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Research paper

Flow-based sorting of neonatal lymphocyte populations for transcriptomics analysis

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ABSTRACT

Rationale: Emerging data suggest an important role for T lymphocytes in the pathogenesis of chronic lung disease in preterm infants. Comprehensive assessment of the lymphocyte transcriptome may identify biomarkers and mechanisms of disease.
Methods: Small volume peripheral blood samples were collected from premature infants enrolled with consent in the Prematurity and Respiratory Outcomes Program (PROP), at the time of discharge from the hospital. Blood samples were collected at two sites and shipped to a central laboratory for processing. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Hypaque gradient centrifugation and separated into individual lymphocyte cell types by fluorescence-activated cell sorting. Gating strategies were optimized to ensure reproducible recovery of highly purified lymphocyte populations over a multi-year recruitment period. RNA was isolated from sorted cells and characterized by high-throughput sequencing (RNASeq).
Results: Blood volumes averaged 2.5 ml, and sufficient PBMCs were collected from 165 of the 246 samples obtained (67%) from the 277 recruited subjects to complete sorting and RNASeq analysis on the resulting sorted cells. The number of total lymphocytes per ml of blood in the neonatal subjects was approximately 4 million/ml. Total lymphocyte frequencies recovered following sort varied widely among subjects, as did the frequency of individual lymphocyte and NK cell sub-populations. RNA yield from sorted cells varied according to cell type, but RNA of sufficient quantity and quality was recovered to enable RNASeq.
Summary: Our results describe a validated procedure for the generation of genome-wide expression data from isolated lymphocyte sub-populations obtained from newborn blood.

1. Introduction

Premature birth is defined as birth before completing 37 weeks of gestation. According to the Centers for Disease Control (CDC), in 2013 nearly 450,000 babies were born prematurely in the United States (Martin et al., 2015). Prematurity-related deaths accounted for 36% of all infant deaths in 2013 (Matthews et al., 2015). Recent evidence indicates that expression of inhibitory receptors of innate and adaptive immune cells differs in cord and circulating neonatal blood compared to adult blood, which could potentially lead to functional consequences on the neonatal immune response against infection (Walk et al., 2012). Alterations in CD8 + T cell repertoire in neonates could affect response to infections (Rudd et al., 2013; Grosek et al., 2013). Similarly, decreases in CD4 + T, CD8 + T, CD19 + B and CD56 + NK cells have been reported in preterm infants, relative to full term controls (Correa-Rocha et al., 2012; Ma et al., 2014). Therefore, a comprehensive assessment of lymphocyte sub-populations, and their respective phenotypes, may enable the identification of disease-related biomarkers and pathogenic mechanisms. Very few publications focus on the characteristics of pediatric blood cell populations from infants, and even fewer focus on blood cells isolated from infants who were born preterm.

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Recent advances in genomics research have included improved whole transcriptome analysis of cell populations using next generation RNA sequencing (RNASeq) technology (Zeng and Mortazavi, 2012). The utility and challenges of generating high throughput data sets from immune cells has recently received much attention, which includes the development of new methodologies for sample preparation (Anon., 2013; Oberg et al., 2015; Lavinder et al., 2015; O'Connor et al., 2014). RNASeq can be reliably used to identify gene signatures associated with disease patterns from small amounts of RNA, which makes it possible to study immune cell populations with great resolution from limiting number of cells isolated from low volumes of blood. Thus, studies that were not feasible due to limiting amounts of blood and/or cell numbers, as is the case with neonatal study subjects, are now possible (Dowling and Levy, 2014).

Here, we describe the development of a protocol to ensure consistent separation of T, B, and NK lymphocyte populations and recovery of RNA from these FACS-purified lymphocytes obtained from neonatal infant blood samples, collected at multiple research sites, over the course of a multi-year study. Our results provide a valuable guide for researchers interested in studying purified lymphocyte sub-population gene expression, when blood volumes are limited, using flow cytometry based methods.

2. Methods

2.1. Protocol overview

Preterm infants were screened and consented for enrollment in the Prematurity and Respiratory Outcomes Study at two separate sites. The Golisano Children's Hospital at the University of Rochester Medical Center (Institutional Review Board (IRB# 00037933) and the Children's Hospital of Buffalo (IRB# 612707) are located approximately 75 miles apart. Blood was obtained at the time of infant discharge from the hospital, which corresponds to 36–41 weeks of corrected gestational age at birth. Standard biosafety level 2 (BSL2) procedures were observed at all times. All blood processing was completed within 24 h as our previous work showed minimal change in T cell phenotype within this time period (Scheible et al., 2012).

An outline of the protocol for handling and processing of these blood samples is described in Fig. 1. Briefly, blood was collected, maintained at room temperature, and immediately delivered to a central processing laboratory at the University of Rochester. Approximately 6–12 h after collection at either site, peripheral blood mononuclear cells (PBMCs) were isolated by density-gradient centrifugation. Isolated PBMCs were stained using a panel of antibodies developed to facilitate fluorescence-activated cell sorting of PBMC sub-populations. Sorted cell populations were processed for the recovery of RNA, which was subjected to high-throughput sequence analysis. The complete, detailed standard operating procedure (SOP) is provided as supplemental information.

2.2. Neonatal blood draw and transport

Venous blood was drawn by clinical care staff. Sodium heparin was chosen over EDTA as an anticoagulant, as the use of heparin is associated with higher cell viability (Carter et al., 1992). At Site I, blood was routinely drawn from a peripheral vein using a 23 g butterfly catheter (BD Biosciences, San Jose, CA, #367279) attached to a 3 cm^3 heparinized syringe (Smiths Medical, Dublin OH, #4043-2). The blood was subsequently delivered from the syringe to a 3 ml heparinized tube (BD #367671) via a Blood Transfer Device Female Luer Adapter (BD # 364880). The blood was delivered to an on-site central processing laboratory and maintained at room temperature for approximately 6–12 h, until processing was continued. At Site II, venous blood was dripped from a 25 g angiocatheter directly into the 3 ml heparinized tube. The blood samples was maintained at room temperature and transported to the Site 1 central processing laboratory (approximately 75 miles away) within approximately 6–12 h, using a local medical courier service.

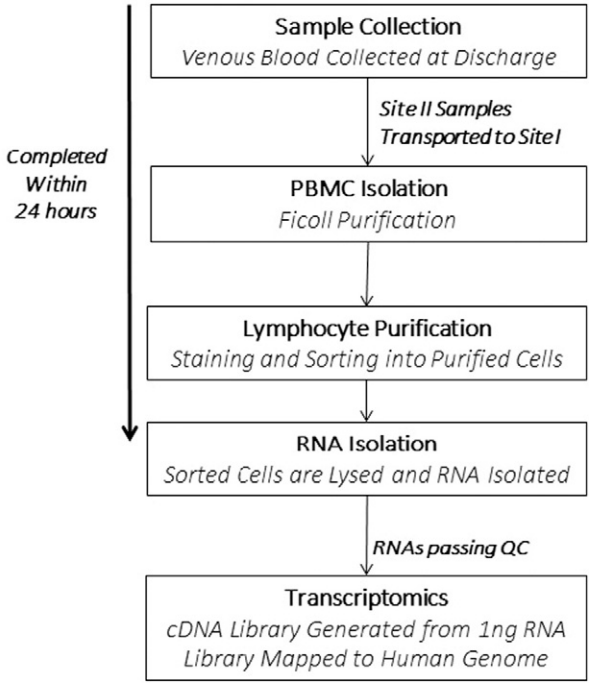

Fig. 1. Procedure diagram. Summary of the critical steps involved in the standard operating procedure (SOP) developed for this study. A detailed SOP is included in the Supplement.

2.3. Tracking of instrument performance

We validated two BD FACSAria cell sorters, in order to ensure availability of an instrument at all times; a 3-laser instrument, capable of detecting 13-fluorescence parameters, and a 4 laser instrument capable of detecting 18-fluorescent parameters were used. The availability of two validated cell sorters minimized the likelihood of missing the opportunity to sort a sample due to an instrument being unavailable. Supplemental Tables 1 and 2 describe the filter configuration and properties of each instrument, which were compatible with the sorting panel design.

We carefully monitored instrument performance using Peak 6 beads (Spherotech #RPC-30-5A), which allowed us to track variability in the detection of fluorescent signal for each run, and to track instrument performance over time. Peak 6 beads were run prior to each set of single colored positive controls to ensure signal was observed in each fluorescent channel. Representative histograms for fluorescence in each channel used in our sort panel are shown for both cell sorting instruments in Supplemental Fig. 1a and b, for staining antibodies and Peak 6 beads, respectively. We further ensured performance over time by performing bridging experiments when switching lots of Peak 6 beads (Supplemental Fig. 2). Interestingly, our data revealed a wide range for coefficient of variation (8–36%) of mean fluorescence intensity depending on the detection PMT, for each of the lots of beads used in our studies (Supplemental Table 3). This observation supported the importance of our bridging experiments.

Major instrument maintenance repairs or baseline calibrations were performed multiple times during the course of this 3-year study. For calibration of instruments, we utilized the fluorescence values in each channel from Peak 6 beads. The mode value of fluorescence from each channel was recorded for each of the runs between service dates, and mean values were calculated. These mean values were then used as targets when setting new voltages for each detector so that the