Published in final edited form as:

Cytotherapy. 2019 February ; 21(2): 234–245. doi:10.1016/j.jcyt.2018.12.004.

Improved expansion of T-cells in culture when isolated with an equipment-free, high-throughput, flow-through microfluidic module versus traditional density gradient centrifugation

Briony C. Strachan¹, Hui Xia¹, Eszter Vörös¹, Sean C. Gifford², and Sergey S. Shevkoplyas^{1,*}

¹Department of Biomedical Engineering, University of Houston, Houston, TX 77204

²Halcyon Biomedical Incorporated, Friendswood, TX 77546

Abstract

Background: The isolation of lymphocytes – and removal of platelets (PLTs) and red blood cells (RBCs) – from an initial blood sample prior to culture is a key enabling step for effective manufacture of cellular therapies. Unfortunately, currently-available methods suffer from various drawbacks – including low cell recovery, need for complex equipment, potential loss of sterility, and/or high materials/labor cost.

Methods: A newly-developed system for selectively concentrating leukocytes within precisely-designed, but readily-fabricated, microchannels was compared to conventional density gradient centrifugation with respect to: (i) ability to recover lymphocytes while removing PLTs/RBCs, and (ii) growth rate and overall cell yield once expanded in culture.

Results: In the optimal embodiment of the new microfluidic approach, recoveries of CD3+, CD19+, and CD56+ cells (85%, 89%, and 97%, respectively) were significantly higher than for paired samples processed via gradient-based separation (51%, 53%, 40%). While the removal of residual PLTs and RBCs was lower using the new approach, its enriched T-cell fraction nevertheless grew at a significantly higher rate than the gradient-isolated cells, with approximately twice the cumulative cell yield observed after 7 days of culture.

Discussion: The standardization of each step of cellular therapy manufacturing would enable an accelerated translation of research breakthroughs into widely-available clinical treatments. The high-throughput approach described in this study – requiring no ancillary pumping mechanism nor expensive disposables to operate – may be a viable candidate to standardize and streamline the

*Corresponding author: Sergey S. Shevkoplyas, Ph.D., University of Houston, Department of Biomedical Engineering, 3605 Cullen Blvd, Houston, TX 77204-5060; phone: +1 (713) 743-5696; fax: +1 (713) 743-0226; sshevkoplyas@uh.edu.

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Conflicts of Interest

SCG and SSS are inventors on U.S. Patent #9,789,235 "Separation and concentration of particles" describing this technology, and also part-owners of Halcyon Biomedical Incorporated, a company which would benefit from its commercialization. BCS, HX, and EV declare no conflict of interest.

initial isolation of lymphocytes for culture, while also potentially shortening the time required for their expansion into a therapeutic dose.

Graphical Abstract

Keywords

leukapheresis; T-cell enrichment; microfluidics; density gradient

Introduction

The use of cellular therapies to treat a wide array of acute and chronic conditions continues to rapidly expand [1]. Particularly exciting are adoptive cellular immunotherapies based on genetically modified T-cells engineered to express chimeric antigen receptors (CARs) that have emerged as potentially curative treatments for large B-cell lymphoma [2], acute lymphoblastic leukaemia [3], multiple myeloma [4], and other types of cancer [5].

The process of manufacturing a CAR T-cell therapy typically involves: collecting lymphocyte-rich mononuclear cell (MNC) concentrates from peripheral blood of patients via leukapheresis, further enrichment of the collected lymphocytes, activating and transducing CD3⁺ T-cells, and finally expanding the modified cells in culture [6, 7]. MNC concentrate purification is necessary as these products contain significant (and quite variable) amounts of residual platelets (PLTs) and red blood cells (RBCs), as well as other types of white blood cells (WBCs), that may interfere with transduction and/or diminish the ability of modified cells to expand in culture – which would ultimately reduce the efficacy of the manufactured therapy [8].

Leukapheresis units are often processed using density gradient separation – performed either with a high-volume centrifuge manually, or with an automated cell processor [9]. Manual density gradient centrifugation is labor-intensive, subjects cell to lengthy processing, and – as it is an open system procedure – must be performed in a sterile hood or ISO 5 cleanroom facility to minimize incidents of microbial contamination. Automated density gradient separation is significantly less laborious, faster, and can be performed as a closed system. However, this requires a large capital expenditure and expensive disposable inserts to operate – features which are also shared by automated elutriation-based equipment. Importantly, lymphocytes enriched using density gradients typically also need to undergo

several washing steps to remove the mildly-toxic medium after the separation [10, 11], and those gathered via elutriation must still undergo a chemical lyse step to remove the majority of residual RBCs, followed by similar washing [12, 13]. Each of these processes is a source of potential damage to the desired WBC population.

The isolation of CD3+ lymphocytes directly from MNC concentrates using antibody-coated magnetic beads is another common method of isolating T-cells. While it has the advantage of very high purity, it typically has a much lower relative yield compared to the above methods and has a much higher cost per cell acquired, particularly when large (200–300 mL) leukapheresis units are used as starting material in order to harvest the vast quantity of cells required to manufacture a therapeutic dose for clinical treatment [12–14]. Additionally, a ‘pre-washing’ step is often required to remove the majority of PLTs from the unit, which may otherwise interfere with binding of beads to their cellular targets [15].

Here we describe passive, high-throughput ‘meso-fluidic’ devices, based on recently-developed *controlled incremental filtration* (CIF) technology [16–18], capable of removing the vast majority of PLTs and RBCs from MNC-rich leukapheresis units while retaining ~85% of T-cells, without the need for extensive dilution or any washing, and at processing speeds of ~4 mL/min with no electromechanical equipment required (or at speeds exceeding 50 mL/min, with use of a simple syringe pump). Most importantly, the T-cells produced by the equipment-free CIF method of cell separation showed significantly higher growth rates in culture as compared to those from conventional density gradient processing, resulting in a cumulative ~94% increase in total cell yield when using this new approach. We therefore suggest that CIF-based technology may offer an economical path to a simple closed-system for lymphocyte purification, which could help streamline and standardize CAR T-cell manufacturing.

Materials and Methods

Fabrication of microfluidic devices

The design and fabrication of microfluidic devices based on controlled incremental filtration (CIF) technology have been described previously in detail [16–18]. Briefly, CIF device designs were generated using custom software (MATLAB®, The MathWorks Inc., Natick, MA) and transferred from chrome-on-glass photomasks (Photo Sciences, Inc., Torrance, CA) into photoresist (SU8 3050; MicroChem Corp, Newton, MA) spun onto 4" silicon wafers (University Wafer, South Boston, MA) using UV (i-line) exposure (ABM-USA Inc., San Jose, CA). The ~140 µm deep structures were created by first applying two sequential layers of photoresist, followed by exposure and development of the photoresist.

For devices to be driven by syringe pumping, master wafers with bas-relief SU8 device features were replicated in poly(dimethylsiloxane) PDMS in a three-step procedure. First, 1.5 mL of 8:1 (base:crosslinker) standard PDMS (Sylgard® 184, Dow Corning Corp, Midland, MI) were dispensed to cover the devices’ inlet/outlet areas on the master wafer and cured for 15 minutes at 75°C. Second, a custom mixture consisting of 5-parts of 8:1 standard PDMS and 1-part of 1:1 (Part A:Part B) hPDMS (Gelest, Morrisville, PA) was spread over the devices in a thin (~1 mm) film, vacuumed for 10 minutes, and cured for 1 hour at 60°C.

Finally, a 7 mm layer of standard PDMS was applied over the semi-cured film, and then cured overnight at 60°C. Device replicas were peeled off, baked at 200°C for 30 minutes to complete hardening of the thin hPDMS/PDMS layer, and then bonded to 102 × 83 mm pieces of cleaned glass, using oxygen plasma (Plasmalab 80 Plus, Oxford Instruments, Abingdon, United Kingdom). To complete assembly, an 8 mm thick PDMS layer (10:1, cured at 80°C for 2 hours) containing large channels for collecting the product effluent from the individual CIF units was bonded onto the device replica layer. For devices to be driven by simple hydrostatic pressure, no hPDMS was necessary thus replicas were cast in 8:1 (base:crosslinker) PDMS alone and bonded to a PDMS-coated petri dish. Inlet and outlet ports were created using biopsy punches of appropriate size for tubing connections. Assembled devices were treated with 1% (w/v) aqueous solution of mPEG-silane (Laysan Bio, Inc., Arab, AL) as previously described [17, 18].

MNC leukapheresis samples

Leukapheresis units (n = 6, ~200 mL each) were purchased from Key Biologics, LLC (Memphis, TN). Key Biologics performed leukapheresis on healthy consenting volunteers using the Fenwal Amicus Separator system (Fresenius Kabi USA, Lake Zurich, IL), and shipped collected leukapheresis units with refrigerated gel packs to our laboratory overnight. Upon receipt, each unit was passed through a filter (SQ40, Haemonetics, Braintree, MA) to remove any large aggregates, as well as non-cellular debris that may be present in this type of apheresis product. All leukapheresis units were processed within 24 hours of collection.

Density gradient separation method

MNCs were purified from ~6mL of each leukapheresis unit via density gradient centrifugation following the manufacturer's protocol (Histopaque®–1077 Hybri-Max™, Sigma-Aldrich, St. Louis, MO).

Microfluidic separation method

Aggregate-filtered leukapheresis samples were used either as is, or diluted to reduce the concentration of white blood cells (WBCs) to approximately 10⁴/μL in PBS with 1.8mM EDTA (for 'Style A' CIF devices) or approximately 5×10⁴/μL in PBS with 0.8% Kolliphor® P-188 (for 'Style B' CIF devices), when necessary. All chemicals were purchased from Sigma-Aldrich. PBS was purchased from Caisson Labs (Smithfield, UT).

MNC samples were: (i) aliquoted into sterile 60 mL syringes (BD Biosciences, Fairlawn, NJ) for pump-driven experiments (GenieTouch™; Kent Scientific Corp., Torrington, CT), or (ii) hung at a height of about ~5ft above the level of a CIF device in a standard 300 mL blood bag, with or without additional driving pressure created by compression of the bag by a standard infusion cuff (Metpak® 5275; Rudolf Riester GmbH, Jungingen, Germany), as noted. Input samples were connected to the inlet port of a CIF device via polyurethane tubing. Similar tubing connected the outlets of the device to reservoirs for collection of the separated streams of platelet/RBC waste (filtrate outlet) and concentrated WBCs (retentate outlet).

Flow cytometry analyses

All flow cytometry samples were stained in 100µL of appropriate buffer for 20 minutes and analyzed using a FACS Aria II flow cytometer (BD Biosciences). Dyes and buffers were purchased from BD Biosciences, unless otherwise stated. *WBC counts*: ~10⁶ WBCs were stained with 5 µL of anti-CD45-APC (HI30) and 1.25 µL each of anti-CD14-BV421 (MφP9) and anti-CD66B-PE (G10F5) in a Trucount™ tube (BD Biosciences). *RBC:WBC ratio*: Samples of approximately 10⁶ RBCs were stained with 2 µL of anti-CD45-APC and 0.01 µL of anti-CD235a-FITC (GA-R2). *Lymphocyte Classifications*: ~10⁶ WBCs were stained with 5 µL of anti-CD45-APC and either 5µL of anti-CD3-PE (UCHT1) and 1.25 µL each of anti-CD4-BB700 (SK3) and anti-CD8-BV421 (RPA-T8); or 1.25 µL each of anti-CD19-BB700 (SJ25C1) and anti-CD56-BV421 (NCAM16.2). *Lymphocyte Activation*: ~10⁶ WBCs were stained with 5 µL each of anti-CD45-APC and anti-CD69-FITC (FN50); a threshold was set using 5 µL of an isotype control, IgG1-FITC (MOPC-21). *Viability (Apoptosis and Live/Dead)*: ~10⁶ WBCs were stained with 5 µL each of anti-CD45-APC and of Annexin V - Alexa Fluor conjugate (ThermoFisher, Waltham, MA) and 50 ng of Propidium Iodide.

Expansion of separated cells in culture

Isolated lymphocytes were suspended in RPMI 1640 medium (Gibco, Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS; Biochrom, Berlin, Germany), 1% (v/v) of penicillin/streptomycin solution (10,000 units/mL of penicillin, 10,000 µg/mL of streptomycin), 50 µg/mL gentamicin, 2.5 µg/mL amphotericin B, 1 µg/mL phyto-hemagglutinin (PHA) (all from Gibco), and 25mM HEPES (Sigma-Aldrich), and incubated at 37°C with 5% CO₂ for 24 hours. After 24 hours, cells were washed by centrifugation at 250 × g for 10 minutes, re-suspended, and transferred to a new 6-well tissue culture plate. After 3 days, cells were washed and transferred to a new plate containing the same medium above, but with PHA replaced by 20 ng/mL human IL-2 (Gibco), and incubated for 7 days using standard proliferation techniques. Total numbers of viable cells were determined using an automated cell counter with Trypan Blue (Countess II, ThermoFisher).

Lymphocyte migration assay

Lymphocyte migration ability was evaluated at the end of culture using a Boyden Chamber CytoSelect Cell Migration Assay kit with polycarbonate membrane inserts (3 µm pore size; Cell Biolabs Inc., San Diego, CA) according to the manufacturer's protocol.

Statistical analysis

Statistical significance of the differences between separation methods for all measured parameters was calculated using a two-sided paired Student's t-Test. For cell culture growth experiments a one-sided test was used, hypothesizing that the CIF-isolated cells would grow more quickly than cells isolated by the harsher density gradient centrifugation technique. A p value of less than 0.05 was considered to be significant.

Results

Design of microfluidic devices

The approach to designing flow-through devices for the separation of PLTs and RBCs from WBCs in MNC-rich leukapheresis units was based largely on our prior theoretical and modeling work on ‘controlled incremental filtration’ (CIF) [16–18]. In brief, blood cells flowing through a CIF-based device which have an effective size above the threshold ‘critical diameter’ (c.d.) of the device will be retained in its central/retentate channel, while cells below this cutoff size are able to be pulled into the side/filtrate channels by the flow of fluid out of the central channel at each filtration point (Fig. 1a). This is accomplished by precise calculation of the dimensions of each channel required at each point to ensure a consistent prescribed c.d. throughout the device [16].

In cases where the particulate fluid in a device is a multi-component solution such as a leukapheresis sample, cell-cell interactions and other features (e.g. the non-spherical shape and deformability of the cells) will have complex effects on the flow dynamics of the cellular mixture as it passes through a device. RBCs in particular, as highly-deformable biconcave discs, pose a challenge with respect to assigning an effective size during their flow through microchannels. As can be seen in Fig. 1b, RBCs tend to build-up within the channel-delimiting posts of a traditional CIF device, particularly when input hematocrit is low, as is typical with leukapheresis samples. However, we found that by angling these posts, while keeping the same fraction of outward fluid flow from the retentate channel, we could alter the streamlines in the vicinity of the filtration points of a CIF-based device to increase the likelihood of an RBC being preferentially pulled into its filtrate channels, while continuing to retain the vast majority of WBCs (Fig. 1c–d).

For practical purposes, a CIF device is designed to have an integer number of ‘legs’ to allow for straightforward patterning of several devices arranged in parallel on the face of a master mold [17, 18]. In this study, two styles of CIF devices with optimized fluidic filtration per gap and post angles were designed: ‘Style A’ CIF devices had two legs with total length of about 140 mm (Fig. 2a) and were able to concentrate large cells ~18-fold, and ‘Style B’ devices had a single leg of about 78 mm (Fig. 2c) and were able to concentrate large cells ~4.5-fold. In practice, only 12 of Style A or 48 of Style B CIF devices combined in a single module could be fit onto a 4" mold (Fig. 2b, d), creating a tradeoff between achieving a higher degree of filtration/concentration (Style A) versus achieving a higher volumetric throughput at a given driving pressure (Style B).

Figure 3 illustrates the three types of approaches used to drive leukapheresis samples through CIF device modules. Style A modules were operated by a syringe pump (Fig. 3a), as is typical for conventional microfluidic technologies to achieve a relatively high (i.e. mL/min or greater) throughput [19, 20]. The shorter length (lower resistance) and higher copy number of individual devices in a Style B module, however, allowed for the elimination of an active pumping mechanism, while still enabling a multi-mL/min processing rate by simply hanging a bag containing the sample above the module (Fig. 3b). As end users may potentially desire still-lower processing times, we also investigated the performance of such

a module while gently compressing the input sample bag with a standard infusion cuff (Fig. 3c).

Processing of leukapheresis samples with CIF devices as compared to density gradient separation

We completed a split-sample study to compare the separation efficiency and quality of purified WBCs produced with the CIF devices versus a conventional density gradient separation method. For each leukapheresis unit, a 6 mL aliquot was processed via density gradient centrifugation ($n = 6$), and the rest of the sample (~ 190 mL) was perfused through the CIF device modules ($n = 3$ for Style A, and $n = 3$ for Style B). In our preliminary studies, we found that Style A CIF modules could typically enrich WBCs to a concentration of $\sim 250 \times 10^3/\mu\text{L}$ before the increased viscosity of the retentate suspension began to have negative effects on device performance (i.e. increased loss of lymphocytes to the filtrate channels). The initial concentration of WBCs in the leukapheresis units used in this study was $54.0 \pm 19.5 \times 10^3/\mu\text{L}$ (range: $32.4 - 85.4 \times 10^3/\mu\text{L}$), and therefore we diluted the samples to a target initial concentration of $\sim 10 \times 10^3/\mu\text{L}$ (actual concentration: $9.6 \pm 0.8 \times 10^3/\mu\text{L}$), before passing them through Style A device modules in order to have a target final WBC concentration of $\sim 200 \times 10^3/\mu\text{L}$. As Style B device modules were designed to concentrate WBCs only 4.5-fold, the MNC samples were used either as-received or were minimally diluted (resultant concentration: $44.4 \pm 10.3 \times 10^3/\mu\text{L}$) to achieve a similar target final WBC concentration.

Table 1 compares the separation performance of the two CIF device modules ($n = 3$ for each) with that of traditional density gradient centrifugation ($n = 6$; other than CD19 and CD56 measurements, which were $n = 3$). Style A modules were able to process MNC samples at a volumetric flow rate of 17 mL/min (driven via a syringe pump, Fig. 3a), while removing a larger fraction of PLTs, but a somewhat smaller fraction of RBCs, than density gradients. Given the output ratio (filtrate:retentate) of $\sim 19:1$ for the Style A module, the theoretical maximum percent removal of RBCs would be $\sim 95\%$, in addition to any ‘crowding out’ effects due to build-up of larger cells. The observed average value of 86% was lower than this theoretical prediction due to some remaining ‘build up’ of RBCs in the retentate channel (see Fig. 1d). The overall lymphocyte recovery and the recoveries for each individual T-cell subtype were consistently higher for the Style A CIF module than those for density gradient separation (Table 1).

Style B CIF modules were able to process leukapheresis samples at a rate of ~ 4 mL/min, simply by hanging the input sample ~ 5 ft above the CIF module (Fig. 3b). A single pass through a Style B module removed a significantly lower fraction of PLTs and RBCs (due to a relatively low filtrate:retentate ratio), but recovered a consistently higher fraction of lymphocytes than the density gradient method (by 13 percentage points, on average, when comparing to all $n = 6$ gradient-processed samples; and by 18 points when comparing to solely the $n = 3$ paired percentage samples). The recoveries of all T-cell subtypes were substantially higher (by more than 20 points, overall; and by more than 30 points when focusing on paired samples only) after one pass through the Style B CIF module compared to density gradient processing, with all of the differences statistically significant. Finally, the

recoveries of B-cells and natural killer (NK) cells using the Style B module were dramatically higher than those for density gradient separation (Table 1).

To test whether PLT and RBC depletion performance of the Style B CIF device module could be further improved, we passed the sample through the module a second time – average RBC removal increased by an additional 19 percentage points, and average PLT removal increased by 15 percentage points. As an expected tradeoff, the overall lymphocyte recovery and recoveries of individual T-cell subtypes declined after the second pass, although still remaining higher than those for density gradient samples (Table 1). Given the output ratio (filtrate:retentate) of ~4:1 for the Style B module, a theoretical maximum percent removal of PLTs and RBCs would be ~80% after one run, and ~96% after two runs. As little to no sample dilution was required in this case, and no EDTA was added to the samples, the lower depletion of PLTs was likely due to elevated sticking of PLTs to WBCs in the retentate. The higher initial RBC count in these samples, however, served to push RBC removal closer to its theoretical maximum.

Table 2 shows the changes in composition of the leukapheresis samples due to processing. (Note: absolute numbers of cells of each type measured are provided in Table S1 of the Supplementary Information, showing values as 10^3 cells per μL of sample; and in Table S2, showing values as 10^9 cells per unit, assuming that 200 mL of the initial volume of each leukapheresis unit had been completely processed by the given cell separation approach). The overall concentration of WBCs in the sample significantly increased when using either density gradient or CIF-based modules, as expected. Density gradient processing significantly increased the fraction of monocytes, while both Style A and B CIF modules did not change it significantly. Processing via all methods preserved the overall fraction of lymphocytes in the sample. However, the fractions of each measured T-cell subtype in the sample was significantly reduced by density gradient centrifugation as well as by two passes through the Style B CIF device module. Importantly, density gradient processing significantly reduced lymphocyte viability with respect to the initial sample and correspondingly increased the fraction of apoptotic or dead cells. Lymphocyte viability for both Style A and Style B CIF modules was no different than in the initial samples, and was significantly higher than for cells processed via density gradients (Table 2).

Expansion in culture of T-cells enriched with CIF device modules or via density gradient centrifugation

To investigate the effect of processing on the ability of T-cells to expand *in vitro*, we cultured samples produced using Style A CIF modules operated at 17 mL/min (Fig. 3a), Style B modules operated at 50 mL/min (Fig. 3a), and Style B modules operated via gravity-driven flow (~4 mL/min, Fig. 3b), alongside paired samples prepared by conventional density gradient centrifugation. Immediately following processing, samples were placed (at a lymphocyte concentration of $1.5 \times 10^6/\text{mL}$) into culture medium containing PHA, and incubated for 4 days to stimulate T-cell division. After incubation with PHA, cells were washed and placed (at a concentration of $10^6/\text{mL}$) into culture medium containing IL-2, and allowed to expand for 7 days (Figure 4).

As expected, the cell expansion curves for all samples followed an exponential growth pattern (Fig. 4, **lines**). T-cells processed via density gradients had the slowest growth rate, reaching 17.5 ± 4.4 (range: 13.5 – 24.7) fold expansion by Day 7 of IL-2 culture. T-cells processed with the Style B device module driven by gravity grew significantly faster, expanding 22.2 ± 3.2 (range: 19.6 – 25.7) fold by the end of culture. The growth rate for T-cells processed by Style A device modules was lower than those from the gravity-driven Style B device, but still significantly faster than cells isolated via density gradients, resulting in a 19.3 ± 2.7 (range: 17.6 – 22.4) fold expansion. The growth of T-cells produced by Style B modules pumped at 50 mL/min was not significantly different from that of cells from paired samples processed conventionally, yielding a 20.0 ± 3.5 (range: 16.7 – 23.6) fold expansion by the end of the IL-2 culture.

At the end of the culture period, cell migration ability was assessed with a commercial kit, using FBS as chemoattractant. Cells cultured from Style A CIF-processed samples migrated to a significantly higher degree than cells initially isolated via density gradients. There was no statistical difference in migration between gradient- or Style B CIF-isolated cell cultures.

Figure 5 quantifies the cumulative impact of each processing method's separation efficiency and the ability of its isolated T-cells to expand in subsequent culture on overall cell yield. The fold-expansion of T-cells initially present in the sample was calculated by combining the measured data for CD3+ cell recovery (Table 1) with the subsequent growth rate for each separation method (Fig. 4). When starting from the same number of T-cells present in the initial sample, and factoring in the superior recovery during 'Day 0' processing (underlying data of Table 1), and the higher rates of exponential growth in culture (i.e. 0.415 day^{-1} for Style A, and 0.447 day^{-1} for gravity-driven Style B, versus 0.405 day^{-1} for density gradients, on average; Fig. 4), both CIF device modules significantly outperformed conventional density gradient processing through most of the 7-day culture (Fig. 5). In this representation, the total fold-expansion of T-cells initially present in a leukapheresis unit was about 83% higher for Style A, and 94% higher for Style B CIF device modules than for paired samples processed via density gradient centrifugation, after 7 days in culture.

Effect of flowrate through CIF devices on cell separation and viability

A novel mixture of PDMS and h-PDMS was used to increase the rigidity of some of the Style B CIF device modules fabricated during this study, in order to reduce deformation during pressurization while still allowing for ready demolding of the cast modules. As shown in Figure 6, increasing the flowrate through a device beyond 30 mL/min nevertheless appeared to deform the channels of the CIF devices sufficiently to affect their internal flow dynamics. At 60 mL/min a significantly larger fraction of WBCs of all types, but particularly B-cells, leaked out of the module's retentate channels due to this structural deformation during high-speed flow. However, there was little change in device performance when progressing from simple gravity-driven flow ($\sim 4.0 \text{ mL/min}$) to an infusion cuff assisted flow ($\sim 14.6 \text{ mL/min}$) to a moderate-speed pump-driven flow (30 mL/min), which correspond approximately to driving pressures of 2.2 PSI, 8.1 PSI, and 16.7 PSI, respectively.

There was a small but not statistically significant increase in the fraction of apoptotic cells from the initial sample to those collected from the CIF devices of Fig. 5 (range: 7.1 – 9.2%), and no significant change in the fraction of ‘dead’ cells (range: 0.8 – 1.3%).

Discussion

This study represents the first demonstration of a ‘meso-fluidic’ approach to cell separation that can process a meaningful amount of a blood sample per unit time, without needing a pump or other mechanism – besides simple gravity – to drive flow. A recent report using microdevices designed in a ‘deterministic lateral displacement’ (DLD) format averaged just ~1.2 mL/min leukapheresis sample throughput at 10 PSI driving pressure, and required a large amount of sample dilution to operate [19]. Another recent DLD-based study required four pumps working in tandem to process dilute whole blood samples, under what would appear to be quite high pressure and shear, processing the equivalent of $\sim 300 \times 10^6$ cells per second [20]. Here, we showed that a CIF-based device module can achieve 4 mL/min processing speed (or $\sim 50 \times 10^6$ cells per second, for leukapheresis samples with $\sim 250 \times 10^3$ PLTs/ μ L, $\sim 50 \times 10^3$ WBCs/ μ L, and $\sim 500 \times 10^3$ RBCs/ μ L) without any pumping at all, and similarly high-levels of cell separation performance at 30 mL/min (i.e. $\sim 400 \times 10^6$ cells per second) using a single common (low-pressure) syringe pump (Fig. 6). Once modules are manufactured out of less deformable thermoplastic parts, as in the Mutlu et al study [20], and if using a higher-powered pump, effective CIF-based cell separation at processing rates well above the 50–60 mL/min already demonstrated in this study should be readily achievable; and cell per second processing rates would be much higher here if we had processed dilute whole blood rather than WBC-rich leukapheresis samples. One reason for the superior CIF device flowrate (per unit pressure) being that while DLD-style devices invariably have small ($< 5 \mu$ m) in-plane features that are difficult to fabricate into deep 3D channels [19–24], we have purposefully formulated the CIF concept to avoid the use of any features below $\sim 20 \mu$ m in size [16–18].

The pump-free approach of using a basic hand-inflated infusion cuff to drive sample processing (Fig. 3c) would appear to be the most likely use case of the CIF-based technology described. With a flowrate of ~ 15 mL/min, a 200 mL leukapheresis unit can be processed in less than 15 minutes using this technique. Since no pumping machinery is required, the entire system can be exceedingly simple to set-up and operate, without necessitating specialized equipment or its attendant training, bench-space, and maintenance requirements. Further, as the driving pressure needed to achieve this flowrate is relatively low (~ 8 PSI), and there are no components to be spun (i.e. as with elutriation- or centrifugation-based approaches to cell separation), the tolerances required of the CIF modules from a manufacturing standpoint are less demanding – suggesting that their cost will also be less than the disposable insert/cartridge costs of more complex systems (which for some existing equipment can exceed 1000USD per unit). In the case of simply hanging the input sample several feet above the CIF cell separation module, the driving pressure created (~ 2.2 PSI at ~ 5 ft) is sufficient to drive the flow of sample through the device at ~ 4 mL/min, which would still allow 200 mL of input sample to be processed in < 1 hr.

The ability to achieve a high flowrate without an active pumping mechanism also provides the user with increased confidence that sterility will be maintained throughout the cell separation procedure set-up and operation. In total, this would consist of a sterile weld of the leukapheresis sample bag to the input tubing of a CIF module, subsequent to any necessary pre-dilution of the original unit (with sterile saline, PBS, Hank's buffered saline, or other common diluents) to ensure an input WBC count of $<50 \times 10^3/\mu\text{L}$ (in the case of the 'Style B' CIF devices described above). After processing, the output bag for collecting enriched WBCs from the flow-through CIF module (Fig. 3) can be sealed and removed, much like the collection bags of effluent from common leukoreduction filters.

Following CIF-based processing, users would also have a more consistent, WBC-enriched sample from which to either directly culture (as we have done in this study) or for additional processing (e.g. bead-based selection of specific WBC subtypes). While the volume of a leukapheresis unit is typically quite consistent, when following manufacturers' protocols, its cell counts can vary widely. With a high average lymphocyte recovery (89%) and low s.d. ($\pm 4\%$) for 'Style B' CIF devices, and similarly predictable performance for PLT and RBC removal (Table 1), adjustment of the input sample concentration to a standardized starting WBC count (e.g. $50 \times 10^3/\mu\text{L}$) should increase the consistency of the WBC count output of the modules accordingly. For example, a 200 mL leukapheresis product containing $100 \times 10^3/\mu\text{L}$ WBCs and $80 \times 10^3/\mu\text{L}$ lymphocytes would be diluted $2\times$ and, after CIF processing, would be expected to produce a sample with $\sim 178 \times 10^3/\mu\text{L}$ lymphocytes, and just $\sim 24\%$ of its original RBCs and $\sim 29\%$ of its PLTs remaining, in a volume of ~ 80 mL. A 200 mL starting product containing $50 \times 10^3/\mu\text{L}$ WBCs and $40 \times 10^3/\mu\text{L}$ lymphocytes, would require no dilution, and after processing would result in the same expected $178 \times 10^3/\mu\text{L}$ lymphocyte concentration (but now in a volume of just 40 mL).

We have shown that removal of $\sim 71\%$ of PLTs and $\sim 76\%$ of RBCs, while concentrating lymphocytes ~ 4.5 -fold, is more than sufficient to produce a 'cleaned up' leukapheresis sample whose T-cells will grow in culture at rates significantly higher than those isolated via density gradients (Fig. 4). This is likely due to the far gentler treatment that the pump- and centrifugation-free Style B CIF module approach has on these cells, as compared to conventional density gradient processing (and its associated washing steps needed for removing the mildly toxic density gradient material). Note also that this is still a larger degree of RBC removal than a common elutriation/hemolysis protocol, which removed just $\sim 67\%$ of RBCs in a recent study, but was nevertheless shown capable of being a viable approach for obtaining a therapeutic dose of T-cells after their subsequent culture [12, 13]. A similar amount of PLT removal as our Style B CIF result is also recommended to enable improved bead-based selection methods for WBC subtypes (e.g. CD34+ cells) [15]. Therefore, it would appear that the removal of $>90\%$ of either residual cell type, as can routinely be achieved with density gradients (Table 1) or a DLD-based approach [19, 20] does not itself provide a significant advantage in terms of the growth rate during culture, nor is it a requisite for facilitating post-processing steps such as magnetic bead separation. However, for users that may desire higher degrees of PLT or RBC removal, they could simply process the effluent retentate through a CIF module a second time and achieve $\sim 86\%$ PLT removal and $\sim 95\%$ RBC removal, while still recovering significantly more CD3+ T-cells than with density gradient processing (Table 1).

In this study, the cumulative effect of superior initial lymphocyte recovery and culture growth rates for the equipment-free CIF approach was a ~94% higher overall T-cell yield by the 7th day of culture (Fig. 5). In more practical terms, the total cell expansion when using the Style B CIF device module would match the Day 7 yield for the density gradient approach about 1.5 days sooner. Thus the ability to potentially significantly shorten the total time required for expansion of lymphocytes in culture for CAR-T and other cell-based treatments is evident, and would represent a further cost advantage (as well as societal benefit) of employing this high-throughput, yet quite gentle (Table 2; Fig. 6), approach to the initial lymphocyte enrichment step for these and related therapies.

Finally, a limitation of this study is that leukapheresis concentrates obtained from healthy donors, rather than patients, were used to test the devices. In clinical practice, leukapheresis units may be contaminated with a much larger number of granulocytes, and may additionally contain leukemia blast cells and/or myeloid-derived suppressor cells. The devices described in this study were designed solely to deplete the leukapheresis samples of all cells smaller than lymphocytes, such as RBCs and platelets, rather than to separate lymphocytes from other types of WBCs. Conventional centrifugation-based technologies can, however, perform this particular type of separation efficiently (e.g. density gradients for depleting granulocytes, and elutriation for depleting granulocytes and monocytes). Further research and development is ongoing to expand the capabilities of our alternative microfluidic approach to isolate lymphocytes from larger WBCs, in addition to platelets and RBCs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

This work was supported in part by a 2012 NIH Director's Transformative Research Award (NHLBI R01HL117329, PI: SSS). Study sponsor had no role in study design, in the collection, analysis, and interpretation of data, in the writing of the report, and in the decision to submit the paper for publication.

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Highlights

- Flow-through device recovers more lymphocytes than density gradient centrifugation.
- Cell recoveries were 85% CD3+, 89% CD19+, and 97% CD56+ with this new approach.
- Microfluidic-isolated cells grew faster than gradient-isolated cells in culture.
- Overall, the new method gives 2× higher cumulative cell yield after 7-day culture.
- Devices can process 200mL of sample in under 1hr, with no pumping mechanism needed.

Figure 1.

Modified design of CIF-based microfluidic devices enhances removal of RBCs. (a) Schematic illustration of a traditional CIF design with ‘horizontal’ posts. (b) Photograph of a traditional CIF device containing leukapheresis sample (diluted for visualization, with ~10% of WBCs fluorescently-stained). (c) Schematic illustration of the revised CIF design, with posts slanted at 35°. (d) Photograph of the same sample in a CIF device with the modified design. Direction of flow is from top to bottom in all images. Scale bars in panels (b) and (d) are 100 μm .

Figure 2.

CIF device modules. **(a)** Schematic illustration of an individual 'Style A' CIF device. **(b)** An assembled microfluidic module containing 12 multiplexed Style A devices, which selectively concentrate large cells (e.g. WBCs) by ~18-fold. **(c)** Schematic illustration of an individual 'Style B' CIF device. **(d)** An assembled microfluidic module containing 48 Style B multiplexed CIF devices, which concentrate large cells by ~4.5-fold. The dead volume of both styles of modules was ~0.5 mL and the dead volume of the attendant tubing was ~0.7 mL in a typical setup. Arrows indicate direction of flow. A U.S. one cent coin is shown in panels (b) and (d) for size reference.

Figure 3.

Three modes of CIF device module operation. The flow of leukapheresis sample through the modules in this study was driven by: (a) a syringe pump, (b) hanging a bag containing the input sample ~5 ft above the module, or (c) gently compressing the hanging sample bag with a standard infusion cuff (inflated to 300 mmHg).

Figure 4.

Expansion of T-lymphocytes from purified MNC samples. Paired leukapheresis units were processed via density gradient centrifugation or with a CIF device, stimulated with PHA for 4 days, and then expanded in IL-2 culture. Values shown are mean $\pm$ standard deviation. Solid lines are exponential fits to the experimental data: $n = 0.98 \times 10^6 e^{0.405t}$ ($R^2 = 0.9995$) for density gradient; $n = 1.0 \times 10^6 e^{0.415t}$ ($R^2 = 0.9969$) for Style A; $n = 0.95 \times 10^6 e^{0.436t}$ ($R^2 = 0.9985$), for Style B pumped at 50 mL/min; and $n = 0.99 \times 10^6 e^{0.447t}$ ($R^2 = 0.9991$) for Style B driven by gravity. Statistical significance of the differences between samples processed via density gradient centrifugation and CIF modules is indicated by * for CIF Style A, # for CIF Style B driven via syringe pump, and \$ for CIF Style B driven by gravity.

Figure 5.

Cumulative impact of a processing method's T-cell recovery, and the subsequent ability of these cells to expand in culture, on total cell yield over time. Fold-expansion was calculated using experimentally measured lymphocyte recovery (see Table 1) and daily growth rates in culture (see Figure 4). Values shown are mean $\pm$ standard deviation. Statistical significance of the differences between density gradient centrifugation and CIF device modules is indicated by * for CIF Style A, and \$ for CIF Style B.

Figure 6.

Effect of flowrate through Style B CIF modules on cell separation performance. Recoveries of WBCs and lymphocyte (CD3+, CD19+, and CD56+) subgroups tend to decrease as PDMS-cast devices deform at higher flowrates, while removal of RBCs and PLTs rise closer to their theoretical maxima. Note that the ratio of filtrate output to retentate output for each trial decreased (from 4.2:1 to 3.9:1 to 3.7:1 to 3.4:1) as the flowrate increased (from 3.95 to 14.6 to 30 to 60 mL/min), which progressively reduced the maximum theoretical removal of smaller cells in these experiments. Values shown are mean $\pm$ standard deviation, with all input samples taken from a single leukapheresis unit to perform each flowrate trial.

Table 1.

Cell separation performance of the CIF device modules in comparison to density gradient centrifugation. Values shown are mean $\pm$ standard deviation; * indicates statistical significance of the difference between density gradient processing versus the corresponding CIF module.

	Density Gradient	CIF Style A	CIF Style B (after single pass)	CIF Style B (after two passes)
Removal				
Platelets	90 $\pm$ 4%	96 $\pm$ 2%	71 $\pm$ 4% *	86 $\pm$ 4%
RBCs	99 $\pm$ 1%	86 $\pm$ 1% *	76 $\pm$ 3% *	95 $\pm$ 1% *
Recovery				
Monocytes (CD14+)	83 $\pm$ 14%	86 $\pm$ 4%	99 $\pm$ 1%	97 $\pm$ 3%
Lymphocytes (CD45+CD66b-CD14-)	76 $\pm$ 11%	85 $\pm$ 5%	89 $\pm$ 4%	82 $\pm$ 8%
CD3+	62 $\pm$ 17%	78 $\pm$ 6%	85 $\pm$ 6% *	74 $\pm$ 11% *
CD3+CD4+	59 $\pm$ 17%	74 $\pm$ 6%	83 $\pm$ 8% *	71 $\pm$ 15% *
CD3+CD8+	61 $\pm$ 21%	81 $\pm$ 6%	83 $\pm$ 7% *	72 $\pm$ 14% *
CD19+	53 $\pm$ 3%	not acquired	89 $\pm$ 5% *	81 $\pm$ 11% *
CD56+	40 $\pm$ 22%	not acquired	97 $\pm$ 1% *	92 $\pm$ 5%

Table 2.

Impact of cell separation approach on processed sample composition. Values shown are mean $\pm$ standard deviation; † indicates statistical significance of the difference between the initial and processed samples, and * indicates statistical significance of the difference between density gradient centrifugation and the corresponding CIF module.

		Initial Sample	Density Gradient	CIF Style A	CIF Style B (after single pass)	CIF Style B (after two passes)
All white blood cells	WBC [$10^3/\mu\text{L}$]	54.0 $\pm$ 19.5	131.6 $\pm$ 21.1 [†]	172.0 $\pm$ 40.9 [†]	194.1 $\pm$ 40.6 ^{†,*}	209.6 $\pm$ 47.6 [†]
	%Granulocytes (CD66b+)	1.0 $\pm$ 0.8%	0.3 $\pm$ 0.2%	0.4 $\pm$ 0.2% ^{†,*}	1.7 $\pm$ 0.4% [*]	1.7 $\pm$ 0.5%
	%Monocytes (CD14+)	15.7 $\pm$ 4.8%	19.6 $\pm$ 7.1% [†]	16.5 $\pm$ 9.3%	16.6 $\pm$ 4.1%	16.6 $\pm$ 4.5%
	%Lymphocytes (CD45+CD66b-CD14-)	83.3 $\pm$ 5.2%	80.1 $\pm$ 7.2%	83.2 $\pm$ 9.2%	81.7 $\pm$ 4.0%	81.8 $\pm$ 4.6%
	%CD3+	52.0 $\pm$ 3.4%	40.0 $\pm$ 9.1% [†]	44.7 $\pm$ 4.3%	42.7 $\pm$ 4.3% [†]	36.6 $\pm$ 4.5% [†]
	%CD3+CD4+	30.6 $\pm$ 3.1%	22.8 $\pm$ 5.8% [†]	25.1 $\pm$ 5.1%	25.0 $\pm$ 4.9% [*]	20.0 $\pm$ 4.2% [†]
	%CD3+CD8+	16.4 $\pm$ 4.9%	12.8 $\pm$ 5.4%	16.0 $\pm$ 6.9%	12.3 $\pm$ 2.5% [†]	11.0 $\pm$ 2.5% [†]
	%CD19	21.8 $\pm$ 8.7%	15.8 $\pm$ 4.6%	not acquired	18.1 $\pm$ 7.9% [†]	16.9 $\pm$ 7.1%
	%CD56	9.3 $\pm$ 8.2%	8.0 $\pm$ 10.7%	not acquired	10.9 $\pm$ 11.9%	10.0 $\pm$ 13.1%
Lymphocytes	%Alive (PS-PI-)	92.3 $\pm$ 3.8%	88.2 $\pm$ 2.6% [†]	94.1 $\pm$ 2.3% [*]	92.3 $\pm$ 0.7% [*]	91.7 $\pm$ 2.6%
	%Apoptotic (PS+)	6.2 $\pm$ 3.0%	7.8 $\pm$ 2.4%	3.6 $\pm$ 1.1%	5.8 $\pm$ 0.5%	5.8 $\pm$ 1.5%
	%Dead (PI+)	1.5 $\pm$ 1.2%	4.1 $\pm$ 0.5% [†]	2.2 $\pm$ 1.6%	1.9 $\pm$ 0.4% [*]	2.5 $\pm$ 1.1% [*]
	%Activated (CD69+)	0.8 $\pm$ 0.6%	1.6 $\pm$ 1.2%	1.6 $\pm$ 1.3%	0.7 $\pm$ 0.2%	0.8 $\pm$ 0.2%