Biomedical Microdevices (2018) 20:77
https://doi.org/10.1007/s10544-018-0323-3

Low-stress Microfluidic Density-gradient Centrifugation for Blood Cell Sorting

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Abstract
Density gradient centrifugation exploits density differences between different blood cells to accomplish separation of peripheral blood mononuclear cells (PBMCs) from polymorphonuclear (PNM) cells, and erythrocytes or red blood cells (RBCs). While density gradient centrifugation offers a label-free alternative avoiding the use of harsh lysis buffers for blood cell isolation, it is a time-consuming and labor-intensive process during which blood cells are subject to high-levels of centrifugal force that can artifactually activate cells. To provide a low-stress alternative to this elegant method, we miniaturized and automated this process using microfluidics to ensure continuous PBMCs isolation from whole blood while avoiding the exposure to high-levels of centrifugal stress in a simple flow-through format. Within this device, a density gradient is established by exploiting laminar flow within microfluidic channels to layer a thin stream of blood over a larger stream of Ficoll. Using this approach we demonstrate successful isolation of PBMCs from whole blood with preservation of monocytes and different lymphocyte subpopulations similar to that seen with conventional density gradient centrifugation. Evaluation of activation status of PBMCs isolated using this technique shows that our approach achieves minimal isolation process induced activation of cells in comparison to conventional lysis or density gradient centrifugation. This simple, automated microfluidic density gradient centrifugation technique can potentially serve as tool for rapid and activation-free technique for isolation of PBMCs from whole blood for point-of-care applications.

Keywords Density gradient centrifugation · Cell sorting · Cell separations · Microfluidic cell sorting · Leukocyte activation

1 Introduction

Blood cells contain vital information as changes in immune and inflammatory status of the body are almost immediately reflected as changes in the numbers and activation status of leukocyte sub-populations (Hersh et al. 1971; Arras et al. 1998; Mellembakken et al. 2002; Fritsche et al. 2004; He et al. 2010). There has been significant interest in trying to use this information for advanced diagnosis, to predict patient clinical trajectories, and for determination of patient-specific treatment alternatives. The presence of large numbers of erythrocytes or red blood cells (RBCs) presents a significant challenge as unique signatures indicative of injury or disease in specific leukocyte sub-populations are often not distinguishable when dealing with complex heterogeneous mixtures of cells (Feezor et al. 2004). Therefore, at a minimum, depletion of RBCs and isolation of leukocytes or leukocyte sub-populations is necessary to obtain highly specific information that can be used for evaluation of patients. Leukocytes are extremely sensitive to changes in environment and stress (Fukuda et al. 2000; Gibson 2008). Isolation of blood and prolonged maintenance ex-vivo after isolation impacts both viability and activation status of leukocytes in a time dependent manner (Frank 1990; Jude et al. 1994). Even the method of collection can alter gene expression profiles in isolated leukocytes (Frank 1990, Jude et al. 1994). Physical or chemical stress during isolation represents additional stimuli that can also activate leukocytes (Naranbhai et al. 2011; Nieto et al. 2012). Therefore it is critical that leukocyte isolation be accomplished rapidly following blood draw with minimal stress during the isolation process.

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Published online: 28 August 2018

Immuno-affinity techniques provide an elegant method to achieve highly selective isolation of leukocytes and leukocyte sub-populations but the antibody binding event itself can be a source of activation (Krutmann et al. 1990; von Bonin et al. 1998). Further, it is essential to have prior understanding of cell surface markers to enable isolation of target cell populations (Dubois et al. 2011). Therefore, label-free approaches are highly desirable to minimize activation and bias during the isolation process. Two commonly used techniques for label-free isolation of leukocytes from whole blood are **(A)** Density gradient centrifugation (DGC) and **(B)** Erythrocyte or red blood cell (RBC) lysis (LYSIS). DGC is an elegant process that relies on differences in mass densities of different cell types to enable separation in a label-free fashion. DGC is typically accomplished with a density gradient medium like ficoll, percoll, sucrose or dextran which has intermediate mass density between peripheral blood mononuclear cells (PBMCs) and polymorphonuclear cells (PNMs) and RBCs (Hofland et al. 1989; Brosseron et al. 2015). LYSIS utilizes different osmotic lytic agents like deionized water, NaCl buffer, NH_4Cl-$KHCO_3$ buffer to accomplish isolation of total leukocytes by selective lysis of RBCs (Li et al. 2017). While both DCG and LYSIS methods are label-free and do not require the use of antibodies which can be an additional source of activation, DCG is associated with exposure of leukocytes to high levels of stress (~800g) for extended periods of time (~ 20 mins) (English and Andersen 1974) whereas LYSIS exposes leukocytes to osmotic solutions and contact with free hemoglobin (heme and hemin) form lysed RBCs which can both cause leukocyte activation (Helms et al. 2013).

Leukocytes express various surface markers that can be qualitatively and quantitatively evaluated to determine activation status (Newton et al. 1997). Transcriptionally regulated markers of activation require a certain amount of time (i.e. transcription of mRNA and translation into proteins) to be useful in evaluating activation. Therefore we focused on activation markers that do not require transcriptional regulation but are already either expressed on the cell surface and become damaged following activation or are contained in vesicles within the cell and translocate to the cell surface following activation. Monocytes in circulation do not express high levels of integrins on the cell surface. However, immediately following activation the expression of integrins (CD11b/CD18) is transiently upregulated to enable monocytes to roll and attach to the endothelium and transmigrate into the underlying tissue (Weber et al. 1995). Therefore evaluation of CD11b/CD18 can be utilized as a highly sensitive marker of monocyte activation and has been previously been used as a highly sensitive marker to evaluate isolation process induced monocyte and granulocyte activation following RBC lysis (Sethu et al. 2006). There have also been several reports that chemokine (C-C and C-X-C motif) receptors in particular are highly sensitive to stress from isolation processes like DGC. In reviewing literature, we found that expression of CCR2 and CCR4 receptors on lymphocytes and CCR2 on monocytes was significantly reduced following DGC. This reduction in expression was found to be a long-term effect and could not be recovered even with treatment with pro-inflammatory stimuli (Nieto et al. 2012).

Microfluidics deals with manipulation of fluids within devices in the sub-millimeter scale and can significantly enhance efficiency in comparison to conventional macroscale processes. Scaling effects can also be enhanced to exploit phenomena like low Reynolds Number (Re) laminar flow to enable processes not possible in the macroscale. Previously, our group miniaturized RBC lysis process within a microfluidic platform and demonstrated high efficiency isolation of leukocytes with minimal isolation process induced activation (Sethu et al. 2004). However, RBC lysis results in isolation of total leukocytes and evaluation of heterogeneous populations is associated with loss in quality of information in comparison to isolated sub-populations (Nieto et al. 2012). Several groups including ours have previously sought to miniaturize conventional DGC but most demonstrations have been limited to proof-of-concept studies using beads which have significant differences in density (Moen et al. 2016; Sun and Sethu 2017). One group achieved microfluidic separations of blood cells but the platform used requires step-by-step layering of blood over Ficoll and processing of small (< 20 μL) of blood and requires complex operation of valves to be able to fractionate separated layers (Kinahan et al. 2016).

In this study we sought to develop a continuous and automated microfluidic device to accomplish isolation of PBMCs (monocytes and lymphocytes) from RBCs and PNMs (granulocytes, basophils, eosinophils, mast cells) by miniaturizing conventional density gradient centrifugation to transform this technique from a high-stress and lengthy isolation process into a rapid and low-stress alternative. Specifically, we hypothesized that establishment of laminar flow and reducing the separation distances by over an order of magnitude will drastically reduce the time and forces necessary to achieve high efficiency density gradient centrifugation. We ultimately envision this device to be adopted for point-of-care processing of blood from patients for isolation of PBMCs. Our design allows for evaluation of blood without any pre-processing, automated metering and delivery of blood and Ficoll into the device, and seamless collection of different cellular fractions via different outlets. Further, this device requires only a simple rotary platform and both fluid transport and centrifugal force are generated via rotary motion of this platform.

2 Materials and methods

2.1 Device operation

The device design for microfluidic density gradient centrifugation (MICRO) and images of the actual device with blood and Ficoll layers are detailed in Fig. 1a-c. The device is initially primed with Ficoll and rid of any air bubbles. Then blood and Ficoll were loaded into the reservoirs with tubing connections as shown in Fig. 1a and the device is subject to rotary motion. Centrifugal forces generated as a consequence of the rotary motion cause flow of both blood and Ficoll into the microchannels arranged in a spiral fashion. The centrifugal force also acts on the blood and Ficoll streams to establish a continuous density gradient across the width of the channel. Force balance on cells within the microchannels shows that cells experience drag forces in the direction of the fluid flow along with centrifugal and buoyancy forces that act in opposite directions across the width of the channel (Fig.1d). For heavier cells (RBCs and PNMs) the centrifugal force is >> than the buoyancy forces and the net result is movement of cells towards the outer wall and collection in the bottom outlet that fractionates fluid in the lower half of the channel. For lighter cells (PBMCs) the buoyancy force > centrifugal force and the net result is maintenance of cells at the blood/Ficoll interface and collection via the top outlet which fractionates fluid in the upper half of the channel.

2.2 Device design and fabrication

Devices were fabricated using previously established techniques for soft-lithography in our laboratory (Parichehreh et al. 2011; Parichehreh and Sethu 2012; Parichehreh et al. 2013). Briefly, channel architectures were laid out using AutoCAD (Autodesk, San Rafael, CA) and printed as a 2D dark-field mask onto a transparency using a high-resolution printer (Fineline Imaging, Boulder, CO). The mask was then used to define a mold using negative photoresist SU-8 (Microchem, Westborough, MA) that was spun onto a 4" silicon wafer at a thickness of 25 μm and developed using SU-8 developer (Microchem, Westborough, MA). Following creation of mold, the devices were cast using polydimethylsiloxane (PDMS) (Sylgard 184, Dow Corning, Midland, MI), holes for placement of reservoirs and for inlet and outlet tubing were punched and irreversibly bonded to either a 4" glass or silicon wafer following treatment with oxygen plasma. The spiral microchannels have the following overall dimensions: H=25μm, W=3mm and L~60cm).

2.3 Device characterization

Based on estimations of centrifugal forces necessary and upper limits for both Reynolds Number (Re) and Deans Number (De), we evaluated multiple designs and shortlisted 3-4 promising configurations. These configurations were then extensively tested and the locations of the blood and Ficoll reservoirs, lengths and diameters of the inlet and outlet tubing and the spin speed of the rotary platform were all optimized to ensure delivery of a narrow (~ 100 μm) stream of blood over a thick stream (~ 3 mm) of Ficoll, sufficient residence time and force to cause movement of all RBCs to the outer wall and flow rates sufficient to process ~ 30μL/min of blood.

2.4 Microfluidic density gradient centrifugation (MICRO)

Devices were primed with Ficoll and fresh blood purchased from a commercial vendor and Ficoll was loaded in reservoirs and collection tubes were placed at the outlets. The device was mounted on a rotary platform and spun at 3000 rpm for 3 minutes. The samples from each outlet were collected separately and the PBMCs from the inner outlet was washed and resuspended in 1X PBS prior to staining, fixation and evaluation using flow cytometry.

2.5 Conventional density gradient centrifugation (DGC)

For DGC, blood was diluted with PBS at 1:1 ratio and 1 ml of diluted blood was carefully layered on top of 4ml Ficoll-Paque in a 15 mL tube and spun at 700g for 20 minutes in a refrigerated centrifuge. Then the plasma layer was removed and buffy coat layer containing the PBMCs was carefully fractionated. The collected sample was washed and resuspended in 1X PBS prior to staining, fixation and evaluation using flow cytometry.

2.6 RBC lysis (LYSIS)

Isolation of total leukocytes using LYSIS was performed by mixing 1ml of whole blood with 14ml of RBC lysis buffer (NH_4Cl buffer) for 5 minutes. The mixture was then spun at 200g for 4 minutes, the supernatant was discarded and pellet was resuspended in 1X PBS prior to staining, fixation and evaluation using flow cytometry.

2.7 Stain, fixation, isolation protocol (SFI)

To preserve native expression levels of various surface markers, 1 mL of whole blood was first stained with fluorescently labelled antibodies associated with phenotypic and activation markers for ~ 40 minutes at room temperature and immediately fixed using 1% paraformaldehyde solution. This sample was then depleted of RBCs using standard RBC lysis protocol and resuspended in PBS for subsequent analysis.

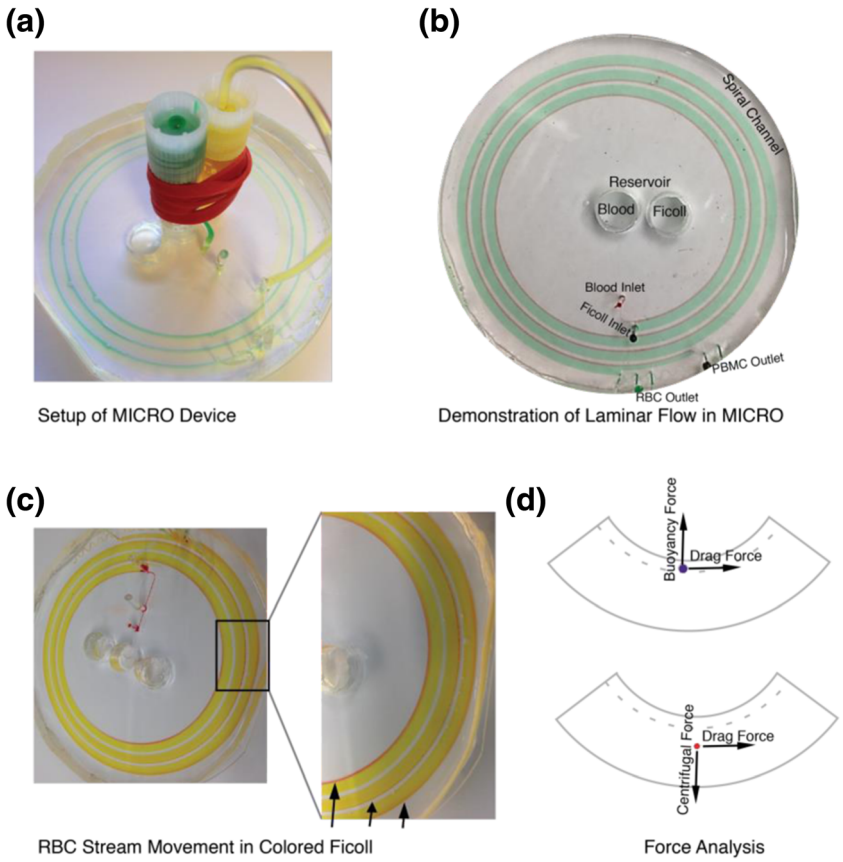

Fig. 1 **a** Actual setup with reservoirs mounted in the center of the device containing the spiral microfluidic channel, **(b)** Demonstration of layering and establishment of laminar flow using saline solution and colored Ficoll solution, **(c)** Proof-of-concept showing movement of RBCs introduced at the top of the channel across a colored stream of Ficoll solution and **(d)** Force balance on cells or particles introduced into the microchannel. For lighter particles Buoyancy Forces > Centrifugal forces which ensures that particles are confined to the top stream whereas for heavier particles the Centrifugal Forces > Buoyancy Forces causing movement towards the outer wall

2.8 Immunolabeling and flow cytometry analysis

Isolated cell populations were stained with antibodies specific to phenotypic markers (Lymphocytes: CD3, CD4 and Monocytes: CD14) and activation markers (Lymphocytes: CCR2, CCR4 and Monocytes CCR2 and CD11b) for ~ 40 minutes at room temperature, immediately fixed using 1% paraformaldehyde and resuspended in flow cytometry buffer for analysis using flow cytometry (FACSCalibur, Becton Dickinson, Franklin Lakes, NJ). Flow cytometry was used to obtain both forward and side scatter information from cells along with expression levels of various phenotype and activation markers. Cell activation was scored using unpaired *t*-tests with two-tailed significance $p<0.05$ for sample sizes of $n>3$.

3 Results

3.1 Establishment of laminar flow and layering of blood over ficoll

In order to facilitate MICRO separation, it is essential that laminar flow is established to ensure layering of blood and Ficoll layers. To accomplish this we ensured that 'Re' < 100 and 'De' was < 40 to ensure laminar flow and minimal impact of rotational secondary flows. Our design with a channel height of 25 μm and width of 3mm at the spin speed of 3000 rpm resulted in laminar flow as evidenced by visualization of distinct streams of water layered over Ficoll (colored to enable visualization) within the device at these conditions (Fig. 1a-d). Our experiments suggest that maintaining high channel aspect ratios (w:h) is critical to ensuring generation minimal levels of rotational forces. We were able to achieve layering for aspect ratios > 1:60 (w:h). Another critical factor for efficient separation is the relative width of the blood and Ficoll streams. With an increase in spin speed, the flow rates of Blood and Ficoll increase in a non-linear fashion and the ratio of the relative widths of the two streams also changes. We experimentally determined that ratio of widths of blood stream to Ficoll needs to be at least 1:5 to achieve efficient separation (Fig. 2).

3.2 Isolation of PBMCS and depletion of RBCs and PNMs

To ensure that the MICRO protocol can efficiently isolate PBMCs while ensuring depletion of more dense RBCs and PNMs we compared cell populations isolated using MICRO with both DGC and LYSIS protocols. DGC should result in isolation of PBMCs with efficient depletion of RBCs and PNMs whereas LYSIS protocol should isolate all leukocytes free of RBCs. A total of 1 mL blood was processed using LYSIS and DGC whereas 100 μL was processed using

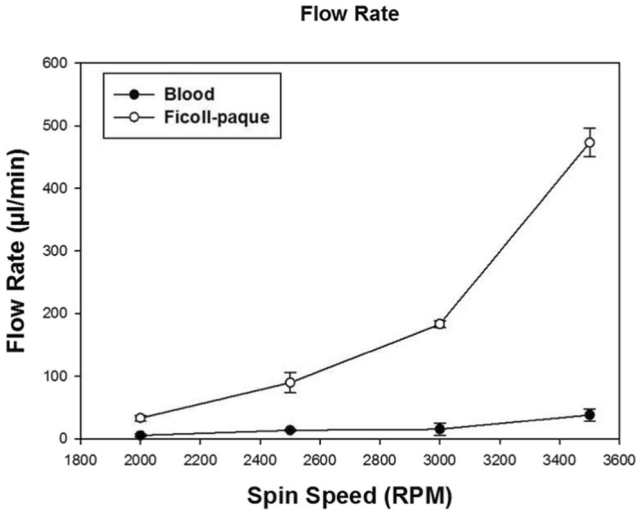

Fig. 2 Flow rate of Blood and Ficoll streams as a function of spin speed of the rotary platform. Experiments were performed at a spin speed of 3000 rpm where the ratio of the widths of the Blood and Ficoll streams were ~ 1:5 resulting in a Blood flow rate of 30-35 μL/min. It is important to note that with an increase in spin speed, the relative increase in flow rates of blood and Ficoll are not linear

MICRO. Flow Cytometric evaluation of various cell populations (Fig. 3) using light scatting suggests that all three techniques result in efficient depletion of RBCs as evidenced by clear identification of leukocyte sub-populations on the forward scatter (FSC) vs. side scatter (SSC) plot with both DGC and MICRO showing depletion of PNMs in addition to the RBCs. Even though a majority of PNMs (> 99%) were depleted using both DGC and MICRO, a small number of PNM contamination is visible from both scatter plots.

3.3 Effect on lymphocyte sub-populations and monocytes

Following confirmation that the MICRO protocol can indeed be used to isolate PBMCs and deplete RBCs and PNMs we sought to confirm if the relative ratios of different PBMC sub-populations isolated using MICRO was comparable to that seen with LYSIS and DGC. To phenotype lymphocyte sub-populations we gated for the lymphocyte region on the FSC vs. SSC plot and evaluated expression of phenotype markers CD3 vs. CD4. To evaluate monocytes we gated the monocyte region on the FSC vs. SSC plot and evaluated expression of monocyte phenotype marker CD14. There are multiple populations of lymphocytes and can be characterized as T-helper cells ($CD3^+CD4^+$), Cytolytic T cells ($CD3^+CD4^-$) and other lymphocytes including B-cells and NK cells ($CD3^-CD4^-$). To establish a control we developed a stain-fix-isolation (SFI) protocol which accomplishes staining and fixation immediately after the blood draw thereby labeling and preserving cells and expression of cell surface markers prior to RBC depletion via lysis. Following LYSIS we see that ~ 33% of lymphocytes are $CD3^+CD4^+$, ~ 23% of lymphocytes are $CD3^+CD4^-$ and 40% of lymphocytes are $CD3^-CD4^-$ (Fig. 4a). With DGC the ratios were different with ~ 26% of lymphocytes are $CD3^+CD4^+$, ~ 20% of lymphocytes are $CD3^+CD4^-$ and 50% of lymphocytes are $CD3^-CD4^-$ (Fig. 4b). MICRO appears to be closer to the ratios obtained with LYSIS with ~ 39% of lymphocytes are $CD3^+CD4^+$, ~ 23% of lymphocytes are $CD3^+CD4^-$ and 34% of lymphocytes are $CD3^-CD4^-$ (Fig. 4c). There was no statistical significance between LYSIS and MICRO for all lymphocyte sub-populations but differences were significant when DGC was compared to both techniques for the $CD3^+CD4^+$ and $CD3^-CD4^-$ populations. Monocyte purity was evaluated by determining the number of cells expressing CD14, a monocyte phenotype marker in the monocyte region on the FSC vs. SSC plot. LYSIS resulted in ~ 70% of $CD14^+$ cells in the monocyte gate whereas this number was > 90% with DGC and > 95% with MICRO (Fig. 5).

3.4 Lymphocyte activation

To determine if the isolation process resulted in activation of lymphocytes, we evaluated expression of chemokine receptors CCR2 and CCR4 which are known to be lost following high stress DGC. Expression of CCR2 and CCR4 was evaluated on all lymphocytes isolated using LYSIS, DGC and MICRO and compared to expression on lymphocytes isolated via SFI (Fig. 6). All lymphocytes in the lymphocyte gate on the FSC vs. SSC plot were considered. Our results confirm that in comparison to SFI, lymphocytes isolated using MICRO and LYSIS appear to have similar number of cells with high and low expression of CCR2 and CCR4 receptors. However, DGC resulted in significant reduction in number of cells with high expression of CCR2 receptor.

3.5 Monocyte activation

To evaluate isolation process induced activation of monocytes, two activation markers that are highly sensitive to stress during the isolation process (CCR2 and CD11b) were selected. $CD14^+$ monocytes isolated using each of the four techniques present in the monocyte gate in the FSC vs. SSC plot were evaluated for expression levels of CD11b and CCR2 (Fig. 7). Our results suggest that even though the mean fluorescence intensity levels were lower following LYSIS, the number of $CD11b^+$ was significantly lower suggesting that there may be selective loss of monocytes expressing CD11b. There was no significant difference in levels of expression CD11b or relative numbers of $CD14^+$ cells using SFI, MICRO and DGC. Evaluation of CCR2 expression on monocytes suggests that LYSIS resulted in significant loss of CCR2 expression on monocytes whereas the relative expression levels on CCR2 on $CD14^+$ monocytes isolated using MICRO and DGC were very similar to that seen with SFI.

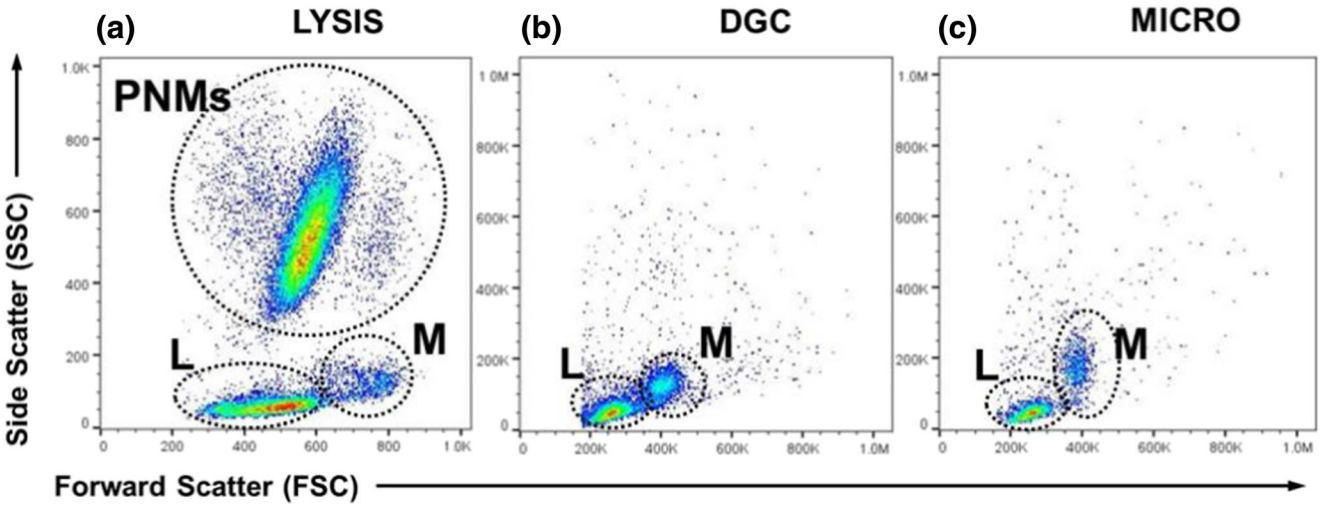

Fig. 3 PBMC Recovery: Flow cytometry scatter plots to distinguish leukocyte sub-populations based on forward scatter (FSC) and side scatter (SSC) on samples isolated using **(a)** RBC Lysis (LYSIS), **(b)** Density Gradient Centrifugation (DCG), and **(c)** Microfluidic Density Gradient Centrifugation (MICRO)

4 Discussion

Inflammation is a key player in our body's defense and satisfactory resolution is critical as chronic sustained inflammation can cause the body significantly more harm. Leukocytes are an integral part of the body's inflammatory response and the numbers and presence of different sub-populations of leukocytes correlate to the body's immediate immune and inflammatory status. Profiling of leukocyte subpopulations therefore provides unique opportunities to monitor and individual's immediate status and provides important information that can be used to diagnose profile, monitor, treat and evaluate effectiveness of treatment. Blood is commonly sampled in the clinical setting and used for rather simplistic evaluation of cell counts and presence of inflammatory secretome in serum. More complex evaluation of leukocytes for expression of markers of activation using techniques like flow cytometry or molecular expression techniques have not been commonly pursued due to complexities associated with ensuring that isolated cells are an accurate representation of the status of the patient.

Given the number of RBCs in whole blood, it is not surprising that evaluation of whole blood samples for molecular expression studies provides significantly lower quality information when compared to isolated leukocytes (Feezor et al. 2004). Therefore, at a minimum, RBC depletion is necessary for analysis of leukocytes and/or leukocyte sub-populations to ensure that samples can provide vital information regarding the state of the body. However, with increased processing involved in separation of leukocytes into different sub-populations, the information contained in the cells has greater potential to be compromised due to time and stress associated with these additional processing steps. Antibody-based approaches that rely on affinity to specific antigens on the surface of the cell to enable isolation provide highly specific alternatives for cell sorting. Concerns relate to the fact that the antibody binding event and steps involved in ensuring binding and subsequent isolation can be a significant source of unnecessary activation (Krutmann et al. 1990). Further, antibody based approaches rely on known antibody-cell interactions and require prior knowledge of molecular expression

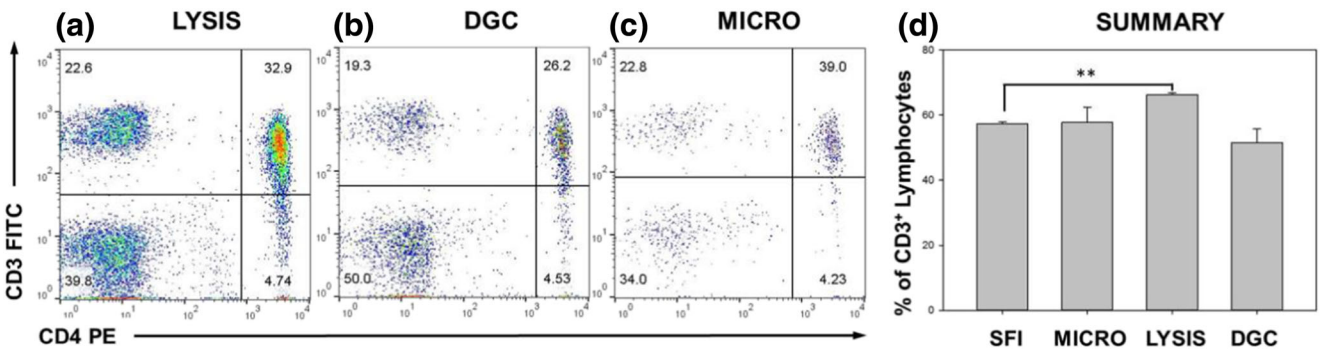

Fig. 4 Lymphocyte Recovery: Flow cytometric evaluation of expression of CD3 vs. CD4 on cells that fall within the lymphocyte gate (i.e. region labeled 'L' in Fig. 3) on the FSC vs. SSC plot for samples isolated using **(a)** RBC Lysis (LYSIS), **(b)** Density Gradient Centrifugation (DCG), **(c)** Microfluidic Density Gradient Centrifugation (MICRO) and **(d)** % $CD3^+$ lymphocytes

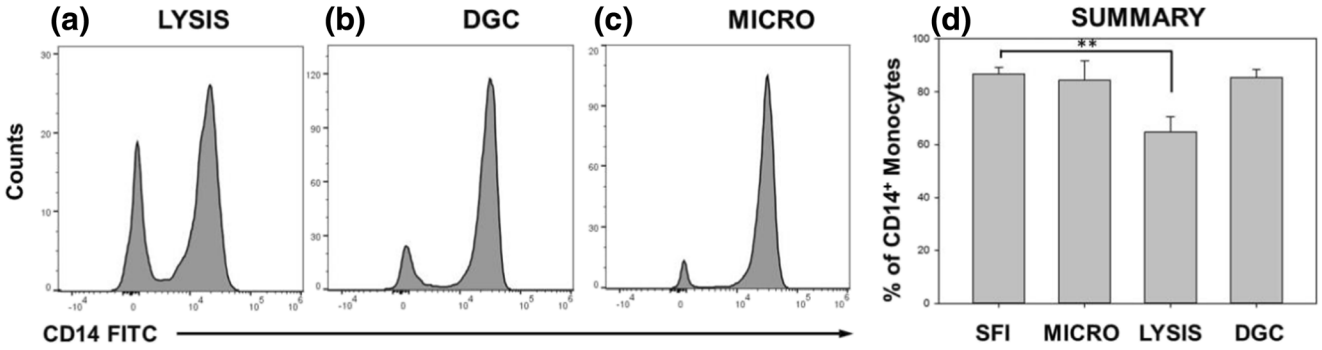

Fig. 5 Monocytes Recovery: Evaluation of expression of CD14 on cells that fall within the monocyte gate (i.e. region labeled 'M' in Fig. 3) on the FSC vs. SSC plot for samples isolated using **(a)** RBC Lysis (LYSIS), **(b)** Density Gradient Centrifugation (DCG), **(c)** Microfluidic Density Gradient Centrifugation (MICRO) and **(d)** % $CD14^+$ Monocytes

patterns on the cell surface. Therefore, label-free approaches like RBC lysis and DGC are more commonly used to ensure unbiased separation of leukocytes and leukocyte sub-populations.

Microfluidics provides unique opportunities to miniaturize conventional separation processes and minimize critical factors like time, stress and sample processing volumes. The RBC lysis has been previously miniaturized and results confirm that isolation high quality total leukocytes with minimal cell loss or activation can be accomplished using this approach (Sethu et al. 2004). This technique is highly suitable for isolation of total leukocytes but subsequent processing is necessary if isolation of leukocyte sub-populations is desired. To enable isolation of leukocyte sub-populations, several groups have attempted to miniaturize conventional DCG using microfluidics (Amasia and Madou 2010; Burger and Ducree 2012; Kinahan et al. 2016; Moen et al. 2016). While there have been some successful attempts to miniaturize DGC, most approaches have demonstrated feasibility using bead solutions but have been unable to translate their approaches for continuous blood sorting. One group used pre-loaded samples to achieve separation of PBMCs from RBCs and PNMs but the capability to process sufficient sample volume was limited due to the small volume of the microfluidic chamber (< 20 μL of blood) (Kinahan et al. 2016). In this manuscript we report a new approach focused on taking microfluidic DGC beyond simple proof-of-concept studies using beads and demonstrating that this effort can be used to isolate PBMCs from whole blood samples without any sample pre-processing. This is not a trivial process as several variables need to be optimized to ensure both laminar flow of Blood and Ficoll streams and sufficient centrifugal force to ensure clean separation of RBCs and PNMs. To accomplish this, we optimized the design including reservoirs, flow control elements (resistances at

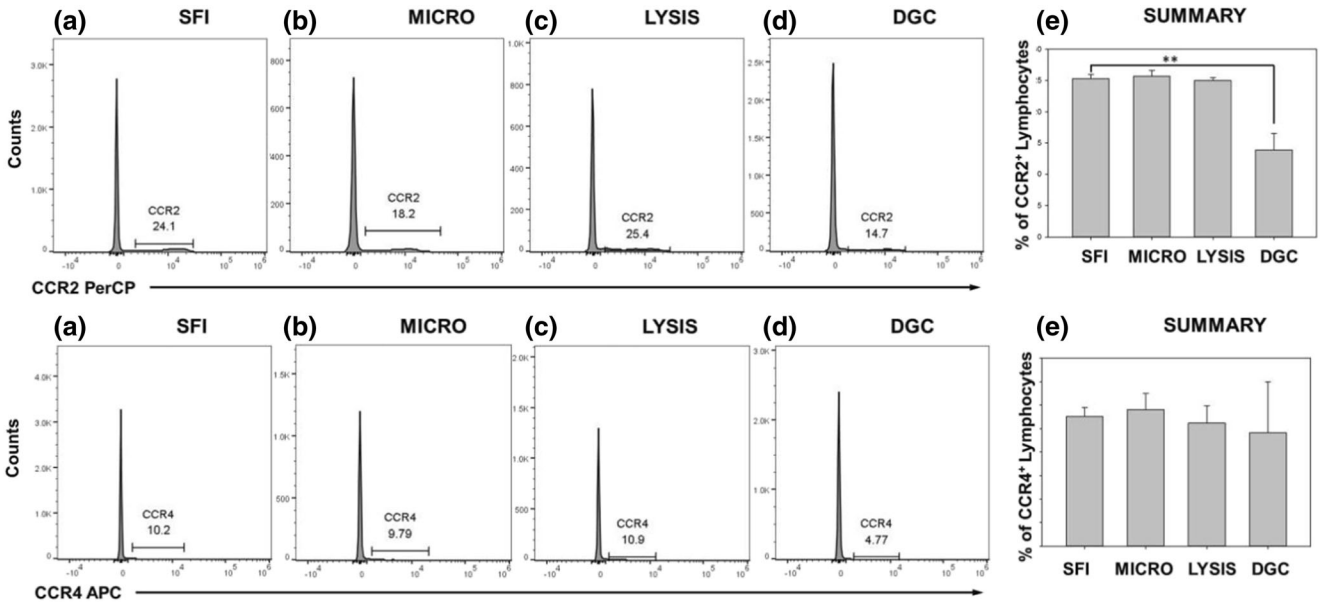

Fig. 6 Lymphocyte Activation: Determination of activation status of Lymphocytes evaluated using expression of chemokine receptors CCR2 **(top)** and CCR4 **(bottom)** for samples isolated using **(a)** Stain-Fix-Lyse protocol (SFI) **(b)** RBC Lysis (LYSIS), **(c)** Density Gradient Centrifugation (DCG), **(d)** Microfluidic Density Gradient Centrifugation (MICRO) and **(e)** Summary of data shown in Scatter Plots

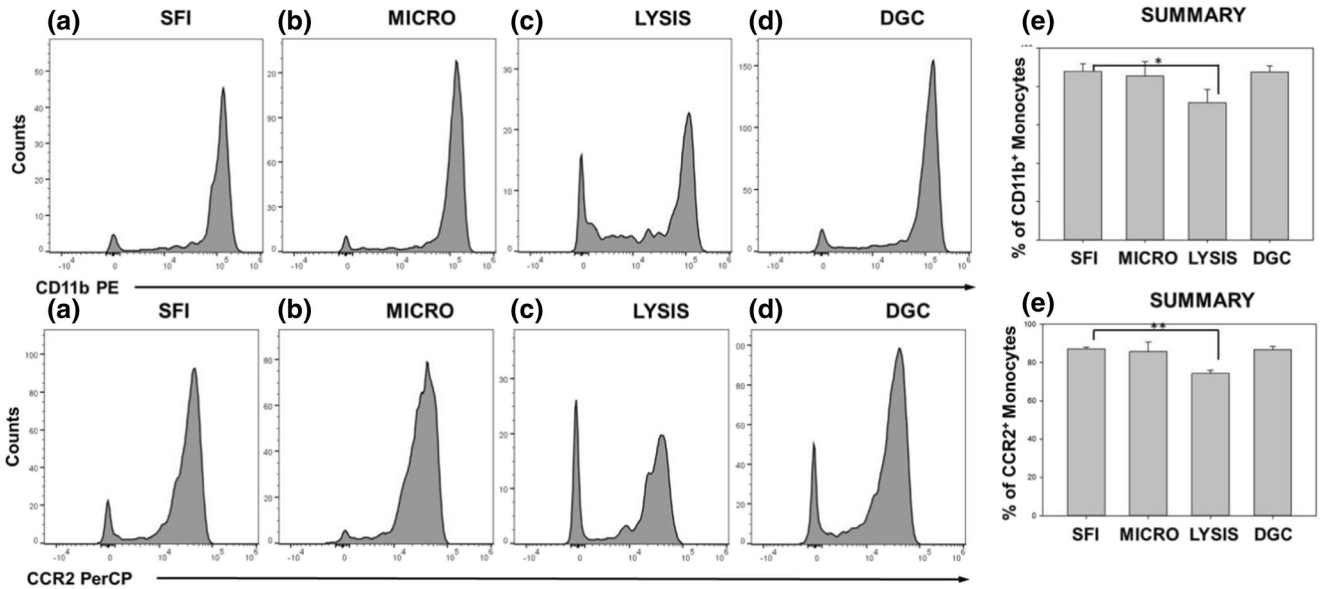

Fig. 7 Monocyte Activation: Determination of activation status of Monocytes evaluated using expression of integrin CD11b **(top)** and chemokine receptor CCR2 **(bottom)** for samples isolated using **(a)** Stain-Fix-Lyse protocol (SFI) **(b)** RBC Lysis (LYSIS), **(c)** Density Gradient Centrifugation (DCG), **(d)** Microfluidic Density Gradient Centrifugation (MICRO) and **(e)** Summary of data shown in Scatter Plots

inlets and outlets), microfluidic channel dimensions and outlets to ensure that the device does not require any external manipulation and the rotary platform can provide both the centrifugal force necessary for separation and the driving force necessary for fluid flow. Following separation, the collection tubes can also be centrifuged to remove the supernatant and resuspend cells in physiological buffers for whole/live cell analysis or with lysis buffers for gene and protein expression analysis. The use of a single rotary platform to perform all necessary functions involved with cell separations ensures that this process can be automated and adapted for point-of-care deployment.

Our results validate our initial hypothesis that microfluidic adaptation of DCG will result in a faster and gentler process for isolation of PBMCs which ensures recovery of cells with minimal loss and minimal isolation process induced activation. Comparison of MICRO with LYSIS and DGC confirms that both lymphocytes and monocytes can be isolated without contamination with RBCs or granulocytes. Further evaluation of lymphocytes using phenotypic markers CD3 and CD4 suggests that the relative ratios of T-helper cells ($CD3^+CD4^+$), Cytolytic T cells ($CD3^+CD4^-$) and other lymphocytes including B-cells and NK cells ($CD3^-CD4^-$) is consistent with both LYSIS and MICRO but DGC produced higher number of $CD3^-CD4^-$ cells and lower numbers of $CD3^+CD4^+$ cells suggesting either selective enrichment of $CD3^-CD4^-$ cells or selective depletion of $CD3^+CD4^+$ cells. Evaluation of cells within the monocyte gate for CD14, a monocyte phenotype marker shows that the number of $CD14^+$ monocytes was high with both DGC and MICRO but significantly lower with LYSIS. This suggests either preservation of cells that are not monocytes within the monocyte gate or damage to monocytes that causes changes to the expression of CD14 on these monocytes.

Our activation studies focused on highly sensitive markers of PBMC activation and rely on expression of chemokine receptors CCR2 and CCR4 on lymphocytes and CCR2 on monocytes which are known to be extremely sensitive to stress along with profiling of expression of CD11b on monocytes which is an integrin that is upregulated following activation in monocytes. To obtain a standard for comparison, we developed a new stain-fix-isolate technique which ensured staining of leukocytes in whole blood immediately following blood draw, fixation and then removal of RBCs via lysis. This accurately preserves the molecular expression signatures prior to the isolation and ensures that the isolation process itself does not change expression of cell surface markers as the cells are fixed during the RBC lysis process. Evaluation of lymphocytes isolated with different techniques suggests that in comparison to the SFI technique, CCR2 and CCR4 expression on lymphocytes was impacted following isolation with DGC but not with MICRO or LYSIS but only loss of CCR2 on lymphocytes isolated with DGC was statistically significant. This is consistent with prior studies that suggest that CCR2 receptors are lost on lymphocytes following high stress DGC and cannot be recovered even with stimulation (Nieto et al. 2012). Evaluation of monocyte populations for expression of CCR2 and CD11b shows that in comparison to SFI, LYSIS results in loss of expression of both CCR2 and CD11b which could either be damage of these surface markers or loss of cells expressing these markers. In comparison, both MICRO and DGC were similar to SFI. These results collectively suggest

that MICRO is the only technique that preserves cellular populations and expression patterns seen in whole blood as evidenced by the close similarity between MICRO and SFI in these activation studies.

In summary, we developed a microfluidic protocol for isolation of PBMCs from whole blood using density gradient centrifugation within microfluidic channels. This required significant design and validation to ensure laminar flow, layering and generation of sufficient centrifugal forces to facilitate separation of PBMCs from contaminating RBCs and PNMs. Evaluation of numbers and activation status of isolated cells suggests that this approach can potentially preserve monocytes and different lymphocyte sub-populations with minimal isolation process induced activation. Finally, the use of a simple rotary platform to deliver samples, facilitate separation and sample collection is ideal for transformation of this approach into a point-of-care device for isolation of PBMCs for rapid evaluation of patient blood samples.

Acknowledgements YS was supported by an Alabama EPSCoR Graduate Research Scholars Program. This work was supported by the NSF CAREER Award # 1149059 to PS and the Comprehensive Cardiovascular Center and the Division of Cardiovascular Disease at the University of Alabama at Birmingham.

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