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MIF Family Cytokines in Innate Immunity and Homeostasis

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The Editors, Profs. Bucala and Bernhagen, dedicate this volume to their spouses, Anne and Aphrodite, with thanks for their long-standing support to their scientific work on MIF.

Contents

MIF- and CD74-Dependent Mechanisms

Shirly Becker-Herman, Naama Gil, Lihi Radomir, and Idit Shachar

Abstract CD74 is a type II cell surface protein that was previously shown to play a role in antigen presentation and as a receptor for the cytokine macrophage migration inhibitory factor (MIF). Studies from recent years demonstrate an important role for CD74 in maintenance of innate and adaptive immune cells. This chapter discusses the MIF/CD74-dependent role in regulating cell survival, metabolism, adhesion, and response to hypoxia in health and disease.

1 CD74

The CD74 gene is located on human chromosome 5 (q32), and was first identified in 1979 by Jones et al. [1]. However, it was not until 1989 that CD74 was shown to have a role in antigen presentation [2]. CD74 is a non-polymorphic type II integral membrane protein (thus also called invariant chain), which exists in different isoforms defined by its primary amino acid sequence. There are four isoforms of CD74 in humans: p33, p35, p41, and p43. CD74 p33 and p41 are distinguished by alternative splicing of the CD74 transcript, where the p41 isoform contains an extra exon (exon 6b). These two isoforms yield two additional protein products due to an N-terminal cytoplasmic extension of 16 residues, which results from an alternative translation initiation site. The major human p33 isoform has an N-terminal cytosolic tail of 30 amino acids, a transmembrane (TM) domain consisting of amino acid 31–56, and a C-terminal 160 residue luminal domain [3].

This chapter discusses MIF's receptor CD74 and their central position in linking innate and adaptive immune response in health and disease.

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The murine CD74 gene encodes two polypeptide chains, one of relative molecular mass of 31 kD (p31) and another less abundant 41 kD (p41) species [4, 5]. Exon 6b is alternatively spliced into the mRNA coding for the p41 isoform [6].

2 CD74 Function

Two main functions were described for CD74:

1. An MHC class II chaperone
2. Cell surface receptor for MIF

2.1 MHCII Chaperone

MHC class II molecules are heterodimeric complexes that present foreign antigenic peptides on the cell surface of antigen-presenting cells (APCs) to CD4+ T cells [7–9].

MHC class II synthesis and assembly begins in the endoplasmic reticulum (ER) with the non-covalent association of the MHC α and β chains with trimers of CD74. Three MHC class II αß dimers bind sequentially to a trimer of CD74 to form a nonameric complex $(\alpha\beta CD74)_3$, which then exits the ER [10]. After being transported to the *trans*-Golgi, the αßCD74 complex is diverted from the secretory pathway to the endocytic system and ultimately to acidic endosome/lysosome-like structures called MHC class II compartments (MIIC or CIIV) or to the cell surface. Cell surface CD74 is modified by the addition of chondroitin sulfate (CD74-CS) at amino acid position 201, and this form of CD74 is associated with MHC class II on the surface of antigen-presenting cells [11–13]. Surface expression of newly synthesized CD74 followed by its rapid internalization to the endosomal pathway has also been known for many years. Experiments investigating cell surface CD74 are complicated by the fact that CD74 on the cell surface is characterized by a very rapid turnover [14–16].

The N-terminal cytoplasmic tail of CD74 contains two extensively characterized dileucine-based endosomal targeting motifs [17–19]. These motifs mediate its internalization from the plasma membrane and from the *trans* Golgi network. In the endocytic compartments, CD74 is gradually proteolytically processed, leaving only a small fragment, the class II-associated Ii chain peptide (CLIP), bound to the released αß dimers. The final step for MHC class II expression requires interaction of αßCLIP complexes with another class II-related αß dimer, called HLA-DM in the human system, and H2-M in mice. Binding of this molecule drives out the residual CLIP, rendering the αß dimers ultimately competent to bind antigenic peptides, which are mainly derived from internalized antigens and are also delivered to the endocytic pathway [20, 21]. The peptide-loaded class II molecules then leave this compartment, by an unknown route, to be expressed on the cell surface and surveyed by CD4+ T cells. Thus, CD74 was thought to function mainly as MHC class II chaperone, which promotes ER exit of MHC class II molecules, directs them to

endocytic compartments, prevents self-peptide binding in the ER, and contributes to peptide editing in the MHC class II compartment [22].

2.2 CD74 as Cell Surface Receptor

A small proportion of CD74 is modified by the addition of chondroitin sulfate (CD74-CS), and this form of CD74 is expressed on the cell surface [11–13, 23]. This cell surface expression of CD74 is not strictly dependent on class II MHC [24, 25], and numerous non-class II positive cells express CD74 where it can serve as a receptor for the initiation of different signaling cascades [26, 27]. The cytokine macrophage migration inhibitory factor (MIF) was found to be the natural ligand of CD74. MIF binds to the extracellular domain of CD74 with high affinity ($KD = 1.40 \times 10^{-9}$ M) and initiates a signaling cascade [28]. In addition, the MIF homologue, D-dopachrome tautomerase (D-DT; MIF-2), binds CD74 with high affinity [29].

MIF and D-DT binding to CD74 induces various cell type-specific signals that result in cell survival, regulated metabolism, adhesion, and pre-inflammatory pathways.

3 MIF/CD74 in Health

3.1 Regulation of Cell Survival

In murine B cells, CD74 expression is directly involved in shaping the B cell repertoire by regulating mature B cell survival [22, 25, 30, 31]. MIF binding to CD74 induces a signaling pathway that involves the Syk tyrosine kinase and the PI3K/Akt pathway [25, 31], induction of CD74 intramembrane cleavage, and the release of the CD74 intracellular domain (CD74-ICD) [32, 33]. It was recently shown that CD74–ICD interacts with the transcription factors RUNX (Runt related transcription factor) and NF-kB and binds the chromatin in proximal and distal regulatory sites enriched for genes involved in apoptosis, immune response, and cell migration. CD74-ICD binding to the chromatin leads to regulation of expression of these genes [34]. CD74-ICD translocates to the nucleus where it induces activation of transcription mediated by the NF-κB p65/RelA homodimer and its coactivator, TAFII105, which in turn upregulates TAp63 transcription and expression [35]. Binding of TAp63 to the Bcl-2 promoter induces the expression of the Bcl-2 protein which inhibits apoptosis. Thus, the MIF/CD74/NF-κB/TAp63 axis shapes the mature B cell repertoire resulting in regulation of the humoral immune response [35].

MIF was found to regulate cell entry into the S-phase in a CD74- and CD44-dependent fashion, by elevating cyclin E levels, resulting in cell proliferation. In addition, this cascade augments Bcl-2 expression, further supporting cell survival [25, 31, 35–38].

Cell surface receptor CD44 has been implicated in the regulation of activation of the tyrosine kinase receptor c-Met [39–41] although the precise mechanism of their interaction is unknown.

c-Met is a unique disulfide-linked α–β heterodimeric receptor tyrosine kinase with a versatile role in regulating numerous biological functions in response to its natural ligand, hepatocyte growth factor/scatter factor (HGF). HGF is a multifunctional cytokine with a domain structure and proteolytic mechanism of activation similar to that of the serine protease plasminogen. Activation of the HGF/c-Met signaling pathway, which requires phosphorylation of various specific tyrosine residues on c-Met itself, leads to cellular responses, including increased motility, proliferation, morphogenesis, and cell survival [42–48].

Following MIF stimulation, c-Met engages with CD74 and CD44 on the cell membrane and, together with HGF, triggers an additional signaling pathway, which is necessary to initiate the MIF-induced survival signaling cascade [37]. The HGF-induced survival pathway controls proliferation and survival of peripheral B cell subsets. HGF enhances the survival of the mature B cell population in the spleen, whereas there is no change in the cell death of the immature population.

c-Met activation results also in increased expression of the cytokine midkine (MK). MK is an heparin-binding cytokine and its activities include anti-apoptosis, mitogenesis, transformation, angiogenesis, and chemotaxis [49]. MK induces a signaling cascade that involves Syk and Akt phosphorylation, leading to the expression of Bcl-2 and enhanced cell survival of the mature B cell population [38].

MK activated several receptors [50]. It was shown that RPTPζ is expressed in mature B cells and its expression is required for the MIF/CD74- and HGF/c-Met-induced survival cascade [38].

The mammalian bone marrow (BM) is the major site of adult hematopoiesis. Importantly, the recent advent of advanced imaging studies has led to the identification of unique niches that provide a highly specialized microenvironment for distinct developmental processes. These include anatomically defined niches for hematopoetic stem cells [51, 52], and for B cell development [53].

The BM harbors dendritic cells (bmDC) that function as myeloid BM cells and display an activated phenotype. Most intriguingly, these cells are concentrated into unique peri-vascular clusters that wrap a distinct set of sinusoids and venules [36]. Conditional ablation of bmDC results in the specific loss of both endogenous and adoptively transferred mature B cells from the BM immune niches. This failure of bmDC-depleted BM to support B cell engraftment could be overcome by the overexpression of the anti-apoptotic factor, Bcl-2, in the mature B cells, suggesting that bmDC provide a unique survival factor. Studies using mixed BM chimeras subsequently showed that this factor is MIF. Thus, mature B cell maintenance requires MIF-producing bmDC [36]. Newly formed mature B cells emerge from the spleen and circulate in the body. In the BM, a survival signal induced by MIF and secreted from bmDC is essential for B cell maintenance.

The role of CD74 as a survival receptor is not limited to B cells. An important feature of MIF's biologic action is its ability to sustain monocyte/macrophage

activation [54]. Both CD74 and CD44 are necessary for MIF protection from p-53-dependent apoptosis in cells of the myeloid lineage.

CD74 is also expressed on normal colon epithelial cells. Similar to its role in B cells, MIF stimulation of CD74 expressed on colon epithelial cells induces a signaling cascade leading to upregulation of cyclin E and Bcl-2 expression, resulting in their survival. These events also required the simultaneous participation of CD44 [27].

3.2 Control of Cell Metabolism

MIF binding to CD74 in macrophages induces a signaling cascade characterized by the protein kinase A-dependent phosphorylation of the CD74 intracellular domain at Ser6 and Ser8 followed by the recruitment of CD44 and its regulated phosphorylation at Ser291, Ser316, and Ser325 [55]. These events are succeeded by the activation of Src-family kinases and further downstream by activation of ERK1/2.

Several biologic activities of MIF are proceeded via ERK1/2 activation; these include arachidonic acid metabolism, prostaglandin production (via cytoplasmic phospholipase A2 and cyclooxygenase-2), and the activation of the Ets family of transcription factors that regulate the expression of Toll-like receptors [56].

3.3 MIF/CD74 in Cell Adhesion

Recent information suggests that the intracellular MIF-binding protein, JAB1, regulates the sustained phase of MIF-induced ERK phosphorylation [57]. MIF-induced ERK phosphorylation also exhibits adhesion dependence [58], which may be correlated with the CD44's known role in cell-extracellular matrix interaction [59]. Cell adhesion also results in an autocrine MIF release response and to a pathway for ERK activation involving the sequence of Rho GTPase, myosin light chain kinase, and focal adhesion kinase [58, 60].

3.4 MIF/CD74 in Hypoxia

Cellular responses to changes in oxygen tension during normal development or pathological processes, such as cardiovascular disease and cancer, are ultimately regulated by the transcription factor hypoxia-inducible factor (HIF) [61]. Rapid growth of tumor cells usually creates a hypoxic environment, which induces cell-adaptation responses, such as HIF-dependent survival pathways and angiogenesis. HIF transcription factors have been implicated in controlling the expression of a wide variety of genes involved in apoptosis, angiogenesis, invasion, and metastasis [61]. HIFs are basic helix-loop-helix–PER–ARNT–SIM (bHLH–PAS) proteins that

form heterodimeric complexes comprising an O_2-labile α-subunit (HIF1α, HIF2α, or HIF3α) and a stable β-subunit (HIF1β, also known as ARNT). Although HIF-1β is constitutively expressed, HIF-1α is rapidly induced by hypoxia. In hypoxia state, these subunits together bind hypoxia-responsive elements (HREs), activating the expression of numerous hypoxia-response genes [62, 63]. Hypoxic HIF activity is controlled primarily through post-translational modification and stabilization of HIF1α and HIF2α subunits. HIFα subunits are modified by HIF-specific prolyl-hydroxylases (PHDs) in the presence of O_2, which leads to normoxic proteasomal degradation that is mediated in part by the von Hippel–Lindau (VHL) tumor suppressor protein [63]. Another mechanism of inhibiting HIF-1α function is mediated by "factor inhibiting HIF" (FIH), which prevents the transcriptional activation of HIF-1α by blocking the interaction between the coactivators p300 and CREB-binding protein [64]. During hypoxia, HIF-1β binds HIF-1α, preventing proteasomal degradation, and the complex is transported to the nucleus, where it binds HIF response elements (HREs). The binding of HIF-1α/HIF-1β to HREs assists in the recruitment of coactivator molecules that form transcription initiation complexes to enhance the expression of its target genes that mediate cellular and physiologic responses to hypoxia [65].

The functional relationship between HIF-1 and MIF has been investigated, indicating the HIF-dependent, as well as the HIF-independent, induction of MIF [66, 67], and an indirect protein interaction between HIF and MIF [68, 69]. T helper cells are capable of inducing HIF-1α and HIF-1 target genes under hypoxia. The induction of MIF under hypoxia is a manifestation of HIF-1 activity. MIF, in turn, is a key regulator of hypoxia-induced HIF-1α protein expression, a process mediated by the MIF receptor, CD74, thus forming an autocrine positive-feedback loop [70].

Recently, the role of MIF/CD74 was analyzed in hypoxia/serum deprivation (SD)-induced apoptosis of mesenchymal stem cells (MSC). This study revealed an attenuated hypoxia/SD-induced apoptosis in an MIF-dependent manner. MIF protected MSCs from hypoxia/SD-induced apoptosis by interacting with CD74 to stimulate c-Met, leading to downstream PI3K/Akt-FOXO3α signaling and decreased oxidative stress [71].

3.5 Osteoclastogenesis

MIF and CD74 are involved in downregulated osteoclast-like (OCL) cell formation. Upon MIF binding, the MIF/CD74/CD44 complex activates phosphorylation of Lyn during osteoclastogenesis. Subsequently, phospho-Lyn downregulates RANKL-induced activation of the Gab2/JNK-1/c-Jun cascade and the Syk/phospholipase Cγ (PLCγ) pathway to suppress the transcription factor NF-ATc1. These results indicate that MIF inhibits osteoclastogenesis by activating Lyn, which in turn downregulates RANKL-mediated osteoclast differentiation by suppressing NF-ATc1 and AP-1 [72].

4 CD74 in Disease

MIF and its receptor CD74 have been associated with tumor progression. MIF is overexpressed in malignancies including solid tumors [73], and it supports the growth of malignant cells. MIF mediates tumor-associated angiogenesis in a murine colon cancer cell line [74]. MIF is frequently overexpressed in primary breast cancer tissues, where it plays a role in tumor–stroma interactions [75]. In addition, in different tumor models anti-MIF antibody treatment has been shown to suppress tumor growth [76, 77]. MIF's receptor, CD74, is overexpressed in various hematopoietic and solid tumors [78–84] including chronic lymphocytic leukemia (CLL) [85, 86]. Its expression in many cancers may serve as a prognostic factor, with higher relative expression of CD74 being associated with tumor progression [87]. A humanized anti-CD74 mAb (milatuzumab; hLL1) is presently in clinical development and has therapeutic activity in multiple myeloma, perhaps by inhibiting CD74-dependent pathways of cell survival [88].

4.1 CD74 in Tumors

4.1.1 Tumor Survival

B Cell Malignancies

CLL

The expression of MIF and its receptor CD74 are upregulated on CLL cells [89]. In these cells, stimulation of CD74 induces a signaling cascade leading to IL-8/CXCL8 secretion, regardless of the patient clinical status [89, 90]. The secreted IL-8 autocrinely, via its receptor CXCR2 to which MIF can also signal, regulates the expression of the anti-apoptotic protein, Bcl-2, and thus controls a survival pathway. Blocking MIF, CD74 (with milatuzumab), or IL-8 results in significant decrease in Bcl-2 expression and induction of apoptosis. Increased serum levels of IL-8 have negative prognostic significance in CLL [91], lending further support to the importance of this pathway in the pathogenesis and progression of disease. Thus, IL-8 secreted following CD74 activation results in an autocrine/paracrine survival response.

Stimulation of CD74 expressed on CLL cells also induces the expression and secretion of MK. Binding of MK to its receptor RPTPζ elevates the expression of Bcl-2 and inhibits caspase 3 and 7 activity. Moreover, blocking RPTPζ activity resulted in inhibition of the MIF/CD74-induced survival cascade and induction of cell death. Together, these results show that the MK/RPTPζ pathway plays a major role in the MIF/CD74 survival cascade in CLL [38]. In CLL patients, MK serum levels are relatively higher when compared to normal individuals, regardless of the

disease stage [38]. These findings are in agreement with other studies in which MK was significantly elevated in serum from cancer patients [92–95].

Similarly to its effect on normal B cells, TAp63 expression was shown to play an important role in CLL survival. Blocking CD74 using milatuzumab specifically downregulates TAp63 expression [35]. Moreover, the lowering of p63 levels in CLL cells specifically inhibits the MIF-induced elevation of Bcl-2 mRNA levels. Thus, the MIF/CD74-induced survival cascade is mediated through TAp63 [90].

MIF and CD74 thus mediate an important survival mechanism in CLL that appears to operate from the very early stages of the disease. This cascade involves secretion of IL-8 and MK, which in turn bind to their receptors to induce a signaling cascade that regulates TAp63 expression resulting in Bcl-2 expression and cell survival.

To define the molecules whose expression is modulated by CD74, thereby regulating CLL survival, we searched for CD74 target genes. One molecule whose expression was strongly upregulated by CD74 activation is CD84. CD84 is a member of the SLAM immunoglobulin superfamily. It is a single chain cell-surface protein with an extracellular portion of 199 aa, which contains four potential N-glycosylation sites. CD84 is predominantly expressed by B cells, T cells, platelets, monocytes, and dendritic cells (DCs) and early in hematopoiesis; its function is versatile, but not fully understood [96]. It was recently shown that CD84 is required for prolonged T cell: B cell contact, optimal T follicular helper function, and germinal center formation in vivo [97]. In addition, murine CD84 was shown to be involved in the modulation of signaling pathways downstream of TLR4, and in regulation of macrophage cell-fate decisions and effector function [98]. Our studies showed that activation of cell surface CD84 initiates a signaling cascade, which enhances cell survival. Both immune-mediated neutralization (using supernatant of a hybridoma raised by us against the CD84 extracellular loop) and blockade of CD84 (using a truncated recombinant CD84 fragment, CD84ECD, that can bind to CD84, thereby blocking the ability of the receptor to engage in further interactions) induce cell death in vitro and in vivo [99].

B Cell Lymphoma

B cell lymphoma cell lines BJAB, Ramos, Raji, and Daudi express CD74. Removal of CD74 sensitizes cells to Fas-mediated apoptosis and subsequently also to Fas-dependent chemotherapies, doxorubicin, and edelfosine. The increased sensitivity to Fas-mediated apoptosis in cells lacking CD74 was due to increased activation/cleavage of the initiator caspase-8 and correspondingly increased activation of effector caspase-3. Thus, the enhancement of Fas-mediated apoptosis occurs at an immediate early step of Fas signaling at the plasma membrane—the activation of death-inducing signaling complex (DISC). MIF signaling through CD74 mediates activation of NF-κB, which is known to regulate expression of cFLIP, a well-known inhibitor of a DISC component caspase-8. Removal of CD74 significantly increases the levels of Fas receptor at the cell surface and thus the amount of the Fas receptor available for activation. Together, these results suggest that specific targeting of the CD74 on the cell surface will sensitize CD74-expressing cancer cells to

Fas-mediated apoptosis, and thus will increase effectiveness of chemotherapy regimens for hematological malignancies [100].

Multiple Myeloma (MM)

Multiple myeloma (MM) is a malignancy of terminally differentiated plasma cells, typically occurring in older patients and producing a monoclonal immunoglobulin protein or fragment. CD74 is strongly expressed on MM cells [101, 102].

Treatment of cells with the humanized anti-CD74 antibody (hLL1) showed in vitro great growth inhibitory effects on myeloma cell lines and in vivo therapeutic effects on established myeloma in SCID mouse models, where administration of hLL1 led to a 45% increase in median survival of myeloma-bearing severe combined immunodeficiency (SCID) mice [88].

Solid Tumors

Melanoma

Melanoma, the most aggressive skin cancer, is believed to be a highly immunogenic tumor. Recent developments in immunotherapies are promising. IFN-γ produced by immune cells has a crucial role in tumor immune surveillance; however, it has also been reported to be pro-tumorigenic. It was recently shown that IFN-γ enhances the expression of CD74, which interacts with its ligand MIF and thereby activates the PI3K/AKT pathway in melanoma, promoting tumor survival. IFN-γ increased phosphorylation of AKT Ser473 and upregulated total cell surface expression of CD74 in human melanoma cell lines tested. CD74 was highly expressed in melanoma tissues. Moreover, the expression of CD74 on tumor cells correlated with plasma IFN-γ levels in melanoma patient samples. Blockade of CD74–MIF interaction reduced Akt phosphorylation and expression of pro-tumorigenic molecules, including IL-6, IL-8, and BCL-2. Inhibition of CD74–MIF interaction significantly suppressed tumor growth in the presence of IFN-γ in our xenograft mouse model. Thus, IFN-γ promotes melanoma cell survival by regulating CD74–MIF signaling [84].

Gastrointestinal Cancers

Expression of CD74 within gastrointestinal carcinomas showed a statistically greater expression than in the normal tissue counterparts [103]. CD74 stimulation by MIF enhances Akt phosphorylation, Bcl-2 expression, and colon carcinoma survival. Thus, CD74 is a survival receptor expressed on colon carcinoma cells [27].

Glioblastoma

CD74 has been further described as one of the most upregulated molecules in human glioblastomas. Fractionation of glioblastoma cells and glioma-associated microglia/macrophages (GAMs) from primary tumors revealed that CD74 is

restricted to GAMs in vivo, while being absent in tumor cells, the latter strongly expressing its ligand MIF. Most interestingly, a higher amount of CD74-positive GAMs was associated with beneficial patient survival constituting an independent prognostic parameter and with an anti-tumoral M1 polarization. In summary, CD74 expression in human gliomas is restricted to GAMs and positively associated with patient survival. In conclusion, CD74 represents a positive prognostic marker most probably because of its association with an M1-polarized immune milieu in high-grade gliomas [104].

4.1.2 MIF and CD74 in CLL Homing and Adhesion

BM stroma provides survival niches for both normal and leukemic mature B cells. Adhesion of CLL cells to BM niches has been demonstrated to rescue these lymphocytes from apoptosis and to extend their life span [36, 105, 106]. The increased accumulation of CLL cells in the BM in the advanced stages of disease also suggests a change in the migratory and homing pattern of the cells, and this phenomenon is supported mechanistically by the increased expression of the integrin VLA-4 in late compared to early stage cells [90, 107–109].

MIF and CD74 play a significant role in regulation of VLA-4 expression on CLL cells and therefore affect the homing and survival of these cells [90]. Since MIF may be released from virtually all types of cells, CLL cells are stimulated by this cytokine in all compartments. During progression to advanced disease, MIF stimulation may elevate VLA-4 cell surface expression to levels that support their homing to the BM by an as yet unknown mechanism. Thus, threshold levels of VLA-4 expression are required for homing of CLL cells to the BM. These levels enable the retention and survival of CLL in the BM in an environment that is enriched with the VLA-4 ligands, VCAM-1 and fibronectin, and supports survival. The VLA-4-fibronectin interaction has been shown to have a significant effect on CLL cell survival [110] as well as a protective effect against fludarabine (the backbone drug used for the treatment of CLL) induced cell death [111]. This situation may create a cycle that can promote disease-associated bone marrow failure. It is possible that CLL exposure to systemic MIF redirects circulating CLL cells back to the BM, where they may further elevate their VLA-4 expression and retention on stromal VLA-4 ligands. Accordingly, MIF or CD74 blockade may provide an additional approach to regulate CLL survival by inhibition of their homing to the BM.

Together, novel therapeutic strategies aimed at blocking MIF/CD74-induced cell survival, and/or alteration of CLL disease progression by decreasing bone marrow homing, and occupation of normal hematopoietic niches could lead to enhanced and better targeted eradication of this lymphoid malignancy.

4.1.3 CD74 in Hypoxia

In transformed cells, MIF was shown to modulate and to be modulated by HIF-1 [69]. In addition, MIF overexpression in human breast cancer cell lines was found to promote hypoxia-induced HIF-1α stabilization [68]. The MIF receptor CD74 was shown to mediate HIF-1 activation by MIF [68]. Moreover, hypoxia-induced VEGF expression is significantly reduced in MIF-deficient cells and increased in MIF-overexpressing cells, consistent with its contribution to HIF-1α stabilization [68, 69].

4.2 Autoimmunity and Inflammatory Response

4.2.1 Systemic Lupus Erythematosus (SLE)

MIF has been implicated in the pathogenesis of numerous inflammatory and autoimmune disorders [112]. Systemic lupus erythematosus (SLE) is a multisystem autoimmune disease that is characterized by the loss of immune tolerance and the production of autoantibodies to nucleic acids and nucleoproteins [113]. The immunopathology associated with SLE results primarily from immune complex deposition in the small vessels of the skin, kidney, and other organs; this leads to the activation of complement and Ig Fc receptors and the recruitment of neutrophils and monocytes. In addition, SLE is characterized by impaired B-cell and T-cell functions and is associated with serological and clinical manifestations that involve multiple organ systems and abnormal B-cell activation and differentiation [113].

B lymphocytes from SLE-afflicted mice express elevated levels of CD74, compared with B cells from healthy mice [114]. Two lupus-prone mouse strains manifest a time-dependent elevation in circulating MIF at ages that correspond with disease progression and the development of glomerulonephritis [115]. MIF [115], CD74, and CD44 [114] mRNA and protein expression in kidneys is also increased significantly in parallel to inflammatory progression in lupus-prone mice. In B cells from the diseased mice, MIF expression is also upregulated [114]. For the specific treatment of SLE, a peptide designated hCDR1, which is based on the sequence of the complementarity-determining region (CDR)-1 of an autoantibody [116], was designed and shown to ameliorate lupus manifestations in both spontaneous and induced models of SLE [117, 118]. Induction of the MIF/CD74 pathways in B cells of SLE-diseased mice is associated with their increased survival. Treatment with hCDR1 diminishes the expression of CD74 and CD44 molecules to the levels generally detected in young healthy mice, resulting in reduced B-cell survival [114]. Furthermore, treatment with (S,R)-3-(4-hydroxyphenyl)-4,5-dihydro-5-isoxazole acetic acid methyl ester (ISO-1), an MIF antagonist, reduces the MIF-dependent proinflammatory cytokine production and leukocyte recruitment and ameliorates immune-mediated renal injury [115]. These results suggest that the MIF/CD74 pathway plays an important role in survival of pathogenic B cells and in lupus pathology.

4.2.2 Multiple Sclerosis

Multiple sclerosis (MS) is a complex demyelinating disease with an autoimmune origin. Monocytes and macrophages play a pathogenic role in multiple sclerosis [119]. EAE, a well-established model of MS, is characterized by extensive lymphocytic infiltration into the central nervous system (CNS). Several reports have demonstrated that monocytes are involved in the exacerbation of EAE, with monocyte depletion resulting in a marked suppression of clinical disease [120, 121]. Blocking of MIF binding to CD74 or CD74 cell surface expression by the HLA-DRα1 domain results in reduced axonal damage and reversal of ongoing clinical and histological signs of EAE [122, 123]. Blocking CD74 enhanced apoptosis, increased random migration of activated monocytes, and reduced the secretion of pro-inflammatory cytokines [122].

4.2.3 MIF/CD74 in Viral Arthritis

Old world alphaviruses are important causes of viral arthritis and arthralgia worldwide. Alphaviruses are members of the *Togaviridae* family and include Ross River virus (RRV), chikungunya virus (CHIKV), mayaro virus, and o'nyong-nyong virus [124]. These viruses circulate in both endemic and epidemic patterns and can cause widespread outbreaks of polyarthritis and arthralgia [124–126] frequently involving tens of thousands to millions of cases.

MIF and CD74 were shown to be involved in alphavirs infections [127, 128]. In comparison to wild-type mice, $CD74^{-/-}$ mice developed only mild clinical features and had low levels of tissue damage. Leukocyte infiltration, characterized predominantly by inflammatory monocytes and natural killer cells, was substantially reduced in infected tissues of $CD74^{-/-}$ mice, but production of pro-inflammatory cytokines and chemokines was not decreased. CD74 deficiency was associated with increased monocyte apoptosis, but had no effect on monocyte migratory capacity. Consistent with these findings, alphaviral infection resulted in a dose-dependent upregulation of CD74 expression in human peripheral blood mononuclear cells, and serum MIF levels were significantly elevated in humans with RRV or CHIKV infections [128]. These findings suggest that both MIF and CD74 play a critical role in mediating alphaviral disease, and blocking these factors with novel therapeutic agents can substantially ameliorate pathology.

4.2.4 CD74 as a Bacterial Receptor and Gastrointestinal Inflammation

H pylori is a Gram-negative spiral bacterium that colonizes the human gastroduodenal mucosa. Infection with *H pylori* usually begins in childhood and persists for decades if untreated. *H pylori* is recognized as a major contributor to chronic gastritis and peptic ulcer formation and is strongly associated with gastric carcinoma and lymphoma. *H pylori* adhesion to the gastric epithelium is important in successful

colonization of the gastric mucosa. Adherent strains survive in the gastric mucosa, reach high bacterial densities, and can re-colonize, whereas non-adherent strains are cleared [129]. The urease protein of *H pylori* utilizes CD74 to attach to gastric epithelial cells (GEC) [130, 131]. MIF or *H pylori* binding to CD74 induces NF-κB and subsequent cellular responses, such as the secretion of proinflammatory cytokines [132]. This interaction is particularly interesting because many bacteria express urease, so the possibility exists that there might be wider applications of this type of interaction with CD74 depending on urease sequence variation between bacteria.

CD74 expression is increased in vivo. Most of the *H pylori*-infected samples and the samples with gastritis for reasons other than *H pylori* infection had much higher expression of CD74 than uninfected samples not exhibiting signs of gastritis. Other studies have further shown the expression of CD74 increased in ulcerative colitis and Crohn's Disease [133]. Increased CD74 expression could then go on to intensify inflammation by providing more free receptors for MIF or *H pylori* attachment.

4.2.5 CD74 in Atherosclerosis

CD74 also promotes atherosclerosis, a lipid-triggered chronic inflammatory condition of the arterial vessel wall. Cd74-/- mice show reduced atherosclerosis and CD74 contributes to atherogenic leukocyte recruitment responses via the MIF/CXCR axis [134, 135].

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HSP90-Stabilized MIF in Oncogenesis and Cell Growth Control

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Abstract Cancer is a complex interplay of diverse genetic aberrations resulting in core phenotypes, summarized as the hallmarks of cancer. The macrophage migration inhibitory factor (MIF) promotes the majority of these hallmarks such as sustaining proliferative signals, evading growth repressors, resisting apoptosis, activating invasion, inducing angiogenesis, and evading immune surveillance. As pro-inflammatory cytokine, MIF also promotes tumor-promoting inflammation. Pleiotropic acting MIF triggers cell proliferation via major survival pathways including PI3K-Akt and MEK/ERK, blocks apoptosis by one of the main tumor suppressors, namely, p53, and controls cell cycle through E2F family members. Importantly, MIF not only acts in the epithelial cancer cell compartment, but also in the associated stromal fibroblasts and cells of the immune system. The tumor-promoting activities of MIF correlate with tumor aggressiveness and poor clinical prognosis. Independent of the histological tumor origin, MIF is highly stabilized in cancer cells via the heat-shock protein 90 chaperone machinery (HSP90). Pharmacological inhibition of tumoral HSP90, which is highly activated in cancers compared to normal tissues, results in MIF degradation in several cancer cell types. Thus, MIF is an HSP90 client. This provides a new way to target elevated MIF by HSP90 inhibition, along with a large number of other critical tumor-promoting proteins that are destabilized by HSP90 inhibition, overall resulting in a profound block of tumor growth.

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Early on in MIF's history, it was already shown that cancer cells harbor elevated intracellular MIF levels. Consequently, MIF knockout cancer models were established to show that MIF has an important role in promoting tumor growth across many different tumor types. For example, MIF deletion in Eμ-Myc transgenic mice protects from B-lymphoma development [1]. In Eμ-TCL1 mice, a mouse model for chronic lymphocytic leukemia (CLL), MIF deletion impairs leukemia progression and extends survival compared to MIF harboring wildtype mice [2]. In fibrosarcoma, MIF loss generates smaller tumors with a lower mitotic index [3]. In bladder tumorigenesis, MIF triggers bladder cancer by increasing cell proliferation and angiogenesis which leads to more aggressive tumors [4]. MIF deletion in Apc MIN/+ mice reduces the growth of small intestinal adenomas [5]. Moreover, MIF loss delays skin cancer progression in response to chronic UVB exposure [6]. Furthermore, MIF deletion in an ErbB2-driven breast cancer mouse model extends survival and suppresses tumor growth [7]. Also, xenograft mouse models confirm MIF's pro-tumorigenic role. The tumor burden of nude mice transplanted with colorectal carcinoma [8] or prostate carcinoma [9] was reduced after anti-MIF therapeutic treatment. Stable MIF knockdown in ovarian carcinoma cells increases overall survival in xenografts [10]. Xenograft models of hepatocellular carcinoma (HCC) cells show that MIF knockdown reduces the tumor growth rate [11]. These multiple mouse models, together with numerous in vitro studies and clinical correlation studies in human cancer samples, strongly support a critical tumor-promoting role for MIF. Cancer cells are "addicted" to stabilized MIF, making it attractive to target MIF during tumor formation and maintenance. For example, patients with moderate to high levels of MIF in their breast cancer tissues show a worse disease-free survival compared to patients with weak MIF expression [12]. MIF is expressed at elevated levels in hypopharyngeal squamous cell carcinoma compared to adjacent normal epithelium, and elevated expression positively correlates with tumor progression [13]. Moreover, plasma levels of MIF are strongly increased in CLL patients compared to healthy controls, with further rise in MIF plasma levels in advanced-stage CLL [2]. In clear cell renal carcinoma (CCRC) patients, MIF expression is dramatically elevated in the serum compared to healthy controls, suggesting MIF as a potential diagnostic marker in CCRC [14]. Moreover, increased MIF expression is associated with poor survival in patients with resected pancreatic ductal adenocarcinoma [15]. Moreover, high MIF levels significantly correlate with high-grade tumors and tumor relapse in glioma patients [16] and hepatocellular carcinoma patients [17]. While MIF levels in patients with primary melanoma were not associated with clinical outcome, higher levels of MIF in metastatic lesions were significantly associated with faster disease progression [18]. In non-small cell lung cancer (NSCLC) patients, the risk of recurrence was associated with high levels of MIF [19]. Also in colorectal cancer patients, MIF levels positively correlate with tumor differentiation and grade [8], as well as with nodal status [20]. In addition, serum levels of MIF are significantly increased in patients with colorectal cancer [21–23]. Finally, high MIF concentrations in the serum were identified in patients with oral squamous cell carcinoma (OSCC), but disappeared after tumor resection [24].

1 Elevated MIF Levels in Cancer Cells

Although immune and other normal cells often contain readily detectable MIF protein, tumor cells of most if not all cancer types contain elevated intracellular levels of MIF. In breast cancer patients, tumoral MIF is frequently elevated [25, 26]. Moreover, MIF was one of the hotspots of a characteristic 32-spot pattern in a breast cancer proteomics study and discriminated mammary epithelial neoplasia from its normal counterpart [27]. A study with 121 breast cancer patients showed that MIF expression is more prominent in cancer tissues compared to normal breast tissues [12]. In high-grade hypopharyngeal squamous cell cancers, MIF levels are higher compared to matched normal tissues [13]. Intracellular MIF protein level, as well as secreted MIF, is strongly increased in tumor cells of CLL patients compared to B cells from healthy donors [2]. Also, high MIF expression is strongly associated with high-grade gliomas [16]. In tumor tissue microarrays of CCRC, MIF is moderately to highly expressed [14]. In primary ovarian cancer, intracellular MIF is elevated compared to normal ovarian tissue [10]. In prostate cancer tissues, MIF is elevated compared to normal prostate tissues [28, 29]. In cervical cancer, immunohistological analysis confirms that MIF is strongly elevated compared to normal cervical tissue [30, 31]. In hepatocellular carcinoma (HCC), MIF levels are significantly upregulated in tumor tissue compared to adjacent normal tissue [11, 17]. Expression of MIF protein is significantly higher in human melanoma cells than in normal cultured melanocytes [32]. Moreover, MIF expression increases during progression of melanocytic lesions to advanced-stage melanoma [18]. Elevated MIF levels are also found in human bladder cancers [33] and in gastrointestinal cancers [5, 8]. In sum, the list of elevated MIF protein expression in cancer is long and strongly suggests that cancer cells need MIF stabilization for progression and/or maintenance. The long-standing question was then how cancer cells elevate MIF protein.

2 MIF Is Stabilized via the HSP90 Machinery

While some cancer types were reported to show a minor to moderate increase in MIF mRNA levels compared to normal cells [29, 30, 34–38], the main mechanism responsible for the robust elevation of MIF protein in tumor cells remained unknown for a long time. In normal healthy cells, HIF1α ICBP90, and SP1 are the only known transcription factors to induce MIF expression [39–42]. However, in a panel of cancer cell lines HIF1α failed to increase MIF protein further beyond the elevated levels they already have (our unpublished data). Thus, another mechanism seemed to be responsible for elevated MIF. We identified that MIF is mainly upregulated via increased protein stabilization [7]. We showed that MIF stabilization is mediated by binding to the tumor-activated HSP90 chaperone machinery that ensures proper conformational folding and protects MIF from degradation (Fig. 1). Pharmacological inhibition of tumoral HSP90 activity by small-molecule inhibitors or HSP90

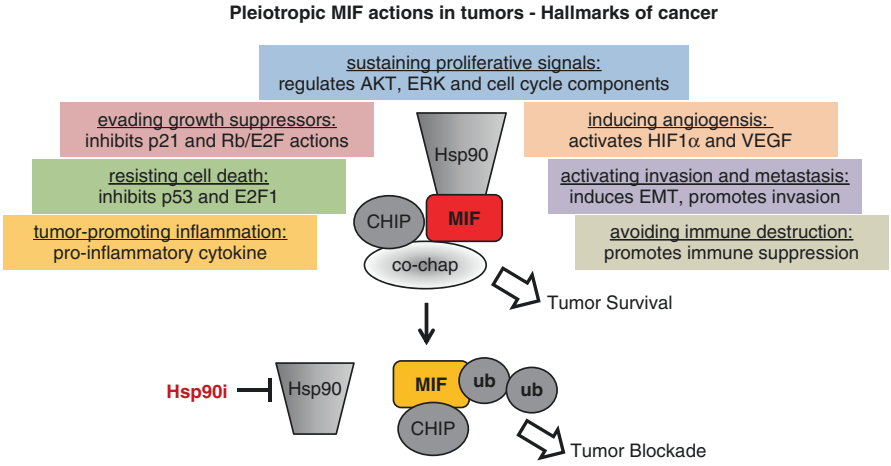

Fig. 1 Pleiotropic MIF actions in tumors—hallmarks of cancer. HSP90 inhibition targets all MIF functions. HSP90 stabilizes client proteins such as MIF, protecting them from proteasomal degradation, thereby promoting tumor survival (*top*). HSP90 inactivation induces the dissociation of HSP90 complex, leading to client release and activation of E3-ubiquitin ligases with subsequent client degradation. Client degradation promotes a profound block in tumor growth (*bottom*)

depletion via siRNAs induces strong MIF degradation with subsequent suppression of tumor cell proliferation and induction of apoptosis in a spectrum of human cancer cells. Importantly, studies in the ErbB2 mouse model of breast cancer fully confirm MIF as a tumor-promoting and HSP90-stabilized client in vivo, since HSP90 inhibition reduces MIF protein and blocks tumor growth in mice [7].

In normal cells, the HSP90 machinery controls folding, maturation, and proper activation of proteins. Stress-damaged, mutated, and conformationally or stoichiometrically aberrant client proteins will be targeted by E3-ubiquitin ligases for proteasome-mediated degradation [7, 42, 43]. Importantly, the HSP90 chaperone cycle in normal cells is dynamic with transient and low-affinity client-HSP90 complexes. Chaperones are expressed at basal levels. During cell transformation and oncogenesis, a cell has to sustain perennial proteotoxic stress. This includes an adverse microenvironment such as hypoxia and acidosis, and cell-intrinsic stress such as conformationally aberrant oncoproteins, high levels of ROS, massive oxidative stress, high levels of DNA damage, and genomic instability. Cells react and adapt to proteotoxic stress with upregulation of the inducible heat-shock chaperone response, most prominently the multiprotein HSP90 machinery [44, 45]. The master transcriptional regulator for the synthesis of inducible heat-shock proteins is heat-shock transcription factor 1 (HSF1). In the classical chaperone displacement model, in unstressed cells HSF1 is maintained in an inactive state by direct association with chaperones, most prominently the HSP90 chaperone. In cells subjected to heat-shock or other proteotoxic stress, the general increase in misfolded proteins liberates HSP90 from HSF1, thereby allowing HSF1 trimerization. Further

activating steps, mostly post-translational modification and translocation, lead to active HSF1 as a phosphoactivated trimer in the nucleus [44]. Activated HSF1 in turn induces expression of stress-inducible chaperones including Hsp90α, Hsp70, and numerous co-chaperones and adaptor proteins. Since cancer cells are under perennial proteotoxic stress, they activate their inducible heat-shock response in a constitutive manner [42, 43, 46–48]. Consequently, cancer cells show massive, near-obligatory upregulation of stable active high-order multicomponent HSP90 chaperone complexes which enable and maintain malignant transformation by stabilizing a portfolio of hundreds of mutant, overexpressed, or conformationally aberrant client proteins which would normally be degraded by the proteasome machinery [42, 43]. Many oncoproteins and tumor-promoting signaling proteins are among tumoral HSP90 clients including receptor tyrosine kinases (EGFR, ErbB2/Her-2) [49], signaling kinases (Bcr-Abl, Akt) [50], c-Raf [51], mutant p53 [47, 52], MIF [7], and monomeric HSF1 itself [53]. Importantly, HSF1 knockout mice (inhibited HSP90 system) are markedly resistant to tumorigenesis, as is impressively shown by in vivo mouse cancer models [48, 54–56]. Moreover, HSF1 is one of the main determinants of oncogenesis in breast cancer [57], not only by inducing the adaptive proteotoxic stress response, but also by modulating the expression of a broad set of genes involved in cell cycle regulation, signaling, metabolism, and protein translation [58]. Even more important, pharmacologic HSP90 inhibitors at least of the geldanamycin class are more active towards tumoral rather than normal HSP90, possibly through their much higher affinity to higher-order multi-HSP90 chaperone complexes typical for tumors. In normal cells, HSP90 inhibitors do not bind adequately to the transient, low-affinity HSP90 chaperone complex. This differential opens a therapeutic window with less toxicity in normal tissues [43, 59, 60]. Recent findings also show that growth receptor signaling including the ERBB network is also able to activate the HSF1-HSP90 axis [46, 61]. In Her-2-positive breast cancer, overexpressed Her-2 constitutively activates HSF1. Inhibiting Her-2 pharmacologically leads to inhibition of phosphoactivated HSF1, and subsequently blocks the activity of the HSP90 machinery, leading to destabilization of clients including MIF [46].

In sum, the HSF1-HSP90 machinery of normal cells is dramatically subverted during oncogenesis into a powerful anti-apoptotic and pro-survival co-oncogenic promoter, as shown by numerous in vitro and in vivo studies [43, 59, 60]. Since cancer cells require constant and massive HSP90 chaperone support to deal with proteotoxic stress, targeting MIF by inhibiting the tumoral HSP90 system provides an attractive—since pleiotropic and tumor specific—therapeutic window due to its pleiotropic and tumor-specific action [62, 63].

One open question is whether the MIF-HSP90 interaction prevents MIF secretion from tumor cells. In cancer cells, the intracellular MIF-Jab1 interaction seems to store MIF for possible secretion [64]. Importantly, MIF can be secreted by some, but not all cancer cells. However, this aspect was not exhaustively examined in a broad panel of cell lines and tumor explants to definitively establish that some cancer types are truly unable to secrete. Types of cancer cell lines which do secrete MIF

are ovarian [65], cervical [30], bladder [33, 66], prostate [29, 67], colon [68, 69], and metastatic breast cancer [70]. MIF secretion can induce autocrine actions such as stimulating survival pathways, as discussed below. More importantly, MIF also acts intracellularly within cancer cells to promote tumorigenesis. It would be important to know whether specific epithelial cancer cells also secrete MIF, since secreted MIF could contribute to an inflammation-associated malignant transformation mechanism of the epithelial cells. Of note, stromal and immune cells are minor sources of MIF, while tumor cells themselves are the major source of tumor-associated MIF, as shown for colonic epithelium [5, 69, 71].

3 Pleiotropic MIF Functions in Cancer

Over the years it became apparent that constitutively stabilized MIF does not simply acquire one main function in oncogenesis, but acts as a highly pleiotropic tumor promoter that covers almost all of the so-called hallmarks of cancer (Fig. 1) [72–74]. As discussed above, MIF can act intracellularly and as secreted cytokine during tumorigenesis and thus in an autocrine and paracrine manner. This potential duality is strongly context dependent.

4 Cell Cycle and Apoptosis

MIF's action contributes to tumor cell evasion from growth suppressors and resistance to cell death and/or also promotes the cell cycle, depending on cellular context (Fig. 1). As one major underlying mechanism, MIF inhibits the key tumor suppressor p53. p53 governs cell cycle, DNA damage response, senescence, and apoptosis if a cell is irreversibly damaged [75, 76]. Moreover, the E2F family of transcription factors is also deregulated by MIF [77, 78]. E2F family members are downstream effectors of the tumor suppressor retinoblastoma protein (Rb) and play a major role in cell cycle progression via the G1/S checkpoint [75, 79]. Cyclin-dependent kinases (Cdks), activated by their corresponding cyclins, phosphorylate and inactivate Rb, which in turn releases E2Fs from their repressive Rb/E2F complexes, resulting in transcription of critical S-phase enzymes and regulators. Thus, the phosphorylated Rb status determines proliferation by governing S-phase entry and S-phase progression. Thus, the phospho-Rb status functions in a broad range of biological processes, for example, DNA replication, DNA damage and repair, differentiation, and apoptosis. The best-studied E2F family member, E2F1, reveals tumor-suppressive activities by inducing cell cycle arrest and apoptosis [75, 79]. Notably, the Rb–E2F pathway and p53 pathway are massively defective in most, if not all, human tumors, which underscore the pivotal role of both pathways in regulating tumorigenesis. Thus, the fact that MIF regulates both of these major pathways in part explains that MIF acts in a pleiotropic manner.

The first evidence that MIF can suppress apoptosis and bypass growth arrest came from experiments with murine embryo fibroblasts (MEFs), where ectopic MIF bypassed p53 growth arrest and apoptosis by suppression of p53 transcriptional activity [80]. The observation that MIF as pro-inflammatory cytokine is capable of functionally inactivating this key tumor suppressor provided a new molecular link between inflammation and tumorigenesis [80]. Interestingly, p53 inactivation is also seen within the inflammatory context. After endotoxin administration, MIF sustains macrophage viability by suppressing p53-induced apoptosis [81]. In support, MIF knockout MEFs showed slower growth properties under normal conditions and earlier growth arrest following DNA damage, compared to wildtype MEFs [82]. The reduced cell growth of $MIF^{-/-}$ fibroblasts seems to be partly p53 dependent since simultaneous co-deletion of MIF and p53 partially rescues the $MIF^{-/-}$ growth delay of these cells [3, 78]. Furthermore, expression of c-myc, which is known to induce p53, potentiates the $MIF^{-/-}$ growth defects [82]. In addition, the E2F member E2F1 is upregulated in normal $MIF^{-/-}$ MEFs, whereas E2F regulators such as p130 and p107 are deregulated, resulting in $MIF^{-/-}$ growth delay [82]. In serum-starved $MIF^{-/-}$ fibroblasts, some E2F-responsive genes including cyclin E and CDC6 are delayed [82]. Generally, E2F1–3 members control p53-dependent mechanisms that in turn control E2F-mediated repression which is crucial for normal cellular proliferation [75]. Thus, MIF seems to control both major growth-regulatory pathways. In sum, MIF deregulates normal cellular proliferation by interfering with both p53 and E2F/Rb pathways, leading to abnormal growth behavior of normal cells.

Stronger evidence that MIF plays a prominent role in tumorigenesis came from transformed MEFs [3, 78, 82]. Here, MIF deletion in MEFs induces resistance to Ras- and c-myc-mediated transformation [82] which are dependent on p53 [3]. In support, a simultaneous co-deletion of p53 and MIF rescues the transformation defects of $MIF^{-/-}$ MEFs [3]. Growth differences also increase after cell transformation, in that transformed $MIF^{-/-}$ MEFs cycle much slower than their wildtype counterparts [78, 82]. The E2F pathway is also impaired in Ras-transformed $MIF^{-/-}$ MEFs [82]. Here, interference with E2F DNA-binding activity by introducing a binding-defective E2F1 mutant in Ras-transformed $MIF^{-/-}$ fibroblasts blocks the transformation defects produced by MIF deletion. This rescue is likely due to the lost cross talk between the E2F and p53 pathways. To further elucidate the link between E2F and MIF, independent of p53, $p53^{-/-}$ compared to $p53^{-/-}$ $MIF^{-/-}$ (DKO) Ras-transformed fibroblasts were characterized [78]. The complete inactivation of p53 is necessary because p53-containing cells do not tolerate an engineered deregulation of the E2F pathway and answer with massive cell death, further pointing to the strong interplay between these two pathways. Of note, in p53-deleted and transformed MEFs, MIF deletion alters the DNA-binding properties of E2F1 and E2F3 and affects the Rb/E2F complex, which leads to a deregulation of cell cycle components including CDC2 and CDC6. In support, MIF deficiency in the same system also impairs tumor formation in allograft experiments [78]. Importantly, changes in E2F-binding properties after MIF deletion are confirmed in *c-myc*-induced lymphomagenesis in vivo [1]. Here, the reduced

E2F-binding properties impair entrance into S-phase, leading to reduced lymphoma growth and enhanced survival of mice. Of note, stabilized tumoral MIF alone is not able to transform cells on its own. A concomitant tumor inducer is required to reveal MIF's tumor-promoting role.

Given this tumor-promoting role, it is important to answer the question whether MIF depletion in established cancer cells can reduce cell proliferation and induce cell death. Several studies using human cancer cell lines, xenografts, and in vivo cancer mouse models addressed this question. MIF depletion by inhibitors or siRNAs does reduce cell proliferation and/or induces apoptosis in cancer cells. For example, siRNA-mediated MIF depletion triggers apoptosis and reduces the clonogenicity of human colorectal cancer cells, also seen in p53-deficient cells [7]. Mechanistically, MIF depletion in wtp53-containing cancer cells leads to p53 accumulation with induction of p21 and Noxa, whereas the mechanism in p53-deficient colorectal cancer cells has not been studied yet [7]. Also, MCF7 breast cancer cells show reduced clonogenicity after siMIF [83]. In an androgen-independent prostate cancer cell line inhibition of MIF via specific inhibitors, anti-MIF antibodies or siRNAs all reduce tumor cell growth by downregulation of cyclin D1 and cyclin E [28]. Knockdown of MIF in melanoma cells significantly decreases proliferation and clonogenicity, while increasing apoptosis [18]. Here, effects are associated with decreased cyclin D1 and CDK4, and increased p27 expression, resulting in reduced numbers of cells entering S-phase. Furthermore, MIF knockdown in HCC cells reduces proliferation by downregulating cyclin D1 expression and induces apoptosis by BIM and Bax upregulation and caspase-3 activation [11]. In clear cell renal carcinoma (CCRC) cell lines, depletion of MIF by small hairpin RNAi (shRNA) led to a significant reduction in growth rate and clonogenic survival by deregulation of $p27^{Kip1}$ [14]. In human lung adenocarcinoma cell lines, the combined loss of MIF and D-DT/MIF-2, the only known homolog of MIF, via siRNA leads to dramatically reduced cell cycle progression and clonogenicity, and increased apoptosis compared to D-DT/MIF-2 or MIF alone [84]. Mechanistically, MIF and D-DT/MIF-2 synergistically inhibit p53 activation and reduce p53 target genes such as p21. Interestingly, p53-deficient lung cancer cells are only partly rescued from the MIF/D-DT-induced cell growth defects, indicating that other pathway(s) besides p53 are also involved in the cancer growth phenotype [84]. In a panel of human cancer cell lines (breast, colorectal, and bone), silencing of MIF results in p53 accumulation and induction of p53 targets including p21, MDM2, and Bax [85]. Here, the regulation of p53 activation seems to derive from a direct interaction between p53 and MIF proteins. Whether inactivation of p53 activity always comes from a direct interaction of MIF with p53 remains to be further confirmed. Well known and accepted is that MIF regulates p53 activity in human cancer cells. Accordingly, in the MMTV-ErbB2 mouse model, genetic MIF loss delays cancer progression by activating p53 and p21 which lead to reduced cell proliferation [7]. Furthermore, in UVB-induced murine skin cancer, MIF loss increases p53 activity leading to reduced proliferation of tumors [6]. Moreover, Eμ-myc-driven $MIF^{-/-}$ B lymphoma frequently contains alterations in the ARF-p53 axis, reinforcing that the p53 pathway is important in the context of MIF [1].

MIF also seems to influence autophagy, because MIF is identified as a target of steroid receptor coactivator-3 (SRC-3), which regulates autophagy in breast cancer cells [83].

Currently, it remains unclear how MIF increases cell growth in p53-deficient cancer cells. The question of MIF-regulated pathways is strongly context dependent, and a single "key" function of MIF has not been identified. What has been established beyond a doubt is that MIF is a powerful driver of oncogenesis, and cancers cells rely on MIF for maximal cell growth and survival.

5 Survival Pathways

MIF sustains proliferative signals (Fig. 1) and these signals transmit directly into the cell cycle and/or regulate apoptosis. MIF acts as autocrine and paracrine activator of survival pathways including PI3K/Akt, MEK/ERK, and NFkB mainly by activation of its cell surface receptor CD74 (see book Chap. 1, Idit Shachar). Activation of these pathways triggers proliferation, blocks apoptosis, and promotes invasion and angiogenesis to boost tumor growth [86].

The role of MIF in PI3K/Akt-mediated survival was first shown by Lue et al. [64]. In their study, recombinant MIF in primary MEFs directly promoted PI3K/Akt activation in an autocrine manner depended on the MIF receptor CD74 [64]. Another study used normal colorectal epithelial cells and showed that ectopic MIF promotes survival by upregulation of cyclin E and anti-apoptotic Bcl-2. This regulation was also seen in CT26 colorectal cancer cells [87] and indicates that MIF function is maintained and elevated in cancer cells. The enhanced survival was again dependent on MIF's cell surface receptor CD74, confirming that MIF acts in an autocrine manner in this system. Furthermore, proliferation of gastric cancer cells by recombinant human (rh) MIF is increased via PI3K/Akt activation, as well as upregulation of cyclin D1 and downregulation of $p27^{KIP1}$ [88]. Moreover, in cervix carcinoma cells and various breast cancer cell lines recombinant MIF also enhances Akt activation to promote survival [64]. Interestingly, this study confirmed that Jab1/CSN5, known as a coactivator of AP-1 transcription and cell cycle regulator through $p27^{KIP1}$ degradation, interacts with MIF and leads to intracellular sequestration in cancer cells. The MIF-Jab1/CSN5 binding was first shown in fibroblasts, where MIF co-localizes with Jab1/CSN5 in the cytosol [89]. Here, MIF inhibits Jab1-mediated AP-1 activity and reduces phospho-c-Jun levels. In cancer cells, MIF-Jab1 binding seems to store MIF for its possible secretion [64].

MIF is also able to regulate the activation of MEK/ERK signaling [90]. In bladder cancer, rhMIF increases proliferation by enhancing ERK activation, which is blocked by specific inhibitors for ERK and MIF [66]. In support, in hepatocellular carcinoma cells, MIF knockdown reduces proliferation by downregulation of p-ERK, but also p-Akt [11]. Studies with androgen-independent prostate cancer cell lines confirm that inhibition of MIF via specific inhibitors, anti-MIF antibodies, or siRNAs attenuates tumor cell growth involving the ERK pathway [28] as well as the

PI3K-Akt pathway [9]. In colorectal and gastric cancer cells, chronic MIF exposure promotes cell proliferation by increasing again both Akt and ERK signaling [20]. Also in untransformed gastrointestinal fibroblasts, rhMIF treatment upregulates Akt and ERK phosphorylation leading to enhanced proliferation. The cross talk between the two major survival pathways is well known, but context dependent. In fibroblast, an inhibition of PI3K also results in downregulation of the transient ERK activation, meaning that PI3K acts upstream of ERK [64]. But in Her-2-overexpressing breast cancer cells, an inhibition of PI3K has no consequence for the ERK signaling [46]. Of note, cancer cells constitutively activate survival pathways, and differences between transient and constitutive ERK activation can course such discrepancies. Anyway, MIF seems to affect both pathways in a cell type-dependent manner.

Another pathway that is not only involved in inflammation and immune responses but also in cell survival is the NFκB pathway. In chronic lymphocytic leukemia (CLL), activation of CD74 by MIF initiates a CD74-MIF-NFkB-IL-8 signaling cascade that contributes to tumor progression [91].

Pathways involved in nutrient- and metabolic processes also promote survival. AMP-activated protein kinase (AMPK), for example, acts as metabolic stress sensor, and stress-induced AMPK activation induces cell cycle arrest and/or cell death [92, 93]. Given the aberrant microenvironment of solid cancers, cells have developed mechanisms to evade AMPK activation. One mechanism involves MIF and D-DT/MIF-2, which antagonize AMPK activation cooperatively via their shared receptor CD74 in non-small cell lung carcinomas (NSCLC) [94].

Thus, the known MIF functions in normal cells including activation of survival pathways are further boosted in cancer cells, mainly via excess of MIF generated by the stabilizing interaction with HSP90, which enhances MIF's auto- and paracrine actions.

6 Angiogenesis

Angiogenesis is a normal physiological process where new blood vessels are formed from pre-existing vessels. Angiogenesis is mediated by triggering the stabilization and activation of one of the most important regulators of angiogenesis, HIF1α, and induction of its target genes including vascular endothelial growth factor (VEGF) [12, 41, 95, 96]. It is also a central step in the transition of tumors from a benign to a malignant state and from a nascent to an established viable tumor (angiogenic switch).

MIF's chemokine-like functions have a positive impact on angiogenesis. Additionally, MIF regulates Cxcl8/IL-8 expression and/or secretion which have been shown to play an important role in tumor growth and tumor angiogenesis [91, 97–99]. Conversely, in response to hypoxic conditions MIF expression in normal cells is induced by HIF1α, as shown in MEFs [41, 95], human endothelial cells (ECs) [100], and human vascular smooth muscle cells (VSMCs) [39]. Moreover, exposing human ECs to hypoxia led to secretion of MIF that participated in the recruitment and migration of endothelial progenitor cells [40]. Compared to liquid tumors, solid tumors in particular have to overcome hypoxic conditions and thus

exhibit high HIF1α levels. In support, hypoxic conditions in cancer cells induce expression of MIF mRNA and secretion of MIF protein [95], as was also shown for pancreatic and squamous cell carcinoma [101] and glioblastoma [36]. Interestingly, however, cancer cells do not markedly elevate their intracellular MIF protein after hypoxia (unpublished data), possibly because they already possess elevated stabilized MIF protein. Thus, because the HSP90 system already stabilizes MIF protein constitutively, cancer cells may not rely on HIF1α-induced MIF expression. This is in contrast to normal cells. In normal VSMCs, hypoxia-induced proliferation and cell migration are inhibited by MIF depletion [39]. Also MIF-depleted MEFs do not survive hypoxia (unpublished data), indicating that normal cells depend on MIF under hypoxic conditions. However, whether MIF-deficient cancer cells are impacted after hypoxia has not been extensively studied, and it is currently unclear how dependent cancer cells are on HIF1α-regulated MIF expression.

At any rate there is strong evidence that MIF regulates angiogenesis. Aside from Cxcl8, MIF was repeatedly shown to regulate VEGF. For example, in NSCLC cells autocrine MIF and D-DT cooperate to activate JNK, c-jun phosphorylation, and AP-1 transcription factor activity, resulting in expression of Cxcl8 and VEGF. This action in turn is dependent on MIF receptor CD74 and indicates MIF involvement in angiogenesis [102]. Furthermore, in breast cancer cell lines exogenous MIF increases VEGF and Cxcl8 secretion [12]. In the same study, the level of MIF correlates positively with expression of Cxcl8 and microvessel density (MVD) in patient-derived samples. In studies with lung cancer patients, MIF correlates strongly with levels of angiogenic CXC chemokines and vessel density, and the risk of cancer recurrence was associated with high CXC, MIF, and VEGF levels [19]. Some MIF knockout mouse studies confirmed a correlation between MIF and angiogenesis. Small intestinal tumors have a reduced MVD after MIF depletion [5]. In UVB-induced murine nonmelanoma skin cancer, MIF deletion leads to less angiogenesis [6]. In bladder cancer, $MIF^{-/-}$ mice have lower stromal vessel density than wildtype mice [4]. Moreover, BBN-induced bladder tumors reduce their vessel density upon MIF inhibition [66]. These models strongly point to an involvement of MIF in angiogenesis.

However, whether and to what extent cancer cells rely on MIF for angiogenesis is not fully understood, and the definitive proof-of-principle remains elusive.

7 Invasion

During recent years, some evidence for MIF's invasive potential was found in diverse human cancer cell lines. For example, in pancreatic cancer cells, MIF overexpression induces epithelial-to-mesenchymal (EMT) transition by regulating EMT-responsive genes and EMT cell characteristics [15]. Moreover, MIF was identified as a critical factor for the invasive and metastatic potential of drug-resistant human colon cancer cells [103]. In contrast, chronic MIF treatment of gastrointestinal fibroblasts leads to a mesenchymal-to-epithelial transformation (MET) [20].

Anyway, both processes contribute to metastasis. In all cases, the underlying signaling pathways and mechanisms remain elusive. Also, whether MIF in general is an important factor for invasion has not been adequately addressed yet. The fact that benign tumors also elevate their MIF level without being invasive suggests a minor role for MIF in invasion. For confirmation of MIF's invasive potential, lots of studies are still needed and in vivo model systems will be more meaningful for clarification.

8 Immune Suppression and Inflammation

Interestingly, some of the newly added "new hallmarks of cancer" [73] were originally described as MIF functions because MIF is clearly a pro-inflammatory cytokine with a strong role in the innate immune response and inflammation and probably link inflammation and cancer [104–108]. Since other chapters of this book address the role of MIF within the immune system in great detail, we will just mention some evidence that MIF also regulates the immune system in the immediate tumor environment. This effect includes both sustained anti-tumor immunosuppression and tumor-promoting inflammation.

MIF was hypothesized for a long time to be a mediator of inflammation-associated tumorigenesis, albeit without a clear mechanism of action within the complex interplay of cell types in the immune response. Recent studies started to clarify MIF's role in immune activities with respect to cancer cells. First, recombinant MIF is known to regulate pathways involved in inflammatory processes including the NFκB pathway [91] and the JNK pathway [109]. Importantly, both signaling pathways are dependent on MIF's cell surface receptor CD74 and lead to upregulation and/or secretion of the well-known inflammatory chemokine Cxcl8, reflecting MIF's role in inflammatory processes. In support, out of 22 genes MIF scored in co-cultured tumor cells associated with JNK- and NFκB-mediated inflammation [110]. The same study also found that MIF produced by tumor cells increases their invasive potential and that this involves interaction with macrophages. Tumor cell-derived MIF activates macrophages and the release of MMPs (matrix metalloproteinases) that play crucial roles in invasion. Another study also showed that MIF controls the tumor microenvironment. In an aggressive metastatic breast cancer mouse model, tumor-derived MIF triggers tumor growth and metastasis through activation of myeloid-derived suppressor cells (MDSCs). MDSCs are known to strongly enhance tumor growth and metastasis by suppression of T cell and NK cell function, effectively inhibiting anti-tumor immunity [70]. In further support, expression of MIF in neuroblastoma leads to inhibition of anti-tumor T cell reactivity and again to suppression of anti-tumor immunity [111]. In this study, MIF-depleted tumor cells were more effectively rejected in immune-competent mice than MIF-proficient cells. As for the cellular basis of increased rejection, MIF-depleted tumor cells in immune-competent mice were associated with increased infiltration of CD8+ and CD4+ T cells, macrophages, and B cells.

Importantly, in immune-deficient mice, the increased rejection of MIF-depleted cells was stopped, confirming the involvement of the immune system. In sum, MIF loss in tumor cells triggers anti-tumor immunity by regulating the host immune response. This is also the conclusion of another study that involves MIF, but derived from tumor-associated macrophages (TAMs), which regulate immunosuppressive activities of T cells [112]. Compared to TAMs from tumor-bearing wildtype mice, TAMs isolated from tumor-bearing MIF-deficient mice showed a higher pro-inflammatory cytokine profile and a reduced T cell immunosuppressive gene profile and activity. Thus, MIF-deficient immune cells impair tumor outgrowth. Importantly, in this study tumor growth was analyzed independent of tumor cell-derived MIF. It would be interesting to see what happens if tumor cells also lose their MIF. This question was addressed in another study using the Eμ-TCL1 mouse model for chronic lymphocytic leukemia (CLL) [2]. First, MIF loss in CLL cells results in lower macrophage infiltration into CLL homing organs like spleen or bone marrow. Moreover, MIF loss also sensitizes CLL cells to apoptosis. Even more interesting is the fact that both tumor-derived and macrophage-derived MIF are important for maximum cell viability in co-cultures. This strongly suggests that MIF expression from both sources, that is, tumor cells and immune cells, are critical for maximum tumor growth.

Although these first studies did analyze the complex interplay between tumor cells and their microenvironment, more research is still needed to better understand MIF's role in the immune system with its diverse and multifaceted interplay of different cell types and compartments. Tissue-specific and cell type-specific analyses in vivo are needed to fully address MIF's function in the anti-tumor immune response. Also, it is not well understood how on the one hand cancer-associated MIF promotes immune suppression and on the other hand normal and/or tumor cell-associated MIF is a pro-inflammatory cytokine in the innate immune response. Importantly, to our knowledge an experimental in vivo cancer model too would support a role of MIF in inflammation-associated cancer remains elusive. To our knowledge, almost all mouse models deal with MIF's role in immune suppression.

9 Proteotoxic Stress

An additional hallmark of cancer is the presence of perennial stress conditions including proteotoxic stress [74]. Cancer cells respond by massive constitutive upregulation and hyperactivation of the HSF1-HSP90 axis, which effectively buffers proteotoxic stress and prevents cell death due to protein aggregation of quantitatively and qualitatively aberrant misfolded proteins. As a result, cancer cells are addicted to the HSP90 machinery and, conversely, are hypersensitive to HSP90 inhibition compared to normal cells, which provides a significant therapeutic window [42, 43, 47, 48]. Targeting this hallmark is strongly associated with a profound block in tumor growth. As discussed above, MIF protein in tumor cells is highly stabilized by HSP90 [7, 46].

10 MIF: A Rational Target in Cancer Therapy

Research during the last decades impressively demonstrated that targeting MIF in cancer provides an attractive and pleiotropic therapeutic window. So far all specifically designed small-molecule MIF inhibitors block MIF's tautomerase activity. However, it is currently an open question whether MIF's enzymatic activity, originally discovered by structural homology and biophysical studies, is critical during *oncogenesis*. Within a nonmalignant purely inflammatory context, MIF tautomerase inhibition indeed shows promising results in experimental sepsis and chronic inflammatory diseases [104, 105, 113, 114]. In contrast, a requirement for MIF's tautomerase activity during oncogenesis remains controversial and/or is dependent on cancer type and whether the immune system is involved in cancer progression [70, 115], depriving the clear rationale for using enzyme-targeting small-molecule inhibitors to block MIF's pleiotropic tumor-promoting activities. In a one-stage benzo[α]pyrene-induced skin tumor mouse model, MIF's tautomerase activity seems to be dispensable [115]. In this study, enzymatically inactive MIF knockin (MIF^{P1G}) mice exhibit an intermediate phenotype between WT and MIF null mice. The authors proposed that MIF's tautomerase activity but not structural features of this site are is dispensable for growth-regulatory properties and instead suggest a role for MIF's N-terminal region in protein–protein interactions. In contrast, metastatic breast cancer cells depend on its tautomerase activity [70]. Here, tumor-derived MIF with its intact tautomerase is important to control the host immune system and subsequently tumor growth. In another model, murine Lewis lung carcinoma cells injected into either enzymatically inactive MIF^{P1G} mice or MIF null mice exhibit the same growth inhibition, in contrast to cells injected into wildtype mice, suggesting that lung cancer depends on MIF's tautomerase action, especially when MIF comes from the microenvironment [116]. Of note, in this study, the Lewis lung carcinoma cells contained properly functioning intracellular MIF level. And, since epithelial cells are the major source of tumoral MIF, MIF's tautomerase should also have to be inhibited in lung carcinoma cells to adequately address MIF's tautomerase function during oncogenesis, in addition to stromal and immune cells. Indeed, tautomerase-targeting small-molecule inhibitors strongly reduce tumor growth in the above lung carcinoma allograft model [116]. Other cancer types may only partly depend on MIF's enzymatic activity since MIF inhibition by such drugs only causes slight-to-moderate tumor growth suppression in xenograft models of colorectal cancer [8], bladder cancer [66], and prostate cancer [28]. However, as a cautionary note in the assessment whether and to what degree MIF's enzymatic activity plays a role in tumors, these studies did not analyze the scenario of complete MIF protein inhibition. Such complete MIF protein inhibition, not only MIF's tautomerase activity, could further increase tumor-inhibitory effects, which is most likely the case.

Of note, cancer mouse models with a genetic MIF knockout often show clearer inhibitory effects on tumor growth rates [1, 2, 7] than mouse models using tautomerase-based inhibitors [8, 28, 66]. Interestingly, stable MIF knockdown in ovarian carcinoma cells decreases tumor burden and increases overall survival in a syngeneic allograft model [10], again supporting inactivation or elimination of the

complete MIF protein. Overall, the efficacy of tautomerase-based MIF inhibitors in anti-cancer therapy remains limited and difficult to assess. Because of these inconsistent results, we suggest that selectively targeting MIF's tautomerase activity is not sufficient as a clinically relevant anti-cancer therapeutic. Additional work to better delineate this scenario is needed, especially in the context of the immune system versus cancer, since immune cells seem to respond stronger to MIF's tautomerase inhibition (see the other book chapters).

Some xenograft studies with neutralizing MIF antibodies, for example, in colorectal cancer, only found slight suppression of tumor growth [8]. One reason for such minor effects of neutralizing MIF antibodies could be their difficulty in infiltrating into solid tumor microenvironments. However, in PC-3 prostate cancer xenografts, anti-MIF antibodies seem to induce strong tumor suppression [9]. In general, the importance of extracellular MIF for tumor development is clearly testified by autocrine activation of survival pathways, the interplay with the immune system, elevated serum MIF levels in cancer patients, and studies with anti-MIF antibodies. The development of inhibitors that completely inhibit MIF's many pleiotropic actions during oncogenesis, including intracellular events such as p53 and E2F regulation, should be a future direction.

Currently, in the absence of available inhibitors against the entire MIF protein and/or action profile, the alternative strategy of directly or indirectly degrading excess levels of intratumoral MIF is the more realistic route. Targeting MIF through HSP90 inhibition is a straightforward and effective way to curb tumor growth [62, 63]. HSP90 inhibitors represent a promising and powerful new class of anti-tumor drugs, despite—or more likely because of—their pleiotropic effects interfering with a broad range of oncogenic molecular networks, rather than targeting a narrowly defined signaling pathway. Currently there are over 30 active clinical oncology trials involving HSP90 inhibitors. Major advances came with second-generation synthetic HSP90 inhibitors such as ganetespib (STA-9090) that are more potent and less toxic than the first-generation geldanamycin-based inhibitors [47, 117]. STA-9090 (Ganetespib) is currently tested in phase II/III trials for various cancer types including anaplastic lymphoma kinase (ALK)-driven NSCLC cancer [118] and triple negative breast cancer [119]. Overall, conventional anti-cancer chemotherapeutics (DNA-damaging agents, S-phase inhibitors, and antimitotics) combined with less toxic HSP90 inhibitors are promising strategies for anti-cancer therapies since they also target a central pleiotropic pro-survival anti-apoptotic hub that stabilizes numerous oncoproteins including MIF, Her-2, Akt, Bcr-Abl, and others. Such pleiotropic strategies should make it harder for cancer cells to bypass signaling pathways and acquire resistance.

11 Conclusion

During the last decade, strong evidence for MIF's tumor-promoting role in the formation and maintenance of tumors has mounted and is now widely accepted. A unique feature of MIF in oncogenesis is its broad expression in different cell types.

MIF is simultaneously highly expressed in tumor cells (both intracellularly and as secreted cytokine) and in stromal cells including immune cells. Thus, MIF exerts dual growth control to promote tumors. On the one hand, MIF controls epithelial tumor cell growth via intracellular pathways, but also in an autocrine and paracrine manner. On the other hand, MIF controls stromal and immune cells, mainly as secreted cytokine. Hence, MIF has the ability to directly link the tumor microenvironment with the epithelial tumor cell compartment. Related to this multifunctionality, MIF acts in a pleiotropic manner to regulate different pathways involved in tumor cell apoptosis, survival, invasion, angiogenesis, metastasis, and in the stromal tumor immune response.

To better understand MIF's role in the complex interplay of tumor cells with their microenvironment, improved in vivo models are needed, since only an intact organism can fully answer such complex questions. Up to now, most mouse studies deal with either stromal/immune cell-derived MIF or with tumor cell-derived MIF. To our knowledge, no single model can manipulate both cell types independently and simultaneously of each other. Such a model could answer the important open question of what is more important, stromal-derived MIF or tumor-derived MIF, or a combination of both? Addressing this stromal/epithelial interplay is also important with regard to developing the most effective MIF-based anti-cancer therapies. It is currently an open question whether MIF's enzymatic activity is critical during oncogenesis. Immune cells seem to be more responsive to available MIF tautomerase inhibitors. In contrast, epithelial tumor cells are hardly (if at all) responsive to MIF tautomerase inhibition and if so, it appears dependent on cancer type and whether the immune system is promoting cancer progression. A possible worst-case scenario with MIF tautomerase inhibitors could be that only the antitumor microenvironment is inhibited, but not the tumor cells themselves, paradoxically promoting their unbridled growth. Eliminating the entire MIF protein appears to be a safer route. A specific MIF inhibitor that blocks the MIF protein per se remains elusive. However, all tumorigenic MIF functions, whether mediated by intracellular or secreted MIF, are indirectly blocked by Hsp90 inhibition via destabilizing MIF protein. Thus, Hsp90 inhibition is a clinically feasible and rational way to inhibit MIF function, together with simultaneously destabilizing many other tumor-promoting Hsp90 clients. Developing a specific blocker of whole MIF protein, at least theoretically, is the right route to inhibit MIF action in cancer. However, based on its dual intracellular and secreted localization and its pleiotropic actions, it is a daunting task. In the meantime, we identified a druggable mechanism that inhibits whole MIF protein indirectly by degrading it.

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CD74, MIF and Breast Tumorigenesis: Insights from Recent Large-Scale Tumour Genomics and Proteomics Studies

Metodi V. Metodiev

Abstract MIF and other cytokines are frequently detected at elevated levels of abundance in solid tumours. Their involvement in tumour biology has been studied for many years, and, with the advent of postgenomic tools such as next-generation DNA and RNA sequencing, and mass spectrometry-driven protein profiling, the underlying mechanisms can be studied in a systematic and quantitative way. This chapter discusses recent studies by our group that have shown that MIF and CD74 are mechanistically involved in breast cancer progression. Analysis of recently released data from the Cancer Genome Atlas (TCGA) as well as our proteomics data is presented and discussed. TCGA data show that MIF and CD74 are rarely mutated in cancer but are consistently overexpressed at the level of mRNA. Furthermore, using high-resolution mass spectrometry to analyse tumour protein abundance, we have identified MIF and CD74 among the proteins that are overexpressed in metastatic triple-negative breast tumours. A cell-based model showed that when CD74 is overexpressed, it interferes with the function of a known tumour suppressor, Scribble, leading to enhanced invasion, possibly because the functions of Scribble in maintaining cell polarity are compromised. The underlying mechanism, yet to be fully elucidated, involves deregulation of Scribble phosphorylation on specific sites in its C-terminal proline-rich domain.

1 Introduction

Tumour cell invasion and metastasis, the one facet of cancer that is most deleterious to patients, are driven by the interaction of multiple molecular pathways that can only be fully understood if studied on a system-wide basis. The causative events for

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metastasis are genomic and epigenomic alterations, which manifest as abnormal expression of specific sets of (mutant) proteins and/or abnormal and sustained loss or increase of catalytic activities, activities which are normally transient and tightly controlled in non-cancerous cells. With all the new tools enabled by the sequencing of the human genome and the development of various deep sequencing technique, it is tempting to try to trace these functional manifestations of the altered tumour genome purely ab initio, starting from the genomic sequence derived from a particular tumour tissue. However, metastasis is not only a function of the evolution of the tumour genome. It also depends on the interaction of the tumour with the host immune system, which in turn is affected by complex environmental factors, which in their turn can act differently in different genomic contexts. The host immune system might on one hand act to suppress tumour growth and even eliminate incipient tumours, but, on the other hand, it can also shape the tumour phenotype into acquiring metastatic properties and direct its evolution into more aggressive and malignant types.

Breast cancer is a particularly heterogeneous type of malignant disease with many subtypes, which are defined either by gene expression signatures, specific mutations and pathophysiological characteristics. This rational stratification is useful in epidemiological studies and in some cases can also help in the process of selecting efficient therapeutic strategies. The latter is particularly true in cases where the molecular hallmarks that define the tumour subtype are also mechanistically involved in tumour growth, invasion, metastasis and/or resistance to chemo or radiotherapy. For example, a subset of breast tumours overexpress the human epidermal growth factor receptor ErbB2 and because of this are sensitive to monoclonal antibodies and tyrosine kinase inhibitors that target ErbB2 [1]. Another subset of breast tumours that makes up about 60% of all newly diagnosed cases express steroid hormone receptors and depend on their activity for survival: patients in this group often respond well to hormonal therapies with drug such as tamoxifen and aromatase inhibitors. However, a significant proportion of the newly diagnosed patients have breast tumours that express neither the ErbB2 growth factor receptor nor the steroid hormone receptors ER and PgR. These are usually denoted as triple-negative breast cancer (TNBC). They are one of the most aggressive and hard to treat breast malignancies [2–4]. Patients diagnosed with TNBC are more likely to be premenopausal women and women of African and African-American origin. TNBC is associated with poor prognosis although patients frequently respond well to systemic chemotherapy [3, 5]. Approximately 20–25% of all newly diagnosed breast cancer cases worldwide belong to the triple-negative subtype. This is why it is a subject of intense investigations aiming to identify new candidate drug targets and to elucidate the molecular pathways that underlie the invasiveness and poor prognosis of triple-negative breast cancer.

MIF and its receptor CD74 [6] have been implicated in cancer in general and in breast cancer in particular by a number of studies. MIF is overexpressed in many solid tumours. In 1986 Gutman et al. reported that functional macrophage migration inhibition test can discriminate between benign and malignant breast lumps with better than 70% specificity [7]. Since these early reports, multiple studies using molecular assays have confirmed this phenomenon, but until very recently lack of system-wide expression profiling data limited the extent to which the significance

of this overexpression can be elucidated. Now such data is available: hundreds of breast tumours have been deep sequenced and the corresponding data recently published along with useful clinical information allowing for identification of genes that are involved in metastasis and correlate with important clinical outcomes such as overall and disease-free survival and response to therapy.

2 Mutational Profiles of MIF and CD74 in TCGA Breast Tumour Data

Over the last decade dramatic advances in sequencing technology provided unparalleled insights into the genomic landscape of cancer. In particular, exome sequencing now allows comprehensive and cost-effective identification of the mutations which drive tumour growth and dissemination. For example, the recently released TCGA datasets contain 1081 breast tumour exome datasets containing more than 100,000 curated somatic mutations (available at https://tcga-data.nci.nih.gov).

This data was generated from tumour and matched peripheral tissue. In the workflow genomic DNA is first extracted from frozen tissue, amplified to generate sequencing libraries, which are then sequenced on an Illumina HiSeq instrument. The raw output files contain short 50–150 bp sequence reads and quality encoding giving the confidence of base calling. These are then processed by a computational pipeline, which maps the sequence reads onto a reference genome sequence. Finally, germ line and somatic mutations and their frequencies are determined by comparing the sequences derived from the tumour and from the peripheral tissue sample.

The analysis of the TCGA data released to date shows that MIF and CD74 are rarely mutated in breast tumours. There is no detectable mutations in the coding sequence of MIF and only two mutations in the CD74 gene, one in a splicing site and another is a missense. Similar very low frequency of genomic mutations is observed in other tumours: out of 114,469 mutations mapped in 469 colon tumours, only three missense mutations were mapped to CD74 and none to MIF; in stomach tumours out of more than 200,000 somatic mutations mapped to 441 tumours, only four were mapped to CD74 and only one to MIF. The low frequency of mutation detected for the two genes suggests that their involvement in tumorigenesis and cancer invasion and metastasis is driven by transcriptional and post-transcriptional mechanisms.

3 CD74 and MIF Expression in Breast Tumours as Determined by RNA Sequencing

In addition to exome and genome sequencing, deep RNA sequencing (RNA-Seq) has recently emerged as the preferred methodology for gene expression analysis replacing microarrays in many applications. It is technically similar to exome and genome sequencing, but instead of preparing sequencing libraries from genomic DNA, RNA-Seq works by first preparing cDNA from RNA and then amplifying it

to prepare the sequencing library. The computational pipelines used to analyse RNA-Seq data map the sequence reads to the reference genome, and the number of sequences mapped to a specific gene is used as raw estimate of the corresponding mRNA abundance.

TCGA contains 1098 breast tumour and matched periphery RNA-Seq datasets as of December 2015. The methodology for sample acquisition and sequencing has been described in several publications by the TCGA consortium, and the data is available at the TCGA website in several formats. A summary of the CD74 and MIF statistics computed from the 1098 datasets is shown in Table 1. MIF is detected with a median gene count of 3717. CD74 mRNA is much more abundant; median gene count is 30,408.

The table shows quartile gene counts calculated from normalized count as reported by TCGA in the individual RNA-Seq data files.

The patterns of expression of MIF and CD74 are characterized by asymmetrical right-tailed distribution frequently seen in tumours.

Figure 1 shows box plots of MIF and CD74 gene counts in tumour and peripheral tissue samples. MIF is significantly more abundant in tumours compared to peripheral tissue with a p-value of 1.14×10^{-40} by the non-parametric Wilcoxon-Mann-Whitney test. This is based on data from 112 peripheral tissue samples and 1043 tumour samples. CD74 shows similar but less dramatic difference in expression between tumour and peripheral tissue with a p-value of 0.00107.

Table 1 MIF and CD74 mRNA abundance in 1043 breast tumours measured by RNA-Seq

Quantile/gene	0% (minimum)	25%	50% (median)	75%	100% (maximum)
MIF	421	2439	4095	6658	69,408
CD74	1135	18,134	31,752	51,492	260,086

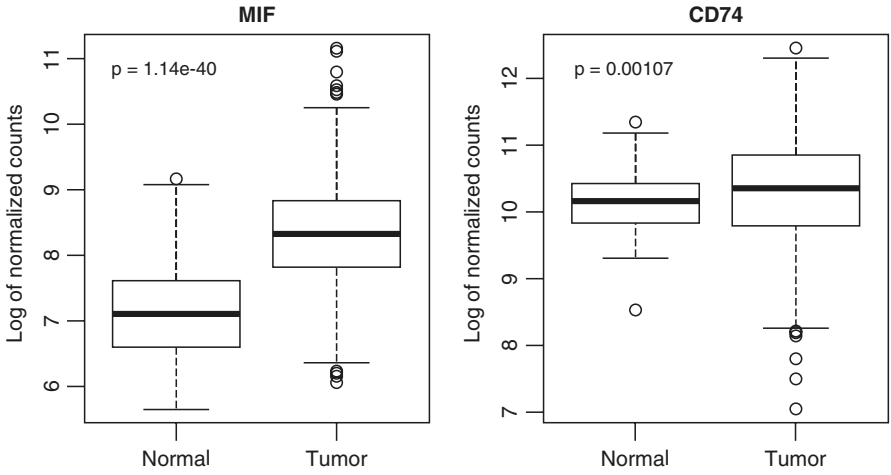

Fig. 1 Comparative analysis of MIF and CD74 expression in breast tumours ($n = 1043$) and normal breast tissue peripheral to the tumour ($n = 112$). Normalized gene counts are log transformed to produce the box plots. The p-values were calculated using the non-parametric Mann-Whitney test

Furthermore, MIF and CD74 expression was dependent on oestrogen receptor status. Statistical analysis using the non-parametric Mann-Whitney two-sample test returned a *p*-value of 0.00011 for MIF and 0.0304 for CD74. In both cases mRNA abundance was higher in ER-negative tumours ($n = 227$) compared to ER-positive tumours ($n = 771$).

Figure 2 shows results from cluster analysis, in which MIF and CD74 gene counts in tumours were used to cluster the breast cancer patients using the hierarchical Ward's algorithm. Two-cluster split of the patients was then used to assess the effect of higher MIF and CD74 expression on overall survival of breast cancer patients.

The analysis showed that higher MIF and CD74 expression in tumours is positively correlated with overall survival. The log-rank test produced a *p*-value of 0.00097. A simple and somewhat trivial explanation of this result is that, in general,

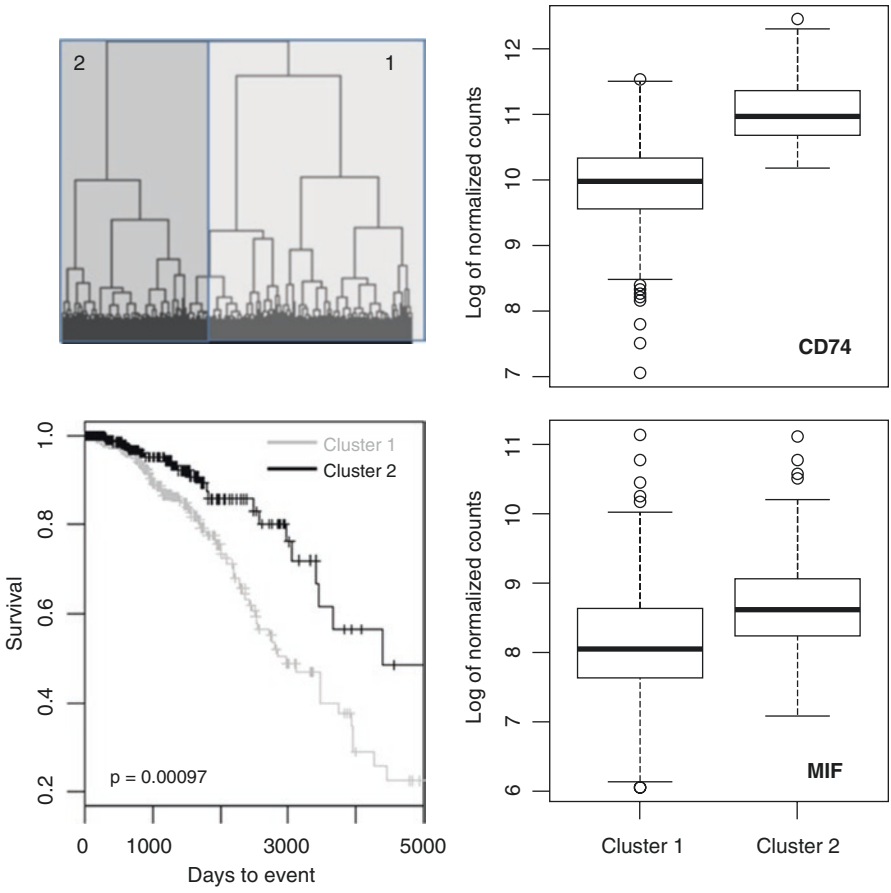

Fig. 2 Clustering of breast tumours by MIF and CD74 expression and survival analysis. *Top left*: Hierarchical clustering using Wards' algorithm. *Bottom left*: Kaplan-Meier survival curves for cluster 1 and cluster 2. The log-rank test was used to calculate the *p*-value shown on the plot. *Right*: box plots of MIF and CD74 expression in the two clusters shown in the top right and bottom right panel

increased inflammation and immune cell infiltration are the causes for increased MIF and CD74 mRNA abundance in the profiled breast tumour tissue samples. However, if the survival analysis is restricted only to ER-positive or ER-negative tumours, the significance is preserved only for ER-positive tumours with a *p*-value of 0.02. The significance is lost for ER-negative tumours where the log-rank *p*-value becomes 0.482 when the same clustering algorithm is used to split the samples. This, and the observed correlation between MIF and CD74 expression and the ER status determined by histochemical assays, suggests that there might be additional more-mechanistic explanations of the expression pattern of the two genes. Higher resolution analysis utilizing micro-dissected samples would be needed to address these issues.

3.1 Identification of Genes Co-regulated with CD74 and MIF in Breast Tumours

To find genes which are co-expressed, and potentially co-regulated with CD74 or MIF, Spearman rho was calculated as described in the appendix. Then a stringent criterion that correlation coefficient (rho) should be larger than 0.8 was used to select candidate genes. Table 2 shows the genes selected for CD74.

The Spearman correlation coefficient rho was calculated in R as shown in the appendix. A stringent cut-off of 0.8 was used to select the genes shown in the table.

Not surprisingly expression of CD74 correlates with other genes involved in antigen presentation. For example, CD74 and HLA-DMA and HLA-DRA have rho greater than 0.95. A scatter plot illustrating this is shown in Fig. 3.

Pathway analysis of the genes from Table 2 using the Kyoto Encyclopedia of Genes and Genomes (KEGG) showed very high enrichment of genes involved in the following partially overlapping immune system-related pathways: "antigen processing and presentation", eight genes, adjusted *p*-value (AdjP) = 2.78×10^{-14}; "*Staphylococcus aureus* infection", six genes, adjP = 5.31×10^{-11}; "cell adhesion molecules (CAMs)", seven genes, adjP = 9.42×10^{-11}; "phagosome", seven genes, adjP = 1.31×10^{-10}; "allograft rejection", five genes, adjP = 3.42×10^{-10}. These adjusted *p*-values were calculated using the hypergeometric test on the basis of numbers of genes in the human genome in the pathway of interest and the numbers of genes from the list in the pathway of interest.

A similar analysis but using transcription factor target enrichment revealed that the list of genes, which are highly correlated with CD74 in primary breast tumours, is enriched of targets of the following transcription factors: ETS2, recognition sequence RYTTCCTG, 11 genes, adjP = 1.84×10^{-8}; PU1, recognition sequence RGAGGAARY, six genes, adjP = 5.67×10^{-5}; MAZ, recognition sequence GGGAGGRR, eight genes, adjP = 0.0030.

The analysis of genes co-expressed with MIF showed that only one gene, DDTL, has rho larger than 0.8. DDTL is located on the same chromosome and near MIF locus. It encodes a MIF-like protein with similar structure and predicted tautomerase

Table 2 Genes correlating with CD74 in 1043 breast tumours

Names	corCD74
HLA-DMA	0.95411718
HLA-DRA	0.929124362
HLA-DPA1	0.899917275
HLA-DMB	0.897878643
HLA-DPB1	0.894288792
CIITA	0.87315557
LST1	0.857794667
WAS	0.855668908
CD37	0.849964171
SELPLG	0.844592954
AIF1	0.839641568
TNFAIP8L2	0.838742339
ARHGAP9	0.835303476
CD4	0.834781085
SPI1	0.833978089
SASH3	0.830311254
NCF4	0.822771032
PTPN7	0.82275615
HCST	0.821739981
HCLS1	0.819566641
FERMT3	0.818709484
CD53	0.817655875
MYO1F	0.817379001
HLA-DRB1	0.815325054
IL12RB1	0.81268804
PARVG	0.811925945
TYROBP	0.811334758
FMNL1	0.808902205
LAPTM5	0.808467535
RASAL3	0.804834639
CORO1A	0.804086301
IL2RG	0.80359508
CARD11	0.802558826
CYTH4	0.802159501

activity. On the other hand, while analysis of CD74 showed that there are no genes with significant negative correlations, the smallest rho was −0.38; correlative expression analysis with MIF as reference identified a group of genes that were clearly negatively correlated with MIF. For negative correlation a cut-off rho < −0.5 was chosen resulting in a list of 64 genes, notably enriched of targets of FOXO4, adjP = 1.98×10^{-6}, 16 genes.

It appears that increased MIF expression in breast tumours is associated with decreased expression of FOXO4 targets, which is intriguing since FOXO4 is known to regulate cell cycle and longevity and is frequently deregulated in cancer via

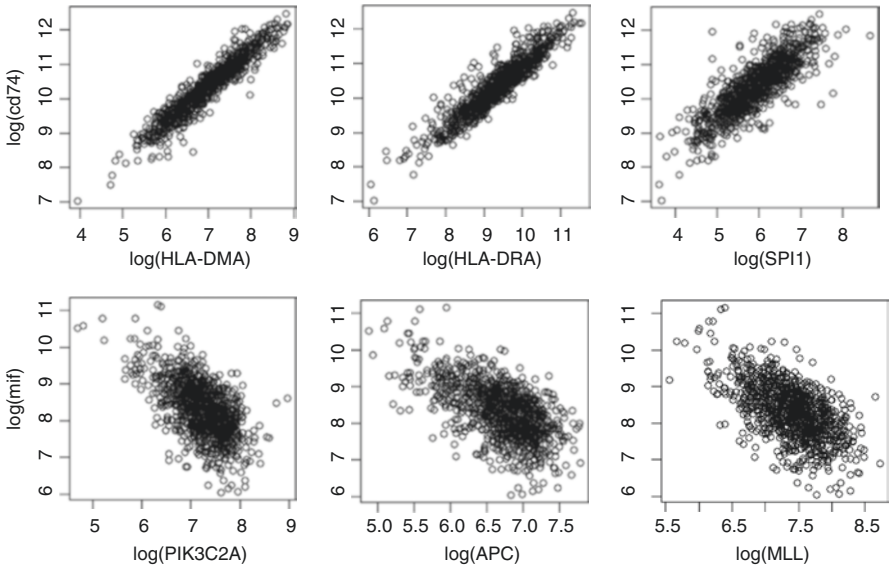

Fig. 3 Correlation of CD74 and MIF expression with genes expressed in breast tumours determined by RNA-Seq. *Top*: positive correlation of CD74 with HLA-DRA, HLA-DMA and SPI1. *Bottom*: negative correlation of MIF with the three FOXO4 target genes PIK3C2A, APC and MLL. In all six plots the correlation test returns p-values smaller than 2.2×10^{-16}. The plots were produced using log-transformed normalized gene counts from 1043 primary breast tumours as reported by TCGA

pathways, which activate the phosphoinositide 3-kinase and AKT/PKB. It has been suggested that FOXO4 inhibits epithelial-to-mesenchymal transition in certain types of cancer, which links it to invasion and metastasis [8, 9]. Thus, from one hand, increased MIF in the tumour correlates with better overall survival, probably because it is associated with more inflammation and lymphocyte infiltration. On the other hand, the negative effect on FOXO4 target expression might indicate that such increased inflammation is a double-edged sword and in some tumours it might trigger invasion and metastasis through inactivation of FOXO4. Consistent with this notion are the results from large-scale proteomics experiments and mechanistic studies discussed in the next section.

4 CD74 and MIF Expression in Breast Tumours at Protein Level

In addition to genomics, protein-level analysis of tumour tissue samples and cultured cancer cell lines has contributed evidence for the involvement of MIF and CD74 in the regulation of tumour growth and metastasis. In one such study we used high-resolution mass spectrometry to profile the proteomes of triple-negative breast tumours and identify proteins that might be linked to increased metastatic propensity [10–12]. In such experiments tumour tissue is used to extract the proteins, which are then digested with sequence-specific protease such as trypsin. This

generates very complex mixtures of short peptides, which are analysed by nanoscale liquid chromatography coupled with detection by mass spectrometry. In our studies CD74 showed specific overexpression in the lymph node metastatic tumours. We have first analysed pooled samples from node-positive and node-negative tumours, which led to the identification of MIF, CD74, STAT1, MX1 and several other proteins implicated in immune responses as overexpressed in the metastatic tumour sample pool. We have then carried out additional experiments with individual tumour samples, which confirmed that CD74 is indeed significantly more abundant in lymph node metastatic tumours [11, 13].

In another set of experiments we knocked down CD74 expression in a triple-negative breast cancer cell line and observed significant decrease in the cell's ability to close the wound in wound-healing assays [13]. It is worth noting that CD74 was also identified among the proteins that are more abundant in the membrane fractions isolated from metastatic variants of a breast cancer cell line compared to samples isolated from an isogenic but non-metastatic cell line variants [14].

Furthermore, immunochemical staining of formalin-fixed tumour samples showed that CD74 is overexpressed in the malignant cells of approximately 50% of the studied triple-negative breast tumours, suggesting that it could be a good marker for prognosis and/or drug target candidate [13]. These observations, together with the already recognized involvement of CD74 in the pathology of some haematological malignancies [15–17], prompted us to investigate the role of CD74 in breast cancer metastasis in a more-mechanistic manner. To this end we generated a collection of cell lines that express CD74 in a highly regulated way under the control of tetracycline-inducible promoter. We then carried out quantitative analyses of the total proteome and the phospho-proteome in cells induced to express CD74 and in control cells.

5 CD74-Dependent Deregulation of the Tumour Suppressor Protein Scribble

To precisely quantify the proteome and phospho-proteome changes induced by CD74 overexpression, we used the stable isotope labelling approach, in which peptides labelled with stable isotopes of carbon and nitrogen are used as internal standards in experiments utilizing detection by electrospray ionization mass spectrometry. The internal standards are conveniently generated by labelling by amino acids in culture, a technique pioneered by Mathias Mann and his group at Max Plank Institute [18–20]. The use of internal standards overcomes a known limitation of electrospray mass spectrometry: the ionization efficiency of the peptides generated by digestion depends on their sequence and can vary greatly, which does not allow direct quantification based on the intensity of the signal. This can be achieved only relative to an internal standard having the same chemical structure (sequence) and hence the same ionization efficiency.

The quantitative proteomics approach based on SILAC labelling is summarized in Fig. 4.

To prepare labelled cultures of cells expressing CD74 we used two cell lines: the non-cancer HEK293 and the breast cancer MCF7. HEK293 cells were engineered to

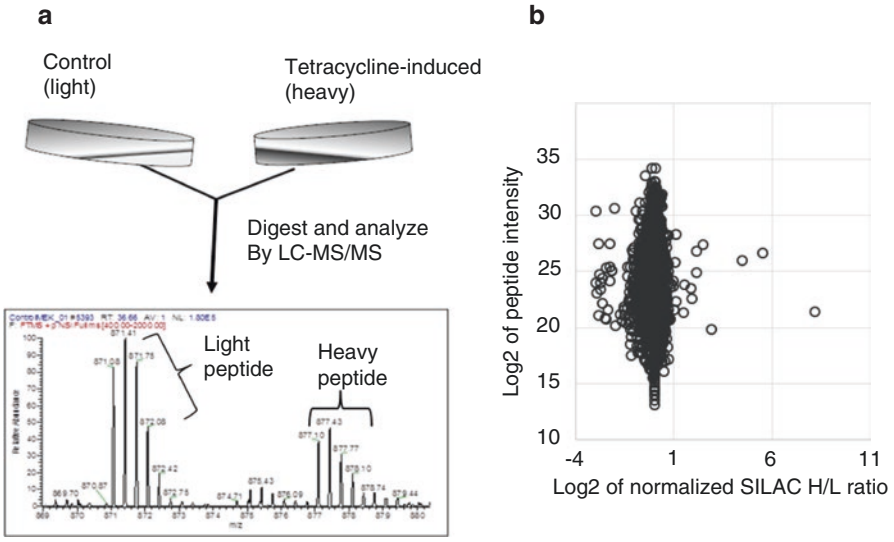

Fig. 4 Quantitative proteomics using stable isotope labelling by amino acids in culture (SILAC). (**a**) Labelling and detection/quantitation of the SILAC peptide pairs by high-resolution mass spectrometry. Note the well-resolved isotope envelopes of the heavy and light peptides. Such resolution is essential for efficient peptide detection, identification and accurate quantitation in complex peptide samples. (**b**) To identify proteins, which differ between the heavy and light sample, the log-transformed normalized H/L SILAC ratio is plotted against log-transformed intensity. Note that vast majority of proteins cluster around Log2(H/L) = 0 showing that only a small number of proteins are affected by the treatment. The same analysis is performed at phosphopeptide level to identify differentially phosphorylated sites

express CD74 under the control of tetracycline-inducible promoter, while MCF7 cells were transiently transfected with a plasmid expressing CD74 under the control of a strong constitutive promoter. The cells were first grown in labelling media containing heavy lysine (six C13 atoms) and heavy arginine (eight C13 and two N15 atoms) for several generations to ensure complete labelling. Use of both arginine and lysine ensured that every tryptic peptide would carry labelled amino acid at its C-terminus, which is important for comprehensive phospho-proteome analysis. The labelled cells were induced to express CD74 for 24 h and then an equal number of labelled and unlabelled control cells were mixed and used to prepare protein samples for analysis. The details of the analytical procedure and data analysis are described in Metodieva et al. [13].

The most significant result that was reproduced in all independent experiments and in both HEK293 and MCF7 cells was the dramatic change in the phosphorylation profile of the tumour suppressor protein Scribble. CD74 overexpression downregulated phosphorylation of Scribble on four serine sites in the C-terminal proline-rich domain of the protein. These sites were S1306 and S1309, detected on a single tryptic peptide, S1348 and S1448 detected on individual tryptic peptides. All four of the sites are followed by proline meaning that the responsible kinases are proline directed—either cyclin-dependent kinases or kinases of the MAPK family. The four phosphorylation sites have been reported previously by large-scale phospho-proteomics studies [19, 21].

These results suggest a model in which CD74 overexpressing breast tumours acquires increased metastatic propensity at least in part via destabilization of Scribble. Scribble has been discovered in *Drosophila* where it is important for the maintenance of polarity. When its function is lost, cells are prone to forming clumps and tumour-like structures, especially when Scribble knockout is combined with inhibition of apoptosis [22–24]. Scribble is very frequently either lost or overexpressed or aberrantly localized in breast tumours [25, 26]. Thus, when CD74 is overexpressed, it would change the phosphorylation profile of Scribble leading to changes in the network of protein-protein interactions that mediate Scribble functions leading to loss of polarity, abnormal division and increased invasion and metastasis. The exact mechanisms, by which CD74 overexpression affects the phosphorylation of Scribble, remain to be elucidated.

6 Concluding Remarks

Recent advances in postgenomic technologies have provided tools for comprehensive analysis of gene expression and protein abundance and protein post-translational modifications in tumour tissues and in cells isolated from tumours. Statistical analysis of the available deep sequencing data from TCGA showed that MIF and CD74 are overexpressed in tumour tissue compared to matched periphery. This can be explained in at least two ways: tumour-induced inflammation is responsible for increased infiltration of immune cells in the tumour. These immune cells natively express CD74 and MIF contributing to increased abundance of CD74 and MIF mRNA detected in tumour tissue by RNA-Seq. Alternatively, or in addition, in some tumours the malignant tumour cells can express increased amount of CD74 and MIF because they are exposed to cytokines released by the infiltrating immune cells. Such model of tumour cell expression of the two genes is supported by in vitro experiments which show that breast cancer cells exposed to interferon gamma express increased amount of CD74. In addition, there is cancer type-specific pattern of CD74 and MIF expression. ER-negative breast tumours are more likely to overexpress the two genes compared to the more frequent ER-positive tumours.

Intriguingly, correlative analysis of the RNA-Seq data from TCGA highlighted a group of genes, targets of the transcription factor FOXO4, which are negatively correlated with MIF expression. FOXO4, which activity is controlled by the PI3K/Akt pathway, regulates the expression of p27/kip1, an important cyclin-dependent kinase inhibitor. FOXO4 downregulation has been implicated in cancer progression and epithelial-to-mesenchymal transition, which is essential part of cancer cell metastasis.

Thus, it would appear that tumours overexpressing MIF have decreased FOXO4 activity and decreased expression of its target genes. Would this mean that such tumours could have higher propensity to metastasize? Such notion is supported by protein-level analysis of lymph node metastatic versus non-metastatic triple-negative breast tumours: quantitative proteomics showed that MIF, CD74, Stat1 and some other immune effector proteins are more abundant in the metastatic tumours [11]. Immunohistochemical staining then demonstrated that such increased amounts of CD74 are found in the malignant cells of these metastatic tumours suggesting that lymphocyte infiltration cannot be the sole explanation for the observed expression patterns.

Finally, overexpression of CD74 in a cell-based model led to surprizing down-regulation of several specific phosphorylation sites in the C-terminus of the tumour suppressor Scribble [13]. This links CD74 to another important aspect of cell biology: the regulation of the apical-basal and the planar polarity of the epithelial cells, which is maintained by Scribble.

Further studies in cell lines and utilizing tumour tissue would be necessary to elucidate the mechanisms behind this CD74 deregulation of Scribble. When elucidated, these mechanisms might provide targets for novel diagnostic assays and more effective personalized therapies for breast cancer.

Appendix

R code to merge individual RNA-Seq data files, cluster by CD74 and MIF expression, perform survival analysis and find genes which are co-regulated with MIF and CD74 in breast tumours. RNA-Seq data files are downloaded along with clinical information from TCGA (https://tcga-data.nci.nih.gov). The individual files are then merged and transposed to create a data frame containing patient data in rows and genes in columns. This is merged with the clinical information table using patients' barcodes. Survival analysis is then performed using the R package "Survival". Co-regulated genes are identified by calculating the Spearman rank-based correlation coefficient.

```
#RNA-seq import and merging
#Download data files from TCGA site. Unpack into working
directory
temp = list.files(pattern="*.genes.normalized_results")
myfiles = lapply(temp, read.delim)
data<- NULL
data<- myfiles[1]
for (i in 2:length(myfiles)){
    data<-merge(data, myfiles[i], by="gene_id")
}
names(data)<- c("gene_id", temp))
write.csv(data, "RNAseq_breast.csv")
rownames(data)<-data$gene_id
data$gene_id<-NULL
data.breast<-t(data)
```

```
#Download clinical data and import into R. Merge with the RNA-
seq data using patients barcodes to produce a #dataframe called
"data.tumors.only
#Then get MIF and CD74 expression levels:

cd74<- data.tumors.only[,3483]     #CD74 is column 3483 in the
table
mif<- data.tumors.only[,11053]     #MIF is column 11053 in the
table

#Cluster patients by expression of CD74 and MIF


dataCD74MIF<-data.frame(cd74,mif)
hc<- hclust(dist(scale(log(dataCD74MIF))), "ward.D2")
plot(hc)     #Look at the clustering and save as graphics file
cl<- cutree(hc, 2)
#Use clustering to do survival analysis
library(survival)
survival<-    as.numeric(ifelse(data.tumors.only$days_to_last_
followup!="[Not Available]",
       data.tumors.only$days_to_last_followup,    data.tumors.
only$days_to_death))
vital<- ifelse(data.tumors.only$vital_status!="Dead", 0,1)
surv<-Surv(survival,vital)
survdiff(surv~cl)    #calculate p-value
fit<- survfit(surv~cl)
plot(fit, col=c("grey", "black"), xlim=c(0,5000), ylim=c(0.2,1),
cex=0.5, xlab="Days to event",     ylab="Survival")

#Analysis of co-expressed proteins: calculate Spearman rho for
all proteins against CD74 and MIF


corMIF<-  apply(data.tumors.only[,-c(1:110)],  2,  function(x)
cor(x, mif, method="spearman"))
corCD74<-  apply(data.tumors.only[,-c(1:110)],  2,  function(x)
cor(x, cd74, method="spearman"))
write.csv(cbind(names, corMIF), "corMIF.csv", row.names=F)
write.csv(cbind(names, corCD74), "corCD74.csv", row.names=F)
```

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MIF-Dependent Regulation of Monocyte/Macrophage Polarization

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Abstract Despite its clearly important and central functional contributions governing monocyte and macrophage activation and differentiation, MIF is highly unconventional. Unlike other cytokines, MIF is not tightly regulated at the expression level, has a well-conserved nonphysiologic keto-enol tautomerase enzymatic activity, lacks a traditional secretory leader sequence, and has, for its primary cell surface receptor, a protein that shuttles MHC class II proteins between the Golgi and the plasma membrane. MIF is historically and best known for its regulatory actions that dictate pro-inflammatory monocyte/macrophage activation ultimately leading to a variety of chronic inflammatory and autoimmune disease pathologies. More recent studies, however, suggest that MIF may be equally important in dictating anti-inflammatory, immunosuppressive monocyte/macrophage functions in certain disease states. Most notably, studies investigating immune-dysregulation in cancer models support a conclusion that MIF is a necessary determinant of macrophage alternative activation and myeloid-derived suppressor cell (MDSC) differentiation/immune suppression. This chapter will summarize both the pro- and anti-inflammatory activities and functions associated with this nonclassical cytokine and will provide some speculation as to how MIF may mechanistically operate and its applicability as a therapeutic target for clinically relevant diseases.

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1 Introduction

Despite its clearly important and central functional contributions governing monocyte and macrophage activation and differentiation, MIF is highly unconventional. Unlike other cytokines, MIF is not tightly regulated at the expression level, has a well-conserved nonphysiologic keto-enol tautomerase enzymatic activity, lacks a traditional secretory leader sequence, and has, for its primary cell surface receptor, a protein that shuttles MHC class II proteins between Golgi and the plasma membrane. MIF is historically and best known for its regulatory actions that dictate pro-inflammatory monocyte/macrophage activation ultimately leading to a variety of chronic inflammatory and autoimmune disease pathologies. More recent studies, however, suggest that MIF may be equally important in dictating anti-inflammatory, immunosuppressive monocyte/macrophage functions in certain disease states. Most notably, studies investigating immune-dysregulation in cancer models support a conclusion that MIF is a necessary determinant of macrophage alternative activation and myeloid-derived suppressor cell (MDSC) differentiation/immune suppression. This chapter will summarize both the pro- and anti-inflammatory activities and functions associated with this nonclassical cytokine and will provide some speculation as to how MIF may mechanistically operate and it's applicability as a therapeutic target for clinically relevant diseases.

2 Pro-inflammatory Functions of MIF

Most early studies related to the function of MIF focused on its purported role as a pro-inflammatory cytokine [1]. By way of background, MIF was first described in the early 1960s as a product of activated lymphocytes that inhibits the random migration of guinea pig macrophages [2, 3]. Despite its "age", relatively little was known about its functional contributions in normal and/or disease physiology until the early 1990s when the Bucala group reported that antibody-mediated neutralization of MIF prevents gram-negative endotoxemia [4]. Subsequent functional studies using a variety of *in vivo* autoimmune and chronic inflammation models revealed centrally important contributing roles for MIF in the development of acute respiratory distress syndrome [5], rheumatoid arthritis [6], glomerulonephritis [7], malarial anemia [8], chronic enterocolitis [9], and gram-negative/gram-positive endotoxemia [4, 10, 11]. The consensus conclusion from these studies was that MIF is an upstream determinant of detrimental acute and chronic innate inflammatory responses [1]. Consistent with innate inflammatory mediators, genetic loss or inhibition of MIF was shown to confer greater susceptibility to pneumococcal [12], mycobacterial [13], fungal [14], and parasitic [15, 16] infections.

Further support of a critical regulatory role for MIF in promoting innate inflammation stems from the fact that loss or inhibition of MIF very consistently leads to reduced levels of monocyte/macrophage TNFα [10, 13, 17] cyclooxyegenase-2

(Cox-2) [18, 19], inducible nitric oxide synthase (iNOS) [20], and numerous other pro-inflammatory mediators. One of the first mechanisms linking MIF to monocyte/macrophage pro-inflammatory functions came from the Calandra laboratory when they demonstrated that MIF is functionally necessary for the transcriptional regulation of the gram-negative bacterial endotoxin receptor, toll-like receptor 4 (TLR4) [21]. MIF was found to maintain TLR4 expression in resting monocytes by modulating the transcriptional activities of the ETS family of transcriptional activators [21]. The net result of this positive regulation on TLR4 expression by MIF was proposed to enable macrophages to be more responsive to gram-negative bacterial by-products and to subsequently clear gram-negative bacterial infections more efficiently [21].

An alternative, but perhaps unifying, mechanism to account for the contribution of MIF to inflammatory responses was later proposed by the Bucala laboratory [18]. Endogenous, macrophage-derived MIF was found to be necessary for maintaining macrophage viability during times of infection or activation [18]. Macrophages lacking MIF were sensitized to p53-dependent activation-induced apoptosis, while cells containing MIF were significantly more resistant. This counter-regulatory action for MIF on p53-dependent macrophage apoptosis was suggested to ultimately result in a more robust and sustained inflammatory response in vitro and in vivo [18].

In addition to MIF-dependent modulation of macrophage transcription, activation, and viability, T lymphocytes have also been shown to be targets of MIF actions. Bacher and colleagues demonstrated that mitogen and antigen-induced Th2 lymphocyte activation and IL-2 expression is largely dependent upon autocrine-acting MIF [22]. More recent studies describe an intriguing role for MIF in the generation of CD4+ regulatory T cells (Treg) in tumor-bearing mice [23]. Interestingly, another study describes an important inhibitory role for MIF in antagonizing Th1-dependent cytotoxic T lymphocyte (CTL) antitumor responses [24]. Because antitumor immunity is thought to rely heavily on Th1-associated tumor cell cytolytic activity [25], overexpression of MIF in a tumor microenvironment may provide a selective growth and protective advantage for developing neoplasms.

3 Tumor-Associated Macrophages (TAMs)

Solid tumors are infiltrated by inflammatory leukocytes, and strong correlations have been observed between increased numbers of intratumoral macrophages and poor prognosis in a variety of human malignancies [26]. As such, both the recruitment and activation of stromal tumor-associated macrophage (TAMs) are considered to be pivotal to solid tumor progression. TAMs derive from circulating immature monocytes that are actively recruited to the tumor microenvironment by the chemokines CCL2, CCL5, CCL7, CCL8, and CXCL12 as well as tumor-derived growth factors and cytokines like vascular endothelial-cell growth factor (VEGF), platelet-derived growth factor (PDGF), and IL-10 [26, 27]. Once recruited into the

tumor stroma, TAMs secrete a variety of paracrine-acting factors that functionally promote neo-angiogenesis, matrix remodeling, and immune suppression [28].

Macrophages can be polarized into at least two functionally distinct phenotypes. These include the classically activated (M1) and alternatively activated (M2) subtypes. M1 macrophages express pro-inflammatory cytokines and effector molecules, such TNF-α, iNOS, IFN-γ, and Cox-2 [26, 29, 30]. In contrast, alternatively activated M2 macrophages express primarily anti-inflammatory cytokines and enzymes such as IL-10, TGF-β, and arginase-1 (ARG-1) [31, 32]. When TAMs are recruited into the tumoral microenvironment, they are rapidly polarized into alternatively activated M2 phenotypes that, in turn, induce antigen-specific lymphocyte non-responsiveness and skew T-cell responses from a pro-tumoral, Th1 phenotype, to an antitumoral, Th2 phenotype [26, 30, 33]. Perhaps more importantly, M2 TAMs are critically important in dictating and maintaining intratumoral neoangiogenesis through the coordinated expression of VEGF, CCL2, FGF2, CXCL8, CXCL1, and CXCL2 [26, 34]. Moreover, TAM-derived matrix metalloproteases (e.g., MMP-2 and MMP-9) promote matrix remodeling, tumor metastatic dissemination, and colonization [35, 36].

4 MIF is Necessary for TAM Alternative Activation

Because of the profound pro-tumorigenic contributions that M2 TAMs provide to developing neoplasms, there has been significant interest in developing therapies that can functionally skew TAMs from a pro-tumoral M2 phenotype toward an antitumoral M1-like phenotype [37]. However, to date, very few target molecules have been identified that can orchestrate this process *and* be therapeutically targeted. Studies from our laboratory reveal that stromal macrophage-derived MIF (as opposed to tumor cell-derived MIF) polarizes TAMs toward an M2 phenotype that—in turn—promotes an immunosuppressive, pro-angiogenic microenvironment within malignant melanoma lesions [38]. Implantation of high MIF-expressing melanoma cell lines into syngeneic MIF-deficient mice results in significant reductions in both subcutaneous melanoma outgrowth and metastatic melanoma lung colonization compared to MIF wild-type mice. Extensive marker analyses revealed that MIF-deficient F4/80$^+$ TAMs isolated from subcutaneous B16-F1 or lung metastatic B16-F10 tumors have significantly reduced expression levels of M2 markers and increased expression levels of M1 markers [38]. Specifically, expressions of the M2 alternative activation markers, ARG-1, Fizz1, MRC, CD206, CD23, and IL-10, were all significantly lower in F4/80$^+$ TAMs as well as CD11b$^+$ peritoneal exudate cells (PECs) isolated from tumors (TAMs) or the peritoneum (PECs) of melanoma-bearing MIF-deficient mice. Intriguingly, not only do MIF-deficient TAMs and PECs express dramatically lower levels of M2, alternative activation markers, they appear to be repolarized into a much more classically activated M1 state [38]. MIF-deficient TAMs and PECs express exceptionally higher levels of TNFα, IL-12, iNOS, Cox-2, IRF5, CD11c, CD80, and CD86 [38]. It should be noted that

monocyte/macrophage repolarization attributed to loss of MIF was found to be responsible for the antitumor effects associated with the loss of MIF observed. In subsequent unpublished studies, our laboratory demonstrated that MIF-deficient naïve monocyte/macrophage PECs were able to impart significant antitumor activities when injected into metastatic melanoma-bearing $MIF^{+/+}$ mice, while $MIF^{+/+}$ naïve monocyte/macrophage PEC adoptive transfer into $MIF^{-/-}$ mice substantially reversed the protective phenotype normally associated with stromal MIF-deficiency (Mitchell laboratory, unpublished observations). These results support a conclusion that $MIF^{+/+}$ monocyte/macrophage populations promote melanoma disease progression and also provide justification and rationale for therapeutic targeting of MIF in malignant disease states.

Despite the intriguing findings demonstrating that MIF-deficient TAMs and PECs exhibit altered M2 and M1 polarization profiles and actively antagonize metastatic melanoma disease progression, it remains important to determine whether MIF-dependent TAM polarization is a result of defective differentiation or whether fully and efficiently polarized M2 TAMs actively require MIF to *maintain* their alternatively activated polarization state. To test this, we utilized our well-characterized MIF small-molecule suicide antagonist [39–42] and treated $MIF^{+/+}$ (wild type) TAMs and PECs isolated from tumors/tumor-bearing mice (respectively) with 4-IPP ex vivo. Intriguingly, 4-IPP treatment of fully polarized TAMs and PECs fully recapitulates MIF-deficiency and dramatically reduces M2 marker expression while simultaneously inducing the re-expression of M1 markers [38]. More importantly, 4-IPP treatment also recapitulates MIF-deficiency and actively reduces established subcutaneous *and* metastatic melanoma tumor burdens in MIF wild-type mice and significantly extends their lifespans. Importantly, 4-IPP treatment of MIF-deficient melanoma-bearing mice had no effect on relative tumor burden or relative macrophage polarization, as expected [38].

In a potential landmark study by the Lyden group [43], MIF was found to be an essential determinant of macrophage recruitment and polarization in the development of pancreatic adenocarcinoma metastatic liver niches. Pancreatic cancer is unique in that it disseminates at a very early stage in its progression [44]. Exosomal MIF—produced at higher levels in pancreatic intraepithelial neoplasia (PanIN) and later-stage pancreatic ductal adenocarcinoma (PDAC) patients compared to healthy donors—was found to be necessary for reprogramming both liver-specific macrophages (Kupffer cells) and bone marrow-derived macrophages that are, in turn, necessary for matrix deposition and niche formation. ShRNA knockdown of MIF in pancreatic cancer cells resulted in MIF-deficient exosomes which were unable to induce appropriate liver niche formation and ultimately resulted in a profound reduction of metastatic lesions in the liver [43].

What is particularly intriguing about this malignant disease-associated MIF-dependent macrophage expression program is that it is essentially the polar opposite of the MIF-dependent expression signature found in acute and chronic inflammatory disease states described above. In other words, MIF serves to promote the expression of pro-inflammatory cytokines, chemokines, enzymes, and macrophage cell surface markers during times of acute or chronic infections or autoimmune

disorders [5–9] but promotes a primarily anti-inflammatory gene expression signature in monocytes/macrophages during malignant disease progression [38]. Although it is currently unclear what mechanistic underpinnings dictate this particular dichotomy in different disease states, it appears that MIF essentially supports the monocyte/macrophage polarization state that is actively being elicited by the disease microenvironment. For example, in tumor-bearing animals, tumor-derived growth factors, cytokines, and chemokines drive macrophage polarization toward an M2, alternatively activated phenotypic state, and endogenous, macrophage-derived, MIF serves to support this phenotype. In contrast, during active autoimmunity or as a consequence of bacterial, fungal, or parasitic infection, M1 and Th1 cytokines and chemokines drive an M1, classically activated, pro-inflammatory macrophage polarization state, and endogenous, macrophage-derived, MIF clearly serves to support this phenotype.

Several laboratories are actively delineating the mechanisms by which MIF may differentially promote monocyte/macrophage polarization phenotypes. Of particular interest are MIF's cognate and associated cell surface receptors. MIF's primary cell surface receptor, CD74, was first identified by the Bucala laboratory as a high-affinity cell surface binding protein for MIF that is responsible for extracellular MIF-dependent activation of a variety of intracellular signaling pathways [45, 46]. Interestingly, in macrophages and other antigen presenting cells, CD74 functions as the invariant chain of the MHC class II receptor. In this context, CD74 ferries class II proteins from the endoplasmic reticulum to the Golgi [47]. Extracellular MIF binds CD74's extracellular, C-terminal domain which initiates CD74 signaling either by intramembrane cleavage resulting in NF-κβ activation or by co-activating CD44 [45, 48, 49] or the co-activating chemokine receptors, CXCR2, CXCR4, and/or CXCR7 [50–53]. Given the large number of potential MIF receptor/co-receptor pairings and complexes, it will be interesting going forward to evaluate whether differential co-receptor complex formation on M1 versus M2 cells exists and/or whether differential signaling occurs from the same complex in classically, versus alternatively, activated macrophages.

5 MIF Promotes TAM-Mediated Angiogenesis

As mentioned above, one important way that TAMs dictate solid tumor disease progression is by promoting increased microvessel density within the tumor stroma [28]. Following recruitment of $CCR2^{+}$ immature TAMs into oxygen-deprived tumoral microenvironments, hypoxia and glycolytic metabolites induce further M2 TAM alternative activation leading to the increased expression and release of several pro-angiogenic mediators including but not limited to VEGF-A, urokinase-type plasminogen activator (uPA), adrenomedullin, basic fibroblast growth factor (bFGF), and MMP-9 [28, 54, 55]. In some cases, angiogenic cytokines and/or growth factors serve to recruit Tie-2 expressing monocytes (TEMs) into the tumor stroma where they align with developing blood vessels through interactions with the Tie-2 ligand, angiopoietin-2 (ANG-2), which is expressed on endothelial cells [56].

Cumulatively, these processes are intimately involved in the angiogenic switch that occurs in rapidly growing solid tumors and is essential for their continued progression and survival [57].

Consistent with a contributory role for MIF in promoting the angiogenic phenotype of alternatively activated TAMs, prior studies demonstrated that lung adenocarcinoma-derived MIF promotes CXCL8 (IL-8) and VEGF expression in human monocytes [58, 59]. More recently, stromal bone marrow-derived macrophage MIF was found to be a necessary determinant of intratumoral angiogenesis that is required for murine teratoma formation [60]. These findings are consistent with a study by the Dranoff group demonstrating that melanoma patients showing durable anti-melanoma immune responses to an experimental therapeutic (consisting of the immune checkpoint inhibitor ipilimumab—anti-CTLA-4—combined with GM-CSF-expression autologous melanoma vaccine) had high levels of anti-MIF autoantibodies. Intriguingly, these autoantibodies act to specifically neutralize MIF-dependent Tie-2 and MMP-9 expression in TAMs, leading to disrupted tumor vasculature, lymphocyte/granulocyte infiltrates, and, by extension, a significantly improved prognosis [61]. Further validating these findings, this laboratory recently discovered that MIF-deficient TAMs exhibit significant reductions in the expression of a number of pro-angiogenic growth factors and enzymes. The expression of Tie-2, stabilin, VEGF-A, and MMP-9 are all significantly lower in $MIF^{-/-}$ TAMs and PECs, and 4-IPP was found to completely recapitulate these reductions in $MIF^{+/+}$-treated melanoma TAMs. Importantly, functional studies confirmed that MIF deficiency or small molecule inhibition results in significant reductions in the ability of TAM supernatants to promote endothelial cell migration and tube formation in in vitro angiogenesis assays [38].

6 MIF Promotes TAM-Mediated Immune Suppression

As discussed above, TAMs preferentially localize to hypoxic areas of tumors that, in turn, serve to promote TAM-elicited angiogenic cytokines, growth factors, and enzymes [54, 55]. Hypoxia has profound effects on TAM functions including their migration into tumors and patterns of gene expression. Hypoxia induces TAM gene expression primarily through the stabilization of the HIF-1α and HIF-2α transcription factors [54, 62]. In addition to the pro-angiogenic mediators induced by HIF-driven transcription, hypoxia also upregulates the expression of anti-inflammatory and immune suppressive determinants such as IL-10, arginase-1, Cox-2, and programmed death ligand-1 (PD-L1) [62, 63]. All of these are thought to contribute to the highly immunosuppressive environment found within solid tumor lesions [56]. Given that macrophages make up the bulk of stromal cells found within tumor microenvironments, it is highly likely that these TAM-associated effectors—individually and combined—are essential for maintaining immune privilege within tumor microenvironments.

$F4/80^{+}$ TAMs isolated from subcutaneous and metastatic melanoma lesions from MIF-deficient or 4-IPP-treated mice were 60–70% less immunosuppressive than

TAMs isolated from $MIF^{+/+}$ wild-type or vehicle control-treated mice (respectively) [38]. Moreover and, again, suggestive of a requirement for MIF in mediating TAM polarization/immune suppressive activity in fully polarized M2 TAMs, 4-IPP treatment of melanoma TAMs ex vivo was dramatically reduced the immune suppressive activity of these TAMs against antigen-specific T lymphocyte proliferation [38]. Although the exact mechanisms responsible for this defect in MIF-deficient or inhibited TAMs is still unresolved, it is likely that the substantial reductions in the expression of IL-10 and Arg-1 are at least partially involved. Arg-1, in particular, is a very important determinant of innate immune cell-mediated inhibition of effector T-cell activation [28, 64].

As discussed above, several groups are actively investigating the relative contributions of individual and coordinated receptor activation in dictating MIF-dependent macrophage alternative activation phenotypes. What is less clear, however, is what downstream signaling effector pathways might be involved. Certainly, one intriguing possibility is that of HIF-1α. MIF is a direct transcriptional target of HIF, and, perhaps more importantly, MIF functionally promotes HIF-1α stabilization [65–68]. Studies are currently underway to determine whether MIF-deficiency or small molecule antagonism disrupts HIF-1α-dependent transcription in tumor-associated monocytes and macrophages. Given the profound importance of hypoxia-driven immune-regulation, the identification of a central role for MIF in mediating these processes would be highly impactful to the field.

In addition to HIF-1α, NF-κB-dependent transcription is also considered to be an important determinant of TAM transcriptional programs [26]. TAMs from advanced tumors exhibit defective NF-κB activation in response to pro-inflammatory signals [30, 31], and this is correlated with impaired expression of TNF-α, IL-1β, and IL-12 [26]. Interestingly, the energy homeostatic AMP-activated protein kinase (AMPK) pathway may functionally inhibit inflammatory responses induced by NF-κB in alternatively activated M2 TAMs [69]. Because MIF promotes AMPK activation in a CD74-dependent manner in other cell types [70–72], it is not unreasonable to speculate that MIF-dependent AMPK activation may be responsible, at least in part, for MIF-mediated M2 alternative activation. In fact, preliminary studies from our group indicate that $MIF^{-/-}$ and 4-IPP-treated melanoma TAMs have significantly reduced AMPK pathway activation compared to control macrophages. Studies are currently underway to identify the precise mechanisms of action for TAM alternative activation in the context of MIF, AMPK, and HIF-1α focusing on both upstream and downstream effectors.

7 Myeloid-Derived Suppressor Cells

In addition to tumor-infiltrating TAMs, circulating, splenic, and intratumoral myeloid-derived suppressor cells (MDSCs) represent another innate immune source of potent immunosuppressive activity in malignant diseases [28]. MDSCs are a heterogeneous population of immature myeloid and neutrophil lineage cells

with suppressive properties that preferentially expand in cancer. MDSCs potently inhibit T-cell proliferation and cytotoxicity, inhibit NK cell activation, and induce the differentiation and expansion of regulatory T cells (Treg) [28, 73]. Like TAMs there is also some evidence that MDSCs are capable of promoting tumor-associated neoangiogenesis, tumor cell extravasation, and metastatic dissemination [74].

Murine MDSCs are characterized by the expression of Gr-1 and CD11b. $CD11b^{+}Gr\text{-}1^{+}$ cells represent only 2–4% of nucleated splenocytes in healthy animals, but numbers can increase to up to 50% of splenocytes in tumor-bearing mice [75, 76]. These cells are a mixture of immature myeloid cells, immature granulocytes, monocytes-macrophages, dendritic cells (DCs), and myeloid progenitor cells. Murine MDSCs are further subdivided into two major groups: $CD11b^{+}Gr\text{-}1^{high}$ granulocytic MDSC (also identified as $CD11b^{+}Ly6\text{-}G^{+}/Ly6C^{low}$ MDSC) and $CD11b^{+}Gr\text{-}1^{low}$ monocytic MDSC (also identified as $CD11b^{+}Ly6\text{-}G^{-}/Ly6C^{+}$MDSC) [76].

Mouse MDSCs inhibit T effector activation by several mechanisms. In particular, the activities of inducible nitric oxide synthase (iNOS) and arginase-1 (ARG1)—both of which are highly expressed in functionally active MDSCs isolated from tumor-bearing mice—are, arguably, the most important. Because both of these enzymes utilize L-arginine as a substrate, this amino acid is rapidly and preferentially catabolized in MDSC-rich environments [77]. In T lymphocytes, L-arginine is necessary to maintain the expression of the ζ-chain in the T-cell receptor (TCR) complex, so, in the presence of L-arginine catabolizing MDSCs, antigen-induced proliferation is ablated [77, 78]. Another T-cell suppressive mechanism employed by MDSCs is the generation of reactive oxygen species (ROS), nitric oxide, superoxide, and peroxynitrite. These ROS prevent lymphocyte activation in a number of ways—one of which is through the nitrosylation and subsequent inhibition of the TCR on CD8 effector cells [79, 80].

Simpson and colleagues demonstrated that tumors derived from MIF shRNA-expressing 4 T1 cells contain significantly fewer monocytic MDSCs than control tumors [81]. In this model, tumor-derived MIF was found to impart significant functional activity toward the efficient differentiation of myeloid cells into immunosuppressive MDSCs. Interestingly, reconstitution of MIF-depleted tumor cells with wild-type MIF cDNA was able to fully restore the MDSC tumor infiltration/differentiation phenotype, whereas a tautomerase-inactive MIF mutant cDNA failed to reconstitute the MDSDC phenotype. Based on these findings, the authors concluded that the tautomerase activity of tumor-derived MIF is important for its effects on MDSCs and tumor metastasis [81].

Confirming a dominant regulatory role for MIF in mediating MDSC differentiation and activity, splenic MDSCs isolated from melanoma-bearing MIF-deficient mice were significantly less immunosuppressive than those isolated from MIF wild-type mice [38]. Similar to the observations with melanoma TAMs, the small molecule MIF inhibitor, 4-IPP, reduced MDSC immunosuppression and corresponding melanoma disease progression in mice [38]. It is important to note that, unlike the study by Simpson and colleagues, MIF-dependent contributions to melanoma MDSC immune suppression were tumor independent. In other words, loss of

endogenous monocyte/macrophage MIF results in substantial and dramatic reductions in MDSC-mediated immune suppression despite the fact that the implanted melanoma cell lines express and secrete very high MIF levels. Current efforts are focused on delineating the molecular mechanisms driving the MDSC-dependent tumor-promoting effects of MIF. Our recent unpublished studies confirm the requirements for MDSC-derived MIF in mediating MDSC differentiation/immunosuppression. For example, in vitro differentiated bone marrow-derived MDSCs require MIF for maximal Arg-1 and iNOS expression and MIF-deficient bone marrow-derived monocytic MDSCs possess substantially reduced MDSC immunosuppressive activity (*manuscript under review*).

Low oxygen tensions found within the stroma of developing tumors was recently found to be a necessary stimulus for inducing the functional differentiation of tumor-infiltrating MDSCs into mature M2, alternatively activated, TAMs [82]. Hypoxia-induced HIF-1α acts to alter the function of MDSCs within the tumor microenvironment and redirects their differentiation toward a more mature, TAM-like, phenotype. These findings not only provide a mechanistic link between different myeloid suppressive cells in the tumor stroma but also suggest an additional source of TAMs not previously known [82]. One critical question that remains unanswered, however, is what role—if any—does MIF play in influencing the HIF-1α-dependent MDSC → TAM differentiation paradigm.

Compared to murine MDSCs, human MDSCs are still poorly characterized. The best marker for human MDSCs remains their T lymphocyte suppressor function, which can be either direct or indirect through the induction of Tregs [28]. Human MDSCs are defined as cells that express common myeloid markers $CD14^+$, $CD11b^+$, and $CD33^+$, but are usually negative/low for HLA-DR and lack the expression of lineage-specific antigens (Lin) such as CD3, CD57, CD19. Human monocytic MDSCs are characterized by a $CD14^+CD33^+HLADR^{low/-}$ immunophenotype and are generally considered to be analogous to the murine monocytic $CD11b^+Ly6\text{-}G^-/Ly6C^+$ MDSCs. Human granulocytic MDSCs are $CD15^+CD33^+HLADR^{low/-}$ and are most closely analogous to $CD11b^+Ly6\text{-}G^+/Ly6C^{low}$ MDSC subpopulations in mice. Monocytic or granulocytic MDSCs are present in patients with melanoma [83], multiple myeloma [84], hepatocarcinoma [85], NSCLC [86], and prostate cancer [87], among others. In contrast to the superior immune suppressive activity observed with tumor-infiltrating MDSCs in murine models [88], most human studies of MDSCs have focused on peripheral blood. Peripherally circulating MDSCs in human are highly immunosuppressive, and their numbers correlate with tumor burden, stage, and grade in a variety of cancers [89, 90].

Depletion of L-arginine and L-cysteine; increased production of nitric oxide, superoxide, and peroxynitrates; and expression of anti-inflammatory cytokines have all been shown to participate in human MDSC T-cell-suppressive functions [72]. In the head and neck cancer patients, both tumor-infiltrating and circulating MDSC immune suppressive activity are closely associated with activated STAT3-mediated events [91]. Additionally, Mao and colleagues demonstrated that monocytic MDSCs from melanoma patients inhibit autologous T-cell activation and proliferation in a COX-2/PGE_2-dependent manner [92].

In order to assess whether MIF participates in human melanoma-induced MDSC differentiation and/or immune suppression, we recently evaluated $CD14^+CD11b^+HLA\text{-}DR^{low/-}$ monocytic MDSCs—a population that is significantly expanded in the periphery of all advanced melanoma patients [83]. Our findings indicate that MIF is preferentially overexpressed in patient monocytic MDSCs compared to $CD14^+$ normal donor monocytes and that MIF inhibition significantly reduces the suppressive properties of $CD14^+HLADR^{low/-}$ MDSCs isolated from late-stage metastatic melanoma patients (*manuscript under review*).

Accumulations of phenotypically immunosuppressive MDSCs are associated with decreased numbers of DCs in the peripheral blood of the head and neck, lung, and breast cancer patients [93]. Functionally, MDSCs isolated from peripheral blood of HLA-A2-positive cancer patients attenuate the production of $CD8^+$ T-cell IFN-γ induced by peptide-pulsed DCs [93]. Thus, circulating MDSCs may provide a developing neoplasm with the ability to induce antigen-specific $CD8^+$ T-cell nonresponsiveness. It would therefore follow that the targeted elimination of MDSCs in cancer patients would be expected to significantly enhance antitumor immune responses in patients. A promising approach that is actively being pursued to accomplish this is the use of agents that induce differentiation of MDSCs into DCs. Retinoic acids, ligands of the retinoic acid receptors [RAR; retinoid X receptor (RXR)], have been shown to stimulate the differentiation of myeloid progenitors into myeloid DCs [94, 95]. Administration of *all-trans* retinoic acid (ATRA) into tumor-bearing mice significantly reduces the numbers of MDSCs while actively inducing the expansion of $CD11c^+$MHC class II^+ myeloid DCs, macrophages, and granulocytes [96]. Importantly, treatment of metastatic renal cell carcinoma patients with ATRA resulted in a decrease in the numbers of $Lin^-HLA\text{-}DR^-CD33^+$ cells which was accompanied by an increase in tetanus toxoid-specific T-cell responses [97].

Initial studies performed by our group using human melanoma MDSC models lend strong support to the hypothesis that the therapeutic targeting of MIF in human melanoma may represent a clinically viable and attractive approach to enhancing antitumor immunity (Fig. 1). Two questions that are currently being explored are (1) how to use MIF antagonists therapeutically to eliminate MDSCs in cancer patients and (2) whether MIF inhibition can be used to effectively induce differentiation of MDSCs into DCs with concomitant improvement in myeloid/lymphoid DC ratio, DC function, and antigen-specific T-cell-mediated immune responses in cancer patients. Studies are currently underway to rigorously answer both of these questions.

8 Conclusions

There is little question as to whether MIF is sufficiently relevant to be therapeutically targeted in clinical disease settings [98–100]. Most MIF-associated clinical sequelae are attributed to its central upstream regulatory role as a pro-inflammatory

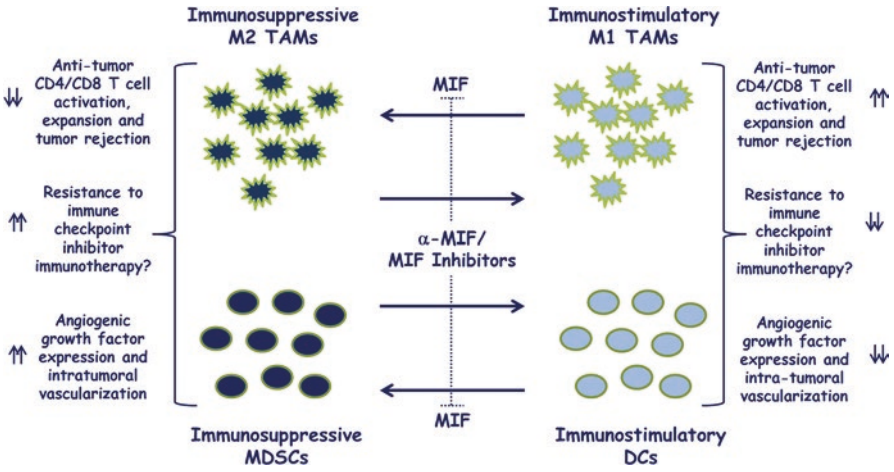

Fig. 1 MIF controls TAM M2 polarization and MDSC differentiation resulting in increased immunosuppressive activity, angiogenic potential and, ultimately, tumor burden. Inhibition of MIF in advanced stage cancer patients is expected to reduce immunosuppression and angiogenic potential of TAMs and MDSCs while simultaneously inducing immunostimulatory M1 TAMs and functionally active dendritic cells which may alleviate TAM and/or MDSC-associated immune checkpoint inhibitor resistance

determinant [101]. However, more recent studies indicate that MIF is also instrumental in driving pathologies associated with diseases characterized by excessive anti-inflammatory and immune-suppressive activities [38, 102, 103]. Despite these intriguing findings, numerous questions remain regarding mechanisms of action, intracellular versus extracellular functions, paracrine versus autocrine activities, and whether MIF's vestigial enzymatic activity is necessary for mediating its biological activities. Consequently, there is still a great deal to be learned about this highly unusual—and equally intriguing—cytokine known as MIF.

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Advances in Understanding the Role of MIF in the Pathogenesis of Autoimmune Diseases

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Abstract Macrophage migration inhibitory factor (MIF) is an immunoregulatory cytokine that was first noted several decades ago for its property to arrest the random movement of macrophages. It is now known that MIF has pluripotent effects including promoting the expression of proinflammatory cytokines, having chemokine-like functions, and promoting cell migration and recruitment. Moreover, it plays an important role in sustaining immune cell survival by inhibiting activation-induced apoptosis. The cell surface receptor for MIF has been identified as the CD74 molecule, and functional interactions with the CXCR2/4 chemokine receptors also occur. CD74 is present on multiple cell types; thus, the function of MIF is defined by the cell type on which it acts. MIF is a critical upstream regulator of both innate and adaptive immune responses. Because of its broad range of activities, MIF has been implicated in the pathogenesis of a variety of infectious, inflammatory, and autoimmune disease conditions including rheumatoid arthritis, multiple sclerosis, and systemic lupus erythematosus. During autoimmune diseases, the overproduction of MIF may potentiate inflammation and priming of autoreactive cells and enhance detrimental effects on affected tissues. In addition, MIF has a unique relationship with corticosteroids (CSs); it can override the effects of CSs and may be important in corticosteroid resistance. In vitro and in vivo evidence has focused attention on MIF as a new therapeutic target for autoimmune diseases by selectively neutralizing MIF with antibodies or specific small molecule inhibitors.

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1 Macrophage Migration Inhibitory Factor (MIF) and Autoimmunity

MIF was independently described in 1966 by two groups as a soluble factor released into cultures of antigen-sensitized lymphocytes that could inhibit random macrophage movement [1, 2]. Based on this property, this cytokine was termed **macrophage migration inhibitory factor**. MIF, unlike most other cytokines, is constitutively expressed and stored in intracellular pools within the cytoplasm [3]. MIF is produced by a variety of immune and nonimmune cells, including macrophages [3], T cells [4], and B cells [5]. MIF has a broad range of activities including inhibition of macrophage migration, enhancement of TNF-α and nitric oxide production by macrophages [6], and activation of T cells [4]. CD74 has been identified as the receptor for MIF [7]. In addition, MIF is known to be a noncognate agonist of the chemokine receptors CXCR2 and CXCR4 [8]. MIF is the only proinflammatory cytokine that is induced by corticosteroids (CSs) and is known to counter-regulate CS-mediated inhibition of macrophage and T cell activation, proinflammatory cytokine production, and apoptosis [9–11]. MIF has been implicated in the pathogenesis of several autoimmune diseases including multiple sclerosis, autoimmune myocarditis, rheumatoid arthritis, type 1 diabetes mellitus, etc., some of which we will be discussing in the following section. Autoimmunity is believed to arise due to dysregulation of central tolerance in the thymus or lack of peripheral tolerance. Autoimmune diseases are sustained by continued activation of inflammatory processes which otherwise protect the host from infection [12]. Several studies have demonstrated that MIF is located upstream of many inflammatory mediators such as TNF-α and IL-1β and induces their production by macrophages [13], thereby leading to persistent inflammatory responses that are known to contribute to autoimmune pathology (Fig. 1).

2 The Role of MIF in Multiple Sclerosis (MS)

MS is an autoimmune disease of the central nervous system (CNS) where neuroantigen-reactive T cells, B cells, as well as cells of the innate immune system contribute to disease pathology [14–20]. Data derived from experimental autoimmune encephalomyelitis (EAE), the widely accepted animal model of human MS, show that neuroantigen-specific T cells migrate from the periphery to the CNS where they encounter neuroantigen presented by antigen-presenting cells (APCs) and promote disease pathology. The pathological hallmarks of this disease include demyelination and axonal transection, and CNS damage is mediated by proinflammatory cytokines such as IL-17, IFN-γ, TNF, and GM-CSF [21–24]. Evidence supports that MHC II expression by professional APCs in the CNS is indispensable for the induction of CD4+ T cell-mediated EAE [24]. However, both CD4+ and CD8+ T cells can mediate EAE [14, 18]. Although an important role for in situ

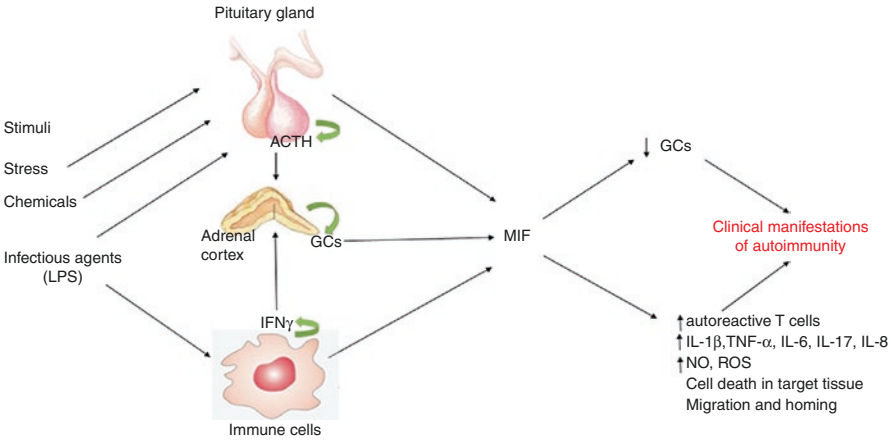

Fig. 1 MIF at the central point of the immunoinflammatory cascade. Adapted from [12, 13]. MIF occupies an upstream position in the events promoting dysregulated inflammatory responses that contribute to autoimmune pathology. Corticosteroids (CSs) and MIF are connected in a tightly regulated balance, and MIF is secreted upon CS induction and then counter-regulates CS effects. Dysregulation of this balance may be a key mechanism in sustaining autoimmune pathology via overexpression of MIF and proinflammatory cytokines via a CS—MIF amplificatory feedback loop. *ACTH* adrenocorticotrophic hormone, *NO* nitric oxide, *ROS* reactive oxygen species

production of reactive oxygen species (ROS) has been suggested in the pathogenesis of MS and EAE, the exact mechanisms leading to demyelination and axonal damage are not fully understood [25–27]. The contribution of MIF to MS comes from several lines of evidence. It was observed that MIF expression increased significantly in the brain in response to LPS-induced neuroinflammation [28, 29]. Furthermore, the levels of MIF are elevated in the cerebrospinal fluid (CSF) of MS patients during relapses compared with the levels during remission, and importantly its increased levels have been associated with clinical worsening and relapses [30]. Subsequent studies in EAE showed that treatment with neutralizing anti-MIF mAb reduced the clinical severity of disease and accelerated recovery by impairing the entry of neuroantigen-specific T cells into the CNS via downregulation of the expression of VCAM-1 [31]. Furthermore, anti-MIF mAb treatment decreased the clonal sizes of neuroantigen-specific T cells and increased their activation threshold. The development of MIF knockout mice [10, 32] further advanced the mechanistic study of this factor in a number of diseases including EAE and led to a better understanding of its role in corticosteroid treatment resistance. CSs have been shown to shift the cytokine profile from Th1 cytokines (IL-2 and IFN-γ) to Th2 (IL-4 and IL-10) which are thought to have protective effects in MS and EAE. Powell et al. reported that $MIF^{-/-}$ mice displayed elevated levels of corticosterone and decreased expression of inflammatory cytokines (TNF-α, IFN-γ, and IL-6) in EAE [33]. Ji et al. showed that $MIF^{-/-}$ mice displayed significantly decreased expression of the transcription factor T-bet [34], known to be critical for the

effector function of encephalitogenic T cells [35]. Progressive disease in MS has been attributed to several factors including mounting evidence for a role of microglia and macrophages [36, 37]. Studies have reported significantly reduced infiltration of macrophages in the brains of MIF inhibitor-treated mice [38]. Also, MIF was shown to induce the microglial transcription factor C/EBP-β which regulates myeloid cell function and plays a role in neuroinflammation [39]. Additionally, in the absence of MIF, microglia are not sufficiently activated [39], thereby leading to reduced activation of T cells in the CNS of MIF-deficient mice [40, 41]. Microinjection of MIF in MIF-deficient mice upregulated inflammatory mediators in microglia which then potentiated EAE-mediated pathology. Further, MIF was shown to be necessary to sustain chronic inflammation in the CNS as loss of MIF bioavailability in $MIF^{-/-}$ mice correlated with decreased pathology [39].

3 The Role of MIF in Systemic Lupus Erythematosus (SLE)

SLE is a systemic autoimmune disease that is characterized by loss of immune tolerance and production of autoantibodies, immune complex deposition, leukocyte infiltration, and chronic inflammation in different organs such as skin, kidneys, brain, and joints [42]. Several studies have reported increased serum MIF levels in SLE patients compared with healthy individuals, and these levels correlated with SLE disease damage (SLICC/ACR index) [43–45]. In addition, increased expression of MIF mRNA was detected in the kidneys of patients with lupus nephritis [46], and another study reported increased concentrations of MIF in urine [47]. Sanchez et al. reported that the MIF 173*C allele, and particularly the MIF 173*C-$CATT_7$ haplotype, was associated with an increased susceptibility to SLE [48]. MRL/*lpr* mice develop spontaneous autoimmune disease resembling human SLE that is characterized by hyperglobulinemia, production of anti-double stranded DNA Abs, accumulation of lymphocyte subsets, and inflammatory skin lesions [49]. Of note, lupus-prone MRL/*lpr* mice showed abundant expression of MIF in the renal cortex [50], which was most evident in tubular epithelial cells which are immunologically active resident cells that can interact with other immune effector cells [51–53]. SLE is characterized by infiltration of macrophages in the kidneys, and they play an important role in amplifying inflammation [54, 55]. Studies conducted by Hoi et al. in $MIF^{-/-}$MRL/*lpr* mice showed reduced renal macrophage recruitment which was associated with reduced urinary levels of the monocyte chemoattractant protein (MCP-1) [50]. MRL/*lpr* mice showed upregulation of MIF in skin lesions compared with non-diseased control mice (MRL/MpJ). $MIF^{-/-}$MRL/*lpr* mice exhibited prolonged survival and reduced renal and skin manifestations of SLE compared with MRL/*lpr* mice. Moreover, inhibition of MIF in the NZB/NZW F1 lupus mouse model showed significantly reduced plasma MCP-1 and TNF-α levels. Also, reduced intrarenal mRNA levels of MCP-1, IL-1β, and TNF-α were

observed. These findings are consistent with MIF's upstream role in the expression of these proinflammatory mediators [56, 57] and may account for the reduction in expression of these tissue-damaging cytokines (IL-1β, TNF-α) upon inhibition of MIF in models of lupus nephritis [58, 59]. However, MIF antagonism did not affect T and B cell activation and autoantibody production suggesting that MIF inhibition specifically reduced excessive proinflammatory cytokine responses [50]. MIF acts as a noncognate ligand for the chemokine receptor CXCR4, and it may thereby modulate recruitment of leukocytes to tissues [8] which is an early event in autoimmune kidney injury. Consistent with this view, treatment with a CXCR4 peptide antagonist show prolonged survival and reduced intrarenal leukocyte trafficking in the B6.Sle1Yaa mouse model of SLE [60]. Taken together, these results implicate MIF as a critical effector of end organ injury in SLE.

4 The Role of MIF in Autoimmune Myocarditis

Myocarditis and its sequelae is an inflammatory disease of the myocardium which is widely underdiagnosed and has an unfavorable prognosis and poor long-term outcome in a significant number of patients [61–63]. It is a common cause of sudden death in up to 20% of young adults [64–66]. Although a wide range of infectious and toxic agents (bacteria, protozoa, alcohol, etc.) have been implicated as causes of myocarditis, it is frequently triggered by viral infections (commonly Coxsackie B virus) which can result in a persistent autoimmune response against heart muscle tissue. An autoimmune mechanism leading to persistent myocarditis is supported by the association with particular human MHC class II antigens such as HLA-DR4 [67], the presence of myocardium-specific autoantibodies [68], and the ability to induce myocarditis in animal models with T cells specific for myocardium-derived antigens [69]. Myocarditis is characterized by the presence of inflammatory infiltrates associated with degeneration or necrosis of cardiomyocytes. The clinical appearance of myocarditis is highly variable ranging from asymptomatic to symptomatic patients and clinical presentations ranging from nonspecific systemic symptoms such as fever, fatigue, and palpitations to fulminant hemodynamic collapse and sudden death. The patients with subacute forms of myocarditis frequently go on to develop chronic-progressive heart failure and dilated cardiomyopathy [65, 70]. EAM is an animal model of post-infectious myocarditis [69], and it can be induced by active immunization of susceptible mouse strains with myosin antigens or adoptive transfer of myosin-reactive T lymphocytes. Romina et al. reported significantly higher levels of MIF in patients with chronic Chagas disease compared with asymptomatic Trypanosoma cruzi-infected and uninfected individuals [71]. One study reported elevated levels of MIF in the hearts of rats with EAM. Yutaka et al. demonstrated that early treatment with anti-MIF mAb markedly delayed the onset of EAM and significantly reduced EAM disease severity in rats [72]. Normal heart shows

weak to moderate expression of MIF in cardiac myocytes, and the above study showed that MIF expression was enhanced in cardiac myocytes around inflammatory foci and in infiltrating cells in EAM hearts treated with control IgG compared with anti-MIF mAb-treated animals. These results implied a role for MIF in recruitment of T cells and macrophages to the heart. The cytokines TNF-α and IL-1β are known mediators for the development of EAM. Treatment with anti-MIF mAb showed significantly decreased protein levels of TNF-α and IL-1β in the heart tissue of EAM rats [72], suggesting that inhibition of TNF-α and IL-1β by anti-MIF mAb treatment could be a mechanism for disease suppression. Also, blockade of MIF leads to a reduction in the expression of VCAM-1 by endothelial cells and infiltration of inflammatory cells in the heart [72]. Additionally, IL-1 has been reported to be a potent inducer of VCAM-1 [57], and inhibition of IL-1 expression by anti-MIF treatment could be a probable mechanism for suppression of VCAM-1 expression during EAM. We observed that MIF-knockout mice showed decreased expression of MIP-1α (macrophage inflammatory protein) in the EAM hearts compared with controls (SN, TGF, unpublished), suggesting that MIF induces the expression of MIP-1α thereby recruiting inflammatory cells to the myocardium. Collectively, these results imply that MIF might be an important mediator of myocardial injury in EAM.

5 The Role of MIF in Rheumatoid Arthritis (RA)

RA is a chronic autoimmune disease characterized by synovial proliferation, impaired apoptosis of resident synoviocytes, inflammatory infiltrates (i.e., monocytes, macrophages, and T cells), destruction of articular joints, and autoantibody production (against rheumatoid factor) [73]. Inflamed joints show increased expression of cytokines (e.g., TNF-α, GM-CSF, IL-1, IL-6, and IL-8) and activation of matrix metalloproteinases [74, 75]. Of note, elevated levels of MIF have been detected in the synovial fluid and synovial tissues of RA patients [76, 77]. Liu et al. observed that the −173 C allele in the MIF promoter region was associated with increased susceptibility to RA [78]. Radstake et al. reported that single nucleotide polymorphisms in the MIF promoter were associated with increased MIF expression and accelerated joint damage in patients with RA [79]. Singh et al. found that adoptive transfer of wild-type macrophages into MIF-deficient mice restored the ability to develop arthritis and also restored serum IL-1β and IL-6 levels [80]. Several studies have explored the role of MIF in animal models of arthritis including collagen-induced arthritis (CIA), adjuvant-induced arthritis, and passively transferred models. Mikulowska et al. were the first to report that treatment with neutralizing antibodies to MIF before immunization with type II collagen led to delayed onset of arthritis and decreased IgG2a responses to type II collagen in a murine CIA model [81]. The severity of histological arthritis was found to be decreased in $MIF^{-/-}$ mice; these mice also showed reduced cartilage damage [82, 83]. Also, Leech et al. were the first to demonstrate the regulatory effect of MIF on p53 and apoptosis in vivo [83]. They observed reduced

proliferation, increased p53 expression, and apoptosis of synoviocytes in the absence of MIF. In vitro studies using human RA synoviocytes showed that MIF induced the proliferation of synoviocytes and inhibited p53 expression and apoptosis in these cells thereby providing pro-apoptotic signals [84]. MIF is expressed by macrophages, $CD4^+$ T cells, and fibroblast-like synoviocytes (FLS) in the RA synovium. MIF produced by FLS upregulates the release of monocyte TNF-α, thereby sustaining the proinflammatory environment within the rheumatoid synovium [85]. MIF is also know to activate the expression of proinflammatory mediators such as prostaglandins via induction of phospholipase A_2 and cyclooxygenase (COX-2), cytokines (IL-1, IL-6, IL-8), and matrix metalloproteinases (MMP-1 and MMP-3) from macrophages and FLS; these mediators may lead to cartilage destruction and tissue degradation in RA [86–88]. Gregory et al. observed reduced synovial leukocyte trafficking in the joint space of $MIF^{-/-}$ mice [89]. Joint destruction in RA is closely related to osteoclastogenesis, and RANKL is the major inducer of osteoclasts. Kim et al. reported that MIF enhanced osteoclastogenesis by upregulating RANKL expression by FLS in patients with RA [76]. MIF is known to activate MAPKs, including extracellular signal-regulated kinase (ERK) and p38 MAPK in synoviocytes [90]. Additionally, MIF has shown to promote chemotactic neutrophil recruitment [91] and T cell activation via regulation of ERK MAPK phosphorylation in RA [92]. Thereby, MIF contributes to the pathology of RA by mediating bone and cartilage injury.

Taken together, extensive evidence suggests a key role for MIF in RA.

6 The Role of MIF in Type 1 Diabetes Mellitus (T1D)

T1D is characterized by autoimmune destruction of insulin-producing pancreatic beta cells (β cells) mediated by autoreactive T cells and macrophages [93, 94]. Evidence supports a role for infiltration of inflammatory cells in and around pancreatic islets early in the pathogenesis of T1D. In inflammatory insulitis, islet-infiltrating immune cells as well as resident cells produce cytokines such as IL-1β, TNF-α, and IFN-γ, IL-17 and other proinflammatory mediators including nitric oxide (NO) that promote the destruction of β cells [95, 96]. Of note, circulating levels of MIF were found to be decreased in patients with recent onset T1D with multiple islet-specific autoantibodies [97], but it remains unresolved how these levels compared with healthy individuals. In contrast to the decreased levels of MIF in patients, its expression has been found elevated in nonobese diabetic (NOD) mice during development of disease, and administration of recombinant MIF has been shown to exacerbate disease development [98]. These conflicting results have not been fully reconciled; however, MIF is known to regulate glucose homeostasis, and it upregulates insulin secretion which is released from the islets in response to glucose [99]. $MIF^{-/-}$ mice show slightly impaired glucose tolerance due to β cell hypoactivity compared to wild-type controls. However, genetic deletion of MIF does not result in overt diabetes implying

that MIF has a redundant role in glucose homeostasis [100]. Grujicic et al. reported that anti-MIF mAb treatment had prophylactic effects on accelerated forms of diabetes in spontaneously diabetic NOD mice [100]. In addition, in $MIF^{-/-}$ mice, the development of MLD-STZ (multiple low doses of streptozotocin)-induced diabetes was suppressed. Apoptosis is one of the critical mechanisms leading to destruction of pancreatic β cells in T1D [101]. It is believed that inducible nitric oxide synthase (iNOS) which leads to the production of NO contributes to apoptosis of β cells [102]. Cvetkovic et al. and Stojanović et al. demonstrated that in vivo neutralization of MIF activity suppressed iNOS enzyme expression and NO release thereby, inhibiting β cell apoptosis. [103, 104]. Additionally, apoptosis was not observed in islets of $MIF^{-/-}$ mice, suggesting that MIF antagonism abrogates β cell apoptosis. Though apoptosis is known to be a late effector mechanism, recent studies have established a role for β cell apoptosis in initiation and amplification of an autoimmune response through (auto) antigen cross-presentation [105]. Therefore, absence of MIF might not only limit apoptosis in β cells but also impair activation of autoreactive T cells. Neutralization of MIF using the small molecule MIF inhibitor ISO-1, downregulated the secretion of proinflammatory mediators, TNF-α, IFN-γ, IL-1, and NO, while augmenting the anti-inflammatory cytokine IL-10 [103]. Based on previous studies, IL-23 is required for autoimmune destruction of β cells in MLD-STZ-induced autoimmune diabetes [106], and IL-17 stimulates iNOS-dependent toxicity in β cells [107]. Neutralization of MIF is known to severely impair IL-23 production which is important for the generation of IL-17 [108]. Hence, impaired activation of IL-23/IL-17 axis in $MIF^{-/-}$ mice may be responsible for resistance to MLD-STZ-induced autoimmune diabetes [100]. MIF contributes to clonal expansion of T cells during T1D based on results that anti-MIF therapy leads to decreased expression of IL-2 receptor by splenocytes and a lower rate of lymphocyte proliferation in vitro [100, 103]. As mentioned earlier, MIF is known to induce the expression of MMPs in RA. A link between MMPs and T1D has been shown, and inhibitors of MMP-2 delay the onset of adoptively transferred diabetes and partially reverse spontaneously developed disease in NOD mice by limiting cleavage of cell surface adhesion receptors, thereby preventing recruitment of diabetogenic T cells into the pancreas [109, 110]. Conceivably, MIF could therefore be involved in the upregulation of MMPs in T1D by promoting the migration of autoreactive T cell into the pancreas. Taken together, the above results from animal models suggest a central role for MIF in the development of autoimmune diabetes.

7 Pharmacological MIF Inhibition as a Therapy for Autoimmune Diseases

The levels of MIF in human plasma range from 2–6 ng/mL; however, during chronic autoimmune diseases such as RA, MS, etc., the concentration of MIF in plasma increases. Elevated levels of MIF may therefore serve as a marker of disease

severity [30, 46, 71, 76, 77]. MIF's upstream role in the immune response suggests that blocking its action would downregulate multiple inflammatory pathways and offer novel therapeutic benefits not similarly achievable with other anti-cytokine treatment strategies [111]. Moreover, MIF's ability to counteract the immunosuppressive action of CSs should make inhibition of MIF a powerful pharmacological target for inflammatory and autoimmune diseases, particularly for those conditions that involve resistance to steroid therapy [9, 112]. Therapeutic approaches to inhibiting MIF in autoimmune diseases have focused on neutralizing anti-MIF mAb, mitigating MIF signaling via CD74, and using small molecule inhibitors of MIF (summarized in Table 1).

Table 1 MIF inhibitors or neutralizing antibodies tested in in vivo and in vitro autoimmune disease models

Disease model	MIF inhibitor	In vivo dose/route of administration or in vitro concentration	Findings
Multiple sclerosis (MS), mouse model for MS- experimental autoimmune encephalomyelitis (EAE)	CPSI-1306 or CPSI-2705 (ISO-1 derivatives)	1.0 mg/kg × 21 d (day)/ oral	Drug administration decreased mean clinical scores of disease and cumulative disease index, decreased leukocyte migration to brain, and mildly increased certain regulatory T cell subsets [38]
Systemic lupus erythematosus (SLE), mouse models for SLE-MRL/lpr and NZB/W F1 mice	ISO-1	MRL/lpr mice—daily for 10 weeks at 40 mg/kg i.p (intraperitoneal). NZB/W F1 mice—daily for 12 weeks at 40 mg/kg i.p.	Drug administration reduced functional and histological indices of glomerulonephritis, inhibited CD74+ and CXCR4+ leukocyte recruitment, and lowered levels of circulating TNF-α in MRL/lpr mice and CCL2 in NZB/NZW F1 mice; mRNA for TNF-α, IL-1β, and CCL2 in kidney was reduced in both strains of lupus-prone mice following treatment [50]
Autoimmune myocarditis, mouse model for autoimmune myocarditis-experimental autoimmune myocarditis (EAM)	**Anti-MIF antibody**	10 mg/kg i.p.—daily 1–20 days or days 13–20	**Anti-MIF antibody** delayed EAM onset, or significantly reduced disease severity (day 13–20 treatment), decreased the expression of VCAM-1, TNF-α, and IL-1β and the migration of T cells and macrophages in the EAM heart [72]

(continued)

Table 1 (continued)

Disease model	MIF inhibitor	In vivo dose/route of administration or in vitro concentration	Findings
Rheumatoid arthritis (RA), mouse models for RA- collagen-induced arthritis (CIA) and adjuvant-induced arthritis (AIA)	**Anti-MIF antibody**	In vitro RAW264.7 cell-induced osteoclast formation/differentiation	**Anti-MIF antibody** reduced RANKL-induced osteoclastogenesis via deregulating RANKL-mediated NF-kB and NFATc1 transcription factor activation [113]
		In vitro stimulated fibrocyte-like synoviocytes	**Anti-MIF antibody** led to a marked reduction in MMP expression in Th1-and Th2-stimulated fibroblast-like synoviocytes [114]
		400 μg i.p.—1 h before immunization and days 3, 6, 9 after immunization	**Anti-MIF antibody** treatment before immunization with collagen type II led to delayed onset and lowered frequency of arthritis in mice [81]
		6 mg i.p.—day 0 and days 3, 6, 8, 10, 12 after immunization	**Anti-MIF antibody** treatment in adrenalectomized adjuvant-induced arthritis (AIA) rats conferred protection from lethality during arthritis and also decreased arthritis [115]
Type 1 diabetes (T1D), mouse model for T1D- multiple low dose-STZ model	ISO-1	3.5–35 mg/kg × 3 d/i.p.	Drug administration decreased hyperglycemia and insulitis; decreased lymphocyte islet-antigen-specific proliferative responses and cell-cell adhesion; decreased ex vivo proinflammatory mediators (TNF-α, IFN-γ, nitric oxide), while increasing anti-inflammatory cytokine IL-10 [103]

8 Anti-MIF mAb

Initially, studies were performed to block the effects of MIF using neutralizing anti-MIF antibodies. Many of these studies showed therapeutic efficacy in animal models of autoimmune diseases such as EAE [31], T1D [100, 103], RA [81], and EAM [72]. In vitro and in vivo studies showed that T cell proliferation can be inhibited using MIF antibodies [4]. Also, a study by Onodera et al. in murine models of RA explored a MIF DNA vaccine. Mice that received the MIF DNA vaccine produced high titers of autoantibodies that reacted to native MIF and were able to ameliorate collagen-induced arthritis [116].

9 Soluble CD74 Receptor and Anti-CD74 mAb

Leng et al. showed that the recombinant soluble form of the CD74 receptor that binds to MIF inhibits MIF-mediated ERK activation in defined cell systems [7]. A study by Schwartz et al. demonstrated that anti-CD74 mAb blocked MIF-mediated AKT activation in Jurkat T cells [117]. As MIF is known to signal through CD74 and mediate several inflammatory processes, blocking CD74 could serve as a potential therapeutic approach.

10 Agonists to CD74/Antagonists to MIF

Recently Benedek et al. demonstrated for the first time that partial MHC II constructs with covalently bound antigenic peptides (referred to as recombinant T cell receptor ligands; RTLs) bind to CD74, thereby blocking accessibility (by downregulating CD74 cell surface expression) and availability of CD74 for MIF binding and preventing its effects on downstream inflammatory activity [118, 119]. This group also reported that agonistic activity of DRα1-MOG35-55 for CD74 resulted in reduced axonal damage and reversal of ongoing clinical and histological signs of EAE. Additionally, the study showed that enhanced expression of CD74 on monocytes in mice with EAE and patients with MS was downregulated by humanized RTLs. Thus, these agonists to CD74 may have the potential to treat autoimmune diseases such as MS.

11 Small Molecule Inhibitors to MIF

Anti-MIF and anti-CD74 mAbs are currently being humanized for clinical applications. However, although neutralizing antibodies and recombinant proteins have the advantage of biological specificity, they are not ideal approaches for drug therapy. Protein-based biological agents require parenteral administration and come with a high cost for production and administration [120, 121]. Hence, nonprotein-based anti-MIF therapies focused on small molecule inhibitors seem an attractive alternative approach for new pharmacological agents [122]. Small molecule MIF inhibitors would be non-immunogenic on repeat administration, and they could be administered orally after optimization of formulation.

The three-dimensional x-ray crystallographic structure of MIF shows the presence of a catalytic site which serves as a small-molecule binding pocket [123]. This binding pocket is of great interest for pharmacological development because it provides access to the protein's surface for design of low molecular weight MIF inhibitors. Also the structure of MIF does not share homology with any other known cytokines, and the small molecule inhibitors that have been developed target the catalytic site of MIF, which has a nonessential enzymatic function [124, 125]. Most of the inhibitors specifically bind to the active site of MIF that contains highly conserved amino acid residues known to be essential for MIF's proinflammatory activity [126]. These inhibitors may

cause altered binding of MIF to its receptor and other cellular protein partners, thereby inhibiting MIF's biologic activities. The first synthesized compound was an aromatic amino acid Schiff's base, 2-[(4-hydroxybenzylidene) amino]-3-(1H-indol-3-yl) propionic acid methyl ester, which prevented the interaction of MIF with its receptor CD74 [127]. Another small molecule compound, N-acetyl-p-benzoquinone imine (NAPQI), a metabolite of acetaminophen, induced a conformational change in MIF thereby affecting its binding to CD74 [122]. Moreover, among the best characterized of these small molecule MIF inhibitors, the administration of (S,R)-3-(4-hydroxyphenyl)-4, 5-dihydro-5-isoxasole acetic acid methyl ester, ISO-1 has shown to prevent or reverse T1D [100] and experimental allergic neuritis (EAN) [128]. Recently ISO-66, a novel and highly stable small molecule MIF inhibitor and an analogue of ISO-1 with improved characteristics, has shown efficacy in melanoma and colon cancer models [129]. However, clinical trials are needed to determine whether these orally active small molecule MIF inhibitors can be developed to treat human autoimmune diseases.

12 Conclusions

Mounting evidence indicates that MIF plays an important role in the development of inflammatory tissue damage and autoimmune diseases via counter-regulation of immunosuppressive CSs effects. MIF promotes immune-cell effector functions and production of a range of Th cell-polarizing and proinflammatory cytokines, regulates immune-cell migration and homing via upregulation of adhesion molecules and/or chemokines, and enhances survival and/or clonal expansion of inflammatory and autoreactive cells. Neutralizing the proinflammatory effects of MIF with mAbs or small molecule inhibitors has been effective in the control of several models of autoimmune diseases. Thus, more than ever, MIF represents a promising potential pharmacologic target for the treatment of inflammatory and autoimmune diseases.

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Role of MIF in Experimental Autoimmune Encephalomyelitis and Multiple Sclerosis

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Abstract Macrophage migration inhibitory factor (MIF) and its receptor, CD74, are pivotal regulators of the immune system. In this chapter we review the roles of MIF and CD74 in multiple sclerosis (MS) and its mouse model, experimental autoimmune encephalomyelitis (EAE). MIF is produced by a number of cells that are involved in MS pathology, such as monocytes and T cells. MIF effects on MS and EAE have been attributed to disease progression, as it was shown to prolong and enhance the pro-inflammatory functions of these cells. In addition, CD74 was also shown to be involved in this process. We and others demonstrated that blocking MIF signaling either by targeting MIF or CD74 could prove very beneficial in inhibiting disease progression. As such, partial MHC class II constructs, that include the HLA-DRα1 domain, could bind to CD74 and block MIF binding and signaling. This results in reversal of clinical signs of EAE and promotion of neuroprotection, thus, pointing to the therapeutic potential of regulating MIF and CD74 in MS.

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1 Introduction

Multiple sclerosis (MS) is a chronic, immune-mediated demyelinating disease of the central nervous system (CNS) [1–3]. MS is categorized into subtypes according to its clinical course. Most patients diagnosed with multiple sclerosis (MS) start with a relapsing-remitting course (RRMS). However, 10–15 years after disease onset, over 50% of RRMS patients develop secondary progressive MS (SPMS). MS patients not initially diagnosed with RRMS have primary progressive MS (PPMS) in which there is gradual progression of impairment and disability from disease onset without an initial relapsing-remitting phase. Furthermore, while MS is 2–3 times more common in women than men, this ratio decreases with older age, and there is an equal sex ratio among PPMS patients [4–7]. RRMS is dominated by infiltration of the CNS by lymphocytes and monocytes, edema, and the physiologic actions of cytokines. Progressive MS is characterized by axonal degeneration in the absence of overt extrinsic acute inflammatory lesions [8–10], although episodic focal inflammatory lesions still occur, but at a much lower frequency than in RRMS. The pathogenesis of the progressive axonal loss in MS is uncertain, but the toxic effects of reactive oxygen species and other soluble mediators of inflammation released by activated microglia may be critical to this stage of the disease [8, 11, 12]. Neurodegeneration is apparent throughout the CNS including the brain, spinal cord, and optic nerves [13–15].

In this chapter, we will focus on the role of macrophage migration inhibitory factor (MIF) and its receptor, CD74, as key inflammatory factors in MS pathogenesis and its mouse model, experimental autoimmune encephalomyelitis (EAE), as well as therapeutic agents that could target the MIF/CD74 pathway.

2 MIF and Multiple Sclerosis

MIF is secreted from various cell types in different tissues. Baseline expression of MIF was detected in brain tissues of naïve rats with elevated expression after LPS stimulation. It was further suggested that during neuroinflammation, macrophages and microglia are one of the main sources of MIF in the CNS [15]. MIF involvement in inflammatory disease is well established. However, although MIF was one of the first cytokines to be discovered, its involvement in MS was demonstrated long after its identification. Niino et al. were among the first to determine that the concentration of MIF in cerebrospinal fluid (CSF) of MS subjects was significantly elevated during disease relapse compared to healthy control subjects [16]. Interestingly, three studies evaluated MIF levels in serum or plasma of MS subjects and in active MS lesions [17–19]. Hagman et al. demonstrated a positive correlation between MIF levels and Expanded Disability Status Scale (EDSS) score of MS subjects. Furthermore, MIF levels were shown to be increased in MS subjects with disability progression compared to subjects with stable disease [18]. In a different study, it was reported that MIF was found to be concentrated at the edge of active white matter lesions, thus suggesting its involvement in MS demyelination [17].

MIF expression is regulated by both genetic and epigenetic factors, such as post-transcriptional modification by histone deacetylases. As for the genetic regulation

of MIF expression, there are two major functional polymorphisms located in the MIF gene: The alleles of the −794 CATT variable number of tandem repeats (VNTR) and the −173 G/C single nucleotide polymorphism (SNP). These polymorphisms have been reported to modulate MIF promoter activity and to correlate with MIF expression levels. It was shown that MIF promoter activity is proportional to the number of the CATT repeats at position −794. In addition, it has been shown that the −173 C allele also increases MIF promoter activity [20]. The correlation between MIF promoter polymorphism and inflammatory diseases was demonstrated in multiple studies. Such associations were reported in autoimmune diseases such as rheumatic arthritis (RA), type 1 diabetes (T1D), and systematic lupus erythematosus (SLE), in different populations [21–23]. However, so far only two contradicting studies about the association of MIF polymorphisms with MS were published, both in the Turkish population [24, 25].

3 MIF and Experimental Autoimmune Encephalomyelitis

Experimental autoimmune encephalomyelitis is a rodent model of CNS inflammatory demyelinating disease, which is widely used for studying MS pathogenesis. Denkiger et al. were the first to demonstrate that anti-MIF treatment of mice with acute EAE impairs the VCAM-dependent homing of encephalitogenic T cells to the CNS, thus reducing disease severity and accelerating recovery [26]. Later on, Caroline Whit acre's group demonstrated that MIF deficient mice (MIF−/−) exhibited only acute signs of EAE without further disease progression [27]. MIF supports EAE progression through its ability to activate and recruit CNS macrophages and microglia, which could impact CNS repair mechanisms during chronic EAE [17].

MIF also has been demonstrated to promote expression of pro-inflammatory cytokines that contribute to MS pathogenesis. This cytokine response is significantly reduced in MIF-KO mice and in EAE mice treated with anti-MIF antibodies [26, 27]. The enhancement of the pro-inflammatory response also was attributed to the MIF anti-apoptotic effect which enables activated macrophages to continue to secrete pro-inflammatory cytokines [28, 29]. Thus, MIF contributes to the pathogenesis of EAE by promoting leukocyte recruitment to the CNS, inhibiting apoptosis of activated monocytes, and enhancing the secretion of pro-inflammatory cytokines [29]. These studies demonstrated MIF involvement in CNS inflammation. However, the role of MIF in demyelination and remyelination is not completely understood.

CD74 (HLA-class II invariant chain) is a type II transmembrane glycoprotein containing a trimerization domain flanked by two highly unstructured regions [30]. Earlier models suggested that the homotrimeric structure could bind up to three αβ MHC class II heterodimers to form a nonameric complex, $Ii_3(\alpha\beta)_3$ [31, 32]. More recent models propose a pentameric complex, with the Ii homotrimer chaperoning a single MHC class II heterodimer from the ER to the cell surface [33]. While the structure of MHC class II bound to CD74 has not yet been solved, interactions between CD74 and the αβ MHC class II heterodimer have been mapped to at least three discrete extracellular locations and the transmembrane domains [34–37]. In addition to its role as a chaperone for MHC class II, it was reported that 2–5% of the cellular CD74 is expressed on

the cell surface independently of MHC class II [38]. CD74, in combination with CD44 and CXCR2/4, has been reported to transduce MIF signaling [39–41], thus indicating an accessory role in immune cell stimulation [41]. Although it has been shown that MIF binds to CD74 extracellular domains, the actual binding site is not known.

We recently demonstrated enhanced CD74 cell surface expression on monocytes in mice with EAE, which implicates its involvement in the disease course. CD74 was upregulated in peripheral blood monocytes by 3 h after EAE induction, ~10 days before the appearance of definite clinical signs. In contrast, CD74 upregulation on resting and activated microglia and infiltrating monocytes in the CNS was correlated with the appearance of definite clinical signs of EAE [42] (Fig. 1). This

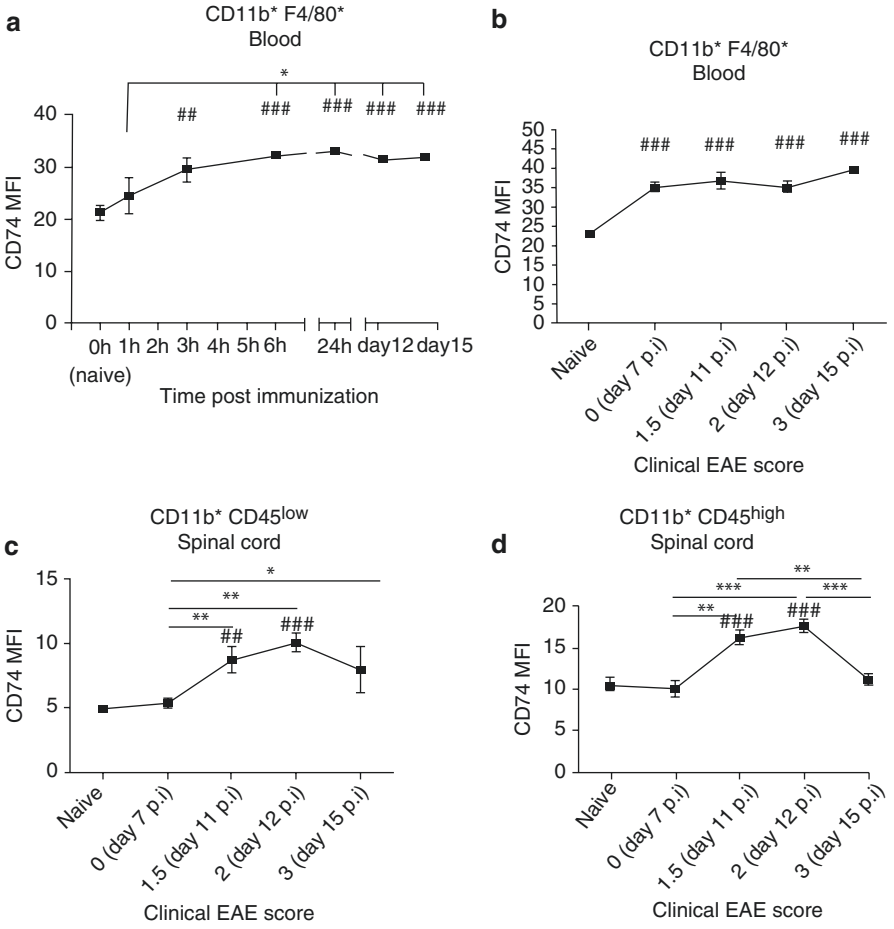

Fig. 1 CD74 expression is upregulated in EAE: (**a**, **b**) Blood macrophages (F4/80^{+}CD11b^{+}), (**c**) resting microglia (CD11b^{+}CD45^{low}) and (**d**) activated microglia and macrophages (CD11b^{+}CD45^{high}) in the spinal cord were analyzed for CD74 expression at different time points ($n = 3$ at each time point) (**b**–**d** Days post immunization in parentheses). Data are shown as mean ± SD and are representative of two independent experiments performed. ## $p < 0.01$, ### $p < 0.001$ vs. control, *$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, Mann Whitney U test

upregulation of CD74 expression in the CNS is associated directly with induction of inflammation by blood-borne monocytes. Ajami et al. demonstrated that the detection of infiltrating monocytes in the CNS of EAE mice was correlated with substantial clinical disability [43].

4 Treatment of EAE by Targeting the MIF/CD74 Pathway

Although significant progress has been made in understanding disease mechanisms in RRMS, our knowledge of the processes that lead to disease progression is limited. Furthermore, while there are several FDA-approved therapies for the treatment of RRMS, the only approved drug for SPMS is a chemotherapy drug, mitoxantrone, which has limited benefit and is highly toxic [44]. Since MIF was shown to be involved in disease progression, as described above, inhibiting MIF signaling might be most beneficial, especially for subjects with progressive MS.

Whitacre and collaborators demonstrated that administration of a small-molecule inhibitor of MIF stopped EAE progression and reduced the migration of leukocytes into the CNS of treated mice. Treatment of EAE in SJL/J and C57BL/6 mice that develop relapsing–remitting and chronic courses of EAE, respectively, with isoxazolines (CPSI-1306 and CPSI-2705), reduced disease severity in C57BL/6 mice and reduced relapses and increased remission length in SJL/J mice, suggesting that inhibition of MIF could prevent or inhibit disease progression. Interestingly, although it was previously reported that these small molecules inhibit the tautomerase activity of MIF, it is not clear whether this catalytic activity of MIF is related to its inflammatory functions. However, it appears from this study that CPSI-1306 and CPSI-2705 can inhibit the inflammatory properties of MIF as well [45].

A different approach to inhibit the MIF/CD74 pathway would target the MIF receptor. Our laboratory discovered and is developing partial MHC class II constructs (pMHC) as a possible immunotherapy for MS. pMHC containing the extracellular domains of the MS risk factor, HLA-DR2, linked covalently to the encephalitogenic myelin oligodendrocyte glycoprotein (MOG)-35–55 peptide (pDR2/MOG-35-55) can reverse CNS inflammation and clinical signs of MOG peptide-induced EAE in DR2 transgenic mice [46–48]. The same construct (termed RTL1000) was recently used successfully in a phase 1 safety trial in MS subjects [49]. We recently discovered that pMHC can bind specifically to the invariant chain of MHC class II (CD74), downregulating its expression on the monocyte cell surface and blocking the inflammatory effects of MIF [42, 50].

Consistent with the prior literature that studied intracellular interactions between CD74 and HLA-DRα during peptide loading of nascent MHC class II molecules [33, 51–55], we recently demonstrated that the DRα1 domain, but not the DR2β1 domain, could bind to immunoprecipitated CD74 [50] (Fig. 2). Hence, we utilized this construct to study cell surface interactions of DRα1 with CD74 and its possible effects on MIF binding and signaling. We demonstrated that increased concentrations of DRα1 (in the absence of a bound antigenic peptide) could down-modulate CD74 levels on monocytes. We further showed that this binding of DRα1to immunoprecipitated CD74 could inhibit binding of MIF to CD74 and its downstream

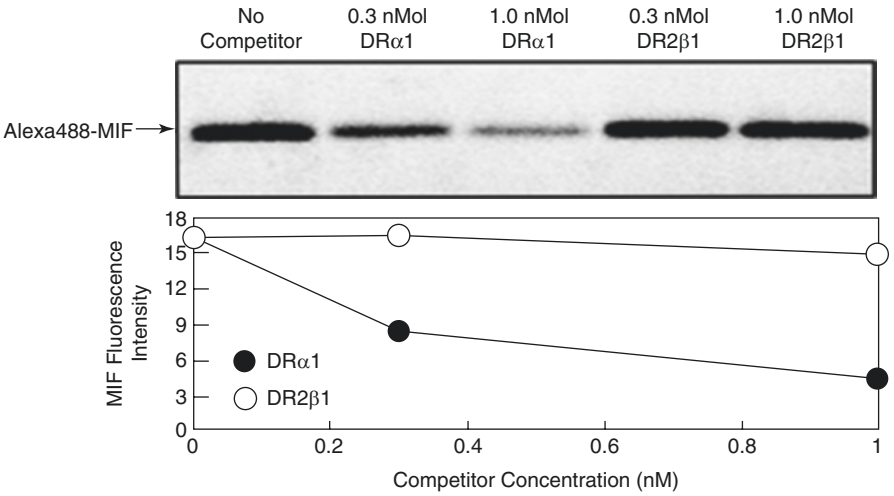

Fig. 2 DRα1 directly inhibits MIF binding to CD74**.** Unlabeled DRα1, unlabeled DR2β1, A488-hMIF (10 pmol), or a mixture of A488-hMIF and unlabeled constructs was bound for 16 h at 4 °C to immunopurified CD74, washed and eluates visualized in 10–20% SDS-PAGE, and quantified by relative fluorescence intensity

signaling effects. And we have demonstrated that DRα1 can inhibit cell migration into the CNS which contributes to the reversal of clinical signs of EAE, similar to the two domain parental construct. Furthermore, disease treatment potency of the DRα1 domain could be destroyed by trypsin digestion but enhanced by addition of a peptide extension (MOG-35-55 peptide) that provided secondary structure not present in DRα1. Because the DRα1 domain is present in all humans and thus would not be recognized as foreign, treatment using DRα1 constructs would not require HLA screening of potential recipients and could be used for treatment of all MS subjects, even those who do not express the HLA-DR2 risk factor [56].

We would thus speculate that the binding of the DRα1 construct to CD74 and subsequent blocking of MIF binding and signaling might represent a natural immunoregulatory role for the DRα chain in terminating MIF-dependent inflammation. This novel concept is supported by the binding of the *Staphylococcal* toxic shock syndrome toxin 1 (TSST-1) to the DRα chain, wherein the TSST-1 binding site on HLA-DRα1 partially overlaps with the DRα1/CD74 binding site [57]. These data, coupled with the report by Calandra et al. showing that MIF is a mediator of the activation of immune cells by TSST-1 [58], suggest the possibility that TSST-1 is blocking the binding of HLA-DRα to CD74, which in turn makes more CD74 available to bind MIF, thus inducing the toxic shock.

An important aspect of treating MS is the ability to stop and reverse ongoing axonal damage. Most of the available drugs for MS have only anti-inflammatory properties; however, partial MHC class II constructs might also promote remyelination and

be effective in treating progressive MS subjects. Such constructs were shown to reduce demyelination, axonal loss, and ongoing damage in SJL/J mice [48]. Furthermore, disease progression that might be affected by MIF is the M1/M2 macrophage/microglia balance in the CNS during EAE [59, 60]. Depending on the type of stimulation, activated macrophages can secrete various cytokines and express different surface markers. It is currently possible to characterize at least two opposing activation states: The classically activated macrophages (M1) express high levels of CD86, CD80, and MHC class II on their cell surface and are very potent in priming T cells and recruiting them to the CNS. These cells are predominantly present in the early stages of EAE. On the other side of the activation spectrum, alternatively activated macrophages (M2) express high levels of CD206, CD163, and arginase1 and low levels of CD40, CD86, and MHC class II [61]. Several studies suggested that M2 macrophages have a beneficial function in EAE, both in inhibiting inflammation and in inducing remyelination by phagocytosis of myelin debris and inducing oligodendrocyte differentiation [62–64]. It is important to note that MIF was shown to be involved in M1 polarization of macrophages [65], and we recently demonstrated that DRα1-mMOG-35-55 reversed EAE clinical signs in C57BL/6 mice (Fig. 3), inhibited infiltration of activated monocytes and $CD4^+$ T cells into the CNS, and increased the frequency of $CD11b^+$ $CD206^+$ (M2) monocytes in the spinal cord (Fig. 4). Furthermore, microarray analysis of spinal cords of DRα1-mMOG-35-55 treated DR*1501-Tg mice with EAE revealed that the expression of pro-inflammatory genes was dramatically reduced after DRα1-mMOG-35-55 treatment relative to vehicle treatment, while the expression of myelin basic protein (MBP) and other genes that were shown to be involved in remyelination and axonal survival and regeneration was upregulated [66].

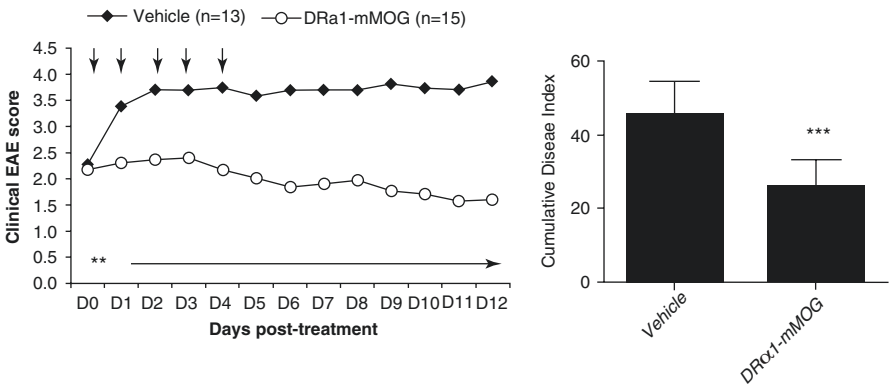

Fig. 3 DRα1-mMOG-35-55 treats clinical EAE in C57BL/6 male WT mice. EAE daily scores in male C57BL/6 mice (treatment days indicated by *black* arrows) and mean clinical EAE daily disease scores (*left*) and cumulative disease index scores (*right*) are shown. $^{**}p < 0.01$, $^{***}p < 0.001$. Daily mean scores were analyzed by Mann Whitney U and mean CDI by Student's *t*-test

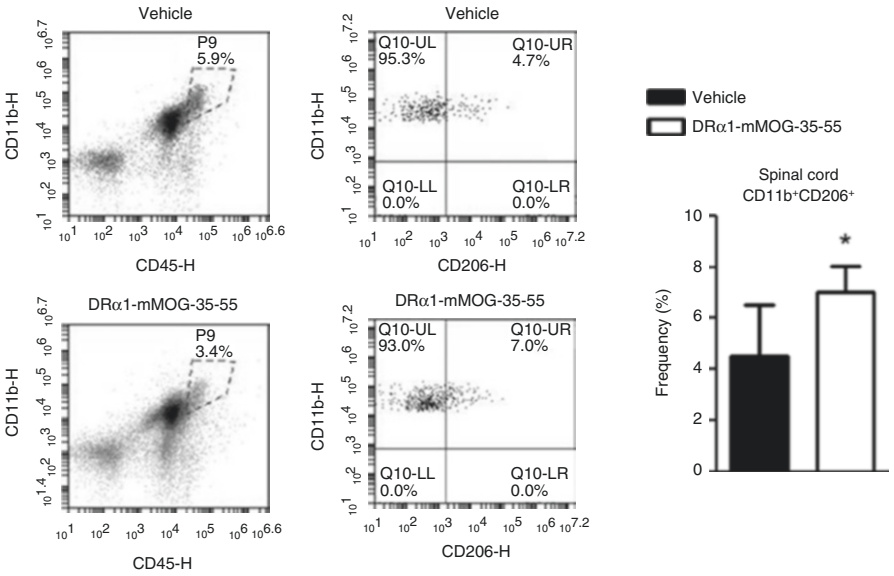

Fig. 4 DRα1-mMOG-35-55 enhances the frequency of $CD11b^{+}CD206^{+}$ cells in the spinal cord**.** Frequency of $CD206^{+}$ on $CD11b^{+}CD45^{hi}$ cells from spinal cords of DRα1-mMOG-35-55- ($n = 6$) vs. vehicle-treated ($n = 6$) C57BL/6 male WT mice with EAE

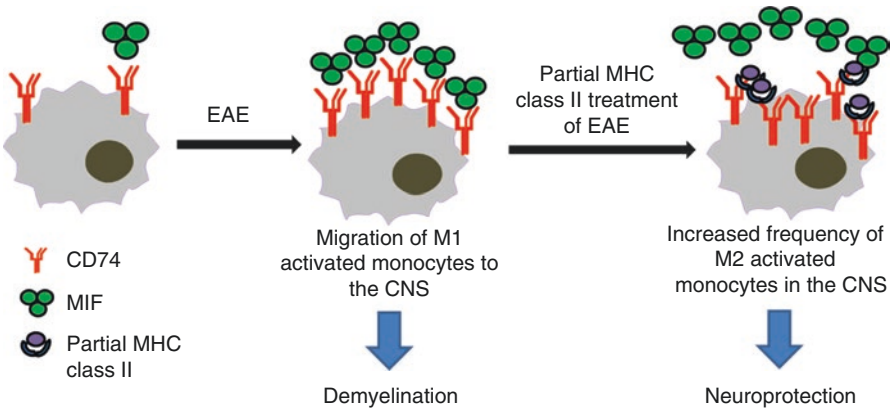

Fig. 5 Partial MHC class II mechanism of action

5 Conclusions

The involvement of CD74 and MIF in immune response during MS is becoming more evident making them potential treatment targets. Small molecules that directly inhibit MIF activity or partial MHC class II constructs that target CD74 could inhibit leukocyte migration into the CNS and attenuate ongoing inflammation and demyelination during MS (Fig. 5). These effects could greatly benefit both RRMS

and SPMS subjects. However, the role of MIF/CD74 in the remyelination process, which is crucial in reversing MS damage, is not known yet and remains to be studied.

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Role of MIF in Hepatic Inflammatory Diseases and Fibrosis

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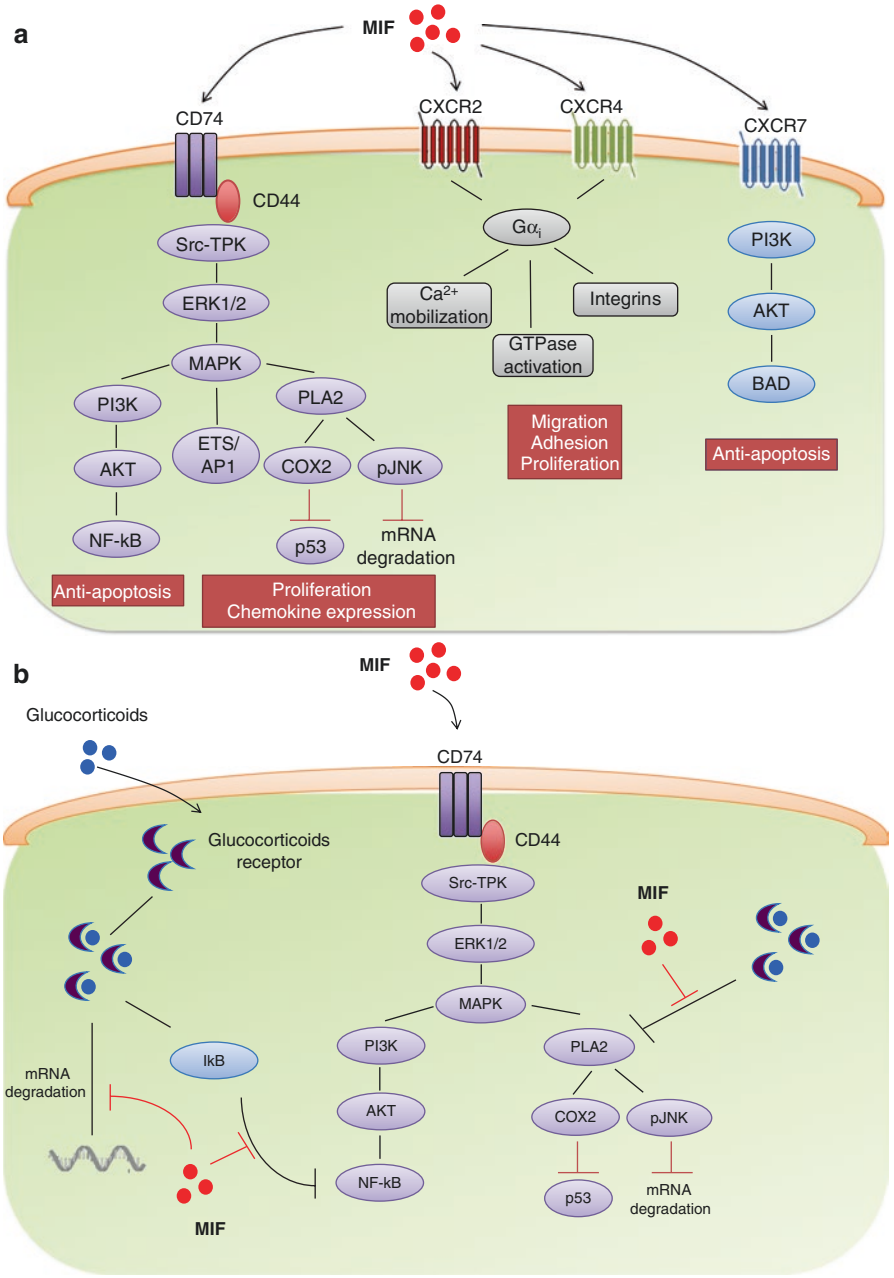
Abstract Macrophage migration inhibitory factor (MIF) is a pluripotent cytokine/chemokine that is an important component of the innate immune response. Recent studies have identified multiple roles for MIF in the progression and resolution of different stages of inflammatory and fibrotic response to liver injury. Here we review the basic functions of MIF and its cognate and non-cognate receptors in hepatic injury and repair, with an emphasis on alcoholic and nonalcoholic liver disease. Specific functions of MIF and its receptors in hepatocytes, Kupffer cells (the resident macrophage in the liver), and hepatic stellate cells are discussed in the context of hepatocyte injury, inflammatory responses and fibrogenesis. Finally, we analyze the potential for MIF as a therapeutic target for hepatic inflammatory and fibrotic diseases.

1 Overview of MIF Pathway

Macrophage migration inhibitory factor (MIF) is a pleiotropic inflammatory mediator. MIF was the first discovered active cytokine [1], but the protein was not cloned and characterized until the 1990s [2]. MIF was initially found to inhibit the random migration of macrophages and was associated with macrophage phagocytosis. MIF is constitutively expressed in both immune and nonimmune cells including various peripheral tissues, and its tissue distribution is almost ubiquitous. Besides its effect on monocyte/macrophage mobility, MIF is also known to be an upstream regulator of immunity and has a chemokine-like function, promoting the directed migration and recruitment of leukocytes into infectious and inflammatory sites [3] (Fig. 1). Its most critical functions encompass the regulation of macrophage function [4, 5], lymphocyte immunity [6, 7], and endocrine functions [7–9]. In contrast to other pro-inflammatory cytokines that are generally suppressed by glucocorticoids, MIF is a unique counter regulator of the immunosuppressive and

Fig. 1 Mode of action of MIF. (**a**) Interactions of MIF with its receptors and activation of signaling pathways. MIF binds a multicomponent receptor comprising two transmembrane proteins: the CD74 ligand-binding protein and the CD44 signal transducer. The MIF-CD74-CD44 complex formation activates Src-family protein tyrosine kinase and ERK1/2-MAPK, ultimately leading to the regulation of transcription factors controlling gene expression involved in cell cycle control, cellular proliferation and cell death, as well as expression of chemokines. MIF activation of CD74/CD44 also activates c-jun N-terminal kinase/stress-activated protein kinase (pJNK) increasing the post-transcriptional stability of mRNAs for pro-inflammatory cytokines such as TNF. MIF also induces signal transduction by binding to CXCR2 and CXCR4, triggering cytosolic Ca2+ influx, integrin activation, and GTPase activation via Gαi, resulting in cell adhesion, migration and proliferation. MIF binds directly to the CXCR7 inducing downstream activation of PI3K-Akt and the phosphorylation-mediated inactivation of pro-apoptotic protein BAD exerts anti-apoptotic effects. (**b**) MIF counter-regulation of glucocorticoids. Glucocorticoids inhibit NF-κB activation, in part, by increasing the expression of IκBα. Glucocorticoids also mediate mRNA destabilization. MIF inhibits IκB-induced synthesis, counteracts inhibition of NF-κB and stabilizes mRNA. Glucocorticoids inhibition of PLA2 activity and arachidonic acid production is also blocked by MIF

anti-inflammatory activities of glucocorticoids [10, 11] (Fig. 1b). MIF plays a nonredundant role in several inflammatory diseases, including sepsis [9, 12], rheumatoid arthritis [13], obesity [14], and atherosclerosis [15] and is associated with pathology of multiple liver diseases including viral hepatitis [16, 17], nonalcoholic (NAFLD)



[18] and alcoholic liver disease (ALD) [19, 20], cirrhosis, and hepatocellular carcinoma (HCC) [21].

There is a single MIF gene in the human genome (22q11.2), and both the exonic structure and DNA sequence of MIF are highly conserved across phylogeny. A remarkable feature of the human MIF gene is the presence of a microsatellite repeat (CATT 5–8) within the 5′ promoter region. This tetranucleotide repeat lies within a predicted pituitary-1 (Pit-1) transcription factor binding site, and both model gene reporter assays and human clinical studies indicate that repeat number is associated with higher MIF expression [13]. The human MIF gene has also a G/C single-nucleotide polymorphism (SNP) at position -173 in the 5′ promoter region [13] that is strongly linked to the tetranucleotide repeat.

MIF monomeric molecular weight is 12.5 kDa, with two antiparallel alpha-helices and six beta-pleated sheets forming an extended secondary structure of the molecule. MIF is a homotrimeric molecule in its active form, and it has been demonstrated to have at least two distinct catalytic activities, i.e., a tautomerase and an oxidoreductase activity [22]. MIF is produced by a variety of cell types including immune, endocrine, endothelial, and epithelial cells [23]. It is constitutively expressed and stored in preformed intracellular pools that are rapidly released upon stimulation [24].

MIF effects are mediated mainly by interactions with three distinct receptor proteins (Fig. 1a). The first is cluster of differentiation 74 (CD74), a single-pass type II transmembrane protein, which forms heterodimeric complexes with CD44 [25]. CD44 phosphorylation then activates Src family non-receptor tyrosine kinases, leading ultimately to ERK1/2 phosphorylation. MIF binding of CD74 is a major determinant of its pro-survival and proliferative functions on immune and tumor cells [26]. Moreover, MIF has been identified as a non-cognate ligand of CXC chemokine receptors (CXCRs) including CXCR2 and CXCR4. In fact, MIF has been classified in the emerging category of cytokines with chemokine-like functions. MIF binding to CXCR2 and CXCR4 activates inflammatory and atherogenic leukocyte recruitment [15]. MIF also stimulates release of MCP-1 from endothelial cells and recruits monocytes by way of CCR2 signaling [27]. Emerging evidence also indicates that effects of MIF recruitment involve functional receptor hetero-oligomerization, and CD74 can form complexes with both CXCR2 and CXCR4. CXCR2/CD74 heterodimers have been implicated in monocyte recruitment, and CXCR4/CD74 complexes have been described to promote activation of the AKT survival pathway in response to MIF [28]. CXCR7, recently renamed atypical chemokine receptor (ACKR)-3, can also bind MIF. CXCR7 belongs to the family of seven transmembrane receptors and shares high structural homology with related CXCRs such as CXCR4 [29]. The MIF/CXCR7 interaction contributes to MIF-triggered B-cell chemotaxis and ERK1/2 activation, and CXCR7 can also form complexes with previously known MIF receptors in both ectopic overexpression and endogenous conditions [30]. These upstream actions in the inflammatory cascade indicate that MIF plays a central role in different acute and chronic inflammatory diseases and could be a biomarker for different clinical applications.

Studies have shown that in several autoimmune and inflammatory disorders, such as rheumatoid arthritis [31], asthma [32], and systemic sclerosis [33], the

predominant impact of high-expression MIF alleles is on the severity of the clinical phenotype. Because most of these diseases were ameliorated by genetic MIF deletion or MIF neutralization, anti-MIF therapies have been considered to be of potential clinical value [34].

2 MIF and the Progression of Hepatic Inflammation and Fibrosis

2.1 Progression of Hepatic Inflammation and Fibrosis

The liver is the largest internal organ in the body performing many activities crucial to the health of its host [35]. It receives a mixed blood supply from venous and arterial sources with the predominant supply originating in the gut. The blood conducted from the gut via the portal vein is rich in nutrients, bacterial products, as well as ingested environmental toxicants [35, 36]. As such, the liver can be injured due to unresolved microbial infection, as well as acute and chronic exposures to noxious stimuli. The liver is normally quite resilient, however, due to its intrinsic ability to regenerate and complex interplay between the multiple resident cell types found in the liver. The detoxification, synthetic, and metabolic functions of the liver are mainly performed by the hepatic parenchymal cells, the hepatocytes, comprising nearly 70% of the cells found in the liver. The nonparenchymal cells make up the balance, including liver sinusoidal endothelial cells (LSEC), Kupffer cells (KC), natural killer cells, and lymphocytes that are critical components of the robust inflammatory functions associated with the liver [35, 36] (Fig. 2a).

The ability of the liver to effectively remove gut-derived pathogens from the circulation is a routine but essential function of the liver. Kupffer cells, the resident macrophages of the liver, are essential players in pathogen clearance. KCs are found in the sinusoidal lumen, making them the proximal responders to pathogens transported from the gut. Pathogen-associated molecular patterns (PAMPs) are components of pathogens, such as bacterial lipopolysaccharide (LPS), a cell-wall component of gram-negative bacteria [35, 36]. Many PAMPs, like LPS, activate KCs and other cells through pattern recognition receptors, the toll-like receptors (TLRs). TLR activation in response to bacterial and viral components induces a myriad of responses in KCs [37], such as increasing phagocytosis as well as cytokine and/or chemokine release [35, 37]. In addition to PAMPs, endogenous molecules can be released from stressed or dying cells, termed damage-associated molecular patterns (DAMPs), that activate TLRs on KCs and other cells in the liver [38]. DAMPs can therefore activate or further enhance inflammatory responses in cells of the liver. The importance of the inflammatory response in the liver cannot be overstated, given the prominence of immune cells residing in the liver and that virtually all forms of liver disease involve the inflammatory response [36, 39, 40].

The role of KCs has been studied in a variety of models of liver injury. KC depletion or neutralization by various techniques confers degrees of protection in animal models of sepsis, acetaminophen overdose [41], early alcohol-induced liver injury

[42], and chemically induced fibrosis [43]. Interestingly, depending upon the manner in which KCs were manipulated can demonstrate dichotomous roles for KCs in liver injury, such as studies wherein KC inactivation [41] versus KC depletion [44] confers protection or exacerbated injury following acetaminophen overdose, respectively. What is clear from these studies is the profound role KCs play as sensors of tissue injury that orchestrate the complex, interconnected web of signals that contribute to liver injury. In response to infectious and sterile immune stimuli, the KC-derived cytokine tumor necrosis factor-alpha (TNF) is critical to augmenting liver injury [35, 36, 39]. TNF can act to directly induce hepatocyte apoptosis, alter hepatic circulation through effects on endothelial cells, and facilitate immune cell entry into the liver [39]. The current understanding of acute and/or chronic liver disease therefore involves multiple cell types in the liver acting in response to noxious stimuli (Fig. 2b).

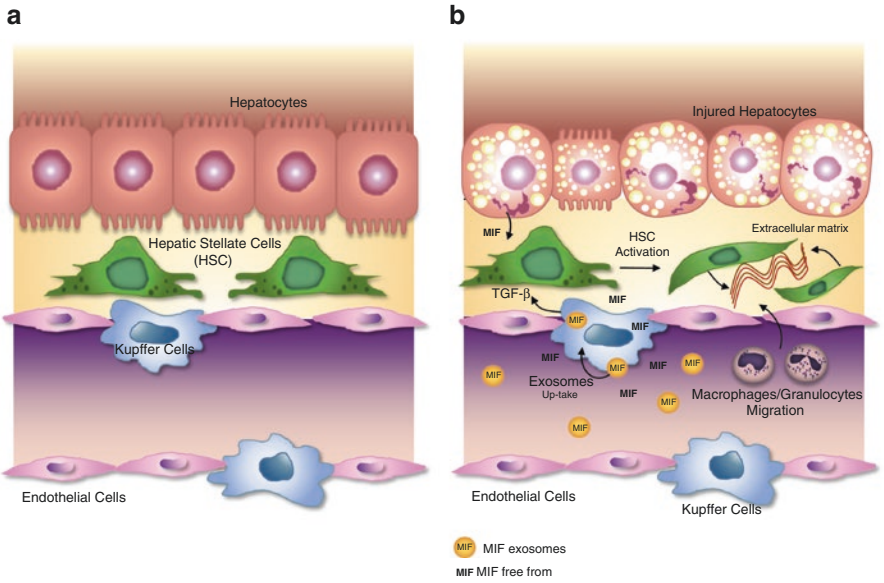


Fig. 2 Interactions of MIF with parenchymal and non-parenchymal cells in the liver. (**a**) Cellular architecture in the healthy liver: In normal conditions in the liver, there is a close interaction between hepatocytes and non-parenchymal cells, including Kupffer cells, the resident macrophage in the liver, quiescent hepatic stellate cells, and liver sinusoidal endothelial cells. (**b**) MIF influences inflammatory and fibrotic processes in the injured liver: In response to liver injury, e.g., in response to heavy, chronic alcohol consumption, obesity and metabolic syndrome, or endotoxemia, MIF concentrations in exosomes or in the plasma is increased in the hepatic sinusoid and can activate signaling in parenchymal and non-parenchymal cells. MIF can activate Kupffer cells (KCs) to promote both a pro-inflammatory milieu by increasing expression of pro-inflammatory cytokines and chemokines, as well as activate fibrotic responses via the release of TGF-β. This pro-fibrotic cytokine induces the activation of hepatic stellate cells (HSCs); activated HSCs acquire a myofibroblastic phenotype, leading to the synthesis and accumulation of extracellular matrix components. ECM deposits subsequently promote the arrest of bone marrow-derived macrophages and neutrophils in the liver, completing the formation of a pre-metastatic niche

The local induction of inflammation within the liver in autocrine/paracrine fashion is but one aspect in the development of liver disease. The recruitment of immune cells to the site of infection or injury is dependent upon an interconnected group of small molecular weight proteins, chemokines, and their receptors. Chemokine expression is upregulated in almost every form of tissue injury [40], and this signals for infiltration of immune cells to either resolve the injury or may lead to maladaptive responses to injury, such as fibrosis [39]. Chemokines can be secreted by many cell types in the liver, and this occurs in both time- and injury-dependent fashions. Chemokines are classified into four different families based upon cysteine residues near their N-terminal, so-called CC, CXC, CX3C, and C, and immune cell recruitment occurs across a concentration gradient created by the cellular source(s) of the chemokine.

CCL2 (MCP-1) is a well-studied chemokine that recruits monocytes and macrophages to the liver in response to acetaminophen intoxication, carbon tetrachloride-induced fibrosis, and chronic alcohol [45]. MCP-1 is produced by hepatocytes, hepatic stellate cells (HSCs), KCs, and LSECs. The CXC chemokines are produced in hepatocytes, HSCs, LSECs, and KCs in response to models of acetaminophen overdose, ischemia/reperfusion injury, and early alcoholic hepatitis [46] and predominantly mediate neutrophil migration to the liver. CX3CL1, the CX3C chemokine, is reported to be involved in monocyte/macrophage recruitment to the liver during hepatitis C infection and fibrosis [39]. What remains a subject of great speculation is the effects of chemokines on the hepatic parenchyma independent of immune cell chemotactic functions. CXCL8, for example, can bind both CXCR1 and CXCR2 on hepatocytes [39, 40]. Expression of both CXCR1 and CXCR2 on hepatocytes appears both time- and injury-dependent and may confer differing effects on hepatocyte survival and proliferation [40]. The roles chemokines play in liver disease are quite diverse and represent promising avenues for therapeutic discovery.

The understanding of chemokines in liver injury is therefore expanding from immune cell recruitment to include differentiation, survival, apoptosis, and fibrogenesis. Moreover, although chemokine expression in the liver can be studied in human samples, such as in CXCR2 ligands in alcoholic hepatitis ([47, 48], the mechanisms underlying the increased expression as well as downstream effects of these chemokines remain a subject under investigation. In addition, despite the well-established role of inflammation in liver disease, specific therapies to alleviate hepatic inflammation and liver disease remain elusive.

MIF, therefore, is an intriguing focus for research aimed at understanding the mechanisms for the progression of liver disease and the development of pathophysiology-based therapeutics for treating liver disease. Increased MIF expression and circulating MIF are associated with many liver diseases, suggesting that MIF plays a role in the progression of these diseases. As MIF is known to polarize macrophages and affect hepatocyte survival and immune cell recruitment, further research into MIF as an upstream regulator of hepatic inflammation and subsequent liver disease progression warrants further consideration.

2.2 Alcoholic Liver Disease

Alcohol use disorder affects nearly 20 million individuals in the United States and is a major cause of preventable morbidity and mortality worldwide [49]. Continuous, chronic alcohol abuse underlies the initiation and progression ALD attributable to 18,000 deaths in 2013 in the United States. ALD is a spectrum disorder encompassing steatosis, alcoholic hepatitis (AH), cirrhosis, and even progressing to liver cancer [49, 50]. The pathogenesis of ALD in humans remains poorly understood, and therapeutic options remain unchanged for decades [50]. Rates of alcohol-related liver morbidities have been constant for the past decade [49, 50]. The proximal, mild stage of ALD, steatosis, is largely reversible in people who abstain from alcohol consumption. AH is a more severe inflammatory condition that presents due to chronic alcohol abuse or acute alcohol binge episodes superimposed on a background of chronic alcohol abuse. Up to 70% of patients who present with AH progress to cirrhosis. Furthermore, patients with AH who abstain from alcohol may still go on to develop cirrhosis. Worldwide, cirrhosis and subsequent mortality or liver transplantation is primarily due to viral hepatitis and chronic alcohol abuse [51].

The cornerstone of managing patients with ALD is abstinence from further alcohol consumption [50]. If alcohol cessation is impossible in patients with ALD, pharmacological antioxidant and anti-inflammatory therapies are ineffective [49, 50]. Increased cytokines such as TNF are known to contribute to ethanol-mediated liver injury in animal studies, but antibody-mediated neutralization of TNF increased AH patient mortality due to severe bacterial infections. Corticosteroid treatment in patients with AH is a standard therapy for decades, but only half of those treated respond, and long-term survival of these patients is unaffected [49, 50]. Despite a lack of effective therapies to treat ALD, studies from human samples and in animal studies demonstrate a robust role for inflammation underscoring the progression ALD.

MIF is an intriguing focus for studying the underlying etiology of ALD, due to the roles that MIF plays in chronic inflammatory diseases such as rheumatoid arthritis [52], asthma [32], and atherosclerosis [53]. Further, understanding the role of MIF in response to current therapeutic options for the treatment of AH is of interest. A critical issue is to identify the molecular determinants in patients nonresponding to corticosteroids. Interestingly, corticosteroids increase MIF expression and release, and MIF can counter corticosteroids' anti-inflammatory function [54]. This complex interaction between MIF and steroid function has not been investigated in the context of AH, but should be the focus of future studies.

MIF has several biologic functions, including macrophage activation, pro-inflammatory cytokine synthesis, and immune cell chemotaxis, that are likely influences in the progression of ALD [49, 50] (Fig. 3). Indeed, a few reports demonstrate a connection between MIF, ALD, and the liver in humans [20, 55]. Kumagi et al. showed that plasma levels of MIF in patients with AH and alcoholic cirrhosis are increased.

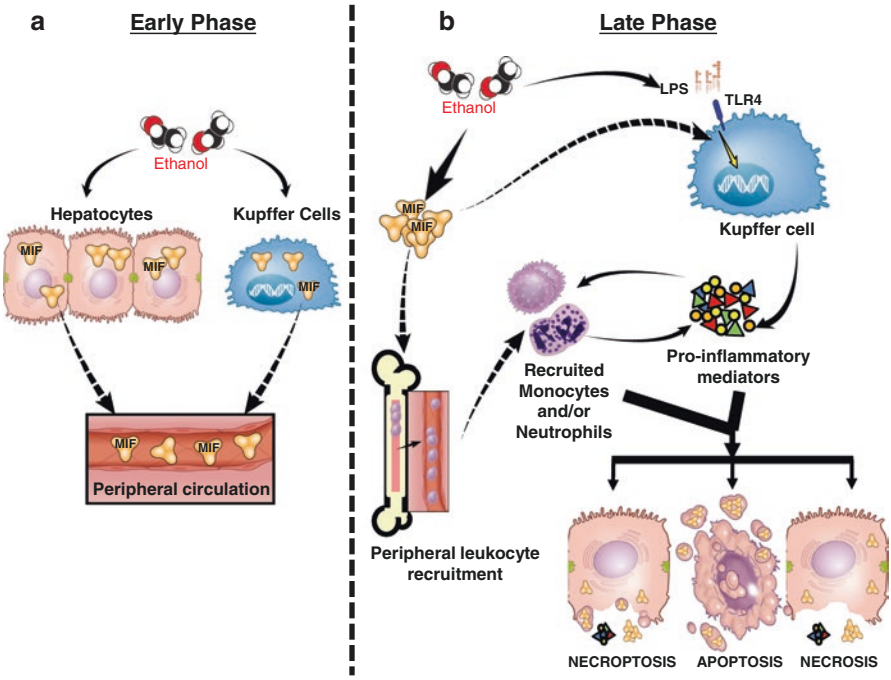


Fig. 3 Role of MIF in alcoholic liver disease (ALD). (**a**) Early phase of ethanol-induced inflammation in the liver. Ethanol consumption increases concentrations of MIF in the circulation, likely released by both hepatocytes and Kupffer cells within the liver. (**b**) Later phase of ethanol-induced inflammation in the liver. Increased circulating MIF increases leukocyte recruitment to the liver and synergizes with the ethanol-induced increase in plasma LPS to increase the expression of pro-inflammatory cytokines by Kupffer cells in the liver. The recruited leukocytes also secrete pro-inflammatory mediators (cytokines/chemokines), generating a pro-inflammatory milieu that contributes to hepatocyte injury via regulated apoptosis and necroptosis, as well as nonregulated necrosis

Moreover, plasma MIF levels in AH patients correlated to markers of liver damage such as γ-GTP and AST [20]. This study also showed that MIF expression was localized to infiltrating cells in the livers of AH patients as well as around stressed and/or dying hepatocytes [20]. Taken together, these studies suggest a role for MIF that contributes to immune cell migration and hepatocellular damage in ALD patients.

Studies in animals reinforce the role of MIF in the progression of ALD [19, 56]. Rats intragastrically infused with ethanol had increased MIF expression in the liver, and this was accompanied by increased expression of pro-inflammatory cytokines such as TNF and interferon-gamma, as well as increased liver damage [56]. The most extensive animal study to date demonstrated MIF as a likely contributor to the early stages of ALD [19]. MIF expression in the liver was increased in ethanol-fed mice, and TNF expression in the liver was decreased in MIF-deficient mice prior to the onset of ethanol-induced liver injury, suggesting that MIF could be a sensitive, upstream regulator of ethanol-induced inflammation and liver damage (Fig. 3).

Further analysis indicated that MIF-deficient mice were protected from liver injury following chronic ethanol feeding, associated with a protection from ethanol-induced inflammatory gene expression (TLR4/TNF) and monocyte infiltration into the liver. MIF-deficient mice were hyporesponsive to inflammatory stress following chronic ethanol feeding, as indicated by blunted expression of chemokines (e.g., MCP-1, CXCL10), adhesion markers (e.g., ICAM-1, E-Selectin), and inflammatory foci [19]. These studies suggest that MIF is a pivotal upstream regulator of ethanol-induced increases in inflammatory stress associated with ALD.

To date, studies of the role of MIF in ALD in animal models have only focused on the early stages of injury. Future studies, modeling more severe inflammatory responses and liver failure, are necessary to fully understand the contribution of MIF to ALD. In particular, future studies could uncover specific mechanisms by which MIF leads to and/or protects from hepatocellular injury and leukocyte infiltration, particularly relevant to severe AH in humans, a condition characterized by neutrophil infiltration [48–50]. In addition, insights into the possible role of genetic polymorphisms in the MIF promoter, including the number of CATT repeats and the −179 G/C single-nucleotide polymorphism, in the progression and/or severity of ALD could represent a significant advancement in determining individual susceptibility to ALD, similar to what has been discovered in patients with systemic lupus erythematosus [54, 57].

2.3 Nonalcoholic Liver Disease

Nonalcoholic fatty liver disease (NAFLD) is a complex spectrum of diseases ranging from benign steatosis (usually asymptomatic) to more severe alterations like nonalcoholic steatohepatitis (NASH), which can evolve toward cirrhosis and, in some cases, HCC [58]. The onset of NAFLD is tightly associated with obesity and type 2 diabetes mellitus (T2DM). The increasing prevalence of obesity and type 2 diabetes mellitus in children and adolescents [59, 60] suggests that the incidence of NAFLD will continue to rise. The mechanisms for the pathogenesis of NAFLD are still uncertain; however, inflammation has a key role in the progression of simple steatosis to the more advanced phases of NAFLD, as well as driving the development of comorbidities, such as cardiovascular complications, associated with obesity and T2DM [61].

While NAFLD is defined as the hepatic manifestation of metabolic syndrome, it is likely that additional organs are involved in parallel with the development of hepatic dysfunction [62]. In particular, adipose tissue seems to be the principal source of metabolic inflammation. Nutrient excess and weight gain, typical of obesity, determine an expansion of adipose tissue mass and adipocytes size [63]. The main consequences of this status are an increase in free fatty acid release and a parallel micro-hypoxia, due to a decrease in oxygen production. These alterations lead to adipocyte apoptosis and an increased infiltration of macrophages, contributing to increased inflammatory cytokine secretion by the adipose tissue [63]. Thus, adipose contributes to an increase in systemic adipokines and cytokines, including

adiponectin, MCP1 [64], TNF-α, IL-6, and IL-8 [65]. These mediators modify the hepatic inflammatory and immune system [66].

MIF is also likely to play a role in inflammation associated with obesity and NAFLD. A number of studies have investigated the relationship between circulating MIF and obesity. Obese patients have significantly higher MIF plasma levels compared with lean subjects, with a positive correlation between this cytokine and body mass index (BMI) [67]. This correlation between MIF and higher BMI or an increased waist circumference has also been reported in studies conducted in children and adolescents (5–17 years old) [68, 69].

A correlation between glucose intolerance and MIF has been observed in many studies; Japanese [70], Chinese [71], and Mexican [72] cohorts each demonstrate a positive relationship between the circulating amount of MIF and impaired glucose tolerance. Moreover, Herder et al. [73] observed an increased MIF serum levels in subjects with impaired glucose tolerance, which was further increased in subjects with T2DM.

The role of MIF in obesity and insulin resistance has also been investigated in animal models of diet-induced obesity. Saksida et al. [74] analyzed the release of this cytokine in a mouse model of high-fat diet-induced obesity; mice on this diet develop an obese phenotype and high levels of insulin, as well as circulating free fatty acids. MIF concentrations are increased in association with expansion of adipose tissue mass and adipocyte size, highlighting a role of this organ as a source of MIF. Interestingly, MIF is secreted together with insulin, and it acts as an autocrine factor to stimulate insulin release [75], and MIF-knockout mice develop obesity and glucose intolerance at 6 months of age [76]. MIF mRNA expression is also significantly higher in pancreatic islets of obese mice, and challenge of islets with palmitic acid induced the production of MIF and also led to apoptosis. MIF appeared to contribute to palmitic acid-induced apoptosis, as MIF-deficient mice were completely protected and resistant to palmitic acid-induced apoptosis [74].

In conclusion, available data indicate that MIF likely plays a role in obesity and diabetes, contributing to the progression of NAFLD. However, given the differential role of MIF at different stages of liver disease progression, with likely contributions to inflammation, hepatoprotection, and fibrosis, it will be important to carefully investigate the role of MIF at each stage of NAFLD, in order to understand potential protective versus detrimental function. Interestingly, there may also be an interaction of MIF and physical exercise, another factor in the development of obesity and T2DM. For example, MIF is increased in the hippocampus by long-term voluntary exercise [77], as well as in the liver of mice during physical exercise [78].

2.4 Hepatic Fibrosis

Liver fibrosis is the result of a persistent or deregulated wound healing, which is a complex process in which a number of cell types and factors interplay to tightly regulate hepatic response to injury. One of the first events to take place after liver

injury is the activation of resident inflammatory liver cells, mainly Kupffer cells, which secrete cytokines and chemokines that amplify the inflammatory response. These inflammatory mediators, together with damage-associated molecular patterns and factors secreted by parenchymal and non-parenchymal cells, promote the activation of HSC and the acquisition of a myofibroblastic phenotype. If injury persists, such as in excessive alcohol consumption, viral infection, or nonalcoholic fatty liver disease, accumulation of extracellular matrix (ECM) increases, leading to fibrogenesis and eventually cirrhosis, which is characterized by a severe alteration of the liver architecture and impaired liver function [79, 80].

HSCs are located in the space of Disse surrounding the liver sinusoids. In the healthy liver, HSCs participate in retinol metabolism, Vitamin A storage, and in the turnover of normal liver ECM. However, after liver injury, HSCs are activated, acquiring myofibroblastic characteristics including proliferative, migration, and contractile properties. Moreover, activated HSCs produce and respond to pro-fibrogenic and pro-inflammatory mediators and are responsible for the production of fibrillar ECM that constitute liver fibrosis. HSCs are the main cell type responsible for the deposition of ECM in the liver, and their activation is a key event in the development of liver fibrosis [79, 80].

Liver fibrosis is the result of an imbalance of the synthesis and degradation of ECM, with an increase in the expression of pro-fibrogenic and pro-inflammatory mediators and a reduction of extracellular degrading factors [79]. Moreover, liver fibrosis is characterized by the expression of a number of cytokines and chemokines which play a critical role in the pathogenesis of liver diseases and progression of fibrosis [81]. Inflammatory drivers secreted by resident and infiltrating inflammatory cells target HSC and promote their activation. At the same time, activated HSCs also display inflammatory functions, secreting immunologically active factors, including cytokines and chemokines, that participate in the progression of liver fibrosis [82]. In this context, expression of MIF is also upregulated in a number of hepatic fibrotic conditions. Assis et al. observed that serum and liver MIF expression was elevated in patients with autoimmune hepatitis and primary biliary cirrhosis, although in this study a correlation was not found between the level of MIF expression and the stage of fibrosis [83]. MIF expression has also been found to be increased in other diseases such as ALD [84] and NASH; in some instances, increased expression of MIF by mononuclear cells in the liver correlated with stage of fibrosis [18, 19, 85].

Hepatic and circulating MIF levels are rapidly increased after liver injury and fibrosis progression. Exposure of mice to carbon tetrachloride (CCl_4), a hepatotoxin that induces fibrosis, rapidly increases the expression of MIF in the liver, with subsequent increases in circulating MIF [84]. This coincident increase in local hepatic expression of MIF, along with increased circulating MIF concentrations in serum, has been described in multiple models of fibrotic liver injury and disease, suggesting that MIF may play a local, as well as systemic role, in generating inflammatory responses to the liver [19]. The hepatocellular source of MIF and the level of MIF expression are dependent on the type of injury and disease development. Both

hepatocytes and infiltrating inflammatory cells appear to be major MIF-producing cell types in the liver [19]. In a rat model of thioacetamide-induced fibrosis, Hori et al. showed that MIF was mainly expressed in degenerating hepatocytes adjacent to fibrotic areas, as well as in scar tissue, but not in non-parenchymal cells [86].

Although in most conditions MIF expression is associated with inflammation and enhanced pro-inflammatory response, its effect on fibrosis development is not completely understood. The effect of MIF on liver fibrosis is dependent not only on its effect on HSC but also on other non-parenchymal cells, inflammatory cells, and hepatocytes. Some reports have shown that MIF plays a protective role in inflammation-induced acute liver injury, such as concanavalin A-induced liver injury [87]. However, MIF expression does not appear to be hepatoprotective in chronic CCL_4-induced liver injury, the best characterized fibrosis in animal model [84].

Two recent studies assessed the mechanistic role of MIF in the development and resolution of liver fibrosis associated with inflammation. The first study addressing the direct effect of MIF on the progression of liver fibrosis clearly showed that, despite having a pro-inflammatory role in liver injury, MIF could also participate in the wound-healing response, similar to that described in other organs, such as the heart and the kidney. Heinrichs et al. showed that in two animal models of liver injury and fibrosis, CCl_4- and thioacetamide-induced liver fibrosis, deficiency of MIF or its receptor CD74 enhanced the development of liver fibrosis [88]. MIF-deficient mice treated with CCL_4 had an increased expression of pro-fibrogenic genes and ECM components, such as TIMP1, TGFβ, or collagen1A1, as well as increased α-SMA expression and collagen deposition. Moreover, they were able to rescue the phenotype by systemically adding recombinant MIF, which ameliorated fibrogenesis in vivo. These results highlight the potential effect of increased serum levels of MIF in liver diseases. Surprisingly, in this report the number of infiltrated cells from main inflammatory cell populations was not impacted by MIF, except for a higher number of NKT cells in CD74-deficient mice. Importantly, this study shows that CD74 is the major MIF receptor involved in the anti-fibrogenic effect of MIF in liver fibrosis. In contrast to the role for CD74, fibrosis progressed similarly in CXCR4 +/− and wild-type mice. Importantly, MIF directly targeted activated HSC through CD74 receptor, decreasing their activation and pro-fibrogenic response (see cell-specific effects section below).

While Heinrichs et al. reported a direct anti-fibrogenic effects of MIF/CD74 on HSC, Barnes et al. showed that the anti-fibrogenic effect of MIF is also mediated by infiltrating monocytes and scar-associated macrophages. In this regard, the results obtained by Barnes et al. add complexity to our understanding of the role of MIF in liver fibrogenesis and propose that MIF may have multiple roles during fibrogenesis, first to inhibit HSC activation, but also to enhance ECM degradation and fibrosis resolution.

Recruitment of scar-associated macrophages plays a critical role in progression and resolution of hepatic fibrosis [89]. Barnes and collaborators showed that treatment of mice with acute or chronic CCL_4, a well-known model of liver fibrosis,

promoted the hepatic expression of MIF and increased its circulating levels. Interestingly, induction of fibrosis in MIF-deficient mice had different effects on male and female mice. While HSC activation and proliferation were enhanced in male MIF-deficient mice, both parameters were reduced in female MIF-deficient animals treated with chronic CCL_4, suggesting that the impact of MIF on fibrosis may be affected by sexual dimorphism [84, 88]

Interestingly, although HSC activation was reduced in female MIF-deficient mice, no differences were observed in the amount of ECM deposition between MIF-deficient and wild-type animals. In order to understand the apparent discrepancies between enhanced HSC numbers and unchanged collagen synthesis, Barnes et al. explored the resolution of fibrosis in MIF-deficient animals. MIF was found to be required for the recruitment of restorative macrophages, as defined by the expression of $CD11b^{+}Ly6G^{-}$, $Ly6C^{lo}$ markers. Consequently, MIF-deficient mice showed reduced recruitment of restorative macrophages, associated with a reduced expression and activity of MMP13, a major MMP involved in matrix degradation [84]. Together with the study by Heinrichs et al. [88], these results suggest that MIF can both directly target HSC and repress HSC activation as well as promote the resolution of fibrosis by promoting ECM degradation.

2.5 MIF and Hepatocellular Carcinoma

There is mounting evidence that MIF plays a role in several types of cancer including in the breast, colon, pancreas, and lungs. Human and experimental data indicate that MIF also participates in primary liver cancer. MIF is overexpressed in HCC, correlates with cyclin D1 expression, and mediates proliferation of HCC cell lines [90]. Moreover, overexpression of MIF is significantly associated with HCC size, intrahepatic metastasis, vascular invasion, and TNM stage [91]. MIF knockdown inhibits the expression of growth-related proteins and induces the expression of apoptosis-related proteins. Finally, genetic variations of MIF (i.e., MIF-794CATT microsatellite repeats) predict HCC surgical prognosis [92]. Overall, these studies support a role for MIF as a novel therapeutic target for HCC. Besides its pathogenic role, MIF serum levels are markedly elevated in HCC patients, and its levels decreased after tumor ablation, suggesting that plasma MIF levels have potential as a diagnostic and prognostic factor for HCC [93].

2.6 MIF and Hepatitis B Infection

Little is known on the role of MIF in chronic hepatitis B infection, which is particularly common in Asia. MIF serum levels are increased in chronic hepatitis B [17], and a genetic epidemiology study revealed that MIF-173 C/C polymorphism is associated with increased risk of chronic hepatitis B in China [16].

3 Cell-Specific Effects of MIF in the Liver

The liver is a complex organ with multiple cell types interacting within a highly structured architecture. Here we will review the role of MIF and its receptors within both parenchymal and non-parenchymal cells residing in the liver.

3.1 Hepatocytes

In the liver, MIF is produced mainly by hepatocytes and Kupffer cells [94], and, upon LPS stimulation, it is rapidly released from the preformed hepatocellular pools. However, it is unclear if MIF released in the liver has autocrine and/or paracrine effects. Studies to date have been primarily in hepatoma cells, which may not reflect the activity of healthy hepatocytes. In tissue from subjects with HCC, MIF protein appeared to be located in the cytoplasm of the tumor cells, whereas adjacent noncancerous tissues exhibited negative or weak MIF staining. MIF expression was directly associated with the overexpression of cyclin D1, and silencing of MIF in hepatocytes induced a significant downregulation of cyclin D1 [90]. Knockdown of MIF with siRNA in a human HCC xenograft model significantly reduced tumor size compared with the control siRNA-treated group [90], consistent for a role of MIF in proliferation and/or apoptosis of HCC cells. MIF knockdown inhibits the expression of growth-related proteins and induces the expression of apoptosis-related proteins.

Hepatocytes are also known to be responsive to MIF, likely dependent on expression of CD74. MIF, along with a variety of other cytokines, is secreted in extracellular vesicles (EVs) by inflamed adipose tissue [95]; these EVs act as potent inducers of systemic insulin resistance (IR). In a recent study, EVs from omental adipose tissue directly desensitized insulin signaling in the liver [95]. The degree of Akt phosphorylation in hepatocytes was associated with IL-6 and MIF levels in the omental adipose tissue EVs, suggesting a role for MIF in the regulation of insulin signaling in hepatocytes. MIF can also regulate the synthesis of acute-phase proteins by hepatocytes; MIF dose dependently increased the synthesis of C-reactive protein (CRP) by primary human hepatocytes [96].

In summary, while it is clear that hepatocytes produce, store, and release MIF, the molecular mechanisms involved in the regulation of this process are not understood. Further, it is also clear that hepatocytes respond to MIF, but again the precise receptor interactions and signaling mechanisms involved in the response have not been well studied.

3.2 Resident Hepatic Macrophages and Recruited Immune Cells

MIF is produced by a variety of cells, such as macrophages [23], recognized as peripheral source of this cytokine in 1994 by Calandra et al. [4]. The role that MIF plays in macrophages has been studied by multiple investigators.

Roger et al. [97, 98] hypothesized that MIF could regulate innate immune responses through modulation of Toll-like receptor 4 (TLR4). They observed that MIF-deficient macrophages were hyporesponsive to lipopolysaccharide (LPS) and gram-negative bacteria, associated with a reduced expression of TLR4. The LPS stimulation of TLR4 induces the release of critical pro-inflammatory cytokines that are necessary to activate potent immune responses [99]. Thus, MIF contributes to the identification of endotoxin-containing bacteria by inducing macrophages to activate host antimicrobial defense systems, a crucial step in immune surveillance against bacteria. These observations lead to the hypothesis that MIF is constitutively expressed in cells that are in close contact with external environment, such as macrophages [23].

Kupffer cells, the resident hepatic macrophages, are important for triggering the inflammatory response in the liver. Liu et al. [100] reported that during liver ischemia/reperfusion injury, MIF serum levels and hepatic expression were rapidly and markedly increased. MIF signaling is known to activate CD74, which is expressed by immune cells, including monocytes, B cells, activated T cells, and dendritic cells, as well as KC and HSC [101]. CD74 is a high-affinity binding protein for MIF, and it is necessary for MIF-induced pro-inflammatory effects [102]. MIF, released by ischemic hepatocytes, could activate KC and peripheral circulating macrophages, acting as an early indicator of the inflammatory response. Another recent study by Costa-Silva et al. [103] demonstrated that MIF can be also released by other organs (such as the pancreas or adipose tissue) in form of exosomes. Within the liver, exosomal MIF is taken up by KC and contributes to activation of fibrotic and pro-inflammatory pathways. Exosomally derived MIF induces the release of tumor growth factor-β (TGF-β) by KC, which in turn promotes fibronectin production by HSC. Fibronectin deposits subsequently promote the arrest of bone marrow-derived macrophages and neutrophils in the liver, facilitating the formation of a pre-metastatic niche (Fig. 1).

The interplay between MIF and macrophages is perhaps best understood in the context of atherosclerosis; studies from atherosclerosis suggest that macrophage recruitment to the plaque was proportional to circulating MIF concentrations [104]. Similarly, in the liver, MIF is required to maintain KC numbers during the progression of chronic liver injury. For example, Barnes et al. [19] reported that chronic ethanol feeding induced apoptosis in resident KC; MIF-dependent recruitment of $Ly6C^+$ monocytes was required to restore and sustain macrophage numbers in the liver after ethanol feeding. Bernhagen et al. [105] demonstrated that MIF acts as noncanonical ligand for chemokine receptors CXCR2 and CXCR4. By activating both CXCR2 and CXCR4, MIF displays chemokine-like functions and acts as a major regulator of inflammatory cell recruitment. While it is clear that KCs express CD74, much less is known about the interactions between MIF and its noncanonical receptors in maintaining macrophage function in the liver. Continued studies will likely reveal the complex regulation of MIF and its receptors within the context of innate immune function in the liver; this will likely be particularly relevant to metabolic liver diseases that result in a compromised intestinal barrier, allowing activation of resident Kupffer cells to bacterial products, such as endotoxin.

3.3 Hepatic Stellate Cells

In the context of liver injury, activated HSCs respond to a number of inflammatory mediators. Although many cytokines and chemokines promote HSC activation, others such as IFN-γ, IL-22, IL-10, and CXCL9 actually reduce or modulate fibrogenic responses [82]. Inflammatory and pro-fibrogenic mediators rarely target HSC as single mediators. On the contrary, HSC response to inflammatory and pro-fibrogenic substances should be seen as a balance between mediators promoting activation and those preventing activation to maintain a quiescent phenotype. There are few reports addressing the direct effect of MIF on HSC. As described in the previous section on fibrosis, MIF is upregulated in a number of fibrogenic conditions, including liver diseases, which plays an important role in the wound-healing response of the liver.

The expression of MIF and its receptors has been studied only in activated HSC, and no information is available in the quiescent state. In the activated phenotype, CD74 is the most highly expressed receptor, while CXCR4 is expressed at a lower level and CXCR2 is absent [88]. In HSC, CD74 expression is upregulated in response to lymphocyte T cytokines such as interferons [101]. Moreover, Assis et al. described that in response to IFN-γ, CD74 is cleaved and released into the media of immortalized human HSC; truncated CD74 can then bind to MIF and neutralize its signal transduction activity [83]. These results support the notion that HSC may be the source of truncated CD74 which is found to be elevated in the circulation in autoimmune and biliary diseases [83]. Interestingly, although not the major source of MIF in the liver, activated HSCs also express MIF. Copple et al. reported the expression of MIF in isolated primary mouse HSC under hypoxic conditions; MIF was expressed together with several mediators of angiogenesis, including VEGF, PDGF, and AngL4. Interestingly, they found that hypoxia induced MIF in both quiescent and activated HSC, and this induction was prevented in HSCs isolated from HIF-1α-deficient mice [106]. Together these studies suggest that MIF could have a paracrine effects on HSC, modulating HSC activation and pro-fibrogenic response.

MIF exerts a direct inhibitory effect on HSC activation and the pro-fibrogenic phenotype, which is mainly mediated by the CD74 receptor [88]. In vitro, recombinant MIF promoted AMPK phosphorylation in HSC, which inhibited PDGF-induced HSC activation. MIF stimulation did not induce migration or proliferation in cultured HSC, but had an inhibitory effect on PDGF-induced proliferation and migration. Moreover, CD74 inhibitory antibody blocked the effects of MIF on PDGF-stimulated HSC, indicating that CD74 is the major receptor driving the effects of MIF on HSC.

MIF can also modulate HSC response to pro-fibrogenic mediators. Patel et al. explored the pro-fibrogenic effect of human immunodeficiency virus (HIV) and particularly HIV-1 Vpu accessory protein on the LX2 HSC cell line. Interestingly, while coculture of monocyte cells expressing Vpu increased the expression of pro-fibrogenic markers in a HSC cell line, LX2, the addition of MIF in the supernatant reduced the expression of TGF-β in monocytes and attenuated the pro-fibrogenic effects on LX2 cells and the expression of COL-1, MMP2, and TGF-β [107].

In summary, HSC can express MIF under certain conditions, and in turn, HSC can respond to both autocrine- and paracrine-derived MIF via its cognate and non-cognate receptors. These interactions will clearly influence the progression of fibrosis in metabolic liver diseases. The dichotomous roles of MIF on hepatocytes, innate immune cells, and HSC contribute to the complex regulation of liver homeostasis both in health and disease progression.

4 MIF as a Target to Treat Liver Diseases

MIF is regarded as a major regulator of inflammation and a mediator that counter-regulates the inhibitory effects of glucocorticoids within the immune system. Therefore, MIF is currently considered a therapeutic target for inflammatory and autoimmune diseases [54]. In addition, MIF is implicated in cancer pathogenesis. Current therapeutic strategies for targeting MIF focus on inhibiting its signaling activity by small molecules or modulating its biological activities using anti-MIF-neutralizing antibodies [108].

Although the role of MIF in liver diseases is not fully understood, recent evidence supports a potential role as therapeutic target. Regardless of the cause, most types of chronic liver disease are characterized by hepatocellular damage, inflammatory cell infiltrate in the hepatic parenchyma, and tissue remodeling, ultimately resulting in progressive fibrosis and cirrhosis. Infiltrating inflammatory cells at the sites of liver injury are directed to remove apoptotic cells, but in addition they secrete a number of chemokines that can induce cell death and stimulate HSCs to secrete collagen. Therefore, chemokines including MIF are considered key drivers of liver damage and fibrogenesis and are currently consider potential targets for therapy [109]. However, while some studies clearly show that MIF mediates inflammation and fibrosis in animal models of chronic liver injury [19], other studies support a hepatoprotective role of MIF in obesity-induced liver injury [61] and fibrosis [84, 88]. Therefore, targeting MIF may have opposite effects depending on the type and degree of liver injury.

Based on its biological properties, it has been suggested that MIF blocking agents could be useful to treat autoimmune diseases. There is some evidence that MIF may also participate in the pathogenesis of AIH, since genetic variation of MIF and its serum levels are associated with this condition [83]. Preclinical studies in animal models are required before this strategy can be recommended to patients with severe AIH. This strategy could be particularly useful in patients with corticoid-dependent AIH, in order to avoid side effects. It is unknown, however, if continuous blockade of MIF could predispose patients to infections, a well-known side effect of corticosteroids. A recent study showed that treatment of animals with experimental sepsis with a small-molecule inhibitor of MIF improves survival by reducing inflammation and improving bacterial control [110]. This study suggests that in the setting of sepsis, MIF could mediate the systemic inflammatory response. Whether chronic MIF blockade predisposes to infection and/or cancer should be explored in preclinical and clinical studies.

The role of MIF in steatohepatitis is controversial. Steatohepatitis can arise from both alcohol abuse and metabolic syndrome (NAFLD). In experimental alcoholic steatohepatitis (ASH), MIF is an important mediator in the regulation of chemokine production and immune cell infiltration in the liver and promotes ethanol-induced steatosis and hepatocyte damage [19]. These results clearly suggest that targeting MIF could ameliorate ALD. The role of MIF as a target in NAFLD is controversial and needs further clarification. Obesity is associated with a chronic low-grade inflammatory state that drives the development of obesity-related comorbidities such as insulin resistance/type 2 diabetes, NAFLD, and cardiovascular disease [111]. This metabolic inflammation is thought to originate in the adipose tissue, which becomes inflamed and insulin resistant when it is no longer able to expand in response to excess caloric and nutrient intake. The production of inflammatory mediators by dysfunctional adipose tissue is thought to drive the development of more complex forms of disease such as type 2 diabetes and NAFLD. MIF is likely an important factor contributing to metabolic inflammation [112]. Increasing evidence suggests that MIF is released by adipose tissue in obesity and that it is also involved in metabolic and inflammatory processes that underlie the development of obesity-related pathologies [61]. Although many of the findings support a pro-inflammatory role of MIF in NAFLD development, recent reports also provide indications that MIF may exert protective effects under certain conditions.

MIF expression is significantly increased especially by mononuclear cells in liver tissue of patients with NASH secondary to inflammation [18]. MIF deficiency partially protects from high-fat diet-induced insulin resistance by attenuating macrophage infiltration, ameliorating adipose inflammation, which improved adipocyte insulin resistance ex vivo. Therefore, MIF represents a potential therapeutic target for treatment of high-fat diet-induced insulin resistance [113]. In contrast to these studies, recent data suggest that MIF exerts a hepatoprotective role through the CD74/AMPK pathway in metabolic models of liver injury [85]. These data indicate that depending on the stage of the disease, MIF can have dual effects in promoting or protecting against liver injury. Further studies should clarify this intriguing issue.

In addition to its potential role in chronic inflammation, MIF has also been proposed as an appealing target to treat several types of cancer such as colorectal, lung, or breast [114]. As described above, MIF has been potentially implicated in the pathogenesis of HCC, a frequent and lethal cancer [90]. Preclinical studies in animal models of HCC are warranted before this strategy can be tested in clinical trials. Also, the potential bioavailability of MIF blocking small molecules in the cirrhotic liver, as well as its safety, should be carefully evaluated.

Finally, targeting MIF receptors, such as CD74, represents an alternative strategy to treat inflammatory processes and cancer [115]. It is well known that cancers expressing CD74 such as non-Hodgkin lymphoma could respond to antibodies targeting this receptor (i.e., milatuzumab) [116]. There are few reports assessing milatuzumab in liver diseases. A recent study showed that milatuzumab prevents hepatic graft-versus-host disease [117]. This study supports a potential role for MIF in ischemia-reperfusion injury in the liver [100]. In contrast, other studies suggest

that CD74 mediates the protective (anti-fibrotic) effects of MIF in experimental fibrosis [88]. These discrepant results reinforce the notion that the MIF-CD74 pathways could exert both beneficial and detrimental effects in chronic liver diseases. This dual biological effect of MIF-CD74 pathway should be taken into account when considering MIF as therapeutic target for liver diseases.

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MIF and Pulmonary Disease

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Abstract As the organ responsible for gas exchange, the lung represents the largest interface between the external and internal environments. Most of the lung's surface area is a delicate lattice of epithelial-endothelial interfaces that permit the efficient exchange of oxygen and carbon dioxide. To maintain its integrity, the lung requires a complex network of defenses against external toxins and pathogens. Macrophage migration inhibitory factor (MIF) is a multifunctional cytokine that serves as a critical regulator of the innate immune response and mediates protection from oxidative stress in the lung. Both pathologic and protective roles for MIF in lung disease have been described. This chapter will focus on the role of MIF in the pathogenesis of pulmonary disease.

1 MIF, Pneumonia, and Acute Respiratory Distress Syndrome

MIF is secreted into the alveolar space as part of the antimicrobial response to infection. MIF is a critical mediator of host defense and inflammation; however, MIF can be maladaptive when infections lead to excessive inflammation and overwhelming lung injury.

Numerous murine models and clinical studies have demonstrated a protective role for MIF in the context of pneumonia. *Mif*-knockout mice show decreased clearance of *Streptococcus pneumoniae* colonization, increased vulnerability to *Klebsiella*

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pneumoniae, impaired killing of gram-negative bacteria by macrophages, and an impaired ability to clear secondary bacterial infections [1–3]. Additionally, MIF is responsible for the transcription of the pattern recognition receptor, dectin-1, which mediates the clearance of *Mycobacterium tuberculosis* [4]. Human *MIF* alleles associated with decreased MIF expression have been associated with increased susceptibility to community-acquired pneumonia [5]. Similarly, there was significant enrichment of the low-expressing *MIF* allele among older individuals with gram-negative sepsis compared with healthy controls [6]. In these conditions, MIF is important for the clearance of infectious agents associated with pneumonia.

However, under other conditions or in the setting of infection by specific organisms, MIF has been demonstrated to be deleterious. *Mif*-knockout and MIF-inhibited mice show lower levels of inflammation and improved survival against lethal doses of LPS and gram-positive enterotoxins [7, 8]. Similarly, MIF elevation is associated with pathogenicity of *Pseudomonas* pneumonia, and patients infected with *Burkholderia pseudomallei* show increased MIF expression [9]. Furthermore, neutralization of MIF in animal models improves bacterial clearance of *Burkholderia pseudomallei* [10]. In general, neutralization of MIF or *Mif*-knockout has been shown to improve outcomes in murine models of sepsis [11, 12].

Acute respiratory distress syndrome (ARDS) is a life-threatening condition characterized by widespread inflammation of the lungs. ARDS commonly occurs as a consequence of pneumonia or non-pulmonary infections that are complicated by systemic involvement. ARDS has an associated mortality of 25–30%, and currently, the only treatment for this disease is mechanical ventilation and supportive care [13].

MIF is elevated in the plasma, immune cells, and endothelial cells of patients with ARDS, and circulating MIF levels correlate with clinical severity [14–16]. A role for MIF and its receptor CD74 in acute lung injury (ALI) has been suggested by numerous studies that correlate decreased MIF activity with attenuated neutrophil migration and thus increased protection from damage-induced lung inflammation. In a study that used ex vivo human macrophages from ARDS-affected patients, MIF was demonstrated to mediate injurious inflammation and override glucocorticoid anti-inflammatory activity. In this same study, neutralizing MIF attenuates pro-inflammatory cytokine production, illustrating the potential for therapeutic use of anti-MIF therapy in ARDS [17].

In animal models, attenuating MIF activity results in a decreased pulmonary inflammatory response and less severe organ injury [7, 8, 11, 12]. The use of anti-MIF and anti-CD74 antibodies in such studies decreased neutrophil migration in lipopolysaccharide (LPS)-induced ALI [18–20]. Similar findings have been reported in ventilator-induced ALI models and ARDS induced by gram-positive exotoxins [20–22]. As an alternative to anti-MIF antibodies, heme oxygenase-1 expression by administration of cobalt protoporphyrins has been shown to negatively regulate lung MIF and TLR4-induced inflammation in response to LPS [23].

Conversely, MIF has been demonstrated to have a protective effect in certain sterile injury models. Hyperoxia (exposure to 100% oxygen) is a commonly used ALI model in which *Mif*-knockout and *Cd74*-knockout mice demonstrate increased sensitivity to hyperoxia-induced lung injury and decreased median survival relative to WT mice [24]. In neonatal mice, exposure to hyperoxia causes

bronchopulmonary dysplasia (BPD), and *Mif*-knockout^{-} and *Cd74*-knockout pups are similarly susceptible to hyperoxia-induced BPD [25, 26]. BPD is a respiratory disorder that occurs in premature neonates in which prolonged delivery of supplemental oxygen causes alveolar septal injury. Genetic studies have associated low-expression *MIF* alleles with increased susceptibility to BPD. Finally, older $Mif^{-/-}$ mice demonstrate increased susceptibility to radiation-induced lung injury, an effect attributed to the lack of MIF-mediated NRF-2 activation? MIF upregulation of nuclear factor erythroid 2-related factor 2 (NRF-2) in murine endothelial cells [27].

2 MIF and Pulmonary Arterial Hypertension

Pulmonary arterial hypertension (PAH) is the narrowing and thickening of blood vessels, involving proliferation of lung vascular endothelial and smooth muscle cells, that ultimately leads to hypoxemia and right ventricular failure. Circulating MIF is elevated in patients with idiopathic and scleroderma-associated PAH [28, 29]. In rodent models of PAH, MIF was shown to promote the proliferation of pulmonary arterial smooth muscle cells and activate anti-apoptotic and pro-inflammatory signaling in pulmonary endothelial cells in a CD74-dependent manner. Inhibition of MIF-CD74 interaction using ISO-1, in multiple rodent models, resulted in decreased pulmonary vascular remodeling, cardiac hypertrophy, and right ventricular systolic pressure [6, 28, 30]. These results indicate a potential therapeutic effect of MIF inhibition for patients suffering from PAH.

3 MIF and Chronic Pulmonary Inflammatory Disease

3.1 Chronic Obstructive Pulmonary Disease

COPD is the third leading cause of death in the United States. Emphysema, a hallmark pathologic finding in COPD, is characterized by an imbalance of lung tissue injury and repair. Emphysema is associated with an increase in cellular senescence, oxidative stress, and DNA damage [31–34].

Several studies evaluating circulating MIF in relation to COPD disease severity have revealed similar trends in MIF concentration and disease pathogenesis. The cumulative data from three studies suggest that MIF is significantly increased in "healthy" smokers or smokers with mild disease. However, in severe disease, circulating MIF concentrations are diminished [35, 36]. These findings have been recapitulated in experimental animal models where mice exposed to cigarette smoke for 3 months exhibited increased MIF concentration in bronchoalveolar lavage (BAL), but at 6 months of exposure—a time course consistent with COPD development in mice—BAL and circulating MIF concentration were decreased [35–38]. Both *Mif*-knockout and *Cd74*-knockout mice spontaneously develop airspace enlargement, and *Mif*-knockout mice are prone to cigarette-induced DNA

damage, cellular senescence, apoptosis, and emphysema [35, 36, 39]. The role for diminished MIF in the pathogenesis of emphysema is unclear, but several factors have been shown to contribute to the severe disease phenotype. First, MIF may promote the expression of a critical lung antioxidant, NRF-2, such that, low MIF levels could increase susceptibility to cellular oxidative damage [40]. Additionally, MIF is a repressor of the p16-RB and p53–21 cellular senescence pathways [36]. Increased cellular senescence is implicated in the secretion of pro-inflammatory cytokines and proteases involved in the pathogenesis severe COPD. Finally, *Mif*-knockout mice show reduced vascular endothelial growth factor (VEGF) VEGF signaling in response to oxidative stress, which results in reduced vasculogenesis, a finding implicated in the pathogenesis of COPD [41–43]. Ultimately, these findings suggest a central role for MIF in mitigating the consequences of oxidative damage in the injured lung and suggest a possible avenue for therapeutic intervention with MIF in patients with severe COPD.

3.2 Asthma

Asthma is a common type of chronic airway inflammation characterized by variable, reversible airway obstruction and bronchospasm. Symptoms include wheezing, coughing, chest tightness, and dyspnea resulting from the contraction of tracheobronchial smooth muscle, hypersecretion of mucus, and mucosal edema [43].

Unlike COPD, expression of MIF is inversely correlated to clinical outcomes in asthma, as illustrated by a study in which MIF concentration was increased in the BAL of asthma patients relative to controls [44, 45]. MIF is stored in circulating eosinophils and contributes to the release of cytokines in response to physiologic asthma stimuli, such as interleukin-5 [46]. Additionally, staining of sputum cells revealed that MIF was co-localized with eosinophil peroxidase in the cytoplasm [47]. Functional MIF alleles that contribute to higher basal and stimulated MIF promoter activity are associated with more severe disease phenotypes [48, 49]. Notably, severe asthma is associated with corticosteroid resistance, and MIF has been shown to override the anti-inflammatory effects of corticosteroids, suggesting a potential therapeutic role for MIF antagonism in this disease [50]. Notably, there are both distinct and overlapping features of asthma and COPD, and the study of MIF in these disease reveals an interesting paradigm where increased MIF results in the deleterious inflammatory consequences seen in asthma and airway predominant COPD, whereas decreased MIF causes cellular senescence, apoptosis, and vascular attrition commonly observed in emphysema.

4 Cystic Fibrosis

Cystic fibrosis (CF) is a common and fatal genetic disorder caused by mutations in the cystic fibrosis transmembrane regulator (CFTR) gene. This disease is characterized by chronic buildup of thick mucus in the airways of the lung, followed by infections with *Pseudomonas* sp. and *Burkholderia* sp. gram-negative bacteria.

As discussed previously, *Mif*-knockout mice were able to clear, but not kill, gram-negative bacteria more effectively than in WT mice. Additionally, MIF activity results in the delayed apoptosis of neutrophils, thus promoting the survival of activated leukocytes that contribute to the inflammatory response [51]. Furthermore, there is a significant correlation between the *Mif* promoter polymorphism and clinical severity of cystic fibrosis. Those individuals with the low-expressing *MIF* allele showed decreased *Pseudomonas* sp. colonization, while those with the higher MIF producing alleles showed increased lung injury [52]. The tautomerase enzymatic activity of MIF is believed to be critical to the inflammatory response in the lung [53]. The pathologic finding of excessive inflammation and the positive clinical outcomes associated with reduced MIF expression suggest that targeting MIF may yield beneficial outcomes when treating the infectious consequences of CF.

4.1 Lung Fibrosis

Lung fibrosis is a respiratory disease characterized by lung tissue scarring. The causes of fibrotic lung disease are commonly genetic, idiopathic, secondary to autoimmune disease, or secondary to drug reactions. MIF is increased in the BAL of patients with idiopathic pulmonary fibrosis (IPF), and immunohistochemical analysis of lung tissue from patients with IPF demonstrated increased MIF in the epithelium and fibroblastic foci [54, 55].

In a mouse model of IPF, administration of the fibrogenic agent bleomycin results in increased *Mif* expression. Although an anti-MIF antibody was able to mitigate the acute effects of bleomycin-induced lung injury, there was no difference in hydroxyproline content or histopathological lung fibrosis scoring [56]. In a radiation-induced lung injury model, aged *Mif*-knockout mice are more susceptible than age-matched control mice. This finding was associated with decreased antioxidant production [57]. In murine models for hepatic fibrosis and chronic liver injury, the *Mif*-knockout mice showed decreased PDGF activation and increased protection from injury [58]. Currently, the role of MIF in lung fibrosis remains uncertain.

4.2 MIF and Lung Cancer

Lung cancer is the most common fatal malignancies in the developed world, accounting for over one million deaths annually. Chronic inflammatory diseases are associated with enhanced risk of cancer, and MIF may be a link between lung inflammation and cancer development.

Histologic studies of lung cancer have suggested a pathogenic role for MIF. In normal lung tissue, MIF mRNA and protein are observed in the bronchial and alveolar epithelium, endothelium, vascular smooth muscle, and alveolar macrophages. Conversely, in tissue derived from primary lung adenocarcinoma, MIF is more heavily concentrated in the alveolar epithelium relative to normal tissue concentrations [59]. Likewise, the presence of MIF in the nuclei of non-small cell lung

cancer (NSCLC) is correlated with a worse prognosis compared to malignancies without MIF. It was subsequently demonstrated that NSCLC that produce high levels of MIF mRNA were derived from patients who were heavy smokers [60]. Furthermore, MIF and CD74 are so prevalent in malignant pulmonary carcinoma that increased immunohistochemical staining of MIF and CD74 could potentially be a biomarker of the disease [61, 62].

There are multiple mechanisms by which MIF's biological function can lead to pulmonary malignancies. MIF expression induces AKT and ERK 1/2 activation, contributing to tumor growth, survival, and invasion. MIF also upregulates VEGF, resulting in increased angiogenesis. Implicated in this proangiogenic process is a CXC chemokine induced by peripheral blood monocytes [63]. MIF can act together with its homolog, D-dopachrome tautomerase, to promote CXC8 and VEGF activity in NSCLC [64]. Finally, MIF negatively regulates the cell senescence and tumor suppressor gene p53 and the Rb-E2F signaling pathway, resulting in increased cell proliferation and reduced growth limitation [36, 65–69]. MIF regulates cyclin-dependent kinases and E2F transcription during cell cycle and growth and may play a role in regulating the DNA damage response [70]. Interesting preliminary data shows that *Mif*-knockout mice exhibit increased levels of DNA damage relative to controls [35, 71].

5 Conclusion

There is a growing body of evidence that highlights the critical role of MIF in various respiratory disorders. MIF acts as a stress-mediated cytokine, activating cellular pathways to mitigate harm during certain infections or under conditions of oxidative stress. High levels of MIF may perpetuate pulmonary conditions in which chronic inflammation becomes detrimental. It may be that MIF is implicated in so many pulmonary diseases because it functions as a rheostat for critical biologic processes in the lung. Therefore, timing, context, and degree determine if MIF serves a beneficial or pathologic role. Therapeutic intervention upon the MIF signaling pathway will require a better understanding of the cell-specific consequences of MIF as well as the various downstream signaling pathways regulated by MIF. However, once elucidated MIF-based strategies offer immense diagnostic and therapeutic potential.

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MIF Mediates Pelvic Inflammation and Pain

Fei Ma, Katherine L. Meyer-Siegler, Karin N. Westlund, and Pedro L. Vera

Abstract Macrophage migration inhibitory factor is stored in releasable pools by urothelial cells. Inflammatory stimuli in the bladder elicit a quick release of MIF from the urothelium and upregulation of MIF receptors in the urothelium. Binding of released MIF to urothelial MIF receptors starts a signaling cascade that promotes bladder inflammation and pain. In addition, recent evidence shows that released MIF is capable of eliciting bladder pain without accompanying bladder inflammation.

1 Introduction

Inflammation is an innate protective response that aims to rid a particular site or organ of pathogens, remove injured tissue, and initiate the repair process. The five cardinal signs of inflammation are heat, redness, edema, pain, and loss of function. Inflammation is an orchestrated series of events that involve host cells, secretion of proteins/cytokines, and activation of afferent nerve endings. Acute inflammatory processes are protective, beneficial, and self-limiting. Chronic inflammation, particularly in the absence of offending stimuli, is maladaptive, may result in tissue

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injury and dysfunction, and can have a significant impact on the quality of life, especially if chronic pain is a prominent component.

Macrophage migration inhibitory factor (MIF) is well recognized as promoting inflammation in a variety of disease states [1]. More recent evidence indicates that MIF is also involved in mediating pain, not as a secondary effect of inflammation but as a direct effect, and this effect may manifest at several places along the neuraxis.

This chapter will review recent experimental evidence indicating that MIF is intimately involved in acute inflammation and pain, particularly in bladder pain. Moreover, MIF may mediate bladder pain even in the absence of inflammation. MIF and MIF receptors may be an interesting novel therapeutic target in disrupting or ameliorating painful chronic conditions, such as interstitial cystitis/painful bladder syndrome.

2 Role of MIF in Both Inflammatory and Neuropathic Pain

There is growing evidence in the clinical literature for an association between MIF and painful conditions. Elevated serum levels of MIF have been reported in patients with a severe attack of pancreatitis [2], painful diabetic neuropathy [3], chronic pelvic pain syndrome [4], and endometriosis (especially in those patients reporting active pain) [5]. In addition, intrauterine endometrial tissue from women with endometriosis who suffered from pelvic pain also showed elevated levels of MIF [6] (Fig. 1). While most of the studies associate increased circulating levels with painful conditions, a recent study reported that plasma MIF levels were significantly lower in patients with chronic non-malignant (spinal) pain than in controls (regardless of gender) [7]. These clinical studies all document a possible role for MIF in painful conditions; however the different disease

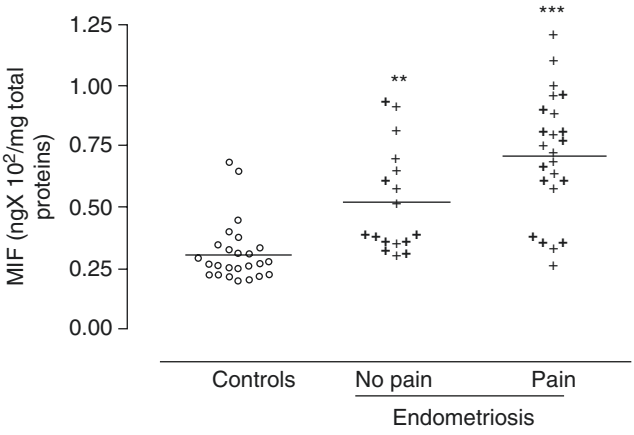

Fig. 1 Differences in MIF concentration in the endometrial tissue of controls (with no pain) and patients suffering endometriosis with/without pain (Adapted from [6])

states and the different measures (systemic vs. organ level) may make it difficult to ascertain whether the changes in MIF levels described are causative or secondary.

More direct evidence comes from experimental studies that indicate that MIF is a pivotal factor in the nociceptive process. For example, mechanical and thermal hyperalgesia elicited by chronic constriction of the sciatic nerve (a common model of neuropathic pain) or by plantar injections of formalin (a common model of inflammatory pain) were prevented by intrathecal injections of a MIF antagonist (ISO-1, (S,R)-3-(4-hydroxyphenyl)-4,5-dihydro-5-isoxazole acetic acid, methyl ester) [8, 9] (Fig. 2).

In addition, these investigators showed that levels of MIF and of the MIF receptor, CD74, were increased in the ipsilateral spinal cord after these injury models. MIF is localized in neurons in the spinal cord (and presumably the major source of the MIF increase seen after constriction nerve injury [9]), while microglial cells co-localized CD74 and are thus, presumably, the targets for increased neuronal MIF levels after constriction nerve injury. Meanwhile, astroglial cells in the spinal cord were reported to be the source of increased MIF levels after plantar formalin injection [8]. Therefore, it appears that different pain models result in different localization, expression, and alterations of MIF and MIF receptor levels along the neuraxis and may point to different mechanisms for MIF-mediated pain.

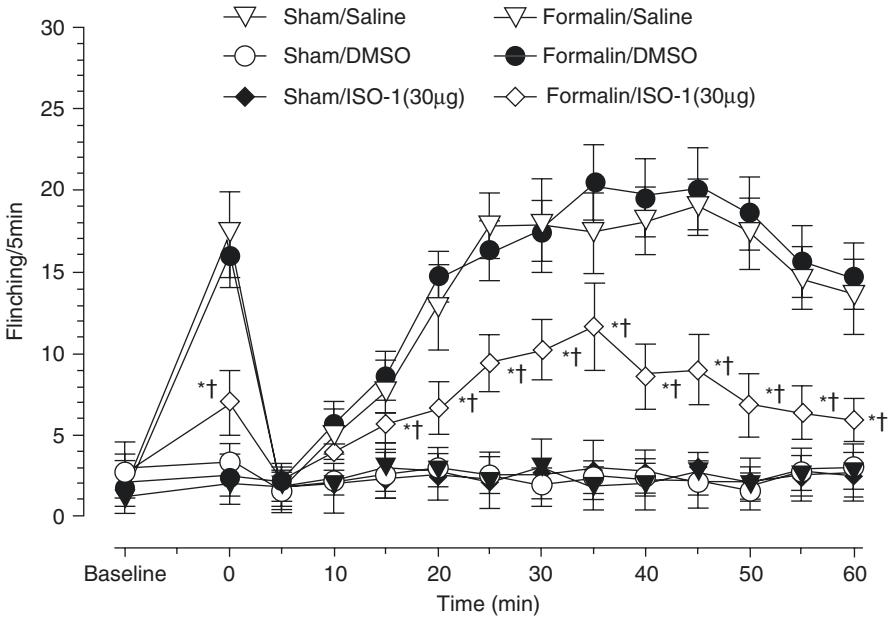

Fig. 2 Decreased pain behaviors (flinching/5 min) seen after intrathecal administration of a MIF antagonist (ISO-1) after plantar formalin injections (Adapted from [8])

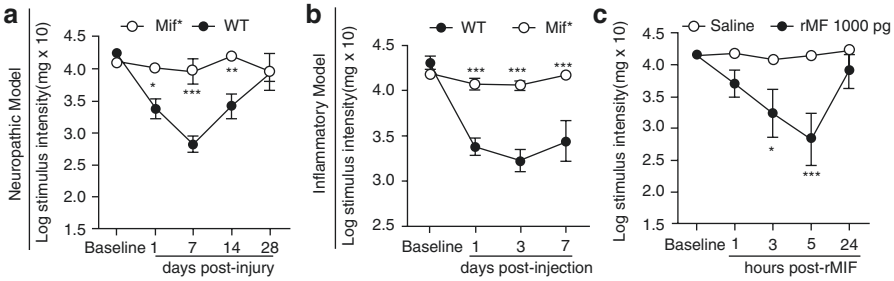

Fig. 3 MIF knockout mice do not develop mechanical hypersensitivity in nerve injury model (**a**) and inflammatory model (**b**). Intraplantar injection of recombinant MIF produces mechanical hypersensitivity (**c**) (Adapted from [10])

Further evidence for a crucial role of MIF in mediated pain behaviors comes from studies using MIF deficient mice. These mutant mice with a *Mif* deletion do not develop pain behaviors in response to a different neuropathic pain model (spared tibial nerve injury model) or to intraplantar injections of Freund's adjuvant (another inflammatory pain model) [10] (Fig. 3a, b).

In addition, pharmacological inhibition of MIF in wild-type mice also reduced pain behavior in this model [10]. Thus, MIF plays a crucial role in the development of pain in these two models tested. Moreover, administration of recombinant MIF elicited microglia activation and increased dorsal root ganglion neuron excitability in vitro [10]. Lastly, in what remains the strongest evidence for a role of MIF in nociception, these investigators showed that intraplantar administration of recombinant MIF results in pain behaviors similarly elicited by other inflammatory models [10] (Fig. 3c).

These studies indicate that MIF may be mediating nociception at several levels of the nervous system. MIF is expressed in neurons in the brain [11], in neurons and glial cells in the spinal cord, in neurons in the dorsal root ganglia conveying sensory information into the spinal cord from the periphery [12], and even in axons and Schwann cells of peripheral (sciatic) nerves [13]. The contribution of each locus of MIF expression in regulating development of pain is likely to be complex and may point to specific target areas to disrupt MIF-mediated pain.

3 MIF Expression in the Bladder

MIF is constitutively expressed in bladder urothelial cells and smooth muscle cells [12] (Fig. 4). Urothelial cells synthesize *Mif* [14] and there's a gradient of MIF protein in the urothelium. Cells in the basal layer show the strongest MIF immunostaining, followed by cells in the intermediate layer. Terminally differentiated urothelial cells (umbrella cells) are either weakly immunostained or show no MIF immunostaining. In addition to MIF, urothelial cells also constitutively express CD74 (canonical MIF receptor) and CXCR4 [14, 15].

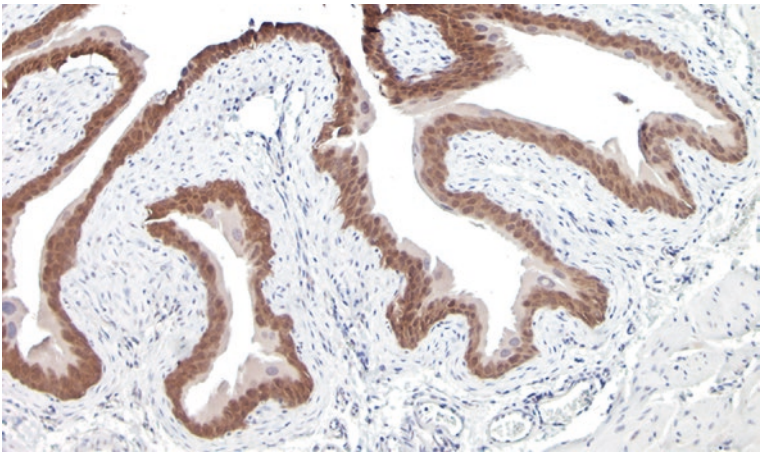

Fig. 4 MIF immunostaining in the urothelium of a mouse bladder. Notice that umbrella cells are either lightly stained or devoid of MIF. Nuclei counterstained with hematoxylin

4 Release of Urothelial MIF Mediates Bladder Inflammation and Pain

Inflammatory stimuli elicit a quick release of stored urothelial MIF [16, 17] along with upregulation of MIF and increased urothelial surface expression of MIF receptors [14, 15, 17–19]. Once released into the intraluminal fluid, MIF binds to urothelial MIF receptors [14] to stimulate a pro-inflammatory cascade in the bladder [20] (Fig. 5).

In a rodent model of neurogenic inflammation in the bladder, systemic substance P decreased MIF in bladder urothelium and increased MIF in the intraluminal fluid as early as 15 min after substance P administration [16]. In addition, inflammatory stimuli also increased MIF mRNA expression in the urothelium as well as increased mRNA expression of MIF receptors, CD74 and CXCR4 [14, 15]. Moreover, local inflammation caused by installation of capsaicin (nociceptive stimulus that results in pain and local inflammation) into the bladder also increased MIF release from bladder urothelium indicating MIF involvement in bladder pain and inflammation [21]. MIF antagonism or MIF receptor antagonism prevents an inflammatory cascade in the bladder and prevents bladder inflammation [20, 22]. Further support for the inflammatory role of MIF in the bladder comes from the observation that MIF was increased in the urine of patients with urinary tract infection [23], a condition resulting in bladder inflammation and bladder pain.

Once released by the urothelium, MIF binds to urothelial MIF receptors to promote an inflammatory cascade in the bladder [14, 20, 22]. It should be noted that nerve growth factor, a pivotal pro-nociceptive factor [24], is also one of the inflammatory mediators upregulated subsequent to MIF release from urothelial cells [16].

Furthermore, MIF antagonism reduced abdominal mechanical hypersensitivity in a commonly used chemical cystitis model in rodents [22]. Thus, our experimental

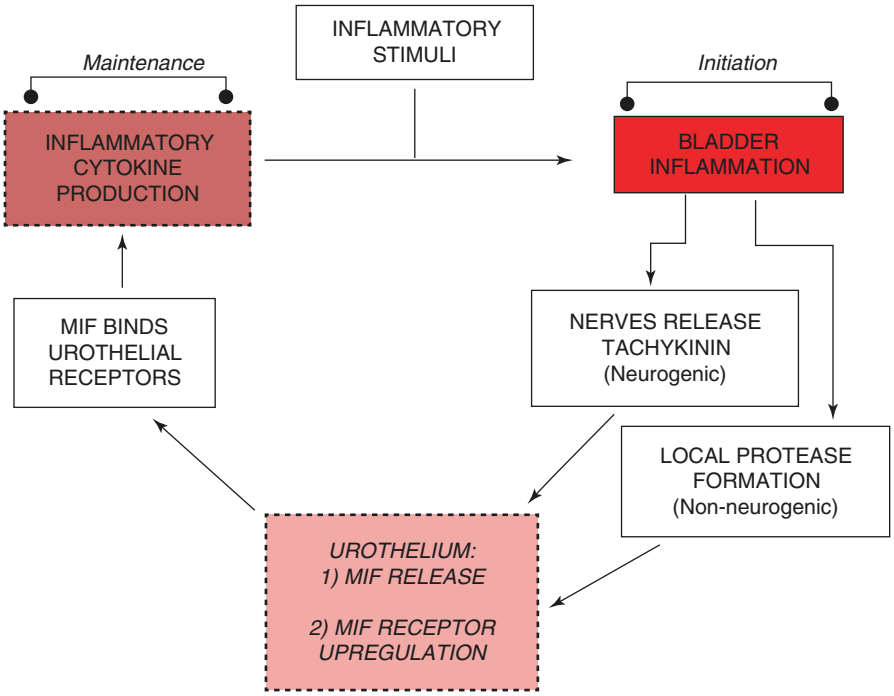

Fig. 5 Proposed model for how MIF initiates and maintains inflammation in the bladder. Urothelial cells release MIF after inflammatory stimulation. Released MIF then binds urothelial MIF receptors to mediate inflammatory changes in the bladder

evidence showed that MIF mediates bladder inflammation and accompanying bladder pain. Since pain is often associated with bladder inflammation, it is difficult to determine if the antinociceptive effects of MIF antagonism are secondary to decreasing bladder inflammation or are they acting primarily to reduce bladder pain.

5 MIF Mediates Bladder Pain Independent of Inflammation

Protease-activated receptors (PAR) are G-protein receptors that carry their own ligands [25]. These ligands are cleaved by naturally occurring proteases and then bind to the receptor to mediate signaling. All PAR (PAR1–PAR4) have been localized to the urothelium [26, 27], and PAR1 was reported to mediate bladder inflammation in mice [27]. Stimulation of PAR1 or PAR4 urothelial receptors was also shown to elicit MIF release from urothelial cells [28, 29] (Fig. 6).

In addition, stimulation of urothelial PAR1 or PAR4 receptors resulted in increased abdominal mechanical hypersensitivity (particularly in the case of PAR4 activation) without overt inflammation detected in the bladder and without any

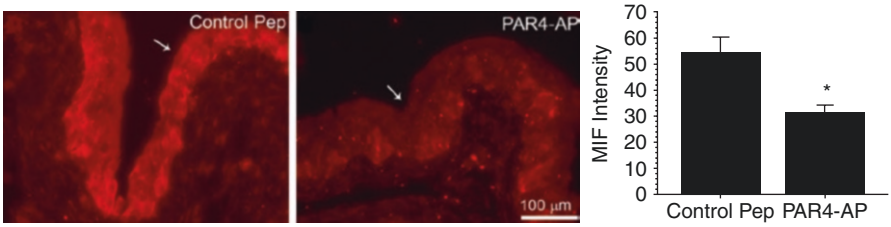

Fig. 6 Stimulation of urothelial receptors with intravesical instillation of PAR4-activating peptide (PAR4-AP) resulted in a decrease in the MIF staining intensity (suggesting MIF release) in the urothelium (*middle panel*) when compared to treatment with a control peptide (*right panel*). This difference was statistically significant (*right panel*) [28]

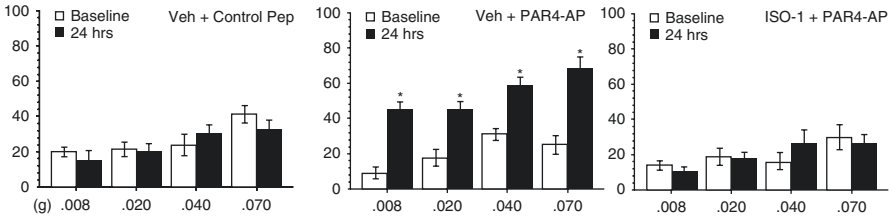

Fig. 7 Comparison of percent responses to ventral abdominal stimulation with graded von Frey filaments, before and after treatment. Left panel shows that intravesical instillation of a control peptide (control pep) had no effect on responses. Intravesical instillation of PAR4-activating peptide (PAR4-AP) resulted in an increase in responses (*middle panel*) indicating increased mechanical sensitivity in the area. This effect is prevented by pretreatment with a MIF antagonist (ISO-1, right panel), indicating that MIF mediates bladder pain after stimulation of urothelial PAR4 receptors [28]

changes in micturition parameters [28, 29]. Moreover, this effect was eliminated by MIF inhibition with ISO-1 and partially reduced by inhibiting CXCR4 (Fig. 7). Urothelial MIF released after PAR stimulation appears to play a key factor in mediating bladder pain, at least partly, through interaction with its receptor (CXCR4). Therefore, these results show that activation of urothelial PAR produces bladder pain in rodents without overt inflammation.

6 MIF Mediates Bladder Pain Through Urothelial Release of High-Mobility Group Box 1 (HMGB1)

HMGB1 is a nuclear chromatin-binding protein that is secreted by cells during infection or injury [30]. HMGB1 can mediate both inflammation and pain [31, 32]. In fact, blocking HMGB1 prevents bladder pain after cyclophosphamide (a common chemical cystitis model resulting in frank inflammation, pain, and hemorrhagic cystitis) in rodents [33].

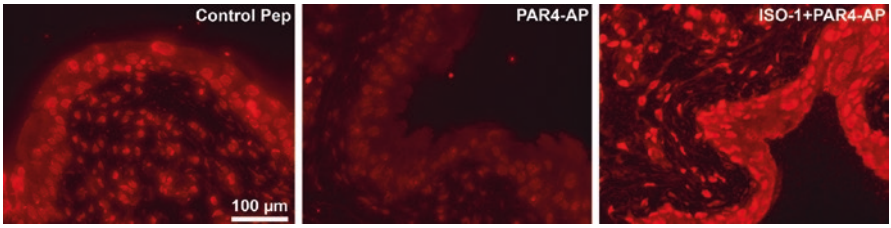

Fig. 8 Stimulation of PAR4 urothelial receptors results in release of HMGB1 from urothelial cells. Left panel shows HMGB1 immunostaining in the urothelium of mice treated with intravesical control peptide. Middle panel shows reduced HMGB1 immunostaining after intravesical PAR4-activating peptide (PAR4-AP). This effect is prevented by pretreatment with a MIF antagonist (ISO-1, right panel) and indicates that HMGB1 release is dependent on MIF release (Adapted from [34])

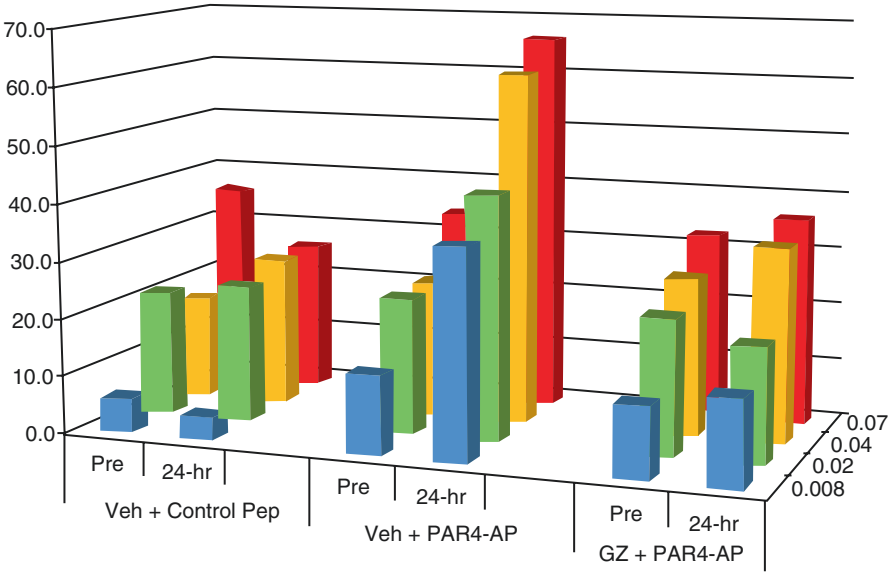

Fig. 9 Comparison of percent responses to ventral abdominal stimulation with graded von Frey filaments, before and after treatment. Left group shows that intravesical instillation of a control peptide (control pep) had no effect on responses. Intravesical instillation of PAR4-activating peptide (PAR4-AP) resulted in an increase in responses (*middle group*), and this effect was prevented by pretreatment with an HMGB1 inhibitor (glycyrrhizin (GZ), *right group*) indicating that HMGB1 mediates bladder pain after stimulation of urothelial PAR4 receptors (Adapted from [34])

Our recent evidence shows that activation of urothelial PAR4 elicits bladder pain due to release of urothelial MIF (as described in Fig. 7). We now have evidence that activation of urothelial PAR4 also elicits HMGB1 release and this effect downstream of MIF since MIF antagonism prevents MIF release (Fig. 8) [34]. Lastly, pain elicited by activation of urothelial receptors with PAR4 can be prevented by blocking HMGB1 (Fig. 9) [34].

Our experimental evidence shows that inflammatory stimuli or activation of urothelial PAR4 results in release of stored pools of MIF from urothelial cells. Released MIF can then bind to MIF urothelial receptors to activate signaling mechanisms that

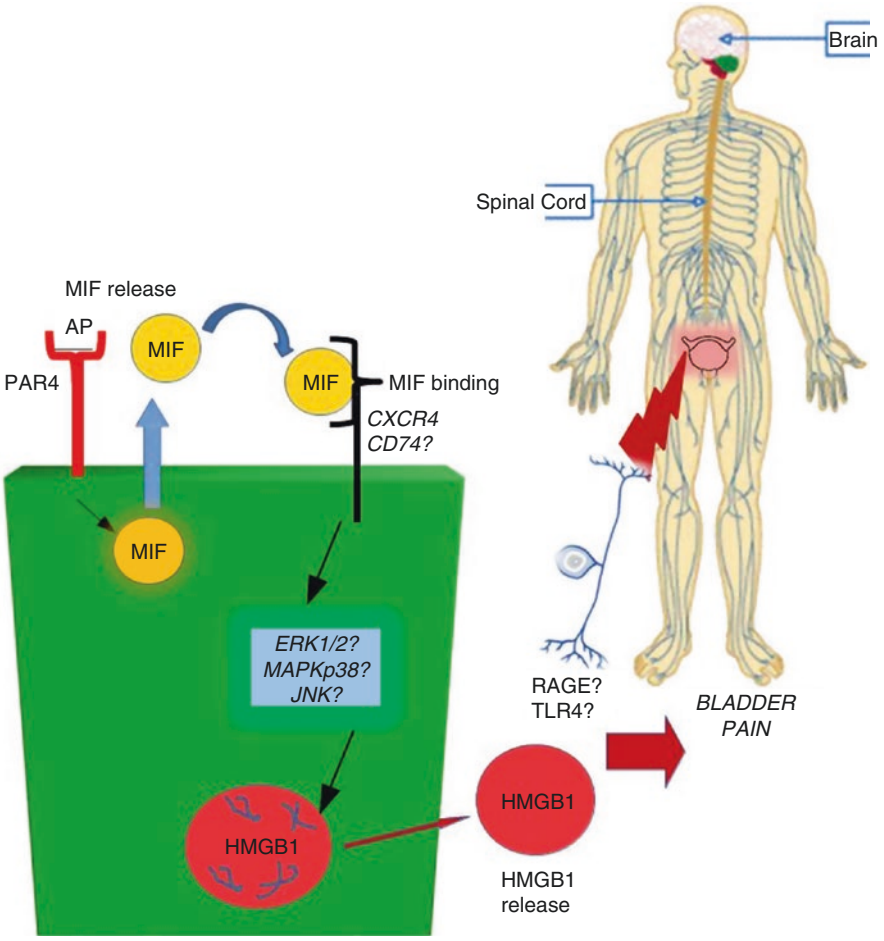

Fig. 10 Proposed model for MIF-mediated bladder pain. Activation of urothelial PAR4 by inflammatory stimuli or proteases elicits release of urothelial MIF. Released MIF binds to urothelial MIF receptors to mediate release of HMGB1. HMGB1 then binds to HMGB1 receptors, presumably on nerve endings, to mediate bladder pain

result in release of urothelial HMGB1. Extracellular HMGB1 mediates bladder pain likely through interaction with HMGB1 receptors in nerve endings innervating the bladder (Fig. 10).

7 Conclusions

Release of urothelial MIF mediates inflammation and also pain, even in the absence of inflammation. These findings add to emerging evidence that MIF plays an active role in mediating pain in general and bladder pain in particular. The link between MIF-mediated bladder pain and HMGB1 is novel and suggests that there may be

multiple possible targets for interrupting bladder pain (and perhaps other types of pain) circuits. Further investigation will likely outline the feasibility of targeting MIF, receptors for MIF, and/or receptors for HMGB1 as a viable path for ameliorating chronic pain conditions.

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MIF Family Proteins in Cardiac Ischemia/ Reperfusion Injury

Christian Stoppe and Jürgen Bernhagen

Abstract For the majority of patients with heart disease, cardiac surgery reduces mortality and remains the preferred therapy option. However, open-heart surgery still remains associated with the development of organ dysfunction and disconcerting complication rates, which results from myocardial ischemia/reperfusion injury and the perioperative inflammatory response. MIF is a structurally unique inflammatory cytokine with chemokine-like activities that acts as a key mediator of the innate and acquired immune response. Dysregulated MIF expression has been demonstrated to contribute to various acute and chronic inflammatory conditions. Although its activities in cardiovascular disease were initially defined in the context of systemic inflammation and exacerbation of inflammation in atherosclerosis, an increasing body of evidence suggests that MIF exerts a more fundamental role in the metabolic response to environmental stress. In the heart, MIF is released by ischemic cardiomyocytes and acts by autocrine/paracrine signaling. Recent studies have identified a unique role for MIF as a local cardioprotective cytokine, which thus may be of particular relevance for patients exposed to myocardial I/R and systemic inflammation during or following cardiac surgery. Moreover, the MIF homolog D-dopachrome tautomerase (D-DT, MIF-2) also is involved in the complex interplay during the inflammatory response and protects the heart from ischemia in mouse models, whereas its clinical relevance remains largely elusive.

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Here we summarize the promising characteristics of MIF suggesting an overall cardioprotective role in the setting of I/R and cardiac surgery and discuss the cellular and molecular mechanisms suggested to underlie this function. Clinical evidence is presented and discussed. Moreover, the less known role of MIF-2 in cardiac protection is scrutinized. Together, this book chapter gives an account of the available evidence on the role of MIF family proteins in cardiac ischemia/reperfusion injury and its clinical potential.

1 Introduction

Despite substantial technical advances in medicine, coronary heart disease still represents the leading cause of morbidity and mortality worldwide, accounting for about 7.3 million deaths annually according to the World Health Organization [1]. The restoration of coronary blood flow is known to trigger deleterious processes, which may even cause a significant higher degree of injury when compared to ischemia alone.

Macrophage migration inhibitory factor (MIF) is a structurally unique inflammatory cytokine with chemokine-like properties that acts as a key mediator of the innate and acquired immune response. Owing to its inflammatory potential, MIF is known as a critical mediator involved in the development of various acute and chronic inflammatory diseases, including atherosclerosis [2–5]. To this end, MIF also is a component of the inflammatory response that is initiated during myocardial ischemia and reperfusion (I/R) injury, e.g., promoting leukocyte infiltration into the damaged heart. However, emerging evidence suggests a time-dependent dual role for MIF in myocardial I/R [6–8]. Inflammatory activities of leukocyte-derived MIF in the post-ischemic heart or during later phases in I/R stress are preceded by a remarkable early cardioprotective effect of local cardiomyocyte-derived MIF. Mechanistically, this encompasses MIF-mediated maintenance of cardiac redox homeostasis and activation of cardioprotective signaling through the MIF receptor CD74. MIF/CD74 signaling promotes adenosine monophosphate-activated protein kinase (AMPK)-dependent myocardial glucose uptake, while inhibiting the pro-apoptotic JNK pathway, which has been implicated in reperfusion injury [6, 7, 9–11]. Intriguingly, these protective MIF pathways are impaired in the senescent heart [10]. Recent evidence also suggests that a homolog of MIF, D-dopachrome tautomerase (D-DT/MIF-2), modulates similar pathways. These characteristics of MIF proteins may give rise to translational benefit for patients exposed to myocardial ischemia/reperfusion and sequelae as they also occur during cardiac surgery procedures.

This chapter summarizes the functional role, mechanisms, and clinical significance of MIF proteins and their receptors in cardiac ischemia.

2 Myocardial Ischemia/Reperfusion Injury and Inflammation

The pathological consequences of heart disease arise from the deleterious effects of acute myocardial ischemia and following reperfusion, which frequently results in cardiomyocyte death, cardiac failure, arrhythmias, and patient death. The major clinical settings in which the heart is subjected to acute ischemia/reperfusion injury (IRI) include myocardial infarction, cardiac surgery, or transplantation.

During myocardial I/R, cardiomyocytes show a high oxygen demand to maintain the continuous production of adenosine 5′-triphosphate (ATP). As ischemia decreases cellular oxidative phosphorylation and interrupts constant oxygen supply, this may further contribute to a failure to resynthesize energy-rich phosphates, including phosphocreatine and ATP. The restoration of blood flow leads to a reperfusion of the ischemic coronary vessels, resulting in a reestablishment of oxygen supply in the tissue. However, the reperfusion of ischemic regions is also known to cause detrimental effects that may significantly exceed the injury, which is induced by ischemia alone [12]. In the past, various studies identified the excessive release of oxygen species, calcium overload, and the activation of phagocytes to represent the most harmful determinants during myocardial I/R. Oxidative stress further leads to oxidation of proteins, lipids, and DNA and alteration of cell necrosis and apoptosis and catalyzes the inflammatory response [13]. To this end, reactive oxygen species (ROS) induce the activation of the NF-κB pathway, which in turn increases the expression of adhesion molecule, inflammatory cytokine, and chemokine gene expression, resulting in an overwhelming inflammatory response [14]. The ensuing immigration of neutrophils, monocytes, and T cells to the ischemic zone further amplifies the inflammatory response and production of ROS [15]. In patients exposed to myocardial ischemia, reperfusion-specific deleterious effects are frequently observed that cover myocardial and peripheral disease including acute kidney injury, life-threatening ventricular arrhythmias, and persistent contractile dysfunction (myocardial stunning). These complications have been suggested to predominantly result from the oxidative stress upon reflow [12].

Despite these potential complications, today's gold standard therapy for acute myocardial infarction (AMI) is the early reperfusion of the ischemic regions. In fact, the first experimental studies were published almost four decades ago and demonstrated that reperfusion not only salvages infarcted myocardium but may also induce damage, accounting for up to 50% of the final infarct size in animal models [16]. Therefore, reperfusion injury constitutes a promising target in the therapy of AMI [12]. However, to date translation of several promising strategies from animal models into clinical practice has failed to reduce I/R injury. This mandates further detailed investigations to understand the mechanisms underlying the pathophysiology of myocardial I/R injury that may eventually help to improve translation from bench to bedside. Against this background, the emerging evidence

on the important role of MIF family proteins in modulating myocardial I/R injury may be of particular relevance and may ultimately lead to novel therapeutic concepts for AMI patients. Moreover, as most cardiac surgery procedures result in I/R stress of the heart, such strategies may become beneficial in improving surgical side effects.

3 Ischemia/Hypoxia Stimulates the Secretion of MIF

Ischemia/hypoxia is a potent regulator for a variety of biological processes, including angiogenesis, vascular contractility, and erythropoiesis [17], and various adaptive response mechanisms to hypoxia are mediated by the hypoxia-responsive transcription factor hypoxia-inducible factor (HIF)-1α [18].

Hypoxia-induced expression and secretion of MIF have been repeatedly reported in the past. Baugh and co-workers showed a HIF-1α-dependent increase of MIF mRNA levels that was further amplified by activation of CREB in HeLa cells upon hypoxic treatment [19]. Given the importance of MIF in chronic and acute inflammation, the authors proposed that the HIF-1α pathway could be a potential therapeutic target for the modulation of MIF expression during hypoxia [19]. Oda and co-workers also demonstrated MIF's ability to reciprocally control HIF-1α activity in a p53-dependent manner via the MIF receptor CD74 [20]. In an experimental in vitro model, hypoxia triggered a biphasic release of MIF from endothelial cells. Furthermore, glyburide, a known pharmacological inhibitor of ATP-binding cassette transporter family A, member 1 (ABCA1) transporter, inhibited hypoxia-induced endothelial MIF secretion in contrast to cycloheximide and the HIF-1α blocker echinomycin. Accordingly, the observed initial MIF peak was suggested to result from a fast release of preformed cytoplasmic pools that were independent of de novo synthesis, while the second peak is likely due to HIF-1α-induced upregulation of MIF expression [21, 22]. Similarly, MIF secretion, together with that of other angiogenic chemokines, is triggered in endothelial progenitor cells (EPCs) following hypoxic stimulation [23]. Hypoxia-induced MIF expression/secretion from endothelial cells and/or EPCs may have beneficial but also detrimental effects, depending on the context and disease, with applications ranging from tumor angiogenesis to neovascularization during ischemia in cardiovascular diseases [23].

A major source of released MIF in the ischemic heart is the cardiomyocyte. This was first shown in an in vitro rat cardiomyocyte model [24] and has since been demonstrated in numerous in vitro and experimental in vivo studies [10, 11, 25–30]. Cardiomyocyte secretion after ischemic stress also was demonstrated for MIF-2/D-DT [31]. As outlined in the following chapters, cardiomyocyte-derived MIF, via intra-, auto-, and paracrine mechanisms, significantly contributes to the cardioprotective effects that have been associated with MIF in the early phase of I/R injury.

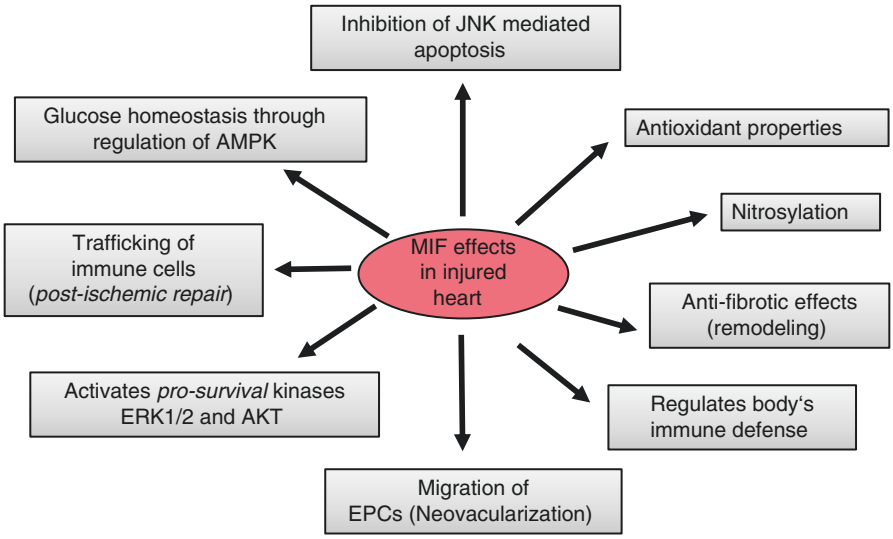

Fig. 1 Schematic overview of MIF effects in the injured heart. Metabolic and signaling effects are highlighted as well as effects of MIF on immune cell recruitment, heart remodeling and biochemical modifications of MIF in the ischemic heart

4 MIF Controls Several Critical Signaling Pathways During Myocardial Ischemia/Reperfusion

Cardioprotection by MIF is a multifactorial phenomenon mediated via several signaling processes and by MIF's intrinsic antioxidant properties [6, 7]. Moreover, MIF-mediated neovascularization through recruitment of EPCs [23, 32] and anti-fibrotic effects of MIF [33] may convey cardioprotection in the mid- to long-run recovery phase after myocardial ischemia/reperfusion. Figure 1 summarizes the reported effects of MIF on signaling pathways in the heart.

4.1 MIF Proteins Promote Protective AMPK Signaling in Experimental Models of Myocardial Ischemia/Reperfusion

In myocardial ischemia/reperfusion stress, MIF activates the cardioprotective AMP-activated protein kinase (AMPK) signaling processes [34] in a CD74-dependent manner. Miller et al. first demonstrated the role of MIF as an auto-/paracrine activator of AMPK, which provides protection in the heart against ischemic injury and apoptotic cardiomyocyte death [11]. The AMPK pathway regulates various metabolic pathways in the heart and controls glucose and lipid uptake, storage, and use [34]. Intriguingly,

AMPK activation and glucose uptake were significantly blunted during ex vivo global I/R in *Mif* deficient. These findings were supported by an impaired post-ischemic ventricular function and an increased infarct size that were observed in *Mif*$^{-/-}$ mice or *Cd74*-deficient mice, underscoring the direct role of the MIF/CD74 signaling axis. Furthermore, Ma et al. investigated the role of the MIF/CD74/AMPK axis in the senescent heart [10]. In an experimental mouse model, the authors found an age-dependent decrease of AMPK activation that was linked with MIF expression. In line with prior studies, myocardial I/R triggered a significant release of MIF into the coronary effluent, whereas MIF levels were significantly decreased in aged hearts compared to young hearts. Interestingly, endogenous MIF-mediated AMPK activation in the stressed heart can be pharmacologically augmented by small molecule MIF agonists that increase MIF signaling through CD74/AMPK to limit cardiac ischemic injury [28].

Moreover, while the situation in human cardiac surgery patients appears to be more complex (see below), MIF-2/D-DT exhibited clear-cut cardioprotective activities in the ischemic heart in murine experimental models. Both antibody-based MIF-2/D-DT neutralization and cardiomyocyte-specific conditional deletion of the *Mif-2/D-dt* gene led to a profound impairment of cardiac function and an increased infarct size in a mouse model of myocardial I/R [31]. Mechanistically, cardioprotection by MIF-2/D-DT also involves the CD74/AMPK pathways, but upstream activation of AMPK appears to be dependent on CaMKK2, whereas LKB1 has been suspected to be the relevant upstream kinase when AMPK is activated by MIF [31]. Thus, cardiomyocyte secretion of MIF-2/D-DT, similar to that of MIF, has important autocrine/paracrine effects during ischemia/reperfusion that protect the heart against injury.

4.2 MIF Inhibits JNK Signaling and Cardiomyocyte Apoptosis in Myocardial I/R Injury

Besides the AMPK pathway, Qi and co-workers reported that MIF is capable of inhibiting c-Jun N-terminal kinase (JNK)-mediated apoptotic processes during myocardial I/R injury. JNK is a member of the mitogen-activated protein kinase (MAPK) family and controls inflammation, proliferation, cell differentiation, and apoptosis [35]. MIF has previously been found to either promote or inhibit JNK signaling, depending on the cellular or disease context [36–38]. In the experimental study performed by Qi and co-workers, *Mif* deficiency was associated with increased JNK activation after global myocardial I/R, which could be reversed by application of extracellular recombinant MIF [9]. Furthermore, cardiomyocytes exposed to I/R injury showed upregulated JNK activation that was accentuated in *Mif*$^{-/-}$ mice compared with WT mice in vivo. Importantly, the (therapeutic) administration of recombinant MIF in *Mif*$^{-/-}$ mice led to decreased infarct sizes and an improvement of left ventricular function. Furthermore, human fibroblasts homozygous for the *MIF* -794 $CATT_5$ allele (5/5 cell lines), which show the lowest level of MIF promoter activity, exhibited an increased activation of the pro-apoptotic JNK pathway when exposed to hypoxia and following reoxygenation [9].

MIF regulation of JNK activity also is linked to MIF's intracellular functions. Unlike most other cytokines and chemokines, substantial concentrations of

preformed MIF protein are stored intracellular in the cytosolic compartment, implying cytosolic effects of this mediator. In fact, cytosolic MIF or exogenous MIF following cellular uptake, endocytosis, and cytosolic translocation engages in several protein-protein interactions with intracellular redox- or cell cycle-related proteins. Kleemann and colleagues demonstrated that MIF interacts with c-Jun activation domain-binding protein-1 (JAB1/CSN5), a subunit of the COP9 signalosome, which functions as a transcriptional co-activator of AP1 transcription and binds to and activates JNK. MIF inhibits JNK activation via its interaction with JAB1/CSN5, affecting JNK-mediated stress and apoptotic responses [36]. Interestingly, this interaction critically depends on MIF residue Cys-81, which was found to be *S*-nitros(yl)ated during myocardial I/R in vivo [27]. *S*-nitros(yl)ation of this cysteine residue leads to a decreased interaction between MIF and JAB1/CSN5 during myocardial I/R in vivo, and *S*-NO-MIF exhibits an increased anti-apoptotic capacity for cardiomyocytes compared to wild-type MIF [27]. Although, the link between MIF-JAB1 interaction and MIF's inhibitory effect on JNK has not yet been functionally tested in myocardial I/R models in vivo, these data suggest that intracellular MIF/JAB1 signaling could be a second signaling route through which MIF controls detrimental JNK activity in the ischemic heart. It remains to be seen whether and how this pathway can complement or amplify the JNK inhibition pathway in cardiomyocytes that is afforded by extracellular (autocrine/paracrine)-directed MIF via the CD74 receptor route [9]. Moreover, it is attractive to speculate that the CD74 route might be further enhanced by the alternative MIF receptors CXCR4 or CXCR2, which have been found to form heteromeric complexes with CD74 that are expressed on ischemic cardiomyocytes and that regulate JNK signaling in model cell lines. It certainly will be of importance to further investigate the detailed mechanism of MIF-mediated apoptosis regulation during myocardial I/R in the future.

4.3 MIF Regulates the Pro-survival Kinases ERK1/2 and Akt

Conditioning of the myocardium is known to activate various cytoprotective pathways and confer protection against the deleterious sequels after ischemia and reperfusion (I/R). The underlying mechanisms of conditioning result from release of substances such as adenosine, bradykinin, endogenous opioids, growth factors, and possibly also neuronal wiring and chemokines [39]. These mediators bind to cell surface receptors, which in turn activate pro-survival signaling cascades, a set of kinases termed as "reperfusion injury salvage kinase" (RISK) pathway [40]. The activation of the pro-survival signaling cascade has been suggested to provide protection against ischemia and reperfusion injury and involves the key regulator proteins PI3K/AKT and ERK1/2. Both kinases are capable of inhibiting the opening of the mitochondrial permeability transition pore (mPTP) by their actions on several downstream signaling molecules that directly modulate the transition state of the pore (e.g., endothelial nitric oxide synthase (eNOS), p53, glycogen synthase kinase (GSK)-3β) or indirectly influence pore permeability by affecting the balance of pro- and anti-apoptotic transcription factors (e.g., Bcl-2, Bax, Bad) [40]. Interestingly, Yellon and colleagues recently demonstrated that AMD3100, a specific inhibitor of

CXCR4/CXCL12 signaling, was capable of inhibiting the protective effects of remote ischemic preconditioning [41]. As CXCR4 also is a receptor for MIF that is able to activate the pro-survival kinase AKT and JNK through CXCR4 and as AMD3100 partially interferes with CXCR4/MIF signaling [37, 42], it has been suggested that MIF might be a mediator of remote preconditioning [2, 43, 44].

The fact that MIF is rapidly released in response to various stimuli and shares many characteristics that overlap with the mechanisms of preconditioning, including the activation of the protective kinases ERK1/2, AKT, and PKC, renders this cytokine a potential mediator of myocardial conditioning. In fact, recent data confirm the notion that MIF is a crucial mediator in conditioning, leading to an activation of ERK, AKT, and PKC [45]. In accordance with the biphasic secretion behavior of MIF observed in endothelial cells after hypoxic stimulation [22], MIF was found to be released from cardiomyocytes in a comparable manner, suggesting similar underlying mechanisms. Furthermore, our group demonstrated a roughly twofold increase in MIF mRNA after a hypoxic stimulus. As MIF protein is expressed preformed in most cell types and MIF mRNA is only moderately regulated, the observed increase in MIF mRNA was suggested to contribute to the observed delayed MIF release about 24 hours after conditioning [45]. The data further showed an activation of ERK, AKT, and PKC throughout the observation period with maximal activation after termination of preconditioning. Hence, the parallel increase in MIF levels and cardioprotective kinases supports the hypothesis that MIF could be a novel player in myocardial conditioning.

5 MIF Exhibits Antioxidative Properties

Besides the activation of different protective protein kinases, MIF directly exerts cardioprotective effects through its antioxidant capacity. These characteristics are related to its intrinsic thiol-protein oxidoreductase (TPOR) activity, which is linked to a so-called Cys-Xaa-Xaa-Cys (CXXC) redox motif that MIF carries and shares with other TPOR proteins [27, 46–49]. CXXC-based catalytic antioxidative activity can quench reactive oxygen species to exert cell protection [49, 50]. Accordingly, in experimental myocardial injury by ischemia/reperfusion (I/R), MIF-deficient hearts exhibited higher levels of oxidative stress than wild-type hearts, as measured by higher oxidized glutathione levels, increased protein oxidation, reduced aconitase activity, and increased mitochondrial injury [46]. This increased myocardial oxidative stress correlated with larger infarct sizes in MIF-deficient hearts. The study gave first in vivo evidence in support of a cardioprotective role of MIF in the ischemic heart by reducing oxidative stress. Moreover, the redox activity of MIF protects the myocardium when exposed to hemodynamic stress. In a mouse model of myocardial hypertrophy induced by transverse aortic coarctation (TAC), growth of the MIF-deficient hearts was significantly greater compared with wild-type TAC hearts; similarly, fibrosis was increased. This correlated with increased circulating levels of MIF, an elevated expression of CD74, and increases in ROS-generating mitochondrial NADPH oxidase, mitochondrial superoxide dismutase 2 (SOD2), and mitochondrial

aconitase activities, together indicating enhanced oxidative burden in the hypertrophied *Mif*-deficient ventricle. Also, in the hemodynamically stressed *Mif*-deficient heart, nuclear p21(CIP1) and cytosolic phospho-p21(CIP1) were elevated compared with wild-type TAC hearts. Thus, the MIF antioxidant activity extends to conditions of myocardial hypertrophy and fibrosis in response to hemodynamic stress [33].

As mentioned above, we recently reported that MIF's favorable action in the heart is further enhanced by *S*-nitrosylation during myocardial I/R [27]. This post-translational modification occurs on the third Cys-residue of MIF, i.e., Cys-81, and further increases the cardioprotective and antioxidative TPOR activity of MIF—likely via inducing a conformational change. The selective S-nitrosothiol formation at Cys-81 led to a doubling of the oxidoreductase activity of MIF and to a decrease in cardiomyocyte apoptosis in the reperfused heart. A recent study measured the kinetics of *S*-NO-MIF formation and its role in intracellular versus extracellular cardioprotective activities of MIF. The authors discovered a rapid decrease of cardiac MIF that was specific to the "early" phase of reperfusion. *S*-NO-MIF formation prevented this rapid decrease, leading to a targeted intracellular accumulation of MIF in this phase of reperfusion. Intracellular MIF preserved the ability of MIF to reduce oxidative stress, i.e., blunted hydrogen peroxide levels and improved aconitase activity, and modulated MIF/CSN5 interactions. It appears that under conditions of MIF *S*-nitrosylation, intracellular cardioprotective MIF-driven mechanisms overcompensate the cardioprotective signaling pathways (via CD74/AMPK) promoted by extracellular, non-*S*-nitrosylated, MIF [30]. Figure 2 summarizes the hypothesized balance between the extracellular versus intracellular cardioprotective

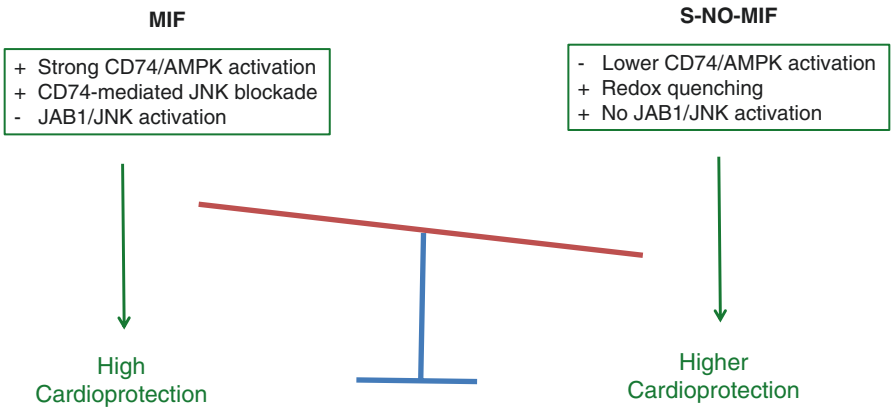

Fig. 2 Scale cartoon delineating the cardioprotective effects of extracellular versus intracellular and non-modified versus S-nitroso (SNO)-modified MIF in the heart during ischemia-reperfusion injury. *Left*, cardioprotective activities and potency of secreted extracellular MIF via interaction with cardiac CD74 receptors. MIF/CD74 signaling in the heart promotes AMP kinase (AMPK) activation and blocks the c-Jun N-terminal kinase (JNK) pathway. These cardioprotective activities are in part counteracted by an activation of JNK through the COP9 signalosome subunit JAB1/CSN5. In conjunction, this leads to high cardioprotection. *Right*, S-nitrosylation of MIF during myocardial I/R leads to intracellular cardiac retainment of MIF. While compromising the cardioprotective MIF/CD74 signaling axis, this promotes redox quenching by intracellular SNO-MIF and attenuates the activation of JNK by JAB1. In sum, these effects provide enhanced cardioprotection

mechanisms of MIF. Targeted intracellular accumulation of MIF by *S*-nitrosation may offer a novel therapeutic approach in the treatment of myocardial I/R injury.

We investigated the significance of perioperative MIF release on redox homeostasis and organ dysfunction in patients that underwent cardiac surgery. Interestingly, we revealed an enhanced antioxidant capacity in patients with highly elevated MIF levels, as assessed by the measured activity of endogenous peroxidase activity (EPA) and thioredoxin (Trx) levels. In contrast, we did not observe an increase of MIF and/or EPA levels in those cardiac surgical patients, which underwent off-pump cardiac surgery, without use of CPB and who were thus not exposed to extensive myocardial I/R. On the other hand, we revealed significantly increased MIF levels in patients undergoing on-pump surgery, which were associated with a reduced occurrence of postoperative atrial fibrillation and acute kidney injury. The reasons were suggested to be due to an enhanced antioxidant capacity in those patients [8].

Overall, antioxidant properties of MIF may represent a relevant mechanism especially in patients exposed to increased oxidative stress during myocardial I/R [8].

6 Clinical Significance of MIF in Myocardial Infarction

Patients with myocardial ischemia frequently demonstrate reperfusion-specific deleterious effects (e.g., life-threatening ventricular arrhythmias after reperfusion, acute kidney injury, and persistent contractile dysfunction (myocardial stunning)), which are suggested to result predominantly from oxidative stress upon reflow [51]. Early reperfusion is today's gold standard therapy for patients with acute myocardial infarction (AMI). Almost four decades ago, first experiments showed that reperfusion may not only salvage infarcted myocardium but also induce damage which may account for up to 50% of the final infarct size in animal models [12, 16]. As translation of protective strategies failed to translate these new approaches into clinical practice, the underlying pathophysiology of myocardial I/R injury needs further mechanical investigations.

Given MIF's characteristics as a fast reacting upstream mediator or the host's immune response and its capacity to drive cardioprotective signaling pathways, several studies have investigated MIF's functional role and clinical relevance in patients exposed to myocardial ischemia/reperfusion. Figure 3 summarizes the position of MIF and inflammation in myocardial injury.

During the past decade, accumulating evidence has indicated a pivotal role for MIF in patients suffering from the pathophysiological processes of myocardial infarction. Müller and colleagues demonstrated that patients with acute coronary syndrome (ACS) have increased serum levels of MIF and that these are associated with the accompanying inflammatory response [52]. While elevated serum levels of MIF were considered a potential risk factor for the development of heart failure in patients with type 2 diabetes mellitus [53], no correlation was found between elevated MIF levels and the risk for the development of coronary heart disease (CHD).

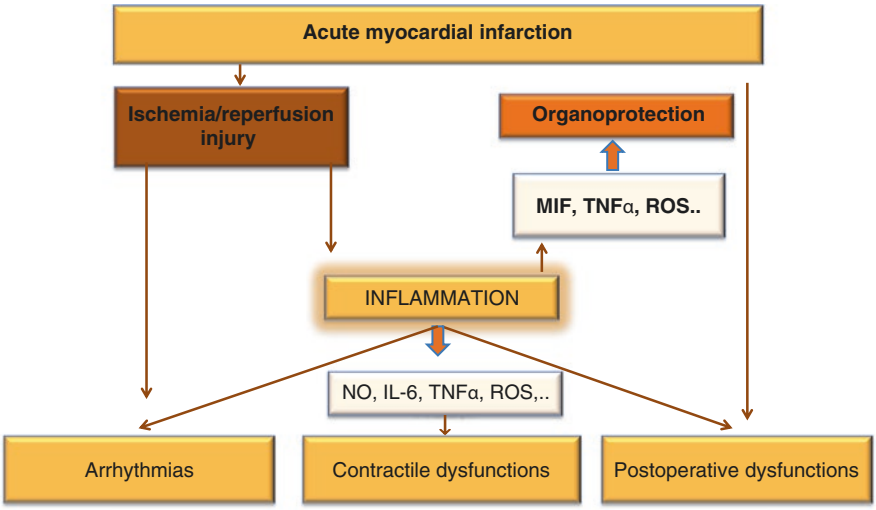

Fig. 3 Role of inflammation in acute myocardial infarction including consequences for clinical outcomes. The dichotomous role of inflammation as exemplified by the inflammatory mediators MIF, tumor necrosis factor (TNF), nitric oxide, interleukin-6, and reactive oxygen species (ROS) in acute myocardial infarction is shown, highlighting the interplay between ischemia/reperfusion (I/R) injury, organoprotection, and clinical outcomes such as arrhythmias, contractile dysfunctions, and postoperative dysfunctions

Importantly, human epidemiological studies revealed an increased risk for CHD patients with a single-nucleotide polymorphism (SNP) (G/C transition) at position −173 of the MIF promoter gene [54, 55] that is accompanied by an increased MIF expression. In the same vein, this transition in the MIF promoter was associated with the development of cardiomyopathy in children [56]. In addition the GG SNP (rs1007888) was shown to increase the risk of myocardial infarction in female patients [57].

Over a decade ago, Takahashi and colleagues first described the kinetics and potential biological functions of MIF in patients with myocardial infarction and in an in vivo model of cultured rat cardiomyocytes [24]. The authors detected increased circulating MIF levels in patients with acute myocardial infarction. Furthermore, they demonstrated that MIF expression and release from cardiomyocytes were stimulated in response to hypoxia and oxidative stress (hydrogen peroxide) in an atypical protein kinase C (PKC)-dependent manner, which further resulted in an activation of ERK1/2 [58]. Yu and colleagues confirmed these findings in an animal study and demonstrated an upregulation of myocardial MIF after infarction, which triggered the subsequent accumulation of macrophages in the infarcted region [59].

The excessive release of MIF after myocardial infarction was further demonstrated in patients successfully resuscitated after cardiac arrest. A majority of patients exhibited an overwhelming release of MIF, whereas circulating MIF levels correlated with the duration of myocardial ischemia, which was measured via time

from cardiac arrest until start of resuscitation [60]. However, the small number of included patients limited the ability of this study to evaluate the significance of MIF release on patient's outcome.

7 Clinical Significance of MIF in Patients Undergoing Cardiac Surgery

For many patients with heart disease, cardiac surgery reduces mortality and remains the preferred therapy option. However, complex cardiac surgical procedures remain associated with disconcertingly high complication rates when measured as short- or long-term outcomes. Recent analyses of large patient databases indicated that major complications including death, myocardial infarction, cardiac arrest and failure, renal failure, stroke, gastrointestinal complications, and respiratory failure occur in up to 16% of all patients during the initial hospital stay [61]. Therefore, a more comprehensive understanding of the underlying aggravating and protective mechanisms is needed to identify promising strategies to protect the vulnerable patient that is exposed to myocardial ischemia/reperfusion in the clinical context of myocardial infarction or cardiac surgery.

The perioperative inflammatory response during cardiac surgery is considered as major contributor to surgery-associated complications. With the recognition of the significance of inflammatory processes in the development of complications and organ dysfunctions, several studies have been conducted to explore the role of MIF in this setting [8, 47, 48, 60, 62, 63]. While initial studies demonstrated MIF to mediate disease-exacerbating effects, emerging evidence suggests an overall cardioprotective role for MIF during I/R, especially in the early phase [9, 11, 31, 46–48], which may be of particular relevance for patients exposed to increased oxidative stress following myocardial I/R. At least in mouse models, this was also found for MIF-2/D-DT, while studies in humans suggest are a more complex picture [31, 47]. Notably as discussed above, MIF also has been demonstrated to antagonize myocardial hypertrophy and the development of fibrosis by attenuating stress-induced activations of hypertrophic signaling pathways through phosphorylation of cytosolic glycogen synthase kinase-3α [33]. Since the post-ischemic heart is frequently exposed to pathologic changes in its myofibrillar contractile function ("remodeling"), MIF release might thus counter-regulate these mechanisms and control the postoperative repair and adaptation mechanisms. In the past, several studies repeatedly demonstrated a significant intraoperative increase of circulating MIF levels in patients who were exposed to an increased inflammatory response and oxidative stress that result from myocardial I/R [8, 62, 64, 65]. The early intraoperative increase in MIF levels was accompanied by a subsequent MIF decrease after termination of surgery. Although the use of cardiopulmonary bypass surgery (CPB) is known to trigger an extensive inflammatory response, recent findings suggest that the MIF release mainly results from the myocardial I/R stress response [8]. Interestingly, the majority of clinical trials showed significantly elevated MIF levels

already prior to surgery in comparison to healthy volunteers, indicating correlations with prior disease status [3]. As the majority of included patients presented with preexisting coronary artery disease, the increased MIF levels may be due to the pro-atherogenic status of these patients [2, 4].

Besides these findings, elevated MIF levels after termination of surgery were inversely correlated with the extent of organ dysfunction as assessed by the well-established organ dysfunction scores SAPS II and SOFA that adequately reflect the severity of disease. Moreover, MIF values on postoperative day 1 (POD1) were closely associated with the measured cardiac output as reflected by the calculated cardiac power index. As MIF has previously been shown to regulate glucose uptake in ischemic cardiomyocytes in an AMPK-dependent manner [11], it may be concluded that the perioperative MIF release enhances cardiac contractility. In addition, postoperative MIF levels showed a significant inverse correlation with the measured troponin T levels at admission to the ICU and at POD1. This might be due to the fact that release of endogenous MIF from cardiomyocytes is known to inhibit the JNK pathway [9, 46], which significantly influences the myocardial damage after ischemia and reperfusion. Accordingly, animal studies with MIF knockout mouse hearts showed an increased extent of myocardial damage after I/R when compared to MIF wild-type mice [9, 11, 27, 46]. In contrast, de Mendonça-Filho and colleagues demonstrated that postoperative MIF values were increased and significantly higher in patients that developed septic complications with microbiologically proven infection [63], indicating a complex behavior of this pleiotropic cytokine in the perioperative context.

8 First Evidence About the Role of MIF-2 and the Soluble Receptor CD74 During Myocardial I/R

While both experimental and clinical studies unanimously suggest that MIF is a protective factor in the injured myocardium—at least in the early phase after I/R—little is known about the more recently characterized MIF family protein member D-dopachrome tautomerase (D-DT/MIF-2). Interestingly, recent data indicates that MIF-2 has MIF-like functions in septic conditions [66, 67] and provides protective effects during myocardial ischemia/reperfusion in an experimental study [31]. These data are in apparent contrast to clinical correlations from cardiac surgery patients that indicate that MIF-2 protects hearts against injury and contractile dysfunction after I/R [47]. The protective autocrine/paracrine effects of MIF-2, as determined in the experimental study, were mediated in a comparable manner as for MIF through activation of AMPK in a CD74-dependent manner [31]. The mechanistic reasons for the diverging effects of MIF and MIF-2 in the clinical setting in the cardiac surgery cohort may be due to MIF and MIF-2's different antioxidant capacities. Of note, MIF-2 lacks two of three conserved cysteines (Cys-60 and Cys-81) that mediate MIF's redox activity and contribute to several of MIF's functional and cardioprotective properties [47, 68]. Furthermore, both cytokines are known to have chemokine-like functions on leukocyte motility, whereas the mechanisms and

subtype-specific profiles may differ. In fact, MIF-2 lacks the *pseudo*-(E)LR motif known to be required for MIF/CXCR2-mediated leukocyte recruitment responses [69]. While molecular evidence is still elusive, the effects of MIF-2 on leukocyte recruitment, if significant, may thus be mediated not through CXCR2 or CXCR2/CD74 complexes but indirectly by an induction of secondary chemotactic mediators such as downstream chemokines. Alternatively, CXCR4-mediated mechanisms could play a role. Lastly, MIF's effect on subacute inflammatory cell recruitment into the injured cardiac tissue may be overcompensated by its metabolic or redox-mediated cardioprotective effects, whereas for MIF-2, which intrinsically lacks the protective antioxidant properties, metabolic actions would predominate [31]. These mechanistic differences also may differ in human versus murine tissues. Additional experimental studies are needed to mechanistically address the discussed underlying functional differences in the future.

The MIF protein family has recently been studied more broadly in myocardial I/R settings. Analyses were expanded to encompass the circulating—shedded—soluble form of the MIF receptor CD74, i.e., the CD74 ectodomain (sCD74). Following up on initial evidence about the functional role and clinical relevance of sCD74 in liver disease in humans [70], serum levels of sCD74 and the sCD74/MIF complex were measured in cardiac surgery patients. Importantly, patients in whom sCD74/MIF complexes were detected exhibited a reduced incidence of acute kidney injury and overall organ dysfunctions in the postoperative course. Mechanistically, it appeared that sCD74/MIF complex formation was linked with an enhanced antioxidant capacity of MIF, and the interaction between sCD74 and MIF increased the TPOR activity of MIF, leading to an increased reduction of the small molecule thiol substrate 2-hydroxyl-ethyl disulfide (HED) and improving redox stress-challenged cardiomyocyte survival [47].

9 Clinical Significance of the MIF Genotype

Protein data on the MIF family as discussed above are accompanied by an intriguing genotype-phenotype relationship between commonly occurring variant MIF alleles and clinical outcomes in cardiac surgery patients [47]. The repeat number of the *MIF* promoter microsatellite (-794 $CATT_{5\text{-}8}$, *rs5844572*) and a nearby single-nucleotide polymorphism (SNP) (-173 G/C, *rs755622*) are associated with higher *MIF* expression resulting in higher serum levels of MIF in humans [71–73]. Cardiac surgery patients carrying the high expression *MIF* genotype showed a significantly higher intraoperative release of MIF protein into the circulation and experienced a significantly lower incidence of organ dysfunction during the postoperative course. Furthermore, no postoperative infections were documented in the patients with the high expression *MIF* genotype. These findings were supported by experimental and clinical findings that showed the crucial function of MIF for bacterial killing in macrophages. Calandra and colleagues demonstrated that *Mif*-deficient cells exhibited an impaired killing of gram-negative bacteria [74], and subjects with high expression *MIF* alleles have been shown to have reduced gram-negative bacteremia [75] and to

have improved intensive care unit (ICU) survival from shock due to community-acquired pneumonia [73]. Therefore, these findings suggest that the pleiotropic role of MIF has to be interpreted cautiously in different clinical settings but nevertheless may assist in the future in the preoperative risk stratification of cardiac surgery patients.

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The MIF-CD74 Inflammatory Axis in Alphaviral Infection

Lara J. Herrero, Ali Zaid, Margit Mutso, and Suresh Mahalingam

Abstract The expansion of mosquito-borne viral (arboviral) arthritis poses a significant threat to human health worldwide. Clinical reports show that arboviral arthritis can be persistent and debilitating, with evidence of bone pathology. As part of the Togaviridae family, alphaviruses are mosquito-borne viruses that are widely distributed throughout the globe causing extensive morbidity and mortality. Despite this, very little is known about the pathogenesis of disease caused by alphaviruses. It has been shown that macrophages play a crucial role in the development of alphaviral arthritis. Infection causes macrophage activation and the release of macrophage inhibitory factor (MIF), which subsequently plays a pivotal role in alphavirus-induced arthritis by regulating the expression of pro-inflammatory factors. This chapter discusses the role of the MIF-CD74 axis in the development of alphavirus arthritis and the therapeutic potential of antagonists in the treatment of alphaviral arthropathies.

1 Alphaviruses

Alphaviruses belong to the family of Togaviridae and genus *Alphavirus* containing approximately 30 different viruses. Their host range is very wide, ranging from invertebrates to vertebrates. Alphaviruses are arboviruses and are therefore transmitted via invertebrate vectors, mainly mosquitoes.

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1.1 Virology

Alphaviruses are rather small viruses with a virion size around 70 nm. The viral particle contains one single-stranded RNA with positive polarity, the genome size being around 12,000 nucleotides. Similarly to messenger RNA, the 5′ end of the virus genome contains a cap structure and the 3′ end a poly (A) tail and is translated using the cellular translation machinery. The viral genome serves as a template for primary translation of viral proteins. Translated proteins form a replication complex to replicate and transcribe the genome and subgenomic RNA, which is needed for synthesizing viral structural proteins. Replication and transcription are very strictly regulated by modulating the structure of the replication complex. Capsids are assembled in the cytoplasm and transported to the plasma membrane. The generation of new viral particles ends with the capsids budding out of the plasma membrane, taking the membranous layer packed with viral glycoproteins with it [1, 2].

1.2 Epidemiology

Alphaviruses are divided into two major groups by their pathology and geographical distribution. New World alphaviruses like western (WEEV), Venezuelan (VEEV), and eastern equine encephalitis virus (EEEV) are mainly associated with encephalitis and are distributed in Southern and Northern America [3]. Old World alphaviruses like chikungunya virus (CHIKV), Ross River virus (RRV), o'nyong-nyong virus (ONNV), Mayaro virus (MAYV), Sindbis-like viruses (SINV), and Barmah Forest virus (BFV) can result in arthritis, myalgia, arthralgia, rash and, in very rare cases, encephalitis may evolve [4].

CHIKV has been extensively expanding its geographical range for the past 10 years and has now reached pandemic proportions. One of the first recorded episodes of CHIKV infection was in 1952 in Tanzania. The virus has since circulated endemically in Africa before reemerging in La Réunion in 2005 and spreading rapidly to Oceania and South-East Asia. In 2013 it reached America and began a rapid spread in the Caribbean and South America [5]. The alphaviruses RRV and BFV are mainly limited to Australia and Papua New Guinea [6] and cause considerable morbidity. The main clinical pathology and major significance to public health caused by these viruses is chronic arthritis.

1.3 Clinical Disease

Alphaviruses are transmitted via a mosquito bite and typically have an incubation time between 2 and 6 days. Around 15% of CHIKV and 50% of RRV infections are asymptomatic, while others can develop fever, rash, myalgia, and/or arthritis.

Alphavirus infection is characterized by high viremia with viral titres ranging from 10^5 to 10^{12} pfu/mL, which is usually cleared within 5 days. Most of the patients clear the symptoms after several weeks, but in some cases the symptoms (especially arthritis) can last for months or even years. Alphavirus-induced arthritis affects primarily peripheral joints: fingers, wrists, elbows, toes, ankles, and knees [7, 8]. Approximately 40% of infected patients develop a chronic disease [9]. Chronic arthritis is more common in elderly people and patients who have higher viral loads during the acute phase of infection [10]. Additionally, patients with previous history of rheumatic arthritis or joint injuries are more prone to suffer from a severe CHIKV infection and to develop chronic arthritis [11].

2 Alphavirus Pathogenesis

Alphavirus-induced arthritis is thought to occur mainly due to the viral ability to infect and replicate in skeletal tissues and joints. This idea is supported by the fact that RRV or CHIKV has been found in the RNA of knees and synovial tissues [12]. The inflammatory response and histopathology of joints during alphavirus infection have many similarities with rheumatic arthritis [13].

2.1 Antiviral Immune Response

Infection with alphaviruses is followed by a robust antiviral immune response. The acute infection is accompanied by high activation of dendritic/NK/$CD4^+$/$CD8^+$ cells and extensive cytokine production (IFN-α, IL-1, IL-6, IL-4, IL-10, IL-12, monocyte chemoattractant protein 1 [MCP-1, or CCL2], IL-6, IL-8, MIP-1α (or CCL3), MIP-1β (or CCL4), macrophage inhibitory factor [MIF]) [10, 14–16]. The chronic phase of alphavirus infection associated with persistence of virus in synovial macrophages is accompanied by an inflammatory response. High levels of specific IgM and elevated levels of IFN, IL-12, MCP-1, IL-6, IL-8, MIP-1α, and MIP-1β have been noted to persist for months [10, 17].

2.2 MIF and Other Soluble Factors

In 2000, a study by Lidbury et al. used a mouse model to determine that depletion of macrophages by silica or carrageenan prior to alphavirus inoculation (in this case RRV) ablated disease. When macrophage-driven inflammation was identified as a basis for RRV myositis and arthritis, subsequent studies were directed specifically at determining the role of cytokines and chemokines with known functions in monocyte/macrophage homeostasis [18].

In 2008, macrophage depletion studies were furthered using clodronate and demonstrated reductions in joint/muscle tissue levels of reactive nitrogen intermediates (RNI), IFN-γ, TNF-α, and MCP-1. These pro-inflammatory proteins were also elevated in synovial fluid collected from patients with active RRV polyarthritis, compared to control samples [19].

The role of pro-inflammatory host factors in mediating alphavirus-induced musculoskeletal disease has been the focus of ongoing studies over the past decade. This led, for instance, to the characterization of CCL2/MCP-1 as a major chemokine involved in the recruitment of monocytes and macrophages to sites of viral replication in the muscle and joint, where the use of a CCL2-inhibitor, Bindarit, saw disease symptoms and mononuclear cell infiltration dramatically reduced [20, 21]. This early study into the role of host factors in alphaviral pathogenesis paved the way for the dissection of an increasingly complex network of cytokines found to have an important role in cell migration and tissue infiltration in viral arthritis and myositis.

Recent advances in the field of immunopathology have enabled a more refined understanding of the way immune host factors regulate pathogenesis through their activating—or inhibitory—role on effector cells in the context of disease. Similarities found between pathogenic mechanisms in rheumatoid arthritis (RA) and alphavirus-induced arthritis provided a working model enabling the characterization of inflammatory factors that may be at play in viral disease [13, 20, 22]. Macrophage inhibitory factor (MIF), a cytokine found in elevated levels in the serum and synovial fluid of rheumatoid arthritis (RA) patients, is a pleiotropic inflammatory molecule known to facilitate recruitment and activation of immune cells [23, 24]. In addition, MIF is upregulated in patients presenting with dengue virus (DV) [25] and West Nile virus (WNV) infections [26]. These studies suggested a potential association between MIF and viral disease, but were limited in confirming the exact role MIF has in disease pathogenesis. In 2011, a study using the mouse model of RRV disease revealed increased levels of MIF in the acute phase of infection and showed that lack of this cytokine was associated with reduced disease symptoms (Fig. 1), due to a marked decrease in immune cell infiltration into the muscle and joint tissue (Fig. 2) [15]. In this study, RRV-infected wild-type (WT) mice showed an increase in MIF expression in serum and tissues, which corresponded to severe inflammation and tissue damage. MIF-deficient ($MIF^{-/-}$) mice and RRV-infected wild-type mice treatment with the MIF ISO-1 antagonist developed mild disease signs accompanied by a reduction in inflammatory infiltrates and muscle destruction in the tissues, despite having minimal differences in viral titres. The ISO-1 antagonist showed efficacy in both preventing disease if given as a prophylactic and in treating disease if administered as a therapy given at disease onset. Interestingly, as a proof-of-concept, reconstitution of MIF into $MIF^{-/-}$ mice rescued the disease phenotype and addition of MIF into wild-type mice intensified RRV disease. Together, these results showed MIF to be a critical soluble factor in driving the clinical severity of alphavirus-induced musculoskeletal disease and potentially a target for the development of antiviral pharmaceuticals.

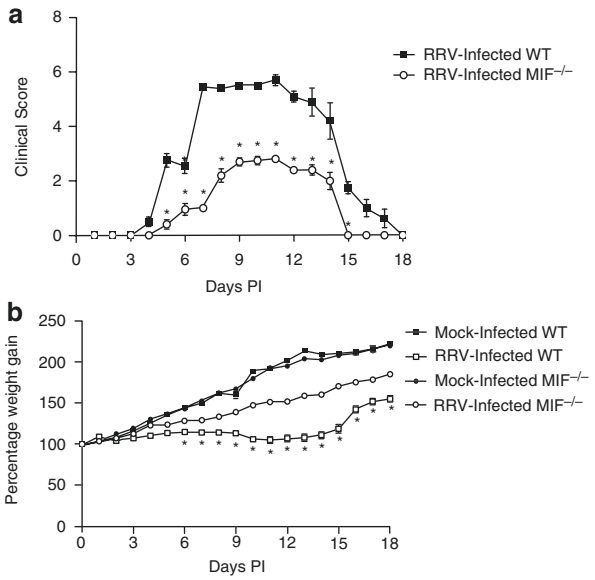
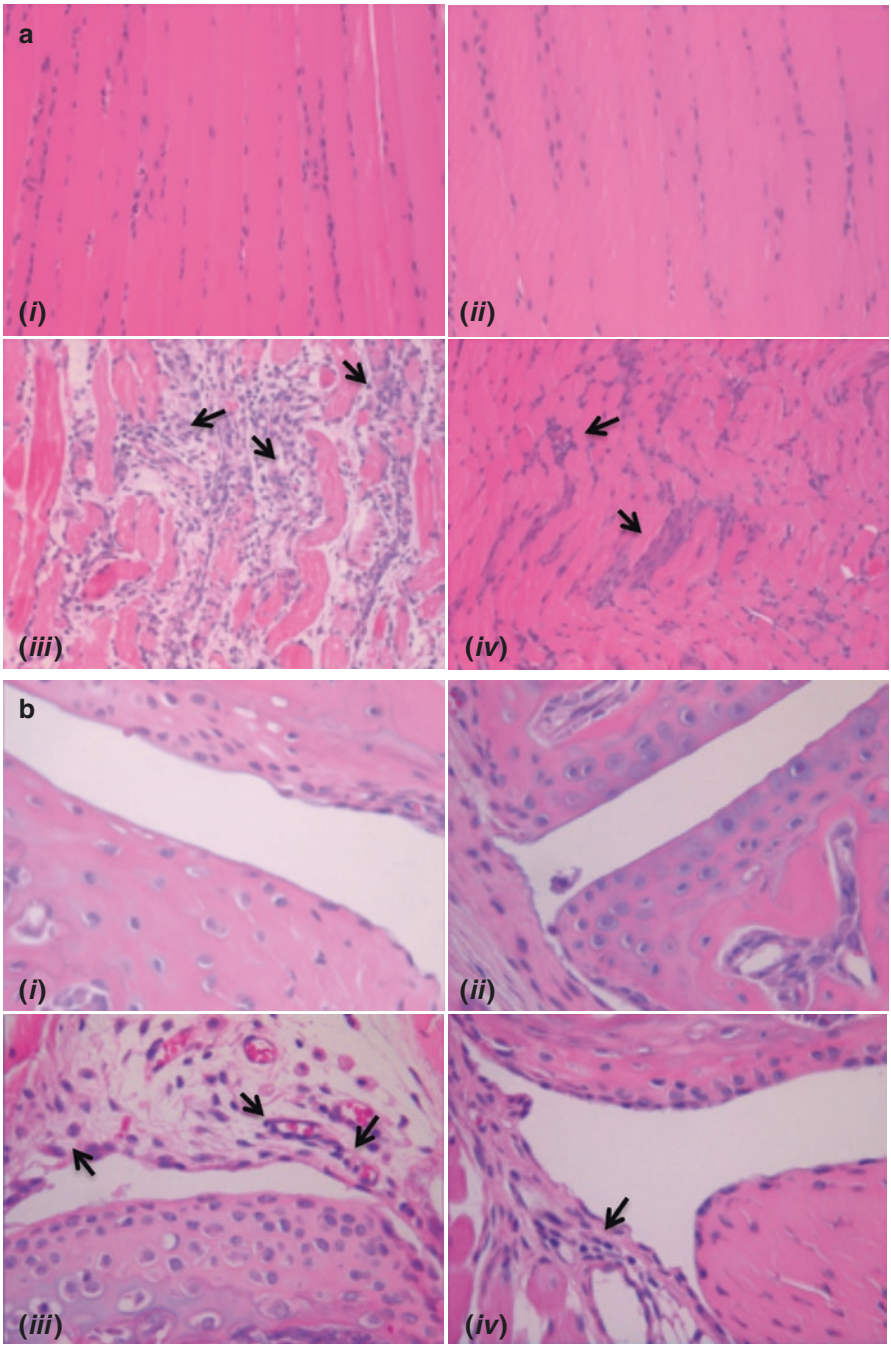


Fig. 1 RRV-induced disease is less severe in MIF$^{-/-}$ mice. Twenty-day-old C57BL/6 WT or MIF$^{-/-}$ mice were infected s.c. with 10^4 pfu RRV or mock-infected with diluent alone. (**a**) Mice were scored for the development of hind-limb dysfunction. (**b**) Mouse weight was monitored at 24-h intervals. Mock-infected mice were scored zero for the duration of the experiment. Each data point represents the mean +/− SEM of five to ten mice and is representative of four independent experiments. *$p < 0.05$ using a Mann–Whitney test (**a**). *$p < 0.05$ compared to RRV-infected MIF$^{-/-}$ using two-way ANOVA with Bonferroni posttest, and RRV infection significantly reduced weight gain compared to mock-infected controls, with $p < 0.05$ for all time points after day 5 (WT) or day 5 p.i. (MIF$^{-/-}$) (**b**). Figure obtained from Herrero LJ, Nelson M, Srikiatkhachorn A, Gu R, Anantapreecha S, Fingerle-Rowson G, Bucala R, Morand E, Santos LL, Mahalingam S. Critical role for macrophage migration inhibitory factor (MIF) in Ross River virus-induced arthritis and myositis. Proc Natl Acad Sci U S A. 2011 Jul 19;108(29):12048–53. doi: 10.1073/pnas.1101089108. Epub 2011 Jul 5. Permission to reproduce this figure was granted by the publishers

2.3 The MIF-CD74 Axis

Given the important function of MIF in alphaviral disease, its receptor, CD74, became the focus of intense scrutiny. In 2003, the cloning of a cell-surface receptor for MIF left an open question with regard to the mechanisms by which the MIF-CD74 axis operates in vivo and in a disease setting [27]. Further investigations showed that upon binding of MIF, signal transduction through CD74 required CD44, an intracellular molecule known to activate tyrosine kinases and now considered an integral part of the CD74 receptor complex [28]. While this further refined our understanding of how the MIF-CD74 signalling mechanism may work, its precise role in alphaviral pathogenesis remained elusive.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Recently, Herrero et al. used CD74$^{-/-}$ mice to understand the role of CD74-MIF signalling in the development of alphavirus-induced muscle and joint inflammation using both RRV and CHIKV disease models [29]. However, while the group's previous study had identified a clear role for MIF in the development of RRV disease, the effect of MIF on the inflammatory and migratory profile of immune effector cells had not been clearly defined. The general consensus on the role of CD74 in disease was loosely established upon its ability to regulate macrophage chemotaxis and tissue migration [30, 31], and studies demonstrated that CD74 could cooperate with chemokine receptors CXCR2 and CXCR4, which in turn enabled them to respond to MIF-dependent activation [30, 32].

In mouse models, RRV disease (RRVD) is characterized by acute myositis and arthritis, whereas CHIKV disease symptoms are scored by the extent of foot swelling and tissue inflammation [33–35]. The study by Herrero et al. found that in both RRV (Fig. 3) and CHIKV (Fig. 4) disease models of infection, CD74$^{-/-}$ mice exhibited reduced clinical disease symptoms and no weight loss at the peak of disease compared to wild-type mice. Typical mononuclear cell infiltration was not observed in the quadriceps and ankle joints of RRV-infected CD74$^{-/-}$ mice or in the chondroskeletal tissue of the feet of CHIKV-infected mice. In the case of RRVD, this finding further reinforced the notion that MIF signalling was critical in the development of myoskeletal inflammation; more importantly, this was the first study showing a role for CD74 in CHIKV pathogenesis, an alphavirus of great topical significance.

A hallmark of alphavirus-induced inflammation is the presence of replicating virus in affected tissues. RRV has been shown to replicate in muscle and joint tissues during the acute phase of infection, and the authors posited that a reduction in viral titres would be concomitant with reduced tissue inflammation they observed in CD74$^{-/-}$ mice. However, viral titres in serum and quadriceps were similar between CD74$^{-/-}$ and WT mice in the early phase of disease, prior to the appearance of clinical signs. Interestingly, higher titres were recovered from the ankles of CD74$^{-/-}$ mice, suggesting that effector cells that depend on CD74 signalling are more effective at viral clearance in particular tissues. However, in the later phases of the

Fig. 2 RRV-induced inflammation is less severe in MIF$^{-/-}$ mice. Twenty-day-old C57BL/6 WT or MIF$^{-/-}$ mice were infected s.c. in the right thorax with 10^4 pfu RRV or mock-infected with diluent alone. At 10 days p.i. mice were sacrificed, perfused with 4% PFA, (**a**) quadriceps and (**b**) ankle tissues removed, paraffin-embedded, and 5 μm sections generated. Sections were stained with H&E. Panels (*i*) mock-infected WT, (*ii*) mock-infected MIF$^{-/-}$, (*iii*) RRV-infected WT, (*iv*) RRV-infected MIF$^{-/-}$. Annotations: (B) bone, (C) cartilage, (P) periosteum, (M) muscle. Images are representative of at least five mice per group (magnification 100×), and *arrows* indicate abundance of inflammatory infiltrates. Figure obtained from Herrero LJ, Nelson M, Srikiatkhachorn A, Gu R, Anantapreecha S, Fingerle-Rowson G, Bucala R, Morand E, Santos LL, Mahalingam S. Critical role for macrophage migration inhibitory factor (MIF) in Ross River virus-induced arthritis and myositis. Proc Natl Acad Sci U S A. 2011 Jul 19;108(29):12048–53. doi: 10.1073/pnas.1101089108. Epub 2011 Jul 5. Permission to reproduce this figure was granted by the publishers

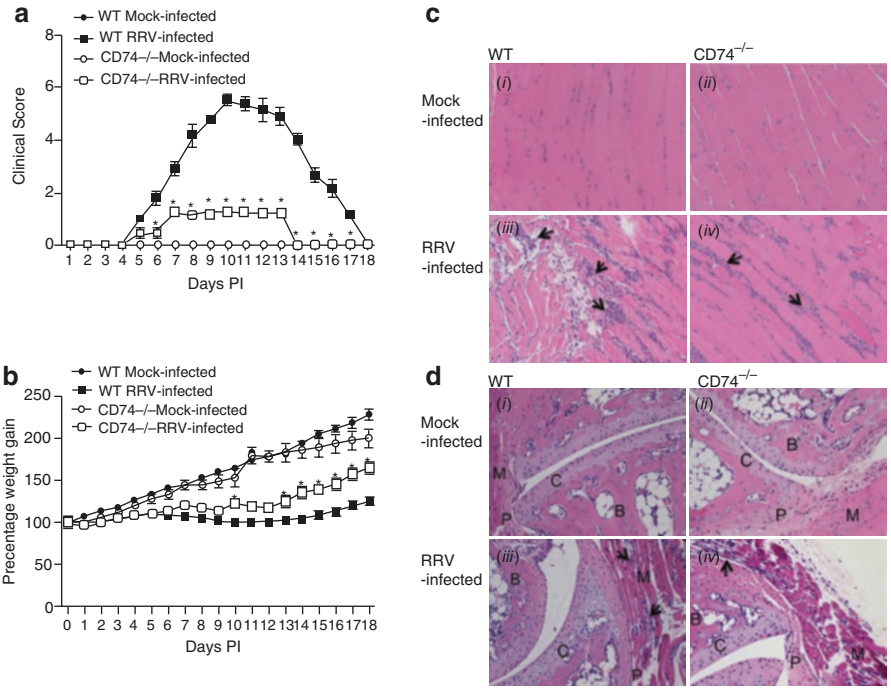

Fig. 3 RRV-induced disease is less severe in CD74$^{-/-}$ mice. Twenty-day-old C57BL/6 WT or CD74$^{-/-}$ mice were infected s.c. with 10^4 pfu RRV or mock-infected with diluent alone. (**a**) Mice were scored for the development of hind-limb dysfunction. (**b**) Mouse weight was monitored at 24-h intervals. Mock-infected mice were scored zero for the duration of the experiment. Each data point represents the mean +/− SEM of five to ten mice and is representative of four independent experiments. *$p < 0.05$ using a Mann–Whitney test (**a**). *$p < 0.05$ compared to RRV-infected CD74$^{-/-}$ using two-way ANOVA with Bonferroni posttest, and RRV infection significantly reduced weight gain compared to mock-infected controls, with $p < 0.05$ for all time points after day 3 (WT) or day 7 (CD74$^{-/-}$) (**b**). For histological analysis, mice were sacrificed at 10 days p.i., perfused with 4% PFA, (**c**) quadriceps and (**d**) ankle tissues removed, paraffin-embedded, and 5 μm sections generated. Sections were stained with H&E. Panels (*i*) mock-infected WT, (*ii*) mock-infected CD74$^{-/-}$, (*iii*) RRV-infected WT, (*iv*) RRV-infected CD74$^{-/-}$. Annotations: (B) bone, (C) cartilage, (P) periosteum, (M) muscle. Images are representative of at least five mice per group (magnification 100×), and *arrows* indicate abundance of inflammatory infiltrates. Figure obtained from Herrero LJ, Sheng KC, Jian P, Taylor A, Her Z, Herring BL, Chow A, Leo YS, Hickey MJ, Morand EF, Ng LF, Bucala R, Mahalingam S. Macrophage migration inhibitory factor receptor CD74 mediates alphavirus-induced arthritis and myositis in murine models of alphavirus infection. Arthritis Rheum. 2013 Oct;65(10):2724–36. doi: 10.1002/art.38090. Permission to reproduce this figure was granted by the publisher John Wiley and Sons

disease, where clinical signs become more prominent, significantly higher viral titres were recovered from quadriceps and ankle tissues of CD74$^{-/-}$ mice compared to WT mice, while no difference was observed in serum titres. Overall, these findings indicated that whilst CD74$^{-/-}$ mice displayed reduced disease severity, this was unlikely to be associated with higher viral clearance in inflamed and infected myoskeletal tissues.

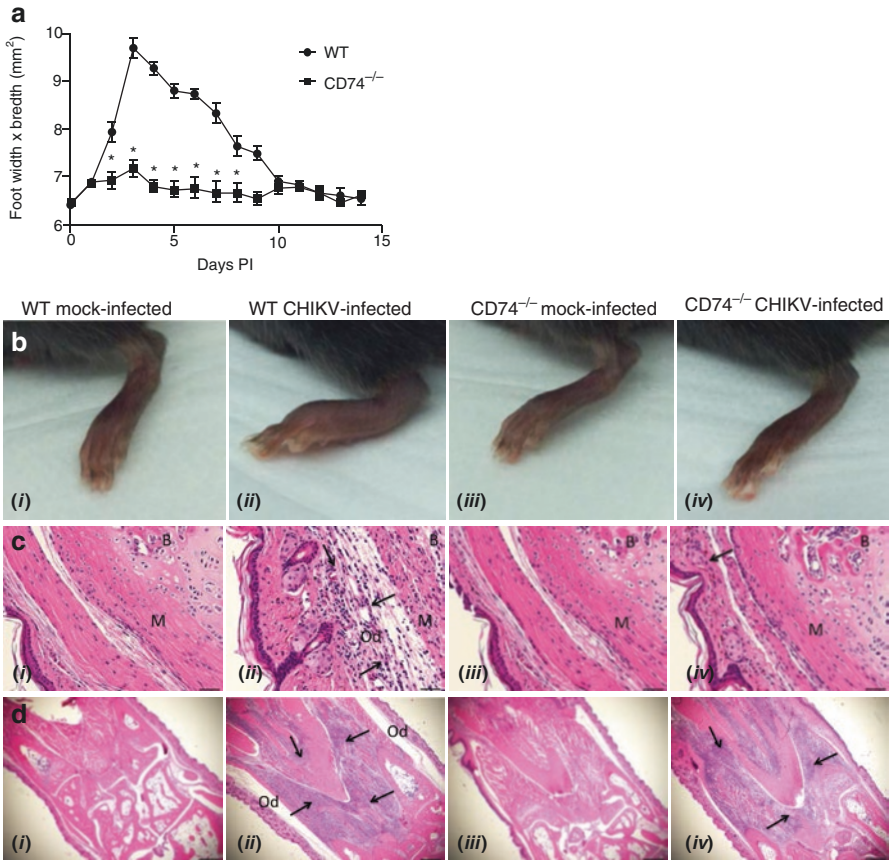

Fig. 4 CHIK-induced inflammation is reduced in CD74$^{-/-}$ mice. Twenty-day-old C57BL/6 WT or CD74$^{-/-}$ mice were infected s.c. in the footpad with 10^4 pfu CHIKV or mock-infected with diluent alone. (**a**) Mice were monitored daily for joint inflammation with foot width and breadth measured over time. Each data point represents the mean +/− SEM of five to ten mice and is representative of two independent experiments. (**a**) *$p < 0.05$ using a Mann–Whitney test. (**b**) Peak swelling at day 3 shown in (*i* and *ii*) WT and (*iii* and *iv*) CD74$^{-/-}$ mice. For histological analysis, mice were sacrificed at 3 days p.i., perfused with 4% PFA, (**c**) ankle tissues and (**d**) footpads removed, paraffin embedded, and 5 μm sections generated. Sections were stained with H&E. Panels (*i*) mock-infected WT, (*ii*) RRV-infected WT, (*iii*) mock-infected CD74$^{-/-}$, (*iv*) RRV-infected CD74$^{-/-}$. Annotations: (B) bone, (M) muscle, (Od) subcutaneous edema. Images are representative of at least five mice per group (magnification (**c**) 200× (**d**) 40×), and *arrows* indicate abundance of inflammatory infiltrates. Figure obtained from Herrero LJ, Sheng KC, Jian P, Taylor A, Her Z, Herring BL, Chow A, Leo YS, Hickey MJ, Morand EF, Ng LF, Bucala R, Mahalingam S. Macrophage migration inhibitory factor receptor CD74 mediates alphavirus-induced arthritis and myositis in murine models of alphavirus infection. Arthritis Rheum. 2013 Oct;65(10):2724–36. doi: 10.1002/art.38090. Permission to reproduce this figure was granted by the publisher John Wiley and Sons

An additional hallmark feature of myoskeletal inflammation in alphavirus-induced disease is the upregulation of a defined set of pro-inflammatory cytokines, namely, MCP-1/CCL2, IFN-γ, and TNF-α; the roles played by these soluble factors in the development of RRV disease had been demonstrated previously [19, 36].

Since CD74 had been shown to (*i*) form complexes with CXCR2 in myeloid cells and CXCR4 in T cells, (*ii*) associate with CD44, an activation marker displayed by antigen-experienced T cells, and (*iii*) be required for MCP-1/CCL2-dependent macrophage migration, the authors investigated whether the reduced disease severity and tissue inflammation they observed in CD74$^{-/-}$ mice were the result of an altered effector cytokine milieu [24, 30, 31, 37]. At the peak of disease, they found that CD74$^{-/-}$ mice had reduced IL-10 expression in both quadriceps and ankle joints, but that IFN-γ and TNF-α levels were elevated in the tissues of CD74$^{-/-}$ mice compared to those of WT mice. Surprisingly, they reported that the levels of MCP-1/CCL2, IL-6, and IL-1β remained unchanged in CD74$^{-/-}$ mice. These results suggested that the production of—and an impaired response to—pro-inflammatory cytokines was not the cause of reduced disease severity in RRVD.

In the context of alphavirus-induced disease, Herrero et al. hypothesized that if reduced clinical severity in CD74$^{-/-}$ mice was neither due to enhanced viral clearance nor reduced pro-inflammatory cytokine production, a difference in migratory kinetics of specific innate inflammatory cell subsets elicited during infection could explain their observations. The authors proceeded to analyse tissue-infiltrating cell subsets and found a substantial reduction in the overall number of CD45^{+} leukocytes in the quadriceps muscle of infected CD74$^{-/-}$ mice in the acute phase of disease. A more refined subset analysis revealed that the number of Gr-1^{hi} CD11b^{hi} inflammatory monocytes, as well as DX5^{+} NK cells and CD3^{+} T cells, were all significantly reduced in the muscle of CD74$^{-/-}$ mice compared to their WT counterparts. To determine whether this reduction was due to a general defect in migration as a result of CD74 deficiency, they injected RRV intraperitoneally into WT and CD74$^{-/-}$ mice and analysed the composition of leukocytes recruited into the peritoneal cavity 22 h later. Interestingly, they found no difference in the proportions or the total number of recruited F4/80^{+} Gr-1^{hi} inflammatory monocytes and F4/80^{+} Gr-1$^{lo/int}$ macrophages. They further showed that migratory properties of monocytes following intraperitoneal infection were indeed impaired in MIF$^{-/-}$ mice, confirming that if a recruitment defect were observed, it would be more likely to be a ligand-dependent, rather than a signalling-dependent process. This finding is consistent with the notion that adhesion of circulating monocyte to the vascular endothelium, and subsequent extravasation, would be curtailed in the absence of MIF in an inflammatory context [30, 38].

Nevertheless, the reason behind an overall decrease in the number of inflammatory infiltrates in the quadriceps of CD74$^{-/-}$ mice remained unexplained. This pointed towards the likelihood of yet another immunoregulatory function of CD74. Indeed, when associated with CD44, CD74 has been shown to mediate MIF-dependent protection from apoptosis [37]. CD74 is known to be involved in anti-apoptotic signalling cascades that promote cell survival and proliferation, either through interference with Fas receptor or via upregulation of pro-survival factor BCL-X_L [39, 40]. Using the intraperitoneal RRV infection and recruitment assay described earlier, the authors quantified cellular apoptosis in peritoneal infiltrates using Annexin V staining. They found that in CD74$^{-/-}$ mice, a significantly higher proportion of F4/80^{+} Gr-1^{hi} cells were indeed apoptotic compared to cells recovered from WT mice. This provided some explanation, at least in part,

to the reduced number of cells found in inflamed quadriceps muscle of RRV-infected CD74$^{-/-}$ mice at the peak of disease. In addition, the authors showed that CD74 cell-surface expression on Ly6C^{hi} CD11b^{hi} splenic monocytes of RRV-infected WT mice was being upregulated during the progression of RRV disease, indicating that CD74 expression on monocytes can be correlated with disease severity. This clarified the intrinsic role played by CD74 as a potential key enhancer of cellular migration in the context of RRV-induced tissue infiltration and inflammation.

3 Conclusions

These results, taken together with the previous report on the role of MIF in alphavirus-induced arthritis, further cemented the notion that the MIF-CD74 signalling axis plays a crucial role in the development of alphavirus-induced musculoskeletal disease, through a combination of effects exerted on inflammatory monocyte recruitment, migration, and survival. Through the use of a mouse disease model of alphavirus infection, and access to samples from patients infected with an emerging alphavirus, this study provided valuable insight into disease mechanisms while pointing towards potential therapeutic avenues.

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MIF in Eosinophilic Inflammation

Marcelo T. Bozza, Claudia N. Paiva, and Priscilla C. Olsen

Abstract Eosinophils are granular leukocytes known to have a central role in the effector arm of Th2 immune responses elicited in allergic diseases and parasitic inflammation. Macrophage migration inhibitory factor (MIF), a proinflammatory cytokine that not only contributes to the immune response to infection but also promotes tissue damage in sterile inflammation, and infectious conditions, is important in Th2 immune responses. Activated Th2 cells have increased MIF mRNA and protein, while eosinophils have mRNA and the preformed protein and secrete high quantities of MIF upon stimulation. In animal models of eosinophilic inflammation such as asthma, rhinitis, dermatitis, eosinophilic esophagitis, and helminth infection, the blockage or the genetic lack of MIF causes a significant reduction of the cardinal signs observed in these diseases. Importantly, atopic patients have increased MIF in affected tissues. MIF also affects several aspects of eosinophil physiology including differentiation, survival, activation, and migration. In this chapter, we reviewed the current knowledge of the role of MIF in eosinophil biology and in eosinophilic inflammatory conditions.

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1 Contribution of MIF to the Pathogenesis of Allergic Diseases

1.1 Eosinophilic Inflammation

Eosinophils are differentiated bone marrow-produced inflammatory cells that transit the blood and are recruited to mucosal tissue, especially during the course of inflammatory conditions such as allergy and parasitic infections. IL-5 produced by Th2 cells, among other cells, is critical to eosinophil proliferation and terminal differentiation in the bone marrow, as well as their release in the blood. Along with IL-5, IL-3 and GM-CSF, may prime eosinophils to respond to chemoattractants such as eotaxin-1 (CCL11) produced at inflammatory sites [1]. Upon inflammation, eosinophils are stimulated and activated leading to selective secretion of a multitude of cytokines and chemokines, as well as lipid mediators and eosinophil cationic protein, a major basic protein, eosinophil peroxidase, and eosinophil-derived neurotoxin [2, 3]. Although the effector activity of these cells is considered key to protect host tissue from parasites, the release of eosinophil granule contents may often contribute to tissue damage as well as remodeling in type-2 inflammatory responses [4].

1.2 The Role of MIF in Allergic Asthma and Rhinitis

The first observations suggesting that MIF could participate in allergic processes came from clinical studies with asthmatic and atopic dermatitis patients [5–8]. MIF concentrations are higher in the bronchoalveolar lavage (BAL) and sputum from asthmatic patients compared to healthy controls [7, 8]. Production and secretion of MIF by human eosinophils indicate that these cells are one of the several potential sources of MIF in asthmatic patients [7]. In fact, airway epithelial cells, macrophages, mast cells, and Th2 lymphocytes also produce and secrete MIF and likely contribute to the MIF produced during allergic episodes [9–12]. Overall, these studies added MIF to a long list of inflammatory mediators found in atopic patients with allergic asthma, which are produced by cells involved in the pathogenesis of this complex inflammatory condition. Moreover, polymorphisms in MIF gene have been associated with allergic diseases such as asthma and atopic dermatitis [13–15].

Studies using animal models of atopic asthma consistently demonstrated that MIF is increased in the BAL fluid and has a critical role in allergic inflammatory response, contributing to tissue eosinophilia, mucus production, and airway hyperreactivity (AHR) [12, 14, 16, 17]. MIF expression is also increased in the lungs in a mouse model of chronic asthma, both in epithelial cells and in infiltrating leukocytes [18]. The exact mechanism by which MIF promotes the pathogenesis of asthma is not completely understood. The lack of MIF has been shown to affect the allergic response in the models of ovalbumin-induced asthma in distinct ways (Fig. 1).

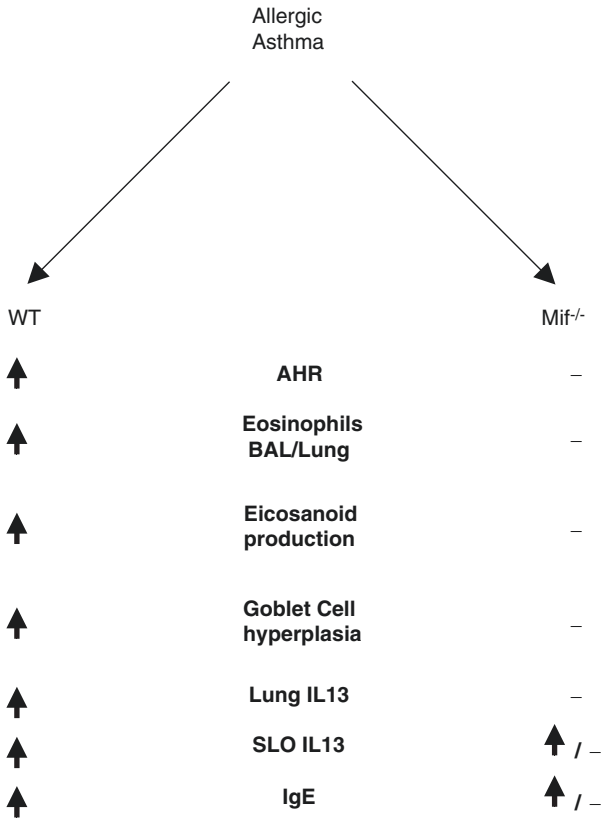

Fig. 1 MIF has a critical role in allergic asthma pathogenesis

In some studies, the abrogation of the cardinal signs of asthma in the absence of MIF is associated with an impaired adaptive immune response, as demonstrated by reduced antigen-specific lymphocyte activation, Th2 cytokine production, and IgE concentrations [12, 14, 19]. Mizue and coworkers attributed the reduced activation of T lymphocytes and reduced production of Th2 cytokines to an impaired antigen presentation function of antigen-presenting cells obtained from T cell-depleted splenocytes from *Mif*$^{-/-}$ compared to wildtype (WT) mice [14]. Another study also demonstrating reduced antigen-specific T cell activation, Th2 cytokine production, and IgE concentrations in mice deficient in *Mif* indicates that macrophages and mast cells, but not DCs, from these animals are defective in activating antigen-specific CD4+ T lymphocytes [12]. Reconstitution of ovalbumin-sensitized *Mif*$^{-/-}$ mice with mast cells from WT mice restored the recruitment of eosinophils to the airways and the serum concentrations of IgE, indicating a role of mast cell-derived MIF in the priming phase of the adaptive immune response.

Using a model of epicutaneous sensitization with ovalbumin in the absence of adjuvant, *Mif*$^{-/-}$ mice have an impaired T cell response with reduced antigen-induced T cell proliferation, Th2 cytokine production, total IgE, and ovalbumin-specific IgG1 serum concentrations as well as lung eosinophilic inflammation upon

intranasal challenge [19]. This study demonstrated that MIF contributes to both the sensitization and the elicitation phases of T cell activation. Interestingly, upon epicutaneous sensitization with ovalbumin, *Mif*$^{-/-}$ mice have an increased number of CD4+Foxp3+ T regulatory cells (Tregs) in draining lymph nodes [19]. Mice deficient in CD74, a MIF receptor, are also defective in generating a Th2 response to epicutaneous sensitization. Interestingly, MIF from T cells, but not from antigen-presenting cells, is essential for T cell activation. *Mif*$^{-/-}$ DO11.10 TCR transgenic T cells are defective in their proliferation and IL-2 production when compared with transgenic T cells derived from WT mice, independent of whether the antigen-presenting cells come from WT or *Mif*$^{-/-}$ mice [19].

A study using an experimental model of asthma induced by ovalbumin demonstrates that *Mif*$^{-/-}$ mice present a profound reduction of AHR, lung eosinophilia, mucus metaplasia lung inflammatory cytokines (IL-13, IL-5, and eotaxin), and lipid mediators (Cys-leukotrienes) despite high serum IgE and Th2 cytokine concentrations in draining lymph nodes [17]. Moreover, allergic WT mice show an increase of eosinophil numbers in the blood and bone marrow, which is not observed in the *Mif*$^{-/-}$ mice. Consistently, treatment of mice and rats with antiMIF antibodies in the challenge phase reduces AHR and tissue eosinophilia without affecting Th2 differentiation and IgE concentrations [16, 17, 20]. These features are also observed in transgenic mice over-expressing Thioredoxin-1 [21]. These transgenic mice compared to WT mice present reduced concentrations of MIF, IL-13, and eotaxin in the lungs, abrogation of the cardinal features of asthma but a preserved systemic Th2 response and IgE concentrations. Thioredoxin-1 binds MIF with high affinity and increases extracellular MIF internalization, suggesting that Thioredoxin-1 expressed on the cell surface serves as one of the MIF-binding molecules and inhibits MIF-mediated inflammatory signals [22]. Interestingly, MIF also belongs to the thioredoxin family of proteins, demonstrating thiol reductase activity [23–25].

In a model of allergic inflammation, MIF is also essential to lipid body biogenesis and leukotriene C4 synthesis in recruited eosinophils, demonstrated by a marked reduction in *Mif*$^{-/-}$ compared with WT mice [26]. Likewise, in vivo administration of recombinant MIF induces eosinophil recruitment to the site of installation and production of leukotriene C4 within newly formed lipid bodies, as demonstrated by EicosaCell methodology. Thus, the critical role of MIF in allergic asthma prevailed with or without impairment of the antigen-specific immune response. In fact, it is not clear why in some studies MIF is essential to Th2 differentiation, while in others it critically contributes to allergic inflammation without affecting the generation of antigen-specific Th2 lymphocytes or IgE concentrations. These observed differences are likely related to variations in the experimental protocols, genetic background, and housing conditions in the different studies.

Tissue remodeling is a characteristic of many chronic inflammatory diseases and an important feature of type-2-mediated pathologies. In asthmatic patients, the cumulative structural changes, including collagen deposition, increased thickness of the subepithelial reticular basement membrane, airway smooth muscle proliferation, goblet cell metaplasia, and mucus plugs, gradually cause progressive loss of pulmonary functions [27]. Although the mechanisms of tissue remodeling are not

well defined and the participation of inflammation in this process is under dispute, eosinophils might contribute to tissue remodeling in some allergic conditions, including some groups of asthmatic individuals [27, 28]. Others, however, observed that eosinophils are dispensable for remodeling and inflammation in an experimental model of asthma induced by house dust mite (HDM) [29]. HDM is a complex and clinical relevant antigen associated with several aspects of asthma pathogenesis, including the activation of lung epithelial cells, dendritic cells, and basophils that culminate in the production of cytokines that affect innate lymphoid cells and T lymphocytes [30, 31].

Considering the abrogation of tissue eosinophilia in experimental models of asthma in the absence of MIF, it would be expected that in chronic asthma, tissue remodeling could be partially dependent on MIF. In fact, the use of a MIF small molecule antagonist, ISO-1, significantly reduces eosinophilic inflammation and prevents changes in airway remodeling in a mouse model of chronic asthma induced by long-term sensitization and challenged with ovalbumin [18]. The inhibitory effect of ISO-1 on airway remodeling is comparable to that of dexamethasone (DEX). The ability of ISO-1 to inhibit eosinophil infiltration and TGF-β expression in the lung tissue of ovalbumin-sensitized mice might have contributed to inhibition of airway remodeling [18]. Whether the observed effects are exclusively related to the blockage of MIF or to the effects of ISO-1 on another target requires further investigations.

In patients with atopic rhinitis, MIF expression is increased in biopsy specimens of the nasal mucous membrane, and markedly in infiltrating eosinophils [32]. Signs of allergic rhinitis induced by alum and ovalbumin immunization and ovalbumin intranasal challenge are abrogated in *Mif*$^{-/-}$ compared to WT mice [33]. These include a significant reduction of eosinophil infiltration and TNF expression in the nasal mucosa of *Mif*$^{-/-}$ mice.

1.3 The Role of MIF in Atopic Dermatitis

Atopic dermatitis is a chronic inflammatory condition that affects the skin, characterized by the presence of eczematous lesions and intense itching. It is a lifelong pathological condition that usually starts at early age, presents with acute flares and is associated with other atopic manifestations such as asthma, rhinitis, and food allergy [34]. Defects of the epidermal barrier with increased exposure to microbial and environmental stimuli are currently considered key to the pathogenesis. Atopic dermatitis is associated with a type-2 immune response with local infiltration of eosinophils and Th2 cells into skin lesions. These infiltrating Th2 lymphocytes are the main sources of IL-4, IL-5, and IL-13, which, together with eotaxin are considered to be important to the pathogenesis [34, 35].

Increased expression of MIF mRNA and protein is observed in inflammatory skin lesions and in sera from atopic dermatitis patients [6, 36]. Importantly, the serum concentrations of MIF in these patients decrease with clinical improvement, suggesting

that MIF might be a marker of disease severity or a mediator that contributes to the inflammatory response and to the pathogenesis [5]. Studies using mouse models of atopic dermatitis indicate that MIF participates in the inflammatory response inducing Th2 cytokines and eotaxin in the skin, and promoting eosinophil recruitment [37]. Transgenic MIF mice have an increased Th2 response and tissue eosinophilia when sensitized and challenged with ovalbumin compared to WT mice. Conversely, the expression of inflammatory cytokines and skin eosinophilic infiltration observed in WT mice after repeated epicutaneous challenge with ovalbumin are virtually absent in *Mif*$^{-/-}$ mice [37]. Moreover, the use of a MIF-DNA vaccination protocol, that elicited the production of endogenous antiMIF antibodies, significantly reduced the inflammatory skin manifestations in mice sensitized and challenged with ovalbumin [38].

The use of more relevant allergens such as ragweed pollen or Japanese cedar pollen confirmed the critical role of MIF in atopic dermatitis, more specifically in a model of conjunctivitis [39]. In this model, mice are systemically immunized with pollen and alum and challenged with pollen via eye drops, after tape-striping the eyelid area. The numbers of conjunctiva- and eyelid-infiltrating eosinophils are significantly increased in pollen-sensitized MIF transgenic mice when compared with WT mice. This change correlates with increased mRNA expression of IL-5, IL-13, and eotaxin in the eyelid skin sites of MIF transgenic mice. Fibroblasts obtained from MIF transgenic mice have a significantly increased expression of eotaxin mRNA and protein upon stimulation with IL-4 compared to WT mice [39]. Conversely, fibroblasts from *Mif*$^{-/-}$ mice showed negligible expression of eotaxin upon stimulation with IL-4. Moreover, stimulation of mouse fibroblasts with recombinant MIF, IL-4, or IL-13 causes an increase in the expression of eotaxin in a mechanism dependent on CD74.

1.4 The Role of MIF in Eosinophilic Esophagitis

Eosinophilic esophagitis (EoE) is a chronic atopic disease, associated with a type-2 immune response, often triggered by environmental agents including food and aeroallergens. Esophageal eosinophilia is the main characteristic of human EoE, but the mechanisms involved in cell accumulation in the tissue and the exact role of eosinophils in disease pathogenesis are not fully understood. EoE patients have an increased deposition of subepithelial collagen fibers and thickening of the basal cell layer due to an increased proliferation of epithelial cells [40, 41].

In esophageal samples from patients with EoE, MIF expression correlates with the number of tissue eosinophils and is detected mostly within the cytosol of cells of the immune system, especially eosinophils [42]. MIF expression is remarkably high in biopsy samples from EoE, but an increase of MIF is also observed in Gastroesophageal Reflux Disease (GERD) patients relative to controls. The inflammatory response of EoE, based on a type-2 immune response, in contrast with the predominant type-1 response in GERD [43], creates an environment in which eosinophils are attracted to and persist within the inflamed mucosa.

In vitro MIF has the ability to directly promote eosinophil chemotaxis in a mechanism dependent on CXCR4 [42], a chemotactic receptor that interacts with

SDF-1α/CXCL12 in addition to MIF [44, 45]. The results indicate that eosinophils constitute a major source of MIF at inflammatory sites in atopic diseases and that MIF influences eosinophil recruitment. Considering that MIF is present mostly in eosinophils within the esophageal mucosa, probably MIF is not a primary trigger in EoE but rather an inflammatory mediator in the effector phase of the disease.

Mif$^{-/-}$ mice are resistant to the increase in eosinophilic infiltration, collagen deposition, and IL-13 expression observed in WT mice in a model of allergic EoE [42]. In vivo administration of recombinant MIF increases tissue eosinophilia and collagen deposition in ovalbumin-sensitized mice. Treatment with antiMIF monoclonal antibody or with a CXCR4 antagonist (AMD3100) in the challenge phase is also highly effective in preventing the signs of EoE, further indicating that the axis MIF/CXCR4 is critically important in the effector phase of the allergic response. CXCR4 is expressed on eosinophils and basophils [46, 47], cells considered essential to the pathogenesis of EoE [48, 49]. Thus, it will be important to characterize the role of MIF and CXCR4 on basophil function and their specific role in the pathogenesis of EoE. Moreover, the contribution of SDF-1α/CXCL12, the cognate ligand of CXCR4, on EoE is unknown and deserves investigation. Together, these results suggest that targeting MIF or the CXCR4 receptor might constitute a therapeutic option to treat patients with EoE. Importantly, AMD3100 (Plerixafor) is approved by the FDA for short-term treatment, and a clinical trial using a long-term and low-dose protocol presented promising results [50].

2 MIF and Helminth Infestation

In past years, several studies analyzed the participation of MIF in helminth infection, pathological conditions frequently characterized by type-2 immune responses. Treatment of mice infected with *Schistosoma japonicum* with antiMIF has no effect in the area of the granuloma compared with IgG-treated controls [51]. Impairment of the Th2 polarization can inhibit eosinophilopoiesis, reduce the size of granulomas and fibrosis on *S. mansoni* infection, as observed in *IL-5*$^{-/-}$ mice or in IL-13/IL4 double-deficient mice [52, 53]. In fact, these studies indicate a profibrotic role of IL-13. In infection with the murine cisticercosis helminthic parasite Taenia crassiceps, IgE concentrations are preserved in *Mif*$^{-/-}$ mice compared to WT mice, suggesting no impairement of the Th2 response in the absence of MIF [54]. *S. mansoni*-infected WT and *Mif*$^{-/-}$ mice have similar plasma concentrations of IL-5 and IL-13 and IgE concentration was even greater in infected *Mif*$^{-/-}$ mice compared to WT mice. *Mif*$^{-/-}$ mice infected with *S. mansoni* present greatly decreased egg granuloma sizes [55]. These granulomas contain fewer eosinophils, which is a phenomenon that is paralleled by decreased bone marrow eosinophilopoiesis in *Mif*$^{-/-}$ mice infected with *S. mansoni*. Together, these results indicate that in *S. mansoni* infection, MIF orchestrates bone marrow eosinophil differentiation and recruitment to granulomas. Although the area of granulomas is decreased in *S. mansoni*-infected *Mif*$^{-/-}$ mice, they present no decrease in fibrosis, the main cause of portal hypertension [55]. This result contrasts with reduced tissue remodeling in mice lacking MIF in models of

allergic asthma and eosinophilic esophagitis, in which there is a significant reduction in IL-13 [18, 42].

In *S. mansoni* infection, similar numbers of adult forms and eggs are present in WT and *Mif*$^{-/-}$ mice [55]. Interestingly, eosinophils are efficient to kill the *S. mansoni* parasite in vitro [56] and to invade schistosomes in vivo [57], indicating that they might participate in defense mechanisms against the parasite. In vivo, however, two mouse lineages deficient in eosinophils have no gross alterations in worm burden or liver fibrosis upon *S. mansoni* infection [58]. In contrast, treatment of mice with neutralizing antibodies against MIF promotes an increased parasite burden and fertility on *S. japonicum* infection compared to control antibody-treated animals [51]. This effect is observed when treatment starts late after infection, but not before infection, suggesting that MIF promotes the control of infection during a determined time window. Moreover, *Mif*$^{-/-}$ mice have previously been shown to poorly control infection with *T. crassiceps* [54]. A recent study has shown that MIF deficiency in a murine infection with *Nippostrongylus brasiliensis*, a helminth similar to the human hookworm, reduced the intestine parasite burden and increased the Th2 response specifically in the draining lymph nodes but not in the spleens [59]. Enhanced Th2 response in this scenario was directly related to a decreased activation of NF-κB and consequent reduction in IL-6 expression in T CD4 *Mif*$^{-/-}$ cells. The MIF tautomerase inhibitor, sulforaphane, used in WT mice infected with *N. brasiliensis* also led to an enhanced Th2 response and clearance of parasite burden, suggesting that MIF's enzymatic activity might have a role in helminth infestation [59]. It is possible that the different outcomes of helminthic infections in the absence of MIF reflect the susceptibility of these parasites to the different defense mechanisms in which MIF interferes, which are currently poorly established.

3 MIF Orthologues from Helminths

Several parasites have orthologues of vertebrate MIF that are likely involved in escape mechanisms [60]. *Brugia malayi*-MIF is the first identified cytokine orthologue from helminth parasites that has the ability to modify host immune responses promoting parasite survival. In fact, MIF orthologues from *Brugia malayi* induce eosinophil inflammation and affect other parameters of the inflammatory response dependent on macrophage activation [61, 62]. Moreover, in association with IL-4, *Brugia malayi*-MIF causes the induction of alternatively activated macrophages [63]. Exposure of monocytes to a MIF orthologue from *Strongiloidis ratti* causes the release of IL-10 instead of TNF, suggesting the involvement of the secreted parasite MIF in immune evasion mechanisms [64]. A MIF orthologue from the nematode *Anisakis simplex* also causes an increase in IL-10 production. This effect is associated with an increased recruitment of CD4+CD25+Foxp3+ regulatory T cells (Tregs) and inhibition of allergic airway inflammation, indicating an opposite role of this MIF in asthma [65]. The induction of IL-10 and Tregs by the MIF orthologue is dependent on TLR2 [66, 67].

4 MIF in Eosinophil Biology

4.1 MIF Is Produced by Eosinophils

Eosinophils contribute to tissue damage and tissue remodeling in type-2 inflammatory responses. Previous studies demonstrated that MIF is produced by a variety of cells and eosinophils constitute an important source of MIF in allergic inflammation and helminth infection [68–70]. Unstimulated human eosinophils have preformed MIF protein and secrete high quantities of MIF upon stimulation with the inflammatory mediators C5a or IL-5 [69]. Similarly, stimulation with PMA causes an early and sustained secretion of MIF in a PKC-dependent manner. At later time points, the production of MIF requires neosynthesis as indicated by reduced secretion in cyclohexamide-treated eosinophils. In vivo, eosinophils present in the inflamed tissue of patients with eosinophilic esophagitis have preformed MIF in the cytosol, constituting the main cell population expressing MIF in this disease [42]. A number of studies have shown important effects of MIF on eosinophil biology (Fig. 2).

4.2 MIF Promotes Eosinophil Recruitment and Chemotaxis

MIF has the ability to directly promote human eosinophil chemotaxis and this effect occurs in a similar concentration range as that of eotaxin [42]. Both, ISO-1, a MIF antagonist, and AMD3100, a CXCR4 antagonist, significantly inhibit the chemotactic effect of MIF. Similarly, MIF promotes the chemotaxis of mouse eosinophils dependent on CXCR2 and CXCR4 [55, 71]. Intrapleural administration of recombinant MIF in mice induces the recruitment of eosinophils [71]. Similarly, intranasal administration of MIF to mice increases the number of eosinophils in the esophagus

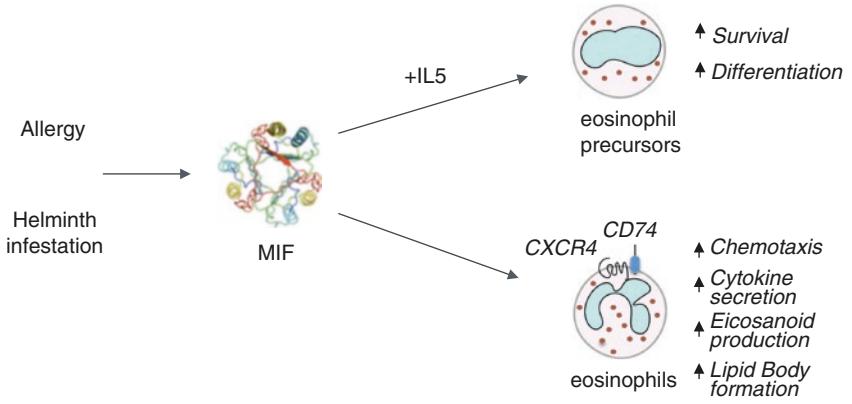

Fig. 2 MIF affects eosinophil development and mature eosinophil biology

[42]. As previously discussed, mice lacking MIF and the blockage of MIF or its receptors (eg. CD74, CXCR4) cause a profound reduction of eosinophil numbers in inflamed tissues in experimental models of allergic and parasitic diseases, demonstrating the general importance of MIF in eosinophil recruitment.

4.3 MIF Causes the Formation of Lipid Bodies and Production of Inflammatory Mediators by Eosinophils

Stimulation of human eosinophils with recombinant MIF induces an increase in the numbers of cytoplasmic lipid bodies and enhanced production of eotaxin and leukotriene C4 [71]. The generation of lipid bodies by MIF is dependent on CD74 expressed on eosinophils. MIF-induced eotaxin acts in an autocrine/paracrine fashion contributing to the generation of lipid bodies dependent on CCR3. Conversely, stimulation of human eosinophils with eotaxin causes the generation of lipid bodies, an effect reverted by antiCD74 neutralizing antibody. Similarly, treatment with eotaxin causes a reduced generation of lipid bodies in eosinophils from *Mif*$^{-/-}$ mice compared to eosinophils from WT. Suboptimal concentrations of eotaxin and MIF have a synergistic effect to induce the generation of lipid bodies [71]. Together, these results suggest that a crosstalk between MIF and eotaxin causing activation of eosinophils.

4.4 MIF Contributes to Eosinophil Maturation Induced by IL-5

IL-5 is a cytokine critically involved in the terminal differentiation of committed eosinophil precursors [72]. Using an in vitro system of eosinophil maturation induced by IL-5, bone marrow from *Mif*$^{-/-}$ mice has a profound defect in generating eosinophils even in the presence of high IL-5 concentrations [55]. Inclusion of rMIF in the *Mif*$^{-/-}$ cultures fully restores the ability of IL-5 to promote accumulation of eosinophils to the numbers achieved in WT. Treatment of *Mif*$^{-/-}$ cell cultures with the pan-caspase inhibitor zVAD restores the number of eosinophils accumulated in the presence of IL-5 to the number of WT controls, indicating that in the absence of MIF the eosinophil precursors are more prone to die by apoptosis [55]. However, in mature eosinophils, the antiapoptotic effect of MIF is minor when compared with GM-CSF [73]. These results indicate that MIF acts as a cofactor necessary to allow optimal IL-5-driven eosinophilopoiesis through the protection of eosinophils, during terminal differentiation induced by IL-5, from programmed cell death. These results also suggest that reduced blood and tissue eosinophilia of *Mif*$^{-/-}$ mice upon type-2 immune responses could in part be due to a defect in eosinophilopoiesis.

5 Perspectives

In recent years several important discoveries regarding the intricate mechanisms of allergic inflammation, especially asthma pathogenesis, have been made with the use of complex and clinically relevant antigens such as house dust mite. It became clear that lung epithelial cells triggering the immune response to allergens by secreting IL-33, IL-25, and TSLP are important. These cytokines contribute both directly and indirectly to the recruitment and activation of type-2 innate lymphoid and Th2 cells, also critically involved in asthma pathogenesis. In order to further establish the role of MIF in the physiopathology of asthma, it will be essential to use better experimental models, including the use of house dust mite. Moreover, future studies should address the role of different MIF cell sources and targets of MIF action with the use of mouse strains with cell-specific deletion of MIF and its receptors. Finally, the use of antiMIF neutralizing antibodies, MIF antagonists, and CXCR4 antagonist in clinical trials of asthma and other allergic diseases will define the importance of MIF in the pathogenesis of human allergy and the putative beneficial effects of blocking MIF in these conditions.

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The Role of MIF in Parasitic Infections

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Abstract Macrophage migration inhibitory factor (MIF) is a pleiotropic molecule with extensive reach and numerous important roles in shaping the immune response to a large variety of infections and inflammatory diseases. MIF was first identified as a factor capable of preventing random macrophage migration in vitro in 1966 (Proc Natl Acad Sci U S A 56(1):72–77, 1966; Science 153(3731):80–82, 1966). Subsequent efforts to characterize the function of MIF have shown that the roles of this molecule extend far beyond the purview of macrophage migration and into antigen-specific responses (Cell Immunol 1:133–145, 1970) macrophage activation and survival (Proc Natl Acad Sci U S A 99(1):345–350, 2002), modulation of glucocorticoid activity to promote inflammation (Ann N Y Acad Sci 210–220, 1999; Nature 377(6544):68–71, 1995), T cell activation (Proc Natl Acad Sci U S A 93(15):7849–7854, 1996), and macrophage phagocytosis (Immunology 92(1):131–137, 1997). MIF also is involved in the coordination between the innate and adaptive immune response. Due to the sheer number of functions performed by MIF during the immune response, its role during parasitic infections has come under increased scrutiny. Interestingly, MIF has been found to be a critical mediator of immunity against a broad range of parasite infections. Here we summarize the findings relevant to the role of MIF during parasitic infections.

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1 The Role of MIF in Malaria

Malaria is a vector-borne parasitic disease which is spread by the bite of female mosquitoes and is caused by organisms of the genus *Plasmodium*. According to the WHO, there were just under 165 million cases of malaria in 2013, with an estimated 854,000 deaths, making malaria one of the most severe infectious diseases in the world. The vast majority of malaria-related deaths were children under the age of 5 [1]. *Plasmodium* infects hepatocytes and erythrocytes in humans and by infecting these cells survives and avoids excessive exposure to the immune system [2–4].

Innate immunity to *Plasmodium* is minimal at best, and acquired immunity is often only observed in those with repeated exposure to the parasite [5, 6]. Complete resistance to malaria infection is often associated with genetic factors, as genes for sickle cell anemia as well as thalassemia and glucose-6-phosphate dehydrogenase deficiency are commonly associated with resistance [7]. Due to the lack of effective human resistance and the hemolytic nature of the disease, the parasite is often able to spread systemically and cause complications including cerebral malaria (CM) and severe anemia, especially in children who have not developed resistance through repeated exposure [8].

Experimental models of malaria have emphasized that the early immune response to *Plasmodium* has the potential to change the course of the disease and prevent or promote complications such as CM and anemia. Numerous studies have suggested a protective role against blood-stage *Plasmodium* parasites for pro-inflammatory, Th1-associated molecules in blood serum such as IL-12 [9], IFN-γ [10–12], and TNF-α [13]. These pro-inflammatory cytokines are primarily responsible for the induction of reactive oxygen species (ROS) and reactive nitrogen species (RNS) by macrophages and monocytes, which play an active role in the elimination of blood-stage parasites during erythrocyte phagocytosis in the spleen [11, 13]. While there is a large amount of evidence to suggest a protective role for the generation of Th1-type inflammation, numerous studies have paradoxically implicated higher serum concentrations of IFN-γ, IL-12, and TNF-α with enhanced pathology [6]. One study links high serum concentrations of the anti-inflammatory molecule IL-10 with protection from CM and anemia [14]. Indeed, some studies have indicated that mortality in children may not be due to a lack of exposure [15] and could be instead due to a systemic overproduction of inflammatory factors such as IFN-γ during reinfection [16, 17]. Thus elimination of *Plasmodium* requires a delicate balance in the immune response to avoid damage to the host and efficiently eliminate the parasite.

One of the factors which underlie this balance is macrophage migration inhibitory factor (MIF). MIF has been shown to function directly in promoting activation and survival of innate immune cells [18–21] and has also been demonstrated to promote activation of T cells [22]. MIF's activity has come under increased scrutiny due to research suggesting that protective immune responses to *Plasmodium* may be dependent on the early immune response by circulating lymphocytes [23]. Importantly, MIF has been shown to be essential in promoting systemic inflammation during the septic shock response [24, 25], underlining its potential importance

during blood-stage *Plasmodium* infections. Numerous studies have shown the importance of MIF during malaria. One study of severe malaria in children associated pathology with a decrease in circulating MIF and MIF transcripts and noted that enhanced MIF expression correlated with high concentrations of IFN-γ and IL-12 in plasma [26]. A separate study of Indian malaria patients noted that genetic abnormalities in the MIF-encoding region and subsequent decreases in MIF expression were correlated with susceptibility [27]. This finding was corroborated by a second genetic study in Kenyan children which focused on mutations in the promoter of the MIF genetic locus, with a noted increase in rates of severe parasitemia among those with mutations in the MIF promoter [28]. Additionally, a study of children with histories of mild or severe malaria found that cases of mild malaria were associated with higher overall concentrations of MIF and MIF transcripts in blood [29, 30]. These studies have outlined a potentially critical role for host-derived MIF expression in limiting complications during malaria.

Interestingly, one study of circulating lymphocyte populations during experimental infections with *P. falciparum* showed that MIF concentrations in sera were reduced precipitously along with concentrations of circulating lymphocytes at the start of the blood-stage of parasitic infection [31]. This raises the possibility that the parasite could downregulate concentrations of host-derived MIF during the start of its symptomatic phase, when it is exposed to circulating monocytes and lymphocytes and therefore vulnerable to immune attack.

As is the case with other pro-inflammatory molecules, MIF expression has also been correlated with the appearance of severe symptoms such as CM and anemia. A study of Indian cerebral malaria patients indicated that higher serum concentrations of MIF in peripheral blood were associated with mortality [32]. Interestingly, in experimental models of malarial anemia, MIF also had an important role. In this model, MIF expression was enhanced by macrophages after hemozoin (malarial pigment) uptake and was correlated with inhibition of erythropoiesis during malaria. This study concluded that MIF expression likely contributed to the observed anemia in malaria patients by inhibition of erythropoiesis [33]. A separate study showed that mice genetically deficient in MIF were more resistant to malarial anemia and displayed a decreased mortality rate in comparison with wild-type controls [34]. However a study in humans showed that MIF levels decreased, while anemia severity augmented during the course of the infection. Diminished levels of MIF were associated with reduced MIF production by monocytes after hemozoin uptake [29, 30]. Thus MIF may play a fundamental role in anemia caused by malaria and also a detrimental inflammatory role during cerebral malaria.

Interestingly *Plasmodium*, like several of the other parasites discussed in this chapter, express a MIF orthologue [35, 36] which is often referred to as *Plasmodium* MIF or PMIF. The expression of this orthologue has become a major topic among malaria researchers because of its potential to modulate the immune response through mimicry or nullification of MIF activity [37]. Recent work with murine infections with *P. yoelii* genetically deficient in MIF has revealed that these MIF orthologues may play a major role in regulation of the parasite's growth, particularly during the liver stage of infection. This study noted that PMIF−/− parasites

were viable and replicated normally in the mosquito vector but also found that the parasite's infection of hepatocytes and subsequent establishment of infection were interrupted [38]. Murine infections with transgenic *P. yoelii* which overexpressed PMIF supported these findings and showed that the parasites were able to establish liver-stage murine infections but developed lower parasitemias with decreased mortality [39].

The mechanisms by which PMIF might modulate the immune response during *P. yoelii* infection were recently investigated using recombinant PMIF. One study by Zhang et al. noted that in vitro incubation with recombinant PMIF inhibited random migration of $CD11b^+$ cells, as is MIF's canonical function, but noted that the release of IL-12, IL-10, TNF-α, and IL-6 by these cells was neither elicited by PMIF incubation nor modulated by PMIF when it was incubated alongside LPS. Additionally, this study reported that the recruitment of inflammatory monocytes from the spleen was altered when PMIF was administered prior to *P. yoelii* infection and noted that populations of activated inflammatory monocytes in both the spleen and serum of infected mice were greatly enhanced by immunization against PMIF [39]. A study by Shao et al. determined that PMIF, like mammalian MIF, elicits a chemotactic response by monocytes in vitro [40]. Another study by Cordery et al. determined that treatment of monocytes with PMIF from *P. falciparum* decreased membrane expression of TLR2 and TLR4, as well as the costimulatory molecule CD86. Additionally, monocyte migration was inhibited in PMIF-treated monocytes. Importantly, this study also noted that PMIF itself was not sufficient to elicit cytokine release from monocytes, which matched the trends observed by Zhang et al. [35, 39]. Localization of PMIF showed that PMIF was excreted from *P. falciparum* during its intraerythrotic stage and accumulated in red blood cells prior to hemolysis. This finding points to an immunomodulatory role for the PMIF which is released when the erythrocyte bursts and the parasite once again comes into contact with the host immune system [35].

In human infections, the study by Cordery et al. showed that PMIF elicits an antibody response but noted that the serum concentration of PMIF antibodies did not correlate with the stage of the disease. The antibody titers of patients followed over the course of this study fell significantly after the resolution of the infection, which suggests that humoral immunity to PMIF may be transient at best [35]. However, a study by Han et al. showed that higher concentrations of PMIF were associated with increased disease severity in human infections with *P. falciparum* and *P. vivax* [41].

While the role of MIF in *Plasmodium* infections is complex and further complicated by the presence of both human and *Plasmodium* orthologues, the research surrounding MIF has indicated that its role is quite extensive. The exact mechanisms of MIF and PMIF activity have yet to be fully elucidated, but early work has shown that MIF's role in counteracting glucocorticoid suppression may be crucial to the development of a balanced immune response to *Plasmodium* parasites. The numerous roles and responsibilities which have been attributed to MIF have ensured that it will be a major topic of investigation by malaria researchers for years to come.

2 The Role of MIF in Toxoplasmosis

Toxoplasma gondii is an intracellular protozoan parasite which infects most warm-blooded animals, including humans, and is the causative agent of the disease toxoplasmosis. Cats and other felines serve as the definitive host in which the sexual cycle of the parasite takes place. In humans, the parasites invade a broad range of tissues, including the brain and muscles, and are usually found forming cysts [42, 43]. It is estimated that almost one third of the human population is infected with *Toxoplasma* [44]. Disease transmission is accomplished by several means, including the consumption of undercooked or contaminated meat, consumption of contaminated water, and exposure to contaminated cat feces, and also by congenital transmission [43, 45].

Most *Toxoplasma* infections in humans are asymptomatic, owing largely to the parasite's intracellular lifestyle and its adaptation to life inside of the human host. Symptomatic infections occur in approximately 10–20% of patients, with common clinical manifestations including lymphadenopathy, fever, headaches, and generalized myalgias [42]. Immunocompromised patients, as well as pregnant women and newborns, are at increased risk of severe complications. In severe cases, toxoplasmosis can disseminate to other areas of the body and damage the brain, heart, skeletal muscle, and intestines [44].

Once the parasite has infected the host, *T. gondii* is able to infect phagocytic cells and nonprofessional phagocytes [46]. The parasite infects the cell in an active invasion process which mediates the formation of the parasitophorous vacuole which is related with successful infection. Also, phagocytosis of the parasite is a common route of infection for professional phagocytes [47, 48].

The immune response which confers protection against toxoplasmosis is largely associated with an optimal CD4+ and CD8+ T cell response. The innate-derived cytokine IL-12 is a major player in resistance against toxoplasmosis. This cytokine is mainly released by dendritic cells and plays a fundamental role inducing IFN-γ by natural killer (NK) cells and CD4+ and CD8+ T cells [49]. IFN-γ promotes host protection via multiple mechanisms including induction of immunity-related GTPases (IRGs) and guanylate-binding proteins (GBPs). The release of IFN-γ also triggers the induction of nitric oxide and reactive oxygen species and is responsible for changes in host metabolism that restrict *T. gondii* replication [50].

Factors which modulate inflammation are critical to the clearance of *Toxoplasma* from the host. MIF, as a potent regulator of the inflammatory response, has been shown to be important in resistance against this parasite. Animal studies conducted using both BALB/c and C57BL/6 mice genetically deficient in MIF have shown a possible protective role for MIF during *T. gondii* infection. MIF−/− mice from both genetic backgrounds were more susceptible to intraperitoneal infection with both the highly virulent RH and the moderately virulent ME49 strains of *T. gondii*. Additionally, it was noted that MIF-deficient mice develop significantly greater liver and brain pathology than wild-type controls [51]. Another study using orally infected BALB/c MIF−/− mice showed similar results, demonstrating that MIF is

essential to resistance in both intraperitoneal and oral parasitic inoculations. Interestingly in MIF-deficient mice, dendritic cells from mesenteric lymph node displayed a less mature phenotype (decreased MHCII, CD80, and CD86 expression) and low IL-12 production compared with WT DCs. In vitro complementary studies by this group showed that bone marrow-derived dendritic cells lacking MIF had an impaired response to *Toxoplasma*-soluble antigens in vitro, strongly suggesting that autocrine MIF production is required for an efficient response of DCs to this pathogen since exogenous MIF restored MIF−/− DC function [52]. In agreement with these findings, oral infection with *T. gondii* in C57BL/6 MIF−/− mice resulted in increased parasitic loads in the intestine accompanied with reduced intestinal inflammation. Of note, oral infection with *T. gondii* in C57BL/6 mice causes inflammatory bowel disease, and the mice died after 15 days of infection, contrary to BALB/c mice which survive [53]. Surprisingly the absence of MIF in C57BL/6 mice also resulted in increased survival compared with MIF-sufficient mice, which died early and showed intestinal inflammation and pathology [54]. This indicates that in this genetic background, MIF can be deleterious for the host, promoting exacerbated inflammation. Interestingly, patients who died from cerebral toxoplasmosis showed little MIF expression in the brain [51].

Because of the potential for severe complications in pregnant women and fetuses, there is significant interest in understanding the immune response to *T. gondii* during pregnancy. Studies have shown an important role for MIF in controlling *Toxoplasma* in placental explants. In vitro infections of human placental explants from the first trimester displayed increased concentrations of MIF and a reduction in the total number of *Toxoplasma* parasites. Explants from the third trimester were more susceptible than those from the first trimester and did not produce MIF. Addition of exogenous MIF was sufficient to decrease parasite loads in both first and third trimester explants [55]. It has also been observed that MIF acts on trophoblasts to control *T. gondii* infection in vitro. Addition of exogenous MIF to infected trophoblasts resulted in reduced parasitic loads when MIF was added at higher concentrations [56].

Overall, the experimental data suggest that MIF is an important mediator of protection during toxoplasmosis. MIF could act enhancing microbicidal mechanisms of macrophages and also increasing their response to cytokines related with protection, for example, by overexpression of TNF-αR and IFN-γR [53]. Together these studies shown that MIF is an important molecule mediating resistance against *Toxoplasma gondii* infection.

3 The Role of MIF During Trypanosomiasis

Trypanosomiasis is a vector-borne disease caused by protozoan parasites of the genus *Trypanosoma*. *Trypanosoma cruzi* is the causative agent of Chagas disease in the Americas, and *Trypanosoma brucei* causes African sleeping sickness. *T. cruzi* transmission occurs when infected triatomine feces contaminate a bite site or mucous membranes. Entering trypomastigotes invade macrophages and differentiate into the

replicative amastigote form before disseminating into the heart, skeletal muscles, and brain. *T. brucei* is transmitted by the bite of an infected tsetse fly. Following the bite, trypomastigotes enter the bloodstream where they replicate extracellularly.

Resistance to *T. cruzi* is dependent on the activation of the innate and adaptive immune responses as mediated by NK cells and macrophages and CD4+ T cells, CD8+ T cells, and B cells, respectively [57, 58]. Pro-inflammatory cytokines that regulate NK cell and macrophage activity like IFN-y, IL-12, and TNF-α play an important role in disease resolution, as does NO production [59–61].

Several groups have examined the role of MIF during trypanosomiasis. Using genetically MIF-deficient mice, Reyes et al. found that MIF−/− BALB/c mice infected with *T. cruzi* displayed increased mortality and higher levels of parasitemia in skeletal muscle and cardiac tissue. Serum from MIF−/− infected mice showed reduced production of the pro-inflammatory cytokines IL-12, TNF-α, IFN-y, IL-1β, and IL-18, with the most pronounced differences at early time points of the infection. Despite severe pathology, hearts from MIF−/− mice had lower mRNA expression of iNOS, IL-12 p35, IL-12 40, and IL-23, suggesting that decreased production of pro-inflammatory cytokines may be responsible for susceptibility. A deficiency in Th1 polarization was reflected by decreased titers of IFN-γ-associated IgG2a [62]. Interestingly, serum levels of the anti-inflammatory cytokines IL-10 and IL-4 were not elevated in MIF−/− mice, eliminating the possibility that a switch of a protective Th1 to Th2 permissive response was the cause of the observed increase in mortality. These data suggest that MIF is necessary for the upregulation of pro-inflammatory cytokines and the generation of a Th1 response during experimental Chagas disease.

Terrazas et al. corroborated the finding that MIF−/− BALB/c mice infected with *T. cruzi* develop increased parasitemia and mortality. In this study, the authors evaluated the early response against *T. cruzi*. Interestingly, MIF levels in serum were increased as early as 12 h post-infection and gradually rose during the first 3 days of infection. In line with early production of MIF during *T. cruzi* infection, other pro-inflammatory cytokines such as IL-1β, IL-12, TNF-α, and IFN-γ were induced early during the infection; however the serum levels of all these cytokines were significantly reduced in MIF-deficient mice.

As a possible cause of the initial impaired inflammatory response in MIF-deficient mice, they found that MIF had an important role during DC activation in vivo and found that DCs had reduced expression of MHC-II, CD80, CD86, OX40L, and IL-12 in the absence of MIF. The authors confirmed these findings in MIF−/− bone marrow-derived DCs in vitro, showing that MIF-deficient DCs had the same reduction in costimulatory molecules as well as impaired IL-12 and TNF-α production in response to *T. cruzi* antigen. Interestingly, addition of exogenous MIF to MIF−/− DCs restored the ability of these cells to efficiently respond to *T. cruzi* antigens in a p38 MAPK-dependent pathway. The inability of MIF-deficient DCs to fully mature and produce IL-12 could help to explain the impaired Th1-associated response observed in mice [63]. However, it is still unknown whether MIF regulates the accumulation or trafficking of DCs or their precursors in vivo or whether MIF affects the activation of a particular subset of DCs.

A study looking at expression profiles in cardiac and skeletal muscle tissue of *T. cruzi*-infected BALB/c mice found high levels of MIF mRNA at early and late

time points. In agreement with the study conducted by Terrazas et al., high MIF expression was observed prior to the induction of TNF-α, IFN-y, or iNOS, suggesting that MIF is part of the primary immune response to *T. cruzi* and is upstream to the expression of pro-inflammatory genes. Histological analysis identified infiltrating lymphocytes as major producers of MIF [64]. Further study is needed to elucidate the role of MIF in different immune cell populations.

Fewer studies have examined the role of MIF in *T. brucei* infection. During *T. brucei* infection, a strong Th1 immune response is required for initial parasite control; however, a persistent pro-inflammatory immune response during the chronic stage is associated with tissue damage, anemia, and increased pathogenicity [65–67]. During the acute phase of *T. brucei* infection in C57BL/6 mice and rats, MIF has been found to be upregulated along with pro-inflammatory genes [68].

A single study by Stijlemans et al. examined the role of MIF in chronic *T. brucei* infection. Similar to *T. cruzi* infection, C57BL/6 MIF−/− mice infected with *T. brucei* had lower serum concentrations of pro-inflammatory IFN-γ and TNF-α at all time points. MIF−/− mice lived an average of 10 days longer than WT mice before succumbing to infection, although no difference was observed in the degree of parasitemia. The same results were achieved using anti-MIF IgG treatment. During the chronic stage of infection, MIF−/− mice had higher serum IL-10 production, reduced liver pathology, and reduced infiltration of inflammatory monocytes and neutrophils into the liver. The altered cell populations were likely due to the reduced expression of the chemokines CCL2, CXCL1, and CXCL5 [69]. It is worth noting that another study looking at the acute stage of *T. brucei* infection showed no reduction of monocyte egress in MIF-deficient mice at 6 days post-infection [70]. Stijlemans et al. isolated neutrophils and monocytes from WT mice with chronic *T. brucei* infection and adoptively transferred them into infected MIF−/− mice (24 dpi). Adoptive transfer of WT neutrophils, but not inflammatory monocytes, into MIF−/− mice increased MIF concentrations and restored MPO activity and TNF-α production. Transfer of WT neutrophils also increased ALT and AST levels in the sera, indicating increased hepatocyte damage. These data suggest that the mechanism for liver injury is MIF dependent for neutrophils, but not for inflammatory monocytes. Additionally, MIF−/− mice had reduced red blood cell death, increased iron bioavailability, and more robust erythropoiesis during later time points of infection. Overall, this study showed a modestly detrimental role of MIF during the chronic stage of *T. brucei* infection [69]. These studies demonstrate an important role for MIF to control *T. cruzi* infection however also indicate that MIF production can result in pathology during *T. brucei* infection.

4 The Role of MIF in Leishmaniasis

Leishmania is a protozoan parasite transmitted by the bite of the sand fly of the genus *Phlebotomus* and *Lutzomyia*. *Leishmania* promastigotes are subdermally inoculated into the host by infected sand flies and are rapidly phagocytosed by

recruited neutrophils. Infected neutrophils then die by apoptosis and are phagocytosed by macrophages or dendritic cells [71]. Once inside the macrophage, the parasite undergoes a morphological switch to its amastigote form, which replicates inside of the phagocyte and causes ulcers in the skin (cutaneous leishmaniasis) or spreads to the bone marrow, spleen, and liver (visceral leishmaniasis) depending of the *Leishmania* species. It is well accepted that the elimination of the parasite relies on the ability of the macrophage to produce nitric oxide. However, the levels of nitric oxide are usually low when the macrophages are infected with *Leishmania* in vitro. The elimination of the parasite requires further stimulation of the infected macrophage with IFN-γ, which triggers nitric oxide production and subsequent elimination of the parasite [72–74]. Studies in experimental models of leishmaniasis have indicated the importance of MIF in parasite elimination. In vitro experiments first demonstrated that stimulation of macrophages with recombinant MIF enhanced parasite killing of *Leishmania major.* The leishmanicidal activity of MIF was dependent on enhanced TNF-α and nitric oxide production [75]. In agreement with this report, MIF-deficient mice were susceptible to *L. major* infection, displaying larger lesions and higher parasite numbers than control mice. Interestingly, the absence of MIF did not affect IFN-γ and IL-4 production during the course of the disease. However, MIF-deficient macrophages displayed reduced nitric oxide and superoxide production in response to IFN-γ compared to MIF-sufficient macrophages. These defects were reflected in the impaired ability of MIF−/− macrophages to eliminate *L. major* in vitro [76]. Thus, in this model, MIF did not affect the Th1 or Th2 response but rather affected macrophage function. Importantly, MIF-producing CD4+ T cells were shown to play an important role mediating protection against *Leishmania pifanoi* after experimental vaccination [77]. The presence of MIF during leishmaniasis has been corroborated in humans. In one study on patients with cutaneous leishmaniasis, MIF levels in serum were elevated [78]. Also, patients with active visceral leishmaniasis caused by *Leishmania infantum* had elevated levels of MIF in serum; however, those patients in remission showed decreased levels of MIF in circulation [79]. As with other parasites, *Leishmanias* also express MIF orthologues. *L. major* secretes two isoforms of MIF that bind to CD74 on macrophages and induce ERK1/ERK2 activation. This pathway reduces apoptosis of infected macrophages and extends parasite survival [80]. Together, these studies show an important role of MIF during leishmaniasis where MIF not only acts by promoting accumulation of macrophages but also has an active role inducing parasite killing.

5 The Role of MIF in Helminth Infections

Helminthes are macroparasites which commonly cause nonlethal disease. However, they usually establish chronic infections in their host. They invade a broad range of organs including the intestine, lungs, liver, lymph nodes, and brain. Despite significant helminth diversity, the immune response mounted against helminth parasites is

most frequently a polarized Th2 response, with elevated production of IL-4, IL-5, and IL-13. These parasites also enhance the production of the regulatory cytokine IL-10. The accumulation of eosinophils and the polarization of macrophages toward an alternatively activated state is also hallmark of helminth infections [81]. Despite the importance of MIF in different infections, there are few reports about how MIF participates in immunity against these macroparasites.

MIF was first reported to be produced in response to experimental schistosomiasis by splenocytes after 8 weeks of infection; however MIF levels were not sustained and decreased over the time [82]. The importance of MIF during schistosomiasis was tested later, where administration of blocking antibodies against MIF resulted in elevated numbers of adult worms but decreased ova production. This effect was seen only when MIF was blocked after 4–6 weeks post-infection [83]. The importance of MIF has been also investigated during cestode infection. During experimental cysticercosis with the metacestode *Taenia crassiceps*, MIF-deficient mice displayed enhanced susceptibility, presenting increased parasitic burdens after 8 weeks post-infection. Interestingly, MIF−/− mice produced similar levels of IL-4 and IFN-γ than their WT counterparts. The susceptibility of MIF-deficient mice against this helminth was associated with the inability of MIF−/− macrophages to produce inflammatory cytokines and nitric oxide [84]. A well-known mechanism of defense against pathogens is the formation of the granuloma, which is commonly structurated with macrophages at its core and surrounded with a variety of immune cells. This structure restricts the spreading or migration of the invading organisms. Interestingly, the granulomas formed in the liver of *S. japonicium*- and in the brain of *T. crassiceps*-infected mice showed enhanced MIF expression [85]. As of yet, it is not known whether MIF deficiency affects granuloma formation or function during helminth infections and has to be further investigated. Apart of the mammalian host, MIF is also important for protection against helminth infection in other organisms. Recently, MIF was detected in snails, a host for schistosomas. Of note, circulating hemocytes expressed MIF, and knockdown of MIF expression in the snail resulted in increased parasite burdens [86], indicating the preserved evolutionary function of MIF as an important molecule for immune function.

Similar to protozoan parasites, helminth parasites express MIF orthologues. Helminth parasite-derived MIF has been shown to interact with the immune system and exert immunomodulatory functions. The filarial parasite *Brugia malayi* secretes MIF, which has 40% homology to human MIF. Similar to human MIF, filarial MIF has the ability to inhibit macrophage migration and also function as a monocyte/macrophage chemoattractant [87]. In addition, *B. malayi* MIF was able to upregulate *Ym1* expression, an alternatively activated macrophage-associated gene, and enhance the recruitment of eosinophils in vivo [88]. Filarial MIF also upregulated the expression of IL-4 receptor on macrophages, thus enhancing their response to IL-4 [89].

In line with the anti-inflammatory properties of helminth-derived MIF, the administration of secreted MIF from *Anisakis simplex* had a beneficial effect in a model of allergic airway inflammation and colitis by augmenting IL-10 production in mice [90, 91]. Also, *Anisakis*-derived MIF downregulated the production of Th2

cytokines in an in vitro culture of peripheral blood mononuclear cells from asthmatic patients [92]. Interestingly, host-derived MIF plays a detrimental role during inflammation, by exacerbating the immune response [93]. In this context, it is possible that parasite-derived MIF competes for the receptors of mammalian MIF, blocking its inflammatory activity. This could be a strategy for the parasite to dampen the host immune response and survive. An evidence of this thought is that a DNA-based vaccine expressing *Trichinella spiralis* MIF promoted a Th1 response and conferred partial protection against *T. spiralis* infection [94], strongly suggesting that parasite-derived MIF could be one of the multiple mechanisms of immune evasion used by helminths. Interestingly, there are no data regarding the role of MIF during helminth intestinal infection, which represents the more common manifestation associated with these organisms. Together these findings identify MIF as an important molecule in the immune response against helminth parasites.

6 Final Remarks

Macrophage migration inhibitory factor (MIF) which is expressed by a variety of immune cells such as monocytes, macrophages, dendritic cells, B cells, T cells, neutrophils, eosinophils, mast cells and basophils was discovered in 1966 and studied as a mediator of delayed type hypersensitivity reactions [95–99]. Here we have presented evidence of the key role of MIF during parasitic infections. In most scenarios, MIF is important to efficiently eliminate parasitic infections, mainly by promoting an efficient response by macrophages and dendritic cells. However, during malaria or *T. brucei* infection, MIF production appears to have an especially complex role, enhancing the inflammatory response and promoting tissue damage. The source of MIF during parasitic infections is still a matter of investigation, but the available evidence points to an early innate immune cell population which initially encounters the pathogen, triggering the release of MIF and promoting an appropriate expression of pattern recognition receptors and the subsequent activation of the cytokine response. Finally, detailed mechanisms of how MIF helps to restrain parasite infections remain to be investigated.

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Parasite MIF Orthologs

Thomas Holowka and Richard Bucala

Abstract Orthologs of macrophage migration inhibitory factor (MIF) have been identified in dozens of protozoan and nematode parasites, and the properties of many of these have been investigated and reported upon. Crystallographic analyses have revealed close structural similarity of these with human MIF, and many of these parasite MIFs are able to bind directly to the human MIF receptor CD74. Additionally, most parasite MIFs demonstrate tautomerase activity similar to that of the human protein. Parasite MIFs demonstrate many of the pro-inflammatory activities of human MIF, including the ability to regulate macrophage migration, stimulate signal transduction pathways, inhibit apoptosis, and promote production of inflammatory cytokines including IL-8 and TNF-α. These properties indicate that parasite MIFs may be involved in altering the immune response during infection, a role that has been confirmed in a handful of animal model studies. These suggest a mechanism in which parasite MIFs signal to host macrophages to influence the ensuing adaptive immune response in order to promote parasite persistence and transmission. Because of the pathogenic impact of parasite MIFs, therapeutic interventions including small molecule inhibitors and immunizations have been explored. It is hoped that therapies targeting parasite MIFs will provide a novel treatment for an array of chronic, difficult-to-treat infectious diseases.

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1 Introduction

Macrophage migration inhibitory factor (MIF) was originally described in 1966 as a soluble factor produced by human lymphocytes and capable of regulating cellular chemotaxis [1]. It subsequently has been characterized as an upstream promoter of inflammation, affecting numerous cellular pathways involved in signal transduction, immune activation, and apoptosis [2]. The multifaceted role of MIF in the mammalian immune system might suggest that this cytokine is uniquely utilized by the immune response of mammals; however it is now appreciated that this is not at all the case. MIF-like molecules, also known as MIF orthologs, are found in organisms at nearly every position of the evolutionary tree. MIF orthologs have been identified in numerous vertebrates including various mammals, birds, and jawed and jawless fish, as well as in invertebrates including shellfish, insects, and snails. Furthermore, MIF is found in organisms outside of the animal kingdom including plants, nematodes, and protozoans [3, 4]. Among these are a variety of parasitic microorganisms, which produce MIF orthologs that are often remarkably similar in structure and function to the MIF molecules employed by the immune systems of the host species.

Besides MIF, there are few reports of parasite-encoded molecules with chemokine- or cytokine-like activity. The best documented parasite protein with cytokine-like activity is cyclophilin 18, which is secreted by *T. gondii.* This cyclophilin was found to bind CCR5 on dendritic cells and macrophages and induce cell migration, proliferation, and production of inflammatory cytokines and NO. The pro-inflammatory activity of this protein was proposed to recruit putative cellular hosts and preserve the host organism from excessive infection [5–7]. The opposite role was found for a chemokine-binding protein secreted by *S. mansoni*, which was observed to have an anti-inflammatory effect on the host response [8]. Neither cyclophilin 18 nor the *S. mansoni* secreted protein resembles any mammalian proteins; however *S. mansoni* and several nematodes including *C. elegans* and *B. malayi* encode proteins that have homologous domains to members of the TGF-β signaling pathway. Both *C. elegans* and *B. malayi* encode TGF-β-like proteins, and *C. elegans* expresses several TGF-β receptors. In all these helminthes, TGF-β signaling appears to act strictly in worm development, and there is no evidence for an effect of these molecules on the host response [9]. To date, the only parasite proteins known to closely mimic the structural, biochemical, and immunological properties of mammalian cytokines or chemokines are the parasite MIF orthologs. The properties of these parasite-encoded MIFs and their impact on the host immune system have been described in dozens of publications, and the findings of these studies will be discussed in the ensuing chapter.

2 MIF-Like Molecules

Before reviewing the current understanding of parasite-encoded MIFs, it is important to touch on the MIF-like molecules that have been discovered in nonpathogenic microorganisms. Soon after discovery of the crystal structure of MIF, it was

recognized to bear close similarity to the bacterial enzymes CHMI (5-carboxymethyl-2-hydroxymuconate isomerase) and 4-OT (4-oxalocrotonate tautomerase), the latter of which dimerizes to form a structure very similar to the MIF monomer. CHMI and the 4-OT dimer additionally trimerize in the same manner as MIF and have tautomerase activity similar to MIF [10, 11]. These bacterial proteins share the N-terminal proline of MIF but otherwise have no sequence homology with the cytokine; thus they cannot be considered true homologs. Nonetheless, CHMI, 4-OT, and MIF are considered principal members of the MIF structural superfamily [11].

MIF orthologs have been identified in a diverse range of eukaryotic organisms, including organisms as simple as protozoans and nematodes. However, MIF is not found in several model organisms including *Drosophila melanogaster* and *Saccharomyces cerevisiae*, indicating that it is not essential in all eukaryotes [11]. Nevertheless, MIF appears to play an important developmental role in *Caenorhabditis elegans*, which encode genes for four distinct MIF orthologs. These orthologs have 15–32% amino acid identity with each other and 22–35% identity with human MIF. Two of these genes, *ce-mif-2* and *ce-mif-3*, are strongly upregulated when the worm enters the dauer stage in response to outside stress [12]. These findings suggest that MIF may have an adaptive function in *C. elegans* during periods of environmental adversity, perhaps including infections; however this possibility has not been fully explored.

Similar to *C. elegans*, many organisms encode multiple MIF-like molecules. For instance, humans and other mammals in fact encode two MIF-like proteins, MIF and D-dopachrome tautomerase (D-DT). The protein D-DT (also referred to as MIF-2, encoded by the *DDT* gene) is a structural homolog of MIF with 34% amino acid sequence identity [13, 14]. D-DT has tautomerase activity similar (though slightly reduced) to that of MIF, but it lacks the amino acid motif known as the "*pseudo*-(E)LR domain" that is necessary for CXCR2 binding [13, 15]. However, D-DT binds CD74 and facilitates much of the same effects on cell signaling and biologic activity, including ERK1/ERK2 activation and upregulation of inflammatory mediators. Similar to MIF, D-DT neutralization protects mice in an experimental model of endotoxic shock, and D-DT has been found to be upregulated in human patients suffering from sepsis [16]. The properties of D-DT and its implication in human disease is only beginning to be investigated, but its extreme similarity to MIF suggests the potential importance of the activity of these two mammalian MIF family members.

Most parasitic organisms that produce MIF-like molecules encode at least two MIF orthologs. In parasitic nematodes, these are divided into MIF-1-type sequences similar to *Ce*-mif-1 and MIF-2-type sequences similar to *Ce*-mif-2 [9]. The MIF-1- and MIF-2-type proteins identified in *C. elegans* are likely to be ancestrally related to the MIF and D-DT found in mammals. Accordingly, the MIF-1-type sequences are somewhat more similar to human MIF, whereas the MIF-2-type orthologs are more similar to the human D-DT in that they universally lack the *pseudo*-(E)LR domain [9, 16]. MIF proteins have been identified in at least two dozen parasitic nematodes, and many of these have been characterized in terms of structural, biochemical, and immunological properties (see Table 1) [9, 17–19]. Additionally, a range of protozoan pathogens including *T. gondii* and species of *Plasmodium,*

Table 1 Properties of parasite MIFs

	Crystal structure	Tautomerase activity	CD74 binding	Chemotactic activity	Signaling/ cytokine stimulation	Animal model studies	References
Parasitic nematodes							
Ancylostoma ceylanicum	X	X	X	X		X	[17, 55]
Anisakis simplex						X	[30, 34, 35]
Brugia malayi	X	X		X	X	X	[18, 23, 25, 33]
Brugia pahangi		X					[24]
Onchocerca volvulus		X*					[23, 29]
Ostertagia ostertagi		X	X		X		[28]
Strongyloides ratti		–			X		[27, 29]
Trichinella spiralis	X	X		X		X	[19, 24, 26, 52, 53]
Trichuris muris		X					[24]
Wuchereria bancrofti		X*					[23, 31, 32]
Parasitic protozoans							
Eimeria acervulina			X	X	X	X	[3, 46–48]
Giardia lamblia	X						[41]
Leishmania major	X	X	X	X	X		[20, 42]
Neospora caninum		–	–				[37]
Plasmodium berghei	X	X*	X		X	X	[21, 39, 44]
Plasmodium falciparum	X	X*	X		X		[21, 39, 44, 45, 51]
Plasmodium vivax	X	X			X	X	[45]
Plasmodium yoelii	X	X			X	X	[38, 43, 50]
Toxoplasma gondii	X	X			X		[22]
Trichomonas vaginalis		X	X		X		[40]

(X) denotes a documented property. (–) denotes absent enzymatic activity or CD74 binding. (X*) denotes oxidoreductase activity

Eimeria, and *Leishmania* also express MIF proteins. Several of these have been found to closely mimic the molecular and biochemical properties of human MIF (see Table 1) [3, 20–22]. However, the role that parasite MIFs play in interactions between the pathogen and host is only beginning to be revealed.

3 Biological and Immunological Properties of Parasitic Nematode MIF Orthologs

The first parasite MIF orthologs were reported in separate publications by Pennock et al. and Pastrana et al. in 1998 [23, 24]. In the former report, MIF-like tautomerization activity was identified in soluble extracts from the parasitic nematodes *Trichinella spiralis*, *Trichuris muris*, and *Brugia pahangi.* The researchers were able to purify MIF orthologs from each of these organisms (*Ts*MIF, *Tm*MIF, *Bp*MIF), and these were found to share 36–47% sequence identity with human MIF [24]. At nearly the same time, Pastrana et al. identified MIF orthologs in *Brugia malayi*, *Onchocerca volvulus*, and *Wuchereria bancrofti* and went on to clone and express the *B. malayi* MIF [23]. Subsequent publications revealed the presence of two MIF orthologs in *B. malayi*, *Bm*MIF-1 and *Bm*MIF-2 [25].

Since the earliest reports of MIF orthologs in *Trichinella*, *Trichuris*, and *Brugia*, additional MIF orthologs have been identified and characterized in over a dozen species of parasitic nematodes [9]. Sequence similarity of these varies somewhat, with MIF-1-type sequences sharing 28–49% identity with *ce-mif-1* and MIF-2-type sequences sharing 28–65% identity with *ce-mif-2*. Western blot and RT-PCR techniques have demonstrated that MIF orthologs are expressed at all life stages of each parasitic nematode tested, with expression greatest in larval stages of *Ancylostoma ceylanicum* and *Strongyloides ratti,* and expression greatest in adult stages of *B. malayi*, *Ostertagia ostertagi*, and *T. spiralis* [17, 18, 26–28]. It was further demonstrated that MIF localized within the hypodermal muscle and/or uterine wall of *B. malayi*, *O. volvulus*, and *T. spiralis* [23, 26, 29]. Additional studies have described the presence of MIF orthologs in soluble extracts and excretory isolates of *A. simplex, B. malayi*, *B. pahangi*, *O. ostertagi, S. ratti*, *T. spiralis,* and *T. muris* [23, 24, 27, 28, 30]. Additionally, antibodies directed against parasitic MIF orthologs have been identified in the sera of humans infected with *O. volvulus*, *W. bancrofti*, and *Strongyloides stercoralis*, in cattle infected with *Onchocerca ochengi*, and in rodents infected with *Litomosoides sigmodontis* and *S. ratti* [27, 29, 31]. These findings further support the excretion of MIF orthologs into extracellular space during infection and also suggest that they are targets of the host immune response.

The crystal structures of three such parasitic nematode MIFs have been solved, including those of *Ancylostoma ceylanicum* (*Ace*MIF), *T. spiralis* (*Ts*MIF), and *B. malayi* (*Bm*MIF-2), at 1.1 Å, 1.65 Å, and 1.8 Å, respectively [17–19]. Each was found to have very similar three-dimensional structures to each other and to human MIF, with trimeric assembly of individual monomers to produce a central pore flanked by β-sheets (see Fig. 1) [18]. However, in each case, critical difference was observed in

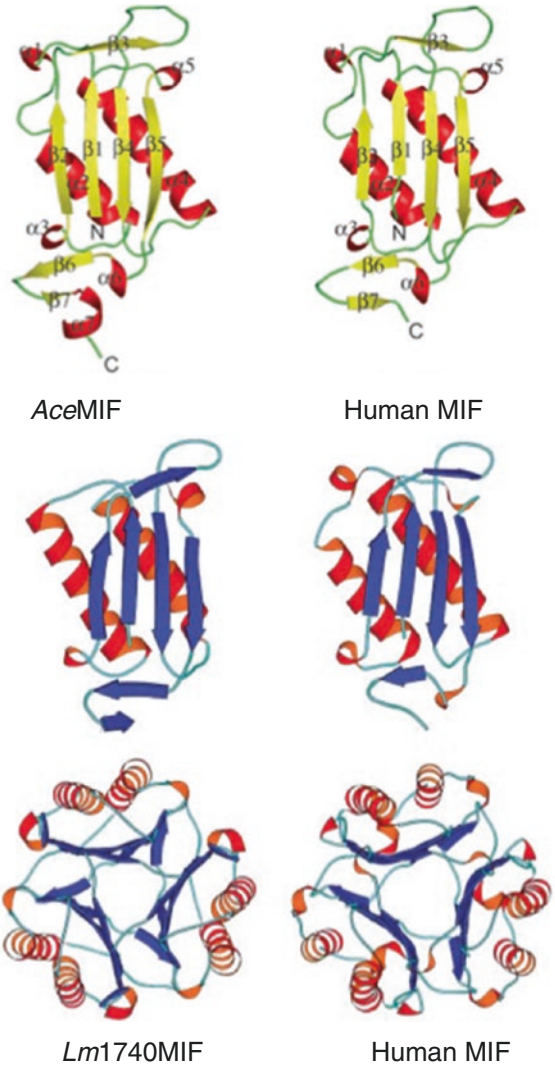

Fig. 1 Parasite MIF crystal structures. Comparisons of crystal structures of *Ace*MIF with human MIF (monomers only) [17] and *Lm*M1740MIF with human MIF [20] (monomers and trimmers)

the identity of residues surrounding the active catalytic site when compared to human MIF. Regardless all were able to catalyze the tautomerization of L-dopachrome methyl esterase with specificity similar to that of human MIF [17–19].

Tautomerase activity has been described in the MIF orthologs of at least eight different parasitic nematodes, including *Onchocerca volvulus*, *Ostertagia ostertagi*, and *Wuchereria bancrofti* (*Ov*MIF-1 and *Ov*MIF-2; *Oos*MIF-1.1, *Oos*MIF-1.2, and *Oos*MIF-2; *Wb*MIF-1 and *Wb*MIF-2) [17–19, 24, 27–29, 31, 32]. Of these, *Ov*MIF-1, *Wb*MIF-1, and *Wb*MIF-2 also demonstrated oxidoreductase activity

using insulin or hydroxyethyldisulfide reduction assays [29, 31, 32]. Oxidoreductase activity has not been demonstrated in most parasite MIF orthologs, despite the conservation of the required C-X-X-C domain in MIF-1-type orthologs. One noted exception is *Wb*MIF-2, which lacks this domain but still functioned as an oxidoreductase [32]. Of all parasitic nematode MIFs that were investigated, only that of *Strongyloides ratti* (*Sra*-MIF) failed to demonstrate any enzymatic activity [27].

In addition to the structural and biochemical properties of parasitic nematode MIFs, the ability to signal to and directly regulate the activity of host cells has been investigated. *Ace*MIF was found to bind highly specifically to the human MIF receptor CD74 with K_d 2.14×10^{-8} M (versus 9.0×10^{-9} M for human MIF) [17]. Similarly, *Oos*-MIF-1.1 was found to bind specifically to human CD74, while a recombinant *Oos*-MIF-1.1 with a mutated N-terminal proline did not [28]. The ability of certain parasitic nematode MIF orthologs to signal directly to host cells can also be inferred by findings that *A. ceylanicum, B. malayi*, and *T. spiralis* MIFs regulate human monocyte migration in vitro [17–19]. *Bm*MIFs-1 and *Bm*MIFs-2 were additionally found to induce expression of TNF-α, IL-8, and host MIF from human monocytes and TNF-α, IL-6, IL-10, and IL-12p40 from mouse bone marrow-derived macrophages [18, 33].

The ability of *Bm*MIF to regulate pro-inflammatory cytokine production is indicative of a role for this protein in regulating the host immune response. Indeed, recombinant His-tagged *Bm*MIF-1 was found to promote recruitment of eosinophils in vivo when injected intraperitoneally into BALB/c mice. Furthermore macrophages isolated from the peritoneal space upregulated Ym1, a marker of alternative activation [25]. In a subsequent study, *Bm*MIFs administered in the presence of IL-4 were inhibited in their ability to upregulate pro-inflammatory cytokines in mouse macrophages in vitro and instead induced markers of alternative activation including Ym1, RELMα, IL-4Rα, and Arginase-1 [33]. These findings suggest that the impact of parasite MIF orthologs may depend on the disease context, such that the normally pro-inflammatory activity of the molecule may be inverted to have an anti-inflammatory effect.

A MIF ortholog from the parasitic whale worm *Anisakis simplex* (*As*MIF) had a surprising impact on the immune response in various disease contexts. When recombinant *As*MIF was administered to mice in an experimental model of OVA/alum-induced asthma, disease pathology and T_H2 cytokine production were greatly reduced. The impact of *As*MIF on airway disease was concurrent with enhanced recruitment and activity of T_{reg} cells in the spleen and lungs and was dependent on TLR2 expression [30]. In a similar study, recombinant *As*MIF administered in a mouse model of ulcerative colitis was found to recruit T_{reg} cells, reducing the production of inflammatory cytokines IFN-γ, IL-6, and IL-13 and ameliorating overall disease pathology [34]. Additionally, *As*MIF was observed to directly promote IL-10 production in vitro in mouse epithelial cells concurrent with upregulation of TLR2 [34, 35]. These findings suggest a surprising anti-inflammatory activity of parasite MIF orthologs, an effect mirrored in a separate study demonstrating that *Strongyloides ratti* MIF induced upregulation of IL-10 but not TNF-α in human

monocytes [27]. These studies make it clear that parasitic nematode MIFs may exert a complex influence on the host immune response, which demands further study in the various disease contexts associated with these pathogens.

4 Biological and Immunological Properties of Parasitic Protozoan MIF Orthologs

Shortly after the identification of MIF orthologs in species of parasitic nematodes, a MIF ortholog was found in the genome of *Plasmodium falciparum*, a protozoan parasite responsible for malaria in humans [36]. MIF orthologs were later identified in the genomes of a variety of apicomplexan parasites, including additional species of *Plasmodium*, *Toxoplasma gondii*, and *Neospora caninum*, and species of the avian parasite *Eimeria* [3, 37]. Crystal structures have since been solved for MIF orthologs from *T. gondii* (*Tg*MIF, at 1.82 Å), *P. falciparum* (*Pf*MIF, at 2.2 Å), *P. berghei* (*Pb*MIF, at 1.8 Å), and *P. yoelii* (*Py*MIF, at 1.8 Å), and all show close structural similarity to mammalian MIF [21, 22, 38]. These orthologs each demonstrated tautomerase activity similar to, but somewhat diminished from, mammalian MIF, most likely due to structural differences in residues at the catalytic site. Oxidoreductase activity was absent from *Tg*MIF; however *Pb*MIF and *Pf*MIF were catalyzed reduction of 2-hydroxyethyldisulfide despite major alterations in the positions of cysteine residues critical for oxidoreductase activity of mammalian MIFs [21, 22, 38, 39]. Interestingly, neither oxidoreductase nor tautomerase activity was identified in *N. caninum* MIF [37].

In addition to the apicomplexans, MIF orthologs have been identified and characterized in other parasitic protozoan organisms, including *Giardia lamblia*, *Trichomonas vaginalis*, and different species of *Leishmania* [20, 40, 41]. The crystal structures for *G. lamblia* MIF (*Gl*MIF) and both *Leishmania major* MIFs (*Lm*1740MIF and *Lm*1750MIF) have been solved (*Gl*MIF at 2.3 Å, *Lm*1740MIF at 1.03 Å, and *Lm*1750MIF at 1.9 Å) and all have close structural similarity to mammalian MIF (see Fig. 1) [20, 41, 42]. However, the shape of the central pore running through the *Gl*-MIF trimer was found to have critical differences with that of the *Plasmodium* MIFs and human MIF. Tautomerization activity was present, but reduced, in *T. vaginalis* MIF (*Tv*MIF) and *Lm*1740MIF and absent in *Lm*1750MIF [20, 40, 42].

While several publications have attempted to localize MIF within parasites using immunofluorescence, these studies were often performed with parasites expressing fusion proteins that may not localize in the same fashion as native protein [39, 40]. However, separate reports using antisera demonstrated localization of both *Eimeria acervulina* MIF (*Ea*MIF) and *Nc*MIF to the apical end of the organism near the secretory organelles [3, 37]. These observations suggest active secretion of MIF orthologs, a possibility supported by the identification of these proteins in the excretory products and/or soluble extracts of *P. berghei, P. yoelii E. acervulina, N. caninum*, and *T. vaginalis* [3, 37, 39, 40, 43]. Additionally, MIF orthologs of *P. falciparum*

and *P. vivax* have been found and quantified in the blood of infected patients [44, 45].

The *Ea*MIF protein produced by *E. acervulina* was among the first protozoan MIF orthologs characterized, and it was found to be expressed most highly in the merozoite stage responsible for infecting epithelial cells in the intestine of the host organism, the chicken [3]. *Ea*MIF was found to bind chicken CD74 and regulate migration of chicken monocytes and furthermore enhance LPS-induced inflammatory cytokine production from chicken PBMCs [46–48]. Interestingly, the MIF produced by the chicken host (*C*MIF) differently impacts cell migration and upregulates a separate set of inflammatory cytokines than *E. acervulina* MIF. Additionally, there appeared to be an additive impact of *Ea*MIF and *C*MIF on overall cytokine production [46, 48]. This finding suggests that evolutionary divergence between host and parasite MIFs may impact their activities in a manner that allows the parasite to utilize the host MIF signaling pathways for its own benefit.

T. gondii is an apicomplexan parasite very similar to *Eimeria*, and recombinant *Tg*MIF demonstrated a pro-inflammatory activity similar to *Ea*MIF in that it was able to stimulate IL-8 production in human PBMCs and activate macrophage ERK1/ERK2 signaling [22]. Other parasitic protozoan MIF orthologs that demonstrate pro-inflammatory activity include *Tv*MIF and *Lm*1740MIF, both of which bind directly to human CD74 and regulate ERK1/ERK2 activation and migration of human monocytes [20, 40, 42]. *Tv*MIF additionally stimulates IL-8 production from monocytes and can activate Akt and BAD phosphorylation in prostate epithelial cells. *Tv*MIF activation of signaling pathways was hypothesized to aid prostate tumor growth, and indeed proliferation and invasion by prostate epithelial cells was promoted in the presence of *Tv*MIF [40]. *Lm*1740MIF demonstrated a related ability to promote cell survival by phosphorylating p53, thereby inhibiting cell-intrinsic apoptosis of cells in the presence of nitrogen radicals [20]. The activities of *Tv*MIF and *Lm*1740MIF suggest that parasite MIF orthologs may serve to promote cell growth and survival in a manner that aids parasite survival and/or promotes disease pathogenesis.

Among the protozoan parasite MIF orthologs, those produced by species of *Plasmodium* have been most extensively characterized in vitro and in vivo. *P. falciparum* and *P. vivax* are each known to cause malaria in humans, and each encodes MIF orthologs, *Pf*MIF and *Pv*MIF, respectively, that have been detected in human serum using specific ELISA assays [44, 45]. Concentrations of these MIFs in serum were found to correlate with disease severity, as elevated *Pv*MIF correlated with increased body temperature and high levels of *Pf*MIF was predictive of cerebral malaria. Both cytokines were found to correlate with increased levels of serum pro-inflammatory cytokines including TNF-α, IL-10, and MCP-1 [44, 45]. Serum *Pv*MIF levels additionally correlated with serum human MIF. It was also observed that serum *Pf*MIF and *Pv*MIF levels correlated with overall parasitemia and that the drop in parasitic load posttreatment occurred with an attendant drop in *Plasmodium* MIF levels [45]. Thus, *Plasmodium* MIFs may be both involved in and predictive of disease pathogenesis during malaria in humans.

A Biacore analysis demonstrated direct binding of *Pf*MIF with human CD74, and *Pf*MIF and *P. berghei* MIF (*Pb*MIF) have been observed to bind CD74 in a pull-

down assay and inhibit AP-1 activation in a manner similar to human MIF [39, 44]. Separate studies have reported on the activities of recombinant *Py*MIF produced in its native form by the rodent parasite *P. yoelii* and demonstrated its various abilities to bind mouse CD11b$^+$ splenocytes, activate ERK1/2 signaling, inhibit apoptosis, and regulate the migration of mouse macrophages in vitro [38, 43, 49]. Both *P. yoelii* and *P. berghei* have been shown to express MIF throughout the parasite life cycle, including in the mosquito vector as well as during liver and blood stages in the murine host. Furthermore, in both cases, it has been reported that *Plasmodium* MIF is secreted into the host hepatocyte and/or red blood cell and then into the extracellular milieu when the cell ruptures and merozoites escape [39, 43, 50].

Several publications have reported on the activity of *Py*MIF in mouse models of infection with very different conclusions. In one such study, immunization against *Py*MIF was protective, resulting in better recruitment of CD11b$^+$ monocytes to the spleen and reduced parasitemia [49]. A separate study found a distinct role for *Py*MIF in parasite pathogenesis in vivo, demonstrating that mutant *P. yoelii* deficient in *Py*MIF were inhibited in their ability to progress through the sporozoite stage in the liver, thus suggesting a developmental role for MIF in this organism [50]. Additional studies have found a possibly conflicting, paradoxical role of *Py*MIF in limiting disease pathogenesis. In one of these, addition of exogenous *Py*MIF to infected mice promoted TNF-α and IL-6 production and actually reduced parasitemia [38]. These findings are supported by a separate publication in which transgenic *P. yoelii* that overexpressed *Py*MIF achieved a lower peak parasitemia and reduced mortality of infected mice but a prolonged course of disease [43]. It is difficult to form a solid conclusion on the activity of *Py*MIF in vivo based on these studies, but this may be indicative of multiple roles for this parasite cytokine during different stages of infection.

Another species of *Plasmodium* that infects rodents, *P. berghei*, also has been investigated in mouse models of infection. A mutant strain of *P. berghei* lacking MIF was not impacted in its ability to survive in a mosquito vector or in a mouse host, and its virulence was unchanged [39]. However, mice infected with the MIF-deficient *P. berghei* produced less inflammatory cytokines and showed reduced apoptosis of active CD4 T cells. These T cells persisted and differentiated into memory cells specific to *P. berghei*. Thus, cured mice that were initially infected with MIF-deficient parasites were able to mount a protective CD4 T cell memory response to subsequent infection, whereas mice initially infected with wild-type parasites lacked a strong memory response and succumbed to disease [44]. This startling finding suggests a role for *Plasmodium* MIF in repressing the adaptive immune response and preventing long-term immunity.

5 Role of Parasite MIFs in Modulating the Host Immune Response

Despite the growing body of research on numerous parasitic MIF orthologs, an overriding paradigm for their role in pathogenesis has yet to be proposed. It is possible MIF functions in the development of some of these organisms, as was previously described for *C. elegans*; however the extreme diversity of biology of these pathogens suggests that developmental roles would be very different among their respective MIF orthologs [12]. Additionally, mutant *Plasmodium* parasites lacking MIF did not show basic developmental defects, demonstrating that MIF is not necessary for growth of all parasites [39, 50]. On the other hand, inflammatory and/or immunomodulatory activity has been described for at least a dozen parasitic MIF orthologs. Thus, it appears that the role of parasite MIF orthologs is primarily at the interface with the host immune system during infection.

MIF is known to function as an upstream promoter of inflammation in the mammalian immune response, and indeed a number of parasitic MIF orthologs have demonstrated very similar pro-inflammatory activity in vitro. Regulation of monocytes and/or macrophages has been described for the MIF orthologs of the nematode parasites *A. ceylanicum*, *B. malayi*, and *T. spiralis* and the protozoan parasites *E. acervulina* and *L. major* [17–20, 46]. It is possible that this activity aids the infecting organism either by blocking effective recruitment of monocytes and macrophages or attracting putative hosts for intracellular parasites such as *L. major*. In either case, it is clear that the ability to regulate the migration of host phagocytes could help a variety of parasitic organisms avoid immune destruction.

Several protozoan parasitic MIFs have demonstrated an ability to block host cell apoptosis in a manner similar to mammalian MIF. *Lm*1740MIF was found to inhibit p53 phosphorylation and prevent macrophage apoptosis, an activity proposed to preserve the intracellular niche for amastigote stage *L. major* [20]. *Py*MIF was similarly found to block apoptosis of macrophages via upregulation of Bcl-2 family molecules, while *Tv*MIF stimulated phosphorylation of Akt and BAD, which participate in anti-apoptotic signaling. In both these cases, prevention of apoptosis was proposed to contribute to sustaining inflammatory responses that promote disease pathogenesis [38, 40].

A number of parasitic MIF orthologs directly promote the production of pro-inflammatory cytokines from macrophages and monocytes in vitro. These include MIF orthologs of the parasitic protozoans *E. acervulina*, *P. berghei, T. gondii*, and *T. vaginalis* and of the parasitic nematodes *B. malayi* and *O. ostertagia* [22, 25, 28, 33, 40, 44, 48]. Many of these MIF orthologs stimulate IL-8 production, suggesting a role in directing leukocyte chemotaxis. Stimulation of pro-inflammatory cytokine production was further suggested by studies of the human malaria parasite *P. falciparum* and *P. vivax* in which levels of *Pf*MIF and *Pv*MIF correlated with increased levels of a variety of cytokines related to inflammation and disease pathogenesis [44, 45]. These data represent the sole published studies of the impact of parasite

MIFs in human patients, and they confirm a pro-inflammatory activity of these molecules.

The pro-inflammatory properties of protozoan parasite MIFs in particular have been verified in several animal model studies. The presence of excessive levels of *Py*MIF in mice infected with *P. yoelii* resulted in increased levels of serum pro-inflammatory cytokines. Curiously, lower peak parasitemia and increased survival time were seen in separate studies where *Py*MIF was overexpressed in parasites or given to mice exogenously [38, 43]. These results might suggest that *Py*MIF enhances the immune response in order to limit pathogenesis in the host and allow the parasites to persist for a lengthened period time, increasing the potential window for transmission. A separate study of *Pb*MIF in a mouse model suggested a distinct mechanism in promoting the parasite life cycle. Mice infected with mutant *P. berghei* lacking *Pb*MIF had reduced levels of a variety of pro-inflammatory cytokines, and this reduced inflammatory state correlated with improved survival of CD4 T helper cells. As a consequence, mice infected with *Pb*MIF-deficient parasites were able to develop effective T-cell memory and generated a specific, protective response to a subsequent infection with *P. berghei* [44]. This study suggests the remarkable ability of *Pb*MIF to promote a hyper-inflammatory environment that restricts T-cell memory development and prevents the establishment of protective immunity to subsequent infection.

A role for parasite MIF orthologs in the inflammatory modulation of T cells also has been observed in the setting of *L. major* infection in a mouse model. In addition to preventing apoptosis, *Lm*MIF was found to promote inflammatory activation of macrophages and dendritic cells in vitro. Mutant *L. major* lacking both *Lm*MIF genes were observed to cause attenuated disease pathogenesis in mice concurrent with a more robust T helper cell response (Holowka and Bucala, in preparation). These findings further support a role for protozoan parasite MIFs in the ability to promote an inflammatory environment that restricts the host adaptive immune response and promotes parasite persistence and transmission.

While the protozoan parasite MIFs appear to perturb the immune response through elevated inflammation, several of the parasitic nematode MIFs demonstrate a surprising anti-inflammatory potential. MIF orthologs of *B. malayi* and *S. ratti* have each been observed to upregulate IL-10 production in macrophages, and *A. simplex* MIF was seen to upregulate both IL-10 and TGF-β in dendritic cells [25, 27, 34]. In the case of *A. simplex*, it was observed that administration of recombinant *As*MIF in several inflammatory disease models in mice induced recruitment of T_{reg} cells, possibly downstream of TLR2-dependent IL-10 production. In this manner, administration of *As*MIF was demonstrated to ameliorate disease in murine models of asthma and ulcerative colitis [30, 34, 35]. It is unclear whether *As*MIF plays a similar immunosuppressive role during an actual infection; however it is easy to imagine that such an activity could be highly beneficial in attenuating the immune response and promoting *A. simplex* pathogenesis.

Similar to *A. simplex* MIF, administration of recombinant *Bm*-MIF proteins from *B. malayi* demonstrated counterintuitive effects on the immune response. Injection of *Bm*-MIF into the peritoneum of mice provoked eosinophil recruitment and the upreg-

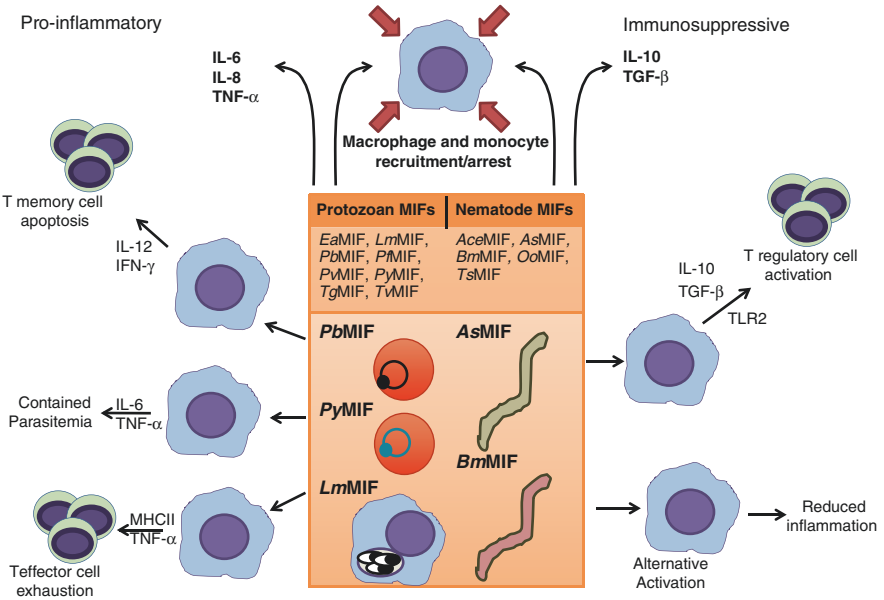

Fig. 2 Immunomodulatory properties of parasite MIFs. Parasite MIFs signal through mononuclear phagocytes to regulate chemotaxis and stimulate inflammatory or immunosuppressive cytokines. Downstream effects may be pro-inflammatory or immunosuppressive and typically impact the T cells of the adaptive immune response

ulation of various markers associated with alternative activation of macrophages. Additionally, treatment of mouse macrophages with *Bm*MIF in vitro enhanced IL-4-induced alternative activation and downregulation of inflammatory cytokine production [25, 33]. These findings suggest that *Bm*-MIF, and possibly other MIF orthologs, function in a context-specific matter such that they may have a pro-inflammatory effect in certain disease settings or an anti-inflammatory effect in others.

There is not a straightforward mechanism for how all parasite MIFs modulate the immune response during disease; however certain unifying principles can be discerned (see Fig. 2). There is good evidence that parasite MIFs function in much the same manner as mammalian MIF, typically binding the host receptor on mononuclear phagocytes and inducing many of the same signaling pathways resulting in effects on chemotaxis, apoptosis, and inflammatory cytokine upregulation. However, the impact of this activity on the course of disease is highly dependent on the ongoing pathology and host immune response during infection. In most cases, it appears that parasite MIF-driven activation of mononuclear phagocytes ultimately impacts the ensuing T cell response, presumably in a manner that is beneficial to the parasite. These effects may include enrichment of cells associated with a T_H2- or T_{reg}-type response that suppresses immunological clearance or inflammatory exhaustion and/or deletion of T helper cells that are critical to generating a protective response and subsequent immunity. Thus, it is proposed herein that MIF orthologs are utilized by parasites to signal to macrophages and monocytes in order to regulate the ensuing T-cell-mediated adaptive response and prevent parasite clearance.

6 Parasite MIF-Based Interventions

The ability of parasite MIF orthologs to potentially support parasite growth and development and simultaneously modulate the host innate and adaptive immune responses makes them intriguing targets for therapy. Intervening in the activity of parasite MIFs could be predicted to aid in clearance of chronic infection and perhaps improve immunity to reinfection of a variety of pathogenic nematodes and protozoans. The therapeutic strategies that have been explored include immunization against parasite MIFs and development of inhibitors that specifically bind to parasite MIF molecules [34, 51–53].

Structural differences among the various MIF orthologs suggest the potential to produce small molecule inhibitors capable of targeting MIFs produced by parasites but not those of humans. Among the parasite MIFs for which a crystal structure has been solved, the tertiary and quaternary structure is very similar to that of human MIF [17, 18, 20–22, 38, 41, 42]. However, nearly all of these were found to have critical difference in residues surrounding the catalytic tautomerization site, affecting the accessibility and/or electrostatic character of this region and the ability to bind model substrate hydroxyphenylpyruvate [17, 18, 20–22, 38, 42]. These structural differences have been implicated in different catalytic activities and reduced susceptibility to human MIF inhibitors. ISO-1, an inhibitor of human MIF tautomerization activity, was found to have a reduced or absent inhibitory effect on MIF orthologs produced by *A. ceylanicum*, *L. major*, *O. ostertagia*, and *T. gondii* [17, 20, 22, 28, 42]. These findings suggest that slight structural differences impact the binding of small molecule inhibitors to different MIF orthologs.

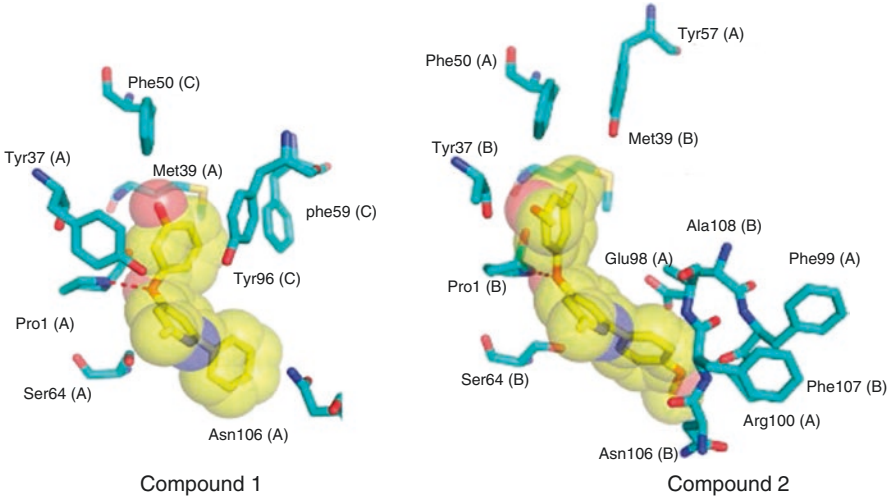

Fig. 3 Specific inhibitors of *Pf*MIF. Interactions of two separate small molecule inhibitors (Compound 1, 3-[(2-methyl-6-phenylpyridin-4-yl)oxy]phenol and Compound 2, 4-(3-methoxy-5-methylphenoxy)-2-(4-methoxyphenyl)-6-methylpyridine) with residues in the tautomerase active site of *Pf*MIF [55]

Several publications have identified small molecules that bind specifically to parasite MIFs and not human MIF. A high-throughput screen against *Ace*MIF-1 identified several selective inhibitors that blocked tautomerization, CD74 binding, and chemotactic potential without impacting human MIF activity. These were additionally found to kill *A. ceylanicum* in vitro [34]. A separate study used virtual screening to identify molecules that inhibited the tautomerization activity of *Pf*MIF at the nanomolar level without inhibiting human MIF [51]. The crystal structures of two of these compounds complexed to *Pf*MIF were solved, and these were demonstrated to block *Pf*MIF binding to human CD74 (see Fig. 3) [54]. These publications serve as proof of concept of the discovery of small molecule inhibitors that may target parasite MIF orthologs without impacting the activity of human MIF.

A separate strategy for targeting parasite MIFs is to vaccinate with recombinant protein in order to provoke a neutralizing antibody response. A previous study demonstrated that vaccination against *Ace*MIF protected hamsters from weight loss and anemia associated with hookworm infection [55]. Separate publications reported vaccination of mice with a DNA plasmid expressing *Ts*MIF alone, alongside the *T. spiralis* MCD-1 protein, or with a *Ts*MIF-*Ts*MCD-1 fusion protein. These vaccines induced a strong T_H1 response and partial protection against *T. spiralis* infection [52, 53]. The plausibility of targeting parasite MIF orthologs for vaccination in humans is confirmed by the presence of immunoreactive antibodies against parasite MIFs in patients infected with *O. volvulus*, *W. bancrofti, S. stercoralis, and P. falciparum* [27, 29, 31, 45].

Studies with mutant *P. berghei* and *P. yoelii* lacking MIF have demonstrated attenuation in parasite pathogenesis and an improved immune response in infected mice [39, 44, 50]. These findings suggest that parasite MIF neutralization by vaccination or with small molecule inhibitors should improve outcomes for the host and potentially bolster the immune response to subsequent infections. Parasitic protozoans and nematodes typically cause chronic infections that may be difficult to treat and do not elicit subsequent protective immunity. Therapeutic inhibition of parasite MIFs is a novel strategy to address both these concerns and may prove useful in the treatment of a wide variety of parasitic pathogens that persist in their hosts due to an ineffective host immune response.

7 Summary

Numerous parasitic nematodes and protozoans produce orthologs of the mammalian cytokine MIF, an upstream regulator of innate immunity and inflammation. Many of these parasite MIFs show structural, biochemical, and immunological properties that are very similar to those of mammalian MIF. While it is not entirely clear how these MIF orthologs are utilized to aid in parasite growth and pathogenesis, a growing body of literature points to an important role in modulating the host immune response. Several parasite MIF orthologs have been studied in

animal models of infection, and they have shown very different mechanisms for altering host immunity, in some cases functioning to enhance inflammatory pathogenesis and in others limiting the development of a protective adaptive response. Animal studies are still lacking in the case of many of the parasite MIF orthologs, as are data regarding their function in human patients. However, current evidence points to a critical role for these molecules in regulating the immune response in order to promote parasite persistence and disease pathogenesis, and as such they are being studied as therapeutic targets. Specific inhibitors of *Plasmodium* MIFs and the development of vaccines against several parasitic nematode MIFs have demonstrated proof of concept that these therapies are viable. Many of the protozoan and nematode parasites that produce MIF orthologs are responsible for chronic, debilitating diseases that do not induce natural immunity and are difficult to treat. Thus, targeting these parasite MIFs may prove to be a critical advance in the battle against this diverse and difficult-to-treat group of human pathogens.

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Index

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