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Role of macrophage migration inhibitory factor in ovalbumin-induced asthma in rats

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Running title: Role of MIF in allergen-induced airway inflammation

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Abstract

Macrophage migration inhibitory factor (MIF) is a pro-inflammatory cytokine and reportedly counteracts the anti-inflammatory effect of endogenous glucocorticoids. There have been only a few reports that demonstrate a potential link between MIF and bronchial asthma. In an attempt to further clarify the precise role of MIF in asthma, we examined the effect of anti-MIF antibody on airway inflammation and airway hyperresponsiveness in an ovalbumin-immunized rat asthma model. Actively immunized Brown-Norway rats received ovalbumin inhalation with or without treatment of anti-MIF antibody. The levels of MIF in bronchoalveolar lavage fluid were significantly elevated after the ovalbumin challenge. An immunohistochemical study revealed positive immunostaining for MIF in bronchial epithelium, even in nonsensitized rats, and the MIF staining in bronchial epithelium was enhanced after the ovalbumin challenge. Anti-MIF antibody significantly decreased the numbers of total cells, neutrophils, and eosinophils in the bronchoalveolar lavage fluid of the ovalbumin-challenged rats, and also attenuated the ovalbumin-induced airway hyperresponsiveness to ovalbumin and methacholine. However, anti-MIF antibody did not affect the level of serum ovalbumin-specific IgE, suggesting that anti-MIF antibody did not suppress immunization itself. These results indicate that MIF plays a crucial role in airway inflammation as well as airway hyperresponsiveness in asthma.

Key words: asthma, macrophage migration inhibitory factor, ovalbumin, eosinophil, airway hyperresponsiveness, airway inflammation

Introduction

Macrophage migration inhibitory factor (MIF) was first described as one of the earliest cytokines to be derived from activated T cells and to prevent the random migration of macrophages (1, 2). Cloning of human MIF cDNA has led to extensive studies using purified recombinant MIF (3) and this protein has been postulated to function as a proinflammatory cytokine (4, 5). Donnelly and colleagues (6) reported that the levels of MIF in bronchoalveolar lavage (BAL) fluid were increased in patients with acute respiratory distress syndrome. We subsequently demonstrated that anti-MIF antibody attenuated both lipopolysaccharide-induced neutrophil accumulation in rat lungs (7) and bleomycin-induced acute lung inflammation and mortality in mice (8). These data support the idea that MIF is a proinflammatory cytokine involved in lung injury.

MIF is now known to be constitutively expressed in a variety of cells, including macrophages, T cells, and bronchial epithelial cells in the lungs (4, 7, 9). MIF has the unique feature of overriding the anti-inflammatory and immunosuppressive effects of glucocorticoids (5, 10). MIF also plays an important regulatory role in the activation of T cells induced by mitogenic or antigenic stimuli (11). The strong induction of MIF mRNA and protein has been observed from Th2 clones but not from Th1 clones (11). Accordingly, MIF is considered to be a pleiotropic peptide, functioning as a cytokine and/or a hormone.

Only a few reports have examined the potential role of MIF in asthma (12-14). Rossi et al. first reported that MIF levels were increased in BAL fluid from asthmatic patients and also that circulating eosinophils could produce MIF upon stimulation *in vitro* (12). However, one subsequent animal study could not support the role of MIF in asthma because anti-MIF serum did not affect the allergic airway inflammation in mice (14). In this study, we thus attempted to further clarify the role of MIF in asthma using rats. We here demonstrate that anti-MIF antibody evidently inhibits ovalbumin (OA)-induced airway inflammation as well as airway hyperresponsiveness in Brown-Norway rats, which have been used as a model of atopic asthma (15-17).

Materials and Methods

Animals and immunization

This research adhered to the Declaration of Helsinki and was approved by the Ethical Committee on Animal Research, Hokkaido University. Specific pathogen-free 6-week-old male Brown-Norway rats (weight range, 160-200 g) were purchased from Japan Charles River Co. (Yokohama, Japan). They were actively immunized to OA by subcutaneous injection with 1 mg OA containing 200 mg aluminum hydroxide. An adjuvant consisting of 1×10^9 heat-killed *Bordetella pertussis* organisms was intraperitoneally injected at the same time.

Preparation of rabbit polyclonal antibody against MIF

Polyclonal anti-rat MIF serum was generated by immunizing New Zealand White rabbits with purified recombinant rat MIF. Rat MIF was expressed in *E.coli* and purified to homogeneity as described in our previous publications (18). In brief, the rabbits were inoculated intradermally with 100 mg of MIF emulsified in complete Freunds' adjuvant (Wako Pure Chemical Industries., Osaka, Japan) at Weeks 1 and 2, and with 50 mg of MIF diluted in incomplete Freunds' adjuvant (Wako Pure Chemical Industries., Osaka, Japan) at Week 4. The immunoglobulin G (IgG) fraction was prepared using Protein A-Sepharose according to the manufacturer's protocol.

Experimental protocol

The rats were divided into three groups: Naive group, OA group, and OA+anti-MIF Ab group. The Naive group did not receive immunization and did not have any treatments. The OA and OA+anti-MIF Ab groups were actively immunized on day 0 and intraperitoneally injected with 2 mg of the non-immunized rabbit IgG or the anti-MIF polyclonal antibody every 2 days from day 0 to day 16. In our preliminary study, we had confirmed that non-immunized rabbit IgG caused no changes in inflammatory cells of the OA-immunized lungs. Either total cell counts or eosinophil counts in BALF were not significantly different between OA immunized+untreated group and OA immunized+non-immunized IgG group （$8.99\pm1.70 \times 10^6$ vs. $7.05\pm0.89 \times 10^6$, $5.79\pm0.76 \times 10^6$ vs. $4.67\pm0.80 \times 10^6$, n=3, 3, respectively） (unpublished data). We thus used the OA immunized+non-immunized IgG group as control in this experiment. We thought that administration of non-immunized rabbit IgG would be desirable to more specifically examine the effect of anti-MIF antibody. On day 14, the rats inhaled 2 % w/v OA for 15 minutes in an exposure chamber. Three days after OA inhalation, broncoalveolar lavage (BAL) was performed, blood samples and lung tissues were taken, and the airway response to OA or methacholine (Mch) was measured.

Bronchoalveolar lavage and cell counting

The lungs were washed three times with 15 ml total of sterile saline. After the lavage, the lungs were fixed with an intrabronchial infusion of 10% neutral

formalin at a constant pressure of 25 cmH_2O for 48-h period. The lavage fluid was centrifuged and the cells were counted and processed for differential cell analysis. The supernatant was used for the measurement of MIF, eotaxin, or IL-13 concentrations.

Measurement of bronchial responsiveness to methacholine and ovalbumin

Three days after OA challenge, another set of three groups were anesthetized with an intraperitoneal injection of pentobarbital sodium (50 mg/kg). Intratrachial intubation was then performed with a metallic tube. The rats were mechanically ventilated (Rodent Ventilator Model 683, Harvard Apparatus, Hollliston, USA). A pressure transducer (TP-602T, Nihon Kohden Co., Tokyo, Japan) was connected to a side port of the metallic tube, and airway opening pressure (Pao) was continuously measured. An aerosol of Mch or OA was administered through a reservoir box connected to the ventilator system. After measurement of baseline Pao, an aerosol of saline followed by Mch or OA was administered.

Immunohistochemical study

Immunohistochemistry was performed on paraffin embedded tissue using a Catalized Signal Amplification kit (DAKO Japan, Kyoto, Japan) according to the manufacturer's protocol. The primary antibody was anti-MIF antibody diluted at 1: 200 with phosphate-buffered saline. The tissue sections were counterstained with methyl green and mounted. The anti-MIF antibody used for

immunohistochemical study was the same as the antibody administered for treatment of rats.

Measurement of MIF levels by ELISA

The levels of MIF in the BAL fluid were quantitated by the enzyme-linked immunosorbent assay (ELISA) method as described in our previous publication (19). The anti-rat MIF antibody administered for treatment of rats was used in ELISA. Briefly, the anti-rat MIF antibody was added to each well of a 96-well microtiter plate. Wells were incubated with biotin-conjugated anti-MIF antibody for 1 h at room temperature. Avidin-conjugated horseradish peroxidase was added after washing, then substrate solution was added to each well. The reaction was terminated with 4 N sulfuric acid. The absorbance was measured at 492 nm on an automated ELISA plate reader. The detection limit of this system was 1.5 ng/ml.

Ovalbumin-specific IgE antibody assay

The levels of OA-specific IgE in serum were quantitated using an ELISA method as previously described (20). Briefly, the 96-well microtiter plates were coated with anti-rat IgE monoclonal antibody (Zymed, South San Francisco, USA) at 4°C for 24 h. The plate was washed and incubated with standard serum or sample serum for 1 h at room temperature. After washing, horseradish peroxidase-streptavidin was plated into each well. After final washing,

o-phenylenediamine solution containing 0.035% hydrogen peroxide was added to each well. The enzyme reaction was stopped by the addition of 4 N sulfuric acid and the absorbance was measured at 490 nm on a plate reader. The absorbance of standard serum diluted 1:100 was arbitrarily defined as U/ml.

Measurement of eotaxin and IL-13 concentrations by ELISA

Because of the high degree of similarity maintained in chemokines across species, a mouse ELISA kit containing a polyclonal antibody that recognizes mouse eotaxin was used to detect the rat cognate. Thus eotaxin levels in BAL fluid were determined using a mouse ELISA kit (R&D Systems Inc., Minneapolis, U.S.A.) according to the manufacturer's instructions. IL-13 levels in BAL fluid were determined using a rat specific solid phase sandwich ELISA kit (Biosource International, Camarillo, USA). The minimum detectable concentration of eotaxin was 3 pg/ml, IL-13 was 1.5 pg/ml.

Statistical analysis

Data are expressed as means ± SE. Statistical analyses were performed on the data through single-factor ANOVA among the three groups and with Student's unpaired t-test for comparisons of two groups. *P* values <0.05 were assumed to be significant.

Results

Expression of MIF in ovalbumin-induced airway inflammation

To investigate whether the expression of MIF in airways is enhanced in this model, we measured the levels of MIF in BAL fluid 3 days after the OA challenge. The levels of MIF in BAL fluid were significantly elevated in the OA group compared with those in the Naive group (14.7±1.4 ng/ml in the OA group vs. 1.3±1.1 in the Naive group, $p<0.05$, Fig. 1).

Immunohistochemical localization of MIF in lungs

Histological examination using the lung tissue confirmed that OA inhalation induced widespread peribronchiolar inflammation in OA-sensitized rats, which is characteristic of asthma. Positive immunostaining for MIF was observed within the bronchial epithelium, even in the Naive group (Fig. 2A). There was a significant increase in immunostaining of the bronchial epithelial cells, epithelial submucosa, and inflammatory cells in the alveoli of the OA group 3 days after the OA challenge (Fig. 2B).

Effect of anti-MIF antibody on airway inflammation

Total and differential cell counts 3 days after the OA challenge are shown in Figure 3. In the OA group, the numbers of total cells, macrophages, eosinophils, and neutrophils were significantly elevated compared with those of the Naive group. Treatment with anti-MIF antibody significantly decreased the numbers of total cells, eosinophils, and neutrophils compared with those of the OA group

(total cells: 15.0±3.5 x 10^6 in the OA group vs. 10.5±2.4 in the OA+anti-MIF Ab group, p<0.01; eosinophils: 10.5±2.7 in the OA group vs. 6.2±2.7 in the OA+anti-MIF Ab group, p<0.01; neutrophils: 1.4±1.2 in the OA group vs. 0.16±0.27 in the OA+anti-MIF Ab group, p<0.01) and thus significantly attenuated airway inflammation.

Effect of anti-MIF antibody on antigen-specific airway contraction and nonspecific airway hyperresponsiveness

To investigate whether anti-MIF antibody suppressed airway hyperresponsiveness, OA -specific and Mch-induced airway contraction were measured. After measurement of the baseline pressure, an aerosol of OA was administered. The airway pressure was significantly increased in the OA group (Fig. 4A) but not in the OA+anti-MIF Ab group (Fig. 4B).

Similarly after measurement of the baseline pressure, an aerosol of Mch was administered for 1 min in progressively doubled concentrations from 0.0625 mg/ml. In the OA group, the airway pressure was significantly increased. In contrast, the OA+anti-MIF Ab group did not respond to Mch (up to 16.0 mg/ml). The Naive group did not respond to either 5% OA or Mch (up to 16.0 mg/ml) (data not shown).

Effect of anti-MIF antibody on the development of humoral immune responses

Elevated levels of IgE are known to be important in the development of an allergen-induced airway response (21). The results described above may be a consequence of suppression of OA immunization by treatment of anti-MIF antibody; therefore, we examined the possibility that anti-MIF antibody might have influenced OA-specific IgE levels in serum. As shown in Figure 5, the levels of OA-specific IgE in serum were significantly elevated, as expected (16), in the OA group compared with the levels in the Naive group (124.0±41.3 U/ml in the OA group vs. 18.6±5.7 in the Naive group, $p<0.05$). Treatment with anti-MIF antibody similarly caused the elevation of OA-specific IgE in serum (153.3±39.6 U/ml).

Effect of a single administration of anti-MIF antibody before airway challenge

Next, we wondered whether the single administration of anti-MIF antibody before the OA challenge might explain the results described above. A 2-mg aliquot of anti-MIF antibody or nonimmunized rabbit IgG was injected only once 2 hours before the OA challenge and BAL was performed 3 days after the OA challenge. As shown in Figure 6, a single administration of anti-MIF antibody did not change either the number of total cells or the differential cell counts in BAL fluid.

Effect of anti-MIF antibody on eotaxin levels in BAL fluid

To investigate the mechanism by which anti-MIF antibody attenuated eosinophil accumulation in the lungs, we measured the levels of eotaxin, a potent

chemokine of eosinophils, in BAL fluid. In the study series up to 24 h after the OA challenge, the levels of eotaxin in BAL fluid began to increase at 4 h and reached peak levels at 8 h in the OA group; however, no appreciable increase was seen in the levels of the Naive group (data not shown). No significant difference was seen in eotaxin levels at 8 h after the OA challenge between the OA group and the OA+anti-MIF Ab group (8.24±1.5 pg/ml in the Naive group, 127.3±38.0 in the OA group, and 160.0±23.3 in the OA+anti-MIF Ab group, Fig. 7A).

Effect of anti-MIF antibody on IL-13 levels in BAL fluid

We also measured the levels of IL-13 in BAL fluid. The levels of IL-13 were significantly elevated at 8 h after OA challenge in the OA group compared with the Naive group. However, no significant difference was seen in IL-13 levels between the OA group and the OA+anti-MIF Ab group (31.2±5.2 pg/ml in the Naive group, 63.0±16.9 in the OA group, and 72.4±8.1 in the OA+anti-MIF Ab group, Fig. 7B).

Discussion

In this study, we first demonstrated that OA-sensitized rats had increased levels of MIF in BAL fluid and enhanced expression of MIF in airway epithelium after the OA challenge. These results are consistent with the previous observation in a human study in which BAL fluid from patients with asthma contained significantly elevated levels of MIF as compared to normal volunteers (12). In addition, we clearly demonstrated that treatment with anti-MIF antibody significantly suppressed airway inflammation and airway hyperresponsiveness, both of which are characteristic features in this rat model of atopic asthma. These results indicate that MIF plays a potent role in the pathogenesis of allergen-induced airway inflammation and that anti-MIF antibody may have a therapeutic potential for bronchial asthma.

The present study does not agree with a previous study in which anti-MIF serum did not affect the allergic inflammation of the airway in mice (14). In that study, mice were exposed to OA once daily for 7days following active immunization by OA injection and were treated with anti-MIF serum every 3 days from the day before the first allergen challenge to the end of the experiment. Such treatment did not significantly reduce the number of eosinophils either in lung tissues or in BAL fluid. The discrepancy between the two studies with regard to the effect of anti-MIF on eosinophil recruitment into the airway requires some explanation. First, the eosinophilic inflammation induced in the other study was milder than that observed in the present study; the percentage of eosinophils

in BAL fluid was nearly 30% in the other study and 64.9 ± 3.7% in our study. The small number of eosinophils in the other study might have obscured the inhibitory effect of anti-MIF antibody. Second, researchers in the other study used anti-MIF serum rather than anti-MIF antibody in their experiment, and the total dose of anti-MIF serum given might not have been sufficient. Indeed, although the previous study also investigated the effect of anti-MIF serum on lipopolysaccharide-induced neutrophilic airway inflammation, those researchers could not demonstrate the effect of the anti-MIF serum either. In contrast, we previously demonstrated that anti-MIF antibody significantly inhibited lipopolysaccharide-induced neutrophil accumulation in rat lungs (7). Taken together, the anti-MIF serum used in the other study may not have had enough potency or may not have been given in a sufficient amount to exert a discernable effect. A less likely possibility for the discrepancy between the two studies is that the role of MIF in animal models of asthma may be different among species.

MIF is known to be constitutively expressed in bronchial epithelium (7, 9). In the present study, the immunohistochemical study clearly demonstrated that expression of MIF was enhanced in airway epithelium after the OA challenge in OA-sensitized rats. This is the first study to demonstrate that bronchial epithelium is a potent source of MIF in an asthma model. Previously, Rossi et al. suggested that eosinophils might be a potential source of MIF in human asthma because circulating eosinophils even from normal volunteers were shown to produce MIF with phorbol myristate acetate stimulation (12). Indeed, the majority

of inflammatory cells in BAL fluid were eosinophils in the present study. Accordingly, bronchial epithelium as well as eosinophils may jointly contribute to the increased levesl of MIF in BAL fluid in our rat asthma model.

Because 60% to 70% of total cells in BAL fluid in OA-sensitized rats were eosinophils, the attenuation of the number of total cells by treatment with anti-MIF antibody is mostly attributed to the attenuation of the number of eosinophils. It has been reported that the eotaxin levels are highly elevated in BAL fluid from patients with asthma (22) and that eotaxin is associated with airway hyperresponsiveness (23); thus, eotaxin may play an important role in the pathogenesis of bronchial asthma. Therefore, we wondered whether the effect of the anti-MIF antibody on airway inflammation might be at least in part explained by its effect on eotaxin. We found that the level of eotaxin in BAL fluid was certainly elevated after the OA challenge compared with that in naïve rats. However, no significant difference was observed in the levels of eotaxin in BAL fluid between OA group and OA+anti-MIF Ab group. In animal models, IL-13 induced airway hyperresponsiveness and airway eosinophilia (24, 25). It is also possible that IL-13-dependent AHR occurs by mechanisms that are independent of airway eosinophilia (26). In the present study, the levels of IL-13 in BAL fluid was elevated after the OA challenge, however, there was no significant difference between OA group and OA+anti-MIF Ab group. We also measured the expression of IL-5 mRNA and MIP-1α mRNA using tissue homogenates after OA challenge. These chemokines are known to have a role in the recruitment of

eosinophils to airways in asthma. However, the level of mRNA for MIP-1α did not increase after antigen challenge and that of IL-5 was under detection limits even after antigen challenge in this model (data not shown). A previous study reported that MIF significantly delayed spontaneous neutrophil apoptosis in vitro and also eosinophil apoptosis to some extent (27). Thus, the anti-MIF antibody might reduce the number of eosinophils and neutrophils in BAL fluid by enhancing apoptosis of those cells.

The anti-MIF antibody dramatically reduced the number of neutrophils in BAL fluid in the present study. Neutrophils are known to be increased in the airways of patients with status asthmaticus (28) and during exacerbations of asthma (29), and also in sputum from subjects with severe asthma (30); however, the role of neutrophils in asthma is not fully understood. The attenuation of the number of neutrophils may be partially attributed to the anti-inflammatory effect of anti-MIF antibody in our model. We have previously reported that anti-MIF antibody inhibits lipopolysaccharide-induced neutrophil accumulation in rat lungs via its suppressive effect on MIP-2, a powerful neutrophil chemokine (7); therefore, the suppression of MIP-2 might cause the attenuation of the number of neutrophils in our rat asthma model.

In the present study, the anti-MIF antibody did not affect antigen-specific IgE in serum, which led us to investigate whether a single dose of anti-MIF antibody could exert its effect before OA inhalation. A single administration of anti-MIF antibody did not reduce the number of total cells and differential cell counts in

BAL fluid, suggesting that the serial injection of the anti-MIF antibody from OA sensitization to 2 days after OA inhalation are necessary for its suppressive effect to be exerted. The total amount of anti-MIF antibody might be important for exertion of its effect. We thus concluded that anti-MIF antibody suppressed OA-induced airway inflammation by an independent mechanism of OA-sensitization.

Glucocorticoids are currently the most effective anti-inflammatory agent in the treatment of asthma (31). However, it is well recognized that a small proportion of patients, who are often named as steroid-resistant asthmatics, fail to respond to glucocorticoids. MIF might play a role in the blunt response to endogenous or exogenous steroids (5, 10). These consideration leads to the speculation that anti-MIF therapy might not only have direct anti-inflammatory effects, but also act by recovering the function of endogenous and/or exogenous glucocorticoids.

Finally, we should make some comments on the weakness of our experimental protocol in this study. First, we did not perform quantitative assessment of airway hyperresponsiveness particularly for Naïve rats and OA+anti-MIF Ab rats, so that we were not sure how much anti-MIF antibody attenuated airway hyperresponsiveness in the OA-immunized lungs. This is because we were only interested in assuring that enhanced airway hyperresponsiveness by OA immunization and inhalation was actually attenuated by anti-MIF antibody. Second, we used airway pressure for assessing airway hyperresponsiveness, which is influenced by changes in both airway resistance and lung compliance.

As the increased airway pressure was confirmed to return to base line in a short time, the change of compliance, which is more likely caused by lung parenchymal injury, could be negligible in our study (data not shown).

In summary, we demonstrated that MIF is certainly involved in the asthmatic response in the OA-sensitized rat asthma model. We also demonstrated that bronchial epithelium is a potent source of MIF in this asthma model. The anti-MIF antibody significantly attenuated OA-induced airway inflammation and airway hyperresponsiveness as well. Although these data support the concepts that MIF plays an important role in asthma and anti-MIF antibody may have a therapeutic potential for asthma, further investigations are necessary to clarify the mechanism of the effect of anti-MIF antibody on asthma pathology and to examine the therapeutic potential of the anti-MIF antibody in human asthma.

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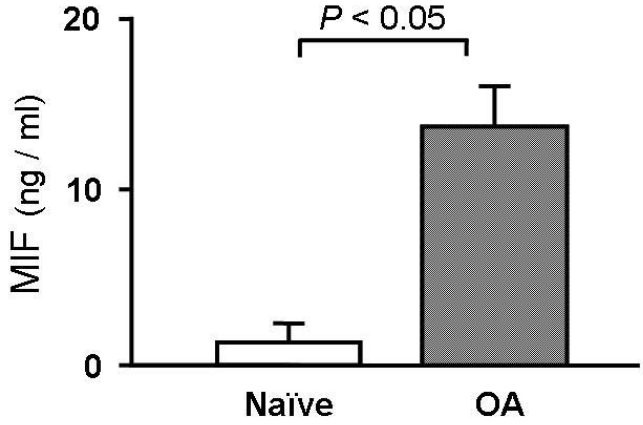

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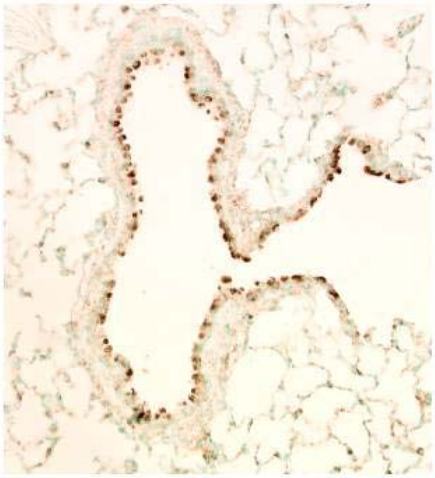
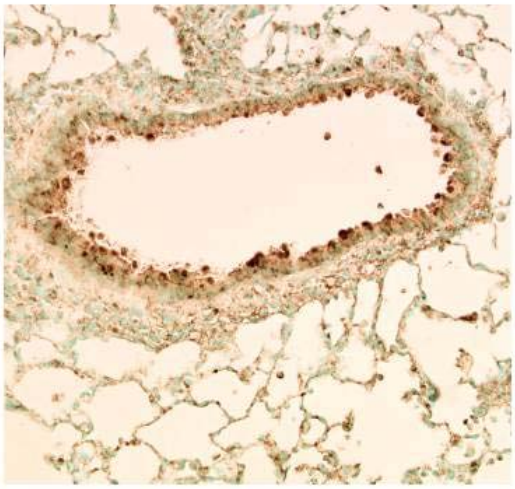
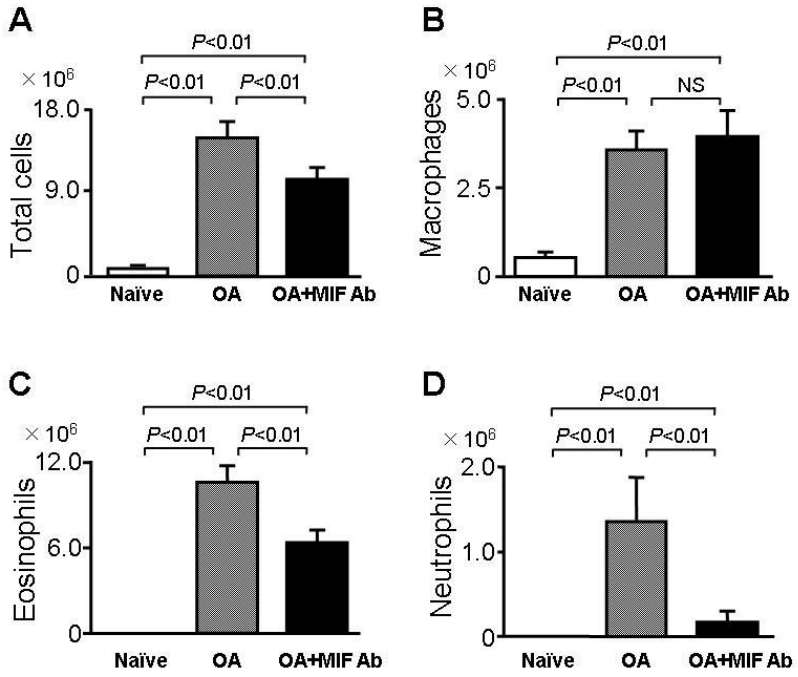
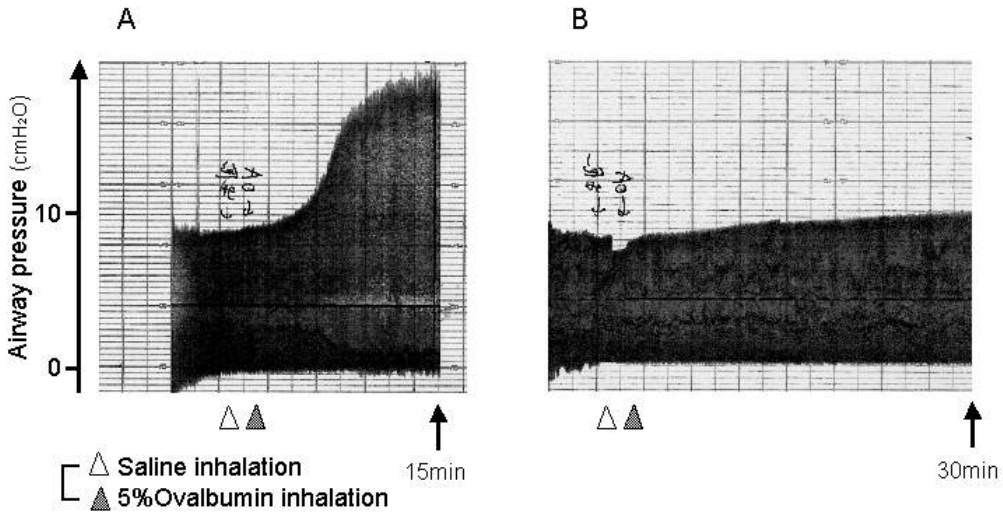
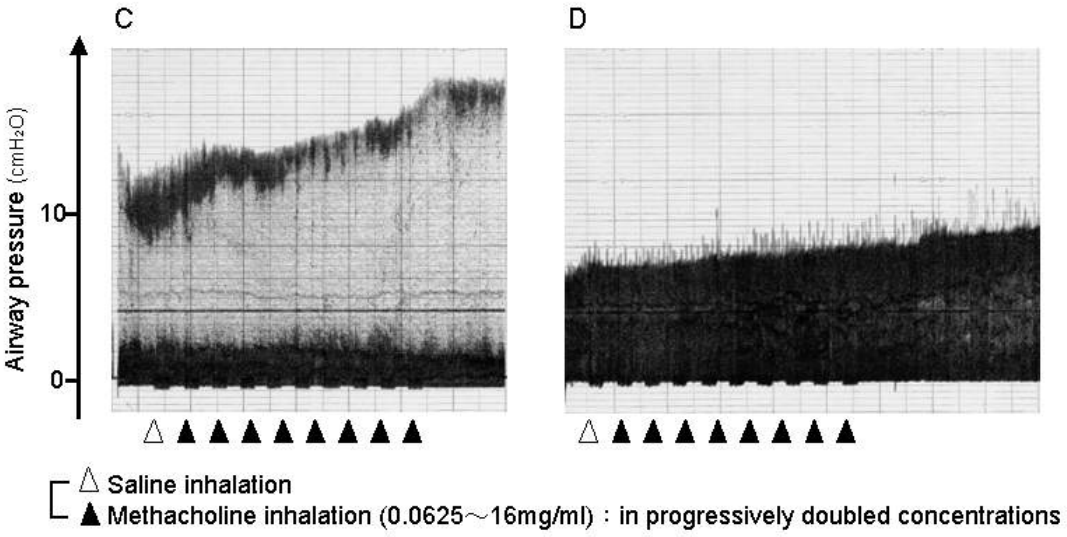


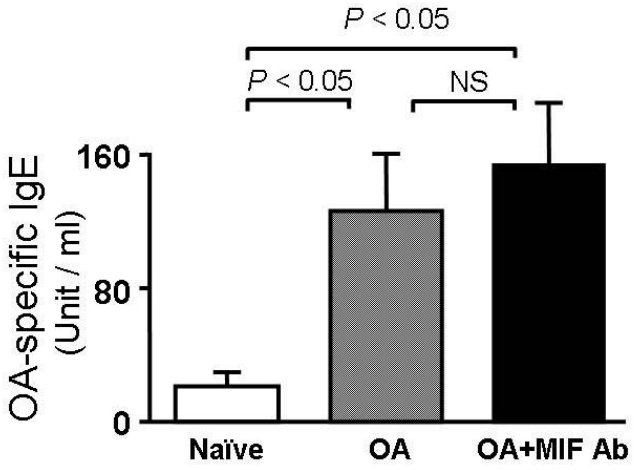
B

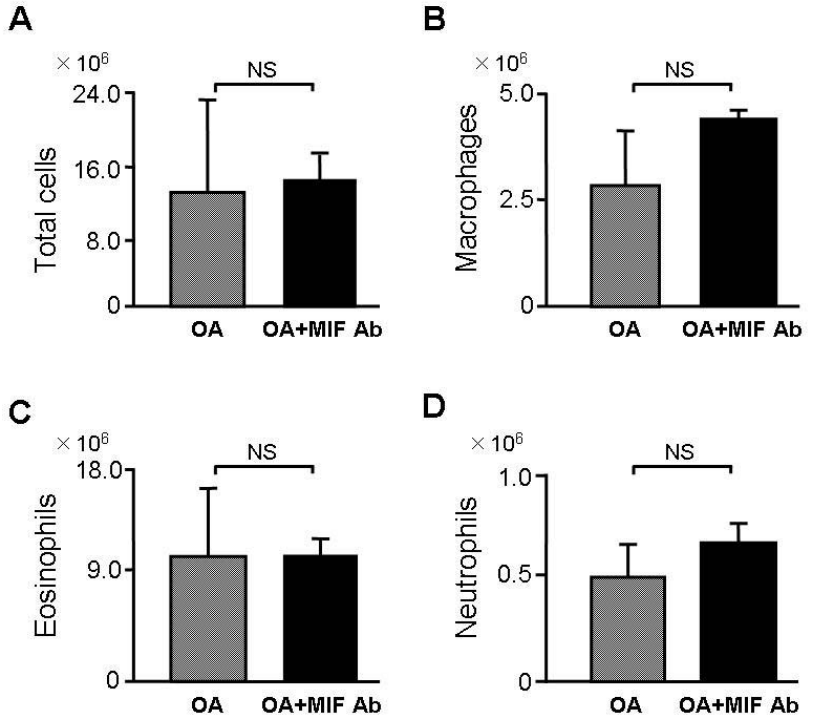


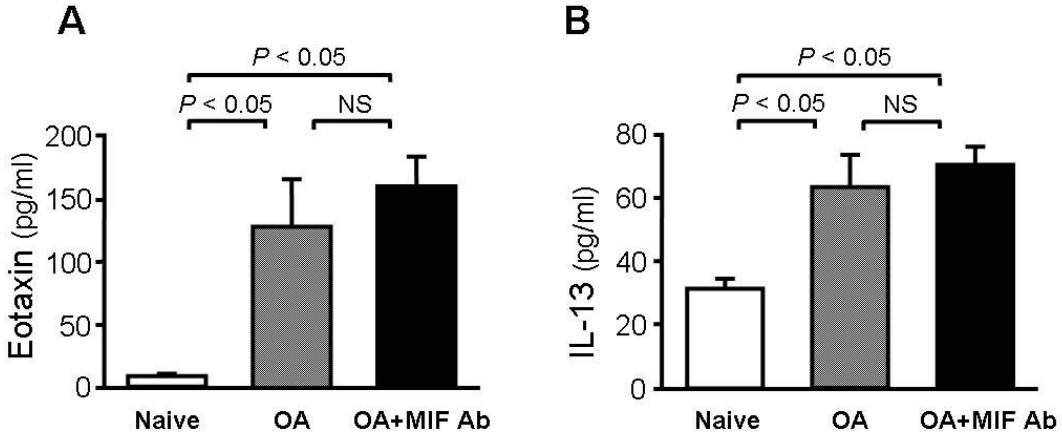

A
P<0.01
P<0.01
P<0.01
×10^6
18.0
9.0
0
Total cells
Naïve
OA
OA+MIF Ab
B
P<0.01
P<0.01
NS
×10^6
5.0
2.5
0
Macrophages
Naïve
OA
OA+MIF Ab
C
P<0.01
P<0.01
P<0.01
×10^6
12.0
6.0
0
Eosinophils
Naïve
OA
OA+MIF Ab
D
P<0.01
P<0.01
P<0.01
×10^6
2.0
1.0
0
Neutrophils
Naïve
OA
OA+MIF Ab


A
B
Airway pressure (cmH2O)
10
0
15min
30min
Saline inhalation
5%Ovalbumin inhalation


C
D
Airway pressure (cmH2O)
10
0
△ Saline inhalation
▲ Methacholine inhalation (0.0625～16mg/ml) : in progressively doubled concentrations


P < 0.05
P < 0.05
NS
OA-specific IgE
(Unit / ml)
160
80
0
Naïve
OA
OA+MIF Ab


A
B
C
D
× 10^6
Total cells
Macrophages
Eosinophils
Neutrophils
NS
24.0
16.0
8.0
0
5.0
2.5
18.0
9.0
1.0
0.5
OA
OA+MIF Ab


A
B
P < 0.05
NS
Eotaxin (pg/ml)
IL-13 (pg/ml)
200
150
100
50
0
80
60
40
20
Naive
OA
OA+MIF Ab