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*J Immunol* published online 23 April 2014
http://www.jimmunol.org/content/early/2014/04/23/jimmunol.1302209

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MIF Promotes B Cell Chemotaxis through the Receptors CXCR4 and CD74 and ZAP-70 Signaling

Christina Klasen,* Kim Ohl,† Marieke Sternkopf,* Idit Shachar,‡ Corinna Schmitz,* Nicole Heussen,¶ Elias Hobeika,¶,†† Ella Levit-Zerdoun,¶,†† Klaus Tenbrock,†† Michael Reth,¶,* Jürgen Bernhagen,* and Omar El Bounkari*

Macrophage migration inhibitory factor (MIF) is a proinflammatory cytokine with chemokine-like functions that plays a pivotal role in the pathogenesis of inflammatory diseases by promoting leukocyte recruitment. We showed that MIF promotes the atherogenic recruitment of monocytes and T cells through its receptors CXCR2 and CXCR4. Effects of MIF on B cell recruitment have not been addressed. In this study, we tested the involvement of MIF in B cell chemotaxis and studied the underlying mechanism. We show that MIF promotes primary murine B cell chemotaxis in a dose-dependent manner, comparable to the B cell chemokines CXCL13 and CXCL12. Splenic B cells express CXCR4 and the receptor CD74 but not CXCR2. Inhibition of CXCR4 or CD74 or a genetic deficiency of Cd74 in primary B cells fully abrogated MIF-mediated B cell migration, implying cooperative involvement of both receptors. MIF stimulation of B cells resulted in a rapid increase in intracellular Ca²⁺ mobilization and F-actin polymerization. Intriguingly, the tyrosine kinase ZAP-70 was activated upon MIF and CXCL12 treatment in a CXCR4- and CD74-dependent manner. Pharmacological inhibition of ZAP-70 resulted in abrogation of primary B cell migration. Functional involvement of ZAP-70 was confirmed by small interfering RNA–mediated knockdown in Ramos B cell migration. Finally, primary B cells from ZAP-70 gene–deficient mice exhibited ablated transmigration in response to MIF or CXCL12. We conclude that MIF promotes the migration of B cells through a ZAP-70–dependent pathway mediated by cooperative engagement of CXCR4 and CD74. The data also suggest that MIF may contribute to B cell recruitment in vivo (e.g., in B cell–related immune disorders). The Journal of Immunology, 2014, 192: 000–000.

Chemokine-directed migration of leukocytes plays a fundamental role in many physiological and pathophysiological processes (1–3). Among inflammatory disease processes, chemokine-mediated leukocyte recruitment has been particularly well studied in atherosclerosis development (4). Also, inhibition of leukocyte migration has been recognized as a potential therapeutic approach in inflammatory disorders (4, 5). Hence, a detailed characterization of the molecular mechanisms that regulate the migration of the various leukocyte cell types is essential to understanding the underlying inflammatory processes.

Received for publication August 20, 2013. Accepted for publication March 24, 2014.

This work was supported by Deutsche Forschungsgemeinschaft Grants DFG-GRK1508 and DFG-FOR809 (to J.B.) and DFG-GRK1508 (to C.K.) and by the START funding program of Rheinisch-Westfälische Technische Universität (RWTH) Aachen Medical School (START 691138 to O.E.B.).

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Abbreviations used in this article: B-CLL, B cell chronic lymphocytic leukemia; CTX, chemotactic index; MFI, mean fluorescence intensity; MIF, macrophage migration inhibitory factor; PSS, penicillinstreptomycin; PFX, peritoxin toxin; siRNA, small interfering RNA; SLE, systemic lupus erythematosus; WT, wild-type.

Macrophage migration inhibitory factor (MIF) is a proinflammatory cytokine with chemokine-like functions that has been recognized to play a central role in several acute and chronic inflammatory diseases (6–11). MIF-triggered signals are mediated by its interaction with CD74, a single-pass type II membrane protein that is also known as the MHC class II chaperone invariant chain. In conjunction with signal-transducing coreceptor proteins, cell surface–expressed MHC class II chaperone invariant chain/CD74 functions as a MIF receptor (12–15).

We demonstrated that MIF is a noncognate ligand of the CXCRs CXCR2 and CXCR4, and biochemical evidence suggests that these chemokine receptors could act as additional signal-transducing CD74 coreceptors upon MIF stimulation (16–19). MIF promotes monocyte and T cell chemotaxis and arrest through CXCR2 and CXCR4, respectively, whereas CXCR2/CD74 complexes are essential in neutrophil migratory responses (9, 16, 20, 21).

MIF is a potent proatherogenic factor and, in vivo, MIF-CXCR2/CXCR4–mediated cell migration has been correlated with monocyte and T cell accumulation in atherosclerotic and neointimal lesions (9, 16, 22–25). Of note, unlike neutralizing anti-CXCL1 or anti-CXCL12 strategies, Abs directed against MIF induced plaque regression in a mouse model of chronic atherosclerosis, suggesting that dual activation of the CXCR2 and CXCR4 axes is critical in atheroprogession in vivo (16). Moreover, MIF/CXCR-mediated signaling mediates hepatic carcinoma cell migration (26), fibroblast migration (20), endothelial progenitor cell recruitment into hypoxic areas (27, 28), and colon cancer cell metastasis (29).

The role of B cells in atherogenesis has been underestimated, but recent evidence indicates that B cells, depending on the context, may have important pro- and anti-atherogenic effects (30, 31). MIF was observed to promote the survival of recirculating B cells in bone marrow niches in mice (32), whereas it promotes human
B cell chronic lymphocytic leukemia (B-CLL) cell survival via the CD74 pathway (33). Moreover, MIF-induced activation of CD74 results in an enhanced proliferation/survival of murine B lymphocytes (12). The role of MIF in B lymphocyte migration and recruitment has not been examined.

ZAP-70 is a protein tyrosine kinase that is a well-known component of the active TCR and plays a crucial role in the initiation of T cell signaling (34). ZAP-70 has not been as well studied in B cells, but it is known that, in B-CLL, ZAP-70 is involved in IgM-BCR signaling (35, 36). Moreover, ZAP-70 was shown to be expressed in normal bone marrow B lineage progenitors, mature splenic B cells, and in some subsets of activated B cells (37–40). Additionally, ZAP-70 is expressed in pro/pre B cells and appears to be important for B cell development (41). A high expression of ZAP-70 was detected in chronic lymphocytic leukemia, and ZAP-70 is recognized as a potential marker for this disease (42–44).

Only a few studies have linked ZAP-70 to cell migration responses. For example, ZAP-70 was suggested to be involved in chemokine receptor signaling and to play a critical role in T cell migration (45, 46). Recent evidence also suggests that ZAP-70 plays a role in the regulation of B-CLL migration (47–50). However, the functional significance of ZAP-70 in mediating the migration of nonmalignant B cells remains poorly documented.

Based on flow cytometric data demonstrating CD74 and CXCR4 surface expression on primary murine B cells, in this study we assessed whether the potent promigratory effects of MIF on monocytes and T cells would extend to B cells. We found evidence that MIF promotes B cell chemotaxis comparable to well-known B cell chemokines, such as CXCL12 and CXCL13, and then set out to scrutinize the underlying mechanisms. Our data identify novel pathways of MIF/MIF receptor–mediated B cell migration responses that could have important implications for various disease settings.

Materials and Methods

Animals

In all experiments, splenic B cells were isolated from 8–12-wk-old male or female wild-type (WT) C57BL/6 mice (Jackson Laboratory, Bar Harbor, ME), CD74−/− mice (51), or ZAP-70−/− mice (52). All experiments and procedures were in compliance with the institutional animal care committee of the Medical Faculty of Rheinisch-Westfälisch Technical University (RWTH) Aachen (TVA-10915A4; TVA-10780G1).

Reagents, Abs, and inhibitors

Biologically active and endotoxin-free recombinant mouse or human MIF was expressed in Escherichia coli and purified as previously described (7, 53). CXCL12 (SDF-1α) was purchased from PeproTech (Hamburg, Germany), and rCXCL13 was from R&D Systems (Wiesbaden, Germany). Anti-human CD74 (LN2), anti-ZAP-70, PE-conjugated anti-CXCR4, and FITC-conjugated anti-CD74 were purchased from BD Biosciences (Heidelberg, Germany). PE-conjugated anti-CXCR2 and allophycocyanin-conjugated rat monoclonal anti-mouse CD74 were obtained from BD Biosciences (Heidelberg, Germany). Phosphorylation site-specific mAbs against ZAP-70 (Y493 and Y319) were purchased from Cell Signaling Technology (Frankfurt am Main, Germany). Murine monoclonal anti-MIF (NIH/IID 9.9) was produced from ascites, and anti-IgG1 was purchased from R&D Systems. Mouse monoclonal anti-actin was obtained from MP Biomedicals (Heidelberg, Germany). Anti-CD74 (C-16), anti-ERK1/2, and anti–phospho-ERK1/2 were purchased from Santa Cruz Biotechnology (Heidelberg, Germany). AMD3100, pertussis toxin (PTX), L-α-Lysophosphatidylcholine, and paraformaldehyde were purchased from Sigma-Aldrich (Hamburg, Germany). P2 was obtained from BD Biosciences. Alexa Fluor 488–labeled phalloidin was from Invitrogen (Karlsruhe, Germany).

Cell culture and small interfering RNA ZAP-70 cell transfection

Raji and Ramos cells were purchased from PromoCell (Heidelberg, Germany). Cells were cultured in RPMI 1640 medium supplemented with 10% FCS and 1% penicillin/streptomycin (P/S) and maintained at 37°C in a humidified atmosphere of 5% CO2. Media and supplements were purchased from Life Technologies (Darmstadt, Germany). Medium was routinely replaced every 2–3 d, and cells were passaged at a dilution of 1:5 or 1:10 when they were 90–95% confluent. Transfection of Ramos cells (2 × 106 cells) was done with small interfering RNA (siRNA) using a Cell Line Nucleofector Kit (Lonza, Cologne, Germany), according to the manufacturer’s protocol. Briefly, Ramos cells were incubated with 300 nM human ZAP-70 siRNA (SMARTpool, M-005398-04; Thermo Scientific/Dharmacon, Schwerzenacker, Germany). PE-conjugated anti-CXCR2 and allophycocyanin-conjugated rat monoclonal anti-mouse CD74 were obtained from R&D Systems. Phosphorylation site-specific mAbs against ZAP-70 (Y493 and Y319) were purchased from Cell Signaling Technology (Frankfurt am Main, Germany). MIF promotes B cell chemotaxis comparable to well-known B cell chemokines, such as CXCL12 and CXCL13, and then set out to scrutinize the underlying mechanisms. Our data identify novel pathways of MIF/MIF receptor–mediated B cell migration responses that could have important implications for various disease settings.

Isolation of primary B cells

Primary B lymphocytes were isolated from the spleen of mice following established procedures. Briefly, the spleen was homogenized using 40-μm cell strainers (BD Falcon, Heidelberg, Germany). RBCs were lysed using RPMI 1640 containing 10% FCS, 2 mM l-glutamine, and 1% P/S. Splenic B cells were purified by negative depletion using the Pan B Cell Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany), according to the manufacturer’s protocol. Purify of the isolated B cells was analyzed by flow cytometry (FCS) following each purification, using an anti-B220 Ab (Miltenyi Biotec), and was estimated to be between 95 and 99%.

Chemotaxis assay

Transmigration of primary B or Ramos cells was assessed in 6.5-mm-diameter 24-Transwell chemotaxis chambers (Costar, Cambridge, MA) with a pore size of 5 μm, as previously described (16). Briefly, freshly isolated B cells were suspended in medium (RPMI 1640, 10% FCS), and a total of 100 μl, containing 1 × 105 cells, was loaded in the upper chamber of the Transwell culture insert. Filters were transferred into the wells containing medium in the presence or absence of MIF, CXCL13, or CXCL12. The chambers were incubated for 4 h at 37°C in 5% CO2. After incubation, cells that had migrated into the lower chamber were collected and counted using a CASY Cell Counter (Roche Innovatis, Bielefeld, Germany). Results are presented as chemotactic index (CTX), which was calculated by dividing the number of migrated cells in the presence of the chemotactrant by the number of migrated cells in the absence of the chemotactrant (control).

Intracellular calcium mobilization

Calcium mobilization measurements were performed using the fluorescent probes Fluo-3, AM and Fura Red, AM (Invitrogen). Freshly isolated murine B cells (5 × 106 cells) were washed, suspended in 1 ml calcium-free PBS, and incubated for 30 min at 37°C with 1.3 μM Fluo 3, AM and 2.7 μM Fura Red, AM in the presence of 0.02% Pluronic F-127 (Invitrogen). After incubation, cells were washed gently and resuspended in RPMI 1640 containing 0.1% BSA and incubated for 10 min at 37°C. Cells were stimulated with 200 nM CXCL12, 200 nM piceatannol, or 300 nM non-targeting control siRNA (28). The Fura Red ratio, as a measure of the released cytosolic Ca2+ concentration, was monitored by FACS analysis. The quantification of the measurements was analyzed using FlowJo software (TreeStar, Ashland, OR).

Cell stimulation and Western blotting

Ramos cells, Raji cells, or isolated primary B cells from WT or Cd74−/− mice were seeded in 24-well plates containing an average of 1 × 106 cells in 1 ml assay medium (RPMI 1640, 10% FCS, 1% P/S). Cells were incubated for 1 h at 37°C. Subsequently, isolated B cells were loaded into the upper chamber, as described above, and subjected to MIF-mediated chemotaxis.

To test the effect of the inhibitors AMD3100 and piceatannol or neutralizing anti-mouse CD74 Ab (C-16) on chemotaxis in response to MIF, CXCL12, or CXCL13, primary B cells (1 × 105 cells) were cultured in medium (RPMI 1640, 10% FCS, 1% P/S) and incubated for 1 h in the presence or absence of the inhibitors or neutralizing Abs (anti-mouse CD74 [C-16], 10 μg/ml; piceatannol, 50 μM; AMD3100, 10 μg/ml; or DMSO as a control). Subsequently, the cells were subjected to the chemotaxis assay in response to chemotactrant.

All chemotaxis assays were plotted as CTXs normalized to spontaneous migration of B cells in medium.
were stimulated in the presence or absence of MIF or CXCL12. Concentrations used and time periods are indicated in the figure legends. For the analysis of the effect of the inhibitors (AMD3100, the G, protein inhibitor PTX, and the Src kinase inhibitor PP2) or anti-human CD74 (LN2) on the phosphorylation of ZAP-70 cells were incubated with the inhibitors for 2 h at 37˚C and stimulated thereafter with MIF or CXCL12. After stimulation, cells were washed and lysed with LDS electrophoresis loading buffer (Invitrogen). Lysates were analyzed by Western blotting. For the analysis of the effect of the inhibitors (AMD3100, the G, protein inhibitor PTX, and the Src kinase inhibitor PP2) or anti-human CD74 (LN2) on the phosphorylation of ZAP-70 cells were incubated with the inhibitors for 2 h at 37˚C and stimulated thereafter with MIF or CXCL12. After stimulation, cells were washed and lysed with LDS electrophoresis loading buffer (Invitrogen). Lysates were analyzed by Western blotting. Equal amounts of proteins were loaded on 12% SDS-polyacrylamide gels and transferred onto polyvinylidene difluoride membranes (Carl Roth, Karlsruhe, Germany). Membranes were blocked in PBS-Tween containing 2% BSA for 1 h. Proteins of interest were detected using specific primary Abs, including anti–ZAP-70, anti-phospho–ZAP-70 (Y493), anti-phospho–ZAP-70 (Y319), anti–ERK1/2, anti–phospho-ERK1/2, and anti–β-actin. After overnight incubation, the blots were washed and incubated with a species-specific secondary Ab coupled to HRP for 1 h at room temperature. Blots were visualized by enhanced chemiluminescence (Super Signal West Femto; Pierce Biotechnology, Bonn, Germany), according to the manufacturer’s protocol.

**F-actin polymerization assay**

Isolated splenic B cells (1.5 × 10⁶ cells) were incubated in RPMI 1640 supplemented with 10% FCS and 1% P/S and stimulated in the presence or absence of MIF or CXCL12 for various lengths times at room temperature. Cells were incubated in assay buffer (4 × 10⁻⁷ M Alexa Fluor 488–labeled phalloidin, 125 μg/ml L-α-Lysophosphatidylcholine, and 4% paraformaldehyde in PBS) for 10 min. Fixed cells were analyzed by flow cytometry on a FACSCanto II (BD Biosciences), and the mean fluorescence intensity (MFI) was determined for each sample. All time points were plotted relative to the MFI of the sample before addition of the chemokine.

**Flow cytometry**

Analysis of the cell surface expression of the MIF receptors CXCR2, CXCR4, and CD74 on isolated splenic B lymphocytes, Ramos cells, or Raji cells was monitored by FACS analysis. Briefly, 1 × 10⁶ cells were washed with PBS supplemented with 0.5% BSA and 0.1% sodium azide and stained with PE-conjugated anti-CXCR4, FITC-conjugated anti-CXCR2, or FITC-conjugated anti-CD74. Specificity of staining was confirmed using the corresponding isotype controls. Stained cells were washed and analyzed by a FACSCanto II (BD Biosciences). The quantification of the measurements was analyzed using FlowJo software (TreeStar).

**Statistical evaluation**

Statistical analysis was assessed using the Student t test with Welch correction (with GraphPad Prism software). A p value ≤ 0.05 was considered statistically significant.

**Results**

**MIF induces the chemotactic migration of primary murine B lymphocytes in a dose-dependent manner**

The potential involvement of MIF in B cell chemotaxis was assessed using isolated B cells from the spleen of WT C57BL/6 mice.

**FIGURE 1.** MIF triggers B cell chemotactic migration in a dose-dependent manner. (A) Cell surface expression of MIF receptors on primary murine B cells. Purified murine splenic B lymphocytes were stained with allophycocyanin- or PE-conjugated Abs directed against CD74, CXCR4, or CXCR2. Surface expression of the receptors was analyzed by FACS. Specific staining is shown as colored lines. Shaded lines indicate measurements using isotype control IgG. (B) MIF triggers the chemotaxis of primary murine B cells. In vitro migration assays were performed using Transwell chambers containing different concentrations of recombinant mouse MIF. Isolated B lymphocytes from the spleen of WT C57BL/6 mice were added to the upper chamber and subjected to a 4-h migration period. (C) Effect of neutralizing anti-MIF mAb on B cell migration in response to MIF. Recombinant MIF (200 ng/ml) was added to the lower chamber and incubated with anti-MIF (36 μg/ml) or IgG1 control (36 μg/ml) for 1 h at 37˚C. Subsequently, isolated B cells were loaded in the upper chamber and allowed to migrate for 4 h. (D) The chemotactic effect of MIF on B cells is similar to that of CXCL12 and CXCL13. Isolated B cells from WT mice were subjected to Transwell chemotaxis assay in response to MIF (200 ng/ml), CXCL12 (100 ng/ml), or CXCL13 (1000 ng/ml). Data are expressed as CTX (mean ± SD) and are representative of three (B and C) or five (D) independent experiments. *p < 0.05, **p < 0.01. ns, not significant.
First, we analyzed the cell surface expression of the MIF receptors CXCR2, CXCR4, and CD74 on isolated B lymphocytes. In agreement with a previous report (54), isolated splenic B cells expressed CD74 and CXCR4 but not CXCR2 (Fig. 1A). The migratory capacity of the B cells in response to MIF, CXCL12, or controls was assayed using a Transwell migration device. MIF showed a dose-dependent chemotactic effect on B cells, with a bell-shaped dose-response behavior typically observed for chemokines. Maximal MIF-induced chemotaxis (CTX = 2.1) was seen using 200 ng/ml of MIF (Fig. 1B). Therefore, this concentration was used for all subsequent chemotaxis assays. To further verify the specificity of the MIF-mediated effect, we blocked MIF using a neutralizing Ab and determined whether this interfered with the MIF-mediated chemotactic B cell response. To this end, recombinant murine MIF (200 ng/ml) was incubated with an anti-MIF mAb or anti-IgG, as control in the lower chamber of the migration device for 1 h at 37˚C. Subsequently, isolated B cells from WT mice were subjected to the chemotaxis assay. As shown in Fig. 1C, the chemotactic response toward MIF was strongly reduced by the anti-MIF mAb. In contrast, anti-IgG did not influence MIF-induced B cell migration. Additionally, MIF-driven B cell chemotaxis was directly compared with that of two well-known B cell chemokines, CXCL12/SDF-1α and CXCL13, which are both known to regulate B cell homing and migration under various physiological and pathophysiological conditions. Of note, CXCL12 triggers chemotactic cell responses through CXCR4, whereas CXCL13 engages CXCR5, a prototypical B cell chemotaxis receptor also prominently expressed on our isolated murine B cells (see below). The chemotactic response of the B cells toward CXCL12 (CTX = 2.8) was similar to that of MIF (CTX = 2). Also, the chemotactic effects mediated by MIF or CXCL12 were comparable to that of CXCL13, which, however, showed a higher chemotactic potency (CTX = 3.8; Fig. 1D). Taken together, these findings suggest that MIF prominently promotes the chemotactic migration of primary B cells.

**MIF-enhanced B cell migration is regulated by CXCR4 and CD74**

Next, we examined the influence of MIF receptors CXCR4 and CD74 on the chemotaxis of B cells toward MIF. To this end, we first analyzed the impact of AMD3100, a selective and competitive pharmacological inhibitor of CXCR4, on the chemotactic migration of B lymphocytes in response to MIF or the other B cell chemokines. Freshly isolated B cells were treated with AMD3100 and subsequently subjected to CXCL12- or CXCL13-triggered chemotaxis. As expected, inhibition of CXCR4 by AMD3100 was associated with a substantial reduction in CXCL12-mediated migration (Fig. 2A). In contrast, the migratory potential of CXCL13, which signals through CXCR5, was not affected (Fig. 2B). Of note, the chemotaxis response induced by MIF was fully abrogated when CXCR4 was blocked with AMD3100 (Fig. 2C), clearly indicating that engagement of CXCR4 by MIF is involved in the regulation of B cell chemotaxis elicited by MIF.

We next wished to examine whether CD74 also contributes to MIF-induced chemotaxis of B cells. WT primary murine B lymphocytes were incubated with a neutralizing Ab against mouse CD74 (C-16) or IgG control and subsequently assessed for their ability to migrate in response to MIF. As shown in Fig. 3A, the chemotactic response toward MIF was fully inhibited after blockade of CD74. In contrast, treatment of cells with IgG did not influence B cell migration in response to MIF, indicating that the second B cell–expressed MIF receptor (CD74) is required for MIF-triggered B cell migration. To further confirm this notion, the migratory capacity of isolated Cd74-deficient primary murine B lymphocytes from Cd74-deficient mice were subjected to a 4 h chemotaxis experiment toward MIF (200 ng/ml) or CXCL13 (1000 ng/ml). (B) Cd74-deficient B cells do not migrate in response to MIF but are responsive to CXCL13. Isolated B lymphocytes from Cd74-deficient mice were subjected to a 4 h chemotaxis experiment toward MIF (200 ng/ml) or CXCL13 (1000 ng/ml). (C) Transmigration assay of Cd74−/− B lymphocytes in response to different concentrations of MIF (0–1000 ng/ml). The data (mean ± SD) are from four independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001, ns, not significant.
spleen B lymphocytes was assessed in response to MIF or CXCL13. Cd74−/− B cells completely failed to migrate in response to MIF, whereas they migrated essentially normally toward CXCL13 (Fig. 3B). To exclude that Cd74−/− B cells only exhibited a shifted dose-response behavior, we subjected them to an expanded concentration range of MIF. However, Cd74−/− B cells were unable to migrate toward any of the tested MIF concentrations (Fig. 3C). Taken together, these data strongly suggested that both CXCR4 and CD74 contribute to MIF-driven B cell chemotaxis. Moreover, the observation that blockade of either CD74 or CXCR4 led to an essentially complete abrogation of the MIF-triggered chemotactic response insinuated that both receptors engage in some kind of cooperative mechanism in controlling MIF-driven B cell migration. Normal migration behavior of AMD3100- and anti–CD74-treated and Cd74−/− B cells to CXCL13/CXCR5-driven chemotaxis indicated that blockade or deletion of these receptors does not lead to generalized migration defects.

MIF-triggered B cell chemotaxis is accompanied by Ca2+ mobilization and F-actin polymerization

It is well known that binding of chemokines to their receptors is associated with intracellular calcium mobilization (55). Our previous studies in neutrophils and fibroblasts showed that MIF also is able to trigger calcium influx through its receptors CXCR2 and CXCR4 and that this is associated with MIF-driven rapid integrin activation and monocyte and T lymphocyte chemotaxis (16). Thus, we examined the ability of MIF to induce changes in intracellular calcium in B lymphocytes. For this purpose, primary B cells were stimulated with 200 ng/ml MIF, a concentration previously shown to be optimal in eliciting B cell chemotaxis (Fig. 1B). For comparison, cells were stimulated with 100 ng/ml CXCL12. Triggered Ca2+ transients were measured by Fluo 3, AM– and Fura Red, AM–based flow cytometry assay. As shown in Fig. 4A, MIF stimulation resulted in a transient and rapid increase in intracellular free calcium, and MIF’s effect on calcium was comparable to that mediated by CXCL12.

Because cell migration is associated with a dynamic remodeling and polarization of the actin cytoskeleton that is necessary for effective directional migration of immune cells (56), we also investigated whether MIF influenced F-actin polymerization. To this end, isolated B cells from WT mice were stimulated for 1, 2, or 3 min with MIF (200 ng/ml) and subsequently fixed and stained with fluorescently labeled phallolidin. The amount of polymerized F-actin in the cells was determined by a FACS-based procedure. MIF stimulation of B cells caused a rapid and transient polymerization of the actin network, with an observed increase in the F-actin content ~20% within 1–3 min. These responses were highly reproducible and reached statistical significance. Again, the MIF effect was compared with that of CXCL12. Using an optimal concentration for both chemokines, MIF and CXCL12 induced actin polymerization to a similar extent (Fig. 4C). These findings indicated that MIF regulates calcium mobilization and actin polarization, most likely involving CXCR4, and that the cellular changes are associated with the MIF-mediated chemotactic response in B cells.

MIF induces ZAP-70 phosphorylation through CXCR4 and CD74

To gain more insight into the mechanism through which MIF mediates B cell chemotaxis and to further explore the apparent

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**FIGURE 4.** MIF enhances intracellular calcium release and F-actin polymerization. (A) MIF and CXCL12 promote intracellular calcium influx. Freshly isolated splenic B cells from WT mice were labeled with the fluorescent calcium indicator Fluo 3, AM and stimulated with MIF (200 ng/ml) or CXCL12 (100 ng/ml); sodium phosphate buffer (pH 7.2; NaP) was used as control. MFI, as a measure of the released cytosolic Ca2+ concentration, was monitored by FACS analysis. (B) Intracellular F-actin polymerization of primary B cells is enhanced by MIF. Isolated B cells from WT mice were stimulated with MIF (200 ng/ml) for 1, 2, or 3 min and subsequently stained with Alexa Fluor 488–labeled phallolidin. Results are presented as percentage of intracellular F-actin relative to the values before stimulation. (C) Comparison of MIF- and CXCL12-mediated F-actin polymerization. Similar experiment as in (B), except that the effect of MIF (200 ng/ml) was compared with that of CXCL12 (100 ng/ml) after 2 min. Data represent mean ± SD values of three (B) or five (C) independent experiments. *p < 0.05, **p < 0.01.
cooperative involvement of CXCR4 and CD74, we sought to characterize key molecules involved in the downstream-signaling cascade. ZAP-70 has been associated with cell migration responses in T cells, as well as B lymphoma cells, and links to chemokine activation have been proposed (45, 46).

We hypothesized that MIF may induce the phosphorylation and activation of ZAP-70 kinase. We first used the human B lymphoma cell line Raji. These cells were stimulated with different MIF concentrations, and cell lysates were subjected to immunoblotting analysis using an Ab that specifically recognizes the phosphorylated regulatory tyrosine residue (Tyr$^{319}$) required for ZAP-70 kinase activation. As shown in Fig. 5A, MIF led to an increase in ZAP-70 phosphorylation. This effect was substantial, concentration dependent, and reached a maximum at 800–1000 ng/ml of recombinant human MIF. Kinetic analysis showed that MIF treatment resulted in a transient ZAP-70 phosphorylation response that peaked at 20 min and persisted for up to 50 min, rapidly disappearing thereafter (Fig. 5B). Of note, stimulation of Jurkat T cells with recombinant MIF revealed a similar ZAP-70 phosphorylation pattern as that seen in the B cells (data not shown). This indicated that MIF-induced ZAP-70 activation is not limited to B cells but also occurs in T cells. To further confirm and characterize ZAP-70 activation elicited by MIF in B cells, we examined the phosphorylation reaction of another tyrosine residue (Tyr$^{493}$) in ZAP-70. This residue is located in the kinase domain and is known to be phosphorylated during activation of ZAP-70 as well. Using primary splenic B cells, we confirmed that ZAP-70 activation by MIF also occurs in primary B cells and found that MIF induced the phosphorylation of both tyrosine residues, Y319 and Y493, required for the catalytic activity of ZAP-70 (Fig. 5C).

The effect of CXCL12 on ZAP-70 phosphorylation has not been studied. Next, we asked whether CXCL12 also induced ZAP-70 phosphorylation. In fact, stimulation of Raji cells with CXCL12 (100 ng/ml) enhanced the phosphorylation of ZAP-70, but the kinetics appeared to be shifted to shorter time periods compared with that elicited by MIF (Fig. 5D). Moreover, CXCL12 also induced ZAP-70 phosphorylation on both tyrosine residues (Y319 and Y493) in a similar manner as MIF in primary B cells (Fig. 5E).

To gain further information about MIF-induced ZAP-70 phosphorylation in B lymphocytes, we asked whether the MIF receptors CXCR4 and CD74 are required for this activation. Accordingly, MIF-mediated ZAP-70 activation in Raji cells was analyzed in the presence versus absence of AMD3100 and/or the LN2 mAb (a neutralizing anti-human CD74 Ab). Blockade of CD74 with LN2 or CXCR4 with AMD3100 led to a partial reduction in MIF-
mediated ZAP-70 phosphorylation (anti-CD74: ~50% reduction; AMD3100: ~80% reduction), whereas simultaneous blockade of both receptors completely abrogated MIF-triggered activation of the kinase (Fig. 5F). Moreover, preincubation of cells with PTX, an inhibitor of Gα proteins and, thus, most CXCR4-signaling responses, also substantially reduced MIF-mediated activation of ZAP-70 (Fig. 5F). The strongest inhibitory effect was seen with PP2, a potent selective inhibitor of Src family kinases (Fig. 5F). Furthermore, stimulation of Cd74−/− B cells with MIF or CXCL12 was associated with a reduction in MIF-induced ZAP-70 activation compared with CXCL12 (Fig. 5G), in line with the notion that MIF activation of ZAP-70 also involves CD74, whereas CXCL12 signals solely through CXCR4.

Together, the signaling analysis showed that MIF-mediated ZAP-70 activation is regulated by the CXCR4/CD74 axis and involves Gα and Src signaling.

**ZAP-70 phosphorylation is functionally involved in MIF-mediated chemotaxis of B cells**

Given that MIF induces ZAP-70 phosphorylation and that ZAP-70 activity, at least indirectly, has been implicated in T lymphocyte and B lymphoma migration, we next asked whether MIF-induced ZAP-70 activity would functionally influence the migration of B cells in response to MIF. Isolated primary B cells were treated with piceatannol (50 μM), a Syk/ZAP-70–selective inhibitor, or with DMSO (vehicle) for 1 h and were subsequently stimulated with MIF (200 ng/ml) for 20 min. Piceatannol treatment, but not the DMSO vehicle, fully abolished MIF-induced ZAP-70 phosphorylation (Fig. 6A). Moreover, piceatannol preincubation also strongly affected the MIF-stimulated phosphorylation of the mitogen-activated kinase ERK1/2 (Fig. 6A), indicating that, in B cells, MIF-triggered ERK phosphorylation required the activation of ZAP-70. Pretreatment of cells with either piceatannol or DMSO did not influence cell viability, as monitored by the trypan blue exclusion assay (data not shown).

We also examined whether piceatannol influenced ZAP-70 phosphorylation induced by CXCL12. In fact, piceatannol also attenuated the stimulatory effect of CXCL12 on ZAP-70 phosphorylation. As observed before (Fig. 5), the stimulatory effect of CXCL12 on ZAP-70 was somewhat smaller than that of MIF. Additionally, the inhibitory effect of piceatannol was less pronounced, whereas this compound almost fully blocked CXCL12-induced ERK1/2 phosphorylation (Fig. 6B).

To begin to determine whether ZAP-70 activity is functionally involved in MIF-driven B cell chemotaxis, primary B cells were preincubated with piceatannol or DMSO and subjected to the transmigration assay in response to MIF. As displayed in Fig. 6C, the migratory capacity of cells in response to MIF (CTX = 2.2) was completely ablated in the presence of piceatannol, whereas DMSO vehicle had no effect. Similarly, piceatannol resulted in an impaired ability of cells to migrate in response to CXCL12 (Fig. 6D).

Even when exhibiting high target kinase specificity, pharmacological inhibitors, such as piceatannol, may have unknown off-target effects. Thus, to further verify the functional contribution of ZAP-70 to MIF-induced B cell migration, we determined whether silencing of ZAP-70 by an siRNA approach also would reduce the chemotaxis of B cells in response to MIF. Ramos B lymphoma cells were used because primary murine B cells were not amenable to appreciable siRNA oligonucleotide transfection efficiencies. We first assessed the chemotactic migration ability of these cells in response to different concentrations of MIF. As shown in Fig. 7A, MIF did not activate Ramos cell chemotaxis as markedly as seen for the primary B cells, but significant chemotactic effects of MIF, with a CTX of 1.7, were observed between 400 and 600 ng/ml. Also, stimulation of these cells with

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**FIGURE 6.** Piceatannol impairs ZAP-70 phosphorylation and transmigration of B cells in response to MIF or CXCL12. Blocking effect of piceatannol on ZAP-70 phosphorylation after MIF (A) or CXCL12 (B) stimulation of primary B cells. Primary B cells were isolated from spleen of WT C57BL/6 mice and subsequently preincubated with 50 μM piceatannol or DMSO (control) for 1 h. Cells were then stimulated with MIF (200 ng/ml) (A) or CXCL12 (100 ng/ml) (B) for 20 min, lysed, and subjected to immunoblot analysis using anti-phospho–ZAP-70 (Y493), anti–phospho-ERK1/2, anti–ZAP-70, anti-ERK1/2, and anti-actin. Piceatannol impairs B cell chemotaxis in response to MIF (C) or CXCL12 (D). Isolated B lymphocytes from WT C57BL/6 mice were preincubated with 50 μM piceatannol or DMSO (control) for 1 h and subjected to chemotaxis in response to MIF (200 ng/ml) (C) or CXCL12 (100 ng/ml) (D) for 4 h. Migrated cells were collected and counted by CASY. The data shown represent mean ± SD of six (C) or three (D) independent experiments. *p < 0.05, **p < 0.01. ns, not significant.
recombinant MIF led to a time-dependent ZAP-70 phosphorylation response (Fig. 7B). Because these experiments supported a role for MIF in Ramos B cell chemotaxis, siRNA knockdown was performed next. Forty-eight hours posttransfection, ZAP-70–specific siRNA led to a 85% decrease in ZAP-70 expression compared with a control RNA-transfected cell batch (Fig. 7C, inset), whereas cell viability was not influenced by siRNA treatment (data not shown). To determine the effect of ZAP-70 depletion on the migration behavior of Ramos cells toward MIF, a Transwell migration assay of transfected cells was assessed in response to 600 ng/ml MIF. The chemotactic migration of ZAP-70–depleted cells was completely abrogated compared with control siRNA-transfected cells (Fig. 7C), confirming that MIF-mediated B cell migration requires the presence of active ZAP-70 kinase.

Finally, and to further verify this notion, we compared the migratory responses of primary B cells isolated from WT versus ZAP-70−/− mice. When exposed to a chemotactic stimulus of 200 ng/ml recombinant MIF or 100 ng/ml CXCL12, control-primary B cells showed CTXs of 1.9 and 2.6, respectively. In contrast, ZAP-70−/− B cells did not migrate in response to MIF; the chemotactic effect of CXCL12 was also fully abrogated (Fig. 7D).

In summary, the various B cell migration studies, in conjunction with ZAP-70 functional impairment, strongly suggested that ZAP-70 activation is a mandatory signaling module in MIF- or CXCL12-driven B cell chemotaxis (Fig. 8).

**Discussion**

MIF has been recognized as a critical mediator of several inflammatory diseases (6, 7). Control of leukocyte recruitment and infiltration has been recognized as an important mechanistic facet of how MIF contributes to the involved inflammatory sequelae (8, 16, 24). Moreover, MIF is proatherogenic, mediating the recruitment of monocytes and T cells into atherosclerotic lesions through CXCR2 and CXCR4, respectively (16, 57, 58). MIF also promotes neutrophil migration (16, 59), but its effect on the migration and recruitment of B cells, which more recently have been recognized to also regulate atheroprogression, is largely unknown.

In the current study, we addressed this issue, primarily focusing on the role of MIF in the chemotactic migration of primary murine B lymphocytes. Our data characterize MIF as a novel B cell chemokine that triggers B cell chemotaxis, which is comparable in extent and kinetics to the classical B cell chemokines CXCL12 and CXCL13. Our study also offers novel mechanistic information about the molecular pathways involved. Accordingly, the MIF receptors CXCR4 and CD74, but not CXCR2, act cooperatively to activate a ZAP-70–mediated pathway that is associated with MIF-driven calcium, ERK1/2, and migration responses.

MIF was reported to promote the survival of murine recirculating B lymphocytes in bone marrow–immune niches (32), as well as human B-CLL cells through CD74 signaling (33). Overall, CD74 has been considered to be primarily responsible for MIF-mediated cell survival and proliferation responses (15), but it also has been

**FIGURE 7.** ZAP-70 siRNA knockdown or ZAP-70 deficiency attenuates B cell migration in response to MIF or CXCL12. (A) Dose-dependent chemotaxis of Ramos cells in response to MIF. Ramos cells were subjected to chemotaxis in response to different concentrations of MIF (0–1000 ng/ml) for 4 h. Data are expressed as CTX. (B) MIF-induced time-dependent ZAP-70 phosphorylation in Ramos cells. Ramos cells were stimulated with MIF (600 ng/ml) for the indicated times. After stimulation, cells were lysed, and lysates were analyzed by Western blotting using anti-phospho–ZAP-70 (Y319) and anti–ZAP-70 Ab. (C) Chemotaxis assay of Ramos cells after downregulation of ZAP-70 by siRNA approach. Ramos cells were transfected with ZAP-70–specific siRNA or control RNA (300 nM each). Forty-eight hours later, the efficiency of the ZAP-70 knockdown was evaluated by immunoblotting using anti–ZAP-70. Transfected cells were then subjected to chemotaxis assay in response to MIF (600 ng/ml). CTXs are normalized to the control RNA-treated cells in the absence of MIF stimulus. (D) Impaired migration of ZAP-70–deficient B cells toward MIF or CXCL12. Primary B lymphocytes were isolated from spleens of WT (upper panel) or ZAP-70−/− (lower panel) mice and subjected to transmigration assay in response to MIF (200 ng/ml) or CXCL12 (100 ng/ml) for 4 h. Data are expressed as CTX and are mean ± SD of three (B and D) or six (C) independent experiments. *p < 0.05. ns, not significant.
B cells from Cd74 difference could be due to the well-known more immature state of respectively. Although not scrutinized further in this study, this yet somewhat lower, migration response toward CXCL13 com-

Interestingly, inhibition of CXCL12/CXCR4 interactions abolished the MIF/CXCR4 and CXCL12/CXCR4 axis in atherogenesis (16). Additionally, CXCR4 is mediated by CXCR4 and CD74. However, blockade of both receptors (“cooperative action”) in MIF-mediated B cell migration and ZAP-70 activation could either involve heteromeric receptor complex formation between CXCR4 and CD74 or a cross-talk between the MIF/CXCR4- and MIF7 CD74-signaling pathways. For simplicity, the data on CXCL12-activated ZAP-70 activation and B cell migration are not shown. Piceatannol blockade of ZAP-70 is depicted and is representatively shown to indicate functional inactivation of ZAP-70 by various approaches.

Our results clearly show that blockade of CD74 by neutralizing Ab or genetic deletion substantially reduced the chemotactic migration of primary murine B cells in response to MIF. Interestingly, we demonstrated previously that the MIF/CD74 pathway controls the expression of Tap63 and VLA-4 integrin, resulting in an enhanced homing of B-CLL cells to the bone marrow (33). We noticed that B cells from Cd74−/− mice exhibited a significant, yet somewhat lower, migration response toward CXCL13 compared with primary B cells from WT mice (CTX = 1.8 and 3.7, respectively). Although not scrutinized further in this study, this difference could be due to the well-known more immature state of B cells from Cd74−/− mice (62).

Intriguingly, we also identified CXCR4 as a receptor mediating MIF-driven B cell migration. As expected, CXCR4 also mediates B cell migration in response to CXCL12. The data suggest a prominent role for the MIF/CXCR4 axis in regulating B cell chemotaxis and, thus, would place the B lymphocyte next to the T cell, in which the migratory response is also regulated by both the MIF/CXCR4 and CXCL12/CXCR4 axis in athogenesis (16). Interestingly, inhibition of CXCL12/CXCR4 interactions abolished the migration and survival of B-CLL cells (63, 64). Additionally, MIF recently was reported to have a role in the development of chronic lymphocytic leukemia (65). Whether the MIF/CXCR4 pathway plays a similar role in the migration of B-CLL cells remains to be investigated.

One revealing mechanistic observation in our study is the finding that the blockade of either CD74 or CXCR4 led to a complete abrogation of MIF-induced B cell chemotaxis. This clearly is suggestive of some degree of cooperativity between these two receptor pathways, which may be explained either by physical receptor complex formation between CXCR4 and CD74 or by some kind of signaling cross-talk between the CXCR4- and CD74-mediated pathways. Receptor dimerization/oligomerization has been implicated in the fine-tuning of several signaling pathways that promote or impair different biological responses and, homodimeric and even heterodimeric dimerization is frequently observed for chemokine receptors (19). Along these lines, CXCR4 was found to form a heterodimeric complex with CXCR7 (66), as well as to engage the TCR (67). In both cases, receptor complex formation resulted in an enhanced cell migration response to CXCL12 (68, 69). We reported previously that CXCR4 and CD74 heterodimerize upon overexpression, can complex in monocytes under endogenous conditions, and cooperate in MAPK signaling in T cells (17). Hence, it is conceivable that CXCR4 and CD74 form a receptor complex in B cells as well and that CXCR4/CD74 complexes might be responsible for the observed MIF-induced B cell migration response. Additionally, CD74/CD44 complexes may contribute to MIF-enhanced B cell migration. CD74/CD44 complexes were reported to initiate signaling cascades in B cells that regulate B cell survival and proliferation (12, 14, 62). Interestingly, CD44 was found to be required for hematopoietic and leukemia cell migration through interaction of its cytoplasmic tail with the actin cytoskeleton involving ankyrin and ezrin-radixin-moesin proteins (70, 71). Recent data also demonstrated that CD44 forms a complex with CD49d that affects the migration, survival, and homing of leukemia cells (72). Suitable biochemical and biophysical studies will be required to further interrogate these possibilities in B cells.

ZAP-70 is a protein tyrosine kinase that is critically involved in the development of T cells. Several studies demonstrated an (indirect) involvement of ZAP-70 in the regulation of chemokine receptor signaling (45, 46). Furthermore, tyrosine phosphorylation of ZAP-70 is induced through activation of the CXCL12/CXCR4 and CXCL10/CXCR3 pathways (73, 74). In this article, we show that the stimulation of B cells with MIF or CXCL12 induces the phosphorylation of ZAP-70 in a dose- and time-dependent manner in both tyrosine residues Y319 and Y493 required for ZAP-70 activation in primary B cells. Inhibitory studies targeting CXCR4 and CD74 suggested that MIF-induced ZAP-70 phosphorylation is mediated by CXCR4 and CD74. However, blockade of the individual receptors only partially reduced ZAP-70 phosphorylation, whereas full inhibition was seen when both receptors were blocked simultaneously or when upstream Src kinase activity was blocked. Involvement of CXCR4 was further supported by the inhibition experiment with PTX, which exerted a partial blockade similar in extent to that seen using AMD3100. In T cells, activation of the Src family kinase Lck is necessary for the phosphorylation of ZAP-70 (75). Such a link has not been reported in B cells, but our PP2 experiment is indicative of a prominent involvement of an upstream Src kinase in MIF-stimulated B cells, as well.

ZAP-70 has been implicated in T cell migration following chemokine stimulation, as well as in B-CLL migration (45–50, 73, 76, 77). In this article, we demonstrate that ZAP-70 is functionally involved in the regulation of MIF- and CXCL12-mediated primary B cell chemotaxis. The evidence comes from an observed
inhibition of ZAP-70 by piceatannol that led to decreased MIF- and CXCL12-induced B cell chemotaxis, from ZAP-70–siRNA knockdown experiments in Ramos cells that showed an attenuation of MIF-induced Ramos B cell chemotaxis, and from chemotaxis experiments with B cells from ZAP-70−/− mice, which exhibited an ablated MIF-induced B cell chemotactic response. Our study is among the few to investigate ZAP-70 in B cells; consequently, little is known about the effect of ZAP-70 deficiency on B cell morphology and function. Interesting aspects that need to be addressed in future studies include questions about the B cell repertoire and the homing properties of ZAP-70−/− B cells in vivo in response to MIF, as well as other B cell migratory signals.

Together, these data establish a role for the MIF/CXCR4/CD74/ZAP-70 and CXCL12/CXCR4/ZAP-70 axes in B cell chemotaxis (Fig. 8). However, despite our identification of a coactivation of ERK1/2 signaling, calcium influx, and actin rearrangement, the precise mechanistic link between the ZAP-70 pathway and the specific chemotactic process needs to be assessed in future studies. Observations made by other investigators, including a suggested role for ZAP-70 in regulating the β2 integrin lymphocyte function-associated Ag-1 (LFA-1) (78, 79), β1 integrin (80) and Talin (77), as well as the requirement of ZAP-70 for CXCL12-promoted α4β1 integrin activation in cell adhesion (81), offer important hints about how to pursue these studies.

It will be of great interest to learn to which B cell migratory processes in vivo and/or to which in vivo pathologies the identified MIF/CXCR4/CD74/ZAP-70 axis may contribute. Processes might include the repartitioning of B cells around T cell areas following T cell−dependent Ag triggers. Autoimmune diseases featuring prominent B cell responses, such as systemic lupus erythematosus (SLE), could be candidate diseases involving MIF-dependent B cell migratory processes. In fact, MIF was reported to play a role in the pathogenesis of SLE (10). However, direct effects of MIF on B cell migration and infiltration in this disease have yet to be elucidated. Leng et al. (82) noted that neutralization of MIF by a pharmacological inhibitor or a neutralizing Ab attenuated renal inflammation in cell adhesion (81), offer important hints about how to pursue these studies.

References

Disclosures
J.B. is a coinventor on patent applications covering anti-MIF strategies in inflammatory diseases. In 2012, J.B. received a consultancy fee exceeding $5000 from Carolus Therapeutics (San Diego, CA), a company pursuing chemokine-based strategies in immune and inflammatory diseases.


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