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Original Article

Chitin induces steroid-resistant airway inflammation and airway hyperresponsiveness in mice

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Abbreviations:

AM, alveolar macrophage; DAPI, 4',6'-

diamidino-2-phenylindole;

APC, allophycocyanin;

DEX, dexamethasone; HE, hematoxylin and

eosin; LLN, lung-draining lymph node;

NLRP3, NLR family, pyrin domain-

containing 3; PAS, periodic acid-Schiff;

PMA, phorbol 12-myristate 13-acetate;

PRR, pattern-recognition receptor; qRT-

PCR, quantitative real-time reverse

transcription-PCR; Rrs, respiratory system

resistance

ABSTRACT

Background: Previous reports have shown that pathogen-associated patterns (PAMPs) induce the production of interleukin (IL)-1 β in macrophages. Moreover, studies using mouse models also suggest that chitin, which acts as a PAMP, induces adjuvant effects and eosinophilic infiltration in the lung. Thus, we investigated the effects of inhaled chitin in mouse models.

Methods: We developed mouse models of inhaled chitin particle-induced airway inflammation and steroid-resistant ovalbumin (OVA)-induced airway inflammation. Some experimental groups of mice were treated additionally with dexamethasone (DEX). Murine alveolar macrophages (AMs), which were purified from bronchoalveolar lavage (BAL) fluids, were incubated with chitin, and treated with or without DEX.

Results: The numbers of total cells, AMs, lymphocytes, eosinophils, and neutrophils among BAL-derived cells, as well as the IL-1 β levels in BAL fluids and the numbers of IL-1 β -positive cells in lung, were significantly increased by chitin stimulation. Airway hyperresponsiveness (AHR) was aggravated in mice of the chitin inflammation model compared to control animals. The production of IL-1 β was significantly increased in murine AMs by chitin treatment, but DEX administration did not inhibit this chitin-induced IL-1 β production. Furthermore, in mouse models, DEX treatment inhibited the OVA-induced airway inflammation and AHR but not the airway inflammation and AHR induced by chitin or the combination of OVA and chitin.

Conclusions: These results suggest that inhaled chitin induces airway inflammation, AHR, and the production of IL-1 β . Furthermore, our findings demonstrate for the first time that inhaled chitin induces steroid-resistant airway inflammation and AHR. Inhaled chitin may contribute to features of steroid-resistant asthma.

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Introduction

Allergy is acknowledged as a major risk factor for asthma, and sensitization by specific allergens such as insects, mites, or fungi is

fundamental to the development of asthma. These antigens known as pathogen-associated molecular patterns (PAMPs) are recognized by dendritic cells (DCs) and macrophages through pattern-recognition receptors (PRRs).¹ Chitin, a huge insoluble polymer consisting of repeating units of β -(1-4)-poly-N-acetyl-D-glucosamine, is, after cellulose, the second most abundant polysaccharide on earth and acts as a PAMP. Chitin is found in the cell walls of bacteria and fungi, mushrooms, the exoskeleton of crustaceans (e.g., crabs and shrimps), as well as insects.² Chitin is a common constituent of house dust samples, and exposure to it in the

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workplace and at home is associated with asthma, suggesting that it might be an important environmental inhalant antigen and contribute to the development of asthma.^{3–5} In previous reports using mouse models, chitin particles induce eosinophilic infiltration in the lung. The adjuvant effects of chitin in the mouse model of ovalbumin (OVA)-induced asthma have also been observed and involved the tissue accumulation of innate immune cells such as eosinophils.^{6–9} Moreover, inhaled chitin induced the expression of interleukin (IL)-25, IL-33, and thymic stromal lymphopoietin (TSLP), which activated type 2 innate lymphoid cells (ILC2s) and CD4⁺ type 2 helper T cells (Th2 cells) in the lung.^{9–11}

Many PAMPs induce the production of IL-1 β , IL-18, and IL-33 by a mechanism dependent on the activation of the NLR family, pyrin domain-containing 3 (NLRP3) inflammasome in macrophages. These IL-1 family members are highly inflammatory cytokines constitutively expressed in mucosal and barrier cell types, acting as regulators of innate and acquired immune responses. Secretion of IL-1 β requires at least two signals; the first signal is cell priming that results in the intracellular synthesis of pro-IL-1 β protein, whereas the second signal is the inflammasome-dependent proteolysis of pro-IL-1 β to secretory and bioactive IL-1 β .^{2,12,13} Recent clinical and experimental reports implicate NLRP3 inflammasome activation and IL-1 β responses in the pathophysiology of neutrophilic asthma.^{10,14–17} The gene expression levels of NLRP3, caspase-1, and IL-1 β are upregulated in patients with neutrophilic asthma.¹⁴ Moreover, IL-1 β mediates Th17 differentiation and IL-17 production that induces steroid-resistant neutrophilic airway inflammation and airway hyperresponsiveness (AHR) in mice.^{16–19}

The involvement of chitin in asthmatic diseases that are mediated by Th2 cells and ILC2s, however, remains unclear. Although alveolar macrophages (AMs) act as the first line of defense to encounter inhaled particulates and pathogens and play an essential role in the inflammatory responses in the pulmonary immune system, little is known about AM responses induced by chitin. In the current study, we investigated whether inhaled chitin particles induced airway inflammation, the production of IL-1 β in AMs, steroid-resistant airway inflammation in mouse models.

Methods

Preparation of chitin particles

Purified chitin from shrimp shells (Sigma–Aldrich, St. Louis, MO, USA) was suspended in distilled deionized water. Chitin was sonicated at 25% output power three times for 5 min using a sonicator (Bioruptor, Cosmo Bio, Tokyo, Japan). The suspensions were then filtered with 40 μ m sterile cell strainers (BD Biosciences, San Jose, CA, USA). Following centrifugation (2800 $\times$ g, 10 min), small chitin pellets (<40 μ m) were freeze-dried for sample preservation (Flexi-Dry Freeze Drier, FTS Systems, Stone Ridge, NY, USA) and autoclaved. Before utilization, the chitin particles were suspended in the desired volume of sterile PBS or medium. LPS contamination was <1 EU/mg as determined by the ToxinSensor Chromogenic LAL (Limulus amoebocyte lysate) Endotoxin Assay Kit (Genscript, Piscataway, NJ, USA).

Mice

Female BALB/c mice were purchased from CLEA Japan (Tokyo, Japan). All mice were kept under specific pathogen-free conditions during the experiments. All animal experiments were approved by the Juntendo University Animal Experimental Ethics Committee, and they complied with the National Institutes of Health guidelines for animal care.

Induction of airway inflammation and steroid-resistant inflammation

BALB/c mice were intranasally injected on three consecutive days 25 μ g of small chitin (<40 μ m) or PBS as negative control after anesthesia using isoflurane. After 24 h from the last injection, bronchoalveolar lavage (BAL) fluids were collected. The mouse model of OVA-induced allergic airway inflammation and chitin-induced airway inflammation was combined to induce steroid-resistant airway inflammation. Briefly, BALB/c mice were immunized by i.p. injection of 25 μ g of OVA (Sigma–Aldrich) with 1 mg of alum adjuvant (Thermo Fisher Scientific, Yokohama, Japan) (OVA/alum) on days 0 and 7. On days 20, 21, and 22, the mice were challenged intranasally with 10 μ g of OVA alone or in combination with 25 μ g of chitin and were treated with or without dexamethasone (DEX, i.p. injection, 3 mg/kg). Negative control animals were immunized with OVA/alum, challenged with PBS, and i.p. injected with PBS in a similar manner. In these different mouse models, BAL fluids were collected on the day following the last injection as described below.

Characterization of BAL fluid and preparation of AMs

BAL fluids were harvested as previously described.²⁰ Briefly, the trachea was cannulated with a polyethylene tube through which the lungs were gently lavaged with 0.5 mL of PBS containing 10% fetal calf serum four times. The total number of cells was determined by Turk dye exclusion. BAL fluid-derived cells were incubated at a concentration of 2×10^6 cells/mL in tissue culture dishes for 2 h at 37 °C, 5% CO₂, followed by extensive washing with pre-warmed RPMI 1640 to remove non-adherent cells. The adherent cells, defined as AMs, were collected by scraping with a cell scraper (Sumitomo Bakelite, Tokyo, Japan). This procedure yielded a purity of >95% AMs as confirmed by morphology in cytopins with Diff-Quik (Sysmex International Reagents, Kobe, Japan) and by flow cytometry using cell surface markers (CD11c, Siglec-F, and F4/80).^{20,21} The viability of the AMs was confirmed by trypan blue dye exclusion.

RNA isolation and quantitative RT-PCR

Total cell RNA was isolated from mouse whole-lung, AMs using the RNeasy plus mini kit (Qiagen, Valencia, CA, USA) with DNase treatment, followed by cDNA synthesis using the SuperScript VILO cDNA Synthesis kit (Thermo Fisher Scientific) according to the manufacturer's instructions. THUNDERBIRD SYBR qPCR Mix (Toyobo, Osaka, Japan) and a StepOnePlus Real-Time PCR System (Thermo Fisher Scientific) were used for quantitative real-time reverse transcription-PCR (qRT-PCR) with the gene-specific primer pairs listed in Table 1. For data analysis, the comparative threshold cycle (CT) value for GAPDH was used to normalize loading variations in real-time PCRs. A $\Delta\Delta C_T$ value then was obtained by subtracting control ΔC_T values from the corresponding experimental ΔC_T values. The $\Delta\Delta C_T$ values were converted to fold differences as compared to the control by raising two to the $\Delta\Delta C_T$ power.

Table 1
Primer sequences for real-time PCR.

cDNA	Forward	Reverse
IL-1 β	TGCCACCTTTTGACAGTGATGA	TGATGTGCTGCTGCGAGATT
NLRP3	ATCAACAGGCGAGACCTCTG	GTCTCTGGCATAACCATAGA
GAPDH	AACCTTGCCATTGTGGAAGG	GGATGCAGGGATGATGTTCT

AHR measurements

Mice were anesthetized with pentobarbital and xylazine, and then intubated with metal 18-gauge catheters via a tracheotomy. The needle was then immediately connected to the flexiVent™ (SCIREQ, Montreal Qc, Canada). The mice were mechanically ventilated with an average breathing frequency of 150 breaths/min. After measurement of baseline resistance (saline inhalation), the mice were challenged with increasing concentrations (0, 6, 12, 24, and 48 mg/mL) of methacholine aerosol generated with an in-line nebulizer and administered directly through the ventilator for 5 s.

IL-1 β measurements

Cell-free supernatants of BAL fluids were assayed for IL-1 β using an ELISA kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

Histological analysis of lung sections

Lungs were removed from mice that were not subjected to the bronchial lavage procedure. Lungs were inflated and fixed by intratracheal instillation of 10% buffered formalin (pH 7.4) at a constant pressure of 25 cm H₂O for 48 h. After routine processing of successive dehydration, the lungs were embedded in paraffin and sectioned in a coronal plane. Sections of 5 μ m thickness were stained with hematoxylin and eosin (HE) and periodic acid–Schiff (PAS) as a measure of mucus production.

Immunofluorescence stainings of lung sections and AMs for IL-1 β

Mouse AMs were seeded on slide glasses at 2.4×10^4 cells/slide and fixed in 4% paraformaldehyde. These slide glasses and lung sections stained with goat anti-IL-1 β polyclonal antibodies (R&D System, used at a concentration of 10 μ g/mL) followed by secondary antibodies (donkey anti-goat IgG conjugated with Alexa Fluor 488; Invitrogen, Carlsbad, CA, USA, used at 1:250 dilution). Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) (Vectashield H-1200, Vector Laboratories, Burlingame, CA, USA). Slides were visualized with a Leica TCS SP5 confocal microscope (Leica Microsystems CMS, Wetzlar, Germany), and images were analyzed using the LAS AF software (Leica Microsystems CMS). In immunofluorescence stainings of lung sections, all fields were analyzed per sample, and the field with the highest concentration of cells was chosen for consistency. The number of IL-1 β -positive cells in each field was enumerated and the mean number per field per section determined.

In Vitro lung-draining lymph node cells culture and cytokine measurements

Lung-draining lymph node (LLN) cells (6×10^5 cells/well) were cultured with 50 μ g/mL OVA in RPMI-1640 medium containing 10% FCS, 10 mM HEPES, 2 mM L-glutamine, 0.1 mg/mL penicillin and streptomycin, and 50 mM 2-mercaptoethanol. To determine the production of cytokines, cell-free supernates were collected at 24 h. IL-4, IL-5, and IL-13 were assayed by the multiplex bead array assays (MILLIPLEX Map Kit, Merck, Darmstadt, Germany) and IL-17A was assayed by ELISA kit (R&D Systems), according to the manufacturer's instructions.

Flow cytometric analysis of whole lung cells

Lung tissues from mice were removed and minced after 24 h from the last injection. The samples were digested with collagenase

and DNase I (Roche Biochemicals, Mannheim, Germany) in RPMI 1640 medium for 30 min at 37 °C, and then filtered to obtain single-cell suspensions. These cells were stimulated with phorbol 12-myristate 13-acetate (PMA, Sigma–Aldrich) (50 ng/mL) and ionomycin (Sigma–Aldrich) (1 μ g/mL) for 4 h in the presence of a protein transport inhibitor containing brefeldin A (GolgiPlug, BD Biosciences) for the last 2 h of stimulation. After stimulation, cells were preincubated with unlabeled anti-CD16/32 mAb (clone 93, BioLegend, San Diego, CA, USA) to avoid nonspecific binding of antibodies to the Fc receptor. Then, cells were stained with Allophycocyanin (APC)–Cy7-conjugated anti-CD3 (17A2, BioLegend) and APC-conjugated anti-CD4 (RM4-5, eBioscience, San Diego, CA, USA) mAbs. After appropriate washing, the cells were fixed and permeabilized using Cytofix/Cytoperm Kit (BD Biosciences), followed by intracellular staining with phycoerythrin-conjugated anti-IL-17A mAbs (TC11-18H10.1, BioLegend). Dead cells were identified by using a Zombie Aqua Fixable Viability Kit (BioLegend), followed by doublet exclusion on forward scatter and side scatter. Stained cells were analyzed by LSR Fortessa (BD Biosciences) and data were processed by FlowJo software (FlowJo).

Statistical analysis

Sample normality was examined using the D'Agostino–Pearson test. Comparisons between multiple groups were made by one-way ANOVA with Tukey's multiple comparisons test using the program GraphPad Prism version 7 (GraphPad Software, San Diego, CA, USA). Differences were considered to be statistically significant when *p*-values were 0.05 or less.

Results

Chitin-induced airway inflammation, AHR, and production of IL-1 β in the lung

Small-size chitin (<40 μ m) was prepared and used for this study, because previous reports suggested that small-size chitin induces TNF- α , IL-17, and IL-10 production in macrophages.^{7,12} We first investigated whether chitin could induce airway inflammation in mice. Mice were intranasally injected with chitin particles on three consecutive days, and BAL fluids were collected 24 h after the last injection. Figure 1 demonstrates that the chitin-induced airway inflammation model had a higher number of total cells, AMs, lymphocytes, eosinophils, and neutrophils in BAL fluids compared to control mice (Fig. 1A). The histological examination showed a cellular infiltration around the bronchioles in the mice stimulated by chitin (Fig. 1B). Chitin administration also induced AHR which is one of the important features of asthma (Fig. 1C). These results suggested that inhaled chitin induced airway inflammation and AHR in the mouse model. In this chitin-induced airway inflammation model, the secreted protein levels of IL-1 β in BAL fluids and the numbers of IL-1 β -positive cells in histological lung sections were increased in comparison to those of control mice, suggesting that inhaled chitin induced the production of IL-1 β in the lung (Fig. 1D–F).

Chitin induces production of IL-1 β in mouse AMs

In this mouse model of chitin-induced airway inflammation, the majority of the infiltrated cells in BAL fluids were AMs that are one of the cell types that interact with PAMPs, including chitin particles (Fig. 1). Thus, we next examined whether chitin could induce IL-1 β production in AMs. Chitin upregulated the mRNA expression levels of IL-1 β and NLRP3 in AMs derived from the mouse model of chitin-induced airway inflammation compared to those from

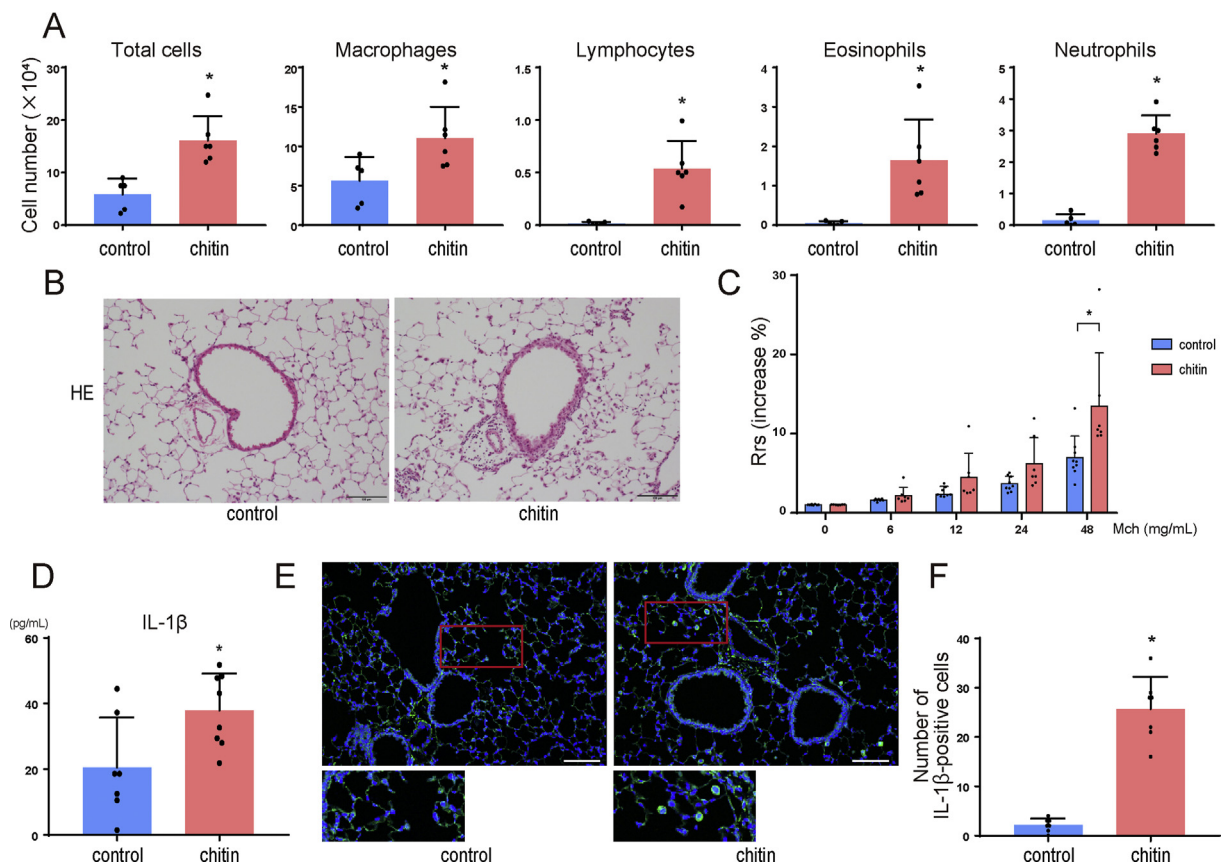


Fig. 1. Chitin induces airway inflammation, AHR, and the production of IL-1 β in the lung. BALB/c mice were intranasally injected with chitin particles or PBS as control on three consecutive days, and BAL fluids were collected 24 h after the last injection. (A) The cellular compositions of airway infiltrates are shown. (B) Lung tissue was stained with HE ($\times 100$). (C) On the day following the last injection, individual mice were assessed for AHR. Results are presented as the mean respiratory system resistance (Rrs) $\pm$ SD in each group after exposure to increasing concentrations of inhaled methacholine. (D) IL-1 β concentrations in BAL fluid were determined by ELISA. (E) Lung tissue was immunostained for IL-1 β (green), and nuclei were visualized with DAPI (blue) ($\times 100$). The lower panels were enlarged inside the red line in the upper panels. (F) Graph showing the number of IL-1 β -positive cells in the lung. Scale bars: 100 μ m. Data represent the means $\pm$ SD. $n = 7$ to 9 mice per group. * $p < 0.05$ compared with control mice.

control mice (Fig. 2A). Moreover, immunofluorescence stainings of BAL-derived AMs revealed that AMs from the mouse model of chitin-induced airway inflammation were stained more intensely for IL-1 β compared to those from control mice (Fig. 2B, C). These results suggested that chitin induced the production of IL-1 β in murine AMs.

It has been reported that *Chlamydia* spp. and *Haemophilus influenzae* induce steroid-resistant IL-1 β responses in the mouse model of OVA-induced allergic airway inflammation.¹⁶ We next examined whether DEX inhibits the production of IL-1 β in AMs and BAL fluids from the mouse model of chitin-induced airway inflammation. Figure 3A and B demonstrates that DEX administration could not inhibit the chitin-induced upregulation of IL-1 β mRNA in murine AMs and IL-1 β protein expression levels in BAL fluids. Treatment with DEX also did not have any effects on the inhibition of IL-1 β production in IL-1 β stainings of murine AMs from the chitin-induced inflammation model (Fig. 3C, D), suggesting that the chitin-induced production of IL-1 β in AMs was resistant to steroid treatment.

Chitin induces steroid-resistant airway inflammation and AHR in a mouse model of asthma

To examine whether chitin could induce steroid-resistant airway inflammation, we developed a mouse model of inhaled chitin particle-induced steroid-resistant ovalbumin-induced airway inflammation and AHR. Mice were immunized with

OVA and alum adjuvant on days 0 and 7. Then, mice were challenged intranasally on days 20, 21, and 22 with OVA alone or in combination with chitin particles and were treated with or without DEX. BAL fluids were collected on the day following the last injection. DEX administration significantly inhibited the OVA-induced airway inflammation including the increases in the numbers of total cells, macrophages, lymphocytes, eosinophils, and neutrophils in BAL fluids compared to control mice (Fig. 4A–E). However, DEX treatment had no statistically significant effect on the increases in numbers of macrophages, lymphocytes, eosinophils, and neutrophils, except total cell number, induced by chitin alone or the combination of OVA and chitin (Fig. 4A–E). Similarly, the results of AHRs and histological examinations showed that DEX treatment inhibited the OVA-induced airway inflammation but not the airway inflammation induced by chitin or the combination of OVA and chitin (Fig. 4F, G). These results suggested that inhaled chitin mediated steroid-resistant airway inflammation and AHR and that this mouse model shared the hallmark features of patients with steroid-resistant asthma.

We next investigated whether DEX administration could inhibit the inhaled-chitin particle-induced IL-1 β production in the mouse model of chitin-induced steroid-resistant ovalbumin-induced airway inflammation. Immunofluorescence stainings for IL-1 β demonstrated that DEX treatment significantly inhibited OVA-induced IL-1 β production (Fig. 4G, H). However, the administration of DEX did not have any inhibitory effects on the IL-1 β

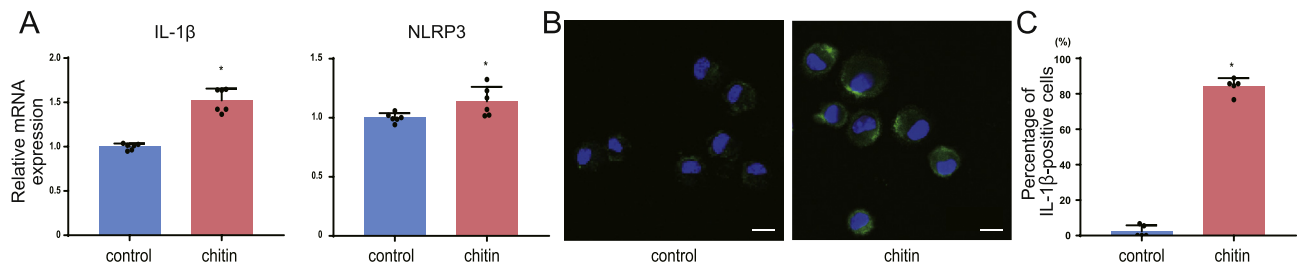


Fig. 2. Chitin induces the production of IL-1 β in murine AMs. BAL fluid-derived cells were incubated in tissue culture dishes for 2 h, followed by extensive washing to remove non-adherent cells. The adherent cells, defined as AMs, were collected. (A) The mRNA levels of IL-1 β and NLRP3 were analyzed by qRT-PCR. Expression levels were normalized to the housekeeping gene GAPDH and calculated as fold induction in comparison to the control (PBS). (B) AMs were immunostained for IL-1 β (green), and nuclei were stained by DAPI (blue). Scale bars: 10 μ m. (C) Graph showing the percentage of IL-1 β -positive cells in AMs. Data represent the means $\pm$ SD. Similar results were obtained in two independent experiments (n = 5 to 7 mice per group). * p < 0.05 compared with control mice.

production in the airway inflammation induced by chitin or the combination of OVA and chitin (Fig. 4G, H). Moreover, immunofluorescence stainings for IL-1 β , CD11c, and Siglec-F demonstrated that CD11c and Siglec-F double-positive cells are IL-1 β producing cells (Supplementary Fig. 1), suggesting that AMs are one of the IL-1 β producing cells because AMs occupy most of CD11c and Siglec-F double-positive cells in the alveolar cavity.^{20,21} Furthermore, the number of IL-1 β -positive cells in CD11c and Siglec-F double-positive cells tended to be similar to the result of the number of IL-1 β -positive cells (Fig. 4G, Supplementary Fig. 1B). These findings suggested that chitin-induced IL-1 β production was steroid-resistant and that they may depend on chitin-induced AM increase and its steroid-resistant.

Next, an anti-IL-1 β neutralizing antibody was intranasally injected into the chitin-induced airway inflammation model to confirm whether chitin-induced airway inflammation depended on IL-1 β . The neutralizing antibody suppress the numbers of total cells, but not macrophages, lymphocytes, eosinophils, and neutrophils, in BAL fluids induced by chitin stimulation (Supplementary Fig. 2). These neutralization experiments suggested that chitin-induced airway inflammation require not only IL-1 β but also other cytokines.

Chitin-induced steroid-resistant airway inflammation and AHR may have associated with the Th17 differentiation and IL-17A production. To address this possibility, LLN cells were prepared on Day 23, and Th2 cytokine (IL-4, IL-5, and IL-13) and IL-17A production against OVA were assessed. The airway inflammation induced by OVA or the combination of OVA and chitin developed production Th2 cytokines in LLN cells, and DEX treatment inhibited these productions (Fig. 5A–C). In contrast, the production of IL-17A in LLN cells that was induced by OVA or the combination of OVA and chitin were not affected by DEX treatment (Fig. 5D). Moreover, to investigate whether Th17 cell differentiation is steroid-resistant

as well as IL-17A production, minced whole lung cells were prepared on Day 23. Flow cytometric analysis showed that IL-17A⁺ CD4⁺ T cells in the lung increased by the combination of OVA and chitin, and DEX treatment had no statistically significant effect on this increase (Fig. 5E). These findings suggested that the combination of OVA and chitin-induced IL-17A production in lymphocytes may be steroid-resistant.

Discussion

To our knowledge, this is the first study to show that inhaled chitin particles induce the production of IL-1 β in murine AMs and steroid-resistant airway inflammation and AHR in mice. Several mouse models present a chitin-induced eosinophilic inflammation in the lung demonstrating that chitin has a potential role as an adjuvant in the mouse model of OVA-induced asthma.^{6–9} In the current study, we revealed that inhaled chitin particles can induce the development of features of steroid-resistant airway inflammation with neutrophil infiltration and AHR, suggesting that inhaled chitin may cause the development of features of steroid-resistant asthma.

In previous reports, the effects of chitin in mouse models were dependent on the activation of ILC2s, Th2 cells, and $\gamma\delta$ T cells via chitin-induced production of IL-25, IL-33, TSLP, monocyte chemoattractant protein-1 (CCL2), and IL-1 β .^{9–11} ILC2s produce IL-5 and IL-13 in response to acute signals emanating from epithelium and mediate the local infiltration of eosinophils in the lung, as well as the alternative activation of M2 macrophages.^{4,6,10,22,23} The terminal differentiation of tissue effector Th2 cells during adaptive immune responses to antigens is induced by similar activation signals in the lung.¹¹ Chitin particles also induce IL-1 β production and activate lung-resident $\gamma\delta$ T cells that produce IL-17 and mediate neutrophil accumulation.¹⁰ Our experiments with chitin-induced

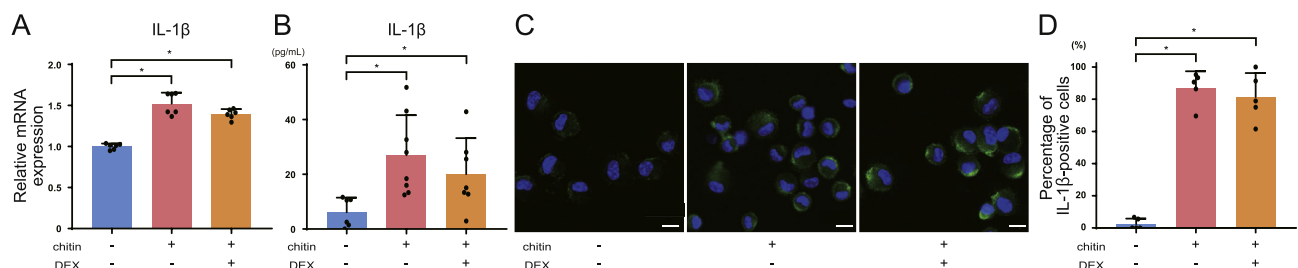


Fig. 3. No inhibitory effects of DEX administration on chitin-induced IL-1 β production. BALB/c mice intranasally injected with chitin particles or PBS as control on three consecutive days were treated with or without DEX. BAL fluids were collected on the day following the last injection. (A) The levels of IL-1 β mRNA in AMs were analyzed using qRT-PCRs. Expression levels were normalized to the housekeeping gene GAPDH and calculated as fold induction in comparison to the control. (B) IL-1 β concentrations in BAL fluid were determined by ELISA. (C) AMs were immunostained for IL-1 β (green), and DAPI was used to visualize nuclei (blue). (D) Graph showing the percentage of IL-1 β -positive cells in AMs. Scale bars: 10 μ m. Data represent the means $\pm$ SD. Similar results were obtained in two independent experiments (n = 5 to 7 mice per group). * p < 0.05.

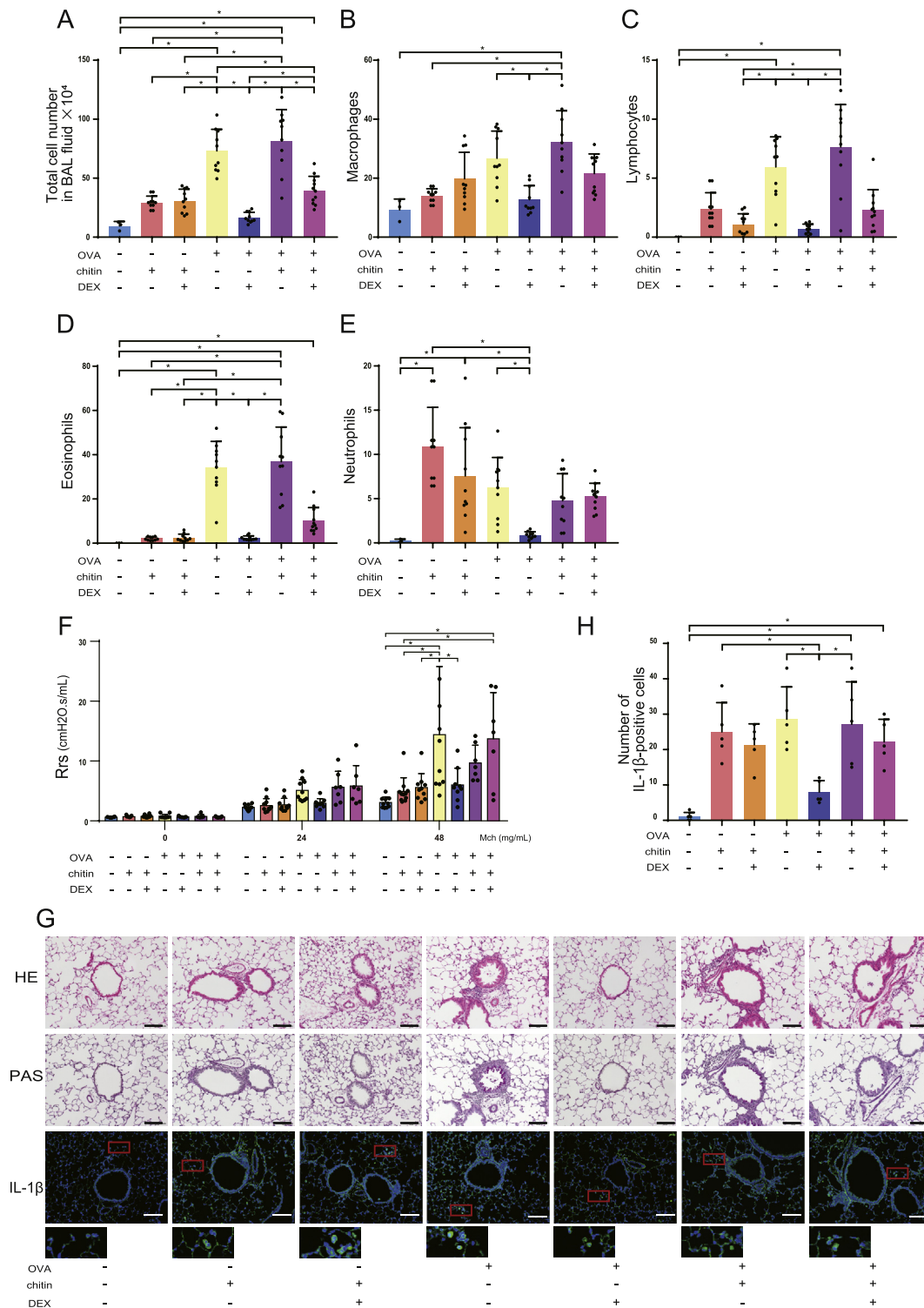


Fig. 4. Chitin induces steroid-resistant airway inflammation and AHR in mice. BALB/c mice were immunized with OVA and subsequently challenged intranasally with OVA alone or in combination with chitin particles with or without DEX treatment. BAL fluids were collected on the day following the last injection. (A–E) The cellular compositions of airway infiltrates are shown. (F) On the day following the last injection, individual mice were assessed for AHR. Results are presented as the mean respiratory system resistance (Rrs) $\pm$ SD in each group after exposure to increasing concentrations of inhaled methacholine. (G) Lung tissue samples were stained with hematoxylin and eosin ($\times 100$), periodic acid–Schiff (PAS) ($\times 100$), or stained with immunofluorescent antibodies against IL-1 β (green) and DAPI marking nuclei (blue). Scale bars: 100 μ m. The lower panels of immunofluorescent staining were enlarged inside the red line in the upper panels. (H) The mean number of IL-1 β -positive cells in each lung section. Data represent the means $\pm$ SD. $n = 7$ to 10 mice per group. $*p < 0.05$.

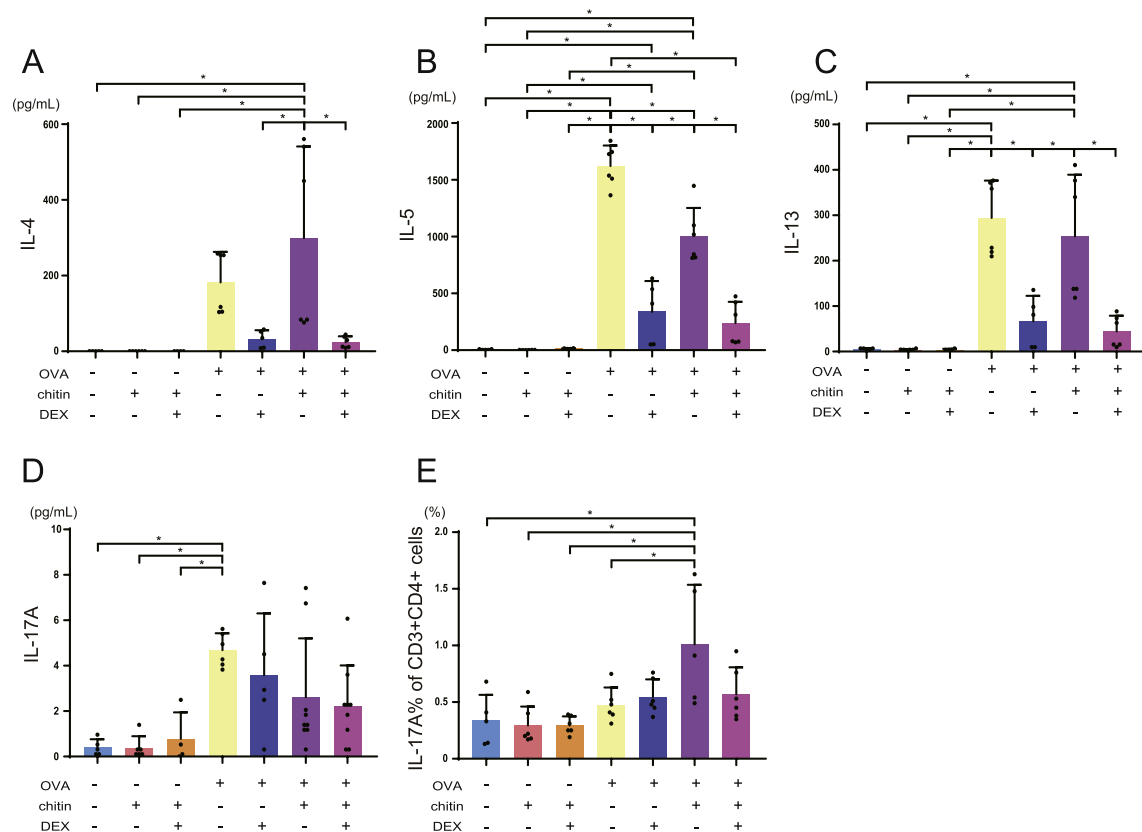


Fig. 5. Cytokine measurements in culture supernates of lung-draining lymph node cells and IL-17⁺ CD4⁺ T cells in lung. BALB/c mice were immunized with OVA and subsequently challenged intranasally with OVA alone or in combination with chitin particles with or without DEX treatment. Lung-draining lymph node (LLN) cells were collected on the day following the last injection and were cultured with 50 μ g/mL OVA. The culture supernates were collected at 24 h. IL-4 (A), IL-5 (B), IL-13 (C), and IL-17A (D) were determined by multiplex bead array assays and ELISA. (E) Lung tissues from mice were removed and minced after 24 h from the last injection. Single-cell suspensions were stimulated with phorbol 12-myristate 13-acetate and ionomycin. The induction of IL-17⁺ CD4⁺ T cells was determined by staining with anti-CD3, anti-CD4, and anti-IL-17A mAbs. Data represent the means $\pm$ SD. $n = 5$ to 7 mice per group. $*p < 0.05$.

airway inflammation demonstrated for the first time that IL-1 β is produced in the murine lung, specifically in AMs. These results might suggest the association with chitin-induced airway inflammation and IL-1 β production by AMs. However, the anti-IL-1 β neutralizing antibody had little effect in inhibiting chitin-induced inflammation. Taken together with previous reports indicate that the effects of chitin in mouse models require a variety of cells including not only IL-1 β producing cells but also ILC2s and Th2 cells. Further studies are needed to investigate whether chitin-induced airway inflammation requires the production of IL-1 β in AMs.

Our findings demonstrate for the first time that a steroid does not have any effects on the inhibition of IL-1 β production in mouse AMs derived from a chitin-induced airway inflammation model and on the inhibition of IL-1 β production, airway inflammation, and AHR in a mouse model of chitin exposure or the combination treatment with OVA and chitin. A previous report indicates NLRP3 inflammasome-mediated IL-1 β -dependent responses in steroid-resistant asthma.¹⁶ Taken together, our findings suggest that inhaled chitin induces steroid-resistant airway inflammation and AHR. The main limitation of our study is that we could not reveal an association between the secreted protein expression levels of IL-1 β and steroid-resistant airway inflammation induced by the combination of OVA and chitin. Further studies are needed to investigate whether chitin-induced steroid-resistant airway inflammation requires IL-1 β -dependent responses via the NLRP3 inflammasome and whether a combination treatment of steroids and inhibitors of IL-1 β inhibit chitin-induced steroid-resistant airway inflammation.

In conclusion, inhaled chitin particles induce airway inflammation and AHR and induce the production of IL-1 β in mouse AMs. We revealed a novel function of chitin by demonstrating in a mouse model its role in the induction of steroid-resistant airway inflammation and AHR. These findings suggest that chitin may contribute to features of steroid-resistant asthma and influence the development and exacerbation of asthma.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.alit.2020.12.004>.

Conflict of interest

NH reports personal fees from AstraZeneca and GlaxoSmithKline, and grants from Kyorin, outside the submitted work. KT reports grants and personal fees from AstraZeneca, Boehringer Ingelheim, Chugai, Eli Lilly, Kyorin, MSD, Nobelpharma, Novartis, Ono, Pfizer, Teijin, and grants from Astellas, GlaxoSmithKline, Kyowa Hakkō Kirin, MiZ, Mochida, Nipro, Nippon Shinyaku, Taiho, Toyama Chemical, Tsumura, Sanofi, Shionogi, Torii, and personal fees from Bristol-Myers, Eisai, Meiji Seika, Mitsubishi Tanabe, Otsuka, Parexel, Sumitomo Dainippon, outside the submitted work. The rest of the authors have no conflict of interest.

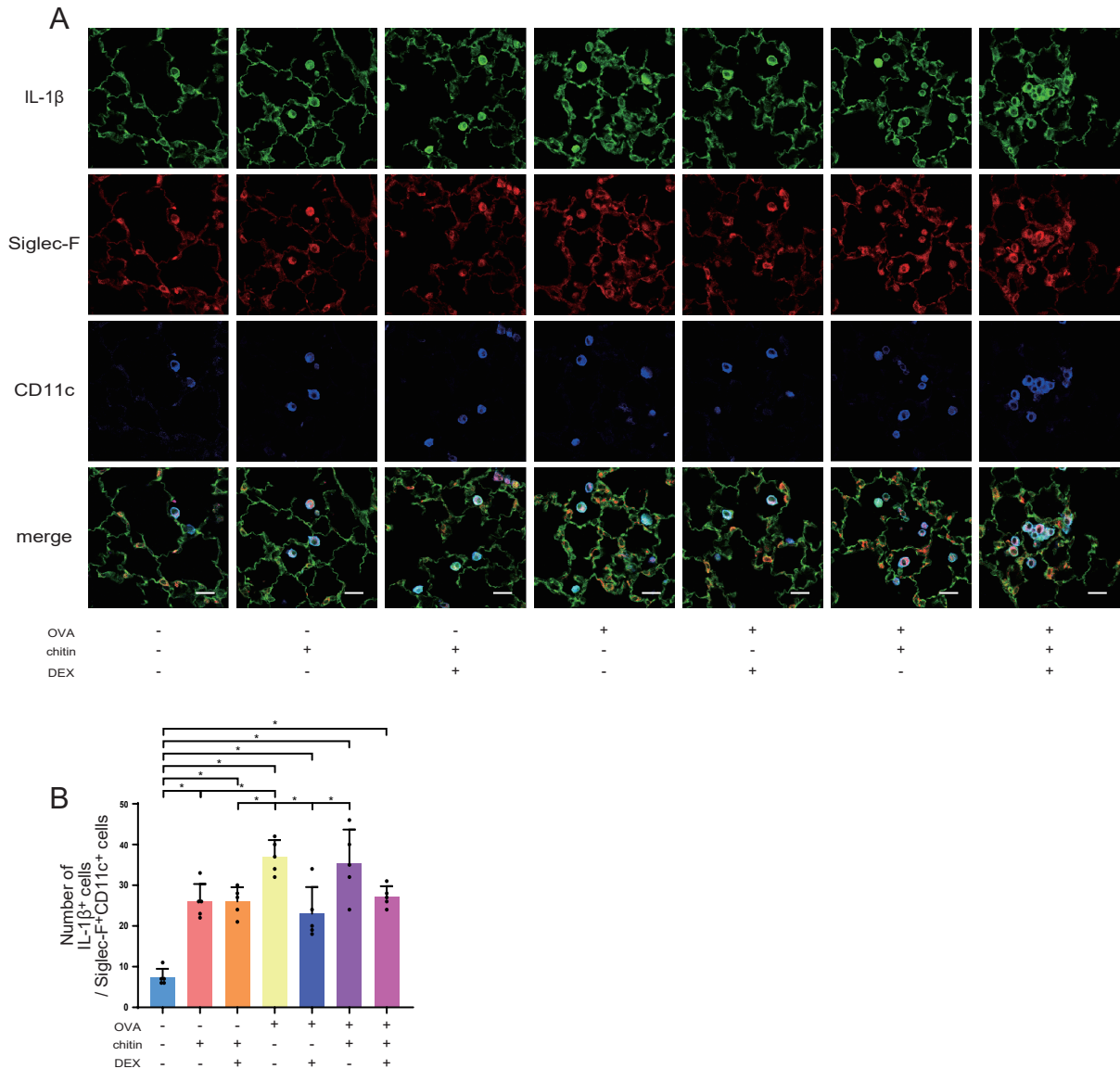
Authors' contributions

TT participated in the design of the study, carried out the molecular studies and mouse studies, and drafted the manuscript. SH, AI, YK, KM, FM, and JI carried out the qRT-PCR and immunofluorescence stainings and performed the statistical analysis. TT, SH, HS, and YS performed additional experiments for revised manuscript. RA, HA, and KT participated in the design of the study. NH conceived the study, participated in its design and coordination, carried out the molecular studies, and helped to draft the manuscript. All authors read and approved the final manuscript.

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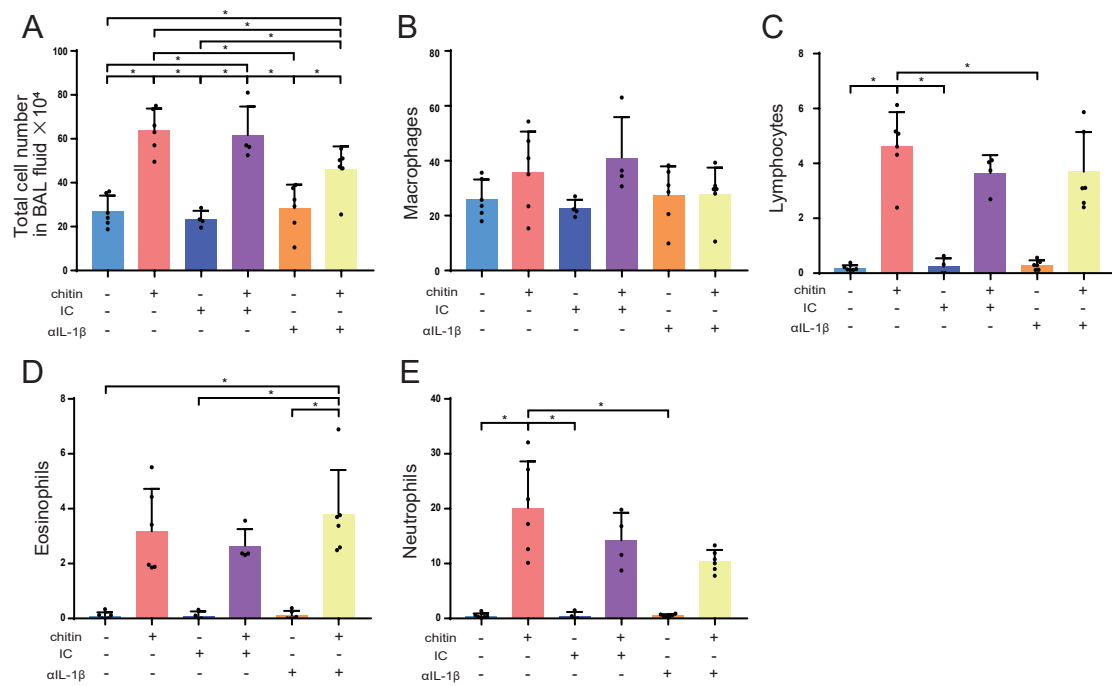
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Supplementary Figure 1.



Supplementary Fig. 1. The production of IL-1 β in murine AMs. BALB/c mice were immunized with OVA and subsequently challenged intranasally with OVA alone or in combination with chitin particles with or without DEX treatment. (A) Lung tissue samples were stained with immunofluorescent antibodies and demonstrated single-color and merged color display with IL-1 β (green), Siglec-F (red), and CD11c (blue). Scale bars: 20 μ m. (B) The mean number of IL-1 β -positive cells per CD11c and Siglec-F positive cells in each lung section. Data represent the means $\pm$ SD. n = 7 to 10 mice per group. *p < 0.05.

Supplementary Figure 2.



Supplementary Fig. 2. No inhibitory effects of anti-IL-1 β neutralizing antibody on chitin-induced airway inflammation. BALB/c mice intranasally injected with chitin particles or PBS as control on three consecutive days were treated with or without DEX. Some groups of mice were intranasally injected with anti-IL-1 β neutralizing antibody or control goat IgG as isotype control (IC) on days -1, 1, and 3. BAL fluids were collected 24 h after the last injection. The cellular compositions of airway infiltrates are shown. Data represent the means $\pm$ SD. $n = 4$ to 6 mice per group. * $p < 0.05$.

Supplementary Methods

IL-1 β neutralization

IL-1 β neutralization was performed in the chitin-induced airway inflammation model using an IL-1 β neutralizing antibody (AF-401-NA; R&D system, Minneapolis, MN, USA), which was adopted from previously published literature (16, 24-26). BALB/c mice were intranasally injected on three consecutive days 25 μ g of small chitin (< 40 μ m) or PBS as control after anesthesia using isoflurane. Some groups of mice were intranasally injected with 10 μ g of neutralizing antibody or control goat IgG control (R&D system) on days -1, 1, and 3. After 24 h from the last injection, bronchoalveolar lavage (BAL) fluids were collected. BAL fluids were collected on the day following the last injection.

Immunofluorescence stainings of lung sections and AMs for IL-1 β , CD11c, and Siglec-F

Lung sections stained with goat anti-IL-1 β polyclonal antibodies (R&D System, used at a concentration of 10 μ g/mL), rabbit anti-CD11c mAbs (D1V9Y, Cell Signaling Technology, Beverly, MA, USA, used at a concentration of 500 ng/mL), and phycoerythrin-conjugated rat anti-Siglec-F mAbs (E50-2440, BD Biosciences, San Jose, CA USA, used at a concentration of 40 μ g/mL) followed by appropriate secondary antibodies (donkey anti-goat IgG conjugated with Alexa Fluor 488;

Invitrogen, Carlsbad, CA, USA, used at 1:250 dilution, and donkey anti-rabbit IgG conjugated with Alexa Fluor 647, Abcam, Cambridge, MA, USA, used at 1:200 dilution). Slides were visualized with a Leica TCS SP5 confocal microscope (Leica Microsystems CMS GmbH, Wetzlar, Germany), and images were analyzed using the LAS AF software (Leica Microsystems CMS GmbH) and ImageJ software. In immunofluorescence stainings of lung sections, all fields were analyzed per sample, and the field with the highest concentration of cells was chosen for consistency. The number of IL-1 β -positive cells in each field was enumerated and the mean number per field per section determined.

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