Macrophage migration inhibitory factor (MIF) has recently been forwarded as a critical regulator of inflammatory conditions, and it has been hypothesized that MIF may have a role in the pathogenesis of asthma and chronic obstructive pulmonary disease (COPD). Hence, we examined effects of MIF immunoneutralization on the development of allergen-induced eosinophilic inflammation as well as on lipopolysaccharide (LPS)-induced neutrophilic inflammation in lungs of mice. Anti-MIF serum validated with respect to MIF neutralizing capacity or normal rabbit serum (NRS) was administered i.p. repeatedly during allergen aerosol exposure of ovalbumin (OVA)-immunized mice in an established model of allergic asthma, or once before instillation of a minimal dose of LPS into the airways of mice, a tentative model of COPD. Anti-MIF treatment did not affect the induced lung tissue eosinophilia or the cellular composition of bronchoalveolar lavage fluid (BALF) in the asthma model. Likewise, anti-MIF treatment did not affect the LPS-induced neutrophilia in lung tissue, BALF, or blood, nor did it reduce BALF levels of tumor necrosis factor-α (TNF-α) and macrophage inflammatory protein-1α (MIP–1α). The present data suggest that MIF is not critically important for allergen-induced eosinophilic, and LPS-induced neutrophilic responses in lungs of mice. These findings do not support a role of MIF inhibition in the treatment of inflammatory respiratory diseases.

Key words: Macrophage migration inhibitory factor (MIF), Allergen-induced eosinophilia, LPS-induced neutrophilia, Mouse models of inflammatory lung diseases

Introduction

Macrophage migration inhibitory factor (MIF) is considered to be a critical regulator of various inflammatory conditions. For example, MIF is a pivotal mediator in the host response to endotoxic shock,¹ and plays an important role in the development of the delayed type hypersensitivity reaction and collagen-induced arthritis in mice.²,³ MIF may further have a proinflammatory role in the development of human acute respiratory distress syndrome.⁴ MIF was described originally to be a T-cell product, which inhibited the random migration of macrophages.⁵,⁶ Interestingly, recent data indicate that MIF is predominantly expressed by Th2-like T-cells.⁷ However, MIF also exists preformed in monocytes/macrophages, eosinophils, B-cells, airway epithelial cells, and corticotrophic cells within the anterior pituitary gland, and is released in response to various proinflammatory stimuli.¹,³,⁸–¹² MIF shares with other cytokines sensitivity to inhibition by therapeutic concentrations of glucocorticoids. However, at low physiological glucocorticoid concentrations MIF expression is induced.¹³,¹⁴ MIF has the property of counteracting anti-inflammatory and immunosuppressive actions of glucocorticoids.¹³,¹⁴ Anti-MIF therapeutic strategies are thus under development with the aim to increase the immunosuppressive and anti-inflammatory properties of endogenously released glucocorticoids, thereby reducing the requirement for steroid therapy in a variety of inflammatory conditions. It has further been suggested that drugs inhibiting MIF would be effective also in inflammatory conditions that exhibit steroid resistance.¹⁴

Airway mucosal inflammation in allergic asthma is characterized by infiltration and/or activation of eosinophils, macrophages, T-lymphocytes, and mast cells.¹⁵ Since all these immune cells can produce MIF in significant quantities, and since elevated levels of MIF have been detected in BALF from asthmatic patients compared with controls, it has been forwarded that MIF may have a role in eosinophilic airway disease.⁹

Recent studies have shown that genetically MIF-deficient mice are resistant to the lethal effects of a high systemic dose of LPS, but susceptible to a
combination of a low-dose of LPS and D-galactosamine. These mice exhibit no impairment of neutrophil migration to the peritoneum elicited by thioglycollate; despite diminished neutrophil accumulation in BALF they clear *Pseudomonas aeruginosa* instilled into the trachea better than wild type mice. Makita *et al.* recently demonstrated that immunoneutralization of MIF attenuates pulmonary neutrophil influx and acute lung injury induced by intraperitoneal (i.p.) administration of LPS in rats. These effects were associated with reduced BALF levels of macrophage inflammatory protein–2, a powerful neutrophil chemokine. Moreover, it has been demonstrated that MIF induces alveolar cells/macrophages to secrete TNF-α and IL–8, two cytokines widely thought to be critically important for neutrophil infiltration in pulmonary inflammatory conditions such as chronic obstructive pulmonary disease (COPD). In an attempt to further explore the hypothesis that MIF may have a role in bronchopulmonary eosinophilic and neutrophilic inflammation, we examined effects of MIF inhibition both in an established murine model of allergic asthma, and in a model involving LPS-induced neutrophilic inflammation in the lungs of mice. Specifically, anti-MIF serum was administered repeatedly during allergen aerosol exposure of immunized mice, or given once before instillation of a low dose of LPS into the lower airways of mice.

**Materials and methods**

**Animals**

Male C57BL/6 mice (*n* = 184, 6–8 weeks of age), were purchased from Bomholtgaard, Denmark. All mice were kept in well-controlled animal housing facilities and had free access to tap water and pelleted food throughout the experimental period. All animals were used under protocols approved by the Ethics Committee of the Faculty of Medicine at the University of Lund.

**Induction of allergic eosinophil-rich airway inflammation**

We have used a protocol slightly modified from that developed by Brusselle and colleagues. On the first day of the experiment (Day 0), all mice were actively immunized by i.p. injection of 10 μg chicken OVA (Grade III, Sigma, St Louis, MO, USA), adsorbed to 1 mg of alum adjuvant. Starting 14–16 days after immunization the mice were exposed once daily during 7 days to aerosolized saline (SAL) or OVA over a 30-min period by placing groups of 5–10 awake mice in an exposure chamber. The aerosol was generated into the chamber using a nebulizer (Bird 500 ml Inline Micronebulizer driven at 4 bar, Bird Co., Palm Springs, CA). The concentration of OVA in the nebulizer was 1% w/v. Animals were sacrificed by i.p injection of pentobarbital 8 h after the last aerosol exposure.

**Induction of neutrophil-rich airway inflammation**

Groups of mice received one intratracheal instillation of a low dose of *Escherichia coli* LPS (Difco Lab., MI, USA, 4 μg/kg, i.e., ~0.08 μg/animal, diluted in ~20 μl saline) or SAL. The present LPS dose is comparable with occupational levels. For example, it has been estimated that cotton mill workers are exposed to 60 μg endotoxin per day. In preliminary dose–response experiments our selected dose of LPS was shown to induce submaximal responses regarding TNF-α levels and total cell numbers in BALF. For the instillation procedure, animals were anaesthetized with enflurane, and a blunt cannula was introduced perorally into the trachea. Animals were sacrificed by i.p injection of pentobarbital 4 or 24 h after LPS or SAL administration.

**In vivo neutralization of MIF**

Starting the day before the first allergen aerosol challenge, mice (*n* = 20) were injected i.p. with 200 μl of rabbit anti-murine MIF serum; this treatment was then repeated every 3 days until termination of the experiment. Control mice (*n* = 20) were injected with a similar volume (200 μl) of NRS. Other groups received no treatment at all. Groups of mice subjected to intratracheal LPS instillation received one i.p. injection of anti-MIF serum (200 μl), NRS, or SAL 12 h before LPS challenge (*n* = 8 in each group). This anti-MIF treatment has previously been shown to neutralize MIF *in vivo* using the same dose level and administration route in mice as in this study. Components in serum may have some capacity to downregulate inflammatory responses, underscoring the need for proper control groups in the evaluation of experiments using anti-serum. Thus, in this study groups of mice treated with anti-MIF serum were compared with corresponding groups of mice treated with NRS.

In additional experiments we explored whether anti-MIF treatment might be effective via the local route. Groups of mice (*n* = 4 in each group) received 15 μl anti-MIF serum or NRS administered intratracheally, alone or together with LPS solution (4 μg/kg) in a total volume of ~20 μl. The present batch of anti-MIF serum was checked for bioactivity at our laboratory using a protocol, which has previously been employed to analyse the role of MIF in endotoxaemia. Groups of mice (*n* = 5–6 in each group) received one i.p. injection of anti-MIF serum (200 μl), NRS, or SAL 2 h before i.p. injection with a high dose of LPS (25 mg/kg). Ninety minutes after LPS challenge blood was collected by
cardiac puncture and placed into EDTA tubes. As an indicator of LPS-induced plasma extravasation, the haematocrit was determined by an automated haematology analyser (Sysmex K–4500, TOA Medical Electronics Co., Kobe, Japan).

Histological analysis of allergic airway inflammation

Lung tissue specimens obtained 8h after the last OVA or SAL exposure were immersed overnight in Stefanini's fixative (2% paraformaldehyde and 0.2% picric acid in 0.1 M phosphate buffer, pH 7.2), rinsed repeatedly in buffer (Tyrode's buffer supplemented with 10% sucrose), frozen in mounting medium (Tissue-Tek, Miles Inc, Elkhart, IN, USA), and stored at −80°C until sectioning. Eosinophils were detected by histochemical visualization of cyanideresistant eosinophil peroxidase (EPO) activity. Briefly, cryosections (10 μm) were incubated for 8 min at room temperature in PBS buffer (pH 7.4) supplemented with 3,3-diaminobenzidine tetrahydrochloride (60mg/100ml, Sigma), 30% H2O2 (0.3ml/100ml), and NaCN (120mg/100ml). Slides were then rinsed in water and mounted in Kaiser's medium (Merck, Darmstadt, Germany). Eosinophils were identified by their dark-brown reaction product. For evaluation of the number of eosinophils in pulmonary tissue, 40 randomly selected areas (0.04mm2 each) in one lung section from each animal were examined. The number of eosinophils in the 40 areas was counted at a magnification of 400, and the mean was expressed as eosinophils/unit area. Cell counts were made in a blinded fashion. For assessment of general airway morphology sections were stained with haematoxylin and erythrosin. Lung tissue specimens and tracheobronchial lymph nodes immersed in buffered 4% paraformaldehyde (pH 7.2), dehydrated, and embedded in paraffin, were used for immunohistochemical visualization of MIF-expressing cells. Sections were incubated overnight in 4°C in a moist chamber with a 1:800 dilution of the rabbit anti-murine MIF serum. The anti-MIF serum used in the present study has previously been used for immunohistochemistry. NRS at a dilution of 1:800 or PBS were used in control sections. The site of the antigen-antibody reaction was revealed by application of fluorescein isothiocyanate-conjugated swine antisera directed at rabbit immunoglobulins (DAKO, Glostrup, Denmark) diluted 1:80 for 1 h at room temperature. In control sections, omitting the primary antibody, only slight yellowish auto-fluorescence was found.

Analysis of cells in peripheral blood and BALF, and measurement of TNF-α and MIP–1α levels in BALF and plasma

Animals were anaesthetized with an i.p. injection of pentobarbital. The chest was opened and a blood sample was collected via the still beating heart. A tracheal cannula was inserted via a midcervical incision. The airways were lavaged once (LPS-challenged mice) or twice (OVA-exposed mice) with 0.7ml of PBS (Life Technologies, Paisley, UK). The BALF was immediately centrifuged (10 min, 4°C, 160 × g). Cell pellets were resuspended in 250 μl PBS for total and differential cell counting and the supernatants were rapidly frozen. Differential counting was performed on May-Grünwald-Giemsa stained cyto spins and blood smears. Between 200 and 500 cells were counted on each cyto spin, and 100 cells were counted on each blood smear. Commercial ELISA kits (R&D systems, MN, USA) were used to measure levels of TNF-α and MIP–1α in the BALF of LPS and SAL challenged mice. TNF-α levels were also measured in plasma obtained from LPS challenged mice. The limit of detection was 5.1 pg/ml for TNF-α, and 1.5 pg/ml for MIP–1α.

Statistics

Data are expressed as mean ± SEM. To calculate significance levels between treatment groups, the Student's t-test was used throughout the study. ELISA values below detection limits were assigned the value of the detection limit. Probabilities <0.05 were used as the generally accepted level of statistical significance for differences between mean values.

Results

Additional validation of the present anti-MIF sera

Exposure of a high dose of endotoxin is known to cause plasma extravasation and subsequent loss of circulating plasma volume. In order to ascertain a
preserved activity of the anti-MIF serum, the haematocrit was determined 90 min after i.p. LPS challenge as a measure of plasma extravasation. The haematocrit was significantly increased in LPS-challenged mice compared with SAL-challenged mice (53.1 ± 4.0% vs. 45.2 ± 6.2%, \( P < 0.001 \)). This response was inhibited in mice treated with anti-MIF serum before LPS challenge (45.7 ± 2.1% vs. 50.8 ± 5.0% in corresponding NRS-treated mice, \( P < 0.01 \)).

**Effect of anti-MIF treatment on allergen-induced airway inflammation**

To assess the role of MIF in allergic airway inflammation, lung tissue eosinophilia and cellular composition of BALF were determined in anti-MIF-treated and NRS-treated mice 8 h after last allergen aerosol exposure. The number of eosinophils in lung tissue was similar in allergen aerosol exposed anti-MIF-treated and NRS-treated mice (Fig. 1). Likewise, total cellular content (data not shown) and the percentage of eosinophils, neutrophils, lymphocytes, and macrophages in BALF did not differ significantly between anti-MIF-treated and NRS-treated mice after allergen aerosol exposure (Fig. 2).

Untreated SAL and OVA exposed mice were also included in the study, to check that the present inflammation was specifically induced by OVA challenge. Histologic analysis of lungs taken from OVA-exposed mice revealed the presence of peribronchial and perivascular infiltrates, whereas lung tissue taken from SAL-exposed animals showed normal lung histology. Allergen aerosol exposures caused a marked eosinophilia in pulmonary tissue (12.1 ± 2.0 vs. 3.0 ± 0.5 eosinophils/unit area in SAL exposed mice, \( P < 0.01 \)). A significant increase in the percentage of eosinophils (44.4 ± 12.2% vs. 2.2 ± 1.6% in SAL-exposed mice, \( P < 0.05 \)), neutrophils (1.7 ± 0.5% vs. 0.1 ± 0.1% in SAL-exposed mice, \( P < 0.05 \)), and lymphocytes (7.5 ± 1.6% vs. 0.8 ± 0.3% in SAL-exposed mice, \( P < 0.01 \)) was demonstrated in response to OVA challenge.

Immunohistochemistry was used to visualize the MIF-expressing cells in the pulmonary infiltrates and tracheobronchial lymph nodes of OVA-challenged mice. A majority of the leukocytes in the perivascular and peribronchial pulmonary infiltrates were MIF-positive (Fig. 3a). Interestingly, large, intensely MIF-positive cells with dendritic shape were seen in the superficial cortex of the tracheobronchial lymph nodes in OVA-challenged mice (Fig. 3b). Lymphocytes, mainly located in the cortex, were also stained for MIF, although less intensely (Fig. 3c). The exact identity of the MIF-positive cells was not further evaluated in the present study. No staining except for a yellowish auto-fluorescence (compare Fig. 3a and d) was observed in control sections where NRS or PBS was used instead of the anti-MIF serum (not shown).

**Characterization of the present ‘low dose’ LPS model**

Intratracheal instillation of a minimal dose of LPS into the lower airways of mice induced a significant increase in total cellular content of BALF 4 h after challenge (51.9 ± 5.5 vs. 10.6 ± 2.6 BALF cells \( \times 10^4 \)/ml in SAL challenged controls, \( P < 0.001 \)). This increase remained 24 h after LPS instillation (33.8 ± 4.6 vs. 12.6 ± 1.8 BALF cells \( \times 10^4 \)/ml in SAL challenged controls, \( P < 0.01 \)). The percentage of
FIG. 3. Immunohistochemistry. Visualization of MIF-expressing cells in the pulmonary infiltrates (a) and tracheobronchial lymph nodes (b and c) of OVA challenged mice, and visualization of MPO-positive cells in the lungs of LPS-challenged mice (d). A majority of the leukocytes in the pulmonary infiltrates (arrows) of OVA-challenged mice were stained with anti-MIF serum (a). Interestingly, large, intensely MIF-positive cells with dendritic shape (arrow) were seen in the superficial cortex of the tracheobronchial lymph nodes in OVA-challenged mice (b). Lymphocytes, mainly located in the cortex, were also stained for MIF, although less intensely (c). In deeper portions of the lymph nodes many cells were unstained (c). A multifocal perivascular and peribronchial MPO-positive (neutrophilic) distribution (arrows) was seen at 4 h after intratracheal LPS-challenge (d). In (a) and (d) a slight yellowish auto-fluorescence, mainly located in the lung parenchyma, is observed. B = bronchus, V = blood vessel. Original magnification × 250.
neutrophils in BALF was strikingly increased at both 4h and 24h after LPS challenge (77.3 ± 2.7% and 53.1 ± 5.9%, respectively, vs. 6.8 ± 4.3% and 8.3 ± 5.9%, respectively, in SAL-challenged controls, P<0.001). The percentage of lymphocytes remained low 4h after LPS challenge (0.8 ± 0.2% vs. 1.4 ± 0.3% in SAL-challenged controls). However, an increase of lymphocytes was observed 24h after LPS instillation (2.3 ± 0.5% vs. 0.9 ± 0.3% in SAL-challenged controls, P<0.05).

The percentage of polymorphonuclear leukocytes (PMN) in peripheral blood increased rapidly after intratracheal LPS instillation (48.8 ± 3.1% vs. 21.2 ± 3.1% in SAL-challenged controls at the 4h time point, P<0.001). At 24h after LPS instillation the percentage of PMN in peripheral blood had returned to baseline levels (23.1 ± 1.6% vs. 20.1 ± 3.5% in SAL-challenged controls).

Histological analysis of lungs taken 4 hours after LPS challenge demonstrated a moderate neutrophilia perivascularly and peribronchially (Fig. 3d). Neutrophils were also detected in alveolar walls and spaces. A similar, but reduced, distribution of neutrophils was observed 24 hours post LPS instillation (not shown).

High levels of TNF-α were detected in BALF 4h after LPS challenge (3302.7 ± 437.9 pg/ml versus 17.0 ± 7.0 pg/ml in SAL-challenged controls, P<0.001). A small increase in levels of TNF-α could still be detected 24h after LPS challenge (16.3 ± 3.1 pg/ml vs. 5.3 ± 0.2 pg/ml in SAL-challenged controls, P<0.01). Intratracheal instillation of LPS also induced a significant increase of MIP–1α levels in BALF 4h after challenge (3942.6 ± 280.4 pg/ml vs. 26.2 ± 14.9 pg/ml in SAL-challenged controls, P<0.001).

Effect of anti-MIF treatment on LPS-induced airway inflammation

To examine the role of MIF in LPS-induced airway inflammation, cellular profile in BALF and neutrophilia in lung tissue and blood were determined in anti-MIF-treated and NRS-treated mice. At 4h and 24h after intratracheal instillation of a low dose LPS total cellular content (data not shown) and the percentage of neutrophils, lymphocytes, and macrophages in BALF were similar in LPS-challenged anti-MIF-treated and NRS-treated mice (Fig. 4a and b). Consistent with the findings in BALF, the number of PMN in blood did not differ significantly between anti-MIF-treated and NRS-treated animals at either time points (Fig. 4a and b). Also, no obvious difference in lung tissue neutrophilia was observed between anti-MIF-treated and NRS-treated animals at 4h and 24h after LPS challenge (data not shown).

Since MIF has been reported to modulate the expression of TNF-α and chemokines in models of endotoxaemia and acute lung injury, TNF-α and MIP–1α levels were measured in LPS-challenged anti-MIF-treated and NRS-treated mice. At 4h after LPS instillation mice treated with anti-MIF serum exhibited similarly increased levels of TNF-α and MIP–1α in BALF as NRS-treated mice (Fig. 5). Equally low levels of TNF-α in BALF were observed in anti-MIF-treated and NRS-treated mice at 24h after LPS challenge (12.5 ± 2.6 pg/ml and 15.9 ± 4.8 pg/ml, respectively). Plasma levels of TNF-α were below detection limit in both anti-MIF-treated and NRS-treated mice at 4h after LPS challenge (data not shown).
Effect of intratracheal anti-MIF treatment on LPS-induced airway inflammation

LPS-challenged mice treated with topical intratracheal anti-MIF serum or NRS exhibited a similar neutrophilia in BALF at the 4h time point (86.5 ± 1.4% and 82.4 ± 2.0% neutrophils in BALF, respectively). Also 24h after LPS challenge no significant difference in BALF neutrophilia was observed between the intratracheally treated mice. Anti-MIF-treated and NRS-treated mice exhibited 68.0 ± 1.6% and 71.0 ± 4.8% neutrophils in BALF, respectively. The BALF neutrophilia in mice receiving intratracheal doses of anti-MIF serum or NRS together with LPS was somewhat increased when compared with that observed in BALF from animals receiving LPS challenge only. In accord, SAL-challenged mice treated intratracheally with NRS demonstrated a mild neutrophilia at both the 4- and 24h time points (35.9 ± 13.0 and 39.4 ± 11.8 % neutrophils in BALF, respectively).

Discussion

This study demonstrates that anti-MIF treatment does not have any major effects on the eosinophil-rich airway inflammation occurring in a murine model of allergic asthma. Similarly, anti-MIF treatment did not change the neutrophilic inflammatory response seen after instillation of a low dose of LPS into the lower airways of mice. Although we cannot exclude the possibility that MIF may regulate other indices of pulmonary inflammation than measured in this study, the present data do not support the view that MIF is critically involved in pulmonary eosinophilic or neutrophilic inflammatory conditions.
the present LPS challenge produced a potentially useful model of COPD. 29

In contrast to most cytokines, MIF mRNA and protein are expressed constitutively in a variety of cell types, such as monocytes/macrophages, T-cells, airway epithelial cells, and pituitary endocrine cells. Proinflammatory stimuli, including LPS, are known to increase MIF mRNA expression above the level present constitutively. 3,12 LPS administrated systemically (i.p.) in high doses has been used in earlier studies to elucidate the role of MIF in endotoxaemia and acute lung injury. 1,11,12,16 For example, treatment of mice with anti-MIF serum conferred full protection to the lethal effects of LPS (17.5 mg/kg) administrated i.p. 1 Consistent with these previous findings and confirming the validation of the employed anti-MIF serum, the present study showed that anti-MIF treatment inhibits plasma extravasation in response to a high systemic dose of LPS.

In a rat model of acute lung injury, Makita et al. 11 demonstrated that anti-MIF treatment reduced the number of neutrophils per alveolus and the BALF neutrophilia, induced by a high dose of LPS given systemically. In mice, i.p. administration of LPS (1–20 mg/kg) does not induce transpulmonary neutrophil migration and infiltration of neutrophils into the alveolar space, but only neutrophil sequestration within the lung vasculature. 36–38 In this study, a fraction of the previously employed i.p. doses of LPS (250–5000-fold less) was given locally into the airways. Reflecting the low dose and route of administration, plasma levels of TNF-α were below detection limit at 4 h after LPS challenge. In apparent contrast to the important role of MIF in host responses to high systemic doses of LPS, anti-MIF treatment did not change the neutrophil-rich inflammatory response induced by this mode of LPS exposure. To explain these data it is suggested that the importance of MIF in different models of LPS-induced host reactions may vary depending on the dose and/or the administration route of LPS. In accord, it has previously been shown that the mechanisms behind host responses to LPS may be completely different in models using high or low doses of LPS. 39 Genetically MIF-deficient mice are also resistant to the lethal effects of a high systemic dose of LPS, but susceptible to a combination of a low dose of LPS and D-galactosamine. 16 The demonstration that MIF is not involved in the present pulmonary neutrophilic inflammation may reduce the promise of anti-MIF compounds as future COPD drugs.

In conclusion, the present data suggest that MIF is not critically important for allergic eosinophilic, or LPS-induced neutrophilic inflammation, in airways of mice. If translatable to human disease conditions, 40 these findings do not support the notion that MIF inhibitors will be effective against eosinophilic or neutrophilic respiratory diseases, such as allergic asthma and COPD.

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