

Title	Synergistic effect of carbon nuclei and polyaromatic hydrocarbons on respiratory and immune responses
Author(s)	Chowdhury, Pratiti H.; Kitamura, Gaku; Honda, Akiko; Sawahara, Takahiro; Hayashi, Tomohiro; Fukushima, Wataru; Kudo, Hitomi; Ito, Sho; Yoshida, Seiichi; Ichinose, Takamichi; Ueda, Kayo; Takano, Hirohisa
Citation	Environmental Toxicology (2017), 32(9): 2172-2181
Issue Date	2017-9
URL	<a href="http://hdl.handle.net/2433/243826">http://hdl.handle.net/2433/243826</a>
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Type	Journal Article
Textversion	author

1 **Synergistic effect of carbon nuclei and polyaromatic hydrocarbons on respiratory and**  
2 **immune responses**

3

4 Chowdhury, Pratiti Home; Kyoto University, Department of Environmental Engineering

5 Kitamura, Gaku; Kyoto University, Department of Environmental Engineering

6 Honda, Akiko; Kyoto University, Department of Environmental Engineering

7 Sawahara, Takahiro ; Kyoto University, Department of Environmental Engineering

8 Hayashi, Tomohiro; Kyoto University, Department of Environmental Engineering

9 Fukushima, Wataru; Kyoto University, Department of Environmental Engineering

10 Kudo, Hitomi; Kyoto University, Department of Environmental Engineering

11 Ito, Sho; Kyoto University, Department of Environmental Engineering

12 Yoshida, Seiichi; Department of Health Sciences, Oita University of Nursing and Health

13 Sciences

14 Ichinose, Takamichi; Department of Health Sciences, Oita University of Nursing and Health

15 Sciences

16 Ueda, Kayo; Kyoto University, Department of Environmental Engineering

17 Takano, Hirohisa; Kyoto University, Environmental Health Division,

18 Department of Environmental Engineering, Graduate School of Engineering

19

20 **ABSTRACT**

21 **Background:** Particulate matter with aerodynamic diameter  $\leq 2.5$   $\mu\text{m}$  (PM<sub>2.5</sub>) is generally  
22 composed of carbon nuclei associated with various organic carbons, metals, ions and biological  
23 materials. Among these components, polyaromatic hydrocarbons (PAHs) such as  
24 benzo(a)pyrene (BaP) and quinones have detrimental effects on airway epithelial cells and  
25 immunodisrupting effects, which leads to the exacerbation of respiratory allergies. The effects  
26 of PAHs and the carbon nuclei, separately as well as in combination, remain to be established.

27 **Objective:** We investigated the effects of BaP, 9,10-phenanthroquinone (9,10-PQ) and 1,2-  
28 naphthoquinone (1,2-NQ) and their combined effects with heated diesel exhaust particle (H-  
29 DEP) as carbon nuclei of typical PM<sub>2.5</sub>.

30 **Methods:** We exposed human airway epithelial cells (BEAS-2B), murine bone marrow-  
31 derived antigen-presenting cells (APCs), and murine splenocytes to BaP, 9,10-PQ, or 1,2-NQ  
32 in the presence and absence of H-DEP. Several important inflammatory cytokines and cell  
33 surface molecules were measured.

34 **Results:** PAHs alone did not have apparent cytotoxic effects on BEAS-2B, whereas combined  
35 exposure with H-DEP induced noticeable detrimental effects which mainly reflected the action  
36 of H-DEP itself. BaP increased CD86 expression as an APC surface molecule regardless of the  
37 presence or absence of H-DEP. None of the BaP, 9,10-PQ or 1,2-NQ exposure alone or their  
38 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<sub>2.5</sub>, especially via APC activation.

42

### 43 **Introduction**

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

51 PAHs, including benzo[a]pyrene (BaP) and quinones, are among the most prominent  
52 compounds found in PM<sub>2.5</sub>. 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 *ortho*-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 *ortho*-quinone (Fig. 1).

62       Regarding the health effects of PM<sub>2.5</sub>, 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<sub>2.5</sub> 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 PM<sub>2.5</sub>.

71

## 72 **Methods**

### 73 *Cell preparation*

74 The experiment was performed on human airway epithelial cells (BEAS-2B) and immune cells  
75 including splenocytes and bone marrow-derived cells (APCs). Ten-week-old NC/NgaTendCrlj  
76 male mice (Charles 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<sub>2</sub> 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

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

100 After aspiration of the media, the cells were resuspended in culture medium R10, which  
101 was GIBCO RPMI 1640 medium (Invitrogen, Grand Island, NY) supplemented with 10% heat-  
102 inactivated fetal bovine serum (FBS; MP Biomedicals, Eschwege, Germany), 100 U/ml  
103 penicillin, 100 µg/ml streptomycin (Sigma, St. Louis, MO), and 50 µM 2-mercaptoethanol  
104 (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 ( $4 \times 10^5$  /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 400g 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  $1 \times 10^6$  cells/ml.

117

#### 118 *Splenocyte isolation*

119 The spleen was removed from the mice, crushed and pushed through a 200-mesh stainless steel  
120 sheet. The cells were suspended in RPMI-1640 media and centrifuged at 400g for 5 min at  
121 20°C. After being treated with hemolytic reagent, the cell suspension was centrifuged one more  
122 time. The numbers of viable cells were determined by the trypan blue exclusion method. The  
123 final concentrations of the cell suspension and exposure medium were appropriately calculated  
124 to achieve the final concentration of  $1 \times 10^6$  cells/ml.

125

#### 126 *Exposure of airway epithelial cells and immune cells*

127 We examined BaP (purchased from Sigma-aldrich), 9,10-PQ (purchased from Sigma  
128 aldrich), and 1,2-NQ (purchased from Tokyo chemical Industry) as exposure reagents. To  
129 understand the synergistic effects of carbon nuclei and PAHs in DEP as representative of PM<sub>2.5</sub>,  
130 we used heated-DEP (H-DEP) where PAHs were excluded by heat at 360°C for 30 min. We  
131 characterized the H-DEP particles and measured various components including several PAHs  
132 consisting of four- five- and six-membered rings. The particle did not have any PAHs  
133 associated with it. Heating at 360°C caused complete evaporation of PAH. However, metals



134 and ions such as, Ca, Na, Zn, Fe, Mn, As and  $\text{SO}_4^{2-}$  had been detected. Cells were exposed to  
135 BaP (0, 0.1, 1.10  $\mu\text{M}$ ) or 9,10-PQ or 1,2-NQ (0, 0.01, 0.1, 1  $\mu\text{M}$  for both) in the presence or  
136 absence of H-DEP (50  $\mu\text{g}/\text{mL}$ ) for 24 or 72 h. The results of our pre-experiments using quinone  
137 showed that doses higher than the aforementioned doses are highly cytotoxic and thus not  
138 appropriate for the immune study.

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

147

#### 148 *Cell viability*

149 Cell viability was assessed by WST-1 assay. The BEAS-2B cell suspension of  $7.5 \times 10^4$  cells/mL  
150 was seeded at 70  $\mu\text{L}/\text{well}$  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  $\mu\text{L}/\text{well}$ . 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  $\mu$ 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  $-80^{\circ}\text{C}$ . 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*

175 The cell surface molecules were measured by a FACS analysis. The following monoclonal  
176 antibodies were used to react with specific antigens: Mouse BD Fc Block™ purified anti-  
177 mouse CD16/CD32 (Becton Dickinson), DEC205 (NLDC-145, PE-conjugated, BioLegend,  
178 San Diego, CA), Rat IgG2a, κ Isotype Control (RTK2758, PE-conjugated, BioLegend), CD86  
179 (GL-1, PE-conjugated, Becton Dickinson), Rat IgG2a, κ Isotype Control (R35-95, PE-  
180 conjugated, Becton Dickinson), Hamster Anti-Mouse TCR-β Chain (H57-597, FITC-  
181 conjugated, Becton Dickinson), Hamster IgG2, λ1 Isotype Control (Ha4/8, FITC-conjugated,  
182 Becton Dickinson), Rat Anti-Mouse CD19 (1D3, PE-conjugated, Becton Dickinson), and Rat  
183 IgG2a, κ Isotype Control (R35-95, PE-conjugated, Becton Dickinson). To determine the  
184 antigen-positive zones in the FACS histogram, we compared stained samples with un-stained  
185 and isotype controls (Fig. 2).

186 After exposure for 24 hr, the immune cells were collected and resuspended in 50 μL  
187 phosphate-buffered saline (PBS) with 0.3% bovine serum albumin and 0.05% sodium azide  
188 (Wako Pure Chemical Industries, Osaka, Japan). Depending on the objective marker, the  
189 appropriate antibody was added into it and maintained for 45 min at 4°C before a wash with  
190 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  $\mu$ M and 10  $\mu$ 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.  
208 4A). The IL-8 expression did not change significantly with BaP exposure either (Fig. 4D). The  
209 quinones' single exposure did not result in any changes in IL-6 or IL-8 expression. The PAHs

210 with or without H-DEP also did not result in any noticeable pro-inflammatory changes (Fig. 4).

211

212 *Effects on immune responses*

213 Effects on APCs

214 BaP single exposure decreased the viability of APCs (Fig. 5A). The quinones alone had a feeble  
215 decreasing effect on cell viability. H-DEP alone did not change cell viability as well. BaP + H-  
216 DEP caused low cell viability compared to the control. 9,10-PQ + H-DEP and 1,2-NQ + H-  
217 DEP exposure both did not show any change in viability compared to control except highest  
218 dose of the later, which lowered the viability (Fig. 5B,C).

219 BaP single exposure showed high CD86 expression (Fig. 6A) while quinones did not  
220 have any effect on any dose. H-DEP alone also did not show any noticeable effect. BaP+H-  
221 DEP had an effect on the elevation of CD86 expression as compared to control. But combined  
222 exposure to the quinones and H-DEP did not have any significant effect (Fig. 6B and 6C). On  
223 the other hand, PAHs with or without H-DEP did not cause any change in the expression of  
224 DEC205 (Fig. 7). IL-10 expression for all of the PAHs and their combined exposure showed  
225 the result under detection level.

226

227 Effects on splenocytes

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

229 Both of the quinones did not show any difference in single exposures except at 1  $\mu$ M dose of  
230 9,10-PQ that showed significantly decreased viability (Fig. 8B,8C). H-DEP alone tended to  
231 decrease cell viability. BaP + H-DEP showed the significant detrimental effect on cell viability  
232 as compared to control. 9,10-PQ + H-DEP and 1,2-NQ + H-DEP tended to decrease cell  
233 viability as compared to control.

234 When we checked the proliferation of splenocytes after 72 hr of exposure, we observed  
235 that BaP single exposure (1  $\mu$ M and 10  $\mu$ M) decreased proliferation (Fig. 8D). Neither 9,10-  
236 PQ nor 1,2-NQ single exposure had a significant effect. H-DEP alone as well as BaP + H-DEP,  
237 9,10-PQ + H-DEP and 1,2-NQ + H-DEP had declining effects on cell proliferation compared  
238 to single exposure (Fig. 8).

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

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

248 9F).

249

## 250 **Discussion**

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

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

263 In 2008, Goulaovic et al. used monocyte leukemia cells to observe the pro-inflammatory  
264 cytokine reaction of macrophages under carbon black + PAHs. They found that BaP has pro-  
265 inflammatory effects whereas ultra-fine particles (14 nm dia.) have immunotoxic effects. They  
266 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  $\mu\text{M}$  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.

284 Experimental studies with DEP or ambient  $\text{PM}_{2.5}$  indicated that pro-inflammatory  
285 responses such as IL-6 and IL-8 release occur in airway epithelial cells (Streerenberg, 1998).



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  $\mu$ M 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

305 was 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  
307 upregulation or downregulation, it is possible that 9,10-PQ at 1  $\mu$ M and combined-exposure  
308 quinones with H-DEP may sometimes follow the same pathway. Alfaro-Moreno et al.2009  
309 reported a sharp IL-8 decrease and an IL-6 increase in airway epithelial cells exposed to  
310 particulate matter, which somewhat resembles our results with BaP single-exposure and 9,10-  
311 PQ + H-DEP (except at 0.01  $\mu$ M).

312         Respiratory allergies such as asthma are mediated by Th2 responses, in which upon their  
313 recognition of an invasive allergen, APCs process the allergen into small peptides for  
314 presentation on major histocompatibility complex (MHC) and migrate to secondary lymphoid  
315 tissue. Subsequently, the matured and activated APC-allergen combination attaches to specific  
316 TCRs on T cells and induces the proliferation of lymphocytes (T and B cells). On the other  
317 hand, APCs are known to produce IL-10, which is essential for a Th2 response.

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

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

329

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

337 According to the findings of Kadkhoda et al., 2005, IL-10 from APCs is essential for the  
338 Th2 response, and the IL-10 expression increased under BaP exposure. In contrast, Hwang et  
339 al.2007 reported that IL-10 production was impaired under BaP exposure. Our present  
340 investigation did not reveal either of those variations (data not shown). IL-10 secretion is an  
341 antigen-specific reaction (Yanaba et al., 2008). We therefore suspect that the PAHs examined  
342 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.

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

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

373

374 **Acknowledgement:** This study was supported by the Environmental Research and Technology  
375 Development Fund (5-1457) by the Ministry of the Environment, Japan.

376

### 377 **Reference**

378 Alfaro-Moreno E, Torres V, Miranda J, Martínez L, García-Cuellar C, Nawrot TS,  
379 Vanaudenaerde B, Hoet P, Ramírez-López P, Rosas I, Nemery B, Osornio-Vargas AR. 2009.

380 Induction of IL-6 and inhibition of IL-8 secretion in the human airway cell line Calu-3 by urban  
381 particulate matter collected with a modified method of PM sampling. *Environ Res* 109:528-35.  
382 doi: 10.1016/j.envres.2009.02.010.

383

384 Andrysik Z, Machala M, Chramostova K, Hofmanova J, Kozubik A, Vondracek J. 2016.  
385 Activation of ERK1/2 and p38 kinases by polycyclic aromatic hydrocarbons in rat liver  
386 epithelial cells is associated with induction of apoptosis. *Toxicol Appl Pharmacol* 211:198-208,  
387 doi: 10.1016/j.taap.2005.06.007.

388

389 Beamer CA, Shepherd DM. 2013. Role of the aryl hydrocarbon receptor (AhR) in lung  
390 inflammation. *Semin Immunopathol* 35:693-704: doi: 10.1007/s00281-013-0391-7.

391

392 Fuentes-Mattei E, Rivera E, Gioda A, Sanchez-Rivera D, Roman-Velazquez FR, Jimenez-  
393 Velez BD. 2010. Use of human bronchial epithelial cells (BEAS-2B) to study immunological  
394 markers resulting from exposure to PM(2.5) organic extract from Puerto Rico. *Toxicol Appl*  
395 *Pharmacol* 243:381-389. doi: 10.1016/j.taap.2009.12.009.

396

397 Goulaouic S, Foucaud L, Bennasroune A, Laval-Gilly P, Falla J. 2008. Effect of polycyclic  
398 aromatic hydrocarbons and carbon black particles on pro-inflammatory cytokine secretion:  
399 impact of PAH coating onto particles. *J Immunotoxicol* 5:337-45. doi:

400 10.1080/15476910802371016.

401

402

403 Gurbani D, Bharti SK, Kumar A, Pandey AK, Ana GR, Verma A, Khan AH, Patel DK, Mudiam

404 MK, Jain SK, Roy R, Dhawan A. 2013. Polycyclic aromatic hydrocarbons and their quinones

405 modulate the metabolic profile and induce DNA damage in human alveolar and bronchiolar

406 cells. *Int J Hygiene Env. Health* 216:553-565. doi: 10.1016/j.ijheh.2013.04.001.

407

408 Hiyoshi K1, Takano H, Inoue KI, Ichinose T, Yanagisawa R, Tomura S, Kumagai Y. 2005.

409 Effects of phenanthraquinone on allergic airway inflammation in mice. *Clin Exp Allergy*

410 35:1243-8.

411

412 Honda A, Sawahara T, Hayashi T, Tsuji K, Fukushima W, Oishi M, Kitamura G, Kudo H, Ito

413 S, Yoshida S, Ichinose T, Ueda K, Takano H. 2016. Biological factor related to Asian sand dust

414 particles contributes to the exacerbation of asthma, *J Appl Toxicol* (accepted) doi:

415 10.1002/jat.3395.

416

417 Hu T, Pan Z, Yu Q, Mo X, Song N, Yan M, Zouboulis CC, Xia L, Ju Q. 2016. Benzo(a)pyrene

418 induces interleukin (IL)-6 production and reduces lipid synthesis in human SZ95 sebocytes via

419 the aryl hydrocarbon receptor signaling pathway. *Environ Toxicol Pharmacol* 43:54-60. doi:

420 10.1016/j.etap.2016.02.011.

421

422 Hwang JA, Lee JA, Cheong SW, Youn HJ, Park JH. 2007. Benzo(a)pyrene inhibits growth and  
423 functional differentiation of mouse bone marrow-derived dendritic cells — Downregulation of  
424 RelB and eIF3 p170 by benzo(a)pyrene. *Toxicol Lett* 169: 82-90.

425

426 Inoue KI, Takano H, Hiyoshi K et al. Naphthoquinone enhances antigen-related airway  
427 inflammation in mice. 2007. *Eur Respir J* 29:259-267. doi: 10.1183/09031936.00033106.

428

429 Kadkhoda K, Pourfathollah AA, Pourpak Z, Kazemnejad A. 2005. The cumulative activity of  
430 benzo(a)pyrene on systemic immune responses with mite allergen extract after intranasal  
431 instillation and ex vivo response to ovalbumin in mice. *Toxicol Lett* 157:31-9.

432

433 Kadkhoda K, Pourpak Z, Akbar Poufathallah A, Kazemnejad A. 2004. The ex vivo study of  
434 synergistic effects of polycyclic aromatic hydrocarbon, benzo(a)pyrene with ovalbumin on  
435 systemic immune responses by oral route. *Toxicology* 199:261-265.

436

437 Koike E, Yanagisawa R, Takano H. 2014. Toxicological effects of polycyclic aromatic  
438 hydrocarbons and their derivatives on respiratory cells. *Atm Env* 97:1-8.



439

440 Kumagai Y, Taguchi K. Toxicological effects of polycyclic aromatic hydrocarbon quinones  
441 contaminated in diesel exhaust particles. *Asian J Atm Env* 2007;1-1:28-35. doi:  
442 10.5572/ajae.2007.1.1.028.

443

444 Li JG, DU YM, Yan ZD, Yan J, Zhuansun YX, Chen R, Zhang W, Feng SL, Ran PX. 2016.  
445 CD80 and CD86 knockdown in dendritic cells regulates Th1/Th2 cytokine production in  
446 asthmatic mice, *Exp Ther Med* 11:878-884. doi: 10.3892/etm.2016.2989.

447

448 Liang XD, Shi HZ, Qin XJ, Deng, JM, 2006, Increase in Concentration of Soluble CD86 After  
449 Segmental Allergen Challenge in Patients With Allergic Asthma, *Chest* Vol. 130: 4(2006), Pg.  
450 1048–1054, doi:10.1378/chest.130.4.1048

451

452 Lordan J, Djukanovic R, Holgate ST. 2000. Pathophysiology and clinical aspects of respiratory  
453 allergies with reference to asthma. *Revue francaise d'allergologie et d'immunologie Clinique*  
454 40:33-45. doi: 10.1016/S0335-7457(00)80018-0

455

456 Lutz MB, Kukutsch N, Ogilvie AL, Rössner S, Koch F, Romani N, Schuler G. 1999. An  
457 advanced culture method for generating large quantities of highly pure dendritic cells from

458 mouse bone marrow. *J Immunol Methods* 223:77-92.

459

460 Ring J, Eberlein-Koenig B, Behrendt H. 2001. Environmental pollution and allergy. *Ann*  
461 *Allergy Asthma Immunol* 87:2-6.

462

463 Rodríguez-Cotto RI, Ortiz-Martínez MG, Rivera-Ramírez E, Mateus VL, Amaral BS, Jiménez-  
464 Vélez BD, Gioda A. 2014. Particle pollution in Rio de Janeiro, Brazil: increase and decrease  
465 of pro-inflammatory cytokines IL-6 and IL-8 in human lung cells. *Environ Pollut* 194:112-120.  
466 doi: 10.1016/j.envpol.2014.07.010.

467

468 Sahu D, Kannan GM, and Vijayaraghavan R. 2014. Carbon black particle exhibits size  
469 dependent toxicity in human monocytes. *Int J Inflamm.* vol. 2014. Article ID 827019,10.  
470 doi:10.1155/2014/827019.

471

472 Steerenberg PA, Zonnenberg JA, Dormans JA, Joon PN, Wouters IM, van Bree L, Scheepers  
473 PT, Van Loveren H. 1998. Diesel exhaust particles induced release of interleukin 6 and 8 by  
474 (primed) human bronchial epithelial cells (BEAS 2B) in vitro. *Exp Lung Res* 24:85-100.

475

476 Tobiszewski M, Namiesnik J. 2012. PAH diagnostic ratios for the identification of pollution  
477 emission sources. *Environ Pollut* 162:110-119. <http://dx.doi.org/10.1016/j.envpol.2011.10.025>

478

479 Wang Y, Wang W, Wang L, Wang X, Xia J. 2012. Regulatory mechanisms of interleukin-8  
480 production induced by tumour necrosis factor- $\alpha$  in human hepatocellular carcinoma cells. *J*  
481 *Cell Mol Med* 16:496-506. doi: 10.1111/j.1582-4934.2011.01337.x.

482

483 Yanaba K, Bouaziz JD, Haas KM, Poe JC, Fujimoto M, Tedder TF. 2008. A regulatory B cell  
484 subset with a unique CD1dhiCD5+ phenotype controls T cell-dependent inflammatory  
485 responses. *Immunity* 28:639-50. doi: 10.1016/j.immuni.2008.03.017.

486

487 Yanagisawa R, Koike E, Win-Shwea TT, Ichinose T and Takano H. 2016. Low-dose  
488 benzo[a]pyrene aggravates allergic airway inflammation in mice, *J Appl Toxicol* 36:1496-504.  
489 doi: 10.1002/jat.3308.

490

491 Yanagisawa R, Takano H, Inoue KI, Ichinose T, Sadakane K, Yoshino S, Yamaki K, Yoshikawa  
492 T, Hayakawa K. 2006. Components of diesel exhaust particles differentially affect Th1/Th2  
493 response in a murine model of allergic airway inflammation. *Clin Exp Allergy* 36:386-95.

494

495

#### 496 **Figure legends**

497 **Fig. 1.** Structures of benzo(a)pyrene, 9,10-phenanthroquinone (9,10-PQ) and 1,2-  
498 naphthoquinone (1,2-NQ).

499

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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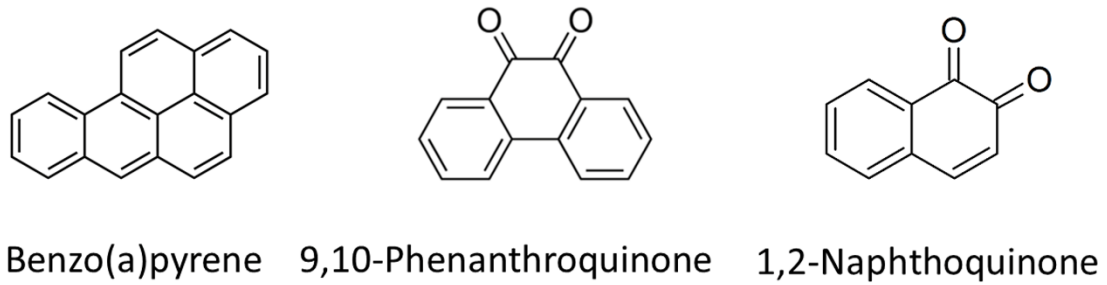
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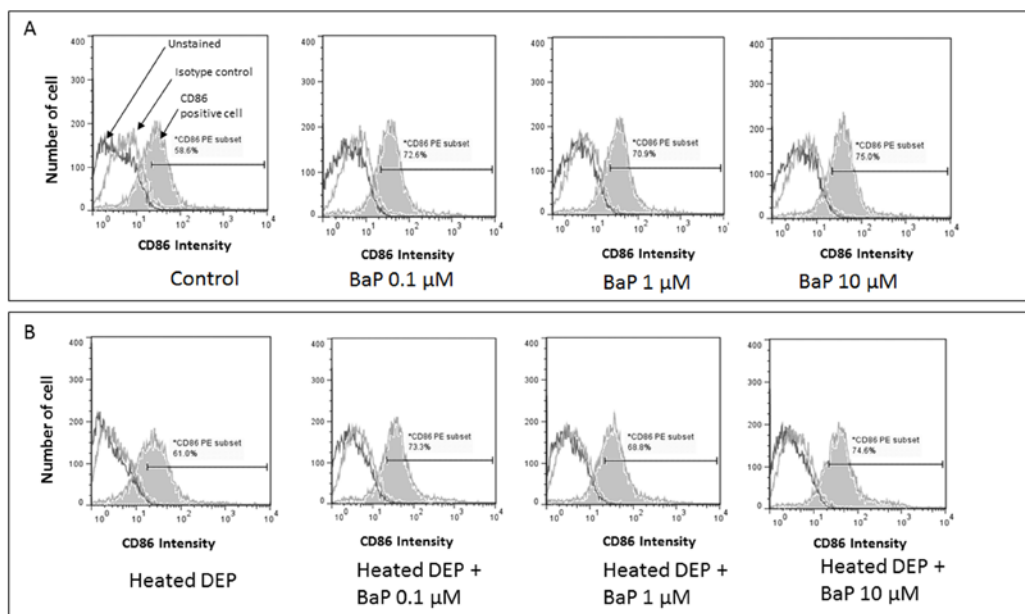
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533 Fig.1



534

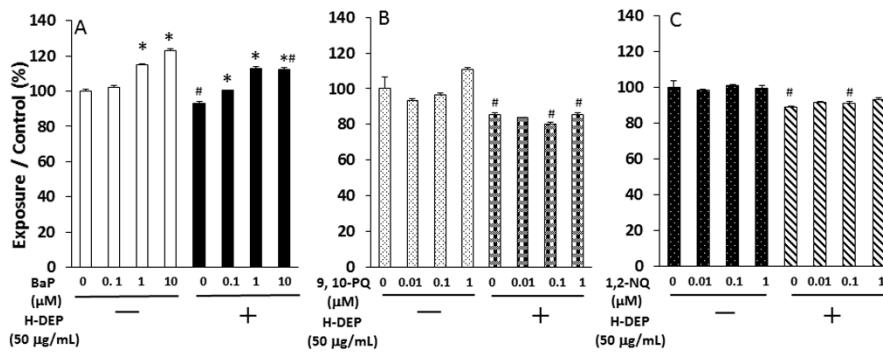
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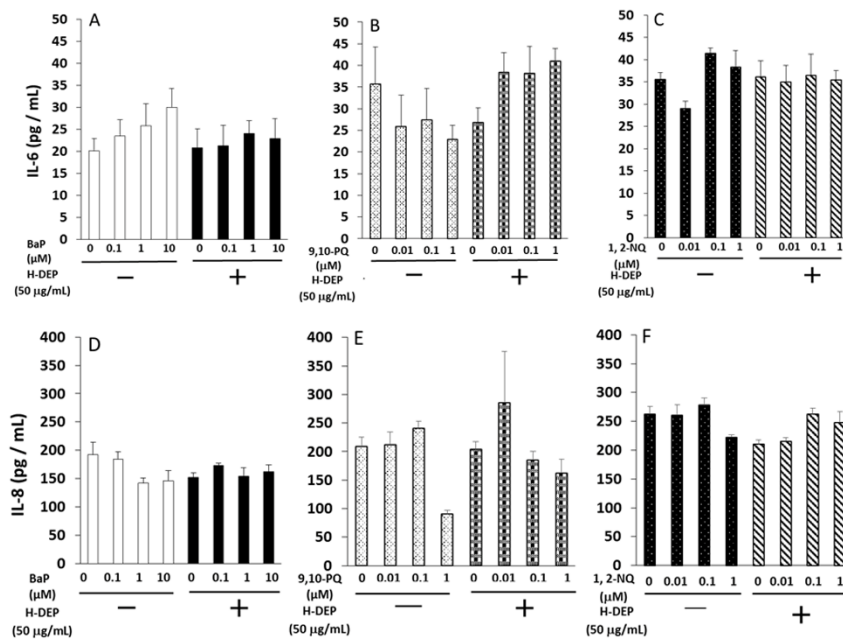
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538 Fig.3



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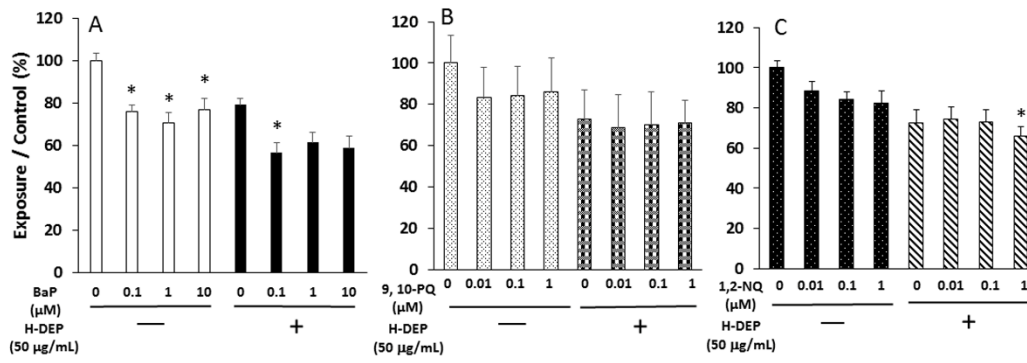
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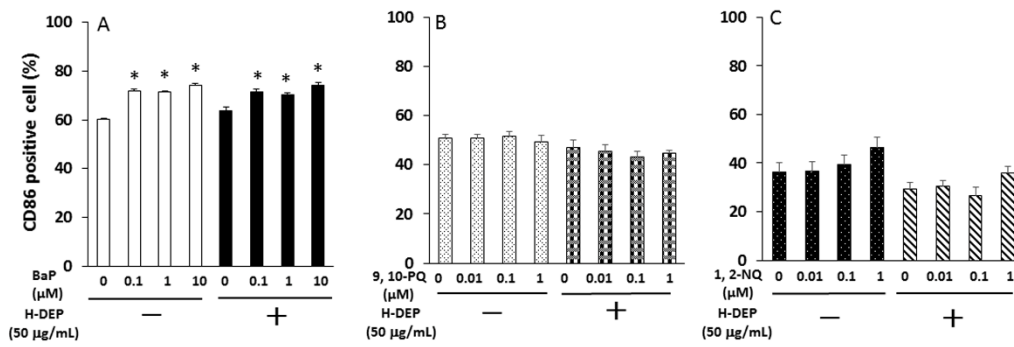
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543 Fig.5



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545 Fig.6

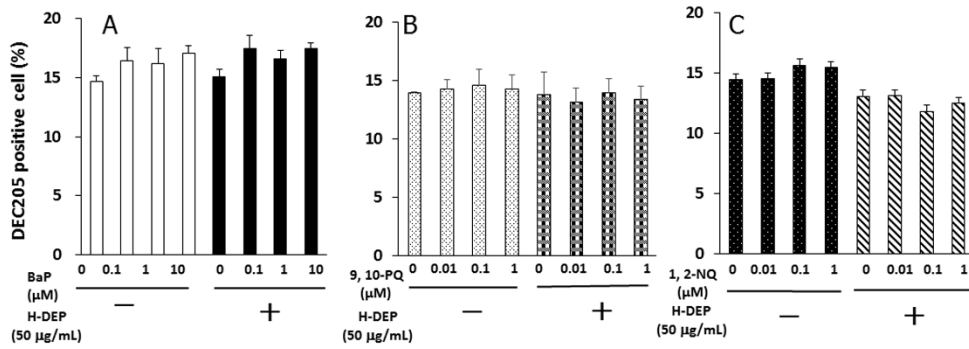


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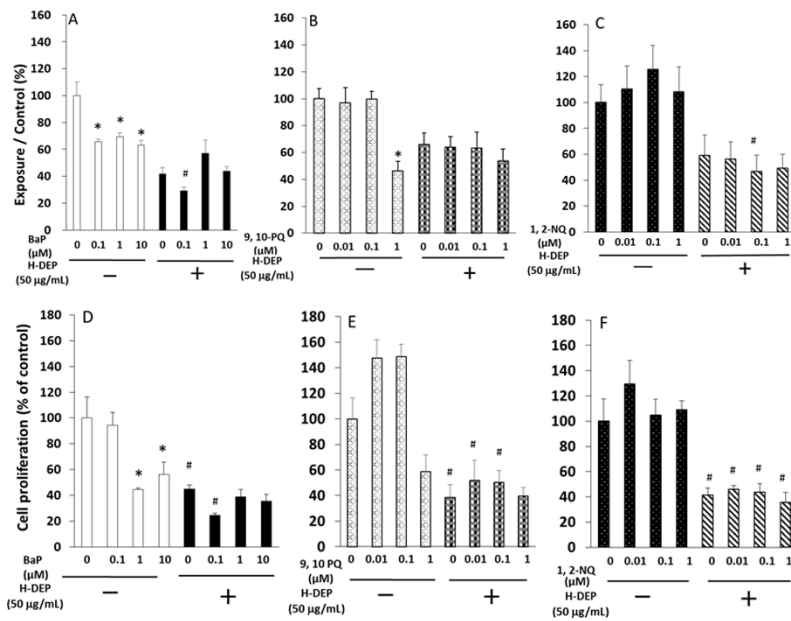


548 Fig.7



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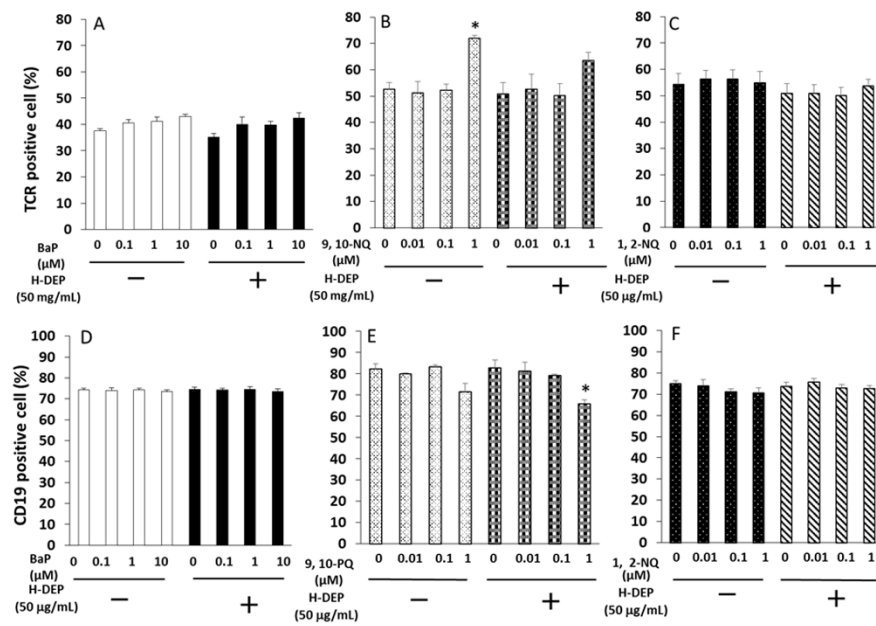
550 Fig.8



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553 Fig.9



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