Ambient fine and coarse particles in Japan affect nasal and bronchial epithelial cells differently and elicit varying immune response

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Abstract

Ambient particulate matter (PM) epidemiologically exacerbates respiratory and immune health, including allergic rhinitis (AR) and bronchial asthma (BA). Although fine and coarse particles can affect respiratory tract, the differences in their effects on the upper and lower respiratory tract and immune system, their underlying mechanism, and the components responsible for the adverse health effects have not been yet completely elucidated. In this study, ambient fine and coarse particles were collected at three different locations in Japan by cyclone technique. Both particles collected at all locations decreased the viability of nasal epithelial cells and antigen presenting cells (APCs), increased the production of IL-6, IL-8, and IL-1β from bronchial epithelial cells and APCs, and induced expression of dendritic and epithelial cell (DEC) 205 on APCs. Differences in inflammatory responses, but not in cytotoxicity, were shown between both particles, and among three locations. Some components such as Ti, Co, Zn, Pb, As, OC (organic carbon) and EC (elemental carbon) showed significant correlations to inflammatory responses or cytotoxicity. These results suggest that ambient fine and coarse particles differently affect nasal and bronchial epithelial cells and immune response, which may depend on particles size diameter, chemical composition and source related particles types.

Keywords: ambient particulate matter, cyclone technique, respiratory cells, immune cells, inflammatory responses

Capsule: We showed for the first time in the world that ambient fine and coarse particles

collected from Japan by the new technique using cyclone have different effects on the epithelium cells of the upper and lower respiratory tract and elicit varying immune response, which may depend on particles size diameter, chemical composition and source related particles types.

1. Introduction

The health effect of ambient particulate matter (PM) is still a problem worldwide. PM is a complex mixture of particles having different chemical components such as solid and liquid materials that contain elemental carbon (EC), organic carbon (OC), inorganic salts, and metals and biological components such as endotoxin and β-glucan and has a compound effect on biological reactions (Schins et al., 2004; Cachon et al., 2014; Honda et al., 2017). Generally, the fine fraction of PM (aerodynamic diameter < 2.5 μm) in urban atmosphere is a complex mixture of primary particles emitted from combustion sources and secondary particles that form in the atmosphere from gaseous components (Marcazzan et al., 2001; Sharma et al., 2007; Sevastyanova et al., 2008; Zerbi et al., 2008). The coarse fraction of PM (aerodynamic diameter > 2.5 μm) generally includes mineral particles of crustal material, sea salt particles, fly ash, and adsorbed species such as endotoxin (Schins et al., 2004; Perez et al., 2007). These components can differ depending on the sources, geographical areas, and seasons. In addition, PM composition depends on factors such as atmospheric photo-chemical reaction and physical redistribution (Vecchi et al., 2004; Samoli et al., 2008).

PM epidemiologically exacerbates respiratory and immune health such as allergic rhinitis (AR) and bronchial asthma (BA) (Tecer et al., 2008) in addition to cardiovascular diseases

and cancer (Kappos et al., 2004). Clinically, AR and BA have a close relationship: about 80% of patients with BA have complications of AR (Bachert et al., 2002). In general, coarse particles and limited fine particles can affect upper respiratory tract, whereas fine particles and limited coarse particles can affect lower respiratory tract (Heyder J., 1986). However, the difference in the effects of fine and coarse particles on the upper or lower respiratory tract and immune responses related to them, as well as their underlying mechanisms have not yet been clarified. Moreover, the components of PM responsible for the adverse health effects have not yet been elucidated owing to their complexity (Lindbom et al., 2006; Hong et al., 2016).

A large amount of fine and coarse particles is needed to evaluate the adverse health effects by *in vivo* and/or *in vitro* studies. However, it is difficult to collect a sufficient amount of PM by conventional filter collection method with extraction. Because of different extraction efficiency and loss of PM constituents, the exposure experiment using PM extracts has a possibility that would not reflect the actual biological response. Our previous study disclosed extracts efficiency of PM2.5 and discussed the problem (Chowdhury et al., 2018). On the other hand, the cyclone technique enables collection of a sufficient amount of PM (fine and coarse particles themselves) for *in vivo* and/or *in vitro* assays enabling the analysis of the effects of ambient particles on respiratory health without the use of a filter or extraction process (Okuda et al., 2015, 2018).

In this study, we investigated the effects of ambient fine and coarse particles collected at three Japanese locations by cyclone technique on nasal epithelial cells (RPMI-2650), bronchial epithelial cells (BEAS-2B), and bone marrow derived antigen presenting cells

(APCs) from NC/Nga mice. Our aim was to estimate the different effects of ambient fine and coarse particles on respiratory and immune cells, their underlying mechanism, and the components which can be responsible for the respiratory and immune health such as AR and BA.

2. Materials and Methods

2.1. Sampling of PM

Samples of fine and coarse particles were collected at an urban area in Fukuoka City, at a suburban of the metropolitan area in Kazo City, Saitama Prefecture (Saitama), and a capital area in Yokohama City in Japan (Suppl. Figure S1) during February to March 2017. The particles as references were obtained by National Institute for Environmental Studies in Japan. One reference (CRM#8) is ethanol-treated vehicle exhaust particulates (Okamoto., 1987) and another (CRM#28) is irradiated atmospheric dust collected by a ventilation filter of the building in Beijing (Mori et al., 2008). Okuda (2013) has indicated CRM#8 consists mainly of fine (or ultrafine) particles, while CRM#28 consist mainly of coarse particles.

The collection was conducted with a high-volume PM sampler using the virtual impactor and cyclone technique with no filter or extraction process (Okuda et al., 2018). The air flow volume per given time for the inlet (virtual impactor) is 1,300 L/min. The total volume of air sampled was determined from the measured volumetric flow rate and the sampling time. The mass concentration of particles in the ambient air was computed as the total mass of collected particles divided by the total volume of air sampled. After sampling, the particles in the amber bottles were collected using a stainless spatula. We previously confirmed size distribution

and morphology of ambient particles collected by cyclone (Suppl. Figure S2). Particles were dissolved in sterile phosphate-buffered saline (PBS) and ultrasonicated at the concentration of 10 mg/mL. Finally, we adjusted at concentrations of 0, 7.5, and 75 μg/mL using medium, PBS (1%) and Dimethyl sulfoxide (DMSO) (0.1%) for the cell exposure experiment in this study. Medium for BEAS-2B cells is serum-free. Similarly, we did not add serum in medium for RPMI-2650 cells to evaluate under the same condition of exposure.

2.2. Chemical, mineralogical and biochemical investigation

The collected particles was characterized by ion chromatography for Aion species (Cl^-, NO_3^-, and SO_4^{2-}) and cation species (Na^+, NH_4^+, K^+, Mg^{2+}, and Ca^{2+}), thermal-optical method (IMPROVE protocol) for OC1-4 and EC1-3, high performance liquid chromatography (HPLC) for polycyclic aromatic hydrocarbons (PAHs) (Chrysene, Benz[a]anthracene, Benzo[b]fluoranthene, Benzo[k]fluoranthene, and Benzo[a]pyrene), and inductively coupled plasma mass spectrometry (ICP-MS) for metals (Al, Si, Ti, V, Cr, Mn, Fe, Ni, Cu, Zn and Pb). The procedure of chemical characterization mentioned above were generally described in several previous papers (Okuda., 2013; Okuda et al., 2013, 2014). Endotoxin and β-glucan have induced inflammatory responses from respiratory cells and immune cells (Veranth et al., 2004; Carmona et al., 2010; Neveu et al., 2011). In this study, we investigated the effect of endotoxin and β-glucan as substances derived from biological components in PM. We performed an endotoxin test and a β-glucan test (both from Associates of Cape Cod, Falmouth, MA, USA) following the manufacturer's instructions.

2.3. Cell Cultures and PM exposure

2.3.1. Upper and Lower Respiratory cells

The RPMI-2650, derived from squamous cell carcinoma of nasal septum was used as model of human nasal epithelial cells which are cells of the upper respiratory tract. These cells display consistent growth and high stability throughout continued culturing *in vitro* with no alteration to the normal diploid karyotype (Moorhead, 1965). The cell line was purchased from the European Collection of Cell Cultures (Salisbury, Wiltshire, United Kingdom) and maintained in Eagle's minimal essential medium (DS Pharma Biomedicals, Osaka, Japan) supplemented with 10% heat-inactivated fetal bovine serum (MP Biomedicals, Eschwege, Germany), 2 mM L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin (Sigma, St Louis, Missouri). As representative of the cells of the lower respiratory tract, the BEAS-2B, derived from human bronchial epithelial cells, was purchased from the European Collection of Cell Cultures and maintained in LHC-9 medium (Thermo Scientific, Waltham, Massachusetts) which is serum-free medium containing Gentamicin. RPMI-2650 cells and BEAS-2B cells were maintained by subculture in 37°C at 5% CO_2 in medium.

2.3.2. Immune cells

Ten-week-old male SPF NC/NgaTndCrlj mice were purchased from Charles River (Osaka, Japan). NC/Nga mice are atopy-prone mice. APCs were obtained after sacrificing mice by cervical dislocation and exsanguination. The procedures used in all animal studies were approved by the Animal Research Committee at Kyoto University. APCs were differentiated using a modification of the protocol provided by Lutz et al (1999). We confirmed APCs by

the expression of about 80% of CD11c which is a molecule specifically expressed in dendritic cells. Bone marrow cells (4×10^5/mL) were cultured in R10 which is RPMI 1640 (Thermo Scientific) supplemented with 10% heat-inactivated fetal bovine serum (MP Biomedicals), 100 U/mL penicillin, 100 μg/mL streptomycin (Sigma), and 50 mM 2-mercaptoethanol (Thermo Scientific) containing Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF).

2.4. Experimental Protocol

The RPMI-2650 cells, BEAS-2B cells, and APCs were exposed to ambient fine and coarse particles at concentrations of 0, 7.5, or 75 μg/mL and reference particles at 75 μg/mL only for 24 h. We evaluated the cell viability, the cytokine release, and dendritic and epithelial cell (DEC) 205 on the cell surface. All control cells were treated with each medium.

2.4.1. Cell Viability

We measured the viability of the RPMI-2650 cells, BEAS-2B cells, and APCs by WST-1 assay using the Premix WST-1 Cell Proliferation Assay System (TaKaRa Bio, Shiga, Japan) as previously described (Honda et al., 2017). The results are expressed as the percentage of exposed group to control cells (0 μg/mL).

2.4.2. Quantification of Pro-Inflammatory Cytokines in the Culture Supernatants

The amounts of IL-6 and IL-8 release in the supernatants from the RPMI-2650 cells and BEAS-2B cells and those of IL-6 and IL-1β release in the supernatants from APCs were

177 measured by ELISA (Thermo Scientific), according to the manufacturer's protocol as
178 previously described (Honda et al., 2017). The detection limits of IL-6 and IL-8 from RPMI-
179 2650 cells and BEAS-2B cells, and IL-6 and IL-1β from APCs were <2.2 pg/mL, <9.8 pg/mL,
180 <1.9 pg/mL, <1.6 pg/mL, <16 pg/mL and 10 pg/mL, respectively.
181
182 ***2.4.3. Expression of DEC205 on APCs cell surface***
183 We measured the expression of DEC205 on the APCs' surface by the FACS analysis, the
184 following monoclonal antibodies were used: Mouse BD Fc Block purified anti-mouse
185 CD16/CD32 (Becton Dickinson), DEC205 (NLDC-145, PE-conjugated; Bio-Legend, San
186 Diego, California), Rat IgG2a, k Isotype Control (RTK2758, PE-conjugated; BioLegend).
187 The fluorescence was measured by a FACSCalibur (Becton Dickinson) as previously
188 described (Honda et al., 2017).
189
190 **2.5. Statistical Analysis**
191 The data are presented as the mean ± standard error of the mean (SEM) for each experimental
192 group (n =3 or 4). The significance of variation among different groups was determined by
193 one-way analysis of variance. Differences among groups were analyzed using Tukey's
194 multiple comparison test. A P-value < 0.05 was considered to indicate a significant difference.
195 Relationships between components in PM and cell viability or cytokine release were tested
196 using Pearson's correlation, with a two-tailed significance study using SPSS software. A P <
197 0.01 and R > 0.9 was shown as a high degree of correlation.
198

3. Results

3.1. The characterization of collected ambient particles

This cyclone system achieved 50% collection efficiency with components having the following aerodynamic cut-off diameters: virtual impactor, 2.4 μm; fine-particle cyclone, 0.18-0.30 μm; and coarse-particle cyclone, 0.7 μm. Particles smaller than 2.4 μm flowed to the fine side at the virtual impactor part, and thus, fine particles were 0.30-2.4 μm and coarse particles were 2.4 μm or more in size. The mean concentrations of fine particles in Fukuoka, Saitama, and Yokohama were 3.0, 5.9 and 9.9 $\mu g/m^3$, respectively while those of coarse particles were 1.5, 4.3 and 14.2 $\mu g/m^3$, respectively. Note that these mass concentrations of fine and coarse particles were expressed as the weights of particles collected by the cyclones per sampled air volume. The concentrations of components of both particles in the prepared solution are shown (Suppl. Table S1-S3). Both particles had different proportions of metal components between Fukuoka and the other locations. In Fukuoka, instead of a small amount of metal components, amounts of Na^+, Cl^- and SO_4^{2-} were high (Suppl. Figure S3).

The content of endotoxin in fine and coarse particles was 0.080 and 0.060 (Fukuoka), 2.50 and 2.97 (Saitama), 4.29 and 6.59 (Yokohama) EU/mL, respectively. Endotoxin in both particles at Fukuoka was lower than those collected at Saitama and Yokohama. The highest content of endotoxin was found in coarse particles at Yokohama. The contents of β-glucan in fine and coarse particles were 317.3 and 1106 (Fukuoka), 294.4 and 666.0 (Saitama), 774.6 and 1060 (Yokohama) pg/mL, respectively. β-glucan in fine particles collected at all locations was lower than those in coarse particles at each location. The highest content of β-glucan was present in coarse particles at Fukuoka.

221

222 **3.2. Biological effects of ambient particles on the Nasal Epithelial Cells**

223 We examined the effects of exposure (24 h) to ambient fine and coarse particles on the
224 viability of RPMI-2650 cells. A significant decrease ($p < 0.01$ vs. control) in the viability of
225 cells was seen upon exposure to both particles collected at all locations in a concentration-
226 dependent manner as compared to the control (unexposed cells). No difference in cytotoxicity
227 caused by ambient fine and coarse particles from each location was detected except for
228 sample collected at Yokohama at a concentration of 7.5 μg/mL (Figure 1).

229 We investigated the effects of 24 h exposure of ambient fine and coarse particles on the pro-
230 inflammatory responses via release of IL-6 and IL-8 from RPMI-2650 cells. None of the
231 particles, at any location were able to evoke detectable cytokine release from RPMI-2650
232 cells.

233

234 **3.3. Biological effects of ambient particles on the Bronchial Epithelial Cells**

235 We examined the effects of exposure to ambient fine and coarse particles for 24 h on the
236 viability of BEAS-2B cells. BEAS-2B cells showed no decrease in viability upon exposure
237 to both particles collected at all locations at any concentration, when compared to control
238 (unexposed cells) (Figure 2A).

239 We investigated the effects of ambient fine and coarse particles on the pro-inflammatory
240 responses via release of IL-6 and IL-8 from BEAS-2B cells after exposure to each particle
241 for 24 h. Both particles collected at all locations increased IL-6 release, in a dose-dependent
242 manner, especially at the concentration of 75 μg/mL (Figure 2B; $p < 0.01$ vs. control).

243 Comparison between fine and coarse particles at the same location indicated that coarse
244 particles at Fukuoka induced higher production of IL-6 than fine particles (Figure 2B; $p <$
245 0.01 vs. fine particles at 75 μg/mL). Comparison among the three locations indicated that
246 fine particles collected at Saitama or Yokohama had greater effect than those at Fukuoka
247 (Figure 2B; $p < 0.01$ vs. fine particles at Fukuoka at 75 μg/mL). Coarse particles collected at
248 Fukuoka had significantly larger effect than those collected at Saitama (Figure 2B; $p < 0.05$
249 vs. coarse particles at Fukuoka at 75 μg/mL). The production of IL-8 showed a pattern similar
250 to that of IL-6. The results of IL-8 different from IL-6 are shown. Coarse particles collected
251 at Fukuoka, fine particles collected at Saitama and both particles collected at Yokohama
252 significantly increased the levels of IL-8 at a concentration of 7.5 μg/mL (Figure 2B; $p < 0.05$
253 or 0.01 vs. control). The fine particles collected at Yokohama showed significantly marked
254 induction of IL-8 than coarse particles (Figure 2B; $p < 0.05$ vs. fine particles at 75 μg/mL).
255 Fine particles collected at Yokohama had greater effect than those collected at Saitama
256 (Figure 2B; $p < 0.01$ vs. fine particles at Saitama at 75 μg/mL).
257
258 **3.4. Biological effects of ambient particles on the APCs**
259 We examined the effects of ambient fine and coarse particles on the viability of APCs after
260 exposure to PM for 24 h. Exposure to both particles resulted in decrease in viability of APCs
261 in a dose-dependent manner as compared to control (unexposed cells) (Figure 3A). A
262 significant decrease in the viability of cells was seen upon exposure to both particles collected
263 at all locations at a concentration of 75 μg/mL and for those at Saitama and Yokohama at the
264 concentration of 7.5 μg/mL (Figure 3A; $p < 0.05$ or 0.01 vs. control). Coarse particles

265 collected at Fukuoka slightly decreased the viability of cells at a concentration of 7.5 μg/mL
266 (Figure 3A; $p < 0.05$ vs. control). No difference in cytotoxicity was detected between fine
267 and coarse particles collected at each location.
268 We investigated the effects of ambient fine and coarse particles on the pro-inflammatory
269 responses analyzed as IL-6 and IL-1β released from APCs after exposure to each particle for
270 24 h. Both particles collected at all locations increased IL-6 release in a dose-dependent
271 manner especially at a concentration of 75 μg/mL (Figure 3B; $p < 0.01$ vs. control). Both
272 particles collected at Saitama and Yokohama also significantly increased the levels of IL-6
273 at the concentration of 7.5 μg/mL (Figure 3B; $p < 0.01$ vs. control). Coarse particles collected
274 at Fukuoka induced higher production of IL-6 than the fine particles (Figure 3B; $p < 0.01$ vs.
275 fine particles at 75 μg/mL). Both particles collected at Saitama and Yokohama induced higher
276 production of IL-6 than that collected at Fukuoka (Figure 3B; $p < 0.05$ or 0.01 vs. fine
277 particles at Fukuoka at 7.5 and 75 μg/mL). Both particles at all locations increased IL-1β
278 release, in a dose –dependent manner, especially at the concentration of 75 μg/mL similar to
279 what was observed in case of IL-6 (Figure 3B; $p < 0.01$ vs. control). Coarse particles collected
280 at Saitama and both particles at Yokohama at a concentration of 7.5 μg/mL increased the
281 levels of IL-1β (Fig 3B; $p < 0.05$ or 0.01 vs. control). Coarse particles collected at Fukuoka
282 induced lower production of IL-1β than the fine particles (Figure 3B; $p < 0.05$ vs. fine
283 particles at 75 μg/mL). Both particles at Fukuoka resulted in greater induction of IL-1β than
284 those at Saitama and Yokohama (Figure 3B; $p < 0.05$ or 0.01 vs. both particles at Fukuoka at
285 75 μg/mL, respectively).
286 The expression patterns of DEC205 in APCs were examined in order to evaluate the effects

of exposure to ambient fine and coarse particles for 24 h on the maturation and activation of APCs. DEC205 is a member of the macrophage mannose receptor family. This molecule is known to mediate the capture and internalization of ligands for subsequent processing and presentation by APCs (Jiang et al., 1995). Both particles collected at all locations increased the ratio of DEC205-positive cells at concentrations of 7.5 and 75 μg/mL (Figure 3C; $p < 0.01$ vs. control). Although, there were no significant differences in the ratio of DEC205-positive cells exposed to the both particles collected at the same location, fine particles collected at Yokohama significantly increased the ratio of DEC205-positive cells when compared with those at Fukuoka (Figure 3C; $p < 0.05$ vs. fine particles at Fukuoka at 7.5 μg/mL).

3.5. Correlation between ambient particles components and biological responses

Determination of the PM components responsible for impacts on examined cell lines by assessing cytotoxicity, pro-inflammatory cytokine release is very important. We evaluated Pearson's correlation coefficients between both particles compounds and the cell viability of RPMI-2650 cells and APCs, and both particles compounds and cytokine release from BEAS-2B cells and APCs. Our analysis showed negative correlations between the cell viability of RPMI-2650 cells and Ca^{2+}, Zn, and OC (Suppl. Figure S4). Positive correlations were observed between IL-6 and IL-8 release from BEAS-2B cells and IL-6 release from APCs and multiple components, including EC, OC, and metals such as Ti and Co (Suppl. Figure S5, S6A). Whereas, positive correlations between IL-1β from APCs and multiple components, including ions, EC, OC, and metals such as As and Pb were shown (Suppl.

309 Figure S6B).

310

311 **4. Discussion**

312 In the present study, we conducted experiments using ambient fine and coarse particles
313 collected by cyclone technique. Ambient both particles decreased the cell viability of RPMI-
314 2650 cells and APCs, and induced production of pro-inflammatory cytokines from BEAS-
315 2B cells and APCs. There was a significant difference in the inflammatory response elicited
316 by fine and coarse particles at Fukuoka. Inflammatory responses induced by particles
317 collected at Saitama and Yokohama were similar to each other but different from those
318 observed for particles collected at Fukuoka. Ambient both particles also induced expression
319 of DEC205 on APCs. Viability of RPMI-2650 cells correlated negatively with Ca^{2+}, Zn, and
320 OC3 and OC4. IL-6 and IL-8 release correlated positively with Ti, Fe, Co, Cr, Mn, V, and Zn,
321 and OC3, OC4, and EC2, whereas IL-1β release correlated positively with As, Pb, and OC2
322 and EC1.

323 Ambient both particles at all locations decreased viability of RPMI-2650 cells but did not
324 induce pro-inflammatory cytokines. Nasal epithelial cells are the first epithelial barrier in the
325 nasal cavity imparting protection from inhaled xenobiotics. Previous studies have indicated
326 that diesel exhaust particles (DEP) or PM10 do not show cytotoxicity nor induce production
327 of pro-inflammatory cytokines from RPMI-2650 cells (Lindbom et al., 2006) and that DEP
328 does not reduce cell viability but decrease the barrier function by reducing zonula occludens
329 -1 (ZO-1) expression in RPMI-2650 cells (Fukuoka et al., 2015). If the barrier function
330 decreases even without cytotoxicity, there is a possibility that PM further weaken the barrier

function due to cytotoxicity. It is possible that particles collected in this study weakened the barrier function, thereby allowing easy invasion by allergens.

It has been reported that primary nasal epithelial cells have lesser Toll-like receptor (TLR) expression compared to alveolar epithelial cells, and more distribution of Toll-interacting protein (TOLLIP; an inhibitor of TLR signaling) which may be one of the reasons for non-induction of pro-inflammatory cytokines by these cells (Moncayo et al., 2014). The low inflammatory responses due to the low expression of TLR and the high expression of TOLLIP on nasal epithelial cells may lead to colonization and coexistence of many resident bacterial groups in the nasal cavity environment. The epithelial cells in the nasal cavity are always exposed to external environment and various bacteria or chemicals therein. Hence, tolerance to these bacteria without induction of inflammation is required to maintain homeostasis.

In case of BEAS-2B cells, though ambient both particles at all locations elevated the levels of IL-6 and IL-8 release, none of them decreased the cell viability. Ambient both particles strongly induced pro-inflammatory cytokine production compared to both reference particles. Bronchial epithelial cells also act as a physical barrier and generate biological and immunological responses against inhaled xenobiotics. IL-6 and IL-8 are the major pro-inflammatory cytokines induced by response to environmental insults and are important key molecules causing acute inflammation in the respiratory system by stimulating lymphocytes (Thacker, 2006). Previous studies have indicated that various chemicals and allergens stimulate IL-6 or IL-8 production from BEAS-2B cells (Honda et al., 2014; Totlandsdal et al., 2012; Park et al.,2009). Ambient both particles collected by cyclone technique can

353 exacerbate allergic inflammation by inducing inflammatory cytokines.

354 Extracts from PM on filter have been used for experiments dealing with health effects of PM.
355 To our knowledge, this is the first experimental demonstration of the effects of allergic
356 inflammation by ambient fine and coarse particles collected by cyclone technique.

357 Fuentes-Mattei et al. (2010) have showed that PM2.5 organic extracts from urban area in
358 Puerto Rico at 50 μg/mL decrease about 20% of cell viability and produce about 650 pg/mL
359 of IL-6 and 235 pg/mL of IL-8 from BEAS-2Bcells, but those from rural area do not decrease
360 cell viability and induce the lower production of IL-6 compared to those from urban area.
361 Gualtieri et al. (2010) have showed that PM2.5 and PM10 aqueous extracts from urban area
362 in Milan at 10 μg/cm^2 (in this study, 75 μg/mL=13.7 μg/cm^2) do not decrease cell viability
363 and produce about 80 pg/mL and 400 pg/mL of IL-8 from BEAS-2B, respectively. We have
364 previously indicated that exposure to PM2.5 collected in winter and subjected to organic
365 extraction rather than aqueous extraction causes an inflammatory response via IL-6
366 production from bronchial epithelial cells and PM2.5 extracts at Fukuoka subjected to
367 organic extraction, produced about 110 pg/mL of IL-6 at a concentration of 75 μg/mL which
368 was about 2 times greater than the level of control (Honda et al., 2017). These same extracts
369 collected in another season produced about 15 pg/mL of IL-6 and about 200 pg/mL of IL-8
370 from bronchial epithelial cells, and the levels of these cytokines were less than control
371 (Chowdhury et al., 2018). In the present experiment, fine particles collected by cyclone
372 technique at Fukuoka at 75 μg/mL produced about 250 pg/mL of IL-6 and about 670 pg/mL
373 of IL-8 in BEAS-2B cells. The levels of IL-6 and IL-8 produced by fine particles collected
374 using cyclone technique was about 6 and 2.5 times more than the level of control, respectively.

375 Although we could not strictly compare these results because it is not exactly the same
376 particle, it is suggested that ambient particles collected by cyclone technique could induce
377 larger inflammatory response than that induced by PM collected by the conventional filter
378 technique with extraction. Cyclone technique is an efficient method for collecting particles
379 that are subjected to exposure studies. Currently, there exist only a few assessments of health
380 effect conducted using ambient particles collected by cyclone technique and hence, further
381 research in this area should be encouraged (Ogino et al., 2017).
382 In the present study, ambient both particles collected at all locations induced expression of
383 DEC205 on APCs. The current study is the first report that ambient particles collected by
384 cyclone technique activates APCs via DEC205 expression. Several studies have shown that
385 carbon black nanoparticles and Asian dust particles can promote the maturation/activation
386 and function of DEC205 on APCs and may be related to their enhancing effects on allergic
387 diseases or responses (Honda et al., 2014; Koike et al., 2008). Ambient both particles induced
388 pro-inflammatory cytokines such as IL-6 and IL-1β from APCs as well as BEAS-2B cells.
389 Ambient both particles strongly induced pro-inflammatory cytokine production compared to
390 both reference particles. IL-1β has been described as a potent pro-inflammatory cytokine and
391 a mediator of a wide range of systemic human diseases (Dinarello, 2005; Koh et al., 2006;
392 Allantaz et al., 2007; James et al., 2011). The difference in reaction of IL-6 and IL-1β
393 observed in this study may be due to the difference in the transcription factors of cytokines
394 or in the sensitivity to exposure components. APCs play important roles in allergens-related
395 airway inflammation (Lambrecht et al., 2012). APCs are activated upon invasion of the upper
396 or lower respiratory tract by ambient particles collected by cyclone technique. As a result,

ambient particles can affect allergic inflammation not only by induction DEC205 expression, but also through induction of pro-inflammatory cytokines.

Some experimental and epidemiological studies have indicated that PM10 may exhibit a similar or higher pro-inflammatory potential than PM2.5 and lead to adverse pulmonary responses, which may require hospitalization (Monn et al., 1999; Becker et al., 2005; Brunekreef et al., 2005; Gerlofs et al., 2007; Camatini et al., 2008). One of the reasons for this may be attributed to the greater presence of microbial factors such as endotoxins or β-glucan in PM10 as compared to PM2.5. Previous studies have shown endotoxin and β-glucan to be associated with the inflammatory effects of PM both in *in vitro* and *in vivo* (Douwes et al., 2003; Becker et al., 2005; Jalava et al., 2008). The U.S. Environmental Protection Agency (1995) has noted that PM10 deposits in the upper airways of the lungs and may be more relevant for asthmatic responses and irritation. On the other hand, PM2.5 is more often the cause of lower respiratory symptoms such as cough and sputum compared to PM10 (Schwartz et al., 2000). In this way, although both PM can cause harmful health effects, the one that causes greater harm has not been elucidated. In the present study, almost no difference of cytotoxicity on RPMI-2650 cells and APCs was observed between the fine and coarse particles. However, coarse particles collected at Fukuoka induced higher production of IL-6 and IL-8 from BEAS-2B cells and IL-6 from APCs than fine particles, but lesser production of IL-1β from APCs. Fine particles collected at Yokohama induced higher production of IL-8 from BEAS-2B cells than coarse particles. Our analysis did not show strong correlations between pro-inflammatory cytokine release and microbial factors. Previous studies have suggested that endotoxin at 2000 EU/mL and βglucan at the

419 concentration of “μg/mL” induces pro-inflammatory responses in airway epithelial cells or
420 APCs (Carmona et al. 2010, Veranth et al. 2004). As the level of endotoxin and βglucan in
421 our study was very low compared to those of the previous studies, we suspect that the level
422 of endotoxin and βglucan in our study failed to noticeable correlation with inflammation in
423 the cells. Apart from microbial factors, previous studies have suggested that co-exposure of
424 SO_4^{2-} or constituent of cedar pollen enhances inflammatory responses (Hiyoshi et al., 2005;
425 Ichinose et al., 2005; Yamada et al., 2012). Not only biological factors but also other
426 compounds contained in PM or allergen substance can contribute to the production of pro-
427 inflammatory cytokines.
428 Yokohama is the most populated city and located in one of the three major industrial areas in
429 Japan. Saitama is close to Yokohama, and relatively the secondary particle tends to be the
430 main component of the formation of fine particles, so secondary air pollution is considered.
431 (Takegawa et al., 2006; Miyakawa et al., 2008). Yokohama and Saitama are located in
432 urban/suburban and industrial areas, respectively. Fe, Cr, Mn, Zn and Co are emitted by the
433 steel industry and V is mainly emitted by oil combustion (Lin et al., 2005; Querol et al., 2006;
434 Japan Ministry of the Environment, 2014). Titanium originates from the chemical industry
435 dealing with nanomaterial particles such as titanium dioxide (Chao et al., 2011). As these
436 metals in particles were correlated with IL-6 and IL-8 production in Yokohama and Saitama,
437 anthropogenic metals might contribute to respiratory disorders. On the other hand, Fukuoka
438 is closest to Mainland China, therefore there are a possibility that the influence of
439 transboundary pollution from China. (kaneyasu et al.,2014; Takami et al., 2016). It has been
440 reported that As and Pb are abundantly contained in coal which is the main energy source in

441 China (Hioki et al., 2009). Generally, atmospheric concentration of As in Japan is very low,
442 and there are few specific sources of As (Taniguchi et al. 2016). At least, the present study
443 demonstrated that As contained much in the particles in Fukuoka showed a high correlation
444 with IL-1β release. This indicated that Fukuoka may be influenced by transboundary
445 pollution from China. However, the other biological responses induced by the particles
446 collected in Fukuoka was lower than the particles in other locations. Hence, particles caused
447 by transboundary contamination may have lower biological activity than particles having a
448 source in our country.
449 Previous studies have reported that metals and their compounds mentioned above induce IL-
450 6, IL-8 or IL-1β *in vivo* and/or *in vitro* studies. For example, Ti or $CoCl_2$ induce the
451 production of IL-6 or IL-8 from bronchial epithelial cells or human lung microvascular
452 endothelial cells, and IL-1β are induced from alveolar macrophages by intratracheal
453 administration of As in mice (Huaux et al., 1995; Carter et al., 1997; Schmalz et al., 1998;
454 Ming et al., 2000; Pascal et al., 2004; Kyoung et al., 2006; Eun-Jung et al., 2008, 2010). It is
455 possible that metals such as Ti, Fe, Cr, Mn, Co, V, Zn, Pb and As can induce IL-6, IL-8 or
456 IL-1β from BEAS-2B cells and APCs, but further investigation is needed to identify the
457 responsible components. The components in OC and EC have not yet been well elucidated
458 (Grabowsky et al., 2011; Ikemori et al., 2009). However, low- and less- volatile organic
459 carbons (OC3 and OC4) may play an important role in the inflammatory reaction in each cell.
460 Previous studies have reported metals or ions such as Fe, Zn, Cr, Mn, V and Cu induce
461 toxicity of airway epithelial cells (Riley et al., 2003; Honda A., 2015). The concentrations of
462 Fe, Cr, V and Cu in our study was low compared to those of the previous studies, but Mn and

Zn was equivalent concentrations. In this study, we showed only high correlation between Zn and toxicity, however it is a possible that the other metals such as Fe, Cr, Mn, V and Cu also induce toxicity of airway epithelial cells.

5. Conclusion

Exposure to ambient particles collected by cyclone technique reduced cellular viability in RPMI-2650 cells and APCs, induced pro-inflammatory responses in BEAS-2B cells and APCs, and induced the maturation/activation of APCs. There was correlation between of some chemical components and biological responses. These chemical components affected differently between nasal and bronchial epithelial cells and elicited varying immune response. In addition, these effects can differ depending on the diameter of the particles and/or collection locations.

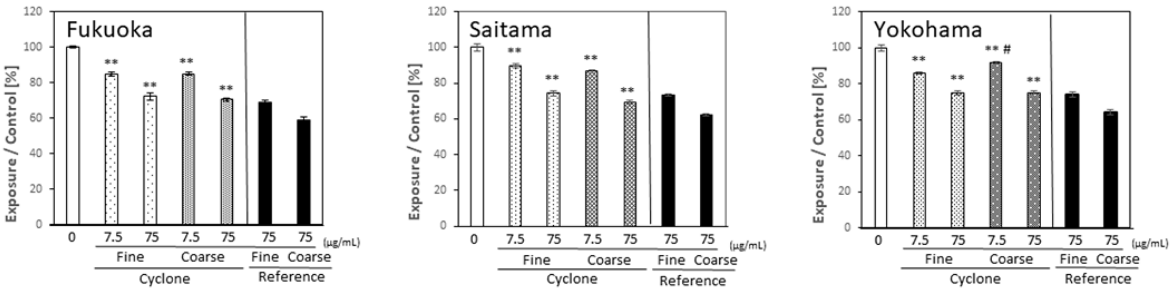

Figure 1. Effects of ambient particles and reference particles on the viability of RPMI-2650 cells. Data are presented as the percentage of the viability of the control. Data are mean ± standard error of the mean (SEM) of 4 individual cultures. *P<0.05, **P<0.01 vs. 0 μg/mL, #P<0.05 vs. Fine particles at 7.5 μg/mL.

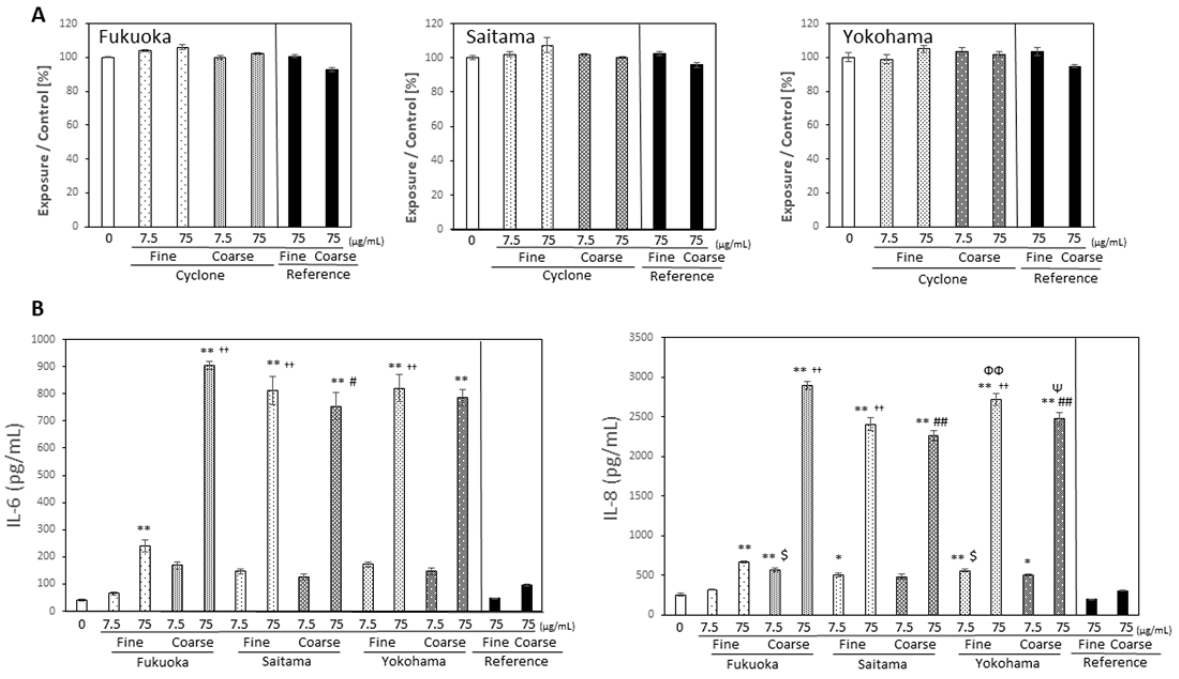

Figure 2. (A): Effects of ambient particles and reference particles on the viability of BEAS-2B cells. Data are presented as the percentage of the viability of the control. (B): IL-6 and IL-8 production from BEAS-2B cells in response to ambient particles and reference particles. Date are mean ± standard error of the mean (SEM) of 4 individual cultures. *P<0.05, **P<0.01 vs. 0 μg/mL, $^{\$}$P<0.05 vs. Fine particles at Fukuoka at 7.5 μg/mL, ††P<0.01 vs. Fine particles at Fukuoka at 75 μg/mL, $^{\#}$P<0.05, $^{\#\#}$P<0.01 vs. Coarse particles at Fukuoka at 75 μg/mL, $^{\Phi\Phi}$P<0.01 vs. Fine particles at Saitama at 75 μg/mL, $^{\Psi}$P<0.05 vs. Fine particles at Yokohama at 75 μg/mL.

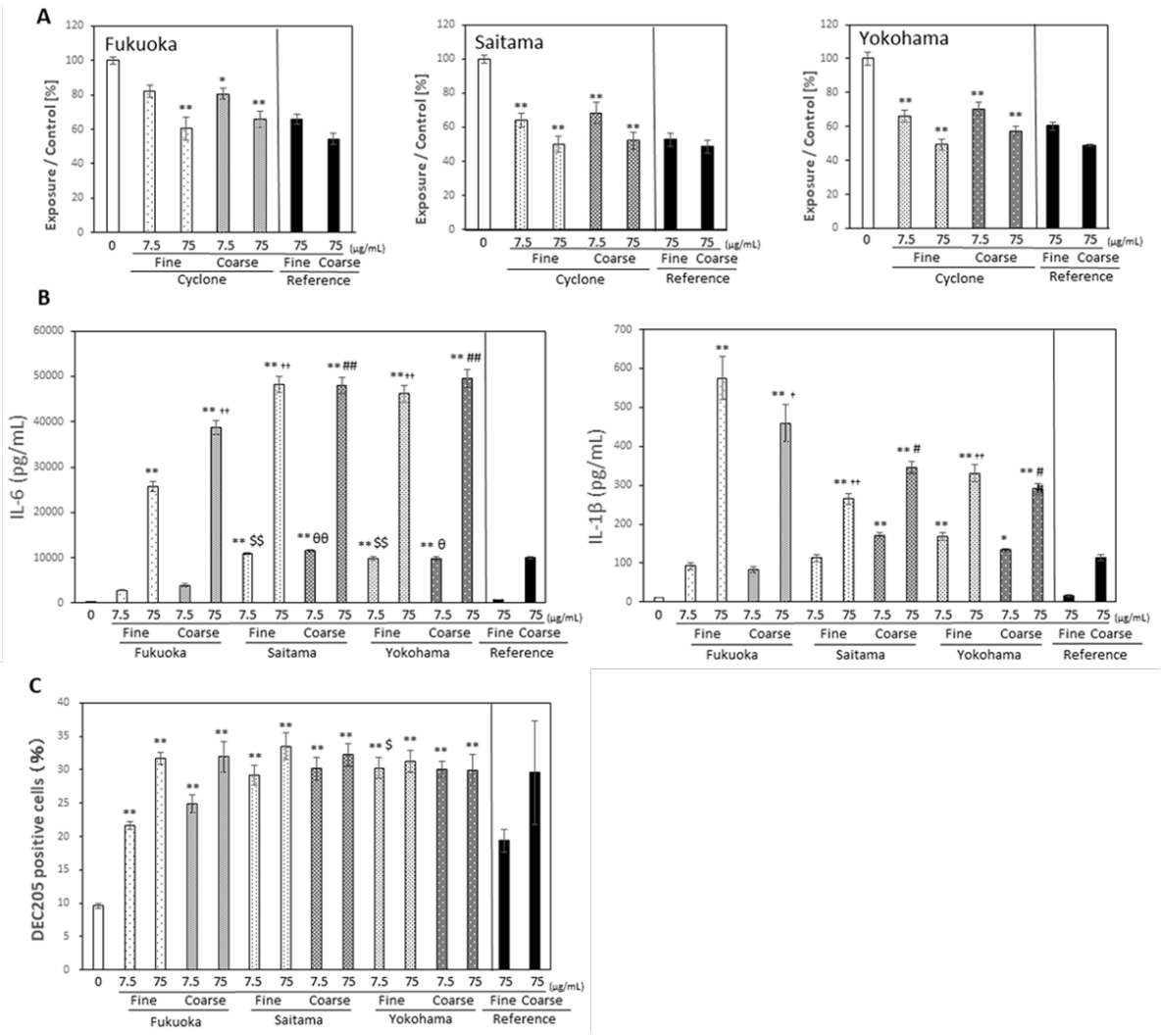

Figure 3. (A): Effects of ambient particles and reference particles on the viability of APCs. Data are presented as the percentage of the viability of the control. (B):IL-6 and IL-1β production from APCs in response to ambient particles and reference particles. (C): Effects of ambient particles and reference particles on the DEC205 expression of APCs. Date are presented as positive cells expressed % events. Date are mean ± standard error of the mean (SEM) of 4 individual cultures. *P<0.05, **P<0.01 vs. 0 μg/mL, $P<0.05, $$P<0.01 vs. Fine particles at Fukuoka at 7.5 μg/mL, †P<0.05, ††P<0.01 vs. Fine particles at Fukuoka at 75

μg/mL, $^{\theta}P<0.05$, $^{\theta\theta}P<0.01$ vs. Coarse particles at Fukuoka at 7.5 μg/mL, $^{\#}P<0.05$, $^{\#\#}P<0.01$ vs. Coarse particles at Fukuoka at 75 μg/mL..

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