# Adapting T cells for the Tumor Microenvironment (TME) During Manufacturing for Improved Anti-Tumor Potency

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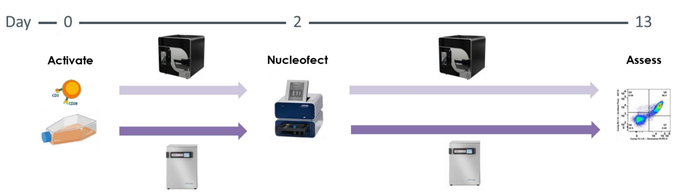
## Introduction

The microenvironment of solid tumors is very challenging for anti-tumor T cells. Low oxygen, low pH, inhibitory signals (e.g. cell surface receptors, metabolites, lipids), and high intratumoral pressure drive infiltrating T cells toward inhibition, exhaustion and death. Improved T cell fitness in this environment is critical to efficacious and durable T cell therapies. The goal of this work was to improve the fitness of therapeutic T cells in the tumor microenvironment by changing the conditions under which a T cell therapy is manufactured. To this end, we utilized a low-oxygen, high-pressure environment created by the Avatar incubator system and AmplifyBio's small-scale Non-viral Gene Editing (NVGE$^{TM}$) platform to generate TCR-T cells with a metabolic program that improves their fitness and potency in the harsh TME.

## Methods




| | |
|---|---|
| Tumor microenvironment | 1% $O_2$ / 5 psi (Avatar incubator) |
| Typical tissue | 5% $O_2$ / 5 psi (Avatar incubator) |
| Tissue culture | 18.6% $O_2$ / 0 psi (normal incubator) |

On day 0, isolated CD4 and CD8 T cells were activated using CD3/CD28 beads then placed in either the Avatar incubator at two different low oxygen, high pressure conditions or in a standard incubator system at normal tissue culture conditions. On day 2, cells were nucleofected with a TCR construct specific for a peptide in the HPV E7 protein then placed back in the same conditions for expansion with IL-7 and IL-15. On day 13, cells were harvested for phenotypic and functional testing.

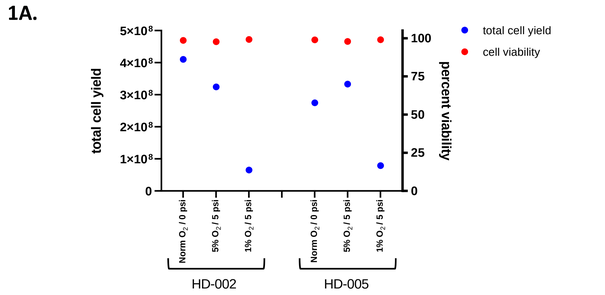
Note: psi is gauge pressure.

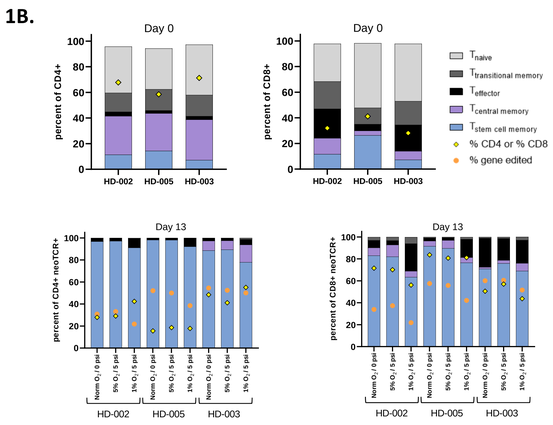
## Conclusions

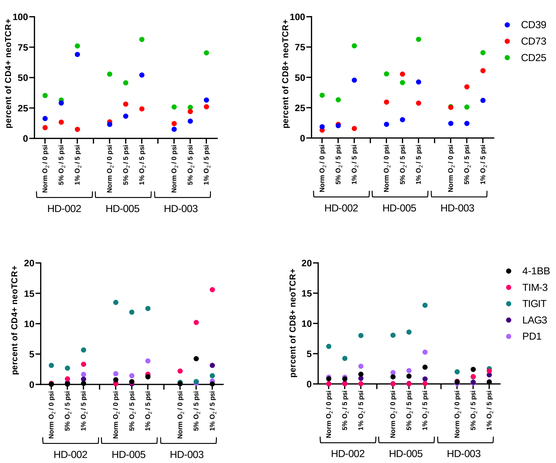
- TCR-T cells generated using AmplifyBio's small-scale NVGE$^{TM}$ platform in the Avatar at 5% $O_2$ / 5 psi (similar to a typical tissue environment) show growth, gene editing rates, memory subsets, and activation/exhaustion phenotypes similar to TCR-T cells expanded under standard tissue culture (normoxic) conditions.
- The TCR-T cells grown in a tissue environment exhibit changes in their transcriptome indicative of adaptation to lower oxygen levels, a shift toward more glycolytic metabolism while conserving their overall spare respiratory capacity, and consistently improved anti-tumor cell cytotoxicity at low effector-to-target ratios in a hypoxic environment relative to cells expanded under normoxic conditions.
- Growing TCR-T cells under extreme hypoxia (1% $O_2$ / 5 psi), as found in a tumor, was deleterious to all measures of phenotype and function.
- These results suggest there is a benefit in anti-tumor efficacy to manufacture TCR-T therapies under more physiological tissue oxygen and pressure conditions.

## Results

### *Figure 1. Cell Growth Characteristics, Memory Subsets, and Phenotype of TCR-T cells During Hypoxic Growth*



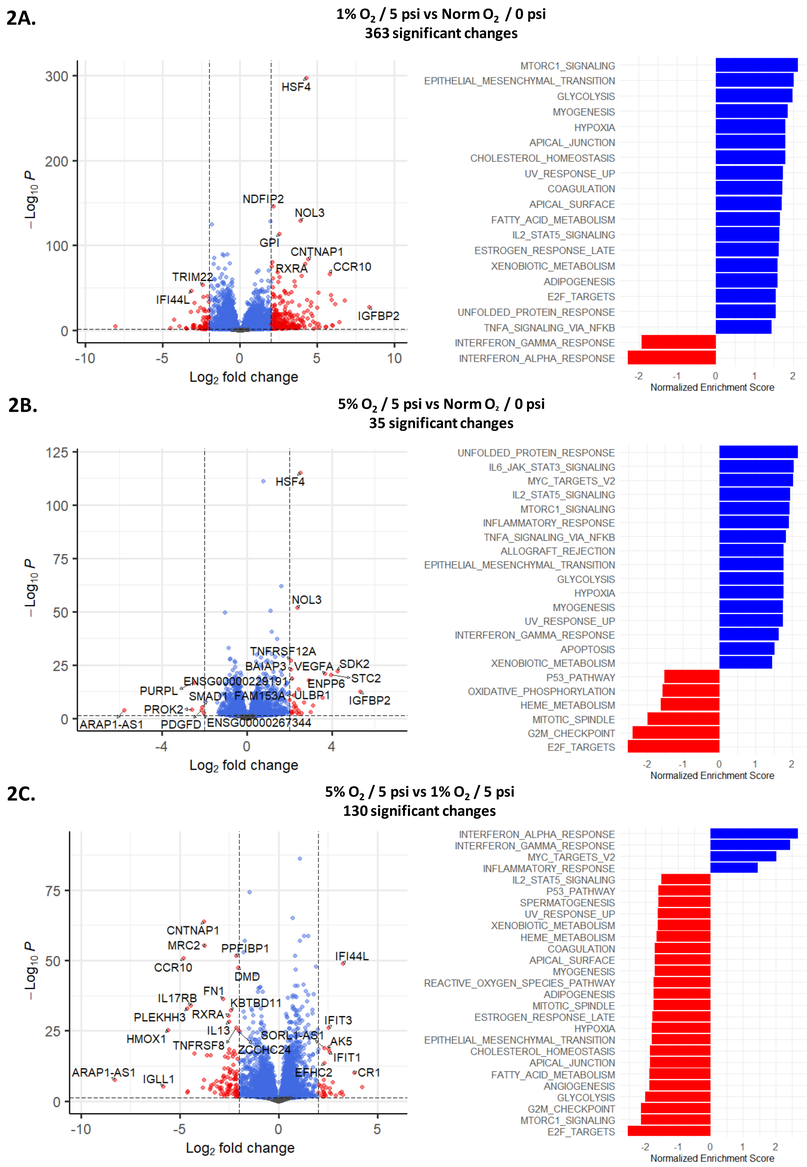




**1A.** Total cell yield and viability at the end of the process (day 14) for 2 different healthy donors demonstrates that cells grown at 5% $O_2$ / 5 psi have similar cell yield and viability compared to the norm $O_2$ / 0 psi condition while the 1% $O_2$ / 5 psi condition results in reduced cell yield. Cell count and viability determined on an NC-200 instrument.

**1B.** Flow cytometry-based assessment of T cell subsets in 3 different healthy donors demonstrates similar gene editing rates, CD4:CD8 ratio, and memory subsets for cells grown at norm $O_2$ / 0 psi and 5% $O_2$ / 5 psi while cells grown in the 1% $O_2$ / 5 psi condition have reduced gene editing, a lower fraction of CD8 T cells, and a higher proportion of $T_{eff}$ at the end of process (day 14).

**1C**. Flow cytometry-based assessment of surface receptors of T cell activation, exhaustion, and metabolism in neoTCR expressing CD4 and CD8 T cells from 3 different healthy donors shows increased CD39 and CD25 expression in the 1% $O_2$ / 5 psi condition relative to the other conditions.

### *Figure 2. Transcriptome Changes During Hypoxic Growth of TCR-T cells*

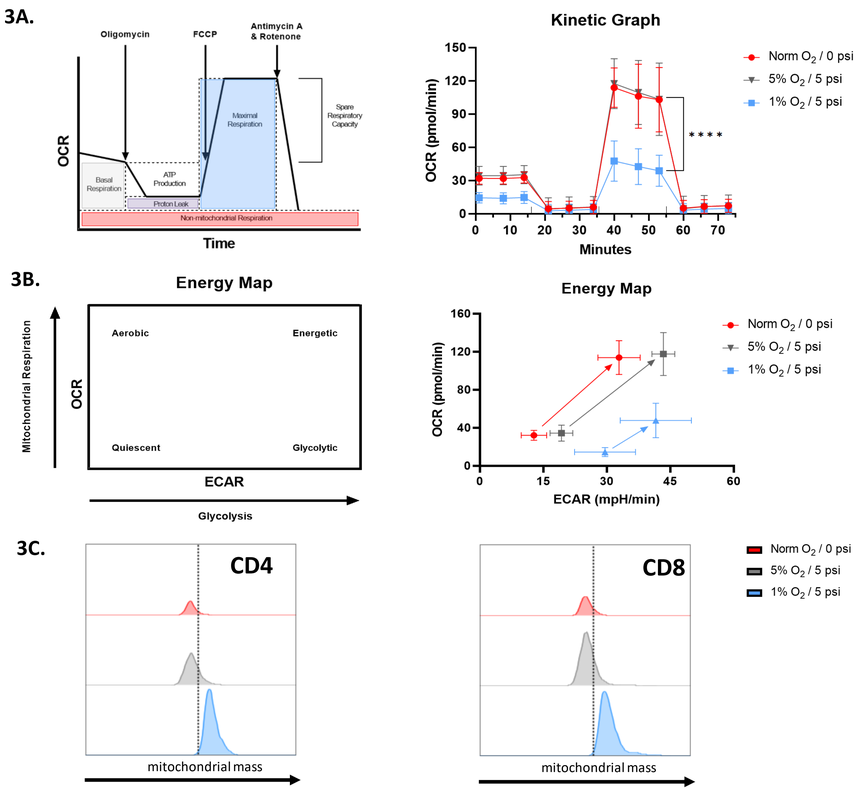



**2A.** Volcano plots of gene expression levels demonstrate the 1% $O_2$ / 5 psi condition has the largest number of significant changes in mRNA expression relative to the norm $O_2$ / 0 psi and 5% $O_2$ / 5 psi conditions including upregulation of known hypoxia-inducible genes like HSF4, IGFBFP, VEGFA and NOL3. Normalized Enrichment Scores of pathway gene expression indicate that TCR-T cells expanded in 1% $O_2$ / 5 psi exhibit increased mRNA expression in pathways related to hypoxia, glycolysis, and cell stress, and decreased expression of the IFN-γ pathway.

**2B.** The number of transcriptome differences between 5% $O_2$ / 5 psi and norm $O_2$ / 0 psi conditions was 35 total genes, suggesting that this condition does not perturb the T cells as much as the full hypoxia condition. Nevertheless, many of the same pathways were upregulated as in the 1% $O_2$ / 5 psi condition. However, significant upregulated pathways included anti-tumor and inflammatory pathways such as IL-2, IFN-γ, and TNF-α, suggesting improved function.

**2C.** Comparison of the TCR-T cells expanded in either 5% $O_2$ / 5 psi or 1% $O_2$ / 5 psi confirms the upregulation of inflammatory and IFN-γ pathways in the 5% $O_2$ / 5 psi condition, and down-regulation of glycolysis in the 1% $O_2$ / 5 psi condition. RNA-seq was used to assess the effect of the different conditions on the transcriptomes of the TCR-T cells (2 donors) at day 14. Differential expression analysis was performed using DESeq2, with a $|\log_2(\text{foldchange})| > 2$ considered significant. Using Gene Set Enrichment Analysis (GSEA), Hallmark pathways from the Human MSigDB Collection were analyzed for up- or down-regulation at day 14, with an adjusted p value < 0.01 defining significance.

### *Figure 3. Metabolic Profiling of TCR-T Cells Generated Under Hypoxic Conditions*

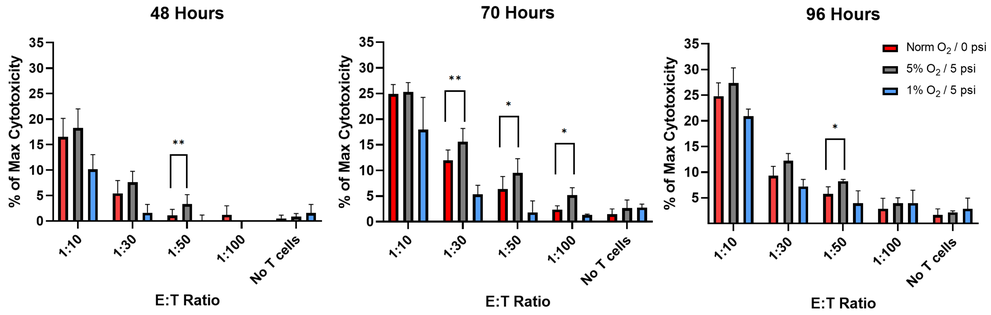



**3A.** Diagram of kinetic oxygen consumption rate (OCR) measured in the Seahorse assay (left panel). TCR-T cells from 2 healthy donors demonstrate similar OCR for cells grown at norm $O_2$ / 0 psi and 5% $O_2$ / 5 psi while cells grown in the 1% $O_2$ / 5 psi condition have a reduced OCR and significantly lower spare respiratory capacity (SPR, right panel).

**3B.** Diagram of Energy Map depicting T cell metabolic phenotype (left panel) prior to editing and at the end of the manufacturing process (directionality shown by arrow). TCR-T cells grown at 5% $O_2$ / 5 psi (from 2 healthy donors) exhibit a right shifted metabolic profile demonstrating more adaptation to lower oxygen environments but with a similar overall oxygen consumptive rate as the cells grown at norm $O_2$ / 0 psi. Cells grown in the 1% $O_2$ / 5 psi condition have a more glycolytic metabolic phenotype and a much lower oxygen consumption rate suggesting less energetic potential (right panel).

**3C.** Flow cytometry-based acridine orange staining of mitochondrial membranes after 72 hr coculture of TCR-T cells with CaSki tumor cells show that the TCR-T cells grown in the 1% $O_2$ / 5 psi condition have a greater increase in mitochondrial mass relative to the other culture conditions.

### *Figure 4. Tumor Cell Killing Under TME Conditions with TCR-T Cells Grown in Hypoxic Conditions*




TCR-T cell cytotoxicity against CaSki tumor cells tested under TME conditions (1% $O_2$ / 2 psi) shows that TCR-T cells cultured at 5% $O_2$ / 5 psi demonstrate consistently higher cytotoxicity at multiple E:T ratios relative to norm $O_2$ / 0 psi and 1% $O_2$ / 5 psi conditions. Data shown are the average plus standard deviation of 3 separate donors. Significance assessed by a paired Student's *t* test at each E:T ratio. Cytotoxicity of the CaSki cells assessed by release of lactate dehydrogenase (LDH) in a plate-based format.