Published in final edited form as:

J Immunol Methods. 2014 September ; 411: 23–36. doi:10.1016/j.jim.2014.06.002.

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

Tiffany Hensley-McBain^a, Antje Heit^a, Stephen C. De Rosa^{a,b}, M. Juliana McElrath^{a,b,c}, and Erica Andersen-Nissen^{a,d,*}

^aVaccine and Infectious Diseases Division, Fred Hutchinson Cancer Research Center, Seattle, WA 98109 USA

^bDepartment of Laboratory Medicine, University of Washington, Seattle, WA 98195 USA

^cDepartment of Medicine, University of Washington, Seattle, WA 98195 USA

^dCape Town HVTN Immunology Laboratory, Hutchinson Center Research Institute of South Africa, Cape Town, South Africa

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 hours of draw to 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.

Keywords

Trucount; multiparameter flow cytometry; clinical trial; experimental medicine trial; assay optimization; phenotyping; enumeration

© 2014 Elsevier B.V. All rights reserved.

*Corresponding Author: Erica Andersen-Nissen, Ph.D., Cape Town HVTN Immunology Laboratory, First Floor, Wembley 3, McKenzie Street, Gardens, Cape Town 8001, +27 (0)21 202 2224, eanderse@hcrisa.org.za.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

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. 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 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; 100µl 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) (Figure 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 (Figure 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 (Figure 1, iii) were further gated to identify myeloid and plasmacytoid dendritic cells. From the non-lymphocyte population (Figure 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 (Figure 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 samples tested. During the analysis of individual clinical trial samples, some adjustment of standardized gates was required to accommodate variation in expression levels in cells and these were applied to all samples collected from that particular participant to ensure validity of longitudinal comparisons.

A fluorescence minus one (FMO) control for each reagent in the staining panel was performed to guide the location of the gates in the gating scheme (Supplementary Figure 1). The gates, with the exception of CD45-AmCyan, showed virtually no events in the gated population of interest in the FMO control. For the CD45-AmCyan, there was no concern about other colors interfering with this channel, but the gate was set lower than dictated by the FMO control. This resulted in 5.4% of the cells in the CD45 positive SSC high gate in the FMO. The reason for setting the CD45 gate lower was due to the issue that the standardized CD45 gate must be drawn quite wide to accommodate significant shifts in CD45 expression between participants.

2.4. Staining and storage test conditions

To ensure accurate staining, we investigated the stability of the prepared antibody staining cocktail TP1 over time. TP1 was prepared once per week during the eight weeks before staining and stored at 4°C in the dark until use. Blood was drawn from a single healthy volunteer, stained in triplicate with each prepared cocktail, and then immediately collected on the flow cytometer for analysis.

To determine the effect of varying the amount of time from venipuncture to staining on the maintenance of consistent cell concentrations and activation frequencies, blood was drawn from 3 healthy volunteers and stained in triplicate with TP1 at 2, 4, 6, 8 and 24 hours after blood collection. Blood remained in the ACD collection tube at room temperature between collection and staining, and the collection tube was inverted several times to mix immediately prior to removal for staining at each time point.

To enable testing of stained Trucount samples at a central laboratory located hours away from the collection site, we tested the effect of shipping stained samples at 4°C and –80°C. Fresh whole blood from a healthy volunteer was stained and fixed in sextuplicate with TP1 within 4 hours of venipuncture; three samples were shipped overnight at 4°C or –80°C from Seattle, WA to Birmingham, AL. Once received at the Birmingham lab, the cold packs (4°C) or dry ice (–80°C) were replaced and the samples were shipped overnight back to Seattle for analysis. For comparison, the other three samples remained at the Seattle lab location and were stored at either 4°C or –80°C. All samples (shipped and stored) were evaluated for expression of these markers by flow cytometry immediately upon return of the shipped samples to the Seattle lab location (two days after staining).

Tests were also done to determine the effect of storage temperature and storage duration on previously stained samples. Preliminary experiments showed that if TP1-stained samples were stored longer than 13 days at 4°C, multicolor flow cytometry analysis was not possible due to increased autofluorescence in some channels, with reduced separation between positive and negative populations resulting in insufficient gating of different populations. To

directly compare the impact of storage at 4°C or –80°C on the maintenance of staining quality, we stained fresh whole blood with TP1 in triplicate from 3 volunteers and analyzed the samples after storage at 4°C for 3, 6, or 13 days; or at –80°C for 3, 6, 13, 23, 30, or 120 days.

Lastly, we performed technician proficiency certification tests to determine how concentration data varied between samples stained by different technicians. A single blood draw was stained and erythrocytes lysed and fixed with TP1 in duplicate by five processing laboratory technicians at Columbia University, frozen at –80°C overnight, and shipped the following day on dry ice to the HVTN laboratory in Seattle for analysis. Samples were thawed upon arrival and analyzed (two days after staining).

2.5. Complete blood counts (CBC)

Blood was collected in EDTA anticoagulant and shipped to a local hematology laboratory for a complete blood count with differential at the same visits for which the TP1 staining was performed.

2.6. Statistical analyses

Blood drawn from 1–3 participants was stained in duplicate or triplicate with TP1 for each condition and/or timepoint tested. Using GraphPad Prism software, unpaired T tests were performed to analyze changes in cell concentrations, frequency of the parent population, and/or median fluorescence intensity (MFI). The following 13 populations were examined for changes in the cell/μl concentrations for each condition and/or timepoint tested: granulocytes, CD3+ lymphocytes, CD4+ T cells, CD8+ T cells, B cells, CD56 bright NK cells, CD56 dim NK cells, CD56 negative NK cells, non-classical monocytes, intermediate monocytes, classical monocytes, myeloid dendritic cells (mDC), and plasmacytoid dendritic cells (pDC). In addition, monocyte activation marker expression was examined as the frequency of each monocyte population (non-classical, intermediate, and classical) expressing high levels of both HLA-DR and CD86 (referred to as the frequency of parent). Due to the limited sample size for these experiments and large number of conditions or timepoints examined for each procedural variation, detecting significant differences after adjusting for multiple comparisons was difficult. Any significant differences that were detected were sporadic and did not represent conclusive trends. The exception to this was the shipping comparison test, which had limited conditions and therefore a smaller adjustment for multiple comparisons. For these reasons, with the exception of the shipping test, we report observed trends rather than statistically significant differences.

GraphPad Prism software was also used to calculate the coefficient of variation (CV) for different cell population concentrations between technicians and to determine the Spearman rank correlation coefficient between cell concentrations obtained by CBC or using the TP1 panel assay.

3. Results and discussion

3.1. The effect of staining, shipping, and storage procedure variations on the enumeration of major leukocyte populations

3.1.1. TP1 antibody staining cocktail can be stored at 4°C for at least 8 weeks before use without a detrimental effect on standardized gating or cell enumeration

In a clinical trial setting with multiple participating laboratories that perform uniform whole blood staining, a central laboratory that coordinates preparation and shipment of a standardized antibody staining cocktail can be crucial to maintain reagent consistency. The ability to centrally prepare these staining cocktails in bulk and store them until use would reduce the workload for individual laboratories as well as limit the variability that may be introduced by minor pipetting errors. To examine the feasibility of a common bulk TP1 antibody staining cocktail, we first tested the effects of increasing storage time at 4°C prior to use. Whole blood from a single blood draw was stained in triplicate with TP1 either made fresh (week 0) or stored at 4°C for 1–8 weeks prior to staining. Immediately after staining, samples were analyzed by multicolor flow cytometry. Figure 2A shows representative dot plots for CD45+ leukocytes and CD3+ T cells, CD19/HLA-DR+ B cells and CD56+ NK cells over time and indicates that staining remains comparable when using bulk antibody staining panels stored for various amounts of time. Similar patterns were observed in samples from our clinical trials using more than 20 volunteers at multiple timepoints (data not shown) and suggest that standardized gates may be used to identify populations stained with a centrally prepared and distributed, bulk multicolor antibody staining cocktail that has been stored for up to 8 weeks before use.

We then wanted to understand the impact of extended TP1 storage on cell concentrations and monocyte activation frequencies. Overall trends suggested only small variations occurred in the calculated concentrations of these cell types as TP1 was stored over time (Figure 2B). Consistency in the cell concentrations over time was observed for abundant cell populations such as CD3+ lymphocytes, as well as for more rare populations such as mDCs and CD56 bright NK cells (Figure 2B). In addition to examining markers that distinguish cell lineages, we also examined the more sensitive activation markers CD86 and HLA-DR. Figure 2C shows that the percentage of monocytes expressing high levels of CD86 and HLA-DR also remained unaffected for the three monocyte subpopulations distinguished by their expression of CD14 and CD16. In addition, the median fluorescence intensities of fluorophores in TP1 remained sufficiently stable over time (Supplementary Table 1) to enable the application of standardized gates to the samples when fluorescence was plotted on a log scale. It should be noted, however, that some variation in median fluorescence intensity was observed and therefore any standardized gates applied should be checked for accuracy across samples.

3.1.2. Increasing the time elapsed from blood draw to staining affects enumeration of some cell populations as well as the frequency of activated monocyte subsets

Increasing the period of time between blood draw and leukocyte isolation has been reported to affect cell recovery, viability, and functionality (Gulati et al., 2002; Bull et al., 2007). In a multicenter clinical trial setting this time can be especially

variable, since multiple staff members are involved in blood draw, handling, transport and processing activities. In addition, the processing laboratory may be located several hours away from the clinic where the venipuncture takes place. We therefore investigated the stability of cell counts and activation phenotypes from blood stored at room temperature before staining for 2 to 24 hours post-blood draw. We found that the concentrations of most cell populations measured showed stable trends following storage of up to 24 hours. Exceptions to this included myeloid dendritic cells (Figure 3A) and CD56 negative NK cells (not shown), which each showed a trend towards increased concentrations measured between 8 and 24 hours after blood draw. In addition, nonclassical (not shown) and intermediate monocyte counts began to vary as little as 4 hours after collection in some volunteers, and classical monocytes showed a trend towards a slight decrease in counts between 8 and 24 hours after collection (Figure 3A). Alterations in cell concentrations over time are likely related to cell death, changes in the adhesion of cells to the collection tube, and changes in expression of cell surface markers that result in misclassification of cell types (Gulati et al., 2002). The proportion of classical monocytes expressing high levels of CD86 and HLA-DR increased markedly between 8 and 24 hours, and variability in the levels of activation marker expression was observed in all three monocyte subsets beginning as early as 4 hours after collection (Figure 3B). Taken together, these data indicate that concentrations and activation frequencies of innate cell subsets such as monocytes and dendritic cells are particularly sensitive to increased time between blood draw and staining using this method.

3.1.3. Transport of stained blood specimens at -80°C is superior to 4°C to maintain cell counts—Standardized acquisition parameters are vital for collection of consistent clinical trial data, yet standardization across different flow cytometers and laboratories presents many obstacles that can be difficult to overcome (Maecker et al., 2010; Perfetto et al., 2012). One way to circumvent these hurdles is to ship previously stained samples to a central laboratory for acquisition and analysis. In multicenter clinical trials where samples must be shipped, it is important to take careful consideration of potential shipping conditions. We therefore compared a previously described packaging method (Hensley et al., 2012) that ensures shipping of stained Trucount tubes at -80°C to shipment of samples at 4°C . We stained samples from a single blood draw with TP1 and then either stored or shipped the fixed samples at 4°C or -80°C , as described in the methods. Immediately upon receipt of the shipped samples (two days after staining), all shipped and stored samples were analyzed on the multicolor flow cytometer. We found significantly lower cell counts for 11 of the 12 cell populations examined in stained samples that were shipped at 4°C compared to samples stored at 4°C , whereas shipping at -80°C preserved all cell counts (Table 2). Interestingly, these differences were not observed for the percentage of CD86/HLA-DR high monocytes. The reduction in cell counts observed when shipped at 4°C and concomitant maintenance of activation percentages suggests that shipping in liquid form causes agitation of the sample and cell lysis. If the reduction in cell concentrations had instead been due to antibody dissociation, activation marker staining would also have been lower and resulting activation frequencies would have been reduced. Taken together, freezing the samples after fixation and shipping on dry ice mitigates the observed decreases in cell concentration and is optimal for use in multicenter clinical trials.

3.1.4. Storage of TP1-stained, erythrocyte-lysed whole blood at –80°C preserves staining and cell concentrations obtained for up to 120 days, but has a detrimental effect on activation marker frequencies after 13 days—After shipping of stained specimens to the central laboratory, specimens need to be stored and analyzed on the flow cytometer in batches for optimal laboratory workflow. Previous studies have determined that freezing whole blood at –80°C prior to staining has little effect on lymphocyte counts (Fiebig et al., 1997), but the effect of long term storage at –80°C or 4°C after staining on all of the major leukocyte populations has not been systematically assessed. To determine the maximal length of time specimens could be stored after staining, we stained and stored blood at either 4°C or –80°C for up to 120 days before flow cytometry analysis.

In a first step, we determined whether standardized common gates could be applied to the flow cytometry data after specimen storage. We observed reduced separation between positive and negative populations in samples stored for as little as 6 days at 4°C for the V450-conjugated antibodies (Figure 4A, CD19 V450 shown), as well as for CD45 Am Cyan staining (not shown). In contrast, staining integrity remained consistent for up to 120 days (with only a slight shift in the V450 staining at day 120) when the samples were stored at –80°C (Figure 4A).

We then examined changes in the cell population concentrations with increasing storage time at the two temperatures. Granulocyte, CD3+ lymphocyte, CD8+ T cell, CD56 dim and CD56 bright NK cell, and classical monocyte concentrations remained consistent after storage for 13 days at 4°C and 120 days at –80°C (Figure 4B). In addition, CD56 negative NK cells showed no change for the duration of storage at either temperature (not shown). Concentrations of B cell, mDC, pDC, and non-classical monocyte populations, however, decreased while intermediate monocyte concentrations increased between 6 and 13 days of storage at 4°C (Figure 4B; pDC and non-classical monocytes not shown). These changes in cell concentrations were due to reduced separation between the positive and negative populations in the V450 channel, which affected the accuracy with which populations could be gated as early as the lymphocyte gate (Figure 1A) and compromised subsequent downstream separation, such as the further gating of CD19 positive and negative cells (Figure 4A). In addition, the increase in fluorescence of CD14 negative cells in the V450 channel led to a shift in cells from the non-classical monocyte gate to the intermediate monocyte gate, likely causing the resulting concentration changes (Supplementary Figure 2). Storage at –80°C maintained these cell concentrations overall, with slight decreases observed only in the mDC population beginning at Day 23 (Figure 4C). Many cell types showed a trend of declining concentrations after being stored between 30 days and 120 days at –80°C, but additional longitudinal data would be required to confirm this trend.

We also examined the effect of storage on the activation markers CD86 and HLA-DR. All monocyte populations showed a decreased frequency of cells expressing high levels of CD86 and HLA-DR beginning as early as 13 days after storage at both 4°C and –80°C (Figure 4C). This could be due to unstable expression of these surface markers; alternatively, tandem dyes have been shown to be less stable than other fluorophores (Hulspas et al., 2009; Le Roy et al., 2009), which could cause the fading fluorescence

observed for both HLA-DR ECD and CD86 PE Cy5 (Supplementary Figure 2B). It should be noted that selective effects on the concentrations of some cell populations suggest that this was not a result of Trucount bead loss or aggregation. In addition there was no adverse effect of long-term storage at either temperature on the Trucount bead fluorescence (not shown).

The resulting data from the optimization experiments suggest that the effects of procedural variations on observed cell concentrations vary by cell type and in some cases are specific to the antibodies used in the panel, as well as to the storage and shipping conditions applied. Table 3 summarizes these findings for the TP1 assay and provides a recommendation for implementing the procedure in a multicenter clinical trial setting based on the results. It is important to note that concentration decreases observed between 23 and 120 days when samples were stored at -80°C cannot be attributed specifically to the cell marker or the fluorophore, so any changes to the antibodies used in the staining panel should be similarly tested to ensure long term staining stability.

3.2. The optimized TP1 assay yields consistent data between technicians in a multicenter clinical trial setting

An additional concern for implementation of this assay in a multicenter clinical trial setting is the comparability between data from samples stained by different laboratory personnel. To address this issue, we analyzed assay proficiency data from one of our trained leukocyte processing laboratories to determine the variability between samples stained by different technicians. Five technicians at a distant site (Columbia University, New York City) stained samples in duplicate from a single blood draw and then stored and shipped them to Seattle using the optimized procedure described above. The coefficient of variation (CV) between the mean concentrations of samples stained by all five technicians was under 25% for twelve of the thirteen cell populations examined (Figure 5A). With a CV of 46.5%, the CD56 negative NK cell population was the only cell subset that showed variation above our acceptable threshold of 30%, but gating of this very rare population away from the relatively abundant CD56 dim population can be variable and is a likely explanation. In addition, the activation frequencies for all three monocyte subsets showed low variation, with CVs well below the 30% threshold (Figure 5B). Overall, these data show that the assay provides cell concentration data and monocyte activation frequencies that are comparable between samples stained by different laboratory personnel when the optimized staining, storage, and shipping procedures are used.

3.3. Cell concentration data obtained using the TP1 assay highly correlates with CBC data obtained from participants in a multicenter HIV vaccine clinical trial

The Trucount cell enumeration method has previously been compared with automated hematology methods in controlled experiments (Nicholson et al., 1997; Vuckovic et al., 2004), but comparability of data obtained using the two methods in a multicenter clinical trial setting has not been documented. We adopted the optimized TP1 assay conditions from the tests outlined above and performed whole blood staining and flow cytometry analysis for an HIV vaccine phase I clinical trial. We then compared cell concentrations obtained using the TP1 assay with CBC data obtained at the same visits for five blood draw visits (one pre-

vaccination and four post-vaccination) for 20 participants. Spearman correlations for concentrations of neutrophils, monocytes and lymphocytes showed the data was highly correlated between the two assays and the slopes of the regression lines are also shown (Figure 6). Interestingly, in contrast with neutrophil and lymphocyte measurements where the CBC measured slightly higher counts than the TP1 assay (Figure 6, respective slopes of 0.891 and 0.884), the monocyte concentrations showed the opposite trend with lower numbers measured by CBC compared to TP1 (Figure 6, slope 1.19). This monocyte phenomenon can be explained by the fact that there is a window of up to 24 hours in which the CBC assay can be performed after blood draw in our clinical trials. As we show in Figure 3A, the overall concentration of classical monocytes (the predominant monocyte population in the blood) declines over time after blood draw, possibly due to adherence of these cells to the surface of the blood tube. Together, the high level of correlation between TP1 assay and CBC counts implies that the staining, shipping, and storage conditions that we have implemented based on the optimization experiments maintain the accuracy of the data and that utilizing our method in a multicenter clinical trial setting provides reliable cell concentrations. Although the TP1 assay method is more expensive and time consuming than CBC, it produces data on subpopulations of leukocytes beyond those normally distinguished by CBC. Table 4 shows the median and ranges of baseline cell concentration and activated monocyte frequencies obtained from this study.

4. Conclusions

In this study, we define the whole blood phenotyping panel assay (TP1 assay) as a valid tool for use in multicenter experimental medicine trial settings to generate accurate and consistent profiles of multiple cellular subsets in the peripheral blood. The TP1 assay can be used to provide a more complete view of the *in vivo* state of the immune system as compared with use of data from CBCs. We identify blood draw, staining, shipping and storage parameters that provide the most consistent data for each leukocyte population of interest. Our data indicate that modifications in the antibody composition of the staining panel require careful testing to guarantee the generation of valid data. Taken together, use of the TP1 assay has the potential to implicate key players in the immune response that would be missed by only analyzing changes in major leukocyte populations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Research reported in this publication was supported by the Bill and Melinda Gates Foundation Collaboration for AIDS Vaccine Discovery Grant 38645 (to M.J.M.) and by the National Institute of Allergy and Infectious Disease of the National Institutes of Health under award number UM1AI068618. The content is solely the responsibility of the authors and does not necessarily represent the official views of the Bill and Melinda Gates Foundation or the National Institutes of Health.

The authors would like to thank Jessica Jones, Sarah Johnson, Erica Clark, and Terri Stewart. In addition, we thank Stephen Voght for critical reading of the manuscript. We thank the James B. Pendleton Charitable Trust for their generous equipment donation.

Abbreviations

CBC	complete blood count
NK cell	natural killer cell
mDC	myeloid dendritic cells
pDC	plasmacytoid dendritic cells
TP1	Trucount panel 1

References

- Andersen-Nissen E, Heit A, McElrath MJ. Profiling immunity to HIV vaccines with systems biology. *Curr Opin HIV AIDS*. 2012; 7:32–37. [PubMed: 22134340]
- Bull M, Lee D, Stucky J, Chiu YL, Rubin A, Horton H, McElrath MJ. Defining blood processing parameters for optimal detection of cryopreserved antigen-specific responses for HIV vaccine trials. *J Immunol Methods*. 2007; 322:57–69. [PubMed: 17382342]
- De Rosa SC, Carter DK, McElrath MJ. OMIP-014: validated multifunctional characterization of antigen-specific human T cells by intracellular cytokine staining. *Cytometry A*. 2012; 81:1019–1021. [PubMed: 23081852]
- Fiebig EW, Johnson DK, Hirschhorn DF, Knappe CC, Webster HK, Lowder J, Busch MP. Lymphocyte subset analysis on frozen whole blood. *Cytometry*. 1997; 29:340–350. [PubMed: 9415417]
- Gulati GL, Hyland LJ, Kocher W, Schwarting R. Changes in automated complete blood cell count and differential leukocyte count results induced by storage of blood at room temperature. *Arch Pathol Lab Med*. 2002; 126:336–342. [PubMed: 11860310]
- Haynes BF, Gilbert PB, McElrath MJ, Zolla-Pazner S, Tomaras GD, Alam SM, Evans DT, Montefiori DC, Karnasuta C, Sutthent R, Liao HX, DeVico AL, Lewis GK, Williams C, Pinter A, Fong Y, Janes H, DeCamp A, Huang Y, Rao M, Billings E, Karasavvas N, Robb ML, Ngauy V, de Souza MS, Paris R, Ferrari G, Bailer RT, Soderberg KA, Andrews C, Berman PW, Frahm N, De Rosa SC, Alpert MD, Yates NL, Shen X, Koup RA, Pitisuttithum P, Kaewkungwal J, Nitayaphan S, Rerks-Ngarm S, Michael NL, Kim JH. Immune-correlates analysis of an HIV-1 vaccine efficacy trial. *N Engl J Med*. 2012; 366:1275–1286. [PubMed: 22475592]
- Hensley TR, Easter AB, Gerdts SE, De Rosa SC, Heit A, McElrath MJ, Andersen-Nissen E. Enumeration of major peripheral blood leukocyte populations for multicenter clinical trials using a whole blood phenotyping assay. *J Vis Exp*. 2012:e4302. [PubMed: 23007739]
- Hulspas R, Dombkowski D, Preffer F, Douglas D, Kildew-Shah B, Gilbert J. Flow cytometry and the stability of phycoerythrin-tandem dye conjugates. *Cytometry A*. 2009; 75:966–972. [PubMed: 19777547]
- Le Roy C, Varin-Blank N, Ajchenbaum-Cymbalista F, Letestu R. Flow cytometry APC-tandem dyes are degraded through a cell-dependent mechanism. *Cytometry A*. 2009; 75:882–890. [PubMed: 19739089]
- Maecker HT, McCoy JP Jr, Consortium FHI, Amos M, Elliott J, Gaigalas A, Wang L, Aranda R, Banchereau J, Boshoff C, Braun J, Korin Y, Reed E, Cho J, Hafler D, Davis M, Fathman CG, Robinson W, Denny T, Weinhold K, Desai B, Diamond B, Gregersen P, Di Meglio P, Nestle FO, Peakman M, Villanova F, Ferbas J, Field E, Kantor A, Kawabata T, Komocsar W, Lotze M, Nepom J, Ochs H, O'Lone R, Phippard D, Plevy S, Rich S, Roederer M, Rotrosen D, Yeh JH. A model for harmonizing flow cytometry in clinical trials. *Nat Immunol*. 2010; 11:975–978. [PubMed: 20959798]
- Nicholson JK, Stein D, Mui T, Mack R, Hubbard M, Denny T. Evaluation of a method for counting absolute numbers of cells with a flow cytometer. *Clin Diagn Lab Immunol*. 1997; 4:309–313. [PubMed: 9144369]

- Perfetto SP, Ambrozak D, Nguyen R, Chattopadhyay PK, Roederer M. Quality assurance for polychromatic flow cytometry using a suite of calibration beads. *Nat Protoc.* 2012; 7:2067–2079. [PubMed: 23138348]
- Rerks-Ngarm S, Pitisuttithum P, Nitayaphan S, Kaewkungwal J, Chiu J, Paris R, Prensri N, Namwat C, de Souza M, Adams E, Benenson M, Gurunathan S, Tartaglia J, McNeil JG, Francis DP, Stablein D, Birx DL, Chunsuttiwat S, Khamboonruang C, Thongcharoen P, Robb ML, Michael NL, Kunasol P, Kim JH, Investigators M-T. Vaccination with ALVAC and AIDSVAX to prevent HIV-1 infection in Thailand. *N Engl J Med.* 2009; 361:2209–2220. [PubMed: 19843557]
- Scriba TJ, Tameris M, Mansoor N, Smit E, van der Merwe L, Isaacs F, Keyser A, Moyo S, Brittain N, Lawrie A, Gelderbloem S, Veldsman A, Hatherill M, Hawkrige A, Hill AV, Hussey GD, Mahomed H, McShane H, Hanekom WA. Modified vaccinia Ankara-expressing Ag85A, a novel tuberculosis vaccine, is safe in adolescents and children, and induces polyfunctional CD4+ T cells. *Eur J Immunol.* 2010; 40:279–290. [PubMed: 20017188]
- Vuckovic S, Gardiner D, Field K, Chapman GV, Khalil D, Gill D, Marlton P, Taylor K, Wright S, Pinzon-Charry A, Pyke CM, Rodwell R, Hockey RL, Gleeson M, Tepes S, True D, Cotterill A, Hart DN. Monitoring dendritic cells in clinical practice using a new whole blood single-platform TruCOUNT assay. *J Immunol Methods.* 2004; 284:73–87. [PubMed: 14736418]
- Zak DE, Andersen-Nissen E, Peterson ER, Sato A, Hamilton MK, Borgerding J, Krishnamurthy AT, Chang JT, Adams DJ, Hensley TR, Salter AI, Morgan CA, Duerr AC, De Rosa SC, Aderem A, McElrath MJ. Merck Ad5/HIV induces broad innate immune activation that predicts CD8(+) T-cell responses but is attenuated by preexisting Ad5 immunity. *Proc Natl Acad Sci U S A.* 2012; 109:E3503–E3512. [PubMed: 23151505]

Highlights

- We develop one antibody staining panel that enumerates all major blood leukocytes.
- We define assay variables relevant to clinical trials that affect cell counts.
- Some assay variables have a greater effect on certain cell populations than others.
- Data from the staining panel correlates highly with complete blood count data.
- The panel can be used in experimental medicine trials to profile immune responses.

Figure 1. Example of gating to distinguish major leukocyte populations as shown on blood from a healthy volunteer drawn into ACD anticoagulant and stained with the 11 color antibody staining panel (Table 1)

Trucount beads (i) are gated and excluded from cells. Granulocytes (ii) are delineated based on side scatter and lymphocytes and monocytes are divided into 3 populations based on SSC and CD14/CD19 expression: CD14/CD19 negative cells (iii), high SSC non-lymphocytes (iv), and low SSC lymphocytes (v). CD14/CD19 negative cells are gated to distinguish myeloid (vi) and plasmacytoid (vii) dendritic cells by gating on CD3, CD56, and CD16 negative and HLA-DR positive cells. Non-classical (viii), intermediate (ix), and classical (x)

monocytes are distinguished from the non-lymphocyte population after excluding cells expressing CD56 and CD3. The frequency of each monocyte subset with high expression of the activation markers CD86 and HLA-DR is then determined. SSC low lymphocytes (v) are then gated to distinguish CD4+ T cells (xi), CD8+ T cells (xii), B cells (xiii), CD56 bright NK cells (xvi), CD56 dim NK cells (xv), and CD56 negative NK cells (xvi). Note: Black ovals show that downstream gates appropriately exclude potential contaminating neutrophils.

Figure 2. Extended storage of TP1 at 4°C has little impact on the quality of staining
Whole blood from a single blood draw was stained in triplicate with TP1 made fresh (week 0) or stored at 4°C for 1 – 8 weeks prior to staining. **A.** Example flow cytometry plots with standardized gates from the common gating strategy (see Figure 1) showing consistency of staining using TP1 prepared either fresh or stored for 4 or 8 weeks. **B.** Representative cell populations show consistency in cell concentrations obtained using TP1 stored for 1–8 weeks versus TP1 made fresh (week 0). Concentrations of pDC and non-classical monocyte populations were also stable (data not shown). CD56 negative NK cell concentrations were

not included in this analysis due to their very low frequency in the participant tested, causing an inability to reliably gate the events. **C.** The percentage of monocyte populations expressing high levels of the activation markers CD86 and HLA-DR remain consistent when using TP1 stored for up to 8 weeks before staining.

Figure 3. Variations in the concentration of individual cell populations and the frequencies of activated monocyte subsets in whole ACD blood over time prior to staining

Blood drawn from three healthy donors into ACD anticoagulant was stained with TP1 in triplicate after leaving the blood in the collection tube at room temperature for 2, 4, 6, 8 or 24 hours post-collection. Different symbols represent 3 different donors stained in triplicate; lines connect the means for each donor. **A.** The concentrations of granulocytes, CD3+ lymphocytes, CD8+ T cells, B cells, and CD56 bright and dim NK cells showed little variation when the blood remained in the collection tube for up to 24 hours prior to staining,

but myeloid dendritic cell concentrations and CD56 negative NK cells tended to increase at 24 hours post-collection and monocyte subsets showed some variability over time. **B.** The frequency of monocyte subsets expressing high levels of activation markers CD86 and HLA-DR began to vary as little as 4 hours after collection, with a marked increase observed in the classical monocytes between 8 and 24 hours.

Figure 4. Storage of stained whole blood at -80°C preserves staining and cell concentrations obtained for most subsets for up to 120 days, yet has a detrimental effect on monocyte activation marker frequencies after 13 days

Blood drawn into ACD anticoagulant from three healthy donors was stained in triplicate with TP1 and stored for various numbers of days at -80°C or 4°C before analysis. Previous experiments showed gating of stained samples was not possible beyond 13 days at 4°C . **A.** Reduced separation between positive and negative populations occurred in samples stored for as little as 6 days at 4°C for the V450 conjugated antibodies (CD19 V450 shown) as well as for CD45 Am Cyan staining (not shown). TP1 staining integrity remained for up to

120 days, with a slight shift in the V450 staining at day 120, when the samples were stored at -80°C . Red gates show common gating based on Day 3 staining that were used to obtain cell concentrations. Dashed grey gates show common gates based on Day 3 staining that had to be moved (adjusted gates not shown) in order to obtain accurate concentrations. Note: CD14 is also stained by a V450 conjugated antibody in this panel, but CD14 positive cells are excluded before this point in the gating scheme so this staining represents CD19 only. **B.** Granulocyte, CD3+ lymphocyte, CD8+ T cell, CD56 dim and CD56 bright NK cell, and classical monocyte concentrations also remained constant after storage at 13 days at 4°C and 120 days at -80°C . In addition, CD56 negative NK cells showed no change for the duration of storage at both temperatures (not shown). Marked changes in the concentrations of mDC, pDC (not shown), B cell, and intermediate monocyte, and non-classical monocyte (not shown) concentrations were observed after storing the samples for 13 days at 4°C . Storage at -80°C maintains these cell concentrations, with slight decreases observed only in the mDC and pDC (not shown) populations beginning at day 23. **C.** All monocyte populations show a decreased frequency of cells expressing high levels of the activation markers CD86 and HLA-DR beginning as early as 13 days after being stored at 4°C or -80°C .

Figure 5. Data from samples stained by different trained laboratory technicians is comparable with low variation for most leukocyte populations

Blood drawn into ACD anticoagulant from a single healthy donor was stained with TP1 in duplicate by five different technicians to assess variation between samples stained by different trained laboratory personnel. **A.** Graphs of representative cell populations showing the low variation between cell concentration data obtained from samples stained by different technicians. pDC, CD56 negative NK cell, and non-classical monocyte concentrations had slightly higher variation with CVs of 23.3%, 46.5%, and 28.0%, respectively (not shown).

B. The frequency of monocyte subsets expressing high levels of CD86 and HLA-DR show relatively low variation between samples stained by different technicians.

Figure 6. Clinical trial data shows that cell concentrations obtained using the TP1 assay highly correlates with the data obtained by Complete Blood Count

Five blood-draw visits (one pre-vaccination and four post-vaccination) for 20 participants were analyzed using the TP1 assay and Complete Blood Count. Neutrophils distinguished by flow cytometry phenotyping refer to cells with high side scatter and high CD16 expression. Monocytes were defined as cells negative for CD3, CD19 and CD56, and positive for CD14 with intermediate side scatter. Lymphocytes refer to all CD14 negative cells with low side scatter.

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)

Table 2

Comparison of cell concentrations and activated monocyte frequencies of samples shipped and stored at 4°C and -80°C for two days.^a

Cell Type	Parameter	4° Stored	4°C Shipped	-80°C Stored	-80°C Shipped
Granulocytes	cells/μl whole blood	3958	2730	4192	4230
CD3+ lymphocytes	cells/μl whole blood	1196	932	1181	1177
CD4+ T cells	cells/μl whole blood	812	634	805	801
CD8+ T cells	cells/μl whole blood	327	251	323	322
CD56 ^{high} NK cells	cells/μl whole blood	11.8	9.1	11.2	11.4
CD56 ^{dim} NK cells	cells/μl whole blood	142	112	132	133
B cells	cells/μl whole blood	249	186	245	245
mDC	cells/μl whole blood	6.1	4.2	5.8	6.7
pDC	cells/μl whole blood	7.4	5.7	5.1	8.1
Classical monocytes	cells/μl whole blood	271	176	277	288
Intermediate monocytes	cells/μl whole blood	12.5	7.5	14.4	14.5
Non-classical monocytes	cells/μl whole blood	11.7	6.7	9.6	10.4
CD86 ^{high} HLA-DR ^{high} classical monocytes	Frequency of parent	1.39	1.42	1.18	1.4
CD86 ^{high} HLA-DR ^{high} intermediate monocytes	Frequency of parent	46.7	49.6	44.7	46.8
CD86 ^{high} HLA-DR ^{high} non-classical monocytes	Frequency of parent	44.6	46.8	48.3	47

^aThe table reports the means of samples stained in triplicate for each condition (one donor blood draw, representative of data seen for 3 individuals). Shaded cells indicate a significant difference between the data from stored and shipped samples for the given temperature based on unpaired t-tests and using a p-value<0.025 (Bonferroni-corrected for multiple comparisons).

Table 3

Summary of findings for each leukocyte population examined for each condition tested and subsequent recommendations for implementing the TP1 assay in a multicenter clinical trial.

Cell Type	Parameter	Antibody Staining Panel Storage at 4°C (0–8 weeks)	Time between blood draw and staining (2–24 hours)	Stained samples shipped at 4°C or –80°C (compared to 2 day storage at same temperature)	Storage of stained samples at 4°C or –80°C (3–120 days) ^b
Granulocytes	Cells/μl whole blood	No Effect	No Effect	4°C causes decrease; No effect at –80°C	No Effect
CD3+ lymphocytes	Cells/μl whole blood	No Effect	No Effect	4°C causes decrease; No effect at –80°C	No Effect
CD4+ T cells	Cells/μl whole blood	No Effect	No Effect	4°C causes decrease; No effect at –80°C	No Effect
CD8+ T cells	Cells/μl whole blood	No Effect	No Effect	4°C causes decrease; No effect at –80°C	No Effect
B cells	Cells/μl whole blood	No Effect	No Effect	4°C causes decrease; No effect at –80°C	Decrease at 13 days when stored at 4°C ; No effect at –80°C
mDC	Cells/μl whole blood	No Effect	Increase at 24 hours	4°C causes decrease; No effect at –80°C	Decrease at 13 days when stored at 4°C ; Slight decrease beginning at 23 days at –80°C
pDC	Cells/μl whole blood	No Effect	No Effect	4°C causes decrease; No effect at –80°C	Decrease at 13 days when stored at 4°C ; Slight decrease beginning at 23 days at –80°C
CD56 ^{bright} NK cells	Cells/μl whole blood	No Effect	No Effect	4°C causes decrease; No effect at –80°C	No Effect
CD56 ^{dim} NK cells	Cells/μl whole blood	No Effect	No Effect	4°C causes decrease; No effect at –80°C	No Effect
CD56-NK cells	Cells/μl whole blood	---	Increase at 24 hours	4°C causes decrease; No effect at –80°C	No Effect
Classical monocytes	Cells/μl whole blood	No Effect	Decrease at 24 hours	4°C causes decrease; No effect at –80°C	No Effect
Intermediate monocytes	Cells/μl whole blood	No Effect	Vary beginning at 4 hours	4°C causes decrease; No effect at –80°C	Increase at 13 days when stored at 4°C; No effect at –80°C
Non-classical monocytes	Cells/μl whole blood	No Effect	Vary beginning at 4 hours	4°C causes decrease; No effect at –80°C	No Effect
CD86 ^{high} HLA-DR ^{high} classical monocytes	Frequency of Parent	No Effect	Increase at 24 hours	No effect at either temperature	Increase at 13 days when stored at 4°C ; Decrease beginning at 13 days at –80°C

Cell Type	Parameter	Antibody Staining Panel Storage at 4°C (0–8 weeks)	Time between blood draw and staining (2–24 hours)	Stained samples shipped at 4°C or –80°C (compared to 2 day storage at same temperature)	Storage of stained samples at 4°C or –80°C (3–120 days) ^b
CD86 ^{high} HLA-DR ^{high} intermediate monocytes	Frequency of Parent	No Effect	Vary beginning at 4 hours	No effect at either temperature	Decrease beginning at 13 days for both temperatures
CD86 ^{high} HLA-DR ^{high} non-classical monocytes	Frequency of Parent	No Effect	Vary beginning at 4 hours	No effect at either temperature	Decrease beginning at 13 days for both temperatures
Protocol Recommendation		Prepare antibody staining panel in large batches and store for up to 8 weeks at 4°C.	Stain blood within 4 hours of blood draw if interested in monocyte populations and 8 hours for consistent data on DC or NK cell populations.	Ship samples overnight on dry ice at –80°C.	Store samples at –80°C and collect them on the flow cytometer within 2 weeks of staining if interested in DC concentrations or monocyte activation. A reduction in the CD86 PE Cy5 MFI indicates that decreased activation percentages are likely due to loss of CD86 PE Cy5 staining.

^a CD56–NK cell counts were not able to be reliably gated due to very low frequency in the participant tested in this experiment.

^b Samples were only stored at 4°C up to 13 days because preliminary testing showed that gating was not possible past this point due to increased autofluorescence and reduced separation in some channels.

Table 4

Median and ranges of baseline cell concentration and activated monocyte frequencies obtained from a multicenter clinical trial (n=20).

Cell Type	Parameter	Median	Range	Interquartile Range
Granulocytes	Cells/ μ l whole blood	3653	1907–6299	2655–4294.5
CD3+ lymphocytes	Cells/ μ l whole blood	1269	694–1746	931–1502.5
CD4+ T cells	Cells/ μ l whole blood	821	416–1241	580–982.5
CD8+ T cells	Cells/ μ l whole blood	326	188–671	267.5–395
B cells	Cells/ μ l whole blood	229	81.7–522	167–284.5
mDC	Cells/ μ l whole blood	10.85	6.79–19.1	8.86–16.125
pDC	Cells/ μ l whole blood	7.43	2.88–16.1	5.6325–8.7475
CD56 ^{bright} NK cells	Cells/ μ l whole blood	9.72	4.67–17.9	7.9175–12.65
CD56 ^{dim} NK cells	Cells/ μ l whole blood	103	24.5–245	77.4–172.75
CD56-NK cells	Cells/ μ l whole blood	7.08	2.05–32.9	5.2–10.875
Classical monocytes	Cells/ μ l whole blood	330	147–579	250.5–409.5
Intermediate monocytes	Cells/ μ l whole blood	13.05	3.91–36.4	8.14–18.85
Non-classical monocytes	Cells/ μ l whole blood	9.65	4.06–18.6	7.49–11.475
CD86 ^{high} HLA-DR ^{high} classical monocytes	Frequency of Parent	0.996	0.0445–6.85	0.523–2.46
CD86 ^{high} HLA-DR ^{high} intermediate monocytes	Frequency of Parent	29.9	3.17–59.6	21.3–44.65
CD86 ^{high} HLA-DR ^{high} non-classical monocytes	Frequency of Parent	40.4	5.11–77	23.8–62.3