Manipulation of epithelial-to-mesenchymal transition reveals metastatic breast cancers support immune suppression via heme metabolism

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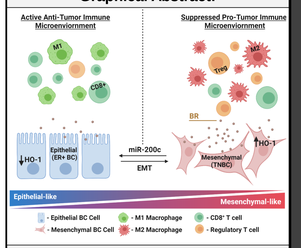
Introduction

- Triple negative breast cancer (TNBC) has often undergone oncogenic epithelial-to-mesenchymal transition (EMT).
- EMT in BC models is associated with increased immune suppression and altered metabolism. (Dongre A et al. *Can Res*, 2017; Reviewed: Terry S et al. *Mol Oncol*, 2017; Jai D et al. *J of Clin Med*, 2019; Sun X et al. *Font Cell Dev Biol*, 2020)
- EMT reversal via miR-200c revealed enzymes, such as heme oxygenase-1 (HO-1), that may simultaneously support metastatic TNBC cell survival and immune suppression. (Rogers TJ et al. *MCR*, 2019)
- ***However, impact of the immune modulatory HO-1 metabolite bilirubin on the TNBC tumor microenvironment had not been studied.***

Graphical Abstract:



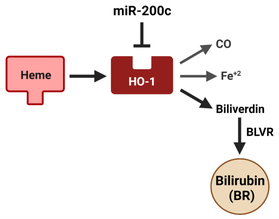
Graphical Abstract: MiR-200c revealed that EMT induces HO-1 in BC cells. This project tests whether HO-1 supports immune suppression by metastatic TNBC cells via its metabolite bilirubin (BR).

Hypothesis

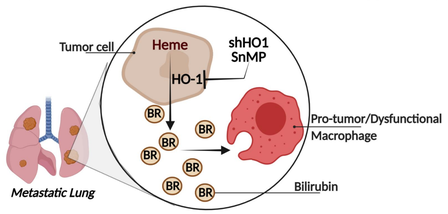
We postulate that tumor cell-HO-1 activity and subsequent bilirubin secretion enhance TNBC metastasis by supporting a pro-tumor immune microenvironment.

Project Model

A. Heme metabolism by HO-1



B. Role of HO-1/BR in metastatic TNBC immune suppression



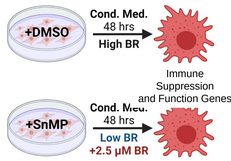
Project Model. A. Heme is broken down by HO-1 into three immunomodulatory products, iron (Fe^{+2}), carbon monoxide (CO) and biliverdin that is converted to bilirubin (BR). **B.** This project tests the impact of HO-1 on TNBC progression via BR-mediated immune suppression. We assess the effects of BR on macrophage immune suppression and dysfunction by blocking tumor cell-HO-1 activity with the FDA approved HO-competitive inhibitor tin mesoporphyrin (SnMP) or shRNA (shHO1).

Methods

- We assessed immune suppressive and efferocytosis genes in RAW264.7 mouse macrophages via qRT-PCR after direct treatment with bilirubin or treatment with conditioned medium (CM) from HO-1 inhibited mammary carcinoma cells.
- Macrophage PD-L1 expression and efferocytic capacity, dead cell engulfment, were observed by flow cytometry and IncuCyte live cell imaging (Essen Bio).
- HO-1 was depleted in 66Cl-4 mammary carcinoma cells using shRNA. 66Cl-4 shHO1 cells were then injected orthotopically into immune-competent hosts and we assessed primary tumor growth and lung metastatic capacity.
- The Cancer Genome Atlas (TCGA) breast cancer specimens were analyzed via CIBERSORT to predict relative immune cell abundance. (The Caner Genome Atlas Network. *Nature*, 2012)

Fig 1. Tumor cell-HO-1 alters immune suppressive and efferocytosis macrophage genes via secreted bilirubin.

A. Experimental design



B. RAW264.7 Macrophage Gene Expression

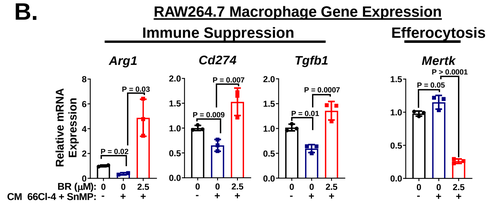
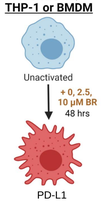


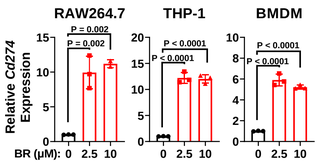
Figure 1 A. Macrophages were cultured with and without 2.5 µM BR delivered in control or bilirubin-depleted conditioned medium (CM) collected from mammary carcinoma cells treated with 10 µM tin mesoporphyrin (SnMP). **B.** mRNA was isolated and tested for immune suppressive (*Arg1, Cd274*/PD-L1, *Tgfb1*) and functional efferocytosis (*Mertk*) genes.

Fig 2. Bilirubin enhances PD-L1 expression in human-derived and primary mouse macrophages.

A. Experimental Design



B. PD-L1 Gene Expression



C. PD-L1 Protein Expression

Treatment	MFI
0 µM BR	4.2x10^5
2.5 µM BR	4.7x10^6
10 µM BR	2.0x10^7
100 ng/mL IFNγ + LPS	3.6x10^8

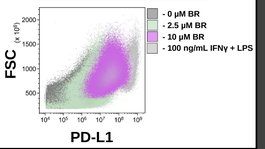
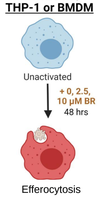


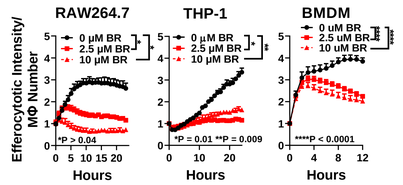
Figure 2. A. RAW264.7 and bone marrow-derived (BMDM) mouse and THP-1 human-derived macrophages were treated with a dose escalation of BR for 48 hours (0-20 µM, select doses are shown). **B-C.** Gene (**B**) and protein (**C**) expression of PD-L1 was assessed by qRT-PCR and flow cytometry. 100 ng/mL IFNγ + LPS was a positive control.

Fig 3. BR decreases mouse and human macrophage engulfment of dead cells (efferocytic capacity).

A. Experimental Design



B. Macrophage Efferocytic Activity



C. RAW264.7, 24 hours

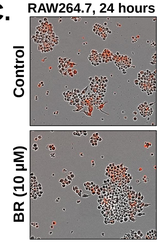
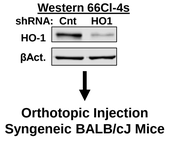


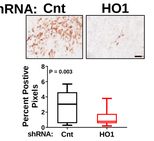
Figure 3. A-C. RAW264.7 and BMDM mouse and THP-1 human-derived macrophages were treated with 2.5 or 10 µM BR for 0-24 hours (**A**). Efferocytosis was measured using an IncuCyte efferocytosis assay (**B**) where dead 66Cl-4 cells were dyed with a marker that fluoresces bright red in the high pH environment of macrophage lysosomes (**C**).

Fig 4. Inhibition of tumor cell-HO-1 limits lung metastatic capacity.

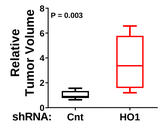
A. HO-1 Knockdown



B. IHC Primary Tumors



C. Tumor Volume



D. Lung Metastatic Capacity

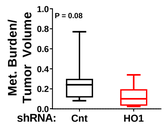
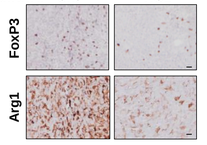


Figure 4. A. HO-1 was genetically depleted from 66Cl-4 mammary carcinoma cells (shHO1) that were then injected into the mammary fatpads of syngeneic BALB/cJ mice in a preliminary study. **B-C.** 8 weeks later, primary tumors were analyzed for HO-1 expression via IHC (**B**), and tumor volume (**C**) and metastatic capacity (**D**) were assessed.

Fig 5. HO-1 correlates with markers of a suppressed tumor microenvironment.

A. IHC Primary 66Cl-4 Tumors



B. CIBERSORT TCGA Breast Cancer Specimens

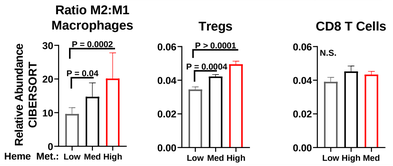


Figure 5. A. IHC conducted on 66Cl-4 shHO1 mammary tumors for FoxP3 (T regulatory cell marker) and Arg1 (immune suppressive macrophage marker). **B.** CIBERSORT analysis conducted on breast cancer specimens from the Nature, 2012 dataset stratified based on expression of heme metabolism genes (*HMOX1/2* and *BLVRA/B*).

Conclusions/Future Directions

- Restoration of miR-200c revealed heme metabolism via HO-1 as a possible immune modulatory pathway in breast cancer.
- Follow-up analysis demonstrated tumor cell-HO-1 inhibition altered expression of macrophage immune suppressive and efferocytosis genes via bilirubin.
- Bilirubin increased PD-L1 expression but decreased macrophage efferocytic capacity.
- Inhibition of tumor cell HO-1 limited lung metastatic capacity and expression of clinically relevant immune suppressive markers.
- ***Summary: HO-1 targeting with FDA approved SnMP may activate anti-tumor macrophages via bilirubin depletion and limit TNBC metastasis.***

- Future studies will continue to test the impact of HO-1 inhibition (shRNA or SnMP) on immune suppressive T cells (exp. Tregs) and macrophage function (exp. expression of efferocytosis receptors) in experimental metastasis models to the lung and liver.

Funding

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