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Cutting Edge: Deficiency of Macrophage Migration Inhibitory Factor Impairs Murine Airway Allergic Responses

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Increased levels of macrophage migration inhibitory factor (MIF) in serum, sputum, and bronchoalveolar lavage fluid (BALF) from asthmatic patients and time/dose-dependent expression of MIF in eosinophils in response to phorbol myristate acetate suggest the participation of MIF in airway inflammation. In this study, we examined inflammation in OVA-sensitized mouse lungs in wild-type and MIF-deficient mice (MIF<sup>-/-</sup>). We report increased MIF in the lung and BALF of sensitized wild-type mice. MIF<sup>-/-</sup> mice demonstrated significant reductions in serum IgE and alveolar inflammatory cell recruitment. Reduced Th1/Th2 cytokines and chemokines also were detected in serum or BALF from MIF<sup>-/-</sup> mice. Importantly, alveolar macrophages and mast cells, but not dendritic cells or splenocytes, from MIF<sup>-/-</sup> mice demonstrated impaired CD4<sup>+</sup> T cell activation, and the reconstitution of wild-type mast cells in MIF<sup>-/-</sup> mice restored the phenotype of OVA-induced airway inflammation, revealing a novel and essential role of mast cell-derived MIF in experimentally induced airway allergic diseases. The Journal of Immunology, 2006, 177: 5779–5784.

Macrophage migration inhibitory factor (MIF) was one of the first cytokines described, based on its role in delayed-type hypersensitivity (1). Subsequent investigations demonstrated its participation in various human diseases (2). Although MIF was initially thought to be expressed primarily in T lymphocytes (1), recent investigations have revealed that other tissues or cell types such as pituitary cells, astrocytes, macrophages (M<sub>d</sub>), smooth muscles cells (SMC), endothelial cells, and mast cells also express this cytokine under inflammatory (3) or antigenic stimulation (4). An involvement of MIF in allergic responses was suggested from observations of increased MIF levels in bronchoalveolar lavage fluid (BALF) from asthmatic patients (5). Eosinophils, the hallmark cells of asthma, release MIF in a time- and concentration-dependent fashion in response to phorbol myristate acetate (5). Further, MIF levels are significantly higher in serum and sputum from asthmatic patients and correlate with the production of eosinophil cationic protein (6), a marker for eosinophilic inflammation of airways in bronchial asthma. It is uncertain, however, whether MIF in asthmatic serum or sputum is a nondiscriminative marker of airway inflammation or directly participates in asthma pathogenesis. In this study, we examined the role of MIF deficiency in a murine model of allergic lung inflammation and demonstrate a pathogenic role for mast cell-derived MIF in airway inflammation and allergy.

Materials and Methods

Animal protocol

BALB/c MIF-deficient mice C.129S4(B6)-Mif<sup>tm1Dwd</sup> (MIF<sup>-/-</sup>) were generated by backcrossing MIF<sup>-/-</sup> mice in C57BL/6J background (7) to BALB/c mice (>12 generations). To induce airway allergic responses, a 12-wk-old mouse was immunized i.p. with 50 μg of OVA in 10 mg of Al(OH)<sub>3</sub>, on days 0, 1, 2, 7, and 14, followed by intranasal challenge with 1 mg of OVA on days 21, 22, and 23 (8). On day 24, mouse serum collection, BALF harvesting, total leukocyte counting, and Diff-Quik staining (cell typing) were performed as detailed elsewhere (8).

Serum and BALF IgE and cytokine level determination

Serum total IgE was determined with a sandwich ELISA (8). Serum and BALF eosinophil cationic protein (ECP) (Cell Sciences), MCP-3 (Cell Sciences), IL-4 (Pierce), IL-5 (Pierce), IL-13 (Peprotech), IFN-γ (BioSource International), TGF-β1 (BioSource International), and TNF-α (BioSource International) were determined using ELISA kits according to the manufacturers’ instructions. MIF was measured by a murine-specific, capture ELISA (detection limit of 0.16 ng/ml) (9).

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Preparation of lung dendritic cells, splenocytes, CD4+ T cells, and mast cells

Mouse lung dendritic cells (DC) were isolated as described previously (10). DC were verified by FACS analysis for CD11c, DEC-205, I-A\(^{\delta}\), CD11b, CD14, F4/80, and Gr-1. Isolated lung DC showed positive staining for the cell surface markers of CD11c, DEC-205, and I-A\(^{\delta}\), and negative cell surface staining for CD11b, CD14, F4/80, and Gr-1. Splenocytes and CD4+ T cells were isolated from C57BL/6J or DO11.10 mouse spleens as we reported previously (11). Mast cells were derived from bone marrow from both MIF\(^{-/-}\) and MIF\(^{+/+}\) mice in the presence of murine IL-3 (PeproTech) as previously reported (12). PWM-stimulated mast cells were prepared by differentiating bone marrow cells in medium containing PWM-stimulated, splenocyte-conditioned medium as described (13).

Mast cell reconstitution

Bone marrow-derived mast cells (BMMC) from MIF\(^{+/+}\) mice were washed with calcium-free PBS and resuspended in PBS. Cells were immediately injected into the tail veins of 5-wk-old MIF\(^{-/-}\) mice (1 x 10\(^7\) cells/mouse). These mice were used in the allergic response model 7 wk after BMMC reconstitution, when BMMC appeared in most recipient mouse lungs (14). Reconstituted BMMC were examined using rabbit anti-murine MIF polyclonal Ab-mediated immunostaining.

Preparation of mouse MIF polyclonal Abs

Full-length mouse MIF cDNA was subcloned into pCRT/NT-TOPO vector and expressed in BL21 (Invitrogen Life Technologies). Poly(His)\(_6\)-MIF fusion proteins were purified over a Hi-Load Quick column (Novagen), and purified proteins were used for immunizing rabbits to produce polyclonal Abs (Proteintech Group). Ab specificity was verified by MIF lysate immunoblot analysis and immunostaining of lung paraffin sections from both MIF\(^{+/+}\) and MIF\(^{-/-}\) mice.

Lung histology

Mouse lungs were removed and fixed in 10% buffered formalin. Paraffin sections (4 \(\mu\)m) were prepared and used for immunostaining of MIF (rabbit anti-mouse MIF polyclonal Ab; 1/2000) and cell proliferation marker Ki67 (rabbit anti-mouse polyclonal Ab; 1/1000, NovoCastra).

MLR and Ag presentation

MLR was carried as previously described (11) in RPMI 1640 on 96-well plates with 2 x 10\(^5\) T cells per well from C57BL/6J mice and different amounts APC, including lung DC, alveolar Mø, resting BMMC, and PWM-stimulated BMMC from BALB/c mice. Ag presentation was performed with 2 x 10\(^5\) DO11.10 T cells mixed with 2 \(\mu\)g/ml OVA\(_{323-339}\) peptide and different amount of APC. Plates were incubated for 2 days followed by the addition of 1 \(\mu\)Ci of \(^3\)H-thymidine and another 6 h of incubation before harvesting.

Results and Discussion

MIF deficiency reduces airway allergic responses

In accord with what has been found in human asthmatic patients (5), the BALF of OVA-sensitized MIF\(^{+/+}\) mice contained higher amounts of MIF than the saline-sensitized controls (Fig. 1A), whereas the BALF of OVA-sensitized MIF\(^{-/-}\) mice contained no MIF (data not shown). Immunohistochemical analysis using an anti-murine MIF polyclonal Ab also revealed increased production of MIF in the airway epithelial cells and in peribronchial inflammatory cells, including mast cells. There was a clearly increased density of peribronchial inflammatory cells in the experimentally induced lungs (Fig. 1B). These initial data suggested a correlation between MIF expression and murine airway inflammation and that a lack of MIF reduces such allergic responses. This hypothesis was examined using MIF\(^{-/-}\) mice. In the same model, OVA-sensitized MIF\(^{-/-}\) mice showed evidence of reduced total leukocyte infiltration (Fig. 1C), including eosinophils (Fig. 1D) and polymorphonuclear (PMN) cells (Fig. 1E) in the BALF when compared with those in OVA-sensitized MIF\(^{+/+}\) mice. The reductions in Mø (Fig. 1F) and lymphocytes (Fig. 1G) were not statistically significant (ANOVA t test), suggesting that MIF is not essential for Mø or lymphocyte recruitment but plays a more dominant role in lymphocyte activation and cytokine production. Eosinophils play a critical role in allergic airway remodeling (15) and...
often serve as hallmarks of allergic airway inflammation. Reduced eosinophil infiltration in MIF−/− mice thus suggested a direct participation of MIF in airway allergic responses. Along with reduced eosinophil infiltration, serum total IgE levels also were significantly lower in MIF−/− mice after OVA immunization (Fig. 1H). Reduced IgE was not due to altered B cell function, because splenocytes from MIF−/− mice showed similar levels of cell surface I-A^d expression, LPS-induced proliferation, and DO11.10 T cell activation as those from MIF+/+ mice (data not shown). These data in MIF−/− mice unequivocally demonstrated that MIF is not simply a molecule marker of inflammation but rather plays an essential role in the pathogenesis of allergic airway diseases.

MIF-deficient BALF or serum contains reduced levels of chemokines

Reduced inflammatory cell infiltration in MIF−/− mice suggested reductions in chemokine production. We anticipated that MIF may affect the production of chemoattractants and indirectly impair leukocyte or eosinophil infiltration into the lung. To test this hypothesis, we measured both eotaxin and MCP-3 in serum and BALF. Eotaxin acts via the CCR3 receptor to mediate the chemotaxis of both eosinophils and mast cells in allergen-sensitized airway inflammation and hyperresponsiveness (16). An eotaxin ELISA demonstrated a significant reduction in this chemokine in BALF, but not in serum, from OVA-sensitized MIF−/− mice as compared with OVA-sensitized MIF+/+ mice (Fig. 2A). MCP-3 is important for eosinophil infiltration and activation and acts via CCR1, CCR2, and CCR3 (17). Consistently, serum but not BALF from OVA-sensitized MIF−/− mice showed a significant reduction of this chemokine (Fig. 2B). Therefore, MIF appears to regulate the production of chemokines to control migration and activation of eosinophils (Fig. 1D) or other leukocytes such as PMN (Fig. 1E).

Another hallmark of airway inflammation is increased cell proliferation mediated by TGF-β from eosinophils (18) or airway SMC (19). In vitro experiments demonstrated that TGF-β induces MIF expression in murine colon carcinoma cells (20) and, in turn, MIF regulates TGF-β expression in mesangial cells (21). Therefore, it is possible that reduced numbers of eosinophils in MIF−/− mice may lead to a reduction of BALF TGF-β levels, which may explain reduced peribronchial inflammatory cell densities in MIF−/− mice (Fig. 1B) by an impairment in their proliferation. A TGF-β1 ELISA affirmed this hypothesis and demonstrated a significant reduction of this multifunctional growth factor in BALF but not in the serum of saline- or OVA-sensitized MIF−/− mice (Fig. 2C). These data suggest a role for MIF in eosinophil and/or airway SMC TGF-β production and consequent airway inflammatory cell proliferation. Indeed, the numbers of Ki67-positive cells were reduced in OVA-sensitized MIF−/− lungs relative to OVA-sensitized MIF+/+ lungs (Fig. 2D).

MIF deficiency impairs Th1 and Th2 cytokine production

In addition to leukocyte infiltration, T cell activation and cytokine production also play critical roles in the pathogenesis of allergic airway diseases such as asthma. Although asthma is mediated by Th2-type T cells, which produce a repertoire of cytokines including IL-4, IL-5, and IL-13, there is a clear role for Th1 cytokines such as IFN-γ in both the establishment and direction of the allergic phenotype. Increased MIF levels in human asthmatic serum, sputum, and BALF (5, 6), as well as OVA-sensitized MIF+/+ mouse BALF (Fig. 1A), suggest a role of MIF in maintaining airway inflammation and T cell activities. Therefore, a lack of MIF may lower airway inflammation, as reflected by a reduction of BALF total leukocytes (Fig. 1C) or eosinophils (Fig. 1D) and reduce T cell activity. To test this hypothesis, we measured both the Th1 cytokines IFN-γ and TNF-α and the Th2 cytokines IL-4, IL-5, and IL-13. BALF but not serum IFN-γ levels were significantly reduced in MIF−/− mice (Fig. 3A), although BALF TNF-α was undetectable (data not shown) and serum TNF-α levels remained the same (Fig. 3B). Whereas serum levels of IL-4 were undetectable (data not

**FIGURE 2.** MIF-deficiency reduced chemokine and TGF-β1 levels and airway inflammatory cell proliferation. *(A)* OVA-sensitized MIF−/− mouse BALF but not serum contained lower levels of eotaxin. *(B)* Decreased serum but not decreased BALF MCP-3 levels were detected in MIF−/− mice. *(C)* TGF-β1 levels also were reduced in MIF−/− mice BALF but not in serum. *(D)* Ki67-positive peribronchial inflammatory cells were much less in OVA-sensitized MIF−/− mouse lungs. *, p < 0.05.
and those of IL-5 were the same between MIF+/− and MIF−/− mice (Fig. 3D), BALF IL-4 (Fig. 3C), BALF IL-5 (Fig. 3D), and serum and BALF IL-13 (Fig. 3E) were all reduced in MIF−/− mice. Thus, MIF appears to act on both Th1- and Th2-type cells in this model of airway inflammation.

MIF deficiency reduces T cell activation

Th1- and Th2-type T cells play essential roles in airway allergic responses (22). Increased levels of Th1- and/or Th2-type cytokines in the serum or BALF of subjects with asthma or Ag-sensitized airway inflammation suggest an enhancement of T cell activation. Thus, reduced levels of T cell cytokines in MIF−/− mice (Fig. 3) suggested a suppression of T cell activation presumably due to the lack of MIF. Indeed, MIF is well known to stimulate T cells both in vitro and in vivo (4). T cell activation can be mediated directly by cytokines or via APC such as B cells, DC, Mϕ, and mast cells. To test these possibilities, we performed MLR using C57BL/6J CD4+/− T cells and Ag presentation using

FIGURE 3. Th1 and Th2 cytokine profiles. A, IFN-γ levels were reduced in OVA-sensitized MIF−/− mouse BALF but not in serum. B, Serum TNF-α levels were undetectable and BALF TNF-α levels showed no differences between OVA-sensitized MIF+/− and MIF−/− mice. C, IL-4 levels were reduced in OVA-sensitized MIF−/− mouse BALF, whereas serum IL-4 levels were undetectable. D, IL-5 levels also were reduced in OVA-sensitized MIF−/− mouse BALF but not in serum. E, Both serum and BALF IL-13 were reduced in OVA-sensitized MIF−/− mice. *p < 0.05.

FIGURE 4. MLR (A, C, E, and G) and Ag presentation (B, D, and F). Lung DC from MIF+/− and MIF−/− mice were equally potent in activating C57BL/6 CD4+ T cells (A) and DO11.10 CD4+ T cells (B). In contrast, compared with those from MIF+/− mice, alveolar Mϕ (C and D) and BMMC (E and F) from MIF−/− mice showed impaired capability in activating C57BL/6 CD4+ T cells in MLR assay (C and E) and OVA323–339 peptide-mediated DO11.10 CD4+ T cell activation (D and F). PWM-stimulated BMMC from MIF−/− also demonstrated reduced CD4+ T cell proliferation relative to those from MIF+/− mice (G).
DO11.10 T cells in the presence of OVA\textsubscript{323-339} peptide and different APC obtained from MIF\textsuperscript{−/−} mice, including lung DC, alveolar Mφ, and BMMC. Surprisingly, DC from MIF\textsuperscript{−/−} mice activated T cells as well as DC from MIF\textsuperscript{+/+} mice in both assays (Fig. 4, A and B). In contrast, both Mφ (Fig. 4, C and D) and BMMC (Fig. 4, E and F) from MIF\textsuperscript{−/−} mice and MIF\textsuperscript{+/−} mice differed in their ability to elicit T cell activation, although cell surface I-A\(^d\) levels in DC, Mφ, and BMMC were not affected by MIF deficiency (data not shown). Further, PWM-stimulated MIF\textsuperscript{−/−} BMMC also showed a reduction in T cell activation (Fig. 4G). Impaired T cell activation by MIF\textsuperscript{−/−} BMMC was not due to an alteration in their intrinsic development or function, as we did not detect significant differences in either morphology or IgE-induced degranulation in BMMC from MIF\textsuperscript{−/−} and MIF\textsuperscript{+/−} mice according to an established protocol (23) (data not shown). These observations suggested a novel function for mast cell and MIF in T cell activation and potentially airway inflammation. However, MIF from other cells such as eosinophils, airway SMC, airway epithelial cells (Fig. 1B), and even T cells themselves may be equally important to T cell activation and airway allergic responses.

Mast cell-derived MIF is required for airway inflammation

Our MLR and Ag presentation assays demonstrated that MIF from Mφ and BMMC, but not from DC, are essential for in vitro CD4\(^+\) T cell activation (Fig. 4). Nevertheless, lung DC are important in initiating and maintaining allergic airway inflammation by polarizing naive T cells into either Th1 or Th2 effector cells, and they establish T cell memory and tolerance to inhaled Ags (24). It is possible that DC express insufficient MIF and, therefore, the MIF-related effects are not strongly mediated by these cells. Moreover, it is known that other cells such as eosinophils and airway epithelial and SMC also produce MIF (18, 19) (Fig. 1B) and, therefore, the role of BMMC- or Mφ-derived MIF in T cell activation/proliferation observed from our in vitro pure cell population assay (Fig. 4) may be compensated by MIF released from other cell types in vivo.

Mast cells are important cellular effectors in asthma or acute or chronic airway inflammation, and their role in other inflammatory/autoimmune pathologies is gaining increasing prominence (2). Mice lacking mast cells are resistant to allergen-induced airway inflammation, and the reconstitution of BMMC into mast cell-deficient mice restores the inflammatory phenotype (12, 25). It is thought that mast cells contribute to airway inflammation by enhancing proliferation and cytokine production of multiple T cell subsets via direct mast cell-T cell interactions and by undefined soluble factors (26) such as MIF. Indeed, we detected 52 ± 2.1 ng/ml MIF in the conditioned medium of 1 × 10\(^6\) resting BMMC, and mast cells in normal lung were also immunoreactive for MIF (data not shown). To examine this hypothesis in vivo, we injected MIF\textsuperscript{+/−} BMMC into MIF\textsuperscript{−/−} mice and induced airway inflammation. MIF-positive BMMC can be detected within reconstituted and OVA-immunized MIF\textsuperscript{−/−} lung parenchyma (Fig. 5A) and adjacent to the airway (data not shown), but not in nonreconstituted MIF\textsuperscript{−/−} lungs (Fig. 1B, left panel). OVA-sensitized, BMMC-reconstituted MIF\textsuperscript{−/−} lungs contained higher numbers of Ki67-positive cells in the peribronchial tissues than those in nonreconstituted OVA-sensitized MIF\textsuperscript{−/−} lungs (Fig. 5B), providing direct evidence for mast cell-derived MIF in airway inflammatory cell proliferation. Consistent with increased cell proliferation, BMMC reconstitution also restored the airway inflammatory phenotype, including the recruitment of total leukocytes (Fig. 5C), eosinophils (Fig. 5D), and PMN cells (Fig. 5E), although Mφ numbers were not affected (Fig. 5F).

Inversely, BMMC reconstitution reduced total lymphocyte numbers in MIF\textsuperscript{−/−} mice (Fig. 5G), similar to what was seen previously (25). Such reduction in BALF lymphocytes did not

**FIGURE 5.** BMMC reconstitution. A, MIF polyclonal Ab immunostaining demonstrated MIF-positive mast cells in wild-type BMMC-reconstituted MIF\textsuperscript{−/−} lung parenchyma. B, BMMC reconstitution restored high numbers of Ki67-positive proliferating peribronchial inflammatory cells in MIF\textsuperscript{−/−} mice. C–G, Total leukocytes (C), eosinophils (D), and PMN (E) were also restored after BMMC reconstitution (+ MCs), although the numbers of Mφ (F) were not changed while those of lymphocytes were reduced after BMMC reconstitution (G). H, BMMC reconstitution significantly restored serum IgE levels in MIF\textsuperscript{−/−} mice (*, p < 0.05).
affect the Ag-sensitized airway immune responses. Indeed, reconstituted MIF−/− mice produced comparable amounts of serum total IgE as MIF+/+ mice (Fig. 5H), suggesting that the lymphocytes in BMMC-reconstituted mice acted comparably to those in MIF+/+ mice. Phenotype recovery in reconstituted mice was not due to excessive mast cells in their lung. Methylene blue staining demonstrated similar numbers of mast cells per lung section in BMMC-reconstituted mice vs wild-type mice was not due to excessive mast cells in their lung. Methyl-
tate to aluminum-OVA-induced airway inflammation. Further, MIF from both mast cells and Mφ participates directly in allergic airway inflammation by enhancing inflammatory cell recruitment and activating lymphocytes to show impaired eosinophil infiltration relative to their wild-
type controls after aluminum-OVA sensitization. Therefore, more complex mechanisms may be involved.

In summary, our study demonstrates that MIF production in the lungs, in part from intrapulmonary mast cells and Mφ, participates directly in allergic airway inflammation by enhancing inflammatory cell recruitment and activating lymphocytes to promote the release of Th1/Th2 cytokines and chemokines.

Disclosures
The authors have no financial conflict of interest.

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