CXCL4-Induced Macrophages: A Novel Therapeutic Target in Human Atherosclerosis?

Christian A. Gleissner and Christian Erbel
University of Heidelberg
Germany

1. Introduction

Atherosclerosis and its consequences (i.e. myocardial infarction and cardiac death) remain the major cause of morbidity and mortality in Western countries (Roger et al. 2011). Despite clinical advances that have substantially improved outcomes in patients suffering from coronary artery disease, including pharmacological interventions (e.g. novel anti platelet therapies, statins, etc.) as well as interventional and surgical therapies (e.g. drug-eluting stents), there is still a huge demand for improved diagnostic tools to identify patients at risk for adverse events as well as therapeutic means to prevent adverse events in these patients. Biomarkers such as high sensitivity CRP (Ridker 2007) or high sensitivity troponin T (Kurz et al. 2011) have brought some improvement in identifying patients requiring more intense treatment; however, the clinical need for better tools remains.

An important concept that may help to improve clinical care for patients with coronary artery disease is the inducement of plaque stability. Atherosclerotic lesions can show features of plaque stability or plaque instability (Naghavi et al. 2003a, Naghavi et al. 2003b). Stable plaques are characterized by a thick fibrous cap and a small necrotic core. By contrast, unstable plaques display a thin fibrous cap and a large necrotic core consisting of apoptotic macrophages, foam cells, and smooth muscle cells. Unstable plaques are more likely to rupture, and plaque rupture may subsequently result in thrombosis and occlusion of the vessel leading to a myocardial infarction or stroke.

A promising approach to identify potential markers of plaque instability may be the study of atherogenesis on a cellular and molecular level. During the development of atherosclerotic lesions, blood monocytes adhere to the activated endothelium, transmigrate into the subendothelial space, and differentiate towards macrophages, dendritic cells, or foam cells (Galkina & Ley 2009). Among the various leukocyte types involved in atherogenesis, monocytes and monocyte-derived macrophages represent the major fraction. The monocyte-macrophage differentiation process is affected by the extracellular matrix as well as by the combination of chemokines and cytokines representing the micromilieu of the plaque (Shashkin et al. 2005). In addition, cell-cell interactions may also affect the fate of monocytes within the atherosclerotic plaque. Monocyte-derived cells secrete chemokines, cytokines, and other mediators, leading to attraction of other immune cells and thereby promoting plaque progression and plaque instability (Shashkin et al. 2005). While it was initially thought that monocyte-derived macrophages represent a homogenous population

of cells, substantial heterogeneity of human plaque macrophages has been recognized for almost two decades (Poston & Hussain 1993). However, only recently has the pathophysiological importance of macrophage heterogeneity aroused further scientific interest (Bouhlel et al. 2007, Boyle et al. 2009, Gleissner et al. 2010a, Waldo et al. 2008).

Platelets and platelet-derived mediators represent important players potentially promoting macrophage heterogeneity in atherosclerosis. It has been long known that platelets play an important role in inflammatory processes reaching far beyond their function in hemostasis (Ross 1999). Especially in the context of atherosclerosis, platelets and platelet-derived molecules are heavily involved. Thus, platelet-derived chemokines may interact with the endothelium as well as with various leukocyte types (Gleissner et al. 2008). Accordingly, activated platelets promote atherogenesis as shown in *Apoe-/-* mice (Huo et al. 2003). This effect may in part be chemokine-mediated by promotion of monocyte adherence to the endothelium (von Hundelshausen et al. 2001, von Hundelshausen et al. 2005), but it may also be a consequence of chemokine-induced monocyte-macrophage differentiation (Gleissner et al. 2010a, Gleissner et al. 2010b, Scheuerer et al. 2000).

The current review will discuss novel insights into the effects of CXCL4, one of the most abundant platelet chemokines, on macrophage differentiation in the context of atherogenesis. It attempts to summarize what is known about CXCL4-induced macrophages, put it into context with knowledge of macrophage heterogeneity in human atherosclerosis, and try to answer the question of whether specifically targeting these CXCL4-induced macrophages may be a promising approach to prevent or treat atherosclerosis in humans.

2. Macrophages and atherogenesis

2.1 Studying macrophages in human and murine models

Studying myeloid cells and especially monocyte-derived macrophages in humans is a difficult task. While monocytes can easily be isolated from human peripheral blood and differentiated towards macrophages in order to perform *in vitro* studies, the possibility of studying human macrophages within atherosclerotic lesions is restricted to the analysis of *post mortem* tissues or explanted tissues, e.g. of carotid endatherectomy specimens or explanted hearts after heart allograft transplantation. Even though these materials will allow studying macrophages within their pathophysiological environments, the systems may still be biased by post mortem processes or effects due to prolonged ischemic time resulting in changes that do not allow correctly assessing the *in vivo* situation.

On the other hand, using mouse models to study monocytes and monocyte-derived macrophages offers great potential by using differently-labeled or genetically modified monocyte subsets (e.g. using the CXC3CR1 FITC mice) that allow the generation impressive *in vivo* tracking models that have helped to identify differential roles of specific monocyte subsets (Auffray et al. 2007, Geissmann et al. 2003). However, these systems have one notable problem: it is extremely difficult to transfer the information gained from these mouse models to the human system. Thus, some of the markers used to identify murine monocyte subsets (e.g. Gr-1. Ly6C, etc.) do not exist in the human system or do not have a human marker that clearly corresponds to them. Furthermore, in many aspects, murine monocyte-derived macrophages behave differently from human macrophages. Thus, treating murine macrophages with lipopolysaccharide (LPS) leads to increased uptake of modified LDL (Fitzgerald et al. 2000). Conversely, treating human macrophages with LPS

prevents uptake of modified LDL (own unpublished observation). Considering that uptake of modified LDL represents a crucial step during macrophage foam cell formation with great impact on atherogenesis, it becomes questionable as to how relevant results from murine experiments may be for the human situation.

A recent extremely valuable study has compared the transcriptomes of human and murine monocyte subsets (Ingersoll et al. 2010). In this study, the authors used CCR2 and CXC3CR1 to identify the two murine monocyte subsets, whereas CD14 and CD16 were used to differentiate between the human monocyte subsets. About 270 genes in humans and 550 genes in mice were differentially-expressed between the monocyte subsets. Interestingly, only about 130 were conserved between species and a substantial number of genes were found to be differentially-expressed in a cross-species comparison. Among those, the authors identified genes extremely important in atherogenesis, including the scavenger receptors CD36 or macrophage scavenger receptor-A, as well as other functionally important molecules such as receptors for apoptotic cells. Again, this supports the notion that there are considerable differences between the human and murine system, and that results from murine experiments should be taken with care when drawing conclusions for human atherosclerosis.

Based on these considerations, the current article will specifically focus on data derived from studies on human macrophages wherever possible. Murine data will only be referred to when no data on human cells are available.

2.2 Macrophage heterogeneity
2.2.1 General concept of macrophage plasticity and polarization

A central feature of macrophages is their plasticity. *Macrophage plasticity* is defined as the capacity of the cell to readily change its phenotypic and functional capacities depending on the external conditions. Thus, the repertoire of surface receptors, the ability to generate reactive oxygen species, and the potential to migrate towards specific chemoattractants can differ substantially depending on the external conditions. By contrast, the term *macrophage polarization* is used for defined states of macrophage plasticity, which can be induced by specific conditions (e.g. combination of cytokines) and is defined by the presence of specific surface markers or functional capacities.

In vivo, the conditions inducing or preventing macrophage plasticity and polarization are probably determined primarily by the micromilieu. In fact, the presence of differentially-polarized macrophages has been postulated early on (Poston & Hussain 1993) and has been confirmed within human atherosclerotic lesions by various groups including ourselves (Bouhlel et al. 2007, Boyle et al. 2009, Gleissner et al. 2010a, Waldo et al. 2008). These findings make clear that macrophages within one atherosclerotic plaque can not be considered to be a uniform cell population but may have different functions depending on their specific polarization type.

2.2.2 Accepted models of macrophage polarization

There is currently a well defined scheme of macrophage polarization in vitro and in vivo. The first to describe an "alternative" macrophage activation were Gordon et al., who demonstrated that upon stimulation with IL-4, macrophages express high levels of mannose receptor CD206 (Stein et al. 1992). Based on this finding, the paradigm of "classically" activated M1 macrophages and "alternatively" activated M2 macrophages became accepted (Fig.1).
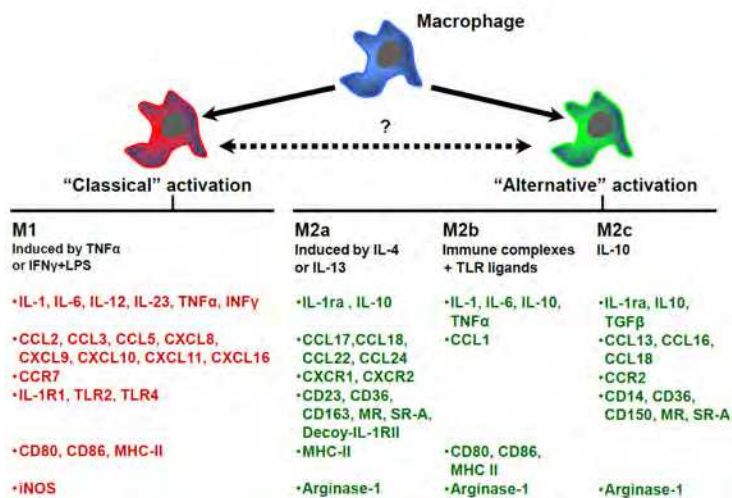
Fig. 1. The established paradigm of macrophage polarization. While M1 macrophages are thought to be involved in type I inflammation (bacterial killing or tumor resistance), M2 macrophages are associated with type II inflammation (allergy, parasital killing). For each polarization type, typical cytokines, chemokines, chemokine receptors, other receptors, surface molecules involved in antigen presentation, and intracellular enzymes are indicated.

According to this paradigm, M1 macrophages can be induced by a combination of interferon-γ and LPS or by tumor necrosis factor-α and are characterized by expression of pro-inflammatory cytokines like interleukin-1, interleukin-6, tumor necrosis factor-α, or interferon-γ. Furthermore, M1 macrophages express chemokines like CLL2 or CCL5, surface receptors like TLR2 or TLR4, and enzymes like iNOS. It is thought that M1 macrophage polarization mirrors the Th1 response of T cells, i.e. they represent a rather pro-inflammatory type of macrophage.

By contrast, M2 macrophages can be induced by Th2 cytokines like IL-4. They can express Th2 cytokines like IL-10 and are specifically characterized by the expression of surface receptors like CD163, CD206 (mannose receptor), or scavenger receptor-A and CD36. Since the first description of alternative macrophage activation has been published, several additional types of M2 macrophages have been identified, now defined as M2a (induced by IL-4 or Il-13), M2b (induced by immune complexes and TLR ligands), and M2c (induced by Il-10). Overall, M2 macrophages reflect the Th2 response and can be considered rather anti-inflammatory.

M1 and M2 macrophage polarization have been extensively studied including a comprehensive transcriptomic analysis of both macrophage polarization types (Martinez et al. 2006). In this gene array analysis, Martinez et al. confirmed the basic M1-M2 paradigm. Interestingly, monocyte-macrophage differentiation induced by MCSF alone leads to expression of many M2 genes, suggesting that basal conditions favour a default shift toward M2. Beyond this novel finding, the authors identified specific gene clusters involved in lipid metabolism, specific clusters of G protein-coupled receptors, and specific chemokines clusters in both M1 and M2 macrophages (Martinez et al. 2006). Both M1 and M2 macrophage polarization have been excellently reviewed by Gordon et al. as well as by Mantovani (Gordon 2003, Mantovani et al. 2009).

3. Platelet factor-4 (CXCL4), macrophages, and atherogenesis

3.1 CXCL4 – A platelet-derived chemokine in atherogenesis

Platelets and platelet-derived chemokines are important factors during atherogenesis (Gleissner et al. 2008, von Hundelshausen et al. 2007, Weber 2005, Weber 2008, Zernecke et al. 2008). In 2003, Ley et al. were able to demonstrate that activated platelets are able to promote atherogenesis (Huo et al. 2003). Upon activation, platelets release various chemokines from their alpha granules. Furthermore, activated platelets present or deposit chemokines and thereby induce recruitment of other cells to the arterial wall.

Among the plethora of chemokines released from activated platelets, CXCL4 (formerly known as platelet factor-4) is one of the most abundant. Accordingly, CXCL4 is released in human blood in micromolar concentrations upon platelet activation (Brandt et al. 2000). CXCL4 has been considered an "enigmatic" chemokine as it lacks the ELR domain typically seen in CXCL chemokines (Gear & Camerini 2003) and has been demonstrated to have effects on various cell types including endothelial cells, lymphocytes, neutrophils, and monocytes (Gleissner et al. 2008). However, the mechanisms by which CXCL4 exerts its effects, including receptors and signal transduction, have not fully been elucidated. Notably, CXCL4 has been demonstrated to induce monocyte adhesion to endothelial cells in conjunction with CCL5 (RANTES) (von Hundelshausen et al. 2005) and to promote macrophage differentiation from human peripheral blood monocytes (Scheuerer et al. 2000). Both mechanisms may be relevant during atherogenesis.

3.2 CXCL4 and macrophage differentiation

A role for CXCL4 for monocyte-macrophage differentiation has been suggested by Scheuerer et al. in 2000 (Scheuerer et al. 2000). Their data demonstrate that CXCL4 not only acts in an anti-apoptotic manner on human peripheral blood monocytes, but also promotes their differentiation towards macrophages in a dose-dependent manner as demonstrated by increased myeloperoxidase expression. The fact that these CXCL4-induced macrophages do not express any detectable CD86 levels on their cell surface suggested that they may be different from those induced by the typically used growth factor macrophage colony-stimulation factor MCSF (Scheuerer et al. 2000).

Interestingly, these first data had been abandoned for almost a decade. One reason for this may be the fact that up to now, nobody has been able to identify the CXCL4 receptor on human monocytes and macrophages. While on T cells and microvascular endothelial cells CXCR3 or splice variants thereof like CXCR3B (Lasagni et al. 2003) have been clearly identified, it is still not clear by which receptor and signal transduction CXCL4 acts on human myeloid cells. Accordingly, it was not until 2010 that our own group did a comprehensive gene expression screen on these cells revealing novel insights into the potential role of CXCL4-induced macrophages (Gleissner et al. 2010b).

3.3 Potential role of CXCL4-induced macrophages in human atherosclerosis
3.3.1 General considerations

Monocyte-derived macrophages are present in atherosclerotic lesions at very early stages of the disease (Galkina & Ley 2009). Activated platelets have been demonstrated to promote atherogenesis, an effect mediated by chemokines released from their alpha granules (Huo et al. 2003). CXCL4 is one of these platelet chemokines and is released from platelets upon activation (Brandt et al. 2000). CXCL4 is known to prevent monocyte apoptosis (Scheuerer et

al. 2000) and to promote differentiation of a specific macrophage phenotype distinct from previously identified macrophage polarizations (Gleissner et al. 2010b). Taking into account that the genetic deletion of CXCL4 in *Apoe-/-* mice is accompanied by reduced atherogenesis (Sachais et al. 2007), and that in human atherosclerotic lesions the presence of CXCL4 has been associated with plaque progression and clinical symptoms (Pitsilos et al. 2003), it seems extremely reasonable to hypothesize that CXCL4-induced macrophages significantly contribute to atherogenesis and progression of atherosclerosis. An important mechanisms of this action may represent the induction of CXCL4-specific, pro-atherogenic macrophages.

3.3.2 Features of CXCL4-induced macrophages – The M4 macrophage

To identify potential mechanisms by which CXCL4-induced macrophages are induced to act in a pro-atherogenic manner, we studied their transcriptome and compared it to that of MCSF-induced macrophages from the same donors. In this analysis, we found that both MCSF- and CXCL4-induced macrophages have strong phenotypic similarities. Thus, they both express similar mRNA and surface levels of CD45, CD14 or intracellular levels of CD68. Their transcriptomes show a strong correlation clearly indicating that they both represent macrophages (Gleissner et al. 2010b).

Interestingly, while CXCL4-treated human monocytes display phenotypic and functional characteristics of macrophages after six days in culture, they also have characteristics that clearly distinguish them from MCSF-induced macrophages. We found 375 genes differentially-expressed between MCSF- and CXCL4-induced macrophages, 206 of them being over-expressed in CXCL4-induced macrophages. Interestingly, CXCL4-induced macrophages displayed neither typical markers of M1 nor of M2 polarization (Gleissner et al. 2010b). Accordingly, using different statistical approaches including gene set enrichment analyses (Subramanian et al. 2005), modified principal components analysis (PCA), and hierarchical clustering (based on transcriptomic data from MCSF-treated, M1-polarized and M2-polarized macrophages (Martinez et al. 2006)), we were able to demonstrate that CXCL4 induces a transcriptome distinct from every macrophage polarization type described thus far (Gleissner et al. 2010b). In all statistical models, it turned out that in relation to their corresponding MCSF-control macrophages, M1 (induced by LPS and interferon-γ) and M2 macrophages (induced by IL-4) clustered together more closely than CXCL4-induced macrophages.

These findings suggest that CXCL4 induces a macrophage phenotype with specific phenotypic and functional characteristics, which will be discussed in more detail below. Based on these results, we have suggested calling these macrophages M4 – a term that we believe reflects both the fact that these cells are distinct from M1 and M2 macrophages and that they can be induced by CXCL4. Accordingly, in the following paragraphs CXCL4-induced macrophages will be referred to as M4 macrophages. Fig. 2 summarizes some of the findings described below in more detail.

3.3.3 Gene expression of matrix metalloproteinases in M4 macrophages

Considering that CXCL4 promotes atherogenesis in *Apoe-/-* mice, we hypothesized that M4 macrophages would over-express genes that are involved in atherogenesis as compared to their MCSF-induced counterparts. Therefore, it was a surprise to discover that M4 macrophages cannot generally be considered pro-atherogenic: some genes implicated in atherogenesis showed higher expression levels, others lower expression levels as compared to MCSF-induced macrophages.
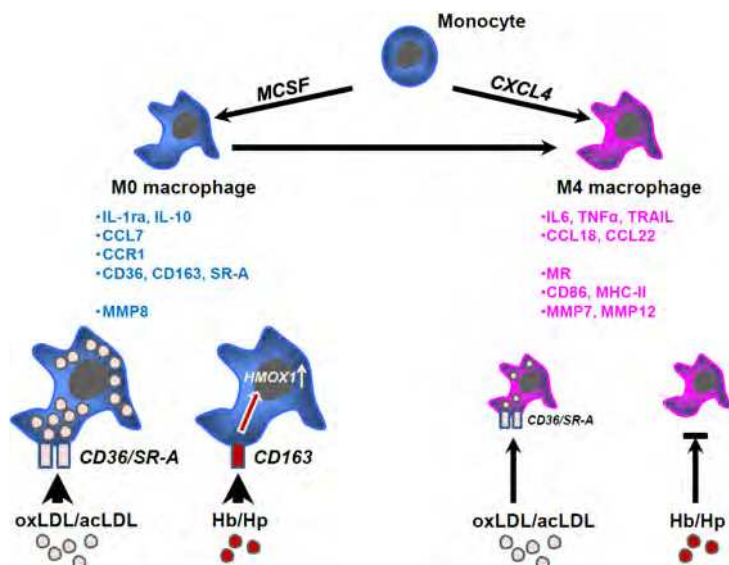
Fig. 2. Features of MCSF- and CXCL4-induced macrophage (M0 and M4 macrophages). For each polarization type, typical cytokines, chemokines, chemokine receptors, other receptors, surface molecules involved in antigen presentation, and intracellular enzymes are indicated. The figures at the bottom indicate functional differences regarding scavenger receptor expression, uptake of modified LDL and the differential potential to clear hemoglobin-haptoglobin complexes resulting in heme oxygenase-1 upregulation.

This ambiguous picture was also seen with matrix metalloproteinases (MMP), of which some showed higher expression in M4 macrophages (e.g. *MMP7* or *MMP12*) while others were expressed more highly in MCSF-induced macrophages (e.g. *MMP8*). Of course, gene expression data do not necessarily reflect protein expression and in the case of MMPs, enzyme activities may be also differentially-regulated; the regulation of MMPs is furthermore affected by activation through cathepsins, which in part are also differentially-regulated (cathepsin B and K significantly higher in M4 macrophages) (Newby 2008).

Taken together, due to the lack of functional data at this stage, it is impossible to judge whether differential-expression of MMPs and potentially different MMP activity in M4 macrophages represent a pro-atherogenic feature of M4 macrophages.

3.3.4 Foam cell formation in M4 macrophages

Based on previous data, that CXCL4 promotes atherogenesis in *Apoe-/-* mice, we hypothesized that CXCL4-induced macrophages would be more prone to foam cell formation induced by modified (i.e. acetylated or oxidized) low density lipoprotein (LDL). Interestingly, the opposite was the shown to be true. While cholesterol efflux transporter ABCA1 and ABCG1 were not differentially-expressed on the mRNA level, scavenger receptors involved in uptake of modified LDL showed significantly lower expression in CXCL4-induced macrophages. Specifically, CD36 and macrophage scavenger receptor-A (which both account for the vast majority of uptake of modified LDL during macrophage foam cell formation) showed significantly lower mRNA expression, while the differences

measured by flow cytometry were only significant for CD36. Interestingly, both uptake of acetylated and oxidized LDL (acLDL and oxLDL) was significantly lower in M4 macrophages, suggesting that CXCL4-induced macrophages have a lower tendency to differentiate towards foam cells. This was somewhat unexpected and supports the notion that the pro-atherosclerotic effects of CXCL4 as shown in *Apoe⁻/⁻* likely reflects a multitude of different effects, including those on cells other than macrophages (i.e. endothelial cells, T cells, and potentially smooth muscle cells).

3.3.5 CD163 and heme oxygenase-1 in M4 macrophages

When analyzing the transcriptome of M4 macrophages, we found *CD163* mRNA coding for the hemoglobin-haptoglobin scavenger receptor to be significantly down-regulated as compared to MCSF-induced macrophages (Gleissner et al. 2010a). CD163 binds hemoglobin-haptoglobin complexes (and hemoglobin with lower affinity). Binding of the ligand to CD163 results in upregulation of heme oxygenase-1 (Schaer et al. 2006). Heme oxygenase-1 (and specifically heme oxygenase-1 expressed in bone marrow-derived cells like monocytes and monocyte-derived macrophages) is thought to be atheroprotective as demonstrated in several mouse models (Juan et al. 2001, Orozco et al. 2007, Yet et al. 2003).

Further experiments studying gene and protein expression in freshly isolated blood monocytes and macrophage induced either by MCSF or CXCL4 confirmed the significant differences. Thus, while MCSF treatment resulted in further up-regulation of CD163 expression, exposure to CXCL4 resulted in complete loss of the receptor within hours. This loss was not mediated by shedding of the receptor, which is one mechanism by which CD163 surface expression can be down-regulated (e.g. after treatment with LPS (Buechler et al. 2000)). Functionally, loss of CD163 resulted in loss of heme oxygenase-1 up-regulation upon exposure to hemoglobin-haptoglobin complexes confirming the functional relevance of our findings.

To assess whether these *in vitro* findings play a role *in vivo*, we studied CD163 protein expression in human atherosclerotic lesions and found that it is differentially-expressed on CD68⁺ macrophages (Gleissner et al. 2010a). Furthermore, in a cohort of 18 consecutive patients undergoing carotid endatherectomy we found an inverse correlation between gene expression of *PF4* (coding for CXCL4) and *CD163*. Considering that *PF4* is exclusively expressed in megakaryocytes and platelets and *CD163* expression is restricted to myeloid cells, we concluded that the presence of large amounts of CXCL4 is associated with low levels of CD163 supporting that this mechanism does play a role *in vivo.*

3.4 M4 macrophages as therapeutic target in atherosclerosis?

As described above, macrophages present in human atherosclerotic lesions do not represent a homogeneous entity, but are composed of different subsets that are characterized by differential-expression of cytokines, chemokines, surface receptors and their enzymatic repertoire. We believe that the platelet-derived chemokine CXCL4 may represent an important inducer of macrophage heterogeneity as it (I) is present in the atherosclerotic lesions, (II) is associated with disease progression, and (III) promotes monocyte differentiation towards a distinct macrophage polarization type.

Based on these considerations, it was surprising to discover that CXCL4-induced M4 macrophages do not display a clearly pro-atherogenic phenotype. In fact, some of their features seem clearly pro-atherogenic (e.g. loss of CD163), while others are ambiguous (e.g.

MMP expression) or may even be anti-atherogenic (e.g. reduced uptake of modified LDL). However, many of these functional data have been obtained *in vitro* on isolated macrophages. Thus, it is possible that in the context of an atherosclerotic lesion consisting of extracellular matrix as well as various other cells types, CXCL4-induced macrophages may have a more clearly pro-atherogenic phenotype. On the other hand, it is possible that the pro-atherogenic effects of CXCL4 represent the summary of various effects on various cell types.

The possibility to specifically address the interaction between a chemokines and its partner (in this case CXCL4 and CCL5 (RANTES)) in the context of atherogenesis has been demonstrated in a very elegant study by Koenen et al., who have designed a small molecule that specifically inhibits the interaction between those two chemokines (Koenen et al. 2009). When treating *Apoe$^{-/-}$* mice with this substance, lesion development was significantly reduced. Even though at this point it is unclear to what extent this effect was due to direct inhibition of CXCL4 effects on macrophages, it still demonstrates that tackling pro-atherogenic chemokines represents a promising approach to prevent or even treat atherosclerosis.

4. Macrophage heterogeneity and the novel M4 macrophage

4.1 Unresolved questions on macrophage heterogeneity

There are a number of unresolved questions regarding macrophage heterogeneity, which make it difficult to assess the specific role of M4 macrophages in this context. Firstly, in vitro models usually do not account for the diversity of peripheral blood monocytes. Thus, CD14$^+$ cells are used to generate macrophages and it is unknown whether certain monocyte subsets (Geissmann et al. 2010, Ziegler-Heitbrock 1989) may be more likely to differentiate towards a specifically polarized macrophage type than others. Secondly, the concept of macrophage polarization is somewhat artificial as it considers the effects of isolated soluble factors (like TNF-alpha or IL-4), whereas in vivo the local micromilieu may be composed of a combination of various cytokine and chemokines. Thirdly, when studying macrophage polarization *in vitro*, dynamics over time are rarely being taken into account. Even though Mantovani et al. have studied the transcriptomes of monocytes, and monocyte-derived macrophages after three or six days' treatment with MCSF (plus additional treatment with LPS/interferon-γ (M1) or IL-4 (M2)) (Martinez et al. 2006), more detailed information on time courses are lacking at this point. Finally, we do not know exactly which of the thus far defined macrophage polarization types represent final states of differentiation and which of them are temporary, i.e. whether induction of a specific polarization type is reversible or not.

4.2 Macrophage polarization beyond M1 and M2

The recent data on macrophage heterogeneity in human atherosclerosis is interesting and puzzling at the same time. Thus, while Bouhlel *et al.* were able to demonstrate gene expression of both M1 and M2 markers in different areas of human atherosclerotic plaques suggesting that both polarization types may exist *in vivo* (Bouhlel et al. 2007), other groups including ourselves have described macrophage phenotypes *in vitro* and *in vivo* that do not necessarily fit into the established scheme of macrophage polarizations (Boyle et al. 2009, Gleissner et al. 2010a, Waldo et al. 2008).

Waldo et al. compared MCSF- and GMCSF-induced macrophages (even though the latter are sometimes considered dendritic cells) and found significant differences in the expression of CD14, which was virtually absent in GMCSF-induced cells (Waldo et al. 2008). Furthermore, these cells were less likely to spontaneously accumulate modified LDL. The authors were able to confirm the existence and the functional characteristics of these macrophages *in vitro* as well as in human atherosclerotic lesions.

Boyle et al. described a novel macrophage that is induced by haemoglobin-haptoglobin (Hb-Hp) complexes and is characterized by high levels of CD163 and low levels of HLA-DR (Boyle et al. 2009). *In vitro*, these cells cleared Hb-Hp complexes more efficiently and showed reduced oxidative stress (Boyle et al. 2009). Mechanistic data show, that this phenotype is induced by autocrine effects of IL-10 suggesting a certain similarity to M2c macrophages.

Our own group has studied the effects of oxidized LDL on human monocyte-derived macrophages and found that these cells express markers that have been associated with dendritic cells (Cho et al. 2007). Thus, these cells up-regulate genes coding for MHC-II, CD11c, and DC-STAMP, suggesting that there may be phenotypic and functional overlap between foam cells and dendritic cells within atherosclerotic lesions.

Finally, as mentioned above, we have studied the phenotypic and functional characteristics of CXCL4-induced macrophages and found features that clearly distinguish these macrophages from any polarization type described thus far (Gleissner et al. 2010b).

4.3 Suggestion for a novel nomenclature of macrophage polarization

Based on this evidence, we feel the necessity to develop a novel classification of macrophage polarization that includes the novel macrophage types described *in vitro* and *in vivo* over the past years. Therefore, we here propose a novel framework that tries to systematically cover those macrophage types that have been unequivocally identified in human atherosclerotic plaques. Many of these polarization types have been extensively characterized, sometimes including transcriptomic analysis. Accordingly, we suggest assigning the "novel" macrophage polarization types names that consist of "M" (for macrophage) and a second number, or letter, or combination of both that is related to the inducers of this polarization type.

Our proposed system has several advantages: Firstly, it allows keeping the established terms M1 and M2 and thereby not only respects the pioneering work by Gordon et al., but also avoids confusion associated with the introduction of new terms. Secondly, for "novel" macrophage polarization types the proposed names hint as to what inducers are involved. Finally, the system allows flexibility as based on the above-mentioned principles, so further addition of novel macrophage polarization types remains possible in the future.

Notably, the proposed nomenclature is restricted to monocyte-derived cells that occur during pathology, i.e. it does not cover tissue macrophages like Kupffer cells in the liver or alveolar macrophages in the lung. By including dendritic cells (DC) in the system, we specifically address myeloid dendritic cells derived from monocyte precursors. Plasmacytoid DCs or DCs constitutively present in certain tissues like Langerhans cells of the skin are again not covered by the proposed system. Accordingly, our system may have some overlap with other classifications of DCs. Table 1a and b give an overview of the proposed system.

	M1	M0	M2a	M2b	M2c
Inducers	MCSF + IFNγ/ LPS *or* TNF-α	MCSF	IL-4 *or* IL-13	Immune complexes + TLR ligands	IL-10
Prominent markers (selection)	CCR7, TLR2, TLR4, MHC-II, iNOS	CD14, CD16, CD163	CD36, CD163, MR, SR-A, MHC-II	CD80, CD86, MHC-II	CCR2, CD14, CD36, MR, SR-A
Functional characteristics	Pro-inflammatory	Inflammation resolution?	Inflammation resolution	Inflammation resolution	Tissue repair
Transcriptome	Yes	yes	yes	no	no
In vivo evidence in athero-sclerosis	Gene expression	Gene expression	Gene expression	no	no
References for transcriptomic analysis	(Martinez et al. 2006)	(Cho et al. 2007, Gleissner et al. 2010b, Martinez et al. 2006, Waldo et al. 2008)	(Martinez et al. 2006)	-	-

Table 1a. Proposal for a novel macrophage classification (established polarization types). The columns are ordered in a way that reflects similarity based on transcriptomic analysis where available.

	M-Hb	M4	M-ox	M-GM	M-DC
Inducers	MCSF + hemoglobin/ haptoglobin	CXCL4	MCSF + oxLDL	GMCSF	GMCSF + IL-4
Prominent markers (selection)	CD163 MHC-II^{low}	CD163$^-$	CD206, MHC-II	CD1a, CD1c, CCR7 CD14$^-$	CD206, MHC-II
Functional characteristics	High clearance of Hb/hp, reduced oxidative stress	Defective Hb/hp clearance, reduced foam cell formation	Ag presentation?	Less spontaneous cholesterol accumulation, unclear	Ag presentation, T cell activation
Transcriptome	No	Yes	Yes	Yes	Yes
In vivo evidence in athero-sclerosis	Histology	Gene expression, histology (CD163)	Histology	Histology (CD68)	Histology
References	(Boyle et al. 2009)	(Gleissner et al. 2010b)	(Cho et al. 2007)	(Waldo et al. 2008)	(Bobryshev & Lord 1995, Jeffrey et al. 2006)

Table 1b. Proposal for a novel macrophage classification (novel polarization types). The columns are ordered in a way that reflects similarity based on transcriptomic analysis where available

5. Conclusions

In summary, we have discussed the role of the platelet-derived chemokine CXCL4 for macrophage differentiation in the general context of macrophage heterogeneity in atherogenesis. There is good evidence that CXCL4 promotes atherogenesis, even though at this point, the relevance of CXCL4-induced macrophages for this process is not entirely understood. While CXCL4-induced macrophages display some potentially pro-atherogenic features, their pro-atherogenic nature is not as evident as expected. Thus, we believe that it will be necessary to study these cells in more detail to elucidate the mechanisms involved in CXCL4-induced macrophage differentiation, but also to study these cells in their pathohpysiological environment, i.e. within the human atheroclerotic plaque.

As CXCL4 is not the only mediator of monocyte macrophage differentiation and as an increasing number of polarized macrophages clearly distinct from M1 and M2 macrophages with potential implication for atherogenesis have been identified *in vitro* and *in vivo*, we have proposed a novel macrophage classification schema, that both respects the established M1 and M2 paradigm and takes into account novel macrophage phenotypes and their inducers.

Taken together, even though our knowledge on macrophage heterogeneity in general and the impact of CXCL4 on macrophage heterogeneity in atherogenesis is growing, we still need more insight into the mechanisms involved in order to use this knowledge as the basis for novel, specific therapies for atherosclerosis.

6. Acknowledgments

7. References

Auffray C, Fogg D, Garfa M, Elain G, Join-Lambert O, Kayal S, Sarnacki S, Cumano A, Lauvau G, Geissmann F. (2007) Monitoring of blood vessels and tissues by a population of monocytes with patrolling behavior. *Science,* Vol.317, No.5838, 2007, pp. 666-670, 1095-9203 (Electronic).

Bobryshev YV, Lord RS. (1995) Ultrastructural recognition of cells with dendritic cell morphology in human aortic intima. Contacting interactions of vascular dendritic cells in athero-resistant and athero-prone areas of the normal aorta. *Arch Histol Cytol,* Vol.58, No.3, 1995, pp. 307-322, 0914-9465 (Print).

Bouhlel MA, Derudas B, Rigamonti E, Dievart R, Brozek J, Haulon S, Zawadzki C, Jude B, Torpier G, Marx N, Staels B, Chinetti-Gbaguidi G. (2007) Ppargamma activation primes human monocytes into alternative m2 macrophages with anti-inflammatory properties. *Cell Metabolism,* Vol.6, No.2, 2007, pp. 137-143, 1550-4131 (Print).

Boyle JJ, Harrington HA, Piper E, Elderfield K, Stark J, Landis RC, Haskard DO. (2009) Coronary intraplaque hemorrhage evokes a novel atheroprotective macrophage phenotype. *American Journal of Pathology,* Vol.174, No.3, 2009, pp. 1097-1108, 1525-2191 (Electronic).

Brandt E, Petersen F, Ludwig A, Ehlert JE, Bock L, Flad HD. (2000) The beta-thromboglobulins and platelet factor 4: Blood platelet-derived cxc chemokines with

divergent roles in early neutrophil regulation. *J Leukoc Biol,* Vol.67, No.4, 2000, pp. 471-478,

Buechler C, Ritter M, Orso E, Langmann T, Klucken J, Schmitz G. (2000) Regulation of scavenger receptor cd163 expression in human monocytes and macrophages by pro- and antiinflammatory stimuli. *J Leukoc Biol,* Vol.67, No.1, 2000, pp. 97-103,

Cho HJ, Shashkin P, Gleissner CA, Dunson D, Jain N, Lee JK, Miller Y, Ley K. (2007) Induction of dendritic cell-like phenotype in macrophages during foam cell formation. *Physiological Genomics,* Vol.29, No.2, 2007, pp. 149-160,

Fitzgerald ML, Moore KJ, Freeman MW, Reed GL. (2000) Lipopolysaccharide induces scavenger receptor a expression in mouse macrophages: A divergent response relative to human thp-1 monocyte/macrophages. *The Journal of Immunology,* Vol.164, No.5, 2000, pp. 2692-2700, 0022-1767 (Print).

Galkina EV, Ley K. (2009) Immune and inflammatory mechanisms of atherosclerosis. *Annual Reviews in Immunology,* Vol.27, No.27, 2009, pp. 165-197,

Gear AR, Camerini D. (2003) Platelet chemokines and chemokine receptors: Linking hemostasis, inflammation, and host defense. *Microcirculation,* Vol.10, No.3-4, 2003, pp. 335-350, 1073-9688 (Print).

Geissmann F, Jung S, Littman DR. (2003) Blood monocytes consist of two principal subsets with distinct migratory properties. *Immunity,* Vol.19, No.1, 2003, pp. 71-82, 1074-7613 (Print).

Geissmann F, Manz MG, Jung S, Sieweke MH, Merad M, Ley K. (2010) Development of monocytes, macrophages, and dendritic cells. *Science,* Vol.327, No.5966, 2010, pp. 656-661, 1095-9203 (Electronic).

Gleissner CA, von Hundelshausen P, Ley K. (2008) Platelet chemokines in vascular disease. *Arteriosclerosis, Thrombosis and Vascular Biology,* Vol.28, No.11, 2008, pp. 1920-1927, 1524-4636 (Electronic).

Gleissner CA, Shaked I, Erbel C, Bockler D, Katus HA, Ley K. (2010a) Cxcl4 downregulates the atheroprotective hemoglobin receptor cd163 in human macrophages. *Circulation Research,* Vol.106, No.1, 2010a, pp. 203-211, 1524-4571 (Electronic).

Gleissner CA, Shaked I, Little KM, Ley K. (2010b) Cxc chemokine ligand 4 induces a unique transcriptome in monocyte-derived macrophages. *The Journal of Immunology,* Vol.184, No.9, 2010b, pp. 4810-4818,

Gordon S. (2003) Alternative activation of macrophages. *Nat Rev Immunol,* Vol.3, No.1, 2003, pp. 23-35, 1474-1733 (Print).

Huo Y, Schober A, Forlow SB, Smith DF, Hyman MC, Jung S, Littman DR, Weber C, Ley K. (2003) Circulating activated platelets exacerbate atherosclerosis in mice deficient in apolipoprotein e. *Nature Medicine,* Vol.9, No.1, 2003, pp. 61-67,

Ingersoll MA, Spanbroek R, Lottaz C, Gautier EL, Frankenberger M, Hoffmann R, Lang R, Haniffa M, Collin M, Tacke F, Habenicht AJ, Ziegler-Heitbrock L, Randolph GJ. (2010) Comparison of gene expression profiles between human and mouse monocyte subsets. *Blood,* Vol.115, No.3, 2010, pp. e10-19, 1528-0020 (Electronic).

Jeffrey KL, Brummer T, Rolph MS, Liu SM, Callejas NA, Grumont RJ, Gillieron C, Mackay F, Grey S, Camps M, Rommel C, Gerondakis SD, Mackay CR. (2006) Positive regulation of immune cell function and inflammatory responses by phosphatase pac-1. *Nat Immunol,* Vol.7, No.3, 2006, pp. 274-283, 1529-2908 (Print).

Juan S-H, Lee T-S, Tseng K-W, Liou J-Y, Shyue S-K, Wu KK, Chau L-Y. (2001) Adenovirus-mediated heme oxygenase-1 gene transfer inhibits the development of atherosclerosis in apolipoprotein e-deficient mice. *Circulation,* Vol.104, No.13, 2001, pp. 1519-1525,

Koenen RR, von Hundelshausen P, Nesmelova IV, Zernecke A, Liehn EA, Sarabi A, Kramp BK, Piccinini AM, Paludan SR, Kowalska MA, Kungl AJ, Hackeng TM, Mayo KH, Weber C. (2009) Disrupting functional interactions between platelet chemokines inhibits atherosclerosis in hyperlipidemic mice. *Nat Med,* Vol.15, No.1, 2009, pp. 97-103, 1546-170X (Electronic).

Kurz K, Giannitsis E, Becker M, Hess G, Zdunek D, Katus H. (2011) Comparison of the new high sensitive cardiac troponin t with myoglobin, h-fabp and ctnt for early identification of myocardial necrosis in the acute coronary syndrome. *Clinical Research in Cardiology,* Vol.100, No.3, 2011, pp. 209-215, 1861-0684.

Lasagni L, Francalanci M, Annunziato F, Lazzeri E, Giannini S, Cosmi L, Sagrinati C, Mazzinghi B, Orlando C, Maggi E, Marra F, Romagnani S, Serio M, Romagnani P. (2003) An alternatively spliced variant of cxcr3 mediates the inhibition of endothelial cell growth induced by ip-10, mig, and I-tac, and acts as functional receptor for platelet factor 4. *J Exp Med,* Vol.197, No.11, 2003, pp. 1537-1549,

Mantovani A, Garlanda C, Locati M. (2009) Macrophage diversity and polarization in atherosclerosis: A question of balance. *Arteriosclerosis, Thrombosis and Vascular Biology,* Vol.29, No.10, 2009, pp. 1419-1423, 1524-4636 (Electronic).

Martinez FO, Gordon S, Locati M, Mantovani A. (2006) Transcriptional profiling of the human monocyte-to-macrophage differentiation and polarization: New molecules and patterns of gene expression. *J Immunol,* Vol.177, No.10, 2006, pp. 7303-7311,

Naghavi M, Libby P, Falk E, Casscells SW, Litovsky S, Rumberger J, Badimon JJ, Stefanadis C, Moreno P, Pasterkamp G, Fayad Z, Stone PH, Waxman S, Raggi P, Madjid M, Zarrabi A, Burke A, Yuan C, Fitzgerald PJ, Siscovick DS, de Korte CL, Aikawa M, Airaksinen KE, Assmann G, Becker CR, Chesebro JH, Farb A, Galis ZS, Jackson C, Jang IK, Koenig W, Lodder RA, March K, Demirovic J, Navab M, Priori SG, Rekhter MD, Bahr R, Grundy SM, Mehran R, Colombo A, Boerwinkle E, Ballantyne C, Insull W, Jr., Schwartz RS, Vogel R, Serruys PW, Hansson GK, Faxon DP, Kaul S, Drexler H, Greenland P, Muller JE, Virmani R, Ridker PM, Zipes DP, Shah PK, Willerson JT. (2003a) From vulnerable plaque to vulnerable patient: A call for new definitions and risk assessment strategies: Part II. *Circulation,* Vol.108, No.15, 2003a, pp. 1772-1778, 1524-4539 (Electronic).

Naghavi M, Libby P, Falk E, Casscells SW, Litovsky S, Rumberger J, Badimon JJ, Stefanadis C, Moreno P, Pasterkamp G, Fayad Z, Stone PH, Waxman S, Raggi P, Madjid M, Zarrabi A, Burke A, Yuan C, Fitzgerald PJ, Siscovick DS, de Korte CL, Aikawa M, Juhani Airaksinen KE, Assmann G, Becker CR, Chesebro JH, Farb A, Galis ZS, Jackson C, Jang IK, Koenig W, Lodder RA, March K, Demirovic J, Navab M, Priori SG, Rekhter MD, Bahr R, Grundy SM, Mehran R, Colombo A, Boerwinkle E, Ballantyne C, Insull W, Jr., Schwartz RS, Vogel R, Serruys PW, Hansson GK, Faxon DP, Kaul S, Drexler H, Greenland P, Muller JE, Virmani R, Ridker PM, Zipes DP, Shah PK, Willerson JT. (2003b) From vulnerable plaque to vulnerable patient: A call for new definitions and risk assessment strategies: Part I. *Circulation,* Vol.108, No.14, 2003b, pp. 1664-1672, 1524-4539 (Electronic).

Newby AC. (2008) Metalloproteinase expression in monocytes and macrophages and its relationship to atherosclerotic plaque instability. *Arterioscler Thromb Vasc Biol*, Vol.28, No.12, 2008, pp. 2108-2114, 1524-4636 (Electronic).

Orozco LD, Kapturczak MH, Barajas B, Wang X, Weinstein MM, Wong J, Deshane J, Bolisetty S, Shaposhnik Z, Shih DM, Agarwal A, Lusis AJ, Araujo JA. (2007) Heme oxygenase-1 expression in macrophages plays a beneficial role in atherosclerosis. *Circ Res*, Vol.100, No.12, 2007, pp. 1703-1711,

Pitsilos S, Hunt J, Mohler ER, Prabhakar AM, Poncz M, Dawicki J, Khalapyan TZ, Wolfe ML, Fairman R, Mitchell M, Carpenter J, Golden MA, Cines DB, Sachais BS. (2003) Platelet factor 4 localization in carotid atherosclerotic plaques: Correlation with clinical parameters. *Thromb Haemost*, Vol.90, No.6, 2003, pp. 1112-1120,

Poston RN, Hussain IF. (1993) The immunohistochemical heterogeneity of atheroma macrophages: Comparison with lymphoid tissues suggests that recently blood-derived macrophages can be distinguished from longer-resident cells. *J Histochem Cytochem*, Vol.41, No.10, 1993, pp. 1503-1512, 0022-1554 (Print).

Ridker PM. (2007) C-reactive protein and the prediction of cardiovascular events among those at intermediate risk: Moving an inflammatory hypothesis toward consensus. *Journal of the American College of Cardiology*, Vol.49, No.21, 2007, pp. 2129-2138, 0735-1097.

Roger VL, Go AS, Lloyd-Jones DM, Adams RJ, Berry JD, Brown TM, Carnethon MR, Dai S, de Simone G, Ford ES, Fox CS, Fullerton HJ, Gillespie C, Greenlund KJ, Hailpern SM, Heit JA, Ho PM, Howard VJ, Kissela BM, Kittner SJ, Lackland DT, Lichtman JH, Lisabeth LD, Makuc DM, Marcus GM, Marelli A, Matchar DB, McDermott MM, Meigs JB, Moy CS, Mozaffarian D, Mussolino ME, Nichol G, Paynter NP, Rosamond WD, Sorlie PD, Stafford RS, Turan TN, Turner MB, Wong ND, Wylie-Rosett J, Committee obotAHAS, Stroke Statistics Subcommittee, Disease ObotAHAH, Stroke Statistics Writing Group. (2011) Heart disease and stroke statistics--2011 update: A report from the american heart association. *Circulation*, Vol.123, No.4, 2011, pp. e18-209,

Ross R. (1999) Atherosclerosis--an inflammatory disease. *N Engl J Med*, Vol.340, No.2, 1999, pp. 115-126, 0028-4793 (Print).

Sachais BS, Turrentine T, Dawicki McKenna JM, Rux AH, Rader D, Kowalska MA. (2007) Elimination of platelet factor 4 (pf4) from platelets reduces atherosclerosis in c57bl/6 and apoe-/- mice. *Thromb Haemost*, Vol.98, No.5, 2007, pp. 1108-1113,

Schaer CA, Schoedon G, Imhof A, Kurrer MO, Schaer DJ. (2006) Constitutive endocytosis of cd163 mediates hemoglobin-heme uptake and determines the noninflammatory and protective transcriptional response of macrophages to hemoglobin. *Circ Res*, Vol.99, No.9, 2006, pp. 943-950,

Scheuerer B, Ernst M, Durrbaum-Landmann I, Fleischer J, Grage-Griebenow E, Brandt E, Flad HD, Petersen F. (2000) The cxc-chemokine platelet factor 4 promotes monocyte survival and induces monocyte differentiation into macrophages. *Blood*, Vol.95, No.4, 2000, pp. 1158-1166,

Shashkin P, Dragulev B, Ley K. (2005) Macrophage differentiation to foam cells. *Current Pharmaceutical Design*, Vol.11, No.23, 2005, pp. 3061-3072,

Stein M, Keshav S, Harris N, Gordon S. (1992) Interleukin 4 potently enhances murine macrophage mannose receptor activity: A marker of alternative immunologic macrophage activation. *J Exp Med*, Vol.176, No.1, 1992, pp. 287-292, 0022-1007 (Print).

Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA, Paulovich A, Pomeroy SL, Golub TR, Lander ES, Mesirov JP. (2005) Gene set enrichment analysis: A knowledge-based approach for interpreting genome-wide expression profiles. *Proc Natl Acad Sci U S A,* Vol.102, No.43, 2005, pp. 15545-15550, 0027-8424 (Print).

von Hundelshausen P, Weber KS, Huo Y, Proudfoot AE, Nelson PJ, Ley K, Weber C. (2001) Rantes deposition by platelets triggers monocyte arrest on inflamed and atherosclerotic endothelium. *Circulation,* Vol.103, No.13, 2001, pp. 1772-1777,

von Hundelshausen P, Koenen RR, Sack M, Mause SF, Adriaens W, Proudfoot AE, Hackeng TM, Weber C. (2005) Heterophilic interactions of platelet factor 4 and rantes promote monocyte arrest on endothelium. *Blood,* Vol.105, No.3, 2005, pp. 924-930,

von Hundelshausen P, Petersen F, Brandt E. (2007) Platelet-derived chemokines in vascular biology. *Thromb Haemost,* Vol.97, No.5, 2007, pp. 704-713,

Waldo SW, Li Y, Buono C, Zhao B, Billings EM, Chang J, Kruth HS. (2008) Heterogeneity of human macrophages in culture and in atherosclerotic plaques. *American Journal of Pathology,* Vol.172, No.4, 2008, pp. 1112-1126, 0002-9440 (Print).

Weber C. (2005) Platelets and chemokines in atherosclerosis: Partners in crime. *Circ Res,* Vol.96, No.6, 2005, pp. 612-616, 1524-4571 (Electronic).

Weber C. (2008) Chemokines in atherosclerosis, thrombosis, and vascular biology. *Arterioscler Thromb Vasc Biol,* Vol.28, No.11, 2008, pp. 1896, 1524-4636 (Electronic).

Yet SF, Layne MD, Liu X, Chen YH, Ith B, Sibinga NE, Perrella MA. (2003) Absence of heme oxygenase-1 exacerbates atherosclerotic lesion formation and vascular remodeling. *FASEB J,* Vol.17, No.12, 2003, pp. 1759-1761, 1530-6860 (Electronic).

Zernecke A, Shagdarsuren E, Weber C. (2008) Chemokines in atherosclerosis: An update. *Arterioscler Thromb Vasc Biol,* Vol.28, No.11, 2008, pp. 1897-1908, 1524-4636 (Electronic).

Ziegler-Heitbrock HW. (1989) The biology of the monocyte system. *Eur J Cell Biol,* Vol.49, No.1, 1989, pp. 1-12, 0171-9335 (Print).
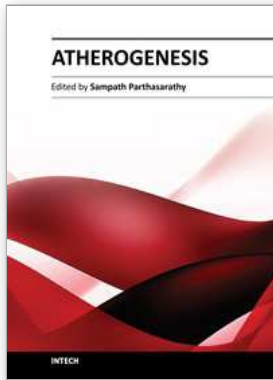
Atherogenesis

Edited by Prof. Sampath Parthasarathy

This monograph will bring out the state-of-the-art advances in the dynamics of cholesterol transport and will address several important issues that pertain to oxidative stress and inflammation. The book is divided into three major sections. The book will offer insights into the roles of specific cytokines, inflammation, and oxidative stress in atherosclerosis and is intended for new researchers who are curious about atherosclerosis as well as for established senior researchers and clinicians who would be interested in novel findings that may link various aspects of the disease.

How to reference

In order to correctly reference this scholarly work, feel free to copy and paste the following:

Christian A. Gleissner and Christian Erbel (2012). CXCL4-Induced Macrophages: A Novel Therapeutic Target in Human Atherosclerosis?, Atherogenesis, Prof. Sampath Parthasarathy (Ed.), ISBN: 978-953-307-992-9, InTech, Available from: http://www.intechopen.com/books/atherogenesis/cxcl4-induced-macrophages-a-novel-therapeutic-target-in-human-atherosclerosis-

INTECH
open science | open minds