Award Number: W81XWH-19-1-0766

TITLE: Immunologic and Microbial Correlates and Mechanisms of Complete Response to Neoadjuvant Chemotherapy in Muscle-Invasive Bladder Cancer

PRINCIPAL INVESTIGATOR: Philip Abbosh, M.D., Ph.D.

CONTRACTING ORGANIZATION:

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13. SUPPLEMENTARY NOTES

14. ABSTRACT

Bladder cancer is often treated with cisplatin-based chemotherapy. Based on our preliminary data, we believe that the immune system has a significant impact on the responder status outcome of patients receiving chemotherapy. We localized this putative immune response to chemotherapy to the CD8 effector T cells. To further characterize this response, we will be quantifying the cytotoxicity and expression profile from perivesical lymph nodes. In addition, we will experimentally test the possibility that CD8 effector T cells impact the chemoresponse using orthotopic bladder cancer mouse models.

We have also identified a microbiome within tumors and within urine samples from bladder cancer patients which may further impact the responder status. We will compare the tumor and urine microbiomes of patients receiving chemotherapy to determine their overlap and their associations with response. We will then test these correlations in an orthotopic bladder cancer mouse model to determine if these are merely correlations or causative of response. It is also possible that we will uncover connections between the microbiome and immune system that further are causative of chemoresponse or chemoresistance.

15. SUBJECT TERMS

Muscle-invasive bladder cancer, neoadjuvant chemotherapy, CD8 T cell, neoantigen, metagenomics, 16s rRNA

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DOD Final Report

Principal Investigator: Philip Abbosh, M.D., Ph.D.
Institution: The Research Institute of Fox Chase Cancer Center
Grant Number: W81XWH-19-1-0766

INTRODUCTION:

Muscle invasive bladder cancer (MIBC) is optimally treated with neoadjuvant cisplatin-based chemotherapy followed by radical cystectomy (RC). With this treatment, up to 40% of patients will have achieved a pathologic complete response at the time of RC. The purpose of the research is to understand the potential immunologic and microbiologic inputs into chemoresponse. I aim to study patient-derived biospecimens in order to characterize the immunological infiltrate and associated microbiome of MIBC samples and then to use what we learn in these studies to test our findings in mouse models of bladder cancer.

KEYWORDS:

Muscle-invasive bladder cancer, neoadjuvant chemotherapy, CD8 T cell, neoantigen, metagenomics, 16s rRNA.

ACCOMPLISHMENTS:

What were the major goals of the project?

Major Task 1: Sample acquisition
To be completed by month 24
Percentage of completion: 100%

Major Task 2: Quantify differences in exhaustion in CD8+ effector T cells between responders vs nonresponders
To be completed by month 36
Percentage of completion: 75%
The final conclusions here are pending the analysis of the last T cell RNA seq libraries, which is ongoing.

Major Task 3: Transcriptionally characterize activation and exhaustion states in nodal T cells
To be completed by month 36
Percentage of completion: 75%
The final conclusions here are pending the analysis of the last T cell RNA seq libraries, which is ongoing.

Major Task 4: Determine whether CD8+ effector T cells traffic between lymph nodes and tumors
To be completed by month 36
Percentage of completion: 75%
The final conclusions here are pending the analysis of the last T cell RNA seq libraries, which is ongoing.

Major Task 5: Determine if immune correlates of chemoresponse are causative of anti-tumor immune responses
To be completed by month 24
Percentage of completion: 50%
The work on human samples is nearly complete with data analysis of the last Tcell sequencing libraries underway. However, the mouse model was not able to get off the ground as described below.

Major Task 6: Discover taxons which associate with pathologic response
To be completed by month 18
Percentage of completion: 100%

In addition, we are extending these analyses into the gut microbiome of chemotherapy patient as well.

Major Task 7: Determine if the urine microbiome is a proxy for the tumor microbiome
To be completed by month 21
Percentage of completion: 80%
We have finished the 16S sequencing of FFPE tumor tissue, FFPE peripheral tissue (as a control) and the urine and now are comparing them to determine the representativeness.

Major Task 8: Determination of the normal C57B6 bladder microbiome
To be completed by month 15
Percentage of completion: 100%

Major Task 9 was not listed in the SOW and is a clerical error.

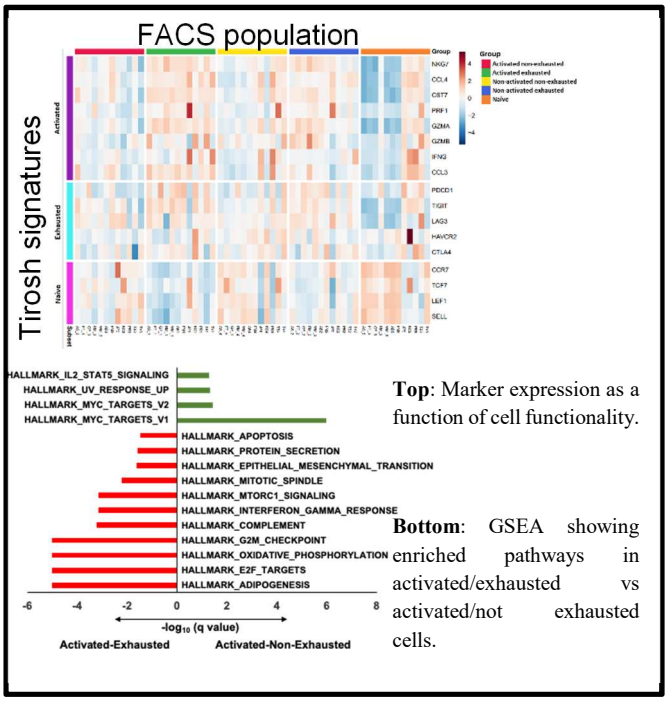
Major Task 10: Optimization of colonization by candidate OTU
To be completed by month 36
Percentage of completion: 50%
Again, we were not able to launch the mouse model.

Major Task 11: Determine if microbial correlates of chemoresponse are causative of chemoresponse
To be completed by month 36
Percentage of completion: 50%

We were unable to launch the mouse model, however, we pursued evaluation of the gut microbiome of mice receiving bladder carcinogen BBN instead as described below.**What was accomplished under these goals?**

The Abbosh Lab has accomplished significant progress in both aims for the project.

For Aim 1, our work has zeroed in on the nodal Tcells. These have been a rich data source. We have now analyzed 66 nodes in total using multicolor FACS. We have generated RNAseq libraries from 20 lymph nodes but have only sequenced 12 of them. Libraries from the other 8 have been assembled and are in queue for the sequencer. RNAseq data was to be used for two purposes: gene set enrichment analysis (GSEA)/transcriptional profiling and TCR sequencing. We have also analyzed TCR repertoires from 19 cystectomy and/or TURBT specimens but the overlap analysis is still pending. Partial results are described below:



Top: Marker expression as a function of cell functionality.

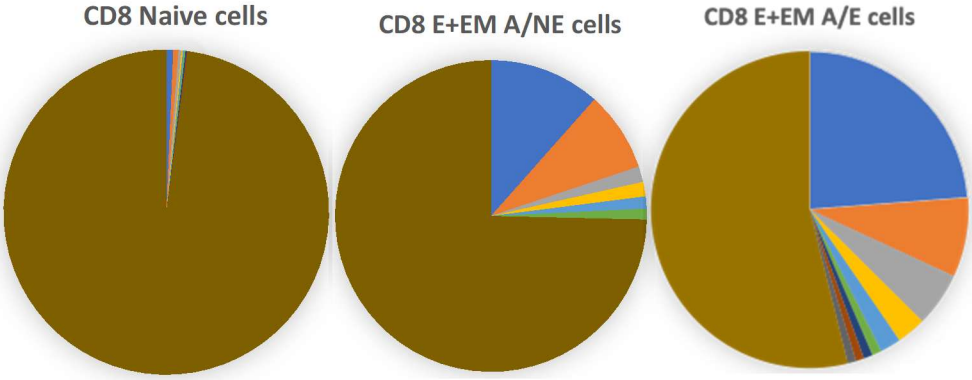
Bottom: GSEA showing enriched pathways in activated/exhausted vs activated/not exhausted cells.

GSEA/transcriptional profiling

Effector and effector memory CD8 Tcells were sorted according to PD1 (exhaustion) and CD69 (activation) status and collected for RNAseq. Naïve CD8 Tcells were also collected for RNAseq. We first evaluated the transcriptional profile described by Tirosh for exhaustion level based on the cell surface markers, confirming Tirosh's findings that exhausted cells have strong expression of activation markers (**figure**, right/top). One key finding here is that Myc pathway upregulation seems to be a key difference that is absent in exhausted effector CD8 Tcells.

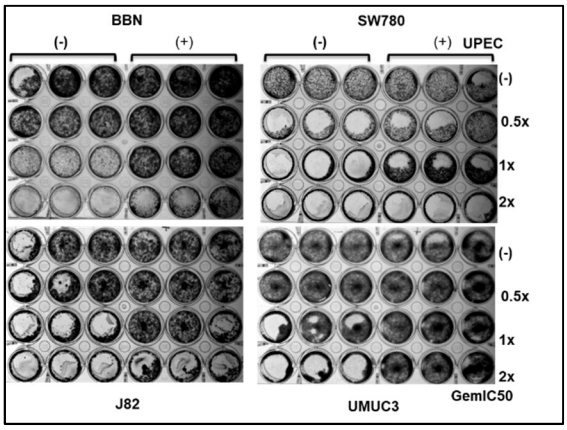
TCR repertoire

I proposed using TCR sequence analysis as a way of 'tracking' T cell movement between perivesical lymph nodes and the tumors. To this end, we initially used the TRUST software, as proposed to catalog TCR sequences in nodal population. However, this algorithm did not give reliable/reproducible results in control experiments. We pivoted to use MIXCR, a similar application that is reported to perform better and faster at calling TCR from NGS data. MIXCR did outperform TRUST in our hands. This approach worked well but analysis is still ongoing as we have not completed the RNAseq of all the lymph node samples yet which we will use to extract the TCR repertoire. To catalog TCR repertoire in tumors, we used Immunoverse PCR as proposed. We do find some interesting features though. Namely, that effector and effector memory TCR clones identified by CDR3 region expand as they become activated then exhausted within the nodal population (**figure**, right).



Left to right are depictions of the TCR repertoire from the effector and effector memory CD8 T cells from one cystectomy patient's lymph node. Dominant clonotypes represented a small percentage of the naïve cells which increased as they became activated then activated and exhausted.

For Aim 2, we are continuing to explore the connection between gemcitabine-metabolism and intratumoral bacteria. Gammaproteobacteria contain a cytidine deaminase enzyme which can detoxify gemcitabine. When four bladder cancer cell lines are infected with uropathogenic *E.coli* or lab strains of *E.coli*, it takes more gemcitabine to reduce colony formation (**figure**, right). In an unproposed experiment, we are using PRIME CRISPR to knockout the function of the enzyme in *E.coli* to further prove that this is the mechanism. We have been able to introduce an edit into the *cdds* locus (C129A), although it was not the intended edit. This edited ORF did not reduce the concentration of gemcitabine in media or increase the concentration of its metabolite. We are in the process of making different *cdds* edits, hopefully ones that result in a stop codon as we had intended. Once we have the right allele, we will test it to determine if it reduces gemcitabine levels and whether that is associated with restoration of gemcitabine sensitivity.

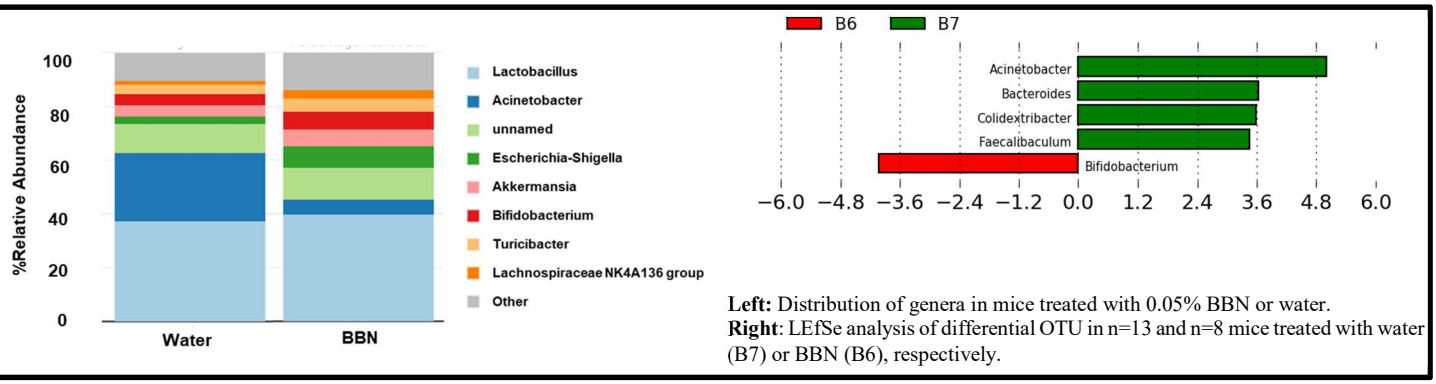


As noted previously, we continued to have trouble developing a faithful intravesical model of bladder cancer to use to infect with OTU-of-interest. Neither our primary approach nor the alternative approaches proposed in the original submission nor those proposed in last year's progress report resulted in tumor take. We therefore switched gears slightly to analyze the microbiome of the one viable and reliable mouse bladder cancer model: the BBN carcinogen. BBN is a tobacco smoke metabolite that is excreted in urine. When given to mice, it causes bladder cancer with histological and genetic resemblance to human bladder cancer.

Rationale for the experiment: Through my lab's work with Dr. Bukavina, we found that both urine from bladder cancer and bladder cancers themselves contain 6 OTU with the specific metabolic capacity to metabolize polycyclic aromatic hydrocarbons (PAH). Interestingly, *Psuedomonas aeruginosa* (also a gammaproteobacteria that can metabolize gemcitabine!) is among the most commonly present PAH metabolizer and a well known although somewhat infrequent uropathogen. Through Dr. Bukavina's work, we were able to see that this bacteria was not infecting the tumor cells at any significant frequency but were most abundant in intratumoral monocytes. As PAHs are common tobacco smoke pathogens and more than a dozen are excreted into urine, and because BBN

is a faithful carcinogen model of tobacco-driven bladder carcinogenesis, we sought to evaluate the bladder and gut microbiome of mice being treated with BBN.

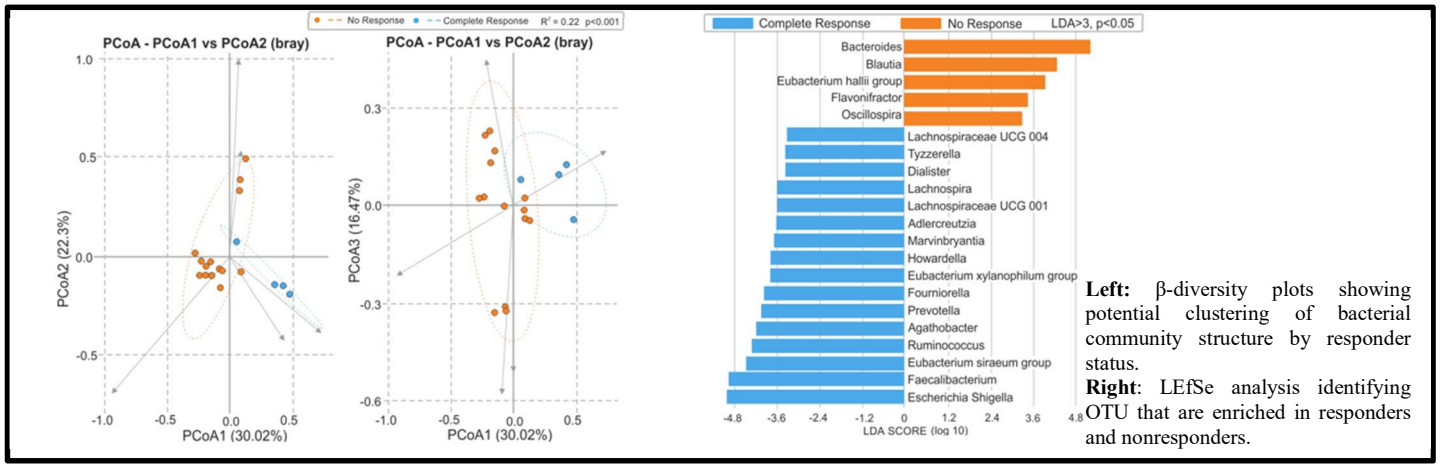
The main findings are that *Bifidobacterium* seems to be enriched in BBN-induced tumors whereas several taxa are enriched in benign mice (**figure**, below). This is consistent with the finding that *Bifidobacterium* was also enriched in human tumors in our TCGA analysis as presented in prelim data from the proposal. There were no



Left: Distribution of genera in mice treated with 0.05% BBN or water.
Right: LEfSe analysis of differential OTU in n=13 and n=8 mice treated with water (B7) or BBN (B6), respectively.

significant differences in alpha or beta diversity metrics (not shown). We unfortunately did not find that *Pseudomonas* was enriched as we had hypothesized. This may be because BBN is not a PAH. There is not currently a PAH-associated model for bladder cancer.

In addition, we also began collecting stool samples from patients who were receiving neoadjuvant AMVAC chemotherapy cocktail. These were collected pretreatment, after the second cycle was completed, and at the time of cystectomy. As a form of internal control, we also collected a stool sample from a live-in partner at each time point to determine if to potentially control for confounders from the environment (e.g. pets). There were not significant changes in alpha or beta diversity that occurred during or after chemotherapy (not shown). However, we did find that there were associations between chemoresponse and gut microbiome profile (**figure**, below)



Left: β-diversity plots showing potential clustering of bacterial community structure by responder status.
Right: LEfSe analysis identifying OTU that are enriched in responders and nonresponders.

Curiously, *E.coli* is a gemcitabine metabolizer but is found to be associated with chemoresponse when found in the gut. However, gemcitabine is not part of the AMVAC regimen and was also found to be enriched in the urine of responder patients (not shown).

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

Philip Abbosh, MD-PhD: I continue to participate in the Research Review Committee as outlined in my career development plan. I felt that the experience of reviewing/critiquing clinical trials for Fox Chase prepared me to design a clinical trial. Therefore, I did also submit a clinical trial concept to the NCI EDDOP program. It was

not selected for funding but did receive favorable reviews. The reviewers stated that more preclinical data was needed to move forward with the concept.

I completed an online course through Temple University to learn programming in R with focus on analysis of RNAseq data. I am not a proficient scripter at this point though.

Rashida Ginwala, PhD: Dr. Ginwala was a funded postdoctoral associate in my lab. She has since 'graduated' and moved to industry at the end of the funded period. She presented her work at the 2022 AACR annual meeting.

Laura Bukavina, MD: Dr. Bukavina joined my lab as a clinical fellow during the research year of her Society for Urologic Oncology fellowship. Her salary is supported entirely by her clinical fellowship but has intersected with this project in a couple ways. She has a strong interest in the microbiome related to bladder cancer as well as the immune microenvironment within tumors. She has little prior lab experience. Since joining the lab, she has learned how to perform sterile tissue culture, make 16S libraries/QC them/superficially perform their analysis, and perform confocal microscopy experiments. In addition, she is performing mouse model experiments. She presented her work on microbiome projects related to (but not overlapping with) the work in this proposal at the SUO, AUA, and ASCO-GU meeting. Her work was selected for podium presentation at the SUO meeting. In addition, she is enrolled in the Master's program at Drexel University in Philadelphia. She performed well in the first semester.

How were the results disseminated to communities of interest?

Our works on these topics were presented at the AACR, SUO, AUA, and ASCO-GU meetings.

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

Not applicable.

IMPACT:

What was the impact on the development of the principal discipline(s) of the project?

I still think our most significant finding/development is the finding of widespread UPEC in urine samples and its ability to detoxify gemcitabine. Gemcitabine is used as both systemic treatment and intravesical therapy. We are now collecting urine specimens from patients receiving intravesical gemcitabine before the drug is instilled and then collecting the sample after it has been instilled. Our goals are to (1) quantify the amount of gammaproteobacteria in the urine sample and (2) quantify the amount of de-aminated gemcitabine in the effluent and then (3) correlate the two quantities. If they do appear correlated, the natural next steps would be to determine if there is an impact on clinical outcome and if so, whether eradication of the gammaproteobacterial would improve outcome through the form of clinical trial.

We had previously thought that *E.coli* had also detoxified vinblastine, but found that this was a spurious result.

Although it is preliminary, the work that has sprouted out of this project related to the possibility of PAH metabolism by pseudomonas in the genesis of bladder cancer is exciting and potentially impactful. PAHs are well known carcinogens that are highly relevant to service members because of exposure to diesel fumes and cigarette smoke. There are several hypotheses we will test to determine the relevance, nature, significance, and impact of the finding of intratumoral *Pseudomonas*.

What was the impact on other disciplines?

Nothing to report.

What was the impact on technology transfer?

Nothing to report.

What was the impact on society beyond science and technology?

Nothing to report yet.

CHANGES/PROBLEMS:

Changes in approach and reasons for change

As mentioned above, we changed gears regarding the mouse model.

Actual or anticipated problems or delays and actions or plans to resolve them

Described above. In brief, we analyzed the bladder and gut microbiome of mice exposed to BBN in drinking water for 6, 12, and 22 weeks with matched mice drinking regular drinking water from the same source.

Changes that had a significant impact on expenditures

Nothing to report.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

We obtained approval to use BBN for murine microbiome experiments from the institutional IACUC. This was not considered a significant change. We had already had approval to use BBN on our mouse protocol when we wrote the project so no modifications were needed.

Significant changes in use or care of human subjects

We also obtained approval to collect human stool samples from the FCCC IRB. These were not considered significant changes. The IRB that supported studies during the funded period was written to satisfy the DOD requirement of having a single protocol to support all studies. This was done because, pre-existent to the application, three separate IRB protocols had been used to collect biosamples that were used to support this project. One of the protocols already supported the collection and use of stool samples, so no modification was needed for the single IRB protocol that supported these studies.

Note that all samples were de-identified samples from different IRB protocols, but the risks or care of subjects in those protocols is similar (i.e. negligible).

Significant changes in use or care of vertebrate animals.

As mentioned above.

Significant changes in use of biohazards and/or select agents

Nothing to report.

PRODUCTS:

Nothing to report.

Publications, conference papers, and presentations

Journal publications. These are still in preparation.

Books or other non-periodical, one-time publications. None.

Other publications, conference papers, and presentations.

AACR 2022 (New Orleans, LA): Changes in the gut microbiome upon exposure to bladder carcinogen N-butyl-N-(4-hydroxybuytl) nitrosamine

ASCO-GU Symposium (San Francisco, CA) Characterization and functional analysis of microbiome in bladder cancer: assessment of neoadjuvant response by gut and urine microbiome. **presenter won ASCO Merit Award.

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.

PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	*Philip Abbosh*
Project Role:	*Principal Investigator*
Researcher Identifier (e.g. ORCID ID):	*0000-0003-3611-9532*
Nearest person month worked:	*4.00*

Contribution to Project:	*Principal Investigator; Oversaw project progress and dissemination of data. Helped plan (and in some cases perform) experiments.*
Funding Support:	*This award supported 75% of Dr. Abbosh's salary/effort. Dr. Abbosh has a 0.4 FTE research appointment.*
Name:	*Alexander Metz*
Project Role:	*Scientific Technician*
Researcher Identifier (e.g. ORCID ID):	*N/A*
Nearest person month worked:	*4.00*
Contribution to Project:	*Performance of FACS analysis of lymph nodes, cell culture experiments, cloning for PRIME-CRISPR editing.*
Funding Support:	*Alex received partial salary/effort on this project.*
Name:	*Rashida Ginwala, Ph.D.*
Project Role:	*Postdoctoral Associate*
Researcher Identifier (e.g. ORCID ID):	*N/A*
Nearest person month worked:	*6.00*
Contribution to Project:	*Performance of FACS analysis of lymph nodes, RNA sequencing/analysis, 16S metagenomics/analysis, cell culture experiments, PRIME-CRISPR editing of E.coli.*
Funding Support:	*Dr. Ginwala's salary/effort is entirely supported through institutional funds.*

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

Nothing to report.

What other organizations were involved as partners?

Organization Name: *Institut Pasteur*
Location of Organization: *Paris, France*
Partner's contribution to the project *Molly Ingersoll serves as my co-career guide*
Financial support *none*
In-kind support *Molly Ingersoll serves as my co-career guide*
Facilities *none*
Collaboration *yes*
Personnel exchanges *no personnel exchanges, but we do have lab meeting together reliably now by teleconference.*

Organization Name: *Johns Hopkins University*
Location of Organization: *Baltimore, MD*
Partner's contribution to the project *Performance of 16S metagenomics*
Financial support *none*

In-kind support *none*
Facilities *none*
Collaboration *yes*
Personnel exchanges *none*
Other *none*

Organization Name: *Respherabio*
Location of Organization: *Baltimore, MD*
Partner's contribution to the project
Financial support *none*
In-kind support *none*
Facilities *none*
Collaboration *yes*
Personnel exchanges *none*
Other ***this collaboration has been discontinued due to a significant price increase in their service.*

Organization Name: *Upwork*
Location of Organization: *San Francisco, CA*
Partner's contribution to the project
Financial support *none*
In-kind support *they perform some metagenomics analysis for this project*
Facilities *none*
Collaboration *yes*
Personnel exchanges *none*
Other *none*

Organization Name: *Novogene*
Location of Organization: *Sacramento, CA*
Partner's contribution to the project
Financial support *none*
In-kind support *they perform Tcell RNAseq*
Facilities *none*
Collaboration *yes*
Personnel exchanges *none*
Other *none*

SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: Not applicable.

QUAD CHARTS: Not applicable.

APPENDICES: Please see Award Chart attached.

CA181178: Immunologic and Microbial Correlates and Mechanisms of Complete Response to Neoadjuvant Chemotherapy in Muscle-Invasive Bladder Cancer

PI: Philip Abbosh, Institute for Cancer Research, PA **Budget:** $658,800

Topic Area: Peer Reviewed Cancer Research Program **Mechanism:** FY18, PRCRP, Career Development Award

Award Status: 9/1/2019 – 8/31/2022

Study Goals:

1. To identify the immune inputs that lead to chemoresponse in bladder cancer.
2. To discover the bacteria that are found within bladder tumors and urine and to determine if they are associated with chemoresponse.

Specific Aims:

Aim 1: To dissect $CD8^+$ effector T cell activation and exhaustion states associated with pCR.
Aim 2: To define microbial ecosystems in bladder cancer and their association with chemoresponse.

Key Accomplishments and Outcomes as related to the SOW:

Regarding Aim 1, We show that exhausted and activated T cells have many of the same genes expressed but also identify key pathways which distinguish the two states, especially Myc target genes. We are still cross-referencing the nodal repertoire with the tumor repertoire to identify overlapping clones. We have however seen that the same clones can be represented in exhausted and activated populations –they are moving through their activation life cycle.

Regarding Aim 2, we have preliminarily identified taxons in urine and stool that are associated with chemoresponse. We have shown that gammaproteobacterial such as Ecoli are able to metabolize and detoxify gemcitabine. We also identify Bifidobacterium as a key enriched genus in mouse bladder tumors.

Publications: none
Patents: none
New Funding Obtained:
U01 CA260369 (PI: Abbosh) “Optimization of Urinary DNA Deep Sequencing Tests to Enhance Clinical Staging of Bladder Cancer Patients” 8/1/2021 - 7/31/2026