Adaptation of headless CAR T cells to tumor microenvironment induces anti-tumor activity against solid cancers.

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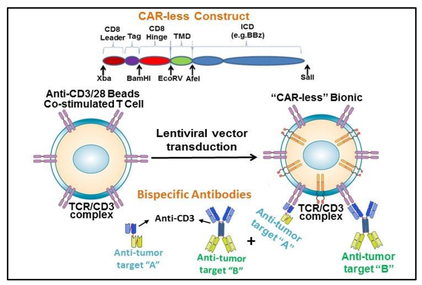
ABSTRACT

The success of chimeric antigen receptor (CAR) T cell therapy in treating several hematopoietic malignancies has been difficult to replicate in solid tumors, in part because of T cell exhaustion and eventual T cell dysfunction. The tumor microenvironment (TME), which includes hypoxia and high interstitial pressure, has a determinative effect on T cell function. T cell reprogramming to adapt to TME is likely to represent a novel and important aspect to enhance anti-tumor activity in the TME. AVATAR Odyssey incubators (Xcell Bio) were used to precisely control oxygen concentration and pressure to simulate TME conditions. Effector T cells including untransduced (UTD) activated T cells (ATC) or bispecific antibody armed UTD and unarmed headless CAR T cells (hCART) or bispecific antibody armed hCART (ahCART) were grown under normal environment (NE) or TME conditions before coculturing with target cells. The effect of NE or TME conditions were determined on T cell proliferation, phenotypic changes, and functional activity of UTD and hCARTs. Functional readouts were measured by flow cytometry based cytotoxicity assay, phenotype using flow cytometry and cytokine profiles by luminex assay under NE or TME-like conditions. The phenotypic changes of UTD and hCARTs showed no changes co-inhibitory receptors (LAG2, LAIR, PD1, TIGIT, CTLA-4, FOXP3, GATA3 and BTLA) on CD4 or CD8 T cells under NE and TME conditions. Interestingly, co-stimulatory receptor expression was enhanced (LIGHT, HVEM, NFATc1 and GITR) on CD8 T cells. Cytotoxicity by UTD and armed or unarmed hCARTs under TME showed enhanced cytotoxicity by ahCARTs at low effector to target (E:T) ratios of 2:1 or 1:1 against MCF-7, SKBR3 and PANC1 cell lines. Cytokine profile of ahCART showed differential cytokine pattern of GM-CSF, IFN-γ, and TNF-α under TME compared NE. These data suggest that manipulating the TME conditions during *ex vivo* T cell expansion could be a promising approach to improve its effectiveness, especially in the context of solid tumors. TME acclimation has clinical potential to enhance anti-tumor activity of cellular therapeutics.

APPROACH

Bispecific Antibody Armed Headless CART (hCART)



- The headless CAR T cells (hCART) were grown in the AVATAR Odyssey to recapitulate the TME conditions to enhance effector functions in the TME.
- Arming T cells with bispecific antibody re-direct T cells to tumor target. This strategy creates an artificial antibody receptor and converts every T cell into a specific cytotoxic T cells.
- Tumor engagement of bispecific antibody armed headless CAR T cells (ahCART) with tumor cells not only exhibit non-MHC-restricted cytotoxicity but also releases cytokines/ chemokines.

RESULTS

hCARTs: Growth Conditions, Phenotype and Functional Assay

Cell Growth Conditions

- Cells were thawed and stimulated with soluble anti-CD3 antibody (OKT3) in and 100 IU of IL-2 overnight and transduced in the following conditions
- Standard Incubator (21% O_2 and 0 psi, NE)
- TME1-15% O_2 and 5 psi
- TME2-10% O_2 and 5 psi
- TME3-5% O_2 and 5 psi
- Cells were expanded for 8 days in standard incubator or AVATAR Odyssey and phenotyped for CAR expression
- Cells were phenotyped for T cells memory subsets, GLUT-1 and CD69 expression

Functional Assay

- T cells were armed with OKT3/HER2 bispecific antibody prior to using in functional assay
- HER2-armed hCAR T cells used in cytotoxicity assays were performed under normal environment (NE) in the standard incubator or under TME in the AVATAR Odyssey (1% O_2, 2 psi) against HER2-expressing cell lines (LNCaP, PANC-1, SKBR3, and MCF7)
- T cells were titrated 2-fold starting at 8:1 effector to target (E:T) ratio
- Co-culture incubated for 72 hours and cytotoxicity was observed via flow cytometry

hCAR Expression, Proliferation and Phenotype of hCART Under NE or Hypoxic TME

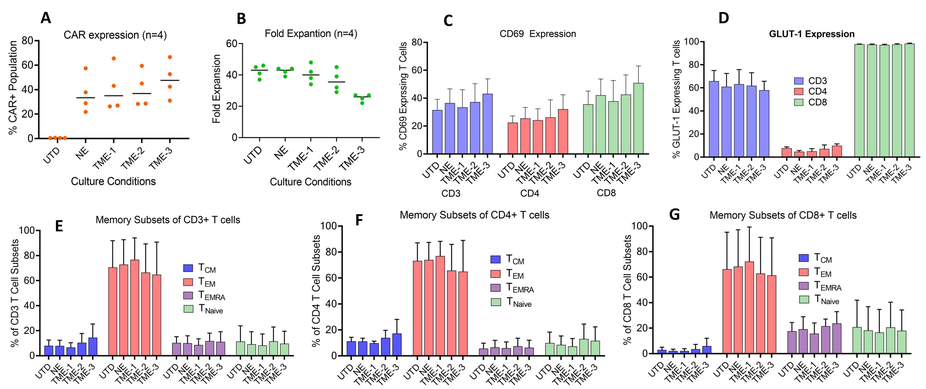


Figure 1. A. The median hCAR expression in T cells was 50% under TME-3 (5% O_2 and 5 psi) compared to NE or other hypoxic conditions ((TME-1,15% O_2 and 5 psi or TME-2, 10% O_2 and 5 psi). **B.** T cell fold expansion was gradually reduced from NE (42 fold), TME-1 (40 fold), TME-2 (38 fold) to TME-3 (26 fold). **C and D.** Show phenotype of CD3, CD4 and CD8 untransduced T cells (UTD), T cells transduced with hCAR grown under normoxic-NE (21% O_2 and 0 psi) or TME conditions on day 8 for the activation marker CD69 expression and marker for metabolic fitness GLUT-1 expression. **E-G.** The untransduced T cells (UTD), T cells transduced with hCAR grown under normoxic-NE (21% O_2 and 0 psi) or TME conditions were phenotyped on day 8 for central memory (T_{CM}), effector memory (T_{EM}), effector memory re-expressing RA (T_{EMRA}) and naïve T cells in CD3+, CD4+ and CD8+ T cells. The CD3+ and CD4+ T cells showed higher percentage of T_{CM} while CD8+ T cells showed higher percentage of T_{Naive} cells and T_{EMRA} T cells regardless of cells grown under NE or TME conditions.

Cytotoxicity by ahCARTs in the Tumor Microenvironment Measured by Flow Cytometry

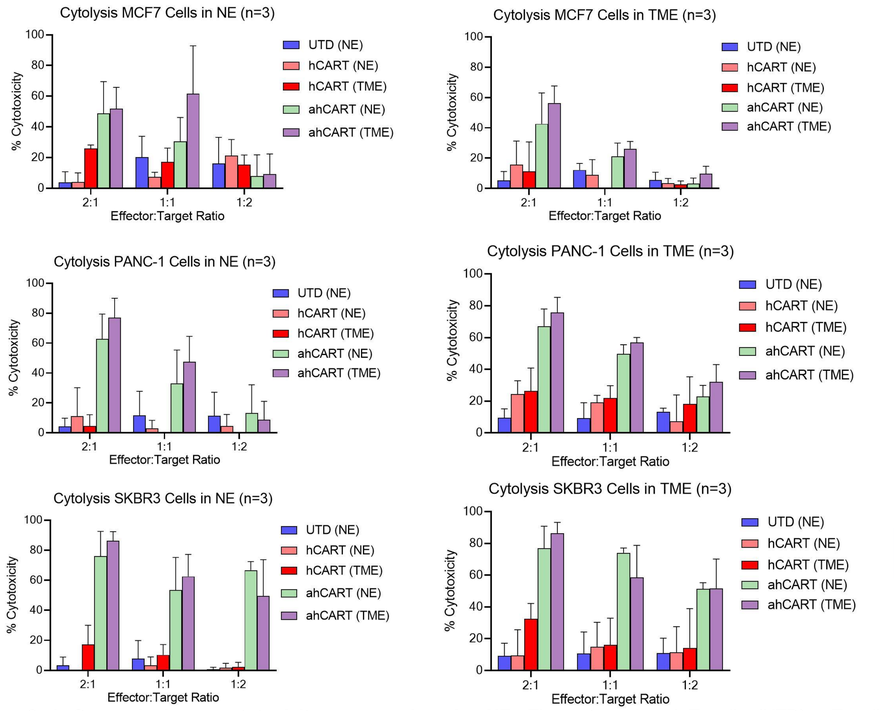


Figure 2. Left panel shows cytotoxicity measured under NE: The untransduced T cells (UTD), T cells transduced with hCAR grown under normoxic-NE (21% O_2 and 0 psi) or 5% O_2 and 5 psi to recapitulate the TME condition were either left unarmed untransduced T cells (UTD) or hCART and anti-CD3 x anti-HER2 bispecific antibody armed UTD (aUTD) or ahCART were tested for cytotoxicity against MCF-7, SKBR-3 (breast cancer), and PANC-1 (pancreatic cancer) cell lines at effector to target ratios at 2:1, 1:1 and 1:2. Cytotoxicity was measured by flow cytometry. **Right panel shows cytotoxicity measured under TME (1% O_2 and 2 psi):** T cells were either left unarmed (NE or TME) or armed with anti-CD3 x anti-HER2 bispecific antibody (ahCART). Armed hCARTs were denoted by NE* or TME* were tested for cytotoxicity at various effector to target ratios. The cytotoxicity by ahCARTs under TME showed enhanced cytotoxicity compared NE condition.

Costimulatory and Coinhibitory Subsets of CD4 and CD8 hCART$_{41BBz}$

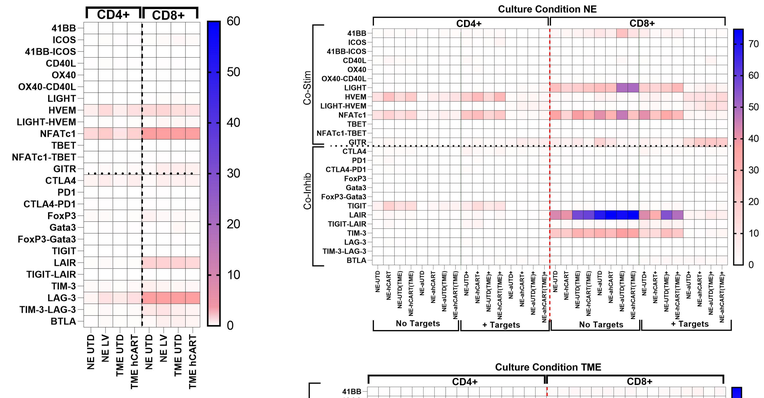


Figure 3. Left panel shows the qualitative analysis of exhaustion and activation markers in CD4+ and CD8+ T cell subsets at baseline (Day 0) using flow cytometry. **Right Upper** and **lower panels** show percentage of CD4+ or CD8+ T cells at day 13 either grown under NE or TME conditions and co-cultured with tumor target in standard incubator (**Right upper**) or AVATAR Odyssey (**Right lower).** The heat map show higher percentage of activation markers measured by flow cytometry. in CD8+ T cells co-culture under TME (1% O_2 and 2 psi).

Engagement of Armed hCART with Tumor Cells Induces Th_1 Cytokines/Chemokines

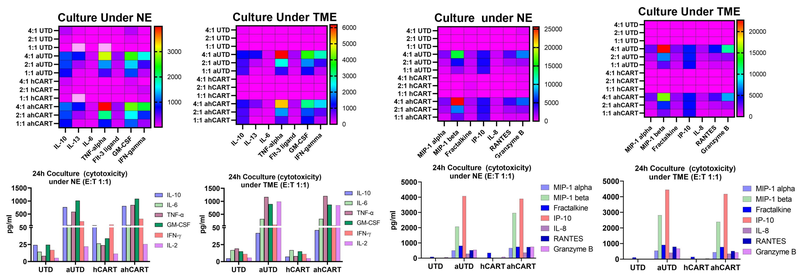


Figure 4. Cell-free supernatants were collected after 18 hours of co-cultures of MCF-7 cells with untransduced unarmed ATC, untransduced bispecific antibody armed ATC (BATs), hCARTs, or ahCARTs at E:T ratios of 4:1, 2:1 and 1:1 for cytokine/chemokine profiling. The heat maps show induction of inflammatory (IL-6), Th_1 (IFN-γ, TNF-α, GM-CSF,IL-2), Th_2 (IL-10) cytokines and chemokines (MIP-1α, MIP-1β, Fractalkine, IP-10, IL-8, RANTES) produced by T cells in co-cultures with MCF-7 cell line.

SUMMARY

- **hCAR Expression was higher (median 50%) in T cells under TME (5% O_2 and 5 psi) compared to NE condition**
- **CD8+ T cells showed higher percentage of T_{EMRA} and $T_{Naïve}$ cells when grown under NE or TME conditions**
- **Bispecific antibody armed hCARTs induced immune modulating and tumor killing cytokines and chemokines upon tumor engagement**
- **CD8+ T cells co-culture under TME (1% O_2 and 2 psi) showed higher percentage of activation markers measured by flow cytometry.**

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