



FULL-LENGTH ARTICLE

Manufacturing

Enrichment of T-lymphocytes from leukemic blood using inertial microfluidics toward improved chimeric antigen receptor-T cell manufacturing



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ARTICLE INFO

Article History:

Received 29 February 2024

Accepted 5 May 2024

Key Words:

acute lymphoblastic leukemia
 CAR-T
 manufacturing
 microfluidic
 phenotype
 purification

ABSTRACT

Chimeric antigen receptor cell therapy is a successful immunotherapy for the treatment of blood cancers. However, hurdles in their manufacturing remain including efficient isolation and purification of the T-cell starting material. Herein, we describe a one-step separation based on inertial spiral microfluidics for efficient enrichment of T-cells in B-cell acute lymphoblastic leukemia (ALL) and B-cell chronic lymphocytic leukemia patient's samples. In healthy donors used to optimize the process, the lymphocyte purity was enriched from 65% (SD \pm 0.2) to 91% (SD \pm 0.06) and T-cell purity was enriched from 45% (SD \pm 0.1) to 73% (SD \pm 0.02). Leukemic samples had higher starting B-cells compared to the healthy donor samples. Efficient enrichment and recovery of lymphocytes and T-cells were achieved in ALL samples with B-cells, monocytes and leukemic blasts depleted by 80% (SD \pm 0.09), 89% (SD \pm 0.1) and 74% (SD \pm 0.09), respectively, and a 70% (SD \pm 0.1) T-cell recovery. Chronic lymphocytic leukemia samples had lower T-cell numbers, and the separation process was less efficient compared to the ALL. This study demonstrates the use of inertial microfluidics for T-cell enrichment and depletion of B-cell blasts in ALL, suggesting its potential to address a key bottleneck of the chimeric antigen receptor-T manufacturing workflow.

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Introduction

Chimeric antigen receptor (CAR) T-cell therapy is one of the most compelling recent advances in cancer treatment. The clinical effectiveness of CAR-T cells has led to the U.S. Food and Drug Administration (FDA) approval of six drugs and their commercialization [1]. However, due to the inherent personalized nature and many complex steps involved in the preparation of CAR-T cells, large-scale implementation of this cutting-edge immunotherapy remains challenging, and it is broadly accepted that further manufacturing advances are urgently required to realize its full potential [2–7].

Efficient enrichment of T-cells from peripheral blood is an essential initial step in CAR-T cell manufacturing. The percentage of T-cells after the initial separation step suffers from large patient-patient variation [8,9] which depends on many factors including the type of cancer,

prior treatments, disease burden and method used for T-cell separation. In most cases, T-cell enrichment efficiency is much lower in cancer patients than in healthy individuals, as reported in several clinical trials [10,11] and by the oncologic advisory committee of the FDA [9].

This variability in the starting T-cell fraction significantly affects the time needed to produce the required CAR-T cell dose [12,13] and also makes it very difficult to standardize the manufacture process. The variability in leucocyte compositions within the starting cellular material was found to have significant effects on the final T-cell product, encompassing aspects such as expansion, genetic modification, phenotypic composition and clinical performance. For example, the presence of monocytes and red blood cells (RBCs) was shown to increase the percentage of CD4 T-cells, while high quantities of neutrophils decreased the percentage of CD4 T-cells [14]. The presence of granulocytes was shown to reduce the proliferation of T-cells and cytokine synthesis [15], while monocytes induce T-cell apoptosis [16] as well as interfere with magnetic bead binding and activation of T-cells [17]. Myeloid-derived suppressor cells and natural killer cells were also shown to interfere with T-cell activation and expansion [18].

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Most recently, the importance of depleting cancerous lymphocytes from healthy T-cells has been demonstrated, as these cells were shown to interfere with the transduction and expansion processes, and were associated to exhaustion of the CAR T-cell product [19–21]. The addition of B-cell receptor inhibitors during expansion was proposed to mitigate cancerous B-cells-induced T-cell exhaustion [22]. Furthermore, a study highlighted that the transduction of contaminating leukemic B-cells could lead to treatment resistance [23]. This landmark study also reported that in leukemic patient's apheresis products, the percentage of leukemic B-cells varies significantly, and can reach up to 90%.

There is clearly a need for improved T-cell enrichment technologies able to yield high T-cell purity while being compatible with cost-effective large-scale manufacturing. Current T-cell enrichment methods used in contemporary CAR-T cell manufacturing have their own advantages and limitations [24,25]. Ficoll separation-based density fractionation of cellular populations is a well-established method that is still used occasionally in clinical trials [26]. It is however incapable of separating cells of similar densities as lymphocytes and monocytes [27]. A more clinically adopted separation method for T-cells is counterflow centrifugal elutriation, such as the Rotea and Terumo Elutra systems. Elutriation devices are used to produce several approved CAR-T cells products, namely Kymriah, Yescarta and Abecma. Elutriation separates lymphocytes from white blood cells in the leukapheresis product based on differences in cell size and density. Elutriation devices achieve acceptable recovery of the lymphocytes with high throughput [24] but have not been reported to separate T cells from cancerous B cells. Conversely, immunomagnetic T-cell separation is also commonly utilized for the manufacturing of CAR-T cells. For example, immunomagnetic enrichment of CD3+ T cells is used for Tecartus and Carvykti while separate enrichment of helper CD4+ T cells and cytotoxic CD8+ T cells is used in Breyanzi. Despite their ability to enrich specific T-cell subsets with high purity, immunomagnetic selection suffers from several drawbacks, including lack of robustness, high cost, limited throughput (less than 10^9 cells/hour) and variable recoveries depending on the magnetic protocol used and donor disease state [10]. Additionally, the necessity to remove residual beads is a noted issue [28].

Toward addressing the limitations of conventional lymphocyte and T cell enrichment approaches, several microfluidic principles have been investigated, including electrokinetic methods [29], acoustophoresis [30–32] and magnetophoresis [33]. Microfluidics offers many advantages in the manufacturing of cellular products [34]. For example, microfluidic cell enrichment technologies can be readily fitted within sterile closed devices compliant with GMP standards and manufactured in the format of disposable cartridges [35]. Compared to large nonmicrofluidic cell sorters, they have lower void spaces and offer greater flexibility in processing volumes. This makes them very desirable in pediatric leukapheresis applications where low extracorporeal volumes are processed [36]. Illustrating the potential of advanced microfluidic approaches, Curate Biosciences recently commercialized its microfluidic deterministic cell separation system [37–39] and the Draper lab also signed an agreement with Kite Pharma to integrate their T-cell enrichment acoustic chips in the company cell therapy manufacturing platform [40,41]. Sony Biotech [42] has developed and commercialized a fluorescent activated cell sorting microfluidic chips for GMP-compliant cell manufacture. Other companies developing closed Good Manufacturing Practice (GMP)-compliant systems include Aenitis Technologies [43] and Cellular Highways [44].

To address the critical bottleneck in the CAR-T cell manufacturing process, we evaluated the performance of inertial microfluidics in enriching T-cells and depleting other cells including leukemic blasts toward an efficient, cost-effective and high-throughput enrichment of T-cells in B-cell acute lymphoblastic leukemia (ALL) patients' blood samples. Our method relies on spiral inertial microfluidics, which is

an established size and density-based microfluidic cell sorter. Inertial microfluidic devices have been successfully implemented for the enrichment of circulating tumor cells from blood [45] and other cell types [46–49] and have been proven to be a high-throughput and versatile platform for cellular bioprocessing [50].

Materials and methods

Device design, fabrication and operation

A low aspect ratio spiral microchannel device is comprised of two inlets and six outlets as illustrated in Figure 1. Two designs were tested, one with four turns (inner radius = 0.8 cm, outer radius = 1.2 cm) and one with eight-turns (inner radius = 0.8 cm, outer radius = 2 cm). The polydimethylsiloxane spiral microchannel devices were fabricated using standard soft lithography from a SU-8 mold which was designed and fabricated by photolithography at the Australian National Fabrication Facility. Different microchannel heights were tested to modulate the flow conditions ranging from 95 to 130 μm . Briefly, the devices were prepared by mixing the base and curing agent (Sylgard 184, Dow Corning Inc.) in a 10:1 ratio, pouring into the mold, degassing and then curing at 60°C. The cured devices were released from the molds and input and output ports were punched using a 14-gauge puncher and then bonded to glass slides using air plasma (Harrick Plasma, USA). Two 15 cm inlet tubing were attached to the device and a syringe pump. Prior to cell separation, devices were treated with 1% bovine serum albumin to minimize nonspecific fouling [45]. Cells were mainly collected in outlets 2–6 whereas in outlet 1 only debris was collected and thus was not used in calculations thereafter. The recovery and purity of the different cell types (cell lines or a leukocyte fraction) in outlets 2–5 were calculated as follows:

Percentage Recovery of the Cell Type in outlets 2–5

$$= \frac{\text{Sum of the Cell Number of the Cell Type in Outlets 2 – 5}}{\text{Sum of the Cell Number of the Cell Type in all outlets}} \times 100$$

Percentage Purity of the Cell Type in outlets 2–5

$$= \frac{\text{Sum of the Cell Number of the Cell Type in Outlets 2 – 5}}{\text{Sum of Cell Number of all Cells in Outlets 2 – 5}} \times 100$$

Initial device optimization with cell lines

Cell lines were initially tested to assess the device operation and performance. Raji cells (CCL-86, ATCC, USA) and A549 cells (CCL-185, ATCC) were cultured in complete RPMI medium (RPMI 1640 medium, 10% FBS, 1% glutamate, 1% penicillin/streptomycin) (Life Technologies, Australia). Raji cells were sub-cultured and every 2 days, the cells were collected by centrifugation and then resuspended in fresh medium at 4×10^5 viable cells/mL. A549 cells were cultured in complete DMEM (DMEM medium, 10% FBS, 1% glutamate, 1% penicillin/streptomycin) (Life Technologies) and subcultured at 70% confluence after dissociating the cells with 0.025% trypsin and 0.01% EDTA (in PBS). All cells were cultured in T-75 culture flasks at 37°C in a humidified atmosphere containing 5% (v/v) CO₂.

Device processing of leukocytes of healthy blood samples

Blood samples from healthy donors were collected in compliance with the University of South Australia Human Research Ethics Committee (protocol number: 201980) and then separated using Ficoll Paque Premium (GE Healthcare, USA) according to the manufacturer instructions to collect leukocytes. The blood was diluted with an equal volume of Dulbecco Modified PBS and then layered carefully on top of the Ficoll Paque medium. The tubes were centrifuged at $400 \times g$ for 40 minutes at 20°C. In order to simulate apheresis starting material (usually contains a mix of lymphocytes, monocytes and

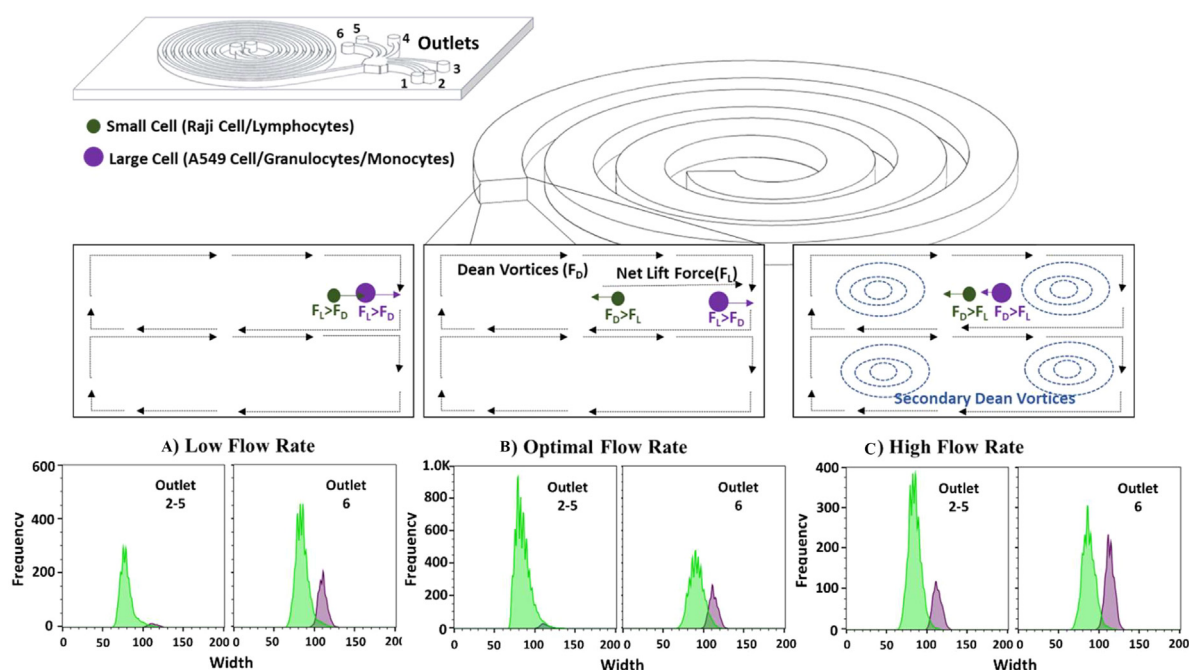


Fig. 1. Schematic of forces acting on cells in inertial microfluidics. (A) At low flow rates, lift forces acting on both small and large-sized cells are high, leading to inefficient separation and reduced recovery of small cells. (B) At optimum flow rates, lift forces acting on larger cells/particles only dominate, providing efficient separation. Larger cells were collected in the innermost channel (outlet 6), while smaller cells (e.g., lymphocytes) were enriched in outlets 2–5. (C) At high flow rates, strong dean vortices with Dean force predominating for both small and large cells/particles resulting in poor separation and reduced purity of small cells. Typical cell size histograms for Raji (green) and A549 (violet) model cells (the gating strategy used is shown in [Supplementary Figure S1](#)). (Color version of figure is available online.)

granulocytes) during optimization, both the mononuclear layer at the interface of the plasma (mainly lymphocytes and monocytes) as well as the granulocyte layer, located as a thin layer above the RBC pellet, were collected and combined in the initial study. Cell suspensions were transferred to new tubes and washed with PBS + 1% FBS. Devices of different dimensions were tested but the eight-turn device with a wall height of 100 and 130 μm was selected for the rest of this study. Ficoll paque was used for leukocyte separation to remove RBC and platelet contaminants which were not the main focus of this study.

Device separation of leukemic blood clinical samples

Cryopreserved B-cell chronic lymphocytic leukemia (CLL) and ALL leukocyte samples were obtained from the South Australian Cancer Research Biobank (SACRB). These studies were approved by the University of South Australia Human Research Ethics Committee and the SACRB Specimen Access Committee (HREC 201777/SACRB-APP-028). All patients provided informed consent in accordance with the Declaration of Helsinki.

Imaging flow cytometry

Cells were stained for immune cell surface markers, including the pan lymphocyte anti-CD45 markers; FITC-anti-CD45 (Raji cells) and leukocyte marker antihuman CD45 EF450 (clone HI30) (patient samples), T-cell marker; antihuman CD3 FITC (clone SK7), B-cell marker; antihuman CD19 APC (clone HIB19), monocyte marker antihuman CD14 PE (clone D3) (Life Technologies). Analyses were performed using either a BD Accuri flow cytometer or an ImageStreamx Mark II imaging flow cytometer (AMNIS, Seattle, WA, USA). Staining was performed in siliconized polypropylene tubes after resuspending cell pellets in ice-cold PBS, 10% FBS and 1% sodium azide at a concentration of 2×10^7 cells/mL. 0.1–10 $\mu\text{g/mL}$ of the primary labeled antibodies were added and then incubated for 45 minutes at room temperature, after which the

cells were washed and suspended in ice-cold PBS, 10% FBS and 1% sodium azide and used for flow cytometry. Analysis of the cellular populations was performed using the IDEAS software Version 6.1 (AMNIS) and FlowJo V10 (FLOWJO, USA). Cell size distributions were calculated using an adaptive erode mask (M04 CH04 77) on the AMNIS software, which allows a more accurate calculation of cell diameters (as shown in [Figure 4C](#)).

Cell viability and proliferation

Leukocyte viability was assessed before and after inertial microfluidic processing using Trypan Blue and PI exclusion staining, according to the manufacturer's instructions. Cell proliferation was determined by staining with the proliferation dye Carboxyfluorescein succinimidyl ester (CFSE) (Biolegend, Australia). Leukocytes were suspended at 2×10^6 cells/mL in PBS and labeled with 10 μM CFSE for 10 minutes at 37°C. The reaction was stopped by adding an equal volume of FBS and incubation for 2 minutes at room temperature. The cells were then washed twice, and the CFSE-labeled cells were cultured for 48 hours at 37°C and 5% CO_2 in 96-well microtiter plates. To stimulate the proliferation of primary cells, especially the T-cells, the cells were activated using anti-CD3, anti-CD28 and IL-2 and the proliferation of the nonenriched leukocytes and the enriched leukocytes after separation was compared over 72 hours at 24-hour intervals. The proliferation index was then calculated using FlowJo software, which calculates it as the total number of divisions divided by the number of cells that went into division.

Statistical analysis

All experiments were repeated at least three times and analyzed nonparametrically using GraphPad Prism. The data was analyzed using a two-way ANOVA mixed model. For single variable experiments, the nonparametric Wilcoxon paired *t*-test was performed.

Results and Discussion

Optimization of size-based fractionation

Inertial microfluidics fractionates cells/particles based on how they experience different forces, depending on their physical characteristics, when flowing in spiral microfluidic devices, namely the net lift force (F_L) and the transverse Dean drag forces (F_D) [51]. The net lift force (F_L) increases with the diameter, while the counter Dean vortices, which are symmetric at the top and bottom of the microchannel, increase as the flow rate and channel height increases. The F_L/F_D experienced by cells/particles is proportional to the cube of the diameter (a_p^3). For larger cells/particles, the lift forces dominate, and they are focused near the inner wall. For lift forces to dominate, the ratio of the diameters to the channel height (a_p/H) is required to be larger than 0.07 [51,52]. The lower lift forces applied to smaller cells allow the Dean drag forces to dominate, resulting in their enrichment away from the inner channel wall. The forces acting under different flow rate conditions are summarized in Figure 2.

Toward optimizing the operation of the inertial microfluidic devices used in this study, we first evaluated the separation of a (2:1) mixture of two cell lines differing in size; Raji cells and A549 cells using an eight-turn inertial spiral microfluidic device (with wall height of 130 μm). Raji cells, a B-lymphocyte-derived cell line were used to mimic the smaller lymphocyte population in blood, while epithelial-derived A549 cells were used to mimic the larger blood cells population, namely granulocytes and monocytes. Results following the testing of different flow rates ranging from 1.2 to 2.5 mL/min confirmed the separation of larger and smaller size cells, with the larger A549 cells, typically collected as expected in the innermost channel (outlet 6), and the smaller Raji cells collected mainly in outlets 2–5 (Supplementary Figure S1 and Figure 1A–C). At flow rates between 1.8 and 2.2 mL/min, we achieved the highest purity and recovery of the smaller cell in outlets 2–5, with a recovery of 89% (SD \pm 0.14) and purity of 82% (SD \pm 0.16) at 2.2 mL/min (Supplementary Figure S1). At these optimal flow rates, the large-sized cells experience mainly the lift forces, whereas smaller cells will be pushed by the dean drag forces away from the innermost wall, resulting in efficient separation, as illustrated in Figure 1B. Lower flow rates (1.2–1.8 mL/min) led to a decreased recovery of Raji cells in outlets 2–5, as more Raji cells shifted to outlet 6 (Figure 1A), due to the predominant lift forces acting on both small and large cells. At higher flow rates (2.5–2.8 mL/min), the effect was the opposite, more A549 cells were collected with the Raji cells in outlets 2–5, resulting in lower purities, due to the emergence of secondary dean vortices (Figure 1C). Building on this data, we next proceeded to systematically fine-tune the methodology using leukocytes collected from healthy donors.

Enrichment of lymphocytes from leukocytes in healthy donors

To fine-tune inertial microfluidic lymphocyte enrichment, leukocytes isolated from the blood of healthy donors were collected. First, the leukocyte composition and cell diameters were determined using imaging flow cytometry by staining with the pan lymphocyte anti-CD45 marker, anti-CD19- for B-cells and anti-CD3 for T-cells (Figure 2). Gating strategy for T- and B-cell populations is shown in Supplementary Figure S2. The mean diameter of T-cells was 7.3 μm (SD \pm 0.06), 7.2 μm (SD \pm 0.06) for B-cells and 8.6 μm (SD \pm 0.25) for other white blood cells (monocytes and granulocytes and some other cells in the lymphocyte population).

We next determined the optimal design and operating conditions to achieve the highest lymphocyte recovery and purity. To this end, leukocytes were resuspended in PBS+1%FBS and processed in inertial microfluidic devices under different conditions. High flow rates (1.8–2.2 mL/min) operation were not found to be optimal for Raji cell separation and therefore for smaller-sized lymphocytes, hence we re-optimized the parameters focusing on lower flow rates. We first tested the effect of different flow rates ranging from 1 to 1.5 mL/min in two device designs fabricated with heights of either 100 or 130 μm and calculated the purity and recovery of the lymphocytic fraction (outlet 2–5) (Figure 3, the lymphocytic fraction determined based on forward side scatter as explained in Supplementary Figure S3). At a flow rate of 1 mL/min, we achieved a higher purity (91% SD \pm 0.06) using the 130 μm device compared to the 100 μm device (80% SD \pm 0.05), although it was not statistically significant. However, the 100 μm device yielded a significantly lower recovery ranging from 16 to 57%, compared to a recovery ranging from 69 to 82% ($P < 0.05$) for the 130 μm device at all three flow rates tested (Figure 3A,B). In the smaller channel height 100 μm device, the a_p/H ratio is higher than in the 130 μm device, causing higher lift forces to act on the smaller-sized cells, leading to the shift of the smaller-sized cells into the larger-sized cell outlet and a reduction in purity.

As the 130 μm channel height device yielded more efficient separation than the 100 μm device, we performed further optimizations using the 130 μm device. The effect of the number of turns of the device was compared for different flow rates (Figure 3C). The longer eight-turn device resulted in a significantly higher purity, compared to the 4-turn device at all flow rates ($P < 0.05$), with the highest purity of 91% SD \pm 0.06 achieved for a flow rate of 1 mL/min. Efficient focusing of cells or particles of varying sizes in inertial microfluidics necessitates a specific downstream channel length. This length is dependent on both the size of the cells/particles and the hydraulic diameter, which, in turn, is influenced by the channel's width and height. [53]. Based on this data, the eight-turn device with a height of 130 μm was selected and used in the subsequent work.

The typical white blood cell concentrations in a typical apheresis product are 1×10^7 – 1×10^8 cells/mL [54]. To determine the cell

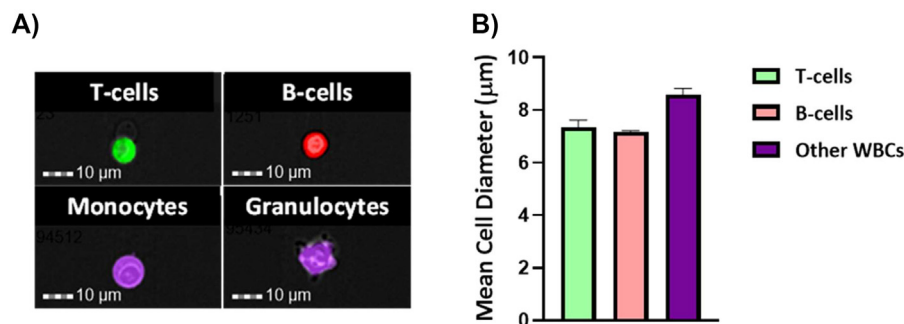


Fig. 2. Leukocyte size distributions in healthy donors. Imaging flow cytometry: (A) Composite images of T-cells (green, CD45⁺CD3⁺CD19⁻), B-cells (red, CD45⁺CD3⁺CD19⁺) and all other leukocytes (violet, CD45⁺CD3⁺CD19⁻). (B) Size distributions were calculated using an adaptive erode mask (M04 CH04 77) on the AMNIS software. (Color version of figure is available online.)

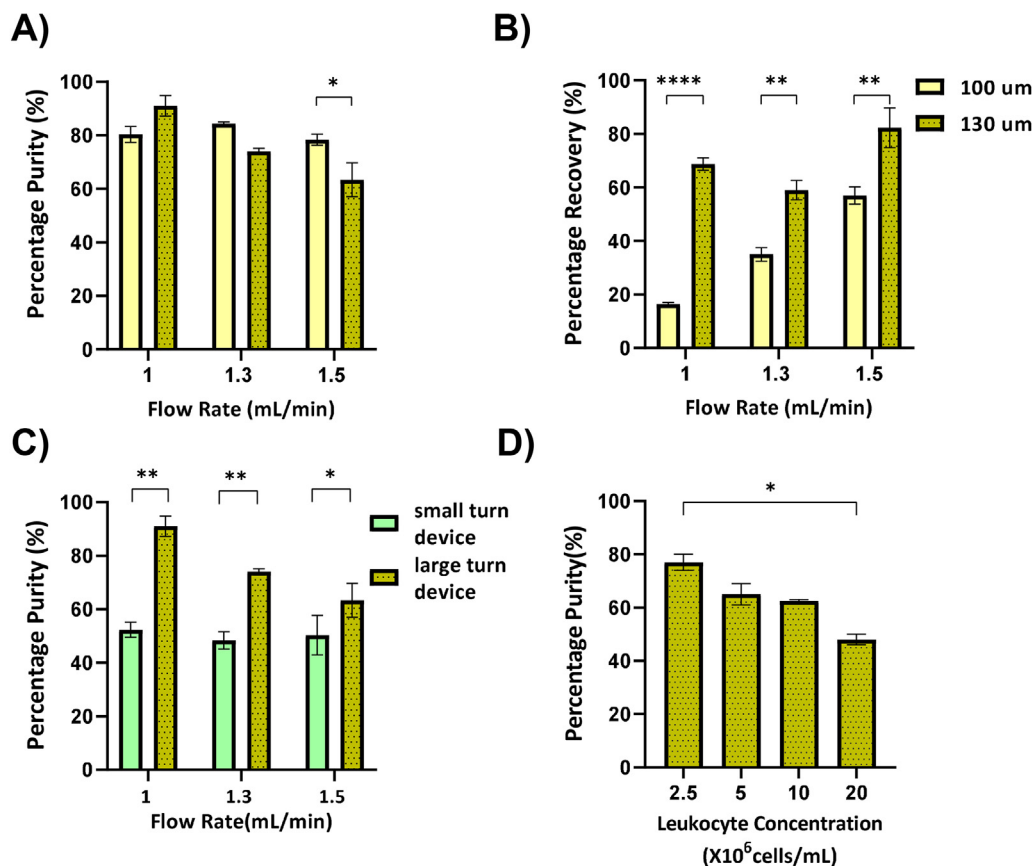


Fig. 3. Optimization of the recovery and purity of lymphocytes from leukocytes isolated from healthy donors. Leukocytes in PBS+1%FBS were processed in the inertial microfluidic device and the percentage recoveries and purities for the lymphocytic enrichment outlets (outlets 2–5) are shown. Effect of flow rate in 100 and 130 μm channel height eight-turn devices on (A) purity and (B) recovery. (C) Effect of flow rates and devices' number of turns on purity (130 μm channel height). (D) Effect of leukocyte concentration on purity in a 130 μm eight-turn device ($n = 3$, * $P < 0.05$, ** $P < 0.005$, **** $P < 0.00005$). (Color version of figure is available online.)

concentration that can be operated in our inertial device, we tested cell concentrations ranging from 2.5×10^6 to 20×10^6 cells/mL (Figure 3D). As expected, the lower concentration of 2.5×10^6 cells/mL resulted in the highest lymphocyte purity (77% SD \pm 0.04), which was significantly greater compared to that obtained at the higher concentration of 20×10^6 cells/mL (48% SD \pm 0.03 $P < 0.05$). The efficient separation of cells in inertial microfluidics relies on their neutral buoyancy. At higher concentrations, cell-cell interactions become more pronounced, causing disruptions in the focused streams of cells with varying sizes. As a result, the separation process becomes less effective [53].

Under these optimal conditions (eight-turn device with 130 μm channel height operated at a flow rate of 1 mL/min and with a feed leukocyte concentration of $2.5\text{--}5 \times 10^6$ cells/mL), we achieved optimal recoveries and purities. From a starting lymphocyte purity of 65% (SD \pm 0.2) and T-cell purity of 45% (SD \pm 0.1), inertial microfluidic processing yielded a lymphocyte purity of 91% (SD \pm 0.06) and T-cell purity of 73% (SD \pm 0.02) and recoveries of 63% (SD \pm 0.04) and 60% (SD \pm 0.22), respectively (Figure 4B). While T-cells and B-cells were both collected in outlets 2–5, most of the other larger leukocytes (monocytes, granulocytes and large lymphocytes) were collected in outlet 6 (Figure 4B), yielding a depletion of 89% (SD \pm 0.04). The mean cell diameter collected in outlets 2–5 was 7.2 μm (SD \pm 0.2), compared to a mean diameter of 8.7 μm (SD \pm 0.4) for cells collected in outlet 6. The extent of T-cell enrichment in outlet 2–5, as compared to outlet 6 before and after separation is shown in Figure 4B. These results confirmed that efficient inertial microfluidic lymphocyte enrichment can be achieved with a good recovery rate and depletion of larger leukocytes (monocytes and granulocytes) in healthy donor samples.

Inertial microfluidic T-cell enrichment in B-cell ALL and CLL patient samples

Leukemic samples are highly contaminated with cancerous cells and have highly variable ratios of the different white blood cell populations, which can be attributed to immunological responses to the disease [55]. To validate the efficiency of inertial microfluidics enrichment of T-cells from leukemic blood, we next assessed the utility and efficacy of our device using clinically relevant samples from donor patients including B-lymphocyte ALL ($n = 6$) and CLL ($n = 3$) samples. Despite the target was to get hold of patient apheresis samples (having a higher percentage of monocytes and granulocytes) however most of the collected leukemic samples were frozen vials of PBMCs (thence had a higher starting lymphocyte percentage of 88% compared to the healthy donor ones in this study 65%).

As expected, the starting composition of the ALL and CLL samples varied between samples, but all had significantly larger numbers of B-cells over T-cells, in comparison to healthy donor samples (Figure 5). In healthy blood samples, B-cells comprise about 10% of the PBMC fraction [56]. In contrast, much higher percentages of B-cells (60% SD \pm 0.3) and lower percentages of T-cells (30% SD \pm 0.2) were found in the ALL samples (Figure 5B). Similarly, for the CLL samples, B-cells made up the majority 95% (SD \pm 0.03) and only very low T-cell numbers were measured (5% SD \pm 0.05), as shown in Figure 5C.

We next determined the T-cell and B-cell diameters in the ALL and CLL samples using imaging flow cytometry. The mean T-cell diameter in healthy blood samples (7.3 μm SD \pm 0.3) was not statistically different to that of T-cells of the ALL (6.9 μm SD \pm 0.7) and CLL (7.1 μm SD \pm 0.3) samples. In contrast, the mean B-cell diameter of the ALL samples (8.3 μm SD \pm 0.8) was significantly higher than that

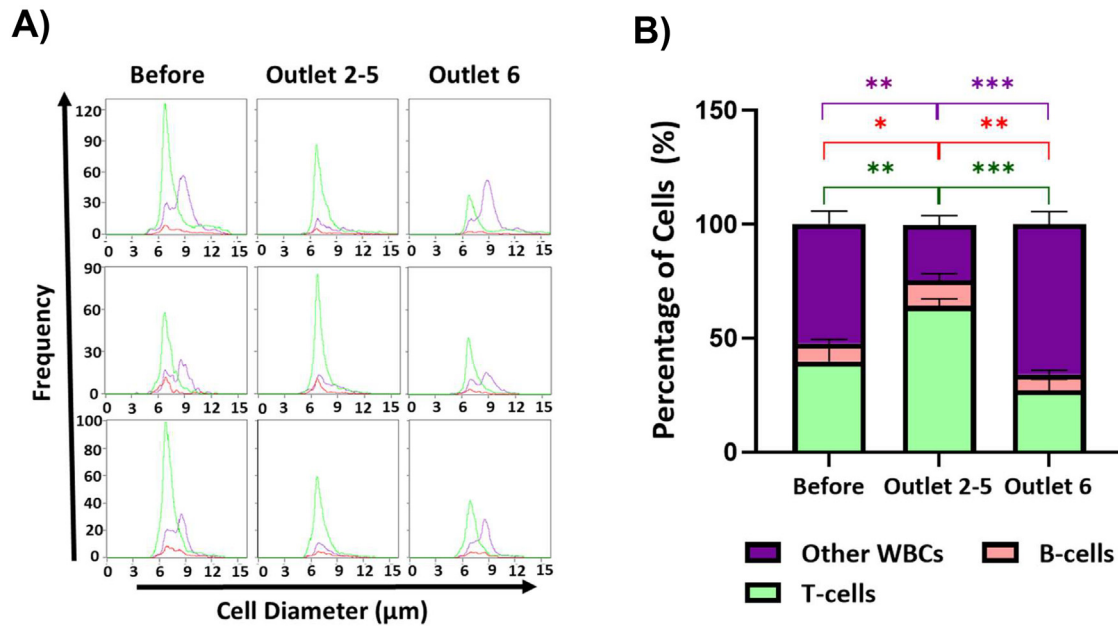


Fig. 4. Size distributions and enrichment of different leukocyte populations. Leukocytes isolated from healthy donors were resuspended in PBS+1%FBS and processed in the inertial microfluidic device under optimized conditions (A) representative histograms of size distributions of the leukocyte subsets; T-cells (green), B-cells (red) and other WBCs (violet) before and after separation in the various device outlets as determined by imaging flow cytometry (shown for 3 independent donor batches). (B) Cellular percentages before and after separation in the device showing enrichment of T-cells in outlets 2–5 and outlet 6 ($n = 7$, $*P < 0.05$, $**P < 0.005$, $***P < 0.0005$, Two-way ANOVA mixed model statistical analysis was used). (Color version of figure is available online.)

measured in healthy samples ($7.2 \mu\text{m}$ SD ± 0.05 , $P < 0.05$). In contrast, B-cells from the three tested CLL samples only displayed a minor size increase ($7.5 \mu\text{m}$ SD ± 0.3). Importantly, the B-cell diameter ($8.3 \mu\text{m}$ SD ± 0.8) in ALL samples was significantly larger than the T-cell diameter ($6.9 \mu\text{m}$ SD ± 0.7 , $P < 0.05$), which provided the impetus for not only efficient inertial microfluidic enrichment of T cells but also depletion of cancerous B cells.

To confirm this hypothesis, the patients' samples were processed using the previously optimized conditions. For the ALL samples, the initial lymphocyte purity ranged from 70 to 99% with a mean of 88% (SD ± 0.2) and the T-cell purity ranged from 7 to 53% with a mean of 36% (SD ± 0.2). After inertial microfluidic processing, we achieved a similar lymphocyte purity of 87% (SD ± 0.1) but significantly increased T-cell purity of 57% (SD ± 0.2) with a 70% (SD ± 0.1) T-cell recovery

(Figure 6A). Remarkably, a 77% (SD ± 0.09) depletion of B-cells was determined. In four of these ALL samples, the T-cell purity increased by $\sim 20\%$ (from ~ 36 to $\sim 57\%$), while in two of the patients the purity increased by $\sim 40\%$ (from ~ 24 to $\sim 63\%$). These two patients also had a 90% depletion of B-cells. Variations in separation effectiveness among patients can be attributed to the distinct size distributions of T-cells and B-cells. The majority of the B-cells in these two samples were large and therefore better differentiated from the T-cells, which allowed efficient separation. In the case of the remaining four samples, there was a range of overlap observed, as illustrated in Supplementary Figure S4. As noted above, the percentage of other white blood cells in the ALL samples was very low, however, in agreement with the data obtained with healthy blood samples, there was a significant depletion of monocytes (89% SD ± 0.1) and other white blood cells (60% SD ± 0.1).

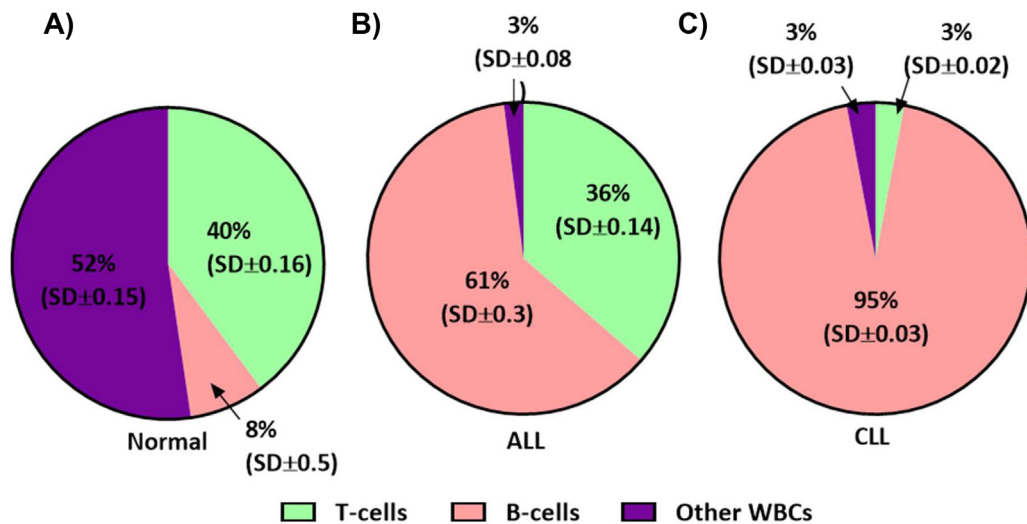


Fig. 5. Pie charts comparing the percentage of leukocyte populations. Starting composition of isolated leukocytes used for isolation in the device (A) Healthy donor sample ($n = 7$); (B) ALL donor samples ($n = 6$); (C) CLL donor samples ($n = 3$). (Color version of figure is available online.)

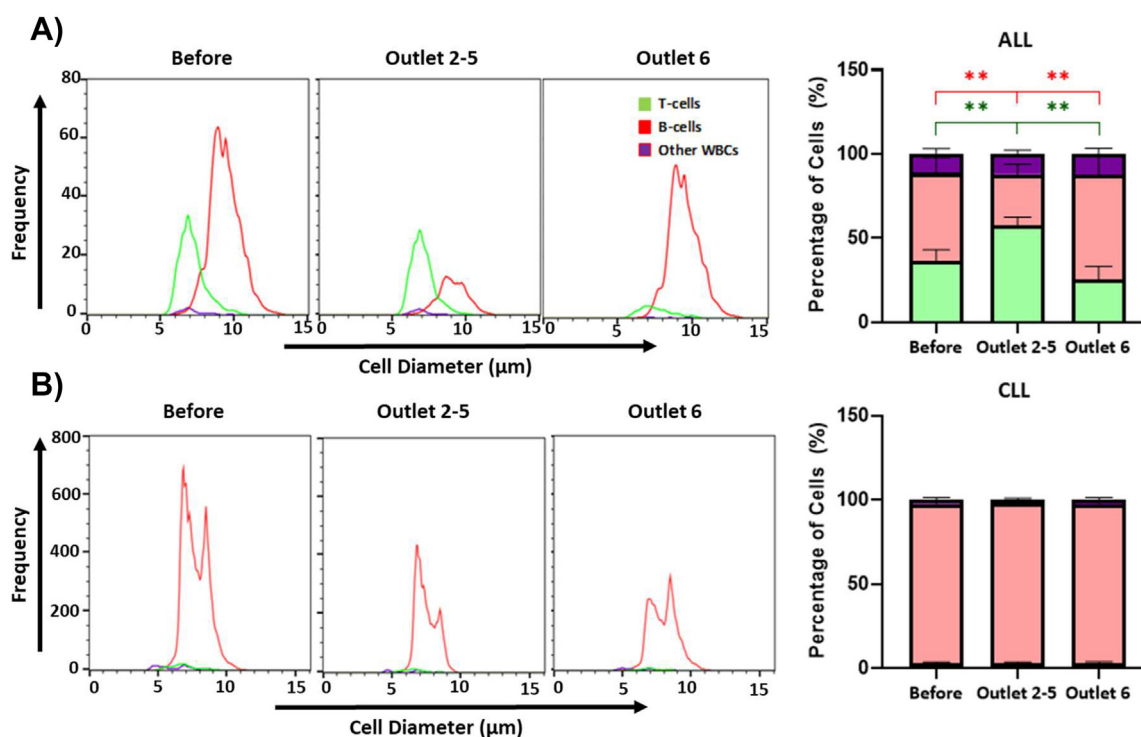


Fig. 6. Relative enrichment of leucocyte populations in ALL and CLL donor samples. Leukemic PBMCs were resuspended in PBS+1%FBS and processed in the inertial microfluidic device under optimized conditions. Representative size distribution and cellular percentages showing the relative enrichment/depletion of leucocytes for (A) ALL and (B) CLL samples before and after separation in the various device outlets as determined by imaging flow cytometry. ($n = 6$ for ALL and $n = 3$ for CLL) Two-way ANOVA mixed model for paired data followed by Tukey multiple comparisons test was used, $**P < 0.005$. (Color version of figure is available online.)

Inertial microfluidic processing of the CLL samples was far less effective as shown in Figure 6B. In these samples, even though there was a good depletion of the nonlymphocytic white blood cell fraction (66%), only 55% of the B-cells were depleted. This is in agreement with the size measurements, as well as the accumulation of small, nonapoptotic mature lymphocytes that have very low proliferation rate found in the blood of CLL patients [55,57–59]. In addition, the T-cells in the CLL samples are also slightly activated and adopt a dysregulated exhaustive phenotype [60], which may explain the relatively poor recovery of T-cells (47%). On the other hand, ALL is characterized by the presence of highly proliferating leukemic blasts that are significantly enlarged [61,62]. We therefore next endeavored to confirm that the significant enrichment of T-cells in the ALL samples originates in the specific depletion of leukemic blasts during inertial microfluidic processing.

Depletion of leukemic blasts in ALL patient samples

Lymphoblasts were identified by CD45 dim/low side scatter characteristics according to Lacombe et al. [63], which was confirmed by morphological analysis on the imaging flow cytometry. The number of leukemic blasts was investigated before and after inertial microfluidic processing. As shown in Figure 7, there was a large variation in the initial frequency of cancerous lymphoblasts of the total B-cell compartment (12–88%) between patients' samples but in all cases, the number of blasts decreased significantly with an average depletion of 74% ($SD \pm 0.09$, $P < 0.005$) ranging from 56 to 84% across the 6 ALL samples tested. The average B cell blast diameter was $8.8 \mu\text{m}$ ($SD \pm 0.5$). It was also worth noting the presence of B-cells outside the blast gate, which were also large and efficiently depleted. Based on previous studies, these B-cells may also be blasts present outside of the blast gating [62,64,65] or highly proliferating nonmalignant B-cells [66–68].

The substantial reduction in ALL lymphoblasts observed in this context holds significant potential importance, given their prevalence in leukemic apheresis products, along with other white blood cells [65,69]. The percentage of blasts differs according to the type of leukemia, stage of disease, administration of prior treatments, as well as patients' age [70]. It is now well recognized that their presence negatively interferes with T-cell activation, transfection and expansion as well as results in T-cell exhaustion [23,71–73]. To address this specific issue, Kite Pharma recently developed a novel manufacturing process (XLP) designed to enrich for T-cells, especially in malignancies with high burden of circulating B-lymphoblasts [74,75].

Effect of inertial microfluidic processing on the viability and proliferation of T-cells

To confirm that inertial microfluidic processing had no detrimental effects on T-cell viability and proliferation, we investigated the viability and proliferation of T-cells collected from outlets 2–5 at 0, 24 and 48 hours post device separation compared to the starting T-cell population (Figure 8). There was no difference in T-cell viability at all tested times (Figure 8A). Similarly, T-cell proliferation was also not affected (Figure 8B).

Effect of inertial microfluidic processing on T-cell phenotypes

It is now well established that the phenotypes of CART-cell product influence to some extent clinical outcomes and that T-cells with less differentiated phenotypes are more preferable for manufacturing [76]. For example, naïve and central memory phenotypes displayed better *in vivo* persistence [77], while central memory phenotype were shown to have higher transfection efficiencies [78]. We therefore assessed the phenotypes of T-cells enriched from four ALL leukemic samples by staining for markers of helper CD4⁺ T-cells, cytotoxic

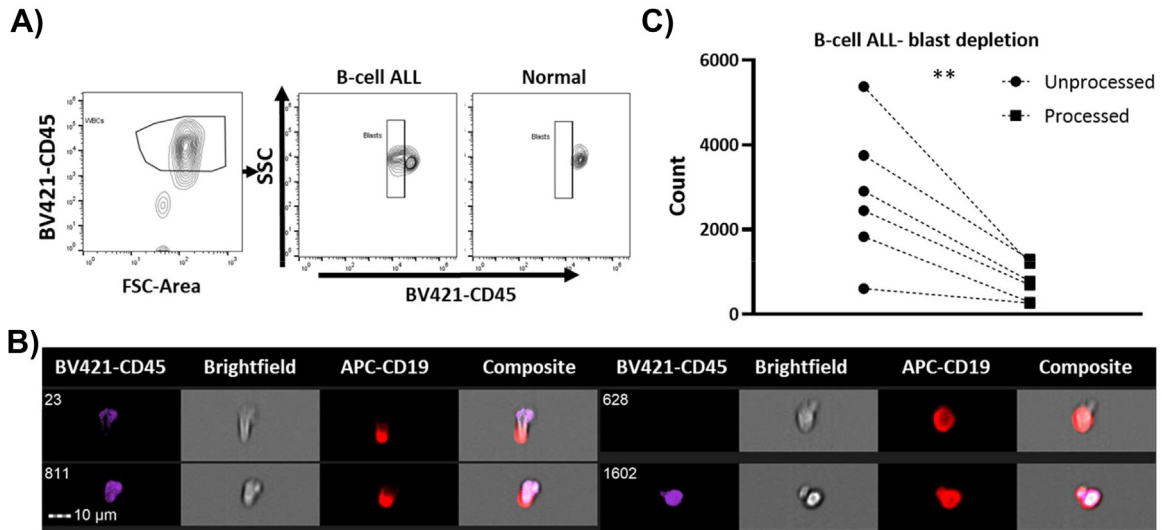


Fig. 7. Depletion of leukemic blasts from B-cell ALL samples. (A) Gating strategy for identifying the leukemic blast population based on CD45 dim/low side scatter characteristics. (B) Morphological confirmation of the leukemic blast population by imaging flow cytometry. (C) Frequency of blasts before and after separation (nonparametric Wilcoxon paired t-test analysis was performed; ** indicates statistically significant depletion at $P < 0.005$, $n = 6$). (Color version of figure is available online.)

CD8⁺ T-cells. We also stained for CD45RO and CCR7 to identify naïve (CD45RO⁺CCR7⁺), central memory (CD45RO⁺CCR7⁺), effector memory (CD45RO⁺CCR7⁻) and terminally differentiated effector cells (CD45RO⁺CCR7⁻ EMRAs). CD4⁺T-regulatory cells (T-regs) and CD8⁺T-suppressor cells (CD25⁺high, FOXP3⁺, CD127⁺ low) before and after inertial microfluidic processing were also assessed. Figure 9 provides a summary of the findings, highlighting notable variations among patients across different samples. While no statistically significant differences were measured in regards to the overall phenotypical compositions before and after processing, it is noteworthy that there was a substantial depletion of CD4 T-regs in some of the samples, with an 87% depletion of T-regs in 2 of the 4 samples tested, while only 20% depletion was measured in the other two samples. It was also noted that CD8 T-suppressor cells were consistently depleted in all samples with an average 68% (SD \pm 0.3) depletion. The substantial patient-to-patient variation in the depletion of T-reg and T-supp is likely due to differences in disease state and prior treatments for these patients, which affects the more activated and expanding cells in the immunosuppressive environment of the leukemic disease as reported by others [13,79–82]. ALL patients have a high concentration of T-regs in the blood due to the immunosuppressive nature of the cancer [83,84]. The presence of T-regs during *ex vivo* expansion can greatly affect the proliferation of antigen-active T-cells and the necessary development of effector T-cells, as shown

in previous *ex vivo* and *in vivo* studies due to their immunosuppressive nature [85–88]. The potential of inertial microfluidic processing to deplete such phenotypes may be an added benefit and requires further investigation.

Translational potential in CAR-T cell manufacturing

The optimized inertial microfluidic process developed in this study efficiently enriched T-cells and depleted B-cell blasts in ALL samples. The process also efficiently depleted larger-sized white blood cellular contaminants as monocytes and granulocytes. Compared to other recently commercialized microfluidic platforms, the inertial microfluidic device was able to achieve a greater enhancement in T-cell purity compared to the Curate chip [39] while offering a greater recovery than that obtained with the Draper acoustophoretic chip [89] in addition to its ability in leukemic blast depletion. Importantly, the observed significant depletion of cancerous B cells is anticipated to perform well in other blood cancers where large B-cell blasts predominate, including acute myeloid leukemia [90], diffuse large B-cell lymphoma and other non-Hodgkin lymphomas [91]. We tested the effect of inertial microfluidic separation on the proliferation of the enriched T-cells and, in agreement with the relevant literature, we found no negative impact. We have also previously shown the feasibility of inertial microfluidic depletion of dead cells from a

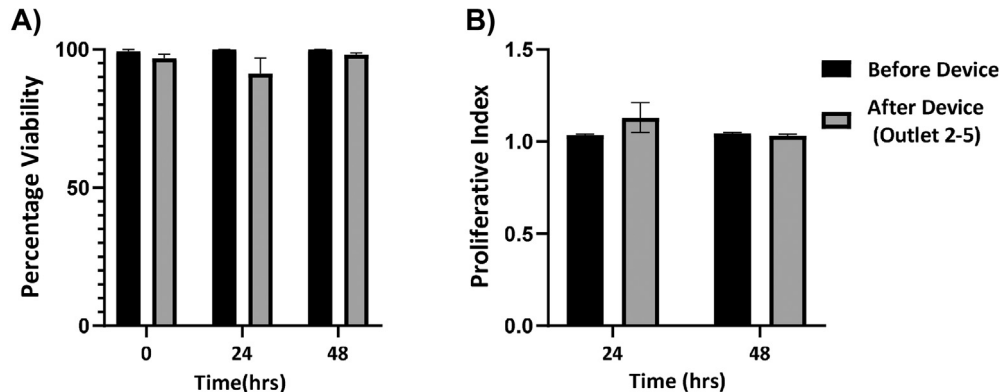


Fig. 8. Effect of inertial microfluidic processing on T cells viability and proliferation. (A) Cell viability, as measured by PI exclusion and (B) cell proliferation, as tested by CFSE staining of CD3 positive T-cells before and after device separation of outlet 2–5 (no statistically significant difference using paired t-test at $P < 0.05$, $n = 3$).

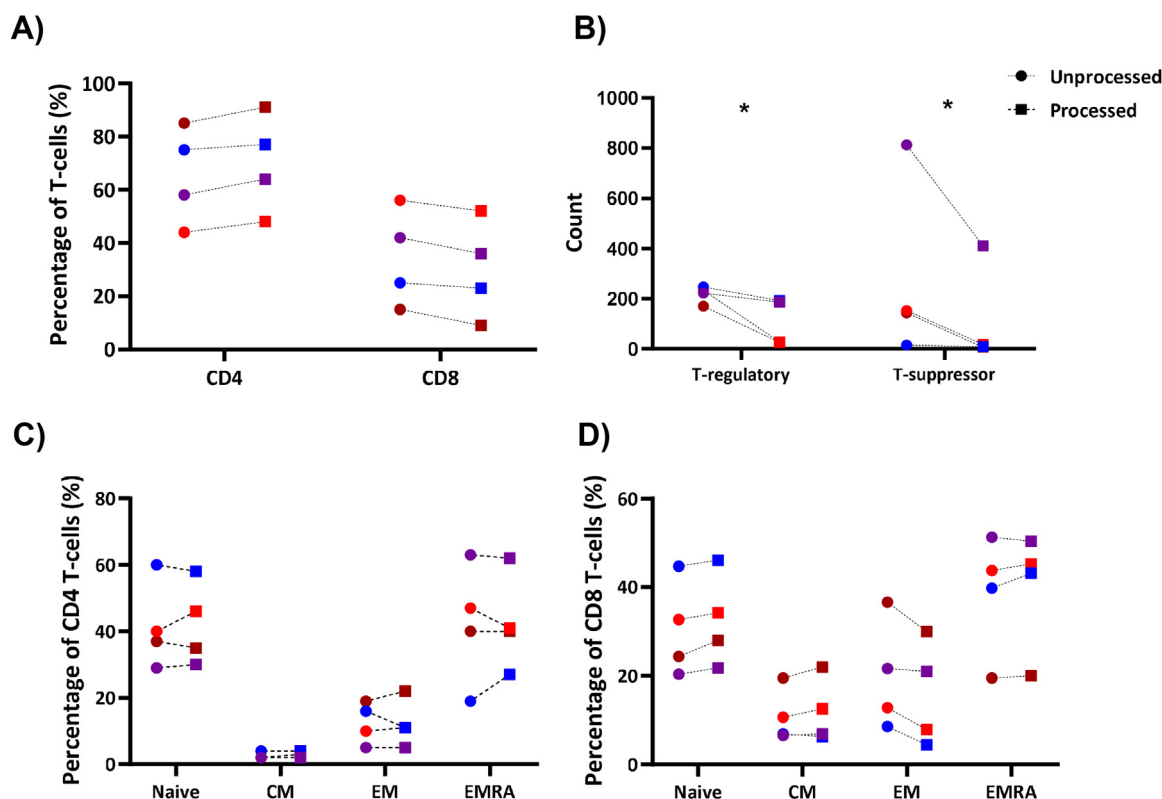


Fig. 9. Effect of inertial microfluidic processing of B-cell ALL samples on the T-cell phenotypes. T-cell phenotypes before and after device separation of outlet 2–5 as determined by imaging flow cytometry. (A) Percentage of CD4 and CD8; (B) number of CD4 T-regulatory and CD8 T-suppressor cells; (C and D) percentages of Naive, central memory (CM), effector memory (EM) and terminally differentiated effector cells (EMRA) for (C) CD4 and (D) CD8. (Mixed model two-way ANOVA for paired data statistical analysis was used and no statistical significance was observed at $P < 0.05$, $n = 4$.) (Color version of figure is available online.)

preclinical CAR-T cell product. Importantly, inertial microfluidic processing of CAR-T cells did not affect their cytotoxicity and percentage of CAR-positive T-cells [92]. While we have not attempted to prepare CAR-T cells from the T cell fraction enriched from ALL patient samples, we anticipate no significant detrimental impact based on the available data and the brief and very mild conditions experienced by the cells during inertial microfluidic processing.

Inertial microfluidics is arguably a good compromise in regard to performance, cost and throughput between elutriation-based lymphocyte enrichment approaches and immunomagnetic bead selection. Microfluidic depletion of B-cell blasts offers a bead-less alternative to bead-based depletion of B-cell contaminants, although further work is warranted to establish the relative performance of inertial microfluidics against immunomagnetic selection. Microfluidic cell processing has the potential to facilitate decentralizing the manufacturing of cellular products, which could reduce cost by one-seventh in the context of automation in a closed system with lower needs for personnel and clean room facilities [93–96]. While it is difficult to price a commercial device meeting the strict requirements associated to the manufacturing of a clinical-grade cellular product, it is well established that single-use disposable microfluidic devices can cost as little as 20 dollars per chip [97,98]. We therefore anticipate that in a scale-up commercial product would not add substantially to the overall cost of CAR-T cell manufacturing compared to current technologies such as elutriation-based lymphocyte enrichment systems [97,98].

In order to appraise the translational potential of inertial microfluidics, one should consider the volumes of starting material being processed in typical CAR-T cell manufacture, which is usually in the order of 200–600 mL [71,99]. Inertial microfluidic devices, despite their relatively limited processing volumes, offer a noteworthy advantage. By stacking multiple devices, it becomes possible to

efficiently process large volumes. This approach has been successfully applied in the enrichment of circulating tumor cells, resulting in a remarkable 15-fold reduction in processing time. [45]. Using a similar approach would allow 15 mL/min processing, that is, 200–600 mL of apheresis product would be processed within an acceptable time of 15–40 minutes. The concept of device stacking is commonly employed in cell separation to increase the throughput as had been presented by Curate Biosciences [38] and Draper [100].

Conclusion

An inertial microfluidic device could significantly increase the purity of T-cells from an average of 45–73% in white blood cell suspension mimicking apheresis-like starting materials from healthy donors. Despite the large variation in the cellular composition of the leukemia samples highly contaminated with B-cells, T-cells were effectively enriched from an average of 36–57% in ALL samples with over 80% depletion of leukemic B-cell blasts. Considering the relative simplicity of the process, the low cost of the underpinning microfluidic device and the anticipated relative ease of integration within closed GMP-compliant system enabling automation, inertial microfluidics provide a compelling alternative to current T-cell enrichment methods used in the CAR-T cell manufacturing process.

Declaration of competing interest

The authors declare no conflict of interest.

Acknowledgments

The authors acknowledge funding from the Australian Research Council Center of Excellence in Convergent Bio-Nano Science and

Technology and the Cell Therapy Manufacturing Cooperative Research Center. This work was performed in part at the South Australian node of the Australian National Fabrication Facility under the National Collaborative Research Infrastructure Strategy to provide nano and microfabrication facilities for Australia's researchers.

Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.jcyt.2024.05.005.

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