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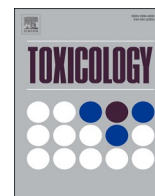
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# Exposure of RAW264.7 macrophages to exhaust emissions (gases and PAH) and non-exhaust emissions (tire particles) induces additive or synergistic TNF- $\alpha$ production depending on the tire particle size

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## ABSTRACT

Road traffic is a major contributor to air pollution and consequently negatively affects human health. Car pollution originates both from exhaust emissions (EE) and non-exhaust emissions (NEE, such as tire and brake wear particles, erosion of road surfaces and resuspension of road dust). While the toxicity of EE and NEE has been characterized separately, their combined effects are poorly documented. However, we are constantly exposed to a mixture of pollutants and their interactions should not be neglected as they may significantly impact their toxicological profile resulting in additive, synergistic or antagonistic effects. To fill this gap, we investigated *in vitro* the combined toxicity of exhaust gases and benzo[a]pyrene (representative of EE) and tire particles (representative of NEE). Macrophages from the RAW264.7 cell line were exposed for 24 h to tire particles (TP) of variable size (6–113  $\mu\text{m}$ ), alone or in combination with exhaust gases ( $\text{CO}_2$ , CO, NO,  $\text{NO}_2$ ) and benzo[a]pyrene (B[a]P) as an archetype of polycyclic aromatic hydrocarbon (PAH). The cell response was assessed in terms of cytotoxicity, proinflammatory response and oxidative stress. TP, gases and B[a]P, alone or in combination triggered neither cytotoxicity nor oxidative stress. On the contrary, a proinflammatory response was elicited with two different profiles depending on the size of the TP: TNF- $\alpha$  production was either slightly (with the finest TP) or strongly (with coarse TP) increased in the presence of gases and B[a]P, suggesting that the effects of TP, gases and B[a]P were either additive or synergistic, depending on TP size.

## 1. Introduction

Air pollution is a major public health issue worldwide. It has been shown to be responsible for many adverse health effects such as respiratory and cardiovascular diseases and cancers (Amigou et al., 2011; Beelen et al., 2014; Brunekreef and Holgate, 2002; Cazzolla Gatti, 2021; Charloux et al., 1997; Dockery et al., 1993; Germanova and Kernozhitskaya, 2017; Greim, 2019; Hamra et al., 2014; Kurt et al., 2016; Mayoralas-Alises and Diaz-Lobato, 2012; Miller et al., 2012; Miller and Newby, 2020; Nishida and Yatera, 2022; Raaschou-Nielsen et al., 2013; Shah et al., 2013; Tseng et al., 2019; Wong, 2014), resulting in millions of premature deaths per year (European Environment Agency, 2023; Miller et al., 2012; Miller and Newby, 2020; Son et al., 2020; WHO, 2014).

Air pollution consists of a complex mixture of gaseous, volatile, semi-volatile and particulate matter (PM), containing organic and inorganic compounds (Guo et al., 2021; Miller et al., 2012; Miller and Newby, 2020). Methane, benzene, naphthalene, formaldehyde, polyaromatic hydrocarbons (PAH), and alkanes are among the various semi-volatile organic chemicals of air pollution. The numerous gaseous pollutants found in ambient air include carbon dioxide ( $\text{CO}_2$ ) and monoxide (CO), ozone ( $\text{O}_3$ ), nitrogen dioxide ( $\text{NO}_2$ ) and sulphur dioxide ( $\text{SO}_2$ ) (Miller et al., 2012; Miller and Newby, 2020). Different mass-weighted categories of PM have been established based on their size: coarse particles with a diameter less than 10  $\mu\text{m}$  ( $\text{PM}_{10}$ ), fine particles with a diameter less than 2.5  $\mu\text{m}$  ( $\text{PM}_{2.5}$ ) and ultrafine particles with a diameter less than 100 nm ( $\text{PM}_{0.1}$ ).

Road traffic is a major contributor to air pollution, in particular in

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urban areas. Two types of emissions can be distinguished: exhaust emissions (EE) and non-exhaust emissions (NEE) (Lewis et al., 2019). The former originate from the tailpipe and result from fuel oxidation and lubricant volatilization during the combustion process while the latter arise from the mechanical abrasion of brakes and tires, erosion of road surfaces and resuspension of road dust into the atmosphere by passing traffic (Fussell et al., 2022; Harrison et al., 2021; Piscitello et al., 2021). While EE have been well documented, NEE have been less studied and few data are available on their potential impact on human health (Bouredji et al., 2023; Forest and Pourchez, 2023). However, we have recently observed that tire particles (TP) were able to induce a size-dependent proinflammatory response (TNF- $\alpha$  production) in a model of macrophages (RAW264.7 cells) while they triggered neither significant cytotoxicity nor enhanced ROS production.

Populations living near roads are continuously exposed to a wide variety of pollutants. However, the impact of each pollutant is usually assessed individually and the interactions between the contaminants and their resulting combined toxicity are often overlooked, partly due to technical challenges (Forest, 2021). This can lead to seriously misleading conclusions when attempting to extrapolate the overall toxicity of co-pollutants from their individual, separately characterized toxicity, since the interactions between these pollutants may cause unexpected effects (Silins and Högberg, 2011; Zheng et al., 2012). In fact, such interactions can notably alter the toxicological profile of co-pollutants, either positively or negatively, *i.e.* through additive, synergistic or antagonistic responses. This argues for a joint toxicity assessment (Deville et al., 2016; Yang et al., 2018), not considering anymore pollutants individually but shifting to an integrated multi-pollutant approach (Yun et al., 2015) to be more representative of real-life conditions.

In this context, the aim of the present study was to assess the *in vitro* combined toxicity of EE and NEE pollutants, by incubating macrophages with a combination of tire particles and exhaust gases with benzo[a]pyrene (B[a]P, as a model of PAH). Therefore, one novelty of this study is to consider a more realistic combination of car pollutants for a better and more reliable sanitary risk assessment.

## 2. Materials and methods

### 2.1. Tire particles (TP)

TP were produced via cryogenic grinding of tire tread bands taken from commercial tires with a maximum wear rate of 40 % (>5 mm residual texture depth). In order to avoid overheating, the samples were slowly cut from three tires of the same brand using a ceramic blade. They were cleaned of surface incrustations using a damp cloth, ultrapure water and compressed air. Three grams of tire particles (fragments of  $\approx$  1 mm size) were put within a zirconium oxide tank and submitted to 10 grinding cycles using a CryoMill from Resch (Verder International BV, Netherlands). Each grinding cycle lasted 2 minutes at a frequency of 30 Hz under liquid nitrogen, preceded and followed by a cooling phase. The obtained TP powder underwent a size-based separation through a sieving process to achieve a diverse range of particle sizes. This procedure utilized a 4-sieve tower with separation sizes set at 70  $\mu$ m, 30  $\mu$ m, 15  $\mu$ m and 5  $\mu$ m in conjunction with a dedicated recovery tank. The sieving operation was performed in 10-minute cycles, at a speed of 2 mm/G, in the presence of ethanol.

TP have previously been characterized extensively. Briefly, TP morphology was analyzed using scanning electron microscopy (SEM) performed with a Zeiss ULTRA-Plus microscope. The elemental composition was assessed by Energy-Dispersive X-Ray Spectroscopy (EDS) and by Inductively Coupled Plasma Mass Spectrometry (ICP-MS). Particle size distribution was evaluated using a laser diffraction size analyzer (Mastersizer 3000, Malvern Instruments Ltd, Worcestershire, UK) (ethanol was used as the solvent) and the specific surface area was determined by the Brunauer Emmett Teller (BET) measurement (by the

analysis of the adsorption of krypton, performed on a Micrometrics TRI-STAR 3000 analyzer).

### 2.2. Exhaust gases and PAH

In this study, we used a mixture of gases that simulates the proportions of the major compounds in real-world exhaust emissions (Hoekman and Robbins, 2012; Rymaniak et al., 2021). We therefore used the following mixture: CO<sub>2</sub> (35,000 ppm), CO (1500 ppm), NO (400 ppm), NO<sub>2</sub> (40 ppm), N<sub>2</sub> (96.31 %) (compressed gas bottle provided by AirProduct, Aubervilliers, France). Please note that although cells will be exposed to a mixture of several exhaust gases, for simplification purposes we will talk about the “gas” condition.

We selected benzo[a]pyrene (B[a]P) as an archetype of polycyclic aromatic hydrocarbon (PAH) (Bukowska et al., 2022; Dussert et al., 2020). PAH, and especially B[a]P, is a ubiquitous environmental pollutant, present in the atmospheric particulate matter as a consequence of incomplete combustion of organic matter as well as coal or petroleum distillation.

### 2.3. Cell culture and exposure to pollutants

The RAW 264.7 cell line derived from mice peritoneal macrophages transformed by the Abelson murine leukemia virus and was provided by ATCC Cell Biology Collection (Promochem, LGC, Molsheim, France). We chose a model of macrophages as these cells from the immune system are ubiquitous and represent the first line of defense of the organism. They are specialized in the recognition and capture of foreign bodies and have a high phagocytosis capacity. The RAW264.7 cell line was chosen in particular because it is widely used in toxicological studies. Cells were cultured in Dulbecco Modified Eagle Medium (DMEM) supplemented with 10 % fetal calf serum and 1 % penicillin-streptomycin at 37°C under a 5 % carbon dioxide humidified atmosphere.

Cells were incubated either with TP alone or with TP in the presence of the exhaust gases and B[a]P for 24 h. As controls, cells were also incubated with gases or B[a]P separately. For TP/cell contact, cells were seeded in 96-well-plates (Fisher Scientific, Illkirch, France, 100,000 cells in 50  $\mu$ L of medium per well) and were allowed to adhere overnight. TP powders were resuspended in de-ionised water to reach a 1600  $\mu$ g/mL concentration. Samples were sonicated in ultra-sound bath for 30 min at 130 kHz and for 5 min at 70 % amplitude with a Digital Sonifier (Branson S-250, Thermo Fisher, France). TP were diluted in cell culture medium to obtain the following final concentrations: 15, 30, 60 and 120  $\mu$ g/mL. The TP-loaded culture medium was then added to the well containing cells and further incubated for 24 h.

For incubation with gas, 500 mL of cell culture medium was placed in a tricol bottle and the gas was introduced at a constant flow rate using a gas line comprising the gas mixing bottle described above, flexible pipework elements and a Coriolis flowmeter allowing the gas to be bubbled into the cell culture medium contained in the tricol flask at a flow rate of 3 L/h. During gas introduction, the tricol bottle was maintained at 4°C since the equilibrium ratio between the diluted gas mixture and the part remaining in the tricol in the gas phase is more favorable at lower temperature, allowing a higher diluted gas mixture concentration. To determine the gas concentration in solution, the same test was reproduced with water instead of cell culture medium and the total inorganic carbon concentration (corresponding to the CO<sub>2</sub> concentration) assessed by the Total Organic Content (TOC) method (with the Vario TOC, Elementar, France). Assessment of diluted gas was performed in water instead of culture cell medium since this last one contains different organic molecules that would prevent correct assessment of diluted CO<sub>2</sub> concentration. Mean assessed CO<sub>2</sub> concentration was 7.4 mg/L. Finally, 150  $\mu$ L of culture medium containing the dissolved gas was added to cell culture (either alone or in combination with TP and B[a]P).

Finally, for incubation with B[a]P, we chose a 20  $\mu$ M concentration

based on literature data (Gao et al., 2022; Ji et al., 2013; Lin et al., 2020). B[a]P was dissolved into cell culture supernatant.

## 2.4. *In vitro* toxicity assays

### 2.4.1. Cytotoxicity

To evaluate cell membrane integrity, the cellular release in the supernatant of cytoplasmic lactate dehydrogenase (LDH) was assessed using the CytoTox-96™ Homogeneous Membrane Integrity Assay (Promega, Charbonnières-les-Bains, France) according to the manufacturer's instructions. The optical density of the samples was determined using a microplate reader (Multiskan RC; Thermolabsystems, Helsinki, Finland) set to 450 nm. Three independent experiments were performed in quadruplicate and the activity of the released LDH was reported to that of negative control cells (unexposed cells). A positive control consisted in the maximal cellular LDH released after cells lysis.

### 2.4.2. Proinflammatory response

After incubation with the pollutants, the production of TNF- $\alpha$  was assessed in the supernatant using a commercial ELISA kit (Quantikine® Mouse TNF- $\alpha$  Immunoassay; R&D Systems, Lille, France) according to the manufacturer's instructions. The optical density of each sample was determined using a microplate reader (Multiskan RC; Thermolabsystems, Helsinki, Finland) set to 450 nm. A standard curve was established and results were expressed in picograms of TNF- $\alpha$  per milliliter of supernatant. Three independent experiments were performed in quadruplicate and the production of TNF- $\alpha$  was reported to that of control cells (unexposed cells).

### 2.4.3. Oxidative stress

A large array of ROS activity can be assessed with the OxiSelect™ ROS Assay kit (Euromedex, Mundolsheim, France). The assay uses the conversion of a non-fluorescent substrate, 2,7'-dichlorodihydrofluorescein diacetate that can easily diffuse through cell membranes and be converted into a fluorogenic molecule 2',7'-dichlorodihydrofluorescein (DCF) in the presence of ROS, fluorescence amount is directly related to ROS level. Fluorescence was detected using a Fluoroskan Ascent fluorometer (Ex: 480 nm, Em: 530 nm, Thermolabsystems) after a 90 min and a 24 h incubation of cells with the pollutants. The 90 min time point was included as ROS are extremely reactive and can be rapidly transformed. A positive control was included incubating cells with H<sub>2</sub>O<sub>2</sub> (1 mM). Three independent experiments were performed in quadruplicate and the generation of ROS was reported to that of the negative control (unexposed cells).

## 2.5. Statistical analysis

Analyses and graphs were performed on Prism software (GraphPad, San Diego, CA). Statistical significance was declared when  $p < 0.05$ , as calculated with two-way ANOVA tests and Tukey post-tests. Each data point represents the mean of at least three independent experiments and is presented with the arithmetic standard error of the mean ( $\pm$  SEM).

## 3. Results

### 3.1. Physicochemical characterization of the tire particles

Table 1 summarizes the main physicochemical features of the different TP size-fractions. The TP fractions are called based on the average particle size of the fraction.

### 3.2. Toxicological profile

The toxicological profile of TP has been previously determined in terms of cytotoxicity, oxidative stress and proinflammatory response (unpublished data). The latter results are reported here for comparison

**Table 1**

Main physicochemical characteristics of the TP. D10: means that 10 % of the sample is smaller than or equal to the D10 value. D90: means that 90 % of the sample is smaller than or equal to the D90 value.

	Mean TP size ( $\mu$ m)	D10 ( $\mu$ m)	D90 ( $\mu$ m)	Chemical composition (ppm)		
TP6	6	2	15	S: 12505	Ca: 1197	Zr: 49
TP22	22	10	37	Zn: 3905	Fe: 357	Cu: 2
TP55	55	32	87	Na: 3055	Si: 92	Pb: 2
TP113	113	40	212	Al: 1241	Ti: 63	

with the results observed for the particles incubated in the presence of gas and B[a]P.

### 3.2.1. Cytotoxicity

After a 24 h exposure of cells to TP alone or in the presence of gas and B[a]P, cytotoxicity was assessed by the level of lactate dehydrogenase (LDH) released into the culture medium. As shown in Fig. 1, the release of LDH was not significantly enhanced when cells were incubated either with the gas, the B[a]P, the TP (whatever their size), or the TP incubated in combination with the gas and B[a]P. This suggests that no cytotoxicity was induced irrespective of the condition of exposure of cells.

### 3.2.2. Oxidative stress

After a 90 min and a 24 h exposure of cells to TP alone or in the presence of gas and B[a]P, the production of ROS was assessed. As shown in Fig. 2, no significant ROS production was triggered compared to control cells, whatever the condition of exposure. This suggests that the gas, B[a]P and TP, alone or in combination, did not trigger oxidative stress.

### 3.2.3. Proinflammatory response

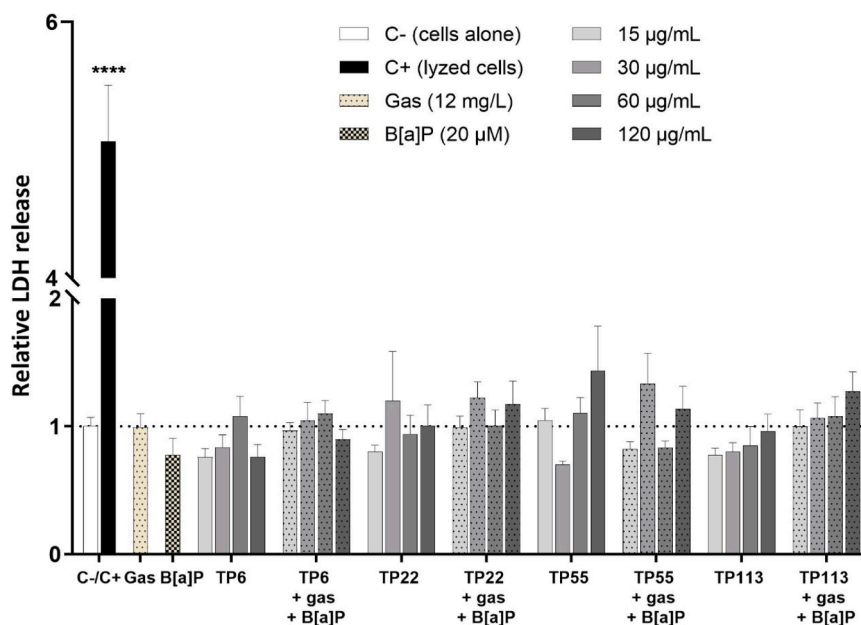
Fig. 3 reports the relative production of the TNF- $\alpha$  proinflammatory cytokine.

We previously observed that exposure to TP alone induced a size-dependent proinflammatory effect. As a matter of fact, while TP6 and TP113 showed a TNF- $\alpha$  production that was not significantly different from that of control cells, TP22 and TP55 (only at the highest dose) triggered an enhanced and dose-dependent response. Exposure to gas alone did not have a significant impact as the TNF- $\alpha$  level was similar to that of control cells. In comparison, exposure to B[a]P alone induced a significantly enhanced proinflammatory response compared to control cells.

More interestingly, when considering the combined effects of TP and gas and B[a]P we observed two types of response depending on the size of TP. First, a TNF- $\alpha$  production slightly increased in the presence of gas and B[a]P suggesting that the effects of TP, gas and B[a]P are additive. This is the case for TP6, TP22 and TP55. The second profile, observed for TP113, showed a sharp and disproportionate increase in TNF- $\alpha$  production in the presence of gas and B[a]P, suggesting that in this case, the effects of TP, gas and B[a]P are synergistic. To confirm this hypothesis, we reported in the Supplementary Data a complementary statistical analysis where we numerically added the effect of B[a]P to that of TP and compared the result with the measured response of TP + gas + B[a]P (Figure S1). On the one hand, the differences between the calculated TP6, TP22 and TP 55 values and the corresponding measured TP + gas + B[a]P values + B[a]P were no longer significant, confirming the additive effects of TP and B[a]P. On the contrary, the difference remained statistically significant when comparing the calculated TP113 to TP113 + gas + B[a]P, further arguing for a synergistic effect of this association.

## 4. Discussion

In the present study, we aimed to assess the *in vitro* combined toxicity of EE and NEE pollutants using a realistic combination of TP, exhaust gas



**Fig. 1.** Cytotoxicity induced by the different TP size-fractions in the absence (solid bars) or in the presence (dotted bars) of gas and B[a]P after a 24 h of cell exposure. Relative LDH release calculated as the ratio of LDH released from treated cells to LDH released by control (unexposed) cells. Results are means of three independent experiments, each performed in quadruplicate and are expressed with the standard error of the mean. C-: Negative control (cells alone). C+: Positive control (lyzed cells). For comparison, cells were also incubated with gas alone or B[a]P alone.

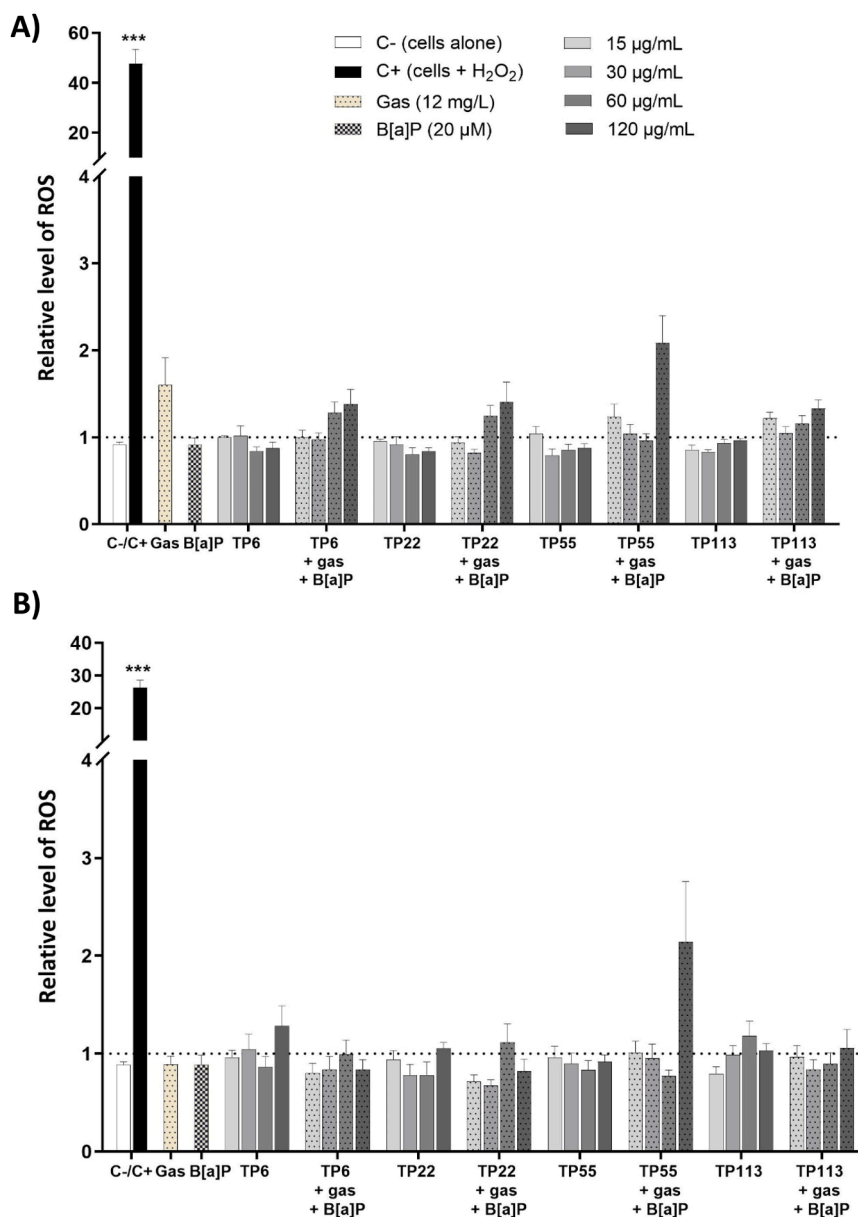
and B[a]P, a main representative of PAH. First, we found that TP (whatever their size), gas and B[a]P, either alone or in combination, were not able to induce cytotoxicity nor oxidative stress. Yet, these pollutants triggered different types of proinflammatory responses: exposure to B[a]P alone induced a significantly enhanced TNF- $\alpha$  production compared to control cells while the exposure to TP alone induced a size-dependent proinflammatory effect. Interestingly, the combined effects of TP, gas and B[a]P depended on TP size as the proinflammatory effects of the finest TP (6–55  $\mu\text{m}$ ) and B[a]P were found to be additive, while those of coarse TP (113  $\mu\text{m}$ ) and B[a]P were synergistic.

To better understand this finding, it may be noteworthy to come back to the mechanism of toxicity of B[a]P. B[a]P (along with other PAHs) is formed during the burning of fossil fuels, wood, and other organic materials and is thus released into the atmosphere as a component of smoke from forest fires, industrial processes, vehicle exhaust, cigarettes, and through the burning of fuel (such as wood, coal, and petroleum products), and it represents a significant component of air pollution (US EPA National Center for Environmental Assessment, 2017). Its toxicity has been well documented and it has been clearly evidenced that B[a]P is carcinogenic (classified Group 1) (IARC, 2012), genotoxic, mutagenic, epigenotoxic, teratogenic, and neurotoxic, and it impairs fertility (Bukowska et al., 2022). The mechanisms of B[a]P action are multiple and involve the formation of DNA adducts, generation of ROS, activation of aromatic hydrocarbon receptor (AhR), and various epigenetic changes. The first step in this series of events is the metabolism of B[a]P by cytochrome P450 (CYP 450) to carcinogenic metabolite: 7 $\beta$ , 8 $\alpha$ -dihydroxy-9 $\alpha$ ,10 $\alpha$ -epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene (BPDE), which creates DNA adducts, causing mutations and malignant transformations (Bukowska et al., 2022). Furthermore, it has been shown that B[a]P could increase the expression of proinflammatory cytokines through the activation of AhR (Bukowska et al., 2022). Once activated by B[a]P, AhR, which is a transcription factor able to respond to various chemicals, translocates from the cytoplasm to the nucleus where it can induce the transcription of numerous genes involved in immune regulation, detoxification, and proliferation, resulting for instance in the generation of ROS and proinflammatory cytokines (Bukowska et al., 2022). Although mechanistic studies are needed to

explore how TP and B[a]P interaction results in additive or synergistic effects, we may hypothesize that the above-mentioned pathways could be involved. To better understand the mechanism of the combined toxicity of TP and B[a]P, one could equally consider the effects of B[a]P inhibitors such as pinocembrin (Alzahrani and Rajendran, 2021) or carvedilol (Shahid et al., 2023). We may also assume that a sensitizing effect caused by TP and gas may occur. Indeed, it is plausible that in case of combined exposure to different pollutants, one of them can make the cells more sensitive to the second one (Forest, 2021). This has been exemplified by the study of Jiang et al. where intracellular perturbations caused by black carbon made the cells susceptible to lead toxicity (Jiang et al., 2020). Similarly, Li et al. showed that by accumulating in cells, zinc oxide nanoparticles altered cell membranes and enhanced the toxicity of co-exposed copper nanoparticles (Li et al., 2015).

Some studies have assessed the *in vitro* or *in vivo* effects of a co-exposure to B[a]P and another pollutant such as arsenic (Wang, 2021), gypsum dust (Wang et al., 2024), or Fe<sub>2</sub>O<sub>3</sub> particles (Garçon et al., 2001). These studies have shown that the effects of B[a]P were generally enhanced in the presence of a co-contaminant. As a matter of fact, co-exposure to Fe<sub>2</sub>O<sub>3</sub> and B[a]P resulted in a more pronounced induction of inflammatory mediators while the toxic effect of B[a]P was significantly increased by loading it into gypsum dust, and the AhR signaling pathway induced inflammation and apoptosis of 16HBE cells. Similarly, the co-exposure of human bronchial epithelial cells (BEAS-2B) to silica nanoparticles and B[a]P resulted in both synergistic and additive cytotoxic and genotoxic effects (Wu et al., 2019, 2016). On the other hand, antagonist effects have also been reported when human epithelial lung cells (A549) were co-exposed to short-multiwalled carbon nanotubes (S-MWCNTs) and B[a]P, or acid functionalized multi-walled carbon nanotubes (F-MWCNTs) and B[a]P as these combinations resulted in decreased cytotoxicity compared to individual exposure (Azari et al., 2019; Rezazadeh Azari et al., 2019).

Regarding TP, very few data are available in the literature on their potential impact on human health (Bouredji et al., 2023), let alone in combination with other pollutants. This is all the more worrying as TP represent extremely hydrophobic solid phases on which poorly water-soluble organic pollutants such as B[a]P (*i.e.* log octanol-water partition coefficient of 6.0 at 25 °C (Mallon and Harrison, 1984)) are



**Fig. 2.** Relative ROS production after a 90 min (A) or 24 h cells/particles contact (B) in the absence (solid bars) or in the presence (dotted bars) of gas and B[a]P. Results are expressed as the ratio of ROS produced by treated cells to ROS released by control (unexposed) cells, and represent the means of three independent experiments, each performed in quadruplicate with the standard error of the mean. C-: negative control (cells alone). C+: positive control (cells incubated with 1 mM H<sub>2</sub>O<sub>2</sub>). For comparison, cells were also incubated with gas alone or B[a]P alone.

likely to accumulate and thus come into direct contact with macrophages. In addition, TP contain soluble additives, including toxic anti-oxidants and anti-ozonants (Bergmann et al., 2024; Fohet et al., 2023; Rauert et al., 2022). Due to the volume dependence, the load of additives carried by TP increases non-linearly with particle diameter, which may explain the size threshold observed for synergistic B[a]P proinflammatory effects to occur. It is therefore highly probable that TP additives would generate a more widespread and long-lasting diffuse layer of soluble co-contaminants around coarse particles. This would include both metals (Zn, Cu, Al, etc., Table 1) and organics (6PPD, 6PPD-Q, PAH, etc.) (Ihnetu et al., 2024; Mayer et al., 2024).

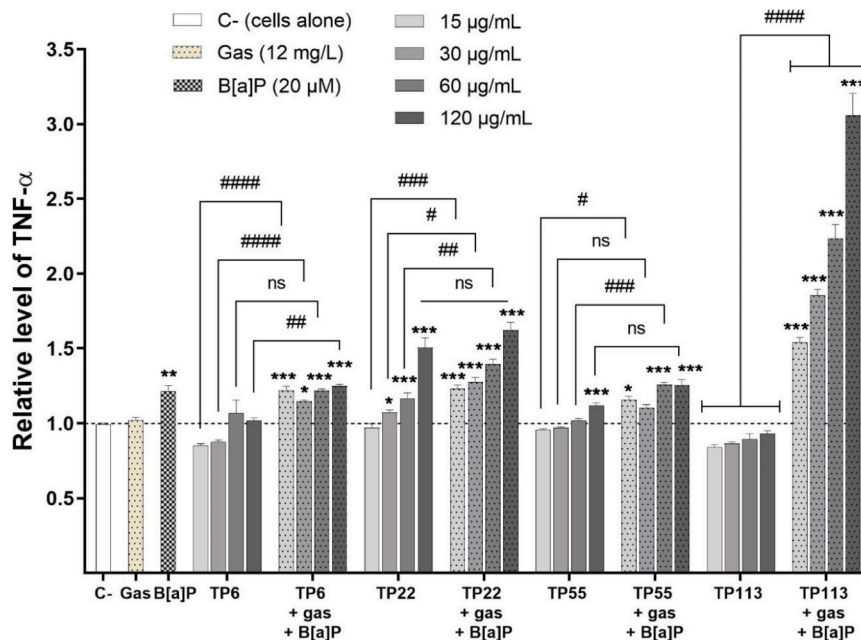
## 5. Conclusion

The more representative characterization of the additive and synergistic effects of exhaust and non-exhaust emissions on population

health is a major, yet critical, challenge. In developed countries, the contributions of exhaust and non-exhaust emissions to ambient particulate pollution are currently of the same order of magnitude. This situation calls attention to the increasing interactions between the TP emissions and ambient pollutants (including from transportation, energy and industry, construction, waste management sectors...). With the present study, we have made a first step towards this characterization by assessing the *in vitro* hazard of a realistic combination of vehicle pollutants to which urban populations are commonly exposed. Further mechanistic studies are needed to explore how TP and B[a]P interaction results in additive or synergistic effects.

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**Fig. 3.** Proinflammatory response induced by TP as assessed by TNF- $\alpha$  levels measured after 24 h of exposure, in the absence (solid bars) or in the presence (dotted bars) of gas and B[a]P. Results are expressed as the ratio of TNF- $\alpha$  produced by treated cells to that produced by control (unexposed) cells, representing means of three independent experiments, each performed in quadruplicate with the standard error of the mean. Statistical significance was determined by a two-way ANOVA analysis and Tukey post-tests. \*  $p < 0.05$ ; \*\*  $p < 0.005$ ; \*\*\*  $p < 0.0005$ ; comparison to the negative control. ns: not significant, #  $p < 0.05$ ; ##  $p < 0.005$ ; ###  $p < 0.0005$ ; ####  $p < 0.0001$ : indicated inter-group comparisons. C-: negative control.

#### CRedit authorship contribution statement

**Valérie Forest:** Writing – original draft, Validation, Supervision, Resources, Project administration, Methodology, Conceptualization. **Riadh Lakhmi:** Writing – review & editing, Validation, Supervision, Resources, Methodology, Formal analysis, Conceptualization. **Abderahmane Bouredji:** Writing – review & editing, Visualization, Investigation, Formal analysis. **Jérémie Pourchez:** Writing – review & editing, Validation, Supervision, Resources, Methodology, Conceptualization. **Bogdan Muresan-Paslaru:** Writing – review & editing, Resources, Investigation.

#### Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests, Abderrahmane Bouredji reports financial support was provided by The French Agency for Ecological Transition. Bogdan Muresan reports a relationship with Michelin that includes: funding grants. Jeremie Pourchez reports a relationship with Michelin that includes: funding grants. Valerie Forest reports a relationship with Michelin that includes: funding grants. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

#### Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.tox.2024.153990](https://doi.org/10.1016/j.tox.2024.153990).

#### Data Availability

Data will be made available on request.

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