Macrophage migration-inhibitory factor (MIF)-based therapeutic concepts in atherosclerosis and inflammation

Dzmitry Sinitski[1]*; Christos Kontos[2]*; Christine Krammer[1]*; Yaw Asare[3],

Aphrodite Kapurniotu[2#]; Jürgen Bernhagen[1,4,5,#]

[1]Department of Vascular Biology, Institute for Stroke and Dementia Research (ISD), Klinikum der Universität München (KUM), Ludwig-Maximilians-University (LMU), 81377 Munich, Germany; [2]Division of Peptide Biochemistry, Technische Universität München (TUM), 85354 Freising, Germany; [3]Department of Translational Medicine, Institute for Stroke and Dementia Research (ISD), Klinikum der Universität München (KUM), Ludwig-Maximilians-University (LMU), 81377 Munich, Germany; [4]Munich Heart Alliance, 80802 Munich, Germany; [5]Munich Cluster for Systems Neurology (SyNergy), 81377 Munich, Germany.

*contributed equally

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[#]Shared last authorship and correspondence:

Professor Jürgen Bernhagen, PhD

Chair of Vascular Biology

Institute for Stroke and Dementia Research (ISD)

Klinikum der Universität München (KUM)

Ludwig-Maximilians-University (LMU) Munich

Feodor-Lynen-Straße 17, 81377 Munich, Germany

Tel.: 0049-89 4400 - 46151

Fax: 0049-89 4400 - 46010

E-Mail: juergen.bernhagen@med.uni-muenchen.de

Professor Aphrodite Kapurniotu, PhD

Division of Peptide Biochemistry

Technische Universität München (TUM)

Emil-Erlenmeyer Forum 5, 85354 Freising, Germany

Tel.: 0049-8161-713 542

Fax: 0049-8161-713 298

E-mail: akapurniotu@wzw.tum.de

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ABSTRACT

Chemokines orchestrate leukocyte recruitment in atherosclerosis and their blockade is a promising anti-atherosclerotic strategy, but few chemokine-based approaches have advanced into clinical trials, in part owing to the complexity and redundancy of the chemokine network. Macrophage migration-inhibitory factor (MIF) is a pivotal mediator of atherosclerotic lesion formation. It has been characterized as an inflammatory cytokine and atypical chemokine (ACK) that promotes atherogenic leukocyte recruitment and lesional inflammation through interactions with the chemokine receptors CXCR2 and CXCR4, but also exhibits phase-specific CD74-mediated cardioprotective activity. The unique structural properties of MIF and its homolog MIF-2/D-DT offer intriguing therapeutic opportunities including small molecule-, antibody-, and peptide-based approaches that may hold promise as inhibitors of athero-sclerosis, while sparing tissue-protective classical chemokine pathways. In this review, we summarize the pros and cons of anti-MIF protein strategies and discuss their molecular characteristics and receptor specificities with a focus on cardiovascular disease.

Key Words: MIF / chemokine receptor / atypical chemokine / small molecule drug (SMD) compound / peptide / antibody

NON-STANDARD ABBREVIATIONS AND ACRONYMS

Apoe$^{-/-}$	Apolipoprotein E
ACK	Atypical chemokine
ACKR	Atypical chemokine receptor
CXCL12	CXC motif chemokine 12
CXCR	CXC motif chemokine receptor
CXCR4	CXC motif chemokine receptor 4
MIF	Macrophage migration-inhibitory factor
SDF-1α	Stromal cell-derived factor-1alpha

INTRODUCTION

Atherosclerosis is a chronic inflammatory disease of our arteries that is characterized by the development of lipid-rich inflamed plaques in the vessel wall. Lesion progression and plaque rupture may result in detrimental cardiovascular events such as acute myocardial infarction and ischemic stroke (1, 2), leading causes of death worldwide (3). Influenced by genetic and environmental risk factors such as hyperlipidemia, atherosclerosis is initiated by endothelial dysfunction, followed by an accumulation of oxidized low-density lipoproteins (oxLDL) and an inflammatory cell infiltrate dominated by monocytes and T cells into the atherogenic vessel wall. Infiltrating monocytes differentiate into macrophages and lipid-laden foam cells. Lesion progression also involves vascular smooth muscle cell (VSMC) proliferation, necrotic core formation, and wall remodeling that may eventually lead to plaque destabilization, rupture, and thrombosis (4).

These processes are mediated by inflammatory cytokines and chemokines at all stages. Some 50 classical chemokines interact with 18 G-protein-coupled receptor (GPCR)-type chemokine receptors. This network is characterized by a high degree of redundancy and promiscuity and chemokines are divided into CC-, CXC-, CX_3C-, and C-type sub-classes and correspondingly-termed receptors (5, 6).

Due to their causal role in atherogenesis, anti-cytokine/-chemokine approaches are pursued as therapeutic strategies to attenuate atherosclerosis (7). A number of chemokine-blocking antibodies and chemokine receptor-inhibiting small molecule compounds (SMDs) are in advanced pre-clinical testing and (early) clinical trial phases (7-11). Importantly, the promising results obtained with an interleukin-1 β (IL-1β)-blocking antibody in the CANTOS trial have validated the inflammatory hypothesis in atherosclerosis and demonstrated the power of cytokine-based anti-inflammatory drugs in patients with established atherosclerotic disease (12).

Macrophage migration-inhibitory factor (MIF) is an inflammatory cytokine with chemokine-like characteristics and unique structural properties and is classified as a prototypical member of the emerging family of ACKs (13-16). ACKs lack the typical chemokine-

fold and conserved N-terminal cysteines of classical chemokines (6), but exhibit chemotactic activity and bind to classical chemokine receptors (16). MIF is upregulated in human atherosclerotic lesions (17) and its levels correlate with coronary artery disease (CAD) (18, 19). *Mif* gene deletion (*Mif-KO*) and antibody-based neutralization of MIF in experimental atherosclerosis suggest it is a major driver of atheroprogression during several stages of the disease (14, 18).

Here we discuss molecular strategies to inhibit MIF and its structural homolog D-dopachrome tautomerase (D-DT), also termed MIF-2, in atherosclerosis and other inflammatory diseases. We cover antibody-based strategies, small molecules directed at the unique MIF catalytic pocket around N-terminal proline-2 or at allosteric sites, and emerging peptide-based approaches. The pros and cons of these strategies, potential side-effects, and envisaged receptor pathway specificities are compared.

MIF IS A CHEMOKINE-LIKE INFLAMMATORY MEDIATOR THAT PROMOTES ATHEROSCLEROSIS

MIF is one of the first cytokines to be discovered. It was originally described in 1966 by John David as a soluble factor produced by human lymphocytes that was capable of inhibiting the random migration of macrophage-like cells out of capillary tubes, while earlier reports on myeloid cell migration even date back to 1932 (15, 20, 21). MIF is a 12.5 kD protein containing 114 amino acids that crystallizes as a trimer, but equilibria between monomers, dimers, and trimers are observed under physiological solution conditions (22, 23). Today, MIF is known as a pleiotropic inflammatory mediator that is structurally distinct from other cytokines, but shares structural homology with bacterial tautomerases/isomerases, suggesting evolutionary conservation (15, 23, 24). It is broadly expressed, but regulated secretion that occurs from semi-constitutive cytosolic stores by a p115-dependent non-conventional mechanism is predominantly seen in cells of the immune system as well as endothelial and tumor cells (15, 25, 26). MIF is the founding member of the MIF protein family that also comprises D-DT/MIF-2 and MIF-like orthologs in numerous species. MIF is an upstream regulator of the host innate

and adaptive immune response, but -if dysregulated- it is a driver of inflammatory diseases as well as cardiovascular diseases including atherosclerosis. Contrary to its eponymous name, MIF has been classified as an ACK that, similar to arrest chemokines such as CXCL1/8, enhances atherogenic leukocyte chemotaxis and arrest. It has been suggested that inhibition of random macrophage migration as observed in the historic experiments, is likely to represent a desensitization effect as well-known for chemokines (14, 16).

Serving as an inflammatory, chemokine-like cytokine and upstream regulator of innate immunity, it is not unexpected that MIF has a key role in numerous inflammatory and autoimmune conditions, including septic shock, rheumatoid arthritis, systemic lupus erythematosus, Crohn's disease, obesity, glomerulonephritis, and inflammatory and allergic lung conditions (reviewed in: (15, 27-30). Owing to the close mechanistic links between chronic inflammation and cancer, MIF also has been identified as a pro-tumorigenic factor in several tumor entities, enhancing cancer cell proliferation, promoting tumor angiogenesis, and modulating anti-tumor immunity (15, 31-34).

Its chemokine-like and inflammatory properties render MIF a potent regulator of the atherogenic process. MIF expression is upregulated in human and murine atherosclerotic lesions with peak levels observed in advanced plaques (17, 19). It is not only upregulated in the atherogenic endothelium and infiltrating leukocytes, but also in vascular smooth muscle cells (VSMCs) and platelets following inflammatory stimulation (17, 35, 36). Antibody-mediated neutralization in *Apoe-/-* mice resulted in reduced lesional immune cell content and lowered levels of inflammatory mediators associated with atherosclerosis (37). Similarly, *Mif*-deficient *Ldlr-/-* mice showed reduced atherosclerotic plaque areas compared to controls (14, 38). Targeting MIF with neutralizing antibodies resulted in significant plaque regression (14). The pro-atherogenic activity of MIF is predominantly mediated via non-cognate interaction with the chemokine receptors CXCR2 and CXCR4, leading to monocyte and T cell recruitment, respectively (14). This is accompanied by an upregulation of adhesion molecules like ICAM-1 and release of atherogenic chemokines such as CCL-2 (39, 40). Moreover, MIF stimulates oxLDL uptake to promote foam cell formation. Foam cells undergo apoptosis and form a

necrotic core surrounded by a fibrous cap (41). MIF is associated with plaque instability as it induces matrix degradation through matrix metalloproteinases (MMPs), followed by fibrous cap thinning resulting in plaque rupture (42). MIF also promotes intra-plaque inflammation by stimulating macrophages to secrete inflammatory mediators such as TNF-α or IL1-β (43). The role of MIF-2 in chronic atherogenesis is subject to current investigations.

Pro-atherogenic effects of MIF are supported by observational clinical studies in CAD patients. For the G/C SNP rs755622 at position -173, a higher susceptibility to develop CAD has been observed for C allele carriers (44-46). Moreover, the *MIF* gene features a tetranucleotide CATT repeat polymorphism ('the $CATT_{5\text{-}8}$ microsatellite') at position -794 that was initially identified in rheumatoid arthritis patients and controls gene expression from the *MIF* promoter (47). $CATT_7$ or $CATT_{non\text{-}5}$ MIF high-expressers show an increased severity of coronary artery atherosclerosis (CAA) and patients carrying the rs755622 C allele and $CATT_7$/C haplotype are more prone to develop CAD (48). This correlates with associations between plasma MIF and CAD, e.g. in acute coronary syndrome (18, 19, 49, 50). Moreover, MIF plasma levels were found to be elevated in a high proportion of ST elevation myocardial infarction (STEMI) patients, were suggested to be an early marker of acute STEMI, and STEMI patients with high admission MIF level experienced a poorer recovery of cardiac function and worse long-term adverse outcomes (19, 51-53). Moreover, the role of MIF in myocardial ischemia/reperfusion (I/R) injury has become apparent from clinical studies in cardiac surgery patients and mouse models. The cardiac surgery procedure recapitulates the ischemic and reperfusion stress seen in myocardial infarction patients, but in contrast to the endogenously occurring myocardial infarction pathology in STEMI patients subjected to percutaneous coronary intervention (PCI), the onset of inflammation and oxidative injury in cardiac surgical patients is predictable as cardiac surgery with the cardioplegia-induced myocardial arrest, assistance of cardio-pulmonary bypass (CPB) and the following myocardial reperfusion, reproducibly elicits an ischemia-reperfusion sequelae. Intriguingly, the increase in peri-operative MIF levels in cardiac surgery patients, as well as ratios of MIF and its soluble receptor CD74 (sCD74), suggest a cardioprotective role of MIF in the ischemic and early reperfusion

phase after myocardial infarction (54-56). In fact, cardioprotection by MIF in myocardial ischemia/reperfusion injury (MI/RI) is confirmed in numerous mouse models (19, 57-62). Along the same lines, the myocardium-specific conditional knockout of *D-dt/Mif-2* exacerbates MI/RI, while MIF-2 levels positively correlated with worse outcome in cardiac surgery patients (54, 63). Interestingly, experimental models addressing the later post-ischemic phase indicated that the role of MIF in cardiac ischemia is complex, with phase-dependent cardioprotective and exacerbating effects observed (57-59, 61, 64). MIF is initially released by ischemic cardiomyocytes or endothelial cells and triggers a cardioprotective autocrine/paracrine signaling response in cardiomyocytes. Here, MIF not only binds to the chemokine receptors CXCR2 and CXCR4, but also to CD74, the surface-expressed form of the MHC class II invariant chain, which serves a secondary function as a high-affinity MIF receptor (65). Cardiac-derived – 1st wave – MIF interacts with cardiomyocyte-expressed CD74 in the ischemic and early reperfusion phase of myocardial I/R to trigger cardioprotective signaling through AMP kinase metabolic reprogramming, an increase in glucose uptake via membrane translocation of GLUT4, and the AKT and ERK survival pathways, while pro-apoptotic JNK signaling is attenuated (58, 60). MIF-2 also mediates cardioprotection in this phase via CD74/AMPK signaling (63). MIF/CD74/AMPK-mediated ischemic recovery is impaired in the senescent heart, suggesting that this protective mechanism could be dampened in aged CHD patients (66). MIF's antioxidant capacity that is based on its redox-active CXXC motif and that it shares with thiol-protein oxidoreductases such as thioredoxin (Trx) (67) also contributes to cardioprotection in the early phase of I/R stress (57, 59, 62). In contrast, MIF's role in the later phase of I/R stress in the heart is an "inflammatory" one that is mediated by CXCR2/CXCR4-dependent recruitment of monocytes and neutrophils. Second wave MIF is additionally and abundantly produced by infiltrating inflammatory cells to amplify the inflammatory response (64). MIF's chemokine receptors serve a dual role in this phase with both protective (cardiomyocyte-expressed CXCR2/4) and pro-inflammatory (CXCR2/4 expressed on infiltrating myeloid cells) activity (68, 69).

MIF PROTEINS AND THEIR RECEPTORS

MIF binds to CXCR2 and CXCR4, representing non-cognate interactions between an ACK and classical chemokine receptors. MIF also binds to CD74, the surface-expressed form of invariant-chain. All three receptors have important roles in atherosclerosis and cardiac disease (see above). Recent evidence also suggests engagement of CXCR7-mediated pathways by MIF (70-72).

Binding of MIF to CXCR2 drives atherogenic recruitment of monocytes and neutrophils (14, 73, 74). Mechanistically, binding of MIF to CXCR2 is similar but not identical to that of the cognate ligand CXCL8 and requires an N-like loop and pseudo-ELR motif (16, 74-76). While data are not yet available, it has been speculated that MIF-2 does not activate CXCR2 as it lacks the pseudo-(E)LR motif of MIF. MIF/CXCR4 binding supports the recruitment of atherogenic T cells (14), but has also been implicated in cancer metastasis and endothelial progenitor cell recruitment (16). Recent evidence suggests an important role of the MIF/-CXCR4 axis in B cell migration that may also contribute to the pro-atherogenic phenotype of MIF (70, 77, 78). CXCR4 is one of the few GPCRs for which an X-ray structure has been elucidated (79, 80) and recent structure-activity studies revealed that the MIF/CXCR4 interface involves an extended N-like loop of MIF, an RLR motif at position 87-89, and the N-terminal Pro-2 (81, 82).

Interestingly, both CXCR2 and CXCR4 are able to form receptor complexes with CD74, offering unexpected mechanistic options as to the fine-tuning of MIF-driven pathways in atherogenesis. In fact, MIF-mediated CXCR2/CD74 signaling has a role in atherogenic leuko-cyte recruitment (14, 83), while CXCR4/CD74 complex formation and/or cross-talk is necessary for MIF-driven B cell migration responses and elicits downstream ZAP-70 signaling (77, 84).

Intracellular CD74/invariant-chain acts as an MHC II chaperone facilitating antigen loading to class II complexes in the endoplasmic-reticulum (85). However, CD74 may be also expressed in class II-negative cells, i.e. upon inflammatory stimulation, and exhibits a major role as cytokine receptor for MIF and MIF-2 (65, 86). MIF and MIF-2 bind to the extracellular

domain of CD74, but as CD74 exhibits a short cytoplasmic domain, signal transduction necessitates accessory molecules such as CD44 or CXCR2/4 (14, 16, 65, 87). A soluble form of CD74 (sCD74) was identified in patients with autoimmune liver disease (88) and as outlined above, evidence from cardiac surgery patients suggests that circulating sCD74 levels correlate with better outcome (54, 56). sCD74-derived strategies could thus represent interesting MIF-targeting approaches in the future.

ANTIBODY-BASED ANTI-MIF STRATEGIES

Antibody-based anti-cytokine/-chemokine strategies are promising therapeutic approaches in inflammatory and cardiovascular diseases. Prominent examples are the IL-1β antibody canakinumab, which was shown in the CANTOS trial to reduce vascular inflammation accompanied by a lower rate of recurrent cardiovascular events (12), the anti-TNF-α antibody infliximab, successfully used in RA (89), and numerous chemokine antibodies such as anti-CCL2 (CNTO888/ABN912), which are in phase 1/2 clinical studies for various inflammatory conditions and cancer (90, 91). Chemokine antibodies such as anti-CCL2 have been efficacious in preclinical models of atherosclerosis (7), suggesting their potential in CAD patients.

Neutralization of MIF by blocking antibodies has improved disease exacerbation in numerous preclinical inflammation models including atherosclerosis (18, 37, 92, 93). The most widely used antibody has been the monoclonal NIH/IIID.9, which was raised against full-length mouse MIF. It was initially tested in immunologically-induced kidney disease (94) and has been demonstrated to potently block disease progression in numerous inflammatory, autoimmune and cardiovascular conditions (14, 16, 18, 19, 37, 95). The epitope recognized by NIH/IIID.9 has not been characterized, but it has been suggested that it recognizes a solvent-exposed region of MIF in the middle part of the sequence (95) similar to mAb clone 1C10 (Bernhagen et al., unpublished), but unlike clone F11, which blocks cecal-ligation-and-puncture-induced sepsis and is directed against the N-terminal of murine MIF (96).

From the existing anti-MIF antibodies successfully tested in pre-clinical inflammation, cancer, and atherosclerosis models, only the MIF-antibody Imalumab has so far advanced into

phase 1/2a clinical trials (NCT01765790) against colorectal cancer and lupus nephritis (97). Imalumab has an anti-inflammatory capacity as it reduces circulating TNF-α, MCP-1 and IL-6, and attenuates disease progression in mouse models of glomerulonephritis and cancer. Based on biochemical and immunochemical experiments, it was suggested that this antibody recognizes an oxidized form of MIF with the oxidoreductase motif of MIF trapped in an oxidized state, and that Cys-81 serves as a molecular redox switch between the latent reduced form of MIF and its oxidized state (93, 98, 99). While the antibody was reported to detect an oxidized MIF species termed 'oxMIF' in tumor tissue from patients with colorectal, pancreatic, ovarian, and lung cancer (100), the claim that oxMIF is the pathophysiologically-relevant MIF species appears speculative and convincing evidence that Imalumab targets pathogenic oxidized MIF species is missing (67, 95).

MIF-2/D-DT shares with MIF a pronounced pro-inflammatory activity profile and has been reported to promote endotoxemia, adipose tissue inflammation, and tumorigenesis similar to MIF. As discussed, MIF-2 also is involved in early cardioprotection after M/I and regulates kidney regeneration, but its role in atherosclerosis has not been studied (19, 63, 86, 101-103). Functional studies on MIF-2 have capitalized on *Mif-2* gene deletion (63) and a neutralizing antibody (86). While this polyclonal antibody shows good blocking potency in pre-clinical models (86), a monoclonal antibody has not been published.

Cytokine/chemokine pathways may be targeted by neutralization of the ligand or may rely on strategies to block the receptor binding site of the ligand and/or receptor signaling. Antibodies against CD74 recapitulate many of the effects seen with neutralizing MIF antibodies, but differences have also been noted. As both MIF and MIF-2 bind to CD74, these may be due to MIF-2-triggered responses that are inhibited by anti-CD74 but not anti-MIF strategies. No other endogenous ligands than MIF or MIF-2 have been identified for CD74. Yet, the blocking phenotype of anti-CD74 may differ from a combined anti-MIF/anti-MIF-2 strategy due to class II-associated functions of endolysosomal-expressed CD74/invariant chain. In fact, specific peptide-based strategies have been developed to block class II-associated functions of CD74 in multiple sclerosis (104-107) (see below).

A neutralizing CD74 mAb has shown potent inhibitory activity in hematologic cancers such as chronic lymphocytic leukemia (CLL) and multiple myeloma (108, 109). One of these mAb clones, a humanized anti-CD74 monoclonal termed Milatuzumab/hLL1, is in clinical trials for CLL and multiple myeloma treatments. However, although gene deletion of CD74 significantly attenuates atherosclerosis in atherogenic *Ldlr-/-* mice (110) and although CD74 serves as an accessory molecule in MIF-driven CXCR2/4-mediated leukocyte recruitment responses (14), neutralizing CD74 antibodies have not been studied in atherosclerosis. It should be emphasized that anti-CD74 strategies may be intrinsically limited regarding their translational potential in atherosclerotic disease due to cardiac protection mediated via the CD74/AMPK pathway in cardiomyocytes following ischemic stress (58). It rather seems that MIF-based strategies in cardiovascular disease should aim at sparing CD74-mediated pathways (19).

CXCR2 and CXCR4 are *bona fide* GPCRs, but antibody development against GPCRs has been delayed. In fact, of the numerous GPCR-modulating agents available, most are small molecules or peptides. Recently, the first GPCR-directed antibody (Erenumab), an antibody against calcitonin-gene-related peptide-receptor (CGRPR), was FDA-approved for treatment of migraine (111). Meanwhile, the development of additional anti-GPCR antibodies including antibodies against CC and CXC chemokine receptors is underway (111).

CXCR4 antibodies are in advanced clinical trials for hematologic malignancies (112), and anti-CXCR4 SMDs such as the bicyclam Plerixafor/AMD3100 which promotes CXCR4-dependent hematopoietic stem cell egress from bone marrow is clinically used in autologous stem cell transplantation of cancer patients (113). Nevertheless, anti-CXCR4 antibody strategies as a means to block MIF-driven pathogenic pathways in atherosclerosis should be pursued with caution. The CXCR4/CXCL12 axis has important homeostatic functions in development and physiology that render generalized anti-CXCR4 strategies difficult. Moreover, disrupting the CXCL12/CXCR4 axis in a mouse model of atherosclerosis promoted lesion formation through dysbalanced neutrophil homeostasis (114) and a recent study

demonstrated a potent atheroprotective effect of vascular CXCR4 via maintaining arterial integrity, endothelial barrier function, and preserving contractile VSMC functions (115).

Cxcr2 gene deficiency reduces the progression of advanced atherosclerosis in mice and in fact, CXCR2 has been one of the first chemokine receptors implicated in atherogenesis (116, 117). CXCR2 also is involved in MIF-elicited atherogenic monocyte and neutrophil recruitment (14, 16, 75), emphasizing the significance of the MIF/CXCR2 axis in leukocyte arrest and atherogenesis. Moreover, anti-MIF antibodies proved superior to anti-CXCL1 (and anti-CXCL12) in an atherosclerosis regression model (14). An anti-CXCR2 antibody therapy is considered a translatable strategy in solid cancer, e.g. through improving the efficacy of checkpoint blockade by preventing trafficking of myeloid-derived suppressor cells (MDSCs) to the tumor site (118) and anti-CXCR2 strategies are pursued in various clinical trials (119), including acute coronary syndrome (ACS) (120). An anti-CXCR2 biparatopic nanobody is in phase I development for the treatment of inflammation (111). Antibody strategies for epitopes specifically targeting MIF/CXCR2 receptor pathways in vascular inflammation and atherosclerosis have not been pursued.

Table 1 summarizes published antibody-based strategies directed at MIF proteins and/or their receptors.

SMALL MOLECULE (SMD)-BASED ANTI-MIF STRATEGIES

MIF proteins are structurally unique among cytokines/chemokines in harboring a conserved catalytic tautomerase cavity that contains the unusually acidic Pro-2 residue. This offers the opportunity to target pathogenic activities of MIF by small molecule approaches. Capitalizing on efficient drug discovery pipelines including *in silico* and high throughput screening, numerous anti-MIF SMDs have been identified that bind into or modulate the tautomerase pocket of MIF and/or MIF-2/D-DT by covalent or non-covalent mode.

Small molecule MIF inhibitors have been compiled in several recent review articles (24, 95, 121, 122). Here, we discuss some of these compounds with a focus on their potential utility in atherosclerosis. **Table 2** summarizes the key features of these inhibitors.

Mechanistically, small molecule MIF inhibitors are classified into different categories: i) competitive inhibitors that non-covalently bind into the cavity; ii) suicide inhibitors that covalently bind into the cavity; iii) allosteric inhibitors that disrupt the active-site through dissociation of the MIF trimer or an otherwise-induced conformational switch; iv) allosteric inhibitors that prevent higher-order MIF oligomers (123, 124). In addition, stabilizers of the MIF monomer have been proposed as inhibitors through prevention of re-association into trimer (123). These may only qualify as inhibitors of MIF/CD74 interactions, which involve trimeric MIF, whereas it has been assumed that the interaction between MIF and CXCR2/CXCR4 is a function of the monomer (14). While most inhibitors have been developed against MIF, some also block MIF-2, although significant differences in K_i and IC_{50} values have been noted (125). A recent study has identified a selective MIF-2 inhibitor that exhibits 13-fold higher binding to MIF-2 than MIF (125).

The MIF tautomerase activity is highly conserved across kingdoms, but to date, physiological substrates in mammalians have not been identified, raising the possibility that it is an evolutionary remainder with no function in humans. Thus, it is now thought that by binding to the tautomerase site, these compounds induce conformational changes in MIF that subsequently alter its receptor-binding properties (82, 126-129) and therefore 'indirectly' influence MIF activities.

The structures of the small molecule MIF inhibitors have been extensively reviewed (121, 122). Briefly, with the isooxazoline compound ISO-1 serving as a reference MIF inhibitor in various inflammation models (130), they can be grouped into isooxazolines (examples: ISO-1, ISO-66, CPSI-1306), chromenes (examples: Orita-13, Kok-17), iminoquinones (example: N-acetyl-p-benzoquinone imine (NAPQI)), triazoles (example: Cisneros-3i), benzoxazolones (example: MIF098), pyrimidazoles (example: K664-1), and isocoumarins (e.g. SCD-19) (121, 122, 131). Allosteric MIF inhibitors encompass pyrazolopyridines (example: clinically-used PDE4 inhibitor Ibudilast), benzoisoselenazolones (example: anti-inflammatory drug Ebselen), and azo compounds (example: p425 or Chicago Sky Blue 6b) (73, 123, 124). Of note, Ibudilast, which can cross the blood-brain-barrier, was recently demonstrated in a phase II clinical trial

in progressive multiple sclerosis, to be associated with slower progression of brain atrophy than placebo (132). Isothiocyanates such as phenethylisothiocyanate (PEITC), are a reactive class of 'natural' MIF inhibitors that are present in appreciable amounts in broccoli and water cress. They covalently bind to the acidic Pro-2 residue in the catalytic site of MIF, attenuate MIF antibody binding, and inhibit inflammatory MIF activities *in vitro*, but have not yet been studied in atherosclerosis (123, 133).

While most of these compounds have not yet been studied in atherosclerosis-relevant test systems or preclinical models, some of them may hold promise as pathway-specific MIF inhibitors in atherogenesis. However, a 'black-and-white' categorization into receptor-specific blockers appears too simplistic. For example, CD74 is a receptor mediating cardio- and tissue-protective MIF activities (19, 58); on the other hand, it drives pro-proliferative functions of MIF and can interact with the MIF chemokine receptors, which would be pro-atherogenic (14, 70, 109). CXCR4 mediates pro-atherogenic lymphocyte recruitment by MIF (14, 78), but also exhibits homeostatic and atheroprotective activities through CXCL12 (115). Notwithstanding, it may be speculated that MIF trimerization inhibitors such as Ebselen might have specificity for MIF/chemokine receptor pathways, while sparing MIF trimer-dependent CD74 signaling (123), which could lead to an overall atheroprotective effect. Ibudilast was found to block MIF/CD74 interactions *in vivo* by preventing astrocyte-derived MIF from interacting with CD74+ microglia during the colonization process of brain metastatic tumors resulting in reduced secondary brain tumor loads (134). Similarly, ISO-1 reduces MIF/CD74 binding albeit at relatively weak IC_{50} values (121), but interestingly, was recently also shown to partially interfere with MIF binding to CXCR4, opening up the possibility that certain MIF inhibitors may have utility in cardiovascular disease. However, given their small interaction surface, it remains questionable whether they would sufficiently differentiate between receptor pathways.

Table 2 summarizes a selection of the developed small molecule MIF inhibitors from different structural classes and compares them to MIF chemokine receptor inhibitors. To this end, AMD3100/Plerixafor and Reparixin are established CXCR4 and CXCR2 inhibitors, respectively. AMD3100 also was shown to be a partial -allosteric- inhibitor of MIF/CXCR4

signaling (82); however, its preferential targeting of CXCL12/CXCR4 responses and its limited application window in autologous transplantation and HIV, probably limits an efficacious usage as a specific anti-MIF compound. Reparixin has so far only been studied in MIF-dependent *in vitro* inflammatory assays (135).

PEPTIDE-BASED ANTI-MIF STRATEGIES

Peptide therapeutics are a powerful alternative to small molecule and antibody strategies with over 60 peptide drugs approved worldwide. The peptide therapeutic landscape has been reviewed in excellent recent reviews (136-138). Advantages of peptide-based inhibitors are: i) good selectivity and potency, ii) good interaction surface coverage, iv) favorable safety, and v) comparatively low production costs due to standard synthesis protocols. On the other hand, they are prone to degradation and oxidation, but this can be improved by smart mimic chemistry (136, 139).

Even though peptide-based inhibitors have been pursued as potential therapeutics in atherosclerosis, e.g. targeting lipid-regulating and inflammatory pathways such as apolipoproteins, NF-κB, and the IL-4 receptor (140-142), peptide approaches targeting MIF-specific pathways are still in its infancies. Interestingly, a recent approach established designed stable peptide inhibitors that specifically disrupt proinflammatory CCL5-CXCL4 interactions, attenuating monocyte recruitment and reducing atherosclerosis. Targeting of CCL5-CXCL4 heteromers avoids side-effects of generalized anti-CCL5 strategies which would compromise systemic host immunity (143, 144), thus underscoring the potential of anti-chemokine peptide strategies.

Anti-MIF peptides have been examined *in-vitro* and partially in preclinical disease models. Peptides targeting both the MIF/CD74 and MIF/chemokine receptor axes have been considered (**Table 3**); some of them are derived from mapping studies of the interfaces between MIF and its receptors.

MIF-derived peptides targeting interactions with CD74 have not yet been tested in disease models. This is probably due to the complex nature of the MIF/CD74 interaction

surface, encompassing all three subunits of the MIF trimer and discontinuous epitopes within an MIF monomer. Nevertheless, a MIF epitope scan for reactivity of the CD74 ectodomain identified MIF peptide 79-86. This octapeptide was able to compete for biotinylated MIF binding to plate-bound CD74 (88), indicating its principal inhibitory utility. A screening for anti-melanoma peptides derived from conserved complementarity-determining region (CDR) sequences of different immunoglobulins identified peptide C36L1, a 17-mer peptide that binds to CD74 on tumor-associated macrophages and dendritic cells and blocks immunosuppressive activities in melanoma models (145, 146). Other inhibitors that target the MIF-CD74 interaction have been developed based on antigenic peptide-loaded fragments of class II resulting in reduced severity of EAE, the experimental mouse model of multiple sclerosis (104, 105, 107). One such peptide is the DR2-restricted myelin determinant mouse (m) myelin oligodendrocyte glycoprotein (MOG)-35-55 covalently linked to a human leukocyte antigen (HLA)-DRα1 domain (the 'DRα1-MOG-35-55' construct) and has been found to reduce CNS inflammation and tissue injury in models of multiple sclerosis, ischemic stroke, and traumatic brain injury (104, 105, 107, 147). RTL1000 is a variant of this construct additionally containing the β1 domain of DR1 ('DRα1β1-MOG-35-55') and is in clinical studies for multiple sclerosis (148, 149). It is thought that these peptide constructs interfere with MIF/CD74-driven neuro-inflammation (150).

A note of caution should be sounded regarding their potential application in atherosclerosis and cardiovascular disease settings due to the protective role of the MIF/CD74 axis in ischemic heart disease (19, 58).

As discussed above, structure-activity studies (SAR) have identified the motifs and residues contributing to the interface between MIF and CXCR2 and CXCR4 and highlighted differences compared to the classical chemokine ligands CXCL8/1 and CXCL12, respectively. The two-side binding mechanism for MIF and CXCR2 is similar but not identical to that for CXCL1/8, while significant differences were noted for the binding interface of MIF/CXCR4 versus CXCL12/CXCR4 (14, 16, 74-76, 81, 82). Peptides served as important tools in these studies and some of them might become templates for peptide-based anti-MIF strategies in

atherosclerosis and inflammation. For example, MIF peptide 47-56, spanning the N-like loop that contributes to site 1 binding with CXCR2, competes with MIF binding to CXCR2 and MIF-mediated atherogenic leukocyte arrest (16, 76). Stabilized variants of this peptide or related ones spanning MIF regions contributing to site 1 or 2 binding might qualify as interesting templates for the future development of MIF-based peptide drugs against atherogenic inflammation.

MIF acts as a partial allosteric agonist of CXCL12, consistent with the notion that the binding interface between MIF and CXCR4 differs from that of CXCL12 and CXCR4 (82). Major differences are the contributions of the extended N-like loop and the cavity around Pro-2 in MIF (81, 82) and the REFFESH motif in CXCL12 (80, 151). This SAR information as well as the crystal structures of MIF and of CXCR4 in complex with small molecules and cyclic peptides give valuable hints as to the development of peptide inhibitors that may specifically block MIF-driven responses (80).

Of note, CXCR4 pathways have already been targeted by peptide inhibitor strategies. CVX15, a 16-residue cyclic peptide analog of the horseshoe crab peptide polyphemusin was co-crystallized with CXCR4 (80) and characterized as an HIV-inhibiting and anti-metastatic agent (152, 153). Polyphemusin II-related synthetic peptides T22, T140, and FC131 were pioneered by Fujii and co-workers to adopt a β-hairpin conformation stabilized by disulfide bonds, resulting in high-affinity CXCR4 inhibitory peptides with a low nanomolar IC_{50} (153-155). This principle was developed further by Kessler and colleagues, who furthered the principle of protein-epitope mimetics and devised novel classes of super-high-affinity CXCR4-targeting cyclopeptides (156, 157), e.g. by “freezing” the conformation of a CXCR4 ligand into a single-active conformation by using a ‘peptoid’ motif (156). In another approach, peptide inhibitors were derived from the N-terminal of CXCL12 and further optimized and stabilized to give rise to sub-nanomolar serum-stable CXCL12/CXCR4 inhibitors with anti-metastatic activity *in vitro* (158, 159). Furthermore, peptides based on the sequence of CXCR4 were linked in an attempt to mimic the ecto-surface of CXCR4 and shown to compete with HIV-gp120 and HIV entry (160, 161). MIF is not able to inhibit HIV entry (82) and peptides targeting

the MIF/CXCR4 axis have not been systematically studied, although a peptide spanning the RLR motif of MIF competes with MIF-mediated lymphocyte migration (81).

CONCLUSION AND OUTLOOK

MIF is a pivotal mediator of atherosclerosis, MIF-2/D-DT shares critical inflammatory activities with MIF, and the MIF receptors CD74, CXCR2, and CXCR4 have all been implicated in atherosclerosis, suggesting that it will be important to develop therapeutic strategies against MIF proteins in atherosclerosis. Importantly, the MIF network is amenable to targeting by all major inhibitor classes, i.e. small molecule compounds, antibodies, and peptides (**Figure 1**). In fact, inhibitors against MIF and/or its receptors from all three classes are in clinical development and the CXCR4 blocker AMD3100, which is a partial inhibitor of MIF/CXCR4 binding, is an FDA-approved drug in cancer. However, to make such strategies applicable for cardiovascular disease, MIF pathway-specific concepts need to be developed that specifically target the atheroprogressive activities of MIF.

Considering the Janus-faced effects of MIF proteins in cardiovascular complications and the complex homeostatic and inflammatory roles of their receptors, this is a challenging task. One strategy might be to specifically target the MIF/CXCR2 interface which is atheropromoting and largely detrimental in the myocardium during an ischemic insult. Both antibodies and peptide-based compounds such as stabilized derivatives of MIF(47-56) could potentially qualify as MIF/CXCR2-specific agents. Similarly, inhibitor strategies specifically targeting the MIF/CXCR4 interaction could be envisioned, although great caution would need to be taken to spare the various protective activities of CXCR4 in the atherogenic vasculature and the ischemic-stressed heart. On the other hand, the cardioprotective effect of MIF and MIF-2 observed in the early phase following myocardial I/R provides a narrow albeit critical therapeutic window to pharmacologically promote the cardioprotective function of MIF before late-phase inflammatory responses kick in. This could especially be relevant in cardiac surgery patients and one relevant agent is the small molecule agonist MIF-20, which binds near MIF's tautomerase pocket and has been reported to have protective effects in an experimental model

of cardiac ischemic injury (162). Such a 'pharmacological augmentation' strategy would be selective to MIF proteins due to their structurally unique tautomerase cavity and might become particularly important in cohorts of patients identified as MIF 'low-expressers' (19, 58). Therapeutically, a combinatorial treatment approach might be considered, in which the cardioprotective effect of MIF in the early-phase of myocardial I/R is carefully and phase-specifically enhanced, followed by phase-specific pharmacological inhibition in the late -inflammatory- phase of I/R injury. Whether any anti-MIF strategy would qualify as a treatment regimen to 'prevent' or 'reverse' chronic atherogenesis similar to canakinumab will have to be subject to comprehensive future investigations. Reversal of atherosclerotic lesions as observed in an experiment mouse model of plaque regression applying anti-MIF but not anti-CXCL12 or anti-CXCL1 antibodies is a promising start in this direction (14).

In conclusion, for applications in atherosclerotic cardiovascular disease, MIF pathway-specific concepts would need to i) specifically target the atheroprogressive activities of MIF, ii) preserve homeostatic effects of intracellular MIF, iii) take into account the cardioprotective functions of CD74, iv) and/or spare CXCL12/CXCR4-dependent vascular protection pathways. Molecular characteristics of such agents would need to account for the necessities of chronic treatment over the course of lesion development and/or phase-specificity in the sequelae related to acute cardiac ischemia.

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CONFLICT OF INTEREST

JB is a co-inventor of patents covering anti-MIF strategies in inflammatory and cardiovascular diseases. Authors declare no additional competing financial interests.

TABLES

Table 1. Antibodies targeting MIF proteins or their receptors

Antibody	Target / Antigen	Application / Utility in Atherosclerosis	References
NIH/IIID.9 (mAb)	Mouse MIF (full-length)	Research and preclinical models; blocks athero-genic effects of MIF	(14, 37, 94)
Imalumab (Bax69) (humanized mAb)	Oxidized form of human MIF	Phase IIa trial for metastatic colorectal cancer	(97)
BaxB01, BaxG03, BaxM159	Oxidized form of human, mouse, or rat MIF	Research and preclinical models	(93, 98-100, 163)
NbE10-NbAlb8-NbE10 (half-life-extended nanobody)	Human and mouse MIF	Research and preclinical sepsis model	(164)
Anti-MIF-2/D-DT	Mouse MIF-2/D-DT (full-length)	Research and preclinical models	(86)
Milatuzumab	CD74	Multiple myeloma, NHL, CLL	(108, 165)
i-bodies (AM3-114, AM4-272, AM3-523; single domain antibody)	CXCR4	Research and preclinical models	(166)
MEDI3185	CXCR4	Research and preclinical models	(112)
Anti-CXCR2 biparatopic nanobody	CXCR2	Preclinical models and phase I clinical trial	(111)
MAB331	CXCR2	Research and preclinical models	(14, 167)
*sCD74	Human and mouse MIF	Research and preclinical models	(56, 65, 88)

Legend: *sCD74; is not an antibody, but the soluble ectodomain of MIF receptor CD74.

Table 2. Small molecules targeting MIF proteins or their receptors

Small Molecule (SMD) Inhibitor	Target / Binding Mode	Ki	IC_{50} / EC_{50}	References
NAPQI N-acetyl-p-benzoquinone imine	MIF Pro-2 Covalent	N/A	IC_{50} (dopachrome) = 40 µM	(131, 168)
4-IPP 4-iodo-6-phenylpyrimidine	MIF/D-DT Pro-2 Covalent	N/A	IC_{50} (HPP) = 0.2-0.5 µM	(169, 170)
ISO-1 4,5-Dihydro-3-(4-hydroxyphenyl)-5-isoxazoleacetic acid methyl ester	MIF Tautomerase site Competitive	Ki (HPP) = 24 µM	IC_{50} (dopachrome) = 7 µM	(130, 171)
SCD-19 Isocoumarin	MIF Tautomerase site Competitive	Not tested	100% inhibition at 100 µM	(172, 173)
4-CPPC 4-(3-Carboxyphenyl)-2,5-pyridine-dicarboxylic acid	MIF-2/D-DT C-terminus V114−L118	Ki (HPP) = 33 ± 0.7 µM	---	(125)
Ebselen 2-Phenyl-1,2-benzisoselenazol-3(2H)-one	MIF trimer Cys-81 Covalent	Ki (HPP) = 0.57 µM	IC_{50} (dopachrome) = 2.4 µM	(123)
p425 6,6'-[(3,3-Dimethoxy[1,1'-biphenyl]-4,4'-diyl)bis(azo)]bis[4-amino-5-hydroxy-1,3-napthalenedisulphonic acid]	MIF trimer Allosteric	Ki (HPP) ≤ 12 µM	IC_{50} (CD74 inhibition) = 0.81 µM	(124)
Ibudilast AV411; 3-isobutyryl-2-isopropyl-pyrazolo-[1,5-a]pyridine	MIF Tyr-37 Allosteric	Ki (HPP) = 30.9 µM	---	(73, 132, 134)
Plerixafor/AMD 3100 (1-[4-(1,4,8,11-Tetraza-cyclotetradec-1-ylmethyl)phenyl]-methyl)-1,4,8,11-tetrazacyclo-tetradecan	CXCL12/CXCR4 Orthosteric anta-gonist MIF/CXCR4 Partial allosteric antagonist	---	IC_{50} (CXCR4) = 0.65 µM EC_{50} (HIV entry) = 0.4-2 µM	(80, 82, 156, 174)
IT1t Isothiourea-1t 6,6-dimethyl-5H-imidazo[2,1-b][1,3]thiazol-3-yl)methyl N,N'-dicyclohexylcarbamimidothioate	CXCL12/CXCR4 Orthosteric anta-gonist MIF/CXCR4 Partial allosteric antagonist	---	IC_{50} (gp120 inhibition) = 8 nM	(80, 82, 174)
Reparixin (αR)-α-methyl-4-(2-methylpropyl)-N-(methylsulfonyl)-benzeneacetamide	CXCL8/CXCR2 Allosteric anta-gonist MIF/CXCR2 (?)	---	IC_{50} (neutrophil migration) = 1 nM	(174, 175)

Legend: HPP, hydroxy-phenylpyruvate.

Table 3. Peptides and peptide mimics targeting MIF proteins or their receptors

Peptide Inhibitor	Target / Binding Mode	Application / Utility in Athero-sclerosis (IC_{50})	References
MIF(79-86) (mouse) LCGLLSDR	MIF/CD74 interface	IC_{50} = ca. 2-3 µM	(88)
MIF(47-56) (human) LMAFGGSSEP	MIF Competitive	EC_{50} = ca. 1-2 µM	(76)
MIF(50-65) (human) FGGSSEPCALCSLHSI	MIF Competitive	Not determined	(176, 177)
Conserved CDR peptide C36L1 KSSQSVFYSSNNKNYLA-NH2	CD74	IC_{50} = upper µM range	(146)
RTL1000 Class II-derived Drα1β1MEVGWYRSPFSRVVHLYRNGK	CD74 trimer MIF/CD74 axis Competitive	IC_{50} = nanomolar range	(107)
DRα1-MOG-35-55 Class II-derived DRα1MEVGWYRSPFSRVVHLYRNGK	CD74 trimer MIF/CD74 axis Competitive	IC_{50} = nanomolar range	(104, 107)
$CXCL12(22\text{-}29)_2$ KGVSLYR-K-RYSLVGK	CXCL12/CXCR4 axis	--- Not known	(178)
CXCL12a(1-9[P2G]) dimer MNAKVVVVL-S-S-LVVVVKANM	CXCL12/CXCR4 axis	IC_{50} = 2.6 µM Not known	(178)
Ac-Arg-Ala-[D-Cys-Arg-Phe-His-Pen]-COOH Derivative of CXCL12 N-terminal	CXCL12/CXCR4 axis	IC_{50} = 1.5 nM	(158, 159)
CVX15 16-residue cyclic peptide analog of the horseshoe crab peptide polyphemusin	CXCR4	Not known	(80)
MCoTI-based cyclotides	CXCR4	IC_{50} = 20 nM EC_{50} (HIV entry) = 2 nM	(179)
Peptides T22 and T140 Polyphemusin II-related synthetic 14-16-meric derivatives	CXCR4	IC_{50} = 17 nM	(153, 154)
Cyclopentapeptide FC131 Head-to-tail-cyclized variant of T140	CXCR4	IC_{50} = 8 nM	(154)
Peptoid 8 Peptoid derivative of FC131	CXCR4	IC_{50} = 40 pM EC_{50} (HIV entry) = 29 nM	(156)

Legend: IC_{50} refers to replacement of ligand (MIF, CXCL12) from receptor (CD74, CXCR4).

FIGURE LEGENDS

Fig. 1. Overview of inhibitory approaches to target the MIF/receptor network in atherosclerosis. The potential utility of all three classes of anti-MIF network inhibitors, i.e. antibodies, small molecule compounds (SMD), and peptides, in attenuating atherosclerosis and/or atherogenic inflammation is indicated with respect to MIF and MIF-2/D-DT, as well as the MIF receptors CXCR4, CD74, and CXCR2. The pros and cons for each inhibitor-type regarding each target ligand/receptor or pathway are indicated by scoring their properties (e.g. specificity) with +, +/–, or –. The outcome boxes are color-coded (red, pro-atherogenic; green, athero-/cardioprotective).

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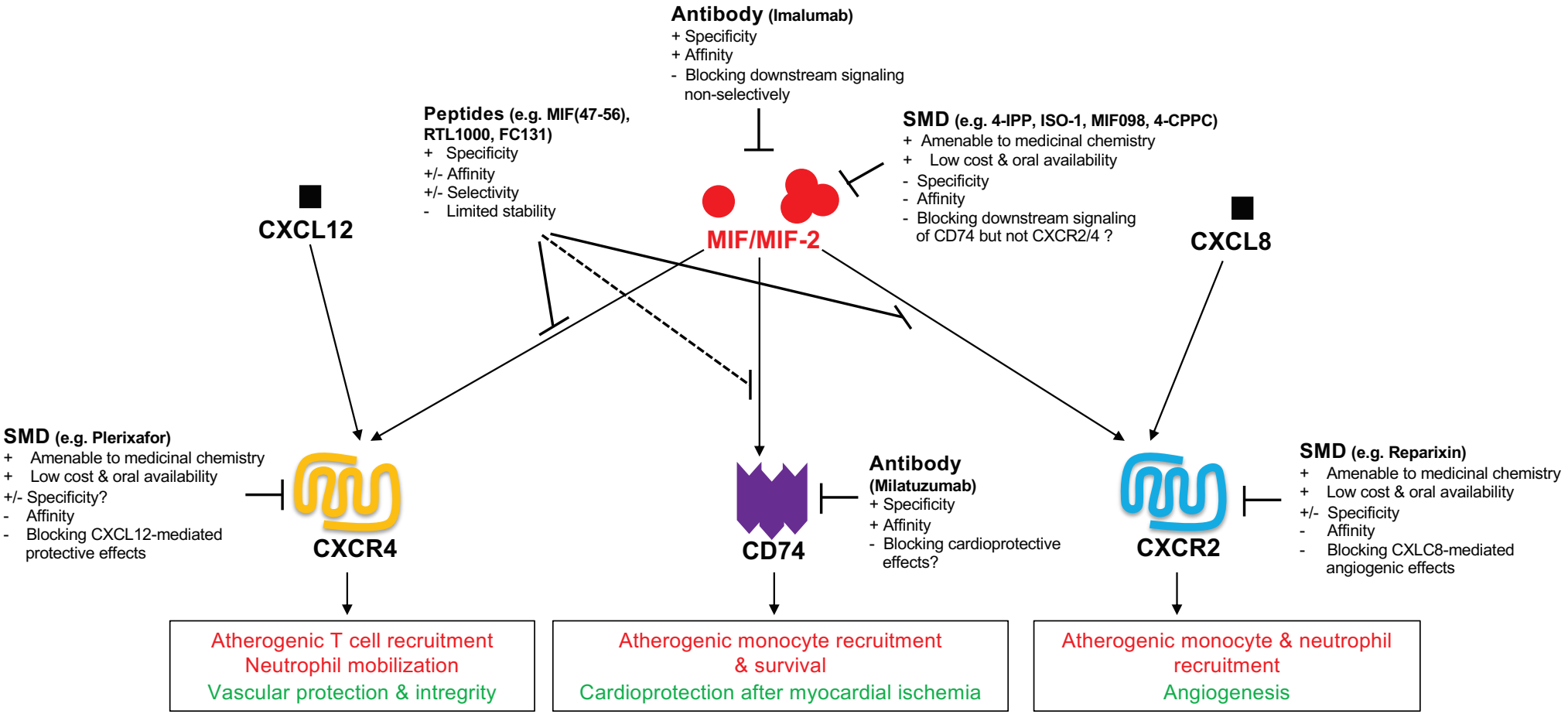
Antibody (Imalumab)
+ Specificity
+ Affinity
- Blocking downstream signaling non-selectively
Peptides (e.g. MIF(47-56), RTL1000, FC131)
+ Specificity
+/- Affinity
+/- Selectivity
- Limited stability
SMD (e.g. 4-IPP, ISO-1, MIF098, 4-CPPC)
+ Amenable to medicinal chemistry
+ Low cost & oral availability
- Specificity
- Affinity
- Blocking downstream signaling of CD74 but not CXCR2/4 ?
CXCL12
MIF/MIF-2
CXCL8
SMD (e.g. Plerixafor)
+ Amenable to medicinal chemistry
+ Low cost & oral availability
+/- Specificity?
- Affinity
- Blocking CXCL12-mediated protective effects
CXCR4
CD74
Antibody (Milatuzumab)
+ Specificity
+ Affinity
- Blocking cardioprotective effects?
CXCR2
SMD (e.g. Reparixin)
+ Amenable to medicinal chemistry
+ Low cost & oral availability
+/- Specificity
- Affinity
- Blocking CXLC8-mediated angiogenic effects
Atherogenic T cell recruitment
Neutrophil mobilization
Vascular protection & intregrity
Atherogenic monocyte recruitment & survival
Cardioprotection after myocardial ischemia
Atherogenic monocyte & neutrophil recruitment
Angiogenesis