

RESEARCH ARTICLE

Aggravating effects of Asian sand dust on lung eosinophilia in mice immunized beforehand by ovalbumin

Miao He^{1,2}, Takamichi Ichinose¹, Seiichi Yoshida¹, Hirohisa Takano³, Masataka Nishikawa⁴, Ikuko Mori⁴, Guifan Sun², and Takayuki Shibamoto⁵

¹Department of Health Sciences, Oita University of Nursing and Health Sciences, Oita, Japan, ²Department of Environmental and Occupational Health, College of Public Health, China Medical University, Shenyang, China, ³Environmental Health Division, Department of Environmental Engineering, Graduate School of Engineering, Kyoto University, Kyoto Daigaku-Katsura, Nishikyo-Ku, Kyoto, Japan, ⁴Environmental Chemistry Division, National Institute for Environmental Studies, Tsukuba, Ibaraki, Japan, and ⁵Department of Environmental Toxicology, University of California, Davis, CA, USA

Abstract

Asian sand dust (ASD) event may result in a significant influence on an asthmatic patient. However, for obvious reasons, there is no experimental study in which asthmatic patients are exposed to ASD. This study was undertaken to clarify the effects of ASD on lung eosinophilia in mice immunized beforehand by ovalbumin (OVA). CD-1 mice were instilled intratracheally with OVA four times at 2-week intervals. Simultaneous intratracheal administration of OVA and ASD (OVA + ASD sim) at the last OVA treatment or intratracheal administration with ASD 1 day before (OVA + ASD pre) /after (OVA + ASD post) the last OVA treatment was performed to investigate the effects of OVA and ASD exposure timing. The three kinds of treatment (OVA + ASD pre; OVA + ASD sim; OVA + ASD post) aggravated allergic lung inflammation and proliferation of goblet cells in the airway epithelium in mice, as evidenced by the cellular profile of bronchoalveolar lavage fluid (BALF) and pathological examination. As an overall trend, these changes were paralleled with the expression of Th2-associated effector molecules and eosinophil relevant cytokine chemokines in BALF as well as the production of OVA-specific IgG1 compared with OVA treatment alone. OVA + ASD sim aggravated lung eosinophilia remarkably compared with the other treatments. The order of the potency of the aggravation was OVA+ASD pre < OVA+ASD post < OVA+ASD sim. These results indicate that ASD has a potent effect in activating lung eosinophilia in mice immunized beforehand by OVA. The simultaneous exposure of asthmatic patients to ASD and its antigen may have serious consequences for such individuals.

Keywords: Asian sand dust, asthmatic model, lung eosinophilia, ovalbumin, murine

Introduction

Asian sand dust (ASD) originates in the Gobi Desert and the Ocher Plateau in West China and is transported to East China, the Korean Peninsula, and Japan as well as across the Pacific Ocean to the United States (Duce et al., 1980; Husar et al., 2001; Kim et al., 2001). Moreover, it is reportedly transported one full circuit around the globe (Uno et al., 2009).

ASD contains various chemical species, including sulfate (SO_4^{2-}) and nitrate (NO_3^-) derived from air

pollutants (SO_2 , NO_2) and microbial agents, such as bacteria, fungi, fungal spores, and viruses (Chen et al., 2010; Kobayashi et al., 2010; Maki et al., 2010). Therefore, an ASD event may cause adverse effects on respiratory health, which in turn increases mortality and morbidity. Epidemiologic studies have demonstrated that ASD events have caused an increase in hospitalization for pneumonia in China (Meng and Lu, 2007). ASD is closely associated with an increase of daily mortality and causes respiratory and cardiovascular problems in Korea (Kwon

Address for Correspondence: Takayuki Shibamoto, Department of Environmental Toxicology, University of California, Davis, CA 95616, USA.
Tel: +1-530-752-4523. E-mail: tshibamoto@ucdavis.edu

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et al., 2002; Yoo et al., 2008) and Taiwan (Chen et al., 2004; Chen & Yang, 2005). In Japan, ASD has attracted attention as aerosol affecting patients with allergies. For example, there are reports on the exacerbation of Japanese cedar pollinosis and seasonal allergic rhinitis (Sato, 2009) as well as of adult asthma (Watanabe et al., 2011a, 2011b) occurring during a dust storm event. In Toyama, Japan Heavy ASD events also increase hospitalization of children ages 1–15 due to asthma attacks (Kanatani et al., 2010).

Recently, we have reported that ASD was correlated with cases of pneumonia induced by *Klebsiella Pneumoniae* (He et al., 2012). Our previous experimental studies used the mouse asthma model to demonstrate that intratracheal instillation of ASD and ovalbumin (OVA) to healthy mice caused significant aggravation of lung eosinophilia (Ichinose et al., 2008a, 2008b; He et al., 2010a). However, we have not studied the effect of ASD on asthmatic patients. Allergic patients would be exposed to ASD and the antigen in various timing sequences. For example, patients may simultaneously inhale ASD and the antigen into their lungs, or they may inhale ASD before/after exposure to the antigen.

There is no experimental study to examine the difference in aggravating effects of lung eosinophilia in relation to the timing of exposure to the ASD and antigen, even though an ASD occurrence may have a significant impact on patients with allergies. Therefore, experimental studies to confirm the epidemiological results, suggesting that ASD causes the aggravation of asthmatic symptoms in patients with asthma, are in order.

In the present study, one-time intratracheal instillation of ASD to the mice immunized beforehand by OVA was performed as experiment based on a hypothetical asthmatic patient. Simultaneous exposure to ASD and OVA at the last OVA treatment or exposure to ASD 1 day before/after the last OVA treatment was performed to investigate the effects of the timing of the ASD and OVA exposure on lung eosinophilia. The investigation included assessment of any pathologic changes in the mice lungs, cytological alteration in bronchoalveolar lavage fluid (BALF), and levels of inflammatory cytokines/chemokines in BALF and OVA-specific IgE and IgG1 antibodies in serum.

Materials and methods

Animals

A total of 75 male CD-1 mice (5 weeks of age) were purchased from Charles River Japan, Inc. (Kanagawa, Japan). After abnormal body weight and sick mice were examined for 1 week and removed from the pool of subjects, the remaining, healthy mice (72 mice) were used at 6 weeks of age. They were fed a commercial diet CE-2 (CLEA Japan, Inc., Tokyo) and given water ad libitum. Mice were housed in plastic cages lined with soft wood chips. The cages were placed in a room air conditioned at 23°C and 55–70% humidity with a light/dark (12h/12h) cycle. CD-1male mice were used because

of their moderate responsiveness to airway inflammation caused by OVA or mite allergen treatment (Ichinose et al., 1997; Ichinose et al., 2003). The study adhered to the U.S. National Institutes of Health Guidelines for the use of experimental animals. The animal care method was approved by the Animal Care and Use Committee at Oita University of Nursing and Health Sciences in Oita, Japan.

Asian sand dust particles

The present study used ASD previously collected from Iki-island, Japan on March 21st–22nd, 2002 after a massive 3-day dust storm event occurred in East Asia. As previously reported, the chemical composition of this ASD sample was 61.8% SiO₂, 13.6% Al₂O₃, 5.7% Fe₂O₃, 5.4% CaO, 3.3% MgO, 0.01% TiO₂, and 2.6% K₂O. The size distribution peak of ASD was observed at 4.7 µm (He et al., 2010a).

Study protocol

CD-1mice were divided into six groups ($n = 12$, each group) according to the treatment with particles as Figure 1: (1) Control: intratracheal instillation with 0.1 mL of normal saline per mouse four times at 2-week intervals; (2) ASD: intratracheal administration with normal saline three times at 2-week intervals and ASD at the last administration; (3) OVA: intratracheal treatment with OVA four times at 2-week intervals; (4) OVA + ASD_{pre}: intratracheal treatment with OVA four times at 2-week intervals, and intratracheal administration with ASD 1 day before the last OVA treatment; (5) OVA + ASD_{sim}: intratracheal treatment with OVA four times at 2-week intervals, and simultaneous intratracheal administration with ASD + OVA at the last OVA treatment; (6) OVA

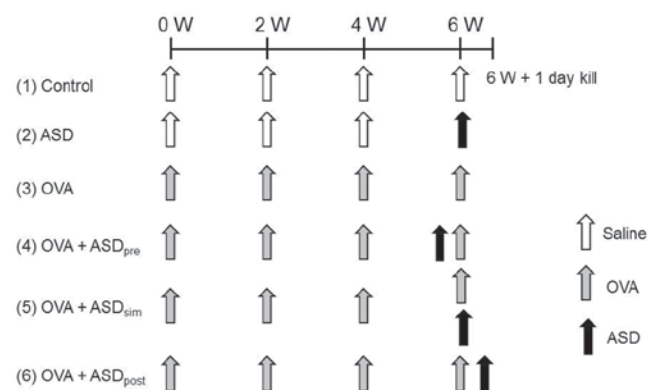


Figure 1. Experimental protocol. (1) Control: instilled intratracheally with 0.1 mL of normal saline per mouse four times at 2-week intervals; (2) ASD: intratracheal treatment with normal saline three times at 2-week intervals and ASD once at the last treatment; (3) OVA: intratracheal instillation with OVA four times at 2-week intervals; (4) OVA + ASD_{pre}: intratracheal delivered with OVA four times at 2-week intervals, and intratracheal administration with ASD on the day before the last treatment of OVA; (5) OVA + ASD_{sim}: intratracheal inhalation with OVA four times at 2-week intervals, and intratracheal administration with ASD at the same time with the last treatment of OVA; (6) OVA + ASD_{post}: intratracheal treatment with OVA four times at 2-week intervals, and intratracheal administration with ASD on the day after the last treatment of OVA.

+ ASD_{post}: intratracheal treatment with OVA four times at 2-week intervals, and intratracheal administration with ASD 1 day after the last OVA treatment. The ASD particles were suspended in normal saline (0.9% NaCl) for instillation (Otsuka Co, Kyoto, Japan). This suspension was sonicated for 5 min with an ultrasonic disrupter, UD-201 type with micro tip (Tomy, Tokyo, Japan), under cooling conditions. OVA was dissolved in the same saline. The instillation dose of ASD was 0.2 mg per mouse and the treatment dose of OVA was 3 µg per mouse (Ichinose et al., 2008a, 2008b; He et al., 2010a, 2010b). Mice were intratracheally instilled with these particles through a polyethylene tube under anesthesia with 4% halothane (Takeda Chemical, Osaka, Japan). One day after the last intratracheal administration, the mice from all groups (age = 12 weeks) were killed by exsanguination under deep anesthesia by intraperitoneal injection of pentobarbital.

Pathological evaluation

Six out of the 12 mice from each group were used for a pathologic examination. The lungs were fixed by 10% neutral phosphate-buffered formalin. After separation of the lobes, 2 mm thick blocks were taken for paraffin embedding. Embedded blocks were sectioned at a thickness of 3 µm, and then were stained with hematoxylin and eosin (H&E) to evaluate the degree of infiltration of eosinophils or lymphocytes in the airway from proximal to distal. The sections were also stained with periodic acid-Schiff (PAS) to evaluate the degree of proliferation of goblet cells in the bronchial epithelium. A pathological analysis of the inflammatory cells and epithelial cells in the airway of each lung lobe on the slides was performed using a Nikon ECLIPSE light microscope (Nikon Co, Tokyo, Japan). The degree of proliferation of goblet cells in the bronchial epithelium was graded on the following scale: 0, not present; 1, slight; 2, mild; 3, moderate; 4, moderate to marked; and 5, marked. "Slight" was defined as less than 10% of the airway with goblet cells stained with PAS; "mild" as 20–30%; "moderate" as 40–50%; "moderate to marked" as 60–70%; and "marked" as more than 80% (Ichinose et al., 2008a, 2008b; He et al., 2010a, 2010b). Pathological changes were assessed on one slide stained with PAS per mouse. This evaluation procedure was performed by two pathologists who cross-checked the data in blinded specimens. The correlation value between the two pathologists was $r = 0.967$, $p < 0.001$ ($n = 108$). All values were expressed as mean $\pm$ SE ($n = 6$).

Bronchoalveolar lavage fluid

The remaining six mice were used for an examination of the free cell contents from BALF. BALF and cell counts were conducted using a previously reported method (Ichinose et al., 2006; 2008a, 2008b; He et al., 2010a, 2010b). In brief, the tracheas were cannulated after the collection of blood. The lungs were lavaged with two injections of 0.8 mL of sterile saline at 37°C by syringe. The lavaged fluid was harvested by gentle aspiration. The

average volume retrieved was 90% of the amount instilled (1.6 mL). The fluids from the two lavages were pooled, cooled to 4°C, and centrifuged at 1500 rpm for 10 min. The total amount of lavages collected from individual mice was used in order to measure the protein levels of cytokines and chemokines in the BALF. The total cell count of a fresh fluid specimen was determined using a hemocytometer. Differential cell counts were assessed on cytologic preparations. Slides were prepared using a Cytospin (Sakura Co, Ltd, Tokyo, Japan) and stained with Diff-Quik (International Reagents Co, Kobe, Japan). A total of 300 cells were counted under oil immersion microscopy. The BALF supernatants were stored at –80°C until analyzed for cytokines and chemokines.

Quantitation of cytokines and chemokines in BALF

The cytokine protein levels in the BALF were determined using enzyme-linked immunosorbent assays (ELISA). Interleukin (IL)-1 β , IL-4, IL-6, IL-13, interferon (IFN)- γ , eotaxin, keratinocyte chemoattractant (KC), monocyte chemoattractant protein (MCP)-1, and macrophage inflammatory protein (MIP)-1 α were measured using an ELISA kit from R&D Systems Inc. (Minneapolis, MN). IL-5, IL-12 were measured using an ELISA kit from Endogen (Cambridge, MA). MCP-3 was measured using an ELISA kit from Bender MedSystems (Burlingame, CA).

Antigen-specific IgE and IgG1 antibodies

OVA-specific IgE and IgG1 antibodies were measured using a Mouse OVA-IgE ELISA kit and a Mouse OVA-IgG1 ELISA kit (Shibayagi Co, Shibukawa, Japan). According to the manufacturer's protocol, 1 U of the anti-OVA IgE is defined as 1.3 ng of the antibody, and 1 U of the anti-OVA IgG1 as 160 ng of the antibody. The absorption at 450 nm (sub-wave length, 620 nm) for OVA-specific IgE and IgG1 antibodies was measured using a microplate reader (Spectrafluor, Tecan, Salzburg, Austria).

Statistical analysis

Statistical analyses of the pathologic evaluation in the airway, cytokine and chemokine proteins in BALF were conducted using the Tukey Test for Pairwise Comparisons (KyPlot Ver.5, Kyens Lab Inc, Tokyo, Japan; <http://www.kyenslab.com/en/>). Differences among groups were determined as statistically significant at a level of $p < 0.05$. The correlation between cytokine/chemokine and cell numbers in BALF was performed using the correlation coefficient test (KyPlot Ver.5, Kyens Lab Inc, Tokyo, Japan).

Results

Enhancement of cell numbers in BALF by ASD

To estimate the effects of ASD on the magnitude of airway inflammation caused by OVA, the cellular profile of BALF was examined (Figure 2). ASD alone increased the number of neutrophils ($p < 0.01$) and total cells ($p < 0.001$) as compared with the control. OVA alone increased the number of

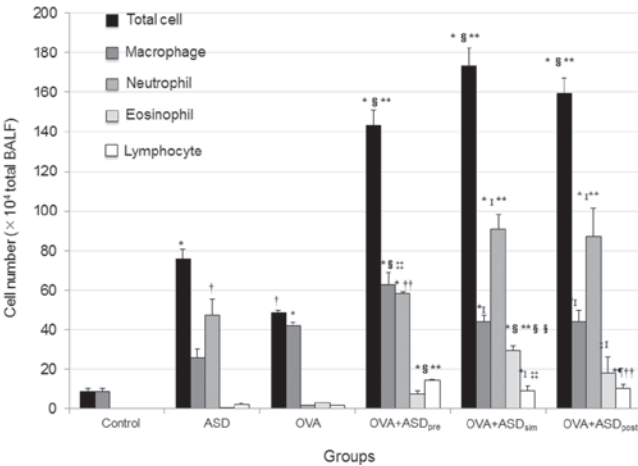


Figure 2. Cellular profile in bronchoalveolar lavage fluid (BALF). All values expressed as mean $\pm$ SE. * $p < 0.001$ vs. Control, $^{\dagger}p < 0.01$ vs. Control, $^{\ddagger}p < 0.05$ vs. Control, $^{\S}p < 0.001$ vs. ASD, $^{\P}p < 0.01$ vs. ASD, $^{\text{I}}p < 0.05$ vs. ASD, $^{**}p < 0.001$ vs. OVA, $^{++}p < 0.01$ vs. OVA, $^{++}p < 0.05$ vs. OVA, $^{SS}p < 0.01$ vs. OVA + ASD_{pre}, $^{**}p < 0.05$ vs. OVA + ASD_{pre}.

macrophages ($p < 0.001$) and total cells ($p < 0.01$) as compared with the control. There was a marked increase in the number of eosinophils in OVA + ASD_{sim} group as compared with the control ($p < 0.001$), OVA ($p < 0.001$), ASD alone ($p < 0.001$) and OVA + ASD_{pre} ($p < 0.01$). OVA + ASD_{post} also increased the number of eosinophils as compared with the control ($p < 0.05$) and ASD alone ($p < 0.05$). The number of eosinophils was greater in the OVA + ASD_{sim} group than in that of the OVA + ASD_{post} group. However, the number of eosinophils in the OVA + ASD_{pre} group did not increase.

OVA + ASD_{pre} treatment significantly increased the number of neutrophils in BALF as compared with the control ($p < 0.001$) and OVA ($p < 0.01$). Furthermore, the combined instillation of OVA + ASD_{sim} and OVA + ASD_{post} resulted in a further significant increase in neutrophils in BALF as compared with the control ($p < 0.001$), OVA ($p < 0.05$) and ASD alone ($p < 0.001$). In addition, OVA + ASD_{pre} treatment increased the number of macrophages in BALF as compared with the control ($p < 0.001$), OVA ($p < 0.05$) and ASD alone ($p < 0.001$); OVA + ASD_{sim} and OVA + ASD_{post} treatment also increased the number of macrophages as compared with the control ($p < 0.001$) and ASD alone ($p < 0.05$). Intratracheal instillation of OVA and ASD resulted in a significant increase in the infiltration of lymphocytes as compared with the control ($p < 0.001$), OVA ($p < 0.05$) and ASD alone ($p < 0.05$). The combined exposure to intratracheal inhalation of OVA and ASD induced a significant increase in total cells in BALF as compared with the control and OVA or ASD alone ($p < 0.001$).

Enhancement of pathologic changes in the airway by ASD

To determine the effects of ASD on lung pathology-related exposure, the lung specimens stained with PAS and HE were evaluated. Table 1 shows the pathologic changes caused by the ASD in the murine airway, and Figures 3 and 4 illustrate the effects of the ASD on pathological changes in the lungs.

Table 1. The evaluation of pathologic changes in the murine airway.

Group*	Animals (n)	Pathological changes		
		Lymphocytes	Eosinophils	Proliferation of goblet cells
Control	6	ND	ND	ND
ASD	6	1.08 $\pm$ 0.35	ND	0.17 $\pm$ 0.17
OVA	6	1.08 $\pm$ 0.30	0.42 $\pm$ 0.20	1.25 $\pm$ 0.46
OVA + ASD _{pre}	6	1.83 $\pm$ 0.48 [§]	1.25 $\pm$ 0.44 ^{†,††}	1.67 $\pm$ 0.46 ^{†,††}
OVA + ASD _{sim}	6	2.50 $\pm$ 0.29 [†]	2.25 $\pm$ 0.31 ^{†,†,††}	2.75 $\pm$ 0.31 ^{†,†,††}
OVA + ASD _{post}	6	2.00 $\pm$ 0.42 [§]	1.70 $\pm$ 0.37 ^{§,††,††}	2.30 $\pm$ 0.41 ^{†,††}

*The control group was instilled intratracheally with 0.1 mL of normal saline four times at 2-week intervals. The ASD alone group was intratracheally instilled with 0.2 mg ASD per mouse four times at 2-week intervals. The OVA alone group was intratracheally instilled with 3 μ g OVA per mouse four times at 2-week intervals. The OVA + ASD_{pre} group was intratracheal delivered with OVA four times at 2-week intervals, and intratracheal administration with ASD on the day before the last treatment of OVA; The OVA + ASD_{sim} group was intratracheal inhalation with OVA four times at 2-week intervals, and intratracheal administration with ASD at the same time with the last treatment of OVA; The OVA + ASD_{post} was intratracheal treatment with OVA four times at 2-week intervals, and intratracheal administration with ASD on the day after the last treatment of OVA. All values were expressed as mean $\pm$ SE.

[†] $p < 0.001$ versus Control.

[§] $p < 0.01$ versus Control.

^{††} $p < 0.05$ versus Control.

^{†††} $p < 0.001$ versus ASD.

^{††††} $p < 0.01$ versus ASD.

^{†††††} $p < 0.05$ versus ASD.

^{††††††} $p < 0.001$ versus OVA.

^{†††††††} $p < 0.05$ versus OVA.

No pathologic alterations were found in the lungs of the control group (Figures 3A and 4A). The single treatment with ASD alone caused slight bronchitis along with proliferation of bronchial cells and alveolitis with neutrophilic inflammation (Figures 3B and 4B). The treatment with OVA alone caused very slight goblet cell proliferation in the bronchial epithelium (Figure 3C) along with very slight infiltration of eosinophils in the submucosa of the airway (Figure 4C). OVA + ASD_{pre} caused mild increased proliferation of goblet cells in the bronchial epithelium (Figure 3D) and eosinophil infiltration in the submucosa (Figure 4D). OVA + ASD_{pre} caused a significant increase in goblet cell proliferation compared with the control ($p < 0.05$), ASD ($p < 0.05$), and eosinophil infiltration compared with the control ($p < 0.05$), ASD ($p < 0.05$) (Table 1). OVA + ASD_{post} caused mild to moderate increased proliferation of goblet cells (Figure 3F) and eosinophil infiltration in the submucosa (Figure 4F). This treatment caused a significant increase in goblet cell proliferation compared with the control ($p < 0.001$), ASD ($p < 0.01$), and eosinophil infiltration compared with the control ($p < 0.01$), ASD ($p < 0.05$) and OVA ($p < 0.001$) (Table 1). However, OVA + ASD_{sim} caused moderate to marked increased proliferation of goblet cells (Figure 3F) and eosinophil infiltration in the submucosa (Figure 4F). This treatment caused a significant increase of goblet cell proliferation as compared with the control ($p < 0.001$),

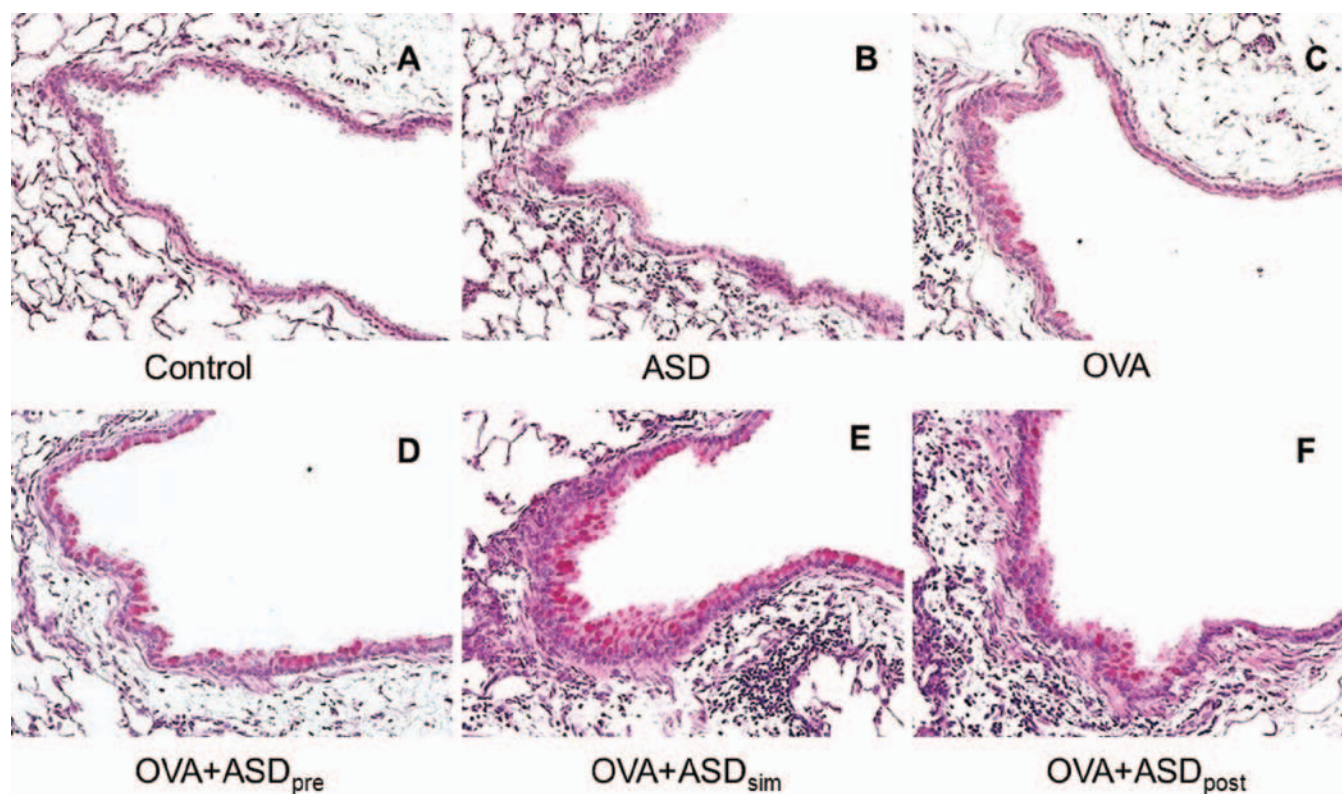


Figure 3. Effects of ASD on pathological changes in the lungs. (A) No pathological changes in lungs treated with saline. (B) Slight bronchitis along with slight proliferation of bronchial cells. (C) Slight proliferation of goblet cells that have mucus stained pink with PAS solution in the airway epithelium treated with OVA alone. (D) Mild proliferation of goblet cells and slight infiltration of inflammatory cells into connective tissue around the airway treated with OVA + ASD_{pre}. (E) Marked proliferation of goblet cells and numerous inflammatory cells in the airway treated with OVA + ASD_{sim}. (F) Moderate proliferation of goblet cells in the airway and moderate infiltration of inflammatory cells into connective tissue around the airway treated with OVA + ASD_{post}. (A–F) PAS stain. (See colour version of this figure online at www.informahealthcare.com/ihf)

ASD ($p < 0.001$) and OVA ($p < 0.05$), and eosinophil infiltration as compared with the control ($p < 0.001$), ASD ($p < 0.001$) and OVA ($p < 0.001$) (Table 1). Combination treatments caused moderate to marked accumulation of lymphocytes in the airway (Figure 4D, 4E and 4F). The degree of the accumulation was higher in OVA + ASD_{sim} than in that of the other two groups (OVA + ASD_{pre}; OVA + ASD_{post}) (Table 1).

Enhancement of cytokines and chemokines in BALF by ASD

To investigate the effects of ASD on the expression of cytokines caused by OVA, the protein levels of IL-1 β , IL-4, IL-5, IL-6, IL-12, IL-13, and IFN- γ in BALF were measured (Table 2). The single treatment of ASD alone increased the expression of IL-1 β , IL-6 and IL-12 as compared with the control ($p < 0.001$). Both OVA + ASD_{sim} and OVA + ASD_{post} induced an increase in IL-1 β and IL-6 as compared with the control and OVA alone ($p < 0.001$), but these levels were almost the same as ASD alone. Only OVA + ASD_{sim} showed any significant elevation of Th2 cytokines such as IL-5 and IL-13 in BALF compared to the other groups ($p < 0.05$). OVA + ASD_{pre} caused a significant increase in Th1 cytokine —IL-12 compared with ASD alone ($p < 0.001$), but the level of IL-12 in the OVA + ASD_{sim} group was lower than that of the ASD-alone group.

In addition, there was no significant increase of Th1 cytokine —IFN- γ , which tended to increase in the OVA + ASD_{pre} group. To characterize the effects of ASD on the expression of chemokines caused by OVA, the presence of eotaxin, KC, MCP-1, MCP-3 and MIP-1 α were assayed (Table 3). ASD alone caused an increase of neutrophil relevant chemokines, such as KC and MIP-1 α in BALF as compared with the control ($p < 0.001$). The expression of eosinophil relevant chemokines, such as eotaxin and MCP-3, was found more in the OVA + ASD_{sim} group than in any of the other groups ($p < 0.05$). Combined administration of OVA and ASD exacerbated the expression of MCP-1 as compared with the control, and OVA alone ($p < 0.05$), and the level of OVA + ASD_{sim} group was greater than that of the OVA + ASD_{pre} group and the OVA + ASD_{post} group. Combined administration of OVA + ASD_{sim} and OVA + ASD_{post} markedly increased the expression of KC and MIP-1 α as compared with the control, ASD and OVA alone ($p < 0.05$).

Enhancement of OVA-specific antibody in serum by ASD

To confirm the effects of ASD on antigen-induced Ig production, the expression of OVA-specific IgE and IgG1 was performed (Figure 5). As shown in Figure 5, combined treatment of OVA and ASD tended to increase

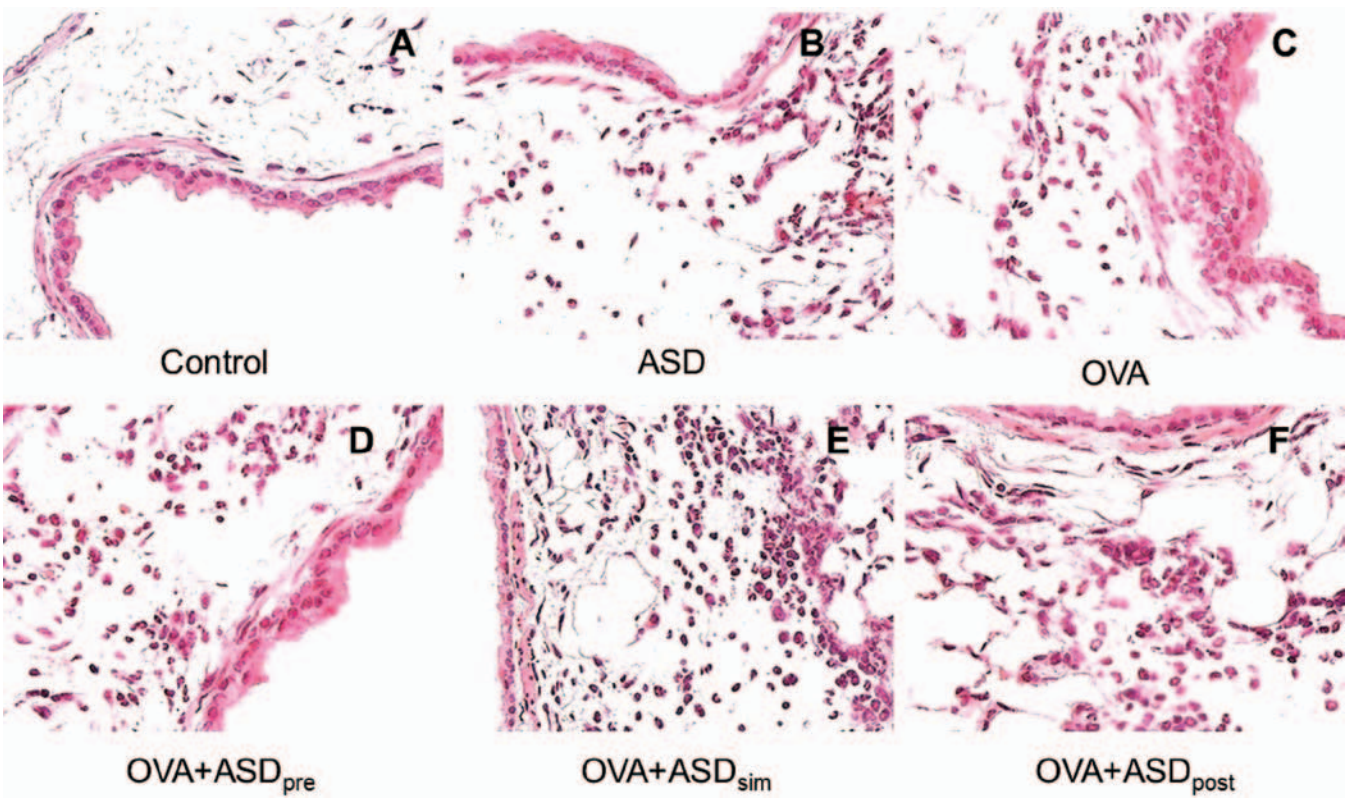


Figure 4. Effects of ASD on infiltration of inflammatory cells in the airway. (A) No pathological changes treated with saline. (B) Slight infiltration of neutrophils into the airway submucosa treated with ASD alone. (C) Slight infiltration of eosinophils and lymphocytes into the airway submucosa treated with OVA alone. (D) Slight to moderate infiltration of eosinophils and lymphocytes into the airway submucosa treated with OVA + ASD_{pre}. (E) Moderate to marked infiltration of eosinophils and lymphocytes into connective tissue in the airway treated with OVA + ASD_{sim}. (F) Moderate infiltration of eosinophils and lymphocytes into connective tissue in the airway treated with OVA + ASD_{post}. (A–F) H&E stain. (See colour version of this figure online at www.informahealthcare.com/ihf)

Table 2. Expression of cytokines in bronchoalveolar lavage fluid.

Group*	Animals (n)	Cytokines (pg protein/total BAL supernatants)						
		IL-1 β	IL-4	IL-5	IL-6	IL-12	IL-13	IFN- γ
Control	6	ND	ND	ND	ND	26.0 $\pm$ 2.10	ND	ND
ASD	6	335 $\pm$ 26.0 [†]	ND	ND	90.0 $\pm$ 9.73 [†]	615 $\pm$ 55.1 [†]	ND	5.90 $\pm$ 5.90
OVA	6	ND	ND	27.4 $\pm$ 13.4	ND	64.2 $\pm$ 7.33	1.67 $\pm$ 1.67	ND
OVA+ASD _{pre}	6	204 $\pm$ 39.5 [§]	ND	24.5 $\pm$ 11.8	35.3 $\pm$ 4.10	1769 $\pm$ 104 ^{†,¶,}	3.98 $\pm$ 2.52	20.6 $\pm$ 10.9
OVA+ASD _{sim}	6	403 $\pm$ 98.2 ^{†,¶}	ND	92.7 $\pm$ 19.0 ^{†,¶, ,¶¶,¶¶}	75.5 $\pm$ 14.3 ^{†,¶, ,¶¶}	514 $\pm$ 105 ^{†,¶, ,¶¶,***}	23.4 $\pm$ 6.10 ^{†,¶, ,¶¶,¶¶}	5.13 $\pm$ 5.13
OVA+ASD _{post}	6	312 $\pm$ 43.5 ^{†,¶¶}	ND	31.7 $\pm$ 9.56	95.5 $\pm$ 16.9 ^{†,}	860 $\pm$ 108 ^{†,¶,}	5.79 $\pm$ 2.54	5.00 $\pm$ 5.00

*The control group was instilled intratracheally with 0.1 mL of normal saline four times at 2-week intervals. The ASD alone group was intratracheally instilled with 0.2 mg ASD per mouse four times at 2-week intervals. The OVA alone group was intratracheally instilled with 3 μ g OVA per mouse four times at 2-week intervals. The OVA + ASD_{pre} group was intratracheal delivered with OVA four times at 2-week intervals, and intratracheal administration with ASD on the day before the last treatment of OVA; The OVA + ASD_{sim} group was intratracheal inhalation with OVA four times at 2-week intervals, and intratracheal administration with ASD at the same time with the last treatment of OVA; The OVA + ASD_{post} was intratracheal treatment with OVA four times at 2-week intervals, and intratracheal administration with ASD on the day after the last treatment of OVA. All values were expressed as mean $\pm$ SE.

[†] p < 0.001 versus Control.

[§] p < 0.05 versus Control.

[¶] p < 0.001 versus ASD.

^{||} p < 0.001 versus OVA.

^{¶¶} p < 0.01 versus OVA.

^{***} p < 0.001 versus OVA + ASD_{pre}.

^{¶¶¶} p < 0.01 versus OVA + ASD_{sim}.

^{¶¶¶¶} p < 0.05 versus OVA + ASD_{post}.

OVA-specific IgG1, and OVA + ASD_{sim} markedly elevated the production of OVA-specific IgG1 compared with the control and ASD (p < 0.05). Combined administration of OVA and ASD did not increase the production of OVA-specific IgE.

Correlation between cytokine/chimokine and cellular response

To clarify the pattern of inflammatory response with each treatment (OVA+ASD_{pre}; OVA+ASD_{sim}; OVA+ASD_{post}), correlation analysis between cytokine/chimokine and

Table 3. Expression of chemokines in bronchoalveolar lavage fluid.

Group*	Animals (n)	Cytokines (pg protein/total BAL supernatants)				
		Eotaxin	KC	MCP-1	MCP-3	MIP-1 α
Control	6	ND	30.1 $\pm$ 4.05	ND	ND	ND
ASD	6	3.54 $\pm$ 0.39	584 $\pm$ 62.5 [†]	176 $\pm$ 31.6	33.5 $\pm$ 6.06	102 $\pm$ 10.2 [†]
OVA	6	3.94 $\pm$ 1.86	68.7 $\pm$ 12.8	5.52 $\pm$ 3.63	4.09 $\pm$ 1.82	ND
OVA+ASD _{pre}	6	8.53 $\pm$ 2.41	240 $\pm$ 40.4	544 $\pm$ 124 ^{†,§§}	296 $\pm$ 112	36.6 $\pm$ 5.44 [§]
OVA+ASD _{sim}	6	21.1 $\pm$ 5.34 ^{†,††,§§,††}	640 $\pm$ 79.8 ^{†,¶,***}	1020 $\pm$ 166 ^{†,††,¶,††}	864 $\pm$ 221 ^{†,††,¶,††,¶¶}	103 $\pm$ 10.9 ^{†,¶,***}
OVA+ASD _{post}	6	10.3 $\pm$ 2.54	603 $\pm$ 88.9 ^{†,¶,†††}	761 $\pm$ 125 ^{†,††,¶}	301 $\pm$ 60.2	72.7 $\pm$ 9.36 ^{†,¶,††}

*The control group was instilled intratracheally with 0.1 mL of normal saline four times at 2-week intervals. The ASD alone group was intratracheally instilled with 0.2 mg ASD per mouse four times at 2-week intervals. The OVA alone group was intratracheally instilled with 3 μ g OVA per mouse four times at 2-week intervals. The OVA + ASD_{pre} group was intratracheal delivered with OVA four times at 2-week intervals, and intratracheal administration with ASD on the day before the last treatment of OVA; The OVA + ASD_{sim} group was intratracheal inhalation with OVA four times at 2-week intervals, and intratracheal administration with ASD at the same time with the last treatment of OVA; The OVA + ASD_{post} was intratracheal treatment with OVA four times at 2-week intervals, and intratracheal administration with ASD on the day after the last treatment of OVA. All values were expressed as mean $\pm$ SE.

[†] $p < 0.001$ versus Control.

^{††} $p < 0.01$ versus Control.

[§] $p < 0.05$ versus Control.

^{†††} $p < 0.001$ versus ASD.

[¶] $p < 0.001$ versus OVA.

^{§§} $p < 0.01$ versus OVA.

^{***} $p < 0.001$ versus OVA + ASD_{pre}.

^{††††} $p < 0.01$ versus OVA + ASD_{pre}.

^{†††††} $p < 0.05$ versus OVA + ASD_{pre}.

^{¶¶} $p < 0.01$ versus OVA + ASD_{post}.

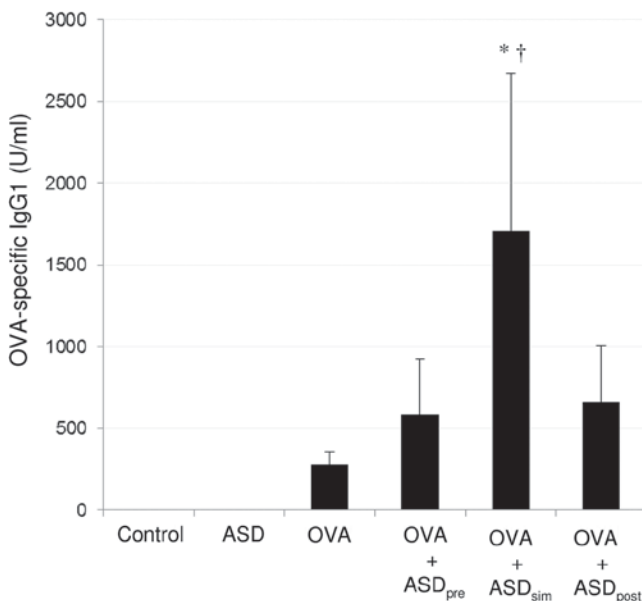


Figure 5. Effect of ASD on OVA-specific IgG1 production in serum. According to the manufacturer's protocol, 1 U of the anti-OVA IgG1 as 160 ng of the antibody. Results are expressed as mean $\pm$ SE. * $p < 0.05$ versus Control, [†] $p < 0.05$ versus ASD.

cellular response in BALF were performed. The amount of IL-5 and Eotaxin were significantly correlated with the number of eosinophils (Figure 6 A: $r = 0.6598$, $p < 0.01$; Figure 6 B, $r = 0.5107$, $p < 0.05$, $n = 18$). The amount of MCP-3 also was significantly correlated with the number of eosinophils ($r = 0.692$, $p < 0.01$, $n = 18$). The amount of IL-12 showed a significant reverse correlation with the number of eosinophils (Figure 6 C, $r = -0.690$, $p < 0.01$, $n = 18$). The amount of IL-12 showed a significant reverse

correlation with IL-13 (Figure 6 D, $r = -0.6083$, $p < 0.01$, $n = 18$). The amount of KC showed no significant correlation with the number of neutrophils ($n = 18$), but KC and neutrophils showed a significant correlation with each other when data of the ASD alone group was added ($r = 0.443$, $p < 0.05$, $n = 24$).

Discussion

ASD may result in aggravation of asthmatic symptoms in patients with child asthma or adult asthma, when those patients are exposed to allergens and ASD in various sequences. In the present study, the aggravating effects of ASD on lung eosinophilia in mice immunized beforehand by OVA were examined as the experiment presupposing an asthmatic condition. In addition, the effects of ASD and OVA exposure timing on lung eosinophilia were also investigated.

The present study has demonstrated that ASD has a potent activating effect on allergic inflammation in mice immunized beforehand by antigens. The effect was, moreover, dependent on the timing of the exposure to ASD relative to the treatment with OVA. The degree of the aggravation of lung eosinophilia was OVA + ASD_{pre} < OVA + ASD_{post} < OVA + ASD_{sim}. The ASD-only one-time treatment caused bronchitis and alveolitis, and clearly increased neutrophils along with its relevant chemokines MIP-1 α (Standiford et al., 1995), KC (Akahoshi et al., 1994), Th-1 trigger cytokine IL-12 (Hsieh et al., 1993), and Th-1 cytokine IFN- γ (Adamthwaite & Cooley, 1994) in BALF, in line with ASD-only four-time treatments reported in previous studies (Ichinose et al., 2008a, 2008b; He et al., 2010a).

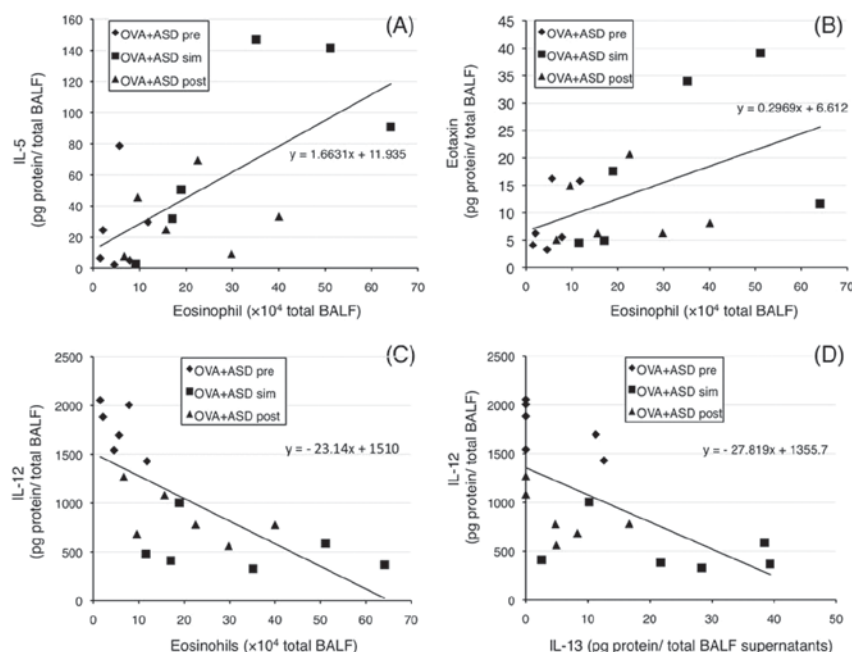


Figure 6. Correlation between cytokine/chimokine and cellular response. (A) Correlation between IL-5 and Eosinophils ($r = 0.6598$, $p < 0.01$). (B) Correlation between Eotaxin and Eosinophils ($r = 0.5107$, $p < 0.05$, $n = 18$). (C) Correlation between IL-12 and Eosinophils ($r = -0.690$, $p < 0.01$, $n = 18$). (D) Correlation between IL-12 and IL-13 ($r = -0.6083$, $p < 0.01$, $n = 18$).

Eosinophils, Th2 lymphocytes and their released inflammatory mediators play a crucial role in human allergic asthma (Pearlman, 1999). IL-5, generated from Th2 cells attracts and activates eosinophils, which are implicated in tissue destruction in allergic asthma (Kidd, 2003). Because toxic eosinophil derived proteins, like most major basic proteins, can cause bronchial mucosal damage in asthmatic airways that may contribute to the symptoms of asthma (Bradley et al., 1991).

Interleukin-4, IL-5, and IL-13 released from Th-2 lymphocytes are well known to stimulate B cells and to lead to the production of antigen specific antibodies (Mossman & Coffman, 1989; Kühn et al., 1991). IL-13 promotes mucous secretion and production of mucous cells such as goblet cells in the bronchial epithelium (Tesfaigzi, 2008). In this study, the three kinds of treatment (OVA + ASD_{pre}; OVA + ASD_{sim}; OVA + ASD_{post}) aggravated allergic inflammation and proliferation of goblet cell in the airway epithelium in mice, which is evidenced by the cellular profiles of BALF and pathological examination. As an overall trend, these changes were paralleled by the expression of Th2-associated effector molecules and eosinophil relevant cytokine chemokines in BALF as well as the production of OVA-specific IgG1. As shown in Figure 6, a significant correlation was observed between IL-5 and eosinophils (A) and between Eotaxin and eosinophils (B) in BALF.

OVA + ASD_{pre} caused fewer pathological changes like eosinophils and goblet cells in the airway and numbers of neutrophils and eosinophils in BALF compared with other treatments. Regarding the induction of cytokines and chemokine, OVA + ASD_{pre} also caused lower induction of neutrophil relevant inflammatory mediators, KC,

MIP-1 α , IL-6 and IL-1 β , and eosinophil relevant inflammatory mediators, IL-5 (Robinson et al., 1993; Foster et al., 1996), eotaxin (Rothenberg et al., 1997) and MCP-3 (Nagase et al., 1999) in BALF compared with other treatments. In contrast, OVA + ASD_{pre} caused higher induction of IFN- γ and IL-12 in BALF than other treatments. The Th1 cytokine IFN- γ is well known to act as a suppressor of the production of Th-2 cytokines (IL-5, IL-13) and to inhibit antigen-induced eosinophil recruitment (Iwamoto et al., 1993). Therefore, the expressions of these inflammatory mediators in the OVA + ASD_{pre} group may be suppressed by the increased IFN- γ . The induction of IFN- γ may weaken allergic reactions induced by post treatment of OVA. The Th-1 relevant cytokine IL-12, through its capacity to induce Th-1 cells, may be capable of weakening allergic responses by OVA. Previous studies have shown that systemic administration of IL-12 abolishes the airway hyperreactivity and pulmonary eosinophilia in a mouse model of allergic asthma (Gavett et al., 1995; Kips et al., 1996). In this study, a significant negative correlation was observed between IL-12 and eosinophils (Figure 6C) and between IL-12 and IL-13 (Figure 6D) in BALF. Thus, OVA + ASD_{pre} is influenced by the Th-1 relevant immune response triggered by ASD pretreatment. Regarding the remarkable induction of IL-12 in OVA+ASD_{pre}, OVA could promote the release of IL12 from antigen presenting cells activated by the ASD pretreatment. However, the response mechanism is not clear at this time.

OVA + ASD_{post} can elicit allergic reactions in mice immunized beforehand by antigen, and the pro-inflammatory mediator inducing ability was higher than OVA + ASD_{pre}. The ASD post treatment can induce Th-2

cytokines by the lower induction of Th-1-associated cytokines, IL-12 and IFN- γ than in the case of OVA + ASD_{pre}. The Th-2 predominant environment created by the four-time pretreatment with OVA may diminish Th-1 cytokine production. It seems that the pathological changes in the OVA + ASD_{post} group appeared more strongly than in the OVA + ASD_{pre} group in terms of this phenomenon. This result indicates that more serious illness develops when an asthmatic is subjected to ASD exposure. Interestingly, the aggravating effects were more remarkable in the OVA + ASD_{sim} group and the induction of Th-2 and eosinophil relevant cytokines and chemokines, and eosinophil number in BALF were two to three times higher than the OVA + ASD_{post} group. The simultaneous administration of OVA and ASD may be able to induce allergic inflammation without the influence of pre- or post-treatment of ASD. In a previous study, we have reported that microbial materials such as β -glucan adsorbed onto ASD may contribute to the exacerbation of lung eosinophilia related to allergen *via* the activation of TLR2, which is a receptor for zymosan (β -glucan), because ASD increases the expression of TLR2 mRNA, but not TLR4 mRNA in *in vitro* study (He et al., 2010a). TLR2-ligands can activate an OVA-associated Th2-biased immune response, such as the induction of Th2-associated effector molecules like IL-13, IL-5, and OVA-specific IgG₁ and IgE. TLR2-ligands can have an effect on the development of Th2-associated diseases such as experimental asthma (Redecke et al., 2004). We speculate that the simultaneous administration of OVA and ASD in the present study enhances a Th-2 based immune response in conjunction with TLR2 expression induced by ASD.

A massive Asian dust storm event occurred in East Asia from March 20–22, 2002. The average density of the ambient particulate matter (total suspended particle: TSP) was 672 μg –796 $\mu\text{g}/\text{m}^3/\text{day}$ in Nagasaki, Iki-island, Japan and 10 $\text{mg}/\text{m}^3/\text{day}$ in Beijing, China on March 20 (Sun et al., 2004). Over the last 2 years, similar heavy Asian dust densities has been observed in Japan on March 20–21, 2010 and May 1–3, 2011.

The instillation dose of ASD in this study was 0.2 mg per mouse. When the body weight of the mice is reached about 36 g, the tidal air volume was approximately 0.15 mL and the breathing rate was about 200 breaths per min. The amount of ASD deposited in the lungs of a single mouse per day (4.32 μg) was calculated using the inhaled value for suspended particulate matter (SPM) (0.1 mg/m^3) set by the Japanese national air quality standard (JNAQS), for SPM (0.1 mg/m^3), the tidal volume, and the breathing rate. If 100% of 10 $\text{mg}/\text{m}^3/\text{day}$ – which is the concentration in previous record of Beijing – is accumulated in a lung (432 μg), the instillation dose (200 $\mu\text{g}/\text{mouse}$) of particles used in the present study would be 0.46 times the amount accumulated in a lung. In the case of the Human Respiratory Tract Model for Radiological Protection (James et al., 1994), the deposition rate into alveoli is approximately 3% for a 6 μm diameter particle.

The 0.2 mg particle instillation dose in this study was approximately 15.4 times the amount of 3% deposition (approximately 13 μg). It is not clear whether ASD can elicit the aggravation of inflammatory reaction in the airway at 13 μg or lower doses. However, it may be difficult to compare this mouse model with an asthmatic patient by these low doses directly, because susceptibility or sensitivity of the airway of the patient and this animal model may be different. Even though the atmosphere level of Japan in an ASD event is lower than in China and the Korean Peninsula, ASD may result in serious consequences for an asthmatic patient as shown recent reports (Kanatani et al., 2010; Watanabe et al., 2011a, 2011b).

Conclusions

This study demonstrates that ASD acts strongly to activate lung eosinophilia in mice immunized beforehand by OVA. This result indicates that ASD can activate acquired immunity by an antigen. Allergic patients are typically exposed to allergens including pollen, mold, mites, and animal dander year-round in daily life and are then exposed to ASD in the spring. Although ASD can aggravate antigen-related lung eosinophilia no matter when the exposure occurs, the simultaneous exposure of ASD and antigen may have serious effects on asthmatic patients. The experimental findings in the present study may serve as a warning about the ill effects of airborne sand dust on patients with asthma.

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Declaration of interest

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