**PROGRESS REPORT TEMPLATE**

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Co-applicant name (if applicable): NA

Grant title: Targeting Occult Cancer Cell Subpopulations Driving Metastases Growth

Date of Award: 5/6/2023

RFP name of award: METAvivor Translational Research Award.

Date of submitting the report: 03/21/2024

**Summary of the project goal** (1 paragraph)

The goal of this study is to identify a targeted therapy aimed at clinically relevant cancer cell subpopulations that can be used in combination with currently approved therapy regimen for metastatic TNBC. We have shown that a small subpopulation of cancer cells that drives and maintains metastatic growth without being selected as the dominant population. This cell population displays an enhanced transcriptional activity at TEAD motifs, a transcription factor implicated in cancer progression and metastasis. Using novel TEAD inhibitors (iTEAD) that are currently in clinical trials, lung metastases were significantly decreased in TNBC mouse models. Pathway analysis upon iTEAD treatment revealed a global decrease in inflammatory pathways and specifically in Toll Like Receptor 4 (TLR4) signaling pathways in the lungs. Thus, we aim to test iTEAD in metastatic TNBC models in combination with standard of care chemotherapy reagents to assess their efficacy against established lung metastases. Additionally, we will examine the effect of using TLR4 inhibitors in combination with current TNBC therapies on metastatic lesions to determine their efficacy at boosting responses to therapy. These combination therapies could improve outcome and survival of patients with lung metastases.

**Specific Aims**

***Specific Aim 1****: Uncover chemotherapeutics that synergize with TEAD inhibitors to eliminate metastases.*First, we will test TEAD inhibitors currently entering the clinic in combination with standard of care (SOC) chemotherapy for metastatic TNBC to determine drug synergism on Patient Derived Xenografts (PDX). Next, selected drug combinations will be tested in 3D organoid invasion assays. Finally, promising drug combinations will be tested in mice models with established lung metastases from PDX implants.

Aim1a: Determine synergistic combinations of iTEAD and chemotherapeutics in 2D and 3D *in vitro* models.

To determine which TNBC subtype would better respond to our proposed combination therapy, we have started testing TEAD inhibitors on human breast cancer cell lines that represent different subtypes of TNBC human disease. iTEAD treatment is shown to decrease 3D invasion in two models of TNBC; mesenchymal and baselike-2 (Figure1). Next, we tested the impact of iTEAD (VT107 and VT3989) with chemotherapy (Paclitaxol and Doxorubicin) on the proliferation of these cell lines to determine any change in drug sensitivity. No significant shift was observed in the IC50 of doxycycline or paclitaxel combination treatment with iTEAD (Figure2 and 3). It is possible that the drug synergism is not observed on the proliferation of the cells but rather on their metastatic and distant site colonization ability. Thus, we will next perform *in vitro* 3D invasion assays with combination therapy and proceed to corresponding subtypes of PDX to assess metastasis in mouse models.



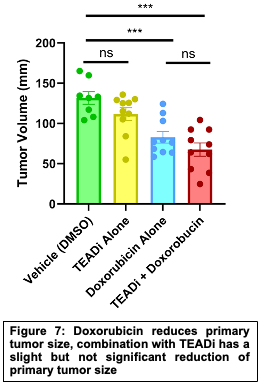


Aim1b: Examine synergism of iTEAD and chemotherapeutics combination in vivo.

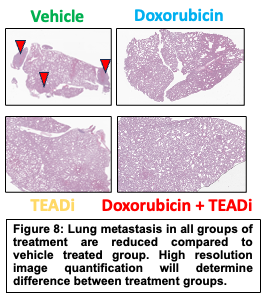
Next, we used the triple negative basal-like cell line 4T1 that is syngeneic to BALB/c mice to test combination therapy in an immune competent mouse model. First, we tested the effect of iTEAD treatment alone on 3D invasion of 4T1 spheres. Invasion in 3D was significantly decreased consist with what we observed with other TNBC cell lines (Figure 4). *In vitro*, 4T1 cells were more sensitive to chemotherapy agents (doxorubicin and paclitaxel) when in combination with iTEAD treatment resulting in a shift of their IC50 (Figure 5).

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*In vivo*, there was no effect on tumor size after iTEAD alone treatment as we have seen previously with other models, yet as expected doxorubicin treatment significantly reduced the primary tumor size. When in combination, iTEAD and Doxorubicin treatment showed a modest reduction in tumor size (Figure 6 and 7). Lung metastasis was observed in all groups yet large nodules were mostly seen in the vehicle treated group. Further histological analysis will determine the effect of treatment on the metastatic load (Figure 8). Tumors and lung tissue will be sequenced to determine gene expression changes upon different treatments.

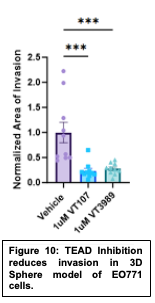
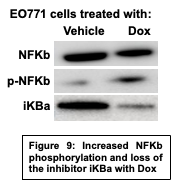


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***Specific Aim 2****: Investigate TLR4 inhibitor synergy with chemotherapeutics and impact on the metastases’ microenvironment.* First, chemotherapeutics used for metastatic TNBC will be tested with TLR4 inhibitor, currently in clinical trials, in 2D viability assays and in 3D invasion assays. Selected combination treatments will be tested in syngeneic mouse models of metastatic mammary tumors to assess changes in established lung metastases as well as inflammation markers and cytokines production between treatment arms.

Aim2a: Determine synergistic combinations of TLR4 inhibitor and chemotherapeutics in 2D and 3D *in vitro* models.



First, we confirmed that NFKb pathway activation, downstream effector of TLR4 signaling, increases with doxorubicin treatment in EO771 cells (Figure 9). Additionally, iTEAD treatment of EO771 spheres in 3D invasion assay showed a significant decrease in cells invading into the matrix (Figure 10). Now we will test TLR4 inhibitor, resatorvid effect on the ability of these cells to invade in 3D models with chemotherapy currently used as standard of care.

Aim2b: To test synergism of TLR4 inhibitor and chemotherapy combination *in vivo*.

No experiments have been performed for this subaim yet.

**Goals for the upcoming funding period/research in progress**

Aim 1: In Addition to AIB1D4 expression, we will determine the PDX samples that correspond with the TNBC subtypes that were most responsive to iTEAD in combination with chemotherapy treatment from our experiments with cell lines already tested. PDX spheres will then be tested in *in vitro* 3D invasion models followed by *in vivo* animal metastasis models. We will also consider staggering the treatment of the two therapies to examine if higher synergy is possible.

Aim2: We will determine the synergistic effect of TLR4 inhibitor and chemotherapeutics combination in 2D proliferation assays and 3D invasion models *in vitro* of EO771 cells. Then, we will examine the effect of combination therapy on established lung metastasis in an immune competent mouse model. Based on the data we obtained in Aim1 with 4T1/ BALBc metastasis model, we will consider using this model with TLR4 inhibitors as a second model to confirm the findings with TLR4 inhibitor.

**Publications/Presentations**

Please list any publications or presentations if applicable that resulted from work funded by METAvivor.

A manuscript titled “ TEAD inhibition blocks metastasis by promoting an anti-tumor niche in the lungs“ is in preparation. Below is the abstract:

Metastases are more aggressive than the primary tumors they originated from, resistant to therapy, and the major cause of patients’ fatality. Cancer cells may disseminate during early-stage disease and seed in distant organs yet are not clinically detectable. Metastatic outgrowth is sustained via cellular crosstalk at the new microenvironment to achieve immune evasion, recruitment of pro-tumorigenic cells, and extracellular matrix remodeling. Therapeutic interventions to disrupt the intricate co-evolution of metastases and their microenvironment are still lacking. Recently, we identified a pioneer subpopulation of cancer cells in metastatic breast cancer that seeds in the lungs and enables metastasis and colonization of other cancer cells without becoming the dominant population. Here, we revealed an enhanced transcriptional activity from the TEAD family of transcription factors and upregulation of the Toll-Like Receptor 4 (TLR4) signaling pathway in these subpopulations. Small molecule inhibitors targeting TEAD activity inhibited the development of lung metastases and, more importantly, halted the outgrowth of cancer cells that had seeded in the lungs in several mouse models. Additionally, immune profiling by flow cytometry revealed a shift in monocyte/macrophage subpopulations, namely an increase in resident alveolar macrophages and a decrease in infiltrating macrophages. Lung infiltrating CD8+ T cells increased in the lungs of iTEAD-treated mice. This suggests that iTEAD-modulated microenvironment- perhaps through an alternative macrophage phenotype- may have reversed cytotoxic T-cells exclusion from the lungs as seeded cancer cells grow to form metastases. TEAD inhibition may serve as the brakes on the metastatic manifestation of already seeded cancer cells by altering macrophage phenotypes in the metastatic niche.

**\*\*Required for METAvivor’s board: Lay Description of Important Outcomes**

**NOTE: this section may be shared on METAvivor’s website and/or in donor publications as it is important for us for donor transparency – thank you!**

Please briefly summarize main project achievements or outcomes so far in lay terms. Please emphasize any clinical trials and/or important findings with potential to impact metastatic breast cancer treatment (now or in the future) stemming from the funded work.

A manuscript highlighting the role TEAD inhibitors play at halting metastasis is in preparation. Our data so far suggest a potential synergistic effect between TEAD inhibitor and chemotherapeutic reagents currently used as standard of care in metastatic TNBC. This effect was observed on both the primary tumor size as well as lung metastases, yet not all TNBC subtypes seem to have equivalent sensitivity. These observations will aid in determining the cohort of patients that would benefit the most from this new combination therapy regimen. Further mechanistic studies will better our understanding of how these agents work together to improve outcome and patient’s survival.