Apoptotic Neutrophils Release Macrophage Migration Inhibitory Factor upon Stimulation with Tumor Necrosis Factor-α

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Macrophage migration inhibitory factor (MIF) is an important cytokine involved in the regulation of innate immunity and present at increased levels during inflammatory responses. Here we demonstrate that mature blood and tissue neutrophils constitutively express MIF as a cytosolic protein not associated with azurophil granules. Functionally active MIF, but not proteases stored in azurophil granules, was released from apoptotic neutrophils following short term tumor necrosis factor (TNF)-α stimulation in a caspase-dependent manner and prior to any detectable phagocytosis by monocyte-derived macrophages. Moreover, TNF-α-mediated MIF release was blocked by glyburide and propenicide, both inhibitors of ATP-binding cassette-type transporters, suggesting that this transporter system is activated during neutrophil apoptosis. Taken together, apoptotic mature neutrophils release MIF upon short term TNF-α stimulation. Therefore, apoptosis may not always occur without the induction of pro-inflammatory mechanisms.

Macrophage migration inhibitory factor (MIF) was one of the first cytokines to be identified (1, 2). Previously published work suggested that MIF is a major regulator of immune responses. MIF is generated by multiple cells of the immune system, such as T cells (1, 2), neutrophils (3, 4), and eosinophils (5), as well as monocytes and macrophages (6). As the name indicates, MIF inhibits the migration of macrophages (7). However, MIF also promotes the production of proinflammatory cytokines and mediators, such as TNF-α, interferon γ, IL-1β, IL-2, IL-6, IL-8, nitric oxide, and prostaglandin E2 (8). Therefore, MIF is considered to be an important amplifier of cytokine production within the host antimicrobial defense system. The crucial antibacterial role of MIF has been demonstrated in MIF−/− mice, which failed to control the growth of Salmonella typhimurium, an intracellular pathogen (9).

In addition, MIF seems to play a key role in sepsis. Patients suffering from severe sepsis or septic shock exhibit enhanced MIF levels in the blood, and MIF levels appeared to correlate with disease severity (10, 11). In in vivo models, MIF increased and anti-MIF-neutralizing antibodies protected against lethality in endotoxic shock (12, 13). Moreover, MIF−/− mice decreased the pro-inflammatory activity and improved survival in an experimental model of sepsis (14). Besides sepsis, MIF may also amplify immune response in other inflammatory diseases (8).

Although MIF is expressed constitutively by many different immune cells, the molecular mechanisms leading to its release are largely unknown. Here we demonstrate that TNF-α-activated apoptotic blood neutrophils release biologically active MIF in a caspase- and ATP-binding cassette (ABC)-type transporter-dependent manner, and we exclude the possibility that MIF release is the consequence of plasma membrane disruption, which occurs at later stages of neutrophil apoptosis under in vitro conditions, in a process called secondary necrosis (15). Interestingly, MIF release preceded any detectable uptake of apoptotic neutrophils by macrophages in vitro. These findings suggest that apoptotic neutrophils might, at least under certain conditions, be able to promote an inflammatory response and challenge the concept that apoptosis is always a non-inflammatory event.

EXPERIMENTAL PROCEDURES

Reagents—The caspase inhibitors N-benzylxycarbonyl (Z)-VAD-fluoromethyl ketone (fmk) (pan-caspase inhibitor), Z-DEVD-fmk (caspase-3 inhibitor), and Z-LEHD-fmk (caspase-9 inhibitor) were purchased from Becton Dickinson Biosciences (Basel, Switzerland). Human GM-CSF was from Novartis Pharma GmbH (Nürnberg, Germany), and complement factor 5a (C5a) was from Calbiochem-Novabiochem Corp. (distributed by Juro Supply GmbH, Lucerne, Switzerland). IL-8 and TNF-α were from R&D Systems (Abingdon, UK). A functionally active recombinant MIF protein (MIF-MBP fusion protein) was generated as previously described (3). The ABC transporter inhibitors glyburide and probenicid were from Sigma-Aldrich (Buchs, Switzerland).

Antibodies—Anti-Fas agonistic monoclonal antibody (mAb; CH11) was obtained from LabForce AG (Nunningen, Switzerland). Goat anti-human MIF Ab, goat anti-human GM-CSF Ab,
and control goat IgG were purchased from R&D Systems. Anti-human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mAb was obtained from Chemicon International, Inc. (Temecula, CA). Rabbit anti-human cleaved caspase-3 Ab was from Cell Signaling Technology, Inc. (BioConcept, Allschwil, Switzerland). Anti-human cathepsin B mAb (clone CA10) was from Oncogene Research Products (distributed by Juro Supply GmbH), anti-human cathepsin D mAb (clone CTD-19) from Sigma-Aldrich, and anti-human CD15 mAb from Becton Dickinson Biosciences. Rabbit anti-human myeloperoxidase (MPO) Ab was obtained from DakoCytomation AG (Baar, Switzerland). Goat horseradish peroxidase-conjugated secondary Ab was from Amersham Biosciences (Dübendorf, Switzerland). R-phycocerythrin-conjugated, rhodamine-tetramethyl rhodamine isothiocyanate-conjugated, and fluorescein isothiocyanate-conjugated goat anti-rabbit and donkey anti-mouse secondary Abs were purchased from Jackson ImmunoResearch Laboratories (Milan Analytica AG, La Roche, Switzerland). Anti-TNF-receptor 1 mAb (anti-TNF-R1; clone H398) was from Alexis (Lausen, Switzerland).

Cells—Isolation of blood neutrophils (16–21) was performed as described previously. Neutrophil purity was always higher than 95% and was assessed by staining with Diff-Quik (Baxter, Düdingen, Switzerland) and light microscopy analysis. For macrophage phagocytosis and activation, as well as for the mono-cyte migration experiments, we isolated peripheral blood mononuclear cells by Ficoll-Hypaque centrifugation (3).

Neutrophil Cultures—Neutrophils were cultured at 3 × 10⁶/ml in the presence or absence of agonists and/or inhibitors for the indicated times using complete culture medium (RPMI 1640 containing 10% fetal calf serum from Invitrogen, Basel, Switzerland). If not indicated otherwise, cytokines were used at 25 ng/ml, caspase inhibitors at 50 μM, anti-Fas mAb at 1 μg/ml, LPS at 100 ng/ml, C5a at 10⁻⁸ M, glyburide at 2.5 μM, and probenecid at 2 μM. The blocking anti-TNF-R1 mAb was used at 10 μg/ml and added 30 min before TNF-α stimulation.

Determination of Apoptosis—Apoptosis was assessed by annexin V staining assay (16–19).

Immunoads—MIF and elastase concentrations were measured in neutrophil supernatants by using a commercial ELISA kit (MIF, Chemicon International, Inc.; elastase, Bender MedSystems, RUWAG Diagnostik, Zurich, Switzerland). TNF-α and IL-8 concentrations were measured in macrophage supernatants using a cytometric bead array assay (human inflammation CBA kit, Becton Dickinson Biosciences). All assays were performed according to the manufacturer’s recommendations.

Confocal Laser Scanning Microscopy—Cytospins were prepared from freshly purified blood neutrophils or cultured neutrophils (19). Cells were fixed in 4% paraformaldehyde at room temperature for 10 min and washed four times in phosphate-buffered saline, pH 7.4. Permeabilization of cells was performed with 0.05% saponin in phosphate-buffered saline and with acetone at −20 °C for 15 min. To prevent nonspecific binding, slides were incubated in blocking buffer (10% bovine serum albumin, 10% human polyvalent serum, 10% normal serum matching the secondary Ab that is used in the staining in phosphate-buffered saline) at room temperature for 1 h. Indirect immunostainings were performed at 4 °C overnight using the following primary Abs: anti-MIF Ab (1:50; diluted in blocking buffer), anti-GM-CSF Ab (1:50), anti-cathepsin B mAb (1:200), cathepsin D mAb (1:200), anti-MPO Ab (1:500), and caspase-3 Ab (1:25). Besides control Abs, we preincubated anti-MIF Ab with MIF and GM-CSF, respectively, before the staining procedure to further demonstrate specificity.

Immunofluorescent stainings were also performed on 4-μm-thick paraformaldehyde-fixed paraffin-embedded tissue sections from appendicitis and ulcerative colitis patients (19, 21). Slides were dried at 52 °C for 2 h and deparaffinized using Neo-Clear Solution (Merck, Darmstadt, Germany), ethanol (100, 90, 80, 60, and 40), and water at room temperature. Following microwave treatment in TE buffer (10 mM Tris, pH 8.0, 1 mM EDTA), slides were washed in water, blocked, and stained with primary anti-MIF Ab as aforementioned and anti-CD15 mAb (1:25).

After incubation with primary Ab, cells and tissues, respectively, were incubated with appropriate tetramethyl rhodamine isothiocyanate- and fluorescein isothiocyanate-conjugated secondary Abs (1:100) in the dark at room temperature for 1 h. The anti-fading agent Moviol (Calbiochem) was added, and the cells were covered by coverslips. The slides were analyzed by confocal laser scanning microscopy (LSM 510, Carl Zeiss, Heidelberg, Germany) equipped with argon and helium-neon lasers.

Image Analysis—With an interval of 0.1 μm, slices were taken throughout the z-axis (z stacks) with 50 and 70 slices per stack in order to show cells in their full extension. The z stacks were deconvoluted with Huygens-2 software (Scientific Volume Imaging, Hilversum, The Netherlands) using the classic maximum likelihood estimation. Deconvoluted stacks were then used for image analysis by Imaris software (Bitplane AG, Zürich, Switzerland), operating with Imaris Surpass (volume and isosurface rendering analysis), to visualize and locate MIF protein expression in neutrophils. For colocalization studies, unprocessed, unfiltered, and undeconvoluted data sets were analyzed by using the Imaris software (Bitplane AG), considering every singular layer of a stack separately. Quantitative data of colocalization events were determined by the statistics modules in the colocalization and Voxelshop software of the Imaris package. Intensities were given as the sum of all colocalizing voxels in a data set, and a computer image was generated. For quantitative analysis of colocalization, the Pearson’s correlation coefficient was calculated.

Immunoblotting—Neutrophils (1 × 10⁶) were lysed in 2 × loading buffer (Invitrogen) with sonication on ice. Samples were boiled and subjected to gel electrophoresis on 12% NuPage-Gels (Invitrogen). Separated proteins were electrotransferred onto polyvinylidene difluoride membranes (Immobilon-P, Milipore). The filters were blocked by blocking solution (Tris-buffered saline, 0.1% Tween 20 containing 5% nonfat milk) and subsequently incubated with the primary Abs in Tris-buffered saline, 0.1% Tween 20, 3–5% nonfat dry milk overnight at 4 °C. The primary anti-MIF Ab was diluted 1:1000. For loading controls, stripped filters were incubated with anti-GAPDH mAb (1:15000). Filters were washed in Tris-buffered saline, 0.1% Tween 20 for 30 min and incubated with the appropriate horseradish peroxidase-conjugated secondary Ab (1/2000) at
room temperature for 1 h. After another washing step, filters were developed by an ECL technique (ECL-Kit, Amersham Biosciences) according to the manufacturer’s instructions.

Macrophage Phagocytosis Assay—Uptake of apoptotic neutrophils by monocyte-derived macrophages was investigated as described previously (22, 23) with slight modifications. Briefly, monocytes were enriched from peripheral blood mononuclear cells by adhesion (24). Approximately $1 \times 10^5$ monocytes were cultured in complete culture medium on a glass coverslip in 24-well tissue culture plates (VWR International AG, Dietikon, Switzerland) in the presence of 20 ng/ml GM-CSF for 7 days. At day 3, cells received fresh GM-CSF-containing medium. $1 \times 10^5$ neutrophils cultured in the presence and absence of death factors for the indicated times were washed and added to macrophages at 37 °C for 30 min. After coincubation, cells were washed with cold 0.9% NaCl, fixed with 1% acetone-formalin, and stained for MPO activity with dimethoxybenzidine in the presence of hydrogen peroxide. Cells were lightly counterstained with Harris’ hematoxylin. The numbers of macrophages containing one or more neutrophils were counted by two independent investigators in at least five fields (minimum of 500 macrophages were evaluated) and photographed under a Zeiss Axiovert microscope at a magnification of ×630.

MIF Bioassays—The functional activity of MIF present in the supernatants of apoptotic neutrophils was tested by a monocyte migration assay using 48-well chemotaxis chambers with 5-µm pore diameter poly(vinylpyrrolidone)-free polycarbonate membranes (3). Briefly, wells in the bottom plate were filled with 28 µl of RPMI 1640 medium in the presence or absence of neutrophil supernatants. As a positive control, recombinant MIF-MBP fusion protein (10 µg/ml) was used. For MIF inhibition, goat anti-human MIF Ab (20 µg/ml) was preincubated with supernatants for 20 min before adding to the well. Peripheral blood mononuclear cells (50 µl of $1.8 \times 10^6$ cells/ml) were placed in the top wells and incubated for 3 h in humidified air with 5% CO₂ at 37 °C. Membranes were removed, air-dried, and stained with Diff-Quik. Migration of monocytes was evaluated microscopically by counting 10 randomly chosen ×400 fields.

**FIGURE 1.** Neutrophils express MIF as assessed by confocal microscopy. A, MIF but not GM-CSF was readily detected in blood neutrophils (upper panel). Preincubation of the anti-MIF Ab with MIF (middle panel) but not GM-CSF (lower panel) resulted in a complete loss of the fluorescence signal in CD15-positive blood neutrophils. Note that CD15 negative cells (most likely eosinophils) remained positive despite MIF preincubation, suggesting that this signal represents natural autofluorescence or nonspecific binding. Bars, 10 µm. The insets represent single cells using a larger magnification. Results are representative of at least three independent experiments. B, tissue neutrophils from patients with appendicitis (upper panel) and ulcerative colitis (lower panel) demonstrated evidence for MIF expression in vivo. Neutrophils were specifically detected using an anti-CD15 mAb. Some MIF-positive neutrophils are indicated in all panels (white arrows). Bars, 10 µm. The results are representative of at least three independent experiments.
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To demonstrate that neutrophil-derived MIF could act as a pro-inflammatory cytokine, we stimulated monocyte-derived macrophages (22, 23) with supernatants of apoptotic neutrophils for 6 h and subsequently harvested the newly generated culture supernatants. To understand the contribution of MIF, we used goat anti-human MIF Ab (20 μg/ml) for MIF neutralization. The pro-inflammatory cytokines TNF-α and IL-8 were analyzed in macrophage supernatants by an immunoassay.

Statistical Analysis—Results are expressed as means ± S.D. for the indicated number of independent experiments. Student’s t test was used to identify statistical significant differences. The calculated p values (*, p < 0.05; **, p < 0.01) are indicated in the figures.

RESULTS

MIF Is Expressed in Blood and Tissue Neutrophils—In initial experiments, we studied MIF protein expression by confocal microscopy. Blood neutrophils were stained with anti-MIF Ab or control Abs. Pre-incubation of the anti-MIF Ab with MIF but not GM-CSF resulted in loss of the fluorescence signal, demonstrating specificity of the anti-MIF staining. CD15 was used as a neutrophil lineage-associated marker. MIF was expressed in the cytosol of neutrophils (Fig. 1A). We also quantified MIF expression by ELISA. The total MIF content observed when neutrophils were cultured for 4 or 8 h (data not shown). To demonstrate MIF expression in neutrophils under in vitro conditions, we analyzed neutrophils in tissue sections of patients suffering from ulcerative colitis or appendicitis. Neutrophils were identified using an anti-CD15 mAb. In both inflammatory diseases, we obtained evidence for MIF expression in most of the infiltrating neutrophils (Fig. 1B). Taken together, MIF is expressed in neutrophils under ex vivo and in vivo conditions.

Apoptotic Neutrophils Release MIF in Vitro—Normal mature blood neutrophils were stimulated with several agonists known to activate surface receptors on neutrophils. Short term (6 h) treatment of the cells with TNF-α or agonistic anti-Fas mAb resulted in a dramatic reduction of the intracellular MIF levels as assessed by immunoblotting (Fig. 2A). In contrast, GM-CSF, C5a, IL-8, and LPS did not appear to have significant effects on cellular MIF levels. Similar results were observed when neutrophils were cultured for 4 or 8 h (data not shown). Note that TNF-α reduced MIF levels in ~40% of the

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blood donors only, whereas MIF reduction following anti-Fas stimulation was a consistent finding. The analysis of TNF-R1 and TNF-R2 surface expression revealed that the TNF-α-mediated lowering of cellular MIF levels was associated with higher expression of both receptors compared with the “non-responders” (data not shown). Therefore, all subsequent experiments presented in this report were performed on TNF-α-responding neutrophil populations. Reduced intracellular MIF levels in neutrophils upon TNF-α stimulation were also shown by confocal microscopy (Fig. 2B).

To investigate whether the reduced intracellular MIF levels of neutrophils following death receptor stimulation were indeed the consequence of MIF release, we analyzed the supernatants in this in vitro system. As assessed by ELISA, we detected small amounts of MIF in supernatants of untreated 9-h neutrophil cultures, but little MIF was released for the first 6 h. Markedly elevated MIF levels, however, were seen in supernatants of TNF-α-treated neutrophils already after a 3-h stimulation (Fig. 2C). The MIF levels further increased at later time points. We also performed dose-dependent experiments and observed that concentrations of less than 1 ng/ml TNF-α had no effect on MIF release by neutrophils (data not shown). In anti-Fas mAb-treated neutrophils, we observed evidence for significant MIF release within 6 h of stimulation (Fig. 2C).

Because TNF and Fas receptors are known to transduce death signals in sensitive cells, we analyzed redistribution of phosphatidylserine (PS), a characteristic feature of apoptotic neutrophils (16–19). In 6-h neutrophil cultures, 99% of untreated cells were non-apoptotic, suggesting that spontaneous apoptosis was not initiated after this short culture period. In contrast, both anti-Fas mAb and TNF-α induced redistribution of PS in the absence of propidium iodide uptake in a subpopulation of neutrophils, demonstrating apoptosis induction (Fig. 2D).

Although these data implied that apoptotic neutrophils release MIF, there was still the possibility that TNF-α or anti-Fas mAb stimulate MIF release and apoptosis in different cells. To investigate MIF release and apoptosis at the single-cell level, we stained TNF-α-stimulated neutrophils with anti-cleaved caspase-3 (indicative for apoptosis) and anti-MIF Abs. Confocal microscopic analysis revealed that the large majority of neutrophils were either MIF-positive and -negative for cleaved caspase-3 (viable cells) or MIF-negative but caspase-3-positive (apoptotic cells), demonstrating that MIF is exclusively released from apoptotic neutrophils (Fig. 2E).

MIF Released by Apoptotic Neutrophils Exhibits Functional Activity—The functional activity of MIF present in supernatants of TNF-α and anti-Fas mAb-stimulated neutrophils was tested by a bioassay. MIF-containing supernatants blocked the migration of monocytes, which appeared to correlate with the amounts of MIF present in the supernatants. The inhibitory activity on monocyte migration was completely blocked by neutralizing anti-MIF Ab (Fig. 3), demonstrating that the supernatants of apoptotic neutrophils contain biologically active MIF. As a positive control, we used a functional active MIF-MBP fusion protein in this system (3). Negative controls were supernatants from non-apoptotic neutrophils cultured in medium that had no effect (data not shown).

MIF Is Not Located in Azurophilic Granules and Its Release Is Both Caspase- and ABC Transporter-dependent—Subcellular localization analysis of MIF expression using confocal microscopy and subsequent imaging analysis revealed that MIF is unlikely to be associated with azurophilic granules of neutrophils (Fig. 4A, upper panel). In these experiments, we used two anti-cathepsin mAbs and an anti-MPO Ab to localize azurophilic granules. Interestingly, although apoptosis induction was again associated with reduced intracellular MIF levels, it did not affect cathepsin B (Fig. 4A, lower panels) or cathepsin D (data not shown) levels, suggesting that apoptotic neutrophils release MIF in the absence of azurophilic granule release and/or secondary necrosis.

We also analyzed elastase, which is, like MPO, cathepsin B, and D, present in azurophilic granules of neutrophils, in supernatants of neutrophil cultures by ELISA. Both GM-CSF and LPS stimulation of neutrophils resulted in elastase release, indicating that the azurophilic granules were mobilized under these conditions. In contrast, TNF-α was unable to release elastase, further demonstrating that the release of MIF was somehow regulated and not the consequence of necrotic cell death (Fig. 4B).

Caspases play a critical role in neutrophil apoptosis pathways (15). Therefore, we investigated whether the release of MIF is caspase-dependent. The pan-caspase inhibitor Z-VAD completely blocked both TNF-α and anti-Fas mAb-MIF releases in a concentration-dependent manner (Fig. 5A), further supporting that MIF is released from apoptotic neutrophils. MIF release and apoptosis (data not shown)
mediated by TNF-α were also completely blocked by an anti-TNF-R1 mAb (Fig. 5A), suggesting that TNF-R1 mediates these effects in neutrophils.

In addition to Z-VAD, we also used specific caspase-3 (Z-DEVD) and caspase-9 (Z-LEHD) inhibitors, which both completely blocked MIF release from TNF-α-stimulated neutrophils (Fig. 5B). Because MIF release was previously reported to involve the ABC transporter subfamily 1 (ABCA1) (25, 26),

FIGURE 4. MIF released from neutrophils upon TNF-α stimulation is not the consequence of mobilization of azurophilic granules. A, upper panel, MIF, cathepsin B, and MPO localization analysis. Overlay of all scanned focal planes in the z direction was visualized using volume rendering analysis, and the colocalized areas were superimposed using isosurface rendering (Imaris software). The colocalization analysis between MPO and cathepsin B was used as a positive control. Right, the numerical analysis was performed on 10 cells in each group, and the correlation coefficient was calculated. Same results were obtained using anti-cathepsin D mAb. Lower panel, intracellular MIF but not cathepsin B levels decreased upon TNF-α stimulation (6-h cultures). The results are representative of three independent experiments. Bars, 10 μm. B, ELISA. The effects of GM-CSF, TNF-α, and LPS on the release of MIF and elastase from neutrophils are shown. Neutrophils were stimulated for 6 h. n = 5; *, p < 0.05; **, p < 0.01.

FIGURE 5. MIF released from neutrophils upon death receptor activation is caspase- and ABC-type transporter-dependent. A, ELISA. TNF-α and anti-Fas mAb-mediated MIF releases (6-h cultures) were blocked by the pan-caspase inhibitor Z-VAD in a concentration-dependent manner. n = 3; **, p < 0.01. B, ELISA. Optimal concentrations of caspase-3 (Z-DEVD) and caspase-9 (Z-LEHD) inhibitors completely blocked TNF-α-mediated MIF release (6-h cultures). Moreover, an anti-TNF-R1 mAb blocked TNF-α-induced MIF release. n = 3; **, p < 0.01.
we also applied the two pharmacological inhibitors glyburide and probenecid that both blocked the MIF release in our system (Fig. 5B). These data point to the possibility that the process of apoptosis and perhaps even caspases themselves, either directly or indirectly, activate ABCA1.

**A Possible Pro-inflammatory Role of MIF Released from Apoptotic Neutrophils**—We next explored the possibility that MIF is released from apoptotic neutrophils before their uptake by monocyte-derived macrophages. Similar assays have previously been used to study mechanisms of phagocytosis of apoptotic neutrophils (22, 23). Interestingly, although TNF-α stimulation of neutrophils resulted in detectable release of MIF after 3 h (Fig. 2C), no phagocytosis of neutrophils was seen at this early time point (Fig. 6A). Neutrophils needed to be cultured in the presence of anti-Fas mAb or TNF-α for at least 6 h in order to see any phagocytic uptake. Moreover, if neutrophil culture periods were increased, additional macrophage phagocytosis was triggered. Based on these data, we cannot exclude the possibility that apoptotic neutrophils release MIF even prior to phagocytosis by macrophages, at least under certain in vitro conditions.

To demonstrate that the MIF released from apoptotic neutrophils might be able to initiate a pro-inflammatory response, we stimulated monocyte-derived macrophages with neutrophil supernatants containing significant amounts of MIF. MIF-containing supernatants induced the releases of both TNF-α and IL-8 from macrophages that were partially blocked by neutralizing anti-MIF Ab (Fig. 6B), pointing to a potential pro-inflammatory role of neutrophil-derived MIF. Because MIF was previously described as a neutrophil survival factor (3), we also cultured TNF-α-stimulated neutrophils in the presence of anti-MIF Ab. Neutralization of MIF resulted in accelerated cell death, suggesting that MIF also exerted an anti-apoptotic role on at least a sub-population of neutrophils in this system (data not shown). These data further confirm that the supernatants of apoptotic neutrophils contain biologically active MIF.

**DISCUSSION**

We demonstrate in this report that mature neutrophils, which are present in high numbers at inflammatory sites, release biologically active MIF upon apoptosis induction. Previously published work demonstrated the release of the anti-inflammatory cytokines IL-10 (27) and TGF-β (28) by apoptotic T cells. On the other hand, apoptotic cells were shown to induce IL-10 in monocytes following uptake of apoptotic cells (29). It was hypothesized that both mechanisms contribute to the generation of an immunosuppressive milieu, which may be required to prevent an inflammatory response during apoptosis. Furthermore, it was speculated that the immunosuppressive effects mediated by apoptotic cells have pathological relevance in viral infections and cancer (28, 29).

MIF, however, is a pro-inflammatory cytokine (8–14), and we provide evidence that it is released from neutrophils in a caspase-dependent manner during early stages of apoptosis. Although MIF was also released from neutrophils undergoing spontaneous apoptosis, we concentrated in this study on death receptor-mediated apoptosis because we wanted to work in a system that provided significant numbers of apoptotic neutro-
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phils in the absence of secondary necrosis. This way we were able to convincingly show that neutrophils release MIF in the absence of any detectable plasma membrane damage. Moreover, when neutrophils were stimulated with TNF-α, we were able to obtain evidence for MIF release by apoptotic neutrophils prior to their phagocytosis by monocyte-derived macrophages. Although our results were generated in vitro and are limited by the sensitivity of the assays, they imply that apoptotic cells might be able to contribute to inflammatory responses, at least under certain pathological conditions. For instance, MIF release by apoptotic neutrophils may occur if the time required for their uptake is too long, in particular under conditions of massive neutrophil apoptosis and/or phagocyte defects. Further work is required to determine under which conditions apoptotic neutrophils release MIF in vivo.

There are contrasting reports regarding the effect of TNF-α on neutrophil apoptosis. Both anti-apoptotic and pro-apoptotic roles have been suggested (30). Moreover, two groups recently described TNF-α-mediated caspase-independent death pathways (31, 32). However, the observations in these two studies were most likely due to the usage of unusually high concentrations of caspase inhibitors (≥100 mM Z-VAD) and were also compound-specific (33). The results of our study confirm the view that TNF-α-induced neutrophil death is apoptosis. Moreover, our observation that neutrophil populations vary among different donors regarding TNF responsiveness may also explain, at least in part, the different results previously reported regarding TNF-α-mediated effects on neutrophil apoptosis.

Another pro-inflammatory cytokine released by apoptotic mouse neutrophils was shown to be IL-1β (34). Similarly to MIF, IL-1β release was blocked by pharmacological caspase inhibition but was independent of caspase-1. Therefore, the authors of this previous report concluded that other caspases may also be able to process IL-1β (34). Here we provide evidence that MIF release is also caspase-dependent. Another similarity between IL-1β and MIF is that their secretion does not involve the classical endoplasmic reticulum/Golgi pathway. Instead both pro-inflammatory cytokines use elements of the ABC transporter system for their secretion (25, 26). In this report, we provide pharmacological evidence that MIF release from apoptotic neutrophils is also utilized by an ABC transporter.

A recent report suggested that C5a induces MIF secretion from non-apoptotic mouse neutrophils (4). In contrast, we observed no MIF release from human neutrophils upon C5a stimulation. Besides differences in the experimental conditions, it is possible that the regulation of ABC transporters differs among species and may or may not involve additional specific signaling pathways. Clearly, further studies are required to understand the exact molecular details responsible for MIF release in neutrophils. For instance, although our data point to the possibility that certain biochemical events during neutrophil apoptosis are somehow able to activate ABCA1, it remains to be investigated whether caspases are directly involved in this process.

In summary, we report that apoptotic neutrophils release MIF. Because neutrophils represent the most common leuka-
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