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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

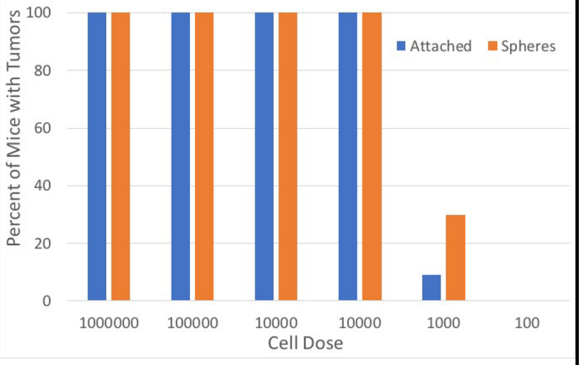
Many women with EOC show an initial response to chemotherapy, often achieving complete remission, but a majority of them will relapse[1, 2]. Such transient clinical response may be attributable, in part, to cancer stem cells, also referred to as tumor initiating cells (TICs). TICs are proposed to be 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.

- Successful marking of ID8 cells with Firefly luciferase has permitted in vivo assessment of ID8 sphere cell tumorigenicity compared to attached cultures. ID8 spheres are more tumorigenic than attached ID8 cells, particularly at the cell dose of 10^3 cells implanted intraperitoneally per mouse. Only one of 11 mice injected with attached ID8 cells formed tumors, but three of 10 mice injected with the same dose of ID8 spheres formed tumors.
- ID8 tumors and ascites have been harvested from a large-

Figure 1: Tumorigenicity of ID8 Spheres Versus Attached ID8

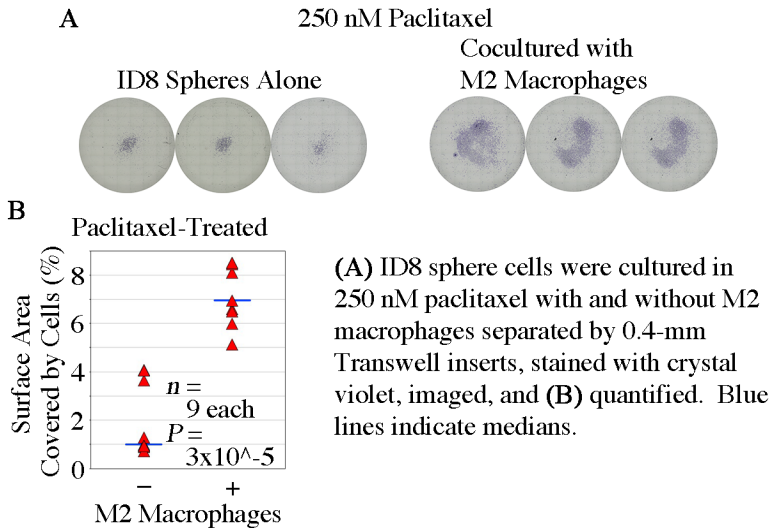


Stated cell doses were injected intraperitoneally into C57BL/6 mice then assessed for tumor formation 17.5 weeks postimplant using bioluminescence imaging.

scale in vivo experiment. Characterization of the ALDH1^{high} cells and tumor associated macrophages is still pending.

- TAMs have been found to increase the numbers of ID8 sphere cells surviving chemotherapy treatment, but do not reproducibly impact migration or invasion.

Figure 2: Macrophages Enhance Cancer Stem Cell Chemoresistance

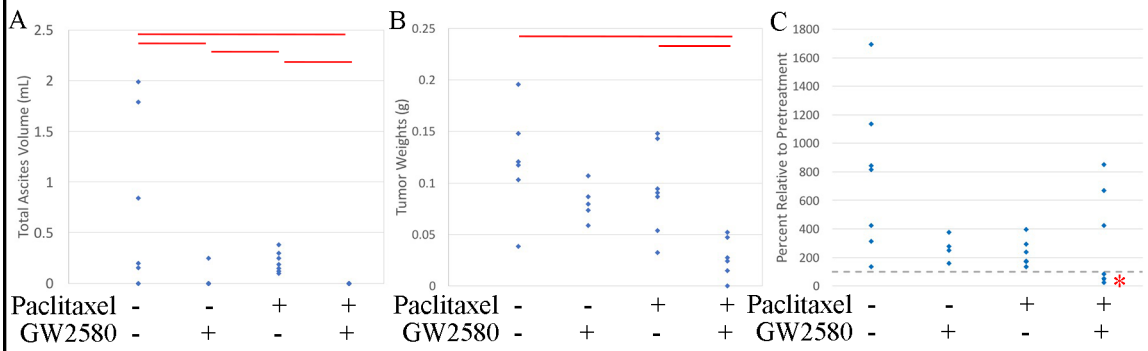


(A) ID8 sphere cells were cultured in 250 nM paclitaxel with and without M2 macrophages separated by 0.4-mm Transwell inserts, stained with crystal violet, imaged, and **(B)** quantified. Blue lines indicate medians.

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

- Preliminary experiments found that the CSF1R inhibitor PLX5622 was less effective at producing a therapeutic effect in ID8 models than the CSF1R inhibitor GW2580. Therefore, subsequent experiments used GS2580 instead.
- The combination of paclitaxel and GW2580 treatment in the in vivo ID8 model of ovarian cancer proved superior to either monotherapy. GW2580 alone and in combination with paclitaxel reduced ascites volumes. Combination therapy reduced tumor weights relative to untreated and paclitaxel-treated mice. Combination therapy, further, was the only treatment group to show reduction in bioluminescence output by tumors relative to pretreatment values (4 out of 7 mice). Immunohistochemical evaluation of resulting tumors from each treatment group will be initiated immediately.

Figure 3: Therapeutic Efficacy of Paclitaxel Combined with CSF1R Inhibition



Intraperitoneal implantation of Firefly-luciferase-marked ID8 cells into C57BL/6 mice was followed by treatment with paclitaxel with the CSF1R inhibitior GW2580 when robust tumor formation was observed. The combination therapy was compared to vehicle only and each therapy in monotherapy. After 4.5 weeks of treatment, (A) ascites volume and (B) tumor weight were measured. (C) Total flux of bioluminescence imaging at endpoint was compared to pretreatment values. In (A) and (B), statistical significance ($p < 0.05$ by ANOVA with post-hoc Holm) between groups is indicated by red, horizontal lines. In (C), the proportion of mice showing reduction in total flux in the combination therapy group was significant by Fisher's Exact Test ($p < 0.015$).

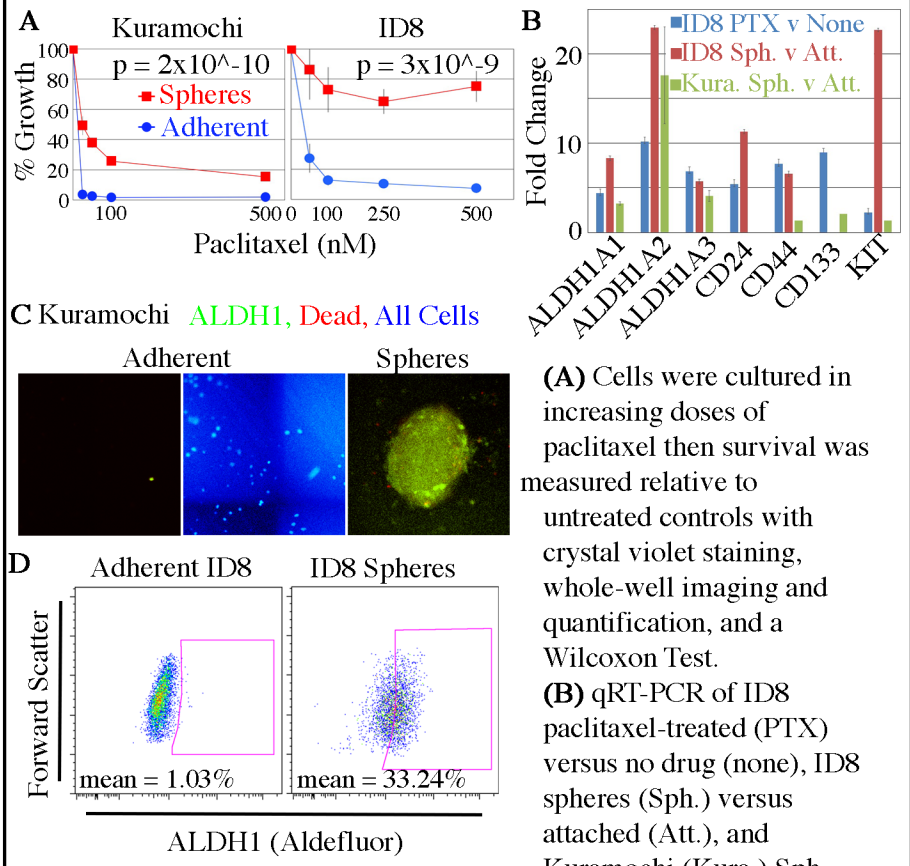
Specific Aim 2a: To investigate the influences of TAMs on TICs and therapeutic benefits of CSF1R inhibition in combination with paclitaxel in intraperitoneal disseminated human SKOV3 EOC model.

- While regenerating the paclitaxel-resistant SKOV3 cell line, recent data was brought to our attention that SKOV3 cells are not representative of human serous ovarian carcinoma. We have, therefore, ceased work with this cell 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.

- OVCAR3 cells appear to overrepresent TICs, failing to leave adequate differentiated cells for comparison. Because we could not reliably validate the TIC phenotype, work with these cells ceased as well. We, therefore, initiated work with the Kuramochi cell line in its place and successfully enriched for TICs that are chemoresistant and have high expression of ovarian cancer stem cell markers, like the sphere cells of the ID8 cell line. Ongoing work indicates that TAMs may similarly increase the number of Kuramochi TICs surviving chemotherapy treatment, like in the ID8 model.

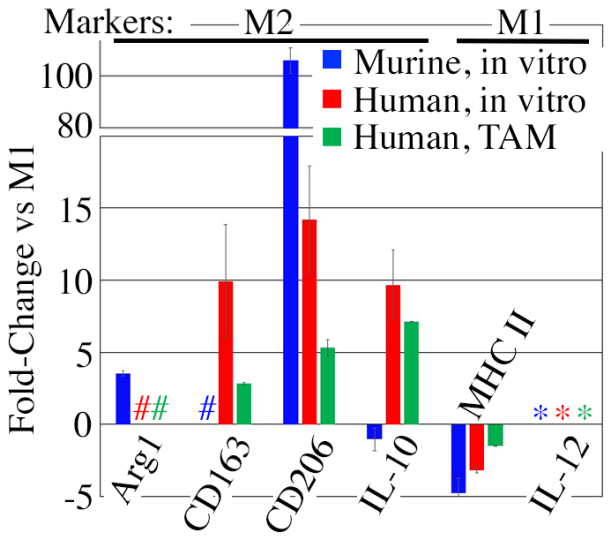
Figure 4: Enrichment of Ovarian Cancer Stem Cells in Kuramochi and ID8 Cell Lines



(A) Cells were cultured in increasing doses of paclitaxel then survival was measured relative to untreated controls with crystal violet staining, whole-well imaging and quantification, and a Wilcoxon Test. **(B)** qRT-PCR of ID8 paclitaxel-treated (PTX) versus no drug (none), ID8 spheres (Sph.) versus attached (Att.), and Kuramochi (Kura.) Sph. versus Att. **(C)** Fluorescence microscopy of Kuramochi cells stained for ALDH1 (Aldefluor) in green and dead cells displayed in red. Matched adherent image shows total cells with DAPI in blue. All images use the same fluorescence intensity scale. **(D)** Aldefluor staining of adherent and sphere ID8 cells. Gating was set with a 0.1% background level in the matched DEAB negative control.

- Optimization of the in vitro differentiation of human TAMs has been completed showing similar expression patterns as isolated TAMs from primary ovarian serous carcinoma and in vitro differentiated murine TAMs used for coculture experiments presented above.

Figure 5: Macrophage Polarization



qRT-PCR validation of in vitro M2 polarization compared to isolated human tumor-associated macrophages (# not tested, * not expressed in M2 cells).

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

- The samples for this analysis were never received.

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

- Implantation of tumor pieces into the ovarian bursa proved difficult to monitor the tumor growth of. We, therefore, have incorporated published culture conditions for ovarian spheroid generation to maintain ovarian cancer cells in vitro long enough to mark the cells with Firefly luciferase. Mice implanted with these cells are being monitored for tumor growth.
- We have successfully engrafted human tumor pieces onto the chicken chorioallantoic membrane model as an alternative for tumor expansion.

Difficulty Encountered:

- We never received samples from clinical trials with PLX3397, which has prevented initiating Specific Aim 3a.
- Tumor engraftment has proven laborious and slow in murine models. We have, therefore, attempted engraftment using the chicken chorioallantoic membrane model.
- Recent work has shown that SKOV3 cells to not reliably represent human ovarian serous carcinoma. We, therefore, abandoned work with this cell line.
- Recent work determined that OVCAR3 cells do not faithfully provide both TICs and non-TIC ovarian cancer cells. We, therefore, abandoned work with this cell line.

Key Research Accomplishments

- Coculture experiments with in vitro differentiated macrophages have shown a strong influence of TAMs on TIC chemoresistance.
- Combination therapy of CSF1R inhibition with paclitaxel shows reduced ascites volume, tumor weight, and bioluminescence output versus pretreatment values.
- In vitro differentiation of human and mouse macrophages has been successfully optimized.

Reportable Outcomes

The engraftment of ovarian cancer cell lines and primary tumors into the chicken chorioallantoic membrane model was published by the Journal of Visualized Experiments in January 2020. The findings regarding the impact of TAMs on TICs is anticipated for publication in early 2021.

Sharrow AC, Ishihara M, Hu J, Kim IH, Wu L. Using the Chicken Chorioallantoic Membrane In Vivo Model to Study Gynecological and Urological Cancers. J Vis Exp. 2020 Jan 28; (155), e60651.

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. Optimization of ID8 and Kuramochi TIC expansion and in vitro TAM differentiation have been completed, permitting in vitro and in vivo testing of this hypothesis with the ID8 model. In vitro analysis of the Kuramochi cell line is ongoing. In vitro experiments have showed increased chemoresistance in TICs cocultured with TAMs, supporting our hypothesis. Furthermore, disrupting these interactions reduces ascites volume, tumor weight, and bioluminescence output relative to pretreatment values.

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Appendices

None.

Supporting Data

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