

# MIF Signal Transduction Initiated by Binding to CD74

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## Abstract

Macrophage migration inhibitory factor (MIF) accounts for one of the first cytokine activities to have been described, and it has emerged recently to be an important regulator of innate and adaptive immunity. MIF is an upstream activator of monocytes/macrophages, and it is centrally involved in the pathogenesis of septic shock, arthritis, and other inflammatory conditions. The protein is encoded by a unique but highly conserved gene, and X-ray crystallography studies have shown MIF to define a new protein fold and structural superfamily. Although recent work has begun to illuminate the signal transduction pathways activated by MIF, the nature of its membrane receptor has not been known. Using expression cloning and functional analysis, we report herein that CD74, a Type II transmembrane protein, is a high-affinity binding protein for MIF. MIF binds to the extracellular domain of CD74, and CD74 is required for MIF-induced activation of the extracellular signal-regulated kinase-1/2 MAP kinase cascade, cell proliferation, and PGE<sub>2</sub> production. A recombinant, soluble form of CD74 binds MIF with a dissociation constant of  $\sim 9 \times 10^{-9} K_d$ , as defined by surface plasmon resonance (BIAcore analysis), and soluble CD74 inhibits MIF-mediated extracellular signal-regulated kinase activation in defined cell systems. These data provide a molecular basis for MIF's interaction with target cells and identify it as a natural ligand for CD74, which has been implicated previously in signaling and accessory functions for immune cell activation.

Key words: cytokine • invariant chain • macrophage migration inhibitory factor • MAP kinase • receptor

## Introduction

Macrophage migration inhibitory factor (MIF)\* is one of the first cytokine mediators to have been described. Its activity was defined in the mid 1960s by immunologists who sought to replicate, in vitro, key features of cell-mediated immunity (1). MIF was identified to be a soluble, T cell-derived factor in 1966 (2, 3), but the protein product resisted biochemical characterization until its cloning in 1989 by David and colleagues (4). A mouse homologue was de-

scribed soon thereafter as a result of investigations into systemically expressed regulators of glucocorticoid action (5). Within a few years, both bioactive MIF protein and neutralizing monoclonal antibodies were produced, and in vitro and in vivo studies established MIF to play an important role in the inflammatory cascade (6, 7).

MIF promotes monocyte/macrophage activation and it is required for the optimal expression of TNF- $\alpha$ , IL-1, and PGE<sub>2</sub> (8–10). MIF-treated macrophages are more phagocytic and better able to destroy intracellular pathogens, such as *Leishmania* (11, 12). These activating functions have been verified by papers in MIF-knockout mice (9, 13, 14), which have also revealed new activities such as the regulation of TLR4 expression (15). MIF's role in adaptive immunity is less well-characterized, but immunoneutralization of

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\*Abbreviations used in this paper: Alexa-MIF, Alexa-488-modified MIF; ERK, extracellular signal-regulated kinase; MIF, macrophage migration inhibitory factor; sCD74, soluble CD74.

MIF inhibits delayed-type hypersensitivity, T cell priming, and antibody production in vivo (16, 17). MIF expression contributes significantly to the immunopathology that results from excessive inflammation and autoimmunity (6, 7, 18), and its role in human disease has been emphasized by the recent description of high-expression *Mif* alleles that are linked to severe rheumatoid arthritis (19, 20).

MIF's molecular mechanism of action appears to be unique among proinflammatory cytokines. MIF broadly counter-regulates the immunosuppressive effects of glucocorticoids (21–24), and at the subcellular level, it induces a sustained pattern of extracellular signal-regulated kinase (ERK)-1/2 MAP kinase activation (25) and maintains proinflammatory function by inhibiting p53-dependent apoptosis (10, 26). Despite evidence for an extracellular mode of action, no cellular receptor for MIF has been described. These circumstances have fueled interest in non-classical mechanisms for ligand activation that have included the role of an intrinsic catalytic activity (27–29) and an endocytic pathway leading to a direct interaction between MIF and the transcriptional coactivator, Jab1 (30).

Using expression cloning and functional analysis, we report the identification of CD74, the cell surface form of the class II-associated invariant chain, as a cell surface binding protein for MIF. MIF binds to CD74 by a high-affinity interaction, and CD74 expression is required for MIF-mediated ERK-1/2 phosphorylation, PGE<sub>2</sub> production, and cell proliferation.

## Materials and Methods

**Cytokines, Antibodies, and Mice.** Human recombinant MIF was prepared from an *Escherichia coli* expression system and purified free of endotoxin by methods described previously (31). Conjugation of MIF to Alexa-488 (32) was performed by the manufacturer's protocol (Molecular Probes). Reaction conditions were optimized to give an average dye/MIF (homotrimer) ratio of 1:1, which was determined by matrix-assisted laser desorption ionization mass spectrometry (33). Recombinant human IL-6 and IFN- $\gamma$  were obtained from R&D Systems.

Human anti-human CD74 mAbs (clones LN2 and M-B741) were obtained from BD Biosciences, and dialyzed free of sodium azide for MIF functional studies. Control studies established that these antibodies do not cross react with recombinant MIF. CD74-KO (34) and wild-type controls were obtained from Jackson ImmunoResearch Laboratories.

**MIF Activity Assays.** MIF-dependent phosphorylation of ERK-1/2 (p44/p42) was measured by Western blotting of cell lysates using specific antibodies directed against phospho-p44/p42 or total p44/p42 (25). MIF-mediated suppression of apoptosis was assessed in serum-deprived, primary fibroblasts by immunoassay of cytoplasmic histone-associated DNA fragments (Roche Biochemicals; references 10, 26). MIF's tautomerase activity was measured by visible spectrophotometry using L-dopachrome methyl ester as a substrate (35). MIF-induced secretion of PGE<sub>2</sub> secretion into medium was measured by specific ELISA (10). Proliferation studies were performed by a modification of previously published procedures (25). Human Raji B cells (American Type Culture Collection) were cultured in RPMI 1640/10% FBS, plated into 96-well plates (500–1,000 cells/

well), and rendered quiescent by overnight incubation in RPMI/0.5% FBS. The cells were washed, the RPMI 1640/0.5% FBS was replaced, and the MIF and antibodies were added as indicated. After an additional overnight incubation, 1  $\mu$ Ci [<sup>3</sup>H]thymidine was added and the cells were harvested 12 h later. Fibroblast mitogenesis was examined in normal human lung fibroblasts (CCL210; American Type Culture Collection) cultured in DMEM/10% FBS, resuspended in DMEM/2% serum, and seeded into 96-well plates (1,500 cells/well) together with MIF and antibodies as shown (Fig. 9 C). Isotype control or anti-CD74 mAbs were added at a final concentration of 50  $\mu$ g/ml. Proliferation was assessed after overnight incorporation of [<sup>3</sup>H]thymidine into DNA.

**Flow Cytometry, Binding Analyses, and Confocal Microscopy.** 2.5  $\times$  10<sup>5</sup> cells/ml THP-1 cells were cultured in RPMI 1640/10% FBS with or without 1 ng/ml IFN- $\gamma$  for 72 h. After washing, 5  $\times$  10<sup>5</sup> cells were resuspended in 0.1 ml of ice-cold PBS, pH 7.4, and incubated with 200 ng of Alexa-488-modified MIF (Alexa-MIF) at 4°C for 45 min. The cells were washed, maintained in ice-cold conditions, and subjected to flow cytometry analysis (FACSCalibur™; Becton Dickinson). In selected experiments, THP-1 monocytes or COS-7 transfectants were incubated with Alexa-MIF together with 50  $\mu$ g/ml anti-CD74 mAb or an isotypic control mAb(36).

Confocal fluorescence microscopy of Alexa-MIF binding to cells was performed with a laser scanning instrument (model LSM 510; Carl Zeiss MicroImaging, Inc.). THP-1 cells were incubated with IFN- $\gamma$  for 72 h and washed three times with PBS/1% FBS before staining for 30 min (4°C) with 2 ng/ $\mu$ l Alexa-MIF or Alexa-MIF plus 50 ng/ $\mu$ l of unlabeled MIF. For double immunofluorescence confocal microscopy, IFN- $\gamma$ -treated THP-1 cells were resuspended in 0.5 ml PBS, pH 7.4, and 20  $\mu$ g/ml Alexa-MIF and 15  $\mu$ g/ml anti-CD74 mAb (clone LN2) and were added at 4°C for 1 h. After washing in ice-cold PBS/2% FBS, the cells were resuspended in 0.1 ml PBS and a rhodamine-conjugated anti-mouse IgG was added at 0°C for 1 h. The samples were washed, resuspended in 0.2 ml PBS, and 30- $\mu$ l aliquots were added to poly-L-lysine-coated slides followed by anti-fade mounting medium (Vectashield; Vector Laboratories). Images were analyzed by Metamorph Intensity Analysis software (Universal Imaging). The percent colocalization for each image was derived from the number of positive pixels common to both images (Alexa-MIF/anti-CD74), divided by the number of positive pixels within the Alexa-MIF image (37). Six cells were analyzed and the significance was determined by the paired Student's t test (independent variables).

**cDNA Library Construction, Expression, and Cell Sorting.** cDNA was prepared from the poly(A)<sup>+</sup> RNA of IFN- $\gamma$ -activated, THP-1 monocytes, cloned into the  $\lambda$ ZAP-CMV vector (Stratagene), and 2.5  $\mu$ g/ml DNA aliquots were transfected into 1.5  $\times$  10<sup>7</sup> COS-7 cells by the DEAE-dextran method (38). The transfected cells were incubated with Alexa-MIF for 45 min at 4°C, washed, and the positively staining cells were isolated with a cell sorter (Moflo; DakoCytomation; reference 36). In a typical run, 1.5  $\times$  10<sup>7</sup> cells/ml were injected and analyzed at a flow rate of 10<sup>4</sup> cells/s. Recovery was generally >90%. Plasmid DNA was extracted from sorted cells using the Easy DNA kit (Invitrogen) and transformed into *E. coli* XL-10 gold (Stratagene) for further amplification. Purified plasmid DNA was retransfected into COS-7 cells for an additional round of sorting. After four rounds of cell sorting, 250 single colonies were picked at random and the insert size was analyzed by PCR. Clones with inserts >1.4 kb were individually transfected into COS-7 cells, and the MIF

binding activity was reanalyzed by flow cytometry and confocal microscopy.

**Protein-Protein Interaction Studies.** Full-length and truncated recombinant CD74 products were generated by PCR and subcloned into the pcDNA 3.1/V5-HisTOPO expression vector (Invitrogen). The fidelity of vector construction was confirmed by DNA sequencing. For pull-down experiments (30), a full-length (V5-CD74<sup>1-232</sup>), NH<sub>2</sub>-terminal-truncated (V5-CD74<sup>46-232</sup>), membrane-truncated (V5-CD74<sup>1-72</sup>), or vector control plasmid was transfected into  $5 \times 10^5$  COS-7 cells using cytofectin (Bio-Rad Laboratories). The cells were harvested 48 h later, lysed with RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% SDS, and 50 mM Tris, pH 7.5), and the lysates were centrifuged at 10,000 *g* for 20 min. Supernatants containing the V5-His-tagged CD74 proteins were incubated with 20  $\mu$ l Ni-NTA-agarose beads (QIAGEN), rocked at 4°C for 1 h, and the beads were collected by centrifugation. After resuspension in PBS, pH 7.4, the bound complexes were incubated with 2  $\mu$ g/ml MIF for 4 h at 4°C. The beads were spun down, resuspended in 1 ml PBS, and washed an additional four times. After SDS-PAGE on 4–20% polyacrylamide gels, the precipitated complexes were analyzed by Western blotting for CD74 (V5 epitope) and MIF.

In vitro transcription and translation was performed using the TNT Reticulocyte Lysate system (Promega). Full-length CD74 (1–232 aa) and three truncated CD74 constructs (1–72 aa, 1–109 aa, and 1–149 aa) were used as templates for coupled transcription and translation in the presence of <sup>35</sup>S-containing amino acids. The binding of <sup>35</sup>S-labeled CD74 to immobilized MIF was assessed by a 3-h incubation at room temperature, as recommended by the TNT protocol (Promega).

**Expression, Purification, and Activity Studies of Soluble CD74 (sCD74).** Truncated, sCD74 proteins comprising the extracellular domain (sCD74<sup>73-232</sup>) and the intracellular/transmembrane domain (sCD74<sup>1-72</sup>) were amplified by PCR and ligated into the pCR T7/CT TOPO *E. coli* expression vector (Invitrogen). After verification of the correct structures by DNA sequencing, the recombinant CD74 proteins were expressed in *E. coli* BL21(DE3)pLysS under IPTG induction. The sCD74 proteins were isolated from *E. coli* lysates by standard methods involving DEAE cellulose chromatography and Ni-NTA affinity chromatography (39). Both sCD74<sup>73-232</sup> and sCD74<sup>1-72</sup> showed single bands by SDS-PAGE and silver staining.

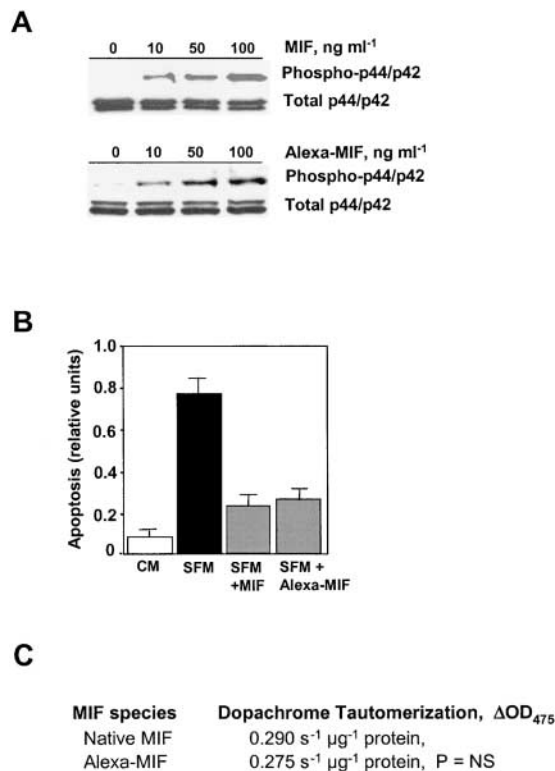
The sCD74 proteins were evaluated for MIF binding by first assessing their ability to inhibit MIF detection in an MIF sandwich ELISA (40). In brief, 96-well plates were coated with an anti-MIF mAb (R&D Systems) at 20 ng/well. After washing, MIF was added together with sCD74<sup>1-72</sup> (intracellular and transmembrane domains) and sCD74<sup>73-232</sup> (extracellular domain) as shown (Fig. 5 C). After incubation at 4°C, the wells were washed, blocked, and a biotinylated anti-MIF pAb (R&D Systems) added. The bound complexes were detected after incubation and washing by adding streptavidin-conjugated alkaline phosphatase (1:60) and *p*-nitrophenylphosphate as substrate.

Real-time binding of MIF to CD74 was measured by surface plasmon resonance using an optical biosensor (model BIA 2000, BIAcore; Amersham Biosciences). The SA sensor chips, amine coupling kit, and BIA Evaluation software were obtained from Amersham Biosciences. MIF or sCD74 was immobilized onto the SA chips in accordance with prescribed methods (41). A surface reference to which no ligand was bound, or to which a membrane protein control (G protein  $\beta\gamma$ ) was bound, was included in the analysis. The derivatized sensor chips were washed and equilibrated in PBS (pH 7.4, 20  $\mu$ l/min), and the ligands

were introduced at five serial dilutions in BIAcore running buffer (150 mM NaCl, 20 mM HEPES, pH 7.4, 2.5 mM MgCl<sub>2</sub>, 1 mM EDTA, 1 mM DTT, and 0.005% P20) in 60- $\mu$ l injection volumes and at a flow rate of 20  $\mu$ l/min. Binding was measured at 25°C for 3 min, followed by 17 min of dissociation. Sensorgram response data were analyzed in the BIA Evaluation Kinetics package and the equilibrium affinity constant calculated (41).

## Results

**Alexa-MIF is Bioactive and Binds to Human Monocytes.** Our initial attempts to prepare an <sup>125</sup>I-labeled MIF species suitable for cell binding studies were frustrated by a loss of MIF bio-activity. We found that linking the fluorescent dye Alexa 488 (32) to recombinant MIF at low-molar density produced an MIF conjugate with full activity in two cell-based assays: (a) stimulation of ERK-1/2 phosphorylation (Fig. 1 A), and (b) protection from apoptosis (Fig. 1 B). Additionally, Alexa conjugation of MIF did not significantly influence MIF's intrinsic tautomerase activity (Fig. 1 C), which is a useful surrogate for the retention of native MIF structure (35).



**Figure 1.** Alexa-488-modified MIF (Alexa-MIF) shows retention of MIF biological activity in established assays. (A) Dose-dependent activation of the p44/p42 (ERK-1/2) MAP kinase cascade in IFN- $\gamma$ -pretreated THP-1 monocytes (25). (B) Suppression of p53-dependent apoptosis in primary human fibroblasts (reference 26; CM, complete medium; SFM, serum-free medium). MIF or Alexa-MIF was added at 50 ng/ml. Data shown are mean  $\pm$  SD of triplicate wells and are representative of three independent experiments. (C) No difference in MIF's intrinsic tautomerase activity was observed in Alexa-MIF versus native (unconjugated) MIF using L-dopachrome methyl ester as a substrate (35).

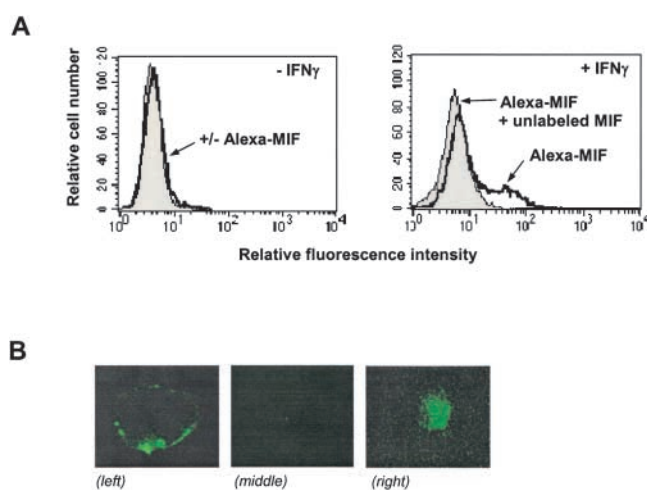
We observed the binding of Alexa-MIF to a subpopulation of IFN- $\gamma$ -activated human monocytes by flow cytometry, and this binding activity was competed by the addition of excess, unlabeled MIF (Fig. 2 A). Confocal microscopy and direct visualization of IFN- $\gamma$ -treated monocytes at 4°C also showed surface binding of Alexa-MIF that was competed by a 25-fold excess of unlabeled MIF. Cell-bound Alexa-MIF was internalized upon raising the temperature to 37°C (Fig. 2 B).

**Expression Cloning of Cell Surface MIF-binding Proteins.** Having prepared a labeled, bioactive form of MIF and identified a cellular source of MIF binding activity, we next constructed a mammalian expression library in the  $\lambda$ ZAP-CMV vector using cDNA from IFN- $\gamma$ -activated, THP-1 monocytes. Library aliquots representing a total of  $1.5 \times 10^7$  recombinants were transfected into COS-7 cells, which we had established previously to exhibit minimal detectable binding activity of MIF (unpublished data), and the transfectants analyzed for Alexa-MIF binding by flow cytometry. Positively staining cell fractions were purified by high-speed cell sorting, and the cDNA clones collected, amplified, and retransfected for additional rounds of cell sorting (Fig. 3 A). Enumeration of the positively staining, sorted cells showed a >400-fold enrichment in MIF binding activity. After four rounds of selection, single colonies were prepared in *E. coli*, and 250 colonies were randomly picked for analysis. We sequenced 50 clones bearing cDNA inserts of >1.4 kb and found that 10 encoded the surface form of the class II-associated invariant chain, CD74 (CD74), a 31–41-kD Type II transmembrane protein(42). The individual clones differed with respect to their total length, but each

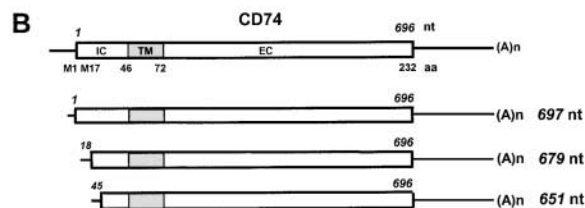
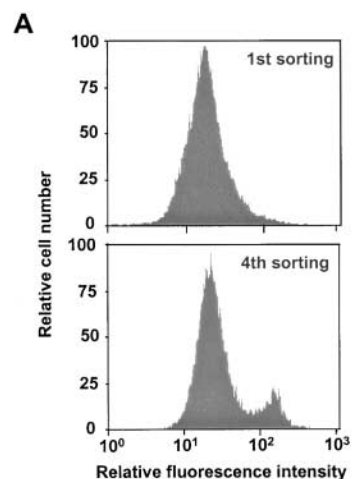
was in the sense orientation and encoded a complete extracellular and transmembrane domain (Fig. 3 B).

**Structural Verification of MIF Binding to CD74.** To verify that CD74 is a cell surface binding protein for MIF, we analyzed the binding of Alexa-MIF to COS-7 cells transfected with a CD74 expression plasmid or a vector control. The binding of Alexa-MIF to the CD74-expressing COS-7 cells was inhibited by excess, unlabeled MIF (unpublished data), and by an anti-CD74 mAb directed specifically against the extracellular portion of the protein (Fig. 4 A). Two-color immunofluorescence confocal microscopy of THP-1 cells showed that MIF colocalized with CD74 in a spacio-temporal specific manner (Fig. 4 B), and the percent colocalization was calculated by Metamorph image analysis to be  $69.2 \pm 12.0$  ( $P = 0.031$ ,  $n = 6$  cells).

We next sought biochemical evidence for an association between CD74 and MIF by performing “pull-down” experiments in cells expressing CD74. MIF was detected by Western blotting of protein complexes precipitated from cells that expressed a V5-tagged, full-length CD74 (V5-CD74<sup>1–232</sup>), an NH<sub>2</sub>-terminal truncated CD74 (V5-



**Figure 2.** Fluorescence analysis of Alexa-MIF binding to cells. (A) Flow cytometry analysis of the binding of Alexa-MIF to THP-1 monocytes. Competition for Alexa-MIF binding was performed in the presence of 20  $\mu$ g/ml of unlabeled MIF. (B) Direct visualization of Alexa-MIF binding to THP-1 monocytes by confocal microscopy. THP-1 cells were incubated with 1 ng/ml IFN- $\gamma$  for 72 h, and stained with Alexa-MIF (left) or with Alexa-MIF plus a 25-fold excess of unlabeled rMIF (middle). Cell-bound Alexa-MIF was rapidly internalized upon shifting cells from 4°C to 37°C for 15 min (right). Magnification: 630.

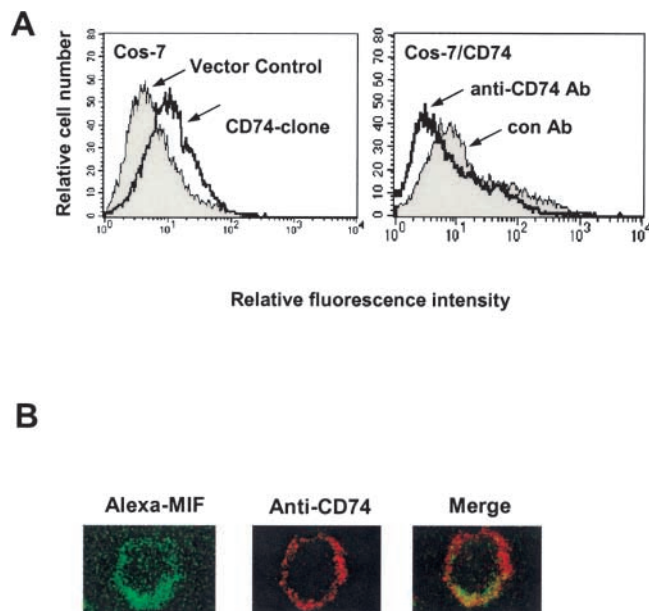


**Figure 3.** Identification by expression cloning of CD74 as a cell surface binding protein for MIF. (A) Progressive enrichment by fluorescence-activated cell sorting of COS-7 cell transfectants showing MIF binding activity. (B) Structure of CD74 (35-kD isoform), and 3 of 10 representative CD74 cDNA clones with MIF binding activity. IC, TM, and EC are the intracellular, transmembrane, and extracellular domains, respectively. M1 and M17 refer to two sites of alternative translation initiation (42).

CD74<sup>46-232</sup>), but not a membrane-truncated CD74 lacking the extracellular domain (V5-CD74<sup>1-72</sup>; Fig. 5 A). [<sup>35</sup>S]-CD74 protein prepared by a transcription and translation-coupled, reticulocyte lysate system also bound to MIF *in vitro*, and a 40 amino acid region within the CD74 extracellular domain (residues 109–149) appears important for MIF binding activity (Fig. 5 B).

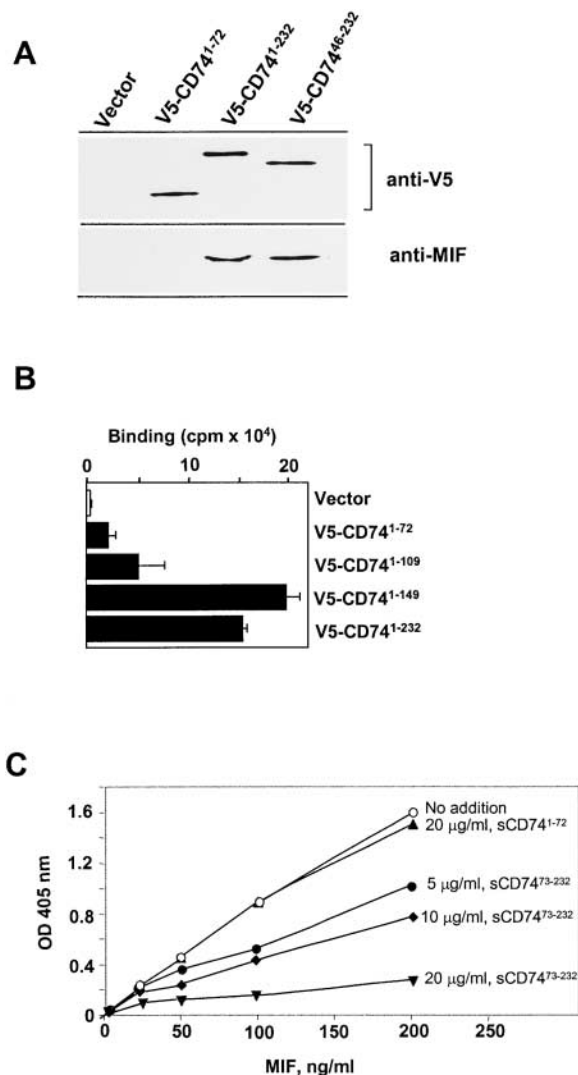
To further validate a significant binding interaction between CD74 and MIF, we expressed in *E. coli* and purified to homogeneity truncated, soluble CD74 proteins (sCD74) comprising the membrane-truncated extracellular domain (sCD74<sup>73-232</sup>), or the intracellular plus transmembrane domains (sCD74<sup>1-72</sup>), and tested their ability to inhibit MIF recognition by a sensitive, (two-antibody) sandwich ELISA system. As shown in Fig. 5 C, the addition of sCD74<sup>73-232</sup>, but not sCD74<sup>1-72</sup>, inhibited MIF detection in a dose-dependent fashion.

**Real-time Binding Analysis of MIF to CD74 (BIAcore Analysis).** We determined the equilibrium rate constant for MIF binding to CD74 by surface plasmon resonance, a technique that measures real-time binding interactions by changes in the refractive index of a biospecific surface (41). Optical biosensor surfaces, or protein “chips,” were prepared and BIAcore analysis of the binding interaction be-



**Figure 4.** Fluorescence analysis of Alexa-MIF binding to CD74-expressing cells. (A) Flow cytometry analysis of the binding of Alexa-MIF to CD74-transfected versus control vector-transfected COS-7 cells (left), and binding of Alexa-MIF to CD74-transfected COS-7 cells incubated with anti-CD74 mAb (clone LN2) versus an isotopic mAb control (right, con Ab). The anti-CD74 mAb, LN2, is reactive with an epitope residing within 60 amino acids of the extracytoplasmic, COOH terminus of the protein (48). mAbs were added at 50  $\mu\text{g/ml}$ , and the data shown are representative of at least three independent experiments. (B) Confocal microscopy images of a representative THP-1 cell (IFN- $\gamma$ -pretreated) double stained with Alexa-MIF (left) and a rhodamine-labeled anti-CD74 mAb (middle). The merged images with yellow areas indicate colocalization of MIF and CD74 (right). The percent colocalization was calculated to be  $69.2 \pm 12.0$  ( $P = 0.0308$ ,  $n = 6$  cells).

tween MIF (surface-bound MIF) and sCD74<sup>73-232</sup> revealed an equilibrium dissociation constant  $K_d$  of  $9.0 \times 10^{-9}$  M (Fig. 6). Complementary binding analysis using MIF in the



**Figure 5.** Biochemical evidence of MIF binding to CD74. (A) Cell-expressed CD74 binds MIF as detected by CD74 pull-down. Membrane-truncated CD74 (V5-CD74<sup>1-72</sup>), full-length (V5-CD74<sup>1-232</sup>), and NH<sub>2</sub>-terminal truncated CD74 (V5-CD74<sup>46-232</sup>) cDNAs were expressed in the pcDNA 3.1/V5-HisTOPO expression vector, transfected into COS-7 cells, and the protein products were precipitated by their expressed His tag. CD74 expression and recovery were monitored by Western blotting with anti-V5 (top). MIF was detected by Western blotting with anti-MIF (bottom). Vector: cells transfected with an empty vector control. (B) MIF binds to the extracellular domain of CD74 *in vitro*. <sup>35</sup>S-CD74 protein was prepared in a coupled transcription and translation reaction using the different CD74 expression plasmids shown. Protein-protein interaction was assessed by measuring bound radioactivity in 96-well plates that were precoated with MIF ( $n = 6$  wells per experiment). The data shown are representative of three experiments. (C) Soluble, extracellular domain CD74 (sCD74<sup>73-232</sup>) but not membrane-truncated CD74 (sCD74<sup>1-72</sup>) inhibits MIF detection by sandwich ELISA. Increasing concentrations of MIF were captured by an immobilized anti-MIF mAb, followed by the addition of the sCD74 species shown and a biotinylated anti-MIF pAb (43). The bound complexes were detected with streptavidin-conjugated alkaline phosphatase and *p*-nitrophenylphosphate as a substrate.

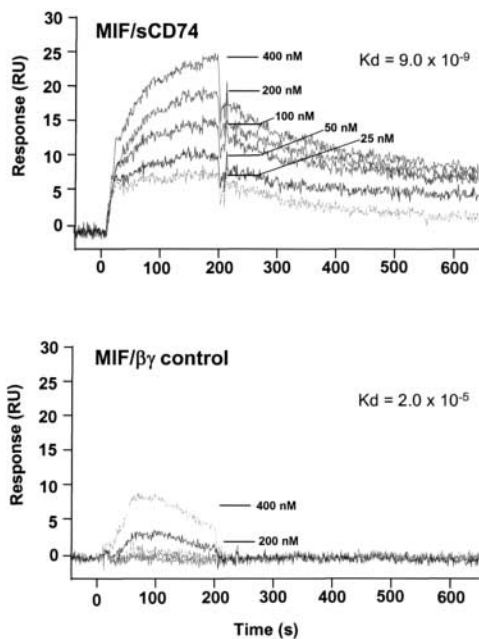
mobile phase and surface bound sCD74<sup>73-232</sup> revealed a  $K_d$  of  $2.3 \times 10^{-10}$  M (unpublished data). These values are in a range that would be expected given the nanomolar concentrations of MIF that have been measured in the circulation (40, 43). Nevertheless, these binding constants may be somewhat lower than the values in vivo because native CD74 is a trimer, and the sCD74<sup>73-232</sup> construct we prepared lacks the transmembrane domain that is implicated in protein trimerization (44).

**CD74 Mediates MIF Induction of ERK-1/2 Phosphorylation, PGE<sub>2</sub> Production, and Proliferation.** MIF has been shown to play an important role in the activation responses of macrophages and fibroblasts, in part by inducing sustained activation of the p44/p42 (ERK-1/2) protein kinase cascade (10, 45). To assess the functional significance of MIF binding to CD74, we examined the capacity of MIF to stimulate p44/p42 phosphorylation in macrophages obtained from mice genetically deficient in CD74<sup>34</sup>. MIF induced the phosphorylation of ERK-1/2 in CD74<sup>+/+</sup> macrophages, but not in CD74<sup>-/-</sup> macrophages (Fig. 7). Moreover, there was no MIF-dependent increase in PGE<sub>2</sub> production in CD74<sup>-/-</sup> macrophages when compared with CD74<sup>+/+</sup> macrophages.

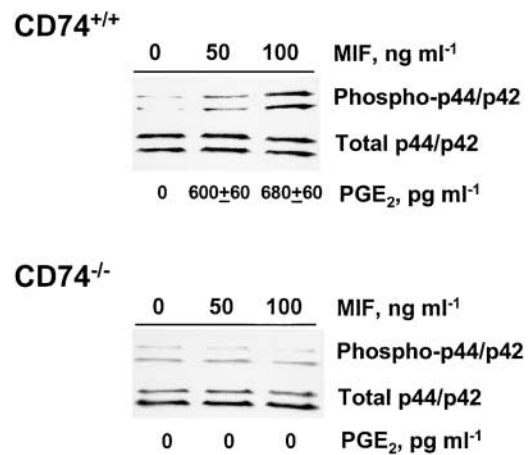
Activation of the p44/p42 kinase cascade is an early event in a signaling pathway leading to mitogenesis, and indeed MIF can stimulate the proliferation of different cell types under conditions of induced quiescence, or growth arrest (10, 45–47). We examined the ability of MIF to induce ERK-1/2 activation and downstream proliferative responses in the human Raji B cell line, which expresses

abundant cell surface CD74 (48). MIF stimulated the phosphorylation of ERK-1/2 in quiescent Raji cells, and this effect was inhibited by two different anti-CD74 mAbs, as well as by sCD74 (sCD74<sup>73-232</sup>, but not sCD74<sup>1-72</sup>; Fig. 8, A and B). The inhibitory effect of anti-CD74 on ERK-1/2 phosphorylation was associated with a significant decrease in the MIF-stimulated proliferation of these cells (Fig. 8 C). Of importance, control studies established that neither of these two anti-CD74 mAbs (clone LN2 nor M-B741) cross react with recombinant MIF (unpublished data). As an additional control for this experiment, we tested the impact of anti-CD74 on the known pathway of IL-6 induction of the ERK-1/2 MAP kinase cascade (49). Western blot analysis showed that there was no effect of anti-CD74 on the increased phospho-ERK-1/2 content of IL-6 stimulated cells (unpublished data).

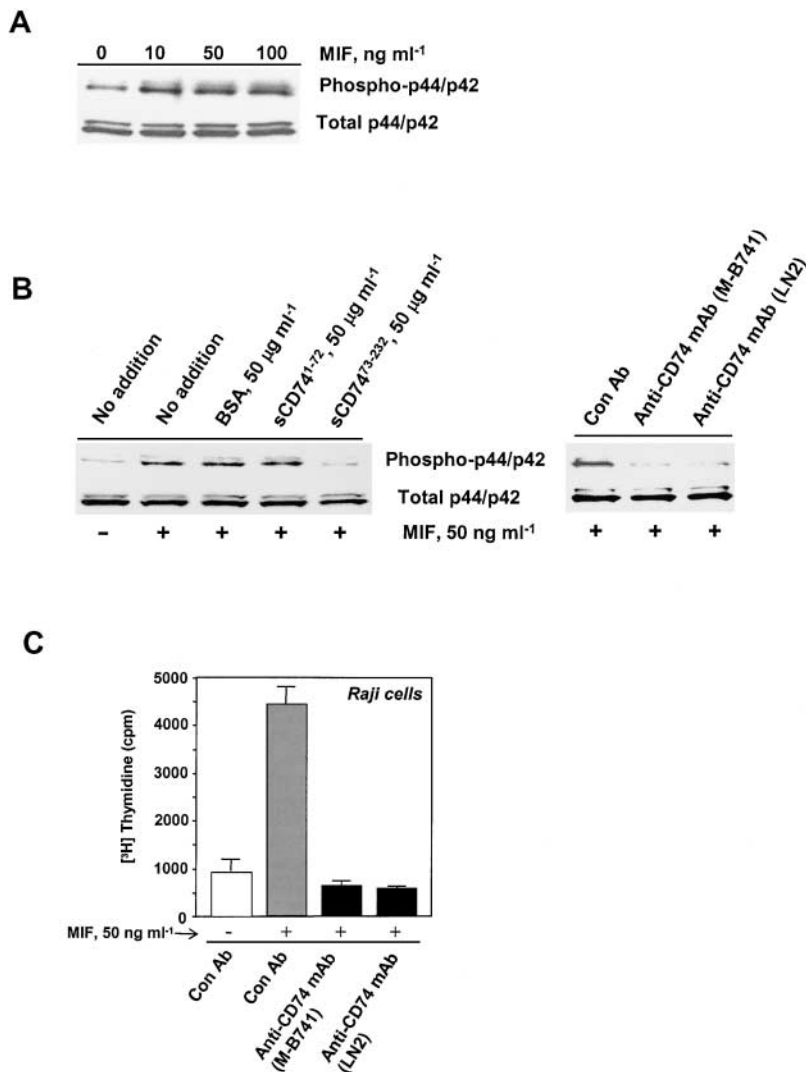
We also sought to evaluate the potential role of the MIF-CD74 stimulation pathway in cells outside the immune system. MIF addition extends the lifespan of primary murine fibroblasts (26), and both MIF's mitogenic effects and its induction of the ERK-1/2 signal transduction cascade have been characterized previously in this cell type (25). Fibroblasts express CD74 (50, 51), and we confirmed by flow cytometry the surface expression of CD74 in CCL210 human lung fibroblasts (unpublished data). MIF stimulates ERK-1/2 phosphorylation in these cells, in agreement with published papers (25, 52), and we found anti-CD74 mAb to significantly inhibit both MIF-induced ERK-1/2 phosphorylation and mitogenesis (Fig. 9 A–C). Together, these data indicate that the binding of MIF to CD74 is a required step in the stimulation of ERK-1/2 phosphorylation and cellular proliferation by MIF.



**Figure 6.** High-affinity binding of MIF to CD74 measured by real-time, surface plasmon resonance (BIAcore analysis). Representative biosensorgrams of the interaction between sCD74 (sCD74<sup>73-232</sup>) and an MIF sensor chip as described in Materials and Methods (top). A control of MIF interaction with the membrane-associated G protein,  $\beta\gamma$  (bottom).



**Figure 7.** CD74 mediates MIF stimulation of p44/p42 (ERK-1/2) phosphorylation and PGE<sub>2</sub> production in wild-type but not CD74-KO macrophages. Thioglycolate-elicited peritoneal macrophages were obtained from CD74<sup>+/+</sup> and CD74<sup>-/-</sup> mice, and  $6 \times 10^5$  cells were stimulated with the indicated concentrations of MIF for 2.5 h. Cells were harvested, and the lysates were quantified for phospho-p44/p42 and total p44/p42 using specific antibodies as described in Materials and Methods. Supernatant PGE<sub>2</sub> concentrations were measured by ELISA (10). Data shown are representative of three independent experiments.



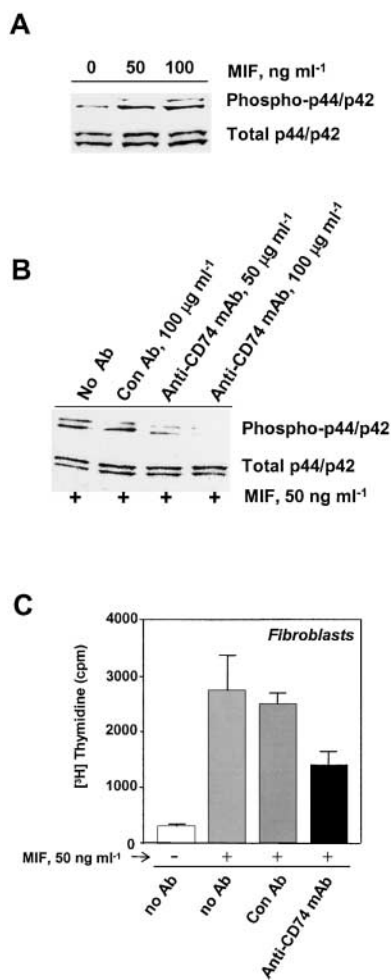
**Figure 8.** CD74 mediates MIF stimulation of ERK-1/2 (p44/p42) phosphorylation and proliferation of human Raji B cells. (A) MIF initiates ERK-1/2 phosphorylation, and (B) sCD74<sup>73-232</sup> and anti-CD74 mAb inhibit MIF-induced ERK-1/2 phosphorylation in Raji cells. Raji cells were stimulated with 50 ng/ml MIF for 2.5 h in the presence of an irrelevant protein (BSA), membrane-truncated CD74 (sCD74<sup>1-72</sup>), extracellular domain CD74 (sCD74<sup>73-232</sup>), an isotype control antibody (Con Ab), or two anti-CD74 mAbs (clones M-B741 or LN2, each added at 50 µg/ml). Anti-CD63 mAb, which is directed to an irrelevant Raji cell surface protein (63), also did not block MIF-stimulated p44/p42 phosphorylation when compared with anti-CD74 mAb (not depicted). (C) Anti-CD74 mAb inhibits MIF-induced Raji cell proliferation. Raji cells were cultured as described in Materials and Methods, and stimulated with rMIF as shown in the presence of 50 µg/ml of the indicated antibodies. Anti-CD74 antibodies showed no effect on Raji cell proliferation in the absence of added MIF (not depicted).

## Discussion

Although the first biological activity attributed to MIF was described in the mid 1960s, information regarding MIF's precise role in cell physiology and immunity has emerged only in the last few years. Among recent findings has been the determination that MIF is expressed by many cell types including the monocyte/macrophage (8), which historically had been considered to be the "target" of MIF action (2, 3). MIF is present preformed in macrophages (and in T cells) and it exerts important, autocrine/paracrine activating effects upon its release (8, 17). Antibody neutralization and signal transduction papers have supported the view that MIF acts by engaging a cell surface receptor (8, 25, 26), however, the lack of information regarding candidate receptors has prompted investigations into nonclassical or specialized modes of action. These have included the biological role of an intrinsic tautomerase activity (27, 53), which may be vestigial (28), and an endocytic pathway that involves a direct interaction between MIF and the transcriptional regulator, Jab1 (30).

We experienced considerable difficulty in preparing a bioactive, <sup>125</sup>I-radiolabeled MIF, and in biosynthetically labeling the protein to a sufficiently high specific activity for cell binding studies. Radioiodination methods result in the adventitious oxidation of MIF's free cysteine residues, which need to be in a reduced state for cytokine bioactivity (54). In contrast, we found that modification of MIF by Alexa-488 under mild conditions produced a fully bioactive protein that enabled the expression cloning of CD74 as a high-affinity, cell surface binding protein for MIF.

This work provides the first insight into a membrane receptor for MIF, and the proximate steps for signal transduction may now be considered in the context of the molecular biology of CD74. A role for CD74 in the transport of class II proteins from the endoplasmic reticulum to the Golgi complex has been established (55); however, it also is known that 2–5% of cellular CD74 is expressed on the cell surface (48, 56). CD74 surface expression occurs independently of class II and in a variety of different cell types



**Figure 9.** CD74 mediates MIF stimulation of ERK-1/2 (p44/p42) phosphorylation and proliferation of CCL210 human lung fibroblasts. (A) MIF stimulates ERK-1/2 (p44/p42) phosphorylation, and (B) anti-CD74 mAb inhibits ERK-1/2 phosphorylation and proliferation of CCL210 human lung fibroblasts. Fibroblasts were stimulated with 50 ng/ml MIF for 2.5 h in the presence of an isotype control antibody (Con Ab) or the anti-CD74 mAb (clone LN2). (C) Anti-CD74 inhibits MIF-induced proliferation of human fibroblasts. Cells were stimulated for 2.5 h with 50 ng/ml rMIF in the presence of a Con Ab or anti-CD74 mAb (clone LN2), each at 100  $\mu$ g/ml. Proliferation results are the mean  $\pm$  SD of triplicate assays and are representative of at least three separate experiments. Anti-CD74 antibodies showed no effect on the proliferation of lung fibroblasts in the absence of added MIF (not depicted).

(50, 56). Of note, CD74-KO mice are developmentally immunocompromised and show lymphoid abnormalities beyond what would be expected from the protein's function as a class II chaperone (34). Recent works have identified an accessory role for CD74 in immune cell stimulation, and this function requires a chondroitin-sulfate-dependent interaction between CD74 and CD44 (57, 58). CD44 is a widely expressed and a polymorphic transmembrane protein with known tyrosine kinase activation properties (59), and the horizontal recruitment of CD44 into an MIF-CD74 complex may be necessary for MIF signal transduction in some cell types. CD74 surface expression is

also known to be regulated by the length of the protein's NH<sub>2</sub>-terminal, intracellular domain, which varies depending on which of two in-phase initiation codons are used (60). Whether this differential translation of CD74 mRNA mediates cellular sensitivity to MIF will also be important to investigate.

The intracellular portion of CD74 lacks sequence domains that might be predicted to interact with downstream signaling molecules. Thus, it is noteworthy that the expression of a truncated, CD74 intracellular domain alone has been shown to initiate p65-RelA-dependent transcriptional activation (61). The activating ligand for CD74 was not been defined by these papers, and this activation pathway appears to require the recruitment of additional intracellular proteins (61). Like MIF, CD74 is a homotrimer (62), and MIF engagement of CD74 may act to effect the oligomerization or the stabilization of the intracellular domain that is necessary for downstream signaling. Thus, the MIF binding activity of CD74 provides insight into the biology of CD74 outside of its role in the transport of class II, and supports those papers that have defined an accessory signaling function for CD74 in immune cell physiology (57, 58, 61).

Whether the binding of MIF to CD74 accounts for all of MIF's cellular actions is unknown, and perhaps unlikely in light of experiments suggesting a pathway for MIF internalization and binding to Jab1 (30), and continued interest in the biological function of MIF's NH<sub>2</sub>-terminal, catalytic domain (28). Nevertheless, recent *in vitro* and *in vivo* works have placed MIF in a pivotal position for the control of innate immunity. MIF regulates the expression of TLR4 (15), which is the receptor for gram-negative endotoxin, and the MIF release sustains proinflammatory function by inhibiting activation-induced, p53-dependent apoptosis (10, 26). MIF's importance in the pathophysiology of infection also has been affirmed in experimental animal models of sepsis, where anti-MIF protects from death even when administered 8 h after infectious insult (40). The recent finding that human MIF is encoded by four functionally distinct alleles, and that the high-expression alleles are associated with severe rheumatoid arthritis (19) further emphasize this cytokine's importance in human inflammatory disease. Pharmacological interference in the MIF-CD74 interaction may offer an important new approach to the modulation of pathologic inflammatory processes.

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