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REVIEW

Insight into the biology of Macrophage Migration Inhibitory Factor (MIF) revealed by the cloning of its cell surface receptor

Lin Leng[1], Richard Bucala[1]

[1]*Departments of Medicine and Pathology, Yale University School of Medicine, The Anlyan Center, S525, 300 Cedar Street, New Haven CT 06520-8056, USA*

The recent cloning of MIF receptor fills an important gap in our understanding of the molecular biology and immunology of MIF. The MIF receptor, like MIF, does not fall into any established family of protein mediators, providing both new challenges and opportunities for the structural and functional analysis of MIF signal transduction.

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Introduction

The protein mediator known as macrophage migration inhibitory factor (MIF) is considered to be one of the first cytokines to be discovered. In the late 1950s, "MIF" described an activity elaborated by activated lymphocytes that arrested the random movement of monocytes/macrophages [1]. This activity correlates with delayed-type hypersensitivity *in vitro*, and it induced significant interest among immunologists for representing a soluble, but non-immunoglobulin, factor that could be studied and manipulated *in vitro*. By 1966, John David and Barry Bloom independently described that MIF was most likely a protein that was secreted into the conditioned medium of activated lymphocytes [2, 3], and immunologists soon measured MIF's eponymous activity by a capillary tube assay. However, MIF was difficult to isolate or clone, so as various other lymphokines (T cell growth factor/IL-2), monokines (endogenous pyrogen/IL-1; tumor necrosis factor/lymphotoxin), and interferons were systematically cloned and produced recombinantly and additional proteins, such as interferon-g and IL-4, were also observed to exhibit migration inhibitory activity, immunologic interest in MIF waned. It wasn't until 1989 that a discrete gene sequence was determined to be associated with MIF [4] and in 1993, pure recombinant protein became available for biologic and structure-function studies [5]. The three-dimensional crystal structure of MIF was solved by three laboratories in 1996, thus revealing a new protein fold and structural superfamily with MIF as its defining member [6-8]. These studies also provided insights into the likely native form of the protein - a homotrimer with a MW of 37.5 kDa (Figure 1).

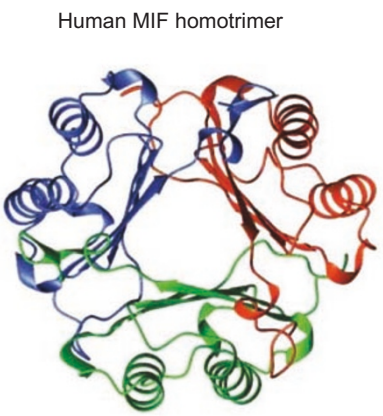

Figure 1 Three-dimensional ribbon diagram of human MIF, revealing its homotrimeric subunit structure. From ref [7]. Each color denotes one monomer.

Correspondence: Lin Leng
E-mail:Lin.Leng@Yale.edu

Abbreviations: Alexa-MIF (Alexa-488-modified MIF); ERK-1/2 (extracellular signal-related kinase); KO (gene knock-out); LPS (lipopolysaccharide); MAPK (mitogen-activated protein kinase); MIF (macrophage migration inhibitory factor); RIP (regulated intramembrane proteolysis); sCD74 (soluble CD74 ectodomain); TLR-4 (Toll-like receptor 4).

The crystal structure determination of MIF showed its marked topologic similarity with certain small bacterial tautomerases such as 4-oxalocrotonate tautomerase. Independent and simultaneous studies by Rorsman and colleagues, who were investigating the enzymatic pathway leading to melanin production, provided evidence that MIF catalyzed the tautomerization of the non-physiologic stereoisomer *D*-dopachrome, a precursor of melanin [9]. Although no physiologic enzymatic activity could be identified, this observation fueled interest into the possible catalytic function of MIF, which together with the lack of molecular information about a cell surface receptor, suggested that MIF mediated its immunologic action via an enzymatic reaction [10]. Site-directed mutagenesis of the protein's N-terminal, putative catalytic proline eliminated bioactivity in some studies [11, 12], but not in others [13-15]. This cast further doubt on the requirement for a cell surface receptor.

The powerful neutralizing action of soluble anti-MIF antibody, both *in vitro* and *in vivo*, together with the retention of MIF biologic activity in many studies of genetically-engineered mutant proteins lacking catalytic activity prompted us to continue the pursuit of a specific, cell surface receptor for MIF. We first addressed this question by studying the signaling pathways that might be activated by MIF stimulation of cells. Beforehand, MIF's effect on cells had been defined biologically; the mediator had significant pro-inflammatory and activating properties on immune cells such as the augmentation of cytokine and NO production and enhanced killing of intracellular pathogens. Many of these actions were attributed to a broad, counter-regulating action on glucocorticoid-mediated immunosuppression of inflammatory responses [16].

We examined phosphorylated substrates within the cytosolic lysates of MIF-stimulated cells. To obviate concerns about contaminating LPS- which has the property of broadly activating diverse signaling pathways in monocytes/macrophages, we performed initial work in MIF-stimulated fibroblasts, which are cells that are relatively insensitive to LPS. We detected an MIF-induced increase in the phosphorylation and activation of the p44/p42 extracellular-signal-regulated kinases (ERK-1/2) family of MAP kinase proteins but not the p38 and SAPK family members [17]. Small molecule inhibitors of ERK-1/2 also were observed to inhibit MIF-dependent ERK-1/2 phosphorylation. The ERK-1/2 proteins are proline-directed, serine/threonine kinases that are generally activated in response to growth or differentiation signals [18, 19]. ERK phosphorylation was detected as early as 30 min after MIF addition but, remarkably, was sustained for a period of at least 24 h. This was surprising because in physiological processes mediated by Ras protein, ERK-1/2 activation is almost always terminated in < 90 min [19, 20]. Notable exceptions include transformation by oncogenic *ras*, and co-stimulation pathways mediated by prolonged integrin ligation [21-23].

An important concept regarding ERK-1/2 signal transduction is that the temporal pattern of ERK-1/2 activation (transient versus sustained) critically determines the fate of the ensuing cellular response [19]. While ERK-1/2 kinase has been best characterized for its role in growth control, ERK-1/2 also activate several downstream effector proteins that are involved in the inflammatory response, such as transcription factors (*c-myc*, NF-kB, and Ets), cytoskeletal proteins mediating membrane activation and phagocytosis, and other protein kinases [19, 24]. Activation of cells by MIF leads to the phosphorylation of ELK-1 [17], which is a member of the Ets family of transcription factors that has gained recent interest because of its role in mediating the expression of Toll-like receptor 4 (TLR-4) [25]. TLR-4 is the receptor for endotoxin (LPS), and it is expressed in reduced levels in mice genetically-deficient in MIF [25]. Among ERK-1/2 effector proteins, cytoplasmic phospholipase A_2 ($cPLA_2$) is an important component of the inflammatory cascade and its enzymatic product, arachidonic acid, is the precursor for the synthesis of prostaglandins and leukotrienes [26]. Arachidonic acid also activates the *c-jun* N-terminal kinase, which is required for the efficient translation of TNFα mRNA [27]. $cPLA_2$ is an important regulatory target for the anti-inflammatory action of glucocorticoids [28], and our experiments showed that MIF could fully "override" glucocorticoid inhibition of $cPLA_2$ activation [17], thus providing one node of molecular control by which MIF-via sustained ERK-1/2 activation - counter-regulates glucocorticoid effects on macrophages. Another target of interaction between MIF and glucocorticoids has been provided by recent studies of MAPK phosphatase (MPK-1) which acts to de-phosphorylate ERK-1/2 and down-regulate the MAPK activation response [29]. MIF downregulates MPK-1, which may contribute to the sustained pattern of MIF activation and glucocorticoid insensitivity of cells.

Cloning of the MIF receptor

The specific ability of MIF to induce cellular ERK-1/2 activation [17] strongly affirmed to us that MIF interacts with a specific cell surface receptor. Our initial attempts to quantify high-affinity cell surface binding sites for MIF were frustrated by the loss of MIF biologic activity upon radioiodination with either tyrosine-directed (lactoperoxidase) or amine-directed (Bolton-Hunter) methodologies. Radioiodination likely results in the oxidation of MIF's cysteine residues, which may need to be in a reduced state

for cytokine activity [30]. MIF that was metabolically labeled with ^{35}S and purified biochemically could be shown to bind to monocytes in a saturable and competitive manner, but the specific activity of binding was too low for the further purification or selection of high-affinity binding sites. We succeeded in creating a fluorescently-labeled MIF species that showed retention of biologic activity (*i.e.* proliferation, inhibition of apoptosis) by linking MIF to the dye Alexa-488 (8-(6-aminohexyl)amninoademosine-3',5'cyclicmonophosphate, bis(triethylammonium)) using mild reaction conditions optimized to produce an average ligand /MIF (homotrimer) ratio of 1:1.

We observed the specific binding of Alexa-MIF to a subpopulation of human THP-1 monocytes by flow cytometry (Figure 2) [31]. High affinity binding sites were increased by the pre-activation of THP-1 monocytes with interferon-γ. Quantitative binding studies performed with increasing concentrations of MIF and analyzed by flow cytometry provided data consistent with two apparent classes of cell surface receptors on THP-1 monocytes. The higher affinity binding activity showed a Kd of 3.7×10^{-8} M , and the lower affinity binding showed a Kd of 3.5×10^{-7} M. Confocal microscopy and direct visualization of human THP-1 monocytes at 4ºC also showed surface binding of Alexa-MIF, and cell-bound Alexa-MIF was internalized upon shifting temperature to 37ºC.

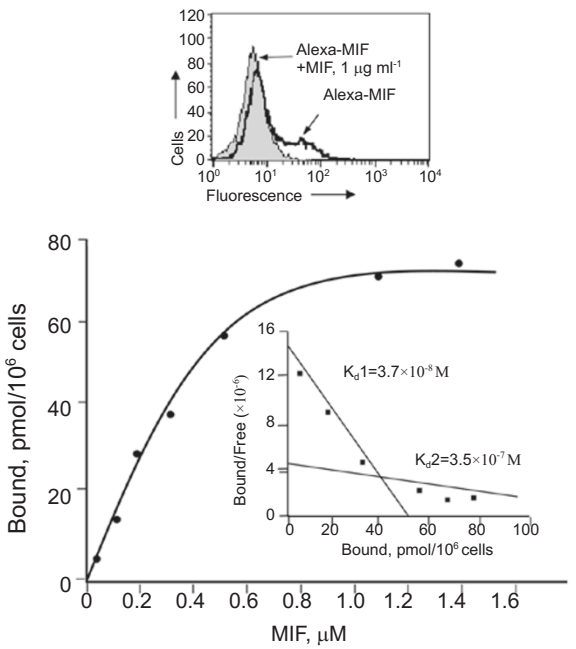

Figure 2 Co-culture of THP-1 human monocytes with IFNã produces a subpopulation of cells with high affinity binding for MIF. Binding data were generated using CellQuest Software (Becton Dickinson, San Jose, CA) from flow data of total THP-1 cells incubated with Alexa-MIF and increasing concentrations of MIF.

To identify the MIF cell surface receptor, we employed an expression cloning strategy based on high-speed fluorescence-activated cell sorting and enrichment of positively-staining clones by re-amplification (Supplementary 1). We constructed a mammalian expression library using cDNA prepared from human THP-1 monocytes. Library aliquots were transfected into COS-7 cells, and the cells which stained with Alexa-MIF purified by fluorescence cell sorting. The cDNA clones then were amplified and re-transfected for additional rounds of cell sorting. After four rounds of FACS selection, single colonies were prepared in *E. coli* and the plasmid DNA isolated. We sequenced 50 clones and found that the majority encoded CD74, the cell surface form of the Major Histocompatibility Class II-associated invariant chain, that is a 31-41 kDa Type II transmembrane protein (Figure 3). Invariant chain has been well-studied for its role as a Major Histocompatibility Class II chaperone [32], and the cell surface CD74 form has a previously described role in immune cell co-stimulation [33, 34]. It is notable that like MIF, cell surface CD74 is expressed as a homotrimer [35].

To assess the functional significance of MIF binding to CD74, we examined the capacity of MIF to stimulate p44/p42 phosphorylation in primary macrophages obtained from mice genetically deficient in CD74 [36]. MIF increased the phosphorylation of the p44/p42 (ERK-1/2) proteins in $CD74^{+/+}$, but not in $CD74^{-/-}$ macrophages, and there was no MIF-dependent increase of PGE_2 production in the $CD74^{-/-}$ macrophages. Transfection of a mammalian CD74 expression plasmid into the COS-7 cell line (which are $CD74^{-/-}$) also reconstituted MIF induction of ERK-1/2 phosphorylation [31].

We measured the binding constant between recombinant CD74 ectodomain ($sCD74^{73-232}$) and MIF by surface plasmon resonance (BIAcore), which quantifies real-time binding interactions by changes in the refractive index of a biospecific surface. BIAcore analysis of the binding interaction between MIF and $sCD74^{73-232}$ revealed an equilibrium Kd of $9.0 \times 10^{-9} - 2.3 \times 10^{-10}$, depending on which molecule of the pair was immobilized to the solid surface. This Kd value may be somewhat lower than the dissociation constant *in vivo* because native CD74 is a trimer and removal of the CD74 transmembrane domain (to facilitate cloning and expression) may inhibit native CD74 oligomerization [37]. Nevertheless, it is well within the range expected for the nanomolar concentrations of MIF that exist in the circulation.

These studies thus established that MIF binds to cells by a specific interaction with the extracellular domain of CD74 to initiate ERK-1/2 activation. This finding was unexpected because the molecular biology of CD74 had been

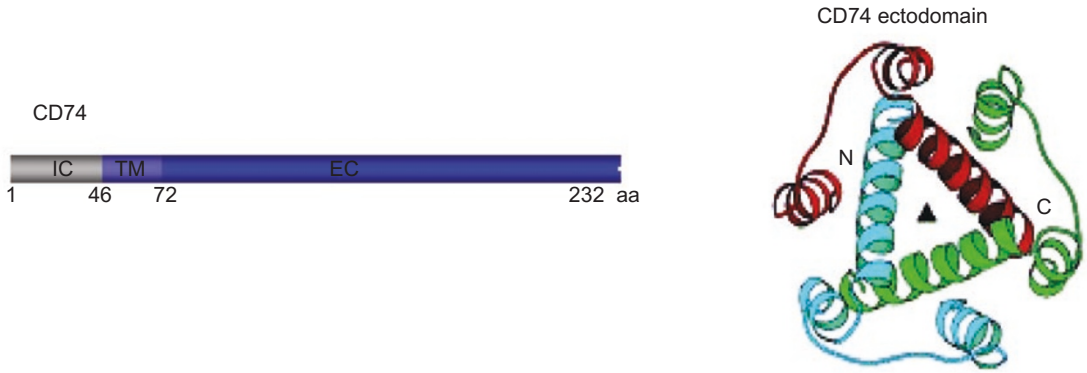

Figure 3 Diagram of human CD74 showing its intracytoplasmic (IC), transmembrane (TM), and extracellular domains (EC). Ribbon diagram of CD74 derived from its three-dimensional crystal structure [35]. Each color denotes one monomer.

so well-defined in the context of its intracellular form as the Class II invariant chain. Nevertheless, it is known that approximately 2-5% of cellular Ii is expressed on the cell surface as CD74 [38,39] and that CD74 expression occurs independently of class II in diverse cell types including monocytes, B cells, activated T cells, and fibroblasts [40, 41]. CD74 surface expression is also known to be regulated by the length of the protein's N-terminal, intracellular domain, which varies depending on which of two in-phase initiation codons are utilized [42]. Whether this differential translation of CD74 mRNA mediates cellular sensitivity to MIF will be important to investigate.

How does the MIF receptor (CD74) explain MIF action?

An evident question is the extent to which CD74-KO mice mimic the phenotype of MIF deficiency. CD74-KO mice were created almost ten years ago [36], and MIF-KO mice have been developed recently by different targeting strategies [43-46]. The MIF KO's display strain-dependent variation in immunologic phenotype, but the recent description of an impaired T_H2 response in these mice mimics what has been reported in mice deficient in CD74 [47, 48]. CD74-KO mice have developmental abnormalities in their B cell compartment, although Class II expression appears unchanged. Nevertheless, the B cell abnormalities in CD74-deficient mice extend beyond what may be expected from the protein's function as a class II chaperone [36]. Idit Shachar and her colleagues have followed up on these observations by studying more the relevant role of CD74 in B cell maturation. Interestingly, the protein has been discovered to undergo regulated intramembrane proteolysis (RIP) [49, 50], which is a recently characterized pathway for the mobilization of dormant transcription factors. Transcription factors of this class are synthesized initially in an inactive form that "nest" as integral membrane precursor proteins. Following cleavage, an active domain is released from the membrane and translocates into the nucleus to activate gene transcription. RIP is known for its conservation in eukaryotic and prokaryotic systems, and it functions to control of diverse biological processes and in response to a variety of signals [51, 52]. In most examples of RIP that have been studied, cleavage occurs by a two-step sequential proteolytic process, with the first step involving the cleavage of the extracytoplasmic segment to shorten the ectodomain to less than 30 aa. A second proteolytic event then occurs within the transmembrane domain, most likely to permit accessibility to the second protease, which releases the product from the lipid bilayer into the cytosol. To determine whether CD74 is processed by RIP, Shachar and colleagues have followed the processing and cleavage of the CD74 extracellular domain, and the release of CD74 intracellular domain followed by its translocation to the nucleus. Entry of the CD74 intracytoplasmic domain into the nucleus leads to the activation of transcription mediated by the NF-kB p65/RelA homodimer and its coactivator, $TAF_{II}105$ [49, 53]. To date, this CD74-dependent activation pathway has been studied exclusively in B cells, and whether there is a role for a ligand, such as MIF, in this process remains to be studied.

How does MIF mediate signal transduction through its receptor?

From a structural perspective, the signaling properties of a CD74 homotrimer are not apparent. The cytosolic domain of CD74 is only 29-46 aa in length - depending on which of two alternative, in-phase initiation codons are translated

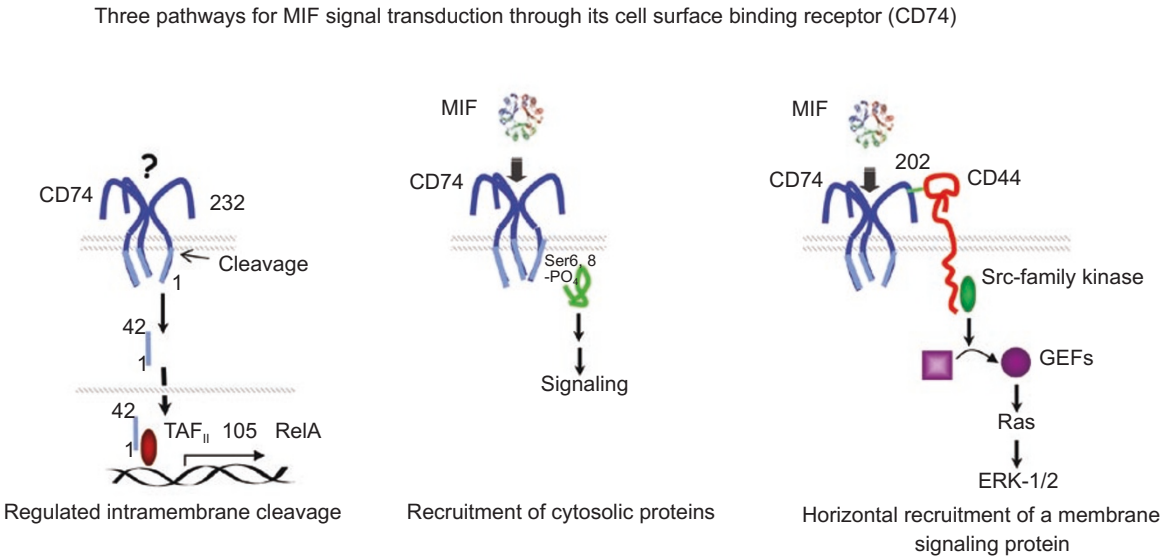

Figure 4 Proposed pathways for MIF signaling based on the known molecular biology of the MIF receptor (CD74).

[42], and it lacks homology with the catalytic domains of tyrosine or serine/threonine kinases, or with domains that might interact with non-receptor tyrosine kinases, or nucleotide binding proteins. The intracytoplasmic portion of CD74 is known to undergo phosphorylation (Ser6, Ser8), in a protein-kinase dependent manner, but evidence supporting ligand activation in this process has not been reported [54]. It nevertheless is noteworthy that recent studies have identified an accessory role for CD74 in T cell stimulation, and by a function that requires a chondroitin-sulphate-dependent interaction between CD74 and CD44 [33, 34]. CD44 is a widely-expressed, polymorphic transmembrane protein with known tyrosine kinase activation properties [55]. Recent evidence in support of a molecular complex between MIF, CD74 and CD44 was provided recently by Meyer-Siegler *et al.* [56], who utilized an immunoprecipitation approach in cultured carcinoma cells.

Figure 4 summarizes our current hypotheses regarding the potential mechanisms for MIF signal transduction via CD74 based on the known molecular biology of these proteins. CD74 RIP is an interesting and well-worked out mechanism for signaling in B lymphocytes. Activation of the RelA/NF-kB family of transcription factors has yet been reported to be a feature of MIF action however, and MIF action in B lymphocytes has not yet been studied. The phosphorylation of the CD74 intracytoplasmic domain is strongly suggestive of a signal transduction function requiring contact with second messengers. We believe that the recruitment of a second transmembrane protein, such as CD44, is a likely mechanism for MIF signaling, especially since CD44 is known to activate Src-family kinases leading to downstream ERK phosphorylation. Receptor activation by such "horizontal" recruitment is typical of proteins that span the membrane only once, and it is the structural basis for signal transduction by several known cytokine receptors [57]. The IL-6 receptor family, for instance, does not contain a functional cytoplasmic or intracellular domain, and is incapable of transducing a signal directly. Rather, the IL-6/IL-6 receptor complex interacts with a second membrane protein, gp130, which then transduces its signal [58]. The highly polymorphic nature of the CD44 ectodomain, which additionally has a role in cell adhesion, also lends itself to cell-specific pleiotropism [59]. Such a pathway is within the expectation for MIF's broad regulatory role in cell survival and apoptosis [46, 60, 61] and suggests that MIF's actions, which have long been considered to be extremely broad for a classical "pro-inflammatory" cytokine, may be productively regulated by the interaction between a cell surface binding protein (i.e. CD74) with a polymorphic, signal transduction molecule (CD44).

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