Background Studies in non-cancer diseases revealed that bilirubin, a metabolite of the heme-degrading enzyme heme oxygenase-1 (HO-1/HMOX1), suppresses normal macrophage function. Although HO-1 expression is often elevated in aggressive cancers, such as triple-negative breast cancer (TNBC), the effects of bilirubin on the tumor microenvironment (TME) remain unknown. We hypothesized that tumor cell-HO-1 activity and subsequent bilirubin secretion enhance TNBC metastasis by supporting immune suppressive, pro-tumor macrophage function.

Methods We tested the impact of bilirubin on human and mouse macrophage immune suppression and efferocytic capacity (engulfment of dead tumor cells) using qRT-PCR, flow cytometry, and live cell imaging (n=6–9). Primary tumor growth, lung metastatic burden, macrophages, and T cells were assessed in syngeneic, immunocompetent mice harboring 66Cl-4 mammary tumors with HO-1 genetic (shHmox1, n=8/group) or pharmacologic inhibition (tin mesoporphyrin or SnMP, 25 mg/kg/daily, n=10/group).

Results Treatment with exogenous bilirubin enhanced macrophage PD-L1 mRNA and protein levels by over 6-fold. In contrast, bilirubin decreased expression of the efferocytosis genes Mertk and Tryo3 by at least 50% and nearly ablated macrophage efferocytic capacity. To assess whether tumor produced bilirubin supports metastatic progression, we evaluated lung metastasis after 66Cl-4-Hmox1 knockdown. Spontaneous lung metastasis from shHmox1 tumors was decreased when compared to shCnt tumors. Global inhibition of HO-1 via SnMP, an FDA approved HO-1 enzymatic inhibitor, decreased tumor cell-bilirubin levels by 82%, lung metastatic burden by 35%, and the number of pro-tumor CD206+ macrophages by 74%. Macrophages isolated from 66Cl-4 mammary tumors treated with SnMP were unable to suppress the expression of cytotoxic molecules granzyme-B and perforin in stimulated CD8 T cells. Interestingly, data mining of KM Plotter showed that high expression of genes required to generate bilirubin predict a poor overall survival of several cancer types treated with Pembrolizumab. To test if HO-1-inhibited tumors are primed for response to T cell directed therapies, 66Cl-4 mammary carcinomas were treated with the combination of SnMP and anti-PD-1. The number of pro-tumor macrophages expressing Arg1 decreased by 35%, while the number of CD8 T cells expressing cytotoxic molecules increased by 63% in 66Cl-4 mammary tumors that received combination treatment compared to anti-PD-1 alone.

Conclusions Tumor cell-HO-1 may support macrophage immune suppression and dysfunction during TNBC progression via bilirubin. Since HO-1 inhibitors including SnMP are FDA approved for treatment of other diseases, these findings could be rapidly translated to provide an alternative immunotherapy approach in aggressive TNBC directed at suppressive, pro-tumor macrophages.

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Ethics Approval All animal experiments were performed using humane procedures in accordance with the protocols #00407 and #01056 as approved by the University of Colorado Institutional Animal Care and Use Committee (IACUC).

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