

Antigen-specific airway IL-33 production depends on Fc γ R-mediated incorporation of the antigen by alveolar macrophages in sensitized mice

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Summary

Although interleukin (IL)-33 is a candidate for the aggravation of asthma, the mechanisms underlying antigen-specific IL-33 production in the lung are unclear. Therefore, we analysed the mechanisms in mice. Intra-tracheal administration of ovalbumin (OVA) evoked increases in IL-33 and IL-33 mRNA in the lungs of both non-sensitized and OVA-sensitized mice, and the increases in the sensitized mice were significantly higher than in the non-sensitized mice. However, intra-tracheal administration of bovine serum albumin did not increase the IL-33 level in the OVA-sensitized mice. Depletion of neither mast cells/basophils nor CD4⁺ cells abolished the OVA-induced IL-33 production in sensitized mice, suggesting that the antigen recognition leading to the IL-33 production was not related with either antigen-specific IgE-bearing mast cells/basophils or memory CD4⁺ Th2 cells. When a fluorogenic substrate-labelled OVA (DQ-OVA) was intra-tracheally administered, the lung cells of sensitized mice incorporated more DQ-OVA than those of non-sensitized mice. The lung cells incorporating DQ-OVA included B-cells and alveolar macrophages. The allergic IL-33 production was significantly reduced by treatment with anti-Fc γ R2/3 mAb. Depletion of alveolar macrophages by clodronate liposomes significantly suppressed the allergic IL-33 production, whereas depletion of B-cells by anti-CD20 mAb did not. These results suggest that the administered OVA in the lung bound antigen-specific IgG Ab, and then alveolar macrophages incorporated the immune complex through Fc γ R2/3 on the cell surface, resulting in IL-33 production in sensitized mice. The mechanisms underlying the antigen-specific IL-33 production may aid in development of new pharmacotherapies.

Keywords: airway inflammation; B-cells; IgG; interleukin-33; macrophages.

Introduction

Interleukin (IL)-33, a member of the IL-1 family of cytokines,¹ is expressed at higher levels in the lungs of human asthma patients than in those of control subjects.² The expression of IL-33 was particularly evident in the lungs of patients with severe asthma, including steroid-resistant asthma.^{2–4} Indeed, it has been reported that IL-33 activates a wide variety of immune cells, including mast cells, basophils, macrophages, dendritic cells, Th2 cells and type 2 innate lymphoid cells (ILC2), through the cell surface IL-33 receptor, ST2, leading to the production of several pro-

inflammatory molecules.^{1,5–8} As such, IL-33 may function as a key molecule bridging innate and acquired immune cells in the pathogenesis of asthma. Thus, elucidation of the mechanisms underlying allergic IL-33 production and its functional roles may be useful for the development of new pharmacotherapies for asthma. Extensive research has been conducted to understand the functional roles of IL-33 in the asthma pathogenesis,^{2–8} but the mechanisms of allergic IL-33 production remain unclear.

IL-33 was originally identified as a nuclear protein in endothelial cells, and then found to be produced by not only endothelial cells, but also epithelial cells, smooth

muscle cells, fibroblasts and haematopoietic cells, especially macrophages.^{1,5} It has been reported that IL-33 is released during necrosis.^{1,6} In allergic diseases, the use of murine pollinosis models has clarified that IL-33 is constitutively expressed in the conjunctival epithelial cells,⁹ and that challenge with a topical ragweed allergen elevated IL-33 mRNA levels in conjunctival tissue 24 hr after the challenge.⁹ Using a murine model of asthma, we and others demonstrated that IL-33 is produced in the lung upon exposure to antigens such as ovalbumin (OVA)^{10–13} and house dust mite.¹⁴ These findings strongly suggested that antigen challenge induces the transcription of IL-33 mRNA, leading to the synthesis of the IL-33 protein in the lung. However, it was also reported that antigen exposure-induced IL-33 production in the airway epithelial cells was dependent on the innate immune response through toll-like receptor (TLR) 4.¹⁴ Collectively, it is unclear whether mechanisms underlying antigen-induced IL-33 production include specific antigen-dependent acquired immune responses, and it is unknown which immune component cells are involved in the antigen recognition leading to airway IL-33 production.

The purpose of the present study was to elucidate: (i) whether mechanisms underlying antigen-induced airway IL-33 production in the sensitized mice are specific antigen-dependent; and (ii) which immune cells and molecules are involved in recognition of the specific antigen in the lung to lead the allergic IL-33 production.

Materials and methods

Sensitization and challenge

As reported previously,^{11,15–18} 5-week-old BALB/c mice (Japan SLC, Hamamatsu, Japan) were sensitized by intraperitoneal (i.p.) injections with OVA (Grade V; Sigma Chem., St Louis, MO) adsorbed to Al(OH)₃ at a dose of 50 µg OVA/2 mg Al(OH)₃/0.5 ml saline/animal on days 0, 14 and 28. Then, the sensitized mice were challenged on day 35 under inhalation anaesthesia with isoflurane with 2% OVA at a volume of 25 µl intra-tracheally. In place of OVA, 2% bovine serum albumin (BSA; Sigma Chem.) was intra-tracheally administered at a volume of 25 µl. For non-sensitized groups, saline or Al(OH)₃ was i.p. administered on days 0, 14 and 28.

This animal study was approved by the Experimental Animal Research Committee at Setsunan University and Kyoto Pharmaceutical University.

Treatment with anti-FcεRI mAb, anti-CD4 mAb, anti-CD20 mAb, anti-FcγRII/III mAb or clodronate liposomes

Sensitized mice were treated with anti-FcεRIα mAb, MAR-1 or hamster IgG (Thermo Fisher Scientific,

Waltham, MA), or phosphate-buffered saline (PBS) three times at 25, 13 and 1 hr before the challenge at a dose of 10 µg/animal (i.p.). We previously reported that both mast cells and basophils were depleted by a protocol conducted according to the present method.¹⁸

A cell line producing rat IgG2b mAb that recognizes the murine CD4 molecule (YTS191-1-2) was kindly provided by Prof. David D. Chaplin (University of Alabama at Birmingham, Birmingham, AL). The hybridoma cells were cultured and grown, and then i.p. injected into severe combined immunodeficiency mice that had been treated with pristane. Ascites fluid was collected 10–14 days after the injection. IgG was purified by protein G affinity chromatography, followed by desalting using PD-10 column chromatography. The purified anti-CD4 mAb, rat IgG (Sigma Chem.) or PBS was i.p. administered once 18 hr prior to the antigen challenge at a dose of 0.6 mg/animal. We have reported near complete depletion of CD4⁺ cells in the lung, spleen and peripheral blood using this protocol.¹⁶

For depletion of B-cells, anti-CD20 mAb, 5D2,¹⁹ kindly donated by Genentech (San Francisco, CA), was intravenously (i.v.) administered 3 days before the challenge at a dose of 0.4 mg/animal. PBS or rat IgG was administered for control groups.

A cell line producing rat IgG2b mAb that recognizes the murine FcγRII/III molecule (2-4G2) was purchased from American Type Culture Collection (Manassas, VA). The anti-FcγRII/III antibody was collected and purified in the same manner as the anti-CD4 mAb. As previously reported,²⁰ the anti-FcγRII/III antibody was used to specifically block cell surface FcγRII/III. The anti-FcγRII/III mAb, rat IgG or PBS was i.p. administered once 24 hr prior to the antigen challenge at a dose of 0.5 mg/animal.

For reduction of alveolar macrophages, clodronate liposomes (Clophosome®; FormuMax Scientific, Sunnyvale, CA) were intra-tracheally administered twice at a volume of 25 µl/animal in a 6-hr interval 3 days before the challenge. PBS or control liposomes (FormuMax Scientific) were administered for control groups.

Measurement of the amount of IL-33 and IL-33 mRNA in the lungs

The lungs were isolated under anaesthesia with pentobarbital and xylazine before or 4 hr after the OVA challenge. The right lobes were homogenized in 1 ml of protease inhibitor cocktail tablet (Complete, Mini®; Roche, Mannheim, Germany)-containing tissue protein extraction reagent (T-PER; Thermo Fisher Scientific). Following centrifugation, the amount of IL-33 in the supernatant was assayed by the ELISA kit (Thermo Fisher Scientific).

The isolated left lobes were homogenized in 0.5 ml of RNAiso Plus® (Takara, Otsu, Japan). Total RNA was extracted by isopropyl alcohol precipitation after

extraction with chloroform. Genomic DNA in the samples was then removed by treatment with deoxyribonuclease (DNase; RT grade; Nippon Gene, Tokyo, Japan) in the presence of ribonuclease (RNase) inhibitor (human placenta, recombinant, solution, Wako, Osaka, Japan). After the removal of DNase by a mixture of phenol/chloroform/isoamyl alcohol (25/24/1; Nippon Gene), total RNA was extracted by ethanol precipitation in the presence of excess amounts of sodium acetate salt. Reverse transcription was performed using ReverTra Ace[®] (Toyobo, Osaka, Japan) with dNTP Mix (Promega, Tokyo, Japan), Random Primers (Promega), and RNase inhibitor at 30° for 10 min, 42° for 40 min and 99° for 5 min. Quantitative polymerase chain reaction was performed with LightCycler[®] Nano (Roche). cDNA samples were mixed with SYBR Premix Ex Taq[™] (Takara), and the forward and reverse primers for IL-33 mRNA²¹ and the house-keeping gene, 36B4 mRNA (Nippon Gene),¹¹ and the mixture were heated at 95° for 30 seconds to activate SYBR Premix Ex Taq[™]. Denaturation (95°, 30 seconds), and annealing and extension (63°, 34 seconds) were repeated for 45 cycles. The melting curve of each primer set was then acquired by increasing the temperature from 60° to 95° using the LightCycler Nano 1.1 software (Roche). The relative standard curve method was used to analyse the expression level of IL-33 mRNA.

Detection of alveolar macrophages, dendritic cells and B-cells in the lung

The lobes of the lungs were isolated under anaesthesia with pentobarbital and xylazine before and 4 hr after the OVA challenge. The tissue was cut into approximately 1 × 1 × 1-mm pieces, followed by incubation with 3 mg/ml collagenase type-I (220 U/mg; Thermo Fisher Scientific) at 37° for 1 hr. Cells were dispersed using a syringe, and then filtrated through 70-µm nylon mesh. After centrifugation, contaminating erythrocytes were lysed with ACK lysing buffer.

Lung cells were treated with an anti-CD16/32 (FcγRII/III) antibody (2-4G2) to prevent the non-specific binding of subsequently used antibodies to the cells. After washing with PBS, the cells were stained with eFluor[™] 780-conjugated Fixable Viability Dye (Thermo Fisher Scientific) to stain dead cells for elimination from the subsequent FACS analyses. After washing with 2% fetal bovine serum (FBS)-containing PBS, cells were incubated with eFluor[®] 660-conjugated anti-mouse F4/80 antibody (BM8), APC-conjugated anti-mouse MHC class II (I-A/I-E) antibody (M5/114.15-2), PE-Cy7-conjugated anti-mouse CD11c antibody (N418) and/or eFluor[®] 660-conjugated anti-mouse CD3 antibody (17A2; all from Thermo Fisher Scientific), and/or Alexa Fluor[®] 647-conjugated anti-mouse B220 antibody (RA3-6B2) and/or PerCP/Cy5.5-conjugated anti-mouse CD45 antibody (30-F11; both from BioLegend, San Diego, CA) for 20 min at 4°.

After washing with 2% FBS-containing PBS, cells were analysed using FACSARIA[™] Fusion (Becton Dickinson, San Jose, CA) and FACSDiva[™] version 8.0.1 (Becton Dickinson).

Based on previous reports,^{11,12,22–25} alveolar macrophages and dendritic cells in the lungs were identified as CD45⁺ CD11c⁺ F4/80⁺ cells and CD45⁺ CD11c⁺ I-A/I-E⁺ cells, respectively. B-cells in the lungs were identified as CD45⁺ B220⁺ cells.

Incorporation of a fluorogenic substrate-labelled OVA, DQ-OVA, by lung cells in vivo

DQ-OVA (Thermo Fisher Scientific) is a fluorogenic substrate-labelled OVA that is metabolized to a fluorescent substance after digestion by intracellular proteases. The mice were sensitized by i.p. administration of saline, Al(OH)₃ or OVA+Al(OH)₃ on days 0, 14 and 28 as described above, and then challenged with intra-tracheal administration of DQ-OVA (10^{−4} g/ml) at a volume of 25 µl/animal. As a negative control, 10^{−4} g/ml DQ-BSA (Thermo Fisher Scientific) was administered at a volume of 25 µl/animal in mice sensitized by i.p. administration of saline, Al(OH)₃, OVA+Al(OH)₃ or BSA+Al(OH)₃ on days 0, 14 and 28 as described above. The left lobe of the lung was isolated under anaesthesia with pentobarbital and xylazine 30 min after the challenge. This tissue was digested with collagenase as described above, followed by treatment with ACK lysing buffer. After incubation with the anti-CD16/32 (FcγRII/III) antibody, the cells were stained following the above protocol for FACS analyses. Gated DQ-OVA⁺ cells were analysed in expression of CD45, CD11c, F4/80, I-A/I-E and B220 to measure numbers of alveolar macrophages, dendritic cells and B-cells in the lungs as CD45⁺ CD11c⁺ F4/80⁺ cells, CD45⁺ CD11c⁺ I-A/I-E⁺ cells and CD45⁺ B220⁺ cells, respectively.

Incorporation of DQ-OVA by lung cells in vitro

As described above, the lobes of the lungs were isolated under anaesthesia before the OVA challenge. The tissue was cut into approximately 1 × 1 × 1-mm pieces, followed by incubation with 3 mg/ml collagenase type-I (220 U/mg) at 37° for 1 hr. Cells were dispersed using a syringe, and then filtrated through 70-µm nylon mesh. After centrifugation, contaminating erythrocytes were lysed with ACK lysing buffer. The single cells were suspended in 10% FBS-containing RPMI1640 medium at 5 × 10⁵ cells/250 µl/well. Pooled serum collected from the saline-treated or OVA+Al(OH)₃-sensitized mice was added to the single cell suspension at a volume of 2.5 µl/well. Ten minutes after the serum addition, DQ-OVA was added to make a final concentration of 10^{−6} g/ml, followed by incubation at 37° for 30 min. Then, the incubated cells

were washed twice with PBS. Flow cytometry detection of DQ-OVA-incorporated alveolar macrophages, dendritic cells and B-cells were conducted as described above.

Immunohistochemical detection of IL-33-producing alveolar macrophages

Before and 4 hr after the OVA challenge, the lung was perfused with approximately 5 ml PBS, and then approximately 100 ml 10% buffered formalin under the anaesthesia with pentobarbital and xylazine. Then, the left lobes of the lungs were isolated, and cut into three sections, followed by immersion in 10% buffered formalin. The fixed tissues were embedded in paraffin, and cut into 4- μ m-thick sections. IL-33- and F4/80-positive cells were detected using sequentially cut sections by immunohistochemistry. Antigenicity in the tissue sections was generally activated by immersion of the sections on slides in Histo VT one (Nacalai Tesque, Kyoto, Japan). After washing, the sections were treated with 3% hydrogen peroxide-methanol solution. Following washing, the sections were treated with diluted rabbit normal serum (Vector Lab., Burlingame, CA) at room temperature for 30 min in

order to prevent non-specific binding of the secondary antibody. After washing, tissue sections were stained with anti-mouse IL-33 (AF3626, 5 μ g/ml) or normal goat IgG (AB-108-C, 5 μ g/ml; R&D Systems, Minneapolis, MN), or anti-mouse F4/80 (BM8, 12.5 μ g/ml) or rat IgG2a, k isotype control (RKT2758, 12.5 μ g/ml; BioLegend) at room temperature for 1 hr. After washing, sections were stained with biotinylated anti-goat IgG antibody (1:200) or biotinylated anti-rat IgG antibody (1:200; Vector Lab.) at room temperature for 30 min. After washing, tissue sections were stained with a mixture of reagent A (avidin DH) and reagent B (biotinylated horseradish peroxidase H; both from Vector Lab.) at room temperature for 30 min, followed by colour development using ImmPACTTM DAB (Vector Lab.), and then counterstaining with haematoxylin solution.

Statistical analyses

A one-way analysis of variance (ANOVA) was performed. If significant differences were detected, individual group differences were determined by Dunnett's tests. The unpaired *t*-test was used to compare between two groups.

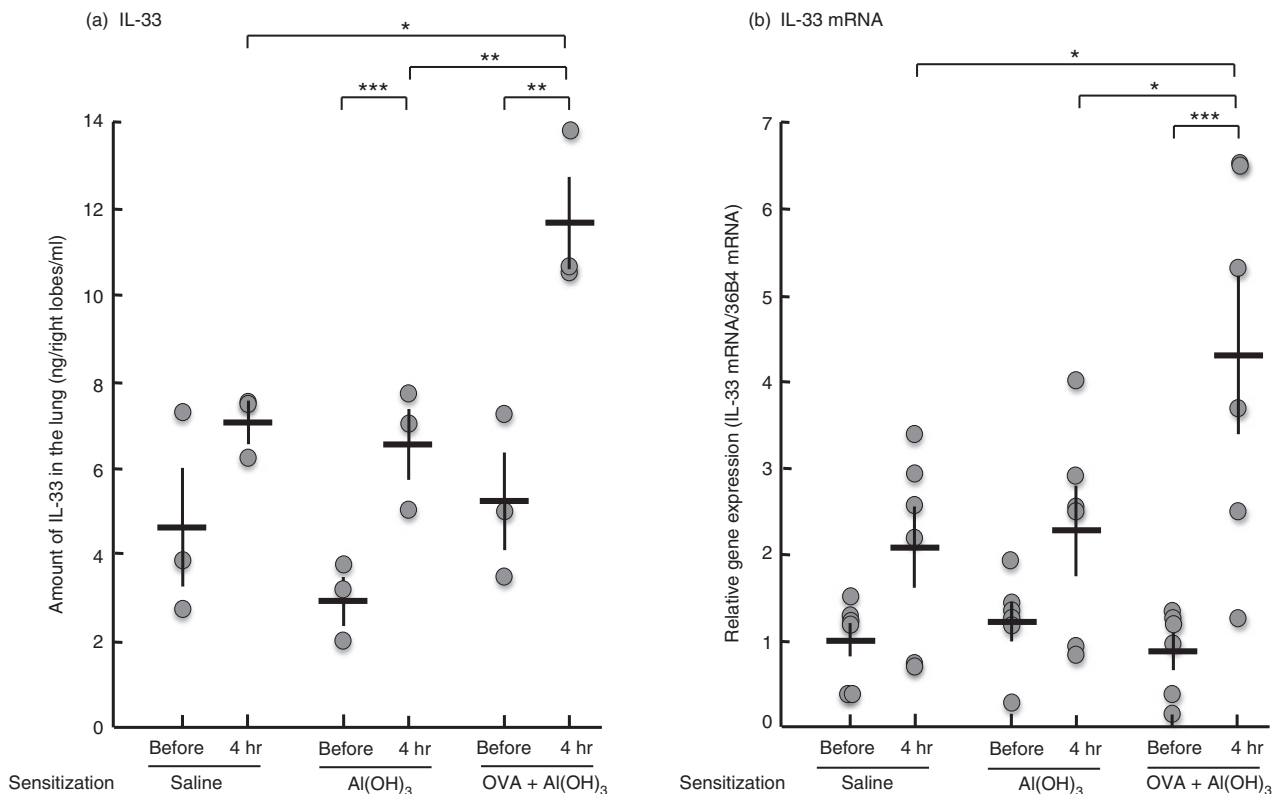


Figure 1. Intra-tracheal ovalbumin (OVA) challenge-induced increases in interleukin (IL)-33 protein (a) and IL-33 mRNA (b) in the lungs of non-sensitized [saline and Al(OH)₃] and sensitized [OVA+Al(OH)₃] mice. Mice were intraperitoneally (i.p.) treated with saline, Al(OH)₃ or OVA+Al(OH)₃ on days 0, 14 and 28, and then OVA was intra-tracheally administered on day 35. Before or 4 hr after the OVA administration, the lungs were isolated for measurement of IL-33 and IL-33 mRNA. Each point represents individual value. Mean $\pm$ SEM of three–six animals are also shown. **P* < 0.05; ***P* < 0.01; ****P* < 0.001.

A probability value (P) below 0.05 was considered significant. These statistical analyses were conducted using JMP® Pro 11.2.0 (SAS Institute Japan, Tokyo, Japan).

Results

Antigen challenge-induced production of IL-33 in sensitized mice

Single intra-tracheal administration of OVA induced increases in both IL-33 protein and mRNA levels in the lungs of non-sensitized mice, although not significantly (Fig. 1a,b). However, when the OVA+Al(OH)₃-sensitized

Table 1. Intra-tracheal BSA challenge-induced increases in IL-33 in the lungs of non-sensitized [saline and Al(OH)₃] and OVA+Al(OH)₃-sensitized mice

	Amount of IL-33 (ng/ml) Treatment (i.p. on days 0, 14 and 28)		
	Saline	Al(OH) ₃	OVA+ Al(OH) ₃
Before BSA administration	4.6 ± 1.4	3.0 ± 0.9	5.2 ± 1.9
4 hr after BSA administration	4.1 ± 0.7	3.8 ± 0.3	5.9 ± 1.9

Mice were intraperitoneally (i.p.) sensitized with saline, Al(OH)₃ or OVA+Al(OH)₃ on days 0, 14 and 28, and then bovine serum albumin (BSA) was intra-tracheally challenged on day 35. Four hours after the BSA administration, the lungs were isolated for measurement of IL-33. Each value represents the mean ± SEM of three animals. IL, interleukin; OVA, ovalbumin.

mice were challenged with OVA, significantly more IL-33 protein and mRNA were produced than in OVA-challenged, non-sensitized mice (Fig. 1a,b).

On the other hand, when BSA was used as a challenge antigen in place of OVA, IL-33 was not produced in any non-sensitized or OVA+Al(OH)₃-sensitized mice (Table 1).

Effects of depletion of mast cells/basophils or CD4⁺ cells on the antigen challenge-induced production of IL-33

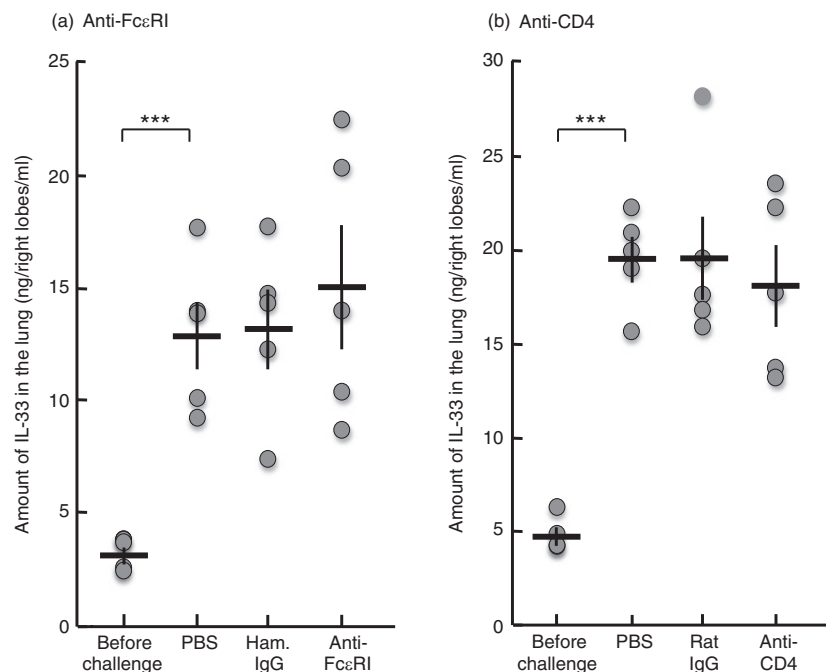
The anti-FcεRI mAb MAR-1 was administered 1 day before the OVA challenge. We previously reported that this protocol led to depletion of mast cells and basophils in the lungs of sensitized mice.¹⁸ However, the antigen-induced IL-33 production in the lungs was not affected (Fig. 2a).

On the other hand, we also reported that treatment with the anti-CD4 mAb YTS191.1.2 1 day before the OVA challenge depleted CD4⁺ cells in the lungs, spleen and peripheral blood in sensitized mice.¹⁶ Even after depletion of CD4⁺ cells, the antigen-induced airway IL-33 production was not affected (Fig. 2b).

Incorporation of OVA by lung cells of the sensitized mice

Using DQ-OVA as a probe, we examined whether OVA was antigen-specifically incorporated into the lung cells of sensitized mice *in vivo*. Lung cells of the sensitized mice incorporated markedly higher amounts of DQ-OVA in

Figure 2. Effects of depletion of mast cells/basophils by anti-FcεRI mAb (a), and of Th2 cells by anti-CD4 mAb (b) on the ovalbumin (OVA) challenge-induced increase in interleukin (IL)-33 in the lungs of OVA+Al(OH)₃-sensitized mice. Mice were intraperitoneally (i.p.) sensitized with OVA+Al(OH)₃ on days 0, 14 and 28, and then OVA was intra-tracheally challenged on day 35. Before or 4 hr after the OVA administration, the lungs were isolated for measurement of IL-33. Anti-FcεRI mAb (MAR-1, 10 µg/animal/time), hamster IgG or phosphate-buffered saline (PBS) was i.p. administered three times at 25, 13 and 1 hr before the OVA challenge. Anti-CD4 mAb (YTS191.1.2, 0.6 mg/animal), rat IgG or PBS was i.p. administered once 18 hr prior to the challenge. Each point represents individual value. Mean ± SEM of five animals are also shown. *** P < 0.001.



comparison with those of the non-sensitized mice (Fig. 3a,c). The lung cells were digested by collagenase, and which cells incorporated OVA was analysed (Fig. 3c, d). As a result, $24.7 \pm 3.5\%$ ($n = 5$) of the OVA-incorporating cells were B-cells ($CD45^+ B220^+$ cells). In addition, $6.8 \pm 2.7\%$ ($n = 5$) and $10.2 \pm 6.3\%$ ($n = 5$) of the cells were alveolar macrophages ($CD45^+ CD11c^+ F4/80^+$ cells) and dendritic cells ($CD45^+ CD11c^+ I-A/I-E^+$ cells), respectively (Fig. 3a,d). Unfortunately, other cell types incorporating the OVA protein have not been clarified.

On the other hand, DQ-BSA was not incorporated in OVA+Al(OH)₃-sensitized mice, whereas the lung cells of BSA+Al(OH)₃-sensitized mice incorporated DQ-BSA with statistical significance (Fig. 3b).

Effects of anti-FcγRII/III mAb on the incorporation of OVA and antigen challenge-induced production of IL-33

We next examined whether the phagocytosis of DQ-OVA was mediated by FcγRII/III on the cell surface of B-cells, alveolar macrophages and dendritic cells. Blocking of

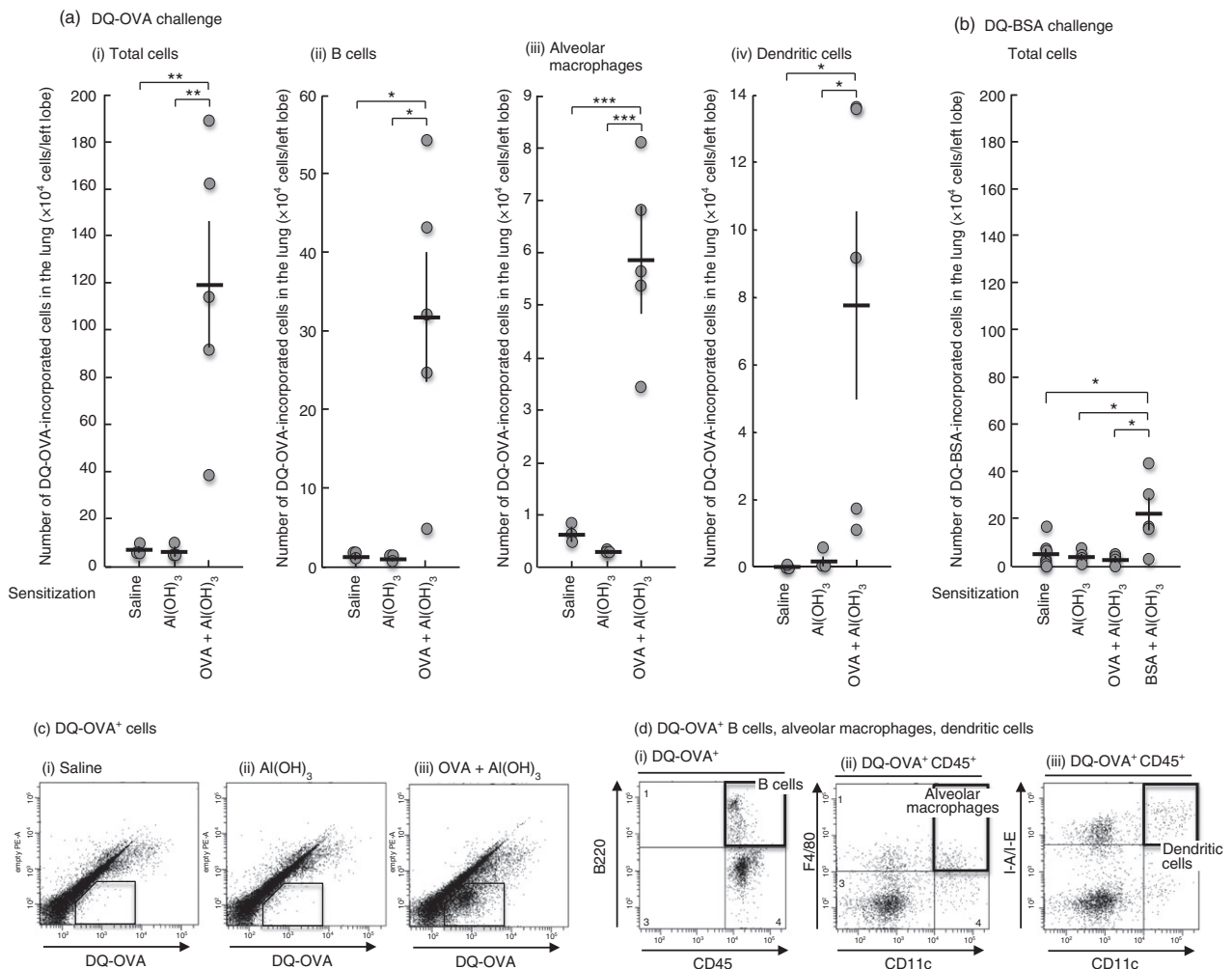


Figure 3. Incorporation of fluorogenic substrate-labelled antigen (DQ-OVA) by the lung cells of non-sensitized [saline and Al(OH)₃] and sensitized [OVA+Al(OH)₃] mice. (a) Amounts of incorporated DQ-OVA in total cells (i), B-cells ($CD45^+ B220^+$ cells) (ii), alveolar macrophages ($CD45^+ CD11c^+ F4/80^+$ cells) (iii), and dendritic cells ($CD45^+ CD11c^+ I-A/I-E^+$ cells) (iv) in the lungs are shown. Mice were intraperitoneally (i.p.) treated with saline, Al(OH)₃ or OVA+Al(OH)₃ on days 0, 14 and 28, and then DQ-OVA (10^{-4} g/ml, 25 μ l/animal) was intra-tracheally administered on day 35, followed by isolation of the lung 30 min after the DQ-OVA administration. The lung was enzymatically digested, and the obtained lung cells were analysed by flow cytometry to detect DQ-OVA⁺ cells. (b) Amounts of incorporated DQ-BSA in total lung cells of the non-sensitized, OVA+Al(OH)₃-sensitized and BSA+Al(OH)₃-sensitized mice. DQ-BSA (10^{-4} g/ml, 25 μ l/animal) was intra-tracheally administered in place of DQ-OVA. Each point represents individual value. Mean $\pm$ SEM of three–seven animals are also shown. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. (c) Typical flow cytometric profiles for detection of DQ-OVA⁺ cells in the lungs of saline-treated (i), Al(OH)₃-treated (ii) and OVA+Al(OH)₃-sensitized (iii) mice. (d) Typical flow cytometric profiles for detection of DQ-OVA⁺ B-cells ($CD45^+ B220^+$ cells) (i), DQ-OVA⁺ alveolar macrophages ($CD45^+ CD11c^+ F4/80^+$ cells) (ii) and DQ-OVA⁺ dendritic cells ($CD45^+ CD11c^+ I-A/I-E^+$ cells) (iii). OVA, ovalbumin.

Fc γ RII/III by the anti-Fc γ RII/III mAb 2-4G2 almost completely abolished the incorporation of DQ-OVA in B-cells, alveolar macrophages and dendritic cells (Fig. 4a). Furthermore, the OVA-induced IL-33 production was also attenuated by the anti-Fc γ RII/III mAb (Fig. 4b).

Effects of depletion of B-cells on the antigen challenge-induced production of IL-33

As B-cells antigen-specifically incorporated OVA (Fig. 3a), we examined whether B-cells were involved in the antigen-specific production of IL-33 in the lungs. As reported previously,¹⁹ the anti-CD20 mAb 5D2 nearly depleted the lungs of all B-cells (CD45⁺ B220⁺ cells; Fig. 5a). The number of B-cells (CD45⁺ B220⁺ cells) in the spleen was also markedly decreased by the anti-CD20 mAb (Fig. 5a). However, the IL-33 production was not affected even by the near complete depletion of B-cells (Fig. 5b).

Effects of reduction of alveolar macrophages on the antigen challenge-induced production of IL-33

As alveolar macrophages also antigen-specifically incorporated OVA (Fig. 3a), the relationship between alveolar

macrophages and the antigen-induced IL-33 production in the lungs was examined. As expected, intra-tracheal administration of clodronate liposomes significantly reduced the number of alveolar macrophages (CD45⁺ CD11c⁺ F4/80⁺ cells) in the lung (Fig. 6a), whereas dendritic cells (CD45⁺ CD11c⁺ I-A/I-E⁺ cells) were unaffected by clodronate liposomes (Fig. 6b). OVA-induced IL-33 production was significantly suppressed by the reduction of alveolar macrophages in the lung (Fig. 6c).

In vitro incorporation of OVA by lung cells in the presence of sensitized mouse serum

It was examined whether the incorporation of OVA by alveolar macrophages can be reproduced *in vitro*. Unfortunately, the lung cells including alveolar macrophages isolated from the OVA+Al(OH)₃-sensitized mice did not incorporate DQ-OVA in higher quantity than the cells isolated from saline- and Al(OH)₃-treated animals (Fig. 7a,b). However, interestingly, when serum collected from the OVA+Al(OH)₃-sensitized mice was added to the culture system, the OVA incorporation was dramatically increased (Fig. 7a,b). Degrees of OVA incorporation in

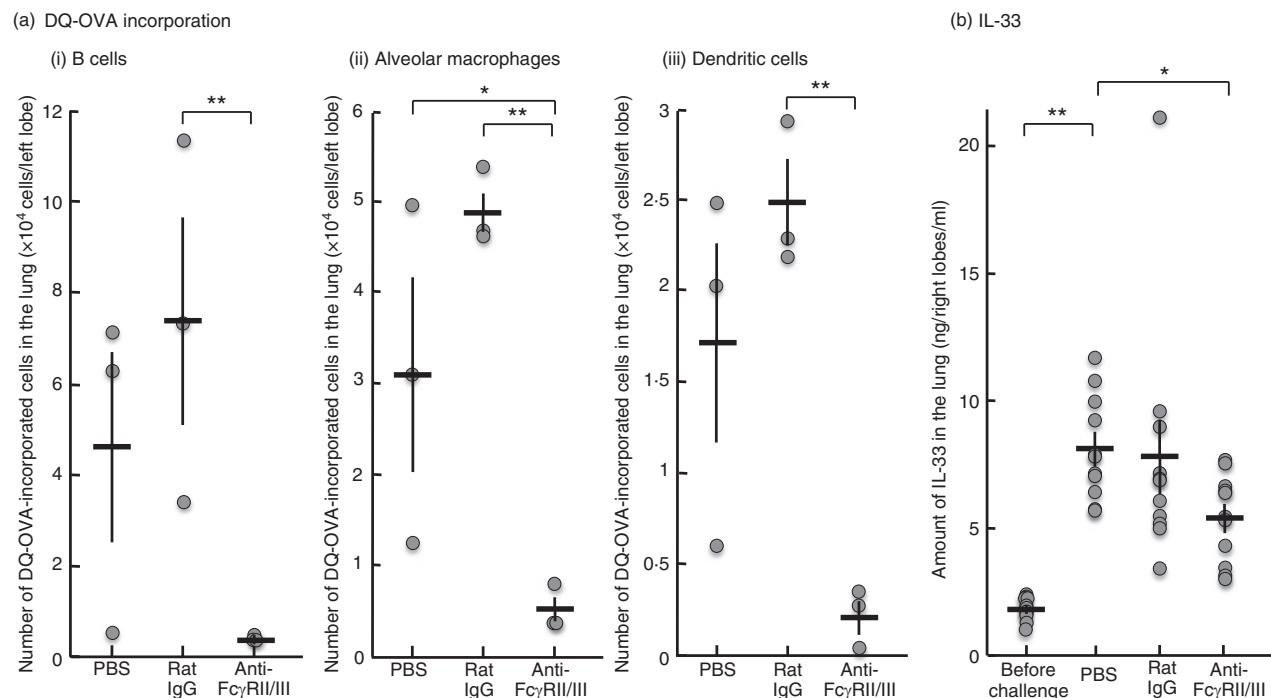


Figure 4. (a) Effects of anti-Fc γ RII/III mAb on the incorporation of DQ-OVA by B-cells (CD45⁺ B220⁺ cells) (i), alveolar macrophages (CD45⁺ CD11c⁺ F4/80⁺ cells) (ii) and dendritic cells (CD45⁺ CD11c⁺ I-A/I-E⁺ cells) (iii) in the lungs of OVA+Al(OH)₃-sensitized mice. (b) Effects of anti-Fc γ RII/III mAb on the OVA challenge-induced interleukin (IL)-33 production in the lungs of OVA+Al(OH)₃-sensitized mice. Mice were intraperitoneally (i.p.) sensitized with OVA+Al(OH)₃ on days 0, 14 and 28, and then DQ-OVA or OVA was intra-tracheally challenged on day 35. Thirty minutes and 4 hr after the respective DQ-OVA and OVA administration, the lungs were isolated for analyses of DQ-OVA⁺ cells and measurement of IL-33, respectively. The anti-Fc γ RII/III mAb (2-4G2, 0.5 mg/animal), rat IgG or phosphate-buffered saline (PBS) was i.p. administered once 18 hr prior to the antigen challenge. Each point represents individual value. Mean $\pm$ SEM of three (a) and 11 (b) animals are also shown. * P < 0.05; ** P < 0.01. OVA, ovalbumin.

the presence of the sensitized mouse serum by the lung cells including alveolar macrophages were not significantly different among saline-treated, Al(OH)_3 -treated and OVA+ Al(OH)_3 -treated groups (Fig. 7a,b).

Immunohistological detection of IL-33-producing cells in the lung

Compared with the lung histology of the non-challenged mice (Fig. 8a), after the antigen challenge, the immunoreactivity of IL-33 was potentiated in the bronchial epithelial cells and smooth muscle cells, and leucocytes at the alveolar site (Fig. 8b). As shown in the tissue sections that were sequentially cut (Fig. 8b,iii,iv), the IL-33-immunoreactive leucocytes were in part F4/80-positive cells (macrophages).

Discussion

First, we assessed whether the mechanisms underlying the intra-tracheal antigen exposure-induced airway IL-33 production were antigen-specific. Airway OVA exposure

evoked increases in IL-33 expression at both protein and mRNA levels even in non-sensitized mice. Compared with the levels of IL-33 and IL-33 mRNA in OVA-exposed, non-sensitized mice, those in OVA+ Al(OH)_3 -sensitized mice were significantly higher. Moreover, BSA did not promote IL-33 production in the lungs of the sensitized mice. Thus, the OVA-induced airway IL-33 production in the sensitized mice included mechanisms associated with not only innate immunity but also antigen-specific acquired immunity. Hammad *et al.*¹⁴ demonstrated that airway exposure to house dust mites induced IL-33 production in mice, and that the airway IL-33 production was dependent on TLR4 activation in airway epithelial cells. Like house dust mites, the OVA used in the present study was not endotoxin-free. Thus, OVA-induced airway IL-33 production even in the non-sensitized mice was induced by innate immune responses associated with TLR4 activation. In this study, we focused on the acquired immune mechanisms involved in further promotion of IL-33 production in the OVA+ Al(OH)_3 -sensitized mice by OVA.

The involvement of mast cells/basophils and Th2 cells in the OVA-induced airway IL-33 production in

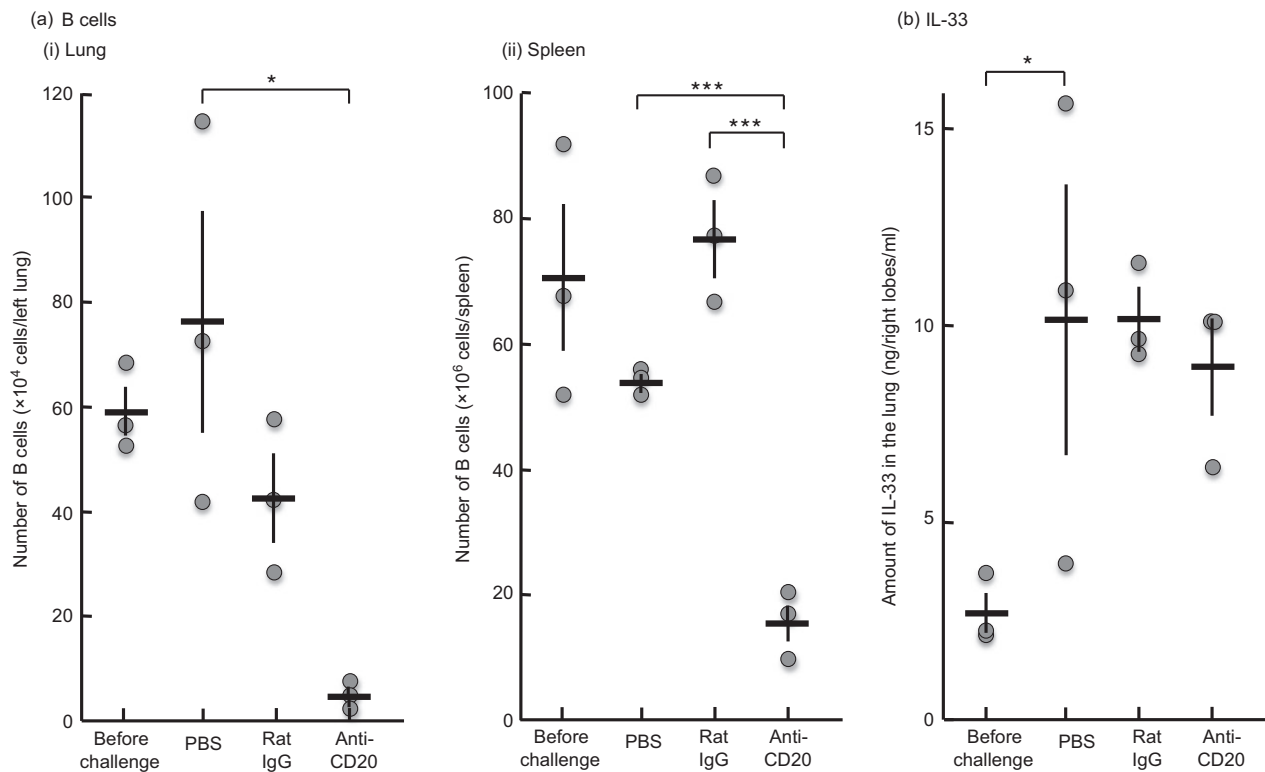


Figure 5. Effects of depletion of B-cells by anti-CD20 mAb on the ovalbumin (OVA) challenge-induced increase in interleukin (IL)-33 in the lungs of OVA+ Al(OH)_3 -sensitized mice. (a) Numbers of B-cells ($\text{CD45}^+ \text{B220}^+$ cells) in the lungs (i) and spleen (ii) of anti-CD20 mAb-treated or non-treated mice. (b) Effects of anti-CD20 mAb on the OVA challenge-induced increase in IL-33 in the lungs of sensitized mice. Mice were intraperitoneally (i.p.) sensitized with OVA+ Al(OH)_3 on days 0, 14 and 28, and then OVA was intra-tracheally challenged on day 35. Before or 4 hr after the OVA administration, the lungs and spleen were isolated for measurement of the number of B-cells or IL-33 level. Anti-CD20 mAb (5D2, 0.4 mg/animal), rat IgG or phosphate-buffered saline (PBS) was intravenously (i.v.) administered once 3 days before the challenge. Each point represents individual value. Mean $\pm$ SEM of three animals are also shown. * $P < 0.05$; *** $P < 0.001$.

sensitized mice was evaluated. Unexpectedly, the depletion of neither mast cells/basophils nor CD4⁺ cells affected the OVA-induced airway IL-33 production in the sensitized animals. As previously reported,^{26–28} the anti-FcεRI mAb and anti-CD4 mAb successfully depleted mast cells/basophils and CD4⁺ cells in mice, and eliminated the functions of these cells.^{16,18} According to these results, neither antigen-specific IgE-bearing mast cells/basophils nor antigen-specific Th2 cells being presented the antigen by antigen-presenting cells were involved in the acquired immune mechanisms associated with the airway IL-33 production.

However, it was unclear how the immune component cells recognized specific antigens in the lungs. Thus, we focused on phagocytosis of OVA by the lung cells. DQ-OVA was used as an OVA probe because it becomes a fluorogenic substance once incorporated and digested by intracellular proteases. Similar with antigen-induced IL-33 production, the lung cells of sensitized mice incorporated

more DQ-OVA than those of non-sensitized mice. FACS analyses clarified that the lung cells incorporating DQ-OVA included B-cells, alveolar macrophages and dendritic cells. The results indicated that antigen-specific mechanisms existed prior to the incorporation of antigens by the lung cells.

In the OVA-sensitized mice, i.p. sensitization three times with OVA+Al(OH)₃ produced large amounts of not only antigen-specific IgE antibody but also antigen-specific IgG antibody in the serum.¹⁵ Thus, the OVA–IgG antibody immune complex may be incorporated into the lung cells, including B-cells, alveolar macrophages and dendritic cells, through the IgG receptor FcγRII/III on the cell surface. We next examined the effects of anti-FcγRII/III mAb on the incorporation of DQ-OVA by the lung cells and airway IL-33 production in the sensitized mice. As expected, the neutralization of FcγRII/III by the mAb almost completely abolished the incorporation of DQ-OVA into B-cells, alveolar macrophages and dendritic

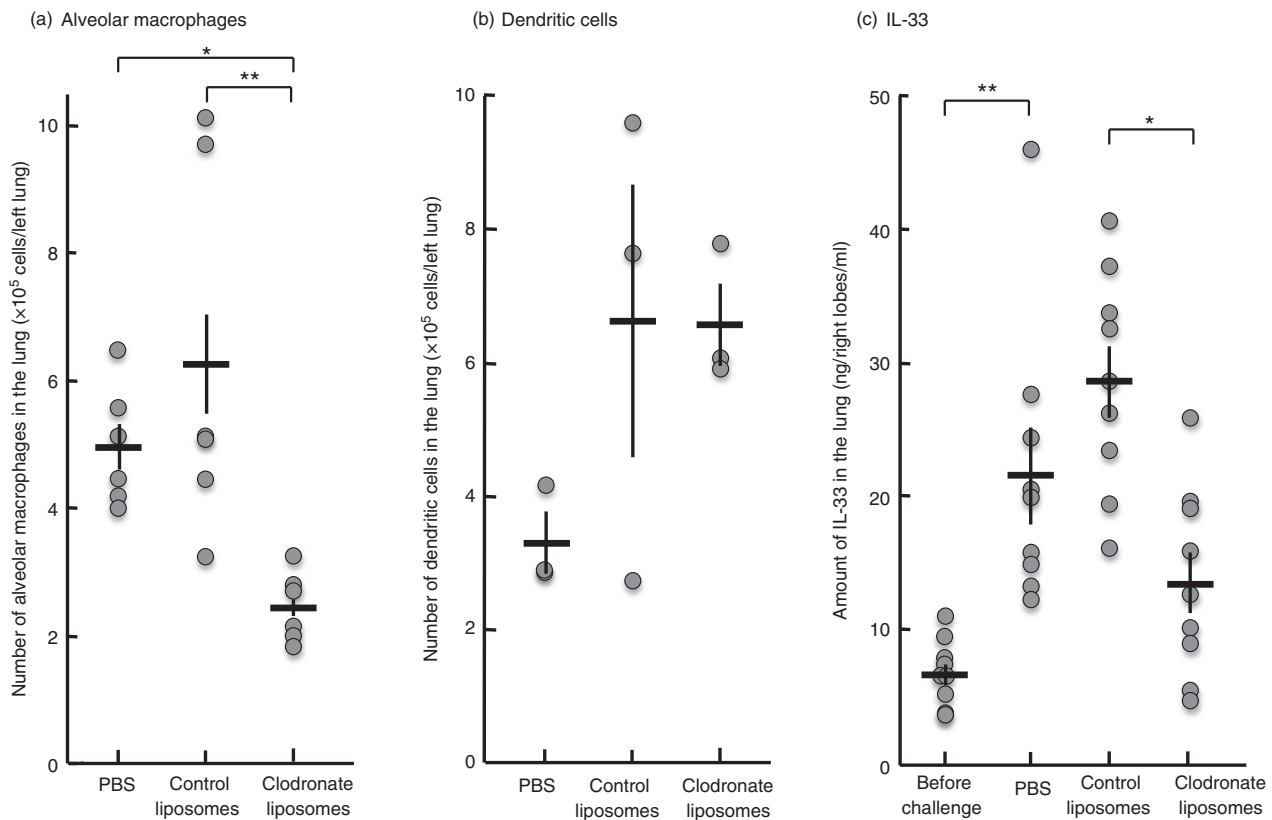


Figure 6. Effects of reduction of alveolar macrophages by clodronate liposomes on the ovalbumin (OVA) challenge-induced increase in interleukin (IL)-33 in the lungs of OVA+Al(OH)₃-sensitized mice. (a) Numbers of alveolar macrophages (CD45⁺ CD11c⁺ F4/80⁺ cells) (i) and dendritic cells (CD45⁺ CD11c⁺ I-A/I-E⁺ cells) (ii) in the lungs of clodronate liposome-treated or untreated mice. (b) Effects of clodronate liposomes on the OVA challenge-induced increase in IL-33 in the lungs of sensitized mice. Mice were intraperitoneally (i.p.) sensitized with OVA+Al(OH)₃ on days 0, 14 and 28, and then OVA was intra-tracheally challenged on day 35. Before (a) or 4 hr (b) after the OVA challenge, the lungs were isolated for measurement of the numbers of alveolar macrophages and dendritic cells (a), and IL-33 level (b), respectively. Clodronate liposomes, control liposomes or phosphate-buffered saline (PBS) was intra-tracheally administered twice in a 6-hr interval 3 days before the challenge. Each point represents individual value. Mean ± SEM of three–six (a and b) and nine (c) animals are also shown. **P* < 0.05; ***P* < 0.01.

cells, and partially suppressed the OVA-induced IL-33 production in the lungs. The incomplete inhibition of IL-33 production by the anti-Fc γ RII/III mAb should be due to the innate mechanisms as shown in the present study, in which intra-tracheal administration of OVA induced IL-33 production even in the non-sensitized mice. Therefore, the administered OVA was likely captured by the antigen-specific IgG antibody, and the antigen-antibody immune complex was then incorporated by the B-cells, alveolar macrophages and dendritic cells through Fc γ RII/III on their cell surface. This may be a substantial antigen-specific mechanism in the allergic IL-33 production.

In this study, it was also demonstrated that when the lung cells of the OVA+Al(OH) $_3$ -sensitized mice were incubated with DQ-OVA *in vitro*, the lung cells incorporated only small amounts of DQ-OVA, whereas when serum collected from the OVA+Al(OH) $_3$ -sensitized mice was added to the *in vitro* culture, the incorporation of DQ-OVA was markedly upregulated. Therefore, serum factors, such as antigen-specific IgG antibodies, may be required for the phagocytosis of OVA by lung cells. Additionally, the requirement of covalent interaction between complement C3b and the antigen-IgG immune complex has been suggested.²⁹

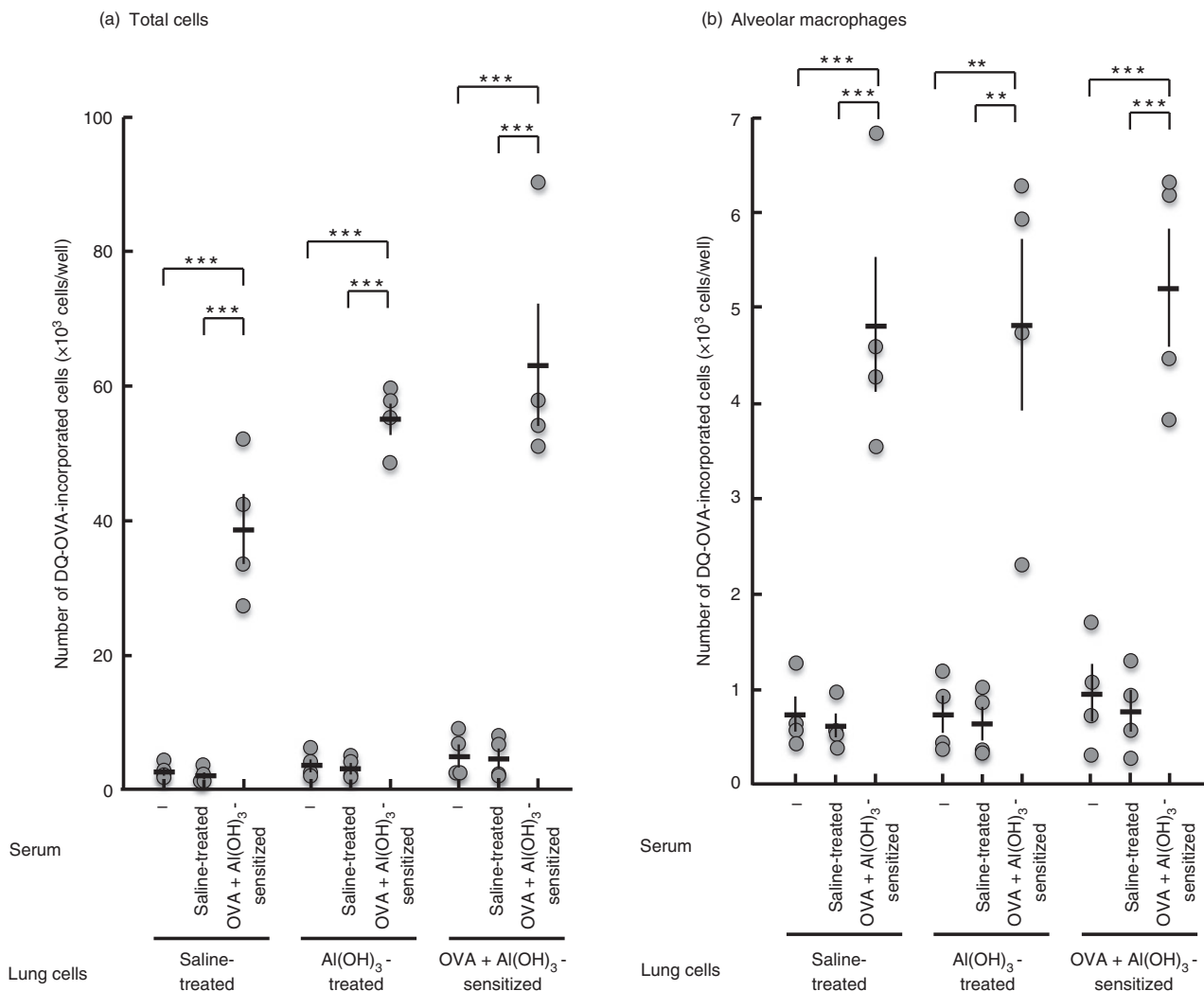


Figure 7. *In vitro* incorporation of fluorogenic substrate-labelled antigen (DQ-OVA) by the lung cells isolated from non-sensitized [saline and Al(OH) $_3$] and sensitized [OVA+Al(OH) $_3$] mice in the absence or presence of serum collected from non-sensitized (saline) and sensitized [OVA+Al(OH) $_3$] mice. Amounts of incorporated DQ-OVA in total cells (a) and alveolar macrophages (CD45 $^+$ CD11c $^+$ F4/80 $^+$ cells) (b) are shown. Mice were intraperitoneally (i.p.) treated with saline, Al(OH) $_3$ or OVA+Al(OH) $_3$ on days 0, 14 and 28. Then, the lungs were isolated followed by enzymatic digestion. Single cell suspension of the lung cells was incubated with DQ-OVA at 37°C for 30 min. Pooled serum collected from the saline-treated or OVA+Al(OH) $_3$ -sensitized mice was added to the cell suspension at a volume of 2.5 μ l/250 μ l cell suspension/well. DQ-OVA-incorporated cells including alveolar macrophages were analysed by flow cytometer. Each point represents individual value. Mean $\pm$ SEM of four animals are also shown. ** $P < 0.01$; *** $P < 0.001$. OVA, ovalbumin.

The three types of phagocytic cells, B-cells, alveolar macrophages and dendritic cells, antigen-specifically incorporated OVA in the lung. Thus, we depleted B-cells and alveolar macrophages in the lungs. Although depletion of B-cells by the anti-CD20 mAb did not affect the antigen-induced IL-33 production, reduction of alveolar macrophages by clodronate liposomes partially but significantly suppressed IL-33 production. Taken together, it was suggested that alveolar macrophages incorporated the OVA-IgG antibody complex through Fc γ RII/III on the cell surface, leading to IL-33 production. On the other hand, the number of dendritic cells was not reduced by clodronate liposomes. Uptake of liposomal-encapsulated clodronate has been demonstrated with monocytes and macrophages, and results in selective apoptosis.³⁰ Wang *et al.*³¹ recently reported that clodronate liposomes selectively depleted immature dendritic cells but not mature dendritic cells. In our present study, dendritic cells were identified as CD45⁺ CD11c⁺ I-A/I-E⁺ cells, as previously reported.^{11,12,22,23,25} As shown in Fig. 3d, the expression of I-A/I-E molecules on the dendritic cells was high, suggesting that the dendritic cells may have been a mature class of dendritic cells. The effect of clodronate liposomes

on the dendritic cells suggests that the mature dendritic cells were not involved in the antigen-induced airway IL-33 production. However, the roles of the different dendritic cell subtypes in IL-33 production need to be clarified.

The present immunohistological analyses showed that the bronchial epithelial cells and smooth muscle cells, and alveolar macrophages produced IL-33 after the intra-tracheal antigen challenge. Our previous immunohistological study using the same kind of anti-IL-33 antibody showed that the bronchial epithelial cells were a major cellular source of IL-33 after the antigen challenge.¹¹ In the present study, the lung was perfused with PBS and then a large volume of 10% buffered formalin, before immersion in 10% buffered formalin although the perfusion procedure was not executed in our previous study.¹¹ The perfusion of the lung with 10% buffered formalin should have improved immunostainability of IL-33 especially in the bronchial smooth muscle cells and leucocytes. Tashiro *et al.*³² also suggested that macrophages themselves are a cellular source of IL-33 in a murine model of allergic airway inflammation. Regarding the smooth muscle cells, to our knowledge, this is the first report to demonstrate that

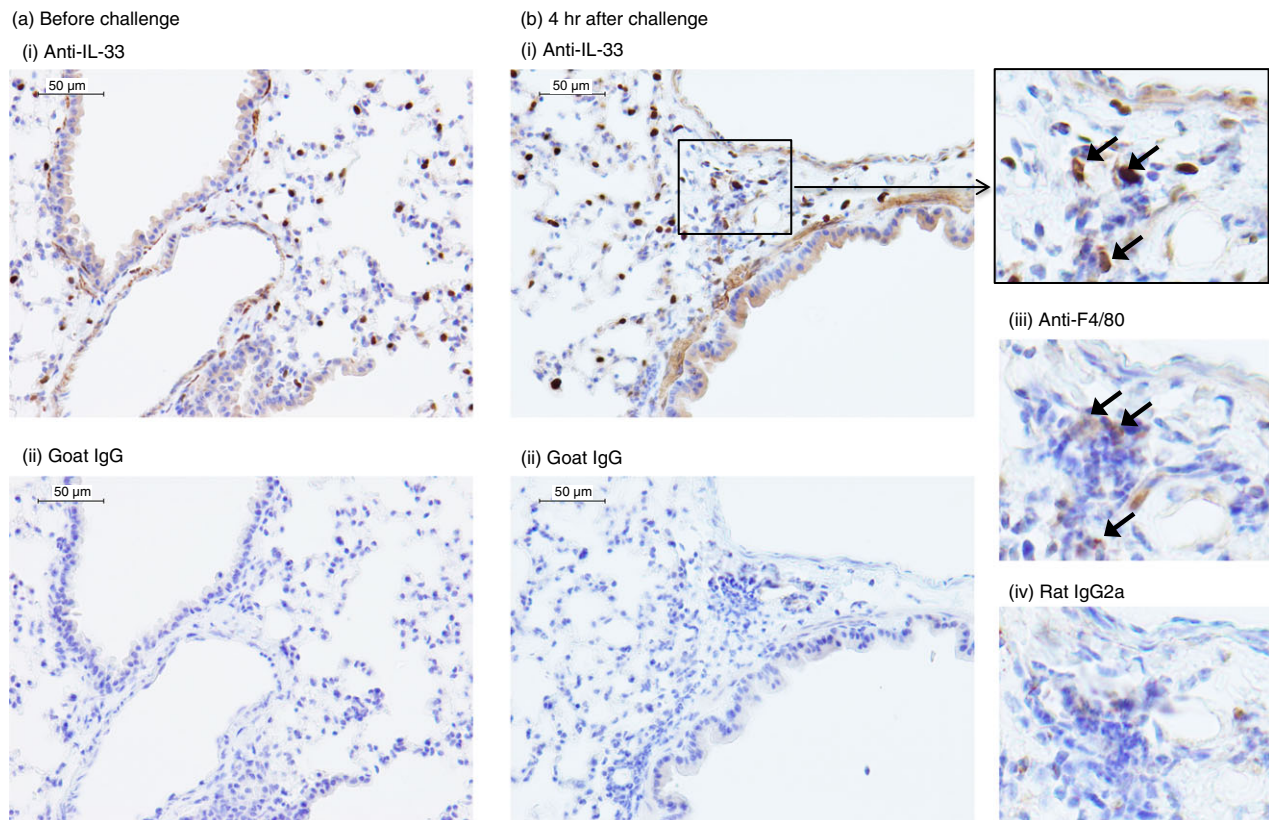


Figure 8. Immunohistological detection of interleukin (IL)-33-immunoreactive cells in lung tissue isolated from sensitized mice before (a) and 4 hr after (b) the intra-tracheal ovalbumin (OVA) challenge. Isolated lung tissues were fixed with 10% formalin, embedded in paraffin and cut into 4- μ m-thick sections, followed by staining with an anti-IL-33 antibody (i) or goat IgG (ii). Sequentially cut sections were stained with an anti-F4/80 antibody (iii) or rat IgG2a (iv) to detect macrophages in the IL-33-producing leucocytes. Scale bar: 50 μ m.

IL-33 was produced from bronchial smooth muscle cells in murine model of allergic airway inflammation *in vivo* even though airway smooth muscle cells of severe asthma patients expressed IL-33.² From the results of the present study, alveolar macrophages incorporating OVA may have directly produced IL-33. Alternatively, the activated alveolar macrophages may directly or indirectly activate airway epithelial cells and smooth muscle cells to produce IL-33. In human bronchial epithelial cells *in vitro*, IL-33 is produced by stimulation with a major macrophage-derived cytokine, TNF- α .³³ It was reported that TNF- α increased the expression of IL-33 in the airway smooth muscle cells *in vitro*.² Oxidative stress may also serve as a key event for IL-33 release by the human airway epithelium.³⁴ The exact cellular sources of IL-33 associated with antigen-specific production need to be clarified.

In this study, it was suggested that IL-33 production from alveolar macrophages, airway epithelial cells and smooth muscle cells were augmented by the acquired immune system. It has become clear that IL-33 plays quite important roles in induction of type 2 inflammation via activation of IL-33 receptor, ST2 on ILC2 as well as Th2 cells, leading to production of type 2-associated cytokines, IL-5 and IL-13.^{1,5–7} In addition, IL-4 is also produced from ILC2 under a certain condition in the presence of Ca²⁺ influx signal (Moro *et al.*, unpublished data). Therefore, IL-33 production from airway cells should be one of indispensable mechanisms underlying pathogenesis of severe asthma.

In conclusion, intra-tracheally administered OVA was bound by the antigen-specific IgG antibody in the lung, and the immune complex was likely incorporated by alveolar macrophages through Fc γ RII/III on the cell surface. This antigen-specific mechanism was involved in the antigen-induced IL-33 production in the lungs of sensitized mice. This mechanism may be useful for development of new pharmacotherapies for asthma.

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Disclosures

The authors have no financial conflicts of interest.

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