Aqueous and organic extract of $PM_{2.5}$ collected in different seasons and cities of Japan differently affect respiratory and immune systems

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

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ABSTRACT (278 words)

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

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

Introduction

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

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

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

71 $PM_{2.5}$ can be transported by air to distant locations. Some parts of Japan have
72 suffered from transboundary pollution such as urban and industrialized particulate matter
73 (Pan et al. 2016). Since Japan is one of the developed countries where asthma and
74 respiratory allergies are consistently increasing (Fukutomi et al. 2011), it is of concern
75 that $PM_{2.5}$ from both inside and outside the country affects the increasing rates of
76 respiratory diseases.

77 Several studies demonstrated that $PM_{2.5}$ extracts have detrimental effects on
78 respiratory health (Cachon et al. 2014; Alfaro-Moreno et al. 2009). Fuentes-Mattei et al.
79 (2010) have suggested that organic extract of $PM_{2.5}$ possibly suppress the role of pregnane
80 X receptor (PXR) and CYP3A5 on human epithelial cells which trigger an inflammatory
81 response. An epidemiological study demonstrated that in winter, the outbreaks of

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

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

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

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

Material and methods

$PM_{2.5}$ sampling sites

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

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

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

$PM_{2.5}$ sampling and extraction

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

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

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

159 and aqueous extracts was performed using ion chromatography, and that of polycyclic
160 aromatic hydrocarbons (PAHs) only in organic extract was done by Gas Chromatography/
161 Mass Chromatography (GC/MS), and that of elemental carbon (EC) and organic carbon
162 (OC) in organic and aqueous extracts were done by the interagency monitor of protected
163 visual environments (IMPROVE) method. To measure the biological components of the
164 $PM_{2.5}$ extracts, we performed an endotoxin test and a β-glucan test (both from Associates
165 of Cape Cod, Falmouth, MA, USA) per the manufacturer's instructions.
166
167 *Cell culture and $PM_{2.5}$ exposure*
168 Airway epithelial cells
169 The airway epithelial cell line BEAS-2B was purchased from the European Collection of
170 Cell Cultures (Salisbury, Wiltshire, UK) and maintained by subculture in 37°C at 5% CO_2
171 in LHC-9 medium. Cells were exposed to an aqueous or organic extract of $PM_{2.5}$ at the
172 concentrations of 0, 7.5, 22.5 or 75 μg/mL for 24 hr. We measured the cell viability and
173 the secretion of the cytokines IL-6 and IL-8 from the airway epithelial cells after 24 hr of
174 exposure to the aqueous or organic extract by conducting a Water Soluble Tetrazolium
175 Salts (WST-1) assay and quantikine Enzyme Linked Immuno Sorbent Assay (ELISA),
176 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 splenocites 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 μg/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

195 marker; CD19, a B-cell marker) were measured by a WST-1 assay, 5-Bromo-2'-
196 deoxyuridine (BrdU), ELISA, and FACS analysis, respectively.
197
198 *Experimental protocol*
199 Cell Viability
200 We measured the cell viability by WST-1 assay using the Premix WST-1 Cell Proliferation
201 Assay System (TaKaRa Bio, Shiga, Japan). WST-1 reagent was added to each well of a
202 96-well plate in 1/10 of volume of cell suspension and mixed well by gently rocking the
203 plate. Cells were incubated with WST-1 reagent at 37^{o}C for 3 hr (BEAS-2B), 30 min
204 (APC) and 4 hr (splenocytes). After the incubation, absorbance was measured on an
205 iMark Microplate Absorbance Reader (Bio Rad Laboratories, Hercules, California) with
206 the wavelength at 450 nm and a reference wavelength at 630 nm. The results are expressed
207 as the percentage of viable cells compared to untreated cells (0 μg/mL).
208
209 Quantitation of Pro-Inflammatory Proteins in the Culture Supernatants
210 After exposure to extracts of $PM_{2.5}$, the medium was harvested and centrifuged at 300g
211 for 5 minutes to remove floating cells. The supernatants were stored at -80^{o}C for further
212 analysis. The levels of IL-6 and IL-8 (Thermo Scientific, Waltham, Massachusetts) in the

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

Fluorescence-Activated Cell Sorter Analysis

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

231 measured by a FACSCalibur (Becton Dickinson). For each sample, fluorescence data of
232 10,000 cells were collected, and positive cells expressed as the percentage events were
233 calculated.
234
235 Cell Proliferation
236 Cell proliferation was measured with a Cell-ProliferationELISA Kit (Roche Molecular
237 Biochemicals, Mannheim, Germany), according to the manufacturer's instructions. This
238 technique is based on the incorporation of the pyrimidine analogue BrdU instead of
239 thymidine into the DNA of proliferating cells. 5-Bromo-2´-deoxyuridine incorporated
240 into DNA is measured by a sandwich-type enzyme immunoassay using monoclonal anti-
241 BrdU antibodies. Splenocytes were exposed to extracts of $PM_{2.5}$ for 72 hours, and cell
242 proliferation was measured by adding BrdU to each well 20 hours before the measurement.
243 Absorbance was measured on the iMark Microplate Absorbance Reader with the
244 wavelength set at 450 nm and a reference wavelength at 630 nm.
245
246 *Statistical analysis*
247 The data are presented as the mean ± standard error of the mean (SEM) for each
248 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 μg/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 μg/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 μg/mL exposure, the aqueous extracts collected in the summer and

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

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

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

The Kawasaki extracts

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

Regarding the IL-8 secretion from the airway epithelial cells, the aqueous extracts did not have any effect. In contrast, the organic extracts from summer lowered the IL-8 secretion compared to the control (Fig. 4C). Similarly, at 22.5 μg/mL, the organic extracts 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.

Cell surface molecules

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

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

The $PM_{2.5}$ Extracts' Effects on Splenocytes

Cell viability

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

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

Cell proliferation: Kawasaki extracts

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

357 extract of the same (Fig. 7C). The proliferation results for the fall and winter samples of
358 aqueous extracts were also significantly higher than those of the same dose (7.5 μg/mL)
359 of organic extracts (Suppl. Fig. S6C).
360
361 Cell proliferation: Fukuoka extracts
362 Unlike the Kawasaki extracts, the Fukuoka samples had significant effects on cell
363 proliferation (Fig. 7D). Interestingly, while the aqueous samples from spring (47.2%),
364 summer (50.2%), and fall (76.5%) significantly increased the proliferation, the organic
365 extracts lowered the proliferation; by season: summer (23.43%), fall (31.2%) and winter
366 (36.3%) compared to the control. The 7.5 μg/mL dose aqueous samples increased the
367 proliferation at fall sample, but the organic extracts from spring, summer and fall lowered
368 the proliferation compared to aqueous sample samples (Suppl. Fig. S6D).
369
370 Cell surface molecules
371 The aqueous extracts from Kawasaki did not have much effect on the TCR
372 expression, with the exception of the 75 μg/mL fall sample (Fig. 8A), which significantly
373 increased the TCR expression. At the lower doses of 7.5 μg/mL, no effect was observed
374 (Suppl. Fig. S7A). The spring and summer 75 μg/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).

Discussion

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

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

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

414 In contrast, Huang et al. (2014) have shown that an aqueous extract of $PM_{2.5}$ had a
415 toxic effect on airway epithelial cells. A study of components of $PM_{2.5}$ aqueous extracts
416 collected from Baghdad, Iraq showed that trace elements such as V and Ni correlated with
417 the reactive oxygen species (ROS) production of alveolar macrophages (Hamad et al.
418 2016). Our present findings indicated that aqueous extracts from Kawasaki, especially
419 those collected during the fall, contain components that affect cellular viability. It is
420 possible that the decrease in cell viability induced by these components permit the
421 invasion of inhaled xenobiotics including allergens, which can contribute to the
422 exacerbation of respiratory diseases such as asthma. On the other hand, organic extracts
423 from Fukuoka increased the cell viability compared to the control in all seasons, which
424 may relate with carcinogenic effects of $PM_{2.5}$ (Bayram H et al 2013, Bayram H et al,
425 2006).

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

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

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

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

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

451 On the other hand, the number of CD86-positive cells was increased in the summer
452 sample in both the aqueous and organic extracts from Kawasaki in the present study.
453 CD86 is a crucial cell surface molecule for antigen presentation for asthmatics, and it is
454 associated with late reaction to allergens (Balbo et al. 2001). CD86 is an important and
455 sensitive cell surface molecule for PAHs, as we recently have observed (Chowdhury et al.
456 2017). Moreover, our previous studies revealed aqueous extract from Kawasaki and
457 Fukuoka were able to increase the CD86 expression on the cells (Honda et al, 2017).
458 Hulette et al, 2005 showed that aqueous soluble chemicals including nickel sulphate and
459 hydroquinone are capable to induce CD86 expression in human dendritic cells. Taking
460 the above findings together, it seems that $PM_{2.5}$ can activate APCs via DEC205 and CD86,
461 and this phenomenon could be related to their enhancing effects on allergic diseases or
462 responses.

463 The viability of splenocytes was also diminished in the aqueous extract samples
464 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

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

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

516 Kawasaki, as an industrial area, is thus more likely to contain higher concentrations
517 of metal ions in its $PM_{2.5}$ extracts than Fukuoka (Figs. 1), which can explain our
518 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 μg/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

561 **Acknowledgement**

562 The study was performed as part of a study project on $PM_{2.5}$ by Japan Ministry of the
563 Environment. We are indebted to Mari Tsuboi for her immense help in the data analysis.

564

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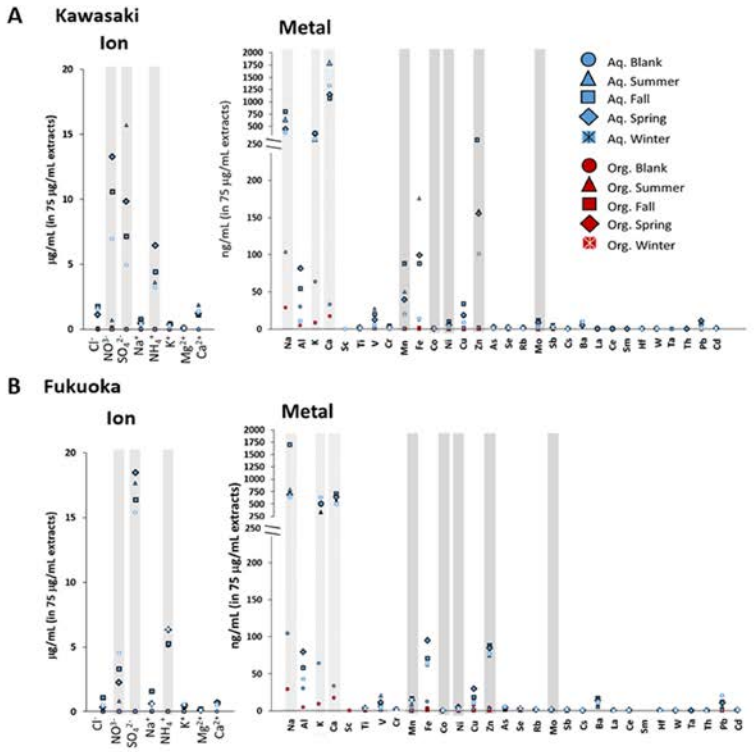

A Kawasaki
Ion
Metal
μg/mL (in 75 μg/mL extracts)
ng/mL (in 75 μg/mL extracts)
Aq. Blank
Aq. Summer
Aq. Fall
Aq. Spring
Aq. Winter
Org. Blank
Org. Summer
Org. Fall
Org. Spring
Org. Winter
B Fukuoka
Ion
Metal

Fig. 1

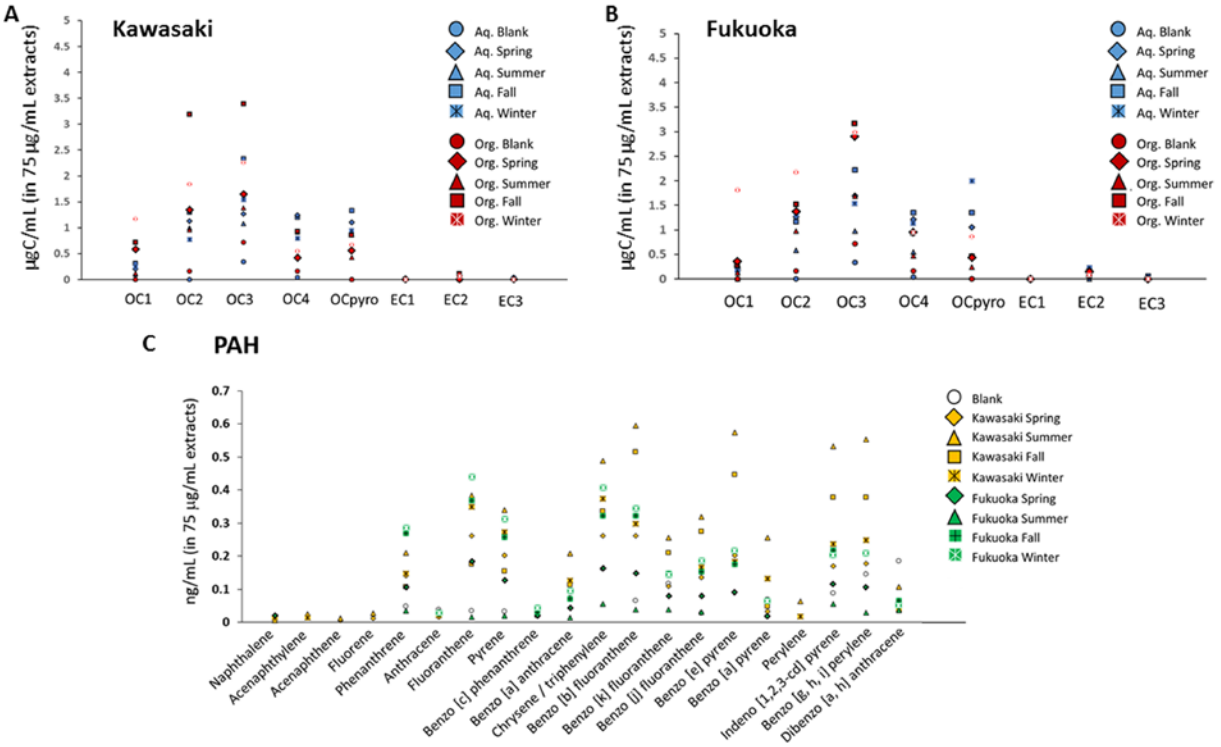
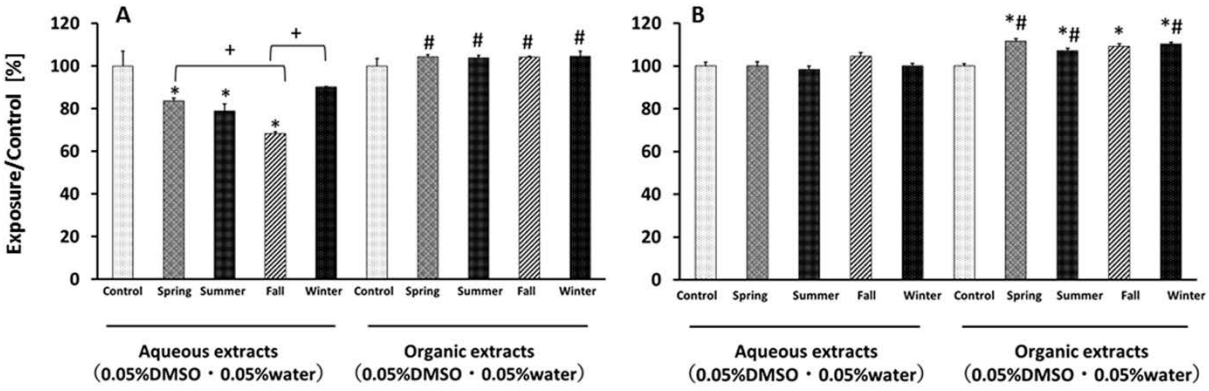

A
Kawasaki
μgC/mL (in 75 μg/mL extracts)
Aq. Blank
Aq. Spring
Aq. Summer
Aq. Fall
Aq. Winter
Org. Blank
Org. Spring
Org. Summer
Org. Fall
Org. Winter
OC1
OC2
OC3
OC4
OCpyro
EC1
EC2
EC3
B
Fukuoka
C
PAH
ng/mL (in 75 μg/mL extracts)
Blank
Kawasaki Spring
Kawasaki Summer
Kawasaki Fall
Kawasaki Winter
Fukuoka Spring
Fukuoka Summer
Fukuoka Fall
Fukuoka Winter
Naphthalene
Acenaphthylene
Acenaphthene
Fluorene
Phenanthrene
Anthracene
Fluoranthene
Pyrene
Benzo [c] phenanthrene
Benzo [a] anthracene
Chrysene / triphenylene
Benzo [b] fluoranthene
Benzo [k] fluoranthene
Benzo [j] fluoranthene
Benzo [e] pyrene
Benzo [a] pyrene
Perylene
Indeno [1,2,3-cd] pyrene
Benzo [g, h, i] perylene
Dibenzo [a, h] anthracene

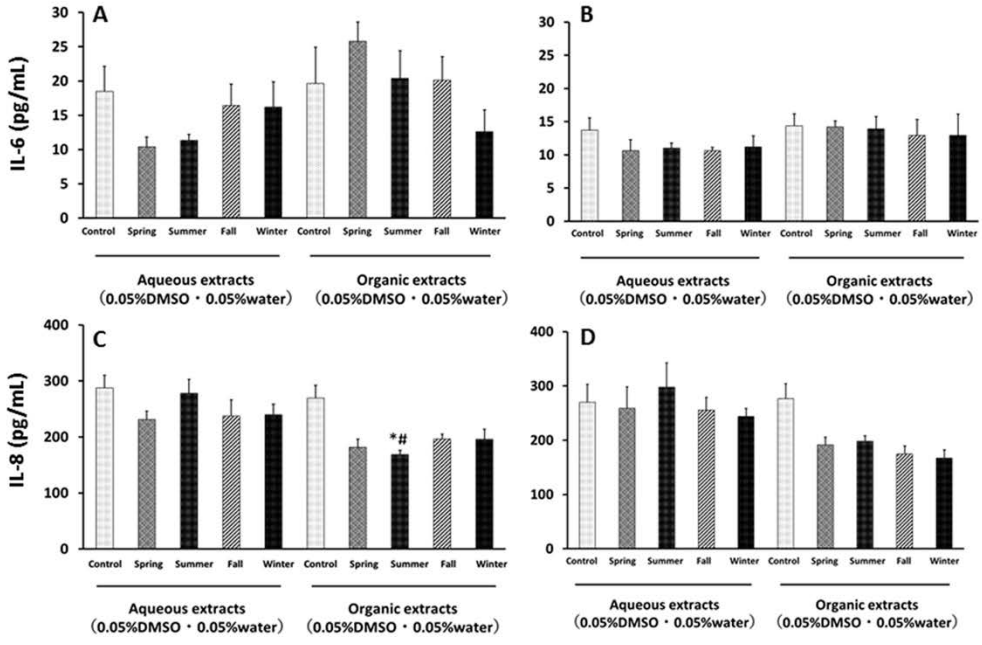
Fig. 2

Fig. 3



Kawasaki Fukuoka

Fig. 4



Kawasaki Fukuoka

Fig. 5

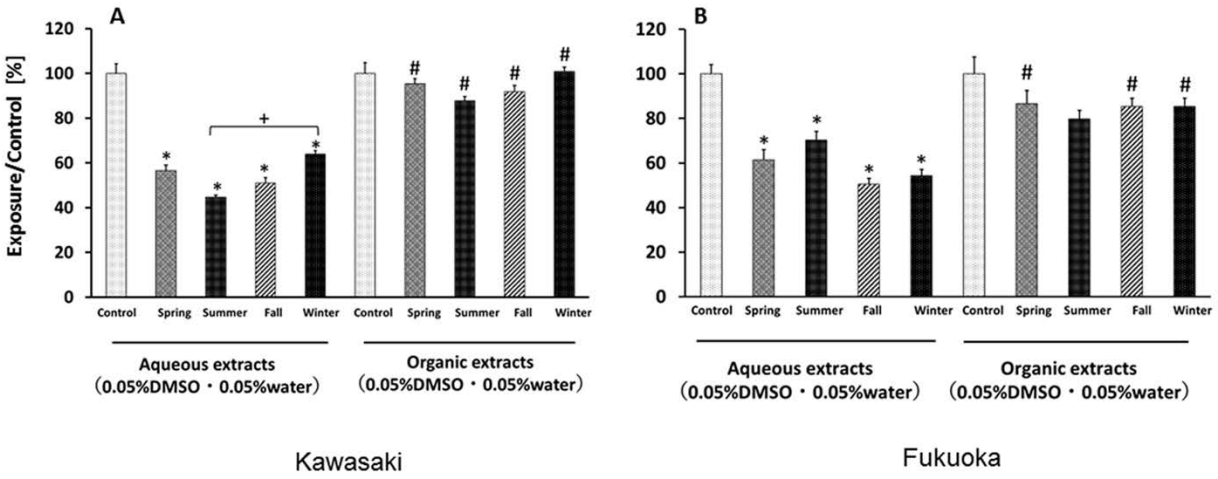

A
B
Exposure/Control [%]
Control
Spring
Summer
Fall
Winter
Aqueous extracts
(0.05%DMSO・0.05%water)
Organic extracts
(0.05%DMSO・0.05%water)
Kawasaki
Fukuoka

Fig. 6

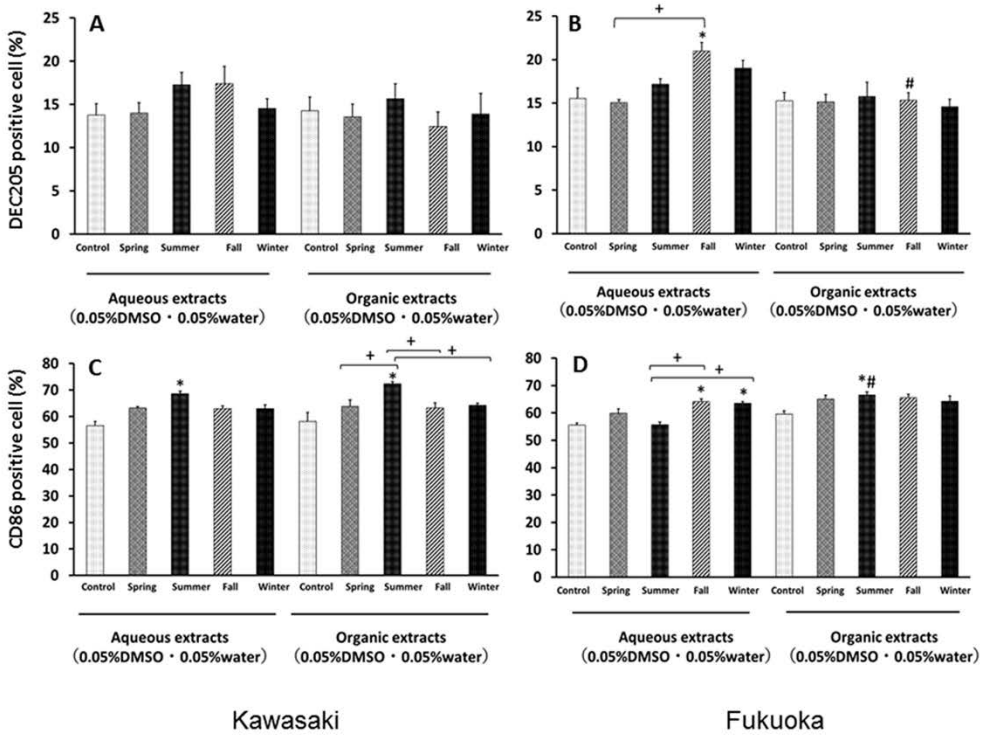

A
B
C
D
DEC205 positive cell (%)
CD86 positive cell (%)
Control
Spring
Summer
Fall
Winter
Aqueous extracts
(0.05%DMSO・0.05%water)
Organic extracts
(0.05%DMSO・0.05%water)
Kawasaki
Fukuoka

Fig. 7

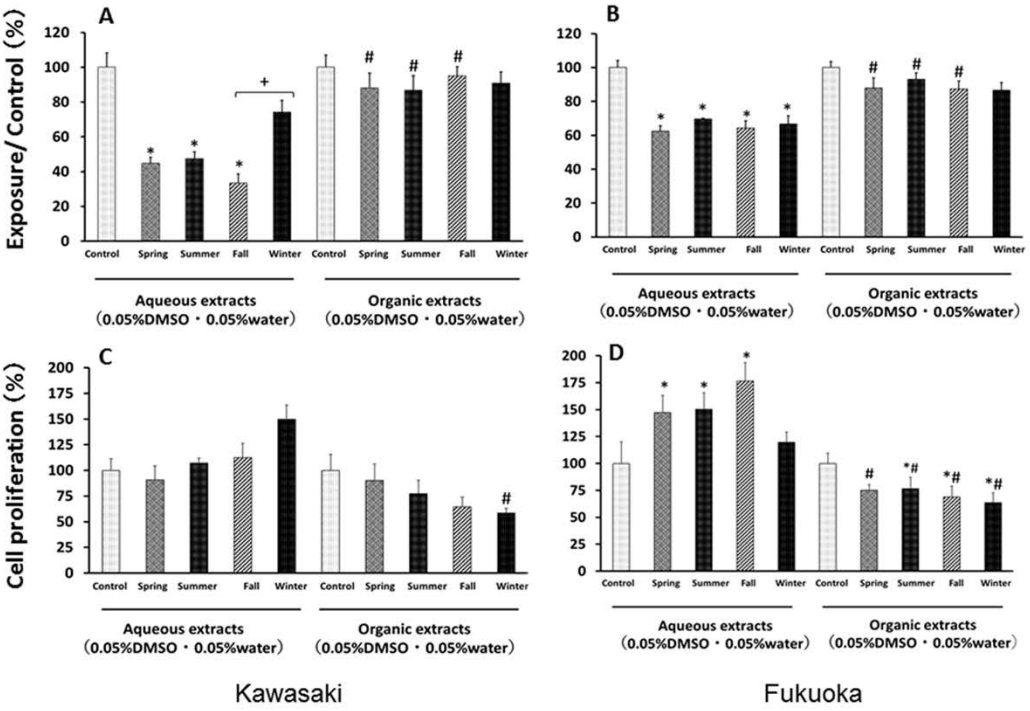
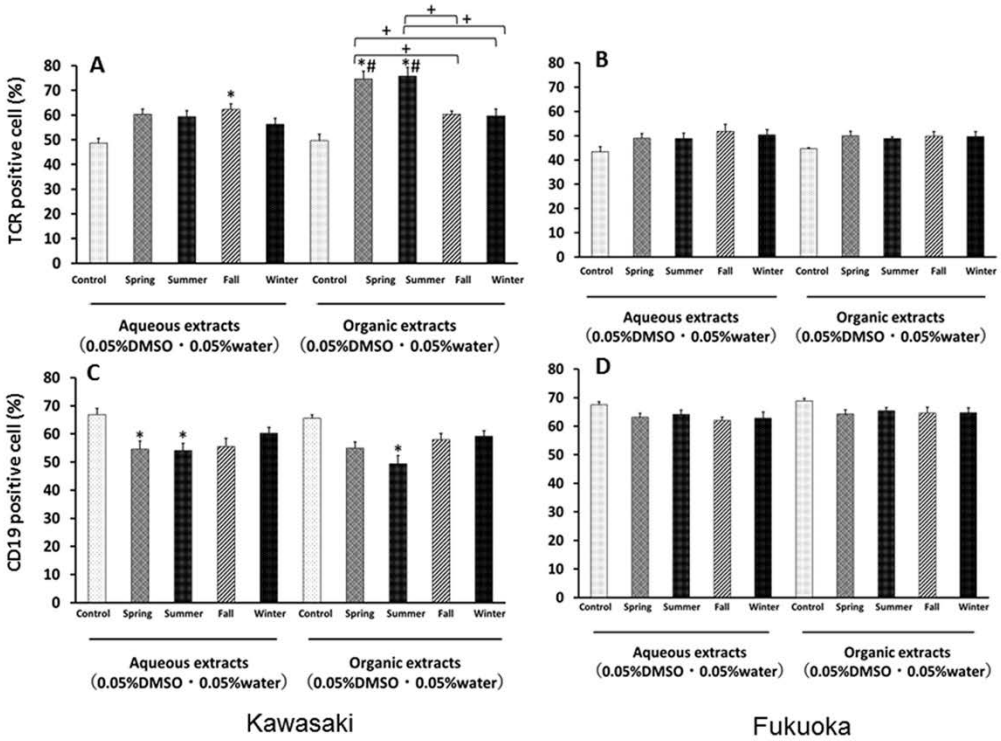
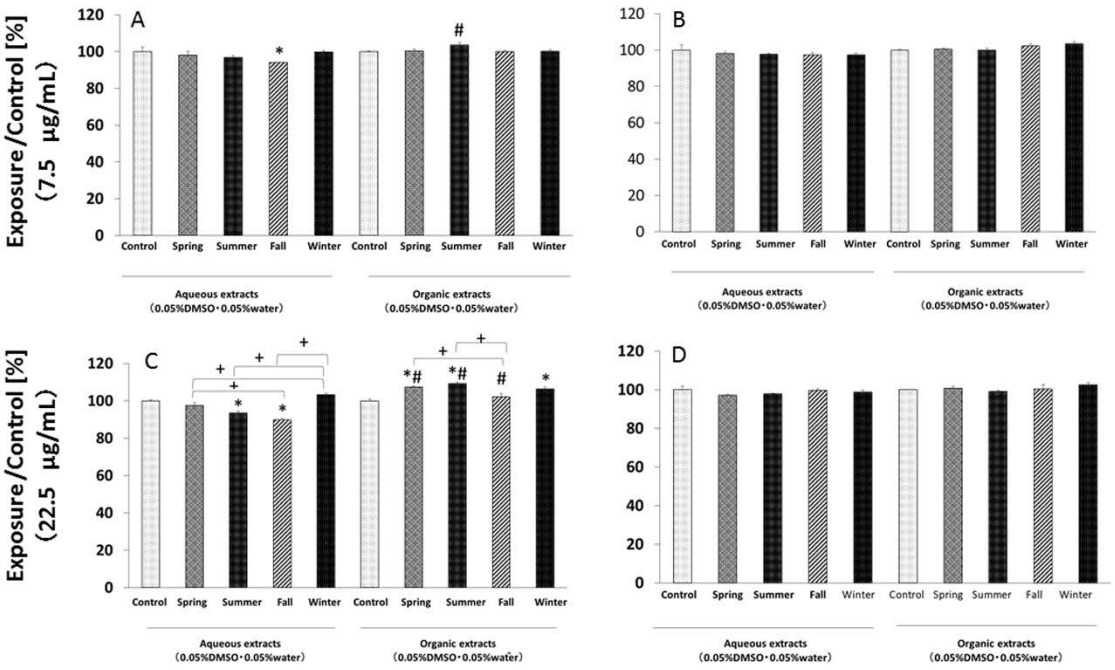

A
B
C
D
Exposure/ Control (%)
Cell proliferation (%)
Control
Spring
Summer
Fall
Winter
Aqueous extracts
(0.05%DMSO・0.05%water)
Organic extracts
(0.05%DMSO・0.05%water)
Kawasaki
Fukuoka

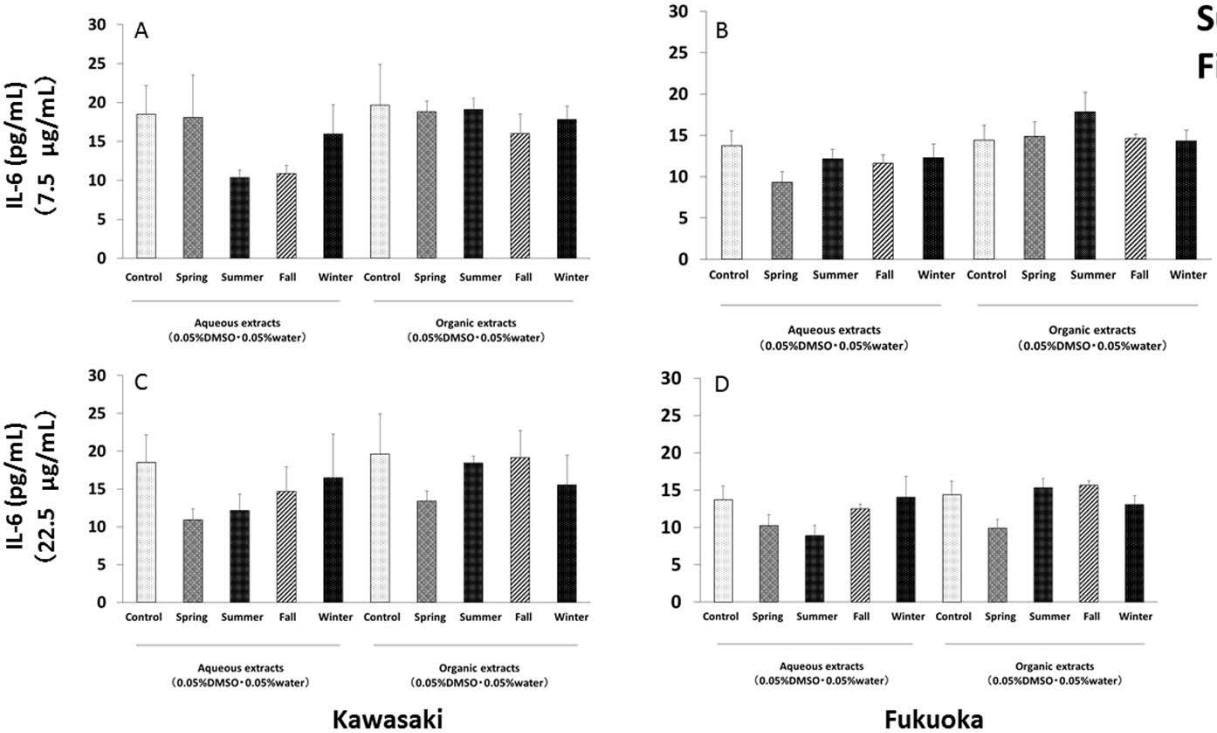
Fig. 8


A
B
C
D
TCR positive cell (%)
CD19 positive cell (%)
Control
Spring
Summer
Fall
Winter
Aqueous extracts
(0.05%DMSO・0.05%water)
Organic extracts
(0.05%DMSO・0.05%water)
Kawasaki
Fukuoka

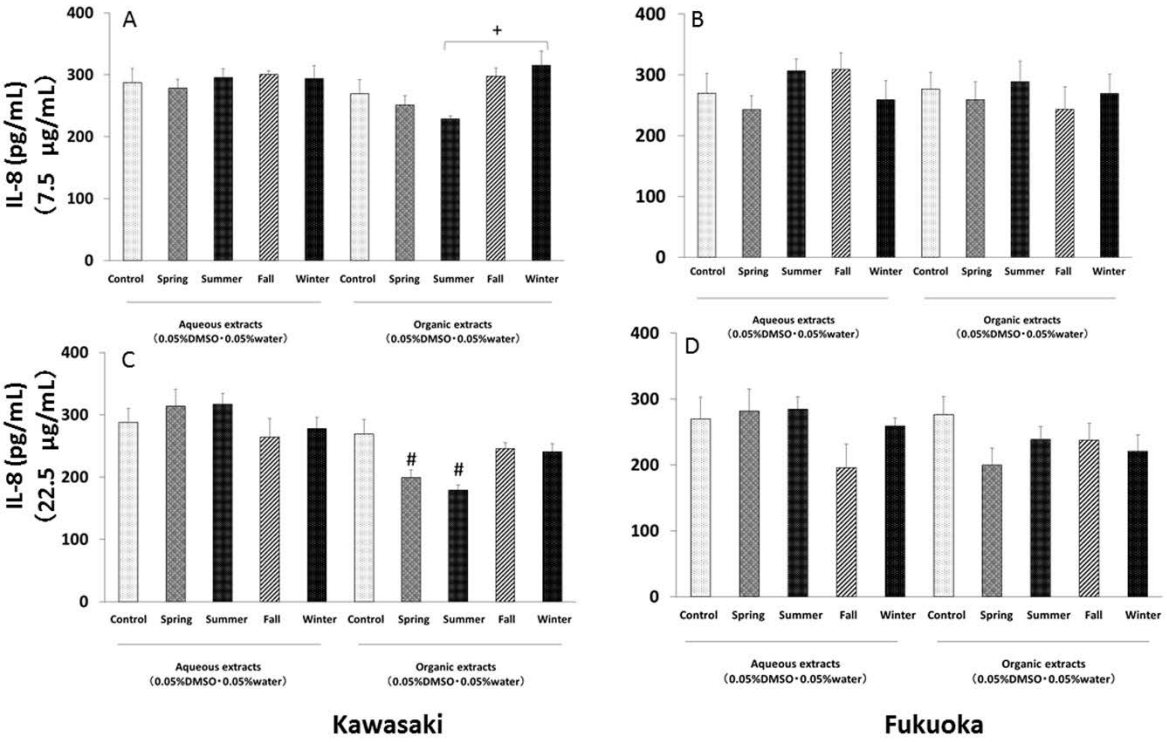
Suppl. Fig. S1.



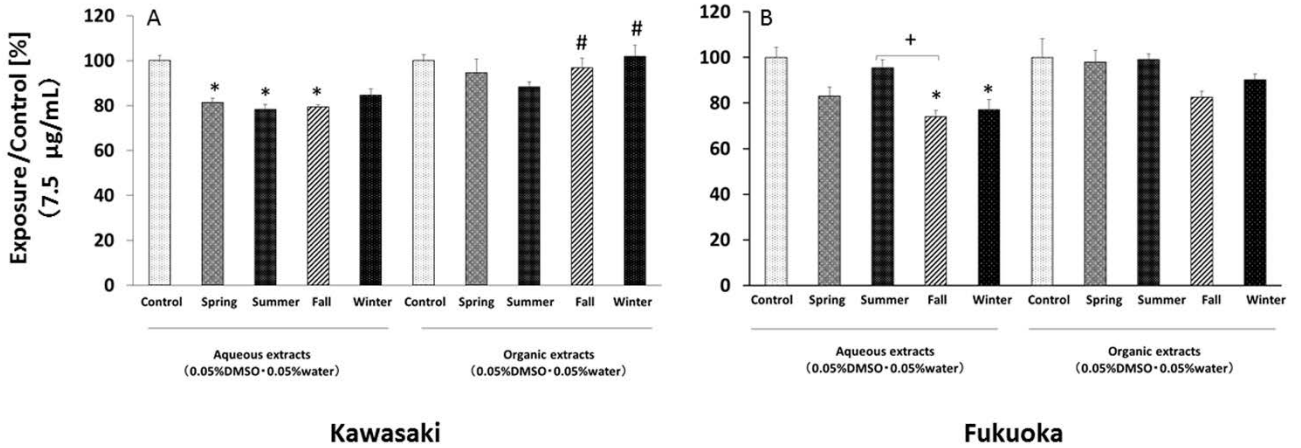
Suppl. Fig. S2.



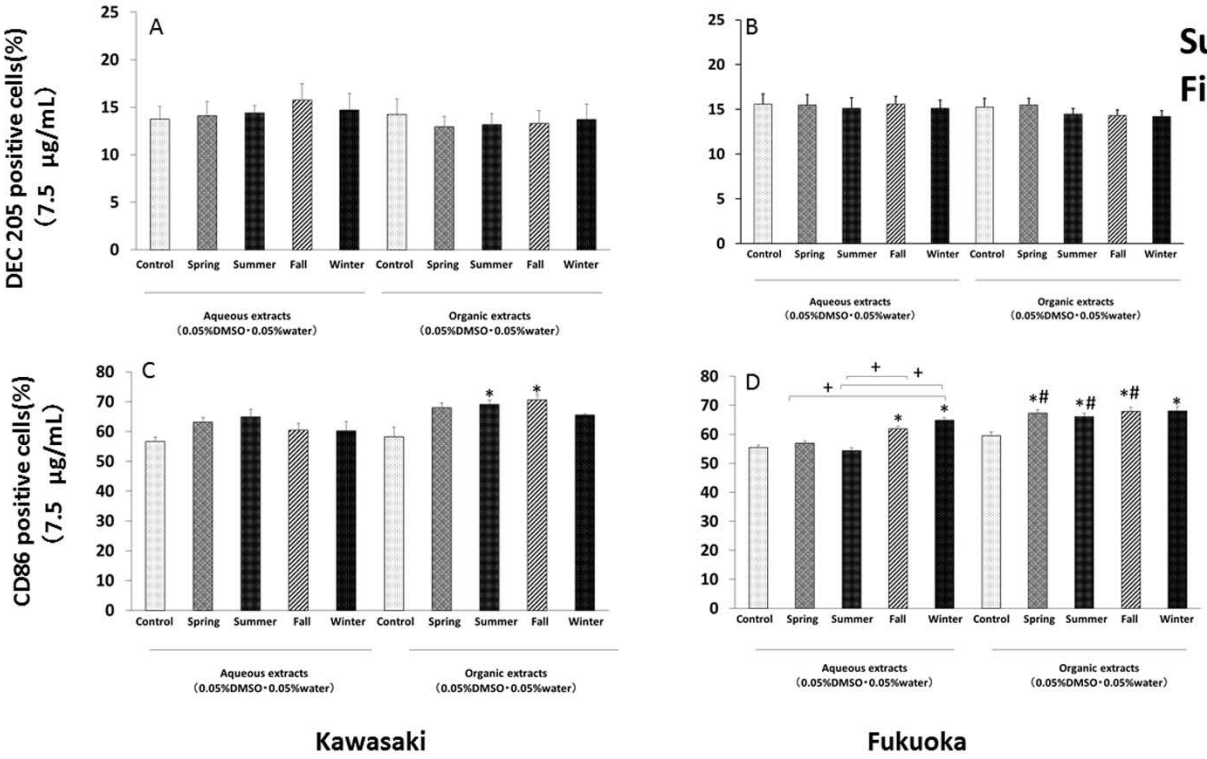
Suppl. Fig. S3.



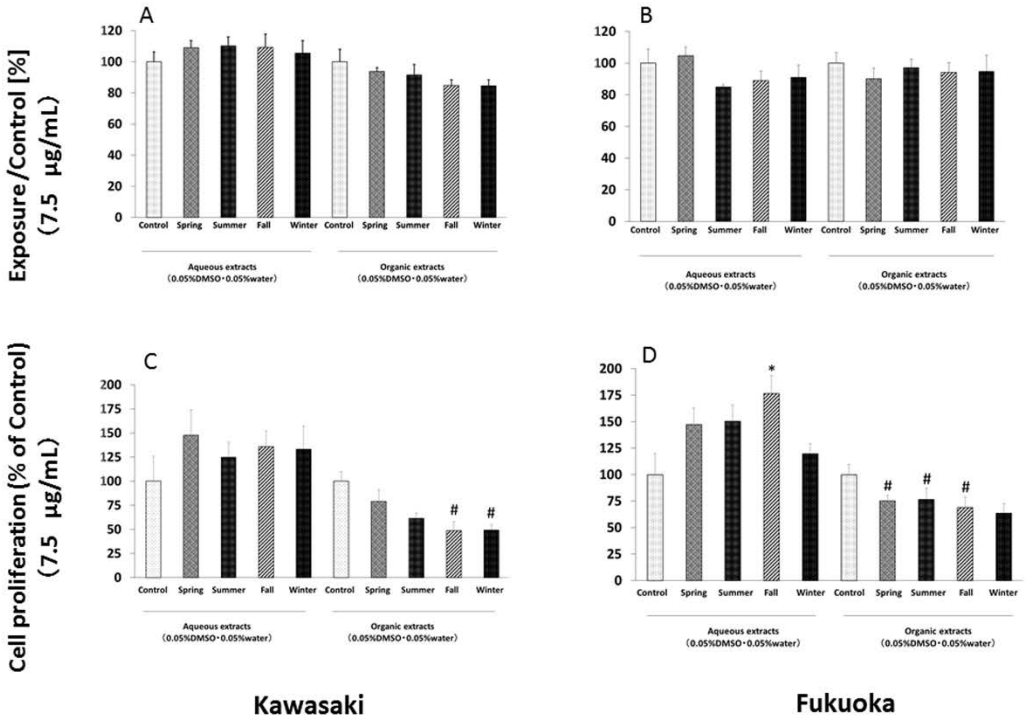
Suppl. Fig. S4.



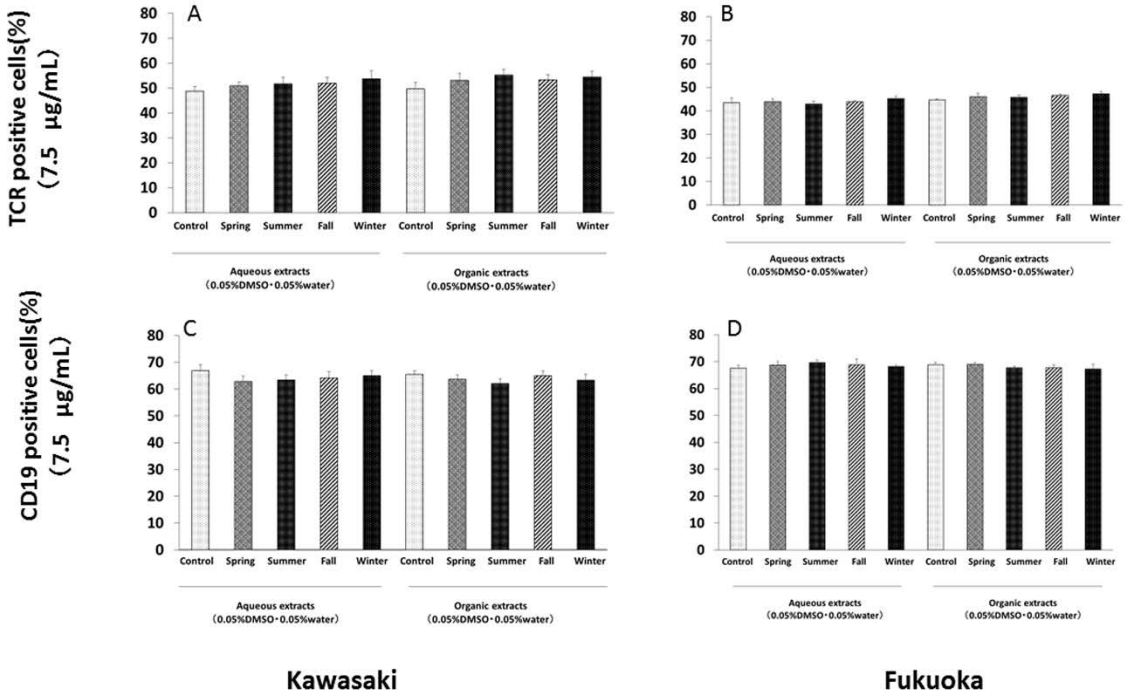
Suppl. Fig. S5.



Suppl. Fig. S6.



Suppl. Fig. S7.



Suppl. Table 1. Corresponding dose of total $PM_{2.5}$ mass by using data on extraction efficiency (μg/m^3)

	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