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Article type : Letter to the Editor

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

To the editor,

1 Asthma is a highly prevalent chronic inflammatory disease of the airways characterized by airway
2 hyperresponsiveness (AHR) and mucus hyperproduction. In the last decades, asthma has been affecting
3 approximately 20% of the population worldwide and genetic changes alone cannot explain this rapid
4 increase. During the same period, increased vehicular traffic and other combustion processes have
5 resulted in a significant increase in ambient particle matter (PM) that can bind polycyclic aromatic
6 hydrocarbons (PAHs) on their surface. PAHs from diesel exhaust and other sources were shown to play a
7 role in the exacerbation of allergic immune responses in human.⁽¹⁾ In acute asthma models, co-exposure to
8 the PAH, benzo(a)pyrene (B(a)P), and ovalbumin enhances the production of allergen-specific IgE,
9 systemic Th2 response, and airway inflammation in mice.⁽²⁾
10 Nanoparticles ($\leq 0.1 \mu\text{m}$), that represent only 2.3% of total PM mass, contribute to 23-30% of the PAHs
11 alveolar deposition coming from roadside sources. Moreover, their small size allows evading clearance
12 from the lung, leading to long-term retention.⁽³⁾ This suggests that nanoparticles are significant contributors
13 of PAHs deposition in the lung and thus, may contribute to acute and chronic inflammation. However, few
14 studies have evaluated the impact of chronic exposure to this pollutant on allergic asthma. Therefore, we
15 established a murine allergic asthma model using the house dust mite (HDM) allergen, to explore the
16 impact of chronic exposure to nanoparticles coupled to PAHs on airway inflammation (Figure S1A for
17 exposure model). In this study, we used carbon black nanoparticles from printers uncoated as reference
18 (NP-Ø) and B(a)P-coated (NP-B(a)P) as a model of nano-particulate pollutant.
19 We analyzed the effects of chronic exposure to NP-B(a)P on different asthma parameters. As expected,
20 exposure to HDM induced allergic asthma including increased AHR (Figure S1B), HDM-specific IgE and

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21 IgG1 in sera (Figure S1C) and pulmonary inflammation, characterized by elevated total cell numbers in the
22 broncho-alveolar lavage (BAL) composed of eosinophils, neutrophils, lymphocytes and macrophages
23 compared to PBS control mice (Figure 1A). Moreover in this model, we did not observe airway remodeling
24 (data not shown). Neither NP-Ø nor NP-B(a)P nanoparticles alone induced airway inflammation. However,
25 NP-B(a)P but not NP-Ø increased AHR in non-sensitized mice, suggesting that B(a)P has a specific effect
26 on AHR independently of HDM. In HDM-sensitized mice, HDM-induced AHR was abolished by NP-Ø and
27 decreased by NP-B(a)P although this one remained increased compared to the PBS control group.
28 Surprisingly, both nanoparticles co-exposed with HDM did not modify inflammatory cell recruitment in the
29 BAL (Figure 1A) and did not induce bronchial remodeling (data not shown). However this result was not
30 supported by cellular infiltration of lung tissue as shown by hematoxylin and eosin histological stain (Figure
31 S1D) and total lung single cell suspension (Figure 1B). Indeed, increased total cell numbers were
32 enhanced in the lungs of HDM+NP-B(a)P mice compared to HDM-sensitized mice (Figure 1B). This cellular
33 infiltration was mainly due to a significant increase of eosinophils, Ly6C⁺ monocytes/macrophages and
34 CD4⁺ T cells in HDM-sensitized mice compared to the PBS group (Figure 1B). Interestingly, NP-B(a)P
35 significantly modifies the HDM-induced cell recruitment. Indeed, Ly6C⁺ as well as Ly6C⁺
36 monocytes/macrophages were significantly elevated in HDM+NP-B(a)P compared to HDM-sensitized mice.
37 Moreover, neutrophils, NKT-like cells and CD8⁺ T cells, not recruited in HDM-sensitized mice, were
38 significantly increased in lungs from HDM+NP-B(a)P mice (Figure 1B). NKT-like cells differ from classical
39 NKT by recognizing antigen presented through major histocompatibility complex and not through CD1d,
40 and can be either CD4⁺, CD8⁺, or double-negative T cells expressing NK-cell markers. In our study, NKT-
41 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
42 increasing the inflammatory infiltration due to an additional response to it and not a shift of HDM-induced
43 response. Since pulmonary inflammation was increased in HDM-sensitized mice exposed NP-B(a)P, we
44 analyzed the expression of cytokines in the lung tissue to better characterize the NPs-induced
45 inflammation. As the nanoparticles adsorbed cytokines, we were unable to measure them by conventional
46 enzyme-linked immunosorbent assay, so we use quantitative RT-PCR. Levels of mRNA coding for IFN-γ
47 and IL-17 cytokines were not modulated between HDM groups (figures 2A and 2B). This suggests that Tc1
48 and Tc17 CD8⁺ T cells did not participate in the increase of CD8⁺ T cells observed in HDM+NP-B(a)P
49 exposed mice. However, type 2 cytokines including IL-4, IL-5, IL-13 and IL-10 were significantly increased
50 in HDM-sensitized mice compared to PBS control group and NP-B(a)P exposure facilitated these increased
51 expressions in HDM+NP-B(a)P compared to HDM group (Figure 2C-F). These results suggest that NP-
52 B(a)P can exacerbate the type 2 response induced by HDM and that an increase in CD8⁺ T cells could
53 include a population of Tc2 cells since CD4⁺ T cells were not increased. As IL-13 and IL-4 participate in
54 mucus production by bronchial epithelium, lung sections were stained with periodic acid-Schiff reagent and
55 mRNA encoding for Muc5b and Muc5ac mucins were evaluated. Mucus production was induced but not
56 significantly different between all HDM groups (Figure S2).

57 In human, higher neutrophil counts were associated with high level of IL-6 regardless of eosinophils in
58 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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121

122 **Conflicts of interest**

123 Pr. Scherpereel reports personal fees from Astra-Zeneca, BMS, MSD, Roche, outside the submitted work.

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126 ALK-Abello, AstraZeneca, Boehringer Ingelheim, Chiesi, GlaxoSmithKlein, MEDA Pharma, Medexact,
127 Novartis, Pierre Fabre, Pfizer, outside the submitted work. All others authors declare that they have no
128 relevant conflicts of interest.

129 **Authors' contribution**

130 JC, PdN, SL, CC, AT and AS contributed to conception and design of the study. JC, MP and PdN
131 performed in vivo experiments and analysis. PM performed in vivo experiments. NV performed nanoparticle

132 coating, drafted the associated methods, and revised the manuscript. JC and PdN drafted the manuscript.
133 MP, CC and AT have substantially revised the manuscript. All authors read and approved the manuscript.

134

135 **Figure legends**

136

137 **Figure 1: HDM+NP-B(a)P chronic administration modifies lung tissue cells but not BAL cell count.**

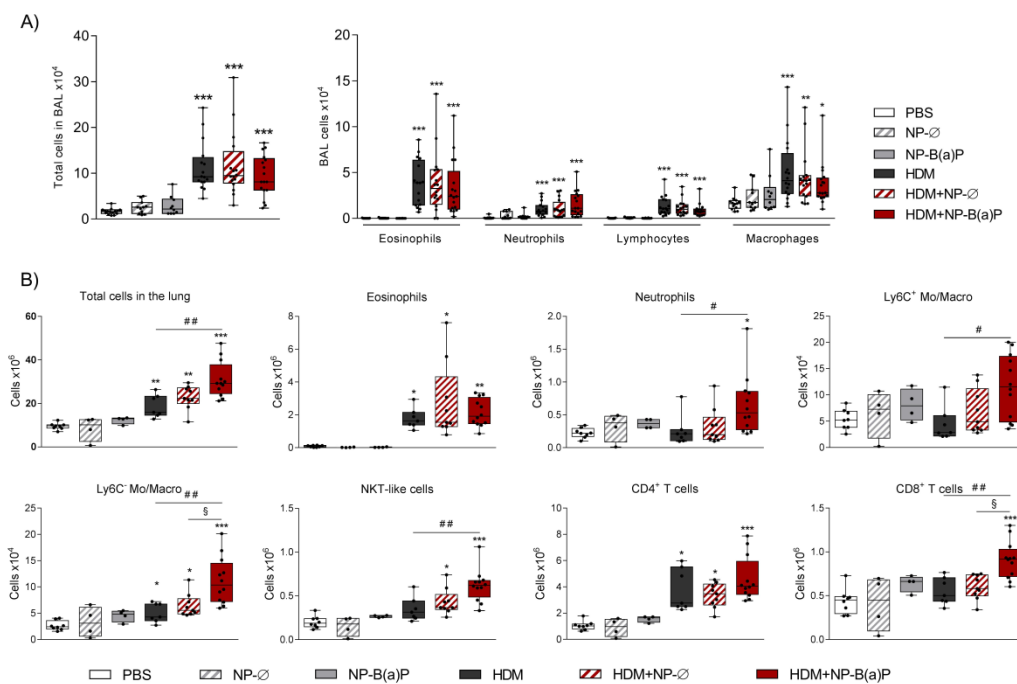
138 (A) Total cell, eosinophils, neutrophils, lymphocytes and macrophages absolute numbers in BAL. (B) Total
139 cells from lung single cell suspensions and eosinophils, neutrophils, Ly6C⁺ and Ly6C⁻
140 macrophages/monocytes, NKT-like, CD4⁺ and CD8⁺ T cells identified by flow cytometry. Data are
141 representatives of two independent experiments (n=10-21 mice per group) and expressed as median min.
142 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-
143 B(a)P, # p<0.05, ## p<0.01, #### p<0.001 between HDM and HDM+NP-B(a)P).

144

145 **Figure 2: HDM+NP-B(a)P chronic administration increases Th2 cytokine expression in the lung.**

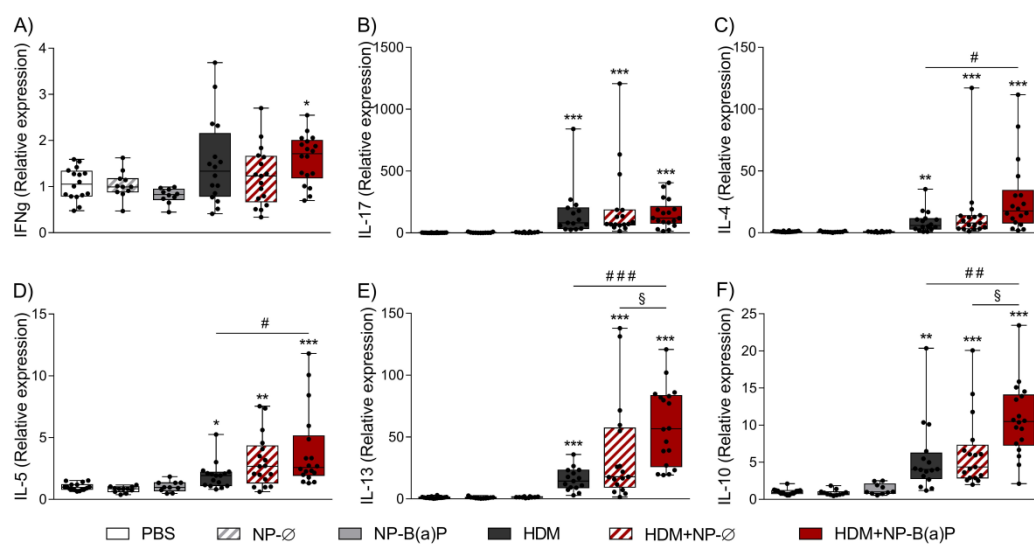
146 Relative expression of *IFN-γ* (A), *IL-17a* (B), *IL-4* (C), *IL-5* (D), *IL-13* (E) and *IL-10* (F) mRNA in lung
147 tissues compared to *rplp0* housekeeping gene. Data are representatives of two independent experiments
148 (n=10-21 mice per group) and expressed as median min. to max. and plots. * p<0.05, ** p<0.01, ***
149 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
150 between HDM and HDM+NP-B(a)P)

Figure 1_J. Carrard_ALL-2020-00679.R1



all_14619_f1.tiff

Figure 2_J. Carrard_ALL-2020-00679.R1



all_14619_f2.tiff

Figure S1_J. Carrard_ALL-2020-00679.R1

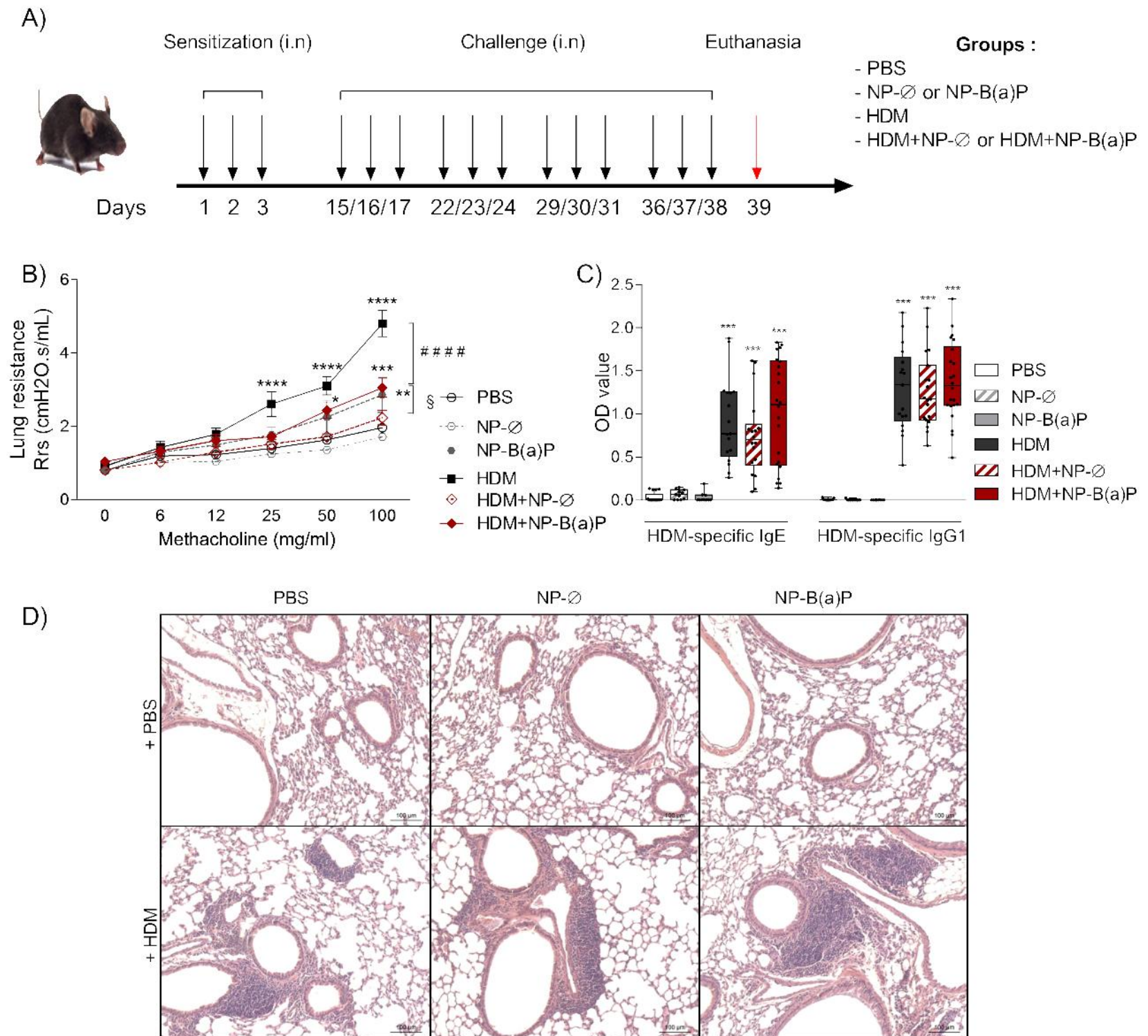


Figure S1: Experimental model and impact of nanoparticles on allergic asthma features.

(A) Experimental model of asthma and chronic exposure to nanoparticles coupled to benzo(a)pyrene in mice. (B) Respiratory resistance (Rrs) following increasing doses of methacholine. (C) HDM-specific IgE and IgG1 in sera. (D) Paraffin-embedded sections of lung stained with H&E (scale bar: 100 μ m). Data are representatives of 2 independent experiments and expressed as median min. to max. and plots from 10 to 21 mice per group; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$, **** $p < 0.001$ vs PBS; § $p < 0.05$ between HDM+NP- $\emptyset$ and HDM+NP-B(a)P; #### $p < 0.001$ between HDM and HDM+NP-B(a)P.

Figure S2_J. Carrard_ALL-2020-00679.R1

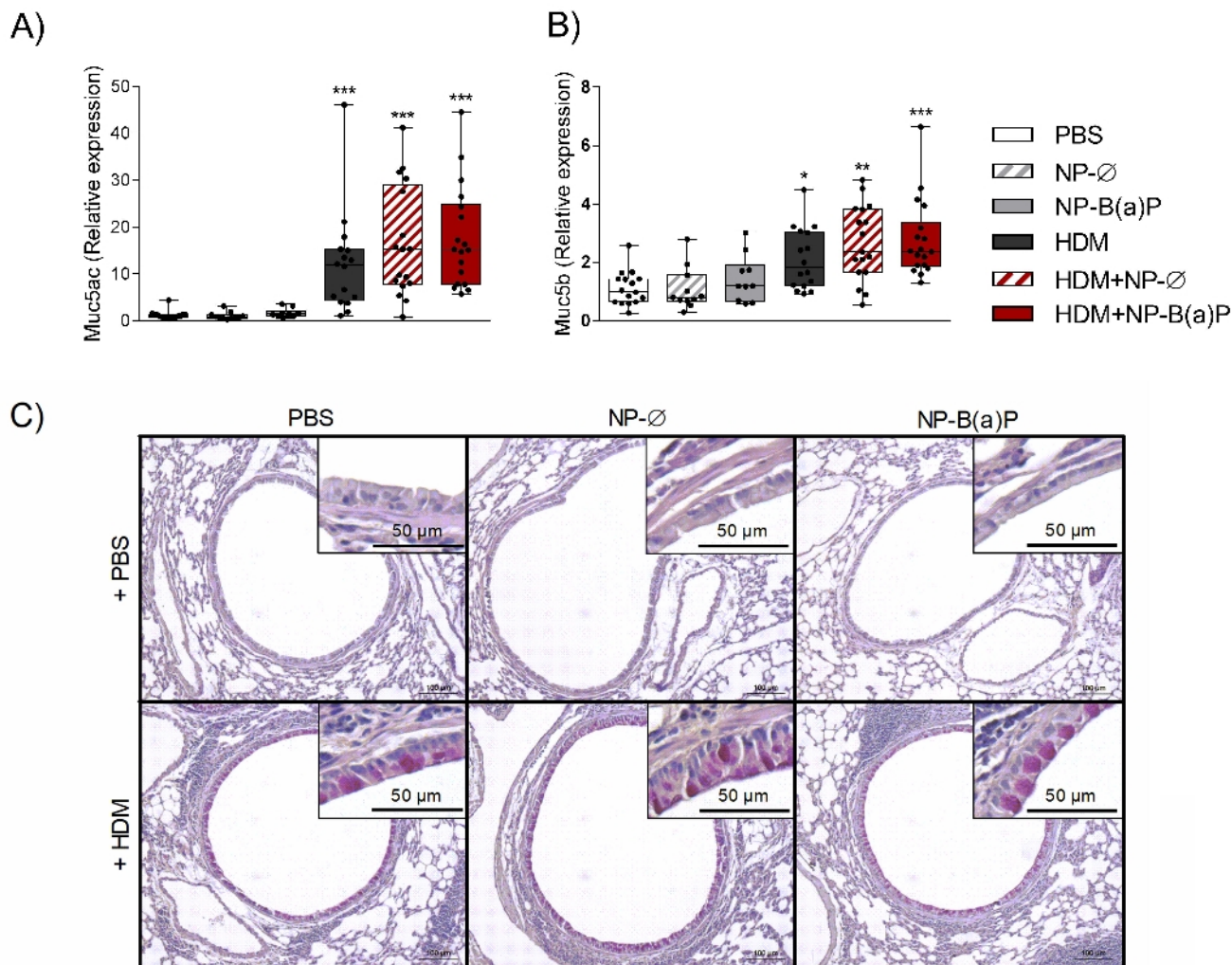


Figure S2: Effects of HDM+NP-B(a)P chronic administration on mucus production.

Relative expression compared to rplp0 house keeping gene of Muc5ac (A) and Muc5b (B) mRNA measured in the lung tissues. (C) Paraffin-embedded lung sections were stained with Periodic acid Schiff (PAS) to analyze mucus production (scale bar :100 µm). Data are representatives of 2 independent experiments and expressed as median min. to max and plots from 10 to 21 mice per group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$ vs PBS.

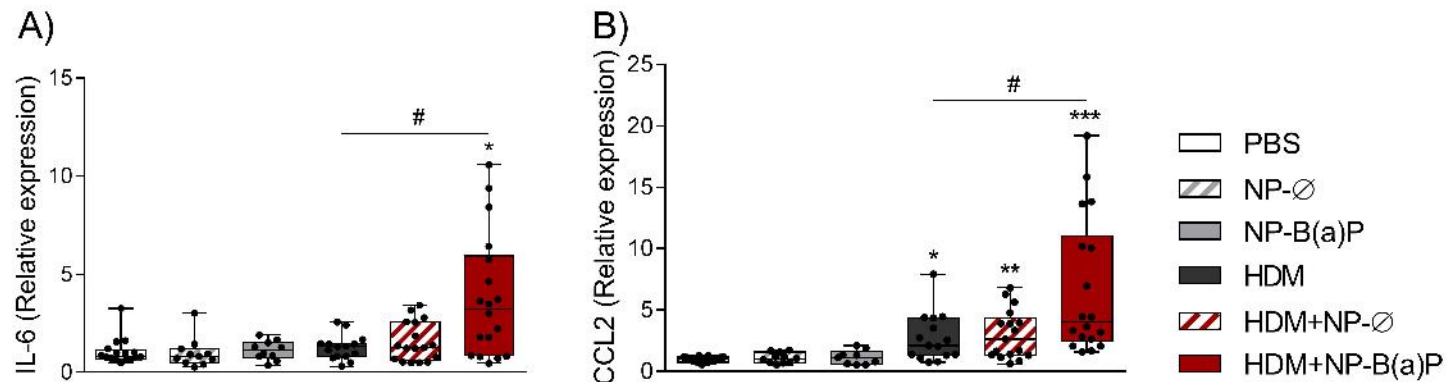


Figure S3: HDM+NP-B(a)P chronic exposure increases IL-6 and CCL2 expression in the lung.

Relative expression compared to rplp0 house keeping gene of IL-6 (A) and CCL2 (B) mRNA measured in lung tissues. Data are representatives of two 2 independent experiments and expressed as median min. to max and plots from 10 to 21 mice per group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$ vs PBS. # $p < 0.05$ between HDM and HDM+NP-B(a)P.

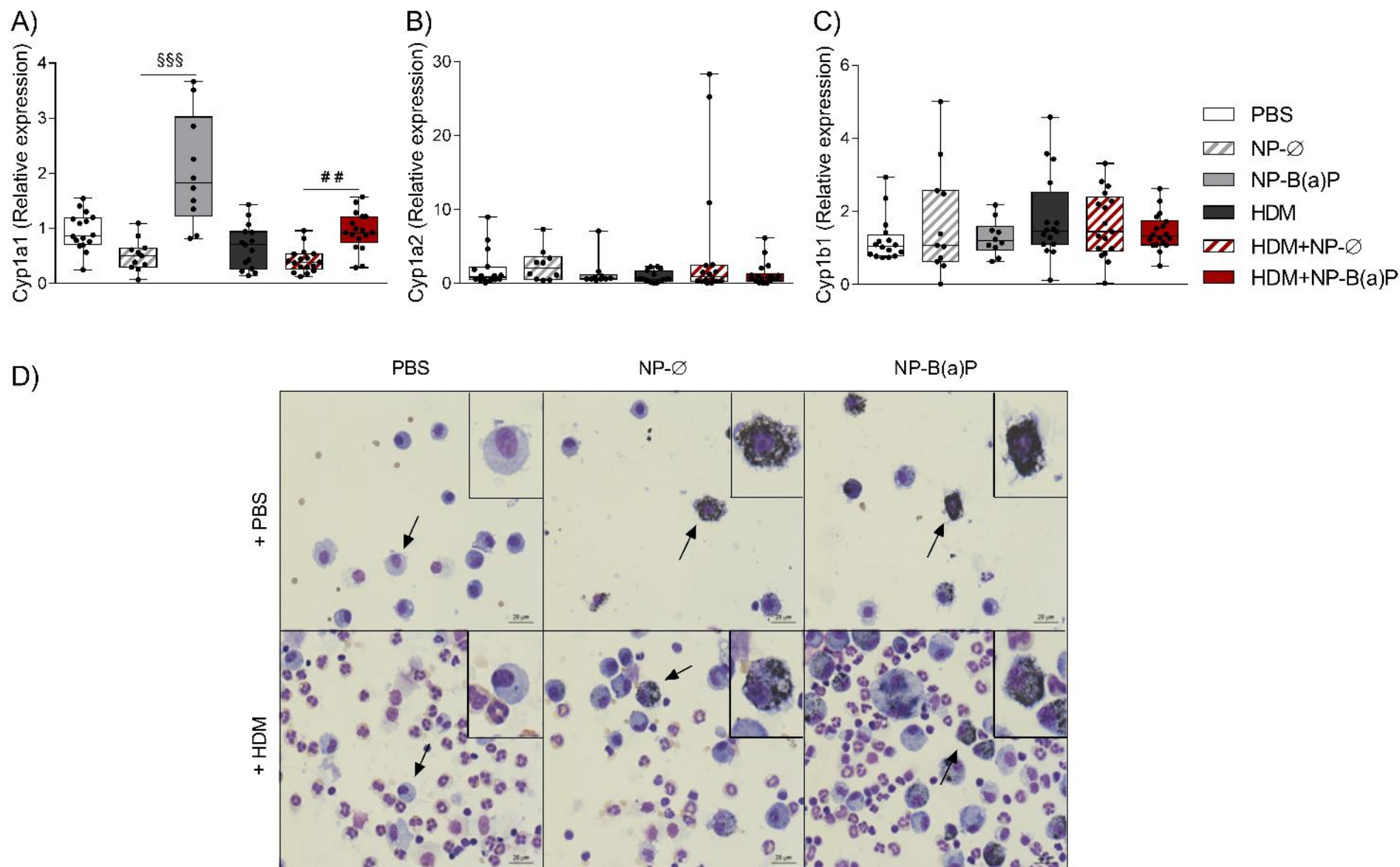


Figure S4: impact of nanoparticles on cytochrome genes expression and detection of nanoparticles in BAL macrophages

Relative expression compared to rplp0 house keeping gene of Cyp1a1 (A), Cyp1a2 (B), Cyp1b1 (C) mRNA in the lung tissues measured by qRT-PCR. (D) Inflammatory cells in BAL were identified by cytocentrifugation and staining with May Grünwald-Giemsa coloration (scale bar: 100µm). The values are expressed as the median min.to max. and plots (Data from 2 separate experiments (n=10–21mice per group)); §§§ p<0.005 between NP-Ø and NP-B(a)P and ## p<0.01 between HDM+NP-Ø and HDM+NP-B(a)P.

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

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MATERIAL AND METHODS

Carbon black nanoparticle coating

Carbon nanoparticles were kindly provided by Dr Sophie Lanone from Institut Mondor de Recherche Biomédicale and previously characterized by Belade *et al.*¹ A part of these nanoparticles was coupled to B(a)P. A solution of B(a)P (Sigma-Aldrich, Lyon, France) at a concentration of 5.55 mg/ml was prepared with dichloromethane (Sigma Aldrich) as solvent. A mass of 1 g of carbon nanoparticles was mixed with 10 ml of this solution, and the solvent was completely evaporated at room temperature in a rotary evaporator (about 3 hours for total evaporation). After recovery of the coated nanoparticles, a control of the amount of B(a)P remaining on the walls of the flask of the rotary evaporator was carried out by GC-MS analysis (GC Perkin Elmer 680, MS Perkin Elmer 600C). The B(a)P not present on the walls is supposed to cover the particles; direct analysis of the B(a)P adsorbed on the nanoparticles is indeed made difficult by its non-total extraction on this support. The final B(a)P concentration on the nanoparticles was 55.5 mg/g. Nanoparticles were stored in brown vial at -20°C.

Animals

C57BL/6 J Female mice (6 weeks old) were obtained from Janvier Labs (Le Genest-Saint-Isle, France). Animal study was approved by the local investigational review board (N° APAFIS#7874-2016070417344442v3) in an accredited establishment at the Institut Pasteur de Lille according to governmental guidelines N°86/609/CEE and the European directive 2010/63/EU. Mice were housed under standard conditions in a pathogen-free facility.

Experimental protocol

After isoflurane gas anesthesia, mice were sensitized intranasally by 25 µg of HDM (Stallergenes Greer, Boston, USA) for 3 consecutive days with or without the presence of 18 µg of uncoated or B(a)P-coupled nanoparticles corresponding to 1 µg of B(a)P. Twelve days after, mice were challenged intranasally in the same conditions 3 days a week during 4 weeks. Mice were euthanized 24 hours after the last intranasal by injection of Euthasol lethal dose. The model for chronic exposure is shown in figure S1A. BAL fluids and cells, blood samples and lung tissues were collected for further analyses.

BAL fluid and cell collection

A total volume of 1 mL of ice-cold phosphate buffer saline (PBS) (Fisher Scientific, Courtaboeuf, France) was used to wash the lungs. Total leukocyte numbers were counted, cytocentrifuged (Shandon cytopsin 4; ThermoFisher Scientific, Waltham, USA) and stained with May-Grünwald Giemsa (Microm Microtech, Brignais, France). Cells were identified as macrophages, eosinophils, neutrophils and lymphocytes by standard hematological procedures and at least 300 cells were counted under x400 magnification.

Serum antibodies measurement

HDM-specific IgE and IgG₁ levels were determined by enzyme linked immunosorbent assay (ELISA) using 100 µg/ml HDM (Stallergenes Greer) coated in wells. After incubation with diluted serum, specific IgE and IgG₁ antibodies were detected by biotinylated goat anti-IgE (Gentaure) or IgG₁ (ThermoFisher scientific). Binding of Streptavidin-Horse Radish Peroxidase was revealed by TMB substrate solution (Sigma-Aldrich, St Louis, USA) and the OD value at 450 nm was determined.

Airway hyperresponsiveness measurement

Airway hyperresponsiveness (AHR) was assessed 72 hours after the last instillation using flexiVent invasive method (SCIREQ, Montreal, Quebec, Canada). Mice were anesthetized (5 ml/kg body weight of 10% medetomidine (Pfizer) and 10% ketamine (Merial)) and immediately intubated with an 18-gauge catheter, followed by mechanical ventilation. Respiratory frequency was set at 150 breaths/min with a tidal volume of 0.2 ml, and a positive-end expiratory pressure of 2 ml H₂O was applied. Mice were exposed to nebulized PBS followed by increasing concentrations of nebulized methacholine (0-100mg/ml in PBS) (Sigma-Aldrich, St Louis, MO, USA) using an ultrasonic nebulizer (Aeroneb, Aerogen, Galway, Ireland). For each dose, 10 cycles of nebulization and measurements were performed. Nebulization was done during the first cycle and consisted of 20 puffs per 10 s, with each puff of aerosol delivery lasting 10 ms. For each cycle, measurements were obtained for 15 s followed by ventilation for 5s. Baseline lung resistance (R_{rs}) was restored before administration of the subsequent doses of methacholine. Only resistance values corresponding to COD values > 0.95 were kept. For each dose, the maximum resistance value measured was taken.

Histology on lung tissue sections

Left lung was fixed in Antigenfix (Diapath, Microm Microtech, France) during 4 hours at room temperature followed by alcohol (30-100%) and Diasolv (Microm Microtech) baths for dehydration. After

paraffin inclusion, Paraffin-embedded lungs were sliced (5 μ m) with a Microtom (Microm HM355S Thermoscientific). Sections were stained using periodic acid–Schiff (Microm Microtech) for mucus production and Hematoxylin & Eosin (Diapath, Microm Microtech) for cellular infiltration. Images were acquired on an AxioPlan2 (Zeiss) light microscope with Zen Pro software.

RNA Isolation and Quantitative RT-PCR

RNA was extracted from lung with a nucleospin RNA mini kit (Macherey-Nagel, Hoerdtt, France) according to the manufacturer's instructions. Extracted RNA was reverse-transcribed with the High-Capacity cDNA Archive kit (Applied Biosystems, Foster City, USA), according to the manufacturer's instructions. The real-time RT-qPCR was performed in Prime time assay PCR Master Mix (Integrated DNA technologies, Leuven, Belgium) using QuanStudio 12K Flex Real-Time PCR System (Thermofisher Scientific). The quantitative RT-PCR cycling were: one cycle at 95°C for 3 min followed by 45 cycles composed by 95°C for 5 s and 60°C for 30 s. Relative mRNA levels ($2^{-\Delta\Delta C_t}$) were determined by comparing the PCR cycle thresholds (C_t) for the gene of interest and *rplp0* (ΔC_t) and ΔC_t values for treated and control groups ($\Delta\Delta C_t$). The primers (Integrated DNA technologies) used for RT-qPCR are shown in Table S1. Ribosomal Protein Lateral Stalk Subunit P0 gene (*Rplp0*) was used as a reference gene in order to normalize the transcript levels.

Flow Cytometry experiments

Circulation was washed by heart perfusion with 10 ml of PBS. Total lungs were digested by 1 mg/mL type IV collagenase (Life Technologies, Courtaboeuf, France) in RPMI (Life Technologies) for 30 min at 37°C. Cells were washed with PBS with 2% fetal bovine serum (FBS) (Eurobio scientific, Courtaboeuf, France) and centrifuged at 2000 rpm for 15 min on a Percoll density gradient ($D = 1075$ g/mL; Amersham Pharmacia Biotech, Piscataway, NJ). Erythrocytes were lysed in lysis buffer (155 mmol/L NH_4Cl , 10 mmol/L KHCO_3 , and 0.5 mmol/L EDTA). Cells were washed with PBS/FBS 2% and filtered on a 100- μ m-pore membrane. Alive total lung cells were counted after Trypan Blue staining. Cells were incubated with viability marker (Zombie aqua™ Fixable Viability Kit, Biolegend, Ozyme, Saint-Cyr-L'Ecole, France) and incubated for 30 min at 4°C with conjugated antibodies as presented in supplemental data Table S2 and S3. The cells were washed and resuspended in PBS/Fetal Bovine Serum 2% and analyzed by flow cytometry (LSRFortessa®, BD Biosciences, San Diego, CA, USA) with the BD FACSDiva™ Software.

Statistical analysis

The data are presented as median min. to max. and plots. All experiments were carried out at least twice. The comparisons between groups were performed using Kruskal-Wallis nonparametric test with Dunns post-hoc test in GraphPad Prism between HDM groups or between HDM and controls groups (GraphPad software, Inc., La Jolla, CA, USA). p -value < 0.05 were considered as statistically significant.

SUPPLEMENTARY RESULTS

Nanoparticles coupled to B(a)P activate AhR signaling pathway

We first aimed at characterizing the molecular pathway triggered by NPs coupled to B(a)P. B(a)P is known to activate AhR signaling pathway and especially cytochrome transcription.^{S2} Therefore, we evaluated the expression of three *cyp* genes *cyp1a1*, *cyp1a2* and *cyp1b1* in total lung extract. NP-B(a)P significantly increased *cyp1a1* but not *cyp1a2* and *cyp1b1* expression compared to uncoated NPs (NP-Ø). Interestingly, chronic exposure to HDM alone did not induce *cyp* genes transcription (Figure S4). The co-exposure of mice with NP-B(a)P and HDM (HDM+NP-B(a)P) also increased *cyp1a1* but not *cyp1a2* and *cyp1b1* expression compared to NP-Ø with HDM (HDM+NP-Ø). However, the presence of HDM tended to decrease *cyp1a1* expression compared to NP-B(a)P alone (Figure S4). These results show that B(a)P fixed on the NPs is able to activate AhR signaling pathway.

SUPPLEMENTARY REFERENCES

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SUPPLEMENTARY TABLES

Supp. Tab. S1: Prime time assay used for the quantitative PCR

Target	Catalogs	Target	Catalogs
<i>cyp1a1</i>	Mm.PT.58.32054918	<i>il-10</i>	Mm.PT.58.13531087
<i>cyp1a2</i>	Mm.PT.58.18171461	<i>il-13</i>	Mm.PT.58.31366752
<i>cyp1b1</i>	Mm.PT.58.43705524	<i>il-17</i>	Mm.PT.58.6531092
<i>ifn-γ</i>	Mm.PT.58.41769240	<i>muc5ac</i>	Mm.PT.58.42279692
<i>il-4</i>	Mm.PT.58.7882098	<i>muc5b</i>	Mm.PT.58.30457752
<i>il-5</i>	Mm.PT.58.41498972	<i>rplp0</i>	Mm.PT.58.43894205

Supp. Tab. S2: Flow cytometry antibodies for T cells staining

Protein Target	Conjugate	Species Target	Company
TCRβ	APC-Vio770	Mouse	Miltenyi Biotec
TCRγδ	Vioblue	Mouse	
CD4	PE-Vio770	Mouse	
CD25	APC	Mouse	
CD8a	BV605	Mouse	Ozyme (Biolegend)

CD5	PerCP-Cy5.5	Mouse	
NK1.1	FITC	Mouse	
CD45	PE-Cy5	Mouse	

Supp. Tab. S3: Flow cytometry antibodies for antigen presenting cells staining

Protein Target	Conjugate	Species Target	Company
LY6C	BV605	Mouse	BD Biosciences
CD64	BV786	Mouse	
CD103	PerCP-Cy5.5	Mouse	
CD86	AF700	Mouse	
CD11c	PE-Cy7	Mouse	
CD11b	V450	Mouse	
CD45	PE-Cy5	Mouse	
Siglec F	PE-CF594	Mouse	
F4/80	PE	Mouse	Miltenyi Biotech
CMHII	FITC	Mouse	
CCR2	BV650	Mouse	Ozyme (Biolegend)
CD206	APC	Mouse	
LY6G	APC-Cy7	Mouse	