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Inflammatory mediators in atherosclerotic vascular disease

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Abstract An impressive body of work has established the current paradigm of atherosclerosis as an inflammatory process that promotes lesion development and progression. Early atheroma formation is characterized by leukocyte recruitment and expression of inflammatory mediators which is confounded in the context of hyperlipidemia. Evidence for an involvement of both innate and adaptive immunity in lesion formation has emerged, supporting a causal relation between the balance of pro- and anti-inflammatory cytokines and atherogenesis. The function of chemokines in distinct steps during mononuclear cell recruitment to vascular lesions has been studied in genetically deficient mice and other suitable models, and displays a high degree of specialization and cooperation. The contribution of platelet chemokines deposited on endothelium to monocyte arrest, differences in the presentation and involvement of chemokines between native and neointimal lesion formation, and related functions of macrophage migration inhibitory factor, a cytokine with striking structural homology to chemokines are of note. A novel role of chemokines in the recruitment of vascular progenitors during neointimal hyperplasia and in the recovery of endothelial denudation underscores their relevance for atherosclerotic vascular disease. The functional diversity of chemokines in vascular inflammation may potentially allow the selective therapeutic targeting of different atherosclerotic conditions.

Key words Cytokines – chemokines – atherosclerosis – inflammation

Abbreviations

ApoE	apolipoprotein E
CCL	CC chemokine ligand
CCR	CC chemokine receptor
CD	cluster of differentiation
CXCL	CXC chemokine ligand
CXCR	CXC chemokine receptor
EC	endothelial cell
GRO	growth-related oncogene
IFN-γ	interferon-γ
IL	interleukin
KC	keratinocyte-derived chemokine
LDL-R	low density lipoprotein receptor
MCP	monocyte chemotactic protein
MIF	macrophage migration inhibitory factor
Mig/IP10	monokine induced by IFN-γ/IFN-γ-inducible protein-10
RANTES	regulated on activation normal T cell expressed and secreted
SDF	stromal cell-derived factor
SMC	smooth muscle cell
TGF-β	transforming growth factor-β
TNF	tumor necrosis factor

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Introduction

An impressive body of work has established and advanced the current paradigm of atherosclerosis as an inflammatory process that couples dyslipidemia to atheroma formation, intitiates lesion development and promotes its progression to the point of acute thrombotic complications and clinical manifestations [43]. Early atherogenesis is characterized by the inflammatory recruitment of leukocytes and the expression of inflammatory mediators confounded in the context of hyperlipidemia. Convincing evidence has emerged for a crucial involvement of innate immunity based on the detection of pathogen-associated molecular patterns by respective recognition receptors, including phagocytes, complement and proinflammatory cytokines in atherogenesis. In addition, disease activity and progression is modulated by adaptive immunity involving T cells, antibodies and immunomodulatory cytokines [32]. The synopsis in Fig. 1 depicts some of the most eminent cytokines involved.

Pro- and anti-inflammatory cytokines in atherogenesis

Recent research has established a causal relation between pro-inflammatory (e.g. TNF-α, IFN-γ, CD40 ligand) and anti-inflammatory cytokines (e.g. IL-10, TGF-β) and their intricate cross-talk and the inflammatory pathogenesis of atherosclerosis. For instance, the blockade or genetic deletion of CD40 ligand (CD40L) has been shown to interfere with the progression of atherosclerosis in hyperlipidemic mice [48, 50]. As evident by genetic deletion of its receptor, IFN-γ has been found to potentiate the formation of less stable atherosclerotic lesions, but can also elicit atherosclerosis in the absence of immunocytes by enhancing smooth muscle cell (SMC) mitogenesis [27, 82]. It has initially been reported that the blockade of TNF-α unlike that of IL-1 appeared to only protect against atherosclerosis in female mice [18]. More recently, however, the genetic deletion and neutralization of TNF-α leading to reduced lesion formation in hyperlipidemic apolipoprotein E-deficient (apoE$^{-/-}$) mice has revealed an active involvement of TNF-α in the pro-

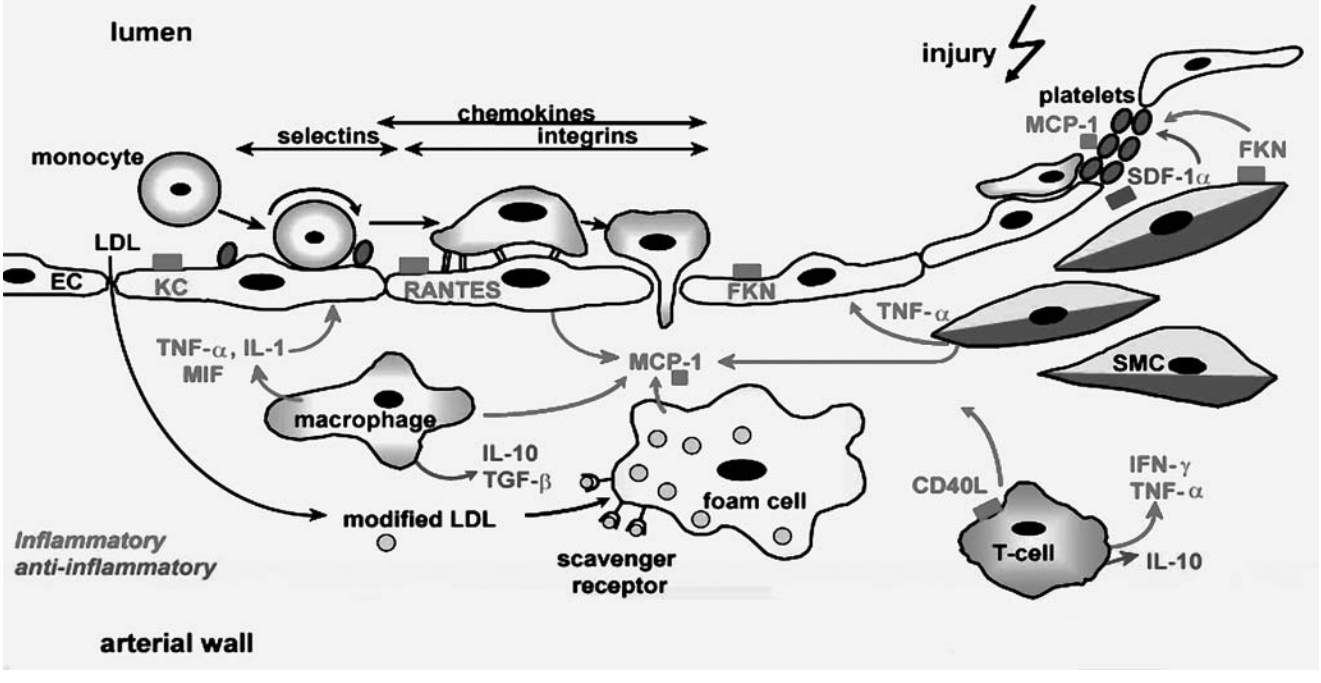

Fig. 1 Pro- and anti-inflammatory cytokines and chemokines regulate mononuclear cell recruitment to vascular lesions. According to the multistep paradigm, leukocyte trafficking is initiated by selectin-mediated rolling interactions followed by integrin-dependent arrest on cytokine-activated endothelium and transmigration induced by chemokines. Activated platelets deposit chemokines, such as RANTES, on endothelial cells (EC) lining early atherosclerotic lesions, triggering the arrest of monocytes. Endothelial fractalkine (FKN) and immobilized KC induce the firm adhesion of monocytes via CX_3CR1 and CXCR2, respectively. Smooth muscle cells (SMCs) can express FKN and SDF-1α, and secrete MCP-1, which is presented on adherent platelets, to trigger monocyte arrest. MCP-1 (via CCR2) and soluble FKN can mediate the subsequent subintimal immigration of monocytes or T cells. Extravasated macrophages accumulate modified LDL and transform into foam cells, characteristic of fatty streak lesions. Intimal mononuclear cells contribute to atherosclerosis through the synthesis of proinflammatory cytokines (TNF-α, IL-1, MIF, IFN-γ, CD40L), but also anti-inflammatory mediators (IL-10).

gression of atherosclerosis [8]. Contradictory reports that genetic deletion of TNF-α fails to reduce atherosclerosis, while deletion of its receptor p55 may even accelerate atherosclerosis [73, 74] may be explained by differences in models and analysis or attributable to a role for TNF-α receptors in the balance of pro- and antiapoptotic signaling during atherogenesis, respectively. On the other hand, deficiency in the anti-inflammatory cytokine IL-10 increased lipid accumulation, T cell infiltration and IFN-γ expression in atherosclerotic lesions [51]. Similarly, a disruption of TGF-β signaling in T cells resulted in accelerated atherosclerosis, a more vulnerable phenotype and increased IFN-γ levels [65], underscoring the decompensated balance between pro- and anti-inflammatory mechanisms in atherosclerosis. While increased circulating levels of these inflammatory markers, for instance CD40L, can serve as markers for cardiovascular risk and predictors of acute vascular syndromes [4], their mechanisms of actions are highly pleiotropic and often share common pathways. For instance, most inflammatory cytokines, such as CD40L, induce the expression and secretion of chemoattractant cytokines (chemokines) in endothelial cells [34]. Despite the apparent redundancy in their expression, chemokines may allow even more specific mechanistic clues and may serve as more specific markers for different stages and types of atherosclerotic lesions.

Inflammatory leukocyte infiltration during atherosclerosis

Expressed in atheroma, adhesion molecules and chemokines guide the early subintimal infiltration with mononuclear cells as the first morphological sign of inflammation in the arteries [62, 76, 79]. The mononuclear cells found in the lesions are comprised of about 80% monocyte-derived macrophages, which transform into foam cells characteristic for fatty-streak lesions, and of about 10–20% lymphocytes predominantly of the Th1 helper subtype of T memory cells [31]. In addition, the presence of dendritic cells interacting with T cells has been convincingly revealed in atherosclerotic lesions as part of the adaptive immunitiy [5, 31]. Moreover, the recruitment of a blood-borne progenitor cell subpopulation eventually giving rise to neointimal smooth muscle cells (SMCs) and endothelial cells (ECs) has been directly demonstrated in various models of atherosclerosis [67, 70].

Chemotactic cytokines (chemokines)

The multistep paradigm of leukocyte trafficking involves sequential and overlapping interactions with the vessel wall, initiated by selectin-mediated rolling interactions followed by the firm integrin-dependent arrest and transendothelial migration towards a chemokine gradient (see Fig. 1). This leukocyte cascade can be hierarchically controlled by functionally specialized chemokines, i.e. chemokines can activate integrin adhesiveness to convert rolling into firm arrest or induce directed migration of monocytes [78]. These functions are to some degree mutually overlapping, for instance, integrins can mediate rolling interactions, while selectin-interactions can serve as a prerequisite for transmigration, and some chemokines, such as fractalkine, can mediate arrest independent of and possibly prior to integrin interactions. The functional specificity of chemokines may also be associated with differences in their presentation and immobilization to the endothelial surface, which appears crucial for the efficacy of their arrest function and has also been implicated in the migratory response under shear flow (chemorheotaxis) [12, 23, 89, 92]. Chemokines constitute a family of structurally related and secretable basic chemotactic cytokines, which are classified in subgroups (CC, CXC, C, CXXXC) according to the position of the N-terminal cysteines.

MCP-1/CCR2 in native and accelerated atherosclerosis

As the first and prototypic CC chemokine, MCP-1 (CCL2) has been detected in human atherosclerotic lesions and is induced primarily in medial and neointimal SMCs as well as in monocytes/macrophages in animal models of atherosclerosis with dietary-induced hypercholesterolemia [56, 95, 96]. Given the pivotal role of monocytes in the process of lesion formation, it was not surprising that the direct evidence for the critical function of chemokines in atherogenesis came from the genetic deletion of the CC chemokine MCP-1 or its receptor CCR2, which mediates the attraction of monocytes but not neutrophils. The absence of MCP-1 or CCR2 in an atherogenic, i.e. either LDL receptor- ($LDLR^{-/-}$) or $apoE^{-/-}$ background, protects mice from developing atherosclerotic lesions [7, 16, 25]. Anti-MCP-1 gene therapy attenuated lesion formation in $apoE^{-/-}$ mice [57]. Moreover, transplantation of apoE3-Leiden mice with bone marrow deficient in CCR2 confirmed the pivotal role of CCR2 expressed on monocytes in the process of atherogenesis [26]. Interestingly, an up-regulation of CCR2 on monocytes has been described in the context of hyperlipidemia [30], which may account for the over-recruitment on monocytes into the vessel wall [30, 55, 81].

A central role of the MCP-1/CCR2 axis in monocyte recruitment and lesion formation was also demonstrated in more complex models of accelerated atherosclerosis after vascular injury. In hyperlipidemic $apoE^{-/-}$ mice,

wire-induced injury of the carotid artery causes the rapid up-regulation of MCP-1 levels in serum and in medial SMCs. In addition, locally secreted MCP-1 can be retained and presented by platelets adhering to the injury site [72]. This is in accordance with *in vitro* studies revealing low affinity binding of MCP-1 to platelets despite a lack of functional CCR2, and extends evidence that endogenous chemokines can be concentrated on the surface of activated platelets, possibly via binding to proteoglycans [13, 22, 39]. Although MCP-1 or CCR2 are not involved in monocyte accumulation on early atherosclerotic endothelium in uninjured carotid arteries, blockade of MCP-1 profoundly inhibited monocyte arrest in denuded apoE$^{-/-}$ carotid arteries perfused *ex vivo* [36, 72]. This suggests a differential and distinctive contribution of MCP-1 to monocyte arrest after endothelial denudation, which may require its local concentration by binding to adherent platelets at the injury site. A causal relationship between early MCP-1-dependent monocyte arrest on denuded vessels and neointimal hyperplasia is inferred by reduced neointimal plaque area and macrophage content in hypercholesterolemic rabbits following angioplasty [52, 53] and in hyperlipidemic apoE$^{-/-}$ mice with genetic deficiency in CCR2, while the relative content of neointimal SMCs is expanded [72]. The function of the MCP-1/CCR2 axis in vascular repair and monocyte recruitment, however, appears to differ between normo- and hyperlipidemic models and the role of MCP-1/CCR2 in macrophage recruitment after arterial injury is less well established in normolipidemia. While monocyte accumulation was reduced after arterial cuff placement or stent placement in normolipidemic animals [17, 35], neointimal SMC content was decreased and macrophage content was unaffected in CCR2$^{-/-}$ mice or MCP-1 antibody-treated rats after endothelial denudation [20, 66]. It could therefore be hypothesized that MCP-1 expression by medial SMCs after endothelial denudation is aggravated in the context of hypercholesterolemia [96], so that sufficient concentrations may be achieved for immobilization on adherent platelets and triggering monocyte recruitment in denuded vessels.

The platelet chemokine RANTES in atherogenesis

The presence of RANTES (CCL5) has been revealed on the luminal surface of carotid arteries with early atherosclerotic endothelium or on neointimal lesions following arterial injury in apoE$^{-/-}$ mice [87]. Subsequently, it could be demonstrated that activated platelets can deliver RANTES and PF4 to the endothelial lining of early atherosclerotic and neointimal lesions, as well as to the surface of monocytes via a mechanism involving platelet P-selectin [36, 71]. The deposition and immobilization of platelet-derived RANTES has been shown to trigger enhanced recruitment of monocytes on activated aortic endothelium [87, 71]. The concept that the deposition of RANTES may be an important mechanism underlying the involvement of platelets in native lesion formation is corroborated by findings that the long-term treatment with Met-RANTES reduced atherosclerotic lesion formation in apoE$^{-/-}$ mice [84]. Alternatively, this could be explained by a blockade of RANTES produced in mononuclear cells infiltrating the lesions or by modulating other chemokine receptors, e.g. by decreasing CCR2 mRNA [84]. While the RANTES receptor CCR1 but not CCR5 mediates the RANTES-induced arrest of monocytes, activated T cells and Th1-cells, CCR5 is demonstrated to support their spreading and both CCR1 and CCR5 to contribute to the transendothelial chemotaxis of these cells triggered by RANTES [91]. Interestingly, the targeted disruption of the RANTES receptor CCR5, failed to protect from atherosclerotic lesion formation [40], and data on a deletion of the other RANTES receptors CCR1 and CCR3 are not yet available. This demonstrates that the engagement of different receptors by the same chemokine ligand can be associated with dramatically distinct functions, further extending the selectivity of specialization. After arterial injury, a blockade of RANTES with the Met-RANTES receptor antagonist did not only inhibit RANTES-mediated arrest *in vitro* but also neointimal macrophage infiltration and hyperplasia in hyperlipidemic mice [71].

Other CC chemokines expressed in atherosclerotic lesions

The CC chemokines TARC (CCL17), PARC (CCL18), and MDC (CCL22) have been identified in macrophage-rich areas of atherosclerotic lesions [24, 63], while ELC (CCL19) expression has been detected in both SMCs and monocyte-derived macrophages of human plaques [24]. Also, the presence of the typically T cell-derived CC chemokines MIP-1α (CCL3), MIP-1β (CCL4) and I-309 (CCL1) could be demonstrated in atherosclerotic plaques [33, 93]. In addition, a novel pathway of vascular inflammation has also been suggested by identifying the overexpression of eotaxin and its receptor CCR3 in atherosclerotic lesions [28].

CXC chemokines in atherogenesis

On the other hand, CXC chemokines with (IL-8/CXCL8, GRO-α/CXCL1) or without ELR motif (Mig/CXCL9, IP10/CXCL10, I-TAC/CXCL11, SDF-1α/CXCL12) are detectable in atherosclerotic lesions [1, 24, 49, 88]. As mice deficient in KC and CXCR2 are not viable or are

extremely susceptible to infection, respectively, the repopulation of atherosclerosis-prone LDLR$^{-/-}$ mice with bone-marrow deficient in CXCR2, the receptor for the neutrophil chemokines IL-8 and GRO-α, resulted in a substantial reduction of atherosclerosis [6]. Since neutrophils are not present in atherosclerotic lesions, this study implied an involvement of CXCR2 in the atherogenic recruitment of monocytes or other bone marrow derived cells. Indeed, CXCR2 is expressed on monocytes [11, 54], and the CXCR2 ligands IL-8 and GRO-α have been shown to enhance adhesion of monocytes on activated endothelial cells and matrix proteins or ECs activated with modified lipoproteins, respectively [47, 75]. Moreover, these ELR CXC chemokines have been implicated in angiogenesis [2, 38] and by promoting plaque neovascularization; this may provide an alternative explanation for the contribution of CXCR2 to atherosclerosis. The blockade of KC in the apoE$^{-/-}$ mice after arterial wire-injury inhibits re-endothelialization and aggravates neointimal growth, but does not affect monocyte recruitment after mechanical injury [44].

The expression of the CXCR3 ligands Mig, IP10 and I-TAC has been detected in atheroma-associated cells, including ECs, as well as an expression of their receptor CXCR3 on lesional T cells [24]. Although a direct involvement of CXCR3 ligands in atherogenesis and atherogenic recruitment of mononuclear cells has not yet been reported, their functional role in recruitment of T cells is strongly inferred by findings that CXCR3 mediates the rapid and shear-resistant arrest of effector T lymphocytes triggered by IP10 and Mig on stimulated ECs [60]. More recent clues for a specific contribution of CXCR3 to atherogenesis indeed indicate a function in early T cell-driven lesion formation not overlapping with a more prominent role of CCR2 in advanced lesions. Non-redundant roles of CCR2 and CXCR3 are also indicated by the more pronounced protection against overall lesion formation in CCR2$^{-/-}$ CXCR3$^{-/-}$ apoE$^{-/-}$ triple knock-out as compared with apoE$^{-/-}$ mice or deletion of either receptor in apoE$^{-/-}$ mice alone [86].

The role of the CXC chemokine SDF-1α in SMC progenitor recruitment

The CXC chemokine SDF-1α is essential for stem cell mobilization, bone marrow engraftment and homing, as well as organ system vascularization [41, 59, 60, 80]. It is also expressed in human atherosclerotic plaques and effectively activates platelets *in vitro* [1]. Reduced SDF-1α plasma levels are associated with symptomatic coronary artery disease, suggesting an anti-inflammatory role for SDF-1α in stabilizing the phenotype of native atherosclerotic plaques [15]. Since bone marrow-derived cells have been shown to contribute to neointimal SMC content in native atherosclerosis or after arterial injury [29, 67, 70] and circulating SMC progenitors have been found in human blood [77], this was highly suggestive of a participation of SDF-1α in human atherothrombotic disease and the response to vascular trauma. Indeed, SDF-1α plasma levels were transiently elevated after wire-injury of carotid arteries in apoE$^{-/-}$ mice and mediated the marked expansion of sca-1^{+} lineage^{-} peripheral blood progenitor cells [70]. Neutralizing SDF-1α markedly reduced the neointimal area and the relative content of SMCs but not macrophages [70] and thus inferred an instrumental role in neointima formation after injury in apoE$^{-/-}$ mice by recruiting circulating SMC-progenitors. Thus, it is conceivable that the expression of SDF-1α in native lesions may also regulate plaque composition by sustaining a chronic influx of SMC progenitors at low levels and thus, in contrast to its adverse effects in neointima formation, may attenuate the inflammatory progression and rather promote stabilization of native atherosclerotic lesions.

The CX_3C chemokine fractalkine

The transmembrane chemokine fractalkine (CX_3CL1) is detectable in atherosclerotic lesions [81], in lesional ECs and most robustly in SMCs located directly beneath lesional macrophages but not in macrophages themselves [24, 42, 94]. Fractalkine is a structurally distinct chemokine fused to a transmembrane mucin stalk [3] and represents another candidate engaged in atherogenic leukocyte recruitment. While the transmembrane protein acts as an efficient adhesion molecule capturing monocytes and T cells on activated endothelium under flow conditions by an integrin-independent mechanism [19, 97], cleavage of the mucin stalk can produce a soluble form of fractalkine with chemoattractant activity for these cells [21, 83]. Migration but not arrest induced by fractalkine, although both mediated through CX_3CR1 is sensitive to pertussis toxin, supporting the concept that specialized functions in arrest and migration require distinct signal transduction pathways [3, 19, 46, 97]. Recently, a novel role for membrane-bound fractalkine has been described in platelet activation and adhesion [68], which may sustain recruitment mechanisms involving the delivery of platelet-derived chemokines. A role of fractalkine in atherogenesis was convincingly derived from the genetic deletion of its receptor CX_3CR1 in atherosclerosis prone apoE$^{-/-}$ mice, which lead to a reduced macrophage infiltration and retarded lesion development in the aorta [14, 42].

The chemokine-like function chemokine MIF regulates plaque formation

An upregulation of the pleiotropic inflammatory T cell and macrophage cytokine MIF has been observed in ECs, SMCs and macrophages during the progression of atherosclerosis in humans and in hypercholesterolemic rabbits [9, 45]. The genetic deletion of MIF in LDLR$^{-/-}$ mice has been shown to reduce lipid deposition and intimal thickening in the aorta [58]. This retardation of native atherogenesis was accompanied by a decrease in lesional cell proliferation, protease expression and activity.

The role of MIF was also studied in models of accelerated atherosclerosis after vascular injury [10, 69]. After wire- injury of carotid arteries in apoE$^{-/-}$ mice, an early upregulation of MIF expression was detected in SMCs but predominantly found in ECs and macrophage-derived foam cells at later stages. Neutralizing MIF markedly reduced neointimal macrophage content and inhibited their transformation into foam cells while increasing the content of SMCs and collagen in the neointima, amounting to a slight reduction in neointimal area [69], and reflects a remarkable shift in the cellular composition of neointimal plaques towards a stabilized phenotype. Moreover, blocking MIF in a study of arterial injury in LDLR$^{-/-}$ mice inhibited neointimal hyperplasia and macrophage infiltration as well as SMC proliferation, confirming an important role of MIF in plaque formation [10].

In vitro, short-term incubation of aortic ECs with MIF triggers monocyte arrest under flow conditions and MIF mediates the monocyte arrest induced by oxidized LDL [69]. This observation supports a model where MIF directly affects endothelial-monocyte interactions by a novel mechanism resembling the function of immobilized chemokines in both native atherogenesis and after injury. Thus, the contribution of MIF to atherogenesis may at least in part be due to a chemokine-like function.

Conclusions

With the apparent redundancy of chemokine expression and function in atherosclerotic lesions in mind, it is not easily conceivable why a deletion of individual chemokines or their receptors would each lead to a marked reduction in lesion formation and is not compensated by other chemokine-receptor pairs. This would indicate that single chemokines do not act independently but rather in concert to efficiently recruit circulating monocytes into lesions, and further insinuates that a functional specialization of chemokines and their receptors for different mononuclear subsets and precursors of other vascular cell types at distinct steps of the atherogenic recruitment process exists to allow for non-redundant roles (see Fig. 1). This serves to distinguish the classical proinflammatory cytokines, such as TNF-α, IL-1 or CD40L, which share pleiotropic effects on different vascular and mononuclear cell types, e.g., the NF-κB-dependent induction of multiple CC and CXC chemokines. The complexity of chemokine induction is increased by the counter-balance provided by anti-inflammatory cytokines, but also by notable exception that some proinflammatory chemokines can only be upregulated by a combination of cytokines, as exemplified by Mig/IP10 induction by TNF-α and IFN-γ [60]. Since the expression of different inflammatory mediators correlates with the amount of inflammatory cells, an imbalance between pro- and anti-inflammatory mediators might play a causal role in atherosclerosis [85]. In addition to the rather crude regulation of expression, remarkable differences exist in the presentation and functional involvement of chemokines between native atherogenesis and neointima formation after injury, as well as throughout the course of plaque formation, further contributing to the highly elaborate specialization of chemokines in mononuclear cell recruitment [90].

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