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Aqueous and organic extract of PM2.5 collected in different seasons and cities of Japan differently affect respiratory and immune systems

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1 **Aqueous and organic extract of PM_{2.5} collected in different seasons and cities of**

2 **Japan differently affect respiratory and immune systems**

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10 **Capsule:** Respiratory health effects of PM_{2.5} extracts depend on their components

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18 **ABSTRACT** (278 words)

19 Particulate matter with diameters $<2.5 \mu\text{m}$ (i.e., $\text{PM}_{2.5}$) has multiple natural and
20 anthropological sources. The association between $\text{PM}_{2.5}$ and the exacerbation of
21 respiratory allergy and asthma has been well studied, but the components of $\text{PM}_{2.5}$ that
22 are responsible for allergies have not yet been determined. Here, we elucidated the effects
23 of aqueous and organic extract of $\text{PM}_{2.5}$ collected during four seasons in November 2014–
24 December 2015 in two cities (Kawasaki, an industrial area and Fukuoka, an urban area
25 affected by transboundary pollution matter) of Japan on respiratory health. Ambient $\text{PM}_{2.5}$
26 was collected by high-volume air samplers and extracted into water soluble and lipid
27 soluble components. Human airway epithelial cells, murine bone marrow-derived
28 antigen-presenting cells (APC) and splenocytes were exposed to $\text{PM}_{2.5}$ extracts. We
29 measured the cell viability and release of interleukin (IL)-6 and IL-8 from airway
30 epithelial cells, the DEC205 and CD86 expressions on APCs and cell proliferation, and
31 TCR and CD19 expression on splenocytes. The water-soluble or aqueous extracts,
32 especially those from Kawasaki in fall, had a greater cytotoxic effect than the lipid-
33 soluble or organic extracts in airway epithelial cells, but they caused almost no pro-
34 inflammatory response. Extract of fall, especially the aqueous extract from Fukuoka,
35 increased the DEC205 and CD86 expressions on APC. Moreover, aqueous extracts of fall,

36 summer, and spring from Fukuoka significantly increased proliferation of splenocytes.
37 Organic extract of spring and summer from Kawasaki significantly elevated the TCR
38 expression, and organic extract of summer from Kawasaki decreased the CD19
39 expression. These results suggest that PM_{2.5} extract samples are responsible for
40 cytotoxicity in airway epithelial cells and for activating APCs and T-cells, which can
41 contribute to the exacerbation of respiratory diseases such as asthma. These effects can
42 differ by PM_{2.5} components, collection areas and seasons.

43

44

45

46 Introduction

47 According to a World Health Organization (WHO) factsheet issued in 2016, air
48 pollution is causing alarming health hazards around the world, and approx. 88% of the
49 premature deaths due to air pollution have occurred in low- and middle-income countries
50 (<http://www.who.int/mediacentre/factsheets/fs313/en/>). Several epidemiological studies
51 have shown a close association between ambient particulate matter (PM) in the air and
52 mortality (Lo et al. 2017, Zeng et al. 2017). Silva et al. (2013) have estimated that 2.1
53 million premature respiratory deaths are due to cardiopulmonary diseases and lung cancer
54 related to anthropogenic PM with diameters $<2.5 \mu\text{m}$ (i.e., $\text{PM}_{2.5}$). Their study also
55 indicated that mortality due to $\text{PM}_{2.5}$ in East Asia and North America has increased
56 recently due to the anthropogenic emission burden and partially due to climate change.

57 Air pollution and its ability to increase respiratory disorders such as asthma have
58 been studied at various cities in different European countries and United States (Pope and
59 Dockery 2006). For example, an epidemiological study demonstrated that $\text{PM}_{2.5}$
60 exacerbates nasal inflammation in asthmatic children (Nikasinovic et al. 2006). Mirabelli
61 et al. (2016) have described a model which derived exacerbation of asthma in asthmatic
62 individuals may begin to increase when the $\text{PM}_{2.5}$ level is $\geq 7.07 \mu\text{g}/\text{m}^3$, and also the
63 prevalence of asthma symptoms increases by 0.5% with each $1.0 \mu\text{g}/\text{m}^3$ increase in $\text{PM}_{2.5}$.

Naser et al. (2008) have showed that the $PM_{2.5}$ level in an urban area of Saitama, Japan is profoundly affected by the $PM_{2.5}$ from vehicular sources. Gautam et al. (2016) have compared multiple Asian cities in which high levels of $PM_{2.5}$ were generated mostly from cooking and heating with solid fuel and vehicular movements. Liang et al. (2016) have reviewed multiple cities' $PM_{2.5}$ sources and observed that industrial activities, coal combustion, vehicular sources, soil crust, biomass burning, dust storm and more were the main sources of $PM_{2.5}$ emission.

$PM_{2.5}$ can be transported by air to distant locations. Some parts of Japan have suffered from transboundary pollution such as urban and industrialized particulate matter (Pan et al. 2016). Since Japan is one of the developed countries where asthma and respiratory allergies are consistently increasing (Fukutomi et al. 2011), it is of concern that $PM_{2.5}$ from both inside and outside the country affects the increasing rates of respiratory diseases.

Several studies demonstrated that $PM_{2.5}$ extracts have detrimental effects on respiratory health (Cachon et al. 2014; Alfaro-Moreno et al. 2009). Fuentes-Mattei et al. (2010) have suggested that organic extract of $PM_{2.5}$ possibly suppress the role of pregnane X receptor (PXR) and CYP3A5 on human epithelial cells which trigger an inflammatory response. An epidemiological study demonstrated that in winter, the outbreaks of

respiratory allergy and asthma are higher than those in other seasons (Habre et al. 2014).
Kurai et al. (2016) reported that winter PM samples from western Japan augmented
respiratory allergy symptoms in mice. Takemura et al. (2016) found no correlation
between seasonal respiratory symptoms' variation and common allergens as pollens,
house dust mites, molds, and dog or cat dander. They suggested that the worsening of
these symptoms may be associated with environmental conditions and pollution.

However, very few studies have identified the precise correlations between
characteristics of seasonally variable $PM_{2.5}$ and their effects on the respiratory and
immune systems. The characteristics of ambient $PM_{2.5}$ vary with location and season, and
so do their effects (Mirowsky et al. 2015). However, the contributing factors of $PM_{2.5}$ for
respiratory allergy and their biological responses have not been fully understood. It is
necessary to determine whether different types of components of seasonally variable
ambient $PM_{2.5}$ extracts affect the human respiratory and immune systems.

We conducted the present study to identify the association between seasonal
variations of $PM_{2.5}$ and the effects on respiratory allergy and inflammation in two cities
of Japan. First, aqueous and organic extracts of $PM_{2.5}$ were collected from Kawasaki (an
industrial area) and Fukuoka (an urban area affected by transboundary PM) during the
spring, summer, fall and winter seasons. We then exposed human airway epithelial cells,

100 murine bone marrow-derived antigen-presenting cells (APC), and murine splenocytes to
101 the aqueous and organic extract of PM_{2.5}. We examined the cell viability, proliferation,
102 cytokines, and cell surface markers associated with respiratory allergy and inflammation.
103 We also characterized possible contributing components of PM_{2.5} that affect respiratory
104 diseases such as asthma.

105

106 **Material and methods**

107 *PM_{2.5} sampling sites*

108 The city of Kawasaki has long been one of the premium industrial and business hubs in
109 Kanagawa Prefecture, a coastal prefecture south of Tokyo. It is known internationally for
110 its global industrial enterprises. Honda et al. (2017) demonstrated that a PM_{2.5} extract
111 from Kawasaki had a pro-inflammatory effect on airway cells and activated immune cells,
112 causing respiratory allergy.

113 In contrast, the city of Fukuoka is situated in southwestern Japan, on an island
114 different from Japan's largest/main island, at approx. 545 miles (878 km) from Kawasaki.
115 Fukuoka Prefecture has suffered from transboundary emission from China. Emissions
116 from China are most likely to be transported to Japan during the monsoon season, i.e.,
117 winter and spring (Yoshino et al. 2016). Moreover, as the city of Fukuoka is one of the

118 largest cities in Japan and also a commercial and industrial hub, the local emissions are
119 also a significant contributor to the ambient air.

120

121 *PM_{2.5} sampling and extraction*

122 A high-volume air sampler (Sibata Scientific Technology, Saitama, Japan) equipped with
123 a PM_{2.5} impactor (Tokyo Dylec, Tokyo) was installed at one collection point of each cities
124 of Kawasaki and Fukuoka for 4–5 days at a flow rate of 740 L/min.

125 During each of the four seasons, namely spring (March, 2015), summer (July–August,
126 2015), fall (Nov.–Dec., 2014), and winter (January, 2015), ambient PM_{2.5} was collected
127 by the air samplers' quartz-fiber filters (one filter/each season) and later divided for the
128 preparation of aqueous extraction and organic extraction. Water-soluble fractions were
129 extracted from half-cut PM_{2.5}-collected quartz-fiber filters using sonication and distilled
130 water (deionized and RNase free, Wako Pure Chemical Industries, Osaka, Japan) at 65°C.
131 The temperature helps to stop bacterial growth in the extract (Ministry of Health and
132 Welfare Ordinance, 1951). The aqueous crude extracts were centrifuged.

133 Lipid-soluble fractions were extracted from the rest of the half-cut filters using
134 Soxhlet and dichloromethane (dioxin analysis-grade, Kanto Chemicals, Tokyo) for 16 h.
135 The organic crude extracts were centrifuged at 4,800 rpm 60 min. Both fractions were
136 evaporated and then set under a gentle stream of nitrogen gas flow until they were dry.
137 Blank filters were also handled in the same manner.

The dried extracts were resuspended in water/dimethylsulfoxide (DMSO, molecular biology-grade, Wako) (1:1) to make the organic extracts and the aqueous extracts at a concentration of 75 mg/mL using weight of PM_{2.5} collected on filter and stored at 4°C in darkness until the bio-assay. The reason we used DMSO and water in both extracts as solvent is to make the background same as far as possible both in organic and aqueous extracts and to improve solubility of both extracts after dry by adding DMSO and water.

At the time of the bioassay, organic or aqueous extracts of PM_{2.5} were diluted to give a final concentration of 0, 7.5, 22.5, or 75 µg/mL in media (0.05% DMSO, 0.05% water). The doses were selected based on our prior literature (Honda et al. 2017).

The percentages of mass concentration of organic extracts and aqueous extracts in that of PM_{2.5} mass on filter from Kawasaki were 10.8% and 63.4% (spring), 7.0% and 44.3% (summer), 17.3% and 51.4% (fall), 12.4% and 38.6% (winter), respectively. The percentages of mass concentration of organic extracts and aqueous extracts in that of PM_{2.5} from Fukuoka were 17.4% and 59.7% (spring), 9.3% and 44.8% (summer), 14.2% and 58.5% (fall), 13.4% and 56.8% (winter), respectively. Corresponding dose of total PM_{2.5} mass by using data on extraction efficiency is shown in suppl. table 1.

Chemical and biological analyses

Chemical characterization was done following the protocol from the Japan Ministry of the Environment. The heavy metal analysis in organic and aqueous extracts was done by inductively coupled plasma mass spectrometry (ICP-MS). The analysis of ions in organic

and aqueous extracts was performed using ion chromatography, and that of polycyclic aromatic hydrocarbons (PAHs) only in organic extract was done by Gas Chromatography/Mass Chromatography (GC/MS), and that of elemental carbon (EC) and organic carbon (OC) in organic and aqueous extracts were done by the interagency monitor of protected visual environments (IMPROVE) method. To measure the biological components of the PM_{2.5} extracts, we performed an endotoxin test and a β -glucan test (both from Associates of Cape Cod, Falmouth, MA, USA) per the manufacturer's instructions.

Cell culture and PM_{2.5} exposure

Airway epithelial cells

The airway epithelial cell line BEAS-2B was purchased from the European Collection of Cell Cultures (Salisbury, Wiltshire, UK) and maintained by subculture in 37°C at 5% CO₂ in LHC-9 medium. Cells were exposed to an aqueous or organic extract of PM_{2.5} at the concentrations of 0, 7.5, 22.5 or 75 μ g/mL for 24 hr. We measured the cell viability and the secretion of the cytokines IL-6 and IL-8 from the airway epithelial cells after 24 hr of exposure to the aqueous or organic extract by conducting a Water Soluble Tetrazolium Salts (WST-1) assay and quantikine Enzyme Linked Immuno Sorbent Assay (ELISA), respectively.

177

178 Immune cells (APCs and splenocytes)

179 Single-cell suspensions at the final density of 1.0×10^6 /mL for APCs and splenocytes
180 were prepared after sacrificing NC/NgaTendCrlj male mice (Chares River Japan, Osaka,
181 Japan) by cervical dislocation and exsanguination. The procedures used in all animal
182 studies were approved by the Animal Research Committee at Kyoto University.

183 APCs were maintained in RPMI 1640 basal medium (Invitrogen, Grand Island,
184 NY) containing Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF) to
185 induce dendritic cell during cell culture. Splenocytes were also incubated in basal RPMI
186 1640 medium. APCs and splenocytes were exposed to the same doses of aqueous or
187 organic extracts of PM_{2.5} as those used for the airway epithelial cells, at the concentration
188 of 0, 7.5 and 75 μ g/mL. The details of the cell preparations were as described previously
189 (Chowdhury et al. 2017).

190 After 24 hr of exposure to each extract, we measured the cell viability and the
191 expression of two cell surface molecules of APCs (i.e., DEC205, a dendritic cell marker;
192 CD86, an APC marker) by performing a WST-1 assay and fluorescence-activated cell
193 sorter (FACS) analysis, respectively. The cell viability, cell proliferation, and the
194 expression of two cell surface molecules of splenocytes (T-cell receptor [TCR], a T-cell

marker; CD19, a B-cell marker) were measured by a WST-1 assay, 5-Bromo-2'-deoxyuridine (BrdU), ELISA, and FACS analysis, respectively.

Experimental protocol

Cell Viability

We measured the cell viability by WST-1 assay using the Premix WST-1 Cell Proliferation Assay System (TaKaRa Bio, Shiga, Japan). WST-1 reagent was added to each well of a 96-well plate in 1/10 of volume of cell suspension and mixed well by gently rocking the plate. Cells were incubated with WST-1 reagent at 37°C for 3 hr (BEAS-2B), 30 min (APC) and 4 hr (splenocytes). After the incubation, absorbance was measured on an iMark Microplate Absorbance Reader (Bio Rad Laboratories, Hercules, California) with the wavelength at 450 nm and a reference wavelength at 630 nm. The results are expressed as the percentage of viable cells compared to untreated cells (0 µg/mL).

Quantitation of Pro-Inflammatory Proteins in the Culture Supernatants

After exposure to extracts of PM_{2.5}, the medium was harvested and centrifuged at 300g for 5 minutes to remove floating cells. The supernatants were stored at -80°C for further analysis. The levels of IL-6 and IL-8 (Thermo Scientific, Waltham, Massachusetts) in the

213 supernatants were measured by ELISA, according to the manufacturer's instructions.
214 Absorbance was measured on the iMark Microplate Absorbance Reader with the
215 wavelength set at 450 nm and a reference wavelength at 550 nm. The detection limits of
216 IL-6 and IL-8 were <1 pg/mL and <2 pg/mL respectively.

217

218 Fluorescence-Activated Cell Sorter Analysis

219 For the FACS analysis, the following monoclonal antibodies were used: Mouse BD Fc
220 Block purified anti-mouse CD16/CD32 (Becton Dickinson), DEC205 (NLDC-145, PE-
221 conjugated; BioLegend, San Diego, California), Rat IgG2a, k Isotype Control (RTK2758,
222 PE-conjugated; BioLegend), CD86 (GL-1, PEconjugated; Becton Dickinson), Rat IgG2a,
223 k Isotype Control (R35-95, PE-conjugated; Becton Dickinson), Hamster AntiMouse
224 TCR-βChain (H57-597, FITC-conjugated; Becton Dickinson), Hamster IgG2, l1 Isotype
225 Control (Ha4/8, FITC-conjugated; Becton Dickinson), Rat Anti-Mouse CD19 (1D3, PE-
226 conjugated; Becton Dickinson), and Rat IgG2a, k Isotype Control (R35-95, PE-
227 conjugated; Becton Dickinson). After the exposure of the PM_{2.5} extracts, the cells were
228 resuspended in 50 μL phosphate-buffered saline (PBS) with 0.3% bovine serum albumin
229 and 0.05% sodium azide (Wako) and then incubated with 0.05 to 1 μg of each antibody
230 for 45 minutes at 4°C. After incubation, the cells were washed, and the fluorescence was

measured by a FACSCalibur (Becton Dickinson). For each sample, fluorescence data of 10,000 cells were collected, and positive cells expressed as the percentage events were calculated.

Cell Proliferation

Cell proliferation was measured with a Cell-ProliferationELISA Kit (Roche Molecular Biochemicals, Mannheim, Germany), according to the manufacturer's instructions. This technique is based on the incorporation of the pyrimidine analogue BrdU instead of thymidine into the DNA of proliferating cells. 5-Bromo-2'-deoxyuridine incorporated into DNA is measured by a sandwich-type enzyme immunoassay using monoclonal anti-BrdU antibodies. Splenocytes were exposed to extracts of PM_{2.5} for 72 hours, and cell proliferation was measured by adding BrdU to each well 20 hours before the measurement. Absorbance was measured on the iMark Microplate Absorbance Reader with the wavelength set at 450 nm and a reference wavelength at 630 nm.

Statistical analysis

The data are presented as the mean $\pm$ standard error of the mean (SEM) for each experimental group (n=4). Differences among groups were analyzed using Tukey's test

(Excel Statistics 2012, Social Survey Research Information, Tokyo). A p -value <0.05 was considered significant. Relationships between components in aqueous extract and cell viability were tested using Pearson's correlation.

Results

Ions, Metals, OC, EC and PAHs in the Aqueous and Organic PM_{2.5} Extracts

The chromatography results revealed the ions, metals, and OC and EC from both the aqueous and organic extract samples. The ion and metal concentrations are illustrated for the extracts from Kawasaki (Fig. 1A) and Fukuoka (Fig. 1B). It was noticeable that the aqueous extracts contained higher concentrations of ions, among which SO_4^{2-} , NO_3^- and NH_4^+ were particularly high in both cities. On the other hand, Na, K and Ca were high in aqueous extracts during almost all seasons at both locations. OC3 was highest in the concentration in both cities especially in organic fall extract followed by OC2. Level of EC1, EC2 and EC3 were low compared to OC (Figure 2 A,B)

As shown in Figure 2C, the concentrations of PAHs including Benzo[b]fluoranthene, Benzo[e]pyrene, Indeno[1,2,3-cd]pyrene, and Benzo[g,h,i]pyrene were particularly high in the summer and fall samples from Kawasaki.

The endotoxin level was under the detection limit (0.0078 EU/mL) for almost all samples. β -glucan was detected in the aqueous extracts from both cities. The β -glucan levels in aqueous extracts (75 μ g/mL) from Kawasaki collected in the spring, summer, fall and winter were 494.60, 648.26, 330.56 and 359.63 pg/mL, respectively. Those from Fukuoka collected in the spring, summer, fall and winter were 473.84, 764.53, 436.46 and 181.06, respectively. The β -glucan level in a blank filter was 5.97 pg/mL, and the level in almost all of the organic extracts from both cities were under the detection level (data not shown).

The PM_{2.5} Extracts' Effects on Airway Epithelial Cells

Cell viability

Under exposure to the 75 μ g/mL aqueous extract of PM_{2.5} from Kawasaki, the viability of the airway epithelial cells was significantly decreased in spring (16.43% lower than the control value), summer (21.14% lower) and fall (31.68% lower) but not in the winter (Fig. 3A). The organic extract exposure at the same dose did not show much variation with seasons, and cell viability was only significantly higher with aqueous extract (Fig. 3A).

At dose 22.5 μ g/mL exposure, the aqueous extracts collected in the summer and

285 fall lowered the cell viability, whereas the organic extracts collected in the spring, summer
286 and winter increased the cells' viability (Suppl. Fig. S1C). The 7.5 µg/mL aqueous extract
287 collected in the fall showed a detrimental effect on cell viability, whereas the organic
288 extract at the same dose did not show any effect compared to the control (Suppl. Fig.
289 S1A).

290 The aqueous extracts from Fukuoka did not show any effect, whereas the organic
291 extract at 75 µg/mL increased the cell viability compared to the control in all seasons (Fig.
292 3B, Suppl. Fig. S1B,D).

293

294 *The Secretion of the Cytokines IL-6 and IL-8*

295 The Kawasaki extracts

296 No extracts of Kawasaki were able to make any significant difference in IL-6 release at
297 75µg/mL dose (Fig. 4A). Similarly, the lower doses as 22.5 and 7.5 µg/mL also did not
298 have any significant effect on IL-6 expression (Suppl. Fig. S2A,C).

299 Regarding the IL-8 secretion from the airway epithelial cells, the aqueous extracts
300 did not have any effect. In contrast, the organic extracts from summer lowered the IL-8
301 secretion compared to the control (Fig. 4C). Similarly, at 22.5 µg/mL, the organic extracts
302 from spring and summer samples lowered the IL-8 secretion slightly (Suppl. Fig. S3A,C).

303

304 The Fukuoka extracts

305 The organic extracts from Fukuoka had no effect on IL-6 and IL-8 secretion (Fig. 4B,D,
306 Suppl. Fig. S3B,D)

307

308 *The PM_{2.5} Extracts' Effects on APCs*

309 Cell viability: Kawasaki extracts

310 The cell viability of the APCs was significantly lowered by exposure to the 75 µg/mL
311 dose of aqueous extracts of Kawasaki from all seasons compared to control: 43.46%
312 reduced for spring, 55.41% for summer, 49.11% for fall and 36.05% for winter (Fig. 5A).
313 The lower dose of 7.5 µg/mL also showed a reducing effect (Suppl. Fig. S4A).

314

315 Cell viability: Fukuoka extracts

316 Similarly, the 75 µg/mL aqueous extracts of Fukuoka from all seasons also significantly
317 decreased the viability of APCs (Fig. 5B). The 7.5 µg/mL dose of the fall and winter
318 samples also significantly lowered the viability compared to the control (Suppl. Fig. S4B).
319 The 75 µg/mL dose of the organic extract did not reduced the cell viability compared to
320 control. The 7.5 µg/mL organic extract showed no change in viability.

321

322 Cell surface molecules

323 Kawasaki aqueous and organic extract did not change the number of DEC205 positive
324 cells at a 75 µg/mL (Fig. 6A). Fukuoka aqueous extracts at 75 µg/mL also showed a higher
325 expression of DEC205-positive cells in fall. The Fukuoka organic extracts showed no
326 effect on the number of DEC205-positive cells (Fig. 6B). The lower dose of 7.5 µg/mL
327 failed to cause any changes in DEC205 expression (Suppl. Fig. S5A,B).

328 The 75 µg/mL dose of both the aqueous and organic extracts collected from
329 Kawasaki in the summer significantly increased the CD86-positive cell expression (Fig.
330 6C). Moreover, the 7.5 µg/mL summer and fall organic extracts also significantly
331 increased this expression compared to the control (Suppl. Fig. 5C). The Fukuoka aqueous
332 extracts collected in the fall and winter significantly increased the CD86 expression (Fig.
333 6D), and the Fukuoka organic extract collected in the summer induced CD86 expression.
334 Similarly, at the lower dose of 7.5 µg/mL, the Fukuoka aqueous extracts from fall and
335 winter caused elevated CD86 expression, and the organic extracts from all the seasons
336 also increased it (Suppl. Fig. S5D).

337

338 *The PM_{2.5} Extracts' Effects on Splenocytes*

339 Cell viability

340 The 75 µg/mL Kawasaki aqueous extracts from all four seasons lowered the splenocytes'
341 cell viability: spring (decreased 68.17%), summer (69.64%), fall (81.67%) and winter
342 (23.90%) (Fig. 7A). Except for winter, these reductions in cell viability were significant
343 compared to the control as well as compared to the corresponding seasons' organic extract
344 at the same dose. At the lower dose (7.5 µg/mL), no noticeable changes were observed
345 (Suppl. Fig. S6A). The Kawasaki organic extracts at all doses and from all seasons had
346 no significant effect on the splenocytes' cell viability.

347 The 75 µg/mL Fukuoka aqueous extracts had a detrimental effect on the
348 splenocytes' viability: spring (45.47% reduction), summer (34.30%), fall (47.73%) and
349 winter (46.09%) (Fig. 7B). At the lower dose, the Fukuoka aqueous extracts produced no
350 changes (Suppl. Fig. S6B).

351

352 Cell proliferation: Kawasaki extracts

353 After 3 days of incubation with the 75 and 7.5 µg/mL doses of Kawasaki aqueous extract,
354 the splenocytes' proliferation was not significantly different from that of the control. The
355 Kawasaki organic extracts at both doses also had little effect on the proliferation. However,
356 the cells' proliferation in winter aqueous extracts was significantly higher than organic

extract of the same (Fig. 7C). The proliferation results for the fall and winter samples of aqueous extracts were also significantly higher than those of the same dose (7.5 $\mu\text{g/mL}$) of organic extracts (Suppl. Fig. S6C).

Cell proliferation: Fukuoka extracts

Unlike the Kawasaki extracts, the Fukuoka samples had significant effects on cell proliferation (Fig. 7D). Interestingly, while the aqueous samples from spring (47.2%), summer (50.2%), and fall (76.5%) significantly increased the proliferation, the organic extracts lowered the proliferation; by season: summer (23.43%), fall (31.2%) and winter (36.3%) compared to the control. The 7.5 $\mu\text{g/mL}$ dose aqueous samples increased the proliferation at fall sample, but the organic extracts from spring, summer and fall lowered the proliferation compared to aqueous sample samples (Suppl. Fig. S6D).

Cell surface molecules

The aqueous extracts from Kawasaki did not have much effect on the TCR expression, with the exception of the 75 $\mu\text{g/mL}$ fall sample (Fig. 8A), which significantly increased the TCR expression. At the lower doses of 7.5 $\mu\text{g/mL}$, no effect was observed (Suppl. Fig. S7A). The spring and summer 75 $\mu\text{g/mL}$ organic extracts also increased the

375 TCR expression (Fig. 8A), whereas the 7.5 µg/mL extracts showed no noticeable effects
376 (Suppl. Fig. S7A). None of the extracts from Fukuoka produced any difference in TCR
377 expression, from any season or at any doses (Fig. 9B, Suppl. Fig. S7B).

378 Regarding the CD19 expression, 75 µg/mL Kawasaki aqueous extracts from the
379 spring and summer significantly decreased the CD19 expression (Fig. 8C), and the 7.5
380 µg/mL extracts produced no difference (Suppl. Fig. S7C). Besides, Kawasaki organic
381 extract only from summer lowered CD19 expression (Fig. 8C). None of the Fukuoka
382 aqueous or organic extracts had any effect on the CD19 expression, at any doses (Fig. 8D,
383 Suppl. Fig. S7D).

384

385 *Correlation between cell viability and the PM_{2.5} components*

386 Determining the correlations between components of PM_{2.5} extracts at 75 µg/mL dilution
387 and cell viability is important to understand the potential cytotoxicity of the extract
388 samples. As our results indicated that the aqueous extracts affected the viability of the
389 airway epithelial cells, we evaluated the Pearson's correlation coefficients for the cell
390 viability and extract components. Our analysis revealed negative correlations between the
391 viability of BEAS-2B cells and several heavy metals in the aqueous extracts including
392 Mn, Mo, Zn, Co and Ni, W, Cr, Cu, Fe, Al and more (Table 1).

393

394 **Discussion**

395 In the present study, the aqueous extracts, especially those collected during fall in
396 Kawasaki, had more cytotoxic effects than the organic extracts in airway epithelial cells,
397 but none of the extracts cause any pro-inflammatory response. The aqueous extracts from
398 Fukuoka, especially those collected during fall, increased the expressions of DEC205 and
399 CD86 on APCs. Aqueous extracts from both cities significantly decreased the viability of
400 splenocytes apart from the winter extract from Fukuoka. In addition, the Fukuoka aqueous
401 extract samples from spring, summer, and fall significantly increased the proliferation of
402 splenocytes. The Kawasaki organic extracts collected during the spring and summer
403 significantly elevated the TCR expression, whereas those collected during the summer
404 decreased the CD19 expression. Negative correlations were observed between the
405 viability of airway epithelial cells and metal components in the aqueous extracts such as
406 Mn, Mo, Zn, Co and Ni .

407 To understand the active/direct effects of PM_{2.5} on respiratory damage, the
408 cytotoxicity of PM_{2.5} in airway epithelial cells is a key issue. In our study, the BEAS-2B
409 cells suffered cytotoxicity from the Kawasaki aqueous extract collected in fall. In
410 previous studies, PM_{2.5} (Zhou et al. 2015) and its extract (Rodríguez-Cotto et al. 2014)

both clearly lowered cell viability. In a study of an urban area of Puerto Rico (Fuentes-Mattei et al. 2010), a polar organic extract lowered the viability of BEAS-2B cells dose-dependently whereas a non-polar organic extract showed no significant effect.

In contrast, Huang et al. (2014) have shown that an aqueous extract of PM_{2.5} had a toxic effect on airway epithelial cells. A study of components of PM_{2.5} aqueous extracts collected from Baghdad, Iraq showed that trace elements such as V and Ni correlated with the reactive oxygen species (ROS) production of alveolar macrophages (Hamad et al. 2016). Our present findings indicated that aqueous extracts from Kawasaki, especially those collected during the fall, contain components that affect cellular viability. It is possible that the decrease in cell viability induced by these components permit the invasion of inhaled xenobiotics including allergens, which can contribute to the exacerbation of respiratory diseases such as asthma. On the other hand, organic extracts from Fukuoka increased the cell viability compared to the control in all seasons, which may relate with carcinogenic effects of PM_{2.5} (Bayram H et al 2013, Bayram H et al, 2006).

IL-6 and IL-8 are the two most prominent pro-inflammation mediators (Martin et al. 1997; Richman-Eisenstat et al. 1993). Our previous study (Honda et al. 2017) based on Kawasaki and Fukuoka have shown that organic extracts had more pro-inflammatory

effect via IL-6 than aqueous extracts. As the IL-6 and IL-8 expression did not increase at all in the present study, we did not observe any noticeable pro-inflammatory effect by any extract.

Rodriguez-Cotto et al. (2014) have found that both IL-6 and IL-8 were decreased when BEAS-2B cells were exposed to an aqueous extract of PM_{2.5}. Although they noted that the result was dependent on the complex mixture of components, they could not explain the underlying mechanism. As the components of PM_{2.5} extracts differ by collection days, the components can cause different health effects. For instance, it has been seen that the after festival days the mortality and morbidity become high due to fireworks and associated causes (Thakur et al. 2010). Fireworks and other burning activities produce significantly higher PM_{2.5} as well as black carbon (Lin et al. 2016).

Here we observed that the aqueous extracts from both Kawasaki and Fukuoka significantly lowered the viability of APCs, as was observed for BEAS-2B cells. DEC205-positive cells was high in the fall extract of Fukuoka. DEC205 is an important cell surface molecule for antigen uptake, processing, and presentation of the antigen by dendritic cells (Tel et al. 2011). Previous investigations indicated that environmental pollutants including PM_{2.5} extract, carbon black nanoparticles, Asian sand dust particles, and di-(2-ethylhexyl) phthalate can promote the maturation/activation and function of

DEC205 on APCs (Honda et al. 2017; Koike et al. 2008, 2009; Honda et al. 2014). From our experimental results, it can be concluded that aqueous PM_{2.5} extracts from Fukuoka induce the maturation/activation of DEC205. The components contained in aqueous extracts such as metals may contribute to DEC205 expression.

On the other hand, the number of CD86-positive cells was increased in the summer sample in both the aqueous and organic extracts from Kawasaki in the present study. CD86 is a crucial cell surface molecule for antigen presentation for asthmatics, and it is associated with late reaction to allergens (Balbo et al. 2001). CD86 is an important and sensitive cell surface molecule for PAHs, as we recently have observed (Chowdhury et al. 2017). Moreover, our previous studies revealed aqueous extract from Kawasaki and Fukuoka were able to increase the CD86 expression on the cells (Honda et al, 2017). Hulette et al, 2005 showed that aqueous soluble chemicals including nickel sulphate and hydroquinone are capable to induce CD86 expression in human dendritic cells. Taking the above findings together, it seems that PM_{2.5} can activate APCs via DEC205 and CD86, and this phenomenon could be related to their enhancing effects on allergic diseases or responses.

The viability of splenocytes was also diminished in the aqueous extract samples from both Kawasaki and Fukuoka, as we also observed for the BEAS-2B cells.

Interestingly, the aqueous extracts from Fukuoka significantly increased the splenocytes' proliferation but decreased their viability. This may be due to the longer incubation time (72 hr) for cell proliferation than cell viability assay (24 hr). Moreover, the ability of metal components to cause aberrant cell proliferation and altered apoptosis is well known (Waalkes et al. 2000). PAHs were also found to be responsible for cell proliferation via AhR ligand activation in mouse liver cells (Chramostová et al. 2004), whereas they suppressed the growth of B and T cells (Allan et al. 2006; Davila et al. 1996).

In our study, wherever TCR-positive cells increased in number, CD19 tended to decrease in the same cases. For instance, the number of TCR-positive cells was increased by organic extract of spring and summer while and the number of CD19-positive cells was decreased by the same extracts. Hence, in the organic extract of spring and summer of Kawasaki city, significantly more T lymphocytes than B lymphocytes were activated. As organic summer extract of Kawasaki had high PAH concentration we suspect the PAH alone (in case of summer) and synergistically with other component (in case of spring) can induce TCR expression. Previous publication also indicated the responsibility of organic extracts from PM_{2.5} for inducing TCR positive cell (Honda et al, 2017). However, we cannot exactly identify the responsible factor yet. Thus, the aqueous extracts proved to be more cytotoxic to B cells while organic extracts may responsible for T cell activation.

To analyze the correlation between biological responses and components of PM_{2.5} correctly, larger regression coefficient is needed. In brief, large variation of biological responses is required. It is clear from our present findings that the responsible factor for cytotoxicity of PM_{2.5} extract affect all three types of cells, i.e., human airway epithelial cells, murine bone marrow-derived APCs, and splenocytes. A notable finding is the decrease of the viability of airway epithelial cells induced by the components in the aqueous extracts, because it is possible that a lower viability of airway epithelial cells will permit the invasion of inhaled xenobiotics including allergens. Therefore, we investigated the correlation between viability of airway epithelial cells and the components in aqueous extracts including ion, metal, EC and OC.

A negative correlation was revealed between some heavy metals and cell viability, and we selected the five most highly correlated metals for a discussion of their cytotoxicity: Mn, Mo, Zn, Co and Ni (Table 1). Previous study Honda et al. (2015) showed that TC50 values (concentration that reduces cell viability to 50%) of Mn⁺² in airway epithelial cells were as low as 3.0μM. Mn has a proven neurological effect and also increased the risk of lung cancer (Mirmohammadi 2014). The mechanism of Mn cytotoxicity is likely to be associated with the formation of ROS in dopamine-producing cells (Stredrick et al. 2004), but Mn cytotoxicity in respiratory system has not yet been

studied. Ott et al. (2004) proposed that a chronic inhalation of Mo may induce subclinical alveolitis. Moreover, Honda et al. (2015) concluded that Ni and Zn are also responsible for low cell viability in a certain dose range, which supports our present findings of negative correlations for these two metals as well. An occupational health study established that Co associated with other metals has a respiratory effect as allergic hypersensitivity (Cugell et al. 1990). Our data showing that Mn, Mo, Zn, Co and Ni affect the airway agree with these previous findings.

Considering the effect of the differences in geographic areas on the viability of airway epithelial cells, Suvarupa and Baek (2016) pointed out that the heavy metal concentration is sometimes higher in industrial areas than residential or commercial areas. They noted that Mn comes into the air from soil and resuspended dust, industrial processes and break wear. On the other hand, Zn emissions are associated with industrial processes and break wear, the Co emission source is mostly coal combustion, and Ni emission is also from break wear. Mo is also an industrial pollutant frequently used in hard metals (Ott et al. 2004).

Kawasaki, as an industrial area, is thus more likely to contain higher concentrations of metal ions in its PM_{2.5} extracts than Fukuoka (Figs. 1), which can explain our observation of low viability of BEAS-2B cells in the Kawasaki samples.

519 We also found that the aqueous extracts collected from Kawasaki in the winter did
520 not lower the cell viability, as they contain fewer metals compared to other seasons.
521 Suvarupa and Baek also suggested that the PM_{2.5} concentration is high in the summer in
522 a few cases, mostly because of solar radiation and the consequent secondary aerosol
523 formation. Moreover, the easy suspension of crustal elements during the summer may
524 contribute to high concentrations of heavy metal in the ambient air. This may explain our
525 finding of fewer metals in the winter, which induces higher viability in airway epithelial
526 cells.

527 β -glucan did not appear to be a significant contributor to any of the present results.
528 β -Glucan was detected only in the aqueous extracts from both cities. The levels were high
529 in the summer: 764.53 pg/mL in Fukuoka and 648.26 pg/mL in Kawasaki. β -Glucan
530 profoundly increased the IL-6 and IL-8 expressions in airway epithelial cells *in vitro* and
531 in an animal model (Carmona et al. 2010, Neveu et al. 2011). It was also reported that the
532 cell viability of macrophages was significantly decreased at a 300 μ g/mL dose of β -glucan
533 (Chang et al. 2009). We did not observe any significance difference of IL-6 and IL-8
534 expression in the present study. As the level of β -glucan in our study was very low
535 compared to those of the previous studies, we suspect that the level of β -glucan in our
536 extracts failed to cause any noticeable changes in the cells.

Getting different extraction efficiency is a limitation of conventional method to collect particulate matter on filter paper. The extraction efficiency can largely depend on composition of PM_{2.5}. Moreover, the extraction of PM_{2.5} on filter cause loss of a part of components of PM_{2.5} and eventual difference of extraction efficiency among samples. To avoid the problem, new techniques without extraction may be needed to evaluate health effects of PM_{2.5} in future research.

Conclusion

Our results indicate that aqueous extracts, especially those collected in fall from Kawasaki, had more cytotoxic effect than organic extracts in airway epithelial cells, although the aqueous extracts caused almost no pro-inflammatory response. The correlation analysis showed that heavy metals such as Mn, Mo, Zn, Co and Ni in PM_{2.5} may be associated with airway epithelial degeneration.

Both the aqueous and organic extract collected during the summer from Kawasaki were capable of activating APCs via CD86 expression. In contrast, the fall Fukuoka aqueous extract activated APCs via the expression of both CD86 and DEC205. Aqueous extract of fall, summer and spring from Fukuoka significantly increased cell proliferation of splenocytes. Organic extract of spring and summer from Kawasaki probably activate

555 T-lymphocytes more than B-lymphocytes.

556 Therefore, in conclusion, the adverse effects of both the aqueous and organic
557 extracts of PM_{2.5} on respiratory health can occur via the activation of APCs and
558 concomitantly T cells, whereas metal components as Mn, Mo, Zn, Co and Ni in aqueous
559 extracts from industrial cities are cytotoxic to airway epithelial cells.

560

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564

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Fig. 1

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Suppl.
Fig. S1.

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Fig. S2.

Suppl. Fig. S4.

Suppl.
Fig. S7.

Suppl. Table 1. Corresponding dose of total PM_{2.5} mass by using data on extraction efficiency (µg/m³)

	Season	Kawasaki	Fukuoka
Aqueous	Spring	22.95	10.21
	Summer	18.12	10.39
	Fall	10.90	7.20
	Winter	9.42	11.47
Organic	Spring	3.91	2.98
	Summer	2.86	2.16
	Fall	3.67	1.75
	Winter	3.03	2.71