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RESEARCH ARTICLE

Technological and Manufacturing Innovation Drive Improved Access to Engineered T Cell Therapies

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ABSTRACT

The creation of autologous gene-modified cell products, such as CAR-T cells (chimeric antigen receptor T cells) has met with clinical success but has been severely restricted by cost, availability, and current commercial models of central manufacturing. Moreover, the inability to produce CAR-T cells at reasonable cost in all but the largest centers slow innovation. CAR-T cells are unique in that these *ex vivo* expanded effector T cell populations express activation receptors comprised of immunoglobulin-like binders, or other immune ligands, bypassing the restriction of expanding and appropriately activating effector cells that arise by recombination of V-D-J genetic elements. However, the use of a single binding moiety to recognize leukemia target cells has selected for the generation of escape mutants, or for cryptic clones to expand that were not initially detected upon diagnostic work-up. To meet this challenge, we have engineered both B cell malignancy-specific and HIV surface antigen-specific CAR-T cells that express multiple binding moieties, thereby reducing the chance of immune escape. The creation of a therapeutic CAR-T cell population also requires a complex set of procedures that includes procurement of a large number of patient T cells, most often by leukapheresis, activation of the T cell population using matrix-associated antibodies targeting the T cell receptor and an immune co-receptor, a gene vector to permanently transduce T cells, and a bioreactor that can accommodate the expansion of the engineered cell population to numbers suitable for infusion. This complex set of procedures, combined with the current central processing model, has led to a complex chain of custody and expensive temperature-controlled shipping requirements. We present here a model whereby production of CAR-T cells at the point of care, using simplified cell procurement, purification, and expansion, can reduce the time and expense of CAR-T cell generation, with the aim to expand the use of these therapies in both the majority world, and in managed care or publicly funded systems. The CAR-T populations produced in this point-of-care ready process are highly active, viable, and have preferred CAR-T phenotypic characteristics associated with clinical efficacy.

Introduction:

Cancer immunotherapy has pioneered the use of multi-agent chemotherapy approaches in order to avert escape mechanisms due to the genetic variation inherent in the tumor. Hodgkin lymphoma was one of the first diseases to be effectively treated by multi-agent chemotherapy[1]. In 2023, new advances combining multi-agent chemotherapy therapy with immune checkpoint blockade show improved outcomes for Hodgkin lymphoma as well[2]. Nevertheless, for most hematologic malignancies' resistance to CAR-T therapy is ascribed to variation in target antigen structure or number[3]. Therapy for HIV infection was similarly ineffective when single agents were tested, as resistance to therapy quickly arose. Combination therapy with multiple agents is highly effective, is able to meet the challenge of viral diversity and mutation, and has changed the face of the disease entirely[4]. The use lentiviral (LV) or retroviral gene vectors to engineer primary human T cells from peripheral blood with chimeric antigen receptors (CARs) has changed the course of treating advanced B cell leukemias and lymphomas[5]. For one of the most common adult hematologic malignancies, B cell lymphoma, it has become clear that CAR-T therapy is superior as a second-line therapy in comparison to current standard of care[6].

However, the approved products for B cell leukemia and lymphoma all target a single cell surface protein, CD19, and leukemic clones may use either alternative splicing or evolve mutations that prevent anti-CD19 CAR T-cells from recognizing the malignancy[7]. To meet this challenge, we have begun to explore novel CAR structures and expression vectors that can target multiple ligands. In the first instance we developed a tandem CAR-T cell product that expresses anti-CD19 and anti-CD20 immunoglobulin derived binders linked by a simple amino acid linker[8]. This construct functioned as an "or" gate and targeted B cell malignancies expressed either single or both of the target antigens, and thereby proved highly effective in clinical trials[9, 10].

The initial push for a CD19 CAR-T cell based therapies, and the amazing use of a pediatric study for the very first approval, was due to the lack CD20 expression of pediatric pre-B ALL, even as anti-CD20 antibody-based therapy being tested an found effective in adult malignancy[11]. The second major effective CAR-T cell product developed for hematologic malignancy targeted another B cell antigen expressed on pediatric pre-B ALL, and was initially used to treat CAR-19 treatment failures, namely CD22[12, 13]. The creation of a tandem (two binders on a single chain) CD22 CAR-T cell product has proven difficult due the sensitivity of the m971 CD22 binder to structural changes[14]. Thus, alternative approaches have featured bi-cistronic or dual transduction (using two lentiviral expression vectors, one for each CAR), resulting in two separate glycoproteins on the T cell surface, each with a single binder[15, 16]. In order to develop a single lentiviral vector (LV) that would target both pediatric and adult indications, we developed an approach targeting three B cell antigens at the same time, CD19, CD20 and CD22[8, 17]. In this construct, the clinically validated CD19-CD20 tandem CAR was combined with the validated m971 CD22 binder. In contrast to leukemia, no CAR-T cell product has been approved for therapy of HIV. Our own research has demonstrated that that multiple binding moieties are able to overcome HIV escape mechanisms in model systems[18, 19]. The concern with multiple antigen binder-expressing LV is the stability of the large vector product, and the ability to generate LV of sufficient titer. In this report we demonstrate that larger format LV that express advanced CAR structures can readily be generated.

One of the most vexing challenges before the treatment community, which includes patients, physicians, managed care networks, and national governments, is the cost of engineered T cell therapies. CAR-T cell therapies are desired because of their high degree of effectiveness. Their cost, however, has put them out of the reach of many[20 21]. Retrospective analysis has demonstrated that CAR-T recipients tend to be white and located near major medical centers[22]. The problem is even more acute when considering deploying these technologies in low- and middle-income countries[23]. Thus, in addition to economic barriers, geography is a major issue. One approach to lowering costs is using local manufacturing of CAR-T to avoid the cost, regulatory challenges and logistics of shipping cell products to central manufacturing plants. Producing the CAR-T at the facility where it will be employed is referred to as "point-of-care" manufacturing[24]. In this scenario a hospital purchases the essential equipment, trains staff, and creates controlled lab space or clean-room for CAR-T manufacture[25]. Using the CliniMACS Prodigy® platform, local manufacturing in India has now begun in earnest[26]. However, this approach still requires costly reagents and a single application dedicated instrumentation. Thus, development of low-cost universal workflows, that can be standardized to meet regulatory requirements, remains a primary driver in cellular engineering for those interested in equitable access. Presented here is an approach that can be widely adopted for point-of-care manufacture of engineered immune

cell products, focusing on CAR-T. While the use of an advanced instrument that requires dedicated tubing sets is still required, the Sepax™ C-Pro, its multi-use programs and flexibility for numerous cell processing steps allows for continued innovation and new approaches to decrease overall process and facility cost.

Methods:

T cell enrichment: Enriched T cells were generated from either fresh whole blood or leukopak products. When fresh whole blood was used as starting material, total blood volume was first reduced using the SmartRedux program on the Sepax™ C-Pro (Cytiva, Marlborough, MA). Then the reduced blood product was co-cultured with Caring Cross Rosette cocktail (T-Pure) for 20 min at room temperature, followed by processing using the NeatCell program on Sepax C-Pro for negative T cell selection utilizing Ficoll-Hypaque gradient centrifugation. When starting with a fresh quarter leukopak product, a matched volume of diluted O- red blood cells (Innovative Research, Novi, MI) were added to the leukopak product to allow for sufficient rosette formation. Subsequent staining and purification procedures were the same as for the whole blood product, without the volume reduction step.

CAR-T manufacturing: Fresh enriched T cells were transferred to culture vessels and immediately supplemented with anti-CD3 and anti-CD28 coated particles for activation, using either Miltenyi Biotec (T Cell TransAct™, AM1) or GeneScript (Enceed™, AM2) products, and cultured at 1 x10^6/ml in X-vivo™15 (Lonza) supplemented with 2% KnockOut™ serum replacement (ThermoFisher) and 30 U IL-2/ml (Miltenyi Biotec), unless noted otherwise. For small scale transduction either T75 flasks or G-Rex®6M (Wilson Wolf, St. Paul, MN) were used. Lentiviral vector (LV) encoding multi-specific CARs was added to cells at a multiplicity of infection (MOI) of 40 on Day 0 of activation, and either a complete medium exchange or a 1:10 medium dilution was carried out on Day 3. For large-scale transduction a G-Rex®100M vessel was used, with the same protocol as for small-scale CAR-T manufacturing, with medium dilution on Day 3. Cell cultures were expanded to day 8, with analysis carried out in-process on Day 6 and at harvest on day 8.

Human Cells and Cell Lines: Human whole blood or leukapheresis products were commercially obtained from Stemcell Technologies (Vancouver, BC, Canada). Human RBCs were purchased from Innovative Research (Novi, MI). Cell lines were purchased from ATCC, (Manassas, VA), unless otherwise noted. The Raji Burkitt lymphoma cell line was purchased from the Leibniz Institute DSMZ (Braunschweig, Germany). Cell lines were cultured in RPMI-1640 medium (Thermo-Fisher) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Hyclone, Logan UT). HEK293 cells were propagated in DMEM and 10% FBS (Thermo-Fisher). Single-cell clones of luciferase-expressing target cell lines for functional assays were created by transduction with firefly luciferase-expressing LV.

Lentiviral vector production: Lentiviral vector (LV) was produced as described earlier[17-19]. In brief, HEK293 were transfected with plasmids encoding VSV-G, Gag-Pol, REV, and LTR/SIN-LTR bounded CAR sequence under the control of an EF-1α promoter, using PEI (Polyplus), induced with sodium butyrate at 24 h and supernatant collected and concentrated 200-fold at 48-72 h post-transfection with Lenti-C concentrator (Takara Bio), aliquoted and frozen at -80°C. LV functional titer was determined by dilution series, transduction quantified by flow cytometric analysis of CAR expression on SupT1 cells.

Flow Cytometry: For CAR detection, CAR T cells were stained with recombinant human CD19 Fc (R&D systems, 9269-CD-050) and human recombinant CD22 His-tag (Thermo Fisher, A42609) for 10 min at room temperature. After 2 washes, cells were stained with goat anti-human Fc Alexa Fluor 647 (Jackson ImmunoResearch, 109-606-098), anti-His PE (Miltenyi Biotec, 130-120-718), anti-CD4-Vioblue (Miltenyi Biotec, 130-113-219), and 7-AAD (Miltenyi Biotec, 170-081-088) for 20min at 4°C. Activated but un-transduced T cells were used as control for CAR expression. For T cell memory phenotyping, anti-CD45RA APC/Vio770 (Milltenyi Biotec, 130-113-353), anti-CCR7 PE/Cy7 (BD Biosciences, 567313), and anti-CD95 FITC (Miltenyi Biotec, 130-124-214) were used. FMO controls of CD45RA, CCR7, and CD95 were used to gate different memory populations. Stained cell samples were analyzed using a CytoFlex flow cytometer (Beckman-Coulter), and data was analyzed by FlowJo.

Cytotoxic T cell (CTL) activity: Un-transduced (UTD) and transduced T cells were co-incubated with either 5x10^3 Raji-luciferase cells or 5x10^3 293T-luciferase cells at 20:1, 10:1, 5:1, 2.5:1 ratios in triplicate. After 24 hr of co-culture, Steady-Glo substrate (Promega) was added into each sample well, positive control wells (tumor alone), and negative control wells (lysed tumor). Luminescence expression was read by Envision (Perkin Elmer) and calculated as follows:

$$Lysis\% = \left(1 - \frac{Lsample - Lneg.}{Lpos. - Lneg.}\right) \times 100\%$$

$Lsample: Luciferase\ of\ sample$
$Lpos.: Average\ Luciferase\ of\ positive\ ctrls$
$Lneg.: Average\ Luciferase\ of\ negative\ ctrls$

Culture supernatants from T cell (effector cell) vs. Raji (target cell) co-cultures were collected prior to Steady-Glo addition, and analyzed by for ELISA of human IL-2, IFN-g, and TNF-a (Thermo-Fisher) on the same day as luminescence reading for cytolysis.

Statistical Analysis: Statistical Analysis was carried out using GraphPad Prism software (v10.0) Statistical significance was determined by average triplicate sample wells and standard deviations calculated, multi-group comparisons were analyzed by one-way ANOVA, and paired t-test for comparison between two groups.

Results:

To decrease the cost of CAR-T manufacture, our first goal was to simplify the processes currently employed and to create protocols amenable to point-of-care manufacturing. Currently, CAR-T manufacturing begins with leukapheresis. Leukapheresis is then followed by some form of T cell selection. This can be performed using antibody embedded in degradable biomaterials seeded with paramagnetic ferrous nanoparticles on a device like the CliniMACS® Plus (Miltenyi) or by an "on-bead" technique where large format activation beads (i.e. DynaBeads™, Thermo Fisher), are used to separate T cells during the activation step on a magnetic table. Large format beads must be removed prior to clinical use, adding another process step. Following T cell isolation, reagents for T cell activation, a gene vector, and growth media containing cytokines must be added.

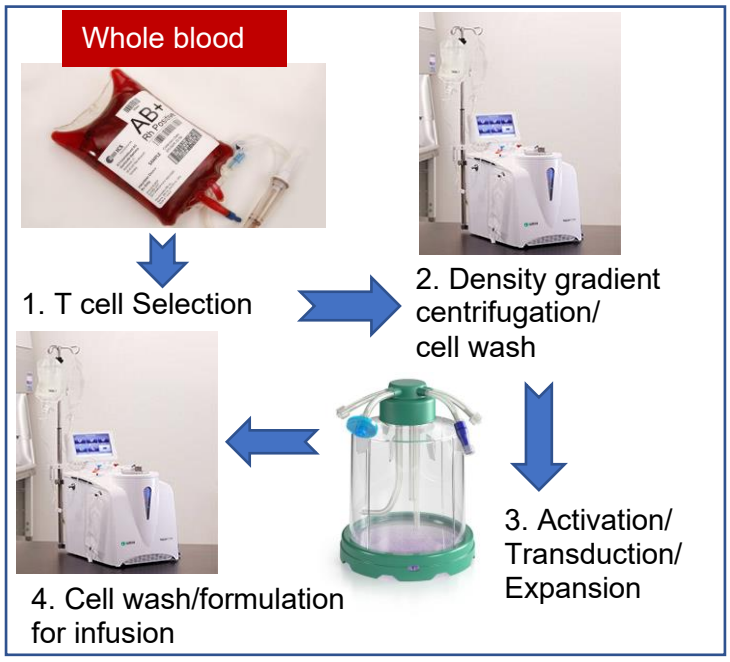


Figure 1. Production of CAR-T Cells using a simplified workflow. *CAR-T manufacturing begins with securing a blood product, depletion of unwanted cell types by density gradient centrifugation, using RBC rosetting to deplete B and myeloid cells, introduction into a culture flask and a final formulation step, again achieved by a closed-system centrifugation step.*

T cells transduced by the gene vector are then expanded in culture. During the production process, as well as at harvest, quality assays must be carried out to insure sterility and CAR expression. Although not required for early-stage development, as CAR-T cells move toward commercial approval, potency assays also need to be developed. An obvious first step to simplify CAR-T manufacturing would be to remove the need for apheresis and the attendant specialized equipment, trained operators, clinical suite, and medical oversight required. We therefore developed a process using whole blood as a starting material, Figure 1.

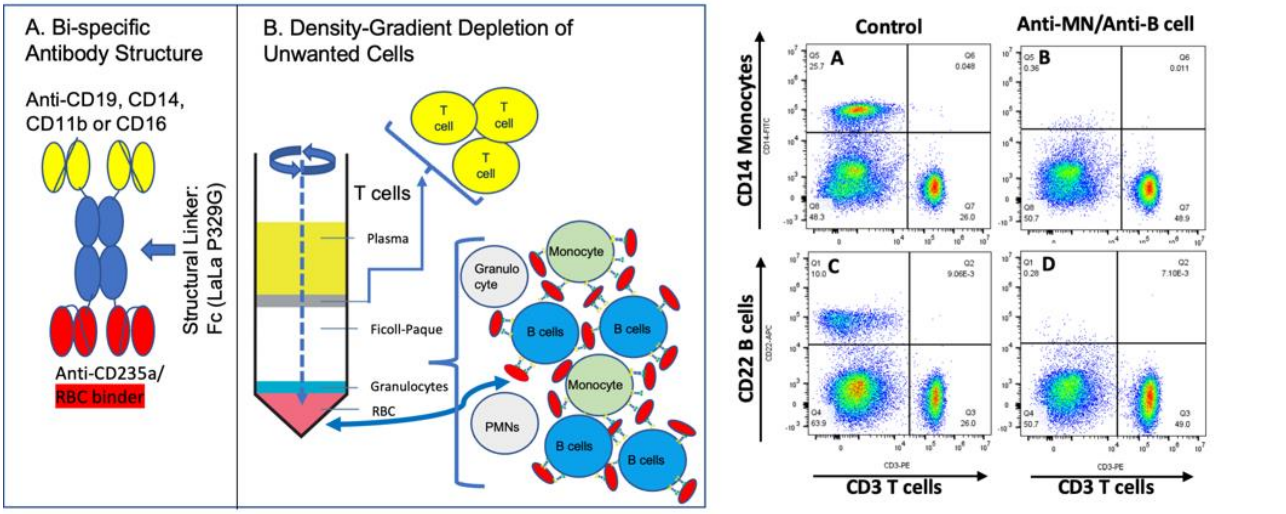
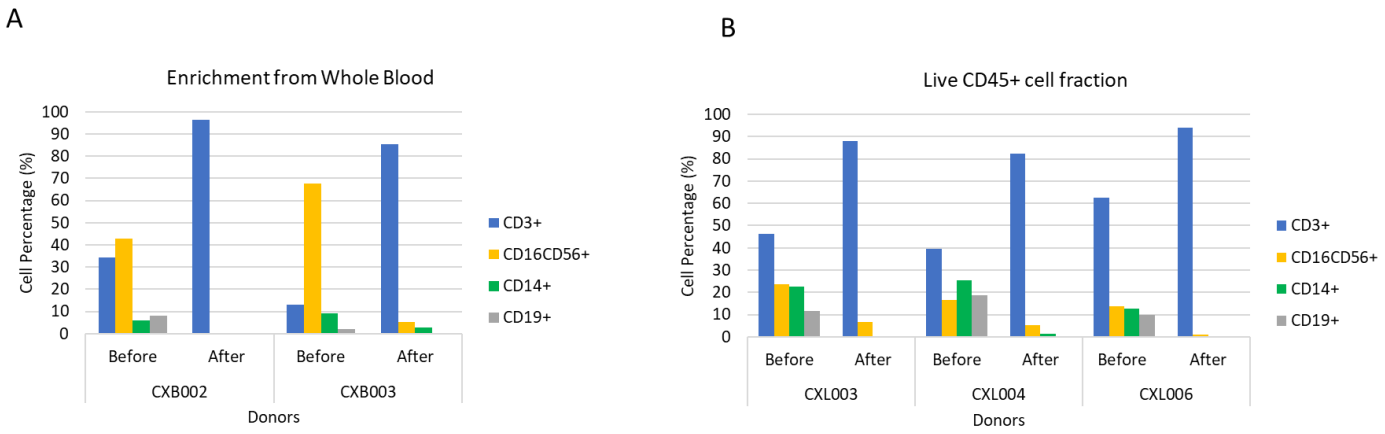


Figure 2. Rosetting purifies T cells by negative selection. *Bi-specific antibody structure used to purify away unwanted cell types (left panel, A) features binding to CD235a and either CD19, 14, 11b, or CD16. Unwanted cell types are rosetted by cross-linking to RBCs, which then allows the unwanted cell types to be removed by centrifugation over a Ficoll-Paque density gradient (left panel, B). Depletion of unwanted cell types using either no depleting antibody (right panel, control column) or anti-MN (CD14) or anti-B cell (CD19) bispecific antibody. The persistence of the resulting T cell population in the lower right quadrant of each panel indicates that T cells have been preserved in the interface (grey band in (B)), and unwanted cells spun to the bottom of the gradient along with RBCs and granulocytes and removed from subsequent culture.*

Given the frequency of T cells in peripheral blood, a standard blood donation amount of 450 ml, or approximately 1/10th of total blood volume, yields more than enough T cells for CAR-T manufacturing. The process described here can also be adapted for leukapheresis, but then requires the addition of a small amount of whole blood in order to supply sufficient RBC for the rosetting process. If leukapheresis is required, we plan to use autologous RBC, collected at the time of apheresis, although use of a type O-neg universal donor could be envisaged. Critical to the process was the creation of a set of bi-specific antibodies. Ig sequences derived from anti-CD19, -CD20, -CD11b, -CD14, or -CD16 were linked by an immunoglobulin constant region (Fc, LaLa P329G) which does not interact with FcRs (Figure 2). This allows for rosetting driven only by CD235a expression on RBCs and the target antigen (Figure 3).



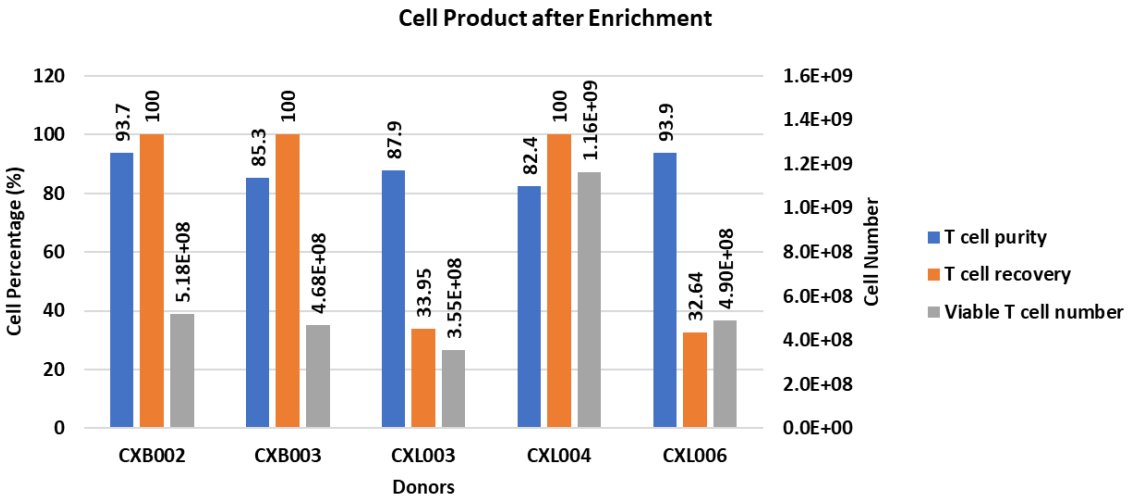
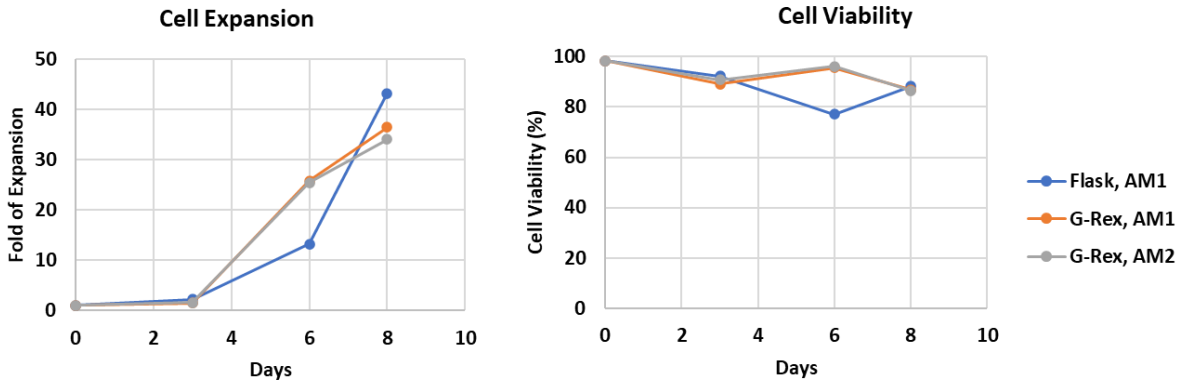


Figure 3. Clinically compliant T cell purification by rosetting. *T cells were enriched from either 2 peripheral blood (**A,** CXB) or 3 quarter leukopak (**B,** CXL) donors. Resulting cell populations were analyzed by flow cytometry, gated on viable CD45+ cells, and analyzed for expression of CD3, CD14 (monocytes), CD19 (B cells), and CD16/CD56 (myeloid and NK cells). Lymphocyte phenotypes prior to purification (before) and after (after) are indicated for each donor.* **C**) *T cell purity, percentage of recovery, and actual T cell number recovered after each enrichment.*

The purification process was notable for depleting the 2 most unwanted cell types: myeloid cells which can inhibit T cell expansion, and B cells which may prove a hazard if transduced with the CAR. Once purified by negative selection, the purified T cells were used for CAR-T manufacturing. In all cases more than 1 x10^8 T cells were isolated, exceeding the desired target cell number for clinical CAR-T manufacturing. Using either a standard tissue culture flask as a control, 20 x10^6 cells total, or a G-Rex vessel, 10 x10^6 cells total, and two different commercially available T cells activation matrices (AM1, AM2), PBMC were transduced with a CD19.20.22 CAR expression LV at an MOI of 40. The initial flask size used was 75 cm^2, which was then increased to 150 cm^2 as the culture expanded. A single G-Rex vessel was used. Cells expanded equally with either AM1 or AM2 in the G-Rex vessel, expanding 25-fold to day 6, and 35-fold to day 8 (Figure 4A). Expansion was somewhat slower in flasks, yielding approximately 12-fold on day 6, but then exceeding 35-fold by day 8 (Figure 4A). Similar CAR expression values were seen in all three conditions on Day 6 (Figure 4B) and Day 8 (not shown).

A



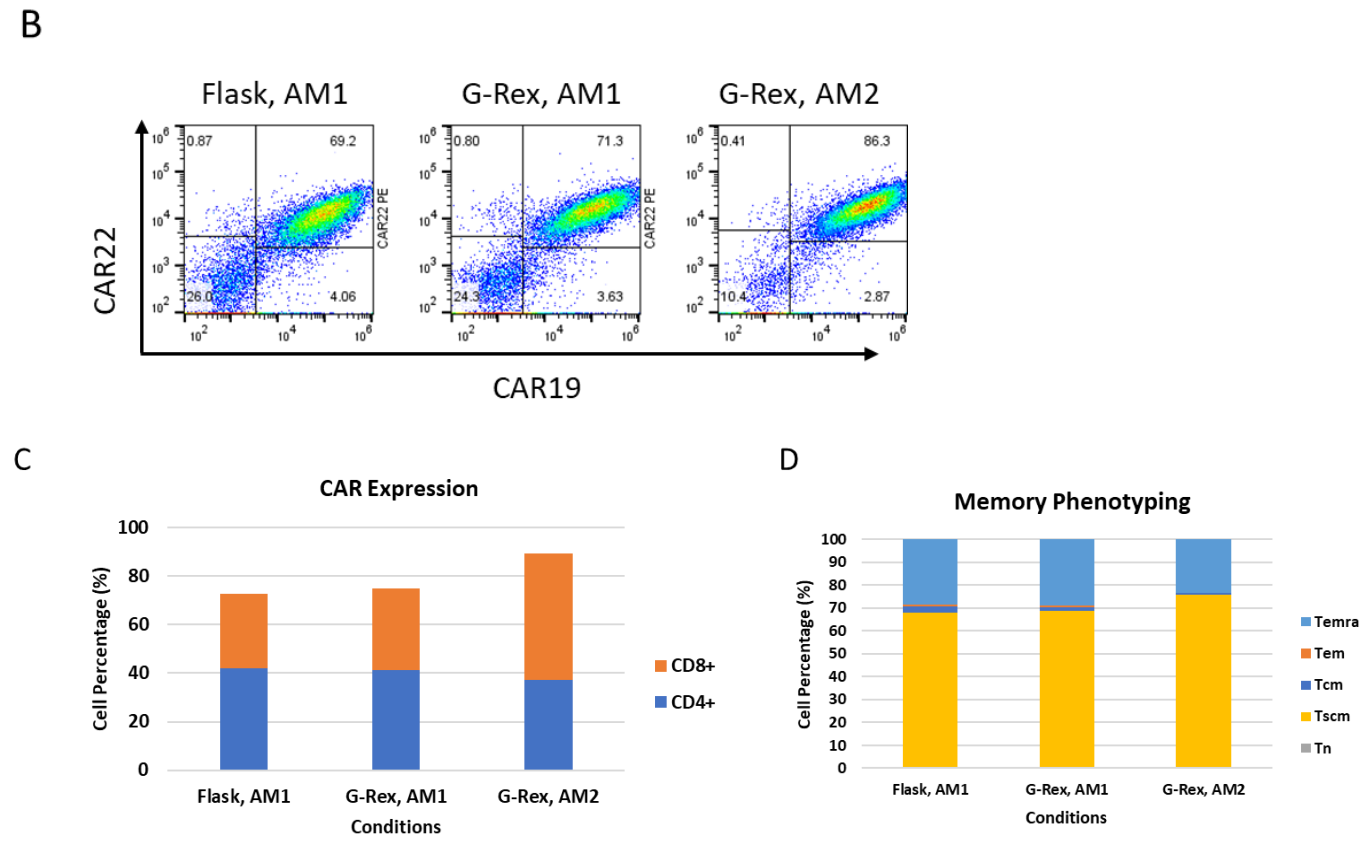


Figure 4. Comparison of tri-specific CAR expression with different vessels. **A.** *CAR-T Cell expansion in G-Rex 6M and traditional flasks.* **B.** *Demonstration of CAR expression by flow cytometry with cells harvested in-process on Day 6 of activation. Double positive expression of αCD19 and αCD22 CARs on T cell surface was shown in the flow plots,* **C.** *Percent CAR expression from panel 4B, gated and quantified for CD4 and CD8 populations.* **D.** *Expression of T cell memory phenotypes. Cell populations generated were almost exclusively T-stem cell memory (yellow, Tscm, CD45RA+, CCR7+, CD95+) or T-effector memory (blue, Temra, CD45RA+, CCR7-) with minimal generation of T-effector memory (Tem, CD45RA-, CCR7-), T-central memory (Tcm, CD45RA-, CCR7+) or T-naïve (Tn, CD45RA+, CCR7+, CD95-).*

Using AM1 and standard tissue culture flasks, the expression of CARs on the surface of transduced T cells was equivalent whether CAR-T cultures were expanded in the G-Rex culture flask, using either AM1 (Figure 4B, center panel) or AM2 (Figure 4B, right panel). This presents new options for clinical CAR-T manufacture. A key attribute of CAR-T activity is the preservation of a stem-cell associated phenotype[27]. It is also essential that there is not significant skewing between the CD4 and CD8 effector cell populations.[28] Using an abbreviated phenotypic analysis, CAR-T cell products were evaluated by flow cytometry for total CAR expression in the CD4 and CD8 T cell subsets (Figure 4C), and for the expression of surface markers associated with a Tscm phenotype, CD45RO-, CCR7+, CD95+ (Figure 4D) were examined. Highly favorable Tscm phenotype CAR-T cells were generated using rosette-purified T cells.

Having confirmed the phenotype and expansion characteristics in different vessels and with different activation matrices of T cells purified using negative-selection by means of antibody mediated rosetting, we examined in detail further approaches to simplify CAR-T manufacturing using rosetting and the G-Rex expansion platform (Figure 5). Additionally, as AM2 is a relatively new product, we performed further direct comparisons to insure its suitability. The next reduction in total protocol steps we instituted was to remove a culture wash at day 3, by simply increase culture media in the culture vessel. The TransAct matrix (AM1) is known to degrade over time, but we did not know if AM2 would perform similarly. Figure 5A demonstrates that for either matrix simple increase the culture volume, 10-fold on day 3, instead of introducing the process step of a wash (requiring some form of centrifugation and media removal) was sufficient to insure both CAR-T cell viability and expansion. We

then explored the impact of omitting the wash step on CAR-T phenotype with regard to CD4/CD8 ratio and memory/differentiation markers (Figure 5B). Here again, the substitution of a wash step for a simple increase in culture volume did not impact the CD4/CD8 ratio or the memory phenotype. In fact, it was the time in culture that appeared to impact the CD4/CD8 ratio more, a characteristic of the serum-free system and supplementation with IL-2 used.

Following phenotypic analysis, we moved to functional CAR-T assays to ensure the new manufacturing approach did not impact CAR-T cell cytolytic or cytokine production activity.

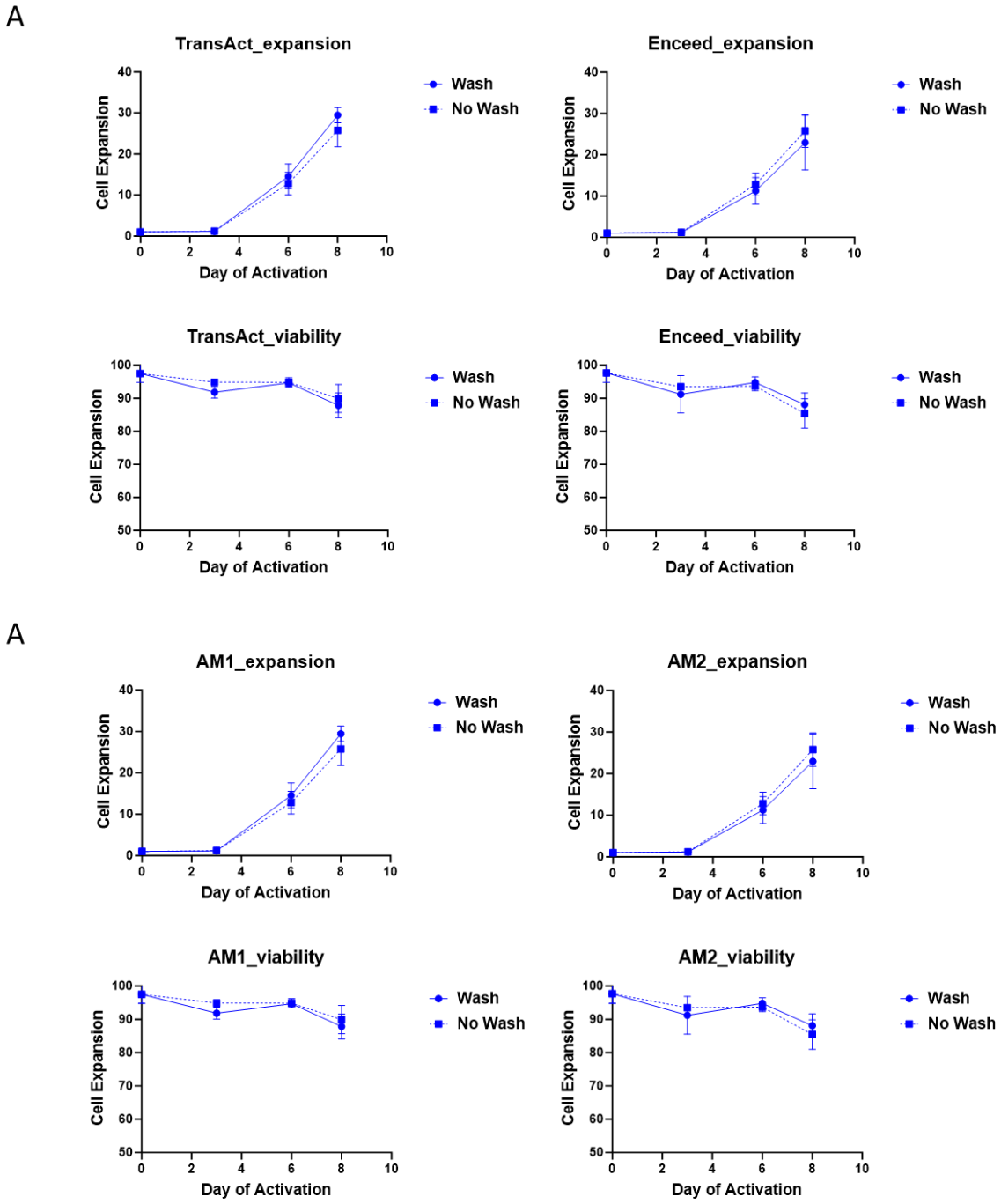


Figure 5. Analysis of streamlined CAR-T transduction protocol. A) *Cell expansion with or without medium exchange when different activation reagents were used. The CAR-T production protocol included a wash step with a complete medium exchange on day 3 (wash) or without a wash step and instead increasing the cell culture volume 10-fold with pre-warmed fresh medium with IL-2 on day 3 (no wash).* B) *CAR expression and memory phenotyping at different days of CAR-T expansion, x-axis, when different transduction protocols were followed, as in figure 4, a representative data set of 3 individual donors tested is shown.*

When AM1 and AM2 were compared head-to-head, with and without a culture wash step on day 3, cytolysis of the Raji lymphoma cell line was not only unaffected, the omission of a wash step gave slightly higher activity, Figure 6A, on both in process day 6 and final harvest cultures, day 8.

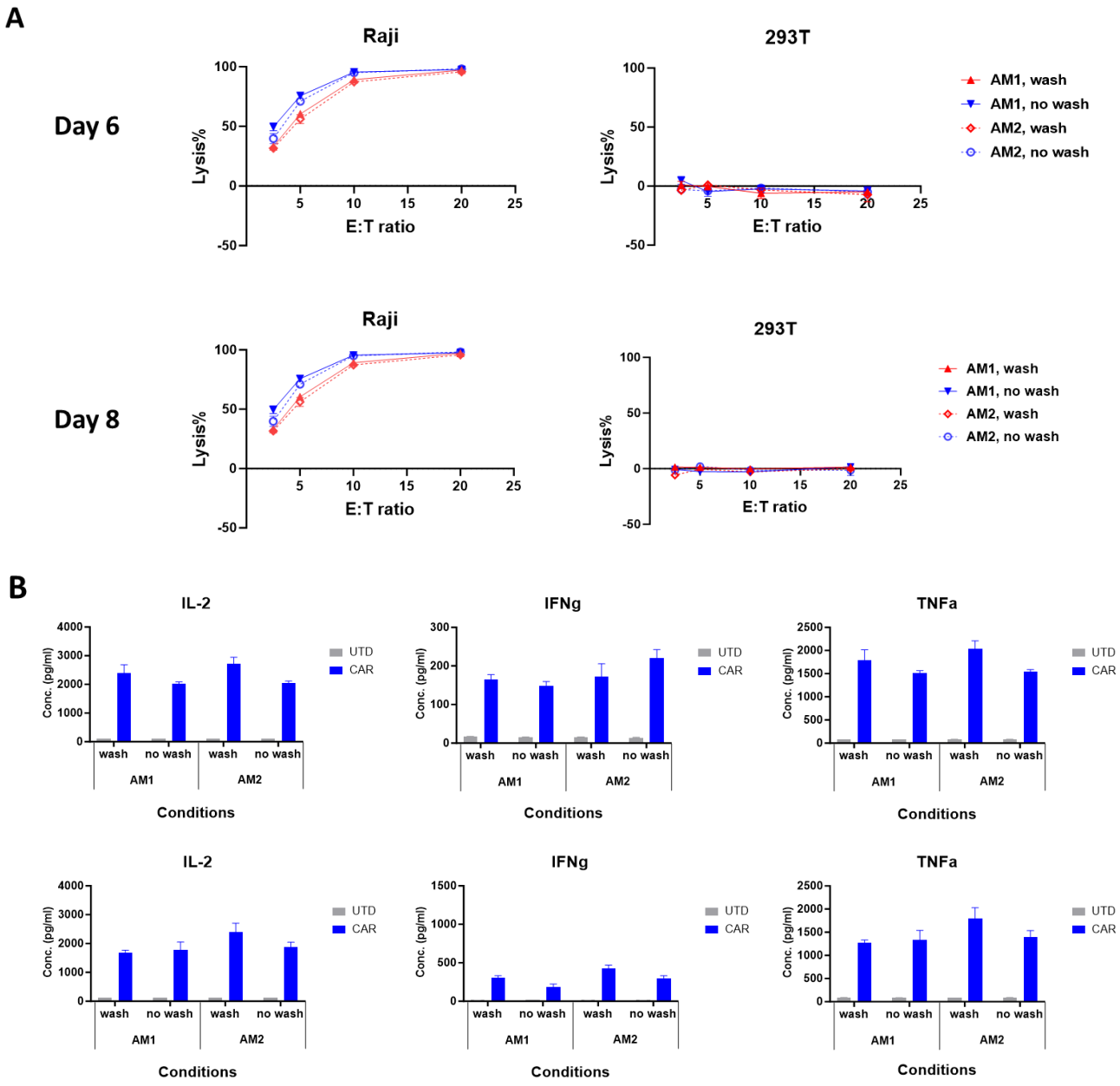


Figure 6. CTL Activity of CAR-T cells transduced in G-Rex 6M. **A.** *Overnight cytolysis assay performed using CAR T cells co-incubated with either Raji-luc cells (positive tumor line) or 293T-luc cells (negative tumor line) at E:T=20:1, 10:1, 5:1, 2.5:1 (x-axis).* **B.** *Cytokine production in the co-culture media was also tested, with supernatant of T cell/Raji co-cultures (CAR) or non-transduced but activated and cultured T cells (UTD), at an E:T=10:1, analyzed by ELISA for IL-2, IFN-g (IFNg), and TNF-a (TNFa) on day 6 (top row) and day 8 (bottom row). Data presented is representative of three individual donors tested.*

When CAR-mediated cytokine release was tested, Figure 6B, small differences were seen between samples, but all expressed high levels of the three cytokines used to ascertain T cell cell function. As with cytolysis there is no negative impact of using an alternate activation matrix or omission of a wash step. Our final verification of this new process for CAR-T manufacturing was to scale-up to the vessel size and format that can be used for clinical CAR-T production.

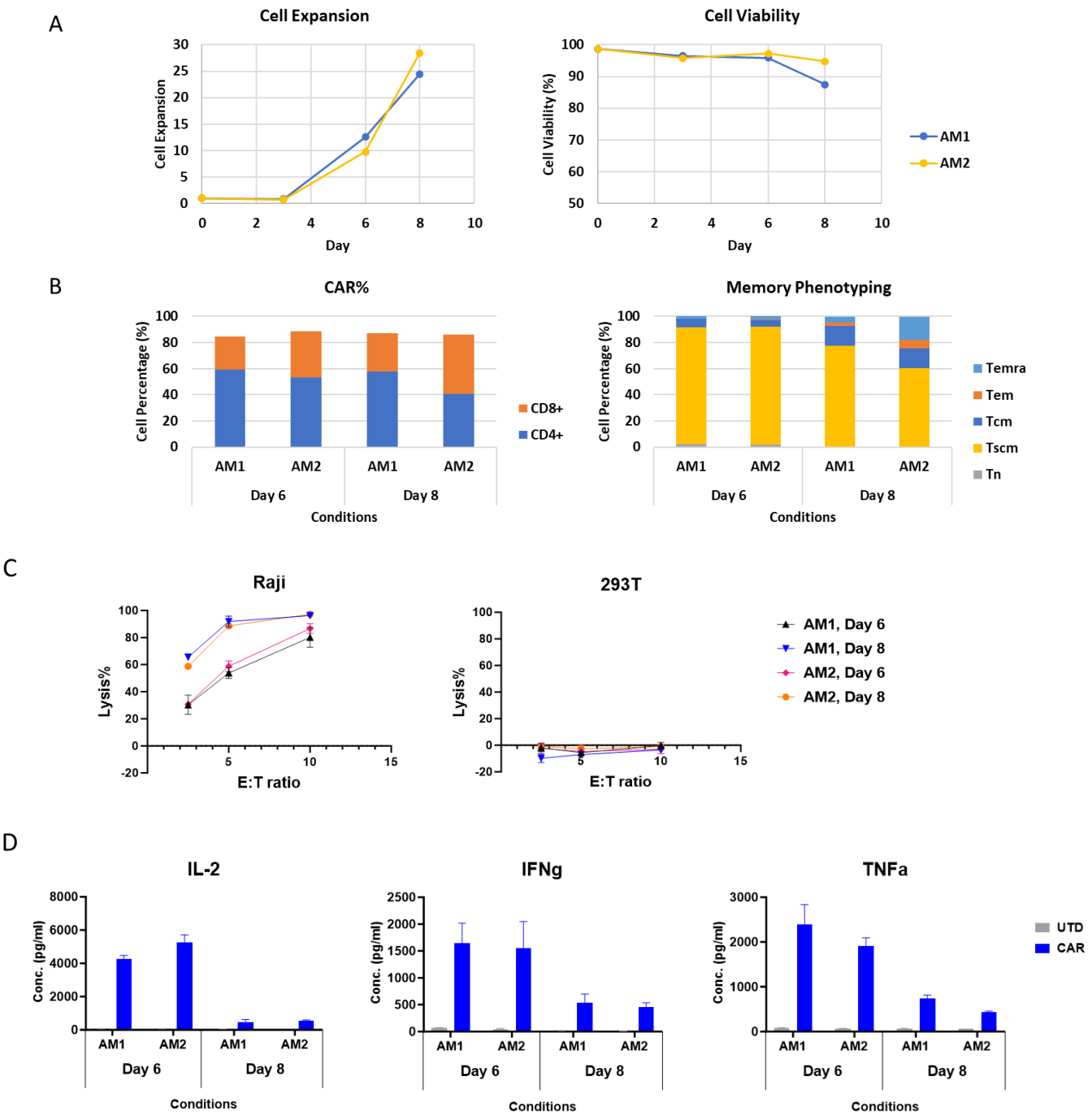


Figure 7. Large-scale CAR-T transduction in large-format static culture. A) *Cell expansion (left) and viability (right) after the activation and transduction of 1x10⁸ T cells in G-Rex100M with no wash streamlined protocol, comparing the two available activation matrices for CAR-T manufacturing (AM1, blue, AM2, yellow).* B) *CAR expression in CD4 and CD8 cells (left) and memory phenotyping (right) of CAR T cells manufactured at large scale and tested in process, day 6, and at harvest, day 8.* C) *Cytolytic activity of CAR-T cells for target cell (Raji, left panel) or control cell line (293T, right panel) of CAR T cells at E:T=10:1, 5:1, 2.5:1 (x-axis) tested on day 6 (triangles), day 9 (circles), and with either AM1 (black, blue) or AM2 (red, orange).* D) *Expression of IL-2, IFN-g and TNF-a upon co-culture with Raji target cell line, at a 10:1 ratio, by CAR-T cells (blue) or control T cells (UTD).*

It was interesting to observe that cultures tested on day 6 had superior cytokine production, while those on day 8 had superior lytic activity, independent of activation matrix. As with the smaller scale process, the clinical scale process produced a CAR-T cell population that was favorable with regard to both expression of the CAR on the T cell surface, and preserving a considerable population of Tscm, favoring resistance to exhaustion in vivo activity.

Discussion:

The currently approved CAR-T products are created from T cells transduced with retroviral (RV) or lentiviral vectors (LV) expressing binders targeting either CD19 or BCMA. We have moved the field forward through creating multi-targeting vectors that can target either three B cell malignancy antigens (CD19, CD20, CD22) or multiple sites on the HIV envelope (CD4 and CCR5 binding site)[17, 19]. However, advancing the breadth or potency of the CAR itself is not sufficient to make this very expensive therapy more broadly and equitable. Here the primary issue is cost. The debut of the CliniMACS Prodigy® device was initially proposed as a means to lower cost, as multiple steps in the process of CAR-T production and the need for large clean-room facilities were obviated[25]. However, the technology remains somewhat restrictive, unless it is embedded within a larger manufacturing program at a national level, whereby corporate restrictions have been addressed. This has been demonstrated by the CAR-T manufacturing program that originated in Barcelona and has now become a national-level program for Spain[29]. This point of care, academic-based approach has been so effective that it is moving forward to gain regulatory approval by the Spanish Agency for Medicines and Health Products (AEMPS), designated as ARI-0001, and now moving forward to European approval[30, 31]. Along these lines we have designed an even lower-cost approach that no longer requires dedicated devices, but incorporates the Cytiva's Sepax™ C-Pro, which can be utilized for multiple process in a clinical cell processing lab and has open, non-restrictive programming options.

Beyond devices, the highest impact we have proposed in lowering the technological and financial barrier in CAR-T manufacturing is to replace the expensive process of T selection from the harvested lymphocyte population using immunomagnetic beads. Furthermore, our process opens the possibility of removing apheresis entirely. RBC-based rosetting has long been recognized as a way to employ the common procedure of density-gradient centrifugation to remove unwanted cell types. Although laboratory-based kits have been long employed, such as the Human T cell isolation kits manufactured by STEMCELL™ Technologies, there is no GMP-compliant option[32]. We demonstrated that depletion of unwanted cell types by a new cocktail set of bi-specific antibodies was able to efficiently create a B cell and myeloid depleted mononuclear cell product, almost exclusively composed of T cells, that was a suitable substrate for the manufacture of next-generation multi-specific CAR-T cells (Figure 2). Significantly, the creation of this approach may allow for whole blood as opposed for apheresis products to be used in CAR-T manufacture. The studies carried out thus far have been from healthy peripheral blood donations. We have not yet verified the amount of T cells that can be derived from leukemia patients using whole blood donation. We anticipate most will be able to donate, and if not, we can use leukapheresis with peripheral blood add-back in order to initiate rosetting (Figure 3). When Korell, F., et al., examined this very question in a group of lymphoma and acute leukemia patients, apheresis was sufficient for almost all patients even with an extremely low peripheral lymphocyte count of 180 (per uL)[33]. Only 4 of 45 patient aphereses required a second leukapheresis, at which time the target cell number was met. Using this reported value for peripheral lymphocyte counts, even the patients with the lowest counts would yield 0.5 $x10^8$ cells per 400 mL donation. Another major driver of cost is the requirement for a GMP-grade activation reagent. With the entry of a second such reagent onto the market, our hope is that further competition will address the cost of the reagent. Our data demonstrate functional equivalency (Figure 4). We have streamlined standard CAR-T manufacture and simplified the process even further by removing from the protocol the need for a wash step on day 3 (Figure 5). Each technical step requiring cell manipulation adds cost, minimally the need for a new sterile tubing set to transfer the CAR-T expansion culture from the culture vessel to the centrifuge. The use of a large format G-Rex device allows for all major steps to be carried out on the day of initiation (Figure 1 and Figure 6), and only sterility and product qualification steps near the end of the culture period would require further technical handling. While a clinical protocol incorporating the advances presented here is yet to implemented in a clinical trial, we present here all the steps required to build a low-cost CAR-T point-of-care production pipeline, at scale, that can be implemented in a global setting either in a dedicated clean-room area or in a mobile clean-room setting.

One of the major advantages of a point-of-care workflow is the marked decrease in the amount of time required to generate a therapeutic CAR-T cell product. Specifically, we are referring to the true "vein to vein" time. Ghassemi, S., et al., published a 24-hour CAR-T manufacturing process that showed efficacy in mouse models[34]. Although only presented thus far in meeting abstracts, Novartis has developed a single-day CAR-T manufacturing process for CD19 and BCMA-specific CAR-T products called T-Charge [35, 36]. The motivation for a single day approach was to generate cells with the least time in culture, and thereby more stem-like properties. This process still requires freezing of the product and full testing, and thereby may not significantly impact vein to vein time, especially in a centralized manufacturing model. Our own data shows that even between day 6 and day 8 changes in T cell activity occur (Figure 6,7). There currently is no agreed upon characteristic of a CAR-T culture that predicts patient outcome, and this has complicated the true definition of a potency assay for this living drug. Even so, our process, because it uses fresh product, manufactured at the point of care, is indeed the fastest process possible. Manufacturing and infusing a freshly produced product cannot be increased beyond 6 days because of the requirement of a washout-our period for the cyclophosphamide-fludarabine based lymphodepletion that CAR-T therapy requires. Until this step is removed, a process shorter than 6 days does not represent a savings in time. In data that surprised the field, both Kite/BMS and Novartis sought to gain second-line approval for CAR-T therapy. In the clinical trials carried out to gain approval, only the Kite study met the threshold required for second-line approval[37, 38]. While there is no direct evidence, and patient groups often vary, in this discussion we propose that increased manufacturing and vein to vein times may indeed play a role. The longer a patient waits to get a CAR-T cell product when indicated, the more advanced their disease becomes. Thus, in addition to the cost and access issues that local manufacturing can solve, it also may prove to be the most effective means to give CAR-T therapy to patient, when disease burden is lower, to it makes this therapy available to patients whose rapidly progressing disease would not allow for central manufactured products to be generated.

Finally, the new low-cost process we have described creates a highly functional CAR-T cell product. Even though we tested alternative activation matrices, removed the wash step normally incorporated after addition of activation matrix and gene vector, and used a low-cost static culture vessel, cell expansion and viability remained optimal. The ability to lyse target cell lines and produce cytokine upon co-culture with target cells was also consistent with a high-quality effector T cell product. Upon making such changes, one concern would be that the T cells would be of lower quality, that is, there would be lower transgene expression in the transduced T cell population and the quality of the cells, with regard to metabolic fitness would be reduced. However, neither of these was the case. The expression of the CAR was not reduced, the CD4 to CD8 ratio was not altered, and the phenotype exhibited by the newly created CAR-T populations was favorable. Most notably, in the large-format static culture process, the vast majority of the CAR-T population exhibited the T-stem cell memory (Tscm) phenotype, Figure 7B. This gives confidence that the point-of-care adaptable process we have described here is suitable for clinical development.

Conclusion:

Advanced medicinal products, such as gene modified T cells are ushering in a new therapeutic era for cancer and infectious disease. Manufacturing chimeric antigen receptor (CAR)-modified T cells is an expensive commercial process that restricts access to underserved populations in developed nations, as well as to the majority world. The creation of a low-cost CAR-T manufacturing process using easily obtained and multi-functional pieces of equipment geared toward point-of-care manufacturing significantly decreases the barriers of logistics, the challenges present in the global supply chain, and the need for a long-distance cold chain for product delivery. The process we have developed is adaptable to both established and mobile clean-rooms and delivers a product superior in expressing multi-antigen specific CAR products, characterized by high in vitro activity and favorable phenotypic profiles associated with resistance to in vivo exhaustion upon clinical testing.

COI statement: BD serves as CEO of Vector BioMed. RJO serves on the scientific advisory boards of Galapagos NV, and Umoja Biopharma.

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