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Streamlined production of genetically modified T cells with activation, transduction and expansion in closed-system G-Rex bioreactors

CHRISTINE GAGLIARDI, MARIAM KHALIL & AARON E. FOSTER

Bellicum Pharmaceuticals, Houston, Texas, USA

Abstract

Background: Gas Permeable Rapid Expansion (G-Rex) bioreactors have been shown to efficiently expand immune cells intended for therapeutic use, but do not address the complexity of the viral transduction step required for many engineered T-cell products. Here we demonstrate a novel method for transduction of activated T cells with Vectofusin-1 reagent. Transduction is accomplished in suspension, in G-Rex bioreactors. The simplified transduction step is integrated into a streamlined process that uses a single bioreactor with limited operator intervention. *Methods:* Peripheral blood mononuclear cells (PBMCs) from healthy donors were thawed, washed and activated with soluble anti-CD3 and anti-CD28 antibodies either in cell culture bags or in G-Rex bioreactors. Cells were cultured in TexMACS GMP medium with interleukin (IL)-7 and IL-15 and transduced with RetroNectin in bags or Vectorfusin-1 in the G-Rex. Total viable cell number, fold expansion, viability, transduction efficiency, phenotype and function were compared between the two processes. *Results:* The simplified process uses a single vessel from activation through harvest and achieves 56% transduction with 29-fold expansion in 11 days. The cells generated in the simplified process do not differ from cells produced in the conventional bag-based process functionally or phenotypically. *Discussion:* This study demonstrates that T cells can be transduced in suspension. Further, the conventional method of generating engineered T cells in bags for clinical use can be streamlined to a much simpler, less-expensive process without compromising the quality or function of the cell product.

Key Words: *autologous, bioreactors, cell therapy, chimeric antigen receptor T cells*

Introduction

Manufacturing autologous T-cell therapies can be a complex process. Gene transfer by viral transduction to generate chimeric antigen receptor (CAR) T cells, for example, has commonly been performed by transferring activated T cells to bags or plates coated with RetroNectin (Takara Bio USA, Inc., Mountain View, CA, USA). The coating step requires dilution, transfer and incubation of the RetroNectin solution with subsequent washing and viral incubation before T cells are added. After transduction, cell suspensions are washed and transferred to additional culture vessels. Several of these steps are often open, labor-intensive and cumbersome.

Alternatively, transduction of T cells in suspension could simplify viral gene transfer and reduce time, cost and risk of errors. Vectofusin-1 (Miltenyi Biotec Inc., Auburn, CA, USA) is a synthetic peptide that can enhance viral transduction when added in solution with virus and cells [1]. Vectofusin-1 forms α-helical nanofibrils that associate with viral particles that enhance virus-cell interactions [2]. Transduction in solution with Vectofusin-1 eliminates the need for surface coatings, expanding the potential vessels in which T cells might be transduced.

Gas Permeable Rapid Expansion (G-Rex; Wilson Wolf Corporation, Saint Paul, MN, USA) bioreactors are disposable single-use vessels specifically engineered to culture and expand immune cells. Like gas-permeable bags, G-Rex vessels are dependent on incubation in standard cell culture incubators. G-Rex technology relies on a silicone gas-permeable membrane on which cells reside and through which oxygen and carbon dioxide can be passively, efficiently exchanged [3,4].

The G-Rex has been shown to be effective in expanding tumor-infiltrating lymphocytes (TILs) [5,6], natural killer (NK) cells [7–9], Vγ9Vδ2 T cells [10], regulatory T cells [11] and virus-specific T cells (VSTs) [12–15]. In the context of genetically modified cells, such as CAR T cells, G-Rex bioreactors do

Correspondence: **Christine Gagliardi**, PhD, Bellicum Pharmaceuticals, 2130 W. Holcombe Blvd Suite 800, Houston, Texas, USA. E-mail: cgagliardi@bellicum.com

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not directly address the added complexity of gene transfer, however, viral transduction in solution with Vectofusin-1 may allow for an integrated process in which all unit operations are carried out in a single G-Rex bioreactor.

The objective of this study was to simplify and optimize the transduction and expansion steps of manufacturing genetically modified T cells for clinical use. We demonstrate a streamlined, novel method for transducing T cells with retrovirus directly in a G-Rex bioreactor using the Vectofusin-1 reagent, followed by robust expansion with no need for operator intervention until harvest.

Materials and Methods

Starting material

Buffy coats from healthy donors were purchased through the Gulf Coast Regional Blood Center (Houston, TX, USA). Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coat using density gradient centrifugation with Lymphoprep medium (STEMCELL Technologies Inc., Cambridge, MA, USA) per the manufacturer's protocol. PBMCs were cryopreserved at 5×10^7 cells/mL in fetal bovine serum with 10% dimethyl sulfoxide and stored in liquid nitrogen until needed.

Culture imitation and T-cell activation

Cryopreserved PBMCs were thawed and washed in cell culture medium. Small-scale experiments aimed at understanding growth kinetics on the G-Rex membrane were cultured and activated in a 50:50 mix of Gibco RPMI 1640 medium (Thermo Fisher Waltham, MA, USA) and Clicks Medium EHAA (FUJIFILM Irvine Scientific, Santa Ana, CA, USA) with 10% fetal bovine serum. For transduction and large-scale experiments, TexMACS GMP medium (Miltenyi Biotec) was used.

Cells were counted and resuspended at $1-2 \times 10^6$ cells/mL in medium with 15 ng/mL interleukin (IL)-7, 5 ng/mL IL-15, 0.2 μg/mL anti-CD3 antibody and 0.5 μg/mL anti-CD28 antibody. Cytokines and antibodies were from Miltenyi Biotec. Cell suspension was transferred to VueLife "C" bags (Saint-Gobain Cell Therapy, Gaithersburg, MD, USA) and incubated at 37°C, 5% CO_2 for 2 days.

For activation in G-Rex 6M 10 cm^2 plates (Wilson Wolf Corporation), PBMCs were thawed, washed and counted. Aliquots of 1×10^7 viable cells were resuspended in 2.0, 4.0 or 10.0 mL medium with 15 ng/mL IL-7, 5 ng/mL IL-15, 0.2 μg/mL anti-CD3 antibody and 0.5 μg/mL anti-CD28 antibody. The cell suspensions were transferred to individual 10-cm^2 wells and incubated at 37°C, 5% CO_2 for 2 days.

Transduction

Two high-titer retroviral constructs were used for this study. One virus, used in the small-scale experiments, carries a rapamycin-induced, caspase-9 safety switch (iRC9) with a truncated CD19 marker that was designed to work in combination with rimiducid-controlled elements [16]. The other, used in the large-scale process, carries a first generation prostate stem cell antigen (PSCA) CAR with rimiducid-inducible co-stimulation (inducible MyD88/CD40 [iMC]) and a CD34 epitope that can be used to measure transduction efficiency [17–19].

For transduction in bags, 20 μg/mL RetroNectin (Takara) in phosphate-buffered saline was incubated in VueLife "AC" bags (Saint-Gobain) overnight at 4°C. The RetroNectin solution was removed and viral supernatant diluted in cell culture medium was incubated in the bag for at least 30 min at room temperature. Activated T cells were washed, resuspended in culture medium with 15 ng/mL IL-7 and 5 ng/mL IL-15 and added to the viral supernatant in the bag. Bags were incubated at 37°C, 5% CO_2 for 1 h, then flipped and incubated for 18–24 h. After incubation, cell suspensions were washed in cell culture medium, resuspended at $0.5-1.0 \times 10^6$ cells/mL in medium with 15 ng/mL IL-7 and 5 ng/mL IL-15, transferred to VueLife "C" bags and incubated at 37°C, 5% CO_2.

For transduction in G-Rex bioreactors, Vectofusin-1 (Miltenyi Biotec) was diluted to 1 mg/mL in sterile water. Vectofusin-1 (10 μg/mL final concentration) was combined with retroviral supernatant at multiplicity of infection (MOI) 1–30. The solution was added to activated T cells in the G-Rex and incubated at 37°C, 5% CO_2 for 18–24 h. After incubation, cell culture medium with IL-7 and IL-15 was added to the maximum volume of the vessel (10 mL/cm^2) and placed back in the incubator.

Expansion

For expansion in bags, samples were taken daily to measure glucose and lactate concentrations, viable cell density and viability. Cell suspensions were diluted to $0.5-1.0 \times 10^6$ cells/mL with fresh medium when the cell density exceeded 2×10^6 cells/mL.

For expansion in G-Rex bioreactors, samples were taken daily to measure glucose and lactate concentrations. If sampled for count and viability, 90% of medium was removed, cells were pipetted into suspension, volume was measured and a sample was

taken. Fresh medium with cytokines was added to the maximum volume.

In-process monitoring

Glucose concentration was measured with a CVS Health Advanced Blood Glucose Meter (CVS Health, Woonsocket, RI, USA). Lactate concentration was measured with the Lactate Plus Meter (Nova Biomedical, Waltham, MA, USA). Cell count and viability were measured on the Nucleocounter NC-3000 (Chemometec, Allerod, Denmark).

Flow cytometry

Flow cytometry was performed on a Novocyte 3000 (ACEA Biosciences, Inc., San Diego, CA, USA) and analyzed with ACEA NovoExpress software. Cells were analyzed for transgene expression, CD4:CD8 ratio and memory population distribution. The following antibodies were used in the flow panel: CD34-PE (ABNova, Walnut, CA, USA) or CD19-PE for transgene detection, CD4-PerCP, CD45RA-PE Cy7 (BD Biosciences, San Jose, CA, USA), CD62L-APC, CD3-Alexa Fluor 700 and CD8-BV510. Unless noted, antibodies were from BioLegend. Zombie yellow fixable viability dye (BioLegend, San Diego, CA, USA) was used to remove dead cells from the analysis. BD Biosciences, San Jose, CA, USA.

Apoptosis assay

Cells from small-scale experiments were incubated for 4 h with or without 10 nmol/L temsirolimus to test function of the iCasp-9 transgene. After incubation, cells were stained with Annexin V-FITC, CD19-PE, CD3-BV421 and zombie yellow fixable viability dye in Annexin V binding buffer. All reagents were from BioLegend. $CD3^+$ $CD19^+$ cells were plotted on Annexin V against viability dye graphs to show viable cells (Annexin V^-/viability dye^-), apoptotic cells (Annexin V^+/viability dye^-) and non-viable cells (Annexin V^+/viability dye^+).

Cytokine secretion assay

Cryopreserved aliquots of cells generated in the G-Rex and bag large-scale processes were thawed, washed and counted. Cells were resuspended at 2×10^6 viable cells/mL in culture medium with IL-7 and IL-15 and incubated at 37°C with 5% CO_2 overnight. On the same day, 1×10^4 $PSCA^+$ human pancreatic cancer cells (ATCC, Manassas, VA, USA) were seeded per well of a 96-well plate and placed in the incubator. The next day, T cells were harvested, washed, counted and resuspended in medium at 2×10^6 cells/mL. Then, 1×10^4 T cells were plated in each well of the 96-well plate containing the $PSCA^+$ cells for an effector to target ratio of 1:1. Each sample was tested with and without 10 nmol/L rimiducid, which triggers the co-stimulation. After 48-h incubation, 25 μL supernatant was collected for cytokine analysis with the Human Cytokine/Chemokine Magnetic Bead kit (Millipore) according to the manufacturer's instructions.

Closed-system process

For the good manufacturing practice (GMP)-compatible G-Rex (100 cm^2) closed-system process, PBMCs were thawed and activated in the G-Rex100MCS in 40 mL (0.4 mL/cm^2) on day 0. On day 2, transduction reagents, including viral supernatant (MOI 5) and Vectofusin-1, were added. On day 3, medium was added to the maximum volume of the vessel (1 L, 10 mL/cm^2). Samples of the supernatant were taken daily for glucose and lactate concentration measurement through the sampling port. Cells were harvested on day 11 with the GatheRex device (Wilson Wolf Corporation). A parallel bag-based process was run for comparison. Briefly, PBMCs from the same donors were thawed and activated in bags, followed by transduction at MOI 5 in RetroNectin-coated bags on day 2. Cells were washed and transferred to cell culture bags for expansion. Samples were taken to measure viable cell density and determine when cultures required dilution. Total viable cells (TVCs), total transgene

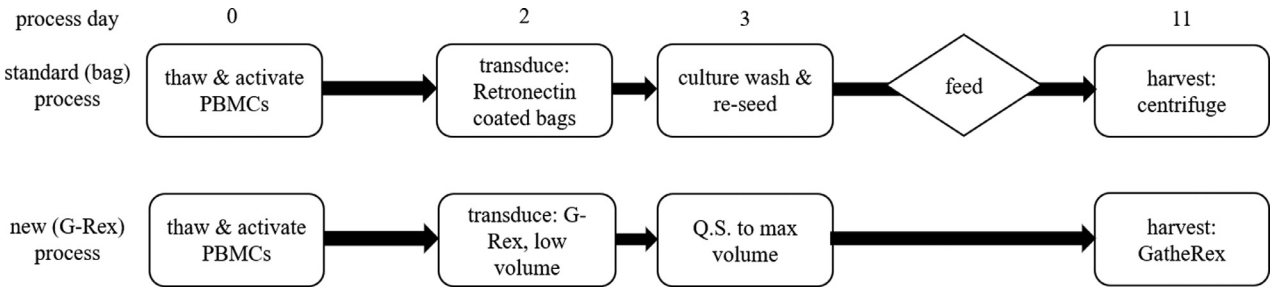

Figure 1. Bag and G-Rex–based process overview.

positive cells, percent transgenic cells, fold expansion, phenotype and cytokine secretion were compared between processes. Process flow charts are shown in Figure 1.

Statistics

Statistics were calculated in GraphPad Prism. Version 7.03. Data are presented as mean ± standard error of the mean. Unpaired Student's *t*-test was used to compared differences between groups. One-way analysis of variance was used compare multiple groups.

Results

Evaluation of T-cell expansion in G-Rex bioreactors

To observe kinetics of expansion in the G-Rex, 1 day after bag transduction, T-cell cultures were washed, resuspended in fresh medium and seeded in 10 cm^2 G-Rex 6-well plates at $0.03-1.00 \times 10^6$ cells/cm^2 (i.e., $0.3-10.0 \times 10^6$ cells/well). Then, 1×10^7 cells were cultured in a bag for comparison. Cells were maintained in culture up to 3 weeks with count and viability measurement and medium exchange every 3–4 days. In all conditions, cell proliferation plateaued at 10–14 days before expansion and viability decreased (Figure 2). Lower seeding densities allowed for higher fold expansion, whereas higher densities resulted in more total viable cells. The maximum number of cells in one well (seeded at 1×10^6 cells/cm^2) peaked at 2.5×10^8 total cells, or 2.5×10^7 cells/cm^2, which corresponds to 25-fold expansion. Cells from the same donor, seeded a lower density (0.06×10^6 cells/cm^2), expanded up to 42-fold to 2.6×10^7 total cells. There was no difference it total cells at any timepoint between conditions starting with 1×10^7 in bags or G-Rex.

To determine the maximum density of the G-Rex membrane, transduced T cells were plated at $0.5-2.0 \times 10^6$ cells/cm^2 (i.e., $5-20 \times 10^6$ cells/10 cm^2 well). G-Rex plates with maximum medium volumes of 4 mL/cm^2 and 10 mL/cm^2 were tested to determine if there is any benefit of the increased medium height. Daily samples of supernatant were taken for glucose monitoring, but the cell layers were left undisturbed until harvest. To monitor proliferation, triplicate wells were harvested 3, 6 or 9 days after seeding. Cytokines were added every 3 days to replicate the environment in cell culture bags where fresh medium is added as cell density is diluted. Cell density at harvest was higher for cells cultured in the 6M format (10 mL/cm^2 medium; Figure 3). A 5×10^5 cells/cm^2 seeding density resulted in harvest density of 2.5×10^7 cells/cm^2 (2.5×10^8 TVCs on 10 cm^2) in the smaller G-Rex compared with 4.2×10^7 cells/cm^2 (4.2×10^8 TVCs) in the 6M G-Rex. There was little difference in harvest among

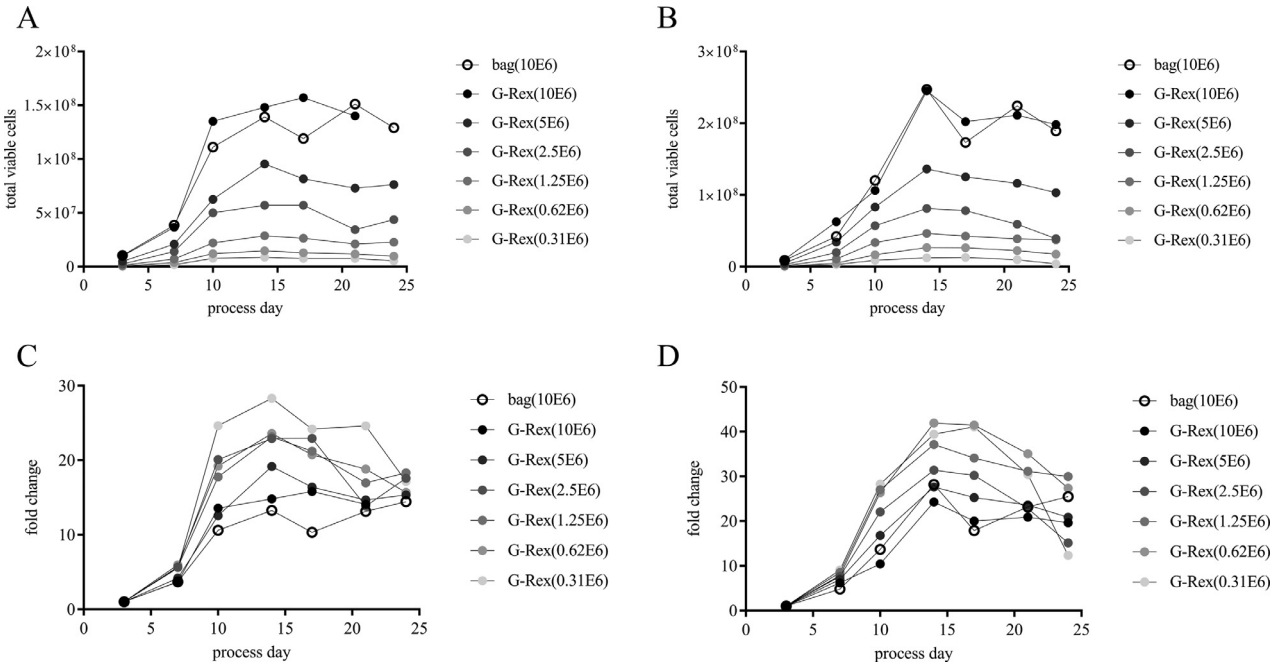

Figure 2. Kinetics of transduced T-cell proliferation, sampling to count every 3–4 days. Total viable cells (A, B) and fold expansion (C, D) for 2 donors (A, C and B, D) grown in bags or 10 cm^2 G-Rex wells seeded with $0.3-10.0 \times 10^6$ cells/well ($0.03-1.0 \times 10^6$ cells/cm^2). Total viable cells or fold change from day of seeding (y-axis) are plotted against process day (x-axis). Open circles represent data from bags; closed circles represent data from G-Rex wells. Marker color is shaded according to seeding density. All conditions were resuspended and counted every 3–4 days.

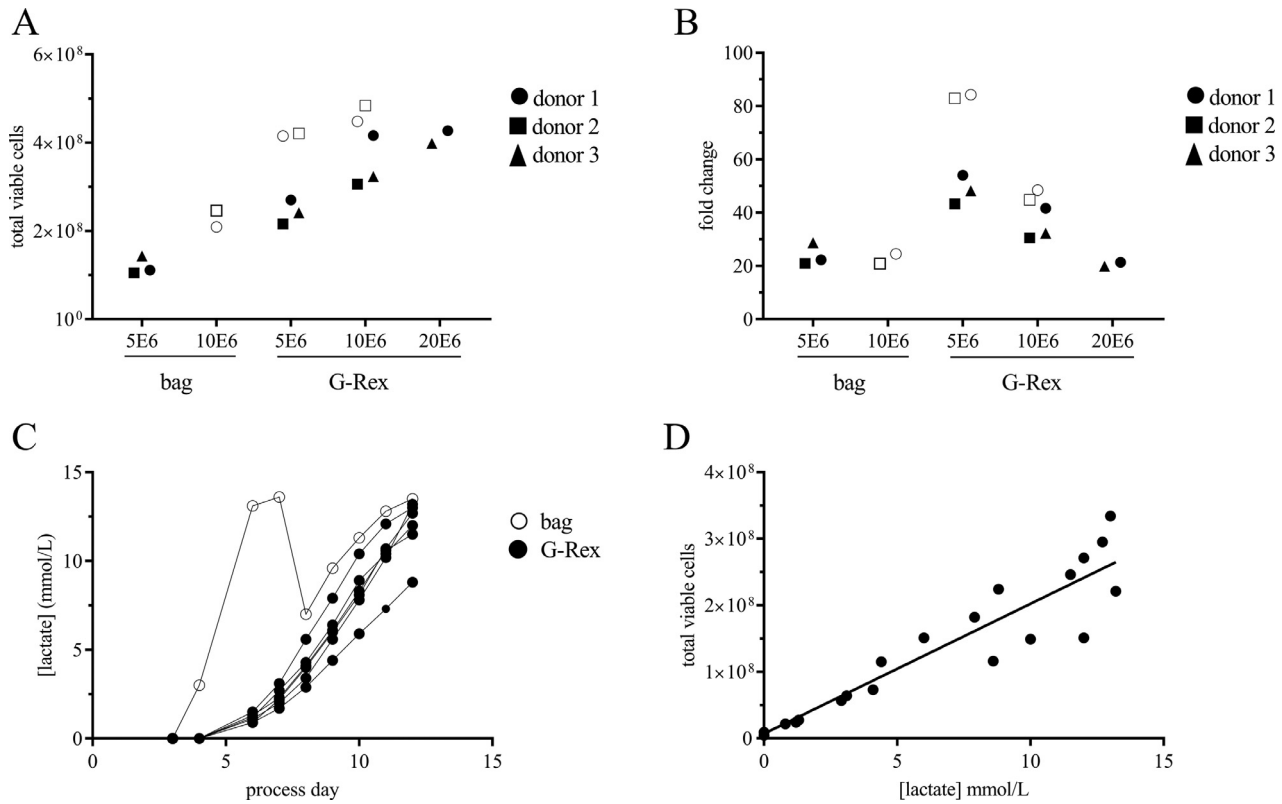

Figure 3. G-Rex maximum supported cell density and lactate accumulation. Total viable cells (A) and fold change (B) of transduced T cells expanded in bags or G-Rex starting with 5×10^6, 1×10^7 or 2×10^7 cells. Black markers represent experiments with 4 mL/cm^2 G-Rex plates; white markers represent experiments with 10 mL/cm^2 G-Rex 6M plates. (C) Sample plot from 1 experiment of lactate concentration (y-axis) plotted against process day (x-axis) for 7 individual culture conditions. Open markers represent bag culture; closed markers represent G-Rex 6M culture. (D) Total viable cells (y-axis) plotted against lactate concentration (y-axis). Data points collected from multiple donors and experiments. Solid line represents linear regression calculated in GraphPad; $R^2 = 0.9081$.

seeding 5×10^5 or 1×10^6 cells/cm^2 ($42 \pm 0.3 \times 10^6$ and $47 \pm 1.8 \times 10^6$ TVCs) in the 6M plates and 2.0×10^6 ($41 \pm 1.5 \times 10^6$ cells/cm^2) in the smaller plates (Figure 3).

To further optimize and simplify culture in the G-Rex, the cytokine feeding schedule was evaluated. The standard condition was supplementing cultures with 15 ng/mL IL-7 and 5 ng/mL IL-15 every 3 days. Additional schedules were 45 ng/mL IL-7 and 15 ng/mL IL-15 given on the day of seeding (i.e., 3x concentration once), 30 ng/mL IL-7 and 10 ng/mL IL-15 given on the day of seeding (i.e., 2x concentration once) and 15 ng/mL IL-7 and 5 ng/mL IL-15 on the day of seeding and 5 days later (i.e., 1x concentration twice in 9 days). There was no difference in TVCs or viability in cultures given 3x the usual amount of cytokines (45 ng/mL IL-7 and 15 ng/mL IL-15) on the day of seeding compared with giving the standard concentration three times over the course of expansion. Data from the same experiments revealed a strong correlation between lactate concentration in the G-Rex and the TVC number (Figure 3).

Optimization of transduction in G-Rex bioreactors

PBMCs activated in bags were washed and transduced with an MOI of 1 in 2.0, 4.0, or 10.0 mL in 10 cm^2 G-Rex 6M plates (i.e., 0.2 mL/cm^2, 0.4 mL/cm^2 or 1.0 mL/cm^2). Transductions were successful at all volumes, ranging from $25 \pm 5\%$ at 0.2 mL/cm^2 to $15 \pm 2\%$ at 1.0 mL/cm^2 (Figure 4A). At 0.4 mL/cm^2, transduction efficiency increases with increased MOI. A MOI of 1, 5, 10 and 30 resulted in transduction efficiencies of $35 \pm 5\%$, $58 \pm 4\%$, $84 \pm 1\%$ and 82%, respectively (Figure 4B). Expansion of transduced T cells was negatively impacted by high MOI (Figure 4C). The average fold expansion of samples transduced with MOI of 1 and 5 was 37 ± 3-fold compared with 21 ± 3-fold at MOIs of 10 and 30.

PBMCs were also activated directly in G-Rex bioreactors before transduction. To maintain a low volume for transduction without having to wash or remove supernatant, cells were activated in 2.0, 4.0 or 10 mL in 10 cm^2 G-Rex 6M plates (i.e., 0.2 mL/cm^2, 0.4 mL/cm^2 or 1.0 mL/cm^2). On the day of transduction, Vectofusin-1 was mixed with retrovirus (MOI 1), and then added to the activated cells.

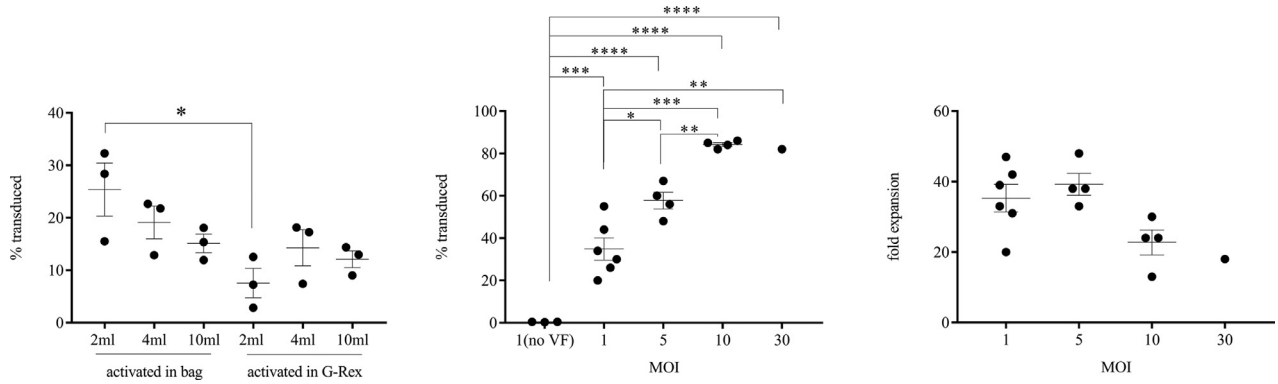

Figure 4. Evaluation of transduction volume and MOI in 10 cm^2 G-Rex. (A) Percentage of transduced cells plotted against mL medium/cm^2 for cells activated in bags or G-Rex before transduction in G-Rex. (B) Percentage of transduced cells plotted against MOI for cells activated in bags and transduced in G-Rex. No VF represents conditions incubated with MOI 1, but without Vectofusin-1 reagent. (C) Fold expansion plotted against MOI. $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$, $^{****}P \leq 0.0001$.

Overall, transduction efficiency was lower than comparable conditions transduced after bag activation and culture wash (Figure 4A). Nonetheless, 14 ± 3% transduction was achieved after activation in 0.4 mL/cm^2. Transgenic cells, expressing an inducible caspase-9, generated by both methods and transduced in the G-Rex were functionally active and responded as expected when incubated with temsirolimus to induce apoptosis (Figure 5). Viability of cells expressing the transgene was reduced 60% with treatment, whereas viability did not change in the non-transduced population.

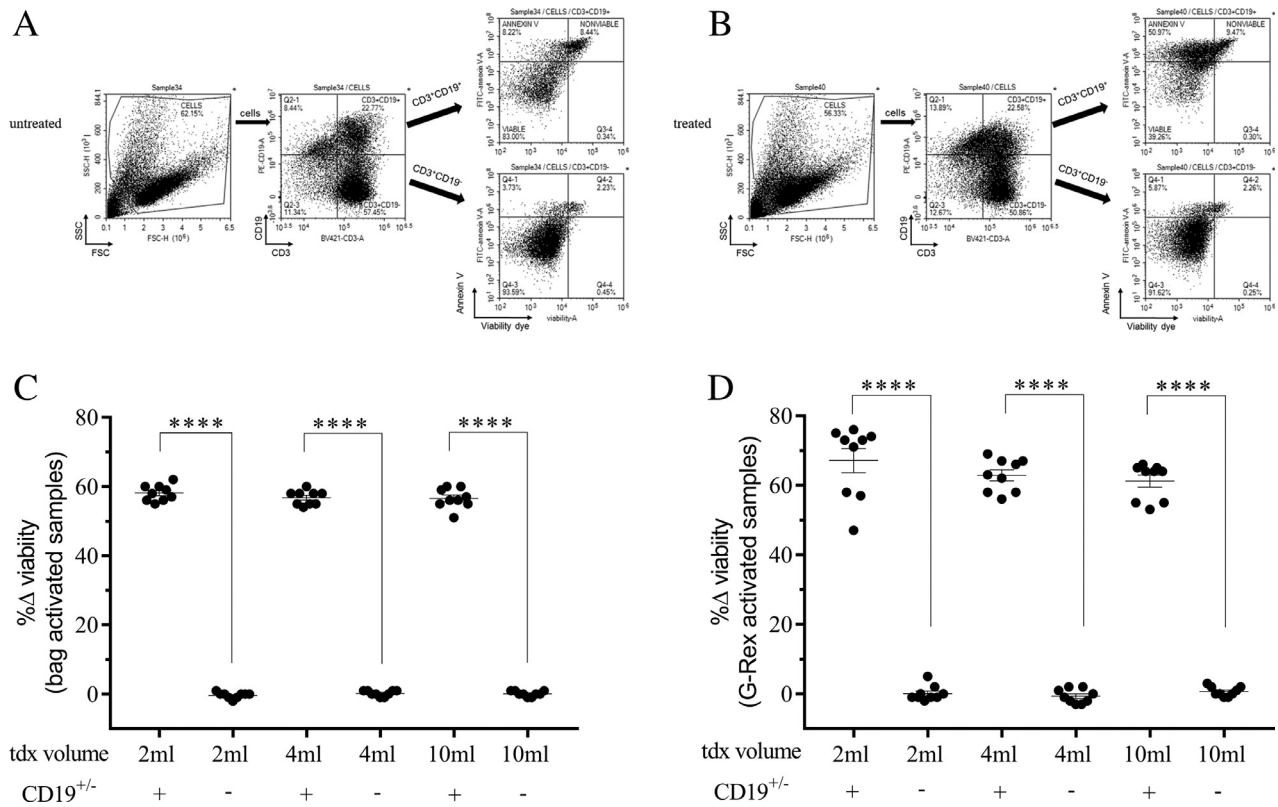

Figure 5. Transgene function after transduction in 10 cm^2 G-Rex. Example flow plots from one sample of untreated cells (A) or cells incubated for 4 h with drug to induce apoptosis (B). The inducible caspase transgene is detected with the CD19 marker. Change in viability for transduced ($CD19^{+}$) and non-transduced ($CD19^{-}$) fractions plotted against treatment group for cells activated in bags (C) or in G-Rex (D) and then transduced in the G-Rex. Change in viability is calculated by the formula (untreated viability − treated viability)/untreated viability. $^{****}P \leq 0.0001$.

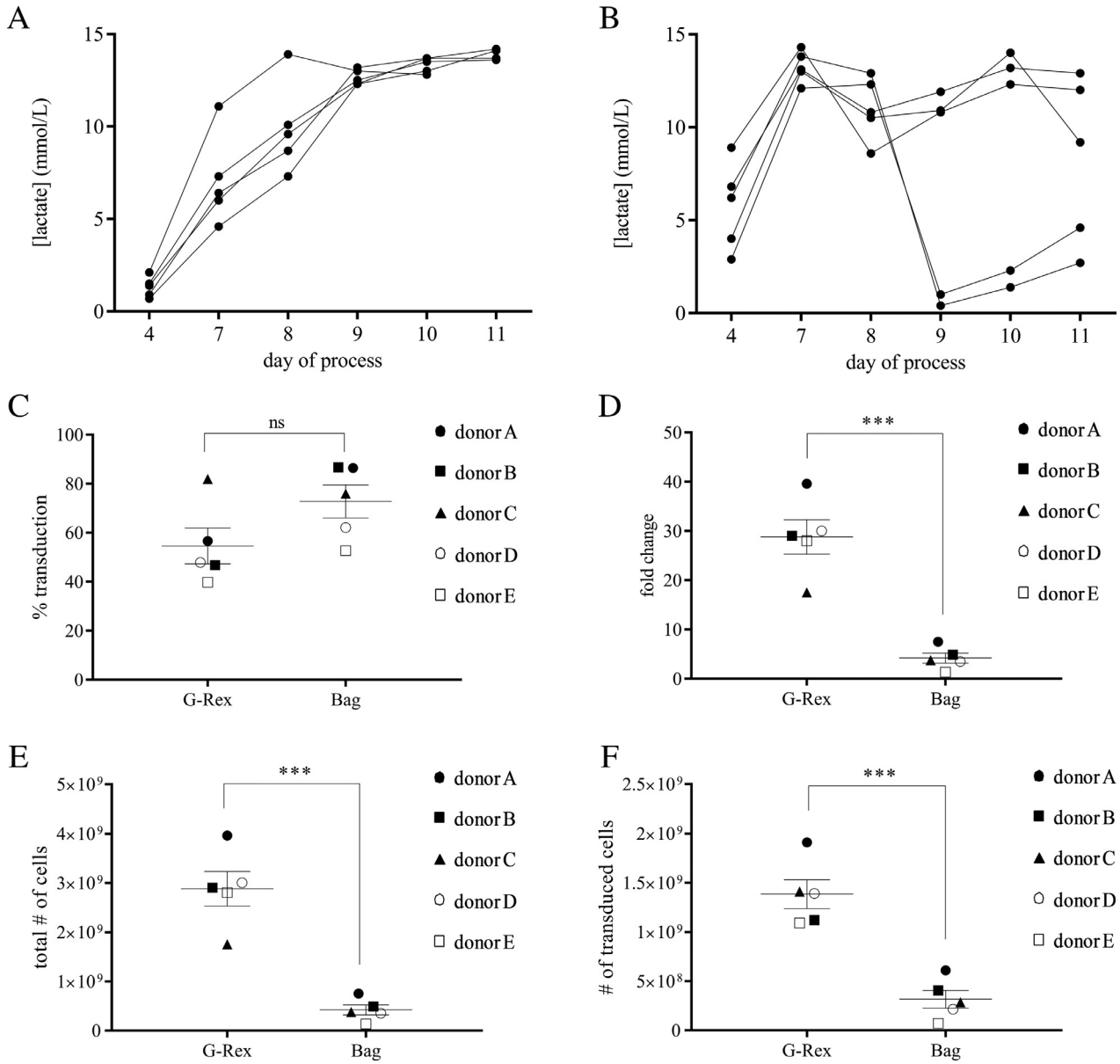

Figure 6. Large-scale G-Rex process compared with bag process lactate, transduction and expansion. (A, B) Lactate concentration (y-axis) plotted against process day (x-axis) for large-scale G-Rex100MCS (A) and bag cultures (B). (C–F) Comparison of transduction percentage (A), fold change (B), total cells at harvest (C) and total transduced cells at harvest (D) of cells generated in the G-Rex and bag-based processes. Each marker represents 1 donor. ***$P \leq 0.001$, not significant $P > 0.05$.

Closed-system G-Rex–based process

In-process monitoring of lactate buildup was used as a surrogate marker of cell proliferation in the G-Rex. There was a slow accumulation of lactate over time in the G-Rex (Figure 6A) compared with rapid increase in the bag with reduction after medium addition (Figure 6B). Transduction efficiency in the G-Rex 100MCS was 55 ± 7%, compared with 73 ± 7% in the RetroNectin-coated bag process (Figure 6C). Significantly more total viable cells ($2.8 \pm 0.4 \times 10^9$ vs $4.2 \pm 1.0 \times 10^8$) and transgenic cells ($1.4 \pm 0.1 \times 10^9$ vs $3.2 \pm 0.9 \times 10^8$) were harvested from the G-Rex compared with the bag process (Figure 6E and 6F). There was no difference in the distribution of CD45RA and CD62L cell expression or CD4:CD8 ratio between cells generated in the two processes (Figure 7). Incubation of the G-Rex–cultured cells, transduced with a PSCA CAR with inducible co-stimulation, on $PSCA^+$ target cells resulted in a functional response measured by secretion of cytokines (Figure 8) that did not differ from that of cells produced in the conventional process.

The cost of reagents and consumables to generate equivalent batch sizes from each process was estimated based on currently available list prices

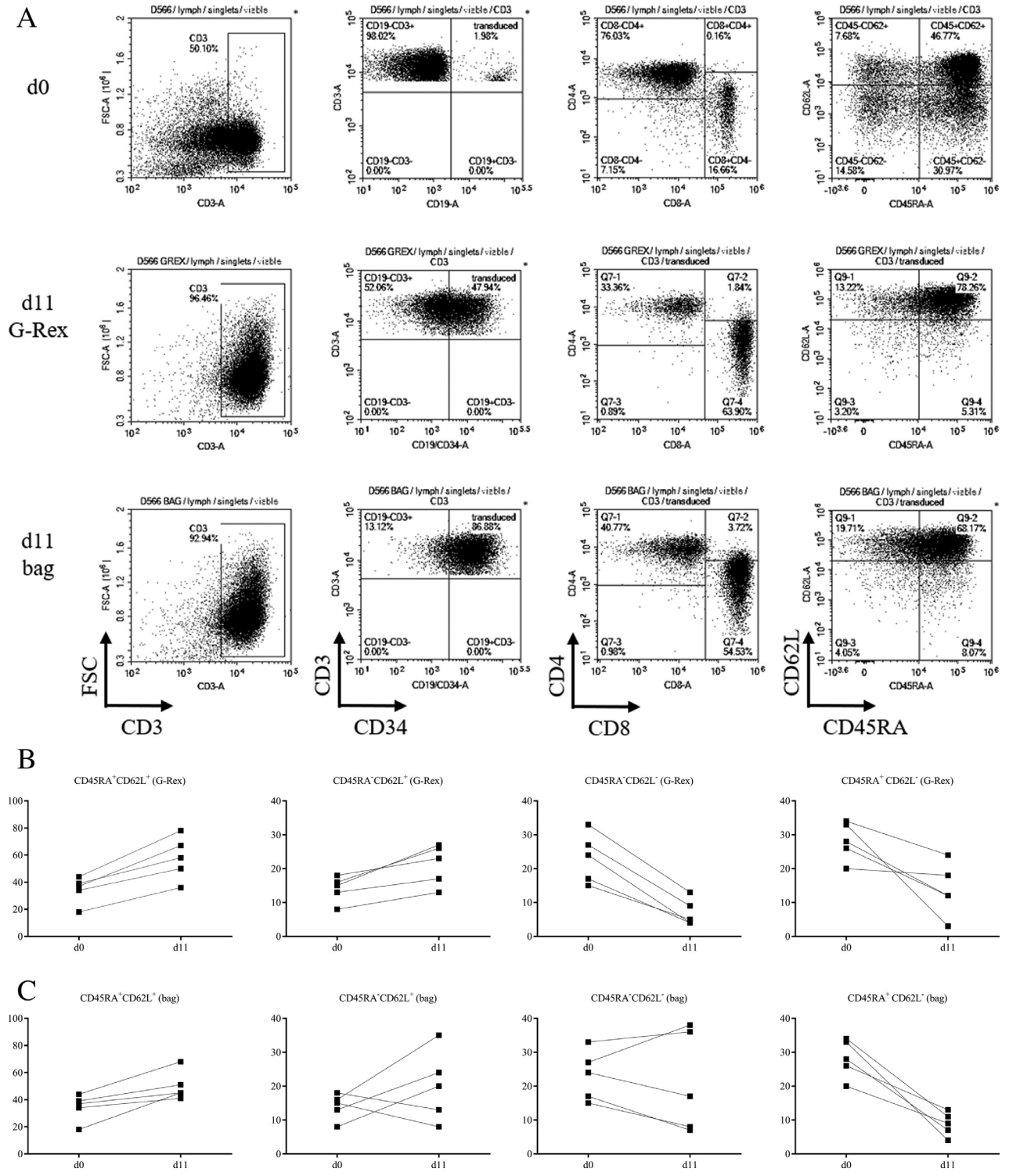

Figure 7. Phenotype of cells generated in the G-Rex and bag process is not different. (A) Sample flow plots for one donor on the initial day of the process (d0), and after harvest from the G-Rex and bag. The transgene is detected using a CD34 marker. (B, C) Distribution of CD45RA and CD62L expression on d0 and d11 for the G-Rex (B) and bag (C) processes. (D) Percentage of $CD45RA^{+}CD62L^{+}$ (Tn), $CD45RA^{-}CD62l^{+}$ (Tcm), $CD45RA^{-}CD62L^{-}$ (Teff) and $CD45RA^{-}CD62L^{-}$ (Tem) from day 0 (checkered bar), G-Rex harvest (white bar) and bag harvest (black bar). Two-tailed, unpaired *t*-test was used to compare G-Rex harvest to bag harvest, with no significant difference between the 2 for any population. (E) CD4:CD8 ratio at harvest from G-Rex or bags.

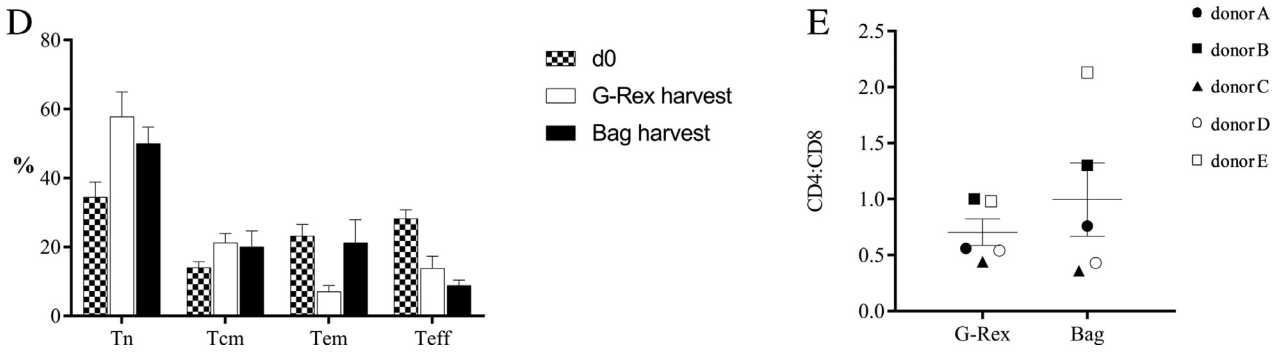

Figure 7 Continued.

(Table 1). According to these calculations, materials for the newly developed process would cost approximately 38% less than that for a conventional process. Most of the cost reduction is due to the efficiency of cell expansion in the G-Rex, which reduces the starting cell number by 4-fold. A comparison between the time required per operation for each process was also made (Table 2), demonstrating a significant reduction of hands-on time for the G-Rex–based process.

Discussion

A major obstacle to manufacturing engineered T cells is introduction of transgenes such as CARs. Here we demonstrate a simple method for transducing T cells in suspension and show integration of the novel transduction step into a streamlined manufacturing process that relies on a single G-Rex bioreactor throughout. Although

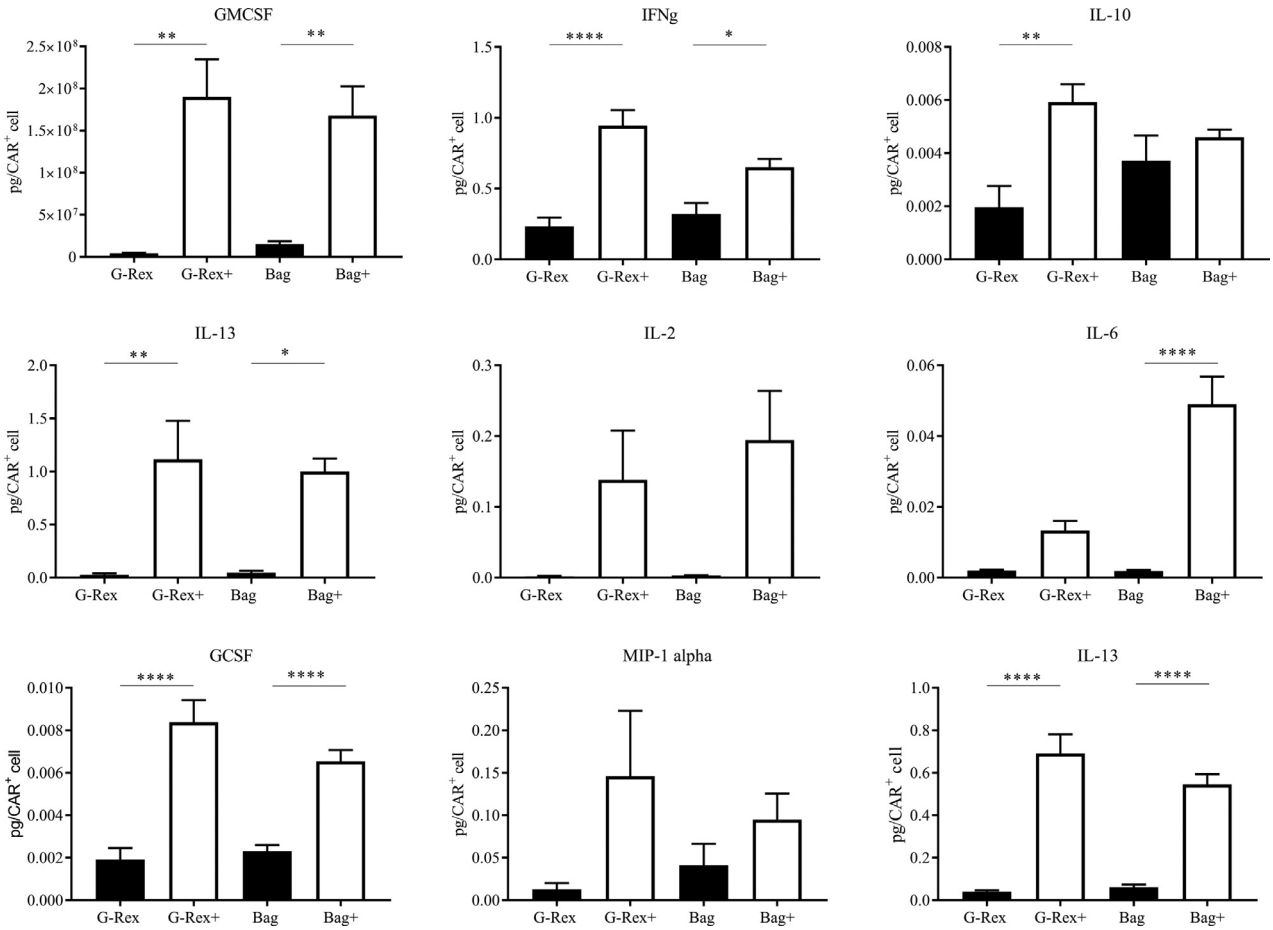

Figure 8. Similar function of transgene in cells generated in the G-Rex and bag processes. Cytokine secretion, normalized to pg/CAR$^+$ cell, of CAR T cells produced in the G-Rex and bag large-scale process after incubation with target cells ± drug to provide co-stimulation. Black bars represent conditions without drug; white bars represent conditions with drug. *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$, ****$P \leq 0.0001$.

Table 1. Bag and G-Rex process cost comparison.

Item	Manufacturer	Catalog no.	Size	List price	Bag-based process		G-Rex–based process	
					Required per run	Cost per run	Required per run	Cost per run
TexMACS GMP medium	Miltenyi Biotec	170-075-306	2 L	$ 305.00	1.00	$ 305.00	1.00	$ 305.00
MACS GMP Vectofusin-1	Miltenyi Biotec	170-076-165	1 mg	$ 875.00	0.00	$ -	1.00	$ 875.00
G-Rex 100MCS	Wilson Wolf Corp	81100-CS	3 pack	$ 729.84	0.00	$ -	0.33	$ 243.28
MACS GMP CD3 pure	Miltenyi Biotec	170-076-116	1 mg	$ 1750.00	0.58	$ 1015.00	0.08	$ 140.00
MACS GMP CD28 pure	Miltenyi Biotec	170-076-117	0.5 mg	$ 895.00	1.45	$ 1297.75	0.20	$ 179.00
hIL-7, premium grade	Miltenyi Biotec	130-095-363	100 μg	$ 1150.00	0.13	$ 149.50	0.45	$ 517.50
hIL-15, premium grade	Miltenyi Biotec	130-095-765	100 μg	$ 1150.00	0.04	$ 46.00	0.15	$ 172.50
Retronectin, GMP grade	Takara	T202	2.5 mL	$ 1442.00	0.50	$ 721.00	0.00	$ -
Cell culture bags	Saint Gobain	290-C	10 pack	$ 993.51	0.30	$ 298.05	0.00	$ -
Transduction bag	Saint Gobain	290-AC	10 pack	$ 993.51	0.10	$ 99.35	0.00	$ -
						$3931.65		$2432.28

The cost of generating 1.4×10^9 transduced cells in the bag- and G-Rex–based process. Cost for the bag process was calculated based on scaling up to a process that could generate as many cells as the G-Rex–based process.

Table 2. Bag and G-Rex process time comparison.

Operation	Bag-based process		G-Rex-based process	
	Method	Time (h)	Method	Time (h)
Activation	Anti-CD3/CD28 in solution	1.00	Anti-CD3/CD28 in solution	1.00
Transduction	Culture wash, transfer to Retronectin-coated bags	4.00	Vectofusin-1 in solution	0.50
Transduction stop	Culture wash, transfer bag	2.00	Medium addition	0.50
Expansion	Feed and transfer, as needed	4.00	NA	0.00
Harvest	Centrifuge	2.00	GatheRex	0.50
In-process monitoring	Mix cell suspension, sample for count (x6 d)	3.00	Sample supernatant for lactate (x6 d)	1.50
		16.00		4.00

The estimated time, in hours, required for each operation in the bag- and G-Rex–based process. Estimates are based on a single operator working in a non-GMP setting.
NA, not applicable.

functionally closed and efficient for expanding cells [3–15], the G-Rex bioreactors have not, to date, addressed the practical challenges of genetic modification of T cells. The first goal of this study was, therefore, to develop a transduction protocol that would work for transducing T cells in suspension in the G-Rex.

Transduction of T cells has typically relied upon RetroNectin-coated cell culture bags or plates. Given that RetroNectin coating of the silicone membrane is not possible, Vectofusin-1 reagent, which is added in solution, was tested as an alternative for transduction in the G-Rex. Incubation of retrovirus with activated T cells in low volumes to increase cell/virus interaction, but without transduction-enhancing reagents, resulted in transduction efficiency of <1%. Adding Vectofusin-1 increased transduction efficiency to 8–25% at MOI 1. Transduction efficiency was increased with increasing MOI, and that increase reached a maximum at MOI 10 (>80%). Medium addition after transduction, without washing, allowed for expansion of the transduced T cells in the G-Rex, although expansion tended to be negatively impacted at MOI ≥10.

Transduction and expansion of cells in the G-Rex eliminate the complexity of transduction with RetroNectin, but still require initial activation, with a wash and transfer step. To further simplify the process, activation in the G-Rex prior to transduction was also evaluated. PBMCs activated in the G-Rex in 0.4

mL/cm^2 were transduced with 15% efficiency. This is slightly lower than cells activated in bags, but also significantly reduces operator time and eliminates wash and transfer of cells between activation and transduction, reducing risk of contamination or other errors.

These data demonstrate that the transduction step can be streamlined to simple addition of reagents to cells rather than RetroNectin coating, washing and transferring cells pre- and post-transduction. This method has the potential to reduce clean room time, operator input and risk of contamination.

Using data from the small-scale experiments, suggesting an optimal seeding density of $0.5-1.0 \times 10^6$ cells/cm^2 and an activation volume of 0.4 mL/cm^2, a GMP-compatible process was devised. Prioritizing simplicity over other parameters, PBMCs were activated, transduced and expanded in a single G-Rex bioreactor. Five runs each of the simplified G-Rex–based process and the standard bag process were performed in parallel. There was no difference in phenotype or function of the cells generated in the two processes. There was a trend toward higher transduction in the bag, although that difference was not statistically significant. Expansion in the G-Rex bioreactor resulted in significantly more total viable cells and transgenic cells at harvest. The average harvest from the G-Rex process was $1.4 \pm 0.1 \times 10^9$ transgenic cells. A hypothetical 80-kg patient who needs a dose of 5.0×10^6 cells/kg could receive >3 doses from one manufacturing process with the G-Rex. In comparison, a bag process, starting with the same number of cells, results in barely enough transgenic cells for a single dose of the same size. Further, the G-Rex–based process reduced the cost of materials by 38% and hand-on time by 75% to generate a batch of 1.4×10^9 transgenic cells.

This study demonstrates that T cells can be transduced with retroviral vectors in solution in the G-Rex bioreactor with addition of Vectofusin-1 as a transduction enhancer. The simplified transduction step allow for an entire process, from activation to harvest, to be carried out in a single vessel. Clinically relevant levels of transgene expression and cell numbers can be achieved by combining reagents in the G-Rex, without complicated time-consuming coating steps of traditional transduction.

The streamlined procedure reduces the hands-on time of transduction to minutes rather than hours and eliminates all cell transfer and wash steps. Further, the increased output per starting material reduces cost of materials compared with the standard method. Cells can be expanded in the G-Rex with limited operator intervention and without specialized equipment.

Declaration of Competing Interest

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Author Contributions

Conception and design of the study: CG. Acquisition of data: MK, CG. Analysis and interpretation of data: MK, CG. Drafting or revising the manuscript: MK, CG, AEF. All authors have approved the final article.

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