Novel visualization techniques towards identification of atherosclerotic patients at risk

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CHAPTER 5

Macrophage folate receptor β (FR-β) expression in auto-immune inflammatory rheumatic diseases: a forthcoming marker for cardiovascular risk?

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CHAPTER 5

Abstract

In patients with systemic auto-immune inflammatory rheumatic diseases (AllRD) like rheumatoid arthritis the prevalence of cardiovascular disease (CVD) is increased. In the pathogenesis of AllRD and atherosclerosis many similarities can be found in the process underlying CVD. Accumulation of inflammatory cells, in particular macrophages at the site of inflammation producing inflammatory mediators serve as a prominent feature in both systemic inflammation and atherosclerosis.

Two different subtypes of macrophages have been described in recent literature namely classically activated macrophages (M1) and alternatively activated macrophages (M2). Alternatively activated macrophages are characterized by low CD14 and high CD163 expression. Macrophages expressing CD14 (M1) have been identified within atherosclerotic plaques, whereas CD14 low macrophages are abundant in vessels without atherosclerosis. Depending on the environment and responses to different stimuli, macrophages in plaques can express diverse pro and anti-atherogenic functions. The balance of these different activation profiles influences atheroma evolution and outcome.

Nowadays, influx of macrophages is recognized as a very important feature of the pathogenesis of plaque formation. Activated macrophages accumulate at the sites of inflammation and can therefore be exploited to better visualize inflammatory responses in atherosclerosis. Furthermore, activated (but not resting) macrophages possess a functionally active receptor for folate (FR-β), but it is not completely clear which subtype of this activated macrophages expresses this receptor and whether the expression of FR-β is restricted to only one of the macrophage subsets. Although future research needs to be done to investigate FR-β expression and function within inflamed tissues, the expression of functional FR-β on tissue macrophages likely occurs during activation. Therefore, expression of FR-β on activated macrophages holds a promising potential for early diagnosis and better analysis of optimal treatment regiments of vascular diseases in association with systemic diseases.

Keywords: auto-immune inflammatory rheumatic diseases; atherosclerosis; macrophage plasticity; vulnerable plaques; FR-β

Highlights:

- Macrophages play an important role in the pathogenesis of both AllRD and atherosclerosis and might link these diseases
- Macrophages can be distinguished in M1 and M2 subtypes
- The balance between M1 and M2 macrophages will influence plaque vulnerability
- FR-β, a marker for M2 macrophages, could be a tool for early detection of atherosclerosis
1. Atherosclerosis in patients with systemic inflammation

1.1 General introduction
Until a view decades ago, atherosclerosis was thought to be a collection of cholesterol and accumulated smooth muscle cells within the arterial vessel wall. In the 1960s, macrophages were identified in atherosclerotic plaques (1) but investigations into the pathophysiology of this condition focused on diet and metabolism, as there was overwhelming evidence that cholesterol leads to the development of atherosclerosis (2). Although an additional link between adipose tissue and rheumatoid arthritis was found (3), inflammation, another player in the field may even have more influence on accelerating atherosclerosis. The importance of inflammation in arterial disease was first pointed out by Virchow (4). New insights into the importance of inflammatory cells in the pathogenesis of atherosclerosis emerged, based on the observation of similarities between atherosclerosis and systemic auto-immune inflammatory rheumatic diseases (AIIRD). Various autoimmune rheumatic diseases such as rheumatoid arthritis (RA) and SLE (systemic lupus erythematosus) accelerate atherosclerosis. (5) Early in the atherosclerotic process as well as at sites of inflammation endothelial cells change from an anti-inflammatory phenotype that resist adhesion and promote fibrinolysis, into a pro-inflammatory phenotype. The latter, activated endothelial cells are characterized by increased expression of endothelin and leukocyte adhesion molecules (ICAM-1, VCAM-1, E-selectin) (6). This facilitates binding and subsequent infiltration and activation of inflammatory cells (in particular monocytes, T-cells and mast cells), and production of pro-inflammatory cytokines such as tumor necrosis factor (TNF-alpha) and interleukin (IL)-6 (7,8). Chemotactic stimuli, provided by pro-inflammatory proteins, such as chemo attractant protein-1 (MCP-1), further promote the recruitment of monocytes by diapedesis through interaction with different receptors, such as monocyte chemokine receptor CCR2 (6). Within the intima, monocytes change into macrophages with help of the monocyte maturation mediator macrophage colony-stimulating factor (M-CSF). M-CSF can induce scavenger receptor expression and as such promote a differential shift from monocytes into macrophages (9). Macrophages internalize oxLDL by the macrophage scavenger receptors which lead to the formation of foam cells (10) (Figure 1).

1.2 Pathogenesis of atherosclerosis and inflammation; the importance of the macrophage
Increased numbers of macrophages producing inflammatory mediators in the synovial fluid are a prominent feature in patients with AIIRD. In atherosclerotic plaques, also a higher number of macrophages is present and this feature supposedly links both diseases (11). Interestingly, in patients with RA, the primary site of inflammation is synovial tissue, from which cytokines can be released into the systemic circulation. These circulating cytokines alter function of distant tissues, in order to generate a spectrum of pro-atherogenic changes
including endothelial dysfunction and damage rendering patients at risk for overt CVD (12-14). For example, TNF-α, secreted by activated macrophages is increased in plasma of SLE patients with CVD, compared to age-matched patients without manifest CVD (15). The above mentioned facts indicate the importance of inflammation, and in particular macrophage activation in the development of atherosclerosis in patients with AllIRD. Macrophages interact with cells in the vessel wall including T-cells and vascular smooth muscle cells (VSMC), directly or via release of cytokines. This interaction results in an enhanced inflammatory response due to subsequent cell activation. Activation induced cell death, through apoptosis, may occur. Apoptosis of macrophages appears to result from cell–cell interactions and the local cytokine environment within the arterial wall, involving the actions of pro- and anti-apoptotic proteins that include death receptors, proto-oncogenes, and tumor suppressor genes (12). Apoptotic macrophages are rapidly cleared by phagocytosis. However, when this process is insufficient, apoptotic cells will turn into secondary necrotic cells with accumulation of insoluble lipids and other cellular contents, a characteristic of advanced lesions (16). Macrophages will also release metalloproteinases (MMPs) which degrade the extracellular matrix (ECM) proteins. MMPs lead to plaque instability and rupture (17-19). Apart from being the source of MMPs, macrophages have been strongly implicated in atherosclerotic plaque rupture through several other mechanisms. Production of death ligands, reactive oxygen species including nitric oxide and proteases can cause apoptosis of SMC (20), which in turn leads to the fibrous cap thinning (21). Also, death of foam cell macrophages (FCM) provokes lipid core expansion, especially in advanced plaques. Finally, macrophages stimulate invasion of vasa vasorum into a base of the plaque, which will further promote intraplaque hemorrhage due to growth of the core (22). Plaque rupture leads to the activation of the coagulation cascade, fibrin deposition, platelet activation and recruitment to form a thrombus (16,23).

1.3 Heterogeneous subpopulations of macrophages within atherosclerotic plaques

Macrophages have been identified as a heterogeneous population of cells with a variety of physiological and pathophysiological functions (24,25). Stein and colleagues were the first to describe “classically” (M1) activated macrophages as having a different phenotype from what are now called “alternatively” (M2) activated macrophages (26). M1 macrophage polarization is induced mainly by bacterial moieties such as LPS and the Th1 cytokine interferon–gamma (IFN-γ). M1 macrophages contribute to the inflammatory response and tissue damage by producing copious amounts of reactive oxygen species, nitrogen intermediates and pro-inflammatory cytokines such as TNF-alpha and IL-6 (24,27). In contrast, “alternatively” activated M2 macrophages originate from monocytes upon stimulation with Th2 cytokines, such as IL-4 (26), as depicted in Figure 1.
Figure 1. Subsets of activated macrophages in human atherosclerotic plaques. Monocytes migrate into the developing atheroma lesion guided by chemokines and adhesion molecules. Infiltrated monocytes become macrophages or differentiate into dendritic cells or osteoclasts. Macrophages in response to the different stimuli further differentiate into M1 (pro-inflammatory type) macrophage which promotes the lesion development or M2 (anti-inflammatory type) that are immune regulatory and upon the stimuli modify the development of atherosclerotic plaques.

In contrast to M1 macrophages, M2 macrophages display a Th2–like phenotype, promoting ECM construction, cell proliferation and angiogenesis (28,29). The precise properties of M2 macrophages vary, depending on their activating conditions, and M2 macrophages have been divided into M2a, M2b and M2c subsets (25) (Table 1).
Although the phenotype and the functions of anti- and pro-inflammatory macrophages are different, both subsets are important components for the innate and adaptive immune response.

Interestingly, nowadays there is a growing body of evidence suggesting presence of a heterogeneous population of macrophages in human atherosclerotic lesions (30-33). Initially, M1 and M2 could be hardly discriminated in plaque tissue due to lack of appropriate markers to distinguish these specific macrophage subsets in tissue specimens. Later on, CD163, the haptoglobin / haemoglobin scavenger receptor was identified as a marker for alternatively activated macrophages (32,34). These M2 macrophages were diffusely present within intimal atherosclerotic lesions (28). CD163 positive macrophages also have been identified in haemorrhaged atherosclerotic plaques. They expressed low
levels of human leukocyte antigen- DR (HLA- DR) and were unlikely classical lipid core macrophages, because CD163 is a marker for alternatively activated macrophages (34). Hence, these macrophage types were thought to suppress the impact of haemorrhage on atherosclerotic progression. M2 macrophages have been also characterized by low CD14 (monocyte differentiation antigen) expression. Macrophages with low levels of CD14 express 5 times more PPAR gamma than macrophages expressing high levels of CD14, and PPAR gamma is associated with an M2 – anti-inflammatory phenotype (33). Regarding the low CD14 expression and M2 macrophage activation, Waldo and colleagues have shown that CD14 and CD68 double positive macrophages could be identified within atherosclerotic plaques, whereas, CD14low macrophages are abundant in normal intimal regions of coronary arteries (31). A recent study by Khallou-Laschet et al. evaluated the phenotype of macrophages associated with progression of atherosclerosis in the apolipoprotein E (ApoE) knockout mouse model. Their study demonstrated that bone marrow-derived macrophages submitted to M1 and M2 polarization specifically expressed arginase II and I, respectively. They also found lesion progression in atherosclerotic disease was correlated with the dominance of M1 over the M2 macrophage subtype (35). M-CSF differentiated macrophages (M2 subtype) express all three activating Fc gamma receptors (FcγR); FcγRIII, FcγRIIa and FcγRI (CD16, CD32 and CD64 respectively) (36-38). Though opinions are divided about this, concerning Lennartz et al. found M1 macrophages express elevated levels of FcγRI, FcγRIIa and FcγRIII, while M2 macrophages express a lower amount of these receptors (39). Given the complexity of atherosclerosis, it is difficult to generalize the findings obtained from murine model in human situation. However, these observations provide evidence that plaque pathogenesis and evolution are influenced by macrophage activation and polarization. Depending on environmental factors and responses to different stimuli, macrophages in plaques can express diverse pro- and anti-atherogenic functions. As a result, the balance of these different activation profiles will influence atheroma evolution and outcome. Recently, folate receptor-β expression (FR-β) has been found on M-CSF polarized M2 macrophages. As such FR-β might serve as a new marker for M2 macrophages (40).
2. Folate receptor-β

2.1 Structure and function of FR-β
Folate (vitamin B9 or folic acid) is critical for the synthesis of nucleotide bases via carrying the methyl groups within cells. Folates are hydrophilic anionic molecules that do not cross biological membranes by diffusion alone, so sophisticated membrane transport systems have evolved for facilitating folate accumulation by mammalian cells and tissues. In mammalian cells, folate is transported through two major mechanisms. One involves a transmembrane transporter protein, which is the reduced folate carrier (a member of the SLC19 family of facilitative carriers) which contributes to anion exchange with high affinity for low folate. The other implies proton coupled folate transporter which belong to the membrane–spanning proteins and mediate bidirectional transfer of reduced folate across the plasma membrane (41,42).

Folate can be internalized by the folate receptor (FR) that is expressed on a limited number of cells and can mediate unidirectional transport of folate into the cells. Three major forms of the folate receptor exist: FR-α, FR-β and FR-γ (43). Among these, the β isoform belongs to the glycosylphosphatidylinositol (GPI) anchored proteins that binds to folate with high affinity (KD<1 nM). FR-β quantitatively recycles, within minutes, between the cell surface and intracellular compartments (44). FR-β is of great importance when folate supply is low or rapid cell growth requires elevated uptake of folate. FR-β is mostly expressed in hematopoetic cells of myelomonocytic lineages (45-47).

2.2 FR-β as a selective cellular marker for activated macrophages
Expression of FR on nonmalignant hematopoetic cells (primarily the beta isoform) appears to be limited to activated macrophages and their precursors (48,49). Previous studies in animal models of RA have shown that activated macrophages express FR-β (40), in particular M2 alternatively activated macrophages (50). The quantitation of activated macrophages in systemic inflammation, in particular RA joint tissues might consequently be of diagnostic value, because activated macrophages content correlates well with articular destruction and poor prognosis in human (51). Since FR expression coincides with macrophage activation, it seems reasonable to image arthritic joints using folate-derived imaging agents. Turk et al. demonstrated that 99mTc-folate can indeed selectively target activated macrophages in vivo, in an adjuvant-induced arthritis mouse model, and that folate-linked imaging agents can facilitate the non-invasive analysis of inflammatory activity (51).

Nowadays, presence of (coronary) atherosclerosis (in RA patients) can be determined using recently developed imaging techniques such as echocardiographic measurement of coronary flow reserve and tissue Doppler imaging, which can detect subtle cardiovascular abnormalities (52). Because atherosclerosis is also characterized by a localized accumulation of activated macrophages, it is important to determine whether folate conjugates might
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prove useful in imaging atherosclerosis as well. Using apolipoprotein E knockout (apoE-/-) mice raised on high-fat diet, Ayala-Lopez et al. documented selective uptake of 99m Tc-EC20 by macrophages present within atherosclerosis lesions by whole-animal radio imaging (53). Consequently, folate conjugates proved to be useful also in imaging atherosclerosis progression in vivo. Although progress has been made in employing FR-targeted imaging of macrophages within atherosclerotic plaques, the findings obtained from mouse studies are difficult to extrapolate to humans. Very recently, Low and colleagues developed an antibody with high specificity to human FR-β and demonstrated that this antibody is able to target FR-β positive cells including macrophages from RA patients (54). The given findings suggest that high affinity FR-β specific antibody can be applied as a research tool for effective targeting of activated macrophages during inflammatory disease progression, which can be used also for early detection of atherosclerosis.

Recent evidence suggests that FR-β expression is not limited only to activated macrophages. It is also present in membrane lysates and on the cell surface of M2 macrophages generated in the presence of M-CSF. The existence of FR-β in M-CSF primed in vitro macrophages is in agreement with its up-regulation in human uterine macrophages that exhibit an immunosuppressive phenotype and whose gene expression profile closely corresponds to that of M2-polarized phenotype (55). The presence of functional FR-β on M-CSF primed macrophages is difficult to reconcile with its expression and function in synovial macrophages from RA patients, which are embedded in an inflammatory environment. So far, few studies have specifically examined the role of M2 in pathogenesis of RA and further studies are needed to correlate FR-β expression and function in macrophage subsets within inflamed tissues. However, it is tempting to speculate that synovial tissue of RA patients might exhibit a mixture of M1 and M2 phenotype and an explanation that would be compatible with the high levels of M-CSF generated macrophages found in RA patients synovia (56).

Like in RA, heterogeneous macrophage populations have been also reported within atherosclerotic lesions and their phenotype has been correlated with plaque stability (29). Because FR expression has been linked to macrophage activation and M2 differentiation, it will be informative to explore whether the abundance of FR-positive macrophages at sites of atherosclerotic lesions is associated with plaque vulnerability. If such association emerges, it would support further exploration of the use of FR-targeted agents for early detection of unstable plaques in chronic inflammatory diseases, hence identifying individuals at risk for overt CVD.

3. Challenges and future prospects

Patients with atherosclerosis are at high risk to develop cardiovascular disease already at disease onset (57). This increased risk cannot be fully explained by traditional cardiovascular risk factors alone (58). The current paradigm places inflammation at the very heart of the
pathogenesis of atherosclerosis. The presence of chronic inflammation in autoimmune diseases such as RA appears to be an important source of cellular and molecular processes that drives the development of atherosclerotic lesions. Macrophages which play a crucial role in the development of chronic inflammatory conditions are present in all stages of atherogenesis, from the nascent fatty streak lesion to the culmination in cardiovascular events. Nowadays medicine face challenges in establishing the algorithms for cardiovascular disease risk assessment based on high chronic inflammatory burden. Challenges appear in early diagnosis of atherosclerosis in patients with autoimmune diseases.

Since plaque rupture is a major cause of cardiovascular events, attention has been directed on evaluation of biological processes that determine plaque vulnerability. A number of non-invasive imaging techniques have been developed to evaluate vascular wall in autoimmune diseases in an attempt to identify so-called vulnerable atherosclerotic plaques which are prone to rupture (59). In recent years, the potential of molecular imaging to characterize the process involved in the initiation and progression of atherosclerotic lesions has been explored. In particular, radionuclide-based molecular imaging, intravascular ultrasound, computed tomography (CT) and magnetic resonance imaging (MRI) have been evaluated in preclinical and clinical studies. Despite their high sensitivity and accuracy in detection of plaque vulnerability, substantial technical and methodological constraints remain unsolved, such as time-consuming preparation, slow plasma clearance, spatial resolution and high costs (59, 60). As such, much remains to be improved before their wide application in clinics.

Currently different macrophage subpopulations have been identified within chronic inflammatory diseases as well in atherosclerosis. It has been proposed that macrophage polarization is not irreversible and macrophages can switch between M1 and M2 phenotype, which is referred as macrophage plasticity [23, 24]. That would suggest that cells that initially promote an inflammatory response (M1) could have later anti-inflammatory properties (35). So far, classically activated macrophages seem the most obvious culprits in atherosclerotic plaque disruption and hence acute coronary events because they have an enhanced ability to kill neighboring cells and destroy extracellular matrix (24). Subsequently, alternatively activated M2 macrophages could suppress the impact of hemorrhage on atherosclerosis progression. However, the effects of M2 macrophages in vulnerable lesions are by definition too little and too late (24,32). Due to the lack of the markers that identify macrophage populations involved in the pathogenesis of autoimmune disorders and lack of knowledge about their precise functions, little is known about to what extend subsets of macrophages contribute to the subsequent atherosclerotic plaque progression. The primary issues concern the plasticity of the macrophages in vivo, correlations between in vitro polarization and their in vivo behavior, what is serving as robust marker for macrophage polarization in disease and how polarization can be manipulated to alter the outcome of chronic inflammatory conditions (16,24). Elucidating the exact role of macrophage subpopulations within lesions and the
molecular clues that drive differentiation and activation, would undoubtedly aid in the development of novel strategies for early diagnosis, stabilization or even regression of vulnerable atherosclerotic plaques.

With recent discovery of FR-beta expression on activated macrophages, folate targeting and imaging of chronic inflammatory disorders and atherosclerosis can now be envisioned (48,49). Expression of FR-beta on distinct sets of the macrophages could present an important morphological marker for the detection of the patients who are at high-risk for developing atherosclerotic plaques. Assessment of FR-beta positive macrophages among the individuals with chronic inflammatory diseases may aid in identification of the patients with vulnerable plaques non-invasively. Hence, FR-beta targeted imaging may harbor tremendous potential in clinical applications in a large population of subjects at risk of cardiovascular events.

**Concluding remarks** (take home message)

The fact that macrophage subsets can be employed as biomarkers for chronic inflammatory diseases and that they might represent treatment targets will undoubtedly continue to provide fertile ground for further investigation. Some exogenous cell trackers/reagents such as antibody against FR-β would selectively image macrophage subset activity associated with atherosclerotic plaque progression that may be clinically translatable. Therefore it may be useful to employ FR-β as *in vivo* imaging tool to non-invasively and rapidly assess prognosis of atherosclerosis in patients with chronic inflammatory diseases and implicate it as a forthcoming marker for cardiovascular risk detection. Moreover, this approach might determine whether a direct relation between progression of atherosclerosis and disease activity exists, and whether this effect can be influenced by therapeutic intervention.
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