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Improving cell viability using counterflow centrifugal elutriation

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ABSTRACT

Background: Cell viability is an important release criterion in the manufacturing of cell therapy products. Low cell viability can have significant impact on product quality and manufacturing efficiency. Counterflow centrifugation technology has been applied to the manufacturing of cell therapy products, to enable cell separation based on size and density. This study evaluated the utility of counterflow centrifugation technology for dead cell removal to improve cell viability of the final product.

Methods: Jurkat cell cultures with low and high dead cell burden were subjected to counterflow centrifugal elutriation to determine the correlation between process parameters (e.g., flow rate, centrifugal force) and processing outcomes (i.e., cell recovery and viability). Subsequently, the optimized parameters were applied to dead cell elutriation using expanded T cells and freshly isolated human amniotic epithelial cells (hAECs). The efficiency of dead cell removal, cell function and post-thaw viability were compared.

Results: Elutriation using a low flow rate allowed better control of viable cell recovery from both low and high dead cell burden cultures of Jurkat cells. The viability of T cells and hAECs was improved by counterflow centrifugal processing, from $80.67\% \pm 2.33$ to $94.73\% \pm 1.19$ and $79.19\% \pm 5.35$ to $90.34\% \pm 3.59$, respectively. Processing increased the proliferation rate of T cells, while the metabolic activity of hAECs was unchanged.

Conclusion: Counterflow centrifugal elutriation can be added as an integrated step to the automated wash-and-concentrate protocol for cell manufacturing to remove dead cells and improve cell viability of the final product.

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Introduction

Cell viability is a critical quality attribute in cell therapy products. Reduced cell viability may result from prolonged time in transit, tissue digestion and manipulation, gene transduction, cryopreservation and cell thawing [1–4]. Optimization of the manufacturing process is probably the most effective way to control cell viability. Nevertheless, manufacturing outcomes may vary as a result of donor-to-donor variability or other factors [5].

Currently, cell viability is a significant issue in the manufacturing of chimeric antigen receptor T (CAR-T) cells. The anti-CD19 CAR-T therapy, Tisagenlecleucel, requires a minimum of 80% viability for commercial use in the United States. A retrospective analysis of the

clinical trial manufacturing data revealed that ~12% to 16% of the products were found to have <80% viability, a problem which continued through to commercial settings [6]. Cell viability issues are not restricted to CAR-T manufacturing. We have been manufacturing human amniotic epithelial cells (hAECs) for clinical use in bronchopulmonary dysplasia for preterm infants [7,8] and have observed that low cell viability is the leading cause of batch failure.

One argument for requiring higher cell viability is that the presence of dead cells may be associated with a higher chance of adverse events or poor efficacy [9–11]. Therefore, it is appealing to have a dead cell-removal step in cell manufacturing. Currently, there are limited options for dead cell removal before cryopreservation. A method developed in research settings is removal of dead cells using magnetic bead-based separation [12]. Cell washing and dead cell removal are sometimes performed before infusion in the clinical settings. Previous studies investigated the use of automated devices in removing cryopreservatives such as dimethyl sulfoxide (DMSO) and

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cellular debris from hematopoietic stem cell preparations before infusion [13–15]. However, such an approach creates an extra manufacturing burden, as it requires additional instrumentation and training onsite, which is not ideal for centralized manufacturing and distribution chains.

Counterflow centrifugal technology has been applied to cell manufacturing for the purpose of cell washing, concentration and selection [16]. Traditional centrifugation pellets cells by accelerating the sedimentation process. Counterflow centrifugation allows cells to travel from the narrow end of the conical chamber toward the wider end against the forces of sedimentation velocity such that cells accumulate and form a fluidized bed. Additionally, there are a counterflow velocity gradient and sedimentation velocity gradient across the chamber. The velocity gradient allows larger or denser cells to accumulate toward the narrow end of the chamber while smaller or less dense cells accumulate at the wider end. Smaller cells can then be elutriated out of the chamber by decreasing the centrifugal force or by increasing the flow rate.

The relationship between the centrifugal and counterflow velocities of the particles can be expressed as a ratio, i.e., the counterflow centrifugal force to flow rate (C:F) ratio. The elutriation process is generally facilitated by a low C:F ratio, which allows expansion of the fluidized cell bed, thereby pushing the smaller cells toward the wider end of the chamber. One of the common challenges with counterflow centrifugal elutriation is the sensitivity of elutriation to cell types, media density and processing temperature. The protocol designing software can predict the retention or elutriation of cells based on information about cell size, cell density and media density. However, some of this information is not readily available, since cell and media density are not routinely measured. Therefore, this study aimed to identify critical process parameters to provide a method of establishing optimal elutriation settings for counterflow centrifugal elutriation processes.

Methods

T cell isolation and expansion

Jurkat cells (clone E6-1, TIB-152; ATCC) were cultured in RPMI 1640 (11875119; Thermo Fisher Scientific, Waltham, MA) supplemented with 10% fetal bovine serum (FBS, A31606; Thermo Fisher Scientific) and 1% antibiotic-antimycotic (15240096; Thermo Fisher Scientific). Human peripheral blood mononuclear cells (PBMCs) were obtained from buffy coats (Australian Red Cross Lifeblood) under the approval of the Monash Health Human Research Ethics Committee (18227). PBMCs were isolated using Ficoll-Paque (17144003; Cytiva,

Marlborough, MA) density separation [17]. Cells were cryopreserved in 5% DMSO and 95% FBS using alcohol-free freezing containers (Cool-Cell; Corning, Corning, NY), which freezes cells at a rate of $-1^{\circ}\text{C}/\text{min}$ in a -80°C freezer overnight before transfer into liquid nitrogen. The cryopreserved PBMCs were thawed and enriched using $\text{CD3}^{+}\text{CD28}^{+}$ Dynabeads (11132D; Thermo Fisher Scientific) supplemented with 0.1 mg/mL DNase I solution (07900; Stemcell Technologies, Vancouver, Canada). The CD3 cells were initially cultured in RPMI with 10% (v/v) FBS (Thermo Fisher Scientific), 1% (v/v) antibiotic-antimycotic and interleukin (IL)-2 (20 IU/mL; 200-02; PeproTech, Rocky Hill, NJ) for 8 days. Cells were continued in culture without IL-2 supplements for an extra 3 to 4 days to allow T cell anergy and apoptosis. CD3 cell number and viability were determined using an automated propidium iodide (PI)/acridine orange cell counter, NucleoCounter NC-202 (Chemometec, Copenhagen, Denmark).

Human amniotic epithelial cell isolation

Healthy, term placental tissues were obtained from elective cesarean delivery in accordance with guidelines and approval from Monash Health Human Research Ethics Committee (01067B). hAECs were isolated as previously described [18]. Cell numbers and viability were measured by an automated PI/acridine orange cell counter (Chemometec).

Counterflow centrifugal elutriation

Dead cell removal was performed using the Rotea standard single-use counterflow centrifugal kit (Thermo Fisher Scientific). The processing kit was set up as the standard wash and concentrate process as previously described [19]. Cells were washed and elutriated using 0.9% (w/v) saline (Fresenius Kabi, Bad Homburg, Germany) supplemented with 0.5% (w/v) bovine serum albumin (BSAS-AU; Bovogen Biologicals, Melbourne, Australia) in Jurkat cell experiments or 0.5% (v/v) human serum albumin (CSL Behring, Melbourne, Australia) in T cell and hAEC experiments.

Dead cell elutriation using low and high dead cell burden culture

Jurkat cells were expanded and subsequently treated with low (0.4-mM) or high (1.2-mM) concentrations of hydrogen peroxide for 15 h to create cultures with low or high dead cell burdens. The apoptotic Jurkat cell cultures were subjected to low (25-mL/min) or high (50-mL/min) flow rate elutriation, and 10^8 viable cells were subjected to the elutriation process in each run (Figure 1). Cell recovery and cell

Figure 1. Experimental design for identifying critical parameters in dead cell elutriation. Jurkat cells were expanded in culture and treated with low or high hydrogen peroxide (H_2O_2) concentrations (0.4 or 1.2 mM, respectively) for 15 h to produce low and high dead cell burden cultures. Each type of culture was then subjected to elutriation processes using low or high flow rates. (Color version of figure is available online.)

viability were determined by trypan blue exclusion assay using Countess II (Thermo Fisher Scientific) automatic cell counter.

Flow cytometry of the apoptotic cell culture

Cell apoptosis was measured by flow cytometry using the dead cell apoptosis kit (V13242; Thermo Fisher Scientific) following manufacturer's instructions. Jurkat cells from unstimulated low and high dead cell burden cultures were stained for Annexin V and PI, and the cells were analyzed using BD FACS Canto II flow cytometer. Results were obtained from three separate batches of cultured cells.

T cell proliferation assay

Primary T cells from six donors were culturally expanded, and cells from each donor were split into the processed (elutriation) group and unprocessed group. Cells were cryopreserved after elutriation. Cells from both groups were subsequently thawed and labeled with CellTrace Violet (CTV) cell proliferation kit (C34557; Thermo Fisher Scientific) following manufacturer's instructions. Cells (8×10^5) were cultured with CD3⁺CD28⁺ Dynabeads (11132D; Thermo Fisher Scientific) in a 1:1 cell-to-bead ratio in 96-well plates. Cells were cultured in RPMI supplemented with 10% FBS, 1% antibiotic-antimycotic and IL-2 (20 IU/mL). The cells were collected on day 3 and stained for CD3 marker conjugated with allophycocyanin (APC; clone UCHT1, 17-0038-42; Thermo Fisher Scientific) and PI (P3566; Thermo Fisher Scientific). The samples were then analyzed on Fortessa flow cytometer following sequential gating on single cells, live cells, CD3⁺ T and CTV-labeled cells. Proliferation index was calculated using the proliferation modeling tool from FlowJo software (version 10).

Cell metabolic assay

Human amniotic epithelial cells were cultured (5×10^5 cells per well in triplicate) in Dulbecco's modified Eagle medium (Nutrient Mixture F-12 [DMEM: F12, 11330-057; Thermo Fisher Scientific] medium supplemented with 10% FBS and 1% antibiotic-antimycotic) for 24 hours. Cell metabolic activity was determined by the colorimetric changes after 2 h of incubation with the CellTiter MTS solution (G3582; Promega, Madison, WI). Absorbance was measured at 490 nm using the SpectraMax i3 system (Molecular Devices, San Jose, CA).

Statistical analysis

Comparisons between cultures with low and high dead cell burdens were analyzed using the Mann–Whitney *U* test. Comparisons between processed and unprocessed samples and cell viability at different time points were analyzed using the Wilcoxon matched-pair signed-rank test. Data were analyzed using Prism 9 software (GraphPad, San Diego, CA). The results are presented as mean $\pm$ standard deviation (SD).

Results

Establishment of low and high dead cell burden in Jurkat cell cultures

Two groups of apoptotic cell cultures were established to identify the critical processing parameters in dead cell elutriation. Jurkat cells were subjected to 0.4 or 1.2 mM H₂O₂ treatment for 15 h to produce low or high dead cell burden cultures (Figure 1). Flow cytometric analysis showed that both low and high dead cell burden cultures contained a mixture of viable cells (Q4), early apoptotic cells (Q3), late apoptotic cells (Q2) and necrotic cells (Q1) (Figure 2A–C). Cell viability was measured by trypan blue exclusion. Cell viability of the

low dead cell burden culture was $\sim 25\%$ higher than that of the high dead cell burden culture ($75.59\% \pm 4.96$ vs. $49.75\% \pm 7.13$, $P < 0.001$) (Figure 2D). The high H₂O₂ concentration also induced a subtle change in cell size: the average viable cell diameter was lower in the high dead cell burden culture ($13.02 \pm 0.86 \mu\text{m}$ in low dead cell burden culture vs. $12.64 \pm 0.61 \mu\text{m}$ in high dead cell burden culture, $P = 0.11$) (Figure 2E). However, this difference was not obvious when visualized under the microscope (Figure 2F, G).

Dead cell elutriation in low and high dead cell burden culture

Dead cell elutriation was visualized in real-time and captured on camera as shown in Figure 3. During the washing step before elutriation, viable and dead cells accumulated within the middle and narrow end of the chamber (Figure 3A). The washing step was typically completed within 2 min when a volume of buffer equivalent to 3 to 4 times chamber volume was passed through the system (Figure 3B). The elutriation step took place as the fluidized cell bed expanded with the decreased C:F ratio. Dead cells thus migrated toward the wider end of the chamber and were elutriated, during which the wider end of the chamber became cloudy (Figure 3C). The elutriation process typically took place over a longer period of time than washing. The wider end of the chamber was cleared after elutriation with ~ 200 mL buffer (Figure 3D).

Dead cell elutriation is generally facilitated by a low C:F ratio. However, viable cells may also be elutriated with a decreasing C:F ratio. Therefore, we first determined the minimum C:F ratio required to retain the viable cells using a C:F ratio reduction test. Jurkat cells (10^8 viable cells) were elutriated at a low (25-mL/min) or high (50-mL/min) flow rate with a stepwise reduction of C:F ratio. Each C:F ratio tested was elutriated with 20 mL of the wash buffer. The number of viable cells elutriated from each C:F ratio was quantified ($n = 3$, Figure 4A, B). The number of viable cells elutriated was comparable between low and high flow rate elutriation in the low dead cell burden group (Figure 4A). There was minimum cell loss observed until the C:F ratio was reduced to 12. The percentage of viable cell loss was $>10\%$ of viable cell loss when the C:F ratio was reduced to 10. Elutriating cells using this C:F ratio would result in unacceptable cell recovery rates based on our experience. Therefore, the minimum feasible elutriation C:F ratio in this experiment was determined to be 12. The elutriation profile remained the same when the high dead cell burden group was subjected to low-flow-rate elutriation (Figure 4B). However, the curve shifted left when the high dead cell burden group was subjected to elutriation at a high flow rate (Figure 4B), during which $>20\%$ of the viable cells were elutriated at the C:F ratio of 14.

We then performed dead cell elutriation with either low or high flow rate over 280 mL wash buffer ($n = 3$, Figure 4C–F). At a low-flow-rate elutriation, the viable cell recovery was gradually decreased from $95.18\% \pm 1.52$ to $56.13\% \pm 34.9$ (low dead cell burden culture) or $89.09\% \pm 11.18$ to $45.06\% \pm 19.80$ (high dead cell burden culture) with reducing C:F ratio (Figure 3C, D). Cell viability was improved from $88.13\% \pm 0.34$ to $93.30\% \pm 1.87$ (low dead cell burden group) and $85.34\% \pm 3.68$ to $91.32\% \pm 1.39$ (high dead cell burden group) (Figure 3E, F). In comparison, high-flow-rate elutriation had an unexpected loss of cell recovery at the C:F ratio of 14 (low dead cell burden group) or 16 (high dead cell burden group), at which viable cell recovery was reduced from $\sim 75\%$ to undetectable (Figure 4C, D).

Furthermore, the minimum feasible C:F ratio was found to be 12 in both low and high dead cell burden cultures from the C:F ratio reduction test using the low flow rate (Figure 4A, B). Dead cell elutriation using the minimum feasible C:F ratio resulted in $\sim 50\%$ of cell recovery in both low and high dead cell cultures after 280-mL elutriation (Figure 4C, D). In comparison, the minimum feasible C:F ratios using the high flow rate were 12 and 16 in low and high dead cell burden cultures, respectively (Figure 4A, B). The last detectable results for high-flow-rate elutriation were at C:F ratios of 16 and 18

Figure 2. Characteristics of Jurkat cell cultures with low and high dead cell burden. Jurkat cells were subjected to 0.4- or 1.2-mM hydrogen peroxide treatment for 15 h. (A–C) Representative FACS plots of untreated and low and high dead cell burden cultures from three experimental repeats. (D) Average cell viability in the cultures with low and high dead cell burden was 75.59 ± 4.96 and 49.75 ± 7.13 . (E) The average diameter of viable cells in the low dead cell culture was $13.02 \pm 0.86 \mu\text{m}$, which was slightly higher than the $12.64 \pm 0.61 \mu\text{m}$ in the high dead cell culture. (F and G) Representative bright-field images of trypan blue staining from low and high dead cell culture, respectively. Data are presented as mean $\pm$ SD, scale bar = $100 \mu\text{m}$. FSC, forward scatter; SSC, side scatter; PI, propidium iodide. (Color version of figure is available online.)

(Figure 4C, D), which did not match the minimum feasible C:F ratio observed in the C:F ratio reduction test (Figure 4A, B). Therefore, the low flow rate elutriation can produce more predictable outcomes and is less sensitive to cell viability.

The optimal elutriation volume for dead cell removal was investigated. The optimal elutriation volume should maximize dead cell removal while minimizing loss of viable cells from the chamber. Hence the elutriation process should stop once dead cell elutriation reaches a

plateau. To understand the relationship between dead cell burden, flow rate and C:F ratio, the number and viability of elutriated cells were determined. The cumulative percentage of dead cells reached $\sim 90\%$ after 160- to 200 mL buffer was elutriated at a low flow rate (Figure 5A, B). The cumulative percentage of dead cells plateaued at 200 to 280 mL of elutriation. This suggests that there is no benefit to increasing elutriation volume beyond that point. A similar pattern was also observed from both low and high dead cell burden groups elutriated with high

Figure 3. Representative real-time images of counterflow centrifugal chamber during dead cell elutriation. (A) Viable and dead cells accumulated toward the narrow end of the chamber during volume reduction and buffer exchange steps. (B) The medium was removed after 30 mL of buffer exchange. (C) Viable cells continued to stay toward the narrow end of the chamber, while dead cells migrated toward the wider end. The wider end of the chamber appeared to be cloudy during the first 100 mL of elutriation. (D) The wider end of the chamber became clear after 200 mL of elutriation. (Color version of figure is available online.)

flow rate (Figure 5C, D). These data indicate that high flow rate did not result in more efficient removal of dead cells (Figure 5C, D).

Dead cell elutriation from expanded T cell culture

Low cell viability is one of the manufacturing challenges in CAR-T therapy, in which the most common cell type used is expanded primary T cells. Expanded T cells (150×10^6 cells) were subjected to the C:F ratio reduction test to determine the minimum C:F ratio for retaining the viable cells. There was minimum viable cell loss until the C:F ratio reached 20 and $\sim 15\%$ of viable cell loss at a C:F ratio of 18 ($n = 3$) (Figure 6A). Similar to the earlier experiment in which $>10\%$ of cell loss indicated that the C:F ratio was too low for efficient elutriation, the minimum feasible elutriation C:F ratio was determined to be 20. Based on the results obtained using Jurkat cells, the C:F ratio required to achieve 80% to 90% cell recovery was six C:F ratio units higher than the minimum feasible C:F ratio (Supplementary Table 1). Therefore, a C:F ratio of 26 was chosen for this experiment. Cell viability was significantly improved, from $80.67\% \pm 2.33$ to $94.73\% \pm 1.19$ ($n = 6$, $P = 0.031$) (Figure 6B). Cell recovery was $93.39\% \pm 2.06$, which was higher than the 80% to 90% recovery observed in the Jurkat cell experiments (Figure 4C). The recovered cells were cryopreserved in 5% (v/v) DMSO in FBS for post-thaw assessment. Thawed cells were assessed for proliferation capacity and post-thaw viability. Here we observed that dead cell removal resulted in an increased proliferation index compared with the unprocessed control group ($n = 6$, $P = 0.0313$) (Figure 6C). The post-thaw viability of T cells from both elutriated and unprocessed groups was maintained for >4 h at room temperature (Figure 6D).

Dead cell elutriation after tissue digestion

hAECs have been used in clinical trials for bronchopulmonary dysplasia in preterm infants, and low cell viability is the leading cause of manufacturing failure. hAECs (30×10^6 cells) were subjected to the C:F ratio reduction test in which $>10\%$ of viable cell loss took place at the C:F ratio of 6; hence a C:F ratio 8 was the minimum feasible C:F ratio ($n = 3$) (Figure 7A). Similar to the previous experiment, we were aiming to achieve 80% to 90% viable cell recovery after elutriation for hAECs. Therefore, a C:F ratio of 14, six units higher than the minimum feasible C:F ratio, was chosen for this experiment. Average cell viability significantly improved from $79.19\% \pm 5.35$ to $90.34\% \pm 3.59$ by elutriation ($n = 8$, $P = 0.0078$) (Figure 7B). Average cell recovery was $88.12\% \pm 7.61$ ($n = 8$). The recovered cells were cultured for 24 h, and metabolic activities were assessed. There was no significant difference between the processed ($n = 9$) and unprocessed cells ($n = 5$, $P = 0.73$) (Figure 7C). Cells were cryopreserved with 5% DMSO FBS and thawed. Post-thaw viability from both processed and unprocessed groups was maintained for >4 h at room temperature (Figure 7D).

Discussion

Counterflow centrifugal elutriation has been a valuable tool for scientific research [20–22] and manufacturing cell therapy products [23–29]. We aimed to identify the critical process parameters in dead cell elutriation to develop an elutriation protocol that maximizes cell recovery with an $\sim 10\%$ increase in cell viability. We first examined the impact of various processing parameters on dead cell elutriation using Jurkat cells, in which the C:F ratio, flow rate and elutriation volume were found to be the critical process parameters.

Figure 4. Comparisons of low and high flow rate elutriation. The number of viable cells exiting the chamber was measured at each incremental reduction of C:F ratio. (A) The elutriation profile of low dead cell burden culture was similar between low- and high-flow-rate elutriation. (B) The high-flow-rate elutriation profile shifted toward the higher C:F ratio in comparison to low flow rate in high dead cell burden culture. (C–F) Cells were subjected to a constant C:F ratio over a volume of 280 mL. The number of recovered viable cells decreased with reducing C:F ratio in low dead cell culture. The trends were similar in high dead cell culture, although high-flow-rate elutriation recovered cells only at C:F 18 and 20. (E and F) Cell viability increased with reducing C:F ratio. Data are presented as mean $\pm$ SD. ND, not detectable. (Color version of figure is available online.)

We then applied the identified parameters from Jurkat cells to expanded primary T cells and hAECs. The cell viability improved from an average of $\sim 80\%$ to $\sim 90\%$ while maintaining cell recovery at $\geq 90\%$. Previous study reported that $\sim 12\%$ to 16% of CAR-T product did not meet the release criterion of $>80\%$ viability; most of the “out of specification” products had a cell viability of 70% to 80% [6]. The 10% improvement in cell viability is arguably sufficient to prevent the manufacturing failure due to low cell viability. Additionally, one of the critical criteria in autologous cell manufacturing is to achieve the target number of viable cells [30]. The high cell recovery observed in this study showed minimum cell loss after the elutriation process.

The proliferation capacity of T cells was improved after dead cell elutriation (Figure 6C). This result suggests that elutriation did not cause cell damage, and the proliferation rate of T cells increased when fewer apoptotic cells were present in the culture. A previous study reported that the proliferation of T cells improved after removal of monocytes by elutriation [28]. It was suggested that soluble factors produced by monocytes, such as prostaglandins, may suppress T cell proliferation [31]. Whether soluble factors produced by

apoptotic cells had the same suppressive effect on T cell proliferation *in vitro* requires further investigation in future studies.

The C:F ratio was found to be the key parameter in controlling the trade-off between cell recovery and viability during dead cell elutriation. There is a small overlap in size between the viable cells and dead cells (Figure 2B, C). Jurkat cells elutriated at the minimum feasible C:F ratio could result in $\leq 94\%$ cell viability, with most of the dead cells elutriated. However, small viable cells were also elutriated at the minimum feasible C:F ratio, which resulted in $\sim 50\%$ viable cell recovery. In contrast, a high C:F ratio could result in 90% recovery with $\sim 85\%$ cell viability. We also observed that high-flow-rate elutriation resulted in the loss of more viable cells in high dead cell burden culture at the same C:F ratio than in low dead cell burden culture (Figure 4B–D). The average viable cell size from the high dead cell burden culture was slightly lower than the viable cells size from the low dead cell burden culture, although the difference was not statistically significant (Figure 2E). This relative difference in cell size may account for the increased loss of viable cells. It has been established that the elutriation profile varies among different cell types [22]. Historically, elutriation is performed at a constant centrifugal force with

Figure 5. Elutriation volume required for dead cell removal. The number of dead cells elutriated in each 40-mL fraction was quantified for each elutriation setting. (A and B) The cumulative percentages of elutriated dead cells converged after 160 mL low-flow-rate elutriation in low dead cell burden culture and high dead cell burden culture. (C and D) The dead cell elutriation pattern remained unchanged in low dead cell culture and high dead cell culture when subjected to high-flow-rate elutriation. (Color version of figure is available online.)

Figure 6. Dead cell elutriation in expanded T cells. Expanded T cells (1.5×10^8 cells) were subjected to elutriation. (A) The number of viable cells exiting the chamber was measured at each incremental reduction of C:F ratio. The minimum C:F ratio for retaining CD3 cells was 20. (B) Cell viability was improved from 80.67 ± 2.33 to 94.73 ± 1.19 after the elutriation. (C) The unprocessed T cells had significantly lower proliferation index compared with the processed cells measured by flow cytometry (1.02 ± 0.03 and 1.13 ± 0.13 , respectively). (D) Post-thaw cell viability was maintained for >4 h at room temperature. * $P < 0.05$ between the indicated groups as assessed by Wilcoxon matched-pairs signed-rank test. (Color version of figure is available online.)

Figure 7. Dead cell elutriation in human amniotic epithelial cells after tissue digestion. Human amniotic epithelial cells were isolated from placental membrane, and 4×10^7 viable cells were subjected to elutriation in each run. (A) The number of viable cells exiting the chamber was measured at each incremental reduction of C:F ratio. The minimum C:F ratio for retaining hAECs was 8. (B) Cell viability improved from $79.19\% \pm 5.35$ to $90.34\% \pm 3.59$ after elutriation. (C) The metabolic activity measured by MTS assay was comparable between unprocessed and processed cells (0.34 ± 0.07 and 0.35 ± 0.07 , respectively). (D) Post-thaw cell viability was maintained for >4 hours at room temperature. (** $P < 0.01$ between the indicated groups as assessed by Wilcoxon matched pairs signed rank test. (Color version of figure is available online.)

stepwise increments of the flow rate in the JE-6 elutriator system (Beckman Coulter, Brea, CA) [22]. The same operation principle is applied in the Elutra system (Terumo BCT, Tokyo, Japan), a counter-flow centrifugal system designed for processing apheresis material in cell manufacturing [27–29]. In both JE-6 and Elutra systems, cells are typically elutriated into multiple fractions. The operator then decides to keep or discard fractions based on the yield and purity of each fraction. The overall process can be time-consuming, especially if only two cell fractions are required. The manufacturer's default protocol of the Elutra system, optimized for lymphocyte and monocyte separation, may not be suitable for processing material beyond blood products. The elutriation setting has to be optimized for each cell type or buffer, as either will influence the processing parameters. The C:F ratio reduction test described in this study (Figures 6A and 7A) allows users to perform elutriation on various cell types with little experience. In some cases, cell size can change in response to stimulation or activation [32]. The C:F ratio reduction test allows for a tailored protocol to be developed for different manufacturing steps.

We also compared dead cell elutriation using 25 (low) and 50 mL/min (high) flow rates at different C:F ratios. The results showed a gradual improvement in viability at the cost of cell recovery when elutriated with a low flow rate. In comparison, high-flow-rate elutriation drastically decreased viable cell recovery from ~75% to undetectable (Figure 4C, D). Although this cell loss could be avoided by elutriating at a higher C:F ratio, high-flow-rate elutriation is not ideal when high cell viability is required. Elutriation with a low flow rate allowed for more precise adjustments to achieve a target cell recovery. Previous studies using the Elutra system used a changing flow rate from 60 to 120 mL/min or 30 to 90 mL/min to elutriate

lymphocytes and separate them from monocytes [27–29]. However, the number of cells and chamber volume in these studies were both higher ($\sim 10^9$ in a 40-mL chamber) in comparison to the current study ($\sim 10^8$ in a 10 mL chamber). Our previous study identified that the stability of the fluidized cell bed increases with increasing cell numbers, and a lower percentage of cells are lost after processing using the same flow rate [19]. It is plausible that the optimal elutriation flow rate may be higher when processing with a higher number of cells or larger chambers. The Rotea chamber is able to accommodate $\sim 10^9$ T cells. However, the impact of a 10-fold-higher cell number on dead cell elutriation was not determined in the current study.

Lastly, the elutriation volume required for ~90% dead cell removal was found to be 160 to 200 mL, which was 16 to 20 times the volume of the chamber (Figure 5). The protocols described in previous counterflow centrifugal elutriation studies typically elutriated each fraction over 20 to 25 times of the chamber volume regardless of the elutriation flow rate [22,27]. The results of the current study suggest that the elutriation volume was critical to the process, in that at least 16 times the chamber volume was required for sufficient dead cell removal.

In summary, the current study identified C:F ratio, flow rate and elutriation volume as the critical process parameters in dead cell elutriation. We demonstrated that the viability of T cells and hAECs could be improved through automated washing and concentration with the integration of dead cell elutriation. It is an efficient process that can be applied before cell expansion or formulation to improve product quality. Future studies may explore the application of dead cell elutriation processes in other cell types or manufacturing steps such as after gene transfection.

Conflict of interests

DJ is the CEO of Scinogy, responsible for development and manufacturing of the Rotea Counterflow centrifuge. Other authors have no conflicts of interest.

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Supplementary materials

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