

The Role of Macrophage Migration Inhibitory Factor in Alzheimer's Disease

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Previous studies have shown that amyloid β protein ($A\beta$), the essential molecule for the formation of toxic oligomers and, subsequently, Alzheimer plaques, has been associated *in vivo* with the immune modulator, macrophage migration inhibitory factor (MIF) (17). To further investigate this association *in vivo* we used the APP transgenic mouse model. Serial brain sections of transgenic APP mice were stained for $A\beta$ plaques and MIF and we observed MIF immunolabeling in microglial cells in association with $A\beta$ plaques in the transgenic mouse brain sections. In addition, functional studies in murine and human neuronal cell lines revealed that $A\beta$ -induced toxicity could be reversed significantly by a small molecule inhibitor of MIF (ISO-1). Finally, to elucidate the role of MIF in Alzheimer's Disease (AD) we measured MIF levels in the brain cytosol and cerebrospinal fluid (CSF) of AD patients and age-matched controls. Our results demonstrate a marked increase of MIF levels within the CSF of AD patients compared with controls. Combined, our results indicate a strong role for MIF in the pathogenesis of AD and furthermore suggest that inhibition of MIF may provide a valuable avenue of investigation for the prevention of disease onset, progression and/or severity.

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INTRODUCTION

An increasing body of evidence indicates that inflammatory processes are involved in the pathophysiology of Alzheimer's Disease (AD) (1–3). A central event in these processes appears to be the activation of microglia by a variety of factors, including β -amyloid ($A\beta$) and proinflammatory cytokines (4). Activated microglia in turn release proinflammatory cytokines, such as interleukin (IL)-1-beta, IL-6 and TNF- α , that may lead to neuronal death and dysfunction by a variety of mechanisms, including enhancement of glutamate-induced excitotoxicity (5), inhibition of long-term potentiation (which limits functional plasticity after

neuronal injury [6,7]) and inhibition of hippocampal neurogenesis (8).

Macrophage migration inhibitory factor (MIF) is a proinflammatory cytokine that is highly expressed in many tissues and disease states (9). Its cellular actions include glucocorticoid counter-regulation (10), sustained MAP kinase activation (11), inhibition of p53-dependent growth arrest (12,13) and control of Jab1 transcriptional effects (14). There is a significant level of baseline MIF expression in the neurons of the hippocampus as well as in other regions of the brain, and proinflammatory stimuli lead to a marked upregulation of neuronal MIF mRNA and protein (15). MIF's physiological functions in the brain

are not understood, but its intrinsic tautomerase activity has suggested a possible role in the detoxification of oxidized catecholamines (16). Interestingly, MIF also has been isolated in association with the β -amyloid peptide (17), which is the main constituent of AD plaques, thereby supporting an emerging theory of a proinflammatory etiology for this neurodegenerative disease. In a recent report, we were the first to show that MIF levels were elevated significantly in comparison to age matched healthy control patients in CSF samples of AD patients (18). Of note, in this study we obtained the highest MIF level in patients with mild cognitive impairment (MCI). MCI may be considered as the prodromal phase in the development of AD. The possible involvement of MIF in AD also has been provided by a recent report showing a significant upregulation of CD74, which acts as a receptor binding site for MIF (19). In the present study, we were interested in elucidating this first functional link between MIF activation and $A\beta$ dependent toxicity in different cell types of the CNS.

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MATERIAL AND METHODS

Animals

Female APP23 transgenic mice (20) were used, which express the human APP751 isoform with the Swedish double mutation (K670N–M671L) under control of a murine Thy1.2 expression cassette (courtesy of Matthias Staufenbiel, Novartis Institutes for Biomedical Research Basel, Switzerland). The animals were hemizygous or littermate controls and had been backcrossed with C57BL/6J mice for at least eight generations. All procedures and protocols met the guidelines for animal care and animal experiments in accordance with national and European (86/609/EEC) legislation and were approved by the local animal welfare committee.

Human Recombinant MIF

Human MIF cDNA was cloned into the pET-17b vector (Novagen, Madison, WI, USA) and expressed in *Escherichia coli* BL-21 (DE3) strain after induction with 0.4 mmol/L isopropyl-D-thiogalactopyranoside (IPTG) and purified from the soluble fraction of the cell lysate by two-step high-pressure liquid chromatography (HPLC): (i) size exclusion HPLC on Bio-Sil TSK 250 (Bio-Rad, Munich, Germany) and (ii) ion-exchange HPLC on Ultropac TSK CM-3SW (LKB/Pharmacia, Freiburg, Germany).

MIF Inhibitor

(*S,R*)-3-(4-Hydroxyphenyl)-4,5-dihydro-5-isoazole acetic acid methyl ester (ISO-1) has been shown recently to covalently bind to the Dopachrome tautomerase activity site of MIF, and this interaction has been reported to inhibit several biological activities of MIF (21).

Western Blotting

Concentration of cell supernatants was necessary for detection of MIF by Western blotting. A 1-mL sample volume was concentrated with a Centricon-10 (Amicon, Beverly, MA, USA) at 4,600g for 70 min. Western blotting was performed by the NuPAGE electrophoresis system (Novex, San Diego, CA, USA) using 4% to 12%

N,N-methylenebisacrylamide-Tris gels. Proteins were transferred onto Optitran BA-S83 membranes (Schleicher & Schuell, Dassel, Germany). The antibodies used were polyclonal anti-MIF rabbit immunoglobulin G (IgG) diluted 1/5,000 in PBS–0.5% Tween 20 and peroxidase-labeled goat anti-rabbit IgG diluted 1/250,000 in PBS–0.5% Tween 20 (Pierce; distributed by Perbio, Bonn, Germany). The bands were visualized by an enhanced chemiluminescence detection system, as recommended by the manufacturer (SuperSignal ULTRA; Pierce; distributed by Perbio).

MIF-Enzyme-Linked Immunosorbent Assay (ELISA)

For measurement of MIF protein, CSF samples from AD patients and age-matched healthy individuals were analyzed by sandwich ELISA, using a monoclonal antihuman MIF capture antibody, and biotinylated goat antihuman MIF IgG detection antibody (R&D Systems, Wiesbaden, Germany), and purified human rMIF as a standard.

Toxicity Assays

MTT toxicity assay was performed as described (22) using SH-SY5Y human neuroblastoma cells. SH-SY5Y cells were received from DSMZ (ACC 209). The cultivation of the cells was carried out at 37°C and under a 5% CO₂ atmosphere. Cells were grown in RPMI 1640 medium (Cambrex, Taufkirchen, Germany) with 10% FBS (Cambrex) and 1% Pen-Strep (Cambrex) in 250-mL cell culture flasks.

Cells were treated with A β oligomers (22) at a final concentration of 2.5 μ mol/L, samples with ISO-1 were incubated 15 min prior to treatment. Each data point was determined in hexaplicate. Alternatively, BV-2 cells were seeded on a 24-well plate and cultivated in 0.5 mL DMEM medium containing 10% FBS, 1% Pen-Strep and 1% L-glutamine for 48 h at 37°C and 5% CO₂. In parallel, 250 μ g β -amyloid 1–40 (Bachem H-1194) was dissolved in 150 μ L HFIP and diluted with 450 μ L H₂O. The β -amyloid solution was stirred magnetically for 48 h at RT. For stimulation, the medium was replaced

by 0.5 mL DMEM with 1% L-glutamine but without phenol red or any other supplements. β -Amyloid 1–40 was added to the medium in concentrations of 5 μ mol/L and 10 μ mol/L. ISO-1 was added at concentrations of 50 μ mol/L and 100 μ mol/L.

After cultivation for a further 48 h, LDH- and MTT-assays were carried out. The LDH assay (Roche, nr 11 644 793 001) was performed as described in the manufacturer's manual.

For MTT assay, supernatant was removed from the cells and new DMEM with 1% L-glutamine and 0.5 mg/mL MTT (minus phenol red and other supplements) was incubated with the BV-2 cells. Subsequent to incubation for 30 min at 37°C with 5% CO₂ in the dark, the supernatant was removed and cells were lysed in 0.5 mL DMSO. Absorption was measured at 570 nm with a Tecan Infinite M200 plate reader. All samples were processed in triplicate.

Analysis of AD and Human Control Brain Tissue

All cases for the analysis of brain cytosol were collected at the Institute of Neuropathology, University Hospital, Muenster. Prior to autopsies, consent was obtained from patients' families to use samples for research. The neuropathological diagnosis of AD was made according to established criteria. (23,24). The control group consisted of brains from cases without neurological and neuropathological abnormalities.

Protein was harvested from frozen post mortem samples of the frontal gray matter of each of the 10 individuals with AD and age-matched controls.

Brain tissues were ground and lysed in T-PER Tissue Protein Extraction Reagent (Perbio, Bonn, Germany) containing proteinase inhibitor mix. Subsequently, the protein concentrations were determined with the BCA-Kit (Perbio); 1 μ g protein per sample was used in Western blot or MIF-ELISA (R&D Systems) and was carried out on 96-well ELISA plates as described in the manufacturer's manual.

There were no significant differences between mean ages ($P = 0.94$, ANOVA) and mean intervals between death and freezing of the brain ($P = 0.92$, ANOVA).

MIF and A β -Immunostaining

Immunohistochemistry was performed for detection of the co-localization of MIF-protein and A β -plaques. Half brains were fixed in 3.7% formaldehyde for 24 h and tissue blocks from the frontal and temporal cortex were embedded into paraffin. Paraffin sections of 3 μ m thickness were dewaxed in xylene and rehydrated using a descending ethanol series. Neighboring sections were incubated with a mouse anti-human amyloid β antibody (Clone 6F3D, 1:50, DAKO, Glostrup, Denmark), and a rabbit polyclonal antibody against murine MIF (1:500). Antigen retrieval was carried out as follows: 70% formic acid for 20 min for the amyloid antibody and 10 mmol/L citrate buffer pH 6.0 for 20 min at 480W in the microwave. Immunostaining was carried out using Shandon coverplate technology, incubation of the primary antibodies at 37°C /1 h and the detection system employed was the Vector Peroxidase kit and the VECTASTAIN ABC anti-rabbit kit according to the manufacturer's instructions (Vector Laboratories, Inc, Burlingame, CA, USA).

Selection of Patients and Controls for MIF Measurement in CSF Samples

We studied 14 individuals who were recruited from a specialized outpatient clinic for cognitive disorders from the Dementia Research Section and Memory Clinic, Rheinische Friedrich-Wilhelms-University Bonn. The group consisted of seven patients (mean age: 66.57; male: 5) and seven control subjects (mean age: 66.71; male: 5). The subjects with AD met clinical diagnostic criteria for probable AD according to the National Institute of Neurological and Communicative Disorders and Stroke and Related Disorders Association (25) and DSM-IV criteria for dementia of the Alzheimer type (26). The diagnoses were based on neuropsychological and clinical evaluation and were made by a consensus conference of psy-

chiatrists and neuropsychologists prior to the MIF measurements.

Control subjects had neither history of cognitive decline nor significant decline or impairment in cognition on clinical examination. They had no history or evidence of neurological disease with potential to affect cognition. All individuals (or, for significantly cognitively impaired individuals, their legal guardian or caregiver with power of attorney) gave written informed consent. The ethics committees of the Rheinische Friedrich-Wilhelms-University Bonn, the Philipps-University Marburg and the University Hospital Muenster approved all experiments performed with human samples.

CSF Collection

CSF samples were taken in the morning in the L3-4 or L4-5 interspaces after obtaining appropriate informed consent. Routine CSF examination included cell counting, protein content, CSF/serum glucose ratio and CSF/serum albumin ratio [albumin CSF/albumin serum] $\pm 1,000$), and were determined by standard procedures. No contamination of erythrocytes was seen in any of the samples. Aliquots were then stored at -80°C until biochemical analysis. Albumin and IgG concentrations were determined in serum and CSF by immunoprecipitation nephelometry. The CSF albumin/serum albumin quotient was used to evaluate the integrity of the CSF-blood barrier. All CSF samples had standard laboratory values within normal ranges.

Statistical Analysis

To evaluate differences between groups, nonparametric tests (Mann-Whitney U test) and ANOVA were performed using SPSS statistical software (SPSS Inc., Munich). Data are presented as mean \pm SD. Significance was evaluated at $P < 0.05$.

RESULTS

MIF Protein Is Associated with A β Deposits in Murine APP 23 Transgenic Mice

Since the initial report on MIF and A β was based on a screening approach to

look for new A β -binding partners using an A β affinity column and subsequently identifying MIF in a soluble fraction of the cerebral cortex of AD brain by immunoprecipitation (17), we sought to identify MIF protein distribution in association with A β deposits by immunohistochemistry. We choose to analyze murine APP 23 as an APP deposition model. Staining serial sections of APP 23 mouse brain for MIF and A β revealed a distinct MIF accumulation at sites of A β plaques (Figure 1). We could demonstrate that one cellular source for the enhanced MIF immunolabelling in association with amyloid deposits is microglia near the plaques.

Blocking MIF in Neuroblastoma Cells Prevents A β -Dependent Neurotoxicity

To establish a functional link between MIF and A β , we used an A β -dependent *in vitro* neurotoxicity assay. We used toxic A β preparations as described previously (22).

Figure 2 shows a profound toxic effect of A β oligomers 1-40 and 1-42 respectively. The A β -mediated toxic effect, measured by MTT activity, was almost completely abolished when the cells were coincubated with the small molecule MIF inhibitor ISO-1. This suggests that MIF alone plays an essential role in the A β -dependent neurotoxicity of neuroblastoma cells. We also were able to show that ISO-1 was able to reduce the toxic effects of A β in a murine microglial cell line (BV2). The best effect, combining three individual experiments, was detected by A β treatment of 10 μ mol/L, which reduced the cell viability to 60% and was restored by ISO-1 to around 90% (Figure 3) of the control. However, due to the fact that the results did not reach statistical significance, this can only be interpreted as a trend.

Elevated Levels of MIF Protein in CSF Samples of AD Patients

Based on our *in vitro* data showing that A β induced MIF protein expression in neuroblastoma cells (27), we were interested to address the critical issue of whether this would also reflect the relevant situation in AD patients. Seven sam-

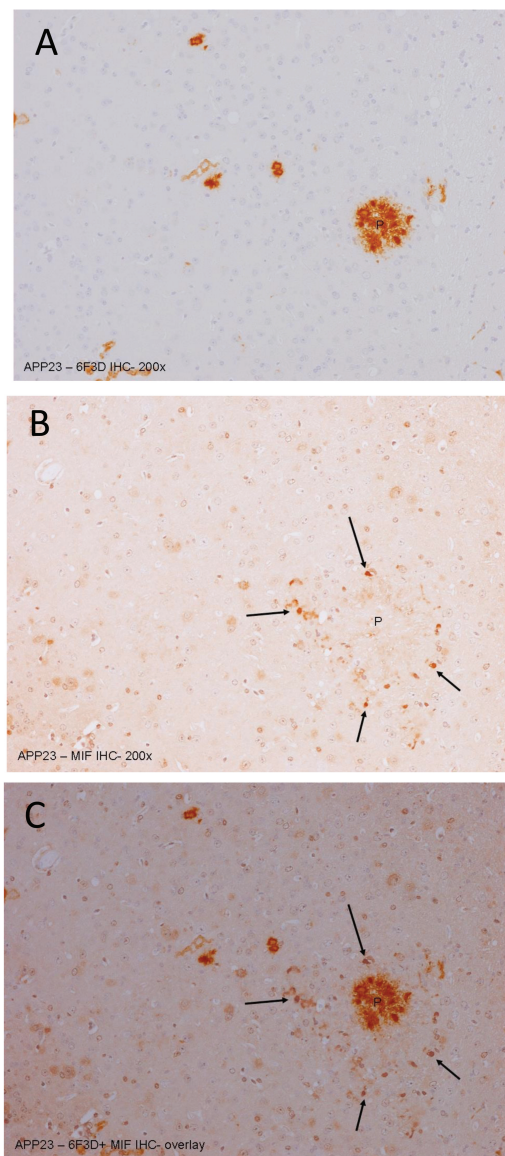


Figure 1. MIF immunostain in association with amyloid plaques. Serial section of plaque bearing APP 23 mice were stained for amyloid deposits (A) and for MIF proteins (B) using the mentioned antibodies in the appropriate dilution. The overlay (C) of both photographs displays the plaque dependent MIF staining (arrows indicate the MIF positive cells, P: plaque). A similar staining pattern was obtained in a total of eight sections from four different transgenic APP mice.

ples from each group, that is, AD patients and age-matched healthy individuals, were randomly taken out of a larger patient or control group. CSF samples of AD patients compared with controls showed a significant increase ($P < 0.001$, Mann-Whitney U) of MIF protein in CSF samples (Figure 4A). In contrast to the marked elevation of MIF in CSF samples,

we did not observe a significant difference in the high basal MIF content in AD patients versus normal controls. MIF levels were assessed using ELISA (Figure 4B) or Western blot (data not shown).

DISCUSSION

The recent report of a physical association and colocalization of MIF with the

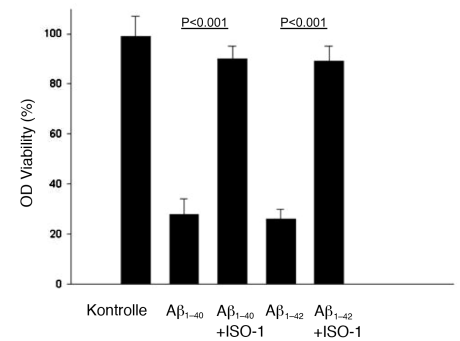


Figure 2. The MIF specific small molecule inhibitor ISO-1 counter-regulates the neurotoxic effect of A β_{1-40} , A β_{1-42} in neuroblastoma SHSY cells. SHSY cells (30,000 cells per well) were incubated alone or in the presence of 2 μ mol/L oligomerized A β_{1-40} , A β_{1-42} , or in the presence of A β_{1-40} , A β_{1-42} in the presence of 200 nmol/L MIF specific inhibitor ISO-1. The viability of the cells was determined by MIT assay. The values of the control cells were set as 100% viability.

A β protein combined with the ability of MIF to form amyloid-like fibrils (17,28) suggests that MIF may play an important role in plaque formation and may be involved in the inflammatory process surrounding the plaques.

MIF is highly expressed in neurons within different regions of the brain, including the cerebral and cerebellar cortex, the hippocampus and the hypothalamus (15). The expression of MIF protein in neuronal tissue is both constitutive and inducible, as revealed by studies following intracisternal endotoxin injection (15). MIF's precise function in the brain is unknown. MIF-KO mice display no major central nervous system abnormalities (29,30). Nevertheless, MIF's ability to sustain ERK-1/2 MAP kinase activation and override p53-mediated apoptosis suggests a potentially important role for this protein in neuronal survival (11,13).

Our initial MIF immunostain using a transgenic APP mouse model confirmed and extended the observation of a colocalization of MIF and A β plaques. A possible cellular source for the MIF is activated microglia near A β plaques. The clustering of microglia at sites of amyloid deposition suggests that microglia are

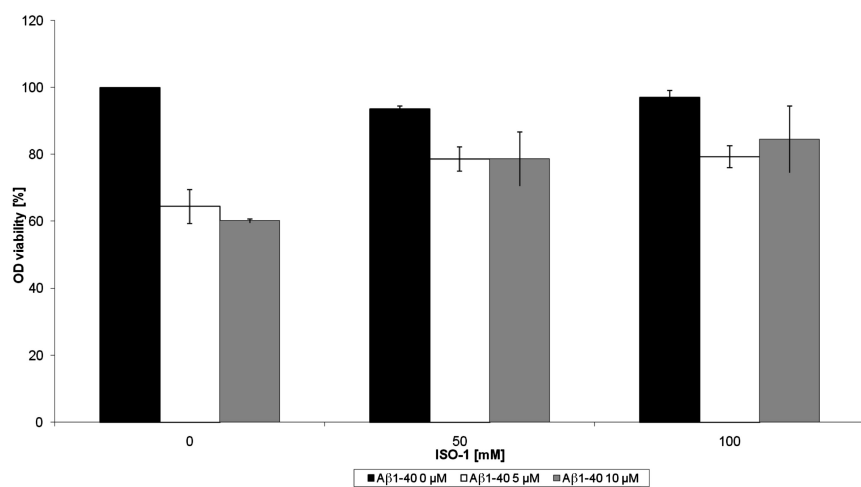


Figure 3. The MIF specific small molecule inhibitor ISO-1 counter-regulates the neurotoxic effect of A β_{1-40} at concentrations of 5 μ mol/L or 10 μ mol/L in murine BV2 microglial cells. At a concentration of 5 μ mol/L, A β viability of the cells was reduced to 73% of the control. This effect was even more prominent when the cells were treated with 10 μ mol/L of A β . Here, we obtained a toxic A β effect of around 60% in comparison to the control cells. That effect was reduced with 50 μ mol/L ISO-1 to around 80% and further reduced to around 90% with 100 μ mol/L of ISO-1. The graph displays the measurements of the mean from three separate experiments, performed in quadruplicate. The results did not reach statistical significance and can therefore only be interpreted as a trend.

drawn to these sites and attempt to remove the amyloid protein. However, due to the insoluble nature of amyloid and the fact that it is present in substantial quantities, it is likely that microglia are unable to clear it. Thus, amyloid deposits remain and continue to attract microglial cells over prolonged periods of time.

It has been shown that MIF markedly induces the phagocytotic ability of macrophages (31). Therefore, enhanced release of MIF by microglia might reflect the cellular response of these cells toward amyloid deposits, ultimately causing the cells to "burn out" and die. On the other hand, an overly robust macrophage response can lead to pathological sequelae that contribute significantly to tissue damage.

The scope of this study was to provide a functional link between MIF and A β -induced neurotoxicity. Our *in vitro* data using a classical neuroblastoma cell line provided clear evidence that the inhibition of endogenous MIF using a small molecule specific MIF inhibitor almost completely antagonized the neurotoxic ef-

fects of A β . A similar effect of ISO-1 was detected in murine BV2 microglial cells. Although in these cells the toxic effect of A β was not as effective as in the neuroblastoma cells, we also obtained an almost complete dose-dependent reversal of the A β -induced toxicity. This suggests that increased release of MIF might not only contribute to the neuronal damage and loss during the pathogenesis of AD in humans, but also might impair the function of microglial cells. Furthermore, the measurement of MIF in CSF samples revealed significantly higher levels of MIF in AD patients compared with age-matched controls. This observation is consistent with our recent independent study on a larger group of MCI and AD patients also showing increased levels of MIF within the CSF (18). In this report, highest MIF levels were detected in MCI samples. That might suggest that microglial activation is a key event already during the early phase of AD development.

Specific manipulation of proinflammatory cytokine levels, such as MIF, is a tempting therapeutic target and could be

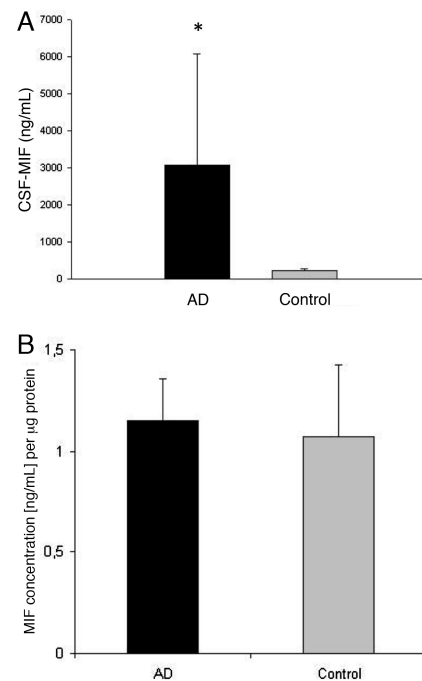


Figure 4. (A) Detection of MIF in CSF of AD patients ($n = 7$) and age-/gender-matched controls ($n = 7$). (B) MIF-ELISA with lysed brain tissues collected from AD patients ($n = 10$) and healthy individuals as control ($n = 9$). We performed a Mann-Whitney U test for nonparametric distributed values to test group differences in MIF concentrations and found significantly elevated MIF levels ($P < 0.001$) in AD patients in comparison to healthy controls (A).

advantageous in comparison to general microglia deactivation. Although microglial activation is clearly and consistently associated with senile plaques and the presence of A β in AD, there is evidence that these activated microglia also may be important to clear A β from plaques and thus be homeostatic rather than neurotoxic (32). In fact, the presence of activated microglia may be necessary for the clearance of A β by passive immunization in AD (33).

A few recent studies including patients with mild cognitive impairment (MCI), a group at high risk to develop Alzheimer-type dementia over time (34), strongly suggest that the inflammatory process may precede the dementia stages (35–38). Furthermore, a study in a transgenic

mouse model of AD has demonstrated very early focal glia activation in conjunction with increased expression of IL-6 and IL-1 β , as well as increased BACE1 production and activity (39). Together, these results provide evidence that neuroinflammation occurs early, at predementia stages of AD. Thus, anti-inflammatory therapeutic intervention at this time may be useful to prevent severe cerebral damage and clinical progression to dementia. Further studies, including the assessment of MIF in MCI, are necessary to evaluate whether MIF is involved in the neuroinflammation at this disease stage, and could be a target for early therapeutic intervention in AD.

In summary, this is the first report providing a functional link between the proinflammatory MIF and the toxicity of A β aggregates, which are considered to play a central role during the course of AD. Blocking the endogenous MIF in neuronal and microglial cells prevented the A β -dependent toxicity in both cell types. Our results clearly suggest that a similar link between MIF and the progression of AD might also exist *in vivo* and that the toxicity of A β can be attributed directly to the increased expression of MIF.

DISCLOSURE

The authors declare that they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

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