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*Original article*

# Role of CCL21 and CCL19 in allergic inflammation in the ovalbumin-specific murine asthmatic model



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**Background: Dendritic cells are the most powerful of the antigen-presenting cells and are known to play important roles in sensitization and inflammation in allergen-specific asthma. Various cytokines and chemokines are involved in the maturation and activation of dendritic cells. Among them is CC chemokine ligand (CCL)21, a key chemokine in the entry of naive T cells and antigen-stimulated dendritic cells into the T-cell zones of secondary lymphoid organs, which is a critical process in antigen-specific T-cell activation.**

**Objective: We studied the role of CCL21 in airway inflammation in asthma by using BALB/c-plt/plt (plt) mice, which possess genetic defects in expression of both CCL21 and CCL19.**

**Methods: Plt and control BALB/c mice were immunized with ovalbumin and alum 4 times and thereafter were subjected to a 2-week regimen of ovalbumin inhalation.**

**Results: In plt mice, ovalbumin-specific IgE response was delayed compared with control BALB/c mice, but they had the same level of response after final immunization. Although airway inflammation and response to acetylcholine were significantly reduced compared with BALB/c mice, significant eosinophilic inflammation and hyperresponsiveness were also observed in plt mice after 2 weeks of inhalation. Four weeks after cessation of inhalation, airway inflammation and hyperresponsiveness in plt mice were greater than in BALB/c mice. At the time of resolution of airway inflammation, IL-10 production was enhanced in BALB/c mice but not in plt mice.**

**Conclusion: The chemokines CCL21 and CCL19 were critical for resolution of airway inflammation.**

**Clinical implications: The findings about the chemokines for induction and resolution of inflammation are key to establishing a new strategy for asthma immunotherapy.**

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*Key words: Asthma, airway hyperresponsiveness, CCL21, dendritic cells, airway inflammation*

In allergic asthma, immune response to inhaled antigen is skewed to the $T_H2$-type response.[1-7] For the recognition of antigen, dendritic cells (DCs) work as professional antigen-presenting cells (APCs). Administration of antigen pulsed DCs induces allergic inflammation.[8,9] In addition, DC maturation is accelerated in the asthmatic airway.[10] Immature DCs possess the ability to take up and process antigens but have low antigen-presenting ability.[11] After taking up antigen, DCs become mature, expressing CD86, CD80, CD40, and CC chemokine receptor (CCR).[12,13] Mature DCs migrate to lymph nodes through the interaction of CCR7 and CC chemokine ligand (CCL)21 (secondary lymphoid tissue chemokine), where they effectively present antigen to naive T cells. CCL21 has potent activity in attracting naive and mature T cells, B cells, and DCs.[14,15] CCL21 was expressed in high endothelial venules in the peribronchial area and perivascular lymphatics in the lung. Another ligand of CCR7 is CCL19 (Epstein-Barr ligand chemokine), which also promotes efficient interaction of T cells and DCs at the site of lymph nodes. The process of antigen presentation has been clearly shown at the skin.[9] Antigen that penetrates the skin is taken up by DCs. Subsequently, these professional APCs enter afferent lymph vessels, a process promoted by CCL21. Antigen-laden APCs then induce the activation, expansion, and differentiation of antigen-specific T cells. These processes are efficiently performed only at skin-draining lymph nodes. Although CCL21 is also expressed in peribronchial areas and perivascular lymphatics in the lung,[16] the role of CCL21 and CCL19 in the asthmatic airway has not been clarified.

CCL21 is encoded by 2 genes, SCYA21a and SCYA21b, which encode CCL21-ser and CCL21-leu

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*Abbreviations used*
APC: Antigen-presenting cell
BALF: Bronchioalveolar lavage fluid
CCL: CC chemokine ligand
CCR: CC chemokine receptor
DC: Dendritic cell
PAS: Periodic acid-Schiff
Penh: Enhanced pause
plt: BALB/c-plt/plt

protein, respectively. CCL21-leu (secondary lymphoid tissue) is absent in mice with a paucity of lymph node T cells (BALB/c-plt/plt [plt] mice), which possess a defect in homing of naive T cells in lymph nodes, resulting in impaired secondary lymphoid organs.[17-19] Plt mice are also deficient in CCL19.[18] To test the role of CCL21 and CCL19 in antigen-specific asthma, we investigated the ability of plt mice to exhibit allergen-specific airway inflammation. We found that airway inflammation and airway response to acetylcholine were significantly reduced in plt mice compared with BALB/c mice. However, significant eosinophilic inflammation and hyperresponsiveness were also observed in plt mice after 2 weeks of inhalation. Interestingly, after cessation of inhalation for 4 weeks, airway inflammation and airway hyperresponsiveness of plt mice were greater than those of BALB/c, suggesting that in the resolution phase of airway inflammation, CCL21 and CCL19 play a critical role.

## METHODS

### Sensitization

BALB/c mice were purchased from Charles River Japan (Kanagawa, Japan). Plt mice were bred in the animal facilities of Musashino and Teikyo University School of Medicine under Specific Pathogen-Free conditions. Care and use of the animals followed the guidelines of the Principles of Laboratory Animal Care formulated by the National Society for Medical Research. The mice were initially immunized intraperitoneally 4 times with 10 μg ovalbumin + 2 mg aluminum hydroxide gel (on days 0, 28, 35, and 49) as previously reported with slight modifications.[20] After immunization, animals were subjected to inhalation of ovalbumin 3 mL/min every other day for 2 weeks (day 50 to day 63 from the first immunization) using a micromist nebulizer (Devilbiss, Somerset, Pa). Mean particle diameter was 3.0 μm. In some mice, recombinant macrophage inflammatory protein 3β/CCL19 (Epstein-Barr ligand chemokine: 5 μg/mouse), purchased from R&D Systems (Minneapolis, Minn), was administered by using an osmotic pump (Alzet Minipump; Durect Corp, Cupertino, Calif), which was implanted subcutaneously in the anterior back region. Four to 5 mice were used per treatment group in each experiment. Untreated mice were also used as controls.

### Assessment of airway responsiveness

The assessment of airway responsiveness was undertaken 24 hours after the last ovalbumin inhalation, as previously reported.[20] We used barometric whole-body plethysmography (Buxco Electronics, Inc, Troy, NY) as described by Hamelmann et al.[21] Data are expressed as enhanced pause (Penh). Airway responsiveness was determined by calculating the ratio of the Penh to control Penh (%Penh). In some mice, invasive measurements were undertaken as previously reported.[22] Briefly, the anesthetized mice were tracheostomized and injected with pancuronium bromide. The animals were connected to a Harvard ventilator with 0.3 mL tidal volume and a respiratory frequency of 120/min. Next, they were placed in the whole-body plethysmograph (Buxco Electronics) to measure airway resistance.

### Measurements of serum ovalbumin-specific IgE and $IgG_{2a}$

To measure ovalbumin-specific IgE, we coated the plate with ovalbumin using the AlaSTAT system (Diagnostics Products, Los Angels, Calif) with some modification as previously reported.[20] After incubation with samples diluted 10-fold, horseradish peroxidase–labeled rat antimurine IgE specific antibody, purchased from Morinaga Co, Ltd (Kanagawa, Japan), was added to the plate as the second antibody. Color was developed and measured at 450 OD.

For ovalbumin-specific $IgG_{2a}$, plates were coated with 50 μg/mL ovalbumin in 50 mmol/L carbonate buffer, pH 9.0. After blocking with SuperBlock (Pierce, Rockford, Ind), samples diluted 2000−fold were added in the plates. Antibodies were detected by peroxidase-labeled antimouse $IgG_{2a}$ (1:500; Invitrogen, Carlsbad, Calif). The color was developed with TMB+ (Dako Corp, Glodtrup, Denmark) and stopped by 1N $H_2SO_4$. The antibody titers were calculated by comparison with internal standards.

### Bronchoalveolar lavage fluid cell analysis and histologic examination

After measurement of pulmonary function, each mouse was exsanguinated. Bronchoalveolar lavage fluid (BALF) was obtained from selected mice by incubating and washing the lung with 1 mL saline until the recovered volume was 5 mL. BALF was centrifuged at 1500 rpm for 10 minutes at 4°C. Pellets were dissolved in 1 mL PBS, and the number of the cells was counted. Cytospin specimen was obtained by rotating at 640 rpm for 2 minutes. Then, the cells were stained with Diff Quik (International Reagents Corp, Osaka, Japan), and the cell fractions were examined on microscope.

The lungs were fully inflated using 10 cm $H_2O$ pressure and fixed with 10% formaldehyde for hematoxylin-eosin and periodic acid-Schiff (PAS) staining.

### RNA extraction and quantification of mRNAs

Lungs were frozen in liquid nitrogen immediately after isolation and were used for RNA extraction. Lung tissue was homogenized at 4°C, and total RNA was extracted by using ISOGEN, a modified acid guanidium-phenol-chloroform method (Nippon Gene Co, Ltd, Tokyo, Japan). RNA was treated with 10U DNase (Qiagen, Hiden, Germany) following the manufacturer's instructions. The purity of the RNA was established by spectrophotometer using a DNA purity calculating program (Hitachi Seisakusho, Tokyo, Japan). The absorbance OD 260/280 ratio was 1.8. After the amount of total RNA was measured, cDNA synthesis was performed with 0.125 μmol/L oligo-dT (Takara Biochemicals, Tokyo, Japan) as previously described.[20] The levels of mRNA were examined by real-time PCR using the Light Cycler-Fast Start DNA Master SYBR GreenI kit (Roche Diagnostics, Mannheim, Germany). In this system, double-stranded DNA is labeled with SYBR Green I and then detected. Quantification was performed on the basis of the standard curve obtained by using serial dilution of specific PCR products. Results are shown as ratios of the level of mRNAs standardized by the level of β-actin mRNA. The primers used were as follows: β-actin, 5′-CCTGTATGC-CTCTGGTCGTA-3′ 5′-CCATCTCCTGCTCGAAGTCT-3′ 260 bp; IL-13, 5′-GATATGAAGTTGAAGAGGAGAG-3′ 5′-GGCAAA-GAAGTAACAAAAGG-3′ 208 bp; IFN-γ, 5′-TCTTGGATAT-CTGGAGGAAC-3′ 5′-GACCTCAAACTTGGCAATAC-3′ 215 bp.

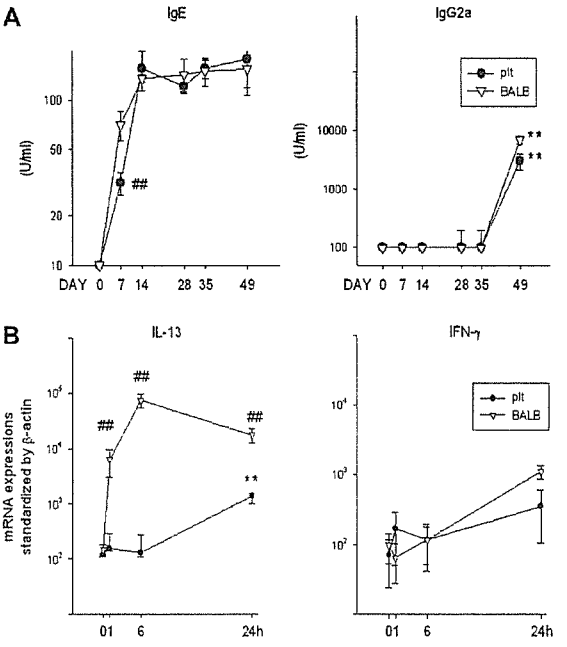


FIG 1. Time course of ovalbumin-specific IgE and $IgG_{2a}$ response (A) and mRNA expressions of IL-13 and IFN-γ (B). Each *bar* represents the mean ± SEM of values obtained from 5 different mice. Five mice per group were examined. Similar results were obtained from 4 other experiments. ##$P < .01$ BALB/c vs plt. **$P < .01$ indicated time vs baseline.

## Quantification of IL-10 production

Lung cells were extracted from some of the mice in which airway responsiveness was measured and used for cell culture. Lungs were cut into small pieces and incubated at 37°C for 1 hour in 150 U/mL collagenase in RPMI. Then, lung pieces were homogenized in 1% penicillin/streptomycin (Gibco Invitrogen, Carlsbad, Calif)/RPMI 1640 using cell strainers with 70-μm pores (BD Falcon, Franklin Lakes, NJ) and centrifuged at 1500 rpm for 10 minutes at 4°C. Cells were then washed twice, and $CD3^+$ cells were separated by using the Dynabead mouse pan T-cell system following the manufacturer's instructions (Dynal Biotic ASA, Oslo, Norway). $CD3^+$ cells with about 5% adherent cells were then suspended in 1% penicillin/streptomycin/10% FCS in RPMI 1640 and cultured with or without ovalbumin (100 μg/mL) in 24-well plates (Corning, Inc, Corning, NY) at $1 \times 10^6$ cells/well at 37°C. After 2 days of culture, the supernatant was collected, and the levels of IL-10 were evaluated by the ELISA Quantising kit (R&D Systems).

## Flow cytometry

Data were collected by using FACSAria (BD Biosciences, San Jose, Calif). A total of $1 \times 10^6$ cells from lung, spleen, blood, and lymph node were stained with indicated antibodies. Antibodies used were antimouse CD16/CD32 (Fc block), phycoerythrin-Cy7–conjugated rat antimouse CD25 antibody, CD4-Alexa 647 (BD Biosciences), and phycoerythrin-labeled CCL19-Fc chimera antibody (Santa Cruz Biotechnology Inc, Santa Cruz, Calif). Cells were first treated with antimouse CD16/CD32 (Fc block) for 10 minutes. Then staining antibodies were added for 30 minutes at 4°C. All plots show log10 fluorescence.

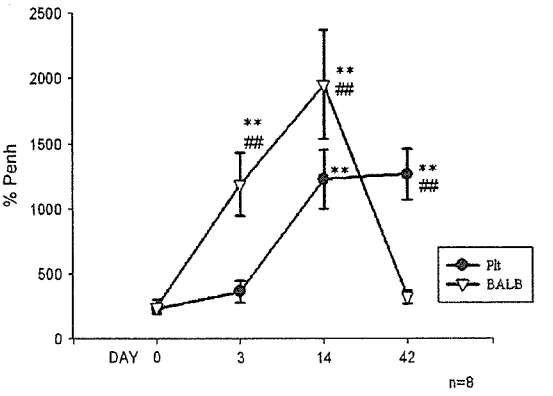


FIG 2. Time course of airway response to acetylcholine. Airway response to 100 mg/mL acetylcholine was expressed as % Penh. Each *bar* represents the mean ± SEM of values obtained from 6 different mice. Similar results were obtained from 3 other experiments. **$P < .01$ indicated day vs day 0. ##$P < .01$ BALB/c vs plt mice.

## Statistics

For comparisons of multiple parameters, we used ANOVA with Bonferroni correction. The level of statistical significance was set at $P < .05$. Data are expressed as means ± SEMs.

# RESULTS

## Ovalbumin-specific IgE and $IgG_{2a}$ synthesis in plt mice

To elucidate the functional role of CCL21 and CCL19 in IgE synthesis, we determined the kinetics of IgE synthesis with immunization of ovalbumin and alum in plt mice and BALB/c mice. Examination of the ovalbumin-specific IgE level on day 7 after the first immunization revealed a significantly lower IgE level in plt mice compared with BALB/c mice (Fig 1, *A*; $P < .01$). On day 14, the difference was no longer evident, a tendency that was continued after the final immunization (Fig 1, *A*). These data suggested that induction of IgE was delayed but that the response was similar to the IgG response reported previously.[23] When we compared $IgG_{2a}$ synthesis, there was significant synthesis on day 49 but no difference between plt and BALB/c mice (Fig 1, *A*).

Next, we examined IL-13 and INF-γ mRNA expression of the lung after ovalbumin inhalation. One hour after ovalbumin inhalation, significant IL-13 mRNA was detected in BALB/c mice but not plt mice (Fig 1, *B*). After 24 hours, IL-13 mRNA expression was detected in plt mice but significantly lower compared with BALB/c mice (Fig 1, *B*). IFN-γ expression was not different between BALB/c and plt mice. These data suggested that plt mice expressed significant $T_H2$ response but lower compared with BALB/c mice.

## Airway hyperresponsiveness and inflammation in plt mice

To determine airway response, we first measured airway resistance after 100 mg/mL acetylcholine

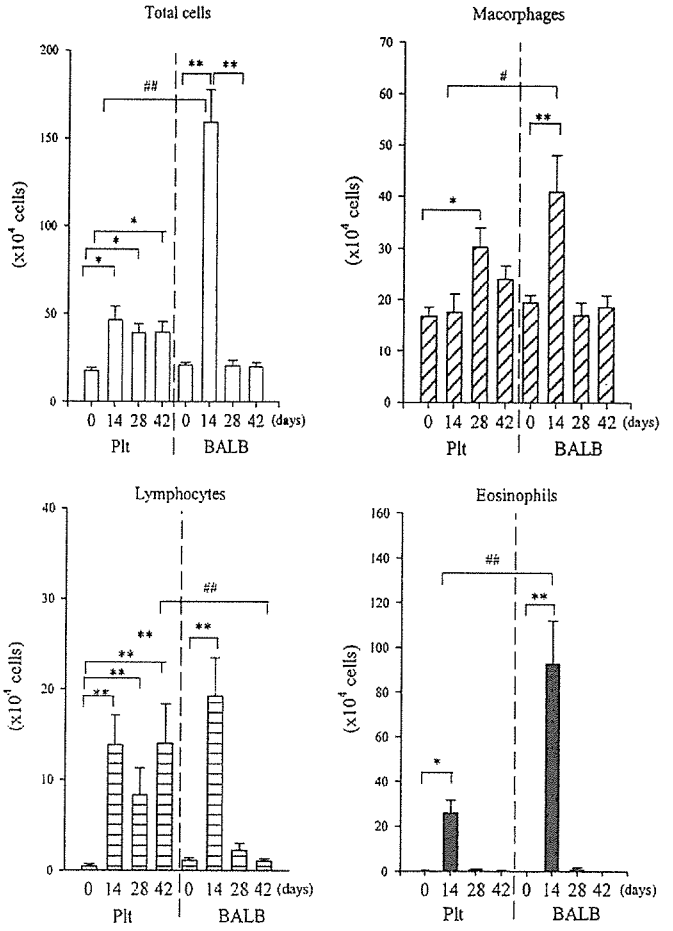


**FIG 3.** BALF cell analysis. Data shown are means ± SEMs of 8 mice per group. *$P < .05$; **$P < .01$ indicated day vs day 0. #$P < .05$; ##$P < .01$ BALB/c vs plt mice.

inhalation (Fig 2). We followed a sensitization system as previously reported.[20] Mice were analyzed 24 hours after the last ovalbumin inhalation at the times indicated in Fig 2. Before ovalbumin inhalation, no increase in airway resistance was observed after acetylcholine inhalation compared with saline inhalation. On day 3, a significant increase in airway response was observed in BALB/c mice but not in plt mice. On day 14, a further increase in airway response was observed in BALB/c mice. On day 14, plt mice also exhibited a significant increase in airway response compared with untreated mice; however, the response was significantly lower than in BALB/c mice. On day 42 (4 weeks after cessation of ovalbumin inhalation on day 14), airway response was decreased in BALB/c mice but not in plt mice. To confirm the results, airway resistance after inhalation of 2.5 mg/mL acetylcholine was also examined by using an invasive measurement on day 42. Airway resistance of plt mice and BALB/c mice was 4.33 ± 0.25 versus 2.94 ± 0.33 cm $H_2O$/L/s, respectively (n = 5 each group; $P < .05$).

To determine airway inflammation, BALF cells were also analyzed in BALB/c mice (Fig 3). On day 14, significant eosinophilia was observed in BALF. Increase in eosinophil and lymphocytes resolved on day 28 (2 weeks after cessation of exposure to ovalbumin). Although the level was lower, plt mice exhibited significant increase in the number of eosinophils on day 14 (Fig 3). In contrast to BALB/c, increases in lymphocytes remained present until day 42 (4 weeks after cessation of exposure to ovalbumin).

Histologic analysis revealed less airway inflammation at day 14 in plt mice than in BALB/c mice (Fig 4, *A*). PAS staining revealed that mucus-producing cells significantly appeared both in plt and BALB/c mice. However, the number of mucus-producing cells was small in plt mice as compared with BALB/c mice (Fig 4, *B*, on day 14). In BALB/c mice, airway inflammation resolved on day 28, but plt mice exhibited significant airway inflammation on day 28 (Fig 4, *A*). These data indicated that airway inflammation was delayed but that significant responses occurred in plt mice compared with BALB/c mice, as is the case with antibody response. It was also noted that resolution of airway inflammation and airway responsiveness was delayed in plt mice.

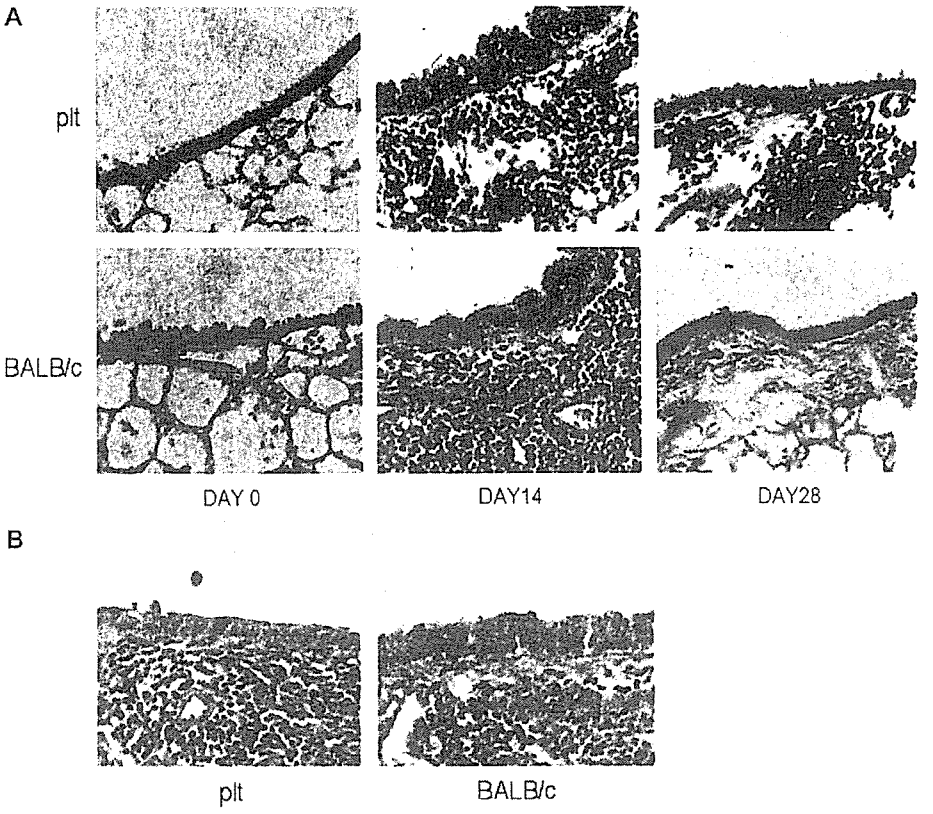


**FIG 4.** Histologic analysis was performed by hematoxylin-eosin staining **(A)** and PAS staining **(B)** in BALB/c and plt mice at the indicated time. Figures are representative data of histologic examination of 5 mice. Original magnification ×200.

## Analysis of CCR7 and CD25 expression

To clarify the mechanisms of delay in resolution of airway inflammation, we performed flow-cytometric analysis to assess CCR7, receptor for CCL19 and CCL21, on T cells, because it has been reported that CCR7 expression is critical for T-cell exit from peripheral tissues.[24,25] $CCR7^+$ cells were present in the $CD4^+$ cells of lung after ovalbumin inhalation both in BALB/c (Fig 5, *A*) and plt mice (Fig 5, *B*). When we examined after cessation of ovalbumin inhalation in plt mice, there were $CD4^+CCR7^+$ cells, but the majority were $CD4^+CCR7^-$ cells (Fig 5, *B*).

Regulatory T cells, which are known to suppress airway inflammation, express CCR7 and are activated by pulmonary DCs at the secondary lymph nodes.[26,27] Therefore, we next examined the expression of CD25 on $CD4^+$ cells. $CD4^+CD25^+$ cells appeared in the BALF after ovalbumin inhalation. Seven days after cessation of ovalbumin inhalation, the number of $CD4^+CD25^+$ cells in the BALF was significantly smaller in plt mice than that in BALB/c mice (Fig 5, *C*). $CD4^+CD25^+$ cells marginally existed in mediastinal lymph node of plt mice (Fig 5, *C*). There were no differences in the number of $CD4^+CD25^+$ cells in the blood and spleen between plt and BALB/c mice (Fig 5, *C*). Although the number of $CD4^+CD25^+$ cells in the BALF was decreased, a significant number of $CD4^+CD25^+$ cells was found in the BALF of plt mice.

## Role of CCL19 in resolution of airway inflammation

To investigate the mechanisms of delayed resolution of allergic inflammation in plt mice, we added CCL19, another ligand of CCR7, for which recombinant protein is available. After 14 days of ovalbumin exposure, we added CCL19 (5 μg/mouse by osmotic pump) continuously. For BALB/c mice, saline was added instead of CCL19. After 1 week of administration, lung lymphocytes were separated and were cultured for 2 days, after which the supernatants were harvested. We measured IL-10 because this protein contributes to the resolution of allergic inflammation. As shown in Fig 6, *A* (in the BALF), and Fig 6, *B* (in the cultured supernatants), IL-10 production in BALB/c mice was significantly greater than in plt mice. The treatment with CCL19 significantly enhanced IL-10 production in plt mice (Fig 6, *B*), suggesting that the defect in CCL19 and CCL21 impairs IL-10 production in lung cells. To assess the effect of CCL19 administration, we also analyzed BALF cells and airway response after 1 week of CCL19 administration. Although lymphocytes in CCL19-treated plt mice did not reach the same level in BALB/c mice, CCL19-treated plt mice exhibited a significantly lower number of lymphocytes in the BALF and Penh level after 100 mg acetylcholine inhalation compared with nontreated plt mice (Fig 6, *C and D*).

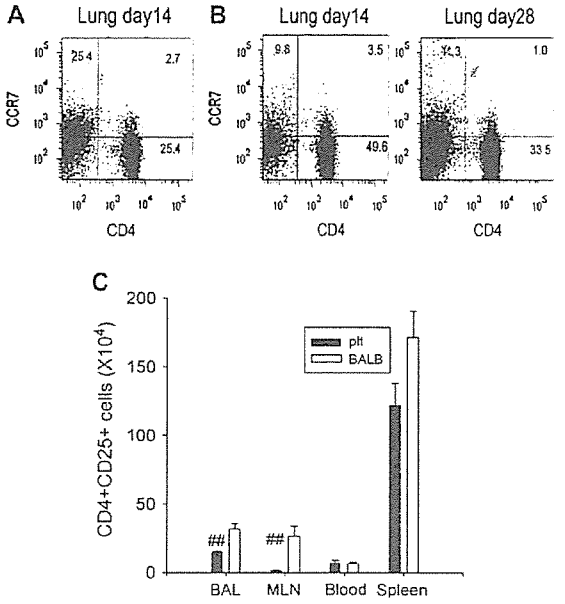


FIG 5. Flow-cytometric analysis of BALF cells, lung, secondary lymph node, blood, and spleen. **A**, CCR7 expression in BALB/c mice. **B**, CCR7 expression in plt mice. **C**, Number of $CD4^+CD25^+$ T cells on day 21 (1 week after cessation of ovalbumin inhalation). *BAL*, Bronchoalveolar lavage; *MLN*, mediastinal lymph node.

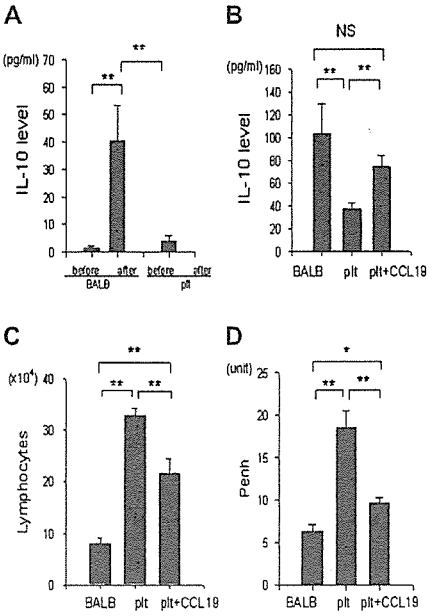


FIG 6. IL-10 production after cessation of allergen inhalation and effect of CCL19 administration. **A**, IL-10 level in the BALF before and after ovalbumin inhalation. **B**, IL-10 level in the cultured cells. Data shown are means ± SEMs of triplicate examination from 4 mice per group. The effect of CCL19 administration on airway inflammation **(C)** and on airway response **(D)**. $*P < .05$; $**P < .01$. *NS*, Not significant.



## DISCUSSION

In the current study, we showed that CCL19 and CCL21 are important but not critical for sensitization and induction of allergic inflammation. In plt mice, which are genetically defective with regard to CCL19 and CCL21,[17] the ovalbumin-specific IgE response was delayed, although the same level of response was noted as in BALB/c mice after the final immunization. Although airway inflammation and airway response to acetylcholine were significantly reduced in plt mice compared with BALB/c mice, significant eosinophilic inflammation and hyperresponsiveness were also observed in plt mice after 2 weeks of inhalation. However, 4 weeks after cessation of inhalation, airway inflammation and airway hyperresponsiveness in plt mice were greater than in BALB/c. At the time of resolution of airway inflammation, IL-10 production was enhanced in BALB/c but not in plt mice.

Enhanced T-cell immune response was observed in plt mice when examined by contact sensitization.[23] CCL21 is present in the luminal surface of venules in peripheral lymph nodes of wild-type mice but not plt/plt.[23] High endothelial venules in plt mice did not express CCL21 and did not support T-cell sticking, resulting in a marked reduction of T-cell homing to peripheral lymph nodes.[23] After immunization of plt mice, T cells and DCs located in the superficial cortex of lymph nodes and in splenic bridging channels.[23] It has been reported that airway inflammation can be induced without secondary lymph nodes.[28] In that study, lymphotoxin α/β knockout mice were used for impairment of secondary lymph nodes. Consistent with previous reports, our data showed that significant airway inflammation in a lesser magnitude was induced without secondary lymph nodes.

Blockade of CCL21 by neutralizing antibody exacerbates lung $T_H1$-dominant inflammation induced by propionibacterium acnes, which is thought to possess linkage to sarcoidosis.[16] Immune response in CCR7 receptor knockout mice was also reported.[12] CCR7 is the receptor for CCL19 and CCL21, and its knockout mice possess impairment of secondary lymph nodes similar to plt mice. However, the impairment of contact sensitivity is severely suppressed compared with plt mice. Antibody response to subcutaneous immunization with complete Freund adjuvant is also depressed in the first 10 days and reaches a level similar to that in wild-type mice after 20 days. Although the immunization protocol and antigens are different, our data with plt mice exhibited similar kinetics.

Plt mice exhibited impaired resolution of airway inflammation. Recently, it has been reported that CCR7 plays a critical role in the lymphocyte exit from peripheral tissue and entry into afferent lymphatics.[24,25] As shown in Fig 5, both $CCR7^+$ and $CCR7^-$ CD4 T cells existed in the lung of both plt and BALB/c mice. Even at the resolution phase, both $CCR7^+$ and $CCR7^-$ cells exist in the lung of plt mice, suggesting that we could not ascribe delayed resolution of inflammation only to impairment of T-cell exit in plt mice. Because IL-10 regulates airway inflammation,[29-31] our data on impairment of IL-10 production in plt mice after cessation of ovalbumin exposure might be

critical in elucidating factors for resolution of airway inflammation. Furthermore, administration of CCL19 resulted in recovery of impaired IL-10 production, suggesting that the defect in IL-10 production was directly related to genetic defects of plt mice. Natural recovery from autoimmune encephalomyelitis has been ascribed to IL-10 producing $CD4^+CD25^+$ regulatory T cells,[32] which are known to suppress airway inflammation and produce IL-10 or TGF-β.[6,7,26,30,33,34] Plt mice also demonstrate defective DC localization,[19] which is a regulator of adaptive immune response as well as a critical inducer.[35] Pulmonary DCs also activate regulatory T cells at the secondary lymph nodes.[27] Although we could not show phenotypical defects in $CD4^+CD25^+$ cells in plt mice, we identify functional impairment of induction of IL-10 production in plt mice, which might be ascribed to functional impairment of regulatory T cells or DCs. Another possible mechanism of delayed resolution is a defect in apoptosis in plt mice, which had been reported previously in $T_H1$-dominant inflammation.[36]



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

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ABSTRACT: **Macrophage migration inhibitory factor (MIF) is a pro-inflammatory cytokine that reportedly counteracts the anti-inflammatory effect of endogenous glucocorticoids. There have only been 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, the present authors examined the effect of anti-MIF antibody (Ab) on airway inflammation and airway hyperresponsiveness in an ovalbumin-immunised rat asthma model.**

**Actively immunised brown Norway rats received ovalbumin inhalation with or without treatment of anti-MIF Ab. 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 nonsensitised rats, and the MIF staining in bronchial epithelium was enhanced after the ovalbumin challenge. Anti-MIF Ab significantly decreased the number 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 Ab did not affect the level of serum ovalbumin-specific IgE, suggesting that anti-MIF Ab did not suppress immunisation itself.**

**The results indicate that macrophage migration inhibitory factor plays a crucial role in airway inflammation and airway hyperresponsiveness in asthma.**

KEYWORDS: **Airway hyperresponsiveness, airway inflammation, asthma, eosinophil, macrophage migration inhibitory factor, ovalbumin**

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]; this protein has been postulated to function as a pro-inflammatory cytokine [4, 5]. DONNELLY *et al.* [6] reported that the levels of MIF in bronchoalveolar lavage fluid (BALF) were increased in patients with acute respiratory distress syndrome. The present authors subsequently demonstrated that anti-MIF antibody (Ab) 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 pro-inflammatory 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]. It 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 T-helper cell (Th) type 2 but not Th1 clones [11]. Accordingly, MIF is considered to be a pleiotropic peptide, functioning as a cytokine and/or hormone.

Only a few reports have examined the potential role of MIF in asthma [12–14]. ROSSI *et al.* [12] first reported that MIF levels were increased in BALF from asthmatic patients and that circulating eosinophils could produce MIF upon stimulation *in vitro*. However, one subsequent animal study could not support this argument of the role of MIF in asthma because anti-MIF serum did not affect allergic airway inflammation in mice [14]. The aim of the present study is to further clarify the role of MIF in asthma using rats. The study will demonstrate that anti-MIF Ab inhibits ovalbumin (OA)-induced airway inflammation as well as airway hyperresponsiveness in brown

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Norway rats, which have been used as a model of atopic asthma [15–17].

## MATERIALS AND METHODS

### Animals and immunisation

The research adhered to the Declaration of Helsinki and was approved by the Ethical Committee on Animal Research (Hokkaido University, Sapporo, Japan). 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 immunised to OA by subcutaneous injection with 1 mg OA containing 200 mg aluminum hydroxide. An adjuvant consisting of $1 \times 10^9$ heat-killed *Bordetella pertussis* organisms was intraperitoneally injected at the same time.

### Preparation of rabbit polyclonal Ab against MIF

Polyclonal anti-rat MIF serum was generated by immunising New Zealand white rabbits with purified recombinant rat MIF. Rat MIF was expressed in *Escherichia coli* and purified to homogeneity, as described in a previous publication of the authors' [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) at week 4. The immunoglobulin (Ig)G fraction was prepared using Protein A Sepharose (GE Healthcare Bio-Sciences, Piscataway, NJ, USA) 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 immunisation and did not have any treatments. The OA and OA+anti-MIF Ab groups were actively immunised on day 0 and intraperitoneally injected with 2 mg of the non-immunized rabbit IgG or the anti-MIF polyclonal Ab every 2 days from day 0 to day 16. In the preliminary study, the present authors had confirmed that non-immunised rabbit IgG caused no changes in inflammatory cells of the OA-immunized lungs. Neither total cell nor eosinophil counts in BALF were significantly different between the OA immunised+untreated group and the OA immunized+non-immunized IgG group ($8.99 \pm 1.70 \times 10^6$ *versus* $7.05 \pm 0.89 \times 10^6$ n=3, and $5.79 \pm 0.76 \times 10^6$ *versus* $4.67 \pm 0.80 \times 10^6$ n=3, 3, respectively; unpublished data). The OA immunized+non-immunized IgG group were thus used as control in this experiment. The authors felt that administration of non-immunized rabbit IgG would be desirable to more specifically examine the effect of anti-MIF Ab. On day 14, the rats inhaled 2 % weight/volume OA for 15 minutes in an exposure chamber. Three days after OA inhalation, broncoalveolar lavage 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 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 interleukin (IL)-13 concentrations.

### Measurement of bronchial responsiveness to methacholine and ovalbumin

Three days after OA challenge, another set of three groups were anesthetised with an intraperitoneal injection of pentobarbital sodium (50 $mg \cdot kg^{-1}$). Intratrachial intubation was then performed with a metallic tube. The rats were mechanically ventilated (Rodent Ventilator Model 683; Harvard Apparatus, Holliston, MA, 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 ($P_{ao}$) was continuously measured. An aerosol of Mch or OA was administered through a reservoir box connected to the ventilator system. After measurement of baseline $P_{ao}$, an aerosol of saline followed by Mch or OA was administered.

### Immunohistochemical study

Immunohistochemistry was performed according to the manufacturer's protocol on paraffin embedded tissue using a Catalized Signal Amplification kit (DAKO Japan, Kyoto, Japan). The primary Ab was anti-MIF diluted at 1:200 with PBS. The tissue sections were counterstained with methyl green and mounted. The anti-MIF Ab used for immunohistochemical study was the same as the Ab administered for treatment of rats.

### Measurement of MIF levels by ELISA

The levels of MIF in the BALF were quantitated using the ELISA method, as described in a previous publication [19]. The anti-rat MIF Ab administered for treatment of rats was used in ELISA. Briefly, the anti-rat MIF Ab was added to each well of a 96-well microtitre plate. Wells were incubated with biotin-conjugated anti-MIF Ab for 1 h at room temperature. Avidin-conjugated horseradish peroxidase was added after washing. Substrate solution was then added to each well. The reaction was terminated with 2 M sulphuric acid. The absorbance was measured at 492 nm on an automated ELISA plate reader. The detection limit of this system was 1.5 $ng \cdot mL^{-1}$.

### OA-specific IgE Ab assay

The levels of OA-specific IgE in serum were quantitated using an ELISA method, as previously described [20]. Briefly, the 96-well microtitre plates were coated with anti-rat IgE monoclonal Ab (Zymed, South San Francisco, CA, 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 2 M sulphuric 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 $U \cdot mL^{-1}$.

### Measurement of eotaxin and IL-13 concentrations by ELISA

Due to the high degree of similarity maintained in chemokines across species, a mouse ELISA kit (R&D Systems Inc.,

Minneapolis, MN, USA) containing a polyclonal Ab that recognizes mouse eotaxin was used to detect the rat cognate. Eotaxin levels in BALF were determined using this kit according to the manufacturer's instructions. IL-13 levels in BALF were determined using a rat-specific solid phase sandwich ELISA kit (Biosource International, Camarillo, CA, USA). The minimum detectable concentration of eotaxin was 3 pg·mL$^{-1}$; IL-13 was 1.5 pg·mL$^{-1}$.

### *Statistical analysis*

Data are expressed as mean±SEM. Statistical analyses were performed on the data using single-factor ANOVA on the three groups and with a Student's unpaired t-test for comparisons of two groups. A p-value of $<0.05$ was assumed to be significant.

## RESULTS

### *Expression of MIF in OA-induced airway inflammation*

To investigate whether the expression of MIF in airways was enhanced in this model, the levels of MIF in BALF were measured 3 days after the OA challenge. They were significantly elevated in the OA group compared with those in the Naive group (14.7±1.4 ng·mL$^{-1}$ *versus* 1.3±1.1 ng·mL$^{-1}$, respectively, $p<0.05$; fig. 1).

### *Immunohistochemical localisation of MIF in lungs*

Histological examination using the lung tissue confirmed that OA inhalation induced widespread peribronchiolar inflammation in OA-sensitised 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 Ab 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

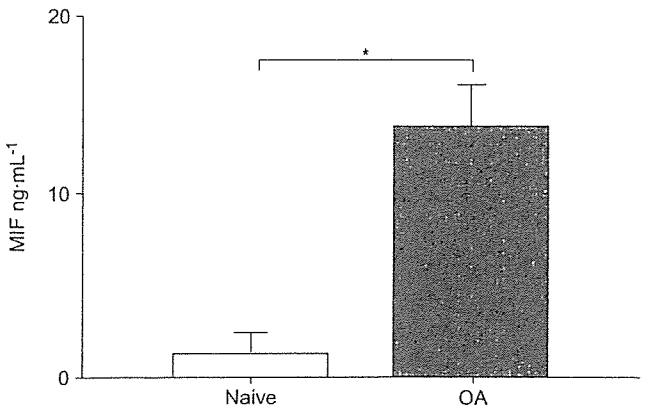


**FIGURE 1.** Levels of macrophage migration inhibitory factor (MIF) in bronchoalveolar lavage fluid (BALF). MIF in BALF significantly increased 3 days after the ovalbumin (OA) challenge in the OA group (n=6) compared with the Naive group (n=3). *: $p<0.05$.


**FIGURE 2.** Immunohistochemistry of macrophage migration inhibitory factor (MIF) in the lung. a) MIF was weakly detected in airway epithelium in the Naive group. b) MIF was prominent in airway epithelium in the ovalbumin (OA) group 3 days after the OA challenge. Scale bars=100 μm.

cells, macrophages, eosinophils and neutrophils were significantly elevated compared with those of the Naive group. Treatment with anti-MIF Ab significantly decreased the numbers of total cells, eosinophils and neutrophils compared with those of the OA group (total cells: 15.0±3.5 ×10$^6$ in the OA group *versus* 10.5±2.4 ×10$^6$ in the OA+anti-MIF Ab group, $p<0.01$; eosinophils: 10.5±2.7 ×10$^6$ in the OA group *versus* 6.2±2.7 ×10$^6$ in the OA+anti-MIF Ab group, $p<0.01$;

neutrophils: $1.4 \pm 1.2 \times 10^6$ in the OA group *versus* $0.16 \pm 0.27 \times 10^6$ in the OA+anti-MIF Ab group, $p<0.01$) and thus significantly attenuated airway inflammation.

### *Effect of anti-MIF Ab on antigen-specific airway contraction and nonspecific airway hyperresponsiveness*

To investigate whether anti-MIF Ab suppressed airway hyperresponsiveness, OA-specific and Mch-induced airway contractions 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 $mg \cdot mL^{-1}$. 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 $mg \cdot ml^{-1}$). The Naive group did not respond to either 5% OA or Mch (up to 16.0 $mg \cdot ml^{-1}$; data not shown).

### *Effect of anti-MIF Ab 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 immunisation by treatment of anti-MIF Ab; the authors therefore examined the possibility that anti-MIF Ab might have influenced OA-specific IgE levels in serum. As shown in figure 5, as expected [16], the levels of OA-specific IgE in serum were significantly elevated in the OA group compared with those in Naive group ($124.0 \pm 41.3$ $U \cdot mL^{-1}$ in the OA group *versus* $18.6 \pm 5.7$ $U \cdot mL^{-1}$ in the Naive group, $p<0.05$). Treatment with anti-MIF Ab similarly caused the elevation of OA-specific IgE in serum ($153.3 \pm 39.6$ $U \cdot mL^{-1}$).

### *Effect of a single administration of anti-MIF Ab before airway challenge*

The authors next considered whether the single administration of anti-MIF Ab before OA challenge might explain the results described above. A 2-mg aliquot of anti-MIF Ab or nonimmunised rabbit IgG was injected only once 2 h before OA challenge and bronchoalveolar lavage was performed 3 days after OA challenge. As shown in figure 6, a single administration of anti-MIF Ab did not change either the number of total cells or the differential cell counts in BALF.

### *Effect of anti-MIF Ab on eotaxin levels in BALF*

To investigate the mechanism by which anti-MIF Ab attenuated eosinophil accumulation in the lungs, the levels of eotaxin, a potent chemokine of eosinophils, in BALF were

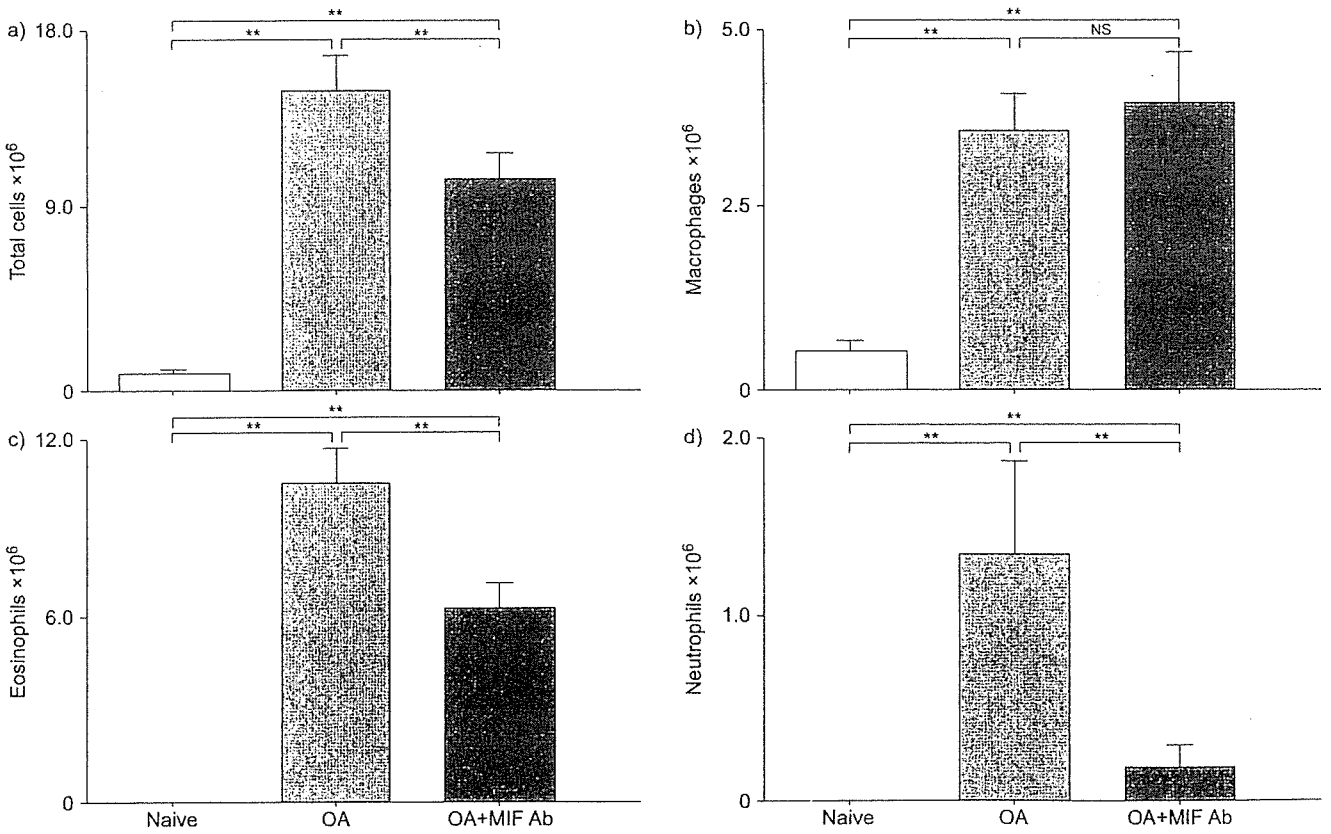


**FIGURE 3.** Total and differential cell counts in bronchoalveolar lavage fluid. Bronchoalveolar lavage was performed 3 days after ovalbumin (OA) challenge. The numbers of a) total cells, b) macrophages, c) eosinophils d) and neutrophils were significantly elevated in the OA group in comparison with the Naive group. Treatment with anti-MIF antibody (OA+MIF Ab) significantly decreased the numbers of total cells (a), eosinophils (c) and neutrophils (d) (n=6). **: $p<0.01$; NS: nonsignificant.

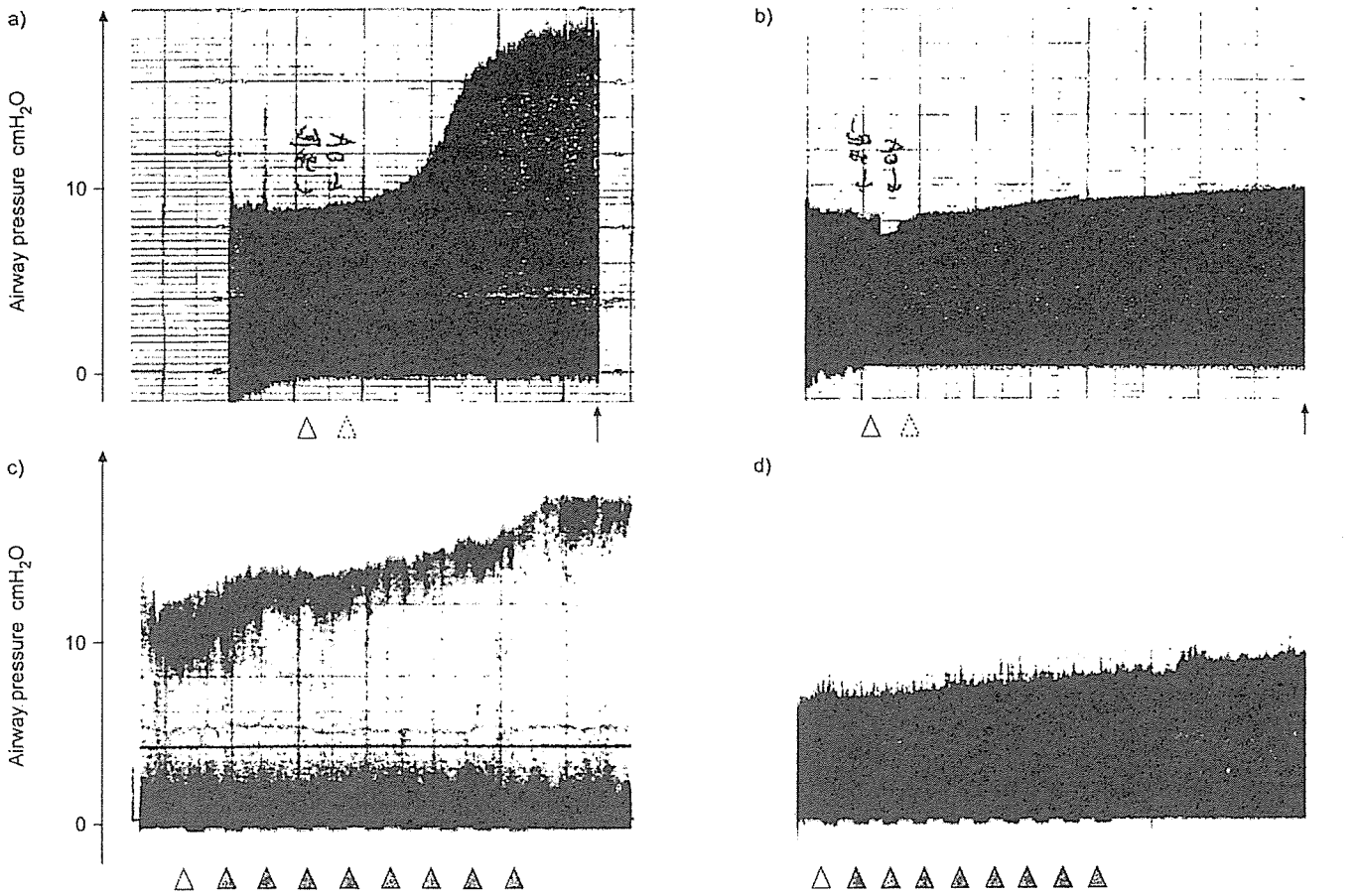


**FIGURE 4.** The effect of anti-macrophage migration inhibitory factor antibody (MIF Ab) on airway contraction. Both ovalbumin (OA)-specific and non-specific airway responsiveness were examined 3 days after OA challenge. After measurement of baseline pressure, an aerosol of 5% OA (indicated by the dotted arrowhead) was administered for 15 min (solid black arrow in a). a) In the OA group, the airway pressure was significantly increased. b) Converesly, the airway pressure was not increased in the OA+anti-MIF Ab group. An aerosol of methacholine (Mch) was administered for 1 min in progressively doubled concentrations from 0.0625 mg·mL$^{-1}$ (grey arrowhead) after measurement of the baseline pressure. c) In the OA group, the airway pressure was significantly increased. d) In contrast, the OA+anti-MIF Ab group did not respond to Mch even at the maximum dose, 16 mg·mL$^{-1}$. Results are representative of three independent experiments. Solid black arrow in b): 30 min; open arrowhead: saline inhalation.

measured. In the study series up to 24 h after OA challenge, the levels of eotaxin in BALF 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 ($8.24\pm1.5$ pg·mL$^{-1}$ in the Naive group, $127.3\pm38.0$ pg·mL$^{-1}$ in the OA group, and $160.0\pm23.3$ pg·mL$^{-1}$ in the OA+anti-MIF Ab group; fig. 7a).

### *Effect of anti-MIF Ab on IL-13 levels in BALF*

The levels of IL-13 in BALF were also measured. The levels 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 ($31.2\pm5.2$ pg·mL$^{-1}$ in the Naive group, $63.0\pm16.9$ pg·mL$^{-1}$ in the OA group, and $72.4\pm8.1$ pg·mL$^{-1}$ in the OA+anti-MIF Ab group; fig. 7b).

## DISCUSSION

This study first demonstrated that OA-sensitised rats had increased levels of MIF in BALF and enhanced expression of MIF in airway epithelium after OA challenge. These results are consistent with the previous observation in a human study in which BALF from patients with asthma contained significantly elevated levels of MIF as compared to normal volunteers [12]. In addition, it has been clearly demonstrated that treatment with anti-MIF Ab 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 Ab 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

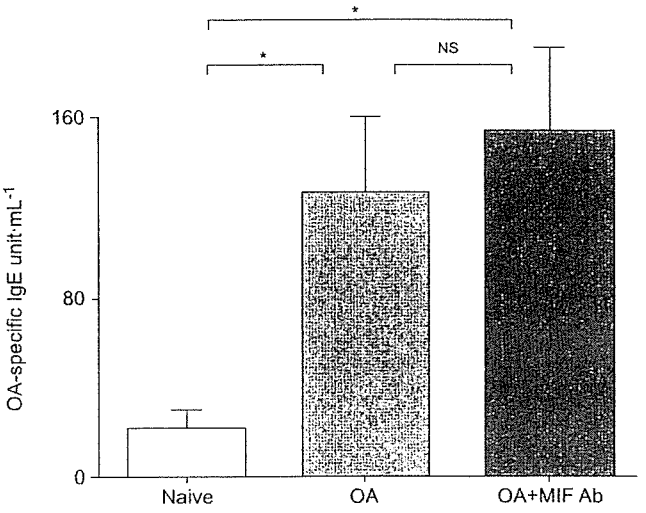


**FIGURE 5.** Effect of anti-macrophage migration inhibitory factor antibody (MIF Ab) on ovalbumin (OA)-specific immunoglobulin (Ig)E levels in serum. Serum was removed from the inferior vena cava 3 days after the OA challenge (n=6). The levels of OA-specific IgE in serum were significantly elevated in the OA group after OA challenge compared with those in the Naive group. There was no significant difference between the OA group and the OA+anti-MIF Ab group. *: $p<0.05$; NS: nonsignificant.

OA once daily for 7 days 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 BALF. 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 BALF was nearly 30% in the other study and $64.9\pm3.7\%$ in the present study. The small number of eosinophils in the other study might have obscured the inhibitory effect of anti-MIF Ab. Secondly, researchers in the other study used anti-MIF serum rather than anti-MIF Ab, 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, the researchers could not demonstrate the effect of the anti-MIF serum either. In contrast, the present authors previously demonstrated that anti-MIF Ab 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 differ among species.

MIF is known to be constitutively expressed in bronchial epithelium [7, 9]. In the present study, the immunohistochemical experiment clearly demonstrated that expression of MIF was enhanced in airway epithelium after OA challenge in OA-sensitised 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.* [12] suggested that eosinophils might be a potential source of MIF in human asthma because even circulating eosinophils from normal volunteers were shown to produce MIF with phorbol myristate acetate stimulation. Indeed, in the present study, the majority of inflammatory cells in BALF were eosinophils. Accordingly, bronchial epithelium as well as eosinophils may jointly contribute to the increased levesl of MIF in BALF in the present rat asthma model.

Because 60–70% of total cells in BALF in OA-sensitised rats were eosinophils, the attenuation of the number of total cells by treatment with anti-MIF Ab is mostly attributed to the attenuation of the number of eosinophils. It has been reported that the eotaxin levels are highly elevated in BALF from patients with asthma [22] and that eotaxin is associated with airway hyperresponsiveness [23]. Eotaxin may therefore play an important role in the pathogenesis of bronchial asthma. As a result, the present authors wondered whether the effect of the anti-MIF Ab on airway inflammation might be at least in part explained by its effect on eotaxin. It was found that the level of eotaxin in BALF was certainly elevated after OA challenge compared with that in naive rats. However, no significant difference was observed in the levels of eotaxin in BALF between the OA group and the OA+anti-MIF Ab group. In animal models, IL-13 has been shown to induce airway hyperresponsiveness and airway eosinophilia [24, 25]. It is also possible that IL-13-dependent airway hyperresponsiveness occurs *via* mechanisms that are independent of airway eosinophilia [26]. In the present study, the levels of IL-13 in BALF were elevated after OA challenge; however, there was no significant difference between the OA group and the OA+anti-MIF Ab group. The authors also measured the expression of IL-5 mRNA and macrophage inflammatory protein (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 the 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*, as well as eosinophil apoptosis to some extent [27]. Thus, the anti-MIF Ab might reduce the number of eosinophils and neutrophils in BALF by enhancing apoptosis of those cells.

The anti-MIF Ab dramatically reduced the number of neutrophils in BALF in the present study. Neutrophils are known to be increased in the airways of patients with status asthmaticus [28], during exacerbations of asthma [29] and 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 Ab in the present model. The authors have previously reported that anti-MIF Ab 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 attenuation of the number of neutrophils in the rat asthma model.

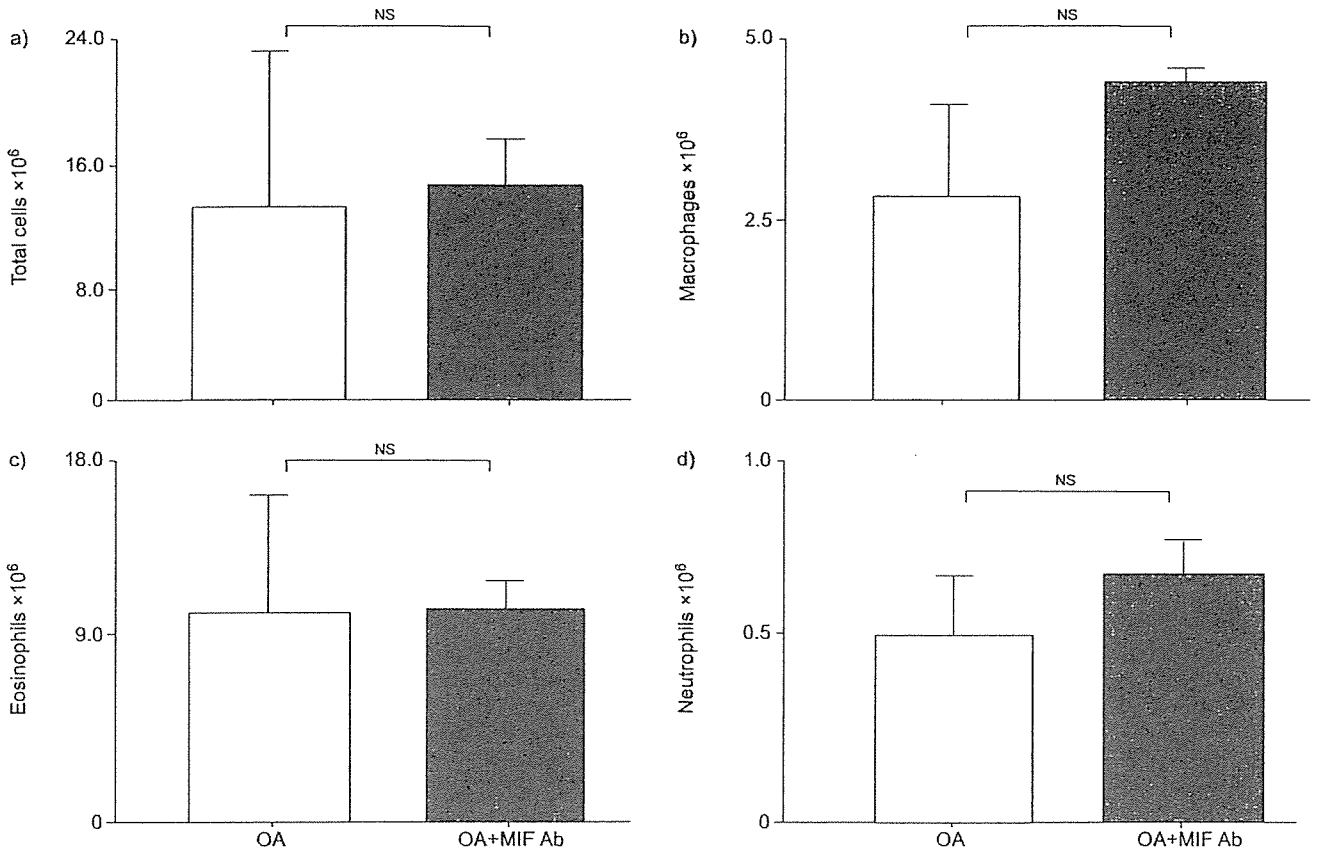


**FIGURE 6.** Effect of single administration of anti-macrophage migration inhibitory factory antibody (MIF Ab) on a) total cells, b) macrophages, c) eosinophils and d) neutrophils in bronchoalveolar lavage fluid (BALF). A 2-mg aliquot of anti-MIF Ab or rabbit IgG was injected once, 2 h before ovalbumin (OA) challenge and bronchoalveolar lavage was performed 3 days after OA challenge. A single administration of anti-MIF Ab did not reduce the number of total and differential cell counts in BAL fluid (n=3).

In the present study, the anti-MIF Ab did not affect antigen-specific IgE in serum, which led to investigation of whether a single dose of anti-MIF Ab could exert its effect before OA inhalation. A single administration of anti-MIF Ab did not reduce the number of total cells and differential cell counts in BALF, suggesting that the serial injection of the anti-MIF Ab from OA sensitisation to 2 days after OA inhalation are necessary for its suppressive effect to be exerted. The total amount of anti-MIF Ab might be important for exertion of its effect. It was therefore concluded that anti-MIF Ab suppressed OA-induced airway inflammation by an independent mechanism of OA-sensitisation.

Glucocorticoids are currently the most effective anti-inflammatory agent in the treatment of asthma [31]. However, it is widely recognised 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]. This consideration leads to the speculation that anti-MIF therapy may not only have direct anti-inflammatory effects, but also act by recovering the function of endogenous and/or exogenous glucocorticoids.

Finally, some comments should be made on the weakness of the experimental protocol in this study. First, quantitative assessment of airway hyperresponsiveness was not performed, particularly for naive rats and OA+anti-MIF Ab rats; this meant it was unclear how much anti-MIF Ab attenuated airway hyperresponsiveness in the OA-immunised lungs. Such assessment was not performed because the authors' specific interest lay in assuring that enhanced airway hyperresponsiveness by OA immunisation and inhalation was actually attenuated by anti-MIF Ab. Secondly, airway pressure was used to assess airway hyperresponsiveness, which is influenced by changes in both airway resistance and lung compliance. As the increased airway pressure was confirmed to return to baseline in a short time, the change of compliance, which is likely to be caused by lung parenchymal injury, could be negligible in the present study (data not shown).

In summary, the present manuscript has demonstrated that macrophage migration inhibitory factor is involved in the asthmatic response in the ovalbumin-sensitized rat asthma model. It has also been shown that bronchial epithelium is a potent source of macrophage migration inhibitory factor in this asthma model. The anti-macrophage migration inhibitory factor antibody also significantly attenuated ovalbumin-induced airway inflammation and airway hyperresponsiveness. Although these data support the concepts that macrophage migration inhibitory factor plays an important

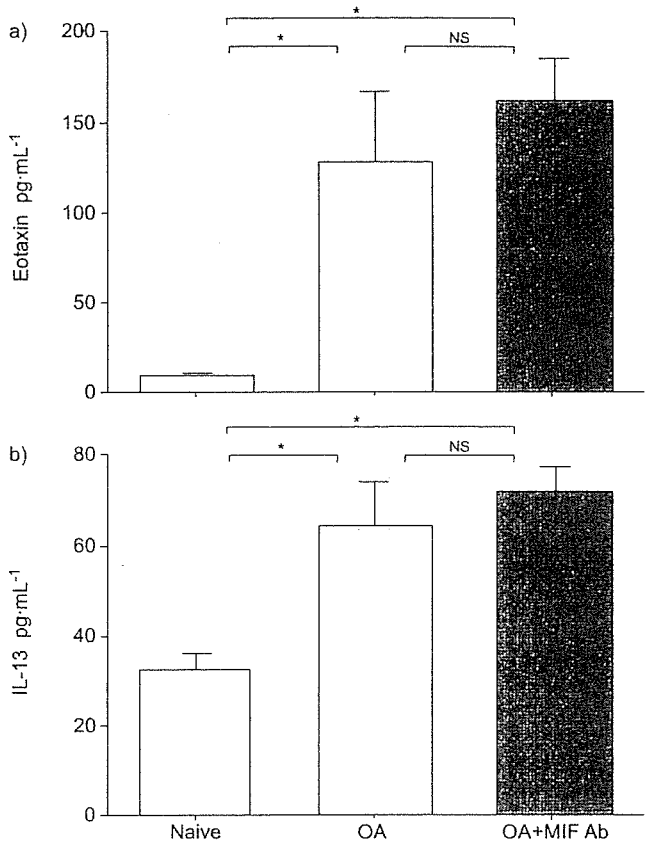


**FIGURE 7.** Effects of anti- macrophage migration inhibitory factory antibody (MIF Ab) on a) eotaxin and b) interleukin (IL)-13 levels in bronchoalveolar lavage fluid (BALF). The levels of eotaxin and IL-13 in BALF were significantly elevated in the ovalbumin (OA) group compared with the Naive group 8 h after the OA challenge. However, there were no significant differences in both chemokines between the OA group and the OA+anti-MIF Ab group (n=3 for each).

role in asthma and anti-macrophage migration inhibitory factor antibody may have a therapeutic potential for asthma, further investigations are necessary to fully understand the mechanism of the effect of anti-macrophage migration inhibitory factor antibody on asthma pathology and to examine the therapeutic potential of the anti-macrophage migration inhibitory factor antibody in human asthma.

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# Polymorphisms in the Muscarinic Receptor 1 Gene Confer Susceptibility to Asthma in Japanese Subjects

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*Rationale:* The human cholinergic receptor muscarinic-1 (CHRM1) is widely distributed in the lungs. In patients with asthma, CHRM1 may be involved in airway constriction, airway epithelial cell proliferation, and airway inflammation. The *CHRM1* gene is located on chromosome 11q13, which is one of the candidate loci for asthma and atopy.

*Objectives:* To determine the role of the *CHRM1* gene polymorphisms in asthma.

*Methods:* We studied nine single-nucleotide polymorphisms (−18379G > A, −9697C > T, −6965T > C, −4953A > G, +267A > C, +1353C > T, +3970C > G, +5418C > G, and +5455G > T) in a case-control study using 326 patients with asthma and 333 healthy control subjects. We also examined functional consequences of the −9697C > T and −4953A > G polymorphisms at the regulatory region using an mRNA reporter assay.

*Measurements and Main Results:* Two common single-nucleotide polymorphisms (−9697C > T and −4953A > G) were associated with asthma. The odds ratio for the TT homozygotes at the −9697C > T polymorphism was 0.29 compared with the CC homozygotes (95% confidence interval, 0.12–0.73; p = 0.008), and the odds ratio for the GG homozygotes at the −4953A > G polymorphism was 1.86 compared with the AA homozygotes (95% confidence interval, 1.04–3.34; p = 0.038). Haplotype analysis showed that the −9697T/−6965T/−4953A haplotype was associated with a lower risk of asthma (p = 0.00055) and the −9697C/−6965T/−4953G haplotype was associated with an increased risk of asthma (p = 0.020). The −9697T/−4953A haplotype was also associated with lower luciferase activity *in vitro* compared with the −9697C/−4953G haplotype.

*Conclusions:* This study, together with an *in vitro* functional study, suggests that the *CHRM1* gene is an important susceptibility locus for asthma on chromosome11q13.

Keywords: case-control studies; IgE; muscarinic cholinergic receptor-1; single-nucleotide polymorphism

The cholinergic nerves are the dominant neural bronchoconstrictor pathway in humans (1). They release acetylcholine onto muscarinic receptors causing cholinergic bronchoconstriction (2), mucous hypersecretion, and edema in the airways. Increases in cholinergic nerve activity and cholinergic hypersensitivity are associated with asthma, and patients with asthma are hypersensitive to the bronchoconstricting actions of muscarinic agonists (3). The human cholinergic receptor muscarinic 1 (CHRM1; Online Mendelian Inheritance of Man database no. 118510) is widely localized in the human lung, including the alveolar walls, bronchial epithelial cells, parasympathetic ganglia, neuromuscular junction, and submucosal glands (4). Studies using pirenzepine, a muscarinic antagonist selective for M1 receptors, have shown that M1 muscarinic receptors are involved in vagally induced bronchoconstriction (5–7). M1 receptor–deficient mice showed increased bronchoconstriction in response to $10^{-8}$ M muscarine in peripheral airways (8), suggesting the existence of an M1 receptor–dependent pathway counteracting cholinergic bronchoconstriction. M1 receptors also play a role in mast cell function (9), epithelial cell proliferation in the trachea (10), release of neutrophil and monocyte chemotactic activity from epithelial cells (11), acetylcholine-induced relaxation of the human pulmonary veins (12), and regulation of water and electrolyte secretion on submucosal glands (13). Taken together, CHRM1 is critically involved in the pathophysiology of asthma.

The gene encoding CHRM1 exists on chromosome 11q13, which has been linked to asthma and asthma-related phenotypes in several genomewide searches (14–17). Given the important role of muscarinic cholinergic mechanisms in asthma, the *CHRM1* gene is biologically an excellent candidate for asthma susceptibility in the region of chromosome 11q13. Thus, in the current study, we examined whether genetic variations in the *CHRM1* gene are associated with asthma. To gain insight into the possible molecular basis of the disease association, we also examined functional consequences of single-nucleotide polymorphisms (SNPs) at the regulatory region of the *CHRM1* gene.

### AT A GLANCE COMMENTARY

#### Scientific Knowledge on the Subject

Genetic studies repeatedly have linked asthma and asthma-related phenotypes to chromosome 11q13, on which several biological candidate genes are located.

#### What This Study Adds to the Field

Gene coding the human cholinergic receptor muscarinic-1 (CHRM1) is an important susceptibility locus for asthma at chromosome 11q13.

## METHODS

*See* online supplement for additional details.

### Study Subjects

A total of 659 unrelated Japanese adults were enrolled in the study (Table 1). Asthma was defined on the basis of recurrent episodes of at least two of three symptoms (cough, wheeze, and dyspnea) that are associated with demonstrable reversible airflow limitation (15% variability in $FEV_1$ or in peak expiratory flow rate either spontaneously or with an inhaled, short-acting $\beta_2$-agonist), or increased airway responsiveness to methacholine, or both, as described elsewhere (18).

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TABLE 1. BASIC CHARACTERISTICS OF 659 JAPANESE SUBJECTS

| | Healthy Control Subjects ($n$ = 333) | Subjects with Asthma ($n$ = 326) | p Value* |
|---|---|---|---|
| Age, yr, median (range) | 41 (18–72) | 45 (16–79) | < 0.0001 |
| Sex, n (male/female) | 208/125 | 148/178 | < 0.0001 |
| Smoking, n (never/ex-/current) | 226/12/95 | 190/69/67 | < 0.0001 |
| Atopy, n (%) | 170 (51) | 237 (73) | < 0.0001 |
| Total serum IgE, log IU/ml, mean (SD) | 1.84 (0.627) | 2.40 (0.622) | < 0.0001 |
| $FEV_1$, % predicted, mean (SD) | — | 69.2 (13.5) | |
| % Reversibility in $FEV_1$, median (range) | — | 16.9 (0–211) | |

* One-way analysis of variance or $\chi^2$ test was used where appropriate.

### Identification of Polymorphisms

Genomic DNA from Japanese subjects was genotyped for +267A > C (rs2067477) and +1353C > T (rs2067480), because an association of these polymorphisms at the muscarinic M1 receptor gene with cognitive function in schizophrenic patients has been reported (19). We selected an additional seven SNPs for genotyping based on the frequency and location of SNPs and the linkage disequilibrium (LD) structure in and around the *CHRM1* gene. We initially obtained genotyping data of 26 HapMap SNPs (spanning 31.6 kb around the gene) from 45 unrelated Japanese subjects at the International HapMap Project (available online at http://hapmap.org/). To select tagSNPs in this region, we used the multimarker predictor method implemented in the Tagger program (20). Tag set was generated (using a threshold $r^2$ of 0.8) using 14 common SNPs with a minor allele frequency of more than 0.05 in the Japanese population.

As the +267A > C and +1353C > T polymorphisms were in a complete LD, we genotyped a total of eight SNPs (−18379G > A [rs1938677], −9697C > T [rs2075748], −6965T > C [rs542269], −4953A > G [rs1942499], +1353C > T [rs2067480], 3970C > G [rs4963323], 5418C > G [rs11601597], and 5455G > T [rs11605665]) for all individuals (n = 659). These SNPs were typed using the assay that combines kinetic (real-time quantitative) polymerase chain reaction (PCR) with allele-specific amplification, as described elsewhere (18). The PCR products were detected using the ABI 7700 Sequence Detection System with the dsDNA-specific fluorescent dye SYBR Green I (Applied Biosystems, Foster City, CA). The −4953A > G polymorphism was typed using TaqMan assay (Applied Biosystems).

### Statistical Analysis

The association of the *CHRM1* gene polymorphism was measured by odds ratio (OR) with 95% confidence intervals (CI) as estimates of relative risk for the development of asthma. We used the Hardy-Weinberg equilibrium (HWE) program (21) to compare observed numbers of genotypes with the numbers of genotypes expected under HWE. For haplotype analyses, we used the Haplo.score program, which adjusts for covariates and calculates simulation p values for each haplotype (22).

### Luciferase Reporter Gene Assay

We constructed two promoter reporter plasmids by placing two haplotypes (−9697C/−4953G and −9697T/−4953A) into the pGL3-Basic vector. Human neuroblastoma IMR32 cells ($1 \times 10^6$) were transiently transfected with 9.5 μg of one of the two constructs and 0.5 μg of the pRL-TK vector, an internal control for transfection efficiency. After 24 h, we measured luciferase activity using the Dual-Luciferase Reporter Assay System (Promega, Tokyo, Japan). Results were expressed as means ± SEM and were compared by paired $t$ test.

### Electrophoretic Mobility Shift Assay

Transcription factor (nuclear factor [NF]-κB or upstream stimulating factor [USF]-1)-DNA binding activity was analyzed using the electrophoretic mobility shift assay (EMSA) kit (Panomics, Redwood, CA), according to the manufacturer's instructions.

## RESULTS

Characteristics of the 333 healthy control subjects and 326 subjects with asthma are shown in Table 1. The median age of subjects with asthma was significantly higher than in healthy control subjects ($p < 0.0001$). There were significantly more females in the asthma group than in the control group ($p < 0.0001$). Subjects with asthma were more likely to be atopic and had higher levels of total serum IgE than did healthy control subjects ($\chi^2$ test or analysis of variance, $p < 0.0001$). More than 50% of the control subjects were atopic, which is consistent with

TABLE 2. COMPARISONS OF ALLELE AND GENOTYPE FREQUENCIES OF EIGHT *CHRM1* SINGLE-NUCLEOTIDE POLYMORPHISMS BETWEEN PATIENTS WITH ASTHMA AND CONTROL SUBJECTS

| SNP | Allele/Genotype | HC $n$ (%) | BA $n$ (%) | p Value* |
|---|---|---|---|---|
| −18379 (rs1938677) | G | 361 (54.5) | 335 (48.1) | 0.33 |
| | A | 301 (45.5) | 311 (51.9) | |
| | GG | 107 (32.3) | 92 (28.5) | 0.56 |
| | GA | 146 (44.1) | 151 (46.7) | |
| | AA | 78 (23.6) | 80 (24.8) | |
| −9697 (rs2075748) | C | 508 (76.3) | 533 (81.7) | 0.015 |
| | T | 158 (23.7) | 119 (18.3) | |
| | CC | 195 (58.6) | 216 (66.3) | 0.039 |
| | CT | 118 (35.4) | 101 (31.0) | |
| | TT | 20 (6.0) | 9 (2.7) | |
| −6965 (rs542269) | T | 491 (73.9) | 472 (73.1) | 0.72 |
| | C | 173 (26.1) | 174 (26.9) | |
| | TT | 184 (55.4) | 175 (54.2) | 0.94 |
| | TC | 123 (37.1) | 122 (37.8) | |
| | CC | 25 (7.5) | 26 (8.0) | |
| −4953 (rs1942499) | A | 477 (71.6) | 434 (66.6) | 0.047 |
| | G | 189 (28.4) | 218 (33.4) | |
| | AA | 174 (52.3) | 147 (45.1) | 0.15 |
| | AG | 129 (38.7) | 140 (42.9) | |
| | GG | 30 (9.0) | 39 (12.0) | |
| +1353 (rs2067480) | C | 615 (92.3) | 608 (93.3) | 0.52 |
| | T | 51 (7.7) | 44 (6.7) | |
| | CC | 284 (85.3) | 287 (88.0) | 0.19 |
| | CT | 47 (14.1) | 34 (10.5) | |
| | TT | 2 (0.6) | 5 (1.5) | |
| +3970 (rs4963323) | C | 533 (80.3) | 524 (80.9) | 0.78 |
| | G | 131 (19.7) | 124 (19.1) | |
| | CC | 216 (65.1) | 211 (65.1) | 0.75 |
| | CG | 101 (30.4) | 102 (31.5) | |
| | GG | 15 (4.5) | 11 (3.4) | |
| +5418 (rs11601597) | C | 414 (62.3) | 385 (59.6) | 0.31 |
| | G | 250 (37.7) | 261 (40.4) | |
| | CC | 132 (39.7) | 113 (35.0) | 0.44 |
| | CG | 150 (45.2) | 159 (49.2) | |
| | GG | 50 (15.1) | 51 (15.8) | |
| +5455 (rs11605665) | G | 538 (81.5) | 496 (77.5) | 0.074 |
| | T | 122 (18.5) | 144 (22.5) | |
| | GG | 218 (66.1) | 192 (60.0) | 0.19 |
| | GT | 102 (30.9) | 112 (35.0) | |
| | TT | 10 (3.0) | 16 (5.0) | |

*Definition of abbreviations*: BA = bronchial asthma; HC = healthy controls.
* $\chi^2$ Test.

recent findings that the prevalence of atopy (as indicated by specific IgE against common inhaled allergens) among Japanese subjects is increasing (23, 24). Prebronchodilator baseline $FEV_1$ at an initial visit to our hospital was examined for 293 subjects with asthma (89.9%), and improvement of $FEV_1$ after bronchodilator therapy (400 μg salbutamol) or after a course of standard asthma medications (inhaled corticosteroids, long-acting $\beta_2$-agonists, theophylline, or leukotriene modulators) was recorded for 214 (65.6%) subjects with asthma (Table 1).

All eight of the SNPs investigated were within the HWE in the control group ($p > 0.05$). The overall success rate for genotyping was 99.6%. Of the eight SNPs, two common SNPs (−9697C > T [rs2075748] and −4953A > G [rs1942499]) in the regulatory region of the *CHRM1* gene had a significant association with asthma (Table 2). Both of these SNPs were significantly associated with asthma when the analysis was adjusted for age, sex, smoking status, and atopic status (Table 3). The OR for the TT homozygotes of the −9697C > T polymorphism was 0.29 compared with the CC homozygotes (95% CI, 0.12–0.73; $p = 0.008$), and the OR for the GG homozygotes of the −4953A > G polymorphism was 1.86 compared with the AA homozygotes (95% CI, 1.04–3.34; $p = 0.038$).

We analyzed data from the eight SNPs with the Haploview program (25) and identified two haplotype blocks (Figure 1) in our case-control population. Haplotype block I comprised three SNPs in the regulatory region (−9697C > T [rs2075748], −6965T > C [rs542269], −4953A > G [rs1942499]), and haplotype block II comprised three SNPs (+1353C > T [rs2067480], +3970C > G [rs4963323], +5418C > G [rs11601597]) in the coding exon and the 3′-UTR. Haplotype analyses were performed in both blocks I and II. The frequency of *CHRM1* haplotypes is shown in Table 4. In block I, the −9697T/−6965T/−4953A haplotype was associated with a significantly lower risk of asthma ($p = 0.00055$) and the −9697C/−6965T/−4953G haplotype was associated with a significantly increased risk of asthma ($p = 0.020$). Inspection of specific haplotypes revealed that this association is most likely due to 9697C > T and 4953A > G, because the same allele for −6965T > C is part of both risk and protective haplotypes. In contrast, none of the haplotypes in block II was associated with asthma.

In the case-only study, associations between asthma-related phenotypes, such as total serum IgE levels and atopy, and the polymorphisms of *CHRM1* were also investigated. We could not find any significant association between the genotypes of the eight SNPs and total serum IgE levels or atopy (*see* Tables E1 and E2 in the online supplement).

The transcriptional activity of the *CHRM1* SNPs at the regulatory region was compared between the −9697C/−4953G haplotype and the −9697T/−4953A haplotype transiently transfected into human neuroblastoma IMR32 cells. Luciferase activity in cell extracts was assessed 24 h after transfection, and was expressed as fold increase in the activity of the *CHRM1* reporter constructs compared with the pRL-TK vector. Figure 2 shows that the reporter plasmid carrying the −9697T/−4953A promoter displayed 37% lower transcriptional activity compared with the plasmid carrying the −9697C/−4953G promoter ($p = 0.019$).

**TABLE 3. GENETIC IMPACT ON ASTHMA OF EIGHT SINGLE-NUCLEOTIDE POLYMORPHISMS IN AND AROUND THE CHRM1 *GENE***

| | | OR (*95% CI*) | |
|---|---|---|---|
| SNP | Genotype | Adjustments (−) | Adjustments (+)* |
| 18379G > A (rs1938677) | GG | 1 (Reference) | 1 (Reference) |
| | GA | 1.20 (0.84–1.72) | 1.14 (0.77–1.71) |
| | AA | 1.19 (0.79–1.81) | 1.33 (0.84–2.12) |
| −9697C > T (rs2075748) | CC | 1 (Reference) | 1 (Reference) |
| | CT | 0.77 (0.56–1.07) | 0.73 (0.51–1.06) |
| | TT | 0.41 (0.18–0.91)† | 0.29 (0.12–0.73)‡ |
| 6965T > C (rs542269) | TT | 1 (Reference) | 1 (Reference) |
| | TC | 1.04 (0.75–1.44) | 1.00 (0.70–1.44) |
| | CC | 1.09 (0.61–1.97) | 1.04 (0.54–2.02) |
| −4953A > G (rs1942499) | AA | 1 (Reference) | 1 (Reference) |
| | AG | 1.28 (0.93–1.78) | 1.38 (0.96–1.98) |
| | GG | 1.54 (0.91–2.60) | 1.86 (1.04–3.34)† |
| +1353C > T (rs2067480) | CC | 1 (Reference) | 1 (Reference) |
| | CT | 0.72 (0.45–1.15) | 0.61 (0.37–1.02) |
| | TT | 2.47 (0.48–12.9) | 1.96 (0.31–12.5) |
| +3970C > G (rs4963323) | CC | 1 (Reference) | 1 (Reference) |
| | CG | 1.03 (0.74–1.44) | 1.12 (0.78–1.62) |
| | GG | 0.75 (0.34–1.67) | 0.78 (0.33–1.86) |
| +5418C > G (rs11601597) | CC | 1 (Reference) | 1 (Reference) |
| | CG | 1.24 (0.88–1.73) | 1.29 (0.89–1.88) |
| | GG | 1.19 (0.75–1.89) | 1.16 (0.70–1.93) |
| +5455G > T (rs11605665) | GG | 1 (Reference) | 1 (Reference) |
| | GT | 1.25 (0.90–1.74) | 1.28 (0.89–1.85) |
| | TT | 1.82 (0.81–4.1) | 1.73 (0.73–4.09) |

*Definition of abbreviations*: CI = confidence interval; OR = odds ratio.

* Adjustment for matching factors and potential confounding factors, including sex, age, smoking status, and atopic status, was performed by unconditional logistic regression analysis.

† $p < 0.05$.

‡ $p < 0.01$.