A closed, autologous bioprocess optimized for TCR-T cell therapies

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Abstract

Autologous cell therapy has proven to be an effective treatment for hematological malignancies. Cell therapies for solid tumors are on the horizon, however the high cost and complexity of manufacturing these therapies remain a challenge. Routinely used open steps to transfer cells and reagents through unit operations further burden the workflow reducing efficiency and increasing the chance for human error. Here we describe a fully closed, autologous bioprocess generating MAGE-B2 TCR-T cells. This bioprocess yielded 5 – 12e9 MAGE-B2-specific TCR-expressing T cells, transduced at low MOIs, within 7 to 10 days, and cells exhibited an enriched memory T cell phenotype and enhanced metabolic fitness. It was demonstrated that activating, transducing, and expanding leuko-apheresed cells in a single bioreactor without a T cell enrichment step promoted lentivirus transduction efficiency while resulting in comparable level of T cell purity (˜97%) as that of leukopak cells that went through CD8+ and CD4+ positive selection. The critical process parameters of the bioreactor, including culturing at a high cell density (7e6 cells/mL), adjusting rocking agitations during phases of scale up, lowering glycolysis through addition of 2-Deoxy-D-glucose (2-DG), and modulating IL-2 levels, were shown to positively regulate TCR expression and cell doubling time, and promote resistance to effector-associated apoptosis of TCR-T cells. The bioprocess described herein supports scale-out feasibility by enabling processing of multiple patients' batches in parallel within a Grade C cleanroom.

Introduction

Autologous T cell therapy has proven to be an effective treatment for hematological malignancies, and there are currently six FDA approved CAR-T therapies for the treatment of leukemia, lymphoma, and multiple myloma (Albinger, Hartmann, & Ullrich, 2021; Costa et al., 2021; Ma et al., 2019; Munshi et al., 2020). Developing effective therapeutics against solid tumors represent an unmet need, and there are several ongoing clinical trials using CAR-T or TCR-T cell therapies for solid malignancies (Morotti et al., 2021). As promising as these personalized T cell therapies are, manufacturing them is challenging due to the high cost of goods manufactured (COGM), inability to scale, complex supply chain logistics, long end-to-end time of bioprocessing plus release testing, and limited patient access. Treating solid tumors presents further challenges; not only must the dose be higher but the engineered T cells must be equipped to penetrate and function within the tumor microenvironment. Doses for solid tumors reach into the 100e8/CAR+ or TCR+/kg cells(Mirzaei, Rodriguez, Shepphird, Brown, & Badie, 2017), therefore a higher yield is required from the manufacturing process. Additionally, producing cells with characteristics that will enable them to home to the tumor site and attack cells expressing the tumor antigen is necessary for an effective treatment.

Typical manufacturing processes for cell therapy rely on isolating white blood cells from the patient through leukophoresis, followed by enriching T cells from a leukopak using magnetic separation, activating the cells with CD3 and CD28 activators, transducing with a viral vector carrying the gene of interest in either static flasks or gas permeable bags and expanding the T cells in a static vessel or rocking bioreactor(Eyles et al., 2019). Many of these steps are open, requiring the technician to handle the patient's cells inside a biosafety

cabinet and use pipettes or syringes to transfer cells into vessels. Repeated physical manipulation of the cells causes sheer stress and can negatively impact cell viability, phenotype and overall fitness(Fowell & Kim, 2021; Mastrogiovanni, Juzans, Alcover, & Di Bartolo, 2020). Due to the high risk of contamination and inability to sterilize the final drug product, these processes must be performed in an ISO 7 clean room. It is also recommended that each patient have a dedicated incubator which increases the manufacturing footprint(Sekiya et al., 2012). Furthermore, each manufacturing step requires costly single-use consumables, increasing COGM and impacting the environmental sustainability as patient numbers increase.

We have developed a fully closed bioprocess for autologous T cell therapies that eliminates the need for syringes, gas permeabilized bags, and static incubators. Through integrating unit operations including activation, transduction of leuko-apheresed cells, and cell expansion in a rocking bioreactor, we achieved high transgene expression and desired T cell yield sufficient for dose escalation studies for solid tumor indications. This bioprocess provides a new approach for TCR-T cell manufacturing resulting in a significant reduction in COGM compared to the state-of-the-art T cell bioprocesses.

Results

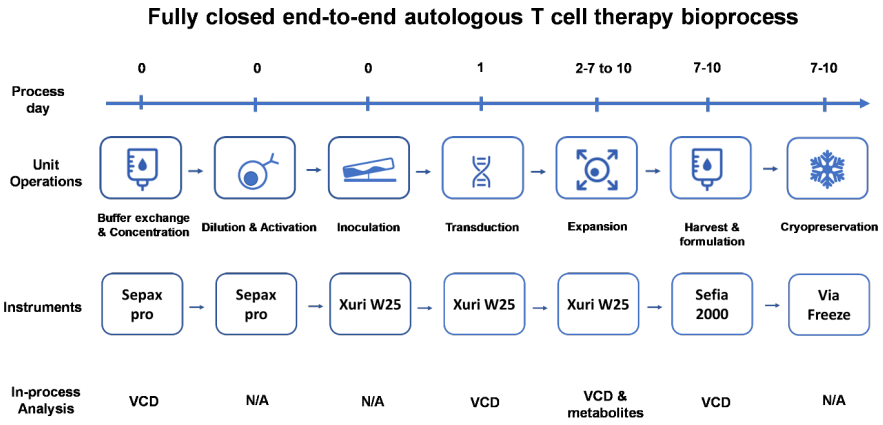
Establishing a fully closed bioprocess to generate autologous TCR-T cells

To streamline our TCR-T autologous bioprocess and lower COGM, we identified the costliest materials and evaluated if they were necessary for generating a high yield of transgene positive T cells. Published T cell bioprocesses enrich T cells from the leukopak prior to activation[5, 9,10], however the CD4 and CD8 magnetic beads and single-use kit used for selection make up a significant portion of the raw material costs of manufacturing. We demonstrated that eliminating the T cell enrichment step resulted in comparable T cell purity following expansion to T cells enriched from a CliniMACS Plus at day8 **(Fig 1B & Supplemental Fig. 1D)** . Unexpectedly, these leuko-apheresed cells also had higher transduction efficiency compared to T cells enriched from CD4+ and CD8+ positive selection independent of whether the transgene was a CAR, GFP or TCR when tested with three different donors in small-scale studies **(Fig. 1A)** .

Many established bioprocesses involve open steps where cells are moved between culture vessels for T cell activation and transduction, mainly due to the use of plate-bound activators and transduction enhancers. Some processes begin expansion in static vessels or bags before transferring to a rocking bioreactor(Eyles et al., 2019; Highfill & Stroncek, 2019). We hypothesized that these separated unit operations and associated open steps adversely affect process performance by subjecting T cells to an unfavorable environment such as deprivation of heterogeneous cell contact, cytokines, and insufficient/timely medium exchange due to lack of perfusion(Chan & Shlomchik, 2000; Deola et al., 2008). We envisioned integrating these steps into one single bioreactor to simplify the workflow and aid in the efficiency of cell engineering and expansion. To enable this, a soluble activator was used to eliminate the need to pre-coat bags. Through optimization of process parameters, we demonstrated that adding ImmunoCult CD3/28/2 to leuko-apheresed cells in a semi-static bioreactor with rocking speed and angle at 2 rpm x 2° is effective in enabling T cell activation **(Supplemental Fig. 1A)** . Cost of lentiviral vector limits the number of cells that can be transduced, and transductions with a multiplicity of infection (MOI) of 5 or higher must be performed in a gas permeable bag due to the low volume necessary for 1-2e8 cells at an optimal cell density. We found a MOI of 1 was efficient for transduction of multiple TCRs**(Supplemental Fig. 1B**). Due to the low amount of lentivirus needed for efficient transduction, we concluded that it is feasible to transduce roughly 7e8 cells in the bioreactor on day1. Together, these findings enabled an integrated workflow for TCR-T cell production by incorporating cell activation, transduction, and expansion in a single bioreactor immediately after the leukopak wash step using the Sepax Pro (**Fig. 2**).

This fully closed bioprocess yields an average of 10e9 T cells from ˜1e9 leukopak cells as starting material within 7-8 days, and a peak of 20-30e9 cells can be reached in 10 days**(Fig. 1C)** . Viability dips midway through the process due to non-T cell death, but rebounds to >97% by day6 of expansion **(Fig. 1D & Supplemental Fig. 1C)** . Variation is observed in growth curve and in-process viability due to donor-to-donor variability, however, end-of-process viability is consistently above 90% **(Fig. 1D)** . Semi-continuous

perfusion ensures timely replenishment of nutrients, removal of undesired metabolites and cell debris and maintains glucose levels above 2g/L and lactate levels below 2 g/L, respectively **(Fig. 1E & 1F)** . Cells harvested at the end of process (EOP) showed TCR expression of CD8+ T cells detected by MHC dextramer, ranging from 30% to 70% with mean expression at 55%**(Fig. 1G)** . Mean TCR expression of CD3+ T cells was 30%, however, due to MHC class I tetramer being only able to detect TCR expression on CD8+ cells **(Fig. 1G)** . Staining with a Vbeta antibody Vβ13.2 targeting the TCR β chain confirmed that CD4+ cells are transduced, but CD4+ TCR expression is undetectable by dextramer staining **(Supplemental Fig. 1C)** . 97% purity of CD3+ T cells was achieved at EOP regardless of CD3+ percentage of leukopak starting material (20-80%) **(Fig. 1H)** . Harvested TCR-T cells showed potent and specific cytotoxicity against MAGE-B2 peptide loaded T2-Luc cells at E:T ratio of 1 after 24 hours during *in vitro* functional assays **(Fig. 1I)** .



Optimizing Xuri W25 bioreactor parameters

To further shorten the end-to-end process time, we evaluated whether several bioreactor parameters, including seeding density, dissolved oxygen (DO) level and rocking agitation, could accelerate cell growth. The same donor apheresis was split into five tested bioreactor conditions listed in **Table 1** .

Xuri#1, the standard condition where cells were seeded at lower density (3.5e6 cells/ml) with lower agitation (3 rpm x 3°), yielded < 4e9 engineered T cells at day9 while Xuri#2, Xuri#3, and Xuri#5 all significantly promoted cell growth to around 10e9 cells through increasing rocking agitation or gassing oxygen directly (DO feedback loop control) to promote a greater level of oxygen transfer**(Fig. 3A)** . Moreover, Xuri#2, Xuri#3, and Xuri#5 with increased rocking speed and angle demonstrated that enhancing agitation also improved in-process cell viability from day6 to day9 compared to Xuri#1 **(Fig. 3B)** . In addition, shortening the bioprocess from 11 days to nine days did not adversely affect T cell purity, as comparable % CD3 T cells were found for all conditions upon harvest**(Fig. 3C)** .

While enhanced oxygen mixing through increasing agitation or direct oxygen input promoted cell growth, those two approaches contrasted in their impact on transduction efficiency. Having direct and steady oxygen delivery through DO feedback loop control from the start (Xuri#3) compromised transduction efficiency and resulted in the lowest TCR surface expression **(Fig. 3D)** . In contrast, DO feedback loop control at a later stage when the bioreactor was fully scaled up (Xuri#5) or enhancing oxygen mixing through rocking agitation at 500 mL volume (Xuri#2) led to TCR levels similar to standard conditions (Xuri#1) **(Fig. 3D)** . In addition, a higher inoculation density at 7e6 cells/ml (Xuri#4) led to a ˜15% increase in transduction efficiency compared with conditions at 3.4e6 cells/ml (Xuri#2) **(Fig. 3D)** . Correlating with higher TCR expression in cells from Xuri#4 and % Tcm for cells from Xuri#2, INF-g release was also modestly enhanced compared with Xuri#1 when co-cultured with MAGE-B2 peptide loaded T2 cells for 24 hours at E:T ratio at 1 **(Fig. 3E)** . No distinct differences were found for % T2 cells lysis at 48 hours due to saturation of T2 cell lysis above 99% **(Fig. 3F)** .

T cell activation and expansion usually comes at the cost of differentiation into effector phenotype (CD45RO+, CCR7-, CD95+) from naïve (CD45RA+, CCR7-, CD95-), stem (CD45RA+, CCR7+, CD95+) and central memory (CD45RO+, CCR7+, CD95+) pool, a phenomenon associated with a shortened life span, T cell exhaustion and compromised therapeutic persistence in the patient(Henning, Roychoudhuri, & Restifo, 2018; Wherry, 2011). Cells from Xuri#2, Xuri#3, and Xuri#5, which had greater oxygen/mass mixing compared to Xuri#1, contained significantly higher % Tcm cells than cells from Xuri#1 and Xuri#4 when compared at yield of 10e9 cells **(Fig. 3G)** . It is also notable that while cells from #Xuri4 had the highest TCR surface expression **(Fig. 3D)** among all the conditions tested, cell growth and %Tcm was lower than its counterpart conditions tested. On the other hand, while cells from Xuri#3 showed higher % Tcm and enhanced cell growth, TCR surface expression of those cells was 3-fold less than cells from Xuri#2**(Fig. 3D)** . This suggests that while oxygen promotes T cell growth, it may negatively impact transduction when supplied in excess during T cell activation. Overall, higher inoculation density and greater rocking agitation adopted at 0.5 L culture volume enables a shorter end-to-end bioprocess time with optimal T cell phenotype and potency.

Inhibiting glycolysis skews T cells towards a more memory phenotype

Upon activation, T cells undergo a metabolic switch from oxidation of fatty acids to glycolysis to enable effector function(Chang & Pearce, 2016; Edwards-Hicks, Mitterer, Pearce, & Buescher, 2020). The switch into effector function is critical for immune surveillance however is undesirable during *ex vivo* manufacturing due to the loss of memory function of T cell and its association with reduced therapeutic efficacy and persistence(Delgoffe et al., 2021; Eyles et al., 2019). We hypothesized that limiting glucose intake could sustain the memory phenotype of T cells and potentially lead to increased efficacy and persistence. Cells were activated and expanded in the presence of a glycolysis inhibitor, 2-DG, at 2mM on day0 until harvest on day9 or gradually removed at day6(Pelicano, Martin, Xu, & Huang, 2006) through semi-continuous perfusion (1L/day) until harvest. No inhibition of cell growth was observed in the presence of 2-DG independent of when 2-DG was removed **(Fig. 4A)** . While cells still continuously differentiated from Tn to Tscm and eventually enriched to a mixed pool of Tcm and Tem, glycolysis inhibition by 2-DG noticeably increased the %Tscm and Tcm at different timepoints during expansion with data shown for two representative donors. **(Fig. 4B)** . % Tscm was higher in conditions with 2-DG on day4 which led to 10% to 20% higher of % Tcm + Tscm on day 7 or day9 compared to the condition lacking 2-DG**(Fig. 4B)** . In addition to enriching total memory T cells, glycolysis inhibition by 2-DG also increased TCR expression by 10% at time of harvest. **(Fig. 4C)** .

Mitochondrial membrane potential (MMP) and mitochondrial mass were also quantified based on the mean fluorescent intensity (MFI) of $DilC_1(5)$ and MitoTracker Red acquired by flow cytometry to investigate whether glycolysis inhibition improved metabolic fitness of TCR-T cells. After encountering target cells, TCR-T cells from all conditions exhibited elevated mitochondrial metabolism as MMP became more polarized and mitochondrial mass increased in order to sustain energetic metabolism for effector function **(Fig. 4D & 4E)** . Interestingly, MMP and mitochondrial mass were less elevated for TCR-T cells activated and expanded in the presence of 2-DG than those cultured without 2-DG. TCR-T cells generated in the presence of 2-DG had similar lysis capacity (E:T=1) **(Fig. 4F)** with lower MMP and mitochondrial mass than cells cultured in the absence of 2-DG. Thissuggests that 2-DG cells have a greater mitochondrial capacity and fitness to meet the increased energy demand for effector function (Sukumar et al., 2016; van der Windt et al., 2012).

Removing IL-2 in-process promotes metabolic fitness and reduced background killing

Over-activation of T cells prior to transduction and expansion can lead to pre-exhaustion of EOP TCR-T cells with compromised persistence and potentially contribute to cytokine release syndrome (CRS) due to high background reactivity(Alizadeh et al., 2019; Gett, Sallusto, Lanzavecchia, & Geginat, 2003; Schluns & Lefrançois, 2003). We hypothesized that in-process IL-2 withdrawal from complete culture media together with semi-continuous perfusion would attenuate activation signaling and generate T cells with lower background activity and enhanced fitness. When evaluated with two donor samples, we found that less than 5% T cells were positive for CD25 upon harvest when IL-2 was withdrawn on day5 or day6 while cells cultured

with IL-2 until harvest had 50-80% CD25+ T cells **(Fig. 5A)** . The delay in CD25 downregulation post IL-2 withdrawal is expected with a semi-continuous perfusion system where IL-2 containing media was slowly perfused out of the bioreactor resulting a gradual decline in IL-2 concentration. Importantly, cells with lower CD25 expression exhibited reduced nonspecific killing while maintaining specific target cell lysis when E:T ratio was increased **(Fig. 5B)** . In the two donors tested, withdrawing IL-2 on day6 or day5 differentially affected TCR expression compared to the control condition (with IL-2), although the differences were not significant. **(Fig. 5C)** .

MMP, ROS, and apoptosis at both resting state and effector state against target cells were measured based on fluorescent intensity (MFI) of $DilC_1(5)$, CellROS Orange, and Annexin of viable T cells using flow cytometry. When encountering target cells, MMP became more polarized, and cells generated more ROS **(Fig. 5D & 5E)** . Spontaneous apoptosis associated with effector function measured by annexin level was elevated upon target cell activation regardless of IL-2 conditions **(Fig. 5F)** . However, T cells from the IL-2 withdrawal condition had lower levels of MMP and ROS at both resting state and effector state compared to T cells that were supplemented with IL-2 throughout the bioprocess **(Fig. 5D & 5E)** .

Discussion

Autologous cell therapy would benefit from a cost-effective, streamlined manufacturing process that yields cell numbers relevant for solid-tumor indications with high transgene expression and the desired T cell phenotype. The raw materials and the complexities of unit operations employed by typical bioprocesses, particularly the costly antibodies for magnetic cell selection and high number of touchpoints requiring skilled labor, drive up COGM(ten Ham et al., 2020). The closed autologous T cell bioprocess described here demonstrates that eliminating T cell enrichment by starting with washed leuko-apheresis material not only reduced COGM without compromising EOP CD3+ T cell purity, but also increased transduction efficiency. Expansion conditions including high seeding cell density (7e6 cells/mL), volume, rocking agitation, and low glycolytic environment (inhibiting glycolysis using 2-DG at 5mM) in the bioreactor also positively regulated TCR expression and T cell doubling time.

T cell manufacturing processes involving open steps require cells to be manually transferred between culture wares or fed in a biosafety cabinet within a Grade B cleanroom(Dietz, Padley, & Gastineau, 2007), increasing the risk of cross-contamination or microbial contamination and negatively affecting the efficiency and robustness of T cell manufacturing due to engineering control constraints. The only commercially available all-in-one T cell processing unit, the CliniMACS Prodigy (Miltenyi Biotec), is also limited by its chamber size, scale-out ability and fixed operation programs. Presented here is a fully closed T cell bioprocessing system utilizing single-use disposable transfer bags for raw material transfer and a single bioreactor for flexible and efficient T cell activation, transduction, and expansion. This approach not only reduces the end-to-end processing time and enhances key critical quality attributes of engineered T cells, it increases manufacturing efficiency by enabling processing of multiple lots in parallel within a Grade C cleanroom.

The critical quality attributes (CQA) of commercial autologous T cell therapy include identity, viability, purity, % transgene positive, potency, absence of impurities and sterility(Aijaz et al., 2018; Lipsitz, Timmins, & Zandstra, 2016). To develop and control an autologous bioprocessing system that meets these specifications while accommodating the inherent donor variabilities requires a delicately designed workflow guided by quality-by-design principles(Lipsitz et al., 2016). The integrated operation developed here contrasted with representative CAR-T cell bioprocess where T cells are enriched from leukopak, activated, and transduced in gas permeable bags, and transferred in a bioreactor for expansion, as depicted in**Supplemental Fig. 2A** . We have found that an integrated operation resulted in superior critical quality attributes of engineered T cells, including TCR-T cell yield, TCR expression, % memory subset, and cytotoxicity (**Supplemental Fig. 2B-I**), during a study using a same donor leukopak. This enhancement of COA is possibly attributed to collective factors from soluble activators and non-T cell population, such as B cells and platelets, and bioreactor parameters(Bieback, Fernandez-Munoz, Pati, & Schafer, 2019; Canestrari, Steidinger, McSwain, Charlebois, & Dann, 2019; Chan & Shlomchik, 2000; Deola et al., 2008). Indeed, we found that activation plays a vital role in transduction efficiency and that ImmunoCult CD3/28/2 (StemCell Technologies) dramatically increased

TCR expression compared to TransAct (Miltenyi Biotech) at the same MOI **(Supplemental Fig. 1F)** .

Stimulation drives differentiation of naïve T cell lymphocytes to effector cells and subjects them to activation-mediated apoptosis(Gett et al., 2003). The fitness of engineered T cells correlates with their therapeutic efficacy and long-term persistence post-transplantation(Gett et al., 2003). It also depends on the cellular characteristics that the manufacturing bioprocess cultivates, mainly through the cellular signals that activation antigen and cytokines trigger and the metabolic and mechanobiological environment of the bioreactor(Franco, Jaccard, Romero, Yu, & Ho, 2020; Rushdi et al., 2020; Scharping et al., 2021; Schluns & Lefrançois, 2003). Consistent with findings that mTOR-driven anabolic growth drives T cell differentiation from a naïve and memory-like state to effector mode after activation(Huang, Long, Zhou, Chapman, & Chi, 2020), we found that limiting glucose, the major carbon source of glycolysis, by using the glucose analog 2-DG effectively attenuated the differentiation of naïve T cells into Tem and preserved Tcm during expansion. 2-DG does not affect glycolysis as a single event but may result in a holistic starvation signal that leads to an overall lowered energetic profile(Sukumar et al., 2013). The slight increase in TCR expression that accompanied the enhanced memory phenotype may be a result of redirection of energetic metabolites from mitochondrial oxidation to biosynthesis. After antigen activation, both endogenous and exogenous IL-2 is engaged and further drive the antigen response while regulating pro-survival molecules, such as B cell lymphoma-2 (Bcl-2), until metabolic homeostasis is reached(Gett et al., 2003; Schluns & Lefrançois, 2003). In turn, the strength of activation signals have a profound impact on T cell fitness related to persistence(Gett et al., 2003; Schluns & Lefrançois, 2003). Increased activation signals lead to sustained activation post-production and could elicit life-threatening CRS(Hay, 2018; Obstfeld et al., 2017). Here it is shown that IL-2 withdrawal from feed medium coupled with semi-continuous perfusion resulted in a gradual decline of the activation state of T cells to less than 5% without expanding the population of FoxP3+ Tregs (data not shown). The low activation state of harvested T cells correlated with reduced non-specific killing of T2 cells without affecting their specific killing of target cells. More importantly, the lowered activation signals via IL-2 withdrawal enhanced metabolic fitness of T cells indicated by less polarized mitochondria and mitochondrial mass when in effector mode(Sukumar et al., 2016). Taken together, these results suggest that fine tuning activation signals during T cell manufacturing can impact the phenotypic characteristics of TCR-T cells. These optimizations enabled a robust manufacturing bioprocess that generated a high yield of quality TCR-T cells with a memory phenotype that has been shown to correlate with increased clinical efficacy while also reducing COGM and eliminating many of the manual touchpoints necessary in typical autologous manufacturing processes.

Methods

Generation of autologous MAGE-B2-TCR-T cells in a fully closed bioprocess

On day0, the fresh leukopak apheresis is washed and concentrated to 150e6 cells/mL in complete culture media using Sepax C-Pro cell processing system (Cytiva) using the program CultureWash-Pro with the CT600 kit. Immediately after sampling and determining the viable cell density (VCD), cells are diluted to 1.2e9 nucleated cells and incubated with ImmunoCult CD3/28/2 activator (StemCell Technologies) at a density of 75e6 cells/mL using the Dilution program on the Sepax C-Pro. After a 1-hour incubation with the activator in a transfer bag at room temperature, the cells are inoculated into a semi-static Xuri W25 bioreactor (Cytiva) pre-equilibrated at 37°C, 5% CO2, 0.1 L/min gas flow rate, and 2 rpm at a 2° angle rocking agitation at seeding density of 4e6 cells/mL with 300 mL complete culture media (CTS OpTmizer T Cell Expansion SFM (Gibco) with 2.5% CTS Immune Cell SR (Gibco) and 300 IU/mL IL-2 (STEMCELL Technologies)). On day1, lentivirus for MAGE-B2-speficic TCR corresponding to MOI = 1 functional titer is transferred into the bioreactor through the feed port via gravity flow after sampling and determining VCD. Until the volume within the Xuri exceeds 300 mL, 150 mL medium is exchanged per day using semi-continuous perfusion to maintain glucose and lactate levels above 2g/L and below 2g/L, respectively. Bioreactor volume is increased to 500 mL or 1 L to maintain cell density at 4e6 cells/mL and above with corresponding rocking agitations and semi-continuous perfusion rates until harvest**(Supplemental Fig. 1G)** . Once cells have expanded to reach the desired yield (10-20e9), cells are harvested and washed with 0.9%

saline (Baxter) supplemented with 0.1% v/v human serum albumin (InVitroCare) (HSA) using the Sefia S-2000 Cell Processing System (Cytiva) under FlexCell program and CT-800.1 Cell Processing kit. Using the same system, TCR-T cells are concentrated to cell density of 100-200e6 cells/mL into two cryobags (Origen Biomedical) and formulated with 50% HyClone cryopreservation media (Cytiva) in 0.9% saline with a final HSA concentration of 0.5% v/v. The cryobags are then frozen in a rate-controlled freezer (VIA Freeze Quad freezer (Cytiva)) at a cooling rate of -1°C/min until the temperature reaches -80°C. Cryobags are then transferred to liquid nitrogen (-140°C) for long term storage.

Lentivirus vector construction and production

DNA sequences encoding MAGE-B2-TCR alpha and beta chains separated by a self-cleaving T2A peptide were cloned into the pALD-Lenti expression vector (Aldevron) by isothermal assembly, with an EF1a promoter driving TCR gene expression. A total of five MAGE-B2 TCRs were tested. DNA sequences encoding CARs or GFP driven by a MSCV promoter were also cloned into a lentiviral expression vector for evaluation. Lentivirus was generated by transient transfection of HEK293 cells with third generation LVV packaging plasmids (p-ALD-Lenti, Aldevron) and the expression construct. Lentivirus was harvested from the supernatant 3 days post-transfection,concentrated using high-speed centrifugation, formulated in a sucrose solution and stored at -80 degC(Gandara, Affleck, & Stoll, 2018). Infectious titers were determined by using a flow cytometry cell-based assay on HEK293 and Jurkat cells(Kutner, Zhang, & Reiser, 2009).

In vitro **T cell activation and lentivirus transduction Grex vessels (WilsonWolf) and Permalife cell culture bags (Origen Biomedical)**

T cells or leuko-apheresed cells were stimulated *in vitro* with ImmunoCult CD3/28/2 activator or Immunocult CD3/28 activator at 25 uL per 1 mL of cells in complete culture media for 16-24 hours before transduction. Prior to transduction, viable cell counts were determined using NC-200. Amount of lentivirus at MOI=1 according to their functional titer were used in all experiments except for the lentivirus titration studies. 24 hours following transduction, culture media were topped to 5 mL in small-scale Grex experiments.

To activate and transduce T cells in Permalife cell culture bags, Permalife cell culture bags were coated with OKT CD3 antibody (Milteniyi Biotech) overnight at 4°C prior to cell inoculation. Enriched T cells were transferred to Permalife cell culture bags with soluble 15E8 CD28 antibody (Milteniyi Biotech) followed by transduction at MOI=1 24 hours after activation. Cells were transferred into Xuri W25 bioreactor 1 day after transduction for scale-up expansion.

T cell enrichment

Using Sepax C-pro, the fresh leukopak was washed and concentrated using CliniMACS PBS/EDTA Buffer (Miltenyi Biotech) supplemented with 0.1% HSA and then incubated with antibodies specific to CD4 and CD8 (Miltenyi Biotec) at room temperature for 30 minutes. Labeled leukopak cells were then transferred on CliniMACS Plus (Miltenyi Biotech) and positive fraction of flow through was collected according to manufacturers' instructions.

Flow cytometry immunophenotyping

Human antibodies specific for CD3 (SK7, Biolegend), CD4 (SK3, Biolegend), CD8 (RPA-T8, BD Biosciences), CD45RA (HI100, Biolegend), CD45RO (UCHL1, Biolegend), CCR7 (REA108, Miltenyi Biotec), CD95 (DX2 , Biolegend) were used for low cytometry acquisition for the determination of Tn, Tcm, Tem, Tte phenotype. 8 color immunophenotyping kit specific for CD3, CD5/16, CD19, CD14, CD45 (Miltenyi Biotec) were used for flow cytometry acquisition for the determination of T cell purity, B cell, NK cells, NKT cells, and monocytes percentage. MAGE-B2 TCR dextramer (Immudex) and anti-human TCR Vβ13.2 (H132, Biolegend) were used to detect MAGE-B2 TCR expression on CD8+ T cells and CD4 + T cells. FACS Symphony or FACS Fortessa (BD Biosciences) was used for flow cytometry acquisition. Samples were analyzed with FlowJo software.

Reactive oxygen species (ROS), mitochondrial membrane potential (MMP), mitochondrial

mass, and Annexin

For analysis of intracellular ROS, TCR-T cells were incubated with CellROX Orange at 5 μM in complete culture media for 30 minutes at 37°C. For detection of effector TCR-T cells intracellular ROS level, TCR-T cells were incubated with MAGE-B2 peptide-loaded T2 Luc Red cells at E:T ratio=1 for 24 hours, cell mixtures were then incubated with CellROS Orange at 5 uM in complete culture media for 30 minutes at 37°C. Cells were then labeled with human antibody against CD3 and CD8 for 15 minutes at 4°C, and ROS level was determined by flow cytometry analysis using mean fluorescence intensity of CellROS Orange gated on CD8+ and CD3+ double positive cells.

Similarly, MMP and mitochondrial mass was measured by labeling cells either by themselves or after co-cultured with T2 Luc Red cells at E:T ratio=1 for 24 hours with $DilC_1(5)$ at 50 nM for 15 minutes at 37°C or MitoTracker Red CMXRos at 25 nM for 15 minutes at 37°C, respectively. Cells were then labeled with antibodies specific for CD3+ and CD8+, followed by flow cytometry analysis.

For analysis of Annexin V, TCR-T cells or cell mixtures of TCR-T cell and MAGE-B2 peptide-loaded T2 Luc Red cells after 24-hour co-incubation were labeled with antibodies specific for CD4+ and CD8+ then washed and labeled with FITC Annexin V in Annexin V Binding Buffer at 1e6 cells/ml for 15 minutes at room temperature in the dark. Cells were diluted with Annexin V Binding Buffer to 0.2e6 cells/mL and analyzed by flow cytometry.

T cell killing assay

T2 cells engineered with luciferase reporter (Luc) were maintained in IMDM supplemented with 2mM L-glu, 20% FBS, 2 ug/mL Blasticidin, and 0.25 ug/mL Puromycin. T2 Luc cells were pulsed with MAGE-B2 peptide at 10 μM for 4 hours. Previously frozen TCR-T cells were thawed and mixed with MAGE-B2 peptide-pulsed T2 Luc cells at various E:T ratio in CTS OpTmizer T Cell Expansion SFM (Gibco) with 2.5% CTS Immune Cell SR (Gibco). Cell mixtures were incubated at 5% CO_2 and 37°C for 48 hours. Steady-Glo® Luciferase (Promega) was added to cell mixture according to manufacturer's protocol. Luminescence was read using EnVision plate reader (PerkimElmer) 5 minutes after reaction.

Human Interferon-gamma (IFN-g)

TCR-T cells were incubated with MAGE-B2 peptide-loaded T2 Luc cells at various E:T ratios for 24 hours in CTS OpTmizer T Cell Expansion SFM (Gibco) with 2.5% CTS Immune Cell SR (Gibco) at 1e6 cells/mL. Supernatant was collected and analyzed for IFN-g using AlphaLISA Human Interferon-gamma Kit (PerkinElmer) according to manufacturer's manual. Sample and standard data was collected using EnVision plate reader (PerkimElmer).

Metabolites, cell counts, and viability measurements

Cell culture supernatant was collected and analyzed for glucose (Glc), Lactate (Lac), pyruvate (Pyr), and lactate dehydrogenase (LDH) using Cedex Bio Analyzer (Roche) according to manufacturer's instructions. Cell counts and viability were collected using NucleoCounter® NC-200 (ChemoMetec) according to manufacturer's protocol.

Statistics

Data sets were compared using 1- or 2-tailed unpaired Student's t test. For all analyses, a P value less than 0.05 was considered statistically significant. * = $P < 0.05$; ** = $P < 0.01$;

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Figure Legends

Fig. 1 . Process and final T cell product characterization of a closed autologous bioprocess. Transduction efficiency of lentivirus coding for CAR, TCR, and GFP measured at day8 (A). T cell purity of leukapheresis at start and following activation and expansion compared to T cell purity following CD4 and CD8 antibody positive enrichment (B). In-process measurements of cell growth (C), viability (D), glucose (GLC & E), lactate (LAC & F). End-of-process TCR expression of CD8 and CD3 T cells detected by PE-dextramer (G). Donor leukopak and EOP CD3 T cell purity (H) and cytotoxicity against MAGE-B2 target cells (I).

Fig. 2 . Fully closed end-to-end bioprocessing system generating TCR-T cell for autologous T cell therapy. Illustration of autologous bioprocess, including washing and activating leukopak apheresis, lentiviral vector transduction and cell expansion in a bioreactor, harvesting and formulating final TCR-T cell product into on desired cell concentration, and finally cryopreservation.

Fig. 3 . Optimization of bioreactor parameters to improve lentiviral vector transduction efficiency and end-to-end process time. In-process cell growth (A), viability (B), CD3 cells percentage (C), TCR expression (D), secretion of IFN-gamma after coculturing with MAGE-B2 peptide loaded T2 cells (E), cytotoxicity against T2 cells pulsed with MAGE-B2 peptide (H), and the expression of CD45RA and CCR7 of CD8+ cells to indicate Tscm, Tcm, and Tem (G).

Fig. 4 . Enrichment of memory T cell subset in final TCR-T cell product via glycolysis inhibition. Glycolysis inhibitor 2-DG at 2mM was added in culture media starting from inoculation until day6 or day9. In-process cell growth (A) was monitored. CD8 T cell differentiation was compared for their percentage of Tscm, Tcm, and Tem subsets in-process at day4 and at harvest for two presentative donors (B). EOP TCR-T cells of CD8+ cells were shown with two representative donors (C). MMP (D), and mitochondrial mass (E) at basal and activated state, and cytotoxicity against MAGE-B2 peptide (+) and (-) T2 cells (F).

Fig. 5. IL2 withdraw from culture medium at day5 or day6 reduced background activation of EOP TCR-T cells with improved metabolic fitness. Impact of IL-2 level on CD25 expression (A), cytotoxicity of TCR-T cells against MAGE-B2 peptide loaded and no peptide loaded T2 Luc cells at E:T ratio 0.2, 1, and 5 (B), EOP TCR expression detected by PE-dextramer (C), levels of mitochondrial membrane potential (D), annexin (F), and ROS (E) of TCR-T cells.

Supplemental Fig. 1. Expression of CD25 of CD8+ T cells following ImmunoCult CD3/28/2 activation in Xuri W25 bioreactor (A). TCR expression at MOI from 0.25 to 10 (B). Vbeta vs Dextramer expression specific to MAGEB2 TCR of CD8 and CD4 cells at harvest (C). Representative percentage of B cells, monocytes, and NK cells of leukocytes following activation in Xuri W25 bioreactor (D). CD8 T cell differentiation as a

function of cell growth (E). TCR expression of CD8+ T cells activated by ImmunoCult CD3/28/2 activator and TransAct at MOI of 1 (F). Rocking agitation parameters optimized at different volumes (G).

Supplemental Fig. 2 . A leukopak was split and processed through either the integrated bioprocess developed in current study or a representative autologous bioprocess. In the integrated and fully closed bioprocess, leukopak cells were washed, and 1.2e9 cells were directly activated with ImmunoCult CD3/28/2 and transduced in a 2L Xuri W25 bioreactor, as described in **Fig. 2 and A** . In the presentative and semi-closed bioprocess, the remaining leukopak from the same donor was enriched for T cells using CD4 and CD8 antibody positive selection on the CliniMACS Plus (Miltenyi Biotec) and 0.5e9 T cells were activated in PL240-2G PermaLife Cell Culture Bag (OriGen Biomedical) with plate-bound CD3 and soluble CD28 antibodies (Miltenyi Biotec) at day0. Cells were transduced at MOI of 1 in both conditions on day1. On day2, T cells activated and transduced in PL240-2G PermaLife Cell Culture Bag were transferred into a Xuri W25 bioreactor, scaled up from 500ml to 1L on day3, followed by semi-continuous perfusion at 500ml -1L/day until harvest **(Fig.3A)** . In-process cell growth (B), CD25 expression (C), viability (D), percentage of CD3 positivity (E), TCR expression were monitored (F). FACS analysis of CD8+ T cell differentiation when cell yield was at 8e9(G). Cytotoxicity against MAGE-B2 peptide pulsed T2 Luc cells at E:T ratio of 1:1 and 1:5 (H). IFN-gamma secretion of TCR-T cells cocultured with MAGE-B2 peptide pulsed T2 cells at 10nM, 1uM, 0uM concentrations (I).

Table 1. Bioreactor parameters tested in optimization study

Bioreactor	Inoculation density (e6cells/ml)	DO (%)	Bioreactor end volume (L)	Agitation
Xuri #1	3.5	None	1	+
Xuri #2	3.5	None	0.5	++
Xuri #3	3.5	100% since Day0	0.5	++
Xuri #4	7	None	0.5	+
Xuri #5	7	100% since Day5	0.5	++

Figure 1

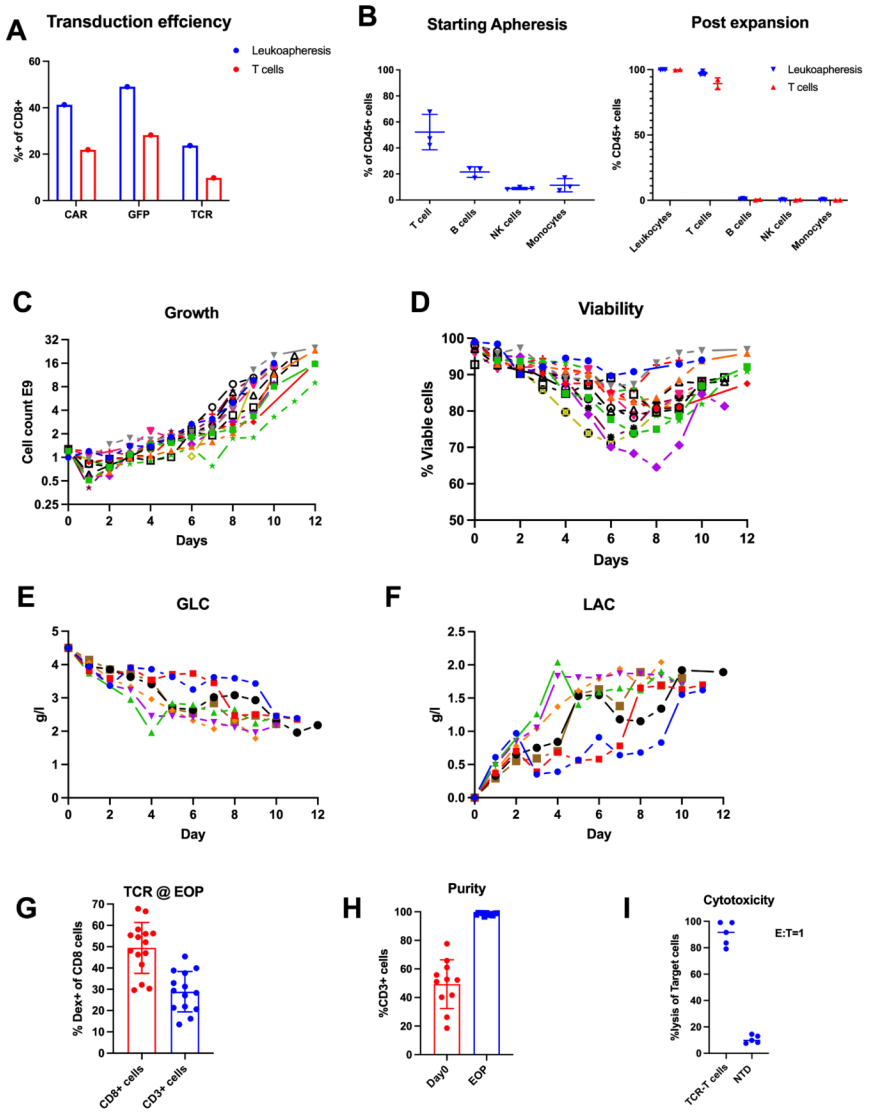


Figure 2

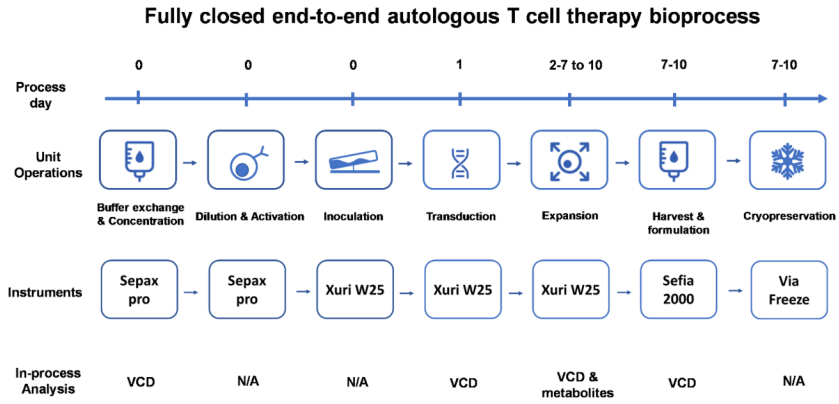


Figure 3

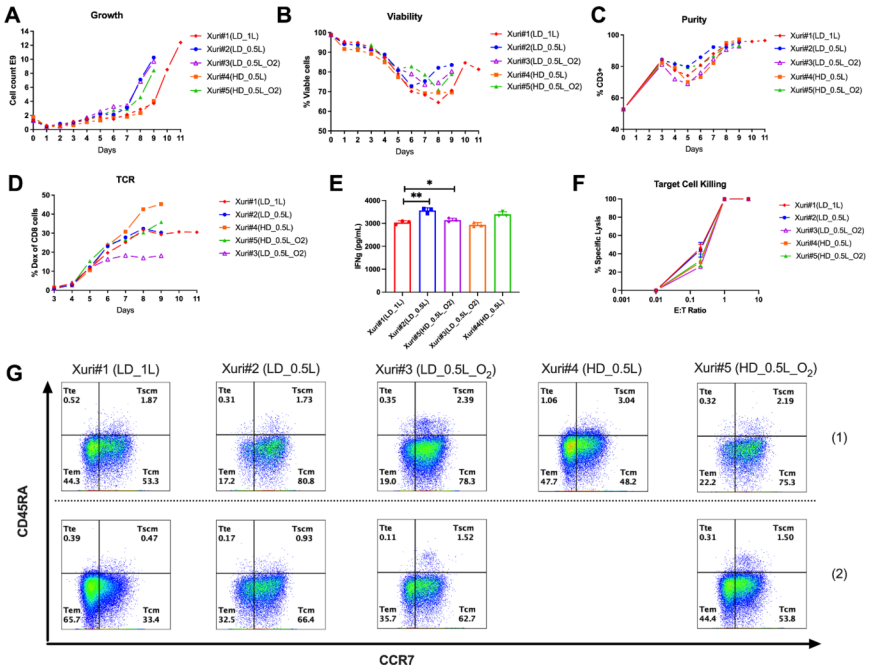


Figure 4

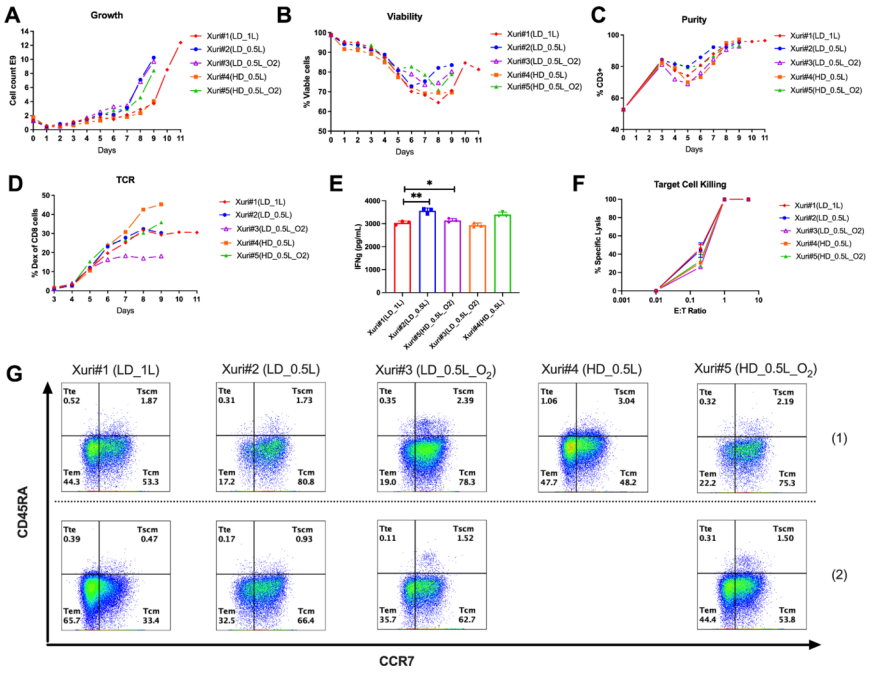
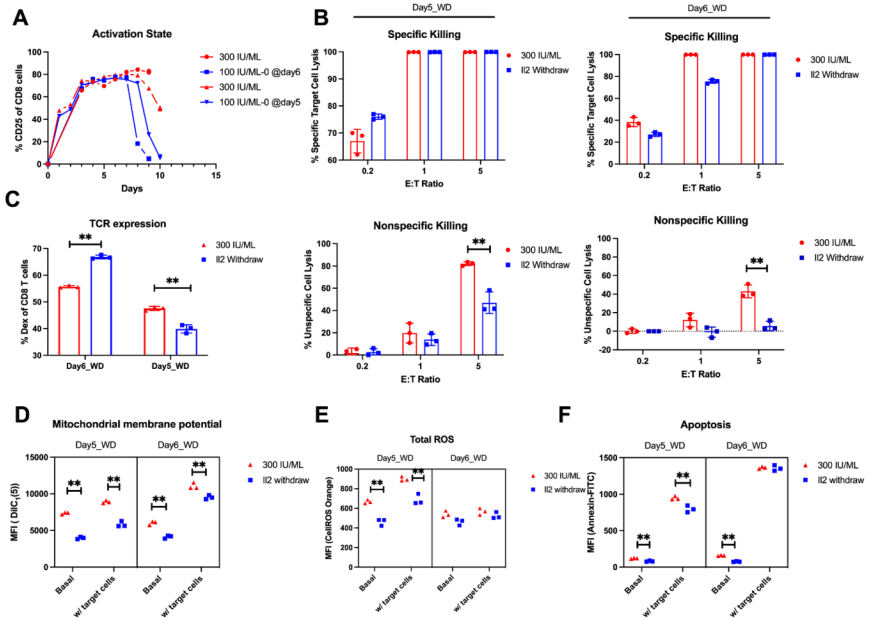
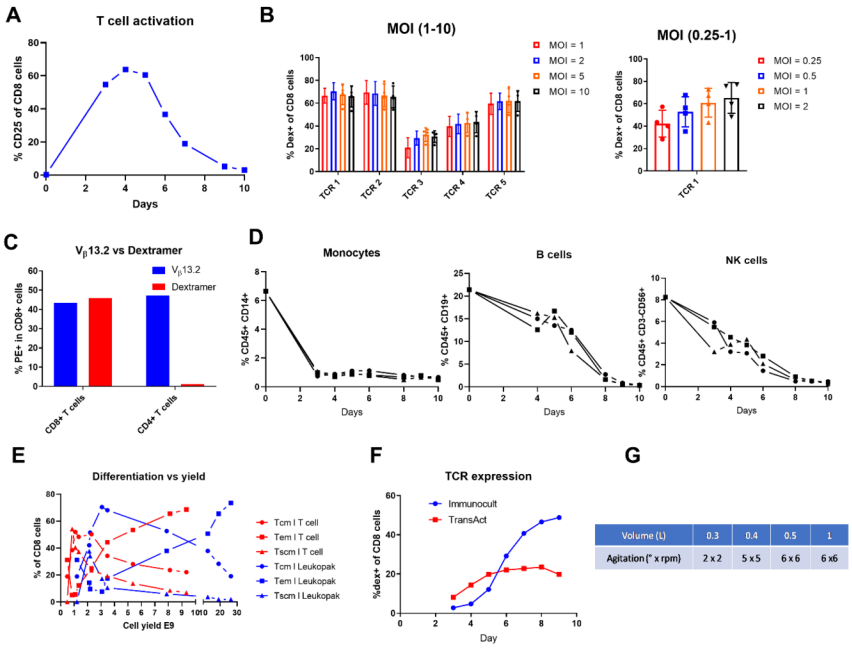


Figure 5



Supplemental Figure 1



Supplemental Figure 2