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14. ABSTRACT

Hypoxia is a potent microenvironmental factor promoting metastatic progression. A critical step in metastatic tumor progression is the ability of tumor cells to evade immune attack. Tumor cells utilize a complex set of mechanisms that prevent the immune system from mounting effective anti-tumor responses. Moreover, the hypoxic tumor microenvironment plays an important role in immune escape by favoring immune suppression and tumor resistance. Tumor hypoxia is thought to promote the immunosuppressive phenotypes of both tumor cells as well as infiltrating immune cells. However, the mechanisms by which hypoxia promotes immunosuppression in ovarian cancer remains to be elucidated and may have important therapeutic implications in the treatment of metastatic ovarian cancer. We hypothesize that hypoxia through HIF-1 signaling in regulatory T cells promotes angiogenic and immunosuppressive phenotypes, each contributing to metastatic ovarian cancer tumor growth. Here we generated mice to directly assess the functional role of HIF-1 in Treg cells in ovarian cancer metastatic tumor growth, angiogenesis, and immunosuppression.

15. SUBJECT TERMS
Ovarian cancer, hypoxia, tumor microenvironment, immune suppression, angiogenesis

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INTRODUCTION:
Metastatic disease is the leading cause of death in ovarian cancer patients. Metastasis is a highly complex and dynamic process that involves critical interactions between tumor cells and the microenvironment. Hypoxia is a potent microenvironmental factor promoting metastatic progression. Clinically, hypoxia and the expression of the hypoxia inducible transcription factors HIF-1, and HIF-2 are associated with increased distant metastasis and poor survival in ovarian cancer. A critical step in metastatic tumor progression is the ability of tumor cells to evade immune attack. Tumor cells utilize a complex set of mechanisms that prevent the immune system from mounting effective anti-tumor responses. Moreover, the hypoxic tumor microenvironment plays an important role in immune escape by favoring immune suppression and tumor resistance. Tumor hypoxia is thought to promote the immunosuppressive phenotypes of both tumor cells as well as infiltrating immune cells. However, the mechanisms by which hypoxia promotes immunosuppression in ovarian cancer remains to be elucidated and may have important therapeutic implications in the treatment of metastatic ovarian cancer. We hypothesize that hypoxia through HIF-1 signaling in regulatory T cells promotes angiogenic and immunosuppressive phenotypes, each contributing to metastatic ovarian cancer tumor growth. Here we propose to directly assess the functional role of HIF-1 in Treg cells by utilizing a genetic approach to dissect the functions of HIF in the context of ovarian cancer metastatic tumor growth, angiogenesis, and immunosuppression.

KEYWORDS: Hypoxia, tumor microenvironment, ovarian cancer, regulatory T cell, HIF-1, angiogenesis, therapy, metastasis, immune suppression.

ACCOMPLISHMENTS:
The major goals of this project are to determine the functional role of hypoxic HIF signaling in regulatory T cells and the impact on ovarian cancer metastasis. In aim 1 we propose to determine the role of HIF-1 deletion in Treg cells in ovarian tumor metastasis. In the second aim, we will determine the role of HIF-1 deletion in regulating proangiogenic activities of Treg cells. In the third aim, we will test the role of HIF-1 in mediating the suppressive function of Treg cells. This project investigates the role of hypoxia inducible factors in driving the metastatic phenotype of ovarian cancer and proposes to block these factors and associated pathways as therapeutic strategies for the treatment of ovarian cancer.

The major goals of the project during this funding period are as stated in the approved SOW are as follows:
TASK 1. To determine the role of HIF-1 deletion in Treg cells on metastatic ovarian cancer growth (years 1 and 2).
Task 1a. Generate FOXP3-Cre and FOXP3-HIF-1mice with existing FOXP3-Cre and HIF-1 floxed homozygous. Two rounds of breeding are required and we need a total of 50 female mice to be generated FOXP3-Cre and FOXP3-HIF-1 (n = 10 each, July 31 2015- July 31 2016).
The goal in the first reporting period (July 31, 2015-July 31, 2016) was to generate mice in which we could investigate the functional role of HIF-1 signaling (inactivation) in regulatory T cells (Tregs) and its impact on ovarian cancer metastasis. To test the functional importance of HIF-1 in Treg cells on metastatic ovarian cancer growth, we have utilized a genetic approach in which conditional deletion of HIF-1 in Treg cells will be achieved using Cre-loxP mediated recombination with a Treg specific promoter. The conditional allele for HIF-1 contains loxP sites that flank exon 2 which encodes the bHLH DNA binding domain resulting in an out-of-frame deletion of exon 2 and inactivation of HIF-1 upon Cre-mediated recombination (Ryan et al., 1998). HIF-1 floxed mice on the C57BL/6 background were a gift from Dr. Randall Johnson and have been part of our breeding colony for many years (Rankin et al., 2012). FOXP3-YFP/Cre mice express a knocked in yellow fluorescent protein/Cre-recombinase fusion protein from the Foxp3 locus without disruption endogenous Foxp3 expression. These mice were recently purchased from the Jackson Laboratory on a C57BL/6 background and have been

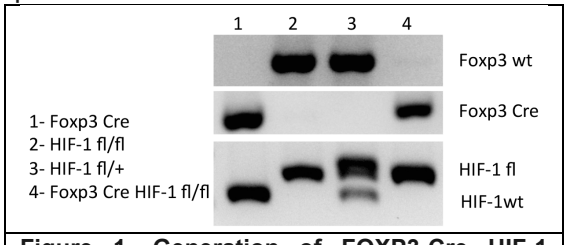

Figure 1. Generation of FOXP3-Cre HIF-1 deficient mice. Genomic PCR of the FOXP3-Cre, HIF-1 floxed alleles in control and FOXP3-HIF-1 deficient mice.

previously used to study the functional role of specific factors in Treg cells (Rubtsov et al., 2008). Mice homozygous for the HIF-1 conditional allele (floxed/floxed) were crossed to FOXP3-Cre mice to generate FOXP3-HIF-1 heterozygous male and female mice. These mice were bred to generate control (FOXP3-Cre) and FOXP3- HIF-1 floxed/floxed deficient female mice that have FOXP3-Cre present on both X alleles (Fig.1). We conclude that FOXP3-HIF-1 deficient mice are viable and the number of Tregs within the spleen and mesenteric lymph node of FOXP3-Cre
control and FOXP3-HIF-1 deficient mice were not significantly different under homeostatic and tumor bearing conditions (Fig. 2).

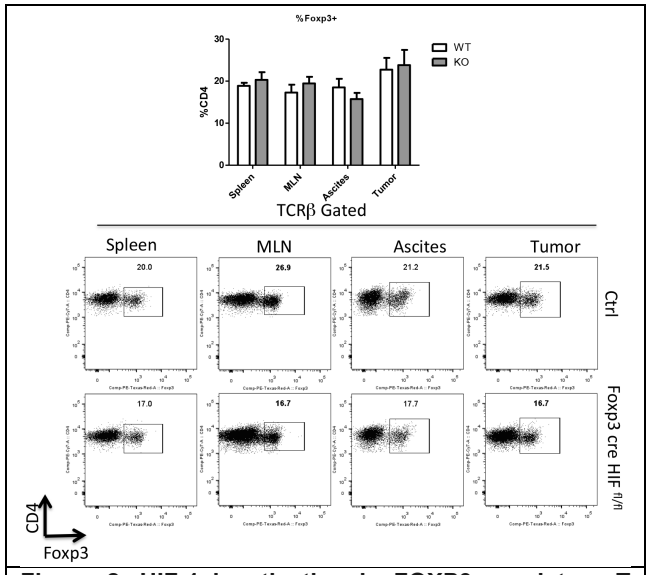

Figure 2. HIF-1 inactivation in FOXP3 regulatory T cells (Tregs) does not affect the frequency of Tregs in the spleen, mesenteric lymph node, ascites, or tumor of ID8 tumor bearing mice. FOXP3-Control (WT) or FOXP3-HIF-1 deficient mice were injected i.p. with 10^6 ID8-Ascites cells. Mice were monitored daily. 30 days after injection mice developed symptoms of ovarian cancer. Ascites volume was measured using a syringe. Macroscopic tumors evident in the omenta of mice were weighed. The frequency of TCRB, CD4, and FOXP3 positive Tregs within each tissue was determined by FACS analysis.

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

This grant is a career development grant where I am an active member and participant of the Ovarian Cancer Academy. During this funding period (July 31, 2015- July 31, 2016) I have attended the kick off DOD Ovarian Cancer Academy (DOD OCA) meeting in Bethesda (July, 2015) in which I had the opportunity to network and meet with the Deans of the Academy, Drs. Nita Maihle and Doug Levine, as well as all of the other early career investigators within the Ovarian Cancer Academy. Additionally, I attend and participate in monthly DOD OCA webinars where I have had the opportunity to present my work and receive feedback, learn about others work to identify collaborations, and receive career development lectures. Finally, I have also had the opportunity to attend and present a poster on our work at the Marsha Rivkin Ovarian Cancer meeting in Orlando (October 2015). Additional professional development activities include starting an Ovarian Cancer Focus Group meeting at Stanford University where Ovarian cancer researchers (Oliver Dorigo, Jonathan Berek, Mickey Hu, Nelson Teng, and Wendy Fantl) present their work in an informal setting to establish collaborations and receive constructive feedback for their work. For my training activities, I meet with my mentor, Dr. Jonathan Berek, monthly to discuss the progress and growth of my ovarian cancer research and identify opportunities for growth. As a result of these meetings, I have applied and received extramural funding from the Marsha Rivkin Center for Ovarian Cancer Research and the Mary Kay Foundation to support my ovarian cancer research.

How were the results disseminated to communities of interest?

While I do not have significant scientific results to report in the first reporting period, I have reached out to the greater Stanford community to make them aware of my project activities and involvement with the DoD Ovarian Cancer Academy. I was interviewed by the Stanford Medicine Scope Blog, an online publication for the Stanford Community and donors, where I described the need for ovarian cancer research, the goals of the DoD Ovarian Cancer Academy, as well as my professional and research goals within this program.

What do you plan to do during the next reporting period to accomplish the goals?
Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.

The goal of the research in the next reporting period is to:

Task 1b. Evaluate ID8 metastatic tumor growth in 6-8 week old FOXP3-HIF-1 mice generated above (July 31 2016-July 31 2017).

We have obtained an highly metastatic derivative of the ID8 syngeneic ovarian cancer cell line, ID8-ascites that was generated by Dr. Katherine Fuh at Washington University. Intraperitoneal injection of ID8 cells results in the development of ascites and solid tumor lesions within the omentum and peritoneum within 30 days post injection (Fig. 3). We have began pilot studies to compare the metastatic tumor growth of ID8-ascites cells in FOXP3-Control and FOXP3-HIF-1 deficient mice. Preliminary studies suggest that HIF signaling in FOXP3 Treg cells does not significantly impact ID8 ascites tumor metastasis as determined by ascites volume and total tumor volume (Fig. 3). Additional studies with more animals per group are needed to confirm these findings and will be performed within the next reporting period.

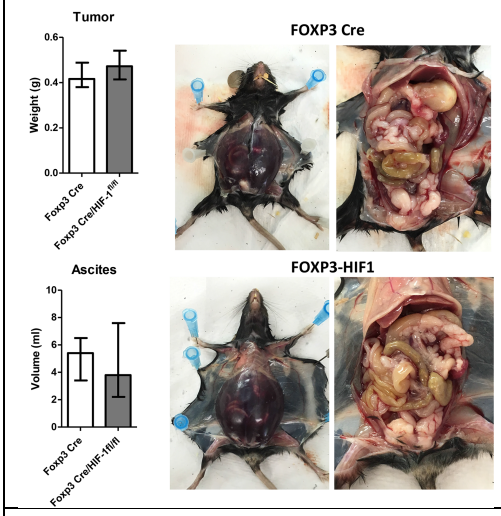

Figure 3. Metastatic tumor burden in the ID8-ascites model of ovarian cancer. FOXP3-Cre control and FOXP3-HIF1 deficient female mice were injected i.p. with $1X10^6$ ID8-Ascites cells. Mice were monitored daily. At 30 days of injection mice developed symptoms of ovarian cancer. Ascites volume was measured using a syringe. Macroscopic tumors evident in the omentum of mice were weighed.

IMPACT:
What was the impact on the development of the principal discipline(s) of the project?
Nothing to Report.

Describe how findings, results, techniques that were developed or extended, or other products from the project made an impact or are likely to make an impact on the base of knowledge, theory, and research in the principal disciplinary field(s) of the project. Nothing to Report.

What was the impact on other disciplines?
Nothing to Report.

Describe how the findings, results, or techniques that were developed or improved, or other products from the project made an impact or are likely to make an impact on other disciplines.
What was the impact on technology transfer?
Nothing to Report.

Describe ways in which the project made an impact, or is likely to make an impact, on commercial technology or public use, including: transfer of results to entities in government or industry; instances where the research has led to the initiation of a start-up company; or adoption of new practices. What was the impact on society beyond science and technology?
Nothing to Report.

Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:
Nothing to Report.

CHANGES/PROBLEMS:
Describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipated.
Nothing to report.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents.
Nothing to report.

PRODUCTS:
Publications, conference papers, and presentations
Rankin EB, Giaccia AJ; Hypoxic control of metastasis; Science; 352: 2016; 175-80; published; acknowledgement of federal support (yes).

Other Products:
We have generated FOXP3-HIF-1 mice in which HIF-1 is conditionally inactivated in regulatory T cells (Tregs). These mice can be useful for a variety of applications investigating the impact of HIF-1 signaling in Treg function.

PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

Name:	Erinn Rankin
Project Role:	Primary Investigator
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	3
Contribution to Project:	Dr. Rankin has designed and assisted Ms. Soriano in all proposed experimental design and execution.
Funding Support:	N/A

Name:	Jonathan Berek
Project Role:	Mentor
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	1.2
Contribution to Project:	Dr. Berek mentors Dr. Rankin by ensuring that Dr. Rankin's research and career development is progression.
Funding Support:	N/A

Name:	Michaela Soriano
Project Role:	Research Assistant
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	7.44
Contribution to Project:	Ms. Soriano has performed all proposed experiments with Dr. Rankin.
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?
Nothing to Report.

What other organizations were involved as partners?
Nothing to Report.

SPECIAL REPORTING REQUIREMENTS
Nothing to Report.

APPENDICES
Nothing to Report.