Chronic exposure to benzo(a)pyrene-coupled nanoparticles worsens inflammation in a mite-induced asthma mouse model

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To the editor,

Asthma is a highly prevalent chronic inflammatory disease of the airways characterized by airway hyperresponsiveness (AHR) and mucus hyperproduction. In the last decades, asthma has been affecting approximately 20% of the population worldwide and genetic changes alone cannot explain this rapid increase. During the same period, increased vehicular traffic and other combustion processes have resulted in a significant increase in ambient particle matter (PM) that can bind polycyclic aromatic hydrocarbons (PAHs) on their surface. PAHs from diesel exhaust and other sources were shown to play a role in the exacerbation of allergic immune responses in human.[(1)] In acute asthma models, co-exposure to the PAH, benzo(a)pyrene (B(a)P), and ovalbumin enhances the production of allergen-specific IgE, systemic Th2 response, and airway inflammation in mice.[(2)]

Nanoparticles (≤0.1 µm), that represent only 2.3% of total PM mass, contribute to 23-30% of the PAHs alveolar deposition coming from roadside sources. Moreover, their small size allows evading clearance from the lung, leading to long-term retention.[(3)] This suggests that nanoparticles are significant contributors of PAHs deposition in the lung and thus, may contribute to acute and chronic inflammation. However, few studies have evaluated the impact of chronic exposure to this pollutant on allergic asthma. Therefore, we established a murine allergic asthma model using the house dust mite (HDM) allergen, to explore the impact of chronic exposure to nanoparticles coupled to PAHs on airway inflammation (Figure S1A for exposure model). In this study, we used carbon black nanoparticles from printers uncoated as reference (NP-Ø) and B(a)P-coated (NP-B(a)P) as a model of nano-particulate pollutant.

We analyzed the effects of chronic exposure to NP-B(a)P on different asthma parameters. As expected, exposure to HDM induced allergic asthma including increased AHR (Figure S1B), HDM-specific IgE and IgG1 in sera (Figure S1C) and pulmonary inflammation, characterized by elevated total cell numbers in the broncho-alveolar lavage (BAL) composed of eosinophils, neutrophils, lymphocytes and macrophages compared to PBS control mice (Figure 1A). Moreover in this model, we did not observe airway remodeling (data not shown). Neither NP-Ø nor NP-B(a)P nanoparticles alone induced airway inflammation. However, NP-B(a)P but not NP-Ø increased AHR in non-

sensitized mice, suggesting that B(a)P has a specific effect on AHR independently of HDM. In HDM-sensitized mice, HDM-induced AHR was abolished by NP-Ø and decreased by NP-B(a)P although this one remained increased compared to the PBS control group. Surprisingly, both nanoparticles co-exposed with HDM did not modify inflammatory cell recruitment in the BAL (Figure 1A) and did not induce bronchial remodeling (data not shown). However this result was not supported by cellular infiltration of lung tissue as shown by hematoxylin and eosin histological stain (Figure S1D) and total lung single cell suspension (Figure 1B). Indeed, increased total cell numbers were enhanced in the lungs of HDM+NP-B(a)P mice compared to HDM-sensitized mice (Figure 1B). This cellular infiltration was mainly due to a significant increase of eosinophils, $Ly6C^-$ monocytes/macrophages and $CD4^+$ T cells in HDM-sensitized mice compared to the PBS group (Figure 1B). Interestingly, NP-B(a)P significantly modifies the HDM-induced cell recruitment. Indeed, $Ly6C^+$ as well as $Ly6C^-$ monocytes/macrophages were significantly elevated in HDM+NP-B(a)P compared to HDM-sensitized mice. Moreover, neutrophils, NKT-like cells and $CD8^+$ T cells, not recruited in HDM-sensitized mice, were significantly increased in lungs from HDM+NP-B(a)P mice (Figure 1B). NKT-like cells differ from classical NKT by recognizing antigen presented through major histocompatibility complex and not through CD1d, and can be either $CD4^+$, $CD8^+$, or double-negative T cells expressing NK-cell markers. In our study, NKT-like cells may participate in the increase of $CD8^+$ T cells in HDM+NP-B(a)P mice.[4] Thus NP-B(a)P is increasing the inflammatory infiltration due to an additional response to it and not a shift of HDM-induced response. Since pulmonary inflammation was increased in HDM-sensitized mice exposed NP-B(a)P, we analyzed the expression of cytokines in the lung tissue to better characterize the NPs-induced inflammation. As the nanoparticles adsorbed cytokines, we were unable to measure them by conventional enzyme-linked immunosorbent assay, so we use quantitative RT-PCR. Levels of mRNA coding for IFN-γ and IL-17 cytokines were not modulated between HDM groups (figures 2A and 2B). This suggests that Tc1 and Tc17 $CD8^+$ T cells did not participate in the increase of $CD8^+$ T cells observed in HDM+NP-B(a)P exposed mice. However, type 2 cytokines including IL-4, IL-5, IL-13 and IL-10 were significantly increased in HDM-sensitized mice compared to PBS control group and NP-B(a)P exposure facilitated these increased expressions in HDM+NP-B(a)P compared to HDM group (Figure 2C-F). These results suggest that NP-B(a)P can exacerbate the type 2 response induced by HDM and that an increase in $CD8^+$ T cells could include a population of Tc2 cells since $CD4^+$ T cells were not increased. As IL-13 and IL-4 participate in mucus production by bronchial

epithelium, lung sections were stained with periodic acid-Schiff reagent and mRNA encoding for Muc5b and Muc5ac mucins were evaluated. Mucus production was induced but not significantly different between all HDM groups (Figure S2).

In human, higher neutrophil counts were associated with high level of IL-6 regardless of eosinophils in sputum.[5] Moreover, IL-6 can play a role on monocyte recruitment *in vivo* through induction of C-chemokine ligand 2 (CCL2).[6] $Ly6C^+$ monocytes also called classical monocytes express the CCL2 receptor and are highly recruited in lungs of mice co-exposed with HDM and NP-B(a)P. Accordingly, we have observed a significant increase in *il-6* and *ccl2* mRNA expression in the lung in response to HDM+NP-B(a)P exposure (Figure S3A and B). Systemic IL-6 inflammation and metabolic dysfunction are associated with more severe asthma[7], the induction of IL-6 by HDM+NP-B(a)P could reflect a switch towards an IL-6-high severe asthma endotype. In human studies, airway neutrophils and $CD8^+$ T cells were found to be increased in severe forms of asthma and these cell types are described as resistant to corticosteroids.[8, 9] Therefore, the recruitment of these cells suggests that HDM+NP-B(a)P co-exposure may participate in the induction of severe cortico-resistant asthma endotype.

In conclusion, our results suggest that chronic exposure to NP-B(a)P can have a summative effect with allergen exposure in potentiating type 2 inflammation thereby inducing a neutrophil, NKT-like, and $CD8^+$ T cell lung inflammatory response. A better understanding of mechanisms responsible for these modifications is required in order to develop effective drugs to treat specifically this asthma endotype.

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Authors

Julie Carrard[1], Philippe Marquillies[1], Muriel Pichavant[1], Nicolas Visez[2], Sophie Lanone[3], Anne Tsicopoulos[1,4], Cécile Chenivesse[1,4], Arnaud Scherpereel[5], Patricia de Nadaï[1]*

1. Univ Lille, CNRS, Inserm, CHU Lille, Institut Pasteur de Lille, U1019 – UMR9017 – CIIL – center for infection and Immunity of Lille, France
2. Univ. Lille, CNRS, UMR 8522 - PC2A - Physicochimie des Processus de Combustion et de l'Atmosphère, Lille, France
3. Univ Paris Est Créteil, INSERM, IMRB, F-94010 Créteil, France
4. CHU Lille, Service de Pneumologie et Immuno-allergologie, Centre constitutif des Maladies Pulmonaires Rares, Lille, France ; CRISALIS/F-CRIN network
5. Pulmonary and Thoracic Oncology Dept, CHU Lille, France.

* : corresponding author : Dr Patricia de Nadaï
Center for Infection and Immunity of Lille,
Institut Pasteur de Lille, 1 rue du Pr Calmette,
59000, Lille, France

Phone: (33) 3 20 87 71 83

e-mail :patricia.de-nadai@pasteur-lille.fr

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Conflicts of interest

Pr. Scherpereel reports personal fees from Astra-Zeneca, BMS, MSD, Roche, outside the submitted work. Pr. Chenivesse reports grants from AstraZeneca, Santélys, personal fees from ALK-Abello, AstraZeneca, Boehringer Ingelheim, GlaxoSmithKlein, Novartis, Roche, Sanofi, and TEVA, non-financial support from ALK-Abello, AstraZeneca, Boehringer Ingelheim, Chiesi, GlaxoSmithKlein, MEDA Pharma, Medexact, Novartis, Pierre Fabre, Pfizer, outside the submitted work. All others authors declare that they have no relevant conflicts of interest.

Authors' contribution

JC, PdN, SL, CC, AT and AS contributed to conception and design of the study. JC, MP and PdN performed in vivo experiments and analysis. PM performed in vivo experiments. NV performed nanoparticle coating, drafted the associated methods, and revised the manuscript. JC and PdN drafted the manuscript. MP, CC and AT have substantially revised the manuscript. All authors read and approved the manuscript.

Figure legends

Figure 1: HDM+NP-B(a)P chronic administration modifies lung tissue cells but not BAL cell count. (A) Total cell, eosinophils, neutrophils, lymphocytes and macrophages absolute numbers in BAL. (B) Total cells from lung single cell suspensions and eosinophils, neutrophils, $Ly6C^+$ and $Ly6C^-$ macrophages/monocytes, NKT-like, $CD4^+$ and $CD8^+$ T cells identified by flow cytometry. Data are representatives of two independent experiments (n=10-21 mice per group) and expressed as median

min. to max. and plots. * p<0.05, ** p<0.01, *** p<0.005 vs PBS; [§] p<0.05 between HDM+NP-Ø and HDM+NP-B(a)P , [#] p<0.05, [##] p<0.01 [####] p<0.001 between HDM and HDM+NP-B(a)P).

Figure 2: HDM+NP-B(a)P chronic administration increases Th2 cytokine expression in the lung. Relative expression of *IFN-g* (A), *IL-17a* (B), *IL-4* (C), *IL-5* (D), *IL-13* (E) and *IL-10* (F) mRNA in lung tissues compared to *rplp0* housekeeping gene. Data are representatives of two independent experiments (n=10-21 mice per group) and expressed as median min. to max. and plots. * p<0.05, ** p<0.01, *** p<0.001 vs PBS; [§] p<0.05 between HDM+NP-Ø and HDM+NP-B(a)P , [#] p<0.05, [##] p<0.01, [####] p<0.001 between HDM and HDM+NP-B(a)P)