Enriching central memory T cells using novel bioreactor design for T cell manufacturing

Sixun Chen[1], Akshaya V Prabhu[1], Ahmad Amirul Bin Abdul Rahim[1], Kang-Zheng Lee[1], Dan Liu[1]*

[1]Bioprocessing Technology Institute BTI, Agency for Science, Technology and Research (A*STAR), 20 Biopolis Way, Singapore 138668, Singapore

* Corresponding author

E-mail: liu_dan@bti.a-star.edu.sg

Abstract

The manufacturing of T cell therapies aims to achieve high yields of product with potent phenotypes. We have developed a novel bioreactor, Bioreactor with an Expandable Culture Area – Dual Chamber (BECA-D), which has previously demonstrated functionality for scaled T cell manufacturing. In this study, incorporation of a stirring mechanism into the double-chamber bioreactor design was tested to homogenize the media components between the two chambers. In addition to the improved media homogenization, the stirring culture was also observed to have higher yield and enrichment of central memory T cells, a T cell subpopulation that has been associated with improved therapeutic efficacy, as compared to a static control. BECA-D with a stirring mechanism (BECA-DS+), was evaluated for its performance in culturing T cells in comparison to a static control, BECA-D, and an industry benchmark, G-Rex10. BECA-DS+ was able to preferentially promote the enrichment of central memory T cells compared to the static cultures, indicative of the effect of the stirring mechanism. By achieving high T cell yields with a favorable subpopulation profile, the mechanical method of incorporating stirring into a double-chamber bioreactor such as BECA-D carries great potential as a useful research and manufacturing tool to support advanced T cell therapy manufacturing.

Keywords: Biomanufacturing, Cell Therapy, Memory T cells, Central Memory T cells, Immunotherapy, Bioreactor

Introduction

In the last decade, autologous T cell therapy has demonstrated its clinical efficacy, earning approvals from multiple regulatory agencies around the world [1–3]. Despite advancements in manufacturing technologies, scaled manufacturing of autologous cell therapies remains challenging, limiting their accessibility and driving up costs [4]. Early understanding of T cell manufacturing targeted high cell viability and yields. However, recent studies have shown that T cell subtypes are also important for the efficacy and persistence of the therapy [5]. The enrichment of less differentiated T cells - naïve T cells (T_N), stem memory T cells (T_{SCM}), and central memory T cells (T_{CM}) – in a T cell therapy product has been observed to confer similar efficacy at a significantly lower dose than a matched non-enriched product [6]. This translates to a shorter culture period, potentially saving manufacturing costs and shortening turnaround times.

Process controls can be implemented during manufacturing to direct T cell population differentiation and proliferation towards an enrichment of less differentiated T cells. The most common method would be the media supplementation of cytokines, such as IL-7 and IL-15 [7,8], and IL-21 [9,10], due to the ease of implementation and the availability of good manufacturing practice (GMP)-grade reagents. Media supplementation of other metabolites such as lactate [11,12] and amino acids [13,14] have also been observed to affect T cell memory population, however these methods are not as established as compared to cytokine supplementation. Apart from media supplementation, environmental control of the culture, notably control of pH [12,15] and dissolved oxygen (DO_2) [16,17] levels of the culture, has shown potential in directing T cell differentiation. Currently, there are no mechanical methods that have been described in literature that can consistently enrich these desired memory T cell populations.

We have previously reported on our work in developing a novel bioreactor, Bioreactor with Expandable Culture Area – Dual Chamber (BECA-D), to meet the specific culture needs of T cells and simplify the culture process (Figure 1A) [18]. This study describes our work in improving the performance of BECA-D with a stirring mechanism to promote the homogenization of media between the chambers within the bioreactor and enrich for central memory T cells in the culture. The incorporation of the stirring mechanism was first tested with a simple double-chamber prototype bioreactor with fixed culture area, which resulted in an improved effect on T cell culture expansion yield and change in heterogeneity of memory subtypes towards the enrichment of T_{CM} cells. The approach was then validated in BECA-D, and results compared with matched culture in G-Rex10, an industry benchmarked culture vessel for T cell culture [19–21], to establish the robustness of the mechanical method in the enrichment of T_{CM} cells.

Materials and Methods

Fabrication of a Double-chamber prototype Bioreactor

The double-chamber prototype bioreactor (Figure 1A) was fabricated from hard plastics (polycarbonate and polypropylene). It consisted of a cell chamber sitting atop support struts, within a containing device with a lid. The cell chamber has a filter support base with polyethylene terephthalate (PET) membrane having pore size of 1.0 µm (Sterlitech), attached to it. The area beneath and surrounding the cell

chamber forms the media chamber. Post-assembly sterilization was performed through autoclave at 121°C, for 20 minutes. All materials used in the construction of the prototype were tested in-house for biocompatibility with Peripheral Blood Mononuclear Cell (PBMC) culture.

Culture of PBMCs in the Double-Chamber Prototype Bioreactor with (Stirring) and without (Static)

The complete culture medium consisted of RPMI-1640 (Thermo Fisher Scientific), 10% heat inactivated Fetal Bovine Serum (FBS) (HyClone), and 2 mM L-glutamine (Thermo Fisher Scientific). This complete culture medium was used for all the experiments described in this manuscript.

For both Static and Stirring culture, 95 mL of complete culture medium was added into the Media Chamber with 50 IU/mL IL-2 (STEMCELL Technologies). PBMCs (STEMCELL technologies) were thawed and seeded at 0.5 x 10^6 cells/cm^2 in 6 mL of complete culture medium into the Cell Chamber with 50 IU/mL IL-2. 150 µL of activators (ImmunoCult™ Human CD3/CD28/CD2 T Cell Activator (STEMCELL Technologies)) was added to the Cell Chamber. The culture was then incubated at 37°C under 5% CO_2. On Day 7, half media change of the Media Chamber was performed. Culture was re-activated by the addition of 150 µL of activators to the Cell Chamber. 50 IU/mL IL-2 was also added on Day 3 or 4, 7, 10 or 11 to the respective chambers based on their media volume.

For Stirred cultures, an autoclaved polyetrafluoroethylene (PTFE) magnetic stirbar was placed into the Media Chamber on Day 0. The culture was transferred onto a stir plate (60 rpm) one day after activation (Day 1 and Day 8). The cultures were stirred for the periods of Days 1-7 and Days 8-14.

Fabrication of BECA-D

BECA-D (Figure 1A) was fabricated as described in a previous study [18]. Briefly, it was made from injection moulded virgin polystyrene and assembled via ultrasonic welding under an ISO 13485 certified environment. The plunger consists of a polycarbonate core with silicone rubber outer wall. The porous membrane was made from PET with a pore size of 1.0 µm. All materials used in the construction of BECA were tested in-house for biocompatibility with PBMC culture.

Culture of PBMCs in BECA-D with stirring (BECA-DS+) and without stirring (BECA-D)

For both BECA-D and BECA-DS+, 228 mL of complete culture medium was added to the Media Chamber with 50 IU/mL IL-2. PBMCs were thawed and seeded at 0.5 x 10^6 cells/cm^2 in 6 mL of complete culture medium into the Cell Chamber with 50 IU/mL of IL-2. 150 µL of activators was added to the Cell Chamber. The culture was then incubated at 37°C under 5% CO_2. On Day 7, half media change of the Media Chamber was performed. Culture area of the Cell Chamber was calculated based on the cell population and adjusted to maintain a cell density of 0.5 x 10^6 cells/cm^2. Culture was re-activated by the addition of 1:40 culture volume of activators to the Cell Chamber. 50 IU/mL IL-2 was also added on Day 3 or 4, 7, 10 or 11 to the respective chambers based on their media volume.

For BECA-DS+, an autoclaved PTFE magnetic stirbar was placed into the Media Chamber on Day 0. BECA-DS+ was transferred onto a stir plate (60 rpm) one day after activation (Day 1 and Day 8). The cultures were stirred for the periods of Days 1-7 and Days 8-14.

Culture of PBMCs in G-Rex10

PBMCs were thawed and seeded at 0.5 x 10^6 cells/cm^2 in 40 mL of complete culture medium with 50 IU/mL of IL-2 and 1 mL of activators. The culture was then incubated at 37°C under 5% CO_2. On Day 7, half media change of the culture was performed. Culture was re-activated by the addition of 1 mL of activators. 50 IU/mL IL-2 was also added on Day 3 or 4, 7, 10 or 11.

Cell Count and Viability

Cell counts and viability were determined based on Trypan Blue exclusion method by diluting a sample of culture with 1:1 volume of 0.4% Trypan Blue Solution (Thermo Fisher Scientific), loaded onto a hemacytometer, and counted under an inverted microscope. Cell counts reported in this study are based on live cells.

Glucose and Lactate Measurements

Media samples were collected and analysed for glucose and lactate levels using the Cedex Bio Analyzer (Roche Diagnostics) with accordance to the manufacturer's protocol.

Phenotyping of memory T cells

On Day 14, cells were harvested and processed for flow cytometry analysis. Cells were stained with Viobility 405/520 Fixable Dye, CD3-VioBlue, CD56-PE-Vio770, CD4-APC-Vio770, CD8-FITC, CD45RA-PerCP-Vio 700, and CD197 (CCR7)-PE (Miltenyi Biotec). MACSQuant Analyser 10 (Miltenyi Biotec) was used to acquire the data and MACSQuantify Software 2.13.3 (Miltenyi Biotec) was used for analyses.

Statistical Analysis

Statistical analyses were performed using Microsoft Excel (Office 365). Paired Student's two-tailed t-tests were performed to assess the statistical significance of differences between two groups within each of the donor sets. One sample two-tailed t-test was performed to assess the statistical significance of differences between a group and a defined value. A p-value of < 0.05 obtained from the test indicates a significant difference.

Results

Incorporation of a stirring mechanism within the double-chamber prototype bioreactor improved media exchange and cell yield

We previously developed a novel bioreactor, BECA-D, consisting of two separate chambers – the Cell Chamber (CC) with an expandable culture area, and the Media Chamber (MC), separated by a porous membrane (Figure 1A). Its performance in the culture of virus specific-T cells was validated in a previous study [22]. In that study, it was observed that the diffusion of glucose and lactate across the porous membrane to be inefficient, possibly hindering cell proliferation. We hypothesised that the incorporation of a stirring mechanism in the MC could improve homogenisation of glucose and lactate between the chambers (Figure 1B). This mechanism is expected to allow for glucose in the MC to be more available to the cells in the CC and to dilute lactate from the CC more efficiently to facilitate continuous cell growth leading to a higher cell yield. To study this, we fabricated a pair of simple double-chamber prototype bioreactors with a fixed 12 cm^2 culture area (Figure 1A) – one prototype with a stirbar added into the MC (Stirring) and one prototype without a stirbar as a control (Static).

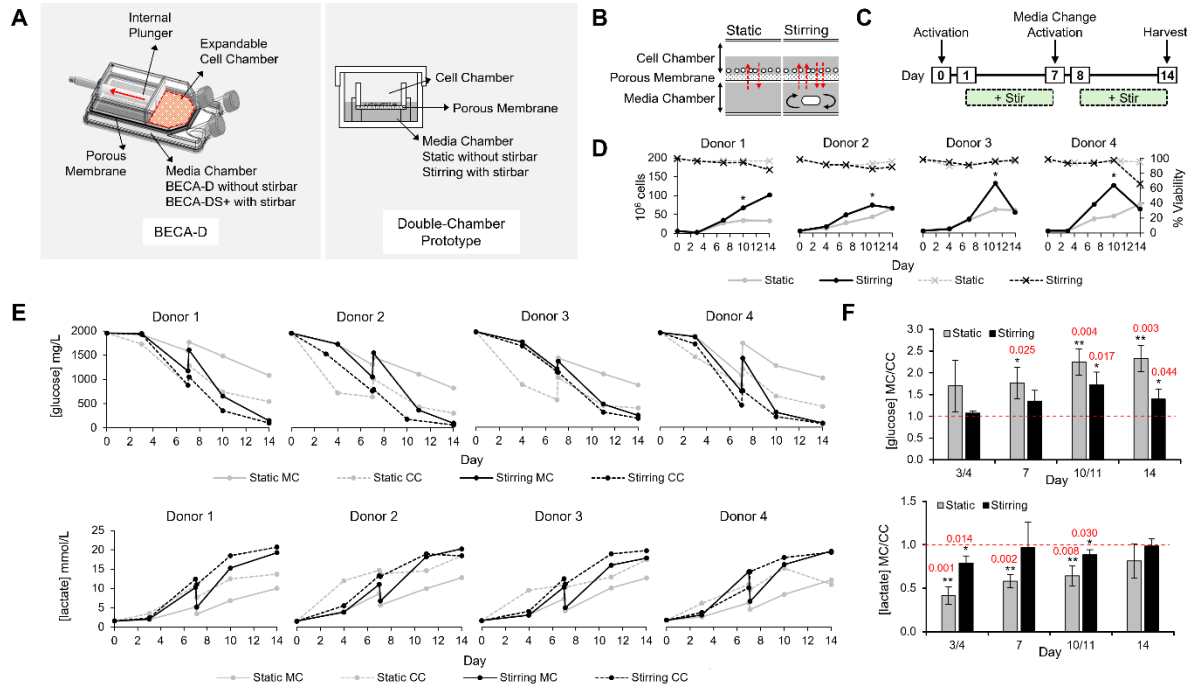


Fig 1. Incorporation of a stirring mechanism in a double-chamber bioreactor improves media exchange within bioreactor and promotes cell proliferation (A) Left: Design of BECA-D bioreactor. Red shaded area indicates area where cells are seeded and cultured. Red arrow indicates direction of expansion of culture area via pull of the internal plunger. Right: Design of double-chamber prototype bioreactor. (B) Side view of media exchange (red arrows) between the cell and media chambers through the porous membrane. Introduction of stirring mechanism via a stirbar enhances media exchange. (C) Experimental plan for comparison of Static and Stirring double-chamber prototype bioreactors (D) Cell counts (solid lines, left axis) and viability (dashed lines, right axis) for both Static and Stirring cultures across Donor 1 to 4. * indicates significant difference in cell count values

between Static and Stirring cultures with p-value <0.05. (E) Glucose and lactate concentrations in the cultures MC (solid lines) and CC (dashed lines) (F) Ratio of glucose and lactate concentrations between MC and CC. Red line indicates the ideal mixing ratio of 1.0. * In dicates a p-value of <0.05 and ** indicates a p-value of <0.01 for a one-sample T-test with the determined value of 1.0. Significant p-values are indicated in red above the data bars. (1.5 column fitting image)

The prototypes were tested with four different sets of donor PBMCs (Donor 1-4) in a 14-day culture. Cells were seeded at 0.50 x 10^6 cells/cm^2 with activation on Day 0 and Day 7, and a 50% media change on Day 7 (Figure 1C). The stirring mechanism was switched on from Day 1 to Day 7 and Day 8 to Day 14 (Figure 1C). In all donors, the Stirring culture achieved higher cell yield compared to Static on Day 14 for Donor 1 and Day 10/11 for Donor 2-4 (Figure 1D). Paired T-test analysis indicated a significant difference (p-value = 0.024) on Day 10/11 cell numbers between Stirring (101.18±34.60 x 10^6 cells) and Static (46.81±12.37 x 10^6 cells) cultures. Viability in the cultures remained high (>80%) in all four donors across the 14-day culture with the exception of Donor 4 Day 14 where the viability dropped to 66%, mirroring the drop in cell population between Day 10/11 to 14 (Figure 1D).

Lactate and glucose levels in both chambers were measured on days 0, 3 or 4, 7, 10 or 11, and 14, showing a steady decrease of glucose levels and increase of lactate levels in the cultures, indicative of T cell proliferation (Figure 1E). Levels of glucose and lactate in the chambers was observed to be more closely matched in Stirring cultures compared to Static cultures. One sample t-test was performed to compare the ratio of glucose and lactate concentration in the chambers to the ideal value of 1.0 (indicating perfect homogenization) (Figure 1F). Both the ratio of glucose and lactate levels in the Static cultures were observed to be significantly different from the ideal value of 1.0 on three days (Day 7, 10/11, and 14 for glucose and Day 3/4, 7, and 10 for lactate) while those in Stirring cultures were observed to be significantly different on two days (Day 10/11 and 14 for glucose and Day 3/4 and 10/11 for lactate). In all statistical analyses performed, the p-value calculated from comparing the chambers in Static cultures were lower than those from Stirring cultures. It was also observed that the glucose level in Stirring for Donors 2, 3, and 4 fell to below 0.5 mg/cell on Day 10/11, indicating that the glucose levels in these cultures might have been insufficient to support the growing culture resulting in the sharp drop in cell numbers observed on Day 14,

Stirring mechanism within the double-chamber prototype bioreactor enriched both CD4 and CD8 Central Memory T cells (T_{CM})

The percentage of CD3 population in the cultures were assessed on Day 0 and Day 14, showing high percentage of CD3 cells in the culture (>90%) in both Stirring and Static cultures and no significant difference between the two types of cultures (Figure 2A). The same observation was made for the levels of CD4 helper and CD8 cytotoxic T cells indicating that the stirring mechanism did not influence the development and proliferation of bulk CD4 and CD8 T cells (Figure 2B). CD45RA and CCR7 expression for T cell memory subtypes in CD4 and CD8 T cell populations were assessed on Day 0 and Day 14 with flow cytometry. These memory subtypes include naïve (T_N), central memory (T_{CM}), effector memory (T_{EM}), and terminally differentiated effector memory (T_{EMRA}) T cells (Figure 2C). In all cultures, T_N and T_{EMRA} populations decreased from Day 0 to Day 14 and T cells primarily expressed T_{EM} and T_{CM} markers on Day 14 (Figure 2D). Stirring cultures were observed to have significantly higher percentage of T_{CM} cells compared to Static cultures in both CD4 (p-value = 0.031) and CD8 (p-value = 0.010) subpopulations. The opposite trend was observed for T_{EM} population where Stirring cultures had significantly lower percentage of T_{EM} cells compared to Static cultures in both CD4 (p-value = 0.028) and CD8 (p-value = 0.012) subpopulations (Figure 2E).

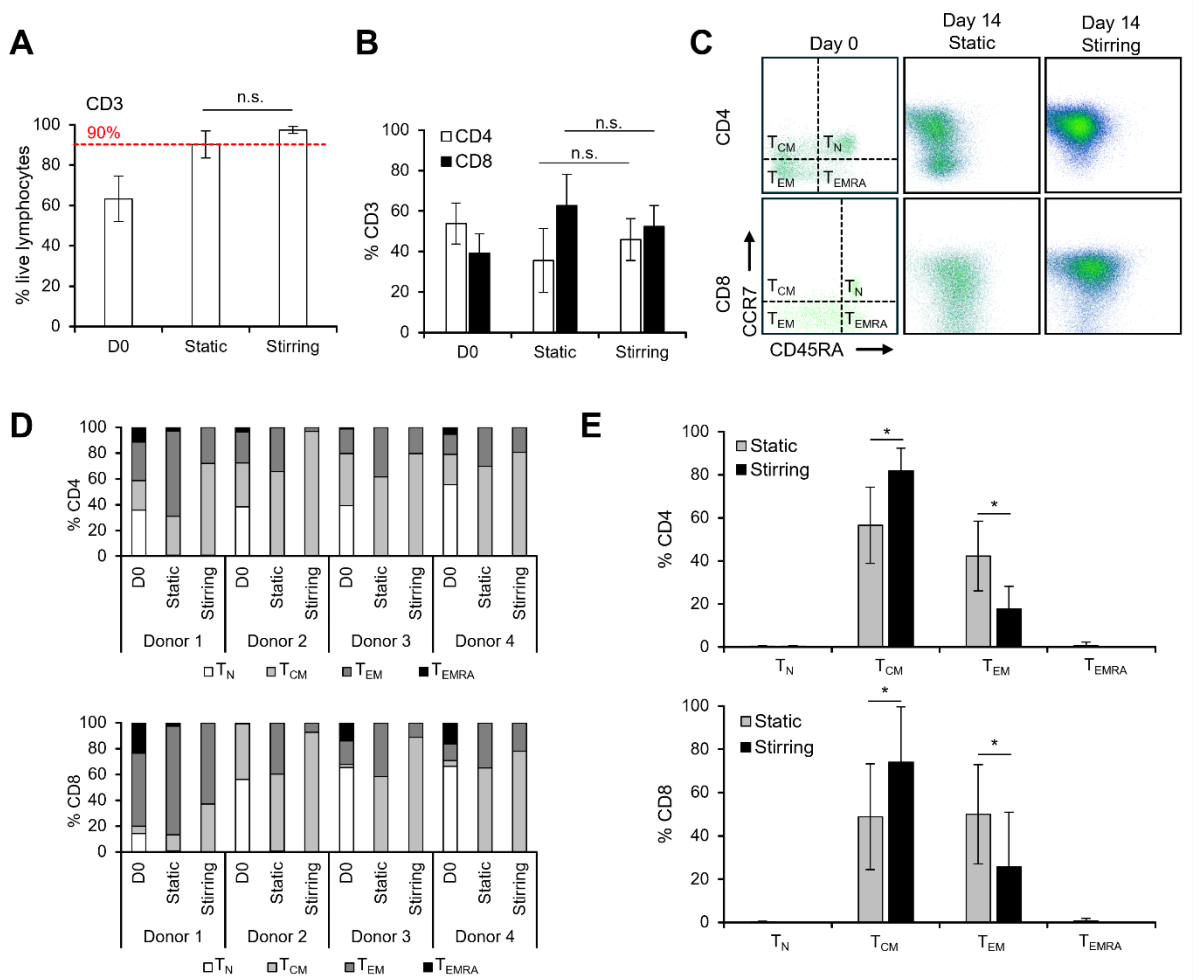


Fig 2. Stirring mechanism enriched for Central Memory T cells in culture. (A) Percentage CD3 population of PBMCs on Day 0 and in Static and Stirring cultures on Day 14. Red line indicates 90% population of live lymphocytes in culture. (B) Percentage CD4 and CD8 population of CD3 PBMCs on Day 0 and in Static and Stirring cultures on Day 14. (C) Representative flow cytometry plot (Donor 2) for assessment of CCR7 and CD45RA markers in the Day 0 PBMC culture and Day 14 Static and Stirring cultures. Representative gating for T_N, T_{CM}, T_{EM}, T_{EMRA} in the CD4 and CD8 population are as shown. (D) Percentage of each T cell memory subtype in CD4 and CD8 population. (E) Percentage of T_{CM} and T_{EM} cells in CD4 and CD8 population. P-values indicated were calculated using paired t-test. n.s. not significant * $p<0.05$ (single column fitting image)

Stirring mechanism in BECA-D improved cell proliferation and media homogenization

To validate the robustness of the method, we repeated the experiment with the same four donor PBMCs (Donor 1 – 4) in the BECA-D bioreactor with a stirbar placed into the Media Chamber of one bioreactor (BECA-DS+) and its static control without the stirbar (BECA-D). Cell cultures were also evaluated with another T cell bioreactor (G-Rex10) that does not have a double-chamber design.

The cells were seeded at 0.50 x 10^6 cells/cm^2 and cultured using the same protocol with an additional step of expanding the culture area in BECA-D and BECA-DS+ on Day 7 (Figure 3A). The culture areas of BECA-D bioreactors were adjusted based on the cell numbers on Day 7 to decrease the cell density back to 0.5 x 10^6 cells/cm^2 (Supplementary Figure 1A and 1B). Across all four donors, BECA-DS+ achieved the highest maximum yield compared to BECA-D and G-Rex10 (Figure 3B). Similar to the Stirred culture in the double-chamber prototype bioreactor (Figure 1D), BECA-DS+ experienced a decrease in culture growth for Donor 2-4 from Day 10/11 to 14. BECA-DS+ was able to improve cell proliferation with a significantly higher yield on Day 10/11 compared to BECA-D (p-value = 0.013) and G-rex10 (p-value = 0.037) (Figure 3C). Viability in the cultures remained high (>80%) in all four donors across the 14-day culture in all bioreactors (Figure 3B).

The stirring mechanism in BECA-DS+ allowed for improved homogenization of media between the two chambers (Supplementary Figure 1C). The ratios of glucose and lactate levels in BECA-D were determined to be significantly different from the ideal value of 1.0 on Day 7 and 10/11 (glucose) and Day 3/4, 7, 10/11 and 14 (lactate). In BECA-DS+, the ratios were significantly different for only one day

for glucose (Day 7) and two days for lactate (Day 3/4, 7) (Supplementary Figure 1D). Glucose availability per cell was calculated to determine whether the level of glucose of media could explain the slowing of cell growth in BECA-DS+ and G-Rex10 cultures from Day 10/11 to 14 in Donor 2, 3, and 4 (Supplementary Figure 1E). Surprisingly, glucose availability in this study did not correlate to the culture growth in BECA-DS+. The only instance between Day 0 and 14 where the glucose/cell fell below 0.5 mg/cell in BECA-D cultures was in Donor 2 on Day 11, however, this did not result in a drop in cell population as observed in the double-chamber cultures. In addition, the culture in Donor 4 on Day 10 had higher glucose availability and yet had a decrease in cell population. The G-Rex10 cultures consistently had lower glucose availability due to G-Rex10's lower media capacity (40 mL, starting glucose level: 78.5±0.3 mg for 5.0 x 10^6 cells) compared to BECA-D (234 mL, starting glucose level: 458.9±1.8 mg for 6.0 x 10^6 cells) but this did not hinder the culture growth. For these cultures, it appears that glucose availability does not sufficiently explain the slowing down of cell proliferation from Day 10/11 to 14.

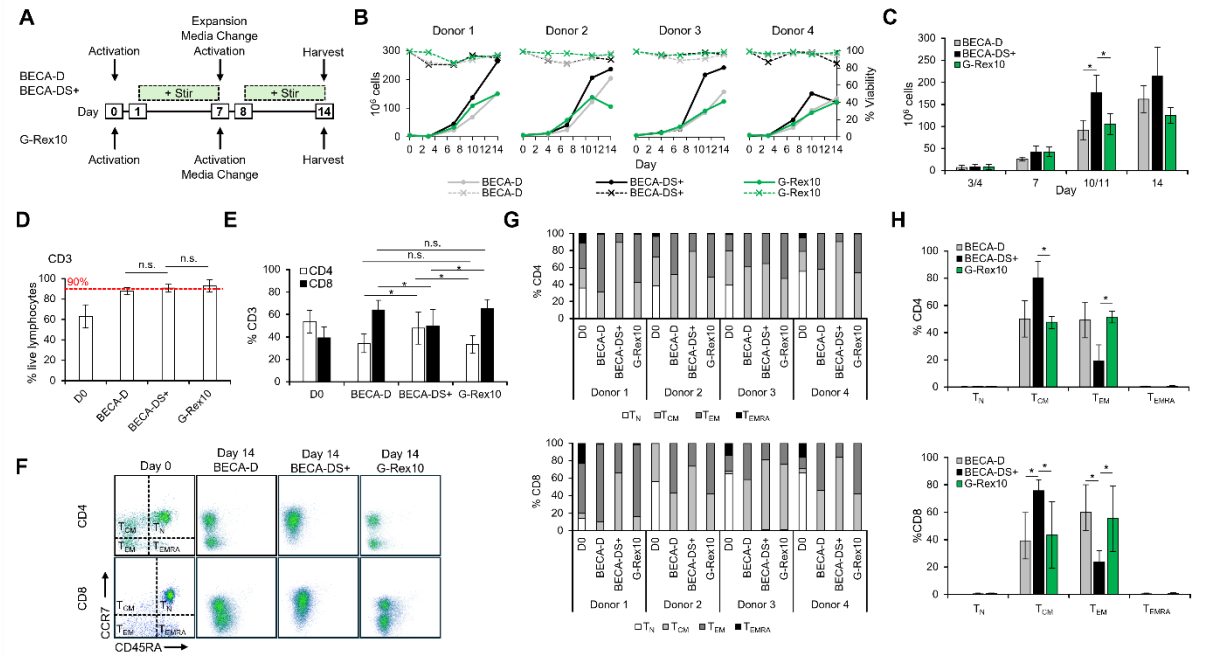


Fig 3. BECA-DS+ preferentially promoted Central Memory T cell (T_{CM}) proliferation. (A) Experimental plan for comparison of BECA-D, BECA-DS+, and G-Rex10 cultures. (B) Cell counts (solid lines, left axis) and viability (dashed lines, right axis) across Donor 1 to 4. (C) Average cell counts across Donor 1 to 4 for each day of culture (3 or 4, 7, 10 or 11, 14). (D) Percentage CD3 population of PBMCs on Day 0 and in the three bioreactors on Day 14. Red line indicates 90% population of live lymphocytes in culture. (E) Percentage CD4 and CD8 population of CD3 PBMCs on Day 0 and in the three bioreactors on Day 14. (F) Representative flow cytometry plot (Donor 4) for assessment of CCR7 and CD45RA markers in the Day 0 PBMC culture and Day 14 cultures. Representative gating for T_N, T_{CM}, T_{EM}, T_{EMRA} in the CD4 and CD8 population are as shown. (G) Percentage of each T cell memory subtype in CD4 and CD8 population. (E) Percentage of T_{CM} and T_{EM} cells in CD4 and CD8 population. P-values indicated were calculated using paired t-test. n.s. not significant * p<0.05. (1.5 column fitting image)

Stirring mechanism in BECA-D enriched both CD4 and CD8 Central Memory T cells (T_{CM})

The cultures were assessed for their T cell population phenotype via flow cytometry analysis on Day 14. The percentage of CD3 cells in the cultures for three bioreactors was high (>85%) with cultures in BECA-DS+ and G-Rex10 having >90% of live lymphocytes being CD3+ cells (Figure 3D). Previously, we observed subtle but not statistically significant difference in CD4 and CD8 population in the Stirring and Static cultures in the double-chamber prototype bioreactor. In this experiment, the difference was more pronounced. There was a statistically significant difference between BECA-D and BECA-DS+ CD4 (p-value = 0.04) and CD8 (p-value = 0.03) populations and same was observed between BECA-DS+ and G-Rex10 CD4 (p-value = 0.05) and CD8 (p-value = 0.04) population (Figure 3E). Interestingly, there was no significant difference in the CD4 and CD8 populations between BECA-D and G-Rex10 (CD4 p-value = 0.61, CD8 p-value = 0.63) suggesting that the stirring mechanism in BECA-DS+ did skew the T cell population to have higher CD4 and lower CD8 population in culture.

In line with the findings from the double-chamber prototype bioreactor study, assessment of CD45RA and CCR7 expression revealed that the T cells primarily expressed T_{CM} and T_{EM} markers on Day 14 and that the stirred culture, BECA-DS+, has enriched T_{CM} population and diminished T_{EM} population compared to static cultures – BECA-D and G-Rex10 (Figure 3F and 3G). The enrichment of CD8 T_{CM}

population in BECA-DS+ cultures was statistically significant compared to BECA-D (p-value = 0.015) and G-Rex10 (p-value = 0.045). BECA-DS+ also has statistically significant lower percentage of CD8 T_{EM} population compared to BECA-D (p-value = 0.013) and G-Rex10 (p-value = 0.044). However, the same was not true for CD4 subpopulation. The higher percentage of T_{CM} and lower percentage of T_{EM} cells were only statistically significant when comparing BECA-DS+ with G-Rex10 cultures (T_{CM} p-value = 0.012, T_{EM} p-value = 0.012) and not when comparing BECA-DS+ with BECA-D cultures (T_{CM} p-value = 0.071, T_{EM} p-value = 0.071) (Figure 3H).

Discussion

Our previous study demonstrated the effectiveness and ease of use of BECA-D in the culture of Epstein-Barr Virus-Specific T cells [10]. We hypothesized that enhancing the media exchange between the cell and media chambers could improve culture outcomes. In this study, we explored our hypothesis with a double-chamber prototype bioreactor and developed an iteration of BECA-D, BECA-DS+, which improved media exchange between the cell and media chambers through a stirring mechanism. The performances of the stirred cultures in both the double-chamber prototype bioreactor and BECA-DS+ was compared to static controls across a 14-day PBMC culture. As expected, the stirring mechanism did improve the glucose and lactate distribution between the chambers and resulted in a higher maximum cell yield on Day 14 for Donor 1 and Day 10/11 for Donors 2, 3, and 4. The cell yield in BECA-DS+ was observed to be higher than that in G-Rex10, however, the performance of G-Rex10 could have been impaired by the non-ideal feeding scheme executed in this study. The glucose availability of G-Rex10 was measured to be consistently low at <0.5 mg/mL from Day 10/11 to 14. It is possible that adjustment of the feeding scheme with more frequent and higher volume media changes might result in an equivalent cell yield as BECA-DS+.

Analysis of the T cell subtypes within the cultures revealed stark differences between the stirred cultures (Stirring and BECA-DS+) and static cultures (Static, BECA-D, and G-Rex10). Surprisingly, the CD4 and CD8 ratios (higher levels of CD8 cells) were consistent within the static cultures, whereas, the stirred cultures had a more balanced distribution of CD4 and CD8 cells. Assessment of T cell memory subtypes showed an enrichment of T_{CM} cells in stirred cultures compared to static cultures. The observation that T cell subtype distributions differ between bioreactors is not novel as bioreactors have key features that may affect T cell metabolism and proliferation, such as improved gaseous exchange in gas permeable bioreactors [23] and high cell density in hollow fibre reactors [24]. It was unexpected to us that the static cultures had a more similar T cell subtype distribution considering that there are major design differences between the two static bioreactors, BECA-D and G-Rex10. The results reinforced the dominant effect of an ‘indirect mixing’ of a separate media chamber on the T cell culture, both in CD4 CD8 distribution as well as enrichment of T_{CM}.

The enrichment of T_{CM} cells, a less-differentiated T cell memory subtype, has been correlated with improved clinical outcomes and T cell persistence [7,25–28]. The improved efficacy of the less-differentiated T cell population could possibly reduce the cell dosage per therapy and accelerate the manufacturing of T cell therapy products. Supplementation of cytokines in media (typically a cocktail involving permutations of IL2, IL7, IL-15, and IL-21) is a common practice to drive T cell expansion towards an enriched T_{CM} population (personal communication) [29]. Other culture parameters have also been observed to affect T_{CM} population including other media components [13,14], pH [12,15], and low DO_2 levels (hypoxia) [16,17]. Compared to these methods, especially in those that requires temporal control, the double-chamber bioreactors (including BECA-DS+) adopt a direct distinct mechanical approach which does not require the use of additional GMP-grade reagents (in contrast to cytokine and media components methods) or specialized equipment (in contrast to hypoxia methods). In addition, cultures in the double-chamber bioreactors did not experience a decrease in proliferative capacity compared to cultures under hypoxia [16,17,30]. The use of the double-chamber bioreactor compared to the above methods for the enrichment of T_{CM} population in culture can possibly reduce culture process complexity and cost for the manufacturing of less-differentiated but more efficacious T cell therapy products.

The stirring mechanism affects the availability of multiple culture media components across the chambers. This study reported only on the glucose and lactate levels in the media, and the improved diffusion of these metabolites does not sufficiently explain the effect on T cell differentiation observed. The availabilities of other media components have been reported to affect T cell differentiation, especially IL-2 [31,32] and activators [33–35]. Additional studies measuring these would be crucial in the elucidation of mechanism behind the T_{CM} enrichment. Assessment of T cell subpopulations in earlier timepoints in the 14-day culture can also facilitate to determine whether the enrichment was due to an

increase in proliferation potential of T_{CM} cells, a decrease in proliferative potential of T_{EM} cells, or a decrease in differentiation potential of the culture. Understanding these details in the mechanism would assist in creating a simpler process which could shorten the culture duration to enable shorter turnaround times in manufacturing.

This study has demonstrated that a mechanical stirring mechanism introduced to a double-chamber bioreactor was able to consistently enrich less-differentiated T cell subpopulations while achieving yields similar to the static controls and commonly used industry T cell bioreactor, G-Rex10. The current execution for this method relies on a magnetic stirbar to engage the stirring mechanism which would not be compatible with processes involving the use of magnetic bead-based reagents such as the Dynabeads™ activators. The current chamber designs also have a substantially large media reservoir compared to G-Rex10 (101 and 234 mL vs 40 mL) hence increasing the cost for media and reagents consumed per run. As we elucidate the mechanism of action for this method, the design of the bioreactors will be improved upon to circumvent these restrictions and facilitate more workflows at a lower cost.

As more cell therapies are expected to be approved in the next decade and the indications for T cell therapy expand, there is an urgent need to lower the cost and improve accessibility of these therapies. Recently, multiple bioreactor systems with advanced culture parameter controls have been launched to help address the manufacturing hurdles for the field (e.g. AVATAR from Xcell Biosciences, IRO from Ori Biotech, and Sefia expansion system from Cytiva). Towards the same goal, we report our bioreactor system, BECA-DS+, as a potential and effective manufacturing solution with unique advantage of enriching T_{CM} cell production. Cell therapy developers and manufacturers can consider the use of these novel bioreactors to cater not only to the increase demand in manufacturing, but also the variety of culture needs for different therapies (e.g. low seed bioreactors for tumour-infiltrating T cells, small bioreactor capacity of highly efficacious cells). Bioreactors with different tuneable features available for T cell culture would be crucial in the endeavour to evaluate critical process parameters (CPPs) and critical quality attributes (CQAs) during therapy process and product development. The expansion in the variety of bioreactors in the market will hopefully result in a more vibrant industry where cell therapy developers and manufacturers have an arsenal of tools available to produce the most efficacious therapy at the lowest cost that can be easily accessed by patients.

Conflicts of Interest

The authors declare no conflicts of interest.

Funding

This work was supported by the Integrated Manufacturing Programme for Autologous Cell Therapy (IMPACT), an Industry Alignment Fund—Pre-Positioning Programmeby Agency for Science, Technology and Research (A*STAR) [Grant Number: H18/01/a0/022].

Author Contributions

D.L. and S.C. conceptualized the study. A.A.A.R. and D.L. designed and developed the bioreactor used in the study. S.C. and D.L. designed the experiments. A.A.A.R., A.V.P., and K.L. performed the experiments. All authors discussed the results and analysis. S.C. wrote the manuscript and prepared the figures with input from all authors.

References

[1] U.S. Food & Drug Administration. Approved Cellular and Gene Therapy Products 2024.

[2] European Medicines Agency. CAT quarterly highlights and approved ATMPs 2024;26:0–3.

[3] Lu J, Xu L, Wei W, He W. Advanced therapy medicinal products in China: Regulation and development. MedComm 2023;4. https://doi.org/10.1002/mco2.251.

[4] Krishna D, Rittié L, Tran H, Zheng X, Chen-Rogers CE, McGillivray A, et al. Short Time to Market and Forward Planning Will Enable Cell Therapies to Deliver R&D Pipeline Value. Hum Gene Ther 2021;32:433–45. https://doi.org/10.1089/hum.2020.212.

[5] López-Cantillo G, Urueña C, Camacho BA, Ramírez-Segura C. CAR-T Cell Performance: How to Improve Their Persistence? Front Immunol 2022;13:1–16. https://doi.org/10.3389/fimmu.2022.878209.

[6] Dickinson MJ, Barba P, Jäger U, Shah NN, Blaise D, Briones J, et al. A Novel Autologous CAR-T Therapy, YTB323, with Preserved T-cell Stemness Shows Enhanced CAR T-cell Efficacy in Preclinical and Early Clinical Development. Cancer Discov 2023;13:1982–97. https://doi.org/10.1158/2159-8290.CD-22-1276.

[7] Xu Y, Zhang M, Ramos CA, Durett A, Liu E, Dakhova O, et al. Closely related T-memory stem cells correlate with in vivo expansion of CAR.CD19-T cells and are preserved by IL-7 and IL-15. Blood 2014;123:3750–9. https://doi.org/10.1182/blood-2014-01-552174.

[8] Cieri N, Camisa B, Cocchiarella F, Forcato M, Oliveira G, Provasi E, et al. IL-7 and IL-15 instruct the generation of human memory stem T cells from naive precursors. Blood 2013;121:573–84. https://doi.org/10.1182/blood-2012-05-431718.

[9] Loschinski R, Böttcher M, Stoll A, Bruns H, Mackensen A, Mougiakakos D. IL-21 modulates memory and exhaustion phenotype of T-cells in a fatty acid oxidation-dependent manner. Oncotarget 2018;9:13125–38. https://doi.org/10.18632/oncotarget.24442.

[10] Chen Y, Yu F, Jiang Y, Chen J, Wu K, Chen X, et al. Adoptive Transfer of Interleukin-21-stimulated Human CD8+ T Memory Stem Cells Efficiently Inhibits Tumor Growth. J Immunother 2018;41:274–83. https://doi.org/10.1097/CJI.0000000000000229.

[11] Feng Q, Liu Z, Yu X, Huang T, Chen J, Wang J, et al. Lactate increases stemness of CD8 + T cells to augment anti-tumor immunity. Nat Commun 2022;13. https://doi.org/10.1038/s41467-022-32521-8.

[12] Cheng H, Qiu Y, Xu Y, Chen L, Ma K, Tao M, et al. Extracellular acidosis restricts one-carbon metabolism and preserves T cell stemness. Nat Metab 2023;5:314–30. https://doi.org/10.1038/s42255-022-00730-6.

[13] Nabe S, Yamada T, Suzuki J, Toriyama K, Yasuoka T, Kuwahara M, et al. Reinforce the antitumor activity of CD8+ T cells via glutamine restriction. Cancer Sci 2018;109:3737–50. https://doi.org/10.1111/cas.13827.

[14] Geiger R, Rieckmann JC, Wolf T, Basso C, Feng Y, Fuhrer T, et al. L-Arginine Modulates T Cell Metabolism and Enhances Survival and Anti-tumor Activity. Cell 2016;167:829-842.e13. https://doi.org/10.1016/j.cell.2016.09.031.

[15] Davern M, Donlon NE, O'Connell F, Gaughan C, O'Donovan C, Habash M, et al. Acidosis significantly alters immune checkpoint expression profiles of T cells from oesophageal adenocarcinoma patients. Cancer Immunol Immunother 2023;72:55–71. https://doi.org/10.1007/s00262-022-03228-y.

[16] Berahovich R, Liu X, Zhou H, Tsadik E, Xu S, Golubovskaya V, et al. Hypoxia selectively impairs CAR-T cells in vitro. Cancers (Basel) 2019;11:1–16. https://doi.org/10.3390/cancers11050602.

[17] Song HW, Prochazkova M, Shao L, Traynor R, Underwood S, Black M, et al. CAR-T cell expansion platforms yield distinct T cell differentiation states 2024;26:757–68. https://doi.org/10.1016/j.jcyt.2024.03.003.

[18] Chen S, Bin Abdul Rahim AA, Wang WW, Cheong R, Prabhu A V., Tan JZY, et al. In-situ scalable manufacturing of Epstein–Barr virus-specific T-cells using bioreactor with an expandable culture area (BECA). Sci Rep 2022;12. https://doi.org/10.1038/s41598-022-11015-z.

[19] Vera JF, Brenner LJ, Gerdemann U, Ngo MC, Sili U, Liu H, et al. Accelerated production of antigen-specific T cells for preclinical and clinical applications using gas-permeable rapid expansion cultureware (G-Rex). J Immunother 2010;33:305–15. https://doi.org/10.1097/CJI.0b013e3181c0c3cb.

[20] Ludwig J, Hirschel M. Methods and Process Optimization for Large-Scale CAR T Expansion Using the G-Rex Cell Culture Platform. Methods Mol. Biol., vol. 2086, Humana Press Inc.; 2020, p. 165–77. https://doi.org/10.1007/978-1-0716-0146-4_12.

[21] Gagliardi C, Khalil M, Foster AE. Streamlined production of genetically modified T cells with activation, transduction and expansion in closed-system G-Rex bioreactors. Cytotherapy

2019;21:1246–57. https://doi.org/10.1016/j.jcyt.2019.10.006.

[22] Chen S, Bin Abdul Rahim A, Cheong R, Prabhu A, Tan J, Wang W, et al. Culture expansion of epstein-barr virus-specific cytotoxic T-lymphocytes (EBV-CTLs) using bioreactor with expandable culture area (BECA). Cytotherapy 2021;23:S170. https://doi.org/https://doi.org/10.1016/S146532492100565X.

[23] Forget MA, Haymaker C, Dennison JB, Toth C, Maiti S, Fulbright OJ, et al. The beneficial effects of a gas-permeable flask for expansion of Tumor-Infiltrating lymphocytes as reflected in their mitochondrial function and respiration capacity. Oncoimmunology 2016;5. https://doi.org/10.1080/2162402X.2015.1057386.

[24] Yoo SM, Lau VWC, Aarts C, Bojovic B, Steinberg G, Hammill JA, et al. Manufacturing T cells in hollow fiber membrane bioreactors changes their programming and enhances their potency. Oncoimmunology 2021;10. https://doi.org/10.1080/2162402X.2021.1995168.

[25] Louis CU, Savoldo B, Dotti G, Pule M, Yvon E, Myers GD, et al. Antitumor activity and long-term fate of chimeric antigen receptor-positive T cells in patients with neuroblastoma. Blood 2011;118:6050–6. https://doi.org/10.1182/blood-2011-05-354449.

[26] Kalos M, Levine BL, Porter DL, Katz S, Grupp SA, Bagg A, et al. T cells with chimeric antigen receptors have potent antitumor effects and can establish memory in patients with advanced leukemia. Sci Transl Med 2011;3. https://doi.org/10.1126/scitranslmed.3002842.

[27] Fraietta JA, Lacey SF, Orlando EJ, Pruteanu-malinici I, Gohil M, Lundh S, et al. Determinants of response and resistance to CD19 chimeric antigen receptor (CAR) T cell therapy of chronic lymphocytic leukemia. Nat Med 2018;24:563–71. https://doi.org/10.1038/s41591-018-0010-1.

[28] Deng Q, Han G, Puebla-Osorio N, Ma MCJ, Strati P, Chasen B, et al. Characteristics of anti-CD19 CAR T cell infusion products associated with efficacy and toxicity in patients with large B cell lymphomas. Nat Med 2020;26:1878–87. https://doi.org/10.1038/s41591-020-1061-7.

[29] Silveira CRF, Corveloni AC, Caruso SR, Macêdo NA, Brussolo NM, Haddad F, et al. Cytokines as an important player in the context of CAR-T cell therapy for cancer: Their role in tumor immunomodulation, manufacture, and clinical implications. Front Immunol 2022;13:1–13. https://doi.org/10.3389/fimmu.2022.947648.

[30] Cunha PP, Minogue E, Krause LCM, Hess RM, Bargiela D, Wadsworth BJ, et al. Oxygen levels at the time of activation determine T cell persistence and immunotherapeutic efficacy. BioRxiv 2022:2022.11.25.517976.

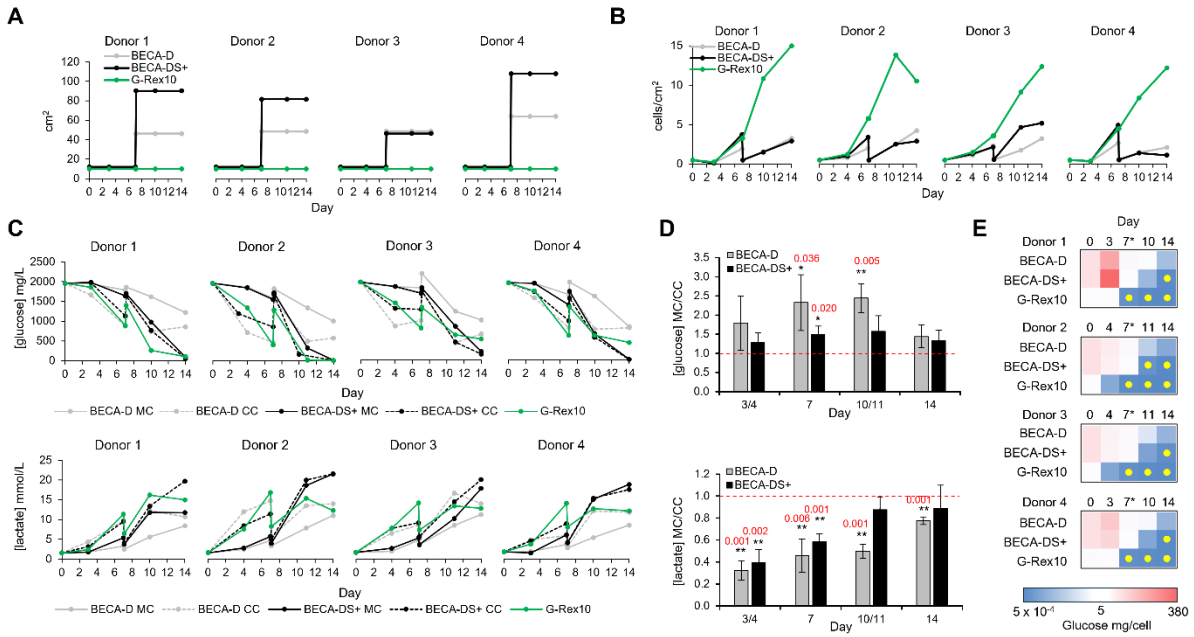
[31] Kaartinen T, Luostarinen A, Maliniemi P, Keto J, Arvas M, Belt H, et al. Low interleukin-2 concentration favors generation of early memory T cells over effector phenotypes during chimeric antigen receptor T-cell expansion. Cytotherapy 2017;19:689–702. https://doi.org/10.1016/j.jcyt.2017.03.067.

[32] Zhang X, Lv X, Song Y. Short-term culture with IL-2 is beneficial for potent memory chimeric antigen receptor T cell production. Biochem Biophys Res Commun 2018;495:1833–8. https://doi.org/10.1016/j.bbrc.2017.12.041.

[33] Zanon V, Pilipow K, Scamardella E, De Paoli F, De Simone G, Price DA, et al. Curtailed T-cell activation curbs effector differentiation and generates CD8+ T cells with a naturally-occurring memory stem cell phenotype. Eur J Immunol 2017;47:1468–76. https://doi.org/10.1002/eji.201646732.

[34] Alvarez-Fernández C, Escribà-Garcia L, Vidal S, Sierra J, Briones J. A short CD3/CD28 costimulation combined with IL-21 enhance the generation of human memory stem T cells for adoptive immunotherapy. J Transl Med 2016;14:4–13. https://doi.org/10.1186/s12967-016-0973-y.

[35] Kagoya Y, Nakatsugawa M, Ochi T, Cen Y, Guo T, Anczurowski M, et al. Transient stimulation expands superior antitumor T cells for adoptive therapy. JCI Insight 2017;2:1–13. https://doi.org/10.1172/jci.insight.89580.



Supplementary Fig 1. (A) Culture areas of BECA-D, BECA-DS+, and G-Rex10 across four donors during the 14-day culture period. (B) Culture densities of the three bioreactors across four donors during the 14-day culture period. (C) Glucose and lactate concentrations in the cultures MC (solid lines) and CC (dashed lines) of BECA-D and BECA-DS+, and in G-Rex10. (D) Ratio of glucose and lactate concentrations between MC and CC in BECA-D and BECA-DS+. Red line indicates the ideal mixing ratio of 1.0. * indicates a p-value of <0.05 and ** indicates a p-value of <0.01 for a one-sample T-test with the determined value of 1.0. Significant p-values are indicated in red above the data bars. (E) Heatmap of glucose per cell (mg/cell) in the three bioreactors across four donors during the 14-day culture period. Yellow dots indicate a value of <0.5 mg glucose/cell. (1.5 column fitting image)