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Alendronate Attenuates Eosinophilic Airway Inflammation Associated with Suppression of Th2 Cytokines, Th17 Cytokines, and Eotaxin-2

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Bisphosphonates (BPs) have been widely used to treat osteoporosis. They act by inhibiting farnesyl diphosphate synthase in the mevalonate pathway. This resembles the action of statins, whose immune-modulating effect has recently been highlighted. In contrast, the effect of BPs on immune responses has not been elucidated well. In this study, we examined the effect of alendronate (ALN), a nitrogen-containing BP, on allergic airway inflammation in a mouse model. BALB/c mice were sensitized twice with OVA and challenged three times with nebulized OVA to induce eosinophilic airway inflammation. ALN was administered by an intragastric tube before each inhalation. ALN strongly suppressed airway eosinophilia and Th2, as well as Th17 cytokine production in the lung. ALN also attenuated eotaxin-2 production in the lung. Immunohistochemistry demonstrated that the major cell source of eotaxin-2 was peribronchial/perivascular macrophages, and flow cytometrical studies confirmed that ALN decreased eotaxin-2 expression in these macrophages. Furthermore, ALN attenuated eotaxin-2 production from mouse pleural macrophages and human monocyte/macrophage-like THP-1 cells in vitro. These results suggest that ALN suppressed Ag-induced airway responses in the mouse model. The suppression of eotaxin-2 production from macrophages appears to be one of ALN's immunomodulatory effects, whereas the mechanism by which ALN suppressed Th2 and Th17 responses could not be fully elucidated in this study. Although a clinical study should be conducted, ALN could be a novel therapeutic option for asthma. *The Journal of Immunology*, 2013, 191: 000–000.

Bisphosphonates (BPs) are widely used for the treatment of osteoporosis in the elderly and for other bone diseases such as bone cancer. Second-generation “nitrogen-containing BPs (N-BPs), such as alendronate (ALN) and risedronate, inhibit farnesyl pyrophosphate synthase in the mevalonate pathway. The mechanism of their action is similar to that of statins, the inhibition of HMG-CoA reductase.

The immune-modulating effect of statins has recently been elucidated (1). The suppressive effects of statins have been reported in many animal models of immune-mediated disorders (2–6). For example, several studies have been conducted using simvastatin (7, 8) or pravastatin (9) in models of allergic airway inflammation. We also showed that pravastatin suppressed allergic airway inflammation partly by suppressing the Ag-presenting capacity of dendritic cells (10). Based on their similar mechanism of action to statins,

N-BPs may affect allergic airway inflammation; however, this possibility has not yet been examined.

N-BPs are mainly distributed in the bones. Few reports have examined the tissue distribution of BPs in the respiratory system. Hisaka and coworkers (11) reported that ALN was retained in the trachea for up to 72 h after repeated oral administration. Mochizuki et al. (12, 13) described a similar tissue affinity for the trachea after the single or repeated i.v. administration of ALN. These findings raise the possibility that ALN could suppress allergic airway inflammation. In contrast, there are no findings on the distribution of other BPs in the lung.

Based on these findings, our study was conducted to examine the effect of ALN on allergic airway inflammation in mice. Mice were sensitized and challenged three times with the OVA Ag. ALN was administered by an intragastric tube 1 h before each challenge. ALN strongly suppressed airway eosinophilia and Th2 cytokine production in the lung and thoracic lymph nodes. ALN also decreased the Th17-type response of immunocytes in the lung and thoracic lymph nodes. In addition, ALN strongly suppressed eotaxin-2 production in the lung. We clarified that macrophages were the main source of eotaxin-2 in the lung, and that production was suppressed by the ALN treatment. These results clearly demonstrate that ALN could attenuate Ag-induced allergic immune responses in the lung.

Materials and Methods

Mice

Male BALB/c mice, 7 wk of age, were obtained from Charles River Breeding Laboratories Japan (Kanagawa, Japan). Mice were housed under conventional conditions in a specific pathogen-free setting. All of the animal experiments were approved by the Animal Research Ethics Board of the Department of Allergy and Rheumatology (University of Tokyo, Tokyo, Japan).

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Abbreviations used in this article: AHR, airway hyperresponsiveness; ALN, alendronate; BALF, bronchoalveolar lavage fluid; BP, bisphosphonate; CMC, carboxyl methyl cellulose; FCM, flow cytometry; FOH, farnesol; GGOH, geranylgeraniol; i.g., intragastric; Mch, methacholine chloride; N-BP, nitrogen-containing bisphosphonate; NC, negative control; PC, positive control; PI, propidium iodide.

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Induction of allergic airway inflammation

BALB/c mice were sensitized with 2 µg OVA (Sigma-Aldrich, Poole, U.K.) in 2 mg alum (Serva, Heidelberg, Germany) i.p. on days 0 and 11. Control mice received i.p. injections of saline on days 0 and 11. Sensitized mice were then challenged with a 3% w/v OVA aerosol in PBS delivered by a nebulizer for 10 min every day on days 18–20. Control mice received the PBS aerosol delivered by a nebulizer for 10 min on days 18–20.

In vivo treatment with ALN in the effector phase of Ag-induced airway inflammation

Mice were sensitized twice and challenged with the OVA solution, as described earlier. Some of the mice received an intragastric (i.g.) injection of ALN (0, 8, 25 mg/kg, respectively), or the vehicle, 1% w/v carboxyl methyl cellulose (CMC) in distilled water, alone 1 h before the OVA challenge on days 18–20. Control mice received i.g. injections of the vehicle 1 h before the PBS challenges. On day 21 or 22, mice were subjected to analyses. Bronchoalveolar lavage fluid (BALF), thoracic lymph nodes, and lung tissues were obtained as reported previously (14–17). The cell count and cell differentials in BALF were calculated.

BALF analyses

BALF analyses were performed as described previously (14, 18). In brief, the lungs were lavaged four times with physiological saline (0.5 ml each). The cell suspension was centrifuged at $300 \times g$ for 10 min at 4°C, and the supernatants were collected and stored at –70°C to measure the concentration of cytokines. Cells were resuspended in 1 ml physiological saline with 1% BSA (Wako, Osaka, Japan), and the total cell number was counted with a hemocytometer. Cytospin samples were prepared by centrifuging the suspensions at 300 rpm for 10 min. On the basis of the findings obtained with Diff-Quick staining (Kokusai-Shiyaku, Kobe, Japan), cell differentials were counted with at least 300 leukocytes in each sample.

Histopathological examination

Formalin-fixed, paraffin-embedded lung sections cut at a thickness of 3 µm were stained with H&E or periodic acid–Schiff. Images were acquired on a BX51 Olympus microscope using its software.

Cytokine production in the lung homogenate, thoracic lymph node cells, and spleen cells

The left lungs were homogenized in 1.0 ml PBS containing 0.5% Triton X-100 and complete protease inhibitor mixture (Roche, Basel, Switzerland). The lung homogenates were cleared of debris and cells by centrifugation at $10,000 \times g$ for 10 min. Cytokine concentrations in the lung homogenate were measured by an ELISA. Single-cell suspensions of the thoracic lymph nodes or spleens were stimulated with OVA (1 µg/ml for lymph nodes, and 10 or 100 µg/ml for spleens) for indicated periods at 37°C, 5% CO₂, and 90% humidity, as described earlier. Cytokine production in the culture supernatant was measured by ELISA.

ELISA and cell proliferation assay

Concentrations of mouse IL-4, IL-5, IL-13, IFN-γ, and total IgE (BD Pharmingen, San Diego, CA), mouse eotaxin-1, mouse eotaxin-2, and human eotaxin-2 (R&D Systems, Minneapolis, MN) were measured using an ELISA kit, following the manufacturer's directions. Mouse IL-17 was measured by ELISA using a purified rat anti-mouse IL-17 mAb for capture and biotinylated rat anti-mouse IL-17 mAb for detection (BD Pharmingen) (10). The titers of samples for IL-17 were calculated by comparisons with internal standards. Cell proliferation was measured based on the incorporation of BrdU using an ELISA kit (Roche, Mannheim, Germany). Data were analyzed with Microplate Manager III, version 1.45 (Bio-Rad, Tokyo, Japan).

Conditions for cell culture

Throughout the study, complete DMEM was used for the mouse cell incubation as reported previously (18). In brief, DMEM (Life Technologies BRL, Grand Island, NY) was supplemented with 10% heat-inactivated FBS, 10 mM HEPES (Life Technologies BRL), 0.1 mM nonessential amino acid (Life Technologies BRL), 1 mM sodium pyruvate, 2 mM sodium glutamate (Sigma-Aldrich), 100 U/ml penicillin (Sigma), 100 µg/ml streptomycin (Sigma), and 50 µM 2-ME (Sigma). Human THP-1 cells were grown in RPM 1640 medium supplemented with 10% heat-inactivated FBS. Cells were incubated in a 96-well flat-bottom microtiter assay plate in an incubator (37°C, 5% CO₂, 90% humidity) for the specified periods.

Preparation of single-cell suspensions from thoracic lymph nodes and spleen cells

Single-cell suspensions for thoracic lymph nodes and spleens were prepared as reported (14). In brief, thoracic lymph nodes were collected and incubated at 37°C for 20 min after treatment with 0.033% (w/v) collagenase (Sigma-Aldrich) in complete DMEM. Incubation for spleens was at 37°C for 15 min after treatment with a 0.1% (w/v) collagenase solution. The spleens were then minced, and single-cell suspensions were prepared by passage through a cell strainer. RBCs were removed by hypotonic lysis using RBC lysing buffer (Roche, Mannheim, Germany). After two washes in HBSS, cells were resuspended in DMEM at 1.25×10^6 cells/ml for lymph nodes and 2.5×10^6 cells/ml for spleen cells, and were cultured for further experiments.

Real-time PCR analysis

Mice were sensitized twice and challenged with the OVA solution, as described earlier. On day 22, mRNA was extracted from the left lung by the acid-guanidinium phenol chloroform method using Isogen (Nippon Gene, Toyama, Japan). A total of 1.5 µg of each RNA was then reverse transcribed at 37°C for 50 min using Superscript II Reverse Transcriptase and an Oligo (dT) primer. The reverse transcriptase was then inactivated by heating at 70°C for 15 min, and RNase was added to remove the template RNA. Eotaxin-1 and eotaxin-2 mRNA levels were determined by real-time quantitative PCR with a standard protocol on LightCycler 480 Instrument (Roche, Rotkreuz, Switzerland) using a LightCycler 480 SYBR Green I Master (Roche). β-Actin was used as a control for normalization. The sequences of the PCR primer pairs (5' to 3') used for each gene were: eotaxin-1, 5'-A-GAGCTCCACAGCGCTTCT-3' (forward) and 5'-GCAGGAAGTTGGGA-TGGAG-3' (reverse); eotaxin-2, 5'-GCAGCATCTGTCCCAAGG-3' (forward) and 5'-GCAGCTTGGGGTCACTACA-3' (reverse); β-actin, 5'-GG-ATGCAGAAGGAGATTACTGC-3' (forward) and 5'-CCACCGATCCA-CACAGAGCA-3' (reverse).

Immunohistochemistry

Sections were cut at a thickness of 3 µm from paraffin-embedded lung blocks, deparaffinized, boiled with unmasking solution (H-3300; Vector Laboratories, Burlingame, CA), blocked with Fc-block (553142; BD Pharmingen), and stained with anti-eotaxin-1 (1:20, AF420-NA; R&D Systems) or anti-eotaxin-2 (1:20, AF528; R&D Systems) polyclonal Ab at 4°C overnight. Secondary biotinylated rabbit anti-goat Ab (Vector Laboratories, Burlingame, CA) was applied for 60 min followed by incubation with the ABC complex solution and nickel-diaminobenzidine (Biolegend, San Diego, CA). Images were acquired on a BX51 Olympus microscope using its software.

Immunofluorescence

Sections were cut at a thickness of 3 µm from paraffin-embedded lung blocks, deparaffinized, Ag-retrieved, blocked with Fc-block as described earlier, and stained with goat anti-mouse eotaxin-2 (1:50, AF528; R&D Systems) polyclonal Ab or goat anti-mouse eotaxin-1 (1:50, AF420-NA; R&D Systems) polyclonal Ab, and rat anti-mouse F4/80 (1:20, MCA497GA [clone CI:A3-1]; AbD Serotec, Raleigh, NC) mAb or rat anti-mouse CD68 (1:200, ADMCA1957T; AbD Serotec) mAb at 4°C overnight. Secondary Ab donkey anti-goat DyLight 488 (1:200, 705-486-147; The Jackson Laboratory, Bar Harbor, ME) and donkey anti-rat DyLight 594 (1:200, 712-516-153; The Jackson Laboratory) were applied for 30 min. Images were acquired on a BX51 Olympus microscope using its software.

Flow cytometry and intracellular cytokine staining

Mice were sensitized twice and challenged with the OVA solution, as described earlier. On day 21, lung cells were obtained according to previously reported methods with a slight modification (18–21). In brief, lung tissues were minced and then treated with 0.033% w/v collagenase/complete DMEM for 30 min. RBCs were removed by hypotonic lysis using RBC lysing buffer (Roche, Mannheim, Germany). After two washes in PBS, cells were counted and adjusted to 1.0×10^6 /ml aliquots for further experiments. BALF cells were obtained as described earlier. Lung and BALF cells were treated with Fc-block (553142; BD Pharmingen) for 5 min. To detect F4/80⁺ macrophages, we stained lung and BALF cells with biotinylated rat anti-mouse F4/80 mAb (123106 [clone BM8]; Biolegend) for 30 min, followed by washing and incubation with streptavidin-PE/Cy5 (eBioscience, San Diego, CA) for 30 min. Cells were then incubated with intracellular fixation buffer (eBioscience) for 20 min. After being washed with permeabilization buffer (eBioscience), the cells were stained with goat anti-mouse eotaxin-2 (AF528; R&D Systems) polyclonal Ab or goat anti-mouse eotaxin-1 (AF420-NA; R&D Systems) polyclonal Ab for 30 min, followed by washing and incubation with donkey anti-goat Alexa 488 (705-546-147;

The Jackson Laboratory). Isotype control Abs were biotinylated rat anti-mouse IgG2a κ (13-4321-81; eBioscience) and normal goat IgG (SC-3887; Santa Cruz, Santa Cruz, CA). Stained cells were then analyzed by flow cytometry (FCM; EPICS XL System II; Coulter). To analyze macrophages, we gated cells according to PE/Cy5-F4/80 parameters. Data analysis was performed using WinMDI2.8 software.

In vitro experiments with pleural macrophages

Pleural macrophages were basically collected as previously reported (22) using a conventional method for the isolation of peritoneal macrophages (23). In brief, naive mice were anesthetized and killed by cutting the abdominal aorta, and pleural cavities were washed with 1 ml DMEM. Pleural fluid cells (2.4×10^5 cells/ml) were incubated at 37°C for 90 min. During this incubation, macrophages stuck to the coverslip. Nonsticking cells were then removed by washing twice with DMEM. An examination of the adhering cells demonstrated that >90% were macrophages based on their morphology on cytopins after the Trypsin-EDTA (Life Technologies BRL) treatment. Macrophages were incubated with ALN (0, 1 μ M/l; kindly supplied by MSD, Tokyo, Japan [previously Banyu Pharmaceutical, Tokyo, Japan]), *trans,trans*-farnesol (FOH; 0, 3 μ M/l; Sigma-Aldrich), all-*trans*-geranylgeraniol (GGOH; 0, 3 μ M/l; Sigma-Aldrich), recombinant mouse IL-4 (0, 20 ng/ml; R&D Systems), and recombinant mouse IL-13 (0, 20 ng/ml; PeproTech, Rocky Hill, U.K.), and were incubated for a further 48 h.

In vitro experiments with THP-1, a human monocyte-like cell line

The human monocytic THP-1 cell line (ECACC) was purchased from DS Pharma Biomedical (Osaka, Japan). THP-1 cells were stimulated with PMA and IL-4/13 to produce eotaxin-2 as previously reported with a slight modification (24, 25). In brief, THP-1 cells (5.0×10^5 cells/ml) were cultured with PMA (50 ng/ml; Sigma-Aldrich), recombinant human IL-4 (0, 20 ng/ml; Wako, Richmond, VA), recombinant human IL-13 (0, 20 ng/ml; Wako, Richmond, VA), and ALN (0, 3 μ M/l; kindly supplied by MSD, Tokyo, Japan [previously Banyu Pharmaceutical]), *trans,trans*-FOH (0, 3 μ M/l; 277541, Sigma-Aldrich), and all-*trans*-GGOH (0, 3 μ M/l; G3278, Sigma-Aldrich) for 48 h.

Detection of apoptosis

Apoptosis was examined by the Annexin/propidium iodide (PI) assay. Mouse BALF cells were obtained as described earlier. Mouse pleural macrophage cells or human THP-1 cells were obtained and stimulated as described earlier, cultured with ALN (1, 3, 10 μ M) for 48 h, and were then treated with 0.25% trypsin EDTA (Sigma) for 5 min at 37°C. The obtained cells were resuspended, washed twice with PBS, and stained with Annexin V-FITC and PI for 15 min at room temperature using the TACS Annexin V-FITC Apoptosis Detection Kit (Trevigen, Gaithersburg, MD). Stained cells were then analyzed by FCM. Annexin V⁻ and PI⁻ staining indicated live and healthy cells. Annexin V⁺ and PI⁻ staining indicated early apoptotic cells. Annexin V⁺ and PI⁺ staining indicated late apoptotic or necrotic cells. Annexin V⁻ and PI⁺ staining indicated damaged, but living cells.

Evaluation of airway hyperresponsiveness

Mice were sensitized twice and challenged with the OVA solution, as described earlier. On day 21, airway hyperresponsiveness (AHR) to methacholine chloride (Mch; Sigma-Aldrich) was measured by the enhanced pause (Penh) system (Buxco, Troy, NY) as reported previously (14–16). In brief, at first the baseline value of Penh was measured after the inhalation of saline. Increasing concentrations of Mch were then delivered by the nebulizer, the percent Penh compared with the baseline value was calculated, and AHR was evaluated by the change in Penh to Mch.

Ag-presenting capacity of lung CD11c⁺ cells

Mice were sensitized twice and challenged with the OVA solution, as described earlier. On day 21, CD11c⁺ APCs in the lungs were positively selected, as reported previously (14, 20). In brief, lung tissues were minced and then treated with 0.033% w/v collagenase/complete DMEM solution for 30 min. Single-cell suspensions of the tissues were obtained, and CD11c⁺ cells were positively selected using MACS CD11c microbeads (Miltenyi Biotec, Auburn, CA). The population of cells from lung tissues was routinely ~70% CD11c⁺, and there was no significant difference in the purity of CD11c⁺ cells between the different groups of mice. These CD11c⁺ cells were exposed to 3000 Gy gamma radiation. In addition, CD4⁺ T cells were obtained from spleen cells of DO11.10 mice using anti-mouse CD4 colloidal superparamagnetic microbeads (Miltenyi Biotec), as previously reported (14, 20). The purity of CD4⁺ cells, confirmed by FCM, was >95%. To measure

Ag-presenting capacity, we cocultured lung CD11c⁺ cells (0.25×10^4 , 0.8×10^4 , and 2.5×10^4 cells/ml) obtained from each group of mice with CD4⁺ T cells (2.5×10^5 cells/ml) selected from the spleens of DO11.10 mice. After a 5-d coculture, the proliferation of CD4⁺ T cells was measured using a BrdU cell proliferation Kit (Roche, Mannheim, Germany).

In vitro and ex vivo experiments with OVA-stimulated spleen cells

In the *in vitro* experiments, mice were sensitized with OVA/alum on day 0 and sacrificed on day 14. Spleen cells were obtained and incubated with 0, 0.1, 0.3, 1, 3, or 10 μ M/l ALN solution in DMEM with OVA (10, 100 μ g/ml) for 5 d. In the *ex vivo* experiments, mice were sensitized with OVA/alum on day 0, and ALN (25 mg/kg) was administered on days 0–13. On day 14, mice were sacrificed. Spleen cells were obtained and restimulated with OVA (10, 100 μ g/ml) in DMEM for 4 d.

In vivo experiments with BALF cells from mice without sensitization or challenge

The BALF cells of naive mice were analyzed as follows. On days 0–2, mice were administered with the vehicle (CMC) or 25 mg/kg ALN. On day 3, BALF was collected. Cytology was evaluated with Diff-Quick staining, and cell viability and apoptosis were analyzed with Annexin V and PI staining followed by FCM as described earlier.

Statistical analyses

Values are expressed as the mean $\pm$ SD. Dunnett's test was used to compare group means in which all test groups were tested against a reference group. The significance of differences between two groups was calculated by the unpaired two-tailed Student *t* test. The *p* values < 0.05 were considered to be significant. We used the R statistical package (<http://www.r-project.org>) for analyses (26).

Results

Treatment with ALN significantly reduced eosinophil numbers in BALF

Intragastric treatment with ALN in the effector phase significantly reduced eosinophil numbers in BALF (Fig. 1A). Eosinophilic airway inflammation was more prominent with OVA sensitization and OVA challenges (positive control [PC]) than with saline injections and PBS challenges (negative control [NC]). Treatment with ALN (8 or 25 mg/kg) attenuated the number of eosinophils in BALF. In contrast, no significant effect was observed on serum total IgE levels by ALN (Fig. 1B).

Treatment with ALN suppressed the development of allergic airway inflammation

We also examined the effect of ALN on inflammation in the lung by histological examination. Treating mice with 25 mg/kg ALN appeared to reduce inflammatory cell infiltration into lung tissue (Fig. 1C) and the production of mucus in the airway epithelium (Fig. 1D).

ALN attenuated the production of Th2 cytokines in the lung

We then examined the immunosuppressive effect of ALN by analyzing cytokine production in the lung homogenate (Fig. 1E–I). ALN significantly attenuated IL-4, IL-5, and IL-13 levels. However, no significant effect was observed on the amount of IFN- γ levels. ALN also significantly reduced IL-17 levels.

ALN attenuated cytokine production in regional lymph nodes

We examined cytokine production in the supernatant of OVA-stimulated regional lymph nodes (Fig. 1J–N). ALN significantly attenuated IL-4, IL-5, and IL-13 levels, but not IFN- γ levels. ALN also significantly reduced IL-17 levels.

ALN attenuated eotaxin-2 production in the lung

We examined the mechanism of ALN that attenuated eosinophil recruitment into the lungs. We analyzed eotaxin production in lung

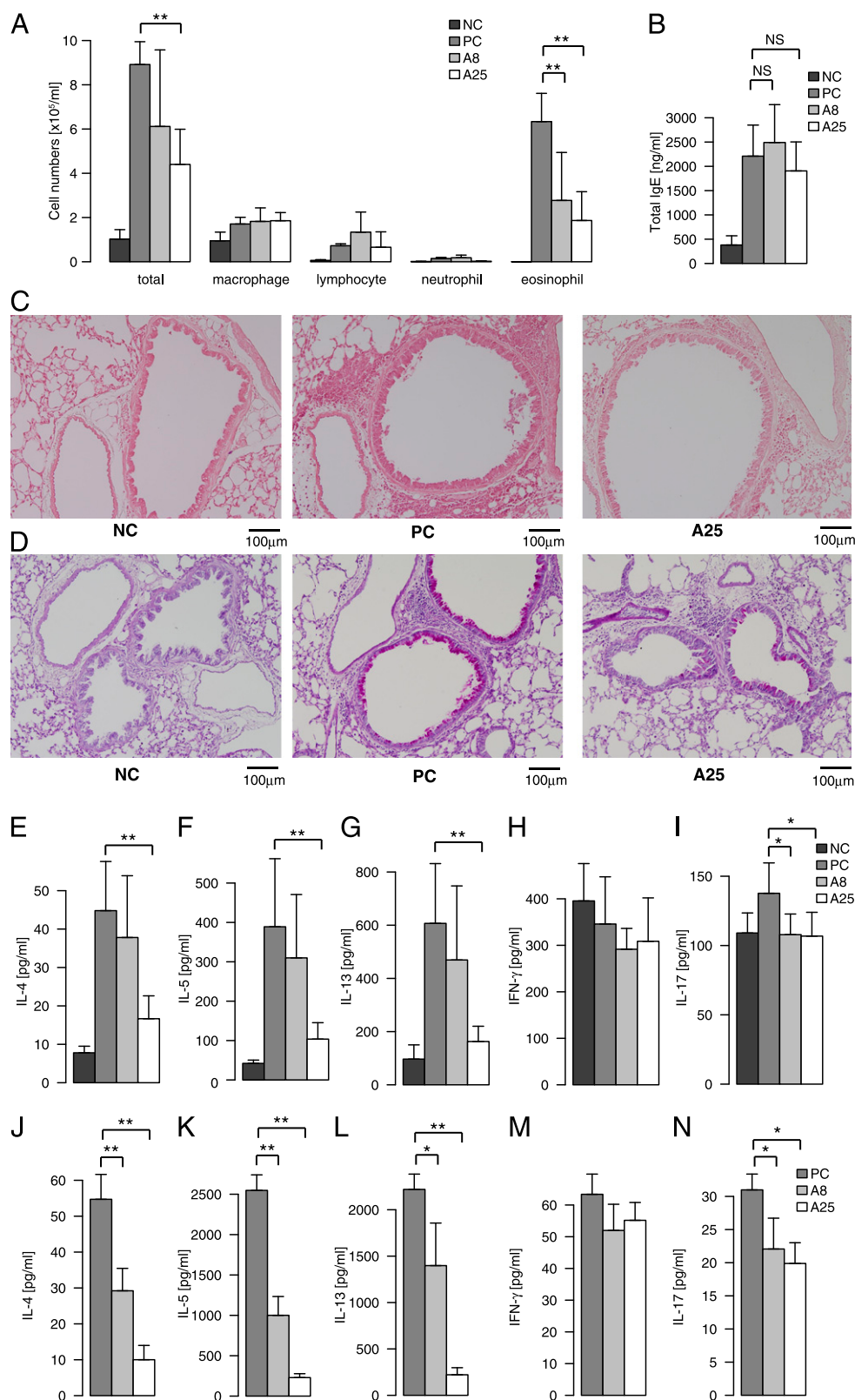


FIGURE 1. Treatment with ALN markedly reduced BALF cell numbers and significantly attenuated cytokine production in the lung and lymph nodes. Mice were sensitized and challenged with OVA as described in the *Materials and Methods*. On days 18–20, they received an i.g. injection of ALN or the vehicle. On day 22, BALF, blood samples, lungs, and lymph nodes were collected. Mice with sensitization (i.p.)/administrations (i.g.)/challenges (nebulization) are represented as follows: saline/vehicle/PBS, NC; OVA/vehicle/OVA, PC; OVA/ALN/OVA, 8 (A8) or 25 mg/kg ALN (A25). **(A)** BALF cytology. **(B)** The concentration of serum total IgE. Representative H&E staining **(C)** and periodic acid-Schiff staining **(D)** of lung tissue. Scale bars, 100 μ m. Production levels of the cytokines IL-4 **(E)**, IL-5 **(F)**, IL-13 **(G)**, IFN- γ **(H)**, and IL-17 **(I)** in the lung homogenate. Data are expressed as the mean $\pm$ SD for $n = 4-7$ mice per group. Production levels of IL-4 **(J)**, IL-5 **(K)**, IL-13 **(L)**, IFN- γ **(M)**, and IL-17 **(N)** in thoracic lymph node cells after 2 d of incubation with OVA (1 μ g/ml). Data were obtained from three wells per group of mice and expressed as the mean $\pm$ SD. Data are representative of three independent experiments with similar results. * $p < 0.05$, ** $p < 0.01$.

tissue. No significant effect was observed on the eotaxin-1 mRNA and protein levels by ALN (Fig. 2A, 2C). In contrast, it significantly decreased eotaxin-2 mRNA (Fig. 2B) and protein levels (Fig. 2D) of eotaxin-2. ALN had no significant effect on eotaxin-2 protein concentrations in BALF (data not shown).

Eotaxin-2 was expressed in peribronchial/perivascular inflammatory cells in the lung

We then elucidated the local expression of eotaxin-2 in the lung. Immunohistochemical analyses revealed that OVA generated eotaxin-2⁺ inflammatory cells among the peribronchial/perivascular spaces. In contrast, epithelial cells were rarely stained (Fig. 2E, 2F, PC). Peribronchial/perivascular immunocytes were positive for eotaxin-1, and airway epithelial cells were also positive even in the NC (NS i.p./vehicle i.g./PBS challenged; data not shown).

A major source of eotaxin-2 was the peribronchial/perivascular macrophages in the lung

We attempted to determine the source of the eotaxin-2 in the lung. Several reports have shown that the major source of eotaxin-2 in the lung in vivo is the macrophage (27–29). Immunofluorescence analysis of PC mice showed that almost all eotaxin-2⁺ peribronchial/perivascular immunocytes were also positive for CD68 and F4/80, respectively, and airway epithelial cells were not positive for eotaxin-2 (Supplemental Fig. 1A, 1B). Most eotaxin-1⁺ immunocytes were also positive for CD68 and F4/80, and airway epithelial cells were mildly positive for eotaxin-1 (Supplemental Fig. 1C, 1D).

ALN attenuated the eotaxin-2⁺ fraction in F4/80⁺ macrophages in the lung

We attempted to confirm the effect of ALN on eotaxin-2 production by macrophages in vivo using FCM. Intracellular staining revealed

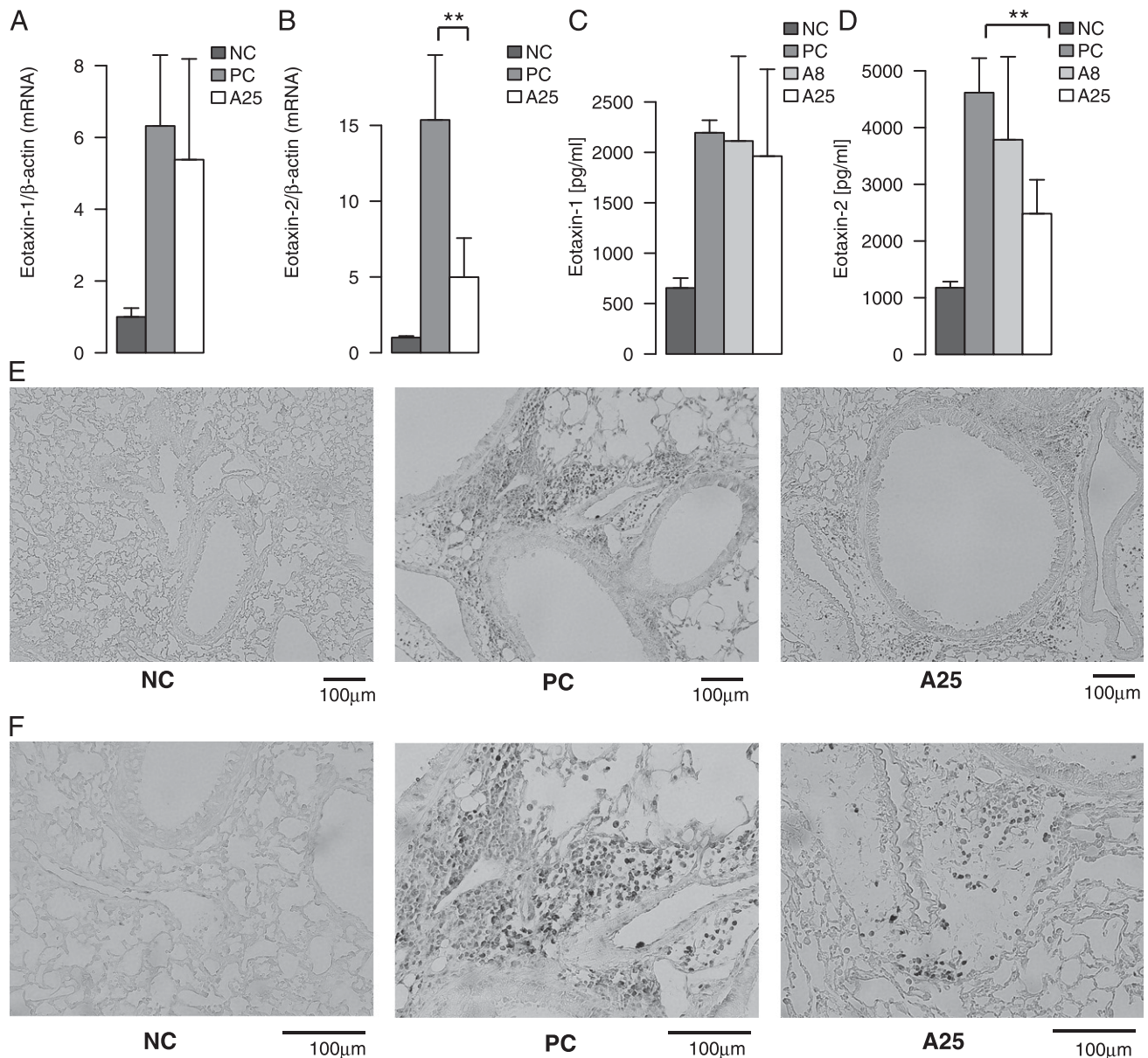


FIGURE 2. Treatment with ALN attenuated eotaxin-2 production in the lung. Mice were sensitized and challenged with OVA, and treated with ALN or the vehicle as described in *Materials and Methods*. On day 22, lungs were extracted. Mice with sensitization (i.p.)/administrations (i.g.)/challenges (nebulization) are represented as follows: saline/vehicle/PBS, NC; OVA/vehicle/OVA, PC; OVA/ALN/OVA, 8 (A8) and 25 mg/kg ALN (A25). mRNA levels of eotaxin-1 (**A**) and eotaxin-2 (**B**) in the left lung were measured and normalized with β-actin. Production levels of eotaxin-1 (**C**) and eotaxin-2 (**D**) were measured in lung homogenate. (**E** and **F**) Representative immunohistochemical staining of lung tissue. Original magnification, ×100 (**E**), ×200 (**F**). Scale bars, 100 μm. Data are representative of three independent experiments with similar results and expressed as the mean ± SD for $n = 4-7$ mice per group. ** $p < 0.01$.

that ALN significantly attenuated the eotaxin-2⁺ fraction in F4/80⁺ macrophages in the lung (Fig. 3C, 3E, 3F). ALN did not have a significant effect on the eotaxin-2⁺ fraction in BALF or on the eotaxin-1⁺ fraction in the lung (Fig. 3B, 3D–F).

ALN attenuated the production of eotaxin-2 by macrophages in vitro

We further investigated whether ALN affected eotaxin-2 production by macrophages in vitro. Pleural macrophages were selected by the plate adhesion method and stimulated by IL-4 and IL-13 for 48 h. Because N-BPs mainly act by inhibiting farnesyl pyrophosphate synthesis in the mevalonate pathway, the intermediates *trans,trans*-FOH or all-*trans*-GGOH were also added to determine whether ALN's effect may be rescued. An ELISA of the supernatants revealed that ALN significantly reduced eotaxin-2 production and this was reversed by GGOH (Fig. 4A), which indicated that ALN acted on farnesyl pyrophosphate synthase. In this in vitro protocol, we evaluated the percentage of live cells and apoptotic cells by staining with Annexin V and PI; however, ALN did not cause significant cell death or cell damage up to 10 μ M (Fig. 4C). We also examined eotaxin-1 production with the same protocol, and eotaxin-1 was not detected even in the PC wells (data not shown).

ALN attenuated the production of eotaxin-2 by THP-1, a human monocyte/macrophage-like cell line, in vitro

The human monocyte/macrophage-like cell line, THP-1, was reported to produce eotaxin-2 when stimulated with PMA, IL-4, and IL-13 in vitro (24, 25). Using this cell line, we attempted to confirm whether ALN could also attenuate human macrophage function. ALN significantly attenuated the production of eotaxin-2 from THP-1 cells, and this effect was reversed by GGOH (Fig. 4B). Cell viability was not significantly affected up to 10 μ M of ALN (Fig. 4D).

ALN did not alter AHR in the lung

We evaluated the effect of ALN on AHR in vivo using the enhanced pause (Penh) system. Treatment with ALN on days 18–20 did not have a significant effect on AHR (Supplemental Fig. 2A), although a slight suppressive tendency was observed.

ALN did not alter Ag presentation in the lung

We examined the effect of ALN on Ag presentation in vivo. Treatment with ALN during the effector phase appeared to have no significant effect on the Ag-presenting capacity of MACS-isolated CD11c⁺ cells in the lung (Supplemental Fig. 2B, 2C).

ALN did not exhibit suppressive effects on Th2-mediated responses in ex vivo and in vitro experiments with spleen cells

We conducted in vitro and ex vivo experiments with OVA-stimulated spleen cells, as described in *Materials and Methods*. No significant effect was observed by ALN on spleen cells either in vitro (Supplemental Fig. 2D–G) or ex vivo (Supplemental Fig. 2H–K).

The suppressive effects of ALN were weaker when it was administered in the sensitization phase (days 0–17) than in the effector phase (days 18–20) in vivo

We also evaluated ALN's effect on the sensitization phase in vivo. In this protocol, ALN was administered throughout the sensitization phase (ALN, days 0–17) or the effector phase (ALN, days 18–20), and mice were sacrificed on day 21. No significant effect was observed on the number of eosinophils in BALF or serum total IgE levels in the ALN (days 0–17) group (Supplemental Fig. 3A, 3B). The ALN (days 0–17) group showed a moderate but less suppressive effect than the ALN (days 18–20) group on the pro-

duction of Th2 cytokines in the lung and OVA-stimulated lymph node cells (Supplemental Fig. 3C–G, 3J–N). ALN (days 0–17) suppressed eotaxin-1 production, but not eotaxin-2 production, in the lung homogenate (Supplemental Fig. 3H, 3I).

ALN did not change the cell populations or viability of BALF obtained from mice without sensitization or challenge in vivo

To evaluate ALN's baseline effect, we analyzed the cytology and viability of BALF cells obtained from mice without sensitization or challenge. Mice were administered with the vehicle (CMC) or 25 mg/kg ALN on days 0–2, and BALF was collected on day 3. The BALF cell population was evaluated with Diff-Quick staining, and the cell viability was evaluated with Annexin V and PI staining followed by FCM. ALN did not have any significant effects on the cell populations or viability of BALF cells (Supplemental Fig. 3O, 3P).

Discussion

The results of this study clearly demonstrated that intragastric delivery of ALN suppressed Th2-mediated allergic immune responses in the airway. ALN, administered during the effector phase, suppressed eosinophilic airway inflammation and the production of Th2 cytokines both in the lung and regional lymph nodes. The production of IL-17 was also reduced in the lung and regional lymph nodes. In addition, ALN significantly suppressed eotaxin-2 production in the lung, the major source of which was the peribronchial/perivascular macrophages, as confirmed by immunohistochemical examinations and FCM. ALN also reduced the production of eotaxin-2 from mouse pleural macrophages and human monocytic/macrophage cells (THP-1). Few studies have examined the effect of BP on asthma or asthma-related cells. To the best of our knowledge, ours is the first study to demonstrate the suppressive effect of BP on Ag-induced allergic airway inflammation in vivo.

In this study, ALN clearly suppressed Th2 responses in the both lung and regional lymph nodes (Fig. 1E–N). This effect is considered to be indirect, as BPs mainly distribute to the phagocytes and not to lymphocytes because of their poor membrane permeability (30–33). ALN did not have systemic suppressive effects in either the in vitro or ex vivo study with spleen cells (Supplemental Fig. 1D–K). Furthermore, we could not confirm a definite effect on CD11c⁺ APCs in the lung in vivo (Supplemental Fig. 1B, 1C). We cannot currently explain the mechanism by which ALN suppressed T cell responses. Because ALN is selectively incorporated by phagocytes, we assume that the direct effector cell was mainly the macrophage. ALN may have altered some functions of the macrophage, including the Ag-presenting capacity and IL-33/ST2 axis, which led to the suppression of T lymphocytes. However, there remains the possibility of ALN's effect on various immunocytes such as lung epithelial cells and mast cells. Another explanation may be that ALN suppressed the amplification of Th2 cell-mediated allergic immune responses caused by eotaxin-2.

In this study, ALN strongly suppressed the transcription and protein production of eotaxin-2 in the lung (Fig. 2B, 2D). The eotaxin/CCR3 axis is critical in allergic airway inflammation (27, 34), although there is a discrepancy in the degree of its contribution (35, 36). In several allergic models, eotaxin-1 was shown to be involved in the early phase (6–24 h) (37–40), whereas eotaxin-2 was involved in the late phase (24 h or more) (38, 39), and thus plays a dominant role in chronic allergic airway inflammation (27, 39). Eotaxin-2 is generally produced by airway epithelial cells and macrophages upon stimulation with IL-4 or IL-13 in vitro (41–43), or after an allergen challenge in vivo (44–46). It promotes AHR and IL-13 production in the airway in cooperation with IL-5 (47), resulting in a vicious cycle that amplifies allergic airway inflammation. ALN

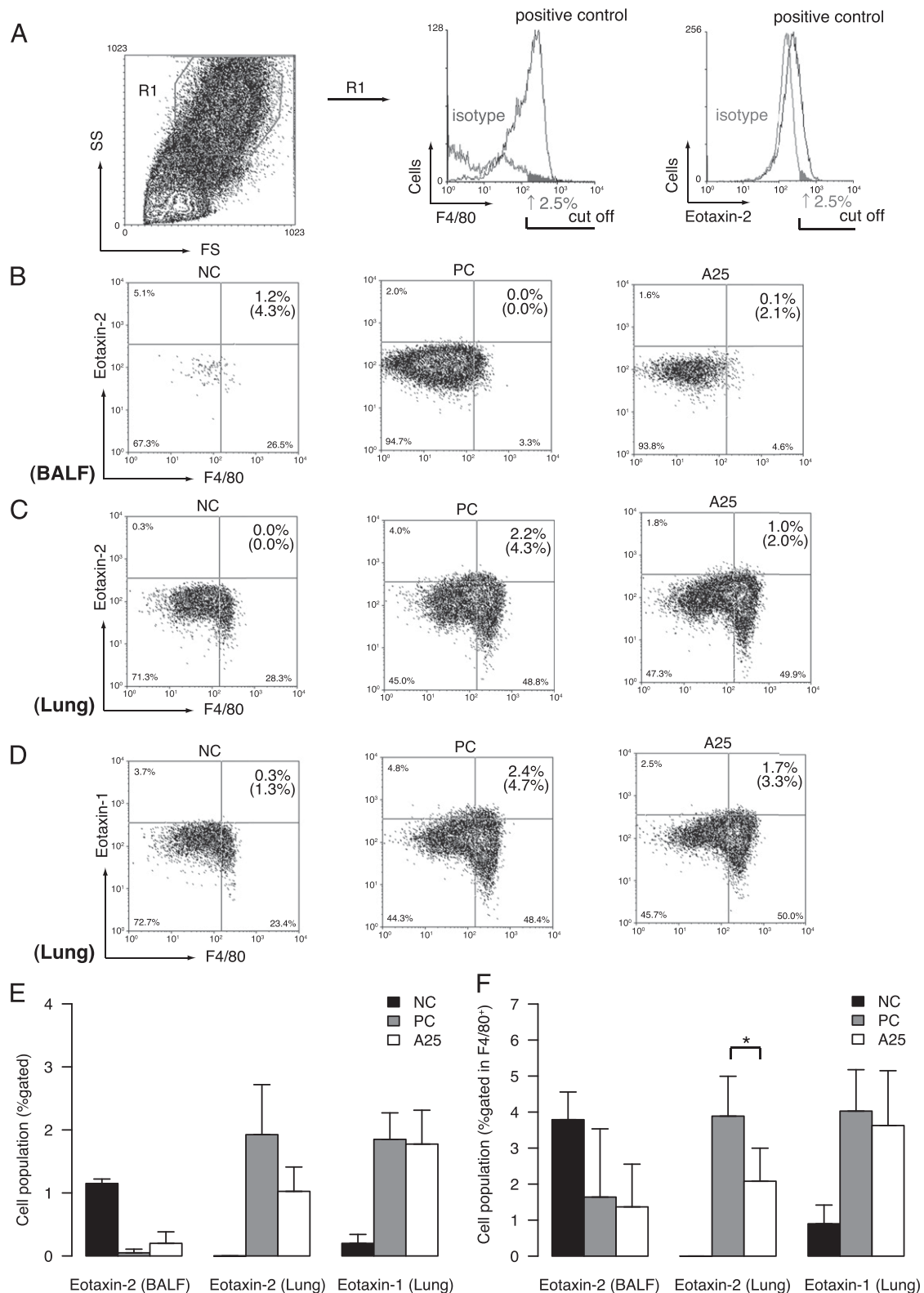


FIGURE 3. Treatment with ALN reduced the intracellular eotaxin-2⁺ fraction in F4/80⁺ macrophages. Mice were sensitized and challenged with OVA and treated with ALN as described in *Materials and Methods*. On day 21, BALF and lungs were collected. BALF and digested lungs were stained with F4/80-biotin-streptavidin-PE/Cy5 and with eotaxin-1-Alexa 488 or eotaxin-2-Alexa 488 after permeabilization. Mice with sensitization (i.p.)/administrations (i.g.)/challenges (nebulization) are represented as follows: saline/vehicle/PBS, NC; OVA/vehicle/OVA, PC; OVA/ALN/OVA, 25 mg/kg ALN (A25). **(A)** Representative flow cytometric isolation of intracellular eotaxin-2⁺ macrophages determined by F4/80. **(B–D)** Representative contour plots demonstrating gating quadrants of intracellular eotaxin-1/eotaxin-2⁺ F4/80⁺ macrophages of BALF (B, eotaxin-2; C, eotaxin-1) and the lung (D, eotaxin-1). Percentages in brackets represent the double-positive ratio to F4/80⁺ macrophages in the R1 region. **(E and F)** The cell populations of intracellular eotaxin-1/eotaxin-2⁺ F4/80⁺ macrophages, the ratio to whole cells in the R1 region (E), and the ratio to F4/80⁺ macrophages in the R1 region (F). Data are representative of three independent experiments with similar results and are expressed as the mean $\pm$ SD for $n = 2$ –4 mice per group. * $p < 0.05$.

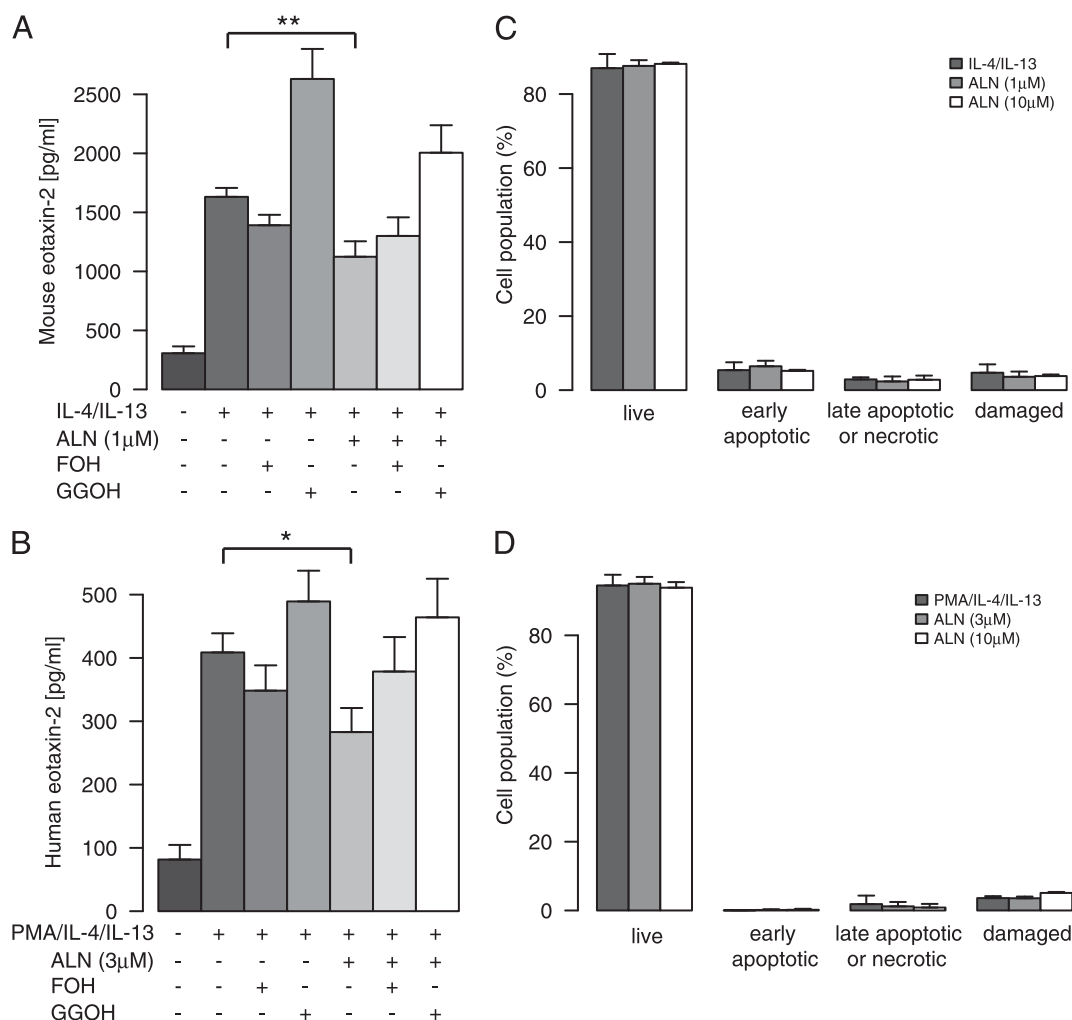


FIGURE 4. ALN attenuated the production of eotaxin-2 by macrophages in vitro. **(A)** Mouse pleural macrophages were isolated by the plate adhesion method and stimulated with IL-4 (20 ng/ml) and IL-13 (20 ng/ml) for 48 h as described in *Materials and Methods*. In some experimental groups, cells were supplied with ALN (1 μM), FOH (3 μM), and GGOH (3 μM). The level of eotaxin-2 in the supernatant was measured. **(B)** Human monocyte/macrophage-like (THP-1) cells were stimulated with PMA (50 ng/ml), IL-4 (20 ng/ml), and IL-13 (20 ng/ml) for 48 h as described in *Materials and Methods*. In some experimental groups, cells were supplied with ALN (3 μM), FOH (3 μM), and GGOH (3 μM). The level of eotaxin-2 in the supernatant was measured. **(C and D)** The percentage of live cells (Annexin V-FITC⁺/PI⁻), early apoptotic cells (Annexin V-FITC⁺/PI⁺), late apoptotic or necrotic cells (Annexin V-FITC⁺/PI⁺), and damaged cells (Annexin V-FITC⁺/PI⁺) in IL-4 (20 ng/ml)/IL-13 (20 ng/ml)-stimulated mouse pleural macrophages supplied with ALN (0, 1, 10 μM) (C) and PMA (50 ng/ml)/IL-4 (20 ng/ml)/IL-13 (20 ng/ml)-stimulated human THP-1 cells supplied with ALN (0, 3, 10 μM) (D). Data are representative of three independent experiments with similar results and expressed as the mean ± SD for three wells per group, respectively. **p* < 0.05, ***p* < 0.01.

may have broken this vicious cycle and then suppressed Th2 responses in the lung.

In our model, eotaxin-1 production was also detected in the peribronchial/perivascular macrophages in the immunohistochemical study (Supplemental Fig. 1C, 1D), whereas ALN did not consistently reduce eotaxin-1 concentrations in the lung homogenate (Fig. 2A, 2C, Supplemental Fig. 1H) or eotaxin-1⁺ fraction of F4/80⁺ macrophages in the lung (Fig. 3D–F). There are several possible explanations for this. First, eotaxin-1 is produced by cells other than macrophages, which ALN may not be able to affect. Bronchial and small airway epithelia, endothelial cells, alveolar macrophages, and airway smooth muscle cells are all potential sources of eotaxin-1 in vivo (27, 44, 45, 48, 49). Second, eotaxin-1 is known to be involved in the early phase (6–24 h) of allergic inflammation (37–40), and our protocol (sacrifice 24 or 48 h after the final challenge) may have failed to evaluate ALN's effect on the production of eotaxin-1 in the lung. According to previous in vitro reports, eotaxin-1 production is limited to airway smooth muscle cells, epithelial cells,

and fibroblasts (43, 50, 51). We could not detect eotaxin-1 from pleural macrophages under any conditions (with LPS and/or IL-4, -17, -33) in vitro (data not shown); therefore, we could not fully confirm ALN's effect on the production of eotaxin-1 by macrophages. The effect of ALN on eotaxin-1 production should be studied further.

The mechanism of the action of BPs is similar to that of statins. Both of them are inhibitors of the mevalonate pathway. We previously reported that pravastatin suppressed eosinophilic airway inflammation by attenuating the function of CD11c⁺ APCs (mainly dendritic cells and partly macrophages) in the lung (10). There are also in vitro reports showing that zoledronic acid, another N-BP, inhibited the function of dendritic cells by altering their differentiation and maturation (52, 53). Therefore, we hypothesized that ALN may also have altered the function of CD11c⁺ APCs to attenuate Th2 responses. We examined the OVA-Ag-presenting capacity of lung CD11c⁺ cells in the effector phase (day 21), and found no significant difference between the control and ALN-treated groups

(Supplemental Fig. 2B, 2C). We also conducted a quantitative PCR analysis on *in vivo* lung mRNA for CCL17 (TARC), a potent chemoattractant for Th2 cells produced mainly by dendritic cells (29, 54–56); however, ALN did not have a significant effect on its transcription (data not shown). Therefore, we consider the contribution of dendritic cell suppression in this study to be weak.

There are limited numbers of reports regarding ALN's effect on the immune system both *in vitro* and in animal models *in vivo*. Sansoni et al. (57) reported that ALN inhibited the Ag-presenting capabilities and LPS-induced generation of IL-1 β , IL-6, and TNF- α in activated human monocytes *in vitro*. Jiang et al. (58) reported that ALN inhibited β_2 -adrenergic receptor internalization and the downregulation of human airway smooth muscle cells *in vitro*. ALN's effects are controversial in *in vivo* studies. Deng et al. (59) reported that an i.p. injection of 40 μ mol/kg (13 mg/kg) ALN induced IL-1-dependent inflammatory reactions in the spleen, lung, and liver. In contrast, the oral administration of 25 or 75 mg/kg ALN, a similar dosage to that used in this study (8 or 25 mg/kg), decreased colonic damage in a model of inflammatory bowel disease in rats (60). Differences in the dosage as well as the route of administration may influence whether ALN reveals a proinflammatory or anti-inflammatory effect.

A critical factor influencing the effect of ALN on immune responses is tissue distribution. When administered, BPs are generally distributed to the bones and remain there for months to years. Their distribution in the respiratory system has seldom been studied. Only two reports have examined this issue (11–13). In these reports, ALN was distributed to the trachea even 72 h after multiple oral administrations (5 mg/kg, 7 d) or a single i.v. administration (0.05 mg/kg) in the rat. In contrast, its distribution to other organs such as the liver, spleen, kidney, and lymph nodes was very low. This high affinity of ALN for bronchial tissue may contribute to a suppressive effect specifically in the lung and could explain why ALN failed to suppress Th2-mediated systemic immune responses to OVA *in vivo* and *in vitro* experiments with spleen cells (Supplemental Fig. 1D–K). ALN moderately suppressed Th2 cytokine production in the lung and lymph nodes when administered during the sensitization phase (days 0–17) *in vivo* (Supplemental Fig. 3). This may have been caused by the maintenance of ALN in the trachea 72 h after the last administration until the effector phase (days 18–20).

In this study, we adopted an intragastric route of administration for the following reasons: 1) ALN is usually administered orally in clinical use, 2) ALN was reported to distribute to trachea after oral administration (11), and 3) oral administration of ALN was reported to attenuate colonic damage in a model of inflammatory bowel disease (60). We mainly used the dosage of 25 mg/kg because 25 or 75 mg/kg of oral administration of ALN was reported to decrease bowel inflammation in a rat model (60), and because 25 mg/kg oral ALN appeared to be more effective than 8 mg/kg in our model (Fig. 1). The mean bioavailability of ALN after oral intake was 0.64% relative to its i.v. equivalent (61). Therefore, the relatively low dose of ALN in the bronchus in this study, delivered by an intragastric tube, may have demonstrated an anti-inflammatory effect in the lung. The intratracheal administration of ALN may be a potential alternative to decrease the dose required for the immunosuppressive effect on allergic airway inflammation.

Regarding its clinical use, ALN has been administered at 0.2 mg/kg (10-mg tablet) daily or 1.4 mg/kg (70-mg tablet) weekly, and a single administration of 70 mg achieved maximal plasmatic concentrations as 82.37–110.71 ng/ml, relevant to 0.25–0.34 μ M (62). After the single or repeated (7 d) oral administration of 5 mg/kg to rats, the concentration of ALN in the trachea was shown to be $\sim$ 4 or 20 times higher than maximal plasmatic concentrations,

respectively (11). Therefore, the *in vitro* ALN concentrations used in this study (1–3 μ M) do not appear to differ from those achievable in the clinical use. However, the *in vivo* dose used in this study (25 mg/kg 3 d) was 125 times higher than that used clinically, and blood concentration data at this dosage were not reported in any species.

BPs are known to induce apoptosis in macrophages at high concentrations. The i.v. administration of liposomal clodronate is a conventional method of depleting macrophages (63). ALN was shown to induce apoptosis in mouse macrophage-like J774.1 cells at a very high concentration (100 μ M) only *in vitro* (64), which is >30 times higher than that which inhibited the production of eotaxin-2 in mouse pleural macrophages (1 μ M) or the human monocytic cell line (THP-1; 3 μ M) in this study (Fig. 4A, 4B). Furthermore, FCM evaluation with Annexin V and PI revealed no significant difference in the viability and apoptosis of mouse macrophages or human THP-1 cells after incubation with up to 10 μ M ALN for 48 h from the PC *in vitro* (Fig. 4C, 4D). BALF cells (mainly macrophages) of nonsensitized and nonchallenged mice administered the vehicle (CMC) or 25 mg/kg ALN exhibited no significant difference in cytology or viability *in vivo* (Supplemental Fig. 3O, 3P). Therefore, we consider that ALN exhibited a suppressive effect without inducing cell death or cell damage in macrophages.

In summary, the oral administration of ALN during airway inflammation effectively suppressed Ag-induced airway responses in a mouse model. ALN attenuated the production of eotaxin-2 from lung macrophages, which may contribute to suppress allergic airway inflammation *in vivo*. The mechanism by which ALN suppressed Th2 and Th17 responses has not been fully clarified. Our results with human monocyte/macrophage-like (THP-1) cells appear to support ALN's anti-inflammatory effect on humans. Therefore, ALN could be a novel therapeutic option for asthma.

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Disclosures

The authors have no financial conflicts of interest.

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Figure Legends

SUPPLEMENTAL FIGURE 1.

The major source of eotaxin-2 is the peribronchial/perivascular macrophages.

Mice were sensitized and challenged with OVA as described in the Materials and Methods. On days 18-20, mice received vehicle. On day 22, lung tissue was extracted. (A) Representative immunofluorescence for a positive control mouse.

Left (Eotaxin-2), middle (CD68), right (merge; Eotaxin-2 + CD68). (B)

Representative immunofluorescence for a positive control mouse. Left

(Eotaxin-2), middle (F4/80), right (merge; Eotaxin-2 + F4/80). (C) Representative

immunofluorescence for a positive control mouse. Left (Eotaxin-2), middle

(CD68), right (merge; Eotaxin-1 + CD68). (D) Representative

immunofluorescence for a positive control mouse. Left (Eotaxin-2), middle

(F4/80), right (merge; Eotaxin-1 + F4/80). Scale bars: 100 μ m.

SUPPLEMENTAL FIGURE 2.

Alendronate did not significantly change AHR, the antigen presentation capacity

of lung CD11c⁺ cells in the *in vivo* study, or the systemic inflammatory response of splenocytes in the *in vitro* or *ex vivo* study. (A-C) Mice were sensitized and challenged with OVA and treated with ALN or the vehicle as described in the Materials and Methods. Mice with sensitization (i.p.)/administrations (i.g.)/challenges (nebulization) are represented as follows: saline/vehicle/PBS, NC; OVA/vehicle/OVA, PC; OVA/ALN/OVA, A25 (25mg/kg). (A) On day 21, AHR was examined by the change in Penh to Mch. Data are expressed as the mean $\pm$ SD for n=8 mice per group. (B and C) On day 21, lungs were extracted. CD11c⁺ cells in the lung were positively selected, irradiated, and cocultured with CD4⁺ T cells from DO11.10 mice. Cell proliferation was measured after 5 days of coculture,. Data are expressed as absorbance (B) or as a percentage of the response relative to that induced by CD 11c⁺ cells obtained from positive control mice. (E-G) *In vitro* study with splenocytes. Mice were sensitized with OVA on day 0. Spleen cells were obtained on day 15, restimulated with OVA (10 or 100 μ g/ml) $\pm$ ALN for 5 days, and the concentrations of cytokines in the supernatant were measured by ELISA. (H-K) *Ex vivo* study with splenocytes.

Mice were sensitized with OVA on day 0, and administered vehicle (PC) or ALN 25mg/kg (A25) on days 0-14. On day 15, spleen cells were obtained and cultured with OVA (10 or 100µg/ml) for 4 days. Cytokine concentrations in the supernatant were measured by ELISA.

SUPPLEMENTAL FIGURE 3.

Treatment with ALN in the sensitization phase (days 0-17) did not significantly affect BALF cytology, but moderately suppressed cytokine production in the lung and lymph nodes. (A-N) Mice were sensitized and challenged with OVA as described in the Materials and Methods. Mice with sensitization (i.p.)/administrations (i.g.)/challenges (nebulization) are represented as follows: saline/vehicle/PBS, NC; OVA/vehicle/OVA, PC; OVA/ALN (25mg/kg)/OVA, ALN (days 0-17) or ALN (days 18-20). On day 21, BALF, lungs, lymph nodes were collected. (A) BALF cytology. (B) The concentration of serum total IgE. (C-I) Production levels of the IL-4 (C), IL-5 (D), IL-13 (E), IFN- γ (F), IL-17 (G), eotaxin-1 (H), and eotaxin-2 (I) in the lung homogenate. Data are expressed as

the mean $\pm$ SD for n=3-7 mice per group. (J-N) Production levels of IL-4 (J), IL-5 (K), IL-13 (L), IFN- γ (M), and IL-17 (N) by lymph node cells were measured after 2 days of incubation with OVA (1 μ g/ml). Data were obtained from three wells per group of mice, and expressed as the mean $\pm$ SD. *, p<0.05; **, p<0.01. (O and P) Treatment with ALN did not significantly effect the cytology or viability of BALF cells. Mice were administered vehicle (CMC) or ALN 25mg/kg (A25) on days 0-2. Both groups of mice were not sensitized or challenged with OVA, and BALF was collected on day 3. (O) BALF cytology. (P) The percentage BALF live cells (annexin V-FITC negative/ PI negative), early apoptotic cells (annexin V-FITC positive/ PI negative), late apoptotic or necrotic cells (annexin V-FITC positive/ PI positive), and damaged cells (annexin V-FITC negative/ PI positive). Data are expressed as the mean $\pm$ SD for n=4 mice per group.



A Eotaxin-2 100 μ m CD68 100 μ m Merge (Eotaxin-2 + CD68) 100 μ m



B Eotaxin-2 100 μ m F4/80 100 μ m Merge (Eotaxin-2 + F4/80) 100 μ m

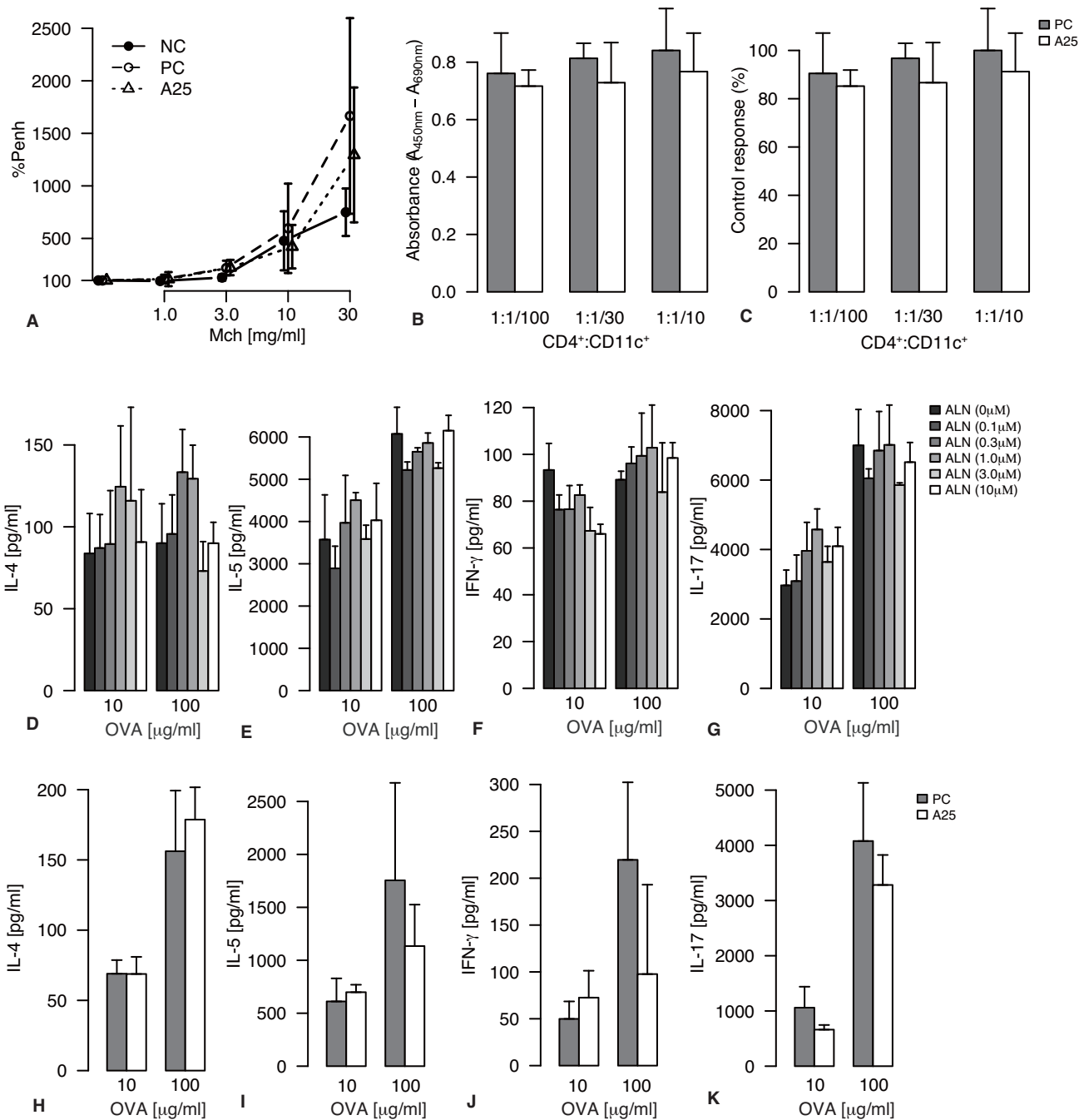


C Eotaxin-1 100 μ m CD68 100 μ m Merge (Eotaxin-1 + CD68) 100 μ m

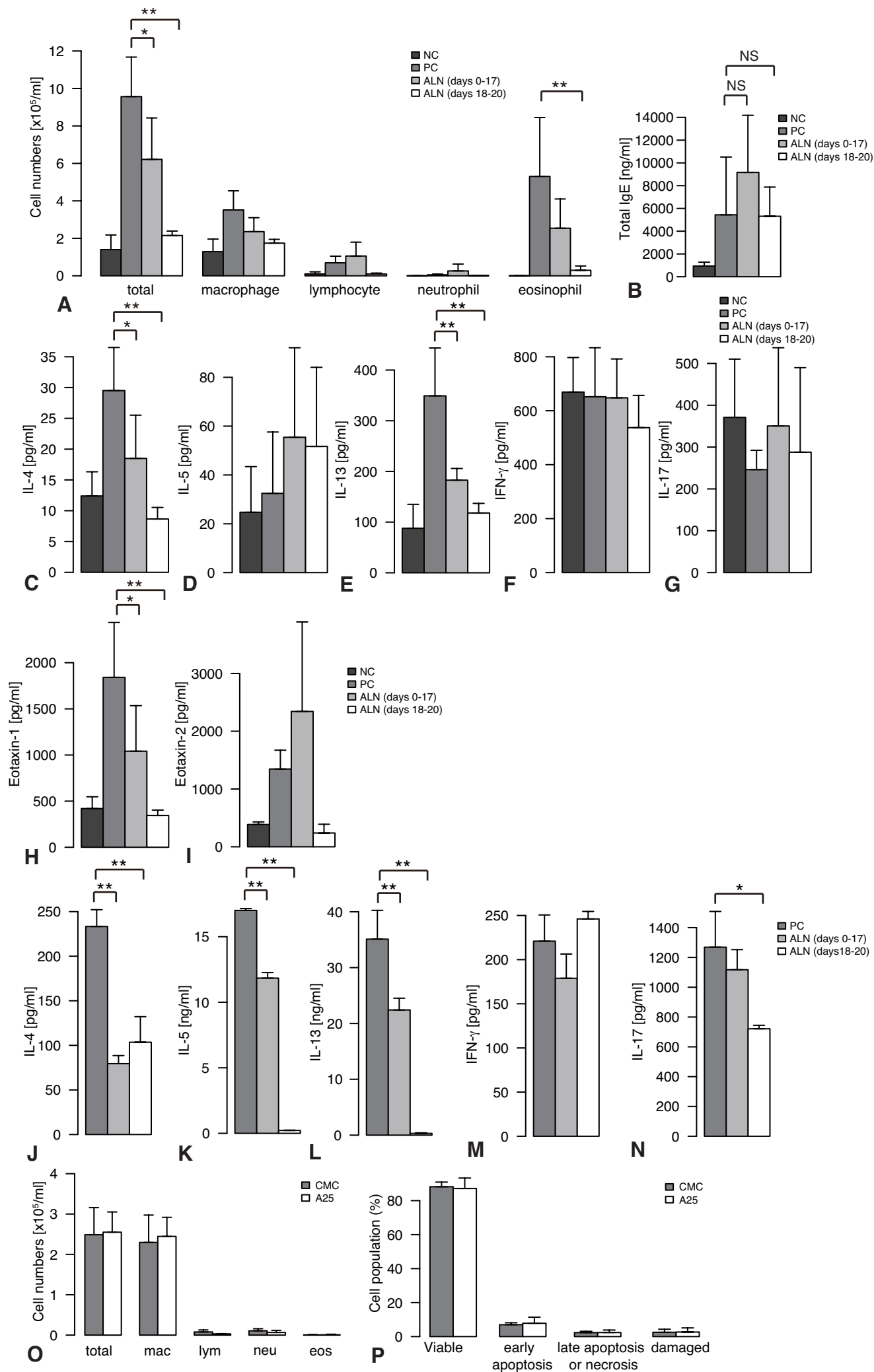


D Eotaxin-1 100 μ m F4/80 100 μ m Merge (Eotaxin-1 + F4/80) 100 μ m

SUPPLEMENTAL FIGURE 1



SUPPLEMENTAL FIGURE 2



SUPPLEMENTAL FIGURE 3