

Detection and Differentiation of Different Monocyte Subsets: Optimization of a Multiparameter Flowcytometric Assay

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Abstract

Peripheral blood monocytes are heterogeneous and it was recently reported that three subsets can be identified based on CD14 and CD16 expression: CD14⁺⁺/CD16⁻ classical monocytes, CD14⁺⁺/CD16⁺ intermediate monocytes and CD14⁺/CD16⁺⁺ non-classical monocytes. The mostly used technique to detect and determine the subtypes is multiparameter flowcytometry, however, there are some technical challenges in the determination of the subsets in flowcytometry, including pre-analytic, analytic and post-analytic aspects. This study aims at validation and optimization the multiparameter flowcytometric determination of monocyte subsets. For this purpose, patients from the outdoor clinic were asked to participate by having an extra EDTA-anti-coagulated blood tube drawn. The stability of the blood sample was investigated by analyzing 20 blood samples directly and 1h, 3h, 4h and 24h after venipuncture. The influence of antibody clone (B73.1 and 3G8; BD Biosciences) against CD16 was investigated making use of another group of 20 blood samples. Reproducibility was tested and three different gating strategies, based on either CD14 back-gating, HLA-DR expression or CD24 and CD56 expression were applied to the flowcytometric results. Samples were analyzed with flowcytometry (FACSCanto II; BD). The relative size of classical monocyte population shows a significant decrease 24h after venipuncture. Using the HLA-DR based gating method, a decrease in the fraction of total monocytes was found. No significant differences were found when comparing the B73.1 and 3G8 antibody clone, however, the use of the 3G8 clone made it possible to make a better distinction between monocytes and granulocytes in the HLA-DR based gating method. All measurements show a good reproducibility (CV < 10%, ICC > 0,8), although the intermediate monocytes show a higher level of variation. In order to make reliable and proper determinations of the different monocyte subsets, sample

preparation and acquisition has to take place within 4 hours after venipuncture. the 3G8 clone is preferred for CD16 labeling and the HLA-DR based gating method is assumed to be the most accurate in identifying the monocyte subsets. Using above mentioned recommendations, the test shows a good reproducibility.

Keywords

Monocyte subsets, flowcytometry, optimalization, sample stability, antibody clone, reproducibility, gating strategy

Introduction

Monocytes constitute 5-10% of peripheral leukocytes, where they circulate for several days in the blood stream and can migrate into tissues to contribute to the macrophage pool. Monocytes and their progeny play important roles in the defense against pathogens, homeostasis and tissue repair, especially in the innate immune system (1).

Monocytes are mononuclear and characterized by a kidney-shaped nucleus. As the main component of the innate immune system, they are responsible for the clearance of bacterial, viral and fungal infections, mainly by the process of phagocytosis (2). Monocytes were considered to be a homogeneous cell population, but in the late 1980s, the report of Passlick and his colleagues (3) identified two phenotypically distinct monocyte subtypes based on differential expression of CD14 and CD16. CD14 is a cell surface receptor involved in the binding of lipopolysaccharide (LPS) and therefore in LPS signaling (4).
[keyword<keyword>Lipopolysaccharides/*chemistry/immunology/pharmacology</keyword><keyword>Molecular Sequence Data</keyword><keyword>Toll-Like Receptor 4/*chemistry/immunology/metabolism</keyword></keywords><dates><year>2013</year></dates><isbn>2092-6413](#) (Electronic. CD16 refers to the low affinity Fc receptor Fcγ-RIII. This receptor binds to the Fc-part of IgG antibodies and thereby activates natural killer (NK) cells resulting in cytotoxicity (5). Over time, it became clear that also the CD16+ monocytes do not represent a homogenous population, but they could be divided into two distinct groups. In 2010, the Nomenclature Committee of the International Union of Immunological Societies recommended a unified nomenclature of the different monocyte subsets. According to this, monocytes are currently divided into CD14+/CD16- classical monocytes, CD14+/CD16+ intermediate monocytes and CD14+/CD16++ non-classical monocytes (6).

The mostly used technique to detect and determine the different subtypes of monocytes is multiparameter flowcytometry. CD16+ monocytes have been implicated in the

pathogenesis of many diseases (1). Therefore, there has been much interest in investigating CD16+ monocytes in disease states. However, NK cells as well as neutrophils share the expression of CD16 with the CD16+ monocytes, which can confuse the flowcytometric analysis. Three different gating strategies are commonly used to identify the monocyte fraction: back-gating, negative and positive gating strategies. The back-gating method is the easiest and cheapest way to select all monocytes. A major drawback of this method is the unwanted admixture with certain lymphocytes and neutrophils (7). Negative or 'exclusion' gating strategies are used to purify the monocyte population utilizing monoclonal antibodies, for example against CD66b or CD15, CD56 and CD19, in order to exclude neutrophils, NK cells and B-cells respectively. Positive or 'inclusion' gating strategies make use of antibodies to CD45, CD115, CD14, CD16 and HLA-DR to select monocytes positively (1).

Another challenge in flowcytometry is the proper gating of the intermediate and the non-classical monocytes. The fractions of these subtypes could be very small, so that a small variation in the gating technique results in a large and hardly reproducible coefficient of variation (CV). A possible solution for this problem is a strong and distinguishable staining expression for the CD16 epitope. In earlier studies of our group, the clones NPK15 and B73.1 were used for immunolabeling of CD16, which both show an intermediate staining level. Most published research in this field, however, made use of the clone 3G8 in the flowcytometric assay. This 3G8 clone is described to have a high staining level, so a better distinction could be made between the intermediate and non-classical monocytes.

Next to the above described major challenges in the flowcytometric determination of the different monocyte subsets in peripheral blood, there are also some pre-analytical and analytical aspects unknown which can influence the outcome of the test. One important aspect is the stability of the blood sample, referring to the time from blood drawn till the start of the preparation of the sample. Another aspect is the reproducibility as well as the intra –and inter assay variations.

The aim of this thesis project is to validate and to optimize the multiparameter flowcytometric determination of the different monocyte subsets. For this purpose, the most recent and often cited publications of large investigator groups in this field will be used as a guidance. Next to the pre-analytical aspect of blood sample stability, analytical aspects, such as the influence of different antibody clones for CD16 immunolabeling, reproducibility and intra –and inter-assay variations, will be investigated. In addition, three different gating strategies will be examined post-analytically. We hypothesize that blood

sample age will not influence the flowcytometric results (8, 9). The use of the 3G8 clone against CD16 is expected to give a higher staining level with respect to the B73.1 clone and therefore a better distinction between intermediate and non-classical monocytes will be possible. Both the positive and negative gating strategies are expected to be more accurate compared to the CD14 back-gating method.

Materials and methods

Patients and Samples

Patients from the outdoor clinic were asked to participate in the study by having an EDTA anti-coagulated blood tube drawn. Only patients who gave informed consent were included in the study. A complete blood count (CBC) was performed for all samples using the XE5000 (Sysmex).

For the purpose of examining the stability of the blood sample, EDTA anti-coagulated blood samples from 20 different patients were used. The analysis was started immediately after venipuncture and after 1h, 3h, 4h and 24h. To assess the influence of type of antibody clone against CD16, EDTA blood samples from a second group of 20 patients were used. From each patient, a sample was incubated with the B73.1 clone while simultaneously another sample was incubated with the 3G8 clone. This test was started within 10 minutes after venipuncture. In order to investigate the reproducibility of the test, another set of blood samples from 10 patients was used. The intra-assay variability was tested by repeated measurements of the same sample. The inter-assay variability was assessed by preparing two different samples of the same patient, followed by measurement on one single flowcytometer.

Three gating strategies were compared in this study. The first method was already described by Hristov et al. (10) and is based on the pre-selection of CD14 positive and low events and the back-gating of this selection. The second gating method as described by Abeles et al. (1) is based on CD14, CD16 and HLA-DR. This gating method is used to make a better distinction between monocytes and lymphocytes and neutrophils. The third method is the most recently described by Selimoglu-Buet et al. (11). This negative gating method makes use of antibodies against CD24 and CD56, to purify the monocyte population from CD24 expressing granulocytes and B cells and CD56 expressing NK cells.

Labeling Procedure

One hundred μ l of EDTA-whole blood was transferred into a 5 ml polystyrene round-bottom tube (BD Biosciences) and incubated for 15 minutes in the dark at RT, simultaneously with conjugated antibodies against CD64 (FITC; clone 10.1; BD Biosciences, San Jose, USA), CD11b (PE, clone D12; BD), HLA-DR (PerCP-Cy5.5; clone L243; BD), CD16 (PE-Cy7; clone B73.1; BD), CD14 (APC; clone M Φ Pg; BD) and CD45 (APC-H7; clone 2D1, BD). For the examination of the influence of antibody clone against CD16, the 3G8 clone (PE-Cy7; BD) was used, next to the B73.1 clone. Erythrocytes were lysed (FACS lysing solution, #349202, BD Biosciences, diluted 1:10 with water) for 10 minutes in the dark at RT. The tube was centrifuged for 4 min at 300G, the supernatant was discarded and the cells were resuspended in 2 ml CellWash (BD). This wash and centrifugation procedure was repeated and the sample was analyzed using flow cytometry (BD FACSCanto flow cytometer; BD Biosciences) and FACSdiva software version 6.1.3 (BD Biosciences).

Statistical Analysis

For the analysis of the sample stability, the coefficient of variation (CV) was calculated of all time points with respect to the 0h sample. The CV of the flow cytometry test must be smaller than 20%. Significance was determined with a one-way ANOVA test. Linear regression analysis and a t-test were used to determine significant differences between the CD16 clones and gating strategies. For the reliability of the repeated measures, CV values and inter- and intra-class variation coefficients (ICC) were calculated. Single and average ICCs were determined, where >0.8 denotes excellent agreement, $0.6-0.8$ denotes good agreement, $0.4-0.6$ denotes fair agreement and <0.4 denotes poor agreement of the results across repeated measures. Data analysis was performed in Microsoft Excel (Microsoft Office), Prism 5.0 (Graphpad) and SPSS Statistics 17.0.0. Statistical significance was defined at the 95% level.

Results

Post-analytic Variable: Gating Strategy

When comparing the CD14 back-gating method with the HLA-DR based gating method, an increase in total monocyte population as percentage of all white blood cells is found when using the CD14 back-gating method. However, this decrease is not significant. The fraction of classical monocytes appeared to be significantly smaller using this gating method in comparison with the HLA-DR based gating method (Figure 1, left). When comparing the same CD14 back-gating method with the CD24 CD56 based gating method, especially the fraction of intermediate monocytes is found to be larger in the CD14 back-gating method.

This difference, however, is not significant. The means of the total monocyte population, the classical monocytes and the non-classical monocytes were neither significant different between the CD24 CD56 gating and CD14 back-gating method. When comparing CD24 CD56 based gating method with the HLA-DR based gating method, an increase in total monocytes as well as in intermediate monocytes is observed using the CD24 CD56 based method. These differences are however not significant. The fraction of classical monocytes is not significant decreased in this method.

Pre-analytic Variable: stability of the Test

The stability of the blood sample as examined using 20 different patient blood samples. For the analysis, the HLA-DR based gating method is used to specify the different monocyte subsets. A significant decrease of classical monocytes was found after 24 hours, compared to 1h and 3h, with a p-value of 0.0006 and 0.0021 respectively (Figure 1, right). Using the same results of the stability test, the coefficients of variation (CV) values were calculated for all monocyte groups at time-points 1h, 3h, 4h and 24h, related to the 0h sample. The medians of all samples at the different time-points are within the 20% range. However, a large variation is observed, especially within the intermediate monocyte population.

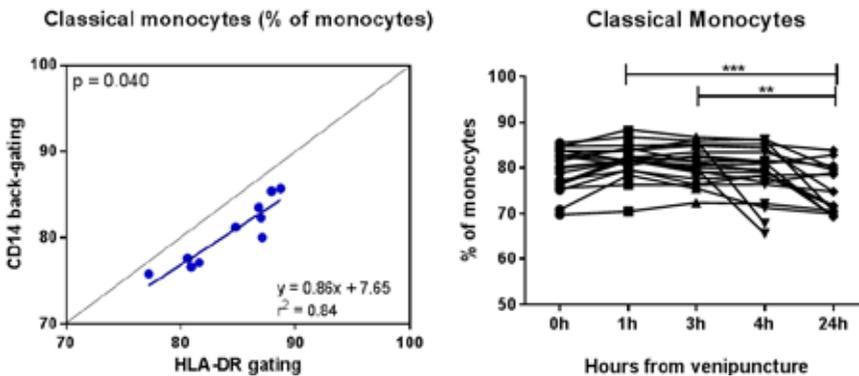


Figure 1. Left: Linear regression analysis of the HLA-DR based gating method and the CD14 back-gating method for the fraction of classical monocytes. Right: stability of the classical monocyte population at different time-points.

Analytic Variable: CD16 Immunolabeling

For CD16 immunolabeling, the B73.1 and 3G8 clone were compared using 20 patient samples. Using the 3G8 clone, a slight decrease in total monocyte population as a percentage of white blood cells is observed, however not significant. The fractions of

classical and non-classical monocytes show a good identity between the two clones, whereas the intermediate monocyte fraction is slightly higher when using the B73.1 clone against CD16.

Analytic Variable: Reproducibility

To determine the intra-assay variation of two measurements performed of the same flowcytometric tube, the CV values were calculated. All intra-assay CVs are beneath the margin of 20% with respect to the mean of both measurements (Table 1). The CVs belonging to the intermediate monocyte population show higher values compared to the other groups, but still below the 20% cut-off value. To determine the inter-assay variation, CV values were calculated of the measurements of two independently prepared flowcytometric tubes. The CVs for all monocyte groups are beneath the margin of 20% and the inter-assay CV of the intermediate monocyte population is again higher compared to other monocyte groups. Next to the CVs, the intra –and inter-assay correlation coefficients (ICC) were determined for all groups of monocytes. All ICC values are above 0.8 and therefore all denote excellent agreement across the repeated measures.

Table 1. Intra-assay CVs (%) of tube 1 and 2 and the inter-assay CV between tube 1 and 2 for the total monocyte (TM) population, classical monocytes (CM), intermediate monocytes (IM), and non-classical monocytes (NCM).

	TM	CM	IM	NCM
Intra-assay CV tube 1	0,84 %	0,42%	7,09%	1,89%
Intra-assay CV tube 2	1,48%	0,99%	8,10%	3,26%
Inter-assay CV tube 1-2	1,63%	0,75%	8,84%	4,47%

Discussion

In the early days, monocytes were considered to be a homogeneous cell population (6). According to the consensus of the Nomenclature Committee of the International Union of Immunological Societies in 2010, monocytes are currently divided into CD14+/CD16- classical monocytes, CD14+/CD16+ intermediate monocytes and CD14+/CD16++ non-classical monocytes. Especially the CD16 positive subsets are known to be implicated in the pathogenesis of many inflammatory diseases (1). Changes in the relative proportions and phenotype of monocytes may have implications for monitoring disease progression and therefore monocytes may be important biomarkers (1). However, the detection and determination of the different monocyte subsets with flow cytometry brings along some technical challenges.

Post-analytic Variable: Gating Strategy

In previous studies of our group (7) a back-gating method was used. A drawback of this method is the unwanted admixture of lymphocytes and neutrophils, which also express CD16. In order to identify the monocyte population and subsets more accurately, three different gating strategies were tested in this study. When comparing the CD14 back-gating method with the HLA-DR based gating method, the total monocyte population shows a decrease in the HLA-DR based gating method. The expected explanation for this is that HLA-DR negative cells, such as lymphocytes and neutrophils are excluded. The classical monocytes show a significant increase using the HLA-DR based gating method. A reason for this could be that not all classical monocytes are selected using the CD14 back-gating method. When creating the back-gate around the monocyte population, there may be some difficulties in distinguishing monocytes at the top of the monocyte population from granulocytes at the bottom of the granulocyte cloud.

The comparison between the CD14 back-gating method with the CD24 CD56 based gating method also shows a decrease of the classical monocyte population in the CD14 back-gate method. The fraction of intermediate monocytes is increased in the CD24 CD56 based gating method, which may be explained by the fact that this method assigns to many intermediate monocytes. The same increase in intermediate monocytes is observed in the CD24 CD56 gating method compared to the HLA-DR gating, which makes it more likely that this gating method may be less accurate in the determination of intermediate monocytes. Also in comparing these two gating methods, a decrease in total monocytes is observed when using the HLA-DR gating method, which may refer to the better purification of the monocyte population.

Pre-analytic Variable: Stability of the test

The results of the stability experiment only show a significant decrease of classical monocytes as a percentage of the total monocyte population after 24h from venipuncture. After 24 hours, a diminishing in cell complexity occurred in all patient samples, which could be visualized in SSC dot plots. This reduction in complexity leads to problems in defining the different leukocyte populations based on scatter properties. Next to this, it was shown in previous research of our group (9), that the binding capacity of cell surface directed antibody-fluorochrome-conjugate CD14-APC and CD16-FITC was reduced after 24h. This may be an explanation for the decreased relative size of the classical monocyte population after 24h. Regarding the CV at the different time points, they were within the predefined cut-off range of 20%. However, the dispersion in CV was large, especially for the

intermediate monocytes. This can be explained by the fact that it is a small cell population within the total monocyte population, and next to this, there is hardly consensus on how to gate and define this subpopulation (12).

Analytic Variable: CD16 Immunolabeling

The comparison of the B73.1 clone with the 3G8 clone used for CD16 labeling does not show prominent differences according to all monocyte groups. Although not significant, a somewhat higher percentage of total monocytes was observed when using the 3G8 clone, which may be the result of the higher staining level of the 3G8 clone. When staining the cell population with the 3G8 clone, granulocytes appeared to be more CD16 positive when compared to the B73.1 clone. This finding is already confirmed by Heimbeck et al., who described the strong neutrophil staining capacity of the 3G8 clone (13). With the appliance of the HLA-DR based gating method, this extremely CD16 positive granulocytes are much easier to identify in a HLA-DR/CD16 dot plot in comparison with the same dot plot with the B73.1 clone. Therefore, immunolabeling with the 3G8 antibody clone against CD16 results in a better distinction between granulocytes and monocytes when using the HLA-DR gating method. Some difficulties may arise in the determination of intermediate monocytes when using the 3G8 clone. Where granulocytes become more CD16 positive, non-classical and intermediate monocytes become less CD16 positive, which results in a down-shift the characteristic monocyte arc. This makes it harder to discriminate between the individual monocyte subsets, with especially the border between the classical and intermediate monocytes hard to establish.

Analytic Variable: Reproducibility

The CV values of the reproducibility experiment, as well as the CV values of the stability experiment demonstrate that the relative size of the intermediate monocytes shows a larger dispersion than the total monocyte population and the other subsets. This larger spread is probably due to the small population size of the intermediate monocytes, as described before. Overall, the CV values of the intra-assay as well as the inter-assay variability show good results with all CV values lower <10%. The ICC values of both variability tests are all above 0.8 and therefore denote excellent agreement.

A limitation of this study is that probably not all participants can be assigned as healthy. Blood samples were drawn from patients from the outdoor clinic and no medical information is available of these patients. A large dispersion may therefore occur between the subtypes and the relative monocyte population sizes may deviate from

previous results concerning the size of the different populations. Another limitation is the small size of the experimental groups used in this study. The use of more participants for each experiment will probably result in more significant and reliable finding.

From this study, it can be concluded that sample preparation and acquisition has to take place within 4 hours after venipuncture in order to make reliable determinations of the monocyte subsets. Next to this, the 3G8 clone is preferred over the B73.1 clone for CD16-epitope labeling and the HLA-DR based gating strategy is assumed to be the most accurate to identify the monocyte subsets. A good reproducibility was found, with all CV values <10%, when making use of the parameters described above.

Role of the student

Imke Demers was an undergraduate student biomedical sciences, with a specialization in molecular life sciences, working under the supervision of M.P.G Leers when the research in this report was performed. The topic was proposed by the supervisor. The processing and analysis of the blood samples, as well as all data analysis and interpretation were done by the student. The writing of the report was also done by the student.

Referencens

1. Abeles RD, McPhail MJ, Sowter D, Antoniadou CG, Vergis N, Vijay GK, et al. CD14, CD16 and HLA-DR reliably identifies human monocytes and their subsets in the context of pathologically reduced HLA-DR expression by CD14(hi) /CD16(neg) monocytes: Expansion of CD14(hi) /CD16(pos) and contraction of CD14(lo) /CD16(pos) monocytes in acute liver failure. *Cytometry A*. 2012;81(10):823-34.
2. Ghattas A, Griffiths HR, Devitt A, Lip GY, Shantsila E. Monocytes in coronary artery disease and atherosclerosis: where are we now? *J Am Coll Cardiol*. 2013;62(17):1541-51.
3. 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.
4. Park BS, Lee JO. Recognition of lipopolysaccharide pattern by TLR4 complexes. *Exp Mol Med*. 2013;45:e66.
5. Janeway C. Appendix II. CD antigens. *Immunobiology*. 2001;5 ed.
6. Idzkowska E, Eljaszewicz A, Miklasz P, Musial WJ, Tycinska AM, Moniuszko M. The role of different monocyte subsets in the pathogenesis of atherosclerosis and acute coronary syndromes. *Scand J Immunol*. 2015.
7. Leers MP, Keuren JF, Frissen ME, Huts M, Kragten JA, Jie KS. The pro- and anticoagulant role of blood-borne phagocytes in patients with acute coronary syndrome. *Thromb Haemost*. 2013;110(1):101-9.
8. Hübl W, Ziegler-Heitbrock LHW, Bayer PM. A Simple Method for Measurement of CD14weakCD16-strong Monocytes in Peripheral Blood. *Immunobiology*. 2000;202(1):2-10.
9. Japink D, Nap M, Sosef MN, Nelemans PJ, Coy JF, Beets G, et al. Reproducibility studies for experimental epitope detection in macrophages (EDIM). *J Immunol Methods*. 2014;407:40-7.

10. Hristov M, Schmitz S, Schuhmann C, Leyendecker T, von Hundelshausen P, Krotz F, et al. An optimized flow cytometry protocol for analysis of angiogenic monocytes and endothelial progenitor cells in peripheral blood. *Cytometry A*. 2009;75(10):848-53.
11. Selimoglu-Buet D, Wagner-Ballon O, Saada V, Bardet V, Itzykson R, Bencheikh L, et al. Characteristic repartition of monocyte subsets as a diagnostic signature of chronic myelomonocytic leukemia. *Blood*. 2015;125(23):3618-26.
12. Zawada AM, Fell LH, Untersteller K, Seiler S, Rogacev KS, Fliser D, et al. Comparison of two different strategies for human monocyte subsets gating within the large-scale prospective CARE FOR HOME Study. *Cytometry A*. 2015.
13. Heimbeck I, Hofer TP, Eder C, Wright AK, Frankenberger M, Marei A, et al. Standardized single-platform assay for human monocyte subpopulations: Lower CD14+CD16++ monocytes in females. *Cytometry A*. 2010;77(9):823-30.