Cancer Systems Immunology unravels complexity of reversing immune suppression and predicts beyond RECIST in metastatic breast cancer

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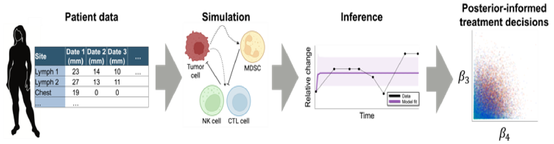
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INTRODUCTION

Combination therapy aimed at modulating the tumor microenvironment (TME) is a robust strategy to improve response to checkpoint inhibition and broaden indications for use in patients with metastatic breast cancer. The epigenetic modulator, entinostat when combined with dual checkpoint blockade; nivolumab and ipilimumab demonstrated promising results in our Phase Ib trial (NCI-0944) and in our recent meta-analysis (AACR-3225, 2025). Use of a cancer systems immunology approach accelerated discovery of the complex mechanism of response to treatment. Lastly, we demonstrate the potential for math modeling to predict response similar to RECIST, and inform organ specific response, a powerful tool with potential for use toward a more personalized approach for patients.

METHODS

To identify mechanisms of response to combination therapy in a preclinical model, we used knowledge-guided subclustering of single-cell RNA-sequencing data and cell circuits analysis to predict salient interactions. Multiparametric flow cytometry and ex-vivo immune suppression assays were used to validate preclinical findings. Imaging mass cytometry and bulk RNA sequencing of patient samples were used to validate findings in patients. Derivation of dynamic mathematical models of tumor-immune modulatory responses were then fit through innovative use of both preclinical (sequencing) and clinical (proteomics) data – to predict that a combination of effects on the TME is necessary for response. Adaptation of this mathematical model was then made to determine its potential for use in prediction of site-specific response in metastatic lesions. We introduced methods employing posterior parameter sampling and simulation to create virtual tumor populations, enabling extrapolation beyond the data to predict probabilities of response in metastatic lesions, even when no data exists at that site.



RESULTS

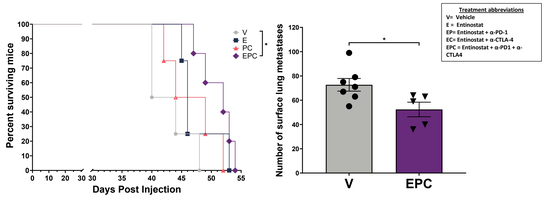


Fig. 1: **Entinostat + anti-PD-1 + anti-CTLA-4 increases survival in the NT2.5-LM model.** Neu-N mice were injected with $1x10^5$ NT2.5-LM cells in the tail vein on day 0. Mice were euthanized as they developed symptoms of lung metastases (e.g. labored breathing). Survival curve for mice treated with different combinations of entinostat (E), anti-PD+1 (P), and anti-CTLA-4 (C; left), and number of surface lung metastases in mice at the time of euthanasia (right).

ACKNOWLEDGMENTS

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RESULTS

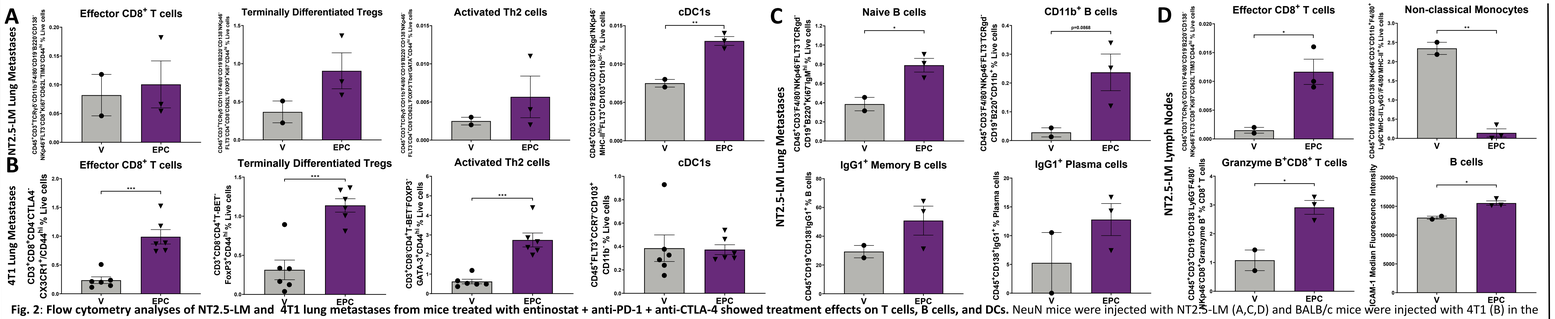


Fig. 2: **Flow cytometry analyses of NT2.5-LM and 4T1 lung metastases from mice treated with entinostat + anti-PD-1 + anti-CTLA-4 showed treatment effects on T cells, B cells, and DCs.** NeuN mice were injected with NT2.5-LM (A,C,D) and BALB/c mice were injected with 4T1 (B) in the 4th mammary fat pad and resulting lung metastases were macro-dissected and analyzed via spectral (A, C, D) or conventional (B) flow cytometry. Cervical lymph nodes were obtained from mice with NT2.5-LM lung metastases and analyzed for cell proportions and ICAM-1 MFI (D). Treatment abbreviations denoted in Fig. 1. The y-axis denotes the cells expressing the specified series of markers as a percent of total single, live cells or the parent population indicated for cell proportions. The Mann-Whitney or unpaired T test was used to determine significant differences between V and EPC. **p=0.01, ***p=0.001.

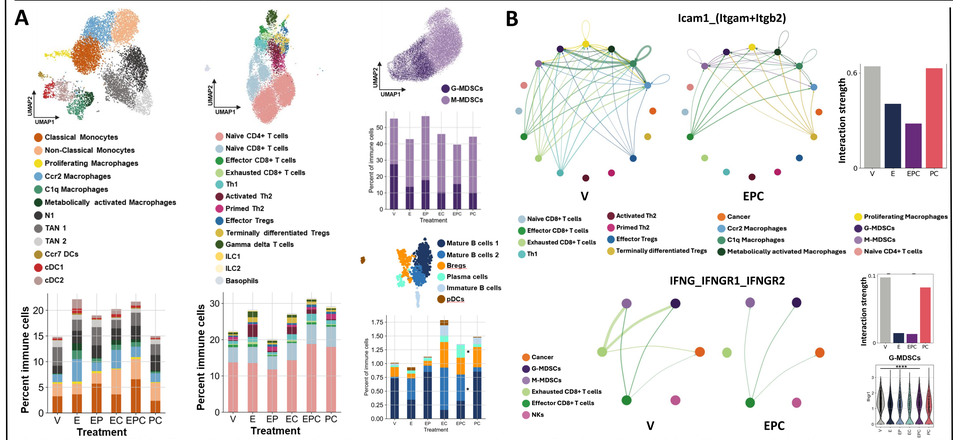


Fig. 3: **scRNA-seq validates flow cytometry results in the NT2.5-LM model and shows decreased ligand-receptor pair interactions associated with immunosuppression in mice treated with entinostat + anti-PD-1 + anti-CTLA-4. A.** UMAP (top) and frequency (bottom) of mature myeloid (left), T cell (center), MDSC (top right), and B cell (bottom right) populations in NT2.5-LM metastases. **B.** Circos plots generated via cell chat analysis showing interactions between immune cell subpopulations in NT2.5-LM metastases from V vs EPC treated mice along with corresponding interaction strengths for interactions between all cells included in the analysis with treatments for Icam1_Itgam_Itgb2 (top) and Ifng_Ifngr1_Ifngr2 (bottom) ligand-receptor pairs (bar plots). The expression of Ifngr1 in G-MDSCs with treatments is shown in the bottom right. ****p=0.0001.

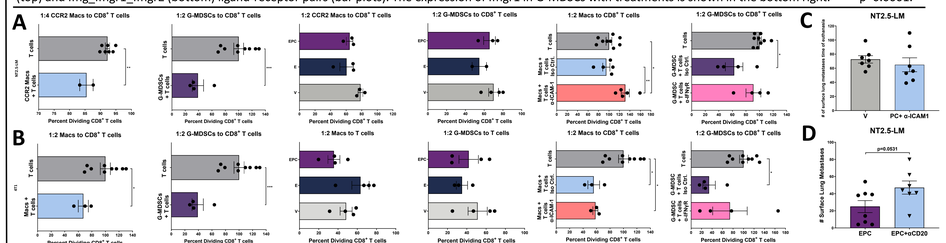


Fig. 4: **G-MDSCs and TAMs suppress T cell proliferation and are unaffected by treatment; blockade of ICAM-1 in TAMs or IFNgR in G-MDSCs stimulates T cell proliferation *ex-vivo*, but *in-vivo* ICAM-1 blockade is ineffective.** G-MDSCs and TAMs were FACS-sorted from NT2.5-LM (**A**) or 4T1 (**B**) lung metastases, co-cultured with CFSE-stained CD8+ T cells, and analyzed via flow cytometry to determine T cell proliferation. **C.** Mice were treated with entinostat, anti-PD-1 + anti-CTLA-4, or anti-ICAM-1 (NT2.5-LM model) and assessed for survival as described in Fig. 1. **D.** Percentage of TAMs from NT2.5-LM lung metastases expressing PD-L1 following treatment with anti-ICAM-1 or isotype control.

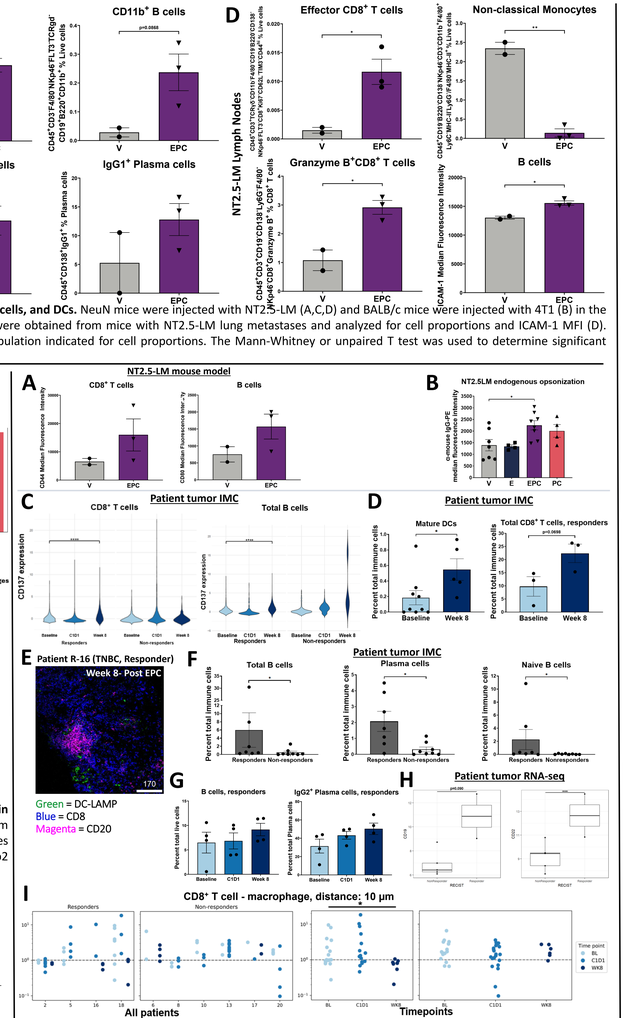


Fig. 5: **EPC demonstrates anti-tumoral effects on B cells and macrophages in patient tissues from the NCI-9844[1] phase 1 clinical trial. A-B.** Expression of CD44 and CD80 activation markers in CD8+ T cells and B cells, and levels of endogenous IgG bound to HER2+ cancer cells within lung metastases, determined via flow cytometry. **C-F.** Expression of CD137 in CD8+ T cells and B cells (C), proportions of mature DCs in all patients and CD8+ T cells in responders (D), visualization of processed signals for DC-LAMP, CD8, and CD20 in a lung sample from patient R-16 at week 8, and proportions of B cells, plasma cells, and naïve B cells in patient tumors determined via imaging mass cytometry (IMC). **G.** Proportions of B cells and IgG2+ plasma cells in blood samples from responders, determined via spectral flow cytometry. **H.** Expression of CD19 and CD22 B cell markers in patient tumors determined via RNA-seq. **I.** Co-occurrence scores at a 10 μm distance between CD8+ T cells and macrophages in patient tumors determined via IMC.

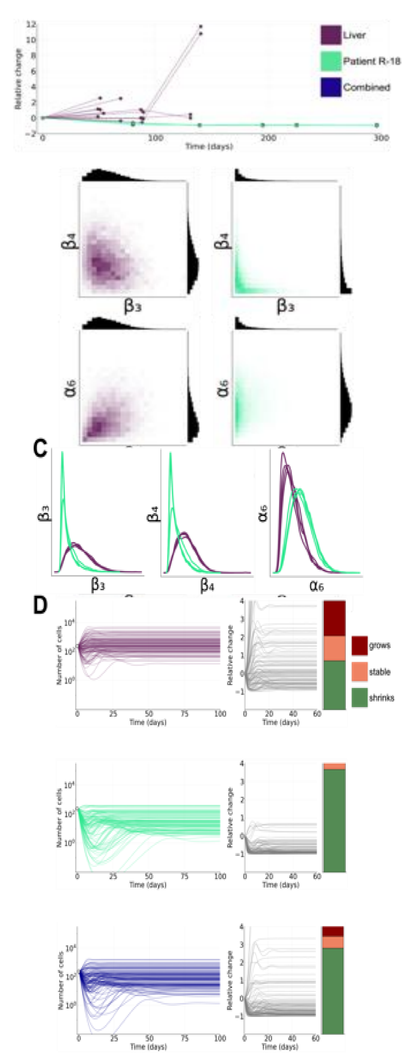


Fig. 6: Site-specific patient-specific tumor prediction enables the analysis of a large virtual patient population undergoing combination treatment. A. Individual tumor data from liver (purple) and patient R-18 (light green). B-C. Posterior parameter distributions from liver and patient R-18. D. 102 tumor simulations for liver tumors, patient R-18 tumors, and liver and patient R-18 tumors (blue). The fraction of tumors that decrease (green), are stable (orange), and increase (red) are also shown.

CONCLUSIONS

The combination of entinostat with anti-PD-1 and anti-CTLA-4 increases survival and decreases lung metastasis in both the NT2.5-LM and 4T1 models. Evidence of activation of CD8+ T cells and B cells was uncovered via scRNA-seq and flow cytometry analyses using models. In addition, EPC treatment was associated with decreased immunosuppressive signaling between macrophages/G-MDSCs and CD8+ T cells, and emulating the effects of EPC via blockade of receptors involved in these signaling pathways resulted in increased proliferation of CD8+ T cells *ex-vivo*. While ICAM-1 blockade was insufficient to elicit a treatment response in combination with ICIs, B cell depletion results in a trending increase in metastasis, suggesting that B cells are required for the treatment response. In support of this, EPC demonstrated increased endogenous opsonization of cancer cells in lung metastases, which correlated with a trending increase in IgG1 production by plasma cells in mice and IgG2 production by plasma cells in patient blood. Analyses of patient tumors revealed increased activation of B cells with EPC and increased proportions of B cells and plasma cells and the presence of mature tertiary lymphoid structures in responders. In addition, EPC was associated with increased proportions of mature DCs, CD8+ T cell activation, and distancing with macrophages. In upcoming studies, we will use animal studies to determine if T/B cell activation and reduction of immunosuppression by myeloid cells are involved as mechanisms of action resulting in the observed EPC treatment efficacy.

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