Clinical immunology

Macrophage migration inhibitory factor is essential for allergic asthma but not for Th2 differentiation

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Macrophage migration inhibitory factor (MIF) is increased in asthmatic patients and plays a critical role in the pathogenesis of asthma. We show here that mice lacking MIF failed to develop airway hyper-responsiveness (AHR), tissue eosinophilia, and mucus metaplasia. Analysis of the bronchoalveolar fluids revealed a substantial reduction of IL-13, eotaxin and cysteinyl-leukotrienes. The lack of these cardinal features of asthma in $MIF^{-/-}$ mice occurs regardless of high concentrations of IL-4 in the lung and OVA-specific IgE in the serum. Antigen-specific lymphocyte proliferation and IL-13 production were similarly increased in the draining lymph nodes of OVA-immunized and challenged $MIF^{-/-}$ mice compared to WT, but were reduced in the spleen of $MIF^{-/-}$, thus indicating differential roles of MIF in these compartments. Stimulation of naive $CD4^+$ cells with anti-CD3 antibody demonstrated that $MIF^{-/-}$ cells produced increased amounts of IFN-γ and IL-4 compared to WT $CD4^+$ cells. Finally, treatment of sensitized BALB/c mice with neutralizing anti-MIF antibody abrogated the development of ARH and airway inflammation without affecting the production of Th2 cytokines or IgE. The present study demonstrates that MIF is required for allergic inflammation, adding important elements to our knowledge of asthma pathogenesis and suggesting that neutralization of MIF might be of therapeutic value in asthma.

Introduction

Allergic asthma is a disorder characterized by chronic lung inflammation, reversible airway obstruction and increases in airway hyper-responsiveness (AHR) to nonspecific stimuli. Several studies have provided compelling evidences that the lung infiltrating leukocytes and the proinflammatory mediators they produce initiate cellular damage, amplify the immune response, cause airway physiological changes and tissue remodeling [1]. The airway inflammation of asthma has a predominance of Th2 $CD4^+$ lymphocytes, eosinophils and mast cells infiltrating the lung interstitium. The induction of allergic asthma, and the cardinal features of

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the disease such as eosinophilia, elevated serum IgE, mucus hypersecretion, and AHR are dependent of Th2 cytokines, in particular IL-4, IL-5, and IL-13 [1, 2]. The role of eosinophils in the physiopathology of the asthmatic reaction is less clear and still controversial. Several studies indicate the existence of a mechanism dependent on IL-5 and eosinophils that induce pulmonary damage and intensify AHR[3–5]. In other studies, however, the induction of AHR was observed despite the absence of infiltrating eosinophils, suggesting dissociation between these phenomena[6–10].

Macrophage migration inhibitory factor (MIF) is a pleiotropic molecule and critical mediator of innate and acquired immune responses[11, 12]. Pre-formed MIF protein is present in many cell types and is released in response to different stimuli, such as infection and cytokine stimulation[12]. MIF exhibits several pro-inflammatory functions, including the induction of TNF-α, IL-1 and NO release from macrophages, and the production of arachidonic acid and eicosanoids through the induction of phospholipase A_2 and cyclooxygenase[13, 14]. A unique property of MIF is its secretion by immune cells in response to physiological increase in glucocorticoid levels. Once released, MIF can counter-regulate the anti-inflammatory effects of steroids on cytokine production[15]. By means of antibody neutralization or gene deletion it has been demonstrated that MIF plays an important role in the pathogenesis of several inflammatory disorders, such as sepsis, glomerulonephritis, arthritis, colitis, encephalomyelitis, leishmaniasis and atherosclerosis [11, 12, 16–23]. Increased MIF expression has also been observed in patients suffering from inflammatory diseases including sepsis, rheumatoid arthritis, acute respiratory distress syndrome (ARDS) and asthma [11, 12, 18, 24–29]. Bronchoalveolar lavage fluids (BALF), sputum and sera from asthmatic patients were found to contain increased levels of MIF [28, 29]. The cell sources of MIF in asthma likely include resident and recently immigrated cells, specially Th2 lymphocytes and eosinophils. In normal lungs, MIF is constitutively expressed by the bronchial epithelium, alveolar macrophages and capillary endothelium [27, 30]. A prominent induction of MIF mRNA and protein has been observed in activated Th2 cells, while eosinophils have pre-formed MIF and are able to secrete high quantities of it upon stimulation [28, 31]. A role of MIF on Th2 pathologies has been demonstrated by the inefficient control of helminthic infection in the absence of MIF [32, 33]. Recent findings suggest that MIF plays an essential role in the physiopathology of experimental pulmonary allergic inflammation and contrary to other studies indicates that MIF is essential for Th2 differentiation [34]. Here, we report that MIF is essential to the induction of AHR, mucus metaplasia, leukocyte infiltration and secretion

of selective inflammatory mediators in the lung. These changes occur despite the increase of antigen-specific IgE concentrations, IL-4 production and lymph node cell proliferation in MIF$^{-/-}$ mice.

Results

Absence of MIF prevented AHR, mucus production, and lung inflammation

Similar to the increased levels of MIF in the BALF of asthmatic patients [28], BALB/c mice sensitized and challenged with OVA [35] presented increased amounts of MIF in the BALF 6 h after the last challenge (Fig. 1A). To characterize the putative role of MIF on AHR and pulmonary allergic inflammation, groups of WT and MIF$^{-/-}$ in the BALB/c background were sensitized and challenged with OVA. Allergic WT animals exhibited a marked AHR in response to increasing doses of methacholine, which was not observed in MIF$^{-/-}$ mice (Fig. 1B). In fact, the response to methacholine in these mice was comparable to that noted in PBS challenged animals. Baseline levels of Penh were similar between groups of mice before inhalation of methacholine. The extent of leukocyte recruitment in the airway lumen was
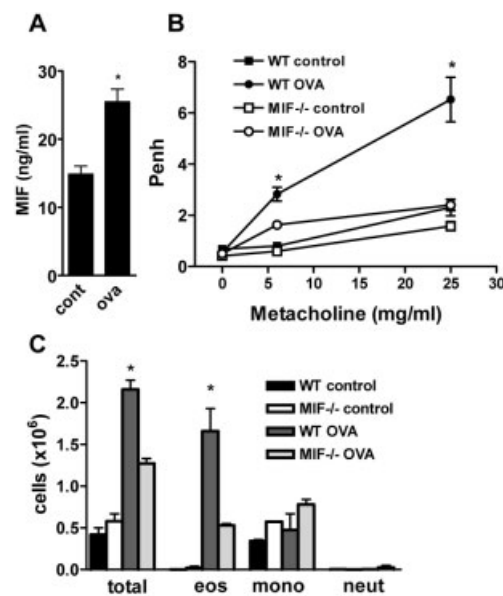

Figure 1. MIF is essential to AHR and airway eosinophilia in allergic mice. (A) MIF concentrations in the BALF of control and OVA-immunized and challenged BALB/c mice p <0.01. (B) Allergic WT and MIF$^{-/-}$ mice and controls were subjected to increasing methacholine concentrations after 6 h of the last challenge. Data represent mean Penh values $\pm$ SEM; n = 8 per group of two independent experiments. *p <0.01. (C) Leukocytes from BALF 6 h after last challenge. Data represent mean $\pm$ SEM; n = 8–9 per group in three separate experiments, *p <0.001.

examined in the BALF, 6 h after the last OVA challenge. Total numbers of cells recovered from the lavage were significantly increased in OVA-sensitized and challenged WT mice, and the differential cell counts demonstrated that the increase in leukocytes was mainly due to the presence of eosinophils in airway lumen (Fig. 1C). In contrast to the effect observed in WT mice, leukocytic inflammation was significantly reduced in the BALF recovered from MIF$^{-/-}$ mice. This reduction of leukocyte numbers in MIF$^{-/-}$ was also observed 24 h after the last challenge with OVA (data not shown).

Lung sections revealed a marked perivascular eosinophilia in allergic WT mice, while only scattered eosinophils were observed in the airway tissue of MIF$^{-/-}$, similar to nonallergic controls (Fig. 2A and B). Quantification of eosinophils in the lung sections confirmed the reduced number of these leukocytes in MIF$^{-/-}$ mice (Fig. 2B). The analysis of airway mucins demonstrated that OVA-induced metaplasia of goblet cells and mucus accumulation were increased in WT mice, while in MIF$^{-/-}$ mice were similar to controls (Fig. 2C). Collectively, the lung pathology induced by OVA was significantly reduced in MIF$^{-/-}$ mice, and was manifested by reduced leukocyte infiltration and mucus secretion.

Reduced blood and bone marrow eosinophilia in MIF$^{-/-}$ mice

Next, we quantified the number of eosinophils in the blood and bone marrow of control and allergic animals. As depicted in Fig. 3A and B, WT and MIF$^{-/-}$ controls presented similar basal numbers of eosinophils in the blood (WT control: 0.6 ± 0.25 vs. MIF$^{-/-}$control: 1.0 ± 0.3, mean $\pm$ SEM, $n = 5$), as well as in the bone marrow (WT control: 3.33 ± 0.36 vs. MIF$^{-/-}$control: 2.92 ± 0.37, $n = 5$). Allergic WT mice showed an increase of eosinophil numbers in the blood that was not observed in the MIF$^{-/-}$ mice (WT OVA: 5.0 ± 0.8 vs. MIF$^{-/-}$OVA: 1.0 ± 0.3, $p < 0.001$, $n = 5$; Fig. 3A). Similarly, we observed a significant difference in the number of eosinophil peroxidase-positive (EPO$^+$) cells in the bone marrow compartment of allergic WT mice compared to MIF$^{-/-}$ mice (WT OVA: 20.60 ± 2.33 vs. MIF$^{-/-}$OVA: 8.78 ± 0.65, $p < 0.01$, $n = 5$; Fig. 3B). To analyze a possible role of endogenous MIF on eosinophil migration, we injected eotaxin in the pleural cavity of naive WT and MIF$^{-/-}$ mice and analyzed the number of eosinophils in the pleural wash. MIF deficiency did not affect the recruitment of eosinophils induced by eotaxin (Fig. 3C). Taken together, these results suggest that the lack of
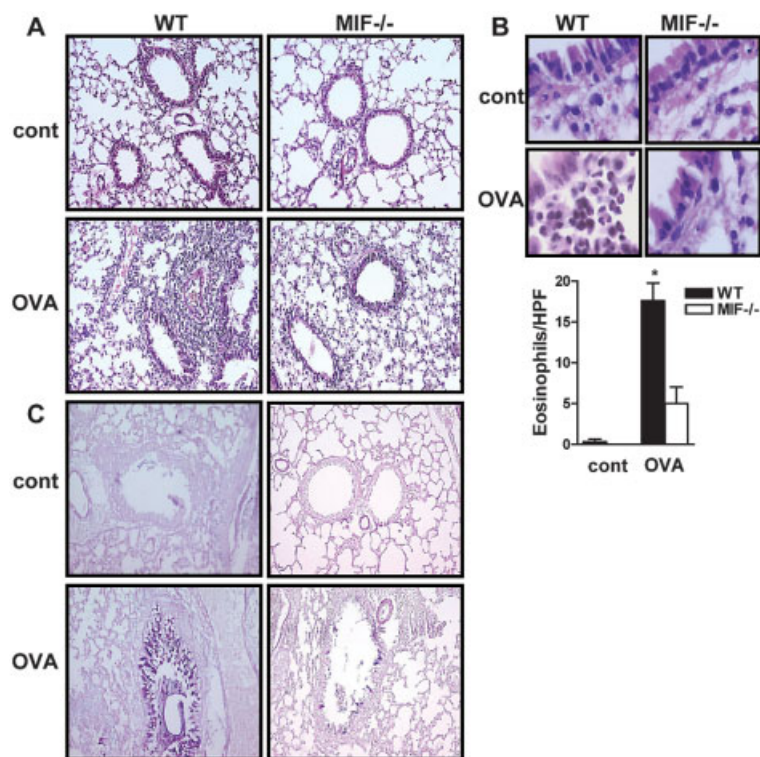

Figure 2. Reduced lung inflammation and mucus production in MIF$^{-/-}$ mice. Mice were sensitized and challenged with OVA and 16 h after the last challenge the lungs were removed and prepared as described in the *Materials and methods*. (A) H&E staining ($\times$ 200). (B) Number of eosinophils in the airway wall per 10 HPF ($\times$ 1000). Data represent mean $\pm$ SEM; $n = 8$–9 per group. *$p < 0.001$. (C) Alcian-blue/ periodic acid-Schiff stain for WT and MIF$^{-/-}$ ($\times$ 200).
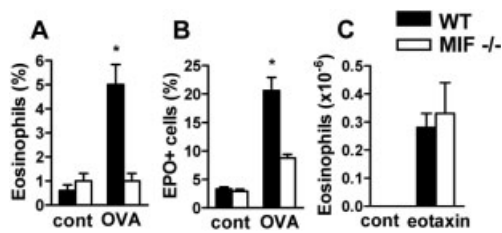
Figure 3. MIF$^{-/-}$ mice have reduced eosinophil production during asthma but normal eosinophil migration *in vivo*. (A) Blood and (B) bone marrow eosinophilia were analyzed 16 h after the last challenge. Data represent mean ± SEM, n = 5–15 per group in two separate experiments.*p <0.001. (C) Recombinant mouse eotaxin (30 pmol/cavity) or PBS were injected in the pleural cavity of naive WT and MIF$^{-/-}$. Eosinophil numbers were determined after 16 h of injection. Data represent mean ± SEM, n = 6 per group.

Figure 4. MIF regulates inflammatory mediator production in the allergic lung. Concentrations of (A) IL-4, (B) IL-5, (C) IL-13, (D) eotaxin and (E) cys-LT were assessed in the BALF from OVA-sensitized WT and MIF$^{-/-}$ mice after 6 h of the last challenge. Data represent mean ± SEM, n = 10 per group in two separate experiments. +p <0.01; *p <0.001 by analysis of variance with Newman-Keuls correction.

pulmonary eosinophilia in MIF$^{-/-}$ mice is not due to an intrinsic defect in eosinophil recruitment, but might be related to a lower production of inflammatory mediators and/or eosinophils.

MIF regulates production of inflammatory mediators in the allergic lung

A recent study demonstrated that MIF$^{-/-}$ mice lack the cardinal features of asthma and display a reduction of IL-4, IL-5 and eotaxin amounts in the lung 16 h after the last challenge [34]. Considering the essential role of Th2 cytokines in eosinophilic inflammation, mucus metaplasia and AHR, and the lack of these features in MIF$^{-/-}$ mice, we measured the concentrations of IL-4, IL-5 and IL-13 in the BALF 6 h after the last challenge. This time was chosen because at later time points the amounts of these cytokines dramatically decrease ([35], and data not shown). As shown in Fig. 4A and B, IL-4 and IL-5 were increased in OVA immunized and challenged WT and MIF$^{-/-}$ mice compared to the control groups, albeit OVA-immunized and challenged MIF$^{-/-}$ mice presented significant less amounts of IL-5 than allergic WT mice. The quantification of IL-13 revealed an almost complete abrogation of this cytokine in MIF$^{-/-}$ mice, while allergic WT mice presented high concentrations in the BALF (Fig. 4C). Similar results were obtained when analyzing the amounts of these inflammatory mediators in lung extracts (data not shown). Next, we quantified eotaxin and cysteinyl leukotrienes (cys-LT) considered important mediators in eosinophil recruitment and asthma pathogenesis [36–38]. MIF$^{-/-}$ mice had a marked reduction of eotaxin and cys-LT compared to allergic WT mice (Fig. 4D and E). Thus, endogenous MIF affects the production of IL-5, IL-13, eotaxin and cys-LT but not IL-4 in the lung of asthmatic animals.
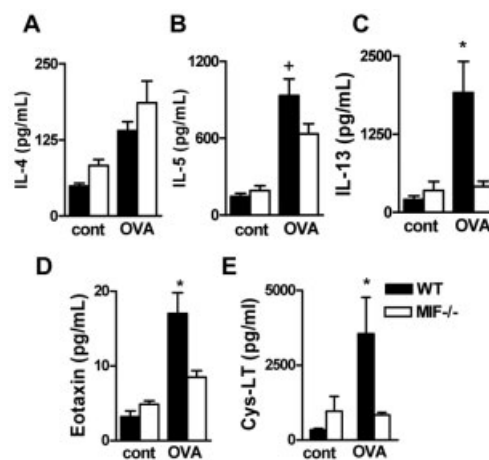
Involvement of MIF on antigen-specific responses

To investigate the role of MIF on sensitization, the serum concentrations of OVA-specific IgE were measured. The concentrations of OVA-specific antibodies were significantly increased in the sera of allergen immunized and challenged WT and MIF$^{-/-}$ mice, compared to controls (Fig. 5A). Previous studies have shown that MIF regulates the activation of T cells induced by mitogenic or antigenic stimuli, inducing IL-2 secretion and lymphocyte proliferation [31, 34]. In fact, MIF$^{-/-}$ mice have a reduced spleen T cell-proliferative response upon OVA restimulation [34]. We also observed that restimulation of spleen cells with OVA caused a reduced proliferative response and IL-13 production in allergen-sensitized and challenged MIF$^{-/-}$ mice compared to WT mice (Fig. 5B, data not shown). However, restimulation of draining lymph node cells with OVA induced a robust proliferation in allergen-sensitized and challenged WT and MIF$^{-/-}$ mice that was not observed in the control animals (Fig. 5C). The quantification of IL-13 in the supernatants of OVA-stimulated lymph node cells revealed similar high amounts in immunized WT and MIF$^{-/-}$ mice, but not in the controls (Fig. 5D). Together, these results indicate that MIF is not essential for antigen-specific IgE production or to the activation of draining lymph node cells, but participates in the activation of OVA-specific spleen cells. Moreover, the observed reduction of IL-13 in the lungs of MIF$^{-/-}$ is likely unrelated to a defect on IL-13 production by lymph node T cells.
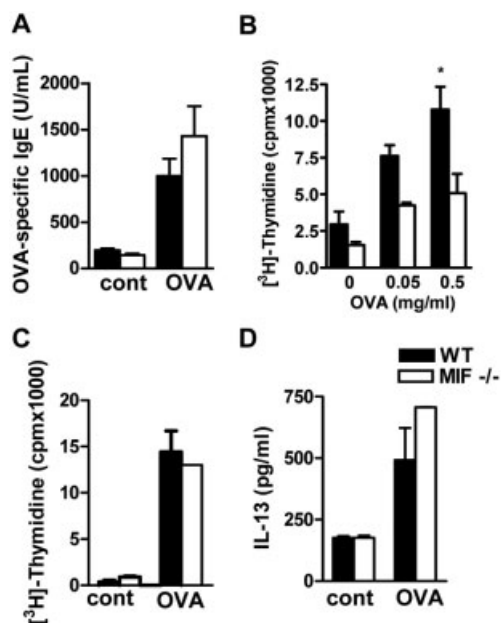
Figure 5. Antigen-driven immune response is not impaired in MIF$^{-/-}$ mice compared to WT mice. (A) OVA-specific IgE titers were evaluated. Results are expressed as the mean ± SEM, n = 12–20 per group in two separate experiments. (B) OVA-induced proliferation of spleen cells in OVA-immunized WT and MIF$^{-/-}$ mice. Results are expressed as the mean ± SEM, n = 3–6 per group in two separate experiments. *p <0.01 by analysis of variance with Newman-Keuls correction. (C) OVA-induced proliferation of draining lymph node cells (D) IL-13 was determined in the supernatants of OVA-stimulated lymph node cells from WT and MIF$^{-/-}$ mice. Results are expressed as the mean ± SEM, n = 3–6 per group in three separate experiments.

MIF regulates IFN-γ and IL-4 production by CD4^{+} lymphocytes

To characterize the role of MIF derived from CD4^{+} cells on Th cell differentiation, we stimulated lymph node-purified naive WT and MIF$^{-/-}$ CD4^{+} cells with anti-CD3 and IL-2, in the absence (default) or presence of polarizing conditions for Th1 or Th2. Strikingly, in three independent experiments the CD4^{+} cells from MIF$^{-/-}$ mice produced significant increased amounts of both IFN-γ (WT: 14.40 ± 6.54 ng/mL $vs.$ MIF$^{-/-}$: 87.77 ± 24.63 ng/mL, p = 0.0451) and IL-4 (WT: 0.38 ± 0.29 ng/mL $vs.$ MIF$^{-/-}$ 4.61 ± 1.23 ng/mL, p = 0.0292; Fig. 6A and B). Increased amounts of IFN-γ and IL-4 were also produced by CD4^{+} cells from MIF$^{-/-}$ mice in Th1 polarizing conditions (Fig. 6A and B). CD4^{+} cells from MIF$^{-/-}$ mice displayed increased concentrations of IL-4, but not of IFN-γ, in Th2 polarizing condition (Fig. 6A and B). Addition of recombinant murine MIF (rMIF) to CD4^{+} cells from MIF$^{-/-}$ mice reduced the amounts of IFN-γ to the concentrations produced by CD4^{+} cells from WT mice (Fig. 6C). Conversely, treatment of WT CD4^{+} cells with
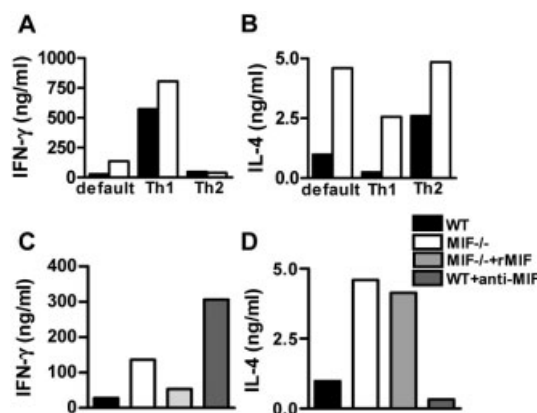

Figure 6. MIF regulates the production of IFN-γ and IL-4 by CD4^{+} lymph node cells. (A) IFN-γ and (B) IL-4 concentrations in the supernatants of lymph node CD4^{+} cells, from WT and MIF$^{-/-}$ mice, stimulated $in\ vitro$ with anti-CD3 and IL-2, in the absence or presence of polarizing conditions for Th1 or Th2. (C) IFN-γ and (D) IL-4 concentrations in neutral conditions supplemented or not with rMIF (100 ng/mL) and anti-MIF (100 μg/mL). Results are expressed as a pool of three to five animals per group of one representative experiment of two or three experiments performed. Considering the variation intrinsic to this type of experiment, only one representative experiment is shown.

anti-MIF caused a robust increase of IFN-γ (Fig. 6C). Analysis of IL-4 concentrations in these very same supernatants demonstrated that addition of rMIF to CD4^{+} cells from MIF$^{-/-}$ mice or anti-MIF to CD4^{+} cells from WT mice did not modified the production of IL-4 (Fig. 6D). Although similar results were obtained in the two experiments in which we used polarizing conditions and treatments with rMIF or anti-MIF, the results have to be considered with caution due to the variation intrinsic of this type of experiment. Together, these results demonstrate a previously unrecognized role of MIF negatively regulating the production of IFN-γ and IL-4 by CD4^{+} lymph node cells.

Treatment with neutralizing anti-MIF mAb inhibited allergic asthma

The lack of the cardinal features of asthma in the absence of MIF prompted us to characterize the effect of anti-MIF treatment of OVA-sensitized mice starting before the allergen challenge. Administration of anti-MIF mAb was able to block the AHR and eosinophil increase in the BALF seen in the OVA sensitization/challenge protocol in BALB/c mice (Fig. 7A and B). Similar to the results obtained with MIF$^{-/-}$ mice, treatment of BALB/c mice with anti-MIF did not affect the serum concentrations of OVA-specific IgE antibodies or the amounts of IL-4 in the BALF (Fig. 7C and D). On the other hand, treatment with anti-MIF had no impact on IL-13, eotaxin or cys-LT (Fig. 7E; data not shown).
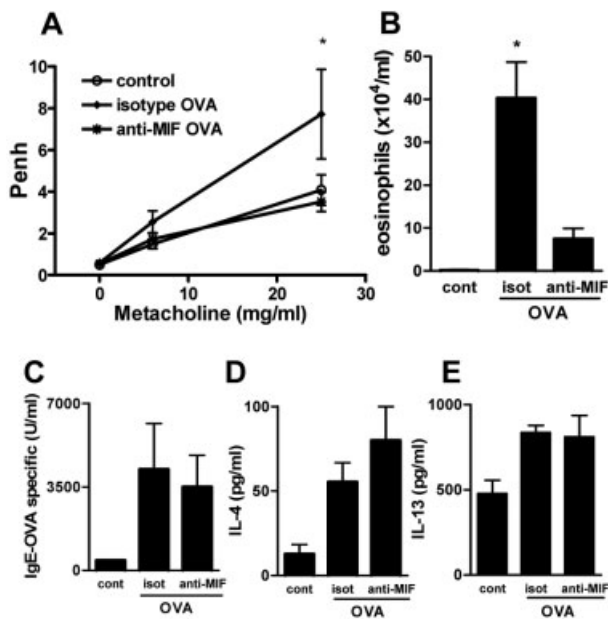
Figure 7. Treatment with anti-MIF abrogates the development of AHR and BALF eosinophilia. OVA-immunized BALB/c mice were treated daily with anti-MIF antibody or isotype control (12.5 μg/g i.p) initiated the day before the first challenge and thereafter. (A) Groups of mice were subject to increasing methacholine concentrations after 6 h of the last challenge. Data represent mean Penh values ± SEM; $n = 8$ per group. *$p <0.01$. (B) Eosinophils from BALF 6 h after last challenge. (C) OVA-specific IgE titers in the serum. Concentrations of (D) IL-4 and (E) IL-13 were assessed in the BALF from OVA-sensitized BALB/c mice treated with anti-MIF antibody or isotype control after 6 h of the last challenge. Results are expressed as the mean ± SEM, $n = 6$ per group.

These results indicate that genetic deficiency of MIF and its neutralization exclusively during the challenge phase have a major impact on AHR and leukocyte inflammation, but different effects on inflammatory mediator production in OVA-induced allergic inflammation.

Discussion

Using a mouse model of asthma, we have demonstrated that MIF$^{-/-}$ mice in the BALB/c background did not develop tissue eosinophilia, mucus metaplasia or AHR, while had reduced amounts of IL-5, IL-13, eotaxin and cys-LT in the lung. These changes occur despite a robust antigen-specific Th2 response observed by increased antigen-specific IgE concentrations, IL-4 production and lymph node cell proliferation. Treatment of immunized BALB/c mice with anti-MIF, exclusively during the challenge phase, inhibited the induction of AHR and eosinophil infiltration without affecting the production of inflammatory mediators such as IL-13, considered essential to the development of AHR and lung allergic inflammation [8, 9, 39, 40]. Thus, the lack of AHR and

eosinophilic inflammation in MIF$^{-/-}$ mice and in BALB/c mice treated with anti-MIF supports and extends an emerging concept that MIF is essential to allergic asthma.

Histopathological analysis revealed a significant decrease in goblet cells metaplasia in MIF$^{-/-}$ compared to WT mice. It is well established that metaplasia of goblet cells and the hypersecretion of mucin in asthma are dependent on IL-13, chemokines and leukotrienes [8, 9, 40–42]. The lower levels of these inflammatory mediators in the lung of MIF$^{-/-}$ mice might constitute an important cause for the observed decreased mucus production in these animals. Interestingly, a recent report demonstrated increased levels of MIF in patients with cystic fibrosis, a pulmonary disease that also displays hypersecretion of mucus [43]. The reduction of tissue eosinophils in MIF$^{-/-}$ mice was not due to an intrinsic defect on eosinophil adhesion and locomotive activities, as pleural injection of eotaxin in naïve WT and MIF$^{-/-}$ induced the infiltration of similar numbers of these leukocytes. The lower number of eosinophils in the lung of MIF$^{-/-}$ during allergic inflammation correlated with reduced numbers of eosinophils in the blood and bone marrow. It is possible that the reduced amount of inflammatory mediators in the lung of sensitized and challenged MIF$^{-/-}$ mice would also affect the production of eosinophils during allergic inflammation. We cannot exclude, however, a more direct role of MIF in the increased eosinophil generation induced by allergic inflammation.

The concentrations of IL-4 in the lung and IgE in the serum induced by OVA immunization and challenge as well as the proliferation of lymph node cells re stimulated with OVA were similar in WT and MIF$^{-/-}$ mice. Another study using a protocol of immunization with OVA and alum to induce allergic rhinitis demonstrated that MIF$^{-/-}$ mice have reduced eosinophil inflammation and mucus metaplasia, but develop a robust Th2 response [44]. Recent studies also revealed a critical role of MIF on AHR, mucus production and lung inflammation in a model of asthma [34, 45]. In these studies, however, using slightly different protocols to induce allergic inflammation endogenous MIF was considered essential for Th2 differentiation and IgE production induced by OVA. The increase of Th2 cytokines in the BALF or in the lung of immunized and challenged WT mice was rather modest when compared to the amounts observed by us [34, 45]. These low levels of Th2 cytokines observed in these studies might be related to the late time of analysis, 16–24 h after the last challenge with OVA [34, 45]. In fact, it has been shown that at late time points after the OVA challenge the concentrations of cytokines suffer a dramatic decrease compared to the peak at 6 h [35]. Considering the role of MIF modulating the expression of TLR4 [46], we cannot exclude that variations in endotoxin

contents present in the OVA preparations, could be responsible for the differential immunizing effects, affecting the production of IgE and Th2 cytokines.

We observed that the proliferation was reduced in $MIF^{-/-}$ spleen cells but not draining lymph node cells after antigen-specific restimulation *in vitro*. It has been proposed that the mechanism responsible for the reduced splenic T cell activation observed on $MIF^{-/-}$ mice upon OVA immunization involves a defect on antigen presentation [34, 45]. In fact, the ability to activate T cells *in vitro* is compromised on macrophages and mast cells but not on DC obtained from $MIF^{-/-}$ mice [45]. It is unclear whether these differences on activation of T cells from spleen and lymph node is related to a preferential involvement of non-DC as APC in the spleen *versus* DC on draining lymph nodes. The MHC class-II invariant chain (CD74) has been shown to be the cell surface receptor for MIF and essential for cell activation under MIF stimulation [47]. Mice deficient in CD74 have a defect on antigen presentation and a reduced Th2 differentiation response with lack of the cardinal features of asthma upon OVA immunization and challenge [48, 49]. These effects have also been attributed to impairment on antigen-presentation capacity in the absence of CD74. However, in opposition to APC from $MIF^{-/-}$ cells, the APC from $CD74^{-/-}$ mice have reduced expression of MHC class II. Interestingly, the lung has been shown to have a marked expression of CD74 both by APC as well as by lung epithelial cells [49, 50], thus suggesting that the effect of MIF could be at least in part dependent on binding to CD74. Future studies are required to clarify the participation of the axis MIF/CD74 on antigen presentation and lung physiology during the asthmatic response.

Using a well-established model of stimulation of purified naive lymph node $CD4^+$ cells with anti-CD3 in the absence or presence of polarizing conditions, we demonstrated an unexpected and previously unrecognized inhibitory role of MIF on IFN-γ and IL-4 production. This effect of endogenous MIF was observed using $MIF^{-/-}$ $CD4^+$ T cells and the reposition of MIF in these cultures reversed the increased production of IFN-γ but not of IL-4, present in the same supernatants. Treatment of WT $CD4^+$ cells with anti-MIF recapitulated the effect of genetic deficiency of MIF on IFN-γ production but not on IL-4 production. One could hypothesize that MIF produced by $CD4^+$ cells acts in an autocrine/paracrine fashion regulating the production of IFN-γ. On the other hand, the effect of MIF on IL-4 inhibition might involve the action of intracellular MIF. The mechanism by which MIF affects Th cells during the priming phase requires further investigation; however, our results clearly demonstrated that MIF is not required for Th2 polarization.

In contrast to what was found in a prior report using neutralizing antibodies [51], our results demonstrated that treatment of mice with anti-MIF in the challenge phase markedly diminished the AHR and the eosinophilic inflammation, but not IgE concentration in the serum or inflammatory mediator concentrations in the BALF. One possibility for this discrepancy is an inefficient neutralization of MIF associated to lower frequency or dose of antibody treatment used. Further studies with different neutralization protocols are required for defining the potential use of anti-MIF in asthma. These results together with the effect of MIF genetic deficiency on inflammatory mediator production suggest that endogenous MIF exerts a regulatory role on inflammatory mediator production during the immunization with OVA but not during the challenge phase. These results also imply that MIF is essential to AHR and eosinophilia independently of its effect on inflammatory mediator production. Our results are similar to the observed effects of MIF neutralization in a model of pulmonary inflammation induced by OVA in rats [52]. In this study, MIF neutralization inhibits AHR and leukocyte infiltration to the lung without affecting IgE concentrations or inflammatory mediator production. Finally, we tested if rMIF would be able to induce an increase in baseline airway reactivity in naïve BALB/c mice using a protocol previously used to demonstrate the involvement of IL-13 on AHR development [9]. Recombinant MIF administered *via* intra tracheal was unable to cause AHR (data not shown), thus indicating that MIF is essential but not sufficient to the induction of AHR.

In conclusion, the present study demonstrates that MIF is critical to the pathogenesis of experimental allergic asthma affecting AHR, inflammation, mucus secretion, and selective inflammatory mediator production. The prior studies showing high levels of MIF in asthmatic patients, with the present results, add important elements to our knowledge of the asthma pathogenesis and suggest that neutralization of MIF might be therapeutic in asthma.

Materials and methods

Mice

The study was approved by the Fiocruz Ethical Committee (CEUA-Fiocruz). We utilized 6–8-week-old $MIF^{-/-}$ in a BALB/c background obtained as previously described [17, 32]. These mice were backcrossed for ten generations onto a BALB/c background.

Sensitization and challenge protocol

Mice were sensitized by two i.p. injections with 10 μg of chicken OVA (OVA Grade III, Sigma Chemical, St. Louis, MO) mixed with 2 mg of alum in 0.2 mL sterile PBS on days 1 and 10. From day 19 to day 24 after the sensitization, mice were challenged daily for 20 min with OVA (5% w/v) in PBS by aerosol [35]. Controls were exposed to aerosol of PBS. In the antibody-based interventions, OVA-sensitized BALB/c mice received a daily dose of 12.5 μg/g of anti-MIF mAb (XIV.15.5)[21] or isotype control *via* i.p. route starting the day before the aerosol challenge. The OVA used throughout the study had approximately 3 EU of endotoxin/ mg of protein.

AHR

The airway responsiveness of OVA-sensitized mice to inhaled methacholine (at concentrations of 6–25 mg/mL during 1 min, with 5 min intervals; Sigma) was measured 6 h after the last OVA challenge in unrestrained conscious mice in a whole-body plethysmograph (Buxco Technology, Sharon, CT). AHR was expressed as enhanced pause (Penh), an indirect measurement that is correlated with airway resistance, impedance; and intrapleural pressure.

Airway histology

Sections of 5 μm were stained with hematoxylin/eosin, or alcian blue with periodic acid-Schiff (PAS). The analysis was carried out using a computer-assisted image analyzer (Image-Pro Plus Version 4.1 for Windows, Media Cybernetics, LP, Silver Spring, MD). Digital photographs of at least ten bronchovascular bundles per tissue section (with bronchioles cross-sectional diameters ranging from 120 to 250 μm) were obtained under light microscopy at ×200 magnification. Eosinophils in the bronchovascular bundles were identified by morphological criteria and quantified as previously described[4].

BALF

The BALF was performed with 1 mL of PBS and total cell count was evaluated microscopically using hemacytometer. Cells were then cytocentrifuged onto glass slides and differential counts were performed on H&E stained cytospin slides by counting 300 cells per slide. The cell-free BALF was obtained and stored at –20°C until used.

Blood and bone marrow eosinophilia

OVA-sensitized WT and MIF$^{-/-}$ mice and control mice were killed, the blood was collected by the retro-orbital plexus and the femurs were removed. Blood smears were stained with Giemsa, and numbers of total leukocytes and eosinophils were calculated. The bone marrow cavity was flushed with RPMI plus 1% FCS (HyClone). The cells were cytocentrifuged and EPO$^+$ cells were counted.

In vivo migration of eosinophils induced by eotaxin

Groups of naive WT and MIF$^{-/-}$ mice were injected in the pleural cavity with recombinant mouse eotaxin (30 pmol/cavity) or PBS. After 16 h, mice were killed and the pleural cavities were washed with 1 mL and total cell count was evaluated microscopically using a hemocytometer. Cells were then cytocentrifuged onto glass slides and differential counts were performed on hematoxilin/eosin-stained cytospin slides by counting 300 cells per slide.

Quantification of cytokines and cys-LT

Cytokine concentrations in BALF, obtained 6 h after the last challenge, were measured using ELISA kits according to the manufacturer's protocol (BD PharMingen, San Jose, CA). Concentrations of cys-LT were measured by EIA according to the manufacturer's protocol (Cayman, Ann Arbor, MI).

Measurement of OVA-specific IgE

Blood samples were collected and serum was obtained and stored at –20°C until measurement of OVA-specific IgE by ELISA (BD PharMingen, San Jose, CA). The antibody titers were determined in relation to pooled standards generated in the laboratory, defined as 1000 U/mL.

Proliferation assay

Draining lymph nodes and spleens were excised and filtered through a nylon mesh. The lymph node cells and spleen cells were cultured in complete RPMI medium with 10% FCS (HyClone) and 10 μM β-mercaptoetanol in the presence or absence of OVA for 48 h. Thymidine incorporation was measured 8 h after addition of 1 μCi of [^{3}H]thymidine.

CD4$^+$ Th cell differentiation

Purified CD4$^+$ T cells from BALB/c or MIF$^{-/-}$ were isolated by negative selection with magnetic beads (Micro Beads, MACS technology) according to the manufacturer's instructions (Miltenyi Biotec, Auburn, CA). Purified cells were analyzed by flow cytometry on a FACScan (BD Biosciences, San Jose, CA). CD4$^+$ T cells were isolated to >96% purity. Purified CD4$^+$ T cells (2×10^6 cells) were cultured and primarily stimulated *in vitro* for 96 h with plate-bound anti-CD3 (1 μg/mL) in the absence or presence of Th1 or Th2 skewed conditions where indicated, or with the addition or blockade of MIF cytokine. Th1 conditions: IFN-γ (100 ng/mL; PeproTech), IL-12 (10 ng/mL; PeproTech) and anti-IL-4 (20 μg/mL; 11B11); Th2 conditions: IL-4 (50 ng/mL; PeproTech) and anti-IFN-γ (XMG1.2; 100 μg/mL). The concentrations of recombinant MIF and anti-MIF (XIV.15.5)[21] were 100 ng/mL and 100 μg/mL, respectively. After 24 h of primary stimulation, IL-2 (20 U/mL) was added to the cultures. On the third day, cells were harvested, washed, and rested for 48 h in the presence of IL-2 alone (20 U/mL). After resting, cells were harvested, washed again and secondarily stimulated for 48 h with plate-bound anti-CD3 (1 μg/mL) in the absence of either cytokines or antibodies. Cell-free supernatants were assessed for IFN-γ or IL-4 by ELISA.

Statistical analysis

Data are presented as mean ± SEM Results were analyzed using a statistical software package (GraphPad Prism 4). Statistical differences among the experimental groups were evaluated by analysis of variance with Newman-Keuls correction or with the *t*-test. Values are expressed as the mean ± SEM. The level of significance was set at $p < 0.05$.

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