, CD8$^+$, and γδ T cells. Patient PBMCs (1 x10^6) labeled with CFSE were cultured with or without the presence of live BCG for 7 days, then incubated with the cocktail of PMA, ionomycin and Golgi blocker for 5 hours, prior to cell-surface staining for CD4, CD8, γδ TCR, followed by intracellular staining for IFN-γ. Shown are representative flow cytometry dot plots for CD4$^+$ (top panels), CD8$^+$ (middle panels), γδ (bottom panels) T cells with percentage of CFSE^{low} IFN-γ$^+$ populations from PBMC samples incubated with live BCG (left panels) versus medium (right panels) for 7 days. corrections.

Progress and re-direction:

We have discovered that BCG's activity is mediated by at least 3 different mechanisms: non-specific (*i.e.,* γδ T cells), tumor-specific (largely mediated by CD8 antigen-specific T cells), BCG-specific (i.e., BCG-specific antigens on the surface of tumor cells are recognized by BCG-specific T cells and elicit immune responses against the tumor). Importantly, it appears that the non-specific (γδ T cells) response is the most important as without the response, antigen-specific and BCG-specific immunity does not happen. What are the implications of these findings? These findings indicate that a robust γδ T cell specific immune response is required for BCG to exert its initial activity. We propose that initial clearance of CIS requires adequate γδ T cell infiltration of the bladder and adequate function of γδ T cells. On the other hand, long-term memory and clearance of tumor long-term is likely to be dependent on memory responses (tumor-specific and BCG-specific immunity). Thus, we propose directing investigations into the nature of γδ T cells (and perhaps NK cells too) as these appear to be principally involved in BCG responses.

Proposed experiments: 1) Characterize the nature of γδ T cells in the bladder of patients first with flow cytometry (including activation status based on surface receptors and proliferation based on Ki67), (2) use RNA-seq to characterize baseline and post-BCG γδ T cells, including percentage of γδ T cell subsets (e.g., V1.01, V10.01, V11.02, V2.02, V3.01, V5P.01, V6.01), V7.01, V8.01.M13434, V8.01.X15273, V9.01, V9.02, VA.01, VB.01) and delta subsets (e.g., V1, V2, V3), activation status, regulatory status, and presence of exhaustion markers (e.g., Tim-3, Lag-3, PD-1), (3) characterize/quantify urine γδ T cell subsets during BCG treatment using flow and single-cell RNA-seq.

We hypothesize that absence of specific γδ T cells in the bladder at baseline or in the urine during BCG treatment will predict CIS persistence at 6 months (*i.e.*, BCG unresponsiveness). This will ultimately be validated using samples from S1602.

Additional testing including correlation of molecular tumor subtype with quantity and types of bladder-infiltrating γδ T cells. For example, we predict that more basal subtypes have less γδ T cells in the bladder.

Actual or anticipated problems or delays and actions or plans to resolve them
We do not anticipate any further delays

Changes that had a significant impact on expenditures
None

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
None. We will be submitting addendums for the new work proposed for Aims 2 and 3

6. PRODUCTS:

Publications, conference papers, and presentations
Nothing to report

Journal publications
Nothing to report

Books or other non-periodical, one-time publications
Nothing to report

Other publications, conference papers and presentations
Nothing to report

Website(s) or other Internet site(s)
Nothing to report

Technologies or techniques
Nothing to report

Inventions, patent applications, and/or licenses
Nothing to report

Other Products
Nothing to report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	Robert Svatek,MD
Project Role:	Co-Principal Investigator
Researcher Identifer:	
Nearest person month worked:	1
Contribution to Project:	Dr. Svatek coordinated activities between the S1602 trial team and the DOD project team. His laboratory work & findings on Aim 3 identified the important role of gamma T cells for this project.
Funding Support:	N/A

Name:	Niannian Ji, PhD
Project Role:	Research Scientist
Nearest person month worked:	5
Contribution to Project:	Dr. Ji conducted the mass and flow cytometry experiments in Dr. Svatek's lab.
Funding Support:	N/A

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Yes, Dr. Svatek's active support has changed. He completed work on three projects and began work on one new project.

NEW – ACTIVE

R01CA231325 (PI: Curiel)	09/19/2018-08/31/2023	0.6 calendar
NCI/NIH	$427,268	

(PQ2) PD-L1 Signals in Aged Hosts Undergoing Cancer Immunotherapy
This proposal combines a team with expertise in aging, tumor immunology, tumor immunotherapy, specific genetically modified animal models and early phase clinical trials with a computational team having great expertise in analyzing and modeling aging of the immune system. We will study age effects on PD-L1/PD-1 signaling in the host and the tumor focusing on melanoma with some bladder cancer work, two tumors that are highly responsive to αPD-1 and/or αPD-L1 as proofs-of-concept and residing in distinct anatomic compartments. Role on project: Co-Investigator.

COMPLETED – INACTIVE

NIH/NCI - K23CA178204 - "Improving immunotherapy in bladder cancer by targeting immune dysfunction" ended 7/31/18 releasing 7.2 calendar months effort.

Voelcker FDN – Young Investigator Award – "Defining mechanisms of mTor inhibition to boost immunotherapy in bladder cancer" ended 6/30/2018 releasing 2.4 calendar months effort.

University of Rochester – 416978-G – "Extracellular vesicle biomarkers for predicting response to BCG Therapy" ended 12/31/18 releasing 0.6 calendar months effort.

What other organizations were involved as partners?
Nothing to report

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: This project is part of a Collaborative Award. Dr. Svatek is the Partnering PI at Site 3.

QUAD CHARTS: Not applicable

9. APPENDICES: None