

Review

Role of macrophages in atherosclerosis

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Received: 27 July 2020/Accepted: 25 August 2020/ Published: 30 September 2020

Abstract: Atherosclerosis is a chronic inflammatory state, which arise from the imbalance in lipid metabolism. Over the last decade, studies have showing the association of macrophages with this maladaptive immune response. Macrophages differentiated from monocytes and populate at the growing atherosclerotic lesions. At the lesion site by accumulating lipid they actively participate in the formation of atherosclerotic plaque. These plaques are very susceptible to rupture which can lead to myocardial infarction or stroke. In future more studies are needed to classify different macrophage populations according to their phenotypic and functional characteristics to identify their roles in the pathogenesis of atherosclerosis. This review highlights several aspects of macrophages activation, diversity, recruitment, and foam cell formation in atherosclerosis.

Keywords: atherosclerosis; macrophages; chemokines

1. Introduction

Atherosclerosis and its associated risk factor hypercholesterolemia causes 16.7 million deaths each year worldwide (Dahlöf, 2010; Lloyd-Jones, 2010). Atherosclerosis has been considered as a slowly progressing chronic inflammatory disease of the arterial walls (Back and Hansson, 2015). The branching points and bends of arteries are particularly susceptible to the formation of atherosclerotic lesion due to the disturbance of local endothelial functions. According to present knowledge, circulating low-density lipoprotein (LDL) molecules acts as a primary source of lipid accumulation in atherosclerotic lesions by entering the vulnerable areas of the arterial vasculature (Krauss, 2010). The accumulation of lipid form plaque at the atherosclerotic lesion which gradually reduce blood flow in the arteries. This eventually causes a complete blockage of the artery (Weber *et al.*, 2008) and result in insufficient supply of oxygen to the heart, or peripheral arteries.

The typical pathology of atherosclerosis is the accumulation of modified lipids, migration of inflammatory cells, formation of foam cells, and changes in the extracellular matrix (ECM) (Gronholdt *et al.*, 1998; Shah, 2002). Over the last few years, after studying a variety of experimental animals of atherosclerosis it has become increasingly clear that inflammation and immunity are tightly associated with atherosclerosis. Knowing the basics of the inflammatory processes is essential for understanding the complex mechanisms involve in atherosclerosis progression, not only in animal models but in humans as well.

Macrophages are the inflammatory cells which are first recruited to the atherosclerotic lesion sites and play a key role at all stages of atherosclerotic lesion progression. It is widely accepted that circulating monocyte-derived cells that enter the atherosclerotic lesions, further differentiate into macrophages. However, some recent studies have reported that macrophages can develop from precursor cells present in adult tissues, independently of the monocytes (Ginhoux and Jung, 2014). Macrophages in atherosclerotic lesions by ingesting the accumulated lipoproteins, can be converted into “foam cells” which are filled with lipid droplets. Notably, these cholesterol-engorged foam cells that accumulate in atherosclerotic plaques appear to have decreased emigrational capacity. Due to this, inflammation in atherosclerotic plaque cannot be easily resolved, and further

progress to more complex atherosclerotic plaque (Randolph, 2014). In these advanced plaques, macrophages further play role as a major contributor by maintaining the local inflammatory response through their secretion of pro-inflammatory mediators (chemokines, cytokines), and by producing reactive oxygen species. Inflammatory cytokines produced by macrophages induce the migration of more inflammatory cells to the lesion. Inflammatory macrophages even after their death play role in the formation of pro-thrombotic necrotic core by releasing lipid contents and tissue factor (Seimon and Tabas, 2009). Rupture of this necrotic core and the intravascular blood clot further contributes to myocardial infarction and stroke.

Although the association of macrophages with atherosclerosis has long been considered, the mechanism that regulates the formation of macrophage foam cells and their retention within the atheroma remains largely unknown (Randolph, 2014). Researchers are only beginning to elucidate the underlying mechanisms that control foam cell formation and retention within the plaque (Libby *et al.*, 2014; Moore *et al.*, 2013). In this review, we will discuss about the central roles of macrophages in the initiation, progression and resolution of atherosclerotic inflammation, by focusing on how the migration, recruitment, and death of macrophages alter the fate of the plaque.

2. Recruitment of circulating monocytes in atherosclerosis

During the past decade, the fact has become clear that hypercholesterolemia, an important risk factor for atherosclerosis, induces expansions of myeloid cells such as monocytes and neutrophils (Qiao *et al.*, 1997). By studying different animal models, e.g. mice, swine and rabbits a significant correlation between augmented number of circulating monocytes and hyperlipidemia has been identified (Gu *et al.*, 1998; Boring *et al.*, 1998). In apolipoprotein E-deficient (ApoE^{-/-}) animals, circulating proinflammatory monocytes are found ~50% higher than in the control animals (Swirski *et al.*, 2007). Hence, mechanisms controlling monocyte recruitment is crucial to understand the contribution of monocyte in atherosclerosis.

Monocytes are bone marrow-derived progenitor cells which do not proliferate in the blood (Geissmann *et al.*, 2003). During the process of atherosclerosis, endothelial injury leads to the migration of circulating monocytes to the intima and subintima (Cros *et al.*, 2010). The rolling of monocytes to the sites of injury usually facilitates by the adhesion proteins of selectin family: P- and E-selectin (Blankenberg *et al.*, 2003; Steffens and Mach, 2004). P-selectin is expressed on the luminal surface of endothelium and interacts with monocyte P-selectin glycoprotein ligand-1 (PSGL-1) (Blankenberg *et al.*, 2003; Quehenberger, 2005). Firm adhesion of monocytes to the luminal surface of activated endothelium further supports by the binding of integrins with vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) (Natarajan and Cai, 2005). Animal model studies have found that genetic deficiencies of VCAM-1 and ICAM-1 are associated with significantly delayed atherosclerosis (Takahashi *et al.*, 2002).

In mice by tracing radiolabeled monocytes at the lesion site it has been recognized that monocytes can differentiate into macrophages (Ginhoux and Jung, 2014). The differentiation of monocytes into macrophages is triggered mainly by two growth factors: granulocyte/macrophage colony stimulating factor (GM-CSF) and macrophage colony-stimulating factor (M-CSF) (Nicola and Metcalf, 1986). Different human and animal model experiments have revealed that M-CSF overexpression play a key role to induce various functions of macrophage in the process of atherogenesis (Qiao *et al.*, 1997). Moreover, delayed atheroma formation also has been documented in mice lacking the expression of M-CSF (Rajavashisth *et al.*, 1998). The event of monocytes differentiation into macrophages also accompanied by a number of morphological and structural changes, such as increase of organelles numbers, altered sensitivity to signalling molecules, and enhanced expression of surface receptors including SR-AI and II, CD (cluster of differentiation) 36, MARCO, SR-PSOX, and CD68 (Kume and Kita, 2001; Minami *et al.*, 2001). Differentiating monocytes show increased activity of lysosomal enzyme, which help them to prepare for the phagocytosis of lipid components (Novoselov *et al.*, 2015). Macrophage after differentiating from monocytes further actively contributes to atherosclerotic lesion regression and tissue remodeling. In summary, monocyte recruitment to the arterial wall initiates the inflammation process (Nathan and Ding, 2010).

3. Role of different macrophages in atherosclerosis

Differentiated macrophages play a central role in atherosclerosis by scavenging lipoprotein particles and ultimately becoming foam cells (Lusis, 2000). Diverse emerging concepts on heterogeneity of macrophages indicate alternative roles of different macrophages in the progression and regression of atherosclerosis (Waldo *et al.*, 2008; Bouhrel *et al.*, 2007).

Existence of two types of macrophage phenotype is widely known- one is classically activated or M1 phenotype, and the other is alternatively activated or M2 phenotype (Johnson *et al.*, 2009). Differentiation of

M1 macrophages is occurred in response to toll-like receptor (TLR), interferon- γ signaling; which is influenced by the availability of pathogen-associated molecular complexes (PAMPs), lipopolysaccharides and lipoproteins. Proinflammatory factors, such as tumor necrosis factor- (TNF-) α , interleukin-1 β (IL-1 β), IL-12, and IL-23, and chemokines CXCL9, CXCL10, and CXCL11 are discharged by this type of macrophages. M1 macrophages produce high levels of reactive oxygen species (ROS) and nitric oxide (NO) that also contribute to the inflammation process (Chistiakov *et al.*, 2015). Again various *in vitro* data show that M1 macrophages not only can trigger apoptosis of smooth muscle cells (SMCs) through activated Fas apoptotic pathway and release pro-apoptotic TNF α and nitric oxide, but also can decrease collagen synthesis in intimal SMCs through the secretion of macrophage-derived matrix metalloproteinases (MMPs) (Fodok *et al.*, 1998).

Anti-inflammatory M2 macrophages are induced by Th2-type cytokines IL4 and IL-13 and secrete anti-inflammatory factors, such as IL-1 receptor agonist and IL-10 (Martinez *et al.*, 2009). Various studies have disclosed inaccuracy of bipolar M1/M2 classification to interpret macrophage diversity and so further division of M2 type into other subgroups was proposed by some authors based on the stimulation and protein expression pattern (Murray *et al.*, 2014; Martinez *et al.*, 2009). M2a, M2c play anti-inflammatory role while M2b play both anti and pro-inflammatory function and M2d is found to be involved in tumor progression (Ferrante *et al.*, 2013).

Apart from M1 and M2 phenotypes, Mox phenotypes can also be evolved when exposed to oxidized phospholipids which are stimulated by functional transcription factor NFE2L2. Some pro-inflammatory markers, such as IL-1 β and cyclooxygenase 2 are being secreted by Mox. In atherosclerotic lesions of mice, Mox macrophages comprise approximately 30% of the total number of macrophages. However, presence of Mox macrophages in human atherosclerotic lesions are yet to be determined (Kadl *et al.*, 2010).

4. Role of cytokines secreted by macrophages in atherosclerosis

Macrophages are the first local inflammatory cells arrive at the atherosclerotic lesions (Gerrity *et al.*, 1979). Macrophages secrete inflammatory cytokines that stimulate the generation of protease, endothelial adhesion molecules, and other mediators, which may enter into the systemic circulation in soluble forms (Galkina and Ley, 2009). Depending on their effects on the formation and progression of the atherosclerotic plaque, cytokines can be classified broadly as pro-atherogenic or anti-atherogenic. Pro-atherogenic cytokines (TNF- α , IL-1, and IL-6) are secreted by macrophages, lymphocytes, NK-Cell and vascular smooth muscle cell and play greater role in atherogenesis by their overexpression (Tedgui and Mallat, 2006). In contrast, anti-atherogenic cytokines act in a protective manner against the atherosclerotic plaque formation such as transforming growth factor- β (TGF- β) (Rajasingh *et al.*, 2006).

TNF- α as a pro-atherogenic cytokines controls several critical cell functions such as cell proliferation, growth, survival, differentiation, and apoptosis. TNF- α binds with TNF receptor (TNFR) (Flynn *et al.*, 1995) and acts as a “master regulator” of other pro-inflammatory cytokine production (Maini *et al.*, 1995). Witsell and Schook (Witsell and Schook, 1992) illustrated that TNF- α also has macrophage differentiation capabilities. The expressions of TNF- α gene transcript occur mainly during the differentiation of bone marrow-derived macrophages.

IL-1 stimulation initiates leukocyte adhesion to endothelial cells for macrophage transmigration and also contributes to slowly progressing inflammatory processes which take place in atherosclerosis (Dinarello, 2011). Previous studies have documented that IL-1 expression induces inflammation in atherosclerosis, whereas Interleukin 1 receptor antagonist (IL-1RA) deficiency significantly promotes the disease (Elhage *et al.*, 1998). The administration of recombinant IL-1RA into Apoe^{-/-} mice (Razin, 1984), or IL-1RA overexpression in Ldlr^{-/-} (Elhage *et al.*, 1998) or Apoe^{-/-} mice, notably suppress plaque burden. On the contrary, IL-1RA knockout C57BL/6J mice fed a high fat diet tended to accumulate foam cells in the aortic wall and, thus, had accelerated disease. Although the circulating levels of IL-1, even in severe inflammatory disease, are unpredictable, with further research anti-IL-1 antibodies will likely be proposed as potential therapy for atherosclerotic patients in the future (Dinarello, 2011).

IL-12 is a heterodimeric cytokine that is produced mainly by plaque macrophages and have influence in T helper type 1 (Th1) differentiation, NK cells and T cells proliferation. Administration of IL-12 showed increased lesion size in Apoe^{-/-} recipients. On the other hand, IL-12 p40-deficient I112b^{-/-} Apoe^{-/-} mice have a 52% reduction of the plaque area at 30 weeks (Davenport and Tipping, 2003). Similarly, as IL-1 and IL-12, IL-8 is a pro-inflammatory cytokine which is secreted by macrophages and after administrating IL-8 antibody in Apoe^{-/-} mice it exacerbates the development of atherosclerotic lesions.

CD40 is produced by macrophages, ECs, and SMCs from atherosclerosis-prone vessels and has led to the hypothesis that the CD40L/CD40 dyad may contribute to atherosclerosis (Galkina and Ley, 2009). CD40L and

CD40 were identified in human atherosclerotic lesions at very early developmental stages and their interaction plays a role in thrombosis, but it also contributes to the modulation of immune response to plaques formation. Treatment with antibodies against CD40L decreases atherosclerosis in *Ldlr^{-/-}* mice, with a concomitant suppression of macrophages and T cells (Mach *et al.*, 1998). Further experiments using *Cd401g^{-/-} Apoe^{-/-}* mice have depicted a pro-atherogenic activity for CD40L in advanced atherosclerosis by developing lipid core formation and plaque destabilization (Zirlik *et al.*, 2007). Although CD40 found critical for the development of advanced lesions in animal experiments, subclinical study conducted on a patient group of atherosclerosis did not find similar result (de Lemos *et al.*, 2005). To successfully target cytokines and cytokine receptors as future therapeutic options, further studies are required to better understand the association of cytokines in atherosclerotic plaque formation.

5. Role of chemokines in monocyte and macrophage migration to the atherosclerotic site

Monocyte and macrophage homeostasis are tightly controlled by the chemoattractants including Complement component 5a (C5a) and (C-C motif) ligand 2 (CCL2) (Figure 1). C5a is one of the most potent inflammatory chemoattractants which enhances monocyte/macrophage recruitment to the site of inflammation (Distelmaier *et al.*, 2009). It has the capability to induce the expression and release of abundant cytokines and chemokines including CCL2, CCL5 and CXCL1 (Buono *et al.*, 2002).

CXCL1 plays major role in classical monocytoysis and induced by high fat diet (Broxmeyer *et al.*, 1996). CCL5 (RANTES) have activity in the recruitment of classical monocytes. With the acceleration of atherosclerosis in response to myocardial infarction, it is interesting to note that a recent clinical study evidenced an interaction between plasma CCL5 levels and progression of atherosclerosis after acute coronary syndrome (Blanchet *et al.*, 2014).

CCL2, also defined as MCP-1, is also thought to be a major chemokine which has role in monocyte and macrophage recruitment during atherosclerosis development. Mouse model lacking CCL2 or its receptor develop smaller atherosclerotic plaques formation in comparison to the control. CCL2 induces monocyte mobilization from the sites of production. The lesion phenotype observed in CCR2-deficient mice showed low circulating monocyte numbers rather than the control mice (Buono *et al.*, 2002).

Receptors of C5a are expressed by various cell types, such as macrophages, SMCs, mast cells, and endothelial cells. Inhibition of C5aR signaling decreased atherosclerosis and neointima formation in *apoe^{-/-}* mice (Manthey *et al.*, 2011). Due to its significant impacts on a neuromas cell types within the atherosclerotic plaque, C5a has identified as one of the key components in plaque destabilization and acute plaque rupture. Study also has been illustrated that enhanced levels of C5a are associated with increased cardiovascular risk in patients with advanced atherosclerosis (Speidl *et al.*, 2005).

6. Foam cell formation, macrophage death and its clearance by efferocytosis

As discussed earlier, macrophage foam cells formation in the intima is a major hallmark of early stages of atherosclerotic plaque development. The significant cause of foam cells generation is the uncontrolled uptake of oxidized low-density lipoproteins (LDL) (Hutchins and Heinecke, 2015). Phagocytosis involved in the lipid internalization that is transported to late endosome/lysosomes, where lysosomal acid lipase (LAL) digests cholesteryl esters releasing free cholesteryl. Free cholesterol in turn is finally processed by acetyl-CoA acetyltransferase (ACAT1), an enzyme that help to transforms cholesteryl to cholesteryl esters (Chistiakov *et al.*, 2016). The endoplasmic reticulum serves as a depot for controlling newly synthesized cholesteryl ester hydrolase (NCEH). In atherosclerosis, pro-inflammatory cytokines influence upregulates the expression of scavenger receptors, especially LOX-1, and downregulate the expression of cholesterol transporters. ACAT1 is also enhanced, while NCEH expression is reduced. This results in deposition of free and esterified cholesterol in macrophages and ultimately generation of foam cells. Furthermore, other cell types, such as endothelial (ECs) and vascular smooth muscle cells (VSMCs), can also resulting in foam cells (Chistiakov *et al.*, 2017).

Formation of lipid rich necrotic cores within vulnerable plaques and macrophage death act as key factors in the promotion of advanced plaque necrosis. On a cellular level, “post-apoptotic,” or “secondary,” necrosis refers to a type of cell perturbation in where membranes become leaky and organelles swell, ultimately results in cellular death. *In vivo* experiment indicates that cell necrosis results when apoptotic cells in which membranes are primarily intact, but organelles are condensed, are not rapidly ingested by neighboring phagocytes. “Necrotic core” is often considered in the literature as “lipid core”, because the dying macrophages are filled with lipid and mostly cholesterol, which ultimately becomes incorporated as extracellular lipid into the areas of plaque necrosis (Chistiakov *et al.*, 2017; Tabas, 2005).

Efferocytosis (Greek for carrying the corpse to the grave) is the removal of apoptotic cells by phagocytosis (Ortega-Gómez *et al.*, 2013). Several stages can be distinguished in efferocytosis such as attraction of phagocytes, recognition, engulfment, and post-engulfment responses. Apoptotic cells (ACs) secrete soluble attraction or “find me” signals in order to attract phagocytes and to initiate their own elimination. One such signal is the lipid lysophosphatidylcholine, which is mainly produced by caspase-3-activated phospholipase A2 and prompt clearance of the death cells (Lauber *et al.*, 2003; Mueller *et al.*, 2007). Sphingosine-1-phosphate (S1P) is formed by following caspase activation (Gude *et al.*, 2008). Production of sphingosine kinase is upregulated in a caspase-dependent manner, which leads to extreme expression of S1P (Gude *et al.*, 2008). Previous studies indicated that S1P is a great attractant for monocytes as well as macrophages (Gu *et al.*, 2011). In fact, S1P has significant role on the regulation of epithelial cell extrusion during apoptosis (Gu *et al.*, 2015). A more complete list of “come and get me” factors which can be found in the recent review by Peter *et al.* (2010). The leaky cell membrane of necrotic or late ACs liberates additional proteins, so-called alarmins, which propagate a danger signal and alert the immune system. Consequently, ACs give electric signals which attract phagocytes, as has been mentioned for endothelial cells (Segundo *et al.*, no date). As previously reviewed, (Thorp, 2010) in atherosclerosis an overload of these “eat me” signals can be found. In addition to “find me” signals, ACs will also express “eat me” signals, i.e., molecules that are upregulated or translocated to the surface to facilitate interactions with phagocytes. In fact, efferocytosis will largely depend on the quantity of positive (“eat me”) and negative (“don’t eat me”) signals. In the end, the “phagocytic synapse” tightly interconnects the phagocyte with the dying cell (zipper like interaction). Therefore, ingestion by the phagocyte, the internalized AC will be processed and degraded through a series of fusions with endosomes and lysosomes (Soehnlein *et al.*, 2013). Finally, as mentioned above, the impact of apoptosis is beneficial in early stages of atherosclerosis, whereas in advanced lesions, it results in plaque instability and thrombogenicity (Broxmeyer *et al.*, 1991). It is assumed that less efficient scavenging of ACs and subsequent secondary necrosis play a significant function in unstable atherosclerotic lesions.

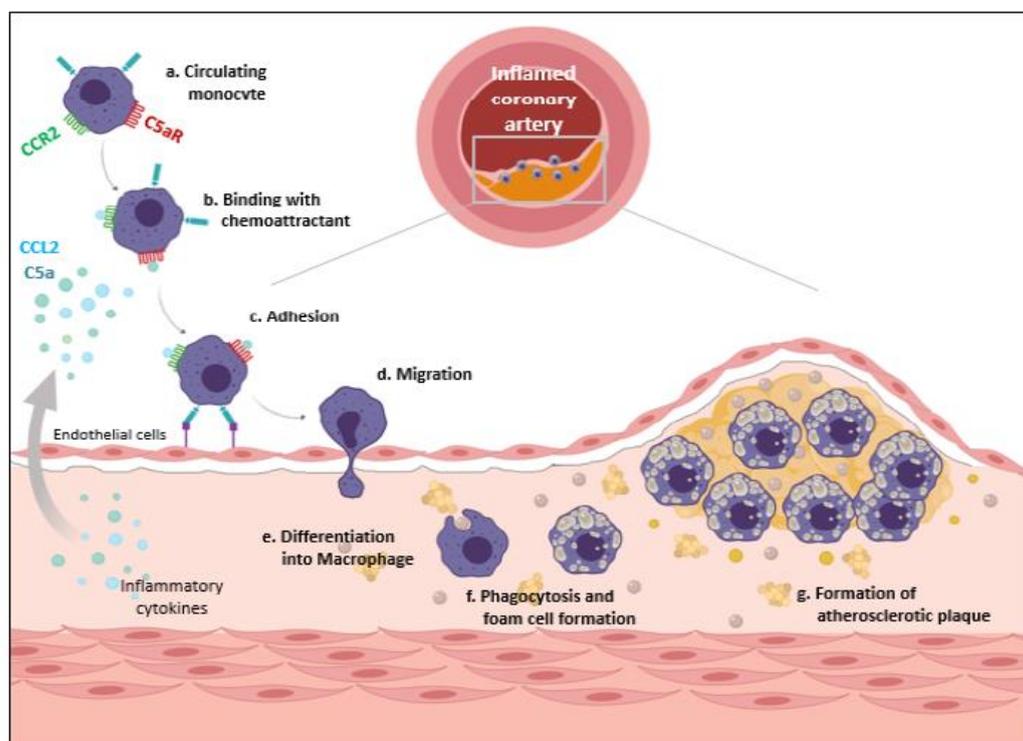


Figure 1. Monocyte trafficking from the circulation to the atherosclerotic plaque. (a) The accumulation of monocytes from the circulation to the atherosclerotic lesion is guided by inflammatory chemoattractants (such as CCL2 and C5a). (b) The engagement of chemoattractant with its receptor on monocyte surface facilitates its chemotaxis to the atherosclerotic endothelium. (c, d) By interacting with atherosclerotic endothelium, inflammatory monocytes then migrate into the atherosclerotic lesion. (e) Once they are recruited to the lesion site monocytes further contribute to inflammation by differentiating into macrophages. (f) These macrophages become foam cells by ingesting cholesterol. (g) These foam cells can form atherosclerotic plaque that may lead to atherosclerosis.

7. Conclusions

Atherosclerosis is a chronic inflammatory disease arising from an imbalance in lipid metabolism and a maladaptive immune response driven by the accumulation of cholesterol-laden macrophages in the artery wall. Concepts on how macrophages and their related cytokines and other factors affect different stages of atherosclerosis is emphasized in this review because highlighting the association of macrophage with atherosclerosis is critical for understanding the prognosis, diagnosis, and treatment of this disease. Macrophages are a vital and versatile lineage having numerous associated effector functions that implicate them in several features of atherosclerosis including lipid phagocytosis, plaque repair, plaque rupture, and the autoimmune phenomena associated with atherosclerosis. After being recruited by circulated monocytes, they take part in LDL uptake and lipid accumulation in the arterial wall becoming foam cell. Macrophage population is heterogenous and several subtypes differ in their function and gene expression profiles. This review provides a brief discussion on the involvement of macrophages in atherosclerosis mainly based on animal model studies which may not completely reflect the process in human. To discover more effective and potential therapeutic options for atherosclerosis, in future more human genetic studies along with mechanism based clinical trials should be performed.

Conflict of interest

None to declare.

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