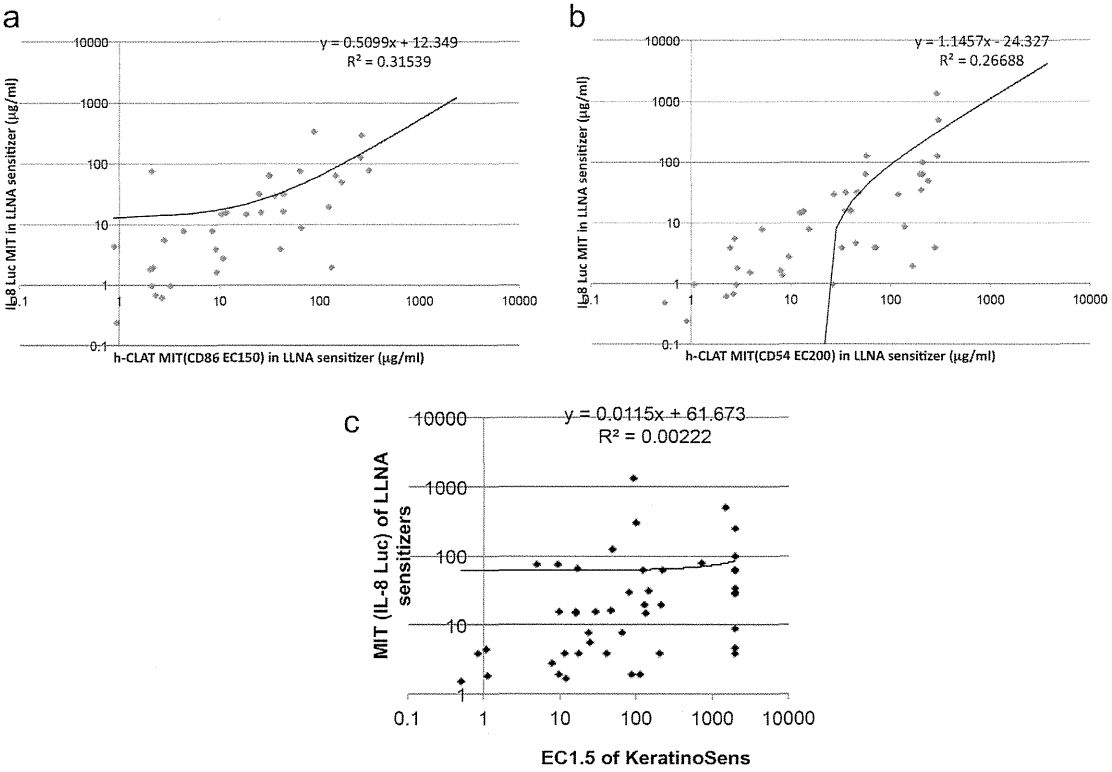

Fig. 7. Correlation between the IL-8 Luc assay and other screening methods (h-CLAT and KeratinoSens). The correlation between the minimum concentration required to induce more than 1.4 of FInSLO-LA in the IL-8 Luc assay (IL-8 Luc assay MIT) and that required to induce more than 150% of CD86 augmentation (h-CLAT MIT (CD86 EC150)) (a), that to induce more than 200% of CD54 in h-CLAT (h-CLAT MIT (CD54 EC200)) (b), and EC 1.5 of KeratinoSens (c).

Table 9
Conversion of the outcome in each single test into scores.

Score	MAX FInSLO-LA/minimum induction threshold (MIT) in IL-8 Luc assay	Avg. score in DPRA	DEREK or times
2	⩾0.1 (Strong positive)	>22.62% (Strong positive)	
1	0.01–0.1 (Weak positive)	6.376–22.62% (Weak positive)	Alert (Positive)
0	<0.01 (Negative)	<6.376% (Negative)	No alert (Negative)

The outcome of each single test was converted to a score, based on the previously reported concept by Jowsey et al. (2006).

method complicated and reduces intra- and inter-laboratory reproducibilities, we would like to include detergents in an exclusion criterion.

It is widely recognized that a single *in vitro* test is insufficient to replace animal testing and that integration of results from different *in vitro* tests, as well as *in silico* methods, is needed for prediction of the skin sensitization potential of chemicals, Since Jowsey et al. (2006) first proposed the integration framework based on the scoring system, weighing the evidence from structure–activity relationships in skin sensitization, penetration, peptide reactivity, and dendritic cell and T-cell activation to evaluate the sensitizing potential as well as the relative potency, a variety of test batteries integrated with different *in vitro* tests and/or *in silico* methods have been reported (Bauch et al., 2012; Jaworska et al., 2011; Natsch et al., 2009; Nukada et al., 2013; Tsujita-Inoue et al., 2014). Most of these approaches substantially improved the accuracy and sensitivity for the potential and potency prediction, compared with LLNA. Therefore, in this study, we also tried to combine the IL-8 Luc assay with other *in vitro* methods to test for skin sensitizing potentials.

Before determining the best combination of test methods, we first examined whether any of the parameters in the IL-8 Luc assay correlate with LLNA potency. Although we could not recognize significant correlation between MIT or FInSLO-LA of the IL-8 Luc assay and LLNA EC3 (data not shown), we found that the MAX FInSLO-LA/MIT values were higher in the group of chemicals with more potent sensitizers. Therefore, we decided to use the MAX FInSLO-LA/MIT with the battery approach.

When we tried score-based battery system and tiered battery system according to the procedure conducted by Nukada et al. (2013), both systems increased accuracy, 87.9% and 90.1%, respectively and the score-based system increased specificity while the tiered-system improved sensitivity. In particular, the chemicals judged as false negative by the IL-8 Luc assay due to the reactivity of chemicals with FBS can be judged as positive by DPRA except for ethylenediamine (Jaworska et al., 2013).

In addition to the yes/no prediction, *in vitro* approach to detect allergenicity of chemicals is required to predict the potency. However, the accuracy in the potency prediction with the scored battery system and the tiered battery system was 67% and 58.2%,

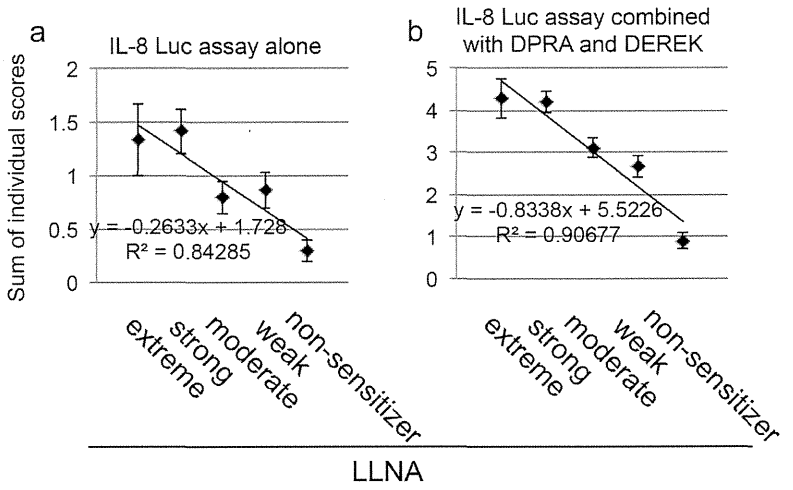

Fig. 8. Score-based battery system based on the IL-8 Luc assay. The mean ± SEM of MAX FInSLO-LA/MIT of chemicals in each group was plotted for different allergenicity groups in the LLNA after conversion of the raw data into a score based on Table 9 (a). The data and the scoring system published by Nukada et al. (2013) and Jaworska et al. (2013) were used for DPRA and DEREK. Then, for the 103 test chemicals, the total battery score between 0 and 5 was calculated by the sum of individual scores (b).

Table 10
Hazard identification by the score-based battery system based on the IL-8 Luc assay.

LLNA	Score-based battery system	
	Positive	Negative
Sensitizers (77)	67	10
Non-sensitizers (26)	5	21
	Sensitivity (%)	87.1
	Specificity (%)	80.8
	Accuracy (%)	85.4

respectively. It is still far from perfect. There is a significant spread for the quantitative data within individual potency classes. LLNA and human data correlate partly with each other, with an R^2 in log–log linear regression between LLNA and human data between 0.45 and 0.75 reported in different studies (ICCVAM, 2011). It means that EC3 in LLNA cannot correctly predict the potency classification of chemicals in humans. In addition, so far, it is not clear how the potency of haptens is determined. Most screening methods do not take T cell response into account. To improve the accuracy, it may need to construct a battery system with a screening method based on T cell response.

Finally, in this study, we explored the factors that impair the performance of the IL-8 Luc assay. Among them, the impacts of FBS on the performance of the IL-8 Luc assay was not negligible. It is a well-known procedure to solubilize water insoluble chemicals in DMSO and dilute the solution with culture medium. This procedure was also employed by h-CLAT and KeratinoSens. Indeed, most of water insoluble haptens we examined in this study significantly increased FInSLO-LA after diluted by this procedure, although we must admit that the IL-8 Luc assay with this procedure produced considerable numbers of false negative results. Surprisingly, the judgment of 5 among 14 haptens in 122 chemical lists we examined was corrected by diluting chemicals with X-VIVO. Saito et al. have reported the similar observation, in which hapten-induced ROS production by THP-1 was significantly attenuated in the presence of FBS (Saito et al., 2013). It is conceivable that FBS suppresses the binding of some haptens with cysteine or lysine residues. Therefore, our novel procedure in which chemicals are diluted with X-VIVO may significantly improve the performance of the IL-8 Luc assay.

There are several advantages in the IL-8 Luc assay. At first, the culture of THP-G8 cells are relatively simple and does not use trypsin or EDTA because THP-G8 cells do not stick to the culture dishes. The second is its simple procedure. At first, chemicals in graded concentrations are added into 96-well culture plate as required in every *in vitro* test method. Then, the cells adjusted to the optimum concentration are seeded to each plate. After 16 h incubation, the plates are set in the luminometer. The process afterward is completely automated except calculating the obtained results in the predesigned Excel sheet. Therefore, the IL-8 Luc assay is considered as a test method that can significantly reduce human errors.

Moreover, the IL-8 Luc assay does not need the step to preculture or determine cell viability after chemical treatment. In the IL-8 Luc assay, since THP-G8 cells can present the promoter activities of IL-8 promoter and GAPDH, a well known house keeping gene, the information of the effects of chemicals on both IL-8 induction and cell viability is obtained simultaneously in each experiment. Therefore, even though 4 experiments are required, one set of experiments can be completed within 4 days. Therefore, the IL-8 Luc assay is a truly high-through method.

Finally, this study succeeded in optimizing the IL-8 Luc assay to predict allergenicity of chemicals and demonstrated that the IL-8

Table 11
Potency classification by the score-based battery system based on the IL-8 Luc assay.

LLNA	Score-based battery system		
	Strong	Weak	Not-classified
Extreme + strong (23)	14	9	0
Moderate + weak (54)	6	38	10
Non-sensitizer (26)	0	5	21
Over prediction rate (%)		10.7% (11/91)	
Under prediction rate (%)		18.4% (19/103)	
Accuracy (%)		70.9% (73/103)	

Table 12
Hazard identification by the tiered system based on the IL-8 Luc assay.

LLNA	Tiered system	
	Positive	Negative
Sensitizers (77)	74	3
Non-sensitizers (26)	10	16
	Sensitivity (%)	96.1
	Specificity (%)	61.5
	Accuracy (%)	87.4

Table 13
Potency classification by tiered battery system based on the IL-8 Luc assay.

LLNA	Tiered system		
	Strong	Weak	Not-classified
Extreme + strong (23)	16	7	0
Moderate + weak (54)	19	32	3
Non-sensitizer (26)	1	9	16
Over prediction rate (%)		28.2% (29/103)	
Under prediction rate (%)		9.7% (10/103)	
Accuracy (%)		62.1% (64/103)	

Luc assay is a promising *in vitro* alternative method. Furthermore, the battery approach of the IL-8 Luc assay combined with DPRA and DEREK could significantly improve the performance.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Transparency Document

The Transparency document associated with this article can be found in the online version.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.tiv.2015.07.006.

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Research Article

Decreased Pulmonary Function in School Children in Western Japan after Exposures to Asian Desert Dusts and Its Association with Interleukin-8

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The objective of the study was to investigate the influence of Asian dust storms (ADS) on pulmonary function of school children and the relationship of this effect with interleukin-8. Morning peak expiratory flow (PEF) was measured daily in 399 children from April to May 2012 and in 384 of these children from March to May 2013. The data were analyzed for an association between ADS events and PEF by linear mixed models. Interleukin-8 transcriptional activity was assessed in THP-G8 cells stimulated by airborne particles collected on ADS days. Seven ADS days were identified: April 23 and 24, 2012; March 8 to 10, 2013; and March 19 and 20, 2013. Changes in PEF after ADS exposure were −8.17 L/min (95% confidence interval, −11.40 to −4.93) in 2012 and −1.17 L/min (−4.07 to 1.74) in 2013, and there was a significant difference between 2012 and 2013. Interleukin-8 transcriptional activity was significantly higher in 2012 at 10.6 ± 2.9-fold compared to 3.7 ± 0.4 in March 8 to 10, 2013, and 2.3 ± 0.2 in March 19 and 20, 2013. The influence of ADS events on pulmonary function of children differs with each ADS event and may be related to interleukin-8 production.

1. Introduction

Asian dust storms (ADS) originating in the deserts of Mongolia, Northern China, and Kazakhstan often disperses dust over East Asia from spring until late autumn and is the second strongest source of dust emission worldwide [1]. An ADS is also a source of air pollutants because the dust contains chemicals, contaminating metals, microorganisms, and ionic components [2–4]. Therefore, ADS is a serious health problem associated with heavy pollution.

Numerous epidemiologic studies have shown that exposure to ADS increases rates of mortality, emergency treatment, and hospitalization for cardiovascular disease and pulmonary disease [5–8]. Other studies have shown that ADS increases the risk of hospitalization and exacerbates pulmonary function and respiratory symptoms in patients with asthma in Japan and South Korea [9, 10]. However, some studies from Taiwan have suggested that there is no significant association of ADS with asthma [11, 12]. Therefore, the influence of ADS on asthma may differ in different regions. This may be associated with differences in the materials attached to ADS airborne particles, which is influenced by the path the particles take [2–4]. We have also shown that effects on lower respiratory tract symptoms in adult patients with asthma differ for each ADS event [13]. Onishi et al. suggested that ADS events can be classified into three types

based on Lidar data: Type 1 events with high counts of air pollution aerosols, Type 2 events with high counts of mineral dust particles, in comparison to air pollution aerosols, and Type 3 events with very low counts of air pollution aerosols [4].

Neutrophils migrate to the lung during acute inflammation induced by exposure to air pollutants [14]. The concentration of interleukin-8 (IL-8) in bronchial lavage fluid and IL-8 mRNA expression in bronchial biopsy tissue from healthy subjects are also increased by air pollutants [15]. IL-8 is increased in the blood of asthma patients during exacerbation [16] and thus is thought to be a key cytokine in exacerbation of asthma. In this context, we found that airborne particles collected on ADS days in Western Japan induced production of IL-8 in THP-G8 cells, whereas this effect did not occur with the original soil of the ADS [17].

In 2012, a study was already conducted to investigate the influence of ADS and air pollutants on pulmonary function of school children in Western Japan. In the current study, to investigate the difference of the influence of ADS events on pulmonary function in children, we conducted an extended survey in 2013, which was to monitor daily peak expiratory flow (PEF) in the same children as the 2012 investigation. Each year, using an IL-8 luciferase assay, these related detrimental effects on pulmonary function and differences in IL-8 promoter activity induced by ADS airborne particles were studied.

2. Materials and Methods

2.1. Subjects. The aim of this longitudinal follow-up study was to examine effects of ADS events on pulmonary function in school children. Daily morning PEF of children was monitored from March to May 2012 and 2013 because ADS events are most frequent in these months. March 2012 was used as trial period to allow the children to familiarize themselves with the monitoring. There was no ADS event in March 2012. The study was performed in Matsue, the capital city of Shimane Prefecture, in Southwest Japan. The population of Matsue is about 200,000 and the area is 530.2 km^2. In March 2012, all 401 fourth grade students aged 8 to 9 years from 4 of 35 elementary schools in Matsue were enrolled in the study. The four elementary schools were within 10 km of each other and all subjects lived within a radius of 1 km of the schools.

The disposition of the children in the study is shown in Figure 1. A total of 401 children were recruited into the study in March 2012. Two were subsequently excluded due to failure to keep a daily record for PEF. Thus, records of daily PEF were analyzed for 399 children in 2012. In March 2013, we recruited the same 401 children, of whom one was excluded due to Moyamoya disease. Sixteen children were subsequently excluded due to failure to keep a daily record for PEF. Thus, records of daily PEF were analyzed for 384 children in 2013.

The subjects recorded their age, gender, height, weight, and presence of asthma, allergic rhinitis, allergic conjunctivitis, atopic dermatitis, and food allergy in March 2012 and March 2013. Subjects were defined as having asthma if they met any of the following criteria in the past 12 months: (1) diagnosis of asthma by a pediatrician, (2) wheezing, (3) use of asthma medication, and (4) visiting a hospital regularly

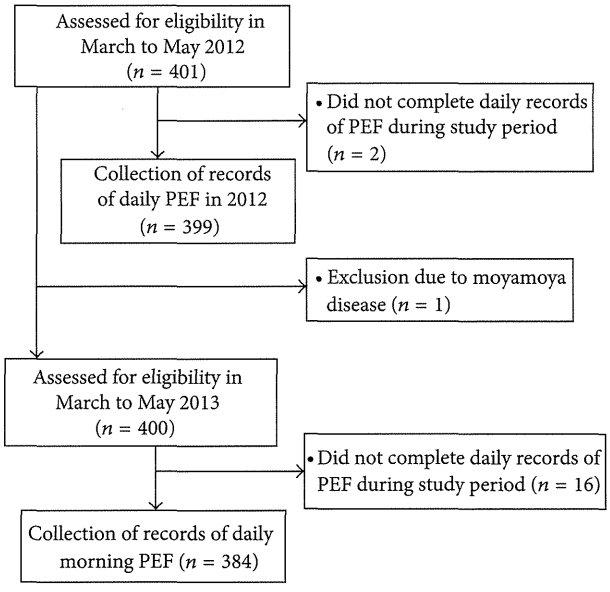


FIGURE 1: Flow chart showing the disposition of children in the study.

for asthma. Similarly, allergic rhinitis, allergic conjunctivitis, atopic dermatitis, and food allergy were judged to be present if the subjects met any of the following criteria in the past year: (1) diagnosis by a pediatrician, (2) use of medication for the disease, and (3) visiting a hospital regularly for the disease.

The study was approved by the institutional ethics committee (Ethics Committee of Tottori University, approval number 1764). We asked the Matsue City Board of Education for their help and received approval to submit the study to the schools. The study was also approved by the Parent Teacher Association (PTA) of each elementary school. Children and their parents were informed by teachers and we obtained formal consent for the study from the Matsue City Board of Education.

2.2. Monitoring of PEF. Before the study, the children and teachers were taught how to measure PEF. All children then measured their morning PEF daily using a peak flow meter (Mini-Wright, Harlow, England, American Thoracic Society Scale) from March to May 2012 and 2013, except for weekends and public holidays. Children recorded their best PEF value from three attempts after arriving at school between 8 a.m. and 9 a.m.

2.3. Definition of ADS Days and Air Pollutant Monitoring. The Japan Meteorological Agency has observatories throughout Japan and defines an ADS day based on a criterion of visibility <10 km due to dust arising from the deserts of East Asia, as determined by meteorological satellites monitoring each area. In this study, we used data from the Matsue observatory and we also referred to data from Light Detection and Ranging (Lidar) to define an ADS day. Lidar depolarization measurements performed simultaneously at two wavelengths can be used to identify nonspherical dust particles, which are mineral dust particles, and spherical aerosols such as organic

aerosols and inorganic sulfates and nitrates [19, 20]. Thus, Lidar can be used to measure levels of mineral dust particles as airborne sand dust particles and nonmineral dust particles as air pollution aerosols in real time. Lidar data are collected continuously in 23 locations in Japan, South Korea, China, Mongolia, and Thailand to detect a potential ADS. Other studies have defined ADS events as a daily (24-hour) average of mineral dust particles $>0.1\,km^{-1}$ [9] or $0.066\,km^{-1}$ (moderate ADS day) and $0.105\,km^{-1}$ (heavy ADS day) [21].

2.4. Preparation of Airborne Particles Collected on ADS Days. On ADS days, dust particles were collected at Tottori Prefectural Institute of Health and Environment, Yurihama, Tottori, which is located 70 km east of Matsue. This is the most suitable area close to Matsue to monitor particulate matter from East Asia because Yurihama is rural and has no source of air pollutants, except for motor vehicles. The observatory in Yurihama is also located away from populated areas. ADS airborne particles were collected in Tottori on April 23 and April 24, 2012; March 8 to 10, 2013; and March 19 and 20, 2013, using a high-volume air sampler (HV-1000R, Shibata, Tokyo, Japan) on the roof of a building. ADS airborne particles were filtered based on their aerodynamic diameters (Andersen Sampler, Shibata) into 5 sizes (<1.1, 1.1–2.0, 2.0–3.3, 3.3–7.0, and >7.0 μm) and each filter was dried in a desiccator before and after sampling to be weighed. ADS airborne particles of 3.3–7.0 μm were subsequently used in the study. Collected airborne dust was sterilized at 121°C for 30 min in an autoclave (Tomy SX-300, Tomy, Tokyo, Japan) to prevent growth of bacteria and fungi and dried at 80°C for 4 h with drying sterilizer (SG600, Yamato Scientific, Tokyo, Japan). The collected airborne dust was then weighed and stored in a freezer at −20°C. Soil from the China Loess Plateau (CJ-1), the original ADS soil from the Tengger Desert and Huining County located in Gansu Province, was obtained from the National Institute for Environmental Studies (Ibaraki, Japan) in 2002. This reference material is certified by the National Institute for Environmental Studies and the National Research Center for Environmental Analysis and Measurement (Beijing, China). To stimulate THP-G8 cells, airborne particles collected on ADS days were diluted to 1 mg/mL with distilled deionized water.

2.5. IL-8 Promoter-Luciferase Gene Reporter Assay and Measurements of IL-8 and Endotoxin. THP-G8 cells are a THP-1-derived reporter cell line that express stable luciferase orange (SLO) and stable luciferase red (SLR) genes under control of the IL-8 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) promoters, respectively [22]. The THP-G8 cell line was kindly provided by the Department of Dermatology, Tohoku University Graduate School of Medicine, Sendai, Japan, and was cultured as described previously [22]. We first stimulated THP-G8 cells (5×10^4 cells/100 μL/well) in 96-well black plates (Greiner Bio-One GmbH, Frickenhausen, Germany) with lipopolysaccharide (LPS) (Wako Pure Chemicals, Osaka, Japan) and examined IL-8 and GAPDH reporter activity for various time periods and concentrations. Luciferase activity was determined using a microplate luminometer with a Phelios multicolor detection system (Atto, Tokyo, Japan) using Tripluc luciferase assay reagent (Toyobo, Osaka, Japan). IL-8 transcriptional activity was assessed from normalized SLO luciferase activity (nSLO-LA), which was calculated as SLO-LA divided by SLR-LA, and the fold induction of nSLO-LA was calculated as the nSLO-LA level of treated cells divided by that of untreated cells [22]. Induction of IL-8 transcriptional activity was measured after THP-G8 cells were stimulated for 5 h with solvent only (negative control), 100 ng/mL lipopolysaccharide (LPS), and ADS airborne particles collected in 2012 and 2013.

IL-8 concentrations in culture supernatants were determined using an enzyme-linked immunosorbent assay (ELISA) kit for IL-8 (R&D Systems, Minneapolis, MN, USA). Samples were run in triplicate and read using an automated ELISA reader (Model 680, Bio-Rad, Philadelphia, PA, USA). The range of the assay was 31.2 to 2000 pg/mL. Endotoxin concentrations in ADS airborne particles were measured using a chromogenic LAL endotoxin assay kit (GenScript, Piscataway, NJ, USA). The range of the assay was 0.01 to 1 EU/mL. The pH of ADS airborne particles was measured with a pH meter (MP220, Mettler Toledo, Schwerzenbach, Switzerland).

2.6. Measurement of Metal Elements in CJ-1 Soil and ADS Airborne Particles. Metal elements in CJ-1 soil and ADS airborne particles collected on April 23 and 24, 2012; March 8 to 10, 2013; and March 19 and 20, 2013 were measured by Oki Engineering (Tokyo, Japan). The concentrations of aluminum (Al), arsenic (As), barium (Ba), calcium (Ca), cadmium (Cd), cobalt (Co), chromium (Cr), copper (Cu), iron (Fe), mercury (Hg), potassium (K), lanthanum (La), magnesium (Mg), manganese (Mn), sodium (Na), nickel (Ni), phosphorus (P), lead (Pb), strontium (Sr), titanium (Ti), and zinc (Zn) were measured by inductively coupled plasma atomic emission spectrometry. Silicon (Si) was measured using electrothermal atomic absorption spectrometry.

2.7. Statistical Analysis. To evaluate the effects of exposure to an ADS on the daily PEF of children, linear mixed models that accounted for correlations among repeated measurements within a subject were used to estimate the effects of exposure to an ADS on the daily PEF of children in April to May 2012 and March to May 2013 [23, 24]. Additionally, to adjust for potential confounding factors, we used linear mixed models with the following general form:

$$Y_{ij} = \beta_0 + \beta_1 x_{1,j} + \sum_{k=1}^{p} \beta_k x_{k,ij} + b_{0,i} + \varepsilon_{ij}. \quad (1)$$

Y_{ij} corresponds to the daily PEF for the ith child at the jth day ($i = 1, 2, \ldots, N$; $j = 1, 2, \ldots, T$). $x_{1,j}$ is an exposure variable of jth day (measurement of air pollution), and $x_{k,ij}$ ($k = 2, 3, \ldots, p$) are potential confounding factors involving individual characteristics (age, gender, height, weight, and presence of asthma, allergic rhinitis, allergic conjunctivitis, atopic dermatitis, and food allergies) and meteorological variables such as daily temperature, humidity, and atmospheric pressure. $\beta_0, \beta_1, \ldots, \beta_p$ are corresponding fixed effects coefficients, and $b_{0,i}$ is the random effect of intercept

Table 1: Characteristics of children.

	2012	2013
Number	399	384
Gender (male/female)	205/194	194/190
Height (cm)	132.3 ± 5.9	137.7 ± 7.0
Male	132.2 ± 5.5	136.9 ± 6.3
Female	132.4 ± 6.4	138.5 ± 7.7
Weight (kg)	29.5 ± 5.8	32.4 ± 6.6
Male	29.6 ± 6.2	32.3 ± 6.8
Female	29.3 ± 5.4	32.6 ± 6.4
Allergic disease		
Asthma	38	45
Allergic rhinitis	78	74
Allergic conjunctivitis	8	15
Atopic dermatitis	44	36
Food allergy	19	20

Data are shown as the mean ± S.D.

for ith child and are assumed to be $b_{0,i} \sim N(0, \sigma_b^2)$. ε_{ij} is the error term, $\varepsilon_{ij} \sim N(0, \sigma^2)$. In addition, effects on PEF were measured from the day of ADS exposure until 3 days after exposure because a dust effect on PEF can persist for up to 3 days [10]. Differences in PEF between the 2012 and 2013 results were also evaluated. The two-pollutant model was applied to different combinations of pollutants to assess the stability of the effects of ADS on PEF after adjustment for individual characteristics (age, gender, height, weight, and presence of asthma, allergic rhinitis, allergic conjunctivitis, atopic dermatitis, and food allergies) and meteorological variables (temperature, humidity, and atmospheric pressure). R version 3.0.3 (R Foundation for Statistical Computing, Vienna, Austria) was used for statistical analysis of PEF values and ADS exposure. Differences of nSLO-LA of THP-G8 cells were analyzed by ANOVA using SPSS Statistics (Japanese version 21.0 for Windows, IBM Japan, Tokyo, Japan). All quoted P values are two-sided and the significance level was set to 0.05.

3. Results

3.1. Profile of the Children. The characteristics of the children in the 2012 and 2013 studies are shown in Table 1.

3.2. Air Pollution Levels and Weather Information on ADS Days and Non-ADS Days. In 2012, April 23 and 24 were identified as ADS days. In 2013, March 8 to 10 and 19 and 20 were similarly identified as ADS days. Non-ADS days were defined as all other days from April 1 to May 31, 2012, and from March 1 to May 31, 2013. Daily levels of mineral dust particles (airborne sand dust particles) and suspended particulate matter (SPM) are shown in each period in Figure 2. The levels of air pollutants and weather during the study periods are shown in Table 2.

3.3. PEF. Changes in PEF after exposure to ADS are shown in Figure 3. In order to show the post-ADS-exposure effects,

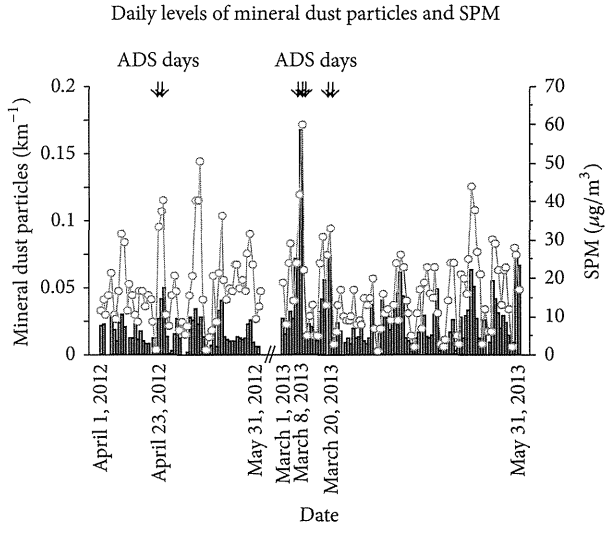


Figure 2: Daily levels of mineral dust particles (airborne sand dust particles) (bar graph) and SPM (line graph). Arrows indicate ADS days.

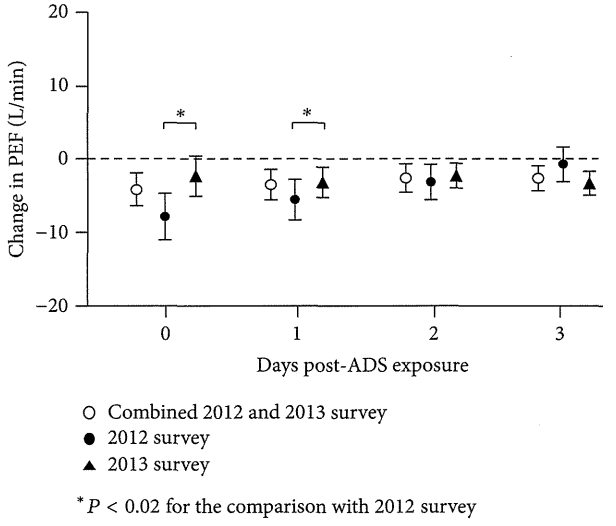


Figure 3: PEF changes caused by an ADS event from 0 (ADS day) to 3 days after ADS exposure in combined 2012 and 2013 (open circles), 2012 (black circles), and 2013 (triangles), with 95% confidence intervals (error bars). Data are controlled for age, gender, height, weight, and presence of asthma, allergic rhinitis, allergic conjunctivitis, atopic dermatitis, and food allergies; meteorological variables such as daily temperature, humidity, and atmospheric pressure; and the linear time trend. There are significant differences in the decrement of PEF on days 0 and 1 between 2012 and 2013 (*P < 0.02).

these changes are shown from 0 (ADS day) to 3 days after ADS exposure. In combining 2012 and 2013, the changes in PEF after exposure to ADS exposure were −4.16 L/min (95% CI, −6.33 to −1.99) on day 0, −2.97 L/min (−5.03 to −0.91) on day 1, −2.23 L/min (−4.12 to −0.35) on day 2, and −2.57 L/min (−4.29 to −0.86) on day 3 after ADS. There were significant decreases in PEF from day 0 to day 3 after ADS exposure. In 2012, the changes in PEF were −7.82 L/min (−10.93 to −4.71)

Table 2: Daily air pollutant levels and weather information on ADS days and non-ADS days in 2012 and 2013.

(a)

Measurement	ADS days		
	April 23 and 24, 2012	March 8 to 10, 2013	March 19 and 20, 2013
Daily average temperature, °C	17.6 ± 1.1	13.6 ± 3.8	12.4 ± 1.4
Daily maximum temperature, °C	23.3 ± 3.0	20.4 ± 0.9	18.6 ± 0.0
Daily minimum temperature, °C	13.1 ± 0.7	8.0 ± 5.8	7.9 ± 4.5
Daily average relative humidity, %	70.0 ± 5.7	64.7 ± 7.6	79.5 ± 3.5
Daily minimum relative humidity, %	48.0 ± 12.7	29.3 ± 10.7	52.0 ± 0.0
Daily average atmospheric pressure, hPa	1010.8 ± 1.0	1008.6 ± 3.1	1007.4 ± 3.0
Daily average mineral dust particles, km^{-1}	0.046 ± 0.006	0.084 ± 0.077	0.075 ± 0.001
Daily average nonmineral dust particles, km^{-1}	0.148 ± 0.097	0.138 ± 0.050	0.097 ± 0.001
Daily average SPM, $\mu g/m^3$	39.5 ± 2.1	44.3 ± 19.0	32.5 ± 5.0
Daily average $PM_{2.5}$, $\mu g/m^3$	17.2 ± 1.3	37.3 ± 16.7	37.8 ± 4.5
Daily average SO_2, ppb	1.3 ± 0.6	2.0 ± 1.1	1.9 ± 1.3
Daily average NO_2, ppb	1.5 ± 0.4	3.4 ± 1.8	3.6 ± 0.6
Daily average O_x, ppb	55.9 ± 5.7	63.1 ± 10.5	49.4 ± 5.3

(b)

Measurement	Non-ADS days	
	2012	2013
Daily average temperature, °C	15.7 ± 3.5	13.2 ± 5.0
Daily maximum temperature, °C	20.9 ± 4.5	18.7 ± 5.7
Daily minimum temperature, °C	10.9 ± 4.0	8.1 ± 5.0
Daily average relative humidity, %	70.4 ± 9.3	69.8 ± 9.8
Daily minimum relative humidity, %	44.9 ± 15.4	42.8 ± 13.6
Daily average atmospheric pressure, hPa	1010.0 ± 5.7	1011.3 ± 5.9
Daily average mineral dust particles, km^{-1}	0.016 ± 0.010	0.024 ± 0.017
Daily average non-mineral dust particles, km^{-1}	0.042 ± 0.037	0.073 ± 0.049
Daily average SPM, $\mu g/m^3$	17.7 ± 10.1	17.8 ± 8.9
Daily average $PM_{2.5}$, $\mu g/m^3$	10.3 ± 5.4	17.5 ± 7.3
Daily average SO_2, ppb	0.9 ± 0.6	1.0 ± 0.8
Daily average NO_2, ppb	2.6 ± 1.2	2.75 ± 1.2
Daily average O_x, ppb	50.0 ± 7.5	50.2 ± 8.4

Data are presented as the mean ± S.D., Non-ADS days were all other days except for ADS days from April 1 to May 31, 2012, and March 1 to May 31, 2013.

on day 0, −5.49 L/min (−8.14 to −2.85) on day 1, −3.15 L/min (−5.54 to −0.75) on day 2, and −0.72 L/min (−3.03 to 1.59) on day 3 after ADS. A significant decrease in PEF persisted for 2 days after ADS exposure in 2012. In 2013, the changes in PEF were −2.33 L/min (−5.09 to 0.44) on day 0, −2.72 L/min (−4.80 to −0.64) on day 1, −2.26 L/min (−3.98 to −0.53) on day 2, and −3.04 L/min (−4.68 to −1.40) on day 3 after ADS. A significant decrease in PEF continued from days 1 to 3 after ADS exposure. On days 0 and 1, the decrease in PEF after exposure to ADS in 2012 was significantly higher than that in 2013. In addition, the 2012 and 2013 forest plots indicate clear differences. Significant differences were observed in PEF on days 0 and 1, and the decrement of PEF in 2012 was higher than that in 2013. In a two-pollutant model adjusted for SPM, $PM_{2.5}$, SO_2, NO_2, and O_x, an ADS event in 2012 alone was significantly associated with a decrease of PEF (Table 3). In contrast, in 2013, a similar model gave no significant relationship between ADS events and PEF in children.

3.4. IL-8 Transcriptional Activity and IL-8 Secretion in THP-G8 Cells. In THP-G8 cells stimulated for 5 h with various LPS concentrations, nSLO-LA (a measure of IL-8 transcriptional activity) reached a plateau at 100 ng/mL LPS (Figure 4(a)). Maximum induction of nSLO-LA by LPS (100 ng/mL) occurred between 4 and 6 h (Figure 4(b)). Based on these results, we subsequently used stimulation for 5 h to investigate the effect of ADS airborne particles on IL-8 transcriptional activity. The concentrations of IL-8 in supernatants of THP-G8 cells stimulated with vehicle, LPS (n = 6, 1 ng/mL), and LPS (n = 6, 100 ng/mL) were 1.2 ± 0.2, 26.6 ± 6.2, and 77.4 ± 10.9 $\mu g/mL$, respectively (Figure 4(c)). This increase in IL-8 secretion is in agreement with the augmentation of nSLO in THP-G8 cells.

The pH values of ADS airborne particles (1 mg/mL) collected on April 23 and 24, 2012; March 8 to 10, 2013; and March 19 and 20, 2013 were 7.9, 7.6, and 7.6, respectively. The nSLO-LA values (IL-8 transcriptional activity) of THP-G8

Table 3: Estimated effects of ADS events on PEF in two-pollutant model after adjustment for SPM, $PM_{2.5}$, NO_2, O_x, and SO_2.

Year	Adjustment	Change in PEF	95% CI	P value
2012 and 2013	Adjusted for SPM	−3.00	−5.31, −0.68	0.011
	Adjusted for $PM_{2.5}$	−3.60	−5.94, −1.27	0.002
	Adjusted for SO_2	−2.14	−4.43, 0.15	0.059
	Adjusted for O_x	−3.49	−5.70, −1.28	0.002
	Adjusted for NO_2	−4.20	−6.37, −2.03	0.001
2012	Adjusted for SPM	−6.04	−9.44, −2.64	0.001
	Adjusted for $PM_{2.5}$	−6.48	−9.78, −3.18	0.001
	Adjusted for SO_2	−7.41	−10.69, −4.13	0.001
	Adjusted for O_x	−3.93	−7.25, −0.62	0.019
	Adjusted for NO_2	−10.04	−13.42, −6.67	0.001
2013	Adjusted for SPM	−1.57	−4.56, 1.43	0.306
	Adjusted for $PM_{2.5}$	−1.97	−5.10, 1.15	0.216
	Adjusted for SO_2	−2.19	−5.02, 0.63	0.128
	Adjusted for O_x	0.19	−2.79, 3.18	0.900
	Adjusted for NO_2	−2.45	−4.38, 1.49	0.085

Calculated for an interquartile by ADS and adjusted for individual characteristics (age, gender, height, weight, and presence of asthma, allergic rhinitis, allergic conjunctivitis, atopic dermatitis, and food allergies) and meteorological variables (temperature, humidity, and atmospheric pressure).
ADS: Asian dust storm, PEF: peak expiratory flow, SPM ($\mu g/m^3$): suspended particle matter, $PM_{2.5}$ ($\mu g/m^3$): particulate matter smaller than 2.5 μm in diameter, NO_2 (ppb): nitrogen dioxide, O_x (ppb): photochemical oxidants, SO_2 (ppb): sulfur dioxide, and CI: confidence interval.

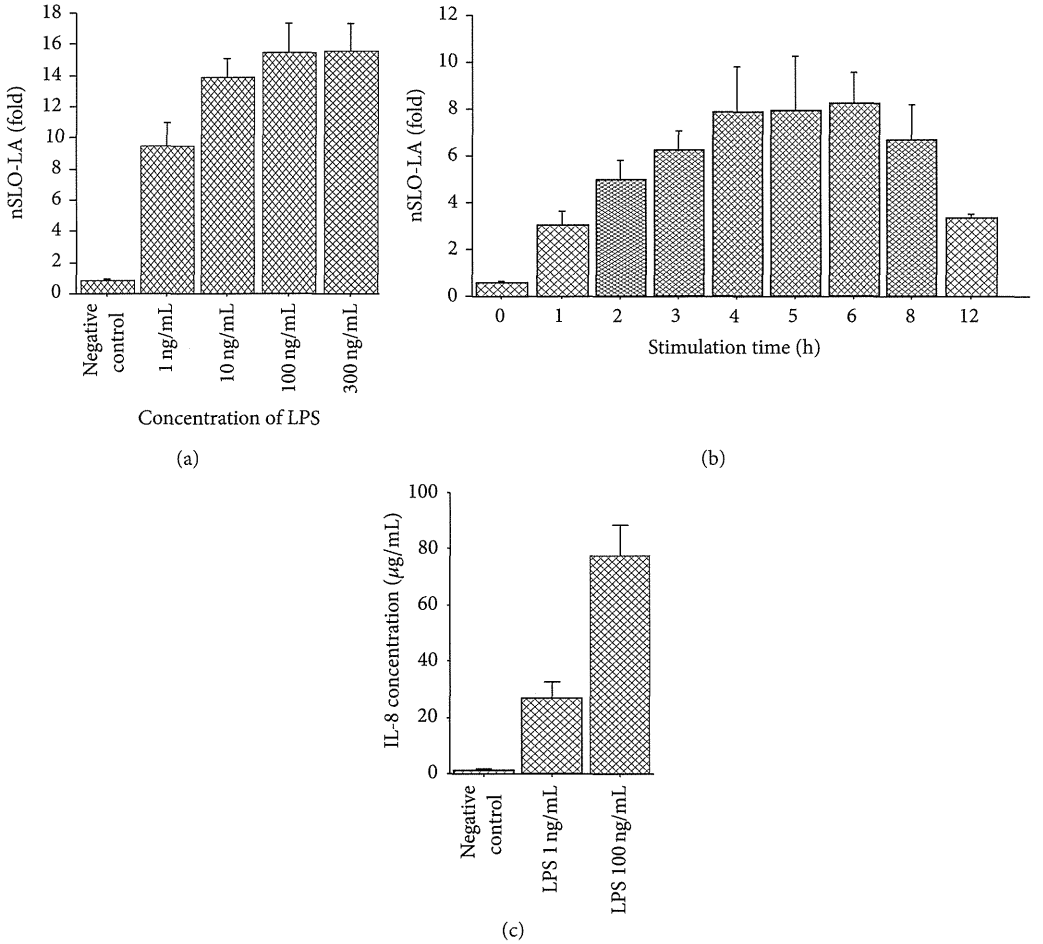
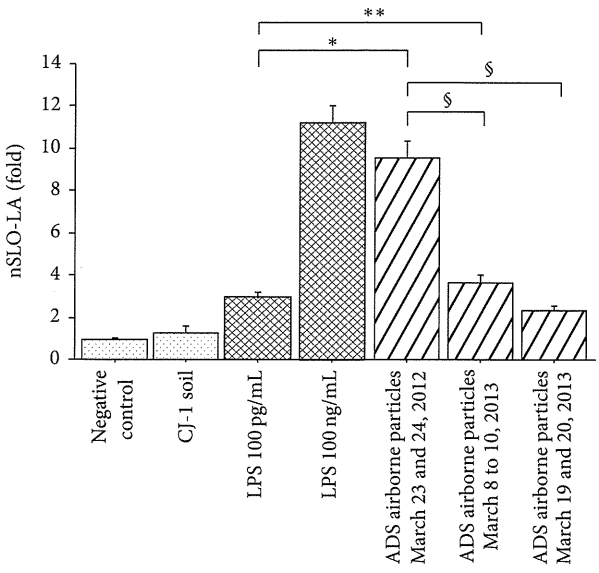


Figure 4: (a) IL-8 transcriptional activity in THP-G8 cells stimulated with LPS at various concentrations (n = 6) for 5 h. IL-8 transcriptional activity is based on normalized SLO luciferase activity (nSLO-LA), which was calculated as SLO-LA divided by SLR-LA. The fold induction of nSLO-LA was calculated as the nSLO-LA of treated cells divided by that of untreated cells [18]. (b) IL-8 transcriptional activity in THP-G8 cells stimulated with 100 ng/mL LPS (n = 6) for various time periods. (c) Concentrations of IL-8 in supernatants of a stable THP-1-derived IL-8 reporter cell line stimulated with solvent only (negative control), LPS (n = 6, 1 ng/mL), and LPS (n = 6, 100 ng/mL). The IL-8 concentration was measured using an ELISA kit. Samples were run in triplicate. The assay range was 31.2 to 2000 pg/mL.



*$P < 0.01$ for the comparison with LPS at 100 pg/mL

**$P < 0.02$ for the comparison with LPS at 100 pg/mL

§$P < 0.01$ for the comparison with ADS airborne particles in April 23 and 24, 2012

FIGURE 5: IL-8 transcriptional activity measured using an IL-8 luciferase assay in a stable THP-1-derived IL-8 reporter cell line. Cells were treated with solvent only ($n = 6$, negative control), LPS ($n = 6$, 100 pg/mL, positive control), LPS ($n = 6$, 100 ng/mL, positive control), and ADS airborne particles collected on April 23 and 24, 2012 ($n = 6$, 1 mg/mL), March 8 to 10, 2013 ($n = 6$, 1 mg/mL), and March 19 and 20, 2013 ($n = 6$, 1 mg/mL). *$P < 0.01$ versus LPS at 100 pg/mL, **$P < 0.02$ versus LPS at 100 pg/mL, and §$P < 0.01$ versus ADS airborne particles from April 23 to April 24, 2012.

cells (Figure 5) changed by 0.95 ± 0.09-fold (vehicle, $n = 6$), 2.87 ± 0.28-fold (LPS, $n = 6$, 100 pg/mL), 11.21 ± 0.28-fold (LPS, $n = 6$, 100 ng/mL), 9.56 ± 0.80-fold (ADS particles from April 23 to 24, 2012, $n = 6$, 1 mg/mL), 3.65 ± 0.36-fold (ADS particles from March 8 to 10, 2013, $n = 6$, 1 mg/mL), and 2.33 ± 0.24-fold (ADS particles from March 19 to 20, 2013, $n = 6$, 1 mg/mL).

The pH value of CJ-1 soil was constant at 8.4 for each event. The pH values of ADS airborne particles (1 mg/mL) collected on April 23 and 24, 2012; March 8 to 10, 2013; and March 19 and 20, 2013 were 7.9, 7.6, and 7.6, respectively. THP-G8 cells were stimulated with CJ-1 soil after adjusting the pH of the soil to 7.8 with 0.1 N sodium hydroxide. The nSLO-LA values (IL-8 transcriptional activity) of THP-G8 cells (Figure 5) changed by 0.95 ± 0.09-fold (vehicle, $n = 6$), 1.48 ± 0.27-fold (CJ-1 soil, $n = 6$, 1 mg/mL), 2.87 ± 0.28-fold (LPS, $n = 6$, 100 pg/mL), 11.21 ± 0.28-fold (LPS, $n = 6$, 100 ng/mL), 9.56 ± 0.80-fold (ADS particles from April 23 to 24, 2012, $n = 6$, 1 mg/mL), 3.65 ± 0.36-fold (ADS particles from March 8 to 10, 2013, $n = 6$, 1 mg/mL), and 2.33 ± 0.24-fold (ADS particles from March 19 to 20, 2013, $n = 6$, 1 mg/mL). nSLO-LA values in THP-G8 cells stimulated by ADS airborne particles differed significantly from those of controls and cells stimulated with 100 pg/mL LPS. nSLO-LA values also differed significantly for each pairwise comparison of airborne particles collected in the three ADS periods.

3.5. Endotoxin Concentration in Airborne Particles Collected on ADS Days. The endotoxin levels in ADS airborne particles (1 mg/mL) collected on April 23 and 24, 2012; March 8 to 10, 2013; and March 19 and 20, 2013 were 0.19, 0.08, and 0.07 EU/mL, respectively. These values were all lower than the level of 0.89 EU/mL found in 100 pg/mL LPS. The endotoxin concentration in 100 ng/mL LPS was out of the range of the assay.

3.6. Concentration of Metal Elements in CJ-1 Soil and Airborne Particles Collected on ADS Days. The concentrations of metal elements in CJ-1 soil and ADS airborne particles collected on April 23 and 24, 2012; March 8 to 10, 2013; and March 19 and 20, 2013 are shown in Table 4.

4. Discussion

To investigate the effect of ADS on pulmonary function, we monitored daily PEF in school children from April to May 2012 and March to May 2013 and found a significant correlation between exposure to an ADS and pulmonary function. The 2012 survey alone also showed this relationship. When differences in PEF between the 2012 and 2013 results were evaluated, the same relationship of PEF with ADS events was not found in 2013, despite the study being conducted in the same children. The decline of PEF upon ADS exposure in 2012 was also significantly higher than that in 2013, and IL-8 transcriptional activity in THP-G8 cells induced by ADS airborne particles collected in 2012 was also significantly higher than that induced by ADS airborne particles collected in 2013. These results suggest that the effect of ADS on pulmonary function in children is associated with enhanced airway inflammation mediated by elevation of IL-8.

Desert sand can reduce pulmonary function in patients with asthma after exposure at a level of PM_{10} ranging from 1500 to 2000 $\mu g/m^3$/hour [25]. This level of PM_{10} is 10 to 20 times higher than that during an ADS event in Japan. In the current study, the level of mineral dust particles on ADS events on March 8 to 10, 2013, and March 19 and 20, 2013 was about twice as high as that in 2012. However, the decrease of PEF after exposure to ADS in 2012 was higher than that in 2013. These results suggest that the decline in pulmonary function of school children during an ADS event has little connection to mineral dust particles (sand dust particles). Nonmineral dust particles were similar on ADS events in 2012 and 2013. ADS events in 2012 had higher counts of air pollution aerosols than those in 2013. Based on Onishi's criteria [4], the ADS event in 2012 can be classified as Type 1 and those in 2013 as type 2. The air pollution aerosols during ADS can be considered a cause of the significant difference in the effect of the ADS on pulmonary function of school children between 2012 and 2013.

The ADS days in 2013 were defined as moderate, while, on April 23 and 24, 2012, the mean daily average concentration of mineral dust particles was 0.046 ± 0.006 km^{-1} in Matsue

Table 4: Concentration of metal elements in CJ-1 soil and airborne particles collected on ADS days.

Metals (μg/mg)	CJ-1 soil	ADS airborne particles in April 23 and 24, 2012	ADS airborne particles in March 8 to 10, 2013	ADS airborne particles in March 19 and 20, 2013
Al	68.00	28.80	22.40	14.80
As	ND	ND	ND	ND
Ba	0.44	0.12	0.18	0.10
Ca	68.00	29.60	44.00	31.20
Cd	ND	ND	ND	ND
Co	0.01	ND	ND	ND
Cr	0.05	ND	ND	ND
Cu	0.03	0.12	ND	0.07
Fe	26.00	22.00	20.80	14.40
Hg	ND	ND	ND	ND
K	0.19	0.38	0.33	0.30
La	0.03	ND	ND	ND
Mg	18.00	14.00	16.80	14.40
Mn	0.70	0.52	0.56	0.40
Na	0.44	33.60	56.00	64.00
Ni	0.03	0.14	0.15	0.11
P	0.68	ND	ND	ND
Pb	0.02	0.06	0.12	0.06
Si	260.00	140.00	108.00	72.00
Sr	0.26	0.16	0.22	0.16
Ti	2.40	0.96	0.92	0.52
Zn	0.07	0.52	0.64	0.60

ADS: Asian dust storm, CJ-1 soil: soil from the China Loess Plateau, the original ADS soil in the Tengger Desert and Huining located in Gansu Province, and ND: not detected.

City. These values are lower than the thresholds defined in previous studies [9, 21]. However, the daily average level of mineral dust particles on ADS days was higher than that on non-ADS days, as required for definition of an ADS day in the Japan Meteorological Agency criteria used in this study.

Many studies have shown that children are susceptible to air pollution such as NO_2, O_x, and SO_2 [18, 26]. Therefore, we used a linear mixed model and a two-pollutant model to adjust for the effects of NO_2, O_x, and SO_2 on pulmonary function. In both models, ADS in 2012 remained significant after inclusion of NO_2, O_x, and SO_2. However, in the 2013 survey, we were not able to find a significant association with ADS and pulmonary function. These results suggest that airborne particles during ADS decrease pulmonary function irrespective of NO_2, O_x, and SO_2.

IL-8 is a key cytokine in air pollutant-induced airway inflammation [15, 16]. Components that adhered to ADS particles can increase release of IL-6 and IL-8 from airway epithelial cells [27]. We showed that ADS airborne particles promote transcriptional activity and production of IL-8 in THP-G8 cells, with a significant increase in IL-8 transcriptional activity in THP-G8 cells treated with ADS airborne particles compared to those treated with original ADS soil (CJ-1 soil). Additionally, we measured the difference in production of IL-8 by particles collected during each ADS event. The IL-8 transcriptional activity of ADS airborne particles collected in 2012 was significantly higher than that for particles collected in 2013. This difference in production of IL-8 by ADS airborne particles may account for the different effects on pulmonary function in school children in 2012 and 2013.

The production of IL-8 induced by ADS airborne particles in 2012 had a significant difference compared to 2013. However, there was no difference between the two ADS airborne particles in 2013. The ADS events in 2013 had happened close together, and we suspect that the route and composition of the ADS airborne particles in the two events were similar. In fact, when we analyzed the effect on PEF between two ADS events in 2013 separately, the decreases in PEF after exposure to ADS were −4.1 L/min (95% CI, −10.6 to 2.4, $P = 0.21$) in March 8 to 10 and −3.3 L/min (95% CI, −10.8 to 4.1, $P = 0.37$) in March 19 and 20. In both ADS events in 2013, there was not a significant decrease of the effects on PEF. Therefore, we presented the combined results in the main analysis. The differences in the substances and the levels of those substances attached to desert sand dusts depend on the route along which desert sand dusts pass and may play an important role in the effect of ADS on pulmonary function in children.

According to the analysis of the concentrations of metal elements, ADS airborne particles in 2012 had more Al, Cu, Fe, K, and Ti compared to those on March 8 to 10 and March 19 and 20, 2013. The amounts of Al, Fe, and Ti in ADS airborne particles in 2012 were lower than CJ-1. Cu and K may play

a causative role in the difference of production of IL-8 induced by ADS airborne particles. However, Kumar et al. indicated that Cu was not a cause of the production of CXCL1 (a mouse functional homologue of IL-8) and IL-6 induced by ambient and traffic-derived particulate matter, but it did indicate that Fe content of airborne particulate matter may be more important in mouse airway epithelial injury [28]. Metal components attached to ADS airborne particles may be one of the causes of the difference between 2012 and 2013, but further study is needed to determine a role of metal components attached to ADS on the effect of production of proinflammatory cytokines.

Ogino et al. found that some proteins contained in ambient particulate matter are important environmental factors that aggravate airway hyperresponsiveness and airway inflammation in mice [29]. An ADS contains different amounts of β-glucan, which can induce airway inflammation [30, 31]. Thus, in addition to chemical substances, anthropogenic metal components, and sulfate, some proteins and β-glucan attached to ADS airborne particles may play important roles in the reduction of pulmonary function during ADS events.

Inhaled LPS is associated with airway neutrophil inflammation in patients with asthma and in healthy subjects [32–34]. Our results show that ADS airborne particles contain endotoxin, and the endotoxin concentration of ADS airborne particle was lower than that in LPS at 100 pg/mL. However, the IL-8 transcriptional activity induced by ADS airborne particle collected on April 23 and 24, 2012 and March 8 to 10, 2013 was significantly higher than that induced by LPS at 100 pg/mL. Endotoxin may augment IL-8 transcriptional activity in THP-G8 cells, in addition to other substances on ADS airborne particles that may induce IL-8.

Park et al. [10] and Yoo et al. [35] found a relationship between ADS events and PEF in Korean children with asthma, while Hong et al. did not find a significant relationship between ADS events and PEF in children without asthma [36]. Patients with allergic diseases may also be more sensitive to air pollution [37–39]. Therefore, in this study, we analyzed the data after adjustment for allergic diseases. This analysis showed that there was a significant decrease of PEF on ADS days in 2012 compared to 2013, regardless of the presence of allergic diseases. However, the number of subjects with each disease was too small to investigate the association of PEF with ADS. Further studies are needed to define the relationship between ADS and PEF in children with allergic diseases.

In this study, children recorded their PEF value after arriving at school but did not record their PEF value on weekends and public holidays. The ADS days March 9, 10, and 20, 2013 lacked PEF data because they were holidays. However, this intermittent missing data is statistically independent of the ADS events. Thus, it would not cause any serious bias in the results. Although it would raise a reduction of statistical power, the significant associations were still observed in the primary analyses.

There are several limitations in the study. First, we did not investigate diseases other than asthma, allergic rhinitis, allergic conjunctivitis, atopic dermatitis, and food allergies. Second, we were unable to diagnose asthma based on airway hyperresponsiveness to methacholine and reversible airflow limitation. In this study, some children were considered to have asthma, when in fact their wheezing may have been caused by respiratory tract infection or other diseases. However, wheezing caused by respiratory tract infection and other diseases is more common in children under 6 years old, and that is younger than those in our study [40]. Additionally, it is difficult to distinguish asthma and reactive airway disease based on the present diagnostic criteria. Third, we were unable to measure the individual amount of exposure to ADS. Fourth, we did not analyze the composition of the ADS airborne particles. Therefore, this study was not able to investigate which components of ADS airborne particles played important roles in reduction of pulmonary function during the ADS and which components induce IL-8. Further studies are needed to define these components.

5. Conclusion

We conclude that the effect of exposure to ADS on pulmonary function in school children differed among ADS events, and that enhancement of IL-8 transcriptional activity also differed among ADS airborne particles collected during the respective events. These findings suggest that substances attached to ADS airborne particles exacerbate pulmonary function of school children. Further studies are needed to identify the substances attached to the ADS airborne particles that play key roles in exacerbation of pulmonary function.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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The Possible Interaction between Receptor Activator of Nuclear Factor Kappa-B Ligand Expressed by Extramammary Paget Cells and its Ligand on Dermal Macrophages

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TO THE EDITOR

A mammary gland is a specific type of apocrine gland located in the subcutaneous fat of the breast (Ackerman *et al.*, 2007). The interaction between receptor of activator nuclear factor kappa-B (RANK) and its ligand RANKL is the main mediator of progesterone-induced proliferation of mammary epithelial cells, and the activation of this pathway promotes mammary tumorigenesis (Bhatia *et al.*, 2005; Gonzalez-Suarez, 2011). Extramammary Paget's disease (EMPD) is an uncommon adenocarcinoma of apocrine origin. It usually affects older patients and is highly metastatic to other organs, including bone (Shiomi *et al.*, 2013). On the basis of these findings, we hypothesized that the interaction between RANK and RANKL has a role in the tumorigenesis of EMPD.

To test this hypothesis, we collected archival formalin-fixed paraffin-embedded skin specimens and cryosections from EMPD patients (Supplementary Table S1 and S2 online) for immunohistochemical and immunofluorescence analysis. This study was approved by the ethics committee of Tohoku University Graduate School of

Abbreviations: Arg1, arginase 1; DC, dendritic cell; EMPD, extramammary Paget's disease; LC, Langerhans cell; MMP-7, matrix metalloprotease-7; RANK, receptor of activator nuclear factor kappa-B; Treg, regulatory T cell

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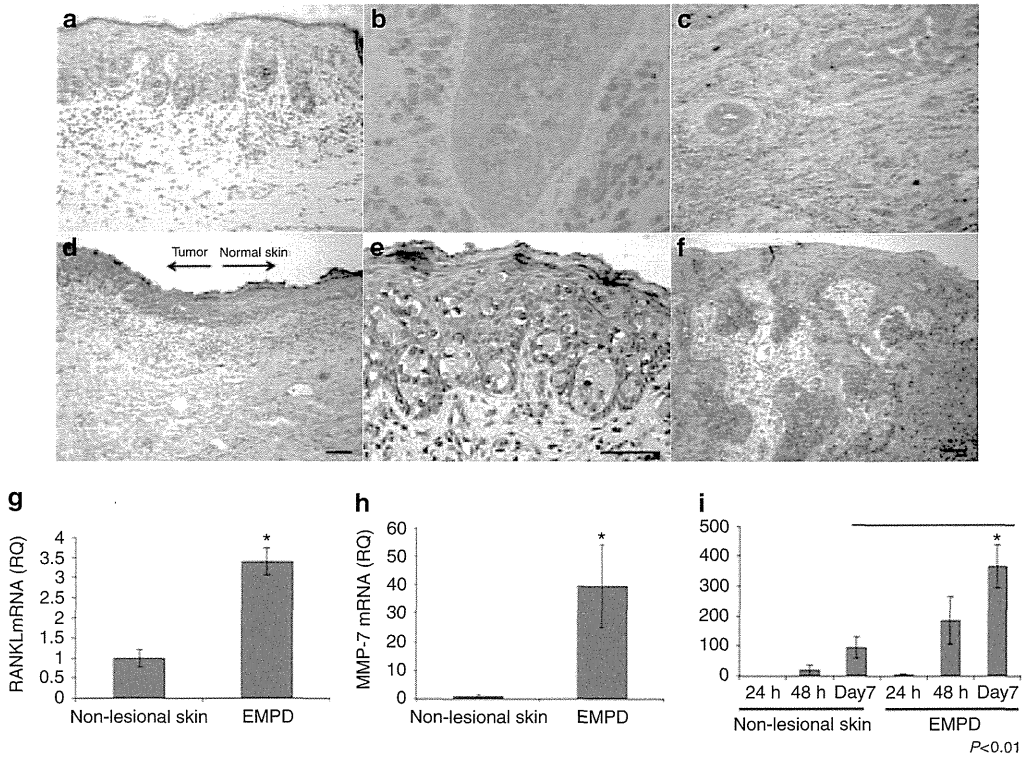

Figure 1. Extramammary Paget's disease lesions, both noninvasive and invasive, express receptor of activator nuclear factor kappa-B ligand and matrix metalloprotease-7. Immunohistochemical staining for RANKL (**a–c**) and MMP-7 (**d–f**). Scale bar = 100 μm. The expression of RANKL mRNA (**g**) and MMP-7 mRNA (**h**) in laser-captured epidermis of EMPD was analyzed by quantitative RT-PCR, using the ΔΔCt method. The mean ± SEM of data from three cases of EMPD and non-lesional skin is presented. The production of sRANKL from EMPD tissue culture was measured using ELISA (**i**). *$P < 0.05$ by Student's *t*-test (**g–i**). RT-PCR, reverse transcriptase–PCR.

Medicine, Sendai, Japan (2013-1-521), and all patients gave written informed consent. The antibodies and antigen retrieval methods used are described in Supplementary Table S3 online. For laser capture microdissection, three samples of EMPD tissue and surrounding normal skin tissue were collected at the time of resection. For the preparation of samples for tissue culture, three samples of EMPD tissue and surrounding normal skin tissue were obtained and cultured in complete medium for 7 days. Full details are available in the Supplementary Methods online.

Figure 1 shows that Paget cells strongly expressed RANKL (Figure 1a–c and Supplementary Table S1 online) and the matrix metalloprotease (MMP)-7, which cleaves RANKL to release a soluble form (sRANKL), (Figure 1d–f and Supplementary Table S1 online) on their surface (Lynch *et al.*, 2005). Notably, keratinocytes of either lesional or normal epidermis did not express RANKL or MMP-7 (Figure 1a and d, and Supplementary Figure S1A online), whereas there were several RANKL-expressing stromal cells scattered in the lesional dermis (Figure 1c). In addition, apocrine glands in the lesional skin of EMPD expressed RANKL and MMP-7 (Supplementary Figure S1B and S1C online), whereas apocrine glands in normal skin did not express MMP-7 (Supplementary Figure S1D online).

To analyze the mRNA expression of RANKL and MMP-7 by EMPD, we performed quantitative real-time reverse transcriptase–PCR using mRNA recovered from the laser-captured epidermis from the lesional skin containing Paget cells and that from the uninvolved skin of three cases of EMPD (Figure 1g and h and Supplementary Figure S1E online). The expression of RANKL mRNA (Figure 1g) and MMP-7 mRNA (Figure 1h) was significantly more abundant in the lesional epidermis of EMPD, suggesting the expression of RANKL and MMP-7 mRNA by Paget cells. As MMP-7 expression by Paget cells suggested that they might release sRANKL into the extracellular space of the lesional skin of EMPD, we cultured the lesional and non-lesional skin of EMPD and measured the concentration of sRANKL in the culture supernatants using ELISA. The results clearly demonstrated that the culture supernatants of the lesional skin contained significantly more sRANKL compared with those of the non-lesional skin (Figure 1i). These results indicate that the extracellular space surrounding EMPD tissue is rich in sRANKL.

To clarify the significance of RANKL expression by Paget cells, we immunohistochemically stained cryosections of EMPD tissue for RANK. RANK was not expressed by Paget cells or surrounding keratinocytes but was instead expressed on scattered dermal cells (Figure 2a), as well as on epidermal dendritic cells (DCs; Figure 2a insert). In addition, RANK mRNA expression in the lesional epidermis of EMPD was lower than that in the non-lesional epidermis, which

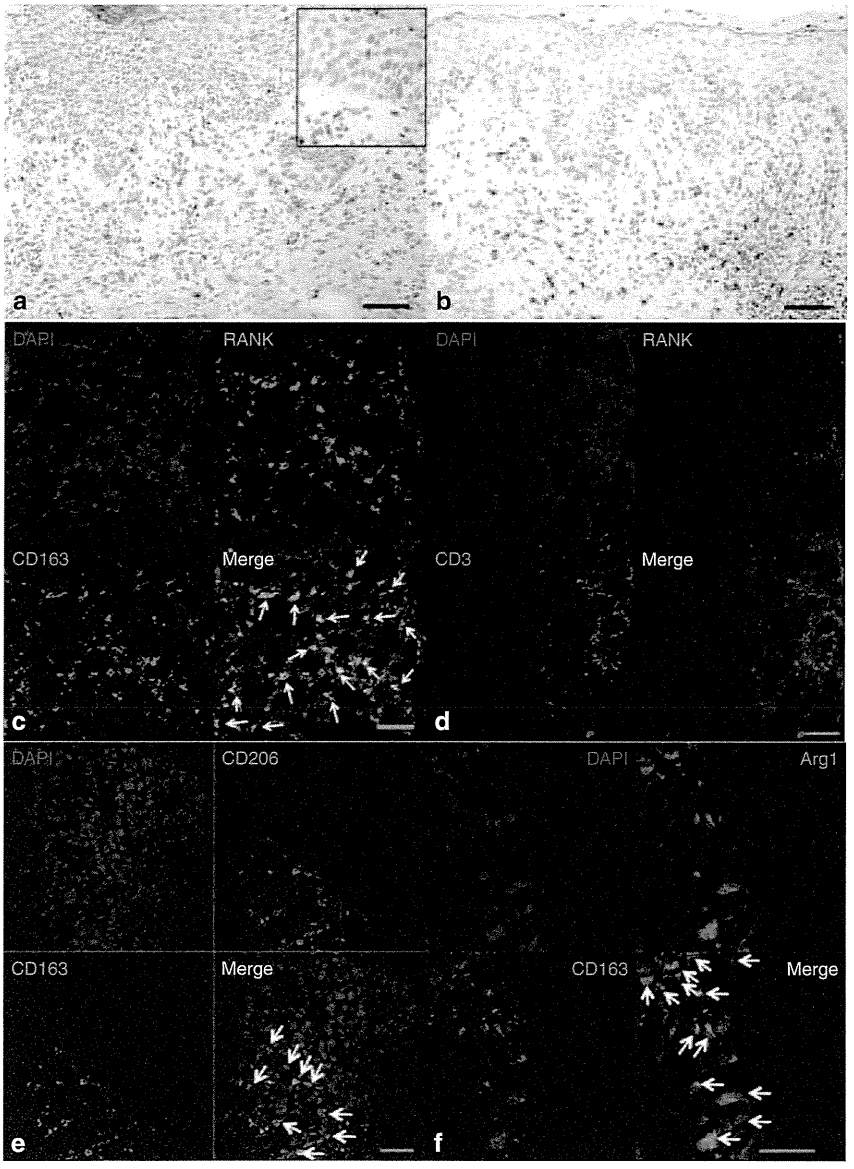

Figure 2. CD163$^+$ CD206$^+$ Arg1$^+$ M2 macrophages express RANK in the lesional skin of extramammary Paget's disease. Immunohistochemical staining of EMPD for RANK (**a**) and CD163 (**b**). Double immunofluorescence staining of EMPD for CD163 and RANK (**c**) (red: CD163, green: RANK, blue: DAPI, yellow: merge), for CD3 and RANK (**d**) (red: CD3, green: RANK, blue: DAPI, yellow: merge), for CD163 and CD206 (**e**) (red: CD163, green: CD206, blue: DAPI, yellow: merge), and for CD163 and Arg1 (**f**) (red: CD163, green: Arg1, blue: DAPI, yellow: merge). Scale bar = 100 μm (**a** and **b**) and 40 μm (**c**–**f**). Arg1, arginase 1; RANKL, receptor of activator nuclear factor kappa-B ligand.

supported the immunohistochemical demonstration of the lack of RANK expression by Paget cells (Supplementary Figure S1F online). Although it is well known that epidermal Langerhans cell (LC)s express RANK (Loser *et al.*, 2006), the origin of the RANK$^+$ cells scattered in the dermis has not yet been clarified. When we immunohistochemically stained the lesional skin of EMPD with several antibodies against macrophages and DCs such as anti-CD163 Ab (Figure 2b), anti-CD1a Ab, anti-CD205 Ab, or anti-CD208 Ab (data not shown), the distribution of the cells expressing CD163 appeared to overlap with that of dermal RANK$^+$ cells (Figure 2b).

Therefore, to clarify the origin of the RANK$^+$ dermal cells, we conducted double immunofluorescence staining of cryosections with combinations of antibodies against RANK and CD163 (Figure 2c), against RANK and CD3 (Figure 2d), against CD163 and CD206 (Figure 2e), and against CD163 and arginase 1 (Arg1; Figure 2f). The average expression of RANK on CD163$^+$ macrophages and that of CD163 on RANK$^+$ cells was 48% and 62%, respectively (Figure 2c, Supplementary Table S2 online). There were a few CD3$^+$ cells that co-expressed RANK (Figure 2d). Moreover, CD163$^+$ macrophages surrounding EMPD expressed both CD206 (Figure 2e) and Arg1 (Figure 2f), which suggests that these CD163$^+$ macrophages including RANK-expressing cells are M2 macrophages (Hao *et al.*, 2012).

We have demonstrated here that RANK is mainly expressed by CD163$^+$ Arg1$^+$ CD206$^+$ macrophages, suggesting that the sRANKL released from Paget cells stimulates these macrophages via RANK. The role of these macrophages in EMPD remains to be clarified. Accumulating evidence has suggested that RANKL-mediated modulation of surface barrier DCs including LCs results in the suppression of autoimmune responses and of detrimental immune responses toward self-antigen, as well as innocuous foreign antigen. As an explanation of this phenomenon, it was reported that RANKL-stimulated LCs induce the expansion of regulatory T cells (Tregs) in UV-irradiated skin (Loser *et al.*, 2006). We previously reported that a significant number of both M2 macrophages and Tregs infiltrate EMPD lesions (Fujimura *et al.*, 2012; Fujimura *et al.*, 2013). However, the interaction between Tregs and these macrophages in EMPD was unclear. Press *et al.* (2011) suggested that increased Tregs are associated with more extensive cases of vulvar EMPD and disease recurrence. Similarly, breast cancers with a high risk for recurrence are accompanied by the recruitment of greater numbers of Tregs to the tumor microenvironment (Bates *et al.*, 2006). Moreover, a high expression level of CD163 on tumor-associated macrophages in breast cancer is associated with reduced overall survival and reduced recurrence-free survival (Medrek *et al.*, 2012). These studies suggested that both Tregs and macrophages have a crucial role in

constituting the immunosuppressive microenvironment that causes poor prognosis in EMPD, as well as in breast cancer. In the accompanying paper, we demonstrate a role for RANKL-stimulated macrophages in recruiting Tregs into the skin.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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AUTHOR CONTRIBUTIONS

TF and SA involved in conception and design; YK, TF, and SF involved in development of methodology; YK, TF, SF, MA, and AK involved in acquisition of data; YK, TF, MA, and SA involved in analysis and interpretation of data; TF and SA involved in writing, review, and/or revision of the manuscript; TF involved in administrative, technical, or material support; TF and SA involved in study supervision.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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