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CXCL4-induced macrophages in human atherosclerosis

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ABSTRACT

Atherosclerosis is considered an inflammatory disease of the arterial wall. Monocytes and monocyte-derived cells (most often termed macrophages) play an essential role in the formation of atherosclerotic lesions, as they take up lipids leading to subsequent foam cell formation accompanied by release of pro-inflammatory cytokines. Similarly, platelets have been discovered to represent an important cell type mediating inflammatory and immune processes in atherogenesis, mainly by secreting chemokines, which are stored in the platelets' alpha granules, upon platelet activation. Therefore, the interaction between monocyte-derived cells and platelets is of exceptional importance. In this review, we specifically focus on the chemokine (platelet factor-4, PF4) and its effects on monocytes and monocyte-derived cells. By formation of heterodimers dimers and -oligomers with CCL5, CXCL4 induces binding of monocytes cells to endothelial cell and thereby promotes diapedesis of monocytes into the subendothelial space. CXCL4 also affects the differentiation of monocytes as it induces a specific macrophage phenotype, which we suggested to term "M4". For example, CXCL4-induced macrophages irreversibly lose the hemoglobin-haptoglobin scavenger receptor CD163. The combination of CD68, S100A8, and MMP7 turned out to reliably identify M4 macrophages both *in vitro* and *in vivo* within atherosclerotic lesions. In human atherosclerotic plaques, M4 macrophages are predominantly present in the adventitia and the intima and their prevalence is associated with plaque instability suggesting that they are a marker of pro-inflammatory activity. Overall, CXCL4-induced M4 macrophages may represent a target for diagnostic and therapeutic interventions in human atherosclerotic disease.

1. Monocyte-derived cells in human atherosclerosis

Atherosclerosis is considered an inflammatory disease of the arterial wall triggered by the immune response to lipids retained in the subendothelial space [1–3]. Monocytes and monocyte-derived cells (most often termed macrophages) play an essential role in this process [3]. Uptake of native and modified (mostly oxidized) low density lipoprotein (LDL) particles by macrophages leads to secretion of chemokines and cytokines thereby promoting an inflammatory environment. This leads to recruitment of further immune cells and vascular smooth muscle cells. In parallel, monocyte-derived cells may assume a dendritic cell-like phenotype allowing interaction with T cells, which in turn leads to involvement of the adaptive immune system [4,5]. In summary, there is excellent evidence supporting a crucial role of monocyte-derived cells in atherogenesis.

Interestingly, monocyte-derived cells retain a large potential of plasticity, i.e. depending on external stimuli provided by the microenvironment, they may undergo phenotypic and functional changes critically affecting the further disease process (Fig. 1). Early on, M1 and M2 markers could be identified within atherosclerotic plaques [6]. *In vitro*, M1 and M2 represent the extremes of a presumable continuum of macrophage polarizations with M1 representing a rather pro-inflammatory phenotype and M2 representing a more anti-inflammatory phenotype associated with limitation of inflammatory processes and inflammation resolution [7]. The implications and underlying mechanisms of this paradigm have been excellently reviewed previously [8–14].

In the meantime, the M1-M2 scheme has been complemented by a plethora of additional polarization types that have been described *in vitro* and *in vivo*, among them hemoglobin-associated Mhem macrophages [15,16] or oxLDL-induced Mox macrophages [17]. These findings have recently been reviewed by several authors [3,18].

2. Platelet-derived chemokines as regulators of atherosclerosis

Besides monocyte-derived cells platelets are known to be major key players in the process of atherogenesis. Rather than representing mere "hemostatic particles", platelets have been discovered to be an important cell type mediating inflammatory and immune processes – mainly by secreting chemokines [19,20].

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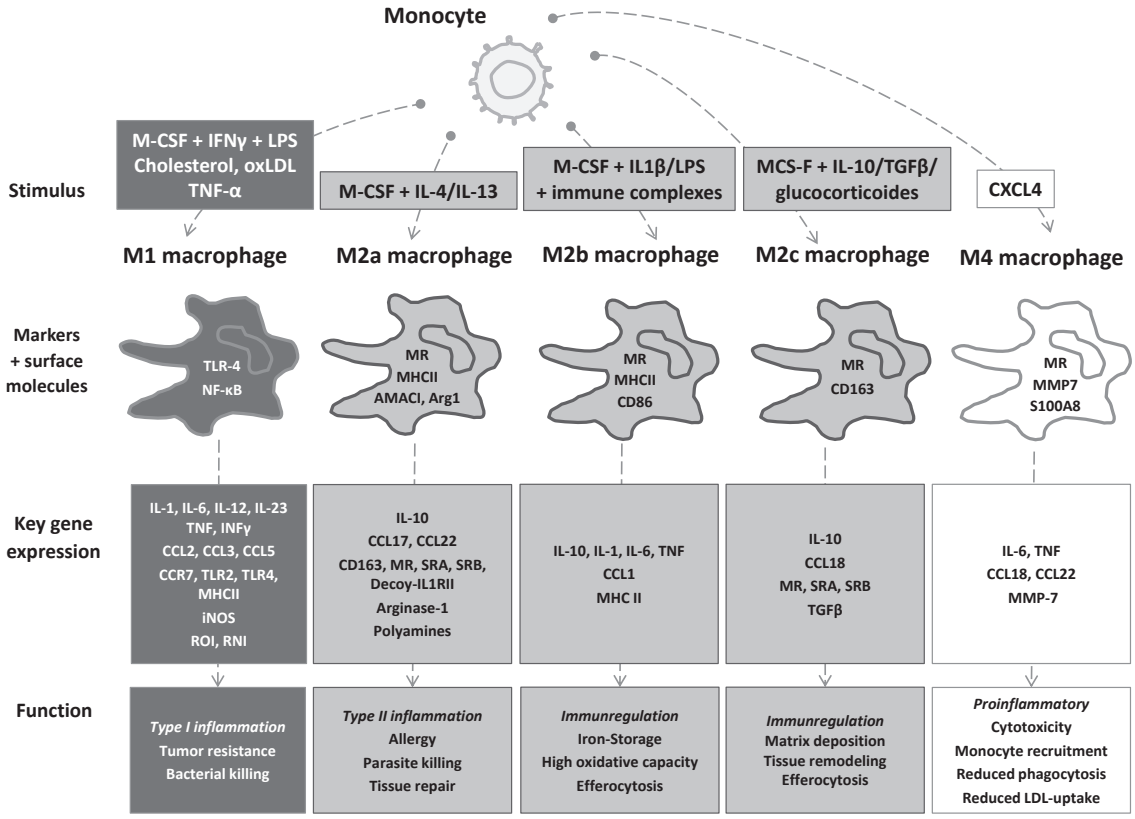

Fig. 1. Differentially polarized macrophages. Based on the stimulus, macrophages may assume different polarization types. The figure indicated typical markers, key genes, and specific functions attributed to M1, M2a/b/c, and M4 macrophages. Arg arginase, CCL CC-chemokine, CCR cc-chemokine receptor, IFN interferon, IL interleukin, iNOS inducible nitric oxide synthase, LPS lipopolysaccharide, M-CSF macrophage colony-stimulating factor, MHC major histocompatibility complex, MMP matrix metalloprotease, MR mannose receptor, NFkB nuclear factor kappa B, oxLDL oxidized low density lipoprotein, ROI reactive oxygen intermediate, RNI reactive nitrogen intermediate, TLR toll like receptor, TNF tumor necrosis factor, SR scavenger receptor, TGF transforming growth factor.

Platelets are formed by shedding from megakaryocytes in the bone marrow – a process called thrombopoeiesis [21]. Platelets are characterized by intracellular alpha granules, in which pro-inflammatory factors (mostly chemokines), including CCL3 (MIP-1alpha), CCL5 (RANTES), CCL7 (MCP-3), CCL17, CXCL1 (growth-regulated oncogene-alpha), CXCL5 (ENA-78), and CXCL8 (IL-8) are stored [22,23]. Upon activation, these mediators can be released immediately without requirement of protein synthesis. The platelets' alpha-granules have been further sub-categorized by electron tomography and vary in their membrane organization and chemokine content [24].

Chemokines are a family of about 45 small (8–12 kDa) peptides that belong to the cytokines. They are primarily involved in the regulation of chemotaxis, i.e. the migration of cells [25], but there have also been implicated in various other developmental, homeostatic and inflammatory processes [26]. Chemokines can be further divided in into four families depending on the positions of their cystein residues (CCL, CXCL, CX3CL1 and CXL1/2) [27,28]. Usually, chemokines bind to chemokine-receptors which belong to the 7-transmembrane G-Protein coupled receptors [27]. Chemokines as well as their receptors are not entirely specific, i.e. chemokines can bind different chemokine receptors, and receptors can be activated by a variety of chemokines. In most cases, CXC-chemokines bind to CXCR receptors, while CCL chemokines bind to CCR receptors [27,28]. In some cases, e.g. for CXCL4, binding to glycosaminoglycans has been described [29].

3. CXCL4 – a "unique" chemokine

In fact, CXCL4 seems to have a special position among the chemokines described: Firstly, it seems to have a plethora of biological functions, including effects on endothelial cells, monocytes, macrophages, dendritic cells, T cells, as well as platelets themselves [19,20]. Secondly, CXCL4 does not possess an ELR acid sequence at its amino terminus and therefore does not bind to CXCR1 or CXCR2 [25]. In this review, we would like to specifically focus on CXCL4 effects on monocytes and monocyte-derived cells.

CXCL4 is released into the blood in concentrations ranging from 0.4 to 1.9 μm from platelet alpha granules upon platelet activation [19,20]. Interestingly, CXCL4 itself has been shown to activate platelets, modulate platelet aggregation and stimulate release of alpha-granule proteins [25].

4. CXCL4-CCL5 interaction in atherosclerosis

One of the effects first recognized was a strong inhibition of angiogenesis. Vice versa binding of CXCL4 by heparin increased tumor angiogenesis *in vivo* [30]. Later on, the effects of CXCL4 on atherogenesis were characterized in further detail [19,20]. While initially it had been believed that CXCL4 acts primarily as a chemotactic factor for monocytes and neutrophils [31] this finding could not be replicated in following studies [32–34]. Still, CXCL4 induced migration of activated T lymphocytes [35].

In vivo a correlation between CXCL4 deposition in carotid plaques from 132 patients with critical carotid stenosis and 6 autopsy specimens, lesion severity and symptomatic atherosclerosis has been found [36]. In 2007, Sachais et al. described that knocking out the murine *Pf4* gene coding for CXCL4 leads to significant reduction of atherosclerotic lesions on the *Apoe*$^{-/-}$ background [37]. Potential mechanisms include reduced deposition of CXCL4-CCL5 heterodimers that may induce adherence of monocytes to the endothelium [38] as well as inhibition of CXCL4-induced monocyte macrophage differentiation [39,40].

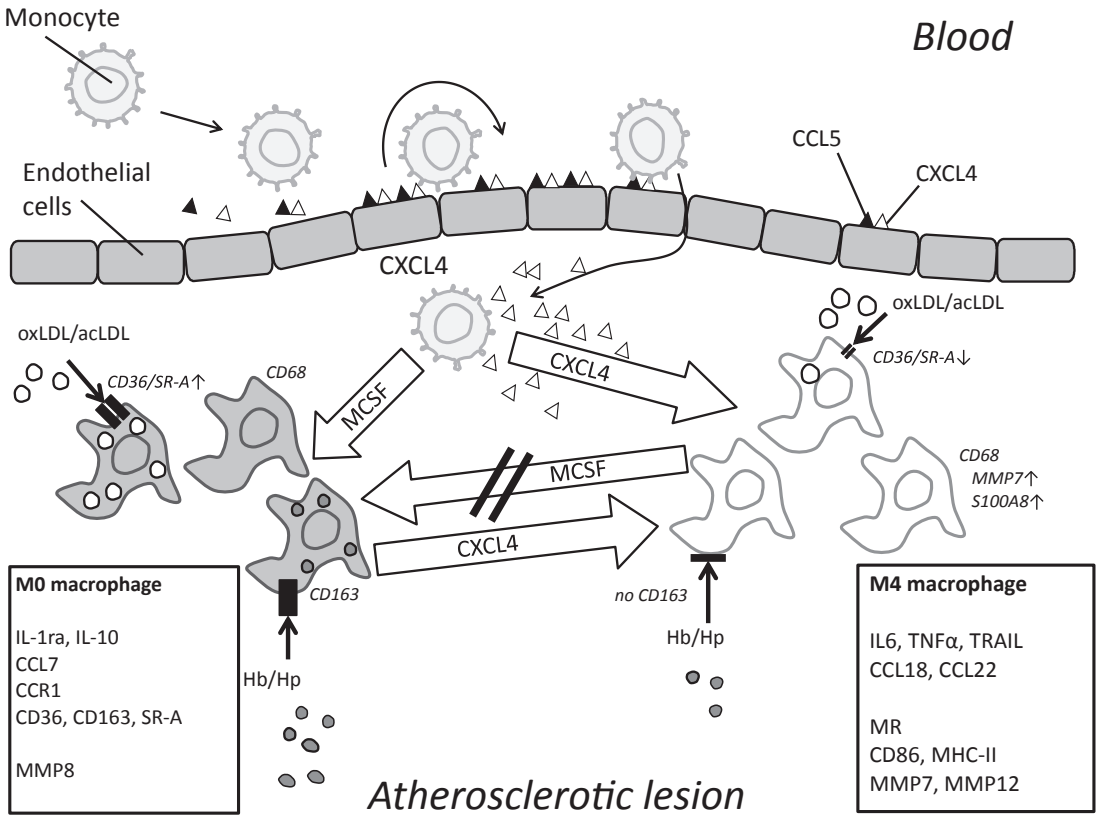

Fig. 2. Mechanisms by which CXCL4 may promote atherogenesis. Platelets may deposit CXCL4 (△) on the vascular endothelium where it may form heterodimers with CCL5 (RANTES) (▲) thereby promoting monocyte adherence to the vascular wall. After transmigration through the endothelium, monocytes exposed to CXCL4 may differentiate towards a specific type of macrophage (M4, right), which is characterized by differential expression of cytokines, chemokines, surface receptors, costimulatory molecules and matrix metalloproteinases as compared to the well characterized MCSF-induced macrophage (M0, left). M4-macrophages can be identified by co-expression of CD68, MMP7 and S100A8 *in vitro* and *in vivo*. Also compared to M0-macrophages, M4-macrophages display reduced expression of scavenger receptors such as CD36 or SR-1, associated with reduced capacity of oxLDL- and acLDL-induced foam cell formation. Most importantly, M4 macrophages lose the hemoglobin surface receptor CD163 and accordingly are unable to upregulate athero-protective heme oxygenase-1 in response to exposure to hemoglobin/haptoglobin complexes – a mechanism, which is preserved in M0 macrophages.

It has been demonstrated that circulating activated platelets can bind to leukocytes, especially monocytes, resulting in platelet-monocyte/leucocyte aggregates [41]. Thereby the chemokines CCL5 (RANTES (regulated on activation normal T cell expressed and secreted)) and CXCL4 are presented on both the monocyte and endothelial cell membrane surfaces. The co-presentation of CXCL4 and CCL5 increased monocyte recruitment to the endothelium more than presentation of CXCL4 or CCL5 alone, respectively [38]. CXCL4 has been shown to enhance the arrest of CCL5-stimulated monocytes and monocytic cells on activated endothelial under flow conditions *in vivo* by a robust heterophilic interaction with CCL5 [38].

Possible explanations for this finding might be that CXCL4 and CCL5 might either bind to different binding sites of the same receptor or that by binding different receptors they might cause these receptors to form heterodimers [38]. The latter notion is supported by observations that inhibition of dimerziation of CXCL4 and CCL5 by a small molecule inhibited atherosclerosis in hyperlipidemic *Apoe*$^{-/-}$ mice [42]. CXCL4 and CCL5 are also known to form stable oligomeric structures larger than dimers (tetramers and polymers, respectively) even in the absence of glycosaminoglycans (GAG) which is essential for binding of chrondroitin sulfate A (CS-A). It is believed that oligomerization might enable these chemokines to bridge multiple sulfation sites along a GAG chain, contributing to their relatively unique ability to bind CS-A as well as their rank as the highest affinity chemokines for heparan sulfate and heparin. Chrondroitin sulfate is most prevalent in cartilage, but can also be found with heparan sulfate on syndecan-1 and syndecan-4 proteoglycans (PGs) on epithelial cells [43]. Dyer et al. could demonstrate that, by simultaneous interaction with CS and HS, CXCL4 and CCL5 possibly induce cross-link formation of GAG chains and thereby enhance localized chemokine presentation [44].

One of the earliest events in the process of atherogenesis is endothelial damage. One mechanism leading to vascular damage by cell death of endothelial cells, primarily by apoptosis, is oxidative stress induced by reactive oxygen species (ROS). Woller et al. investigated the interaction of human monocytes and endothelial cells upon activation of monocytic cells by CXCL4. They could thereby demonstrate in their experiments that CXCL4-activated monocytes become cytotoxic for endothelial but not epithelial cells in a time- and dose-dependent manner as they release high amount of ROS for more than 2 h following stimulation mediated by beta2 integrin ICAM-1 interaction. This causes apoptosis in endothelial cells. Woller et al. proposed that this particular pathway could be of particular importance in the pathomechanism of vascular damage caused by ischemia and reperfusion injury following revascularization [45].

5. CXCL4 effects on macrophage differentiation

Previously, CXCL4 had been found to prevent monocyte apoptosis and promote cell survival [46]. Several groups had shown in the past that CXCL4 in conjunction with GM-SCF promotes differentiation of monocyte into macrophages and inhibits apoptosis of macrophages [46]. Also differentiation of monocytes into specialized antigen-presenting dendritic cells can be induced by CXCL4 in conjunction with IL-4 [47].

Thus, we investigated the effects of the platelet chemokine CXCL4 on macrophage differentiation [40]. Interestingly, up to 2010 it had not

been investigated whether CXCL4-induced macrophages are any different from other macrophages types. We performed transcriptomic analyses of monocyte-derived macrophages induced by either macrophage colony-stimulating factor (M-CSF) or CXCL4 *in vitro* [40]. In fact, after six days cells had undergone the expected morphological changes associated with macrophage differentiation. Also, CD45 and CD68 expression went up, while CD14 expression was downregulated. Furthermore, there was a highly significant correlation regarding gene expression between M-CSF- and CXCL4-induced macrophages ($r = 0.934$ $P < 0.0001$).

However, statistical analysis also revealed differential expression of 375 genes, among them a large number of genes associated with atherogenesis. CXCL4-induced macrophages showed characteristics of both M1 macrophages, induced by lipopolysacchardide/interferon-γ (M1) and M2 macrophages, induced by IL-4, thus, no clear classical or alternative polarization pattern could be identified. Based on gene enrichment analysis as well as modified principal component analysis and hierarchical clustering, we were able to confirm that CXCL4 induces a specific macrophage transcriptome. Based on these findings, we suggested calling these CXCL4-induced macrophages “M4”.

6. Functional aspects of CXCL4-induced M4 macrophages

Functionally, M4 macrophages showed higher expression of a number of pro-inflammatory cytokines (both at the mRNA and/or at the protein level) including interleukin-(IL-)6, tumor necrosis factor-alpha, CCL18, and CCL22. Also they displayed reduced expression of scavenger receptors such as CD36 or SR-1, associated with reduced capacity of oxLDL-induced foam cell formation (Fig. 2).

Interestingly, CXCL4 leads to complete and irreversible loss of CD163 expression independent of shedding [39]. CD163 is the receptor for hemoglobin and hemoglobin-haptoglobin complexes [48]. It is of special importance in case of intra-plaque hemorrhage. Its engagement leads to induction of atheroprotective heme oxygenase-1 [39]. The entire CD163 heme oxygenase-1 pathway was completely abolished, once monocytes/macrophages were exposed to CXCL4. When analyzing human carotid endarterectomy specimens, we found an inverse correlation between *PF4* and *CD163* mRNA expression. As *PF4* mRNA is exclusively expressed in platelets (and megakaryocytes absent from plaques) and *CD163* mRNA expression is restricted to myeloid cells, we concluded that our *in vitro* findings may in fact be of relevance in human atherosclerosis.

Furthermore, CXCL4 has been shown to inhibit binding and uptake of LDL mediated by the LDL receptor. PF4 bound to ox-LDL directly and also increased ox-LDL binding to vascular cells and macrophages. PF4 did not stimulate ox-LDL binding to cells that do not synthesize glycosaminoglycans or after enzymatic cleavage of cell surface heparan and chondroitin sulfates. The effect of PF4 on binding ox-LDL was dependent on specific lysine residues in its C terminus. Addition of PF4 also caused an approximately 10-fold increase in the amount of ox-LDL esterified by macrophages. Furthermore, PF4 and ox-LDL co-localize in atherosclerotic lesion, especially in macrophage-derived foam cells [49] and thereby CXCL4 promotes foam cell formation in atherosclerotic plaques.

To test whether M4 macrophages are actually present in human atherosclerotic lesions, we had to identify suitable markers allowing unequivocal identification of M4 macrophages with high sensitivity and specificity. Thus, based on transcriptional data from differentially polarized macrophages (M1, M2, and M4), we developed a score that enabled us to specifically identify markers with high expression in M4 macrophage that were not overexpressed in M1 or M2 macrophages. Eventually, the combination of CD68, S100A8, and MMP7 turned out to reliably identify M4 macrophages both *in* vitro and *in vivo* within atherosclerotic lesions [50].

Using this marker combination, we investigated coronary arteries from 52 hearts explanted during heart allograft transplantation [51]. Thirty-two patients underwent transplantation for ischemic heart failure (CAD), 19 for dilated cardiomyopathy (controls). In all atherosclerotic coronary arteries, roughly 32% of all $CD68^+$macrophages fulfilled criteria of M4 polarization (i.e. they were $CD68^+MMP7^+S100A8^+$). The highest numbers of M4 macrophages were found in the adventitia, followed by the intima, only very few in the media. Interestingly, prevalence of M4 macrophages both in the intima and the adventitia was significantly associated with plaque stability as determined by Stary class. Thus, unstable plaques displayed more M4 macrophages than stable plaques. The results of a multivariate analysis taking into account additional factors such as gender, age, or cardiovascular risk factors, suggest that the prevalence of M4 macrophages within the atherosclerotic plaque is a surrogate marker of vascular inflammation.

7. The quest for the CXCL4 receptor

The quest for the CXCL4 receptor has been long and only partially successful. On the search of its receptors, Brandt et al. could show in 2000 that in neutrophils, CXCL4 binds to a chondroitin sulfate proteoglycan (REF). In microvascular endothelial cells (HMEC-1), Lasagni et al. found that CXCL4 specifically binds to CXCR3B, a splice variant of the CXCR3 receptor [52]. Chemotactic effects of CXCL4 on lymphocytes are also primarily promoted by CXCR3-B [35]. However, while an activation of microvascular endothelial cells was not affected by pertussis toxin, showing that it is not mediated by a G protein-coupled receptor, incubation of lymphocytes with pertussis toxin partially inhibited the stimulating effects of CXCL4. Therefore it has been postulated that chemotactic activity is stimulated by CXCL4-dependent G-protein coupled receptor activation [35]. Our own data suggest that CXCR3 does not play a role in CXCL4 effects on human macrophages. For example, blocking the CXCR3 receptor by an antibody did not alter CXCL4-induced downregulation of the hemoglobin-haptoglobin receptor CD163 [39]. By contrast, chlorate, an inhibitor of glycosaminoglycan synthesis, mitigated CXCL4-induced macrophage differentiation suggesting binding to a chondroitin sulfate proteoglycan to be the relevant mechanism by which CXCL4 acts on macrophages [39,53]. It can be speculated that these chondroitin sulfates are bound to an unknown core protein. Thus, there are still unresolved questions to be answered in the future.

8. CXCL4-induced intracellular signal transduction

In neutrophils CXCL4 exocytosis is induced by CXCL4 and p38 mitogen-activated protein (MAP) kinase co-stimulation and adherence of neutrophils is stimulated by an CXCL4-dependent activation of src-kinases [54]. In monocytes activation of PI3K, Syk and p38 MAPK by CXCL4 triggers respiratory, while delayed activation of Erk, with a maximum activity after 6 h of stimulation, lead to increased monocyte survival and differentiation. Moreover up-regulation of chemokine and cytokine mRNA as well as protein has been shown to be dependent on a CXCL4-JNK signaling pathway [55].

9. CXCL4 in comparison to its variant CXCL4L1

CXCL4L1 (PFalt, PF4var1), which is also released by platelets, is a nonallelic variant of CXCL4 [56]. When CXCL4 and CXCL4L1 are compared at a genetic level, they show a strong homology suggesting that they developed by duplication of a common origin gene. This differentiation is immanent in humans a there is no orthologue gene sequence coding for CXCL4L1 in the mouse genome [57]. At the protein level, in CXCL4L1 only three amino acids in the C-terminal alpha helix are substituted, compared to CXCL4 [56]. For CXCL4L1, the substitution of leucin by histidine in the position 67 has been attributed to angiostatic effects and binding of heparin, heparan sulfate and chrondroitin sulfate B [58].

While CXCL4 is stored in granules and released upon activation of protein kinase C, CXLC4L1 is synthesized and secreted continuously [59]. Furthermore CXCL4 has first and foremost tissue specific localized effects, CXCL4L1 is highly diffusible [58].

At a functional level, in contrast to M-CSF and CXCL4, CXCL4L1 is not a survival factor for monocytes. CXCL4L1 also did not affect epithelial cell proliferation [60]. Notably, expression of chemokine receptors CCR2, CCR5 and CXCR3 was significantly higher on CXCL4L1-treated monocytes compared to M-CSF- and CXCL4-stimulated monocytes. IL-1 receptor antagonist (IL-1RN) expression was upregulated by CXCL4 and downregulated by CXCL4L1, respectively, whereas both chemokines reduced the expression of the mannose receptor (MRC) [61]. Furthermore, CXCL4L1 has been shown to induce angiostasis and chemotaxis by binding to CXCR3 [62] and angiostatic effect are mediated by its COOH-terminal peptide [63]. Gouwy et al. could demonstrate that through activation of CXCR3, CXCL4L1-stimulated monocytes released significantly higher amounts of CCL2 and CXCL8 compared to CXCL4-treated monocytes, which they suggested shows more pronounced inflammatory traits for CXCL4L1 [61]. Perplexingly, CXCL4L1 inhibited endothelial chemotaxis much more strongly than CXCL4 [56]. Contrastingly, CXCL4L1 was not able to induce monocyte recruitment in conjunction with CCL5, which suggests that CXCL4-CCL5 heterodimerization is appertain to CXCL4.

Also, differences in immature monocyte-derived dendritic cells (iMDDC) upon incubation with either CXCL4 or CXCL4L1 have been found. Especially, CXCL4 was able to increase phagocytotic capacity and lead to downregulation of matrix-metalloproteases in these cells when compared to CXCL4L1 [61]. When evaluating the potential role of CXCL4L1 for atherosclerosis *in vivo* studies provide further insight. De Sutter et al. could show that low PF-4var/CXCL4L1 levels are associated with a poor outcome (that is a significant higher rate of the combines endpoint cardiac death, non-fatal acute myocardial infarction, stroke or hospitalization for heart failure) in patients with stable CAD and preserved LV function [64]. In conclusion, while CXCL4 has mainly proinflammatory effects in atherogenesis, CXCL4L1 may at the same time act as a competitive antagonist with a potential antiinflammatory and plaque-stabilising effect. Although direct evidence is yet missing, CXCL4L1 could hypothetically induce neovascularization at a microvascular level particularly in the myocardium by its angiostatic activity. This could potentially result in a better vascularisation in this area and thereby prevent myocardial ischemia. As CXCL4L1 does not downregulate MMPs when compared to CXCL4, this may prevent abnormal diastolic stiffness due to interstitial fibrosis, and reduced coronary vasodilator reserve associated with medial wall thickening of intramyocardial resistance vessels.

10. Summary and conclusions

In summary, over the past 15 years we and others could demonstrate that the platelet chemokine CXCL4 has significant effects on monocytes and macrophages reflected by induction of a specific macrophage phenotype, which we suggested to term “M4”. M4 macrophages are distinct from previously described macrophage polarization types. Other than previously identified macrophage polarizations, M4 polarization is irreversible. They can be identified in human tissue by co-expression of CD68, MMP7, and S100A8 with good sensitivity and specificity. In human atherosclerotic plaques, M4 macrophages are predominantly present in the adventitia and the intima. Their prevalence is associated with plaque instability; most likely they are a marker of inflammatory activity within the plaque. Future research will have to elucidate the effects of CXCL4 on macrophages on a mechanistic molecular levels leading to definite identification the receptor(s) and signal transduction pathways involved. Furthermore, it will have to be tested whether M4 macrophages represent a suitable target for diagnostic or therapeutic interventions in human atherosclerotic disease. In conclusion, while CXCL4 clearly play a proinflammatory role in atherogenesis, clinical data suggest that CXCL4L1, a variant of CXCL4, might act as a competitive antagonist with a mainly antiinflammatory and plaque-stabilising effects.

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