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ARTICLE

Macrophage migration inhibitory factor inhibits neutrophil apoptosis by inducing cytokine release from mononuclear cells

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Abstract

The chemokine-like inflammatory cytokine macrophage migration inhibitory factor (MIF) is a pivotal driver of acute and chronic inflammatory conditions, cardiovascular disease, autoimmunity, and cancer. MIF modulates the early inflammatory response through various mechanisms, including regulation of neutrophil recruitment and fate, but the mechanisms and the role of the more recently described MIF homolog MIF-2 (D-dopachrome tautomerase; D-DT) are incompletely understood. Here, we show that both MIF and MIF-2/D-DT inhibit neutrophil apoptosis. This is not a direct effect, but involves the activation of mononuclear cells, which secrete CXCL8 and other prosurvival mediators to promote neutrophil survival. Individually, CXCL8 and MIF (or MIF-2) did not significantly inhibit neutrophil apoptosis, but in combination they elicited a synergistic response, promoting neutrophil survival even in the absence of mononuclear cells. The use of receptor-specific inhibitors provided evidence for a causal role of the noncognate MIF receptor CXCR2 expressed on both monocytes and neutrophils in MIF-mediated neutrophil survival. We suggest that the ability to inhibit neutrophil apoptosis contributes to the proinflammatory role ascribed to MIF, and propose that blocking the interaction between MIF and CXCR2 could be an important anti-inflammatory strategy in the early inflammatory response.

KEYWORDS
CXCL8, CXCR2, D-DT, hypochlorous acid, inflammation, MIF-2, MIF

1 | INTRODUCTION

The chemokine-like inflammatory cytokine macrophage migration inhibitory factor (MIF) is an upstream mediator of innate immunity.[1,2] When dysregulated, MIF is a driver of acute and chronic inflammatory conditions, cardiovascular disease, and cancer.[3–6] D-dopachrome tautomerase (D-DT/MIF-2) is a recently described homolog of MIF, but its role in host immunity and disease is less well understood.[7,8] MIF proteins are structurally unique and do not belong to any of the classical cytokine or chemokine families.[8,9] They are broadly expressed, but are best known as mediators released from immune cells such as

Abbreviations: 4-IPP, 4-iodo-6-phenylpyrimidine; D-DT, D-dopachrome tautomerase; MIF, macrophage migration inhibitory factor; oxMIF, HOCl-oxidized MIF; oxMIF-2, HOCl-oxidized MIF-2/D-DT.

monocytes, T cells, and neutrophils, as well as endothelial cells, following inflammatory, atherogenic, and ischemic stress.[2,8] MIF activities have been reported to be both disease exacerbating and tissue protective, depending on the microenvironmental or disease context. Overall, MIF is widely regarded as a proinflammatory mediator due to its ability to recruit immune cells, trigger and amplify the production of other proinflammatory cytokines, prolong macrophage survival, favor polarization of macrophages toward an inflammatory phenotype, and counterregulate the immunosuppressive actions of glucocorticoids.[2,5,10–12] MIF's inflammatory actions are triggered through binding to its receptors CXCR2, CXCR4, CD74, and potentially also CXCR7.[13–18]

Neutrophils are the first inflammatory cells to migrate toward sites of infection and sterile injury, where they phagocytose and kill bacteria and fungi, capitalizing on an armory of oxidants and antimicrobial proteins.[19,20] Neutrophil fate is a critical factor in the resolution or amplification of inflammation. Apoptosis facilitates the removal of these short-lived cells, whereas delayed apoptosis can result in necrotic cell death, involving disintegration and release of proteolytic enzymes and proinflammatory mediators, resulting in tissue injury and ongoing inflammation.[19,21] This phenomenon contributes to nonresolving inflammation and has been implicated in many chronic human inflammatory diseases including atherosclerosis, coronary artery diseases, neuroinflammation, pulmonary inflammation and fibrosis, and rheumatoid arthritis.[22–24] Various inflammatory cytokines such as G-CSF, GM-CSF, IL-1β, IL-4, IL-6, CXCL8 (IL-8), or TNF-α are known to inhibit neutrophil apoptosis,[25–27] but the regulatory loops are poorly defined. A better understanding of the mechanisms promoting neutrophil survival might reveal new anti-inflammatory strategies.

We and others previously reported that MIF is able to prolong neutrophil survival in vitro,[28,29] but the mechanisms underlying its prosurvival effect warrant further investigation. For example, we observed that the ability of MIF to slow neutrophil apoptosis required the presence of PBMCs.[29] The cytokines and mechanisms involved in mediating this indirect effect of MIF on neutrophils were not determined, and it is unknown, which MIF receptors are involved. Moreover, the ability of MIF-2 to regulate neutrophil apoptosis has not yet been investigated.

MIF and MIF-2 have a conserved nucleophilic *N*-terminal proline residue providing it with an intrinsic catalytic tautomerase activity,[30] a structural feature that is unique among cytokines and chemokines but shares homology to a family of bacterial tautomerases/isomerases.[31] Although the physiologic relevance of the catalytic activity of MIF proteins in humans is unknown, with physiologic substrates not yet discovered, the catalytic cavity has served as a promising structural hub to create small molecule inhibitors targeted at MIF proteins.[32] Residues forming the tautomerase catalytic site contribute to the binding of MIF to its receptors CD74 and CXCR4. Accordingly, proline-2 mutants of MIF or MIF pretreated with small molecule tautomerase inhibitors exhibit reduced binding activity for CD74 and CXCR4.[33,34] Given the likely colocalization of MIF and neutrophils at inflammatory sites, we have recently investigated whether neutrophil-derived oxidants such as hypochlorous acid (HOCl) produced by the enzyme myeloperoxidase (MPO) can modify the *N*-terminal proline of MIF.[29,35] Indeed, the *N*-terminal proline was found to be readily oxidized to a proline imine abolishing the tautomerase activity of MIF, but retaining its antiapoptotic activity. The impact of proline oxidation on the efficacy of tautomerase inhibitors such as 4-iodo-6-phenylpyrimidine (4-IPP) is not clear.

Here, we tested the hypothesis that both MIF and MIF-2 and their HOCl-oxidized counterparts (*herein* termed: oxMIF and oxMIF-2) limit neutrophil apoptosis and examined the cytokines, receptors, and cell types involved in mononuclear cell-mediated inhibition of neutrophil apoptosis by MIF proteins.

2 | MATERIAL AND METHODS

2.1 | Reagents

4-Iodo-6-phenylpyrimidine Santa Cruz Biotechnology (Heidelberg, Germany) was purchased from Bio-Strategy (Christchurch, New Zealand). HOCl ($\varepsilon_{292} = 350\ M^{-1}\ cm^{-1}$ at pH 12) was purchased from Sigma–Aldrich (Merck, Darmstadt, Germany) or as commercial chlorine bleach from Household and Body Care (Auckland, New Zealand). Dextran from *Leuconostoc mesenteroides* (average molecular weight: 150,000 Da; Sigma) and Ficoll-Paque (GE Healthcare, Uppsala, Sweden, and Freiburg, Germany) were used for neutrophil isolation. Cells were cultured in RPMI 1640 media supplemented with 1 mM glutamine (Gibco, Thermo Fisher Scientific, Karlsruhe, Germany) and heat-inactivated FCS from Sigma–Aldrich. Annexin V, Alexa Fluor™ 488 conjugate, and Annexin binding buffer (5×) for flow cytometry were purchased from Thermo Fisher Scientific. For isolation of monocytes, total lymphocytes and T cells, CD14, CD16, Glycophorin A (CD235α) MicroBeads, and a Pan T-Cell Isolation Kit (human) were all purchased from Miltenyi Biotec (Bergisch Gladbach, Germany). Anti-CD74, -CXCR2, and -CXCR4 antibodies for flow cytometry analyses were purchased from BD Pharmingen (Heidelberg, Germany). Commercial ELISA kits for IL1-β, IL-6, CXCL8, CXCL12 (SDF-1), G-CSF, and GM-CSF were purchased from R&D Systems (Wiesbaden, Germany). Recombinant human CXCL8, recombinant human TNF-α, anti-IL-8/CXCL8, and anti-IL-6 antibodies (R&D Systems) were purchased through In Vitro Technologies (Auckland, New Zealand) or directly via R&D Systems. Anti-CD74 antibody LN2 was purchased from Abcam (Cambridge, UK), CXCR2 agonist SB225002 from Sigma–Aldrich, and the CXCR4 blocking peptide NBP1-76867PEP Novus Biologicals LLC (Centennial, CO, USA) from In Vitro Technologies. For biotin labeling of MIF, MIF-2, oxMIF, and oxMIF-2, the Biotin Labelling Kit from Roche (Mannheim, Germany) was used.

2.2 | Isolation of human neutrophils and PBMCs from peripheral blood

Blood was obtained from healthy human volunteers with informed consent and with ethical approvals from the Ethics Committee of LMU Munich, Germany, and the Southern Health & Disability Ethics

Committee, New Zealand. Our protocols for obtaining blood abide by the Declaration of Helsinki principles.

Human neutrophil granulocytes and PBMCs were isolated from freshly drawn heparinized blood under sterile conditions by dextran sedimentation followed by a density gradient centrifugation using Ficoll. The intermediate layer containing the PBMCs was separated and washed with RPMI media supplemented with 10% heat-inactivated FCS (v/v). Neutrophils were isolated from the Ficoll pellet by red blood cell lysis in hypotonic buffer.[36] Neutrophils were resuspended in RMPI media supplemented with 10% heat-inactivated FCS (v/v). Flow cytometric analysis of neutrophils using characteristic forward/side scatter (FSC/SSC) verified a purity of 98%–99%.

Monocytes were isolated from the PBMC layer by positive selection using CD14, CD16, and CD235α MicroBeads, and lymphocytes by negative depletion using the same antibodies. Flow cytometry analysis of isolated monocyte and lymphocyte populations showed 95%–99% purity using characteristic forward/side scatter.

T cells were purified from the above-described lymphocyte layer by negative selection using the Pan T-Cell Isolation Kit (human) from Miltenyi Biotech following the manufacturer's protocol.

2.3 | Surface receptor expression on neutrophils and PBMCs

Freshly isolated human neutrophils and PBMCs (1×10^6/ml) were pelleted and resuspended in 200 µl ice cold flow cytometry buffer (PBS containing 0.5% BSA and 0.01% sodium azide, pH 7.2). For each receptor (CD74, CXCR2, CXCR4), cells were incubated with 10 µl of the specific fluorescently labeled antibody and/or isotype controls for 45–60 min at 4°C in the dark. Afterward, cells were washed and resuspended in 300 µl flow cytometry buffer. Flow cytometry analysis was used to quantify the fluorescent signal which was expressed relative to the isotype control.

2.4 | Expression and purification of recombinant human MIF and MIF-2

Recombinant human MIF and MIF-2 were expressed in an *Escherichia coli* BL21/DE3- based system and purified using the protocols described before.[14,29] The concentration of LPS in the MIF preparation was measured by using the Limulus amoebocyte assay (Lonza, Cologne, Germany) following the manufacturer's instructions and was <20–100 pg/µg MIF.

2.5 | Effect of MIF and MIF-2 on neutrophil apoptosis

Freshly isolated human neutrophils (5×10^5/ml) were incubated separately or in the presence of different ratios with human PBMCs, lymphocytes, or monocytes with MIF and MIF-2 (1-10 µg/ml) in RPMI supplemented with 10% heat-inactivated FCS (v/v) in a 24-well plate (Nunc, Thermo Fischer) at 37°C/5% CO_2 in a humidified incubator. In separate experiments, MIF and MIF-2 were treated with a 5-fold molar excess of HOCl for 15 min at RT before adding the oxidized MIF proteins to the cells at 10 µg/ml. Oxidation of MIF proteins by HOCl was performed as described previously,[29] which results in the oxidative formation of an N-terminal proline-imine in MIF or MIF-2; these oxidized isoforms are annotated as "oxMIF" and "oxMIF-2." Polymyxin B (20 µg/ml) was added to all cultures to bind any remaining contaminating traces of LPS present in the recombinant MIF protein preparations. The ability of polymyxin B to block LPS-mediated inhibition of neutrophil apoptosis was verified in a separate experiment by adding it to a neutrophil/PBMC 1:1 coculture containing externally added LPS at a concentration of 1 ng/ml.

After 21 h, phosphatidylserine exposure and viability were measured in duplicate using annexin V/propidium iodide (PI) staining and flow cytometry analysis as described previously.[29,37] For the purpose of this study, PI-negative/Annexin V-negative cells are referred to as "viable neutrophils" and PI-negative/Annexin V-positive cells as "apoptotic neutrophils" (Fig. 1). Although these cells are still viable, that is, they have the ability to exclude PI, they have entered apoptosis and are in the process of losing viability.[38]

PBMCs were also incubated in the absence of neutrophils with increasing concentrations of MIF, MIF-2, and oxMIF and oxMIF-2 (1.0, 2.5, 5.0, 7.5, 10.0 µg/ml), the supernatants were collected and stored at −80°C for further experiments. Supernatants (600 µl/well) were added to freshly isolated neutrophils (5×10^5/ml) and were incubated for 21 h as described above followed by annexin V-FITC / PI labeling and flow cytometry analysis.

The small molecule inhibitor 4-IPP has been shown to block MIF's enzymatic activity and bioactivity through covalent binding to the *N*-terminal proline.[39] We incubated MIF and oxMIF with 10-fold molar excess of 4-IPP for 15 min at RT before adding the proteins to the neutrophil–PBMC coculture system and determining neutrophil apoptosis after 21 h as described above. DMSO was used as a vehicle control at a concentration of 0.1%.

To determine relevant cell surface receptors important for MIF-mediated effects, neutrophils and/or PBMCs (1×10^7/ml) were pretreated with the CXCR2 antagonist SB225002 ("anti-CXCR2," 25 µg/ml), a CXCR4 blocking peptide NBP1-76867PEP ("anti-CXCR4," 25 µg/ml) or an anti-CD74 antibody LN2 ("anti-CD74," 10 µg/ml) for 30 min at RT before incubating both cell types together in the presence of MIF as described above. SB225002 was dissolved in DMSO and therefore DMSO was added to cells as a control at the same final concentration (0.05%). In separate experiments, only PBMCs were pretreated with blocking agents, pelleted by centrifugation and resuspend in fresh RPMI media before adding to a coculture with neutrophils. Neutrophils were also pretreated with receptor blocking agents and then added to supernatants collected from MIF-treated PBMCs. In all cases, neutrophil survival was assessed after 21 h as described above.

To determine the role of CXCL8 in the MIF-mediated inhibition of neutrophil apoptosis, PBMCs were stimulated as described above and the supernatant was collected after 21 h. Anti-CXCL8/IL-8 (1.2 µg/ml

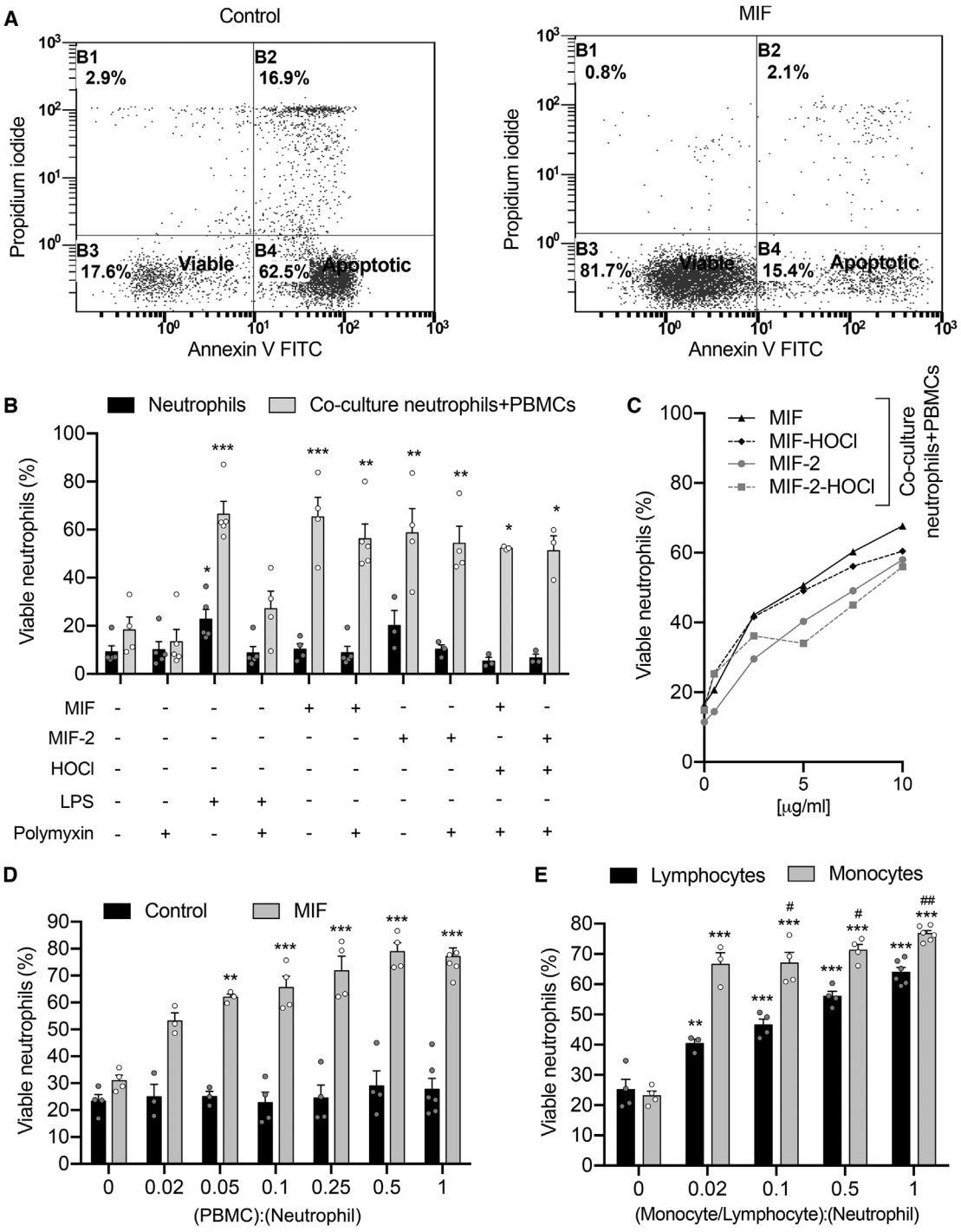

FIGURE 1 MIF and MIF-2 prolong neutrophil survival in presence of mononuclear cells. (A) Representative flow cytometry data showing viable and apoptotic neutrophils after 21 h incubation with (10 μg/ml) and without MIF. Propidium iodide (PI)-negative and Annexin V (Annexin V FITC)-negative cells are labeled as "viable neutrophils" and PI-negative and Annexin V-positive cells as "apoptotic neutrophils." (B) Neutrophils (5×10^5/ml, black bars) or neutrophils and PBMCs (both at 5×10^5/ml, gray bars) were incubated in the presence or absence of LPS (1 ng/ml), MIF (10 μg/ml), MIF-2 (10 μg/ml), MIF and MIF-2 treated with a 5-fold molar excess of HOCl (i.e., oxMIF and oxMIF-2, 10 μg/ml) and polymyxin B (20 μg/ml). After 21 h, the percentage of viable cells was determined. (C) Neutrophils and PBMCs (both at 5×10^5/ml) were incubated with increasing concentrations of MIF, oxMIF, MIF-2, and oxMIF-2 (0–10 μg/ml) for 21 h and viable neutrophils determined as above. A representative dose–response curve is shown. (D) Neutrophils (5×10^5/ml) and different ratios of PBMCs (up to 5×10^5/ml) were incubated in the presence (gray bars) or absence (black bars) of MIF (10 μg/ml) and polymyxin B (20 μg/ml) and viable neutrophils determined after 21 h. (E) Different ratios of lymphocytes (black bars) and monocytes (gray bars) were incubated in the presence or absence of MIF (10 μg/ml) and polymyxin B (20 μg/ml) for 21 h and viable neutrophils determined as for (A). Data points represent independent experiments using separate donors and bars represent the mean ± SEM ($n = 3–5$). Statistical differences to controls containing either no MIF (A), no PBMCs (D), or no monocytes/lymphocytes (E) were determined within each group (black bars or gray bars) using one-way ANOVA and are indicated by $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Statistical differences between monocytes and lymphocytes when present at the same ratio to neutrophils were determined using Mann–Whitney test and are indicated by $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$.

for MIF and 0.4 μg/ml for MIF-2) and/or anti-IL-6 (1 μg/ml for MIF and MIF-2) was added to the supernatants and incubated for 15 min at RT. Monoclonal IgG1 antibody was used as negative control. Neutrophils (5×10^5/ml) were added and incubated for 21 h at 37°C/5% CO_2 and neutrophil survival was measured as described above.

2.6 | Synergistic effect of MIF and CXCL8 on neutrophil survival

To determine a potential synergistic effect of MIF and CXCL8, neutrophils (5×10^5/ml) were incubated for 21 h with either recombinant human CXCL8 (20 ng/ml) and MIF (10 μg/ml) separately or CXCL8 (0.2–20 ng/ml) and MIF (1–10 μg/ml) together. Additionally, neutrophils (5×10^5/ml) were preincubated with TNF-α (100 ng/ml) for 1 h, before incubation with recombinant CXCL8 (20 ng/ml), MIF (10 μg/ml), or MIF (1–5 μg/ml) and CXCL8 (20 ng/ml) together for 21 h. Neutrophil survival was measured as described above.

2.7 | ELISA analysis of supernatants from PBMCs incubated with MIF/MIF-2 and oxidized MIF/MIF-2

Levels of IL-1β, IL-6, CXCL8, CXCL12 (SDF-1α), G-CSF, and GM-CSF released into the cell culture supernatant during the incubation of PBMCs with MIF, MIF-2, oxMIF, or oxMIF-2 for 21 h were measured using commercially available Duo-Set ELISA-Kits (R&D Systems) according the manufacturer's instructions. Supernatants were diluted 1:10 with reagent diluent (1% BSA, 0.05% Tween/PBS) before adding to the plates.

2.8 | Statistical analyses

Graphs were plotted and statistical analysis was performed using GraphPad Prism 8 (GraphPad Software, La Jolla, CA, USA). Differences between groups were determined by one-way ANOVA or Mann–Whitney test as appropriate. A P value < 0.05 was considered significant.

3 | RESULTS

3.1 | MIF and MIF-2 prolong neutrophil survival in the presence of mononuclear cells

Previous experiments indicated that the ability of MIF to attenuate neutrophil apoptosis required the presence of PBMCs.[29] We have examined this observation in more detail and also used oxMIF, MIF-2, and oxMIF-2 to determine if they act in a similar way. The ability of MIF to inhibit neutrophil apoptosis was assessed by flow cytometry with annexin V/PI staining (Fig. 1A). The recombinant proteins used in this study contained low concentrations of endotoxin/LPS (0.02–0.1 ng/μg protein). To exclude any possible effect of traces of LPS,[25] all experiments were performed in the presence of the neutralizing agent polymyxin B. The antiapoptotic effect of LPS was effectively blocked (Fig. 1B). Confirming our earlier observations, MIF only delayed neutrophil apoptosis when neutrophils were cocultured with PBMCs (Fig. 1B and Suppl. Fig. 1). MIF-2 had a similar prosurvival effect on neutrophils that was only observed in the presence of PBMCs (Fig. 1B). Oxidation of both MIF and MIF-2 by HOCl did not significantly diminish their ability to prevent neutrophil apoptosis (Fig. 1B). All 4 MIF isoforms (MIF, oxMIF, MIF-2, oxMIF-2) inhibited neutrophil apoptosis in the presence of PBMCs in a dose-dependent manner over the same concentration range (Fig. 1C).

Initial coculture experiments were performed at a 1:1 cell ratio of PBMCs and neutrophils. Next, we determined the minimum amount of PBMCs required to mediate the prosurvival effect of MIF. A ratio of 0.05 of PBMCs to neutrophils was sufficient for MIF to have a significant effect on neutrophil apoptosis (Fig. 1D). Because PBMCs consist of a mixture of approximately 80% lymphocytes and 20% monocytes, we fractionated the PBMC subpopulations to determine which cell type was responsible for mediating the MIF effect. Both cell populations were capable of mediating MIF-triggered neutrophil survival, but monocytes showed a significantly larger effect at any given ratio of mononuclear cell to neutrophil (Fig. 1E). Thus, monocytes appeared to the predominant PBMC cell type mediating the MIF-triggered prosurvival effect. This notion was confirmed further, when we determined the potential specific contribution of T cells, which make up the largest proportion of lymphocytes. We tested their ability to promote neutrophil survival in the presence of MIF but found that they had no effect (Suppl. Fig. 2). This indicates that monocytes are likely to be the major cell required to promote neutrophil survival in the presence of MIF. In subsequent experiments, we chose to work with mixed PBMC populations to encompass the range of cell types present in vivo.

3.2 | Small molecule inhibitor 4-IPP blocks the neutrophil prosurvival effect of MIF but not oxidized MIF

We previously showed that small molecule MIF inhibitors such as 4-IPP cannot bind to the *N*-terminal proline residue once it is oxidatively modified by HOCl.[29] This suggests that anti-inflammatory drugs targeting the *N*-terminal proline may have decreased efficacy at sites of neutrophilic inflammation, where HOCl is produced by the neutrophil-derived MPO. Here, we tested this hypothesis in our neutrophil survival/apoptosis assay. MIF and oxMIF were incubated with 4-IPP before being added to the PBMC/neutrophil coculture system. Compared with untreated MIF, MIF treated with 4-IPP showed a significantly decreased ability to prolong neutrophil survival (Fig. 2). In contrast, the prosurvival effect was preserved when oxMIF was treated with 4-IPP, indicating that 4-IPP was not able to inhibit the activity of proline-oxidized MIF.

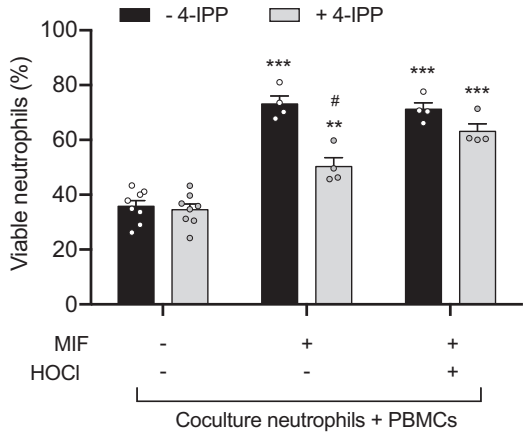

FIGURE 2 Oxidation of *N*-terminal proline prevents inhibition by MIF inhibitor 4-IPP. Neutrophils and mononuclear cells (both at 5×10^5/ml) were incubated in the presence or absence of MIF, oxMIF (both 10 μg/ml), and polymyxin B (20 μg/ml), and viable neutrophils determined as described in Figure 1B (black bars). MIF and oxMIF (latter indicated by "HOCl") (both 10 μg/ml) were also incubated with a 10-fold molar excess of 4-IPP for 15 min before adding the mixture to the coculture (gray bars). Data points represent independent experiments using separate donors and bars represent the mean ± SEM ($n = 4$–8). A statistical difference to the control without MIF within each group was determined using one-way ANOVA and is indicated by $^{**}P < 0.01$ and $^{***}P < 0.001$. Statistical differences between each MIF isoform and its 4-IPP-treated counterpart (black vs. gray bars) were determined using Mann–Whitney test ($^{\#}P < 0.05$).

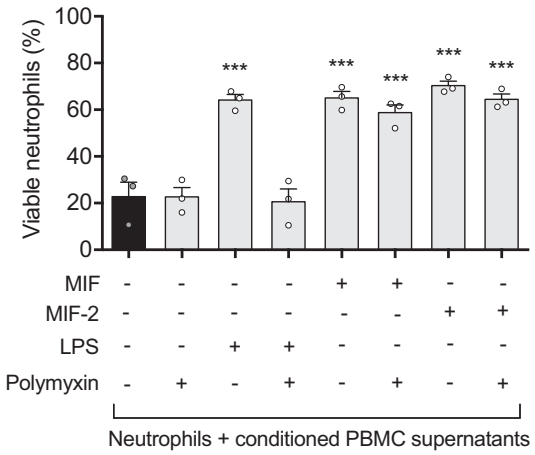

FIGURE 3 Conditioned supernatants of MIF- and MIF-2-treated mononuclear cells prolong neutrophils survival. PBMCs (5×10^5/ml) were incubated in the presence or absence of LPS (1 ng/ml), MIF (10 μg/ml), MIF-2 (10 μg/ml), and polymyxin B (20 μg/ml) for 21 h. Conditioned supernatants were collected and incubated with neutrophils (5×10^5/ml) for 21 h. Viable neutrophils were determined as described in Figure 1. Data points represent independent experiments using separate donors and bars represent the mean ± SEM ($n = 3$). Statistical differences between treated samples (gray bars) and untreated (control black bar) were determined using one-way ANOVA and are indicated by $^{***}P < 0.001$.

3.3 | Mononuclear cells secrete neutrophil survival mediators following exposure to MIF and MIF-2

Next, we determined whether the indirect PBMC/monocyte-mediated effect of MIF proteins on neutrophil survival was due to the release of a soluble factor from PBMCs. PBMCs were incubated with MIF or MIF-2, cells were removed by centrifugation and the supernatants added to neutrophils. PBMC-conditioned cell culture supernatants were able to promote neutrophil survival to the same extent as the coculture system (Fig. 3), suggesting that a soluble survival factor was released from PBMCs in response to MIF or MIF-2 that inhibited neutrophil apoptosis.

Because various cytokines are known to inhibit neutrophil apoptosis,[25–27,40] we measured the levels of the candidate cytokines IL-1β, IL-6, CXCL8, CXCL12, G-CSF, and GM-CSF in PBMC supernatants after incubation with MIF or MIF-2. IL-1β, IL-6, and CXCL8 showed a concentration-dependent increase with increasing concentrations of MIF, CXCL12 peaked at a low concentration of MIF, and G-CSF or GM-CSF levels were not elevated. Statistical significance was reached for IL-1β and CXCL8 at the high end of the MIF dose curve (Fig. 4A). Treatment with oxMIF led to an essentially identical cytokine release profile (Fig. 4C). Interestingly, MIF-2 and oxMIF-2 not only led to an up-regulation of CXCL8 and IL-1β, but also triggered an additional significant release of IL-6 and G-CSF (Figs. 4B and 4D). Thus, although MIF-2 showed the same prosurvival capacity on neutrophils, the cytokine profiles of MIF-2- and oxMIF-2-treated PBMCs differed somewhat from those of MIF and oxMIF. Overall, the most pronounced induction effect of all MIF isoforms was seen for CXCL8 (Fig. 4).

3.4 | CXCL8 and MIF act synergistically to promote neutrophil survival

As CXCL8 was the predominant cytokine released from PBMCs stimulated with MIF or MIF-2, we investigated whether CXCL8 alone was sufficient for the inhibitory effect of MIF-treated PBMCs on neutrophils. Conditioned media from MIF-treated PBMCs were incubated with neutralizing mAbs directed against CXCL8. Compared with an isotype control antibody, a CXCL8-blocking antibody decreased the prosurvival effect of PBMC supernatants by approximately 60% (Fig. 5; dark gray bars).

As blocking CXCL8 did not fully inhibit the antiapoptotic effect of PBMC supernatants and because other cytokines were also found up-regulated in the MIF/MIF-2-stimulated PBMC supernatants, we next tested whether a combination of blocking antibodies against CXCL8 and IL-6, which was released at the second-highest levels after CXCL8, can achieve a greater degree of inhibition. However, antibodies against IL-6, when added to MIF-conditioned supernatants in addition to those against CXCL8, did not further inhibit the antiapoptotic effect compared with the anti-CXCL8 treatment alone and had no significant effect by themselves (Fig. 5). In contrast, in the case of MIF-2-conditioned media, where IL-6 is present at higher levels than in those

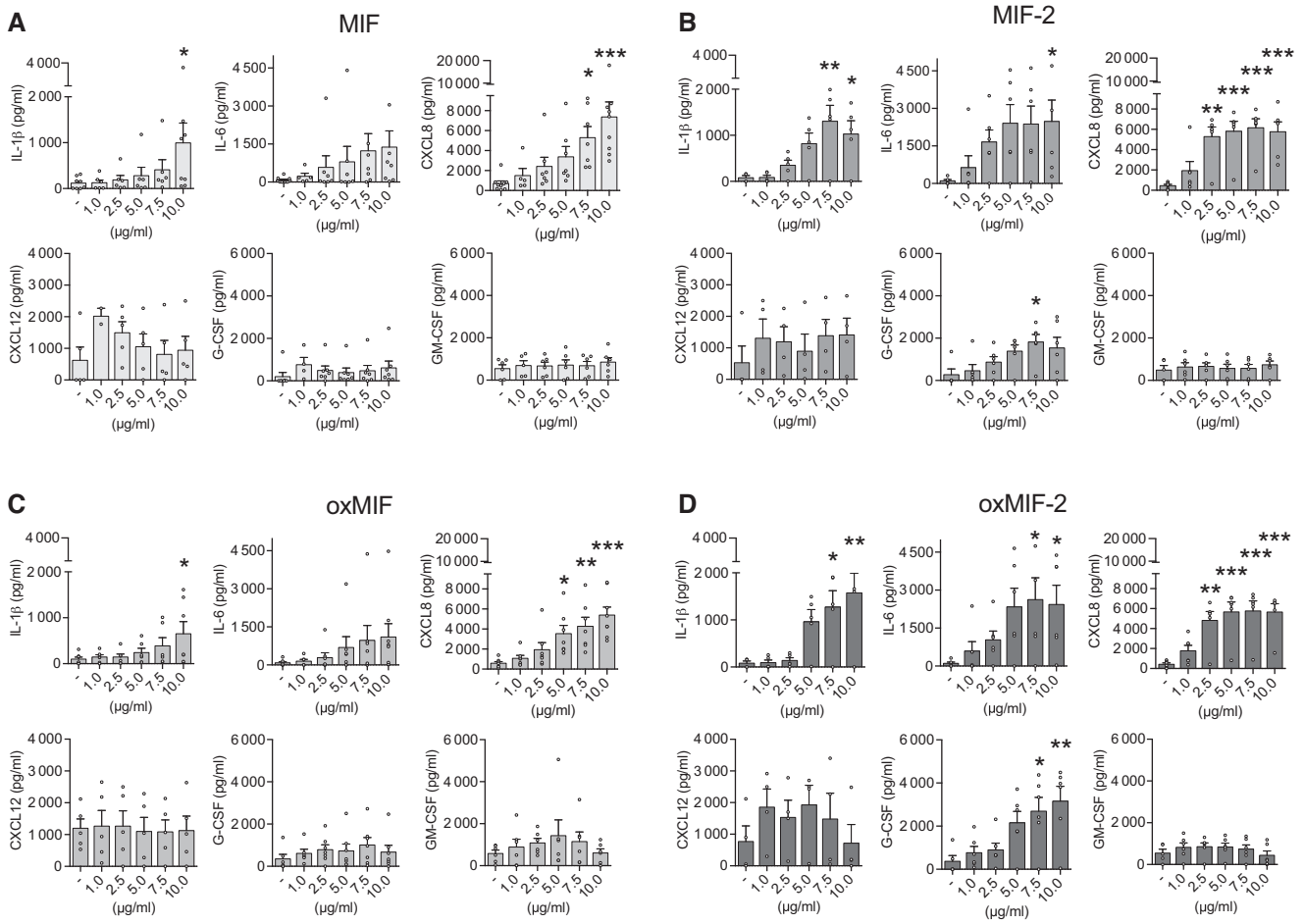

FIGURE 4 Cytokine release profile of mononuclear cells after incubation with MIF isoforms. PBMCs (5×10^5/ml) were treated with increasing concentrations (0–10 μg/ml) of MIF (A), MIF-2 (B), oxMIF (C), and oxMIF-2 (D) as indicated. After 21 h, supernatants were collected and IL-1β, IL-6, CXCL8, CXCL12, G-CSF, and GM-CSF measured by ELISA. Data points represent independent experiments using separate donors and bars represent the mean ± SEM ($n = 6$). Statistical differences between treated samples and untreated controls in each group were determined using one-way ANOVA and are indicated by $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

from MIF-conditioned media and is significantly up-regulated, blocking IL-6 decreased the prosurvival effect to the same extent as blocking CXCL8. Combining blocking antibodies against CXCL8 and IL-6 further attenuated the inhibitory effect on MIF-2-conditioned supernatants by 10%, but not back to control levels. Together, this suggested that the secretion of CXCL8 and of CXCL8/IL-6 from PBMCs in response to MIF and MIF-2, respectively, contributes substantially to the neutrophil prosurvival effect.

To scrutinize the mechanism of MIF-elicited CXCL8-mediated inhibition of apoptosis, we tested whether purified, LPS-free, recombinant human CXCL8 could inhibit neutrophil apoptosis at concentrations measured in the MIF-treated PBMC supernatants, that is, 20 ng/ml. The percentage of viable neutrophils only increased slightly after incubation with CXCL8, and this was not statistically significant (Fig. 6A), suggesting that CXCL8 alone is not sufficient to inhibit neutrophil apoptosis and that a combination of both CXCL8 and MIF may be required to inhibit neutrophil apoptosis. Indeed, when neutrophils were incubated with both recombinant CXCL8 and recombinant MIF, the observed survival effect was comparable to that elicited by PBMCs (Fig. 6A). To explore a possible synergistic effect of MIF and CXCL8 on neutrophil survival, different concentrations of either MIF (1–10 μg/ml) at a constant concentration of CXCL8 (20 ng/ml) (Fig. 6A) or different concentrations of CXCL8 (0.2–20 ng/ml) at a constant concentration of MIF (10 μg/ml) (Fig. 6B) were tested. While the antiapoptotic effect was dependent on the concentration of MIF, the concentration of CXCL8 could vary 100-fold and still elicit a maximal inhibitory response in the presence of MIF. Although the detailed elucidation of the mechanism was beyond the scope of this study, these data suggested that CXCL8 and MIF have a synergistic effect on neutrophil apoptosis.

Next, we determined whether a short exposure to TNF-α, to simulate active microenvironmental inflammation, would influence the effect of MIF and CXCL8 on promoting neutrophil survival. Indeed, apoptosis of neutrophils that were primed with TNF-α was inhibited to a greater extent following exposure to MIF in combination with CXCL8 (Fig. 6C). Primed neutrophils were exquisitely sensitive to MIF with a maximal inhibitory response observed at any of the tested MIF concentrations. In contrast, untreated neutrophils responded to MIF in a

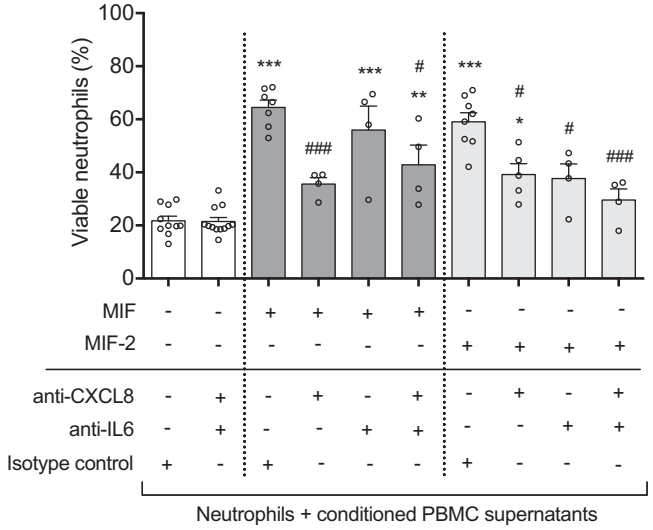

FIGURE 5 CXCL8 is required for the neutrophil survival effect of MIF proteins. (A) Mononuclear cells (5×10^5/ml) were incubated in the presence or absence of MIF, MIF-2 (both 10 μg/ml), and polymyxin B (20 μg/ml) as described in Figure 1B. Conditioned supernatants were collected, a neutralizing mAb against CXCL8 (anti-CXCL8; 0.4 μg/ml for the incubation with MIF-2 and 1.2 μg/ml for that with MIF) and/or against IL-6 (anti-IL-6; 1 μg/ml for incubations with MIF and MIF-2) or an IgG1 antibody as isotype control were added, the mixture incubated with neutrophils (5×10^5/ml) for 21 h, and viable neutrophils determined as described in Figure 1. Data points represent independent experiments using separate donors and bars represent the mean ± SEM ($n = 4–7$). Statistical differences between control and treated samples were determined using one-way ANOVA ($^*P > 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$). Statistical differences between isotype control and blocking antibody treatments in each sample were determined using Mann–Whitney test and are indicated by $^{\#}P < 0.05$ and $^{\#\#\#}P < 0.001$.

concentration-dependent manner with concentrations of > 2.5 μg/ml required to significantly inhibit apoptosis.

3.5 | CXCR2 is a critical receptor in promoting neutrophil survival

Depending on the tissue and disease context, the inflammatory activities of MIF are mediated through CD74, CXCR2, or CXCR4.[14–17,33] In some cases, 2 or 3 of these MIF receptors are activated, with receptor cross-talk and/or complex formation occurring.[13,14]

To determine which MIF receptor is responsible for the observed prosurvival effects, we analyzed receptor levels on neutrophils and PBMCs, and functionally probed their contribution with receptor-specific inhibitors. Flow cytometry showed that CXCR2 is strongly expressed on neutrophils (Suppl. Fig. 3A), confirming previous data that this is an abundant neutrophil chemokine receptor.[20,41] Neutrophils also expressed low levels of CXCR4, while CD74 expression was not detected (Suppl. Fig. 3A), in line with previous observations.[14,41] We found that all 3 MIF receptors are expressed on PBMCs, with the highest expression level seen for CXCR4, followed by CD74, while only very low levels of CXCR2 were detected (Suppl. Fig. 3B). To determine which receptors are involved in facilitating the antiapoptotic effect of MIF on neutrophils, we blocked the receptors using a mAb (LN2), a blocking peptide (NBP1-76867PEP), and a small molecule antagonist (SB225002) directed against CD74, CXCR4, and CXCR2, respectively.[42,43] Blocking CD74 or CXCR4 in the coculture system had no significant effect on MIF-mediated inhibition of neutrophil apoptosis (Fig. 7A). In contrast, blocking CXCR2 strongly decreased the survival of neutrophils elicited by MIF, suggesting that this receptor has a major role in the antiapoptotic effect of MIF on neutrophils in the coculture system (Fig. 7A). Interestingly, a significant decrease of neutrophil survival by blocking CXCR2 was also observed in the absence of exogenously added recombinant MIF, suggesting that endogenous neutrophil- or PBMC-derived MIF contributes to CXCR2-mediated neutrophil survival by an autocrine/paracrine pathway.

To further dissect whether neutrophil- or PBMC-expressed CXCR2 was the predominant driver of the antiapoptotic effect, we blocked CXCR2 in both cell types separately. When neutrophil CXCR2 was blocked before exposing the neutrophils to MIF-conditioned PBMC supernatants, the survival response was ablated (Fig. 7B). Similarly, when CXCR2-blocked PBMCs were incubated with MIF, their supernatants could no longer inhibit neutrophil apoptosis (Fig. 7C). Collectively, these results suggest a key contribution of CXCR2 on both PBMCs and neutrophils during MIF inhibition of neutrophil apoptosis.

4 | DISCUSSION

In this study, we show that MIF, MIF-2, and their *N*-terminal proline-oxidized isoforms oxMIF and oxMIF-2 prolong neutrophil survival, but only when neutrophils are exposed in the presence of mononuclear cells. We demonstrate that the mechanism involves the release of soluble survival factors, chiefly CXCL8, from PBMCs following binding of MIF to one of its known receptors, CXCR2, and a direct effect of MIF on neutrophils via a CXCR2-dependent pathway (Fig. 8).

MIF is highly up-regulated during inflammation and is known to exert a variety of proinflammatory activities,[2,3,10,11,14] but the contribution that MIF makes to neutrophil-mediated inflammation is not well understood. Neutrophils are the first inflammatory cells arriving at sites of inflammation and injury, and their circulating levels increase dramatically during inflammation.[20,44] Previous studies showed that MIF can induce neutrophil transmigration in vitro[14]; therefore, it is likely that neutrophils and MIF are colocalized at inflammatory sites. MIF has previously been reported to prolong neutrophil survival,[28] but subsequent evidence suggested that this may not be a direct effect.[29] Here, we offer in vitro cell data that are in support of a 2-hit model, in which MIF acts on both neutrophils and neighboring mononuclear cells. Our data further suggest that improvement of neutrophil survival by MIF or its isoforms requires an interplay of both cell types involving the release of prosurvival factors by mononuclear cells and their synergistic action together with MIF.

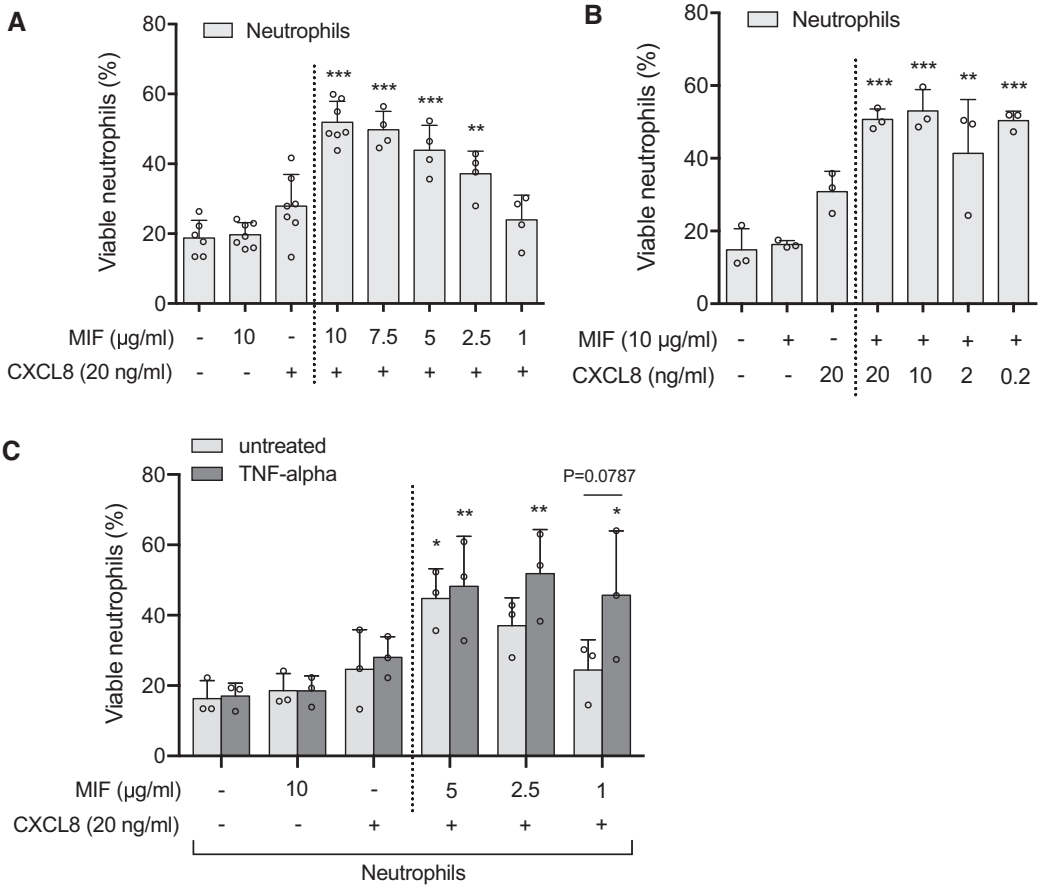

FIGURE 6 Synergistic effect of MIF and CXCL8 promoting neutrophil survival. (A and B) Synergism between MIF and CXCL8. (A) Neutrophils (5×10^5/ml) were incubated with recombinant CXCL8 (20 ng/ml), MIF (10 μg/ml), or MIF (1–10 μg/ml) and CXCL8 (20 ng/ml) for 21 h and viable neutrophils determined. (B) Neutrophils (5×10^5/ml) were incubated with recombinant CXCL8 (20 ng/ml), MIF (10 μg/ml), or MIF (10 μg/ml) and CXCL8 (0.2–20 ng/ml) for 21 h and viable neutrophils determined. (C) TNF-α enhances the synergistic effect (trend). Neutrophils (5×10^5/ml) were preincubated with TNF-α (100 ng/ml) for 1 h (TNF-α-treated; dark gray bars) or not pretreated (untreated; light gray bars), before incubating with recombinant CXCL8 (20 ng/ml), MIF (10 μg/ml), or MIF (1–5 μg/ml), and CXCL8 (20 ng/ml) for 21 h. Viable neutrophils were determined as described in Figure 1. Data points represent independent experiments using separate donors and bars represent the mean ± SEM ($n = 3$–7). Statistical differences between control and treated samples in each group were determined using one-way ANOVA (**$P < 0.01$, ***$P < 0.001$).

During an inflammatory response, monocytes infiltrate after the initial recruitment of neutrophils. The ensuing interaction and cooperation of both cell types through cytokine-mediated crosstalk eventually completes the immune response and results in a resolution of inflammation.[44,45] Depending on the exact time point along the infiltration cascade, different ratios of neutrophils and monocytes/macrophages and different concentrations of inflammatory cytokines, including MIF, will be present. Our study suggests that if MIF (or MIF-2) expression in early inflammatory lesions coincides with neutrophil infiltrates and minimum levels of infiltrated monocytes (i.e., ≥5% of neutrophils), neutrophil apoptosis will be inhibited or delayed, potentially prolonging the inflammatory response.

MIF has previously been shown to stimulate the expression and secretion of IL-8/CXCL8 in lymphocytes and synovial fibroblasts.[16,46,47] We detected significant levels of CXCL8 in the supernatants of MIF-stimulated PBMCs, suggesting it could be a likely survival factor contributing to neutrophil survival. Although CXCL8 is well known as a major chemoattractant of neutrophils, studies investigating the prosurvival effect of CXCL8 have delivered conflicting results.[48,49] Here, we show a synergistic effect of MIF and CXCL8 on neutrophils. It is important to note, however, that when we blocked CXCL8 in the supernatants of MIF-treated PBMCs, the neutrophil prosurvival effect was not fully abrogated, indicating that other cytokines present in the supernatants may, at least partially, compensate for the lack of CXCL8. We tested the involvement of IL-6 and found it did not play a role in the prosurvival effect of MIF-conditioned PBMC supernatants. Further studies are needed to elucidate the role of other cytokines in mediating the effect of MIF on neutrophil survival.

The use of receptor blocking agents in our study clearly indicated that the inhibition of neutrophil apoptosis was mediated by the MIF chemokine receptor CXCR2 that is expressed on the surface of both monocytes and neutrophils (Fig. 8). However, CXCR2 is also the neutrophil receptor for CXCL8,[50] so the inhibitory effect of the CXCR2-blocking small molecule inhibitor may have been the result of abolishing the interaction between CXCL8 and neutrophil CXCR2.

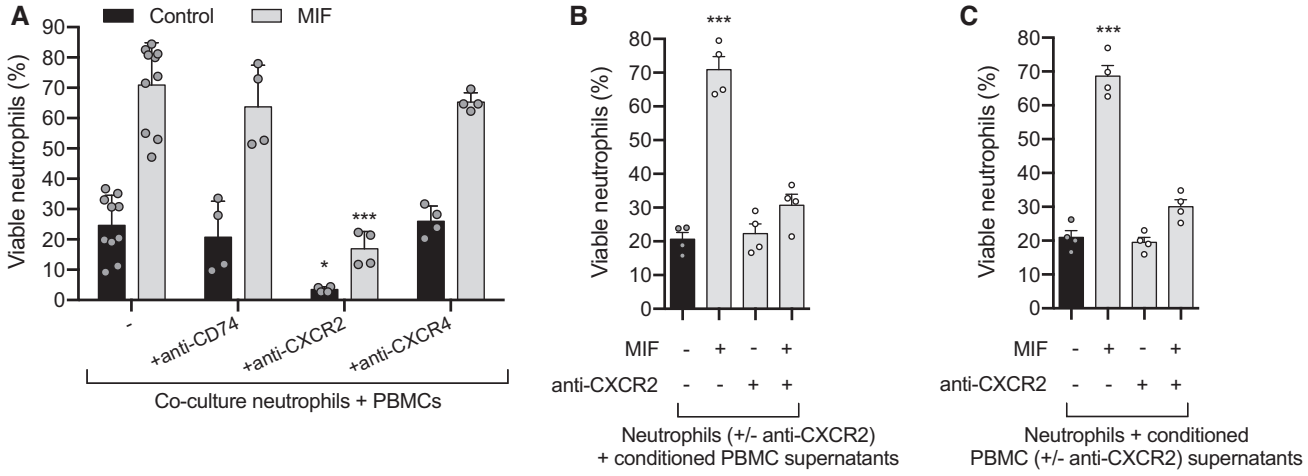

FIGURE 7 Both neutrophil- and mononuclear cell-expressed CXCR2 are required for the antiapoptotic effect of MIF. (A) Neutrophils and mononuclear cells (PBMCs) (both at 5×10^5/ml) were preincubated with a neutralizing anti-CD74 antibody LN2 ("anti-CD74," 10 μg/ml), the small molecule CXCR2 antagonist SB225002 ("anti-CXCR2," 10 μM), or the CXCR4-blocking peptide NBP1-76867PEP ("anti-CXCR4," 25 μg/ml) for 30 min before incubating the cells with MIF (10 μg/ml; also containing 20 μg/ml polymyxin B) for 21 h. Viable neutrophils were determined as described in Figure 1B. (B) Neutrophils (5×10^5/ml) were preincubated with SB225002 (10 μM) for 30 min, then supernatants from MIF-treated PBMCs were added and viable neutrophils determined after 21 h. (C) PBMCs (5×10^5/ml) were preincubated with SB225002 (10 μM) for 30 min, excess of blocking agent was removed by centrifugation and cells resuspended in fresh media containing polymyxin B (20 μg/ml), and incubated for 21 h. The conditioned supernatants were added to neutrophils (5×10^5/ml) and incubated for 21 h and viable neutrophils determined as described in Figure 1. Data points represent independent experiments using separate donors and bars represent the mean ± SEM ($n = 4$–10). Statistical differences between treated samples (gray bars) and untreated controls (black bar) in each group were determined using one-way ANOVA and are indicated by $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

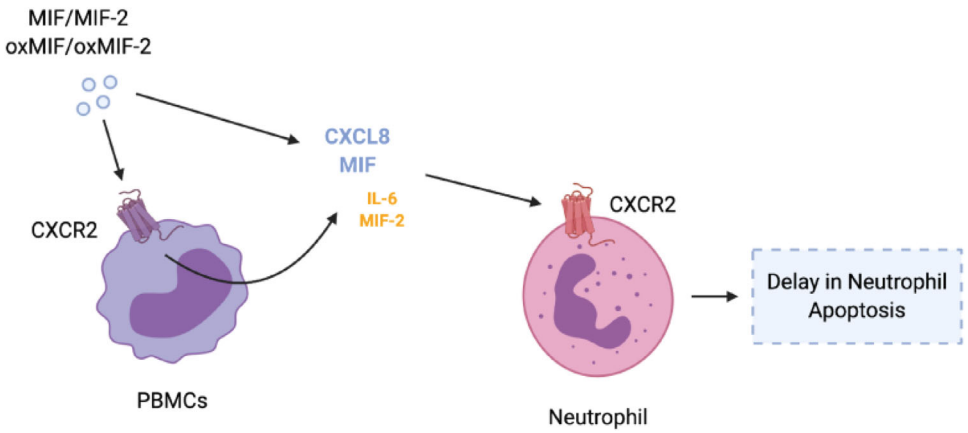

FIGURE 8 Proposed mechanism for the inhibition of neutrophil apoptosis by MIF proteins. MIF and MIF-2 (and their oxidized isoforms; latter not shown) stimulate the release of CXCL8 and IL-6 (and potentially other prosurvival cytokines) from mononuclear cells (PBMCs) via the surface receptor CXCR2. Together, CXCL8 and MIF, and potentially also MIF-2 and IL-6 delay neutrophil apoptosis via the neutrophil surface receptor CXCR2 and potentially another receptor (latter not shown). Created with BioRender.com.

Alternatively, both the CXCL8/CXCR2 and MIF/CXCR2 axis may have been blocked by the CXCR2 inhibitor. We cannot fully exclude the possibility that the effect of MIF on neutrophil survival could be, at least in part, due to another neutrophil receptor. One possibility is CXCR7, a more recently identified noncognate MIF receptor on lymphocytes and tumor cells.[13] Alternatively, a residual MIF/CXCR4 effect cannot be ruled out, as additional CXCR4-blocking inhibitors were not tested in our study.

The synergistic effect on neutrophil survival that we observed between MIF (and MIF-2) and lymphocytes (or their supernatants) may also partially question the sole role of CXCR2. Although lymphocytes produce CXCL8 in response to MIF,[16,47] they do not usually express detectable levels of CXCR2. Although our lymphocyte enrichment protocol reliably led to > 95% purity of the lymphocyte pool, we cannot exclude an effect of remaining scattered monocytes in these preparations.

MIF and CXCL8 (or CXCL1/KC in mouse models) play important roles in acute and chronic inflammatory conditions such as atherosclerosis, inflammatory lung diseases, and rheumatoid arthritis. Neutrophils and monocytes contribute to the initiation and propagation of the inflammatory milieu in these diseases and both MIF and CXCL8 are chemoattractants for neutrophils and monocytes promoting their recruitment into developing lesions.[12,14,51,52] The synergism between MIF and CXCL8 in neutrophil/monocyte cocultures identified in our current study could thus contribute to the inflammatory process in these diseases. On a molecular level, the cooperative/synergistic behavior of MIF and CXCL8 may encompass different mechanisms such as: (i) cross-talk of the MIF/CXCR2 and CXCL8/CXCR2 downstream signaling pathways; (ii) MIF/CXCL8 complex formation; or (iii) sequential activation of pathways with mutual interactions and/or a feed-forward loop functionality. Future in-depth studies into the exact mechanism are warranted. To further mimic the inflammatory environment in our experimental set-up, we exposed neutrophils to the proinflammatory mediator TNF-α, which is expressed in inflammatory lesions and typically up-regulated in nonresolving inflammation, before adding MIF and CXCL8. Interestingly, this priming seemed to further enhance the effect of MIF/CXCL8 on neutrophil survival highlighting its significance in relation to inflammation.

Small molecule MIF inhibitors such as 4-IPP have already been developed and have shown promise in blocking MIF/CD74 signaling. In the present study, we show that 4-IPP can block the ability of MIF to prolong neutrophil survival, indicating that it can also block the interaction of MIF with CXCR2. 4-IPP targets the nucleophilic *N*-terminal proline of MIF, which so far has not been implicated as a determinant of the MIF/CXCR2 interface.[17,53] Reports on inhibitors of the MIF/CXCR2 axis have so far been limited to peptide-based inhibitors[53,54] and the allosteric MIF inhibitor ibudilast.[55] However, the 4-IPP-based modification of MIF may also induce conformational changes similar to ibudilast that affect the CXCR2-binding interface of MIF. Moreover, our group previously showed that this residue becomes oxidized to a proline-imine by neutrophil-derived HOCl.[29] Here, we show that HOCl-treated isoforms of MIF proteins, that is, oxMIF and oxMIF-2, can still trigger the release of cytokines from PBMCs and inhibit neutrophil apoptosis. This indicates that inhibitors targeting the *N*-terminus of MIF proteins might not effectively work in an oxidative microenvironment created during neutrophilic inflammation. However, in vivo proline-imine modification of MIF has yet to be demonstrated.

MIF-2 (D-dopachrome tautomerase/D-DT) is a more recently discovered member of the MIF protein family, which has been suggested to share MIF-like activity such as binding to CD74 and activating the ERK1/2 MAP kinase pathway. Accordingly, overlapping activities of MIF and MIF-2 have been proposed, at least for some physiologic processes and disease settings.[7,56] Regulation of neutrophil biology or pathophysiology by MIF-2 has not been explored. The present study is the first to show that similar to MIF, MIF-2 prolongs neutrophil survival in the presence of PBMCs. Unlike MIF, MIF-2 also caused the release of significant levels of IL-6 from PBMCs, which inhibited neutrophil apoptosis in a similar fashion to PBMC-derived CXCL8. Our results thus indicate that MIF and MIF-2 have distinct effects on PBMCs. To this end, it is interesting to note that MIF-2 lacks the pseudo-ELR (Arg^{12}-Xaa-Asp^{45}) motif of MIF that has been implicated to contribute to the MIF/CXCR2 binding interface,[17] but more in-depth future studies on how the differential responses of MIF and MIF-2 are mediated are warranted.

In conclusion, we show that the atypical chemokine/cytokine MIF, as well as its homolog MIF-2/D-DT, inhibits neutrophil apoptosis through a mononuclear cell-dependent mechanism, which involves the release of the cytokines CXCL8, IL-6, and potentially also G-CSF and GM-CSF. Moreover, we demonstrate that MIF proteins act synergistically together with CXCL8 to delay neutrophil apoptosis. We also provide evidence that the chemokine receptor CXCR2 plays a key role in mediating the ability of MIF to prolong neutrophil survival, suggesting that the MIF/CXCR2 axis could be a pharmacologic target for nonresolving inflammation with utility in a variety of inflammatory diseases.

AUTHORSHIP

L.S. designed and performed the experiments, analyzed the data, and wrote the manuscript. N.D. and J.B. conceived and supervised the study, designed the experiments, wrote and edited the manuscript, and provided funding. L.Z. performed the experiments, provided materials, and reviewed and edited the manuscript. C.K. helped with the experiments and reviewed and edited the manuscript. M.B.H. advised on the study design and interpretation of the results, and edited the manuscript. U.H.-C. and T.R. advised on the study design, provided funding, and reviewed and edited the manuscript.

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DISCLOSURES

J.B. is an inventor on patents covering anti-MIF strategies. The other authors declare no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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