Microfluidic-based Cell Separation Method Improves Workflow for Evaluation of Rare Lymphocytes from Cancer Patient Samples

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Introduction

Isolation of lymphocytes from whole blood is an initial and imperative step for many *in vitro* assays. For example, when developing a cellular immunotherapy, it is crucial to generate a valid method to separate and to expand a distinct lymphocyte population. However, the isolation of rare lymphocytes from the peripheral blood products of both cancer patients and healthy volunteers can be difficult with the standard cell separation methods such as Mononuclear Cell Preparation Tubes with Sodium Heparin™ (CPTs). The cell separation medium (polyester gel and a density gradient liquid) confined within CPTs enable mononuclear cells to be separated from whole blood in a centrifugation step (1). The peripheral blood mononuclear cells (PBMC) are then harvested by carefully pipetting them from the liquid surface (1). In this cell separation method substantial lymphocyte loss occurs, the mechanism of harvest inherently produces inconsistent results due to technician variability, and processing time can be burdensome. To mitigate these issues, an automated microfluidic approach (MicroMedicine's Microfluidic System, or MS) was evaluated for lymphocyte recovery, processing time, and viability. The robustness of this approach to obtain lymphocytes from whole blood provides statistically improved outcomes for recovery and processing time when compared to processing whole blood with CPTs. Based on these improved results, further studies were performed on white blood cells (WBC) isolated using the MS. The MS provided an efficient product from which a rare lymphocyte population was effectively isolated and expanded *in vitro* (demonstrated by immunopheno-typing where applicable). This demonstrates that the MS is a useful processing instrument that can be incorporated in small- to medium-scale *in vitro* assays, especially when applied to pharmaceutical development.

Figure 1. MicroMedicine's Microfluidic System allows for separation of WBCs from 3-75 ml of whole blood using a microfluidic disposable kit.

References

(1) BD Vacutainer CPT: Cell Preparation Tube with Sodium Heparin
(2) Phlebotomy SOP
(3) Positive Selection Immunomagnetic Cell Separation SOP
(4) Small-Scale Culture SOP

Materials & Methods

Patient Samples

The IRB/IEC-approved written informed consent was obtained from each healthy volunteer and cancer patient who provided whole blood for this study prior to the phlebotomy procedure. Once informed consent was obtained, each participant received an identification number that was used for the remainder of the process.

CPTs versus MS

During the comparison of processing CPTs versus MS for lymphocytes, 4 CPTs and 4 BD Vacutainer™ Blood Collection Tubes with ACD-A were obtained from a healthy volunteer. The CPTs were processed as described in "BD Vacutainer CPT: Cell Preparation Tube with Sodium Heparin" and the ACD-A tubes were processed with the MS according to the User Manual. This process was repeated with 5 different healthy volunteers. Lymphocyte recovery, hands-on processing time, and viability were recorded for each repetition. For each of the aforementioned values, mean and standard deviation were calculated. Furthermore, an independent samples t-test was conducted to determine statistical significance when comparing lymphocyte recovery, hands-on processing time, and viability for processing whole blood with CPTs versus MS.

Rare Lymphocyte Isolation and Immunophenotyping

Four ACD-A tubes were obtained from healthy volunteers and cancer patients then processed with the MS. A rare lymphocyte was selected from the MS WBC product (3). In addition to the marker specific to the rare lymphocyte, the following cluster of differentiation (CD) markers were evaluated with flow cytometry for immunophenotyping both before and after the small-scale immunomagnetic selection: CD45, CD3, CD4, CD8. This process was repeated 10 times (4 healthy volunteers and 6 cancer patients). The mean and standard deviation were calculated for each lymphocyte marker (both pre-selection and post-selection) and for target cell recovery.

Rare Lymphocyte Isolation and Expansion

Four ACD-A tubes were obtained from cancer patients then processed with the MS. A rare lymphocyte was selected from the MS WBC product (3). In addition to the marker specific to the rare lymphocyte, CD45 was evaluated with flow cytometry both before and after the small-scale immunomagnetic selection. After selection for the rare lymphocyte population, *in vitro* (4) expansion of these lymphocytes was completed with cell growth being recorded throughout the culture period. This process was repeated with 5 cancer patients.

Results

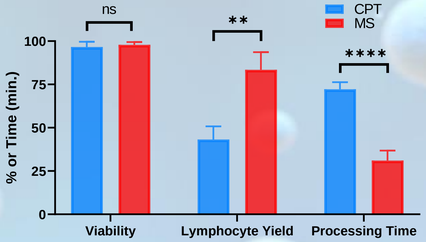

Figure 2. Comparison of lymphocyte isolation methods from whole blood using Cell Preparation Tubes with Sodium Heparin™ (CPTs) versus the Microfluidic System (MS). Results are the mean of 5 healthy donors. Viability of lymphocytes following isolation was comparable between the two methods, at 96.7 ± 3.0% vs. 97.9 ± 1.6%, respectively. Lymphocyte yield was significantly higher using the MS (83.5 ± 10.1%) vs. the CPTs (43.2 ± 7.6%) while processing time was significantly less using the MS vs. the CPTs (31 ± 5.8 vs. 72.2 ± 4.1 min, respectively). Error values were calculated using standard deviation; significance was determined using a two-tailed T-test (NS, $p > 0.05$; **, $p \leq 0.01$, ****, $p \leq 0.0001$).

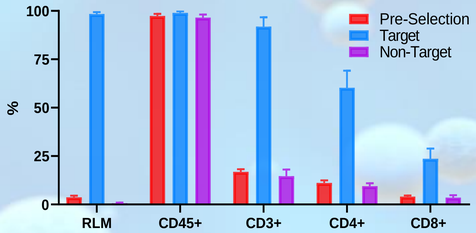

Figure 3A. Immunophenotyping Pre- and Post-Selection of a Rare Lymphocyte Population from MS-derived WBCs from Heathy Volunteers. The results are the mean from four healthy donors; the error bars show standard deviation. The pre-selection analysis shows the rare lymphocyte marker population (RLM) is 3.7 ± 0.8% of the total MS WBC product collected, while 98.4 ± 0.95 % of the rare lymphocytes comprise the post-selection target fraction and the non-target post-selection fraction contains only 0.5 ± 0.4% of the rare cells. Pre-selection (ps), target (t), and non-target (nt) values for different cell types are shown as follows: CD45: ps= 97.4 ± 1.1%, t=99.0 ± 0.8%, nt=96.5 ± 1.6%; CD3: ps=16.9 ± 1.3 %, t=91.9 ± 4.8%, nt=14.7 ±3.4%; CD4: ps=11.1 ± 1.3%, t=60.3 ± 8.8%, nt=9.4 ± 1.6%; CD8: ps=4.0 ± 0.6%, t=23.6 ± 5.3%, nt=3.5 ± 1.3%.

Results

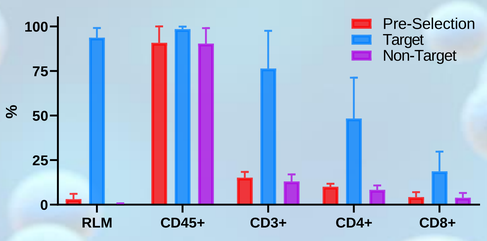

Figure 3B. Immunophenotyping Pre- and Post-Selection of a Rare Lymphocyte Population from MS-derived WBCs from Cancer Patients. The results are the mean from six cancer patients; the error bars show standard deviation. The pre-selection analysis shows the rare lymphocyte marker population (RLM) is 3.1 ± 2.9% of the total MS WBC product collected, while 93.6 ± 5.4 % of the rare lymphocytes comprise the post-selection target fraction and the non-target post-selection fraction contains only 0.4 ± 0.4% of the rare cells. Pre-selection (ps), target (t), and non-target (nt) values for different cell types are shown as follows: CD45: ps= 90.8 ± 9.2%, t=98.5 ± 1.4%, nt=90.4 ± 8.6%; CD3: ps=15.1 ± 3.2 %, t=76.4 ± 21.1%, nt=13.0 ± 3.9%; CD4: ps=10.1 ± 1.6%, t=48.4 ± 22.9%, nt=8.3 ± 2.5%; CD8: ps=4.2 ± 2.8%, t=18.8 ± 11.0%, nt=3.9 ± 2.7%.

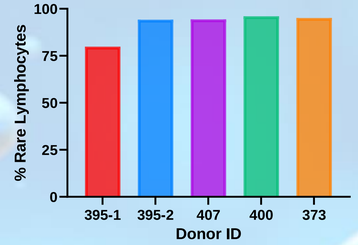

Figure 4. Rare Lymphocyte Purity from Cancer Patients. These data show the purity of five cancer patients' rare lymphocyte population after WBC separation using the MS followed by immunomagnetic isolation. Samples 395-1 and 395-2 were isolated from the same colon adenocarcinoma patient; however, the 395-2 collection was made after the patient started immunotherapy treatment. The selection of the rare lymphocytes for 395-1 had a purity of 79.9% prior to expansion while the purity for 395-2 was 94.2%. Sample 407 was selected from a patient with adrenal cortical carcinoma and had a purity of 94.4%. Sample 400 has a purity of 96.0% and came from a patient with sigmoid colon adenocarcinoma. Sample 373 came from a gastroesophageal adenocarcinoma patient and had a rare lymphocyte purity of 95.2%.

Results

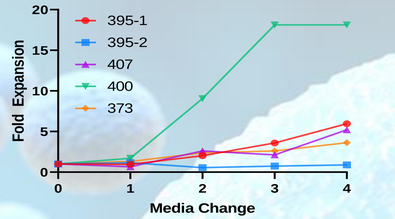

Figure 5. Expansion curve for *in vitro* culture of rare lymphocyte population. The rare lymphocyte population isolated from cancer patients was expanded *in vitro* and the fold expansion over 9-10 days of growth is shown. The cells were activated and 3 media changes were performed. Cells from 395-1 had a 6.0-fold expansion and 395-2 cells had 0.9-fold expansion. Note that both of these samples were isolated from the same patient; however, the 395-2 collection was made after the patient started immunotherapy treatment. The other samples expanded as shown: 3.6-fold for 373, 5.2-fold for 400 and 18.1-fold for 407. These variable expansion rates were typical for the rare lymphocytes isolated from cancer patients using our protocol. Media Change 0=start of culture, 4=Harvest.

Conclusions

Isolating lymphocytes from whole blood is a necessary prerequisite for *in vitro* assays, particularly in molecular- and cellular-based pharmaceutical development. Compared to the standard cell separation method of using CPTs, the MS proved to be a more efficient, robust, and opportune mechanism to perform this function as validated by lymphocyte recovery and hands-on processing time. Further, the MS provides a greater lymphocyte yield, resulting in an increased number of rare lymphocytes for downstream immunomagnetic isolation. Thus, the MS WBC product can be used successfully for selecting and expanding a distinct lymphocyte population. In summary, the MS is suitable for lymphocyte-specific *in vitro* assays because of the following: venipuncture is a simple, low-risk procedure applicable to a wide range of cancer patients; a variable amount of whole blood can be used for processing in the MS; a concentrated, consistent WBC population is obtained in an automated, rapid, easy-to-use method. While leukapheresis is a current, superlative method to enrich for PBMCs that is often used in the development of cellular immunotherapy (in addition to therapeutic and diagnostic applications), the MS provides a complement to leukapheresis, particularly in instances where leukapheresis is either unable to be performed, or small- to medium-scale experiments are required for pre-clinical studies.