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TITLE: Is VISTA:VSIG3 an Actionable Immune Checkpoint Target in Kidney Cancer?

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CONTRACTING ORGANIZATION: Dana-Farber Cancer Institute

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13. SUPPLEMENTARY NOTES

14. ABSTRACT

Hypothesis: The failure of PD-1 blockade in RCC is due to activation of other immune checkpoint pathways, such as VISTA, which may be targeted for greater therapeutic benefit in cancers refractory to PD-1 pathway blockade.

Specific Aims: (1) Determine whether blocking the VISTA immune checkpoint pathway improves the response to PD-1 pathway blockade in the RENCA model. (2) Determine which cell types in human RCC express VISTA, VSIG3, VSIG8 and PSGL-1.

Major Findings: We have developed VISTA and VSIG3 antibodies to test for blocking ability in *in vitro* functional assay and the RENCA mouse model. However in neutral conditions, I was able to reproduce VSIG3 binding with VISTA, but not the binding of VSIG8 with VISTA. Others have reported the the acidic environment of a tumor may change the charge of residues within the histidine-rich extracellular domain of VISTA *in vivo*, thus improving the specificity of clinically relevant binding partners, such as PSGL-1. I have reproduced the human PSGL-1:human VISTA binding in vitro, but this does not appear to be conserved with mouse PSGL-1:mouse VISTA in vitro. We have explored the blocking antibodies that would block both VSIG3 and PSGL-1 binding with VISTA and the lead antibody failed to improve outcomes alone or in combination with PD-1 blockade in the RENCA tumor. Our data suggested that PSGL1:VISTA make be a critical pathway in the immune suppressive environment of the human tumor, but may not be testable in syngenetic mouse models.

15. SUBJECT TERMS

NONE LISTED

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TABLE OF CONTENTS

9. Introduction

For cancers to grow, they must evade destruction by the immune system. Many cancers evade the immune system by expressing molecules that turn off the anti-cancer immune response. Blocking these inhibitory checkpoints, like Programmed cell death-1 (PD-1), has become a standard therapy for treatment of many types of tumors including kidney cancer. Combining immune checkpoint inhibitors has been shown to improve outcomes for patients with kidney cancer. However, many patients fail to respond to currently available immune checkpoint inhibitors. These tumors appear to have a smoldering anti-tumor immune response that is turned off by expression of other negative immune regulators related to PD-1, such as VISTA. VISTA is an immune checkpoint that is highly expressed across kidney cancers. At the time of this original proposal VSIG-3 and VSIG-8 were reported to be VISTA's binding partners; however in the first year of this grant, we were unable to repeat the binding assay for VISTA:VSIG-3 or VISTA:VSIG-8. Others have had difficulty repeating VISTA:VSIG8 binding as well. In the interim, it has been established that PSGL-1 is a receptor for VISTA at acidic pH, such as those found in the tumor microenvironment, while VSIG-3 appears to be more relevant as a receptor at neutral pH.

In the second year of this grant, we optimized the binding assay of VISTA:VSIG-3, which appears to be a relatively weak receptor for VISTA, as well as reproducing the VISTA:PSGL-1 at acidic pH and confirming that at low pH it is a much stronger receptor for human VISTA. Much of the published literature on VISTA expression on lymphocytes and myeloids cells have been performed with lab-specific antibodies (such as MH5A in the Chen labs) rather than those that are commercially available. We compared the different commercially available VISTA antibodies (MH5A, MIH63 and MIH64) available to define what murine lymphocytes and myeloid cells express VISTA. We also compared the staining/binding of our panel of VISTA antibodies and determined the many of our mVISTA antibodies block both the VISTA:PSGL-1 and VISTA:VSIG-3 interaction.

Due to COVID19, our mouse therapeutic experiments and analysis of human nephrectomies for VISTA expression were significantly delayed due to closure of non-COVID research labs and mouse facilities (to non-COVID research) at DFCI during the early surge in Massachusetts, as well as off-loading the facility from having clinical research staff on-site for nephrectomy collections. In the time while wet-lab were on hold, we performed (1) structural analysis of VISTA, related B7 proteins, the VISTA mAbs and its receptors to better illustrate the relationship between the VSIG-3 and PSGL-1 binding site and (2) computational analysis of VISTA expression on available single cell sequences (from melanoma tissue) to assess the level of expression of VISTA in different tumor infiltrating immune cells.

We have reproduced the human PSGL-1:human VISTA binding in vitro, but this does not appear to be conserved with mouse PSGL-1:mouse VISTA in vitro. In the third year studied the difference between mouse and human PSGL-1 and suspected differences in posttranslational modification may abrogate the PSGL-1: VISTA interaction seen in humans. However given the multiple receptors reported for VISTA, we have explored the blocking antibodies that would block both VSIG3 and PSGL-1 binding with VISTA. However the lead antibody failed to improve outcomes alone or in combination with PD-1 blockade in the RENCA tumor. Our data suggested that PSGL1:VISTA may be a critical pathway in the immune suppressive environment of the human tumor, but may not be testable in syngenetic mouse models. Humanized tumor models are out of the scope of this project.

Purpose: My primary goal has been to determine if targeting receptors of the VISTA pathway, VSIG-3 or PSGL-1, can overcome PD-1 resistance in a mouse kidney cancer model. We have developed a panel of mouse antibodies to test this question. By determining if blocking the function of VISTA through VSIG-3 or PSGL-1 overcomes the tumor's ability to evade the immune system in a mouse model, we may be able to develop better therapeutics for blocking this pathway in patients with cancer.

Scope: In addition to investigating VISTA:VSIG-3, VSIG8 or PSGL-1 as a target for fighting cancer, we will study patients' tumors to explore biomarkers that could predict whether the therapy is likely to work. This could help to determine which patients will most likely benefit from VISTA pathway inhibitors and facilitate the

development of new therapies to treat patients with kidney cancer. Studying the clinically relevance of this pathway in murine models is limited by the differences in the function of PSGL-1 in mouse immunology.

10. Keywords

Kidney cancer, VISTA, VSIG-3, PSGL-1, immune checkpoint inhibition

11. Accomplishments

- **What were the major goals of the project? What was accomplished under these goals?**

Specific Aim 1: Aim 1: Determine whether blocking the VSIG3 or VSIG8 immune checkpoint pathway improves the response to PD-1 pathway blockade in the RENCA model.

Major Task 1: Characterize the ability of VSIG3 and VSIG8 antibodies to block this interaction and effect immune cell function *in vitro*

→ We have developed VISTA antibodies and characterized the ability of different mVISTA monoclonal antibody clones to detect VISTA by western blot analysis (clone 10G8) and by flow cytometry (Figure 2) to assess the ability to compare the avidity of the panel of antibodies (see annual report 2020, Figure 2).

→ Given the variability in the expression pattern of VISTA in the literature, we assayed VISTA expression on mouse lymphocytes and macrophage (see annual report 2020, Figure 3). This revealed that VISTA expression on cells can vary depending on site (thymus, spleen vs inguinal lymph node).

→ We had postponed in hRCC nephrectomy analysis in the SOW timeline due to the lag in nephrectomies due to COVID.

Major Task 2: Characterize the ability of VISTA:VSIG3 and VISTA:VSIG8 blocking antibodies to inhibit tumor growth *in vivo*, alone or in combination with PD-1 blocking antibodies

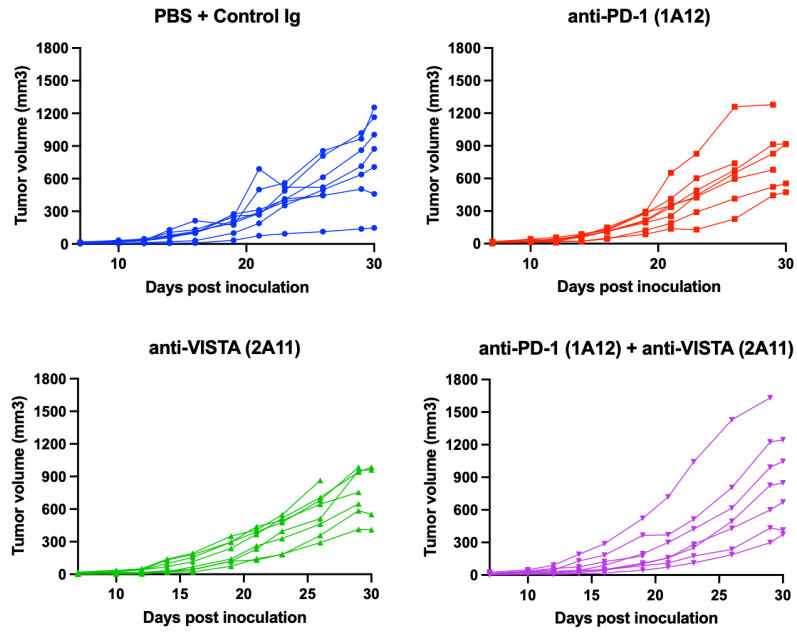

Figure 1. Combination trial of VISTA PD-1 blockade in RENCA tumor in mice showed that VISTA blockade did not overcome resistance to PD-1 blockade in RENCA tumors.

→ (**Figure 1**)

Major Task 3: Identify the subset of cells that VSIG3 and VSIG8 are expressed in the human RCC

→ Due the lag in nephrectomies during, we focused on a computational exploration of VISTA expression on different populations of single-cell RNA of immune cells (see annual report 2020, Figure 4) from RNASeq data available through a colleague (Nir Hacohen, MGH/Broad).

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

Major Task 1: Training and educational development in kidney cancer research

→ Subtask 1: I have attended weekly lab meeting and present monthly in a meeting with my mentor Dr. Gordon Freeman to expand my knowledge and understanding of tumor immunology. We alternate between presenting data and a relevant recently published journal in a journal club format in our small group meeting.
I have published two manuscripts on VISTA during this grant period.
→ Subtask 2: I presented my work from this DOD "Is VISTA an actionable immune checkpoint target in kidney cancer?" at the monthly Kidney Cancer Dana-Farber Cancer SPORE meeting in July of 2020.
I did not attend the Jackson Laboratories Workshop on Techniques in Modeling Human Cancer in Mice in August 2020 due to it being postponed due to COVID19.
→ Subtask 3: I continue attending the monthly DF/HCC Kidney Cancer SPORE meetings and presented my research at SPORE meeting during year 2 (2019).
→ Subtask 4: I did attend a national scientific meeting in relevant scientific field this year: specifically, the annual American Society of Clinical Oncology (ASCO) meeting in June, 2020, which was held online, due to COVID19.

- **How were the results disseminated to communities of interest?**

I presented the aims, preliminary data, and some of the results from the first year of this project at Kidney Cancer Research Summit in Philadelphia, Pennsylvania in September 2019. This meeting was organized by the charitable organizations: Kidney Cancer Coalition and KCAN, to bring together basic and cutting-edge translational researcher supported by the funding from the Department of Defense CDMRP - KCRP. I presented my work from this DOD "Is VISTA an actionable immune checkpoint target in kidney cancer?" at the monthly Kidney Cancer Dana-Farber Cancer SPORE meeting in July of 2020 remotely.

I published an editorial on VISTA and its novel acidity-dependent receptor PSGL-1 in 2020: Mahoney KM, Freeman GJ. Acidity changes immunology: a new VISTA pathway. Nat Immunol. 2020 Jan;21(1):13-16. doi: 10.1038/s41590-019-0563-2.

I published original data from this grant in Yuan L, Tatineni J, Mahoney KM, Freeman GJ. VISTA: A Mediator of Quiescence and a Promising Target in Cancer Immunotherapy. Trends Immunol. 2021 Mar;42(3):209-227. doi: 10.1016/j.it.2020.12.008. Epub 2021 Jan 23.

12. Impact

- **What was the impact on the development of the principal discipline(s) of the project?** Nothing to report.

- **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

13. Changes/Problems

- **Changes in approach and reasons for change**

Due to the slowing of nephrectomy samples 2019 year due to COVID and multiple labs assessing current nephrectomies for other projects (Kidney Cancer Association's Advanced Discovery Award "HHLA2/KIR3DL3 as a novel therapeutic immune checkpoint pathway in renal cancer" and the DFHCC Kidney SPORE), and given the role of cytoreductive nephrectomy nehrectomy has gotten increased scrutiny with recent efficacy of combination therapy clinical trials, we did not have access to sufficient human RCC samples to power our study. We analyzed mouse RENCA samples for VISTA, PSGL-1 and VSIG3, but given

our invitro studies found the PSGL-1:VISTA interaction was not confirmed, we focused on the in vivo mouse VISTA blocking studies rather than single cell RNASeq of the mouse RENCA model.

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

The technicians, Dhouha Daasi and Arul Shanmugan, left the lab to start a postdoctoral fellowship in the fall of 2019 and due to COVID-associated family obligations, respectively. In the second year of this grant, I recruited the masters student Long Yuan to work on this project, and in the third year recruited the assistance of two additional members of the lab: an undergraduate student Jahnavi Tateni and technician Laura Carstensen to perform mouse experiments.

- **Changes that had a significant impact on expenditures**
- **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents** Nothing to Report
- [**Significant changes in use or care of human subjects** Nothing to Report
- **Significant changes in use or care of vertebrate animals.** Nothing to Report
- **Significant changes in use of biohazards and/or select agents** Nothing to Report

14. Products

- **Publications, conference papers, and presentations**
  - i. **Journal publications.**

Mahoney KM, Freeman GJ. Acidity changes immunology: a new VISTA pathway. Nat Immunol. 2020 Jan;21(1):13-16. doi: 10.1038/s41590-019-0563-2.

Yuan L, Tatineni J, Mahoney KM, Freeman GJ. VISTA: A Mediator of Quiescence and a Promising Target in Cancer Immunotherapy. Trends Immunol. 2021 Mar;42(3):209-227. doi: 10.1016/j.it.2020.12.008. Epub 2021 Jan 23.

  - ii. **Books or other non-periodical, one-time publications.** Nothing to Report
  - iii. **Other publications, conference papers, and presentations.** Nothing more 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

15. Participants & Other Collaborating Organizations

- **What individuals have worked on the project?**

Name:	*Kathleen Mahoney*
Project Role:	*PI*
Researcher Identifier (e.g. ORCID ID):	2-3869-6803
Nearest person month worked:	*18*
Contribution to Project:	*Dr. Mahoney has performed work in the planning of experiments in year 3, including binding assays and functional assays, as well as hiring and training of new technician and student for this project.*
Funding Support:	Beth Israel Deaconess

Name:	*Aedin Culhane*
Project Role:	Computational Biostatistician
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	1.00
Contribution to Project:	*Aedin Culhane has performed the computational biostatistics on the human and mouse experiments.*
Funding Support:	Dana-Farber Cancer Institute

Name:	Paul Catalano
Project Role:	Biostatistician
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	1.00
Contribution to Project:	*Paul Catalano has performed the experimental biostatistics on the mouse experiments and human specimen exploratory analysis for potential biomarkers.*
Funding Support:	Dana-Farber Cancer Institute

- **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?** Nothing to Report

16. Special Reporting Requirements

- **COLLABORATIVE AWARDS:** Not applicable
- **QUAD CHARTS:** Not applicable

17. Appendices: Not applicable