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Properties of Human Blood Monocytes I. CD91 expression and log orthogonal light scatter provide a robust method to identify monocytes that is more accurate than CD14 expression

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Abstract

Background—This study was designed to improve identification of human blood monocytes by using antibodies to molecules that occur consistently on all stages of monocyte development and differentiation.

Methods—We examined blood samples from 200 healthy adults without clinically diagnosed immunological abnormalities by flow cytometry (FCM) with multiple combinations of antibodies and with a hematology analyzer (Beckman LH750).

Results—CD91 (α_2 -macroglobulin receptor) was expressed only by monocytes and to a consistent level among subjects (mean MFI = 16.2 ± 3.2). Notably, only $85.7 \pm 5.82\%$ of the CD91⁺ monocytes expressed high levels of the classical monocyte marker CD14, with some CD91⁺ CD16⁺ cells having negligible CD14, indicating that substantial FCM under-counts will occur when monocytes are identified by high CD14. CD33 (receptor for sialyl conjugates) was co-expressed with CD91 on monocytes but CD33 expression varied by nearly ten-fold among subjects (mean MFI = 17.4 ± 7.7). In comparison to FCM analyses, the hematology analyzer systematically over-counted monocytes and eosinophils while lymphocyte and neutrophil differential values generally agreed with FCM methods.

Conclusions—CD91 is a better marker to identify monocytes than CD14 or CD33. Furthermore, FCM (with anti-CD91) identifies monocytes better than a currently used clinical CBC instrument. Use of anti-CD91 together with anti-CD14 and anti-CD16 supports the identification of the diagnostically significant monocyte populations with variable expression of CD14 and CD16.

Keywords

Monocytes; cell counts; leukocyte differential counts; CD91; CD14; CD33; CD16

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Introduction

Blood monocytes are important cells that can differentiate through several pathways leading to cells with distinctly different functions. For example, monocytes can migrate to infected areas and develop into “classically activated” or M1 macrophages with pro-inflammatory and anti-microbial activity. Monocytes can also differentiate into dendritic “antigen presenting cells” that initiate immune responses (1). Monocytes can enter non-infected tissues and develop into “alternatively activated” or M2 macrophages that can remove apoptotic cells in tissues without initiating an immune response and without causing inflammation. This normal and beneficial activity of M2 macrophages can be subverted when monocytes enter tumors and develop into M2 macrophages that can function as “myeloid suppressor cells” that actively inhibit the generation of an immune response. This broad diversity in the potential pathways of monocyte development and differentiation has been extensively examined and documented, *e.g.*, see reviews, (2, 3, 4). However, much less is known about the heterogeneity of blood monocytes themselves. As part of a larger study to characterize the diversity of leukocyte properties in healthy subjects, we have included extensive examinations of blood monocytes. We will report in the second paper of this series that human blood monocytes exhibit heterogeneity within individuals and wide diversity among healthy subjects.

In order to perform flow cytometric examinations of blood monocytes, we needed a robust and reliable way to identify these cells. Monocytes are often identified as cells that express high levels of CD14. However, it has been known for more than 20 years that variable fractions of monocytes express low levels of CD14 and that elevated numbers of this monocyte subset can be clinically significant (5, 6, 7, 8). The expression of CD33 is also an accepted strategy to identify monocytes (9) although the exact function of CD33 on monocytes remains undetermined. On the other hand, CD91 is a multi-functional receptor molecule that binds α_2 -macroglobulin and other ligands (10, reviewed 11) and is known to occur on monocytes. As we will report here, we have compared cells labeled with antibodies to both CD33 and CD91 and found that they were co-expressed on monocytes, including on those monocytes with low levels of CD14. When we compared the variability of expression among individuals, we found that CD91 expression was much more consistent than that of CD33. Therefore, our data indicate that CD91 expression is a robust way to identify monocytes by flow cytometry.

We incorporated anti-CD91 into a mixture of antibodies and determined leukocyte differential counts (LDC) by flow cytometry/ We then compared these results with the LDC values determined in “complete blood counts” (CBC) performed on a clinical hematology analyzer (Beckman Counter LH750). Our results confirmed that the LDC values from the hematology analyzer (that are based on cell volume and light scatter measurements) often disagree with FCM methods that measure both light scatter properties and the expression of monocyte-associated molecules (12). Here in part I of our ‘Properties of Human Blood Monocytes’, we propose that FCM with anti-CD91 should be applied clinically to identify blood monocytes. In part II, we characterize the variability among 200 donors of significant

markers of monocytes, such as CD163 which is selectively expressed by M2 or ‘anti-inflammatory’ macrophages.

Materials and Methods

Blood Collection, Processing & Hematology Analyses

Subjects (142 female, 58 male) were selected by one of the authors (WJD) from his general medical practice. Dr. Diamond excluded children and adolescents under age 20, individuals over age 70 (with one exception), pregnant women and patients with known immunological abnormalities such as HIV infection or autoimmune conditions including multiple sclerosis, rheumatoid arthritis, *etc.* Participants provided informed consent and the study was approved by the University of Nevada Institutional Review Board (Approval #B02/03-34).

Peripheral blood samples were collected from 12 groups of participants, typically consisting of 15-20 subjects, at the site of WJD's medical practice. Blood samples (EDTA Vacutainer Tube #367861 & #367863, BD BioSciences, San Jose, CA) were collected between 7 and 9 am and transported to the University of Nevada laboratories. Blood samples were mixed by inversion and aliquots of 50 μ l were dispensed for labeling with antibodies. A portion of each blood sample was taken to the local LabCorp® clinical laboratory for differential leukocyte and CBC determinations. (At the time of these studies, the LabCorpR facility was using a Beckman-Coulter LH750 hematology analyzer.) The processing of the blood samples, including labeling with antibodies, was typically completed shortly after noon on the day of collection.

Antibodies & Labeling

The antibodies used for this report and its sequel Part II are listed in supplement Table ST1. The volumes of antibodies used for labeling were based on the amounts recommended by the suppliers as scaled to the volumes of blood that were labeled. (Separate titrations indicated that the recommended amounts of antibodies equaled or exceeded maximal labeling.) Antibodies to different antigens were premixed and added to blood samples in a single pipetting step (except for antibodies to intracellular proteins, namely, perforin, granzyme B and TIA-1). Since the cells in whole blood are suspended in plasma with $\sim$ 10mg IgG per ml, *e.g.*, (13), we did not add additional IgG to block Fc binding. In some cases, we were measuring the expression of CD16 so we could not include unlabeled anti-CD16 or “Fc block”. Aliquots of blood plus antibodies were mixed and incubated in the dark at room temperature for 30-45 min at which time 1.0 ml of diluted FACSlysing Solution (#349202, BDBioSciences, diluted 1:10 with water) was added. After the erythrocytes were lysed ($\sim$ 10 min), 3.0 ml of PBS was added, the samples were centrifuged and the pellets resuspended in 1.0 ml of staining buffer (sheath solution as described below supplemented with 1% fetal bovine serum and 0.09% sodium azide and 0.22 μ m filtered). Labeled samples were kept refrigerated until they were run on the cytometer. Separate tests showed that surface labeled samples produced comparable FCM results, *i.e.*, fractions of cells labeled and consistent MFI values, for as long as $\sim$ 72 hours after labeling. These observations indicated that large groups of samples could be processed and examined without compromising the results.

Samples to be examined for both surface and intracellular components were processed differently, according the procedures recommended for the IntraPrep kit (#IM2389, Immunotech, a subsidiary of Beckman-Coulter, Fullerton, CA). The antibodies to surface components were first added as described above. After the initial incubation, the labeled samples were treated with the fixation reagent from the IntraPrep kit, diluted with PBS, centrifuged and then rendered permeable with the IntraPrep perm reagent at which time the antibodies to the intra-cellular components were added. The most critical step in this procedure was vortexing to ensure that the pellets of fixed cells were thoroughly resuspended before adding the perm reagent and the antibody(ies) to intracellular components. After labeling intracellular component(s), these samples were washed by centrifugation and resuspended in 1.0 ml PBS with 1% formalin. We found that the intracellular labels tended to “leak” upon overnight storage so these samples were run on the cytometer as soon as possible after labeling, typically within a few hours.

Flow Cytometry

All samples in these studies were run on a Beckman-Coulter Epics XL/MCL using the carousel with BioSure sheath solution (BioSure, Grass Valley, CA, #1023, 8X concentrate properly diluted with deionized water). The optical filters in the instrument were originally configured to detect cells labeled with fluorescein (FITC), phycoerythrin (PE), PE-Texas Red tandem (ECD) and PE-cyanine 5 tandem (PC5). In order to reduce the amounts of fluorescence spillover, we modified the configuration in order to detect FITC, PE, PC5 and PE-cyanine 7 tandem (PC7) labeled antibodies (see supplement figure S1). PMT detector voltages were set in separate tests to optimize the ratio of “signal” (antibody labeled cells) to “background” (unlabeled cells) and were kept constant throughout the study. Beads (9.2 μ m pink fluorescent beads, Polymer Laboratories, a division of Varian, purchased by Agilent) were run each day to verify proper cytometer operation. Analyses of the data files from these beads demonstrated that the instrument performed comparably throughout the ~4.5 month duration of the study. The XL generates considerable heat and we found previously that elevations in room temperature could reduce the PMT outputs. Therefore, the room temperature was kept at 65-70°F during these studies.

The XL is a fully digital instrument that collects data with 20 bit resolution but stores data in files as 10 bit (0-1023) unsigned integers. In the case of parameters collected with log amplification, values are mathematically converted to a four decade log scale ranging from 10^{-1} (0.10) to 10^3 (1,000). (It should be noted that this intensity scale means that unlabeled or “negative” cells in the first decade have MFI values between 0.10 and 1.0.) In order to utilize the full 20 bit instrument resolution, fluorescence compensation was performed during data collection. The proper compensation values were determined with antibody capture beads (made in house with biotin-antimouse Ig from Jackson ImmunoResearch, West Grove, PA, bound to 6.0 μ m streptavidin beads, Polysciences, Warrington, PA) that were loaded with FITC, PE, PC5 or PC7 antibodies and run without compensation. These files were processed using FlowJo software to determine a compensation matrix, the values of which were then loaded into the XL software (MS-DOS based System II). The XL calculates compensated fluorescence values mathematically using a matrix of spillover values comparable to the way in which FlowJo determines compensation in offline analyses.

Since the data are stored as 10 bit unsigned integers, there is no way to express the negative values that can be produced from compensation. Therefore, cells with negative values accumulate in the lowest or 0.10 channel and were included in analyses. (It should also be noted that since one does not have offline access to the full 20-bit values that the data cannot be effectively transformed to a combined log-linear display as devised by the Stanford group (14) and incorporated into FlowJo.) The quality of some tandem fluorochromes, particularly those with PC7, varied and some had considerable residual PE signal. These residual PE signals were left without “compensation”. However, antibody combinations were designed so that residual PE signals from PC7 were less than any PE signals to be measured on cells that were labeled with PC7 antibodies.

Data files were collected with linear forward scatter (FSC) set with gain 2 and logarithmic amplification on orthogonal scatter (SSC) and on all fluorescence detectors. FSC signals on the XL can be attenuated by sliding a neutral density filter in place. The data in these studies were collected without attenuation. Also, all signals were collected as pulse areas, *i.e.*, the normal mode on the XL. With regard to SSC, it is somewhat unconventional to collect and display log SSC signals so the rationale will be discussed in more detail below. Some antibody combinations were “multiplexed” with the same fluorochrome on more than one antibody, *e.g.*, (15, 16). In these cases, the different populations labeled with the same fluorochrome could be distinguished by SSC differences, by differences in the fluorescence intensity of various populations and/or by differential labeling with another antibody. Examples of multiplexed labeling are depicted and described in Figure 1 and in supplemental Figure S3.

Some blood leukocytes such as eosinophils and to a lesser extent granulocytes had relatively high levels of autofluorescence whereas lymphocytes and monocytes did not have appreciable amounts of autofluorescence. When the blood samples were labeled with mixtures of antibodies that contained an antibody with either fluorescein, PE, PC5 or PC7 that did not react with monocytes then the unlabeled monocytes appeared in the first decade. Since the first decade on the XL intensity scale ranged from 10^{-1} (0.10) to 10^0 (1.00) the MFI values of unlabeled monocytes were typically ~ 0.2 to ~ 0.6 . On the other hand, the monocytes in blood samples labeled with an antibody to a monocyte lineage marker such as anti-CD33 or anti-CD91 appeared in the second or third decades and had MFI values 10-100 fold higher than “background”. Therefore, we did not routinely include “fluorescence minus one” (FMO) isotype controls for monocyte lineage markers such as CD33 or CD91.

Data files were collected with a threshold set on FSC or on CD45 fluorescence (Figure S3). When a threshold was set on FSC, an additional fluorescence and/or SSC “live gate” was set to eliminate debris from lysed erythrocytes that would be eliminated by setting a threshold on CD45 expression. (Antibody to CD45 was excluded from most antibody combinations.) Samples were collected with the XL set on “high” with stop conditions based on acquisition time (*e.g.*, 2 min for the samples for leukocyte differential analyses as depicted in supplemental Figure S3) or on regions that were set around populations of interest. There were no limits placed on the total events collected and the data files typically contained 50,000 to 200,000 total events. The studies here focused on monocytes so that stop conditions were set to include a minimum of 3,500 monocytes per file. (A small number of

samples had too few monocytes to acquire 3,500 as illustrated in the top panel of Supplemental Figure S2.)

Analyses of Data

Data files were analyzed with FlowJo (TreeStar, Ashland, OR) using the Mac versions of the software. Fluorescence intensity values are reported as the “median fluorescence intensity” (MFI) as determined by FlowJo. It should be emphasized again that the intensity scale for the XL data that is mapped to four log decades from 10^{-1} (0.10) to 10^3 (1,000) means that unlabeled cells in the first decade have MFI values between 0.10 and 1.0. Labeled cells in the second and third decades have MFI values from 1.0 to 100. Tabular data from FlowJo tables were transferred to Excel spreadsheets where the built-in statistical functions were used to determine means and other values such as standard deviations and correlations.

Results

Selection of anti-CD91 for FCM identification of monocytes

Multiple molecules are known to be expressed on some or all monocytes including CD4, CD14, CD33, CD36, CD38, CD91, CD244, HLA-DQ and HLA-DR. Of these molecules, both CD14 and CD91 mediate unique monocyte functions and the expression of CD14 is widely used to identify monocytes. Nevertheless, it has been known for over 20 years that some monocytes express low to undetectable amounts of CD14. Although the exact function of CD33 on monocytes remains unclear, the expression of CD33 is also an accepted property to identify monocytes. CD91 mediates important monocyte functions, as will be discussed, but it has not been widely used to identify this population. Therefore, we have compared the use of anti-CD33 and anti-CD91 to identify monocytes. (Detailed discussions of CD33 and the other listed molecules on monocytes will be presented in paper II.) As depicted for one subject in Figure 1, we examined blood samples from 79 subjects that were simultaneously labeled with antibodies to CD91, CD33 and CD14. As illustrated, similar numbers of monocytes within each sample were identified by CD33 or by CD91 expression *vs.* log SSC and these populations both included monocytes with low levels of CD14. When we compared the numbers of monocytes identified within each sample with anti-CD33 or anti-CD91 we found the slope to be 0.974 ($R^2 = 0.94$). Since each sample included both anti-CD91 and anti-CD33, we could also compare the median fluorescence intensity (MFI) for each antibody in the populations defined with either antibody. These MFI values agreed even more closely with the slope of CD91 expression in CD91⁺ *vs.* CD33⁺ monocytes near unity (slope = 1.00, $R^2 = 0.99$) and that of CD33 expression in CD91⁺ *vs.* CD33⁺ monocytes also near unity (slope = 1.02, $R^2 = 1.00$). These comparisons of monocyte counts and MFI values are depicted graphically in Supplemental Figure S2. Thus, within each sample that contained both anti-CD33 and anti-CD91, the two antibodies labeled nearly identical populations of monocytes. It is also important to know how much variability exists among subjects in the intensity of expression of CD33 and CD91 on monocytes. Therefore, we examined samples from all 200 subjects with PE-anti-CD91 or PC5-anti-CD33, identified the monocytes and determined the MFI values for anti-CD91 and for anti-CD33 on the monocytes. The PE-anti-CD91 MFI values from our 200 subjects were mostly clustered within $\pm 20\%$ of the mean with a few outliers above and below this range (mean MFI = 16.16 ± 3.19 , range 3.25 to 24.5,

7.5-fold). These results are illustrated in Figure 2 in which the MFI values are shown on a log scale corresponding to the fluorescence intensity scale from the XL and plotted vs. the ages of the subjects. These results also illustrate that the level of CD91 expression was independent of the age of the subjects. In contrast, the expression of CD33 was considerably more variable among individuals with a standard deviation of $\pm 44\%$ (mean MFI = 17.36 ± 7.69) and the range was more extreme, with MFI values from 2.28 to 39.0, 17-fold (presented in detail in part II). Twenty-four of the donors (12%) had CD33 MFI values of 5.0 or less, indicating that CD33 gating would vary substantially among donors. It should be emphasized again that monocytes incubated with PE or PC5 antibodies that did not label monocytes had PE or PC5 MFI values of ~ 0.2 to ~ 0.6 . Thus, CD91 and CD33 were co-expressed on monocytes but the amounts of CD91 expressed per cell were considerably less variable among individuals than those of CD33.

CD91⁺ monocytes with low/absent CD14

CD14 expression is widely used to identify monocytes, although it is known that CD14 expression varies in monocyte subsets and in states of development and differentiation, *e.g.*, (17, 18). We examined 199 subjects with antibodies to CD91 and to CD14, including the group described above with the example depicted in Figure 1, and found overall that $85.7 \pm 5.82\%$ (range 56.5% to 94.1%) of the CD91⁺ monocytes from those subjects had high CD14 as would typically be used to identify monocytes. In other words, an average of more than 14% of the monocytes of the normal donors would have been omitted by using CD14 as an identifier and, in the most extreme case, 43.5% of the donor's monocytes would have been omitted. As reported above in the comparison of CD33 and CD91 on monocytes, the cells of 79 donors were labeled simultaneously with antibodies to CD3e, CD91, CD14, CD33 and CD16. CD16, the IgG Fc gamma receptor III, supports recognition of pathogens bound with IgG1 and IgG3 antibodies and can facilitate macrophage killing of bacteria and yeast. Figure 3 depicts the expression of CD16 and CD14 by CD91⁺ monocytes from one individual. These typical data illustrate that virtually all of the monocytes with low/absent CD14 are CD16⁺. In order to evaluate CD14 and CD16 expression more completely, CD91⁺ monocytes were identified in each of the 79 subjects as illustrated above in Figure 1. The CD91⁺ monocytes were then divided into two populations, namely, 1) CD14^{bright+} cells, comparable to the typical "CD14⁺ monocytes" and 2) CD14^{low/neg} monocytes. Among this subset of 79 subjects, $11.8 \pm 3.58\%$ (range 4.71% – 20.8%) of the monocytes were CD14^{low/neg}. The expression of CD16 on each of these two populations was then determined. The CD14^{bright+} monocytes had low CD16 expression (mean MFI = 0.24 ± 0.15 , range 0.10 – 0.85) whereas the CD14^{low/neg} cells, as illustrated in Figure 3, had higher CD16 expression (mean MFI = 3.84 ± 4.50 , range 0.10 – 22.70). The CD14^{low/neg} cells that also express CD16 correspond to "non-classical" monocytes (19) and are the cells with prognostic significance in cases of sepsis, *e.g.*, (5). The CD14^{bright+} monocytes can be further divided into CD16^{Neg} "classical" monocytes and CD16⁺ "intermediate" monocytes (19). The potential clinical significance of the "intermediate" population remains to be determined so we have avoided making this distinction. Since there is no clear demarcation in the CD14^{bright+} cells between those that are CD16^{Neg} and CD16⁺, it would require an appropriate "fluorescence minus one" (FMO) control to determine the fraction of CD14^{bright+} cells that are "CD16⁺".

Flow cytometric leukocyte differential counts (LDC)

We used antibody to CD91 to identify monocytes and incorporated some slightly atypical strategies to perform flow cytometric LDC determinations as depicted in Figure S3. First, we used log amplification for orthogonal light scatter (SSC) to keep all the leukocyte populations fully on scale in order to be able to enumerate all the blood leukocytes. The data reported here were all acquired on a single Beckman-Coulter Epics XL/MCL cytometer but we have found that labeled human blood samples produced comparable log SSC patterns on a fully digital Beckman FC500 (with two lasers), a standard analog BDBiosciences FACSscan and on a digital BDBiosciences LSR II. Second, we sometimes used more than one antibody with the same fluorochrome in order to increase efficiency on the four-color flow cytometer that was used. That strategy was illustrated above in Figure 1 and is again depicted in Figure S3 in which FITC-anti-CD3e and FITC-anti-CD15 were used along with PE-anti-CD20 and PE-anti-CD91. Based on light scatter properties and/or labeling intensities one could readily distinguish FITC-CD3e⁺ T lymphocytes from FITC-CD15⁺ granulocytes and eosinophils and likewise one could distinguish PE-CD20⁺ B lymphocytes from PE-CD91⁺ monocytes. In other experiments (not shown), we verified that the eosinophil population identified on the basis of CD15 expression and FSC as depicted in Supplemental Figure S3 had high autofluorescence (20) and had lower CD16 expression than the neutrophils (21). The flow cytometric LDC was determined with the total number of CD45⁺ leukocytes as the denominator in calculating leukocyte frequencies.

Overestimation of monocytes by the LDC from a hematology analyzer

Aliquots of blood from all 200 subjects had “complete blood counts” (CBC) performed on a Beckman Coulter LH750 hematology analyzer. The CBC results included LDC values which are illustrated in comparison with flow cytometric LDC values in Figure 4. The frequencies of monocytes in the LDC values from the Beckman Coulter LH750 hematology analyzer were overall ~10% higher than values obtained by anti-CD91 FCM analyses of CD45⁺ leukocytes (Figure 4). Furthermore, the low correlation coefficient ($R^2 = 0.65$) underscores the differences in results produced by the differing technologies. For ten (5%) of our 200 donors, the over-estimation of monocytes was 50% or higher and the highest was 2.2 fold higher by the hematology analyzer. In contrast, the frequencies of lymphocytes and neutrophils were close by the two methods, with slopes near unity. In addition to monocytes, the hematology analyzer also overestimated eosinophils by ~8% but with a consistent bias ($R^2 = 0.94$).

Absolute monocyte counts

The absolute number of monocytes was estimated from the absolute total leukocyte count derived from the hematology analyzer and the frequency of CD91⁺ monocytes determined in the flow cytometric LDC. The mean monocyte count was 420 ± 140 monocytes/ μ l (range 191 to 960). The 58 male subjects had more monocytes (mean = 466 ± 183 , range 195 to 960) than the 158 female subjects (mean = 402 ± 123 , range 191 to 752), but the difference was not significant ($p = 0.31$; unpaired, two-tailed t test). There was no detectable association between the monocyte counts and the MFI of CD91 labeling as there might have been if the

amount of antibody were limiting. There was also no detectable association between the monocyte count and the ages of the subjects.

Discussion

In order to identify cell types by FCM, the target molecules of lineage-specific antibodies ideally should be 1) present on all the cells of that lineage, 2) uniquely expressed on that lineage and 3) have known function. In the cases of T and B cells, the expression of CD3 or CD19, respectively, fulfills those requirements. In the case of blood monocytes, CD14 is uniquely expressed and monocytes and is involved in mediating monocyte function (22). CD14 is a GPI-linked protein that binds bacterial lipopolysaccharide (LPS) which is further facilitated by serum LPS binding protein (23). LPS bound in this manner is “focused” to Toll-like receptor 4 (TLR4). LPS-binding protein and CD14 together enhance the sensitivity of TLR4 on monocytes and macrophages to bacterial endotoxins by orders of magnitude (24). However, CD14 falls short as an ideal lineage marker since CD14 expression is low or absent on monocytes in some states of differentiation as has been known for approximately 20 years (19). In the present studies, we found overall that an average of ~15% of monocytes identified by light scatter and expression of CD91 or CD33 had low to undetectable levels of CD14. Furthermore, in some conditions, an increased number of monocytes with reduced amounts of CD14 and/or with elevated CD16 expression can provide useful prognostic information as has been reported in cases of bacterial sepsis (5, 6, 7, 8, 25). Therefore, expression of high levels of CD14 is not a reliable monocyte marker and will, in fact, not identify a potentially significant population of monocytes.

In contrast to CD14 expression, CD91 expression does fulfill the criteria to be a lineage marker for blood monocytes. Among blood leukocytes, CD91 is uniquely expressed on monocytes as illustrated in Figure 1. CD91 is also a functional molecule on myeloid cells. It is both the α_2 -macroglobulin receptor and the low density lipoprotein receptor-related protein (LRP) and has several known activities (10, reviewed 11). For example, CD91 recognizes complexes of α_2 -macroglobulin with bound serine proteases such as thrombin (26, 27). CD91 is also a receptor for “heat shock proteins” (HSP) (28, 29) which can serve as chaperones for peptides produced by the lysosomal pathway. Peptides internalized in this way can be “cross presented” to CD8 T cells (30). More recently, it has been learned that CD91 can bind C1q (31) and mannan binding lectin (32). Thus, CD91 is a multifunctional molecule and we found that the expression of CD91 varied relatively little among our 200 healthy subjects. In contrast to CD14, CD91 is not known to be absent on monocyte subsets. Therefore, since CD91 appears to be consistently expressed among normal subjects and since CD91 has known functions on monocytes, we suggest that CD91 expression should be considered as a definitive marker to identify human blood monocytes. The inclusion of antibodies to CD14 and to CD16 would then enable one to identify the “classical”, “intermediate” and “non-classical” monocyte populations (19).

The expression of CD33 or of CD36 has also been used to identify monocytes. The expression of CD33 has long been recognized as a monocyte lineage marker (9). CD33 was subsequently shown to have potential functional activity that could be mediated by an immunoreceptor tyrosine-based inhibitory motif (ITIM) in its cytoplasmic region (33).

Nevertheless, the physiologic ligand(s) for CD33 remain to be identified. As we have demonstrated here, CD33 and CD91 are co-expressed and can be used to identify comparable populations of monocytes (Figures 1 and S2). However, the amount of CD33 expressed on monocytes varied much more among individuals than did that of CD91 as one can see in the CD33 MFI values illustrated in Figure S2. Although the expression of CD91 was relatively invariant among the healthy subjects we examined, there have been reports that CD91 expression may vary in individuals with melanoma (34) or in those infected with HIV (35, 36). The heterogeneity of CD33 expression will be discussed in more detail in the subsequent paper of this series.

CD36 is also a potentially functional molecule on monocytes. It was demonstrated more than 20 years ago that cross-linking CD36 with antibodies could initiate an oxidative burst by monocytes (37). More recently, CD36 has been shown to be involved in the process leading to M2 polarization of monocytes/macrophages and is also expressed at higher levels on M2 polarized cells (38). CD36 expression has also been reported to be elevated on the monocytes of subjects with type 2 diabetes (39). However, the expression of CD36 is not limited to monocytes since it also occurs on platelets (40). Thus, both CD33 and CD36 are potentially functional molecules on monocytes that may be involved in mediating the activities of those cells. However, both CD33 and CD36 are expressed at different levels among individuals. Because of this variability, the level of expression of CD33 and CD36 could provide potentially useful information. In order to measure the expression of CD33, CD36, CD14, CD16, and other molecules variably expressed on monocyte, a more consistently expressed molecule, such as CD91, would be preferable as a lineage marker.

The utility of using log SSC to keep resting and activated lymphocytes (41) or disparate cell types (42, 43) all on scale has been appreciated for more than 20 years. Similarly, the original report by Stelzer et al. (44) demonstrating the utility of examining blood leukocytes labeled with anti-CD45 also used log SSC for the display. However, other reports from about the same time that used CD45 and SSC for analysis used linear SSC, *e.g.*, (45).

Subsequently, the vast majority of flow cytometric studies have reported the use of linear SSC including recent reports describing methods for flow cytometric leukocyte differential counts (46, 47). If one is focused on examining only lymphocytes, then linear SSC is perfectly adequate and may be advantageous since it can make it easier to discern subsets that differ subtly in SSC properties. However, linear SSC does not work well if one needs to identify and enumerate all the leukocytes populations as is required to determine the LDC by flow cytometry. The median SSC values for lymphocytes and granulocytes differ by nearly an order of magnitude (7-8 fold) so it may not be possible to keep all the lymphocytes and all the granulocytes on a linear SSC display making it difficult to obtain a reliable count of both cell types. Our results illustrate that the very simple change of collecting log SSC can keep all the populations of blood leukocytes fully on scale and facilitate their identification.

Using antibody to CD91 to identify monocytes, we have described a labeling and gating protocol to identify the principal populations of blood leukocytes including monocytes, total lymphocytes, T cells, B cells, neutrophils and eosinophils using a single-laser, four-color flow cytometer (Figure S3). The total leukocyte population was determined from a bivariate plot of CD45 vs. log SSC and that value formed the denominator to determine the relative

frequencies of the principal populations. Our protocol also used “multiplexed” antibodies, *i.e.*, more than one antibody per fluorescence detector. Other investigators have employed the same concept with different combinations of antibodies, *e.g.*, (48). Multiplexed antibodies have also been combined with a DNA-binding dye to detect nucleated cells and distinguish them from platelets (49). More recently, Roussel, *et al.*, (46) demonstrated that FCM examination of blood with a more extensive panel of antibodies could produce an extended leukocyte differential count superior to that obtained from a hematology analyzer combined with microscopic examination of blood smears. Subsequently, the same laboratory and other investigators (47) have evaluated different antibody combinations and reported comparable results. If combined with known numbers of beads in a lyse/no wash protocol, any of these strategies can determine both relative and absolute leukocyte differential counts. Our current results indicate that expression of CD91 should be more widely considered as a monocyte lineage marker.

Blood samples from all of the 200 healthy subjects that were examined by FCM were also examined with a Coulter LH750 hematology analyzer. As illustrated in Figure 4, the relative frequencies of neutrophils and lymphocytes determined by these two methods agreed closely. On the other hand, the hematology analyzer appeared consistently to over-count eosinophils by ~8% relative to the frequency determined by FCM. Monocytes were the primary focus of the present studies and we found that the enumeration of monocytes showed the greatest discrepancy between the two methods. Using hematology analyzers that were either the same (50) or different (51, 52, 53) from the one in our studies, other investigators also found that FCM analyses yielded superior results, and that the hematology analyzers overestimated monocytes, *e.g.*, the Sysmex XE2100 overestimated monocytes by 11% (53). This finding is understandable since the cell volume and light scatter measurements utilized by hematology instruments may incompletely resolve monocytes from other leukocytes. Results similar to ours were obtained in a prior study that compared manual differential counts with values from the LH750 (54). Although the frequencies of monocytes and eosinophils were overestimated by the hematology analyzer, the frequencies of the more numerous neutrophils and lymphocytes were comparable in the LDC values from the hematology analyzer and the flow cytometer. In these studies of monocyte properties and in subsequent reports of the properties of NK cells, T and B cells, we will use the LDC values determined by flow cytometry and the absolute leukocyte counts determined by the hematology analyzer to estimate the absolute counts of the lymphocyte populations.

In our extended characterization of monocytes in part II of this study, we found that monocyte-associated molecules such as CD33, CD38, CD86, CD163, HLA-DR, HLA-DQ and TLR2 varied considerably in frequency and/or in the extent of expression (MFI) among the subjects that were examined. Thus, monocytes have a great deal of heterogeneity among healthy individuals.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Literature Cited

1. Hume DA, Ross IL, Himes SR, Sasmono RT, Wells CA, Ravasi T. The mononuclear phagocyte system revisited. *J Leukoc Biol.* 2002; 72(4):621–7. [PubMed: 12377929]
2. Martinez FO, Sica A, Mantovani A, Locati M. Macrophage activation and polarization. *Front Biosci.* 2008; 13:453–61. [PubMed: 17981560]
3. Solinas G, Schiarea S, Liguori M, Fabbri M, Pesce S, Zammataro L, Pasqualini F, Nebuloni M, Chiabrando C, Mantovani A, et al. Tumor-conditioned macrophages secrete migration-stimulating factor: a new marker for M2-polarization, influencing tumor cell motility. *J Immunol.* 2010; 185(1): 642–52. [PubMed: 20530259]
4. Serafini P, De Santo C, Marigo I, Cingarlini S, Dolcetti L, Gallina G, Zanovello P, Bronte V. Derangement of immune responses by myeloid suppressor cells. *Cancer Immunol Immunother.* 2004; 53(2):64–72. [PubMed: 14593498]
5. Ziegler-Heitbrock HW, Strobel M, Fingerle G, Schlunck T, Pforte A, Blumenstein M, Haas JG. Small (CD14+/CD16+) monocytes and regular monocytes in human blood. *Pathobiology.* 1991; 59(3):127–30. [PubMed: 1715711]
6. Fingerle G, Pforte A, Passlick B, Blumenstein M, Strobel M, Ziegler-Heitbrock HW. The novel subset of CD14+/CD16+ blood monocytes is expanded in sepsis patients. *Blood.* 1993; 82(10): 3170–6. [PubMed: 7693040]
7. Rothe G, Gabriel H, Kovacs E, Klucken J, Stohr J, Kindermann W, Schmitz G. Peripheral blood mononuclear phagocyte subpopulations as cellular markers in hypercholesterolemia. *Arterioscler Thromb Vasc Biol.* 1996; 16(12):1437–47. [PubMed: 8977447]
8. Scherberich JE, Nockher WA. CD14++ monocytes, CD14+/CD16+ subset and soluble CD14 as biological markers of inflammatory systemic diseases and monitoring immuno-suppressive therapy. *Clin Chem Lab Med.* 1999; 37(3):209–13. [PubMed: 10353463]
9. Terstappen LW, Hollander Z, Meiners H, Loken MR. Quantitative comparison of myeloid antigens on five lineages of mature peripheral blood cells. *J Leukoc Biol.* 1990; 48(2):138–48. [PubMed: 2196321]
10. Herz J, Strickland DK. LRP: a multifunctional scavenger and signaling receptor. *J Clin Invest.* 2001; 108(6):779–84. [PubMed: 11560943]
11. Lillis AP, Van Duyn LB, Murphy-Ullrich JE, Strickland DK. LDL receptor-related protein 1: unique tissue-specific functions revealed by selective gene knockout studies. *Physiol Rev.* 2008; 88(3):887–918. [PubMed: 18626063]
12. Buttarello M, Plebani M. Automated blood cell counts: state of the art. *Am J Clin Pathol.* 2008; 130(1):104–16. [PubMed: 18550479]
13. Gonzalez-Quintela A, Alende R, Gude F, Campos J, Rey J, Meijide LM, Fernandez-Merino C, Vidal C. Serum levels of immunoglobulins (IgG, IgA, IgM) in a general adult population and their relationship with alcohol consumption, smoking and common metabolic abnormalities. *Clin Exp Immunol.* 2008; 151(1):42–50. [PubMed: 18005364]
14. Tung JW, Parks DR, Moore WA, Herzenberg LA, Herzenberg LA. New approaches to fluorescence compensation and visualization of FACS data. *Clin Immunol.* 2004; 110(3):277–83. [PubMed: 15047205]
15. Buican TN, Hoffmann GW. Immunofluorescent flow cytometry in N dimensions. The multiplex labeling approach *Cell Biophys.* 1985; 7(2):129–56. [PubMed: 2412696]
16. Bradford JA, Buller G, Suter M, Ignatius M, Beechem JM. Fluorescence-intensity multiplexing: simultaneous seven-marker, two-color immunophenotyping using flow cytometry. *Cytometry.* 2004; 61A(2):142–52. [PubMed: 15382027]

17. Ziegler-Heitbrock L, Ancuta P, Crowe S, Dalod M, Grau V, Hart DN, Leenen PJ, Liu YJ, MacPherson G, Randolph GJ, et al. Nomenclature of monocytes and dendritic cells in blood. *Blood*. 2010; 116(16):e74–80. [PubMed: 20628149]
18. Wong KL, Tai JJ, Wong WC, Han H, Sem X, Yeap WH, Kourilsky P, Wong SC. Gene expression profiling reveals the defining features of the classical, intermediate, and nonclassical human monocyte subsets. *Blood*. 2011; 118(5):e16–31. [PubMed: 21653326]
19. Passlick B, Flieger D, Ziegler-Heitbrock HW. Identification and characterization of a novel monocyte subpopulation in human peripheral blood. *Blood*. 1989; 74(7):2527–34. [PubMed: 2478233]
20. Thureau AM, Schylz U, Wolf V, Krug N, Schauer U. Identification of eosinophils by flow cytometry. *Cytometry*. 1996; 23(2):150–8. [PubMed: 8742174]
21. Hartnell A, Moqbel R, Walsh GM, Bradley B, Kay AB. Fc gamma and CD11/CD18 receptor expression on normal density and low density human eosinophils. *Immunology*. 1990; 69(2):264–70. [PubMed: 1968426]
22. Ingalls RR, Heine H, Lien E, Yoshimura A, Golenbock D. Lipopolysaccharide recognition, CD14, and lipopolysaccharide receptors. *Infect Dis Clin North Am*. 1999; 13(2):341–53, vii. [PubMed: 10340170]
23. Hailman E, Lichenstein HS, Wurfel MM, Miller DS, Johnson DA, Kelley M, Busse LA, Zukowski MM, Wright SD. Lipopolysaccharide (LPS)-binding protein accelerates the binding of LPS to CD14. *J Exp Med*. 1994; 179(1):269–77. [PubMed: 7505800]
24. Schromm AB, Brandenburg K, Rietschel ET, Flad HD, Carroll SF, Seydel U. Lipopolysaccharide-binding protein mediates CD14-independent intercalation of lipopolysaccharide into phospholipid membranes. *FEBS Lett*. 1996; 399(3):267–71. [PubMed: 8985160]
25. Nockher WA, Wiemer J, Scherberich JE. Haemodialysis monocytopenia: differential sequestration kinetics of CD14+CD16+ and CD14++ blood monocyte subsets. *Clin Exp Immunol*. 2001; 123(1):49–55. [PubMed: 11167997]
26. Kristensen T, Moestrup SK, Gliemann J, Bendtsen L, Sand O, Sottrup-Jensen L. Evidence that the newly cloned low-density-lipoprotein receptor related protein (LRP) is the alpha 2-macroglobulin receptor. *FEBS Lett*. 1990; 276(1-2):151–5. [PubMed: 1702392]
27. Strickland DK, Ashcom JD, Williams S, Burgess WH, Migliorini M, Argraves WS. Sequence identity between the alpha 2-macroglobulin receptor and low density lipoprotein receptor-related protein suggests that this molecule is a multifunctional receptor. *J Biol Chem*. 1990; 265(29):17401–4. [PubMed: 1698775]
28. Binder RJ, Han DK, Srivastava PK. CD91: a receptor for heat shock protein gp96. *Nat Immunol*. 2000; 1(2):151–5. [PubMed: 11248808]
29. Basu S, Binder RJ, Ramalingam T, Srivastava PK. CD91 is a common receptor for heat shock proteins gp96, hsp90, hsp70, and calreticulin. *Immunity*. 2001; 14(3):303–13. [PubMed: 11290339]
30. Binder RJ, Kumar SK, Srivastava PK. Naturally formed or artificially reconstituted non-covalent alpha2-macroglobulin-peptide complexes elicit CD91-dependent cellular immunity. *Cancer Immun*. 2002; 2:16. [PubMed: 12747761]
31. Duus K, Hansen EW, Tacnet P, Frachet P, Arlaud GJ, Thielens NM, Houen G. Direct interaction between CD91 and C1q. *Febs J*. 2010; 277(17):3526–37. [PubMed: 20716178]
32. Duus K, Thielens NM, Lacroix M, Tacnet P, Frachet P, Holmskov U, Houen G. CD91 interacts with mannan-binding lectin (MBL) through the MBL-associated serine protease-binding site. *Febs J*. 2010; 277(23):4956–64. [PubMed: 21054788]
33. Ulyanova T, Blasioli J, Woodford-Thomas TA, Thomas ML. The sialoadhesin CD33 is a myeloid-specific inhibitory receptor. *Eur J Immunol*. 1999; 29(11):3440–9. [PubMed: 10556798]
34. Stebbing J, Bower M, Gazzard B, Wildfire A, Pandha H, Dalgleish A, Spicer J. The common heat shock protein receptor CD91 is up-regulated on monocytes of advanced melanoma slow progressors. *Clin Exp Immunol*. 2004; 138(2):312–6. [PubMed: 15498042]
35. Stebbing J, Gazzard B, Kim L, Portsmouth S, Wildfire A, Teo I, Nelson M, Bower M, Gotch F, Shaunak S, et al. The heat-shock protein receptor CD91 is up-regulated in monocytes of HIV-1-infected “true” long-term nonprogressors. *Blood*. 2003; 101(10):4000–4. [PubMed: 12531796]

36. Kebba A, Stebbing J, Rowland S, Ingram R, Agaba J, Patterson S, Kaleebu P, Imami N, Gotch F. Expression of the common heat-shock protein receptor CD91 is increased on monocytes of exposed yet HIV-1-seronegative subjects. *J Leukoc Biol.* 2005; 78(1):37–42. [PubMed: 15800028]
37. Trezzini C, Jungi TW, Spycher MO, Maly FE, Rao P. Human monocytes CD36 and CD16 are signaling molecules. Evidence from studies using antibody-induced chemiluminescence as a tool to probe signal transduction *Immunology.* 1990; 71(1):29–37. [PubMed: 1698715]
38. Oh J, Riek AE, Weng S, Petty M, Kim D, Colonna M, Cella M, Bernal-Mizrachi C. Endoplasmic reticulum stress controls M2 macrophage differentiation and foam cell formation. *J Biol Chem.* 2012; 287(15):11629–41. [PubMed: 22356914]
39. Sampson MJ, Davies IR, Braschi S, Ivory K, Hughes DA. Increased expression of a scavenger receptor (CD36) in monocytes from subjects with Type 2 diabetes. *Atherosclerosis.* 2003; 167(1): 129–34. [PubMed: 12618277]
40. Tandon NN, Lipsky RH, Burgess WH, Jamieson GA. Isolation and characterization of platelet glycoprotein IV (CD36). *J Biol Chem.* 1989; 264(13):7570–5. [PubMed: 2468669]
41. Redelman D. Cyclosporin A does not inhibit the PHA-stimulated increase in intracellular Ca²⁺ concentration but inhibits the increase in E-rosette receptor (CD2) expression and appearance of interleukin-2 receptors (CD25). *Cytometry.* 1988; 9(2):156–63. [PubMed: 2896111]
42. Redelman D, Butler S, Robison J, Garner D. Identification of inflammatory cells in bovine milk by flow cytometry. *Cytometry.* 1988; 9(5):463–8. [PubMed: 3180948]
43. Ordog T, Redelman D, Horvath VJ, Miller LJ, Horowitz B, Sanders KM. Quantitative analysis by flow cytometry of interstitial cells of Cajal, pacemakers, and mediators of neurotransmission in the gastrointestinal tract. *Cytometry.* 2004; 62A(2):139–49. [PubMed: 15536638]
44. Stelzer GT, Shults KE, Loken MR. CD45 gating for routine flow cytometric analysis of human bone marrow specimens. *Ann N Y Acad Sci.* 1993; 677:265–80. [PubMed: 8494214]
45. Borowitz MJ, Guenther KL, Shults KE, Stelzer GT. Immunophenotyping of acute leukemia by flow cytometric analysis. Use of CD45 and right-angle light scatter to gate on leukemic blasts in three-color analysis. *Am J Clin Pathol.* 1993; 100(5):534–40. [PubMed: 8249893]
46. Roussel M, Benard C, Ly-Sunnaram B, Fest T. Refining the white blood cell differential: the first flow cytometry routine application. *Cytometry A.* 2010; 77(6):552–63. [PubMed: 20506466]
47. Roussel M, Davis BH, Fest T, Wood BL. Toward a reference method for leukocyte differential counts in blood: Comparison of three flow cytometric candidate methods. *Cytometry A.* 2012; 81A(11):973–982. [PubMed: 22736499]
48. Faucher JL, Lacronique-Gazaille C, Frebet E, Trimoreau F, Donnard M, Bordessoule D, Lacombe F, Feuillard J. 6 markers/5 colors” extended white blood cell differential by flow cytometry. *Cytometry A.* 2007; 71(11):934–44. [PubMed: 17879238]
49. Bjornsson S, Wahlstrom S, Norstrom E, Bernevi I, O'Neill U, Johansson E, Runstrom H, Simonsson P. Total nucleated cell differential for blood and bone marrow using a single tube in a five-color flow cytometer. *Cytometry B Clin Cytom.* 2008; 74(2):91–103. [PubMed: 18061952]
50. Grimaldi E, Carandente P, Scopacasa F, Romano MF, Pellegrino M, Bisogni R, De Caterina M. Evaluation of the monocyte counting by two automated haematology analysers compared with flow cytometry. *Clin Lab Haematol.* 2005; 27(2):91–7. [PubMed: 15784123]
51. Hubl W, Hauptlorenz S, Tlustos L, Jilch R, Fischer M, Bayer PM. Precision and accuracy of monocyte counting. Comparison of two hematology analyzers, the manual differential and flow cytometry. *Am J Clin Pathol.* 1995; 103(2):167–70. [PubMed: 7531938]
52. Hubl W, Andert S, Erath A, Lapin A, Bayer PM. Peripheral blood monocyte counting: towards a new reference method. *Eur J Clin Chem Clin Biochem.* 1995; 33(11):839–45. [PubMed: 8620061]
53. Cherian S, Levin G, Lo WY, Mauck M, Kuhn D, Lee C, Wood BL. Evaluation of an 8-color flow cytometric reference method for white blood cell differential enumeration. *Cytometry B Clin Cytom.* 2010; 78(5):319–28. [PubMed: 20533390]
54. Aulesa C, Pastor I, Naranjo D, Piqueras J, Galimany R. Validation of the Coulter LH 750 in a hospital reference laboratory. *Lab Hematol.* 2003; 9(1):15–28. [PubMed: 12661824]

Abbreviations

CBC	complete blood count
FCM	flow cytometry
FITC	fluorescein isothio-cyanate
MFI	median fluorescence intensity
PE	phycoerythrin
PC5	phycoerythrin-cyanine 5 tandem fluorochrome
PC7	phycoerythrin-cyanine 7 tandem fluorochrome

Figure 1. Antibodies to CD91 or to CD33 identify comparable monocyte populations

Aliquots of blood from 79 subjects were labeled with FITC-anti-CD91, FITC-anti-CD3e, PE-anti-CD14, PC5-anti-CD33 and PC7-anti-CD16. Monocytes were identified by CD91 or by CD33 expression as illustrated and then examined for labeling with anti-CD14. As shown, labeling with anti-CD91 or with anti-CD33 identified comparable numbers of monocytes that included cells with variable expression of CD14. Detailed comparisons of the monocytes identified with anti-CD91 and anti-CD33 are depicted in Supplemental Figure S2

Figure 2. The median fluorescence intensity (MFI) of PE-anti-CD91 on human blood monocytes indicates consistent expression of CD91 regardless of the age of the blood donors

Blood samples from all 200 subjects were labeled with antibody combinations that included PE-anti-CD91. The MFIs of the bound PE-anti-CD91 were determined and plotted on a log scale comparable to that on the XL vs. the subjects' ages. The PE MFI values of monocytes labeled with other antibody combinations that included a PE antibody non-reactive with monocytes was in the range of 0.2 to 0.6. As illustrated, CD91 expression on monocytes was independent of age. The monocytes from one subject expressed an unusually low level of CD91 but overall among the donors the CD91 expression was in a narrow range with a standard deviation of $\sim \pm 20\%$.

Figure 3. CD91⁺ blood monocytes include cells with variable expression of CD14 and CD16
As described and depicted above in Figure 1, aliquots of blood were labeled with FITC-anti-CD91, PE-anti-CD14, PC5-anti-CD33 and PC7-anti-CD16. Monocytes were identified by CD91 expression as illustrated in Figure 1 and then examined for labeling with anti-CD14 and anti-CD16 as shown above.

Figure 4. Leukocyte differential counts from a Beckman Coulter LH750 hematology analyzer overestimated the frequencies of monocytes and eosinophils

Blood samples from 200 subjects were analyzed by LabCorp for complete blood counts (CBC) using a Beckman Coulter LH750 Hematology Analyzer. Aliquots of the same samples were labeled with antibodies to CD45 plus antibodies to monocyte and granulocyte antigens, *e.g.*, CD91 & CD15, respectively, and analyzed by flow cytometry (FCM). Gating for the different cells is illustrated in supplemental figure S3. The leukocyte differential counts from the two platforms were plotted as illustrated with the least squares fitted lines constrained to intersect zero. The hematology analyzer consistently over-estimated the frequency of monocytes compared to monocytes identified by anti-CD91 labeling in the flow cytometric tests by 1.098 fold or ~10% based on the slope of the best fit line. The hematology analyzer also consistently ($R^2 = 0.94$) indicated ~8% higher frequencies of eosinophils. On the other hand, the lymphocyte and neutrophil frequencies from the two platforms were similar with slopes close to 1.00 and lacked any consistent bias.