Acetaldehyde at low concentration synergistically exacerbates allergic airway inflammation as an endocrine disrupting chemical and as a volatile organic compound

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Running head: *Effect of low acetaldehyde concentration on allergic airway inflammation*

Key words: bronchial asthma, acetaldehyde, allergic airway inflammation, endocrine disrupting chemical(s), volatile organic compound(s)

Abstract

Background: Acetaldehyde is an endocrine-disrupting chemical (EDC) and volatile organic compound (VOC). It is also a carcinogen and teratogen that causes bronchoconstriction in a subset of asthmatics. However, the mechanism through which acetaldehyde acts as an EDC/VOC in causing allergic airway inflammation remains unknown.

Objectives: The present study determines the effects of a low concentration of acetaldehyde, which itself did not trigger airway inflammation, on extant allergic airway inflammation in a murine model of allergic asthma.

Methods: Four groups of BALB/c mice [control(Cont), *Dermatophagoides farinae* (*Df*) allergen sensitized (AS), intranasally acetaldehyde injected (ALD), and allergen sensitized and acetaldehyde injected (AS-ALD)] were prepared. We compared airway hyperresponsiveness (AHR) lung pathology, serum IgE, and airway concentration of cytokines among these groups.

Results: Physiological and histological differences were not evident between ALD and Cont mice. The AS mice developed AHR allergic airway inflammation characterized by goblet cell hyperplasia and eosinophilic infiltration. AHR and airway eosinophilia were significantly enhanced in AS-ALD, compared with AS mice. Serum total and *Df*-specific IgE were

39 significantly increased in both AS and AS-ALD mice compared with Cont and ALD mice,
40 but comparable between AS and AS-ALD mice. Mite allergen sensitization significantly
41 increased IL-5 and GM-CSF and decreased IFN-γ in the airways, and injecting ALD into the
42 airway significantly increased IL-5, GM-CSF, and IFN-γ in the airways of AS mice.
43 ***Conclusions:*** Exposure to acetaldehyde can enhance allergic airway inflammation in asthma.
44

Introduction

It is generally accepted that endocrine-disrupting chemicals (EDCs) confer health risks such as toxicity, carcinogenicity, mutagenicity, immunotoxicity, and neurotoxicity in humans [1-5]. A relationship between EDCs and allergic diseases has been described [6-10], but the exact mechanism underlying this relationship remains obscure. Some EDCs are volatile, and these are generally refered to as volatile organic compounds (VOCs). Exposure to VOCs such as formaldehyde or acetaldehyde can cause sick building syndrome (SBS) or bronchial asthma [6-10].

Bronchial asthma is characterized by chronic airway inflammation and airway hypersensitivity [11-15]. Among the various inflammatory cells, type 2 T helper lymphocytes (Th2), which produce Th2 cytokines that regulate allergic airway inflammation, are typically located in the airways of asthma patients [12]. In particular, the Th2 cytokine IL-5 promotes the maturation and activation of eosinophils [11-15]. Interferon (IFN)-γ, a Th1 cytokine, inhibits the biological effects of Th2 cytokines. Th2 immunity is dominant over Th1 immunity in asthma [11, 12]. For both children and adults, the most common trigger of acute exacerbation of asthma is viral respiratory tract infection [16]. Although the precise

underlying mechanism of virus-induced asthma exacerbation remains unknown, viral infection probably exacerbates Th2-dominant allergic airway inflammation [11, 16].

A number of other factors can exacerbate asthma. We have previously reported that alcohol consumption exacerbates asthma in about half of Japanese patients with asthma [17-19]. Acetaldehyde, a metabolite of alcohol, plays a critical role in this alcohol-induced bronchoconstriction via stimulation of mast cells/basophils to produce histamine [20]. Acetaldehyde is not only a metabolite of alcohol but it is also a VOC that is linked to SBS and asthma [6-10], and it might have various adverse effects in humans [1-5]. In fact, acetaldehyde in cigarette smoke inhibits ciliary motility via a phophokinase C (PKC)-dependent mechanisms [21]. Taken together, these findings indicate that acetaldehyde affects airway inflammation as a VOC. Nonetheless, little is known about interactions between acetaldehyde and allergic airway inflammation. The present study investigates the effects of acetaldehyde as an EDC/VOC on extant allergic airway inflammation induced by mite allergens in a novel murine model of asthma.

Material and methods

Acetaldehyde concentration

The concentration of acetaldehyde used in the present study was determined by preliminary experiments based on the findings of published reports [22, 23]. Several concentrations of acetaldehyde were injected intranasally in mice once a day for a week according to the protocol described below. And then, lung specimens were histologically evaluated. We concluded that 50 μg of acetaldehyde does not directly injure the murine airway since this dose did not cause either tissue damage or inflammatory change in this model. This concentration was lower than that used in a previous study of humans [23].

Animals and immunization protocol

An animal model of mite allergen-sensitized asthma was prepared as described [24, 25]. Four groups (n=8 per group) of female BALB/c mice (Charles River Japan, Inc., Yokohama, Japan), 4-6 weeks of age, were housed at the Laboratory Animal Center for Biochemical Research, Nagasaki University School of Medicine. All mice were immunized twice intraperitoneally on days 1 and 14 with 0.5 mg/mouse of *Dermatophagoides farinae* (*Df*, American house dust mite) (LG-5339, Cosmo Bio, Tokyo, Japan) precipitated in aluminum

hydroxide. These mice were then challenged intranasally with 50 μl of phosphate-buffered saline (PBS) (control group, Cont; acetaldehyde inoculated group, ALD) or with 50μg/50μl of *Df* allergen (allergen-sensitized group, AS; allergen sensitized and acetaldehyde inoculated group, AS-ALD) on days 14, 16, and 18 as previously described [25]. The ALD and AS-ALD groups were each intranasally injected with 50 μg of acetaldehyde (Sigma, St. Louis, MO, USA) from days 14 to 20. AHR was determined on day 20 in unrestrained mice using whole-body plethysmography. All mice were sacrificed by dislocation of the cervical vertebrae on day 21, and peripheral blood was collected from each group. Bronchoalveolar lavage fluid (BALF) was obtained from half of the mice in each group using 0.5 ml of ice-cold PBS. Lung tissues were obtained from the other half of each group of mice for pathological evaluation. The procedures were reviewed and approved by Nagasaki University School of Medicine Committee on Animal Research. All experiments were repeated at least three times.

Determination of AHR

We measured AHR in unrestrained mice using whole body plethysmography (PULMOS-I, M.I.P.S., Osaka, Japan) as we previously reported [25]. AHR is expressed as calculated

specific airway resistance (sRAW) which closely correlates with pulmonary resistance measured using conventional, two-chamber plethysmography in ventilated animals. The four groups of mice were exposed for 5 minutes to nebulize PBS and subsequently to increasing concentrations (6, 12, 25, 50 mg/ml) of nebulized methacholine (MCh; Sigma) in PBS using an ultrasonic nebulizer (NE-U17, Omron, Kyoto, Japan) and recordings were taken for 3 minutes after the delivery of each dose.

Pathological evaluation of pulmonary inflammation

Lung sections from each group were stained with hematoxylin and eosin (H&E) evaluated (magnification ×400) at least three times by three different observers in a blinded fashion as described [25]. The number of eosinophils and total number of nuclei in three randomly selected airways was determined. The eosinophil count is expressed as a ratio (%) of the total cells in the airway.

Determination of serum IgE level

The serum concentrations of total IgE and *Df*-specific IgE were measured in duplicate using enzyme-linked immunosorbent assays (ELISA). The total serum IgE concentration was determined using a rat anti-mouse IgE antibody (Ab) (PharMingen, SanDiego, CA, USA) and

biotin-conjugated rat anti-mouse IgE mAb (PharMingen) as described [20]. Other 96-well ELISA plates were prepared to measure *Df*-specific IgE. Plates were coated overnight at 4°C with 5 μg/ml of *Df* extract. Serum samples (1:10) were incubated for 2 hours at room temperature in the *Df*-coated plates before incubation with biotin-conjugated rat anti-mouse IgE mAb. The optical density (OD) at 405 nm was determined using an automatic ELISA plate reader. The total serum IgE level was expressed as μg/ml using a mouse IgE standard (PharMingen). The *Df*-specific serum IgE levels are expressed as OD_{405}.

Analysis of BALF

BALF samples were evaluated using a hemocytometer and light microscopy. Each BALF sample was centrifuged for 10 minutes at 400×g at 4°C, and cytokines were analysed in the supernatants. The cell pellets were resuspended in 1 ml of PBS. The total number of cells in the BALF was counted using a hemocytometer, and cells on cytospin slides were fixed and visualized by May-Giemsa staining. Three observers performed differential counts of 200 cells. Absolute cell numbers were calculated as the product of the total and differential cell counts. The absolute number of eosinophils in the BALF was calculated. The concentrations of IFN-γ, IL-5, and granulocyte macrophage colony-stimulating factor (GM-CSF) in the

BALF supernatants were determined by ELISA (Quantikine, R&D Systems Inc., Minneapolis, MN, USA), as described by the manufacturer.

Statistical analysis

Results are expressed as mean ± standard error of mean (SEM). Data were evaluated using repeated-measures ANOVA with a Bonferroni multiple comparison test. A p value of < 0.05 was considered significant.

Results

Low acetaldehyde concentration enhanced AHR in a murine model of asthma

We measured AHR to inhaled Mch (Figure 1). The sRAW did not significantly increase in response to PBS inhalation in any group, but significantly increased in the AS and AS-ALD groups compared with the Cont group after inhaling 25 and 50 mg/ml of Mch, and in the AS-ALD group after inhaling 50 mg/ml Mch compared with AS group.

Low acetaldehyde concentration synergistically worsened airway inflammation

Figure 2A shows representative pathological features of the four groups of mice. Airway inflammation was not significantly increased in ALD, compared with Cont mice. Goblet cell metaplasia and cellular infiltrate with eosinophils were identified in AS mice. The mean number of infiltrating eosinophils per 10 perivascular areas was significantly increased in AS-ALD compared with AS mice (mean ± standard error; 38.7±12.1 vs. 22.1±9.7, $p<0.05$). Analysis of the cellular components of BALF revealed significantly more airway lymphocytes and eosinophils in AS, than in Cont mice, and airway eosinophilia was more significantly increase in AS-ALD, than in AS mice (Figure 2B).

Acetaldehyde did not change serum IgE levels

Serum total IgE and *Df*-specific IgE levels are shown in Figure 3. Serum total IgE and *Df*-specific IgE were significantly increasesd in AS and AS-ALD mice compared with Cont and ALD mice. Total IgE or *Df*-specific IgE did not significantly differ between Cont and ALD mice.

Acetaldehyde increased IL-5 and GM-CSF concentrations in BALF

Figure 4 shows IL-5, IFN-γ, and GM-CSF concentrations in BALF. Like the pathological lung profile, the cytokine profile was not significantly altered in ALD mice compared with Cont mice, whereas IL-5 and GM-CSF were significantly increased and IFN-γ was significantly decreased in AS, compared with Cont mice. Injection a low dose of acetaldehyde in AS mice significantly increased levels of IFN-γ, IL-5 and GM-CSF.

Discussion

The major findings of the present study are as follows. Intranasal injection of a low concentration of acetaldehyde, which itself did not trigger airway inflammation, worsened AHR, significantly exacerbated extant allergic airway inflammation induced by mite allergens and increased the production of Th1 and Th2 cytokines. Acetaldehyde is commonly encountered in the environment. Cigarette smoke and vehicle exhaust emissions contain both acetaldehyde and formaldehyde [2, 6-8, 21], and these chemicals are also found in paints, plastic products, and adhesives, etc. [2, 7, 9]. In addition, some fruits naturally contain acetaldehyde [2], and acetaldehyde is used as a food additive in some countries. Therefore, continuous exposure to environmental acetaldehyde or formaldehyde might exacerbate asthma. Many reports have described that formaldehyde act as an EDC/VOC and adversely affects health [1-10]. Garrett et al. reported that inoculation with low levels of formaldehyde increased the risk of bronchial asthma in children [7]. Exposure to low levels of formaldehyde also increases IgE levels in humans [26] and mice [10]. Guinea pigs exposed to formaldehyde showed enhancement of allergic sensitization to inhaled allergens [27]. However, the effects of low-level acetaldehyde (as an EDC/VOC) on allergic airway inflammation has not been reported in detail.

Acetaldehyde can trigger acute exacerbations of asthma as it is also a metabolite of alcohol [17-20, 28]. About half of Japanese patients with asthma have experienced exacerbation after alcohol consumption. Many Japanese people have a raised concentration of acetaldehyde in the peripheral blood after alcohol consumption because of having genetically lower or absent activity of aldehyde dehydrogenase (ALDH) 2, which is a primary enzyme involved in acetaldehyde metabolism [17-20]. We previously confirmed that such an increase in the blood acetaldehyde concentration stimulates human mast cells in bronchial epithelial cells to release histamine, causing bronchoconstriction [17-20]. The present findings suggests that acetaldehyde has a proinflammatory effect in the pathophysiology of asthma in addition to a bronchoconstrictive effect.

The immunological effects of alcohol have attracted focus from the viewpoint of infectious diseases [29]. In fact, alcoholism is considered a risk factor for infections such as pneumonia [30]. A growing body of evidence points to alcohol as an important modifier of mucociliary clearance, which is the first line of defense for the lungs [28]. Acetaldehyde activates PKC in airway cells and might be linked to the release of airway oxidants [21]. Aytacoglu et al. reported that alcohol could cause lung damage [31]. In contrast, little is known about interactions between alcohol and allergic inflammation. One possible

mechanisms through which acetaldehyde might enhance allergic airway inflammation is that inhaled acetaldehyde physically injures the airway epithelium, which enhances the penetration of mite allergen into the airway, resulting in an increased IgE response. However, this was not so in the present study. Clarisse et al. measured the indoor air concentrations of aldehydes [32]. The combustion of cigarettes remarkably increases the airborne aldehyde concentration [33]. Thus, smoking tobacco increases the amount of exposure to acetaldehyde compared with the low level generated in the present study, in which levels of mite allergen-specific IgE antibody were comparable between AS and AS-ALD mice. We previously showed that acetaldehyde, but not alcohol, stimulates GM-CSF production from the airway epithelium *in vitro* through the activation of nuclear factor κ-B (NF-κB) in lung tissue from patients with lung cancer [34]. The present study also demonstrated that a low concentration of acetaldehyde significantly increased airway production of GM-CSF induced by mite allergen *in vivo*. Since, GM-CSF is a growth factor for dendritic cells that serve as the primary antigen-presenting cells in the airway, the present findings suggest that the maturation of dendritic cells by acetaldehyde-induced GM-CSF production enhances adaptive immunity and thus exacerbates allergic airway inflammation. Although how acetaldehyde increases GM-CSF production remains uncertain, the present study indicates at

least one mechanism through which allergic airway inflammation is exacerbated by acetaldehyde acting as an EDC.

In conclusion, acetaldehyde might be involved in the pathogenesis of asthma via two pathways. One is that blood levels of acetaldehyde increased as a result of the genetically reduced ALDH2 activity in some Asian populations stimulate mast cells to release histamine after oral alcohol intake, and this causes bronchoconstriction. The other is that inhaled acetaldehyde acting as an EDC enhances allergic airway inflammation.

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336 **Figure legends**

337 **Figure 1.** Acetaldehyde emphasized AHR in AS and AS-ALD group mice.

338 Data are shown as means ± SEM of sRAW (n=8 per group). *$p < 0.01$ vs. Cont, $^{\dagger}p < 0.05$ vs.

339 AS.

340

341 **Figure 2.** Representative photomicrographs (400×) of lung tissue samples of four groups of

342 mice (A), and BALF cell count (B).

343 (a), Control; (b), allergen-sensitized group (AS); (c), acetaldehyde-injected group (ALD); (d),

344 allergen-sensitized and acetaldehyde-injected group (AS-ALD). Eosinophilic airway

345 inflammation and acetaldehyde further exacerbated allergic airway inflammation in AS mice

346 (d). Acetaldehyde alone did not cause histological changes in the murine lung at this

347 concentration (c). Lymphocyte and eosinophil numbers are significantly increased in BALF

348 from AS and AS-ALD groups compared with Cont group. Eosinophil count significantly

349 increased in AS-ALD, compared with the AS group (B). *$p < 0.01$ vs. Cont, $^{\dagger}p < 0.01$ vs. AS.

350

351 **Figure 3.** Serum levels of total IgE and *Df*-specific IgE

Levels of both are significantly increased in AS and AS-ALD, compared with Cont group. Acetaldehyde did not additively affect serum IgE. *$p < 0.01$ vs. Cont.

Figure 4. Cytokine concentrations in the BALF

Levels of IL-5 and GM-CSF are significantly increased in AS and AS-ALD, compared with Cont group, and are significantly higher in the AS-ALD, than in AS group. Acetaldehyde accordingly exerted synergistic effect with antigen sensitization on BALF IL-5 and GM-CSF concentration. Level of IFN-γ is significantly decreased in the AS, compared with all other groups. *$p < 0.01$ vs. Cont, $^{\dagger}p < 0.05$ vs. Cont, $^{\ddagger}p < 0.05$ vs. AS.

Figure 1.

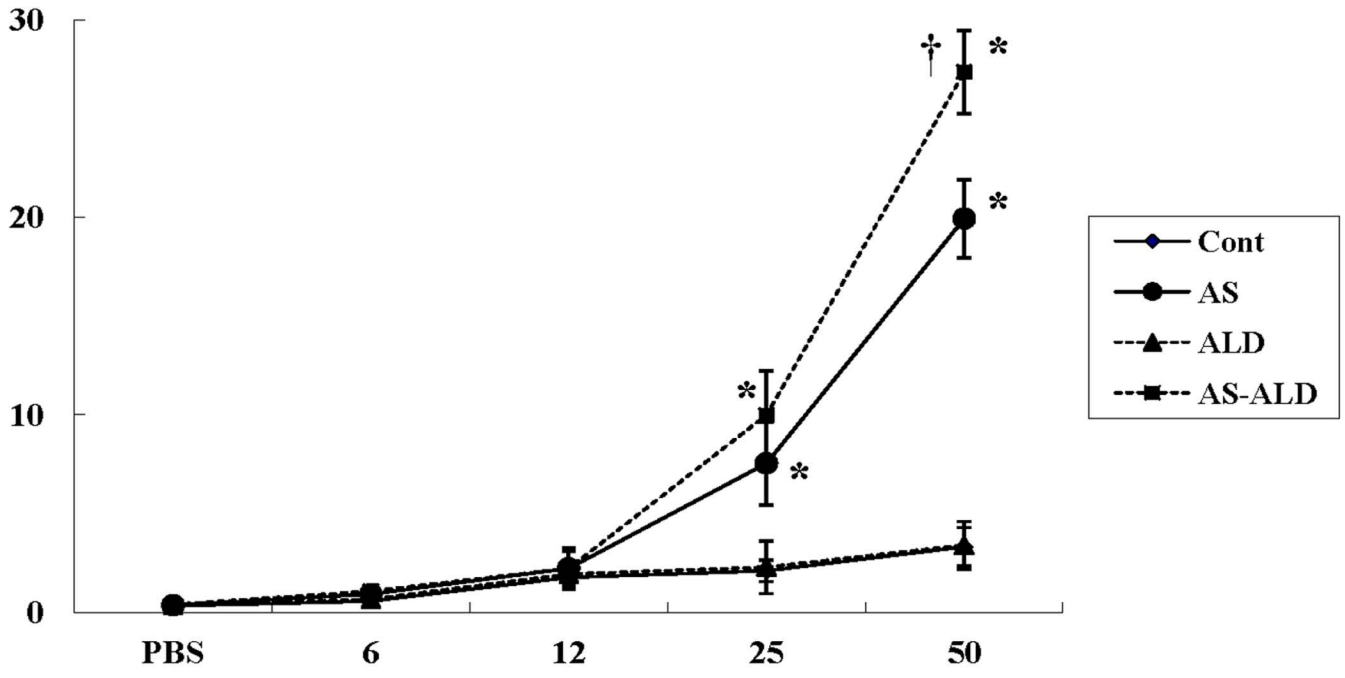

Figure 2.

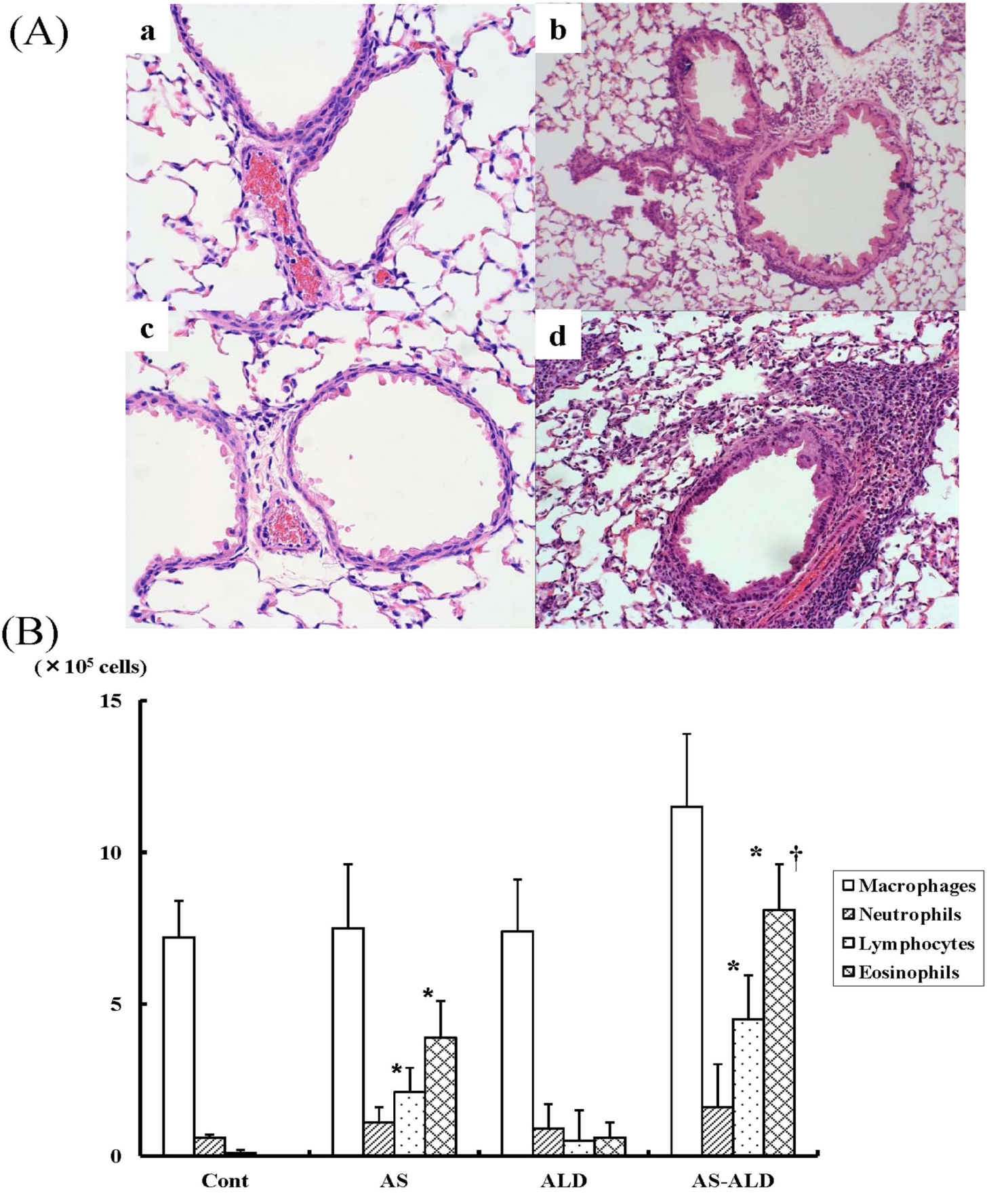
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Figure 3.

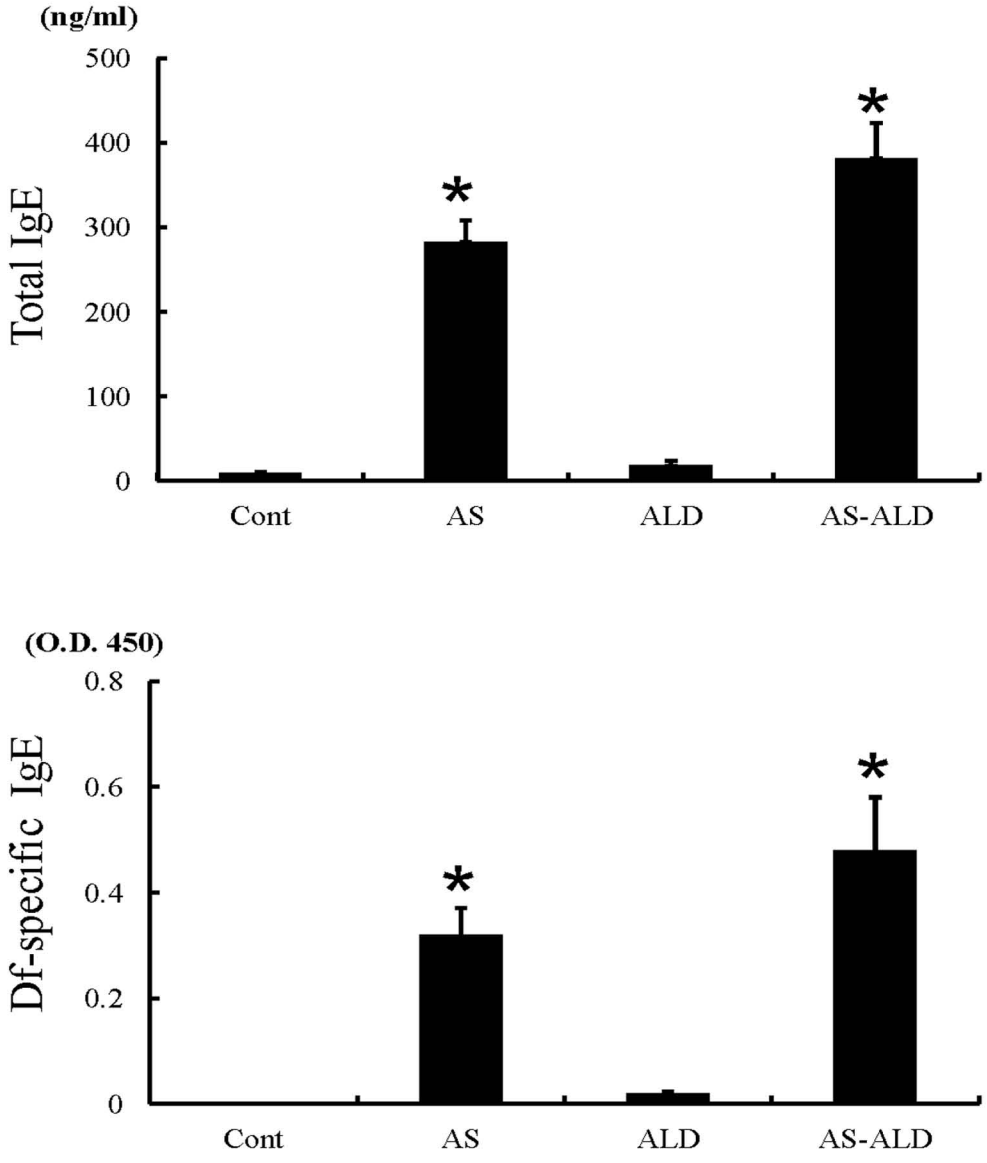
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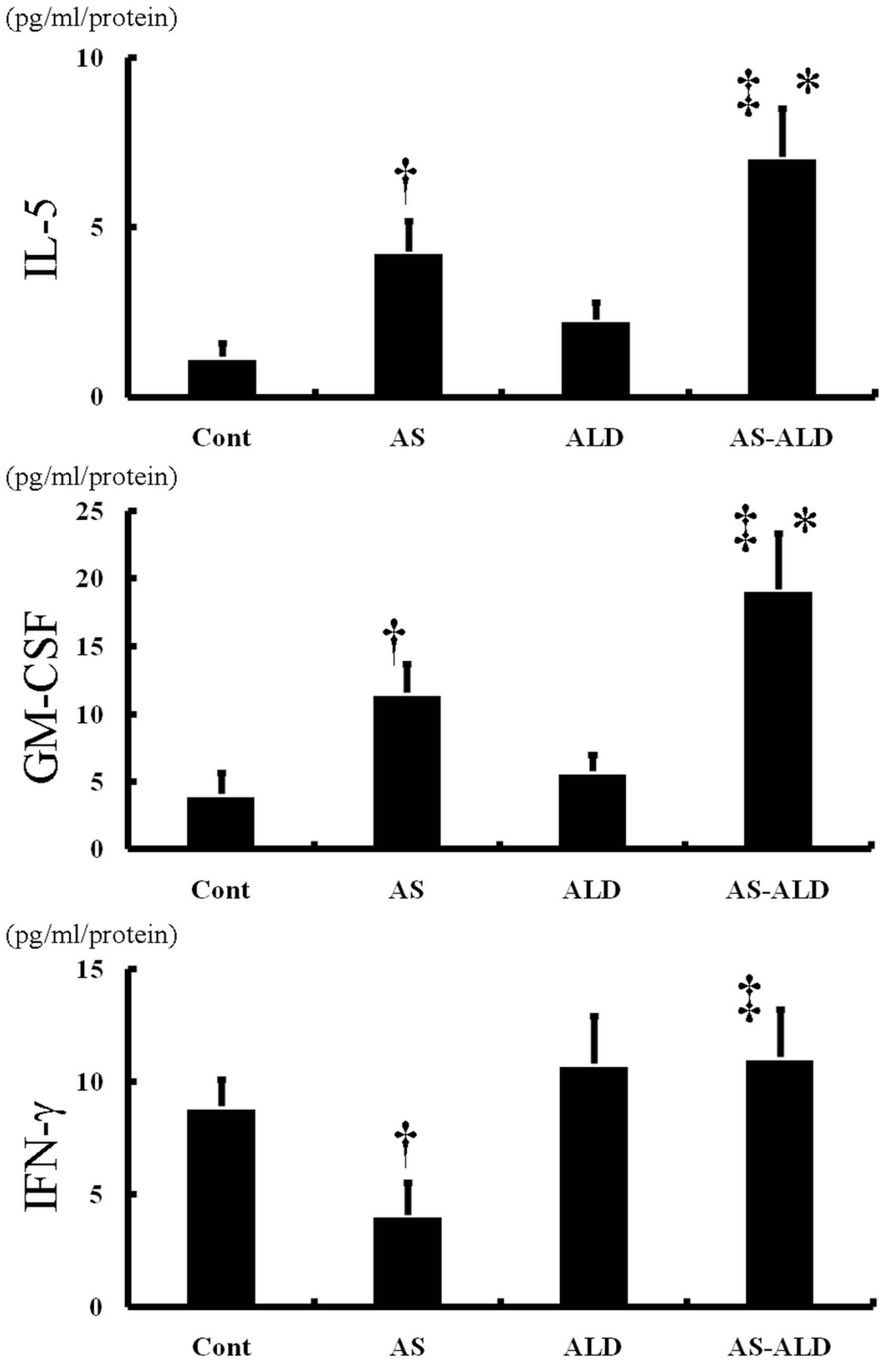
Figure 4.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