

1439 **TERMINALLY EXHAUSTED CD8⁺TILS PROMOTE AGGRESSIVE CANCER STEM CELLS WHILE CONCURRENTLY EVADING ANTI PD1 THERAPY**

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Background Tumor-infiltrated CD8⁺T cell (TIL) heterogeneity is serving as one of the major hurdles in successful PD1 therapy. According to recent reports, among two subpopulations of exhausted CD8⁺TILs, (progenitor-exhausted, CD8⁺T_{PEX}; terminally-exhausted, CD8⁺T_{TEX}), CD8⁺T_{TEX} do not respond to anti-PD1 therapy.¹⁻³ However, functional status of intra-tumoral-CD8⁺T_{TEX} remains elusive. Whether and how they participate in tumor advancement holds immense clinical importance. Given the prominence of Cancer Stem Cells (CSCs) in establishing metastatic cancer progression by evading therapies, we became interested to study CD8⁺T_{TEX} behaviour in terms of CSC regulation.

Methods CD8⁺T_{TEX} (Lin⁻PD1⁺TCF1⁻) and CSC (Lin⁻CD44⁺CD24⁻CSCs) frequency and their co-relation in regards to tumor advancement were analysed in human carcinomas (n=33; from 22 breast and 11 ovarian carcinoma patients). Furthermore, MACS-isolated CD8⁺T-cells from human-PBMC or murine-spleenocytes were repeatedly exposed to tumor-lysate and tumor-supernatant in presence of antigen-loaded DCs for 120h to obtain CD8⁺T_{TEX} (PD1⁺TIM3⁺TCF1⁻CXCR5⁺IFN γ ^{low}) in-vitro. These CD8⁺T_{TEX} were co-cultured with MCF7, MDAMB-231 and 4T1 cells respectively to study the influence of CD8⁺T_{TEX} on CSCs. RT-PCR, colony-formation assay, matrigel-invasion-assay, tumour-sphere assay and in-vivo tumorigenicity assay with Crl:NU-Foxn1nu athymic nude mice were utilized to characterise CD8⁺T_{TEX} influenced CSCs. ELISA, Western-blot, flow-cytometry, immune-staining, pharmacological inhibition or genetic knockdown by in-vitro and in-vivo si-RNA silencing were used to study mechanism behind CD8⁺T_{TEX}-CSC cell-interaction.

Results Screening of human primary tumors disclosed that CD8⁺T_{TEX} cells remain strongly enriched across cold (low-TIL frequency) advanced-carcinomas, compared to hot (high-TIL frequency) advanced-carcinomas (p<0.001). Additionally, CD8⁺T_{TEX} cells positively correlated with CSC frequency (r = 0.8809) throughout cold-advanced carcinomas; suggesting their interdependency on tumor advancement. Furthermore, in-vitro co-culture assay as well as in-vivo adoptive transfer of CD8⁺T_{TEX} resulted in increment of intra-tumoral CSC frequency. However, this upregulation was not brought down by anti-PD1 therapy (p<0.001). Additionally, CD8⁺T_{TEX}-influenced-CSCs exhibited increased tumorigenic and metastatic potential in athymic-nude mice. They showcased invasive and migratory phenotype with long invadopodia by overexpressing CXCR4, MMP7 and Cofilin (figure 1). These CSCs remained sustained by overexpressing OCT4, SOX2, KLF4 and NANOG. Involvement of LAMP3/NRP1-VEGFR2 axis in CD8⁺T_{TEX}-CSC crosstalk was also observed (figure 2).

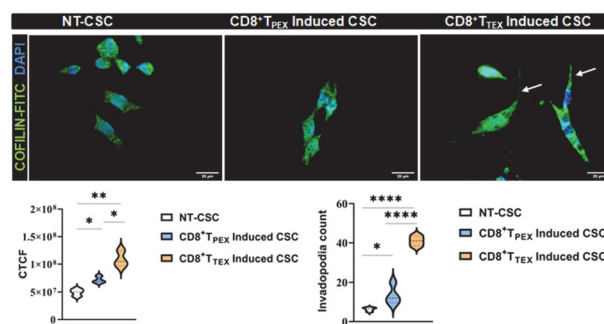
Conclusions Cumulative results counsel against indiscriminate use of anti-PD1 therapy. Rather a prior screening of CD8⁺TIL and CD8⁺T_{TEX} frequency in carcinoma patients would be beneficial. Additionally, LAMP3, NRP1 and VEGFR2 could be utilized as prospective therapeutic targets against CD8⁺T_{TEX}-influenced aggressive CSCs at advanced carcinoma patients with cold-tumor stroma.

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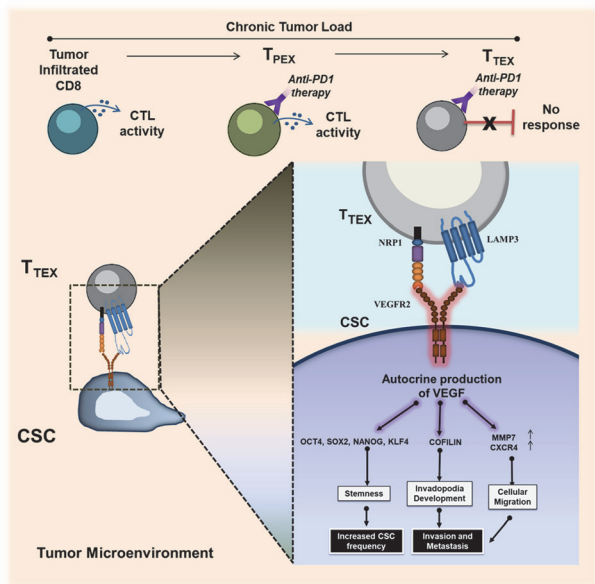
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Ethics Approval All human and animal experiments were approved by Institutional Human and Animal Ethical Committee of Chittaranjan National Cancer Institute, Kolkata, India. Approval numbers are CNCI-IEC-RB-2019-6, IAEC-1774/RB-15/2017/2 and IAEC-1774/RB-19/2017/15. All patients included gave informed consent before taking part in the study.



Abstract 1439 Figure 1 CD8⁺T_{TEX} induce invasive CSC generation Representative immune-fluorescence micrographs at 100x magnification for CSCs stained with Cofilin-FITC. Invadopodia are demarked by white arrows. Corrected total cell fluorescence intensity (CTFI) and number of invadopodia were quantified and displayed as violin graphs (mean±SD). Statistical alpha value was calculated from unpaired t-test (n=4).



Abstract 1439 Figure 2 Model proposing CD8+TTEX mediated aggressive CSC generation
 Due to chronic tumor load, CD8TILs undergo terminal exhaustion and generate CD8+TTEX cells which does not respond to anti-PD1 therapy. These CD8+TTEX cells however take part in tumor advancement by generating an invasive and aggressive CSC variant through LAMP3/ NRP1-VEGFR2 axis.

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