2 immune responses

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20 ABSTRACT

Background: Particulate matter with aerodynamic diameter $\leq 2.5 \ \mu m \ (PM_{2.5})$ is generally 2122composed of carbon nuclei associated with various organic carbons, metals, ions and biological materials. Among these components, polyaromatic hydrocarbons (PAHs) such as 23benzo(a)pyrene (BaP) and quinones have detrimental effects on airway epithelial cells and 24immunodisrupting effects, which leads to the exacerbation of respiratory allergies. The effects 2526of PAHs and the carbon nuclei, separately as well as in combination, remain to be established. **Objective:** We investigated the effects of BaP, 9,10-phenanthroquinone (9,10-PQ) and 1,2-27napthoquinone (1,2-NQ) and their combined effects with heated diesel exhaust particle (H-28DEP) as carbon nuclei of typical PM_{2.5}. 29Methods: We exposed human airway epithelial cells (BEAS-2B), murine bone marrow-30 derived antigen-presenting cells (APCs), and murine splenocytes to BaP, 9,10-PQ, or 1,2-NQ 31in the presence and absence of H-DEP. Several important inflammatory cytokines and cell 3233surface molecules were measured. **Results:** PAHs alone did not have apparent cytotoxic effects on BEAS-2B, whereas combined 34

exposure with H-DEP induced noticeable detrimental effects which mainly reflected the action
of H-DEP itself. BaP increased CD86 expression as an APC surface molecule regardless of the
presence or absence of H-DEP. None of the BaP, 9,10-PQ or 1,2-NQ exposure alone or their
combined exposure with H-DEP resulted in any significant activation of splenocytes.

39 Conclusions: These results suggest that PAHs and carbon nuclei show additive effects, and
40 that BaP with the carbon nuclei may contribute to exacerbations of allergic respiratory diseases
41 including asthma by PM_{2.5}, especially via APC activation.

42

43 Introduction

It is clear from decades-long studies (Ring et al, 2001) that the exacerbation of respiratory allergies is associated with particulate matter with aerodynamic diameters $\leq 10 \,\mu\text{m}$ (PM₁₀) and that with aerodynamic diameters $\leq 2.5 \,\mu\text{m}$ (PM_{2.5}). Particulate matters are usually released into the air from sources such as the burning of coal and wood, the combustion of petrol and diesel, and a variety of industrial processes. The aforementioned emissions included polyaromatic hydrocarbons (PAHs) associated with carbon particle and metal (Tobiszewski and Namiesnik, 2012).

51 PAHs, including benzo[a]pyrene (BaP) and quinones, are among the most prominent 52 compounds found in PM_{2.5}. Diesel exhaust particles (DEPs) contains PAHs that cause the 53 exacerbation of allergic respiratory diseases including asthma. Several studies have shown BaP 54 and quinones also have a strong association with the increased incidents of allergic airway 55 disorder (Kadkhoda et al, 2004, Inoue et al, 2007, Hiyoshi et al, 2005, Kumagai et al, 2007). 56 In addition, quinones are important agents that affect health, and they are considered 57 responsible for modulating the metabolic profile and DNA damage in respiratory cells (Gurbani

58	et al, 2013). Several studies established that <i>ortho</i> -quinone has a higher cytotoxic effect than
59	para-quinone in airway epithelial cells (Koike et al, 2014) which may indicate that ortho-
60	quinone contributes to exacerbations of airway inflammation. 9, 10-phenanthroquinone (9,10-
61	PQ) and 1, 2-naphthoquinone (1,2-NQ) are both examples of <i>ortho</i> -quinone (Fig. 1).
62	Regarding the health effects of $PM_{2.5}$, the synergy of organic components and the nuclei
63	themselves has been reported (Yanagisawa et al, 2006). It is thus necessary to determine the
64	effects of PAHs alone and their co-exposure with a carbon nucleus to understand the
65	mechanisms by which PM _{2.5} leads to the exacerbation of allergic diseases. However, the
66	combined effects of BaP or quinones and carbon nuclei have not been investigated.
67	Our objective in the present study was to observe the effects of BaP, 9,10-PQ and 1,2-
68	NQ and their combined effects with the carbon nuclei on respiratory cells as well as
69	lymphocytes and antigen-presenting cells (APCs), to understand the factors responsible for the
70	vulnerability of respiratory allergy to PM2.5.
71	
72	Methods
73	Cell preparation

The experiment was performed on human airway epithelial cells (BEAS-2B) and immune cells
including splenocytes and bone marrow-derived cells (APCs). Ten-week-old NC/NgaTendCrlj
male mice (Chares River Japan, Osaka, Japan) were sacrificed by cervical dislocation and

77	exsanguinated by cutting the abdominal aorta and vein. Splenocytes and APCs were extracted
78	from each mouse. All animal study procedures were approved by the Animal Research
79	Committee at Kyoto University.
80	
81	Airway epithelial cells
82	The BEAS-2B cell line, which is derived from human bronchial epithelial cells transformed by
83	an adenovirus (12-SV40 hybrid virus) was purchased from the European Collection of Cell
84	Cultures (Salisbury, Wiltshire, UK).
85	To initiate the cell culture, the vial containing the cells was taken out from liquid nitrogen
86	and added into serum-free LHC-9 medium (Life Technologies, Carlsbad, CA, USA). The
87	subculture was maintained in LHC-9 medium in an incubator in a 5% CO ₂ atmosphere at 37°C.
88	For particular experiments, cells were seeded in 96- and 12-well collagen I-coated plates and
89	incubated for 72 hr to reach semi-confluence in LHC-9 medium with the same conditions as
90	those used for the subculture.
91	
92	Bone marrow isolation

93 Cells were isolated from the femur bone of two legs of each mouse. After the surrounding
94 muscle tissue was removed, the bones were left in 70% ethanol for 3 min and washed with
95 RPMI medium. Both ends of the bones were cut and the marrow was flushed with RPMI

medium using a syringe with a 25G needle. The marrow suspension was passed through a
sterile nylon mesh to remove small pieces of bone and debris, and the red blood cells were
lysed with hemolytic reagent (ammonium chloride based). The cells were centrifuged at 400*g*for 5 min at 20°C.

After aspiration of the media, the cells were resuspended in culture medium R10, which
was GIBCO RPMI 1640 medium (Invitrogen, Grand Island, NY) supplemented with 10% heatinactivated fetal bovine serum (FBS; MP Biomedicals, Eschwege, Germany), 100 U/ml
penicillin, 100 µg/ml streptomycin (Sigma, St. Louis, MO), and 50 µM 2-mercaptoethanol
(Invitrogen).

105

106 Differentiation of APCs

107 APCs were differentiated using a modification of the protocol described by Lutz et al, 1999. In 108 brief, bone marrow cells $(4 \times 10^5 \text{ /mL})$ were cultured in R10 medium containing 20 ng/mL 109 granulocyte-macrophage colony-stimulating factor (GM-CSF). On day 3, the same volume of 110 the medium containing 20 ng/mL GM-CSF was added to the culture. On day 6, half the culture 111 medium was replaced with fresh medium. On day 8, non-adherent and loosely adherent cells 112 were collected by gentle pipetting. The differentiated APCs were centrifuged at 400*g* for 5 min 113 at 20°C and then resuspended in fresh medium.

114 The numbers of viable cells were determined by the trypan blue exclusion method. The

115	final concentration of the cell suspension and exposure medium was appropriately calculated
116	to achieve the final concentration as 1×10^6 cells/ml.

118	Splenocyte	isolation
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- The spleen was removed from the mice, crushed and pushed through a 200-mesh stainless steel sheet. The cells were suspended in RPMI-1640 media and centrifuged at 400*g* for 5 min at 20°C. After being treated with hemolytic reagent, the cell suspension was centrifuged one more time. The numbers of viable cells were determined by the trypan blue exclusion method. The final concentrations of the cell suspension and exposure medium were appropriately calculated to achieve the final concentration of 1×10^6 cells/ml.
- 125

126 Exposure of airway epithelial cells and immune cells

We examined BaP (purchased from Sigma-aldrich), 9,10-PQ (purchased from Sigma aldrich), and 1,2-NQ (purchased fom Tokyo chemical Industry) as exposure reagents. To understand the synergistic effects of carbon nuclei and PAHs in DEP as representative of PM_{2.5}, we used heated-DEP (H-DEP) where PAHs were excluded by heat at 360°C for 30 min. We characterized the H-DEP particles and measured various components including several PAHs consisting of four- five- and six-membered rings. The particle did not have any PAHs associated with it. Heating at 360°C caused complete evaporation of PAH. However, metals and ions such as, Ca, Na, Zn, Fe, Mn, As and SO_4^{2-} had been detected. Cells were exposed to BaP (0, 0.1, 1.10 μ M) or 9,10-PQ or 1,2-NQ (0, 0.01, 0.1, 1 μ M for both) in the presence or absence of H-DEP (50 μ g/mL) for 24 or 72 h. The results of our pre-experiments using quinone showed that doses higher than the aforementioned doses are highly cytotoxic and thus not appropriate for the immune study.

To measure the cell viability and the interleukin (IL)-6 and IL-8 releases from airway 139epithelial cells, we performed a water-soluble tetrazolium salt-1 (WST-1) assay and enzyme-140linked immunosorbent assay (ELISA), respectively. We also measured the cell viability and the 141expression of cell surface molecules of APCs (DEC205, a dendritic cell marker; CD86, an APC 142marker) by performing a WST-1 assay and fluorescence-activated cell sorter (FACS) analysis, 143144respectively. The cell viability, cell proliferation and the expression of cell surface molecules 145of splenocytes (T-cell receptor [TCR], a T-cell marker; CD19, a B-cell marker) were measured by WST-1 assay, 5-bromo-2'-deoxyuridine (BrdU) ELISA, and FACS analysis, respectively. 146147

148 Cell viability

149 Cell viability was assessed by WST-1 assay. The BEAS-2B cell suspension of 7.5×10^4 cells/mL 150 was seeded at 70 µL/well in collagen I-coated 96-well plates and cultured for 3 days. On day 151 3, the medium was discarded and exposed to the sample solution at an equal concentration by 152 70 µL/well. After 21 hr of exposure, the WST-1 reagent was added. A WST-1 reagent amount

153	should be 1/10th of the sample volume; for this experiment, it was thus 7 μ L. After 3 hr under
154	WST-1 exposure, the absorbance of the plate was measured at 450 nm using a microplate reader
155	(reference wavelength 630 nm).
156	Similarly, APCs and splenocytes were cultured for 23 hr 30 min and 20 hr, respectively,
157	and then treated with a 1/10th amount of sample volume of WST-1 reagent for 30 min for the
158	APCs and 4 hr for the splenocytes. Thereafter, the absorbance of the plate was measured at 450
159	nm using a microplate reader (reference wavelength 630 nm).
160	
161	Cell proliferation assay
162	To estimate the splenocytes' proliferation, we performed a BrdU analysis. According to the
163	instructions of the BrdU assay kit (Roche Lifescience). The cells were exposed for 3 days and
164	subjected to absorbance measurement at wavelength 450 nm with the reference wavelength
165	630 nm. BrdU was added at 20 hr prior to the measurement.
166	
167	Quantification of cytokines in culture supernatant by ELISA
168	After exposure to a PAH and the combination of PAH + H-DEP, BEAS-2B cells were cultured
169	for 24 hr and centrifuged at $300g$ for 5 min, and the supernatant was collected. The supernatant
170	was stored at -80° C. The levels of IL-6 and IL-8 released from BEAS-2B cells were measured
171	by quantikine ELISA kits (IL-6 and IL-8 from Thermo Scientific). The IL-10 level of the APCs

172 was also determined by a quantikine ELISA kit (Thermo Scientific).

173

174 FACS analysis

The cell surface molecules were measured by a FACS analysis. The following monoclonal 175antibodies were used to react with specific antigens: Mouse BD Fc Block[™] purified anti-176mouse CD16/CD32 (Becton Dickinson), DEC205 (NLDC-145, PE-conjugated, BioLegend, 177San Diego, CA), Rat IgG2a, ĸ Isotype Control (RTK2758, PE-conjugated, BioLegend), CD86 178(GL-1, PE-conjugated, Becton Dickinson), Rat IgG2a, ĸ Isotype Control (R35-95, PE-179conjugated, Becton Dickinson), Hamster Anti-Mouse TCR-B Chain (H57-597, FITC-180 conjugated, Becton Dickinson), Hamster IgG2, $\lambda 1$ Isotype Control (Ha4/8, FITC-conjugated, 181 182Becton Dickinson), Rat Anti-Mouse CD19 (1D3, PE-conjugated, Becton Dickinson), and Rat IgG2a, κ Isotype Control (R35-95, PE-conjugated, Becton Dickinson). To determine the 183antigen-positive zones in the FACS histogram, we compared stained samples with un-stained 184and isotype controls (Fig. 2). 185

After exposure for 24 hr, the immune cells were collected and resuspended in 50 μL phosphate-buffered saline (PBS) with 0.3% bovine serum albumin and 0.05% sodium azide (Wako Pure Chemical Industries, Osaka, Japan). Depending on the objective marker, the appropriate antibody was added into it and maintained for 45 min at 4°C before a wash with FACS buffer. The cell surface molecules were measured with a FACS Calibur (Becton

191	Dickinson). For the analysis, the percentage data of positive cells with a particular marker per
192	10,000 cells was determined.
193	
194	Statistical analysis
195	The experiments for cytotoxicity, the release of cytokines, and the cell surface molecule
196	expression were performed with multiple samples (n=3–4). The average values \pm standard error
197	of the mean (SEM) were calculated for all statistical analyses. Intergroup differences were
198	examined by Tukey's multiple comparison tests. A p-value <0.05 was considered significant.
199	
200	Results
201	Effects on respiratory responses
202	Exposure to BaP alone at higher doses increased the cell viability of the airway epithelial cells.
203	Neither of the two quinones alone changed cell viability. H-DEP alone lowered cell viability.
204	BaP + H-DEP (1 μ M and 10 μ M) exposure resulted in high viability (referred with '+' sign in
205	Fig. 3A) compared to the control. Combined exposure to 9,10-PQ + H-DEP and 1,2-NQ + H-
206	DEP at some doses decreased the cell viability compared to single-exposure (Fig. 3B,C).
207	With BaP exposure, the IL-6 expression did not show any significant difference (Fig.

209 quinones' single exposure did not result in any changes in IL-6 or IL-8 expression. The PAHs

212 Effects on immune responses

213 Effects on APCs

BaP single exposure decreased the viability of APCs (Fig. 5A). The quinones alone had a feeble 214decreasing effect on cell viability. H-DEP alone did not change cell viability as well. BaP + H-215DEP caused low cell viability compared to the control. 9,10-PQ + H-DEP and 1,2-NQ + H-216DEP exposure both did not show any change in viability compared to control except highest 217dose of the later, which lowered the viability (Fig. 5B,C). 218BaP single exposure showed high CD86 expression (Fig. 6A) while quinones did not 219220have any effect on any dose. H-DEP alone also did not show any noticeable effect. BaP+H-221DEP had an effect on the elevation of CD86 expression as compared to control. But combined exposure to the quinones and H-DEP did not have any significant effect (Fig. 6B and 6C). On 222the other hand, PAHs with or without H-DEP did not cause any change in the expression of 223DEC205 (Fig. 7). IL-10 expression for all of the PAHs and their combined exposure showed 224225the result under detection level.

226

227 Effects on splenocytes

BaP single exposure decreased cell viability significantly after 24 hr of exposure (Fig. 8A).

Both of the quinones did not show any difference in single exposures except at 1 μ M dose of 9,10-PQ that showed significantly decreased viability (Fig. 8B,8C). H-DEP alone tended to decrease cell viability. BaP + H-DEP showed the significant detrimental effect on cell viability as compared to control. 9,10-PQ + H-DEP and 1,2-NQ + H-DEP tended to decrease cell viability as compared to control.

When we checked the proliferation of splenocytes after 72 hr of exposure, we observed that BaP single exposure (1 μ M and 10 μ M) decreased proliferation (Fig. 8D). Neither 9,10-PQ nor 1,2-NQ single exposure had a significant effect. H-DEP alone as well as BaP + H-DEP, 9,10-PQ + H-DEP and 1,2-NQ + H-DEP had declining effects on cell proliferation compared to single exposure (Fig. 8).

Figure 9 summarizes the following results. BaP single exposure did not cause changes in TCR expression. However, 9,10-PQ single exposure at 1 μ M resulted in a significantly high expression of TCR (Fig. 9B). 1,2-NQ single exposure did not have any effect. H-DEP alone did not show any change. 9,10-PQ + H-DEP at the 1 μ M dose tended to have an increasing effect compared to the control.

BaP exposure did not change CD19 expression. The single-exposure of quinones also did not result in any change in CD19 expression. H-DEP alone also did not have any noticeable effect. BaP + H-DEP did not have any effect, but 9,10-PQ + H-DEP at 1 μ M decreased the CD19 expression (Fig. 9E). 1,2-NQ + H-DEP exposure did not affect the CD19 expression (Fig.

250 Discussion

Here we observed that the PAHs alone did not have apparent cytotoxic effects on airway epithelial cells, whereas the combined exposure of 9,10-PQ or 1,2-NQ with H-DEP induced a noticeable detrimental effect, which mainly reflected the action of H-DEP. No evidence was obtained indicating that considered PAHs or combined exposures have any pro-inflammatory effect on airway epithelial cells. BaP had a significant impact on the immune response by stimulating APCs via CD86 to enhance co-stimulatory molecules' expression.

H-DEP exposure did not result in any noticeable increase of CD86, but BaP and H-DEP showed synergistic effects (under conditions in which BaP or H-DEP sometimes affected cell viability). 9,10-PQ and 1,2-NQ and their combined exposure with H-DEP did not show any significant activation of APCs. H-DEP inhibited splenocyte proliferation. No significant activation of splenocytes was caused by BaP, 9,10-PQ or 1,2-NQ single-exposure or by combinations of them with H-DEP.

In 2008, Goulaovic et al. used monocyte leukemia cells to observe the pro-inflammatory cytokine reaction of microphages under carbon black + PAHs. They found that BaP has proinflammatory effects whereas ultra-fine particles (14 nm dia.) have immunotoxic effects. They also noted that BaP with ultrafine particles partially amplified the immunotoxic effect. In a very

267	similar way, we conducted our present experiment to investigate the effects of the well-studied
268	PAH, such as BaP compared to the effects of quinones 9,10-PQ and 1,2-NQ, which are not yet
269	widely studied but a potential contributor of respiratory allergy, in the presence and absence H-
270	DEP. In contrast to Goulaouic et al.'s study, our focus was the scope of respiratory allergy, i.e.,
271	airway epithelial cells and immune cells of atopic prone NC/Nga mouse. Based on the previous
272	reports and the present status of our knowledge, the present study is the first to elucidate a
273	synergistic effect in respiratory cells.
274	Airway epithelial cells act as the first line of defense against xenobiotics in the innate
275	immune system. They are capable of secreting cytokines such as IL-6 and IL-8, and thereafter
276	via a trans-signaling mode, they are critically involved in the pathogenesis of inflammatory
277	response. In the present study, we observed that the single exposures of PAHs did not have a
278	significant effect on cell viability, except for BaP, which significantly increased cell viability
279	(p <0.01) at 1 and 10 μ M doses in airway epithelial cells (BEAS-2B) cells. We also observed
280	that most of the combined exposures with H-DEP decreased cell viability compared to the
281	PAHs alone. It has been reported that carbon nano and micron size particles decreased cell
282	viability size and dose-dependently (Sahu et al, 2014), and the combined-effect cytotoxicity in
283	the present study might thus be the result of mainly the carbon nuclei.
994	Experimental studies with DEP or ambient PMas indicated that pro inflammatory



286	Other investigators demonstrated the inhibition of IL-8, but an elevation in IL-6 expression
287	(Fuentes et al, 2010, Rodriguez-cotto, 2014). Depending on the components of exposure, the
288	IL-6 and IL-8 expressions change, because the pathophysiological mechanisms are different.
289	To understand how our chosen PAHs influences the pro-inflammatory effect, we analyzed the
290	IL-6 and IL-8 expressions. Although we did not observe significant changes in IL-6 expression
291	due to any of the PAHs, the steady increase of IL-6 with a single exposure to BaP should not
292	be overlooked. Hu et al., 2016, recently reported that the aryl hydrocarbon receptor (AhR) is a
293	signaling pathway that is a probable cause of the increase of IL-6. It is already established that
294	BaP is an AhR ligand (Beamer and Shepherd, 2013). According to them it seems likely that
295	after entering the body BaP goes through a cascade of intra-cellular reaction with AhR and
296	eventually reaches a range of endpoints including aberrant cytokine secretion.
297	In the present study, none of the PAHs apparently caused inflammatory responses, but
298	BaP might have triggered IL-6 release via some signal transductions as the IL-6 secretion was
299	greater than that of the control. On the other hand, IL-8 did not change significantly but their
300	dose dependent result should not be overlooked. The low dose of 9,10-PQ somewhat elevated
301	the IL-8 secretion but the 1 μM dose downregulated the IL-8 secretion but the change is not
302	statistically significant. The 9,10-PQ+H-DEP exposure resulted in slight IL-8 elevation,
303	whereas Bap + H-DEP showed no such variation. Wang et al. 2012, indicated that IL-8 is
304	regulated by the phosphorylation of Erk1/2, whereas the phosphorylation of p38 inhibits it. It

305was also reported that PAHs including BaP induced ERK1/2 and p38 kinases (Andrysik, 2016). 306 Though it has not been confirmed that PAHs trigger the same biological pathway of IL-8 upregulation or downregulation, it is possible that 9,10-PQ at 1 µM and combined-exposure 307 quinones with H-DEP may sometimes follow the same pathway. Alfaro-Moreno et al.2009 308 reported a sharp IL-8 decrease and an IL-6 increase in airway epithelial cells exposed to 309particulate matter, which somewhat resembles our results with BaP single-exposure and 9,10-310 PQ + H-DEP (except at 0.01 μ M). 311Respiratory allergies such as asthma are mediated by Th2 responses, in which upon their 312recognition of an invasive allergen, APCs process the allergen into small peptides for 313 presentation on major histocompatibility complex (MHC) and migrate to secondary lymphoid 314315tissue. Subsequently, the matured and activated APC-allergen combination attaches to specific 316TCRs on T cells and induces the proliferation of lymphocytes (T and B cells). On the other

In APCs, the expression of CD86 was significant after BaP single and combined exposures in our study. Yanagisawa et al. 2016 obtained similar results in their study, in which the numbers of CD86⁺ and MHC class II⁺ cells feebly increased with BaP exposure of Ovalbumin sensitized mediastinal lymph node (MLN) cells. The MHC class II and costimulatory molecule CD86 is essential for antigen presentation. According to Li et al.,2016 CD86 is a key factor of Th1/Th2 cytokine regulation. It has been noted that APCs express co-

hand, APCs are known to produce IL-10, which is essential for a Th2 response.

stimulatory molecules that include CD86, which is responsible for providing the second signal
for the optimal induction of T-cell activation, division, and differentiation. It is evident that the
patients with allergic asthma has high CD86 in their Bronchoalveolar lavage (BAL) fluid and
it is concluded that CD86 has a role to play in allergen-induced inflammatory process in
asthmatic pathway (Liang et al, 2006).

329

In the present study, BaP rather than H-DEP exaggerated immune responses through the activation of APCs. BaP in the presence and the absence of heated-Asian sand dust particles increased the CD86 expression on APCs (Honda et al., in press). This result is in accord with our novel findings regarding H-DEP; that is, (1) BaP adhered to the particles has greater effects than the particles themselves and (2) BaP and particles have additive/synergistic effects on CD86 expression, which can be expected to be important in the initiation of Th2 responses, which eventually trigger respiratory allergies such as asthma.

According to the findings of Kadkhoda et al., 2005, IL-10 from APCs is essential for the Th2 response, and the IL-10 expression increased under BaP exposure. In contrast, Hwang et al.2007 reported that IL-10 production was impaired under BaP exposure. Our present investigation did not reveal either of those variations (data not shown). IL-10 secretion is an antigen-specific reaction (Yanaba et al., 2008). We therefore suspect that the PAHs examined herein without antigenicity did not trigger the IL-10 molecule. 343 DEC205/CD205 molecule, which is known for mediating the capture and internalization 344 of ligands for processing and presentation by APCs, was not affected by our chosen PAHs alone 345 or by their combined exposure with H-DEP. On the basis of the present results, BaP and the 346 two quinones could not affect the antigen uptake.

Splenocytes' viability and proliferation seem to be linked, as the combined exposure to 347all of the PAHs studied here inhibited both the viability and the proliferation of the splenocytes. 348TCR is an appropriate marker of T cells because it interacts with the MHC antigen peptide 349complex on APCs, and CD28 molecules expressed on the surface of T cells interact with CD80 350and CD86 ligands expressed on the surface of APCs (Li et al, 2016). Considering this 351costimulatory pathway, we measured TCR on splenocytes. Although the cell viability was 352significantly low with the 9,10-PQ exposure at the highest dose (1 µM), the TCR expression 353was highest in that case. These results may be interpreted as indicating that under those doses, 354other kind of cell types in splenocytes had degenerated except T cells, and thus the number of 355TCR-positive cells seemed to increase. 1,2-NQ + H-DEP exposure resulted in a slight negative 356for TCR expression, but the difference was not significant. CD19 deficiency is responsible for 357358a worse inflammatory response (Lordan et al, 2000). It is thus reasonable to infer that 9,10-PQ has the potential to trigger inflammation at a higher dose with H-DEP by inhibiting CD19 359expression. Comparing the TCR and CD19 percentages, the numbers of T cells were increased 360whereas those of B cells were decreased at the high dose of 9,10-PQ, both in single and 361

362 combined exposure. This may explain why the B cells had higher sensitivity against 9,10-PQ
 363 compared to the T cells.

364 In conclusion, our results established that the synergistic exposure to PAHs and the carbon nuclei is notably detrimental compared to a single exposure, at least under in vitro 365conditions. The 9,10-PQ or 1,2-NQ alone may not be the principle contributing factor to the 366airway inflammation. However, in combined exposure with carbon nuclei, they impart 367observable detrimental effects. BaP can contribute to the development and/or exacerbation of 368 369 respiratory allergies such as asthma, especially to the immune system via APC activation. As in ambient air, PM_{2.5} is always associated with PAHs and other material, and we propose that a 370 much more realistic approach to study the effects of particulate matter is to investigate exposure 371372to their combinations.

373

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376

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496	Figure legends
497	Fig. 1. Structures of benzo(a)pyrene, 9,10-phenanthroquinone (9,10-PQ) and 1,2-
498	nephthoquinone (1,2-NQ).

500	Fig. 2. Representative data for unstained (black), isotype control (gray) and CD86-positive
501	cells (filled gray). The histogram shows the percentage of CD86-positive cells with subset bars.
502	
503	Fig. 3. Cell viability of BEAS-2B cells exposed to (A) BaP, (B) 9,10-PQ and (C) 1,2-NQ.
504	*p<0.05 vs. corresponding control. #p<0.05 vs. same concentration.
505	
506	Fig. 4. Levels of IL-6 and IL-8 produced by BEAS-2B cells exposed to (A&D) BaP, (B&E)
507	9,10-PQ and (C&F) 1,2-NQ.
508	
509	Fig. 5. Cell viability of APC was performed by WST-1 Assay to understand the effect of (A)
510	BaP, (B) 9,10-PQ and (C) 1,2-NQ in APC. *p<0.05 vs. corresponding control.
511	
512	Fig. 6. CD86 positive cells in APC during exposure of (A) BaP, (B) 9,10-PQ and (C) 1,2-NQ
513	in APC, measured by FACS. *p<0.05 vs. corresponding control.
514	
515	Fig 7. DEC205 positive cells in APC during exposure of (A) BaP, (B) 9,10-PQ and (C) 1,2-
516	NQ in APC, measured by FACS.
517	

518	Fig. 8. Cell viability of splenocyte and cell proliferation during exposure of (A&D) BaP,
519	(B&E) 9,10-PQ and (C&F) 1,2-NQ. *p<0.05 vs. corresponding control. #p<0.05 vs. same
520	concentration.
521	
522	Fig. 9. TCR and CD19 positive cells in splenocytes during exposure of (A&D) BaP, (B&E)
523	9,10-PQ and (C&F) 1,2-NQ in BEAS-2B cell line was measured by FACS. *p<0.05 vs.
524	corresponding control.
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Benzo(a)pyrene 9,10-Phenanthroquinone 1,2-Naphthoquinone

535 Fig.2





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540 Fig.4



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545 Fig.6







550 Fig.8



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