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Selective Targeting of a Disease-Related Conformational Isoform of Macrophage Migration Inhibitory Factor Ameliorates Inflammatory Conditions

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Macrophage migration inhibitory factor (MIF), a proinflammatory cytokine and counterregulator of glucocorticoids, is a potential therapeutic target. MIF is markedly different from other cytokines because it is constitutively expressed, stored in the cytoplasm, and present in the circulation of healthy subjects. Thus, the concept of targeting MIF for therapeutic intervention is challenging because of the need to neutralize a ubiquitous protein. In this article, we report that MIF occurs in two redox-dependent conformational isoforms. We show that one of the two isoforms of MIF, that is, oxidized MIF (oxMIF), is specifically recognized by three mAbs directed against MIF. Surprisingly, oxMIF is selectively expressed in the plasma and on the cell surface of immune cells of patients with different inflammatory diseases. In patients with acute infections or chronic inflammation, oxMIF expression correlated with inflammatory flare-ups. In addition, anti-oxMIF mAbs alleviated disease severity in mouse models of acute and chronic enterocolitis and improved, in synergy with glucocorticoids, renal function in a rat model of crescentic glomerulonephritis. We conclude that oxMIF represents the disease-related isoform of MIF; oxMIF is therefore a new diagnostic marker for inflammation and a relevant target for anti-inflammatory therapy. The Journal of Immunology, 2015, 195: 2343–2352.

Macrophage migration inhibitory factor (MIF) is a proinflammatory cytokine with a pleiotropic spectrum of biological functions. One particular hallmark of MIF is its ability to override immunosuppressive effects of glucocorticoids, thereby promoting and aggravating local and systemic inflammatory responses mediated by macrophages and monocytes (1–3). Furthermore, MIF upregulates inflammatory responses by inducing the expression of other proinflammatory mediators, such as TNF-α (4), NO (5), and PGE2 (3), and by enhancing chemotaxis and macrophage infiltration (6). In addition, MIF inhibits tumor suppressor p53 (7) and activates the central kinases Akt/PKB and Erk that positively affect survival of immune cells, therefore contributing to the maintenance of inflammatory processes (3, 8). Levels of MIF are elevated in many inflammatory conditions, such as rheumatoid arthritis (9), asthma (10, 11), sepsis (12), psoriasis (13), inflammatory bowel disease (IBD) (14, 15), systemic lupus erythematosus (SLE) (16), and cancer (17). Experiments showed that MIF-deficient mice have reduced susceptibility to experimental sepsis (18), arthritis (19), IB (15), and organ injury caused by SLE (20), suggesting that MIF is a key player in the development of specific diseases. Moreover, animals benefited from treatment with neutralizing polyclonal or monoclonal anti-MIF Abs in models of experimental sepsis or septic shock (21), delayed-type hypersensitivity (22), arthritis (23), IB (15), and other diseases (24).

In contrast with other proinflammatory cytokines, MIF is constitutively expressed by numerous cell types, has a wide tissue distribution (25–27), and is present in the circulation of healthy subjects in the range of 1–15 ng/ml (21, 28, 29). MIF presents a ubiquitous but challenging therapeutic target because it requires the functional neutralization of a protein that is constantly present in healthy and diseased subjects.

We recently described the fully human mAbs BaxB01, BaxG03, and BaxM159 that effectively inhibit the biological activity of MIF in vivo and in vitro (30, 31). These Abs are specific for two distinct epitopes (aa 50–68 or 86–102), which form a β-barrel structure containing the highly conserved Cys-Ala-Leu-Cys-Ser motif (30); this forms the catalytic motif of the thiol protein oxidoreductase linked to MIF’s biologic function (32–34). After more in-depth investigation, we found that this set of Abs is specific for a previously unrecognized, redox-dependent isoform of MIF that we
designated as oxidized MIF (oxMIF). OxMIF is selectively expressed under proinflammatory conditions in patients and animals. The outcomes of our in vivo studies indicate that oxMIF represents a disease-related form of MIF that can be specifically targeted for anti-inflammatory therapy by mAbs.

Materials and Methods
Reagents
BaxB01, BaxG03, BaxM159, BaxH02, and an isotype-matched human control Ab were produced as described previously (30). Polyclonal rabbit anti-MIF Abs were generated by immunization with recombinant MIF and purified from serum over protein A columns (GE Healthcare) and immuno-ribilized recombinant MIF. Recombinant MIF was expressed in Escherichia coli and purified as described previously (30). OxMIF was generated either by adding saturated t-cystine solution (Sigma) to recombinant MIF and subsequent dialysis against PBS or by adding oxidized glutathione (Sigma).

Human sample collection
Plasma samples from healthy subjects (control donors) were collected from volunteers in a normal state of health, with no apparent signs of disease. Plasma samples from sepsis and asthma patients were purchased from Tissue Solutions. Blood samples for FACS analysis were collected from patients with bacteriaemia at the general hospital (Allgemeines Krankenhaus, Vienna, Austria). Plasma samples from psoriasis patients were purchased from Asterand. Urine and plasma samples from SLE patients were collected at the Wilhelminenspital (Vienna, Austria). Plasma samples from IBD patients and biopsies for colonic explant cultures were collected from ulcerative colitis (UC) and Crohn disease patients at the Medical University Innsbruck. Biopsies were taken from areas showing endoscopic signs of high inflammatory activity (lesional) and from endoscopically normal appearing, noninflamed mucosa (nonlesional) (35). The control biopsies were obtained from patients without signs of intestinal inflammation. Colonic biopsies were cultivated for 24 h, and supernatants were harvested for MIF measurements. All patients and control donors signed an informed consent for sample collection. Blood sample collection from patients with bacteremia was approved by the Ethics Committee Medical University Innsbruck, Austria. Urine and plasma sample collection from SLE patients was approved by the hospital ethics committee of the Wilhelminenspital. The IBD study was approved by the Ethics Committee from the Medical University Innsbruck, Austria.

Animal models of IBD
Anti-CD40 into recombination activating gene-1 (−)(−) model of acute colitis. To induce acute colitis, we injected i.p. an agonistic mAb directed against mouse CD40 (FGK45 IgG2a; BioXCell) or an isotype control into recombination activating gene-1 (−)(−) C57BL/6 mice (7–9 wk, male; Taconic). Anti-oxMIF Abs (or isotype control) were administered at the day of disease induction by anti-CD40 Abs and at day 1 post anti-CD40 treatment. Mice were culled on day 7.

IL-10−/− plus piroxicam model of chronic colitis. Spontaneous chronic enterocolitis of IL-10−/− mice (11 described earlier. Total MIF in rodent samples was determined by capturing rabbit anti-MIF Abs and detection of captured reduced MIF and oxMIF by a biotylated rabbit anti-MIF Ab and streptavidin-HRP conjugate (GE Healthcare). Total MIF and oxMIF have been shown to be stable in plasma when stored at 4°C or −80°C (Supplemental Fig. 3).

Surface plasma resonance
Abs were immobilized onto a CMS sensor chip (GE Healthcare). Reduced glutathione (Sigma) and oxidized glutathione were added to HEPES-buffered saline containing EDTA and SurfaceTaq P20 (GE Healthcare) at different ratios. Recombinant MIF (10 µm) was diluted in these buffers and injected into a BIAcore T200 (GE Healthcare). Redox potentials (mV) of the GHG/GSSG buffers were measured using an InLab Redox Microelectrode (Mettler Toledo).

Western blot
Samples were boiled in NuPAGE LDS-Sample Buffer containing 100 mM DTT and proteins were separated on NuPAGE 4–12% Bis-Tris Gels (Life Technologies). Proteins were transferred on nitrocellulose, and MIF was detected by polyclonal rabbit anti-MIF (FL115; Santa Cruz) and anti-rabbit IgG-HRP conjugate (Thermo Scientific) using SuperSignal Chemiluminescent Substrate (Thermo Scientific).

Flow cytometry
Blood from mice was diluted in Cell Staining Buffer (Biolegend) containing either Alexa Fluor 700–labeled anti-CD3ε and PerCP-Cy5.5–labeled anti-Ly6G or allophycocyanin-labeled anti-CD14 and PE-Cy7–labeled anti-CD19 Abs (all from eBioscience). Blood was also incubated with BaxB01, rabbit anti-MIF Ab (FL115; Santa Cruz), and anti-rabbit IgG-HRP conjugate (Thermo Scientific) using SuperSignal Chemiluminescent Substrate (Thermo Scientific).

Immunoprecipitation
Mouse spleens or leukocyte subpopulations have been prepared by Ficol gradient centrifugation (granulocytes and PBMCs) and monocyte separation (lymphocytes and monocytes) by cell culture plate attachment, and 1 × 10⁶ cells were lysed in cell lysis buffer (Cell Signaling Technology). Cleared

OXMIF AS A NEW TARGET IN INFLAMMATORY DISEASES

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lysates were incubated with Ab and immunoprecipitated with Protein G Dynabeads (Life Technologies). Immunoprecipitated MIF was analyzed by Western blot as described earlier.

Immunohistochemistry

Rat kidney cryosections were fixed in 4% formaldehyde and incubated with goat anti-human IgG directly labeled with HRP (Sigma) to detect anti-oxMIF Ab or isotype control Ab. Anti-oxMIF Ab BaxB01 was used to detect total MIF in renal tissue as MIF was denatured during fixation of tissue (Fig. 1F). After Avidin/Biotin block (Vector Laboratories), biotinylated BaxB01 and R.T.U. VECTASTAIN ABC reagent (Vector Laboratories) were used to detect total MIF. The nuclei were counterstained with hematoxylin, and the slides were dried in ascending series of ethanol and xylene. After mounting with VECTAMOUNT Permamount and

FIGURE 1. Redox-dependent binding of anti-MIF Abs to recombinant human MIF. (A) SPR studies using immobilized BaxB01 showed redox-dependent binding of human MIF in different redox buffers. Higher concentrations of oxidized glutathione (GSSG) versus reduced glutathione (GSH) enabled binding of MIF to BaxB01. (B) SPR binding response units plotted against the redox potentials of the GSH/GSSG buffers and evaluated by four-parameter fit. We calculated the half-maximal response to be at ~94 mV, which indicates the redox potential where MIF rearranges its structure from a reduced to an oxidized state. (C) Binding of E. coli–derived recombinant human MIF oxidized with l-cystine (oxMIF), (D) binding of recombinant MIF in its reduced state, and (E) binding of oxMIF reduced with 2 mM DTT to immobilized oxMIF-specific Abs BaxB01, BaxG03, BaxM159, and to a human control IgG; insets show the binding to anti-MIF Ab BaxH02 that was selected from the same phage-display–derived Ab panel as BaxB01 but does not differentiate between the reduced and oxidized form of MIF. (F) Schematic drawing showing the binding of oxMIF-specific Abs to MIF, either induced by redox-mediated reversible conformational changes or by nonphysiological, irreversible conformational changes in the structure of MIF:

oxMIF Ab or isotype control Ab. Anti-oxMIF Ab BaxB01 was used to detect total MIF in renal tissue as MIF was denatured during fixation of tissue (Fig. 1F). After Avidin/Biotin block (Vector Laboratories), biotinylated BaxB01 and R.T.U. VECTASTAIN ABC reagent (Vector Laboratories) were used to detect total MIF. The nuclei were counterstained with hematoxylin, and the slides were dried in ascending series of ethanol and xylene. After mounting with VECTAMOUNT Permamount and

FIGURE 2. Presence of oxMIF in the circulation of patients and healthy controls. (A) Plasma levels of oxMIF in samples from healthy controls and patients with acute or chronic inflammatory diseases. (B) Plasma levels of total MIF in the same samples. Individual values and medians (red lines) are shown. We used the Kruskal–Wallis test followed by Dunn’s multiple correction test for statistical analyses. **p < 0.01, ***p < 0.001. (C) OxMIF levels plotted against total MIF levels for each individual plasma sample. Spearman correlation analysis showed a significant correlation between MIF and oxMIF levels for each disease, but not the healthy control group.
coverslipping, full slide scans were acquired using an Olympus VS120 slide scanning microscope at 20-fold magnification (UPLANSAPO 20x, numerical aperture 0.75; PIKE F505/C Camera; Allied Vision Technologies). Images are presented at 10-fold magnification.

Statistics

Distribution was tested with Kolmogorov–Smirnov tests. If normal distribution was confirmed, data were evaluated by one-way ANOVA followed by Dunnett’s multiple comparison test; otherwise, data were evaluated by Kruskal–Wallis test followed by Dunn’s multiple correction test. Two groups were compared by unpaired two-tailed Student t test (normal distribution) or Mann–Whitney U test.

Results

Two conformational isoforms of MIF are immunologically distinct

Human monoclonal anti-MIF Abs BaxB01, BaxG03, and BaxM159 were shown to neutralize MIF functional activity in cell-based assays and to be protective in animal models (30, 31). However, surface plasmon resonance (SPR; Fig. 1A, 1B) and ELISA studies (Fig. 1C–E) revealed that binding of these Abs to MIF is redox dependent. In vitro oxidation of recombinant human MIF with either l-cystine or oxidized glutathione induced a structural rearrangement and generated a conformational isoform (oxMIF) that was detected by these anti-MIF Abs with nanomolar affinities, whereas recombinant MIF in its reduced state was not detected. The half-maximum redox potential at which MIF underwent a structural rearrangement that enabled Ab binding occurred at $E_{h} = –94$ mV (Fig. 1A, 1B). ELISA studies confirmed the specificity of BaxB01, BaxG03, and BaxM159 for oxMIF (Fig. 1C, 1D), and reduction of oxMIF with DTT abrogated Ab binding (Fig. 1E). BaxB01, BaxG03, and BaxM159 bound with high specificity to recombinant mouse and rat oxMIF (data not shown). Rat and mouse MIF share ~90% homology with human MIF and contain the highly conserved 57Cys-Ala-Leu-Cys60 motif (5, 33, 38).

The reversible and redox-dependent transition of MIF to oxMIF is the only way to achieve binding to Abs BaxB01, BaxG03, or BaxM159 under physiological conditions. However, under non-physiological conditions, such as immobilizing recombinant MIF on solid surfaces (e.g., plastic or glass) or treatment with denaturing

![Figure 3](http://www.jimmunol.org/Download)

**FIGURE 3.** Presence of MIF on the surface and in the cytosol of immune cells. (A) Lymphocytes, monocytes, and granulocytes were purified from blood samples of five different healthy control donors. Immune cell lysates were prepared, pooled, and subjected to immunoprecipitation using the oxMIF-specific (BaxB01, BaxM159) and polyclonal anti-MIF (α-MIF) Abs. Immunoprecipitated MIF was detected by Western blot (a representative Western blot of two independent experiments is shown; images are from different parts of the same blot). (B) FACS analyses of granulocyte (CD45+CD15+), monocyte (CD45+CD14+), B cell (CD45+CD19+), and T+NK cell (CD45+CD56+) populations from healthy control donors using oxMIF-specific Ab BaxB01 (left panel, black line) and polyclonal Ab (anti-MIF) recognizing total MIF (right panel, black dashed line). Isotype IgG from matching species were used as controls (gray lines). Blood samples from 10 different donors were analyzed and 1 representative FACS profile is shown. (C) FACS analyses of granulocyte (CD45+CD15+), monocyte (CD45+CD14+), B cell (CD45+CD19+), NK cell (CD45+CD56+), and T cell (CD45+CD3+) populations from bacteremic patients using oxMIF-specific Ab BaxB01 (left panel, black line) and polyclonal Ab (anti-MIF) recognizing total MIF (right panel, black dashed line). Isotype IgG from matching species were used as controls (gray lines). Blood samples from three different patients were analyzed and the FACS profile of a representative patient is shown.
and/or cross-linking agents (e.g., formaldehyde), MIF irreversibly changes its structure, which also leads to Ab binding (30) (Fig. 1F). In addition, several small molecules that oxidize MIF, for example, 5-chloro-2-methyl-4-isothiazolin-3-one, can be used to efficiently convert MIF into an oxMIF surrogate, and hence lead to Ab binding. Taken together, our data demonstrate the discovery of a redox-dependent isofrom of MIF and a strict specificity of our mAbs for this isofrom.

**OxMIF is detected in plasma samples of patients with acute and chronic inflammatory diseases but not in healthy subjects**

The biological relevance of the two immunologically distinct, redox-dependent conformational isofroms of MIF became evident when plasma samples from patients with different acute and chronic inflammatory diseases were compared with those from healthy control volunteers using ELISA. Two ELISA methods were developed: one that quantitatively detected total MIF, that is, the sum of oxMIF and reduced MIF, and a second that specifically quantitated oxMIF. Whereas the plasma samples of 60 of 69 healthy controls did not contain detectable oxMIF, we found small amounts of oxMIF (up to 7.9 ng/ml) in samples of 9 individuals (9/69, 13% oxMIF+; median 0.0 ng/ml). The plasma levels of oxMIF were significantly elevated in patients with septicemia (40/40, 100% oxMIF+; median 7.2 ng/ml), psoriasis (43/43, 100% oxMIF+; median 3.8 ng/ml), asthma (63/63, 100% oxMIF+; median 9.4 ng/ml), UC (15/20, 75% oxMIF+; median 3.7 ng/ml), and Crohn disease (31/39, 79% oxMIF+; median 3.7 ng/ml; Fig. 2A). Elevated plasma levels of oxMIF were also determined in a subpopulation of SLE patients, namely, patients with systemic exacerbations of SLE without renal involvement (8/9, 89% oxMIF+; median 8.3 ng/ml; Fig. 2A). In contrast, oxMIF was not significantly elevated in the plasma of SLE patients in remission (n = 43; median 0 ng/ml) or in patients with acute lupus nephritis (LN; n = 21; median 0; data not shown).

The same plasma samples from healthy controls showed a baseline level of total MIF (median 5.1 ng/ml), consistent with MIF levels published in the literature (21, 28). In this sample collection, total MIF was significantly elevated only in patients with septicemia (median 13.3 ng/ml) and asthma (median 12.7 ng/ml), but not in patients with psoriasis, IBD, or SLE (Fig. 2B). The degree of statistical significance was markedly higher for elevation of oxMIF levels than for elevation of total MIF levels, as the baseline level for oxMIF in control samples was 0 ng/ml. Interestingly, correlation plots demonstrated that plasma levels of oxMIF and total MIF correlated in these different inflammatory diseases (Fig. 2C). On average, total MIF levels were ~50% higher than oxMIF levels, indicating that oxMIF comprises two thirds of MIF in the circulation of patients. However, neither oxMIF levels nor total MIF levels followed a standard distribution, and both show strong variation with levels of up to 250 ng/ml.

Immune cells are known to store high levels of MIF intracellularly (25–27). We analyzed lysates from granulocytes, monocytes, and lymphocytes derived from healthy control donors by immunoprecipitation to assess whether oxMIF was present in the cytosol of leukocytes. As expected, we detected MIF in each cell type but could not precipitate oxMIF (Fig. 3A). Our studies also revealed that MIF can be detected on the surface of immune cells. We established flow-cytometry methods to analyze membrane-bound total MIF and oxMIF. In samples of human leukocytes isolated from healthy controls, MIF was expressed on the surface of monocytes/macrophages and granulocytes. Within the different lymphocyte subsets, MIF was expressed on the surface of B cells, but barely on T or NK cells. However, oxMIF was never detected on the surface of leukocytes from healthy controls (Fig. 3B). To assess whether oxMIF is expressed on the surface of human leukocytes under disease conditions, we analyzed blood samples from bacteremic patients. Whereas total MIF was detected on the surface of all immune cells (Fig. 3C, right column), oxMIF was detected on the surface of granulocytes, macrophages, and NK cells, but not on the surface of B or T cells (Fig. 3C, left column).

We also investigated immune cells from an experimental sepsis model in mice to support the observation that oxMIF is expressed on the surface of leukocytes under diseased conditions. Total MIF was detected on the cell surface of all immune cell populations...
(granulocytes, monocytes, B cells, and T cells) from challenged and nonchallenged mice. OxMIF was expressed only on granulocytes and monocytes from infected mice and could not be detected on the cell surface of any leukocytes from the healthy control group (Supplemental Fig. 1A, 1B), which was in accordance with the results obtained from analyzing human leukocytes (Fig. 3B, 3C). In addition, we tested for the presence of oxMIF in spleen lysates. Our results show that the cytoplasm of immune cells and other spleen cells does not contain oxMIF, regardless of whether the spleen was derived from healthy mice or mice with peritonitis (Supplemental Fig. 1C). Thus, our data show that during the course of an inflammatory response, increased levels of oxMIF could be detected in the plasma and the cell surface of immune cells.

**OxMIF expression is linked to sites of inflammation in LN and IBD**

We analyzed the urine from patients diagnosed with SLE, with or without renal flares, and from healthy controls to gain further insights into oxMIF expression in different pathologic conditions. SLE samples were classified into three different groups depending on the patient’s diagnosis at the time of sampling, that is, remission (without signs of acute kidney inflammation), systemic flare of SLE (without renal manifestation), or acute LN (characterized by acute renal flares). We found a clear correlation between disease severity and the oxMIF/Cr ratio in the urine in patients with acute LN (Fig. 4A). We did not see any elevation of urinary oxMIF/Cr ratio in patients with systemic SLE without renal manifestation, or in patients in remission (Fig. 4A). Total MIF/Cr ratios indicated that all MIF measured in the urine was oxMIF (Fig. 4B). In plasma, oxMIF was significantly elevated during systemic exacerbations of SLE without renal involvement, when compared with healthy controls (Fig. 2A). In contrast, oxMIF was not elevated in the plasma of patients in remission or in patients with LN (data not shown). Thus, an acute renal flare coincided with local expression of oxMIF in the kidney, which could be detected primarily in the urine, whereas systemic exacerbation of SLE resulted in elevated oxMIF in the circulation.

We analyzed supernatants from colonic explant cultures to address the presence of oxMIF in the colon of patients with IBD (35). We showed that biopsies taken from foci of acute inflammation from patients with UC and Crohn disease contained significantly

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**FIGURE 5.** Effects of anti-oxMIF Abs in mouse models of IBD. In an anti-CD40 Rag-1<sup>−/−</sup> model of acute colitis, reduction of disease activity (A) and reduction of histological signs (B) of enterocolitis were determined after preventive treatment with anti-oxMIF Abs BaxB01 and BaxG03. Each point represents data from an individual mouse with the mean values shown as red lines (n = 6–7 mice/group). Graphs are representative of two independent experiments. Representative 5-μm colon tissue sections from this model were stained with H&E (original magnification ×10) (C). In an IL-10<sup>−/−</sup> piroxicam model of chronic colitis, reduction of disease activity (D) and alleviation of histological signs (E) of colitis were determined after treatment with BaxB01 and BaxG03. Each point represents data from an individual mouse with the mean values shown as red lines (n = 10–12 mice/group, except n = 5 for healthy control group on normal diet). Graphs are representative of two independent experiments. Representative colons from different treatment groups of the IL-10<sup>−/−</sup> piroxicam model are shown (F). Reduction of proinflammatory cytokines TNF-α (G), MCP-1 (H), and IL-6 (I) after treatment with BaxB01 and BaxG03 in the IL-10<sup>−/−</sup> piroxicam mouse model are shown. Data are expressed as mean and SEM. We used the one-way ANOVA followed by Dunnett’s multiple comparison test (against control IgG group) for statistical analyses. *p < 0.05, **p < 0.01, ***p < 0.001.
higher amounts of oxMIF and MIF than those taken from nonlesional sites or biopsies from healthy controls (Fig. 4C, 4D). Levels of TNF-α (Fig. 4E), IL-6 (Fig. 4F), and IL-8 (data not shown) paralleled the presence of oxMIF in colonic explant supernatants. We conclude that oxMIF is expressed in inflamed tissues and subsequently released to, for example, the plasma or urine during acute phases of inflammatory diseases.

**Treatment with oxMIF-specific Abs alleviates inflammation in experimental enterocolitis and crescentic glomerulonephritis**

We addressed whether neutralization of oxMIF is sufficient to reduce inflammation in vivo in animal models of chronic inflammation. First, we assessed the efficacy of oxMIF-specific Abs in two IBD models with different pathogeneses: the anti-CD40–induced acute colitis model (39) and the IL-10−/− piroxicam model of chronic colitis (40, 41). Injection of agonistic anti-CD40 mAb to T cell– and B cell–deficient Rag-1−/− mice induced pathogenic symptoms of acute colitis and intestinal innate inflammatory response that functionally depend on TNF-α, IFN-γ, and IL-12p40 and IL-23p40 pathways (39). Treatment with either BaxB01 or BaxG03 reduced disease activity scores (Fig. 5A) and histological signs of colitis (Fig. 5B, 5C), characterized by little crypt elongation, goblet cell depletion, and few inflammatory cells in anti-oxMIF–treated mice.

We synchronized development of chronic enterocolitis in IL-10−/− mice by feeding with piroxicam, a nonsteroidal anti-inflammatory drug (42, 43). Repeated injections of anti-oxMIF Abs BaxB01 and BaxG03 effectively alleviated signs of chronic enterocolitis as demonstrated by the decrease of the disease activity scores (Fig. 5D) and histology index (Fig. 5E), and reduced thickening of the colon (Fig. 5F) in the treated groups. We also measured a decreased secretion of the proinflammatory cytokines TNF-α, MCP-1, and IL-6 after anti-oxMIF treatment (Fig. 5G–I).

We assessed the efficacy of anti-oxMIF treatment in a rat model of crescentic glomerulonephritis. After injection of nephrotoxic serum targeting the glomerular basement membrane, the animals developed glomerular crescents from tissue injury driven by intraglomerular macrophages, leading to proteinuria and finally to kidney failure (36, 44). We detected urinary oxMIF 4 d after disease induction. Treatment of the animals with BaxB01 starting on day 4 after disease induction significantly reduced proteinuria (Fig. 6A), glomerular macrophage infiltrates (Fig. 6B), and glomerular crescents (Fig. 6C). Urinary oxMIF levels increased significantly during disease progression in the control Ab group but remained 10-fold lower in the BaxB01-treated group (Fig. 6D). In addition, urinary levels of the proinflammatory cytokines TNF-α and IL-1β decreased (Supplemental Fig. 2). Immunohistochemistry staining of the rat kidney showed that the BaxB01 Ab was localized in the glomeruli and the adjacent tubular regions, exactly those regions where inflammation and expression of oxMIF was expected. In contrast, staining for total MIF showed a more uniform distribution that could not be linked to foci of inflammation (Fig. 6E).

The outcomes of these studies demonstrate the anti-inflammatory effect of Abs BaxB01 and BaxG03 in rodent models of IBD and crescentic glomerulonephritis. In previous studies we showed that Abs BaxB01, BaxG03, and BaxM159 have beneficial effects in models of experimental sepsis and contact hypersensitivity (30). Collectively, these data strongly support the concept that mAbs directed against oxMIF alleviate inflammation in vivo.

**In vivo synergy between oxMIF-specific Abs and glucocorticoids**

One specific property of MIF is its ability to override immunosuppressive effects of glucocorticoids (1, 3, 27). Thus, we used the

![FIGURE 6. Effects of the oxMIF-specific Ab BaxB01 in a rat model of crescentic glomerulonephritis. After disease induction, two treatments with anti-oxMIF Ab BaxB01 (40 mg/kg) significantly reduced proteinuria (A), infiltration of ED1 macrophages (Mφ/gcs: macrophages per glomerular cross section) into the glomeruli (B), and the percentage of glomeruli with crescents (C) compared with isotype control IgG (40 mg/kg)-treated animals, and data are shown as median ± interquartile range. We used the one-way ANOVA followed by Dunnnett’s multiple comparison test (against control IgG group) or Student two-tailed t test for statistical analyses (n = 8 rats/group). *p < 0.05, **p < 0.001, ***p < 0.0001. Graphs are representative of four independent experiments. (E) Representative immunohistochemistry staining of kidneys from diseased rats treated with BaxB01 or control IgG (Ctrl IgG) or vehicle. DAB staining and hematoxylin counterstaining. Original magnification ×20. The formalin-fixed kidney cryosections were incubated with polyclonal peroxidase-conjugated anti-human IgG (to detect BaxB01 and control IgG) or BaxB01 and peroxidase-conjugated anti-human IgG (to detect total MIF; red MIF is denatured due to fixation and therefore accessible to BaxB01 binding; see Fig. 1F). Inset shows a control staining with nonimmune human IgG. ns, not significant.**
and glomerular crescents (Fig. 7C). We selected the subtherapeutic dose of 0.025 mg/kg dexamethasone for combination with different doses of BaxB01. Rats received a single dose of dexamethasone on day 4 and treatment with BaxB01 on days 4 and 6 after disease induction. We treated control rats with either dexamethasone or BaxB01 alone. Dose–response curves demonstrated an amplified reduction of proteinuria (Fig. 7D), macrophage infiltration (Fig. 7E), and generation of glomerular crescents (Fig. 7F) when BaxB01 and a subtherapeutic dose of dexamethasone are combined. Furthermore, the results indicated a greater reduction of disease parameters with the combination than expected with the sum of BaxB01 and dexamethasone monotherapy. The effects of anti-oxMIF combined with 0.025 mg/kg dexamethasone were more pronounced than achieved with a 10-fold higher dose of dexamethasone monotherapy, implying that BaxB01/dexamethasone synergy leads to a >10-fold higher apparent activity of dexamethasone. These data demonstrate that an anti-oxMIF Ab treatment potentiates glucocorticosteroids in alleviating crescentic glomerulonephritis.

Discussion

Our data show that MIF occurs in two immunologically distinct conformational isoforms, reduced MIF and oxMIF, which reversibly interconvert, dependent on the local redox potential. Whereas MIF is abundantly expressed in the body and can be detected in the circulation, on the cell surface, and in the cytoplasm of cells isolated from healthy controls, we found oxMIF predominantly expressed in patients with inflammatory diseases. Moreover, we found that oxMIF is produced specifically during acute inflammatory phases and its presence could be linked to sites of inflammation.

MIF is a proinflammatory cytokine that uses different mechanisms, which include: 1) overriding glucocorticoids, 2) triggering the release of other proinflammatory cytokines from monocytes/macrophages, and 3) chemokine functions postulated to play a role in the recruitment of leukocytes into inflamed tissues (1–3, 6, 37, 45, 46). We showed that anti-oxMIF treatment interfered with these three mechanisms. In the mouse colitis models and the rat glomerulonephritis model, the beneficial effects of anti-oxMIF treatment were linked to extenuated immune responses characterized by a reduction of Th1-biased inflammatory cytokines. In the rat glomerulonephritis model, treatment with anti-oxMIF Abs reduced the number of infiltrating macrophages, thus establishing a direct link to the inhibition of MIF chemokine functions. Because combination of dexamethasone with anti-oxMIF Abs not only resulted in additive but also clear amplified treatment effects in the rat glomerulonephritis model, we conclude that anti-oxMIF Abs inhibit inflammatory triggers of the MIF-glucocorticoid axis.

In vitro conversion of reduced MIF to oxMIF is not associated with posttranslational modifications, as described previously (47, 48) (data not shown). Indeed, the molecular basis of the structural rearrangement of MIF under proinflammatory conditions is unknown. Several redox-regulated proteins are known to interact with MIF (33). Other studies have identified hepatopoietin (49), tumor suppressor p53 (50), and peroxiredoxin (51) as intracellular targets for MIF, whereas binding of thioredoxin in the extracellular space regulates internalization and proinflammatory signaling of MIF in vitro (52). However, we suspect that the conversion of abundantly expressed MIF to a disease-related isoform (oxMIF) is a tightly regulated mechanism that stimulates disease progression by activating and triggering proinflammatory signals.

We have previously reported the generation of a highly diverse panel of 145 unique fully human anti-MIF Abs derived from a phage display library (30). This panel has been screened for Abs that neutralize MIF biologic activity in vitro and in vivo. In this previous report (30), we showed that in vivo protective anti-MIF Abs are specific for two distinct epitopes (aa 50–68 or 86–102), which form a β-barrel structure containing the functionally important thiol protein oxidoreductase motif. Exactly the same Abs turned out to be specific for oxMIF. We repeatedly tested anti-MIF Abs from this panel that were not able to discriminate between oxMIF and reduced MIF in animal models, but these Abs failed our screening because we never observed a beneficial therapeutic effect. Our data indicate a remarkable correlation among epitope specificity, specificity for oxMIF, and in vivo protection in models of inflammation. We conclude that specific inhibition of oxMIF is a potent method of interfering with the biological activity of MIF. OxMIF is therefore a relevant new target in inflammation.

FIGURE 7. OxMIF-specific Abs synergize with dexamethasone in a rat model of crescentic glomerulonephritis. Treatment with three different doses of dexamethasone led to a dose-dependent reduction of proteinuria (A), infiltration of ED1 macrophages (MΦ/gcs: macrophages per glomerular cross section) into the glomeruli (B), and percentage of glomeruli with crescents (C) (n = 8 male rats/group). Treatment with different doses of anti-oxMIF Ab BaxB01 allowed the determination of different dose–response curves showing a reduction of proteinuria (D), infiltration of ED1 macrophages (MΦ/gcs: macrophages per glomerular cross section) into the glomeruli (E), and the percentage of glomeruli with crescents (F), dependent on whether animals were treated with BaxB01 alone (dashed lines) or in combination with a subtherapeutic dose of dexamethasone (solid lines). Graphs show the combined results of two independent experiments (in total n = 16 rats/group), and data are shown as mean ± SEM.
Furthermore, the detection of oxMIF in urine and plasma samples from patients with inflammatory diseases and the correlation of oxMIF with disease stages illustrate its potential as a biomarker for initial diagnostic screening and for differentiation between disease stages.

Disclosures

M.T., R.J.K., D.V., P.D., A.S., H.K., M.R., G.C.M., H.E., and F.S. are full-time employees of Baxter; R.J.K., H.E., and F.S. have ownership interest (including patents) in Baxter Healthcare; F.W.K.T., B.J., H.T., and C.T. have a research grant from Baxter and are consultants of the same; and J.K. is a consultant for Baxter. The remaining authors have no financial conflicts of interest.

References

Supplemental Figure 1. OxMIF and total MIF in mice with peritonitis and healthy controls. (A) Representative oxMIF FACS analysis of granulocyte (GR1+), monocyte (CD14+), B-cell (CD19+) and T-cell (CD3+) gated populations from *E. coli* O111:B4 challenged mice 1 hour (E 1h) or 22 hours (E 22 h) post challenge or prepared from control mice 1 hour after PBS injection (Ctr 1 h) using oxMIF specific antibody BaxB01 (black line) and Isotype IgG (grey line). (B) Representative total MIF FACS analysis of granulocyte (GR1+), monocyte (CD14+), B-cell (CD19+) and T-cell (CD3+) gated populations from *E. coli* O111:B4 challenged mice 1 hour (E 1h) or 22 hours (E 22 h) post challenge or prepared from control mice 1 hour after PBS injection (Ctr 1 h) using total MIF specific antibody FL115 (black dashed line) and Isotype IgG (grey line). (C) Spleen cells lysates from *E. coli* challenged and control mice were subjected to immunoprecipitation with oxMIF specific antibodies BaxB01, BaxM159 or an isotype control antibody (Ctr). As positive control, 5-chloro-2-methyl-4-isothiazolin-3-one (P), which converts MIF into surrogate oxMIF, was added during antibody incubation. Intracellular MIF could only be precipitated from spleen cell lysates when 5-chloro-2-methyl-4-isothiazolin-3-one was added to the samples (left and right picture are from different western blots; different parts of the same blot are separated by a parting line in the right picture).
Supplemental Figure 2. Effects of the oxMIF-specific antibody BaxB01 in a rat model of crescentic glomerulonephritis. After 4 days of treatment, repeated injection of anti-oxMIF antibody BaxB01 (40 mg/kg) significantly reduced levels of inflammatory cytokines IL-1β (A) and TNF-α (B). Data are expressed as mean and SEM from two independent experiments (n=16). One-way ANOVA followed by Dunnett’s Multiple Comparison test (against control IgG group) were used for statistical analyses. ** p<0.01; *** p<0.001.
Supplemental Figure 3

**Supplemental Figure 3. Short and long-term stability analysis of oxMIF in plasma.** Three plasma samples from SLE patients were pooled and stored in aliquots at 4°C for 48 hours or at -80°C over a 6 month period. Aliquots were tested at indicated time points for oxMIF (full squares) and total MIF (full circles) concentration by ELISA. The results show that short term storage at 4°C (A) or long term storage at -80°C (B) does not influence oxMIF or total MIF concentration and confirms stability of both MIF isoforms at the storage conditions chosen.