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Review

Variety matters: Diverse functions of monocyte subtypes in vascular inflammation and atherogenesis

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ABSTRACT

Monocytes are important mediators of the innate immunity by recognizing and attacking especially bacterial pathogens but also play crucial roles in various inflammatory diseases, including vascular inflammation and atherosclerosis. Maturation, differentiation and function of monocytes have been intensively explored for a long time in innumerable experimental and clinical studies. Monocytes do not represent a uniform cell type but could be further subdivided into subpopulations with distinct features and functions. Those subpopulations have been identified in experimental mouse models as well as in humans, albeit distinguished by different cell surface markers. While Ly6C is used for subpopulation differentiation in mice, corresponding human subsets are differentiated by CD14 and CD16. In this review, we specifically focused on new experimental insights from recent years mainly in regard to murine monocyte subpopulations and their roles in vascular inflammation und atherogenesis.

1. Introduction into monocyte immune functions

The importance of monocytes and their distinct subsets for vascular inflammation and atherosclerosis is documented by currently several thousand hits in PubMed e.g. [1,2,3] and is the central focus of this review. Several excellent review articles have already been published with regard to monocyte subsets during the course of experimental myocardial infarction. In this event, a bi-phasic recruitment of monocytes into the injured myocardium is described: an initial first phase dominated by large numbers of Ly6C^{hi} monocytes and a later second phase dominated by a much lower number of Ly6C^{lo} monocytes [4]. However, the sequence and origin of recruited monocyte subsets into the chronically inflamed arterial wall is different compared to the acute situation after myocardial infarction. To our best knowledge, no review article exists with respect to the role of monocyte subsets for the different states of experimental atherosclerosis and vascular inflammation. We want to close this gap with the present review. To better understand the role of monocytes in this context, we initially summarize their pivotal role in inflammation and subsequent immune response to combat pathogens. Of note, monocyte functions in vascular disease initially follow the same pattern.

The innate immune system consists of a small humoral and a major cellular component comprising different cell types that combat invading pathogens immediately as the first line of defense. These processes pave the way for the more specific subsequent adaptive immunity with effector cells of the lymphoid lineage and major humoral components. Collectively, these cells are referred to as inflammatory cells. The cellular component of the innate immunity includes leukocytes derived mainly from myeloid progenitors that giving rise to lineages of monocytes, macrophages, dendritic cells, granulocytes and mast cells [5]. Among these, monocytes show the largest plasticity. During hematopoiesis, they initially originate and mature in the fetal liver and later on in the bone marrow. In doing so, they run through different stages of progenitor cells and are finally released into the circulation by a common monocyte progenitor (cMoP) [6]. In the case of infection, monocytes follow a gradient of the chemokine CC-chemokine ligand 2 (CCL2, also known as monocyte chemotactic protein 1, MCP-1). They penetrate the effected tissue from the blood stream, designated as diapedesis, and differentiate into macrophages with extended effector function [7]. In this regard, a timely coordination of interacting processes of circulating monocytes with endothelial cells lining the inner lumen of the vessels is needed. These processes start with monocyte attraction through loose cell contact and firm adhesion and finally lead to transmigration of the monocytes across the endothelial barrier. C—C chemokine receptor type 2 (CCR2) and integrins such as $\alpha_4\beta_1$ integrin on monocytes and adhesion molecules such as vascular cell adhesion molecule 1 (VCAM-1) and intercellular adhesion molecule 1 (ICAM-1) on endothelial cells are responsible for these

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successive processes [8,9]. Invasion of monocytes from the blood stream across the vascular endothelium is required for basic immunological surveillance of tissues, as well as for pathogen clearance. Monocyte-derived tissue macrophages have the ability to phagocyte pathogens and produce reactive oxygen species (ROS) such as superoxide mainly by the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase for pathogen killing due to respiratory burst. Furthermore, they release high levels of cytokines and chemokines to recruit other immune cells which carry on the immune response [10]. Influx of these immune cells into an infected area produces an inflammatory reaction that is characterized by redness, heat, pain and swelling. Besides scavenging macrophages, monocytes can give rise to antigen presenting dendritic cells (DCs). They especially patrol tissues closely in contact with the external environment, such as the skin (here as specialized Langerhans cell), intestine but also the vascular wall. Due to pathogen contact, they become activated and migrate to close-by lymph nodes where they present antigens to T cells and B cells of the adaptive immune response [11]. Of note, during differentiation, monocyte-derived effector cells acquire extended functions, such as migration and phagocytosis. The critical precondition for a successful immune response is a timely recognition of invading pathogens. This is guaranteed by Toll-like receptors (TLRs) – mammalian homologues [12,13] of the Drosophila Toll protein [14,15] – which are highly expressed on immune cells but also on tissue cells including vascular cells. TLRs composed as homo- or heterodimers represent cognate pattern recognition receptors (PRRs) of the innate immunity. They recognize a high diversity of molecules common in pathogens of bacterial and viral origin referred to as pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharide, lipopeptides, peptidoglycan fragments and many more [16,17]. TLR ligation induces the activation of inflammatory pathways such as the mitogen-activated protein kinase (MAPK) cascade or nuclear factor κB (NFκB) finally leading to the expression of cytokines and co-stimulatory molecules [18]. > 10 TLR members have been identified in mammalian [19] that also recognize endogenous ligands released during tissue damage and fibrosis or from apoptotic cells [20]. These ligands include cellular components like polypeptides, polysaccharides and phospholipids or extracellular matrix (ECM) degradation products, which are capable of non-infectious immune cell activation. These ligands include proteins and peptides, polysaccharides and proteoglycans, nucleic acids and phospholipids and are cellular components or extracellular matrix (ECM) degradation products, which are capable of non-infectious immune cell activation. In regard to atherosclerosis, oxidized low density lipoprotein (oxLDL) or minimally modified LDL represent such endogenous ligands. They are derived from LDL particles from the blood and accumulate due to endothelial dysfunction in the vascular wall. Initially, monocytes take up these particles by TLR4 and the co-receptors cluster of differentiation (CD)14 and CD36. Thereafter, they differentiate to macrophages with enhanced uptake properties. By excessive uptake they transform into foam cells, which undergo apoptosis and finally form the necrotic core of the atherosclerotic plaque [21,22]. The biology of monocytes was first described with respect to their important function for the innate immunity. However, it has become evident that monocytes also play a central role in many inflammatory disorders such as rheumatoid arthritis or atherosclerosis independently of infectious pathogens.

2. Monocyte subsets enter the arena – Identification by function and markers

The general understanding of monocyte biology has fundamentally changed by the identification of distinct monocyte subsets. In a similar way, some time ago T cells have been identified as different subpopulations, characterized by their diverse function and markers, such as naïve T cells (CD45A, CCR7), cytotoxic T cells (CD8), T helper cells (CXCR, CCR4), regulatory T cells (CD25), memory T cells (CD127) and several others more [23].

2.1. Murine monocytes subsets

The groundbreaking discovery was made by Geissmann and colleagues in 2003; the group reported that blood monocytes consist of two principal subsets with distinct migratory properties [24,25]. The authors initially characterized these two subsets by the expression of different surface markers. One subset characterized by CX3CR1^{lo} CCR2^{+} Gr1^{+} with a relatively short live span that is recruited to inflamed tissue and a second subset characterized by the expression of CX3CR1^{hi} CCR2^{-} Gr1^{-} that was found in non-inflamed tissues. Gr1 is a glycosylphosphatidylinositol (GPI)-linked protein expressed on granulocytes and macrophages known to be important for myeloid differentiation. Gr-1 is also known as Ly6C and the latter term has established itself in the literature, however, in different spelling variants such as Ly6C^{+}/Ly6C^{-} or Ly6C^{high}/Ly6C^{low}. In this review, we use the short form Ly6C^{hi}/Ly6C^{lo} that is widely accepted but we have used all variants for literature search. Ly6C^{hi} monocytes are also referred to as classical monocytes, whereas Ly6C^{lo} monocytes are referred to as non-classical monocytes.

When infiltrating tissues, the diversity of monocytes is carried forward to macrophage and dendritic cell diversity. Particularly in early inflammation, Ly6C^{hi} inflammatory monocytes are generally thought to preferentially differentiate into pro-inflammatory type I macrophages (M1) whereas Ly6C^{lo} monocytes are thought to develop more likely to anti-inflammatory or pro-reparative type II macrophages (M2) [26]. However, this scenario is not strictly black-and-white and cell type diversity is accompanied by high cell type plasticity. In other words, various cell types can differentiate into different other cell types, often in a bidirectional manner depending on exogenous stimuli and microenvironmental factors. In regard to macrophage and dendritic cell biology this goes far beyond the scope of this review but could be read up in different excellent reviews e.g.[26–28]. Besides LyC6^{hi} and LyC6^{lo} monocytes, the existence of a third intermediate murine subpopulation is disputed among the scientists in the field. This subpopulation is characterized by an intermediate expression of Ly6C therefore referred to as Ly6C$^{+/-}$ or Ly6C^{int} monocytes. They generally possess rather more pro-inflammatory characteristics [29] and may just represent a transitional stage in the differentiation from Ly6C^{hi} to LyC6^{lo} monocytes. Depending on the FACS gating settings, monocytes with intermediate Ly6C expression are sometimes assigned to either Ly6C^{hi} or Ly6C^{lo} monocytes but are frequently completely excluded from the analysis. Since the independent status of Ly6C^{int} monocytes has not yet been definitively clarified and very little is known in terms of their potential role in vascular pathophysiology we do not discuss this subset in our review in detail. In the following chapters, we will depict the current level of knowledge on Ly6C^{hi} and Ly6C^{lo} monocyte maturation and their role in different stages of vascular inflammation and atherosclerosis. Research in the field is progressing fast and this review is more a current snap shot than a final report. This is particularly true for murine monocyte subsets where advanced techniques such as genetic fate mapping experiments are applicable. Recent studies suggest even greater monocyte diversity. Beyond Ly6C, other markers have been identified to characterize highly tissue-specific or disease-specific monocyte subsets. To give just one example, regulatory monocytic myeloid-derived suppressor cells could be distinguished from their granulocytic counterparts by the expression of CD48 [30].

2.2. Human monocyte subsets

Similar to the classification of monocyte subtypes in mice, peripheral blood monocytes in humans can also be divided into different subpopulations with distinct phenotypic and functional properties. However, human monocyte subpopulations are characterized by other markers, namely by the lipopolysaccharide (LPS) receptor CD14 and the FcγIII receptor CD16 and have already been described in 1989 [31]. Based on differential expression of these markers, three functionally

Table 1
Markers and role of murine and human monocyte subsets in vascular inflammation and atherogenesis.

	Markers	Chemokine receptors	Role in vascular inflammation and atherosclerosis
Murine subsets	$Ly6C^{hi}$ (classical)	$CCR2^{hi}CX3CR1^{lo}$	– rolling on the endothelium – recruitment to lesions (more frequently) – differentiation to M1 macrophages (more frequently) – high phagocytic capacity
	$Ly6C^{int}$(intermediate)	*$CCR2^{hi}CX3CR1^{lo}$*	– *existence as distinct monocyte subtype is unclear* – *characteristics similar to $Ly6C^{hi}$monocytes*
	$Ly6C^{lo}$ (non-classical)	$CCR2^{lo}CX3CR1^{hi}$	– patrolling along the endothelium – recruitment to lesions (less frequently) – differentiation to M2 macrophages (more frequently)
Human subsets	$CD14^{++}CD16^{-}$ (classical)	$CCR2^{hi}CX3CR1^{lo}$	– high phagocytic capacity
	$CD14^{++}CD16^{+}$ (intermediate)	$CCR2^{int}CX3CR1^{lo}$	– high capacity of endothelial adherence
	$CD14^{+}CD16^{++}$ (non-classical)	$CCR2^{lo}CX3CR1^{hi}$	– patrolling along the endothelium

diverse subtypes are distinguished [32]. CD14 and CD16 are described as the essential markers delineating human monocytes subsets, but also other cells than monocytes – like neutrophils and NK cells – express these receptors on their cell surface. Thus, it is necessary to use additional pan-monocytic markers like CD86 or HLA-DR to obtain reliable identification of monocyte subpopulations. Several publications dealing with the improvement of FACS gating strategies for the optimal discrimination of monocyte subsets by cytometry have been published so far e.g. [33].

The classical inflammatory monocytes ($CD14^{++}CD16^{-}$) closely resemble the murine $Ly6C^{hi}$ subtype. They originate from the bone marrow or spleen reservoir and comprise ≥92% of monocytes in the human blood. Classical monocytes express high levels of CCR2 as well as the chemokine receptors CXCR1, CXCR2, and CXCR4 [34]. Upon LPS stimulation, they primarily release pro-inflammatory cytokines and chemokines like interleukin (IL)-1β, IL-6, IL-8, Tumor necrosis factor-alpha (TNF-α) and CCL2 [35]. Moreover, these cells express a wide range of genes involved in phagocytosis like CD93, CD64, CD32, CD36, ficolin 1 (FCN1) and signal regulatory protein alpha (SIRPA) and exhibit the highest phagocytic capacity of all monocyte subtypes [36,37].

While it is still undetermined if murine $Ly6C^{int}$ monocytes are a kind of intermediary stage during the conversion from $Ly6C^{hi}$ to $Ly6C^{lo}$ monocytes or a functionally distinct cell population, a defined subtype of intermediate monocytes in humans ($CD14^{++}CD16^{+}$) has been clearly identified. These intermediate monocytes share phenotypic and functional features of both classical and non-classical monocytes. They express CCR1 and CCR2 as well as CXCR2, which are common for classical monocytes, but also CX3CR1 that is mainly expressed on non-classical monocytes. Like classical monocytes, they exhibit CCR5 on their cell surface, indicating that they may also be attracted to atherosclerotic lesions in a CCR5-dependent manner and thus may contribute to cardiovascular diseases [36,38–40]. Intermediate monocytes are involved in the production of ROS and pro-inflammatory mediators like TNF-α and IL-1β. In contrast, it has also been reported that they are the main source of the anti-inflammatory cytokine IL-10 [41]. Furthermore, these cells express Tie2, an angiopoietin receptor normally expressed on endothelial cells and an early marker for angiogenesis indicating phenotypic similarities to TEMs (Tie2-expressing monocytes) [42].

The non-classical monocytes ($CD14^{+}CD16^{++}$) in humans are equivalent to the patrolling $Ly6^{lo}$ monocyte subtype in mice. They do not express CCR2 and CD62L, but high levels of CX3CR1 [43]. Like in mice, they are involved in tissue regeneration and debris removal from the vasculature and are accountable for the vascular integrity. As mentioned above, non-classical monocytes are far more motile than classical monocytes and express high levels of genes associated with cytoskeleton mobility [25,44]. As in the murine system, they exert similarities to tissue macrophages [45]. The expression of genes associated with maturation progressively increases from classical via intermediate to non-classical monocytes. Furthermore, telomere length in non-classical monocytes is shorter indicating that these cells are more mature than classical monocytes [35,46]. In patients with hematopoietic stem cell transplantation, monocyte subpopulations develop from the initially transplanted common precursor cell and gradually increase by the early appearance of $CD14^{++}CD16^{-}$ monocytes, followed by an increasing number of $CD14^{++}CD16^{+}$ and subsequently $CD14^{+}CD16^{++}$ monocytes as the most mature cellular subtype [47]. Similar results were reported in a study that investigated the developmental relationship between all three monocyte subpopulations using deuterium labeling. Following monocytopenia, the restoration of blood monocytes behaves the same way as after stem cell transplantation [48].

With regard to inflammation, non-classical monocytes are thought to release mainly pro-inflammatory cytokines like IL-1β and TNF-α upon LPS stimulation, although these data are still discussed controversially [49,50]. A recent report may give a solution for the controversy as it demonstrated that the highly pro-inflammatory response following TLR stimulation in vitro was associated with high levels of miR-146a, a miRNA that is known to negatively regulate TLR signaling and associated with senescence in other cell types [48]. Here, non-classical monocytes exhibited the clearest hallmarks of senescence including proliferative status, telomere length, cellular ROS levels, and mitochondrial membrane potential, followed by the intermediate and classical subset. The highly pro-inflammatory nature of the non-classical monocytes could be a manifestation of a senescence-associated secretory phenotype (SASP), likely induced by a high basal NF-κB activation. Besides pro-inflammatory cytokine induction, NF-κB also upregulates miR-146a as a negative feedback loop mechanism to limit inflammation. We have summarized the principal markers and chemokine receptors for murine and human monocyte subsets in Table 1.

3. Monocyte mobilization and tissue recruitment

3.1. Monocyte mobilization from the bone marrow and spleen

The development of monocytes in the bone marrow is attributed to a multi-stage procedure of differentiation that originates from hematopoietic stem cells (HSC). While long-term HSCs have self-renewing potential and maintain the pool of pluripotent progenitor cells, short-term and multi-potent HSCs trigger lineage-restricted differentiation of cells [51,52]. Clonogenic common progenitor cells differentiate in either lymphoid lineages (common lymphoid progenitor, CLP) and give rise to all lymphoid blood cells including T, B and natural killer (NK) cells or myeloid lineages (common myeloid progenitor, CMP) [53,54]. From CMPs, megakaryocyte-erythrocyte progenitors as well as granulocyte and macrophage progenitors (GMP) arise which further differentiate to all mature erythroid-lineage and myeloid-lineage cells, respectively [55]. In this process, the differentiation step to GMPs is critical for the development of the myeloid lineage including cells like granulocytes, monocytes, macrophages and dendritic cells [56]. Subsequently, a more specialized monocyte/macrophage and dendritic cell

progenitor cell emerges (MDP) [57,58]. There is evidence that beyond MDPs mixed populations of progenitor cells exist, which have an even more restricted lineage potential. Recent studies identified a common dendritic cell precursor (CDP) that originates from MDPs and does not generate monocytes [59]. Furthermore, the existence of a MDP-derived common monocyte precursor (cMoP) has been confirmed that strictly triggers the differentiation to Ly6C^{hi} and Ly6C^{lo} monocytes, although in different entities [60].

Currently, Ly6C^{hi} and Ly6C^{lo} monocytes are supposed to differ in their maturation status and that Ly6C^{lo} monocytes originate from Ly6C^{hi} monocytes. This consensus is mainly based on the evidence that in monocyte-depleted mice there is a temporal dependency in the de novo regeneration of Ly6C^{hi} monocytes that emerge first before Ly6C^{lo} monocytes appear again [61,62]. These findings were strengthened by detailed lineage mapping studies in which the incorporation of bromodeoxyuridine (BrdU) within the monocyte subsets was investigated [63]. In line with previous studies, Ly6C^{hi} monocytes rapidly incorporated BrdU with only a short delay between bone marrow-derived and blood monocytes. However, the BrdU incorporation by bone marrow-derived Ly6C^{lo} monocytes and more importantly, the appearance of BrdU labeled Ly6C^{lo} monocytes in the periphery took significantly longer. One important objection about these studies is the fact that not only monocytes but also other proliferating cells – in particular stem cells – were labeled in this approach. Therefore, adoptive transfer experiments were performed in which bone marrow-derived Gr1^{hi} (Ly6C/Ly6G^{hi}) monocytes were implanted in mice and the presence of donor-derived Ly6C^{lo} monocytes was proven three days after transplantation [64]. This approach enabled the exclusion of MDP from being BrdU labeled as these cells lack Gr1. However, after discovering the existence of cMoPs as a CD117^{+}Ly6C^{hi} progenitor population further adoptive transfer studies using cMoP cells had to be performed to confirm the concept of Ly6C^{hi} to Ly6C^{lo} monocyte conversion [60]. Nevertheless, although multiple laboratories using different experimental strategies which all support the conversion hypothesis, it cannot be excluded that a still unidentified progenitor cell in the bone marrow exists that directly gives rise to Ly6C^{lo} monocytes.

Besides the bone marrow, the murine spleen serves as a reservoir of Ly6C^{hi} and Ly6C^{lo} monocytes that are also recruited in case of inflammation. As the spleen stores more monocytes than are circulating in the periphery, their release results in a massive increase of monocytes in the circulation that decisively contributes to immune regulation by either inflammatory or regenerative properties [65,66]. Beyond that, the murine spleen comprises a significant amount of myeloid progenitor cells, making the spleen to a site of extramedullary hematopoiesis. Depending on the hematopoietic stress that induces extramedullary hematopoiesis, HSCs are mobilized to sites outside the bone marrow like the spleen where they assemble around sinusoids in the red pulp and expand hematopoiesis [67]. Robbins et al. have demonstrated in an atherosclerotic mouse model that hematopoietic stem and progenitor cells relocate from the murine bone marrow to the splenic red pulp where they – triggered by granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-3 – clonally expand and differentiate to Ly6C^{hi} monocytes. These extramedullary generated Ly6C^{hi} monocytes then invade atherosclerotic lesions and fuel the inflammatory process by secreting ROS and pro-inflammatory cytokines [66].

3.2. Monocyte recruitment to target tissues

The recruitment of monocytes to sites of inflammation is critical for host defense. In this context, changes in the microenvironment such as stress, cholesterol loading, hyperglycemia and infection have a great impact on the expression of different transcription factors and cell surface receptors and decisively determine the fate of lineage differentiation of HSCs. Stress for example enhances bone marrow-mediated monocyte production by diminished expression of C-X-C motif chemokine ligand 12 (CXCL12) which triggers myeloid proliferation [68]. However, hypercholesterolemia has a different kind of mechanism to induce proliferation of CMPs and GMPs thereby triggering monopoiesis. It has been shown that cholesterol accumulation due to an impaired cholesterol efflux in bone marrow HSCs results in an increased formation of lipid rafts that promote clustering of the common β-subunit of the IL-3/GM-CSF receptor within the plasma membrane [69–71]. As a result, these mice exhibit an augmented monocyte accumulation and macrophage burden in atherosclerotic lesions. Comparable results have been demonstrated for hyperglycemia. In diabetic mice, the enhanced number of peripheral monocytes was due to an increased expression of S100A8/S100A9, which induces GMP proliferation through interaction with the receptor for advanced glycation end products (RAGE) [72]. In CMPs, it has been reported that changes in the intracellular glucose metabolism lead to alterations in the expression of the glucose transporter type I, which increases CMP proliferation and likewise monocyte production [73]. With regard to microbial infections, a variety of different studies have demonstrated that monocytosis is induced through a TLR2-mediated increase in CCL2 secretion [74]. While the interaction of the stromal cell-derived factor-1 (SDF-1) and its receptor CXCR4 is required for the retention of precursor cells in the bone marrow [75,76], monocyte chemoattractant proteins have been shown to be important players for the emigration of monocytes from the bone marrow into the bloodstream and the recruitment to affected tissues. Especially CCL2 and its corresponding receptor CCR2 are pivotal in Ly6C^{hi} monocyte tissue recruitment [1,77]. This was demonstrated by CCR2-deficiet mouse models causing a significant reduction in circulating Ly6C^{hi} monocytes but accordingly leading to an accumulation of monocytes and monocyte precursors in the bone marrow of CCR2$^{-/-}$ mice [74,78]. Of note, CCR2-deficient mice are protected from the development of atherosclerosis [1].

It is still uncertain how exactly inflammatory processes in central organs or remote tissues promote emigration of inflammatory Ly6C^{hi} monocytes from the bone marrow. Most probably, cells at the site of inflammation produce high amounts of chemokines that enter the circulation thereby building up a gradient towards the inflamed tissue. This chemokine gradient triggers the release of monocytes from the bone marrow. But this scenario presumes a high amount of secreted chemokines in order to achieve a response in the bone marrow despite the chemokines' dilution in the plasma and their rapid clearance by decoy receptors [79,80]. Moreover, although circulating chemokines can reach high concentrations in the bloodstream at later stages of inflammation, monocytes are recruited to the affected tissue at very early time points [81,82]. It remains to be clarified which exact mechanism is underlying the recruitment of inflammatory Ly6C^{hi} monocytes in the early time of inflammation. Of note, in the absence of inflammation, Ly6C^{hi} monocytes immigrate again into the bone marrow and may differentiate to Ly6C^{lo} monocytes [61,64].

Besides all environmental changes that influence the presence of monocytes in the circulation by either affecting progenitor proliferation or by shaping the release of cytokines that regulate monocyte egress from the bone marrow, it is also conceivable that epigenetic factors might cause a transcriptional reprogramming of myeloid progenitors during disease with crucial effects on their release and final quantity. The key transcription factors controlling monocyte differentiation have been reviewed before [83] and include PU.1 that is critical for early steps of myeloid development [84], runt-related transcription factor 1 (RUNX1) that regulates transcription of PU.1 [85], nuclear receptor subfamily 4, group A, member 1 (Nr4a1, Nur77) which is necessary for Ly6C^{lo} development [86] as well as GATA binding protein 2 (GATA2) that is involved in myeloid progenitor self-renewal and differentiation [87], Friend leukemia integration 1 transcription factor (Fli-1) which negatively regulates myeloid cell development [88], interferon regulatory factor 8 (Irf8) and its direct target gene Kruppel-like factor 4 (KLF4) as well as different CCAAT/enhancer-binding-protein (C/EBP) isoforms. In the context of disease however, it will be crucial to understand how these transcription factors are being further regulated by

noncoding RNAs and miRNAs and how they interact in terms of transcriptional output resulting in the generation of either pro-inflammatory Ly6C^{hi} or reparative Ly6C^{lo} monocytes.

3.3. Tissue-resident macrophages do not originate from monocytes

It is well accepted that HSCs-derived monocyte subsets released from bone marrow or spleen differentiate into macrophages subtypes when invading tissues in the course of inflammation. However, this does not apply for the comparatively modest number of resident, locally self-maintaining macrophages that monitor tissues under homeostasis and contribute to tissue integrity. Recent work using fate mapping experiments identified early progenitors in the yolk sac as the origin of these macrophages. Schulz and coworkers initially reported that the transcription factor Myb was crucial for the development of HSCs and subsequently for all CD11b-positive monocytes and macrophages, but was not necessary for the development of yolk sac-derived F4/80-positive tissue-resident macrophages [89]. These cells have been already established prior to birth and their maintenance during adulthood is independent of the replenishment by blood monocytes [63]. Accordingly, blood monocytes did not make a notable contribution to the development of tissue-resident macrophages that repopulate after depletion in a macrophage colony-stimulating factor (M-CSF) and GM-CSF-dependent manner [90]. Blood monocytes do not necessarily differentiate to macrophages upon tissue invasion. Jakubzick et al. used a series of different techniques showing that blood monocytes can enter tissues without major changes in gene expression profiles and recirculate to lymph nodes without differentiation to macrophages [91]. The myocardium is among the organs in which yolk sac-derived macrophages persisted in adulthood. Recently high myocardial macrophage heterogeneity has been reported by identifying four distinct macrophage subpopulations. Under homeostatic conditions, resident myocardial macrophages were maintained through proliferation. During myocardial inflammation, blood monocytes contributed to all myocardial macrophage subsets, whereas resident macrophages also expanded through proliferation [92]. Perdiguero and colleagues reported that tissue-resident macrophages originate from erythro-myeloid progenitors distinct from HSCs. These progenitors develop in the yolk sac at E8.5 and migrate and colonize the fetal liver before E10.5 [93]. Also tissue-resident arterial macrophages have been recently identified, which originate from the yolk sac as well as from blood-derived monocytes shortly after birth and self-maintain in steady state and after infection [94].

Excellent recent experimental studies have already uncovered a great amount of detail on tissue-resident macrophages and their contribution in physiology and pathology. In this regard, the yolk sac and the fetal liver as embryonic sources of macrophage precursors have been identified from where they colonize tissues via the blood during embryogenesis and shortly after birth [95]. However, the detailed mechanism of tissue-specific migration of these cells remains largely unknown. Since most of the new insights of tissue-resident macrophages are based on advanced genetic techniques in murine models their origin and role in humans is poorly known.

4. Monocyte subsets in vascular disease

With annually 17.7 million cases, cardiovascular diseases (CVD) make up one third of all deaths worldwide (WHO 2018). CVD include coronary artery disease (CAD), cerebrovascular disease and peripheral arterial disease (PAD), which are mainly caused by atherosclerosis. Atherosclerosis itself is a slowly developing, multifaceted inflammatory disease. Not only well-known risk factors like smoking, diabetes, arterial hypertension or augmented plasma lipid levels but also an activated innate immune system with its effector cells such as monocytes and macrophages are of vital importance for the pathology. After the initial development of endothelial dysfunction, monocytes are the very first cells being attracted to these sites and infiltrate into the subendothelial space. Thus, monocytes play a crucial role already during the earliest events of the disease. Accordingly, Saederup et al. observed markedly reduced atherosclerotic lesion size in mice lacking monocyte chemoattractant proteins or their corresponding receptors [96]. In the following section, the different roles and processes of monocyte subsets during atherogenesis will be discussed.

4.1. At the very beginning: endothelial dysfunction and monocyte recruitment

Different factors like oxidative stress, proinflammatory cytokines, viral or bacterial infections, hyperglycemia or elevated blood plasma LDL levels lead to an activated endothelium. If these factors are derailing this entails a dysfunctional endothelium with partial loss of the vascular barrier function, an increased chemokine production and an enhanced expression of leukocyte adhesion molecules [97–99]. In this context, the bioavailability of nitric oxide (NO) is known to be crucial in determining endothelial activation. Among others, NO regulates leukocyte adhesion and migration but also endothelial cell homeostasis by inhibiting platelet aggregation [100]. Upon activation, platelets induce a proinflammatory signaling in monocytes and increase the number of platelet-monocyte complexes which correlate with the occurrence of an acute coronary syndrome [101,102]. In a misbalanced, pro-atherosclerotic and pro-oxidative environment, lipoproteins become redundantly oxidized. Elevated levels of oxLDL were already described in the 1980´s to exert chemotactic effects on monocytes in humans [103] inducing complex formation with platelets and facilitating recruitment and endothelial activation [104].

Activated endothelial cells release chemokines like CCL2 and CCL5 and up-regulate immunoglobulin-like adhesion molecules such as VCAM-1 and ICAM-1 [105]. In this regard, the pivotal role of CCL2 in vascular inflammation has been already introduced earlier. High levels of circulating CCL2 attract particularly CCR2 expressing Ly6C^{hi} monocytes to atherosclerotic lesions [2]. Furthermore, Tsou et al. observed that CCR2 deficiency decreased blood monocytes but conversely increased total monocyte counts in the bone marrow suggesting a critical role for CCR2 in monocyte egress [78]. These data point towards two different modes of action for CCR2: The first one mediates monocyte egress from the bone marrow; the second one mediates monocyte recruitment from the blood into the inflamed vascular wall. Several experimental approaches indicate that circulating CCL2 binds to components of the extracellular matrix like glycosaminoglycans and collagens at specific tissues resulting in a CCL2 gradient [106,107]. However, different other factors have been identified as chemokines for monocytes. For instance, CCL7, another agonist for CCR2 (also referred to as MCP-3) was shown to be induced in rat aortic smooth muscle cells and carotid arteries upon inflammatory stimuli or balloon angioplasty. In this context, CCL7 has been shown to be critical for the recruitment of Ly6C^{hi} monocytes as well as for the aortic and hepatic lipid accumulation [78,108,109]. Circulating Ly6C^{hi} monocytes are recruited along such chemokine gradients. Subsequently, endothelial expressed lectin-like adhesion glycoproteins P- and *E*-Selectin enable initial monocyte capture, deceleration and rolling by interacting with P-Selectin glycoprotein ligand (PSGL)-1. In this regard, An and coworkers observed that Ly6C^{hi} monocytes express PSGL-1 at a significant higher level compared to Ly6C^{lo} monocytes, consequently this subset shows enhanced binding to P- and *E*-Selectin. Furthermore, atherosclerosis-prone apolipoprotein (Apo)E$^{-/-}$ mice lacking PSGL-1 had an impaired recruitment of Ly6C^{hi} monocytes to atherosclerotic lesions [110]. Firm adhesion of Ly6C^{hi} monocytes to the endothelium is mediated via VCAM-1 and ICAM-1 and their interaction with the corresponding integrins expressed on the monocyte surface, namely very late antigen (VLA)-4 and CD11/18. Accordingly, Park et al. showed that administration of a monoclonal antibody that specifically blocks VCAM-1 leads to attenuated monocyte infiltration and plaque formation in ApoE$^{-/-}$

mice [3].

4.2. Proceeding: invasion of monocytes into the vessel wall

After capture and firm adhesion to the endothelium via VCAM-1 and ICAM-1, Ly6C^{hi} monocytes undergo diapedesis and transmigrate into the subendothelial space. This is facilitated by additional vascular adhesion molecules like platelet endothelial cell adhesion molecule (PECAM-1), junctional adhesion molecules (JAMs) or connexins that form intercellular gap junctions. Hashimoto et al. observed a PECAM-1-dependent as well as -independent transmigration of human monocytes near to but also apart from junctions and elucidated the process of transmigration not only through cell-cell junctions but also directly through endothelial cells [111].

It is well established that mainly Ly6C^{hi} monocytes migrate into the subendothelial space at sites of an inflamed and dysfunctional endothelium whereas Ly6C^{lo} monocytes actively patrol along the vasculature [29,112]. In an elegant approach, Soehnlein et al. transplanted bone marrow from ApoE$^{-/-}$ mice and either administered leukocytes with or without Ly6C^{lo} monocytes to recipient ApoE$^{-/-}$ mice showing that mainly Ly6C^{hi} monocytes contribute to the progression of atherosclerosis [113]. However, this scenario is not strictly dogmatic. Monocyte subsets show a slightly different responsiveness to chemokines, partially due to a different surface expression of chemokine receptors. For example, Ly6C^{lo} monocytes are known to respond well to chemokine C-X3-C motif ligand 1 (CX3CL1). During hematopoiesis, the corresponding chemokine receptor CX3CR1 appears first on MDPs and is expressed on Ly6C^{lo} to a much higher extend compared to Ly6C^{hi} monocytes [24]. Consequently, deletion of CX3CR1 leads to diminished patrolling of Ly6C^{lo} monocytes and reduced monocyte survival since CX3CR1 also controls the expression of B-cell lymphoma 2 protein (BCL2) which exerts anti-apoptotic properties [25,114]. CX3CR1-dependent directional motility of Ly6C^{lo} monocytes on the endothelium, referred to as crawling, is mediated by a close receptor-ligand interaction with the β_2-integrin lymphocyte function-associated antigen (LFA)-1 as receptor on monocytes and its ligands ICAM-1 and ICAM-2 on endothelial cells [25]. According to the slightly different purpose, the patrolling of Ly6C^{lo} monocytes is approximately 100 slower (~12 μm/min) than the rolling of Ly6C^{hi} monocytes (~30 μm/s) and independent – different to rolling – of the direction of blood flow [25,115]. Interestingly, Marcovecchio and coworkers recently showed that the patrolling activity of Ly6C^{lo} monocytes is associated with the recognition of oxLDL via scavenger receptor CD36. These data suggest an activation of Ly6C^{lo} monocytes upon oxLDL uptake followed by increased mobilization and patrolling during early atherogenesis. However, they did not observe any changes in monocyte extravasation into the surrounding tissue [116].

Since resident Ly6C^{lo} monocytes are not only located at sites of inflammation but mainly scavenge their environment for microparticles and cell damage they are referred to as 'intravascular housekeepers' [29]. Carlin et al. observed that upon TLR7 stimulation, Ly6C^{lo} monocytes are retained at the endothelium in a G-protein ($G\alpha_i$)-dependent manner and orchestrate neutrophil recruitment and necrosis of endothelial cells followed by removal of cellular debris by phagocytosis [117]. Of note, Ly6C^{lo} monocytes are not restricted to the endothelial surface but also observed in atherosclerotic lesions, however in a significant smaller extend compared to Ly6C^{hi} monocytes [118]. These data not only suggest Ly6C^{lo} monocytes to undergo diapedesis as well, but also indicate that lesional macrophage and dendritic cell heterogeneity is the result of distinct monocyte subpopulations entering the vascular wall and building up the atherosclerotic plaque. Accordingly, Tacke et al. showed that compared to Ly6C^{hi}, monocytes Ly6C^{lo} monocytes more likely express dendritic cell-associated CD11c after entering the plaque. The same group additionally observed that CCR5, which plays a role in Ly6C^{hi} recruitment, is also expressed on the surface of Ly6C^{lo} monocytes and involved in their vascular recruitment. Interestingly, Ly6C^{hi} but not Ly6C^{lo} monocytes exhibit CX3CL1-dependent migration into atherosclerotic lesions pointing towards a differential role of this chemokine during Ly6C^{lo} monocyte patrolling and recruitment [118].

4.3. Finally: differentiation of monocytes to macrophages and foam cells

Although the identification of different mouse monocyte subsets dates back several years [24], less is known about the 'fate' of Ly6C^{hi} and Ly6C^{lo} monocytes with respect to their differentiation after extravasation into atherosclerotic lesions. This lack of information might be due to the fact that recruited monocytes tend to lose their Ly6C expression while becoming tissue macrophages [119]. Thus, other markers for subtype identification need to be used. Since Ly6C^{hi} monocytes are known to be precursors of inflammatory CX3CR1-expressing CD11b^{+} dendritic cells in the intestine, this monocyte subset is suggested to also give rise to inflammatory, classically activated M1 macrophages [120]. Accordingly, Huo et al. observed preferential differentiation of Ly6C^{hi} monocytes into M1 lesional macrophages in Bmal1-deficient mice [121]. In contrast, using a plaque regression model Rahman et al. recently suggested that Ly6C^{hi} monocytes primarily give rise to M2 macrophages [122]. Interestingly, Swirski et al. observed a Ly6C^{hi} monocytosis in hypercholesterolemic mice, which is the result of increased survival, proliferation and impaired conversion from Ly6C^{hi} to Ly6C^{lo} monocytes [123]. Additionally, this group showed a selective accumulation of Ly6C^{hi} monocytes in atherosclerotic lesions upon a high fat high cholesterol western diet and a rapid differentiation into lesional macrophages. However, a particular commitment of monocyte subsets to a distinct macrophage subpopulation remains unclear. As mentioned above, oxLDL drives monocyte recruitment via platelet activation. Badrnya et al. additionally showed that the presence of platelets doubled the amount of oxLDL taken up by monocytes in a P-Selectin-PSGL-1 interaction-dependent manner [104]. Lesional macrophages as well as dendritic cells become foam cells by oxLDL uptake and thus participate in the following necrotic core and plaque development [124].

Since Ly6C^{lo} monocytes are known to extravasate less frequently but are more tempted to differentiate into plaque cells expressing dendritic cell-associated CD11c [118], one can speculate that this monocyte subset preferentially gives rise to atherosclerotic plaque dendritic cells. As mentioned above, monocyte-derived dendritic cells can participate in plaque progression as foam cells or might play a role in the further activation of the adaptive immune system as antigen presenting cells, leading to activation and recruitment of T-cells [125]. Interestingly, Liu et al. observed decreased dendritic cell accumulation in the aortic wall in CX3CR1-deficient mice, pointing towards CX3CR1-expressing Ly6C^{lo} monocytes being the precursor of those intimal dendritic cells [126]. Additionally, it is discussed if Ly6C^{lo} monocytes preferentially give rise to plaque cells supporting the development of a more stable plaque phenotype since Ly6C^{lo} monocytes are known to support cardiac remodeling and wound healing after myocardial infarction by e.g. collagen production [127].

Beside scavenger receptors that enable oxLDL uptake, macrophages and dendritic cells also express TLRs and various other receptors involved in PAMP-recognition. Upon their stimulation, mainly pro-inflammatory cytokines such as TNF-α and IL-6 but also anti-inflammatory cytokines such as transforming growth factor beta (TGF-β) and IL-10 are released. In terms of pro-inflammatory cytokines inflammation and plaque progression is aggravated [128].

Apart from these observations, less is known about the monocyte subpopulations selectively giving rise to M1 and/or M2 macrophages. Furthermore, the question whether a distinct monocyte subpopulation preferentially enters plaque lesions in a certain stadium of plaque development remains unclear. These and other critical questions affecting the functional fate of monocyte subsets after extravasation need to be addressed in future studies. In order to better illustrate the complex

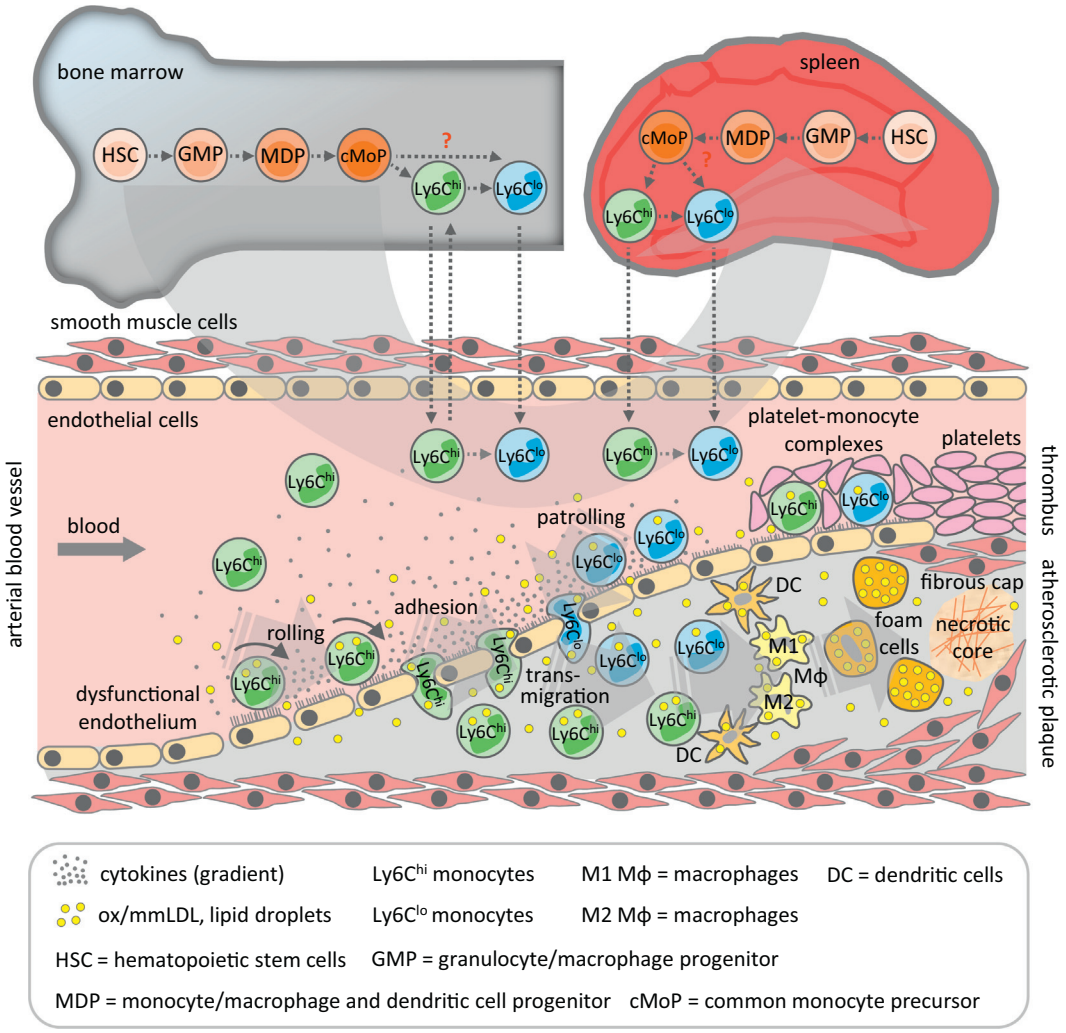

Fig. 1. Simplified scheme of monocyte maturation, differentiation, blood passage and recruitment to the vascular wall/atherosclerotic plaque. Hematopoietic stem cells (HSC), granulocyte-monocyte progenitor (GMP), monocyte/macrophages and dendritic cell precursors (MDP), common monocyte progenitor (cMoP), Ly6C^{hi} monocytes (Ly6C^{hi}), Ly6C^{lo} monocytes (Ly6C^{lo}).

processes of monocyte maturation, differentiation, blood passage and recruitment to the inflamed vascular wall and the growing atherosclerotic plaque we have summarized this scenario in Fig. 1.

As discussed in detail, the majority of macrophages in atherosclerotic plaques are derived from circulating blood monocytes. In this regard, developing lesions mainly recruit macrophages from blood monocytes, whereas macrophages in advanced lesions maintain or increase by local proliferation with less new monocyte recruitment [129]. Even though tissue-resident arterial macrophages have been already identified, the presence of tissue-resident macrophages in atherosclerotic plaques and their role in atherogenesis is still unclear. However, an interaction of tissue-resident arterial macrophages with monocyte subpopulations is conceivable and could potentially influence all processes discussed in this review.

4.4. A side glance on human monocyte subsets in vascular disease

In the development of atherosclerosis, CD16$^+$ monocytes are the focus of interest for several years now. In the beginning it has been shown that CD16$^+$ monocytes correlate with classical cardiovascular risk factors like body mass index, diabetes mellitus or intima-media thickness [130,131]. Moreover, another study conducted in low-risk individuals provided evidence that there is a significant association between CD16$^+$ monocytes and states of obesity [132]. Likewise, increased numbers of CD14$^+$CD16$^+$ monocytes correlate with coronary atherosclerosis and elevated TNF-α serum concentration [133]. However, all mentioned studies characterized entire CD16$^+$ populations without distinguishing intermediate from non-classical monocytes. Recent studies investigated individual monocyte subpopulations and revealed that the intermediate monocyte subpopulation plays a pivotal role in cardiovascular disease. CD14^{++}CD16$^+$ monocytes have been identified as an independent predictor of cardiovascular events [134,135]. There is also evidence that the number of CD14^{++}CD16$^+$ monocytes are associated with the vulnerability of atherosclerotic plaques [136,137]. In line with this, it was demonstrated lately that coronary plaque vulnerability in patients with stable angina pectoris might be due to an upregulation of TLR4 on intermediate CD14^{++}CD16$^+$ monocytes which positively correlated with the remodeling index and negatively correlated with computed tomography attenuation value [138]. Although monocyte frequencies were determined in most of the cases, there is also evidence that the shift of monocyte subsets along their CD14/CD16 continuum rather than the total amount of cells seems to be pivotal for the prediction of cardiovascular events [139]. In comparison to other monocyte subsets, intermediate monocytes adhere with sevenfold higher efficiency to VCAM-1, while the capture efficiency of CD14^{++}CD16$^+$ monocytes isolated from patients with acute myocardial infarction is twice as high as in healthy controls [140]. In line with this, monocyte-platelet aggregates with intermediate

$CD14^{++}CD16^{+}$ monocytes were increased in patients with diffuse CAD and therefore could represent an important contributor to accelerated coronary atherosclerotic plaque progression [141]. Furthermore, it has been shown that intermediate $CD14^{++}CD16^{+}$ monocytes were associated with low serum levels of high density lipoprotein (HDL) cholesterol and low ApoA-I in patients with chronic kidney disease. Additionally, demonstrate a higher binding affinity of enzymatically degraded LDL which results in an accelerated lipid accumulation and foam cell formation [142,143]. We have summarized the suggested roles of murine and human monocyte subset in vascular inflammation and atherogenesis as discussed in this review in Table 1.

Although a lot of research has been already conducted on the impact of human monocyte subpopulations on the development of atherosclerosis, there is still much work to be done if we want to adopt a global perspective reflecting all functions of monocyte subsets in cardiovascular disease.

5. Conclusion and perspectives

Even though the importance of monocytes for vascular inflammation and atherogenesis is known for some time, we have just started to understand the role of distinct monocyte subpopulations in this process. New experimental findings in terms of monocyte subpopulations are released in rapid succession, the appropriate research is in constant flux and we are aware that this review is just a status report. One challenging task for future research in the field is to answer the question about the factual existence of an intermediate monocyte subpopulation in mice. As already mentioned, the existence of such intermediate subpopulation in humans is much better accepted. This may be easily explained by comparing the FACS plots. Despite comparable gating strategies, human intermediate monocytes could be clearly differentiated from the other two subgroups as a defined independent subset. The situation in mice is often not so clear, which nicely correspondents with our own observations. It may be noted that the quantification of monocyte subpopulations from any kind of body fluids by FACS is unproblematic and gives reliable results. This is entirely different when cell populations within tissues are quantified. Mincing of tissue to liberate cells definitely affects cell quality for further analysis and immunohistochemical staining of tissue slides have technical limitations in the restricted use of antibodies. However, technical advancement in the field may overcome these limitations in the near future. Elegant genetical approaches such as subsets-specific fate mapping or advanced molecular biological techniques such as laser-assisted microdissection along with single cell PCR or proximity extension assay (Olink technology) are on the starting blocks to ensure a detailed cell population characterization and quantification within tissues.

Monocyte biology and vascular biology are closely connected since monocyte recruitment to any target site must be enabled by the vascular wall. From our perspective, it is well conceivable that the existing monocyte subpopulations will be further subdivided – beyond Ly6C – by novel surface markers and still to defined functions. The existence of monocyte subpopulations with regulatory function, which are already introduced e.g. [144,145], or memory functions would immunologically make sense, especially in long-term chronic inflammatory disorders such as atherosclerotic vascular disease.

Disclosure

None.

Conflict of interest

All authors declare that no conflict of interest exists.

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