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14. ABSTRACT
We hypothesize that M2 macrophages also support chemotherapy resistance by interacting with tumor-initiating cells. With this proposal, we aim to thoroughly investigate the influence of M2 macrophages on ovarian tumor-initiating cells. To do so, we will combine the chemotherapy drug paclitaxel with a CSF1R inhibitor to block M2 macrophages. Using three models of ovarian cancer: a mouse model, a human cell line implanted into mice, and samples of human ovarian cancer implanted into mice, we will study M2 macrophages and tumor-initiating cells during chemotherapy treatment. We will further explore how interactions between M2 macrophages and tumor-initiating cells support chemoresistance by disrupting M2 macrophages with CSF1R inhibition. We expect that combination therapy of CSF1R inhibition and paclitaxel will render the ovarian tumor-initiating cells more sensitive to chemotherapy treatment.

15. SUBJECT TERMS
Ovarian Cancer, Tumor Initiating Cells, Tumor Associated Macrophages, CSF1R

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Introduction

Many women with EOC show an initial response to chemotherapy, often achieving complete remission, but a majority of them will relapse[1, 2]. Such transient clinical response may be attributable, in part, to cancer stem cells, also referred to as tumor initiating cells (TICs). TICs are proposed to be a subpopulation of cancer cells with stem cell characteristics that allow them to resist chemotherapy and radiation treatment[3, 4]. It is hypothesized that these TICs persist after therapy and regrow the tumor, causing relapse and metastasis. Although direct evidence of this is limited, higher percentages of ovarian cancer TIC correlate with a poorer clinical outcome[5, 6]. Several different ovarian TIC markers have been proposed; however, work by our group and others indicates that high aldehyde dehydrogenase 1 activity (ALDH1high) may serve as a superior ovarian cancer stem cell marker[5, 7, 8]. Recent reports demonstrated reciprocal interactions between TAMs and TICs. Ovarian TICs can polarize naïve macrophages to the M2 phenotype[9, 10]. In other cancers, this recruitment of M2 TAMs supports the stem cell phenotype of TICs and expands their numbers[11, 12]. The M2 macrophages may protect ovarian TICs from chemotherapy treatment, which could have significant clinical implications[13, 14]. Our research showed that blocking TAMs can improve the outcome of conventional therapy, such as chemotherapy, by eliminating the pro-angiogenic and pro-immunosuppressive influences of the TAMs. An additional benefit of targeting TAMs could be weakening their support of TICs and their contribution to chemotherapy resistance. These intriguing crosstalks between TAMs and TICs in the treatment failure of EOC clearly warrants further investigation.

Body

Specific Aim 1a: To fully characterize the TIC, TAM and immune cell population in the original ID8 and ID8-CR model, in its primary tumor as well as metastatic lesions.

- We found that sphere cultures provided superior enrichment in ALDH1^{high} ID8 cells than enrichment with paclitaxel resistance (Figure 1A). Adherent cultures routinely show less than 1% ALDH1^{high} cells, as measured using the Aldefluor reagent. Sphere cultures showed more than 65% of cells with high Aldefluor staining using stringent gating. However, when comparing staining with and without the reversible inhibitor, DEAB, it appears that the entire population is actually shifted

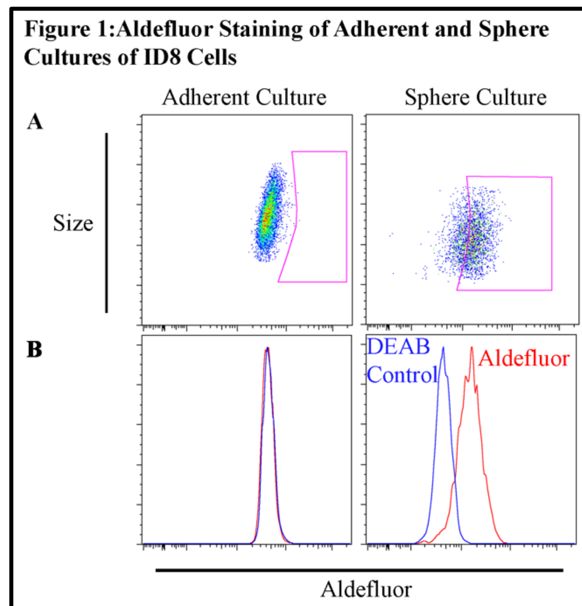
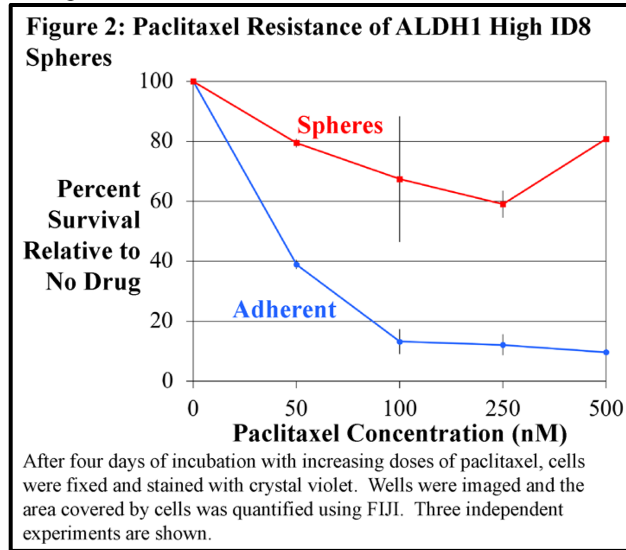


Figure 1:Aldefluor Staining of Adherent and Sphere Cultures of ID8 Cells

and Aldefluor high (Figure 1B). We have decided to proceed using sphere formation to enrich for our TICs rather than paclitaxel resistance.

- ALDH1^{high} ID8 spheres are more resistant to paclitaxel than ALDH1^{low} ID8 adherent cells (Figure 2).



After four days of incubation with increasing doses of paclitaxel, cells were fixed and stained with crystal violet. Wells were imaged and the area covered by cells was quantified using FIJI. Three independent experiments are shown.

- Firefly luciferase marked adherent and sphere cells have been implanted IP into C57BL/6 mice to assess tumorigenicity. These experiments are ongoing.

Specific Aim 1b: To assess the therapeutic effects of paclitaxel without and with CSF1R inhibition (PLX5622) in the ID8 models.

- Completion of one pilot study showed technical difficulties with using Renilla luciferase for our non-invasive bioluminescence imaging. Therefore, we have designed and generated a new lentivirus expression vector containing mStrawberry and Firefly luciferase as a fusion protein. ID8 cells have been transduced to stably express our fusion reporter and sorted for mStrawberry$^+$ cells. To form more imageable primary tumors, we are also testing High Concentration Matrigel instead of the standard formulation. A pilot experiment is underway to confirm that the technical modifications will permit more reliable bioluminescence imaging and testing of therapeutic efficacy.
- To confirm the influence of TAMs on TIC tumor formation, we have adapted a chick embryo model to test tumorigenicity in a more cost-effective manner. We have found that ID8 cells not enriched for TICs only engraft when co-implanted with the murine macrophage cell line, RAW.

Specific Aim 2a: To investigate the influences of TAMs on TICs and therapeutic benefits of CSF1R inhibition in combination with paclitaxel in intraperitoneal disseminated human SKOV3 EOC model.

- We have unfortunately identified mycoplasma contamination within the paclitaxel-resistant SKOV3 cells for this study and are in the process of regenerating the resistant line.

Specific Aim 2b: To investigate the influences of TAMs on TICs and therapeutic benefits of CSF1R inhibition in combination with paclitaxel in intraperitoneal disseminated human OVCAR3 EOC model.

- These experiments have not yet been initiated. Will initiate this aim in 6-12 months.

Specific Aim 3a: To analyze pre- and post-treatment tumor specimen obtained from advanced EOC patients in a clinical trial of PLX3397 plus paclitaxel.

- These experiments have not yet been initiated. Will initiate this aim in 6-12 months.

Specific Aim 3b: To harvest and analyze malignant ascites from patients with high-grade serous ovarian cancers (HGSC) and establish new PDXs.

- To improve engraftment likelihood, we have opted to implant tumor pieces from patients with high-grade serous carcinoma into immunocompromised mice. We expect the preservation of the tumor microenvironment to better support tumor growth. We have implanted tumor pieces onto the ovarian bursa of nude mice and subcutaneously into NSG mice. We are currently monitoring these animals for tumor growth.
- We have optimized tumor digestion conditions to permit flow cytometric cell sorting of high-grade serous ovarian tumors.
- A sorting panel to isolate cancer cells (Figure 3A), TICS, TAMs (Figure 3B) and other immune cells has been designed and tested.

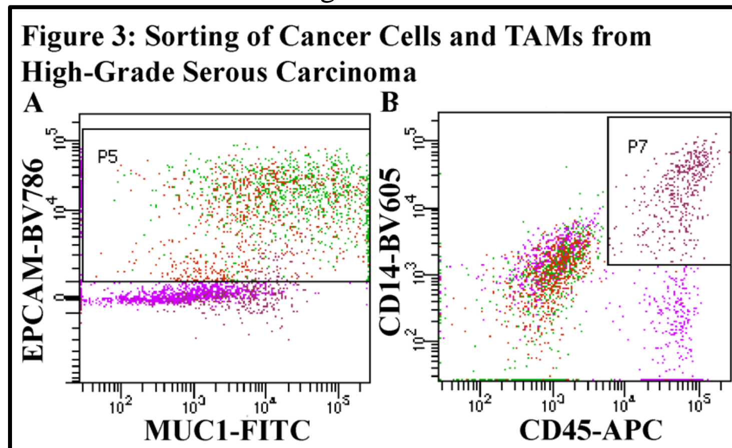


Figure 3: Sorting of Cancer Cells and TAMs from High-Grade Serous Carcinoma

Difficulty Encountered:

- Several difficulties have been encountered that have hampered progress. We observed week-to-week variability in Renilla luciferase bioluminescence imaging that hampered reliable assessment of therapeutic efficacy. We subsequently designed a new expression vector to introduce Firefly luciferase into our cells. The designing, cloning sorting and validation of this marker delayed progress by approximately 6 months.
- It was determined that the paclitaxel-resistant SKOV3 cells that were provided by a collaborator were contaminated with mycoplasma. The regeneration of a stable paclitaxel-resistant subline has delayed progress by approximately one year.

- The key researcher on this project, Dr. Allison Sharrow, celebrated the birth of a child and took maternity leave. This delayed progress by four months.

Key Research Accomplishments

- We have reliably identified a superior method for enrichment of ALDH1^{high} ID8 cells and have confirmed the chemoresistance of these cells.
- We have generated stably transduced ID8 and SKOV3 cells marked with mStrawberry and Firefly luciferase for more reliable *in vivo* bioluminescence imaging.
- We can successfully obtain a single cell suspension from high-grade ovarian serous carcinoma as well as identify and sort relevant cell populations by flow cytometry for further characterization.
- Tumor pieces from high-grade serous carcinoma can be implanted into nude and NSG mice for PDX generation.
- Early findings point to an important role for TAMs in tumor formation.

Reportable Outcomes

None

Conclusion

We hypothesize that crosstalk between TAMs and TICs supports stem cell traits, especially chemoresistance and that disrupting these interactions may improve treatment outcomes in ovarian cancer. We have made significant progress to overcome technical difficulties, which we anticipate will enable us to advance our understanding of this proposal. We can readily enrich for TICs *in vitro* in the ID8 and SKOV3 cell lines, permitting direct studies of these cells and have verified their chemoresistance. We have generated a new expression vector and successfully marked our cell lines with Firefly luciferase to permit more reliable *in vivo* bioluminescence imaging. Our sorting panel has been optimized and verified, permitting direct study of TICs and TAMs from high-grade serous carcinoma samples. Furthermore, our approach for generating PDX has been optimized and we are awaiting tumor engraftment. To more rapidly and cost-effectively begin to address *in vivo* interactions between TAMs and TICs, we have developed a chicken chorioallantoic membrane implantation model. Using this model, we have confirmed that TAMs do enhance the tumorigenicity of ovarian cancer cells and we are working to validate that this effect works through the tumorigenicity of TICs.

References

1. Siegel RL, Miller KD, and Jemal A. *Cancer statistics, 2016.* CA: A Cancer Journal for Clinicians. 66(1): 7-30.
2. Howlader N, Noone A, Krapcho M, Garshell J, Miller D, Altekruse S, Kosary C, Yu M, Ruhl J, Tatalovich Z, Mariotto A, Lewis D, Chen H, Feuer E, and Cronin K

(eds). *SEER Cancer Statistics Review, 1975-2012*. National Cancer Institute. Bethesda, MD. http://seer.cancer.gov/csr/1975_2013/, based on November 2014 SEER data submission. Available from: [accessed October 5, 2015].

3. Lobo NA, Shimono Y, Qian D, and Clarke MF. *The Biology of Cancer Stem Cells.* Annual Review of Cell and Developmental Biology. 23(1): 675-699.
4. Gerber JM, Smith BD, Ngwang B, Zhang H, Vala MS, Morsberger L, Galkin S, Collector MI, Perkins B, Levis MJ, Griffin CA, Sharkis SJ, Borowitz MJ, Karp JE, and Jones RJ. *A clinically relevant population of leukemic CD34+CD38- cells in acute myeloid leukemia.* Blood. April 12, 2012; 119(15): 3571-3577.
5. Silva IA, Bai S, McLean K, Yang K, Griffith K, Thomas D, Ginestier C, Johnston C, Kueck A, Reynolds RK, Wicha MS, and Buckanovich RJ. *Aldehyde Dehydrogenase in Combination with CD133 Defines Angiogenic Ovarian Cancer Stem Cells That Portend Poor Patient Survival.* Cancer Research. June 1, 2011; 71(11): 3991-4001.
6. Wang Y-C, Yo Y-T, Lee H-Y, Liao Y-P, Chao T-K, Su P-H, and Lai H-C. *ALDH1-Bright Epithelial Ovarian Cancer Cells Are Associated with CD44 Expression, Drug Resistance, and Poor Clinical Outcome.* The American Journal of Pathology. March 2012; 180(3): 1159-1169.
7. Abelson S, Shamai Y, Berger L, Shouval R, Skorecki K, and Tzukerman M. *Intratumoral Heterogeneity in the Self-Renewal and Tumorigenic Differentiation of Ovarian Cancer.* Stem Cells. March 2012; 30(3): 415-424.
8. Sharrow AC, Perkins B, Collector MI, Yu W, Simons BW, and Jones RJ. *Characterization of aldehyde dehydrogenase 1 high ovarian cancer cells: Towards targeted stem cell therapy.* Gynecologic Oncology. 8//; 142(2): 341-348.
9. Panni RZ, Sanford DE, Belt BA, Mitchem JB, Worley LA, Goetz BD, Mukherjee P, Wang-Gillam A, Link DC, Denardo DG, Goedegebuure SP, and Linehan DC. *Tumor-induced STAT3 activation in monocytic myeloid-derived suppressor cells enhances stemness and mesenchymal properties in human pancreatic cancer.* Cancer Immunology, Immunotherapy. May; 63(5): 513-28.
10. Jinushi M, Chiba S, Yoshiyama H, Masutomi K, Kinoshita I, Dosaka-Akita H, Yagita H, Takaoka A, and Tahara H. *Tumor-associated macrophages regulate tumorigenicity and anticancer drug responses of cancer stem/initiating cells.* Proceedings of the National Academy of Sciences. 108(30): 12425-12430.
11. Schmid MC, Avraamides CJ, Foubert P, Shaked Y, Kang SW, Kerbel RS, and Varner JA. *Combined Blockade of Integrin-α4β1 Plus Cytokines SDF-1α or IL-1β Potently Inhibits Tumor Inflammation and Growth.* Cancer Research. 71(22): 6965-6975.
12. Beatty GL, Chiorean EG, Fishman MP, Saboury B, Teitelbaum UR, Sun W, Huhn RD, Song W, Li D, Sharp LL, Torigian DA, O'Dwyer PJ, and Vonderheide RH. *CD40 Agonists Alter Tumor Stroma and Show Efficacy Against Pancreatic Carcinoma in Mice and Humans.* Science. 331(6024): 1612-1616.
13. Zhang M, He Y, Sun X, Li Q, Wang W, Zhao A, and Di W. *A high M1/M2 ratio of tumor-associated macrophages is associated with extended survival in ovarian cancer patients.* Journal of Ovarian Research. 7(1): 19.
14. Mitchem JB, Brennan DJ, Knolhoff BL, Belt BA, Zhu Y, Sanford DE, Belaygorod L, Carpenter D, Collins L, Piwnica-Worms D, Hewitt S, Udupi GM, Gallagher

WM, Wegner C, West BL, Wang-Gillam A, Goedegebuure P, Linehan DC, and DeNardo DG. *Targeting Tumor-Infiltrating Macrophages Decreases Tumor-Initiating Cells, Relieves Immunosuppression, and Improves Chemotherapeutic Responses.* Cancer Research. February 1, 2013; 73(3): 1128-1141.

Appendices

None.

Supporting Data

None (relevant data inserted into the body section and appended manuscript).