Co-Expression Of Extracellular Matrix Enzymes Heparanase Or PH-20 Augments The Anti-Tumor Efficacy Of Folate Receptor 1-Targeting CAR T Cells In An Ovarian Cancer Model

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ABSTRACT

Introduction: The tumor extracellular matrix (ECM) is a physical barrier which obstructs trafficking of CAR T cells to tumors. We hypothesize that co-expressing ECM remodeling/degrading enzymes heparanase (HPSE) or hyaluronidase (PH-20) with a CAR directed against folate receptor 1 (FolR1) will overcome the physical tumor microenvironment barrier and improve anti-tumor efficacy of anti-FolR1 CAR in a human ovarian cancer model.

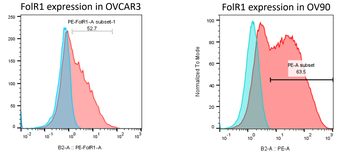
Methods: ECM CARs were constructed by combining anti-FolR1 ScFv with a CD8 hinge and transmembrane domain, 4-1BB, and CD3ζ endodomains, followed by a P2A element and full-length HPSE or GPI-anchored PH-20 sequence. CAR T cells were prepared from CD4+ and CD8+ T cells of healthy donors by lentiviral vector transduction. CAR expression was evaluated by flow cytometry; HPSE and PH-20 expression were measured via ELISA and Western blotting, respectively. CAR-mediated killing and cytokine release were observed in overnight co-culture assays with FolR1-expressing tumor cells. ECM CARs' migration through Cultrex™ and sodium hyaluronate matrices were assessed for HPSE and PH-20 function, respectively. ECM CARs' efficacy was evaluated *in vivo* using a FolR1-expressing subcutaneous OV90 ovarian cancer xenograft model in NOD SCID Gamma (NSG) mice, and representative OV90 tumors were harvested at day 7 post-CAR T treatment for immunohistochemical and multiplex fluorescence staining.

Results: FolR1 expression was confirmed in ovarian cancer cell lines and substrates targeted by HPSE (perlecan, glypican 3, CD138) and PH-20 (hyaluronan) were detected by immunofluorescence in PFA fixed human ovarian tumors' sections. Lentiviral vector-transduced primary T cells yielded robust expression of FolR1 CAR and ECM enzymes. Anti-FolR1 CAR and ECM CARs secreted pro-inflammatory cytokines IL-2, TNFα, IFNγ and lysed OVCAR3 human tumor cells with similar efficacy. ECM CARs with HPSE or PH-20 exhibited enhanced migration through ECM-rich matrices as compared to anti-FolR1 CAR alone. *In vivo*, all anti-FolR1 CAR T cells rejected OV90 tumors and extended survival compared to tumor alone or un-transduced T cells (UTD). However, ECM CARs co-expressing HPSE or PH-20 accelerated tumor regression as compared to anti-FolR1 CAR alone. Tumor histology revealed CAR T cell infiltration (CD3+, CD8+) in mice treated with anti-FolR1 CARs but not UTD, indicating that anti-FolR1 CARs can effectively infiltrate OV90 tumors. IHC and MACSima™ Imaging Cyclic Staining (MICS) studies reveal enhanced function of the ECM CARs in the tumor microenvironment (TME) and provide insights on ECM tumor remodeling.

BACKGROUND

Expression of FolR1 antigen and tumor ECM proteins in human ovarian cancer

A. Human Ovarian Cancer Cell Lines



B. Human Ovarian Adenocarcinoma Tissue Sections

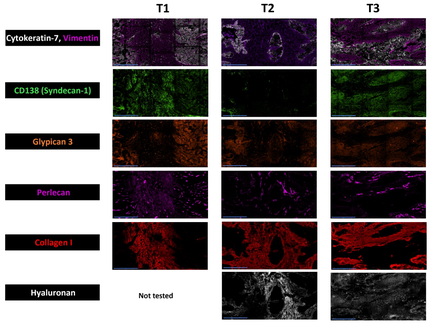
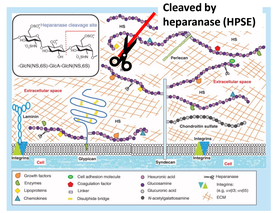


Figure 1. Folate Receptor 1 is expressed in human ovarian cancer cell lines and expression of ECM proteins in human ovarian cancer tissue. A. FolR1 cell surface expression was assessed by flow cytometry in human ovarian cancer cell lines OVCAR3 and OV90. FolR1⁺ – red, isotype control histograms – blue. **B.** Fresh-frozen PFA fixed tissue sections of three patients with ovarian adenocarcinoma were processed for multiplex fluorescence staining and imaged on the MACSima™ platform. Ovarian tumor (Cytokeratin-7) and stroma (Vimentin) regions were identified. ECM proteins were detected in the tumor (CD138 and Glypican 3) and stroma (Perlecan, Collagen I, and Hyaluronan). Scale – 500um.

BACKGROUND

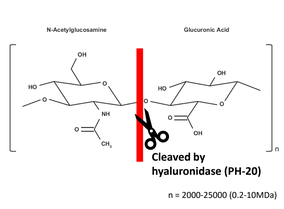
Structure and enzymatic cleavage of heparan sulfate proteoglycans and hyaluronan

A. Heparan Sulfate Proteoglycans



Adapted from Rivara S, Milazzo F, Giannini G 2016 *Fut Med Chem*

B. Hyaluronan



Adapted from Knowles S. et. al. 2021 *Expert Opinion on Drug Delivery*

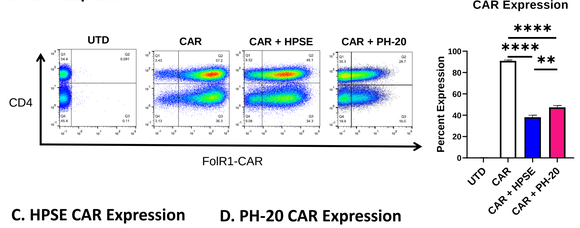
Figure 2. Heparanase enzymatically cleaves heparan sulfate proteoglycans and hyaluronidase degrades hyaluronan. A. The core of proteoglycans like glypicans, syndecans, and perlecans are covalently linked to heparan sulfate side chains. Heparanase cleaves the glycosidic bond between glucuronic acid and N-sulfo glucosamine, depicted by the red line. **B.** Hyaluronidase cleaves the glycosidic bond between glucuronic acid and N-acetyl-glucosamine, depicted by the red line.

RESULTS

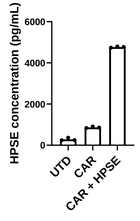
Constructs design and expression of FolR1 CAR and ECM Enzymes

A. CAR Structure

B. CAR Expression



C. HPSE CAR Expression



D. PH-20 CAR Expression

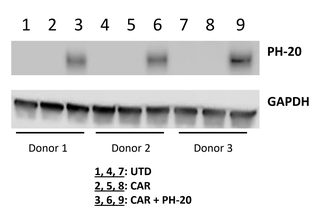
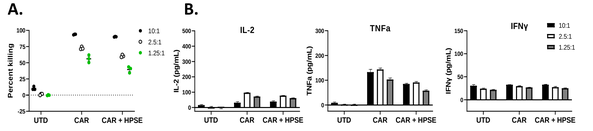


Figure 3. Structure and expression of FolR1 CAR and ECM enzymes in human primary T cells. A. The FolR1 CAR is comprised of a fully human FolR1 ScFv targeting domain, a CD8 hinge and transmembrane domain, a 4-1BB co-stimulatory domain and a CD3ζ activation domain. ECM CARs are comprised of the FolR1 CAR, followed by 2A peptide, in frame to an ECM molecule HPSE or PH-20. **B.** Primary T cells from healthy donors were activated with TransAct™ in the presence of 30 IU/mL IL-2 and transduced with lentiviral vectors encoding CAR constructs. Transduced T cells were assayed for CAR surface expression with FolR1-Fc staining followed by anti-Fc-AF647 with flow cytometry. CD4 staining was included to identify CD4+ and CD8+ populations. UTD – un-transduced control. Mean ± SEM of 3 donors; one-way ANOVA. **p<0.01, ****p<0.0001. **C.** HPSE in supernatant from FolR1 CAR T cells was measured by ELISA on culture day 10. One representative donor out of 3 is shown, mean ± SEM of 3 technical replicates. **D.** PH-20 was measured by Western blots from CAR + PH-20 lysates taken from culture day 10 in 3 donors. 20 ug protein were run on an SDS-PAGE gel and processed for Western blotting to confirm PH-20 expression in the ECM CAR. GAPDH, loading control.

RESULTS

Cytotoxicity analysis and cytokine response of ECM CARs *in vitro*

CAR + HPSE



CAR + PH-20

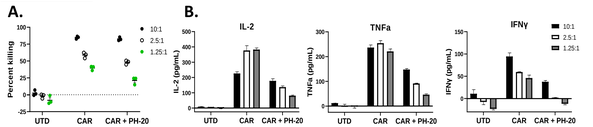


Figure 4. ECM CARs have similar antigen-specific cytotoxicity *in vitro* compared to CAR alone. A. Luciferase-based cytotoxicity assays were performed on FolR1+ tumor line OVCAR3 co-incubated with CAR T cells overnight at the indicated effector to target (E:T) ratios: 10:1, 2.5:1, or 1.25:1. Percent specific target lysis was assessed by luminometry. Data represents one out of 3 separate donors tested. Mean ± SEM of three technical replicates. **B.** Production of IL-2, TNFα, and IFNγ after co-culture was analyzed by ELISA. Mean ± SEM of three technical replicates. Data represents one donor out of 3 separate donors tested.

In vitro matrix migration of ECM CARs

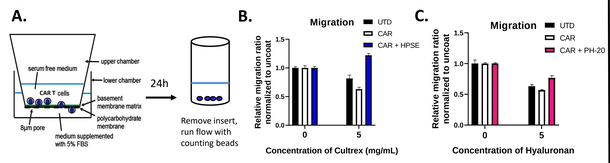


Figure 5. ECM CARs penetrate through ECM-rich matrices exceeding UTD and CAR alone. A. HPSE and PH-20 effect on CAR T cell migration was evaluated by an *in vitro* migration assay using 0 or 5 mg/ml Cultrex™ coated or sodium hyaluronate coated transwells, respectively. Half a million thawed CAR T cells were seeded into precoated transwells. After 24hr, the total CAR T cells that migrated into the bottom chamber were quantified using CountBright™ Absolute Counting Beads and measured by flow cytometry. **B and C:** Relative migration of CAR T cells through Cultrex™ **(B)** or hyaluronan **(C)** coated transwells compared to uncoated transwells was quantified. Mean ± SEM of 3 technical replicates from one representative donor out of 3 donors tested.

***In vivo* efficacy of ECM CARs – OV90 model**

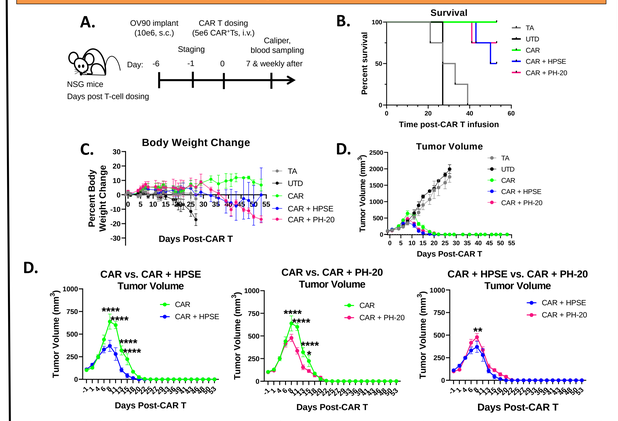
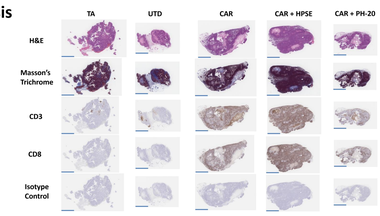


Figure 6. Inclusion of HPSE or PH-20 elements accelerates *in vivo* tumor regression kinetics of FolR1 CARs. A. Female NSG mice bearing subcutaneous OV90 ovarian cancer tumors were treated with 5E6/mouse CAR+ T cells. Tumor size, weight, and peripheral blood analyses were done weekly until D53 post-CAR T dosing. Treatment groups: TA, UTD, CAR alone, CAR + HPSE, CAR + PH-20. **B.** Percent survival of the treatment groups were graphically represented. **C.** Body weight changes of mice during OV90 xenograft study were recorded weekly or bi-weekly. Body weight change was calculated as the percent change from study initiation. **D.** Tumor regression kinetics for FolR1 CAR alone or with ECM elements are shown. Mean ± SEM of 4 mice/group. Two-way ANOVA; *p<0.05,**p<0.01, ****p<0.0001. TA-tumor alone, UTD – un-transduced T cell control.

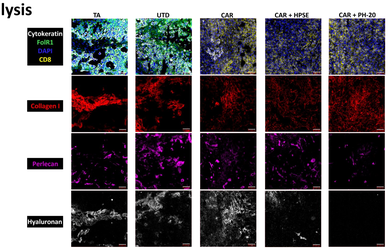
RESULTS

CAR T cell tumor infiltration and ECM composition of OV90 xenograft tumors

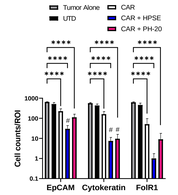
A. IHC analysis



B. MICS analysis



C. Tumor regression



D. CAR T infiltration and ECM remodeling

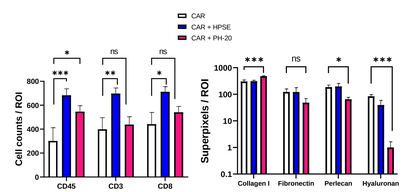


Figure 7. FolR1 CARs infiltrate OV90 tumors and ECM CARs remodel the tumor microenvironment. One mouse per treatment group from the *in vivo* study described in **Fig. 6** was sacrificed on D7 post-CAR T infusion. OV90 tumors were harvested and bi-sectioned where half of the tumor was fixed in FFPE, and the other half was embedded in O.C.T. medium and fresh-frozen. **A.** FFPE-sectioned tumors were stained with H&E, Masson's Trichrome, anti-CD3, anti-CD8, and isotype control. Scale – 2 mm. **B.** Fresh-frozen tumor sections were PFA fixed, processed for multiplex fluorescence staining, and imaged by MICS. Scale – 50 um. **C.** Tumor regression was evaluated as cell count/ROI by MACS® iQ View software. **D.** CAR T infiltration was analyzed by cell count/ROI (left) and ECM proteins were analyzed by superpixel count/ROI (right) on the MACS® iQ View software. Data represents 8 ROIs for each tumor section, mean ± SEM. Statistical differences were detected by one-way or two-way ANOVA *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001; #p<0.05 CAR + ECM enzyme vs CAR alone.

SUMMARY AND CONCLUSION

- We demonstrate the development of highly effective FolR1 CAR T cells with enzymatic function towards tumor extracellular matrix (ECM) proteins: perlecan was measured as a prototype substrate for HPSE and hyaluronan for PH-20
- Co-expression of HPSE or PH-20 enzymes with FolR1 CARs boosted CAR T cell penetration into solid tumor matrix *in vitro*, and in an ovarian tumor model *in vivo,* resulting in improved tumor clearance
- **MICS analysis** confirmed tumor regression on a microscopic level (reduction in EpCAM, Cytokeratin, and FolR1 markers) in all CAR-treated groups as compared to tumor alone and UTD controls
- As compared to FolR1 CAR T cells alone on day 7 post treatment, tumor infiltration was significantly enhanced for CARs with ECM elements, especially in CAR + HPSE
- CAR + HPSE did not reduce net amount of its target perlecan (superpixel count); additional analysis will be performed to evaluate the cleavage rate of heparan sulfate proteoglycans by CAR + HPSE
- CAR + PH-20 treatment reduced the net amount of its substrate hyaluronan, and unexpectedly also HPSE substrate perlecan, and increased net collagen I in ECM, suggesting TME remodeling

In conclusion, the anti-FolR1 CARs boosted with ECM enzymes HPSE or PH-20 demonstrated specific lysis and cytokine release toward FolR1-expressing cell lines, improved penetration through ECM-rich matrices *in vitro*; accelerated tumor infiltration and regression, and ECM remodeling in an ovarian cancer model *in vivo*. Therefore, ECM CARs may help improve clinical outcomes in patients with ovarian tumors.