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Intratracheal exposure to Fab fragments of an allergen-specific monoclonal antibody regulates asthmatic responses in mice

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Summary

Fab fragments (Fabs) maintain the ability to bind to specific antigens but lack effector functions due to the absence of the Fc portion. In the present study, we tested whether Fabs of an allergen-specific monoclonal antibody (mAb) were able to regulate asthmatic responses in mice. Asthmatic responses were induced in BALB/c mice by passive sensitization with anti-ovalbumin (OVA) polyclonal antibodies (pAbs) (day 0) and by active sensitization with OVA (days 0 and 14), followed by intratracheal (i.t.) challenge with OVA on day 1 and days 28, 29, 30 and 35. Fabs prepared by the digestion of an anti-OVA IgG1 (O1-10) mAb with papain were i.t. administered only once 30 min before antigenic challenge on day 1 or day 35. The results showed that i.t. administration of O1-10 Fabs with OVA markedly suppressed the early and/or late phases of asthmatic responses caused by passive and active sensitization. Similar results were obtained when Fabs of anti-OVA IgG2b mAb (O2B-3) were i.t. administered. In contrast, neither i.t. injection of intact 01-10/O2B-3 nor systemic injection of O1-10 Fabs suppressed the asthmatic responses. *In vitro* studies revealed that the capture of OVA by O1-10 Fabs prevented the subsequent binding of intact anti-OVA pAbs to the captured OVA. These results suggest that asthmatic responses may be down-regulated by the i.t. exposure to Fabs of an allergen-specific mAb via a mechanism involving the capture of allergen by Fabs in the respiratory tract before the interaction of intact antibody and allergen essential for the induction of asthmatic responses.

Keywords: asthma; Fab; intratracheal exposure; monoclonal antibody.

Introduction

Allergic asthma is a chronic inflammatory disease in which airway obstruction and airway hyperresponsiveness associated with elevated levels of allergen-specific IgE are distinctive features.[1,2] The asthmatic responses are divided into the early and late phases.[3–5] The early asthmatic response (EAR) is characterized by acute obstruction of the airway beginning within minutes of allergen exposure. IgE-mediated degranulation of mast cells plays a role in EAR.[6] The late asthmatic response (LAR) begins 3–6 hr after allergen exposure and is related to the infiltration of inflammatory cells including macrophages, lymphocytes, neutrophils and eosinophils in the lung parenchyma and airway epithelium.[7–9] However, the mechanism underlying LAR has not been completely defined. For instance, a number of studies have demonstrated that T helper type 2 cytokines such as interleukin-5 (IL-5) and IL-13 and eosinophils appear to play a role in asthma because the production of these cytokines and eosinophilia in the lung are hallmarks of this disease.[2,10–12] However, clinical studies have also shown that asthmatic symptoms in

Abbreviations: BALF, bronchoalveolar lavage fluid; EAR, early asthmatic response; IL-5, interleukin-5; i.p., intraperitoneal; i.t., intratracheal; KC, keratinocyte-derived chemokine; LAR, late asthmatic response; mAb, monoclonal antibody; MIP-2, macrophage inflammatory protein 2; mMCP-1, mouse mast cell protease-1; OVA, ovalbumin; pAbs, polyclonal antibodies; sRaw, specific airway resistance

patients were not ameliorated following treatment with humanized anti-IL-5 monoclonal antibodies (mAbs) that resulted in a marked reduction of eosinophils in the circulation as well as in the airway tissue.[13,14] On the other hand, the role of neutrophils in asthma, particularly in patients with severe asthma, has been shown.[15,16] Our recent studies using a murine model of asthma also revealed that complement activation and neutrophils appear to be at least partly involved in the late phase of the asthmatic responses in mice.[17–19]

Therapeutic drugs for asthma include anti-allergic drugs, bronchodilators and corticosteroids.[20–23] However, these drugs do not act antigen-specifically. Although subcutaneous and sublingual immunotherapy in asthma is currently available as an antigen-specific therapy, 1 or 2 years are often required before patients benefit from this approach.[24–27] It is also known that local and systemic anaphylactic reactions often occur in allergic patients treated with this antigen-specific immunotherapy.[28–30]

Fab fragments (Fabs) produced by the digestion of antibodies with papain maintain the ability to bind specific antigens but lack the binding site for complement as well as the site for binding to receptors on immune and inflammatory cells,[31–34] which play an important role in asthma.[7–9] Therefore, Fabs lacking effector functions may be used as a tool to regulate asthma antigen-specifically. In the present study, we show for the first time that intratracheal (i.t.) exposure to Fabs of an antigen-specific IgG1 or IgG2b mAb can down-regulate asthmatic responses in mice.

Materials and methods

Animals

Male 7-week-old BALB/c mice were obtained from Japan SLC, Inc. (Shizuoka, Japan). The animals were maintained in a temperature-controlled environment with free access to standard rodent chow and water. All animal care and the experimental procedure were conducted according to the guidelines of the Ethics Committee of Kobe Pharmaceutical University.

Induction of asthmatic responses

Mice were passively sensitized by intraperitoneal (i.p.) injection of 200 μl PBS containing 1 mg of polyclonal antibodies (pAbs) to ovalbumin (OVA; Sigma-Aldrich Fine Chemicals, St Louis, MO) from the sera of mice immunized with OVA that had been purified through an OVA-coupled HiTrap NHS-activated HP column (GE Healthcare UK Ltd., Little Chalfont, UK).[35] Twenty-four hours later, under anaesthesia with escain, 20 μl OVA (40 μg) was i.t. administered to induce asthmatic responses. Animals were also actively sensitized by i.p. injection of 200 μl PBS containing 50 μg OVA absorbed by 1 mg alum (Wako, Osaka, Japan) on days 0 and 14. On days 28, 29, 30 and 35, the animals were challenged by i.t. administration of 20 μl OVA under anaesthesia as described previously.[17] Two hundred micrograms of OVA was used as a challenging dose on days 28, 29 and 30, while 40 μg of the antigen was used on day 35. In some experiments, mice were passively sensitized by i.p. injection of 200 μl PBS containing 100 μg anti-OVA IgE mAb (OE-1) on days 0, 1, 2, 7, 8, 9 and 14 followed by i.t. administration of 20 μl OVA on days 1, 2, 3, 8, 9, 10 and 15 to induce IgE-mediated asthmatic responses.[36] Two hundred micrograms of OVA was used as a challenging dose on all days except day 15, when 40 μg OVA was administered.

Measurement of airway resistance

To evaluate the extent of airway resistance, specific airway resistance [sRaw; $cmH_2O \times ml/(ml/seconds)$] was measured in conscious mice before and 10 min to 5 hr after the first or fourth challenge with OVA using a two-chambered, double-flow plethysmograph system (Pulmos-I; MIPS, Osaka, Japan) according to the method of Pennock *et al.*[37]

Analysis of cells in bronchoalveolar lavage fluid

At 5 hr after the fourth antigenic challenge, mice were killed, the trachea was cannulated, and the left bronchi were tied for histological examination. The right air lumen was washed twice with 0·5 ml Hanks' balanced salt solution. The total number of leucocytes in the bronchoalveolar lavage fluid (BALF) was determined by staining with Turk's solution. For differential cell counts, cells were stained with Diff-Quik solution (International Reagents, Kobe, Japan). A minimum of 300 cells were counted under a microscope and classified as macrophages, lymphocytes, neutrophils or eosinophils on the basis of their morphological characteristics.[36]

Histological and immunohistochemical analyses

The left lungs were fixed in 10% neutral-buffered formalin, dissected embedded in paraffin, and then sectioned at 4 μm before staining with haematoxylin and eosin. Immunohistochemical analysis was also performed to detect IL-1β according to a method described previously.[17] In order to measure Gr-1^+ cells in the lung, endogenous peroxidase was blocked with 3% H_2O_2 in water for 30 min. After blocking non-specific binding with diluted normal rabbit serum in PBS for 20 min, lung sections were incubated for 18 hr at room temperature with anti-Gr-1 mAb (Biolegend, San Diego, CA), and for 1 hr with biotinylated anti-rat IgG2b (Biolegend). The slides were stained with

streptavidin–horseradish peroxidase, and the colour was developed using the diaminobenzidine substrate kit for peroxidase (Vector Laboratory, Burlingame, CA). Counterstaining was performed using Mayer's haematoxylin. Histological and immunohistochemical scoring for each section was evaluated on a scale of 0–4 with increments of 0·5 by a blinded observer.

Production and purification of anti-OVA mAbs and Fabs

B-cell hybridomas producing anti-OVA IgG1 (O1-10), IgG2b (O2B-3), and IgE (OE-1) mAbs were established using methods described previously.[38] The hybridoma cells were grown in the CELLine CL1000 with BD-Cell-MAb medium (BD Biosciences, Franklin Lakes, NJ) and the produced mAbs in the medium were purified using an OVA-coupled HiTrap NHS-activated HP column (GE Healthcare). The preparation and purification of Fabs from mAbs were performed according to a previously described method.[35]

Administration of Fabs

Various doses of O1-10 Fabs dissolved in 0·2 ml PBS were i.t. administered 30 min before antigenic challenge applied 24 hr after passive sensitization with anti-OVA pAbs. The O1-10 Fabs were also i.t. administered only once 30 min before the fourth challenge with OVA. PBS and normal IgG Fabs (Sigma-Aldrich) were used as controls. In some experiments, O1-10 Fabs were administered at the time of or 2 hr after OVA challenge.

Measurement of mouse mast cell protease-1 and complement C3a in serum

The serum levels of mouse mast cell protease-1 (mMCP-1) were determined using an ELISA kit (eBioscience, San Diego, CA). C3a in BALF was also detected by ELISA using rat anti-mouse C3a antibody (BD Bioscience) according to a method described previously.[39,40]

Measurement of chemokines and cytokines in the lung

The lung was homogenized in 1 ml T-PER, a tissue protein extraction reagent (Thermo Scientific, Rockford, IL) containing a Complete Mine Protease Cocktail tablet (Roche, Mannheim, Germany). The lung homogenates were centrifuged at 9000 ***g*** for 10 min at 4°. Keratinocyte-derived chemokine (KC), macrophage inflammatory protein 2 (MIP-2), IL-1β, IL-13 (R&D Systems, Minneapolis, MN), IL-6, IL-4, IL-5, IL-17A, and IL-23 (Biolegend) in the supernatants of lung homogenates were measured using quantitative colorimetric sandwich ELISA kits.

Inhibition analysis

To investigate whether an epitope of OVA recognized by O1-10 was the same as or different from that of OE-1, 50 μg/ml intact OE-1 alone or a mixture of 50 μg/ml intact OE-1 and 0·5, 5, 50 and 500 μg/ml O1-10 Fabs was added to OVA coated on 96-well plates; they were incubated at 37° for 1 hr. After washing the plates, alkaline phosphatase-conjugated anti-Fc of IgE (Sigma-Aldrich) was added to the plates and they were further incubated at 37° for 1 hr.[35] Then, the plates were developed with *p*-nitrophenyl phosphate before reading at 405 nm using a microplate reader. Similarly, 50 μg/ml intact O1-10 alone or a mixture of 50 μg/ml intact O1-10 and 0·5, 5, 50 and 500 μg/ml O2B-3 Fabs was added to OVA coated on 96-well plates followed by addition of anti-Fc of IgG to investigate whether these two antibodies recognize the same or different epitopes of OVA. To investigate whether the capture of OVA by anti-O1-10 Fabs in advance could prevent the subsequent interaction between the captured OVA and intact anti-OVA pAbs or OE-1, 0·005, 0·05, 0·5 and 5 μg/ml O1-10 Fabs were incubated with OVA coated on 96-well plates at 37° for 1 hr. After washing, this was followed by the addition of 0·05, 0·5 or 5 μg/ml anti-OVA pAbs to the plates and they were further incubated at 37° for 1 hr. Similarly, 3 μg/ml O1-10 Fabs were incubated with OVA followed by the addition of 1, 3 or 30 μg/ml OE-1. This was further followed by the addition of alkaline phosphatase-conjugated anti-Fc of mouse IgG and IgE to measure anti-OVA pAbs and OE-1 binding to OVA, respectively. Keyhole limpet haemocyanin (Sigma-Aldrich) was used as a control antigen.

Statistics

Statistical analyses were performed by one-way analysis of variance. If significant differences were detected, individual group differences were determined by a Bonferroni–Dunn test. A P value of $< 0{\cdot}05$ was considered statistically significant.

Results

Effects of the i.t. exposure to anti-OVA IgG1mAb (O1-10) Fabs on early and late asthmatic responses

Intratracheal administration of 120 μg O1-10 Fabs 30 min before antigenic challenge with OVA markedly suppressed sRaw in EAR induced by passive sensitization with anti-OVA pAbs (Fig. 1a,b). sRaw in LAR induced by active sensitization with OVA was also significantly suppressed by O1-10 Fabs administered 30 min before the last (fourth) antigenic challenge, although sRaw in EAR tended to be reduced by the Fab fragments

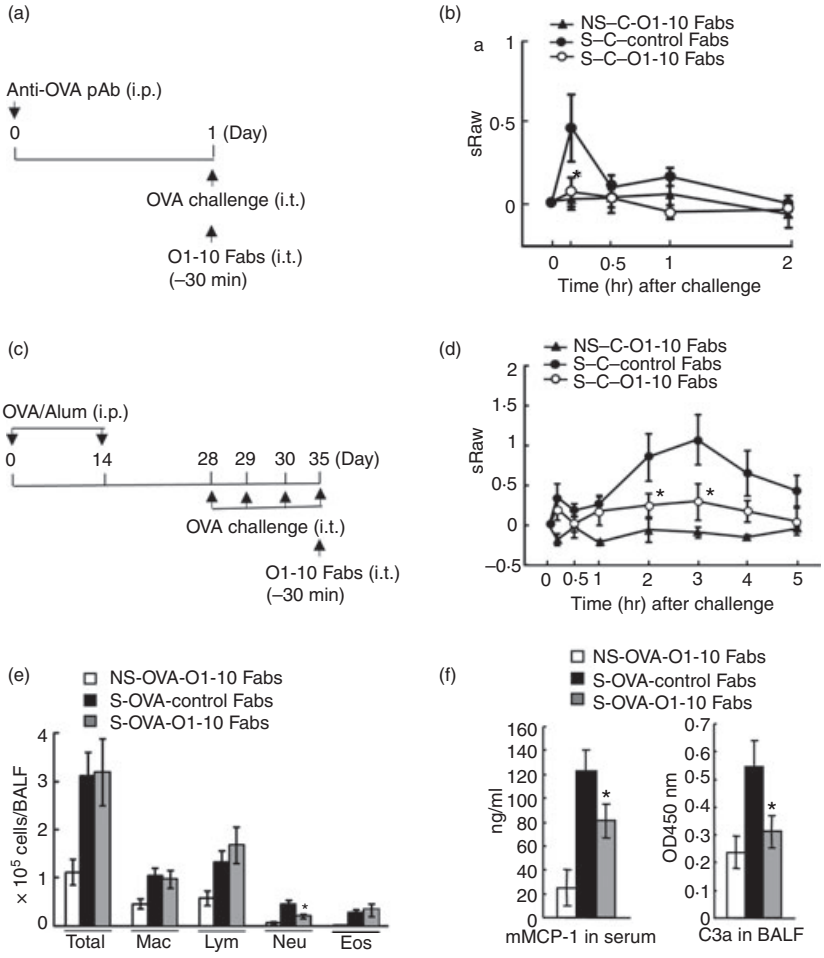

Figure 1. Intratracheal (i.t.) exposure to O1-10 Fabs 30 min before antigenic challenge suppresses asthmatic responses. Mice sensitized (S) with anti-ovalbumin (OVA) polyclonal antibodies (pAbs) on day 0 were i.t. challenged (C) with 40 μg OVA on day 1 (a) followed by measurement of specific airway resistance (sRaw) (b). One hundred and twenty micrograms of O1-10 Fabs (S-C-O1-10 Fabs) and control Fabs (S-C-control Fabs) were i.t. administered 30 min before the antigenic challenge. Non-sensitized (NS) mice given OVA were also i.t. treated with O1-10 Fabs (NS-C-O1-10 Fabs). Mice were also sensitized (S) with OVA on days 0 and 14 and i.t. challenged (C) with 200 μg of OVA on days 28, 29 and 30, and with 40 μg on day 35 (c). After the fourth antigenic challenge, sRaw (d), inflammatory cells including macrophages, lymphocytes, neutrophils and eosinophils in bronchoalveolar lavage fluid (BALF) (e), mouse mast cell protease-1 (mMCP-1) in sera and C3a in BALF (f) were measured. One hundred and twenty micrograms of O1-10 Fabs (S-C-O1-10 Fabs) and control Fabs (S-C-control Fabs) were i.t. administered only once 30 min before the fourth challenge with OVA on day 35. Non-sensitized (NS) mice given OVA were also i.t. administered O1-10 Fabs (NS-C-O1-10 Fabs). Data are means ± SEM of five to seven mice. $*P < 0{\cdot}05$ versus control Fabs. Data are representative of three independent experiments.

(Fig. 1c,d). When the number of inflammatory cells including macrophages, lymphocytes, neutrophils and eosinophils in BALF was measured at 24 hr after the last challenge, i.t. treatment with O1-10 Fabs resulted in significantly reduced infiltration of neutrophils but not other cells (Fig. 1e). There were also significantly lower levels of mMCP-1 in sera as well as C3a in BALF in mice treated with O1-10 Fabs (Fig. 1f). The suppression of sRaw in LAR and the infiltration of neutrophils in BALF by the i.t. exposure to 13, 40 and 120 μg of O1-10 Fabs were dose-dependent (see Supporting information, Fig. S1a–c). Histological examination of the lung of mice with asthmatic responses i.t. treated with O1-10 Fabs appeared to show the reduction of neutrophil infiltration (Fig. 2a–d). The trial of immunohistochemical staining of neutrophils with an anti-Gr-1 mAb also showed that Gr-1$^+$ cells appeared to be decreased in mice treated with O1-10 Fabs (Fig. 2e–h). Treatment with O1-10 Fabs appeared to reduce the production of IL-1β in the lung (Fig. 2i–l). The levels of chemokines including KC and MIP-2, inflammatory cytokines IL-1β, IL-6, T helper type 2 cytokines IL-4, IL-5, IL-13 and IL-17A as well as IL-23 in lung tissues were lower in mice treated with O1-10 Fabs than in those with control Fabs (Fig. 3a–i). The suppressive effect of O1-10 Fabs on asthmatic responses was also observed when the

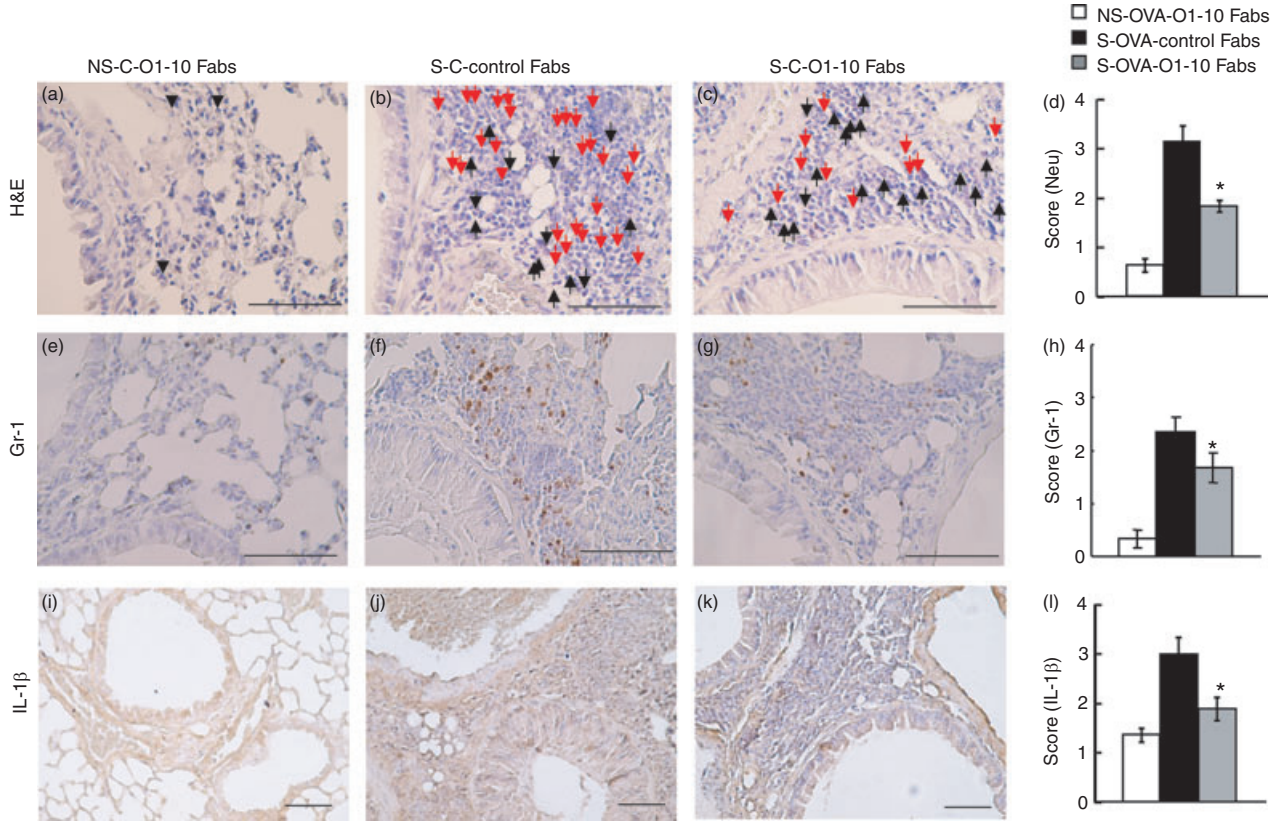

Figure 2. Intratracheal (i.t.) exposure to O1-10 Fabs reduces the infiltration of neutrophils and production of interleukin-1β (IL-1β) in the lung tissue of mice with asthmatic responses. Mice sensitized (S) with ovalbumin (OVA) on days 0 and 14 were i.t. challenged (C) with 200 μg OVA on days 28, 29 and 30, and with 40 μg on day 35 as shown in Fig. 1(a). One hundred and twenty micrograms of O1-10 Fabs (S-C-O1-10 Fabs) and control Fabs (S-C-control Fabs) were i.t. administered once 30 min before the fourth challenge with OVA on day 35. Non-sensitized (NS) mice given OVA were also i.t. treated with O1-10 Fabs (NS-C-O1-10 Fabs). Twenty-four hours after the fourth antigenic challenge, histological changes in the lung (a–c, ×40) were examined by haematoxylin and eosin staining. Neutrophils and eosinophils are shown by red and black arrows, respectively. Gr-1$^+$ cells (e–g, ×40) and IL-1β (i–k, ×20) in the lung were also immunohistochemically stained. Scale bars in all photographs represent 100 μm. Neutrophil infiltration and IL-1β production in the lung tissue were also scored as shown in Materials and methods (d, h, l). Data are means ± SEM of five mice. *$P < 0{\cdot}05$ versus S-C-control Fabs. Data are representative of two independent experiments.

fragments were i.t. administered at the time of OVA challenge (see Supporting information, Fig. S2a–c). However, O1-10 Fabs failed to affect asthmatic responses when given 2 hr after the antigenic challenge (see Supporting information, Fig. S2d,e).

Effects of the i.t. exposure to O1-10 Fabs on anti-OVA IgE mAb (OE-1)-mediated asthmatic responses

To investigate whether O1-10 Fabs can suppress asthmatic responses induced by passive sensitization with antigen-specific IgE antibodies, anti-OVA IgE mAb (OE-1)[18,36] was used. The results showed that the i.t. exposure to O1-10 Fabs markedly down-regulated sRaw in both EAR and LAR (Fig. 4a,b), as well as infiltration of neutrophils in BALF (Fig. 4c). There was significantly reduced production of mMCP-1 in sera and C3a in BALF in O1-10 Fab-treated mice (Fig. 4d). The *in vitro* competitive analysis of OE-1 and O1-10 Fabs to OVA showed that the epitope of OVA recognized by OE-1 was different from that by O1-10 (Fig. 4e).

Effects of the i.t. exposure to anti-OVA IgG2b mAb (O2B-3) Fabs on asthmatic responses

To investigate whether not only anti-OVA IgG1 mAb Fabs (O1-10 Fabs) but also other isotypes of the antigen-specific mAb Fabs down-regulate asthmatic responses, anti-OVA IgG2b mAb (O2B-3) Fabs were administered 30 min before the fourth antigenic challenge. The results showed that O2B-3 Fabs were markedly able to suppress sRaw in EAR as well as LAR (Fig. 5a,b), the infiltration of neutrophils in BALF (Fig. 5c), and the production of mMCP-1 and C3a (Fig. 5d). The suppression of these asthmatic responses by O2B-3 Fabs was dose-dependent (see Supporting information, Fig. S1d,e). *In vitro* studies showed that the epitope of OVA recognized by O2B-3 Fabs was different from that by O1-10 (Fig. 5e).

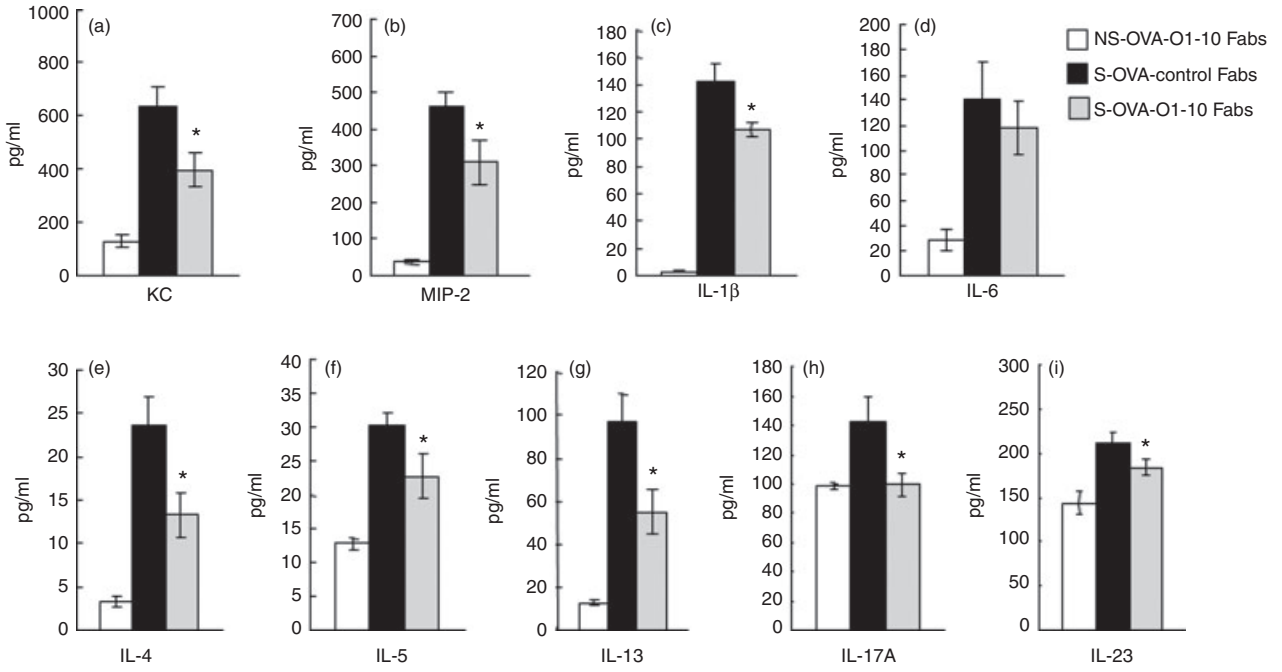

Figure 3. Effect of the intratracheal (i.t.) exposure to O1-10 Fabs on the production of various chemokines and cytokines in the lung of mice with asthmatic responses. Mice were sensitized (S) with ovalbumin (OVA) on days 0 and 14 and i.t. challenged (C) with 200 μg OVA on days 28, 29 and 30, and with 40 μg on day 35 as shown in Fig. 1(a). One hundred and twenty micrograms of O1-10 Fabs (S-C-O1-10 Fabs) and control Fabs (S-C-control Fabs) were i.t. administered only once 30 min before the last (fourth) challenge with OVA on day 35. Non-sensitized (NS) mice given OVA were also i.t. administered O1-10 Fabs (NS-C-O1-10 Fabs). Keratinocyte-derived chemokine (KC) (a), macrophage inflammatory protein 2 (MIP-2), (b), interleukin-1β (IL-1β) (c), IL-6 (d), IL-4 (e), IL-5 (f), IL-13 (g), IL-17A (h), and IL-23 (i) from the lung homogenate obtained 24 hr after the last antigenic challenge were measured as described in the Materials and methods. Data are means ± SEM of five to seven mice. *$P < 0{\cdot}05$ versus control Fabs.

The effect of the i.t. exposure to intact O1-10 and O2B-3 or the systemic injection of O1-10 and O2B-3 Fabs on asthmatic responses

The effects of the i.t. exposure to intact O1-10 mAb and O2B-3 mAbs given 30 min before the fourth challenge on asthmatic responses were also investigated. The results showed that both intact mAbs failed to affect sRaw in EAR as well as LAR and infiltration of inflammatory cells including neutrophils in BALF (see Supporting information, Fig. S3a–c). To examine the effect of the systemic injection of O1-10 and O2B-3 Fabs on asthmatic responses, the mAb Fabs were i.p. administered. The results showed that O1-10 and O2B-3 Fabs failed to influence sRaw in both EAR and LAR as well as infiltration of inflammatory cells including neutrophils in BALF (see Supporting information, Fig. S3d–g).

Effects of the capture of OVA by O1-10 Fabs on the subsequent binding of anti-OVA pAb to the captured antigen

To investigate the mechanism by which asthmatic responses are suppressed by the i.t. exposure to O1-10 Fabs, 96-well plates were coated with OVA followed by the addition of 0·005, 0·05, 0·5 and 5 μg/ml O1-10 Fabs to the plates to capture the antigen. This was followed by the addition of 0·05, 0·5 or 5 μg/ml anti-OVA pAbs to examine the ability of the pAbs to bind to the captured OVA. The results showed that the binding of 0·05 and 0·5 μg/ml anti-OVA pAbs to OVA was reduced by the OVA-capturing O1-10 Fabs in a dose-related fashion (Fig. 6a). However, the binding of OVA and O1-10 Fabs in advance failed to affect the subsequent interaction between OVA and 5 μg/ml anti-OVA pAbs. Similar results were obtained when O2B-3 Fabs were used (data not shown). The IC_{50} of O1-10 and O2B-3 Fabs for anti-OVA pAbs (0·05 μg/ml) were 7·8 and 17·4 μg/ml, respectively. The capture of OVA by O1-10 Fabs was also effective in preventing the subsequent binding between OVA and 1 and 3 μg/ml, but not 30 μg/ml, intact OE-1 (Fig. 6b).

Discussion

The present study suggests that i.t. exposure to Fabs of a pathogenic antigen-specific mAb may be effective in regulating asthmatic responses because either O1-10 (anti-OVA IgG1 mAb) or O2B-3 (anti-OVA IgG2b mAb)

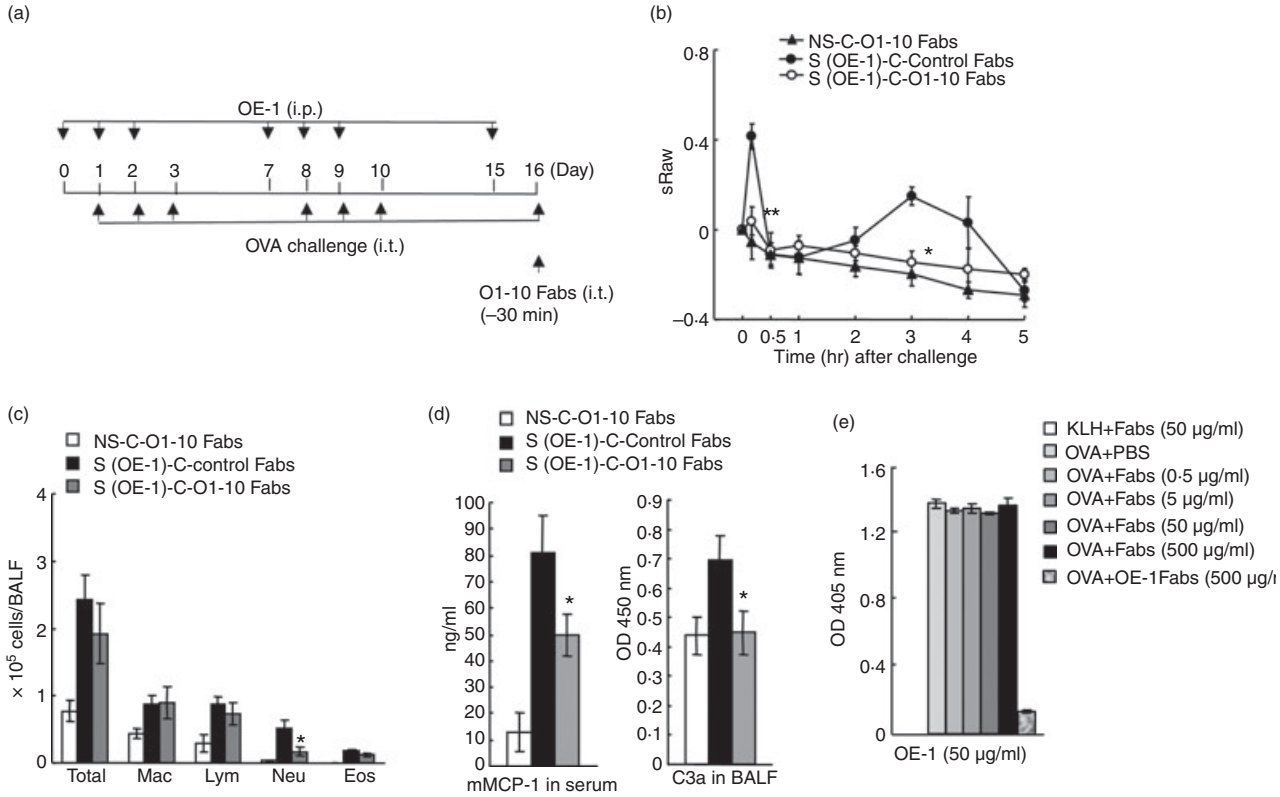

Figure 4. Intratracheal (i.t.) exposure to O1-10 Fabs suppresses anti-ovalbumin (OVA) IgE monoclonal antibody (mAb) OE-1-mediated asthmatic responses. Mice sensitized (S) with 100 μg anti-OVA IgE mAb OE-1 on days 0, 1, 2, 7, 8, 9 and 15 were i.t. challenged (C) with 200 μg OVA on days 1, 2, 3, 8, 9 and 10 and with 40 μg OVA on day 16 (a). After the last (seventh) antigenic challenge, specific airway resistance (sRaw) (b), inflammatory cells including macrophages, lymphocytes, neutrophils, and eosinophils in bronchoalveolar lavage fluid (BALF) (c), mouse mast cell protease-1 (mMCP-1) in sera, and C3a in BALF (d) were measured. One hundred and twenty micrograms of O1-10 Fabs (S-C-O1-10 Fabs) or control Fabs (S-C-control Fabs) were i.t. administered 30 min before the seventh challenge with OVA. Non-sensitized (NS) mice given OVA were also i.t. treated with O1-10 Fabs (NS-C-O1-10 Fabs). Data are means ± SEM of five mice. $*P < 0{\cdot}05$ versus S-C- control Fabs. Either 50 μg/ml OE-1 alone or a mixture of 50 μg/ml OE-1 and 0·5, 5, 50 or 500 μg/ml O1-10 Fabs was added to OVA coated on 96-well plates followed by further addition of anti-Fc of IgE (e). Anti-OE-1 Fabs were used as a positive control. Keyhole limpet haemocyanin was used as a control antigen. Data are representative of two independent experiments.

Fabs i.t. administered to mice 30 min before the antigenic challenge suppressed sRaw in EAR and/or LAR induced by passive sensitization with anti-OVA pAbs as well as by active sensitization with OVA. This is the first report demonstrating the antigen-specific regulation of asthmatic responses by mAb Fabs to the disease-causing antigen. Although hyposensitization for allergy is currently available as an antigen-specific therapy, 1 or 2 years are often required before patients benefit from this approach.[24–27] Local and systemic anaphylactic reactions are also often seen in patients treated with this antigen-specific immunotherapy.[28–30]

The suppression of asthmatic responses by the i.t. exposure to OVA-specific mAb Fabs appears to be due to the unique structure and function of Fabs in that they maintain antigen-binding sites but lack an Fc portion for binding to receptors on immune and inflammatory cells that play an important role in asthma.[6,7] Fabs also lack the other binding part of Fc for complement, resulting in the failure of complement activation. However, a number of studies have shown that there are at least five different immunogenic epitopes of OVA reacting with anti-OVA IgE antibodies from patients with allergy to eggs or from mice sensitized with OVA.[41–45] Therefore, it was an unexpected result that Fabs of O1-10 or O2B-3 recognizing only one epitope of OVA markedly suppressed asthmatic responses induced by either passive sensitization with anti-OVA pAbs or active sensitization with OVA. The suppression of asthmatic responses by O1-10 Fabs was also seen when the *in vivo* responses were induced by sensitization with anti-OVA IgE mAb (OE-1)[18,36] recognizing an epitope different from that by O1-10. It would be expected that asthmatic responses induced by passive sensitization with OE-1 and anti-OVA pAbs are suppressed by Fabs of the mAb and pAbs, respectively. In fact, we previously demonstrated that Arthus type reaction-mediated antigen-induced arthritis in mice[46–49] induced by passive sensitization with anti-OVA pAbs followed by

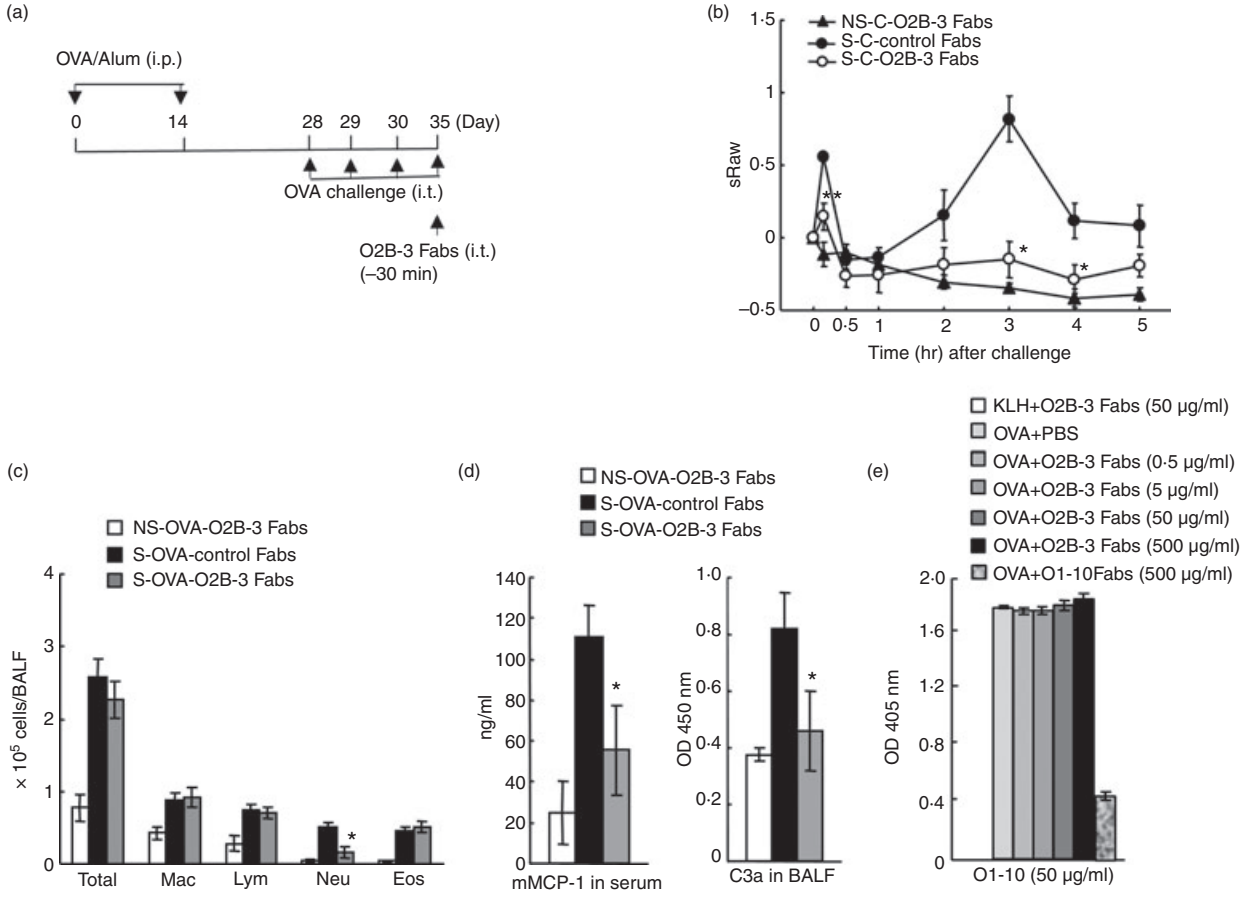

Figure 5. Intratracheal (i.t.) exposure to O2B-3 Fabs suppresses asthmatic responses. Mice sensitized (S) with ovalbumin (OVA) on days 0 and 14 were i.t. challenged (C) with 200 μg of OVA on days 28, 29 and 30 and with 40 μg of OVA on day 35 (a). After the fourth antigenic challenge, specific airway resistance (sRaw) (b), inflammatory cells including macrophages, lymphocytes, neutrophils and eosinophils in bronchoalveolar lavage fluid (BALF) (c), mouse mast cell protease-1 (mMCP-1) in sera, and C3a in BALF (d) were measured. One hundred and twenty micrograms of O2B-3 Fabs (S-C-O2B-3 Fabs) or control Fabs (S-C-control Fabs) were i.t. administered once 30 min before the last antigenic challenge with OVA on day 35. Non-sensitized (NS) mice given OVA were also i.t. treated with O1-10 Fabs (NS-C-O1-10 Fabs). Data are means ± SEM of five mice. $*P < 0{\cdot}05$ versus control Fabs. Fifty micrograms of intact O1-10 alone or a mixture of 50 μg/ml intact O1-10 and 0·5, 5, 50 or 500 μg/ml O2B-3 Fabs was added to OVA coated on 96-well plates followed by further addition of anti-Fc of IgG to the plates (e). Anti-O1-10 Fabs were used as a positive control. Keyhole limpet haemocyanin was used as a control antigen. Data are representative of two independent experiments.

intra-articular injection of the antigen was suppressed by their Fabs.[35] The finding that asthmatic responses are suppressed by Fabs of an anti-OVA mAb is far more important than those of anti-OVA pAbs in terms of their clinical application as a number of mAbs but not pAbs have been used to treat various diseases in humans.[50–53]

The binding of small amounts, but not large amounts, of intact anti-OVA pAbs and OE-1 with OVA was prevented when the antigen was captured by O1-10 Fabs in advance, as shown in our *in vitro* studies. The suppression of asthmatic responses by O1-10 Fabs was seen when the fragments were i.t. administered 30 min before the antigenic challenge. Therefore, the pre-formed OVA and O1-10 Fab complexes in the respiratory tract appeared to block the subsequent interaction between the antigen and the intact pAbs that is essential for the induction of asthmatic responses. The amount of anti-OVA pAbs present in the respiratory tract appears to be relatively small compared with that in serum. This may be supported by the finding that the systemic (i.p.) injection of O1-10 Fabs failed to affect asthmatic responses. Our recent studies also showed that allergic footpad reactions in mice induced by passive sensitization with anti-OVA pAbs followed by subcutaneous injection of OVA into their footpads was suppressed when O1-10 Fabs were locally co-administered with the antigen but not injected systemically (unpublished data). The amount of intact anti-OVA pAbs or OE-1 present around the pre-formed complexes of O1-10 Fabs and OVA appears to be important because, when there is a small amount of these antibodies, they

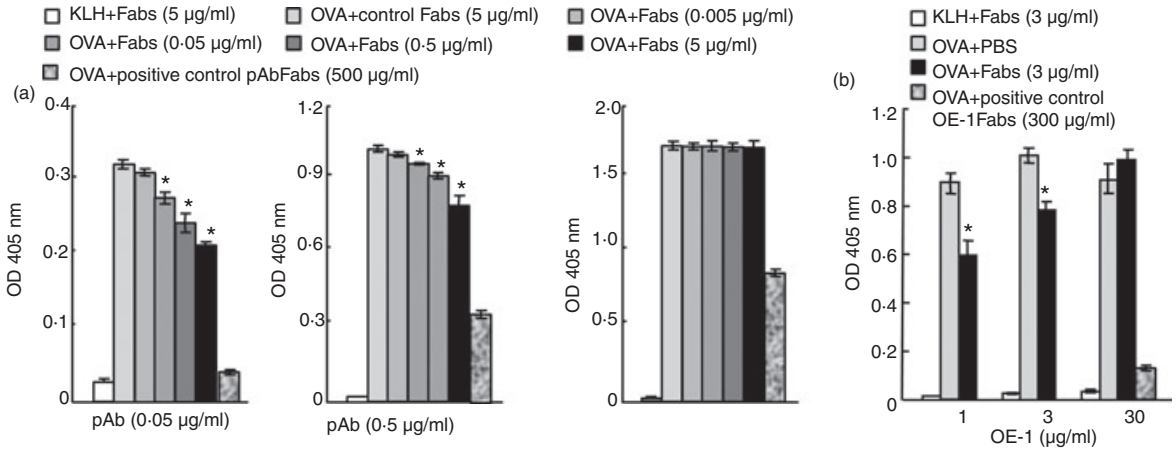

Figure 6. The capture of ovalbumin (OVA) by O1-10 Fabs in advance prevents the subsequent binding of small but not large amounts of intact anti-OVA polyclonal antibodies (pAbs) or intact OE-1 to the captured OVA. The indicated amounts of O1-10 Fabs were added to OVA coated on 96-well plates. This was followed by further addition of 0·05, 0·5 and 5 μg/ml intact anti-OVA pAbs (a) or 1, 3 or 30 μg/ml intact OE-1 (b) to the plates. Then anti-Fc of IgG or IgE was added to investigate the ability of these intact antibodies to bind OVA. Anti-OVA pAb Fabs or anti-OE-1 Fabs were used as a positive control. $*P < 0{\cdot}05$ versus control Fabs. Data are representative of three independent experiments.

have fewer chances to bind the captured OVA, but when there is a large amount of the antibodies, they have more chances to interact with the antigen.

The regulation of sRaw in EAR by O1-10 or O2B-3 Fabs appears to suggest that the fragments lacking Fc were effective in suppressing degranulation of mast cells playing a role in EAR.[6] This is supported by the decreased serum level of mMCP-1 as an indicator of degranulation of mast cells[54–56] in mice treated with O1-10 Fabs. The attenuation of LAR by these Fabs that also lack the binding site for complement suggests that complement activation might have been suppressed in mice given the fragments. In fact, the serum levels of C3a were lower in mice treated with O1-10 Fabs as shown in the present study. The decrease in neutrophils in the BALF from mice administered O1-10 Fabs also appears to support the lower levels of C3a as this complement product is chemotactic for neutrophils.[57–59] The serum levels of KC, MIP-1, IL-17A and IL-33 involved in the recruitment of neutrophils were lower in the Fab treatment group. The decreased infiltration of neutrophils and lower production of inflammatory cytokines including IL-1β and IL-6 in the lung tissue of animals treated with O1-10 Fabs appear to support this further.

The number of eosinophils in BALF as well as in lung tissues was not affected by OVA-specific mAb Fabs. It is well known that accumulation of eosinophils in the lung is characteristic of human asthma.[11,12] There is also evidence of marked T helper type 2 cytokines including IL-5 in airway tissue in asthma.[2,10] However, it is not clear whether eosinophils do play a critical role in asthma. For instance, clinical studies showed that asthmatic symptoms were not ameliorated by humanized anti-IL-5 mAbs that markedly depleted eosinophils in the circulation as well as in the airway tissue.[13,14] Clinical studies have also demonstrated that some asthmatics, especially those with severe asthma, have increased neutrophils in their airways, suggesting a role of this type of inflammatory cell in the disease.[15,16] Furthermore, complement factors including C3a also appear to be involved in human asthma.[60,61] We previously revealed that neutrophils and complement activation play a role in LAR in the asthma model[17–19] that was also used in the present study. However, relatively high doses of OVA were used at the time of the antigenic challenge to induce LAR in this murine model. The use of high doses of the antigen might have caused severe acute inflammation, resulting in infiltration of more neutrophils than eosinophils, as shown in our experiments. Kumar and Foster[62] suggested that the roles of cells and molecules in asthmatic responses in mice were altered by changing the protocol (dose and duration) for challenge.

Alternatively, the failure of eosinophil recruitment to the lung by OVA-specific Fabs might in part be explained by the fact that the fragments were administered only once 30 min before the last (fourth) OVA challenge. The infiltration of eosinophils but not neutrophils was already observed in the lung before the fourth antigenic challenge (unpublished data). The antigen-specific Fabs might have been effective in decreasing eosinophils if the Fab fragments had been administered throughout the first to fourth antigenic challenges because the production of IL-5 as well as IL-4 and IL-13 was suppressed in mice treated with anti-OVA mAb Fabs, as shown in the present study. BALF and lungs were collected to measure eosinophils at 24 hr after the fourth OVA challenge. This

timing of collection of these materials might also explain why there was no difference in the number of this type of cell in control and Fab treatment groups. The suppression of eosinophil recruitment by the antigen-specific Fabs might have been observed if BALF and lungs had been collected later.

The failure of the suppression of asthmatic responses by i.t. administration of intact O1-10 (IgG1) and O2B-3 (IgG2b) appears to indicate that these intact antibodies could interact with mast cells and complement via the sites of their Fc. Mast cells have been shown to express not only FcγRI but also FcγR on their surface, which are also receptors for IgG1 and IgG2b.[63–65]

Antibody Fabs used to treat murine or human diseases include anti-IL-13 mAb and digoxin-specific Fabs. Hacha *et al.*[66] demonstrated that nebulized anti-IL-13 mAb Fab' fragments reduced allergen-induced asthma in mice. Digoxin-specific Fabs have been used to treat patients with potentially life-threatening digitalis toxicity.[31–33] However, no studies have been performed on the applicability of allergen-specific mAb Fabs in diseases in humans as well as animals.

It is noteworthy that the suppressive effects of O1-10 Fabs on asthmatic responses were observed when mice were i.t. treated with the Fabs only once 30 min before the last antigenic challenge, suggesting that asthmatic attacks in humans might also be prevented by the inhalation of antigen-specific antibody Fabs, although a pathogenic antigen causing asthma has not been completely defined. Inhalation of proteins such as pathogenic antigen-specific Fabs would be possible because insulin has been inhaled by patients with diabetes mellitus.[67–70]

In conclusion, asthma may be specifically regulated by i.t. exposure to Fabs of a pathogenic antigen-specific mAb via a mechanism involving the mAb Fabs capturing the antigen in the respiratory tract before the interaction between intact antibodies and the antigen that is required for the induction of asthmatic responses.

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Disclosures

The authors have declared that they have no conflicts of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Dose-related suppression of specific airway resistance and infiltration of inflammatory cells in bronchoalveolar lavage fluid in mice with asthmatic responses by intratracheal (i.t.) exposure to O1-10 or O2B-3 Fabs.

Figure S2. Intratracheal administration of O1-10 Fabs at the same time as but not at 2 hr after antigenic challenge suppresses asthmatic responses.

Figure S3. Neither intratracheal exposure to intact anti-ovalbumin O1-10 and O2B-3 nor systemic injection of their Fabs affects asthmatic responses.