Role and analysis of monocyte subsets in cardiovascular disease

(Joint position paper of the ESC WG on Atherosclerosis & Vascular Biology and the ESC WG on Thrombosis)

Christian Weber[1,2], Eduard Shantsila[3], Michael Hristov[1], Giuseppina Caligiuri[4], Tomasz Guzik[5], Gunnar H. Heine[6], Imo E. Hoefer[7], Claudia Monaco[8], Karlheinz Peter[9], Ed Rainger[3], Agneta Siegbahn[10], Sabine Steffens[1], Johann Wojta[11], Gregory YH Lip[3]

[1]Institute for Cardiovascular Prevention, Ludwig-Maximilians-University (LMU), Munich, Germany

[2]German Centre for Cardiovascular Research, Partner Site Munich Heart Alliance, Munich, Germany

[3] University of Birmingham, Institute of Cardiovascular Sciences, City Hospital, Birmingham, UK.

[4]INSERM, Bichat Hospital, Paris, France

[5]Jagiellonian University, Krakow, Poland & University of Glasgow, Glasgow, UK

[6]Department of Internal Medicine IV, Saarland University Medical Center, Homburg, Germany.

[7]Laboratory of Experimental Cardiology & Laboratory of Clinical Chemistry and Hematology, UMC Utrecht, Netherlands

[8]University of Oxford, Oxford, UK

[9]Atherothrombosis and Vascular Biology, Baker IDI Heart and Diabetes Institute, Melbourne, Australia

[10]Department of Medical Sciences, Clinical Chemistry, Uppsala University, Uppsala, Sweden

[11]Department of Internal Medicine II, Core Facilities, Medical University of Vienna, Vienna, Austria

Abstract

Monocytes as cells of the innate immunity are prominently involved in the development of atherosclerotic lesions. The heterogeneity of blood monocytes has widely been acknowledged by accumulating experimental and clinical data suggesting a differential, subset-specific contribution of the corresponding subpopulations to the pathology of cardiovascular and other diseases. This position paper re-evaluates current nomenclature and summarizes key findings on monocyte subset biology to propose a consensus statement about phenotype, separation and quantification of the individual subsets.

Introduction and preamble

Monocytes represent 3% to 8% of peripheral blood leukocytes. They play an important role in immune defence, inflammation, and tissue remodelling. The key function of circulating monocytes is innate immunity, which is non-specific host protection against foreign pathogens through their prompt detection and elimination. This system is not antigen-specific and depends on pattern recognition receptors for conserved components of various pathogens, including their membrane (e.g., lipopolysaccharides). These pattern recognition receptors include CD14, Toll-like receptors, nucleotide oligomerization domain-like receptors, and retinoic acid-inducible gene I-like receptors, along with other.[1] Monocytes/macrophages can phagocyte pathogens but they also promote pathogen neutralization and elimination by the production of numerous cytokines, antigen processing and presentation.[2]

Monocytes have a remarkable developmental plasticity and following their migration to tissues they can differentiate into various types of macrophages, but also give origin to cells of other lineages at appropriate culture conditions. Monocytes isolated from different anatomical sites show a diversity of phenotypes and function to meet demands of local tissues.[3]

Monocytes contribute to atherogenesis through formation of 'foam' macrophages and promotion of leukocyte recruitment to plaques. Monocytes are also directly involved in a number of chronic inflammatory conditions, such as glomerulonephritis, rheumatoid arthritis and lung fibrosis.[4] High monocyte count and activity could be detrimental to the patient due to a potential of damaging its own tissues and high numbers of monocytes have been linked to higher risk of unfavourable outcomes.[5, 6]

Definition of monocyte subsets

In the past, blood monocytes were analysed using microscopy of stained slides or their light scatter properties in haematology analysers. These approaches are not suitable for analysis of monocyte subsets. Flow cytometry has become the standard method for analysis of monocyte subsets as it provides the capacity of simultaneous analysis of several surface markers combined with the power of fast and observer independent collection of data from a large number of cells.

Following the discovery of several monocyte subsets CD14 and CD16 have emerged as standard markers for definition of monocyte populations.[7] These studies analysed 'classical' monocytes with high expression of CD14 and lacking CD16 (~85%, usually marked as CD14+CD16– subset) as opposed to CD16+ monocytes with lower CD14 expression (~15%, usually designated as CD14low/CD16+). Later it has been found that CD16+ monocytes include two very distinct populations, those with high CD14 and moderate CD16 expression (~9%, CD14++CD16+) and those with low CD14 expression and high CD16 levels (~6%, CD14+CD16++).

The 2010 nomenclature document acknowledged existence of the three subsets and standardized their definition as CD14++CD16– or 'classical', CD14++CD16+ or 'intermediate' and CD14+CD16++ or 'nonclassical' subsets (Table 1).[8] Given that studies of unselected CD16+ monocytes are still reported, it is important to always check for the definition of nonclassical monocytes the authors of a particular publication actually refer to. The 2010 Nomenclature introduced the term of 'intermediate' monocyte subset for the CD14++CD16+ population. The term is based on their CD16 expression and does not fully reflect their developmental, functional or overall phenotypic characteristics. In fact, this subset has the highest rather than intermediate expression of most key monocyte markers. To

avoid ambiguity most recent publications report both the phenotypic definition and the name of the subsets. As the field is rapidly expanding and new monocyte subsets as well as markers for the existing subsets are likely to emerge, it is reasonable to assign numerical codes to the subsets similar to the approach used in other fields in biology (e.g., lymphocytes, cytokines and chemokines). For simplification, we propose the following abbreviations for this document: Mon1 for the CD14++CD16– classical subset, Mon2 for the CD14++CD16+ intermediate subset, Mon3 for the CD14+CD16++ nonclassical subset.[9-12] This designation does not intend to reflect a similarity of Mon1 to M1 macrophages and of Mon2 to M2 macrophages, despite a possibility for some preponderance for polarization[13], and has been used throughout the entire document.

Accumulating evidence clearly demonstrates profound difference between Mon2 and Mon3 in phenotype, function, gene expression and responses to a variety of pathological states. Mon2 are larger and more granular than Mon3 and they have higher expression of multiple receptors including those involved in inflammation/immunity (e.g., Toll-like receptor 4 [TLR4], CCR5, CD11b, HLA-DR, interleukin-6 [IL-6] receptor, integrin β2) and angiogenesis/tissue remodelling (e.g., vascular endothelial growth factor [VEGF] receptors 1 and 2, Tie2, CXCR4) (Table 2).[9, 14, 15] In fact, Mon2 have highest expression of many of those receptors among all monocyte subsets.[9] In contrast, Mon3 have higher expression of integrin α4, SLAN, CX3CR1, sialophorin, CD294, and Siglec10.[9, 15]

Functional studies have shown that Mon2 have a higher antigen presenting capacity (Table 3).[16, 17] Mon2 compared to Mon3 also have a higher phagocytic activity, higher production of tumour necrosis factor (TNF)-α, IL-6 and IL-1β in response to lipopolysaccharide (LPS) and a higher rate of aggregation with platelets.[9, 14] Furthermore, Mon2 were reported to be strong producers of IL-10 and show a high capacity to engulf apoptotic cells similar to Mon3.[9, 18, 19] Mon2 were the only subset able to form cordlike structures in Matrigel after stimulation with

VEGF indicating a potential implication of this subset in angiogenesis.[17] With respect to phagocytosis, Mon2 have been reported to take up carboxylate microspheres at a level between Mon1 and Mon3, whereas for uptake of *Escherichia coli* bacteria, Mon2 showed the highest phagocytic capacity among all 3 subsets.[9, 17, 18]

Global gene expression analysis demonstrated for Mon2 a preferential expression of genes relevant to antigen presentation, phagocytosis and angiogenesis (Tables 4 and 5).[15, 17, 20] Zawada et al. identified 258 genes up-regulated in Mon2, and 301 genes in Mon3 with a $p < 10^{-10}$. The authors noted that Mon2 was distinguished from Mon3 by significantly higher expression of genes linked to defence against microbial pathogens (LYZ, S100A8, CD14, S100A10) and MHC II–restricted antigen processing and presentation (HLA-DRA, CD74, IFI30, HLA-DPB1, CPVL). In contrast, the Mon3 subset expressed higher levels of genes connected to MHC I–restricted processes (HLA-B, B2M), transendothelial mobility (LSP1, LYN, CFL1, MYL6) and cell-cycle progression (CDKN1C, STK10). In another gene expression profiling study Mon2 and Mon3 monocytes strongly differed in the expression of genes involved in cell adhesion, oxidative stress, and phagocytosis, pointing to a distinct transendothelial trafficking potential and a greater capacity of Mon2 for generation of reactive oxygen species as well as for phagocytosis of pathogens.[15]

In view of the above observations, the two subsets differ in their respective characteristics to a degree that their analysis as a single population of CD16+ monocytes is likely to result in misleading conclusions regarding phenotype, function, gene expression and role in pathological conditions for either subset. We therefore conclude that these two subsets should not be analysed as a single population of CD16+ monocytes.

In contrast to ‘classical’ monocytes the primary biological roles of the two minor subsets are still insufficiently understood, but substantial differences in their dynamics and function have been well documented. As opposed to ‘classical’ monocytes ($CCR2^{hi}$Ly6C+ in mouse and

Mon1 in humans), nonclassical monocytes (CX3CR1^{hi}Ly6C– in mouse and Mon3 in humans) showed the capacity of active patrolling of the vascular endothelium under homeostatic and inflammatory conditions.[21] This process is also accompanies by monocyte retention at the endothelial surface and their extravasation under homeostatic state or inflammation to remove damaged cells and debris from the vasculature and contribute to wound healing and tissue recovery.[22, 23] The patrolling behaviour is not unique for nonclassical monocytes as observed in mice. In humans, long-range crawling behaviour has been demonstrated for Mon1 and Mon3. However, this phenomenon was also observed with Mon2, which once again mandates delineation of the three subsets in human studies.[24]

Possible developmental relationships between the different monocyte subsets remain unclear. Classical monocytes are known to acquire CD16 expression during their maturation into macrophages, which may suggest that Mon2 are a more mature or activated form of Mon1.[25] However, analyses of bone marrow samples do not support this hypothesis. In contrast, cells with Mon2 phenotype are the dominant type of bone marrow monocytes.[9, 26] These cells express surface markers typical of intermediate monocytes, show phagocytic and angiogenic properties and produce intracellular oxygen species.[9] These observations indicate that the Mon1 and Mon3 phenotypes are predominantly acquired in circulation by maturation of bone marrow Mon2 cells. In this scenario prominent upregulation of Mon2 in patients with acute cardiovascular and chronic inflammatory states may at least partly reflect accelerated mobilisation from bone marrow.[27, 28] Another study has shown that in people undergoing myloablation and subsequent bone marrow transplantation, the first cells in the peripheral blood are indeed classic monocytes, directly followed by the appearance of intermediate and finally nonclasscial monocytes.[29]

Characterisation and quantification of monocyte subsets by flow cytometry

Sample collection and processing

Monocyte subsets are most often analysed from peripheral venous blood samples. The methodological considerations below are likely to be similarly applicable to analysis of arterial samples. Analysis of monocyte subsets in tissues is seldom done in humans and requires consideration of phenotypic changes due to maturation into different types of macrophages.

Several pre-analytical aspects have to be considered when analysing monocyte subsets. The blood should be obtained ideally by venepuncture or arterial puncture. Ethylenediamine tetraacetic acid (EDTA) anticoagulated blood should be used for quantification of the subsets, but other anticoagulants, such as heparin may be used to assess functional characteristics of monocytes and their interactions with platelets. Anticoagulants have to be specified in the reports for every analysis. The freshly obtained blood should be kept at room temperature and processed immediately (ideally no later than 2 hours) as more prolonged storage may affect monocyte phenotype.[30] For example, two hour delay in sample processing results in appearance of CD16 on Mon1, and numbers of CD16+ monocytes significantly increase after 4 hours of storage at room temperature.[30]

Blood samples should be taken at the same time-of-day, typically in the morning since there is a diurnal variation in Mon2 subset.[30, 31] Before sampling, the donor should be allowed to rest for at least 5 minutes in order to avoid increases of CD16+ monocytes due to preceding exercise.[30, 32] Since excessive stress with catecholamine release may mobilize CD16+ monocytes, blood sampling should be done under low stress conditions.[33, 34]

Density gradient isolated mononuclear cells should not be used for monocyte quantification, but the approach can be used for isolating individual monocyte subsets for *in vitro*

experiments on monocyte function and phenotypic analysis. It should be kept in mind that monocytes are extremely sensitive to any manipulation and changes in expression of surface molecules has been noted after cell preparation procedures.[35] Also monocytes respond vigorously to LPS-contaminated reagents. Thus, short protocols from beginning of processing to sample fixation or immediate acquisition by flow cytometry should be used.

Controlled freezing and storage in liquid nitrogen at -196°C may lead to clumping of monocytes during the recovery process. A comparison of subset numbers and properties of liquid nitrogen-stored monocytes versus fresh monocytes and whole blood monocytes would be required in order to ensure the validity of results with liquid nitrogen-stored cells. The current recommendation is to study blood monocytes with minimal processing using fresh whole blood analysis, wherever possible. Gentle continuous rotation of samples during storage/staining has been used to prevent possible adhesion of monocytes to the wall of sampling tubes and minimise their interaction with other blood cells such as platelets, but benefits of this approach require further assessment.

Staining and enumeration of monocytes

For quantification of monocyte subsets, typically 50-100 μL of anticoagulated whole blood optimally obtained by reverse pipetting is mixed with monoclonal antibodies and incubated for 15-20 min at room temperature. Staining of monocyte subsets on ice has been reported and can be used when inhibition of particular monocyte functions is required (e.g. phagocytosis). Benefits of routine staining on ice are not obvious and such protocols need to allow longer staining time (up to 30 min) to ensure adequate saturation of monocyte epitopes.

Absolute counts of individual subsets (cells per μL or mL) should always be reported for studies of human monocytes. Percentages of the subsets to the total monocytes can be additionally reported when relevant. Single-platform approach using counting beads added to

the whole blood is preferred to the dual-platform approach, when a separate hematoanalyser is used to obtain total leukocyte or monocyte counts with proportions of monocyte subsets obtained by flow cytometry. Since the leukocytes are determined with two different types of instruments the two-platform approach shows lower precision compared to the single-platform. For CD34+ hematopoietic stem cells in blood, the superiority of the single-platform over the dual-platform approach has been clearly demonstrated.[36] Similarly, it is recommended to use absolute counting beads for enumeration of blood monocytes.[37] Either commercial flow cytometry tubes containing lyophilized bead pellets or counting bead suspensions can be utilized. The use of commercial tubes is expensive but more accurate as it eliminates potential pipetting errors related to adding counting beads from suspensions.

After staining, red blood cells are lysed and stained cells typically fixed. Utilisation of lysing solutions containing a fixative agent has putative benefits of shorter exposure of unfixed monocytes to LPS contaminations within reagents. After fixation the samples are directly entered into a multicolour flow cytometer (lyse no-wash strategy). Fixation can be omitted if a flow cytometer is available for immediate analysis (within 1 hour of lysing) as the use of unfixed samples allows proper exclusion of dead cells by their scatter properties.

Separation of monocytes from non-monocytes

The initial gating aims to separate monocytes from other leucocytes, dead cells and debris. This can be achieved using optical properties of monocytes and their expression of certain surface markers (Figure). Admittedly there is a certain scatter overlap between the three types of leucocytes and every care must be taken to minimise loss of monocytes for further quantification, while avoiding contamination by other leucocyte populations. Particular care is needed for discrimination of lymphocytes from Mon3, the smallest and least granular monocyte subset. Several approaches have been used to achieve this.

One approach is to use an additional 'pan-leukocytic' (e.g. CD45) or even a 'pan-monocytic' (HLA-DR or CD86) marker which is solely expressed by monocytes. HLA-DR is an MHC class II molecule expressed at high levels on the key antigen-presenting cells, such as macrophages/monocytes, B-lymphocytes and dendritic cells. In healthy volunteers, HLA-DR showed 2.5-fold lower expression on Mon3 than Mon2.[15] Monocyte expression of the receptor is upregulated under immune stimulation, but the magnitude of these changes specifically on individual monocytes subsets is not known. The receptor is not expressed on granulocytes and is therefore useful for their exclusion. Given that the expression of HLA-DR on B-lymphocytes and dendritic cells, utility of the receptor for separation of monocyte from these cells is less certain, but it will help to remove T-lymphocytes.

CD86 is also expressed on antigen-presenting cells including monocytes and it plays a role in promoting T cell activation and survival.[38] It is also expressed on effector memory T cells.[39] Given that monocytes are the major type of CD86 expressing cells, the marker has been widely used to improve the separation of monocytes from non-monocytes.[40, 41]

As an alternative to additional use of CD86 or HLA-DR a step-wise gating based on scatter and CD14/CD16 has been utilised and it relies on clear discrimination of granulocytes from monocytes using CD14 vs. side scatter plots and an additional negative selection of lymphocytes (CD14– and CD16–) and natural killer cells (CD14– and CD16+), which cluster distinct from monocytes on a preselected CD14 vs. CD16 gate.[9] However, with such an approach, some nonclassical monocytes with low CD14 expression will be excluded from analyses and their counts will be lower than when using pan-monocyte markers for analyses.

Separation of Mon1 from CD16+ monocytes

Monocytes identified by the strategy above described are plotted for their CD14 vs. CD16 expression (Figure). Careful optimisation of the protocol setup is essential to allow accurate discrimination of CD16+ and CD16– monocytes, given that Mon1 and Mon2 do not form distinct clusters when assessed using CD14/CD16. At present, there is no marker to demonstrate distinct clusters of Mon1 and Mon2. The two subsets form a single cluster on CD14 vs. CD16 plot and their discrimination relies on careful setup of a cut-off between CD16+ and CD16– cells.

Separation of Mon2 from Mon3

The Mon2 are discriminated from Mon1 based on CD16 expression, but their differentiation from Mon3 is more difficult. Based on CD14/CD16 expression, Mon2 and Mon3 subsets do not form separate clusters and several approaches have been used to discriminate them. One approach is to draw a vertical or an oblique line placed to the left of the CD14++ population of classical monocytes (see Figure).[42] These lines define CD14++CD16+ (Mon2) and CD14+CD16++ (Mon3) subsets. When defining cells by gating in the CD14/CD16 plot, the Mon2 were shown to have prognostic value with respect to cardiovascular events in CKD patients with or without dialysis and in cardiovascular high-risk patients undergoing coronary angiography.[40, 41, 43] In addition, increases in Mon2 identified by this gating approach have been reported in a broad range of inflammatory and infectious diseases, including sepsis, tuberculosis, Crohn's disease and asthma.[44]

A recently published study based on a large cohort of patients compared monocyte subsets by two gating strategies, a 'rectangular gating strategy' (i.e., using vertical lines) and a 'trapezoid gating strategy' (i.e., using oblique lines). With both gating strategies, higher Mon2 counts had similar strength to predict cardiovascular outcomes. Regardless of the

gating strategy the monocyte subsets shared similar phenotypic characteristics.[45]

Given a degree of subjectivity in this approach, it is essential that firm rules of the delineator positioning are established before analysis and that analyses are performed in a blinded manner. Search for markers for unambiguous identification of Mon2 and Mon3 has suggested CCR2 to be highly expressed on Mon2, but only minimally expressed on Mon3 (8-fold difference was reported in one study[9] while another study suggested no CCR2 expression on Mon3).[14] The best discrimination of the two subsets can be achieved using CCR2 vs. side scatter plot, thus additionally employing differences in granularity between Mon2 (more granular) and Mon3 (less granular).[9]

A recent study tested utility of 6-sulfo LacNAc (SLAN) to delineate Mon2 (SLAN–) and Mon3 (SLAN+).[46] Gene expression profiling identified that SLAN– monocytes had highest expression levels of MHC class II genes, consistent with their attribution as Mon2. Utilisation of SLAN also distinguished Mon3 from CD11c+ dendritic cells. Utility of this marker was further supported by clinical studies showing selective increase in SLAN– Mon2 in sarcoidosis and a 5-fold depletion of the SLAN+ Mon3 in hereditary diffuse leukodystrophy with axonal spheroids.[46]

Characterization of monocyte subsets

In addition to changes in numbers, alterations in phenotype and function may occur in disease. Cell surface markers are best defined by multicolour flow cytometry and their expression level has to be quantified as median fluorescent intensity. It is important to remember some crucial recommendations that should be taken in mind when designing a multicolour experiment such as antigen co-expression and intensity, fluorochrome brightness and spillover, antibody clones and instrument settings. It is thus crucial to keep these settings stable for all samples analysed, with daily calibration of the flow cytometer in use.

It has been shown that decreased HLA-DR on classical monocytes indicates an immune-depressed state,[47] that lack of integrins prevents leukocyte-to-endothelial cell interaction,[48] CD16+ monocytes lacking the CCR2 chemokine receptor do not migrate in response to CCL2[49] and expression of tissue factor (TF) on monocytes, notably CD16+ monocytes may indicate a procoagulant state.[50, 51] Therefore, the study of these and other cell surface markers on monocyte subsets can be informative in a clinical setting. Markers to be analysed for clinical use require standardization and calibration with specific reference beads as has been implemented for HLA-DR expression on monocytes.[47]

Important functions of monocytes include cytokine production, phagocytosis and antigen presentation. Cytokine production requires activation of monocytes and this is best done with whole blood and LPS as a prototypic activator, which activates monocytes via TLR4. In fact, isolation of monocyte subsets from blood can lead to accidental activation of monocytes due to inadvertent contamination of reagents with LPS. A typical read-out system for such functional studies uses whole blood and intracellular staining for cytokines like TNFα. Here the secretion of the cytokine is prevented by blockade of the Golgi system. Production of the respective cytokine can then be assigned to selective monocyte subsets in multicolour flow cytometry.[9, 52]

Phagocytosis is a central function of monocytes. It involves uptake and degradation of microbes and of apoptotic cells and this may culminate in the presentation of antigen to T cells. Phagocytosis tests can be performed with fluorochrome-tagged particles with and without antibody and complement binding to the particle. It has been demonstrated that Mon1 and Mon2 avidly phagocytes *Escherichia coli* whilst Mon3 showing only minimal phagocytic activity.[9] Earlier studies of the two CD16+ subsets without subset differentiation showed conflicting results which may either reflect relative predominance of Mon2 or Mon3

in the conditions tested or involvement of different surface receptors in various phagocytosis assays.[7, 53]

Analysis of antigen presentation with the current technologies requires purification of monocytes including density gradient separation followed by isolation of subsets using magnetic beads or cell sorting, which can activate monocytes and affect the functional properties of the individual subsets. Hence, results from any assay which mandates monocyte (subset) purification have to be interpreted with care. The purified cells can be put into culture with allogeneic T cells or with autologous T cells and soluble antigen. Analysis of T cell proliferation is done either using incorporation of 3H-Thymidine or stable labelling of T cells with lipophilic dyes like CFSE. Collectively, such analyses have shown higher levels of T cell activation in co-cultures with CD16+ monocytes as compared to Mon1.[16, 17]

An important feature of monocytes in the context of cardiovascular disease is the formation of monocyte platelet aggregates (MPA).[54, 55] These aggregates form via interaction of P-selectin on activated platelets and with PSGL1 on leukocytes and with additional adhesion molecules.[56] MPAs are indicative of platelet activation and they may have a role in induction of gene activation in monocytes, such as genes for proinflammatory cytokines and TF. To preserve the labile aggregates in their *in vivo* state, whole blood is sampled with non-chelating anti-coagulants followed by immediate staining at room temperature with CD14 and CD42 to identify monocytes and platelets, respectively. With respect to monocyte subsets, higher proportions of MPAs have been reported for Mon2 as compared to Mon1 and Mon3.[9] On the other hand, several studies revealed that Mon2 have the highest rate of aggregation with platelets.[9, 27, 57] This could be partly due to higher levels of P-selectin glycoprotein ligand-1 on the Mon2 population.[58] These variations likely reflect very complex interactions of monocytes and platelets that involve multiple pathways ranging from widespread (possibly almost constant) temporal interactions between the cells to irreversible

contacts (e.g., in case of phagocytosis of activated platelets by monocytes).[59] It has been demonstrated that 'weak' contacts are more common for Mon1 and irreversible contacts for Mon3.[59] Careful protocol design is essential with particular attention paid to the choice of anticoagulant to match study aims and minimal delay in sample processing.[30, 59] Increase in MPA could be due to increased monocyte counts and/or increase in percentage of monocytes aggregated with platelets. and it thus would be a good practice. Therefore, it is useful to report both absolute MPA values and MPA per cent of monocytes for individual subsets.[27]

Recently, a contribution of monocytes in angiogenesis has been recognized. In this context, cells expressing the angiopoetin receptor Tie2 have been shown to support angiogenesis *in vitro* and *in vivo*.[17, 60, 61] Previously, monocyte/macrophage induced angiogenesis has been reported in the context of tumour vessel formation, but more recently its role in occlusive cardiovascular disease has come into focus.[62, 63] Mouse classical monocytes were shown to improve capillary density, and both classical and nonclassical monocytes improved the angiographic score.[62] Also, human CD16+ monocytes transferred into mice were shown to improve post-ischemic arteriole size.[63] Further analysis of human intermediate and nonclassical monocytes showed a significant differences between these subsets, with Mon2 having higher expression of Tie2, VEGF receptors type 1 and 2 and CXCR4.[9] Hence, different monocyte subsets may have specific angiogenic capacities, which can be crucial for neovascularization in cardiovascular disease.

Other considerations for analysis

Reproducibility of flow cytometry measurement depends on the number of events collected for analysis. It is recommended that a minimum of 400 events for the rarest subset is collected which will result into estimated coefficient of variability of measurement of 5% providing all other standard quality control measures are ensured.[64, 65]

Numbers of CD16+ monocytes where shown to be lower in females and to increase with age.[66 67] Therefore, clinical studies including analysis of monocyte subsets need age and sex matched control groups. Also, the effects of concurrent therapy need to be considered. Glucocorticoids are most relevant, since they deplete Mon3 but may increase counts of Mon1 and Mon2 monocytes.[29] In patients requiring such treatment, sampling for monocyte subset analysis should be done before starting any systemic glucocorticoid therapy or any other treatment (e.g. non-steroid immunosuppressants) that can affect monocyte characteristics.

In patients with major illness including those in intensive care units or patients with acute cardiovascular events, mobilization of monocyte subsets from the marginal pool may occur which might be responsible for the increase in CD16+ monocytes in such diseases.[68] Because catecholamines can mobilize monocytes, it is conceivable that adrenergic blockers may decrease the number of CD16+ monocytes from baseline. Barrisone et al found differences in Mon2 numbers between patients with and without beta-blocker therapy in chronic heart failure but more studies are required in order to settle this point.[69]

Considerations for analysis of monocyte subsets in mice

While human monocyte subsets were defined using monoclonal antibodies and flow cytometry in 1989, the first evidence for the mouse was based on the differential expression of a CX3CR1-promotor-driven GFP transgene in 2001.[7, 70] Since the GFP signal in this mouse model is only slightly different between monocyte subsets, the current approach to mouse blood monocytes is based on the use of fluorochrome-conjugated monoclonal antibodies.

In the mouse, blood for monocyte analysis is typically taken by puncture of the orbital venous plexus or by cardiac puncture under terminal anesthesia. Such conditions represent maximal stress, which will mobilize monocytes from the marginal pool. Compared to man, where

blood is taken under low stress conditions at rest, it therefore can be expected that nonclassical monocytes in mouse blood samples represent a higher fraction of monocytes.

As in man, the recommended approach for analysis in the mouse is to avoid any processing and to use whole blood. Typical markers used are CD115, CD43 and Ly6C.[71] CD115 recognizes the M-CSF-receptor and is a marker used to identify monocytes as such.[72] Both CD43 and Ly6C have a broad distribution among leukocytes, and they show an informative differential expression pattern between classical (Ly6C++CD43+) and nonclassical monocytes (Ly6C+CD43++).[73, 74] The Ly6C reagent is to be preferred over the Gr-1 antibody, since the latter also reacts with Ly6G on granulocytes. Finally, standard beads to determine the absolute count of monocytes in mouse blood need to be added.

Further characterization of mouse monocyte subsets demonstrated lower levels of CCR2 on the nonclassical monocytes, a selective interaction of these cells with the endothelium and a higher level of TLR-ligand induced TNF production.[75-77] While all of these features are shared with the nonclassical monocytes in man, a detailed comparison of gene expression profiles and marker expression demonstrated substantial differences with a reverse pattern for some cell surface molecules between monocyte subsets of man and mouse.[78] CD36, for instance, is exclusively expressed by mouse nonclassical monocytes and in man by classical monocytes. Hence, findings on monocyte subsets in the mouse disease models cannot be directly transferred to man but need confirmation in the respective patients. In a seminal paper on mouse monocyte subsets, the intermediate monocytes have been defined based on Ly6C and CD43 expression patterns but to date no further information on this subset in the mouse has been published.[72] Therefore, analysis of these cells has a lot of potential in mouse models of cardiovascular disease.

Monocyte subsets in cardiovascular disease

Monocyte-derived macrophages are key cells contributing to atherosclerotic plaque development and instability. Accordingly, the relative contribution of the monocytes subsets in atherosclerosis, its risk factors and complications has attracted major interest.[79-81]

In patients with chronic kidney disease (CKD), a condition with high risk of atherosclerosis-related complications, counts of intermediate and nonclassical monocytes are increased compared to healthy people. Moreover, in CKD low Apo-I and low high density lipoprotein (HDL) cholesterol levels were correlated with high Mon2 levels, and Mon2 was the only subset to independently predict cardiovascular events.[82, 83] Likewise, subjects with stable atherosclerosis and high Lp(a) had higher proportions of Mon2.[84]

Mon2 showed *in vitro* augmented lipid accumulation and oxidized low density lipoprotein (LDL) cholesterol uptake with lower cholesterol efflux compared to other subsets.[82] Mon2 revealed high expression of cholesterol scavenging receptors (CD36, CD68), but low expression of ATP-binding cassette transporter (ABCA1) implicated in cholesterol efflux.[82] *In vitro* experiments further demonstrated that native LDL promotes whereas HDL inhibits the Mon1 phenotype.[13] In an older study, a low HDL-cholesterol and the apoE4 allele were associated with higher proportion of blood CD16+ subsets, but this study did not differentiate Mon2 and Mon3.[85] Recently, mice experiments showed that a triglyceride-rich environment changes proportions of circulating monocyte subsets by promotion of the extravasation of $Gr1^{low}$ cells, the subset that has resemblance to human Mon3.[22]

However, roles of monocyte subsets in atherosclerosis are complex and all human monocyte subsets appear to be related to certain patterns of dyslipidaemia. ApoE production, an important process in the prevention of atherosclerosis, was demonstrated for Mon1 and Mon2, but not for Mon3.[86] In patients with stable coronary artery disease (CAD), levels of

small HDL and atherogenic small dense LDL correlated with Mon3 and were inversely associated with Mon1.[87, 88] In a study of subjects with or without carotid atherosclerosis, high counts of Mon1 only were significantly predictive of carotid stenosis, intima-media thickness and advanced plaque neovascularization (a surrogate for high risk of plaque rupture).[89] Precise roles of individual subsets in different aspects of atherogenesis still merit further investigation.

Multiple lines of evidence suggest specific changes in Mon2 in cardiovascular diseases. Patients with acute myocardial infarction, unstable angina, acute heart failure, abdominal aneurysm and stroke have elevated Mon2, which correlated with troponin T levels while Mon3 counts are not significantly different (Table 6).[27] Moreover, Mon3 were reduced in patients with stroke.[90, 91] Mon2 are the only subset independently associated with occurrence of cardiovascular events in patients referred for elective coronary angiography and in those with CKD, but Mon3 did not demonstrate prognostic implications in these patients.[40, 83] Of interest, salt loading led to rise in Mon2 levels and monocyte pro-inflammatory activation, which was reverted by salt depletion.[92] These observations could provide further insight in hypertension, a cardiovascular risk factor closely associated with high dietary salt intake.

Limited data are currently available on the effect of cardiovascular therapeutic interventions on levels, phenotype and activation of individual monocyte subsets. A difference in Mon2 counts appeared to vary depending on beta-blocker use in chronic heart failure.[69]

Twelve weeks treatment with bezafibrate decreased circulating CD16+ monocytes in patients with type 2 diabetes.[93] Temporal withdrawal of statins had no significant impact on monocyte counts but was associated with some phenotypic changes.[94] In patients with familial hypercholesterolemia, Mon3 exhibited an increased uptake of oxidized LDL via CD36, whereas Mon1 preferentially take up native LDL. Mon3 have a higher adherence to activated endothelial cells in response to oxidized LDL and native LDL stimulation.[95]

In a double-blind randomized study of hypercholesterolemic patients, fluvastatin was compared to placebo (both on low lipid diet). Although there was no difference in the proportion of monocyte subsets at the beginning of the study, after 52 weeks of the intervention percentage of Mon1 was lower and percentages of Mon2 and Mon3 were higher in the fluvastatin group.[96] However, the study results show unusually low proportions of Mon1 (means of 55-60%) and unusually high proportions of Mon2 (means of 25-35%). Mon3 were infrequent at the beginning of the study (mean of 7.6%), but their percentages almost doubled at the end of the study. The study results thus need to be interpreted with care (the study report does not provide pictures of the flow cytometric approach used). Another study assessed hypercholesterolemic sedentary who were given rosuvastatin (10 mg daily) and randomly assigned to receive exercise training. These two groups were additionally compared to a separate control group.[97] The study has not found any effect of rosuvastatin on proportions of Mon1 and on a combination of CD16+ subsets. Exercise training reduced percentage of the CD16+ subsets by 20 weeks (but not at 10 weeks). Also in patients with unstable angina, treatment with statins was associated with lower proportions of the two CD16+ subsets analysed together, but there was no relationship between the CD16+ monocytes and LDL or LDL/HDL cholesterol ratio.[98] The conflicting results of these studies may reflect used methodology of monocyte assessment and a prospective randomised study is needed to establish effects of statins on the three monocyte subsets.

In view of expanding data on the supporting role of monocyte subsets in a range of pathological states, development of specific treatments (e.g., biological agents) to modulate the number or function of individual monocyte subsets would be desirable. Until such data are available, individuals should be encouraged to follow general life style recommendations for primary and secondary disease prevention, such as weight reduction and smoking cessation.

Consensus statement

New analyses of CD16+ monocytes must distinguish the CD14++CD16+ intermediate and CD14+CD16++ nonclassical subsets. Flow cytometric analysis of monocyte subsets should include a minimum of CD14 and CD16 with either rectangular or trapezoid gating applied to separate intermediate and nonclassical monocytes. If technical capacity allows, the addition of 'pan-marker' (e.g., CD45, CD86 or HLA-DR) and another marker to separate the two CD16+ subsets (e.g., CCR2 or SLAN) is further advised. The current recommendation is to study blood monocytes with minimal processing using fresh whole blood analysis, wherever possible. Reporting absolute counts of monocyte subsets established using a single-platform approach should become a standard.

REFERENCES

1. Janeway CA, Jr. and Medzhitov R. Innate immune recognition. *Annual review of immunology*. 2002;20:197-216.

2. Kantari C, Pederzoli-Ribeil M and Witko-Sarsat V. The role of neutrophils and monocytes in innate immunity. *Contributions to microbiology*. 2008;15:118-46.

3. Zhao Y, Glesne D and Huberman E. A human peripheral blood monocyte-derived subset acts as pluripotent stem cells. *Proceedings of the National Academy of Sciences of the United States of America*. 2003;100:2426-31.

4. Monie TP, Bryant CE and Gay NJ. Activating immunity: lessons from the TLRs and NLRs. *Trends Biochem Sci*. 2009;34:553-61.

5. Horne BD, Anderson JL, John JM, Weaver A, Bair TL, Jensen KR, Renlund DG, Muhlestein JB and Intermountain Heart Collaborative Study G. Which white blood cell subtypes predict increased cardiovascular risk? *Journal of the American College of Cardiology*. 2005;45:1638-43.

6. Zalai CV, Kolodziejczyk MD, Pilarski L, Christov A, Nation PN, Lundstrom-Hobman M, Tymchak W, Dzavik V, Humen DP, Kostuk WJ, Jablonsky G, Pflugfelder PW, Brown JE and Lucas A. Increased circulating monocyte activation in patients with unstable coronary syndromes. *Journal of the American College of Cardiology*. 2001;38:1340-7.

7. Passlick B, Flieger D and Ziegler-Heitbrock HW. Identification and characterization of a novel monocyte subpopulation in human peripheral blood. *Blood*. 1989;74:2527-34.

8. Ziegler-Heitbrock L, Ancuta P, Crowe S, Dalod M, Grau V, Hart DN, Leenen PJ, Liu YJ, MacPherson G, Randolph GJ, Scherberich J, Schmitz J, Shortman K, Sozzani S, Strobl H, Zembala M, Austyn JM and Lutz MB. Nomenclature of monocytes and dendritic cells in blood. *Blood*. 2010;116:e74-80.

9. Shantsila E, Wrigley B, Tapp L, Apostolakis S, Montoro-Garcia S, Drayson MT and Lip GY. Immunophenotypic characterization of human monocyte subsets: possible implications for cardiovascular disease pathophysiology. *Journal of thrombosis and haemostasis : JTH*. 2011;9:1056-66.

10. Van Craenenbroeck AH, Van Ackeren K, Hoymans VY, Roeykens J, Verpooten GA, Vrints CJ, Couttenye MM and Van Craenenbroeck EM. Acute exercise-induced response of monocyte subtypes in chronic heart and renal failure. *Mediators of inflammation*. 2014;2014:216534.

11. Zeng S, Zhou X, Ge L, Ji WJ, Shi R, Lu RY, Sun HY, Guo ZZ, Zhao JH, Jiang TM and Li YM. Monocyte subsets and monocyte-platelet aggregates in patients with unstable angina. *J Thromb Thrombolysis*. 2014;38:439-46.

12. Rong MY, Wang CH, Wu ZB, Zeng W, Zheng ZH, Han Q, Jia JF, Li XY and Zhu P. Platelets induce a proinflammatory phenotype in monocytes via the CD147 pathway in rheumatoid arthritis. *Arthritis research & therapy*. 2014;16:478.

13. Al-Sharea A, Lee MK, Moore XL, Fang L, Sviridov D, Chin-Dusting J, Andrews KL and Murphy AJ. Native LDL promotes differentiation of human monocytes to macrophages with an inflammatory phenotype. *Thrombosis and haemostasis*. 2015;115.

14. Cros J, Cagnard N, Woollard K, Patey N, Zhang SY, Senechal B, Puel A, Biswas SK, Moshous D, Picard C, Jais JP, D'Cruz D, Casanova JL, Trouillet C and Geissmann F. Human CD14dim monocytes patrol and sense nucleic acids and viruses via TLR7 and TLR8 receptors. *Immunity*. 2010;33:375-86.

15. Wong KL, Tai JJ, Wong WC, Han H, Sem X, Yeap WH, Kourilsky P and Wong SC. Gene expression profiling reveals the defining features of the classical, intermediate, and nonclassical human monocyte subsets. *Blood*. 2011;118:e16-31.

16. Grage-Griebenow E, Zawatzky R, Kahlert H, Brade L, Flad H and Ernst M. Identification of a novel dendritic cell-like subset of CD64(+) / CD16(+) blood monocytes. *European journal of immunology*. 2001;31:48-56.

17. Zawada AM, Rogacev KS, Rotter B, Winter P, Marell RR, Fliser D and Heine GH. SuperSAGE evidence for CD14++CD16+ monocytes as a third monocyte subset. *Blood*. 2011;118:e50-61.

18. Skrzeczynska-Moncznik J, Bzowska M, Loseke S, Grage-Griebenow E, Zembala M and Pryjma J. Peripheral blood CD14high CD16+ monocytes are main producers of IL-10. *Scandinavian journal of immunology*. 2008;67:152-9.

19. Mikolajczyk TP, Skrzeczynska-Moncznik JE, Zarebski MA, Marewicz EA, Wisniewska AM, Dzieba M, Dobrucki JW and Pryjma JR. Interaction of human peripheral blood monocytes with apoptotic polymorphonuclear cells. *Immunology*. 2009;128:103-13.

20. Schmidl C, Renner K, Peter K, Eder R, Lassmann T, Balwierz PJ, Itoh M, Nagao-Sato S, Kawaji H, Carninci P, Suzuki H, Hayashizaki Y, Andreesen R, Hume DA, Hoffmann P, Forrest AR, Kreutz MP, Edinger M, Rehli M and consortium F. Transcription and enhancer profiling in human monocyte subsets. *Blood*. 2014;123:e90-9.

21. Thomas G, Tacke R, Hedrick CC and Hanna RN. Nonclassical patrolling monocyte function in the vasculature. *Arteriosclerosis, thrombosis, and vascular biology*. 2015;35:1306-16.

22. Saja MF, Baudino L, Jackson WD, Cook HT, Malik TH, Fossati-Jimack L, Ruseva M, Pickering MC, Woollard KJ and Botto M. Triglyceride-Rich Lipoproteins Modulate the Distribution and Extravasation of Ly6C/Gr1(low) Monocytes. *Cell reports*. 2015;12:1802-15.

23. Misharin AV, Cuda CM, Saber R, Turner JD, Gierut AK, Haines GK, 3rd, Berdnikovs S, Filer A, Clark AR, Buckley CD, Mutlu GM, Budinger GR and Perlman H. Nonclassical

Ly6C(-) monocytes drive the development of inflammatory arthritis in mice. *Cell reports*. 2014;9:591-604.

24. Collison JL, Carlin LM, Eichmann M, Geissmann F and Peakman M. Heterogeneity in the Locomotory Behavior of Human Monocyte Subsets over Human Vascular Endothelium In Vitro. *Journal of immunology (Baltimore, Md : 1950)*. 2015;195:1162-70.

25. Wang ZQ, Bapat AS, Rayanade RJ, Dagtas AS and Hoffmann MK. Interleukin-10 induces macrophage apoptosis and expression of CD16 (FcgammaRIII) whose engagement blocks the cell death programme and facilitates differentiation. *Immunology*. 2001;102:331-7.

26. Mandl M, Schmitz S, Weber C and Hristov M. Characterization of the CD14++CD16+ Monocyte Population in Human Bone Marrow. *PloS one*. 2014;9:e112140.

27. Tapp LD, Shantsila E, Wrigley BJ, Pamukcu B and Lip GY. The CD14++CD16+ monocyte subset and monocyte-platelet interactions in patients with ST-elevation myocardial infarction. *Journal of thrombosis and haemostasis : JTH*. 2012;10:1231-41.

28. Rossol M, Kraus S, Pierer M, Baerwald C and Wagner U. The CD14(bright) CD16+ monocyte subset is expanded in rheumatoid arthritis and promotes expansion of the Th17 cell population. *Arthritis and rheumatism*. 2012;64:671-7.

29. Rogacev KS, Zawada AM, Hundsdorfer J, Achenbach M, Held G, Fliser D and Heine GH. Immunosuppression and monocyte subsets. *Nephrology, dialysis, transplantation : official publication of the European Dialysis and Transplant Association - European Renal Association*. 2015;30:143-53.

30. Shantsila E, Tapp LD, Wrigley BJ, Montoro-Garcia S, Ghattas A, Jaipersad A and Lip GY. The effects of exercise and diurnal variation on monocyte subsets and monocyte-platelet aggregates. *European journal of clinical investigation*. 2012;42:832-9.

31. Dimitrov S, Lange T, Nohroudi K and Born J. Number and function of circulating human antigen presenting cells regulated by sleep. *Sleep*. 2007;30:401-11.

32. Steppich B, Dayyani F, Gruber R, Lorenz R, Mack M and Ziegler-Heitbrock HW. Selective mobilization of CD14(+)CD16(+) monocytes by exercise. *American journal of physiology Cell physiology*. 2000;279:C578-86.

33. Kittner JM, Jacobs R, Pawlak CR, Heijnen CJ, Schedlowski M and Schmidt RE. Adrenaline-induced immunological changes are altered in patients with rheumatoid arthritis. *Rheumatology*. 2002;41:1031-9.

34. Dimitrov S, Lange T and Born J. Selective mobilization of cytotoxic leukocytes by epinephrine. *Journal of immunology (Baltimore, Md : 1950)*. 2010;184:503-11.

35. Lundahl J, Hallden G, Hallgren M, Skold CM and Hed J. Altered expression of CD11b/CD18 and CD62L on human monocytes after cell preparation procedures. *Journal of immunological methods*. 1995;180:93-100.

36. Gratama JW, Braakman E, Kraan J, Lankheet P, Levering WH, Van Den Beemd MW, Van Der Schoot CE, Wijermans P and Preijers F. Comparison of single and dual-platform assay formats for CD34+ haematopoietic progenitor cell enumeration. *Clinical and laboratory haematology*. 1999;21:337-46.

37. Hristov M, Schmitz S, Nauwelaers F and Weber C. A flow cytometric protocol for enumeration of endothelial progenitor cells and monocyte subsets in human blood. *Journal of immunological methods*. 2012;381:9-13.

38. Chen C, Gault A, Shen L and Nabavi N. Molecular cloning and expression of early T cell costimulatory molecule-1 and its characterization as B7-2 molecule. *Journal of immunology*. 1994;152:4929-36.

39. Jeannin P, Herbault N, Delneste Y, Magistrelli G, Lecoanet-Henchoz S, Caron G, Aubry JP and Bonnefoy JY. Human effector memory T cells express CD86: a functional role in naive T cell priming. *Journal of immunology*. 1999;162:2044-8.

40. Rogacev KS, Cremers B, Zawada AM, Seiler S, Binder N, Ege P, Grosse-Dunker G, Heisel I, Hornof F, Jeken J, Rebling NM, Ulrich C, Scheller B, Bohm M, Fliser D and Heine GH. CD14++CD16+ monocytes independently predict cardiovascular events: a cohort study of 951 patients referred for elective coronary angiography. *Journal of the American College of Cardiology*. 2012;60:1512-20.

41. Rogacev KS, Ziegelin M, Ulrich C, Seiler S, Girndt M, Fliser D and Heine GH. Haemodialysis-induced transient CD16+ monocytopenia and cardiovascular outcome. *Nephrology, dialysis, transplantation : official publication of the European Dialysis and Transplant Association - European Renal Association*. 2009;24:3480-6.

42. Ziegler-Heitbrock L and Hofer TP. Toward a refined definition of monocyte subsets. *Frontiers in immunology*. 2013;4:23.

43. Heine GH, Ulrich C, Seibert E, Seiler S, Marell J, Reichart B, Krause M, Schlitt A, Kohler H and Girndt M. CD14(++)CD16+ monocytes but not total monocyte numbers predict cardiovascular events in dialysis patients. *Kidney international*. 2008;73:622-9.

44. Wong KL, Yeap WH, Tai JJ, Ong SM, Dang TM and Wong SC. The three human monocyte subsets: implications for health and disease. *Immunologic research*. 2012;53:41-57.

45. Zawada AM, Fell LH, Untersteller K, Seiler S, Rogacev KS, Fliser D, Ziegler-Heitbrock L and Heine GH. Comparison of two different strategies for human monocyte subsets gating within the large-scale prospective CARE FOR HOMe Study. *Cytometry Part A : the journal of the International Society for Analytical Cytology*. 2015;87:750-8.

46. Hofer TP, Zawada AM, Frankenberger M, Skokann K, Satzl AA, Gesierich W, Schuberth M, Levin J, Danek A, Rotter B, Heine GH and Ziegler-Heitbrock L. Characterization of subsets of the CD16-positive monocytes: impact of granulomatous inflammation and M-CSF-receptor mutation. *Blood*. 2015.

47. Docke WD, Hoflich C, Davis KA, Rottgers K, Meisel C, Kiefer P, Weber SU, Hedwig-Geissing M, Kreuzfelder E, Tschentscher P, Nebe T, Engel A, Monneret G, Spittler A, Schmolke K, Reinke P, Volk HD and Kunz D. Monitoring temporary immunodepression by flow cytometric measurement of monocytic HLA-DR expression: a multicenter standardized study. *Clinical chemistry*. 2005;51:2341-7.

48. Hayward AR, Harvey BA, Leonard J, Greenwood MC, Wood CB and Soothill JF. Delayed separation of the umbilical cord, widespread infections, and defective neutrophil mobility. *Lancet*. 1979;1:1099-101.

49. Weber C, Belge KU, von Hundelshausen P, Draude G, Steppich B, Mack M, Frankenberger M, Weber KS and Ziegler-Heitbrock HW. Differential chemokine receptor expression and function in human monocyte subpopulations. *Journal of leukocyte biology*. 2000;67:699-704.

50. Funderburg NT, Zidar DA, Shive C, Lioi A, Mudd J, Musselwhite LW, Simon DI, Costa MA, Rodriguez B, Sieg SF and Lederman MM. Shared monocyte subset phenotypes in HIV-1 infection and in uninfected subjects with acute coronary syndrome. *Blood*. 2012;120:4599-608.

51. Giesen PL, Rauch U, Bohrmann B, Kling D, Roque M, Fallon JT, Badimon JJ, Himber J, Riederer MA and Nemerson Y. Blood-borne tissue factor: another view of thrombosis. *Proceedings of the National Academy of Sciences of the United States of America*. 1999;96:2311-5.

52. Belge KU, Dayyani F, Horelt A, Siedlar M, Frankenberger M, Frankenberger B, Espevik T and Ziegler-Heitbrock L. The proinflammatory CD14+CD16+DR++ monocytes are a major source of TNF. *Journal of immunology (Baltimore, Md : 1950)*. 2002;168:3536-42.

53. Nockher WA and Scherberich JE. Expanded CD14+ CD16+ monocyte subpopulation in patients with acute and chronic infections undergoing hemodialysis. *Infection and immunity*. 1998;66:2782-90.

54. Furman MI, Benoit SE, Barnard MR, Valeri CR, Borbone ML, Becker RC, Hechtman HB and Michelson AD. Increased platelet reactivity and circulating monocyte-platelet aggregates in patients with stable coronary artery disease. *Journal of the American College of Cardiology*. 1998;31:352-8.

55. Shantsila E and Lip GY. The role of monocytes in thrombotic disorders. Insights from tissue factor, monocyte-platelet aggregates and novel mechanisms. *Thrombosis and haemostasis*. 2009;102:916-24.

56. Jorissen RN, Reddy GS, Ali A, Altman MD, Chellappan S, Anjum SG, Tidor B, Schiffer CA, Rana TM and Gilson MK. Additivity in the analysis and design of HIV protease inhibitors. *Journal of medicinal chemistry*. 2009;52:737-54.

57. Wrigley BJ, Shantsila E, Tapp LD and Lip GY. Increased formation of monocyte-platelet aggregates in ischemic heart failure. *Circulation Heart failure*. 2013;6:127-35.

58. Ozaki Y, Imanishi T, Teraguchi I, Nishiguchi T, Orii M, Shiono Y, Shimamura K, Ishibashi K, Tanimoto T, Yamano T, Ino Y, Yamaguchi T, Kubo T and Akasaka T. Association between P-selectin glycoprotein ligand-1 and pathogenesis in acute coronary syndrome assessed by optical coherence tomography. *Atherosclerosis*. 2014;233:697-703.

59. Shantsila E, Montoro-Garcia S and Lip GY. Monocytes circulate in constant reversible interaction with platelets in a [Ca2+]-dependent manner. *Platelets*. 2014;25:197-201.

60. De Palma M, Venneri MA, Galli R, Sergi Sergi L, Politi LS, Sampaolesi M and Naldini L. Tie2 identifies a hematopoietic lineage of proangiogenic monocytes required for tumor vessel formation and a mesenchymal population of pericyte progenitors. *Cancer cell*. 2005;8:211-26.

61. Coffelt SB, Tal AO, Scholz A, De Palma M, Patel S, Urbich C, Biswas SK, Murdoch C, Plate KH, Reiss Y and Lewis CE. Angiopoietin-2 regulates gene expression in TIE2-expressing monocytes and augments their inherent proangiogenic functions. *Cancer research*. 2010;70:5270-80.

62. Cochain C, Rodero MP, Vilar J, Recalde A, Richart AL, Loinard C, Zouggari Y, Guerin C, Duriez M, Combadiere B, Poupel L, Levy BI, Mallat Z, Combadiere C and Silvestre JS. Regulation of monocyte subset systemic levels by distinct chemokine receptors controls post-ischaemic neovascularization. *Cardiovascular research*. 2010;88:186-95.

63. Czepluch FS, Bernhardt M, Kuschicke H, Gogiraju R, Schroeter MR, Riggert J, Hasenfuss G and Schafer K. In vitro and in vivo effects of human monocytes and their subsets on new vessel formation. *Microcirculation*. 2014;21:148-58.

64. Carey III JL, McCoy JP, Jr. and Keren G. Flow Cytometry in Clinical Diagnosis (4th edition). 2007;ASCP Press.

65. Darzynkiewicz Z. Essential Cytometry Methods (Reliable Lab Solutions). *Academic Press*. 2009.

66. Heimbeck I, Hofer TP, Eder C, Wright AK, Frankenberger M, Marei A, Boghdadi G, Scherberich J and Ziegler-Heitbrock L. Standardized single-platform assay for human monocyte subpopulations: Lower CD14+CD16++ monocytes in females. *Cytometry Part A : the journal of the International Society for Analytical Cytology*. 2010;77:823-30.

67. Hearps AC, Martin GE, Angelovich TA, Cheng WJ, Maisa A, Landay AL, Jaworowski A and Crowe SM. Aging is associated with chronic innate immune activation and dysregulation of monocyte phenotype and function. *Aging cell*. 2012;11:867-75.

68. Fingerle-Rowson G, Auers J, Kreuzer E, Fraunberger P, Blumenstein M and Ziegler-Heitbrock LH. Expansion of CD14+CD16+ monocytes in critically ill cardiac surgery patients. *Inflammation*. 1998;22:367-79.

69. Barisione C, Garibaldi S, Ghigliotti G, Fabbi P, Altieri P, Casale MC, Spallarossa P, Bertero G, Balbi M, Corsiglia L and Brunelli C. CD14CD16 monocyte subset levels in heart failure patients. *Disease markers*. 2010;28:115-24.

70. Palframan RT, Jung S, Cheng G, Weninger W, Luo Y, Dorf M, Littman DR, Rollins BJ, Zweerink H, Rot A and von Andrian UH. Inflammatory chemokine transport and presentation in HEV: a remote control mechanism for monocyte recruitment to lymph nodes in inflamed tissues. *The Journal of experimental medicine*. 2001;194:1361-73.

71. Ziegler-Heitbrock L. Monocyte subsets in man and other species. *Cellular immunology*. 2014;289:135-9.

72. Sunderkotter C, Nikolic T, Dillon MJ, Van Rooijen N, Stehling M, Drevets DA and Leenen PJ. Subpopulations of mouse blood monocytes differ in maturation stage and inflammatory response. *Journal of immunology*. 2004;172:4410-7.

73. Rosenstein Y, Santana A and Pedraza-Alva G. CD43, a molecule with multiple functions. *Immunologic research*. 1999;20:89-99.

74. Bamezai A. Mouse Ly-6 proteins and their extended family: markers of cell differentiation and regulators of cell signaling. *Archivum immunologiae et therapiae experimentalis*. 2004;52:255-66.

75. Geissmann F, Jung S and Littman DR. Blood monocytes consist of two principal subsets with distinct migratory properties. *Immunity*. 2003;19:71-82.

76. Auffray C, Fogg D, Garfa M, Elain G, Join-Lambert O, Kayal S, Sarnacki S, Cumano A, Lauvau G and Geissmann F. Monitoring of blood vessels and tissues by a population of monocytes with patrolling behavior. *Science*. 2007;317:666-70.

77. Burke B, Ahmad R, Staples KJ, Snowden R, Kadioglu A, Frankenberger M, Hume DA and Ziegler-Heitbrock L. Increased TNF expression in CD43++ murine blood monocytes. *Immunology letters*. 2008;118:142-7.

78. Ingersoll MA, Spanbroek R, Lottaz C, Gautier EL, Frankenberger M, Hoffmann R, Lang R, Haniffa M, Collin M, Tacke F, Habenicht AJ, Ziegler-Heitbrock L and Randolph GJ. Comparison of gene expression profiles between human and mouse monocyte subsets. *Blood*. 2010;115:e10-9.

79. Hristov M and Heine GH. Monocyte subsets in atherosclerosis. *Hamostaseologie*. 2015;35:105-12.

80. Jaipersad AS, Lip GY, Silverman S and Shantsila E. The role of monocytes in angiogenesis and atherosclerosis. *Journal of the American College of Cardiology*. 2014;63:1-11.

81. Ghattas A, Griffiths HR, Devitt A, Lip GY and Shantsila E. Monocytes in coronary artery disease and atherosclerosis: where are we now? *Journal of the American College of Cardiology*. 2013;62:1541-51.

82. Rogacev KS, Zawada AM, Emrich I, Seiler S, Bohm M, Fliser D, Woollard KJ and Heine GH. Lower Apo A-I and lower HDL-C levels are associated with higher intermediate CD14++CD16+ monocyte counts that predict cardiovascular events in chronic kidney disease. *Arteriosclerosis, thrombosis, and vascular biology*. 2014;34:2120-7.

83. Rogacev KS, Seiler S, Zawada AM, Reichart B, Herath E, Roth D, Ulrich C, Fliser D and Heine GH. CD14++CD16+ monocytes and cardiovascular outcome in patients with chronic kidney disease. *European heart journal*. 2011;32:84-92.

84. Krychtiuk KA, Kastl SP, Hofbauer SL, Wonnerth A, Goliasch G, Ozsvar-Kozma M, Katsaros KM, Maurer G, Huber K, Dostal E, Binder CJ, Pfaffenberger S, Oravec S, Wojta J and Speidl WS. Monocyte subset distribution in patients with stable atherosclerosis and elevated levels of lipoprotein(a). *Journal of clinical lipidology*. 2015;9:533-41.

85. Rothe G, Gabriel H, Kovacs E, Klucken J, Stohr J, Kindermann W and Schmitz G. Peripheral blood mononuclear phagocyte subpopulations as cellular markers in hypercholesterolemia. *Arteriosclerosis, thrombosis, and vascular biology*. 1996;16:1437-47.
86. Braesch-Andersen S, Paulie S, Smedman C, Mia S and Kumagai-Braesch M. ApoE production in human monocytes and its regulation by inflammatory cytokines. *PloS one*. 2013;8:e79908.
87. Krychtiuk KA, Kastl SP, Pfaffenberger S, Pongratz T, Hofbauer SL, Wonnerth A, Katsaros KM, Goliasch G, Gaspar L, Huber K, Maurer G, Dostal E, Oravec S, Wojta J and Speidl WS. Small high-density lipoprotein is associated with monocyte subsets in stable coronary artery disease. *Atherosclerosis*. 2014;237:589-96.
88. Krychtiuk KA, Kastl SP, Pfaffenberger S, Lenz M, Hofbauer SL, Wonnerth A, Koller L, Katsaros KM, Pongratz T, Goliasch G, Niessner A, Gaspar L, Huber K, Maurer G, Dostal E, Wojta J, Oravec S and Speidl WS. Association of small dense LDL serum levels and circulating monocyte subsets in stable coronary artery disease. *PloS one*. 2015;10:e0123367.
89. Jaipersad AS, Shantsila A, Lip GY and Shantsila E. Expression of monocyte subsets and angiogenic markers in relation to carotid plaque neovascularization in patients with pre-existing coronary artery disease and carotid stenosis. *Annals of medicine*. 2014;46:530-8.
90. Kaito M, Araya S, Gondo Y, Fujita M, Minato N, Nakanishi M and Matsui M. Relevance of distinct monocyte subsets to clinical course of ischemic stroke patients. *PloS one*. 2013;8:e69409.
91. Urra X, Villamor N, Amaro S, Gomez-Choco M, Obach V, Oleaga L, Planas AM and Chamorro A. Monocyte subtypes predict clinical course and prognosis in human stroke. *Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism*. 2009;29:994-1002.

92. Zhou X, Yuan F, Ji WJ, Guo ZZ, Zhang L, Lu RY, Liu X, Liu HM, Zhang WC, Jiang TM, Zhang Z and Li YM. High-salt intake induced visceral adipose tissue hypoxia and its association with circulating monocyte subsets in humans. *Obesity (Silver Spring, Md).* 2014;22:1470-6.

93. Terasawa T, Aso Y, Omori K, Fukushima M, Momobayashi A and Inukai T. Bezafibrate, a peroxisome proliferator-activated receptor alpha agonist, decreases circulating CD14(+)CD16(+) monocytes in patients with type 2 diabetes. *Translational research : the journal of laboratory and clinical medicine*. 2015;165:336-45.

94. Jaipersad AS, Shantsila E, Blann A and Lip GY. The effect of statin therapy withdrawal on monocyte subsets. *European journal of clinical investigation*. 2013;43:1307-13.

95. Mosig S, Rennert K, Krause S, Kzhyshkowska J, Neunubel K, Heller R and Funke H. Different functions of monocyte subsets in familial hypercholesterolemia: potential function of CD14+ CD16+ monocytes in detoxification of oxidized LDL. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology*. 2009;23:866-74.

96. Rothe G, Herr AS, Stohr J, Abletshauser C, Weidinger G and Schmitz G. A more mature phenotype of blood mononuclear phagocytes is induced by fluvastatin treatment in hypercholesterolemic patients with coronary heart disease. *Atherosclerosis*. 1999;144:251-61.

97. Coen PM, Flynn MG, Markofski MM, Pence BD and Hannemann RE. Adding exercise to rosuvastatin treatment: influence on C-reactive protein, monocyte toll-like receptor 4 expression, and inflammatory monocyte (CD14+CD16+) population. *Metabolism: clinical and experimental*. 2010;59:1775-83.

98. Imanishi T, Ikejima H, Tsujioka H, Kuroi A, Ishibashi K, Komukai K, Tanimoto T, Ino Y, Takeshita T and Akasaka T. Association of monocyte subset counts with coronary fibrous cap thickness in patients with unstable angina pectoris. *Atherosclerosis*. 2010;212:628-35.

99. Shantsila E, Tapp LD, Wrigley BJ, Pamukcu B, Apostolakis S, Montoro-Garcia S and Lip GY. Monocyte subsets in coronary artery disease and their associations with markers of inflammation and fibrinolysis. *Atherosclerosis*. 2014;234:4-10.

100. Leers MP, Keuren JF, Frissen ME, Huts M, Kragten JA and Jie KS. The pro- and anticoagulant role of blood-borne phagocytes in patients with acute coronary syndrome. *Thromb Haemost*. 2013;110:101-9.

101. Wrigley BJ, Shantsila E, Tapp LD and Lip GY. CD14++CD16+ monocytes in patients with acute ischaemic heart failure. *Eur J Clin Invest*. 2013;43:121-30.

102. Subimerb C, Pinlaor S, Lulitanond V, Khuntikeo N, Okada S, McGrath MS and Wongkham S. Circulating CD14(+) CD16(+) monocyte levels predict tissue invasive character of cholangiocarcinoma. *Clinical and experimental immunology*. 2010;161:471-9.

103. Amir O, Spivak I, Lavi I and Rahat MA. Changes in the monocytic subsets CD14(dim)CD16(+) and CD14(++)CD16(-) in chronic systolic heart failure patients. *Mediators of inflammation*. 2012;2012:616384.

104. Berg KE, Ljungcrantz I, Andersson L, Bryngelsson C, Hedblad B, Fredrikson GN, Nilsson J and Bjorkbacka H. Elevated CD14++CD16- monocytes predict cardiovascular events. *Circulation Cardiovascular genetics*. 2012;5:122-31.

105. Ghigliotti G, Barisione C, Garibaldi S, Brunelli C, Palmieri D, Spinella G, Pane B, Spallarossa P, Altieri P, Fabbi P, Sambuceti G and Palombo D. CD16(+) monocyte subsets are increased in large abdominal aortic aneurysms and are differentially related with circulating and cell-associated biochemical and inflammatory biomarkers. *Disease markers*. 2013;34:131-42.

106. Klimek E, Mikolajczyk T, Sulicka J, Kwasny-Krochin B, Korkosz M, Osmenda G, Wizner B, Surdacki A, Guzik T, Grodzicki TK and Skalska A. Blood monocyte subsets and

selected cardiovascular risk markers in rheumatoid arthritis of short duration in relation to disease activity. *BioMed research international*. 2014;2014:736853.

107. Passos S, Carvalho LP, Costa RS, Campos TM, Novais FO, Magalhaes A, Machado PR, Beiting D, Mosser D, Carvalho EM and Scott P. Intermediate Monocytes Contribute to Pathologic Immune Response in Leishmania braziliensis Infections. *The Journal of infectious diseases*. 2014.

108. Moniuszko M, Bodzenta-Lukaszyk A, Kowal K, Lenczewska D and Dabrowska M. Enhanced frequencies of CD14++CD16+, but not CD14+CD16+, peripheral blood monocytes in severe asthmatic patients. *Clinical immunology (Orlando, Fla)*. 2009;130:338-46.

109. Poitou C, Dalmas E, Renovato M, Benhamo V, Hajduch F, Abdennour M, Kahn JF, Veyrie N, Rizkalla S, Fridman WH, Sautes-Fridman C, Clement K and Cremer I. CD14dimCD16+ and CD14+CD16+ monocytes in obesity and during weight loss: relationships with fat mass and subclinical atherosclerosis. *Arteriosclerosis, thrombosis, and vascular biology*. 2011;31:2322-30.

110. Schauer D, Starlinger P, Reiter C, Jahn N, Zajc P, Buchberger E, Bachleitner-Hofmann T, Bergmann M, Stift A, Gruenberger T and Brostjan C. Intermediate monocytes but not TIE2-expressing monocytes are a sensitive diagnostic indicator for colorectal cancer. *PloS one*. 2012;7:e44450.

Table 1. Definition of monocyte subsets

Older definition		2010 Nomenclature definition		Suggested numerical assignment
CD14+CD16– CD14++CD16– CD14highCD16–	Classical	CD14++CD16–	Classical	Mon1
CD14+CD16+ (can be related to both either CD16+ subset) CD14lowCD16+ CD14–CD16+	Nonclassical	CD14++CD16+ CD14++CD16+CCR2+ CD14++CD16+SLAN+	Intermediate	Mon2
		CD14+CD16++	Nonclassical	Mon3

Table 2. Summary of phenotypic characteristics of Mon2 and Mon3 in healthy donors

Parameter	Comment
Size	Mon2 bigger[9, 14]
Granularity	Mon2 more granular[9, 14]
Surface markers with higher expression on Mon2	
CD14	12-fold[9]
CCR2	8-fold[9] (another study suggested no expression on Mon3)[14]
CCR5	8-fold[15]
CD163	7-12-fold[9, 15]
CD64	6-fold[9]
CXCR4	3-fold[9]
CLEC10A	4-fold[15]
GFRA2	4-fold[15]
CD11b	4-fold[15]
CD1d	4-fold[15]
HLA-DR	2.5-fold[15]
VEGF receptor 1	2.5-fold[9]
TLR4	2.4 fold[9]
Ferritin	2-fold[9]
Interleukin-6 receptor	2-fold[9]
CD115	1.7-1.8 fold[15]
Integrin β2	1.7-fold[9]

Tie2	1.5-fold[9]
Surface markers with higher expression on Mon3 subset	
CD16	3-fold[9]
Integrin α4	2-fold[9]
SLAN	7-fold[15]
CD294	2.3-fold[15]
Siglec10	2.8-fold[15]

Table 3. Functional characteristics of Mon2 and Mon3

Parameter	Comment
Aggregates with platelets, %	Higher with Mon2 (1.3-fold)[9]
Phagocytic activity	Higher by Mon2 (3-fold)[9, 14]
TNF-α, IL-6, IL-1β, IL-10 production in response to LPS	Higher inMon2[9, 14]
Form cordlike structures in Matrigel after VEGF stimulation	Mon2 only[17]

Table 4. Genes with at least 2-fold difference in expression by Mon2 and Mon3 (Wong at al.)[15]

Gene expression	Magnitude of difference
Higher by Mon2	
CD14 antigen (CD14)	4-fold
Chondroitin sulfate proteoglycan 2 (CSPG2)	4-fold
Solute carrier family 2 member 3 (SLC2A3)	4-fold
CD9 antigen p24 (CD9)	4-fold
CD163 antigen (CD163)	3-fold
Phospholipase A2 group VII (PLA2G7)	3-fold
mast cell-expressed membrane protein 1 (MCEMP1)	3-fold
C-type lectin domain family 10 member A (CLEC10A)	3-fold
Epithelial V-like antigen 1 (EVA1)	3-fold
Ribonuclease RNase A family 2 (RNASE2)	3-fold
GDNF family receptor-_2 (GFRA2)	3-fold
Aldehyde dehydrogenase 1 family member A1 (ALDH1A1)	3-fold
Lectin galactoside-binding soluble 2 (GALS2)	3-fold
Macrophage receptor with collagenous structure (MARCO)	2-fold
Arachidonate 5-lipoxygenase-activating protein (ALOX5AP)	2-fold
S100 calcium binding protein A12 (S100A12)	2-fold
Glutaminyl-peptide cyclotransferase (QPCT)	2-fold
Folate receptor 3 (gamma; FOLR3)	2-fold

Oncostatin M (OSM)	2-fold
Early growth response 1 (EGR1)	2-fold
Cytochrome P450 family 27 subfamily A polypeptide 1 (CYP27A1)	2-fold
Olfactomedin 1 (OLFM1)	2-fold
Peptidyl arginine deiminase type IV (PADI4)	2-fold
Major histocompatibility complex class II DO_ (HLA-DOA)	2-fold
Angiogenin ribonuclease RNase A family 5 (ANG)	2-fold
H19 imprinted maternally expressed untranslated mRNA (H19)	2-fold
Stearoyl-CoA desaturase (SCD)	2-fold
S100 calcium binding protein A9 (calgranulin B; S100A9)	2-fold
DNA-damage-inducible transcript 4 (DDIT4)	2-fold
Higher by Mon2	
LY6/PLAUR domain containing 2 (LYPD2)	6-fold
SH2 domain containing 1B (SH2D1B)	4-fold
Cadherin-like 23 (CDH23)	4-fold
Vitelline membrane outer layer 1 homolog (chicken; VMO1)	3-fold
Creatine kinase brain (CKB)	3-fold
Secretoglobin family 3A member 1 (SCGB3A1)	2-fold
Surfactant pulmonary-associated protein D (SFTPD)	2-fold

Table 5. Comparison of top genes differentially expressed between Mon2 and Mon3. Fold differences presented as log2(Mon2/Mon3 ratio) (p value $<10^{-44}$ for all parameters) (Zawada et al.)[17]

Gene	Fold difference
MHC complex, class II, DRα (HLA-DRA)	1.5-fold
Lysozyme (LYZ)	2.7-fold
CD74 molecule, MHC class II invariant chain (CD74)	1.1-fold
IFNγ-inducible protein 30 (IFI30)	1.3-fold
S100 calcium-binding protein A8(S100A8)	2.5-fold
MHC complex, class II, DP β1 (HLA-DPB1)	2.1-fold
Thymosin β10 (TMSB10)	1-fold
Secreted and transmembrane 1 (SECTM1)	1.1-fold
Finkel-Biskis-Reilly murine sarcoma virus (FBR-MuSV) ubiquitously expressed (FAU)	1.3-fold
Carboxypeptidase, vitellogenic-like (CPVL)	3-fold
Peptidylprolyl isomerase A (cyclophilin A) (PPIA)	1-fold
CD14 molecule (CD14)	2.8-fold
S100 calcium-binding protein A10 (S100A10)	1.5-fold
murine osteosarcoma viral oncogene (FOS FBJ	4-fold
TNFα-induced protein 2 (TNFAIP2)	1.5-fold
Cofilin 1 (CFL1)	-1.2-fold
IFN-induced transmembrane protein 2 (1-8D) (IFITM2)	-1.3-fold
Guanine nucleotide-binding protein (G protein), α inhibiting activity polypeptide 2 (GNAI2)	-1.5-fold

Niemann-Pick disease, type C2 (NPC2)	-1.4-fold
Myosin, light chain 6, alkali, smooth muscle and non-muscle (MYL6)	-1-fold
Cyclin-dependent kinase inhibitor 1C (p57, Kip2) (CDKN1C)	-1.5-fold
v-yes-1 Yamaguchi sarcoma viral-related oncogene homolog (LYN)	-1.4-fold
Lysosomal protein transmembrane 5 lysosomes (LAPTM5)	-1.1-fold
Ornithine decarboxylase antizyme 1 (OAZ1)	-0.9-fold
MHC, class I, B (HLA-B)	-0.6-fold
β2-microglobulin (B2M)	-0.5-fold
Serine/threonine kinase 10 (STK10)	-1.7-fold
Prosaposin (PSAP)	-0.5-fold
Cystatin B (stefin B) (CSTB)	-1.5-fold
Lymphocyte-specific protein 1 (LSP1)	-1-fold

Table 6. Monocyte subsets in cardiovascular disorders

CV disorder	Mon2	Mon3
Patients referred for elective coronary angiography[40]	Independently predictive of cardiovascular events.	No predictive value
Stable CAD[99]	(i) No changes in counts (vs. healthy)	(i) No changes in counts (vs. healthy)
Acute MI[27]	(i) ↑ count (2.5-fold) (ii) ↑ NFkappaB pathway activity (iii) Correlation with troponin T level	(i) No changes (ii) ↑ NFkappaB pathway activity (iii) No correlation with troponin T
Acute MI[100]	(i) ↑ count and percentage (ii) ↑ intracellular CD41 (more pronounced than Mon3) (iii) ↑ TF expression	(i) No change (ii) ↑ intracellular CD41 (less pronounced than Mon2) (iii) ↑ TF expression
Unstable angina[11]	(i) ↑ percentage (ii) ↑ in intermediate-to-high risk patients vs. low risk patients	(i) No change (ii) No difference with risk severity
Unstable angina[100]	(i) ↑ count (ii) increased intracellular CD41 (more pronounced than Mon3)	(i) No change (less pronounced than Mon2)
Acute heart	(i) ↑ count	(i) No changes

failure[101]	(ii) Negatively associated with cardiovascular events.	(ii) No predictive value
Chronic heart failure[102]	(i) ↑ count (ii) correlate with NYHA class, LVEF, NT-proBNP.	(i) ↑ count (ii) No correlation
Chronic heart failure[103]	(i) No changes (vs. healthy) (ii) No association with end-diastolic dimension	(i) ↑ percentage (vs. healthy) (ii) Inversely associated with end-diastolic dimension
Stroke[90, 91]	(i) ↑ count (ii) Counts inversely related to mortality	(i) ↓ count (ii) Counts inversely related to 'poor' outcome
Randomly selected subjects[104]	↑ count in patients with cardiovascular events (p=0.051)	↑ no relevance to outcome
Abdominal aortic aneurysm[105]	↑ count (vs. healthy)	↑ count (vs. healthy)