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Complement C3a-Induced IL-17 Plays a Critical Role in an IgE-Mediated Late-Phase Asthmatic Response and Airway Hyperresponsiveness via Neutrophilic Inflammation in Mice

Nobuaki Mizutani,* Hirofumi Goshima,* Takeshi Nabe,† and Shin Yoshino*

Allergen-specific IgE plays an essential role in the pathogenesis of allergic asthma. Although there has been increasing evidence suggesting the involvement of IL-17 in the disease, the relationship between IL-17 and IgE-mediated asthmatic responses has not yet been defined. In this study, we attempted to elucidate the contribution of IL-17 to an IgE-mediated late-phase asthmatic response and airway hyperresponsiveness (AHR). BALB/c mice passively sensitized with an OVA-specific IgE mAb were challenged with OVA intratracheally four times. The fourth challenge caused a late-phase increase in airway resistance associated with elevated levels of IL-17$^+$CD4$^+$ cells in the lungs. Multiple treatments with a C3a receptor antagonist or anti-C3a mAb during the challenges inhibited the increase in IL-17$^+$CD4$^+$ cells. Meanwhile, a single treatment with the antagonist or the mAb at the fourth challenge suppressed the late-phase increase in airway resistance, AHR, and infiltration by neutrophils in bronchoalveolar lavage fluid. Because IL-17 production in the lungs was significantly repressed by both treatments, the effect of an anti–IL-17 mAb was examined. The late-phase increase in airway resistance, AHR, and infiltration by neutrophils in bronchoalveolar lavage fluid was inhibited. Furthermore, an anti–Gr-1 mAb had a similar effect. Collectively, we found that IgE mediated the increase of IL-17$^+$CD4$^+$ cells in the lungs caused by repeated Ag challenges via C3a. The mechanisms leading to the IgE-mediated late-phase asthmatic response and AHR are closely associated with neutrophilic inflammation through the production of IL-17 induced by C3a. ***The Journal of Immunology*****, 2012, 188: 000–000.**

Elevated allergen-specific IgE levels in serum are a hallmark of allergic asthma, a disease characterized by allergen-induced early and late asthmatic responses, airway hyperresponsiveness (AHR), and airway inflammation (1–3). IgE plays a primary role in triggering the early asthmatic response through mast cell and/or basophil degranulation followed by chemical mediator-induced acute narrowing of the airway within minutes after exposure to an inhaled Ag (4). Meanwhile, several clinical and experimental investigations have suggested that Ag-specific memory T cells, especially CD4$^+$ T cells, play an integral role in orchestrating chronic asthmatic responses such as the late response, AHR, and airway inflammation through the secretion of a variety of Th2-type cytokines including IL-4, IL-5, and IL-13 (2, 5–8). Additionally, IgE has the capacity to induce the production of Th2-type cytokines and the development of airway inflammation and AHR in mice (9–11), indicating the importance of IgE in chronic asthmatic responses. More interestingly, in clinical studies, the severity of symptoms in some poorly controlled asthmatic patients was improved by treatment with a humanized anti-IgE Ab (12–14). Thus, allergen-specific IgE can be indispensable for the chronic pathogenesis of asthma, creating a vicious cycle in asthmatic patients.

In addition, there has been increasing evidence of important roles for IL-17–secreting CD4$^+$ T cells (Th17 cells) in allergic asthma as well. Increased levels of IL-17 have been found in bronchoalveolar lavage fluid (BALF), sputum, and blood from patients with allergic asthma (15–20). In mouse models of allergic asthma, systemic blockage of IL-17 inhibited the allergen-induced accumulation of neutrophils in the airway and AHR (20–22), suggesting that IL-17 plays an important role during the pathophysiological process of allergic asthma. Furthermore, Lajoie et al. (23) have reported that not only severe AHR but also the increases in IL-17 production and IL-17$^+$CD4$^+$ cells in asthmatic lungs were reduced in complement C3a receptor-deficient mice. However, the role of IL-17 in allergic asthma has not been fully elucidated, especially in the late-phase asthmatic response; furthermore, the relationship between IL-17 and IgE-mediated asthmatic responses has been unknown.

We have previously established a murine model of allergic asthma that shows early and late-phase asthmatic responses and AHR after four repeated intratracheal Ag challenges in BALB/c mice passively sensitized with an i.p. injection of Ag-specific IgE mAb (24). A key feature of this IgE-sensitized model was that the late-phase asthmatic response was not induced by the first to third challenge but required the fourth challenge administered 5 d after the third, suggesting that the first three challenges (induction phase) elicited changes that conditioned the mice for a qualitatively different response as a consequence of the fourth challenge (effector phase). Additionally, treatment with a C3a receptor antagonist at the fourth challenge (effector phase) suppressed the late-phase asthmatic response and AHR, indicating C3a to be involved in their development (24). However, the precise

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Abbreviations used in this article: AHR, airway hyperresponsiveness; BALF, bronchoalveolar lavage fluid; MCh, methacholine; OE-1, OVA-specific IgE mAb; PAS, periodic acid–Schiff; sRaw, specific airway resistance.

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mechanisms underlying the late-phase asthmatic response and AHR in this model remain unclear.

In the current study, we sought to define the cells and molecules essential to the development of the late-phase asthmatic response and AHR in this IgE-sensitized model, focusing on the role of IL-17 in these IgE-mediated responses. First, we examined the role of $CD4^+$ cells in the late asthmatic response by conducting pathological analyses using an anti-CD4 mAb. Second, using a C3a receptor antagonist or anti-C3a mAb, we determined whether C3a produced during the first three challenges (induction phase) is critical for the increases of IL-17^+CD4^+ cells in the lungs at the fourth challenge. Third, we assessed whether the C3a-induced responses at the fourth challenge (effector phase) contribute to the late-phase asthmatic response and AHR by conducting analyses using a C3a receptor antagonist, anti-C3a mAb, anti–IL-17 mAb, and anti–Gr-1 mAb.

Materials and Methods

Animals

Male 7-wk-old BALB/c mice were obtained from Japan SLC. These mice were maintained in a temperature-controlled environment with free access to standard rodent chow and water. All of the experimental procedures were approved by the Experimental Animal Research Committee at Kobe Pharmaceutical University.

OVA-specific IgE mAb

The OVA-specific IgE mAb (OE-1) was derived from a B cell hybridoma producing murine IgE as described previously (25). The hybridoma was grown in the CELLine CL1000 with BD-Cell-MAb medium (BD Biosciences, San Diego, CA) supplemented with 20% heat-inactivated FBS, 1% L-glutamine, and 1% penicillin–streptomycin. OE-1 levels in culture supernatants of hybridoma were assayed by ELISA. OE-1 was detected by using plates coated with anti-mouse IgE Ab and adding biotin-labeled anti-mouse IgE Ab. Alkaline phosphate anti-biotin was added, the plate was developed with *p*-nitrophenyl phosphate, and measurements were made at 405 nm using a microplate reader. OE-1 levels were calculated by comparison with mouse IgE standards (Southern Biotech, Birmingham, AL).

Passive sensitization with a specific IgE mAb

Passive sensitization with OE-1 was performed according to a previously described method (24). As shown in Fig. 1A, BALB/c mice were passively sensitized with repeated i.p. injections of a hybridoma supernatant containing OE-1 (100 μg/mouse) on days 0, 1, 2, and 7. Nonsensitized mice were injected with a culture supernatant of the parental myeloma cell line. Both the sensitized and nonsensitized mice were challenged on days 1, 2, 3, and 8 under anesthesia with escain (Mylan, Osaka, Japan) with 1% OVA (grade V; Sigma-Aldrich, St. Louis, MO) in a volume of 20 μl by intratracheal administration as reported (26, 27). Additionally, the mice sensitized with OE-1 on days 0, 1, 2, and 7 were challenged on days 1, 2, and 3 with 1% OVA, and then with saline on day 8 (OE-1–sensitized nonchallenged group). The dose of OE-1 has been reported to cause early- and late-phase increases in airway resistance and AHR (24).

Furthermore, to investigate whether purified OE-1 also induces asthmatic responses, OE-1 in cultured supernatants was purified with a HiTrap affinity column. In the same protocol as that for Fig. 1A, BALB/c mice i.p. injected with a purified OE-1 (100 μg/mouse) or PBS were repeatedly intratracheally challenged with 1% OVA (Supplemental Fig. 1A).

Treatment with mAbs or a C3a receptor antagonist

A cell line producing rat IgG2b mAbs that recognize the murine CD4 molecule (YTS191.1.2) was kindly provided by Prof. David D. Chaplin (University of Alabama at Birmingham, Birmingham, AL). The anti-CD4 mAb was produced and purified as described previously (28, 29). The purified anti-CD4 mAb or rat IgG2b (eBioscience, San Diego, CA) was i.p. administered 30 min before the first (Fig. 2A) or fourth sensitization with OE-1 at a dose of 0.6 mg/animal (Fig. 3A). It has been reported that the treatment with anti-CD4 mAb 18 h before the first or fourth administration of Ag in mice actively sensitized with OVA plus alum significantly reduced the number of $CD4^+$ cells in BALF, peripheral blood, and spleen at the fourth challenge (28, 29).

A C3a receptor antagonist, SB290157 (0.6 mg/mouse) (Calbiochem, San Diego, CA), was i.p. administered 30 min before each of the challenges on days 1, 2, 3, and 8 (Fig. 4A, multiple treatment). Furthermore, to test whether the first three Ag challenges increase IL-17^+CD4^+ cells in the lungs via C3a, the C3a receptor antagonist was administered 30 min before each of the challenges on days 1, 2, and 3, and the IL-17^+CD4^+ cells in the lungs 5 d after the third challenge (day 8) were measured (Fig. 5A, multiple treatment). In another experiment, SB290157 (0.6 mg/mouse) was i.p. injected only once 30 min before the fourth challenge (Fig. 6A, single treatment). The dose of inhibitor has been reported to cause an almost maximal inhibition of a late-phase increase in airway resistance in OVA plus alum-sensitized mice (27). Furthermore, to corroborate the studies using a C3a receptor antagonist, we examined the effects of neutralizing mAb against C3a (clone 3/11; HyCult Biotechnology, Uden, The Netherlands) on the increase in IL-17^+CD4^+ cells and the asthmatic responses at the fourth challenge. A dose (5 μg/mouse) of anti-C3a mAb or rat IgG2a (R&D Systems, Minneapolis, MN) was intratracheally given 30 min before each of the first three challenges (Fig. 5A, multiple treatment) or the fourth challenge (Fig. 7A, single treatment).

As shown in Fig. 8A, a single dose (150 μg/mouse) of anti–IL-17 mAb (clone 50104; R&D Systems) was administered i.p. 30 min before the fourth challenge in mice sensitized with OE-1 (single treatment). In another experiment, anti–IL-17 mAb (150 μg/mouse) was administered 30 min before each of the challenges on days 1, 2, and 3 (Fig. 6F, multiple treatment). Control mice were given the same amount of rat IgG2a (R&D Systems). The dose of the anti–IL-17 mAb has been reported significantly to reduce the accumulation of neutrophils in BALF in a murine model of asthma (30).

In accordance with a previously reported method (29, 31), an i.p.-administered dose (150 μg/mouse) of an anti–Gr-1 mAb (RB6-8 C5; eBioscience) or rat IgG2b (eBioscience) was given 30 min before the fourth sensitization with OE-1 (Fig. 9A).

Measurement of airway resistance

To evaluate the degree of early- and late-phase increases in airway resistance, specific airway resistance [sRaw; $cmH_2O \times mL/(mL/s)$] was measured in conscious mice before and 10 min to 5 h after the first and fourth challenges using a two-chambered, double-flow plethysmograph system (Pulmos-I; M.I.P.S., Osaka, Japan) according to the method of Pennock et al. (32).

Measurement of AHR to methacholine

AHR to methacholine (MCh) was assessed 24 h after the fourth challenge. Briefly, increasingly higher doses of MCh (3.125, 6.25, and 12.5 μg/ml) in solution were consecutively administered via the intratracheal route to nonsensitized and sensitized mice under escain-induced anesthesia at 30-min intervals. sRaw was measured 2 min after the respective instillations of the three doses of MCh.

Analysis of cells recovered by bronchoalveolar lavage

To evaluate airway inflammation, we examined the accumulation of inflammatory cells in BALF as described previously (24, 27). Animals were killed with diethyl ether. The trachea was cannulated, and the left bronchi were tied for histological or flow cytometric examination. Then, the right air lumen was washed twice with 0.5 ml HBSS. The recovered lavage fluid was centrifuged at $120 \times g$ for 5 min at 4°C. The cell pellet was suspended with a defined volume (200 μl/sample) of HBSS. The total leukocyte count in the lavage fluid was determined by staining with Turk's solution. For differential cell counts, bronchoalveolar lavage cells were stained with Diff-Quik solution (Sysmex International Reagent, Kobe, Japan).

Histological analysis

The left lungs were fixed in 10% neutral-buffered formalin, then dissected, embedded in paraffin, and cut 4 μm thick. Sections were stained with H&E and periodic acid–Schiff (PAS) as described previously (24, 27).

Immunohistochemistry was performed with paraffin-embedded sections. Serial 4-μm-thick sections of lung were mounted on glass slides, dewaxed, and rehydrated with PBS. Endogenous peroxidase was blocked with 3% H_2O_2 in water for 30 min. After the blocking of nonspecific binding with diluted normal rabbit serum in PBS for 20 min, the sections were incubated for 1 h at room temperature with a polyclonal Ab against IL-17 (SC-1251, goat IgG; Santa Cruz Biotechnology, Santa Cruz, CA) or a polyclonal Ab against IL-23 (AF-3626, goat IgG; R&D Systems). The slides were developed using the Vectastain Elite ABC goat IgG kit and the diaminobenzidine substrate kit for peroxidase (Vector Laboratories, Burlingame, CA). Counterstaining was done with Mayer's hematoxylin. As a negative control, goat IgG was used.

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Scoring for each section was evaluated by a blinded observer on a scale of 0–4 with increments of 0.5 for inflammation (H&E), goblet cell hyperplasia (PAS), and immunohistochemistry (IL-17 and IL-23).

Measurement of OVA-specific IgG1 levels in serum

Levels of the OVA-specific IgG1 Ab in serum were measured by ELISA, as described (24, 33). OVA-specific IgG1 was detected by using plates coated with OVA and adding alkaline phosphate-conjugated anti-mouse IgG1. The plates were developed with *p*-nitrophenyl phosphate and read at 405 nm using a microplate reader. Values for serum OVA-specific IgG1 (1:1000) were expressed as absorbance units.

Measurement of cytokines and C3a

Levels of IL-4 and IL-13 in BALF were measured using quantitative colorimetric sandwich ELISA kits (R&D Systems).

Because the amount of IL-17 in BALF was under the level detectable (<8 pg/ml) by ELISA, we measured the amount in supernatants of lung homogenates. The frozen right lobe of lungs (after bronchoalveolar lavage) was homogenized in 1 ml T-PER (Thermo Scientific, Rockford, IL) containing Complete Mini Protease Inhibitor Cocktail tablets (Roche, Mannheim, Germany; 1 tablet/10 ml T-PER stock reagent). Lung homogenates were centrifuged at 9000 × *g* for 10 min at 4°C. The levels of IL-17 and IL-23 in supernatants of lung homogenates were measured using quantitative colorimetric sandwich ELISA kits (BioLegend, San Diego, CA).

The level of C3a in BALF was measured by ELISA. C3a in BALF 24 h after the fourth challenge in mice sensitized with IgE was detected by using plates coated with rat anti-mouse C3a Ab (BD Biosciences) and adding biotin-labeled rat anti-mouse C3a Ab (BD Biosciences). Alkaline phosphatase-conjugated anti-biotin was added, the plate was developed with *p*-nitrophenyl phosphate, and measurements were made at 405 nm using a microplate reader. The value for C3a in BALF was expressed as absorbance units.

Lung cell isolation

The left lobe of the lungs was isolated, cut into 1-mm^3 pieces in digestion buffer [RPMI 1640 containing 150 U/ml collagenase (Wako, Osaka, Japan), 30 μg/ml DNase I (Sigma-Aldrich), and 10 mM HEPES] and incubated at 37°C for 1 h. The resulting single-cell suspension was washed by centrifugation with PBS supplemented with 2% FBS, and cell numbers were determined using staining with trypan blue after treatment with ACK lysis buffer to remove erythrocytes.

Detection of $CD4^+$, $IL\text{-}4^+CD4^+$, and $IL\text{-}17^+CD4^+$ cells by flow cytometry

The numbers of $CD4^+$ cells in the left lobe of lungs of IgE-sensitized mice were measured by flow cytometry, as previously reported (26). In brief, after incubation with an anti-mouse FcγRII/III mAb (clone 2.4G2; BD Biosciences) to block the binding of subsequent Abs to FcγRII/III, leukocytes recovered from collagenase/DNase I-digested lung tissue were incubated with a PE–Cy7–labeled anti-mouse CD4 mAb (clone H129.19; BD Biosciences). After washing, the stained cells were fixed with 4% paraformaldehyde and then analyzed using FACSCalibur (BD Biosciences) and Cell Quest software (version 3.3; BD Biosciences).

The ability of $CD4^+$ cells in the lung to produce IL-4 or IL-17 was assessed. Leukocytes in lung tissue were suspended in RPMI 1640 medium containing 10% FBS, 1% L-glutamine, and 1% penicillin–streptomycin at a concentration of 1×10^6 cells/ml. The cells were stimulated for 16 h with PMA (100 ng/ml) and ionomycin (500 ng/ml), then brefeldin A and

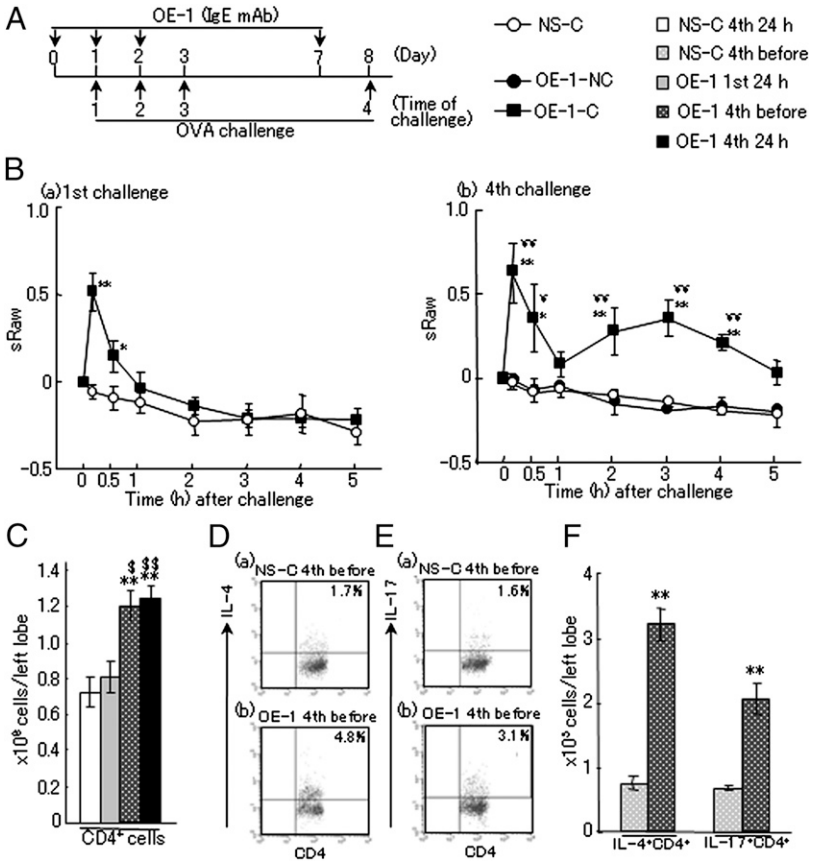

FIGURE 1. Multiple Ag challenges induce a late-phase increase in airway resistance and increase of $CD4^+$ cells in the lungs in IgE-sensitized mice. (**A**) Experimental protocol for sensitization with OE-1 and the challenge with Ag in BALB/c mice. (**B**) Changes in sRaw after the first (**a**) and fourth (**b**) challenge in nonsensitized challenged (NS-C), OE-1–sensitized nonchallenged (OE-1-NC), and OE-1–sensitized challenged (OE-1-C) mice. (**C**) Changes in $CD4^+$ cell numbers in lung tissue 24 h after the fourth challenge in nonsensitized mice (NS-C 4th 24 h), 24 h after the first challenge in OE-1–sensitized mice (OE-1 1st 24 h), and 2 h before (OE-1 4th before) or 24 h after the fourth challenge in OE-1–sensitized mice (OE-1 4th 24 h). (**D** and **E**) Representative dot plot depicting the percentage of $IL\text{-}4^+CD4^+$ (D) and $IL\text{-}17^+CD4^+$ (E) cells in the lungs of the NS-C 4th before (**a**) and OE-1 4th before (**b**) groups. (**F**) Changes in $IL\text{-}4^+CD4^+$ and $IL\text{-}17^+CD4^+$ cell numbers in lung tissue in mice sensitized with OE-1. Results shown are from one experiment representative of two independent trials. Each value represents the mean ± SEM for four to six animals. $^*p < 0.05$, $^{**}p < 0.01$ (compared with the NS-C, NS-C 4th 24 h, or NS-C 4th before group), $^{¥}p < 0.05$, $^{¥¥}p < 0.01$ (compared with the OE-1-NC group), $^{\$}p < 0.05$, $^{\$\$}p < 0.01$ (compared with the OE-1 1st 24 h group).

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monensin were added for 4 h at 37°C in 5% CO_2. The cells were washed twice with PBS supplemented with 2% FBS, incubated with the anti-mouse FcγRII/III mAb, washed once with PBS supplemented with 2% FBS, and incubated with a PE–Cy7–labeled anti-mouse CD4 mAb (BD Biosciences). After three washes with PBS supplemented with 2% FBS, the cells were fixed with 4% paraformaldehyde, made permeable with saponin, and stained with PE-labeled anti–IL-4 mAb (clone 11B11; eBioscience) or PE-labeled anti–IL-17 mAb (TC11-18H10.1; BioLegend). Finally, they were washed three more times with PBS supplemented with 2% FBS. Each sample was analyzed using FACSCalibur and Cell Quest software.

Statistical analyses

Data are shown as the mean ± SEM. Statistical analyses between the two groups were determined using Student *t* test (two-tailed). A probability value of $p < 0.05$ was considered statistically significant.

Results

Multiple challenges induce a late-phase increase in airway resistance and increase of CD4$^+$ cells in the lungs in IgE-sensitized mice

Consistent with our previous finding (24), in OE-1–sensitized challenged mice, the first challenge caused a swift elevation of sRaw that peaked at 10 min followed by a gradual diminution until 1 h after the challenge, with no obvious changes in sRaw observed at 2–5 h (Fig. 1Ba). In contrast, the fourth challenge, which was performed 5 d after the third, induced a significant biphasic elevation of sRaw, peaking at 10 min and 3 h (Fig. 1Bb). Furthermore, sRaw values before the fourth challenge were 2.223 ± 0.030 in the nonsensitized challenged, 2.309 ± 0.056 in the OE-1–sensitized nonchallenged, and 2.241 ± 0.042 in the OE-1–sensitized challenged groups. Throughout the above experiment, no significant differences in sRaw before the challenges were found among the groups.

First, to investigate the role of CD4$^+$ cells in the development of the late-phase increase in airway resistance, we measured the number of CD4$^+$ cells in the lungs at the fourth challenge. Although the level of CD4$^+$ cells in the lungs of the IgE-sensitized mice 24 h after the first challenge was similar to that in the nonsensitized group, it increased until the time of the fourth challenge. This increase was sustained for at least 24 h after the fourth challenge (Fig. 1C). Additionally, there were increases in the percentage and the absolute number of IL-4$^+$CD4$^+$ and IL-17$^+$CD4$^+$ cells at the fourth challenge in comparison with those in nonsensitized mice; furthermore, in IgE-sensitized mice, both increases of IL-4$^+$CD4$^+$ cells were higher than those of IL-17$^+$CD4$^+$ cells (Fig. 1D–F).

Treatment with anti-CD4 mAb at the first challenge inhibits the late-phase increase in airway resistance, airway inflammation, and goblet cell hyperplasia in IgE-sensitized mice

In this IgE-sensitized model, an increase of CD4$^+$ cells in the lungs until the fourth challenge, the time at which the late-phase increase in airway resistance developed, was observed. Therefore,

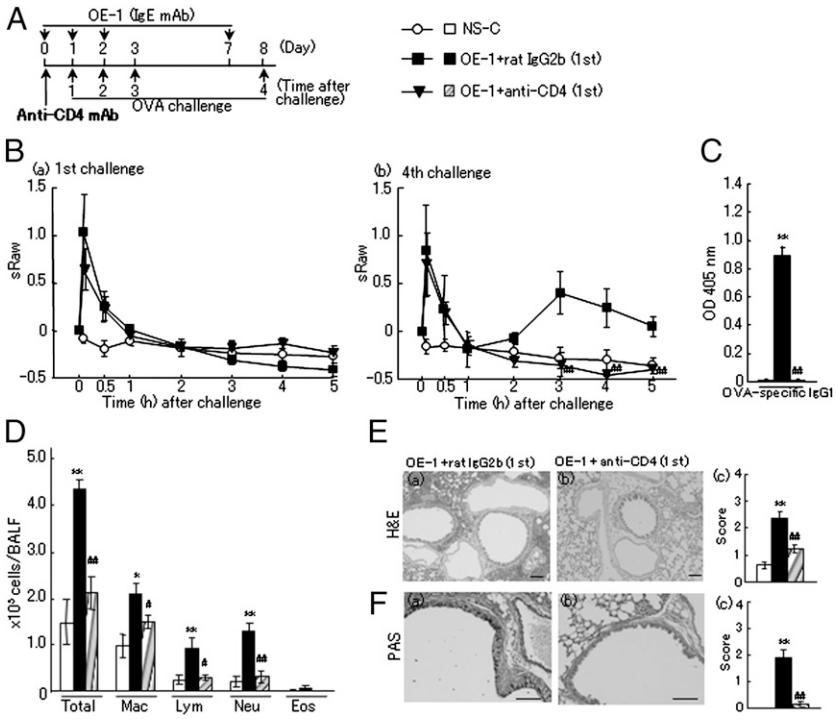

FIGURE 2. Treatment with anti-CD4 mAb at the first challenge inhibits the late-phase increase in airway resistance, airway inflammation, and goblet cell hyperplasia in IgE-sensitized mice. (**A**) Experimental protocol for depletion of CD4$^+$ cells in the induction phase. The mice received an i.p. injection of an anti-CD4 mAb 30 min before the first sensitization on day 0 [OE-1+anti-CD4 (1st)]. Negative and positive controls were nonsensitized challenged (NS-C) and OE-1–sensitized challenged, control rat IgG2b-treated [OE-1+rat IgG2b (1st)] mice, respectively. (**B**) Effect of treatment with the anti-CD4 mAb on changes in sRaw after the first (**a**) and fourth (**b**) challenge in mice sensitized with OE-1. (**C**) Effect of the anti-CD4 mAb on OVA-specific IgG1 levels in serum of mice sensitized with OE-1. (**D**) Effect of the anti-CD4 mAb on increases in inflammatory cell numbers in BALF 24 h after the fourth challenge in mice sensitized with OE-1. (**E**) Changes in inflammation (H&E) in lung tissue 24 h after the fourth challenge in the OE-1+rat IgG2b (1st) (**a**) and OE-1 +anti-CD4 (1st) (**b**) groups. Scale bar, 100 μm. Histological appearance was scored as a measure of inflammation (**c**). (**F**) Changes in goblet cell hyperplasia (PAS) in lung tissue 24 h after the fourth challenge in the OE-1+rat IgG2b (1st) (**a**) and OE-1+anti-CD4 (1st) (**b**) groups. Scale bar, 100 μm. Histological appearance was scored as a measure of goblet cell hyperplasia (**c**). Results shown are from one experiment representative of two independent trials. Each value represents the mean ± SEM for five animals. $^*p < 0.05$, $^{**}p < 0.01$ (compared with the NS-C group), $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ [compared with the OE-1+rat IgG2b (1st) group]. Eos, Eosinophils; Lym, lymphocytes; Mac, macrophages; Neu, neutrophils; Total, All cells.

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we investigated the effect of the administration of the anti-CD4 mAb 30 min before the first sensitization on the development of the late-phase increase in airway resistance (effect on the induction phase). The mAb inhibited the late-phase increase in airway resistance, although it did not affect the early increase in airway resistance (Fig. 2Ba, 2Bb). Numbers of inflammatory cells such as macrophages, lymphocytes, and neutrophils in BALF and OVA-specific IgG1 production in serum were significantly increased after the fourth challenge (Fig. 2C, 2D). However, as previously reported by us (24), no eosinophilia was evoked even after the fourth challenge in the IgE-sensitized mice. The infiltration of leukocytes and OVA-specific IgG1 production were inhibited by the treatment with the anti-CD4 mAb (Fig. 2C, 2D). Although both IL-4 (9.0 ± 3.5 pg/ml) and IL-13 (21.3 ± 1.9 pg/ml) levels were increased in BALF 24 h after the fourth challenge, those in the IgE-sensitized anti-CD4 mAb-treated mice and the non-sensitized challenged mice were under the detectable limit (<2 and 1.5 pg/ml, respectively).

Fig. 2E and 2F show effects of the anti-CD4 mAb on histologically assessed inflammation and goblet cell hyperplasia in the lung. The histological changes were also suppressed by the treatment.

Treatment with anti-CD4 mAb at the fourth challenge inhibits the late-phase increase in airway resistance in IgE-sensitized mice

Subsequently, to investigate the role of $CD4^+$ cells at the fourth challenge (the effector phase), the anti-CD4 mAb was injected 30 min before the fourth sensitization. The late-phase increase in airway resistance and infiltration of lung tissue by lymphocytes and neutrophils was inhibited even by the anti-CD4 mAb treatment before the fourth challenge (Fig. 3B, 3C), whereas neither the early-phase increase in airway resistance nor production of OVA-specific IgG1 was affected (Fig. 3B, 3D). Furthermore, the increased levels of IL-4 and IL-13 in BALF and IL-17 in the lung tissue supernatants 24 h after the fourth challenge in IgE-sensitized mice were also suppressed by the treatment (Fig. 3E, 3F). Conversely, airway inflammation and goblet cell hyperplasia were not inhibited by the treatment (data not shown) because such changes had already been established by the time of the fourth challenge (24).

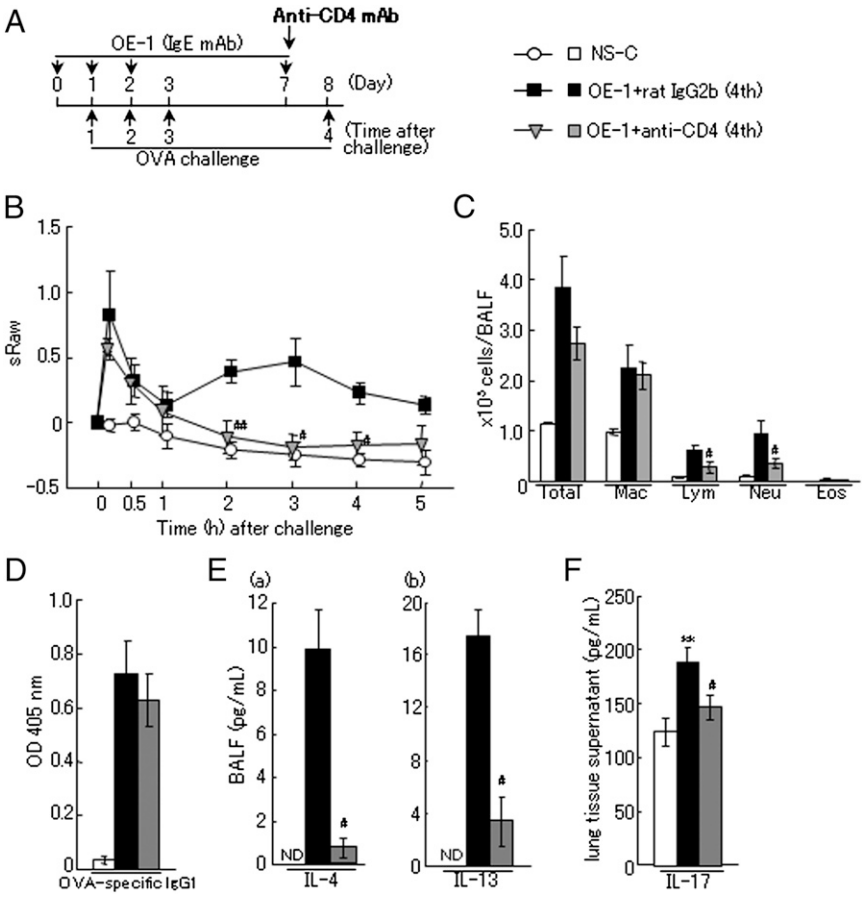

FIGURE 3. Treatment with anti-CD4 mAb at the fourth challenge inhibits the late-phase increase in airway resistance in IgE-sensitized mice. (**A**) Experimental protocol for depletion of $CD4^+$ cells in the effector phase. The mice received an i.p. injection of an anti-CD4 mAb 30 min before the fourth sensitization on day 7 [OE-1+anti-CD4 (4th)]. Negative and positive controls were nonsensitized challenged (NS-C) and OE-1–sensitized challenged, control rat IgG2b-treated [OE-1+rat IgG2b (4th)] mice, respectively. (**B**) Effect of treatment with the anti-CD4 mAb on changes in sRaw after the fourth challenge in mice sensitized with OE-1. (**C**) Effect of the anti-CD4 mAb on increases in inflammatory cell numbers in BALF 24 h after the fourth challenge in mice sensitized with OE-1. (**D**) Effect of the anti-CD4 mAb on OVA-specific IgG1 levels in serum of mice sensitized with OE-1. (**E**) Effect of the anti-CD4 mAb on increases in IL-4 (**a**) and IL-13 (**b**) in BALF 24 h after the fourth challenge in mice sensitized with OE-1. (**F**) Effect of the anti-CD4 mAb on the increase in IL-17 in lung tissue supernatant 24 h after the fourth challenge in mice sensitized with OE-1. Results shown are from one experiment representative of two independent trials. Each value represents the mean ± SEM for five to six animals. $^{**}p < 0.01$ (compared with the NS-C group), $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ [compared with the OE-1+rat IgG2b (4th) group]. Eos, Eosinophils; Lym, lymphocytes; Mac, macrophages; Neu, neutrophils; Total, all cells.

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Multiple treatments with a C3a receptor antagonist inhibit the late-phase increase in airway resistance in IgE-sensitized mice

We have reported that multiple treatments with a C3a receptor antagonist during repeated challenges with an Ag inhibited the development of a late-phase increase in airway resistance in actively sensitized mice (27). Therefore, the effect of such multiple treatments on the late-phase increase in airway resistance was also evaluated in this IgE-sensitized model. The C3a receptor antagonist suppressed the late-phase increase in airway resistance (Fig. 4Bb), numbers of macrophages, lymphocytes, and neutrophils in BALF (Fig. 4C), and the increased level of IL-17 in the lung tissue supernatant (Fig. 4F) but did not affect the early response, the OVA-specific IgG1 level in serum, or the production of IL-4 and IL-13 in BALF (Fig. 4B, 4D, 4E). Additionally, histological analyses revealed that airway inflammation and goblet cell hyperplasia in the lungs of mice sensitized with IgE were inhibited by the multiple treatments (Fig. 4G, 4H).

Multiple treatments with a C3a receptor antagonist or anti-C3a mAb inhibit the increase in IL-17$^+$CD4$^+$ cells in IgE-sensitized mice

It has been reported that the inhibition of C3a signaling suppressed the increase in IL-17$^+$CD4$^+$ cells in the lungs in a murine model of asthma (23). Therefore, we examined whether multiple treatments with the C3a receptor antagonist or anti-C3a mAb during the first three challenges reduced the increased percentage and number of IL-17$^+$CD4$^+$ cells in the lung at the fourth challenge in our IgE-sensitized model. Both multiple treatments inhibited the increased percentage in IL-17$^+$CD4$^+$ cells in the lungs at the fourth challenge; furthermore, suppression of the absolute number of cells was significantly observed (Fig. 5Bi, 5Bii).

Single treatment with a C3a receptor antagonist or anti-C3a mAb at the fourth challenge inhibits the late-phase increase in airway resistance, AHR, and IL-17 production in the lungs in IgE-sensitized mice

To test whether C3a produced by the fourth challenge mediates the late-phase increase in airway resistance, AHR, and IL-17 production, we analyzed the effects of a single dose of SB290157 at the fourth challenge. The treatment inhibited the late-phase increase in airway resistance, AHR, and airway neutrophilia, although neither the early response nor the increased number of macrophages and lymphocytes in BALF was affected (Fig. 6B–D). Moreover, the increased levels of both IL-17 and IL-23 in the lungs were reduced by the single treatment (Fig. 6F–I), although the compound did not affect IL-4 and IL-13 production in BALF (Fig. 6E).

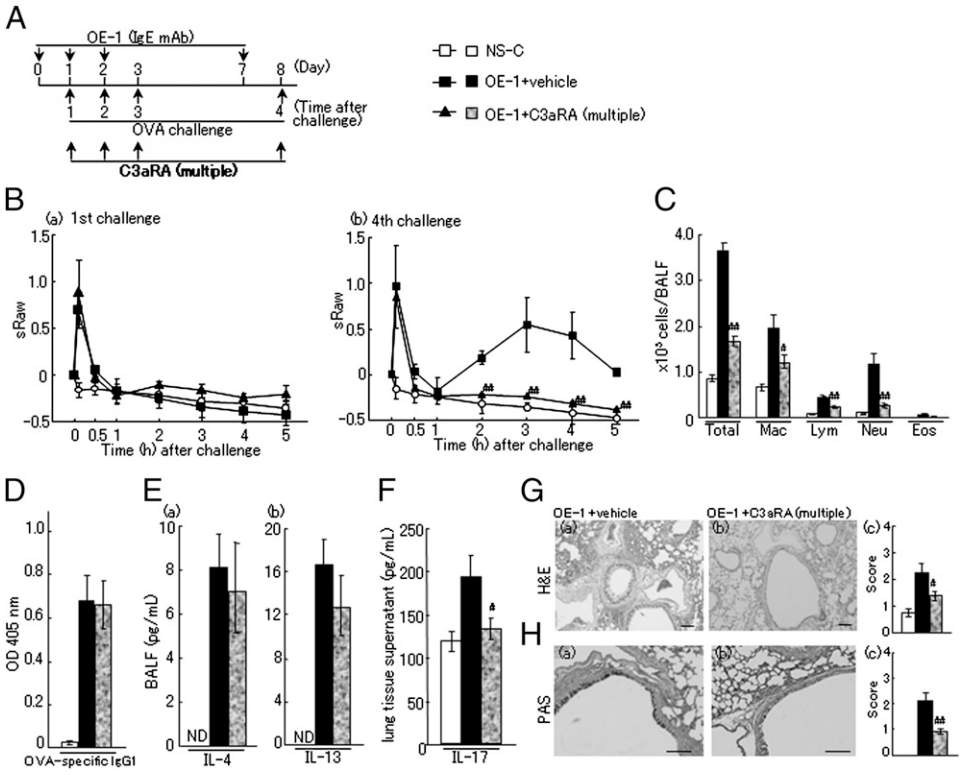

FIGURE 4. Multiple treatments with a C3a receptor antagonist, SB290157, inhibit the late-phase increase in airway resistance in the lung in IgE-sensitized mice. (**A**) Experimental protocol for multiple treatments with SB290157. SB290157 was i.p. administered during the Ag challenges [OE-1+C3aRA (multiple)]. Negative and positive controls were nonsensitized challenged (NS-C) and OE-1–sensitized challenged, vehicle-treated (OE-1+vehicle) mice, respectively. (**B**) Effect of multiple treatments with SB290157 on changes in sRaw after the first (**a**) and fourth (**b**) challenges in mice sensitized with OE-1. (**C**) Effect of multiple treatments with SB290157 on the inflammatory cell number in BALF 24 h after the fourth challenge in mice sensitized with OE-1. (**D**) Effect of multiple treatments with SB290157 on OVA-specific IgG1 levels in serum 24 h after the fourth challenge in mice sensitized with OE-1. (**E**) Effect of multiple treatments with SB290157 on increases in IL-4 (**a**) and IL-13 (**b**) in BALF 24 h after the fourth challenge in mice sensitized with OE-1. (**F**) Effect of multiple treatments with SB290157 on the increase in IL-17 in lung tissue supernatant 24 h after the fourth challenge in mice sensitized with OE-1. (**G**) Changes in inflammation (H&E) in lung tissue 24 h after the fourth challenge in the OE-1+vehicle (**a**) and OE-1+C3aRA (multiple) (**b**) groups. Scale bar, 100 μm. Histological appearance was scored as a measure of inflammation (**c**). (**H**) Changes in goblet cell hyperplasia (PAS) in lung tissue 24 h after the fourth challenge in the OE-1+vehicle (**a**) and OE-1+C3aRA (multiple) (**b**) groups. Scale bar, 100 μm. Histological appearance was scored as a measure of goblet cell hyperplasia (**c**). Results shown are from one experiment representative of two independent trials. Each value represents the mean ± SEM for five to seven animals. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ (compared with the OE-1+vehicle group). Eos, Eosinophils; Lym, lymphocytes; Mac, macrophages; Neu, neutrophils; Total, all cells.

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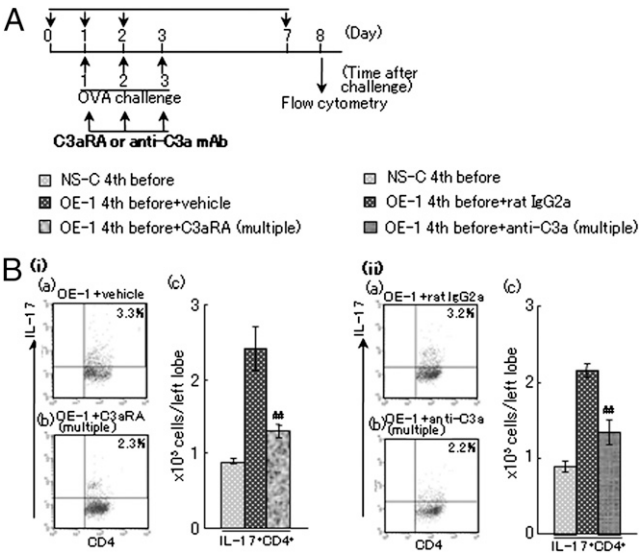

FIGURE 5. Multiple treatments with the C3a receptor antagonist SB290157 or anti-C3a mAb inhibit the increase of IL-17$^+$CD4$^+$ cells in the lungs of IgE-sensitized mice. (**A**) Experimental protocol for multiple treatments with the C3a receptor antagonist or anti-C3a mAb. The mice received an i.p. or intratracheal administration of the C3a receptor antagonist [OE-1 4th before+C3aRA (multiple)] or anti-C3a mAb during the first to third challenges [OE-1 4th before+anti-C3a (multiple)], respectively. Negative and positive controls were nonsensitized challenged (NS-C 4th before) and OE-1–sensitized challenged, vehicle or rat IgG2a-treated (OE-1 4th before+vehicle or rat IgG2a) mice, respectively. (**Bi**) Representative dot plot depicting the percentage of IL-17$^+$CD4$^+$ cells in the lungs of the OE-1 4th before+vehicle (**a**) and OE-1 4th before+C3aRA (multiple) (**b**) groups. The effect of multiple treatments with SB290157 on increases in IL-17$^+$CD4$^+$ cells numbers in lung tissue was evaluated (**c**). (**ii**) Representative dot plot depicting the percentage of IL-17$^+$CD4$^+$ cells in the lungs of the OE-1 4th before+rat IgG2a (**a**) and OE-1 4th before +anti-C3a (multiple) (**b**) groups. The effect of multiple treatments with anti-C3a mAb on increases in IL-17$^+$CD4$^+$ cells numbers in lung tissue was evaluated (**c**). Results shown are from one experiment representative of two independent trials. Each value represents the mean ± SEM for four to five animals. $^{\#\#}p < 0.01$ (compared with the OE-1+vehicle or rat IgG2a group).

Additionally, we used neutralizing mAb against C3a in IgE-sensitized mice. Single treatment with anti-C3a mAb at the fourth challenge inhibited the late-phase increase in airway resistance, AHR, infiltration of neutrophils, and the production of IL-17 and IL-23 in the lung tissue supernatants but not early-phase airway resistance or the production of IL-4 and IL-13 in BALF (Fig. 7). Thus, treatment with anti-C3a mAb showed similar effects compared with those of treatment with a C3a receptor antagonist.

Meanwhile, we attempted to investigate whether purified OE-1 mediated early- and late-phase increases in airway resistance, AHR, and airway inflammation, resulting in the induction of these asthmatic responses. Furthermore, the suppression pattern of the asthmatic responses by single treatment with a C3a receptor antagonist at the fourth challenge in mice sensitized with purified OE-1 resembled those of mice sensitized with a hybridoma supernatant containing OE-1 (Supplemental Fig. 1).

Treatment with anti–IL-17 mAb at the fourth challenge inhibits the late-phase increase in airway resistance, AHR, and neutrophil accumulation in BALF in IgE-sensitized mice

The data shown in Figs. 6 and 7 suggest that C3a-induced IL-17 production could contribute to the development of an IgE-mediated late-phase increase in airway resistance and AHR. Therefore, we investigated effects of an anti–IL-17 mAb. Treatment with the anti–IL-17 mAb before the fourth challenge significantly suppressed the late-phase increase in airway resistance, AHR, and the infiltration by neutrophils but not the early response or the increased numbers of macrophages and lymphocytes in BALF (Fig. 8B–D). Furthermore, the increased level of C3a in BALF 24 h after the fourth challenge was also significantly reduced by the treatment (Fig. 8E).

To investigate the role of IL-17 in the development of airway inflammation and goblet cell hyperplasia, an anti–IL-17 mAb was administered during the first to third challenges. However, neither the airway inflammation nor the goblet cell hyperplasia was affected by the mAb (Fig. 8G, 8H).

Treatment with anti–Gr-1 mAb at the fourth challenge inhibits the late increase in airway resistance and AHR

The results shown in Figs. 6, 7, and 8 indicate the infiltration of lung tissue by neutrophils to be linked to the induction of the late-phase increase in airway resistance and AHR. Therefore, we examined the effect of treatment with an anti–Gr-1 mAb on the late-phase increase in airway resistance and AHR. When the mAb was administered 30 min before the fourth sensitization, the late-phase increase in airway resistance, AHR, and neutrophil numbers was significantly reduced (Fig. 9B–D).

Discussion

Allergen-specific IgE has long been regarded as a major molecular component of allergic asthma. There has also been increasing evidence of a role for IL-17 in the disease. However, the relationship between IgE-mediated asthmatic responses and IL-17 has not yet been defined. In this study, we focused on the role of IL-17 in an IgE-mediated late-phase asthmatic response and AHR in mice repeatedly challenged with Ag and found that IgE mediated the increase of IL-17$^+$CD4$^+$ cells in the lungs through C3a production. Furthermore, single treatment with a C3a receptor antagonist or anti-C3a mAb at the fourth challenge suppressed the late-phase increase in airway resistance, AHR, and neutrophilic inflammation in BALF, as well as the production of IL-17 in the lungs, but not IL-4 or IL-13 production in BALF. These findings prompted us to examine the effect of an anti–IL-17 mAb. The Ab reduced the late-phase increase in airway resistance, AHR, and neutrophilic accumulation after the fourth challenge. Additionally, treatment with an anti–Gr-1 mAb greatly suppressed the infiltration of neutrophils in BALF, the late increase in airway resistance, and AHR. These results clearly show that C3a-induced IL-17 production plays a critical role in the late-phase asthmatic response and AHR through neutrophilic airway inflammation in mice sensitized with IgE.

The increase in CD4$^+$ cells in the lungs of mice sensitized with IgE had occurred by the fourth challenge, the timing of the late-phase increase in airway resistance (Fig. 1), suggesting the increase of CD4$^+$ cells in the airway caused by the first three challenges to be required for the induction of the late-phase increase in airway resistance in response to the fourth challenge. Therefore, we examined the contribution of the CD4$^+$ cells at the fourth challenge (effector phase) to the development of a late-phase increase in airway resistance; treatment with an anti-CD4 mAb at the fourth challenge inhibited the late-phase increase in airway resistance, indicating that the CD4$^+$ cell activation at the effector phase is related to the development of a late-phase increase in airway resistance in this IgE-sensitized model (Fig. 3). Thus, it can be speculated that proinflammatory factors produced by the activation of CD4$^+$ cells after the fourth challenge induced the late-phase asthmatic response.

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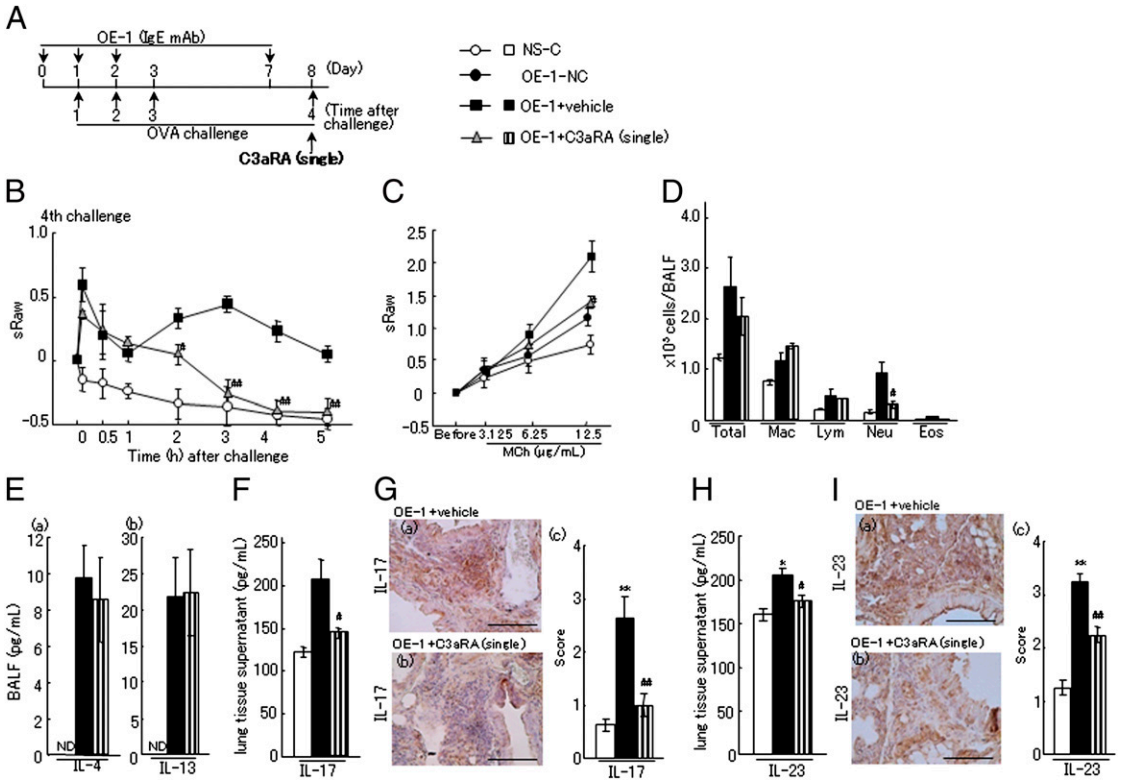

FIGURE 6. Single treatment with the C3a receptor antagonist SB290157 inhibits the late-phase increase in airway resistance, AHR, and IL-17 production in the lungs in IgE-sensitized mice. (**A**) Experimental protocol for a single treatment with SB290157. SB290157 was i.p. administered 30 min before the fourth challenge [OE-1+C3aRA (single)]. Negative and positive controls were nonsensitized challenged (NS-C) and OE-1–sensitized challenged, vehicle-treated (OE-1+vehicle) mice, respectively. (**B**) Effect of a single treatment with SB290157 on changes in sRaw after the fourth challenge in mice sensitized with OE-1. (**C**) Effect of a single treatment with SB290157 on the development of AHR 24 h after the fourth challenge in mice sensitized with OE-1. (**D**) Effect of a single treatment with SB290157 on the inflammatory cell number in BALF 24 h after the fourth challenge in mice sensitized with OE-1. (**E**) Effect of a single treatment with SB290157 on increases in IL-4 (**a**) and IL-13 (**b**) in BALF 24 h after the fourth challenge in mice sensitized with OE-1. (**F**) Effect of a single treatment with SB290157 on the increase in IL-17 in lung tissue supernatant 24 h after the fourth challenge in mice sensitized with OE-1. (**G**) Immunohistochemical localization of IL-17 in lung tissue 24 h after the fourth challenge in the OE-1+vehicle (**a**) and OE-1+ C3aRA (single) (**b**) groups. Scale bar, 100 μm. Histological appearance was scored as a measure of IL-17 expression (**c**). (**H**) Effect of a single treatment with SB290157 on the increase in IL-23 in lung tissue supernatant 24 h after the fourth challenge in mice sensitized with OE-1. (**I**) Immunohistochemical localization of IL-23 in lung tissue 24 h after the fourth challenge in the OE-1+vehicle (**a**) and OE-1+C3aRA (single) (**b**) groups. Scale bar, 100 μm. Histological appearance was scored as a measure of IL-23 expression (**c**). Results shown are from one experiment representative of two independent trials. Each value represents the mean ± SEM for five to seven animals. $^{*}p < 0.05$, $^{**}p < 0.01$ (compared with the NS-C group), $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ (compared with the OE-1+vehicle group). Eos, Eosinophils; Lym, lymphocytes; Mac, macrophages; Neu, neutrophils; Total, all cells.

Lajoie et al. (23) have reported that inhibition of C3a-mediated signaling resulted in fewer lung IL-17^{+}CD4^{+} cells in a murine model of allergic asthma. Consistent with this finding, we showed that multiple treatments with a C3a receptor antagonist or anti-C3a mAb during the first three Ag challenges suppressed the increases of IL-17^{+}CD4^{+} cells in the lungs at the fourth challenge (Fig. 5), suggesting that C3a produced during the repeated challenges is essential for the increase of IL-17^{+}CD4^{+} cells in the lungs. Conversely, as demonstrated in our previous report (24) and in the current study (Fig. 6), single treatment with a C3a receptor antagonist at the fourth challenge suppressed the late-phase increase in airway resistance and AHR in this IgE-sensitized model. Furthermore, increases in IL-17^{+}CD4^{+} cells and IL-17 production in the lungs at the fourth challenge were observed (Figs. 1, 3). On the basis of these findings, we investigated whether the C3a-induced IL-17 production could have contributed to the development of a late-phase increase in airway resistance and AHR. The single treatment with the C3a receptor antagonist or anti-C3a mAb at the fourth challenge inhibited the late-phase increase in airway resistance, AHR, and the production of IL-17 in the lung (Figs. 6, 7); furthermore, treatment with the anti–IL-17 mAb reduced the late-phase increase in airway resistance and AHR (Fig. 8), suggesting that C3a-induced production of IL-17 contributed to the late asthmatic response and AHR. In addition to the C3a-induced IL-17 production, we found that C3a-mediated signaling induced the production of IL-23 after the fourth challenge (Figs. 6, 7). It has been reported that IL-23 is crucial for the maintenance of Th17 cells (34–36) and the full acquisition of an effector function of Th17 cells (36). The major source of IL-23 is several types of APCs such as activated dendritic cells, monocytes, and macrophages after exposure to pathogen-derived molecules (37–40); moreover, C3a receptor signaling promotes IL-23 production by dendritic cells (23). Thus, a C3a-mediated IL-23–Th17 axis, which leads to IL-17 production, may be critical for the development of the late-phase asthmatic response and AHR in this IgE-sensitized model.

The concentration of C3a in BALF was elevated in patients having a late-phase asthmatic response compared with that in nonatopic control patients (41); in this IgE-sensitized model, an increased level of C3a in BALF was also observed (Fig. 8). However, the mechanism of C3a production has been unknown. We have shown an increase in OVA-specific IgG1 production in mice sensitized with IgE, and in experiments in vitro, we have shown that an immune complex composed of OVA-specific IgG1 mAb

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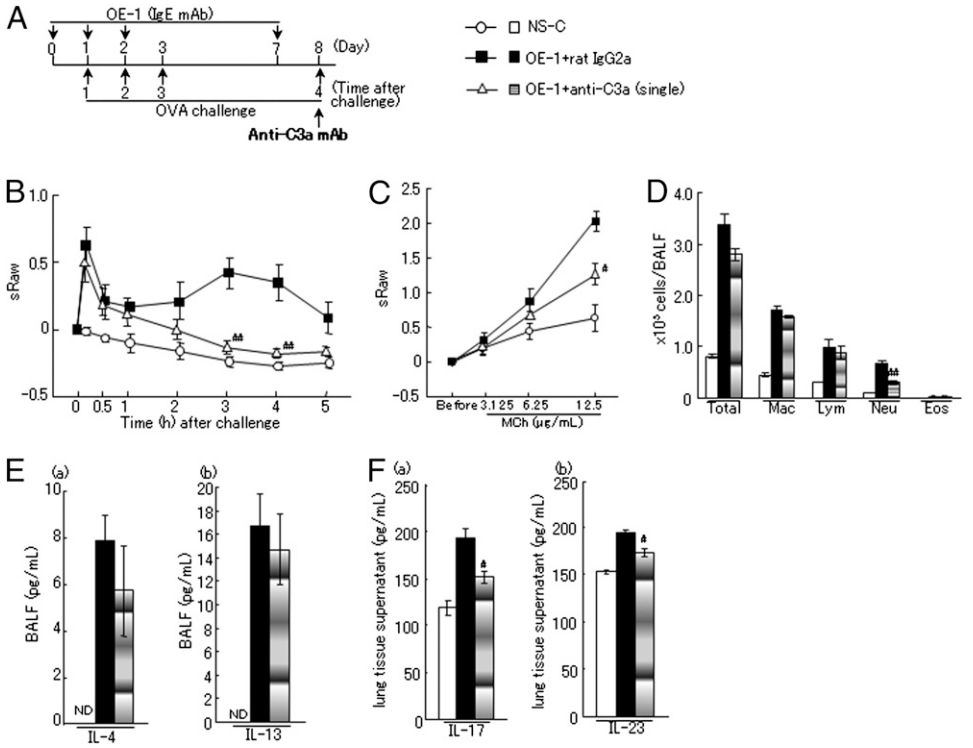

FIGURE 7. Single treatment with anti-C3a mAb at the fourth challenge inhibits the late-phase increase in airway resistance, AHR, and IL-17 production in the lungs in IgE-sensitized mice. (**A**) Experimental protocol for a single treatment with anti-C3a mAb. Anti-C3a mAb was intratracheally administered 30 min before the fourth challenge [OE-1+anti-C3a (single)]. Negative and positive controls were nonsensitized challenged (NS-C) and OE-1–sensitized challenged, control rat IgG2a mAb-treated (OE-1+rat IgG2a) mice, respectively. (**B**) Effect of a single treatment with anti-C3a mAb on changes in sRaw after the challenge in mice sensitized with OE-1. (**C**) Effect of a single treatment with anti-C3a mAb on the development of AHR 24 h after the fourth challenge in mice sensitized with OE-1. (**D**) Effect of a single treatment with anti-C3a mAb on the inflammatory cell number in BALF 24 h after the fourth challenge in mice sensitized with OE-1. (**E**) Effect of a single treatment with anti-C3a mAb on the increases in IL-4 (**a**) and IL-13 (**b**) in BALF 24 h after the fourth challenge in mice sensitized with OE-1. (**F**) Effect of a single treatment with anti-C3a mAb on the increases in IL-17 (**a**) and IL-23 (**b**) in lung tissue supernatant 24 h after the fourth challenge in mice sensitized with OE-1. Results shown are from one experiment representative of two independent trials. Each value represents the mean ± SEM for five to seven animals. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ (compared with the OE-1+rat IgG2a group). Eos, Eosinophils; Lym, lymphocytes; Mac, macrophages; Neu, neutrophils; Total, all cells.

and OVA, but not OVA-specific IgE mAb and OVA, activated C3 (24), suggesting C3a to be produced in response to an immune complex of IgG1 and the Ag in this IgE-sensitized model, although the mouse IgG1 Ab is generally considered a poor activator of complement. Alternatively, another possibility is that a protease released from inflammatory cells such as mast cells and pulmonary macrophages or derived from the allergen proteolytically cleaves C3 to generate C3a (42, 43). Thus, the Ag-triggered release of protease in IgE-sensitized mice could lead to C3a production. Meanwhile, notably, treatment with the anti–IL-17 mAb reduced C3a production in the BALF of mice sensitized with IgE (Fig. 8), indicating that IL-17 led to further C3a production in the lung, meaning that there is positive feedback regulation of IL-17 production, which is mediated by C3a.

Conversely, the single treatment with the C3a receptor antagonist or anti-C3a mAb did not inhibit the production of Th2 cytokines, although it inhibited the late-phase increase in airway resistance and AHR (Figs. 6, 7). These results show that suppression of the late-phase increase in airway resistance and AHR by the antagonist or the mAb was not related to the production of Th2-type cytokines. However, there have been conflicting reports: C3a receptor-deficient mice sensitized with mixed OVA and *Aspergillus fumigatus* (44) or house dust mites (45) showed reduced cytokine levels and AHR. Kumar and Foster (46) have suggested that the roles of cells and molecules in AHR were altered by changing the protocol (dose and duration) for the challenge, indicating that the role of C3a in the pathogenesis of asthma also varies depending on the protocol used.

The recruitment of neutrophils, but not eosinophils, was consistently observed during the late-phase increase in airway resistance after the fourth challenge in this IgE-sensitized model, although only a small number of neutrophils were seen before the challenge (24), suggesting the neutrophilic inflammation to be critical to the induction of the late-phase increase in airway resistance. In clinical cases, allergic asthma has long been considered an eosinophilic bronchitis; however, its symptoms were not ameliorated by a marked reduction in eosinophil numbers in blood and the airway by treatment with anti–IL-5 (47, 48). Furthermore, some asthmatics, particularly those who have severe asthma (48–50) and are resistant to corticosteroids (51), have raised neutrophil counts in their airways, suggesting neutrophils to be a more valid target than eosinophils in certain stages of the pathogenesis. Therefore, we attempted to examine the role of neutrophils in this IgE-sensitized model. When examining the roles of neutrophils in various diseases, the anti–Gr-1 mAb, RB6-8C5, has been extensively used as a tool for depleting the granulocytes in mice (52–54). We have also demonstrated that depletion of neutrophils by the anti–Gr-1 mAb greatly suppressed a late-phase increase in airway resistance in actively sensitized mice (29). In this study, treatment with the anti–Gr-1 mAb before the fourth challenge significantly suppressed late-phase increase in both airway resistance and AHR under conditions of a marked reduction in the

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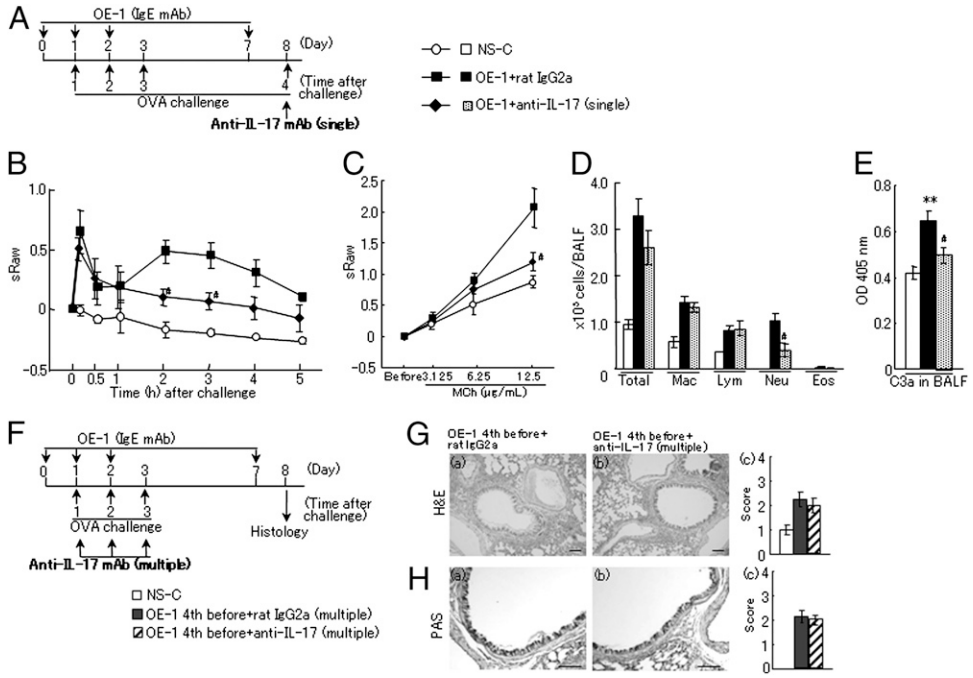

FIGURE 8. Treatment with anti–IL-17 mAb at the fourth challenge inhibits the late-phase increase in airway resistance, AHR, and neutrophil accumulation in BALF in IgE-sensitized mice. (**A**) Experimental protocol for a single treatment with anti–IL-17 mAb. The mice received an i.p. injection of the anti–IL-17 mAb 30 min before the fourth challenge [OE-1+anti-IL-17 (single)]. Negative and positive controls were nonsensitized challenged (NS-C) and OE-1–sensitized challenged, control rat IgG2a mAb (OE-1+rat IgG2a) mice, respectively. (**B**) Effect of a single treatment with the anti–IL-17 mAb on changes in sRaw after the challenge in mice sensitized with OE-1. (**C**) Effect of a single treatment with the anti–IL-17 mAb on the development of AHR 24 h after the fourth challenge in mice sensitized with OE-1. (**D**) Effect of a single treatment with the anti–IL-17 mAb on the inflammatory cell number in BALF 24 h after the fourth challenge in mice sensitized with OE-1. (**E**) Effect of a single treatment with the anti–IL-17 mAb on the increase of C3a in BALF 24 h after the fourth challenge in mice sensitized with OE-1. (**F**) Experimental protocol for multiple treatments with the anti–IL-17 mAb. The mice received an i.p. injection of the anti–IL-17 mAb during the first to third challenges. (**G**) Changes in inflammation (H&E) in lung tissue 5 d after the third challenge in the OE-1 4th before+rat IgG2a (**a**) and OE-1 4th before+anti–IL-17 (multiple) (**b**) groups. Scale bar, 100 μm. Histological appearance was scored as a measure of inflammation (**c**). (**H**) Changes in goblet cell hyperplasia (PAS) in lung tissue 5 d after the third challenge in the OE-1 4th before+vehicle (**a**) and OE-1 4th before+anti–IL-17 (multiple) (**b**) groups. Scale bar, 100 μm. Histological appearance was scored as a measure of goblet cell hyperplasia (**c**). Results shown are from one experiment representative of two independent trials. Each value represents the mean ± SEM for four to seven animals. $^{**}p < 0.01$ (compared with the NS-C group), $^{\#}p < 0.05$ (compared with the OE-1+rat IgG2b group). Eos, Eosinophils; Lym, lymphocytes; Mac, macrophages; Neu, neutrophils; Total, all cells.

airway neutrophil count (Fig. 9), indicating that the late-phase increase in airway resistance and AHR in mice sensitized with IgE was also mediated by neutrophilic airway inflammation. Regarding the mechanisms underlying the recruitment of neutrophils to inflamed sites, various pathways have been reported to date. For example, Th17-type cells activated by Ag stimulation play im-

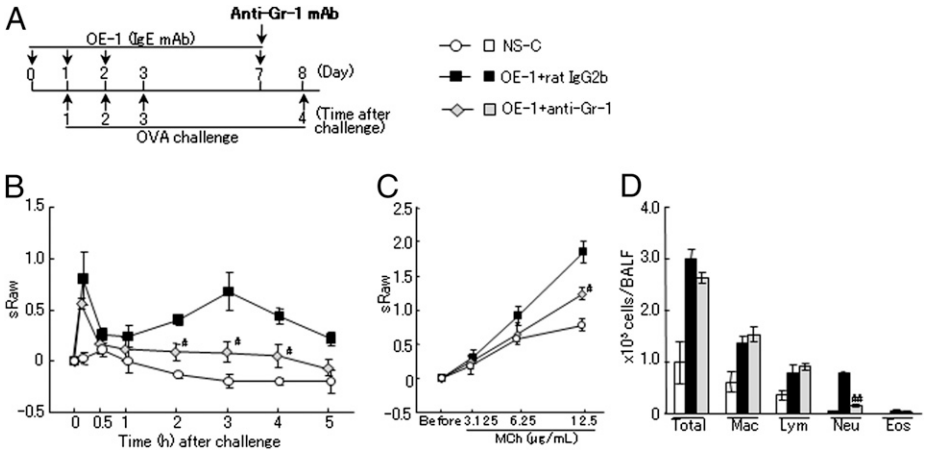

FIGURE 9. Treatment with anti–Gr-1 mAb at the fourth challenge inhibits the late-phase increase in airway resistance and AHR in IgE-sensitized mice. (**A**) Experimental protocol for a single treatment with the anti–Gr-1 mAb. The mice received an i.p. injection of the anti–Gr-1 mAb 30 min before the fourth sensitization (OE-1+anti–Gr-1). Negative and positive controls were nonsensitized challenged (NS-C) and OE-1–sensitized challenged, control rat IgG2b mAb (OE-1+rat IgG2b) mice, respectively. (**B**) Effect of treatment with the anti–Gr-1 mAb on changes in sRaw after the fourth challenge in mice sensitized with OE-1. (**C**) Effect of treatment with the anti–Gr-1 mAb on the development of AHR 24 h after the fourth challenge in mice sensitized with OE-1. (**D**) Effect of treatment with the anti–Gr-1 mAb on the inflammatory cell number in BALF 24 h after the fourth challenge in mice sensitized with OE-1. Results shown are from one experiment representative of two independent trials. Each value represents the mean ± SEM for five animals. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ (compared with the OE-1+rat IgG2b group). Eos, Eosinophils; Lym, lymphocytes; Mac, macrophages; Neu, neutrophils; Total, all cells.

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portant roles in neutrophil infiltration into the lung through IL-17 production (55, 56). In the current study, we also demonstrated that neutralization of IL-17 inhibited the infiltration of neutrophils as well as the late-phase increase in airway resistance and AHR in this IgE-sensitized model (Fig. 8). Furthermore, Wakashin et al. (57) have reported that IL-23–mediated enhancement of Ag-induced neutrophil recruitment in the airway was reduced by the absence of IL-17, suggesting that IL-23 also contributes to neutrophilic airway inflammation through IL-17 production in this model.

Meanwhile, consistent with previous findings in murine models of asthma (8, 58), we showed that the $CD4^+$ cell depletion caused by treatment with the anti-CD4 mAb from the first challenge (induction phase) suppressed the airway inflammation in IgE-sensitized mice (Fig. 2). Furthermore, goblet cell hyperplasia, a characteristic feature of allergic asthma (59–61), was also inhibited by the treatment (Fig. 2), suggesting that the $CD4^+$ cell activation during the first three challenges (induction phase) contributed to the IgE-mediated airway inflammation and goblet cell hyperplasia, whereas the treatment with the anti-CD4 mAb at the fourth challenge (effector phase) did not inhibit these responses. IL-4 and IL-13 have been known to induce airway inflammation and goblet cell hyperplasia in vivo (62, 63). In the current study, airway production of IL-4 and IL-13 was also reduced by treatment with the anti-CD4 mAb at the first challenge. In contrast, multiple treatments with the anti–IL-17 mAb did not affect the development of airway inflammation and goblet cell hyperplasia (Fig. 8), suggesting IL-4 and IL-13, but not IL-17, to be critical for the development of airway inflammation and goblet cell hyperplasia in this IgE-sensitized model. In addition, multiple treatments with the C3a receptor antagonist also inhibited airway inflammation and goblet cell hyperplasia (Fig. 4). It has been reported that C3a contributes to the accumulation of leukocytes (64–66) and induces goblet cell hyperplasia in the lung (67), suggesting not only Th2-type cytokines but also C3a in the induction phase to be important to the airway inflammation and goblet cell hyperplasia in IgE-sensitized mice.

In conclusion, in analyses using this IgE-sensitized model, we demonstrated that 1) $CD4^+$ cells at the fourth challenge (effector phase) are critical for the development of a late-phase asthmatic response; 2) C3a produced during the first three challenges (induction phase) increased the number of $IL\text{-}17^+CD4^+$ cells in the lungs at the fourth challenge; and 3) C3a-induced IL-17 production at the fourth challenge (effector phase) contributed to the late-phase asthmatic response and AHR through infiltration of neutrophils. These findings suggest that IgE contributes to the development of allergic asthma through C3a-driven IL-17 responses and thus enhance insight into the underlying pathogenesis of allergic asthma and a potential therapeutic strategy for the disease.

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Disclosures

The authors have no financial conflicts of interest.

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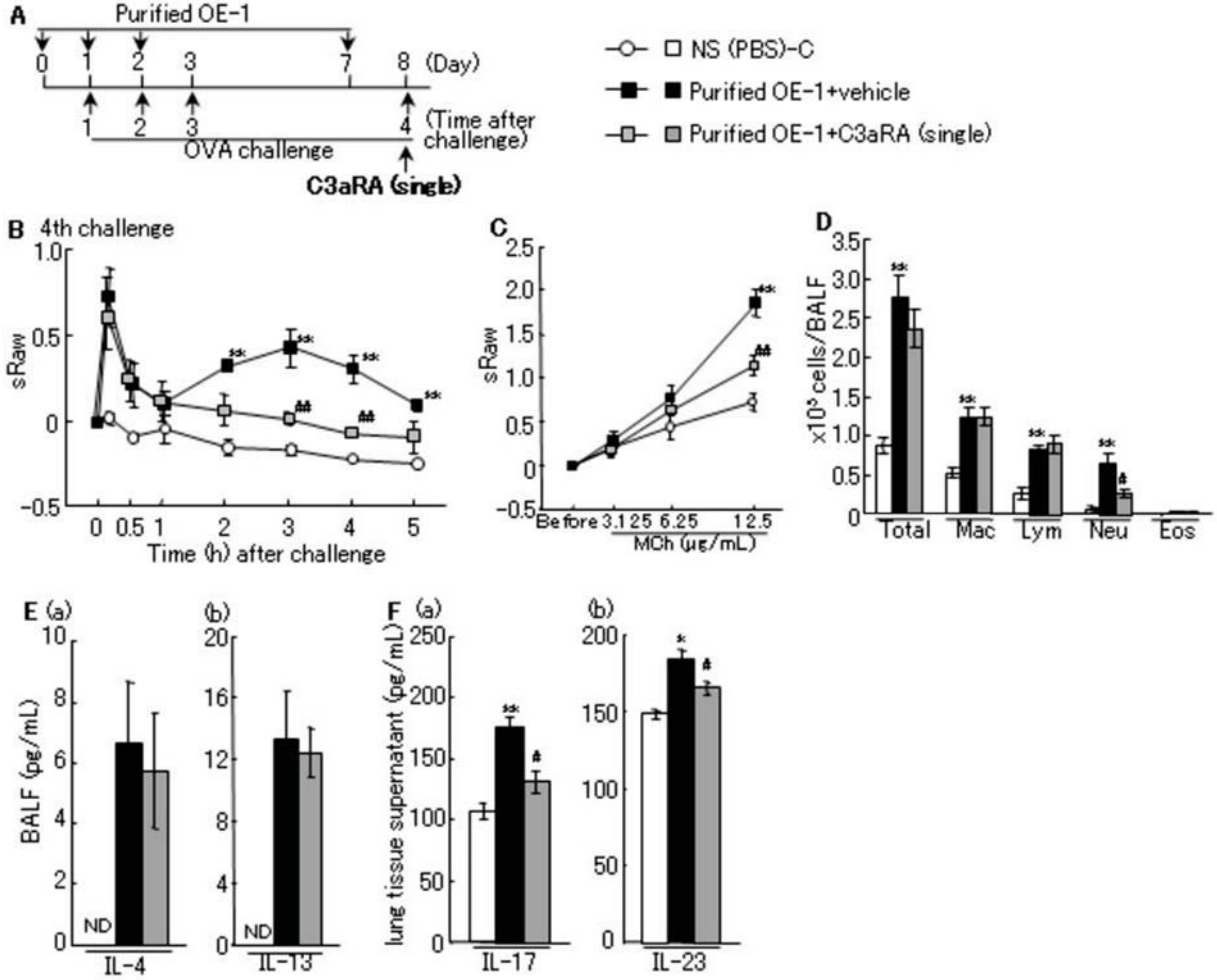
Supplemental Figure 1



Supplemental Fig. 1 Single treatment with the C3a receptor antagonist SB290157 inhibits the late-phase increase in airway resistance, AHR, and IL-17 production in the lungs of purified OE-1-sensitized mice. (A) Experimental protocol for sensitization with purified OE-1 and the challenge with antigen in BALB/c mice. SB290157 was administered 30 min before the fourth challenge. (B) Effect of SB290157 on changes in sRaw. (C) Effect of SB290157 on the development of AHR. (D) Effect of SB290157 on the inflammatory cell number in BALF. (E) Effect of SB290157 on increases in IL-4 (a) and IL-13 (b) in BALF. (F) Effect of SB290157 on the increase in IL-17 (a) and IL-23 (b) in lung tissue supernatants. Each value represents the mean ± SEM for five to seven animals. *, $p<0.05$ and **, $p<0.01$ compared with the NS (PBS)-C group. #, $p<0.05$ and ##, $p<0.01$ compared with the purified OE-1+vehicle group.