Gastrointestinal, Hepatobiliary and Pancreatic Pathology

Essential Role for Macrophage Migration Inhibitory Factor in Gastritis Induced by *Helicobacter pylori*

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Macrophage migration inhibitory factor (MIF) is an upstream regulator of immune and inflammatory responses; however, its role in *Helicobacter pylori* (HP)-associated gastritis remains unknown. We infected MIF knockout (KO) and wild-type mice with SS1 HP and found that 2 weeks after infection, MIF and its receptor CD74 were markedly up-regulated in wild-type mice. This up-regulation preceded the up-regulation of both tumor necrosis factor- α and intercellular adhesion molecule-1, as well as the development of moderate gastritis at 8 weeks, as determined by a significant infiltration of neutrophils, T cells, and macrophages. In contrast, KO mice were protected against HP-induced gastritis by preventing the up-regulation of CD74 and Th1-mediated immune injury, including a reduction in the Th1 transcriptional factor T-bet and the expression of interferon- γ . Additionally, inhibition of skin delayed type hypersensitivity reactions to HP antigens in KO mice also suggested a critical role for MIF in cell-mediated injury. A regulatory role for MIF in Th1-immune responses was further demonstrated by the finding that antigen-primed CD4⁺ T cells lacking MIF failed to differentiate into the Th1 phenotype; these cells were instead promoted to Th2 differentiation after challenge with HP antigen *in vitro*. Results from this study indicated that inhibition of HP-induced innate immune responses and Th1-mediated immune injury may be the key mechanisms by which KO mice failed to develop gastritis after HP infection. (Am J Pathol 2009, 174:1319–1328; DOI: 10.2353/ajpath.2009.080708)

Approximately 50% of the world's population has been infected with *Helicobacter pylori* (HP). HP infection results in the development of gastritis mediated by both innate and adaptive immune responses, including expression of tumor necrosis factor- α (TNF- α), interleukin-1 β (IL-1 β), IL-8, and Th1-dominant cytokines such as interferon- γ (IFN- γ) and IL-12. These mediators act by recruiting neutrophils, macrophages, and T cells to the gastric mucosa, resulting in gastric inflammation.^{1,2}

Migration inhibitory factor (MIF) is a multifunctional cytokine with an upstream regulatory role in inflammation.³ MIF, originally thought to be produced by activated T cells,⁴ now is recognized as a pre-formed cytokine that is stored within the cytoplasm of macrophages and many other cell types within the host.⁵ MIF is released on stimulation by stress, endo- and exotoxins, and infection.^{6,7} Once released, MIF acts as a pro-inflammatory cytokine to induce the expression of other inflammatory cytokines/mediators including IL-1, TNF α , IL-2, IL-6, IL-8, IFN- γ , and inducible nitric oxide synthase, which acts to recruit and activate macrophages and T cells, resulting in inflammation and immune-mediated disease.^{5–7} It now is well established that MIF plays a pathogenic role in many inflammatory and immune diseases, including ulcerative colitis,^{8–10} experimental autoimmune encephalomyelitis,¹¹ experimental autoimmune myocarditis,¹² experimental autoimmune diabetes,¹³ acute distress syndrome,¹⁴ rheumatoid arthritis,¹⁵ atherosclerosis,¹⁶ and kidney diseases.¹⁷

Evidence to support the importance of MIF in gastric inflammation comes from our recent studies that MIF is up-regulated in gastric inflammation and plays a role in gastric ulcer induced by acetic acid.¹⁸ Furthermore, we have also shown that up-regulation of gastric MIF is associated with HP infection.^{19,20} Interestingly, the association

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between functional promoter polymorphisms in the MIF gene and the progression of gastric mucosal inflammation and the development of mucosal atrophy suggests that MIF also is a risk factor for the subsequent development of gastric cancer.²¹ Nevertheless, the exact pathogenic role of MIF in HP-induced gastritis remains unclear.

Emerging evidence from recent studies demonstrates that Th1-mediated immune mechanisms may be critical in the development of gastritis associated with HP infection, in both animal models and human patients.^{22–27} It has been long considered that MIF is a T cell cytokine and mediates Th1 immune response.²⁸ Thus, we hypothesized that MIF may be a critical mediator in regulating Th1-mediated gastritis in response to HP infection. In the present study, we tested this hypothesis and explored the pathogenic role of MIF in HP-induced gastritis in MIF knockout (MIF KO) mice. In addition, the mechanism of MIF in regulating HP-induced Th1 and Th2 phenotype differentiation was studied *in vitro* in HP-antigen-sensitized CD4⁺ T cells.

Materials and Methods

H. pylori Culture and Quantitation

H. pylori, Sydney Strain 1(SS1) was grown on *Brucella* Agar (Oxoid, UK) supplemented with 8% horse blood and selective supplement for *H. pylori* (Oxoid) for 3 days under 37°C before harvest. *Lactobacilli rhamnosus* was obtained from American Tissue Culture Collection (ATCC 7469). The bacteria were grown on Malt Extract Agar (Oxoid) at 37°C for 2 days. A single colony was selected and then cultured in the lactic broth (Oxoid, UK) under 37°C for 1 day with aerobic conditions created by shaking vigorously (300 rpm). A standard curve of OD₆₀₀ versus colony forming unit was used to give a quantity of the bacteria in the suspension.

Animal Model of Gastritis Induced by Inoculation of *H. pylori*

MIF wild-type and MIF KO mice (C57/BL6, age 8 weeks, 20 g) were generated as previously described.²⁹ All mice

A *H. pylori* immunohistochemistry

B Histology

Figure 1. Effect of MIF KO on HP infection and histology. **A:** Immunostaining of anti-HP antibody showing equal levels of HP infection in gastric mucosa in both wild-type (wild-type) and KO mice. **B:** Representative histology sections stained with periodic acid-Schiff. Note that there are no detectable abnormal histological changes at 2 weeks in both MIF wild-type and KO mice (**c, d**) after HP infection. However, a moderate gastritis with many polymorphonuclear leukocytes (inserted picture, **arrows**) and mononuclear cell infiltration is developed in MIF wild-type (**e**), not in KO (**f**) mice at 8 weeks after HP infection. **C:** Quantitative histological score. **D:** Quantitative neutrophils cell infiltration. Each bar represents mean $\pm$ SEM for a group of six mice. *** $P < 0.001$ as compared with wild-type mice without HP infection; ** $P < 0.01$ as compared with the time-matched wild-type mice. Magnification: $\times 1000$ (**A**) and $\times 400$ (**B**).

were housed in the Animal Unit at the University of Hong Kong with free access to food and water. A mouse model of gastritis was induced in groups of six MIF wild-type and KO mice by intragastric injection of HP (SS1, 1×10^7 colony forming units). HP suspended in 500 μ l *Brucella* broth (Oxoid) was administered via gavage tube for three times every other day in 1 week. Based on our pilot experiments, the use of this dose produced a moderate gastritis in mouse antrum of the stomach. Groups of six MIF wild-type or KO mice were euthanized at 2 weeks and 8 weeks after the last HP inoculation. Gastric tissues including the body and antrum of stomach were collected and fixed in methyl Carnoy's solution for 24 hours for histology and immunohistochemistry or snap-frozen and stored at -80°C for RNA extraction. In addition, groups of six normal MIF wild-type and KO mice were used as controls. All experimental procedures were approved by the Hong Kong Hygiene Department and the Committee on the Use of Live Animals for Teaching and Research at the University of Hong Kong.

Immunization of Mice

Mice were sensitized by injection of HP sonicated protein (500 $\mu\text{g/ml}$) mixed with the complete Freund's adjuvant (Sigma-Aldrich, St. Louis, MO) in the ratio of 1:1/vol:vol by sonication. The mixture was injected subcutaneously into both flanks of the mice (50 μ l/each site). Seven days after immunization, skin delayed type hypersensitivity was tested and splenocytes were isolated for culture as described below.

Skin Delayed Type Hypersensitivity Test

Seven days after immunization as described above, skin delayed-type hypersensitivity (DTH) to the HP antigen was tested by subcutaneous injection of 2 μg of HP sonicated protein into the left footpad of the mouse. Two μg of sonicated protein from *L. rhamnosus* was injected into the right footpad of the mouse as a negative control. Skin swelling was measured by electronic caliper (Gilson,

Figure 2. Immunohistochemistry shows MIF and CD 74 expression in gastric tissues in MIF wild-type and KO mice with or without HP infection. **A:** MIF expression in gastric tissues with or without HP infection. Results show that there is a minimal MIF expression in normal gastric tissue in a wild-type (WT) mouse (a), which is markedly up-regulated at 2 and 8 weeks after HP infection (c, e). In contrast, no MIF expression is found in MIF KO mice throughout the disease time course (b, d, f). **B:** CD74 expression in gastric tissues with or without HP infection. Note that similar to MIF expression, a marked up-regulation of CD74 is found at 2 weeks (iii) after HP infection, which remains high at 8 weeks (e). In contrast, no up-regulation of CD74 is seen in mice deficient for MIF (ii, iv, vi). **C:** Quantitative analysis of MIF. **D:** Quantitative analysis of CD74. Each bar represents mean $\pm$ SEM for a group of six mice. *** $P < 0.001$ as compared with wild-type mice without HP infection; ### $P < 0.001$ as compared with the time-matched wild-type mice. Magnification = original $\times$ 400.

MO) after 24 hours and tissues were fixed with methyl Carnoy's solution for 24 hours and embedded in paraffin for immunohistochemical examination.

Splenocyte Culture

Mouse splenocytes were isolated by a sieving method at day 7 after immunization with the HP antigen. After washing, single spleen cells at 2×10^6 were seeded on the 24-well plate in Dulbecco's Modified Eagle medium supplemented with 20% fetal bovine serum, 0.1% penicillin, and streptomycin. Cells were stimulated with HP or *L. rhamnosus* protein at concentrations 0, 5, 10, 25, and 50 $\mu\text{g/ml}$ for 0, 48, and 72 hours. After stimulation, the differentiation of Th1 (CD4+IFN- γ +) or Th2 (CD4+IL-4+) T cells was quantitatively analyzed by two-color flow cytometry as described below.

Histology and Immunohistochemistry

Changes in gastric histology were examined in methyl Carnoy's- fixed, paraffin -embedded tissue sections (4 μm) stained with hematoxylin and eosin or periodic acid-Schiff. Histological changes were scored by updated Sydney System.³⁰ Neutrophils infiltrating the gastric mucosa were identified by their polymorph nuclear morphology and the number of polymorph nuclear cells in the entire antrum was counted under high-power fields ($\times 40$) by means of a 0.025-mm² graticule fitted in the eyepiece of the microscope. Data obtained were expressed as cells per mm².

For immunohistochemistry, sections (4 μm) were stained for over night at 4°C with the primary antibodies, including the goat anti-CD3 (DAKO Corporation, Carpinteria, CA), rat anti-F4/80 (macrophage marker (CD68); Serotec, Raleigh, NC), goat anti-TNF- α , goat anti-IFN- γ , goat anti-HP, rabbit anti-CD74 and MIF (all from Santa Cruz Biotechnology, CA) using the microwave antigen retrieval technique as previously described.^{18,31} After washing, sections were stained with the secondary antibodies with peroxidase-conjugated anti-rabbit or goat antibody followed by goat or rabbit peroxidase anti-peroxidase (Dako Corporation, CA) for 1 hour at room temperature. After being washed, color was developed with 3, 3-diaminobenzidine and counterstained with hematoxylin. An isotype-matched IgG (Sigma) was used as a negative control throughout the study.

Quantitative analysis of immunostaining was performed on coded slides as described previously.¹⁸ Briefly, the number of positive cells for CD3 and F4/80 was counted under high-power fields ($\times 40$) by means of a 0.025-mm² graticule fitted in the eyepiece of the microscope. The entire antrum and area of DTH-tested skin were examined. Positive cells were expressed as cells per mm². Expression of gastric MIF, CD74, IFN- γ , and TNF α in the entire antrum was determined using the quantitative Image Analysis System (Carl Zeiss Microimaging, Thornwood, NY). Briefly, the examined area of antrum was outlined, the positive staining patterns were identified, and the percent positive area calculated. Data were expressed as percent positive area examined.

Real Time Reverse Transcription-PCR

The expression of genes of interest within the tissues was detected by quantitative real-time reverse transcription-PCR. Total RNA was extracted from the gastric tissues or cultured spleen cells by RNA extraction kit and dissolved in RNAase free water. cDNA was prepared from RNA using the Perkin-Elmer Reverse Transcription System (Perkin Elmer Inc., Norwalk, CT) as per manufacturer instruction.

Real-time PCR was run with the Opticon real time PCR machine (MJ Research Inc., Waltham, MA) using IQ SYBR green supermix reagent (Bio-Rad, Hercules, CA). The specificity of the real time PCR was confirmed via routine agarose gel electrophoresis and Melting-curve analysis. The gene for glyceraldehyde-3-phosphate dehydrogenase was used as an internal standard. The primers used in this study were: MIF: 5'-GCAAGCCCCGACAGTACA-3', reverse 5'-CGTTCGTGCCGCTAAAAGTC-3'; CD74: 5'-TTCCGA-AATCTGCCAAACCT-3', reverse 5'-GACATTGGACGCAT-CAGCAA-3'; IFN- γ : forward 5'-TGGATATCTGGAGGAAC-TGGCA-3', reverse 5'-TTTCGCCTTGCTGTTGCTGA-3'; T-bet: forward 5'-AAGCAAGGACGGCGAATGTT-3', reverse 5'-TCCACCAAGACCACATCCACAA-3'; TNF α : 5'-CATGA-GCACAGAAAGCATGATCCG-3', reverse 5'-AAGCAGGA-ATGAGAAGAGGCTGAG-3'; ICAM-1: forward 5'-TCAGGT-ATCCATCCATCCCAGAGA-3', reverse 5'-AGCTCATCTT-

Figure 3. Real-time PCR shows mRNA expression of MIF, CD 74, TNF α , and ICAM-1 in gastric tissues in MIF wild-type (WT) and KO mice with or without HP infection. **A:** MIF mRNA expression at 8 weeks after HP infection. **B** and **C:** CD74 mRNA expression at 2 and 8 weeks after HP infection. **D** and **E:** TNF α mRNA expression at 2 and 8 weeks after HP infection. **F:** ICAM-1 expression at 8 weeks after HP infection. Each bar represents mean $\pm$ SEM for a group of six mice. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ as compared with wild-type mice without HP infection; * $P < 0.05$, ** $P < 0.01$ as compared with the time-matched wild-type mice.

TCAGCCACTGAGTC-3'; and glyceraldehyde-3-phosphate dehydrogenase: forward 5'-TGCTGAGTATGTCGTGGAGTCTA-3', reverse 5'-AGTGGGAGTTTGCTGTTGAAATC-3'.

Flow Cytometry

After culture, splenocytes were collected and fixed for 30 minutes in 2% paraformaldehyde and permeabilized by 0.5% saponin solution for 5 hours under 4°C. Then cells were stained with phycoerythrin-conjugated rat anti-mouse CD4 (e-Bioscience) for 30 minutes at 4°C, followed by a rabbit anti-mouse IFN- γ -fluorescein isothiocyanate (FITC) (Serotec) or IL-4-FITC (e-Bioscience). After being extensively washed, single cells were analyzed by flow cytometry (Cytomic FC500MPL, Beckman Coulter, High Wycombe, UK).

Statistical Analysis

Data obtained from both *in vivo* and *in vitro* studies were expressed as the mean $\pm$ SEM. Statistical analyses were performed using one-way analysis of variance, followed

by the *t*-test from GraphPad Prism 3.0 (GraphPad Software, Inc. San Diego, CA).

Results

Effects of MIF KO on Gastric Histology after *H. pylori* Infection

Both MIF KO and wild-type mice infected with HP exhibited strong positivity for the rapid urease test. Immunohistochemically, both MIF KO and wild-type mouse stomach at 8 weeks after HP infection showed equivalent HP positive immunostaining with the anti-HP antibody (Figure 1A), indicating that mice lacking MIF did not influence the extent of HP infection.

Histologically, periodic acid-Schiff-stained sections showed that although there was not detectable abnormalities in gastric histology in all animals at 2 weeks after HP infection, a moderate gastritis with many polymorph nuclear cells (neutrophils) and mononuclear cells infiltrating the mucosa was developed in MIF wild-type mice at 8 weeks after

Figure 4. Immunohistochemistry shows F4/80+ macrophage and CD3+ T cell infiltration in gastric tissues in MIF wild-type (WT) and KO mice with or without HP infection. **A:** F4/80+ macrophages. Results show that there are a few F4/80+ macrophages (brown, arrows) in normal gastric tissue in both MIF wild-type and KO mice without HP infection (a, b). The number of macrophages is not increased in the gastric tissues in both MIF wild-type and KO mice at 2 weeks (c, d), but is significantly increased in MIF wild-type (e), but not KO (f) mice at 8 weeks after HP infection. **B:** CD3+ T cells. Note that compared with normal (a), 2 weeks after HP infection, wild-type mice show a significant increase in CD3+ T cells (brown, arrows) in gastric tissues (c), which is further increased at 8 weeks (e). In contrast, MIF KO mice show no CD3+ T cell infiltration through out the entire disease time course (b, d, f). **C:** Quantitative analysis of macrophages. **D:** Quantitative analysis of T cells. Each bar represents mean $\pm$ SEM for a group of six mice. ****P* < 0.01 as compared with wild-type mice without HP infection; **P* < 0.05, ****P* < 0.001 as compared with diseased MIF wild-type mice. Magnification = original $\times$ 400.

HP infection, which was absent in mice lacking MIF (Figure 1B). This was further confirmed by histological score and the influx of neutrophils (Figure 1, C and D).

Gastric MIF and MIF Receptor CD74 Are Up-Regulated in MIF Wild-Type, but Not in MIF KO Mice after *H. pylori* Infection

We next examined whether HP infection up-regulated MIF and its receptor CD74 in murine gastric tissue. In uninfected gastric tissue, immunohistochemistry and quantitative real-time PCR showed minimal MIF mRNA and protein expression was constitutively expressed by gastric epithelial cells in wild-type, but not in KO mice (Figure 2, Aa, b, and C; and Figure 3A). The MIF receptor, CD74, was weakly expressed in the gastric tissues in both normal wild-type and KO mice (Figures 2 Ba, b, and D, and 3B). By contrast, both real-time PCR and immunohistochemistry also showed that a marked up-regulation of gastric MIF and CD74 mRNA and protein was found in the wild-type mice at 2 weeks after HP infection, preceding the development of gastric inflammation at 8

weeks as shown histologically in Figure 1, B–D. A further increase in MIF and CD74 in MIF wild-type mice was detected at 8 weeks after HP infection (Figures 2 and 3, A–C). However, mice lacking MIF remained negative for MIF and did not show an increase in CD74 mRNA and protein expression in HP-infected stomach through out the entire disease course (Figures 2 and 3, A–C).

MIF KO Mice Are Protected Against Gastric Inflammation Induced by *H. pylori* Infection

Immunohistochemistry revealed that there were few CD3+ T cells and F4/80+ macrophages in normal gastric mucosa in both MIF KO and wild-type mice (Figure 4, Aa, b, Ba, b, C, and D). At 2 weeks after HP infection, although gastric F4/80+ macrophages remained low, CD3+ T cells was significantly increased in wild-type mice (Figure 4). By 8 weeks after HP infection, all MIF wild-type mice exhibited a prominent macrophage and T cell infiltration with the development of moderate gastritis (Figure 4). By contrast, there was no increase in macrophage and T cell infiltration in the gastric tissues of MIF KO though out the disease course

Figure 5. Immunohistochemistry demonstrates that mice lacking MIF show a prevention of gastric TNF α and IFN- γ expression after HP infection. **A:** Gastric TNF α expression: (a) minimal expression of TNF α protein (light brown) in the normal gastric tissue in a MIF wild-type mouse; (b) minimal expression of TNF α protein (light brown) in the normal gastric tissue in a MIF KO mouse; (c–f) moderate to strong TNF α expression in the gastric mucosa of a MIF wild-type mouse at 2 (c) and 8 (e) weeks after HP infection, which is not prevented in MIF KO mice (d, f). **B:** IFN- γ expression. Similar to TNF α expression, IFN- γ expression is increased, but not statistically significant, in gastric tissues in wild-type mice at 2 weeks after HP infection (c), which is largely up-regulated at 8 weeks (e). In contrast, no up-regulation of gastric IFN- γ is seen in MIF KO mice with HP infection (b, d, f). **C:** Quantitative analysis of gastric TNF α expression. **D:** Quantitative analysis of gastric IFN- γ expression. Each bar represents mean $\pm$ SEM for a group of six mice. * P < 0.05, *** P < 0.001 as compared with wild-type mice without HP infection; # P < 0.05, ### P < 0.01 as compared with the time-matched MIF wild-type mice. Magnification = original $\times$ 400.

Figure 6. Real-time PCR shows that deletion of MIF prevents up-regulation of Th1 cytokine IFN- γ and transcription factor T-bet expression in gastric tissues after HP infection. **A, B:** IFN- γ mRNA expression in MIF wild-type and KO mice at 2 weeks and 8 weeks after HP infection. **C, D:** T-bet mRNA expression in MIF wild-type and KO mice at 2 and 8 weeks after HP infection. Each bar represents mean $\pm$ SEM for a group of six mice. * P < 0.05, ** P < 0.01 as compared with wild-type mice without HP infection; * P < 0.05, ** P < 0.01 as compared with diseased MIF wild-type mice.

after HP-infection, indicating that MIF deficiency resulted in protection from gastric inflammation.

It has been well demonstrated that the pro-inflammatory cytokine TNF α and adhesion molecule ICAM-1 play a

critical role in gastric inflammation associated with HP infection.^{1,2} We examined gastric TNF α and ICAM-1 expression by immunohistochemistry and real-time PCR and observed marked up-regulation of gastric TNF α and ICAM-1 in wild-type mice at 8 weeks after HP infection (Figure 3, D–F and Figure 5, A and C). By contrast, mice lacking MIF exhibited normal levels of TNF α and ICAM-1 expression (Figures 3, D–F and 5, A and C).

MIF KO Mice Are Protected Against Th1-Mediated Immune Injury Induced by *H. pylori* Infection

Th1-mediated immune injury is critical for the development of gastritis associated with HP infection^{22–27} and MIF has been reported to be associated with a Th1-type T cell response.^{7,28} Accordingly, we examined the pathogenic role of MIF in Th1-mediated immune response in gastric tissues after HP infection. As shown in Figure 6C, a Th1 master transcriptional factor T-bet mRNA was significantly up-regulated in MIF wild-type mice at 2 weeks after HP infection, contributing significantly to a marked up-regulation of a Th1 signature cytokine IFN- γ at 8 weeks after infection with HP (Figure 5, B and D, and Figure 6, A and B). By contrast, mice lacking MIF exhibited normal mRNA and protein levels of IFN- γ and T-bet in gastric tissues infected with HP through out the entire disease time course (Figure 5, B and D, and Figure 6,

Figure 7. Mice lacking MIF are protected against a skin DTH response in response to HP antigen challenge. **A:** Skin macrophage (F4/80+) infiltration: (a) a MIF wild-type mouse after control lactobacilli antigen (LB) challenge; (b) a MIF KO mouse after control lactobacilli antigen (LB) challenge; (c) a MIF wild-type mouse after HP antigen challenge; (d) a MIF KO mice after HP antigen challenge; (e) Quantitative analysis of F4/80+ macrophages in the skin. **B:** Skin CD3+ T cell infiltration: (a) a MIF wild-type mouse after control lactobacilli antigen (LB) challenge; (b) a MIF KO mouse after control lactobacilli antigen (LB) challenge; (c) a MIF wild-type mouse after HP antigen challenge; (d) a MIF KO mice after HP antigen challenge; (e) Quantitative analysis of CD3+ T cells in the skin. Positive immunostaining cells are indicated in dark black. Each bar represents mean $\pm$ SEM for a group of 3 mice. * P < 0.05, ** P < 0.01, *** P < 0.001 compared with mice infected with control antigen (LB); ## P < 0.01, ### P < 0.001 compared with diseased MIF wild-type mice. Magnification = original $\times$ 400.

Figure 8. Antigen-sensitized CD4⁺ T cells lacking MIF fail to differentiate into the Th1 phenotype (CD4⁺IFN- γ ⁺) as demonstrated by two-color flow cytometry. **A:** Histogram of CD4-PE, IFN- γ -FITC, and CD4⁺IFN- γ ⁺ in response to lactobacilli (LB) or HP antigen challenge. **B:** A time-dependent response of the CD4⁺IFN- γ ⁺ subset induced by HP or control LB antigens. **C:** A dose-dependent response of the CD4⁺IFN- γ ⁺ subset induced by HP or control LB antigens. Each bar represents mean $\pm$ SEM for a group of three independent experiments. * P < 0.05, ** P < 0.01 as compared with normal control mice (0); # P < 0.05, ## P < 0.01 as compared with MIF wild-type mice.

A–D), supporting a critical role for MIF in the pathogenesis of Th1-mediated gastritis infected with HP.

To further determine the contributory role for MIF in Th1-mediated immune injury associated with HP infection, a classical skin DTH reaction was performed. As shown in Figure 7, HP antigen-primed MIF wild-type mice exhibited a strong skin DTH reaction as demonstrated by a significant increase in F4/80⁺ macrophage and CD3⁺ T-cell infiltration in response to HP antigen challenging (Figure 7, A c and e, and B, c and e), but not to control lactobacilli antigen (Figure 7, A, a and e, and B, a and e). MIF KO mice by contrast showed an unresponsiveness to HP, as well as lactobacilli antigen challenge, with only minimal T cell and macrophage infiltration (Figure 7, A b, d, and e, and B, b, d, and e).

Antigen-Sensitized Spleen CD4⁺ T Cell Lacking MIF Are Failed to Differentiate into the Th1 Phenotype After Challenge with *H. pylori* Antigen in Vitro

To investigate the mechanism by which MIF regulates Th1-mediated gastritis induced by HP infection, we per-

Figure 9. Antigen-sensitized CD4⁺ T cells lacking MIF are promoted to differentiate into the Th2 phenotype (CD4⁺IL-4⁺) as demonstrated by two-color flow cytometry. **A:** Histogram of CD4-PE, IL-4-FITC, and CD4⁺IL-4⁺ in response to HP antigen challenge. **B:** Quantitative analysis of a dose-dependent response of the CD4⁺IL-4⁺ subset induced by HP antigens. Each bar represents mean $\pm$ SEM for a group of three independent experiments. * P < 0.05, ** P < 0.01, and *** P < 0.001, as compared with normal control mice (0); # P < 0.05, and ## P < 0.01 as compared with MIF wild-type mice.

formed studies in HP antigen-sensitized spleen T cells *in vitro*. CD4⁺ T cells expressing the Th1 cytokine IFN- γ or Th2 cytokine IL-4 were examined by two-color flow cytometry. The addition of HP antigen but not a control lactobacilli antigen induced MIF wild-type CD4⁺ T cells to differentiate into the Th1 phenotype in a time and dose-dependent manner, with 25% of the CD4⁺ T cells expressing IFN- γ (Figure 8). By contrast, antigen-sensitized, CD4⁺ T cells lacking MIF failed to differentiate into IFN- γ -producing cells, but was promoted to differentiate into the Th2 phenotype (CD4⁺IL-4⁺) in response to HP antigen challenge in a dose-dependent manner (Figure 9). These data, taken together, support an important role for MIF in regulating the Th1 T cell differentiation response in HP induced gastritis.

Discussion

Emerging evidence shows that MIF plays a critical role in the T cell-mediated immune response in a number of immunologically induced diseases in human and experimental animal models, including experimental colitis and gastric ulcer.^{8–18} In the present study, we demonstrated

a new role for MIF in the pathogenesis of gastritis induced by HP infection. We found that mice deficient for MIF were protected against gastritis induced by HP infection, and this effect was associated with reduced up-regulation of TNF- α , ICAM-1, and inhibition of macrophage and T cell infiltration. Furthermore, we observed a significant suppression of Th1-mediated immunity in the setting of genetic MIF deficiency. HP infection resulted in an up-regulation of the Th1 master transcriptional factor T-bet and the Th1 signature cytokine IFN- γ , and DTH in MIF wild-type, but not in KO mice. The important regulatory role of MIF in HP-induced Th1 immune response was further demonstrated by the findings that MIF KO CD4⁺ T cells failed to differentiate to the Th1 phenotype, but enhanced Th2 differentiation in response to HP antigen challenge *in vitro*.

There are several possible mechanisms by which MIF may regulate gastritis induced by HP infection. First, consistent with the recent report that deletion of MIF suppresses dextran sulfate sodium-induced colitis,⁸ findings from the present study also demonstrated that MIF may act as an important mediator of macrophage-mediated innate immune response. It is known that the presence of receptor CD74 is important for MIF to exert its biological activities in immune and inflammatory response.³² CD74 also is known to interact with HP, leading to the release of the chemokine IL-8.^{33,34} Macrophages are the major cell type that produces MIF and these cells contain abundant pre-formed MIF protein within intracellular pools.⁴ MIF is released in response to stimuli including bacterial endotoxin and pro-inflammatory cytokines such as TNF- α and IFN- γ .³⁻⁶ In the present study, we found that gastric MIF and its receptor CD74 were markedly up-regulated at 2 weeks after HP infection. This preceded the development of gastric inflammation that occurred significantly at 8 weeks, demonstrating a pathogenic role of MIF in initiating the gastric inflammation. Thus, gastric MIF may be released after HP infection. Once released, MIF functions as both a paracrine and an autocrine activator of its receptor CD74 and then binds, and activates CD74 to induce the cascade of gastric inflammation, resulting in up-regulation of TNF α , ICAM-1, and promoting further macrophage and T cell infiltration. This sequence of events may well explain the finding that MIF KO mice express a normal level of MIF receptor CD74 but were nevertheless protected against gastric inflammation.

Secondly, MIF may act by stimulating T cell activation and Th1 immune response to promote a chronic phase of HP-induced gastritis. MIF acts as a critical cofactor in T cell activation in response to both mitogenic and antigenic stimuli. MIF also acts to override glucocorticoid inhibition of T-cell proliferation and activation, thereby promoting the expression of Th-1 cytokines including IL-2 and IFN- γ .³⁵ In dextran sulfate sodium-induced colitis, mice lacking MIF are resistant to colitis,^{8,10} which is associated with an inhibition of Th1-derived cytokines such as IFN- γ .¹⁰ In contrast, mice with transgenic overexpression of MIF exhibit greater susceptibility to experimental colitis.⁹ It is now well accepted that the Th1-mediated immune response is regulated by the transcriptional fac-

tor T-bet³⁶ and that the gastritis associated with HP infection proceeds by a Th1-dependent mechanism.^{1,2} The HP cysteine-rich protein is a Th1 polarizing agent,³⁷ and mice lacking T-bet and IFN- γ have been shown to reduce HP-induced gastric inflammation.^{24,25,27} Findings from the present study, that up-regulation of Th1 transcriptional factor T-bet at 2 weeks after HP infection preceded the development of Th1-mediated gastritis at 8 weeks in MIF wild-type mice but absent in MIF-KO mice, demonstrated a critical role for MIF in Th1-mediated gastritis in response to HP. These observations were further supported by the *in vitro* finding that HP antigen-sensitized CD4⁺ T cells lacking MIF were prevented from differentiating into IFN- γ -producing Th1 cells. Interestingly, we also found that CD4⁺ T cells lacking MIF were promoted to differentiate into IL-4 producing Th2 phenotype in response to the HP antigen, suggesting that enhanced Th2 response may also be a mechanism by which MIF KO mice were protected from HP-induced gastritis. Taken together, all these results support the notion that *H. pylori*-induced gastritis is prevented in mice treated with IL-4, but is exacerbated in IL-4, but not IFN- γ , gene-deficient mice.^{24,38}

Finally, blockade of HP antigen-specific skin DTH reaction in MIF KO mice provided an additional evidence for a role of MIF in cell-mediated immune response in the stomach in response specifically to HP antigen. Skin DTH is long considered as a classical Th1 mediated immune response linked with activation of Th1 subtype T-cell. MIF is produced by activated T cells and indeed is the first cytokine identified in association with DTH response.⁷ It has been considered that the DTH response is a pathological mechanism in many chronic, immunological diseases and is a classical Th1 immune response. Thus, inhibition of HP-induced skin DTH response in MIF KO mice could be supportive to imply that MIF plays a role in T cell-mediated gastritis induced by HP. This was consistent with previous observations that recombinant MIF exacerbates and a neutralizing anti-MIF antibody inhibits the antigen-challenged skin DTH reaction in tuberculosis in mice²⁸ and in primed anti-glomerular basement membrane glomerulonephritis in rats.¹⁷

In summary, the present study has demonstrated that MIF is a critical mediator of both the innate and the Th1 immune response in gastritis induced by HP infection. MIF regulates CD4⁺ T cell differentiation into the Th1 phenotype and promotes Th1-mediated gastritis in response to HP infection via the transcription factor T-bet. Inhibition of Th1-mediated immune injury while promoting Th2 immune response may be a key mechanism by which MIF KO mice are protected against gastritis induced by HP infection.

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