Rationale: Macrophage migration inhibitory factor (MIF) is released on platelet activation. Circulating MIF could potentially regulate platelets and thereby platelet-mediated inflammatory and regenerative mechanisms. However, the effect of MIF on platelets is unknown.

Objective: The present study evaluated MIF in regulating platelet survival and thrombotic potential.

Methods and Results: MIF interacted with CXCR4-CXCR7 on platelets, defining CXCR7 as a hitherto unrecognized receptor for MIF on platelets. MIF internalized CXCR4, but unlike CXCL12 (SDF-1α), it did not phosphorylate Erk1/2 after CXCR4 ligation because of the lack of CD74 and failed in subsequent CXCR7 externalization. MIF did not alter the activation status of platelets. However, MIF rescued platelets from activation and BH3 mimetic ABT-737–induced apoptosis in vitro via CXCR7 and enhanced circulating platelet survival when administered in vivo. The antiapoptotic effect of MIF was absent in Cxcr7−/− murine embryonic cells but pronounced in CXCR7-transfected Madin–Darby canine kidney cells. This prosurvival effect was attributed to the MIF–CXCR7–initiated PI3K–Akt pathway. MIF induced CXCR7–Akt–dependent phosphorylation of BCL-2 antagonist of cell death (BAD) both in vitro and in vivo. Consequently, MIF failed to rescue Akt−/− platelets from thrombin-induced apoptosis when challenged ex vivo, also in prolonging platelet survival and in inducing BAD phosphorylation among Akt−/− mice in vivo. MIF reduced thrombus formation under arterial flow conditions in vitro and retarded thrombotic occlusion after FeCl3–induced arterial injury in vivo, an effect mediated through CXCR7.

Conclusion: MIF interaction with CXCR7 modulates platelet survival and thrombotic potential both in vitro and in vivo and thus could regulate thrombosis and inflammation. (Circ Res. 2014;115:939-949.)

Key Words: Akt • apoptosis • BAD • chemokine • MIF

Platelets are multifunctional cells that play a critical role in wound healing, immune defense, and repair mechanisms at sites of vascular and tissue injury.1,2 Platelets undergo apoptosis3 either as part of a spontaneous senescence program or after activation. Stimulation of platelets with agonists like thrombin or collagen at sites of platelet accumulation induce apoptosis or apoptosis-like events, such as phosphatidylserine externalization, cell shrinkage, loss of mitochondrial transmembrane potential (∆Ψm), or caspase-3 activation.4,5 However, mechanisms that protect platelets against apoptosis to confer survival and implications of this process are incompletely understood. Platelets harbor a wide array of pro- and anti-inflammatory/angiogenic factors in their granular repertoire,6 many of which could regulate vascular injury/inflammation, and which might also influence platelet survival. Recently, we described that platelets express both chemokine receptors CXCR4 and CXCR7,5,6 and that surface expression of CXCR7 is significantly elevated in patients with acute coronary syndrome as compared with stable angina pectoris.8 Platelet CXCR7 surface expression significantly correlates with levels of its ligand CXCL12 and with functional recovery after myocardial infarction. Moreover, CXCL12 induces differential cellular trafficking of CXCR4/CXCR7 through active involvement of Erk1/2 and cyclophilin A and rescues platelets from activation-induced apoptosis.7

Macrophage migration inhibitory factor (MIF), a widely expressed and pleiotropic cytokine and a chemokine-like factor, is a ligand for CXCR4,8 induces monocyte migration and their
MIF can modulate survival of platelets, as described recently by plaque rupture show significantly higher plasma levels of troponin I release. Patients with acute coronary syndrome caused by plaque rupture can induce migration of monocytes along with SDF-1/CXCL12. However, the potential effect of MIF on platelets themselves remained elusive. MIF effects on target cells depending on cell type and functional context are executed through interaction with the receptors CXCR2, CXCR4, CD74, and CXCR7. Platelets are anucleated terminally differentiated cells with limited migratory activity under hemodynamic stress, but regulate hemostasis, inflammation, and regeneration, all of which can be potentially modulated by their relative survival or life span in circulation. In the present study, we evaluated the role of MIF on platelet survival and thrombotic potential.

Methods

A detailed account of materials and methods used in the study is provided in the Online Data Supplement.

Results

Differential Effects of MIF and CXCL12 on Translocation of CXCR4 and CXCR7

CXCL12 is a high-affinity ligand for CXCR4 and CXCR7. Ligation of CXCR4 with CXCL12 results in enhanced surface translocation of CXCR7 on the platelets, which exerts a prosurvival effect. MIF also binds to CXCR4 and triggers leukocyte chemotaxis. Thus, we asked whether MIF could regulate the differential trafficking of CXCR4-CXCR7 and platelet survival as evidenced for CXCL12. MIF, like CXCL12, induced internalization of CXCR4 (Figure 1A and Online Figure 1 for control stainings), but did not induce CXCR7 externalization like CXCL12 (Figure 1A). Therefore, although both MIF and CXCL12 bind to CXCR4 and induce CXCR4 internalization, only CXCL12 leads to CXCR7 externalization, suggesting a ligand-specific CXCR4-mediated signaling in platelets. MIF and another CXCR7-specific ligand, that is, CXCL11, did not affect the CXCL12-induced CXCR4 internalization. However, they counteracted CXCL12-mediated externalization of CXCR7 (Online Figure II). Moreover, MIF did not alter the activation status of platelets either when administered alone or in combination with platelet agonist (Online Figure III) nor did it alter the formation of filopodia/lamellipodia on activation-induced shape change while adhering to fibrinogen-coated surface (Online Figure IV).

CXCL12/CXCR4-ligation leads to Erk1/2 phosphorylation, an essential prerequisite for CXCL12/CXCR4-driven surface translocation of CXCR7. However, MIF, in contrast to CXCL12, did not induce Erk1/2 phosphorylation, either in platelets (Figure 1B) or in primary T lymphocytes (Online Figure VA). Thus, absence of MIF-induced Erk1/2 phosphorylation is part of the differential effect of ligand-specific CXCR4-dependent signaling in platelets accounting for the lack of CXCR7 surface translocation. However, like CXCL12, MIF could induce Erk1/2 phosphorylation in monocytes (Figure 1B).

MIF binds to the chemokine receptors CXCR2, CXCR4, and CXCR7. Both CXCR2-CXCR4 can form functional complexes with the single-pass transmembrane-receptor CD74. CD74 is essential for MIF-dependent Erk1/2 phosphorylation after CXCR4 ligation. Although platelets express CXCR4 and CXCR7, both CXCR2 and CD74 were absent on the platelet surface (Figure 1C). Thus, lack of CD74 in platelets might be a determinant in defining the ligand-specific CXCR4-dependent signaling effects and translocation of CXCR7. Unlike platelets, monocytes expressing CD74 (Online Figure VB) could induce Erk1/2 phosphorylation downstream of CXCR4 ligation by MIF.

MIF Ligates CXCR4 and CXCR7 on Platelet Surface

We further verified MIF ligation to CXCR4-CXCR7 on platelets. MIF could be detected on the surface of untreated platelets possibly coming from an autocrine source, which was substantially (P<0.001) enhanced in platelets treated with rh-MIF (Figure 2A). Binding of rhMIF to the platelet surface was significantly inhibited in the presence of both anti-CXCR4 and anti-CXCR7 antibodies (P<0.001), suggesting receptor ligation. Further, platelets were incubated with rhMIF, and CXCR4-CXCR7/MIF interaction was analyzed by coimmunoprecipitation. We found that MIF coimmunoprecipitated with both CXCR4 and CXCR7, confirming a receptor–ligand interaction (Figure 2B). Immunofluorescence confocal microscopy also revealed that MIF colocalized with CXCR4 and CXCR7 on the platelet surface (Figure 2C). Moreover, CXCL11, another CXCR7-specific ligand, also showed significant (P<0.001) binding to the platelet surface (Figure 2D), confirming the specificity of the experimental approach.

CXCR7 Is the Predominant Receptor for Mediating Ant apoptotic Effects of MIF on Platelets

MIF promotes cell survival. Therefore, we asked whether MIF can modulate survival of platelets, as described recently for CXCL12. Platelets were treated with MIF or CXCL11 for 30 minutes before thrombin-related peptide SFLLRN or BH3 mimetic ABT-737 was added to induce apoptosis. In the
Figure 1. Differential effects of macrophage migration inhibitory factor (MIF) and CXCL12 on CXCR4-CXCR7 trafficking and CXCR4-induced Erk1/2 phosphorylation. **A. Left**, Representative immunofluorescence confocal microscopic images showing relative intracellular localization of CXCR4 (Alexa Fluor 647-red) and CXCR7 (Alexa Fluor 488-green) in untreated and MIF-treated platelets. Internalized CXCR4 among MIF-treated platelets is indicated by a white arrow. **Right**, Bar diagram representing differential translocation of CXCR4 and CXCR7 in response to CXCL12, MIF, and CXCL11 in human platelets as determined by flow cytometry. Data demonstrates a significant (*P < 0.05) increase in the mean fluorescence intensity (MFI) for CXCR7 in CXCL12-treated platelets and a concomitant (**) P < 0.001) decrease in the MFI for CXCR4 owing to receptor internalization in CXCL12- and MIF-treated platelets with respect to controls. CXCL11 did not drive CXCR4 translocation but internalized (*P < 0.05) CXCR7 from the platelet surface. Data represents mean±SEM of 5 independent experiments. **B**, Western blot analysis detecting Erk1/2 and phospho-Erk1/2 in resting (lane-1), MIF-treated (lane-2), and CXCL12-treated (lane-3) platelets and monocytes. **Lower**, Densitometric analysis of the Western blot data showing significant (*P < 0.05) Erk1/2 phosphorylation after treatment with CXCL12 but not MIF in platelets and by both CXCL12 and MIF (**P < 0.05) among monocytes. Data represents 3 independent Western blots. **C. Upper**, Representative immunofluorescence images showing expression of CXCR4 and CXCR7 (Alexa Fluor 488-green), and however, a lack of CXCR2 and CD74 in resting platelets as evident from the overlay with DIC images. **Below**, Magnified images for immunofluorescence analysis of CXCR4 and CXCR7 in platelets. **Overlay**, Magnified images for immunofluorescence analysis of CXCR4 (Alexa Fluor 647-red) and CXCR7 (Alexa Fluor 488-green) expression in platelets. (Bar=2 μm). **Lower**, Flow cytometric histogram overlay showing surface expression of CXCR4, CXCR7, and absence of CXCR2-CD74 in platelets (red lines) with respect to isotype controls (black solid fill). **Lower right**, Bar diagram of the flow cytometric data depicting surface expression of MIF receptors on resting (upper) and activated (lower) platelets with respect to isotype controls. Data shows significant (**P < 0.001) expression of both CXCR4 and CXCR7 on resting platelet surface, and however, lack of CXCR2 and CD74 as compared with isotype. Activation of platelets by agonists like ADP (adenosine di-phosphate, 100 μM), CRP (collagen related peptide, 1 μg/mL), and thrombin-receptor activating peptide (TRAP; 25 μM) did not alter the surface expression of CXCR2 and CD74. Data represents mean±SEM of 5 independent experiments.
Figure 2. Macrophage migration inhibitory factor (MIF) binds to CXCR4 and CXCR7 on platelet surface. A, Flow cytometric histogram overlays showing binding of MIF (detected with anti-MIF-FITC) to untreated (red lines) and rhMIF treated (blue lines) human platelets with respect to isotype controls (black, solid fill) in presence/absence of blocking antibodies against CXCR4/ CXCR7 (brown lines) or respective IgG controls (pink line; upper). Representative immunofluorescence images (center; n=3) showing presence of MIF (Alexa Fluor 647-red) on the surface of platelets either untreated/treated with rhMIF in presence/absence of receptor blocking antibodies and IgG controls (Bar=2 μm). Bar diagram of flow cytometric data (lower panel) representing significant surface binding of MIF (**P<0.001 vs untreated) on platelets, which was significantly abrogated in presence of CXCR4-CXCR7 blocking antibodies (#P<0.001 vs MIF-treated). Data represents mean±SEM of 4 independent experiments. B, Immunoprecipitation followed by immunoblot analysis show coimmunoprecipitation of MIF (12KDa) with CXCR4 (43KDa) and CXCR7 (50KDa) in platelets. Other bands specified include the light chain band corresponding to 26 KD and the heavy chain band corresponding to 55 KD. Equal loading of samples for immunoblot analysis is shown by β-actin and α-tubulin. Data represents 3 independent IP and IB analysis. C, Representative immunofluorescence images showing MIF (Alexa Fluor 647-red) colocalization with both CXCR4 and CXCR7 (Alexa Fluor 488-green) on the platelet surface (Bar=2 μm). D, Flow cytometric histogram overlays showing CXCL11 binding (detected with anti-CXCL11-FITC) to untreated (red lines) and rhCXCL11 treated (blue lines) platelets with respect to isotype controls (black, solid fill) in presence/absence of blocking antibodies against CXCR4/CXCR7 (brown lines) or respective IgG controls (pink line; upper). Bar diagram of the flow cytometric data (lower) representing significant surface binding of CXCL11 (**P<0.001 vs untreated) on platelets, which was significantly abrogated in presence of CXCR7 (alone or in combination with CXCR4 antibody; #P<0.001 vs CXCL11-treated) but not CXCR4-blocking antibody. Data represents mean±SEM of 3 independent experiments.
presence of MIF and CXCL11, thrombin-receptor activating peptide and ABT-737–induced apoptosis in platelets was significantly (P<0.05) attenuated as evidenced by mitochondrial transmembrane potential (ΔΨm) loss (TMRE [tetramethylrhodamine, ethyl ester] fluorescence), change in annexin-V binding, and caspase-3 activation (Figure 3A and 3B).

Because MIF-dependent internalization of CXCR4 did not result in Erk1/2 activation due to the lack of CD74 and CXCR2 being absent on platelets, it was tempting to speculate that MIF might be executing its prosurvival effects through CXCR7. As predicted, in the presence of blocking anti-CXCR7 antibody (but not a respective control or blocking anti-CXCR4), MIF and CXCL11 could not antagonize activation-induced apoptosis (Figure 3C). These prosurvival effects of MIF as mediated through CXCR7 in platelets were further substantiated in cells where CXCR7 is either genetically deleted as in murine embryonic liver cells or is stably overexpressed in Madin–Darby canine kidney cell transfectants (Figure 3D and 3E). MIF could not rescue CXCR7−/− murine embryonic liver cells from ABT-737–induced apoptosis unlike their CXCR7+/− counterparts (Figure 3D). Moreover, in Madin–Darby canine kidney cell lines stably transfected with CXCR7, MIF could rescue from starvation-induced apoptosis (Figure 3E). As an antiapoptotic factor, MIF reduced externalization of phosphatidylinerine (deciphered by Annexin V binding), which is an eat-me signal. This could affect the antiapoptotic effects of MIF on platelets. A, Representative immunofluorescence images showing presence/absence of active-cleaved caspase 3 (in green along with rhodamine-phalloidin in red) in resting and apoptotic platelets after TRAP activation and in presence of MIF/CXCL11 in combination with/without blocking antibodies against CXCR4-CXCR7 as indicated (Bar=2 μm). B, Bar diagram showing mean fluorescence intensity (MFI) for Annexin V-FITC fluorescence among platelets under resting state, after induction of apoptosis by BH3 mimetic ABT-737 in a concentration-dependent manner (0.1–10μM) and a significant (*P<0.05) decrease among apoptotic platelets in presence of MIF/CXCL11, which was abrogated (#P<0.05 compared with MIF/CXCL11-treated) in presence of anti-CXCR7-antibody. Data are mean±SEM from 3 independent experiments. C, Bar diagram showing MFI for Annexin V-FITC and TMRE (tetramethylrhodamine, ethyl ester) fluorescence denoting ΔΨm among resting platelets after induction of apoptosis by thrombin-receptor activating peptide (TRAP) and a significant (*P<0.05) decrease in apoptotic platelets in presence of MIF/CXCL11, which was substantially abrogated (#P<0.05 compared with MIF/CXCL11-treated) in presence of anti-CXCR7, but not anti-CXCR4-antibody. Data are mean±SEM from 3 independent experiments. D, Up, Phase-contrast images of cultured murine embryonic liver cells showing the presence of mature megakaryocytes in cultures derived from CXCR7+/+ and CXCR7−/− fetal liver. Middle, Representative immunofluorescence images showing the presence of CXCR7 in CD42b-positive megakaryocytes derived from CXCR7−/− embryonic cells, whereas absence of CXCR7 among cells isolated from CXCR7−/− embryos. Low, Flow cytometric histogram overlay showing the relative surface expression of CXCR7 (red line) among CXCR7+/+ and CXCR7−/− embryonic liver cells with respect to isotype control (grey fill). Lower most, Bar diagram showing TMRE-MFI perceived among CXCR7+/+ and CXCR7−/− murine embryonic liver cells and the significant (*P<0.05) antiapoptotic effect of MIF against ABT-737–induced apoptosis among CXCR7+/+ embryonic liver cells. Data are mean±SEM. E, Up, Flow cytometric histogram overlay showing surface expression of CXCR7 on MDCK cells stably transfected with CXCR7 or control pcDNA3 vector (red line) with respect to isotype control (solid grey fill). Below, Bar diagram representing the count of Madin–Darby canine kidney (MDCK) cells (stably transfected with CXCR7 or control pcDNA3 vector) showing nuclear condensation after serum deprivation and the significant (**P<0.01) antiapoptotic effect of MIF on FCS-starvation–induced apoptosis. Data are means±SD. F, Bar diagram showing percent of CD42b-positive macrophages denoting the population of macrophages that have phagocytosed CD42b-labeled apoptotic (ABT-737-treated) platelets (*P<0.05 vs resting platelets), which is significantly (#P<0.05 in MIF+ABT-737–treated vs ABT-737–treated platelets) reduced with MIF-treated platelets. Data are mean±SEM from 3 independent experiments.
phagocytic uptake of platelets by macrophages. As predicted, MIF reduced the phagocytic uptake of nonapoptotic (in presence of MIF) as compared with apoptotic (in absence of MIF) platelets by macrophages (Figure 3F). Because MIF rescued platelets from undergoing apoptosis, it did not necessitate their phagocytic uptake by macrophages.

MIF/CXCR7-Initiated Platelet Survival Is Mediated Through Akt-Dependent Phosphorylation of Proapoptotic BAD

To decipher the downstream signaling intermediates involved in MIF-induced platelet survival, we evaluated the effects of various pharmacological kinase inhibitors. In the presence of PI3K (wortmannin) and Akt-inhibitor (SH-6), the antiapoptotic effects of MIF and CXCL11 (Figure 4A) were significantly reduced (P<0.05), an observation strongly suggesting the involvement of the PI3K-Akt pathway in MIF/CXCR7-dependent platelet survival. Further, the importance of Akt in prompting MIF-initiated platelet survival was also evidenced and exemplified in Akt−/− murine platelets. Unlike in wild type Akt+/+ platelets, neither MIF nor CXCL11 induced activation-induced apoptosis (TMRE fluorescence) in Akt−/− platelets (Figure 4B). This lack of the prosurvival effect among Akt−/− platelets was not because of a difference in the basal level of CXCR4/CXCR7 surface expression (Figure 4C) or their differential trafficking in response to CXCL12 and MIF, which was comparable to wild type.

We confirmed MIF-mediated induction of the PI3K-Akt pathway in platelets as reported previously in other cells (Figure 5A and 5B) comparable to that induced by CXCL12 and CXCL11 (Figure 5A, left panel). Moreover, both MIF and CXCL11 induced PI3K (Figure 5A) and Akt (Figure 5B) phosphorylation in platelets, which was substantially (P<0.01) reduced in presence of CXCR7 but not CXCR4-blocking antibody (Figure 5A and 5B), confirming the initiation of the prosurvival PI3K-Akt-signaling cascade downstream of CXCR7-MIF ligation.

Akt activation results in phosphorylation-mediated inactivation of proapoptotic BAD (BCL-2 antagonist of cell death), a BH3-only protein which regulates platelet apoptosis. Thus, we asked whether MIF inactivates (phosphorylates) BAD downstream of CXCR7 ligation. MIF induced BAD phosphorylation in a time-dependent manner (Figure 5C, left), which was inhibited in the presence of CXCR7 but not CXCR4-blocking antibody (Figure 5C, right). Similar results were obtained with CXCR7-ligand CXCL11 (Figure 5C and 5D). MIF- and CXCL11-induced BAD-phosphorylation was significantly (P<0.05) abrogated in the presence of Akt inhibitor-SH-6 (Figure 5C and 5D). Evidently, BAD-phosphorylation among apoptotic platelets in response to MIF was also absent in Akt−/− murine platelets, whereas WT platelets showed BAD-phosphorylation in response to MIF, which was abrogated by blocking CXCR7 and by Akt inhibition (Figure 5E).

MIF Attenuates Thrombus Formation Through CXCR7 Engagement

Platelet activation and enhanced phosphatidyserine surface exposure regulates thrombus formation. Phosphatidyserine exposure is not only an indicator of apoptosis but also of platelet activation, leading to phospholipid scrambling at the plasma membrane. Because MIF exerts an antiapoptotic effect against activation-induced apoptosis and significantly reduces phosphatidyserine exposure (Figure 3B), we presumed that it might modulate thrombus formation. In the presence of MIF (200 ng/mL), platelets formed substantially smaller thrombi with a significantly (P<0.001) reduced thrombus surface coverage (Figure 6A). In the presence of blocking anti-CXCR7 or anti-CXCR4 antibody but not IgG control, the effect of MIF on thrombus formation was significantly (P<0.001) reduced. Immunostaining of thrombi revealed significantly higher TMRE and lower Annexin V signals in MIF-treated sets (Figure 6A). We further evaluated the thrombogenic potential of platelets from Mif−/− mice, which lack an endogenous source of MIF. Mif−/− mice showed enhanced thrombus formation as compared with Mif+/+. Supplementation with rmMIF substantially reversed the effect (Figure 6B).
MIF Prolongs Platelet Survival and Attenuates Thrombus Formation After Arterial Injury In Vivo

Prosurvival effects of MIF against platelet apoptosis in vitro were further substantiated in vivo after injection of recombinant MIF into Akt\(^{+/-}\) and Akt\(^{-/-}\) mice. MIF injection significantly (\(P<0.05\) vs WT) promoted retention of labeled circulating platelets within a span of 5 days among WT but did not have any effect on Akt\(^{-/-}\) mice with respect to control (Figure 7A). Moreover, MIF administration in vivo induced BAD-phosphorylation in circulating Akt\(^{+/-}\) but not Akt\(^{-/-}\) mice platelets substantiating the Western blot data and even more so after ex vivo challenge with thrombin (Figure 7A). Phosphorylation-mediated inactivation of BAD in circulating platelets could contribute to prolonged survival under the influence of MIF. MIF administration in vivo also significantly reduced the thrombotic potential of platelets from Akt\(^{+/-}\) mice platelets as evaluated on day 5 (Figure 7A) in a dynamic flow chamber assay.

We further verified the antithrombotic potential of MIF in vivo by using intravital microscopy and FeCl\(_3\)-induced arterial injury in mice. Time to arterial occlusion after FeCl\(_3\)-induced injury of mesenteric arterioles in mice was significantly prolonged with occlusion time of \(\geq 40\) minutes under the influence of MIF (Figure 7B). Phosphorylation-mediated inactivation of BAD in circulating platelets could contribute to prolonged survival under the influence of MIF. MIF administration in vivo also significantly reduced the thrombotic potential of platelets from Akt\(^{+/-}\) mice platelets as evaluated on day 5 (Figure 7A) in a dynamic flow chamber assay. We further verified the antithrombotic potential of MIF in vivo by using intravital microscopy and FeCl\(_3\)-induced arterial injury in mice.
of MIF compared with control protein showing occlusion at 10 minutes. This effect was counteracted in presence of a blocking antibody against CXCR7 (Figure 7B). Therefore, MIF exerted its antithrombotic effect through CXCR7 engagement after vessel injury.

Discussion

The major findings of the present study are the following: (i) MIF and CXCL12 differentially regulate the relative expression of their receptors CXCR4 and CXCR7 on the platelet surface. MIF can internalize CXCR4 like CXCL12, but fails to induce Erk1/2 phosphorylation-mediated surface translocation of CXCR7. (ii) MIF ligates CXCR7 and counteracts apoptosis in platelets through activation of the PI3K-Akt pathway causing phosphorylation of proapoptotic BAD and also relatively prolongs platelet survival in circulation. (iii) MIF limits phosphatidylserine exposure and consequentially regulates thrombus formation through CXCR7 involvement after arterial injury. Our findings establish CXCR7 as a hitherto unrecognized receptor for MIF on platelets involved in modulating platelet apoptosis (Figure 8) and imply a novel role for MIF in limiting thrombus formation.

Recently, Strüßmann et al deciphered platelets as a novel source of MIF and demonstrated the potential importance of platelet-derived MIF in governing monocyte migration and thus possibly vascular inflammation.17 The potential effect of MIF on platelet function and survival is unknown. MIF ligates CXCR2, CXCR4,9,10,19 and CD74,19,26 which forms a complex with CD44.21,27 Recently, we documented that CXCL12-CXCR7 might contribute to enhance the regenerative efficacy of platelets,8 which can be substantially influenced by the survival of platelets at the site of action. Because CXCR7 is a vital receptor in modulating platelet survival and therefore has relevant clinical consequences,8 we verified the potential effect of MIF in governing platelet survival possibly through CXCR7 (and CXCR4) compared with a CXCR7-specific ligand, that is, CXCL11.

Presently, we describe that similar to CXCL12 and CXCL11, MIF protects platelets from activation and BH3 mimetic-induced apoptosis. Even though the prosurvival effects of CXCL12 and MIF are similar, we observed significant differences in the underlying mechanisms. Previously, the kinetics and pattern of CXCL12 and MIF release from platelets in response to agonists was addressed showing a distinctive difference17; hereby, we provide convincing evidence that after release, both these chemokines could have a differential effect on the relative surface availability of their receptors on platelets, that is, CXCR4-CXCR7. Both MIF and CXCL12 are known ligands for CXCR49,10,19,20 and induce receptor internalization in platelets (7 and Figure 1 herein). Unexpectedly, in contrast to CXCL12, MIF did not stimulate Erk1/2 phosphorylation in platelets (like in T cells but unlike in monocytes) because of
the absence of CD74 and failed in subsequent CXCR7 surface translocation. This suggests a ligand-specific (MIF versus CXCL12) effect on CXCR4-triggered signaling in platelets. Thus, a difference in spatial distribution of MIF and CXCL12 owing to different release kinetics also translates to different signaling mechanisms, possibly to govern distinct cellular functions which require in-depth characterization.

MIF can individually bind to CD74 or CXCR2/CXCR4 with high affinity, independent of the presence of the other receptor partner. MIF binding to CD74 results in activation of Erk1/2, also PI3K/Akt signaling, regulating cell proliferation and survival.19–22 Previously, MIF was identified to promote cell survival by activating the Akt pathway.28,29 In human rhabdomyosarcoma cell lines, which secrete MIF but do not express CXCR2 and CD74 like platelets, MIF triggers phosphorylation of MAPK p42/44 and Akt, enhances adhesion to fibronectin and human umbilical vein endothelial cells (HUVECs), and promotes tumor vascularization. CXCR7 is the predominant receptor to mediate the prosurvival activity of CXCL12.7 Presently, we found that MIF promotes platelet survival in circulation when administered in vivo and rescues platelets from apoptosis predominantly via CXCR7, like CXCL11. This data on platelet is further substantiated by Cxcr7−/− embryonic cells where MIF could not exert its antiapoptotic effect against ABT-737–induced apoptosis. Moreover, MIF could rescue CXCR7 expressing Madin–Darby canine kidney cell transfectants from starvation-induced apoptosis. The MIF/CXCR7-dependent antiapoptotic effect was dependent on the PI3K–Akt pathway as indicated by pharmacological inhibition of PI3K–Akt and the inability of MIF to rescue platelets from apoptosis or prolong circulating platelet survival in Akt−/− mice. Phosphorylation of the downstream target BAD by Akt was the final determinant in executing the prosurvival effects after MIF ligation of CXCR7 both in vitro and in vivo. One might contemplate on the functional relevance of these platelets...
Akt−/−

Akt+/+

MIF bind directly to the CXCR7 and the phosphorylation-mediated inactivation of proapoptotic protein BAD to exert its prosurvival benefits.

Because MIF counterregulated thrombin and collagen/thrombin, glycoprotein VI stimulation, by BH3-mimetic) or after platelet activation with convulxin/gomyelinase enhances activation-dependent degranulation and thrombogenic activity of platelets from MIF-treated mice, which show prolonged survival. Currently, we deciphered the relative thrombotic potential of platelets from Akt−/− mice, which show prolonged platelet survival in circulation under the influence of MIF compared with Akt+/+ mice, where MIF did not exert any significant effect. Interestingly, the thrombogenic activity of platelets from MIF-treated Akt−/− mice was significantly reduced. It would be worthwhile investigating the regenerative capacity of these platelets in circulation, which show prolonged survival.

Because the antiapoptotic effect of the MIF/CXCR7 interaction involves an attenuation of prothrombotic phosphatidylserine exposure on the platelet surface, we hypothesized that MIF/ CXCR7 might also potentially regulate thrombus formation. Previously, we identified acid sphingomyelinase, involved in membrane scrambling and degranulation in regulating platelet secretion and initiation of procoagulant activity. Acid sphingomyelinase enhances activation-dependent degranulation and thrombin generation after phosphatidylserine exposure on platelets, resulting in enhanced arterial thrombus formation without affecting agonist-induced increases of cytosolic Ca2+ and integrin αIIbβ3 activation or aggregation.25 The clinical relevance of a dysregulation in phosphatidylserine exposure is exemplified in Scott syndrome, a bleeding disorder whereby the Ca2+-dependent phospholipid scrambling of platelets and other blood cells is impaired.30 Externalization of phosphatidylserine is either dependent/independent of mitochondrial depolarization resulting from induction of apoptosis (as triggered by BH3-mimetic) or after platelet activation with convulxin/thrombin and collagen/thrombin, glycoprotein VI stimulation, and PAR1-PAR4 activation.30 Because MIF counterregulated not only activation-induced but also ABT-737–induced apoptosis in platelets, it appeared to be a potential candidate in modulating their thrombotic potential. Here we found that MIF attenuates thrombus formation both in vitro and after arterial injury in vivo through CXCR7 involvement. Moreover, MIF prolonged the initiation of platelet procoagulant activity denoted by the time to peak of thrombin generation in a thrombin generation assay using platelet-rich plasma by the calibrated automated thrombography method (data not shown). This is in keeping with the observed prolonged time to occlusion in injured arterioles as seen in MIF-treated mice with occlusion time of ≥40 minutes as compared with 10 minutes in control. MIF neither altered integrin activation (PAC-1 binding) by itself nor that induced by conventional platelet agonist like thrombin-receptor activating peptide. This could bear promising clinical relevance in patients undergoing antiplatelet therapy against cardiovascular syndromes and cerebral ischemia. Continued debates regarding relative efficacy and dosage regimen of antiplatelet therapies warrants novel candidates. Agents that check thrombotic aspects without compromising the hemostatic and regenerative capacity of platelets are wanted, and MIF could potentially emerge as a relevant physiological candidate.

On the other hand, platelet-derived MIF released at the site of platelet activation/adhesion might foster platelet survival to ensure sustained platelet-dependent tissue regeneration and repair. Human platelets are estimated to harbor 3 femtogram of MIF/cell17 and with the substantial number of circulating platelets might contribute significantly to plasma levels. MIF being a proinflammatory chemokine-like cytokine, this could significantly contribute to vascular inflammation. Previously, we reported elevated plasma levels of MIF in patients with acute coronary syndrome as compared with symptomatic coronary artery disease and healthy subjects, which was associated with other systemic inflammatory markers like C-reactive protein and IL-6 and correlated with troponin release.16 Platelets having been acknowledged as a substantial source of MIF, these clinical observations need to be reevaluated to determine the contribution of platelet-derived MIF in these contexts. Nevertheless, MIF coming from a paracrine source at the site of vascular inflammation, injury, atherosclerotic lesions, or other circulating cells like monocytes and macrophages is enough to register the changes as deciphered here even in the absence of platelet-derived MIF. Thus, irrespective of its origin, MIF can exert an autocrine/paracrine effect on platelets and thereby regulate platelet survival and thrombotic properties. Platelets being a readily available source of chemokines, cytokines, growth factors, which modulate platelet-inflammatory cell or platelet-endothelial interactions, their sustained survival might profoundly influence the duration and resolution of inflammatory processes, for example, onset and progression of atherosclerosis, plaque stability, and symptomatic coronary artery disease.

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Disclosures

None.

References


Novelty and Significance

What Is Known?

• Macrophage migration inhibitory factor (MIF) is a prominent pro-inflammatory cytokine, which modulates inflammation and innate immunity.
• MIF regulates the function of inflammatory cells and promotes disease progression.
• Antagonism of MIF is a potential target to control disease states.

What New Information Does This Article Contribute?

• MIF is a hitherto unrecognized ligand of CXCR7.
• MIF interaction with CXCR7 promotes platelet survival.
• MIF inhibits platelet-dependent thrombus formation via CXCR7.

MIF is a prominent cytokine involved in inflammation and innate immunity. At site of inflammation, MIF is released from a variety of cells and regulates chemotaxis and adhesion. Platelets play a critical role in inflammation and thrombosis; however, the role of MIF for platelet function is unknown. The present study shows that MIF regulates platelet survival and thrombosis. We identified the chemokine receptor CXCR7 as a novel receptor for MIF. Interaction of MIF with CXCR7 attenuates platelet apoptosis and prolongs their life span in circulation. We also found that MIF–CXCR7 ligation controls platelet-dependent thrombus formation. These findings link the proinflammatory role of MIF with an anti-thrombotic effect and suggest targeting the MIF–CXCR7 interaction may be a potential strategy to control inflammation.
Macrophage Migration Inhibitory Factor Limits Activation-Induced Apoptosis of Platelets via CXCR7-Dependent Akt Signaling

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Supplemental Material

Materials: Recombinant murine/human/feline SDF-1α/CXCL12, recombinant human CXCL11/I-TAC (rhCXCL11), recombinant murine CXCL11/I-TAC (rmCXCL11) recombinant human MIF (rhMIF), recombinant murine MIF (rmMIF), mouse monoclonal anti-human CXCR4-PE, rat anti-mouse CXCR4-FITC, mouse anti-human/mouse CXCR7-PE, rat monoclonal anti-mouse CXCR4-unconjugated, mouse monoclonal anti-human CXCR4-unconjugated, mouse monoclonal anti-human CXCR7-unconjugated, mouse anti human CXCR2-unconjugated antibodies were procured from R&D systems. Rabbit polyclonal CD74-FITC, rabbit polyclonal MIF-FITC and rabbit polyclonal CXCL11-FITC were from Bioryt, rabbit polyclonal antibody to CXCR7/GPR159 (N-term) was from Acris Antibodies, mouse monoclonal anti-MIF and rabbit anti-human CXCL11 were from Abcam, rabbit monoclonal p42/44MAPK (Erk1/2) and rabbit monoclonal phospho p42/44 MAPK (Erk1/2), rabbit monoclonal-PI3K and rabbit monoclonal-phospho-PI3K, rabbit monoclonal-Akt and rabbit monoclonal-phospho-Akt, rabbit monoclonal-phospho-BAD, rabbit monoclonal-cleaved active caspase 3-Alexa Fluor-488 conjugated antibodies were purchased from Cell Signaling Technology. Rhodamine phalloidin, rabbit anti-mouse-Alexa Fluor-488, goat anti-rabbit-Alexa Fluor-488, donkey anti-rabbit-Alexa Fluor-568 and donkey anti-mouse-Alexa Fluor-647 secondary antibodies were purchased from Invitrogen. X488-fluorescently conjugated–GPIb antibody to label circulating platelets in vivo and rat anti-mouse CD42b-DyLight 649 conjugated antibodies were procured from Emfret Analytics. TRAP (SFLLRN) was purchased from Sigma-Aldrich, Akt inhibitor SH-6, PI3K inhibitor wortmanin, PKC inhibitor Gö6976, from Cell Signaling Technology, Prostaglandin I2 from Calbiochem, tetramethylrhodamine ethyl ester (TMRE) from Invitrogen, FCCP from Abcam Biochemicals, ABT-737 from SelleckChem, Annexin V-FITC conjugated was purchased from Immuno Tools and Annexin V Fluos was purchased from Roche. Recombinant murine thrombopoietin (rm TPO) was obtained from Immunotools and DMEM+GlutamaxTM-I from GIBCO.

Methods:

Animals: Gene-targeted mice lacking Akt/protein Kinase Bα (Akt−/−), and their corresponding wild-type littermates were generated as described previously (18). Studies on Akt−/−mice and their wild type (WT) counterparts were carried out to assess CXCR4-CXCR7 surface expression, their differential trafficking in response to MIF and CXCL12, also to decipher platelet apoptosis and BAD phosphorylation in vitro and the effect of MIF on platelet survival in circulation in vivo, the levels of BAD
phosphorylation among circulating platelets and to evaluate the thrombotic potential of these mice administered with MIF or control protein. Gene-targeted mice lacking MIF (Mif⁻/⁻), and their corresponding wild-type littersmates were generated as described previously (1), and were originally kindly obtained by Dr. R. Bucala, Yale University, New Haven, USA, and Dr. Fingerle-Rowson, University Hospital Cologne, Germany. Studies on Mif⁻/⁻ mice and their wild type (WT) counterparts were carried out to assess the relative potential of thrombus formation under dynamic arterial flow conditions in vitro and whether supplementation with recombinant murine MIF can reverse the effect. Wild type C57BL/6 were used to cause FeCl₃ induced arterial injury and generate occlusive in vivo thrombus formation model in mice and ascertain the impact of MIF on thrombus formation in vivo by intravital microscopy. CXCR7⁻/⁻ embryos and control littersmates were obtained by mating heterozygous mice (2). All animal experiments were conducted according to German law for the welfare of animals and were approved by local authorities of Tübingen, Aachen and Jena.

**Isolation of platelets:** Platelet rich plasma (PRP) and washed platelets were isolated from peripheral human and murine blood as previously described (3-4).

**Isolation of platelets and monocytes:** Monocytes were isolated from peripheral human blood as previously described through density gradient centrifugation in Ficoll and adherence depletion (1).

**Surface expression of CD74, CXCR2, CXCR4 and CXCR7 on platelets:** Platelets (10⁶/sample) were treated with respective fluorochrome conjugated antibodies against CXCR2, CXCR4, CXCR7 and CD74 for 30 mins at room temperature (RT) along with the respective isotype controls. Samples were fixed in 0.5% paraformaldehyde and analyzed by flow cytometry (BD, Biosciences, FACS Calibur) (3). In another set of experiment to decipher whether the activation status of platelets may have an impact on the relative surface availability of CD74 and CXCR2, platelets (PRP) (10⁶/sample) were treated with agonists like ADP (100µM), TRAP (SFLLRN 25µM) and collagen related peptide (CRP 1µg/ml) for 30 min at RT in presence of the respective fluorochrome-conjugated antibodies against CXCR2 and CD74, along with isotype controls. Following the incubation period, samples were fixed in 0.5% paraformaldehyde and analyzed by flow cytometry.

**Surface expression of CD74 on human monocytes:** Monocytes (10⁶/sample) were treated with respective fluorochrome conjugated rabbit anti-human CD74-FITC for 30 min at RT along with the respective isotype controls. Samples were fixed in 2% paraformaldehyde and analyzed by flow cytometry (3).
Intracellular expression of CD74, CXCR2, CXCR4 and CXCR7 by immunofluorescence confocal microscopy: Resting human platelets were fixed with 1% paraformaldehyde, applied to 0.01% poly-L-lysine coated coverslips, and permeabilized with 0.3% Triton X-100. Following blocking with 1% BSA-PBS for 1hr at RT, samples were labelled overnight at 4°C with respective primary antibodies- mouse anti-human CXCR4 (1:50), rabbit anti-human/mouse CXCR7 (1:50), rabbit anti-human CD74 (1:25) and mouse anti-human CXCR2 antibody (1:100). After thorough washing with PBS+0.3% Triton X-100+0.1% Tween-20, samples were incubated with corresponding secondary antibodies (Alexa Fluor 488 rabbit anti-mouse IgG at 1:200, Alexa Fluor 488 goat anti-rabbit IgG at 1:100) for 2hrs at RT, washed and the coverslips were mounted with antifade fluorescence mounting medium (Dako). Expression of CD74 in peripheral blood human monocytes was also evaluated as described for platelets using immunofluorescence labelling and confocal microscopy. Images were acquired using a Zeiss LSM 510 Meta, Axioplan 2 Imaging Confocal Laser Scanning Microscope (Carl Zeiss Micro Imaging) with a 100x ocular (3).

Differential trafficking of CXCR4 and CXCR7 in platelets:

Flow cytometry: Human and murine platelets from Akt−/− mice and their wild type counterparts were treated with recombinant CXCL12 (1µg/ml), rmCXCL11 (100ng/ml) and rmMIF (200ng/ml) as specified at RT for 20 mins and CXCR4 and CXCR7 surface expression was detected flow cytometry (3). To ascertain the effect of CXCL11 and MIF on CXCL12 induced differential trafficking of CXCR4 and CXCR7, platelets were pre-incubated with MIF (200ng/ml) or CXCL11 (100ng/ml) before adding CXCL12 (1µg/ml), stained for surface expression of CXCR4-CXCR7 and analyzed by flow cytometry (3).

Immunofluorescence confocal microscopy: Human platelets were kept under resting condition or treated with rhMIF (200ng/ml) for 20 mins at RT. Thereafter, samples were fixed with 1% paraformaldehyde, applied to 0.01% poly-L-lysine coated coverslips, and permeabilized with 0.3% Triton X-100. Following blocking with 1% BSA-PBS for 1hr at RT, samples were labelled overnight at 4°C with respective primary antibodies- mouse anti-human CXCR4 (1:50) and rabbit anti-human/mouse CXCR7 (1:50). After washing with PBS+0.3% Triton X-100+0.1% Tween-20, samples were incubated with corresponding secondary antibodies (Alexa Fluor 647 donkey anti-mouse IgG at 1:200, Alexa Fluor 488 goat anti-rabbit IgG at 1:100) for 2hrs at RT, washed and the coverslips were mounted with antifade fluorescence mounting medium (Dako). Images were acquired using a confocal laser scanning microscope.
microscope as stated previously (3). Control immunofluorescence stainings are shown with respective IgG controls and secondary antibodies as part of Online Figure I.

**Binding of MIF and CXCL11 to platelet surface receptors:**

**Flow cytometry:** Human platelets were either kept untreated (for basal level of MIF/CXCL11 surface expression) or treated with rhMIF (200ng/ml) or rhCXCL11 (100ng/ml) for 10 min at RT in presence/absence of blocking antibodies against CXCR4-CXCR7 (10µg/ml), or respective IgG controls (10µg/ml) as indicated. Platelets were treated with blocking antibodies/IgG controls for 30 mins at RT before addition of rhMIF/rhCXCL11. Thereafter, cells were treated with anti-MIF-FITC or anti-CXCL11-FITC antibody for 30 mins at RT, fixed in 0.5% paraformaldehyde and analyzed for the surface binding of MIF/CXCL11 by flow cytometry.

**Immunofluorescence confocal microscopy:** Human platelets were either kept untreated or treated with rhMIF (200ng/ml) in presence/absence of blocking antibodies against CXCR4-CXCR7 (10µg/ml) or respective IgG controls (10µg/ml) as stated above and fixed in 1% paraformaldehyde. Thereafter platelets were processed for immunofluorescence labelling (3) with respective primary antibodies—mouse anti-human MIF (1:100), mouse anti-human CXCR4 (1:50) and rabbit anti-human/mouse CXCR7 (1:50) followed by corresponding secondary antibodies (Alexa Fluor 488 goat anti-rabbit IgG at 1:100, Alexa Fluor-488 rabbit anti-mouse IgG at 1:100, Alexa Fluor 647 donkey anti-mouse IgG at 1:200) (7). Samples were mounted with antifade fluorescence mounting medium (Dako). Images were acquired using a confocal laser scanning microscope as stated before (3).

**Co-immunoprecipitation and Western Blot analysis**

Washed human platelets (3) were kept untreated or treated with recombinant human MIF (rhMIF-200ng/ml) for 10 mins at RT, lysed in immunoprecipitation (IP) buffer (15mM Tris-hydrochloride, 155mM NaCl, 1mM EDTA, 0.08mM sodium azide, protease-phosphatase inhibitor cocktail) for 60 min at 4°C. Immunoprecipitation was carried out overnight at 4°C with the following antibodies: mouse monoclonal anti-human MIF (10µg/500µg protein), rabbit polyclonal anti-human CXCR4 (2µg/100µg protein), rabbit polyclonal anti-human/mouse CXCR7 (10µg/100µg protein) and the respective IgG controls followed by 2hrs incubation with washed sepharose beads (GE Health Care) at 4°C. Samples were washed thrice and preheated for 10 mins to 95°C after dilution with 2x Lâmmli buffer + 5% mercaptoethanol. Processed samples were run on a 8.5% SDS-polyacrylamide gel electrophoresis (PAGE) gel for
detection of CXCR4 (43KD), CXCR7 (50KD), 15% SDS-PAGE gel for MIF (12KD) detection. Blotting onto a polyvinylidene difluoride membrane (Immibilon, Millipore) was performed using a SemiDry Transfer Cell System (Peqlab). Membranes were incubated overnight at 4°C with respective primary antibodies. For detection, corresponding secondary fluorochrome-labeled antibodies and the Odyssey infrared imaging system (LI-COR, Bad Homburg, Germany) were used (3).

**Immunoblots for phosphorylation of Erk, PI3K, Akt and BAD:** Platelets were treated with 200ng/ml of MIF or 100ng/ml of CXCL11 or 500ng/ml of CXCL12, cell lysates were processed as previously described (7) and samples were run on a 8.5% SDS-PAGE gel for PI3K and phospho-PI3K, Akt and phospho-Akt, Erk1/2 and phospho-Erk1/2 detection, 15% SDS-PAGE gel for phosphorylated-BAD detection. Blocking antibodies against CXCR4/CXCR7 (10µg/ml) or Akt-inhibitor SH-6 (25µM) were used as pre-treatments for 30 min at RT prior to the addition of MIF/ CXCL11 as indicated. Blotting and detection with a corresponding secondary antibody was done as stated before. Densitometric analysis of blots was carried out using ImageJ software (3).

**Analysis of apoptotic platelets (annexin V binding, mitochondrial transmembrane potential loss (ΔΨm):** Human platelets were triggered to undergo activation induced apoptosis following exposure to TRAP (SFLLRN-25µM) or apoptosis with increasing concentrations of BH3 mimetic ABT-737 (0.1-10µM) and murine platelets from Akt⁻/⁻ mice or their wild type counterparts were activated with thrombin (0.2U/ml), in the absence/presence of MIF (200ng/ml)/CXCL11 (100ng/ml) for 3h and analyzed for apoptotic markers by flow cytometry (3). Interventions like PKC inhibitor Gö6976 (1µM), PI3K inhibitor wortmannin (100nM) and Akt inhibitor SH-6 (25µM) and their respective vehicle controls and blocking antibodies against CXCR4 and CXCR7 (10µg/ml) and the respective IgG controls (10µg/ml) were pre-incubated for 30 min before adding MIF/CXCL11.

**Immunofluorescence confocal microscopic analysis for active cleaved Caspase 3 and phospho-BAD in apoptotic platelets:** Platelets were kept under resting conditions or activated with TRAP (25µM) in the absence/presence of rhMIF (200ng/ml) or rhCXCL11 (100ng/ml). Blocking antibodies against CXCR4/CXCR7 (10µg/ml) or Akt-inhibitor SH-6 (25µM) were used as pre-treatments for 30 min at RT prior to the addition of MIF/CXCL11 as indicated. Thereafter, samples were immunolabelled with rabbit anti-human cleaved caspase 3-Alexa Fluor 488 antibody (1:100) and phospho-BAD antibody (1:200) and rhodamine phalloidin, counterstained with donkey anti-rabbit Alexa Fluor-488 antibody for phospho BAD and then
analyzed by confocal microscopy using a 100x ocular (3). Control immunofluorescence stainings are shown with respective IgG controls and secondary antibodies as part of Online Figure I.

**Analysis of apoptotic [mitochondrial transmembrane potential loss ($\Delta\Psi_m$)] murine embryonic liver cells from CXCR7$^{+/+}$ and CXCR7$^{-/-}$ murine embryos:**

Fetal liver cells were obtained from whole livers taken from Cxcr7$^{+/+}$ and Cxcr7$^{-/-}$ mouse fetuses at embryonic day-14 in accordance with approved ethical guidelines. Single-cell suspensions were prepared by successive passage through 18-, 21- and 23-gauge needles and cultured in Dulbecco’s modified Eagle medium supplemented with 10% fetal bovine serum albumin, 4mM L-glutamine, penicillin/streptomycin and 50ng/ml of recombinant mouse thrombopoetin (TPO) at a cell density of $1 \times 10^6$/ml. Genotyping of the embryos was performed with tissue obtained from paw biopsies. Following 5 days of culture, liver cells were harvested and challenged to undergo apoptosis in response to ABT-737 (25µM) in presence/absence of rmMIF (200ng/ml) for 4 hrs at 37°C. Thereafter cells were stained with TMRE to ascertain $\Delta\Psi_m$ for 30 min at RT and analyzed by flow cytometry. Before harvest, phase contract images were captured from 4 to 5 different microscopic areas (Carl Zeiss, optical objective 20x,) using AxioVision software (Carl Zeiss). To ascertain the expression of CXCR7 in the embryonic cells, surface expression analysis was done by flow cytometry as described above for platelets. Immunofluorescence confocal microscopic analysis with a 100x ocular was done for intracellular expression and localization of CXCR7. For immunofluorescence confocal microscopic studies the embryonic liver cells were counterstained with megakaryocyte marker CD42b (with rat anti-mouse-CD42b antibody) in addition to CXCR7 immunofluorescence staining. Control immunofluorescence stainings are shown with respective IgG controls and secondary antibodies as part of Online Figure I.

**Analysis of apoptotic MDCK cells undergoing serum deprivation induced apoptosis adjudged by nuclear condensation (heteropicnotic nuclei):**

**Culture of MDCK cells:** Madin-Darby canine kidney (MDCK) cells stably transfected with human CXCR7 (pcDNA3.1-CXCR7) or pcDNA3.1 control vector were a kind gift of Dr. A. Rot, University of Birmingham, Birmingham, UK (new address: University of York, York, UK). Generation of these cells has been described before (5) Cells were cultured in DMEM+GlutaMAX, supplemented with 10% FCS, 2mM L-glutamine, 100U/ml penicillin plus 100µg/ml streptomycin and grown in monolayers.
Surface expression of CXCR7 on MDCK cells: Surface expression of human CXCR7 in MDCK-CXCR7 transfectants was verified by flow cytometric analysis before each experimental set. Cells \((3 \times 10^5)\) were resuspended in MDCK medium containing 0.5% FCS, transferred to a reaction tube, centrifuged at 4°C and 400 x g, and the cell pellet was resuspended in 1ml flow cytometry buffer (PBS, pH 7.2, with 0.5% BSA and 0.1% NaN\(_3\)). Cells were washed twice in this buffer and subjected to antibody labeling. For CXCR7 detection, a mouse anti-human CXCR7-PE-labeled antibody was used along with mouse IgG2a as isotype control. 5µl of antibody solution was applied for \(3 \times 10^5\) cells. Samples were washed in flow cytometry buffer and resuspended in 500µl of the same buffer for analysis, which was performed on a FACS Canto II™ Flow Cytometer (BD Bioscience, Heidelberg, Germany). Mock-transfected cells (MDCK-pcDNA3.1) were used as control.

Effect of MIF on serum-deprivation or starvation induced apoptosis in MDCK cells: MDCK cells (stably transfected with human CXCR7 vector or control pcDNA3 vector) were incubated in 6 well plates \((1 \times 10^6\text{cells/well})\) in full medium overnight. The medium was removed 24 hrs later and the cells were washed twice with PBS and cultured in 0.1% FCS to induce serum deprivation-induced apoptosis. Incubations with 10% FCS and 0.5% FCS were performed as control. MIF (100ng/ml) was added to the medium at the same time. Following the 48hrs-incubation period, the cells were stained with 1µg/ml Hoechst 33342 for 20 min and observed under the microscope. Cells exhibiting nuclear condensation were counted and considered as apoptotic cells with characteristic change in nuclear morphology.

Phagocytic uptake of apoptotic platelets by macrophages: THP1 monocytic cell lines were treated with phorbol-myristic acid (PMA-10ng/ml) for 48 hrs to differentiate them into macrophages. Human platelets were triggered to undergo apoptosis following exposure to BH3 mimetic ABT-737 (10µM), in the absence/presence of rhMIF (200ng/ml) for 3hrs and analyzed parallelly for apoptotic marker i.e. annexin V binding by flow cytometry (3). Apoptotic platelets were further labelled with platelet specific marker CD42b-FITC for 30 min at RT and thereafter co-incubated with culture-derived macrophages for 4hrs. Samples were fixed in 0.5% paraformaldehyde and acquired on a flow cytometer in presence/absence of trypan blue to quench for surface fluorescence coming from adherent (but not phagocytosed) platelets and analyzed for CD42b positive macrophage population which have phagocytosed apoptotic platelets. Relative phagocytic uptake of platelets with/without MIF was evaluated as % of CD42b positive macrophages.
Analysis of platelet survival in circulation in vivo: Gene-targeted mice lacking Akt (Akt\(^{--}\)) and their wild type littermates (Akt\(^{++}\)) were injected intravenously via tail vein with X488-fluorescently conjugated–GPIbβ antibody (0.1µg/g body weight) for labelling circulating platelets (6). rmMIF at 2µg/mouse was also administered under the influence of isoflurane anesthesia. Blood was collected at baseline level, 1, 3 and 5 days post injection. The percentage of X-488 GPIbβ (CD42b) labelled platelets (denoting platelets retained in circulation) was deciphered at each time points (at baseline, 1, 3 and 5 days) post MIF injection by flow cytometry for platelets in PRP using FSC-SSC parameters and confirmed by CD42b-DyLight 649 labelling which would label all the platelets in the samples analyzed. The relative % of X-488-CD42b-labelled platelets that were labelled at baseline (day 0) in vivo (and retained in circulation since) were ascertained with respect to total platelet population in each sample (6). Relative status of BAD phosphorylation among Akt\(^{++}\) and Akt\(^{--}\) mice under the influence of MIF administration in vivo was also determined by intracellular staining with anti-phospho-BAD antibody and analyzed by flow cytometry. Briefly platelets from Akt\(^{++}\) and Akt\(^{--}\) mice treated i.v. with rmMIF or control protein were kept untreated or further challenged ex vivo with thrombin (0.2U/ml) for 1 hr at RT. Thereafter platelets were fixed with 1% paraformaldehyde, permeabilized with 0.3% Triton X-100, labelled with rabbit anti phospho-BAD antibody (1:100) for 1hr at RT, washed and counterstained with allophycocyanin (APC) conjugated goat anti-rabbit IgG (at 1:100) for 1hr at RT. Thereafter samples were analyzed for intracellular staining of phospho-BAD by flow cytometry.

Thrombus formation under arterial flow conditions in a flow chamber assay: Whole blood from healthy human subjects was kept untreated or treated with rhMIF (200ng/ml) for 30 min at RT, in presence /absence of blocking antibodies against CXCR4-CXCR7 (10µg/ml) and respective IgG controls (10µg/ml) given as a pretreatment for 30 min before adding MIF. Thereafter blood was perfused through a transparent flow chamber (slit depth 50µm) over a collagen-coated surface (100µg/ml) at arterial (1700 s\(^{-1}\)) wall shear rates for 5 min as described previously (7). After perfusion. the chamber was rinsed for 5 min by perfusion with Tyrode buffer and pictures were taken from 4 to 5 different microscopic areas (Carl Zeiss, optical objective 20x). Analysis was done with AxioVision software (Carl Zeiss, Axiovert 200) and the thrombus area was determined (7). The thrombus thus formed was stained with TMRE and Annexin V (with Annexin V-Fluos) to decipher the relative survival of cells and phosphatidylserine exposure in thrombus under the influence of MIF in presence/absence of blocking antibody against CXCR4-CXCR7 and analyzed by
confocal microscopy as described before. Similar experimentation was carried out with murine blood derived from Mif\(^{+/+}\) and Mif\(^{-/-}\) mice to ascertain their relative thrombogenic potential in presence and absence of rmMIF (200ng/ml) and whether resubstitution with MIF in Mif\(^{-/-}\) mice blood could reverse the observed effect. Thrombus formation under flow chamber arterial shear rates was also evaluated in blood from Akt\(^{+/+}\) and Akt\(^{-/-}\) mice treated with control protein or with rmMIF (2µg/mouse) 5 days post MIF i.v. injections as described.

**Intravital microscopy of thrombus formation in mesenteric arterioles injured with FeCl\(_3\):** C57BL/6 mice (5-6 weeks of age) were treated by i.v. injection with rabbit anti-CXCR7 (10µg/mouse, Abcam) or IgG (10µg/mouse,Santa Cruz ) either 60 mins before injury and 30 mins before injury when anti-CXCR7 administration was followed by MIF (2µg/mouse). Mice were treated with rmMIF (2µg/mouse) or BSA 30 mins before inflicting injury. Mice were anesthetized with medetomidine, midazolam, and fentanyl. After a midline abdominal incision, the mesentery was exteriorized and arterioles free of fat tissue were injured by tropical application of a filter paper saturated with 20% FeCl\(_3\) for 10 sec to generate occlusive *in vivo* thrombus formation model in mice. Thrombus formation was made visible by i.v. injection of acridin and observed with a fluorescence microscope. Time until full occlusion of the vessel (ie, when blood flow had stopped for > 1 min) was measured. Experiments were terminated after 40 min (7).

**Effect of MIF on platelet activation markers CD62P surface expression and PAC-1 binding by flow cytometry:** Platelets (PRP) (10\(^6\)/sample) from healthy donors were treated with increasing concentrations of rhMIF (0-200ng/ml) either alone of in combination with platelet agonist TRAP (SFLLRN -25µM) for 30 min at RT in presence of respective fluorochrome conjugated antibodies against CD62P –FITC and PAC-1-FITC. Following the incubation period, samples were fixed in 0.5% paraformaldehyde and analyzed by flow cytometry.

**Effect of MIF on platelet activation and spreading over fibrinogen-coated surface:**
Cover slips were coated with 1mg/ml human fibrinogen for overnight at 4 °C and then blocked with 1% BSA solution for 1hr. Washed platelets were prepared as described previously (3) and resuspended at a concentration of 1x10\(^5\) platelets/µl in Tyrode’s buffer supplemented with 1mM CaCl\(_2\). Platelets were incubated with rhMIF (200ng/ml) for 30 min at RT, before they were seeded to the fibrinogen coated coverslips and incubated at room temperature for 30 min while images were
captured every 20 sec (x 100, DIC, Carl Zeiss, Aviovert 200) using Axio Vision software. Number of MIF treated or untreated platelets forming lamellipodia and filopodia were counted using ImageJ software.

**Surface expression of CXCR2, CXCR4, CXCR7 and CD3 on T-lymphocytes:**

Primary human T-lymphocytes were isolated from the peripheral blood mononuclear cell (PBMC) fraction (prepared by Ficoll-Paque™ Plus technology) of buffy coat (obtained from donor blood at the Blood Bank of Uniklinik RWTH Aachen) by MACS isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany) using the positive selection approach for CD3+ cells. Isolated T-cells were further differentiated by incubation with 5µg/ml phytohaemagglutinin (PHA) for 4 days with addition of 20ng/ml IL-2 at days 0 and 2. Surface levels of CXCR2, CXCR4, CXCR7, and CD74 were measured by flow cytometry as described above.

**Analysis of Erk1/2 phosphorylation in response to MIF and CXCL12 in T-lymphocytes:**

Primary T-lymphocytes, isolated as described above, were cultured in RPMI 1640 supplemented with 10% FCS, 2mM L-glutamine, 100U/ml penicillin and 100µg/ml streptomycin. For the Erk1/2 phosphorylation assay, cells were seeded in 24-well plates at a density of 0.5x10^6 cells/well and exposed to serum-free medium for 1h. Stimulation with 200ng/ml of rMIF or rCXCL12 was performed for 15 min at 37°C. Afterwards, plates were immediately placed on ice, medium removed with a multi-channel pipetman and replaced 100µl lysis buffer. Lysates were transferred and mixed with Lämmli electrophoresis buffer and subjected to SDS-PAGE/Western blotting as described above. Erk and phosphorylated Erk were detected using Santa Cruz (Heidelberg, Germany) polyclonal antibody C-16 and monoclonal antibody E-4, respectively.

**Statistical Analysis:** Data are presented as mean±S.E.M. All data were tested for significance using GraphPad Prism software (GraphPad Software, Inc. USA) setting statistical significance at P<0.05 with 1-way ANOVA using Bonferroni posthoc test.

**Online Figure legends:**

**Online Figure I: Upper panel-** Representative immunofluorescence confocal microscopic images showing expression of CXCR4 (Alexa Fluor 647-red) and CXCR7 (Alexa Fluor 488-green), with respect to negative control staining performed with mouse IgG followed by donkey anti-mouse-Alexa Fluor-647 and rabbit IgG
followed by goat anti-rabbit-Alexa Fluor-488. **Middle panel-** Representative immunofluorescence confocal microscopic images showing expression of CD42b (Alexa Fluor 488-green) and CXCR7 (Alexa Fluor 568-red) in murine embryonic liver cells derived megakaryocytes, with respect to negative control staining performed with respective control IgG followed by donkey anti-rabbit-Alexa Fluor-568 and rabbit anti-mouse-Alexa Fluor-488 counterstaining. **Lower panel-** Immunofluorescence confocal microscopic images demonstrating presence of phospho-BAD (Alexa Fluor 488-green and rhodamine phalloidin-red) in MIF treated apoptotic (TRAP-induced) platelets which was reduced substantially in presence of CXCR7 blocking antibody and Akt inhibitor. Control staining with rabbit IgG followed by counterstaining with goat anti-rabbit Alexa Fluor 488 and rhodamine phalloidin are shown on the right.

**Online Figure II:** Bar diagram showing the relative effect of rhMIF (200ng/ml) and rhCXCL11 (100ng/ml) on CXCL12 (1µg/ml)-induced differential trafficking or translocation of CXCR4 and CXCR7 in human platelets as determined by flow cytometry. Data demonstrates significant (*P<0.05) increase in the mean fluorescence intensity for CXCR7 in CXCL12-treated platelets, which was affected by pre-treatment of platelets with MIF and CXCL11. However CXCL12-induced CXCR4 internalization (**)P<0.001) remained unaltered. Data presented as mean±S.E.M of 3 independent experiments.

**Online Figure III:** Bar diagram representing the surface expression of platelet activation markers like CD62P (on the left denoting degranulation of α-granules from activated platelets) and PAC-1 binding (on the right) in response to increasing concentrations of rhMIF (0-200ng/ml) and MIF in combination with conventional platelet agonist TRAP (thrombin receptor activating peptide acting through PAR-1 receptor- 25µM). MIF did not alter the activation status of platelets when administered either alone or in combination with TRAP. Data represent mean±S.E.M of 3 independent experiments.

**Online Figure IV:** **Left-** DIC images of untreated and rhMIF (200ng/ml) treated platelets adherent to fibrinogen (1mg/ml) coated surface and forming characteristic filopodia and lamellipodia denoting platelet activation and shape change. **Right-** Bar diagram representing the number of untreated or MIF treated platelets forming filopodia and lamellipodia while adhering to fibrinogen coated surface. Data represent mean±S.E.M of 4 independent experiments.

**Online Figure V: A. Left-** Flow cytometric histogram overlay showing the surface expression of CXCR4, CXCR7, CD3 and the lack of CXCR2 and CD74 surface
expression among human T-lymphocytes isolated from buffy coat (red lines) with respect to isotype controls (grey solid fill). **Right-** Primary T-lymphocytes were isolated from buffy coat and were incubated with MIF (200ng/ml) or CXCL12 (200ng/ml) for 15 min at 37°C. Cell lysates were analyzed by Western blot using anti-phospho-Erk antibody. Anti-Erk served as loading control. Western blot analysis detected Erk1/2 and phospho-Erk1/2 in resting (lane-1), MIF-treated (lane-2) and CXCL12-treated (lane-3) T-lymphocytes. Lower panel-densitometric analysis of the Western blot data showing Erk1/2 phosphorylation following treatment with CXCL12 but not MIF in T-lymphocytes. **B.** Representative immunofluorescence confocal microscopic image showing expression of CD74 (Alexa Fluor 488-green), in monocytes as evident from the overlay with DIC images. (Bar=5µm).

**References**


