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Application Note

CD19 CAR T manufactured under TME culture conditions exhibit superior potency & persistence

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AVATAR Odyssey | RUO Cell Therapy Manufacturing Platform

AVATAR Foundry | GMP Cell Therapy Manufacturing Platform

Introduction

Adoptive cell therapy with T cells, including those reprogrammed to express chimeric antigen receptors (CAR T cells), has been very successful in patients with hematological malignancies. Their therapeutic benefit in solid tumors, however, has been limited. Even for those patients who do respond to this immunotherapy, there remains a risk of relapse due to exhaustion and limited persistence of the transferred T cells. Further, the hostile tumor microenvironment (TME) present within both solid and hematological tumor sites leads to exhaustion and dysfunction of the transferred cells.

CAR T cell persistence is a critical factor in the efficacy of these therapies, as the sustained activity of therapeutic cells in the patient is essential for continuous targeting and elimination of tumor cells, and is associated with clinical remission and survival of recipient patients (Porter et al.,). A related factor associated with therapeutic efficacy is T cell serial killing, or the ability of individual T cells to sequentially recognize and kill multiple target cells (Davenport et al.,). This ability is critical for maximizing the antitumor response, especially when the tumor burden is substantial. Persistent serial killing enables a more efficient and prolonged anti-tumor response, thereby enhancing therapeutic outcomes and potentially reducing the likelihood of tumor escape mechanisms.

Various factors may influence the persistent killing activity of CAR T cells, including the molecular design of the antigen receptor. For example, inclusion of the 4-1BB co-stimulatory domain into the CAR has been shown to generate CAR T cells with improved mitochondrial function leading to enhanced persistence, as compared to CAR T cells containing a CD28 co-stimulatory domain (Kawelekar et al,). *Ex vivo* culture conditions have also been observed to impact CAR T cell persistence. Shortening the duration of cell expansion from the standard 9-14 days to 3-5 days generated CAR T cells with higher antitumor activity and increased persistence in vivo (Gassemi, et al.,). Additionally, substituting the cytokine IL-2 with a combination of IL-7 and IL-15 yields CAR T cells with longer persistence and antitumor activity in vivo (Cieri, et al.,). In this application note we describe a novel method of CAR T expansion that closely replicates the solid tumor microenvironment to produce products with superior potency and persistence.

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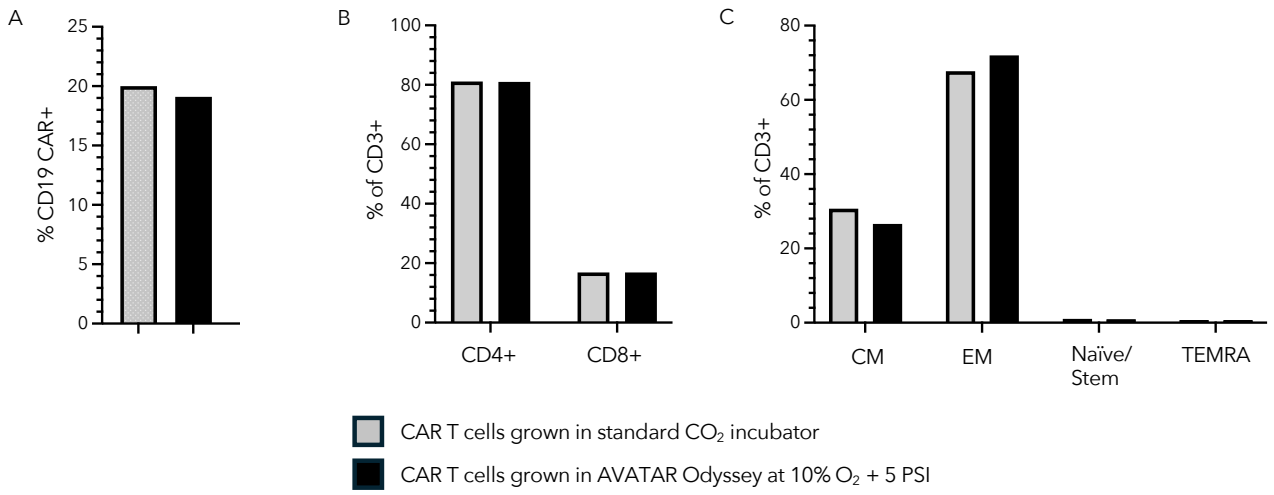

Figure 1 | CAR T cells grown in the AVATAR Odyssey are phenotypically similar to cells grown in a standard CO_2 incubator. A. Bar graphs show the percentage of CD19 CAR+ cells present in the total T cell population grown in a standard CO_2 incubator (gray) vs the AVATAR Odyssey (black). B. Shows the percentage of CD4+ cells (left) and CD8+ cells (right) within CD3+ cells grown in a standard CO_2 incubator vs the AVATAR Odyssey. C. Shows the percentage of Central Memory (CD45RA-CCR7+), Effector Memory (CD45RA-CCR7-), Naïve/Stem Cell Memory (CD45RA+CCR7+) and Terminally differentiated Effector Memory cells (CDRA+CCR7-) within CD3+ cells grown in a standard CO_2 incubator vs the AVATAR Odyssey.

Several groups have shown the beneficial role that hypoxic culture can have on T cell function. Short hypoxic exposure only at the time of T cell activation has been shown to enhance CAR T cell antitumor in vitro and in vivo, and yielded the types of metabolic adaptations associated with improved persistence (Cunha, et al.,). Further, activation of the hypoxia response master regulator, HIF1α, leads to the differentiation of CD69+CD103+ resident memory T cells, which have been shown to persist and retain antitumor function within the tumor microenvironment while resisting exhaustion (Liikanen, et al.,).

The AVATAR platform is a unique incubation technology that enables the precise control of oxygen tension and hyperbaric pressure on cells in culture. We, and others, have shown that culturing CAR T cells at reduced oxygen concentrations and increased pressure leads to the generation of cells with increased persistence and potent serial killing activity. The AVATAR Odyssey is a small, research-use incubator that is used to screen the effects of reduced oxygen and increased pressure on cellular phenotypes. The instrument is compatible with several small-scale, off-the-shelf consumables like well plates and flasks. The AVATAR Foundry is a novel GMP bioreactor developed for cell therapy manufacturing in the context of optimized environmental conditions leading to improved potency. It provides automated fluidics and utilizes a proprietary gas permeable consumable that automatically scales in size from 50mL to 1.5L cell culture capacity.

In this study, we generated CD19 CAR T cells in the AVATAR Odyssey and Foundry instruments and assessed their persistence and serial killing activity in two variations of a cytotoxicity assay: one in which effector to target (E:T) ratios are much lower than typically used in cytotoxicity assays (1:4 to 1:32). Under these conditions, each CAR T cell must kill multiple tumor cells in order to observe measurable rates of cytotoxicity, indicating that the T cells are able to persistently kill without exhaustion. In a variation of this assay, CAR T cells are serially challenged with fresh tumor cells every 72 hours, again testing their ability to escape exhaustion and continue to engage in serial killing activity.

CD19 CAR T cells were manufactured at small-scale at low oxygen conditions in the AVATAR Odyssey system and compared to those grown in a standard CO_2 incubator. Further, CD19 CAR T cells were manufactured at clinical-scale at low oxygen conditions in the AVATAR Foundry system or in a standard CO_2 incubator. Both populations of CAR T cells grown under low oxygen exhibited increased persistent killing, either at very low E:T ratios or under conditions of serial tumor challenge.

Results

To investigate the impact of low oxygen culture on CAR T cell persistent killing activity, we first generated CD19 CAR T cells in the small-scale AVATAR Odyssey system. Odyssey instruments are often deployed in sets of two or four and enable users to screen for the impact of oxygen concentrations and hyperbaric pressures on cellular phenotype and function. We have previously found that growing T cells under conditions of 5 PSI hyperbaric pressure increases their expansion without leading to phenotypic changes. Thus, we performed our CAR T cell manufacturing under conditions of low oxygen (10%) and 5 PSI and compared their function to those manufactured in a standard CO_2 incubator.

Isolated T cells were activated with CD3/CD28 beads and transduced with a commercially available lentivirus encoding a CD19 CAR construct. CAR T cells were grown for a total of 8 days, at which point they were analyzed by flow cytometry to determine their transduction efficiency and cell surface phenotype. Cells manufactured in both approaches exhibited comparable percentages of CAR+ cells and similar frequencies of CD4+ and CD8+ populations (Figure 1a, b). The cells were further analyzed for the relative frequencies of T cell memory populations using expression of CD45RA and CCR7 markers. CAR T cells from both manufacturing approaches exhibited comparable frequencies of central memory, effector memory, naïve and effector memory RA+, terminally differentiated cells (Figure 1c). We next measured the persistent killing activity of both cell populations by first challenging them with tumor cells at low E:T ratios. Under these conditions, T cells must engage in

persistent killing of multiple tumor cells without exhaustion in order to generate measurable cytotoxicity rates. CD19 CAR T cells were incubated with CD19-expressing Nalm6 cells at E:T ratios of 1:4, 1:8, 1:16, and 1:32. Cultures were analyzed for specific lysis using flow cytometry at 24 hours and 72 hours following initiation of coculture. At both time points, CAR T cells manufactured under low oxygen in the AVATAR Odyssey consistently exhibited higher rates of cytotoxicity (Figure 2a, b). To further challenge the T cells, we then added fresh tumor cells to these cultures and allowed them to incubate for another 72 hours before flow cytometric analysis. Though both populations of CAR T cells were able to efficiently kill tumor cells at the highest E:T ratio, only those cells expanded under low oxygen and hyperbaric pressure conditions were able to maintain their persistent killing activity at lower E:T ratios, hinting that cells were better able to engage in serial killing activity (Figure 2c).

These initial experiments were performed at small scale in open consumables in the AVATAR Odyssey system. To bridge to a larger scale, clinical workflow, we performed a similar workflow in the AVATAR Foundry system. This is a GMP closed bioreactor that employs a gas permeable consumable that scales in size from 50mL to 1.5L without the need to transfer or swap culturing vessels. We compared a low oxygen (5%) with 5 PSI AVATAR Foundry workflow to a workflow using the same consumable in a standard CO_2 incubator.

50 million isolated T cells were activated with CD3/CD28 beads and transduced with a lentivirus encoding a CD19 CAR construct. CAR T cells were grown for 15 days until they reached a working volume of 1.5L, at which point they were analyzed by flow cytometry to determine their transduction efficiency and cell surface phenotype. Cells manufactured using both approaches exhibited similar percentages of CAR+ cells. (Figure 3a). Interestingly, cells expanded in the low oxygen condition exhibited increased frequencies of cells with a naïve/stem cell memory phenotype (Figure 3b). This is particularly interesting because of recent work showing that hypoxic culture can enrich for this cell population in cell therapy manufacturing (Song et al.,). Stem cell memory T cells are known to exhibit long-term persistence, proliferative capacity and potent antitumor activity (Wang et al.,).

To measure the persistent killing activity of the CAR T cells, we performed five rounds of repeated tumor challenge. CAR T cells and tumor cells were cocultured at an E:T ratio of 1:4 for 72 hours, at which point fresh tumor cells were added and the coculture continued for another 72 hours. This process of serial challenge was repeated for a total of 5 tumor challenges. While cells generated in both manufacturing approaches were able to efficiently kill tumor cells on the first and second challenges, cells generated in the standard CO_2 incubator started losing their potency by the third challenge and exhibited no tumor lysis by the fifth challenge. In contrast, cells grown at low oxygen and hyperbaric pressure in the AVATAR Foundry, retained the ability to kill almost 100% of tumor cells by the fifth challenge, indicating that these CAR T cells had increased persistence and potent serial killing activity (Figure 3c).

Conclusion

We have shown how growing CD19 CAR T cells under conditions of low oxygen can boost their persistent tumor killing. We first performed small scale CAR T cell manufacturing in the AVATAR Odyssey and showed that these cells were phenotypically similar to their counterparts expanded in a standard CO_2 incubator but exhibited increased cytotoxicity at very low E:T ratios and under a tumor rechallenge condition. Next, we showed that these observations can be translated to a large scale, clinically relevant workflow by performing similar experiments in the AVATAR Foundry GMP bioreactor. This system has been uniquely designed to manufacture cell therapies with improved potency. CD19 CAR T cells were grown at low oxygen up to 1.5L scale and functionally compared to cells grown in a standard CO_2 incubator. This experiment again showcased that CAR T cells grown at low oxygen and hyperbaric pressures exhibited increased persistent killing, as demonstrated by their ability to retain killing activity over 5 repeated tumor challenges without exhaustion. Interestingly, in this workflow we also found that CAR T cells expanded in low oxygen had higher frequencies of naïve/stem cell memory T cells.

These observations have significant relevance because long-term CAR T cell persistence is associated with sustained clinical remission and survival of recipient patients (Porter et al.,). While others have showcased how genetically modified CAR

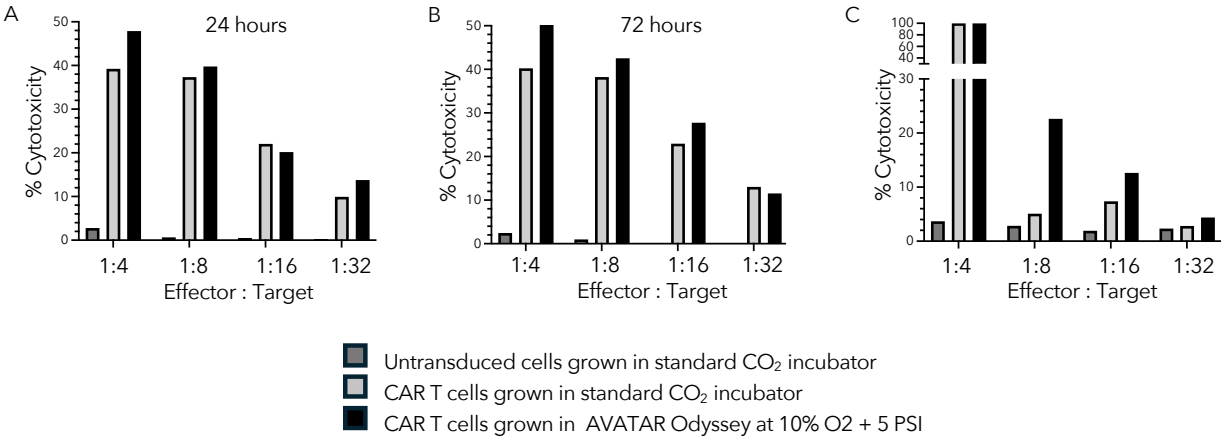

Figure 2 | CAR T cells grown in the AVATAR Odyssey show increased persistent killing as compared grown in a standard CO_2 incubator. **A.** Bar graphs show the percentage of specific Nalm6 cytotoxicity across a range of effector to target ratios after 24 hours of coculture. Dark gray bars were untransduced effector cells grown in a standard CO_2 incubator (negative control), gray bars are CD19 CAR T cells grown in a standard CO_2 incubator and black bars are CD19 CAR T cells grown in the AVATAR Odyssey. **B.** Shows the same data as above after 72 hours of coculture. **C.** Shows the same data as above after CAR T cells were rechallenged with fresh tumor cells for another 72 hours.

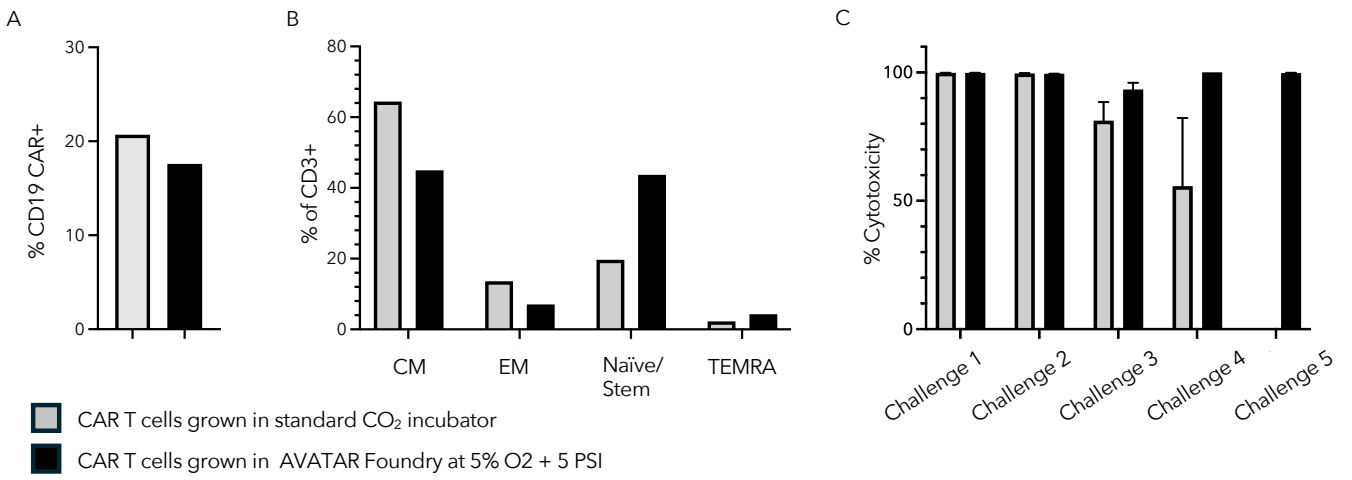

Figure 3 I CAR T cells grown in the AVATAR Odyssey show increased frequencies of naïve/stem cell memory cells and higher persistent killing as compared to cells grown in a standard CO_2 incubator. A. Bar graphs show the percentage of CD19 CAR+ cells within CD3+ cells grown in a standard CO_2 incubator (gray) vs the AVATAR Foundry (black). B. Shows the percentage of Central Memory (CD45RA-CCR7+), Effector Memory (CD45RA-CCR7-), Naïve/Stem Cell Memory (CD45RA+CCR7+) and Terminally differentiated Effector Memory cells (CDRA+CCR7-) within CD3+ cells grown in a standard CO_2 incubator vs the AVATAR Foundry. C. Shows the percentage of specific Nalm6 cytotoxicity across a series of 72 hours-long, serial challenges with fresh Nalm6 tumor cells at a 1:4 effector to target ratio. Gray bars are CD19 CAR T cells grown in a standard CO_2 incubator and black bars are CD19 CAR T cells grown in the AVATAR Foundry.

coreceptors or cytokine formulations can enhance T cell persistence, our work highlights the significant impact that low oxygen culture and hyperbaric pressures can also play in this area (Kawalekar, et al.,) (Cieri, et al.,). We are actively working to investigate the mechanisms underlying these observations and to translate them to in vivo tumor models. We are also particularly interested in repeating these experiments in a solid tumor model, where low oxygen at the tumor site is a significant suppressive force that adoptively transferred cells face. We propose that conditioning CAR T cells to low oxygen and hyperbaric pressures in vitro may improve their potency and persistence when faced with the suppressive forces of the tumor microenvironment.

Methods

CD19 CAR T cell manufacturing in AVATAR Odyssey

Untouched CD3+ T cells were isolated from healthy donor leukopaks using the EasySep™ Human T Cell Enrichment Kit (StemCell Technologies). T cells were activated in 6 well plates with Dynabeads™ Human T-Activator CD3/CD28 for T Cell Expansion and Activation (Gibco) in media containing RPMI-1640 (Gibco), 10% heat inactivated FBS (Gibco) and 40 U/mL recombinant human IL-2 (Peptrotech). Cells were cultured in a standard CO_2 incubator at ~21% O_2 and 0 PSI or in the AVATAR Odyssey system at 10% O_2 and 5 PSI. Temperature was maintained at 37°C and CO_2 at 5% in both incubators. 2 days following activation, cells were transduced with lentivirus encoding a CD19 CAR construct. Cells were transduced at a multiplicity of infection (MOI) of 3 and virus was directly added to wells and gently mixed. Cells were sampled and fed with media every 2-3 days to maintain their concentration.

CD19 CAR T cell manufacturing in AVATAR Foundry

CD3+ T cells were isolated as above and activated in a gas permeable consumable within a standard CO_2 incubator ~21% O_2 and 0 PSI or in the AVATAR Foundry instrument at 5% O_2 and 5 PSI. Temperature was maintained at 37°C and CO_2 was maintained at 5% in both incubators. 2 days following activation, cells were transduced with lentivirus encoding a CD19 CAR construct. Cells were transduced at a multiplicity of infection (MOI) of 3. In the standard incubator, cells were manually resuspended, sampled, and fed with media every 2-3 days to maintain their concentration. In the AVATAR Foundry, cells were automatically resuspended using the rocking function and samples were drawn from the sample port. The automated fluidics were used to feed the culture with appropriate media volumes. Cultures were maintained until they reached 1.5L, the maximum capacity of the AVATAR Foundry consumable. For the standard incubator condition, cultures were manually harvested in a biosafety cabinet and used for downstream analysis. In the AVATAR Foundry conditions, the automated harvest operation was used to harvest the cells into a transfer bag for downstream analysis.

Phenotypic Analysis

After 8-15 days of expansion, cells were analyzed by flow cytometry. Cells were washed and stained for the CD19 CAR in a two-step stain, using biotin-conjugated CD19Fc, followed by streptavidin-conjugated PE. Cells were then stained with the following antibodies: CD3-FITC, CD4-PE/CF594, CD8-BV786, CD45RA-BV421, PD-1-PerCP/Cy5.5, CCR7-AF700, CD69-APC, CD103-BV605 (Biolegend), and Live/Dead-BV510 (Invitrogen). Cells were run on a CytoFLEX LX cytometer (Beckman Coulter). Data was analyzed in FlowJo and Graphpad Prism software.

Functional Assay

Expanded T cells were harvested and Dynabeads were removed. Cells were washed and the percentage of CD19 CAR+ cells was assessed by flow cytometry to calculate the number of T cells to plate per well. E:T ratio calculations were based on the number of CAR+ T cells. T cells were resuspended in media without IL-2 at 4e4 CAR+ cells/mL. This suspension was used for the first E:T ratio of 2:1. A 2-fold dilution series of effector cells was performed 7 times, to a final dilution of 1:32 E:T. CD19-expressing, mCherry labeled Nalm6 cells were harvested, washed, and resuspended to 8e4 cells/mL. Cocultures were set

up in a 96-well U-bottom plate. 100µL of T cells from each titration were aliquoted into wells, yielding 8e3 CAR+ cells/well for the 2:1 E:T condition. 100µL of Nalm6 cells were then dispensed into those wells, yielding 4e3 Nalm6 cells/well. Control wells included those containing CAR T cells alone and those containing Nalm6 cells alone. T cells and tumor cells were incubated in a standard CO_2 incubator for 24 or 72 hours prior to analysis. Following coculture, cells were harvested, washed and stained with the following antibodies: CD3-FITC (Biolegend) and Live/Dead-BV510 (Invitrogen). Cells were run on a CytoFLEX LX cytometer (Beckman Coulter).

If tumor rechallenges were performed, 100µL of media was carefully removed from the top of the assay wells without disturbing the cells. 100µl of Nalm6 cells at 4e4 cells/mL were carefully added back to the wells, yielding 4e3 cells added per well. 100µL of media was added to wells not receiving rechallenge. Control wells containing only the new Nalm6 cells were created as the new baseline for analysis of this challenge. Cocultures were then incubated for another 72 hours in a standard CO_2 incubator. Cells were analyzed by flow cytometry. If wells demonstrated >95% cytotoxicity of tumor cells, CAR T cells were rechallenged with fresh tumor cells.

The percent cytolysis was calculated by using the Live/Dead staining to calculate the percentage of dead Nalm6 cells. A Nalm6-only control well was used to subtract the percentage of spontaneous lysis to generate a calculated specific lysis value. Alternatively, CountBright flow cytometry beads (Thermo Fisher) can be used to calculate exact concentrations of live Nalm6 cells in the control and coculture wells, which can then be used to calculate specific lysis of the Nalm6 cells.

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