Atherosclerosis & inflammation: Macrophage heterogeneity in focus

Stöger, J.L.

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Chapter 2

Macrophage heterogeneity in atherosclerosis: relevance and functional implications

J. Lauran Stöger*, Pieter Goossens*, Menno P.J. de Winther

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* These authors contributed equally
Chapter 2

Abstract

Atherosclerosis is a chronic inflammatory disease involving many cell types with a well-accepted key role for macrophages. A wide array of different properties and functional characteristics are attributed to macrophages present in the atherosclerotic plaque. As an increasing body of evidence confirms the consensus that macrophages comprise a heterogeneous population, several co-existing subtypes with diverse, even opposing specialties have already been described in fields like parasitology, tumour biology and metabolic disorders. However, macrophage heterogeneity within atherosclerotic lesions has not been studied in detail yet. In this review we will introduce the characteristics of macrophage subsets in other pathologies and address the presence and possible roles of distinct macrophage subtypes in the rapidly evolving field of atherosclerosis. Finally, we make an effort to relate these subtypes to disease progression and explore a number of opportunities for novel diagnostic and therapeutic approaches.
Introduction

Atherosclerosis is a multifactorial disease that afflicts the medium and large sized arteries of the body. Formerly perceived as a mere lipid storage disease, the treatment of atherosclerosis in patients has focused mainly on the lowering of plasma cholesterol. Although effective lipid lowering can be achieved pharmaceutically, cardiovascular disease (e.g. myocardial infarction, stroke) still constitutes the main cause of mortality in modern societies. Since these acute clinical events are largely attributable to complications of atherosclerotic lesions, novel strategies for prevention and management of atherosclerosis are in high demand. From this perspective, the fundamental role of inflammation as a mediator of all stages of atherogenesis is being appreciated to an ever-increasing extent. Not only does the chronic inflammatory response associated with atherosclerosis act alongside well-known environmental and genetic risk factors to induce lesion initiation, it also promotes progression and ultimately leads to plaque rupture by reducing lesion stability. Consequently, extensive scientific efforts have been made over the past decade to illuminate the inflammatory processes that underlie atherosclerosis development at the molecular and cellular level.

Atherosclerosis, as an inflammatory condition, involves many cell types and mediators of the immune system. In this regard, monocyte-derived macrophages are imperative as these immune cells partake in innate and adaptive (Th1 and Th2-mediated) immune responses, which are both intricately involved in atherogenesis. As such, macrophages are generally accepted as key players in the pathophysiology of atherosclerosis, which highly determine the development of the atherosclerotic plaque. Not only is it one of the main cellular constituents of the lesions (e.g. as foam cells), but it also serves as a major source of inflammatory mediators. Thereby it determines the inflammatory equilibrium (i.e. the balance between pro- and anti-inflammatory factors) during atherogenesis and has great impact on activation, migration and survival of other cells in the plaque and affects plaque stability. The morphology of macrophages in atherosclerotic lesions can vary dramatically, from a large quiescent lipid-laden foam cell to a small active inflammatory cell. Recent work in the field of innate immunity has led to the identification of macrophage subsets based on their immune polarization. These subsets portray distinct characteristics and identification of specific markers and their functional role in vivo has recently gained strong interest in different fields of inflammatory disease. Knowledge on the in vivo contribution of different macrophage populations to the development of atherosclerosis however is scarce. However, based on their distinct physiological roles, macrophage subsets are expected to highly determine plaque composition, stability and consequently clinical outcome. Taking approaches with experimental animals as lead, we will highlight in this review the characteristics of macrophage subsets and discuss their relevance and contribution in a number of pathologies. Moreover, we speculate on the functional characteristics of macrophage subsets in athero-
sclerosis and about the opportunities that arise in using macrophage subsets for therapeutic targeting and the diagnosis of atherosclerosis.

**Macrophage heterogeneity**

Cells of the mononuclear phagocyte lineage (i.e. monocytes, macrophages) are characterized by a substantial degree of heterogeneity. Especially macrophages are known to express functionally different phenotypes in response to tissue-derived signals and the immunological microenvironment, thus skewing them towards their many tasks in homeostasis, host defence and pathology. Furthermore, macrophages display notable plasticity, as they are capable of rapidly switching between activation states in response to a specific incentive. Accordingly, under inflammatory conditions macrophage populations may initially partake in promoting inflammation and later contribute to its resolution. Over the last decade, a conceptual framework has been devised and gradually expanded to account for the polarized functional properties of different macrophage populations. In reflection of the Th1 and Th2 nomenclature in lymphocytes, polarized macrophage subsets have been initially labelled M1 and M2 macrophages.

Classically activated or M1 macrophages are elicited through stimulation with the Th1 cytokine interferon-γ (IFN-γ) alone, through a concomitant stimulus of IFN-γ with microbial products, such as lipopolysaccharide (LPS) or by activation with the Th1 cytokine TNF. Although these cells show extensive morphological diversity based on their tissue localization, M1 cells share the ability to secrete large quantities of pro-inflammatory cytokines, such as tumour necrosis factor (TNF), interleukin (IL)-1β, IL-6, IL-18 and IL-12. Additionally, they produce high levels of anti-microbial effector molecules (reactive oxygen and nitrogen species). These qualities allow M1 macrophages to act as potent inducers and effectors of polarized type I immune responses, consequently associating them with resistance to pathogens and tumours as well as tissue destruction. Recently, a further subdivision of M1 macrophages in M1a and M1b cells was suggested to distinguish between classical and innate activation of macrophages, where the latter subset is induced through ligation of for instance Toll-like receptors (TLR) by so-called Pathogen-Associated Molecular Patterns (e.g. LPS). While this phenotype somewhat resembles the classical activation profile, the different phagocytic properties and inability to produce functional amounts of IL-12 sets it apart from classically activated M1a macrophages.

In recent years, the term M2 has served as a generic name indicating the various forms of alternatively activated or anti-inflammatory macrophages. As opposed to classical activation by the Th1 cytokine IFN-γ, alternative activation of macrophages is induced by several distinct stimuli and a subdivision of M2 cells has been made accordingly. M2a cells are gen-
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Macrophage heterogeneity in health and disease

Although the contribution of different macrophage subsets to atherosclerosis is very scarcely studied, the concept of macrophage heterogeneity has already been well explored in other pathologies involving macrophages in their pathogenesis (i.e. infectious disease, cancer and metabolic disorders). Logically, knowledge of the lessons learnt in other fields might be useful when applied to atherosclerosis research, taking into account however the different underlying mechanisms by which macrophage malfunction can lead to morbidity. It is important to realize that both the inability of macrophages to react to changes in their environment or an exaggerated reaction can be the cause of this malfunction. Usually, favouring one macrophage subset over the other subsets causes both. Most probably, such an imbalance also plays a role in the pathogenesis of atherosclerosis, making it very relevant to study the changes in subset distribution throughout the disease course and to understand the specialised contribution of each subset to the many different macrophage functions in the atherosclerotic plaque that were until now attributed to a single cell type.
Table I. Simplified overview of the phenotypical characteristics of M1/M2 macrophages

<table>
<thead>
<tr>
<th>M1</th>
<th>M2a</th>
<th>M2b</th>
<th>M2c</th>
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<tbody>
<tr>
<td>IFNγ + LPS or TNF</td>
<td>IL-4, IL-13</td>
<td>IC + IL-1β or TLR-ligands</td>
<td>IL-10 (TGF-β, glucocorticoids)</td>
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<tr>
<td>IL-12&lt;sup&gt;high&lt;/sup&gt;</td>
<td>IL-10&lt;sup&gt;high&lt;/sup&gt;</td>
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<td>TNF</td>
<td>TGF-β</td>
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<td>IL-1β</td>
<td>IL-1</td>
<td>IL-1β</td>
<td>IL-1Ra</td>
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<td>IL-6</td>
<td>IL-6</td>
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<td>IL-18</td>
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<tr>
<td>NO, ROS (iNOS)</td>
<td>Polyamines (Arg-1)</td>
<td></td>
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<tr>
<td>CCL2, CCL3, CCL4, CCL5</td>
<td>CCL17, CCL18, CCL22, CCL24</td>
<td>CCL1</td>
<td>CCL18</td>
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<tr>
<td>MHC-II</td>
<td>MR</td>
<td>CD86</td>
<td>MR</td>
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<td>CD86</td>
<td>Decoy IL1-RI</td>
<td>CD86</td>
<td>CD163</td>
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<td>CD80</td>
<td>SR</td>
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<td>Sr</td>
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<td>IL-1RI</td>
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<tr>
<td>TLR2/TLR4</td>
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Abbreviations: IFNγ, interferon-γ; LPS, lipopolysaccharide; TNF, tumour necrosis factor; IL, interleukin; IC, immune complexes; TLR, Toll-like receptors; TGF-β, transforming growth factor-β; NO, nitric oxide; ROS, reactive oxygen species; iNOS, inducible nitric oxide synthase; Arg-1, arginase-1; IL-1Ra, IL-1 receptor antagonist; CCL, chemokine C-C motif ligand; MHC-II, major histocompatibility complex class II; CD, cluster of differentiation; IL-1RI, IL-1 receptor I; MR, mannose receptor; SR, scavenger receptor.

**Macrophage heterogeneity in infectious disease**

Regulation of the delicate balance between M1 and M2 type polarization has been shown to be critical in host defence against many types of pathogens. Based on the analysis of host transcriptomes in several studies, Jenner et al. identified a set of genes that together comprise the ‘common host response’ of innate immune cells in reaction to a large number of pathogens, including bacteria, viruses and fungi. Especially monocyte-derived macrophages seem to develop a common pattern of gene expression when challenged by bacteria. Moreover, Benoit et al. reported more recently that this shared response of macrophages to bacterial infection is mainly associated with increased expression of genes that are involved in polarization towards a functional M1 program. More specifically, these include the genes that encode for TNF, IL-6, IL-12, MCP-1 and inducible nitric oxide synthase (iNOS). Up-regulation of these genes partially accounts for the enhanced microbial killing activity that is regarded as a typical characteristic of M1 macrophages. Thus, by augmenting the immediate resistance to intracellular bacteria and promoting immunity, M1 polarization serves a protective role in host defence during acute infectious disease. However, an extended period of M1 skewing may lead to a deleterious outcome if not kept in check. This is illustrated by the correlation between the amount of circulation M1-type cytokines and sepsis severity in a human population. In their review, Benoit et al. further provide a detailed account of the
approaches that several bacterial pathogens have developed to hamper the M1 polarization of macrophages in an attempt to endure in their surroundings. Different strategies include inhibition of the oxidative microbicidal capacity of macrophages (NO release), inhibition of the expression and secretion of M1-type cytokines (primarily IL-12 and TNF), the release of virulence factors that interfere with M1-associated cellular signalling pathways and finally, direct promotion of M2 polarization\(^3^4\). Skewing of macrophages towards an M2 phenotype is associated with continuing presence of bacteria in tissues and the conversion of some infectious disease to a chronic state. For instance, replication of Coxiella burnetii, the causative agent of Q-fever, was shown to be related to macrophages expressing a M2 signature that featured up-regulation of arginase-1, YM-1 and the mannose receptor (MR) and down-regulation of iNOS and M1-type cytokines. Activation by IFNγ however, inhibits replication of this pathogen and reprograms macrophages towards M1\(^3^6-3^8\). Additionally, Whipple’s disease, which is caused by Tropheryma whippelii, has also been linked to M2 polarization. Whereas this pathogen is eradicated in an M1-driven immunological microenvironment, it actively replicates in a M2 setting\(^3^9\).

Over the last few years studies on macrophage function in parasite infections have also yielded great knowledge about the existence and function of different macrophage subsets in disease\(^4^0\). Infection of a host organism with both intracellular and extracellular parasites triggers a specific inflammatory response. In the first line of defence that precedes adaptive immunity, macrophages mediate both anti-parasitic immunity, parasite clearance by phagocytosis and immunosuppression. A type 1 pro-inflammatory response by M1 macrophages targeted at parasite elimination is crucial for limiting parasite growth during the acute phase of infection. Following this initial immune reaction, a type II inflammatory response ensues, aimed at resolving the inflammatory process through the effects of M2 macrophages. Any disturbance of the equilibrium between these two phases will worsen the outcome of the infection. Whereas lack of the initial M1 response for example results in uncontrolled parasite colonisation, an insufficient M2 response leads to more efficient parasite killing but on the downside, is accompanied by excessive systemic inflammation, which leads to anaemia and liver damage by reactive oxygen species (ROS) eventually resulting in host death. It is widely accepted that macrophages play a critical role during trypanosomiasis and that their ability to shift towards an anti-inflammatory phenotype determines the susceptibility of the organism for sustained trypanosome infection. The infection with Trypanosoma congolense, the African parasite responsible for sleeping sickness, exemplifies how this fragile balance of macrophage heterogeneity can influence parasite infection and how the parasite exploits changes in the inflammatory response. Upon infection, this extracellular parasite will settle in the liver, spleen, brain and other organs. It is covered by variant-specific surface glycoproteins (VSG) that are very immunogenic and, cause a T-cell dependent B-cell response. However, by frequently transforming their shape, the adaptive response targeted at these molecules is rather useless and makes vaccination against this parasite impossible\(^4^1\). Trypomastomes can be phagocytosed upon binding of antibodies to VSG, but this binding simultane-
ously causes the molecules to be released in the circulation as soluble VSG (sVSG). Initially, this sVSG modulates macrophage function towards systemic macrophage activation with M1 characteristics, which is sustained by T-cell-derived IFNγ and characterized by production of the trypanotoxic agents NO and TNF. Following this early type I cytokine response, there is a transition towards a type II response, mainly involving alternatively activated M2a macrophages, which is characterized by high plasma levels of IL-10, IL-4 and IL-13. The end result is tolerance towards the parasite with a long host survival and limited tissue damage 42-44. C57BL/6 mice however lacking either TNF 45 or IL-10 46 signalling are both susceptible to trypanosomes and die upon infection, by excessive parasite growth or by IFN-γ mediated shock syndrome respectively 47. Concluding, fighting bacterial and parasite infections necessitates an adequate regulation of the balance between M1 and M2 macrophages to ensure proper killing, without complication by excessive tissue damage or prolonged survival of the pathogen in the host.

Tumour-associated macrophages

Another model illustrating the relevance of the macrophage M1/M2 paradigm in pathologic situations consists of the macrophages that are found within tumours 48. A tumour microenvironment not only exists of malignant cells but also includes stromal cells and cells of the immune system. This immune surveillance aims at the elimination of tumour cells through innate and adaptive responses 49 but is evaded by genetic variability of the tumour cells, active immune suppression and secretion of tolerogenic signals. Because of their contribution to both the targeting and the protection of the tumour cells, the tumour-associated macrophages (TAMs) are a great example of the possible consequences of macrophage heterogeneity 8,50,51. Moreover, these even opposing functional characteristics show clearly the threats to be dealt with when contemplating macrophage directed therapies 52.

The macrophages that are initially attracted to tumours by factors like M-CSF and CCL2 53,54 show an M1 phenotype, are cytotoxic through secretion of oxygen radicals and slow down tumour cell proliferation. They also induce an inflammatory environment that attracts and activates cells of the adaptive immune system 55,56. When their activation is sustained over a longer period however, they increase the risk of DNA damage and tissue structure abnormalities, promoting carcinogenesis 57. The tumour cells that are lysed by these M1 macrophages release factors including TGF-β and sphingosine-1-phosphate, which reprogram the macrophages towards an alternatively activated phenotype 58. Subsequently, these M2 macrophages will temper the inflammation and the production of oxygen radicals, and lead to the development of a more tolerogenic environment by secretion of IL-10 and TGF-β 58. Moreover, these cells mediate cell proliferation and tissue remodelling through arginase induction and tumour invasiveness through secretion of chitinase-like proteins, further contributing to tumour development. In addition, another important feature of the alternatively activated
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TAMs is their role in the formation and remodelling of new blood vessels in the tumour area by releasing pro-angiogenic factors such as VEGF (vascular endothelial growth factor), TNF, IL-8 and bFGF (basic fibroblast growth factor) and angiogenesis modulating enzymes such as MMP2-, MMP-7, MMP-9, MMP-12 and COX-2. As the rapid tumour growth demands increasing perfusion to prevent hypoxia due to poor perfusion and subsequent necrosis, angiogenesis is essential for disease progression.

Since TAM density in tumours is correlated with a poor prognosis, blocking the attraction of macrophages to the tumour might seem a promising therapeutic target. Indeed, in experimental models this approach showed to inhibit further tumour growth. However, it also prevents the potential of more M1 polarized macrophages to engage in tumour cell lysis and the promotion of an anti-tumour immune reaction.

The formation of tumour promoting TAMs has been linked directly to the NF-κB signal transduction pathway, which activates transcription factors playing an essential role in regulating inflammation and immunity. Especially, the NF-κB subunit p50 has been identified as an important regulator of macrophage polarization in tumours. The observation that TAMs are defective in their production of the NF-κB target gene IL-12 but rather show higher levels of IL-10 indicated that a regulation at the level of NF-κB can be expected. It was shown that p50 can form inhibitory homodimers that are able to bind the NF-κB consensus binding sites, repressing transcription of a subset of NF-κB dependent inflammatory genes. Indeed, TAMs show a tolerance to LPS and other pro-inflammatory signals as characterised by a defective IL-12, IL-6 and TNF expression and relatively enhanced IL-10 expression. Moreover, TAMs were characterized by massive nuclear overexpression of p50. Interestingly, the anti-inflammatory phenotype of the TAMs was reversed in macrophages lacking p50 and both p50 deficient mice and wildtype mice that received p50 deficient bone marrow displayed a delayed tumour progression with increased survival. Thus activation of the NF-κB subunit p50 seems to induce an M2 phenotype in TAMs. Continuing on the role of NF-κB signalling in regulating TAM function, it was more recently shown that the main kinase mediating NF-κB activation, IKK2 (or IKKβ), inhibits M1 differentiation of macrophages by affecting signal transducer and activator of transcription-1 (STAT1) signalling. Consequently, deletion of IKK2 specifically in macrophages led to a more pronounced M1 phenotype, characterized by high IL-12, iNOS and MHCII expression and low levels of IL-10. This polarization resulted in macrophages that were better capable of actively eliminating tumour cells, both directly by enhanced production of oxygen radicals and indirectly through IL-12 dependent NK cell anti-tumour activity. Thus, NF-κB activation is intricately involved in the induction of anti-inflammatory tumour repressive TAMs at different levels. Moreover, it is clear that induction of macrophage polarization towards an M1 phenotype is a more desirable option for treatment than mere prevention of macrophage attraction to tumours.
**Chapter 2**

**Macrophage heterogeneity in obesity and insulin resistance**

In recent years, mounting evidence suggests that low-grade inflammation contributes to the development of obesity and insulin resistance. More specifically, recent work has shown that obesity and insulin resistance are associated with alterations in macrophage polarization. Particularly the role of adipose tissue macrophages (ATM) has been subject of investigation. It was shown that ATMs in lean mice show characteristics of M2 macrophages with expression of arginase and IL-10. Upon induction of obesity through a high fat diet, ATMs switch their phenotype towards a functional M1 program including expression of markers such as iNOS and TNF. These M1 macrophages accumulate in adipose tissue with progressive obesity, and are recruited by MCP-1 dependent mechanisms. Absence of MCP-1 or its receptor CCR2 abolishes migration of macrophages into adipose tissue thereby preventing adipose tissue inflammation and improving insulin resistance. Moreover, it was shown that IL-10, produced by ATMs in lean mice protects adipocytes from TNF induced insulin resistance.

The M2 phenotype of ATMs in lean mice was subsequently shown to be maintained by the nuclear receptor peroxisome proliferator-activated receptor γ (PPARγ). Induction of alternatively activated macrophages by IL-4 was reduced in the absence of PPARγ and led to reduced arginase-1 activity, decreased suppression of IL-6 and inhibition of fatty acid oxidation in macrophages. Furthermore, macrophage specific deletion of PPARγ in vivo reduced the M2 phenotype of ATMs, led to impaired adipocyte function with reduced mitochondrial oxidation and eventually resulted in enhanced obesity and insulin resistance. In line with these findings, Hevener et al. showed that macrophage PPARγ deficiency leads to impaired insulin sensitivity in muscle, adipose tissue and liver and worsens insulin resistance upon high fat feeding of mice. Thus, PPARγ is necessary for maintenance of the M2 phenotype in adipose tissue of lean individuals and thereby protects against obesity and insulin resistance.

Interestingly, other than in adipose tissue resident macrophages, disruption of PPARγ does not affect Kupffer cells, the liver’s resident macrophages. In these cells, the transcriptional activation of another nuclear receptor, PPARδ, by mono-unsaturated fatty acids, brings the cells in an alternative activation state. Contrary to the adipose tissue macrophages, not the oxidative metabolism changes accompanying this phenotype but rather their anti-inflammatory capacity appear to be of importance. Again here, PPARδ deficiency impairs alternative macrophage activation of Kupffer cells, causing hepatic dysfunction and diet-induced insulin resistance and obesity. Similar observations were described by Kang et al. showing that myeloid specific PPARδ deficiency leads to insulin resistance and severe steatohepatitis.

Hence, macrophage polarization regulated by both PPARγ and PPARδ provides an important incentive in the pathogenesis of metabolic diseases. The anti-inflammatory phenotype of
tissue macrophages in healthy conditions seems to be protective, while the phenotypic switch of macrophages towards a pro-inflammatory M1 phenotype seems to exacerbate changes to obesity and insulin resistance.

Macrophages as central players in atherosclerosis initiation and progression

From the above it is clear that macrophage polarization is a main determinant in the pathological development of many different diseases. As such the macrophage is also a key player in atherosclerotic plaque development and stability (Figure 1).

Figure 1. A central role for macrophages in plaque development. From left to right the development of an atherosclerotic plaque is depicted. Early phases involve the subendothelial retention and accumulation of LDL that becomes minimally modified and induces chemokine secretion and endothelial activation. In response, monocytes are attracted and migrate into the vessel wall where they differentiate into macrophages and scavenge modified lipoproteins. Ultimately, this leads to the development of macrophage derived foam cells, with their characteristic intracellular lipid droplets. Continuing foam cell accumulation results in the formation of a fatty streak. Macrophages in atherosclerotic lesions also secrete lipid modifying enzymes, further promoting LDL oxidation; and chemokines and cytokines, which control the inflammatory balance and regulate further recruitment, activation and proliferation of cells. Upon further progression of atherogenesis T-cells are attracted and activated which propagate the chronic inflammatory state of the lesion. Furthermore, smooth muscle cells proliferate and migrate to form a fibrotic plaque-stabilizing cap. Foam cells die through apoptosis and lack of proper scavenging of apoptotic cells leads to secondary necrosis, which releases cellular debris and gives rise to the formation of a lipid rich necrotic core. Plaque growth can ultimately lead to obstruction of the lumen or result in a rupture of the plaque, leading to the release thrombogenic material, causing formation of a thrombus. The process of rupture is promoted by the local recruitment of macrophages, producing matrix-degrading enzymes.
The main initiating events in atherosclerosis development encompass endothelial dysfunction and modification of low-density lipoprotein (LDL) \(^80\). Aberrant blood flow renders specific areas of the vessel wall more susceptible to atherogenesis, since the altered dynamics increase permeability of the endothelial barrier to macromolecules such as LDL \(^81\). These molecules subsequently accumulate in the subendothelial matrix, where they undergo modification through oxidative or enzymatic processes. Retention of these modified lipoproteins exerts a pro-inflammatory stimulus on the micro-environment, thereby promoting expression of a range of adhesion molecules (e.g. vascular cell adhesion molecule 1; VCAM-1), chemotactic proteins (e.g. monocyte chemoattractant protein 1; MCP-1) and growth factors (e.g. macrophage-colony stimulating factor; M-CSF) by the overlying endothelium \(^82\). This in turn mediates leukocyte recruitment and monocyte migration into the intima at lesion prone sites \(^81\). Upon differentiation by the presence of M-CSF, monocyte-derived macrophages will start to internalize substantial amounts of modified LDL via scavenger receptors (SR) \(^81\text{--}83\). Although this process of foam cell formation may at first serve a protective role in removing pro-inflammatory modified lipids and apoptotic cell debris from the vessel wall, the increasing build-up of these cells ultimately leads to the development of fatty streaks. The progression of a clinically insignificant fatty streak to a more complex lesion is hallmarked by the migration of smooth muscle cells (SMCs) from the media to the subendothelial space of the vessel wall \(^81,82\). In conjunction with a continuous influx of immune cells, these SMCs can proliferate within the lesion, contribute to foam cell formation or start to produce components of the extracellular matrix. Concurrently, foam cells will become apoptotic and improper scavenging of these apoptotic cells will lead to the release of their lipid-filled contents to the growing necrotic core of the plaque \(^80\). Ultimately, an advanced lesion is formed, in which the lipid-rich necrotic core is shielded from the lumen by a fibrous cap of SMCs and extracellular matrix. In disease progression to clinical manifestation, rupture of this fibrous cap is generally thought to elicit thrombosis, as exposure of plaque lipids and tissue factor to blood components will consequently initiate the coagulation cascade \(^81,84\). Interestingly, the balance between trophic and degenerative factors that determine the physical integrity of the fibrous cap can be profoundly affected by inflammatory factors. For instance, the pro-inflammatory stimulus interferon-\(\gamma\) (IFN\(\gamma\)) inhibits extracellular matrix production by SMCs, while macrophages enable the degradation of SMC-secreted matrix proteins by the production of matrixmetalloproteinases (MMPs) \(^80,85\). Hence, cellular and humoral factors of immunity can promote plaque vulnerability.

**Macrophage heterogeneity in atherosclerosis**

As shown above, the functional characterization of macrophage heterogeneity has been ongoing now for several years in numerous different macrophage-related pathologies. It is also clear that the macrophage is a true multifunctional player in atherosclerotic lesion develop-
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ment, contributing to plaque initiation, growth, and stability. Based on their inflammatory characteristics it can be expected that M1 macrophages promote, while M2 macrophages protect in atherosclerosis development. However so far, the functional contribution of macrophage subsets to atherosclerosis has barely been studied. Early on, the heterogeneity of macrophages in human atherosclerotic lesions was already demonstrated. Using differentiation markers and staining for lipids and lysosomal phosphatase it was shown that macrophages localized more centrally in the lesions have more differentiated and matured characteristics then macrophages in the superficial layers of the plaques, which may imply functional differences between macrophage populations in the lesions. Similarly, using a set of macrophage specific monoclonal antibodies, it was shown that macrophages in murine atherosclerotic lesions also show a large degree of heterogeneity as well. More recently, Waldo et al. yet again described heterogeneity of human macrophages in atherosclerotic lesions by discriminating subsets based on their CD14 positivity. Thus, heterogeneity of macrophages in human and mouse atherosclerotic lesions has been established. However data showing a direct link between the M1 and M2 subsets and atherosclerotic lesions is limited.

Recently, Bouhlel et al. were the first to describe expression of M1 and M2 markers in atherosclerotic lesions. Concretely, they demonstrated that genes such as MCP-1, IL-6 and TNF, markers associated with M1 macrophages and mannose receptor, CD163, IL-10 and CCL18, markers of M2 macrophages are expressed in human atherosclerotic lesions. Moreover, they found a direct correlation between expression of M2 markers and PPARγ expression, indicating a role for PPARγ in regulating M2 differentiation, similarly as was described before in mice in relation to diet-induced obesity (see above). Indeed by using cell culture they could confirm that PPARγ activation polarizes human macrophages towards an M2 phenotype. Interestingly, patients treated with PPARγ agonists showed a more pronounced M2 phenotype in their circulating mononuclear cells as well. However, in these patients PPARγ agonists did not change M2 marker expression in atherosclerotic lesions. Although certain questions remain unanswered, this paper describes expression of certain M1 and M2 markers and identifies the lesional localization of mannose receptor, as M2 and MCP-1 as M1 marker. Hereby, this was the first paper to describe M1 and M2 macrophages in atherosclerotic lesions. More recently, Boyle et al. demonstrated that intraplaque haemorrhages, a main contributor to plaque instability, evokes the induction of CD163 positive M2 macrophages in human atherosclerotic plaques in coronary arteries. This may imply CD163, which is a scavenging receptor for haemoglobin-haptoglobin complexes, as a marker for plaque instability. However, so far this report is the only association of M1/M2 subset markers with the phenotype (i.e. stability) of an atherosclerotic lesion.
Macrophage subset effector function and atherosclerosis

From a theoretical perspective and knowing the many specialized functions macrophages exert in all stages of atherogenesis, it can be expected that specific features of the M1 and M2 differentiated macrophage subsets described before in this review contribute to plaque development. First of all, many different macrophage polarization associated cytokines (see table I) have been subject of extensive investigations in atherosclerosis (reviewed by Tedgui and Mallat and Kleemann et al.)\textsuperscript{89,90}. In general, cytokines that are specifically produced by M1 macrophages have a pro-atherogenic effect. Using knockout, transgenic, treatment or inhibitory approaches it is firmly established that cytokines such as TNF, IL-1 and IL-12 promote disease progression in different animal models for atherosclerosis. In contrast M2 associated cytokines are anti-atherogenic, such as IL-10, or are anti-atherogenic and pro-fibrotic, such as TGFβ. These cytokine data fit with the concept that M1 macrophages promote atherosclerosis and M2 inhibit plaque growth and mediate plaque stability.

For chemokine patterns associated with macrophage polarization\textsuperscript{4}, the story is more complex. Both the CCL5 (RANTES) and CCL2 (MCP-1 or JE) are considered M1 chemokines and have been studied to some extent in atherosclerosis\textsuperscript{91}. While CCL5 blockade reduces atherosclerosis\textsuperscript{92}, inhibition of its two receptors has differential effects. CCR5 seems to act proatherogenic and genetic deletion of CCR5 reduces atherogenesis and inhibits Th1 responses\textsuperscript{93-95}, while in contrast CCR1-deficient mice show accelerated atherosclerosis, with increased IFNγ as important mediator\textsuperscript{96,97}. CCL2 and its receptor CCR2 have a more clear role and seem to act really pro-atherogenic\textsuperscript{98-100} and regulate the specific recruitment of inflammatory monocytes to atherosclerotic lesions\textsuperscript{101}. Many other chemokines still needs better association with macrophage subsets or have not been investigated for their role in atherosclerosis development.

In line with the pro-atherogenic function of M1 macrophages and the putative atheroprotective effect of M2 macrophages, the shift of the arginine metabolism in these latter cells could also act beneficial. The induction of arginase by M2 skewing leads to the production of ornithine and subsequently proline and polyamines through conversion of arginine. In macrophages this diverges arginine use away from nitric oxide synthesis through iNOS and mediates the production of precursors for collagen synthesis (i.e. proline). Hereby, M2 macrophage may promote plaque stability and reduce atherogenesis. This was recently substantiated by genetic analysis of two rabbit strains with high and low susceptibility for atherosclerosis, showing that the rabbits which had reduced atherosclerosis development were characterized by elevated levels of Arg1 activity, regulated through a polymorphism in the 3’-untranslated region\textsuperscript{102}.

Although the above all point towards an anti-atherogenic effect of M2 macrophages, some other polarization-associated functions seem to counter this notion. M2 macrophages are
highly phagocytic and express increased levels of scavenging receptors such as scavenger receptor class A (SR-A) and CD36. Both these have been originally described as major mediators of foam cell formation and promoters of atherosclerosis, although some recent investigations have started to question the clear foam cell promoting function of these scavenger receptors and even suggest an inflammation mediating role of SR-A and CD36 in atherogenesis 103,104. Thus, M2 polarization may promote foam cell formation and thereby mediate atherosclerotic lesion growth, but may also be affecting inflammation associated atherosclerotic remodelling by changes in scavenger receptor activity. Finally, especially from the field of tumour biology, it is clear that M2 macrophages are promoting angiogenesis 105. As discussed above, angiogenesis is a key event in tumour growth and progression and the so-called “angiogenic-switch” is also considered to be mediated at least partly by the M2 macrophages found in tumours, the TAMs 105,106. Hypothetically, M2 macrophages in atherosclerotic lesions may thus promote microvessel growth in atherosclerotic lesions too. Since angiogenesis is considered to promote lesion growth, mediate lesional inflammation and contribute to plaque instability through intraplaque haemorrhage caused by lack of proper structural integrity of the newly formed microvessels 107, M2 macrophages may have undesired effects from the angiogenesis point of view. Thus, although the cytokine and chemokine profile seems to predict a pro-atherogenic function for M1 macrophages and M2 macrophages may be protective, other functions of polarized macrophages may have opposing and destabilizing effects on atherosclerotic lesions.

All these specific characteristics imply that an important role for the balance between macrophage phenotypes, both spatially and temporally, might be expected. The characterization of this balance and its regulation provides an interesting challenge since it might open doors towards new methods of atherosclerosis diagnosis and therapy, targeting or favouring specific cell types and functions. One of the major obstacles that will have to be overcome when studying macrophage phenotypes in atherosclerosis is the current lack of a good readout system, with markers that specifically distinguish the different phenotypes in atherosclerotic lesions. Despite the existence of many lists describing typical genes, proteins and metabolic products associated with the different phenotypes, these markers are usually more abundant in, but rarely specific for a certain subset. To be able to recognize the different subsets within one tissue, a more clear-cut readout system would be desirable. Moreover, most of the M1 and M2 associated markers have not been tested for their expression and intraleisional localisation in either human or experimental atherosclerosis.

Hence, to better understand the functions exerted by the different subsets, their characteristics should be further examined both on a molecular and phenotypic level. Moreover, since atherosclerotic plaques contain unique stimuli for the macrophages, namely modified LDL particles, it will be of great interest to study if they alter the response of the macrophages to the typical M1 and M2 inducing signals or even if the lipid-laden foam cells could be seen as yet another phenotype, with characteristics distinct from the current M1 and M2 pheno-
types. Furthermore, since different subsets are present in the plaque and probably co-exist for extended periods within one tissue, it would also be valuable to study the interaction between macrophages with different phenotypes. An important feature to study here is the possibility of macrophages to redifferentiate into another phenotype due to changes in its environment 10.

Macrophage polarizing cytokines in atherosclerosis

To actually study the role of the different macrophage subsets in atherogenesis, specific gene targeting in different murine models of atherosclerosis remains the first method of choice. Up until now, a broad range of studies deleting or over-expressing cytokines with key functions in macrophage polarization, in both ApoE−/− and LDLR−/− mice have been performed. However these investigations never focused directly on how a specific intervention affects macrophage phenotype in the plaque 89.

In the setting of atherosclerosis, IL-10 is one of the best-studied polarizing cytokines. As described earlier, IL-10 is involved in M2c polarization, but has also been shown in several studies to exert athero-protective qualities, especially in early atherogenesis. Several knockout models for this cytokine describe a significant increase in lesion size and inflammation, which is accompanied by reduced plaque stability and an increased influx of T-cells 108,109. In line with these findings, different models of transgenic over-expression of IL-10 demonstrate a reduced lesion size 110-112. However, since IL-10 also favours a Th2 response and has anti-inflammatory capacities on most other cell types present in the atherosclerotic plaque as well, it is still impossible to claim that its anti-atherogenic effect is only mediated by eliciting a shift in macrophage phenotypes. For this reason, additional studies addressing IL-10 signalling specifically in macrophages should be performed that clearly distinguish between the inability of these macrophages to respond to or produce and secrete IL-10. Moreover, such studies may provide novel insights into the expression of markers for macrophage subsets in atherosclerosis and their dependence on polarizing signals.

Another polarizing cytokine, IL-4, rather induces an M2a phenotype in macrophages. Although these are potentially anti-atherogenic, IL-4 is also known to be involved in the up-regulation of VCAM-1 113,114 and MCP-1 115,116 in endothelial cells, both important positive mediators of atherogenesis. This possibly blurs the effect of studies investigating the effects of IL-4 on atherosclerosis in mouse models and may be the reason why ApoE−/−IL-4−/− double KO mice or LDLR−/− mice transplanted with IL-4−/− bone marrow initially rather showed an atherosclerosis promoting effect in a time and site specific manner 117,118. However, an initial study on IL-4 deficiency in C57BL/6 mice showed no effect 119 and more recently King et al. 120 performed an extensive study with both ApoE−/− and LDLR−/− mice with IL-4 knockout or treatment approaches and also found no effect on atherosclerotic lesion formation. These
results raise questions concerning the contribution of M2a macrophages to atherogenesis, but may also be caused by the aforementioned opposing effects on different cellular populations in the plaques. Finally, paradoxical effects of IL-4 may even indicate differences between mouse and man.

Comparable to the difficulties that arise in distinguishing the M2 and Th2 promoting capacities of IL-10, the effect of macrophage polarization by IFNγ on atherogenesis is also difficult to reveal, since this pro-inflammatory cytokine both induces M1 macrophages and Th1 cells. Most experimental studies performed clearly show a reduction in lesion size upon absence of this cytokine or its receptor \(^{121-123}\) or an induction by intraperitoneal recombinant IFNγ administration \(^{124}\), confirming a pro-atherogenic effect of this M1 polarizing cytokine. However, similar as above, direct effects on macrophage function in atherogenesis is lacking and it is unclear which exact cell types mediate the observed effects and whether genetic or treatment modification of IFNγ in the mice actually led to changes in macrophages populations and functionality in the plaque.

Last but not least, the pro-inflammatory TNF is probably the most difficult macrophage polarizing cytokine to study due to its broad range of activities. For example, it not only promotes an M1 phenotype but also induces cell attraction through upregulation of ICAM-1, VCAM-1 and MCP-1 in endothelial cells and influences foam cell formation through regulation of scavenger receptor mediated LDL uptake \(^{125}\). The broad range of possible interactions with several key stages in the atherogenesis implies difficulties in comparing different models. In general however it can be summarized that absence of TNFα results in decreased atherosclerotic lesion size and progression \(^{125-128}\). It would however be very difficult at the moment to translate this to a direct theory on the effect of TNF-induced M1 macrophages on the atherosclerotic plaque because of the described abundance in atherosclerosis regulating effects.

Concluding, the above data clearly shows association of polarizing cytokines with atherosclerosis development, as depicted in Figure 2 as well. However, studies on the direct effect of M1/M2 skewing on atherosclerosis in experimental models are lacking and more specifically, identification of subsets and their functional roles is really scarce. A lot of these studies still need to be performed to achieve some insight, while in the end, the major challenge will still be to translate this knowledge from the mouse atherosclerosis models to the human situation. Especially since both the macrophage heterogeneity \(^{129}\) and atherogenesis \(^{130,131}\) are known to be differently regulated in both organisms.
Below are indicated the processes that are potentially affected by macrophage polarization towards either M1 or M2.

**Figure 2.** Macrophage polarization in atherosclerosis. Overview of the macrophage skewing cytokines and factors associated with the macrophage subtypes that are known to be present in the atherosclerotic plaque. Below are indicated the processes that are potentially affected by macrophage polarization towards either M1 or M2.

**Macrophage polarization: beneficial effects of therapeutics and future opportunities for imaging?**

Numerous studies provide an indication for therapeutic agents, of which the function can at least partly be ascribed to their immunomodulatory properties. Hypothetically, distinct macrophage subsets might be involved in mediating the (pleiotropic) actions of some of these drugs. In turn, these agents might drive macrophage polarization to a certain extent as well and may thereby present ways to manipulate macrophage reprogramming. In addition, imaging of macrophages as a diagnostic tool has recently gained great interest. Since macrophage subsets are likely to be differentially involved in regulating plaque instability, future diagnosis based on identification of culprit macrophages may be very beneficial.
Therapeutic interventions modulating macrophage polarization?

Statins (3-hydroxy-3-methylglutaryl coenzyme A or HMG-coA reductase inhibitors), provide a proficient approach to achieve lowering of serum cholesterol in patients and have accordingly become one of the most commonly used drugs in the primary and secondary prevention of cardiovascular disease\textsuperscript{132}. Over the last decade, several in vitro and in vivo studies established the fact that statins conduct at least part of their beneficial effects through a manner that is independent of their lipid-lowering properties\textsuperscript{133,134}. As a result, statins have gained strong interest in different fields of inflammatory disease, as several of their pleiotropic effects were demonstrated to be attributable to the modulation of inflammatory processes. In 2002, Youssef et al. showed that the HMG-coA reductase inhibitor atorvastatin significantly alters the inflammatory balance in a murine model of auto-immune encephalomyelitis\textsuperscript{135}. Whilst inhibiting secretion of the pro-atherogenic Th1-cytokines IL-12, IFNγ and TNF, atorvastatin promoted a shift towards a Th2-type immune response through the release of cytokines such as IL-4, IL-5, IL-10 and TGF-β. Similarly, a decrease in Th1 bias was reported in human study populations\textsuperscript{136,137}, providing additional in vivo evidence for the immunoregulatory potential of statins. With regard to macrophage polarization, these findings may be of particular interest, since changing the immune response through treatment with statins might thus be affecting the phenotype of macrophage populations. Apart from being acknowledged as anti-atherogenic factors, Th2 effector molecules are evidently engrossed in the skewing of macrophages towards M2 phenotypes\textsuperscript{13}. For this reason, statin-mediated alterations in inflammatory processes might well offer an interesting mechanism for shaping macrophage polarization. Rather than being limited to the development of atherosclerosis, differential involvement of macrophage subsets may potentially be incorporated in other inflammatory disorders as well. As such, statins have been shown to exert anti-inflammatory effects in rheumatoid arthritis\textsuperscript{138} and to inhibit the amount and size of brain lesions in multiple sclerosis (MS)\textsuperscript{139}.

Thiazolidinediones (TZDs), such as rosiglitazone, are PPARγ agonists that are used to increase insulin sensitivity in type 2 diabetes patients. In addition to their metabolic action they have also been shown to have profound anti-atherosclerotic effects in numerous animal models\textsuperscript{140-142}. These anti-atherosclerotic effects are largely attributed to their anti-inflammatory action\textsuperscript{143} since PPARγ can directly interfere with inflammatory signal transduction cascades such as, Nuclear Factor-kB (NF-kB), Signal Transducer and Activator of Transcription-1 (STAT-1) and activating protein-1 (AP-1)\textsuperscript{143}. In line with this anti-inflammatory action rosiglitazone treatment has been shown to reduce plasma levels of C-reactive protein (CRP) in type 2 diabetes patients\textsuperscript{144}. As discussed above, it was shown that PPARγ agonists can directly drive macrophage polarization in both human and mouse cells towards M2 polarization\textsuperscript{30,76}. Thus, an important part of the anti-atherosclerotic action of PPARγ activation may be attributed to polarization of macrophages to an M2 phenotype. In a recent randomized placebo controlled trial in non-diabetic patients rosiglitazone was indeed shown to reduce lesional in-
flammation and increased plaque collagen content and stability. Interestingly, all of these effects are associated features of M2 macrophages.

The macrophage as a diagnostic target

As mounting evidence designates a lesion’s inflammatory burden, rather than the degree of stenosis, as the major determining factor for clinical outcome in atherosclerosis newly developed imaging strategies accordingly aim to assess the key inflammatory components that decide a plaque’s fate. Seeing that polarized macrophage subsets are hypothesized to highly affect plaque stability, supposedly with contrasting outcomes, imaging strategies that target specific macrophage populations might prove valuable tools in the prevention of acute ischemic events.

Above all, magnetic resonance imaging (MRI) is considered to be of great potential in this regard. Due to its excellent soft-tissue contrast, this non-invasive imaging modality is recognized as being supremely adept for serially assessing atherosclerotic vessels. Through application of MRI in experimental animal models (i.e. rabbits and mice) and human research populations, adequate in and ex vivo imaging protocols for the assessment of atherosclerotic plaque composition have been defined. By discriminating plaque constituents based on their respective biophysical and biochemical properties, this technique is able to detect cellular elements, lipid core, fibrosis, and thrombus formation in atherosclerotic lesions with high sensitivity and specificity. Interestingly, the use of contrast enhancement provides additional opportunities for depicting inflammatory mediators in atherosclerosis (e.g. macrophages). One such an approach utilizes ultrasmall superparamagnetic particles of iron oxide (USPIO) as a contrast agent for MRI. Once internalized by macrophages, these nanoparticles cause a focal loss of signal intensity on MRI and thus enable non-invasive localization of macrophages in vivo. At present, this technique has been used to assess atherosclerotic plaques in hyperlipidemic rabbits, ApoE-/- mice and was demonstrated to be a feasible strategy for macrophage detection in a human population. Interestingly, the latter study reported accumulation of USPIO especially in macrophages in ruptured or rupture-prone atherosclerotic lesions. Hence, by appraising plaque macrophage content, USPIO-enhanced MRI might help distinguish between stable and vulnerable lesions.

In a similar fashion, several studies evaluated alternative imaging modalities for their ability to portray macrophages in atherosclerosis. Recently, Hyafil et al. demonstrated that the iodinated contrast agent N1177 is readily taken up by macrophages and subsequently induces an increased density of macrophage-rich tissues on computed tomography (CT) imaging. Upon histological examination this effect co-localized with macrophage infiltration. In addition, Fayad and colleagues reported a CT-based approach to be suitable for plaque characterization. Finally, positron emission tomography (PET) imaging techniques can visualize
macrophages by detecting uptake of $^{18}$F fluorodeoxyglucose (FDG). Macrophage-rich atherosclerotic lesions show augmented uptake of FDG due to the higher metabolic activity of activated macrophages. Interestingly, symptomatic lesions exhibit more FDG accumulation in comparison to their asymptomatic counterparts, further implicating macrophages in plaque complication.

In summary, these studies represent several perspectives by which macrophages may serve as suitable targets for diagnostic purposes and future imaging strategies. Although promising, these methods need to be optimized before routine application in clinical practice can be considered. Accordingly, the current diagnostic approach to atherosclerosis still relies heavily on (invasive) clinical imaging modalities that depict the lumen and vessel wall of atherosclerotic arteries to identify individuals at risk for acute ischemic events. However, our growing insights in the molecular mechanisms that drive atherosclerosis continue to stress the need for imaging techniques that are able to depict functional and biological processes as opposed to anatomical abnormalities. As we further unravel the relations between distinct macrophage subsets and atherogenesis, the development of novel imaging strategies that utilize the concept of macrophage polarization for the identification of macrophage subsets within individual plaques might revolutionize the ways by which we assess atherosclerotic arteries and stratify individual risk.

**Conclusion**

Over the past few years, we gained considerable insight into the inflammatory processes that lie at the core of atherosclerosis development. Macrophages are progressively acknowledged as major determinants of atherosclerosis, joining both innate and adaptive immune responses in its pathogenesis. Currently, several polarized M1 and M2 macrophage subsets have been identified, and both populations were demonstrated to be present in atherosclerotic lesions. Although the differential expression of effector phenotype implies macrophage function in atherosclerosis to be highly ambiguous, evidence supporting their functional implications in vivo is largely lacking. Ensuing, several arising challenges, that complicate the implementation of macrophage polarization in our understanding of the atherosclerotic process, need to be addressed. Firstly, additional markers are required that will allow us to better distinguish the current macrophage subsets and possibly help identify new ones. Preferably, these markers will be adept for characterizing both murine and human macrophage populations, as inter-species variability is becoming increasingly apparent. Any such markers might subsequently facilitate the exploration of means by which the inflammatory balance in atherogenesis can be affected (e.g. pharmaceutical interventions). Additionally, we need direct functional studies in mice that lack a specific macrophage subset to illustrate the in vivo relevance of that particular subtype and possibly provide further insight into
the underlying mechanisms. Ultimately, the current M1/M2 paradigm offers a comprehensive take on the concept of macrophage heterogeneity. However, due to its simplified nature, it is highly unlikely that this digotomy represents the full scope of functional macrophage phenotypes. Instead, the current M1 and M2 states are better regarded as extremes of a continuum that remains to be explored. Hence, if we are to generate a classification that is a more accurate portrayal of in vivo atherosclerotic conditions, we should strive to advance our knowledge regarding this phenomenon. Only by negotiating the aforementioned difficulties will we be able to develop strategies that allow us to skew macrophage polarization in such a manner that it serves our diagnostic and therapeutic goals, be it in the context of atherosclerosis or other macrophage-mediated disorders such as obesity and cancer.
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Chapter 2

Macrophage heterogeneity in atherosclerosis


Macrophage heterogeneity in atherosclerosis


