Role for macrophage migration inhibitory factor in asthma


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Communicated by George J. Todaro, Targeted Growth, Inc., Seattle, WA, August 18, 2005 (received for review May 6, 2005)

Macrophage migration inhibitory factor (MIF) is an immunologic regulator that is expressed in inflammatory and autoimmune disorders. We investigated MIF’s role in asthma using genetic approaches in a mouse model and in a cohort of asthma patients. Mice genetically deficient in MIF that were primed and aerosol-challenged with ovalbumin showed less pulmonary inflammation and lower airway hyperresponsiveness than genetically matched, wild-type controls. MIF deficiency also resulted in lower titers of specific IgE, IgG1, and IgG2a, and decreased pulmonary, Th2 cytokine levels. IL-5 concentrations were lower and corresponded to decreased eosinophil numbers in bronchoalveolar lavage fluid. T cell studies also showed a lower level of antigen-specific responses in MIF-KO versus wild-type mice. In an analysis of 151 white patients with mild, moderate, or severe asthma (Global Initiative for Asthma criteria), a significant association was found between patients with mild, moderate, or severe asthma (Global Initiative for Asthma criteria), a significant association was found between mild asthma and the low-expression, S-CATT MIF allele. Pharmacologic inhibition of MIF may be beneficial and could be guided by the MIF genotype of affected individuals.

G enetic and environmental factors interact to produce the immunopathology of asthma, which includes eosinophilic and monoclonal infiltration into Airways, a dysregulated T cell response, and mucous metaplasia and airway remodeling (1–3). Studies in mice and in human subjects have focused attention on the role of immunoregulatory mediators in asthma pathogenesis. Experiments in models of asthma have supported a role for T helper 2 (Th2) cells (4–6), which mediate allergic inflammation by producing the cytokines IL-4, IL-5, IL-13, and IL-10 (3, 7). IL-4 and IL-13 promote the production of IgE, which is important for antigen-specific responses in the lung (8, 9). IL-5 induces the differentiation, proliferation, and activation of eosinophils and is an important for mobilizing eosinophils and for prolonging eosinophil survival (10–12). IL-13 also mediates eosinophilic inflammation, airway hyperresponsiveness, mucus metaplasia, and airway fibrosis (13–15).

Macrophage migration inhibitory factor (MIF) antagonizes the action of glucocorticoids and functions as an upstream activator of innate responses (16, 17). MIF induces extracellular signal-regulated kinase (ERK) 1/2 phosphorylation (18), up-regulates TLR-4 expression (23), regulates Jab1 transcriptional effects (24), and suppresses p53-mediated apoptosis (21, 22). This effect may sustain inflammatory responses in the face of activation-induced apoptosis (22). MIF also promotes IL-2 and IL-2 receptor expression and memory T cell development, and it may influence Th1/Th2 cell differentiation responses in experimental infections (23, 24).

MIF has been detected in high amounts in the bronchoalveolar lavage fluid (BALF), serum, and sputum in asthma (25). Eosinophils also release MIF upon stimulation with C5a or IL-5 (25). Significant interest in MIF in inflammatory disease has been engendered by the description of functional polymorphisms in the MIF promoter, which have been associated with inflammatory disorders (26).

We investigated MIF’s role in asthma by studying an experimental model of disease in MIF-knockout (MIF−/−) mice. We also analyzed the distribution of high- versus low-expression MIF alleles in a cohort of patients with asthma.

Materials and Methods

Mice. Mice were from The Jackson Laboratory or were bred at the Yale Animal Resources Center. MIF−/− mice (21, 27) were from the BALB/c genetic background were used at generation N8. Studied mice were age-matched females (6–9 wks of age) and were maintained on OVA-free diets in a pathogen-free environment.

Cytokines and Antibodies. Mouse MIF was prepared free of endotoxin as described in ref. 28. Mouse IL-2, IL-4, and IL-12p70 were from R & D Systems. Anti-IL-4, anti-IFN-γ, anti-CD3e, anti-CD28, FITC-conjugated anti-IFN-γ, PE-conjugated anti-IL-4, biotinylated anti-CD4, biotinylated anti-B220, biotinylated anti-Thy1.2, PE-Cy5-conjugated anti-B220, PE-Cy5-conjugated anti-CD8, and streptavidin-PE-Cy5 were from E Bioscience.

Sensitization and Challenge with OVA. Mice were sensitized with an i.p. injection of OVA (20 μg, low endotoxin) in aluminum hydroxide gel and PBS on days 0 and 5. On days 12, 13, and 14, mice inhaled aerosolized OVA or PBS for 40 min in a chamber connected to a NE-U07 nebulizer (OMRON Healthcare).

Measurement of Igs. Igs were measured by specific ELISA (Bethyl Laboratories). OVA-specific IgE, IgG1, and IgG2a were measured in OVA-coated microtiter plates.

Airway Measurements. Airway hyperresponsiveness was assessed by methacholine-induced airflow obstruction of conscious mice placed in a plethysmograph (15). Enhanced pause (Penh) values were measured and averaged (15).

BALF and Histologic Analysis. Lungs were lavaged with PBS 16 h after the last challenge, and the BALF was pooled for analysis. BALF cells were cytospun and stained with May–Grunwald–Giemsa solution. Cells were classified by morphological criteria. Eosinophils were quantitated by eosinophil peroxidase. Lungs were inflated and fixed overnight before staining with periodic acid-Schiff reagent.

 Abbreviations: APC, antigen-presenting cell; BALF, bronchoalveolar lavage fluid; MIF, macrophage migration inhibitory factor; OVA, ovalbumin.

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mRNA Analysis. Total RNA was isolated by using TRIzol (GIBCO/BRL). cDNA was amplified by SuperScript One-Step RT-PCR, using Platinum Taq polymerase (Invitrogen) and specific primers. The RT-PCR of all samples from individual experiments were done in the same reaction and run on the same agarose gel.

Proliferation Assays. Spleen cells were cultured with or without OVA for 3 days (6 × 10⁵ cells per well). CD4⁺ T cells or splenic B cells were purified by positive selection with anti-CD4 mAb or anti-B220 mAb and MACS separation columns (Miltenyi Biotec, Auburn, CA). T cell-depleted splenocytes as APCs were prepared from spleen cells by negative selection with anti-Thy1.2 and streptavidin microbeads. Isolated CD4⁺ T cells, B cells, and APCs were >95% CD4⁺, >95% B220⁺, and <5% Thy1.2⁺, respectively. CD4⁺ T cells from DO11.10H2dTg mice were cocultured with APCs for 3 days.

Cytokine Assays. IL-4, IL-5, IL-10, and IFN-γ were assayed with BD Biosciences ELISAs, and IL-2, eotaxin, and IL-13 were measured with kits from R & D Systems. The MIF ELISA followed a previously reported method (detection limit: 0.16 ng/ml) (29). Intracellular staining of IL-4 and IFN-γ was performed by using the Fixation/Permeabilization Solution kit (BD Biosciences). Flow cytometry analysis was performed on a FACSCalibur (BD Biosciences), and the results were analyzed with FLOWJO software (Tree Star, Ashland, OR).

Patient Selection. Blood samples were collected from adults with asthma (n = 151) attending a hospital outpatient department in Dublin, Ireland, and from disease-free controls (n = 164) matched for ethnicity (white). All patients were assessed by a single physician (S.J.L.) according to the British Thoracic Society (BTS) (30). All patients had pulmonary function studies performed where airflow obstruction and significant reversibility were confirmed. Asthma severity was defined by the Global Initiative for Asthma (GINA) criteria (31). Additional criteria pertaining to asthma severity that were studied included (i) the lowest FEV₁ recorded in case notes, (ii) the number of hospital admissions for asthma, and (iii) the number of days on corticosteroids in the prior 12 months. The control group had similar pulmonary function studies performed, and no evidence of airflow obstruction was found. Atopy was defined as a positive response to an allergen panel.

Genotype Analysis. Analysis for the CATT MIF polymorphism was performed as described in ref. 32. Statistical analysis was performed with the open-source R-package (R Development Core Team, 2004). The associations between categorical variables were explored using tabulations, and analyzed using the Chi² test and log-linear modeling (33).

Fig. 1. Serum (A) and OVA-specific (B) Ig response in MIF⁺/+ (●) and MIF⁻/⁻ (○) mice. Results are the mean ± SD from two independent experiments using four to six mice per group. *P < 0.05; **P < 0.01; ***P < 0.001 [for MIF⁺/+ versus MIF⁻/⁻ by Student’s t test (two-tailed)].

Fig. 2. Airway response curves in OVA-challenged and PBS-challenged mice. MIF⁺/+ (● and □) and MIF⁻/⁻ (○ and □) mice were administered methacholine 12 h after the last challenge. Enhanced pause (Peh) values are expressed as mean ± SEM (n = 13 per group). *, P < 0.05 [Student’s t test (two-tailed)].

Fig. 3. Histologic appearance of lungs 16 h after challenge with OVA or PBS. The sections are representative of five mice in each group. Staining was with periodic acid-Schiff reagent (200×).
Results

MIF−/− Mice Show a Reduced Ig Response to OVA. Murine models of asthma such as the OVA-prime and aerosol challenge model are characterized by the preferential induction of a Th2 immunologic response. We examined first the total and OVA-specific Ig response in MIF−/− mice and genetically matched MIF+/+ mice. Both total and OVA-specific IgM, IgE, IgG1, and IgG2a increased in serum by day 8 of OVA sensitization in the wild-type, MIF+/+ strain (Fig. 1). There were significantly lower levels of these antibodies (with the exception of IgM) in the MIF−/− mice than in the MIF+/+ mice.

MIF−/− Mice Show Decreased Airway Hyperresponsiveness, Peribronchial Infiltration, and Mucus Hyperproduction. We subjected OVA-primed mice to aerosol challenge and measured airway reactivity in response to methacholine administration (Fig. 2). Airway responsiveness to methacholine was reduced in the MIF−/− mice. Lungs obtained from the control, PBS-challenged mice (both MIF+/+ and MIF−/−) showed normal histology, whereas the lungs of the OVA-challenged MIF−/− mice showed peribronchial inflammation, mucous hyperproduction, and goblet cell hypertrophy (Fig. 3). By comparison, peribronchial, cellular infiltration in response to OVA challenge was much reduced in the MIF−/− mice. These functional airway and histologic data support a role for MIF in the development of an asthma phenotype.

Differential Infiltration of Inflammatory Cells into the Lungs of MIF+/+ Mice Versus MIF−/− Mice. BALF analysis showed that total cell numbers were reduced in the setting of genetic MIF deficiency, irrespective of OVA challenge (Fig. 4). The ~2-fold reduction in total and mononuclear cell numbers in BALF supported the observations in lung tissue (Fig. 3). Leukocyte subfractionation...
or PBS-challenged mice were restimulated with OVA or PBS and thymidine incorporation into DNA was measured (mice were incubated with OVA, and thymidine incorporation into DNA was measured. T cells were restimulated with OVA (Fig. 4 and F).

We also measured in BALF the concentrations of IL-5, which mediates eosinophil activation and recruitment, and eotaxin, which is a potent, eosinophil-selective chemokine. Both cytokines were lower in the MIF−/− mice than in the MIF+/+ mice (Fig. 4 G and H).

Cytokine Expression in the Lungs Is Reduced in MIF-Deficient Mice. The mRNA levels for these Th2 cytokines did not differ between the MIF−/− and MIF+/+ mice under control conditions, but the increase in mRNA levels for IL-4, IL-5, and IL-13 in response to OVA challenge was less for the MIF−/− than the wild-type mice (Fig. 5A). The mRNA levels for IL-10, or for IFN-γ, were not markedly different between the MIF−/− and MIF+/+ mice. The small increase in IFN-γ mRNA in the MIF−/− mice was not reflected by a significant change in the levels of IFN-γ protein (Fig. 5C).

We confirmed the production of MIF protein in the lungs of wild-type mice after aerosolized OVA challenge (Fig. 5B), and we observed a significant decrease in IL-4, IL-5, IL-13, and eotaxin production in MIF−/− versus MIF+/+ mice (Fig. 5D, E, G, and H). The concentrations of IFN-γ and IL-10 protein also were not significantly different in the absence of MIF (Fig. 5C and F). These data support a reduction in the expression and production of Th2 cytokines that mediate allergic inflammation in mice deficient in MIF.

OVA-Specific T Cell Activation Is Reduced in MIF−/− Mice. The splenic T cell response to OVA was significantly attenuated in the MIF−/− mice (Fig. 6A), as was the production of IL-2 (Fig. 6B). T cell MIF production in response to antigen stimulation in wild-type mice was rapid, peaked at 24 h, and preceded IL-2 production. Purified B cells proliferated equally well to OVA stimulation whether the cells were from wild-type or MIF−/− mice (Fig. 6C), suggesting that

revealed lower numbers of macrophages (Fig. 4B), lymphocytes (Fig. 4C), neutrophils (Fig. 4D), and eosinophils (Fig. 4E) of MIF−/− mice when compared with MIF+/+ mice; however, this result was significant only for the eosinophil subpopulation. We verified BALF eosinophil numbers by measuring eosinophil-specific peroxidase (Fig. 4 E and F). Neither bone marrow nor circulating eosinophil numbers were influenced by genetic MIF deficiency, in agreement with the hematologic characterization of these mice (21, 27).

Fig. 6. Immune cell activation responses. (A) Splenic cells from OVA-sensitized and -challenged mice were incubated with OVA and thymidine incorporation measured. *, P < 0.05; **, P < 0.01; ***, P < 0.005. (B) MIF and IL-2 in supernatants (***, P < 0.005). (C) Splenic B lymphocytes from OVA-sensitized and -challenged mice were incubated with OVA, and thymidine incorporation into DNA was measured (**, P < 0.005). (D) Proliferative responses of OVA-Tg transgenic T cells cultured with purified APCs from OVA-primed and inhalation-challenged MIF+/+ or MIF−/− mice (***, P < 0.005).

Fig. 7. Th1/Th2 polarization. (A) T cell differentiation and intracellular cytokine staining in splenic T cells from mice sensitized to OVA and aerosol-challenged in vivo. T cells were restimulated with OVA in vitro, and cytokine staining was measured in a CD4+ gated T cell population. (B–F) Splenic T cells from OVA-sensitized and OVA- or PBS-challenged mice were restimulated with OVA or PBS in vitro, and the supernatants were analyzed 72 h later by ELISA. *, P < 0.05; **, P < 0.01.
there is no intrinsic defect in B cell responses in this model of asthma.

We next examined whether the reduction in T cell-proliferative responses to OVA stimulation could be attributed to an impairment in antigen presentation. For this purpose, we used the OVA-TCR transgenic (DO11.10H2dTg) mouse strain (34). CD4\(^+\) T cells from the spleens of DO11.10H2dTg mice were cocultured with APCs isolated from OVA-primed and inhalation-challenged MIF\(^{-/-}\) or MIF\(^{+/+}\) mice. There was a significant reduction in the proliferative response of OVA-specific T cells stimulated with MIF\(^{-/-}\) APCs when compared with MIF\(^{+/+}\) APCs (Fig. 6D), suggesting a role for APC-derived MIF in the adaptive T cell response.

OVA-Specific, T\(_{\text{H}2}\) Cell Responses Are Reduced in MIF\(^{-/-}\) Mice. Splenic T cells were obtained from OVA-sensitized and PBS- or OVA-challenged mice, and restimulated with OVA for the measurement of T cell polarization responses. The intracellular production of IFN-γ (T\(_{\text{H}1}\) response) and IL-4 (T\(_{\text{H}2}\) response) then was measured in the CD4\(^+\) T cell population segregated by flow cytometry (Fig. 7A). CD4\(^+\) T cells from the OVA-sensitized, wild-type mice showed an enhanced IL-4 response and a poor IFN-γ response, in accord with this disease model. The increase in IL-4 production was evident in the OVA-restimulated CD4\(^+\) T cells irrespective of prior challenge. By contrast, the CD4\(^+\) T cells from the OVA-sensitized MIF\(^{-/-}\) mice showed a markedly reduced IL-4 response and no significant induction in IFN-γ production.

Splenic T cells obtained from MIF\(^{-/-}\) mice and stimulated with OVA showed a marked reduction in the secretion of the T\(_{\text{H}2}\) cytokines, IL-4, IL-5, IL-10, and IL-13, when compared with T cells obtained from their MIF\(^{+/+}\) counterparts (Fig. 7 B–F). These data support a reduced T\(_{\text{H}2}\) cell response in MIF\(^{-/-}\) mice in response to OVA sensitization and inhalation challenge in vivo.

Polymorphisms in the Human MIF Gene and Their Relationship to Asthma Incidence and Severity. Functional promoter polymorphisms in human MIF have been identified and recently associated with different inflammatory diseases (26). These polymorphisms include a tetranucleotide sequence, CATT, that is repeated between five and eight times at position \(-794\) in the gene promoter (Fig. 8). An increase in CATT repeat number produces an increase in MIF promoter activity (32).

To obtain an initial assessment of MIF genotype in human asthma, we collected 315 DNA samples (151 asthma and 164 controls) from an ethnically homogenous (white), adult population. The prevalence of the different forms of the MIF CATT polymorphism in the asthmatic and control population is summarized in Table 1 and 2. Initial statistical analysis revealed no differences between the distribution of these alleles in the asthmatic versus the control groups (\(P = 0.42\)), in agreement with a recently published study in a Japanese cohort (35). We next stratified the asthmatic group by disease severity, as defined by Global Initiative for Asthma criteria (31), and observed significant associations between the patient group encoding the MIF 5-CATT polymorphism and less severe clinical disease (5/5 vs. X/X alleles, \(P = 0.007\); and 5/5 + 5/X versus X/X alleles, \(P = 0.05\); where 5 is 5-CATT and X is 6-, 7-, or 8-CATT alleles). A trend was observed between the 5/5 and the combined 5/5 + 5/X groups for both (i) higher recorded FEV\(_1\) (\(P = 0.06\) and \(P = 0.13\), respectively) and (ii) fewer hospital admissions for asthma (\(P = 0.09\) and \(P = 0.08\), respectively). We also collected data on the prevalence of atopy, which was present in 44% of the studied individuals, and we found no evidence for any association (\(P = 0.76\)). These data support a protective role for the low-expression, 5-CATT MIF allele in the development of severe asthma.

**Discussion**

Emerging data have emphasized MIF’s role as an upstream regulator of the innate and adaptive immune response. Its proximate actions are to promote cytokine and Toll-like receptor expression (16–18), and to sustain cellular responses in the face of activation-induced apoptosis (21, 22). An additional feature of MIF action is its ability to counterregulate the immunosuppressive action of glucocorticoids (16). High levels of MIF in serum and in the bronchial alveoli occur in patients with asthma (25), and the observation that MIF expression persists in established disease has raised the possibility that it may sustain inflammation and contribute to clinical steroid resistance (36, 37).

The development of mice genetically deficient in MIF permits a direct test of MIF’s role in models of asthma. Our results in OVA-primed and inhalation-challenged mice support an intrinsic role for MIF in the immune response to OVA. When compared with wild-type mice, MIF\(^{-/-}\) mice showed a reduction in the total and OVA-specific secondary response antibodies, especially IgE. These findings suggest that MIF deficiency results in a selective defect in T\(_{\text{H}1}\)2 priming, or in the B cell response that is necessary for isotype switching. These findings with respect to the humoral response differ from what has been observed during infection with *Taenia crassiceps*, in which IgE levels are preserved in the absence of MIF (24). These latter studies were performed in mice of the same genetic background and T\(_{\text{H}2}\) sensitivity as described here, and the difference in the antibody response may relate to the nature or the duration of the antigenic stimulus by helminthic infection.

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**Table 1. Prevalence of MIF CATT polymorphism in the enrolled asthma and control populations**

<table>
<thead>
<tr>
<th>MIF genotype</th>
<th>5/5</th>
<th>5/6</th>
<th>5/7</th>
<th>6/6</th>
<th>6/7</th>
<th>7/7</th>
<th>7/8</th>
<th>5/5 + 5/X (%)</th>
<th>X/X (%)</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asthma</td>
<td>15</td>
<td>46</td>
<td>9</td>
<td>57</td>
<td>21</td>
<td>2</td>
<td>1</td>
<td>70 (46.4)</td>
<td>81 (53.6)</td>
<td>151</td>
</tr>
<tr>
<td>Controls</td>
<td>7</td>
<td>51</td>
<td>13</td>
<td>69</td>
<td>20</td>
<td>3</td>
<td>1</td>
<td>71 (43.4)</td>
<td>93 (56.7)</td>
<td>164</td>
</tr>
</tbody>
</table>

5, 5-CATT MIF allele; X, 6-, 7-, or 8-CATT MIF allele; N, number of cases.
The reduction in the levels of IgE in the MIF−/− mice was associated with a lower level of IL-5 and eotaxin in BALF, a decrease in IL-4, IL-5, and IL-13 in the lung, and a decrease in the secretion of these cytokines by splenocytes. The decrease in Th2 cytokines is consistent with the pathophysiologic findings in the MIF−/− mice: decreased peribronchial infiltration and eosinophil content in alveoli; decreased mucus production, and decreased airway hyperresponsiveness.

Investigation of the immune cell functional responses in the OVA-prime and aerosol challenge model revealed a significant reduction in the T cell proliferative and IL-2 responses to antigen that were due, at least in part, to a decrease in the antigen-presentation function of MIF-deficient APCs. MIF’s precise mechanism of action in promoting Th2 immunity remains to be elucidated. There is little known about the signaling pathways that may underlie the preferential induction of Th2 immunity, although it is considered that the initial innate response to antigen(s) may be critical (3, 4, 6). In this respect, MIF’s ability to induce sustained ERK-1/2 activation may be of importance (18). Activation of the ERK-1/2 mitogen-activated protein kinase (MAPK) cascade in the differentiation of Th2 cells may be necessary for antigen-induced airway hyperreactivity and eosinophilic inflammation (38).

We observed a significant association between mild asthma, as defined by Global Initiative for Asthma guidelines, and homozygosity or heterozygosity for the low-expression, 5-CATT MIF allele. We did not find an association between particular alleles and asthma incidence, in agreement with the recent report of Hizawa et al. (35), who observed no association between MIF polymorphisms and the incidence of asthma in a group of 287 Japanese patients. Clinical asthma severity was not examined in that study, however.

In summary, the present study assigns an important function for MIF in the immunopathogenesis of asthma via the promotion of Th2 responses. The human genetic data suggest that different MIF promoter alleles, which are prevalent in the population and may exist in a balanced polymorphism (26), play a role in asthma clinical severity. MIF inhibition in asthma may be therapeutically beneficial, and specific intervention may be guided by the MIF genotype of affected individuals.

We thank T. Zheng, Dr. Y.-K. Kim, N. Y. Chen, and L. A. Rabach for technical support; L. Cohn for the DO11.10H2Dtg mice; and J. Griffith for helpful comments. We are indebted, for statistical advice, to A. Staines (University College, Dublin). This work was supported by National Institutes of Health Grants 1R01-AR49610 and 1R01-AR050498 (to R.B.), Sapporo Immunodiagnostics (Y.M.), and the Science Foundation and Health Research Board of Ireland (S.C.D.).

### Table 2. Breakdown of asthma cases by GINA criteria and distribution of the 5-CATT (S) versus non-5-CATT MIF alleles (X)

<table>
<thead>
<tr>
<th>GINA criteria</th>
<th>Asthma cases, %</th>
<th>S/S, %</th>
<th>S/X, %</th>
<th>X/X, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Severe persistent</td>
<td>24</td>
<td>2</td>
<td>6</td>
<td>16</td>
</tr>
<tr>
<td>Moderate persistent</td>
<td>31</td>
<td>1</td>
<td>11</td>
<td>19</td>
</tr>
<tr>
<td>Mild persistent</td>
<td>38</td>
<td>4</td>
<td>17</td>
<td>17</td>
</tr>
<tr>
<td>Intermittent</td>
<td>7</td>
<td>3</td>
<td>2</td>
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</table>