

Research Article

MIF is essential to the establishment of house dust mite-induced airway inflammation and tissue remodeling in mice

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Macrophage migration inhibitory factor (MIF) is present in high amounts in the BALF and serum of asthmatic patients, contributing to the pathogenesis of experimental asthma induced by OVA in mice. Whether MIF contributes to the pathophysiology on a more complex and relevant asthma model has not been characterized. *Mif*-deficient (*Mif*^{-/-}) or WT mice treated with anti-MIF antibody were challenged multiple times using house dust mite (HDM) extract by the intranasal route. HDM-challenged *Mif*^{-/-} mice presented decreased airway hyperresponsiveness, lung infiltration of eosinophils, mucus hypersecretion, and subepithelial fibrosis compared to HDM-challenged WT mice. Amounts of IL-4, IL-5, and IL-13 were decreased in the lungs of *Mif*^{-/-} mice upon HDM challenges, but the increase of CCL11 was preserved, compared to HDM-challenged WT mice. We also observed increased numbers of group 2 innate lymphoid cells and Th2 cells in the BALF and mediastinal LNs (mLN)-induced challenged by HDM of WT mice, but not in HDM-challenged *Mif*^{-/-} mice. Anti-MIF treatment abrogated the airway infiltration of eosinophils, mucus hypersecretion, and subepithelial fibrosis in the lungs of HDM-challenged mice. In conclusion, MIF ablation prevents the pathologic hallmarks of asthma in HDM-challenged mice, reinforcing the promising target of MIF for asthma therapy.

Keywords: allergic asthma · eosinophils · macrophage migration inhibitory factor · house dust mite



Additional supporting information may be found online in the Supporting Information section at the end of the article.

Introduction

Allergic asthma is a chronic inflammatory disease characterized by episodes of respiratory signs and symptoms such as wheezing, dyspnea, cough, and chest tightness triggered by allergens

[1]. Characteristically, asthmatic individuals with the allergic phenotype present lung infiltration of eosinophils and CD4⁺ T cells accompanied by high concentrations of Th2 cytokines, namely IL-4, IL-5, and IL-13 [2]. IL-4 and IL-5 are crucial for Th2 polarization of CD4⁺ T cells and eosinophil activation [3, 4], respectively, while IL-13 is strongly related to airway remodeling and lung function decline [5]. Despite the undoubted relevance of Th2 cytokines in asthma, a myriad of soluble factors

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has been shown to contribute to allergic asthma pathogenesis, as is the case of macrophage migration inhibitory factor (MIF) [6–10].

MIF is a pleotropic molecule that exerts a proinflammatory activity on several diseases such as sepsis, esophagitis, cancer, infections, and allergies [11–17]. Clinical studies demonstrated that asthmatic patients present higher MIF concentrations in the BALF, sputum, and serum than healthy controls [9, 14]. Importantly, eosinophils and Th2 CD4⁺ T cells activated in vitro secrete MIF and these cells are considered a potential source of MIF during Th2-skewed immune responses [13, 14, 18, 19]. By using antibody neutralization or gene deletion, we and others have identified the critical role of MIF in the lung infiltration of eosinophils in OVA-induced asthma model [8, 10, 20]. Further evidence revealed that MIF regulates eosinophil generation in the BM, chemotaxis to the target tissue and activation [8, 13, 21]. Besides, the absence of MIF in OVA-challenged mice is associated with decreased T-cell activation, Th2 cytokine amount, and TGF- β 1 pulmonary expression [8, 10, 22]. Recently, Lan et al. showed that MIF overexpression induces airway muscle cells proliferation in the lung [23]. Based on the MIF capacity to regulate inflammatory and resident cells, it is not surprising that MIF has a profound impact on the development of airway hyperresponsiveness (AHR), mucus hypersecretion, increased smooth muscle thickness, and subepithelial fibrosis in the lung of OVA-challenged mice [10, 22, 24, 25].

OVA-based models are classically used to study asthma, however, OVA is a clinically irrelevant allergen to humans [26]. Furthermore, mouse sensitization is conventionally performed by subcutaneous or intraperitoneal injection of OVA in conjunction with a chemical adjuvant (e.g. Alum), thus, depicting an artificial design [26]. In contrast, house dust mites (HDMs) are a common elicitor of asthma, being reactive in up to 85% of asthmatic patients [27]. The intranasal challenge with HDM extract in mice elicits not only a local sensitization [28], but also the airway epithelial cell activation due to the proteolytic activity of mite proteins [29, 30]. Importantly, HDM-activated airway epithelia induces group 2 innate lymphoid cells (ILC2s), a group of cells that has been identified as initiators of Th2 activation and key producers of IL-5 and IL-13 in humans and mice [31, 32]. Therefore, HDM-induced experimental asthma is a relevant model to understand asthma pathogenesis. Lan et al. recently showed that a MIF antagonist (ISO-1) partially prevents AHR, leukocyte infiltration, and epithelial damage in a mixed granulocytic asthma model induced by HDM [33]. However, the impact of MIF antagonism is not clear in other features of lung remodeling and in the Th2 response driven by ILC2s and Th2 CD4⁺ T cells on a prototypical eosinophilic asthma model induced by HDM. Although relevant for future therapeutic evaluation, possible off-target effects of ISO-1 preclude a firm conclusion of these findings concerning the pathophysiological roles of MIF on HDM-induced asthma.

In this study, we explored the HDM-induced asthma model in mice to examine the impact of MIF genetic deletion or antibody blockade on the generation of airway inflammation, particularly

in the counts of eosinophils, Th2 CD4⁺ T cells and ILC2s, lung remodeling, and pulmonary function.

Results

Absence of MIF prevents AHR and airway inflammation in HDM-challenged mice

Since it is not fully known whether MIF is relevant to the pathophysiology of asthma models induced by complex allergens, we sought to investigate the role of MIF on pulmonary function in experimental asthma induced by HDM. WT and *Mif*^{-/-} mice were challenged nine times with an HDM extract (Fig. 1A), and airway responsiveness in response to methacholine bronchoprovocation 24 h after the last challenge was determined by enhanced pause (Penh). As expected, WT mice exposed to HDM exhibited a marked AHR in response to increasing doses of methacholine inhalation as compared with saline-challenged WT mice (Fig. 1B). The HDM-challenged *Mif*^{-/-} mice presented a response equivalent to that observed in saline-challenged *Mif*^{-/-} or WT mice (Fig. 1B). Importantly, the Penh levels registered before inhalation of methacholine were similar among all groups of mice (Fig. 1B).

The analysis of histological sections evidenced the large cellular infiltrate of eosinophils in the lungs of HDM-challenged WT mice, which was significantly less pronounced in HDM-challenged *Mif*^{-/-} mice (Fig. 1C & D). In addition, we found increased numbers of infiltrating cells in the BALF of WT mice after the last challenge with HDM, compared to saline-challenged WT mice (Fig. 1E). This infiltrate was mostly composed of eosinophils and mononuclear cells (lymphocytes, monocytes, and macrophages) (Fig. 1F & G), followed by increasing numbers of neutrophils (Fig. 1H). The HDM-challenged *Mif*^{-/-} mice presented increased numbers of total cells and eosinophils in the BALF as compared to saline-treated WT-mice, however, the numbers were two to three times lower than those observed in HDM-challenged WT mice (Fig. 1E & F). Numbers of mononuclear cells were significantly decreased in the BALF of HDM-challenged *Mif*^{-/-} mice compared to HDM-challenged WT mice (Fig. 1G). Numbers of neutrophils were increased in both groups challenged with HDM (Fig. 1H). Together, these results indicate that MIF absence ameliorates or prevents the hallmarks of HDM-induced asthma in mice.

Recently, MIF has been implicated in airway epithelial barrier disruption in an HDM-asthma model [33]. Therefore, we sought to investigate the early release of lung epithelial-cell derived cytokines, such as IL-25, IL-33, and TSLP, 6 h after two HDM challenges. There were no significant differences between the two groups: saline- or HDM-challenged in both *Mif*^{-/-} and WT mice (data not shown). There was no significant change either in DCs or IM lung cell counts, although IM CD86 MFI was increased only in WT HDM-challenged mice (Supporting Information Fig. S1). At this early time point, there are still no significant changes in effector cells such as eosinophils, neutrophils, or Th2 cells (Supporting Information Fig. S1).

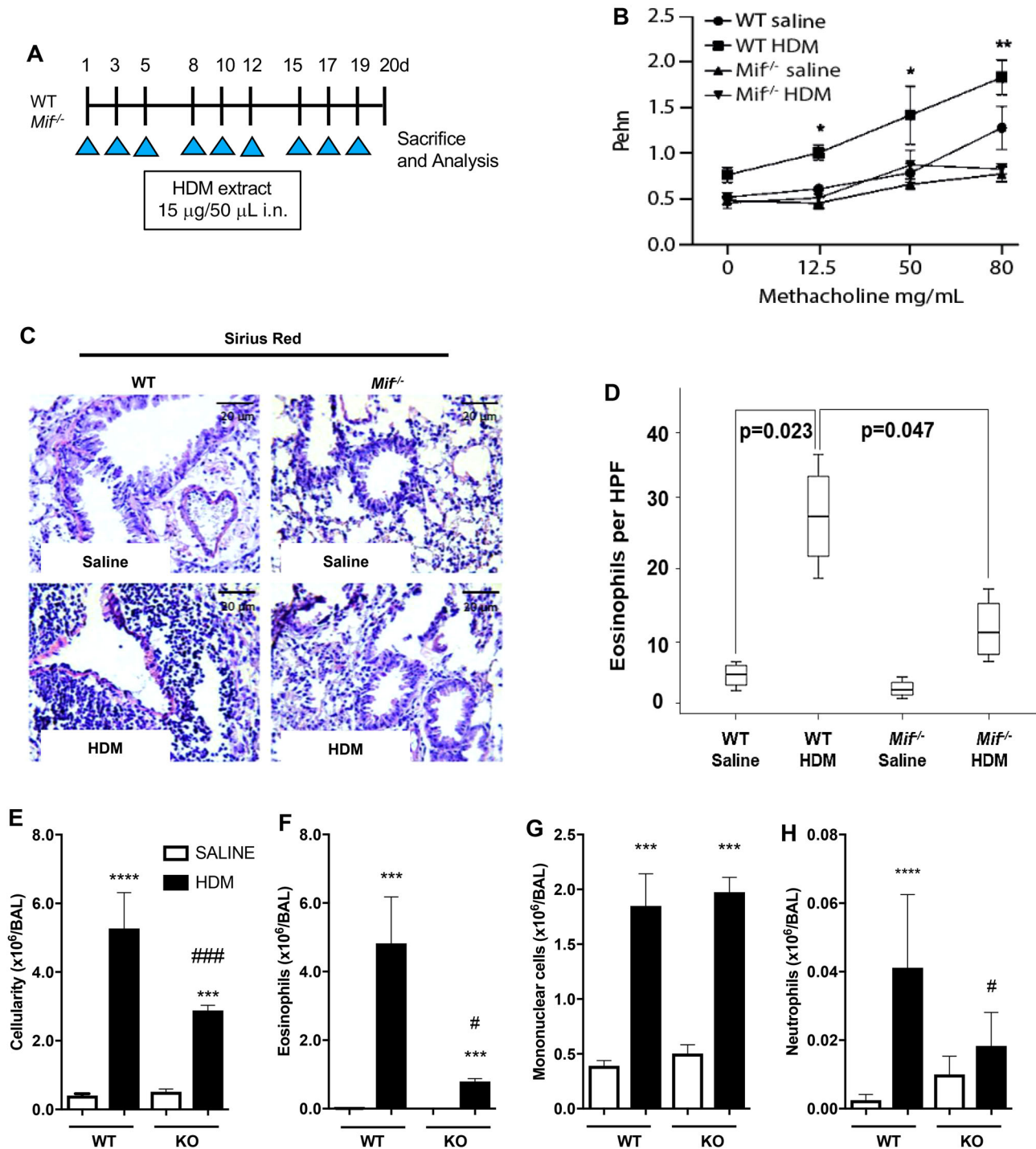


Figure 1. Decreased AHR and lung inflammation in *Mif*^{-/-} mice challenged with HDM. (A) WT and *Mif*^{-/-} mice were challenged nine times on alternate days with HDM (15 µg/50 µL i.n.) or saline and the lungs were evaluated 24 h after the last challenge. (B) Mice in each group were subjected to increasing and cumulative doses of aerosolized methacholine (0, 12.5, 50, and 80 mg/mL), and AHR was measured as Penh values recorded from whole-body barometric plethysmography. (C) Lung sections were stained with Sirius Red to evaluate eosinophil infiltration into the lungs. Scale bar = 20 µm. (D) Semi-quantitative analysis of lung eosinophil infiltration evaluated per high power field (HPF) on Sirius Red-stained sections. (E) Total number of leukocytes, (F) eosinophils, (G) mononuclear cells, and (H) neutrophils retrieved from BALF cells stained with the Fast Panoptic stain kit and evaluated under the light microscopy. Results pooled from two independent experiments are shown as mean ± SD (n = 5), E–H (n = 8–9), or as median with interquartile range ± SD on D (n = 4–7). Data on panels B and E–H were analyzed by one-way ANOVA for multiple comparisons, followed by Tukey's test or unpaired Student's t-test when appropriate. Data on panel D were evaluated with the Kruskal–Wallis ANOVA on ranks test, in which multiple comparisons were carried out using the Dunn's test. Values are significantly different compared to saline-challenged WT or *Mif*^{-/-} mice *p < 0.05; **p < 0.01; ***p < 0.001. Values are significantly different compared to HDM-challenged WT mice #p < 0.05, ###p < 0.0001.

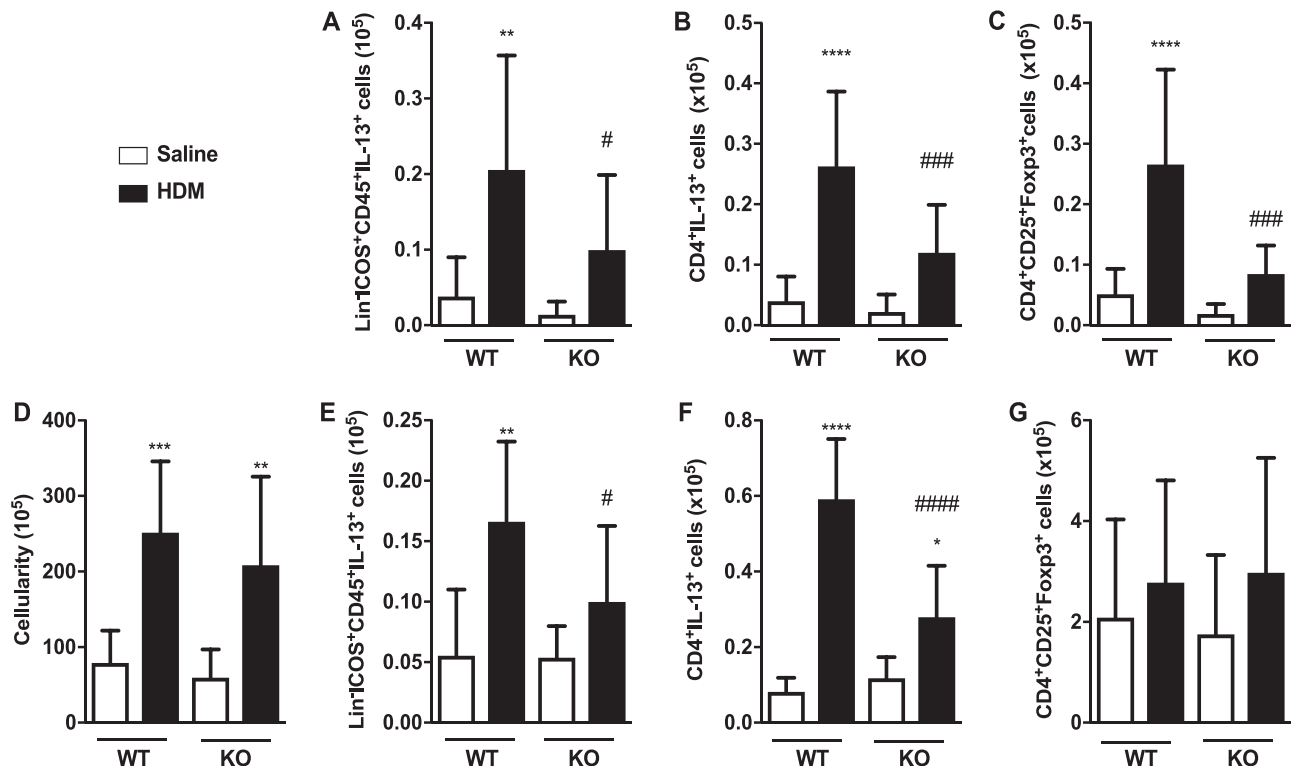


Figure 2. Decreased numbers of ILC2s and IL13⁺ T cells in *Mif*^{-/-} mice challenged with HDM. WT and *Mif*^{-/-} mice were challenged nine times on alternate days with HDM (15 µg/50 µL i.n.) or saline, and the cellular composition in the bronchoalveolar fluid (BALF) and mediastinal lymph nodes (mLNs) were evaluated by flow cytometry 24 h after the last challenge. Counts of (A) ILC2s (Lin⁺CD45⁺ICOS⁺IL-13⁺), (B) IL-13⁺ CD4⁺ T cells, and (C) Treg cells (CD4⁺CD25⁺Foxp3⁺) in the BALF. Total number of (D) leukocytes, (E) ILC2s, (F) IL-13⁺ CD4⁺ T cells, and (G) Treg cells in the mLNs. Results are shown as mean ± SD of eight to nine animals pooled from two independent experiments. Data were analyzed by one-way ANOVA for multiple comparisons followed by unpaired Student's t-test. Values are significantly different compared to saline-challenged WT or *Mif*^{-/-} mice **p* ≤ 0.05; ***p* < 0.01; ****p* ≤ 0.001; *****p* ≤ 0.0001. Values are significantly different compared to HDM-challenged WT mice #*p* ≤ 0.01; ###*p* ≤ 0.0001.

ILC2 and Th2 cell counts are reduced in *Mif*^{-/-} mice upon HDM challenges

ILC2s and CD4⁺ T cells are important sources of key cytokines in allergic asthma, such as IL-4, IL-5, and IL-13, with a positive impact on eosinophil infiltration in the lungs [32, 34]. Indeed, the numbers of ILC2s (Lin⁺CD45⁺ICOS⁺IL-13⁺) and CD4⁺IL-13⁺ cells were increased in the BALF of HDM-challenged WT mice, compared to saline-challenged WT mice, while HDM-challenged *Mif*^{-/-} mice presented a less pronounced increase in the number of these cells (Fig. 2A & B). Foxp3⁺ Treg cell numbers were increased in the BALF of HDM-challenged WT mice when compared to the saline control group (Fig. 2C), which was not observed in HDM-challenged *Mif*^{-/-} mice. Despite the comparable increase of total cell numbers in the mediastinal lymph nodes (mLN) of HDM-challenged mice (Fig. 2D), the numbers of ILC2s and CD4⁺IL-13⁺ cells in the mLN were significantly higher in HDM-challenged WT mice than in *Mif*^{-/-} HDM-challenged mice (Fig. 2E & F). There was no significant difference in numbers of Foxp3⁺ Treg cells in the mLN among the groups (Fig. 2G). These

results reveal an impact of MIF on ILC2s, Th2, and Treg cells in experimental asthma induced by HDM.

MIF regulates the production of inflammatory mediators in the lungs of HDM-challenged mice

We evaluated the impact of MIF absence on Th2 immunity by means of cytokine detection in the BALF. As shown in Fig. 3, the concentrations of IL-4, IL-5, and IL-13 were increased in HDM-challenged WT mice, compared to the saline-challenged WT mice. HDM challenge on *Mif*^{-/-} mice resulted in significantly lower amounts of IL-4, IL-5, and IL-13 in the BALF, compared to HDM-challenged WT mice (Fig. 3A-C). Remarkably, the concentrations of IL-4 and IL-13 in the BALF of HDM-challenged *Mif*^{-/-} mice were comparable to those observed in their respective saline control groups (Fig. 3A & C). The amounts of CCL11 were significantly increased in the same extent in mice challenged with HDM, WT, or *Mif*^{-/-}, compared to their respective saline control groups

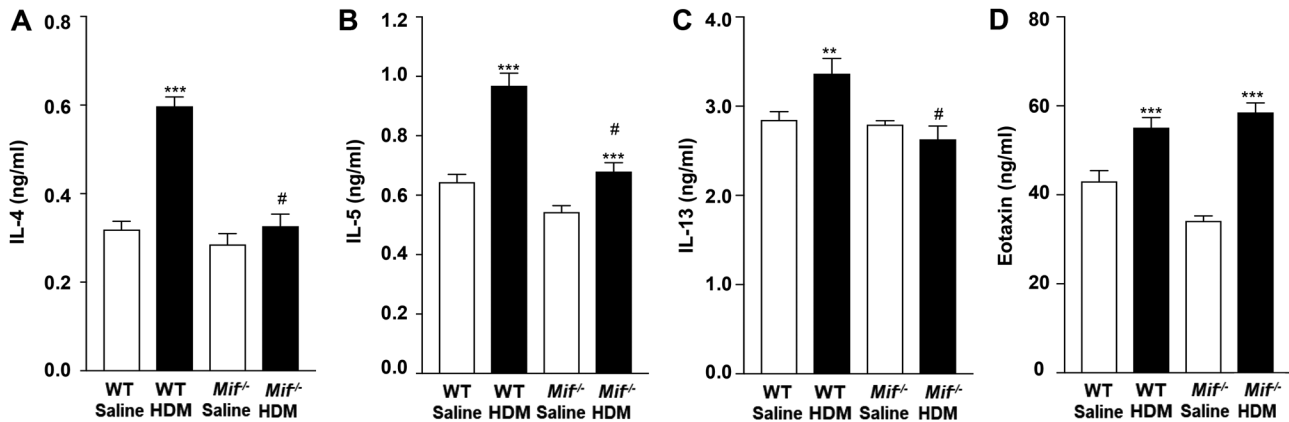


Figure 3. Th2 impairment in *Mif*^{-/-} mice challenged with HDM. WT and *Mif*^{-/-} mice were challenged nine times on alternate days with HDM (15 μ g/50 μ L i.n.) or saline, and cytokine levels were evaluated on homogenized lungs 24 h after the last challenge. Levels of (A) IL-4, (B) IL-5, (C) IL-13, and (D) CCL11 in lung homogenates. Results are shown as mean $\pm$ SD of three to five animals. Data were analyzed by ANOVA for multiple comparisons followed by unpaired Student's t-test. Values are significantly different compared to saline-challenged WT or *Mif*^{-/-} mice ** p < 0.01; *** p $\leq$ 0.001. Values are significantly different compared to HDM-challenged WT mice # p $\leq$ 0.05.

(Fig. 3D). Thus, MIF deficiency impacts IL-4, IL-5, and IL-13 concentrations in the lungs upon HDM challenge, with no effect on CCL11.

MIF regulates airway remodeling in the lungs of HDM-challenged mice

An important feature of the HDM asthma model is the airway remodeling, characteristic of chronic human asthma. HDM challenge induced an increase in the presence of mucus-secreting cells in the lungs of WT mice, compared to WT control group, which was abrogated in HDM-challenged *Mif*^{-/-} mice (Fig. 4A & B). The peribronchiolar fibrotic content increased by HDM challenge in WT mice but was not observed in HDM-challenged *Mif*^{-/-} mice (Fig. 4C & D). Together, these results suggest that MIF impacts airway remodeling induced by HDM.

To assess whether MIF deficiency prevents or delays the generation of airway inflammation and remodeling, analyses were performed 96 h after the last HDM challenge (Fig. 5A). At this later time point, eosinophilic parenchymal infiltrate (Fig. 5B & C), as well as mucus-secreting cells, were still observed in WT HDM challenged mice, but not in HDM-challenged *Mif*^{-/-} mice (Fig. 5D & E). There was no delayed accumulation of lung ILC2, T CD4⁺IL-13⁺ cells or mLN myeloid cells on HDM-challenged *Mif*^{-/-} mice (Supporting Information Fig. S2A–E). These results indicate that MIF, in fact, prevents, rather than delays, the lung inflammatory response observed in HDM-challenged WT mice.

Anti-MIF antibody protects against the development of asthmatic cardinal features induced by HDM

To characterize MIF as a potential therapeutic target, we evaluated whether an anti-MIF antibody prevents the hallmark fea-

tures of asthma in HDM-challenged mice. WT mice were treated with an anti-MIF mAb before each HDM challenge and then analyzed 24 h after the last challenge (Fig. 6A). Treatment of HDM-challenged WT mice using anti-MIF antibody significantly reduced the eosinophil lung infiltration observed in IgG1 isotype control-treated mice (Fig. 6B & C). This treatment also abrogated mucus hypersecretion and collagen deposition in the lungs, which were attenuated due to the treatment of HDM-challenged WT mice, compared to HDM-challenged WT mice treated using the IgG1 isotype or left untreated (Fig. 6D–G). Thus, the anti-MIF antibody prevents the cardinal features of asthma in HDM-challenged mice.

Discussion

It has been shown that MIF is increased in atopic patients [9, 14] and plays a central role in the pathogenesis of OVA-induced asthma [8, 10]. Using the asthma model induced by HDM, we demonstrated that compared to WT mice *Mif*^{-/-} exhibit reduced AHR, an ablation on eosinophil counts, type 2 cytokine concentrations (IL-4, IL-5, IL-13), mucus secretion, and collagen fiber deposition in the lungs. Importantly, when a late time point was analyzed, we confirmed that MIF ablation prevented, rather than delayed the HDM-induced lung allergic response. In addition, the therapeutic use of anti-MIF antibodies during HDM challenges mimics the attenuated asthma phenotype observed in *Mif*^{-/-} mice. Thus, our data indicate that MIF contributes to the development of asthma hallmark features in the HDM model.

Asthma development is greatly impacted by ILC2s, which are known to be induced by activated epithelia, and in turn, activated ILC2s produce type 2 cytokines (IL-4, IL-5, and IL-13) [32, 35, 36]. In contrast to the HDM asthma model, murine studies using OVA as an allergen present contradictory results regarding the activation of ILC2s [37–41]. Here, we show that *Mif*^{-/-} mice are unable to sustain high numbers of ILC2s in the airway and

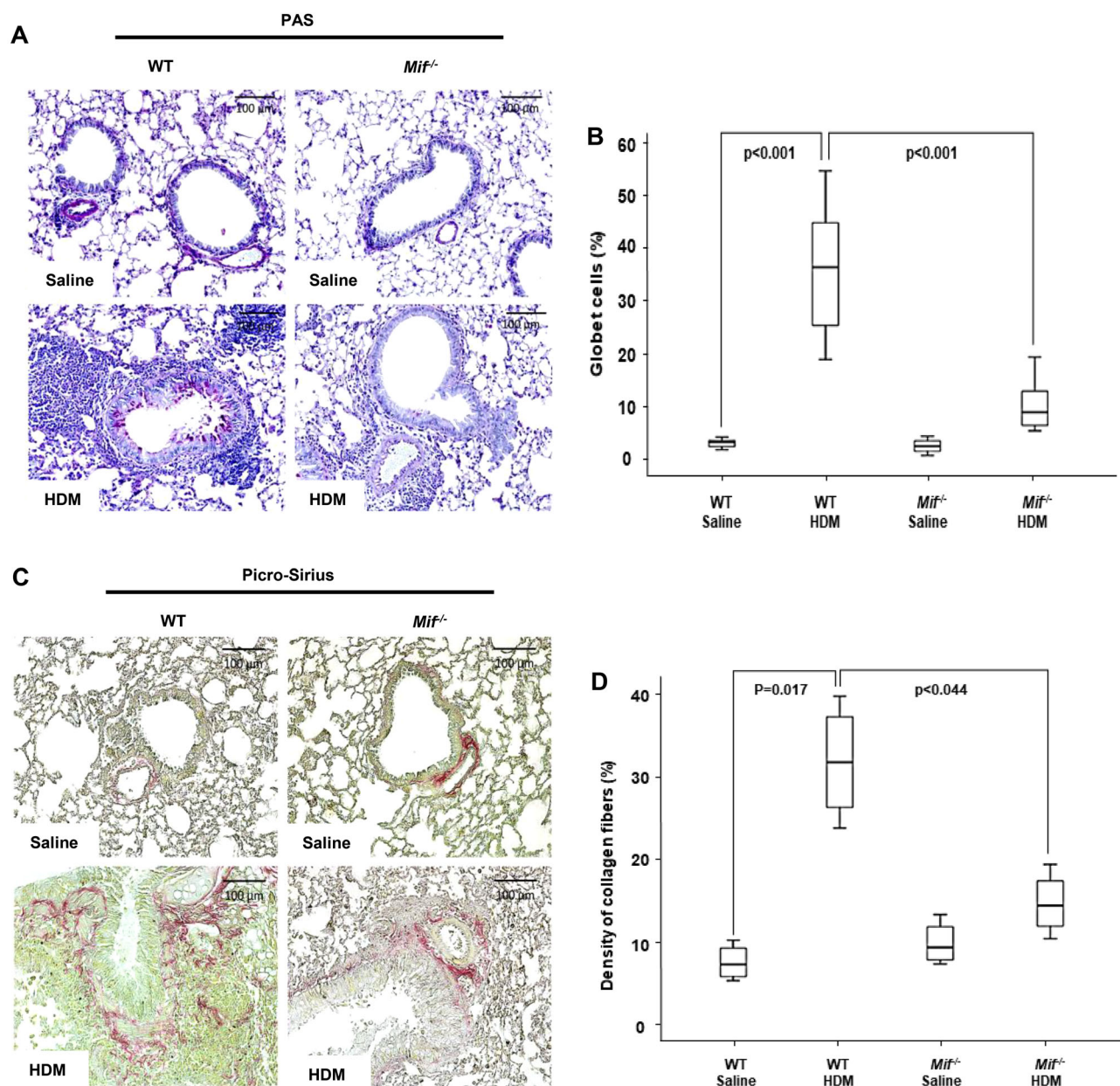


Figure 4. Airway remodeling ablation in *Mif*^{-/-} mice challenged with HDM. WT and *Mif*^{-/-} mice were challenged nine times on alternate days with HDM (15 μ g/50 μ L i.n.) or saline and the lungs were retrieved 24 h after the last challenge, sectioned, and stained. (A) Representative photomicrograph of Periodic Acid-Schiff (PAS)-stained lung sections (scale bar = 100 μ m) and (B) semiquantitative analysis of airway mucus secretion. (C) Representative photomicrograph of Picro-Sirius-stained lung sections (scale bar = 20 μ m) and semiquantitative analysis of (D) peribronchiolar fibrotic content. Results are shown as interquartile range $\pm$ SD of four to seven animals on B and D. Data were evaluated with the Kruskal-Wallis ANOVA on ranks test, in which multiple comparisons were carried out using the Dunnett's test.

mLN upon HDM challenges, as opposed to MIF-sufficient mice. Recently, it has been shown that *Mif*^{-/-} mice exhibit decreased ILC2s during helminthic infection [42, 43]. This decreased ILC2 numbers greatly impact macrophage phenotype and the outcomes of parasitic infection [42]. It still needs to be determined whether MIF affects ILC2s directly in asthma and whether this is critical for the development of airway eosinophilia, Th2 polarization, lung remodeling, and AHR.

Mizue et al. showed that MIF ablation reduces IL-4 amounts, while increasing IFN- ψ , IL-10, and Tregs, which counterbalance the Th2 phenotype and improve OVA-induced asthma [44]. However, we observed that MIF does not affect Th2 differentiation or the concentrations of IL-4 in the lungs of mice challenged with OVA [8]. Here, we show that IL-4 concentrations are reduced in the airway of HDM-challenged *Mif*^{-/-} mice. Importantly, IL-13 producing T-cells numbers are also decreased upon HDM

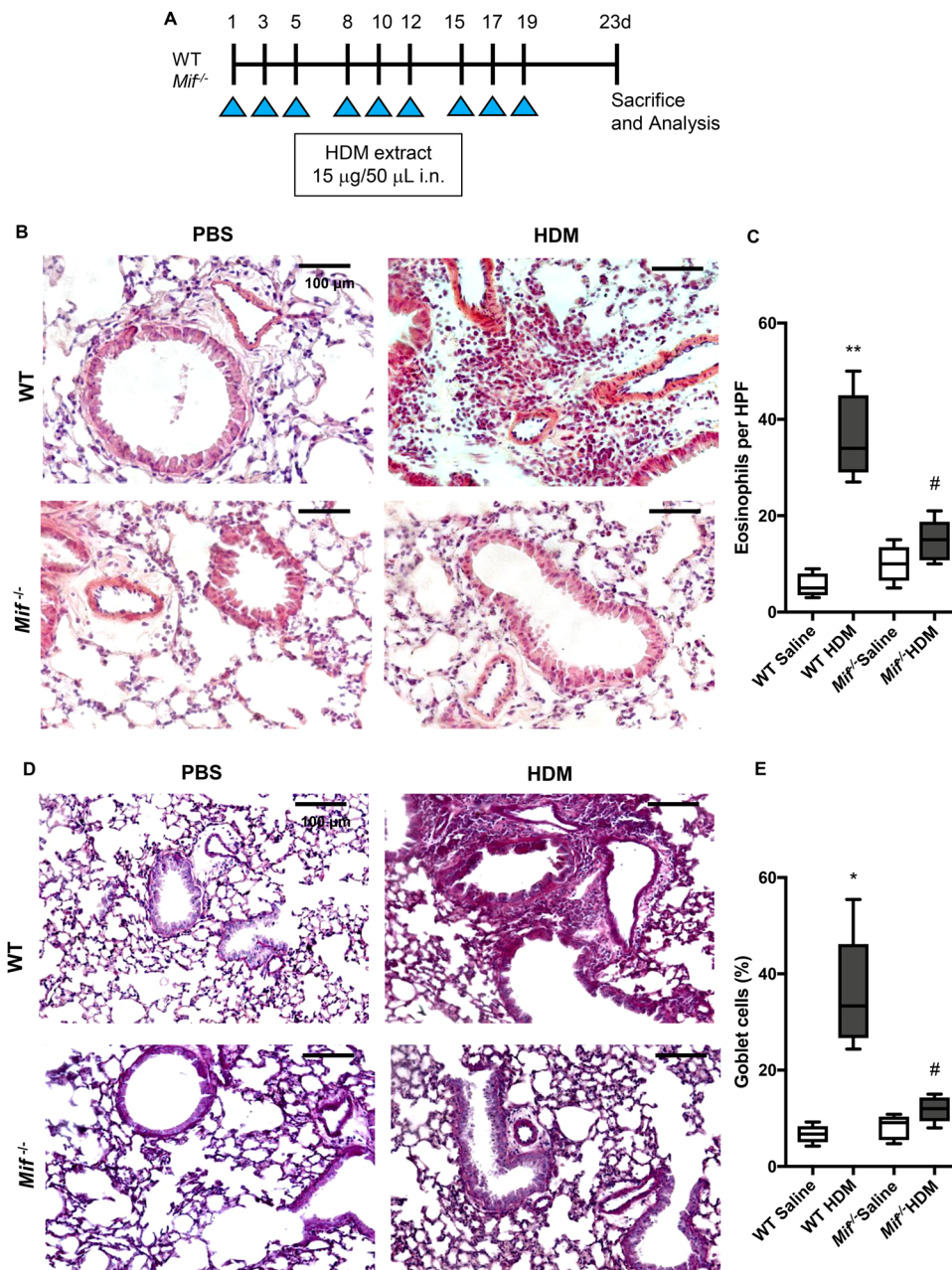


Figure 5. Decreased lung inflammation and remodeling in *Mif*^{-/-} mice challenged with HDM. (A) WT and *Mif*^{-/-} mice were challenged nine times on alternate days with HDM (15 µg/50 µL i.n.) or saline and the lungs were retrieved 96 h after the last challenge, sectioned, and stained. (B) Representative photomicrograph of Sirius Red-stained sections (scale bar = 100 µm) and (C) semiquantitative analysis of lung eosinophils infiltration evaluated per high power field (HPF). (D) Representative photomicrograph of Periodic Acid-Schiff (PAS)-stained lung sections (scale bar = 100 µm) and (E) semiquantitative analysis of airway mucus secretion. Results are shown as interquartile range ± SD of five to six animals on C and E. Data on panel D were evaluated with the Kruskal-Wallis ANOVA on ranks test, in which multiple comparisons were carried out using the Dunnnett's test. Values are significantly different compared to saline-challenged WT or *Mif*^{-/-} mice **p* < 0.05; ***p* < 0.01. The values are significantly different compared to HDM-challenged WT mice #*p* ≤ 0.05.

challenge in *Mif*^{-/-} mice compared to WT challenged with HDM. In conclusion, we observed an impact of MIF on the regulation of the Th2 response induced by HDM lung challenges, which is probably independent of Tregs.

Eosinophils accumulate in the airway of atopic asthmatic individuals, and high numbers of these cells can correlate with dis-

ease severity [45, 46]. The results presented herein showing less eosinophilic airway inflammation on HDM-challenged *Mif*^{-/-} mice are in accordance with previous results on OVA models [8, 44], suggesting that the critical role of MIF on the development of airway eosinophilia occurs independently of the asthma model. Moreover, *Mif*^{-/-} mice present reduced tissue eosinophil numbers

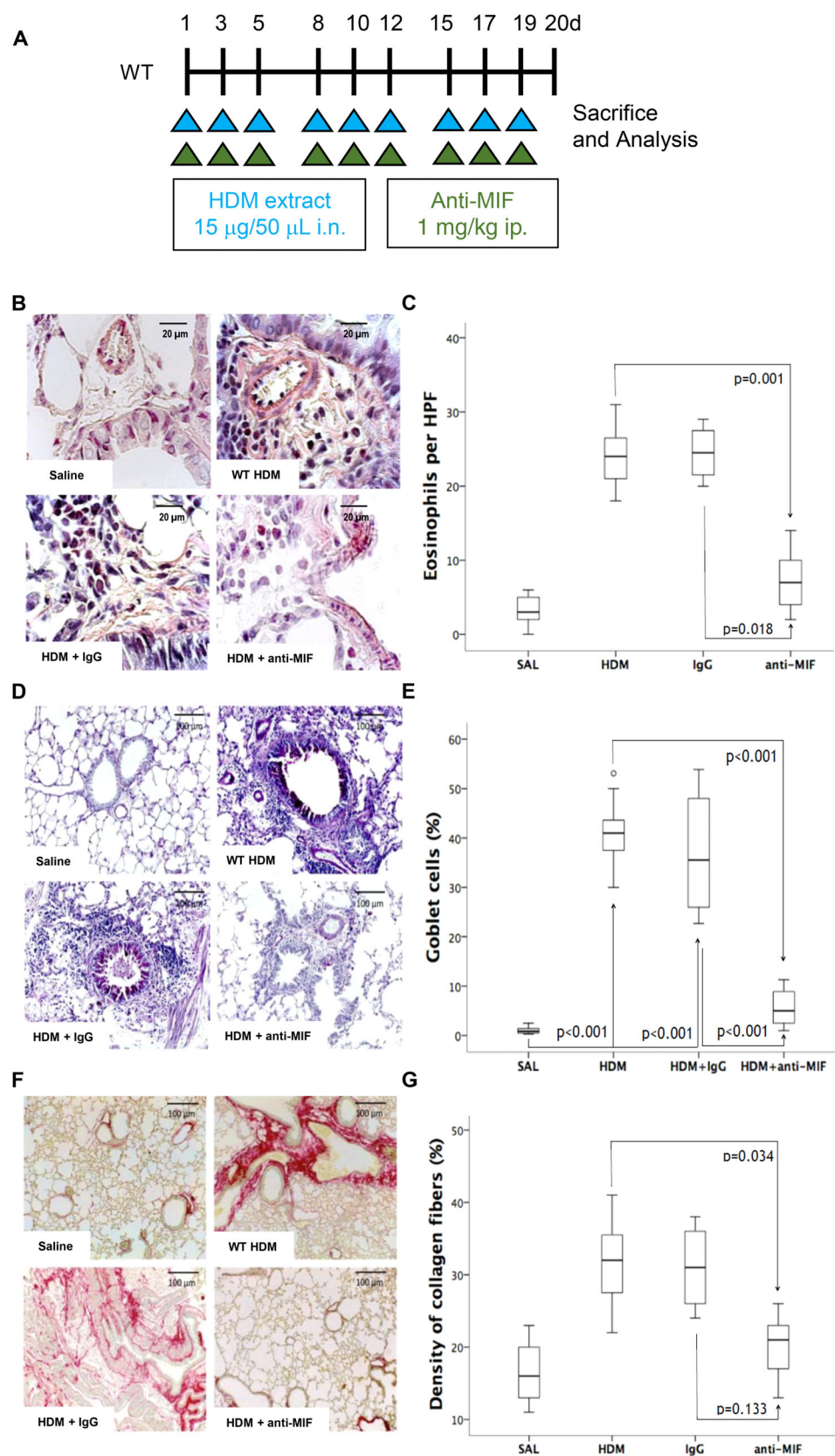


Figure 6. Anti-MIF treatment impairs airway eosinophil infiltration and lung remodeling in HDM-challenged mice. (A) Mice were treated with anti-MIF IgG1 (1 mg/kg, i.p.) or nonrelevant IgG1 isotype control, and after 1 h, mice were challenged with HDM (15 µg/50 µL i.n.), a total of nine times. Lungs were retrieved 24 h after the last challenge, sectioned, and stained. (B) A representative photomicrograph of Sirius Red-stained lung sections (scale bar = 100 µm) and (C) semiquantitative analysis of lung eosinophil infiltration. (D) Representative photomicrograph of Picro-Sirius-stained lung sections (scale bar = 100 µm) and (E) semiquantitative analysis of airway mucus secretion. (F) Representative photomicrograph of Picro-Sirius-stained lung sections (scale bar = 20 µm) and semiquantitative analysis of (G) peribronchiolar fibrotic content. Results are shown as interquartile range ± SD of four to nine animals on C, E, and G. Data on panel D were evaluated with the Kruskal–Wallis ANOVA on ranks test in which multiple comparisons were carried out using the Dunnett's test.

in experimental mouse models of eosinophilic esophagitis and *Schistosoma mansoni* infection [13, 24]. IL-5 and CCL11 are critical to maintain the elevated eosinophil numbers into the tissue [47, 48]. HDM-challenged *Mif*^{-/-} mice present reduced levels of IL-5 in the airway, which is consistent to the decreased eosinophil numbers in the airway. Although it was reported that concentrations of CCL11 are reduced in the lung of OVA-challenged *Mif*^{-/-} mice [8, 44], in our study, CCL11 concentrations are high in the airway of HDM-challenged *Mif*^{-/-} mice. Mould et al. published that CCL11-induced tissue eosinophilia is abrogated in the absence of IL-5, which could partially explain the low numbers of eosinophils even with high concentrations of CCL11 in the lungs [47]. MIF can directly induce eosinophil infiltration in vitro, while the use of recombinant MIF leads to increased mucosal eosinophil infiltration in a model of eosinophilic esophagitis [13]. Therefore, it is possible that MIF ablation *per se* is critical for the eosinophil number reduction in the HDM model as well, and together with IL-5 ablation leads to this drastic reduction in eosinophil counts in the airway, independent of CCL11.

HDM-challenged *Mif*^{-/-} mice presented reduced lung tissue eosinophil counts and IL-13, which was consistent with the decreased airway collagen deposition and mucus secretion observed. Humbles et al. demonstrated that eosinophil ablation is sufficient to avert airway fibrosis and the increase in airway smooth muscle in OVA-based asthma model [49]. This observation was supported by data on experimental models and in human studies [50–52]. However, Fattouh et al. showed that eosinophil depletion is unable to change the degree of mucus production and airway subepithelial fibrosis in a HDM asthma mouse model [53]. In fact, several factors can induce airway remodeling, that is, TGF- β , which can be released by neutrophils and eosinophils, among other cells [5, 54, 55]. Of note, it was demonstrated that eosinophils do not play a critical role in lung function decline neither in HDM nor in OVA models [49, 53], which reinforces that MIF could improve respiratory mechanics in asthma via eosinophil-independent mechanisms.

OVA is classically used to study asthma due to the feasibility of the model, as it is relatively cheap and there is an available protein and genetic models in which specific aspects of adaptive immunity can be achieved [56]. However, OVA is not a potent immunogenic protein, requiring adjuvants during sensitization and alternative routes of administration such as intraperitoneal and subcutaneous [57]. Instead, HDM is one of the most common aeroallergens worldwide and sensitization normally occurs locally in the lungs of the mice, without the need of adjuvants [27]. The local sensitization after HDM exposure is favored by the proteolytic components that HDM carries (e.g. Der p 1), which target the epithelium and trigger innate immune responses [27, 58]. Thus, the HDM model is a more translational approach to study asthma, while OVA-based models do not depict asthma's complexity. Recently, Lan et al. showed that the MIF antagonist ISO-1 improves lung function in mice challenged with HDM, while reducing airway eosinophilia and Th2 cytokine levels [33]. In this study, airway inflammation was characterized by a superimposition of neutrophils over eosinophils [33], in contrast to the exper-

imental model explored in our study, which led to a predominant eosinophilic lung inflammation.

Importantly, treatment with anti-MIF prevents eosinophil infiltration into the airway and lung remodeling. It is important to highlight that the current therapies for asthma affect primarily airway inflammation or bronchoconstriction in a reversible way, having a negligible impact on lung remodeling [59]. By targeting inflammation and tissue remodeling, anti-MIF appears as a promising therapy in asthma.

In summary, we describe here the critical role of MIF in the HDM asthma model in mice, thus, supporting the effects of MIF observed previously on OVA-based models. By revealing the importance of MIF in a clinically relevant asthma model, this study reinforces that targeting MIF can be a good therapeutic approach to control asthma.

Materials and methods

Animals

Female and male C57BL/6 WT and *Mif*^{-/-} mice aged 6 to 8 weeks old were supplied with water and food *ad libitum*. All mice are maintained in ventilated microisolators in the same animal facility. *Mif*^{-/-} mice were obtained as previously described [16, 60], and mice were backcrossed for 10 generations onto a C57BL/6 background. C57BL/6 WT mice used in this study were not littermates of the *Mif*^{-/-} mice. Littermate mice from the same genetic background, female or male, were distributed in HDM-challenged or saline-challenged groups, throughout the experiments. All experimental procedures were approved by the Animals Ethics Committee of the Health Sciences Centre of the Federal University of Rio de Janeiro (IMPPG028 and 017–22 CEUA-UFRJ, Rio de Janeiro, Brazil).

HDM challenge

Mice were challenged intranasally with 15 μ g of HDM extract (*Dermatophagoides pteronyssinus*, Greer Laboratories, Lenoir, NC, USA) diluted in 50 μ L of saline for 3 days a week during 3 weeks, and animals were analyzed 24 h after the last challenge [61]. Control mice were challenged intranasally with 50 μ L of saline. In another set of experiments, mice were intraperitoneally treated with anti-MIF IgG1 monoclonal antibody (clone NIH3L.D950, 1 mg/kg) or nonrelevant IgG1 isotype control (1 mg/kg) 1 h before intranasal challenge with HDM [13]. For short-term experiments, mice were challenged intranasally with the HDM extract for 2 alternate days, and animals were analyzed 6 h after the last challenge. A late time point was also assessed, in which mice were challenged 3 days a week for 3 weeks and analyses were performed 96 h after the last challenge.

Lung function

Airway responsiveness was recorded using a whole-body noninvasive plethysmograph (Buxco Electronics, Sharon, CT, USA) 24 h after the last challenge. Briefly, conscious unrestrained mouse was placed on the plethysmography chamber and then nebulized first with aerosolized PBS (0), then with increasing and cumulative concentrations of aerosolized methacholine (12.5, 50, and 80 mg/mL) during 1 min, with 5-min intervals. The values of lung mechanics were expressed as enhanced pause (Penh), as described previously [62].

Cell recovery and count

BALF was obtained from each mouse by flushing the lung with PBS ($5 \times 300 \mu\text{L}$) through a polyethylene tracheal cannula coupled with a syringe, followed by a gentle aspiration of the injected volume. The total volume recovered was centrifuged (500g for 10 min at 4°C), and the supernatant and cell pellet were used for experiments. mLNs were carefully extracted, placed in 1 mL of PBS, and a cell suspension was obtained after mechanical homogenization. The total number of cells in the BALF and mLN was counted in a hemocytometer. The cytospin smears from the cells in the BALF were used for a differential count using the Fast Panoptic stain kit (Laborelin Ltd., São Paulo, Brazil). A minimum of 300 cells were counted and classified as eosinophils, neutrophils, and mononuclear cells based on morphological criteria.

Flow cytometry assays

Cells recovered from BALF and mLN were blocked with anti-CD16/32 and stained using monoclonal antibodies to detect Th2 CD4⁺ T cells—anti-CD4 APC (GK1.5) and anti-IL-13 Alexa Fluor 488 (eBio13A) or ILC2s—anti-ICOS PE (15F9), anti-CD45 APC/Cy7 (30-F11), lineage negative eFluor450 and anti-IL-13 Alexa Fluor 488, as previously described [63]. Mouse Hematopoietic Lineage Antibody Cocktail (Lin⁻, eBioscience) includes Anti-Mouse CD3 (17A2), Anti-Mouse CD45R (B220) (RA3-6B2), Anti-Mouse CD11b (M1/70), Anti-Mouse TER-119 (TER-119) and Anti-Mouse Ly-G6 (Gr-1) (RB6-8C5). Prior to intracellular staining of cytokines, cells were stimulated with PMA (0.04 ng/mL) and ionomycin (0.012 mM/mL) in the presence of Brefeldin A (0.01 mg/mL) for 3 h at 37°C , and then washed, labeled for surface antigens, fixed, and permeabilized [64]. Treg cells were characterized by staining for CD4, CD25, and Foxp3 according to the manufacturer's instructions of the Mouse Treg Staining Kit (eBiosciences; San Diego, CA). For the characterization of innate immune cell populations in mLNs and lungs, cells were incubated with LIVE/DEAD Fixable Aqua Dead Cell staining (1:1000, BV510; Invitrogen) in PBS for 30 min, before staining with monoclonal antibodies against anti-CD103 BV421 (clone), anti-Ly6G BV605 (clone), anti-CD45 BV785 (30-F11), anti-CD11c FITC (clone), anti-IAb PerCP/Cy5.5 (clone), anti-CD86 PE

(clone), anti-SiglecF APC (clone), anti-CD11b Alexa Fluor 700 (clone), and anti-CD24 APC/Cy7 (clone). All antibodies were purchased from BioLegend and eBiosciences. Cells were acquired on FACS Canto II flow cytometer or on LSR FORTRESSA X-20 (BD Biosciences Immunocytometry Systems, San Jose, CA) and analyzed using FlowJo X 10.0.7 software (Tree Star Inc., Ashland, OR).

Gating strategies for ILC2 and myeloid cells are shown in Supporting Information Fig. S3.

ELISA assays

IL-4, IL-5, IL-13, and CCL11 were detected in lung homogenate at 24 h after the last challenge. ELISAs were performed with commercially available kits (Peprotech, Rock Hill, NJ), following the instructions of the manufacturer.

Histological analysis

Lungs were inflated by the injection of 1 mL of 4% buffered formalin through a tracheal catheter, and then fixed in the same solution overnight. Paraffin-embedded specimens were cut into 5- μm sections and stained with Sirius Red, Periodic Acid-Schiff (PAS) or picrosirius to detect, respectively, eosinophils, mucus-producing cells, and fibrotic content under light microscopy. The images of stained histological sections were captured and analyzed using a computer-assisted image analyzer (Leica QWin Plus V 3.5.1, Leica Microsystems Ltd., Switzerland). Eosinophils were counted in the five areas at $\times 400$ magnifications, and mean was expressed as eosinophils per high-power field. Mucus secretion was quantified by counting the number of goblet cells present in at least 500 cells of the bronchial epithelium and the values were expressed as percentage. The density of collagen fibers was defined by the percentage of stained area in relation to total area observed in at least five bronchovascular bundles per tissue at a magnification of $\times 100$.

Statistical analysis

All data (except histological) were analyzed by one-way ANOVA for multiple comparisons, followed by Tukey's test or unpaired Student's *t*-test when appropriate. Tests were performed on GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA). Data are presented as mean $\pm$ SEM. Analyses of histological data were performed using the statistical software SPSS for Windows version 20.0 (IBM SPSS Inc., Chicago, IL, USA) and the experimental groups were evaluated with the Kruskal–Wallis ANOVA on ranks test, in which multiple comparisons were carried out using the Dunnett's test. Values are expressed as medians with interquartile ranges. For all data analysis, a value of $p < 0.05$ was taken as significant.

Ethics Approval Statement: Animal Studies

All of the work involving the care and use of mice was performed under protocols approved by the UFRJ Animal Ethics Committee (IMPPG028 and 017–22 CEUA- UFRJ, Rio de Janeiro, Brazil).

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Abbreviations: AHR: airway hyperresponsiveness · HDM: house dust mite · ILC2: group 2 innate lymphoid cell · PAS: Periodic Acid-Schiff

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