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14. ABSTRACT

Primary ovarian cancer (OC) represents a complex set of stem cell and cancer cell phenotypes embedded in a mixture of stromal and infiltrating immune cells. This grant develops techniques and approaches using mass cytometry that organize the heterogeneity within and between patient tumors to enlighten mechanisms and clinical opportunities in the apparent chaotic structure of the cancer. Over the past year, we processed and analyzed 22 primary dissociated HG-SOC samples with over 100 antibodies comprising three panels designed to interrogate the tumor cells and tumor-immune infiltrate. We have a streamlined and highly optimized experimental pipeline in place from resection until data analysis of mass cytometry data. In addition to reproducing many of the trends we saw in our pilot studies our data analysis on this recent set of samples so far reveals: i) organization of samples into groups based upon the heterogeneity ii) evidence of epithelial-mesenchymal transition iii) the most comprehensive description of the HG-SOC-immune infiltrate to date. This includes many of the immune checkpoint proteins currently targeted in the clinic.
Data analysis is continuing to elucidate the relationships between these biological findings.

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INTRODUCTION

High-grade serous ovarian cancer (HG-SOC) is the fifth most lethal cancer in women and the most lethal of gynecological malignancies[1,2]. Most often diagnosed at more advanced stages, a great challenge in treating HG-SOC is the apparent large number of disease subclasses based on genetic analyses [1,3,4]. Defective DNA repair mechanisms are characteristic of the disease and are most likely responsible for the extensive genetic abnormalities, most frequent of which are focal copy number alterations and epigenetic modifications [3,4], confounding a systematic approach to successful treatment the disease. Furthermore, given the genetic plasticity of HG-SOC each patient can manifest one disease at diagnosis and other subtypes over time. At present, platinum-based therapeutic regimens are the most commonly used in the clinical settings of first diagnosis and post-relapse. Frequently a more aggressive platinum resistant form emerges.

According to a seminal review by Vogelstein et al. the vast array of genetic events found in cancer all converge on three essential cellular processes, cell fate, cell survival and genome maintenance all regulated by twelve intracellular signaling pathways [5]. This is consistent with cancer having a "structure".

The hypothesis of our DoD proposal is that in spite of the vast range of genetic aberrations detected in HG-SOC, there must exist a unifying architecture that links biology to pathology across these tumors. By dissecting HG-SOC (diagnostic, recurrent and chemo-resistant) into single cells for analysis of their phenotypes and signaling states, at the *deepest possible resolution currently available*, we will provide a unifying vision of *ovarian cancer "systems biology"* to bring about more informed changes to treatment modalities. To accomplish this vision with HG-SOC, we are using a single cell technology, mass cytometry, or CyTOF (**Cy**tometry by **T**ime-**O**f-**F**light), largely developed in our laboratory, for immunologic and cancer cell studies[6-9]. CyTOF uses antibodies conjugated to chelated metal ion tags, allowing for the simultaneous measurement of up to 40 parameters on a cell-by-cell basis, including surface markers and intracellular signaling proteins. CyTOF has been applied to complex tissues such as blood, bone marrow and, recently, ovarian ascites as well as single-cell suspensions derived from primary HG-SOC tumors.

Over the past year, we have greatly expanded the scope of our analysis and performed CyTOF experimentation on a further 22 samples, each with three panels of 40 antibodies. *Thus, each sample was analyzed at the single cell level in over 100 dimensions, a level of detail for proteins not previously seen in HG-SOC or for that matter in any other solid tumor malignancy.* Only minor changes were made to the tumor cell antibody panel. To note the tumor panel was derived from a comprehensive review of the literature as it pertains to ovarian cancer and cancer biology in general. The major changes during this past year were in the design and optimization of two antibody panels against the tumor-immune cell infiltrate. One immune cell panel was focused on deep profiling of dendritic, myeloid and macrophage cells whereas the second panel was focused on NK and T-cells. Specifically,

In addition, our lab and the Neel Lab have initiated the development of functional assays that will be used to understand which cell subsets harbor phenotypes associated with malignancy, metastasis, drug resistance and immuno-suppressive or immuno-enhancive characteristics. Furthermore, the Nolan Lab and Pe'er labs continue to develop new data analysis tools. An overview of our data from the last year will be presented. A brief update (bullet points) for each subtask will be given. Text in blue font is the update of aims for this year's report with updates from our 2014 report, in red font, left for comparison. Within the body of the text, a detailed discussion will be provided for our progress over the last year, which continues to build on **Task1** as well as report on studies initiated for **Task 2** and some exciting new findings in our tumor immune studies in **Task 3.**

KEYWORDS
Serous ovarian cancer, primary tumors, mass cytometry, single cell, antibodies, stem cell, heterogeneity, epithelial mesenchymal transition, immune compartment, clustering, correlation analyses, NK cells, T cells, macrophages, immune checkpoints.

OVERALL PROJECT SUMMARY

A. Background

Single mass cytometry facilitates high-dimensional, quantitative analysis of the effects of bioactive molecules on cell populations at single-cell resolution[6-9]. Datasets are generated with antibody panels (up to 40) in which each antibody is conjugated to a polymer chelated with a stable metal isotope, usually in the Lanthanide series of the Periodic Table [6,8-10]. The antibodies recognize surface markers to delineate cell types, such as immune, epithelial, mesenchymal, and intracellular signaling molecules demarcating multiple cell functions such as survival, DNA damage, cell cycle and apoptosis. By measuring all these parameters simultaneously, the signaling network state of an individual cell can be measured. The ultimate goal of this work, and beyond, will be to assign molecular status and function to cell subsets defined by 40 parameters at the single cell level.

B. Overview of status of tasks

Task 1

Subtask 1a. Establish conditions for dissociation of solid tumors into single cells that maintain cells' ability for functional signaling. **Done with protocols transferred to Indivumed Inc, Hamburg Germany and now routine. We continue to procure de-ideintified samples from Indivumed who are now the preferred provider of quality samples to the NCI.**

Subtask 1b. Select a panel of extracellular modulators with which to measure signaling responses in both tumor cells and peripheral blood cells. **A preliminary list of modulators has been made including but not limited to, TBFβ, BMP2, EGF, TGFα, heregulin, amphiregulinn, LPA, IL6, LPS, IL6, IFNa, and IFNg has been made and protocols for exposing single cell dissociation of primary tumors are in the process of being transferred to Indivumed. Work in progress is prioritizing this list. This next phase will involve transferring our protocols to Indivumed which is planned for later in the year.**

Subtask 1c. Select two panels of ~40 antibodies each. Done. **We constructed two antibody panels in which the second was a variant of the first based on a mass cytometry experiment with six primary samples. *The data from two independent experiments with each panel will be described in the body of the text.* We assembled three panels with 40 antibodies each. One panel was focused on the tumor cells and the two others on the tumor-immune infiltrate (Table 1)**

Subtask 1d. We have submitted the necessary HRPO (IRB) and the ACURO and are awaiting approval. **Done**

Subtask 1e. Acquire 10 primary diagnostic (no treatment) ovarian tumor or ascites samples with matched blood samples. **Done. We have performed two mass cytometry experiments: i) six primary naïve tumors and ten HG-SOC ovarian cell lines described to be genetically most similar to primary HG-SOC [11]. We acquired 22 samples which were all processed for CyTOF with the three antibody panels.**

Subtask 1f. Develop and apply new informatics tools and algorithms to the data generated from subtask 1d (Nolan lab and Pe'er lab at Columbia) (these efforts will be ongoing throughout most

of the duration of this award) **New tools developed: from the Nolan Lab: Citrus [12], X-shift (unpublished), Gatefinder (unpublished), Pe'er Lab: DREMI [13]. A manuscript describing X-shift has been submitted and a manuscript about gatefinder is accepted for publication in nature Biotechnology. We are using these algorithms in combination with standard statistical tools for analyzing our data.**

Subtask 1g. Pending data from subtask 1e modify antibody panels. Titrate any new antibodies (3-36 months. Anticipate continuous low-level activity for this subtask throughout the award period). ***See subtask 1c.*** **Our three new and modified antibody panels include new antibodies which were all conjugated and titrated. Concentrations where signal to noise was maximal were selected for our CyTOF experiments.**

Subtask 1h. Acquire >er than 150 primary diagnostic (Neel lab at UHN Toronto, and Berek at Stanford) serous ovarian cancer samples (from Neel at UHN and Berek at Stanford) and process for mass cytometry with modified panels (6-40 months). Twenty five of these will be processed for xenotransplant (the Neel Lab currently has Research Ethics Board approval to conduct all of the tests described), requiring 10 mice for each subject tumor for 250 mice. **In progress. Continues in progress.**

Subtask 1i. Using SPADE and other algorithms, segregate and aggregate cell subsets in hierarchical pattern with intracellular and cell surface marker combinations. **Using a new deterministic K-nearest neighbor-clustering algorithm, we see important relationships between tumor cell subsets. This information will be presented in the body of the text. We analyzed the new set of 22 HG-SOC samples with this algorithm and noted some new findings especially as they pertain to the tumor and immune cell compartments. This analysis will be presented.**

Subtask 1j. Building of subset space in relationship to therapy/outcome (6-48 months). **We have not run enough samples and also for those we have run, not enough time has elapsed to fully evaluate patient outcome. We are expecting the outcomes data regarding platinum sensitivity in the next couple of weeks and may not be able to incorporate those findings into this report.**

Subtask 1k. Assess relative tumor-initiating properties of cell subsets from subtask 1h with established quantitative xenograft assay (Neel lab, 6-40 months). **In progress.**

Task 2

Previous work from the Nolan group showed that measuring the signaling responses of cancer cells to perturbations is more informative than assessing basal phosphorylation states. This task is focused on measuring signaling responses to extracellular perturbants such as growth factors, cytokines and drugs with relevance to ovarian cancer. In this task, the objective will be to uncover druggable pathways in serous ovarian cell subsets within and across primary samples.

Task 2 has subtasks that are dependent and independent of Task 1. For Task 2 we have set up foundational studies to measure drug responses in HG-SOC cell lines. Specifically, we have set MTT assays (colorimetric readout) to measure the effects of drugs on proliferation, and growth in soft agar assays. We are evaluating carboplatin and paclitaxel and other investigational agents such as PARP inhibitors, JQ1 (an epigenetic modifier) and others that are under evaluation based on our primary tumor work in Task 1. Due to the relative immaturity of these studies, we will focus this report on the 1c, g and i. **We have established growth in soft agar assays using HG-SOC cell lines for evaluating their tumorigenic potential with and without drugs. In the past few months we have focused on PARP inhibitors. The assays is ready for application to cell subsets isolated from primary tumors which is imminent.**

Task 3

Although the presence of infiltrating cytotoxic T cells correlates with good prognosis, whereas regulatory T cells correlate with poor prognosis in SOC, there is limited understanding of the factors that contribute to the generation of these opposing responses. Understanding the mechanisms by which a given tumor microenvironment is able to promote immune surveillance could eventually lead to the clinical development of biomarkers that could select patients responsive to immune therapy. We will use mass cytometry to evaluate the tumor microenvironment in the same SOC samples as above utilizing antibodies against immune cell subsets. **We analyzed 22 HG-SOC primary samples from Indivumed with two panel focused on the immune infiltrate. The panels included checkpoint inhibitors and the data will be presented in detail.**

Subtask 3a*.* Assemble panel of extracellular modulators based on the known biology of the cell types that infiltrate ovarian tumors; immune cells, endothelial cells and stromal cells. **Ongoing**

Subtask 3b*.* Validate reagents to monitor signaling pathways mediated by extracellular modulators in cell lines and peripheral blood. (1-24 months). **We have available a large repository of agents (growth factors, cytokines and drugs [14]) with which to characterize immune cell subsets from peripheral blood taken from HG-SOC patients. We are currently prioritizing which agents to use. Ongoing.**

Subtask 3c*.* Acquire 10 primary serous ovarian cancer samples with which to test response of tumor infiltrating cells to extracellular modulators identified in 3a. **(Ongoing). Ongoing.**

Subtask 3d. Culture tumor-infiltrating lymphocytes from samples in Subtask 3c and characterize them for cytokine and chemokine production. (Ohashi lab 12-24 months). **This subtask has changed and the Nolan Lab is generating enriched immune fractions from primary tumors and establishing in vitro assays to determine immune-suppressive versus immune-enhancive activities of the tumor immune compartment. Based on our data with the tumor immune cell infiltrate (discussed in the body of the text) we are following up with our findings regarding NK cell subsets and performing co-culture experiments between peripheral NK cells and HG-SOC cell lines which are discussed in the text below.**

Subtask 3e*:* Acquire >er than 150 primary serous ovarian cancer samples (Neel lab at UHN Toronto, and Berek at Stanford) with which to test response of tumor infiltrating cells to extracellular modulators identified in 3a. (24-50 months). **In progress.**

Subtask 3f*.* Culture tumor-infiltrating lymphocytes from samples in Subtask 3c and characterize them for cytokine and chemokine production. (Ohashi lab 24-60 months). **See Subtask 3d.**

Subtask 3g*:* Using SPADE and other algorithms, segregate and aggregate tumor infiltrating cell subsets in hierarchical pattern with intracellular and cell surface marker combinations. Build computational models that correlate intracellular signaling responses in tumor infiltrating cell subsets with intracellular signaling responses of tumor cells with clinical outcomes. (12-60 months) **In progress and update will be in body of text. This is a continuous activity for us and the latest data will be discussed in the text below.**

C. Description of studies and results

The bulk of this report will describe the analysis of 22 primary HG-SOC tumor samples with over 100 antibodies providing, to date, the most comprehensive single cell proteomic analysis of HG-SOC.

Introduction and Background

As in years one a two, we continue to pay close attention to obtaining samples of the highest quality, minimizing their ischemic time. With Indivumed Inc. in Hamburg we have highly stringent protocols in place that are now routine. All the primary samples that we evaluated were processed within 4 hours including transit time. *We believe these initial steps—though tedious—*

are critical to "trusting" the data from such precious samples as those obtained from patients with fatal diseases. Furthermore, Indivumed, now the preferred site for high quality sample procurement by the NCI, has sites within the USA at Georgetown, Geisinger Health Network and University of Rochester. Dr. Fantl has been invited to give seminars at all sites to the gynecologic oncologists presenting the work. The idea has been met with enthusiasm and willingness by surgeons to provide many samples for the work in this proposal.

The experiments performed in years one and two, with 6 and 10 primary samples respectively, allowed us to optimize the conditions for the 22-sample experiment we performed in this past reporting year. This has ensured data of the highest quality. Some of the additional controls we performed are included.

Results and analyses

Quality controls

To compare cell characteristics before dissociation with single-cell CyTOF analysis, we performed immunohistochemistry (IHC) *(Dr Alexander Borowsky, UC Davis)* on formalin-fixed paraffin embedded (FFPE) sections prepared by Indivumed within minutes of tumor resection, with the exact same tumors. All IHC was performed manually without the use of automated immunostainers as described [15] with modifications contingent upon optimization of the antibodies for similar antigens used for the CyTOF. Antibody deletion controls were used for every assessed antigen to confirm specific staining. For each marker, we measured the frequency of cells in which it was expressed and the intensity by IHC and CyTOF (E-cadherin, vimentin, CD45, pAKT, FAP and p53). The examples show correlations (Pearson r-value) for the frequency of cells expressing *E-cadherin* (0.41) and *cleaved PARP* (0.52) measured by the platforms (Figure 1 and2). While the data are acceptable, we are continuously evaluating variables, especially from the outliers, to improve the correlations.

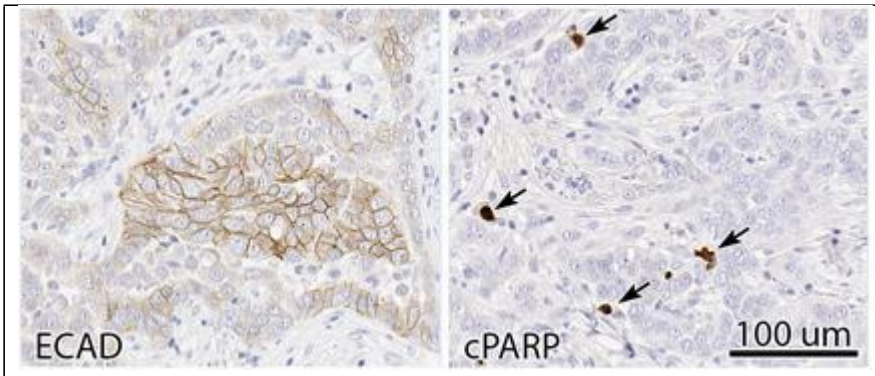

Figure 1: IHC of E-cadherin and cleaved PARP

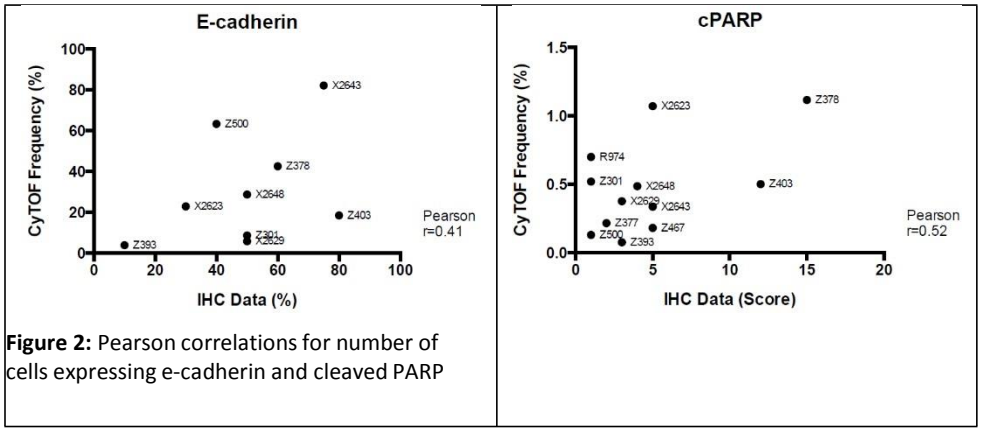

Figure 2: Pearson correlations for number of cells expressing e-cadherin and cleaved PARP

To reduce sample-to-sample variability, mostly due to staining with the antibody panel and differences in machine sensitivity, cells corresponding to each sample were barcoded using six stable palladium isotopes[16,17]. In the barcoding scheme, each isotope is used in binary fashion, high for "1" and low for "0". Hence, the barcode itself is a binary string that is can be decoded for

each cell to determine the sample from which each cell is derived. For the algorithm to be enabled, the maximum number of barcoded samples that can be combined is 20[17]. We also developed two important modifications. One allows us to allow barcode staining, prior to methanol permeabilization, allowing methanol-sensitive surface markers to be assessed in combination with mass cell barcoding multiplexing. This modified protocol relies on transient permeabilization with saponin[16]. The second modification was in the development of a new bar-coding algorithm that takes into account the large range of sizes between individual cells, and the consequent uptake of differing amounts of bar-code, within a complex sample. For example, immune cells are in the order of 15 microns whereas tumor cells are in the size range of >er than 50 microns. In brief, in this new barcoding algorithm we changed the distance metric for discarding outliers. This work is being prepared for publication.

As mentioned above in this latest experiment we analyzed 22 samples with over 100 antibodies against the tumor cells and the immune-cell infiltrate of each individual sample (Figure 3). The choice of antibodies was from an extensive review of the literature, focused on ovarian and cancer biology in general, as well as from our own pilot experiments with HG-SOC cell lines[11] and primary tumor samples. Most antibodies were conjugated in-house and optimized by titrations using biologically relevant cells. ***This experiment represents one of the most comprehensive single-cell proteomic characterizations of HG-SOC ever undertaken.***

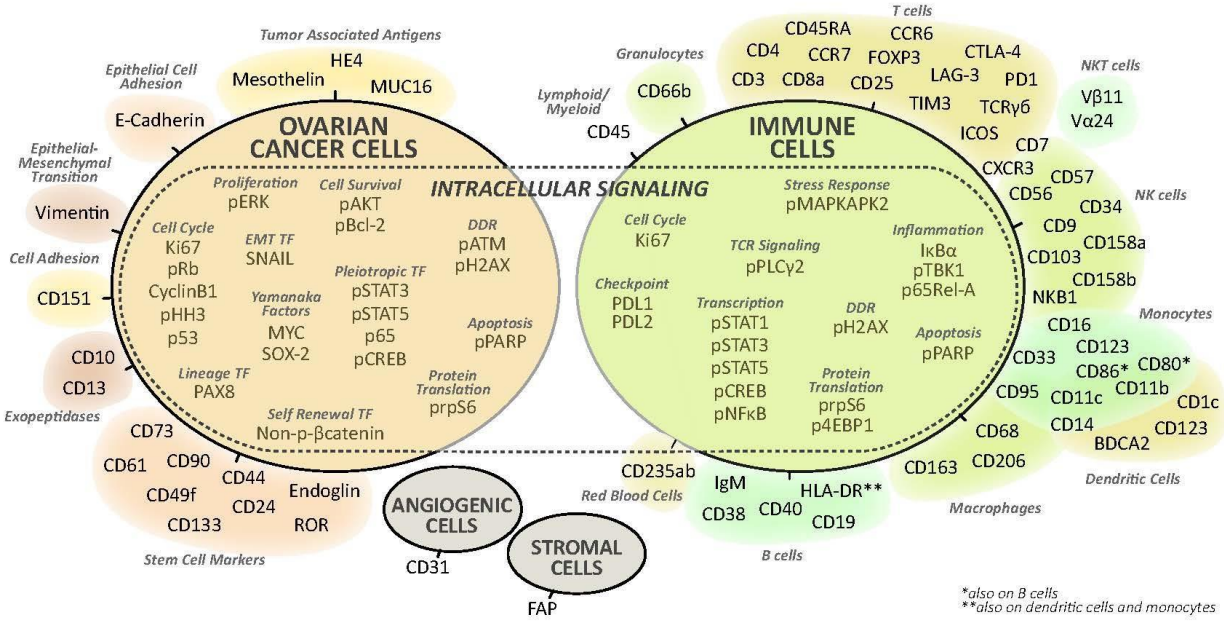

Figure 3: Antibodies against all the proteins depicted were conjugated and optimized for before inclusion into CyTOF panels.

Markers CD45, FAP and CD31 from the tumor antibody panel allow us to enumerate tumor, immune and stroma/angiogenic compartments, respectively. With the caveat that markers are rarely exclusive to a single compartment, for the purposes of this study we defined the **tumor compartment as CD45-/CD31-/FAP-**, the **immune compartment as CD45+/CD31-/FAP.**

Data analysis of tumor compartment

As with our pilot experiments from years 1 and 2, we initiated our data analysis with the gated tumor cells by performing a clustering step using primarily the cell surface markers, exception is vimentin an intermediate filament with epitopes within the cytoskeleton (E-cadherin, MUC16, mesothelin, HE4, CD71, CD49f, CD61, CD24, CD44, CD13, CD90, CD133, CD10, CD151, ROR, endoglin, vimentin). Our "X-shift" unsupervised clustering algorithm X-shift calls out

cell populations by searching for the local maxima of cell event density in the marker space. X-shift uses an optimized algorithm for K-nearest neighbor density estimate, which allows it to perform clustering of large datasets in sub-quadratic runtime. The algorithm has one free parameter, which is the number of nearest neighbors (K) that controls the degree of smoothing of the density estimate and thus, the resulting number of clusters. It can also be run in a fully automated mode, whereby the free parameter value is chosen by the algorithm itself (Samusik and Nolan submitted). X-shift clusters will be cross-checked with other clustering algorithms such as used by the Citrus algorithm [12] as well as manual gating of the CyTOF data. The latter is considered by flow cytometrists as the gold standard for identifying cell subsets by both CyTOF and fluorescence.

X-shift cell clusters (from all 22 samples) were then computationally *arranged* along a ***minimum spanning tree (MST)***, such that clusters with co-expression patterns bearing the most resemblance to each other were placed next to each other. The MSTs can be viewed as an average across all samples or the MST for an individual sample can be extracted from the composite MST. Clusters are represented as bubbles, the *size of which corresponds to the number of cells in the cluster*. *The level of expression of a specific marker in the cluster is color coded from red (high) to blue (background) (Figures 4 and 5).*

HG-SOC is an epithelial cancer whose cells undergo dynamic and reversible transitions between multiple phenotypic states, the extremes of which are defined by the expression of epithelial and mesenchymal proteins. Two proteins that are hallmarks of these states are E-cadherin and vimentin respectively[18-21]. Figures 4 and 5 show "average" MSTs for E-cadherin and vimentin expression patterns. As reported for the first two pilot studies in proceeding years, there is striking exclusivity in their expression profiles. In all cases the data were unsupervised. However, there are a series of central clusters (1 to 14) where E-cadherin and vimentin are co-expressed, suggesting a transitional state between epithelial and mesenchymal (EMT) or

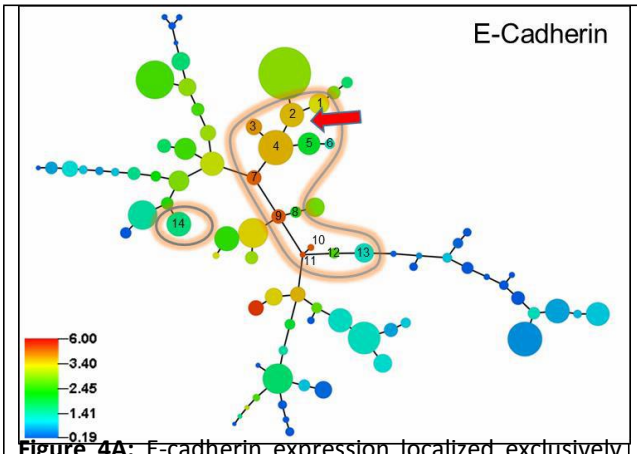

Figure 4A: E-cadherin expression localized exclusively to left-hand side of MST. Co-expression of e-cadherin with vimentin depicted in clusters with orange boundary.

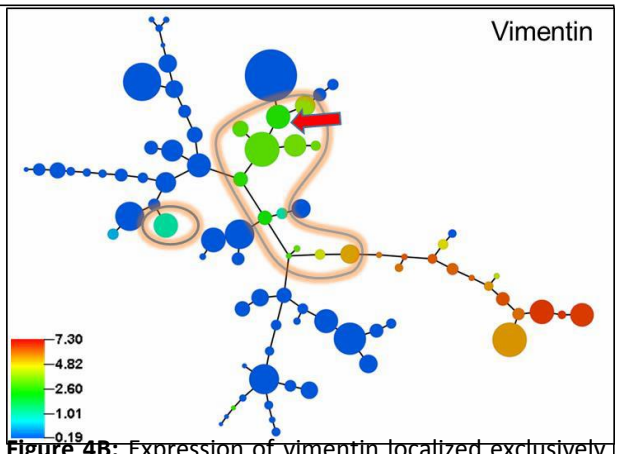

Figure 4B: Expression of vimentin localized exclusively to right-hand side of MST. Co-expression of vimentin with E-cadherin depicted in clusters with orange boundary.

metastatic states. EMT induces epithelial cells to lose polarity and cell adhesion, contributing to the acquirement of cell migration and invasion ability[19,22]. Notably, these clusters have high co-expression levels of stem cell markers (Figure 5), a characteristic described for cells undergoing EMT[23]. In particular cluster #2 co-expressed some of the highest levels of multiple stem cell markers (Figures 4 and 5 red arrow). We are currently analyzing the co-expression of these markers on a per patient basis as well as performing correlations with clusters revealed from the immune panels. Our plan is to sort these cell clusters and perform tumorigenic assays such as

growth in soft agar. At this time it is hard to make correlations with clinical features as almost all the tumors were diagnostic, chemo-naïve from patients with stage IIIC HG-SOC. At the time of this analysis three had relapsed within 6 months after their last cycle of chemotherapy. Thus, at the moment, the numbers are not large enough to make correlations with clinical features.

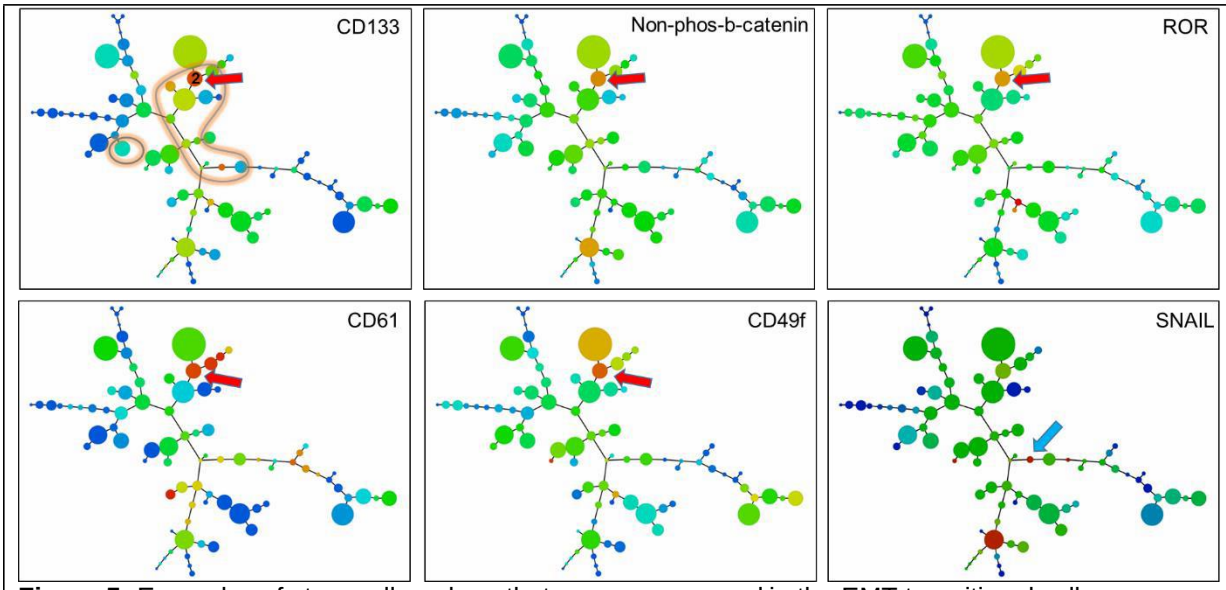

Figure 5: Examples of stem cell markers that are co-expressed in the EMT transitional cells.

In spite of the fact that the specimens were all resected from late stage tumors we wanted to ask whether there were similarities and differences between the clusters of each sample. Thus, in order to address the premise of this work, that the heterogeneity of HG-SOC conforms to a defined set of cell type distributions, the cell populations, identified by their phenotype from X-shift clustering (described above and referred to as "X-shift cell populations") were grouped together based on their co-occurrence across samples by hierarchical clustering (columns in Figure 6A). This analysis revealed groups of 3 to 14 X-shift cell populations that mapped closely to one another and thus recurred to similar extents across samples (dendrogram across columns in Figure 6A). In order to define this structure in greater depth, individual samples were clustered together based on the number and size of X-shift cell populations (rows in Figure 6A). Clustering revealed three groups of HG-SOC samples of seven, four and three samples respectively (dendrogram across rows in Figure 6A). An additional five HG-SOC samples were close to these groups within HG-SOC computational space. Three samples clustered apart and were found to be two granulosa and one mucinous ovarian cancer sample respectively. These data suggest that the heterogeneity of HG-SOC does indeed have "structure" and that ovarian cancers of different histiotypes fall outside those boundaries. We are currently evaluating whether correlations within the HG-SOC sample groups are related to clinical features such as platinum resistance or BRCA1/2 status.

Within each sample, each X-shift cell population was colored for absolute number of cells with greater intensity depicting more cells. Notably, there were three X-shift cell populations that were dominant across HG-SOC samples but absent in the two other ovarian histiotypes. Conversely, X-shift clusters in these latter histiotypes were absent in the HG-SOC samples. The three X-shift cluster that occurred most frequently across all 22 samples and seemed to drive the dendrograms had distinguishing features of higher expression for: **red cluster 1** *CD24, mesothelin, endoglin, pSTAT3 and pSTAT5* **blue cluster 2** *MUC16, Ki67* **green cluster 3** *CD90, CD13* (Figures 6 and 7). These data suggest the possibility of three distinct trajectories for HG-SOC within this patient cohort. We are currently following up with further analysis and plan to sort

these clusters from prospective tumors and perform functional assays to determine tumorigenic potential.

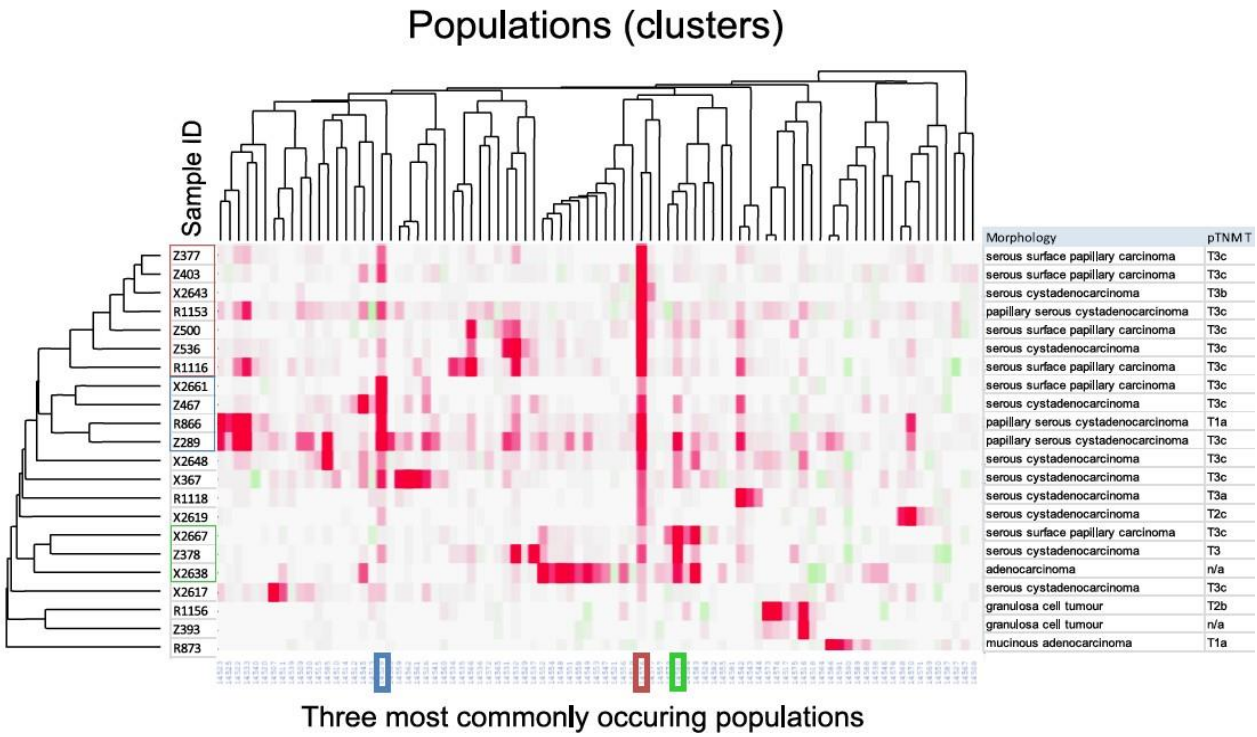

Figure 6: Dendrograms show i) co-occurrence of clusters (horizontal) and ii) similarities between samples based on size and number of clusters in each sample (vertical).

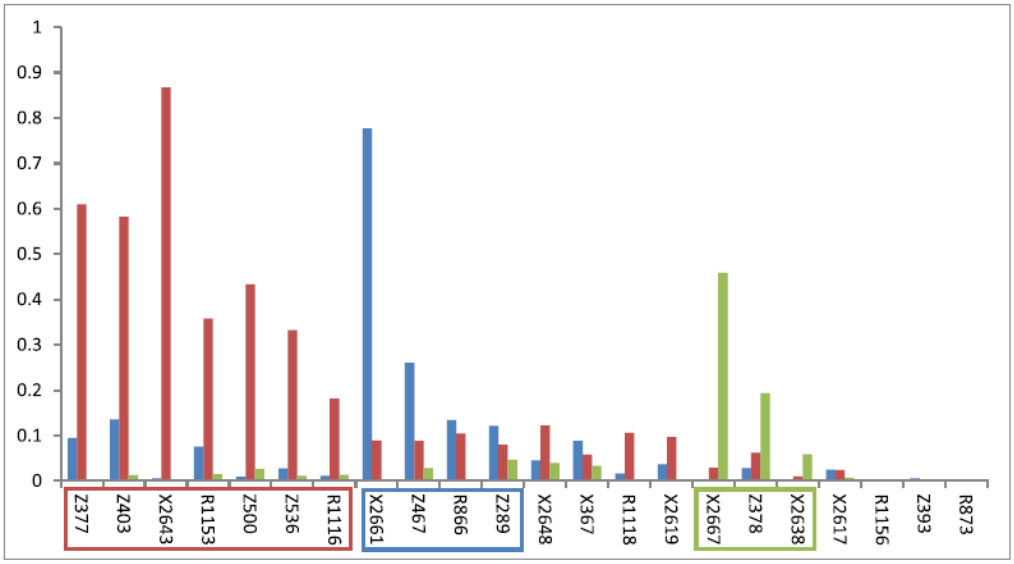

Figure 7: Frequencies of commonly occurring clusters across samples

Work from the Neel Lab characterizing the tumor compartment

During the funding period, we completed sorting and injection of our putative, SOX2+ cell-enriched, TIC clusters from 8 primary, chemo-naïve SOC cases. We are currently monitoring tumor growth in this limiting dilution experiment to determine if these clusters are enriched for TICs. We have also begun further characterization of these stem cell clusters by examining their marker expression and *in vitro* growth properties compared with control clusters. For the latter

experiments, we have developed two cell culture systems, one based on the protocol developed by Liu and Schlegel, which uses mouse fibroblast feeders, as well as a modification of the organoid system of Sato and Clevers.

Using these two culture systems, we have defined sets of conditions to grow viably frozen primary SOC patient samples with a >85% success rate. We have also optimized these conditions to measure organoid and colony formation in primary samples. With these assays and our LDA xenograft assay, we have begun characterizing the sorted stem cell clusters by looking at colony, organoid, tumor formation, and *in vitro* properties. Furthermore, we are passaging these cultures and establishing new stable cell lines from bulk tumors as well as sorted clusters, and testing their tumor-forming capacity. We will use these lines to determine the function of stem cell markers (e.g. SOX2) in tumor formation, proliferation, colony/organoid formation using RNAi or CRISPR. Although the SOX2+ clusters remain the most likely TIC candidate, our CyTOF results also revealed the existence of additional cell clusters that are SOX2- but express other candidate stem cell control factors (e.g., β–catenin). To address the heterogeneity of SOC, specifically the potential heterogeneity in stem cell hierarchies, we are examining other known, non-SOX2, stem-catenin, notch). In this period, we have narrowed down these candidate factors and have identified appropriate cell line controls to interrogate the expression and relevance of these factors/pathways using flow/CYTOF. We are screening primary samples, as well as cultured SOC cells, to evaluate the heterogeneity of expression of these stem cell markers to determine which pathways, and in what proportion, are expressed in SOC. Concurrently, we are exploring extracellular markers expressed variably or in a minority population in SOC, as would be expected in a hierarchy, that we will compare with stem cell TFs to find an extracellular marker profile with which to purify cells expressing particular stem cell pathways (i.e. in the same manner through which we sorted SOX2 enriched cell populations).

Data analysis of the immune compartment (Nolan)

Our working hypothesis is that the heterogeneity of HG-SOC is finite such that boundaries exist. If that is so, it implies that there are correlations, Indeed we reproduced the finding we reported last year with ten samples that there was a strong anti-correlation between the relative size of the immune compartment with the tumor compartment. We also reproduced our finding that showed specific natural killer (NK) cell subsets correlated positively with tumor compartment size. We also hypothesized that the NK cell subsets resembled decidual NK cells that line the uterus and “tolerate” the fetus as well as vascularize the placenta. Consistent with this finding was the finding that, with our new immune antibody panel, the NK cells that correlated positively were CD56mid and expressed CD9, a hall mark of decidual NK cells. We are currently following up with this finding with functional co-culture experiments. Our goal is to determine whether HG-SOC tumors can confer a decidual-like phenotype on NK cells such that they are activated to secrete proangiogenic factors. We have begun to look at this by co-culturing NK cells in healthy peripheral blood mononuclear cells with some HG-SOC cell lines described by Domcke et al.[11] and Figure 8. We also performed a CyTOF analysis on ten of the HG-SOC cell lines and were able to discern that each cell line recapitulated an aspect of HG-SOC, such that some cell lines were more “epithelial”, others more “mesenchymal” and some were a combination (presented in prior reports and unpublished).

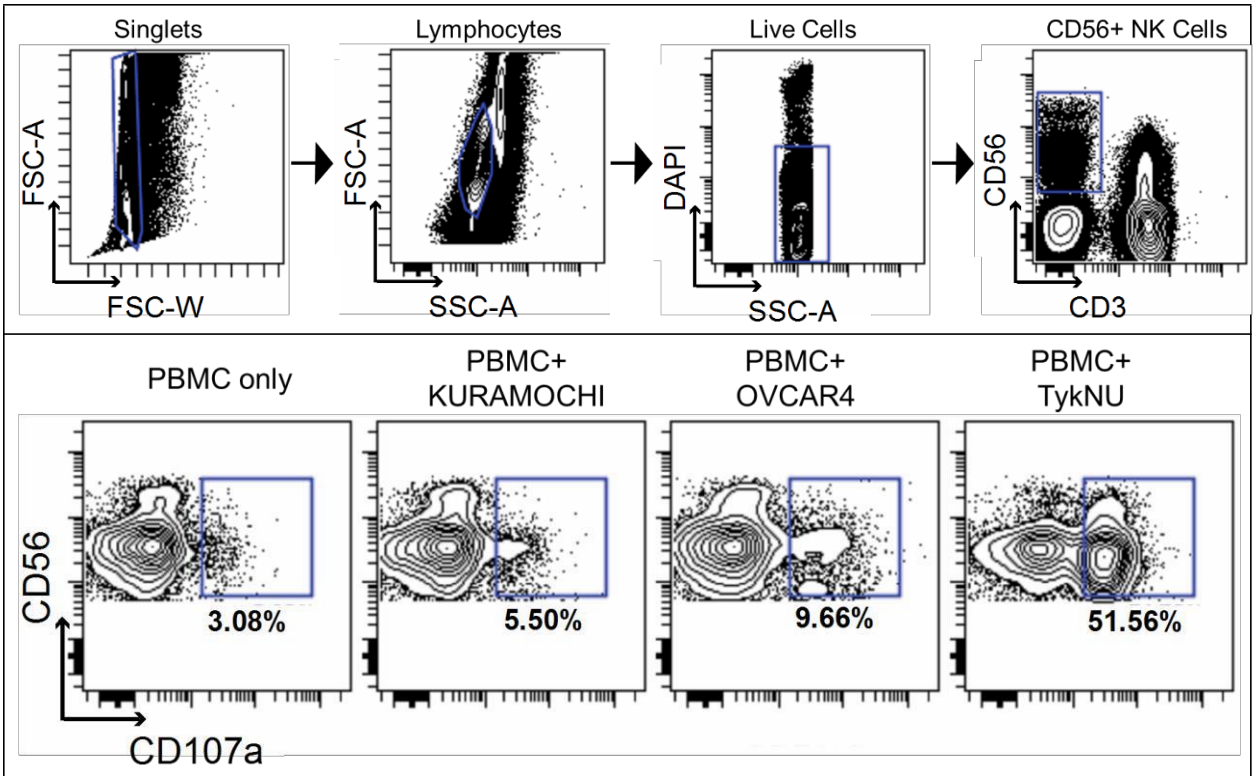

Figure 8: Upper panel depicts gating strategy for CD56bright NK cells. the cancer cell lines recapitulate different aspects of HG-SOC. TykNUs are a more mesenchymal/metastatic HG-SOC cell line and mediate the greatest activation of NK cells, represented here by an increase in CD57

Our new immune cell antibody panels permitted an extensive characterization of T-cell subsets and the expression of immune checkpoint proteins (Figures 9 and 10). Thus we gated out CD8+, CD4+, T regs and γδTCR T cell populations from the CD45+/CD31-/FAP- gate within each tumor sample. The most striking feature was the vast range of expression of the immune checkpoint markers, across the different T cell types. The example shown for PD-1, recently approved (Nivolumab from Bristol Meyers Squib and Pembrolizumab from Merck) for melanoma[24-26] and is in clinical trials for other malignancies including HG-SOC[27]. Our data below strongly advocate a method of screening a patient's tumor before commencing with therapy (Figure 9). This analysis is ongoing.

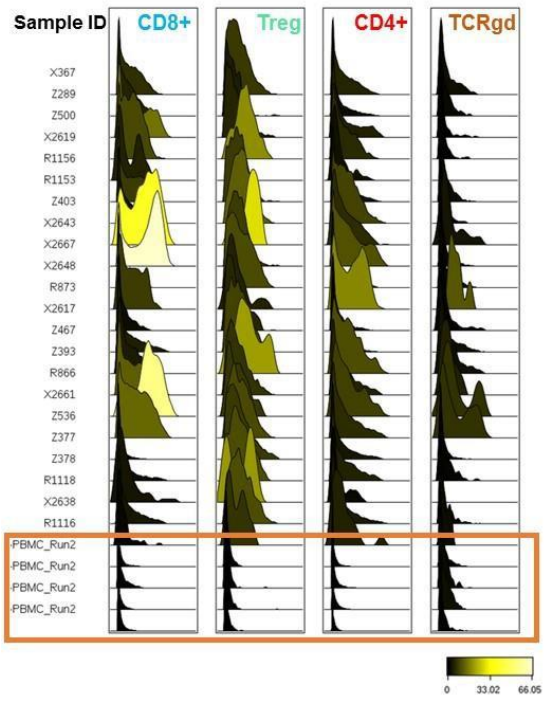

Figure 9: Variable levels of PD1 expression across T-cell subsets

Additionally from the CD4+ and CD8+ T-cell gates and using CD45RA and CCR7 we gated out functional T-cell subsets: **naïve** (CD45RA+CCR7+), **T_{CM}** (CD45RA-CCR7+), **T_{EM}** (CD45RA-CCR7-), **Teff**(CD45RA+CCR7-) cells[28,29] (Figure 10). The data showed that naïve T cells in both CD4+ and CD8+ T-cell populations were mostly absent, suggesting that an immune response had occurred and the T-cells were "educated". Furthermore, Teff cells were also

absent in both CD4+ and CD8+ T cell populations suggesting that an active immune response was not going on. We are analyzing these data to determine whether these "educated" T cells are tumorigenic or "exhausted". The functional gates for both CD4+ and CD8+ T cells demonstrated an increased frequency of **T_{EM}** cells compared to the other functionally classified T cells. However, T_{CM} cells were only observed in the CD4+ T-cells. At this stage of our analysis of the T cell subsets, it appears that an immune response was mounted but that it is not active. This work lays the foundation for understanding how the memory cells could be reactivated and also identifying their T-cell receptor specificities.

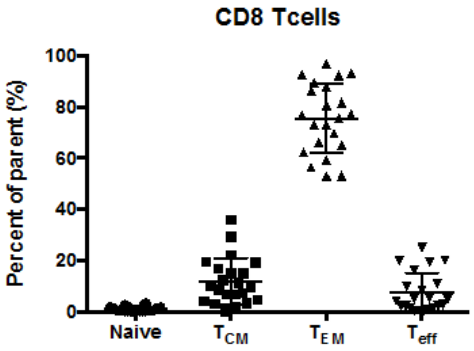

Figure 10A: Higher frequency of T_{EM} CD8+ cells.

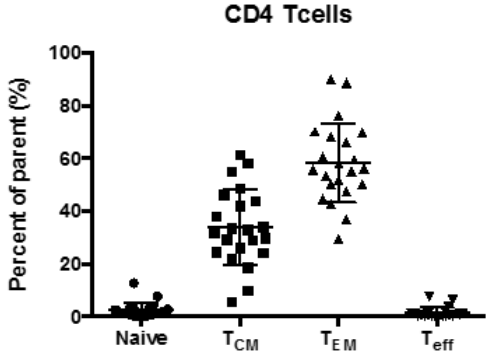

Figure 10B: Higher frequency of TEM and TCM CD4+ cells

We are in the process of analyzing the myeloid/macrophage/B cell panel. Overall, our analysis of the immune and tumor compartment relationships is ongoing.

D. KEY RESEARCH ACCOMPLISHMENTS

- Established three validated antibody panels against the tumor and immune cell compartments.
- Tumor samples analyzed with over 100 antibodies
- Conjugated 500ug of each antibody to ensure an adequate supply for analyzing future tumor specimens.
- Streamlined the experimentation as we now understand what most meaningful controls.
- Established a new de-barcoding algorithm to take into account large range of cell size within complex primary HG-SOC samples
- Landscaped tumor and immune compartments of 22 primary HG-SOC tumors
- Reproduce many of the trends observed in the two pilot studies regarding features of both the tumor and immune compartments
- Developed co-culturing experiments for NK cells with HG-SOC cell lines.
- Combined our new computational tools with standard statistics; both descriptive and inferential as well as multiple hypothesis testing techniques to analyze the data.

E. REPORTABLE OUTCOMES

PUBLICATIONS

1. Zunder ER, Finck R, Behbehani GK, Amir ED, Krishnaswamy S, Gonzalez VD, Lorang CG, Bjornson Z, Spitzer MH, Bodenmiller B, Fantl WJ, Per'er D, **Nolan GP**. *Palladium-based Mass-Tag Cell Barcoding with a Doublet-Filtering Scheme and Single Cell Deconvolution Algorithm*. Nature Protocols. 2015. PMID: 25612231 PMCID: PMC4347881.

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PRESENTATIONS (Nolan)

1. **Nolan:** Medimmune "Science Day", October 2, 2014, **Translational Sciences and Infectious Diseases/Vaccine Research,** Mountain View, CA
2. **Nolan:** Stanford University Cancer Institute, October 6, 2014, **How Precisely Can We Measure and Interpret Intratumor Heterogeneity?** Stanford, CA
3. **Nolan:** RECOMB/ISCB Conference on Regulatory and System Genomics, November 12, 2014, **Systems Biology Keynote: High Parameter Single Cell Analysis Define a Structured Immune System & Cancer Hierarchies,** San Diego, CA
4. **Nolan:** StandUp2Cancer – Google [X] Immunology Summit, January 6, 2015, **Current and Imminent Imaging Technologies,** Mountain View, CA
5. **Nolan:** NIH Immunology Interest Group (IIG), January 28, 2015, **Single Cell Proteomics and Genomics at High Scale,** Bethesda, MD
6. **Nolan:** Stanford Medicine Leadership Retreat, January 31, 2015, **Biotechnology Transfer at Stanford: What's Working? What Could Be Better?** Sausalito, CA
7. **Nolan:** Institute for Cellular and Molecular Biology, University of Texas, April 9, 2015, **Single Cell Systems: Structured View of Cancer and Immunology,** Austin, TX
8. **Nolan:** Fox Chase Cancer Center, April 16, 2015, **A Systems Structured View of Immunity and Cancer-Organization of Heterogeneity Made Easy**, Philadelphia, PA
9. **Nolan:** FDA's Office of Regulatory Science and Innovation (ORSI), April 27, 2015, **Cross-Species Immune System Reference (Human Immune Atlas),** Silver Spring, MD
10. **Nolan:** Johns Hopkins School of Medicine Immunology Forum, May 26, 2015, **A Single Cell Systems Based View of Immunity and Cancer,** Baltimore, MD
11. **Nolan:** Johns Hopkins Rheumatic Disease Research Center, May 28, 2015, **A Systems-Structured View of Cancer and Immunity- Heterogeneity Made to Order,** Baltimore, MD
12. **Nolan:** Koch Institute 2015 Symposium, June 12, 2015, **The Deeper Phenotypes of the Single Cell in Cancer and Immunity,** MIT, Cambridge, MA.
13. **Nolan:** FOCIS 2015, June 26, 2015, **Multiparameter Single Cell Deep Phenotypic and Genotypic Profiling for Studies of Inflammatory Processes**, San Diego, CA
14. **Nolan:** StandUp2Cancer – Google [X] Technology and Analysis meeting, July 8, 2015, **Immune Cell Characterization**, Cambridge, MA.

INVENTIONS PATENTS AND LICENSES

N/A

F. OTHER ACHIEVEMENTS

OC ambassadorship duties

Numerous seminars as above to national and international audiences.

Interaction with the OC community

Movie about the work to the Stanford Women's Cancer Centre (WFantl/JBerek)
Participation in GCIG and COGi conferences (WF/JB).

Your mentoring progress

Continue to mentor graduate students and post-docs in my lab (about 30) and at other institutions (GPN).
Committee member for six thesis committees (GPN).
Student mentor for three graduate students and four post-docs at Stanford (WF). One of the

graduate students now applying part of is project to OC an post-docs are working on OC under guidance of WF and also clinical input from JB.
Pre-major advisor for two Stanford undergraduates with interest in natural sciences and medicine (WF).

Your mentee's progress
During the past year, four of my post-docs received faculty positions (GPN).
One graduate student graduated (GPN).

G. CONCLUSIONS
See tumor diversity between samples, but within *a limited phenotypic hierarchy:*
– For both surface markers and signaling molecules
• See mutually exclusive expression of E-cadherin and vimentin in "epithelial" and "mesenchymal" compartments
• See cells in transitional EMT that also co-express stem cell markers confirming what has been proposed in the literature.

- Stem cell markers scattered throughout compartments: are there many ways to be a stem cell? Functional analysis can help answer this.
- Great diversity in size of immune compartment across samples
- We reproduce correlations between a tumor cell type with immune compartment size and immune cell type with tumor compartment size.
- We reproduced the finding that the relative size of tumor and immune compartments anti-correlated.
- We reproduce the finding that NK cell subsets are positively correlated with tumor compartment size and increase the likelihood that those NK cells could be decidual-like.
- Highly regulated communication between immune and tumor compartments.
- New level of detail regarding the immune compartment revealed by multi-parametric single cell mass cytometry.
- We reproduced many aspects of the data from the two pilot studies which strongly validates our experiments at the technical level allowing us to makes inferences about HG-SOC biology.
- The information is foundational for following up with larger sample cohorts with the potential to inform treatments particularly immune-therapies for HG-SOC.

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