Cross-Talk between Macrophage Migration Inhibitory Factor and Eotaxin in Allergic Eosinophil Activation Forms Leukotriene C_4–Synthesizing Lipid Bodies

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Recent studies have demonstrated an essential and nonredundant role for macrophage migration inhibitory factor (MIF) in asthma pathogenesis. Here we investigate the mechanisms involved in MIF-induced eosinophil activation. By using a model of allergic pulmonary inflammation, we observed that allergen challenge–elicited eosinophil influx, lipid body (also known as lipid droplets) biogenesis, and leukotriene (LT) C_4 synthesis are markedly reduced in *Mif*$^{-/-}$ compared with wild-type mice. Likewise, *in vivo* administration of MIF induced formation of new lipid bodies within eosinophils recruited to the inflammatory reaction site that corresponded to the intracellular compartment of increased LTC_4 synthesis. MIF-mediated eosinophil activation was at least in part due to a direct effect on eosinophils, because MIF was able to elicit lipid body assembly within human eosinophils *in vitro*, a phenomenon that was blocked by neutralization of the MIF receptor, CD74. MIF-induced eosinophil lipid body biogenesis, both *in vivo* and *in vitro*, was dependent on the cooperation of MIF and eotaxin acting in a positive-feedback loop, because anti-eotaxin and anti-CCR3 antibodies inhibit MIF-elicited lipid body formation, whereas eotaxin-induced lipid body formation is affected by anti-CD74 and MIF expression deficiency. Therefore, allergy-elicited inflammatory MIF acts in concert with eotaxin as a key activator of eosinophils to form LTC_4-synthesizing lipid bodies via cross-talk between CD74 and CCR3. Due to the effect of MIF on eosinophils, strategies that inhibit MIF activity might be of therapeutic value in controlling allergic inflammation.

Keywords: macrophage migration inhibitory factor; leukotriene C_4; lipid droplets; allergic inflammation; asthma

Macrophage migration inhibitory factor (MIF) is a pleiotropic molecule that acts as a critical mediator of both innate and acquired immune responses (1, 2). Recently, a role for MIF in pathogenesis of T helper (Th) type 2–driven inflammatory reactions was reported. A central, nonredundant function of MIF in asthma pathogenesis was established using Th2-driven animal models of allergic pulmonary inflammation, where endogenous MIF was found to regulate bronchial hyperresponsiveness, mucus secretion, and eosinophilic inflammation (3–5). Supporting such a role in pulmonary inflammation, patients with asthma were found to have increased concentrations of MIF in the bronchoalveolar lavage (BAL) fluids, sputum, and sera (6, 7). Similarly, a role for MIF was also described in the Th2 response induced by parasitic infections, such as helminthic infections by *Taenia crassiceps*, *Schistosoma japonicum*, and *Schistosoma mansoni* (8–10).

Even though studies concur that MIF participates in the pathogenesis of Th2-driven diseases, such as asthma and parasitic infestations, the MIF-targeted mechanisms involved in regulation of Th2 pathologies are still incompletely understood. Although polarization toward a Th2 response was initially postulated as the targeted pathway, there is no consensus in the literature on this topic (3–5, 10). In regard to the development of Th2-driven disturbances, we hypothesized that MIF acts as a modulator of eosinophil activation. Indeed, MIF has emerged as a potent inflammatory mediator capable of regulating eosinophil differentiation and chemotaxis, as well as leukotriene (LT) C_4 production triggered by allergic challenge (3, 10).

Eosinophil recruitment and subsequent activation at sites of allergic inflammation has the potential to generate and release diverse lipid and protein mediators critical to the development and perpetuation of the allergic process (11–13). Indeed, eosinophils represent a major source of LTC_4 and its extracellular derivatives (LTD_4 and LTE_4), products of the 5-lipoxygenase (5-LO) pathway of arachidonic acid metabolism in allergic diseases (14–16).

Central to the pathogenesis of allergic diseases, these cysteinyl LTs (cysLTs) cause bronchoconstriction, mucous hypersecretion, increased microvascular permeability, bronchial hyperresponsiveness, eosinophil infiltration, and airway remodeling (17–19). Although the enzymatic pathways by which eosinophils synthesize LTC_4 are well established, the key regulatory mechanisms of allergen-induced LTC_4 synthesis, such as its specialized intracellular compartmentalization and the relevant endogenous allergy-triggered stimuli, remain uncharacterized.

The formation of LTC_4 requires the functional interaction of at least four proteins acting in cascade: cytoplasmic phospholi-

CLINICAL RELEVANCE

We demonstrated a cooperative stimulatory loop between macrophage migration inhibitory factor (MIF) and eotaxin in allergy-elicited eosinophil activation, placing MIF as an interesting candidate for new antiallergic therapeutical development.

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pase A_2; 5-LO–activating protein; 5-LO; and LTC_4 synthase (20, 21). Studies from several groups suggest that dynamic compartmentalization of these biosynthetic proteins is a primary mechanism for the regulated synthesis of LTC_4 (for review, *see* Refs. 22, 23). Upon leukocyte activation, three distinct intracellular domains may compartmentalize such macromolecular organization for LTC_4 synthesis: the nuclear membrane (24); phagosomes (25); and/or lipid bodies (26).

Lipid bodies, also known as lipid droplets or adiposomes, are cytoplasmic lipid domains surrounded by a phospholipid monolayer with a unique fatty acid composition, characterized by a neutral lipid-rich core and variable protein composition (27, 28). Characterization of lipid body protein content in different cell types has suggested that lipid bodies are heterogeneous, dynamic, and multifunctional organelles. Proteins compartmentalized within lipid bodies include enzymes involved in lipid metabolism, cytokines, proteins of the Rab family, specific kinases, and small GTPases, thus positioning lipid bodies as potential regulators of lipid metabolism, membrane trafficking, and intracellular signaling (for review, *see* Refs. 28, 29). Likewise, by uncovering that eicosanoid-forming enzymes are compartmentalized alongside esterified arachidonic acid within lipid bodies (26, 30, 31), an additional lipid body function as inflammatory mediator producer was postulated. This was then confirmed by the direct detection of newly synthesized LTC_4 in eosinophil lipid bodies (32, 33).

Lipid body biogenesis is a biological process that has been intensively studied over the past few years. Assembly of new lipid bodies is recognized as an acute, highly regulated cellular event that is stimulus and cell type specific, which takes place at specialized regions of endoplasmic reticulum, and ultimately determines the specific functions of the newly formed lipid bodies. Moreover, increased biogenic activity is a constant feature of leukocyte activation elicited by inflammatory process (for review, *see* Ref. 28), as observed in eosinophils from patients with Crohn's disease (34) and hypereosinophilic syndrome (35), and from animals undergoing allergic inflammation (33, 36, 37).

Although it is recognized that the allergic inflammatory response increases the number of LTC_4-synthesizing lipid bodies within activated eosinophils, the detailed molecular mechanisms controlling their biogenic process remain largely unclear. It was determined by *in vitro* assays that specific agonists, particularly eotaxin and prostaglandin $(PG)D_2$, could trigger signaling mechanisms that rapidly stimulate the formation of new LTC_4-synthesizing lipid bodies within human eosinophils (32, 33). Accordingly, *in vivo* studies conducted by our group showed that both the biogenesis of lipid bodies and the enhanced synthesis of LTC_4 at newly formed lipid bodies triggered by allergic inflammation are partially mediated by eotaxin/regulated on activation, normal T cell–expressed and secreted (RANTES) acting via CCR3 receptors (33) and by PGD_2 (37).

As discussed previously here, MIF mediated the enhancement of LTC_4 production triggered by allergic challenge in a mouse asthma model (3). Therefore, we aimed to investigate the mechanisms involved in MIF-mediated, allergen-elicited LTC_4 synthesis, focusing on the ability of MIF to activate LTC_4-synthesizing machinery in eosinophils by analyzing three key regulatory aspects: (*1*) the intracellular LTC_4-synthesizing compartment; (*2*) the induced biogenesis of such cytoplasmic compartments, named lipid bodies; and (*3*) the potentially stimulatory cross-talk with eotaxin. Our results show that, at sites of eosinophilic inflammation, cross-talk between MIF and eotaxin activates eosinophils, and results in the assembly of lipid bodies with the ability to synthesize LTC_4.

MATERIAL AND METHODS

Reagents

Chicken egg ovalbumin (OVA), BSA, PBS, 1-ethyl-3-(3-dimethylamino-propyl) carbodiimide (EDAC), and mouse IgG1 were purchased from Sigma Chemical Co. (St. Louis, MO). Neutralizing antibodies (Abs) to murine and human eotaxin, recombinant murine (rm)MIF, rmeotaxin, rheotaxin, recombinant human (rh)MIF, and CCR3 were purchased from R&D Systems (Minneapolis, MN), whereas anti-CD74 (clone LN2) was from BD Pharmimgen (San Diego, CA). MK 886 was purchased from Biomol (Plymouth Meeting, PA).

Animals

C3H/He, C3H/HeJ, BALB/c, and MIF-deficient ($Mif^{-/-}$) mice (3, 38) (BALB/c background) ranging from 6 to 8 weeks old, of both sexes, were obtained from the Oswaldo Cruz Foundation breeding unit (Rio de Janeiro, Brazil). Protocols were approved by the Fundação Oswaldo Cruz animal welfare committee.

Model of Allergic Pulmonary Eosinophilia

Allergic inflammation in the lungs of actively sensitized mice was generated as previously described (2). Briefly, mice were sensitized with intraperitoneal injection of OVA (10 μg/mouse) and Al(OH)3 (10 mg/ml) resuspended in sterile saline (0.2 ml) on Days 1 and 10. From Day 19 to Day 24 after sensitization, mice were challenged daily for 20 minutes with OVA (5% in sterile saline) by aerosol. Sterile saline was aerosolized to sensitized mice as a negative control. Aerosolization was performed in a 30 × 20 × 10–cm acrylic chamber, and the aerosol was generated with an ultrasonic nebulizer. At 24 hours after the last aerosolization, animals were killed by CO_2 inhalation, and the tracheas were surgically exposed and cannulated. The BAL fluid was collected by washing the lungs with PBS (1 ml).

Pleurisy Triggered by MIF or Eotaxin

Naive mice received intrapleural injections of either rmMIF (0.1–1 μg/cavity) or rmeotaxin (30 pmol/cavity), each diluted in sterile saline immediately before use. Control animals were injected with the same volume (0.1 ml) of saline. At 6 or 24 hours after stimulation, mice were killed by CO_2 inhalation, and pleural cavities were rinsed with 1 ml of PBS.

Leukocyte Counts

Total leukocytes (diluted with Turk's 2% acetic acid fluid) were counted using Neubauer chambers. Differential counts were performed in cytopins stained by the May-Grunwald-Giemsa method. Counts are reported as total number of eosinophils per BAL fluid or pleural cavity.

Quantification of cysLTs and Eotaxin

Amount of cysLTs found in BAL or pleural fluid were measured by a commercial EIA kit according to the manufacturer's instructions (Cayman Chemicals, Ann Arbor, MI). Eotaxin were measured in the pleural fluid using commercially available ELISA kit according to the manufacturer's protocol (R&D Systems).

EicosaCell for LTC_4 Immunolocalization

LTC_4 was localized to the sites of formation, as previously described (32, 33). Briefly, leukocytes were recovered from the

pleural cavity 6 hours after MIF or saline administration, and 1 hour after zileuton, by washing the cavity with 500 μl of Hanks' balanced salt solution (HBSS). Cells were then immediately mixed with 500 μl of EDAC (1% in HBSS), which is used to cross-link eicosanoid carboxyl groups to amines of adjacent proteins. After a 30-minute incubation at 37°C with EDAC to promote both cell fixation and permeabilization, pleural leukocytes were then washed with HBSS, cytospun onto glass slides, and blocked with HBSS containing 2% normal mouse serum and normal goat serum for 10 minutes. The cells were then incubated with rabbit anti-LTC_4 Abs (Cayman Chemicals) or nonimmune rabbit IgG for 45 minutes. The anti–adipose differentiation-related protein Ab was added for 45 minutes to distinguish cytoplasmic lipid bodies within leukocytes. Cells were washed three times with HBSS for 10 minutes and incubated with Cy2-labeled anti-rabbit IgG in addition to Cy3-labeled anti-guinea pig secondary Abs for 1 hour. The slides were analyzed by confocal laser scanning microscopy on a Zeiss LSM 510-META (Jena, Germany).

Isolation and Stimulation of Human Eosinophils

Peripheral blood was obtained with informed consent from normal donors, and eosinophils were isolated by negative selection using the StemSep system (StemCell Technologies Inc., Vancouver, Canada). resulting in a final population that was more than 99% pure and more than 95% viable (39). The protocol used was approved by the Fundação Oswaldo Cruz ethics review board. Purified eosinophils (2×10^6 cells/ml in $HBSS^{-/-}$) were incubated for 1 hour in a water bath (37°C) with rhMIF (0.05–5 ng/ml), rheotaxin (1–100 ng/ml), or a combination of MIF (0.05 ng/ml) and eotaxin (1 ng/ml). Each *in vitro* experiment was performed in triplicate and repeated at least three times, with eosinophils purified from different donors.

Lipid Body Staining and Enumeration

To quantify lipid bodies, eosinophils were cytocentrifuged (450 rpm, 5 min) onto glass slides., While still moist, cells were fixed in 3.7% formaldehyde (diluted in Ca^{2+}/Mg^{2+}–free HBSS [pH 7.4]), rinsed in 0.1 M cacodylate buffer (pH 7.4), stained with 1.5% OsO_4 for 30 minutes, rinsed in distilled H_2O, immersed in 1.0% thiocarbohydrazide for 5 minutes, rinsed in 0.1 M cacodylate buffer, restained with 1.5% OsO_4 for 3 minutes, rinsed in distilled water, and then dried and mounted. Cell morphology was observed and lipid bodies were quantified by microscopy. A total of 50 consecutively scanned eosinophils was then subjected to blinded evaluation by more than one individual. Results are expressed as the number of lipid bodies per eosinophil.

Treatments

Using the pleurisy model, animals were pretreated with intrapleural injections of anti-murine eotaxin monoclonal Ab (mAb) (10 μg/cavity) 30 minutes before allergic challenge. Animals pretreated with an irrelevant Ab showed no alteration of MIF-induced response (data not shown).

For *in vitro* mechanistic studies, eosinophils were pretreated for 30 minutes with 10 μg/ml of neutralizing Abs against human CD74 (clone LN2, as described in Ref. 40), eotaxin, or CCR3. Isotype-matched nonimmune Abs were used as controls. The anti-CD74 Ab has azide; however, at the concentrations used (final concentration ⩽ 0.002%) it did not affected viability (>95%) or lipid body formation.

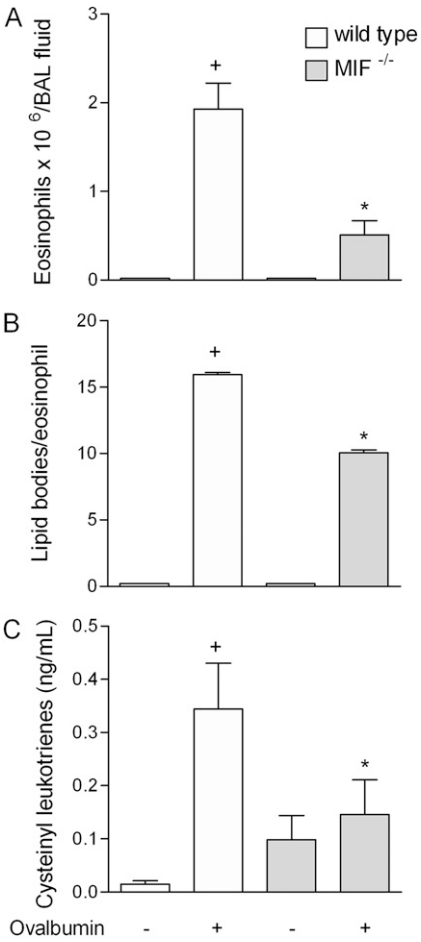

Figure 1. Macrophage migration inhibitory factor (MIF) mediates eosinophil influx (*A*), lipid body formation within recruited eosinophils (*B*), and cysteinyl (cys) leukotriene (LT) C_4 synthesis (*C*) induced by allergen-driven pulmonary inflammation. Analysis was performed on bronchoalveolar fluids harvested 24 hours after the final ovalbumin aerolization. Control groups were immunized and aerosolized with sterile saline. Results are expressed as the mean (±SEM) calculated from at least eight animals. $^{+}P \leqslant 0.05$ compared with saline control group; $^{*}P \leqslant 0.05$ compared with wild-type (WT) stimulated mice.

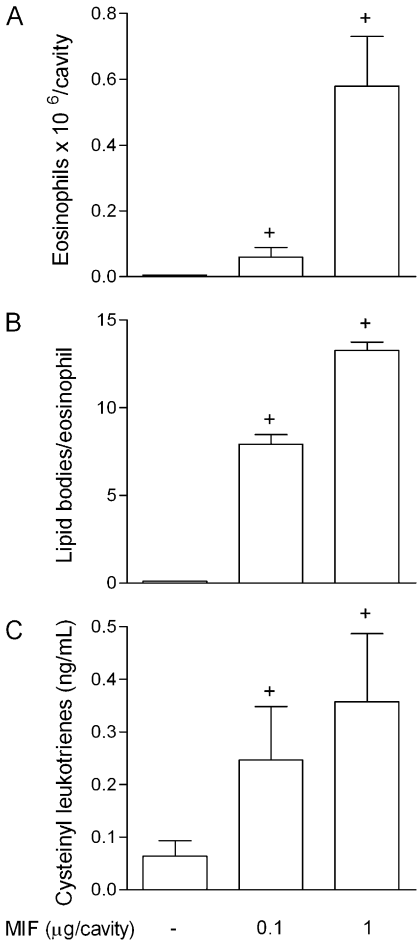

Figure 2. MIF evokes eosinophil influx (*A*), lipid body formation within recruited eosinophils (*B*), and cysLT synthesis (*C*) in a pleurisy model. Analysis was performed on pleural fluids harvested 24 hours after intrapleural injection of recombinant murine MIF (0.1 and 1 μg/cavity). The control group was injected with sterile saline. Results are expressed as the mean (±SEM) calculated from at least eight animals. $^{+}P \leqslant 0.05$ compared with control group.

TABLE 1. *IN VIVO* EFFECTS OF MACROPHAGE MIGRATION INHIBITORY FACTOR ON EOSINOPHIL INFLUX AND LIPID BODY BIOGENESIS ARE NOT DUE LPS CONTAMINATION, BECAUSE ACTIVATION OF TOLL-LIKE RECEPTOR 4 IS NOT INVOLVED IN THESE PHENOMENA

Condition	Animal Strain	Eosinophils/Cavity	Lipid Bodies/Cell
Saline	C3H/He	0.01 ± 0.01	0.50 ± 0.01
rmMIF	C3H/He	0.18 ± 0.05*	4.54 ± 0.05*
Saline	C3H/HeJ	0.01 ± 0.01	0.20 ± 0.01
rmMIF	C3H/HeJ	0.16 ± 0.03*	4.08 ± 0.05*

Definition of abbreviation: rmMIF, recombinant murine macrophage migration inhibitory factor.

Toll-like receptor 4–sensitive (C3H/HeN) and nonsensitive (C3H/HeJ) mice were stimulated *in vivo* with rmMIF (1 μg/cavity) for 24 hours. Results are expressed as means (±SEM) calculated from at least six distinct animals.

* $P \leq 0.01$ compared to saline-stimulated cells.

Statistical Analysis

Results from *in vivo* assays are expressed as means (±SEM), and were analyzed statistically by means of ANOVA, followed by the Newman-Keuls Student test, with the level of significance set at a P value less than 0.05.

RESULTS

MIF Mediates Assembly of Eosinophil Lipid Bodies and Synthesis of LTC_4 Triggered by Allergic Pulmonary Inflammation

We have previously reported that, in addition to the well known induction of LTC_4 production, allergic challenge also triggers the biogenesis of lipid bodies, which function as intracellular compartments for LTC_4 synthesis within recruited eosinophils (33). The mechanisms involved in eliciting the biogenesis of cytoplasmic lipid bodies and priming infiltrating eosinophils for enhanced LTC_4 production in allergic inflammatory sites are not completely known, but involve, at least, the mediation by eotaxin and PGD_2, two well known eosinophilotactic agents (33, 37).

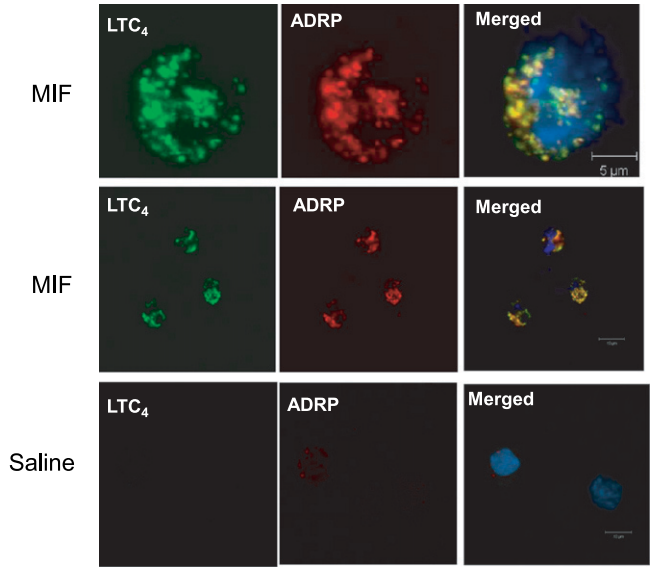

Figure 3. Eosinophil LTC_4 synthesis in MIF-stimulated animals occurs in lipid bodies. EicosaCell images illustrate intracellular immunodetection of newly formed LTC_4 (*green*) and adipose differentiation-related protein (ADRP) (*red*) in cells from MIF-stimulated (*upper* and *middle panels*) and saline-stimulated animals (*lower panels*). Overlaid immunofluorescence microscopy images of identical fields are shown in the *right column*.

Recently, we and others have identified MIF as a key mediator of allergic inflammation (3–5, 28). Here, to investigate whether MIF has any role in eosinophil lipid body–driven LTC_4 synthesis triggered by allergic challenge, a model of acute allergic pulmonary inflammation in wild-type (WT) versus *Mif*$^{-/-}$ mice (BALB/c background) was used. As previously demonstrated (3) and shown in Figure 1A, BAL eosinophilia triggered by aerosol allergic challenge is drastically reduced in MIF$^{-/-}$ mice compared with the response observed in WT mice. Similarly, both formation of new lipid bodies within recruited eosinophil and cysLT generation were significantly decreased in allergen-challenged MIF$^{-/-}$ mice when compared with WT mice (Figures 1B and 1C). Together, these data indicate that endogenous MIF, found at an allergic inflammatory site is capable of eliciting lipid body assembly within infiltrating eosinophils, in parallel to enhancing the production of cysLTs.

MIF Triggers *In Vivo* Biogenesis of Functional LTC_4-Synthesizing Lipid Bodies within Recruited Eosinophils

Because MIF appears to contribute to eosinophil activation during allergic responses, we examined whether MIF was able to elicit eosinophil LTC_4-synthesizing machinery in recruited eosinophils *in vivo*. Intrapleural administration of MIF (0.1 or 1 μg/cavity) in naive mice induced accumulation of eosinophils

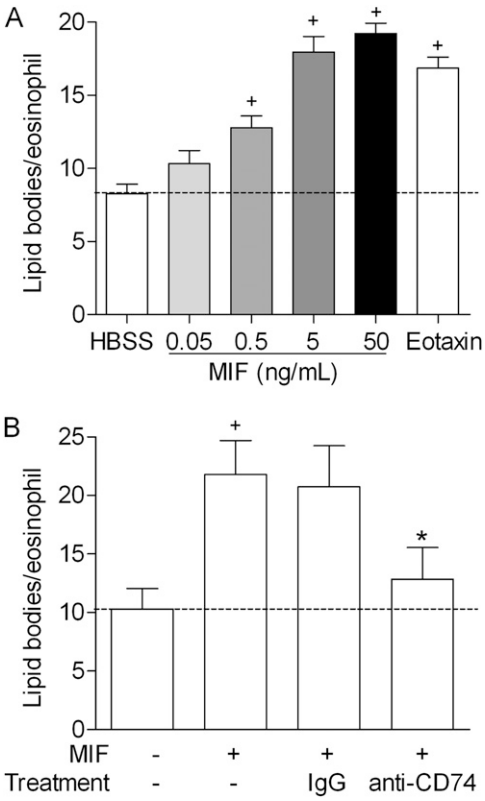

Figure 4. MIF induces lipid body biogenesis in human eosinophils *in vitro*. (*A*) Dose-dependent response of recombinant human (rh)MIF (from 0.05 to 50 ng/ml) on lipid body biogenesis after stimulation of human eosinophils *in vitro* for 1 hour. (*B*) Effect of 30-minute pretreatment with anti-CD74 or isotype control IgG1 on rhMIF-stimulated (50 ng/ml) human eosinophils. Results are expressed as means (±SEM) calculated from triplicate results from one donor, and are representative of results from eosinophils isolated from at least three distinct donors. $^{+}P \leq 0.05$ compared with Hanks' balanced salt solution (HBSS) control group; $^{*}P \leq 0.05$ compared with MIF-stimulated untreated cells.

in a dose-dependent manner (Figure 2A). These eosinophils showed an increased number of cytoplasmic lipid bodies (Figure 2B), thereby demonstrating the ability of MIF to activate the formation of lipid body biogenesis machinery *in vivo*. Of note, MIF-induced eosinophil activation *in vivo* was not dependent on LPS contamination, because C3H/HeJ mice, an LPS-hyporesponsive strain that has an inactivating point mutation within the signal-transducing domain of Toll-like receptor 4 (41), also respond to intrapleural MIF (1 μg/cavity) with eosinophil recruitment and lipid body formation of similar magnitudes to those observed in C3H/HeN mice, which express functional Toll-like receptor 4 (Table 1).

As in allergic inflammation, and concurring with MIF-induced *in vivo* lipid body biogenesis, there was also significant production of cysLTs in MIF-injected animals (Figure 2C). It was determined that this parallel increase in numbers of lipid bodies and levels of secreted cysLTs was the result of a functional relationship between lipid bodies and cysLTs. As illustrated in Figure 3, EicosaCell, a methodology that cross-links and immunolabels LTC_4 at the original sites of synthesis, identified infiltrating eosinophils as the cell population responsible for LTC_4 production during MIF-elicited inflammatory reactions. In addition, a more detailed analysis revealed that the intracellular LTC_4-synthesizing compartment displayed a punctate cytoplasmic pattern proximal to, but separate from, the nucleus, which is fully consistent in size and form with eosinophil lipid bodies. In fact, the compartmentalization of newly formed LTC_4 in eosinophil lipid bodies was ascertained by the colocalization with adipose differentiation-related protein (Figure 3). Virtually no LTC_4 was immunolocalized within cells of saline-injected or MIF-stimulated, MK886-treated animals (data not shown), thus demonstrating that the newly formed lipid bodies of *in vivo*, MIF-stimulated eosinophils are enzymatically suited for effective LTC_4 synthesis.

MIF Activates Eosinophils Directly through Its Receptor, CD74

To investigate whether MIF was indeed capable to directly activating eosinophils and sufficient to trigger the formation of new lipid bodies, we stimulated *in vitro* purified human eosinophils with rhMIF. Figure 4A shows that rhMIF (0.05–50 ng/ml) induced a rapid (1 h) and dose-dependent increase in the numbers of cytoplasmic lipid bodies within human eosinophils isolated from healthy donors. Moreover, rhMIF-induced lipid body biogenesis appeared to be a direct effect of MIF on specific receptors expressed on eosinophil surface, because the neutralizing Ab against CD74, which recognizes the major histocompatibility complex class II invariant chain identified as the MIF receptor (40), significantly inhibited the effects of MIF. Together, this indicates that eosinophils respond directly to MIF stimulation, which, at least in part, depends on CD74 binding. Eosinophils pretreated with isotype-matched, nonimmune Ab displayed expected lipid body biogenesis under MIF stimulation (Figure 4 and data not shown). Moreover, the specificity of the treatment was confirmed by the observation that CD74 Ab neutralization failed to inhibit platelet activating factor (PAF)-induced lipid body formation (from 19.8 ± 1.6 to 17.4 ± 2.8 lipid bodies/eosinophil; $n = 3$ [when nontreated and anti-CD74–treated, PAF-stimulated eosinophils were compared]).

Eotaxin Mediates MIF-Driven Eosinophil Activation

The mechanisms involved in MIF-induced lipid body formation were further investigated by focusing on the putative cross-talk between MIF and eotaxin. It is well established that CCR3 activation by eotaxin in eosinophils stimulates both potent chemoattractant and lipid body biogenic responses, which control allergen-driven LTC_4-synthesizing machinery (for review, *see* Ref. 32). Here, several of our findings show that MIF-induced eosinophil activation, both *in vivo* and *in vitro*, appeared to be dependent on eotaxin-driven autocrine and/or paracrine activity: (*1*) as shown in Figure 5A, *in vivo* pretreatment with a neutralizing anti-murine eotaxin mAb significantly inhibited both eosinophil influx and lipid body biogenesis induced by the i.pl. administration of rMIF; (*2*) increased levels of eotaxin were found in the pleural fluid of mice injected with MIF (1 μg/cavity) (Figure 5B); and (*3*) *in vitro* rMIF-elicited, CD74-dependent lipid body assembly within human eosinophils was blocked by neutralizing anti-human eotaxin mAb, and is significantly inhibited by anti-

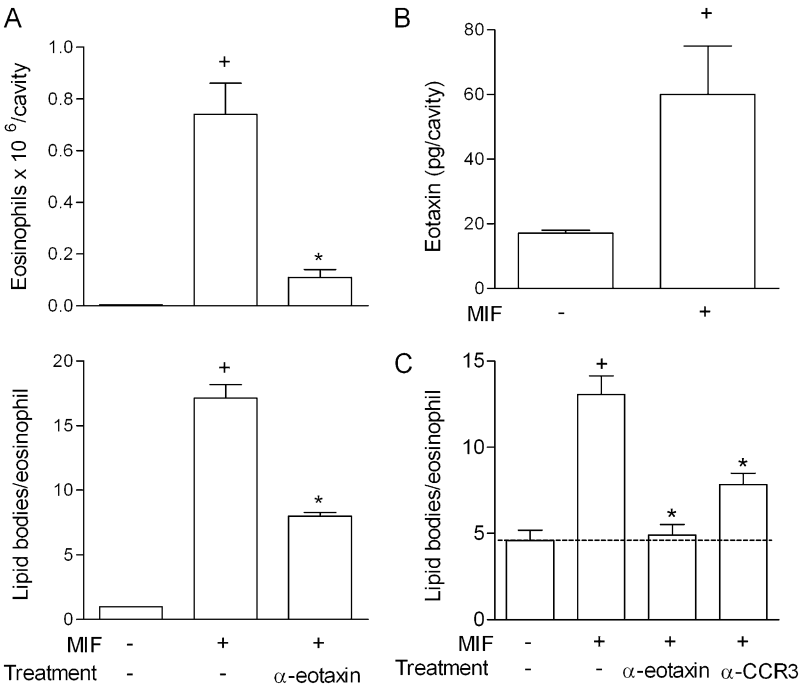

Figure 5. Role of eotaxin in MIF-driven eosinophil activation. (*A*) Mice received an intrapleural injection of recombinat murine (rm)MIF (1 μg/cavity). Analysis of eosinophil influx and lipid body formation were performed 24 hours after MIF administration. (*B*) Pleural levels of eotaxin were analyzed 6 hours after rmMIF i.pl. injection (1 μg/cavity). Results are expressed as the mean (±SEM) calculated from at least six animals. $^{+}P \leqslant 0.05$ compared with control animals; $^{*}P \leqslant 0.05$ compared with rmMIF-injected mice. For *in vitro* analysis of lipid body biogenesis shown in (*C*), purified human eosinophils were preincubated with anti-eotaxin or anti-CCR3 and then stimulated with rhMIF (5 ng/ml) for 1 hour. *In vitro* results are expressed as means (±SEM) calculated from triplicate results from one donor, and are representative of results from eosinophils isolated from at least three distinct donors. $^{+}P \leqslant 0.05$ compared with HBSS control group; $^{*}P \leqslant 0.05$ compared with MIF-stimulated, untreated cells.

human CCR3 mAb (Figure 5C), suggesting that an endogenous eosinophil-derived eotaxin found at the site of the inflammatory reaction could mediate part of the MIF/CD74–induced eosinophil activation response.

MIF Mediates Eotaxin-Driven Eosinophil Activation

To better characterize the role of MIF and eotaxin cross-talk on regulating eosinophil responses, the input of MIF/CD74 on eotaxin-induced eosinophil activation was evaluated. Similar to the MIF-triggered eosinophil activation response mediated by eotaxin, MIF autocrine/paracrine activity appears to mediate the ability of eotaxin to trigger lipid body biogenesis. Although *Mif*$^{-/-}$ mice stimulated with eotaxin (30 pmol/cavity, i.pl.) *in vivo* showed eosinophil influx levels comparable to WT mice, *Mif*$^{-/-}$ mice displayed negligible eosinophil lipid body biogenesis after eotaxin stimulation (Figure 6). This result was confirmed when *in vitro* neutralization of human CD74 on human eosinophils was able to inhibit lipid body formation triggered by rheotaxin (Figure 7A), indicating that eotaxin-stimulated eosinophils rapidly secrete MIF that, acting autocrinally and/or paracrinally on CD74 expressed on eosinophils, triggers assembly of new lipid bodies. Finally, as shown in Figure 7B, suboptimal concentrations of rheotaxin (1 ng/ml) and rhMIF (0.05 ng/ml) acted in a cooperative manner to induce assembly of new lipid bodies. Therefore, in addition to mediating eosinophil activation, physiological levels of eotaxin and MIF found *in vivo* at sites of eosinophilic inflammation may also synergize to effectively activate recruited eosinophils.

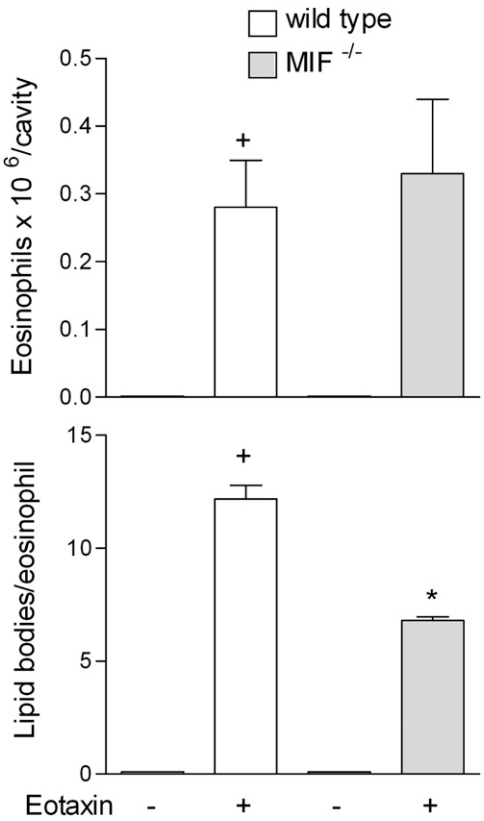

Figure 6. MIF mediates eotaxin-driven eosinophil activation. WT and *Mif*$^{-/-}$ mice received an intrapleural injection of eotaxin (30 pmol/cavity). Analysis of eosinophil influx and lipid body formation were performed 24 hours after eotaxin administration. Results are expressed as the mean (±SEM) calculated from at least six animals. $^{+}P \leqslant 0.05$ compared with nonstimulated WT mice; $^{*}P \leqslant 0.05$ compared with eotaxin-injected WT mice.

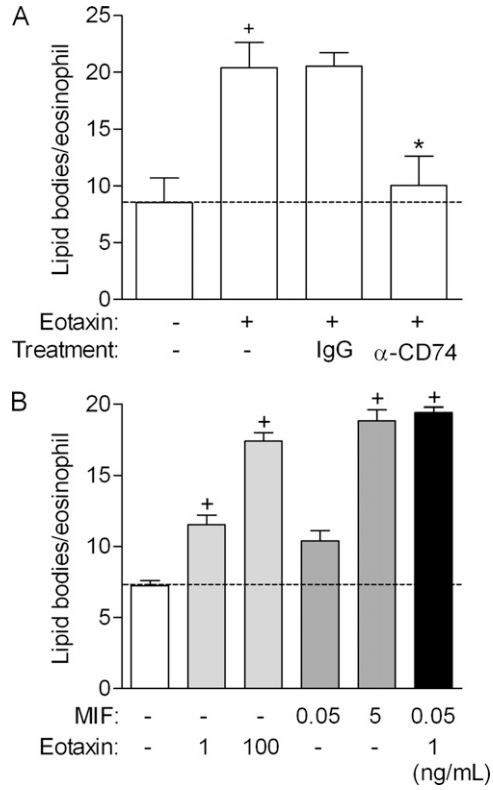

Figure 7. MIF mediates eotaxin-driven eosinophil activation *in vitro*. Lipid body formation in purified human eosinophils that were (*A*) preincubated for 30 minutes with anti-CD74 or isotype control IgG1, and then stimulated with eotaxin (100 ng/ml), or (*B*) stimulated with recombinant human (rh)MIF (0.05 or 5 ng/ml), eotaxin (1 or 100 ng/ml), or costimulated with both eotaxin (1 ng/ml) and rhMIF (0.05 ng/ml) *in vitro*. Analysis of lipid body formation was performed 1 hour after stimulation. Results are representative of three independent experiments. $^{+}P \leqslant 0.05$ compared with HBSS control group; $^{*}P \leqslant 0.05$ compared with eotaxin-stimulated, untreated cells.

DISCUSSION

Recent studies have demonstrated that MIF has an essential role in modulating several aspects of the physiopathology of allergic pulmonary inflammation, including eosinophil recruitment and the production of inflammatory mediators in the lung (3). In addition, it has been previously shown that MIF can activate distinct intracellular signaling pathways, which can enhance cell eicosanoid synthetic capacity (42, 43). Our study investigates the mechanisms involved in the interplay of MIF and cysLTs in allergic pulmonary inflammation, whereby MIF may directly activate and thereby enhance the capacity of eosinophils to synthesize LTC_4. By employing *in vivo* and *in vitro* assays, we characterized the ability of MIF to directly activate eosinophils, eliciting rapid biogenesis of LTC_4-synthesizing lipid bodies, organelles considered as markers of leukocyte activation. Moreover, we demonstrated that MIF functions via activation of its receptor, CD74, and acts in concert with eotaxin in a positive-feedback loop to activate eosinophils in response to allergic pulmonary inflammation.

Biogenesis of cytoplasmic lipid bodies has been noted within cells engaged in inflammatory disorders, and, although they were long viewed as lipid storage depots within cells, it has now become increasingly accepted that these organelles compartmentalize a diverse set of proteins and, therefore, participate in different cell functions, including eicosanoid synthesis (reviewed in Ref. 28). In regard to lipid bodies found in

eosinophils, *in vivo* and *in vitro* studies conducted by our group have shown that an induced, highly orchestrated phenomenon, which is mediated, in part, by CCR3 activation induced by endogenously produced eotaxin and RANTES (32, 36), controls the biogenesis of eosinophil lipid bodies. These newly formed eosinophil lipid bodies are sites of 5-LO–driven LTC_4 synthesis—which contributes to the characteristic enhancement of cysLTs production in response to allergic challenge (33). In this study, we found that MIF has, as with CCR3-activating chemokines, a potent and direct effect on eosinophils to stimulate lipid body biogenesis. Furthermore, the capacity of MIF to regulate lipid body assembly within infiltrating eosinophils during allergic pulmonary pathogenesis was found to impact the ability of eosinophils to generate LTC_4, thereby contributing to enhanced cysLT generation *in vivo*.

The mechanisms involved in MIF-induced eosinophil activation, which culminates in biogenesis of LTC_4-synthesizing lipid bodies, emerged as a two-player receptor engagement system. In regard to allergic inflammation, eosinophil-activating mediators, including PAF (26, 35), IL-5 (35, 44), CC chemokines (32), and PGD_2 (37), act directly on receptors on the eosinophil surface and initiate intracellular signaling cascades that culminate with *de novo* formation of lipid bodies and prime LTC_4 synthesis.

Recently, CD74, the cell surface form of the major histocompatibility complex class II–associated invariant chain, was demonstrated to be a high-affinity MIF receptor with signal-transducing ability (40). We observed a significant inhibition of MIF-induced lipid body formation in human eosinophils *in vitro* using neutralizing anti-human mAb CD74, suggesting that MIF directly activates eosinophils to form lipid bodies, in part by interacting with its specific membrane receptor. Although MIF may have direct effects on eosinophils via CD74 activation, our results demonstrate that MIF cooperates with eotaxin to trigger robust eosinophil activation and form new lipid bodies.

We recently showed that endogenously generated eotaxin partially mediates allergen- and PGD_2-induced formation of eosinophil lipid bodies (36, 37). Moreover, either exogenously administered MIF or endogenously generated MIF, in the course of allergic reaction, are able to elicit eotaxin generation (3). Complementary to this, we verified that inhibition of eotaxin or its receptor, CCR3, either *in vivo* or *in vitro*, substantially reduced MIF-triggered eosinophil lipid body formation. This suggests that part of the mechanism of MIF-induced lipid body formation is due to an indirect effect on CCR3 by eosinophil-derived eotaxin, which is rapidly released in response to MIF stimulation, and contributes to the process in an autocrine/paracrine manner. Of note, eotaxin-driven eosinophil lipid body biogenesis was also partially inhibited by blocking MIF and CD74, suggesting that MIF and eotaxin cooperate to fully activate eosinophils.

Together, our data, as illustrated in Figure 8, demonstrate that, during eosinophilic allergic reactions, MIF controls eosinophil activation by binding to its receptor, and triggers both LTC_4 synthesis within newly formed lipid bodies as well as the rapid release of preformed eotaxin. In response, such an eosinophil-derived chemokine, acting paracrinally and/or autocrinally on eosinophils, binds to the CCR3 receptor to induce both lipid body–driven LTC_4 synthesis and secretion of stored MIF from eosinophils. Therefore, after an initial increase of cell-free eotaxin and/or MIF concentrations within sites of allergic reaction, characterized by local eosinophilia, a system of eosinophil retroactivation appears to take place that results in lipid body–driven LTC_4 production by infiltrating eosinophils. Importantly, among other cytokines, eosinophils are known to store preformed pools of both MIF and eotaxin

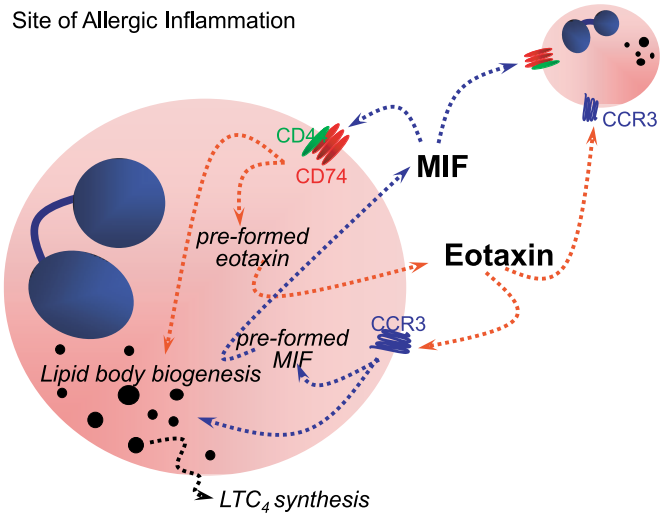

Figure 8. Cross-talk between MIF and eotaxin in regulating eosinophil activation. In sites of allergic inflammatory reactions, physiological concentrations of endogenous MIF and eotaxin (potentially derived from infiltrating eosinophils) act on their cognate receptors expressed on recruited eosinophils (CD74 and CCR3, respectively), and cooperate in an autocrine/paracrine fashion to elicit eosinophil activation, which is characterized by lipid body biogenesis and compartmentalized LTC_4 synthesis.

(reviewed in Ref. 45). Moreover, it is also recognized that properly stimulated eosinophils can rapidly select and secrete small, but biologically active amounts of eotaxin, which can function in an autocrine manner (39).

Eosinophil activation evoked by MIF launches the rapid, but highly regulated biogenic process of lipid body assembly, which forms organelles committed to synthesizing large amounts of LTC_4, a typical result of allergic inflammatory responses. CysLTs are known to be an essential mediator of bronchial hyperresponsiveness, mucus secretion, the production of inflammatory mediators, and eosinophil recruitment—the same allergic features now recognized to be regulated, in part, by MIF. Our data also unveil a cross-talk mechanism between eosinophil-derived eotaxin and MIF that controls eosinophil activation and regulates the amplification of allergy-related LTC_4 synthesis. Therefore, such MIF/eotaxin-elicited, eosinophil lipid body–driven LTC_4 synthesis rises as an interesting candidate for new antiallergic therapeutic approaches.

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