FEBS Letters 551 (2003) 78–86 FEBS 27588

Regulated secretion of macrophage migration inhibitory factor is mediated by a non-classical pathway involving an ABC transporter

Oliver Flieger[a], André Engling[b], Richard Bucala[c], Hongqi Lue[d], Walter Nickel[b], Jürgen Bernhagen[a,d,*]

[a] *Institute for Interfacial Engineering, University of Stuttgart and Fraunhofer Institute of Biotechnology, Nobelstrasse 12, 70569 Stuttgart, Germany*
[b] *Biochemie-Zentrum Heidelberg, Im Neuenheimer Feld 328, 69120 Heidelberg, Germany*
[c] *Department of Medicine and Pathology, Yale University School of Medicine, New Haven, CT 06520-8031, USA*
[d] *Unit of Biochemistry and Molecular Cell Biology, Institute of Biochemistry, University Hospital RWTH Aachen, Pauwelsstrasse 30, 52074 Aachen, Germany*

Received 14 May 2003; revised 4 August 2003; accepted 4 August 2003

First published online 20 August 2003

Edited by Ulrike Kutay

Abstract The cytokine macrophage migration inhibitory factor (MIF) is inducibly secreted by immune cells and certain other cell types to critically participate in the regulation of the host immune response. However, MIF does not contain a N-terminal signal sequence and the mechanism of MIF secretion is unknown. Here we show in a model of endotoxin-stimulated THP-1 monocytes that MIF does not enter the endoplasmatic reticulum and that MIF secretion is not inhibited by monensin or brefeldin A, demonstrating that MIF secretion occurs via a non-classical export route. Glyburide and probenicide but not other typical inhibitors of non-classical protein export strongly block MIF secretion, indicating that the export pathway of MIF involves an ABCA1 transporter.

Key words: MIF; Cytokine; Nonclassical secretion; Leaderless secretory protein; ABC transporter

1. Introduction

Macrophage migration inhibitory factor (MIF) is a pleiotropic multifunctional cytokine with a mostly proinflammatory spectrum of action in the host immune response. As such, MIF is a critical mediator of a number of immune and inflammatory conditions (reviewed in [1–3]). A causal and pivotal role for MIF has been best characterized in the pathogenesis of bacterial septic shock [4], rheumatoid arthritis [5] and tumorigenesis [2]. In these diseases, MIF is released from the involved inflammatory cells and numerous studies applying neutralizing anti-MIF antibodies have shown that released circulating MIF is critical for MIF's immunologic and inflammatory effects [6–11].

MIF is exceptional among cytokines, as it has several functions outside the immune system [3]. MIF can act as a glucocorticoid antagonist and endocrine factor [9,11], it has catalytic properties [12,13] and regulates cellular activity through the coactivator JAB1/CSN5 [14] and the cell surface protein CD74/Ii chain [15]. Thus, besides possibly having intracellular functions, the former findings in conjunction with the data on antibody neutralization of circulating MIF have clearly demonstrated that MIF is effectively secreted upon stimulation. Specific secretion of MIF can be induced by a variety of stimuli, including inflammatory stimuli such as endotoxin (LPS, lipopolysaccharide) and tumor necrosis factor [16], as well as hormones such as corticosteroids [11], adrenocorticotrophic hormone (ACTH) [17], and angiotensin II [18]. MIF secretion is not limited to immune cells but can also be induced in endocrine cells and certain epithelial cells [3]. Unlike for most cytokines, for which expression is tightly regulated by stimulation, substantial levels of preformed MIF are found in several cell types, indicating that secretion of MIF could be due to an enhancement of MIF expression and de novo protein synthesis but could also be caused by an induction of a release from pre-existing MIF stores. MIF release from both origins was shown under conditions of LPS stimulation to occur in corticotrophic pituitary cells [9,17] and in cultured myotubes [19].

As the MIF gene does not encode for an N-terminal signal sequence for the translocation into the endoplasmic reticulum (ER) and because cell staining analysis has indicated that in addition to the cytosol, where MIF is predominantly localized, MIF could only be detected in small vesicles [17,20], the nucleus [21,22], and pinched-off vesicles outside of cells [20], it has been suggested that MIF secretion may occur by a non-classical secretion pathway. However, no biochemical study has yet been undertaken to systematically investigate this suggestion. In particular, the mechanism of MIF secretion following inflammatory or immunologic stimulation has not yet been studied.

Soluble secretory proteins such as hormones and cytokines are usually secreted by the so-called classical or ER/Golgi-dependent secretion pathway [23]. However, a study by Rubartelli and coworkers first demonstrated for a mammalian

*Corresponding author. Fax: +49-241-8082427.
E-mail address: jbernhagen@ukaachen.de (J. Bernhagen).

Abbreviations: ABC transporter, ATP binding cassette transporter; BFA, brefeldin A; BSP, bromosulfalein; CHX, cycloheximide; CCCH, carbonyl cyanide m-chlorophenyl hydrazone; DIDS, 4,4′-diisothiocyanato-stilbene-2.2′-disulfonic acid; ER, endoplasmatic reticulum; IVT, in vitro transcription/translation; FCS, fetal calf serum; LDH, lactate dehydrogenase; LLS, leaderless secretory protein; LPS, lipopolysaccharide; MA, methylamine; MIF, macrophage migration inhibitory factor; Phen, 1,10-phenanthroline; Trx, thioredoxin; Vera, verapamil

0014-5793/03/$22.00 © 2003 Published by Elsevier B.V. on behalf of the Federation of European Biochemical Societies.
doi:10.1016/S0014-5793(03)00900-1

secretory protein that the cytokine interleukin-1β (IL-1β) is not secreted by a classical secretion pathway [24]. Since then, it has become more apparent that a number of soluble proteins are secreted in an ER/Golgi-independent manner. Secretion of these proteins does not seem to occur by a single pathway; rather, diverse distinct mechanisms appear to underly the release of these proteins. Because no unifying transport concept has been identified, these processes have been collectively termed non-classical protein secretion or protein export to distinguish them from the classical ER/Golgi-mediated pathway. To date, more than 20 proteins have been identified that are secreted by non-classical export. While some of them are typical extracellular mediators such as IL-1α, IL-1β, or the fibroblast growth factors FGF-1 and FGF-2, others have previously been viewed as typical intracellular proteins. The enzyme thioredoxin (Trx), gene-regulating proteins such as the transcription factor Engrailed homeoprotein isoform 2, or the chromatin-binding protein high-mobility group binding protein-1 belong to this latter class of non-classically secreted proteins. Both stimulated and apparently uninduced secretion was observed to result in the release of these factors. While non-classically secreted proteins belong to diverse structural classes of proteins and while the secretion pathways taken and the inducing stimuli are diverse, non-classically secreted proteins generally do not have a N-terminal signal sequence. They are therefore also referred to as leaderless secretory (LLS) or signal peptide-less proteins. Evidence on the secretion of LLS proteins and on the potential export routes involved was recently summarized in a comprehensive review [25].

Due to its pivotal role as a mediator of Gram-negative septic shock [9–11], MIF production has been best studied in cellular models of LPS stimulation. Although MIF was initially discovered as a T-cell factor acting on monocytes/macrophages [26], it was later realized that the monocyte/macrophage is the most important source of MIF in vivo [16]. MIF is potently secreted from monocytes/macrophages following LPS stimulation and, once released, participates in the host inflammatory response to the bacterial toxin [9,10,16]. We therefore elected a cellular model of LPS-stimulated monocytes to investigate the secretion mechanism of MIF in detail and to experimentally address the possibility that MIF could be secreted by a non-classical route. While previous studies of MIF induction from LPS-stimulated monocytes had mostly been performed in mouse cells, we have established an experimental system that allows to quantitate LPS-induced MIF secretion from human THP-1 monocytes. We demonstrate that LPS-regulated secretion of MIF is fully functional in the absence of an intact ER/Golgi system. Moreover, we provide evidence that ABCA1 transporters are involved in the overall process of LPS-induced secretion of MIF.

2. Materials and methods

2.1. Cell lines, reagents and antibodies

Human monocytic THP-1 cells were maintained in complete RPMI 1640 medium containing 10% fetal calf serum (FCS), 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. RAW 264.7 mouse macrophages were maintained in complete DMEM medium containing 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. All cell culture media and reagents were from Invitrogen/Life Technologies (Karlsruhe, Germany). Recombinant human MIF (rMIF) and rabbit anti-mouse MIF polyclonal antibody were prepared as described previously [14]. Peroxidase-conjugated anti-rabbit IgG was used as secondary antibody and was from Santa Cruz Biotechnology (Heidelberg, Germany). Alkaline phosphatase-conjugated goat anti-rabbit IgG was from Pierce/KMF (St. Augustin, Germany). Normal goat serum was from Invitrogen. PNGase F was from New England Biolabs (Heidelberg, Germany). All inhibitors used were from Fluka-Sigma-Aldrich Chemie GmbH (Taufkirchen, Germany).

2.2. MIF secretion assay

THP-1 cells were synchronized for 3 h in RPMI 1640 medium containing 1% FCS and 2 mM L-glutamine. Cells were washed once and plated in the same medium at 1×10^6 cells per ml in 12-well plates. For routine assays, inhibitors were added at the indicated concentrations 45 min prior to stimulation with 10 μg/ml LPS. Inhibitors were dissolved as suggested by the supplier. Solvents applied were mostly organic solvents such as dimethylsulfoxide (DMSO) and ethanol and therefore, control incubations with solvent alone were included in all assays to exclude potential toxic effects. All solvents used were also probed directly for potential cell toxicity effects by performing lactate dehydrogenase (LDH) assays on cell incubations treated with solvent alone. These analyses showed that the solvents did not cause any significant toxic effects (see Table 1). Unless stated otherwise, cells were collected after 4 h by centrifugation for 5 min at $400\times g$ and cell-free supernatants stored at −20°C until analysis by enzyme-linked immunosorbent assay (ELISA).

2.3. LDH activity assay

LDH activity was measured according to the recommendations of the supplier (Roche Diagnostics, Mannheim, Germany). Briefly, activity was assayed by mixing 20 μl cell-free supernatants and 980 μl assay buffer (7.6 mM sodium pyruvate, 0.2 mM NADH in 100 mM potassium phosphate buffer, pH 7.0). Absorbance was read at 340 nm for 5 min and activity expressed in mU/ml.

2.4. MIF and TNF ELISAs

Cleared lysates were prepared by lysing the cells in 25 mM Tris–phosphate buffer, pH 7.8, 2 mM dithiothreitol, 2 mM bis-1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid (CDTA), 10% glycerol, and 1% Triton X-100 for 20 min on ice followed by centrifugation for 10 min at 13 000 rpm and 4°C. Cell-free supernatants were assayed directly, while cell lysates were diluted at an appropriate ratio in Tris-buffered saline (TBS) (20 mM Tris–HCl, pH 7.3, 150 mM NaCl) containing 0.1% bovine serum albumin (BSA) and 0.05% Tween 20. Human MIF was assayed by a sandwich ELISA using the MAB289 capture and BAF289 detection antibody from R&D Systems (Wiesbaden, Germany) according to the manufacturer's instructions. Briefly, Immulon II microtiter plates (Dynex Technologies, Denkendorf, Germany) were coated with the capture antibody (2 μg/ml in phosphate-buffered saline, PBS) overnight, washed and blocked with blocking buffer containing 1% BSA and 5% sucrose in PBS for 1 h. 100 μl aliquots of the cell supernatants or diluted lysates were added to each well and incubated for 2 h. The wells were washed and incubated with the biotinylated BAF289 antibody (0.2 μg/ml in TBS, containing 0.1% BSA and 0.05% Tween20) for 2 h. This was followed by the addition of peroxidase-conjugated streptavidin (Roche Molecular Biochemicals, Mannheim, Germany) for 20 min. Following addition of TMB substrate solution (Pierce/KMF), immune complexes were quantified in a standard microtiter plate reader. MIF concentrations were calculated by extrapolation from a standard curve (range 0–100 ng/ml; sensitivity 19 pg/ml) using bacterially expressed rMIF [27] as a standard.

TNF was quantified by R&D System's capture antibody MAB610 and detection antibody BAF210 using essentially the same protocol as above except that the capture antibody was used at 4 μg/ml. TNF concentrations were calculated by extrapolation from a standard curve (range 0–1000 pg/ml, sensitivity 5 pg/ml) using recombinant TNF (R&D Systems).

2.5. N-glycosylation analysis

THP-1 cells were stimulated with 10 μg/ml LPS for 4 h and RAW 264.7 cells were stimulated with 100 ng/ml LPS for 12 h. Cells were

then lysed in RIPA buffer (50 mM Tris–HCl, 150 mM NaCl, 1% Igepal CA-630, 0.5% deoxycholate, 0.1% SDS, 2 mM EDTA, pH 7.5) and cleared by centrifugation at 13 000×*g*. 10 μg cell lysates, 100 μg cell-free supernatants, 10 ng rMIF and 5 ng anti-MIF IgG were denatured in buffer containing 0.5% SDS and 1% β-mercaptoethanol for 10 min at 100°C. Samples were then incubated in 50 mM sodium phosphate buffer, pH 7.5, containing 1% NP-40 in the presence or absence of 100 NEB units PNGase F (New England Biolabs) overnight at 37°C. The reaction was stopped by adding 2× Laemmli electrophoresis buffer and heating to 95°C for 3 min. The samples were separated by denaturing SDS–PAGE. For comparison, *N*-glycosylated MIF was produced in B-16 melanoma cells engineered to stably express a fusion protein containing the interleukin-2 (IL-2) N-terminal signal peptide linked to the mouse MIF gene (kindly provided by Dr. Glen Dranoff, Dana-Farber Cancer Institute and Harvard Medical School, Boston, MA, USA). IL-2-signal peptide–MIF fusion protein expressed and secreted in these cells was analyzed by PNGase F treatment and Western blotting against MIF using the protocols described above, except that digestion was performed with 1000 NEB units for 1 h.

2.6. SDS–PAGE and Western blot analysis

Samples were separated on 18% or 4–12% NuPAGE (for MIF analysis) and 10% (for IgG analysis) SDS–PAGE gels, transferred to nitrocellulose, and immunoblotting analysis performed essentially as described previously [14]. The blot was incubated at room temperature for 2 h with the appropriate primary antibody as specified in the figure legend, and diluted 1:1000 in blocking buffer. Incubation with the corresponding peroxidase-conjugated secondary antibody was performed at room temperature for 2 h at a dilution of 1:10 000 in blocking buffer. As ECL reagent, SuperSignal West Dura (Pierce) was used.

2.7. In vitro translation into microsomes

Human MIF cloned into pET11b as described previously [28] was used as the encoding plasmid and was linearized by digestion with *Eco*RI (New England Biolabs) and the cDNA was transcribed using the Ribomax Large Scale RNA Production System T7 (Promega, Mannheim, Germany). The human MIF transcript or the yeast α-factor glycosylation control mRNA was then translated for 90 min at 30°C using the Flexi Rabbit Reticulocyte Lysate System with or without canine pancreatic microsomal membranes in the presence of RNAsin ribonuclease inhibitor (all from Promega) and ProMix L-[^{35}S] in vitro labeling mix (Amersham Biosciences, Freiburg, Germany). The reaction was stopped by adding 2× Laemmli electrophoresis buffer and heating for 3 min at 95°C. The samples were separated on an 18% SDS–PAGE gel and analyzed as described above.

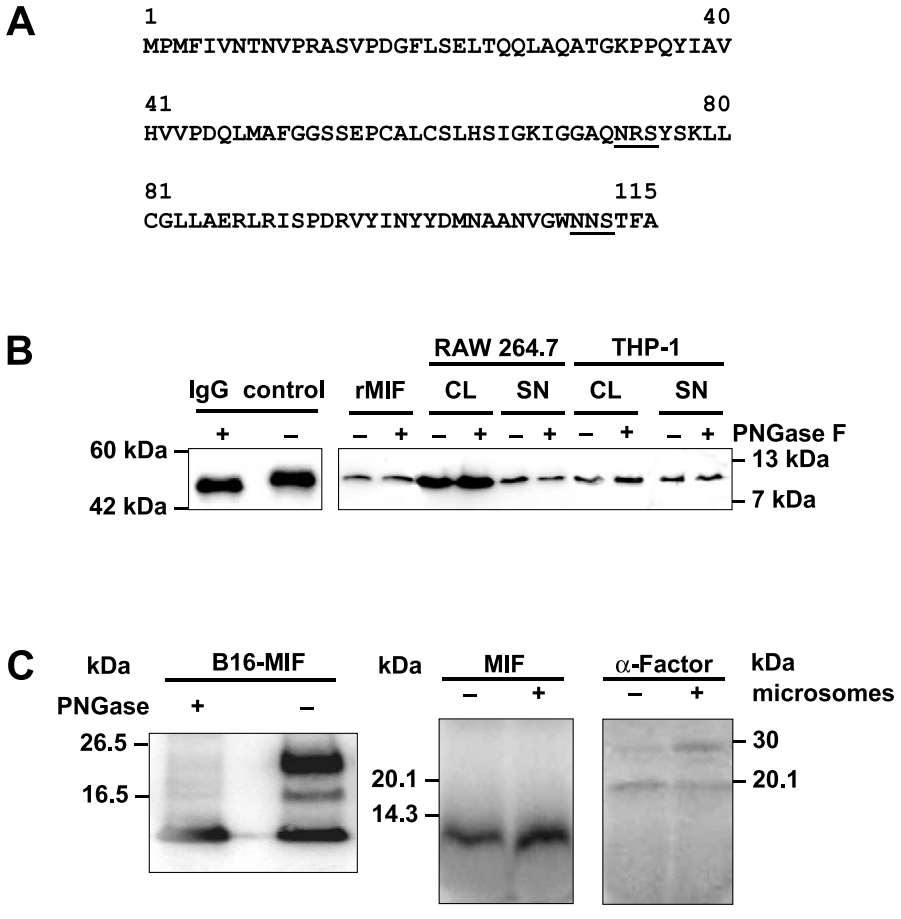

Fig. 1. MIF is not glycosylated and does not enter microsomes. A: Amino acid sequence of human MIF. MIF does not have a classical N-terminal or an internal hydrophobic signal sequence. The two potential *N*-glycosylation sites of MIF are underlined. The Met-1 residue is post-translationally processed in both bacteria and mammalian cells. B: *N*-glycosylation analysis of MIF expressed and secreted in LPS-stimulated monocytes/macrophages. Immunoblot analysis of IgG, rMIF, THP-1 and RAW 264.7 cell lysates (CL) and supernatants (SN) after incubation with (+) or without (−) PNGase F. Left panel, samples were separated on 10% SDS–PAGE and probed with anti-IgG antibody. Right panel, samples were separated on 16.5% Tricine–SDS–PAGE and probed with anti-MIF antibody. C: Autoradiograph of [^{35}S]-labeled, in vitro-translated human MIF (middle panel) or yeast α-factor protein (right panel) in the presence (+) or absence (−) of microsomal membranes. Secreted MIF expressed as an IL-2 signal peptide/MIF fusion construct in B-16 melanoma cells and concentrated from the cell supernatant (B16-MIF) migrates at 24 and 17 kDa (left panel), as evidenced by PNGase F digestion and Western blotting with anti-MIF antibody. The positions of relevant molecular weight markers are indicated.

3. Results

3.1. MIF does not enter the classical ER/Golgi pathway, but is effectively secreted from monocytes/macrophages following LPS stimulation

Numerous reports have shown that MIF is effectively and specifically secreted from several cell types following stimulation. This is somewhat surprising because MIF does not have an N-terminal signal peptide (Fig. 1A). MIF from bovine liver cell lysates does not become post-translationally *N*-glycosylated although two potential *N*-glycosylation sites are present in the MIF cDNA sequence [28] (Fig. 1A), but *N*-glycosylation of secreted MIF had not been investigated in immunologically stimulated monocytes/macrophages. We first wanted to analyze whether MIF expressed and secreted from LPS-stimulated monocytes/macrophages was also not *N*-glycosylated as seen for the liver MIF. Human monocytes (THP-1) and mouse macrophages (RAW 264.7) were stimulated with LPS and intracellular and secreted MIF was probed for *N*-glycosylation by PNGase F treatment and SDS–PAGE/Western blotting analysis (Fig. 1B). While PNGase F treatment of immunoglobulin G (IgG), a known *N*-glycosylated protein, resulted in a band shift, with the faster migrating band corresponding to the size of deglycosylated IgG, no PNGase F effect whatsoever was observed when the MIF-containing monocyte/macrophage cell lysates and supernatants were analyzed by electrophoresis and anti-MIF Western blot. This confirmed that MIF expressed and secreted in LPS-induced monocytes/macrophages was in fact not *N*-glycosylated. To rule out the possibility that potential inhibitory processes occurring in immune-stimulated monocytes/macrophages could interfere with the transport of MIF across the ER membrane, we next tested whether in vitro transcribed and translated (IVT) human MIF was able to enter microsomal vesicles and to become *N*-glycosylated therein. MIF also did not enter the microsomal vesicles, as evidenced by a lack of *N*-glycosylation in this in vitro system (e.g. its unchanged molecular size of ~12.5 kDa, Fig. 1C). By contrast, yeast α-factor, a protein known to be *N*-glycosylated in the ER/Golgi passage, and frequently used as a standard for *N*-glycosylation in IVT systems, entered the ER vesicles and was *N*-glycosylated, as evidenced by a band at 30 kDa that appeared in the presence of microsomes in addition to the 19 kDa band. *N*-glycosylation of MIF would have resulted in a bis-*N*-glycosylated protein species with an apparent size of 24 kDa protein. Also, an apparently mono-*N*-glycosylated form with a band size of 17 kDa can be observed. This was demonstrated by PNGase F digestion and Western blotting of cell supernatants from B-16 melanoma cells that had been constructed to stably express an IL-2-signal peptide–MIF fusion protein targeting MIF into the ER/Golgi pathway (Fig. 1C).

THP-1 monocytes were chosen as an LPS-inducible monocyte cell line to study MIF secretion by MIF-specific ELISA. MIF was potently and specifically released from THP-1 monocytes following stimulation with 10 μg/ml LPS (Fig. 2A). Specificity of MIF release was verified by MIF Western blot analysis (Fig. 2B). MIF secretion was fast, with the peak of MIF secretion occurring by 2–4 h post LPS induction. Significant LPS-stimulated release was seen up to 8 h. LPS-stimulated MIF secretion was not due to cell stress or cell death, as only a minor portion of cells stained positively for Trypan blue ($<5\%$) and because no significant LDH activity was detected in the cell supernatants (Table 1). Uninduced release of MIF occurred only to a low extent (Fig. 2A). LPS stimulation of MIF secretion was concentration-dependent (Fig. 2C). Measurable release of MIF was seen with 0.1 μg/ml of LPS; however, best results were obtained with 10 μg/ml of LPS, which is why subsequent analyses were performed using this LPS concentration.

3.2. Inhibitors of the classical ER/Golgi secretion pathway as well as a variety of other protein secretion inhibitors do not interfere with MIF secretion from LPS-stimulated THP-1 cells

To further probe whether any parts of the ER/Golgi pathway would act to mediate stimulated secretion of MIF, we next analyzed the effects of the inhibitory compounds brefeldin A (BFA) and monensin on the secretion of MIF from LPS-treated THP-1 cells. Both compounds are typical inhibitors of the ER/Golgi secretion pathway. BFA inhibits transport of secretory proteins between the ER and the cis-Golgi [29], whereas monensin predominantly blocks the protein transport at the trans-Golgi apparatus cisternae [30]. Fig. 3A shows that monensin only marginally inhibited LPS-stimulated MIF secretion, when added at 0.5 μg/ml, while no reduction was seen with 5 μg/ml monensin. By contrast, the secretion of the cytokine TNF, which is known to be secreted by the ER/Golgi pathway, was completely abolished in the

Table 1
LDH activity assays of the inhibitor studies and solvent control incubations

Solvent, positive control buffer, stimulus	LDH activity[a] in cell supernatant [mU/ml]	Classical pathway inhibitors/ other agents	LDH activity[a] in cell supernatant [mU/ml]
PBS	6.0	Monensin	32.2
0.5% EtOH	2.0	Brefeldin A	20.1
0.01% NaOH in EtOH	6.0	CCCH	50.2
0.1% DMSO	10.0	Cycloheximide	21.4
1×10^6 lysed cells	221.1	Methylamine	26.1
		A23187	24.1
		ABC transporter inhibitors	LDH activity[a] in cell supernatant [mU/ml]
		Verapamil	60.3
		Benzamidin	n.d.
		Phenantrolin	n.d.
		Glyburide	32.2
		Probenicide	n.d.

[a]LDH activity assay was performed as described in Section 2. Data are the means of four measurements.

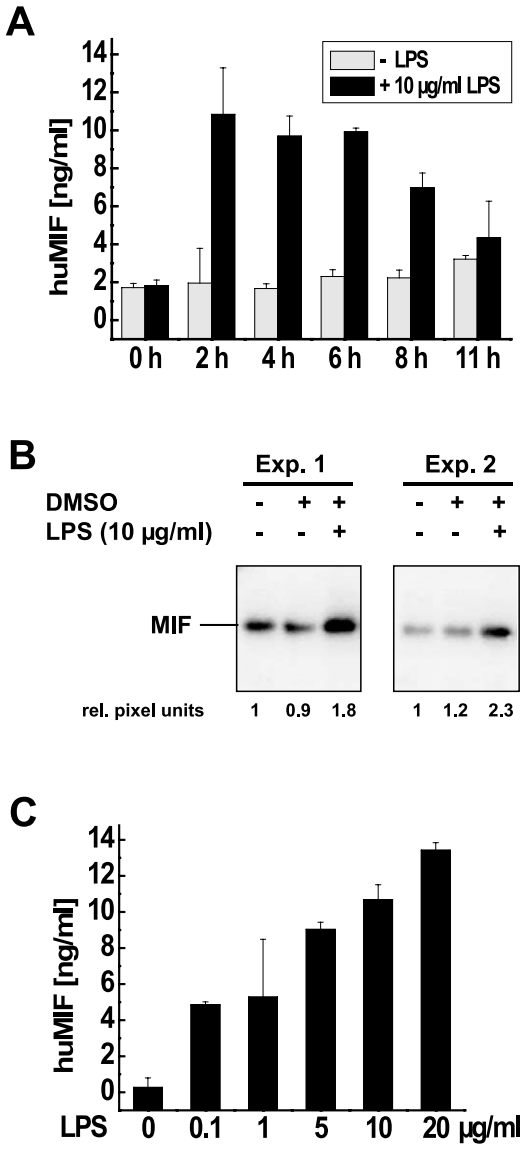

Fig. 2. Secretion of MIF from LPS-stimulated THP-1 monocytes. A: Time course of the secretion of MIF following incubation of cells with and without LPS. Cells were treated with LPS (10 µg/ml) (black bars) or PBS (gray bars), supernatants collected at the indicated time intervals and assayed for MIF concentration by ELISA. Results are expressed as mean values ± S.D. of triplicate measurements from one representative experiment ($n = 3$) of a total of three kinetics performed. B: Western blot verification of LPS-induced secretion of MIF from THP-1 cells. Cells were treated with LPS (10 µg/ml), PBS (buffer control), or DMSO (solvent control) for 4 h, supernatants collected at the indicated time intervals and assayed by NuPAGE electrophoresis and immunoblotting with MIF-specific antibody. Bands were subjected to semi-quantitative analysis using densitometry with NIH image and numbers shown indicate relative pixel intensities. C: LPS concentration dependence. Cells were treated with LPS at the indicated concentrations, supernatants collected after 4 h and assayed for MIF concentration by ELISA. Results are expressed as mean values ± S.D. of triplicate measurements from one dose response ($n = 3$) of a total of three experiments.

presence of monensin (Fig. 3B). Of note, while BFA led to a ~30% decrease in TNF secretion (Fig. 3B), this reagent not only did not inhibit the secretion of MIF, but even led to an apparent stimulation of MIF secretion up to 150% of the effect seen for LPS alone (Fig. 3A). Overinduction by BFA

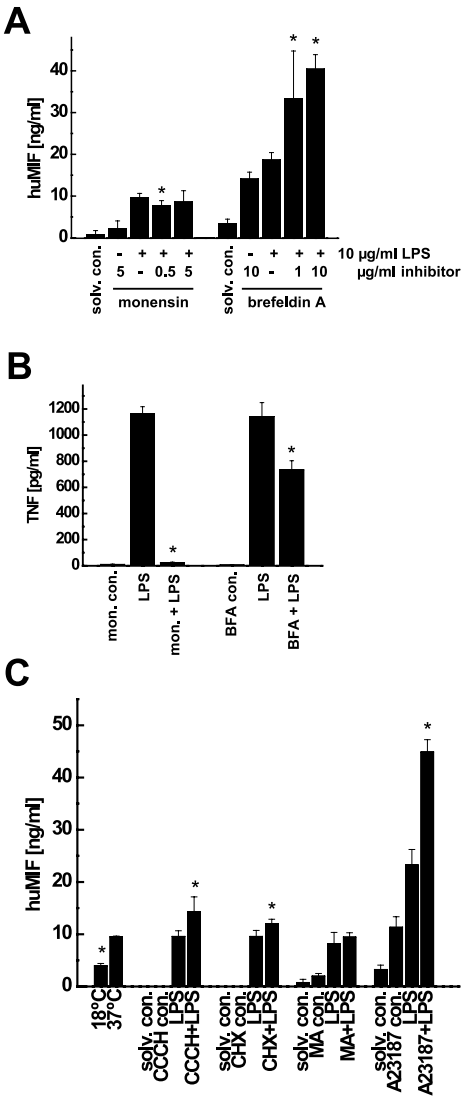

Fig. 3. MIF secretion is neither inhibited by the classical pathway inhibitors brefeldin A (BFA) and monensin nor by typical other protein secretion inhibitors. A,B: Effect of BFA and monensin (abbreviated: mon.) on the secretion of MIF from LPS-stimulated THP-1 cells. Human MIF and TNF ELISAs of THP-1 supernatants after stimulation for 4 h with 10 µg/ml LPS in the absence or presence of the indicated concentrations of BFA and monensin. The concentration of secreted MIF (panel A) or TNF (panel B) as measured by ELISA is shown. Results are expressed as mean values ± S.D. of triplicate measurements from three independent experiments ($n = 9$). Control incubations with the corresponding solvents alone (solv. conc.) or inhibitor alone (for example, 5 µg/ml monensin/no LPS) showed that the solvents did not have inadvertent effects. C: Effect of the endocytosis inhibitor methylamine (MA, 10 mM), the calcium ionophore A23187 (1 µg/ml), the protein synthesis inhibitor CHX (2 µg/ml), the energy uncoupling agent CCCH (10 µM), and an 18°C temperature block (control at 37°C) on MIF secretion from LPS-stimulated THP-1 cells. Results are expressed as mean values ± S.D. of triplicate measurements from two ($n = 6$; for temperature, CHX, and A23187) or three ($n = 9$; for CCCH and MA) independent experiments. Control incubations with the corresponding solvents alone (solv. conc.) or the inhibitor alone (for example, CCCH con.) showed that the solvents or inhibitors did not have inadvertent effects. Stastistical significance was always determined for the 'LPS+inhibitor' data sets in comparison to the corresponding 'LPS' group by unpaired Student's t-test. Significant differences ($P < 0.05$) are indicated by an asterisk on the 'LPS+inhibitor' bars. For the temperature block experiment, the 18°C data set is compared with that of the 37°C treatment.

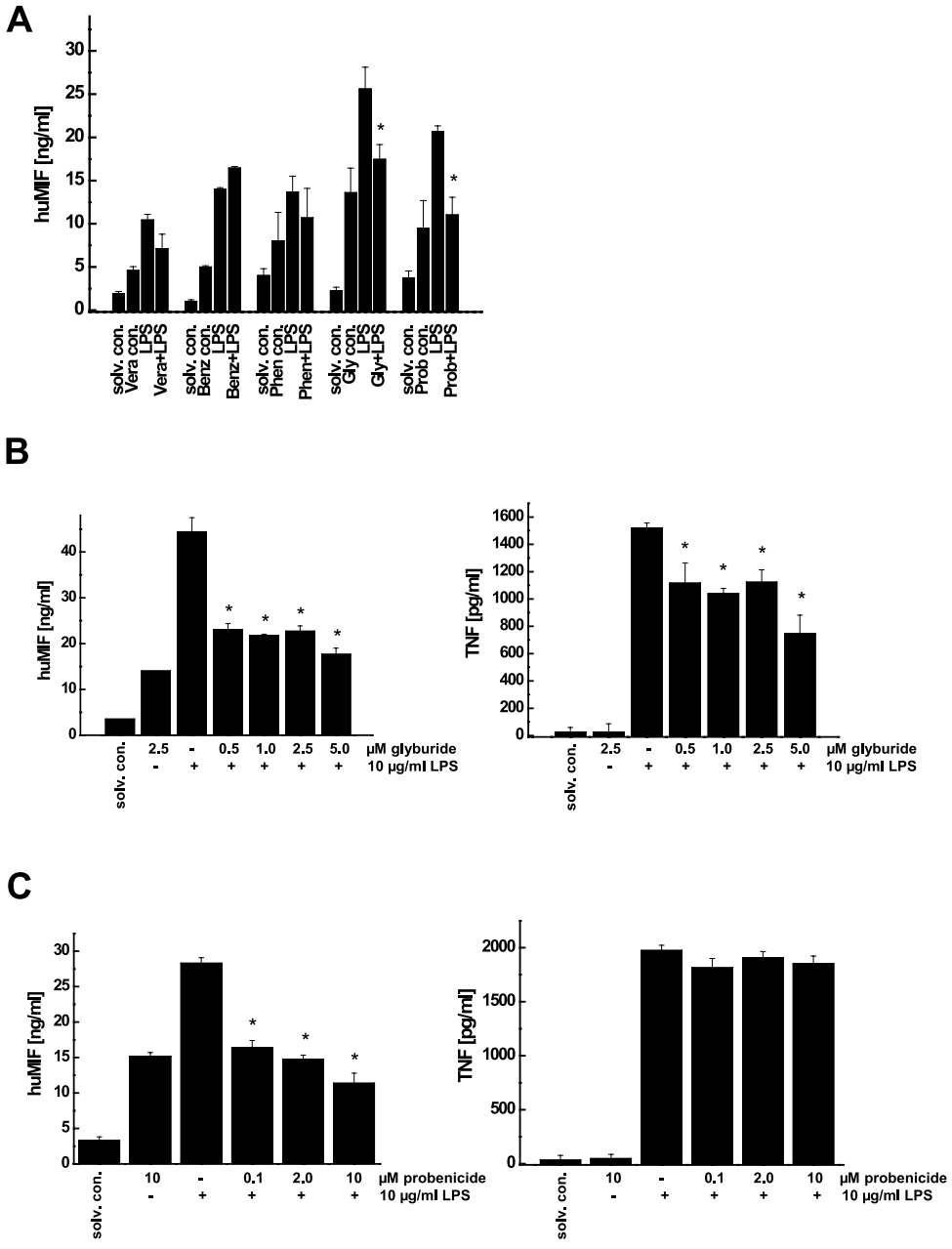

Fig. 4. Inhibitors of ABC transporters inhibit the secretion of MIF. A: Quantitative assessment of the effects of several ABC transporter inhibitors on the secretion of MIF from LPS-stimulated THP-1 cells. Human MIF ELISA of THP-1 supernatants stimulated with 10 μg/ml LPS in the absence or presence of ABC transporter activity inhibitors. BSP (4 μM) and DIDS (500 μM) exhibited non-specific effects on the release of MIF (bars not shown; see text). Vera was added at a concentration of 15 μM, benzamidine HCl (Benz) at 20 μM, Phen at 50 μM, glyburide (Gly) at 2.5 μM, and probenicide (Prob) at 2 μM. Results are expressed as mean values ± S.D. of triplicate measurements from three independent experiments ($n = 9$), except for Vera and benzamidine, with $n = 6$ measurements from two independent experiments. Control incubations with the corresponding solvents alone (solv. con.) or the inhibitor alone (for example, Benz con.) showed that the solvents or inhibitors did not have inadvertent effects. B: Effect of glyburide on the secretion of MIF as compared to its effect on TNF secretion. A concentration dependence as indicated is shown. Results are expressed as mean values ± S.D. of triplicate measurements from three independent experiments ($n = 9$). Control incubations with the corresponding solvents alone (solv. con.) or the inhibitor alone (for example, 2.5 μM glyburide/no LPS) showed that the solvents or inhibitors did not have inadvertent effects. C: Same as panel B, except that cells were treated with probenicide instead of glyburide. Stastistical significance was always determined for the 'LPS+inhibitor' data sets in comparison to the corresponding 'LPS' group by unpaired Student's t-test. Significant differences ($P < 0.05$) are indicated by an asterisk on the 'LPS+inhibitor' bars.

was not due to non-specific cell death, as BFA only led to background LDH activity levels in the cell supernatants (Table 1). Next, a number of other typical inhibitors for which an interference with cellular transport processes had been reported [24] were applied to begin to distinguish and potentially identify the export pathway taken by MIF. Fig. 3C shows that neither the protein synthesis inhibitor cycloheximide (CHX), the respiratory chain uncoupling reagent carbonyl cyanide *m*-chlorophenyl hydrazone (CCCH), the endocytosis inhibitor methylamine (MA), nor the calcium ionophore A23187 inhibited MIF secretion. Rather, stimulated LPS-induced secretion of MIF was even superinduced

by some of these agents. CHX and CCCH led to a slight but significant overstimulation as compared to the effect of LPS alone. The CHX effect, while small, could indicate that short-living proteins produced by de novo protein synthesis are not necessary for LPS-stimulated MIF secretion. Overinduction by CCCH was more pronounced but since CCCH incubations showed an increased LDH activity in the cell supernatants (Table 1), no further conclusions may be drawn from this finding. A23187 led to a marked (two-fold) increase of LPS-induced MIF secretion. A23187, among other effects, can induce calcium-dependent exocytosis, indicating that LPS-induced MIF secretion in monocytes could be a calcium-dependent process. Except for CCCH, none of these inhibitors led to a non-specific release of LDH (Table 1). Applying an 18°C temperature block led to a significant reduction of LPS-stimulated MIF secretion of approximately 50%. An 18°C temperature block is known to interfere with transport processes through the Golgi passage [31].

3.3. *Secretion of MIF from LPS-stimulated THP-1 monocytes is markedly blocked by the ABC transporter inhibitors glyburide and probenicide*

For IL-1β, evidence was obtained that multidrug resistance proteins (MDR) of the ATP binding cassette (ABC) transporter subfamily 1 (ABCA1) are involved in non-classical export of this cytokine [32]. As ABC transporters had also been implicated in the non-classical secretion of FGF2 [33], we next tested whether addition of inhibitors of ABC transporters interfered with the non-classical secretion of MIF. LPS-stimulated THP-1 cells were preincubated with various inhibitors reported previously to block ABC transporter- or P-glycoprotein-mediated pathways (Fig. 4A).

Except for verapamil (Vera), which led to a slight elevation in secreted LDH levels, the ABC transporter inhibitors were not toxic for the THP-1 cells (Table 1). Bromosulfalein (BSP) and 4,4′-diisothiocyanato-stilbene-2.2′-disulfonic acid (DIDS) led to an LPS-independent stimulation of MIF release from THP-1 cells, an observation which made it difficult to further evaluate the potential secretion-interfering effect of these compounds. The P-glycoprotein inhibitor Vera and 1,10-phenantrolin (Phen) slightly inhibited LPS-stimulated MIF secretion, but these effects were not significant. Benzamidine did not exhibit any inhibitory effect.

Most strikingly, these scouting experiments indicated that the putative ABCA1 inhibitors glyburide and probenicide markedly inhibited MIF secretion (Fig. 4A). We therefore analyzed the effect of these two compounds further. Glyburide inhibited LPS-induced secretion of MIF from THP-1 cells over a wide concentration range (Fig. 4B). Strongest inhibition was observed at a concentration of 5 μM. At lower concentrations of glyburide, the inhibitory effect was reduced, however, at 0.5 μM, glyburide inhibition was ~50%. In contrast, as previously noted [32], glyburide inhibited the classical secretion of TNF to a much lesser extent (Fig. 4B) and also did not lead to marked cell toxicity (Table 1). The glyburide findings were confirmed by the concentration curves with probenicide. Fig. 4C shows that probenicide strongly and concentration-dependently inhibited the secretion of MIF, while absolutely no effect of probenicide could be detected with regard to TNF secretion. Neither treatment with glyburide nor probenicide led to a reduction in the intracellular protein concentration and synthesis rate of MIF (data not shown), demonstrating that these inhibitors did not affect the de novo synthesis of MIF. This further suggested that glyburide and probenicide specifically interfere with the non-classical export of MIF.

4. Discussion

Non-classical export of a soluble mammalian secretory protein was first discovered 13 years ago [24]. Since then, several proteins, including cytokines, growth factors, components of the extracellular matrix, as well as certain enzymes and transcription factors that were previously thought to only exhibit intracellular functions were found to be effectively secreted by non-classical export pathways (reviewed in [25]). According to the available evidence, no single transport pathway mediates non-classical export, but several distinct export protein machineries exist. Only for a few factors, including IL-1α, IL-1β, and FGF-1, molecular entities involved in the secretion pathway have been identified. For IL-1β, it appears from inhibitor studies and antisense experiments [32,34] that the overall process of secretion requires the function of an ABC transporter protein. As glyburide, one of the inhibitors that was found to inhibit IL-1β secretion, exerts specificity towards ABCA1 transporters and as anti-ABCA1 antisense oligonucleotides reduce IL-1β secretion from macrophages [34], secretion of this cytokine appears to be associated with the function of an ABCA1 transporter, which is probably located within an endolysosomal compartment [35]. FGF-1 appears to be exported as a multiprotein aggregate with cytosolic p40 domain of the membrane protein synaptotagmin (p40 Syt1) and the Ca^{2+} binding protein S100A13 [36–39]. A copper-mediated oxidation and dimerization process of FGF-1 is required for FGF-1 release and the unprocessed precursor form of IL-1α represses stress-induced FGF-1 export [40]. A very recent publication elucidated the precise role of IL-1α in this process and revealed molecular details of the export of IL-1α itself. Mandinova et al. [41] demonstrated that IL-1α is a copper-binding protein and that stress-induced IL-1α release is copper-dependent and associated with binding to S100A13, which also binds copper. In addition, as shown previously for FGF-1 [42], a molten globule character was demonstrated for IL-1α that could be critical for traversing the plasma membrane. As IL-1 and FGF proteins share several striking structural similarities and as eight of the currently identified 10 members of the IL-1 family lack a signal peptide, it is likely that the molecular mechanism of the secretion of several IL-1 family members is similar to that of FGF-1 [41].

MIF is a key inflammatory factor for which a role in several disease states has been demonstrated [3]. However, although discovered already 4 decades ago [26], the mechanisms underlying the biological effects of MIF are not yet well understood. The signal transduction pathways of MIF have remained ill-defined [3]. Also, although specific secretion of MIF can be induced from several immune and non-immune cells, the machinery mediating intracellular targeting and secretion of MIF has remained unknown. Elucidation of the secretion pathway of MIF would therefore be of high interest and, should it turn out that MIF-specific processes exist, could give rise to novel therapeutic approaches aiming at blocking the inflammatory and disease-promoting properties of this cytokine.

In this study, we have focused on studying the secretion

pathway of MIF in immune-stimulated, i.e. LPS-induced, monocytes, because LPS-induced MIF secretion is likely to represent a (patho)physiologically relevant process [16]. We provide direct evidence that MIF is not secreted by the ER/Golgi pathway in these cells. Instead, we demonstrate that MIF is secreted by non-classical export. Because the pharmacological substances glyburide and probenicide, which interfere with ABC transporter function, strongly blocked MIF secretion while having no effect on MIF synthesis and turnover, we conclude that an ABC transporter is a critical component required for the process of MIF secretion.

It had long been speculated that MIF could be secreted by non-classical ways. MIF does not have an N-terminal signal peptide, nor does it have apparent internal ER-targeting motifs [28]. Furthermore, while a cytosolic and nuclear localization of MIF has been shown [14,21,22,43,44], Eickhoff and coworkers recently demonstrated that MIF occurred in pinched-off vesicles outside the epithelial cells in the lumen of the epididymis, and that this localization was associated with MIF secretion from these cells [20]. On the other hand, the observation that MIF is localized within typical secretion vesicles of endocrine cells such as ACTH-secreting pituitary cells and insulin-secreting β-islet cells [17,45], could be in agreement with a classical secretion pathway. The latter possibility may be supported by the study of Eickhoff et al. [20], who also observed the localization of MIF in vesicles in epididymal epithelial cells. However, in such cells yet another specialized secretion mechanism appears to exist, as a pinching-off of MIF-containing exosomes and a direct transfer to spermatozoa was detected. Also, MIF secretion is induced by a profile of immunological stimuli (i.e. TNF, IL-1, or LPS) that is very similar to that of other cytokines that are secreted by classical pathways. Thus, indications for both a more classical and non-classical mode of MIF secretion had been collected. However, no systematic study had yet addressed this question in a physiologically relevant cellular model.

We first excluded the possibility that MIF would enter the ER/Golgi system by a so far unknown mechanism. We show that MIF does not enter microsomes in vitro, is not glycosylated in monocytes or macrophages in vivo, and that MIF secretion from monocytes is not inhibited by inhibitors of the classical pathway such as BFA or monensin. The overstimulatory effect on MIF secretion seen for BFA was reminiscent of the effect of this inhibitor on the secretion of IL-1β [24]. Other pharmacophores, including the protein synthesis inhibitor CHX, the energy uncoupling reagent CCCH, the endocytosis inhibitor MA, and the Ca^{2+} ionophore A23187, had either no effect or also overinduced MIF secretion. Together, these data unanimously suggested that MIF is secreted by a non-classical pathway.

Different non-classical export pathways exhibiting different molecular machineries have been suggested to exist. These include heat shock-inducible pathways in connection with mechanisms involving protein aggregate formation with p40 Syt1 and S100A13, ABC transporter-mediated pathways, processes dependent on tubulin-mediated transport processes, and yet other pathways (see above and summarized in [25]). We addressed the possibility that non-classical MIF secretion could depend on ABC transporter activity, because the export of two other non-classically secreted cytokines, IL-1β and FGF-2, had already been found to be associated with ABC protein function. Of the corresponding array of inhibitors tested on LPS-induced MIF secretion in monocytes, glyburide and probenicide markedly block MIF secretion in a concentration-dependent fashion. This leads us to conclude that secretion of MIF requires the function of ABC transporter proteins for at least one step during the secretory process. As glyburide and probenicide have been suggested to be somewhat specific in their inhibitory potential for the ABCA1 transporter subclass, we conclude that this sub-category of transporters is involved.

There are at least three potential mechanisms by which ABCA1 transporters could be involved in MIF export. As ABCA1 has been suggested to serve as a regulator of vesicular transport between the TGN and the plasma membrane [46], these proteins could either mediate the entry of MIF into post-Golgi vesicles or could promote MIF vesicle transport. In the latter case, transport of MIF across the vesicle membrane would have to be mediated by an as yet unidentified protein. A possible candidate may be the vesicle-tethering protein, p115 [47]. Of note, ABCA1 was also proposed to function as a channel protein or so-called floppase [46]. However, although the non-classically secreted factors FGF-1 and IL-1α have been suggested to traverse the plasma membrane by direct non-vesicular processes [38,39,41], it is unlikely that MIF is directly transferred across the plasma membrane in an ABCA1-mediated fashion, as the export mechanism for MIF seems to differ from that of FGF-1 and IL-1α. Also, it is currently unclear whether the floppase activity of ABCA1 applies to proteins [48]. Lastly, the effect of ABCA1 on MIF export could be an indirect transporter function. ABCA1 proteins can act to translocate lipids, a process that could lead to an altered membrane structure that in turn could inhibit the proteinaceous and possibly membrane-bound machinery mediating translocation of MIF.

The conclusions drawn in this study that were derived mainly from the application of the pharmacological inhibitors will have to be confirmed by additional biochemical studies, such as MIF–ABCA1 interaction studies or the use of monocytes/macrophages from ABCA1 knock-out mice. In such future studies, the possibility that MIF export is mediated by ABC transporters other than ABCA1 will also need to be addressed, because glyburide also affects ABCC7 function [49] and probenicide has been reported to interfere with several ABCC proteins [50].

Our data suggest that the export pathway taken by MIF could be similar to that suggested to be used by IL-1β. Similar export mechanisms for MIF and IL-1β could mirror the similar pro-inflammatory spectrum of activities that these two cytokines share [51]. MIF also shares a potent angiogenic activity profile with the IL-1 type proteins [7]. However, a pro-angiogenic activity is especially exerted by the FGFs, which together with IL-1α seem to be released by a distinct mechanism. It is of note in this context that MIF is also efficiently secreted by heat and redox stress (J. Bernhagen, unpublished and [52]). In contrast, the mechanisms of LPS-stimulated secretion for IL-6, another typical pro-inflammatory cytokine, appears to be clearly different from that of LPS-stimulated MIF secretion [53]. MIF and Trx share several functional homologies with respect to their thiol-protein oxidoreductase activities and cytokine effects [3]. Trx, like MIF, is secreted by non-classical pathways [54]. However, although MIF and Trx share intriguing functional similarities as cytokines [25] and share several features of their export

pathways, it is unclear whether the mechanisms behind their non-classical export pathways are similar. While IL-1β and Trx secretion is inhibited by MA [24,54], this reagent did not interfere with the export of MIF. ABCA1 inhibitors interfere with the secretion of MIF and IL-1β, but do not affect Trx secretion.

Together, our study provides the first mechanistic evidence that MIF is secreted by a non-classical pathway and suggests that this pathway is dependent somehow on ABCA1 transporter function. Thus, these findings are important for future molecular studies and offer a first basis for the investigation of potential therapeutic approaches targeting the MIF secretion process.

Acknowledgements: We thank G. Dranoff for providing the IL-2-signal peptide–MIF-expressing B16 cells. We are grateful to H. Lue for assistance with intracellular localization studies, and to D. Finkelmeier, G. Geiger, and M.T. Nguyen for help with the THP-1 cell assays. We thank K. Pfizenmaier for helpful discussions and support of the Ph.D. thesis of O.F. This work was supported in part by Grant numbers Be 1977/1-3 and SFB 542/TP-A7 of the Deutsche Forschungsgemeinschaft (DFG) to J.B., by DFG Grant 423/3-1 to W.N., and by the Land Baden-Württemberg Grant 24-720.431 to W.N. and J.B., and NIH 2R01-Al42310 to R.B.

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