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Culturing CAR-T cells under hypoxic and hyperbaric conditions yields increased proliferation and functional potency without changes in phenotype.

Evan Massi, Candy Garcia, Hadia Lemar, Yelena Bronevetsky, Shannon Eaker, James Lim
Xcellbio, San Francisco, California

Abstract: Environmental conditions affects CAR-T potency

Limited research has delved into the impact of increased atmospheric pressure on cell culture dynamics. Certain studies hint at amplified bovine endothelial cell numbers under increased ambient pressure up to 2.3 PSI, as well as CHO cells for protein production. However, the effects appear inconsistent, either fostering or inhibiting cellular proliferation based on cell type. Here we present a novel approach: culturing human primary T cells at an elevated pressure of 5 PSI and reduced oxygen levels in the AVATAR Foundry and Odyssey incubator systems, which precisely controls oxygen, carbon dioxide, and hyperbaric pressure levels. Our findings demonstrate a remarkable improvement in T cell proliferation with minimal alterations observed in cell phenotype, outperforming conventional incubation methods regardless of the culturing vessel – be it multi-well plates, flasks, or gas-permeable bags. Certain low oxygen culture conditions also yielded increased cytotoxic potency of CAR-T cells when compared to cultures grown in a standard CO2 incubator as assessed by CD19 CAR-T/NALM6 co-culture cytotoxicity assays. Specifically, these cells were able to maintain cytotoxic persistence with repeated rounds of tumor challenges without exhaustion. Moreover, CAR-T cells cultured in these low oxygen conditions exhibit increased expression of GLUT1, which has been shown to correlate with increased anti-tumor efficacy, reduced exhaustion and prolonged survival of CAR-T cells. The results we present not only showcase an improved overall yield in cell numbers but also highlight the maintained cellular function and the absence of significant phenotype changes under hypoxic and pressurized conditions.

This groundbreaking observation bears substantial implications in the domain of cell and gene therapy manufacturing, where higher yield and increased potency in a condensed timeframe is vital. Our study adds a compelling dimension on the role of hypoxia and hyperbaric pressure in cell culture, opening new avenues for enhancing productivity without compromising cellular integrity.

Methods & Experimental Design

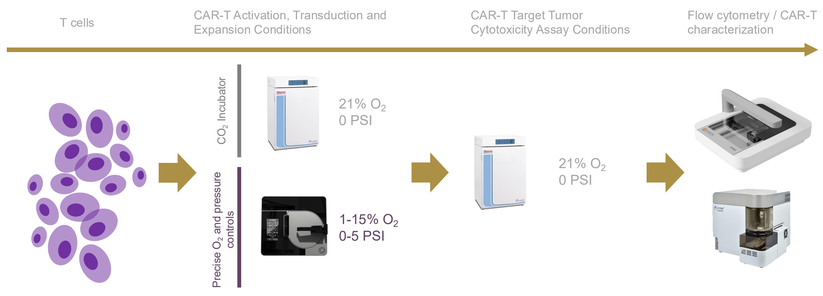
Frozen untouched CD3+ T cells from a healthy donor were thawed and cultured in RPMI containing 10% HI-FBS and in the presence of human T-activator CD3/28 Dynabeads + rhIL2 and cultured under 3 environmental conditions; 1) Normal CO_2 incubator (NI), 2) AVATAR (AVT) 15% O_2 + 5 PSI, and 3) AVATAR (AVT)10% O_2 + 5 PSI. The following day, the cells were transduced with a 3rd generation CD19-CAR lentivirus or STEAP1-CAR lentivirus, returned to their respective incubators, expanded, and cryopreserved.

Cell expansion and viability was measured every 2-3 days via cell counter. **CAR expression and phenotype** was measured by flow cytometry. **Functional activity** was measured with two types of cytotoxicity assays using NALM6-mCHERRY-Luc-Puro target cells:

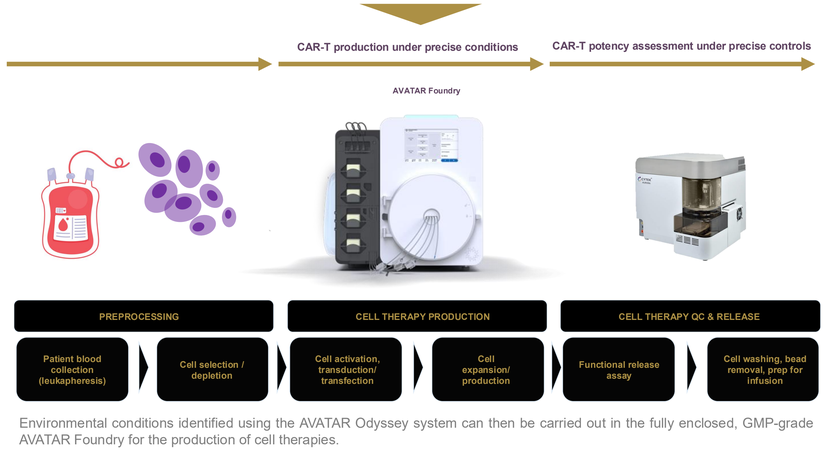
Extended challenge assay: prolonged co-culture of CAR-positive effector and target cells at varying ratios. CAR-specific cytotoxicity is measured via flow cytometry *or* the Axion OMNI live imaging system relative to untransduced (UTD) and NALM6-only control wells and fluorescent counting beads. Assays were run in a normal incubator.

Serial challenge assay: Co-cultures are set up as above. Samples are pulled and measured via flow cytometry and fresh NALM6 cells are reintroduced to co-cultures every 48-72h. Each reintroduction of NALM6 target cells is considered a new "challenge". Effector cells are serially challenged until loss of efficacy and/or exhaustion is observed. Data is visualized by outgrowth of NALM6 cells over time.

AVATAR Odyssey & AVATAR Foundry: Preclinical and GMP cell therapy manufacturing platform

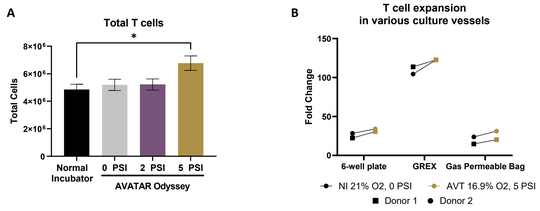


Preclinical discovery: The precisely controlling O_2 levels and pressure within the AVATAR Odyssey system enables the exploration of optimal environmental conditions for enhancing the potency and yield of cell therapies. Additionally, it facilitates testing under conditions that closely mimic the physiological environment, including the tumor microenvironment (TME).



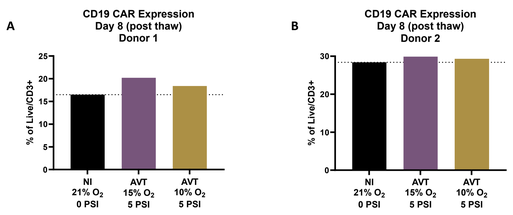
Environmental conditions identified using the AVATAR Odyssey system can then be carried out in the fully enclosed, GMP-grade AVATAR Foundry for the production of cell therapies.

CD19 CAR-T cells expanded in hyperbaric pressure results in increased yield



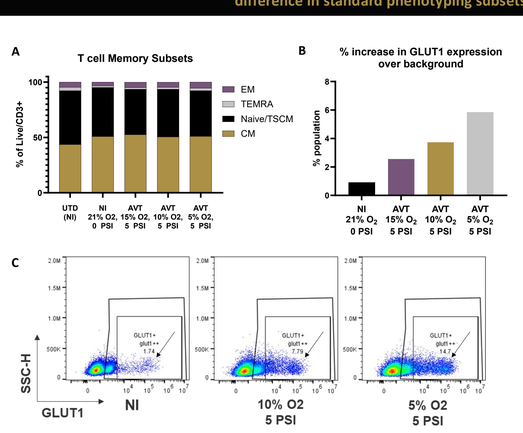
Cell growth of T cells in a normal incubator (NI) versus the AVATAR Odyssey (AVT). A. Healthy donor T cells were expanded in either a normal incubator (NI) or the AVATAR Odyssey at various hyperbaric conditions. Average+SEM of 12 healthy donors. B. T cells were expanded in various cell culture vessels either in a normal incubator or under hyperbaric pressure (5 PSI) for 7 days. Average yields were higher when grown under AVT conditions.

Transduction efficiency of T cells is enhanced or comparable to a normal incubator when cultured in low oxygen and hyperbaric conditions



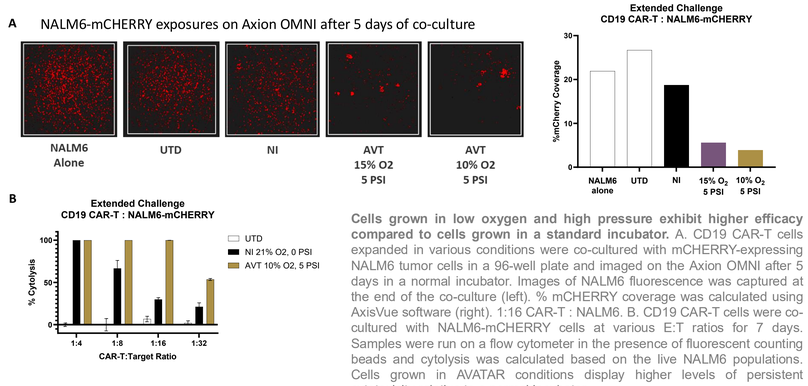
CD19 CAR transduction efficiency of healthy T cell donors. Cells were transduced on day 1 post-activation with a CD19-CAR lentivirus at an MOI of 3. CAR expression was measure by flow cytometry after 8 days cultured in a gas permeable bag in either a normal incubator (NI) or an AVATAR Odyssey (AVT). A. Donor 1. B. Donor 2. Average transduction efficiency was comparable or higher under AVT conditions.

T cells cultured in low oxygen and hyperbaric pressure show increased GLUT1 expression, with minimal difference in standard phenotyping subsets



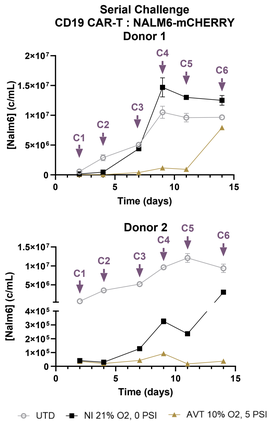
Few changes in phenotype with increasing pressure and shifts in GLUT1 expression with decreasing oxygen. A. High frequencies of CCR7+CD45RA- central memory T cells, and CCR7+CD45RA+ naïve or stem cell memory-like T cells for this donor. No significant difference between conditions observed. B. T cells transduced with STEAP1 CAR lentivirus were cultured for 8 days in various low oxygen conditions. A subset of high-expressing GLUT1+ cells were observed when oxygen was decreased. C. A separate donor was transduced with STEAP1 CAR lentivirus and expanded for 8 days in a cell culture bag. Subpopulation of high-expressing GLUT1+ cells increases with decreasing oxygen levels. High-expressing GLUT1+ cells = "glut1++". Representative dot plots of differing GLUT1-expressing cells.

CD19 CAR-T cells expanded in low oxygen and hyperbaric pressure exhibit superior potency during an extended tumor challenge assay



Cells grown in low oxygen and high pressure exhibit higher efficacy compared to cells grown in a standard incubator. A. CD19 CAR-T cells expanded in various conditions were co-cultured with mCHERRY-expressing NALM6 tumor cells in a 96-well plate and imaged on the Axion OMNI after 5 days in a normal incubator. Images of NALM6 fluorescence was captured at the end of the co-culture (left). % mCHERRY coverage was calculated using AxisVue software (right). 1:16 CAR-T : NALM6. B. CD19 CAR-T cells were co-cultured with NALM6-mCHERRY cells at various E:T ratios for 7 days. Samples were run on a flow cytometer in the presence of fluorescent counting beads and cytolysis was calculated based on the live NALM6 populations. Cells grown in AVATAR conditions display higher levels of persistent cytotoxicity relative to a normal incubator.

CD19 CAR-T cells expanded in low oxygen and hyperbaric pressure exhibit persistent killing activity during a serial tumor challenge assay

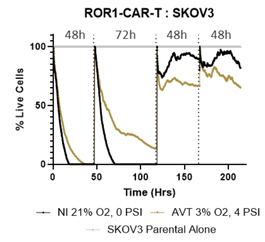


Serial Challenge Assay Flow Diagram

Combine CAR-T cells and NALM6 target cells at varying E:T ratios. → 48-72h → Pull sample to measure by flow cytometry. Rechallenge co-culture wells with fresh NALM6 cells. → 48-72h → Repeat previous step with fresh NALM6 cells until exhaustion is observed.

Low oxygen, high pressure cultures yielded CAR-T cells with increased persistence upon serial challenging with tumor target lines. Donors 1 and 2 were activated for 1 day with a soluble CD3/CD28 activation cocktail and transduced with CD19-CAR lentivirus. Cells were then expanded for 7 days and cryopreserved. CAR-T effector cells were co-cultured with NALM6-mCHERRY target cells at a ratio of 1:2 in a 48-well plate in a normal incubator. After 48h, samples were pulled and run on a flow cytometer in the presence of fluorescent counting beads to determine the concentration of live NALM6 cells in culture. Fresh NALM6 cells and media were added back to each well and co-cultures were incubated for another 72h before pulling another sample and repeating the process. Effector cells were serially challenged 6 times. Cultures expanded under lower oxygen and higher pressure were able to maintain much lower concentrations of NALM6 cells even after repeated challenges compared to cells grown under normal incubator conditions. Each data point represents a separate challenge. Average+SD of duplicates. C1-C6 = Challenge 1 – Challenge 6.

Next steps: *in vivo* and solid tumor modeling, dialing in optimal culture conditions, cytokine analysis, RNA-seq



The ability to acclimate T cell therapies using the AVATAR Odyssey and Foundry systems in the environment they will encounter within a solid tumor is proven to be beneficial. Culturing tumor cells and then performing the functional analysis of the T cell product in a more physiologically relevant environment may lead to more predictive outcomes in animal models and potentially the clinic. A metabolic shift occurs in these acclimated T cells to help them to survive in lower [O_2]. In this experiment, T cells were transduced with a CAR targeting an ovarian cancer marker (ROR1) and expanded in 3% O_2 and 4 PSI. The prostate cancer cell line was acclimated to tumor microenvironment settings of 1% O_2 and 2 PSI. For the cytotoxicity assay, the CAR-T cells were challenged with tumor cells in TME settings and rechallenged with more tumor cells for every 48-72h. T cells grown in the lower [O_2] were more potent at inhibiting tumor growth in later challenges, suggesting exposure to low oxygen could condition them to the harsher conditions of the solid tumor milieu.

Although this data is promising for the future of cell therapy development, there is plenty of work to be done to elucidate benefits seen with hypoxic and hyperbaric conditioning in the AVATAR systems.

- Identifying optimal culture conditions for CAR-T cell or tumor infiltrating lymphocyte (TIL) therapies will be paramount to achieving robust and effective treatments. This includes physiologically relevant culture conditions that are more akin to the native environment of these cells.
- With phenotype lacking as a consistent indicator of function, cytokine analysis of the culture may be a practical alternative. Furthermore, deeper analysis of both intracellular and extracellular markers may allow for predictive efficacy.
- Single cell RNA-seq can potentially provide insights into the functional differences observed between cell therapies grown in a normal incubator compared to more persistent cultures grown under hypoxic and hyperbaric conditions of the AVATAR system.
- Mouse studies utilizing CAR-Ts cultured in low oxygen and hyperbaric conditions are ongoing and showing promising results regarding tumor growth inhibition compared to CAR-Ts cultured in a conventional incubator.

Summary & Conclusions

- Culturing T cells in the AVATAR system at low oxygen and hyperbaric conditions had moderate improvements in proliferation. Specifically, increased pressure leads to increased expansion.
- Transduction efficiency was either improved or unaffected by low oxygen, high pressure culturing conditions. This is largely donor-dependent.
- Little-to-no changes observed in phenotype for the most common T cell subset surface markers.
- CAR-T cells cultured in low oxygen conditions yielded a subset of cells expressing high levels of GLUT1 beyond what is typically seen during normal activation and expansion. It has been reported that overexpression of GLUT1 can lead to increased anti-tumor efficacy and metabolic fitness (Shi 2024).
- CAR-T cells cultured in physiologically relevant conditions led to increased potency and cytolytic persistence when assayed for both an extended time frame and when serially challenged with tumor target cells.

Scan the QR Code for a copy of this poster, and access to materials and methods section. Visit us at Xcellbio.com to find out how you can enhance your cell therapy potency in the solid tumor microenvironment.