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Research paper

Optimization of a whole blood phenotyping assay for enumeration of peripheral blood leukocyte populations in multicenter clinical trials

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ABSTRACT

Vaccination with viral vectors or adjuvants can induce early changes in circulating peripheral blood leukocytes that are predictive of a protective immune response. In this study, we define an 11-color whole blood antibody staining Trucount Panel (TP1) to enumerate and phenotype the major leukocyte populations in a human vaccine experimental medicine trial setting. TP1 can be prepared up to 8 weeks prior to use, enabling bulk preparation at a central laboratory and distribution to clinical sites. Cells in whole blood must be stained within 4 h of draw to accurately detect the major cell populations. Staining of cells with TP1 followed by storage and shipping at −80 °C to a central laboratory has little to no effect on the cell concentrations observed. We also present data from an HIV vaccine multicenter clinical trial obtained using the optimized TP1 assay protocol and show that the data produced accurately correlates with complete blood count (CBC) data. Taken together, these data indicate the optimized TP1 panel assay can be used in a multicenter clinical trial setting to increase our understanding of systemic responses to vaccination or disease.

1. Introduction

The immune response required to prevent HIV infection remains incompletely understood. The RV144 HIV vaccine trial in Thailand found modest vaccine efficacy and has led to the identification of immune responses that may be important for HIV-1 vaccine action (Rerks-Ngarm et al., 2009; Haynes et al., 2012). To better understand these responses and how they can be improved, it is vital to expand the immune response parameters measured after vaccination and provide better coverage of the "immunologic space". The early innate responses induced by vaccination are known to influence the quality and longevity of the adaptive immune response, so assessing these parameters in experimental medicine trials is key for defining early biomarkers of vaccine efficacy (Andersen-Nissen et al., 2012).

We have shown in a small experimental medicine trial that an adenoviral-vectored HIV vaccine induces early dynamic changes in peripheral blood leukocytes that predict the downstream adaptive response (Zak et al., 2012). To increase our power to identify additional biomarkers of vaccine immunogenicity or efficacy, these methods needed to be adapted for use in a multicenter clinical trial setting.

Abbreviations: CBC, complete blood count; NK cell, natural killer cell; mDC, myeloid dendritic cells; pDC, plasmacytoid dendritic cells; TP1, Trucount panel 1.

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Complete blood counts (CBC) with differential are typically used in clinical trials to evaluate safety of the product by providing concentrations for the main leukocyte populations (e.g., lymphocytes, monocytes, and neutrophils). However CBC lack the capability to assess numbers and activation states of other important immune cell populations (e.g., monocyte sub-populations, dendritic and NK cells) and typically require an entire 3–10 ml tube of blood for analysis by a routine clinical laboratory. In contrast, whole blood multicolor flow cytometry phenotyping assays can generate concentration and activation data on many cell populations and have been adapted for use in vaccine trial settings (e.g. (Scriba et al., 2010)). These assays require approximately 100-fold smaller volumes of blood than a CBC, but staining and analysis require more technical expertise relative to CBCs due to the multistep procedure and the complex flow cytometers used to collect the data and are much more difficult to standardize and monitor across multiple clinical sites.

Here, we define an 11 color antibody staining panel ("Trucount Panel 1" or TP1) and associated assay conditions that yield data consistent enough for use in a multicenter experimental medicine trial setting. TP1 can enumerate the major leukocyte populations in the peripheral blood and simultaneously provide information on the composition and activation state of certain innate cell populations. In this study, we demonstrate that TP1 can be prepared ahead of time and stored prior to use. We show how individual cell populations are affected if blood is not stained immediately after venipuncture and how storage and shipping conditions affect the different cell types. We present data to quantify the variability of the assay and demonstrate the high correlation between counts obtained using TP1 or a CBC in a vaccine trial setting. Together, the definition of these conditions led to an optimized protocol that we are currently implementing in multicenter clinical trials in the HIV Vaccine Trials Network (Hensley et al., 2012). This protocol is adaptable for use in different experimental medicine trials and represents a promising tool to better elucidate the early immune responses by assessing trafficking and activation state of key innate cells such as dendritic cells, monocytes and NK cells.

2. Materials and methods

2.1. Study participants

All subjects were enrolled at HIV Vaccine Trials Units located in Seattle, WA; Birmingham, AL; or New York, NY. Unvaccinated, HIV-seronegative control whole blood samples were obtained from volunteers in the Seattle Assay Control cohort protocol or HVTN proficiency protocol 998 at Columbia University. Study participants in the HIV Vaccine Trials Network vaccine protocol were healthy, HIV-1-uninfected adults enrolled in Seattle, WA or Birmingham, AL. Each protocol enrolled men and women $\geq$18 years old. Participants for all protocols provided informed written consent prior to enrollment, and all studies were approved by the relevant Institutional Review Boards.

2.2. Whole blood staining and flow cytometry analysis

Blood was collected from volunteers in ACD anticoagulant; 100 μl of whole blood was transferred to Becton Dickinson (BD) Trucount tubes as detailed previously (Hensley et al., 2012) and stained using the 11 color TP1 antibody staining panel, containing fluorescently-labeled monoclonal antibodies specific for the major cellular populations in blood (Table 1). Staining parameters were varied to determine their effect on staining stability as described in Section 2.4. After staining, erythrocytes were lysed, the remaining cells were fixed using BD FACSLyse (BD Bioscience), and variations in the storage and shipping conditions were tested as described in Section 2.4. The Trucount tubes were then analyzed using a BD LSRII flow cytometer with a configuration as previously published (De Rosa et al., 2012), and the number of cells per microliter of whole blood was calculated as previously described (Hensley et al., 2012).

2.3. Flow cytometry gating scheme

On average, approximately 40% of each tube (monitored as the collection of at least 20,000 Trucount beads) was collected and analyzed. The gating strategy to distinguish subpopulations follows, with slight modifications, the previously published strategy (Hensley et al., 2012) (Fig. 1). Briefly, our gating strategy first used the APC and PE-Cy5 channels to draw an inclusion gate around the Trucount beads to gate them for counting, and an exclusion gate to exclude them from subsequent cellular analysis. The cells were then gated using forward scatter height and forward scatter area to include only single cells for further analysis. Subsequently, side scatter (SSC) and CD45 staining were used to separate lymphocytes and monocytes from granulocytes (Fig. 1, ii). Lymphocytes and non-lymphocytes were then divided using SSC and CD14, which included the separation of $CD19^+$ lymphocytes from $CD14^+$ non-lymphocytes that are stained with the same fluorophore. CD14/CD19 negative cells (Fig. 1, iii) were further gated to identify myeloid and plasmacytoid dendritic cells. From the non-lymphocyte population (Fig. 1, iv), non-classical, intermediate, and classical monocytes were delineated and quadrant gates applied to stratify monocyte subsets expressing high levels of the activation markers HLA-DR and CD86. Lastly, SSC low lymphocytes (Fig. 1, v) were further gated to distinguish $CD4^+$ T cells, $CD8^+$ T cells, B cells, $CD56^{bright}$ NK cells, $CD56^{dim}$ NK cells, and $CD56^{negative}$ NK cells. In addition, to obtain neutrophil counts for comparison to complete blood count data from clinical trial samples, the granulocytes were further gated to distinguish $CD16^+$ neutrophils (not shown). Standardized gates were applied to ensure comparability between all

Table 1
Antibody staining panel "Trucount Panel 1" (TP1) to distinguish all major peripheral blood leukocyte populations and level of monocyte activation.

Marker	Fluorophore	Supplier	Clone
CD45	AmCyan	BD Biosciences	2D1
CD3	AlexaFluor700	BD Biosciences	UCHT1
CD8	PerCP-Cy5.5	BD Biosciences	SK1
CD4	FITC	BD Biosciences	RPA-T4
HLA-DR	ECD	Beckman Coulter	Immu-357
CD14	V450	BD Biosciences	MΦP9
CD19	V450	BD Biosciences	HIB19
CD16	APC-H7	BD Pharmingen	3G8
CD56	PE-Cy7	BD Biosciences	NCAM16.2
CD11c	APC	BD Biosciences	B-ly6
CD123	PE	ebioscience	6H6
CD86	PE-Cy5	BD Pharmingen	2331 (FUN-1)