Monocyte function and trafficking in cardiovascular disease

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Abstract
Monocytes are key effectors of the immune homeostasis and play a crucial role in (vascular) injury repair. Despite their role in immune defense and tissue repair mechanisms, monocytes are also involved in several pathological conditions such as autoimmune and cardiovascular diseases as well as cancer. This suggests that monocytes can be used as diagnostic and as therapeutic targets. A better understanding and characterisation of monocytes and their function in both physiological and pathological situations is thus of great interest. This review focuses on recent advances on the role of monocytes in cardiovascular diseases and describes the value of monocytes as either disease marker or therapeutic target for (cardio)vascular diseases.

Keywords
Monocytes, cardiovascular disease, atherosclerosis, myocardial infarction

Introduction
Monocytes are protagonists in host defense since they participate in the regulation of both the innate and the adaptive immune responses as well as in inflammation. They are circulating mononuclear phagocyte-like cells derived from a common myeloid (macrophage and dendritic cell [DC]) precursor in the bone marrow (BM), which emigrate from the BM in a CCR2 chemokine receptor-dependent manner (1, 2). Monocytes, which are classified in different groups, do not proliferate outside the BM, but they circulate for several days in the blood stream to the peripheral tissues where they patrol blood vessels (3–6). Monocyte recruitment and infiltration into tissues is modulated by proinflammatory as well as metabolic stimuli. Although it was initially believed that monocytes – following their generation – circulate in the bloodstream until they enter tissues and differentiate or until they die, it was recently shown that undifferentiated monocytes can reside in the subcapsular red pulp of the spleen in numbers much higher than those in the circulation (7). Upon tissue infiltration, monocytes can further differentiate irreversibly into tissue macrophages and/or DCs (4, 8). This suggests that monocytes are relatively undifferentiated and that their phenotype is determined by the tissue microenvironment. Interestingly, monocytes can also differentiate into endothelial, epithelial or other types of cells under certain culture conditions. Monocytes play crucial roles in the clearance of bacteria, viruses, toxic substances and in the eradication of apoptotic and necrotic cells. In addition, they play important roles in angiogenesis and arteriogenesis as well as tissue repair after injury (9, 10). However, uncontrolled recruitment of monocytes contributes to the development of inflammatory diseases such as rheumatoid arthritis, atherosclerosis, cardiovascular diseases (CVD) and cancer (9, 11).

In this review, we will focus on the role of different monocyte subsets in vascular repair and CVD. Moreover, we will discuss the mechanisms involved in monocyte functional dysfunction as well as recent advances in the field of monocyte trafficking in vivo.

Human monocyte subsets
Monocytes represent 5–10% of the total human leucocyte cells in the adult, show morphological heterogeneity and they can be categorised into different subclasses. Initially human monocytes were classified according to the expression of two molecules: CD14, which is part of the lipopolysaccharide (LPS) receptor, and CD16, also known as the FcγRIII receptor (10, 12). Human monocytes were first classified in classical CD14<sup>hi</sup>CD16<sup>−</sup>CD16<sup>+</sup>CD16<sup>−</sup> (CD14<sup>++</sup>CD16<sup>−</sup>, 85–90% of the monocytes) and non-classical CD14<sup>lo</sup>CD16<sup>−</sup> (CD14<sup>−</sup>CD16<sup>−</sup>, 10–15% of the total monocytes). Recently, a third subset the intermediate monocytes CD14<sup>hi</sup>CD16<sup>+hi</sup>CD16<sup>−</sup> (CD14<sup>++</sup>CD16<sup>+</sup>) was characterised (13, 14). The CD14<sup>++</sup>CD16<sup>−</sup> monocytes express higher surface levels of monocyte chemotactic protein-1 (MCP-1) receptor CCR2, but lower levels of macrophage inflammatory protein alpha (MIP-1 alpha) receptor CCR5 (15). CD14<sup>−</sup>CD16<sup>−</sup> cells display higher major histocompatibility complex (MHC) class II (16) and lower levels of VEGFR1 expression, and decreased levels of ROS production when compared to the CD14<sup>++</sup>CD16<sup>−</sup> monocyte subset (17–19). Both CD14<sup>++</sup>CD16<sup>−</sup> and CD14<sup>−</sup>CD16<sup>−</sup> monocytes can respond to the Toll-like receptor

Thrombosis and Haemostasis 108.5/2012
(TLR)2 and TLR4 ligands, whereas CD14+CD16+ monocytes respond to stimuli via TLR7 and/or TLR8 (20). The intermediate CD14++CD16+ cells express higher levels of CX3CR1, CXCR4 and CCR2, while CD14−CD16+ express no detectable levels of CCR2 and CCR5. Moreover, intermediate monocytes CD14++CD16+ express angiotensin converting enzyme (ACE, CD143), TIE2, VEGFR2 and endoglin (arguing for an involvement of these cells in the process of angiogenesis), produce high levels of ROS and inflammatory mediators (such as tumour necrosis factor [TNF]-α and interleukin [IL]1β) (11, 21). In addition, CD14++CD16+ cells show lower expression of scavenger receptor-type A (SR-A).

These phenotypic differences of the monocyte subsets predict also their differences in function. CD14++CD16+ monocytes are primarily involved in the inflammatory responses and they were initially designated also as inflammatory monocytes; although, recently it was demonstrated that the non-classical monocytes may have also inflammatory functions. However, the terms "inflammatory monocytes" or "proinflammatory monocytes" are not recommended because this leads to confusion (10). CD14++CD16+ monocytes have increased antimicrobial potential due to their phagocytic ability and their production of antimicrobial proteins. Due to their high CCR2 expression, they are attracted to sites of inflammation where they can infiltrate and differentiate into macrophages and/or DCs. CD14++CD16+ monocytes are considered to be less mature compared to the CD16+ positive cells and they can differentiate into CD14++CD16+ cells in the circulation (9–11). CD16+ non-classical monocytes, when stimulated with TLR2 and TLR4 ligands, produce TNF-α and IL-1 (16, 20, 22). In addition, CD16+ cells produce less IL-10. These results suggested that CD16+ monocytes are potent inducers of inflammation (12). Interestingly it was reported that CD16+ monocytes have increased capacity to differentiate into DCs while CD16− monocytes are prone to differentiate into macrophages (13, 23, 24). After the identification of the intermediate monocyte subset CD14++CD16+, it became clear that this subset produces higher levels of inflammatory cytokines and express markers which suggest that these cells may have proangiogenic potential (11, 21). It was shown that CD14+CD16+ monocytes selectively formed clusters on matrigel after VEGF stimulation. In addition, monocytes expressing the VEGFR2 were shown to have endothelial-like functional capacity (25). Furthermore, monocytes expressing the angiopoetin-2 receptor TIE-2 have been characterised as highly proangiogenic cells specifically linked to tumour infiltration (26).

**Mouse monocytes and their function**

Contrary to human monocytes, mouse monocyte subsets are better characterised. All mouse monocytes are identified by F4/80+CD11b+ expression and they can be subdivided into different subsets according to the expression of the receptors CCR2, CX3C chemokine receptor 1 (CX3CR1) and Ly6C, which is part of the GR1 epitope (3, 4, 15, 27–30). Thus, mouse monocytes have been subdivided into two subsets: the inflammatory monocytes which express high levels of CCR2 but low levels of CX3CR1 and high levels of GR1 and Ly6C (CX3CR1lowCCR2+Ly6Chigh) and the resident monocytes, which do not express CCR2 and GR1 and they have low levels of Ly6C but high levels of CX3CR1 (CX3CR1highCCR2+Ly6Chigh) (3, 4, 15, 27–30). In addition, an intermediate subset of mouse Ly6C cells has been reported to play important role in inflammatory responses (10, 31–33). According to the relative expression of the chemokine receptors, it has been suggested that the mouse inflammatory subset CX3CR1lowCCR2+Ly6Chigh corresponds to the classical monocytes while the non-inflammatory CX3CR1highCCR2+Ly6Chigh subset to the intermediate and non-classical human subsets (Table 1) (10). Although these observations suggested initially that it could be possible to address the in vivo role of human monocytes by studying the mouse system, later studies suggested that there are a lot contradictions between the human and mouse subsets’ function and phenotype. Whether this is due to species differences or incomplete characterisation of the subsets remains to be established.

The CX3CR1lowCCR2+Ly6Chigh monocytes express a number of inflammatory and proteolytic mediators and they are preferentially recruited to inflamed tissues, where they differentiate into macrophages, which are important for clearance of pathogens and for the resolution of inflammation. They can also differentiate into inflammatory DCs (9). The CX3CR1highCCR2+Ly6Chigh monocytes patrol blood vessels and extravasate in response to vascular damage in order to facilitate wound healing and revascularisation (3, 34). Recent studies have suggested that resident monocytes can be also recruited to sites of inflammation, where they can contribute to wound healing by differentiating into alternatively activated macrophages (27).

**Monocytes and vascular repair**

In adult organisms there are two major mechanisms of vessel growth: angiogenesis, the formation of new blood vessels from preexisting ones, and arteriogenesis, the growth of preexisting collateral arterioles into functional collateral arteries, which can compensate the functional loss of occluded arteries (35). In adults physiological angiogenesis is restricted to the female reproductive system; however, blood vessel growth occurs also in pathophysiological conditions such as tissue repair after wounding, tumour growth or inflammatory diseases. Besides endothelial cells (ECs) and smooth muscle cells (SMCs) there is strong evidence that monocytes contribute also to angiogenesis and arteriogenesis (36–39). VEGF is the master regulator of angiogenesis promoting EC activation and sprouting and inhibition of VEGFα results in inhibition of angiogenesis. Nevertheless, VEGFα can affect also monocyte function besides EC function. VEGFα as well as PI GF can induce monocyte recruitment and in this way they could contribute to angiogenesis (39–41). Thus, inhibition of angiogenesis by inhibiting VEGF signalling may not only be due to the effects on EC function but also on monocyte function (37–39, 41). In the case of pathological angiogenesis it was shown that inhibition of monocyte recruitment results in reduced angiogenesis (40, 42).
PlGF has been identified as a specific ligand of VEGFR1, which is the exclusive VEGF receptor on CD14++CD16– monocytes. PlGF KO mice display reduced tumour angiogenesis and tumour growth due to decreased monocyte infiltration (43). In addition, VEGFR1TK-KO transgenic mice expressing a VEGFR1 receptor with an inactivating mutation in the kinase domain display impaired angiogenesis as well as inflammation in different disease models (44). Moreover, monocytes may contribute to angiogenesis in a paracrine fashion. Interestingly, mononuclear cell precursors can give rise to endothelial-like cells which contribute to angiogenesis either by incorporating in the endothelium or by secreting factors which will induce angiogenesis (45–47).

Monocytes play an important role in arteriogenesis. VEGF and MCP1 are cytokines which induce collateral formation. Both of them have been shown to promote monocyte migration suggesting that they can increase collateral growth either by monocyctic or EC recruitment (39, 41, 48). PlGF-deficient mice display reduced collateral formation/arteriogenesis supporting the importance of monocytes in arteriogenesis. It was also demonstrated that PlGF can induce collateral growth in a rabbit model of hind limb ischaemia (49, 50). These effects were shown to be monocyte dependent since depletion of monocytes almost completely abolished the arteriogenic activity of PlGF (49, 50). Likewise, VEGFR1TK-KO-deficient mice show impaired arteriogenesis (51–54) and it was suggested that VEGFR1 exerts these effects by regulating the recruitment of bone marrow-derived monocytes/macrophages, which in turn regulate angiogenesis by secreting various angiogenic growth factors.

Monocytes were shown to play an important role in heart repair following myocardial infarction (MI) (34, 55). In fact, monocytes are the inflammatory cell type dominating the infarcted myocardium. Although studies have shown that anti-inflammatory treatment is beneficial as it can decrease the infarct-size-to-area-at-risk ratio after ischaemia-reperfusion injury (56, 57), complete depletion of monocytes and macrophages may not be always beneficial. These contradictory roles of monocytes can be explained by the involvement of the different subsets. It has been proposed that at the early stages the pro-inflammatory monocytes will contribute to the clearance of debris and dead cells. During later stages the pro-angiogenic monocytes will promote angiogenesis and tissue repair (34). Indeed mouse studies have shown that Ly6C<sup>high</sup> pro-inflammatory monocytes dominate the infarcted myocardium (days 1 to 4) (3, 34, 58, 59) (Fig. 1A). During the later stages the Ly6C<sup>low</sup> reparative monocytes are recruited and promote the resolution of inflammation and tissue repair and it has been suggested that they initiate a response that resembles the one described for M2 macrophages, which are thought to be involved in tissue repair (3, 34). Similarly human studies have shown that CD14++CD16– monocytes are increased during the first days after MI (day 2) while the CD14++CD16– monocytes peak later (day 4) (Fig. 1A) (60, 61). In support of this idea, CD14++CD16– monocytes were negatively related to left ventricular recovery after acute myocardial infarction while the number of CD16+ monocytes has been shown to be higher in patients with stable angina pectoris compared with patients with acute myocardial infarction (60, 62, 63). In agreement with this it was shown that abrogation of the inflammatory phase results in reduced removal of dead cells and debris, whereas abrogation of the repair phase decreases angiogenesis and the deposition of collagen (34). Interestingly, a similar biphasic response was reported for stroke, skin wounds and cancer (34, 64, 65). Recent studies provided evidence that monocytes recruited to sites of injury such as MI live for a short period, and that changes in the rate of cell recruitment and local death results in resolution of inflammation. In a model of murine MI it was demonstrated that monocyte recruitment is not only fostered by cells derived from the BM but also by extramedullary monocytopoiesis in the spleen (7, 65). Inhibition of splenic monocytopoiesis results in defective cardiac repair and accelerated the evolution of heart failure.

### Monocyte function in CVD

Monocytes play important roles in vascular repair and homeostasis and their elimination leads to inadequate angiogenesis and tis-
Figure 1: Role of monocytes in myocardial repair (A) and in atherosclerosis (B). A) Biphasic monocyte responses following MI: in the early phase 1 (day 0–4) there is an inflammatory response during which monocytes contribute to the removal of dead cells and debris. In the repair phase (day 3–8) there is resolution of inflammation and induction of angiogenesis and tissue repair. During late remodelling (week 2–5) there is collagen deposition and development of fibrosis. B) Circulating monocytes are recruited by chemokines and adhere to the activated endothelium. Transendothelial migration of monocytes leads to their accumulation and differentiation into macrophages which transform into foam cells following lipid uptake. Lesion progression occurs by migration of SMCs from the media to the intima, proliferation of resident SMCs and increased synthesis of extracellular matrix.
Atherosclerosis is the main cause of CVD in the Western world and refers to the development of atheromatous plaques in the arterial wall (Fig. 1B). Several studies have suggested that atherosclerosis is an inflammatory disease (66–69). During atherogenesis activation of the endothelium by irritating stimuli (such as dyslipidaemia, hypertension or pro-inflammatory mediators) results in increased adhesion and migration of monocytes into the intima where they mature into macrophages, which take up oxidised lipoproteins and convert to foam cells. Studies in mice provided evidence for the important role of monocytes in the development of atherosclerosis (70, 71). Osteopetrotic (op/op) mice lacking macrophage colony-stimulating factor M-CSF due to a structural gene mutation, display impaired growth and differentiation of monocytes as well as decreased numbers of circulating monocytes. Op/op mice have been crossed to a number of mouse models of atherosclerosis. It was shown that this resulted in inhibition of atherosclerosis development in ApoE-/- as well as in LDL receptor-deficient mice (70, 71). It was demonstrated that Ly6C<sup>high</sup> monocytes are increased in the blood of hypercholesterolaemic ApoE-deficient mice consuming a high-fat diet. Ly6C<sup>high</sup> monocyte adhesion to endothelium was increased and infiltration of Ly6C<sup>high</sup> was increased in the lesions where they became lesional macrophages, while Ly-6C<sup>high</sup> to Ly-6C<sup>low</sup> conversion was impaired (58). Statin treatment resulted in reduced cholesterol and reduced numbers of Ly6C<sup>high</sup> monocytes and as a result in reduced lesion formation further supporting the importance of monocytes in the development of atherosclerosis (58). Interestingly monocytes, which infiltrate atherosclerotic lesions, do not originate only from the BM, but also from the splenic reservoir (7, 72). Other studies demonstrate that both Ly6C<sup>high</sup> and Ly6C<sup>low</sup> monocytes are recruited to early atherosclerotic lesions. Combined inhibition of CCR2, CCR5 and CX3CR1 signalling inhibited monocyte recruitment and almost abolished atherosclerosis in the ApoE-/- mouse model further supporting the role of monocytes in the development of atherosclerosis (58, 73–75). Recently it was shown that efficient degradation of CCR2 mRNA in monocytes, using optimised lipid nanoparticles carrying siRNA for CCR2, prevents their accumulation at sites of inflammation and in atherosclerotic plaques, and reduces infarct size after coronary artery occlusion (76).

Because of their important role in vascular repair, several studies have focused on the analysis of the functional activity of monocytes in CVD. It was shown that monocytes isolated from individuals with diabetes mellitus (DM) fail to adequately respond to various growth factor stimuli (37, 39, 41, 77). The impaired chemotactic response of DM monocytes to VEGF-A is due to a VEGF signal transduction defect secondary to unspecific activation of downstream signalling pathways (37, 39, 41, 77). This is due to activation of RAGE (receptor for advanced glycation end-products) and ROS and inhibition of PTPs (protein tyrosine phosphatases) (77). Monocytes from DM patients do not adequately respond to other chemotactic ligands such as MCP1 or sonic hedgehog (77, 78). In contrast, monocyte migration towards transforming growth factor (TGF)β is not negatively affected by DM, suggesting that DM selectively interferes with specific growth factor signalling pathways. As a consequence, TGFβ may be used as an agent to stimulate collateral formation in DM patients, where other growth factors are resistant (79). Interestingly, monocytes from patients with other cardiovascular risk factors such as hypertension, smoking or hypercholesterolaemia, show a chemotactic defect towards VEGF ligands as well (80, 81). Although future work is awaited to provide more information, abnormalities in monocyte function may be one of the factors responsible for reduced vessel growth and vascular complications in patients with cardiovascular risk factors. Based on their important role in vascular repair and cardiovascular function, monocytes may provide potential targets for the development of diagnostics for cardiovascular risk assessment and for the development of novel cardiovascular therapies (41).

Several studies have tried to characterise the role of different monocyte subsets in CVD. CD14<sup>+</sup>CD16<sup>+</sup> monocytes was correlated to high risk and family predisposition in coronary artery disease (62). In another study it was shown that despite the fact that CD14<sup>+</sup>CD16<sup>+</sup> monocytes can predict future cardiovascular risk independently of other risk factors, however their numbers do not associate with the extent of atherosclerosis (82). In addition increased numbers of CD16<sup>+</sup> monocytes were associated with increased cardiovascular risk (82–84). Moreover, it was shown in stroke patients that CD16<sup>+</sup> monocytes have beneficial effect on clinical outcome, whereas CD14<sup>+</sup>CD16<sup>+</sup> have been associated with poor outcome and higher mortality (85). In line with these results it was suggested that CD16<sup>+</sup> monocytes might have a more protective, and perhaps reparative, rather than plaque-promoting, function. Clearly, more clinical studies are needed to fully understand the role of different monocyte subsets in CVD.

**Monocyte trafficking in vivo**

Several studies have focused on the characterisation of monocyte trafficking employing flow cytometric analysis of peripheral blood or microscopic analysis of the tissues. These types of studies provide information regarding the phenotype of the cells as well as their localisation at specific time points. Considering the dynamics of the immune system, it became apparent that novel approaches are needed for cell tracking in real time for better characterisation of their trafficking, turnover and effector functions. Genetic studies in mice have used reporter genes, e.g. green fluorescent protein or luciferase, under the control of specific promoters such as CX3CR1 or CD11b in order to track monocyte and their derivatives in vivo (3, 27). Although these approaches provided important information regarding monocyte trafficking and function, they have limitations due to inadequate imaging in larger areas or at increased depths. Consequently, these technologies have limited or no clinical translatability. In the clinic, imaging of the cardiovascular system using X-ray coronary angiography provides anatomical imaging and enables percutaneous coronary interventions (PCI) but does not always identify the atherosclerotic plaques at risk for rupture. In patients, following MI, function, perfusion and...
infarct size are measured by serial echocardiography, magnetic resonance imaging (MRI) and nuclear imaging techniques. Nevertheless, these techniques cannot always identify the patients at risk for developing heart failure. Thus, there is a need for better characterisation of the infarcted heart. As a result several investigators have focused on the development of molecular imaging of the cardiovascular system. Molecular imaging focuses on the immunobiology and cell biology of the vasculature and the infarcted area and may lead to an earlier and more precise diagnosis and possibly to the development of novel therapeutic strategies for cardiovascular pathologies (86).

To broaden our knowledge on atherosclerosis and on the role of monocytes therein, several studies have focused on the characterisation of mononuclear cell trafficking in vivo using animal models of atherosclerosis for coronary imaging. Monocytes and macrophages are characteristic cells within atherosclerotic plaques, which have the ability to perform phagocytosis. This characteristic has been used for targeted imaging of these cells. Variated types of nanoparticles labelled with different isotopes such as fluorine-18 (87), copper-64 (88), a variety of nanomaterials such as liposomencapsulated Gd-DTPA (89, 90) and ultrasmaller superparamagnetic iron oxide (USPIO) (91) nanoparticles were used to image monocytes/macrophages after they have phagocytosed these particles. Another approach used to image coronary arteries, already used in the clinic, is PET-CT (positron emission tomography – computed tomography) using the 18F-fluorodeoxyglucose (18F-FDG) PET isotope. 18F-FDG is a glucose analogue and it is taken up by cells with high metabolic activity. Inflammatory atherosclerotic plaques show high signals on PET images. It was demonstrated that the 18F-FDG signal correlates to the number of monocytes/macrophages within the atherosclerotic lesions (92–94). Nevertheless, since 18F-FDG is not specific to leukocytes in the atherosclerotic plaques, there are technical difficulties to be considered when using this imaging technique.

MI triggers an inflammatory response in the myocardium, which initiates the wound healing process. As mentioned earlier, monocyte recruitment plays an important biphasic role in post-MI vascular repair. Therefore imaging approaches, which are able to detect increased and sustained inflammation post MI, would be of great importance for the identification of individuals at high risk for heart failure. Studies in preclinical models have employed ex vivo labelling of purified populations of monocytes with exogenous cell trackers. Labelled monocytes were reinjected into the recipient and then followed in vivo (95, 96). A variety of optical (fluorochromes), nuclear (111In-oxine) and MRI (Feridex, cross-linked iron oxide (CLIO), USPIO) were used as exogenous trackers. Nevertheless, we have to keep in mind that ex vivo labelling of the cells may affect their behavior in vivo. Other trackers, which can be taken up by monocytes/macrophages in vivo, have been used to image MI in vivo. MRI was used to follow the uptake of iron oxide nanoparticles or 18Fluorine-labelled liposomes by macrophages. Myeloperoxidase (MPO) is a biomarker for inflammatory monocytes and neutrophils which was also used in MRI imaging to image monocytes in the infarcted heart (90, 97). In addition, several studies have focused on multimodal imaging such as PET-CT imaging of the atherosclerotic plaques or PET-MRI for imaging of the heart. Despite the advances in the field of molecular and cellular imaging and the promising results, more studies are required for improvement of molecular imaging of the heart. The identification of specific markers for monocyte tracking in vivo will be of great importance and will provide the field of cardiovascular imaging with great opportunities which may lead to clinical translation of molecular imaging and for the development of patients’ individualised risk assessment.

Concluding remarks

Monocytes are emerging as therapeutic targets in a multitude of disorders which involve inflammation. Monocytes are circulating cells and their recruitment to different sites of the body is a dynamic process depending on the locally produced factors. In addition, monocytes can adapt their function according to the micro-environmental changes. Nevertheless defects in monocyte function lead to pathological situations such as atherosclerosis or vascular injury. Despite the advances in our understanding of the role of monocytes in CVD there are still a lot of questions remaining. What is the fate of monocytes in the atherosclerotic plaques or in the myocardium following MI in vivo? Does the monocyte phenotype in blood correlate with the one at the lesions or at the infarcted myocardium? May the deletion of specific types of monocytes be beneficial for therapy? Despite the technical challenges, further characterisation of monocyte function ex vivo, in vivo as well as by molecular imaging will provide novel insights into the differentiation properties, function and trafficking of monocytes, their interactions with other cell types as well as the identification of more specific phenotypic markers. This might help us to elucidate the biological role of human monocyte subsets in the various stages of CVD and may lead to the development of novel therapeutic interventions and disease-monitoring.

Acknowledgements

Our studies on the role of growth factor signaling in CVDs are supported by the Interdisziplinäres Zentrum für Klinische Forschung (IZKF) Münster and by the 'Innovative Medizinische Forschung' (IMF PA121004) program of the Medical Faculty of the University of Münster. Additional markers from the ones indicated have to be used to define the cells as monocytes.

Conflicts of interest

None declared.

References


