Macrophage Migration Inhibitory Factor (MIF) Induces B Cell Survival by Activation of a CD74/CD44 Receptor Complex.

Yael Gore\textsuperscript{1}, Diana Starlets\textsuperscript{1}, Nitsan Maharshak\textsuperscript{3}, Shirly Becker-Herman\textsuperscript{1}, Utako Kaneyuki\textsuperscript{2}, Lin Leng\textsuperscript{2}, Richard Bucala\textsuperscript{2} and Idit Shachar\textsuperscript{1,3}

\textsuperscript{1}Department of Immunology, the Weizmann Institute of Science, Rehovot, Israel, 76100; \textsuperscript{2}Department of Medicine, Yale University School of Medicine, New Haven, Connecticut 06520, USA

\textsuperscript{3}Corresponding Author: Idit Shachar, Department of Immunology, The Weizmann Institute of Science, Rehovot, Israel 76100, Tel: 972-8-934257, Fax; 972-8-9344141, Email: idit.shachar@weizmann.ac.il

Macrophage migration inhibitory factor (MIF) is an upstream activator of innate immunity that regulates subsequent adaptive responses. It was previously shown that in macrophages, MIF binds to a complex of CD74 and CD44, resulting in initiation of a signaling pathway. In the current study, we investigated the role of MIF in B cell survival. We show that in B lymphocytes, MIF initiates a signaling cascade that involves Syk and Akt, leading to NF-κB activation, proliferation and survival in a CD74 and CD44 dependent manner. Thus, MIF regulates the adaptive immune response by maintaining the mature B cell population.

An established paradigm divides the immunologic response into innate and adaptive components, with critical interactions between the two producing normal immunity or immunopathology. The innate response represents the earliest host response to invasive pathogens by cells such as monocytes/macrophages, while the adaptive response includes the development of antigen-specific responses, antibody production, and immunologic memory. Significant attention is currently focused on the molecules that link innate and adaptive immunity, and that may critically regulate immune responses or the development of inflammatory/autoimmune diseases.

CD74 is a non-polymorphic type II integral membrane protein that is expressed on antigen presenting cells including macrophages and B cells. It has a short N-terminal cytoplasmic tail of 28 amino acids (aa), followed by a single 24 aa transmembrane region and an approximately 150 aa lumenal domain. The CD74 chain was considered initially to function mainly as an MHC class II chaperone, which promotes exit of MHC class II molecules, directs them to endocytic compartments, prevents peptide binding in the ER, and contributes to peptide editing in the MHC class II compartment (1). A small proportion of CD74 is modified by the addition of chondroitin sulfate (CD74-CS), and this form of CD74 is expressed on the surface of antigen presenting cells, including monocytes and B cells. Antibody blocking studies additionally have shown that CD74-CS interacts with CD44, which activates a Src-kinase dependent signaling pathway (2). It was previously shown that macrophage migration inhibitory factor (MIF) binds to the CD74 extracellular domain on macrophages, a process that results in initiation of a
signaling pathway (3). MIF accounts for one of the first cytokine activities to have been described (4). MIF promotes monocyte/macrophage activation and is required for the optimal expression of TNF, IL-1 and PGE2 (5-7). MIF-activated macrophages are more phagocytic and better able to destroy intracellular pathogens, such as Leishmania (8,9). These activating functions have been verified in MIF-knockout mice (6,10,11). MIF’s role in adaptive immunity is less well-characterized, but neutralization of MIF using specific antibodies inhibits delayed-type hypersensitivity, T cell priming and antibody production in vivo (12,13). MIF expression contributes significantly to the immunopathology that results from excessive inflammation and autoimmunity (14,15).

CD44 is a broadly-expressed single-pass transmembrane protein with known kinase-activating properties. Recently, CD44 was described as an integral component of the CD74 receptor complex (16,17). While CD74 was sufficient for MIF cell surface binding, CD44 was found to be necessary for MIF signal transduction (17).

In our previous studies, we showed that CD74 expressed on B cells is directly involved in the survival of the mature B cell population (18-20) through a pathway leading to the activation of transcription mediated by the NF-κB p65/RelA homodimer and its co-activator, TAFII105 (21). NF-κB activation is mediated by the cytosolic region of CD74 (CD74-ICD), which is liberated from the membrane (22). We demonstrated that following the removal of the CD74 luminal domain, an intramembranal cleavage event at amino acid 42 occurs, resulting in the release of the CD74 cytosolic fragment (CD74-ICD; aa 1-42). CD74-ICD then translocates to the cell nucleus and activates NF-κB (23). Therefore, CD74 acts as a signaling molecule and requires a processing step to mediate a signal resulting in accumulation of mature B cells. This signal is attenuated by degradation of the active CD74-ICD fragment, and its removal from the cytoplasm (20,22). Moreover, we recently demonstrated that CD74 stimulation with anti-CD74 antibody leads to NF-κB activation, enabling entry of the stimulated B cells into the S phase, an increase in DNA synthesis, cell division, and augmented expression of anti-apoptotic proteins. These findings therefore indicate that surface CD74 functions as a survival receptor (24). However, the natural ligand of CD74 on B cells was not known.

In this study we followed the role of MIF in B cells. We show that CD74 forms a complex with CD44 on the B cell surface, and that MIF can serve as a ligand for the CD74/CD44 complex; this complex is essential for the MIF-induced signaling cascade that results in B cell survival.

**Materials and Methods**

**Cells**

Spleen cells were obtained from C57BL/6, CD44/- (25) or CD74/- (26) mice. All animal procedures were approved by the Animal Research Committee at the Weizmann Institute.

**Cells and B cell separation**

Spleen cells were obtained from the various mice at 6-8 weeks of age, as previously described (27). B cells were then purified from each mouse strain, using CD45R beads (BD Biosciences). The purity of the purified cells (between 96-99%) was analyzed by FACS following each experiment.

**MIF stimulation**

Recombinant murine MIF was purified from an expression system as previously described and contaminating endotoxin removed by C8 chromatography (28). For MIF stimulation, 1x10^7 primary B cells were incubated in RPMI medium containing 0.1% (v/v) FCS at 37°C for 3 h. Next, cells were resuspended in medium containing 100 ng/ml of recombinant MIF and incubated at 37°C for various periods.

**PI staining**

Purified B cells were cultured in 6-well plates at 1x10^7 cells/well in RPMI medium supplemented with 3% FCS, 2 mM glutamate, 100 U/ml penicillin, 100 µg/ml streptomycin, with or without MIF (100 ng/ml) and 0.1 µM of the MIF inhibitor ISO-1 (CalBiochem) for 24 hours. Cells were collected by centrifugation, washed and fixed in 70% cold ethanol and incubated in the presence of RNase (25µg/ml). Propidium iodide (PI; 25µg/ml) (Sigma) was added and analyzed by FACS.

**Luciferase assay for monitoring NF-κB activation**
Subconfluent 293 cells were transfected in a 24-well plate using a total of 1 mg plasmid; empty or CD74 expression vectors (400 ng) were added together with 20 ng of the Gal4 luciferase reporter, 0.5 ng of DBD fusion plasmids, and 1 ng of RSV-Renilla luciferase. The total amount of DNA was kept constant by adding pBabe vector. Cells were incubated for 5 hours and then stimulated with MIF (100 ng/ml) and 0.1uM ISO-1 for 24 hr, harvested, and luciferase and Renilla luciferase activities were measured.

**Proliferation of B cells**

Purified B cells were cultured in 96-well plates at 2x10^5 cells/well in RPMI medium supplemented with 1% FCS, 2 mM glutamate, 100U/ml penicillin, 100 µg/ml streptomycin, in the presence of 100 ng/ml MIF, 0.1 uM ISO-1 or 25 µg/ml LPS from salmonella Thyphosa (Sigma) for 24hr. DNA synthesis was assayed by pulsing the cultures with 1 µCi of [³H] thymidine for the last 18h of culture, after which the cells were harvested and counted.

**RNA isolation and reverse transcription**

Total RNA was isolated from cells using the Tri Reagent kit (MRC). Reverse transcription was carried out using Superscript II RT (Gibco-BRL). Primers that were used included:

- **Cyclin E:** GAAAATCAGACCCAGAGACG
- **BCL-2:** 5’ CAGGGCGATGTTGCTCC
- **HPRT:** 5’ CAGGGTAGGGCTGGCTATGGCT
- **GTTGGATACAGGCGACCTTGTG**

**Preparation of cell extracts**

Stimulated cells were lysed in buffer containing: 25 mM Tris, pH 7.4; 2 mM Vanadate; 75 mM β-glycophosphate, pH 7.2; 2 mM EDTA; 2 mM EGTA; 10 mM NaPPI; and 0.5% NP-40 in the presence of the following protease inhibitors: 10 µg/ml Leupeptin, 10 µg/ml aprotinin, 10 µg/ml pepstatin, 10 µg/ml chymostatin (Roche), 1mM PMSF (Sigma), and 20 mM N-ethylmaleamide (Sigma).

**Cell lysis by hot SDS**

Cell lysates were prepared as described previously (29).

**Tricine gels**

16% (w/v) Tricine-SDS PAGE were performed as previously described (30).

**Immunoprecipitation and Western blot analysis**

To detect changes in protein phosphorylation, lysates or immunoprecipitates were separated by 12%(w/v) SDS-PAGE. The proteins were transferred onto a nitrocellulose membrane and probed with anti p-Tyr (pTyr99; Santa Cruz) followed by horseradish peroxidase-conjugated anti-mouse (Jackson Labs). The membrane then was stripped and reprobed with anti-tubulin antibody (Sigma) followed by peroxidase-conjugated anti-mouse (Jackson Labs).

To detect changes in Akt phosphorylation, the membrane was probed with anti-p-Akt antibody (Cell Signaling Technology) followed by peroxidase-conjugated anti-rabbit (Jackson Labs). The membrane then was stripped and reprobed with anti-tubulin antibody (Santa Cruz) followed by peroxidase-conjugated anti-mouse (Jackson Labs).

To detect CD74 intramembrane cleavage, lysates were resolved by tricine gels, blotted into nitrocellulose and probed as described previously (22).

**Immunoprecipitation**

Protein-G Sepharose beads (Pharmacia) were conjugated to p-Tyr mAb for 2 hrs at 4°C, followed by three washes in PBS. Beads were added to the cell lysates and p-Tyr proteins were immunoprecipitated overnight. The protein G bound material was washed three times with PBS containing 0.1% SDS and 0.5% NP40. Immunoprecipitates were separated by 10% (w/v) SDS-PAGE. The protein bands were transferred onto a nitrocellulose membrane and probed with anti-Syk (4D11, Pharmingen) followed by horseradish peroxidase-conjugated anti rabbit IgG (Jackson Labs).

**Annexin PI staining**

Purified B cells were cultured in 24-well plates at 1x10^5 cells/well in Optimem medium supplemented with 3% FCS, 2 mM glutamate, 100 U/ml penicillin, 100 µg/ml streptomycin, with or without 100 ng/ml MIF and 0.1 µM ISO-1 for 18 hours. Cells were collected by centrifugation, washed and stained with Annexin (BD Biosciences) and PI (Bender Medsystems) for 15 min at room temp.
Annexin and PI staining were analyzed by FACS.

**Hyaluronic Acid stimulation**

Primary B cells (1x10^7) were incubated in RPMI medium containing 0.1% (v/v) FCS at 37°C for 3 h. Next, cells were cultured in 24-well plates at 1x10^7 cells/well in RPMI medium supplemented with 0.1% FCS, and incubated in the presence or absence of 0.1 mg/ml hyaluronic acid (HA) (Sigma), 6 μg/ml anti-CD44 antibody (KM114; BD Biosciences), and 6 μg/ml anti isotype control antibody for 8 hours at 37°C.

**CD44 blocking**

B cells (1x10^7) were incubated with 100 ng/ml MIF. For CD44 blocking, 1x10^7 B cells were incubated in 1 ml RPMI medium containing 0.1% (v/v) FCS in the presence of 6 μg/ml anti-murine CD44 (KM114; BD Biosciences) at 37°C for 8 hr.

**Immunofluorescence and flow cytometry**

Staining was performed on freshly isolated splenocytes. The following antibodies were used: RA3-6B2 anti-CD45R/B220 and anti-CD44 (ebiosciences). Staining was analyzed by FACS. Characterization of B cells: Freshly isolated splenocytes, were stained for RA3-6B2 anti-CD45R/B220, anti-CD21 (CR2/CRII; ebioscience), anti-CD24(HSA) (ebioscience) and anti-CD23(ebioscience) and analyzed by FACS.

**MIF injections**

C57BL/6 mice were IP injected daily with 200 ng of MIF for 8 days. Spleens were collected and splenocytes were analysed for their B cell repertoire and survival.

**Results**

A complex of CD74 and CD44 is essential to initiate a signaling cascade induced by MIF.

It was recently shown that CD44 is an integral member of the CD74 receptor complex leading to MIF signal transduction in monocytes (17). To determine whether in B cells, CD74 forms a similar complex with CD44, we first analyzed its cell surface expression in B cells by FACS analysis. As shown in Fig 1A, B cells derived from control or CD74 deficient mice express cell surface CD44. To analyze CD74-CD44 complexes, control B splenocytes were lysed and CD44 was immunoprecipitated. Immunoprecipitates were separated on SDS-PAGE and analyzed by anti-CD74. As shown in Fig 1 B-D, CD44 specifically pulled down CD74, mainly of the p31 isofrom, showing that CD74 forms a complex with CD44 in B cells. CD44 mRNA levels were unchanged following MIF stimulation in both control and CD74/-/- B cells (data not shown). In addition, the existence of the CD74/CD44 complex was MIF independent, since no change in its formation was detected in cells treated with MIF for short (Fig 1E) or longer (data not shown) periods.

We then wished to determine whether MIF binds to this complex, inducing a signaling cascade. We demonstrated previously that in mouse B cells, CD74 releases its cytosolic domain (CD74-ICD) and initiates a signaling pathway that activates NF-κB and promotes cell survival (24). To determine whether MIF stimulation triggers CD74-ICD release in a CD44-dependent manner, we first followed the intramembrane cleavage and control and CD44/-/- B cells. Cells were lysed with hot SDS, and the release of CD74-ICD was analyzed by Tricine gel. As seen in Fig. 2A, MIF stimulation elevated the degree of intramembrane cleavage and liberation of the CD74 cytosolic domain. However, the specific augmentation of CD74-ICD release was abolished in CD44 deficient cells.

We then followed the MIF-induced signaling cascade. B splenocytes derived from control, CD74/-/- or CD44/-/- mice were incubated in the presence of 100 ng/ml MIF for 0-10 minutes. The cells then were lysed and phosphorylated proteins were analyzed by Western blot analysis. We have previously shown that Syk and Akt phosphorylation is induced following anti-CD74 stimulation. To determine whether these proteins are phosphorylated following MIF stimulation, B cells derived from control, CD74/-/- or CD44/-/- mice were incubated in the presence or absence of MIF for 5 min. Next, the cells were lysed and Syk and Akt phosphorylation were analyzed. As can be seen in Fig 2, while MIF induces Syk (Fig 2B) and Akt (Fig 2E) phosphorylation in the control B cells, it did not affect the activation of these proteins in CD74/-/- (Fig 2C, F) or CD44/-/- (Fig 2D, G) B cells. Thus, MIF initiates a signaling cascade involving Syk tyrosine kinase and Akt proteins through interaction and activation of cell surface CD74 and CD44.
We previously demonstrated that CD74 induces a signaling pathway that results in NF-κB activation and cell survival (21,24). To determine whether MIF induces a similar cascade activating NF-κB in the nucleus, a fusion construct containing the C-terminal transactivation domain of p65/RelA and the DNA-binding domain of the yeast transcription factor Gal4, was co-transfected into HEK 293 cells, along with a luciferase reporter containing the Gal4 binding sites, with CD74, and with the RSV promoter, which was used as a reference (21,24). The cells then were incubated in the presence or absence of MIF and luciferase activity was measured 24 hr later. As demonstrated in Fig 3A, stimulation with MIF significantly increased NF-κB activity. To further verify the role of MIF in NF-κB activation, cells were treated with the MIF antagonist ISO-1. ISO-1 (a cell-permeable isoxazoline compound), a non-toxic inhibitor of MIF that binds to bioactive MIF at a catalytically active tautomerase site (31). As shown in Fig 3A, ISO-1 reduced MIF-induced NF-κB activation to baseline levels. These data support a model whereby MIF activation of CD74 initiates a signaling cascade leading to NF-κB activation in B cells.

**MIF induces proliferation and survival in B cells**

In most cases examined, Akt activation promotes various cellular responses that are associated with cell division, including increased cell size, suppression of apoptosis, inactivation of cell cycle inhibitors, and induction of cyclin and cytokine gene expression (32). In addition, we have recently demonstrated that an activating anti-CD74 antibody induces B cell proliferation and survival (24). To determine whether CD74 stimulation by MIF triggers B cell proliferation and survival, [3H] thymidine incorporation was measured in MIF stimulated and unstimulated cells in B cells derived from control, CD74-/- or CD44-/- mice. A specific elevation in [3H] thymidine incorporation was observed 24h (Fig 3B) following MIF stimulation, while B cells deficient in CD74 or CD44 did not respond (although they were able to respond to LPS stimulation).

Propidium iodide staining showed that the proportion of cells in the S phase of the cell cycle was elevated upon MIF-stimulation of control, but not CD74-/- or CD44-/- B cells. Cell cycle progression is regulated by cyclin dependent kinases (Cdk). Cdks are constitutively expressed during the cell cycle and are activated upon binding to specific cyclins differentially expressed during various stages of the cell cycle. This transient cyclin expression activates Cdk and regulates cell cycle progression (33). To further determine whether MIF regulates cell entry into the S-phase in a CD74- or CD44-dependent fashion, we followed cyclin E, which is expressed upon S-phase initiation. As demonstrated in Figs 4 B-D, cyclin E transcription and expression was upregulated following MIF stimulation. Stimulation of cyclin E was specific and did not occur in CD74 or CD44 deficient B cells. Altogether, these results demonstrate that following MIF stimulation, B cells synthesize DNA, enter S phase, and divide.

To determine whether MIF induces a survival cascade in B cells, the transcription of the anti-apoptotic and survival factors was followed. B splenocytes isolated from control, CD74-/- or CD44-/- mice were incubated the presence or absence of MIF (100 ng/ml) for 8 hrs and Bcl-Xl and Bcl-2 expression followed by RT-PCR. While MIF stimulation of control B cells resulted in augmentation of Bcl-Xl and Bcl-2 transcription, no change was detected in CD74-/- or CD44-/- stimulated B cells (Fig. 5A-D).

To further verify the role of MIF in B cell survival, cells were incubated in the presence or absence of MIF and its antagonist, ISO-1. As shown in Fig 5D, treatment of control B cells with ISO-1 resulted in a significant reduction of MIF-induced Bcl-2 mRNA levels, showing that MIF regulates Bcl-2 expression. The requirement for the CD44 signaling coreceptor in MIF mediated survival signals was tested by stimulating B cells in the presence or absence of anti-CD44, which blocks hyaluronan binding, without inducing shedding or internalization of CD44 (Supplementary data, Fig 1). Bcl-2 transcription was then followed using RT-PCR. As can be seen in Fig 5E, the MIF-induced Bcl-2 transcription was dramatically inhibited by the anti-CD44 antibodies in control B cells, while no change was observed in CD74-/- cells. Furthermore, activation with HA in control B cells elevated

![Image](http://www.jbc.org/DownloadedFrom)
Bcl-2 mRNA levels in a CD44 dependent manner, while in cells lacking CD74, this cascade was inhibited (Fig 5G). Thus, both receptors are essential for the B cell survival cascade.

To directly demonstrate the role of MIF induced cascade in B cell survival, we then followed the effect of MIF and ISO-1 on B cell survival. B cells were incubated in the presence or absence of MIF or ISO-1 and the cells were analyzed for apoptosis by PI Annexin staining. As shown in Fig 6, a reduction in Annexin positive cells and elevation of the live population was detected in control cells stimulated with MIF, while in cells deficient of CD74 or CD44, no change was observed. In addition, treatment with ISO-1 significantly restored the Annexin-positive population, supporting the role of MIF in promoting B cell survival.

Finally, to directly demonstrate the in vivo role of MIF induced cascade in B cell survival, control, CD74-/- or CD44-/- splenocytes were analyzed for their apoptotic population by Annexin staining. As shown in Fig 7A, splenic B cells lacking MIF or its receptors (CD74 and CD44) showed reduced viability and a larger population of apoptotic cells compared to the wild type B population. To determine whether the CD74/CD44 regulates mature B cell survival, we compared the B cell populations in control, CD74-/- and CD44-/- mice. As demonstrated in Fig 7B, mice lacking CD44 showed a reduction in their mature B cell population; however, this downregulation was not as dramatic as in mice deficient for CD74 (Fig 7B; (18)). To directly follow the role of MIF in regulating the B cell survival and repertoire, control mice were injected with PBS or MIF for 8 days and their B cell subpopulations and survival were analyzed. As shown in Fig 7C, MIF stimulation reduced the apoptosis of the B cell population, resulting in a reduced transitional population and an elevation in the percent of mature cells. These results show that MIF induces a survival cascade that is CD74/CD44 dependent, and that enlarges the mature B cell compartment.

Discussion

MIF accounts for one of the first cytokine activities to have been described (4). An important feature of the biologic action of MIF is its ability to sustain monocyte/macrophage activation in the face of activation-induced, p53-dependent apoptosis (7,34). Endotoxemic, MIF-deficient mice exhibit increased macrophage apoptosis than wild-type controls, and this enhanced apoptosis is recapitulated in mice lacking the MIF binding (CD74) or signaling (CD44) receptors (17). MIF recently has been described to be a cognate ligand for CD74 (3). The results presented here demonstrate that in B lymphocytes, MIF initiates a signaling cascade, leading to B cell survival in a CD74 and CD44 dependent manner. Thus, MIF regulates survival of both innate and adaptive immune cells and therefore might serve as a link between these responses.

Our studies show that in B cells, MIF initiates a signaling cascade following binding to the CD74/CD44 complex. Immunoprecipitation studies also support the formation of a complex between CD74 and CD44 in B cells. This ensuing signaling cascade involves the Syk and Akt kinases. Syk belongs to the Syk/ZAP-70 family of PTK and plays a crucial role in B cell development, both during B-cell fate decisions and during antigen processing. Recent findings indicate that expression of Syk in non-hematopoietic cells plays a role in a wide variety of cellular functions and in the pathogenesis of malignant tumors (35). Syk was previously shown to be required for the activation of Akt in a PI3K dependent manner. Indeed, following MIF stimulation, we detected activation of the PI3K effector, Akt (36,37). Akt activation promotes a number of cellular responses that are associated with cell division, including increased cell size, suppression of apoptosis, inactivation of cell cycle inhibitors, and induction of cyclin and cytokine gene expression (32). At the molecular level, expression of activated Akt in T cells correlates with augmented NF-κB function, including the upregulation of Bcl-XL (38,39).

Our results also demonstrated that MIF augmented NF-κB function, which was associated with entry into the S phase, elevation of DNA synthesis resulting in cell division, and increased expression of Bcl-XL and Bcl-2, leading to a suppression of apoptosis both in vitro and in vivo. Together, these results establish that MIF binding to both CD74 and CD44 initiates a survival pathway, resulting in the rescue of the mature B population from death.
Interestingly, both MIF and CD74 have been implicated in pathways important for tumor progression. It has been reported that MIF is over-expressed in solid tumors (40,41), and that expression is associated with the growth of malignant cells (42). Anti-MIF immunoglobulin therapy also has been shown to induce an anti-tumor response (43). Many studies have demonstrated the overexpression of CD74 in various cancers (16,44-48), and CD74 has been suggested to serve as a prognostic factor, with higher relative expression of CD74 behaving as a marker of tumor progression (49). Moreover, a humanized anti-CD74 monoclonal antibody (hLL1) was shown to have a therapeutic action in multiple myeloma, perhaps due to the high level of expression of CD74 in this plasma cell malignancy (50).

CD44 describes a type I transmembrane family of signaling proteins that is encoded by a single, highly conserved gene (51). There is heterogeneity in the structure of the mature protein product that is due in part to post-translational modifications that differ depending on the cell type and growth conditions. In addition, CD44 transcripts are subject to alternative splicing, which predominantly affects the extracellular, membrane-proximal stem structure of the protein (52,53). Studies in which the function of the protein was disrupted indicate roles for CD44 in tumor formation, immune responsiveness and haematopoiesis, as well as in immunity against bacterial infection. The signaling properties of CD44 result for the assembly of intracellular complexes that may vary with the nature stimulus and the structure of the protein’s ectodomain (54). CD44 mediated signals involve non-receptor tyrosine kinases and other intracellular mediators that lead to enhanced cell growth, motility, and survival. Here, we show that the HA binding domain plays a role in MIF binding. The studies described herein thus establish the functional role of an MIF-activated, CD74/CD44 complex in delivering signals important for B cell survival.

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Legends

Fig 1- CD74 and CD44 form a complex in B cells. (A) B splenocytes from C57BL/6, and CD44/- mice were double stained with anti-B220 and anti-CD44. Histogram presents cell surface expression of CD44 on B220 positive cells. (B) Proteins from total B220+ B cell lysates or from B cell lysates after anti-CD44 immunoprecipitation were separated on SDS-PAGE and transferred into nitrocellulose. CD74 was detected by Western blot analysis. (C) Proteins from total B220+ B cell lysates or from B cell lysates after anti-CD44 or isotype control immunoprecipitation were separated on SDS-PAGE and transferred into nitrocellulose. CD74 was detected by Western blot analysis. (D) Proteins from total B220+ B cell lysates derived from CD44/- and CD74/-, or from those B cell lysates after anti-CD44 or isotype-control immunoprecipitation were separated on SDS-PAGE and transferred into nitrocellulose. CD74 was detected by Western blot analysis. (E) CD74 protein was analyzed in total B cell lysates or in CD44 co-immunoprecipitated proteins by Western blot analysis.

Fig 2- MIF induces a signaling cascade that involves Syk and Akt in a CD74 and CD44 dependent manner. (A) B cells from control or CD44/- mice were incubated with or without MIF (100 ng/ml) for 1 h. Cells were then lysed in hot-SDS, and lysates were separated on a Tricine gel and analyzed with the IN1 rat monoclonal antibody, which recognizes the CD74 cytosolic domain, followed by anti-rat HRP antibodies. The arrow indicates the CD74-ICD band. (B-D) B220+ B cells derived from control (B), CD74/- (C), or CD44/- (D) mice were incubated in the presence or absence of MIF (100 ng/ml) for various periods. Immediately after stimulation, cells were washed and fast frozen in liquid N2. The cells then were lysed and an aliquot reserved for total Syk analysis. Phosphorylated proteins from the remaining lysates were immunoprecipitated with an anti-p-Tyr antibody. Immunoprecipitates and total lysate proteins were separated on 10%(w/v) SDS-PAGE and blotted with an anti-Syk antibody as described in Methods. The intensity of the phosphorylated band following each treatment was divided by the intensity of the non-phosphorylated band in each lane. The activation fold ratio in the absence of any treatment was normalized to 1 and the ratio for each treatment was calculated as the intensity of the treated sample relative to 1. The results presented are representative of at least five different experiments. (E-G) B220+ B cells derived from control (E), CD74/- (F) or CD44/- (G) mice were incubated in the presence or absence of MIF (100 ng/ml) for various periods. Immediately after stimulation, cells were washed and fast frozen in liquid N2. Next, the cells were lysed as described in Methods and the lysates were separated on 10%(w/v) SDS-PAGE and blotted with anti-p-Akt or anti-Tubulin antibodies. The results presented are representative of at least three different experiments.

Fig 3- MIF induces NF-kB and B cell proliferation in a CD74 and CD44 dependent manner. (A) NF-κB activation was analyzed by luciferase assay, as described in the Methods. 293 cells were transfected with FL CD74 or an empty vector. The cells then were incubated with or without MIF, and in the presence or absence of ISO-1. Following stimulation, the cells were lysed and NF-κB activation was determined. The results shown represent the average of at least five independent experiments with similar results. (B) B220+ B cells derived from control, CD74/- or CD44/- mice were incubated in the presence or absence of MIF (100 ng/ml) for various periods. Immediately after stimulation, cells were washed and fast frozen in liquid N2. Next, the cells were lysed as described in Methods and the lysates were separated on 10%(w/v) SDS-PAGE and blotted with anti-p-Akt or anti-Tubulin antibodies. The results shown represent the average of at least four independent experiments with similar results.

Fig 4- The MIF downstream signaling cascade leads to B cell entry to S phase in a CD74 and CD44 dependent manner. (A) B220+ B cells derived from control, CD74/- or CD44/- mice were incubated in the presence or absence of MIF (100 ng/ml) and in the presence or absence of ISO-1 for 24 hrs. PI staining was performed as described in Methods. The results presented are representative of three separate experiments. (B-D) B220+ B cells derived from control (B), CD74-/- (C) or CD44-/- (D) mice were incubated in the presence or absence of MIF (100 ng/ml) for various periods. Total RNA was isolated and reverse transcription using primers for cyclin E or HPRT was carried out using Superscript II RT. The results presented are representative of five separate experiments.
Fig 5- The MIF downstream signaling cascade induces transcription of genes that are required for B cell survival.

(A) B220+ B cells derived from control, CD74/- or CD44/- mice were incubated in the presence or absence of 100 ng/ml MIF for 8 hrs. RT-PCR using primers for Bcl-XL or HPRT were performed and activation fold was calculated as described above. (B-D) B220+ B cells derived from control (B), CD74/- (C) or CD44/- (D) mice were incubated in the presence or absence of MIF (100 ng/ml) for various periods. RT-PCR was performed as described in Methods. The intensity of the BCL-2 band following each treatment was divided by the intensity of the HPRT band from the same treatment. The activation fold ratio in the absence of any treatment was normalized to 1 and the ratio for each treatment was calculated as the intensity of the treatment sample relative to 1. The results presented are representative of five separate experiments. (E) Control B cells were stimulated with MIF (100 ng/ml) in the presence or absence of various concentrations or ISO-1. RT-PCR using primers for BCL-2 or HPRT was performed and activation fold was calculated as described above. (F) Control or CD74/- B cells were stimulated in the presence or absence of MIF (100 ng/ml) and anti-CD44. RT-PCR using primers for Bcl-2 or HPRT were performed and activation fold was calculated as described above. The results presented are representative of five separate experiments. (G) Control or CD74/- B cells were stimulated in the presence or absence of HA (0.1mg/ml), anti-CD44 or an isotype control antibody. RT-PCR using primers for Bcl-2 or HPRT were performed and activation fold was calculated as described above. The results presented are representative of three separate experiments.

Fig 6- MIF induces in vitro B cell survival in CD74 and CD44 dependent manner. B220+ B cells derived from control, CD74/- or CD44/- mice were incubated in the presence or absence of MIF and in the presence or absence of ISO-1 (0.1 μM) for 18 hrs. Cell apoptosis was analyzed by PI and Annexin staining as described in the Methods. The results presented are representative of three separate experiments.

Fig 7- MIF induces B cell survival in CD74 and CD44 dependent manner in vivo. (A) Single cell suspensions of spleens from C57BL/6, MIF/-, CD74/- and CD44/- mice were stained with PE-B220. Cell death was measured by Annexin V-FITC and 7AAD staining. (B) Characterization of the mature (M), Transitional II (T2), Transitional I (T1) and the marginal zone (MZ) B cell populations in control, CD74/- and CD44/- spleens. (C) Characterization of the various B cell populations (1) and cell death (2) in mice injected daily for 8 days with MIF or PBS (control).
Fig 1
Figure 2
Fig 3
Fig 4
### A

| Stimuli | Control B cells | CD74-/- B cells | CD44-/- B cells |
|---------|-----------------|-----------------|-----------------|
|         | - MIF           | - MIF           | - MIF           |
| Bcl-X<sub>1</sub> | 1 3.4 | 1 1.01 | 1 1.02 |
| HPRT    | 0.99            | 0.98            | 1.01            |

### B

**Time (h)**

|   | Control B cells |
|---|-----------------|
| MIF | 3 8 10 12 24 |

### C

**CD74-/- B cells**

| Time (h) | 3 | 8 | 10 | 12 | 24 |
|----------|---|---|----|----|----|
| MIF      | - | + | -  | +  | -  |
| Bcl-2    | 1 | 1.06 | 1.2 | 1.2 | 1.13 | 1.1 | 1.34 | 1.27 |
| HPRT     | 1 | 0.92 | 0.93 | 0.97 | 0.8 | 0.84 | 0.86 | 0.88 | 0.93 |

### D

**CD44-/- B cells**

| Time (h) | 3 | 8 | 10 | 12 | 24 |
|----------|---|---|----|----|----|
| MIF      | - | + | -  | +  | -  |
| Bcl-2    | 1 | 1.01 | 1.01 | 1.02 | 0.86 | 0.87 | 0.96 | 0.91 | 0.72 | 0.65 |
| HPRT     | 1 | 1.15 | 1.18 | 1.02 | 1.26 | 1.24 | 1.2 | 1.19 | 1.13 | 1.01 |

### E

| MIF | ISO-1 (µM) | Bcl-2 | HPRT |
|-----|------------|-------|------|
| -   | -          | 1.2  | 1    |
| +   | 0.1        | 2.4  | 0.93 |
| +   | 1          | 0.2  | 0.96 |
| +   | 10         | 0.2  | 1.01 |
| +   | 20         | 0.2  | 1.02 |

### F

**Control B cells**

| Stimuli | - | MIF | MIF+αCD44 |
|---------|---|-----|-----------|
| Bcl-2   | 1 | 3.7 | 0.98 |
| HPRT    | 1 | 0.99 | 0.98 |

### G

**Control B cells**

| Stimuli | - | HA | HA+αCD44 | HA+αCD44+Con ab |
|---------|---|----|----------|------------------|
| Bcl-2   | 1 | 1.7 | 2.3 |
| HPRT    | 1 | 1.05 | 1.01 | 1 |

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**Fig 5**
Fig 6
A

B

C

Fig 7
Macrophage migration inhibitory factor (MIF) induces B cell survival by activation of a CD74/CD44 receptor complex
Yael Gore, Diana Starlets, Nitsan Maharshak, Shirly Becker-Herman, Utako Kaneyuki, Lin Leng, Richard Bucala and Idit Shachar

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