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Physiological manufacturing conditions enhance *in vivo* efficacy of CD19 CAR-T cells in mice

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Abstract: Environmental conditions in the manufacturing process affects CAR-T potency

Since the first chimeric antigen receptor T-cell therapy was approved in 2017, significant progress has been made in manufacturing to improve patient access to these life-saving therapies. For CD19 CAR-T cells, complete response rates of 40-54% have been reported in patients with relapsed or refractory aggressive B cell lymphomas, a staggering result considering the historically poor outcomes associated with this disease. However, roughly half the treated patients did not exhibit the same profound response, owing to the complexity associated with the production of potent autologous cell therapies.

In this study, we examined whether manufacturing conditions can impact the *in vivo* efficacy of CAR-T cells in tumor-bearing mice. Utilizing the AVATAR manufacturing platform from Xcellbio, we expanded healthy donor-derived CD19 CAR-T cells under physiological conditions mimicking the human vasculature system. We achieved this by tightly regulating oxygen and hyperbaric pressure levels during the expansion process. We then compared tumor-bearing mice treated with AVATAR expanded cells against conventionally manufactured CD19 CAR-T cells derived from a standard CO_2 incubator (21% O_2 + 0 PSI). CD19 expressing NALM6-mCh-Luc-Puro tumor cells were implanted intravenously in 48 mice across 6 groups (female NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ (NSG)), and tumor burden was measured via bioluminescence over 29 days post-implant. We report that CD19 CAR-T cells manufactured under conditions mimicking the vascular environment (15% O_2 + 5 PSI) exhibited superior tumor control compared to cells expanded in a conventional CO_2 incubator (1.8e8 vs 6.4e6 photons/second, mean BLI signal on day 29). Furthermore, we observed significant increases in plasma IFNγ levels in animals treated with AVATAR expanded cells (mean 111 pg/mL vs 32 pg/mL; day 19 post dose) indicating potent anti-tumor activity compared to animals treated with conventionally manufactured CD19 CAR-T cells. Blood analysis revealed similar trends, with AVATAR expanded CD19 CAR-T+ cells present in higher numbers (21 cells/μL of blood vs 7 cells/μL; mean day 28 post dose). Also noteworthy was the increased trafficking of AVATAR-manufactured CD19 CAR-T cells to the spleen and bone marrow compared to conventionally expanded CAR-T cells. In summary, physiological culture conditions mimicking the oxygen and pressure levels of the vasculature system significantly enhanced the potency of CD19 CAR-T cells in tumor-bearing mice as measured by decreased tumor burden, higher effector cytokine production, and increased trafficking of CAR-T cells to the lymphoid organs. Manufacturing therapeutic cells in the AVATAR system has the promise to boost the efficacy of CD19 CAR-T therapy and may enable solid tumor-targeting cell therapy, which has long suffered from poor potency

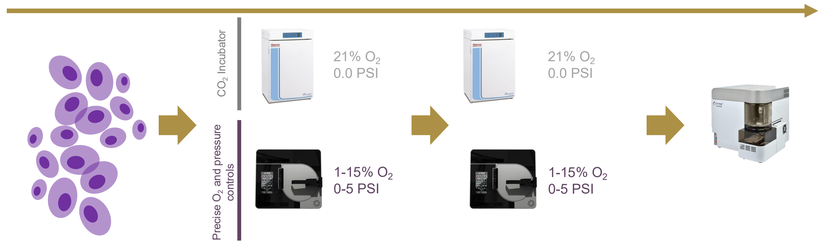
Methods & Experimental design

In vitro: Frozen untouched CD3+ T cells from a healthy donor were thawed in the presence of Human T-activator CD3/28 Dynabeads + rhIL2 and cultured under 3 environmental conditions; 1) Normal CO_2 incubator (NI), 2) 15% O_2 + 5 PSI (15_5), and 3) 10% O_2 + 5 PSI (10_5). The following day, the cells were transduced with a 3rd generation CD19-CAR lentivirus, returned to their respective incubators, expanded, and cryopreserved. CD19-CAR expression was observed by flow cytometry and cytotoxic activity was measured in a killing assay with NALM6-mCh-Luc-Puro target cells.

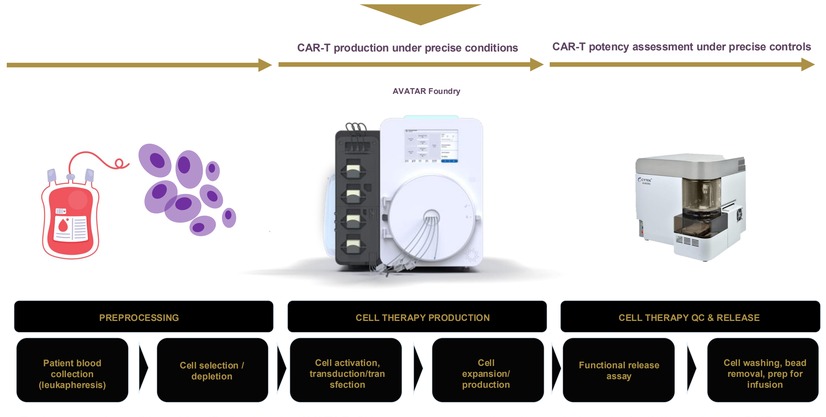
In vivo: Female NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ (NSG) mice were inoculated with 0.5e6 NALM6-mCh-Luc-Puro cells IV. Three days post-inoculation, T cells were administered at 2.5e6 CD19-CAR+ T cells per animal. BLI tumor measurements and blood samples were taken weekly up to 30 days post tumor inoculation. Blood was examined for circulating T cells by flow cytometry using **Labcorp's Custom Expanded Persistence T Memory Panel**. ddPCR, MSD, and flow cytometry methods were used to assess T cell health and function throughout the study. Animal care and use was performed in accordance with applicable animal welfare regulations at an AAALAC International accredited animal program.

****T cell therapy manufacturing was performed at Xcellbio in the AVATAR Odyssey. *In vivo* study was performed at Labcorp**

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

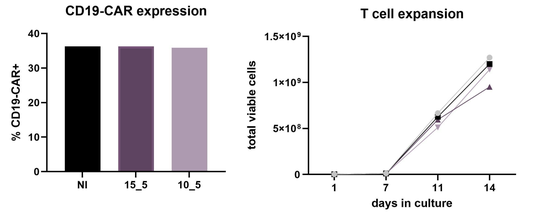


Preclinical discovery: The precise controlling O_2 levels and pressure within the AVATAR Odyssey system enables the exploration of optimal environmental conditions for enhancing the potency and yield of cell therapies. Additionally, it facilitates testing under conditions that closely mimic the physiological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



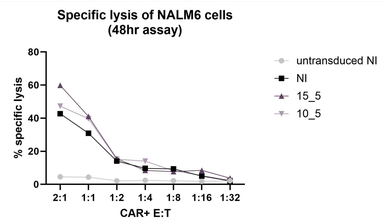
Environmental conditions identified using the AVATAR Odyssey system can then be carried out in the fully enclosed, GMP-grade AVATAR Foundry for the production of cell therapies.

CD19 CAR-T cells grown in decreased oxygen and increased pressure has no significant effect on expression or expansion



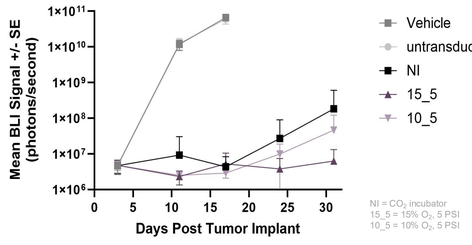
Expression and cell growth of CD19 CAR-T cells. Similar CAR expression was observed across all culture conditions. Overall growth of the T cells was not significantly impacted in lower O_2 and pressurized environmental settings

CD19 CAR-T cells grown under AVATAR conditions show enhanced cytotoxic activity compared to cells grown in a conventional CO_2 incubator *in vitro*



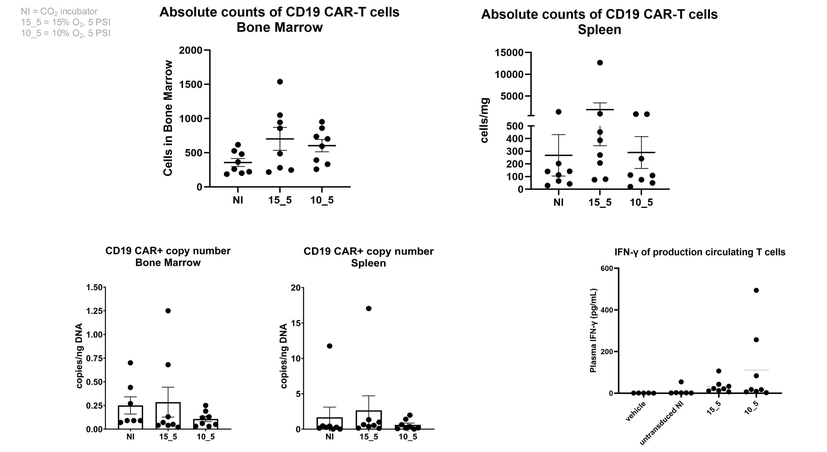
Cytotoxicity assay with NALM6 target cells. T cells were co-cultured with NALM6 cells for 48hr. Specific lysis was measured by flow cytometry. CD19 CAR specific killing was observed in a dose-dependent manner. T cells grown under pressure and decreased O_2 had increased potency over cells grown in a standard incubator

In vivo challenge of CD19 CAR-T cells cultured in the AVATAR system exhibit improved tumor control compared to cells grown in a conventional CO_2 incubator



In vivo evaluation of CD19 CAR-T cells. BLI measurements were taken weekly post-CAR-T treatment. CD19 CAR specific tumor growth inhibition was greater in cells cultured in a pressurized AVATAR system compared to standard incubation. Culturing the cells in a more physiologically relevant environment improved efficacy. 15_5 > 10_5 > NI

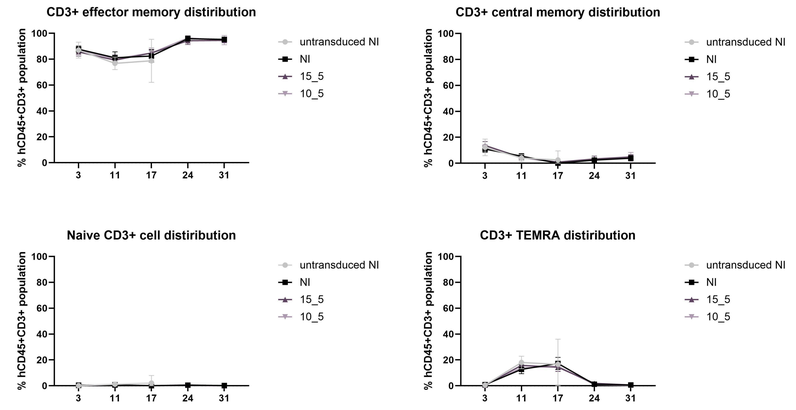
Analysis of circulating CD19 CAR-T cells *in vivo*: single timepoint & endpoint analysis



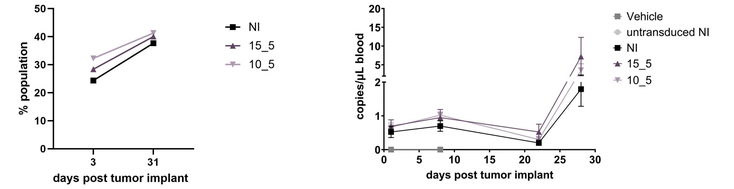
Endpoint analysis showed an increase of CD19 CAR+ T cells trafficking to the bone marrow and spleen. Cells grown in both 15_5 and 10_5 AVATAR conditions had greater migration compared to cells grown in a standard incubator. CAR+ cells were observed by ddPCR. However, these data were not as robust as the total cells found within the tissues.

IFNγ production at day 19: T cells grown at 15_5 or 10_5 in the AVATAR had increased levels of serum IFNγ compared to cells grown in a standard incubator.

Analysis of circulating T cells *in vivo*: changes over time

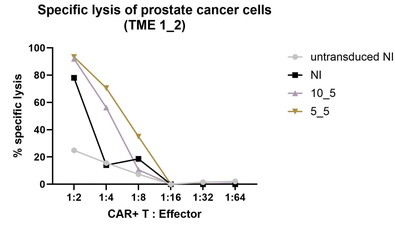


The T cells maintained their phenotype (flow cytometry) through the duration of the study. This particular T cell donor was mostly comprised of effector memory (EM) cells post-*in vitro* expansion No naïve cells were observed, nor did the cells appear terminally differentiated (TEMRA) or exhausted (PD1+/LAG3+TEMRA cells).



Increased CD19 CAR+ cells over time was readily observed in CD8+ T cells (flow cytometry) or total T cells (ddPCR).

Next steps: solid tumor targeting



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 in 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 a prostate cancer marker and expanded in 10 or 5% O_2 and 5 PSI. The prostate cancer cell line was acclimated to tumor microenvironment settings of 1% O_2 and 2 PSI (1_2). For the cytotoxicity assay, the CAR+ T cells were challenged with tumor cells in TME settings for 4 days and rechallenged with more tumor cells for an additional 4 days. T cells grown in the lower [O_2] were more potent at inhibiting tumor growth in both the first challenge and rechallenge compared to T cells grown in the NI, suggesting that acclimation is key to increased efficacy at low E:T ratios.

Summary & conclusions

- Culturing T cells in the AVATAR system at lower [O_2] had no effect on expansion or CAR expression.
- Acclimating T cells to physiologically relevant environmental conditions enhanced potency.
- There was a correlation between *in vitro* and *in vivo* efficacy. This has been observed in several donors with this model. Experiments are planned to determine if this correlation repeats in solid tumor models *in vivo*.
- No significant changes in the phenotype of the T cells at the beginning and end of the *in vivo* study. No exhaustion was observed.
- Increased CAR+ expression (in the periphery by flow cytometry, ddPCR, or absolute counts within tissue) correlated with tumor growth inhibition: 15_5 > 10_5 > NI.
- Our current findings in targeting solid tumors *in vitro* demonstrate the importance of acclimating and functionally testing T cell therapy products in a physiologically relevant environment.

Scan the QR code to learn more about the AVATAR Odyssey and AVATAR Foundry platforms and visit us at xcellbio.com