Tumor cell AADAT suppresses CD8+ T cells by depleting malate, driving progression in Triple-Negative Breast Cancer

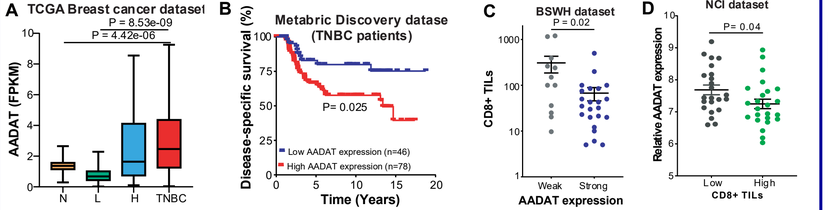
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Background and Rationale

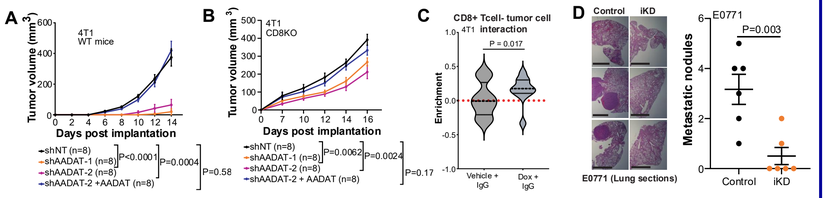
- A significant portion of advanced Triple-negative Breast Cancer (TNBC) patients do not respond to current chemo- and immunotherapies, or they develop resistance over time.
- A significant obstacle to optimizing immunotherapy is the immunosuppressive tumor microenvironment (TME), shaped in part by tumor-intrinsic metabolic programs.
- Many metabolic intermediates e.g. Kynurenine pathway enzymes, directly modulate immune cell differentiation and function, and reprogramming these axes has emerged as a promising avenue to overcome therapeutic resistance.
- One of the less studied KP enzymes, Alpha Amino Adipate Aminotransferase (AADAT) can act as an immuno-metabolic modulator by suppressing CD8+ T cell activity in TME by depleting Malate.
- The objective of the study is to uncover a readily translatable metabolic vulnerability with potential to improve outcomes for patients with aggressive breast cancer subtypes.

AADAT Expression Correlates with Reduced CD8+ TILs and Poor Prognosis in TNBC



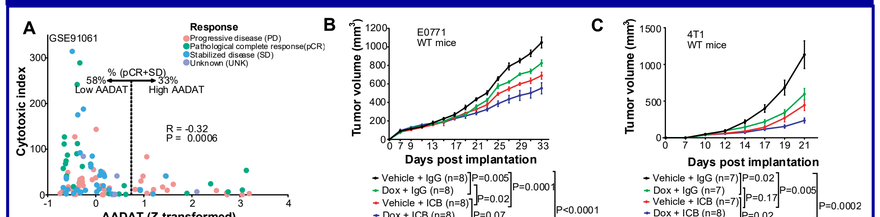
A. *AADAT* mRNA expression in breast tumors from the TCGA dataset across four PAM50 subtypes: Normal-like (n = 113), Luminal (n = 575), HER2-enriched (n =37), and TNBC (n = 115). **B.** Kaplan-Meier survival curves of TNBC patients from the METABRIC Discovery dataset, stratified by the median AADAT expression level. **C.** Infiltration of CD8+ T cells in TNBC tumors in the Baylor Scott and White dataset with weak (n = 12) or strong (n = 23) AADAT IHC staining. **D.** *AADAT* mRNA expression in breast tumors from the NCI dataset (GSE37751), divided into two subgroups of low (n = 23) and high (n = 24) CD8+ TILs divided based on the median number of intra-tumoral CD8+ TILs.

AADAT Promotes Tumor Growth and Suppresses Immune Infiltration in TNBC



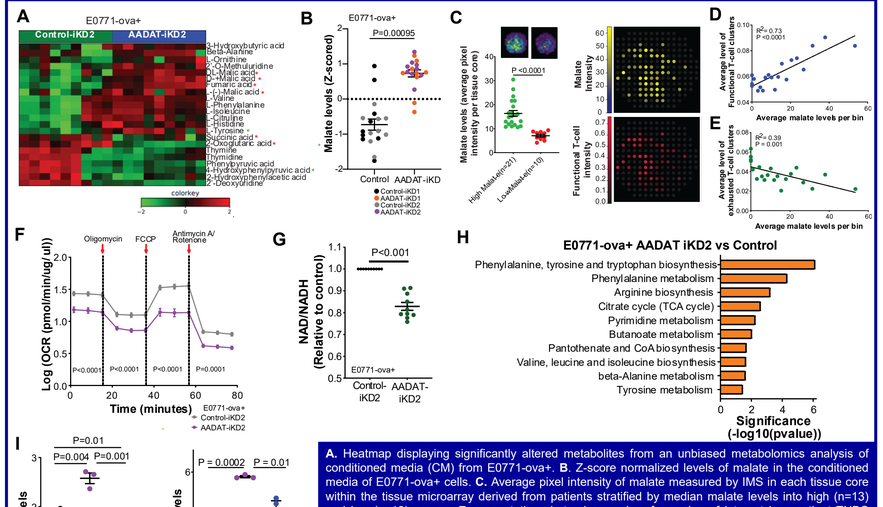
A. Tumor growth analysis of wild-type BALB/cJ mice orthotopically implanted with the syngeneic 4T1 cell lines stably transduced with either control non-targeted shRNA (shNT), Aadat-specific shRNAs (shAadat), and an Aadat re-expression. **B.** Tumor growth analysis of BALB/cJ mice with CD8 knockout (CD8KO), orthotopically implanted with the syngeneic 4T1 cell line. **C.** Violin plot illustrating the spatial co-localization and higher interaction of CD8+ T cells and tumor cells in the AADAT iKD and control tumors. **D.** Left panel: Representative H&E images of lung sections from wild-type C57BL/6J mice four weeks following resection of size-matched E0771 tumors expressing doxycycline-induced Aadat-specific shRNA (n = 6, per treatment group). The primary tumors were resected at 300 mm^3 followed by induction of AADAT KD for 28 days using Doxycycline and quantification of lung metastases. Scale bar: 1 mm.

AADAT Inhibition Sensitizes TNBC to Immune Checkpoint Blockade



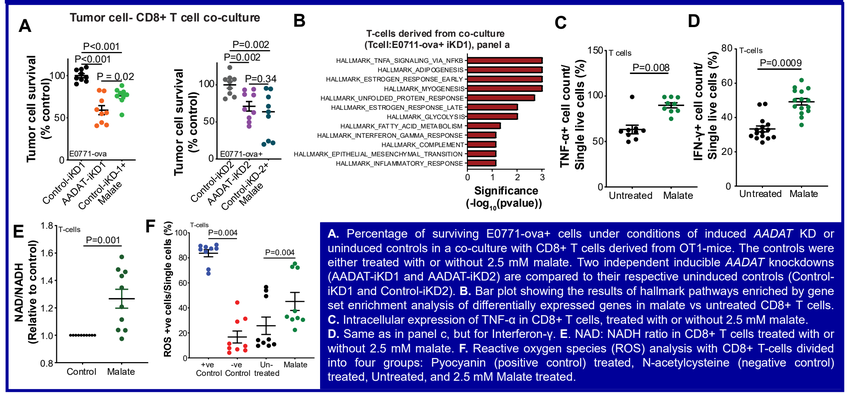
A. Analysis of clinical and transcriptomic data (GSE91061) from melanoma patients treated with immunotherapy, divided into groups with high (n = 27) or low (n = 82) expression of *AADAT*. **B.** Tumor growth analysis of E0771 cells expressing doxycycline-inducible Aadat-specific shRNA (iKD1) orthotopically implanted into syngeneic wild-type C57BL/6J mice. The mice were treated with one of four regimens: 1) vehicle + IgG (control), 2) doxycycline + IgG (genetic KD of *AADAT*), 3) vehicle + ICB (immune checkpoint blockade therapy), and 4) doxycycline + ICB (*AADAT* KD + immune checkpoint blockade therapy). **C.** Tumor growth analysis of 4T1 cells expressing doxycycline-inducible Aadat-specific shRNA, orthotopically implanted into syngeneic wild-type BALB/cJ mice. The mice were treated with either of the above mentioned (Panel B) four regimens.

AADAT maintains CoQ$_{10}$-supported redox homeostasis, malate buildup and export



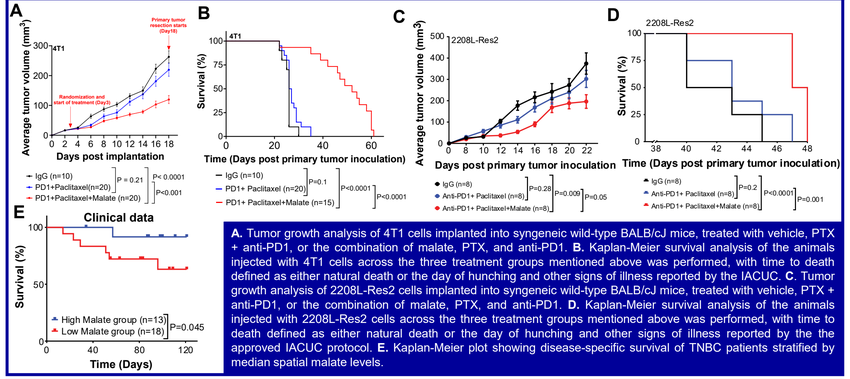
A. Heatmap displaying significantly altered metabolites from an unbiased metabolomics analysis of conditioned media (CM) from E0771-ova+. **B.** Z-score normalized levels of malate in the conditioned media of E0771-ova+ cells. **C.** Average pixel intensity of malate measured by IMS in each tissue core within the tissue microarray derived from patients stratified by median malate levels into high (n=13) and low (n=18) groups. Representative photomicrographs of a region of interest in a patient TNBC tumor show similar spatial clustering patterns for malate (top panel; resolution about 20 cells-IMS) and the CD8+ functional T cell cluster (bottom panel; single-cell resolution- IMC). **D.** The plot demonstrates a positive correlation between average levels of functional T-cell clusters and malate. **E.** Plot demonstrating a negative correlation between exhausted T cells and malate. **F.** Line plot illustrating the oxygen consumption rate (OCR) measured with the Mito-Stress test on a Seahorse Bioanalyzer. **G.** NAD: NADH ratio in E0771-ova+ cells. **H.** Bar plot showing significantly enriched pathways derived from the altered list of metabolites shown in panel a. **I.** Normalized intracellular (left panel) and conditioned media (right panel) malate levels in E0771-ova+ cells.

Malate Recapitulates AADAT KD Effects on T Cell Activation



A. Percentage of surviving E0771-ova+ cells under conditions of induced *AADAT* KD or uninduced controls in a co-culture with CD8+ T cells derived from OT1-mice. The controls were either treated with or without 2.5 mM malate. Two independent inducible *AADAT* knockdowns (AADAT-iKD1 and AADAT-iKD2) are compared to their respective uninduced controls (Control-iKD1 and Control-iKD2). **B.** Bar plot showing the results of hallmark pathways enriched by gene set enrichment analysis of differentially expressed genes in malate vs untreated CD8+ T cells. **C.** Intracellular expression of TNF-α in CD8+ T cells, treated with or without 2.5 mM malate. **D.** Same as in panel c, but for Interferon-γ. **E.** NAD: NADH ratio in CD8+ T cells treated with or without 2.5 mM malate. **F.** Reactive oxygen species (ROS) analysis with CD8+ T-cells divided into four groups: Pyocyanin (positive control) treated, N-acetylcysteine (negative control) treated, Untreated, and 2.5 mM Malate treated.

Malate supplementation Enhances the Efficacy of Standard-of-Care Immunochemotherapy in TNBC Pre-clinical Models and Improved Survival in TNBC Patients



A. Tumor growth analysis of 4T1 cells implanted into syngeneic wild-type BALB/cJ mice, treated with vehicle, PTX + anti-PD1, or the combination of malate, PTX, and anti-PD1. **B.** Kaplan-Meier survival analysis of the animals injected with 4T1 cells across the three treatment groups mentioned above was performed, with time to death defined as either natural death or the day of hunching and other signs of illness reported by the IACUC. **C.** Tumor growth analysis of 2208L-Res2 cells implanted into syngeneic wild-type BALB/cJ mice, treated with vehicle, PTX + anti-PD1, or the combination of malate, PTX, and anti-PD1. **D.** Kaplan-Meier survival analysis of the animals injected with 2208L-Res2 cells across the three treatment groups mentioned above was performed, with time to death defined as either natural death or the day of hunching and other signs of illness reported by the the approved IACUC protocol. **E.** Kaplan-Meier plot showing disease-specific survival of TNBC patients stratified by median spatial malate levels.

Conclusion

AADAT defines a metabolic-immune axis driving immune evasion and therapy resistance in triple-negative breast cancer. Blocking AADAT or administering oral malate reactivates CD8+ T-cell immunity and sensitizes tumors to chemo-immunotherapy. These findings uncover a readily translatable metabolic vulnerability with potential to improve outcomes for patients with aggressive breast cancer subtypes.

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