BJP British Journal of Pharmacology

DOI:10.1111/bph.12154
www.brjpharmacol.org

RESEARCH PAPER

Roles of basophils and mast cells infiltrating the lung by multiple antigen challenges in asthmatic responses of mice

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Keywords
basophils; mast cells; allergic asthma; FcεRI; flow cytometer; early asthmatic response; late asthmatic response; IL-4; eosinophils; neutrophils

Received
11 May 2012
Revised
6 January 2013
Accepted
16 February 2013

BACKGROUND AND PURPOSE
Mast cell hyperplasia has been observed in the lungs of mice with experimental asthma, but few reports have studied basophils. Here, we attempted to discriminate and quantify mast cells and basophils in the lungs in a murine asthma model, determine if both cells were increased by multiple antigen challenges and assess the roles of those cells in asthmatic responses.

EXPERIMENTAL APPROACH
Sensitized Balb/c mice were intratracheally challenged with ovalbumin four times. Mast cells and basophils in enzymatically digested lung tissue were detected by flow cytometry. An anti-FcεRI monoclonal antibody, MAR-1, was i.p. administered during the multiple challenges.

KEY RESULTS
The numbers of both mast cells (IgE^+ C-kit^+) and basophils (IgE^+ C-kit^- $CD49b^+$) increased in the lungs after three challenges. Treatment with MAR-1 completely abolished the increases; however, a late-phase increase in specific airway resistance (sRaw), and airway eosinophilia and neutrophilia were not affected by the treatment, although the early-phase increase in sRaw was suppressed. MAR-1 reduced antigen-induced airway IL-4 production. Basophils infiltrating the lung clearly produced IL-4 after antigen stimulation *in vitro*; however, histamine and murine mast cell protease 1 were not increased in the serum after the challenge, indicating that mast cell activation was not evoked.

CONCLUSION AND IMPLICATIONS
Both mast cells and basophils infiltrated the lungs by multiple intratracheal antigen challenges in sensitized mice. Neither mast cells nor basophils were involved in late-phase airway obstruction, although early-phase obstruction was mediated by basophils. Targeting basophils in asthma therapy may be useful for an early asthmatic response.

Abbreviations
BAL, bronchoalveolar lavage; EIA, enzyme immunoassay; i.t., intratracheal; mAb, monoclonal antibody; MHC, major histocompatibility complex; mMCP, murine mast cell protease; OVA, ovalbumin; PE, phycoerythrin; sRaw, specific airway resistance

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Introduction

Basophils and mast cells bind IgE antibodies via their cell surface expression of FcεRI. Cross-linking of these receptor-bound IgE molecules by multivalent antigen results in immediate activation of these cells to release chemical mediators including histamine, proteases and arachidonic acid metabolites that cause tissue injury (Oettgen and Geha, 1999; Galli, 2000; Galli *et al.*, 2005). Not only mast cells, but also basophils, are capable of producing cytokines and chemokines that can orchestrate chronic allergic responses associated with Th2 type inflammation (Falcone *et al.*, 2000; Schroeder *et al.*, 2001; Voehringer *et al.*, 2004). In addition, it has been recently found in murine studies that basophils expressed major histocompatibility complex (MHC) class II molecules, and that they possessed the machinery to take up, process and present antigens to T cells on MHC class II molecules (Perrigoue *et al.*, 2009; Sokol *et al.*, 2009; Yoshimoto *et al.*, 2009).

In asthmatic patients, there are increased numbers of not only mast cells (Heard *et al.*, 1989; Pesci *et al.*, 1993; Macfarlane *et al.*, 2000; Kassel *et al.*, 2001; Brightling *et al.*, 2002), but also basophils (Koshino *et al.*, 1993; Macfarlane *et al.*, 2000), in the lung tissue. Detection of human mast cells and basophils in isolated lung sections can be achieved by semi-quantitative histological staining (Heard *et al.*, 1989; Koshino *et al.*, 1993; Pesci *et al.*, 1993; Macfarlane *et al.*, 2000; Kassel *et al.*, 2001; Brightling *et al.*, 2002). Although there is considerable evidence concerning the roles of mast cells in the pathogenesis of asthma, little is known about the role of basophils in this disease. It has been reported that chronic airway antigen challenge resulted in the development of mast cell hyperplasia in the lungs of sensitized mice (Ikeda *et al.*, 2003; Yu *et al.*, 2006); however, the potential roles of basophils in murine asthma remain incompletely defined. As a technique to assess the roles of basophils in allergy and helminthic infection, basophil-depleting antibodies have been utilized (Obata *et al.*, 2007; Denzel *et al.*, 2008; Sokol *et al.*, 2008). An anti-FcεRI monoclonal antibody (mAb), MAR-1, is capable of depleting basophils systemically *in vivo* and has contributed to recent researches on basophils (Denzel *et al.*, 2008; Sokol *et al.*, 2008). However, there is a problem in specificity: the anti-FcεRI mAb may affect not only basophils but also mast cells (Min, 2010; Wada *et al.*, 2010; Falcone *et al.*, 2011).

On the other hand, we have developed, using multiple intratracheal (i.t.) antigen challenges, a model of asthma in Balb/c mice that shows early- and late-phase asthmatic responses (Nabe *et al.*, 2005; 2011a,b). Sensitized mice showed only a transient increase in specific airway resistance (sRaw) at 10 min (early asthmatic response) after the first i.t. antigen challenge, whereas both early- and late-phase increases in sRaw (late asthmatic response) were induced, with peaks at 10 min and 2–4 h, respectively, after the fourth i.t. challenge (Nabe *et al.*, 2005; 2011a,b). We demonstrated that eosinophils and $CD4^+$ cells had markedly accumulated in the lungs immediately prior to the fourth challenge (Nabe *et al.*, 2005; 2011a,b). In addition, both IgE^+ C-kit$^+$ cells (most likely mast cells) and IgE^+ C-kit$^-$ cells (at the time, not fully identified) had also increased in the lungs prior to the fourth challenge (Nabe *et al.*, 2005). Altogether, our data suggested that these cells, which are associated with Th2 type inflammation, could contribute to the induction of late-phase airway obstruction after the fourth airway challenge.

In the present study, we attempted to discriminate and quantify mast cells and basophils in the lung tissue using flow cytometry and determine if not only mast cells, but also basophils, are increased in the lungs following multiple antigen challenges. Secondly, using anti-FcεRI mAb, we determined if the accumulated mast cells and basophils are associated with the induction of fourth challenge-induced early and late asthmatic airway obstruction, and airway eosinophilia and neutrophilia.

Methods

Sensitization and challenge

Female Balb/c mice (5–12 weeks) were maintained in a temperature- and humidity-controlled environment and 12-h light/dark cycle with free access to water and rodent chow *ad libitum*. All experimental protocols were approved by the Experimental Animal Research Committee at Kyoto Pharmaceutical University. Every effort was made to minimize animal suffering and to reduce the number of animals used. All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010).

Similar to the method used in our previous report (Nabe *et al.*, 2005), we sensitized 6-week-old Balb/c mice (Japan SLC, Hamamatsu, Japan) by i.p. injection with ovalbumin (OVA; 50 μg, Grade V; Sigma-Aldrich, St. Louis, MO, USA) adsorbed to alum (2 mg in 0.5 mL PBS) on days 0, 14 and 28. The alum was prepared as previously described (Nabe *et al.*, 1997). Sensitized mice were challenged on days 35, 36, 37 and 40 under inhalation anaesthesia using isoflurane (Dainippon Pharmaceutical Co., Osaka, Japan) with 2% OVA administered i.t. in a volume of 25 μL as reported (Ho and Furst, 1973).

Detection of basophils and mast cells by flow cytometry

Mice were killed before and 4 h after the first and fourth antigen challenges by exsanguination via the abdominal aorta under anaesthesia with ketamine (62.5 $mg \cdot kg^{-1}$, i.m.)/xylazine (25 $mg \cdot kg^{-1}$, i.m.). The pulmonary circulation was perfused using PBS, and then the left lobe of the lung was isolated. The isolated lung was cut into 1 mm^3 pieces in digestion buffer (RPMI-1640 containing 150 $U \cdot mL^{-1}$ collagenase [Iwaki Glass, Tokyo, Japan), 30 $\mu g \cdot mL^{-1}$ deoxyribonuclease I (Calbiochem, La Jolla, CA, USA) and 10 mM HEPES] followed by incubation at 37°C for 1 h with continuous agitation in an incubator. At 30 and 60 min during incubation, the suspension was further disaggregated by trituration through an 18 gauge needle. After incubation, the cells were passed through 35 μm nylon mesh. The resulting single-cell suspension was washed by centrifugation with PBS supplemented with 2% FBS, and cell numbers were determined using a haematocytometer after treatment with ACK lysis buffer to remove erythrocytes.

Flow cytometric detection of mast cells and basophils was performed according to a modification of the method of

Luccioli *et al*. (2002) and Min *et al*. (2004). Isolated cells were first incubated with an anti-mouse FcγRII/III mAb (clone 2.4G2; BD Biosciences, San Diego, CA, USA) for 15 min at 4°C to block the binding of subsequent antibodies to FcγRII/III. After washing once with PBS supplemented with 2% FBS, the cells were incubated at 4°C with FITC-labelled anti-mouse IgE mAb (clone R35-72; BD Biosciences) at 5 μg·mL^{-1}, Cy-chrome-labelled anti-mouse CD117 (C-kit) mAb (clone 2B8; eBioscience, San Diego, CA, USA) at 2 μg·mL^{-1}, and phycoerythrin (PE)-labelled anti-mouse CD49b mAb (Clone DX5; BD Biosciences) at 2 μg·mL^{-1} for 25 min. After washing once with PBS supplemented with 2% FBS, the cells were fixed with 4% paraformaldehyde. Each sample was analysed using a FACSCalibur (Becton Dickinson, San Jose, CA, USA) and CellQuest Pro software (version 6.0; Becton Dickinson).

For analysis of the morphology of IgE$^+$ C-kit$^+$ cells and IgE$^+$ C-kit$^-$ CD49b$^+$ cells, the cells were sorted using FACSCalibur, and were then centrifuged onto a glass slide (Settling chamber; Neuro Probe, Cabin John, MD, USA) at 50× *g* for 30 s at 4°C followed by staining with Wright-Giemsa (Diff-Quik solution; Sysmex International Reagent, Kobe, Japan). Cell morphology was observed using light microscopy.

For measurement of histamine contents in IgE$^+$ C-kit$^+$ cells and IgE$^+$ C-kit$^-$ CD49b$^+$ cells, two fractions of cells were collected using a FACSAria II Cell Sorter (Becton Dickinson). The collected cells were disrupted by sonication for 1 min in the presence of 3% perchloric acid and 0.2% Triton X-100. Following centrifugation, histamine concentration in neutralized supernatant was measured by an enzyme immunoassay (EIA) kit (Oxford Biomedical Res., Oxford, MI, USA).

Immunohistochemical staining of the lung tissue

Mice were killed before the first and fourth antigen challenges as described above. The lungs were then isolated and fixed by immersion in 10% neutral buffered formaldehyde for 18–24 h. Then, tissues were embedded in paraffin, followed by the preparation of 5 μm sections. After deparaffinization, antigenicity of sections was activated by Retrievagen A (pH 6.0; BD Biosciences) at 90°C for 10 min. After blocking with 10% normal rabbit serum, sections were stained with anti-mouse IgE polyclonal Ab (5 μg·mL^{-1}; Bethyl Lab., Montgomery, TX, USA) at 37°C for 1 h. Then, after washing with PBS, the sections were stained with either anti-mouse SCFR/C-kit mAb (2.5 μg·mL^{-1}, clone 18067; R&D Systems, Minneapolis, MN, USA) or anti-rat CD49b (10 μg·mL^{-1}, clone Ha1/29; BD Biosciences) at 4°C for 12–18 h. After washing, sections were stained with Alexa Fluor 488-labelled anti-goat IgG Ab (7.5 μg·mL^{-1}; Jackson ImmunoResearch Lab., West Grove, PA, USA) at room temperature for 1 h. Then, after washing, sections were stained with either Alexa Fluor 594-labelled anti-rat IgG+IgM Ab (1.9 μg·mL^{-1}; Jackson ImmunoResearch Lab) or Alexa Fluor 594-labelled anti-Armenian hamster IgG (7 μg·mL^{-1}; Jackson ImmunoResearch Lab.). After washing, nuclei were then stained with 4′,6-diamino-2-phenylindole (DAPI; Dojindo, Kumamoto, Japan), and the sections were coverslipped in Prolong Gold anti-fade reagent (Invitrogen, Carlsbad, CA, USA). Photomicrographs were acquired using a fluorescence microscope (IX71; Olympus, Tokyo, Japan).

Treatment with anti-FcεRI mAb

In experiment 1, as shown in Figure 1A, sensitized mice were treated with anti-FcεRIα mAb, MAR-1 (eBioscience), hamster IgG (eBioscience) or PBS from 1 day before the first challenge twice a day for 4 days at 10 μg·per animal (i.p.). During the first to third antigen challenges, the treatment was conducted 1 h before and 11 h after the respective challenges.

In experiment 2 (Figure 1B), anti-FcεRI mAb, MAR-1, hamster IgG or PBS was i.p. administered during the third to fourth challenges twice a day for 3 days at 10 μg per animal.

Measurement of pulmonary function

sRaw [$cmH_2O \times mL\cdot(mL\cdot s^{-1})^{-1}$] was measured as an indicator of airway resistance before and after the fourth OVA challenge using a double-flow plethysmograph (Pulmos-I. II. III; M.I.P.S., Osaka, Japan), according to a previously reported method (Pennock *et al*., 1979; Flandre *et al*., 2003). Double-flow plethysmography is a non-invasive technology that calculates sRaw by analysing breathing patterns at respective nasal and thoracic air flows. Having the ability to make repeated measurements was essential for analysis of the late asthmatic response. Although other invasive methods can measure actual airway resistance and/or conductance, it is impossible to measure them longitudinally over a prolonged time course. Reliability and reproducibility of measurements made using non-invasive double-flow plethysmography were increased by assuring that all measurements were made in an air-conditioned environment controlled for temperature (22–23°C) and humidity (50–60%).

Analysis of cells recovered by bronchoalveolar lavage (BAL)

Mice were killed 4 h after the fourth airway antigen challenge by lethal i.m. injection using a mixture of ketamine and xylazine. After perfusion of the pulmonary circulation with PBS, the lung was lavaged via a tracheal catheter using two aliquots of 0.8 mL PBS containing 2% FBS. Total leukocyte numbers were counted using a particle count and size analyser (Z2; Beckman Coulter, Brea, CA, USA) after treatment with ACK lysis buffer. To determine differential cell counts, cells were centrifuged onto a glass slide using a cell settling chamber followed by staining with Diff-Quik solution.

Measurement of IL-4, histamine and murine mast cell protease (mMCP)-1 in BAL fluids and/or serum

As an indicator of the activation of basophils, IL-4 in BAL fluids collected 4 h after the fourth challenge was measured using an ELISA kit (eBioscience).

To assess mast cell activation, concentrations of histamine and mMCP-1 in BAL fluids and serum collected before, and 10 min and 4 h after the first and fourth challenges were measured using an EIA or ELISA kit (histamine EIA kit, SPI-Bio, Montigny le Bretonneux, France; mMCP-1 ELISA kit, eBioscience).

Additionally, histamine content in the lung tissue was measured; the lung tissue was isolated before the first challenge. Before the lung was isolated, the pulmonary circulation was perfused with 10 U·mL^{-1} heparin-containing PBS

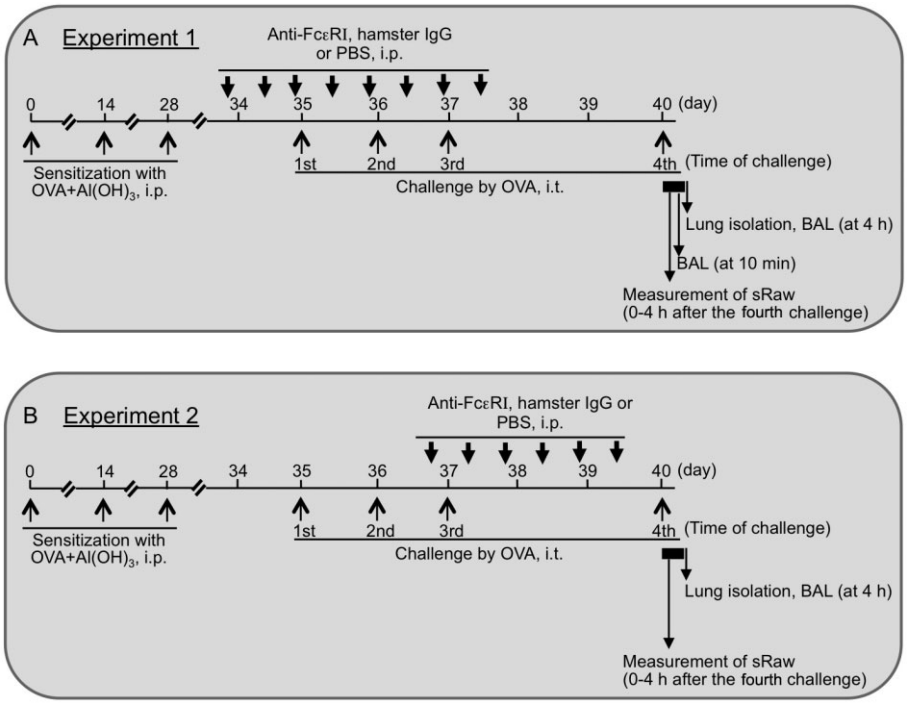

Figure 1

Schedules for treatment with an anti-FcεRI mAb, MAR-1, from the time before the first challenge (Experiment 1, A), and from the time 3 days before the fourth challenge (Experiment 2, B).

to avoid contamination by serum. The isolated lung was disrupted by homogenization using a homogenizer (10 000 rpm, 20 s) in the presence of 3% perchloric acid and 0.2% Triton X-100, and then the homogenate was heated in boiling water for 15 min. After centrifugation, histamine concentration in neutralized supernatant was measured by an EIA kit (Oxford Biomedical Res.).

Antigen-induced IL-4 production of basophils in vitro

Whether basophils infiltrating the lung were capable of producing IL-4 in response to antigen stimulation was assessed. Leukocytes recovered from collagenase/ deoxyribonuclease I-digested lung tissue were suspended in RPMI 1640 medium containing 10% FBS, 100 U·mL^{-1} penicillin and 100 μg·mL^{-1} streptomycin at a concentration of 5×10^6 cells·mL^{-1}, and were then distributed into the wells of a 12-well plate using 1 mL·per well. The cells were incubated with PBS or OVA at final concentrations of 10^{-6}, 10^{-5} or 10^{-4} g·mL^{-1} in the presence of 1.75 μL·per well of a protein transport inhibitor solution containing monensin (BD Golgistop; BD Biosciences) at 37°C in 5% CO_2 for 4 h.

After incubation, cells were washed twice with PBS supplemented with 2% FBS and were then incubated with anti-mouse FcγRII/III mAb for 15 min at 4°C in order to block FcγRs. After washing once with PBS supplemented with 2% FBS, the cells were incubated at 4°C with FITC-labelled anti-mouse IgE mAb (clone R35-72; BD Biosciences) at 5 μg·mL^{-1} and PE-Cy7-labelled anti-mouse CD49b mAb (clone DX5; eBioscience) at 2 μg·mL^{-1} for 25 min. After washing three times with PBS supplemented with 2% FBS, the cells were incubated with a fixation/permeabilization solution (BD Cytofix/Cytoperm; BD Biosciences) at 4°C for 12–18 h. After fixation and permeabilization, the cells were washed twice with a permeabilization/wash buffer (BD Perm/Wash), and were then incubated with 4 μg·mL^{-1} of PE-labelled anti-IL-4 mAb (clone 11B11; eBioscience) at 4°C for 40 min. Finally, the cells were washed three times with PBS supplemented with 2% FBS. Each sample was analysed using FACSCalibur and Cell Quest software.

Statistical analyses

Comparison between values before the first and fourth challenges, and that between before and 4 h after the fourth challenge were performed by the unpaired *t*-test. Statistical analyses among multiple groups in *in vivo* experiments using MAR-1 were performed using one-way ANOVA, followed by the Bonferroni–Dunn test. For statistical analyses of *in vitro* IL-4 production, the paired *t*-test was performed. $P < 0.05$ was considered significant.

Results

Multiple i.t. antigen challenge-induced early- and late-phase asthmatic responses, airway eosinophilia and airway neutrophilia

As we have reported (Nabe *et al.*, 2005; 2011a,b), when i.p. sensitized mice were intratracheally challenged with antigen

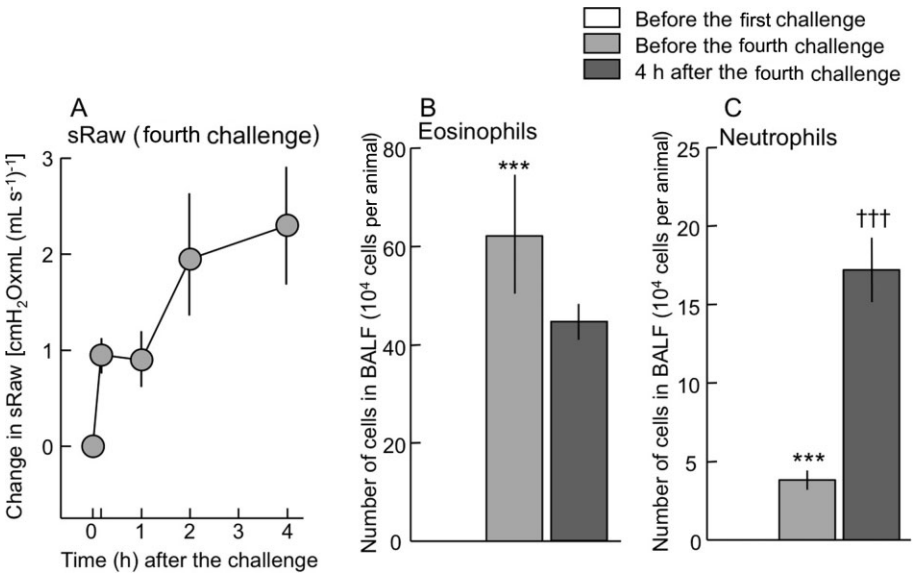

Figure 2

(A) Time-course change in sRaw after the fourth challenge in sensitized mice, and (B and C) time-course changes in numbers of eosinophils (B) and neutrophils (C) in BAL fluid before and/or after the first and fourth challenges. Mice were sensitized by i.p. injection of OVA + $Al(OH)_3$ on day 0, 14 and 28, and then challenged by i.t. administration of OVA solution on day 35, 36, 37 and 40. Each point and column represents the mean ± SE of eight animals. ***$P < 0.001$ versus before the first challenge. †††$P < 0.001$ versus before the fourth challenge.

four times, the fourth challenge produced a late-phase increase in sRaw 2–4 h after the challenge (Figure 2A). The late-phase increase in sRaw was preceded by an increase in sRaw, peaking at 10 min (Figure 2A). Eosinophils accumulated in the lungs until prior to the fourth challenge, whereas the fourth challenge exerted no further increase in the number of eosinophils 4 h after the challenge (Figure 2B). Neutrophils also increased until before the fourth challenge, followed by a further increase after the fourth challenge (Figure 2C).

Increases in mast cells and basophils in the lung following multiple antigen challenges

Consistent with our previous study (Nabe *et al.*, 2005), we identified a population of IgE^+ C-kit^+ cells in suspensions of lung cells harvested immediately before the fourth OVA challenge (Figure 3A). These IgE^+ C-kit^+ cells were sorted and stained with Wright-Giemsa for microscopic evaluation of their morphology. As shown in Figure 3A, IgE^+ C-kit^+ cells were greater than 10 μm in diameter and were rich in cytoplasmic granules. These cells displayed characteristics of mast cells based on both cell surface immunophenotype and their microscopic morphology.

Also consistent with our previous study (Nabe *et al.*, 2005), we detected a prominent population of IgE^+ C-kit^- cells in the lung cell suspensions (Figure 3B). Interestingly, almost all of these IgE^+ C-kit^- cells were positive for the expression of CD49b (Figure 3B), suggesting that they should be basophils (Min *et al.*, 2004; Mukai *et al.*, 2005). Morphologically, the IgE^+ C-kit^- $CD49b^+$ cells showed lobulated nuclei, a limited amount of cytoplasm and few granules (Figure 3B). The morphology of the IgE^+ C-kit^- $CD49b^+$ cells in this study was also similar to that of basophils (Min *et al.*, 2004; Mukai *et al.*, 2005).

Table 1

Time-course changes in total leukocytes in the lung before, and 10 min and 4 h after the first and fourth challenges

	Total cells ($\times 10^4$ cell·per left lobe)
Before the first challenge	654 ± 82
Before the fourth challenge	2430 ± 515**
4 h after the fourth challenge	3693 ± 528

Each value is the mean ± SE of 5 or 7 animals.
**$P < 0.05$ versus before the first challenge.

As shown in Figure 3A, multiple antigen challenges significantly increased the number of mast cells prior to the fourth challenge approximately five times. No further increase in the number of mast cells was induced 4 h after the fourth challenge, when the late-phase asthmatic response was evoked (Figure 3A). The number of basophils was also increased by three antigen challenges, whereas the fourth challenge did not induce significant increase (Figure 3B). Table 1 shows time-course changes in total leukocyte number in the lung tissue. The multiple antigen challenges produced a significant, approximately fourfold, increase in the total number of cells; however, the percentage of mast cells and basophils in the respective total cells did not change so markedly (Figure 3A and B). Thus, the increased numbers of mast cells and basophils depended mainly on the increase in total leukocyte number. This is logical because not only mast cells and basophils but also other leukocytes such as eosinophils and mononuclear cells were increased in the lung by multiple antigen challenges (Nabe *et al.*, 2005; 2011a,b).

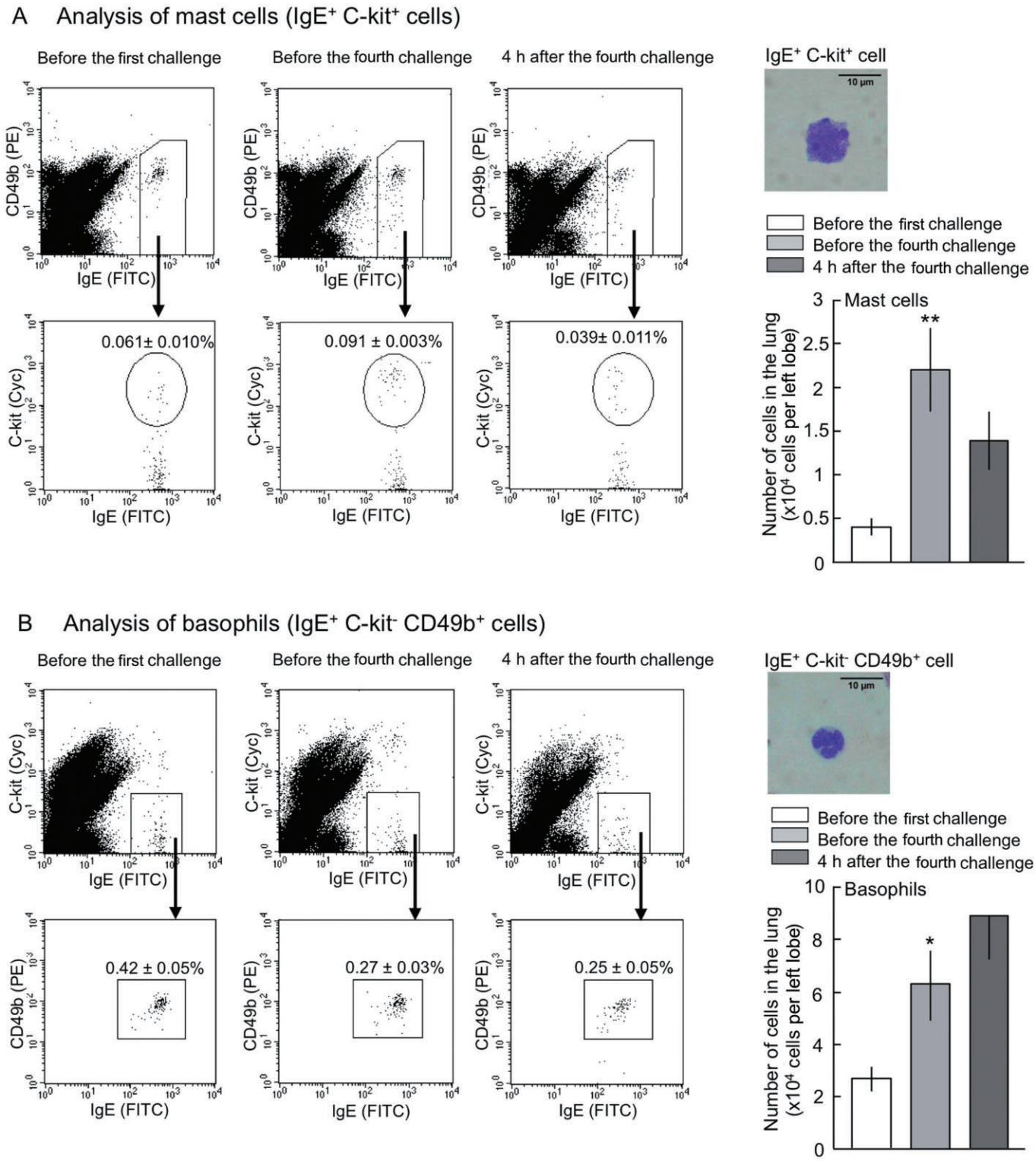

Figure 3

Typical flow cytometric profiles and morphology of IgE$^+$ C-kit$^+$ cells (mast cells, A) and IgE$^+$ C-kit$^-$ CD49b$^+$ cells (basophils, B), and time course of changes in numbers of mast cells (A) and basophils (B) in cell suspensions of the lungs isolated from sensitized mice. The lung tissue was harvested just before the first challenge and just before or 4 h after the fourth OVA challenge. Each column represents the mean ± SE of 5 or 7 animals. *,**$P < 0.05$, $P < 0.01$ versus before the first challenge.

Furthermore, IgE$^+$ C-kit$^+$ cells (mast cells) and IgE$^+$ C-kit$^-$ CD49b$^+$ cells (basophils) were characterized from a point of histamine content. These two populations of cells were collected by a cell sorter, and histamine content was measured after disruption of the cells. As shown in Table 2, histamine content of IgE$^+$ C-kit$^+$ cells and IgE$^+$ C-kit$^-$ CD49b$^+$ cells was 0.50 ± 0.03 and 0.12 ± 0.01 pg·per cell respectively.

Table 2

Histamine content in IgE$^+$ C-kit$^+$ cells (mast cells) and IgE$^+$ C-kit$^-$ CD49b$^+$ cells (basophils) in the lung

	Histamine content (pg·per cell)
IgE$^+$ C-kit$^+$ cells	0.50 ± 0.03
IgE$^+$ C-kit$^-$ CD49b$^+$ cells	0.12 ± 0.01

Each value is the mean ± SE of three experiments.

Immunohistochemical detection of IgE$^+$ C-kit$^+$ cells and IgE$^+$ CD49b$^+$ cells in the lung tissue

Next, IgE$^+$ C-kit$^+$ cells and IgE$^+$ CD49b$^+$ cells in the lung tissue were immunohistochemically detected by using fluorescence-labelled antibodies. Only a few IgE$^+$ C-kit$^+$ cells were detected in the lung tissue isolated before the first challenge (Figure 4A). At the time before the fourth challenge, a relatively large number of IgE$^+$ C-kit$^+$ cells were observed mainly in the alveolar parenchyma (Figure 4B). Similar to the result of IgE$^+$ C-kit$^+$ cells, IgE$^+$ CD49b$^+$ cells were also increased by multiple antigen challenges, and infiltration was observed mainly in the alveolar parenchyma (Figure 4C and D).

Effects of anti-FcεRI mAb, MAR-1 on the asthmatic responses

Experiment 1. As shown in Figure 1A, the anti-FcεRI mAb was i.p. administered during the first to third challenges from the time prior to the first challenge. The treatment almost completely abolished the increase in the numbers of both

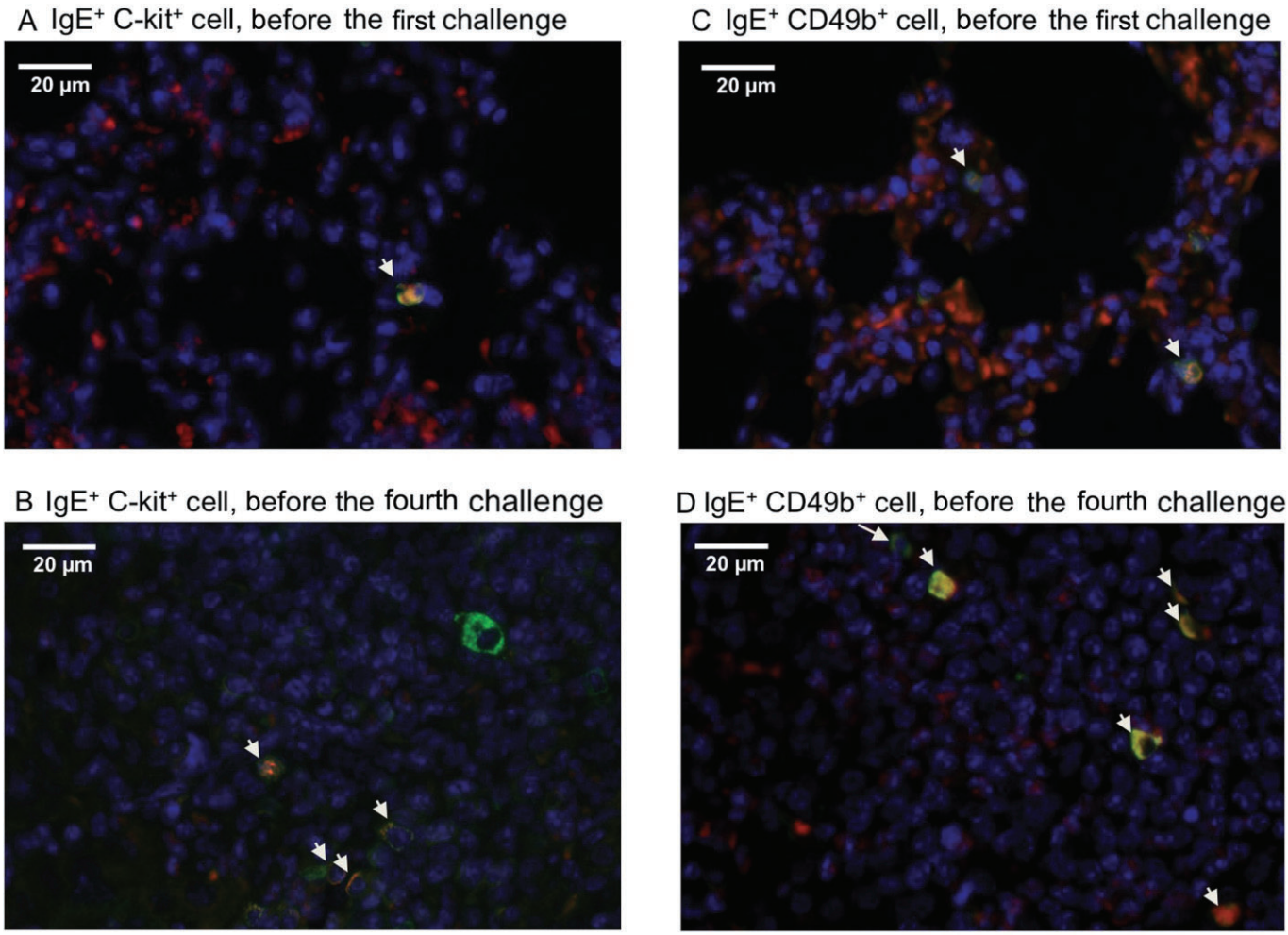

Figure 4

Immunohistochemical staining of IgE$^+$ C-kit$^+$ cells (mast cells, A and B) and IgE$^+$ CD49b$^+$ cells (basophils, C and D) in the lung tissue of sensitized mice. Lung tissue was harvested before the first (A and C) and fourth (B and D) antigen challenges. Arrows: IgE$^+$ C-kit$^+$ cells (A and B) or IgE$^+$ CD49b$^+$ cells (C and D).

mast cells (Figure 5B) and basophils (Figure 5C), although treatment with hamster IgG did not significantly affect these increases (Figure 5B and C). The percentage of mast cells and basophils in the total leukocyte number in the lung was prominently reduced by treatment with anti-FcεRI mAb (Figure 5B and C). In contrast, total leukocyte number in the lung was not significantly affected by the treatment (Figure 5A). Thus, the inhibitory effect of anti-FcεRI mAb depended on the reduction of the percentage of mast cells or basophils in the total leukocyte number in the lung tissue.

However, the increase in sRaw during the late phase (2–4 h after the fourth challenge) was not affected by treatment with anti-FcεRI mAb (Figure 6A and C). The increase in sRaw at 10 min was considerably small in the anti-FcεRI mAb-treated group although it was unexpected that the control hamster IgG-treated group also showed a lowered response 10 min–1 h after the challenge (Figure 6A and B). Neither eosinophilia nor neutrophilia in the airway tissues was influenced by anti-FcεRI mAb treatment (Figure 6D and E).

Next, we examined whether basophil activation was reduced by anti-FcεRI mAb treatment. As a marker of basophil activation, IL-4 in BAL fluid was measured because it has been reported that IL-4 was produced early after an airway antigen challenge in sensitized mice (Luccioli *et al*., 2002). Expectedly, the IL-4 concentration was markedly increased 4 h after the fourth challenge in comparison with that before the fourth challenge (Figure 6F). The increase in IL-4 was significantly suppressed by anti-FcεRI mAb treatment by approximately 50% (Figure 6F).

We assessed whether basophils infiltrating the lung definitely produce IL-4 in response to antigen challenge *in vitro*. The lung tissue was isolated prior to the fourth challenge and enzymatically digested. The single-cell suspension was stimulated with the antigen OVA for 4 h. Flow cytometric analyses revealed that the number of IL-4$^+$ cells in IgE$^+$ CD49b$^+$ cells (basophils) significantly increased in response to the *in vitro* antigen challenge (Figure 7A). The antigen-induced increase of IL-4$^+$ basophils was concentration dependent (Figure 7B).

On the other hand, whether activation of mast cells was evoked after the fourth challenge was also examined *in vivo*. As markers of mast cell activation, concentrations of histamine and mMCP-1 (Mathias *et al*., 2009) in serum before, and 10 min and 4 h after the fourth challenge were measured in comparison with those at the first challenge. As shown in Table 3, the first challenge evoked significant increases in histamine within 10 min and mMCP-1 at 4 h. However, these activation markers in serum were not significantly increased after the fourth challenge although they tended to be increased even before the fourth challenge compared to levels before the first challenge (Table 3). On the other hand, in BAL fluids, neither mMCP-1 nor histamine was increased after the first and fourth challenges (data not shown).

Experiment 2. We assessed whether the fourth challenge-induced asthmatic responses were affected when anti-FcεRI mAb was administered during the third to fourth challenges. Even when FcεRI was neutralized during the relatively close period to the effector phase for the fourth challenge-induced asthmatic response, both mast cells and basophils were completely reduced (Figure 8A and B).

However, the increase in sRaw in the late phase was not affected by the anti-FcεRI mAb treatment (Figure 8C and E). The early-phase increase in sRaw was clearly and significantly suppressed by anti-FcεRI mAb by approximately 70% in comparison with the hamster IgG-treated group, which showed comparable magnitude of the early response to the PBS-treated group in this treatment schedule (Figure 8C and D).

Discussion and conclusions

In the present study, using flow cytometry, we demonstrated that multiple i.t. antigen challenges induced the recruitment not only of mast cells, but also of basophils, into the lungs. This is the first report to demonstrate that allergen challenges induce lung basophilia in an experimental model of asthma. Furthermore, an anti-FcεRI mAb, MAR-1, effectively suppressed the increases in both mast cells and basophils in the airway tissue; however, unexpectedly, the reduction of airway mast cell and basophil numbers produced no inhibition of late-phase airway obstruction. On the other hand, the early-phase increase in sRaw was suppressed by MAR-1. MAR-1 treatment clearly suppressed antigen-induced airway production of IL-4, which should be produced from basophils infiltrating the lungs; however, mast cell activation was not provoked after the fourth antigen challenge because neither histamine nor mMCP-1 was increased after the challenge. Taken together, multiple i.t. antigen challenges produced airway basophilia, which contributed importantly to the induction of early airway obstruction.

Regarding mast cell hyperplasia in allergen-driven murine models of asthma, Ikeda *et al*. (2003), who detected mast cells

Table 3

Increase in amounts of histamine and murine mast cell protease (mMCP)-1 in serum after the first and fourth antigen challenges

	Histamine (ng·mL^{-1})		mMCP-1 (ng·mL^{-1})	
	First challenge	Fourth challenge	First challenge	Fourth challenge
Before	121 ± 20	175 ± 17	30 ± 4	74 ± 18
10 min	387 ± 67***	224 ± 30	53 ± 20	119 ± 33
4 h	144 ± 7	181 ± 21	103 ± 33*	95 ± 24

Each value is the mean ± SE of six animals.
*,***P < 0.05, 0.001 versus before challenge.

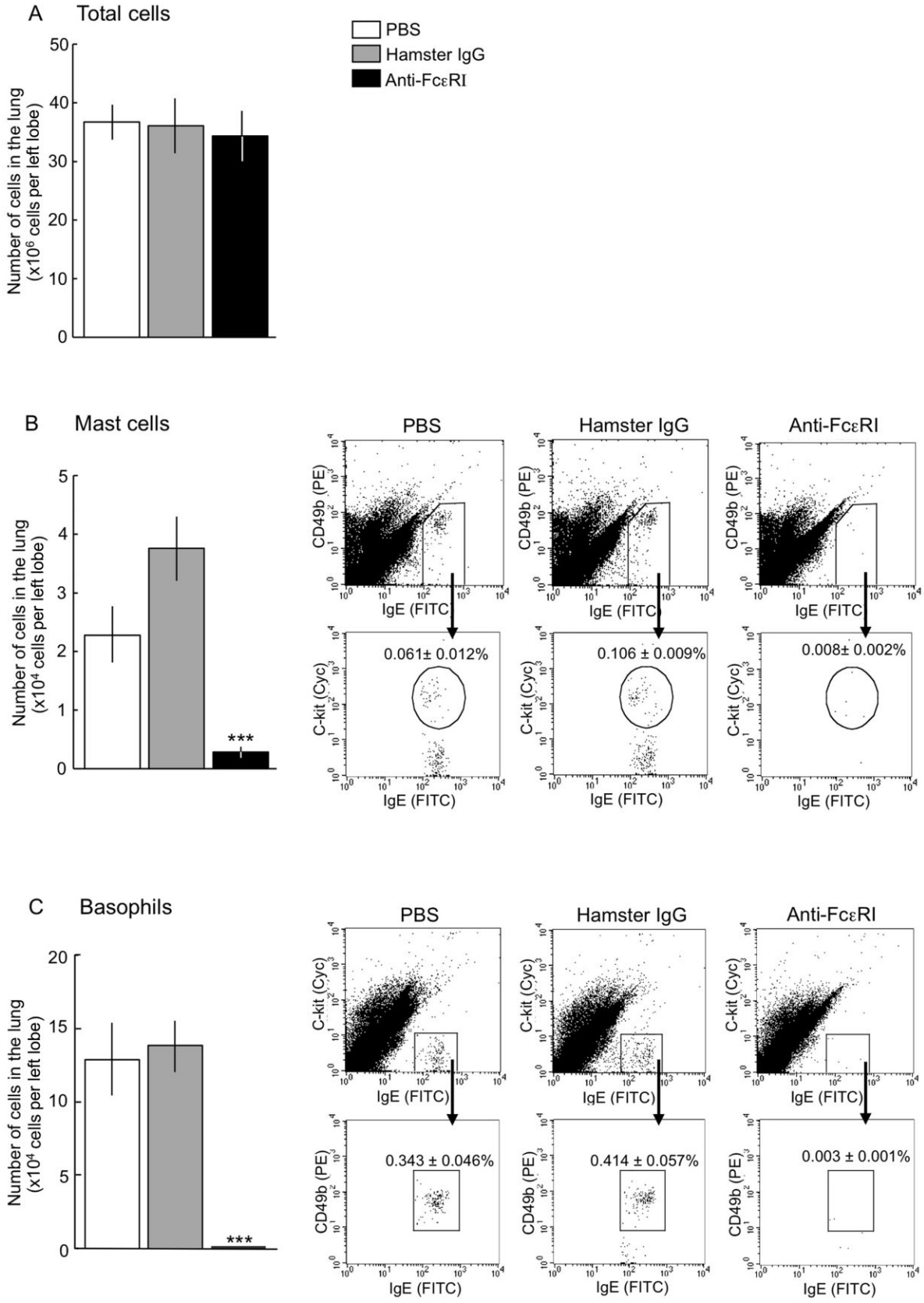

Figure 5

Experiment 1: Effects of an anti-mouse FcεRI mAb, MAR-1, on antigen-induced increases in the numbers of total cells (A), mast cells (B) and basophils (C) in the lung. The anti-mouse FcεRI mAb, hamster IgG or PBS was administered as shown in Figure 1A. The lung was isolated 4 h after the fourth challenge. Each column and value in panels represent the mean ± SE of 7 or 8 animals. ***$P < 0.001$ versus hamster IgG treated.

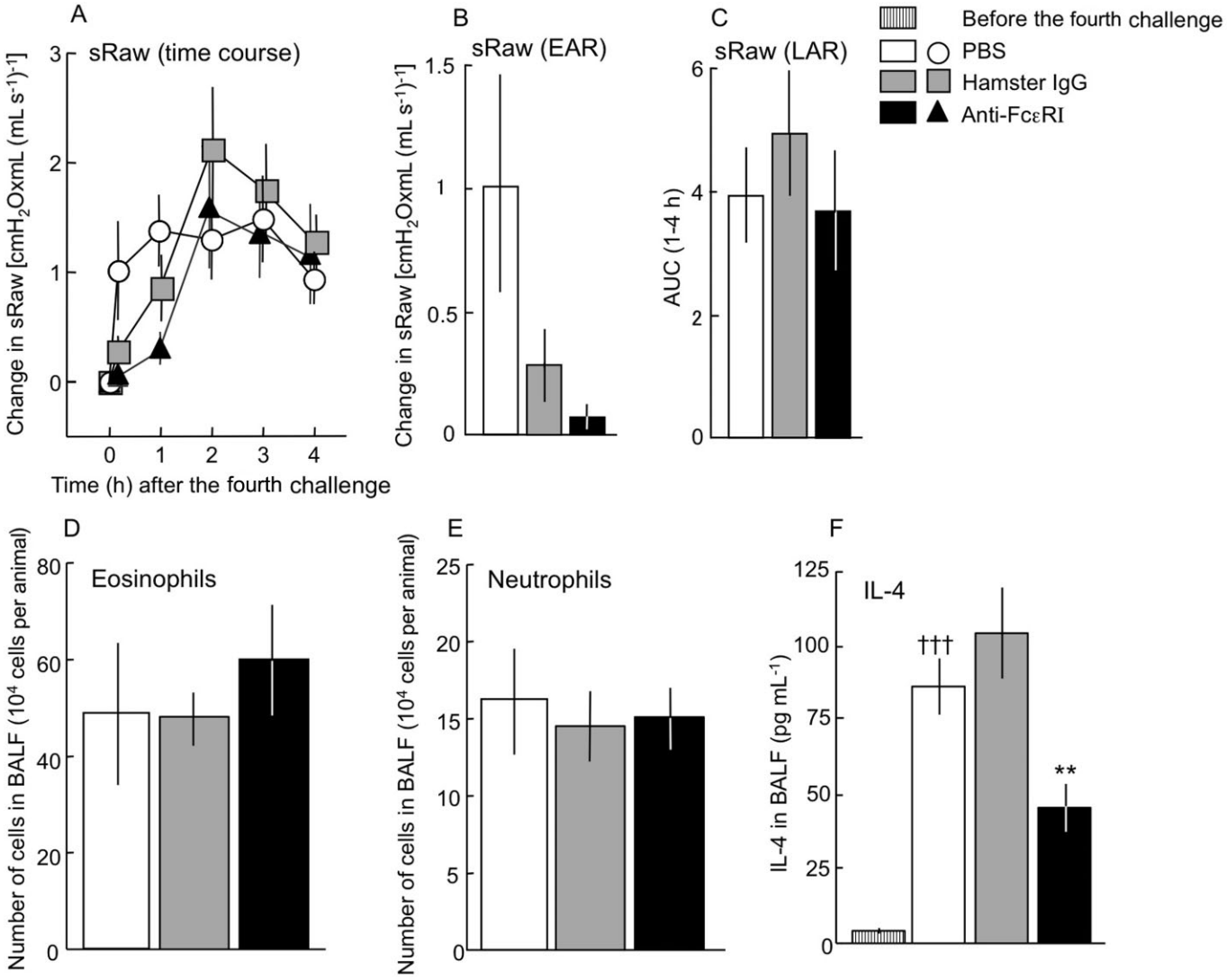

Figure 6

Experiment 1: Effects of an anti-mouse FcεRI mAb, MAR-1, on antigen-induced increase in sRaw after the fourth OVA challenge (A, B and C), increases in the numbers of eosinophils (D) and neutrophils (E) in BAL fluids, and increase in the amount of IL-4 (F) in BAL fluids. The anti-mouse FcεRI mAb, hamster IgG or PBS was administered as shown in Figure 1A. Panel C shows the effect on sRaw as the AUC for sRaw during the late (1–4 h) phase. Each point and column represent the mean ± SE of 7 or 8 animals. $^{**}P < 0.01$ versus hamster IgG treated. $^{\dagger\dagger\dagger}P < 0.001$ versus the value before the fourth challenge.

by toluidine blue staining, reported that chronic intranasal OVA challenge twice a week for 1 month increased numbers of mast cells in the lungs of sensitized mice. Our present study is the first report to detect airway mast cell hyperplasia by flow cytometric and immunohistochemical analyses of IgE and C-kit molecule expression on lung cells. Regarding lung basophilia, because there has been no staining technique in mice, it has been unclear whether antigen challenge causes the recruitment of basophils into the lung tissue. These techniques can be useful for analysis of the mechanisms underlying mast cell hyperplasia and basophilia in the airway tissue and for the development of new therapeutic drugs that affect recruitment of these inflammatory cells.

Mast cells and basophils detected in the lung tissue were characterized from the point of histamine content. These cells were separately collected using a cell sorter and were found to possess histamine at 0.50 and 0.12 pg·per cell respectively. It has been reported that histamine content in human lung mast cells was 3.7 pg·per cell (Patella *et al.*, 1995a) or 10.6 pg·per cell (Patella *et al.*, 1995b). In contrast to the content in human lung mast cells, that in murine lung mast cells was considerably low. However, bone marrow-derived murine mast cells contained a comparable amount (approximately 0.5 pg·per cell) of histamine (Yamamura *et al.*, 1994) to the level in murine lung mast cells in this study. In addition, mast cells dispersed from fetal mouse skin also contained a comparable level of histamine (Yamada *et al.*, 2003). Thus, the pathophysiological roles of histamine in murine airway tissues might be limited compared to those in human airways. On the other hand, there has been no

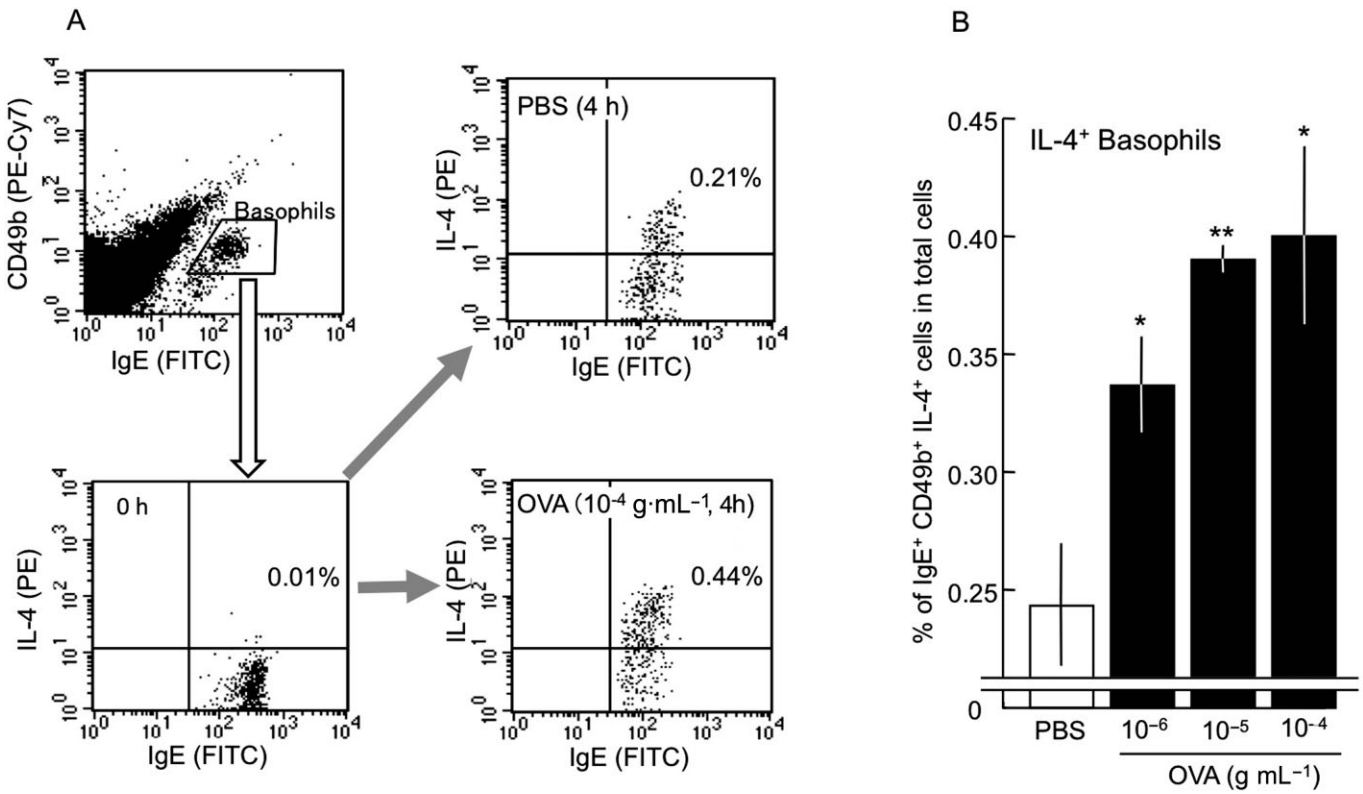

Figure 7

Analysis of IL-4 production by basophils infiltrating the lung tissue. (A) Flow cytometric analysis of antigen (OVA)-induced IL-4 synthesis by basophils in a single-cell suspension from the lungs of sensitized mice. (B) Concentration dependence of OVA-induced IL-4 production by the lung basophils. The lung isolated before the fourth challenge was enzymatically digested to obtain a single-cell suspension. The cells were incubated with the indicated concentrations of OVA, or with control PBS, together with a protein transport inhibitor, monensin, for 4 h prior to flow cytometric analysis. Each column represents the mean ± SE of three experiments. *,**$P < 0.05$, $P < 0.01$ versus PBS treated.

study to measure histamine content in murine basophils collected in the lung. The histamine content (0.12 pg·per cell) was lower than that in human peripheral blood basophils, which possessed 1.1 pg·per cell (Patella *et al.*, 1995a) or 1.3 pg per·cell (Ishmael and MacGlashan, 2010) of histamine. Schneider *et al.* (1999) reported that murine basophil precursors may have histidine decarboxylase and produce histamine. Collectively, in comparison with human mast cells and basophils, these IgE-possessing cells in mice have relatively low levels of histamine.

It has been suggested that there is a restriction in the use of anti-FcεRI mAb, MAR-1, for basophil depletion because effects on mast cells can not be ruled out (Min, 2010; Wada *et al.*, 2010; Falcone *et al.*, 2011). Indeed, Denzel *et al.* (2008) reported that treatment with MAR-1 partially but significantly reduced the number of mast cells (IgE$^+$ C-kit$^+$ cells) in the peritoneal lavage fluid of mice. In the present study, we also demonstrated that increases in not only basophils but also mast cells were markedly suppressed by MAR-1. It was reported that when MAR-1 was injected twice a day for 3 days at a dose of 5 μg·per animal, prolonged and complete depletion of basophils was systemically achieved (Denzel *et al.*, 2008). We applied this protocol of antibody treatment to the murine model of asthma, whereas both basophils and mast cells were only partially reduced in the lung tissue (data not shown). Thus, in the next experiment, a twofold dose (10 μg·per animal) of MAR-1 was injected twice a day for 4 days, as shown in Figure 1A. The increases in dose and total times of MAR-1 treatment resulted in the almost complete abolition of both basophils and mast cells in the lungs. However, alternatively, there is a possibility that anti-FcεRI mAb just occupied FcεRI molecules, so that fluorescence-labelled anti-IgE mAb for flow cytometry could not access the IgE on mast cells and basophils. However, Denzel *et al.* (2008) demonstrated that pre-incubation of bone marrow cells with MAR-1 *in vitro* did not interfere with the detection of surface IgE on basophils by anti-IgE.

There is also a possibility that multiple treatments with anti-FcεRI mAb may have affected the binding of IgE to FcεRI on basophils and mast cells. If this is true, inhibition on early asthmatic response by treatment with anti-FcεRI mAb may not be due to the decrease in the numbers of basophils and mast cells in the lung tissue. However, from the finding reported by Denzel *et al.* (2008) as described above, it can be speculated that anti-FcεRI mAb, MAR-1 and IgE do not compete for identical binding sites in FcεRI molecule. Indeed, it was demonstrated that multiple treatment with MAR-1 caused prolonged depletion of basophils systemically in the peripheral blood, bone marrow, spleen and liver (Denzel *et al.*, 2008). Therefore, it can be considered that FcεRI-

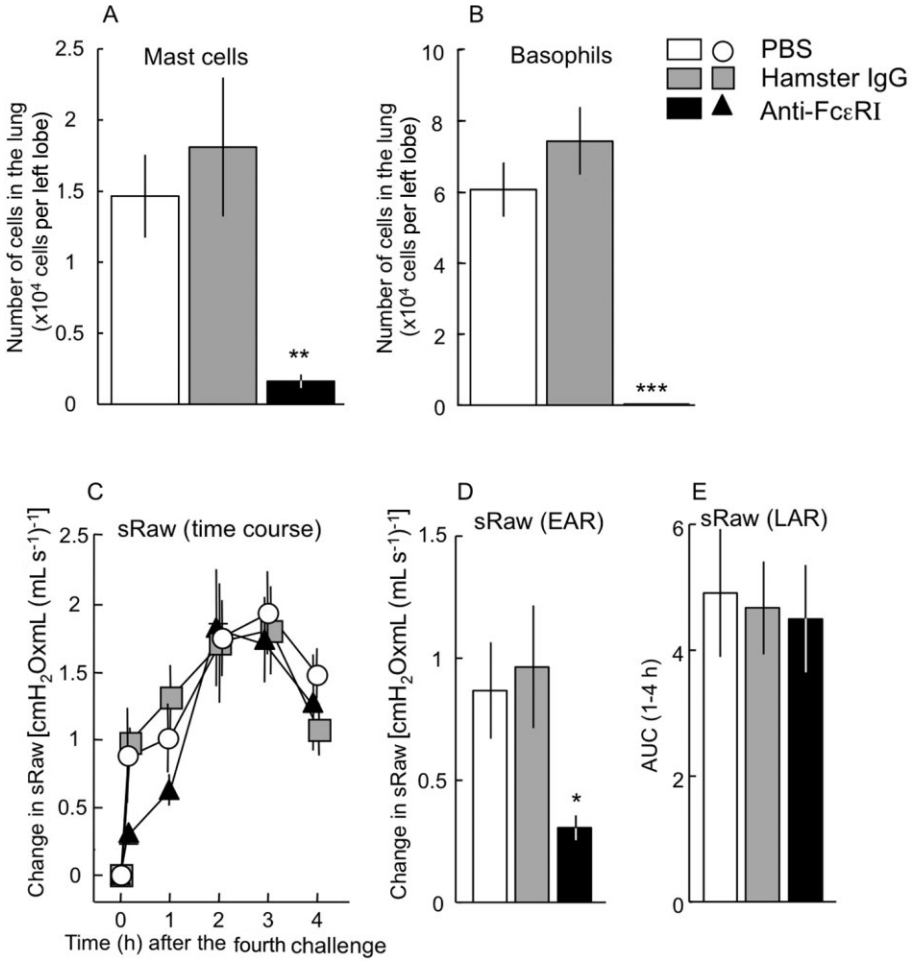

Figure 8

Experiment 2: Effects of an anti-mouse FcεRI mAb, MAR-1, on increases in numbers of mast cells (A) and basophils (B) in the lung, and increase in sRaw after the fourth OVA challenge (C, D and E). Anti-mouse FcεRI mAb, hamster IgG or PBS was administered as shown in Figure 1B. Panel E represents the effect on sRaw as the AUC for sRaw during the late (1–4 h) phase. Each point and column represents the mean ± SE of 4–14 animals. *,** and ***$P < 0.05$, 0.01 and 0.001 versus hamster IgG treated.

bearing cells were depleted by MAR-1 treatment, resulting in the cells actually disappearing from the lung tissue.

Whether activation of basophils and mast cells in the airway was decreased by treatment with MAR-1 was checked. As has been reported *in vivo* (Luccioli *et al.*, 2002), the present *in vitro* study also demonstrated that basophils recruited in the lungs indeed produced IL-4 in response to antigen stimulation. Additionally, treatment with MAR-1 clearly reduced the increase in IL-4 in BAL fluids *in vivo* by approximately 50%, suggesting that MAR-1 definitely reduced the number of basophils in the lungs and/or impaired the function of basophils. The rest of IL-4 produced should have been derived from $CD4^+$ T cells because treatment with anti-CD4 mAb before the fourth challenge also inhibited IL-4 production by approximately 50% (Nabe *et al.*, 2011a).

In contrast to the marked activation of basophils after the fourth challenge, which was manifested by marked IL-4 production, mast cell activation was impaired because neither histamine nor mMCP-1 was increased in serum as well as BAL fluids after the fourth challenge. In contrast, the first challenge clearly increased serum levels of histamine and mMCP-1. The result of mMCP-1 was consistent with that of another report, in which inhalation of a chemical hapten increased mMCP-1 in serum in sensitized mice (Mathias *et al.*, 2009). Thus, desensitization of mast cells was induced by multiple antigen challenges, as has been demonstrated in an *in vitro* study (Shalit and Levi-Schaffer, 1995). From the present result of IL-4 production after the fourth challenge, basophil activation was evoked after the fourth challenge. However, we found that histamine content in lung basophils was low, so it can be considered that the serum level of histamine was not altered after the fourth challenge even though the basophils were activated. On the other hand, identical to other reports (Xu *et al.*, 2006; Falanga *et al.*, 2012), the present study also revealed that the serum level of histamine was considerably high. We measured histamine content in the lung tissue and found that the left lung collected before the first challenge contained 26.0 ± 2.3 ng·per left lung of histamine, which was considerably lower than serum histamine. In a study that measured histamine content

in various organs of Balb/c mice, the stomach, lymph nodes, thymus and spleen contained a large amount of histamine in comparison with the lung (Zimmermann *et al*., 2011). Thus, histamine increased in serum after the first challenge may be derived mainly from organs other than the pulmonary tissues. An anaphylactic response in systemic organs may be simultaneously induced, leading to elevation of the serum level of histamine. Thus, in comparison with the increase in mMCP-1, the histamine level in serum may not be an appropriate activation marker of mast cells in the lung.

Treatment with control hamster IgG from the time before the first challenge showed the inhibition of early-phase airway obstruction, although other asthmatic responses were not affected. This result means that long-term treatment with a foreign protein may affect cells and/or molecules that participate in the early asthmatic response, although it is not clear which cells were affected. Thus, we next examined the effects of MAR-1 by injecting during a relatively close period to the effector phase, as shown in Figure 1B. Consequently, hamster IgG exerted no effect on the early asthmatic response, although MAR-1 clearly inhibited the early response.

At present, it is unclear how basophils participate in the induction of early-phase airway obstruction. It was reported that basophils are one of the major players in IgG- but not IgE-mediated systemic anaphylaxis (Mukai *et al*., 2009): In response to allergen-IgG immune complexes, basophils release platelet-activating factor (PAF) as the major chemical mediator to induce systemic anaphylaxis. We have indeed demonstrated that allergen-specific IgG as well as allergen-specific IgE was markedly increased by multiple antigen challenges in this model (Nabe *et al*., 2005); however, neither a PAF receptor antagonist (Nabe *et al*., unpublished) nor a mAb against FcγRII/III (Nabe *et al*., 2011a), which transduces cellular response to IgG immune complexes, suppressed the early-phase response. Therefore, other mechanisms than IgG immune complex-induced release of PAF from basophils could contribute to this early response. It was recently reported that mMCP-11 is derived preferentially from basophils in mice, and mMCP-11 was capable of inducing microvascular leakage without infiltration of proinflammatory cells, such as eosinophils and neutrophils (Yamagishi *et al*., 2011). Thus, the basophil-derived protease may participate in the induction of early-phase airway narrowing by producing airway mucosal oedema.

Regarding the late-phase airway obstruction, we could conclude that basophils did not play significant roles in the response. We have reported that the late-phase response was induced after the fourth challenge but not after the first to third challenges (Nabe *et al*., 2005). The late-phase response after the fourth challenge was seen in association with the development of high levels of airway inflammation, suggesting that this inflammation was essential for the late-phase response. The fourth challenge-induced late-phase airway obstruction was completely suppressed by reducing $CD4^+$ cells from the time of the first challenge (Nabe *et al*., 2011b); therefore, $CD4^+$ T cells play important roles in the formation of airway inflammation, which is the basis of the late-phase airway obstruction. When focusing on the effector phase (the fourth challenge-induced allergic response) because depletion of neutrophils but not eosinophils significantly attenuated the late-phase response (Nabe *et al*., 2011a; 2012), neutrophils infiltrating the airway could be effector cells causing the late response.

Obata *et al*. (2007) suggested that basophils may function as initiators rather than effectors during IgE-mediated chronic cutaneous allergic inflammation in mice. This hypothesis was based on the fact that elimination of basophils by a basophil-depleting Ab prevented the development of IgE-mediated chronic allergic inflammation as well as the increase in other granulocytes, neutrophils and eosinophils, which were thought to be effector cells in the inflammatory change (Obata *et al*., 2007). In contrast, in the present study, even when MAR-1 was administered from the time before the first challenge, airway infiltration of neither eosinophils nor neutrophils was inhibited by treatment. Although it has been recently reported that basophils function to take up, process and present antigens to T cells on their MHC class II molecules (Perrigoue *et al*., 2009; Sokol *et al*., 2009; Yoshimoto *et al*., 2009), basophils may not function as antigen-presenting cells in this asthma model. Supporting evidence is presented in a report by Hammad *et al*. (2010), who demonstrated that basophils were unnecessary and insufficient antigen-presenting cells for the development of Th2 immunity to house dust mite allergen when it was introduced via the natural inhaled route.

In conclusion, we have demonstrated that multiple antigen challenges markedly induced the infiltration of basophils and mast cells into the lungs. The lung-infiltrating basophils contribute to the induction of early-phase airway obstruction. It is unlikely that basophils function as antigen-presenting cells in the development of late-phase airway obstruction and cellular infiltration into the airway tissues. This study suggested basophils as potential targets for therapy aimed at controlling the early-phase response in asthmatic patients.

Acknowledgements

This study was supported in part by a Grant-in-Aid for Scientific Research (C) (23590093, T. Nabe) from the Japan Society for the Promotion of Science and a grant from The Promotion and Mutual Aid Corporation for Private Schools of Japan. The authors express sincere gratitude to Dr. Shitaro Kurokawa and Dr. Makoto Okumura from the Central Research Laboratories of Kaken Phramaceutical Co. Ltd. (Kyoto, Japan) for operating the FACSAria Cell Sorter, and Ms. Maki Kishima and Mr. Shinya Fujii (Department of Pharmacology, Kyoto Pharmaceutical University) for technical assistance.

Conflicts of interest

None.

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