

Role and analysis of monocyte subsets in cardiovascular disease

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Summary

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 document re-evaluates current

nomenclature and summarises key findings on monocyte subset biology to propose a consensus statement about phenotype, separation and quantification of the individual subsets.

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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 oligomerisation domain-like receptors, and retinoic acid-inducible gene I-like receptors, along with other (1). Monocytes/macrophages can phagocytose pathogens but they also promote pathogen neutralisation 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

Table 1: Definition of monocyte subsets.

Older definition		2010 Nomenclature definition		Suggested numerical assignment
CD14 ⁺ CD16 ⁻ CD14 ⁺⁺ CD16 ⁻ CD14 ^{high} CD16 ⁻	Classical	CD14 ⁺⁺ CD16 ⁻	Classical	Mon1
CD14 ⁺ CD16 ⁺ (can be related to both or either CD16 ⁺ subset) CD14 ^{low} CD16 ⁺ CD14 ⁻ CD16 ⁺	Nonclassical	CD14 ⁺⁺ CD16 ⁺ CD14 ⁺⁺ CD16 ⁺ CCR2 ⁺ CD14 ⁺⁺ CD16 ⁺ SLAN ⁺	Intermediate	Mon2
		CD14 ⁺ CD16 ⁺⁺	Nonclassical	Mon3

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 CD14^{low}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 standardised 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: 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 polarisation (13), and has been used throughout the entire document. A recent nomenclature next to experimental guidelines on macrophage activation and polarisation has highlighted the complexity of the macrophage origin (14). These guidelines recommend that reproducible experiment settings should be employed in macrophage research and CD14⁺ blood monocytes should be used as a standard to produce human M1/M2 macrophages. The document does not specifically address relation of Mon2 and Mon3 to particular profiles of macrophage activation and this area requires further investigation.

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

Parameter	Comment (Ref.)
Size	Mon2 bigger (9, 15)
Granularity	Mon2 more granular (9, 15)
Surface markers with higher expression on Mon2	
CD14	12-fold (9)
CCR2	8-fold (9) another study suggested no expression on Mon3 (15)
CCR5	8-fold (16)
CD163	7–12-fold (9, 16)
CD64	6-fold (9)
CXCR4	3-fold (9)
CLEC10A	4-fold (16)
GFRA2	4-fold (16)
CD11b	4-fold (16)
CD1d	4-fold (16)
HLA-DR	2.5-fold (16)
VEGF receptor 1	2.5-fold (9)
TLR4	2.4 fold (9)
Ferritin	2-fold (9)
IL-6 receptor	2-fold (9)
CD115	1.7–1.8 fold (16)
Integrin 2	1.7-fold (9)
Tie2	1.5-fold (9)
Surface markers with higher expression on Mon3 subset	
CD16	3-fold (9)
Integrin α 4	2-fold (9)
SLAN	7-fold (16)
CD294	2.3-fold (16)
Siglec10	2.8-fold (16)

Table 3: Functional characteristics of Mon2 and Mon3.

Parameter	Comment (Ref.)
Aggregates with platelets, %	Higher with Mon2 (1.3-fold) (9)
Phagocytic activity	Higher by Mon2 (3-fold) (9, 15)
TNF- α , IL-6, IL-1 β , IL-10 production in response to LPS	Higher in Mon2 (9, 15)
Form cord-like structures in Matrigel after VEGF stimulation	Mon2 only (18)

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 β 2) and angiogenesis/tissue remodelling (e.g. vascular endothelial growth factor [VEGF] receptors 1 and 2, Tie2, CXCR4) (► Table 2) (9, 15, 16). In fact, Mon2 have highest expression of many of those receptors among all monocyte subsets (9). In contrast, Mon3 have higher expression of integrin α 4, SLAN, CX3CR1, sialophorin, CD294, and Siglec10 (9, 16).

Functional studies have shown that Mon2 have a higher antigen presenting capacity (► Table 3) (17, 18). Mon2 compared to Mon3 also have a higher phagocytic activity, higher production of tumour necrosis factor (TNF)- α , IL-6 and IL-1 β in response to lipopolysaccharide (LPS) and a higher rate of aggregation with platelets (9, 15). Furthermore, Mon2 were reported to be strong producers of IL-10 and show a high capacity to engulf apoptotic cells similar to Mon3 (9, 19, 20). 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 (18). 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 subsets (9, 18, 19).

Global gene expression analysis demonstrated for Mon2 a preferential expression of genes relevant to antigen presentation, phagocytosis and angiogenesis (► Table 4 and ► Table 5) (16, 18, 21). 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 strongly differed in the expression of genes involved in cell adhesion, oxidative stress, and phagocytosis, pointing to a distinct transendothelial trafficking potential and

Table 4: Genes with at least two-fold difference in expression by Mon2 and Mon3 (Wong at al. [16]).

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
Vitellogenin membrane outer layer 1 homolog (chicken; VM01)	3-fold
Creatine kinase brain (CKB)	3-fold
Secretoglobin family 3A member 1 (SCGB3A1)	2-fold
Surfactant pulmonary-associated protein D (SFTPD)	2-fold

a greater capacity of Mon2 for generation of reactive oxygen species as well as for phagocytosis of pathogens (16).

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 (22). This process is also accompanied 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 (23, 24). 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 (25).

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 (26). 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, 27). 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 (28, 29). Another study has shown that in people undergoing myeloablation 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 nonclassical monocytes (30).

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.

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. [18]).

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
TNF-α-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

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 [h]) as more prolonged storage may affect monocyte phenotype (31). For example, a 2 h delay in sample processing results in appearance of CD16 on Mon1, and numbers of CD16⁺ monocytes significantly increase after 4 h of storage at room temperature (31).

Blood samples should be taken at the same time-of-day, typically in the morning since there is a diurnal variation in Mon2 subset (31, 32). Before sampling, the donor should be allowed to rest for at least 5 minutes (min) in order to avoid increases of CD16⁺ monocytes due to preceding exercise (31, 33). Since excessive stress with catecholamine release may mobilise CD16⁺ monocytes, blood sampling should be done under low stress conditions (34, 35).

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 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 (36). Monocytes respond also 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 vs 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, 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⁺ haematopoietic stem cells in blood, the superiority of the single-platform over the dual-platform approach has been clearly demonstrated (37). Similarly, it is recommended to use absolute counting beads for enumeration of blood monocytes (38). Either commercial flow cytometry tubes containing lyophilised bead pellets or counting bead suspensions can be utilised. 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 h of lysing) as the use of unfixed samples allows proper exclusion of dead cells by their scatter properties.

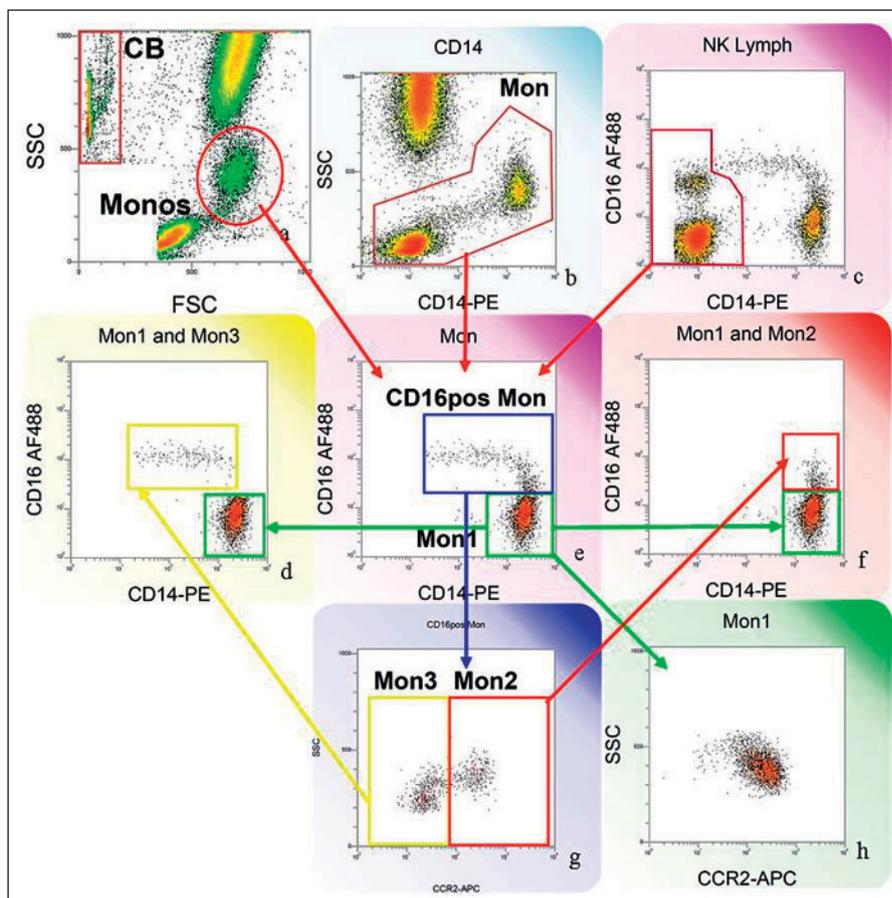
Separation of monocytes from non-monocytes

The initial gating aims to separate monocytes from other leukocytes, dead cells and debris. This can be achieved using optical properties of monocytes and their expression of certain surface markers (► Figure 1). Admittedly there is a certain scatter overlap between the three types of leukocytes and every care must be taken to minimise loss of monocytes for further quantification, while avoiding contamination by other leukocyte 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 (16). 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 (39). It is also expressed on effector memory T cells (40). Given that monocytes are the major type of CD86 expressing cells,

Figure 1: Gating strategies and presentation of monocyte subsets. (a) selection on monocyte region on forward scatter (FSC) vs side scatter (SSC) plot, (b) separation of mononuclear cells from granulocytes, (c) selection of CD14⁻ lymphocytes to separate CD16⁺CD14⁻ natural killer lymphocytes from CD16⁺ monocyte subsets, (e) separation of monocyte subset, that must correspond to regions Monos on (a) and Mon on (b) but must not include lymphocytes from (c), (g) separation of CD16⁺ monocyte subsets on the basis of their CCR2 expression, (h) CCR2 expression of Mon1, (d) and (f) location of Mon3 and Mon2, respectively, on CD14/CD16 plot. Mon 1: classical CD14⁺⁺CD16⁻CCR2⁺, Mon 2: intermediate CD14⁺⁺CD16⁺CCR2⁺ and Mon 3: non-classical CD14⁺CD16⁺⁺CCR2⁻ subsets.



the marker has been widely used to improve the separation of monocytes from non-monocytes (41, 42).

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 1). 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 1) (43). 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 (41, 42, 44). 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 (45).

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 (46).

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 [15]). 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⁺) (47). 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 five-fold depletion of the SLAN⁺ Mon3 in hereditary diffuse leukodystrophy with axonal spheroids (47).

Characterisation 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 (e.g. 3G8 for anti-human CD16) 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 (48), that lack of integrins prevents leukocyte-to-endothelial cell interaction (49), CD16⁺ monocytes lacking the CCR2 chemokine receptor do not migrate in response to CCL2 (50) and expression of tissue factor (TF) on monocytes, notably CD16⁺ monocytes may indicate a procoagulant state (51, 52). 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 standardisation and calibration with specific reference beads as has been implemented for HLA-DR expression on monocytes (48).

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 TNF- α . Here the secretion of the cytokine is pre-

vented by blockade of the Golgi system. Production of the respective cytokine can then be assigned to selective monocyte subsets in multicolour flow cytometry (9, 53).

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 phagocytose *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, 54).

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 (17, 18).

An important feature of monocytes in the context of cardiovascular disease is the formation of monocyte platelet aggregates (MPA) (55, 56). These aggregates form via interaction of P-selectin on activated platelets and with P-selectin glycoprotein ligand-1 (PSGL-1) on leukocytes or with additional adhesion molecules (57). 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, 28, 58). This could be partly due to higher levels of PSGL-1 on the Mon2 population (59). 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) (60). It has been demonstrated that 'weak' contacts are more common for Mon1 and irreversible contacts for Mon3 (60). Careful protocol design is essential with particular attention paid to the choice of anticoagulant to match study aims and minimal delay in sample processing (31, 60). Increase in MPA could be due to increased monocyte counts and/or increase in percentage of monocytes ag-

gregated with platelets. Therefore, it is useful to report both absolute MPA values and MPA per cent of monocytes for individual subsets (28).

Recently, a contribution of monocytes in angiogenesis has been recognised. In this context, cells expressing the angiopoietin receptor Tie2 have been shown to support angiogenesis *in vitro* and *in vivo* (18, 61, 62). 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 (63, 64). Mouse classical monocytes were shown to improve capillary density, and both classical and nonclassical monocytes improved the angiographic score (63). Also, human CD16⁺ monocytes transferred into mice were shown to improve post-ischaemic arteriole size (64). 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 neovascularisation 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 (65, 66).

Numbers of CD16⁺ monocytes were shown to be lower in females and to increase with age (67–68). 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 (30). 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, mobilisation of monocyte subsets from the marginal pool may occur which might be responsible for the increase in CD16⁺ monocytes in such diseases (69). Because catecholamines can mobilise 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 (70).

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, 71). 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 anaesthesia. Such conditions represent maximal stress, which will mobilise 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 freshly obtained whole blood. However, this may not be possible when fixation protocols are required. There is no widely accepted protocol for optimal monocyte fixation and a choice of fixative should be based on the nature of conducted experiments and study aims. As monocytes are extremely sensitive to any manipulation, the utility of such protocols should be first established in order to exclude potential changes in surface molecule expression between fresh and fixed samples. Typical markers used to describe monocyte subsets in mice are CD115, CD43 and Ly6C (72). CD115 recognises the M-CSF-receptor and is a marker used to identify monocytes as such (73). 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⁺) (74, 75). 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 characterisation 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 (76–78). 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 (79). 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 (73). 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 monocyte subsets in atherosclerosis, its risk factors and complications (especially myocardial infarction) has attracted major interest (80–84).

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 (85, 86). Likewise, subjects with stable atherosclerosis and high Lp(a) had higher proportions of Mon2 (87).

Mon2 showed *in vitro* augmented lipid accumulation and oxidised low-density lipoprotein (LDL) cholesterol uptake with lower cholesterol efflux compared to other subsets (85). Mon2 revealed high expression of cholesterol scavenging receptors (CD36, CD68), but low expression of ATP-binding cassette transporter (ABCA1) implicated in cholesterol efflux (85). *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 (88). Recently, mice experiments showed that a triglyceride-rich environment changes proportions of circulating monocyte subsets by promotion of the extravasation of

Gr1low cells, the subset that has resemblance to human Mon3 (23).

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 (89). 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 (90, 91). 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 neovascularisation (a surrogate for high risk of plaque rupture) (92). 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) (28). Moreover, Mon3 were reduced in patients with stroke (93, 94). 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

Table 6: Monocyte subsets in cardiovascular disorders.

CV disorder	Mon2	Mon3
Patients referred for elective coronary angiography (41)	Independently predictive of cardiovascular events.	No predictive value
Stable CAD (102)	(i) No changes in counts (vs healthy)	(i) No changes in counts (vs healthy)
Acute MI (28)	(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 (103)	(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 (103)	(i) ↑ count (ii) increased intracellular CD41 (more pronounced than Mon3)	(i) No change (less pronounced than Mon2)
Acute heart failure (104)	(i) ↑ count (ii) Negatively associated with cardiovascular events	(i) No changes (ii) No predictive value
Chronic heart failure (105)	(i) ↑ count (ii) correlate with NYHA class, LVEF, NT-proBNP	(i) ↑ count (ii) No correlation
Chronic heart failure (106)	(i) No changes (vs healthy) (ii) No association with end-diastolic dimension	(i) ↑ percentage (vs healthy) (ii) Inversely associated with end-diastolic dimension
Stroke (93, 94)	(i) ↑ count (ii) Counts inversely related to mortality	(i) ↓ count (ii) Counts inversely related to 'poor' outcome
Randomly selected subjects (107)	↑ count in patients with cardiovascular events (p=0.051)	↑ no relevance to outcome
Abdominal aortic aneurysm (108)	↑ count (vs healthy)	↑ count (vs healthy)

demonstrate prognostic implications in these patients (41, 86). Of interest, salt loading led to rise in Mon2 levels and monocyte pro-inflammatory activation, which was reverted by salt depletion (95). 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 (70).

Twelve weeks treatment with bezafibrate decreased circulating CD16⁺ monocytes in patients with type 2 diabetes (96). Temporal withdrawal of statins had no significant impact on monocyte counts but was associated with some phenotypic changes (97). In patients with familial hypercholesterolaemia, Mon3 exhibited an increased uptake of oxidised LDL via CD36, whereas Mon1 preferentially take up native LDL. Mon3 have a higher adherence to activated endothelial cells in response to oxidised LDL and native LDL stimulation (98).

In a double-blind randomised study of hypercholesterolaemic 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 (99). 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 hypercholesterolaemic 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 (100). 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 (101). 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 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.

Conflicts of interest

G. Y. H. Lip has served as a consultant for Bayer, Astellas, Merck, Sanofi, BMS/Pfizer, Daiichi-Sankyo, Biotronik, Portola, and Boehringer Ingelheim, and has been on the speakers bureau for Bayer, BMS/Pfizer, Boehringer Ingelheim, and Sanofi Aventis. None of the other authors declares any conflicts of interest.

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