# TARGETING MONOCYTES/MACROPHAGES TO TREAT ATHEROSCLEROTIC INFLAMMATION

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# TARGETING MONOCYTES/MACROPHAGES TO TREAT ATHEROSCLEROTIC INFLAMMATION

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EDITORIAL
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# Editorial: Targeting Monocytes/Macrophages to Treat Atherosclerotic Inflammation

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**Editorial on the Research Topic**

**Targeting Monocytes/Macrophages to Treat Atherosclerotic Inflammation**

By now well into the XXI century, atherosclerosis with its cerebro- and cardiovascular complications continue to represent the leading cause of morbidity and mortality at global level. The identification and control of known risk factors—hypertension, diabetes, cigarette smoking and above all, elevated low-density lipoprotein (LDL) cholesterol—have actually allowed the achievement of a dramatic decrease in the incidence of major cardiovascular diseases in many western countries, and current pharmacological treatments aiming to slow down progression of atherosclerosis are thus almost entirely centered on reducing plasma cholesterol levels. Nevertheless, elevated LDL levels alone cannot account for the entire burden of atherosclerosis, and the concept of atherosclerosis as a *proliferative* disorder independent of cholesterol levels has gained increased significance as a pathogenic pathway since its first proposal back in the '70s (Ross and Glomset, 1973). It is by now widely acknowledged that the pathogenic basis of atherosclerosis extends far beyond intimal infiltration of cholesterol. Animal experiments, observations on human atheromata as well as clinical biomarker studies, all support the importance of immune and inflammatory pathways in the initiation, progression, and eventual thrombotic manifestations of the disease.

The precise identification of the cell types accumulating inside human atherosclerotic lesions had to wait until the advent of monoclonal antibody technology, which eventually allowed to recognize mononuclear phagocytes as the main precursors of "foam cells" typically populating the plaques. The same was then observed for smooth muscle cells of the arterial wall, which can also originate macrophage-like and even typical foam cells through sort of a metaplastic transformation (Bennett et al., 2016). The "proliferative" and the "inflammatory" interpretations of atherogenesis can be thus reunified into one comprehensive theory, viewing atherosclerosis as an extraordinarily complex pathobiological process in which pathways of inflammation are set into motion by several risk factors and in turn promote altered behaviors of arterial wall cells. In this picture, cytokines, chemokines, and adhesion molecules associated with components of the vessel wall, as well as the immune/inflammatory cell types intervening in the lesions—monocytes/macrophages in the first place—become the natural subjects for investigation, being the likely responsible of altered arterial biology. Among these, several potential targets for therapeutic treatments of atherosclerosis have been conjectured. Indeed, the translation of biological insights into new solutions is starting to work, as shown by the encouraging results of antiinflammatory treatments based on anti-IL-1beta monoclonal antibodies [CANTOS trial: (Libby, 2017; Ridker et al., 2017)].

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The reviews and research articles that make up the present Research Topic represent a unique collection, capable of providing an overview of current trends of pharmacological research in the atherosclerosis field. To start with, the paper by Flynn et al. provides an overview of the different types and origin of macrophages and macrophage-like cells contributing to the atherosclerotic disease. The focus is mainly pointed on the influence of diabetes and obesity on myelopoiesis and macrophage activation/accumulation, leading to an overall increased cardiovascular risk. Targeting the production of monocyte-derived macrophages was shown to reduce preclinical atherosclerosis in a number of metabolic and inflammatory diseases. A key role is played by S100A8/A9 heterocomplex, a myeloproliferative factor active both in diabetes and obesity, whose inhibition could represent a strategy to reduce cardiovascular risk in this kind of patients. The following paper by Martinet et al. proposes an interesting insight into the macrophage death modes observed in advanced plaques, from canonical (necrosis, apoptosis) to more exotic ones (efferocytosis, necroptosis, pyroptosis, ferroptosis, parthanatos, as well as autophagic death).

A correct appraisal of these processes is obviously crucial for the development of pharmacological interventions aiming at stabilization of vulnerable, rupture-prone plaques, or possibly even at regression of lesions. The remaining papers in the present series in fact are all examples of this kind of approach. Nikiforov et al. provide, *e.g.,* an overview of studies on the activation status of monocytes in atherosclerotic patients. Monocytes obtained from atherosclerotic patients indeed present with a hyperreactive, "trained" phenotype which significantly correlates with intima-media thickness taken as an index of disease progression. The authors propose thus to employ trained monocytes from atherosclerotic patients as an *ex vivo* model for testing the ability of potential antiatherogenic compounds to attenuate the monocyte hyperreactivity.

Can any clues be derived from epigenetics? In their elegant original article, Luque-Martin et al. have investigated the antiinflammatory effects of an esterase-sensitive histone deacetylase inhibitor. The authors do observe a reduced production of proinflammatory cytokines by isolated peritoneal macrophages. On the other hand results in an *in vivo* knock-out (ldlr-/-) mice model were disappointing, as the inhibitor could not reduce the formation of plaques. Nevertheless, the study overall offers a remarkable example of how efficient experimental strategies can be devised.

The review by Getz and Reardon explores the structure-function relationships of apoproteins (apoE, apoA-I) and serum amyloid A (SAA) with their ability to regulate cholesterol homeostasis within macrophages. Mimetic peptides derived from the three apoproteins are proposed as therapeutic agents. ApoE- as well as SAA-mimetic peptides were shown, *e.g.,* to reduce atherosclerosis in apoE$^{-/-}$ mice. ApoA-1-mimetic is probably the most promising compound, since its antiinflammatory potential has already been shown in other inflammatory disorders such as respiratory and intestinal diseases and chronic arthritis.

Vascular smooth muscle cells (VSMCs) are the subject of the review by Ramel et al. Plasticity of VSMCs during the progression of atherosclerosis, as well as their complex interactions with endothelium and monocytes/macrophages are comprehensively overviewed. A general but detailed picture is thus provided of what the authors call *"a bad dialogue"* taking place between VSMCs and immune cells, capable of modulating plaque stability vs. progression and rupture. Against this background, experimental studies investigating a series of potential molecular targets for therapeutic intervention—IL-1beta, histone H4, chemokine CXCL10, etc.—are reviewed. The paper by Pastore et al. focuses instead on the myeloid-epithelial-reproductive tyrosine kinase (MerTK), a factor involved in shaping macrophages differentiation towards a M2, "reparative" phenotype. The role of MerTK in nonalcoholic fatty liver disease (NAFLD)-associated cardiovascular diseases is highlighted, together with its possible use as an innovative target. Interestingly, both a selective PPAR-γ antagonist and a synthetic agonist for liver "X" receptors (LXRs) can upregulate MerTK expression. The authors overview the small-molecule MerTK inhibitors and monoclonal antibodies currently under evaluation.

The importance of miRNAs in the development and progression of atherosclerosis is receiving increasing attention. The mini-review by Bruen et al. deals with the inhibition of macrophage-specific micro-RNA miR-155 as a viable therapeutic strategy to decrease inflammation. Indeed, conjugated linoleic acid (CLA) as well as PPAR-γ agonists were shown to regulate candidate miRNAs and promote a proresolving atherosclerotic plaque microenvironment. At present however no miRNA-based anti-atherosclerotic therapies have yet entered clinical trials, since the specific delivery of miRNAs to the desired sites of action—which is mandatory in order to prevent off-target effects—is still not feasible.

Last but not least, the contribution by van der Vorst et al. is an appraisal of the G-protein coupled receptors (GPCRs) in the inflammatory process. A selection of GPCRs mainly expressed on myeloid cells are discussed as potential players in progression of atherosclerosis. In particular, GPCRs working as receptors for chemokines and formyl-peptide, chemerin receptor 23, as well as the calcium-sensing receptor are taken into account as potential targets for treatment of cardiovascular diseases.

In conclusion, nobody can tell how long we will have to wait before adequate anti-atherosclerotic treatments become available in the clinic. The complexity of the matter has even led someone to question whether atherosclerosis truly represents a single pathological condition, or rather the term actually comprises many disease "subtypes" (Khera and Kathiresan, 2017). Difficult obstacles in the way of research remain to be overcome. As shown however by the recent literature—including the present Research Topic—the investigative efforts in search of possible leads to therapy are gradually yielding some first translational, intriguing results.

## AUTHOR CONTRIBUTIONS

AC, CG, and AP discussed the contents of the paper. AP drafted the manuscript.

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REVIEW
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# Macrophage Death as a Pharmacological Target in Atherosclerosis

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Atherosclerosis is a chronic inflammatory disorder characterized by the gradual build-up of plaques within the vessel wall of middle-sized and large arteries. Over the past decades, treatment of atherosclerosis mainly focused on lowering lipid levels, which can be accomplished by the use of statins. However, some patients do not respond sufficiently to statin therapy and therefore still have a residual cardiovascular risk. This issue highlights the need for novel therapeutic strategies. As macrophages are implicated in all stages of atherosclerotic lesion development, they represent an important alternative drug target. A variety of anti-inflammatory strategies have recently emerged to treat or prevent atherosclerosis. Here, we review the canonical mechanisms of macrophage death and their impact on atherogenesis and plaque stability. Macrophage death is a prominent feature of advanced plaques and is a major contributor to necrotic core formation and plaque destabilization. Mechanisms of macrophage death in atherosclerosis include apoptosis, passive or accidental necrosis as well as secondary necrosis, a type of death that typically occurs when apoptotic cells are insufficiently cleared by neighboring cells via a phagocytic process termed efferocytosis. In addition, less-well characterized types of regulated necrosis in macrophages such as necroptosis, pyroptosis, ferroptosis, and parthanatos may occur in advanced plaques and are also discussed. Autophagy in plaque macrophages is an important survival pathway that protects against cell death, yet massive stimulation of autophagy promotes another type of death, usually referred to as autosis. Multiple lines of evidence indicate that a better insight into the different mechanisms of macrophage death, and how they mutually interact, will provide novel pharmacological strategies to resolve atherosclerosis and stabilize vulnerable, rupture-prone plaques.

**Keywords: cell death, macrophages, atherosclerosis, apoptosis, necroptosis, pyroptosis, ferroptosis, autophagy**

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## INTRODUCTION

Atherosclerosis is a progressive inflammatory disease of large- and medium-sized arteries that may start as early as childhood (Lusis, 2000). Due to endothelial dysfunction, low density lipoprotein (LDL) particles are able to accumulate in the intima of the vessel wall where they are prone to a wide range of chemical alterations. Modification of LDL into oxidized (ox) LDL activates endothelial cells (ECs) to enhance the expression of various cell adhesion molecules such as cell adhesion molecule 1

(VCAM-1) and intercellular adhesion molecule 1 (ICAM-1), thereby mediating infiltration of inflammatory cells such as monocytes and lymphocytes. Together with the proliferation of vascular smooth muscle cells (VSMCs), lipid deposition and matrix accumulation, vascular inflammation (especially monocytes) drives the build-up of an atherosclerotic plaque (Moore and Tabas, 2011). Indeed, once trapped in the intima, monocytes undergo differentiation into macrophages and turn into foam cells by the uptake of modified LDL. Formation of macrophage foam cells in the intima is a major hallmark of early stage atherosclerotic lesions (Chistiakov et al., 2017). Interestingly, plaque macrophages are very heterogeneous, displaying a variety of subtypes depending on their protein expression patterns and activation stimuli (Wilson, 2010). As a consequence, they exert either beneficial or harmful functions in atherosclerosis. On the one hand, macrophages are capable of scavenging cytotoxic lipoproteins and other harmful substances in the plaque such as dead cells, especially in the early stages of atherogenesis, to avoid cytotoxicity within the developing lesion (Schrijvers et al., 2007). Efficient clearance of dead cells is essential for preventing secondary necrosis and also triggers an anti-inflammatory response through the release of anti-inflammatory cytokines. Moreover, macrophages can enhance tissue repair by promoting extracellular matrix synthesis and VSMC proliferation, which in turn enhances plaque stability. On the other hand, advanced plaques contain large numbers of macrophages with a pro-inflammatory phenotype that secrete matrix degrading enzymes, thereby contributing to plaque destabilization, plaque rupture and thrombotic events. Macrophages in advanced plaques also contribute to death of surrounding cells either by releasing toxic oxygen and nitrogen radicals or via Fas-Fas ligand interactions (Martinet and Kockx, 2001).

The global aim in the treatment of atherosclerosis is the prevention of cardiovascular complications (Libby, 2013). Lifestyle changes such as dietary lipid lowering, regular physical activity, smoke cessation, and weight control are necessary measures in the prevention of atherosclerosis. However, if lifestyle changes are not sufficient to modify risk factors of cardiovascular disease, treatment with lipid lowering drugs (e.g., statins, fibrates or PCSK9 inhibitors) is recommended. Given their high level of plasticity, macrophages represent an attractive alternative target for the development of anti-atherosclerosis therapies (Saha et al., 2009). Distinct anti-inflammatory strategies to treat or prevent atherosclerosis have recently emerged (Back and Hansson, 2015). Because cell death is a prominent feature of advanced atherosclerotic plaques with a major impact on atherogenesis and plaque destabilization (Kockx and Herman, 2000), this review will focus on the pharmacological modulation of macrophage death in atherosclerosis. As outlined in more detail below, plaque macrophages may undergo diverse types of death, ranging from standard mechanisms of death (apoptosis, necrosis) to less-well characterized mechanisms including necroptosis, pyroptosis, ferroptosis, parthanatos and autosis. Growing evidence indicates that pharmacological targeting of these types of death is a promising approach to stabilize vulnerable plaques and may contribute to the beneficial effects of currently applied plaque-stabilizing therapies (Martinet et al., 2011b, 2013; Karunakaran et al., 2016; Sergin et al., 2017).

## TARGETING MACROPHAGE APOPTOSIS

Macrophage apoptosis is an important feature of atherosclerotic plaque development and can be initiated by multiple factors such as oxidant stress, high concentrations of cytokines (e.g., TNFα), activation of the Fas death pathway by Fas ligand and endoplasmic reticulum (ER) stress (Seimon and Tabas, 2009) (**Figure 1**). In the past two decades, research directed at understanding the functional consequences of macrophage apoptosis in atherosclerosis revealed that the effect of macrophage death largely depends on the stage of the atherosclerotic plaque. Apoptosis of macrophages in early stages of the disease is considered beneficial as it limits lesion cellularity and suppresses plaque progression (Seimon and Tabas, 2009). Indeed, several experimental studies in mice suggest an inverse relationship between macrophage apoptosis and early lesion area. Reconstitution of ApoE*3-Leiden mice with $p53^{-/-}$ bone marrow, for example, resulted in reduced macrophage apoptosis, while macrophage content and lesion area increased significantly (van Vlijmen et al., 2001). Another study demonstrated reduced macrophage apoptosis and increased lesion size in $LDLR^{-/-}$ mice reconstituted with bone marrow-derived hematopoetic cells lacking the pro-apoptotic protein Bax (Liu et al., 2005). Moreover, deletion of a macrophage survival protein, known as AIM (apoptosis inhibitor of macrophage) or IKKα, a protein directly associated with two major prosurvival pathways (PI3K/Akt and NF-κB), renders macrophages highly susceptible to oxLDL-induced cell death and reduces early atherosclerosis in $LDLR^{-/-}$ mice (Arai et al., 2005; Babaev et al., 2016). Overall, these findings clearly illustrate that lesional macrophage apoptosis is necessary, at least initially, to reduce the pool of macrophages within the expanding plaques (Rayner, 2017). The results also imply that phagocytic clearance of apoptotic macrophages, better known as efferocytosis, is efficient in early lesions (**Figure 2A**). However, a large body of evidence indicates that efferocytosis in advanced lesions is impaired (Schrijvers et al., 2005) (**Figure 2B**). Several potential mechanisms of defective efferocytosis in atherosclerosis have been reported, but the most likely explanation is that efferocytosis itself becomes defective and/or that lesional apoptotic cells become poor substrates for efferocytosis (Yurdagul et al., 2018). Indeed, not only the expression and function of efferocytosis receptors (e.g., MerTK) and their bridging molecules (e.g., Gas6) are deficient in advanced plaques, dead cells in lesions also express lower amounts of "eat-me" signals (e.g., calreticulin), which prevents efficient clearance by phagocytic cells. Defective phagocytosis of apoptotic cells has a number of consequences that promotes the progression and complications of atherosclerotic plaques. Apoptotic cells that are not ingested, become secondarily necrotic, which can stimulate atherogenesis through induction of inflammation and enlargement of the necrotic core (Martinet et al., 2011a). Along these lines, it has been reported that

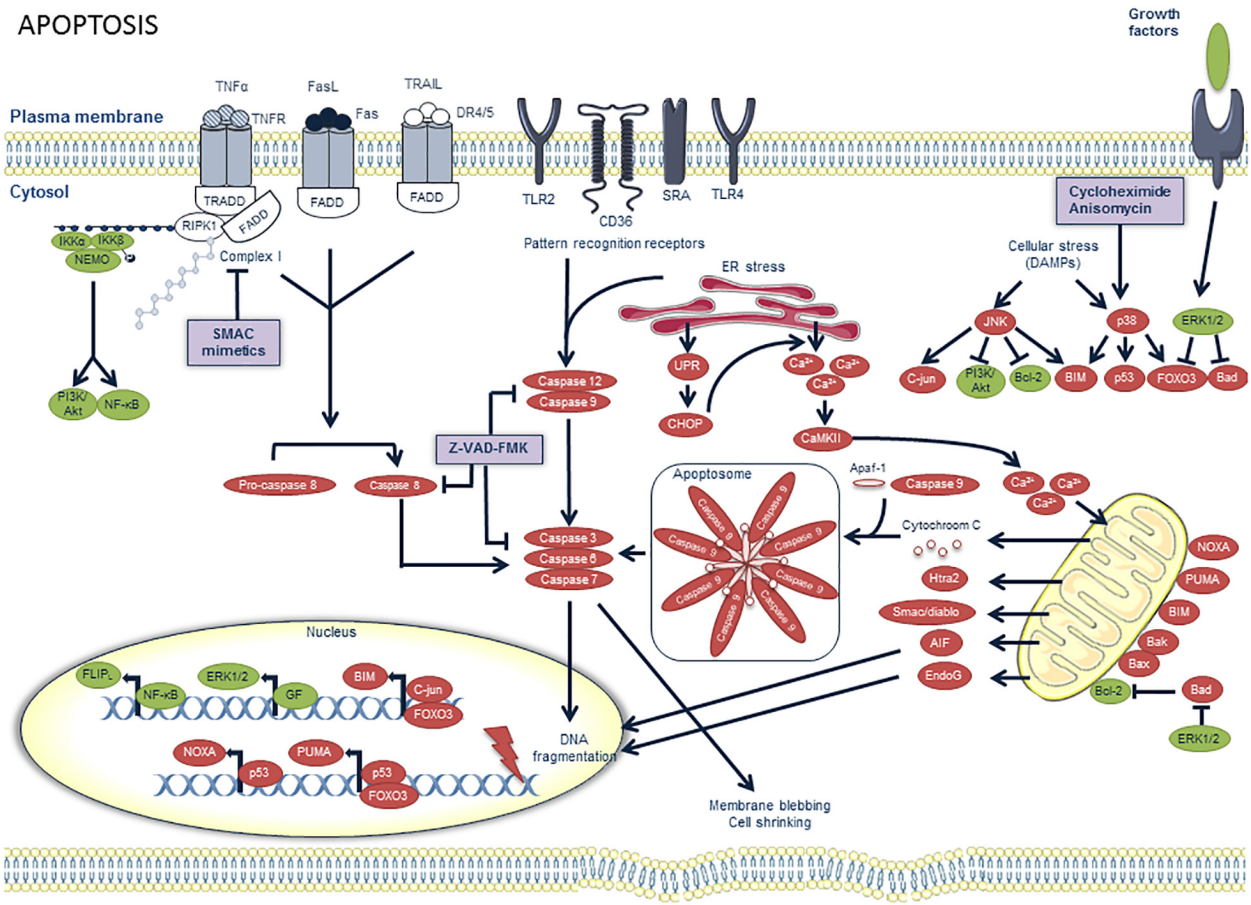


**FIGURE 1 |** Overview of the apoptosis pathway and potential targets for pharmacological modulation. Pro-apoptotic factors are illustrated in red and prosurvival factors in green. Apoptosis can be initiated by multiple factors. Extrinsic pro-apoptotic pathways are activated when death ligands such as Fas ligand (FasL), TNFα and TNF-related apoptosis-inducing ligand (TRAIL) bind on death receptors (DR) such as Fas, TNFR1 and DR4/5, respectively. These receptors contain a death domain (DD) on the cytoplasmic side of the cell membrane, which can interact with other DD-containing proteins. The TNFα pathway is illustrated in more detail in **Figure 3**. Upon binding of FasL to Fas or TRAIL to DR4/5, Fas-associated death domain (FADD) is recruited. Consequently, FADD recruits pro-caspase 8 and converts it to active caspase 8. When caspase 8 is inhibited, for example by nitrosylation (due to high levels of NO production), or by peroxidation resulting from oxidative stress inside the plaque, a switch from apoptosis to necroptosis is observed, as further depicted in **Figure 3**. Other pro-apoptotic pathways include the activation of pattern recognition receptors (PRRs) by pathogen associated molecular patterns (PAMPs) and ER stress, both generating active caspases 9 and 12. Prolonged ER stress can activate the unfolded protein response (UPR), of which CCAAT-enhancer-binding protein homologous protein (CHOP) has been demonstrated to be a crucial pro-apoptotic effector in macrophages. CHOP promotes $Ca^{2+}$ release from the ER, which in turn promotes activation of $Ca^{2+}$/calmodulin-dependent protein kinase II (CamKII). The latter can induce $Ca^{2+}$ uptake by the mitochondria which alters their membrane potential, thereby triggering the release of pro-apoptotic proteins, such as cytochrome C. Cytochrome C forms, together with caspase 9 and apaf-1, a complex called the apoptosome which also activates the effector caspases of apoptosis. Intrinsic pro-apoptotic pathways are activated upon exposure to ER stress, along with other cellular stressors or damage associated molecular patterns (DAMPs). They can activate mitogen-activated protein kinase (MAPK) signaling pathways which provide either pro-apoptotic (through JNK or p38 activation) or prosurvival signals (through ERK1/2 activation). The protein synthesis inhibitors anisomycin and cycloheximide are known to induce macrophage apoptosis through the p38 MAPK pathway. The pro-apoptotic pathways eventually lead to the activation of effector caspases 3, 6, and 7 which induce DNA fragmentation, membrane blebbing and cell shrinkage, all key features of apoptosis. Apoptosis can be pharmacologically inhibited by the pan-caspase inhibitor z-vad-fmk.

apoptotic macrophages localize to sites of plaque rupture (Kolodgie et al., 2000), suggesting that macrophage death itself can promote plaque rupture. Non-cleared apoptotic cells are also an important source of tissue factor, which increases plaque thrombogenicity (Mallat et al., 1999). Accordingly, the impact of macrophage apoptosis in advanced plaques is far more complex as compared to early lesions.

Experimental evidence has demonstrated that inducing or enhancing the capacity of efferocytosis is a promising therapeutic path to promote the resolution of inflammation and to prevent formation of vulnerable plaques that are prone to rupture. One type of approach that may successfully target defective efferocytosis is the use of antibodies that block the anti-phagocytic 'don't eat me signal' CD47. TNFα promotes CD47 overexpression in atherosclerotic plaques via NF-κB and renders vascular cells resistant to phagocytic clearance (Kojima et al., 2016). Atherosclerotic mice treated with anti-CD47 antibodies display less apoptotic debris and develop significantly smaller

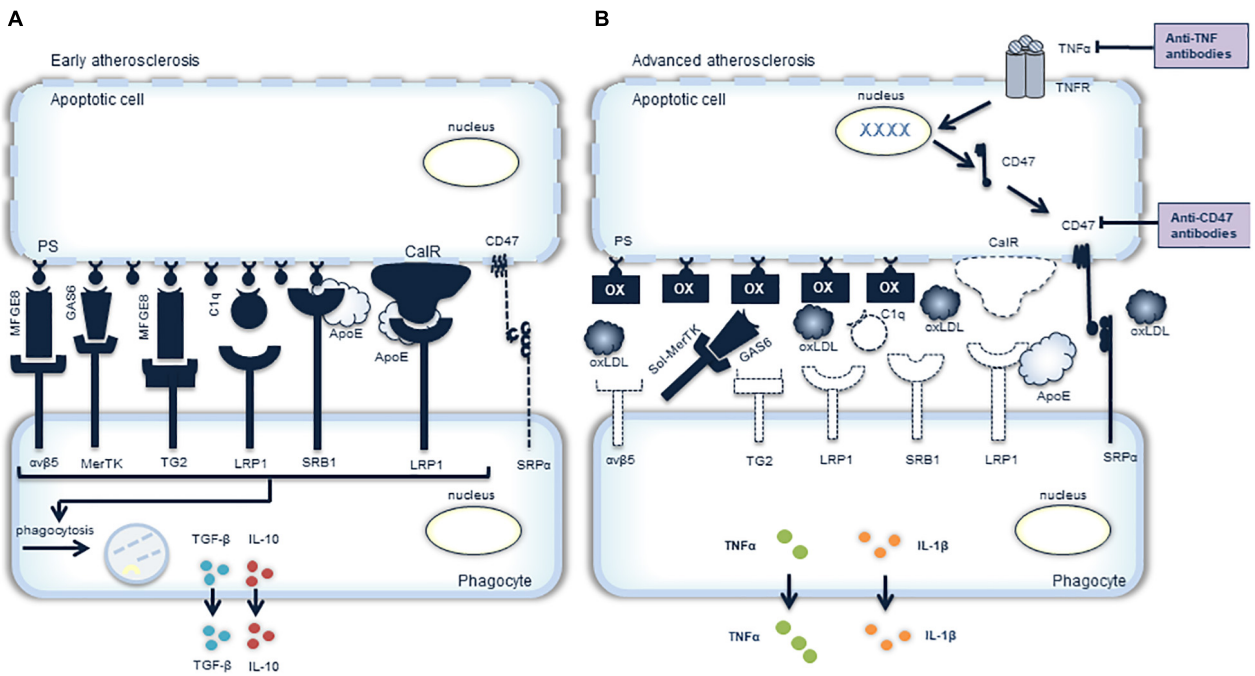


**FIGURE 2 |** Overview of efferocytosis and potential targets for pharmacological stimulation. The clearance of apoptotic cells, a process called efferocytosis, is very important and closely regulated. Phagocytes responsible for efferocytosis have to discriminate viable, healthy cells from dying cells. This is mediated by the expression of "don't eat me" molecules (e.g., CD47) on the surface of viable cells that interact with SIRPα (signal-regulatory protein α) on phagocytes and thereby prevent engulfment of the viable cell **(A)**. Dying cells on the other hand, express "eat me" molecules on their surface, such as calreticuline (calR) and phosphatidylserine (PS). These molecules interact with engulfment receptors on phagocytes (e.g., integrin αvβ5, Mer receptor tyrosine kinase (MerTK), transglutaminase 2 (TG2), low density lipoprotein receptor-related protein 1 (LRP1) and scavenger receptor B (SRB). The interaction between "eat me" molecules and engulfment receptors is mediated by bridging molecules [e.g., milk fat globule-EGF factor (MFGE), growth arrest-specific 6 (Gas6), and complement C1q]. In advanced atherosclerotic plaques, efferocytosis is impaired. This may be attributed to the altered phagocytic capacity of macrophages, VSMCs and dendritic cells. Moreover, dying cells in advanced plaques become poor substrates due to the decreased expression of "eat me" molecules and bridging molecules, as well as TNFα-induced expression of the "don't eat me" molecule CD47 **(B)**. The latter can be inhibited by targeting either TNFα or CD47 with anti-TNFα or anti-CD47 antibodies, respectively. Another factor playing a role in impaired efferocytosis is the abundance of oxLDL in the plaque, which masks the "eat me" molecules and competes with the dying cells for macrophage engulfment. Competitive inhibition is also seen upon cleavage of the receptor MerTK, rendering an inactive, soluble form (sol-MerTK) that competes with the bridging molecules such as Gas6.

atherosclerotic plaques as compared to mice treated with control antibodies (Kojima et al., 2016). Of note, concomitant inhibition of CD47 and TNFα using anti-CD47 antibody therapy and commercially available anti-TNFα antibodies such as infliximab or etanercept offers a synergistic benefit in the clearance of apoptotic cells (Kojima et al., 2016). Given that the risk of future cardiovascular events can be reduced in patients with rheumatoid arthritis undergoing anti-TNFα therapy, there is a strong rationale for combining anti-inflammatory and pro-efferocytic therapies for the treatment of advanced atherosclerosis (Kojima et al., 2017). However, anti-CD47 antibodies also promote erythrophagocytosis so that dose optimization and changes in treatment regimen may be required to avoid anemia. Another type of approach is to enhance the expression of efferocytosis-related genes. Glucocorticoids, the most widely used anti-inflammatory drugs, are known to enhance both short-term and continued efferocytosis, at least *in vitro*. Short-term phagocytosis of apoptotic cells is enhanced mainly by upregulation of the MerTK receptor and bridging molecule C1q expression levels, while continued phagocytosis is promoted via the induction of lipid sensing nuclear receptors (e.g., LXR, PPARδ) and uncoupling protein 2 (UCP2) (Garabuczi et al., 2015). However, glucocorticoids can also enhance efferocytosis by stimulating the release of annexin A1 (Maderna et al., 2005; Scannell et al., 2007), which is highly expressed in resting macrophages. Once secreted, annexin A1 binds to phosphatidylserine (PS) at the surface of apoptotic cells, and thereby serves as a bridging molecule between PS and the annexin receptor on the macrophage surface. Recently, de Jong et al. (2017) demonstrated that annexin A1 levels in the vessel wall and circulating plasma negatively correlate with neointima size, which confirms its reparative role by enhancing efferocytosis. However, the long-term use of corticosteroids is associated with many undesirable and potentially lethal side effects. It may lead to vascular injury and can stimulate experimental atherosclerosis so that prolonged therapy with this type of medication should be avoided whenever possible. Yet another exciting approach to clear apoptotic cells in advanced plaques

would be to increase the production of long-chain fatty acid-derived lipid mediators, called specialized proresolving mediators (SPMs), including resolvin D1 (RvD1), RvD2, RvE1, and RvE2. Resolvins mediate resolution by blocking the production of pro-inflammatory mediators (i.e., chemokines and cytokines) and leukocyte trafficking to sites of inflammation (Fredman and Serhan, 2011), albeit they also promote efferocytosis through p50/p50-homodimer-mediated repression of TNFα production (Lee et al., 2013). Interestingly, levels of SPMs (in particular RvD1) in vulnerable regions of atherosclerotic plaques are significantly decreased (Fredman et al., 2016), possibly through cleavage of MerTK, as mice with cleavage-resistant MerTK have increased circulating levels of SPMs (Cai et al., 2016). Administration of RvD1 to $LDLR^{-/-}$ mice during plaque progression restores RvD1 levels to that of less advanced lesions and improves plaque stability by enhancing lesional efferocytosis (Fredman et al., 2016). Also delivery of other SPMs (e.g., RvD2, Maresin-1, RvE1) favors a proresolving milieu and prevents atheroprogression in mice (Hasturk et al., 2015; Viola et al., 2016). Finally, it should be mentioned that pharmacological inhibition of macrophage apoptosis obviously may help to inhibit the accumulation of non-phagocytized apoptotic debris and plaque development. A recent example is the study of Tian et al. (2017) showing that the apoA-I mimetic peptide D4F protects macrophages from oxLDL-induced apoptosis by suppressing the activation of the Fas/FasL pathway, which reduces plaque formation in atherosclerotic $ApoE^{-/-}$ mice.

Based on the abovementioned findings, we may conclude that induction of macrophage apoptosis in plaques with impaired efferocytosis represents a certain risk. However, macrophages that accumulate in the plaque actively promote degradation of the extracellular matrix and contribute to death of VSMCs. From this perspective, selective removal of macrophages via macrophage-specific initiation of cell death may also have plaque-stabilizing effects. To further clarify this issue, Gautier et al. (2009) developed CD11c-diptheria toxin (DT) receptor (DTR) transgenic mice that show macrophage apoptosis after administration of DT. Sustained macrophage apoptosis was associated with increased plaque inflammation and accelerated plaque progression. These results are different from those of Stoneman et al. (2007), who reported that acute induction of macrophage apoptosis in a similar animal model (CD11b-DTR mice) had no effect on plaque extent or composition. The difference in the final outcome of both studies could be related to the use of two different mouse models (CD11b-DTR versus CD11c-DTR). Because monocytes are $CD11b^{high}$ cells, there is a possibility that they are (more) extensively depleted in CD11b-DTR mice (50% reduction in circulating monocytes) as compared to CD11c-DTR mice so that their recruitment to the lesion and the impact on vascular inflammation and plaque development is significantly impaired. On the other hand, DT-treated CD11c-DTR mice revealed an elevation in plasma cholesterol, which may have contributed to an increase in lesion size (Gautier et al., 2009). In addition to the use of genetically modified mice, our laboratory developed different pharmacological strategies to selectively deplete macrophages in atherosclerotic plaques through apoptosis induction. Plaque macrophages are metabolically highly active, thus also more sensitive to protein synthesis inhibitors as compared to other cell types in the vessel wall including VSMCs and endothelial cells. As a consequence, local administration of the protein synthesis inhibitor cycloheximide to rabbit plaques depletes the macrophage content via p38 MAPK-mediated apoptosis induction without altering VSMC viability or other obvious detrimental effects (Croons et al., 2007). The protein synthesis inhibitor anisomycin provides similar results, with a mechanism of action analogous to cycloheximide (Croons et al., 2009). Other strategies that allow the selective removal of plaque macrophages via apoptosis include the induction of ER stress by exogenous NO donors (De Meyer et al., 2003; Martinet et al., 2007a), or the inhibition of inositol monophosphatase by lithium chloride (De Meyer et al., 2011). Although these strategies are successful in a preclinical setting and look promising, there are several drawbacks that may hamper clinical applications (Croons et al., 2010; Martinet et al., 2011b). First, depletion of peripheral blood monocytes should be avoided, because they play a critical role in both innate and adaptive immunity. Local delivery of macrophage depleting drugs (e.g., with drug-eluting stents or targeted nanoparticles) can decrease or avoid unwanted systemic effects. Second, additional pro-efferocytic therapy will be required to prevent accumulation of free (non-engulfed) apoptotic cells. Because the phagocytic capacity of plaques will be compromised by depleting macrophages, this prerequisite may be hard to accomplish. In addition, combined treatment (for example with lipid lowering drugs such as statins) may be needed to counteract re-infiltration of circulating monocytes after macrophage depletion. Finally, it would be recommendable to conceive therapeutic strategies that selectively promote apoptosis in early lesional macrophages and/or that selectively prevent cell death in advanced lesions, which is again not self-evident.

# TARGETING MACROPHAGE NECROSIS

## Passive Necrosis

Necrotic cell death is characterized by an increased cell volume (oncosis), organelle swelling and chromatin condensation, which eventually culminates in plasma membrane rupture and the release of intracellular compounds (Majno and Joris, 1995). Macrophage necrosis is a key feature in the pathogenesis of atherosclerosis (Crisby et al., 1997; Tabas, 2005), and triggers the formation and enlargement of a central necrotic core, which plays a pivotal role in unstable atherosclerotic plaques (Tabas, 2005). Indeed, 80% of necrotic cores in advanced human atherosclerotic plaques are larger than 1 $mm^2$, which compromises > 10% of the lesion area (Virmani et al., 2007). However, in 65% of plaque ruptures, the necrotic core occupies > 25% of the plaque area (Virmani et al., 2007). Due to a lack of reliable methods to detect and quantify necrosis in tissue, plaque necrosis has not been extensively studied. Plaque necrosis can be triggered by

multiple factors including high levels of oxidative stress, an overload of intracellular $Ca^{2+}$ and cellular ATP depletion. Moreover, as mentioned above, impaired efferocytosis in advanced plaques causes accumulation of apoptotic bodies, which undergo secondary necrosis (Martinet et al., 2011a). Necrotic macrophages do not only contribute to the formation and enlargement of the necrotic core, they are also a source of pro-inflammatory cytokines and damage associated molecular patterns (DAMPs) (Seimon and Tabas, 2009). The release of DAMPs promotes inflammation in the plaque, thereby causing plaque instability. High mobility group box 1 protein (HMGB1) is one of the best studied DAMPs in atherosclerosis. Macrophages in atherosclerotic plaques are a major source of HMGB1 production (Kalinina et al., 2004). Once released in the extracellular space, HMGB1 interacts with different receptors including receptor for advanced glycation end-products (RAGE). Binding of HMGB1 triggers the transcription of pro-inflammatory cytokines in a NF-κB dependent manner, thereby promoting further plaque development (de Souza et al., 2012). Experimental evidence has shown that HMGB1 expression increases during atherogenesis (Kalinina et al., 2004). Consistently, a more recent study has demonstrated that HMGB1 is highly expressed in mouse atherosclerotic plaques. Neutralization of HMGB1 in $ApoE^{-/-}$ mice reduces plaque area through inhibition of immune cell accumulation and macrophage migration (Kanellakis et al., 2011). Interestingly, mounting experimental evidence suggests that statins attenuate plaque formation partly by reducing HMGB1 and RAGE expression (Cuccurullo et al., 2006; Calkin et al., 2008; Yin et al., 2010). Moreover, administration of simvastatin to $ApoE^{-/-}$ mice reduces vascular inflammation via downregulation of the HMGB1-RAGE axis (Liu et al., 2013). Besides inhibiting DAMPs, directly targeting necrotic cell death by using antioxidant therapy could be an alternative strategy for plaque stabilization. Several epidemiological, clinical, and experimental studies have been conducted to evaluate possible beneficial effects of antioxidant vitamins such as vitamins C and E on atherogenesis. However, there is no convincing evidence that anti-oxidant therapy attenuates atherosclerotic plaque progression (Cherubini et al., 2005). Targeting specifically mitochondrial ROS by administration of NecroX-7, which acts as a scavenger of mitochondrial ROS and peroxynitrite, could be an alternative approach to inhibit necrotic cell death (Kim et al., 2010). Recently, our laboratory demonstrated that NecroX-7 treatment reduces the necrotic area without affecting plaque size in $ApoE^{-/-}$ mice (Grootaert et al., 2016). Moreover, NecroX-7 improves important features of plaque stability such as lowering plaque inflammation, reducing oxidative stress and increasing collagen content and fibrous cap thickness (Grootaert et al., 2016). Currently, a phase 2 clinical study is evaluating the efficacy, safety and pharmacokinetics of a single intravenous injection of NecroX-7 before percutaneous coronary intervention in patients with ST-segment elevated myocardial infarction (STEMI) ([1]identifier NCT02770664). As atherosclerosis is a chronic disease, the evaluation of chronic administration of NecroX-7 should be included in future clinical trials.

## Necroptosis

For many years, necrosis has been considered as an uncontrolled way for a cell to die. However, research in the field of cell death drastically changed by the discovery of small molecules, termed necrostatins, which inhibit receptor-interacting protein kinase (RIPK)1 to induce necroptosis in TNFα-treated cells (Degterev et al., 2005, 2008) (**Figure 3**). This discovery led to the characterization of downstream necroptosis mediators, namely RIPK3 and mixed lineage kinase domain-like protein (MLKL) (Cho et al., 2009; He et al., 2009; Zhang et al., 2009). In addition to TNFα, other necroptosis triggers have been identified including TNF-related apoptosis inducing ligand (TRAIL), first apoptotic signal ligand (FasL), interferons, toll-like receptor ligands, and virus-activated pathways (Vanden Berghe et al., 2014). Nonetheless, TNFα-induced necroptosis is currently the best-characterized necroptosis pathway. The response of cells to TNFα is complex. In most cases, binding of TNFα leads to ubiquitination of RIPK1 by cellular inhibitors of apoptosis (cIAP1/2) and linear ubiquitin chain assembly complexes (LUBAC), followed by the activation of cell survival pathways including NF-κB and MAPK pathway. However, de-ubiquitination of RIPK1 promotes cell death signaling pathways, such as caspase-dependent apoptosis and RIPK3-MLKL-mediated necroptosis (Dondelinger et al., 2016). Necroptosis and apoptosis pathways seem to compete with each other. When caspase 8 is inhibited by pharmacological or physiological stimuli, the necroptosis proteins RIPK1 and RIPK3 will be phosphorylated (Declercq et al., 2009). Subsequently, RIPK3 induces phosphorylation of MLKL, which triggers MLKL oligomerization. Finally, MLKL oligomers associate with the plasma membrane, which causes plasma membrane disruption and the release of DAMPs (Dondelinger et al., 2014; Wang et al., 2014). Recently, it has been demonstrated that gene expression of the necroptosis mediators RIPK3 and MLKL is elevated in human atherosclerotic plaques. Moreover, RIPK3 and MLKL mRNA are specifically upregulated in subjects with unstable compared to stable atherosclerotic plaques (Karunakaran et al., 2016). This finding is supported by another study demonstrating that the protein levels of RIPK1 and RIPK3 are increased in advanced human atherosclerotic lesions (Tian et al., 2016). In addition, RIPK3 expression is elevated in advanced plaques of $LDLR^{-/-}$ mice. Interestingly, RIPK3 gene expression in macrophages increases during the development of atherosclerosis, which suggests that macrophage necroptosis plays a role in advanced plaques. Indeed, RIPK3 deficiency in bone-marrow-derived cells reduces advanced atherosclerotic lesions in $ApoE^{-/-}$ and $LDLR^{-/-}$ mice (Lin et al., 2013). Overall, these findings clearly illustrate a role for RIPK3-mediated macrophage necroptosis in atherosclerosis. Besides these genetic studies, Karunakaran et al. (2016) demonstrated that pharmacological inhibition of RIPK1 by Nec-1s reduces plaque size and promotes plaque stability in $ApoE^{-/-}$ mice with established atherosclerotic lesions. Additional *in vitro* experiments have unraveled the underlying mechanism by which necroptosis is induced in

[1]https://clinicaltrials.gov

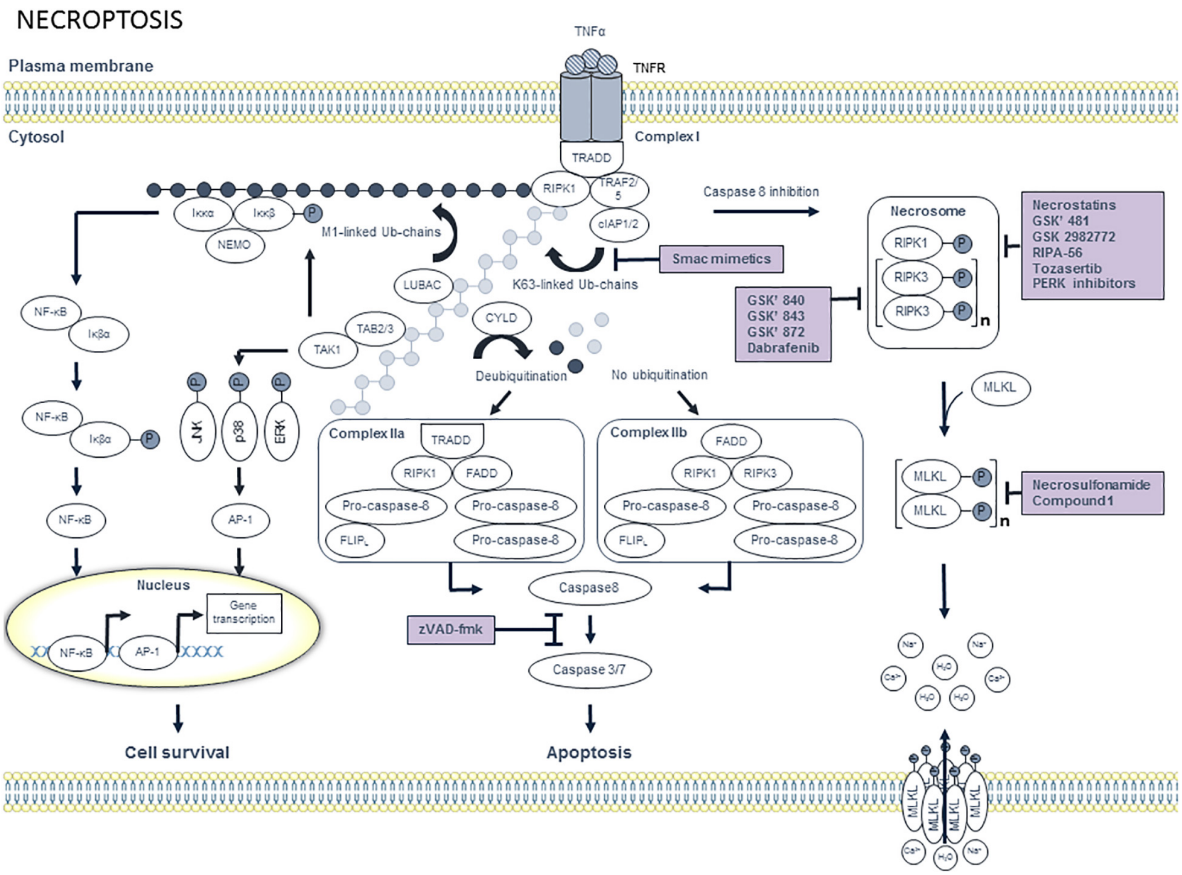


**FIGURE 3 |** Overview of the necroptosis pathway and potential targets for pharmacological inhibition. Binding of tumor necrosis factor α (TNFα) to trimeric tumor necrosis factor receptor 1 (TNFR1) leads to the recruitment of tumor TNFR1-associated death domain (TRADD) and receptor interacting protein kinase 1 (RIPK1). Subsequently, TNFR-associated factor 2 (TRAF2), TRAF5, cellular inhibitor of apoptosis protein 1 (cIAP1) and cIAP2 are recruited to TRADD, thereby forming complex I. CIAP1/2 then ubiquitinates RIPK1 with K63-linked ubiquitin chains, which allows the recruitment of linear ubiquitin chain assembly complexes (LUBAC). Subsequently, M1-linked ubiquitin chains are generated by LUBAC and added to RIPK1. Both M1- and K63-ubiquitin chains serve as a scaffold for the recruitment of IKKα/IKKβ/NEMO and TAB2/TAB3/TAK1, respectively. Subsequently, TAK1 phosphorylates IKKβ as well as the downstream MAPKs including JNK, p38 and ERK, which activate the transcription factor AP-1. Phosphorylated IKKβ activates IκBα, which results in the release of nuclear factor κB (NF-κB). Finally, NF-κB translocates toward the nucleus, where it induces transcriptional upregulation of pro-survival genes. Cell survival can be inhibited by targeting cIAP1/2 with smac mimetics. When RIPK1 is deubiquitinated by cylindromatosis protein (CYLD), RIPK1 dissociates from complex I and engages with FADD, which in turn recruits pro-caspase 8 and FLICE like protein long isoform ($FLIP_L$) heterodimer and pro-caspase 8 homodimer to form complex IIa. Pro-caspase 8 and $FLIP_L$ heterodimer inhibits activation of caspase 8, thereby promoting cell survival. In contrast, pro-caspase 8 homodimer generates active caspase 8, which initiates caspases 3 and 7 to induce apoptotic cell death. When RIPK1 is not ubiquitinated, complex IIb will be formed. Complex IIb consists of RIPK1, RIPK3, pro-caspase 8 and $FLIP_L$ and is also known as the ripoptosome. In contrast to complex IIa, the kinase activity of RIPK1 is crucial to induce apoptosis via complex IIb. Apoptotic cell death can be pharmacologically inhibited by pan-caspase inhibitors such as zVAD-fmk. When RIPK1 deubiquitination is inactivated and caspase 8 is inhibited, RIPK1 will dissociate from complex I. In the cytosol, RIPK1 binds to RIPK3 and subsequently a series of auto- and trans-phosphorylations of RIPK1 and RIPK3 occurs. Phosphorylated RIPK3 then recruits and phosphorylates mixed lineage kinase domain-like protein (MLKL), thereby promoting MLKL oligomerization. Next, MLKL oligomers migrate to the plasma membrane and execute necroptosis by the formation of pores and by the deregulation of sodium and calcium channels. Pharmacological suppression of necroptosis may occur by compounds that inhibit RIPK1, RIPK3, or MLKL.

atherosclerosis. During plaque development, oxidized LDL increases ROS-mediated RIPK3 and MLKL gene expression in macrophages, which leads to necroptosis (Karunakaran et al., 2016). Overall, these data demonstrate that inhibition of macrophage necroptosis could be a promising therapeutic strategy to prevent the development of a vulnerable plaque. During the past decade, several research groups have been focusing on the development of RIPK1 inhibitors. As a result, a phase 2 clinical study is ongoing to evaluate the effect of the RIPK1 inhibitor GSK2982772 on psoriasis, ulcerative colitis and rheumatoid arthritis ([1]identifier NCT 02776033, NCT02903966, and NCT 02858492). However, potential beneficial effects of GSK2982772 on atherosclerosis have not been studied yet.

## Pyroptosis

Macrophages may undergo other types of regulated necrosis, even though their significance in atherosclerosis is not always clear-cut. Among the most well-defined is pyroptosis, a pro-inflammatory form of regulated cell death that is triggered by the activation of caspase-1 (Xu et al., 2018) (**Figure 4**). Activation

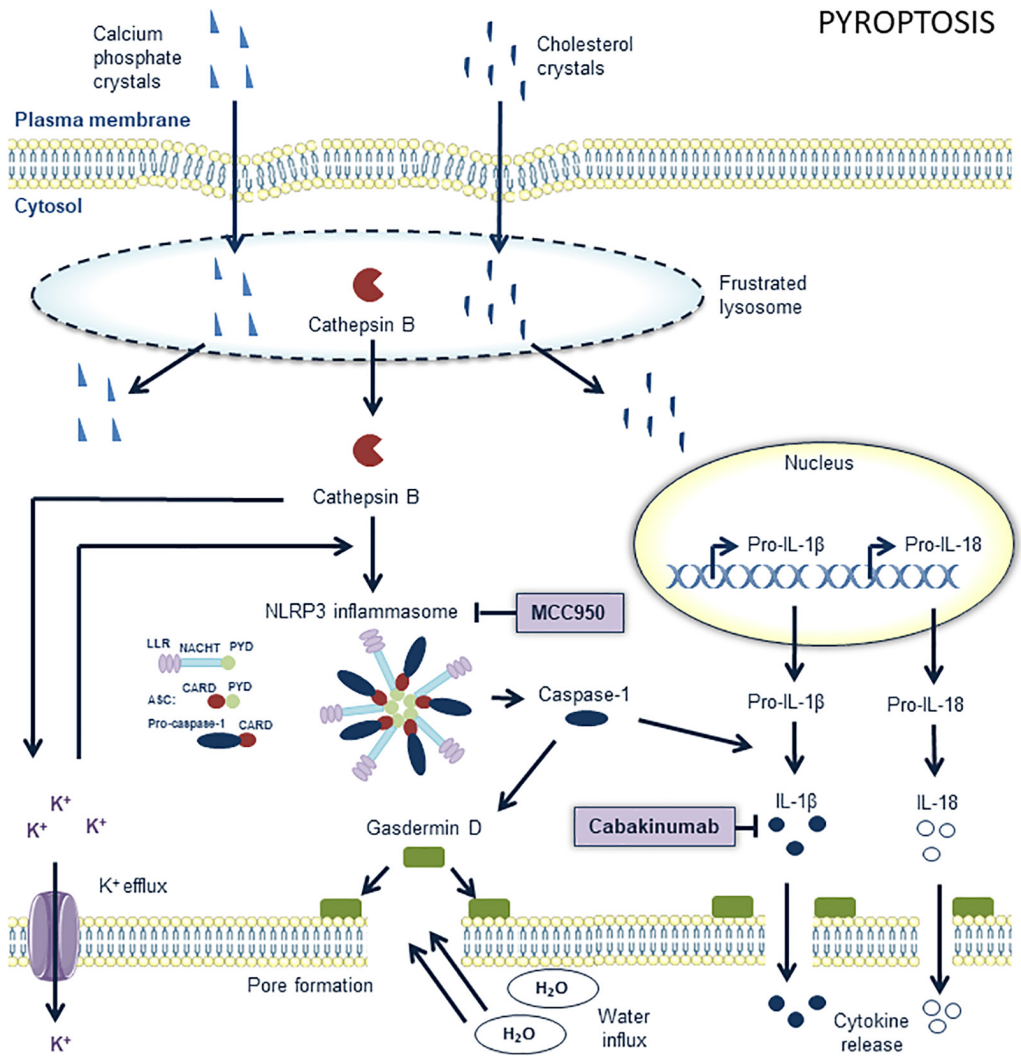


**FIGURE 4 |** Overview of the pyroptosis pathway and potential targets for pharmacological inhibition. The nucleotide-binding oligomerization domain-like receptor (NLR) family, pyrin domain containing 3 (NLRP3) inflammasome is a key component in pyroptosis. It contains procaspase 1 and processes it into active caspase 1, the main effector of pyroptosis. MCC950 is a potent and selective inhibitor of the NLRP3 inflammasome in macrophages in atherosclerotic lesions. The upstream pathways of the NLRP3 inflammasome are not completely elucidated but cholesterol crystals and calcium phosphate crystals are known to play a role. They "frustrate" lysosomes which subsequently lyse and release their content into the cytoplasm. The subsequent release of cathepsin B out of these lysosomes into the cytoplasm promotes potassium efflux. These events promote the assembly of the NLRP3 inflammasome which consists of conserved domains such as C-terminal leucine-rich repeats (LRRs), a central nucleotide domain, called NACHT, and an N-terminal pyrin domain (PYD). Assembly of the inflammasome occurs through the interactions of pyrin domains (PYD) and caspase recruitment domains (CARD). The apoptosis-related speck-like protein containing CARD (ASC) is composed of a PYD and CARD domain and may serve as a link between LRR-NACHT-PYD and CARD bound to procaspase 1. Active caspase 1 cleaves gasdermin D to induce pore formation and processes pro-IL-18 and pro-IL-1β into active interleukins, which are released out of the cells through the formed pores. IL-1β can be pharmacologically targeted with anti-IL-1β antibodies such as canakinumab. Further pore formation by gasdermin D eventually leads to cell swelling and osmotic lysis.

of this inflammatory protease requires a large supramolecular complex, known as an inflammasome. The NLR protein-3 (NLRP3) inflammasome is currently the best characterized inflammasome and consists of NLRP3, ASC, and procaspase-1. Mounting evidence suggests that oxLDL as well as crystals of cholesterol and calcium phosphate in atherosclerotic plaques activate NLRP3 inflammasomes through lysosomal rupture and subsequent cathepsin release (Karasawa and Takahashi, 2017; Grebe et al., 2018), which in turn leads to cleavage and activation of procaspase-1. The activated caspase-1 exerts proinflammatory effects by converting pro-IL-1β and pro-IL-18 into their bioactive form. Activated caspase-1 also leads to processing of gasdermin D and the rapid formation of plasma membrane pores, which in turn allows water influx, cell swelling and osmotic lysis. Interestingly, secretion of IL-1β and IL-18 does not require lysis and is temporally associated with gasdermin D-dependent pore formation, suggesting that these pores are sufficient to mediate cytokine release. Recent studies have shown that components

of the NLRP3 inflammasome in human plaques are mainly expressed in macrophages (Shi et al., 2015), and that pyroptosis in plaque macrophages may promote necrotic core formation and plaque instability in advanced lesions (Xu et al., 2018). Because both NLRP3 and caspase-1 deficiency decreases atherosclerosis in ApoE$^{-/-}$ mice (Gage et al., 2012; Usui et al., 2012; Zheng et al., 2014), therapeutic modulation of NLRP3 or caspase-1 activity is likely to offer significant health benefits for patients with severe atherosclerosis. This assumption is further supported by several epidemiological studies showing that patients with coronary atherosclerosis display high aortic expression of NLRP3, which is directly correlated to disease severity and clinical risk factors for cardiovascular disease (e.g., hypertension, diabetes, smoking, LDL cholesterol) (Zheng et al., 2013). Others reported increased expression of NLRP3, caspase-1, IL-1β, and IL-18 in carotid plaques as compared to non-atherosclerotic arteries with the highest expression levels in unstable lesions (Shi et al., 2015). Besides modulating NLRP3 or caspase-1 activity, direct targeting of IL-1β in atherosclerosis, without affecting cholesterol levels, is an interesting alternative approach that was recently evaluated in the CANTOS trial using canakinumab, a human monoclonal antibody that binds to IL-1β and thereby blocks the interaction of IL-1β with its receptor and subsequent downstream pro-inflammatory signaling events. CANTOS clearly demonstrated that statin-treated patients with residual inflammatory risk, as determined by elevated CRP levels, benefit from additional anti-inflammatory therapy (Ridker et al., 2017), which is in line with previous findings in mice showing that monoclonal antibodies targeting IL-1β inhibit atherosclerotic plaque formation (Bhaskar et al., 2011). However, according to a more recent study, IL-1β neutralization in advanced lesions of ApoE$^{-/-}$ mice induces loss of VSMC and collagen within the fibrous cap, whereas the macrophage content increased (Gomez et al., 2018). This finding indicates that IL-1β has an unexpected atheroprotective role in late-stage atherosclerosis, most likely by stimulating cell proliferation (Libby et al., 1988). Accordingly, it will be critical to determine which patients will benefit most from anti-IL-1β therapy, and more importantly, which patients might develop adverse effects (Gomez et al., 2018).

## Ferroptosis

Ferroptosis is a form of regulated necrosis that is associated with iron-dependent accumulation of lipid hydroperoxides (Dixon et al., 2012; Stockwell et al., 2017) (**Figure 5**). This iron-catalyzed form of necrosis can be induced by erastin (Cao and Dixon, 2016), which inhibits the membrane-bound cystine/glutamate antiporter $X_c^-$. Blockage of this antiporter impairs the cellular uptake of cystine, an essential precursor in the synthesis of the cellular antioxidant glutathione (GSH). This intracellular deficit of GSH triggers the accumulation of reactive oxygen species (ROS), which causes cells to die by excessive oxidation of the membrane lipids (Cao and Dixon, 2016). To the best of our knowledge, ferroptosis has not yet been investigated in atherosclerosis, but is gaining considerable attention. Indeed, given that lipid peroxidation, intraplaque hemorrhages and iron deposition are hallmarks of advanced human plaques, which is indirect evidence for the initiation of ferroptosis, we assume that macrophage ferroptosis has a major role in atherosclerotic plaque destabilization. Glutathione peroxidase 4 (GPX4) can be considered to be one of the most important anti-oxidant enzymes in mammals because of its unique activity to reduce phospholipid hydroperoxides. A study by Seiler et al. (2008) underlined the importance of GPX4 in the prevention of ferroptosis by showing that inducible GPX4 gene inactivation in mice or cultured cells caused significant cell death due to excessive lipid peroxidation, which is a main feature of ferroptosis. Conversely, overexpression of GPX4 removes oxidative lipid modifications and inhibits plaque development in ApoE$^{-/-}$ mice (Guo et al., 2008), thereby confirming the possible role of ferroptosis in cardiovascular disease. Ferrostatin (Fer-1) is the first pharmacological compound that was identified as a selective and potent inhibitor of ferroptosis in *in vitro* experiments (Dixon et al., 2012). It is thought to exert its anti-ferroptosis activity by preventing oxidative damage to membrane lipids. Unfortunately, Fer-1 suffers from inherent stability problems (hydrolysis of ester moiety), which drastically limits the application of these molecules *in vivo*. Recently, Fer-1 analogs with improved potency and ADME properties have been designed (Hofmans et al., 2016). Analogous with other anti-ferroptosis drugs (e.g., liproxstatins, anti-oxidants such as vitamin E), these molecules are able to prevent ferroptotic cell death, though further investigation is needed to determine whether these compounds are able to inhibit atherosclerosis.

## Parthanatos

Another emerging type of regulated leukocyte necrosis in cardiovascular disease is parthanatos (Barany et al., 2017), a form of cell death that is different from the other cell death processes described above (**Figure 6**). Parthanatos is driven by hyperactivation of poly(ADP-ribose) polymerase-1 (PARP-1) and occurs in response to oxidative damage of cellular DNA. By forming poly(ADP-ribose) (PAR) polymers, PARP-1 overactivation depletes the cellular pool of nicotinamide adenine dinucleotide ($NAD^+$) and ATP, yet this does not seem to be the primary cause of cell death (Fatokun et al., 2014). One of the key processes of parthanatos is the binding of poly(ADP-ribose) polymers to apoptosis-inducing factor (AIF), which promotes the release of AIF into the cytosol and its translocation into the nucleus, where it mediates large-scale DNA fragmentation and chromatin condensation. Pharmacological inhibitors of this process can efficiently delay parthanatos in multiple cell types. Among the different therapeutic opportunities in the parthanatos cascade (e.g., inhibition of PARP, PAR, AIF release, or nuclear translocation), only PARP inhibitors are the most advanced in development (Fatokun et al., 2014). Currently, parthanatos is a poorly studied phenomenon in atherosclerosis, though several lines of evidence indicate that parthanatos in plaque macrophages could play a major role in atherogenesis and represents an interesting drug target. First, advanced plaques reveal high levels of oxidative stress and tissue damage through formation of peroxynitrite (Martinet and Kockx, 2001). Once formed, peroxynitrite can oxidize a variety of biomolecules including DNA. Elevated levels of the oxidative DNA damage marker 7,8-dihydro-8-oxoguanine and

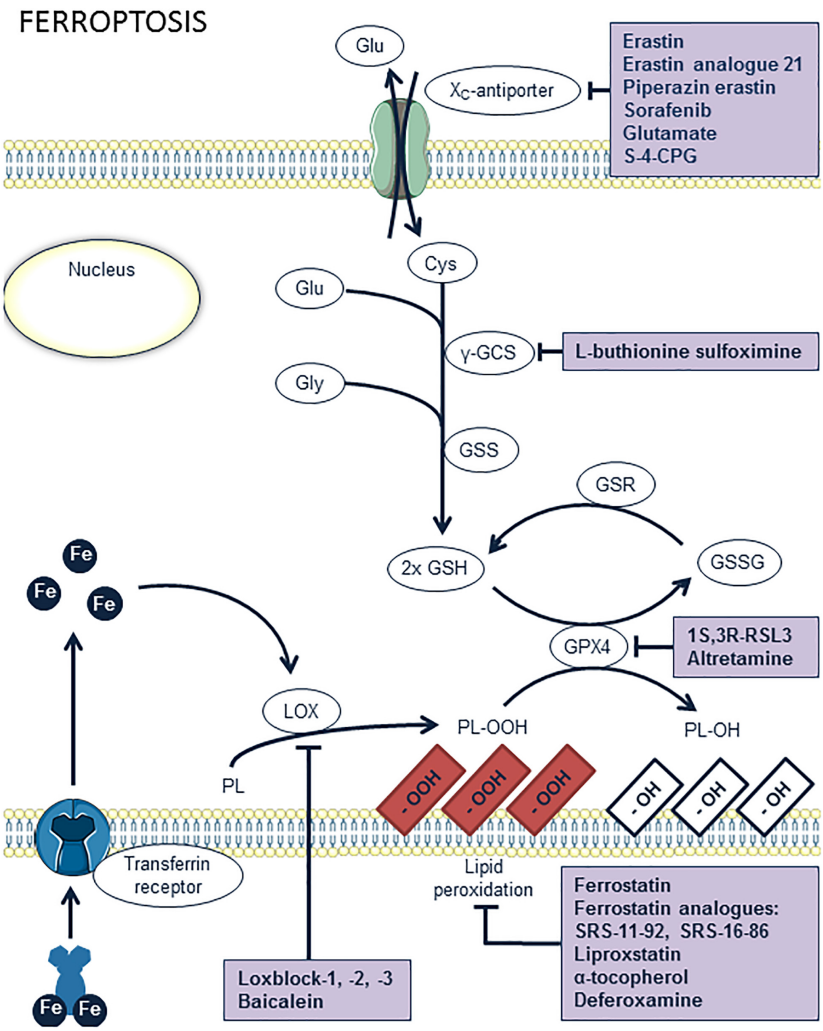


**FIGURE 5 |** Overview of the ferroptosis pathway and potential targets for pharmacological inhibition. Lipoxygenases (LOX) and iron catalyze the peroxidation of phospholipids (PL). Peroxidized lipids are reduced by glutathion peroxidase 4 (GPX4), upon simultaneous oxidation of glutathion (GSH). However, excessive lipid peroxidation of membrane phospholipids induces ferroptosis. This may occur when GPX4 is inhibited, e.g., by 1S,3R-RSL3 or altretamin, or when glutathion is absent. The latter can result from inhibition of glutathion synthesis by L-buthionine sulfoximine or from a lack of cystein inside the cells through inhibition of the $x_c$-antiporter system, e.g., by erastin or analogs, sorafenib, glutamate, or *S*-4-carboxyphenylglycine (*S*-4-CPG). Ferrostatin, liproxstatin, vitamin E, and deferoxamine are antioxidant compounds known to inhibit ferroptosis. Another way of inhibiting ferroptosis is provided by inhibition of lipid peroxidation itself by blocking LOX enzymes with loxblock-1, -2, -3, or baicalein.

an increased number of DNA strand breaks have been reported in both human and experimental plaques (Martinet et al., 2001, 2002). Most interestingly, oxidative DNA damage was associated with the upregulation of PARP-1 (and other DNA repair enzymes), predominantly in the macrophage-derived foam-cells of the plaque. Second, pharmacological inhibition of PARP-1 in atherosclerosis attenuates plaque development in ApoE$^{-/-}$ mice. Moreover, a reduction of PARP-1 activity enhances plaque stability and promotes the regression of pre-established plaques. Inadequate formation of plaques after PARP-1 inhibition may result from impaired translocation of NF-κB into the nucleus, followed by a reduction in inflammatory mediators (e.g., VCAM-1, MCP-1) and monocyte recruitment (Oumouna-Benachour et al., 2007; Xie et al., 2009). PARP-1 inhibition may also have a major impact on endothelial function, foam cell formation, lipid metabolism and the induction of cell death (switch from necrosis to apoptosis), all of which are central to the pathogenesis of atherosclerosis (Xu et al., 2014).

## TARGETING MACROPHAGE AUTOPHAGY

Autophagy is an evolutionary conserved physiological process in the body that maintains intracellular homeostasis by sequestrating unnecessary or dysfunctional cellular components in double membrane structures, called autophagosomes (Dikic and Elazar, 2018) (**Figure 7**). The latter fuse with

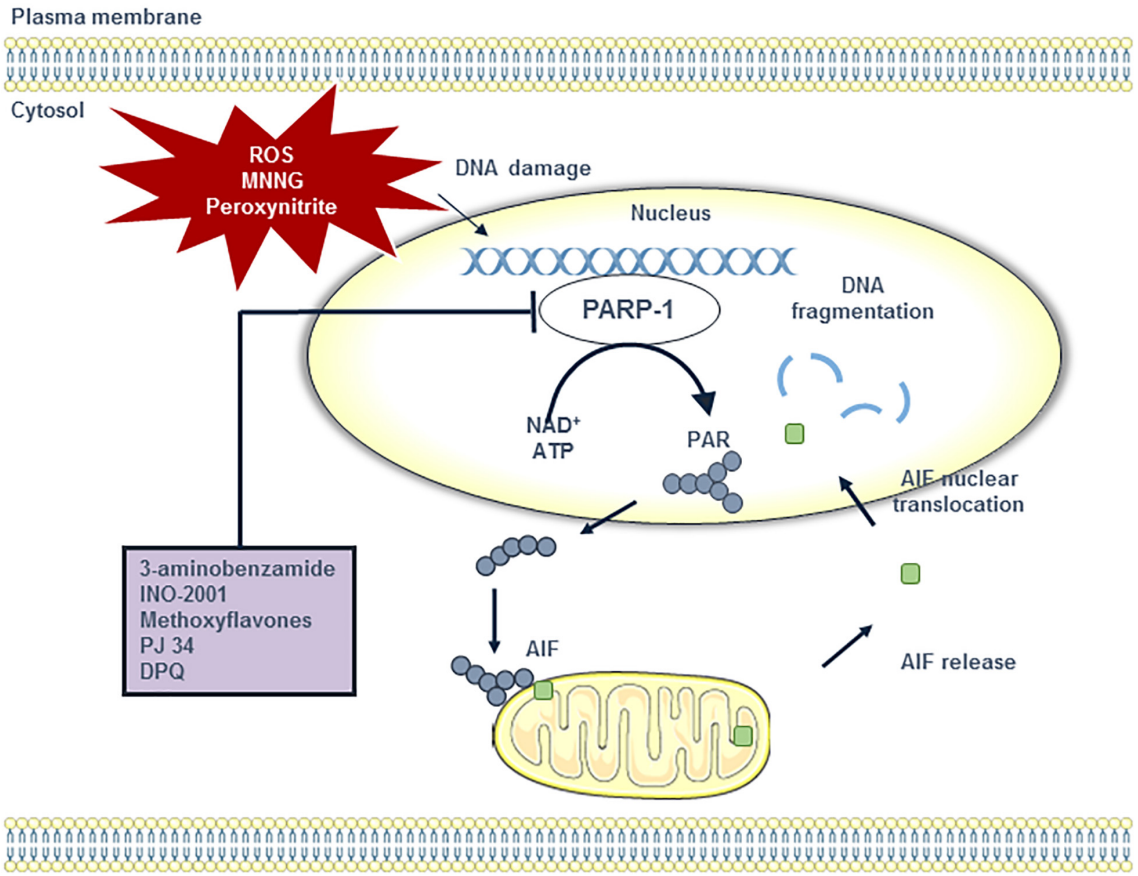


**FIGURE 6 |** Overview of the parthanatos pathway and potential targets for pharmacological inhibition. DNA damage can be caused by different stimuli including ROS, methylnitronitrosguanidine (MNNG) and peroxynitrite. When DNA is damaged, PARP-1 activity will increase in order to repair the DNA. However, poly (ADP-ribose) polymerase 1 (PARP-1) overactivation results in the depletion of the cellular pool of nicotinamide adenine dinucleotide ($NAD^+$) and ATP, which are used to synthesize poly ADP-ribose (PAR) polymer. PAR polymers migrate to the mitochondria, where they directly bind to apoptosis-inducing factor (AIF). AIF is then released from the mitochondria and translocated to the nucleus. Once in the nucleus, AIF causes large-scale DNA fragmentation and chromatin condensation. Parthanatos can be blocked by PARP-1 inhibitors such as 3-aminobenzamide, INO-1001, methoxyflavones, PJ34, and DPQ.

lysosomes,where the contents are degraded by acid hydrolases. As the autophagic process allows recycling of macromolecules and energy, basal autophagy primarily has cytoprotective functions and represents an essential *in vivo* process mediating proper vascular function. During atherosclerotic plaque formation, basal autophagy is stimulated in all major cell types, including macrophages, to blunt inflammatory signaling and to suppress atheroprogression (De Meyer et al., 2015). Indeed, disruption of essential autophagy-related genes (Atg5, Atg7), either in macrophages or VSMCs, accelerates atherosclerotic plaque development in mice (Liao et al., 2012; Razani et al., 2012; Grootaert et al., 2015; Osonoi et al., 2018). Macrophage autophagy not only protects against apoptosis, it also plays a prominent role in the clearance of apoptotic cells by efferocytosis (Liu et al., 2014). Indeed, Martinez et al. (2011) described engagement of MAPLC3A (LC3)-associated phagocytosis (LAP), which is distinct from the classical autophagy pathway but is dependent upon autophagy-specific genes such as Beclin1, Atg5 and Atg7. LAP is required for the engulfment and degradation of apoptotic cells. As a consequence, plaques of macrophage-specific Atg5-knockout mice reveal increased apoptosis, larger necrotic cores and overall lesion complexity (Liao et al., 2012). Moreover, defective autophagy in plaque macrophages is associated with proatherogenic inflammasome activation (Razani et al., 2012). This effect is probably mediated by crystalline cholesterol that accumulates in plaques with a macrophage-specific Atg5 deletion, and/or by the inefficient removal of damaged mitochondria and subsequent superoxide/ROS production (Razani et al., 2012). Additional *in vitro* experiments have shown that macrophage autophagy promotes reverse cholesterol transport and regulates the delivery of lipid droplets to lysosomes in macrophage foam cells, where lysosomal acid lipase-dependent lipolysis leads

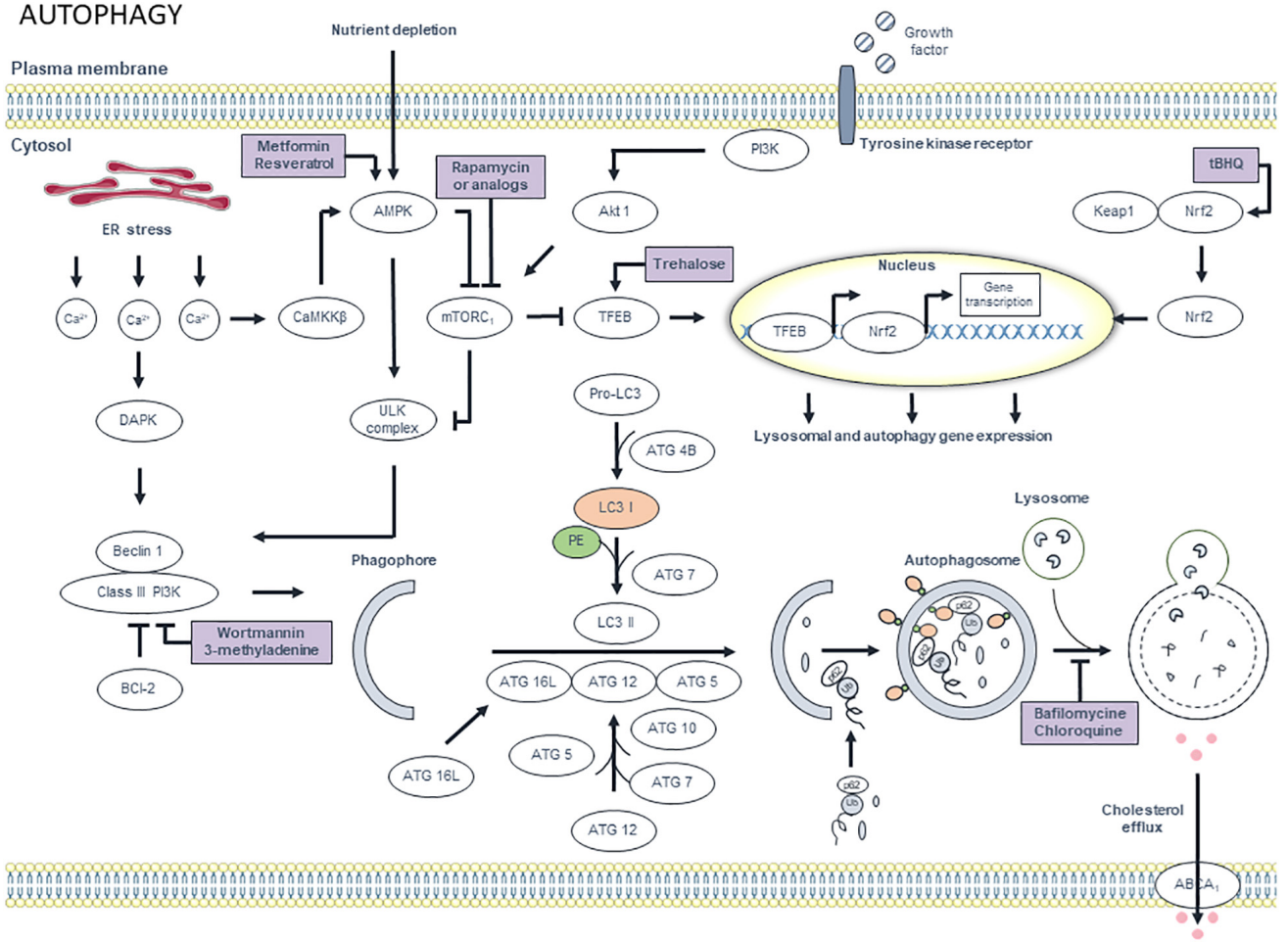


**FIGURE 7 |** Overview of the autophagy pathway and potential targets for pharmacological modulation. Different extracellular signals such as amino acids, growth factors and insulin, activate mammalian target of rapamycin complex 1 ($mTORC_1$) via PI3K and Akt1 signaling, thereby inhibiting autophagy. However, upon deprivation of nutrients and growth factors, AMP-dependent protein kinase (AMPK) is activated. AMPK inhibits $mTORC_1$, which results in the activation of ULK complex. Interestingly, its activation can also occur independently from $mTORC_1$. ULK complex, in turn, activates class III PI3K via phosphorylation of beclin 1. Subsequently, class III PI3K generates phosphatidylinositol 3-phosphates (PI3P), which are essential for the synthesis of the phagophore membrane. Further membrane elongation involves two ubiquitin-like conjugation systems. In the first system, ATG12 is conjugated to ATG5 via a reaction that involves ATG7 and ATG10. Subsequently, ATG12-ATG5 complex binds to ATG16L. ATG12-ATG5-ATG16L associates with the phagophore. The second system involves the conjugation of LC3 to phosphatidylethanolamine (PE). First, pro-LC3 is processed by Atg4B, which results in cytosolic LC3-I. ATG7 then adds phosphatidylethanolamine (PE) to LC3-I to form LC3-II, which associates with the phagophore membrane. Misfolded proteins and aggregates are tagged with ubiquitin chains, which are a substrate for p62. Protein – p62 complexes aggregate and migrate to the autophagosomal membrane, where p62 interacts with LC3. After the formation of the autophagosome, the outer membrane of the phagosome fuses with a lysosome, thereby forming an autolysosome. Finally, the internal material is degraded by lysosomal enzymes and nutrients and metabolites are recycled. Efflux of cholesterol via $ABCA_1$ transporters is promoted by autophagy. Interestingly, autophagy can be regulated at the transcriptional level. $mTORC_1$ inactivation results in nuclear translocation of transcription factor EB (TFEB). Once in the nucleus, TFEB triggers the transcription of autophagy and lysosomal genes. Moreover, oxidant stress can trigger autophagy by disrupting the interaction between nuclear factor erythroid-derived 2-like 2 (Nrf2) and Kelch-like ECH-associated protein 1 (Keap1). Nrf2 then translocates to the nucleus where it upregulates the transcription of different autophagy-related genes including p62. Additionally, calcium homeostasis plays a role in the autophagy pathway. ER stress causes calcium release, which activates proteases such as death-associated protein kinase (DAPK) and $Ca^{2+}$/calmodulin-dependent protein kinase kinase β (CaMKKβ). The latter induces autophagy via inhibition of $mTORC_1$ in an AMPK-dependent manner. DAPK induces autophagy by phosphorylating beclin 1 and thereby activating class III PI3K. Autophagy can be stimulated pharmacologically by targeting AMPK using metformin and resveratrol. Rapamycin (or analogs) induce autophagy via $mTORC_1$ inhibition. Induction of transcription of lysosomal and autophagy genes by targeting Nrf2 and TFEB with tert-butylhydroquinone (tBHQ) and trehalose, respectively, is an alternative approach for autophagy induction. Autophagy can be inhibited by targeting class III PI3K with wortmannin and 3-methyladenine. Alternatively, the autophagic process can be blocked by targeting lysosomes with bafilomycin and chloroquine.

to the generation of free cholesterol for efflux (Ouimet et al., 2011). In this way, macrophage autophagy suppresses foam cell formation and contributes to the regression of atherosclerotic plaques. Several lines of evidence indicate that the uptake and degradation of cellular cargo in lysosomes, a process known as autophagic flux, is functional and atheroprotective during

early atherosclerosis, but is stalled or becomes defective in macrophages of advanced atherosclerotic plaques (De Meyer et al., 2015). The underlying mechanisms are poorly defined, but may include (1) deposition of ceroid, an insoluble and indigestible complex of proteins and oxidized lipids that accumulates in lysosomes, (2) lysosomal dysfunction through permeabilization of the lysosomal membrane and/or the accumulation of lipids, and (3) age-dependent inhibitory effects (Kurz et al., 2007). These findings highlight the need for pharmacological interventions with compounds that stimulate the protective effects of autophagy in the atherosclerotic plaque. Many pharmacological approaches for the induction of macrophage autophagy in atherosclerosis have been proposed (Maiuri et al., 2013; Sergin and Razani, 2014; De Meyer et al., 2015). One straightforward and exciting pathway for maintaining sufficiently high levels of macrophage autophagy is by augmenting transcription factor EB (TFEB), a recently identified master regulator of autophagic activity and lysosome biogenesis. By overexpressing TFEB in macrophages, autophagy-lysosomal dysfunction is restored, which translates into broad atheroprotection (Sergin et al., 2017; Evans et al., 2018). TFEB also stimulates genes involved in endocytosis and phagocytosis. As mentioned above, these processes may help to reduce lesion complexity by clearing apoptotic cells via efferocytosis. Interestingly, TFEB overexpression can be pharmacologically induced by treating mice with the natural occurring disaccharide trehalose (Sergin et al., 2017; Evans et al., 2018), although the precise mechanism by which this occurs is unknown. It is hypothesized that trehalose is taken up by fluid-phase endocytosis and accumulates in the endosome-lysosomal system where it modulates its function. Of note, given the large amounts of trehalase in the gastrointestinal tract, oral dosing of trehalose should be avoided (Sergin et al., 2017; Evans et al., 2018). In future studies, it would be recommendable to use degradation-resistant trehalose analogs or to co-administer trehalase inhibitors. Another similar approach for inducing macrophage autophagy is the activation of nuclear factor (erythroid-derived 2)-like 2 (Nrf2) (Lazaro et al., 2018). This transcription factor is responsible for regulating a broad network of cytoprotective and antioxidant genes, including genes that are part of the autophagy machinery (e.g., SQSTM1/p62, Atg5). Treatment of ApoE$^{-/-}$ mice with the Nrf2 inducer tert-butyl hydroquinone (tBHQ) provides atheroprotective effects, not only through the concerted upregulation of antioxidant and anti-inflammatory mechanisms, but also by enhancing autophagic flux in the vessel wall (Lazaro et al., 2018). Apart from trehalose, several other natural products are able to stimulate autophagy in macrophages including adenosine derivative cordycepin (Li et al., 2017), ursolic acid (Leng et al., 2016) or ginsenoside Rb1 (Qiao et al., 2017), the most abundant active component of ginseng. All these compounds attenuate atherogenesis or promote plaque stability in mice via the activation of macrophage autophagy. Recently, microRNA-33 (miR-33) has been found to suppress macrophage autophagy in atherosclerosis (Ouimet et al., 2017). Inhibition of miR-33 with anti-miR-33 increases expression of its direct target genes in the autophagy pathway (i.e., Atg5, Lamp1, and Prkaa1 in mice), but also promotes AMPK-dependent activation of the FOXO3 and TFEB transcription factors. In this way, treatment of atherosclerotic LDLR$^{-/-}$ mice with anti-miR-33 restores defective autophagy in plaque macrophages, increases lysosomal biogenesis and triggers efferocytosis via an autophagy-dependent mechanism to reduce plaque necrosis (Ouimet et al., 2017). Other approaches that are worthwhile to mention include stimulation of macrophage autophagy via mild induction of ER stress (Ma et al., 2016) and statin therapy, even though the latter strategy is controversial. Some groups claim that statins block the maturation of autophagosomes, even with submicromolar statin concentrations (Miettinen and Bjorklund, 2015), which leads to a reduced basal autophagic flux. This block in autophagosome maturation increases the levels of autophagic markers, which might lead to the incorrect conclusion that statins increase autophagy (Miettinen and Bjorklund, 2016). In this light, it is tempting to speculate that reduced autophagic flux caused by the administration of statins may function as a mechanism for muscle toxicity and statin-induced myopathies (Miettinen and Bjorklund, 2016), which are well-known adverse effects of statin use. However, despite the abovementioned assumptions, growing evidence indicates that cholesterol depletion (via statins or other compounds) induces autophagy (Cheng et al., 2006) and that statin therapy attenuates pro-inflammatory effects and cholesterol accumulation in macrophages via autophagy-dependent signaling pathways (Huang et al., 2015; Han et al., 2018).

Although autophagy is an important subcellular pathway that mediates macrophage survival (Grootaert et al., 2018), exuberant induction of autophagy can stimulate macrophage death via the poorly understood type II programmed cell death, also termed autosis (Liu and Levine, 2015). In atherosclerotic plaques, selective induction of macrophage autosis may occur after stent-based delivery of rapamycin-derivatives (rapalogs) such as everolimus, and leads to a marked reduction of macrophages without altering the plaque VSMC content (Martinet et al., 2007b; Verheye et al., 2007). Rapamycin or rapalogs stimulate autophagy through inhibition of mTOR, a serine/threonine protein kinase that controls a variety of cellular functions including protein translation and cell proliferation. Knockdown of mTOR by mTOR-specific siRNA clears macrophages in a similar way (i.e., via induction of selective autophagy-mediated macrophage death) and inhibits the progression and destabilization of atherosclerotic plaques by downregulating MMP2 and MCP1 expression and by increasing the thickness of the fibrous cap (Wang et al., 2013; Zhai et al., 2014). It has been proposed that autophagy-mediated cell death using high concentrations (μM range) of rapamycin (or a rapalog), unlike apoptosis or necrosis, is the preferred type of death for selective depletion of macrophages in atherosclerotic plaques because macrophages undergoing this type of death literally digest themselves to death, without any major release of cellular content that could evoke an inflammatory response following post-autophagic necrosis (Martinet et al., 2007c). However, more recent studies showed that everolimus-treated macrophages secrete pro-inflammatory cytokines (e.g., IL-6, TNFα) and chemokines (e.g., MCP1) prior to autosis,a

phenomenon that is not autophagy-dependent, but mediated through activation of p38 MAP kinase (Martinet et al., 2012). This finding indicates that everolimus-induced macrophage death is not a harmless event and provides a rationale for combined treatment of atherosclerotic plaques with everolimus and an anti-inflammatory agent (e.g., glucocorticoid) that suppresses inflammatory responses without affecting the ability of everolimus to deplete macrophages in atherosclerotic plaques (Martinet et al., 2012). Interestingly, a recent report showed that relatively low levels of rapamycin (50–100 nM) do not induce macrophage autosis, but facilitate autophagic removal of LPS-induced pro-IL-1β protein and mitochondrial ROS, thereby inhibiting activation of p38 MAP kinase and NF-κB, which in turn downregulates secretion of several pro-inflammatory cytokines including IL-6, IL-8, and MCP1 (Ko et al., 2017). In addition, low levels of rapamycin cause transcriptional upregulation of SQSTM1/p62, which potentiates autophagy and activates the Nrf2 pathway to further suppress mitochondrial ROS (Ko et al., 2017). Overall, these findings suggest that the final concentration of rapamycin (or rapalogs), used either *in vitro* or *in vivo*, is an important factor that determines macrophage fate (autosis or survival) and the cellular pro-inflammatory phenotype.

## INTERRELATIONSHIP BETWEEN CELL DEATH PATHWAYS AND CONCLUDING REMARKS

Research in the past two decades has demonstrated that macrophage death is critically involved in the formation and destabilization of atherosclerotic plaques. The use of pharmacological compounds modulating macrophage death is beneficial not only in preventing atherogenesis, but also in promoting plaque stability and even regression of established plaques. However, the question remains whether we should induce or prevent macrophage death and in which stage of the plaque. Preventing cell death is technically more challenging than the induction of cell death (Hotchkiss et al., 2009). Different forms of cell death can occur simultaneously, particularly in advanced atherosclerotic plaques, because of the coordinated action of multiple death-inducing stimuli. Accordingly, it may be necessary to target multiple death pathways. Another aspect that complicates therapeutic inhibition of cell death is the crosstalk between cell death mechanisms (Nikoletopoulou et al., 2013). Apoptosis, autophagy, and (regulated) necrosis were initially considered to be mutually exclusive states. However, recent findings reveal a balanced interplay between these types of death so that blocking one type of death may sensitize cells to initiate another death pathway. For example, inhibition of caspases by the pan-caspase-inhibitor zVAD is sufficient to prevent apoptosis in many experimental models, but may facilitate the necroptosis program downstream of TNFR. Accordingly, "Death by any other name" (referring to the novel by Daphne Kapsali) as well as "Dosis sola facit venenum" (referring to Paracelsus) seem to be very important issues when targeting macrophage death in atherosclerotic plaques. In line with these findings, several effector molecules and signaling pathways have been identified as key mediators in different types of cell death (Chen et al., 2018).

Macrophage-specific initiation of cell death can have plaque-stabilizing effects. However, the type and timing of cell death induction might be important. Indeed, one must be careful because induction of macrophage apoptosis in plaques with impaired efferocytosis, such as in late stages of atherosclerosis, represents a certain risk. In contrast, pharmacological inhibition of necrosis improves several features of plaque stability such as lowering plaque inflammation, reducing oxidative stress, and increasing collagen content and fibrous cap thickness. Moreover, inhibition of macrophage necroptosis could be a promising therapeutic strategy to prevent the development of a vulnerable plaque. Interfering with other types of regulated necrosis in macrophages, such as pyroptosis, ferroptosis, and parthanatos is not clear-cut at present and deserves further investigation. Last but not least, autophagic flux becomes defective in macrophages of advanced atherosclerotic plaques. A growing body of evidence indicates that treatment with an autophagy inducer can be exploited as a potential strategy to prevent plaque formation and destabilization. Of note, there is currently a strong scientific rationale for recommending combination therapy to treat or prevent atherosclerosis. Indeed, inhibiting various types of cell death simultaneously via combined therapy could be an important emerging concept in the field of atherosclerosis, yet very little experimental evidence exists that supports this approach. In our opinion, treatment with an autophagy inducer is one of the most interesting strategies to stabilize vulnerable plaques, on top of statin therapy (or other lipid-lowering interventions), for several reasons. By clearing damaged organelles and misfolded proteins, autophagy acts as a cellular safeguard that (i) protects vascular cells against apoptosis, (ii) plays a prominent role in the clearance of apoptotic cells by efferocytosis and the prevention of (secondary) necrosis, (iii) blocks inflammation and (iv) enhances cholesterol efflux from macrophage-derived foam cells in different stages of atherosclerosis. Thus by stimulating autophagy, several cell death pathways are impaired. However, inducing autophagy in macrophages to an extent that leads to autophagy-induced death (autosis) might be detrimental due to induction of an inflammatory response. Moderate stimulation rather than excessive stimulation of autophagy is recommended and this issue needs more attention in future studies. It is also noteworthy that besides standard therapy (use of small organic molecules or biological agents such as antibodies) long non-coding RNAs (lncRNAs) and miRNAs are gaining more and more importance in cell death research and clearly affect different types of macrophage death in atherosclerosis. Indeed, recent evidence indicates that macrophage apoptosis is regulated by exosomal lncRNA growth arrest specific transcript 5 (GAS5) (Chen et al., 2017) and several miRNAs including miR-21 and miR-30c-5p (Canfran-Duque et al., 2017; Ceolotto et al., 2017). The latter also modulates pyroptosis (Li et al., 2018), while other LncRNAs represent key regulating factors during efferocytosis

or autophagic flux (Guo et al., 2019; Ye et al., 2019). Although these findings open interesting therapeutic perspectives, several limitations and challenges such as target specificity, route of delivery and stability, should be resolved before these RNA drugs can reach clinical applications.

## AUTHOR CONTRIBUTIONS

IC and PP have made the figures. All authors contributed to the writing of the manuscript.

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# miR-155 in the Resolution of Atherosclerosis

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Atherosclerosis is a chronic progressive inflammatory disease where advanced lesions can eventually completely obstruct blood flow resulting in clinical events, such as a myocardial infarction or stroke. Monocytes and macrophages are the dominant biologically active immune cells involved in atherosclerosis disease and play a pivotal role during initiation, progression, and regression of disease. Altering macrophage inflammation is critical to induce regression of atherosclerosis and microRNAs (miRs) have emerged as key regulators of the macrophage phenotype. MiRs are small noncoding RNAs that regulate gene expression. They are dysregulated during atherosclerosis development and are key regulators of macrophage function and polarization. MiRs are short nucleotide transcripts that are very stable in circulation and thus have potential as therapeutics and/or biomarkers in the context of atherosclerosis. Of relevance to this review is that inhibition of macrophage-specific miR-155 may be a viable therapeutic strategy to decrease inflammation associated with atherosclerosis. However, further studies on these miRs and advancements in miR therapeutic delivery are required for these therapeutics to advance to the clinical setting. Conjugated linoleic acid (CLA), a pro-resolving lipid mediator, is an agonist of the peroxisome proliferator-activated receptor (PPAR)-γ. The biological activities of CLA have been documented to have anti-atherogenic effects in experimental models of atherosclerosis, inducing regression and impacting on monocyte and macrophage cells. Our work and that of others on PPAR-γ agonists and polyunsaturated fatty acids have shown that these mediators regulate candidate miRNAs and promote pro-resolving atherosclerotic plaque microenvironments.

**Keywords: monocytes, macrophages, atherosclerosis, inflammation, miR-155**

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## ATHEROSCLEROSIS

Atherosclerosis is a chronic progressive disease that is characterized by accumulation and deposition of lipids and fibrous elements, coupled with an inflammatory response resulting in the development of lesions. Lesions develop in the tunica intima of large- and medium-sized arteries (Ross, 1993), following endothelial cell (EC) damage due to hyperlipidemia, hyperglycemia, hypertension, and inflammation (Knowles et al., 2000). The earliest clinical hallmark of a developing atherosclerotic lesion is the accumulation of lipid-laden macrophages termed foam cells which aggregate to form the "fatty streak" (Gerhard and Duell, 1999).

## ATHEROSCLEROSIS AND INFLAMMATION

Inflammation plays a pivotal role in atherosclerosis disease progression. Modified lipids such as oxidized low-density lipoprotein (oxLDL) cholesterol stimulate ECs to secrete pro-inflammatory mediators including cytokines and adhesion molecules which facilitate monocyte adhesion and subsequent migration, resulting in macrophage differentiation and expansion.

Monocytes originate from innate precursor myeloid cells in the bone marrow (Thomas et al., 2017). They account for ~10% of all leukocytes and circulate in the blood for 1–3 days (Ziegler-Heitbrock et al., 1993; Ley et al., 2011). Monocytes repopulate macrophage populations, control homeostasis, and participate in inflammatory responses switching on both innate and adaptive immune responses (Gerhard and Duell, 1999; Swirski et al., 2007). Monocytes are a heterogeneous cell population and different subsets are associated with changes in inflammation status (Ley et al., 2011). Murine monocytes are identified by surface expression of CD115, CD11b, F4/80, and chemokine receptors CCR2 and CX3CR1 (Patel and Zhang, 2017), which identify two distinct monocyte populations in mice, inflammatory and patrolling. The three main monocyte (Mo) subsets in humans are described as Mo1 classical, Mo2 intermediate and Mo3 nonclassical or patrolling monocytes.

Classical or pro-inflammatory Mo1 monocytes represent 80–90% of the monocyte population and are typically described as $CD14^{++}$ $CD16^{-}$ in humans (Ley et al., 2011) or $Ly6C^{+}$ in mice (Swirski et al., 2007). Upon stimulation, they secrete high levels of interleukin (IL)-10 (Swirski et al., 2007), and activation with toll-like receptor 4 agonists results in the secretion of tumor necrosis factor (TNF)-α, IL-6, and IL-1β (Ley et al., 2011; Italiani and Boraschi, 2014), whereas stimulation with a toll-like receptor 3 agonist results in interferon (IFN)-α secretion. They extravasate into the blood in a CCR2-MCP-1-dependent manner where they mediate inflammatory responses (Boyette et al., 2017). Mo2 intermediate monocytes are only found in humans characterized by $CD14^{++}$ $CD16^{+}$ and are most similar to human classical and murine $Ly6C^{+}$ monocytes (Ley et al., 2011; Boyette et al., 2017; Patel and Zhang, 2017). They secrete high levels of TNF-α and low levels of anti-inflammatory IL-10 (Italiani and Boraschi, 2014) and are increased in patients with arterial disease compared to healthy controls (Tsujioka et al., 2009). It has been shown that days following myocardial infarction, there is an increase in the classical Mo1 monocyte population, whereas days later, intermediate Mo2 monocytes prevail (Tsujioka et al., 2009). Nonclassical Mo3 monocytes are identified by $CD14^{-/+/lo}$ $CD16^{++}$ in humans (Ley et al., 2011) or $Ly6C^{-}$ in mice and are patrolling or resident monocytes (Ziegler-Heitbrock et al., 1993; Patel and Zhang, 2017). Upon stimulation, they secrete lower levels of TNF-α, IL-6, and IL-1β, and higher levels of IL-10 compared to other subsets (Ley et al., 2011) and they expand under conditions of stress (Patel and Zhang, 2017). Mo3 monocytes migrate to sites of damaged vasculature to promote wound healing (**Figure 1**).

Macrophage contribution to plaque development was identified when the macrophage-colony stimulating factor (M-CSF)-deficient osteopetrotic apolipoprotein E knockout ($ApoE^{-/-}$) mouse was shown to have an 86% decrease in lesion volume (Moore and Tabas, 2011). M-CSF drives the differentiation of monocytes into unpolarized M0 macrophages *in vitro* (Mosser and Edwards, 2008). M1 "classical" macrophages are pro-inflammatory, secreting the pro-inflammatory cytokines IL-1β, IL-6, IL-12, and TNF-α and are also characterized by increased expression of inducible nitric oxide synthase (iNOS), cyclooxygenase-2, and the generation of reactive oxygen species (Butcher and Galkina, 2011). The effects of macrophage-derived pro-inflammatory cytokines on vascular cells is well documented, where they contribute to EC dysfunction, reducing EC secretion of endothelial nitric oxide synthase and driving oxidative stress. M1 macrophages have been implicated in the formation of the necrotic core, plaque destabilization, and thrombus formation due to their ability to phagocytose oxLDL and secrete matrix metalloproteinase (MMP)-1, MMP-3, and MMP-9 (Boyle et al., 2011). M2 "alternative" macrophages were first derived from monocytes using M-CSF and IL-4 (Gordon and Martinez, 2010) and are characterized by expression of CD206. More recently, M2 subsets such as M2a, M2b, and M2c macrophages have been identified, where M2a macrophages are derived from IL-4 and IL-13, M2b macrophages from IL-1β or lipopolysaccharide (LPS), and M2c macrophages from IL-10, transforming growth factor β or glucocorticoids (Wolfs et al., 2011). In atherosclerotic plaques, M2 macrophages promote wound healing, matrix remodeling, efferocytosis, and fibroblast recruitment (Butcher and Galkina, 2011; Huang et al., 2012) and are localized far from the lipid core, in contrast to M1 macrophages. M2 macrophages are unable to efficiently phagocytose oxLDL but are professional efferocytes with the ability to promote secretion of MMP-11 and MMP-12 (Boyle et al., 2011; Huang et al., 2012). This suggests that M2 macrophages mediate pro-resolving roles in the clearance of apoptotic cells in early atherosclerosis but may play a role in plaque destabilization in later stages of disease.

**Abbreviations:** $ApoE^{-/-}$, Apolipoprotein E knockout; BMDM, Bone marrow-derived macrophage; c, cis; CLA, Conjugated linoleic acid; EC, Endothelial cell; IFN, Interferon; IL, Interleukin; iNOS, Inducible nitric oxide synthase; LDLR, LDL receptor; LPS, Lipopolysaccharide; MCP-1, Monocyte chemoattractant protein-1; M-CSF, Macrophage-colony stimulating factor; miR, microRNA; MMP, Matrix metalloproteinase; Mo, Monocyte subset; mRNA, Messenger RNA; oxLDL, Oxidized low-density lipoprotein; PBMC, Peripheral blood mononuclear cell; pHLIP, pH-induced transmembrane structure; PPAR, Peroxisome proliferator-activated receptor; STAT, Singal transducer and activator of transcription; t, trans; TNF, Tumor necrosis factor; WT, Wild type.

## CONJUGATED LINOLEIC ACID AND ATHEROSCLEROSIS

Conjugated linoleic acid (CLA) is a generic term denoting a group of naturally occurring isomers of linoleic acid (18:2, n6), that differ in the position or geometry [i.e., cis (c) or trans (t)]

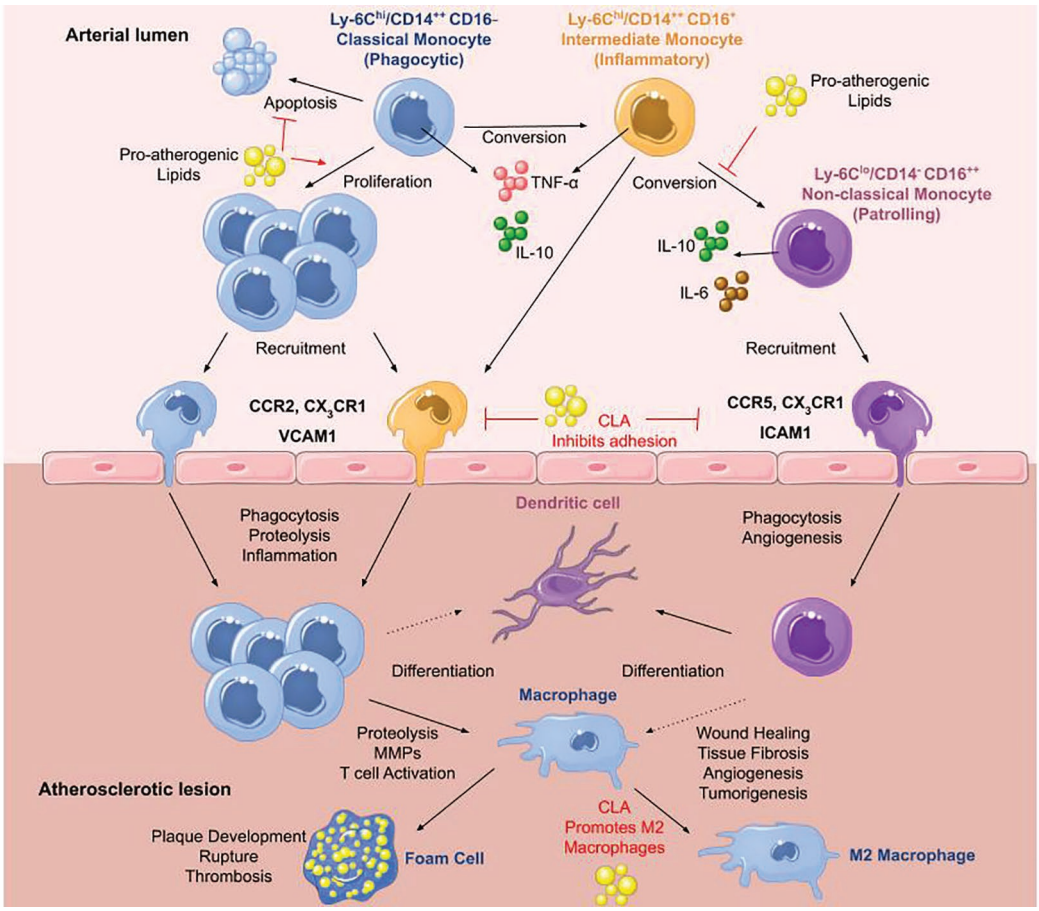


**FIGURE 1 |** Monocytes in atherosclerosis. The three main monocyte subtypes are Mo1, Mo2, and Mo3. Mo1 are classical monocytes defined as inflammatory, phagocytic, CD14$^{++}$ CD16$^{-}$ in humans and Ly6C$^{hi}$ in mice. They infiltrate lesions through CCR2 and differentiate into macrophages that can readily transform into foam cells. They secrete IL-10 and high levels of TNF-α upon stimulation with a toll-like receptor 4 agonist. Mo2 monocytes are termed intermediate monocytes and only found in humans defined by CD14$^{++}$ CD16$^{+}$. They are most similar to murine Ly6C$^{hi}$ inflammatory monocytes. They can convert into Mo3 nonclassical anti-inflammatory monocytes differentiating into dendritic cells or pro-inflammatory macrophages most likely contributing to disease progression. Mo3 patrolling monocytes are identified as CD14$^{-}$ CD16$^{++}$ in humans and Ly6C$^{lo}$ in mice. They secrete high levels of IL-10 and IL-6 with roles in wound healing and angiogenesis. They infiltrate plaques through CCR5. In atherosclerosis, high levels of pro-atherogenic lipids promote proliferation of Mo1 monocytes, inhibiting their apoptosis, and block the conversion of Mo2 intermediate monocytes into Mo3 nonclassical monocytes. Pro-resolving lipid mediators such as CLA can inhibit monocyte adhesion to ECs and promote M2 macrophage differentiation (adapted from Ley et al., 2011).

of their double bonds (Eder and Ringseis, 2010). There are 28 CLA isomers with c9,t11-CLA, which accounts for ~80% of CLA intake in the diet and t10,c12-CLA is the most abundant. The biological activities of CLA have been documented to have anti-atherogenic effects in an experimental model of atherosclerosis when administered in an 80:20 blend of its two most abundant isomers c9,t11-CLA and t10,c12-CLA, respectively (Toomey et al., 2006).

Our previous work, coincident with that of others, has shown that the CLA 80:20 blend induces resolution of pre-established atherosclerosis in ApoE$^{-/-}$ mice. In comparison with controls, CLA-fed mice also had decreased aortic macrophage accumulation, decreased CD36 expression (Toomey et al., 2006), increased aortic peroxisome proliferator-activated receptor (PPAR)-α and PPAR-γ expression, and negative regulation of pro-inflammatory gene expression, suggesting that CLA exerts its pro-resolving effects in part *via* activation of PPARs (McClelland et al., 2010; McCarthy et al., 2013a,b). In more recent studies, it was shown that CLA isomers in an 80:20 blend induce M2 macrophages (de Gaetano et al., 2015).

Furthermore, in the ApoE$^{-/-}$ model of atherosclerosis, CLA promotes a pro-resolving microenvironment, and we have identified that the monocyte/macrophage is the cellular target through which CLA mediates its effect (Toomey et al., 2006). CLA also inhibits monocyte adhesion to ECs, monocyte migration to monocyte chemoattractant protein-1 (MCP-1), and decreases MCP-1 production in part *via* a PPAR-γ-dependent mechanism (McClelland et al., 2010). This implies CLA is a potent inhibitor of monocyte function and may play a role in regulating the migratory monocytes in atherosclerosis.

Monocyte differentiation into macrophage subsets is critical for either promoting development or inducing resolution of

atherosclerosis. The M1 macrophage content of atherosclerotic plaques is associated with the clinical incidence of ischemic stroke and increased inflammation (Brown et al., 2002) and it has been shown that there is an M2 to M1 switch during plaque progression suggesting that interventional tools, able to revert the macrophage infiltrate toward the M2 phenotype, may exert an athero-protective action. CLA in an 80:20 blend of c9,t11:t10,c12-CLA impacts on macrophage polarization by reducing CD68 expression of M1 macrophages and increasing CD163 and CD206 expression associated with M2 macrophages, in human peripheral blood mononuclear cell (PBMC)-derived macrophages (de Gaetano et al., 2015). These findings have been confirmed *in vivo* where CLA supplementation in ApoE$^{-/-}$ mice induced the anti-inflammatory M2 phenotype *via* increasing IL-10 production in atherosclerosis regression (McCarthy et al., 2013a,b). This suggests that CLA primes the monocyte/macrophage toward a pro-resolving M2 phenotype to exert athero-protective effects. Further understanding of the pathways through which CLA mediates its effect on monocytes and macrophages is critical in identifying regulators that drive atherosclerotic regression including microRNAs (miRs) which govern macrophage phenotype. The effects of CLA on miRs have been previously documented in adipose tissue (Parra et al., 2010; Qi et al., 2015), intestinal epithelial cells (Daimiel-Ruiz et al., 2015), and ovarian cancer cells (Shahzad et al., 2018).

## miR FUNCTION

miRs are short noncoding RNAs, approximately 20 nucleotides in length. In a *Caenorhabditis elegans* model, it was demonstrated that *lin-4* transcripts, approximately 22 and 61 nucleotides in length, did not encode for a protein but regulated the messenger RNA (mRNA) of *lin-14* by inhibiting protein translation (Lee et al., 1993). miRs regulate gene expression through inhibition of translation (Bartel, 2004). Through binding with an RNA-induced silencing complex, the nucleotide sequence of the miR allows targeted base pairing with the 3′ untranslated regions of complementary mRNA (Hammond et al., 2000; Martinez et al., 2002). MiR sequence-specific silencing of mRNA can occur *via* two mechanisms: enzymatic cleavage of the transcript occurs if there is sufficient complementarity of the miR-mRNA sequences, or *via* translational repression if there is a lack of complementarity yet still some complementary miR sites present on the mRNA (Zeng et al., 2002, 2003). A single miR can target multiple transcripts and a single gene can be under the control of multiple miRs.

## ANTI-INFLAMMATORY DIETARY COMPOUNDS AND miRs

Although the effects of CLA on miRs in the context of atherosclerosis remain to be elucidated, in the context of myocardial infarction, treatment of mice with CLA in conjunction with nitrite improved heart function and induced miR-499 (Qipshidze-Kelm et al., 2013). Other anti-inflammatory dietary compounds function in part by downregulation of miR-155, a key regulator of inflammation, these include resveratrol (Tili et al., 2010), curcumin (Ma et al., 2017), apigenin (Arango et al., 2015), and quercetin (Boesch-Saadatmandi et al., 2011). The polyunsaturated fatty acids, docosahexaenoic acid and arachidonic acid, significantly decrease miR-155 in murine macrophages stimulated with LPS (Roessler et al., 2017). In addition, PPAR-γ agonists, rosiglitazone and telmisartan, decrease miR-155 in pre-adipocytes and in adipose tissue (Li et al., 2015; Peshdary and Atlas, 2018). Interestingly, CLA, a PPAR-γ agonist, increases aortic IL-10 secretion and increases phosphorylated signal transducer and activator of transcription (STAT)-3 signaling in the resolution of atherosclerosis (McCarthy et al., 2013a,b). It has been documented that IL-10 inhibits the BIC gene which encodes for miR-155 *via* a STAT-3-dependent mechanism (McCoy et al., 2010). miR-155 is one of several miRs that regulates inflammation and may be of clinical significance for novel therapeutics or prognostic indices of atherosclerotic disease progression and regression.

## miRs IN ATHEROSCLEROSIS

Multiple reviews have highlighted miR dysregulation in atherosclerosis progression (Small et al., 2010; Santovito et al., 2012; Condorelli et al., 2014). Several miRs, including miR-155, are upregulated in human cardiac disease (Krishnan et al., 2017) and miR-155 is significantly increased in plasma and plaques in atherosclerotic patients (Li et al., 2016). Given miR stability in circulation and their ability to regulate gene expression, they have potential in diagnostics (Fichtlscherer et al., 2010), prognostics (Karakas et al., 2017), and therapeutics. miRs involved in governing macrophage polarization and the distinct miR profiles of the M1 and M2 macrophage have been reviewed previously (Essandoh et al., 2016; Li et al., 2018) and miR-155 and miR-33 are emerging targets in macrophages to reduce atherosclerosis development. miR-33 inhibition in macrophages enhances cholesterol efflux (Najafi-Shoushtari et al., 2010) and reduces atherosclerotic lesions in mice (Horie et al., 2012; Ouimet et al., 2015). However, miR-155 may be a more *via*ble target given its role in determining macrophage phenotype.

miR-155 is encoded from the BIC gene in response to monocyte and macrophage stimulation. LPS upregulates miR-155 expression in THP-1 monocytes and macrophages (Taganov et al., 2006; Graff et al., 2012). Furthermore, miR-155 is increased during phorbol 12-myristate 13-acetate-stimulated differentiation of THP-1 monocytes to macrophages (Forrest et al., 2010). OxLDL also promotes miR-155 expression in PBMCs (Chen et al., 2009) and THP-1 macrophages (Li et al., 2016). In bone marrow-derived macrophages (BMDMs) from wild type (WT) mice, IFN-β and IFN-γ increased miR-155 expression *via* TNF-α autocrine signaling (O'Connell et al., 2007). Polarization of PBMC-derived macrophages (Graff et al., 2012), THP-1 cells, and BMDMs (Cai et al., 2012) to the M1 phenotype (induced by IFN-γ and LPS) also resulted in increased miR-155 expression. There are multiple targets of miR-155 in macrophages that regulate inflammation (**Figure 2**).

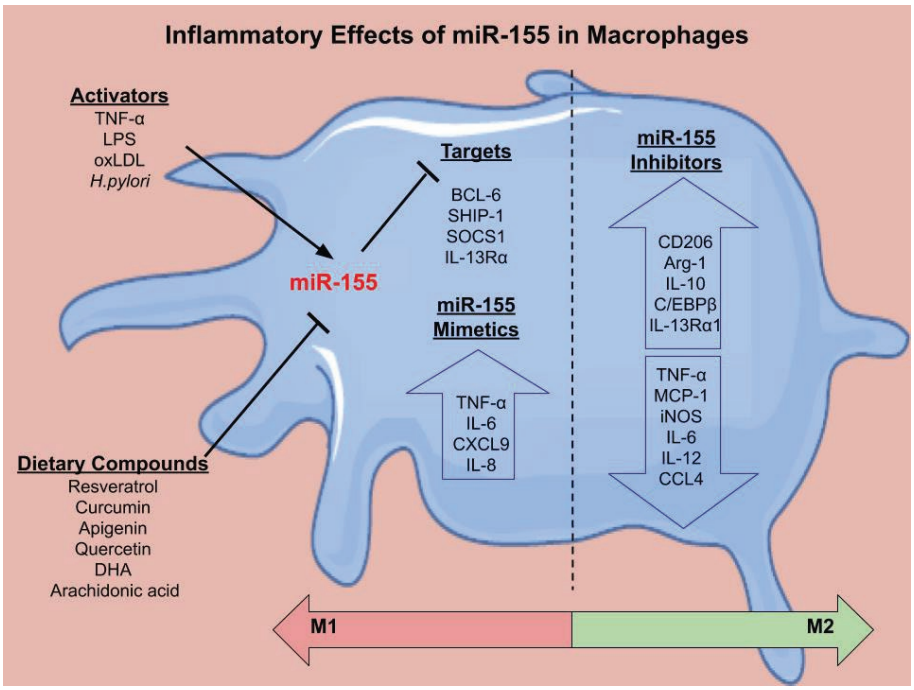


**FIGURE 2 |** The inflammatory effects of miR-155 in macrophages. There are several activators of miR-155 including LPS, TNF-α, oxLDL, and *H.pylroi*. miR-155 can in turn inhibit gene expression. Several dietary compounds including the polyunsaturated fatty acids, docosahexaenoic acid (DHA) and arachidonic acid, can inhibit miR-155. Validated targets of miR-155 in macrophages include B Cell CLL/Lymphoma 6 (BCL-6) (Nazari-Jahantigh et al., 2012), inositol polyphosphate-5-phosphatase D (SHIP-1) (O'Connell et al., 2009), suppressor of cytokine signaling 1 (SOCS1) (Wang et al., 2010), and IL-13 receptor subunit α 1 (IL-13Rα1) (Martinez-Nunez et al., 2011). miR-155 mimetics cause increased pro-inflammatory cytokine secretion (TNF-α, IL-6, CXCL9, and IL-8). miR-155 inhibition decreased pro-inflammatory cytokines and chemokines and increased M2 markers. Overall, the literature suggests that miR-155 mimetics shift macrophages toward an M1 phenotype, while miR-155 inhibition skews the macrophage toward an M2 phenotype, however this can be context dependent.

## miR-155 IN MACROPHAGES AND MOUSE MODELS OF ATHEROSCLEROSIS

miR-155 is upregulated in response to inflammatory stimuli where nuclear factor-κB directly binds to the promoter of pri-miR-155/BIC gene (Li et al., 2016). Several *in vitro* experiments have attempted to elucidate whether miR-155 increases or decreases monocyte/macrophage inflammation. One study showed that miR-155 is anti-inflammatory and mediates its effects through degradation of calcium-regulated heat stable protein 1 which is critical for the stabilization of TNF-α mRNA (Li et al., 2016). Transfection of miR-155 mimic into THP-1 macrophages followed by oxLDL stimulation decreased TNF-α and increased oxLDL uptake, while miR-155 inhibitors had opposing effects (Li et al., 2016).

However, several other studies report that miR-155 has pro-inflammatory effects. Transfection of M0 THP-1 macrophages with a miR-155 mimic resulted in increased IL-6, TNF-α, and CXCL9 (Graff et al., 2012). PBMCs from *Helicobacter pylori*-infected patients had increased miR-155 expression. Transfection of miR-155 mimics into these infected macrophages increased cytokine secretion of IL-10, TNF-α, and IL-8 (Yao et al., 2015).

miR-155 inhibition in BMDMs from WT mice demonstrated that miR-155 is critical in sustaining the pro-inflammatory response. Pro-inflammatory LDL receptor (LDLR)-related protein 1 antagonist activity was blocked by miR-155 inhibition, as measured by decreased expression of TNF-α, IL-6, and CCL4 when stimulated with LDLR-related protein 1 (Mantuano et al., 2016). TNF-α has been shown to increase miR-155 which in turn may function in a positive feedback loop that sustains inflammation. In a separate study, inhibition of miR-155 in WT macrophages increased transcription factor, CCAAT enhancer binding protein beta, and downstream arginase-1 expression which are associated with an M2 phenotype (Arranz et al., 2012). miR-155 inhibition also reduced LPS induction of iNOS and overexpression of miR-155 had directly opposing inflammatory effects (Arranz et al., 2012).

M2 macrophages are essential for regression of atherosclerosis. Inhibition of miR-155 in M1s using antisense oligonucleotides resulted in an increase in M2 markers arginase-1, chitinase 3-like 3, CD206, resistin-like molecule-α, and IL-10, and a decrease in M1 markers TNF-α, iNOS, and IL-12 (Cai et al., 2012). Interestingly, transfection of a miR-155 mimic into M2 macrophages skewed them toward an M1 macrophage profile. M0s treated with pre-miR-155 mimics prior to M2 polarization resulted in a suppressed M2 phenotype following stimulation with IL-4. Depleting miR-155 in M0s followed by stimulation with LPS and IFN-γ resulted in a decreased M1 phenotype (Cai et al., 2012). In addition, miR-155 inhibition in macrophages increased IL-13

receptor alpha subunit 1, which facilitated an IL-13-mediated increase in STAT-6 activation and upregulation of IL-13 regulated genes that are important in the development of the M2 phenotype (Martinez-Nunez et al., 2011). These studies demonstrate that miR-155 is critical in orchestrating the inflammatory response and may be a viable target to promote M2 polarization or to reprogram M1 macrophages to reduce chronic inflammation, which is essential for regression of atherosclerosis (**Figure 2**).

To date, studies on the effects of miR-155 deletion *in vivo* are conflicting. ApoE$^{-/-}$ mice with a leukocyte-specific miR-155 deficiency had decreased plaque size and number of lesional macrophages following partial carotid ligation (Nazari-Jahantigh et al., 2012). Furthermore, macrophages derived from this model had lower levels of MCP-1 when activated. Similarly, ApoE$^{-/-}$miR-155$^{-/-}$ mice had decreased macrophage inflammation and reduced atherosclerotic lesion development. The regulatory effects of miR-155 on leukocyte cells were confirmed through transplantation of miR-155-deficient bone marrow into ApoE$^{-/-}$ mice which also halted atherogenesis (Du et al., 2014). Other studies have demonstrated that injection of antagomir-155 attenuated atherosclerosis development and progression in ApoE$^{-/-}$ mice (Yang et al., 2015; Ye et al., 2016). In contrast, Ldlr$^{-/-}$ mice transplanted with miR-155-deficient bone marrow had increased atherosclerotic plaques, elevated levels of pro-inflammatory monocytes, and decreased IL-10 production from peritoneal macrophages (Donners et al., 2012). These contradictory results may be due to the different animal models used. After 3 months on a Western diet, ApoE$^{-/-}$ mice have elevated plasma cholesterol, larger aortic root lesion with macrophage-dense necrotic cores, and increased smooth muscle cells in comparison to Ldlr$^{-/-}$ mice (Roselaar et al., 1996). Therefore, the ApoE$^{-/-}$ mouse model may be more reflective of advanced atherosclerosis, suggesting that miR-155 may have stage-specific effects during atherosclerotic lesion development.

## miR-155 THERAPEUTICS IN CLINICAL TRIAL AND miR DELIVERY

While no miR therapeutics have entered clinical trial for the treatment of atherosclerosis, two miR-155 inhibitors are currently under development. MRG-106, a miR-155 inhibitor, administered intratumorally, was well tolerated, had on-target activity, and had promising preliminary results in a phase 1 clinical trial in 6 patients with the mycosis fungoides form of cutaneous T-cell lymphoma (Querfeld et al., 2016). MRG-106 is currently in the phase 2 SOLAR trial (NCT03713320). MRG-107 is another anti-miR-155 therapy currently under development for treatment of Amyloid Lateral Sclerosis. However, several miR therapeutic clinical trials have been discontinued, including studies on MRX34 (NCT 01829971), RG-125 (NCT02826525), and RG-012 (NCT02855268).

Delivery of the miR therapeutic to the desired site of action is critical to prevent off-target effects. Cheng et al. combined a peptide with a low pH-induced transmembrane structure (pHLIP) to a peptide nucleic acid anti-miR, specific for miR-155 (Cheng et al., 2015). This delivery vector, pHLIP-anti-miR-155, can only be transported through the plasma membrane under acidic conditions such as those located in solid tumors. In two mouse tumor models, intravenous administration of this construct inhibited miR-155, had no systematic toxicity, and reduced metastasis. RNA-sequencing and bioinformatic analysis showed that 25% of the genes upregulated by pHLIP-anti-miR-155 were associated with cell adhesion and leukocyte transendothelial migration (Cheng et al., 2015). Whether this technology could be effective in targeting atherosclerotic plaques directly remains to be investigated. pHLIP-anti-miR-155 functioned in solid tumors which have a pH of approximately 6. In contrast, plaque pH is heterogenous, lipid-rich regions have a pH 7.15 whereas calcified areas had a pH 7.73 (Naghavi et al., 2002). Young et al. used a "blockmir" technology oligonucleotide-based drug, CD5–2, to selectively inhibit the miR-27a binding site in vascular endothelial-cadherin (Young et al., 2013). Delivery of CD5–2 intravenously was shown to be effective in reducing vascular leak and inflammation in animal models of retinopathy (Ting et al., 2018). Further advancements in miR therapeutic delivery to the desired site of action may facilitate clinical development.

## CONCLUSION

Atherosclerosis is an inflammatory disease where monocyte/macrophage cells are the dominant biologically active immune cells. The athero-protective effects of CLA during an experimental *in vivo* model of atherosclerosis regression identified the monocyte/macrophage as the cellular target. Given the role of miRs in macrophage polarization, they are likely to be critical regulators in the regression of atherosclerosis. miR-155 is upregulated in response to pro-inflammatory stimuli and its inhibition may be a viable strategy to reduce the inflammatory response. However, given some conflicting results, more studies are required to investigate the stage-specific effects of miR-155 inhibition during atherosclerosis progression. However, further development of miRs in clinical trials and improved methods of miR delivery could lead to development of macrophage-specific miR therapeutics in the context of atherosclerosis.

## AUTHOR CONTRIBUTIONS

RB and SF contributed equally to the preparation and writing of this manuscript, supervised by OB who edited the manuscript. All authors approved the final manuscript.

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REVIEW
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# Apoproteins E, A-I, and SAA in Macrophage Pathobiology Related to Atherogenesis

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Macrophages are core cellular elements of both early and advanced atherosclerosis. They take up modified lipoproteins and become lipid-loaded foam cells and secrete factors that influence other cell types in the artery wall involved in atherogenesis. Apoproteins E, AI, and SAA are all found on HDL which can enter the artery wall. In addition, apoE is synthesized by macrophages. These three apoproteins can promote cholesterol efflux from lipid-loaded macrophages and have other functions that modulate macrophage biology. Mimetic peptides based on the sequence or structure of these apoproteins replicate some of these properties and are potential therapeutic agents for the treatment of atherosclerosis to reduce cardiovascular diseases.

**Keywords: macrophage, apoE, apoA-I, SAA, cholesterol efflux, oxidation, mimetic peptides, atherosclerosis**

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## INTRODUCTION

Macrophages are a core cellular element of both early and advanced atherosclerotic lesions in humans and experimental animal models. Many of the macrophages of lesions are derived from blood monocytes entering regions of the arterial wall lined with activated endothelial cells. Most studies of the involvement of macrophages in atherosclerosis are concerned with several features of macrophage pathobiology: the ingress of monocytes into lesions and their differentiation into macrophages; the conversion of macrophages to lipid loaded foam cells; with the genes the macrophages express in the context of the lesions that influence other cells in the lesion including endothelial cells, smooth muscle cells and cells of the adaptive immune system; and with the egress of macrophages from the lesion. The accumulation of macrophages in arterial lesions is not only due to the balance of the ingress and egress of monocytes/macrophages, but also consequent on local macrophage proliferation (Robbins et al., 2013; Williams et al., 2018). In early atherosclerotic lesions the macrophage foam cell containing stored cholesteryl ester droplets is the most obvious biomarker of the process. These features have directed attention to the lipid metabolism of these cells.

The detailed examination of the evolution of lesions is not possible in humans as the mechanistic study of the atherogenic process is difficult to study directly and individual subject exhibit quite different genotypes. Instead the evolution of human atherosclerosis is largely inferred from the analysis of atherosclerotic lesions obtained from patients, living or dead. One of the best examples of this approach is represented by the PDAY study, an autopsy-based study of young individuals deceased as a result of accidents (McMahan et al., 2008). In this study, the extent and distribution of lesions in the vasculature in the subjects was correlated with risk factors (e.g., gender, smoking, hyperlipidemia, hypertension, and diabetes). Given the limitations of the refined examination of

human lesion development, attention has turned to animal models of atherosclerosis, with most studies in the last decades utilizing murine models lacking either the apoE gene (*Apoe*) or the LDL receptor (*Ldlr*) gene (Getz and Reardon, 2012). Indeed, since first being described in 1992 (Plump et al., 1992; Zhang et al., 1992) the $Apoe^{-/-}$ mouse has become the favored model for the study of experimental murine atherosclerosis (Getz and Reardon, 2016). ApoE is a multifunctional protein (Getz and Reardon, 2009) that is normally present on circulating lipoproteins, where it functions as a ligand for lipoprotein uptake, particularly for the removal of intestinal derived chylomicron remnants and hepatic derived VLDL remnants. As a result, $Apoe^{-/-}$ mice are hyperlipidemia even while being fed a low-fat chow diet. This hyperlipidemia is further increased by feeding a high fat, high cholesterol diet Western type diet.

# THE INFLUENCE OF APOE ON MACROPHAGES AND MONOCYTES

## Macrophage Expression of ApoE Reduces Atherosclerosis

ApoE is expressed in many cell types, particularly hepatocytes and macrophages. The only cells that do not express significant levels of this apoprotein are enterocytes (Driscoll and Getz, 1984). The significance of the apoE produced by macrophages for atherogenesis has been highlighted by bone marrow transplantation experiments. Macrophages are a major apoE-producing cell derived from bone marrow precursors, and implicit in these transplantation studies is that the macrophages are the operative cells responsible for the reported results. When $Apoe^{-/-}$ mice were transplanted with wild type bone marrow, apoE levels in the plasma increased and the hyperlipidemia and atherosclerosis exhibited a notable reduction, despite only low levels of apoE in the plasma (Linton et al., 1995). Unfortunately, the co-ordinate reduction of both does not allow for the assessment of the contribution of the macrophage derived apoE to the attenuation of atherosclerosis independent of effects on plasma lipids. However, there are a number of experiments in $Apoe^{-/-}$ mice that point to an independent influence of macrophage derived apoE on atherosclerosis. It appears that the plasma concentration of apoE required for rescue of the dyslipidemia is higher than that associated with the reduction in lesion formation. In one study, retrovirus mediated apoE expression in macrophages rescued early atherosclerotic lesion development with little effect on plasma lipids (Hasty et al., 1999). In these experiments the plasma level of apoE was only about 1% of levels in wild type mice. In another study (Bellosta et al., 1995) the visna virus LTR was employed to drive the transgenic expression of human apoE in macrophages in $Apoe^{-/-}$ mice. Mice with a range of plasma lipids and apoE levels were obtained. When a subgroup of animals was selected that had essentially the same blood lipid levels and lipoprotein profile as $Apoe^{-/-}$ control mice, a significant reduction in lesions was noted in the transgenic mice, again implying that the expression of apoE by macrophages was capable of attenuating lesion development independent of effects on blood lipids. Compatible with this interpretation is the increased early lesion formation observed when bone marrow from $Apoe^{-/-}$ mice was transplanted into high fat, high cholesterol fed wild type mice (Fazio et al., 1997).

## Macrophage ApoE and Cholesterol Efflux

The function of apoE in macrophages that is thought to be most important for its anti-atherogenic role is its ability to enhance cholesterol efflux from the arterial macrophages, thereby reducing their lipid burden and the subsequent downstream production of macrophage products, such as pro-inflammatory cytokines and chemokines that promote atherogenesis (**Table 1**). We have recently reviewed the role of apoE in cellular cholesterol efflux and reverse cholesterol transport (Getz and Reardon, 2018). Most studies of cholesterol efflux *in vitro* and *in vivo* use mouse macrophage cell lines, such as J774A.1 or RAW264.7 cells. However, the role of apoE in promoting cholesterol efflux may be under appreciated since, unlike tissue macrophages including those found in atherosclerotic lesions, these cell lines do not express apoE. Advantage has been taken of the absence of apoE expression by J774 cells to examine differences between exogenous and endogenous apoE in promoting cholesterol efflux. For this, J774 cells were transfected to express apoE under the control of a non-cholesterol responsive promoter. Comparing these cells with untransfected cells it was shown that endogenous apoE is more effective in promoting cholesterol efflux than is exogenous apoE added to the media (Lin et al., 1999). It appears that the efflux promoted by endogenous apoE is qualitatively different than that seen with exogenous apoE (**Figure 1**). Unlike exogenous apoE, endogenous apoE-mediated efflux is not ABCA1 dependent (Huang et al., 2001). Instead, the apoE produced by the cells is found associated with the plasma membrane, bound to either the LDL receptor, heparan sulfate proteoglycan or membrane lipids (Zhao and Mazzone, 1999; Lin et al., 2001). In humans there are three isoforms of apoE, designated apoE2, apoE3, and apoE4. ApoE2 and apoE4 differ from the most prevalent apoE3 isoform by single amino acid; residues 112 and 158 are cysteine and arginine, respectively in apoE3, both residues are cysteines in apoE2 and both are arginines in apoE4. ApoE2 has significantly lower

**TABLE 1 |** Macrophage Related Functions of ApoE, ApoA-I, and SAA.

| Apoprotein | Macrophage Related Function |
|---|---|
| ApoE | Synthesized by macrophages |
| | Promotes cholesterol efflux |
| | Regulates monocytosis |
| | Anti-inflammatory |
| | Antiatherogenic |
| ApoA-I | Promotes cholesterol efflux |
| | Myeloperoxidase mediated modifications reduces efflux capacity |
| | Antiatherogenic |
| SAA | Synthesized by macrophages |
| | Inhibits HDL mediated cholesterol efflux |
| | Proatherogenic |

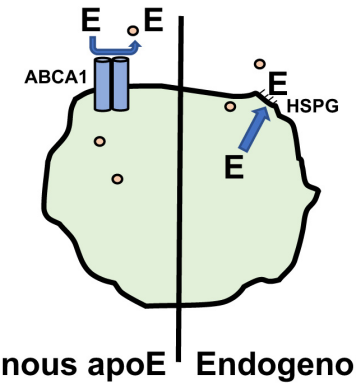


**FIGURE 1 |** Promotion of Cholesterol efflux by apoE. Macrophage cholesterol (○) efflux promoted by exogenous apoE is dependent on ABCA1. Cholesterol efflux promoted by endogenous apoE involves apoE associating with the plasma membrane via heparin sulfate proteoglycans(HSPG) and is ABCA1 independent.

binding affinity for the LDL receptor, while apoE4 has slightly higher binding affinity. Human apoE isoform replacement mice have been created in which each human apoE isoform replaces the endogenous murine apoE. When LDL receptor expression in macrophages from the gene replacement mice was upregulated by simvastatin treatment, apoE protein secretion and cholesterol efflux promoted by apoE4 was reduced while that of apoE2 was unaffected, reflecting their affinity for the LDL receptor (Lucic et al., 2007).

Reverse cholesterol transport is a process by which excess cholesterol in cells, including macrophages, is effluxed and transported via plasma lipoproteins to the liver for excretion in feces. This pathway is frequently examined *in vivo* using radiolabeled cholesterol loaded macrophages to follow the transport of the cholesterol from the macrophages to the feces. Consistent with the *in vitro* cellular cholesterol efflux results reported in the preceding paragraph, macrophages deficient in apoE are not as effective as wild type macrophages in supporting reverse cholesterol transport *in vivo* (Zanotti et al., 2011).

## Regulation of Macrophage ApoE Expression

As macrophage apoE plays a role in cholesterol efflux, it is of interest to understand the regulation of its expression in macrophages. ApoE production increases as monocytes differentiate into macrophages (Werb et al., 1986). In experiments with mouse peritoneal macrophages and human THP1 cells differentiated into macrophages, cholesterol loading of the cells in culture upregulates apoE mRNA and apoE protein secretion (Mazzone et al., 1987, 1989). On the other hand, macrophages derived from human monocytes do not exhibit an increase in apoE synthesis on cholesterol loading but endogenous levels are sufficient to enable cholesterol efflux (Zhang et al., 1996). A more recent study showed a similar up regulation of apoE mRNA in peritoneal macrophages harvested from *Ldlr*$^{-/-}$ mice fed a Western type diet containing 1.25% cholesterol (Spann et al., 2012). This appears to be attributable to activation of the liver X receptor (LXR) by the desmosterol that accumulates in the cholesterol loaded macrophages. The cholesterol mediated induction of apoE gene transcription, but not the basal level of apoE gene transcription, is abolished in the absence of LXR nuclear receptors (Laffitte et al., 2001). The results of Spann et al contrast with the proteomic analysis of proteins secreted from lipid-loaded peritoneal macrophage of *Ldlr*$^{-/-}$ mice fed the same Western type diet (Becker et al., 2010). Forty-six proteins were differentially expressed by peritoneal macrophages from chow and Western diet fed mice. Among these differentially expressed proteins, apoE secretion was profoundly decreased along with other proteins that have been shown to influence atherogenesis like MFGE8 (lactadherin), lipoprotein lipase, and LRP (LDL receptor related protein) from macrophages of Western type diet fed *Ldlr*$^{-/-}$ mice. ApoE appears to be a regulator of this protein network, as a different and attenuated pattern of proteins responsive to cholesterol loading was noted in macrophages from *Apoe*$^{-/-}$ mice. Since Western type diet fed mice are insulin resistant as well as hyperlipidemia, further studies examined the response of the macrophage protein network in the presence and absence of insulin resistance. ApoE and 8 other members of this macrophage protein network were dysregulated in the presence of insulin resistance via a mechanism dependent on IFNγ and independent of changes in their transcript levels. In addition, using *Ldlr-/-* mice fed high cholesterol diets with (insulin resistant) and without (insulin sensitive) high fat content, IFNγ was shown to be an important driver of atherosclerosis in mice with insulin resistance (Reardon et al., 2018). IFNγ modulation of the protein network was also observed in vessel wall macrophages. IFNγ is generally thought to be pro-atherogenic by promoting foam cell formation by stimulating the cell surface expression of scavenger receptors and by polarizing macrophages toward the pro-inflammatory M1 subset (Boshuizen and de Winther, 2015). The reduction in the expression of apoE and other proteins in this network may also contribute to the pro-atherogenic role of IFNγ, especially in the setting of insulin resistance. Parenthetically, it is noteworthy that peritoneal and bone marrow derived macrophages are not phenotypically identical, at least with respect to their response to oxidized LDL (Bisgaard et al., 2016). Clearly, despite much work on the role of apoE in macrophage biology, much remains to be clarified.

## ApoE and Monocytosis

It is now clear that monocytosis is a risk factor for the development of atherosclerotic cardiovascular disease as well as myocardial infarctions (Murphy and Tall, 2016; Swirski et al., 2016). This increased risk is related to dysfunction of cholesterol homeostasis, at least at the level of monocyte progenitor cells in the bone marrow and extramedullary sites such as the spleen. There are two major subsets of circulating monocytes that are distinguished by the level of Ly6C on their cell surface. The pro-inflammatory Ly6C$^{hi}$ monocytes dominate in the monocytosis associated with hypercholesterolemia and these are the cells that preferentially infiltrate the artery wall

and become lesional macrophages (Swirski et al., 2007). Tall and collaborators have explored the role of apoE in the expansion of monocyte precursor pools (Murphy et al., 2011). In a seminal prior study, they showed increased proliferation of hematopoietic stem and progenitor cells (HSPCs) when enriched in cellular cholesterol (Yvan-Charvet et al., 2010). Cholesterol accumulation was enhanced in this study because of an inability of the cells to efflux the sterol as a result of engineered deficiency of the ABC transporters ABCA1 and ABCG1. Subsequent studies demonstrated that the most efficient removal of cholesterol from the HSPCs requires the interaction of cell autonomous apoE (rather than circulating apoE) with the cell surface ABC transporters. This was shown in competitive bone marrow transplant experiments. The cell surface of the cholesterol loaded HSPCs are enriched in the common β-subunit of the IL-3/GM-CSF receptors. GM-CSF is produced by macrophages, T cells and by innate response activator B cells. The latter cells are expanded in secondary lymphoid organs in the context of hypercholesterolemia (Hilgendorf et al., 2014).

In much of the literature it is assumed that the macrophage is loaded with lipid once sequestered in the atherosclerotic lesion. However, recently it has been suggested that murine monocytes may acquire their initial lipid load while still in the circulation (Xu et al., 2015). Hypercholesterolemia in *Apoe*$^{-/-}$ mice results in lipid loading of blood monocytes, producing foamy monocytes. The foamy monocytes are positive for the expression of CD36 and CD11c, which correspond to Ly6C$^{lo}$ monocytes. These monocytes were shown to enter nascent atherosclerotic lesions. This seems to be contrary to the prevailing literature, which concluded that the inflammatory Ly6C$^{hi}$ monocytes represent the major monocyte subclass contributing to the evolving atherosclerotic plaque (Swirski et al., 2007). Of course, it is necessary to consider the stage of atherogenesis under study. Interestingly, Combadiere et al. (2008) reported that the extent of aortic root lesions in *Apoe*$^{-/-}$ mice correlated not only with total blood monocyte levels, but also with the level of Ly6C$^{lo}$ monocytes. To what extent this is a function of the prosurvival signals exhibited by the CX3CR1 expressed by Ly6C$^{lo}$ monocytes remains to be established (Landsman et al., 2009). In the normal artery wall, the resident macrophages mainly have an M2-like phenotype and are found in the adventitia. In early foam cell lesions in the *Apoe*$^{-/-}$ model, M2-like and M1-like macrophages are present in approximately equal numbers, while in advanced lesions M1-like macrophages predominate (Khallou-Laschet et al., 2010; Koltsova et al., 2013). The two subsets are not evenly distributed in human atherosclerotic lesions. M1-like macrophages are located in the shoulders and in the vicinity of the necrotic core that are associated with plaque rupture, with few M2 macrophages in these regions (Stoger et al., 2012). Khallou-Laschet et al. (2010) have suggested that M2 macrophages of the early lesions may be converted to M1 macrophages as the lesions progress, though this suggestion is not universally accepted (Peled and Fisher, 2014). The precise origin and role of the major individual monocyte/macrophage subsets in the evolution of the atherosclerotic plaque is not fully clarified despite a good deal of effort from several laboratories.

## Other Anti-atherogenic Functions of ApoE

While apoE has a major role in the regulation of macrophage cholesterol homeostasis, it also has other anti-atherogenic activities. ApoE is considered to be anti-inflammatory. One mechanism by which apoE may exert its anti-inflammatory function is by promoting the dominance of M2 macrophages, operating through the engagement of either the cell surface VLDL receptor or the apoE receptor 2 on macrophages (Baitsch et al., 2011). This is mediated in part by activating p38 MAP kinase signaling. Ly6C$^{lo}$ monocytes express higher levels of apoE than do Ly6C$^{hi}$ monocytes (Li et al., 2015). This probably extends also to their derived macrophages. M2 macrophages are also relatively enriched in the efferocytosis bridge molecule MerTK (DeBerge et al., 2017), suggesting that these cells in particular may be responsible for the uptake of apoptotic cells derived from free cholesterol overloaded pro-inflammatory macrophages. The pro-inflammatory M1 macrophages express high levels of NFκB while the M2 macrophages are rich in LXR and PPARγ that drive the production of apoE and the ABC transporters responsible for cholesterol efflux (Adamson and Leitinger, 2011). ApoE has also been shown to reduce lipid oxidation and the activation of endothelial cells, suppress innate immunity (Bouchareychas and Raffai, 2018) and reduce the migration and proliferation of smooth muscle cells in the intima (Swertfeger and Hui, 2001; Swertfeger et al., 2002). Recently an additional mechanism has been described accounting for the anti-inflammatory action of apoE (Li et al., 2015). In macrophages apoE induces miR-146a, which inhibits TRAF6 and IRAK 1 and hence NFκB activation. Thus, macrophage apoE has a multitude of actions that can impact atherogenesis only some of which are dependent on its influence on lipid homeostasis.

# MACROPHAGE MYELOPEROXIDASE REDUCES THE CHOLESTEROL EFFLUX PROMOTING ABILITY OF APOA-I

ApoA-I is the major apoprotein found on HDL and one of the major anti-atherogenic functions of apoA-I/HDL is the promotion of cholesterol efflux and reverse cholesterol transport. They also have anti-inflammatory and anti-oxidative properties. Unlike apoE, no significant amount of apoA-I is produced by macrophages. However, plasma HDL can enter the artery wall to promote cholesterol efflux following interaction with ABCG1 on macrophages foam cells. In addition, small amounts of apoA-I may be displaced from the HDL and exist as lipid-poor particles that can promote cholesterol efflux from macrophage foam cells via interaction with ABCA1 to generate nascent HDL or pre-β HDL. These nascent HDL particles can promote further cholesterol efflux from macrophages following interaction with cell surface ABCG1 (Rosenson et al., 2016). Macrophages can influence the functionality of apoA-I. Macrophages, especially M2 macrophages, as well as neutrophils and monocytes release myeloperoxidase, an enzyme that has the capacity to oxidize methionine, tryptophan, and tyrosine residues in apoA-I. The

myeloperoxidase oxidized apoA-I exhibits reduced cholesterol efflux capacity both in culture and *in vivo* (Zheng et al., 2004; Hewing et al., 2014), likely due to impaired interaction with ABCA1. Oxidation of tryptophan 72 appears to account for about 50% of its impaired function (Huang et al., 2014). When oxidized apoA-I is added to the plasma, the modified apoprotein does not bind well to HDL and is found in the lipid-poor fraction (Hewing et al., 2014). Lipid-poor apoA-I is more rapidly cleared from the plasma than is HDL associated apoA-I and this rapid removal could contribute to the low efficacy of modified apoA-I in promoting cholesterol efflux *in vivo* and to the relatively low extent of modified apoprotein found in the plasma. Interestingly, tryptophan 72 oxidized apoA-I is present in lipid-poor form in human atherosclerotic plaques at ∼1,000-fold higher levels than in the plasma. Tyrosine 166 in apoA-I is nitrated by myeloperoxidase and, like the oxidized tryptophan 72 containing protein, lipid-poor apoA-I containing this myeloperoxidase modified amino acid is enriched in the arterial wall lesions compared to the plasma (DiDonato et al., 2014). Thus, it is likely that, at least from the point of view of atherosclerosis, lipid-poor apoA-I is oxidized by macrophage derived myeloperoxidase in the artery wall resulting in impaired reverse cholesterol transport.

# THE INFLUENCE OF SAA ON MACROPHAGES

Serum amyloid A (SAA) and apoE have some broad similarities in properties, though not in detailed functions. They both contain multiple amphipathic helices, though different in overall structure. There are multiple isoforms of both apoE and SAA in humans, but in other species only SAA has multiple isoforms. Both proteins are lipoprotein associated; VLDL and HDL for apoE, and predominantly HDL for SAA. They are both primarily synthesized by the liver, though other cells, including macrophages, are capable of producing the proteins. Finally, both have the capacity to bind proteoglycans. While the range of concentrations of apoE in the plasma is modest, that of SAA is dramatic. It is present in plasma at quite low levels under basal conditions but is greatly increased in conditions of acute inflammation, the so-called acute phase reaction. In situations of chronic inflammation, such as atherosclerosis, plasma levels are modestly increased over basal levels. Indeed, its level in the plasma of patients with cardiovascular disease is useful as a biomarker of cardiovascular disease risk, at least as useful as C-reactive protein (Johnson et al., 2004).

The SAA isoforms are encoded by four genes. SAA1 and SAA2 are acute phase proteins. Their synthesis is stimulated by the cytokines IL-6 and TNFα and their plasma levels are notably elevated (∼1,000 fold) in acute inflammation. SAA1 and SAA2 are neighboring genes that are coordinately transcribed and the mature proteins differ in only 6 of 104 residues. In humans SAA4 is constitutively expressed and SAA3 is a pseudogene. However, in mice SAA3 is expressed, mostly in adipose tissue. Modeling of SAA1 based on structural studies describes a protein that has 4 helices that appear to have different functions. Helices 1 and 3 bind to HDL, helix 2 serves as a bridge between HDL and fibronectin and laminin and helix 4 serves as a bridge between HDL and proteoglycans (Frame and Gursky, 2017). While SAA1/2 are primarily associated with HDL in the acute phase, SAA turns over more rapidly than apoA-I and apoA-II, the major proteins of HDL, suggesting that the lipoprotein does not turnover as an intact particle (Kim et al., 2016).

## SAA and Reverse Cholesterol Transport

During acute inflammation there is a reduction in the reverse cholesterol transfer of macrophage cholesterol via the plasma to feces (McGillicuddy et al., 2009), but this reduction is accounted for by SAA in acute phase HDL only to a limited extent (de Beer et al., 2013). Scavenger receptor class B type I (SR-BI) is a cell surface receptor responsible for selective cholesteryl ester uptake from HDL and both HDL associated SAA and lipid-poor SAA bind to this receptor. But lipid-poor SAA inhibits the ability of SR-BI to promote selective cholesteryl ester uptake (Cai et al., 2005). In accord with the limited role of SAA in reverse cholesterol transport, knocking out SR-BI in mice has limited impact on reverse cholesterol transport (Wang et al., 2007). Thus, these pathways do not have a large quantitative role on the reduced reverse cholesterol transport observed in acute inflammatory states. Given the complexity of the proteome of HDL (Pamir et al., 2016), and the large changes in the composition of acute phase HDL (Vaisar et al., 2015), as well as in LCAT and in ABC transporters during the acute phase (Feingold and Grunfeld, 2010), other possibilities may be entertained. Nonetheless, the study of HDL from several inbred mouse strains under basal conditions revealed an inverse correlation of SAA1 levels on the HDL and its ability to promote ABCA1 dependent cholesterol efflux (Pamir et al., 2016).

## Pro-inflammatory Properties of SAA

Lipid-poor SAA has multiple pro-inflammatory actions that are mediated by a variety of cell surface receptors, including TLR2, TLR4, CD36, FPR2, RAGE, and P2XY (Eklund et al., 2012). The activation of macrophages by SAA stimulates the release of IL-8 and MCP-1 that function to attract neutrophils and monocytes, respectively into sites of inflammation. The secretion of other cytokines and factors known to promote atherogenesis are also increased. The lipid-poor SAA-mediated increase in IL-1β secretion from macrophages appears to be due to increased potassium efflux, cathepsin B activation and reactive oxygen species generation leading to the activation of the NLRP3 inflammasome (Shridas et al., 2018). All of these effects are attenuated by the addition of HDL, possibly due to the sequestering of the lipid-poor SAA. How lipid-poor SAA is generated in tissues such as the artery wall is not clear since little lipid-poor SAA is detected in plasma.

SAA plays a role in the retention of HDL in the arterial wall of atherosclerotic *Apoe*$^{-/-}$ and *Ldlr*$^{-/-}$ mice (O'Brien et al., 2005). SAA containing HDL is retained by binding to proteoglycan, such as biglycan. This results in segregation of the HDL from the cell surface of the macrophage foam cells of the lesion and hence a lower capacity to mediate cholesterol efflux from these cells. Consistent with this, HDL derived from SAA knockout mice

has a much lower capacity for binding vessel wall proteoglycans and an enhanced cholesterol efflux potential (Chiba et al., 2011). Thus, the cholesterol efflux capacity of the intravascular HDL containing SAA is reduced. ApoE is also able to bind to proteoglycan, but in the context of the inflammation associated with atherosclerosis, HDL carries more SAA than apoE.

## SAA and Atherosclerosis

Based on the pro-inflammatory properties of SAA discussed above, one would expect that the overexpression of SAA would augment atherosclerosis and its removal would reduce lesion development. Indeed, lentivirus-mediated overexpression of murine SAA1 increased total aortic atherosclerosis and aortic root lesion in *Apoe*$^{-/-}$ mice (Dong et al., 2011). This was correlated with increased macrophage content of the lesions and an elevation of MCP-1 expression. The development of mice lacking the two major acute phase SAAs (*Saa1/2*$^{-/-}$ mice) has facilitated further studies on the role of SAA. Contrary to expectations, the absence of SAA1/2 in chow fed *Apoe*$^{-/-}$ mice had no effect on atherosclerosis when the vessels were examined at 50 weeks of age (De Beer et al., 2014). The absence of SAA1/2 in the *Ldlr*$^{-/-}$ model fed the Western type diet revealed a reduction of early lesion development in the ascending aortic arch (after 6 weeks of diet), which was no longer evident when the diet was extended to 12 weeks (Krishack et al., 2015). No changes were seen in the other arterial sites at either time. Reciprocal transplantation between *Ldlr*$^{-/-}$ and *Ldlr*$^{-/-}$ *Saa1/2*$^{-/-}$ mice indicates that SAA derived from both systemic production and bone marrow derived cells participate in the early atherosclerosis phenotype. A reduction in atherosclerosis was also observed when the SAA receptor FPR2/ALX was knocked out in the *Ldlr*$^{-/-}$ background (Petri et al., 2015).

The SAA3 isoform may also have a role in murine atherogenesis. The overexpression of SAA3 using adeno-associated virus in *Apoe*-/- mice was associated with an increment in atherosclerosis. In addition, the administration of SAA3 antisense oligonucleotides to *Apoe*$^{-/-}$ *Saa1/2*$^{-/-}$ mice reduced aortic root lesion area. Thus, SAA3, a minor acute phase reactant, is pro-atherogenic (Thompson et al., 2018).

## SAA and Monocytosis

Interestingly, the induction of hyperlipidemia in the *Ldlr*$^{-/-}$ *Saa1/2*$^{-/-}$ mice by feeding a Western type diet lead to an increase in total blood monocytes. Most of this increase was attributable to the level of Ly6C$^{lo}$ monocytes and not Ly6C$^{hi}$ monocytes that have been found to be associated with hyperlipidemia induced monocytosis and increased atherosclerosis (Swirski et al., 2007; Krishack et al., 2016). No change in blood monocyte levels or subclass distribution was observed in chow fed *Ldlr*$^{-/-}$ *Saa1/2*$^{-/-}$ mice indicating that the regulation of monopoiesis is the result of an interaction of SAA status and hyperlipidemia. It is notable that the levels of neutrophils and lymphocytes were not altered. An increase in total monocytes, due largely to increased levels of the Ly6C$^{hi}$ monocyte subset, was noted in the bone marrow of the hyperlipidemic *Ldlr*$^{-/-}$ *Saa1/2*$^{-/-}$ animals. This was accompanied by an increase in the monocyte precursor cells macrophage-dendritic progenitor cells (MDP) and its product CDP. Importantly no change was observed in the most primitive precursor cell (HSPC). This is important because this observation, along with the normal neutrophil count, tends to suggest that the increase in monocytes is not the result of a dysfunction in cholesterol homeostasis in the entire hematopoietic system. The reconciliation of the blood monocytosis, especially the higher Ly6C$^{lo}$ monocytes, with the bone marrow findings requires further study. Such questions as (a) whether both subsets of monocytes are released from the bone marrow at similar rates resulting in a higher Ly6C$^{hi}$ monocyte in the blood; (b) whether Ly6C$^{hi}$ monocytes are rapidly converted to Ly6C$^{lo}$ monocytes in the circulation in the presence of low SAA1/2 level; (c) while it is known that Ly6C$^{hi}$ monocytes leave the blood more rapidly than Ly6C$^{lo}$ monocytes, it is not known if and how SAA may influence this process; and (d) does SAA influence the expression and activity of NR4A1, a transcription factor involved in the survival of Ly6C$^{lo}$ monocytes (Hanna et al., 2011). The relative rates of recovery of monocytes and its subsets after clodronate depletion would be of considerable interest in answering these questions.

# THE ROLE OF MIMETIC PEPTIDES DERIVED FROM THE THREE APOPROTEINS

## ApoA-I Mimetic Peptides

The three apoproteins that are the focus of this review all contain amphipathic α-helical domains. Of these proteins, apoA-I has the most regular repeating helical structure. Mature human apoA-I contains 243 amino acids, with the last 199 amino acids arranged in 10 amphipathic α-helices. Eight of the helices contain 22 amino acids, while the remaining 2 have 11 amino acids. Each helix has a hydrophobic face and a hydrophilic face. Positively charged residues are located at the boundary between the two faces and the negatively charged residues are on the hydrophilic face. Considering the regularity of the amphipathic α-helices in apoA-I, Segrest and colleagues devised an idealized helical peptide from the average components of its eight 22 amino acid helices (**Figure 2**). This ideal helix contained 18 amino acids, designated 18A, and does not contain the amino acids linking adjacent helices (Anantharamaiah et al., 1985). The helicity and stability of the 18A peptide is increased by N-terminal acylation and C-terminal amidation. The 18A peptide replicated the physical structure of the apoA-I α-helices but does not have any sequence homology to any specific helix. Many variants of the model peptide have been developed which mostly modify the hydrophobic face. As 18A contains 2 phenylalanine residues on its hydrophobic face, it has also been designated 2F. The substitution of the hydrophobic residues valine and leucine in 2F by phenylalanine leads to variants of the peptide designated by the number of phenylalanine residues in the peptide. Up until recently the variant most studied is 4F. As the hydrophilic face contains lysine residues, these peptides synthesized with L-amino acids are susceptible to trypsin-like proteolysis, especially when administered by the oral route. The comparison of various doses

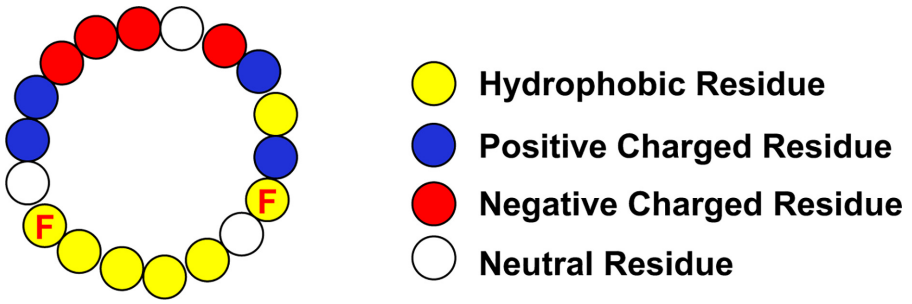


**FIGURE 2 |** Idealized Apo-I Amphipathic α-Helix. The location of the phenylalanine residues (F) on the hydrophobic face are indicated.

of the 4F peptide composed of D-amino acids, and hence resistant to proteolysis, administered either orally or intraperitoneally to mice has generated the hypothesis that a primary site of action of the mimetic peptide is on the small intestine where it modulates the level of bioactive lipids (Navab et al., 2011, 2012). How this relates to macrophage biology in the artery wall is not clear.

Among the atheroprotective functions attributed to apoA-I are its ability to promote cholesterol efflux and participate in multiple steps in reverse cholesterol transport, its anti-oxidative capability due to its ability to sequester oxidized lipids and related to this last function is its capacity to inhibit the chemotaxis of monocytes. Many of the variant mimetic peptides exhibit these functions of apoA-I in *in vitro* assays (Getz et al., 2010). To some extent some of these peptides, by orders of magnitude, are much more powerful on a molar basis than is apoA-I. This is particularly the case for their capacity to bind oxidized fatty acids and phospholipids (Van Lenten et al., 2008b). Both 2F and 4F promote cholesterol efflux (**Table 2**) and are equally effective in binding macrophage ABCA1 and promoting its stabilization (Tang et al., 2006). The peptides also activate JAK2 autophosphorylation that promotes phosphorylation of STAT3 (Liu and Tang, 2012), resulting in decreased secretion of chemokines and cytokines by macrophages. 4F also reduces lipid rafts and Toll-like receptor 4 levels on the surface of macrophages (Smythies et al., 2010). However, 4F, but not 2F, is atheroprotective (Getz et al., 2010). The intraperitoneal administration of 4F peptide reduces early nascent atherosclerosis in *Apoe*$^{-/-}$ and *Ldlr*$^{-/-}$ mice (Navab et al., 2002; Wool et al., 2011) but has little effect on mature lesions in 28 week old animals (Wool et al., 2011).

The studies so far described used monohelical peptides. Since all neighboring helices in intact apoA-I may function co-operatively, tandem amphipathic α-helical peptides have also been studied. Two 18A peptides joined with a single proline residue, designated 37pA, is almost as active as 2F in promoting ABCA1 dependent cholesterol efflux (Tang et al., 2006). But two 4F helices joined by either a single proline or alanine residue or a 7 amino acid sequence derived from the interhelical region between helices 4 and 5 are all more active in facilitating cholesterol efflux than are the monohelical peptides 2F and 4F (Wool et al., 2008). These tandem amphipathic helices are symmetric. However, since the adjacent helices in apoA-I do not have identical sequences, asymmetric tandem peptides have also been studied. Remaley and colleagues generated a peptide, designated 5A, in which the 2F peptide is joined by a single proline residue to a second helix in which 5 of the hydrophobic residues in 2F are replaced by alanine to reduce its lipid affinity (Sethi et al., 2008). This tandem peptide promoted ABCA1 dependent cholesterol efflux with higher specificity than 37pA. Interestingly if the alanine substituted helix is placed at the N-terminal position of the tandem peptide (5A-2 peptide) rather than at the C-terminus its activity in facilitating cholesterol efflux is very much attenuated.

The C-terminal helix (helix 10) of human apoA-I is the helix most responsible for the capacity of the apoprotein to bind lipid (Palgunachari et al., 1996). Ghadiri and colleagues have fashioned a peptide consisting of three copies of this last helix coupled to a bridge scaffold. Even though it is constructed of L-amino acids it is resistant to proteolysis (Zhao et al., 2013, 2014). Indeed, when administered orally to mice, very little peptide is detectable in the

**TABLE 2 |** Apoprotein Mimetic Peptides.

| Apoprotein | Mimetic Peptide | Properties |
|---|---|---|
| ApoE | ATI-5361 (residues 238–266) | Promotes cholesterol efflux |
| | | Atheroprotective |
| | *monomers* | |
| ApoA-I | 2F | Promotes cholesterol efflux |
| | | Binds and stabilizes ABCA1 |
| | | No effect on atherosclerosis |
| | 4F | Promotes cholesterol efflux |
| | | Binds and stabilizes ABCA1 |
| | | Atheroprotective (early lesions) |
| | dimers | |
| | 4F-dimers | More active than monomer in promoting cholesterol efflux |
| | 5A (asymmetrical 2F helices) | More active than monomer or symmetrical 2F peptide (37pA) in promoting cholesterol efflux |
| SAA | SAA2.1 (residues 1–20) | Inhibits acyl cholesterol acyl transferase activity |
| | SAA2.1 (residues 74–103) | Activates neutral cholesteryl ester hydrolase |
| | | Promotes cholesterol efflux |
| | | Atheroprotective |

plasma, suggesting that like D4F its action may be on the intestine (Wool et al., 2014). This trimeric peptide was incorporated into DMPC nanoparticles for daily administration to *Ldlr*$^{-/-}$ mice for 10 weeks along with a Western type diet. This treatment was effective in lowering plasma cholesterol levels, facilitating cholesterol efflux and reducing atherosclerosis in both the whole aorta and the aortic root (Zhao et al., 2014). Interestingly when the monomeric helix was similarly used as nanoparticles for treatment, it was almost as effective as the trimeric peptide.

Other peptides containing the C-terminal helices of human apoA-I are also effective in promoting cholesterol efflux from lipid loaded macrophages. A 33 amino acid peptides containing helix 9, an 11 amino acid helix, with helix 10 (9/10 peptide) or helix 1 (1/9 peptide) are effective in promoting cholesterol efflux (Natarajan et al., 2004). Helix 9 appears to be important for this functionality, though it is not sufficient since the 10/9 peptide with the helices reversed is less effective than the 9/10 peptide and peptides in which helix 9 is joined with other 22 amino acid apoA-I helices are totally ineffective. The apoA-I sequences in inbred strains of mice are not all identical as exemplified in the comparison of apoA-I of the atherosensitive C57BL/6 strain and the atheroresistant FVB/N strain. The proteins differ in their C-termini by two amino acids: Q225K and V226A, C57BL/6 and FVB, respectively. While it is unlikely that these differences play an important role in the relative atherosusceptibility of the two strains, the 9/10 tandem helices of C57BL/6 apoA-I is much more effective in promoting cholesterol efflux from cholesterol loaded macrophages (Sontag et al., 2014).

## ApoE Mimetic Peptides

As discussed above, apoE promotes cholesterol efflux from macrophages. The C-terminus, which contains amphipathic α-helices, is particularly important in its efflux capacity (Vedhachalam et al., 2007). The 26 amino acids encompassing residues 238–266 has been used as the basis for an apoE mimetic peptide designated ATI-5361 (Bielicki et al., 2010; Hafiane et al., 2014). This peptide promotes cholesterol efflux from lipid loaded macrophages *in vitro* and macrophage to feces reverse cholesterol transport *in vivo*. It also reduces atherosclerosis in *Apoe*$^{-/-}$ mice. Because this peptide induced muscle toxicity, a variant of this peptide with substitution of phenylalanine for leucine residues and arginine for citrulline residues was generated. CS-6253 and ATI-5367 have similar *in vitro* properties, including the ability to promote cholesterol efflux, but CS-6253 is not toxic *in vivo* (Hafiane et al., 2015).

## SAA Mimetic Peptides

Peptides derived from SAA have also been reported to be able to enhance macrophage cholesterol efflux (Kisilevsky and Tam, 2003; Tam et al., 2005). An N-terminal peptide (residues 1–20) of SAA2.1, but not SAA1.1, inhibits acyl cholesterol acyl transferase in macrophages, while a C-terminal peptide (residues 74–103) of the same isoform activates neutral cholesteryl ester hydrolase. The net result of the modulation of these enzymatic activities is to liberate free cholesterol from the stored cholesteryl esters in the macrophages, which is then available for efflux to acceptors. Similar effects on these two enzymes and cholesterol efflux is observed with acute phase HDL and SAA2.1 liposomes (Tam et al., 2002). The treatment of *Apoe*$^{-/-}$ mice with a liposomal formulation of these two peptides reduces and reverses lesion formation (Tam et al., 2005).

# CONCLUSION

In this review we have discussed the interaction of three HDL apoproteins, apoE, apoA-I and SAA and the mimetic peptides derived from them, with macrophages *in vitro* and *in vivo*. Much of the action of the proteins and peptides is focused on the regulation of macrophage cholesterol homeostasis. But they have other effects, some of which are independent of cholesterol metabolism. Further work is required to distinguish among these various functions and their cellular interactions with respect to the development of atherosclerosis. The peptides offer the opportunity to explore structure-function of apoprotein interactions with the cells and their progenitors, although it has to be realized that not all of the apoprotein properties are replicated by these small molecules. Although much of this review has been concerned with atherosclerosis, it is notable that at least the apoA-I mimetics have been shown to be useful as potential therapies for other inflammatory disorders, such as respiratory inflammations, intestinal inflammation, chronic arthritis, and even some cancer models (Van Lenten et al., 2008a; Cedo et al., 2016).

# AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REVIEW
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# G-Protein Coupled Receptor Targeting on Myeloid Cells in Atherosclerosis

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Atherosclerosis, the underlying cause of the majority of cardiovascular diseases (CVDs), is a lipid-driven, inflammatory disease of the large arteries. *Gold standard* therapy with statins and the more recently developed *proprotein convertase subtilisin/kexin type 9* (PCSK9) inhibitors have improved health conditions among CVD patients by lowering *low density lipoprotein* (LDL) cholesterol. Nevertheless, a substantial part of these patients is still suffering and it seems that 'just' lipid lowering is insufficient. The results of the *Canakinumab Anti-inflammatory Thrombosis Outcome Study* (CANTOS) have now proven that inflammation is a key driver of atherosclerosis and that targeting inflammation improves CVD outcomes. Therefore, the identification of novel drug targets and development of novel therapeutics that block atherosclerosis-specific inflammatory pathways have to be promoted. The inflammatory processes in atherosclerosis are facilitated by a network of immune cells and their subsequent responses. Cell networking is orchestrated by various (inflammatory) mediators which interact, bind and induce signaling. Over the last years, G-protein coupled receptors (GPCRs) emerged as important players in recognizing these mediators, because of their diverse functions in steady state but also and specifically during chronic inflammatory processes – such as atherosclerosis. In this review, we will therefore highlight a selection of these receptors or receptor sub-families mainly expressed on myeloid cells and their role in atherosclerosis. More specifically, we will focus on chemokine receptors, both classical and atypical, formyl-peptide receptors, the chemerin receptor 23 and the calcium-sensing receptor. When information is available, we will also describe the consequences of their targeting which may hold promising options for future treatment of CVD.

**Keywords: G-protein coupled receptors, myeloid cells, cardiovascular disease, atherosclerosis, therapy**

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## INTRODUCTION

### General Pathology of Cardiovascular Diseases

Cardiovascular diseases (CVDs), with myocardial infarction (MI) and stroke as most common clinical manifestation, remain the leading cause of death worldwide (Hansson, 2005), underpinning the importance of further research into and development of novel therapeutic approaches.

Atherosclerosis, a lipid-driven chronic inflammatory disease, has been recognized as the main underlying cause of CVD (Ross, 1999; Hansson, 2005; Braunersreuther et al., 2007a). Endothelial damage by hemodynamic shear stress is a main initiator of atherosclerosis formation, resulting in increased endothelial permeability and hence increased susceptibility for lipid infiltration (Hansson et al., 2015). This damage enables the infiltration of various lipids, like low-density lipoprotein (LDL), into the intima where it is subsequently modified into oxidized LDL (oxLDL) (Ross, 1999; Braunersreuther et al., 2007a). Together with the endothelial damage, accumulation of these modified lipids triggers an inflammatory response, resulting in progressive inflammatory cell infiltration into the sub-endothelial layer (Ross, 1999; Braunersreuther et al., 2007a). During this mobilization stage (**Figure 1**), predominately monocytes will bind to adhesion molecules on the activated endothelium and subsequently infiltrate into the vessel wall by transmigration (Schumski et al., 2018). Infiltrated monocytes subsequently differentiate into macrophages which phagocyte cell debris and oxLDL, resulting in the formation of foam cells (Hansson, 2005; Charo and Ransohoff, 2006). As foam cells also trigger inflammation by releasing cytokines and chemokines, a vicious circle is created resulting in the continued recruitment and mobilization of leukocytes to the vascular wall. This results in the formation of so called fatty streak lesions which will continue to develop and grow over time. During this progression stage, activated lesional macrophages also secrete matrix metalloproteinases that can digest extracellular matrix components, leading to plaque destabilization. In the end, macrophages become apoptotic due to the continued lipid accumulation and contribute to the formation of necrotic cores (Moore and Tabas, 2011). Besides monocytes and macrophages, also neutrophils have been described to play an important role in the development of atherosclerotic lesions. It has been shown that neutrophils can influence almost every step of this pathology, including endothelial dysfunction, monocyte recruitment, foam cell formation and plaque destabilization (Döring et al., 2015). Eventually, plaque growth or the rupture of lesions resulting in atherothrombosis can cause the artery to occlude. This occlusion will cause ischemia in downstream tissues, resulting in cardiovascular events like stroke or MI (Hansson, 2005).

## Classical CVD-Therapies

Cardiovascular disease-therapy is mostly focussing on mitigation of hyperlipidemia (statins) and management of thrombotic factors (aspirin) to prevent further progression of the disease. Statins are inhibitors of the HMG-CoA reductase, thereby reducing the production of cholesterol and the current golden CVD-therapy (Okopien et al., 2016). A recent meta-analysis of several statin clinical trials indeed confirmed that statin use clearly reduces plasma LDL levels (up to 55–60%) and thereby also resulted in significant reductions in cardiovascular risk (Boekholdt et al., 2014). However, as with a lot of therapies there are also off-target side effects due to the use of statins. It has been shown that statin treatment results in a striking 9% increased risk for the development of diabetes (Preiss et al., 2011). This has led to a debate about the use of statins and especially fuelled the development of adequate alternatives. One of these intriguing new players in the field of hyperlipidemia therapy is monoclonal antibodies against proprotein convertase subtilisin/kexin type 9 (PCSK9). In a physiological condition, PCSK9 interacts with the LDL receptor in the liver to stimulate its degradation and additionally prevention its recycling to the cell membrane (Cohen et al., 2005). Inhibiting PCSK9 thus results in an increased surface expression of LDL receptors that are capable of binding and internalizing LDL particles, thereby reducing the plasma LDL levels. The great potential is demonstrated by the fact that PCSK9 inhibition can cause a 60% reduction of LDL, even on top of the LDL lowering due to statin use, without any indications of serious side effects (Robinson et al., 2015; Stone and Lloyd-Jones, 2015; Zhang et al., 2015). The only major drawback of these monoclonal antibodies is the fact that the production is still very costly and therefore wide-scale usage is not yet feasible.

## Novel CVD-Therapies

Besides above described therapies focussing on lipid modulation, immunomodulation has emerged during the last decades as a promising therapeutic option. Accumulating evidence especially supports the beneficial role of interleukin-1β (IL-1β), tumor necrosis factor (TNF) and IL-6 inhibition (Ridker and Luscher, 2014). All of these cytokines are part of a common pathway. IL-1β is initially produced as an inactive precursor and therefore requires proteolytic cleavage which is mediated by the nucleotide-binding leucine-rich repeat-containing pyrin receptor 3 (NLRP3) inflammasome (Strowig et al., 2012). Inhibition of IL-1β using the monoclonal antibody canakinumab results in the significant reduction of plasma IL-6 and high-sensitivity C-reactive protein (hsCRP) levels, without lowering LDL cholesterol (Ridker et al., 2012). The effect of IL-1β targeting on cardiovascular risk has recently been evaluated in the Canakinumab Anti-inflammatory Thrombosis Outcome Study (CANTOS) trial. This randomized, double-blind, placebo-controlled trial involving stable patients with previous MI showed that canakinumab was effective in reducing plasma hsCRP levels and preventing adverse cardiac events (Ridker et al., 2017). Although this study shows great promise of immunomodulatory therapies, the use of canakinumab was associated with an increased risk of fatal infection or sepsis, despite the exclusion of patients with chronic or recurrent infection. Therefore, more elaborate studies are needed to elucidate the mechanism behind these adverse side effects in order to develop a more specific targeting approach.

## GPCRs as Novel Therapeutic Targets

Although several novel therapies have been explored over the last years, atherosclerosis still cannot be fully reversed by medical treatment, warranting the necessity of innovative therapeutic approaches. Recently, G-protein coupled receptors (GPCRs) have emerged as promising pharmacological targets because of their diverse functions. This is also highlighted by the fact that several recent reviews discussed the targeting of GPCRs in atherosclerosis in a rather general setting (Desimine et al., 2018; Pirault and Back, 2018; Tang et al., 2018; Gencer et al., 2019; Noels et al., 2019). Therefore, in this review we will restrict ourselves to the discussion of the role of GPCRs on myeloid cells

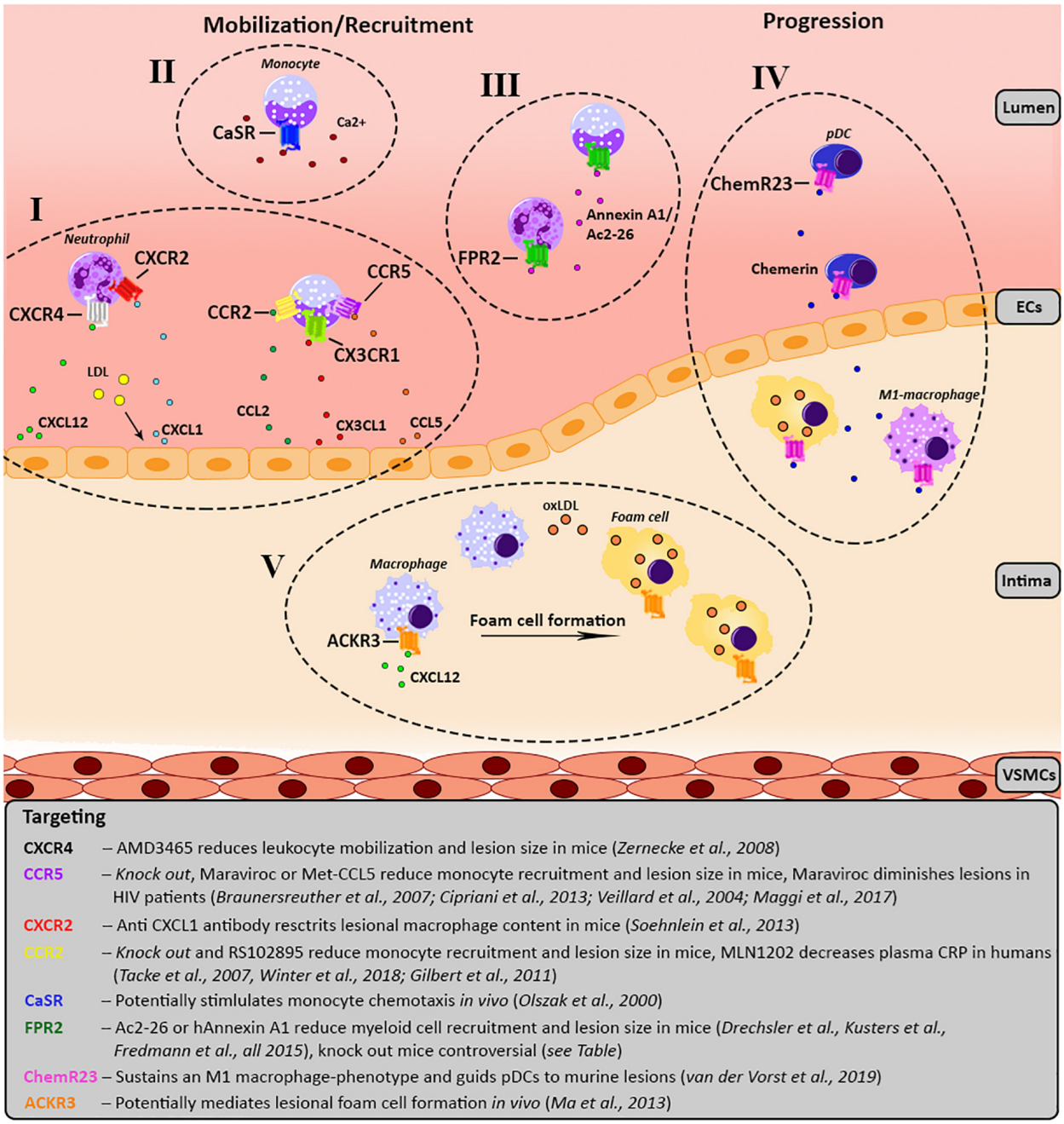


**FIGURE 1 |** Schematic overview of the involvement of the various GPCRs in atherosclerosis development. For each receptor the key processes, as well as agonists/antagonists are summarized and depicted over three main phases of atherosclerosis development; mobilization, leukocyte recruitment and plaque progression. Receptors of the same GPCR-subfamily are clustered together and categorized from I till V. **(I)** Chemokine receptor CXCR4 causes migration of leukocytes toward its ligand CXCL12. Additionally, upon LDL stimulation CXCL1 is released by endothelial cells causing myeloid cells, which carry CXCR2 on their surface to migrate toward the endothelium. CCL2 and CX3CL1 mediate the recruitment of monocytes expressing CCR2 and CX3CR1, respectively. In line with this, monocytes expressing CCR5 are recruited to the lesion by CCL5. **(II)** Monocytes show a CaSR-dependent increase of chemotaxis toward CCL2 upon stimulation with calcium. **(III)** FPR2 is mostly expressed on myeloid cells and has several contradictory effects, please see **Table 1**. FPR2-agonists like Ac2-26, an Annexin A1 peptide, and Annexin A1 reduce monocyte/neutrophil recruitment. **(IV)** ChemR23 maintains a M1 macrophage phenotype and stimulates pDC migration and infiltration into atherosclerotic plaques. **(V)** ACKR3 expression is upregulated in lesional macrophages which engulf modified lipids resulting in foam cell formation. ACKR3, atypical chemokine receptor 3; CAD, coronary artery disease; CaSR, calcium-sensing receptor; CCR, C-C chemokine receptor; CCL, C-C chemokine ligand; ChemR23, chemerin receptor 23; CRP, C-reactive protein; CXCR, C-X-C chemokine receptor; CX3CR1, CX3C chemokine receptor 1; CXCL, C-X-C chemokine ligand; CX3CL1, CX3C chemokine ligand 1; FPR2, formyl-peptide receptor 2; HIV, human immunodeficiency virus; LDL, low-density lipoprotein; oxLDL, oxidized LDL; pDC, plasmacytoid dendritic cell.

in atherosclerosis and CVD and their potential targeting (please also refer to **Figure 1**).

# G PROTEIN COUPLED RECEPTORS (GPCRs)

## General Overview and Classification

G-protein coupled receptors, also known as seven-transmembrane domain receptors or heptahelical receptors constitute, with at least 800 members, the largest family of cell surface receptors (Gloriam et al., 2007; Trzaskowski et al., 2012). These various names can be explained by the fact that these receptors all pass the cell membrane seven times and couple to G proteins to activate internal signal transduction upon activation. GPCRs can bind a wide variety of endogenous ligands, including neuropeptides, amino acids, ions, hormones, chemokines, lipid-derived mediators and ions (Marinissen and Gutkind, 2001; Lagerstrom and Schioth, 2008; Hazell et al., 2012). However, the exact ligands of several receptors remain to be identified, making them orphan GPCRs (Chung et al., 2008).

Classically, the GPCR family was divided into three main classes (A, B, and C) with no detectable sequence homology between these classes (Bjarnadottir et al., 2006). Over the years several subgroups emerged to create a more detailed classification, for example class A which accounts for almost 85% of all GPCRs has been further subdivided into 19 subgroups (A1–A19) (Joost and Methner, 2002). Additionally, the main classification also increased in diversity, creating six main classes based on sequence homology and functional similarity (A-F system) (Foord et al., 2005). Also alternative classification systems have been created, for example the GRAFS system that subdivides the receptors based on phylogenetic analysis into five groups called Glutamate, Rhodopsin, Adhesion, Frizzled/Taste2, and Secretin (Fredriksson et al., 2003).

## Key Pharmacological Concepts

G-protein coupled receptors have distinct binding sites, whereas the main binding site is called the orthosteric binding site, several distinct sites are also susceptible to ligand-binding and are called allosteric binding sites. Depending on the binding site that is used, the ligands can be given the corresponding term orthosteric or allosteric ligand. Binding of allosteric ligands to the receptor will induce a conformational change that influences the affinity or binding potential of orthosteric ligands in a positive (positive allosteric modulator) or a negative (negative allosteric modulator) manner. Before going into detail by discussing the specific GPCRs or sub-families and their respective ligands, it is important for the comprehension of the reader to have a proper definition of several pharmacological concepts (IUPHAR/BPS; Wacker et al., 2017).

An agonist is a ligand or drug that binds to a receptor and alters the receptor state resulting in a biological response. Conventional agonists increase the receptor activity either to the maximum extent (full agonist) or to less than 100% of the maximal response (partial agonist). In contrast, an inverse agonist reduces the receptor activity. For this, the receptor must elicit intrinsic or basal activity already in the absence of the ligand, as the inverse agonist can only then decrease the activity below this basal level. Antagonists do not produce a biological response upon binding to a receptor, but reduces the action of another drug, generally an agonist. Also here, there are two different subtypes being competitive or non-competitive antagonists. Competitive antagonists bind to the same site as the agonist (usually the orthosteric site) on the receptor without causing activation, but thereby blocking the binding of the agonist. This kind of antagonism is reversible by increasing the concentration of agonist to outcompete the antagonist. However, non-competitive antagonists do not compete directly with the binding of the agonist as they bind to an allosteric site on the receptor, resulting in an irreversible effect.

## GPCR Signaling

In the inactive state, GPCRs are bound to a guanosine diphosphate (GDP) associated heterotrimeric G protein complex ($G_{\alpha\beta\gamma}$) (Rosenbaum et al., 2009). Upon activation, the GPCR will undergo a conformational change which induces cytoplasmic signal transduction by influencing the $G_\alpha$ subunit via protein domain dynamics (Hilger et al., 2018). The activated $G_\alpha$ subunit subsequently exchanges guanosine triphosphate (GTP) in place of GDP, triggering the dissociation of the $G_\alpha$ subunit from the $G_{\beta\gamma}$ dimeric subunit and from the receptor. These two dissociated subunits can then interact with other intracellular effector proteins to further activate various signaling cascades (Digby et al., 2006). $G_\alpha$ subunits especially target effectors like adenylyl cyclases, cGMP phosphodiesterase, phospholipase C (PLC), and RhoGEFs (Kristiansen, 2004; Milligan and Kostenis, 2006), while $G_{\beta\gamma}$ recruit kinases to the membrane and regulate potassium channels, voltage-dependent $Ca^{2+}$ channels, adenylyl cyclases, PLC, phosphoinositide 3 kinase and mitogen-activated protein kinases (Smrcka, 2008; Khan et al., 2013). As $G_\alpha$ has intrinsic GTPase activity, the cellular response is terminated once this subunit hydrolyses GTP again to GDP resulting in the reassociation with $G_{\beta\gamma}$. The induced signaling and thus functional consequences of GPCR activation are highly variable and largely depend on the nature and binding efficacy of the ligand (Maudsley et al., 2005; Woehler and Ponimaskin, 2009; Kenakin and Miller, 2010; Zheng et al., 2010; Ambrosio et al., 2011). Currently, there are at least 20 different $G_\alpha$ subunits identified, which based on structural and functional similarities can be divided into four families, i.e., $G_i$, $G_s$, $G_q$, and $G_{12/13}$ (Simon et al., 1991). Members of the $G_i$ family (e.g., $G_{\alpha i}$, $G_{\alpha t}$, $G_{\alpha z}$) mediate primarily the inhibition of adenylyl cyclase or the activation of phosphodiesterase 6, while members of the Gs family (e.g., $G_{\alpha s}$, $G_{\alpha alf}$) facilitate the activation of adenylyl cyclase. Furthermore, the Gq family members (e.g., $G_{\alpha q}$, $G_{\alpha 11}$, $G_{\alpha 14}$) are known to activate the kinase PLC, while the $G_{12/13}$ family members ($G_{\alpha 12}$ and $G_{\alpha 13}$) activate the Rho family of GTPases. Additionally, also G protein-independent interactions have been demonstrated for GPCRs, mainly with β-arrestins (Beaulieu et al., 2005; Lefkowitz and Shenoy, 2005), resulting in the internalization of the receptor into endosomes followed by degradation or recycling of the receptor (Daaka, 2012). Arrestin coupling can also induce activation of downstream effector

proteins like mitogen-activated protein kinases or SRC kinases. Interestingly, some GPCRs are even able to activate both G protein-dependent as well as G protein-independent signaling (Feng et al., 2005).

## Ligand Bias Theory

The classical view is that the binding of an agonist to a particular GPCR elicits its effects through a single mechanism of activation, suggesting a single activated confirmational state of the receptor (Stephenson, 1956; Black and Leff, 1983). Recently, by measuring broader networks of signals stimulated by agonists, it has become clear that agonists do not only show quantitative differences (e.g., partial or full agonist, fitting in the classical view) but also functional selectivity (e.g., one ligand selectively stimulates one signal whereas another ligand selectively stimulates a second signal via the same receptor) which is not fitting with this classical view (DeWire and Violin, 2011). This gave rise to the concept of ligand bias or also termed biased agonism (Michel and Charlton, 2018), which especially during the past decade received more appreciation and support. The concept of functional selectivity and ligand bias has been comprehensively reviewed elsewhere (Kenakin and Miller, 2010), even in the context of cardiovascular pharmacology (DeWire and Violin, 2011).

## Importance of GPCRs for Therapeutics

Due to the large variety in functional effects mediated by GPCRs, they have been implicated in a multitude of processes that play a crucial role in atherosclerosis development. GPCRs are the most "druggable" receptor class, as a striking 30–35% of all medicines currently on the market target one of these receptors (Hauser et al., 2017; Sriram and Insel, 2018). This is mainly caused by the fact that most GPCRs have small ligands and thus the corresponding binding pockets in these receptors are also small and therefore relatively easy to target. However, especially within the chemokine receptor family difficulties arise when a single receptor can bind multiple ligands. A small molecule blocking the binding site of one of these ligands does not necessary also blocks the binding of all others (Wells et al., 2006). Besides the use of small molecules also different targeting approaches are being used or at least evaluated, like the modification of ligands or antibodies against specific receptors. As Hauser et al. (2017) recently published a very elegant review of trends in GPCR drug discovery further elucidating the various GPCR drugs and agents that are in clinical trials, we will keep the discussion of this rather limited.

## Focus of This Review

In this review, we will highlight a selection of GPCRs or receptor sub-families mainly expressed on myeloid cells and clearly linked to atherosclerosis. The chemokine receptors, both classical and atypical, formyl-peptide receptors (FPRs), chemerin receptor 23 and the calcium-sensing receptor (CaSR) will be described in detail as they have been shown to play an important role in chronic inflammation and atherosclerosis (**Figure 1**). When information is available, we will also describe the consequences of their (therapeutic) targeting in CVD.

# CHEMOKINE RECEPTORS

Chemokines (small chemotactic cytokines) and their receptors are multifunctional operators of the immune system facilitating many vital steps of an immune response, such as leukocyte activation, migration, differentiation, phagocytosis and adhesion in addition to their homeostatic roles, such as leukocyte homing (Johnston and Butcher, 2002; Kim, 2004). Chemokines are classified according to their conserved cysteine residues and bind to two types of seven transmembrane receptors: conventional (GPCRs) and atypical chemokine receptors (ACKRs). The main difference between the two types of receptors is the structural inability of ACKRs to couple and thus signal through G proteins (Griffith et al., 2014). With approximately 50 different ligands and 20 receptors, the chemokine/chemokine-receptor family comprises a very complex and also highly dynamic system. Based on the crucial role of this system in various processes that are important in atherosclerosis development and CVD, targeting specific chemokine–chemokine receptors dyads are promising approaches for CVD-treatment (Weber and Noels, 2011).

## Classical Chemokine Receptors

### CXCL1–CXCR2 Axis

As described before, the accumulation of oxLDL in the vessel is one of the initiating steps of atherogenesis. The oxidation of LDL generates lysophosphatidylcholine, which is the main substrate for the enzyme autotaxin. This enzyme subsequently transforms lysophosphatidylcholine into lysophosphatidic acid (LPA). This LDL-derived LPA will induce the release of CXCL1 from endothelial cells (Zhou et al., 2011). Subsequently, CXCL1 interacts with CXCR2 on neutrophils and classical monocytes, thereby stimulating their mobilization into the blood stream and migration to sites of inflammation. In line with this, systemic absence of CXCL1 or hematopoietic CXCR2-deficiency has been shown to be protective against atherosclerosis in mice by reducing the intra-plaque macrophage accumulation (Soehnlein et al., 2013).

### CCL2–CCR2 Axis

Another chemokine-axis that has been shown to play an important role during these initial phases of lesion development is the CCL2/CCR2-axis, especially by mediating the mobilization of classical, inflammatory monocytes. Accordingly, CCR2-deficient mice show reduced atherosclerotic lesion formation due to an attenuation of monocytosis (Swirski et al., 2007; Tacke et al., 2007; Weber and Noels, 2011). Recently, it has been shown that there is a striking circadian control of endothelial and myeloid cell activities. This circadian control is part of the daily rhythms, which are controlled by key proteins like CLOCK and BMAL1 (Zhang et al., 2014). A recent study by Winter et al. (2018) could show that such rhythmic control is also present in chronic inflammatory processes of large vessels, thereby mediating rhythmic myeloid cell recruitment. The recruitment of neutrophils and monocytes to atherosclerotic lesions oscillates with a peak during the transition from the activity to the resting phase (Winter et al., 2018). They could show that this oscillating

recruitment is regulated by the rhythmic release of myeloid cell-derived CCL2, as blockage of this signaling abolished the oscillatory leukocyte adhesion. Interestingly, the adhesion of myeloid cells to the microvasculature is different than the previously discussed macrovascular effects as here the adhesion peak was reached during the early activity phase. This opens up novel opportunities of well-timed pharmacological targeting of CCL2 in order to modulate the effects on atherosclerosis formation, without disturbing the microvascular cell recruitment (Winter et al., 2018).

Interestingly, deletion of both CCL2 and CX3CR1, or CCR2 and CX3CL1 even further decreased atherosclerosis development compared with single deficiencies in the proteins, which could be attributed to a strongly attenuated monocytosis and hence reduced plaque macrophage accumulation (Combadiere et al., 2008; Saederup et al., 2008). Pharmacological targeting could further confirm these results, as administration of a non-agonistic CCL2-competing mutant (PA508) with increased proteoglycan affinity, or siRNA-mediated silencing of CCR2 in mouse models of MI resulted in reduced recruitment of classical monocytes to the infarcted areas (Liehn et al., 2010; Majmudar et al., 2013). Targeting the CCL2–CCR2 axis has already been evaluated in a phase 2 human clinical trial, where blockage of CCR2 with MLN1202, a specific humanized monoclonal antibody that inhibits CCL2 binding, resulted in reduced plasma CRP levels in patients at risk for CVD (Gilbert et al., 2011). Thereby, targeting of this chemokine-axis remains a promising approach for future CVD therapies.

## CCL5–CCR5 Axis

Besides recruitment, chemokines and their receptors also play an important role in leukocyte arrest on the endothelium by integrin activation. For example, activated platelets release CCL5 which is subsequently immobilized on the surface of inflamed endothelium, triggering leukocyte arrest (von Hundelshausen et al., 2001). This CCL5-mediated myeloid cell recruitment has been shown to be dependent on sialylation of the receptors CCR1 or CCR5, as deficiency of sialyltransferase St3Gal-IV in mice resulted in decreased monocyte and neutrophil recruitment and reduced atherosclerotic lesion size in a CCL5-related manner (Döring et al., 2014). The potential of targeting CCL5 receptors as therapeutic approach was further validated by studies where CCR5 deficiency (Braunersreuther et al., 2007b), inhibition of CCR5 with maraviroc (Cipriani et al., 2013) or general blockage of CCL5 receptors using Met-CCL5 (Veillard et al., 2004) all showed clearly reduced atherosclerotic lesion size and lesional macrophage content in mice. As maraviroc is an FDA-approved HIV-entry inhibitor, it is already used in the clinic, where it could be observed that treatment of HIV-patients with maraviroc seemed to lower atherosclerotic lesion growth (Maggi et al., 2017). Furthermore, it is interesting to note that there is a correlation between plasma CCL5 levels and the progression of atherosclerosis in patients after acute coronary syndrome (Blanchet et al., 2014). All by all, the CCL5–CCR5 chemokine axis seems a promising therapeutic target, especially as an inhibitor is already in clinical use and proven reduce the atherosclerotic risk.

## Chemokine Heterodimers

With respect to the development of pharmacological targeting of chemokine receptors, it is intriguing to note that chemokines can also form higher-order complexes with themselves (homomers) or with other proteins (heteromers). For example, CCL5 can form a heteromeric complex with CXCL4 and thereby augmenting the CCL5-stimulated arterial monocyte adhesion (von Hundelshausen et al., 2005). This also has clear implications for atherosclerosis development as selective disruption of the CCL5-CXCL4 heteromer by the cyclic peptide MKEY results in reduced plaque formation in mice (Koenen et al., 2009). Administration of MKEY did not interfere with systemic immune responses, like T cell proliferation of clearance of viral infections, clearly highlighting the potential and specificity of this peptide. Additionally, treatment with MKEY has been shown to preserve heart function and decrease the infarct size in a model of myocardial ischemia/reperfusion injury. Moreover, MKEY treatment resulted in a reduced inflammatory reaction in response to injury, demonstrated by the attenuation of monocyte and neutrophil recruitment. Interestingly, there was also a significant reduction of citrullinated histone 3 in the infarcted tissue, showing that MKEY can also prevent NETosis (Vajen et al., 2018). Another example of a heteromer that stimulates leukocyte adhesion is the complex between neutrophil-borne human neutrophil peptide 1 (HNP1) and platelet-derived CCL5 (Alard et al., 2015). Disruption of this complex with the specific peptide SKY resulted in decreased recruitment of classical monocytes in a murine MI model (Alard et al., 2015). The continued elucidation of the precise physiological and especially pathological functions of various chemokine–chemokine interactions (von Hundelshausen et al., 2017) will further identify novel and interesting targets with clinical potential.

## CXCL12–CXCR4 Axis

Another important chemokine axis in cell homeostasis, mobilization and immunity is the CXCL12–CXCR4 axis (van der Vorst et al., 2015). For example, systemic treatment of atherosclerosis prone mice with the biglycan CXCR4 antagonist AMD3465 resulted in increased atherosclerosis lesion size compared to untreated controls due to increased neutrophil mobilization (Zernecke et al., 2008). Using cell-specific genetic ablation of CXCR4, endothelial CXCR4 has been shown to promote re-endothelialization after vascular injury and prevent neointimal hyperplasia (Noels et al., 2014) and to limit atherosclerosis development by maintaining the endothelial integrity (Döring et al., 2017). This endothelial barrier integrity was mainly promoted by the signaling of CXCL12–CXCR4 to Akt/WNT/β-catenin resulting in enhanced VE-cadherin expression thereby stabilizing the cellular junctions. Additionally, CXCR4 was shown to be crucial in the maintenance of a normal contractile SMC phenotype. In sharp contrast to the clearly atheroprotective role of vascular CXCR4, its ligand CXCL12 seems to be atheroprogressive as endothelial derived CXCL12 promotes lesion development (Döring et al., 2019). Since the current studies only focused

on the role of vascular CXCL12 and CXCR4, it remains to be identified whether and which hematopoietic cells play an important role in the modulation of inflammation by CXCL12–CXCR4. In humans, it has already been shown that both CXCL12 and CXCR4 are associated with CVD. For example, regression analysis demonstrated that the C-allele at *rs2322864* in the CXCR4 locus is associated with an increased risk for coronary heart disease (Döring et al., 2017). Additionally, expression of both CXCR4 and CXCL12 was increased in human carotid atherosclerotic lesions compared to healthy vessels (Merckelbach et al., 2018). Genome-wide association studies further confirmed the importance of CXCL12 by showing that a single nucleotide polymorphism at 10q11 near the CXCL12 locus is independently associated with the risk for coronary artery disease (CAD) (Mehta et al., 2011; Döring et al., 2019). Furthermore, the causal role of CXCL12 as mediator of CAD has been confirmed in the ORIGIN and CARDIoGRAM populations by a mendelian randomization study (Sjaarda et al., 2018). All by all, these data clearly support an important role for the CXCL12–CXCR4 chemokine axis in atherosclerosis development and CVD occurrence.

### Concluding Remarks

Classical chemokine receptors and their corresponding ligands play a key role in the immune system and have been shown to be drivers and regulators of CVD (please refer to **Table 1** for a summary of important studies and their key findings and to **Table 2** for an overview of ligand types involved). Interference with this system seems like a very promising therapeutic approach, although this should be carefully designed and has to be context-specific to avoid unwanted, but almost unavoidable, side-effects.

## Atypical Chemokine Receptors

As mentioned before, ACKRs are unable to signal through G proteins but are known to recruit β-arrestin upon ligand binding and are thereby key directors of chemokine driven immune responses as they regulate the bioavailability, internalization, localization as well as the gradient establishment of chemokines (Patel et al., 2009; Ulvmar et al., 2011; Graham et al., 2012; Cancellieri et al., 2013; Bonecchi and Graham, 2016). Moreover, ACKRs can modify the signaling activity of other chemokine receptors via heterodimer formation, thus may also ultimately influence G-protein signaling pathways (Decaillot et al., 2011). Due to their broad-spectrum immunological functions, ACKRs are promising therapeutic targets for the treatment of inflammatory diseases, such as atherosclerosis (Gencer et al., 2019). So far, four types of ACKRs are well recognized: ACKR1 (DARC), ACKR2 (D6), ACKR3 (CXCR7 or RDC-1) and ACKR4 (CCRL1), whereas new members are subject to further investigation: ACKR5 (CCRL2) and ACKR6 (PITPNM3) (Ulvmar et al., 2011). Three members of this family, ACKR1, ACKR2, and ACKR3, are critical for inflammatory responses and will therefore be discussed in greater detail, whereas ACKR4 seems to be primarily involved in homeostatic processes.

### ACKR1

ACKR1 is expressed on erythrocytes as well as venular endothelial cells and binds plentiful inflammatory chemokines. It is well known that the absence of ACKR1 on erythrocytes causes a Duffy-negative phenotype in African people (Howes et al., 2011; Novitzky-Basso and Rot, 2012; Horuk, 2015). A study by Duchene et al. (2017) showed that Duffy negative individuals exhibited an altered neutrophil phenotype by CCR2, CD16, and CD45 overexpression in comparison to Duffy positive individuals, indicating an amplified defense mode of neutrophils as a result of the lack of ACKR1 on erythrocytes. Considering that ACKR1 binds a wide range of inflammatory chemokines in addition to the characteristic scavenging activity of ACKRs, it is concluded that erythrocyte-specific ACKR1 is a decoy receptor regulating the levels of circulating inflammatory chemokines, such as CCL2 and CXCL8 (Jenkins et al., 2017). Endothelial ACKR1, on the other hand, mediates the internalization of extracellular chemokines and allows their presentation on the cell surface (Novitzky-Basso and Rot, 2012). This process enhances leukocyte recruitment and supports leukocyte-endothelium adhesion, augmenting inflammation. Due to its contrasting roles in different cell types, it is difficult to gauge the impact of systemic ACKR1 deficiency in the context of atherosclerosis. One possibility is that it may lead to a rise in circulating inflammatory myeloid cells, such as monocytes, through an increase in circulating inflammatory chemokines, which would be considered a pro-atherosclerotic event. On the other hand, it may result in a reduction of myeloid cell adhesion to the endothelium, which may in turn decrease lesional macrophage accumulation and thereby limit the development of lesions. Wan et al. (2015) reported an atheroprotective role of ACKR1 deficiency in an apolipoprotein E deficient ($ApoE^{-/-}$) mouse model. This was shown to be a result of decreased lesion sizes observed with a decreased inflammatory phenotype in circulating monocytes and macrophages in addition to decreased T-cells in the aortic vessel wall (Wan et al., 2015). This finding highlights a detrimental role of ACKR1 in atherosclerosis. Another study investigating ACKR1 in the context of inflammation through a bone fracture model in mice reported a significant reduction in macrophage numbers around the fractures in ACKR1 deficient mice (Rundle et al., 2013). This outcome was observed with a concomitant decrease in inflammatory markers, such as IL-1β, IL-6 as well as monocyte chemotactic protein-1, confirming a detrimental role for ACKR1 in macrophage recruitment and inflammation. Taken these findings into account, the inhibition of this receptor might be a therapeutic approach in atherosclerosis treatment.

### ACKR2

Similar to ACKR1, ACKR2 also binds numerous inflammatory chemokines. It is expressed on lymphatic endothelial cells, innate-like B cells and some macrophage subsets (Bonecchi and Graham, 2016). Growing evidence discloses an anti-inflammatory profile for ACKR2 with a central role in the resolution of inflammation (Bonavita et al., 2016; Bideak et al., 2018; Massara et al., 2018). ACKR2 is defined as a scavenger receptor for inflammatory chemokines, because ACKR2 deficient

**TABLE 1 |** Consequences of GPCRs targeting in cardiovascular disease *in vivo*.

| Receptor | Ligand | Species and tissue or model | Pathophysiology | Results | Receptor effect | References |
|---|---|---|---|---|---|---|
| **Classical chemokine receptors** | | | | | | |
| CXCR2 | CXCL1 | Mouse, injection of anti-CXCL1 antibody | Atherogenesis | Reduced lesion size, decreased macrophage content | 👎 | Soehnlein et al., 2013 |
| CCR2 | n.d. | Mouse, *Apoe*$^{-/-}$*Ccr2*$^{-/-}$ | Atherosclerosis | Reduced lesion size, decreased monocytosis | 👎 | Tacke et al., 2007 |
| | n.d. | Mouse, injection of nanoparticle-encapsulated siRNA targeting Ccr2 in *Apoe*$^{-/-}$ | Myocardial infarction model | Attenuated classical monocyte recruitment and infarct inflammation | 👎 | Majmudar et al., 2013 |
| | | Mouse, injection of CCR2 inhibitor RS102895 into *Apoe*$^{-/-}$ | Atherosclerosis | Reduced myeloid cell recruitment | 👎 | Winter et al., 2018 |
| | CCL2 | Mouse, injection of CCL2-competitor PA508 into C57Bl/6 | Myocardial infarction model | Attenuated myocardial ischemia/reperfusion injury, reduced classical monocyte recruitment | 👎 | Liehn et al., 2010 |
| | CCL2 | Human, specific monoclonal antibody MLN1202 treatment | Atherosclerosis | Decreased plasma C-reactive protein levels | 👎 | Gilbert et al., 2011 |
| | CX3CL1 | Mouse, *Apoe*$^{-/-}$*Ccr2*$^{-/-}$*Cx3cl1*$^{-/-}$ | Atherosclerosis | Strongly reduced lesion size, decreased monocytosis and plaque macrophage accumulation | 👎 | Saederup et al., 2008 |
| CX3CR1 | CCL2 | Mouse, *Apoe*$^{-/-}$*Cx3cr1*$^{-/-}$*Ccl2*$^{-/-}$ | Atherosclerosis | Strongly reduced lesion size, decreased monocytosis and plaque macrophage accumulation | 👎 | Combadiere et al., 2008 |
| CXCR3 | n.d. | Mouse, *Apoe*$^{-/-}$*Cxcr3*$^{-/-}$ | Atherosclerosis | Reduced atherosclerotic lesion size | 👎 | Veillard et al., 2005 |
| | n.d. | Mouse, injection of the CXCR3 antagonist NBI-74330 into *Ldlr*$^{-/-}$ | Atherosclerosis | Reduced lesion size, less activated T cells but enrichment of regulatory T cells | 👎 | van Wanrooij et al., 2008 |
| | CXCL10 | Mouse, *Apoe*$^{-/-}$*Cxcl10*$^{-/-}$ | Atherogenesis and atherosclerosis | Decreased lesion formation, reduced accumulation of $CD^{4+}$ T cells | 👎 | Heller et al., 2006 |
| CCR5 | CCL5 | Mouse, *Apoe*$^{-/-}$*St3Gal4*$^{-/-}$ | Atherosclerosis | Reduced CCL5-induced myeloid cell recruitment and plaque size | 👎 | Döring et al., 2014 |
| | n.d. | Mouse, *Apoe*$^{-/-}$*Ccr5*$^{-/-}$ | Atherosclerosis | Reduced lesion size with more stable plaque phenotype | 👎 | Braunersreuther et al., 2007b |
| | n.d. | Mouse, injection of CCR5 antagonist Maraviroc into *Apoe*$^{-/-}$ | Atherogenesis and atherosclerosis | Decreased atherosclerosis formation by reducing macrophage infiltration | 👎 | Cipriani et al., 2013 |

*(Continued)*

**TABLE 1** | Continued

| Receptor | Ligand | Species and tissue or model | Pathophysiology | Results | Receptor effect | References |
|---|---|---|---|---|---|---|
| | n.d. | Mouse, injection of CCR5 antagonist Met-RANTES into *Ldlr*$^{-/-}$ | Atherosclerosis | Reduced plaque formation, correlated with decreased leukocyte infiltration | 👎 | Veillard et al., 2004 |
| | n.d. | Human, CCR5 antagonist Maraviroc treatment of HIV patients | Atherosclerosis | Reduced development of atherosclerosis | 👎 | Maggi et al., 2017 |
| | CCL5 | Human, plasma | Coronary artery disease | Association between elevated plasma CCL5 levels and the progression of coronary artery disease | 👎 | Blanchet et al., 2014 |
| | CCL5–CXCL4 heteromer | Mouse, injection of inhibitory peptide MKEY into *Apoe*$^{-/-}$ | Atherosclerosis | Decreased atherosclerosis formation and attenuated monocyte recruitment | 👎 | Koenen et al., 2009 |
| | CCL5–CXCL4 heteromer | Mouse, injection of inhibitory peptide MKEY into C57Bl/6 | Myocardial infarction model | Decreased infarct size and preserved heart function, attenuated leukocyte recruitment | 👎 | Vajen et al., 2018 |
| | CCL5–HNP1 heteromer | Mouse, injection of inhibitory peptide SKY into C57Bl/6 | Myocardial infarction model | Reduced myeloid cell recruitment | 👎 | Alard et al., 2015 |
| CXCR4 | n.d. | Mouse, injection of CXCR4 antagonist AMD3464 into *Apoe*$^{-/-}$ | Atherosclerosis | Increased lesion size due to enhanced neutrophil mobilization | 👍 | Zernecke et al., 2008 |
| | n.d. | Mouse, *Apoe*$^{-/-}$*Bmx-Cre*$^{+}$*Cxcr4*$^{flox/flox}$ | Wire-induced injury of carotid artery | Increased neointima formation, due to reduced reendothelialization | 👍 | Noels et al., 2014 |
| | n.d. | Mouse, *Apoe*$^{-/-}$*Bmx-Cre+Cxcr4*$^{flox/flox}$ | Atherosclerosis | Increased atherosclerotic lesion formation and disrupted vascular integrity | 👍 | Döring et al., 2017 |
| | CXCL12 | Mouse, *Apoe*$^{-/-}$*Bmx-Cre*$^{+}$*Cxcl12*$^{flox/flox}$ | Atherosclerosis | Reduced plaque size | 👎 | Döring et al., 2019 |
| | n.d. | Human, regression analysis of coronary heart disease cohorts | Coronary heart disease | Associated of the C-allele at rs2322864 with increased risk for coronary heart disease | 👎 | Döring et al., 2017 |
| | CXCL12 | Human, carotid atherosclerotic lesions | Atherosclerosis | Increased expression of CXCR4 and CXCL12 in atherosclerotic lesions compared to healthy vessels | 👎 | Merckelbach et al., 2018 |
| | CXCL12 | Human, genome-wide association studies | Coronary artery disease | Independent association of single nucleotide polymorphism at 10q11 with the risk for coronary artery disease | 👎 | Mehta et al., 2011; Döring et al., 2019 |
| | CXCL12 | Human, mendelian randomization study | Coronary artery disease | CXCL12 is a causal mediator of coronary artery disease in humans | 👎 | Sjaarda et al., 2018 |

*(Continued)*

**TABLE 1** | Continued

| Receptor | Ligand | Species and tissue or model | Pathophysiology | Results | Receptor effect | References |
|---|---|---|---|---|---|---|
| **Atypical chemokine receptors** | | | | | | |
| ACKR1 | n.d. | Mouse, *Apoe*$^{-/-}$ *Ackr1*$^{-/-}$ | Atherogenesis and atherosclerosis | Reduced atherogenesis and atherosclerosis formation, with reduced Ccl2 and Cxcl1 expression in aorta |  | Wan et al., 2015 |
| ACKR3 | n.d. | Mouse, *Apoe*$^{-/-}$*Ackr3*$^{-/-}$ | Wire-induced injury of carotid artery | Increased neointima formation and increased lesional macrophage accumulation |  | Li et al., 2014 |
| | n.d. | Mouse, *Apoe*$^{-/-}$ | Atherosclerosis | ACKR3 expression is upregulated during monocyte-to-macrophage differentiation and thereby enhances phagocytosis |  | Ma et al., 2013 |
| **Formyl-peptide receptors** | | | | | | |
| FPR2 | n.d. | Human, coronary lesions | Atherosclerosis | Upregulation of *FPR2* mRNA expression in human lesions compared to healthy vessels |  | Petri et al., 2015 |
| | n.d. | Mouse, *Ldlr*$^{-/-}$ *Fpr2*$^{-/-}$ | Atherosclerosis | Reduced lesion formation and less macrophage infiltration |  | Petri et al., 2015 |
| | n.d. | Mouse, *Fpr2*$^{-/-}$ bone marrow into *Ldlr*$^{-/-}$ | Atherosclerosis | Decreased atherosclerotic lesion formation and reduced macrophage accumulation |  | Petri et al., 2015 |
| | n.d. | Mouse, *Apoe*$^{-/-}$ *Fpr2*$^{-/-}$ | Atherosclerosis | Aggravated atherosclerosis formation and increased monocyte recruitment |  | Drechsler et al., 2015 |
| | Annexin A1 | Mouse, *Apoe*$^{-/-}$ *AnxA1*$^{-/-}$ | Atherosclerosis | Increased atherosclerosis development and macrophage accumulation |  | Drechsler et al., 2015; de Jong et al., 2017 |
| | Ac2-26 | Mouse, injection of Ac2-26 into *Apoe*$^{-/-}$ | Atherosclerosis | Reduced lesion size and lesion macrophage accumulation |  | Drechsler et al., 2015 |
| | human Annexin A1 | Mouse, injection of Annexin A1 into *Ldlr*$^{-/-}$ | Atherogenesis and atherosclerosis | No effect on atherogenesis, but attenuated progression of existing plaques |  | Kusters et al., 2015 |
| | Ac2-26 | Mouse, injection of Ac2-26 into *Ldlr*$^{-/-}$ | Advanced atherosclerosis | Stabilization of advanced plaques by increasing collagen content while decreasing plaque necrosis |  | Fredman et al., 2015 |
| | Ac2-26 | Mouse, injection of Ac2-26 into *Ldlr*$^{-/-}$*Fpr2*$^{-/-}$ | Advanced atherosclerosis | No beneficial effects of Ac2-26 administration | – | Fredman et al., 2015 |
| | Resolvin D1 | Mouse, injection of Resolvin D1 into *Ldlr*$^{-/-}$ | Advanced atherosclerosis | Enhanced plaque stability by improved efferocytosis, less necrosis and thicker fibrous cap |  | Fredman et al., 2016 |
| | Ac2-26 | Mouse, injection of Ac2-26 into C57/Bl6 *and* *Fpr1*$^{-/-}$ | Myocardial infarction model | Reduced acute myocardial injury |  | Gavins et al., 2005 |

*(Continued)*

**TABLE 1** | Continued

| Receptor | Ligand | Species and tissue or model | Pathophysiology | Results | Receptor effect | References |
|---|---|---|---|---|---|---|
| **Chemerin receptor 23** | | | | | | |
| ChemR23 | Chemerin-9 (C9) | Rat, injection of C9 with/without ChemR23 antagonist CCX832 into Sprague-Dawley rats | Hypertension | ChemR23-dependent increased blood pressure |  | Kennedy et al., 2016 |
| | n.d. | Mouse, *Ob/Ob*$^{-/-}$ and *Db/Db*$^{-/-}$ | Obesity | Increased serum total chemerin and bioactive chemerin | – | Parlee et al., 2010 |
| | Chemerin-15 (C15) | Mouse, injection of C15 into C57Bl/6 | Myocardial infarction model | Reduced heart damage and neutrophil recruitment |  | Cash et al., 2013 |
| | Resolvin E1 | Mouse, eicosapentaenoic acid supplementation of *Apoe*$^{-/-}$ Western Diet of *Apoe*$^{-/-}$ *ChemR23*$^{-/-}$ | Atherosclerosis Atherosclerosis | Reduced atherosclerosis development Increased atherosclerosis development |  | Laguna-Fernandez et al., 2018 |
| | Resolvin E1 | Mouse, *ChemR23*$^{-/-}$ | Intimal hyperplasia | Increased intimal hyperplasia with more pro-inflammatory macrophages and reduced smooth muscle cell proliferation |  | Artiach et al., 2018 |
| | n.d. | Mouse, *Apoe*$^{-/-}$ *ChemR23*$^{-/-}$ *Apoe*$^{-/-}$ *ChemR23*$^{-/-}$ bone marrow into *Apoe*$^{-/-}$ recipients | Atherogenesis and atherosclerosis | Reduced atherosclerosis development, more M2 macrophages, diminished pDC recruitment Reduced atherosclerosis development |  | van der Vorst et al., 2019 |
| **Calcium-sensing receptor** | | | | | | |
| CaSR | n.d. | Rat, injection of isoproterenol in vitamin D3-induced atherosclerotic Wistar rats | Myocardial infarction model | Increased CaSR expression | – | Guo et al., 2012 |
| | NPSR568 | Rat, injection of calcimimetic NPSR568 into spontaneously hypertensive rats | Hypertension | Reduced blood pressure and inhibition of arterial vascular proliferation remodeling |  | Sun et al., 2018 |
| | Astragaloside IV | Rat, injection of astragaloside IV into Sprague-Dawley rats | Myocardial infarction model | Attenuated myocardial injury and cardiomyocyte apoptosis |  | Yin et al., 2019 |
| | Astragaloside IV | Rat, injection of isoproterenol into Sprague-Dawley rat | Myocardial infarction model | CaSR-dependent attenuated cardiac hypertrophy and apoptosis |  | Lu et al., 2018 |
| | Calhex231 | Rat, injection of Calhex231 (CaSR inhibitor) into spontaneously hypertensive rats | Hypertension and cardiac hypertrophy | Reduced heart weight to body weight ratio and CaSR levels |  | Hong et al., 2017 |
| | Calhex231 | Rat, injection of isoproterenol and Calhex231 (CaSR inhibitor) into Wistar rats | Hypertension and cardiac hypertrophy | Amelioration of cardiac hypertrophy and inhibition of autophagy |  | Liu et al., 2016 |

*n.d., not determined.*

**TABLE 2 |** Types of GPCR-ligands discussed in the review.

| Target | Ligand | Type |
|---|---|---|
| CCR2 | CCL1 | Endogenous agonist |
| | MLN1202 | Monoclonal antibody |
| CCR5 | Maraviroc | Antagonist |
| CXCR2 | CXCL1 | Endogenous agonist |
| CXCR4 | AMD3465 | Antagonist |
| $CX_3CR1$ | $CX_3CL1$ | Endogenous agonist |
| CCL5–CXCL4 | MKEY | Antagonist |
| CCL5–HNP1 | SKY | Antagonist |
| ACKR3 | CXCL11 | Endogenous agonist |
| | CXCL12 | Endogenous agonist |
| | Adrenomedullin | Endogenous agonist |
| | Bovine adrenal medulla 22 | Endogenous agonist |
| | TC14012 | Agonist |
| FPR2 | Annexin A1 | Endogenous agonist |
| | fMLP | Agonist |
| | Cathepsin G | Endogenous agonist |
| | Resolvin D1 | Endogenous agonist |
| | Ac2-26 | Agonist |
| | Lipoxin A4 | Endogenous agonist |
| ChemR23 | Chemerin (different lengths depending on enzymatic cleavage) | Endogenous agonist/biased agonist (depending on length of ligand) |
| | Resolvin E1 | Endogenous agonist |
| CaSR | $Ca^{2+}$ | Agonist |
| | $Mg^{2+}$ | Positive allosteric modulator |
| | Cinacalcet | Positive allosteric modulator |
| | NPS R-467 | Positive allosteric modulator |
| | NPS R-568 | Positive allosteric modulator |
| | NPS 2143 | Negative allosteric modulator |
| | Ronacaleret | Negative allosteric modulator |
| | Calhex 231 | Negative allosteric modulator |

mice reproducibly showed increased levels of inflammatory chemokines, like CCL2 (Jamieson et al., 2005; Martinez de la Torre et al., 2005; Whitehead et al., 2007; Collins et al., 2010; Vetrano et al., 2010). The anti-inflammatory properties of ACKR2 are not only limited to its scavenging activities; this receptor is also involved in the regulation of monocyte and macrophage dependent immune responses. For example, ACKR2 deficiency in a murine zymosan A-initiated peritonitis mouse model was shown to promote macrophage efferocytosis, suggesting an important potential function of ACKR2 in atherosclerotic plaques with regards to the efficiency of foam cell efferocytosis (Pashover-Schallinger et al., 2012). Additionally, a *Mycobacterium tuberculosis* disease model lead to rapid death in ACKR2 deficient mice with concomitant increased infiltration of mononuclear cells, e.g., macrophages, into inflamed tissues as well as lymph nodes (Di Liberto et al., 2008). Macrophage infiltration and accumulation in atherosclerotic lesions leads to the progression and eventually growth of plaques. It is therefore of great interest to inhibit these key processes in order to treat atherosclerosis. Considering its roles in macrophage efferocytosis and immune cell infiltration, ACKR2 may be a novel therapeutic target in the research of atherosclerosis treatment. A study conducted by Savino et al. (2012) reported a CCR2-dependent, selective increase in $Ly6C^{high}$ monocyte numbers in circulation as well as secondary lymphoid organs of mice lacking ACKR2 in the non-hematopoietic fragment. This outcome was observed with a delayed graft versus host disease development due to the immunosuppressive activity of the $Ly6C^{high}$ monocytes pointing toward a contrasting role of the receptor in the context of adaptive immune responses. Nevertheless, in the context of atherosclerosis, a rise in inflammatory monocytes in circulation may lead to increased monocyte infiltration and intra-plaque macrophage accumulation, thus result in more advanced lesions. Hence, ACKR2 is a significant immunomodulatory candidate and its roles shall be scrutinized in a cell type and disease model specific manner.

## ACKR3

ACKR3 is expressed in endothelial cells, marginal B cells, neurons as well as mesenchymal and some hematopoietic cells (Massara et al., 2016). It binds two well-known chemokine ligands, CXCL11 and CXCL12, in addition to adrenomedullin and bovine adrenal medulla 22 (BAM22) (Wang et al., 2018). ACKR3 can signal through β-arrestin and activate extracellular signal-regulated kinase (ERK) as well as phosphoinositide 3-kinase (PI3K)-Akt signaling pathways (Ma et al., 2013). Moreover, ACKR3 can control CXCL12 signaling by either regulating its concentrations or heterodimerization with its alternative receptor CXCR4 (Levoye et al., 2009). Although ACKR3 is crucial in vascular and cardiac development, a number of studies demonstrated its detrimental effects in the context of inflammation.

Research suggests that inflammation caused an increased expression of ACKR3 on immune cells, especially myeloid cells. Infiltrating monocytes in a mouse peritonitis model as well as lesional macrophages in aortic atheroma of mice showed increased ACKR3 expression, pointing toward an inflammatory role of ACKR3 (Ma et al., 2013; Chatterjee et al., 2015). Ma et al. (2013) showed that ACKR3 expression was detected in the macrophage positive area defined by F4/80 positivity within atherosclerotic lesions, whereas this was not observed in the vessel wall of healthy aortas. Moreover, this study showed that whilst undifferentiated THP-1 cells expressed CXCR4 but not ACKR3 mRNA, phorbol 12-myristate 13-acetate (PMA) treatment in THP-1 cells (promoting macrophage differentiation) induced the expression of ACKR3 mRNA whilst downregulating CXCR4 mRNA. Further functional analysis of macrophages with regards to ACKR3 activity was assessed by ACKR3 agonists, such as CXCL12 and TC14012. Treatment of macrophages with these agonists showed increased uptake of FITC-labeled *E. coli,* demonstrating a significant increase in cellular phagocytosis. This effect was abolished by siRNA silencing of ACKR3, confirming that the observed phagocytosis was a result of ACKR3 activity. These findings were endorsed by increased uptake of acetylated LDL by the macrophages stimulated with the same ACKR3 agonists. Another study by Chatterjee et al. (2015) showed that monocytes in the peritoneal fluid of mice with peritonitis showed enhanced

CXCR4, ACKR3, and CXCL12 expression, also suggesting that this axis plays an important role in monocyte function during inflammation. Furthermore, ACKR3 was shown to promote monocyte survival and adhesion onto a CXCL12 rich platelet surface as well as the phagocytic activity and foam cell formation of macrophages (Chatterjee et al., 2015). In line with these results, ACKR3 is suggested to support monocyte to macrophage differentiation through CXCL12 activity (Sanchez-Martin et al., 2011). This was supported by the significant reduction of CD136 expression of human monocytes upon both CXCR4 and ACKR3 antagonist treatment. In the same study, monocyte differentiation into $CD136^{+}$ macrophages was shown to be inhibited by means of CXCL12 neutralization as well as CXCR4 and ACKR3 blocking. Moreover, exogenous CXCL12 dependent M-CSF production by the monocytes was partially inhibited by CXCR4 and ACKR3 antagonists, further confirming CXCL12, CXCR4, and ACKR3 dependent regulation of monocyte to macrophage differentiation. Altogether, these findings suggest that ACKR3 promotes atherosclerosis by supporting monocyte and macrophage driven inflammatory processes. Therefore, its inhibition might be a valuable therapeutic target in order to interfere with key events driving atherosclerosis.

### Concluding Remarks

Without a doubt, ACKRs play crucial roles in the regulation of immune responses and therefore offer significant therapeutic targets in order to control the inflammatory processes. Nevertheless, their wide array of functions establishes a great complexity, making it very difficult to determine individual targets. Thus, it is of great importance to scrutinize and understand the biology of ACKRs CVD (please refer to **Table 1** for a summary of important studies and their key findings and to **Table 2** for an overview of ligand types involved).

## FORMYL-PEPTIDE RECEPTORS

Formyl-peptide receptors (FPRs) belong to the group of pattern recognition receptors (PRRs) and comprise a family of chemoattractant GPCRs involved in host defense against bacterial infections and clearance of cell debris. FPRs are well conserved among mammals (Ye et al., 2009) and are mainly present on myeloid cells such as neutrophils (except FPR3) and monocytes (He and Ye, 2017). In addition to myeloid cells, astrocytes, microglia, hepatocytes, and immature dendritic cells express FPR1, whereas FPR2 is also expressed on epithelial cells, hepatocytes, microvascular endothelial cells, and smooth muscle cells (He and Ye, 2017). FPRs were originally discovered as receptors that bind highly conserved *N*-formyl methionine-containing protein and peptide sequences of bacterial and mitochondrial origin (Forsman et al., 2015). For example, one of the most potent agonists for FPR1 is the *Escherichia coli*-derived peptide *N*-formyl methionyl-leucyl-phenylalanine (fMLF) (Ye et al., 2009), while the most prominent bacterial FPR2 ligands are the *staphylococcal*-derived phenol-soluble modulins (PSMs) (Kretschmer et al., 2015). However, it has become evident that FPR1 and FPR2 recognize a variety of structurally diverse ligands including many host-derived endogenous agonists (see also **Table 2**) such as Annexin A1, Resolvin D1, Cathepsin G, and the cathelicidin LL37 (*mouse: Cramp*) all of which have been associated with inflammation and/or resolution in mice and man (He and Ye, 2017; Filep et al., 2018). Hence, FPRs may exert ambivalent effects during leukocyte recruitment and in (chronic) inflammatory conditions such as atherosclerosis.

## Role of FPR2 – Annexin A1 in Atherosclerosis Development

Studies on human atherosclerotic plaque specimens supported the notion of the involvement of FPRs in lesion development by pointing at defective resolution within these lesions (Fredman et al., 2016). Additionally, FPR2 mRNA expression was upregulated in human samples from coronary lesions in comparison to healthy vessels (Petri et al., 2015). Similarly, mice deficient for the low-density lipoprotein receptor (LDLR) and FPR2 exhibited decreased atherosclerosis development and less monocyte infiltration and foam cell formation compared with control animals. Analogous results were obtained in $Ldlr^{-/-}$ mice transplanted with FPR2-deficient bone marrow, here dampened activation of lesional macrophages was also attributed to the lack of FPR2 (Petri et al., 2015). These findings support *in vitro* work from Lee et al. (2013, 2014) showing that oxLDL and serum amyloid-2 mediate foam cell formation via FPR2. Hence, one could argue that agonists, which mediate lesional macrophage activation via FPR2 disturb resolution. However, FPR2 expression on vascular smooth muscle cells (VSMCs) seems to stabilize atherosclerotic lesions suggesting a diverse role of FPR2 on hematopoietic versus vascular cell types. Still, specific agonists or antagonists, which mediate one or the other response, were not investigated in this study (Petri et al., 2015). In contrast, $Apoe^{-/-}$ mice which also lacked FPR2 or Annexin A1 showed enhanced atherosclerotic lesion development, increased myeloid cell recruitment and adhesion to the inflamed vessel wall. One explanation focusses on the observation that Annexin A1/FPR2 interaction seems to tightly control and inhibit integrin activation (Drechsler et al., 2015; de Jong et al., 2017). In line, treatment of $Apoe^{-/-}$ or $Ldlr^{-/-}$ mice with Annexin A1 or the Annexin A1 fragment Ac2-26 reduced atherogenesis by decreasing necrosis, mediating efferocytosis and supporting fibrous cap stability (Drechsler et al., 2015; Kusters et al., 2015). Equivalent results were obtained in $Ldlr^{-/-}$ mice with advanced atherosclerosis, which were treated with the agonist Ac2-26 packed into nanoparticles that targeted type IV collagen to ensure deposition in atherosclerotic lesions. Plaques of animals treated with Ac2-26 nanoparticles displayed reduced macrophage numbers, smaller necrotic core sizes, and higher amounts of anti-inflammatory interleukin 10 compared to control animals. On the contrary, when treating $Ldlr^{-/-}$ $Fpr2^{-/-}$ mice with Ac2-26, the protective effects were abolished suggesting an important role of FPR2 on myeloid cells in mediating arterial (lesional) resolution through interaction with Annexin A1 (Fredman et al., 2015).

## Pro-resolving Lipid Mediators

Specialized pro-resolving lipid mediators (SPMs) including the resolvins are derived from the ω-3 PUFAs eicosapentaenoic acid (EPA) or docosahexaenoic acid (DHA). They have important roles in the resolution of inflammation, either via their own GPCRs or by modulating GPCRs for ω-6 PUFA. For example, resolvin E1 (RvE1) enhances the phagocytosis of apoptotic neutrophils via ChemR23 (please also see section "ChemR23 and Resolvin E1 – Mechanisms of Resolution") and inhibits the infiltration of neutrophils by antagonizing LTB4 or leukotriene B4 receptor 1 (BLT1). Resolvin D1 (RvD1) instead has been shown to bind to two GPCRs, namely, the orphan receptor, GPR32, and the lipoxin receptor, FPR2/ALX through which it mediates its pro-resolving effects (Jannaway et al., 2018). In line with this, if the endogenous agonists RvD1 was administered to *Ldlr*$^{-/-}$ mice during the transition phase of atherosclerotic lesions from early into advanced plaques. Fredman et al. (2016) could show that RvD1 enhanced lesional efferocytosis, and decreased plaque necrosis compared with vehicle controls. Similarly, repetitive administration of endogenous agonists Resolvin D2 to *Apoe*$^{-/-}$ mice prevented atheroprogression, though most likely mediated via the G-protein coupled receptor 18 (Viola et al., 2016). These findings illustrate the therapeutic potential of pro-resolving FPR agonists to restore defective resolution, which is most likely mediated through myeloid cells in atherosclerotic lesions.

## FPR Signaling and MI

Consistently, a protective role of Annexin A1 and its mimetic peptides could also be demonstrated in experimental models of ischemia-reperfusion injury, e.g., in a mouse model of MI (Gavins et al., 2005). Moreover, Ferraro et al. (2019) for example examined to what extent endogenous control of inflammation resolution and its therapeutic stimulation enables improved cardiac function in the absence and presence of Annexin A1. They showed that myeloid cells infiltrating at early stages post MI deliver Annexin A1 hereby terminating inflammation and promoting healing through macrophages with an angiogenic phenotype with release of VEGF-A. They could further reveal similar protective functions of Annexin A1 in a model of MI in pigs, hence demonstrating that Annexin A1 facilitated cardiac angiogenesis and myocardial repair (Ferraro et al., 2019).

## FPR Signaling Complexity

Other FPR agonists such as Cathepsin G (Ortega-Gomez et al., 2016) and LL37/Cramp (Döring et al., 2012; Wantha et al., 2013) clearly mediate pro-atherogenic effects by enhancing monocyte adhesion and recruitment, though one cannot exclude that these functions may partly be mediated by other receptors. As FPRs recognize both pro-inflammatory and pro-resolving signals, the question remains how one receptor can mediate opposing responses. In this context, Cooray et al. (2013) suggested that anti-inflammatory, but not pro-inflammatory signals activate homodimerization of FPR2, which, in turn trigger the release of anti-inflammatory mediators such as interleukin 10. Heterodimers instead can transduce, e.g., pro-apoptotic signals, explaining why the same receptor system may integrate diverse signals (Cooray et al., 2013). Another plausible option is the concept of biased agonism (please also see section "GPCR Signaling") (Michel and Charlton, 2018) describing that agonists/antagonists might activate specific receptor domains, thereby promoting downstream responses, which at least in part do not overlap. As an example, the small lipid lipoxin $A_4$ has been shown to activate FPR2 by interacting with its extracellular loop III (Chiang et al., 2000), while, e.g., serum amyloid A responses were reliant on extracellular loops I and II (Bena et al., 2012). Hence, all of the latter should be considered in the context of designing potential new therapeutics triggering resolution via FPRs.

## Concluding Remarks

Formyl-peptide receptors have evolved to be a class of receptors that recognize a broad range of structurally distinct ligands and are expressed by a variety of cell types. Many studies have also shown that FPR function is not restricted to host defense against microbes, but also impacts on chronic inflammatory disease such as atherosclerosis and autoimmune diseases or even cancer. Most interestingly, FPR2 does not only mediate pro-inflammatory but also resolution processes and return to homeostasis. While these findings greatly expanded the scope of the pharmacology and biology of FPRs, a better understanding of how FPRs recognize and respond to distinct ligands is needed to explore their further potential as therapeutic targets (please refer to **Table 1** for a summary of important studies and their key findings and to **Table 2** for an overview of ligand types involved).

# CHEMERIN RECEPTOR 23

The chemerin receptor 23 (ChemR23; chemokine-like receptor 1, CMKLR1) is a class A (rhodopsin-like) GPCR expressed on the surface of immune cells subtypes such as dendritic cells (Vermi et al., 2005), monocytes and macrophages (Herova et al., 2015). It is therefore expressed in spleen and lymph nodes, but also in the skin, adipose tissue (Goralski et al., 2007; Goralski and Sinal, 2009) and lung (Wittamer et al., 2003; Roh et al., 2007). Functionally, ChemR23 – mostly through its *bona fide* ligand chemerin- mediates immune cell activation and chemoattraction (Carlino et al., 2012; Rourke et al., 2013). The gene encoding for ChemR23 is called *CMKLR1* (non-human = *cmklr1*), first cloned in 1996 by Gantz et al. (1996) and, under the name of ChemR23, in 1998 by Samson et al. (1998). Another gene encoding G-protein coupled receptor 1 (GPR1) was proved to share a common ancestor with *CMKLR1* (Vassilatis et al., 2003) with a sequence identity of 37% (Kennedy and Davenport, 2018). Therefore, it is designated as chemerin receptor 2. The corresponding human sequence for ChemR23 and GPR1 share 80% sequence identity with its corresponding murine genes (Kennedy and Davenport, 2018). ChemR23 has two known ligands in mouse and human, namely chemerin and Resolvin E1 (RvE1). Based on its similarities with GMKLR1, Barnea et al. (2008) were the first to identify chemerin as a ligand for GPR1. GPR1 can act to modify glucose homeostasis during obesity, in

line with known functions of chemerin (Rourke et al., 2015). However, as yet it is largely unknown what G protein pathway it activates, only few studies showed chemerin modestly induced calcium release (Barnea et al., 2008) or RhoA signaling (Rourke et al., 2015), more investigation are warranted to unveil the downstream pathways. In this review, we mainly discuss the role of ChemR23 in CVD.

## Chemerin and Its Functions

The adipokine chemerin is encoded by the *RARRES2* (non-human = *rarres2*) gene (Nagpal et al., 1997; Busmann et al., 2004). After being translated into the circulating pro-chemerin, the protein undergoes extensive enzymatic processing. It has been shown that the serine proteases, cathepsin G and elastase, are the main enzymes responsible for the conversion of pro-chemerin into its active form (Wittamer et al., 2005; Ortega-Gomez et al., 2016). The resulting protein variants differ in length and functional properties (Meder et al., 2003; Wittamer et al., 2003). Depending on the chemerin variant binding to ChemR23, the receptor couples to a different subtype of $G\alpha_i$ or isoform of $G\alpha_o$ (Wittamer et al., 2004). Regarding downstream-signaling via ChemR23, it could be shown that the variants C9 (or chemerin-9) and 13 were more potent in inhibiting G protein-dependent cAMP, but less potent in inducing β-arrestin compared with human chemerin 21-157. In summary injection of C9 into rats increased blood pressure via ChemR23 but not via GPR1 mediated signaling and could be inhibited by applying the ChemR23 specific antagonist CCX832 (Kennedy et al., 2016). This lead to the conclusion that shorter C-terminal fragments of chemerin seem to impose a strong bias toward activating G protein coupled signaling. Therefore, signaling via ChemR23 cannot be pinpointed to induce neither purely pro-inflammatory nor anti-inflammatory effects.

## ChemR23–Chemerin Axis in CAD

Studies in animal models of CAD for example showed that expression of both ChemR23 and chemerin were induced in mice which were fed a high fat diet (Roh et al., 2007). Human studies also show elevated plasma concentrations of chemerin and their association with an increased risk of hypertension (Kennedy et al., 2016; Watts et al., 2018), a higher body mass index and blood pressure in patients with type 2 diabetes mellitus compared to healthy controls (Yang et al., 2010). Increased expression levels of ChemR23 were also described in perivascular fat tissue and correlated with increased blood pressure (Neves et al., 2015). In line, augmented expression levels of both ChemR23 and chemerin have been shown in atherosclerotic plaques of human patients and in mouse models of vascular inflammation and a positive correlation between chemerin expression in perivascular adipose tissue and atheroprogression has already been demonstrated (Kostopoulos et al., 2014). Another hypothesis includes an influence of adipokine expression in the heart and vasculature and subsequent plaque progression (Spiroglou et al., 2010). Specifically, it was already found that chemerin expression in human epicardial adipose tissue was positively correlated with the severity of coronary atherosclerosis (Gao et al., 2011). In addition, plasma chemerin levels are associated with markers of inflammation and are significantly higher in CAD patients, which do not receive low dose aspirin treatment. The latter does also reduce pro-inflammatory cytokine secretion by macrophages, which may lead to reduced chemerin secretion by adipocytes and may be a reason for the lower chemerin levels in the circulation of CAD patients on low dose aspirin (Herova et al., 2014).

In contrast, Cash et al. (2013) reveal a protective role of certain chemerin variants in a model of acute MI by preventing excessive neutrophil infiltration. Chemerin-15 induced signaling via ChemR23 was also described to increase efferocytosis in macrophages *in vitro* and in an *in vivo* model of peritoneal inflammation (Cash et al., 2010) and was shown to reduce acute intravascular inflammatory events in murine cutaneous wounds (Cash et al., 2014). In a recently published dietary intervention study, anti-atherosclerotic effects of ChemR23 were outlined using a ChemR23 knock-out mouse model on an $Apoe^{-/-}$ background (Laguna-Fernandez et al., 2018). A deficiency in ChemR23 ($Apoe^{-/-}$ $ChemR23^{-/-}$) seemed to accelerate atherogenic signaling in macrophages, induced cholesterol uptake and phagocytosis and lead to an increased lesion size and reduced plaque stability, hence claiming that a functional receptor mediates atheroprotective signaling (Laguna-Fernandez et al., 2018). Contradictory, in a very recent publication from our group we saw that hematopoietic ChemR23-deficiency increases the proportion of alternatively activated M2 macrophages in atherosclerotic lesions and attenuates pDC homing to lymphatic organs and recruitment to atherosclerotic lesions, which synergistically restricts atherosclerotic plaque formation and progression (van der Vorst et al., 2019). Nevertheless, $ChemR23^{-/-}$ VSMCs exhibited a significantly lower proliferation rate compared with VSMCs derived from $ChemR23^{+/+}$ mice while ChemR23-deficient peritoneal macrophages from had significantly higher mRNA levels of pro-inflammatory cytokines compared with $ChemR23^{+/+}$ macrophages. Finally, conditioned media (CM) transferred from $ChemR23^{-/-}$ macrophages to VSMCs significantly increased VSMC proliferation compared to treatment with CM from $ChemR23^{+/+}$ macrophages at least *in vitro*. These results assert dual signaling effects to ChemR23 depending on cell type (VSMCs versus macrophages) expressing the receptor (Artiach et al., 2018), pointing at a diverse role of the receptor on hematopoietic versus vascular cells in atherosclerotic lesion development. An alternative hypothesis suggests that anti-inflammatory effects of chemerin in atherosclerosis are exerted via reduced adhesion to the affected vascular endothelium. One study could show a downregulation of vascular cell adhesion molecule – 1 following chemerin treatment in human umbilical vein endothelial cells and rat aorta and consequently, a reduced monocyte adhesion to the arterial wall (Yamawaki et al., 2012). In conclusion, while chemerin/ChemR23 seems to exert more pro-inflammatory effects on hematopoietic cells, its presence on vascular cells seems to point at an anti-inflammatory role of this ligand receptor pair. However, the relative abundance of pro- versus anti-inflammatory ligands which may also compete with each other and their highly tissue specific expression patterns are expected to further shape these diverse cellular

responses in different stages of (chronic) inflammation such as atherosclerosis.

## ChemR23 and Resolvin E1 – Mechanisms of Resolution

RvE1, a metabolite of EPA (a type of polyunsaturated fatty acids), plays an important role in the return to tissue homeostasis (Schwab et al., 2007; Gao et al., 2013; Hasturk et al., 2015) and is suggested to exhibit anti-inflammatory and pro-resolving effects via ChemR23 or leukotriene B4 receptor 1 (BLT1) (Arita et al., 2005b; Arita et al., 2007) (please also see section "Pro-resolving Lipid Mediators"). RvE1-dependent blockage of VSMC migration, a critical process in the progression of atherosclerosis, and switching into a protective anti-atherosclerotic phenotypic in VSMCs, confer an anti-inflammatory role of vascular ChemR23 signaling (Ho et al., 2010). Moreover, RvE1 rescues impaired neutrophil phagocytosis, oxidized LDL uptake and phagocytosis of macrophages, promotes phagocytosis-induced neutrophil apoptosis (El Kebir et al., 2012; Herrera et al., 2015; Artiach et al., 2018), and also attenuates APC functions targeting dendritic cell migration and reducing IL-12 production via ChemR23 (Arita et al., 2005a). Furthermore, RvE1 can restore inflammation induced mitochondrial dysfunction and reduce polymorphonuclear leukocyte infiltration in BLT1 dependent manner (Arita et al., 2007; Mayer et al., 2019), along with ChemR23-mediated counter regulatory actions to mediate the resolution of inflammation. It is also evident that RvE1 suppresses inflammatory cytokine release, facilitating the healing process, and inhibits macrophage migration by activating ChemR23 in a ligation model of acute MI (Liu et al., 2018). Moreover, supplementation of *Apoe*$^{-/-}$ mice with polyunsaturated fats as potentially beneficial intervention was supposed to enhanced interaction of RvE1 with ChemR23 in atherosclerosis prone mice and reduced their lesion size (Laguna-Fernandez et al., 2018). However, one study proposed that RvE1 and chemerin compete for the same recognition site on ChemR23, and RvE1 binding is blocked at the presence of the chemerin peptide (Arita et al., 2005a). Whether above summarized results hold true when RvE1 is not actively administered or otherwise supplemented, most likely exceeding competition for the recognition site on ChemR23 by chemerin, has yet to be fully elucidated.

Taken together, all these findings open a potential new avenue for the modulation of the magnitude of the local inflammatory responses also in chronic inflammatory disease such as atherosclerosis by fine tuning receptor specific responses in a cell and tissue specific manner.

## Concluding Remarks

The chemerin/ChemR23 axis is a complex network involved in the regulation of immune responses contributing to both the onset and the termination of inflammation. However, several studies show that the various chemerin isoforms may exert different actions downstream of ChemR23. Since chemerin has multiple and different actions, the possibility to selectively modulate its activity can become an attractive target for drug development. Thus, the use of substances boosting its anti-inflammatory properties could be a promising target in exploiting new strategies to treat atherosclerosis (please refer to **Table 1** for a summary of important studies and their key findings and to **Table 2** for an overview of ligand types involved).

# CALCIUM-SENSING RECEPTOR

The CaSR belongs to the metabotropic glutamate receptor GPCR subfamily and is most abundantly expressed in the parathyroid gland and kidney (Brown et al., 1993; Aida et al., 1995; Riccardi et al., 1995; Bockaert and Pin, 1999). Here it senses changes in extracellular $Ca^{2+}$ concentrations and couples this to intracellular signaling pathways that modify parathyroid hormone (PTH) secretion and renal calcium reabsorption in order to maintain the $Ca^{2+}$ homeostasis (Diez-Fraile et al., 2013).

## Ligands and Signaling

Although $Ca^{2+}$ is the main agonist for this receptor, CaSR responds to several other cations (e.g., $Mg^{2+}$, $Gd^{2+}$, $Sr^{2+}$, $La^{2+}$, and $Ba^{2+}$) and a variety of other ligands (McLarnon and Riccardi, 2002). Interestingly, $Mg^{2+}$ has the potency to augment CaSR signaling responses in the presence of $Ca^{2+}$ and thus is a positive allosteric modulator, meaning that serum $Mg^{2+}$ will affect $Ca^{2+}$-CaSR signaling in clinical conditions (Ruat et al., 1996). Moreover, $Mg^{2+}$ has been shown to also stimulate CaSR mRNA expression and protein levels whilst CaSR activation decreases $Mg^{2+}$ levels (Ikari et al., 2001), indicating a negative feedback loop between $Mg^{2+}$ and CaSR. Other, non-cation agonists of the receptor include polyamides, such as spermine and spermidine, and various amino acids (Quinn et al., 1997; Conigrave et al., 2000).

Upon ligand binding, CaSR activates several intracellular signal transduction pathways, mainly through Gαi, Gαq, and Gα12/13 G-protein subtypes. CaSR influences several effectors, such as PLC, adenylate cyclase (AC), cytosolic phospholipase A2 (cPLA2), phosphatidylinositol 4-kinase (PI4K), phospholipase D (PLD), and ERK (Kifor et al., 1997; Chang et al., 1998; Arthur et al., 2000; Huang et al., 2002; Huang et al., 2004).

## CaSR in Inflammatory Diseases

Abnormal CaSR activity or expression contributes to the development of CVDs. Following the discovery that CaSR is expressed in the heart tissue of rats, Guo et al. (2012) investigated the relationship between CaSR and MI in atherosclerosis by inducing MI in atherosclerotic rats and non-atherosclerotic controls. Here the authors showed that CaSR expression was significantly increased in the atherosclerotic MI group compared to the MI controls, suggesting that CaSR plays an important role in MI caused by atherosclerosis (Guo et al., 2012). Besides its suspected role in atherosclerosis and MI, CaSR is also important in numerous other inflammatory diseases. For example, a population-wide study and Felderbauer et al. (2006) linked several CaSR polymorphisms and mutations to chronic pancreatitis (CP) and idiopathic CP (Muddana et al., 2008). Furthermore, a study focusing on asthma showed that asthmatic patients and allergen-sensitized mice have higher

expression levels of CaSR (Yarova et al., 2015), linking abnormal CaSR expression to yet another inflammatory disease. Moreover, Cheng et al. (2014) reported a diminished intestinal barrier function and a more inflammatory immune response in intestinal epithelial-specific CaSR knockout mice, all of which increased their susceptibility to chemically induced colitis. Together, these studies clearly show a very broad involvement of CaSR in inflammatory processes.

## Monocyte-Specific CaSR

CaSR is expressed on various inflammatory cells, such as monocytes and macrophages. Expression of CaSR on monocytes has been implicated in chemotaxis, a key process in inflammatory diseases. For example, one study indicated an interrelationship between CCR2 and CaSR and also showed that $Ca^{2+}$ stimulates the chemotaxis of monocytes to CCL2 in a CaSR-dependent manner (Olszak et al., 2000). Paccou et al. (2013) followed up on this study by investigating CaSR expression on monocytes in response to several stimuli. Here, total CaSR expression increased in monocytes upon calcitriol, the biologically active form of vitamin D, stimulation whilst TNF decreased total CaSR expression in a dose-dependent manner (Paccou et al., 2013). Further connecting this receptor to inflammatory diseases, several studies report the activation of the NLRP3 inflammasome by CaSR in monocytes, where the review by Tang et al. (2018) provides an overview of all GPCRs involved in NLRP3 inflammasome activation and inhibition. NLRP3 inflammasome activation consequently leads to caspase-1 activation, which in turn cleaves pro IL-1β and pro IL-18 into their active forms mediating a pro-inflammatory response (Groslambert and Py, 2018). A study by Rossol et al. (2012) showed that monocytes sense changes in extracellular $Ca^{2+}$ concentrations via CaSR signaling, which subsequently leads to the activation of the NLRP3 inflammasome (Rossol et al., 2012). However, another study showed that NLRP3 is also activated by decreased cAMP concentrations, which is in striking contrast to the study by Rossol et al. (2012) where no significant influence of cAMP levels on inflammasome activation could be detected (Lee et al., 2012). Also in a human setting, CaSR could already be linked to atherosclerosis development as Malecki et al. (2013) showed a 1.5-fold increased CaSR expression on peripheral blood monocytes of patients with peripheral artery disease (PAD). Overall, CaSR expression on monocytes seems to enhance pro-inflammatory responses via stimulation of chemotaxis and inflammasome activation.

## Macrophage-Specific CaSR

Focusing more on macrophage-specific CaSR, stimulation of CaSR promotes the release of pro-inflammatory mediators, such as IL-1β and TNF-α, by monocyte derived macrophages (Xi et al., 2010). In line with this, Canton et al. (2016) reports that extracellular $Ca^{2+}$ is sensed by CaSR which subsequently signals through PLC and PI3K to induce constitutive micropinocytosis. Interestingly, two studies also linked macrophage-specific CaSR to the activation of the NLRP3 inflammasome. One study investigated the causal role of CaSR in the activation of the NLRP3 inflammasome via proteolytic pathways. It showed that the receptor activates the NLRP3 inflammasome and proteolytic maturation of IL-1β in differentiated macrophages via a chaperone-assisted degradative pathway (Gutierrez-Lopez et al., 2018). Another study by Lee et al. (2012) focused on the role of extracellular cations in inflammasome activation. It showed that CaSR activates the NLRP3 inflammasome in bone marrow-derived macrophages via increased intracellular $Ca^{2+}$ and decreased cAMP (Lee et al., 2012). Together, CaSR expressed on macrophages also promotes pro-inflammatory responses via increased release of cytokines and inflammasome activation.

## Pharmacological Intervention

As CaSR is implicated in various diseases, it has become an interesting pharmacological target to investigate. The compounds which target the receptor can be divided into two categories: the calcimimetics and calcilytics. Calcimimetic compounds are positive allosteric modulators and include Cinacalcet, NPS R-467 and NPS R-568 (Nemeth et al., 1998, 2004). On the other hand, calcilytics such as NPS 2143, Ronacaleret and Calhex 231 are negative allosteric modulators (Nemeth et al., 2001; Petrel et al., 2003; Balan et al., 2009). Cinacalcet was the first allosteric GPCR modulating compound to be approved for the market (Brauner-Osborne et al., 2007), showing promising results in several case studies. One study reports durable and robust effects of Cinacalcet therapy in patients with neonatal severe hyperparathyroidism (NSHPT). Cinacalcet was offered as an experimental alternative drug in a case of NSHPT, where Cinacalcet was successful in rapidly normalizing the patient's serum calcium levels, thereby improving muscle tone and the overall clinical condition (Gannon et al., 2014). Another study reports an acute increase of urinary calcium excretion in renal transplant recipients with secondary hyperparathyroidism after treatment with Cinacalcet, without showing adverse effects on glomerular filtration rate or renal graft calcium deposits (Courbebaisse et al., 2012). Calcilytics are mostly researched as a potential treatment of osteoporosis, but with limited success. Although clinically safe, no calcilytic to this day has been approved to be used to treat osteoporosis in humans (Kiefer et al., 2011). However, research has suggested the use of calcilytics in other diseases than osteoporosis, as for example Yarova et al. (2015) showed that calcilytics abrogate airway hyperresponsiveness and inflammation in allergic asthma.

## Concluding Remarks

Overall, CaSR plays an important role in various chronic inflammatory diseases, which is underlined by the many pro-inflammatory mechanisms induced by CaSR signaling in monocytes and macrophages. Calcimimetics and calcilytics show great therapeutic potential in other disease types, suggesting a potential of these drugs in the treatment of chronic inflammatory diseases as well. Important to keep in mind is that certain mutations and polymorphisms of CaSR affect the binding affinity of its allosteric modulators (Leach et al., 2013), making various patients less sensitive to pharmacological intervention. The development of compounds which can overcome these obstacles should be a focus point in future pharmacological research (please refer to **Table 1** for a summary of important studies

and their key findings and to **Table 2** for an overview of ligand types involved).

## CLOSING REMARKS

Research in recent decades has improved our understanding of the complex mechanisms of inflammatory processes within atherosclerosis. In addition, the CANTOS trial has clearly shown that the reduction of inflammatory processes has a positive effect on the outcome of CVD. The latter is particularly true for patients with pre-existing inflammatory conditions. In the context of these inflammatory processes, the interaction and activation of immune cells plays an important role. GPCRs are a group of receptors that play a central role in controlling these immune responses, but they are ubiquitously expressed and convey both pro- and anti-inflammatory signals. In addition, many of these receptors detect different ligands, which in turn deliver diverse immune responses depending on context (acute versus chronic), tissue and cell type involved. For example, the expression of CXCR4 seems atheroprotective by retention of neutrophils in the bone marrow and by maintaining arterial endothelial integrity while simultaneously endothelial-derived CXCL12 (*bona fide* ligand of CXCR4) appears pro-atherogenic. Data on FPR2 are similarly contradictory; here *in vivo* studies in mice show both more and less plaque progression in case of deletion of FPR2. In the context of an Annexin A1 supplementation, however, protective effects of the interaction of Annexin A1 and FPR2 on myeloid cells have been described. These examples, in turn, underline the significance of a specific ligand and cell type as part of a particular immune response. It is therefore crucial, in addition to the further characterization of receptor–ligand interactions and their consequences within chronic inflammation, to not draw generalized conclusions, but focus on individual conditions. With regard to therapeutic intervention aimed at GPCR-mediated immune responses, it is therefore also crucial to improve cell-specific drug delivery approaches and to identify other potentially impacting factors such as variation of genetic or epigenetic factors, which may influence therapeutic outcomes. Eventually, improvement of CVD therapy with respect to effective but safe therapeutics does clearly point in the direction of a cell-specific treatment tailored to the individual patient. Further elucidation and understanding of the concept of biased ligands, resulting in different signaling and thus effects of the binding of distinct ligands to the same receptor, could further improve the development of tailored treatment.

## AUTHOR CONTRIBUTIONS

EvdV, LP, MM, SG, YY, and YD drafted the manuscript and made critical revisions. CW made critical revisions.

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REVIEW
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# Role of Myeloid-Epithelial-Reproductive Tyrosine Kinase and Macrophage Polarization in the Progression of Atherosclerotic Lesions Associated With Nonalcoholic Fatty Liver Disease

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Recent lines of evidence highlight the involvement of myeloid-epithelial-reproductive tyrosine kinase (MerTK) in metabolic disease associated with liver damage. MerTK is mainly expressed in anti-inflammatory M2 macrophages where it mediates transcriptional changes including suppression of proinflammatory cytokines and enhancement of inflammatory repressors. MerTK is regulated by metabolic pathways through nuclear sensors including LXRs, PPARs, and RXRs, in response to apoptotic bodies or to other sources of cholesterol. Nonalcoholic fatty liver disease (NAFLD) is one of the most serious public health problems worldwide. It is a clinicopathological syndrome closely related to obesity, insulin resistance, and oxidative stress. It includes a spectrum of conditions ranging from simple steatosis, characterized by hepatic fat accumulation with or without inflammation, to nonalcoholic steatohepatitis (NASH), defined by hepatic fat deposition with hepatocellular damage, inflammation, and accumulating fibrosis. Several studies support an association between NAFLD and the incidence of cardiovascular diseases including atherosclerosis, a major cause of death worldwide. This pathological condition consists in a chronic and progressive inflammatory process in the intimal layer of large- and medium-sized arteries. The complications of advanced atherosclerosis include chronic or acute ischemic damage in the tissue perfused by the affected artery, leading to cellular death. By identifying specific targets influencing lipid metabolism and cardiovascular-related diseases, the present review highlights the role of MerTK in NAFLD-associated atherosclerotic lesions as a potential innovative therapeutic target. Therapeutic advantages might derive from the use of compounds selective for nuclear receptors targeting PPARs rather than LXRs regulating macrophage lipid metabolism and macrophage mediated inflammation, by favoring the expression of MerTK, which mediates an immunoregulatory action with a reduction in inflammation and in atherosclerosis.

**Keywords: monocytes, macrophages, nonalcoholic fatty liver disease, MerTK, inflammation, atherosclerosis, drug targeting**

## METABOLIC ASPECTS OF NAFLD: INSULIN RESISTANCE, METABOLIC SYNDROME, AND TYPE 2 DIABETES

Nonalcoholic liver disease (NAFLD) was firstly described in 1980 (Ludwig et al., 1980) and is currently the most common cause of chronic liver disease worldwide (Li et al., 2018). The global prevalence of NAFLD is estimated to be approximately 25%, with the highest rates in South America (31%) and Middle East (32%), followed by Asia (27%), USA (24%), Europe (23%), and Africa (14%) (Younossi et al., 2016). NAFLD comprises a spectrum of conditions ranging from simple hepatic lipid accumulation without inflammation, defined nonalcoholic fatty liver or NAFL, to nonalcoholic steatohepatitis (NASH), characterized by hepatic fat deposition with hepatocellular damage, inflammation, and fibrosis. This latter form in a smaller proportion of patients may lead to a series of complications including cirrhosis, liver failure, and hepatocellular carcinoma (HCC) (Marra et al., 2008; Ofosu et al., 2018). Cirrhosis may develop after about 15–20 years of chronic hepatocellular damage, and it is mainly characterized by a modified deposition of extracellular matrix components that, in cirrhotic liver, can be up to six times higher than in normal liver (Parola and Pinzani, 2018). In addition, inflammatory response contributes to hepatic encephalopathy, portal hypertension, liver failure, and increased risk of HCC (Tacke and Trautwein, 2015).

The development and progression of NAFLD is a complex and multifactorial process. NAFLD pathogenesis was originally described by the "two-hits hypothesis" (Day and James, 1998). According to this assumption, the "first hit" is represented by an excess intrahepatic lipid accumulation due to high intake of saturated fats, obesity, IR, and excessive fatty acids in the circulation (Marra, 2004). This sensitizes the liver to further insults acting as a "second hit" (Del Campo et al., 2018) including oxidative stress, lipid peroxidation, and mitochondrial dysfunction. These events give rise to a lipotoxic microenvironment, which leads to further damage of the hepatic tissue, consequently promoting inflammation and fibrogenesis (Buzzetti et al., 2016). More recent investigation has hypothesized that appearance of NASH is the result of the effects of signals deriving from multiple sites, including the gut, the adipose tissue, the muscle, and the liver itself, as illustrated as the "multiple-hits" hypothesis (Tilg and Moschen, 2010). The mechanisms underlying liver fibrosis are intricate and involve the interplay of multiple factors. Among these, a key role is played by the cross-talk between various liver-resident and infiltrating cellular subsets, which produce and secrete different soluble mediators (cytokines and chemokines) (Weiskirchen et al., 2018). In most cases, tissue injury induces an inflammatory response involving the local vascular system, immune cells, and release of endocrine and neurological factors. In this context, non-parenchymal cells [endothelial and hepatic stellate cells (HSCs)] and resident or recruited immune cells [macrophages, dendritic cells (DCs), and mast cells] secrete a variety of pro-inflammatory molecules such as tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β), pro-fibrotic factors including transforming growth factor-β (TGF-β) and pro-apoptotic mediators, as well as reactive oxygen species (Tilg and Diehl, 2000). All together, these signals lead to the activation of matrix-producing cells (including HSCs) and consequently to myofibroblast trans-differentiation (Weiskirchen et al., 2018).

NAFLD not only is related to obesity, hypertension, and inflammation but also is closely associated with insulin resistance (IR), metabolic syndrome (MetS), and type 2 diabetes (T2D) (Gentilini et al., 2016). A recent meta-analysis of published prospective studies has investigated for the first time the association between the presence of NAFLD and the risk of developing T2D and MetS. In particular, it has been observed that NAFLD (as diagnosed by either serum liver enzymes or ultrasonography) predicts T2D development over a median follow-up of 5 years in a pooled population of patients from 20 prospective studies. Moreover, NAFLD was also associated with an increase in MetS incidence over a median follow-up of 4.5 years in a pooled population of patients from eight prospective studies (Ballestri et al., 2016). Importantly, IR has been shown to be crucial for NAFLD progression. Indeed, approximately 80% of obese and diabetic patients are affected by NAFLD (Marchesini et al., 1999). IR is defined as the decreased ability of tissues to respond to insulin signals, and diverse types may be distinguished, a systemic and a hepatic insulin resistance. Systemic IR is characterized by the inability of insulin to diminish blood glucose levels in an appropriate manner due to the impairment of GLUT4 receptor translocation to the surface membrane of the muscle cell, leading to insulin-dependent lower glucose uptake (Petersen and Shulman, 2017). Hepatic IR is described by cessation of insulin-induced suppression of hepatic glucose production and increased stimulation of lipogenesis (Petersen and Shulman, 2017).

Interestingly, insulin also controls lipid metabolism, as it enhances fatty acid re-esterification into triglyceride in adipocytes and the liver. Metabolic actions of insulin are mediated by the PI3K–AKT/PKB pathway (Cohen, 2006), which is phosphorylated by the insulin receptor through two major substrates, insulin receptor substrate 1 and 2 (IRS-1 and IRS-2). Well-established AKT/PKB substrates include GSK-3, a

**Abbreviations:** nonalcoholic fatty liver disease (NAFLD), nonalcoholic steatohepatitis (NASH), MerTK (myeloid-epithelial-reproductive tyrosine kinase), hepatocellular carcinoma (HCC), cardiovascular diseases (CVD), insulin resistance (IR), tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), transforming growth factor-β (TGF-β), apoptotic cells (ACs), free fatty acids (FFAs), *de novo* lipogenesis (DNL), sterol regulatory element-binding protein 1c (SREBP-1c), patatin-like phospholipase-3 (PNPLA3), transmembrane 6 superfamily member 2 (TM6SF2), low-density lipoproteins (VLDL), triglycerides (TGs), high-density lipoproteins (HDL), low-density lipoproteins (LDL), growth arrest-specific 6 (Gas-6), carboxyl-glutamic acid (Gla), epidermal growth factor (EGF), sex hormone-binding globulin-like (SHBG), laminin G (LG), visceral adipose tissue (VAT), granulocyte macrophage colony-stimulating factor (GM-CSF), reactive oxygen species (ROS), reactive nitrogen intermediates (RNI), hepatic stellate cells (HSCs), macrophage colony-stimulating factor (M-CSF), growth arrest-specific-6 (Gas-6), matrix metalloproteinases (MMPs), inducible NO synthase (iNOS), hypoxia-inducible factor (HIF), pyruvate kinase M2 (PKM2), peroxisome proliferator-activated receptors (PPARs), liver X receptors (LXRs), retinoid X receptors (RXRs), dendritic cells (DCs), ATP-binding cassette transporter A1 and G1 (ABCA-1 and ABCG-1), reverse cholesterol transport (RCT), apolipoprotein B (APO-B), smooth muscle cells (VSMCs), phosphatidylserine (PtSer), toll-like receptor 4 (TLR4), soluble fragment of MerTK (sol-Mer), specialized pro-resolving mediators (SPMs), suppression of cytokine signaling-1 and 3 (SOCS-1 and SOCS-3), N,N-dimethyl-3β-hydroxycholenamide (DMHCA), methylpiperidinyl-3β-hydroxycholenamide (MePipHCA).

glycogen synthesis regulator, FOXO transcription factors, which upon phosphorylation inhibit transcription of FOXO-dependent gluconeogenic genes (Carter and Brunet, 2007), and sterol regulatory element-binding protein 1c (SREBP-1c), thus enhancing expression of rate-limiting glycolytic and lipogenic enzymes (Foretz et al., 1999; Foufelle and Ferré, 2002).

Also, promoting *de novo* lipogenesis in the liver, mediated by SREBP-1c, IR inhibits lipid export in the form of triglyceride-rich very-low-density lipoprotein (VLDL), hepatic FFA oxidation, and triglyceride (TG) accumulation, the major form of lipids stored in NAFLD patients (Browning and Horton, 2004).

Liver is the principal site of lipid metabolism; hepatic necro-inflammation has a crucial atherogenic role because it exacerbates systemic IR and promotes atherogenic dyslipidemia, with increased triglycerides, decreased high-density lipoprotein (HDL)-cholesterol, and increased low-density lipoprotein (LDL)-cholesterol (Nobili et al., 2010). Moreover, increased levels of highly atherogenic small dense type A LDL-cholesterol and of oxidized LDL-cholesterol are frequently detected in NAFLD. The main alteration in atherogenesis is the TG hepatic overproduction of as well as cholesterol-enriched VLDL particles.

## NAFLD AS A RISK FACTOR FOR CARDIOVASCULAR DISEASES

NAFLD has been recognized as strong predictor of increased carotid intima-media thickness, independent of other known cardio-metabolic risk factors.

Hepatic fat accumulation may be an important determinant of the relationship between NAFLD and atherosclerosis. Recently, it has been proposed that fatty liver is not *per se* a risk factor for atherosclerosis, unless it is associated with metabolic derangements. It has been suggested that there might be two different forms of fatty liver disease: one mainly related to metabolic abnormalities and another due primarily to genetic factors, characterized by higher risk of progressive liver damage (Sookoian and Pirola, 2011; Hamaguchi et al., 2007).

NAFLD is associated with adverse metabolic and atherosclerosis risk profiles (Fox et al., 2007; Neeland et al., 2013). From the metabolic point of view, the biological mechanism responsible for NAFLD-associated atherogenesis could be due to the crosstalk between visceral adipose tissue (VAT), gut, muscle tissue, and liver (Tilg and Moschen, 2010). Indeed, expanded and inflamed VAT releases molecules, such as adipokines, IL-6, and TNF-α, potentially involved in IR and cardiovascular disease (CVD) development (Fargion et al., 2014). Moreover, dietary chylomicrons and *de novo* lipogenesis contribute to the increased hepatic FFA pool as well as the development of NAFLD (Kleiner and Brunt, 2012).

Lipid accumulation in the liver leads to sub-acute inflammation followed by cytokine production *via* the NF-kB pathway. In particular, the activation of NF-kB leads to increased transcription of several pro-inflammatory genes that mediate the progression of systemic and low-grade inflammation. The increase in adipose tissue and chronic inflammation also cause an imbalance in adipokine secretion, in particular a reduction of adiponectin. Adiponectin has been shown to have anti-inflammatory and anti-fibrotic capacity (Di Maira et al., 2018; Marra et al., 2008), and its low levels are associated with high fat content (Bugianesi et al., 2005) and the progression from steatosis and CVD to NASH and CV-atherosclerosis, respectively (Matsuzawa et al., 2004). NASH is involved in atherogenesis through the systemic release of pro-atherogenic mediators (C-reactive protein, IL-6, and TNF-α) and hypercoagulation and hypo-fibrinolysis induction mediated by fibrinogen, factor VII, and plasminogen-activator inhibitor-1 (Kotronen and Yki-Järvinen, 2008; Targher et al., 2008). In this way, the liver becomes a source of pro-atherogenic molecules that amplifies arterial injury. In line with these results, growing evidence indicates that atherosclerosis is proportional to the severity of liver damage (Alkhouri et al., 2010) (**Figure 1**).

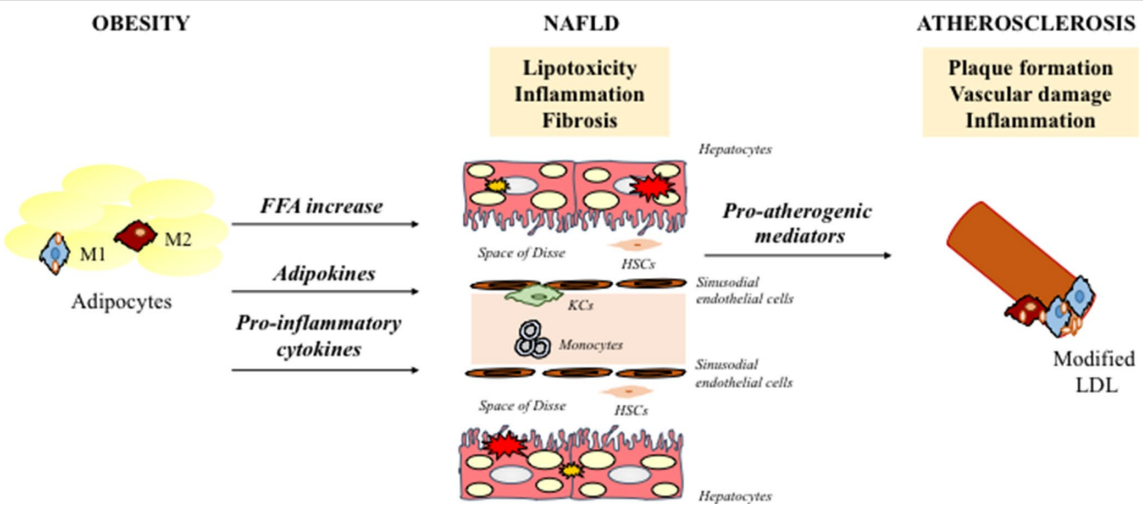



**FIGURE 1 |** Schematic representation of key mechanisms responsible for NAFLD associated-atherosclerosis. NAFLD contributes to a more atherothrombotic risk profile via atherogenic dyslipidemia, hepatic/systematic insulin resistance and increased secretion of several proinflammatory and pro-coagulant mediators. NAFLD, nonalcoholic fatty liver disease; HSCs, hepatic stellate cells; FFA, free fatty acids; LDL, low-density lipoproteins; KCs, kupffer cells.

The importance of NAFLD and its close association with CVD development has been highlighted by two meta-analyses. Notably, in a systematic meta-analysis of 34 cross-sectional and prospective cohort studies, an increase in coronary artery disease, hypertension, and atherosclerosis in NAFLD patients was observed, although no association between NAFLD/NASH and with overall or CVD-related mortality was shown (Wu et al., 2016). Additionally, (Targher et al., 2016) have described a strong correlation between NAFLD and increased risk of fatal and non-fatal CVD events, increased carotid intima-media thickness, increased coronary artery calcification, impaired flow-mediated vasodilation, and arterial stiffness. Indeed, several mechanisms correlated with NAFLD pathogenesis are involved in atherosclerosis and include genetic predisposition, reduced levels of adiponectin, IR, atherogenic dyslipidemia, oxidative stress, chronic inflammation, and altered production of pro- and anti-coagulant factors (Francque et al., 2016). Recently, a systematic review (Zhou et al., 2018) has described a two-fold increase in risk of CVD in diabetic NAFLD patients compared with non-NAFLD group, confirming other previous findings (Targher et al., 2007; Hamaguchi et al., 2007). Likewise, other two independent studies found that TG to high-density lipoprotein cholesterol ratio (TG/HDL-C) could be considered a better NAFLD predictor compared to other several lipid parameters and markers of liver injury (Ren et al., 2019; Fan et al., 2019).

Remarkably, also several polymorphisms associated with predisposition of NAFLD progression have been identified. Among the most validated factors, the Patatin-like phospholipase-3 (PNPLA3)/adiponutrin, rs738409 C > G SNP, I148M (Valenti et al., 2010) variant is involved both in hepatic lipid remodeling and in lipoprotein secretion, determining a greater predisposition to NASH (He et al., 2010; Ruhanen et al., 2014). Indeed, there is dissociation between *de novo* lipogenesis and the severity of hepatic steatosis in carriers of the I148M variant (Mancina et al., 2015). The involvement of the PNPLA3 variant has been observed also in lean subjects, where the presence of PNPLA3 GG genotype is correlated with a more severe liver and cardiovascular damage (Fracanzani et al., 2017). Among NAFLD patients, with minor metabolic alterations, the presence of GG PNPLA3 makes the subjects more susceptible to liver and CVDs, amplifying the effects of environmental factors (Fracanzani et al., 2017). In addition, carotid plaques have been independently associated not only with well-known risk factors for atherosclerosis but also with the PNPLA3 GG genotype (Petta et al., 2013).

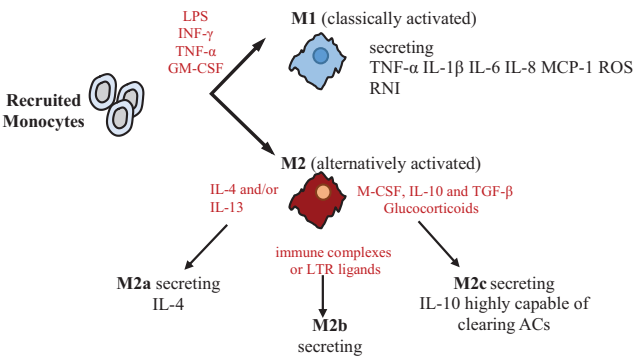
Moreover, the transmembrane 6 superfamily member 2 (TM6SF2) rs58542926 C > T SNP, which encodes the loss of function E167K variant, has been associated with higher risk of NAFLD progression but with lower risk of cardiovascular events (Pirazzi et al., 2012). This protective effect of the E167K variant reflects the reduced circulating levels of atherogenic lipoproteins, because of higher intracellular lipid retention, mainly TGs and cholesterol, in hepatocytes. The mechanism seems related to the reduction of VLDL secretion, thus resulting in TG accumulation and consequent steatosis (Dongiovanni et al., 2015).

## ACTIVATION OF MACROPHAGES DEPENDS ON THEIR METABOLIC STATE

In the liver, resident macrophages, the Kupffer cells are central players in the development of NASH by recruiting inflammatory immune cells and secreting pro-inflammatory cytokines (Sica et al., 2014; Raggi et al., 2015; Raggi et al., 2017). Importantly, the balance between M1 and M2 macrophages (**Box 1**) mediates

**BOX 1 |** Macrophage polarization.

In response to various signals, activated macrophages differentiate into two main subsets: M1 (classically activated) and M2 (alternatively activated). M1 macrophages are stimulated by LPS, INF-γ, TNF-α, and/or granulocyte macrophage colony-stimulating factor (GM-CSF) to produce inflammatory mediators, including TNF-α, IL-1β, IL-6, IL-8, IL-12, chemokine (C-C motif) ligand 2 (CCL2/MCP-1), reactive oxygen species (ROS), and reactive nitrogen intermediates (RNI), promoting inflammatory responses and HSC activation (Nathan, 2002). M2 macrophages regulate inflammatory reactions and tissue repair and can be distinguished in diverse subtypes, each one induced by different cytokines and eliciting different signals. In particular, M2a macrophages (CD206/mannose receptor$^+$ CD209/DC-SIGN$^+$ CD163$^-$ CD16$^-$ MerTK$^-$) are stimulated by IL-4 and/or IL-13 and induce mainly a Th2 response. M2b macrophages are stimulated by immune complexes or LTR ligands and are involved in Th2 activation and immune regulation, producing IL-10 and inflammatory cytokines. Finally, M2c macrophages (CD206$^{high}$ CD209$^-$ CD163$^+$ CD16$^+$ MerTK$^+$) are stimulated by macrophage colony-stimulating factor (M-CSF) plus IL-10 and TGF-β or by glucocorticoids, are characterized by their ability to secrete IL-10, which, in turn, is amplified by Gas-6 secretion in an autocrine manner, *via* MerTK signaling, and are involved in immune suppression, tissue repair, matrix remodeling, and clearance of apoptotic cells (Zizzo et al., 2012; Martinez et al., 2006).




However, this concept is a little too simplistic to describe the polarization of liver macrophages, especially in pathological conditions. In the injured liver, macrophages often express markers of inflammation or resolution simultaneously, and can rapidly change their phenotype depending on the hepatic microenvironment (Tacke, 2017). During the early stages of liver injury, bone marrow-derived monocytes are intensively recruited to the liver and differentiate into inflammatory macrophages (mostly M1) to produce pro-inflammatory and profibrotic cytokines. Subsequently, recruited macrophages switch to an M2 phenotype, which secretes a wide variety of matrix metalloproteinases (MMPs), such as MMP-2, MMP-9, MMP-12, MMP-13, and MMP-14, and anti-inflammatory cytokines such as IL-4, IL-13, and IL-10, aimed to facilitate fibrosis resolution (Pradere et al., 2013).

the progression or resolution of liver fibrosis. Intriguingly, M1-M2 functional changes have been shown to be dependent on underlying metabolic changes (O'Neill and Pearce, 2016).

In the lean adipose tissue, M2 macrophages produce high amounts of ATP (~30 per glucose) through oxidative phosphorylation, a biochemical process slower than glycolysis (O'Neill et al., 2016). In contrast, increased lipid storage in obesity is associated with adipocyte dysfunction and a pro-inflammatory response, with an increase in M1-polarized pro-inflammatory macrophages (Norata et al., 2015). In particular, activation of immune receptors, such as TLRs, IL-1 receptor type I, and TNF-R, results in activation of NF-kB and JNK signaling, which can induce serine phosphorylation of IRS-1 and IRS-2 and thereby inhibition of downstream insulin signaling (McNelis and Olefsky, 2014; Marra, 2008).

In M1 macrophages, upregulation of glycolytic metabolism feeds the pentose phosphate pathway (Van den Bossche et al., 2017). Although glycolysis produces only a small amount of energy (two molecules of ATP per glucose) (Nagy and Haschemi, 2015), this pathway supports inflammatory macrophage responses by generating NADPH, utilized by inducible NO synthase (iNOS) to produce NO or by NADPH oxidase to produce ROS, both necessary to sustain the antimicrobial activity of pro-inflammatory macrophages (Modolell et al., 1995). Moreover, glycolysis generates pyruvate to fuel the tricarboxylic acid cycle. In M1 macrophages, this cycle is interrupted after citrate and succinate (Jha et al., 2015; O'Neill, 2015). Increased synthesis of acetyl coenzyme A from citrate determines the synthesis of free fatty acids (FFAs), lipids, and prostaglandins (Infantino et al., 2011; Infantino et al., 2013). Up-regulation of proteins involved in the uptake [e.g., CD36 (Bassaganya-Riera et al., 2009)], esterification [e.g., diacylglycerol O-acyltransferase (Koliwad et al., 2010)], and oxidation [e.g., long-chain 3-hydroxyacyl-CoA dehydrogenase (Vats et al., 2006)] of FFAs could provide energy for M2 cells to restore tissue homeostasis (Shapiro et al., 2011). These processes would allow a reduction of FFA concentration by reducing IR and inflammation (Vats et al., 2006) (**Figure 2**).

## MERTK IN THE ACTIVATION OF ANTI-INFLAMMATORY M2C MACROPHAGES

MerTK represents the second member of Tyro-3, Axl, and Mer (TAM) receptor tyrosine kinase (RTK) family to be described (Linger et al., 2008).These receptors are characterized by adhesion molecule-like domains in the extracellular region, mimicking the structure of neural cell adhesion molecule important in cell–cell contacts, which contains five Ig domains and two fibronectin type III domains (Yamagata et al., 2003). The best-studied ligands for MerTK are the Vit-K modified-carboxylated proteins growth arrest-specific 6 (Gas-6) and Protein-S (Mark et al., 1996; Anderson et al., 2003). These glycoproteins share ~44% of homology and have analogous domain structure, consisting of an N-terminal-carboxyl-glutamic acid (Gla) domain, four tandem epidermal growth factor (EGF)-like repeats, and a C-terminal sex hormone-binding globulin-like region (SHBG) containing 2 laminin G (LG) repeats (Mark et al., 1996). Gas-6 and Protein-S are biologically active following the carboxylation of the Gla-domain through a vitamin K-dependent reaction (Stenhoff et al., 2004). This domain mediates the $Ca^{2+}$-dependent binding to negatively charged membrane phospholipids, such as phosphatidylserine exposed on the surface of apoptotic cells

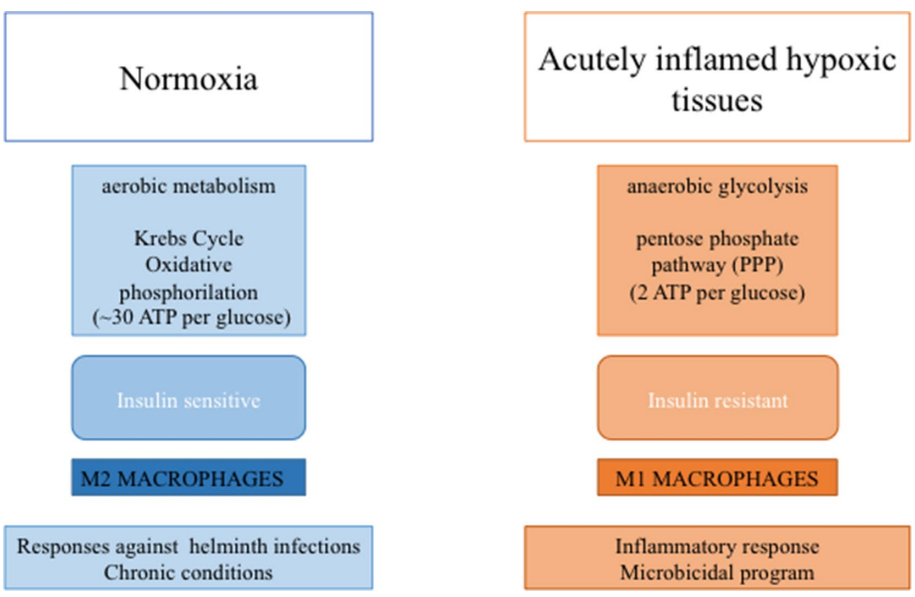



**FIGURE 2 |** Macrophage-mediated functions to metabolic activities. In diagram on the left are depicted tissue features in the lean state associate with M2-macrophage polarization, while in diagram on the right are depicted tissue features during inflammation and insulin resistance associate with M1-macrophage polarization.

(Huang et al., 2003). LG domains are involved in the ligand–receptor interaction by forming a V-shaped structure, stabilized by a calcium-binding site (Sasaki et al., 2002). In human plasma, protein-S is highly concentrated (0.30 μM/L) (Rezende et al., 2004) (approximately 1,000 times higher), compared to Gas-6 (0.16–0.28 nM/L) (Balogh et al., 2005), conceivably due to the involvement of protein-S in the coagulation pathways, where it functions as a co-factor for protein C during factor Va and VIIIa inactivation (Heeb, 2008). Gas-6 is expressed mainly in vascular smooth muscle and endothelial cells, and it is frequently upregulated after tissue damage (Ekman et al., 2010).

MerTK is normally expressed in monocytes/macrophages, DCs, NK cells, NKT cells, HSCs, megakaryocytes, platelets, epithelial tissue, and reproductive tissue (Behrens et al., 2003; Petta et al., 2016). M2c macrophages express MerTK at high levels and display a marked capability to clear apoptotic bodies, a physiological process defined as efferocytosis (Zizzo et al., 2012). Interestingly, it has been demonstrated that M2c polarization is closely associated with MerTK upregulation, and detection of M2c receptors predicts MerTK expression (Zizzo et al., 2012). Indeed, MerTK expression and Gas-6 secretion follow the expression of specific M2c macrophages CD163 and CD16. This specific macrophage phenotype can be induced by M-CSF or dexamethasone, and IL-10 could enhance M-CSF effects. In addition, M2c macrophages are able to release Gas-6, which can, in turn, amplify IL-10 secretion in an autocrine manner, *via* MerTK signaling (Zizzo et al., 2012). Gas-6, linked to the externalized phosphatidylserine, activates MerTK, initiating the phagocytic process and inducing the activation of downstream pathways, such as ERK, P38, MAPK, FAK, AKT, and STAT-6, that mediate transcriptional events, leading to a decrease of pro-inflammatory cytokines, such as IL-12, and increase in inflammatory repressors, including IL-10 and TGF-β, thus generating an anti-inflammatory milieu (Tibrewal et al., 2008).

MerTK signaling plays a central role in the suppression of the innate immune response, as demonstrated in experimental models of endotoxemia, in which MerTK knockout mice exhibit an extreme activation of inflammatory responses and ineffective resolution of inflammation, mediated by elevated levels of TNF-α and IL-1 (Lee et al., 2012). MerTK acts by maintaining both central and peripheral tolerance, through different mechanisms, including efferocytosis. It is already well-described MerTK overexpression in murine models of fibrogenesis and in patients with NASH and severe fibrosis (Petta et al., 2016). Indeed, in genome-wide association studies, it has been reported that the MERTK locus rs4374383 G > A correlates with decreased hepatic MerTK expression, thus protecting against liver fibrosis in chronic hepatitis C and NAFLD (Patin et al., 2012; Petta et al., 2016). The same G > A variant has been found to be associated with cardio-metabolic derangement and nutritionally induced inflammation and could contribute in this way to liver and cardio-metabolic disease (Musso et al., 2017). Moreover, it has been shown that in human NAFLD specimens, MerTK is mainly expressed in macrophages and HSCs loosely aggregated within inflammatory foci (Petta et al., 2016). MerTK signaling has been recently studied also in humans with both acute liver failure syndromes and acute-on-chronic liver failure, where a significant cause of morbidity is sepsis. Triantafyllou et al. (Triantafyllou et al., 2018) have shown an expansion of MerTK-positive cells in circulatory and tissue compartments of patients with acute liver failure compared with healthy and cirrhotic controls, together with a concomitant increase in Gas-6 and in MerTK phosphorylation.

Notably, in response to acute liver injury, MerTK mediates downregulation of inflammatory cascades contributing to hepatic immune regulation by preventing autoreactive T cell development. However, in the context of chronic inflammation, MerTK promotes HSC activation, thus resulting in excessive fibrogenesis by abundant collagen and extracellular matrix proteins secretion (Petta et al., 2016).

Various therapies targeting MerTK are currently under development. Small-molecule tyrosine kinase inhibitors such as UNC569 (Christoph et al., 2013), UNC1062 (Schlegel et al., 2013), and UNC1666 (Lee-Sherick et al., 2015) have been recently described. These compounds competitively bind MerTK in its catalytic site, impeding phosphorylation and activation of the kinase domain. Treatment with these inhibitors causes a decrease in MerTK downstream signaling. Next-generation inhibitors have also been reported, including UNC2025, a potent, orally bioavailable inhibitor (Zhang et al., 2014). Other agents include Mer590, a monoclonal antibody that directly binds to the extracellular domain and induces internalization and degradation of MerTK (Cummings et al., 2014).

Small-molecule inhibitors and monoclonal antibodies are the main drugs that are currently used to inhibit signaling pathways by interfacing with specific molecules. Nevertheless, any targeted therapy has its own limitations. Although identifying a specific molecular target is crucial for NAFLD-associated cardiovascular treatment, targeting only a single molecule may not be completely determinant of these complex diseases. Other limitations include toxicity during the treatment, as well as mechanisms of resistance to molecular-targeted drugs.

## MACROPHAGE NUCLEAR RECEPTORS CONTROL MERTK EXPRESSION IN LIPID METABOLISM NAFLD ASSOCIATED

Macrophage polarization is an important mechanism for the regulation of inflammatory response, and it is finely controlled by the nuclear receptor superfamily members peroxisome proliferator activated receptors (PPARs) (α, β/δ, and γ isotypes) and liver X receptors (LXRs) (LXR-α and LXR-β) (Rigamonti et al., 2008). These transcription factors form heterodimers with retinoid X receptors (RXR) (α and β isotypes) and, upon binding a lipid or synthetic ligand, mediate gene expression through trans-activation (Szanto and Roszer 2008). Nuclear receptors have considerable roles in the modulation of macrophage functions. Their ligands influence the transcription of genes regulating lipid homeostasis, pro-inflammatory cytokine production, resolution of inflammation, and synthesis of mediators that promote tissue healing (Rőszer et al., 2013; Menendez-Gutierrez et al., 2012). PPAR-γ can be activated by metabolic signals (i.e., polyunsaturated fatty acids and lipoproteins) (Nagy et al., 1998), by inflammatory mediators

(i.e., eicosanoids) (Kliewer et al., 1997), or by immunologic signals (i.e., cytokines) (Huang et al., 1999). PPAR-γ activation results in lipid uptake through the scavenger receptor CD36, and β-oxidation of fatty acids (Szanto and Roszer, 2008) is associated to macrophage polarization into M2a cells (Bouhlel et al., 2007). LXRs are oxysterol-activated transcription factors that sense elevated cellular cholesterol (Repa and Mangelsdorf, 2002). PPAR-γ and LXR activities are coordinated, PPAR-γ is in fact able to activate LXRs, but in certain conditions, PPAR-γ and LXRs exert opposing roles (Szanto and Roszer, 2008). In M2a macrophages, IL-4 stimulates the increase of PPAR-γ expression and LXR-α downregulation (Chinetti-Gbaguidi et al., 2011). LXRs are important for both apoptotic cell clearance and suppression of the inflammatory response during their phagocytosis. PPARs and LXRs control the transcription of many receptors, including MerTK.

Accumulation of excess lipoprotein-derived cholesterol in macrophages activates LXRs that, in turn, trigger the induction of ABC transporter, mediating cholesterol efflux (Castrillo and Tontonoz, 2004) and the upregulation of MerTK in mice (A-Gonzalez et al., 2009) and in humans (Zizzo and Cohen, 2015). Gonzalez et al. have demonstrated that phagocytosis of apoptotic cells activates LXRs, probably through the accumulation of membrane-derived cholesterol. LXRs, in turn, activate transcription of MerTK, generating a positive feedback to promote further efferocytosis, a process that mediates the increased expression of ABC transporter genes such as ABCA-1 and ABCG-1, involved in the efflux of the excess cholesterol and immunosuppression (A-Gonzalez et al., 2009). These results indicate that the LXR-dependent regulation of MerTK is important for normal immune homeostasis. MERTK$^{-/-}$ and LXRs DKO mice share a series of features, including amplified pro-inflammatory responses and increased susceptibility to both autoimmunity and atherosclerosis (Ait-Oufella et al., 2008; Cohen et al., 2002).

## ROLE OF MERTK IN ATHEROSCLEROSIS PROCESS

Atherosclerotic lesions are clinically silent, and the acute cardiovascular events can be consequent to evolution to necrotic plaques (Virmani et al., 2000). At first, apoptotic cells are efficiently cleared by neighboring macrophages to limit overall lesion cellularity (Tabas, 2005). Here, efferocytosis is rapid and without inflammation. In physiological conditions, apoptotic cells are engulfed and degraded in phagolysosomes, and macrophages become overloaded with macromolecular constituents and cholesterol. In advanced atherosclerosis, the persistence of chronic inflammatory stimuli promotes lesion destabilization and susceptibility to heart attack and stroke. The role of inflammation in promoting atherosclerosis is well documented. Indeed, in advanced plaques, apoptotic foam cells, induced by chronic endoplasmic reticulum stress, elicit inflammatory responses (Li et al., 2013). In addition, endoplasmic reticulum stress is strongly correlated with plaque rupture (Li et al., 2006). Two processes contribute to post-apoptotic necrosis and defective efferocytosis and are impaired to resolve the inflammation response (Schrijvers et al., 2005; Libby, 2002; Tabas, 2010). Defective efferocytosis may be manifest at multiple levels, including improper presentation of apoptotic bodies ligands, failure to secrete *come find me* recruitment signals, or defects at the level of phagocytes (Vandivier et al., 2006). Efferocytosis is impaired in this last stage, and defective MerTK contributes, at least in part, to expansion of necrotic plaque (Tabas, 2005). In this regard, evidence demonstrates that mice lacking MerTK have shown a defect in efferocytosis, and this correlated with increased inflammation and necrosis within the plaque (Ait-Oufella et al., 2008; Thorp et al., 2008). Moreover, macrophages near the necrotic core of human atheroma showed lower MerTK expression than those in the peripheral lesions (Garbin et al., 2013). Finally, in the advanced atherosclerosis, accumulation of lipids and ROS increases levels of oxidized phospholipids. These lipids can bind to scavenger receptors and may compete for apoptotic cell recognition, compromising efferocytosis mechanisms (Gillotte-Taylor et al., 2001). A recent study shows that in the lesions, prevention of dead cells' uptake is mediated by some apoptotic cells displaying a *don't-eat-me* molecule called CD47, which is usually lost during apoptosis (Kojima et al., 2016).

Efferocytosis can be impaired by inactivation of MerTK under some inflammatory conditions (Sather et al., 2007). In particular, oxidized LDLs induce the expression of toll-like receptor 4 (TLR4), increase secretion of pro-atherogenic cytokines, such as TNF-α and IL-1β, and reduce secretion of anti-inflammatory cytokines, such as TGF-β and IL-10 (Bae et al., 2009). This pro-inflammatory environment impairs efferocytosis, promoting increased lipid uptake, which amplified phagocytosis, and reducing MerTK expression levels on the macrophage surface (Miller et al., 2003). The decrease of MerTK expression is associated with its cleavage by the metalloproteinase ADAM17. In human atheromas, macrophages adjacent to the necrotic core have higher ADAM17 than those in peripheral lesion (Garbin et al., 2013). Multiple athero-inflammatory stimuli, such as oxidative stress, hypoxia, and oxidized ligands, are able to promote ADAM17 activity (Sather et al., 2007; Garbin et al., 2013). Efferocytosis is suppressed by destroying the receptor and creating soluble Mer (sol-Mer), which competes for the binding molecules Gas-6 and Protein-S. Interestingly, oxidized LDLs, promoting MerTK cleavage and defective efferocytosis, can activate necroptotic pathways within advanced plaques, favoring the development of necrotic core (Karunakaran et al., 2016). In a recent study, it has been demonstrated that oxidized LDLs are able to increase sol-Mer levels and decrease MerTK expression in the surface of wild-type macrophages but not in macrophages pre-treated with ADAM17 inhibitor or in macrophages that express cleavage-resistant MerTK (Cai et al., 2016). Of note, MerTK-mediated efferocytosis might be limited by availability of Gas-6. In this regard, vascular smooth muscle cells appear to be a major source of Gas-6 within the lesions (Melaragno et al., 1999; Yin et al., 2000). It has been reported that vulnerable plaques have a paucity of smooth muscle cells in areas next to rupture (Clarke et al., 2006).

## NUCLEAR RECEPTORS AS MACROPHAGE THERAPEUTIC TARGET

Nuclear receptors such as PPARs and LXRs are important transcription factors associated with the specific accessory functions of macrophages. PPAR-γ exhibits great potentiality as a drug target in the therapy of inflammation-related diseases. Thiazolidinediones are insulin sensitizers used to improve glycemic control in T2DM patients. However, they may cause weight gain, fluid retention that can precipitate cardiac failure and bone fractures, and risk of bladder cancer (Cariou et al., 2012). In order to eliminate the onset of these effects, further research into new PPAR-γ modulators is required.

GW9662 is a potent, irreversible, and selective PPAR-γ antagonist. Zizzo et al. have shown that this PPAR-γ antagonist induces macrophage differentiation towards M2c-like ($CD206^+$ $CD163^+$ $CD16^+$) cells and upregulation of the MerTK/Gas-6 axis. It has shown that among the novel small molecules derived from GW9662, BZ-26 has a stronger interaction with PPAR-γ and higher transcriptional inhibitory activity of PPAR-γ compared with GW9662. BZ-26 inhibits inflammatory macrophage differentiation of THP1 human monocytic cell line (Bei et al., 2016). Moreover, BZ-26 attenuates the inflammatory responses in LPS-triggered acute inflammation mouse model down-regulating peripheral TNF-α and IL-6 level. BZ-26 inhibits NF-kB transcriptional activity and abolishes LPS-induced nuclear translocation of P65. These data demonstrate that PPAR-γ, besides being a ligand-activated nuclear receptor implicated in regulation of lipid and glucose metabolism, is a fundamental transcription factor for differentiation and activation of macrophages. PPAR-γ could represent an important therapeutic target to modulate inflammation *via* inhibiting inflammatory macrophages.

LXRs are important regulators of cholesterol, free fatty acids, and glucose metabolism. LXRs drive cholesterol efflux in macrophages (through ABCA-1 and ABCG-1) and support reverse cholesterol transport by cholesterol conversion to bile acids and excretion in the liver. Moreover, their activation is important in regulating immune processes and in inhibiting inflammatory gene expression (Joseph et al., 2003). It has been shown that T0901317, a synthetic LXR agonist, upregulates MerTK expression during the polarization of monocytes to macrophages independently of M2c polarization, with significant effects already occurring at low doses (Zizzo and Cohen, 2015), confirming previously obtained data in mice (A-Gonzalez et al., 2009). Unfortunately, synthetic LXR agonists, such as T0901317, mediate reverse cholesterol transport not only in the macrophage but also in other cell types, including hepatocytes (Grefhorst et al., 2002). Activation of cholesterol efflux from both sources induces the activation of a lipogenic program, mediated by SREBP (Grefhorst et al., 2002), which induces remarkable steatosis and dyslipidemia in the liver in mouse models and human patients (Kirchgessner et al., 2016). These conditions make LXR's therapeutic targeting unsustainable. Recently, Muse et al. have identified two synthetic compounds: N,N-dimethyl-3β-hydroxycholenamide (DMHCA) and methylpiperidinyl-3β-hydroxycholenamide (MePipHCA), which act as potent activators of LXR target genes involved in cholesterol efflux (e.g., ABCA-1 and ABCG-1) in human and murine macrophages, while having no effect on the expression of lipogenic SREBP targets (e.g., Fasn) in the liver (Magida and Evans, 2018). Interestingly, DMHCA and MePipHCA activity on Kupffer cells does not induce target gene activation in the liver (Muse et al., 2018). These two LXR agonists exhibit anti-atherosclerotic activity without causing substantial hypertriglyceridemia in mice; therefore, they might represent a new class of athero-protective agents (Muse et al., 2018). It has been demonstrated the efficacy of DMHCA and MePipHCA in suppressing inflammation without causing liver lipid accumulation or liver injury in mouse models (Yu et al., 2016) (**Table 1**). Further studies will be needed to evaluate the effect of these compounds on macrophage polarization and activation. A limitation of these compounds is the large dose for *in vivo* efficacy. Therefore, the pharmacokinetic profile of these molecules will need to be improved.

**TABLE 1 |** Overview of nuclear receptors functions modulating lipid metabolism in macrophages and actions mediated by their synthetic ligands.

| Receptors | Role in macrophages polarization | Role in lipid metabolism | Agonist or antagonis |
|---|---|---|---|
| PPARγ | Mediates macrophage differentiation via STAT-6 into M2a cells (Huang et al., 1999) | Regulates lipid uptake through the scavenger receptor CD36 (Szanto and Roszer, 2008) Regulates β-oxidation of fatty acids (Vats et al., 2006) | ***PPARγ antagonists:*** **GW9662** induces M2c polarizing effects, with upregulation of MerTK (Zizzo and Cohen, 2015) **BZ-26** attenuates inflammation by inhibiting the differentiation inflammatory macrophages (Bei et al., 2016) |
| LXRs | Promote uptake of ACs through the induction of MerTK (A-Gonzalez et al., 2009) | Act as oxysterols sensors Mediate cholesterol efflux (Szanto and Roszer, 2008) | ***LXRs agonists:*** **T0901317** upregulates MerTK expression (Zizzo and Cohen, 2015) (A-Gonzalez et al., 2009) **DMHCA and MePipHCA** act as potent activators of LXRs target genes involved in cholesterol efflux (Magida and Evans, 2018) |

## CONCLUSIONS AND FUTURE PERSPECTIVES

Although there is a clear association between NAFLD and the progression of atherosclerotic lesions, the underlying mechanisms are only partially delineated. Lipid metabolism plays key roles in the polarization of macrophages, which, in turn, influences the pathogenesis of lipid-related diseases (**Figure 1**). The huge complexity of NAFLD and CVD pathogenesis suggests a multitarget pharmacological approach, in which macrophages represent an intriguing target.

In this scenario, the nuclear receptor-dependent regulation of MerTK is important for immune homeostasis and MerTK regulates the pro-inflammatory responses reducing both autoimmunity and atherosclerosis.

## AUTHOR CONTRIBUTIONS

MP, SG, RMP, GL, CR, SP, and FM contributed to analysis of publications, drafting of the manuscript, and critical revision of the content.

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REVIEW
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# Monocytes, Macrophages, and Metabolic Disease in Atherosclerosis

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Atherosclerotic cardiovascular disease (CVD) is a lipid-driven chronic inflammatory disease, in which macrophages are responsible for taking up these lipids and driving disease progression. Over the years, we and others have uncovered key pathways that regulate macrophage number/function and identified how metabolic disorders such as diabetes and obesity, which are common risk factors for CVD, exacerbate these pathways. This ultimately accelerates the progression of atherosclerosis and hinders atherosclerotic regression. In this review, we discuss the different types of macrophages, from monocyte-derived macrophages, local macrophage proliferation, to macrophage-like vascular smooth muscle cells, that contribute to atherosclerosis as well as myeloid-derived suppressor cells that may have anti-atherogenic effects. We will also discuss how diabetes and obesity influence plaque macrophage accumulation and monocyte production (myelopoiesis) to promote atherogenesis as well as an exciting therapeutic target, S100A8/A9, which mediates myelopoiesis in response to both diabetes and obesity, shown to be effective in reducing atherosclerosis in pre-clinical models of diabetes.

**Keywords: diabetes, obesity, monocyte, macrophage, atherosclerosis**

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## INTRODUCTION

Cardiovascular disease (CVD) remains the leading cause of death worldwide and is of major concern in populations with increasing prevalence of metabolic disease (World Health Organization, 2017). Diabetes and obesity are both independent risk factors for CVD, with diabetic and pre-diabetic patients accounting for 65% of all CVD deaths (Barr et al., 2007; Flint et al., 2010). Complicating the situation further, obesity-associated diabetes accounts for 90–95% of adult diabetes diagnoses (Mozaffarian et al., 2015). Importantly, traditional risk factors, such as dyslipidemia and hypertension, fail to account for the increased risk of CVD in diabetes. Moreover, through the JAPAN-ACS trial, it was found that, while statin therapy is equally effective in lowering cholesterol levels in patients with and without diabetes, patients with diabetes exhibited impaired plaque regression (Hiro et al., 2010).

In recent years, targeting inflammation in CVD with the Canakinumab Anti-inflammatory Thrombosis Outcome Study (CANTOS) demonstrated that inhibiting interleukin-1β (IL-1β) reduces the incidence of secondary cardiovascular events, independent of lipid lowering (Ridker et al., 2017). Further, it has become well established through pre-clinical models that changes in the inflammatory milieu of atherosclerotic plaques, as well as systemic inflammation, play an important role in the development and vulnerability of atherosclerotic plaques to rupture. In particular, macrophages and their circulating precursors, monocytes, have been shown to play an important role in the pathogenesis of atherosclerosis (Murphy and Tall, 2016). This review will discuss the

role of different sources of macrophages in atherosclerosis in the context of diabetes and obesity with a focus on the role of monocyte-derived macrophages and myelopoiesis in promoting atherosclerosis.

## MACROPHAGES IN ATHEROSCLEROSIS

Atherosclerosis is primarily driven by the combination of lipid accumulation and immune cells within the plaque (Moore et al., 2013). In particular, macrophages play a crucial role in the development of atherosclerosis through uptake of modified cholesterol, in particular oxidized low-density lipoproteins (oxLDL), and the subsequent impairment of cholesterol efferocytosis, resulting in lipid-laden macrophages known as foam cells. These foam cells have impaired migratory capacity and thus become trapped within the plaque where they die and form a necrotic core (Pagler et al., 2011). In mice, macrophages are particularly important in promoting the development of early atherosclerotic lesions beginning to accumulate during the formation of fatty streaks; however, they are also involved in the transformation to a mature, inflammatory and unstable lesion, vulnerable to rupture (Moore and Tabas, 2011). In humans, plaque development begins at sites of disturbed flow through the formation of vascular smooth muscle cell (VSMC)-rich adaptive intimal thickening and the retention of modified lipoproteins, which precede immune cell infiltration (Otsuka et al., 2015). Macrophages subsequently accumulate within the lesion and increase throughout disease progression (Otsuka et al., 2015).

During the early fatty-streak stages of atherosclerosis in mice, M2 (pro-resolving) macrophages predominate in the lesion, whereas more advanced plaques are suggested to exhibit a shift towards an M1 (inflammatory)-dominant state (Khallou-Laschet et al., 2010). However, during plaque regression, decreases in macrophage content is accompanied by the restoration of the M2 phenotype (Rahman et al., 2017). In humans, macrophages found to express M1 or M2 markers have both been identified in the lesion, with M1 macrophages predominate within the rupture-prone shoulder regions of the plaque (Stoger et al., 2012). In vulnerable, symptomatic plaques, markers of M1 macrophages (CD68, CD11c) are increased while M2 markers (CD163 and mannose receptor) are decreased (Cho et al., 2013). Furthermore, a myriad of macrophage subtypes and species-specific populations have been defined in both mice and humans and reviewed elsewhere (Bobryshev et al., 2016), all contributing to different roles in atherosclerosis. In diabetic models, hyperglycemia significantly increases the abundance in plaque macrophages, thereby inducing a twofold effect: 1) accelerating plaque formation and 2) hindering plaque regression, even with adequately controlled plasma cholesterol levels. This increase in

**TABLE 1 |** Summary of the sources of macrophages and macrophage-like cells within the plaque and their role in atherosclerosis and metabolic disease.

| Macrophage type | Resident tissue macrophages | VSMC-derived macrophage-like cells | Monocyte-derived macrophages | Monocytic MDSCs |
|---|---|---|---|---|
| *Origin* | Likely to ultimately originate from monocytes | Vascular smooth muscle cells | Bone marrow stem cells *via* medullary or extramedullary (spleen) myelopoiesis | Bone marrow stem cells *via* medullary or possibly extramedullary (spleen) myelopoiesis |
| *Subsets and markers* | CD68, Mac-2<br>$Brdu^+$ following labeling | CD68, Mac-2 | CD68, Mac-2<br>will incorporate Brdu<br>**Monocytes**<br>Mouse: $CD11b^+Ly6G^-$ and $Ly6C^{hi}$ or $Ly6C^{lo}$<br>Human: HLA-DR+ and $CD14^{++}CD16^-$, $CD14^+CD16^+$ or $CD14^{dim}CD16^+$ | Mouse: $CD11b^+Ly6G^-Ly6C^{hi}$<br>Also lack CD11c and MHC class II<br>Human: $CD14^+HLA\text{-}DR^{-/lo}$ |
| *Limitations of markers* | Due to shared markers, lineage tracing studies still required to confirm contribution of these cells | Due to shared markers, lineage tracing studies required to confirm contribution in metabolic diseases | Some studies lack sufficient evidence to rule out potential contributions of macrophage from other sources. Bone marrow transplant studies and monocyte labeling provide good evidence for their contribution. | Most studies in mice fail to discriminate MDSCs from monocytes and/or neutrophils |
| *Function* | Pro-atherogenic | Pro-atherogenic<br>Reduced phagocytic capacity | Pro-atherogenic | Anti-atherogenic?<br>Immunosuppressive |
| *Mouse vs. human* | Minimal proliferation of macrophages identified in humans, unknown macrophage origin | Evidence of co-expression of SMC and macrophage markers. May contribute to macrophage content identified by immunohistochemistry. | Circulating monocytes correlate with disease | Associated with ACS in humans<br>Role in mice unclear |
| *Affected by diabetes/ obesity* | ↑ total plaque macrophage content in diabetic mice<br>Unknown if from resident tissue macrophages | Promoted by hypercholesterolemia and hyperglycemia (*in vitro*), unknown in models of diabetes/obesity | ↑ in circulation in diabetes/obesity<br>↑ lesion entry in diabetes<br>↓ lesion egress in diabetes | ↑ in circulation in T1D patients<br>↑ in obese adipose tissue<br>Not known if altered in the lesion |

*VSMC, vascular smooth muscle cell; MDSC, myeloid-derived suppressor cell.*

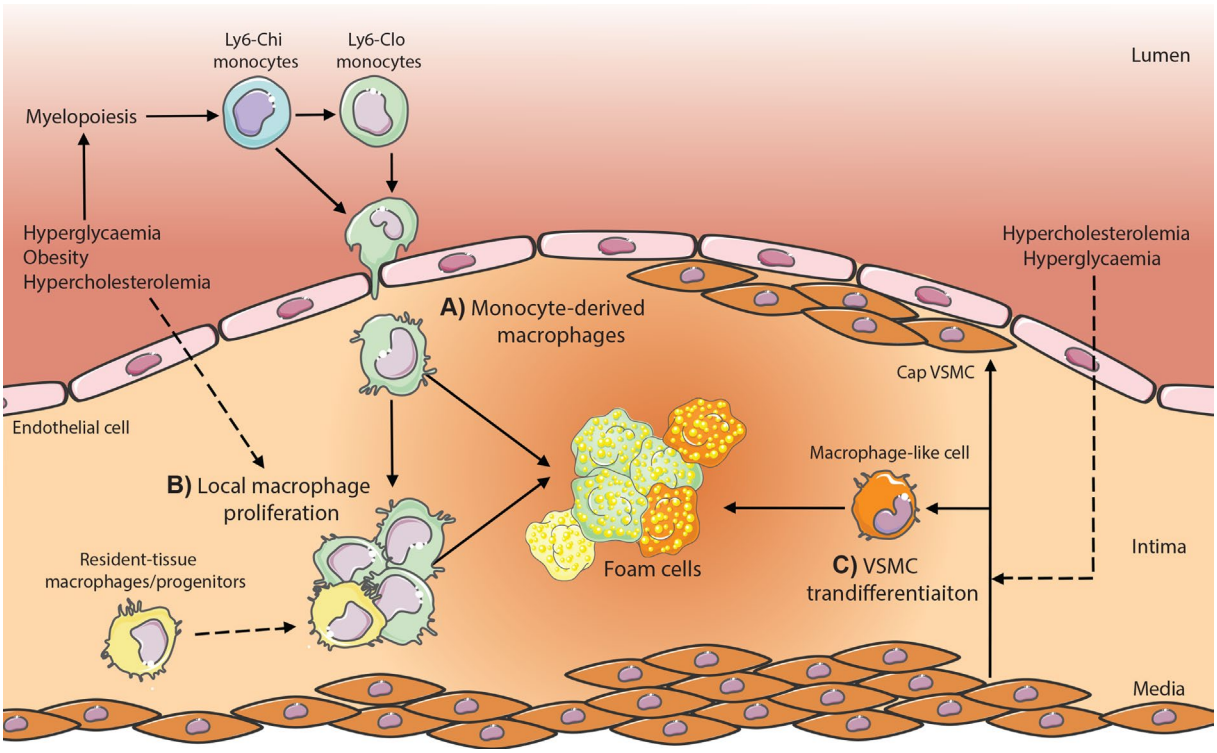


**FIGURE 1 |** Potential contributing macrophage sources in atherosclerotic plaques in metabolic disease. **(A)** Monocyte-derived macrophages are produced through enhanced myelopoiesis in response to hyperglycemia, hypercholesterolemia, or obesity-associated adipose inflammation and infiltrate the plaque where lipid-loading triggers transformation into foam cells. **(B)** Local macrophage proliferation and **(C)** vascular smooth muscle cell (VSMC) transdifferentiation within the plaque contributes also have the potential to produce foam cells; however, whether metabolic dysregulation (hypercholesteremia, hyperglycemia) modulates these processes is yet to be established *in vivo*.

plaque macrophages is primarily caused by a significantly higher proportion of circulating monocytes entering the atherosclerotic lesion (Parathath et al., 2011; Nagareddy et al., 2013; Distel et al., 2014). However, alternative sources of macrophages may also contribute to lesion development, which are discussed below and summarized in **Table 1** and **Figure 1**.

## MONOCYTE-DERIVED MACROPHAGES

Elevated circulating leukocytes are associated with increased CVD risk, with this association primarily driven by monocytes and neutrophils (Olivares et al., 1993; Danesh et al., 1998; Orchard et al., 2003; Coller, 2005). Monocyte abundance is an independent risk factor for CVD, with monocytosis causally linked to both the acceleration of atherosclerotic lesion progression and impaired lesion regression (Swirski et al., 2007; Tacke et al., 2007; Waterhouse et al., 2008; Nagareddy et al., 2013). In atherosclerosis, plaque macrophages are largely derived from circulating monocytes infiltrating the plaque. Endothelial activation induces the arrest of monocytes onto the vessel wall where they transmigrate into the arterial wall, maturing into macrophages. Adoptive transfer of GFP-labeled monocytes into 10-, 20-, and 50-week-old spontaneously atherosclerotic *Apoe*$^{-/-}$ mice shows that the degree of monocyte infiltration is increased throughout disease progression (Swirski et al., 2006). Moreover, impaired monocyte trafficking to plaque sites attenuates atherogenesis, as demonstrated in mice lacking either the monocyte chemotactic protein 1 (MCP-1) or its receptor CCR2, which strongly supports the hypothesis that monocyte infiltration is required for atherogenesis (Boring et al., 1998; Gosling et al., 1999). A seminal study by the Mallat group further demonstrated the importance of monocyte-derived macrophages *via* combined inhibition of CCL2 alongside CX3CR1 and CCR5, which are also required for monocyte trafficking, resulting in a 90% reduction in atherosclerosis in *Apoe*$^{-/-}$ mice correlating with levels of circulating macrophages (Combadiere et al., 2008). In mice, monocytes have been shown to enter into the plaque more readily in the context of diabetes and hypercholesterolemia (Swirski et al., 2007; Tacke et al., 2007; Murphy et al., 2011; Parathath et al., 2011; Nagareddy et al., 2013). Direct evidence of monocyte infiltration through tracking studies showed that monocyte entry is increased in diabetes while simultaneously impairing egress of these cells out of the plaque, resulting in overall increased retention (Parathath et al., 2011; Nagareddy et al., 2013). This corresponds to an increase in macrophage content in the plaques of both diabetic mice and humans (Moreno et al., 2000; Parathath et al., 2011; Nagareddy et al., 2013).

## RESIDENT-TISSUE MACROPHAGES

While macrophages have traditionally been considered to be derived from bone marrow (BM) monocytes, evidence in a number of organs has demonstrated that, under steady-state conditions, monocyte-derived macrophages contribute little to overall macrophage populations (Yona et al., 2013). Myeloid cells, including macrophages, are known to originate from two stages of hematopoiesis during development: 1) primitive hematopoiesis, occurring in the yolk-sac prenatally, and 2) definitive hematopoiesis, beginning in the fetal liver and maintained in the BM throughout life. In a number of organs, resident-macrophage populations derived from primitive hematopoiesis have been shown to be maintained through adulthood in mice by local proliferation. In the heart, steady-state cardiac macrophages have been found through genetic fate mapping to primarily originate from the yolk-sac, maintained by local proliferation (Epelman et al., 2014; Heidt et al., 2014). However, following myocardial infarction (MI), these resident macrophages are replaced by an influx of monocytes that mature into both macrophages with inflammatory (M1) and tissue-repair (M2) phenotypes (Heidt et al., 2014; Hilgendorf et al., 2014).

In the vasculature, a population of $Sca1^{+}CD45^{+}$ adventitial macrophage progenitor cells (AMPCs) have been identified, which are proposed to be derived by local proliferation maintained from prenatal development and which are upregulated in atherosclerosis (Psaltis et al., 2014). Adoptive transfer of $GFP^{+}$ AMPCs into the carotid artery of *Apoe*$^{-/-}$ mice prior to 16 weeks on a Western-type diet demonstrated that these AMPCs contributed to macrophage populations, primarily in the adventitia, but also to a lesser degree in the plaque. However, while this study suggested that the BM and spleen are not able to efficiently reconstitute AMPC populations, definitive fate mapping studies are required to determine whether these cells are indeed true resident tissue macrophages seeded prenatally. The Robbins group performed fate mapping studies showing the contribution of embryonic macrophage precursors and BM HSPCs within the aorta postnatally (Ensan et al., 2016). Through pulse-labeling of CX3CR1 and FLT3, this study identified that while both embryonic and BM-derived precursors maintain the aortic macrophage repertoire, respectively, BM HSPC contribution declines throughout adulthood, at least under homeostatic conditions. While this study demonstrates the interplay between two defined macrophage precursor populations in the steady state, further fate mapping studies during the development and progression of atherosclerosis, as well as in metabolic disease, are required to delineate the significance of the contribution of the resident macrophage pool vs. monocyte-derived macrophages.

## LOCAL MACROPHAGE PROLIFERATION

The atherosclerotic lesion, in theory, requires a constant turnover of macrophages to promote the resolution of this inflammatory pathology of the vascular wall. Within the plaque, macrophages have been suggested to proliferate to maintain macrophage content, particularly within more advanced lesions. Robbins et al. (2013) performed elegant experiments utilizing parabiosis of CD45.1 and CD45.2 mice in combination with BrdU labeling to measure proliferating macrophages within the plaque. From this study, they concluded that, during early lesion development, only ~30% of plaque macrophages were derived from local proliferation, with the remainder derived from monocytes; however, in advanced lesions, the contribution of macrophage proliferation increased to ~87% of total plaque macrophage content. Importantly, however, in the same study, parabiosis experiments conducted over a few months revealed that all locally proliferating macrophages were replaced by recruited monocytes (as we have discussed previously) (Murphy and Tall, 2014). Perhaps plaque monocytes/macrophages undergo a round of proliferation in response to the environment but then either die in or leave the lesion, and hence, new blood-derived monocytes are recruited. This ultimately suggests that monocyte entry is the rate-limiting step in atherosclerotic lesion progression.

In the context of diabetes, Lamharzi et al. (2004) have demonstrated that diabetes-associated hyperlipidemia can exacerbate plaque macrophage proliferation, which appears to require the hyperlipidemic environment as this phenotype was not seen in non-hyperlipidemic diabetic mice. Further, while this study showed that atherosclerotic and hyperglycemic conditions stimulate proliferation *in vitro*, the *in vivo* findings were based on BrdU labeling with prior studies showing that BrdU is incorporated into monocytes, and thus monocyte-derived macrophages, as well as locally proliferating macrophages (Yona et al., 2013). Given the technical caveat of distinguishing $BrdU^{+}$ staining between proliferating local macrophages and BM progenitors, of which monocyte-derived macrophages are derived, there are currently no studies that have conclusively determined the contribution of local macrophage proliferation in diabetes or obesity. Moreover, studies focused on local macrophage proliferation in atherosclerosis rely on macrophage markers that do not necessarily represent "true" (myeloid-derived) macrophages. Fate mapping with confetti mice (Wang et al., 2013) could be employed in atherosclerotic models to fate map clones of hematopoietic-derived monocytes/macrophages. Approaches such as this should help to more faithfully determine the contribution of recruited cells to the atherosclerotic plaque. In human lesions, very few proliferating cells are detected within the lesions by proliferating cell nuclear antigen (PCNA) staining (<1% in the majority of patient samples), with 27.1% of these $PCNA^{+}$ cells identified as macrophages ($HAM56^{+}$) but only 3.9% of these were Mac $387^{+}$ (Gordon et al., 1990). In addition, 15.5% of $PCNA^{+}$ cells in human lesions were found to be $HHF35^{+}$, indicating VSMCs. Importantly, however, PCNA staining only detects cell proliferation at an individual timepoint and may therefore underestimate the percentage of macrophages within the plaque that retain the capacity to proliferate locally.

## VASCULAR SMOOTH MUSCLE CELL-DERIVED MACROPHAGE-LIKE CELLS

More recently, VSMCs have been shown to transdifferentiate into macrophage-like cells in atherosclerotic plaques. The first evidence for this *in vitro* was provided by the Fisher group where

they demonstrated that VSMCs have the capacity to take up and accumulate cholesterol to form foam cells (Rong et al., 2003). This process was associated with a decrease in smooth muscle cell surface markers and gene expression, which coincided with an upregulation of macrophage genes and surface markers including CD68 and Mac-2. However, despite the expression of macrophages markers, these macrophage-like cells have lower phagocytic and efferocytic capacity compared to activated peritoneal macrophages, and gene expression of these cells indicates that their phenotype remains closer to VSMCs than macrophages (Vengrenyuk et al., 2015).

*In vivo*, genetic inducible fate mapping of VSMCs demonstrated that, during atherosclerosis, VSMCs undergo clonal expansion to form the fibrous cap. Within 8 weeks of high-fat feeding, a large number of clonal VSMCs migrate to the plaque core where they undergo transdifferentiation, downregulating SMC markers and upregulating macrophage markers (Misra et al., 2018). While these cells only have approximately a fifth of the proliferative capacity of VSMCs in the cap, these VSMC-derived cells constitute the majority of proliferative cells in the core, indicating that VSMCs and macrophage-like cells could contribute largely to the previously described local "macrophage" proliferation. Transdifferentiation of the VSMC has been shown to require a reduction in integrin β3 (Itgβ3) to induce upregulation of toll-like receptor 4 (TLR4), resulting in an increase in CD36 and a cholesterol-induced phenotypic switch towards a more macrophage-like cell (Misra et al., 2018). Complementing *in vitro* studies, macrophage-like cells contain oxLDL and exhibit foam cell-like morphology *in vivo* (Feil et al., 2014; Misra et al., 2018). However, while in steady-state atherosclerotic models, VSMC transdifferentiation has been shown to contribute to the "macrophage" content of atherosclerotic plaques identified by traditional macrophage markers, the exact role of these macrophage-like cells in the pathogenesis of the disease remains to be determined. Moreover, the contribution of VSMC-derived macrophage-like cells to atherosclerotic plaques in diabetes and obesity have yet to be established. *In vitro*, hyperglycemia has been shown to induce VSMC proliferation, migration, and inflammatory gene expression suggestive of a potential to induce transdifferentiation *in vivo* (Qi et al., 2012).

## MONOCYTE SUBSETS IN ATHEROSCLEROSIS

In mice, monocytes can be divided into two major subsets: classical CCR2$^{hi}$CX3CR1$^{lo}$Ly6C$^{hi}$ monocytes and non-classical CCR2$^{lo}$CX3CR1$^{hi}$Ly6C$^{lo}$ monocytes. Fate mapping and adoptive transfer studies indicate that Ly6C$^{lo}$ monocytes develop from the Ly6C$^{hi}$ subset (Swirski et al., 2007; Yona et al., 2013). Hypercholesterolemia has been shown to expand the Ly6C$^{hi}$ subset and has been proposed to impair their maturation towards Ly6C$^{lo}$ monocytes (Swirski et al., 2007). Ly6C$^{hi}$ monocytes are more inflammatory compared to Ly6C$^{lo}$ monocytes and, in atherosclerosis, Ly6C$^{hi}$ monocytes preferentially infiltrate the arterial wall (Swirski et al., 2007; Tacke et al., 2007; Potteaux et al., 2011). Specific depletion of Ly6C$^{hi}$ monocytes reduced lesion progression and lowered macrophage and apoptotic cell content (Soehnlein et al., 2013). Importantly, CCR2 and CX3CR1 are both required for Ly6C$^{hi}$ monocyte recruitment to plaques, while Ly6C$^{lo}$ recruited monocytes, despite their higher expression of CX3CR1, are independent of these chemokine receptors (Tacke et al., 2007). Deletion of CX3CR1, CCL2, or its receptor, CCR2, has been shown to impede atherosclerotic progression, suggesting that recruitment of the Ly6C$^{hi}$ monocyte subset is responsible for promoting atherogenesis (Kuziel et al., 2003; Combadiere et al., 2008). In contrast, deletion of CCR5, required for Ly6C$^{lo}$, but not Ly6C$^{hi}$, monocyte recruitment, has been shown not to effect atherosclerotic progression, at least in early lesions (Kuziel et al., 2003). However, in mice lacking CCL2 and CX3CR1, inhibition of CCR5 further reduces atherogenesis, indicating that Ly6C$^{lo}$ monocytes contribute to atherogenesis in the absence of Ly6C$^{hi}$ monocyte recruitment (Combadiere et al., 2008). Mice deficient in Nur77 (also known as NR4A1), a transcription factor that is shown to be required for Ly6C$^{lo}$ monocyte differentiation and survival (Hanna et al., 2011), exhibit accelerated atherosclerosis in some studies but not in others (Hamers et al., 2012; Hanna et al., 2012; Chao et al., 2013). Interestingly, however, CCR2 and CX3CR1 have also been shown to be required for the resolution of inflammation and plaque regression, indicating that the recruitment of Ly6C$^{hi}$ monocytes is required for plaque regression (Rahman et al., 2017). However, in the context of diabetes, we and others have previously demonstrated increased Ly6C$^{hi}$ monocyte entry in regressing plaques, associated with increased inflammatory gene expression and reduced M2 polarization, which could suggest that diabetes impairs Ly6C$^{hi}$ monocyte differentiation to M2 macrophages and/or that excess Ly6-C$^{hi}$ monocytes may also play a deleterious role in plaque regression (Parathath et al., 2011; Nagareddy et al., 2013).

In humans, monocyte subsets are defined based on their CD14 and CD16 expression, with classical monocytes (CD14$^{++}$CD16$^{-}$) traditionally recognized as being analogous to the Ly6C$^{hi}$ subset and non-classical monocytes (CD14$^{dim}$CD16$^{+}$) being equivalent to the Ly6C$^{lo}$ subset in mice. Fate mapping in human monocytes has demonstrated that CD14$^{dim}$CD16$^{+}$ monocytes develop from CD14$^{++}$CD16$^{-}$ monocytes by transitioning through a third population of CD14$^{+}$CD16$^{+}$ intermediate monocytes (Korkosz et al., 2012). All three populations are found in the circulation with classical monocytes comprising 80–90% of total circulating monocytes under homeostatic conditions, while non-classical and intermediate monocytes comprise 2–10% and 2–5%, respectively. In contrast to the role of Ly6C$^{hi}$ monocytes in driving atherosclerotic progression in mice, evidence largely supports a role for CD16$^{+}$ monocytes including non-classical monocytes, as well as intermediate monocytes, in increasing CVD risk (Schlitt et al., 2004; Hristov et al., 2010; Rogacev et al., 2010; Rogacev et al., 2011; Rogacev et al., 2014). *In vitro*, all human monocyte subsets polarize to M1 macrophage in response to LPS/IFNγ or GM-CSF or to M2 macrophages in response to M-CSF (Al-Sharea et al., 2016; Boyette et al., 2017). In contrast, IL-4 also induces M2 polarization in classical and intermediate but not non-classical monocytes (Al-Sharea et al., 2016). Importantly, both M1 and M2 macrophage function has been shown to differ based on the monocyte subset from which they were derived, with those from

classical monocytes exhibiting higher phagocytic capacity than that of intermediate or non-classical monocyte-derived macrophages (Boyette et al., 2017).

Further advances in immune cell profiling with the introduction of CyTOF mass cytometry also indicate that the use of additional markers in defining these subsets may help to improve the potential of monocyte subsets as predictive determinants of cardiovascular risk (Thomas et al., 2017). However, although circulating levels of these monocyte subsets are associated with CVD, it remains unknown what capacity these monocyte subsets have to infiltrate the plaque and promote atherosclerosis. The development of new humanized immune system mouse models of atherosclerosis that support all the monocyte subsets could allow for the direct study of human monocyte subsets in the plaque to determine whether these subsets play a causal role in promoting atherogenesis or plaque instability.

## MONOCYTE SUBSETS IN DIABETES AND OBESITY

Diabetes and obesity are associated with increased circulating monocytes (Nagareddy et al., 2013; Nagareddy et al., 2014). In diabetic mice, this is predominantly driven by an increase in the Ly6C$^{hi}$ subset, whereas diet-induced obesity (DIO) results in an expansion of both Ly6C$^{hi}$ and Ly6C$^{lo}$ monocytes (Nagareddy et al., 2013; Nagareddy et al., 2014). It has previously been suggested that hypercholesterolemia may act to inhibit the conversion of Ly6C$^{hi}$ to Ly6C$^{lo}$ monocytes (Swirski et al., 2007), which, given the hypercholesterolemia present in diabetic mouse models, could explain the difference in monocyte phenotypes between diabetes and DIO.

In humans, obesity, measured either as fat mass or by the WHO obesity classification, has been shown to be associated with an increase in intermediate (CD14$^+$CD16$^+$) and non-classical (CD14$^{dim}$CD16$^+$) monocytes (Stansfield and Ingram, 2015). Obese patients with diabetes exhibit an even greater increase in non-classical monocytes but with similar levels of intermediate monocytes compared to obese non-diabetic patients (Poitou et al., 2011). Roux-en-Y gastric bypass surgery—which is known to reduce cardiovascular risk in obese patients with diabetes and is associated with reductions in weight loss and improved glycemic control—significantly reduced both monocyte subsets (Poitou et al., 2011). Moreover, this decrease in the CD14$^+$CD16$^+$ monocyte subset is associated with decreased intima-media thickness, suggesting that the intermediate monocyte subset may contribute to accelerated atherosclerosis and increased risk of CVD in diabetes and obesity. Similar effects on CD14$^+$CD16$^+$ monocytes were also observed in morbidly obese patients following gastric bypass with no difference observed between diabetic and non-diabetic individuals (Cottam et al., 2002).

## MYELOID-DERIVED SUPPRESSOR CELLS

Myeloid-derived suppressor cells (MDSCs) are a population of immune regulatory myeloid cells that have been identified in a number of pathological conditions, most notably in cancer, but also in metabolic disease. MDSCs are defined as either monocytic MDSCs (mMDSCs, low side scatter CD11b$^+$Ly6G$^-$ Ly6C$^{hi}$) or granulocytic MDSCs (gMDSCs, high side scatter CD11b$^+$Ly6G$^+$Ly6C$^{lo}$). As such, mMDSCs share the same markers as Ly6Chi inflammatory monocytes; however, they typically lack the monocytic markers CD11c and MHC class II. These MDSC are known to develop from monocyte and neutrophil precursors in the bone marrow, where a number of cytokines, lipid mediators, and growth factors have been shown to reprogram development toward MDCS. In humans, mMDSCs are classified as CD14$^+$HLA-DR$^{-/lo}$, in contrast to the monocyte subsets, which are HLA-DR$^+$, and are increased in the blood of patients with acute coronary syndrome (ACS) (Wang et al., 2015). However, despite this association, there is little direct evidence for a role of mMDSCs in atherosclerosis. In an attempt to assess the role of MDSCs in atherosclerosis, a study by Foks et al. (2016) utilized repetitive adoptive transfer of bone marrow CD11b$^+$Gr1$^+$ cells, from *Ldlr*$^{-/-}$ mice fed a WTD for 2 weeks and deemed to be MDSCs, into *Ldlr*$^{-/-}$ mice on a WTD for 6 weeks, demonstrating that these cell transfers reduce lesion size. Importantly, however, although the co-culture of these cells with T cells demonstrated suppressive features of MDSCs, the definition of MDSCs in this study does not exclude classical neutrophils or Ly6C$^{hi}$ monocytes, which comprise a large portion of the bone marrow. Moreover, while these cells were tracked to spleen, lymph nodes, and adipose tissue, it was not determined whether these cells could traffic to the plaque. Moreover, while adoptive transfer studies provide a foundation indicating an antiatherogenic role for these cells, the artificial nature of these experiments makes it difficult to assess whether these cells would naturally maintain these immunosuppressive characteristics during normal or pathological trafficking from the bone marrow. Further studies, delineating mMDSCs from Ly6C$^{hi}$ monocytes, using additional markers and lineage tracing, are required to directly track the contribution and causal role of these cells in the development of atherosclerosis. In humans, MDSCs increased the circulation of obese patients and patients with T1D (Whitfield-Larry et al., 2014; Bao et al., 2015). In obese (high-fat-fed ob/ob) mice, CD11b$^+$GR1$^+$ have been found to accumulate in the spleen, liver, and epididymal fat (Xia et al., 2011). Whether obesity or diabetes promotes the accumulation of MDSCs in the plaque remains to be seen. More importantly, while MDSCs may play an anti-atherogenic role, their potential contribution in metabolic disease does not appear to outweigh the pro-atherogenic role of macrophages within the lesion.

## MYELOPOIESIS IN METABOLIC AND INFLAMMATORY DISEASE

Monocytes originate in the bone marrow where they develop from hematopoietic stem cells through a process called myelopoiesis. Myelopoiesis is a subbranch of hematopoiesis specifically involving the production of myeloid cells, which include monocytes as well as dendritic cells, granulocytes, platelets, and red blood cells. Hematopoietic stem cell commitment towards the monocytic lineage involves the progressive differentiation

through a number of myeloid and monocyte precursors including, in order from least to most committed, the common myeloid progenitor (CMP), granulocyte–macrophage progenitor (GMP), monocyte–dendritic cell progenitor (MDP), and common monocyte progenitor (cMOP) (Hettinger et al., 2013). Enhanced myelopoiesis is known to result in monocytosis and accelerates the progression of atherosclerosis, as well as impairing atherosclerotic regression (Swirski et al., 2007; Tacke et al., 2007; Murphy et al., 2011; Nagareddy et al., 2013; Distel et al., 2014). Furthermore, under inflammatory settings, hematopoietic stem and progenitor cells (HSPCs) can mobilize to the spleen where they give rise to additional monocytes through extramedullary myelopoiesis (Robbins et al., 2012).

Myelopoiesis can be modulated by a number of metabolic and inflammatory diseases associated with an increased risk of CVD including hypercholesterolemia, rheumatoid arthritis (RA), MI, as well as both diabetes and obesity. In hypercholesterolemia, myelopoiesis is enhanced by cholesterol loading of stem cells, which increases proliferation of these cells, resulting in augmented monocyte production (Swirski et al., 2007; Tacke et al., 2007; Yvan-Charvet et al., 2010; Murphy et al., 2011). Hypercholesterolemia is also associated with stem cell mobilization and extramedullary myelopoiesis, giving rise to Ly6C$^{hi}$ monocytes, which accelerate atherosclerosis (Robbins et al., 2012). In RA, myelopoiesis is induced *via* a similar mechanism whereby inflammatory signals impair cholesterol efflux mechanisms, resulting in a proliferative stem cell phenotype and extramedullary myelopoiesis (Dragoljevic et al., 2018). Following MI, monocytes are mobilized to the heart with Ly6C$^{hi}$-driven monocytosis, resulting in increased Ly6C$^{hi}$ accumulation within the first 4 days post-MI, while Ly6C$^{lo}$ monocytes were shown to preferentially accumulate 5–7 days post-MI following changes in chemotactic signals (Nahrendorf et al., 2007). MI-induced β3-adrenergic signaling induces the release of stem cells from the BM, following which they migrate to and seed the spleen. The spleen becomes a major organ for extramedullary myelopoiesis, contributing to an already elevated pool of circulating Ly6C$^{hi}$ monocytes that would readily enter the inflamed arteries to accelerate atherosclerosis (Dutta et al., 2012). In contrast, diabetic (both STZ and Akita models) and obese (DIO, db/db and ob/ob) mice exhibit enhanced myelopoiesis within the BM (i.e., medullary), but not extramedullary, suggesting a separate signaling pathway for myelopoiesis in these metabolic diseases (Nagareddy et al., 2013; Nagareddy et al., 2014).

## S100A8/A9 AS A MEDIATOR OF MYELOPOIESIS IN METABOLIC DISEASE

S100A8/A9, a damage-associated molecular pattern protein heterodimer, correlates with the severity of coronary artery disease in diabetic patients as well as with HbA1c levels and body mass index (BMI) (Peng et al., 2011; Nagareddy et al., 2013; Cotoi et al., 2014). Moreover, neutrophils, the primary source of S100A8/A9, are likewise associated with both coronary events and cardiovascular deaths (Cotoi et al., 2014). We have previously shown that S100A8/A9 is responsible for inducing myelopoiesis in both diabetes and obesity; however, the mechanism by which S100A8/A9 mediates this process differs between disease contexts, which we discuss below (**Figure 2**).

## MYELOPOIESIS IN DIABETES

In diabetes, we found that monocytosis is consequential of hyperglycemia-induced upregulation of plasma S100A8/A9 (Nagareddy et al., 2013). In this setting, S100A8/A9 was shown to interact directly with RAGE on CMPs and macrophages in the BM, activating NF-κB signaling within these cells to promote the secretion of M-CSF and GM-CSF, respectively. GM-CSF and M-CSF promote the proliferation and differentiation of GMPs, with M-CSF directing proliferation of CMPs *via* autocrine signaling. S100A8/A9 also directly induces the proliferation CD34$^{+}$ stem cell progenitors from human cord blood *in vitro*. This is likely due to signaling *via* RAGE on CMPs and results in increased CD14$^{+}$ monocytes. Moreover, controlling glucose levels in diabetic mice with a sodium glucose co-transporter 2 inhibitor (SGLT2i) reduces circulating S100A8/A9 and prevents both diabetes-induced myelopoiesis and atherosclerosis. In addition to promoting monocytosis, hyperglycemia-induced S100A8/A9 also results in increased production of highly reactive platelets (through an independent mechanism), which together results in increased platelet–leukocyte interactions (Kraakman et al., 2017). Again, inhibiting S100A8/A9 is effective in dampening this process.

Hyperglycemia also promotes the formation of advanced glycation end products (AGEs), as well as carbonyl intermediates such as glycoaldehyde (GA), glyoxal (GO), and methylglyoxal (MGO), which are elevated in diabetes. Carbonyls (specifically GA and GO) have been shown to destabilize ABCA1 and impair cholesterol efflux in macrophages *in vitro* (Passarelli et al., 2005). ABCA1 upregulation in diabetic mice *via* treatment with the anti-miR33 prevents diabetes-induced myelopoiesis and monocytosis, but not neutrophilia (Distel et al., 2014). Anti-miR33 treatment also prevented diabetes-induced monocyte recruitment and improved M2-switching and macrophage reduction during atherosclerotic regression (Distel et al., 2014). This was likely mediated *via* the reduction in myelopoiesis through stabilization of ABCA1 and HDL-mediated cholesterol transport in CMPs and GMPs as well as the direct upregulation of ABCA1 observed in plaque macrophages, improving cholesterol efferocytosis within the plaque. While plasma HDL-C was increased by anti-miR33 treatment, total cholesterol, triglycerides, and plasma glucose levels were increased in diabetes but not altered by anti-miR33 treatment. Blocking ABCA1 has also been shown to prevent the anti-inflammatory effects of apolipoprotein A-I (apoA-I) from HDL on monocytes, indicating that carbonyl-mediated destabilization of ABCA1 likely impairs effects of HDL on macrophages, CMPs, and GMPs (Murphy et al., 2008). Plasma MGO levels have also been shown to be associated with increased incidence of CVD in patients with type 1 and type 2 diabetes (Hanssen et al., 2017; Hanssen et al., 2018); however, whether MGO influences cholesterol handling in stem or progenitor cells and influences myelopoiesis remains unknown.

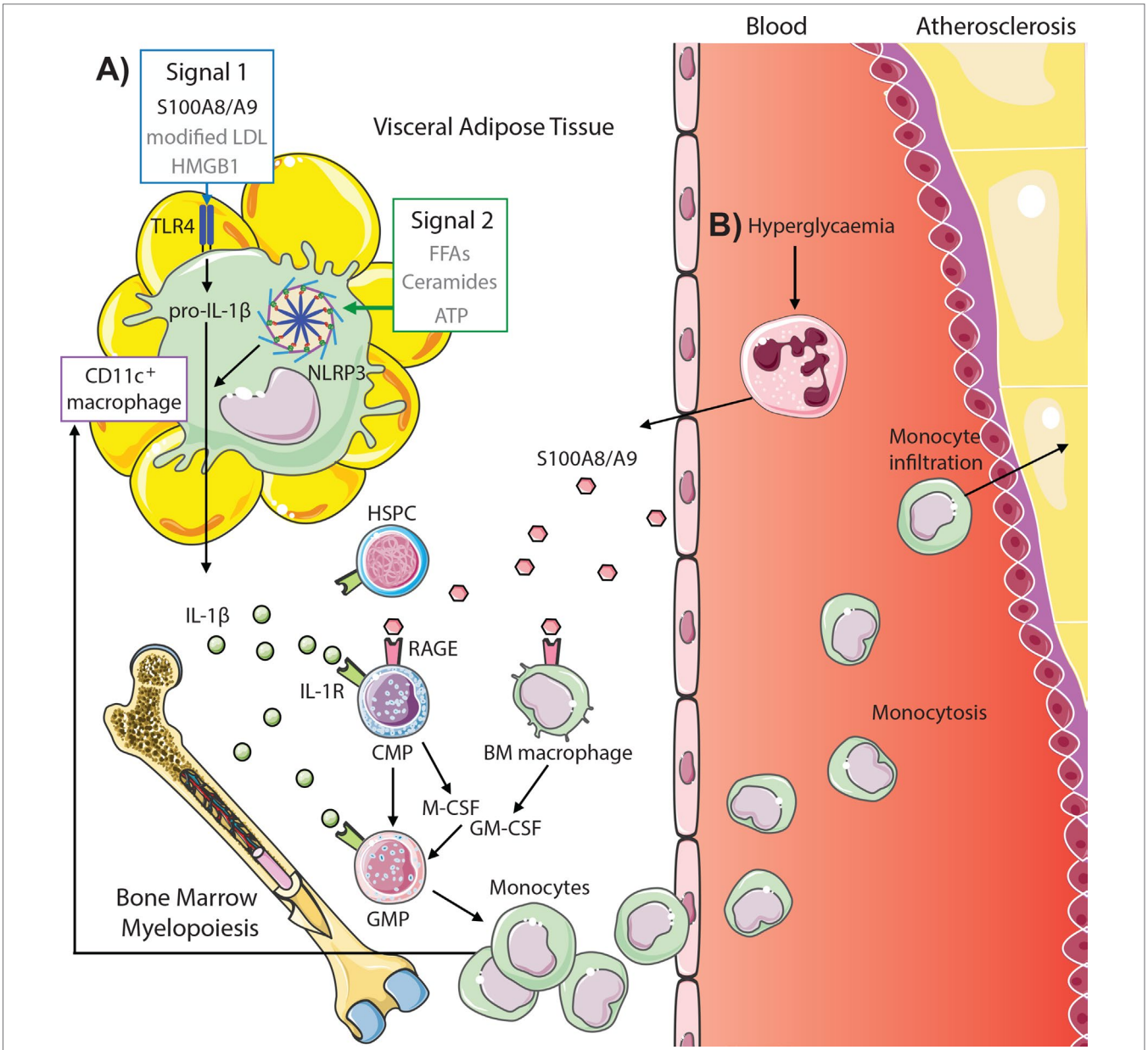


**FIGURE 2 |** S100A8/A9 drives myelopoiesis and monocytosis in diabetes and obesity. **(A)** Adipose tissue inflammation in obesity promotes monocytosis through S100A8/A9-TLR4 signaling on CD11c$^+$ adipose tissue macrophages (signal 1, other potential mediators in gray) and activation of the NLRP3 inflammasome (signal 2) to promote IL-1β, which signals through the IL1R on common myeloid progenitors (CMPs) and granulocyte–macrophage progenitors (GMPs) to induce myelopoiesis. This results in both increased circulating monocytes and feeds back to increase CD11c$^+$ macrophages. **(B)** Hyperglycemia promotes monocytosis by direct signaling of neutrophil-derived S100A8/A9 *via* RAGE on CMPs and macrophages in the bone marrow, promoting CMP and GMP proliferation and differentiation *via* autocrine and paracrine (M-CSF and GM-CSF) signaling, respectively. These monocytes infiltrate atherosclerotic lesions to promote atherogenesis (further detailed in **Figure 1**).

Monocyte levels have also been shown to be associated with plasma norepinephrine in diabetic patients, which has been proposed to modulate extramedullary myelopoiesis (Vasamsetti et al., 2018). In contrast with previous studies that showed no change in GMPs in the spleen (Nagareddy et al., 2013), this study showed increased splenic GMPs in diabetes, which were more proliferative and were shown *via* adoptive transfer experiments to differentiate more readily, generating higher numbers of myeloid cells. Importantly, however, GMPs in this study were gated more strictly, resulting in a much smaller population containing only the most CD34$^{hi}$ subset, which may suggest that further heterogeneity exists within the classically defined GMP population than previously thought. How the GMPs arrived in the spleen is unknown given that these cells have an ~2-week half-life in the spleens of atherogenic mice (Robbins et al., 2012), and it is well known that HSPC mobilization from the BM is severely perturbed in diabetes (Ferraro et al., 2011). However, it should be noted that Vasamsetti *et al.* did reveal that ablation of sympathetic signaling in the spleen

did reduce GMP proliferation. Whether plasma norepinephrine influences the proliferative or differentiative potential of other myeloid progenitors remains unknown.

## MYELOPOIESIS IN OBESITY

Interestingly, in models of DIO, we found that S100A8/A9 did not significantly increase in the plasma but were instead elevated locally within the visceral adipose tissue (VAT) (Nagareddy et al., 2014). This reflected measurements in the plasma and adipose tissue from lean and obese humans. Here, S100A8/A9 signaled through TLR4 on CD11c$^+$ adipose tissue macrophages (ATMs) to promote the production of IL-1β, acting as a myeloproliferative signal in the bone marrow, thereby inducing monocytosis. Alongside S100A8/A9, a number of endogenous TLR-4 ligands including gut-derived LPS, HMGB1, and modified LDL are elevated in the plasma and adipose tissue in obesity and may contribute to ATM priming to produce IL-1β (Erridge, 2010). Obesity is also associated with elevated levels of free fatty acids (FFAs), which were originally postulated to act as a TLR4 agonist, as TLR4$^{-/-}$ mice are protected from obesity-associated inflammation (Davis et al., 2008; Osborn and Olefsky, 2012). However, recent evidence suggests that FFAs such as palmitate promote macrophage inflammation, a process that requires TLR4 signaling, but do not bind to TLR4 directly (Lancaster et al., 2018). TLR4 signaling induces pro-IL-1β production, which requires cleavage by the NLRP3 inflammasome to produce functional IL-1β, and as such, secondary signals are needed to activate the NLRP3 inflammasome (Sims and Smith, 2010). In obesity, FFAs as well as ceramides and extracellular ATP have all been suggested to activate NLRP3 (Vandanmagsar et al., 2011; Wen et al., 2011; Moon et al., 2016). Together, this suggests that, while S100A8/A9 plays an important role in inducing IL-1β production, additional inflammatory signals within the adipose tissue likely co-signal to promote IL-1β and myelopoiesis in obesity.

## TRAINED IMMUNITY IN METABOLIC DISEASE

Given that metabolic diseases are chronic, yet have the ability to be reversed through changes in diet and exercise leading to whole-body metabolism improvement and weight loss, it is important to know the length of the effects on the hematopoietic system. Through serial bone marrow transplants, hematopoietic stem cells from obese mice have been shown to retain an increased potential to produce myeloid cells compared to those originating from lean mice (Singer et al., 2014). These cells produced more CD11c$^+$ BM cells and overall increase in both total and CD11c$^+$ ATMs once re-exposed to DIO. These results suggest that potential epigenetic regulation of stem cells in obesity could promote myeloid skewing. This production of CD11c$^+$ ATMs could also feed-forward to enhance the IL-1β signaling induced by S100A8/A9 in obesity to promote myelopoiesis and monocytosis. A recent study by the Latz group demonstrated that a Western-type diet induces both transcriptomic and epigenomic reprogramming in GMPs towards the "trained immunity" phenotype, mediated *via* NLRP3 signaling (Christ et al., 2018). In β-glucan models of trained immunity, IL-1β acts on HSPCs to modulate myelopoiesis (Mitroulis et al., 2018). It is therefore likely that IL-1β signaling from ATMs could also potentiate trained immunity within the context of obesity and that S100A8/A9 may therefore play a role in trained immunity upstream of IL-1β.

Likewise, in diabetes, it is postulated that hyperglycemia induces long-term effects on the immune system through a process known as hyperglycemic memory, mediated through epigenetic modifications. Supporting this, clinical studies have demonstrated that improved glucose control has sustained effects following the return to usual glucose control (Nathan and Group, 2014). Furthermore, in recent years, it has become apparent that diabetic as well as pre-diabetic patients exhibit severe variations in glycemia, and post-prandial hyperglycemia is an independent risk factor for CVD (Decode Study Group, 2001; Ning et al., 2012; Monnier and Colette, 2015; Hall et al., 2018). Pre-clinically, transient hyperglycemia has been shown to induce epigenetic changes in the vasculature, which was associated with increased NFκB signaling (Brasacchio et al., 2009). It would therefore be interesting to determine whether hyperglycemia also results in trained immunity by inducing epigenetic changes within the stem and progenitor cells in the BM. Moreover, given the presence of S100A8/A9-driven myelopoiesis in the setting of chronic hyperglycemia, it would also be interesting to determine whether fluctuations in glycemia, in particular transient hyperglycemia, could promote S100A8/A9 signaling to induce myelopoiesis and whether this could have downstream effects on atherogenesis.

## EFFECTS OF WEIGHT LOSS ON S100A8/A9 AND MONOCYTOSIS

Despite strong evidence that obesity induces trained immunity, we have previously shown that weight loss reduces monocyte and neutrophil levels in diet-induced obese mice, suggesting that preventing obesity-related inflammatory signals is still effective in reducing myelopoiesis (Nagareddy et al., 2014). Likewise, monocyte and neutrophil levels were also found to be significantly reduced in obese patients following bariatric surgery associated with an average weight loss of 26.7% (Nagareddy et al., 2014). Moreover, a recent study in obese patients with or without type 2 diabetes mellitus (T2DM) showed that plasma S100A8/A9 was significantly reduced following Roux-en-Y gastric bypass surgery independent of diabetes (Lylloff et al., 2017). However, it is important to note that while non-diabetic patients were classified as having Hba1c below 6.5% (48 mmol/mol), Hba1c levels decreased in all groups. Unfortunately, independent associations of plasma S100A8/A9 with Hba1c or BMI were not reported. While monocytes were not specifically measured in this study, total leukocytes were associated with S100A8/A9 levels and were reduced and in obese/diabetic patients following surgery. Together, these data suggest that weight loss reduces S100A8/A9 and monocytosis in humans; however, whether this is a consequence of reduced adiposity, improved glucose tolerance, or both is yet to be delineated.

## S100A8/A9 AS A POTENTIAL THERAPEUTIC TARGET IN METABOLIC DISEASE-RELATED CVD

Given the role of S100A8/A9 in atherosclerosis in both diabetes and obesity-associated myelopoiesis and the subsequent effects of monocytosis on atherosclerosis, targeting S100A8/A9 could be a potential therapeutic target to treat monocytosis and atherosclerosis in metabolic disease. Indeed, we have previously shown that blocking S100A8/A9 signaling, using a small molecular inhibitor ABR-215757 (Paquinimod), in STZ-diabetic mice prevents hyperglycemia-induced atherogenesis and reduces plaque macrophage content (Kraakman et al., 2017). Although it has yet to be explored whether S100A8/A9 promotes myelopoiesis in the context of other metabolic and cardiovascular risk factors, plasma levels of S100A8/A9 are also associated with hyperlipidemia and smoking and are increased following MI, which suggests that S100A8/A9 may be, at least in part, responsible for this increase in circulating myeloid cells and could therefore contribute to plaque macrophage accumulation and atherosclerosis in these patients (Du et al., 2012; Schiopu and Cotoi, 2013).

## CONCLUSIONS AND FUTURE DIRECTIONS

Diabetes and obesity are associated with exacerbated macrophage inflammation, which contributes to an overall increase in cardiovascular risk. Macrophages play a crucial role in the development of CVD by promoting atherosclerosis and contribute to plaque vulnerability. Macrophages within the plaque are a heterogenous population known to be derived from a number of sources, consisting of both true macrophages and macrophage-like cells, which may contribute differently to lesion development or regression. Diabetes and obesity are known to contribute to macrophage accumulation by enhancing myelopoiesis to increase circulating and infiltrating monocytes; however, metabolic abnormalities such as hyperglycemia and hypercholesterolemia may also contribute to macrophage accumulation by influencing the development and proliferation of alternative sources of macrophages. The contributions of locally produced macrophages and monocyte-derived macrophages in promoting macrophage accumulation and promoting atherosclerosis requires further study, particularly in the context of metabolic disease. Targeting the production of monocyte-derived macrophages has proven to be effective in reducing atherosclerosis pre-clinically in a number of metabolic and inflammatory diseases including hypercholesterolemia, RA, obesity, and diabetes. In particular, S100A8/A9 has emerged as a key myeloproliferative factor in diabetes and obesity, and as such, treatment with inhibitors of S100A8/A9 in addition to current therapies may reduce CVD risk in these patient groups.

## AUTHOR CONTRIBUTIONS

All authors contributed to writing and drafting the review.

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# Trained Circulating Monocytes in Atherosclerosis: *Ex Vivo* Model Approach

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Inflammation is one of the key processes in the pathogenesis of atherosclerosis. Numerous studies are focused on the local inflammatory processes associated with atherosclerotic plaque initiation and progression. However, changes in the activation state of circulating monocytes, the main components of the innate immunity, may precede the local events. In this article, we discuss tolerance, which results in decreased ability of monocytes to be activated by pathogens and other stimuli, and training, the ability of monocyte to potentiate the response to pathological stimuli, and their relation to atherosclerosis. We also present previously unpublished results of the experiments that our group performed with monocytes/macrophages isolated from atherosclerosis patients. Our data allow assuming the existence of relationship between the formation of monocyte training and the degree of atherosclerosis progression. The suppression of trained immunity *ex vivo* seems to be a perspective model for searching anti-atherogenic drugs.

**Keywords: atherosclerosis, monocyte, inflammation, activation, training, tolerance, intima, lipoproteins**

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## INNATE IMMUNITY AND ATHEROSCLEROSIS

Atherosclerosis remains a major problem of modern medicine, accounting for a substantial proportion of cardiovascular morbidity and mortality worldwide. Inflammation is one of the key mechanisms of atherosclerosis pathogenesis. Pro-inflammatory cells and molecules are present in atherosclerotic lesions and are currently regarded as potential therapeutic targets. According to current understanding, atherosclerotic lesion development can be regarded as a local chronic inflammatory process (Bentzon et al., 2014; Nikiforov et al., 2017a). At the cellular level, the likely triggering event in atherosclerosis is the accumulation of lipids, mainly cholesterol and its esters, in the cells constituting the intima-media layer of the arterial wall. The resulting foam cells owe their name to the abundant lipid inclusions filling their cytoplasm. The source of accumulating lipids is circulating modified low-density lipoprotein (LDL). Modified LDL particles are prone to formation of large self-associates, which are captured by macrophages. Previous studies by our group have identified the top 10 master regulator genes responsible for intracellular lipid accumulation by analyzing the transcriptome of macrophages that accumulated cholesterol as a result of modified LDL treatment (Orekhov et al., 2018b). The majority of the identified genes were associated with the immune response and inflammation. The obtained results were well correlated with the results reported by other authors, who showed that modified LDL initiated the secretion of pro-inflammatory molecules

(Wiesner et al., 2010; Yang et al., 2014). One unexpected observation was that none of the identified master regulators were directly related to intracellular cholesterol metabolism. This suggested that the immune response may play a key role in foam cells formation.

Recent studies show that local inflammation in tissues can be preceded by earlier events taking place in the circulation (Edsfeldt et al., 2015) or even in the bone marrow (Libby and Ebert, 2018). Study of correlation between cytokines circulating in the blood of patients with atherosclerosis and the same cytokines in the atherosclerotic plaques yielded some interesting results. Concentrations of macrophage inflammatory protein-1b (MIP-1b), tumor necrosis factor (TNF), and fractalkine significantly correlated not only with the contents of the same cytokines in the plaques, but also with the contents of other pro-inflammatory molecules, such as interferon gamma (IFNγ), C-C motif ligand 2 (CCL2), and interleukin 6 (IL-6) (Edsfeldt et al., 2015). It is also known that monocytes isolated from the blood of atherosclerosis patients respond more strongly to lipopolysaccharide (LPS) stimulation, demonstrating increased expression and secretion of inflammatory markers. The facilitated activation of monocytes in patients with atherosclerosis may result from trained immunity (Bekkering et al., 2016).

Monocytes are key cells of the innate immunity present in the circulation that penetrate the arterial wall upon activation. Changes in monocyte inflammatory activation may hinder the resolution of inflammation in atherosclerotic lesions and contribute to the disease progression.

It is well known that atherosclerotic clinical manifestations are associated with increased monocyte activability. Mononuclear cells of unstable angina patients with recurrent phases of instability exhibit enhanced production of IL-6 in response to low-dose of LPS, which is correlated with baseline CRP levels (Liuzzo et al., 2001). The observed higher monocyte sensitivity seems to be a result of enhanced expression of TLR4 in circulating monocytes likewise detected in patients with unstable angina and acute myocardial infarction (Methe et al., 2005). At the same time, the association between CD14+/TLR-4+ monocytes of patients before cardiovascular events and future cardiovascular events was not detected (Lorenzen et al., 2011). These findings indicate that atherosclerotic clinical manifestations effect on CD14+/TLR-4+ monocytes contributing to enhanced pro-inflammatory response. However, the reasons of increased sensitivity of monocytes of atherosclerotic patients without clinical manifestations remain unclear.

## TOLERANCE AND TRAINING: OPPOSITE MANIFESTATIONS OF THE INNATE IMMUNE MEMORY

Tolerance of the innate immunity is one of the mechanisms of the resolution of inflammation. This phenomenon is characterized by the loss of sensitivity of monocytes/macrophages to repeated exposure to the pathogen (Dobrovolskaia and Vogel, 2002; Dobrovolskaia et al., 2003). This loss of sensitivity is manifested by reduced expression and secretion of the major pro-inflammatory cytokines and chemokines TNFα, IL-6, IL-1RA, CX3CR1, IL-10, HLA-DR, IL-8, CCL2, and IL-1, by monocytes/macrophages that become "tolerant" (Medvedev et al., 2006; Biswas and Lopez-Collazo, 2009). Tolerance is believed to be evolutionarily formed to protect body tissues from damage due to hyper-inflammation (Ifrim et al., 2014). However, tolerance can have negative consequences when the body is exposed to pathogens for a long time. LPS is the most studied inducer of tolerance. LPS is recognized by toll-like receptor 4 (TLR4), which triggers two signaling pathways. One of these pathways leads to the activation of IFN regulatory factor 3 (IRF3), while the other activates mitogen-activated protein kinases (MAPKs) and IkB kinase (IKK) complexes, which activate the transcription factors AP-1 and NF-kB, respectively. Together, IRF3, AP-1, and NF-kB transcription factors are responsible for the expression of inflammatory genes induced by LPS (Seeley and Ghosh, 2017).

Prolonged exposure to high doses of LPS provokes tolerance that develops as a result of the interaction of many factors involved in the transmission of signals from toll-like receptors including decreased expression of TLR4 itself, MyD88–TLR4 association, IL-1R-associated kinase (IRAK) activity, IkBa degradation (Medvedev et al., 2000; Nomura et al., 2000), and increased expression of some negative regulators, such as A20 and IRAK-M (Xiong et al., 2011), and some microRNAs that bind to different agents in the signal transmission chain or modulate their expression (Quinn et al., 2012). However, negative regulation of the TLR4 signaling pathway is not the only mechanism of tolerance formation. Recent studies have demonstrated that tolerance induction in macrophages is accompanied by chromatin remodeling, which blocks the access of transcription factors to a number of genes involved in TLR signal transduction (Foster et al., 2007). Other factors are also able to participate in the formation of tolerance, including nucleosome remodeling and DNA methylation and metabolic changes, which may contribute to the duration of the tolerance effect (Seeley and Ghosh, 2017).

Several studies have shown that tolerant macrophages have increased phagocytic activity, which in turn may play an important role in foam cells formation. However, in those studies, latex beads with immobilized components of the bacterial membrane were used to induce phagocytosis (Jing et al., 2013; de Lima et al., 2014).

TLRs are not the only regulators of tolerance formation. It was demonstrated that tolerance to TNFα is characterized by the loss of susceptibility of cells to re-stimulation with TNFα after a prolonged incubation of cells with this cytokine (Zwergal et al., 2006). Interestingly, a so-called "cross-tolerance" between TNFα and LPS has been observed, in which cells lost their sensitivity to TNFα after stimulation with LPS and *vice versa* (Park et al., 2011).

Recently, Netea with co-authors systematically investigated the role of pattern recognition receptors in the induction of long term responses of the innate immune system. It turned out that the interaction of cells with ligands that bind to NOD-like receptors (NOD2 receptors or NOD1 receptors), as well as to the dectin 1 receptor, induce a sensitization effect: repeated interaction of monocytes with the pathogen caused

not a reduced, but increased pro-inflammatory cell activation, compared to the primary effect. Such phenomenon was called "training of innate immunity," which is the exact opposite of tolerance (Ifrim et al., 2014). Interestingly, in some cases, low concentrations of TLR ligands (0.001–10 pg/ml of LPS) not only diminished tolerance, but also promoted training, thereby forcing monocytes to maintain the inflammatory status (Ifrim et al., 2014). Trained immunity can be caused by some pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs), as well as oxidized LDL and Lp (a) (Munoz Villa, 1989; Kleinnijenhuis et al., 2012; Quintin et al., 2012; van der Valk et al., 2016).

Long-term tolerance responses of innate immune cells can be suppressed by both primary and repeated stimulation. Thus, interferons alpha and gamma are capable of such an effect by causing remodeling of the chromatin region responsible for tolerance formation (Bentzon et al., 2014; Shi et al., 2015). It can be concluded that some factors can modulate tolerance and training from one direction to another.

## ARE MONOCYTES TRAINED IN ATHEROSCLEROSIS?

The inflammatory responses of circulating monocytes isolated from atherosclerotic patients have been evaluated in a clinical study that included healthy donors (N = 13) and patients with subclinical atherosclerosis (N = 23). Quantitative diagnostics of atherosclerotic states was performed by ultra-sonographic measurement of intima-media thickness (IMT) of common carotid arteries in high-resolution regimen. For this purpose, the distal portions of right and left carotid arteries were scanned in lateral angle of interrogation. IMT of common carotid arteries was measured on the far wall of the distal 10-mm segment before the area of carotid sinus. To assess the presence of atherosclerotic plaques, the examination also included a scan of the left and right common carotid arteries, the carotid sinus area, as well as external and internal carotid arteries in three fixed projections—anterior, lateral, and posterior. When visualizing atherosclerotic plaque, carotid arterial stenosis was assessed in transverse projection. The measurements of IMT and plaque stenosis were carried out with M'Ath computer software (Metris, SRL, France). The average of two measurements (from right and left arteries in lateral position) was considered an integral indicator of mean IMT. The following plaque score was used for analysis: 0—absence of plaque, 1—stenosis up to 20%, 2—stenosis 20–50%, 3—more 50% stenosis. Stenosis of the carotid artery lumen more than 20% was considered as defined atherosclerotic plaque. Other patient characteristics recorded in the study were age, gender, body mass index (BMI), Tchol, Tg, LDLc, HDLc, and statin usage (**Table 1**). The study protocol has been approved by the Institute for Atherosclerosis Research Committee on Human Research and meets the standards of the Declaration of Helsinki in its revised version of 1975 and its amendments of 1983, 1989, and 1996 (JAMA 1997;277:925–926). All study participants were free of cardiovascular disease. The extent of asymptomatic atherosclerosis was assessed using the data on IMT variability in apparently healthy individuals from the Russian population as described previously (Orekhov et al., 2015). Individuals belonging to the lowest and second quartiles of age-adjusted IMT distribution with no evidence of visible atherosclerotic plaques in any segment of carotid arteries were considered to be non-predisposed to atherosclerosis ("healthy"). Patients belonging to the third and fourth quartiles of IMT distribution with visible atherosclerotic plaques (more than 20% of the arterial lumen) in at least one segment of carotid arteries were considered having subclinical (asymptomatic) atherosclerosis. Patients belonging to the third and fourth quartile of IMT distribution with no atherosclerotic plaques visualized in any segment of carotid arteries were excluded from the study as intermediate. The sample size was sufficient to form statistically significantly different groups of subjects for IMT and monocyte activation.

**TABLE 1 |** Baseline characteristics of the participants and pro-inflammatory response of circulating monocytes.

| Characteristics | Healthy individuals (n = 13) | Subclinical atherosclerosis (n = 23) | p value |
|---|---|---|---|
| Age, y | 63 ± 3 | 69 ± 2 | 0.09 |
| Gender, % male (n) | 8 (1) | 30 (7) | 0.08 |
| BMI (kg/m2) | 25.3 ± 1.1 | 28.7 ± 1.2 | 0.08 |
| IMT | 0.75 ± 0.03 | 0.90 ± 0.03 | 0.00** |
| Plaque score | 0.6 ± 0.1 | 2 ± 0.1 | 0.00** |
| TChol, mmol/L | 6.1 ± 0.3 | 5.8 ± 0.9 | 0.42 |
| Tg, mmol/L | 2.5 ± 1.3 | 1.2 ± 0.1 | 0.36 |
| LDLc, mmol/L | 3.9 ± 0.3 | 3.5 ± 0.2 | 0.34 |
| HDLc, mmol/L | 1.8 ± 0.1 | 1.7 ± 0.1 | 0.42 |
| Statin use, % yes (n) | 15 (2) | 30 (7) | 0.30 |
| TNF expression in non-stimulated monocytes | 0.010 ± 0.003 | 0.016 ± 0.002 | 0.16 |
| TNF expression in LPS-stimulated monocytes | 0.029 ± 0.004 | 0.052 ± 0.009 | 0.02* |
| TNF secretion by non-stimulated monocytes, pg/ml | 689 ± 174 | 494 ± 55 | 0.30 |
| TNF secretion by LPS-stimulated monocytes, pg/ml | 3763 ± 332 | 4623 ± 317 | 0.05* |

*Data are presented as mean ± SD or n (%). BMI, body mass index; IMT, intima-media thickness; TChol, total cholesterol; Tg, triglycerides; LDLc, low density lipoprotein cholesterol; HDLc, high density lipoprotein cholesterol; TNF, tumor necrosis factor; LPS, lipopolysaccharide. Plaque score characterizes the degree of stenosis: 0—no stenosis, 1—less than 20%, 2—20–50%, 3—over 50%. CD14-positive monocytes were isolated from participants and incubated with or without LPS (1 mkg/ml) for 24 h. Then TNF secretion and expression were measured by ELISA and qPCR respectively.*

**p < 0.05; **p < 0.01.*

Monocytes were isolated from patients using magnetic CD14+ separation and incubated with 1 μg/ml of LPS for 24 h. After that, the secretion and expression levels of TNFα was measured using ELISA and qPCR respectively. The secretion and expression levels of TNFα were significantly increased in LPS-stimulated monocytes isolated from atherosclerotic patients compared with healthy participants (**Table 1**). It turned out that plasma TNF level was low and did not exceed 23 pg/ml as well as no significant difference between atherosclerotic patients and healthy individuals was observed. This finding relates well with different study of systematic mediators of inflammation in asymptomatic patients (Montecucco et al., 2010).

Moreover, the significant correlation between TNFα expression by LPS-stimulated monocytes and IMT was observed (**Figure 1A**). Differences between the two groups of subjects correlated well with the results of another study (Patel et al., 2017). However, the key result of the current study was the observation that monocyte susceptibility to activation correlated not only with a discrete parameter (group number) but also with IMT. This may mean that there is a link between the formation of monocyte training and the degree of atherosclerosis progression. Previous studies by our group focused on the expression of TNFα in human lesions corresponding to different stages of atherosclerosis progression and found that it was maximal in lipofibrous plaques that are most enriched in lipids (Orekhov et al., 2018a). It is possible that the formation of monocyte tolerance with plaque thickening that may be responsible for the observed effect.

Interestingly, direct correlations of TNFα expression as well as secretion by non-stimulated and LPS-stimulated monocytes were observed for all participants (**Figure 1C**, **D**). It is therefore possible that the monocyte training effect is observed in response not only to TLR4 stimulation induced by LPS, but also to stressful conditions as a result of cell isolation, attachment, and cultivation.

## MONOCYTE ACTIVATABILITY AND LIPID PROFILE

Surprisingly, secretion and expression of TNFα in LPS-stimulated monocytes demonstrated a strong negative correlation with participant's HDL cholesterol (**Figure 1E**, **F**). Negative correlation between HDL cholesterol and TNFα secretion not only in LPS-stimulated, but also in non-stimulated monocytes (PPC = −0.692**, $p < 0.001$) was observed. Our data suggest that the blood lipid profile may be an important factor determining the degree of the monocyte inflammatory response to a pathogen. At the same time, the lipid profile itself is a poor marker, since patients with atherosclerosis and healthy subjects did not significantly differ in Tchol, Tg, LDLc, and HDLc (**Table 1**). Furthermore, no significant correlation between HDLc and IMT was observed (PPC = −0.245, $p = 0.162$).

A recent report revealed a correlation between the lipid profile of healthy subjects and the ability of classical, non-classical, and intermediate monocyte subpopulations to respond to LPS stimulation (Patel et al., 2017). This study evaluated only intracellular cytokine production. Interestingly, an inverse correlation between HDLc and intracellular production of IL-1β was found. No significant correlation between the intracellular production of TNFα and Tchol, LDLc or HDLc was observed. Large individual differences in monocyte activation were also found. Unfortunately, patients with atherosclerosis did not participate in the study.

It is well known that HDL may have a protective, anti-inflammatory effect on the endothelial cells. However, its effect on monocytes and macrophages is less studied. Murphy et al. (2008) demonstrated that HDL caused a dose-dependent decrease in CD11b activation under the influence of PMA, and apolipoprotein A-I was responsible for the effect. ApoA-I acted *via* ABCA1, whereas HDL acted through several receptors. The ability of HDL to modulate the expression of inflammatory genes has also been studied (Colin et al., 2014). On the one hand, Colin S et al. showed that HDL had no effect on the formation of the alternatively-activated M2 phenotype of macrophages, which suggests that the anti-inflammatory properties of HDL do not manifest themselves through the enhancement of the anti-inflammatory phenotype M2. Finally, Lee et al. (2016) investigated the ability of HDL to modulate the differentiation of monocytes to pro-inflammatory M1 macrophages in the presence of LPS and IFNγ. It turned out that HDL reduced the expression of M1 macrophage surface markers CD192 and CD64, as well as pro-inflammatory genes TNFα, IL-6, and MCP-1 (CCL2). The authors demonstrated that reverse cholesterol transport played an important role in the observed effect.

Recently, we analyzed the transcriptome of HDL-treated monocyte-derived macrophages in order to identify genes that could be upregulated by HDL (Orekhov et al., 2018c). Only three identified genes were significantly up-regulated by HDL-treatment: fatty acid desaturase 1 (FADS1, regulates unsaturation of fatty acids), insulin induced gene 1 (INSIG1, regulates lipid synthesis), and the low-density lipoprotein receptor (LDLR, binds non-modified LDL). In parallel, the role of identified genes in cholesterol efflux was investigated. We found that knockdown of INSIG1 and LDLR using siRNA decreased cholesterol efflux down-regulating the expression of ABCA1 and ABCG1. Thus, HDL particles seem to activate genes involved in lipid biosynthesis and these genes are required for successful cholesterol efflux. FADS1, INSIG1, and LDLR are regulated through SREBP2 pathway likely as a result of the reduction of the cellular cholesterol content. HDL particles may also affect on basic mediators regulating not only lipid biosynthesis but other anabolic processes (Nagao et al., 2017) as well as maintenance and repair reactions (Kimura et al., 2010). Target of rapamycin (mTOR), central mediator of anabolic processes including activation of SREBPs with further lipid biosynthesis, is involved in the induction of trained immunity (Cheng et al., 2014). It is assumed that AMP activated protein kinase (AMPK) plays a critical role in development of immune tolerance (Kim et al., 2014). Thus, we can speculate that HDL particles accelerate cholesterol efflux and lipid biosynthesis affecting on monocyte sensitivity *via* energy mediators (Bauer et al., 2018).

## SUPPRESSION OF TRAINED IMMUNITY *EX VIVO* AS A MODEL FOR SEARCHING ANTI-ATHEROGENIC DRUGS

It appears therefore that trained immunity is an unfavorable phenomenon that can contribute to chronic inflammation. Suitable models are needed to develop approaches to reducing the monocytes reactivity. Previous works by our group used an *in vitro* model to identify the anti-atherogenic and anti-inflammatory properties of various pharmacological agents (Nikiforov et al., 2017b). However, this approach gave no results. The *ex vivo* model turned out to be much more effective. In this model, patients with atherosclerosis were given an

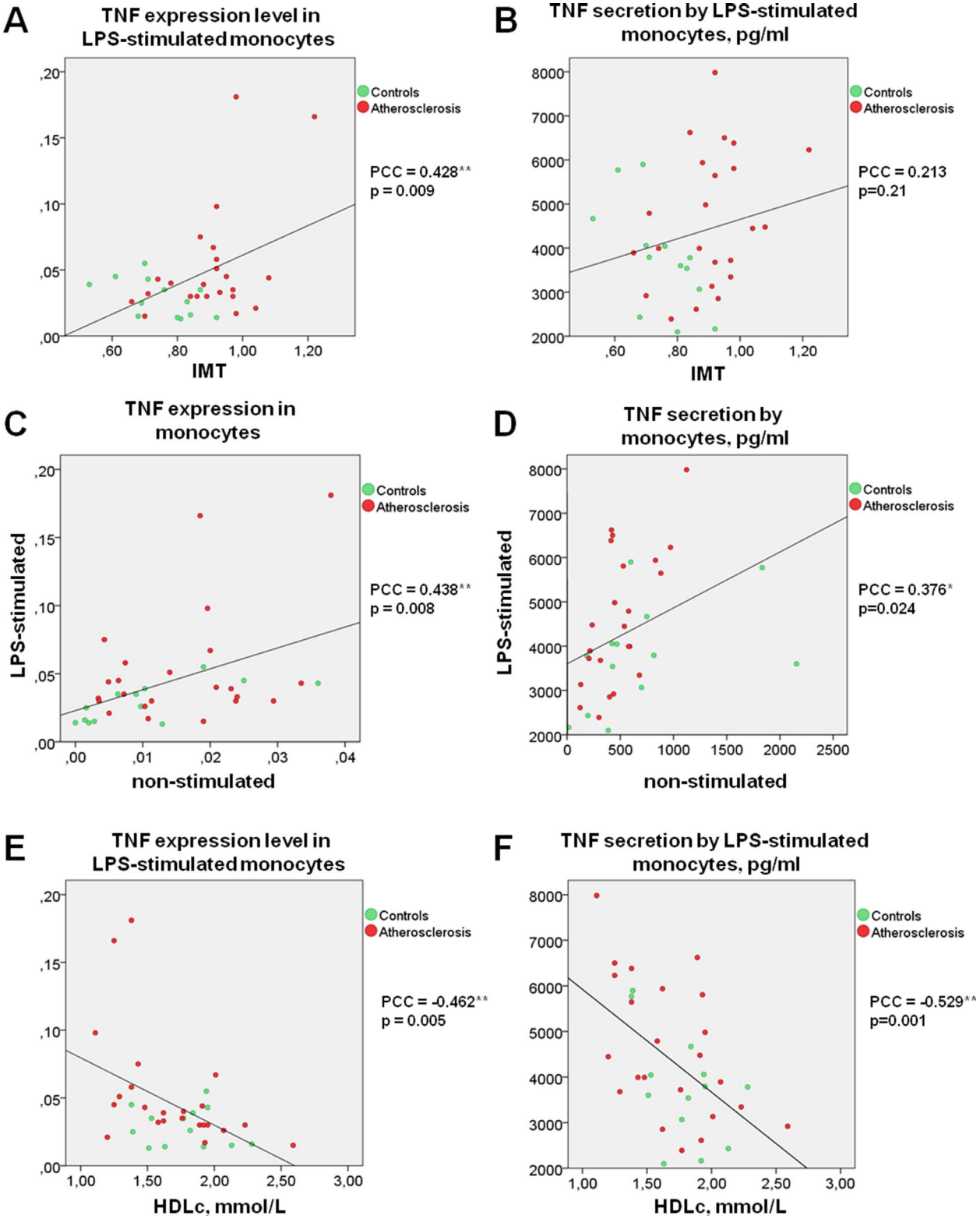


**FIGURE 1 |** TNF secretion and expression level by human monocytes under LPS stimulation correlates directly with IMT and inversely with HDL cholesterol. **(A** and **B)** Correlation between TNF expression **(A)** or secretion **(B)** level by LPS-stimulated monocytes and IMT. **(C** and **D)** Correlation between the abilities of LPS-stimulated and non-stimulated monocytes express **(C)** or produce **(D)** TNF. **(E** and **F)** Correlation between TNF expression **(E)** or secretion **(F)** level by LPS-stimulated monocytes and HDL cholesterol. Each point on graphs corresponds to one patient. PPC, Pearson correlation coefficient.

investigational drug, and blood samples were collected at 0, 2, and 4 h. Monocytes were isolated from blood samples taken before the drug administration (0 hours) and after 2 and 4 h. After that, monocytes were cultivated in the presence of a pro-inflammatory activator (most often IFNγ, less often LPS) for 24 h, after which the TNFα expression in stimulated and non-stimulated cells was measured. In parallel, serum was isolated from blood samples, which were tested for their ability to induce the accumulation of cholesterol in cultured control monocytes isolated from the blood of healthy donors (Nikiforov et al., 2017c). Usually, blood serum of patients with atherosclerosis is atherogenic, i.e., causes the accumulation of cholesterol in cultured monocytes when added at 10% for 24 h. Blood serum of healthy donors, which did not exhibit atherogenic effect, was used as a control (Orekhov et al., 2014).

The described model was used to evaluate four medicinal products: Allicor (INAT-Farma, Russia), CardioHealth (Sweden), Cellex (Pharm-Sintez, Russia), and Vezugen (JSC "Pharm," Russia). It turned out that Allicor and CardioHealth caused a significant decrease of TNFα expression in non-stimulated monocytes isolated from blood taken 4 h after drug administration and a tendency to suppression of response by stimulated cells. Four hours after receiving Allicor or CardioHealth, a significant decrease in the ability of blood serum to cause cholesterol accumulation in a primary macrophage culture was observed as well.

The observed increased pro-inflammatory sensitivity of monocytes from atherosclerotic patients and its correlation with IMT allowed us to assume that trained monocytes can make a significant contribution to chronic inflammation development in the arterial wall. Thus, trained phenotype of circulating monocytes can be considered as a perspective target for anti-atherosclerotic therapy. This *ex vivo* model seems well suited for identifying drugs that have an ability to reduce the reactivity of circulating monocytes and, as a consequence, be of interest for the development of anti-atherosclerotic immune-corrective therapy.

## CONCLUDING REMARKS

In conclusion, the most significant factor associated with IMT appears to be the level of TNFα expression in monocytes. Interestingly, there was a correlation between HDLc and TNFα secretion not only in LPS-stimulated but also in non-stimulated monocytes, which in turn may indicate tolerance induction by HDL independent on TLR4 stimulation. Possibly, stressful conditions caused by the procedure of isolating monocytes and their attachment in culture expressed ability to sensitize monocytes provoking long-term training responses. Of course, this interpretation is still rather speculative, and the mechanism of HDL's influence on the training remains to be elaborated.

It is likely that, due to the ability of HDL to produce cholesterol outflow from circulating cells directly into the bloodstream, there may be a correlation between cholesterol (and cholesterol esters) in circulating monocytes and their ability to be activated. It may turn out that in the logical chain "low HDLc—trained monocytes—high IMT" one link is missing. This link might be directly or indirectly related with the intracellular cholesterol content in circulating monocytes.

## ETHICS STATEMENT

This study was carried out in accordance with the recommendations of Institute for Atherosclerosis Research Committee on Human Research and meets the standards of the Declaration of Helsinki in its revised version of 1975 and its amendments of 1983, 1989, and 1996 (JAMA 1997;277:925–926) with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the Atherosclerosis Research Committee on Human Research.

## AUTHOR CONTRIBUTIONS

NN conceptualized, performed experimental studies, literature search, and supervision of the research project, and wrote the manuscript. TK conducted the patients' recruitment, clinical examination, and clinical data acquisition. Cell culture experiments were performed by AP and MK. Manuscript editing was done by RW and AO.

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# Targeting Histone Deacetylases in Myeloid Cells Inhibits Their Maturation and Inflammatory Function With Limited Effects on Atherosclerosis

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Monocytes and macrophages are key drivers in the pathogenesis of inflammatory diseases. Epigenetic targets have been shown to control the transcriptional profile and phenotype of these cells. Since histone deacetylase protein inhibitors demonstrate profound anti-inflammatory activity, we wanted to test whether HDAC inhibition within monocytes and macrophages could be applied to suppress inflammation *in vivo*. ESM technology conjugates an esterase-sensitive motif (ESM) onto small molecules to allow targeting of cells that express carboxylesterase 1 (CES1), such as mononuclear myeloid cells. This study utilized an ESM-HDAC inhibitor to target monocytes and macrophages in mice in both an acute response model and an atherosclerosis model. We demonstrate that the molecule blocks the maturation of peritoneal macrophages and inhibits pro-inflammatory cytokine production in both models but to a lesser extent in the atherosclerosis model. Despite regulating the inflammatory response, ESM-HDAC528 did not significantly affect plaque size or phenotype, although histological classification of the plaques demonstrated a significant shift to a less severe phenotype. We hereby show that HDAC inhibition in myeloid cells impairs the maturation and activation of peritoneal macrophages but shows limited efficacy in a model of atherosclerosis.

**Keywords: histone deacetylase, atherosclerosis, therapeutic targeting, monocyte, macrophage maturation**

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## INTRODUCTION

Emerging evidence suggests that epigenetics plays a crucial role in regulating immune cell function and may therefore offer many potential therapeutic opportunities for immune-mediated inflammatory diseases. In recent years, the identification of selective inhibitors of epigenetic enzymes and reader proteins has advanced our understanding of chromatin regulation of gene expression leading to renewed therapeutic efforts to reduce disease progression (Tough et al., 2016; Tough and Prinjha, 2017).

Histone deacetylases (HDAC) are a family of proteins that remove acetyl groups from lysine residues on histone tails and other proteins. The removal of these acetyl groups from histones causes DNA to be more compact, leading to a decrease in gene expression. There are 18 HDAC subtypes within the HDAC family that are subdivided into four classes (I, II, III, and IV) based on their homology to yeast proteins (Koeneke et al., 2015). HDACs in monocytes and macrophages are involved in multiple processes, from maturation to inflammatory response (Das Gupta et al., 2016). The classical inhibitors for these proteins broadly target classes I, II, and IV, which include 11 HDACs (New et al., 2012).

Currently, the use of inhibitors in the clinic is limited to oncology patients due to side effects (Rius and Lyko, 2011; McClure et al., 2018; Suraweera et al., 2018; Banik et al., 2019). Since the inhibition of HDACs offers great potential in several immune-mediated inflammatory diseases (Schotterl et al., 2015; Angiolilli et al., 2017; Cao et al., 2019), the specific targeting of immune cells with inhibitors of epigenetic enzymes may be key to success in non-oncology patients.

Carboxylesterase (CES) enzymes transform membrane-permeable esters into charged acids that are less able to cross the membrane (Imai, 2006). CES1 expression in humans is restricted to hepatocytes and cells of the mononuclear myeloid lineage, such as monocytes and macrophages (Su et al., 2004; Li et al., 2005). Based on this expression pattern, small molecules with an esterase-sensitive motif (ESM) are selectively hydrolyzed by CES1, enabling specific targeting of these cells. The ester-drug leads to the generation and retention of the charged acid, which is also pharmacologically active, within CES1-expressing cells. For instance, the combination of ESM technology with an HDAC inhibitor results in an increase of acetylation levels specifically in monocytes (Needham et al., 2011).

The inhibition of HDAC enzymes has shown wide-ranging anti-inflammatory effects (Falkenberg and Johnstone, 2014; McClure et al., 2018) with demonstrated efficacy in mouse models of inflammatory diseases (Lin et al., 2007; Cao et al., 2014; Hoeksema et al., 2014; Van den Bossche et al., 2014). Furthermore, monocytes and macrophages have an important role in the development and initiation of atherosclerosis (Moore and Tabas, 2011; Swirski et al., 2016; Tabas and Bornfeldt, 2016). Atherosclerosis is a lipid-driven disease that involves chronic inflammation. Monocytes and macrophages detect and phagocytose oxidized low density lipoproteins (oxLDL), becoming foam cells and acquiring a pro-inflammatory phenotype (Moore et al., 2013; Chistiakov et al., 2017). Modulating this phenotype should have beneficial effects in the outcome of the disease.

Based on the importance of myeloid cells in atherosclerosis and the efficacy seen with HDAC inhibitors in models of inflammatory diseases, we wished to evaluate whether HDAC inhibition in myeloid cells would be sufficient to drive efficacy. In our studies, we used a previously characterized molecule, CHR-4487 (ESM-HDAC528) (Needham et al., 2011). A related HDAC inhibitor also using ESM technology (Tefinostat) is being evaluated for efficacy in myeloid oncology indications (Zabkiewicz et al., 2016; Knapper et al., 2018). However, the application of ESM technology outside of oncology therapies has not been fully explored. In our studies, we tested whether ESM-HDAC528 targeting would deliver efficacy in a model of atherosclerosis. We found that compound modulated the pro-inflammatory phenotype and maturation of macrophages, with limited effect on reducing severity of plaques in atherosclerosis but no significant improvement in other disease parameters.

## MATERIALS AND METHODS

### Compounds

The compound used in the studies was ESM-HDAC528 (also termed CHR-4487) described in the work of Needham et al., 2011. The structure of the compound is shown in **Supplementary Figure 1A**. For *in vitro* work, the compound was dissolved in DMSO and used at a range of concentrations: 10, 50, and 100 nM, and also 1,000 and 10,000 nM for viability studies. For the *in vivo* studies, the compound was used at 3 mg/kg. The compound was dissolved in PBS without calcium and magnesium (PBS -/-), 5% DMSO and 11.25% cyclodextrin. 100 µl of either vehicle control or compound were injected intraperitoneally (i.p.) daily for 4 days for the thioglycollate model and 4 for weeks for the atherosclerosis model.

### Animals

The human CES1 transgenic mouse (*CES1/Es1e$^{lo}$*) was generated by Genoway (Lyon, France) from C57BL/6 mice by targeted insertion of the expression cassette into the expression permissive *hprt* locus on the X chromosome by homologous recombination. Expression of the *CES1* transgene was driven by the human CD68 promoter, which has previously been shown to direct transgene expression in macrophages of transgenic mice (Gough et al., 2001). These mice were then cross-bred with a naturally plasma esterase-low *Es1e$^{lo}$* mouse (obtained from Jackson Labs USA: strain 000785 - B6;D2-a *Ces1c$^{e}$*/EiJ) at Charles River (Margate, UK). From here on, these animals will be referred to as "transgenic mice" or "*CES1/Es1e$^{lo}$*". Control C57BL/6 wild-type (WT) mice were used in the *in vitro* experiments. In the acute study, twelve 10-week male *CES1/Es1e$^{lo}$* mice were divided in filter-top cages and injected with thioglycolate. Mice were divided in two groups (n = 6 per group) and injected either with 3 mg/kg ESM-HDAC528 or vehicle *via* intraperitoneal (i.p.) injection daily from the day of the thioglycolate injection. On day 3, blood was collected 3 h after i.p. injection, and on day 4, mice were sacrificed 24 h after the last injection for collection of blood and peritoneal cells (PECs).

For atherosclerosis experiments, we made use of low-density lipoprotein receptor knock-out mice (*ldlr*$^{-/-}$), which are prone to develop atherosclerosis. *Ldlr*$^{-/-}$ mice (C57BL/6 non *Es1e*$^{lo}$) were obtained from Jackson laboratories. A bone marrow transplantation (BMT) was performed by transferring bone marrow from *CES1/Es1e*$^{lo}$ mice provided by GlaxoSmithKline into the *ldlr*$^{-/-}$ mice. Forty 10-week-old female *ldlr*$^{-/-}$ mice were allocated to filter-top cages and provided with water containing neomycin (100 mg/L, Sigma, Zwijndrecht, Netherlands) and polymyxin B sulphate (60,000 U/L, Invitrogen, Bleiswijk, Netherlands) from 1-week pre-BMT until 5 weeks post-BMT. The animals received 2x6 Gy total body irradiation on two consecutive days. Bone marrow from *CES1/Es1e*$^{lo}$ mice was resuspended in RPMI-1640 (Gibco, Breda, Netherlands) with 5 U/ml heparin and 2% heat inactivated FCS (Gibco, Breda, Netherlands) and $10^7$ cells were injected intravenously per irradiated mouse. BMT efficiency was determined by qPCR for relative presence of the LDL receptor on DNA isolated from blood (GE Healthcare, Eindhoven, Netherlands). One mouse was excluded from the analysis due to inefficient BMT (<85%). Five weeks after the BMT, the mice were put on a high-fat diet (HFD) (0.15% cholesterol, 16% fat, Arie Blok Diets, Netherlands) for 10 weeks. In week 5, mice were divided in two equal groups by randomization based on weight, cholesterol, and triglyceride levels. One group received 3 mg/kg ESM-HDAC528 and the other received vehicle daily via i.p. dosing for 4 weeks. On week 9, 7 days prior to sacrifice, blood was taken 3 h after i.p. injection of ESM-HDAC528 and on week 10, on the day of the sacrifice, 24 h after i.p. injection of the compound to perform flow cytometry analysis on the blood. After sacrifice, each animal's heart was excised and frozen in Tissue-Tek (DAKO, Eindhoven, Netherlands) for histology. Two mice were sacrificed before the end of experiment as they reached humane endpoints. One additional mouse was excluded from the analysis due to insufficient tissue quality. A total of 17 mice from ESM-HDAC528 group were compared to 19 mice from the vehicle group for the histological analyses and 18 versus 19 for the flow cytometry experiments, where mice with low number of total events were also excluded.

All animal experiments were conducted at the University of Amsterdam and approved by the Committee for Animal Welfare of the Academic Medical Center, University of Amsterdam (permits: DBC242 and 103169-2). All animal studies were ethically reviewed and carried out in accordance with European Directive 2010/63/EEC and the GSK Policy on the Care, Welfare and Treatment of Animals.

## Bone Marrow-Derived Macrophage Culture and Functional Study

Bone marrow was isolated from femurs and tibia of *CES1/Es1e*$^{lo}$ and WT mice by flushing with RPMI-1640. The cells were cultured in RPMI-1640 with 25 mM HEPES and 2 mM L-glutamine, which was supplemented with 10% FCS, penicillin (100 U/ml), streptomycin (100 mg/ml), and 15% L929-conditioned medium as a source of M-CSF for 8 days. On day 8, cells were stimulated with LPS alone (10 ng/ml) or LPS (10 ng/ml) plus IFN-γ (100 U/ml) or left unstimulated for 24 h. Supernatants were collected and IL-6, IL-12(p40), and TNF were quantified by ELISA in accordance with the supplier's protocols (Life Technologies). Nitric oxide (NO) production was measured by $NO_2$ quantification by the Griess reaction. To measure viability, the BMDMs from transgenic mice were pretreated for 30 min with ESM-HDAC528 at 10, 100, 1,000, or 10,000 nM. Afterwards BMDMs were left untreated or stimulated overnight with 20 μg/ml 7-ketocholesterol (7KC; Sigma), 50 μg/ml ox-LDL or 10 μg/ml 25-hydroxycholesterol (25OHC; Sigma) and stained with propidium iodide (PI)/Annexin V-Alexa-Fluor647 according the manufacturer's instructions (Invitrogen). The percentage of viable macrophages (Annexin V-/PI-) was measured using a FACS Canto II.

After overnight ESM-HDAC528 pretreatment at 10 or 100 nM and DiI-oxLDL (Biotrend) treatment (3 h, 10 μg/ml), DiI-oxLDL uptake was measured by flow cytometry. Oxidized LDL uptake by BMDMs from transgenic mice was measured by flow cytometry. For lipid staining, BMDMs were pretreated with the inhibitors for 30 min, stimulated with 50 μg/ml oxLDL (BTI) for 24 h, and stained with LipidTOX Red (Invitrogen) according to the manufacturers' instructions. The median fluorescence intensities (MFI) were calculated with FlowJo software version 10.4.2.

## Peritoneal Macrophages

Four days prior to the sacrifice, mice were injected intraperitoneally with 1 ml thioglycolate medium (3%, Fisher, Bleiswijk, Netherlands). Upon sacrifice, the peritoneum was flushed with 10 ml ice cold PBS and PECs were collected as described previously (Neele et al., 2017). Flushed thioglycolate-elicited cells were cultured at a density of 100,000 cells/well in 100 μl in 96-well tissue culture plates (Greiner Bio-One, alphen a/d Rijn, Netherlands) in RPMI-1640 containing 25mM HEPES, 2mM L-glutamine, 100 U/ml penicillin, and 10% FCS (all Gibco, Breda, Netherlands). After 3 h adherence, non-adherant cells were washed away and the adherent cells were left either unstimulated or stimulated for 24 h with LPS (10 ng/ml) alone, LPS (10 ng/ml) plus IFN-γ (10 U/ml), or 24 h with IL-4 (20 ng/ml). Supernatants were collected and IL-6, IL-12(p40)/IL-12(p70), and TNF were quantified by ELISA in accordance with the supplier's protocols (Life Technologies). NO production was measured by $NO_2$ quantification in a Griess reaction. Cells were harvested using 1x Citrate from a 10X stock solution (1.35M potassium chloride, 0.15M sodium citrate, dilute in 100 ml milliQ and autoclaved) for 5 min at 37°C; the reaction was stopped by adding PBS-/- and cells were detached and washed twice with FACS buffer. Fc receptors were blocked with CD16/CD32 blocking antibody (1:100, eBioscence) in FACS buffer and cells were stained with appropriate antibodies (**Supplementary Table 2**) for 30 min at RT. Cells were then washed with FACS buffer and fluorescence was measured with a CytoFLEX flow cytometer and analysed with FlowJo software version 10.4.2. Cells were gated by excluding doublets, then selecting the macrophages based on FSC-A/SSC-A parameters. Positive peaks for markers were defined based on isotype control antibodies and the MFI was determined. This method was also used to

measure the expression of alternative activation markers (PDL2, CD71, CD206, CD301) *in vitro* in BMDMs from transgenic mice following treatment with ESM-HDAC528 at concentrations of 10, 50, and 100 nM and with IL-4 (20 ng/ml) for 24 h.

PECs were used immediately post-isolation to quantify mature peritoneal macrophages (PEMs) and intracellular lysine acetylation levels within those cells. Lysine acetylation was determined using the same protocol as for blood, minus the red blood lysis step. To evaluate maturation markers, cells were washed with FACS buffer and then stained with appropriate antibodies (**Supplementary Table 3**) for 30 min at RT and Fc receptors were blocked with CD16/CD32 blocking antibody (1:100, eBioscence) in FACS buffer. Cells were then washed with FACS buffer and fluorescence was measured with a CytoFLEX flow cytometer and analysed with FlowJo software version 10.4.2. After removing the doublets, macrophages were defined as $CD11b^+$ and $F4/80^+$ and then maturation markers Ly6C and CD64 were measured in these populations.

## Intracellular Acetylation Flow Cytometry and Triglyceride/Cholesterol Measurement

100 µl of blood were withdrawn from mice at 3 h and 24 h after i.p. injection of ESM-HDAC528. The blood was collected in tubes containing sodium heparin. For the 3 h time point, mice were injected with 3 mg/kg ESM-HDAC528 and their food was restricted for 3 h in order to get an accurate measurement of triglycerides and cholesterol. 50 µl of blood were centrifuged (10 min, 4°C, 2,000 rpm) to separate the plasma from blood cells. Plasma cholesterol and triglyceride levels were enzymatically measured according to the manufacturer's protocol (Roche, Woerden, Netherlands). 50 µl of blood was used for flow cytometry to measure intracellular acetylation at 3 h and 24 h. The blood was mixed 1:1 with PBS -/- and stained with cell surface marker antibodies for 30 min on ice (**Supplemental Table 1**). Red blood cells were lysed and cells were fixed by using BD FACS Lyse/Fix solution following the manufacturer's instructions (BD Pharmingen). After washing the cells twice with FACS buffer (0.5% BSA, 0.01% $NaN_3$ in PBS), cells were permeabilized using Human FoxP3 buffer following manufacturer's instructions (BD Pharmingen) and stained with an antibody for acetylated lysine (PanAck, Biolegend) for 30 min at RT. Cells were washed twice and resuspended in FACS buffer. Data were acquired using a BD Canto II and analysed with FlowJo software version 10.4.2. The cells were gated by excluding doublets, and then $Ly6G^+$ neutrophils were distinguished from monocytes, B, and T cells. Monocytes ($CD11b^+/CD115^+$) were distinguished from lymphocytes. Lymphocytes were further separated in B cells ($B220^+/CD3^-$) and T cells ($B220^-/CD3^+$). The MFI was determined from the positively stained cells (following FMO and isotype control corrections).

## Histochemistry

Atherosclerotic lesions from the heart were cut into 7 mm sections on a Leica 3050 cryostat at −25°C. Cross area sections of 42 mm were stained with toluidine blue (0.2% in PBS, Sigma-Aldrich, Gillingham, UK) to determine lesion size. Total lesion size per section was measured using Adobe Photoshop CS4. Lesion severity was scored (0, 1, 2, 3, 4, 5) by an experienced pathologist as no lesion (score 0) early (intimal xanthoma, scores 1, 2), moderate (pathological intimal thickening, score 3) and advanced (fibrous cap atheroma, scores 4, 5), as described elsewhere (Kanters et al., 2003). Sirius red staining was performed for 30 min to measure collagen content (0.05% direct red in saturated picric acid, Sigma, Zwijndrecht, Netherlands). Images were obtained using a Leica DM3000 microscope and quantified with Adobe Photoshop CS4 where collagen was quantified as the percentage of total lesion size. For immunohistochemistry, slides were fixed in acetone and blocked with Avidin/Biotin Blocking Kit (Vector Laboratories, Burlingame, USA). Hereafter, cells were incubated with MOMA-2 (1:4000, AbD Serotec, Uden, Netherlands) to stain for macrophages, ER-MP58 (1:200, AbD serotec, Uden, Netherlands) for infiltrating monocytes. Necrosis area was measured based on toluidine blue staining by a pathologist and corrected for total plaque size.

## Human Whole Blood Intracellular Acetylation Measurement

All donors provided written informed consent for the use of their samples, and the collection and use of the samples received Institutional Review Board approval. Blood from healthy volunteer donors was collected into tubes containing sodium heparin anticoagulant. 140 µl of blood was treated with compound for 4 h at 37°C after which samples were fixed and lysed for 15 min using FACS lysing solution (BD Pharmingen). Cells were washed with FACS buffer and Fc receptors were blocked using human IgG (Sigma) for 15 min at RT. Samples were stained at RT for 30 min with anti-CD66 (BD Pharmingen 551479) and anti-CD14 (BD Pharmingen 555399), to identify neutrophils and monocytes, after which samples were washed twice in FACS buffer and permeabilized for 30 min at RT using nuclear permeabilization buffer (Biolegend). Samples were then washed once and resuspended in nuclear permeabilization buffer containing anti-acetylated lysine antibody (Biolegend 623404) or a matched isotype control (R&D Systems IC0041P) and incubated at RT for 30 min **(Supplementary Table 4)**. Samples were washed twice in PBS and sample data were acquired using the BD FACS Canto II Flow Cytometer with FACS Diva (BD BioSciences software version 6.1.3.). Cells were gated by excluding doublets and neutrophils and monocytes identified. The remainder of nonstained viable cells were defined as lymphocytes. The MFI (median fluorescent intensity) of acetylated lysine within each population was determined.

## Statistical Analysis

Data represent the mean ± standard error of the mean (SEM). Differences between groups were analyzed using an unpaired student's *t*-test, two-way ANOVA using Bonferroni post hoc test analysis for grouped analysis or Chi-squared test. *P*-values <0.05 were considered statistically significant. Nonlinear curves for concentration-response studies for the data from human whole blood intracellular acetylation experiments were also generated. Data were analyzed using GraphPad Prism version 5.0 (GraphPad software, La Jolla, California).

To assess plaque severity (ranked 0, 1, 2, 3, 4, 5), an average severity score (based on 2–3 sections per animal) was calculated to give a single value for each animal. A nonparametric Mann-Whitney test was applied using Prism version 5.0 (GraphPad software, La Jolla, California) to determine whether the median score differed significantly between the treatments.

# RESULTS

## ESM-HDAC528 Reduces Pro-Inflammatory Cytokine Production

The mouse orthologue of CES1 significantly differs in distribution of expression and substrate specificity (Berry et al., 2009). Therefore, we utilized transgenic mice containing the human *CES1* gene under the control of the *CD68* promoter. Bone marrow-derived macrophages (BMDMs) from transgenic or WT mice were activated with LPS or LPS/IFNγ in the presence of the targeted HDAC inhibitor (ESM-HDAC528). Structurally, the ESM-HDAC528 compound is composed of an HDAC inhibitor conjugated to an ester group. When the ester group is cleaved from the HDAC inhibitor, by the enzyme CES1, in myeloid cells of the transgenic mice, it accumulates within those specific cells **(Supplementary Figure 1A)**.

After stimulation, we observed a concentration-dependent inhibition of the production of pro-inflammatory mediators (IL-6, IL-12p70, NO) but not for TNF **(Figure 1A)**. No inhibition was observed with compound in WT macrophages at these concentrations, likely due to the lack of expression of the human enzyme. Further characterization of functions related to atherosclerosis of BMDMs from the transgenic mice after treatment with ESM-HDAC528 were also performed. Firstly, viability was assessed (**Supplementary Figure 1B**). No effects on viability were observed in the cells after treatment with

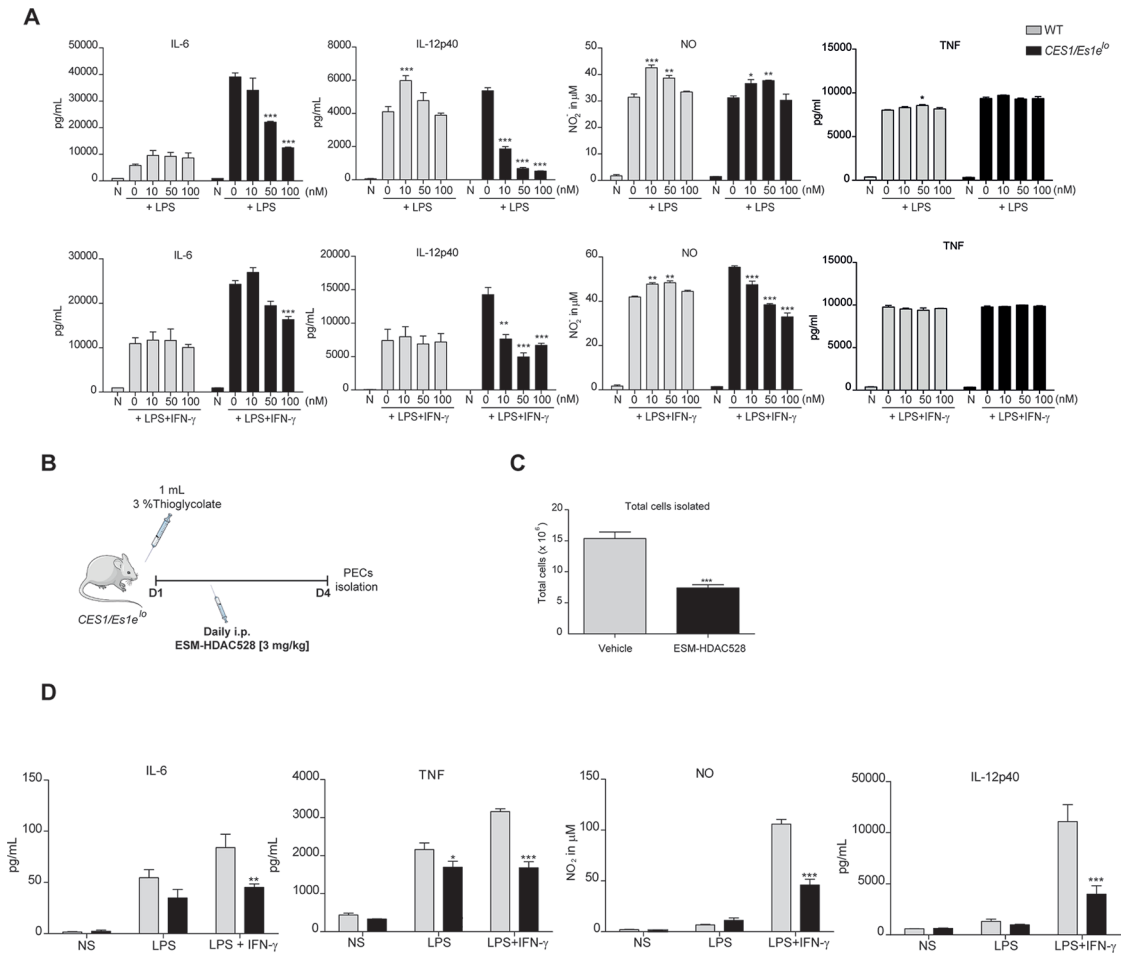


**FIGURE 1 |** ESM-HDAC528 reduces pro-inflammatory cytokine production both *in vitro* and *in vivo*. **(A)** Cytokine production by BMDMs from *CES1/Es1e*$^{lo}$ mice and WT mice after stimulation with LPS (10 ng/ml) or LPS (10 ng/ml) + IFN-γ (10 U/ml) in the presence of increasing concentrations of ESM-HDAC528 for 24 h. n = 3. **(B)** Design of acute thioglycolate model. Transgenic mice were treated for 4 days with daily i.p. injection of 3 mg/kg ESM-HDAC528 (n = 6) or vehicle (n = 6), on day 4, 24 h after i.p. injection PECs were isolated. **(C)** Total number of cells isolated from the peritoneal lavage in each group n = 6 per group. **(D)** Cytokine production by PEMs isolated from the mice (n = 6) of each group attached and then stimulated for 24 h with LPS (10 ng/ml) or LPS (10 ng/ml) + IFN-γ (10 U/ml). Statistical significance was determined by unpaired *t*-test (C) or two-way ANOVA with Bonferroni correction **(A, D)** ($p < 0.05$). All error bars represent the SEM. ns = *p* value > 0.05, **p* value ≤ 0.05, ***p* value ≤ 0.01, ****p* value ≤ 0.001.

10 and 100 nM of ESM-HDAC528. At higher concentrations (1,000 and 10,000 nM), the viability was reduced. Based on these results, subsequent experiments were performed at 10, 50, and 100 nM. The expression of alternative activation surface markers **(Supplementary Figure 1C)** after IL-4 stimulation was determined. No significant changes were observed except for a trend to reduction in the expression of CD206 at higher concentrations. Another important function is lipid uptake **(Supplementary Figure 1D)**; in this case, no effects were observed in uptake of oxidized LDL after treatment with ESM-HDAC528.

Prior to *in vivo* studies, intracellular acetylation of white blood cells was determined in human whole blood treated with either the non-targeted, conventional HDAC inhibitor SAHA (suberanilohydroxamic acid) or ESM-HDAC528 (**Supplementary Figures 1E** and **1F)**. ESM-HDAC528 was more potent than SAHA at increasing intracellular acetylation levels and this phenomenon was selectively observed in monocytes.

To assess if a suppressed macrophage response also manifested *in vivo*, an inflammatory response was initiated in *CES1/Es1e*$^{lo}$ mice by a single i.p. thioglycolate injection **(Figure 1B)**. It has previously been demonstrated that ESM-HDAC528 specifically targets circulating monocytes (Needham et al., 2011) and we wanted to extend this observation to PEMs. In this study, mice were injected i.p. daily for 4 days with 3 mg/kg ESM-HDAC528 or vehicle and on day 4 PECs were isolated. The total number of cells isolated from the ESM-HDAC528 group was significantly reduced compared to the vehicle group (**Figure 1C**).

After 3 h attachment (to enrich for PEMs), cells isolated from both groups were stimulated *in vitro* with LPS or LPS/IFNγ. Interestingly, PEMs from the ESM-HDAC528 group produced lower levels of pro-inflammatory mediators after activation compared to equal numbers of plated PEMs from the vehicle group **(Figure 1D)**. These data indicate that ESM-HDAC528 reduces macrophage activation both *in vitro* and *in vivo*.

## ESM-HDAC528 Modulates the Maturation of Freshly Isolated PEMS and the Expression of Macrophage Activation Markers on Cultured PEMS.

We next wanted to understand whether the effects of an ESM-HDAC inhibitor on cytokine production were due to a change in polarization or maturation. The maturation status was measured in freshly isolated PEMs. Mature PEMs can be defined as a CD11b$^+$ and F4/80$^+$ population (Misharin et al., 2013; Cassado et al., 2015) **(Figure 2A)**. The percentage of this population was different between groups, with a reduction of 28% in the ESM-HDAC528 group compared to vehicle-treated mice. Other markers (Ly6C and CD64) were also measured within the macrophage population (CD11b$^+$ F4/80$^+$). Ly6C is a monocyte marker expected to be higher in immature macrophages (Robbins et al., 2013), whereas CD64 is expressed in mature macrophages rather than in monocytes (Tamoutounour et al., 2012). The population showed an increased percentage of Ly6C$^+$ cells and reduction in CD64$^+$ cells which indicates reduced maturation in the PEMs of the ESM-HDAC528 treatment group **(Figure 2A**). Additionally, in cells that were mature (CD11b$^+$ and F4/80$^+$), the MFI for these maturation markers (CD11b and F4/80) was lower in the ESM-HDAC528 group (**Figure 2B**).

Next, we measured the expression of pro-inflammatory and alternative activation surface markers. The gating strategy used for stimulated cells is shown **(Figure 2C)**. After attachment, the cells are expected to be predominantly PEMs. After stimulation, cells were harvested, doublets excluded, and the PEMs were gated based on FSC-A/SSC-A. Surface markers were detected using either PE- or APC-conjugated antibodies. The positive peaks of those markers were defined using an isotype control and the MFI of the markers was determined from the positive population **(Figure 2C)**. CD80 expression was significantly decreased on both unstimulated and stimulated PEMs from ESM-HDAC528 treated mice compared to vehicle controls. Furthermore, CD86 expression was significantly lower in LPS-treated PEMs **(Figure 2D)**. No effects were observed on PDL2 and CD71, IL-4-induced markers of alternatively activated macrophages **(Figure 2E)**. We conclude that ESM-HDAC528 blocks PEM maturation and inhibits the expression of pro-inflammatory markers.

## ESM-HDAC528 Treatment Does Not Affect Lipid Levels in an Atherosclerosis Model

The preceding experiments demonstrated that ESM-HDAC528 affects the pro-inflammatory and maturation status of macrophages. We next wanted to test if this would be of benefit in a model of atherosclerosis. In this *in vivo* atherosclerosis study, *ldlr* $^{-/-}$ mice were irradiated and transplanted with bone marrow from *CES1/Es1e*$^{lo}$ mice. Mice were fed for 10 weeks on an HFD and treated from week 5 either with 3 mg/kg ESM-HDAC528 or vehicle **(Figure 3A)**.

The efficiency of the BMT, measured by chimerism, was equal and above the threshold of 85% for the mice divided between both analysis groups **(Figure 3B)**. The mean weight remained similar across the study in both treatment groups **(Figure 3C)**. As expected, triglycerides and cholesterol levels increased over the study duration. However, levels of both analyses remained similar in both groups **(Figure 3D)**.

## ESM-HDAC528 Increases Acetylation Specifically in Murine Myeloid Cells

To confirm the targeted activity of ESM-HDAC528, we measured the levels of acetylation in circulating white blood cells using the gating strategy described **(Figure 4A).** In blood samples collected 3 h after ESM-HDAC528 treatment, there was a significant increase in acetylation in monocytes. The acetylation levels in other immune cells were unchanged compared to the vehicle control, showing the specific targeting of this compound to mononuclear myeloid cells **(Figure 4B)**. After 24 h, when compound was no longer systemically detectable, acetylation was modestly increased in neutrophils in this study, although monocyte acetylation had reverted to similar levels in both groups **(Figure 4C)**.

We also determined acetylation in freshly isolated PEMs and found higher levels in the compound group compared to the vehicle,

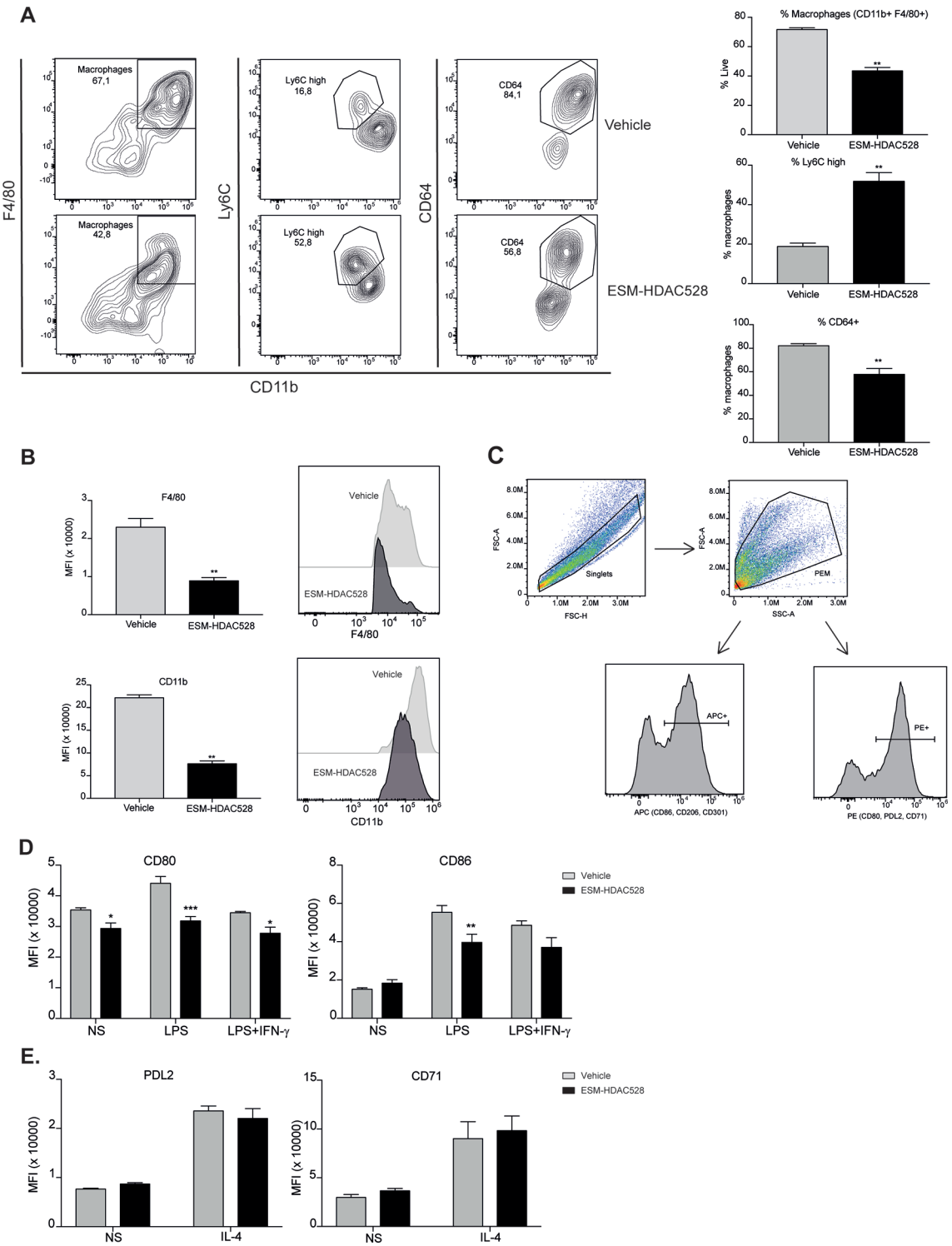


**FIGURE 2 |** ESM-HDAC528 treatment modulates PEM maturation and surface marker expression. **(A)** Percentage of mature macrophages (CD11b+ and F4/80+), and the maturation markers Ly6C and CD64 within macrophage populations in the freshly isolated cells 24 h after injection n = 6 per group. **(B)** MFI of F4/80 and CD11b in the mature macrophages. n = 6 per group. **(C)** General gating strategy for activation marker expression on PEMs after attachment and 24 h stimulation for activation. Antibodies were conjugated to either APC or PE depending on the panel. **(D)**. MFI of the positive peaks for the pro-inflammatory surface markers in PEMs Isolated, attached and stimulated for 24 h with LPS (10 ng/ml) or LPS (10 ng/ml) + IFN-γ (10 U/ml). n = 6 per group. **(E)** MFI of the positive peaks for the alternative activation surface markers in PEMs attached and stimulated for 24 h with IL-4 (20 ng/ml). n = 6 per group. Statistical significance was determined by unpaired *t*-test **(A** and **B)** or two-way ANOVA with Bonferroni correction **(D** and **E)** ($p < 0.05$) All error bars represent the SEM. ns = $p$ value > 0.05, *$p$ value ≤ 0.05, **$p$ value ≤ 0.01, ***$p$ value ≤ 0.001.

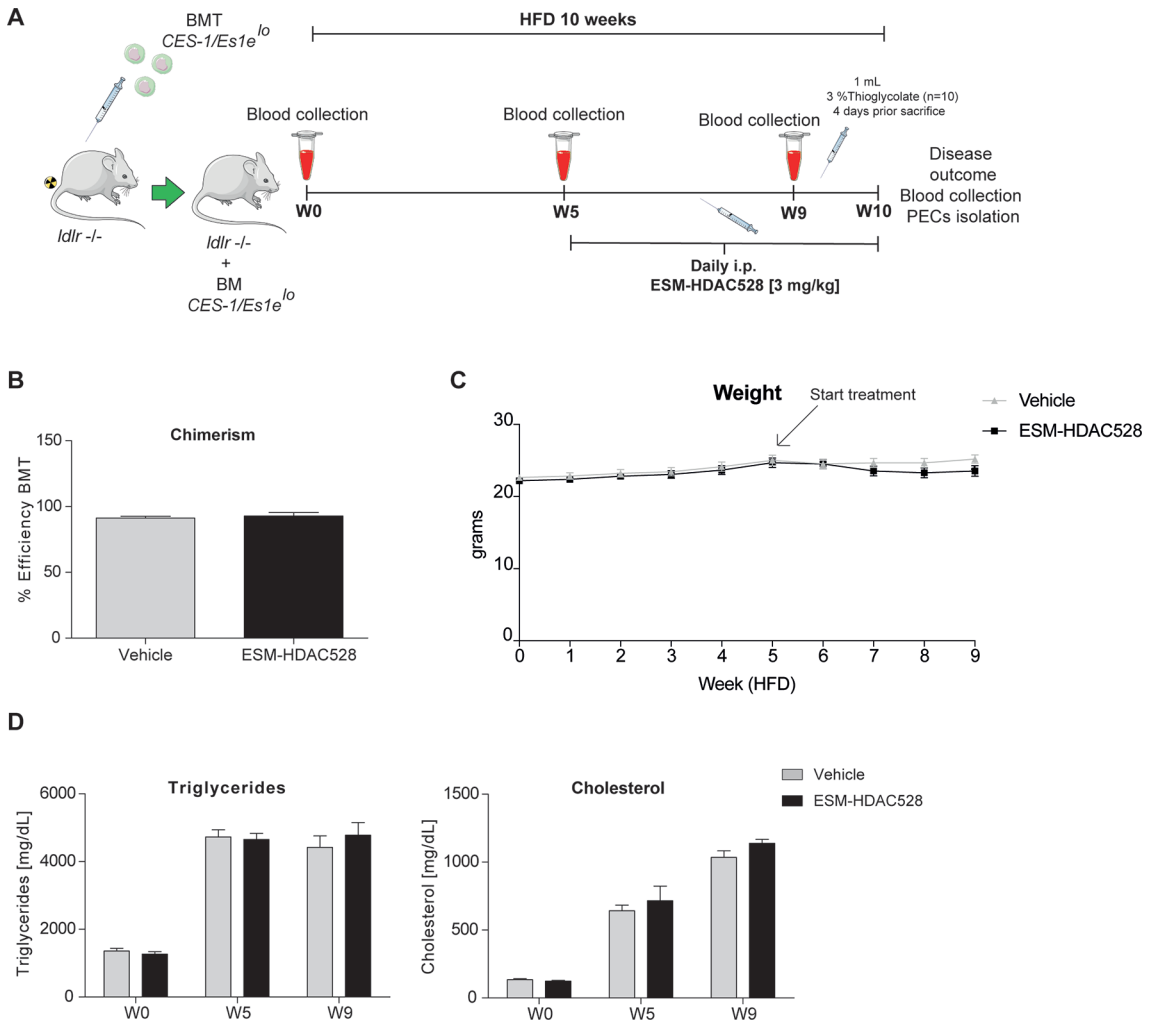


**FIGURE 3 |** ESM-HDAC528 treatment does not affect clinical features in a model of atherosclerosis. **(A)** Design of the atherosclerosis study. The *Idlr* knockout mice were transplanted with bone marrow from *CES-1/Es1e*$^{lo}$ mice. The mice were divided into two groups ESM-HDAC528 (n = 19) and vehicle (n = 19). Mice were on an HFD for 10 weeks. On week 5, the mice were treated with 3 mg/kg ESM-HDAC528 or vehicle by daily i.p. injection. Blood was collected on week 0, 5, 9, and 10. On week 10, mice were sacrificed, the disease outcome was assessed and PECs were isolated. **(B)** Efficiency of the BMT in both groups. n = 19 vehicle vs. n = 18 ESM-HDAC528. **(C)** Weight of the mice from both groups during the study. n = 19 vehicle vs. n = 18 ESM-HDAC528 **(D)** Triglycerides and cholesterol levels of the groups on week 0, 5, and 9. n = 19 vehicle vs. n = 18 ESM-HDAC528. Statistical significance was determined by unpaired *t*-test **(B)** or two-way ANOVA with Bonferroni correction **(C** and **D)** ($p < 0.05$). All error bars represent the SEM.

demonstrating specificity of the compound not only in monocytes but also in macrophages in this mouse model **(Figure 4D)**.

## ESM-HDAC528 Modulates PEM Maturation and Activation to a Lesser Extent in the HFD Atherosclerosis Model

We wanted to evaluate whether ESM-HDAC528 dampened macrophage maturation and activation in the atherosclerosis model to a similar extent to that seen in the acute model **(Figure 2)**. The percentage of mature macrophages ($CD11b^+$ $F4/80^+$) following thioglycolate administration was lower in the compound-treated group **(Figure 5A)**. Within this macrophage population, maturation markers also showed the same trend as previously observed, with CD64 expression being significantly lower in the compound group and a trend for increased Ly6C **(Figure 5A)**. The expression levels of the maturation markers CD11b and F4/80 (as assessed by MFI) were significantly lower for CD11b and there was a trend toward a reduction of F4/80 expression within the mature macrophages in the ESM-HDAC528 treatment group **(Figure 5B)**. Overall, we observed an effect of the compound on the maturation of macrophages, although the magnitude of change was generally weaker than observed in the acute model.

The gating strategy for the measurement of activation markers was as previously defined **(Figure 5C)**. Interestingly, in contrast to our previous observation, in PEMs from atherosclerotic mice stimulated with LPS alone or in addition to IFNγ, an increase of CD80 was seen following ESM-HDAC528 treatment. However, for LPS+IFNγ induced expression of

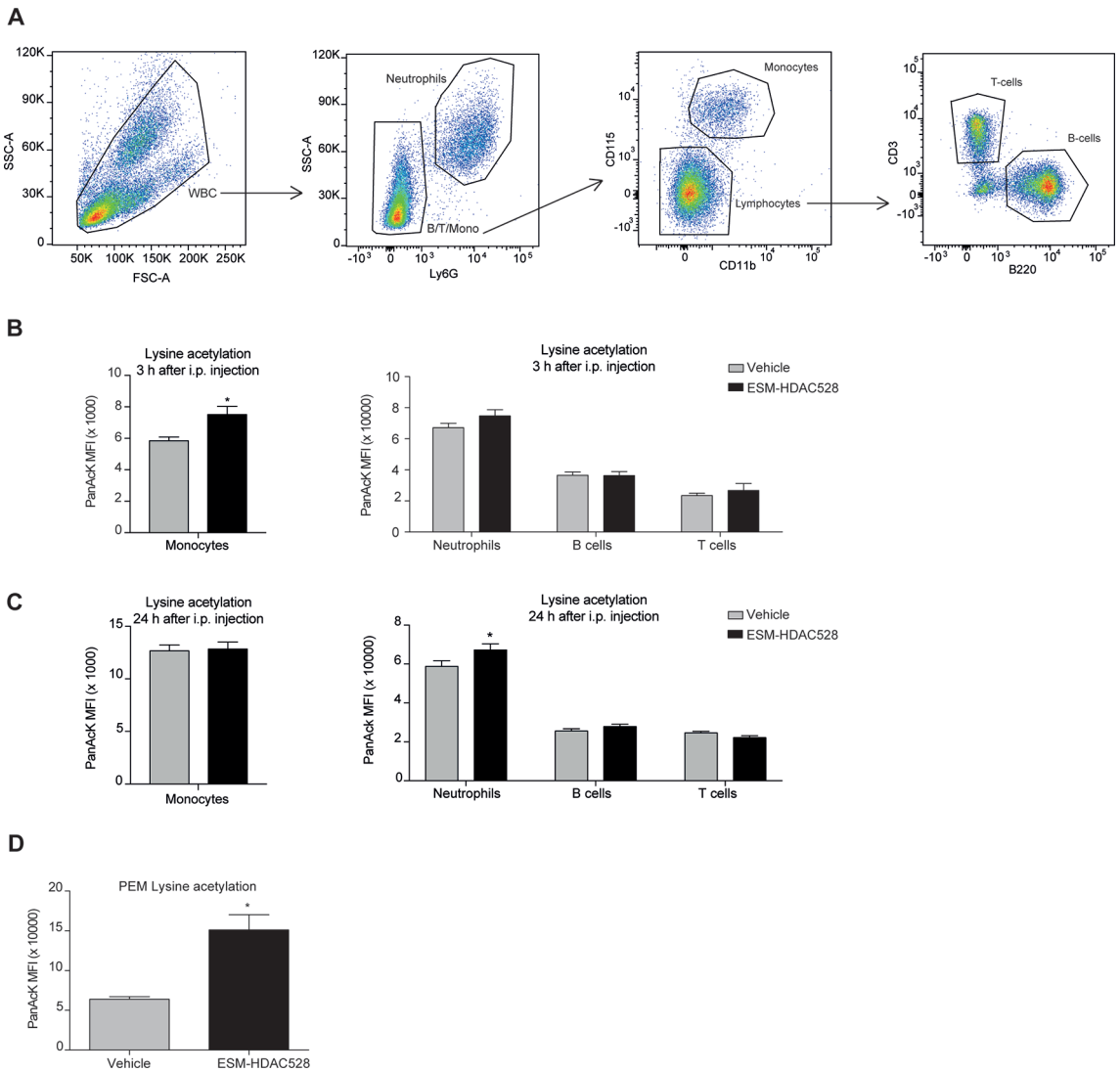


**FIGURE 4 |** ESM-HDAC528 selectively increases acetylation in myeloid cells. **(A)** Gating strategy for white blood cells after doublet exclusion. **(B)** MFI of intracellular acetylation levels in monocytes and other WBC 3 h after i.p. injection. n = 19 vehicle vs. n = 18 ESM-HDAC528. **(C)** MFI of intracellular acetylation levels in monocytes and other WBC 24 h after i.p. injection. n = 19 vehicle vs. n = 18 ESM-HDAC528. **(D)** MFI for intracellular acetylation levels in fresh PEMs isolated 24 h after i.p. injection. n = 5 per group. Statistical significance was determined by unpaired *t*-test **(B** and **C)** or two-way ANOVA with Bonferroni correction **(B**–**D)** ($p < 0.05$). All error bars represent the SEM. **p* value ≤ 0.05

CD86, there was a reduction in the ESM-HDAC528 treated animals **(Figure 5D)**. For the alternative activation markers following IL-4 stimulation, there was an increase of CD71 and PDL2 and a reduction of CD301 with ESM-HDAC528 **(Figure 5E)**. Pro-inflammatory mediators were also measured, and, except for NO, which was reduced in the ESM-HDAC528 group, there was no significant inhibition of the production of pro-inflammatory mediators by macrophages **(Figure 5F)**. In general, ESM-HDAC528 had a reduced ability to inhibit macrophage maturation in this model. Additionally, the effects on pro-inflammatory mediators were milder and polarization markers were inconsistent with inhibiting a pro-inflammatory phenotype.

## Disease Outcome After Treatment With ESM-HDAC528

Considering the characteristics of the macrophages after the treatment with ESM-HDAC528, we wanted to understand the impact on the disease outcome. Therefore, the severity of the atherosclerotic lesions in the mice was scored. We observed a reduction in the percentage of the more severe phenotypes of the plaques (pathological intimal thickening and fibrous cap atheroma) in the ESM-HDAC528 treated mice together with an increase of the less severe phenotype (intimal xanthoma) **(Figure 6A)**. The severity scores of all lesions were combined to give a composite score per animal, showing that the animals treated with ESM-HDAC528 had a significantly reduced

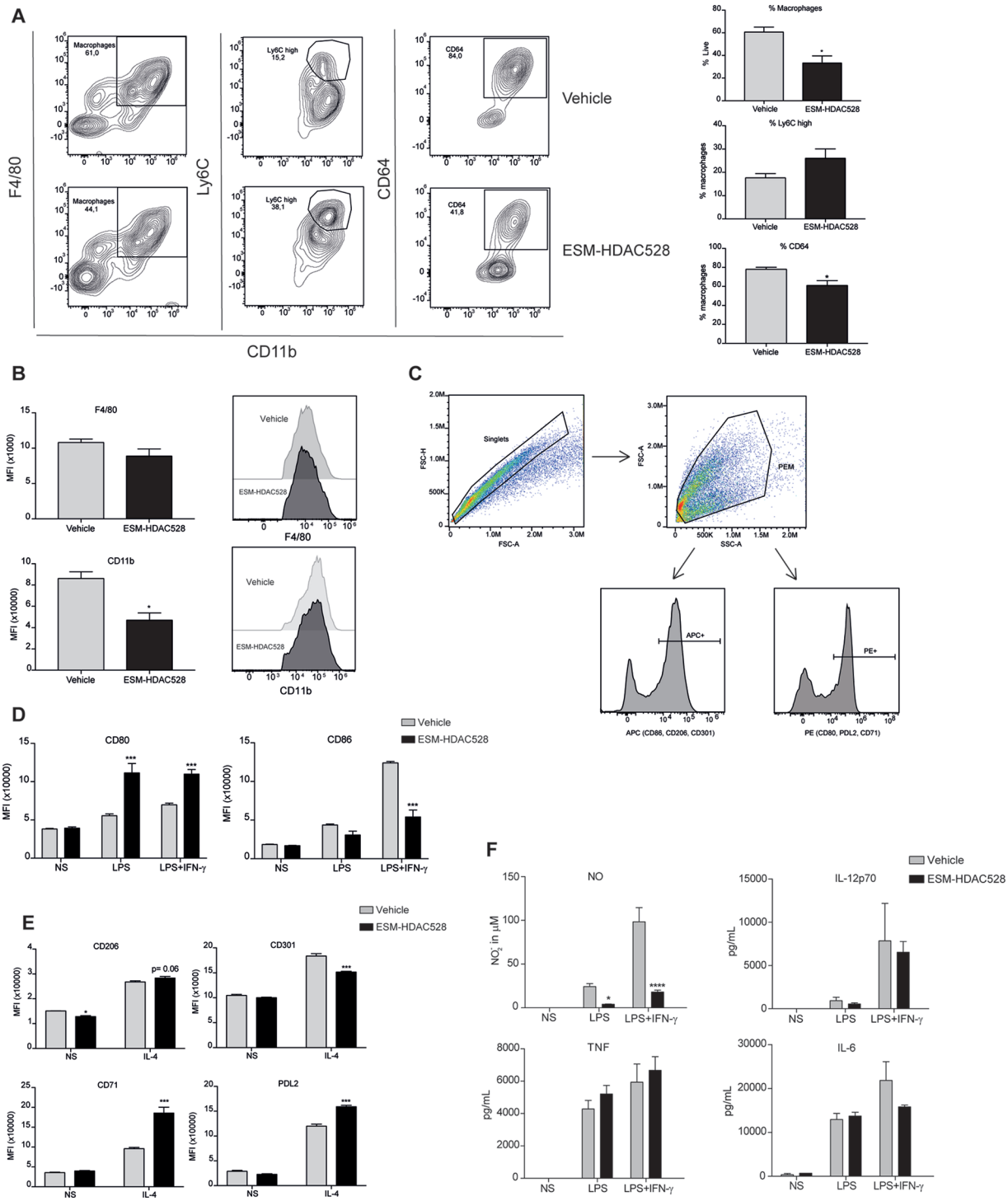


**FIGURE 5 |** ESM-HDAC528 modulates PEM maturation and activation to a lesser extent in an atherosclerosis model. **(A)** Percentage of mature (CD11b+ and F4/80+) macrophages from freshly isolated PECs 24 h after ESM-HDAC528 injection and of cells expressing Ly6C and CD64 within this population. n = 5 per group. **(B)** MFI of F4/80 and CD11b in the mature macrophages 24 h after injection. n = 5 per group. **(C)** General gating strategy for activation marker expression on PEMs after attachment and 24 h stimulation for activation. Antibodies were conjugated to either APC or PE dependent on the panel. **(D)** MFI of the positive peaks for pro-inflammatory surface markers in PEMs Isolated, attached and stimulated for 24 h with LPS (10 ng/ml) or LPS (10 ng/ml) + IFN-γ (10 U/ml). n = 5 per group. **(E)** MFI of the positive peak for alternative activation surface markers in PEMs attached and stimulated for 24 h with IL-4 (20 ng/ml). n = 5 per group. **(F)** Cytokine production by PEMs isolated from the mice (n = 5) of each group stimulated 24 h with LPS (10ng/ml) or LPS (10ng/ml) + IFN-γ (10 U/ml). Statistical significance was determined by unpaired *t*-test **(A** and **B)** or two-way ANOVA with Bonferroni correction **(D**–**F)** ($p < 0.05$). All error bars represent the SEM. ns = *p* value > 0.05, **p* value ≤ 0.05, ***p* value ≤ 0.01, ****p* value ≤ 0.001.

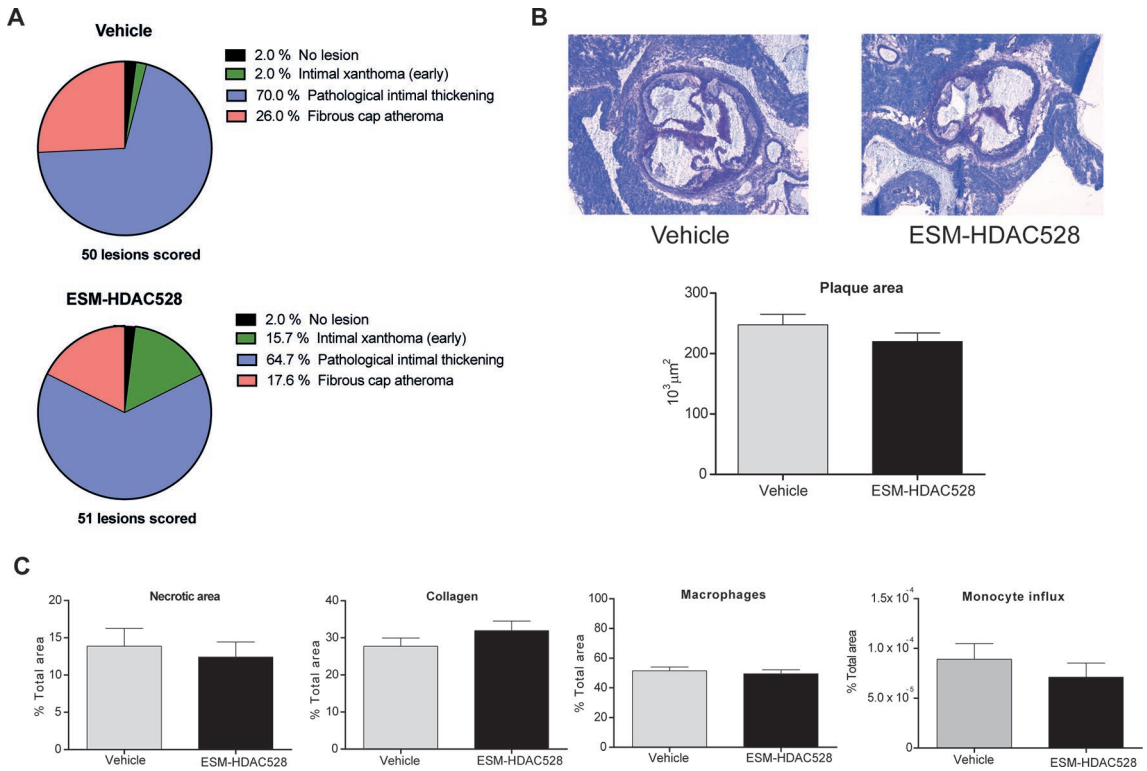


**FIGURE 6 |** ESM-HDAC528 does not reduce plaque formation. **(A)** Severity of the plaque for the different groups, the plaque is rated according to the morphology. n = 50 lesions in vehicle vs. n = 51 lesions in ESM-HDAC528. **(B)** Plaque size for the different groups. Representative images of the plaque area and measurement of plaque area for the different groups. n = 19 vehicle vs. n = 17 ESM-HDAC528. **(C)** Different disease characteristics: necrotic area, percentage of macrophages, collagen, and monocyte influx in the plaques of the different treatment groups. n = 19 vehicle vs. n = 17 ESM-HDAC528. Statistical significance was determined by unpaired *t*-test **(B** and **C)** or Chi-square test **(A)** ($p < 0.05$). All error bars represent the SEM**.**

median severity score upon ESM-HDAC528 treatment compared to vehicle treated mice (ESM-HDAC528 median = 3.00; vehicle median = 3.67; $p = 0.0163$). A trend for reduction in total plaque area was also observed ESM-HDAC528 treated mice **(Figure 6B)**.

For the rest of the disease parameters, there were no significant differences between the groups. Nevertheless, the necrotic plaque area and monocyte influx also showed a tendency to be reduced in the atherosclerosis model and the increase in collagen could indicate a more beneficial phenotype **(Figure 6C)**. In conclusion, in parallel to effects on maturation of the macrophages, atherosclerotic plaque severity was partially improved by the ESM-HDAC528 treatment in this model.

## DISCUSSION

Macrophages play a role in virtually every stage of atherosclerosis and reshaping their dysregulated activation is considered to be the holy grail of macrophage therapeutic targeting (Sica and Mantovani, 2012). The Canakinumab Anti-inflammatory Thrombosis Outcomes Study (CANTOS) recently delivered clinical data demonstrating that inflammation is a key driver of atherosclerosis (Ridker et al., 2017). Therefore, targeting macrophage-mediated inflammation has emerged as an attractive approach for atherosclerosis therapy. Meanwhile, it has become increasingly clear that epigenetic mechanisms are critical regulators of inflammatory responses. Histone deacetylases that regulate the acetylation status of histones and non-histone proteins are of high interest since broad-spectrum HDAC inhibitors are well documented to decrease inflammation and disease severity in multiple diseases (Neele et al., 2015). Moreover, inhibition of HDACs in macrophages has beneficial athero-protective effects *in vitro* (Van den Bossche et al., 2014) but their progression as a potential atherosclerosis therapy was prevented by the observation that the broad-spectrum HDAC inhibitor Trichostatin A (TSA) unexpectedly increased plaque size in a mouse model of atherosclerosis (Choi et al., 2005). However, this could be due to negative effects of TSA on other cell types that are known to affect atherosclerosis such as endothelial cells and smooth muscle cells (Rössig et al., 2002).

Therefore, we reasoned that inhibiting HDACs specifically in macrophages and monocytes would be beneficial in an atherosclerosis setting. To achieve this, we used an ESM-conjugated HDAC inhibitor which is selectively hydrolyzed into a charged molecule and retained within monocytes and macrophages by human carboxylesterase-1 (CES1) (Needham et al., 2011). Accordingly, ESM-HDAC528 had no inhibitory

effect on BMDMs from WT mice but efficiently inhibited inflammatory responses in BMDMs that were derived from transgenic mice that expressed human CES1 driven by the monocyte/macrophage-specific CD68 promoter (*CES1/Es1e*$^{lo}$). ESM-HDAC528 exhibited monocyte-specific activity (lysine acetylation) in human white blood cells. Moreover, we found that ESM-HDAC528 was more potent than the conventional non-targeted HDAC inhibitor SAHA in these cells.

*In vitro* data showed decreased levels of cytokine production in transgenic BMDMs in contrast to WT BMDMs where no decreases were observed. Interestingly, in the case of IL-12p40, NO, and, to a much lesser extent, TNF, a significant induction was observed in WT cells. This phenomenon is not understood and could be addressed in future work with more extended concentration ranges to explore the potential of biphasic responses.

After validating our approach *in vitro*, we next confirmed the efficacy of ESM-HDAC528 *in vivo* and that i.p. injection of the drug into *CES1/Es1e*$^{lo}$ mice efficiently reduced the LPS (+/- IFNγ)-induced production of IL-6, TNF, IL-12, and NO. Interestingly, the total number of PECs and macrophages isolated from ESM-HDAC528-injected mice was reduced and these cells appeared less mature as evidenced by increased Ly6C and decreased CD11b, F4/80, and CD64 expression. Since these distinct ESM-HDAC528-mediated effects could potentially dampen atherosclerosis progression, we next assessed the effect of this drug on atherosclerosis in *ldlr*$^{-/-}$ mice that were transplanted with *CES1/Es1e*$^{lo}$ bone marrow. Acetylation levels in monocytes and PEMs were increased in the ESM-HDAC528-treated group and this was accompanied by reduced macrophage activation. Yet, the effects of HDAC inhibition on inflammatory and maturation endpoints in PEMs were less pronounced in these hypercholesterolemic mice. Although ESM-HDAC528 treatment did not significantly affect plaque size, these plaques were classified as less severe histological phenotypes, with the change being statistically significant and consistent with at least a partial impact on an important disease outcome.

One of the outstanding questions from our observations is how HDAC inhibition impairs monocyte to macrophage differentiation and inflammatory responses, and why the latter effect is less pronounced in a hypercholesterolemic environment. It should be noted that, in this BMT model, ESM-HDAC528 would not be targeted to non-bone marrow derived lineages of macrophages which would not express human CES1. This could explain the limited efficacy seen in the atherosclerosis model. Additionally, it is well described that cell fate decisions within the hematopoietic system are regulated by epigenetic mechanisms and distinct HDACs were shown to be implicated in myeloid development (reviewed in (Das Gupta et al., 2016)). Specific HDACs are differentially regulated and expressed in response to environmental factors, and while HDAC inhibitors mediate anti-inflammatory effects *via* a wide range of mechanisms, they can also amplify inflammatory responses in macrophages. For example, HDAC6 normally acts as a transcriptional activator of the anti-inflammatory cytokine IL-10 and consequently HDAC6 inhibition or genetic knock-down diminishes IL-10 secretion (Cheng et al., 2014). As such, ESM-HDAC528 could potentially inhibit distinct HDACs in normal versus hypercholesterolemic mice and this might explain why broad spectrum HDAC inhibition is less beneficial in the context of atherosclerosis.

Together, our data highlight the potential for drugs that selectively target individual HDACs to improve effectiveness in atherosclerosis treatment. In this context, (macrophage-specific) HDAC3 inhibition may be an attractive target for atherosclerosis therapy since its deletion promotes anti-atherogenic macrophage responses while inhibiting inflammatory macrophage cues (Mullican et al., 2011; Kobayashi et al., 2012). Moreover, myeloid HDAC3 deficiency improved collagen deposition and lipid handling in atherosclerotic plaques and induced a more stable plaque phenotype (Hoeksema et al., 2014). Inhibitors that preferentially inhibit HDAC3 also exert antiatherogenic effects *in vitro* (Van den Bossche et al., 2014) but HDAC3-selective drugs (especially macrophage-specific ones) that are applicable *in vivo* are currently not available.

Overall, we demonstrate that targeting HDACs in monocytes and macrophages with ESM technology inhibits both inflammation and monocyte to macrophage differentiation while only minimally affecting atherosclerosis endpoints. While this is an improvement in comparison to the previously applied non-targeted broad-spectrum inhibitor TSA, our study supports the need for drugs that selectively inhibit individual HDACs in target cells.

## DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

## ETHICS STATEMENT

All animal experiments were conducted at the University of Amsterdam and approved (permits: DBC242 and 103169) by the Committee for Animal Welfare of the Academic Medical Center, University of Amsterdam. All animal studies were ethically reviewed and carried out in accordance with European Directive 2010/63/EEC and the GSK Policy on the Care, Welfare and Treatment of Animals.

## AUTHOR CONTRIBUTIONS

Conceptualization, JB, RL-M, MW, WJ, PM and HL. Methodology, JB, RL-M, RF, SB, SV, MG, CR, AN. Formal Analysis, RL-M, JB, AN, RF, SB, SV, MG and CR. Writing – Original Draft, RL-M, JB. Writing – Review & Editing, RL-M, JB, RF, HL, PM, and MW. Visualization, RL-M, JB. Supervision, MW, PM and JB. Funding Acquisition, MW, WJ, RP and IR. All authors read and approved the final manuscript.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2019.01242/full#supplementary-material

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REVIEW
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# Immune and Smooth Muscle Cells Interactions in Atherosclerosis: How to Target a Breaking Bad Dialogue?

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Inflammation is a well-known pathophysiological factor of atherosclerosis but its therapeutic targeting has long been ignored. However, recent advances in the understanding of the immune mechanisms implicated in atherosclerosis have unveiled several therapeutic targets currently undergoing clinical trials. These studies have also shed light on a dialogue between the immune compartment and vascular smooth muscle cells (VSMCs) that plays a critical role in atherosclerotic disease initiation, progression, and stabilization. Our review focuses on the link between cellular and soluble immune effectors and VSMC behavior at different phases of the pathology. Furthermore, we discuss the potential targeting of these interactions to efficiently prevent cardiovascular diseases.

**Keywords: inflammation, atherosclerosis, smooth muscle cells, therapeutic targets, cardiovascular diseases**

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## MOLECULAR AND CELLULAR DETERMINANTS OF ATHEROSCLEROSIS

Cardiovascular diseases (CVDs) remain the leading cause of death worldwide and are in constant increase in western as well as low and middle-income countries [https://www.who.int/news-room/fact-sheets/detail/cardiovascular-diseases-(cvds)], underpinning the importance of finding novel therapeutic approaches.

The major cause of CVD is atherosclerosis. This pathology involves inflammatory and fibroproliferative mechanisms engaging communication between vascular cells [endothelial cells and vascular smooth muscle cells (VSMCs)] and immune cells. Atherosclerotic plaque progression leads to intimal thickening and culminates in late stages with plaque rupture which can cause myocardial infarctions and strokes.

The first initiating event of atherosclerosis involves shear stress perturbations responsible for endothelial cell dysfunction and inflammation leading to accumulation of low density lipoprotein (LDL) in the subendothelial space. Novel recent data has demonstrated that contrary to what was expected, this mechanism is not a passive movement of LDL across the endothelial barrier. Indeed, it involves the binding of LDL to the SRB-I receptor and subsequent transcytosis involving the guanine nucleotide exchange factor (DOCK4)/Rac pathway (Huang et al., 2019) and specific lipidic compartments (caveolae) found in great quantity in atheroprone regions. This mechanism is responsible for the initiation of the flow-dependent inflammatory priming of cells in atheroprone locations (Ramirez et al., 2019). LDL accumulation and oxidation in the medial area of arteries further amplifies inflammation by inducing chemokine secretion and expression of adhesion molecules at the surface of endothelial cells, such as intercellular adhesion molecule (ICAM)

and vascular cell adhesion molecule (VCAM). This leads to a subsequent modification of the VSMC phenotype and the recruitment of immune cells.

The major involvement of VSMCs in atherosclerosis was revealed decades ago after observation that these cells were the main cellular component of atherosclerotic lesions (Parker, 1960; Imai et al., 1966). Since this discovery, decades of research have modulated our understanding of VSMC function during atherosclerosis. Importantly, recent studies have demonstrated that atherosclerosis development requires a dialogue between VSMCs, endothelial cells, and immune cells. Indeed, neutrophils, monocytes, lymphocytes, and mast cells are recruited to atherosclerotic lesions and interact with vascular cells. These interactions are critical as VSMCs undergo a phenotypic switching, the outcome of which depends on the immune environment.

## VSMC PLASTICITY DURING ATHEROSCLEROSIS

In healthy conditions, VSMCs are mostly quiescent and differentiated, a phenotype called "contractile". In this state, VSMCs express several markers such as 22 kDa actin-binding protein (SM22α/*tagln)*, smooth muscle actin (αSMA) or smooth muscle cell myosin (SM-MHC/*myh11*). Furthermore, they ensure hemodynamic and structural regulation of the vessel wall (Lacolley et al., 2012; Bennett et al., 2016). In addition, VSMCs keep a high potential of dedifferentiation, in response to various external cues (growth factors, inflammation, matrix, lipoproteins, etc.) (Roostalu and Wong, 2018). Indeed, VSMCs are able to shift from a contractile phenotype to a so-called synthetic phenotype whereby cells are able to migrate, proliferate, and remodel the extracellular matrix. Phenotypic switching is characterized by (i) a progressive reduction or total loss of several VSMC lineage markers and (ii) increased proliferation capabilities associated with synthesis of extracellular matrix components and proteases (Ait-Oufella et al., 2006; Johnson et al., 2011; Newby, 2014). In the context of atherosclerosis, VSMC dedifferentiation can reach extreme phenotypes in which they are no longer identifiable as VSMCs. Dedifferentiated VSMCs can express macrophage markers such as CD11b, F4/80, or CD68 and acquire inflammatory cell properties by releasing pro-inflammatory cytokines or monocyte chemoattractants (Allahverdian et al., 2018). This VSMC-to-macrophage phenotypic switching implies genes such as KLF4 (Krüppel-like factor 4) (Alexander and Owens, 2012). Consistently, loss of KLF4 delays phenotypic switching or significantly reduces plaque size (Yoshida et al., 2008). This is associated with increased fibrous cap thickness which is indicative of higher plaque stability. Moreover, VSMC derived macrophage cells have the ability to accumulate lipids and become foam cells, a process which is also KLF4-dependent. A recent study has demonstrated that 60 to 70% of foam cells in mouse atherosclerotic lesions originate from VSMCs (Wang et al., 2019). These findings are consistent with those obtained in human atheromas and clearly indicate a major underestimated VSMC contribution to foam cell formation and atherosclerotic disease progression (Allahverdian et al., 2014). However, compared to classical monocytes, the phagocytic capabilities of VSMC-derived macrophages are reduced. Thus, these cells uptake less lipids as well as other materials such as dying cells or necrotic debris and participate by this way to the development of the necrotic core and intensify inflammation (Chaabane et al., 2014). Moreover, foam cell apoptosis leads to cholesterol deposition, ultimately forming the necrotic core. Amplification of the inflammatory process and growing of the necrotic core area is achieved by further VSMC and immune cell recruitment (Chaabane et al., 2014).

Hence, VSMC phenotypes conditioned by the immune environment (proliferation, apoptosis, matrix degradation, inflammation, or foam cell formation) are key determinants in the etiology of atherosclerosis leading either to a adingleleither to scl to sclerosis key determinants in the mainstability being responsible for plaque rupture and subsequent cardiovascular events.

## IMMUNE CELLS AND THEIR INTERACTION WITH VSMCs DURING ATHEROSCLEROSIS

The first evidence of the role of inflammation in atherosclerosis was suggested in the 1980's with the observation of inflammatory infiltrates in the coronaries of patients with unstable angina (Stratford et al., 1986; Wallsh et al., 1986) also observed in a rabbit experimental model fed a hypercholesterolemic diet (Schwartz et al., 1985). Over the years, numerous analyses have revealed the involvement of different immune cells in human atherosclerosis. The function of these immune cells has been clearly identified thanks to mouse models, such as LDLR$^{-/-}$ and apolipoprotein E (ApoE)$^{-/-}$ mice, that develop atherosclerosis (**Figure 1**).

### Monocytes/Macrophages

The real demonstration of the role of monocytes/macrophages was revealed by using M-CSF deficient mice crossed with ApoE$^{-/-}$ or LDLR$^{-/-}$(Qiao et al., 1997; Rajavashisth et al., 1998). These models presented a dramatic decrease in atherosclerosis development. Moreover, the recruitment of monocytes by their interaction with endothelial cells through adhesion molecules has been extensively described. VSMCs from healthy parts of the artery do not express adhesion molecules whereas ICAM-1, VCAM-1, and fractalkine (CXC3CL1) were found upregulated in VSMCs found in regions of atherosclerotic plaque (O'Brien et al., 1993; Endres et al., 1997; Braun et al., 1999; Barlic et al., 2007). These data suggest that synthetic VSMCs maintain monocytes/macrophages within the vessel through direct interaction between both cell types (Cai et al., 2004a; Cai et al., 2004b).

In fact, most of the communication between monocytes/macrophages and VSMCs has been shown to occur through immune mediators. Among them, interleukin 1β (IL-1β), tumor necrosis factor α (TNF-α), IL-6, and monocyte chemokine protein 1 (MCP-1) are highly secreted and play major roles in atherosclerosis, VSMC dysfunctions, and inflammation of the arterial wall (Bobryshev et al., 2016) (see **Table 1**). Nevertheless,

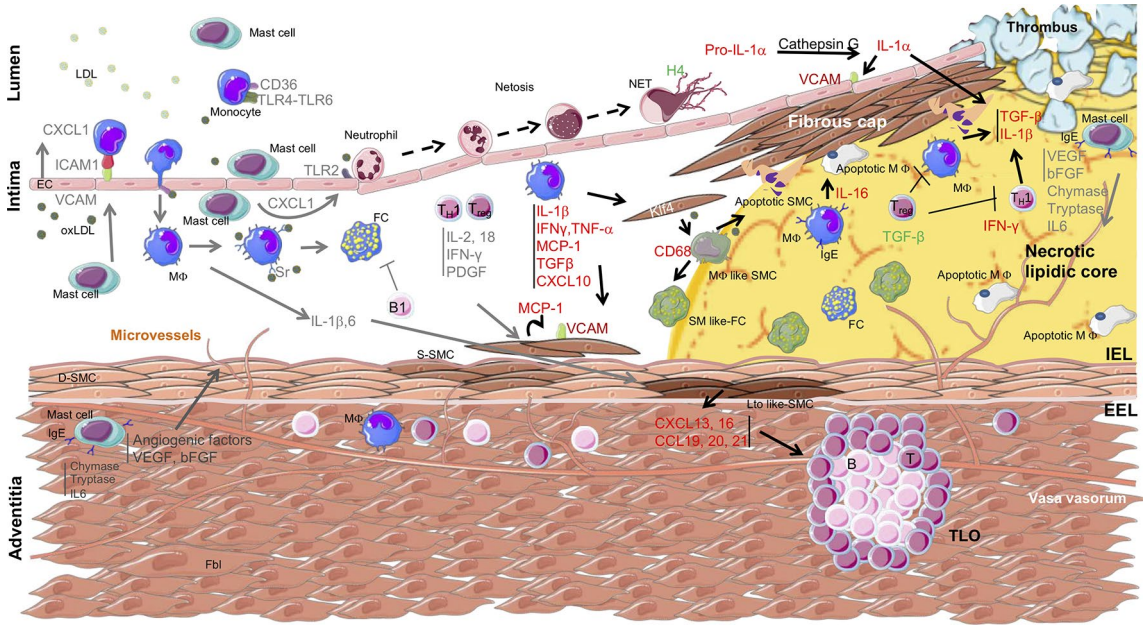


**FIGURE 1 |** Overview of immune cell localization, cytokine secretion, and interaction with smooth muscle cells within the arterial wall during atherosclerosis progression. Arrows indicate the origin and target of immune mediators between the different cell types involved in atherosclerosis. The yellow area indicates the necrolipidic core. D-SMC, differentiated smooth muscle cell; S-SMC, synthetic smooth muscle cell; Lto like-SMC, tissue organizer-like smooth muscle cell; SM like-FC, smooth muscle like-foam cell; MΦ like SMC, macrophage like smooth muscle cell; apoptotic SMC, apoptotic smooth muscle cell; EC, epithelial cell; Fbl, fibroblast; TLO, tertiary lymphoid organ; IEL, internal elastic lamina; EEL, external elastic lamina; TC, lymphocyte T cell; BC, lymphocyte B cell; MΦ, macrophage; FC, foam cell; apoptotic MΦ, apoptotic macrophage; LDL, low-density lipoprotein; oxLDL, oxidized low-density lipoprotein; NETs, neutrophils extracellular traps; TLR, Toll-like receptor; CD, cluster of differentiation; KLF4, Kruppel-like factor 4; IgE, immunoglobulin type E; H4, histone H4; ICAM, intercellular adhesion molecule; VCAM, vascular cell adhesion molecule; IFN, interferon; PDGF, platelet-derived growth factor; MCP1, monocyte chemokine protein 1; TGF, transforming growth factor; CXCL, C-X-C motif chemokine; CCL, chemokine Ligands.

in addition to their immune functions, these secreted molecules could also act on VSMC proliferation and survival. Therefore, targeting these molecules could have some deleterious effects on plaque stabilization. Blocking MCP-1, a chemokine also involved in VSMC proliferation and migration (Fougerat et al., 2012), has been demonstrated to be efficient to limit atherosclerosis progression and to increase plaque stability in the established atherosclerosis ApoE$^{-/-}$ mouse model (Ni et al., 2001; Inoue et al.,

**TABLE 1 |** List of major immune mediators impacting VSMC behavior during atherosclerosis.

| Immune mediator | Cellular source | Major SMC behavior | References |
|---|---|---|---|
| IFN-γ | TH1 | ↗Proliferation<br>CXCL10 secretion | Lupieri et al., 2019 |
| IgE | B cells | SMC apoptosis | Wang et al., 2011 |
| MCP-1 | Monocytes/macrophages,<br>PDGF stim VSMC | ↗Proliferation<br>↗Migration<br>↗Collagen | Ni et al., 2001; Inoue et al., 2002; Fougerat et al., 2012 |
| TGF-β | Macrophage, Treg | ↗Collagen | Mallat et al., 2001b; Lutgens et al., 2002; Robertson et al., 2003 |
| IL-18 | TH1 | ↘VSMC accumulation *in vivo* | Mallat et al., 2001a; Elhage et al., 2003 |
| IL-6 | Monocytes/macrophages | ↗Proliferation | Bobryshev et al., 2016 |
| TNFα | Monocytes/macrophages | ↗Proliferation | Bobryshev et al., 2016 |
| IL-1α | Neutrophils | ↗Adhesion molecules expression | Folco et al., 2018. |
| Fractalkine | VSMC located in lesions | Interaction with monocytes | O'Brien et al., 1993; Barlic et al., 2007 |
| IL-1β | Monocytes/macrophages | ↗VSMC *in vivo*<br>↗collagen content *in vivo* | Gomez et al., 2018 |
| NET, histone H4 | Neutrophils | VSMC lysis<br>Plaque destabilization | Folco et al., 2018; Silvestre-Roig et al., 2019 |

*IFN-γ, interferon γ; IgE, immunoglobulin type E; TGF-β, transforming growth factor β; IL, interleukin; MCP-1, monocyte chemokine protein 1; NETs, neutrophils extracellular traps; H4, histone H4; VSMC, vascular smooth muscle cell; PDGF, platelet-derived growth factor; CXCL, C-X-C motif chemokine.*

2002). However, blocking IL-1β seems to interfere with smooth muscle viability modifying plaque composition (Gomez et al, 2018). Indeed, recent work done by Gomez et al. reported that IL1-ß produced by VSMCs in response to TNFα contribute to fibrous cap formation in advanced atherosclerotic lesions in ApoE-/- mice, hence stabilizing the atherosclerotic plaque. Importantly, while blocking IL-1β had no effect on lesion size, it completely inhibited beneficial outward remodeling (Warner and Libby, 1989; Gomez et al., 2018).

In a similar manner, proteinases, secreted by macrophages, have been shown to have opposing roles in vascular remodeling and plaque rupture in advanced atherosclerosis (Newby, 2014). Indeed, it is clearly established that activation of metalloproteases is a prerequisite to induce matrix degradation, a process necessary to facilitate proliferation and migration of VSMC involved in vascular remodeling (Newby, 2005; Gerthoffer, 2007). Nevertheless, secretion of proteinases by macrophages during late atherosclerosis leads to destruction of extracellular matrix, associated in this case with plaque instability and plaque rupture (for review, see Newby, 2014). Thus, a better understanding of the basis for these opposing roles and a better characterization of the specific role of different proteinases needs to be done to propose effective therapies against myocardial infarction.

## Neutrophils

Adhesion molecules on endothelial cells also participate in the recruitment of neutrophils (Schmidt et al., 2016). Activation of Toll-like receptor 2 (TLR2) through binding of modified LDL has been proposed to play a role in endothelial cell-dependent inflammatory processes (Franck et al., 2017). TLR2 expression at the surface of endothelial cells has been correlated to neutrophil adherence in regions of local flow disturbance leading to superficial erosion (Franck et al., 2018). This supports a role for neutrophils in erosion-associated thrombosis. The activation of neutrophils promotes endothelial cell apoptosis and desquamation, favoring platelet recruitment, thrombin generation, and thrombus formation (Quillard et al., 2017). Moreover, neutrophil activation initiates a specific type of programmed cell death called NETosis which leads to the release of neutrophil extracellular traps (NETs). These are constituted of macromolecular aggregates containing DNA, histones, and granular enzymes. NETs could be responsible for thrombotic complications in mouse intimal lesions, recapitulating features of superficial erosion in humans (Franck et al., 2018). Moreover, exposure of human endothelial cells to NETs increases the expression of adhesion molecules such as ICAM and VCAM-1 as well as tissue factor (TF). Cathepsin G, a serine protease abundant in NETs, has been shown to cleave the pro–IL-1α precursor leading to the release of the more potent mature form of IL-1α responsible for ICAM-1, VCAM-1, and TF expression (Folco et al., 2018). In a model of advanced atherosclerosis with features of instability obtained by inducing a shear stress modifier around the carotid in high fat diet fed ApoE$^{-/-}$ mice, the number of neutrophils was inversely correlated to the number of VSMCs and positively correlated with necrotic core area lesion size and plaque instability (Silvestre-Roig et al., 2019). In this work, a direct interaction between neutrophils and VSMCs has been described (Silvestre-Roig et al., 2019). The authors demonstrate that VSMCs found in the fibrous cap attract neutrophils, triggering the ejection of NET-like histone H4 which is responsible for VSMC lysis, ultimately leading to atheroma plaque destabilization. One question remains as to how neutrophils interact with VSMCs in the fibrous cap. One possibility proposed by (Silvestre-Roig et al., 2019) is that the release of cytotoxic NETs also induces endothelial cell death to favor the rapid infiltration of neutrophils within the fibrous cap.

## Mast Cells

Mast cells could also be involved in the recruitment of neutrophils and other leucocytes to the plaque by stimulating the upregulation of adhesion molecule expression in the endothelium and by secreting CXCL-1 (IL8) (Zhang et al., 2011; Wezel et al., 2015). These cells, first described in atherosclerotic plaques in 1954 (Cairns and Constantinides, 1954), play an important role in atherosclerosis development at different steps of the pathology. Indeed, they are able to take part in foam cell formation by increasing macrophage LDL uptake through granule secretion participating to the initiation of atherosclerosis (Kovanen, 1991). These cells, classically activated by immunoglobulin type E (IgE) through the FcεR1 receptor (i) secrete histamine which increases endothelial cell permeability (Kovanen and Bot, 2017) and (ii) produce a wide range of inflammatory cytokines involved in atherosclerosis initiation and progression (Sun et al., 2007). They are also able to secrete vascular endothelial growth factor and basic fibroblast growth factor, which participate to the formation of neo-vessels associated with plaque hemorrhage in complicated atherosclerosis (Kaartinen et al., 1996; Lappalainen et al., 2004). Mast cell number is significantly elevated in the shoulder region of coronary plaques susceptible to plaque rupture and thrombosis (Kaartinen et al., 1994), suggesting a role of activated mast cells in VSMC apoptosis. Consistent with this observation, it has been demonstrated that mast cell TLR4 activation is responsible for VSMC apoptosis through chymase and IL-6 release (den Dekker et al., 2012). Interestingly, it has also been demonstrated that the FcεR1 receptor is also expressed at the surface of VSMCs under inflammatory conditions. Its activation by IgE leads to cytokine secretion and SMC apoptosis (Wang et al., 2011) indicating that IgE could locally induce atherosclerotic plaque destabilization by a direct action on VSMCs or an indirect effect through mast cell activation.

## Lymphocytes

In addition to monocytes, neutrophils, and mast cells, lymphocytes are highly involved in atherosclerosis regulation. The adaptive immune system invades the atherosclerotic vascular wall from both the arterial lumen side and the adventitial side, playing an important role in immune-vascular cell dialogue during atherogenesis.

## T Cells

T cells have been found in human atherosclerotic plaques from the initiation phase to plaque rupture where they are

found in a higher proportion (Otsuka et al., 2015). While the role of $CD4^+$ T cells has been extensively studied, the role of $CD8^+$ T cells is still unclear. Indeed, the genetic association of CVDs with $CD8^+$ T cells has been described (Davies et al., 2012), however experimental studies in mice have reported contradictory results regarding $CD8^+$ T cell functions (Zhou et al., 1996; Kyaw et al., 2013; van Duijn et al., 2019). Indeed, Kyaw et al. showed that $CD8^+$ T cell depletion using a CD8α or CD8β monoclonal antibody ameliorated atherosclerosis in $ApoE^{-/-}$ deficient mice fed a high-fat diet by reducing lipid and macrophage accumulation, apoptosis, necrotic cores, and inflammatory cytokines such as MCP-1, IL-1β, and interferon γ (IFN-γ). Conversely, a recent study performed by Van Duijn J et *al*. showed that in a mouse model of advanced atherosclerosis (high fat diet-fed $LDLR^{-/-}$ mice), depletion of CD8 increased the Th1 $CD4^+$ T cell fraction in lesions, resulting in increased inflammation and lesion destabilization (van Duijn et al., 2019). This discrepancy could be explained by the difference in the experimental protocol. Indeed, Kyaw et al. depleted CD8+ in 8 week old $ApoE^{-/-}$ mice and then submitted the mice to a high fat diet. However, in their protocol Van Duijn J et al, used $LDLR^{-/-}$ mice fed a high fat diet for 16 weeks and injected depleting antibodies during the last 6 weeks before sacrifice and atherosclerosis analysis (Kyaw et al., 2013; van Duijn et al., 2019). Therefore, further experiments need to be done to clearly define the role of $CD8^+$ T cells during atherogenesis.

The role of $CD4^+$ T cells depends on the subset concerned. It is now well established that Th1 cells secrete pro-inflammatory molecules such as IFN-γ which drives inflammation of the arterial wall (Frostegard et al., 1999; Smirnova et al., 2014), whereas Treg subpopulations dampen the immune system and decrease atherosclerosis development (Ait-Oufella et al., 2006). Consistently, Th1 cytokines are predominantly found in advanced human atherosclerotic plaque cells (Frostegard et al., 1999). Among the cytokines secreted by Th1 cells, IL-18 appears to negatively regulate VSMC accumulation within atheroma lesions in $ApoE^{-/-}$ mice, supporting the notion that IL-18 is an important mediator of Th1-induced atherosclerosis (Mallat et al., 2001; Elhage et al., 2003). IFN-γ, the major Th1 cytokine also plays an important role in VSMC dysfunction. Indeed, IFN-γ positively contributes to VSMC proliferation in $ApoE^{-/-}$ and $LDLR^{-/-}$ mouse models (Gupta et al., 1997; Buono et al., 2003; Leon and Zuckerman, 2005). IFN-γ also contributes to plaque vulnerability by increasing matrix degradation through protease induction or by decreasing matrix synthesis by VSMCs. For example, IFN-γ increases the secretion of cathepsin S in human VSMCs which results in elastin degradation (Sukhova et al., 1998). IFN-γ could also favor the inflammatory response in VSMCs by increasing the expression of FcεR1, hence increasing VSMC ability to respond to IgE (Wang et al., 2011). Finally, IFN-γ directly acts on smooth muscle cells to induce the production of IFN-γ–inducible protein 10 (IP-10 or CXCL10), an important chemokine involved in atherogenesis and plaque destabilization (Heller et al., 2006; Segers et al., 2011). Interestingly, we recently demonstrated that this chemokine also acts directly on the endothelium to delay efficient healing after arterial injury (Lupieri et al., 2019). These data demonstrate a central role for VSMCs in relaying the T-cell response within the artery.

## B Cells

Studies to decipher the role of B cells in atherosclerosis carried out in the past years, controversies remain. These could be explained in part by the opposite functions of subsets of B cells (B1 and B2 cells) identified in human arteries (Tsiantoulas et al., 2015). B1 cells derived from fetal hematopoietic stem cells produce natural IgM independently of Th signals, and play a protective role in atherosclerosis (Lewis et al., 2009; Kyaw et al., 2011). Indeed, it has been proposed that IgM able to recognize oxidized phospholipids such as 1-palmitoyl-2-(5-oxovaleroyl)-sn-glycero-3-phosphorylcholine were produced during atherosclerosis. These antibodies impaired uptake of OxLDL by macrophages and recognized similar oxidation-specific epitopes on apoptotic cells in atherosclerotic lesions (Shaw et al, 2000). On the contrary, B2 cells produce immunoglobulins in response to Th signals which exert a pro-atherogenic action (Kyaw et al., 2010; Kyaw et al., 2012; Sage et al., 2012). Although atherosclerosis-promoting antibodies affect plaque composition and stability (Centa et al., 2019), an interaction between B cells and smooth muscle cells has not yet been reported.

## Tertiary Lymphoid Organs

The accumulation of lymphocytes can also be found in adventitial tertiary lymphoid organs (TLOs) which have been described in mouse and human atherosclerotic plaques. Cellular infiltration of the adventitia in human atherosclerotic arteries was first described by Schwartz et al. in 1962 (Schwartz and Mitchell, 1962). The comparative quantification of cellular infiltration in patients who suffered a myocardial infarction and in patients who died of non-cardiac causes demonstrated a possible correlation between adventitial immune cell infiltration and unstable coronary diseases (Kohchi et al., 1985). Cell aggregate organization in TLOs have been shown to develop in the abdominal aorta lamina adventitia of old $ApoE^{-/-}$ mice, in association with atherosclerotic lesions. The evolution of TLOs has been classified into three different stages and varies from a structure predominantly composed of T cell aggregates (stage I) to a structure with separate T and B cell areas with ectopic germinal centers (stage III) (Grabner et al., 2009; Akhavanpoor et al., 2018). VSMCs are thought to play the role of non-hematopoietic stromal lymphoid tissue organizer-like cells (LTo), required for TLO formation (Lotzer et al., 2010). VSMCs switch to their LTo-like phenotype through the activation of their lymphotoxin β-receptor (LTβR) by a lymphocyte tissue inducer (LTi), like macrophages or immune cells of the intima plaque. LTo-like VSMCs secrete lymphorganogenic chemokines like CXCL13 or CCL21, attracting macrophages, dendritic cells, T cells, B cells leading to TLO organization in the adventitia (Moos et al., 2005; Hu et al., 2015; Srikakulapu et al., 2016). It seems that VSMCs may also contribute to the formation of TLOs *via* an LTβR-independent pathway. In this case, bone-marrow derived-macrophages play the role of LTi to trigger the production of CCL19, CCL20, and CXCL16 by VSMCs, promoting immune cell aggregation in the adventitia (Guedj et al., 2014).

Altogether, these data illustrate that the dialogue between immune cells and VSMCs (summarized in **Table 1** and **Figure 1**) must be taken into consideration to develop effective therapeutic approaches for treating atherosclerosis.

## MOLECULAR CLUES FOR FUTURE THERAPIES

Current therapeutic strategies for atherosclerosis work by lowering cholesterol levels (statins, PCSK9 antibodies), reducing platelet functions, and controlling arterial tone (Zhao and Mallat, 2019). Nevertheless, atherosclerosis development is linked to important inflammatory processes of the arterial wall. Thus, targeting the immune compartment might be useful to fight CVDs and several clinical trials aiming at targeting immune processes have been done. However, to date, these trials were unsuccessful. Hypotheses to explain these adverse outcomes are multiple, including redundant inflammatory pathways or lack of functional data regarding the targeted pathways [reviewed in (Zhao and Mallat, 2019)]. Another possibility is that VSMC status can vary from one plaque to another. Thus, depending on their status, VSMCs might respond differently to a given therapeutic compound. Future therapeutic approaches will have to consider VSMC plasticity to improve their overall efficiency. Here, we will focus on the latest targets identified in clinical and pre-clinical studies that could impact VSMC behavior during atherosclerosis.

### Targeting IL-1β

The implication of the IL-1 pathway in atherosclerosis and VSMC proliferation and activation by inflammation has been extensively described. Numerous *in vivo* studies have demonstrated that inhibition of the NLRP3/IL-1β module decreases plaque development and deepens inflammation (Baldrighi et al., 2017). Altogether, these findings have opened the way to clinical trials targeting this pathway. Anti-IL-1β strategies have been studied in a phase III clinical study called CANTOS (Ridker et al., 2017). This study demonstrated that targeting IL-1β improves cardiovascular outcomes in patients with stable atherosclerosis. Nevertheless, this strategy failed to prevent cardiovascular events in high grade inflammatory patients and increased the number of fatal infections. This could be linked to the fact that the impact of IL-1β inhibition is still unclear. Recent *in vivo* evidence in ApoE$^{-/-}$ mice indicates that IL-1β has atheroprotective functions. Indeed, Gomez et al. have clearly demonstrated that IL-1 signaling is required within VSMCs to prevent their apoptosis, retaining them in the fibrous cap in late stage atherosclerosis (Gomez et al., 2018). Thus, this therapeutic approach might indeed be deleterious and sheds light on VSMC plasticity in the different phases of atherosclerosis.

### Targeting Histone H4

In advanced atherosclerotic lesions, VSMC apoptosis is a hallmark of plaque rupture. One mechanism of VSMC death has been recently elucidated. Indeed, Silvestre-Roig et al. have reported that VSMCs are targeted by histone H4 containing NETs produced by infiltrated bone marrow derived neutrophils into the atheroma (Silvestre-Roig et al., 2019). Histone H4 molecules present at the NET surfaces interact with VSMC plasma membranes through electrostatic interactions and form pores inducing rapid cell death. Due to the importance of VSMC death in plaque stability, the authors developed a therapeutic strategy to prevent this histone H4-mediated effect. Using molecular dynamic simulation, they designed small peptides that disturb histone H4-membrane interactions. This analysis demonstrated that the N-terminal part of histone H4 is critical for membrane interactions. *In vitro*, the histone inhibitory peptide prevented histone H4 from interacting with VMSCs and protected VMSCs from cell death. *In vivo*, administration of this peptide using an osmotic mini-pump to mice carrying pre-existing atherosclerotic lesions (ApoE$^{-/-}$ fed a high fat diet) increased VSMC number and consequently improved plaque stability. Thus, inhibition of histone H4 interactions with membranes could represent a potential therapeutic strategy for the prevention of advanced plaque rupture.

### Targeting CXCL10

C-X-C motif ligand 10 (CXCL10), or IP-10, is a small chemokine belonging to the CXC chemokine family (Luster and Ravetch, 1987). This chemokine mediates several biological functions in different cell types and tissues through binding to its receptor CXCR3. Of note, CXCL10 is responsible for monocyte and lymphocyte chemo-attraction to inflammatory sites. During atherosclerosis progression, endothelial cells, macrophages, and VSMCs express CXCL10 (van den Borne et al., 2014). Consistently, the ApoE$^{-/-}$ mouse model in which CXCL10 or its receptor were invalidated displayed reduced atherosclerosis development (Veillard et al., 2005; Heller et al., 2006). This was also the case using a pharmacological inhibitor of CXCR3 (NBI-74330) in the LDLR$^{-/-}$ mouse model (van Wanrooij et al., 2008). Altogether, these data place CXCL10 as an attractive target for atherosclerosis therapies (van den Borne et al., 2014). Interestingly, several monoclonal antibodies have been tested in phase II clinical trials for auto-immune diseases such as rheumatoid arthritis or ulcerative colitis. However, these antibodies demonstrated limited anti-inflammatory activity despite the major role of CXCL10 in inflammation (Yellin et al., 2012; Mayer et al., 2014). This divergence between clinical and experimental observations can be explained by several factors such as the differences in CXCR3 isoform expression between mice and humans. Indeed, humans express 3 isoforms, CXCR3-A, CXCR3-B, and CXCR3-alt (Lasagni et al., 2003; Ehlert et al., 2004) whereas mice only express one, CXCR3 closely related to CXCR3-A (Lu et al., 1999). Redundancy in the chemokine system could also explain these negative clinical results (Solari et al., 2015). Another factor of ineffective targeting is the amount of chemokines available for the antibodies. Indeed, chemokines such as CXCL10 are sequestered on glycosaminoglycans (GAGs). This interaction with GAGs prevents chemokine diffusion from the production site to the circulation, potentiating their local action. This observation suggests that higher doses of antibodies might be required to adequately inhibit

free as well as GAG-trapped chemokines (Solari et al., 2015). However, in the diabetes RIP-LCMV glycoprotein mouse model, it has been shown that specific targeting of GAG-trapped CXCL10 was less effective in reversing hyperglycemia than antibodies directed against the free chemokine (Bonvin et al., 2017). Thus, further work is required to understand the molecular mechanism underlying CXCL10 functions in atherosclerosis, especially the implication of GAG-trapped versus free CXCL10. Interestingly, we recently demonstrated that after arterial mechanical injury, CXCL10 produced by VSMC in response to T-lymphocyte secreted IFN-γ directly inhibits endothelial healing. Thus, IFN-γ/CXCL10 axis may provide novel strategies to promote endothelial healing and prevent further atherosclerosis complications after therapeutic interventions (Lupieri et al., 2019).

### Targeting the PI3Kγ Signaling Pathway

In contrast to the other PI3K family members which are ubiquitously expressed, PI3Kγ presents a selective expression profile restricted to the hematopoietic and cardiovascular systems. Historically, PI3Kγ was first described for its role in leucocyte functions (Hirsch et al., 2000; Hawkins and Stephens, 2015). Later, we demonstrated that PI3Kγ drives immune-inflammatory processes within the arterial wall leading to atherosclerosis and restenosis (Fougerat et al., 2008; Fougerat et al., 2012; Smirnova et al., 2014). Moreover, PI3Kγ plays a role in VSMCs by being implicated in their migration (Fougerat et al., 2012). Finally, this kinase participates in the VSMC-immune dialogue by acting as a relay between T lymphocytes and endothelial cells during post-injury arterial healing (Lupieri et al., 2019). Thus, PI3Kγ represents an attractive therapeutic target and its inhibition could have a double beneficial impact on atherosclerosis by preventing VSMC phenotypic switching and accelerating endothelial healing. Interestingly, PI3Kγ inhibition is currently being tested in a clinical trial for solid cancers with the assumption that its immunomodulation functions in macrophages could exacerbate anti-tumoral immunity (De Henau et al., 2016; Kaneda et al., 2016). Depending on the upcoming results, such a therapeutic strategy could be considered in the context of CVDs.

## CONCLUDING REMARKS

Since the discovery of VSMC involvement in atherosclerosis, our understanding of how VSMCs contribute to this disease has evolved dramatically. Pioneering studies assumed that VSMCs exert a protective effect against plaque rupture by forming the fibrous cap. In the past decade, this role has been re-evaluated as these cells present high plasticity and contribute to different plaque phenotypes. In this context, communication between immune cells and VSMCs has been shown to play a crucial role in the modulation of VSMC behavior, promoting either plaque stability, progression, or rupture in response to specific stimuli. This aspect of VSMC biology should be investigated more deeply to be able to propose novel therapeutic avenues to fight against CVD.

## AUTHOR CONTRIBUTIONS

DR, SG, AN-S, and ML originally conceived and wrote the manuscript. M-KS and NA designed figures and figure legends. All authors read and approved the final manuscript.

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