

AWARD NUMBER: W81XWH-17-1-0144

TITLE: Immune Infiltrate Dynamics in Cancer Progression

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REPORT DATE: May 2018

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
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REPORT DOCUMENTATION PAGE

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1. REPORT DATE May 2018			3. DATES COVERED 1 May 2017 - 30 Apr 2018		
4. TITLE AND SUBTITLE Immune Infiltrate Dynamics in Cancer Progression			5a. CONTRACT NUMBER		
			5b. GRANT NUMBER W81XWH-17-1-0144		
			5c. PROGRAM ELEMENT NUMBER		
6. AUTHOR(S) Sandra Orsulic E-Mail: orsulics@cshs.org			5d. PROJECT NUMBER		
			5e. TASK NUMBER		
			5f. WORK UNIT NUMBER		
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Cedars-Sinai Medical Center 8700 Beverly Boulevard Los Angeles, CA 90048			8. PERFORMING ORGANIZATION REPORT NUMBER		
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012			10. SPONSOR/MONITOR'S ACRONYM(S)		
			11. SPONSOR/MONITOR'S REPORT NUMBER(S)		
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT The goal of this project is to systematically characterize immune cell enrichment in the microenvironment of ovarian cancers and identify immune cell subsets that are prognostically relevant for patient care and/or can be used to develop more effective therapies targeting the tumor microenvironment. The two main cell types in the tumor microenvironment are fibroblasts and immune cells. An increased presence of fibroblasts is typically associated with poor prognosis while an increased presence of immune cells is an indicator of good prognosis. However, it is unclear if fibroblasts and immune cells affect prognosis independently or through an interdependent interaction. We first developed a computational model to identify immune cell types that are over-represented in fibroblast-rich tumors and under-represented in fibroblast-poor tumors. We found that fibroblast-rich tumors have an increased presence of pro-tumorigenic, and decreased presence of anti-tumorigenic, immune cells. Our comprehensive analyses of the tumor microenvironment in primary, metastatic, and recurrent ovarian tumors confirmed our hypothesis that fibroblasts alter the composition of immune cell infiltrates in tumors to tip the balance toward the pro-tumorigenic immune cell types. Our major finding from the correlative analysis of the tumor microenvironment with clinical outcomes is that the presence of CAFs negates the beneficial effects of immune infiltrates on patient survival.					
15. SUBJECT TERMS Ovarian cancer, tumor microenvironment, tumor progression, cancer-associated fibroblasts, immune infiltrates					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			19b. TELEPHONE NUMBER (include area code)
Unclassified	Unclassified	Unclassified	Unclassified	18	

Table of Contents

	<u>Page</u>
1. Introduction.....	1
2. Keywords.....	1
3. Accomplishments.....	1
Major goals of the project.....	1
Major accomplishments.....	4
Major activities.....	4
Specific objectives.....	4
Significant findings or key outcomes.....	4
Other achievements.....	12
4. Impact.....	12
5. Changes/problems.....	13
6. Products.....	13
7. Participants.....	14
8. Special reporting requirements.....	14
9. Appendix.....	N/A

1. INTRODUCTION:

It is becoming increasingly clear that the tumor microenvironment plays an important role in tumor evolution, including the evolution of immune tolerance. In particular, cancer-associated fibroblasts (CAFs) are emerging as critical regulators of immune cell recruitment and function. CAFs physically hinder trafficking of functional immune cells to the tumor bed. CAFs are also a rich source of biologically active molecules that modify immune cell behavior and attract or repel certain immune cell types. While tumor progression is typically accompanied by the increased presence of CAFs, the configuration of pro-tumorigenic and anti-tumorigenic immune cell types during tumor progression is currently unknown. Our integrative approach is providing a comprehensive view of immune cell enrichment in the tumor microenvironment during tumor progression. Our major finding is that the presence of CAFs negates the beneficial effects of immune infiltrates on patient survival. In addition to identifying combinations of cell subsets that are prognostically significant for patient treatment with existing immunotherapies, our study could prove that immune cell infiltration can be effectively manipulated by targeting CAFs, which may provide a novel approach to immunotherapy. Our spatiotemporal quantitative map of individual immune cell subsets in matched primary, metastatic, and recurrent ovarian tumors could serve as a foundation for therapeutic targeting of the CAF/immune cell interface to develop more effective and less toxic therapies that selectively deplete the tumor-promoting stromal cell subsets in different stages of tumor progression.

KEYWORDS:

Ovarian cancer, tumor microenvironment, tumor progression, cancer-associated fibroblasts, immune infiltrates

ACCOMPLISHMENTS:

- What were the major goals of the project?

2.

Specific Aim 1 (specified in proposal)	Timeline	Percent Completed
Major Task 1 Identify biomarkers and therapeutic targets in the tumor stroma by correlating stromal factors with clinical outcomes.	Months	Cedars-Sinai Medical Center
Subtask 1 Normalize transcriptome data from 5 public Affymetrix-based ovarian cancer datasets and combine into one dataset with transcriptome and clinical information for 1,228 primary ovarian cancer samples.	1-2	100%
Subtask 2 Computationally (implementing the BASE algorithm) deduce hematopoietic cell type enrichment in human ovarian cancer samples using the ImmGen dataset (131 mouse hematopoietic	3-5	90%

cell types). Validate the enrichment of 22 major hematopoietic cell types using the LM22 dataset (leukocyte 547-gene signature that distinguishes 22 major human hematopoietic cell types).		
Subtask 3 Use statistical methods to associate enrichments in individual hematopoietic cell types (or specific groups of cell types) with distinct molecular and clinical phenotypes, such as the molecular subtype, chemosensitivity, metastasis, and survival.	6-7	90%
Subtask 4 Write and publish the computational part of the manuscript.	8-15	40%
Milestone Achieved Identified and validated association between immune cell enrichment and molecular and clinical parameters (molecular subtype, stromal content, overall survival, disease-free survival, and cisplatin sensitivity) in 1,228 primary ovarian cancer samples.	15	
Specific Aim 2 (specified in proposal)	Timeline	Site 1
Major Task 2 Validate the identified stromal factors and generate a spatiotemporal map of stromal activity associated with tumor progression and chemoresistance.	Months	Cedars-Sinai Medical Center
Subtask 1 Amend approved IRB 44852 protocol for local approval and send related material for DoD's approval.	Upon award notice	100%
Subtask 2 Design an immune cell panel for multi-color flow cytometry/immunohistochemistry (IHC)/immunofluorescence (IF); purchase reagents; plan experiments.	1-30	70%
Subtask 3 Prospectively collect surgically isolated matched primary and metastatic ovarian cancer samples from 40 patients. Process tissues in 4 ways: 1) disaggregate into viable single-cell suspensions for flow cytometry; 2) fix in formalin/embed in paraffin (FFPE) for IHC; 3) snap freeze for IF; 4) grow fresh omental metastasis tissue (5-10 patients) in culture for isolation of carcinoma-associated fibroblasts (CAFs).	1-30	30%
Subtask 4 Isolate and culture CAFs from metastasis samples from 5-10 patients.	1-12	10%
Subtask 5 Conduct multi-color flow cytometry and multi-color IHC or IF on prospectively collected samples from 40 patients or on an existing tissue microarray (TMA) of patient-matched primary, metastatic, and recurrent cancer samples from 42 patients.	1-30	30%

Subtask 6 Acquire images from TMAs and process for image analysis.	3-30	50%
Subtask 7 If ambiguous results are obtained with TMA, validate IHC and IF data on full tumor sections.	1-36	5%
Subtask 8 Analyze data from multi-color flow cytometry and multi-color IHC or IF and correlate with stromal content and clinical parameters, including debulking status, cisplatin sensitivity, disease-free survival, and overall survival.	3-36	10%
Milestone Achieved Obtained a detailed spatiotemporal map of immune cell subsets in primary and metastatic tumor samples and correlated data with stromal content and clinical parameters, including debulking status, cisplatin sensitivity, disease-free survival, and overall survival. Identified actionable immune biomarkers and therapeutic targets.	36	
Specific Aim 3 (specified in proposal)	Timeline	Site 1
Major Task 3 Demonstrate that targeted ablation of stromal cell subsets impedes tumor progression.	Months	Cedars-Sinai Medical Center
Subtask 1 Amend approved IACUC 5318 protocol for local approval and send related material for DoD's approval.	Upon award notice	Dr. Sandra Orsulic
Subtask 2 Purchase FVB mice, drugs and reagents; design a panel of mouse immunomarkers for multi-color flow cytometry and IHC/IF; select targeted stromal agents for treatment; plan experiments.	1-2	30%
Subtask 3 Implant FVB mice with mouse ovarian cancer cells. Treat mice with selected targeted stromal agents.	3-25	30%
Subtask 4 Collect and process tissues from experimental and control mice, including ascites, blood, tumor, spleen and lymph nodes, for multi-color flow cytometry and IHC or IF.	5-30	30%
Subtask 5 Conduct multi-color flow cytometry and multi-color IHC or IF on collected tissues from experimental and control mice, including ascites, blood, tumor, spleen and lymph nodes.	5-30	10%
Subtask 6 Analyze data using statistical methods; replicate experiments if necessary; prepare manuscripts.	5-36	10%
Milestone Achieved	36	

Tested the effect of stroma-targeted therapy on immune response. Identified potential immunomodulators for cancer treatment.		
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- **What was accomplished under these goals?**

1) major activities

Our activities focused on obtaining the required regulatory documentation for work with human tissues and mouse models, planning experiments, purchasing reagents, conducting experiments, and analyzing data. We have completed most of the activities proposed in Aim 1 and are currently writing a manuscript describing these data.

2) specific objectives

Aim 1. We identified an association between immune cell enrichment and molecular and clinical parameters (molecular subtype, stromal content, overall survival, disease-free survival, and cisplatin sensitivity) in 1,154 primary ovarian cancer samples.

Aim 2. We generated a detailed spatiotemporal map of immune cell subsets in primary and metastatic tumor samples and correlated data with stromal content and clinical parameters.

Aim 3. We collected tissues from mouse ovarian cancer models treated with two stroma-targeted therapies.

3) significant results or key outcomes, including major findings, developments, or conclusions

Aim 1. Identify biomarkers and therapeutic targets in the tumor stroma by correlating stromal factors with clinical outcomes

Aim 1, major achievement #1. Different molecular subtypes of ovarian cancer are associated with different stromal cell subsets. The hypothesis for Aim 1 is that individual immune cell types associated with specific tumor states and clinical outcomes in ovarian cancer can be identified by superimposing transcriptomes of tumor samples with transcriptomes of isolated hematopoietic cell types. We used unbiased computational analyses of public datasets to test whether the presence of CAFs is correlated with a skewed proportion of immune cell subsets. Transcriptomic analyses have clustered high-grade serous ovarian cancers into 4 main molecular subtypes: immunoreactive, mesenchymal, differentiated, and proliferative (1, 2). Of the 4 molecular subtypes, the immunoreactive and mesenchymal subtypes have been associated with the best and the worst survival, respectively (2). The immunoreactive and mesenchymal subtypes have also been associated with low tumor purity (3, 4) indicating that some of the transcripts defining these subtypes may be derived from various stromal cells, including fibroblasts, adipocytes, endothelial cells, and immune cells. Pathology analyses have confirmed that the mesenchymal subtype of ovarian cancer is enriched for CAFs. It has been shown that the relative contribution of specific stromal cell types in cancer can be deduced by enrichment of cell type-specific transcriptomes generated from purified mouse or human stromal cells (5, 6).

To preliminarily assess the relative content of a wide array of stromal cell types in the 4 molecular subtypes of ovarian cancer, we superimposed the largest existing dataset for ovarian cancer (a compendium of >1000 high-grade serous ovarian cancers curated by Verhaak and colleagues) (2) and the largest collection of purified stromal cell types (231 distinct cell types isolated from mouse tissues by the Immunological Genome Project) (7). Stromal cell enrichment in the 4 molecular subtypes was assessed using the statistical similarity between the gene expression profile of each patient and that of hematopoietic cell types developed by the BASE method (8). Unsupervised hierarchical clustering analysis showed that different molecular subtypes of ovarian cancer are associated with distinct stromal cell enrichment. The immunoreactive and mesenchymal subtypes clustered together and were primarily associated with a high stromal cell content while the proliferative and differentiated subtypes clustered together and were associated with a relatively low stromal cell content (**Fig. 1**).

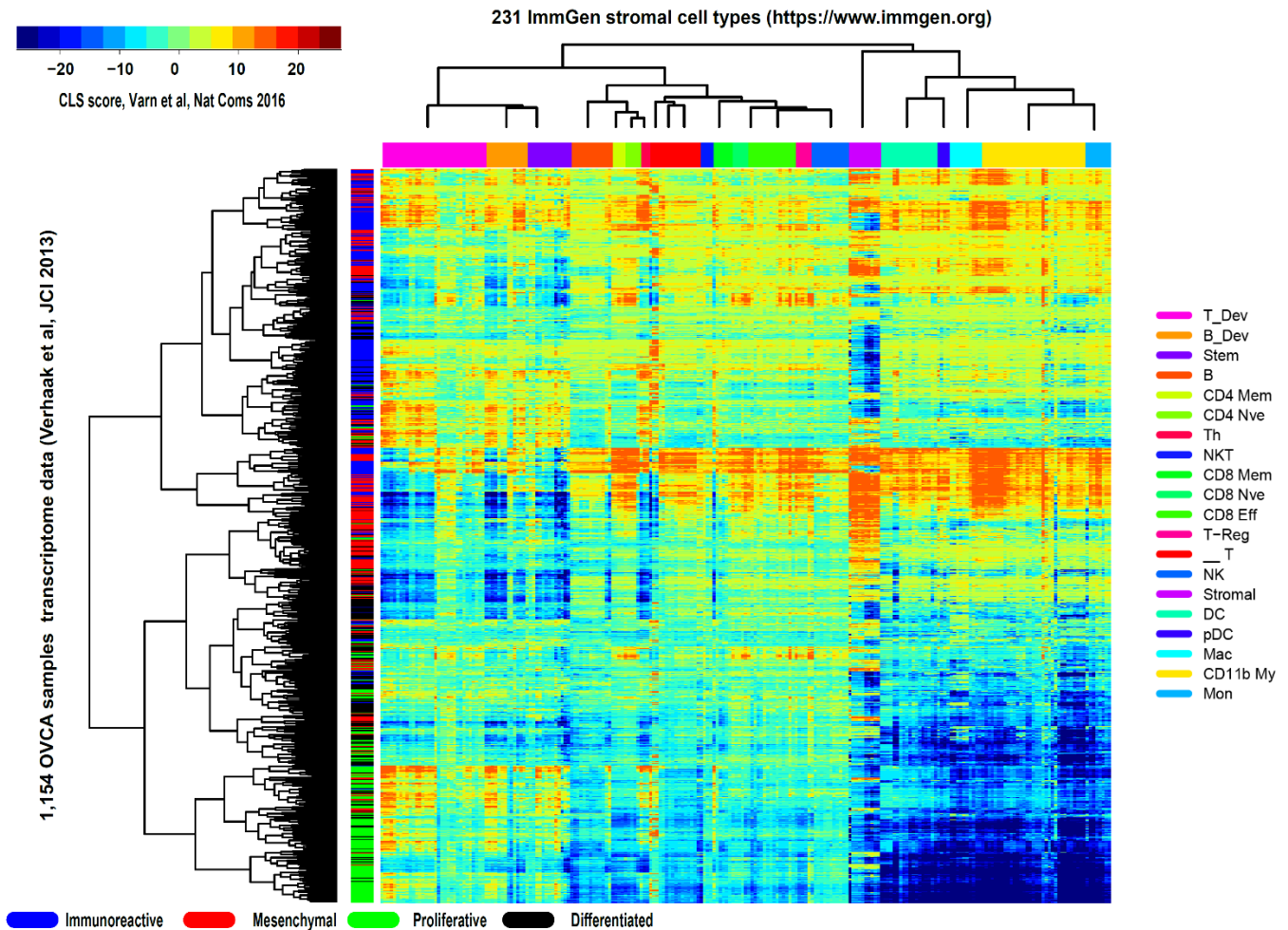


Fig. 1. Molecular subtypes in ovarian cancer are characterized by different stromal cell enrichment. The column shows 231 ImmGen cell types assigned to 20 groups. The row shows 1154 ovarian cancer samples assigned to immunoreactive, proliferative, differentiated, and mesenchymal molecular subtypes.

Aim 1, major achievement #2. The mesenchymal molecular subtype of ovarian cancer is enriched for tolerogenic immune cell subsets. Studies in ovarian cancer have demonstrated that immune cell infiltrates are typically associated with better survival (9). However, not all immune cells are effective in mounting an immune response. Effective immunity requires a highly organized process of maturation and activation of immune cells. To determine whether the 4 molecular subtypes of ovarian cancer in the ovarian TCGA transcriptome dataset are enriched for different phenotypic/functional subsets of immune cells, we evaluated the enrichment of metagenes highly specific for individual differentiation and activation stages of multiple human immune cell types previously defined by Angelova and colleagues (5). Overall, metagenes of immune cell types were enriched in the immunoreactive and mesenchymal molecular subtypes and depleted in the differentiated and proliferative subtypes (**Fig. 2**). This is consistent with the reported high tumor purity (low stromal content) in the differentiated and proliferative molecular subtypes of ovarian cancer. Additionally, the metagenes of most immune cell types, including cytotoxic lymphocytes, effector memory T cells, natural killer (NK) cells, and natural killer T (NKT) cells, were enriched at higher levels in the immunoreactive, rather than the mesenchymal, molecular subtype (**Fig. 2**). The mesenchymal subtype was enriched for the metagenes of typical immunosuppressive cell types, such as T regulatory cells (Treg), T-helper 2 cells (Th2), immature dendritic cells (iDC), plasmacytoid dendritic cells (pDC), mast cells, and monocytes. (**Fig. 2**).

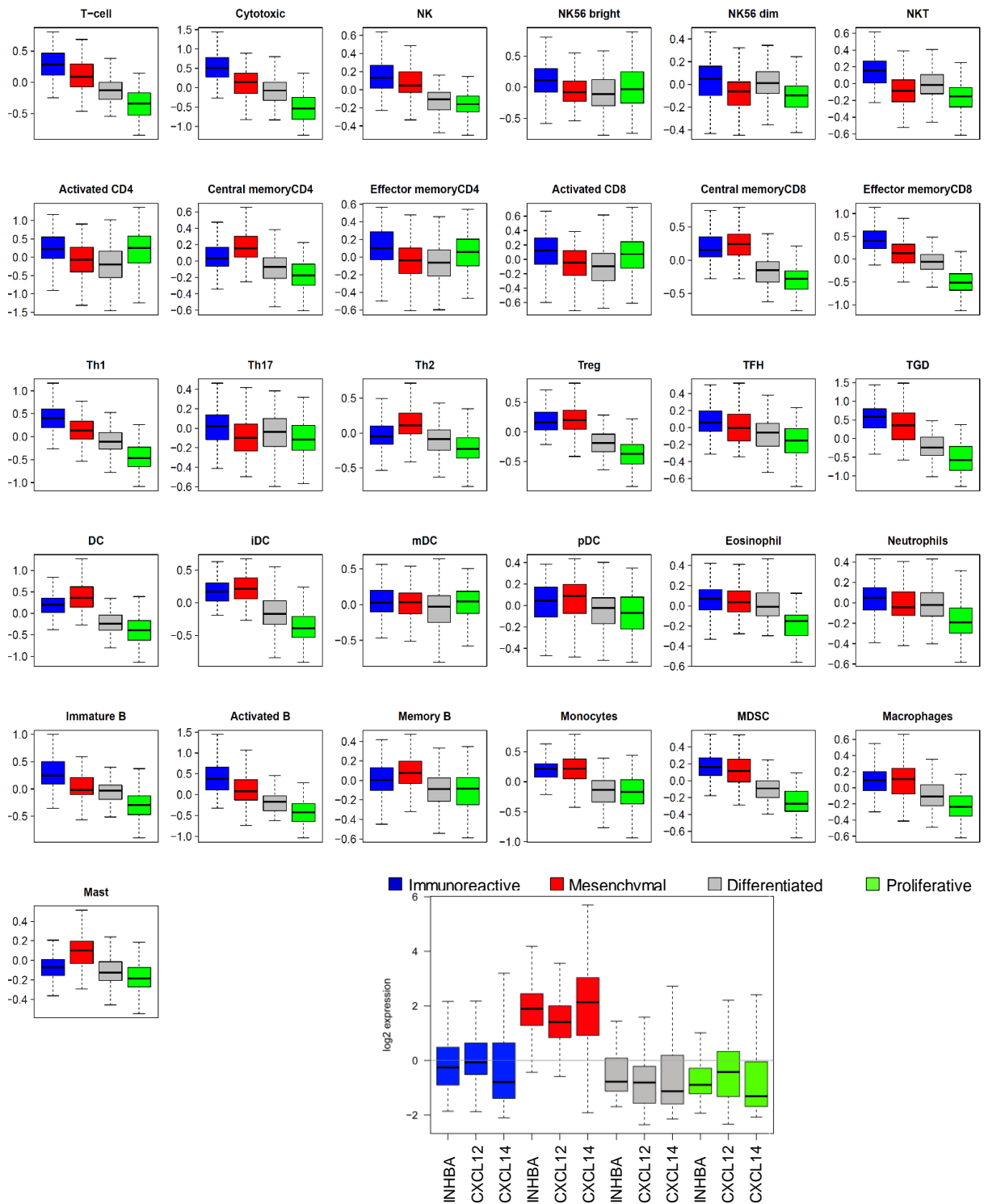


Fig. 2. Molecular subtypes in ovarian cancer are enriched for different subsets of immune cells and secreted molecules that play roles in immune cell attraction, retention, survival, and function.

Aim 2. Generate a spatiotemporal map of stromal activity associated with tumor progression and chemoresistance.

Aim 2, major achievement #1. Generated data on the identity of cell types, extracellular matrix, and soluble factors involved in ovarian cancer progression and metastasis. Ovarian cancer cells have a predilection to metastasize to the omentum although it is still unknown which factors associated with the omentum make this site attractive to ovarian cancer cell seeding (10). With the goal of elucidating expression patterns in the microenvironment of omental metastases, we profiled expression of immune- and cancer progression-related genes in pre-treatment omental metastasis samples from **152 patients** diagnosed with HGSOE. This is the largest study of omental metastasis transcriptome in HGSOE to date (manuscript in preparation). Among other analyses, we quantitated fibroblasts, immune cells, and cancer cells and identified genes correlated with these cell types (**Fig. 3**).

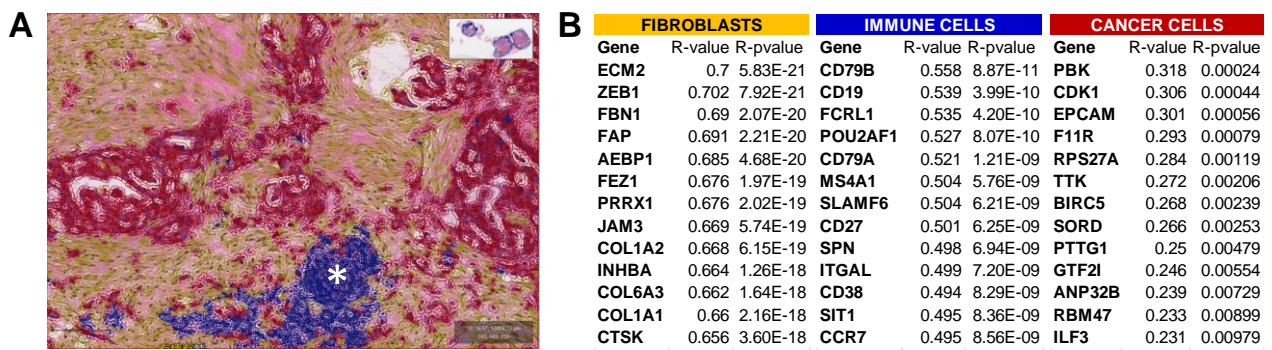


Fig. 3. Identification of genes associated with individual cell types in the tumor microenvironment. (A) Computational image analysis of different cell types in omental metastasis samples from 152 patients with HGSOE. Fibroblasts, immune cells, and cancer cells are shown in yellow, blue, and red, respectively. An asterisk indicates an ectopic lymphoid structure. **(B)** A list of top genes associated with fibroblasts, immune cells, and cancer cells.

In 10 of these patients, we compared the transcriptomes of patient-matched primary cancers, omental metastases, and non-omental peritoneal metastases to evaluate similarities and differences between different tumor sites and to identify genes that may be specifically enriched in individual tumor sites. We found that the transcriptomes of primary tumors are different from those of metastatic tumors and that omental metastases are similar to non-omental metastases regardless of the anatomical location of the non-omental metastasis (**Fig. 4**). Our differential gene expression analysis between patient-matched primary tumors and peritoneal metastases identified many of the genes that have been previously associated with metastasis (11, 12), including POSTN, ADAM12, and COL11A1 (**Fig. 5**), which have been studied in our laboratory in association with suboptimal cytoreductive surgery and poor survival in patients with HGSOE (13-17). We have shown that these genes are expressed by CAFs in human HGSOE (13, 15, 17) and have generated immortalized human CAFs that secrete high levels of POSTN, ADAM12, COL11A1, and other key genes associated with peritoneal metastasis (13, 18).

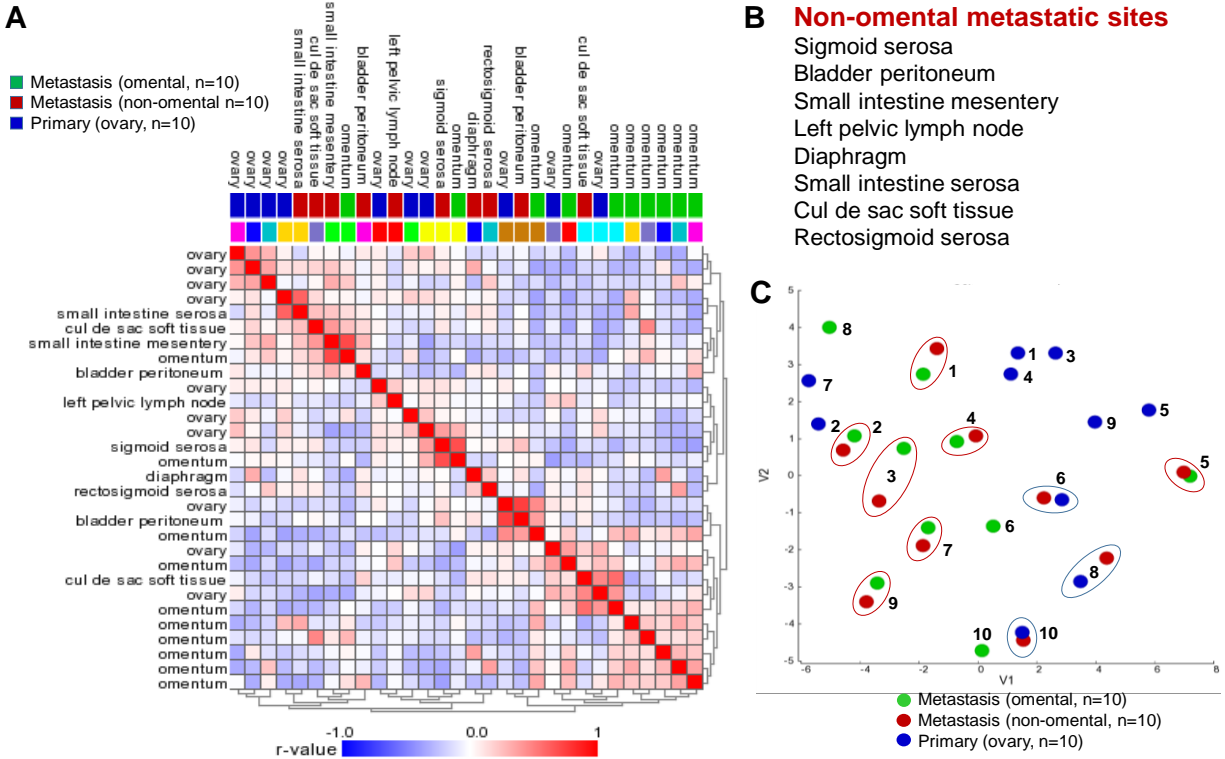


Fig. 4. Molecular profiling reveals a greater similarity between omental and non-omental metastases than between metastases and primary tumors. (A) Sample correlation map (SCM)-rank clustering of 30 patient-matched tumors from 3 different peritoneal sites. **(B)** A list of distinct peritoneal sites of non-omental metastases. **(C)** T-SNE plot (perplexity=9) of 30 patient-matched tumors from 3 different peritoneal sites. In 7 patients, omental and non-omental metastases cluster together, while in the remaining 3 patients, non-omental metastases cluster with the primary tumor.

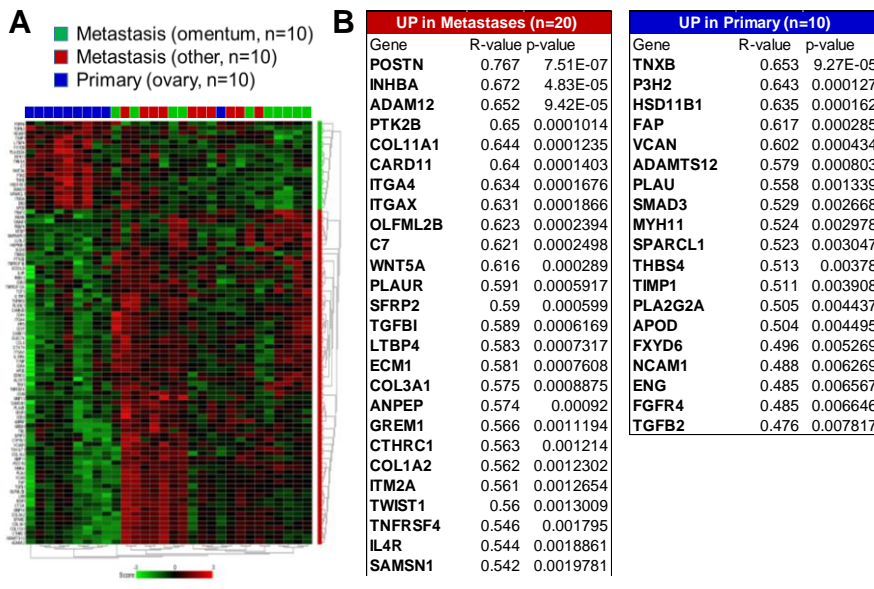
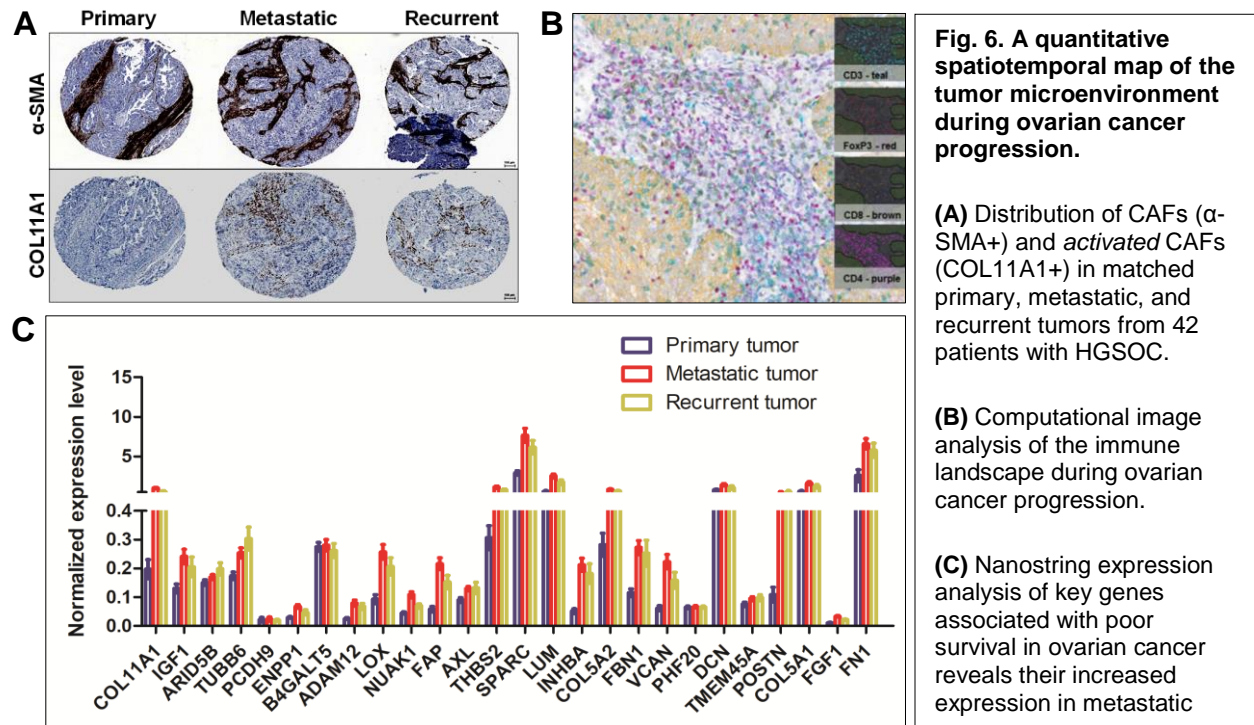


Fig. 5. Identification of genes differentially expressed between patient-matched primary tumors and metastases. (A) Hierarchical clustering of 30 patient-matched tumors from 3 different peritoneal sites shows that omental and non-omental (other) metastases cluster together and away from primary tumors. **(B)** A list of top genes differentially expressed in metastatic and primary tumors.

Aim 2, major achievement #2. Obtained data on the spatial distribution of fibroblasts, immune cells, and cancer cells during ovarian cancer progression. In addition to the 152 samples of omental metastases, we generated Nanostring expression profiles from primary, metastatic, and recurrent tumors from 42 patients with HGSOE and developed a tissue microarray comprising triplicate cores from each of the samples (378 tumor cores + normal tissues). To date, this is the largest collection of clinically and transcriptionally annotated patient-matched primary, metastatic, and recurrent ovarian cancer samples. We are currently analyzing the spatiotemporal dynamics of immune infiltrates and CAFs during tumor progression (**Fig. 6**).



Aim 3. Demonstrate that targeting CAFs improves the immune response to tumors.

At present, we have no significant achievements or findings to report for Aim 3.

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4) *other achievements*

Nothing to report.

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

Nothing to report.

b. How were the results disseminated to communities of interest?

Nothing to report.

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

For Aim 1, we will complete the manuscript and revise based on reviewers' suggestions. For Aim 2 and Aim 3, we will continue our experiments and analyses as planned.

3. IMPACT:

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

Nothing to report.

b. What was the impact on other disciplines?

Nothing to report.

c. What was the impact on technology transfer?

Nothing to report.

d. What was the impact on society beyond science and technology?

Nothing to report.

4. CHANGES/PROBLEMS:

a. Changes in approach and reasons for change. No

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

Dr. Dongyu Jia, one of the two postdoctoral fellows on the project, accepted a tenure-track Assistant Professor position at Georgia Southern University. The recruitment of a new postdoctoral fellow for this project was delayed by several months, but we now have a new postdoctoral fellow to work on the project.

c. Changes that had a significant impact on expenditures.

The delayed recruitment of a postdoctoral fellow reduced overall expenditures.

d. Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents. No

e. Significant changes in use or care of human subjects. No

f. Significant changes in use or care of vertebrate animals. No

g. Significant changes in use of biohazards and/or select agents. No

5. PRODUCTS:

Nothing to report.

a. Publications, conference papers, and presentations

i. Journal publications.

Nothing to report.

ii. Books or other non-periodical, one-time publications. N/A

iii. Other publications, conference papers, and presentations.

Invited talk (acknowledged grant funding): Sandra Orsulic, Tumor Microenvironment. Cancer Biology Seminar, Mayo Clinic, Jacksonville, FL. February 23, 2018.

Invited manuscript (acknowledged grant funding): Marcela Haro and Sandra Orsulic. A paradoxical correlation of cancer-associated fibroblasts with survival outcomes in B-cell lymphomas and carcinomas. Currently under review in *Frontiers in Cell and Developmental Biology*.

b. Website(s) or other Internet site(s). N/A

c. Technologies or techniques. N/A

d. Inventions, patent applications, and/or licenses. N/A

e. Other Products. N/A

6. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

a. What individuals have worked on the project?

Name:	Sandra Orsulic
Project Role:	PI
Nearest person month worked:	2.00
Contribution to Project:	Dr. Orsulic oversaw projects for all three specific aims, including experimental design, execution, and data analysis and interpretation. She submitted one invited manuscript and prepared presentations as well as the progress report.

Name:	Beth Karlan
Project Role:	Collaborator
Nearest person month worked:	0.24
Contribution to Project:	Dr. Karlan advised on the translational aspects of the proposal and participated in experimental design.

Name:	Marcela Haro, PhD
Project Role:	Postdoctoral Fellow
Nearest person month worked:	4.50
Contribution to Project:	Dr. Haro conducted all experiments in this proposal and assisted in data acquisition and analysis. She submitted one invited manuscript and has written parts of the forthcoming manuscript describing the results of analyses conducted in Aim 1.

Name:	Barbie Taylor-Harding, PhD
Project Role:	Research Associate
Nearest person month worked:	3.00
Contribution to Project:	Dr. Taylor-Harding conducted all experiments in Aim 3 that involved testing different combinations of treatments in the immunocompetent mouse model of ovarian cancer and assisted in data acquisition and analysis.

b. Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period? No

c. What other organizations were involved as partners? None

7. SPECIAL REPORTING REQUIREMENTS

a. COLLABORATIVE AWARDS: N/A

b. QUAD CHARTS: N/A

8. APPENDICES: N/A