

Early postnatal, but not late, exposure to chemical ambient pollutant 1,2-naphthoquinone increases susceptibility to pulmonary allergic inflammation at adulthood

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Abstract High diesel exhaust particle levels are associated with increased health effects; however, knowledge on the impact of its chemical contaminant 1,2-naphthoquinone (1,2-NQ) is limited. We investigated whether postnatal and adult exposures to 1,2-NQ influence allergic reaction and the roles of innate and adaptive immunity. Male neonate (6 days) and adult (56 days) C57Bl/6 mice were exposed to 1,2-NQ (100 nM; 15 min) for 3 days, and on day 59, they were sensitized and later challenged with ovalbumin (OVA). Airway hyper-responsiveness (AHR) and production of cytokines, immunoglobulin E (IgE) and leukotriene B₄ (LTB₄) were measured in the airways. Postnatal exposure to 1,2-NQ activated dendritic cells in splenocytes by increasing expressing cell surface molecules (e.g., CD11c). Co-exposure to OVA effectively polarized T helper (Th) type 2 (Th2) by secreting Th2-mediated cytokines. Restimulation with unspecific stimuli (PMA and ionomycin) generated a mixed Th1 (CD4⁺/IFN- γ ⁺) and Th17 (CD4⁺/

IL-17⁺) phenotype in comparison with the vehicle-matched group. Postnatal exposure to 1,2-NQ did not induce eosinophilia in the airways at adulthood, although it evoked neutrophilia and exacerbated OVA-induced eosinophilia, Th2 cytokines, IgE and LTB₄ production without affecting AHR and mast cell degranulation. At adulthood, 1,2-NQ exposure evoked neutrophilia and increased Th1/Th2 cytokine levels, but failed to affect OVA-induced eosinophilia. In conclusion, postnatal exposure to 1,2-NQ increases the susceptibility to antigen-induced asthma. The mechanism appears to be dependent on increased expression of co-stimulatory molecules, which leads to cell presentation amplification, Th2 polarization and enhanced LTB₄, humoral response and Th1/Th2 cytokines. These findings may be useful for future investigations on treatments focused on pulmonary illnesses observed in children living in heavy polluted areas.

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Introduction

Over the past 10 years, there has been sufficient epidemiological and experimental evidence to accept that exposure to urban air pollution, including particulate matter (PM), is associated with increased health effects on distinct age-groups; indeed, vulnerable individuals such as infants and patients with asthma and cardiovascular diseases are likely to be more affected (Lin et al. 2004; Schneider et al. 2010; D'Amato 2011; Jenerowicz et al. 2012).

PM varies in size, composition and concentration, depending on origin and age, and it is formed by a complex mixture of suspended solid and liquid particles in semi-equilibrium with surrounding gases. Fine PM (PM_{2.5} µm) such as those from combusted diesel exhaust (DEP) is present in larger concentrations in the environment (see review: van Berlo et al. 2012). They penetrate deep in the airways, and its surface area has the ability to bind proteins, as well as to act as a carrier of allergens and specific organic chemical compounds such as quinones. Among these chemical compounds, the 1,2-naphthoquinone (1,2-NQ) has been suggested to be present in vehicle exhaust particles and in ambient air samples at significant concentrations (Cho et al. 2004). Quinones are known to possess toxicological and alkylating properties, thus inducing oxidative stress and essential macromolecule damage (Brunmark and Cadenas 1989; Bolton et al. 2000; Lin et al. 2007; Shinkai et al. 2012), which might contribute to DEP-induced biological effects in allergic diseases. Our group has shown that acute intratracheal (i.t.) injection of DEP induces airway inflammation, which is highly influenced by increased concentration of 1,2-NQ, due to neurogenic mechanisms involving up-regulation of transient receptor potential (TRP) vanilloid receptor type 1 (TRPV1) and tachykinin receptors (Teles et al. 2010). Inoue et al. (2007a) also reported that exposure to naphthoquinone (NQ) enhances antigen-related airway inflammation with goblet cell hyperplasia in mice. The same group demonstrated that chronic exposure to NQ in adult mice also increases lung inflammation independently of the presence of a second antigen, and this effect was mediated by augmented levels of mucin-producing gene (MUC5AC) and interleukin 13 (IL-13) (Inoue et al. 2007b). Moreover, it has been shown that chronic exposure to 9,10-phenanthraquinone (9,10-PQ), another quinoid compound involved in DEP, enhances immunoglobulin production and infiltration of inflammatory cells into alveolar spaces that are related to OVA, whereas 9,10-PQ is likely to be partially responsible for DEP toxicity on the allergic airway (Hiyoshi et al. 2005a, b).

Although PM_{2.5} is widely recognized as a significant risk factor for a number of diseases in susceptible individuals, including asthmatic (Tao et al. 2003; Robinson et al.

2011), there has been no evidence where the atmospheric chemical pollutant 1,2-NQ is associated with increasing susceptibility to allergic pulmonary disease, upon either its early (neonatal period) or later exposure (adulthood). On the other hand, asthma sensitization is highly dependent on the level of allergen exposure and normally begins in early childhood after re-exposure to ubiquitous environmental allergens (e.g., house dust mites (HDM), molds, plant pollen or animal dander) (Li et al. 2011). Although the strong rise in the incidence of asthma worldwide has been linked to high PM_{2.5} levels, the contributory role of its chemical contaminant 1,2-NQ in the evolution or exacerbation of this condition in susceptible individuals is still unknown. Therefore, we aimed to investigate whether early postnatal and adult exposures to 1,2-NQ influences allergic reaction in mice, and whether immune cells and mast cells are related.

Materials and methods

Chemicals and preparation of pollutant samples

The chemical 1,2-NQ was obtained from Sigma (St. Louis, MO, USA). Immediately prior to animal exposure, 1,2-NQ (100 nM) was prepared under sonication in a vehicle PBS solution containing 0.001 % DMSO and 0.001 % Tween 80. Isoflurane was obtained from Cristália (Itapira, São Paulo, Brazil). Al(OH)₃ was provided by EMS (São Paulo, Brazil). ELISA kits for cytokines, immunoglobulin E (IgE) and LTB₄ were purchased from R&D Systems (Ann Arbor, MI, USA) and Biolegend (San Diego, CA, EUA), respectively. Antibodies fluorochrome conjugated (FITC, PE, APC or Cy) for CD4, CD11c, CD11b, CD80, CD86, I-Ab, IL-4, IL-17 and IFN-γ were obtained from BD Biosciences® (San Diego, CA). All other products were purchased from Sigma Chemical Co. (St. Louis, MO, USA.).

Animal husbandry and assessment of clinical and behavioral changes

Experiments employed in this study were approved by the Institute of Biomedical Sciences (ICB/University of São Paulo, Brazil) Ethics Committee (Process number 113, book 2/2007) and were carried out in accordance with the Brazilian Council for Control of Animal Experimentation (CONCEA) guidelines.

Both neonate (male and female) and adult male C57Bl/6 mice were bred and maintained at the Pharmacology Department Animal House, Institute of Biomedical Sciences of São Paulo University. Mothers remained with the pups at all times, except during the pups exposure to 1,2-NQ or its vehicle and weighing sessions, until their

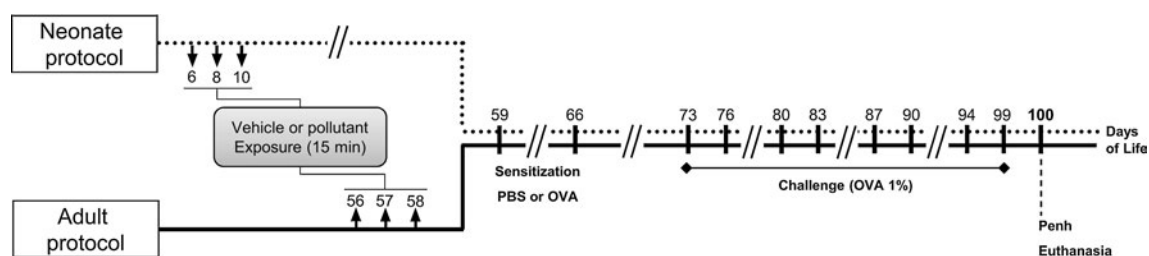


Fig. 1 Pollutant exposure scheme during adulthood and neonatal period. Adult (on days 56, 57 and 58 of age) or neonate (on days 6, 8 and 10 of age) C57Bl/6 mice were exposed to 1,2-NQ (100 nM) or its vehicle (PBS containing 0.001 % DMSO and 0.001 % Tween 80) during a 15-min period each day. On day 59, neonate or adult mice were submitted to subcutaneous ovalbumin sensitization (OVA; 10 μ g emulsified in Al(OH)₃ 1.6 mg and PBS) or vehicle and fur-

ther boosted with similar doses of OVA on day 66. Starting on day 73, mice were challenged with inhaled antigen OVA (1 %, during 20 min), and subsequently twice a week during 4 weeks. After 24 h of the last challenge, mice were submitted to *in vivo* Penh function evaluation, and after euthanasia, the biochemical and cell assays were performed as described under the “Methods” section

weaning at day 23, where male offspring were sex-housed five to a cage under conventional environmental conditions (12-h/12-h light/darkness cycle), and were freely fed laboratory diet and filtered water. Throughout this study, the remaining female mice were removed and used in a different study in our laboratory.

Neonate male mice exposed to 1,2-NQ (or its vehicle) were monitored daily for clinical signs of moribundity (e.g., unusual posture, gait abnormalities, convulsions, tremors and unusual behavior such as writhing and self-mutilation) and mortality. They were weighed at day 5 prior to the pollutant or vehicle nebulization and thereafter at 2, 6, 10, 30, 60, 70, 77, 84, 91 and 100 days of age.

Animal exposure to 1,2-NQ and experimental design

Adult or neonate male C57Bl/6 mice were randomly divided into four groups for each protocol (neonatal or adult): 1) control group (vehicle of pollutant plus PBS challenge), 2) 1,2-NQ plus PBS challenge, 3) vehicle of pollutant plus ovalbumin (OVA) challenge (allergic group) and 4) 1,2-NQ plus OVA challenge. Either neonate (days 6, 8 and 10) or adult mice (days 56, 57 and 58) were housed unrestrained in a polycarbonate cage (surface area of 600 cm²) and exposed to environmental 1,2-NQ atmosphere (10 μ g m⁻³ inside chamber) or vehicle (0.001 % DMSO and 0.001 % Tween in PBS). The pollutant atmospheres were generated from fresh 1,2-NQ stock solution (100 nM, 10 ml) diluted in its vehicle, through a standard ultrasonic nebulizer (Mod Respiramax; NS, São Paulo, Brazil) directly coupled to the cage. The animals were exposed to aerosolized 1,2-NQ (or its vehicle) for 15 min, during 3 days (Fig. 1). In neonate protocol, the exposure to 1,2-NQ was performed during three alternate days in order to avoid excessive animal handling and consequently to minimize the stress to the pups and their mothers.

After the last exposure to the pollutant, neonate mice were returned to the cages and used 59 days later, while the adult mice exposed to 1,2-NQ were submitted to the allergen treatment with OVA (Fig. 1) 24 h later. All the animals were submitted to the unrestrained lung function test (Penh) prior to euthanasia. In order to reduce variations in lung function assessment as a result of circadian rhythms, the pollutant and allergen exposures and the Penh assessment *in vivo* were always performed during the day at the same period.

Determination of aerosol output rate inside the chamber

The aerosol output inside the polycarbonate cage and related weight loss after nebulization period were determined via gravimetric analysis, accordingly (Dennis et al. 1990; Arzhavitina and Steckel 2010). Briefly, the nebulizer glass tube was weighed before and after placing 1,2-NQ solution (100 nM; 10 ml), and immediately after the nebulization process. The nebulized 1,2-NQ mass into the chamber was calculated based on the standard equation [$m_{\text{ng}} = m_{\text{w}_{\text{ng}}}[\text{NQ}]\cdot V_{\text{sol}}$], in which m_{ng} = 1,2-NQ mass nebulized inside chamber, $m_{\text{w}_{\text{ng}}}$ = 1,2-NQ molecular weight (158.15 g/mol), [NQ] = the pollutant solution concentration and V_{sol} = solution volume pollutant placed in the nebulizer tube. The resulting concentration of 1,2-NQ nebulized inside the chamber is approximately 152 ng with 2–3 % of loss.

Allergen sensitization and challenge

Adult mice either at 24 h or 59 days after the last exposure to 1,2-NQ or its vehicle were first sensitized by the allergen OVA (grade V; Sigma-Aldrich Co., St Louis, MO, USA) following standard protocols used to mimic asthmatic subjects (Riffo-Vasques et al. 2007). Briefly, animals were sensitized to OVA via a subcutaneous (s.c.) injection of

10 µg OVA (0.2 ml) emulsified in 1.6 mg Al(OH)₃ in PBS solution (Day 0). After a week (Day 7), animals received a similar dose of OVA as a booster sensitization. Nonsensitized mice received only s.c. injection of Al(OH)₃ in PBS (0.2 ml). From day 14, mice were challenged by inhalation of OVA (1 %) by an aero nebulizer (Mod Respiramax; NS, São Paulo, Brazil) for 20 min and subsequently twice a week for 4 weeks. Animals were used 24 h later for the in vivo assay or euthanized for the in vitro measurements. As controls, both nonsensitized and sensitized mice were challenged with PBS. At 24 h after the last challenge with OVA, the mice were anesthetized using 2 % isoflurane and vaporized with 100 % oxygen, and a sample of peripheral blood was collected from the abdominal aorta, following exsanguination. BAL was retrieved and the femur and spleen were taken, to obtain the bone marrow and spleen cells, respectively, as described below.

Bronchoalveolar lavage (BAL) cell collection and cytology analysis

Mice were terminated by exsanguination under isoflurane anesthesia, and a tracheal cannula was inserted via a mid-cervical incision. The lungs were lavaged five times with 0.3 ml of pyrogen-free heparinized PBS (5 UI/ml, pH 7.4). The recovered bronchoalveolar lavage fluid (BAL) was centrifuged (10 min, at 4 °C, 300g) and the supernatant stored (−80 °C) for further analysis. The resulting pelleted cell was resuspended in 0.2 mL of PBS, and a total (Neubauer) and differential (May-Grunwald-Giemsa stain) cell counts were performed. A minimum of 100 cells were counted and classified as eosinophils, neutrophils and mononuclear cells based on normal morphological criteria.

Bone marrow and peripheral blood collection and hematology

Samples of blood were collected from the abdominal aorta in a syringe containing heparin (50 UI/ml) and centrifuged (800g at 4 °C) for 10 min, and the resulting plasma was collected and stored at −80 °C. The total cells were quantified (Neubauer, Turk solution stain), and blood smears were used to obtain differential cell count. At least 100 cells were counted and classified accordingly based on normal morphological criteria. In parallel, both the femurs from euthanized mice were removed (≈1 cm), and the epiphyses were cut transversely. The bone marrow cells were isolated by flushing the two femurs with PBS (2 ml per femur), and the total (Neubauer) and differential (May–Grünwald–Giemsa stain) cell counts were performed. The recovered lavage fluid was submitted to both the total and differential cell counts. A minimum of 100 cells were counted, and the eosinophilic lineage was classified as immature eosinophils

(myeloblast, promyelocyte, myelocyte and metamyelocyte) or mature eosinophils recognized by the intensely dyed granules and morphological criteria.

Lung morphological analysis

After euthanasia, the mouse chest was opened and the pulmonary artery was perfused using 5 ml heparinized saline (5 UI/ml) and postfixed for at least 24 h in 4 % buffered paraformaldehyde (pH 7.4). Then, lung fragments (the middle third of the caudal aspects of both lungs) were embedded into *Histosec* paraffin (Merck, EUA) and cut transversally into slices (approximately 5 µm) using a microtome (Spencer 820, American Optical Corporation; USA). Specimen sections were mounted onto glass slides and routinely stained with hematoxylin and eosin (H and E). Alternatively, lung specimens were fixed in 4 % paraformaldehyde containing 0.1 M Sörensen phosphate buffer (pH 7.4 at 4 °C) for 2 h and stained with toluidine blue in order to assess the degranulation of mast cells. Images were captured digitally with a Nikon E600 microscope (Nikon, Japan) integrated to Olympus DP-72 digital camera (Olympus, Japan) and analyzed for mast cell degranulation.

Measurement of cytokines, IgE and leukotriene B₄-concentrations in BAL fluid

The BAL samples were thawed, and concentrations of Th1/Th2 cytokines (IL-4, IL-5, IL-10, IL-13, TNFα and IFN-γ; R&D Systems, Ann Arbor, MI, USA) and total IgE and LTB₄ (eBioscience, San Diego, USA) were measured according to the manufacturer instructions. The range of detectable concentration of cytokines varied from 2000 to 15.625 pg/ml for TNF-α, IFN-γ and IL-10, and from 500 to 3,900 pg/ml for IL-4, IL-5 and IL-13.

Determination of respiratory flow (lung function) by whole-body plethysmography

Lung function of the mice was tested at 24 h after the last 1,2-NQ (or its vehicle) exposure or OVA (or related vehicle PBS) challenge. The enhanced pause (Penh) was measured, using a whole-body plethysmography system, as an indicator of thoracic breathing pattern [airway hyper-responsiveness (AHR)] to the aerosolized cholinergic agonist methacholine (MCh) at 24 h post-OVA or PBS challenge in conscious, spontaneously breathing mice. AHR was expressed as enhanced pause (Penh), a calculated unitless value that correlates changes in enhanced pause, tidal volume (Vt) and breathing frequency (breaths/min) or [(expiratory time/relaxation time)−1] : [max. expiratory flow/max. inspiratory flow] within the same animal (Helyes et al. 2009). Briefly, each mouse was placed in a chamber and

allowed to settle for 5 min while the continuous measurements of pressure–time wave in the chamber were registered and calculated via a connected transducer-computer data acquisition system (Mod PLY4211; Buxco; NY, USA). Aerosolized PBS followed by increasing concentrations of MCh (3.125, 6.250, 12.5, 25.0 and 50 mg/ml) was nebulized through an inlet of the main chamber for 180 s to induce changes in the AHR. Readings were taken and averaged for 5 min after each nebulization. Penh was expressed as absolute values and area under curve (AUC) before and after respective MCh administration.

Isolation of spleen cells and flow cytometry analysis

Neonate spleen cells were harvested 24 h after last 1,2-NQ or its vehicle exposure. In parallel, spleen cells were obtained from adult mice at 24 h after last antigen or PBS challenge. Tissues were minced and dispersed into single-cell suspension containing DMEM, and erythrocytes were lysed with Red blood cell lysing buffer (Sigma Chemical Company, EUA), accordingly (Peron et al. 2010). Following centrifugation (450 g for 5 min at 4 °C), the resulting pellet was resuspended in DMEM, and the cells were counted (density of 1×10^6 cells/well) and incubated with monoclonal antibody anti-mouse CD16/CD32 (1:100 for 30 min at 4 °C; BD Biosciences Pharmingen, EUA) to block Fc receptor. After Fc blocking, cells were incubated (30 min; at 4 °C) with anti-mouse monoclonal antibodies conjugated with fluorochrome (FITC, Cy, PE or APC) specific to surface molecules CD11c, CD11b, CD80, CD86 and I-Ab ($0.5 \mu\text{g}/10^6$ cells; Biosciences Pharmingen, EUA). After the incubation period, cells were washed twice with PBS buffer (pH 7.4) containing 3 % fetal bovine serum and further centrifuged (450g for 5 min at 4 °C). The cells obtained were fixed in paraformaldehyde (1 %; 200 μl), and reading was taken in a flow cytometer (Becton and Dickinson, Mountain View, CA). The expression of co-stimulatory molecules analysis was performed after the acquisition of 5,000 events in specific gate for CD11c and CD11b (CellQuest software; Apple, USA). Fluorescence data were expressed as percentage of positive cells. An independent group of neonate or adult mice without any treatment was used as a naïve control.

Spleen cells harvested from neonate mice exposed to 1,2-NQ or its vehicle (density = 1×10^6 cells/well) were cultured in DMEN (37 °C; 5 % of CO_2) in the presence of anti-CD3 ($1 \mu\text{g}/\text{mL}$; unspecific stimulus), OVA ($2 \mu\text{g}/\text{ml}$; specific Th2 stimulus) or forbol miristato acetato (PMA; $50 \text{ ng}/\text{ml}$) plus ionomycin ($1 \mu\text{g}/\text{ml}$; specific to T and B lymphocyte stimulus) and brefeldin A during 24 h. After this period, cells were washed and submitted to flow cytometry staining protocol for surface staining of CD4 ($0.5 \mu\text{g}/10^6$ cells; Biosciences Pharmingen, EUA) as

described above. Later for intracellular staining, cells were fixed and permeabilized with Cytofix/Cytoperm kit (e-biosciences®, San Diego, CA), according to the manufacturer's protocol, and incubated (30 min; at 4 °C) with monoclonal antibodies conjugated with fluorochrome (PE or APC) for IL-17, IL-4 and IFN- γ . Cells were then washed ($2\times$) with Permash (e-bioscience, San Diego, CA) and fixed in 1 % paraformaldehyde, and acquisition was performed via flow cytometer (FacsCanto-BD Biosciences Pharmingen, EUA).

Statistical analysis

Data are expressed as the mean $\pm$ SEM of *n* animals unless otherwise stated. Differences between groups were analyzed by one- or two-way analysis of variance (ANOVA) followed by Bonferroni's modified *t* test or by unpaired Student's *t* test as indicated, by using the program Instat (GraphPad Prism software 4.0). *P* value <0.05 was taken as significant.

Results

Impact of early-life exposure to 1,2-NQ on clinical aspects and ponderal growth (body weight)

The values for growth in offspring exposed to 1,2-NQ or its vehicle are expressed as area under the curve (AUC) over a 100-day period. They show that there were no significant differences between the groups early exposed to 1,2-NQ in the presence or absence of allergic stimulus OVA (256 ± 8 ; 246 ± 6 ; 251 ± 2 ; AUC mean body weight for 1,2-NQ PBS, allergic group only and 1,2-NQ plus OVA, respectively, *n* = 6–7) as compared with control-vehicle group (249 ± 9 AUC mean body weight for control-vehicle group, *n* = 6–7). Neither deaths nor clinical signs of moribundity and change in behavior were found in any of the groups.

Early-life exposure to 1,2-NQ exacerbates allergic lung inflammation

The total cell (and eosinophils) counts in the airways (BAL) were evaluated at 24 h post-OVA challenge in both mice exposed to 1,2-NQ at neonatal and adult period. The cells in BAL fluid from the control (vehicle) group were >99 % mononuclear cells in all mice exposed to the vehicle at adulthood or during the neonatal period (Supplemental Table S1 [A and B]), and also the eosinophil number in BAL fluid and lungs was minimal in these groups (Fig. 2a, b, f, g). Exposure to 1,2-NQ in adults and neonates also did not affect the number of eosinophils (or total cells) in BAL fluid (Fig. 2a, b), but significantly increased the number of neutrophils ($0.83 \pm 0.4 \times 10^4/\text{BAL}$) when compared

with respective vehicle-treated animals, as observed at 24 h post-PBS challenge [Supplemental Table S1 (A and B)].

Challenge with OVA in mice treated with vehicle at adult or at neonatal period significantly increased the number of total cells, characterized by eosinophils (Fig. 2a, b), lymphocytes, neutrophils and macrophages [Supplemental Table S1 (A and B)] in BAL fluid and lung tissue (Fig. 2f, g) in comparison with vehicle-treated animals. The eosinophils and total cells (Fig. 2a, b) in addition to lymphocytes, but not macrophages, numbers in BAL fluid (Supplemental Table S1 [A and B]) and lung tissue from the mice exposed to 1,2-NQ at neonatal period were much greater at 24 h after OVA challenge as compared to its allergic group or control (vehicle) group (Fig. 2a, b, f, g). In contrast, at 24 h after OVA challenge, the eosinophil (and total cells) number in BAL fluid and lung tissue from mice exposed to 1,2-NQ at adult period did not significantly increase when compared with allergic group (Fig. 2a, b, f, g).

Effect of 1,2-NQ on bone marrow and peripheral blood eosinophil counts

Allergic group from neonate or adult protocol showed that the number of immature and mature forms of eosinophils in bone marrow significantly elevated, as expected (Fig. 2c–e). However, the challenge with the antigen OVA in mice exposed to 1,2-NQ at a neonate period, but not at an adult stage, evoked a further increase ($P < 0.05$) in the pattern of mature eosinophils in the bone marrow compared to its allergic group (Fig. 2e). At 24 h post-PBS challenge, the number of mature and immature forms of eosinophils was not significantly higher in mice exposed to 1,2-NQ at neonatal or adult period (1,2-NQ plus PBS group) than in their control-vehicle group (Fig. 2d, e).

In peripheral blood of mice exposed to 1,2-NQ at adult stage the counts of eosinophils at 24 h post-OVA challenge showed an insignificant trend toward an increase when compared to vehicle-treated mice ($0.95 \pm 0.2 \times 10^5/\text{ml}$ and $0.24 \pm 0.1 \times 10^5/\text{ml}$, respectively; $n = 6$). In contrast, at 24 h post-OVA challenge, the peripheral blood of mice exposed to 1,2-NQ at neonatal period exhibited a marked increase ($P < 0.01$) in total cell counts ($46 \pm 4.0 \times 10^5/\text{ml}$; $n = 7$), as well as in the number of eosinophils ($1.0 \pm 0.5 \times 10^5/\text{ml}$; $n = 8$) as compared with its allergic group ($29 \pm 2.5 \times 10^5/\text{ml}$ and $0.5 \pm 0.1 \times 10^5/\text{ml}$, respectively; $n = 7$ – 8).

Effect of early-life exposure to 1,2-NQ on histopathological pattern and mast cell degranulation in lung

The lung specimens analyzed from control-vehicle mice showed normal histological appearance with very few

scattered cell infiltration and typical preserved peribronchial and perivascular appearance (Fig. 2f,g). Likewise, the histological examination of the lungs from the 1,2-NQ plus PBS group (neonate protocol) showed a similar profile to that found in the lung of control-vehicle mice (Fig. 2g). Lungs from mice treated with 1,2-NQ plus PBS at adulthood exhibited an increase in peribronchiolar and perivascular leukocyte influx, comprising mainly of polymorphonuclear cells (Fig. 2f). At 24 h post-OVA challenge, the lung of allergic mice from neonate or adult protocol exhibited exudate and significant leukocyte influx, into the connective tissue surrounding the blood vessels and bronchiolar segments (Fig. 2f, g). Similar peribronchiolar leukocyte influx or exudate appearance found in the allergic group was seen in 1,2-NQ plus OVA group from adult protocol (Fig. 2f). At 24 h post-OVA challenge, mice exposed to 1,2-NQ at neonatal period showed a marked increase in cell influx, comprising mainly of eosinophils and lymphocytes, and exudate compared with its allergic group (Fig. 2g).

At 24 h post-PBS challenge, lungs from control groups exposed to vehicle during adulthood or at neonatal stage all showed intact mast cell and no activated cells. Mice exposed to 1,2-NQ during adulthood or at neonatal period showed a significant reduction in the number of intact mast cells and a concomitant increase in activated mast cells compared to the respective control group (Table 1; Supplemental fig. 1). Likewise, lung of allergic mice from adult or neonatal protocol exhibited reduced intact mast cells and a significant population of mast cell degranulation (Table 1; Supplemental fig. 1). Mast cell degranulation in the mice lung exposed to 1,2-NQ at a neonatal period or at adulthood and challenged with OVA was increased (63 and 100 %, respectively) in comparison with respective control (vehicle) groups, but as compared to each respective allergic group, it showed statistically significant difference just in the adult protocol (86 and 57 %, respectively; Supplemental fig. 1).

Cytokine, IgE and LTB₄ concentrations in the BAL

The cytokine concentration in BAL fluid of control (vehicle) mice from adult and neonatal protocols was unchanged after 24-h challenge with PBS compared with naïve mice (data not shown; $n = 4$). In the BAL fluid of group 1,2-NQ plus PBS from adult protocol, the concentrations of IL-5, IL-13, IFN- γ , TNF- α and IL-10 were enhanced ($P < 0.05$) compared to the control group (Fig. 3a–e, $n = 5$ – 6). Cytokine levels for IL-13 and TNF- α in BAL fluid of 1,2-NQ plus PBS from neonatal protocol were increased when compared with its control group (Fig. 3a–e). Twenty-four hours after OVA challenge, the BAL fluid of allergic mice from neonatal protocol

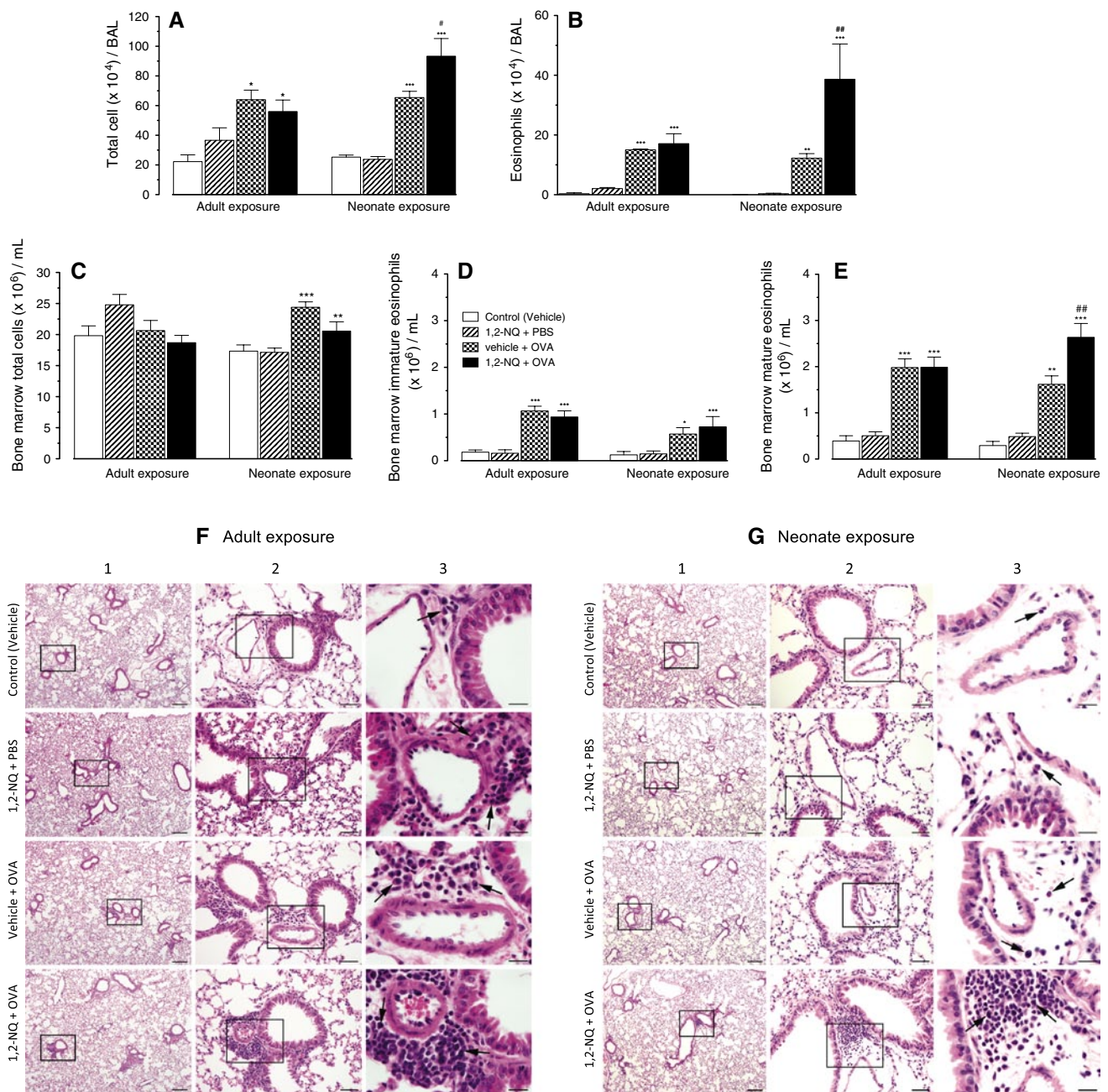


Fig. 2 Comparative effect of adulthood and early postnatal exposure to 1,2-NQ on total inflammatory cells and eosinophils in BAL fluid (**a**, **b**), bone marrow (**c**–**e**) and lung histopathological pattern (**f** and **g**) 24 h after OVA challenge. Each bar represents the values as mean $\pm$ SEM for n animals ($n = 5$ – 7 , $n = 5$ – 6 , $n = 5$ – 7 and $n = 5$ – 7 ; for vehicle, 1,2-NQ + PBS, vehicle + OVA and OVA + 1,2-NQ, for adult and neonate protocols, respectively). * $P < 0.05$, ** $P < 0.01$ and

*** $P < 0.001$ compared with the control group (vehicle). # $P < 0.05$ and ### $P < 0.01$ compared with allergic group (vehicle plus OVA). **f** (1, 2 and 3) and **g** (1, 2 and 3) illustrate high-power fields of lung parenchyma and bronchiolar structures stained with H and E. The black arrows indicate edema and leukocyte influx (mononuclear and/or polymorphonuclear). The scale bars in the pictures under columns 1, 2 and 3 represent 200, 50 and 20 μ m, respectively

presented significant levels of IL-5, IL-13 and TNF- α compared with control group (Fig. 3a–c). The early-life, but not late-life, exposure to 1,2-NQ largely increased the levels of IL-13 and IFN- γ in the BAL fluid of mice at 24 h after antigen challenge (1,2-NQ plus OVA group),

compared with the allergic group (Fig. 3b, d). Marked levels of IL-5, IL-13, TNF- α and IFN- γ (Fig. 3a–d) were also observed in BAL fluid of mice exposed to 1,2-NQ at neonatal period and challenged with OVA compared with its control group (Fig. 3a–d). In the 1,2-NQ plus OVA

Table 1 Effects of early postnatal exposure to 1,2-NQ on mast cell degranulation in lung 24 h after the last OVA challenge

Groups								
Mast cells mm ²	Control (vehicle)		1,2-NQ + PBS		PBS + OVA		1,2-NQ + OVA	
	Adult	Neonate	Adult	Neonate	Adult	Neonate	Adult	Neonate
Intact	1.9 ± 0.5	2.8 ± 0.3	1.0 ± 0.1	1.7 ± 0.3 [#]	1.2 ± 0.2	0.5 ± 0.1***	0* [#]	1.1 ± 0.4***
Activated	0	0	1.2 ± 0.2*	1.2 ± 0.1**####	1.6 ± 0.4*	3.0 ± 0.4***	1.2 ± 0.1*	19 ± 0.3***##
Value in (%)	0	0	54.5	41.3	57.2	85.7	100.0	63.3
Total	1.9 ± 0.5	2.8 ± 0.3	2.2 ± 0.1	2.9 ± 0.3	2.8 ± 0.4	3.5 ± 0.3	1.2 ± 0.1	3.0 ± 0.3

The incidence of intact or degranulated mast cells was calculated as percentage of total mast cell number. Data are expressed as mean ± SEM; *n* = 4 animals/group

* *P* < 0.05, ** *P* < 0.01 and *** *P* < 0.001 when compared with the respective control group (vehicle)

[#] *P* < 0.05, ## *P* < 0.01 and #### *P* < 0.001 when compared with the respective allergic group (vehicle plus OVA). Supplemental histology figure is available and illustrates these findings

group from adult protocol, none of the cytokine levels significantly increased in the BAL fluid compared with allergic group (Fig. 3a–e).

At 24 h after OVA challenge, the concentrations of IgE and LTB₄ were higher in BAL fluid of mice exposed to 1,2-NQ at neonatal period, but not at adulthood as compared with their control or allergic group (Table 2). Likewise, a significant increase in LTB₄ was found in BAL fluid of mice only exposed to 1,2-NQ at a neonatal period, but not at adult period when compared to its control group vehicle (Table 2). In parallel, significant levels of IgE were found in BAL of mice exposed only to 1,2-NQ from adult protocol, but not from neonatal protocol (Table 2). None of the allergic groups from both protocols exhibited significant levels of IgE after 24-h challenge with OVA versus its control group (Table 2).

Pollutant exposure and airway responsiveness

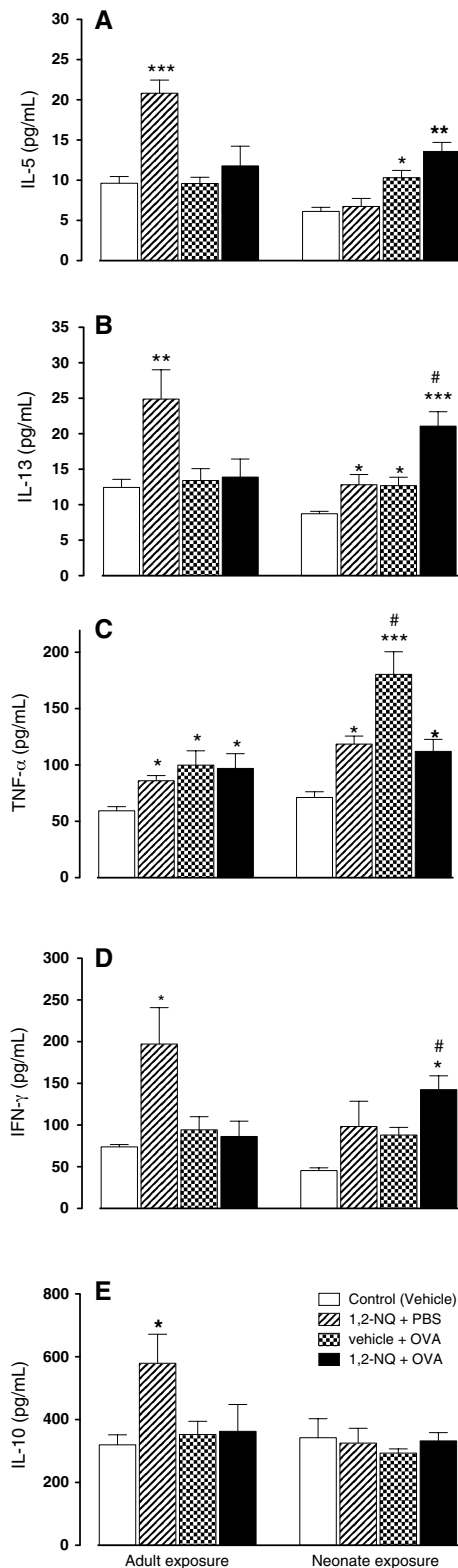
Figure 4 (panels a–d) shows that at 24 h after challenge with PBS, the AHR of control (vehicle) either to adult or to neonatal protocol, as quantified by Penh as well as AUC in response to increasing concentration of MCh challenge, did not show significant differences compared to basal values evoked by PBS (Time 0). Likewise, dose–response curve and AUC in response to MCh in mice exposed to 1,2-NQ during neonatal or adulthood and later to PBS did not exhibit a significant increase compared with control vehicle-treated mice (Fig. 4a–d). In all cases, MCh challenge caused a marked and dose-dependent increase in Penh (and AUC) in allergic mice, indicating AHR as seen in asthmatics (Fig. 4a–d); however, in response to the same concentrations of MCh, mice exposed to 1,2-NQ at neonatal, but not at adulthood, and postchallenged with antigen (1,2-NQ plus OVA) presented a trend to reduction in AHR when compared with their respective allergic mice (Fig. 4a–d).

Effects of early postnatal exposure to 1,2-NQ enhance CD11c⁺ infiltration

After 24-h OVA (or PBS) challenge, the CD11c⁺ antigen-presenting (APC) cell population assessed in spleen cell suspension of mice exposed to 1,2-NQ at neonatal period, but not at adulthood, was greatly increased in comparison with the control-vehicle group (Fig. 5a–e). Likewise, at 24 h after OVA challenge to the animals exposed to 1,2-NQ at neonatal period, there was a significant increase in CD11c⁺ population in comparison with control (vehicle) mice; however, this response was not further increased when compared with animals exposed to 1,2-NQ plus PBS (Fig. 5c–e). The OVA challenge in allergic mice from both protocols did not cause enhancement of CD11c⁺ population in spleen cell suspension when compared with control-vehicle mice (Fig. 5a–e). At 24 h after the last 1,2-NQ exposure at neonatal period, either the CD11c⁺ population or the expression of co-stimulatory molecules CD80 and CD86, but not IA-b (MHC-II), was markedly augmented in splenocytes of these animals as compared to control (vehicle) group (Fig. 6a–d). Splenocyte suspension from both adult and neonate naïve mice exhibited similar levels of CD11c⁺ population and CD80 and CD86 expression (Fig. 5a–e). In both protocols, at either neonatal or adult period, 1,2-NQ exposure did not increase the CD11b⁺ cell population (a macrophage/monocyte marker), as assessed in spleen cell suspension at 24 h after vehicle and OVA challenge, when compared with the allergic or control group (data not shown).

Effects of early postnatal exposure to 1,2-NQ in T lymphocyte population

T lymphocyte population from splenocytes of neonate mice exposed to 1,2-NQ and re-stimulated in vitro with PMA and ionomycin, unlike CD3 re-stimulation, showed a significant



increase in the percentage of Th1 ($CD4^+/IFN-\gamma^+$) and Th17 ($CD4^+/IL-17^+$) phenotype in comparison with the control (vehicle) group (Fig. 7a, b). The percentage of Th2 ($CD4^+/IL-4^+$) lymphocyte population from splenocytes of neonate

Fig. 3 Effects of adulthood or early postnatal exposure of mice to 1,2-NQ on the concentrations of IL-5 (a), IL-13 (b), TNF- α (c), IFN- γ (d) and IL-10 (e) measured in BAL fluids collected 24 h after the last OVA (or PBS) challenge. Each bar represents the values as mean $\pm$ SEM for n animals ($n = 5$, $n = 5-6$, $n = 6-7$ and $n = 6-7$; for vehicle, 1,2-NQ + PBS, vehicle + OVA and OVA + 1,2-NQ, for adult and neonate protocols, respectively). * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ compared with the respective control group (vehicle). ## $P < 0.01$ compared with respective allergic group (vehicle plus OVA)

Table 2 Effects of early postnatal or adult exposure to 1,2-NQ on the concentrations of total IgE and LTB $_4$ in BAL samples collected 24 h after the last OVA challenge

Group	IgE (pg/mL)		LTB $_4$ (pg/mL)	
	Adult	Neonate	Adult	Neonate
Control (vehicle)	48 $\pm$ 6	45 $\pm$ 5	3.5 $\pm$ 0.6	2.3 $\pm$ 0.1
1,2-NQ + PBS	77 $\pm$ 7*	51 $\pm$ 9	4.4 $\pm$ 1.2	3.8 $\pm$ 0.3*
PBS + OVA	52 $\pm$ 8	48 $\pm$ 2	3.0 $\pm$ 0.4	2.3 $\pm$ 0.1
1,2-NQ + OVA	50 $\pm$ 8	103 $\pm$ 11**#	3.5 $\pm$ 0.1	4.2 $\pm$ 0.7**

Data are expressed as mean $\pm$ SEM; n animals ($n = 5-5$, $n = 5-6$, $n = 6-7$ and $n = 6-7$; for vehicle, 1,2-NQ + PBS, vehicle + OVA and OVA + 1,2-NQ, for adult and neonatal protocol, respectively)

* $P < 0.05$ and ** $P < 0.01$ when compared with the respective control group (vehicle)

$P < 0.01$ compared with the respective allergic group (vehicle plus OVA)

mice exposed to 1,2-NQ re-stimulated with both PMA and ionomycin or CD3 did not differ from vehicle group (Fig. 7c). Splenocytes from neonate mice exposed to 1,2-NQ and re-stimulated in vitro with OVA for 24 h showed a significant increase in the percentage of Th2 ($CD4^+/IL-4^+$) population when compared to the control group (Fig. 7c).

Discussion

Increasing air pollution is directly linked to serious health side effects, mainly in susceptible individuals such as children and those with preexisting pathological conditions, such as asthma (Lin et al. 2002; Romieu et al. 2012; Franchini et al. 2012; Jassal et al. 2013) or blepharitis (Malerbi et al. 2012). Traffic-related ambient air pollution, such as DEP, is among the major culprits due to its increasing levels in the air, complexity and its ability to absorb atmospheric chemicals, such as 1,2-NQ (Cho et al. 2004; Krzyzanowski et al. 2005). In fact, we have previously shown that the intratracheal (i.t.) administration of DEP induces a dose-dependent inflammation in the rat airways, which is highly influenced by the co-administration of 1,2-NQ (Teles et al. 2010). However, the detailed understanding of the underlying mechanism(s) of action of this specific

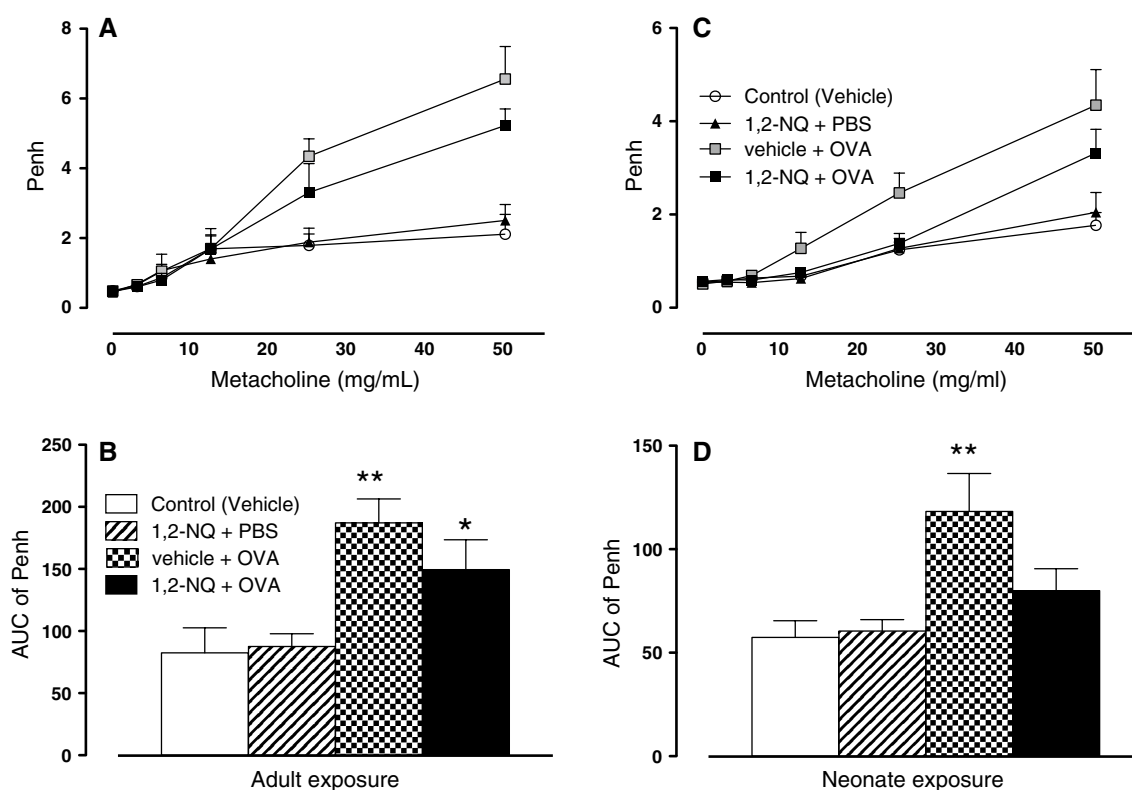


Fig. 4 Airways responsiveness (Penh) to methacholine (MCh) of mice exposed to 1,2-NQ (or its vehicle) at the adulthood (**a, b**) or early postnatal (**c, d**) period. **a, c** illustrate the dose-response curves to MCh, and **b, d**, the calculated AUC values. Data are expressed as

mean $\pm$ SEM for n animals ($n = 5$, $n = 5-6$, $n = 6-7$ and $n = 6-7$; for vehicle, 1,2-NQ + PBS, vehicle + OVA and OVA + 1,2-NQ, for adult and neonate protocols, respectively). * $P < 0.05$ and ** $P < 0.01$ when compared with the respective control group (vehicle)

contaminant is still limited in both healthy and susceptible individuals. In this study, we have comparatively assessed whether the early (neonatal) or later (adulthood) short-term exposure of mice to 1,2-NQ interferes with molecules and cells involved in the host immunological system, and hence the inflammatory response.

We demonstrated that exposure of mice to 1,2-NQ during the neonatal phase, but not during adulthood, exacerbates the antigen response, as characterized by the elevated number of mature eosinophil in the bone marrow and pronounced eosinophilia and lymphocytosis associated with the enhanced production of Th2/IL-13 and Th1/IFN- γ cytokines, humoral response (IgE), LTB $_4$ and the concomitant histopathological changes observed in the airways (such as leukocyte influx and edema), without significant effects on IL-5, TNF- α or MCh-induced AHR when compared with the allergic matched group. It should be emphasized that differences exist between neonate and adult airways, including greater permeability, with a higher number of pulmonary alveoli in neonates than in adults when compared on a body weight basis. Thus, the air uptake $^{-1}$ min, and hence the allergic sensitization, is higher in the former

(Pinkerton and Joad 2006). Also, Th2 phenotype development is higher in children of up to 2 years, due to their ability to suppress Th1 phenotype, thus delaying the Th1 cell maturation (Holt et al. 2005).

On the other hand, the exposure of either neonate or adult mice to 1,2-NQ *per se* failed to induce eosinophilia or lymphocytosis, either peripherally, in the airways or bone marrow. In line with these results, other authors showed that only repeated and high doses of either DEP or 1,2-NQ were capable of enhancing antigen-related and unrelated airway inflammation in rodents (Dong et al. 2005; Takano et al. 2007; Inoue et al. 2007a).

In spite of the lack of significant eosinophilia in the airways or bone marrow, the exposure of adult mice to 1,2-NQ resulted in moderate leukocyte infiltration (neutrophilia) into the lung, associated with an increased humoral response (IgE titers), and neutrophilia associated with production of Th1 (TNF- α , IFN- γ , IL-10) and Th2 cytokines (IL-5, IL-13). Likewise, only the exposure to 1,2-NQ during the neonatal period evoked the significant production of IL-13, TNF- α and LTB $_4$ in the airways, and a trend toward increased IFN- γ , without significantly affecting IgE, IL-5 or IL-10

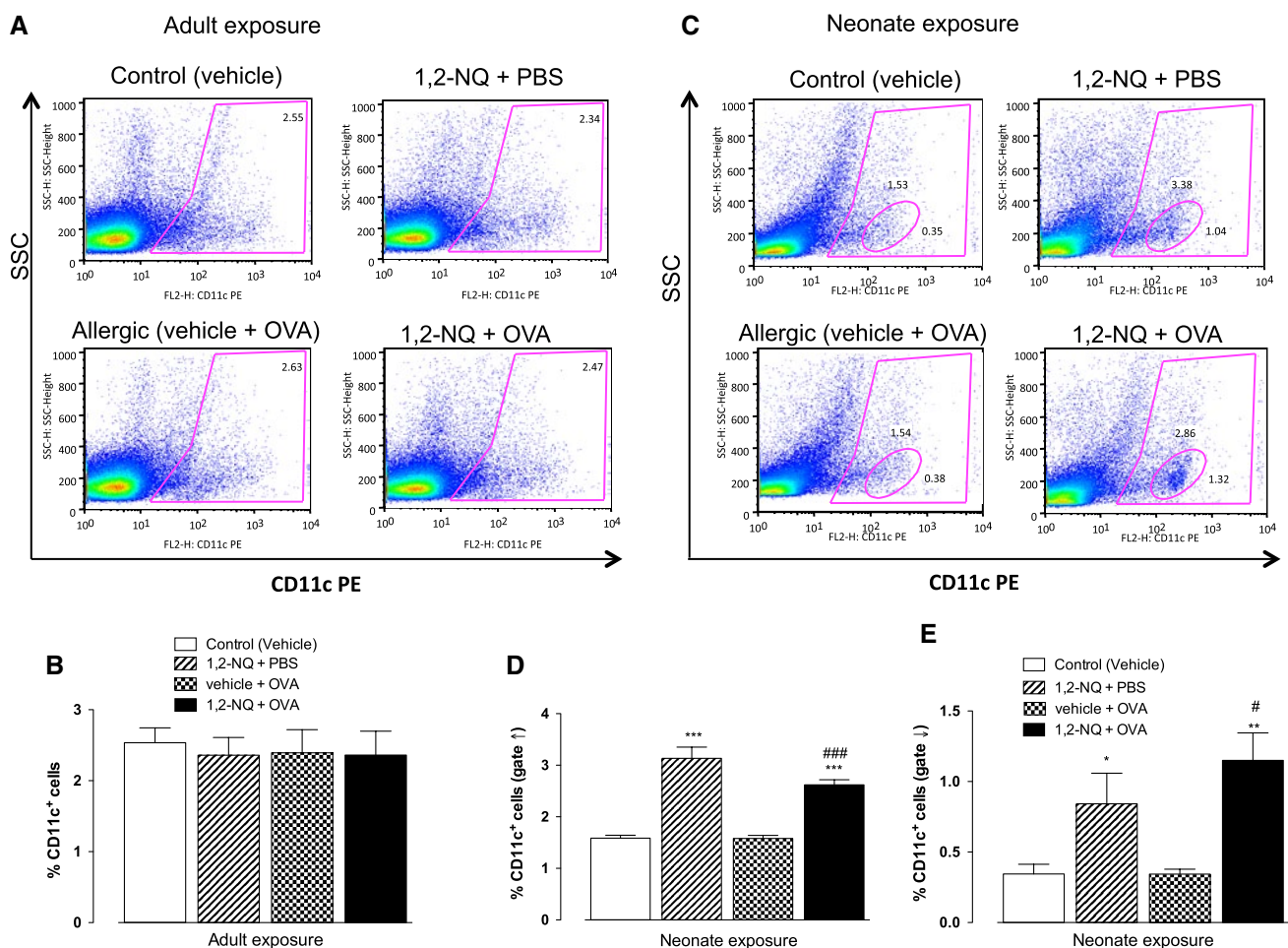


Fig. 5 Effects of adulthood and postnatal exposure of mice to 1,2-NQ on CD11c⁺ presentation in response to OVA challenge. **a, c** illustrate the scattergrams of CD11c⁺ cell population (SSC or percentage value) obtained from splenocytes of mice exposed to 1,2-NQ or vehicle at adulthood or postnatal period, respectively; **b** and **d** show the analysis at high gates of the scattergrams shown in **a, c**; and **e** the analysis of **c** scattergram at low gate. Data are expressed as

mean $\pm$ SEM for n animals ($n = 5-6$, $n = 6-6$, $n = 6-6$ and $n = 6-7$; for vehicle, 1,2-NQ + PBS, vehicle + OVA and OVA + 1,2-NQ, for adult and neonate protocols, respectively). * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ when compared with the respective control group (vehicle). # $P < 0.05$ and ### $P < 0.001$ when compared with the respective allergic group (vehicle plus OVA)

production. We here provide evidence, for the first time, that the short exposure of mice to 1,2-NQ during the neonatal period, and to a lesser extent during adulthood, positively regulates an active and more powerful recruitment of eosinophils (but not neutrophils) from the bone marrow to the airways upon a second contact with a specific antigen (e.g., OVA). Likewise, the exposure of adult mice to DEP did not induce asthma, but enhanced neutrophilia and worsened the experimental allergic asthma phenotype, which was characterized by increased concentrations of Th17/Th2 cytokines and pulmonary eosinophilia (Brandt et al., 2013). These findings are in agreement with epidemiological evidences that show that susceptible individuals (e.g., infants) living in busy traffic areas, and consequently exposed to high levels of DEP (and possibly its extractable chemical 1,2-NQ), are at greater risks for the development of airway diseases.

These results led us to further evaluate the role of cells and genes relevant to the humoral and innate immune response in this model.

Under a range of physiological and pathophysiological stimuli, it is known that CD4⁺ T lymphocytes can be differentiated at least into four lineages of T helper (Th) phenotypes, Th1, Th2, Treg and Th17, which exert distinct biological functions related to either protection against intracellular pathogens or allergic inflammatory diseases (e.g., allergic asthma). While allergic asthma is mainly driven by Th2 lymphocytes, the expansion of Th17 cells, and the consequent production of IL-17A among others, has been linked to a neutrophilic form of asthma (Cosmi et al. 2011) and contributes to DEP-mediated exacerbation of severe asthma symptoms in children (Brandt et al., 2013).

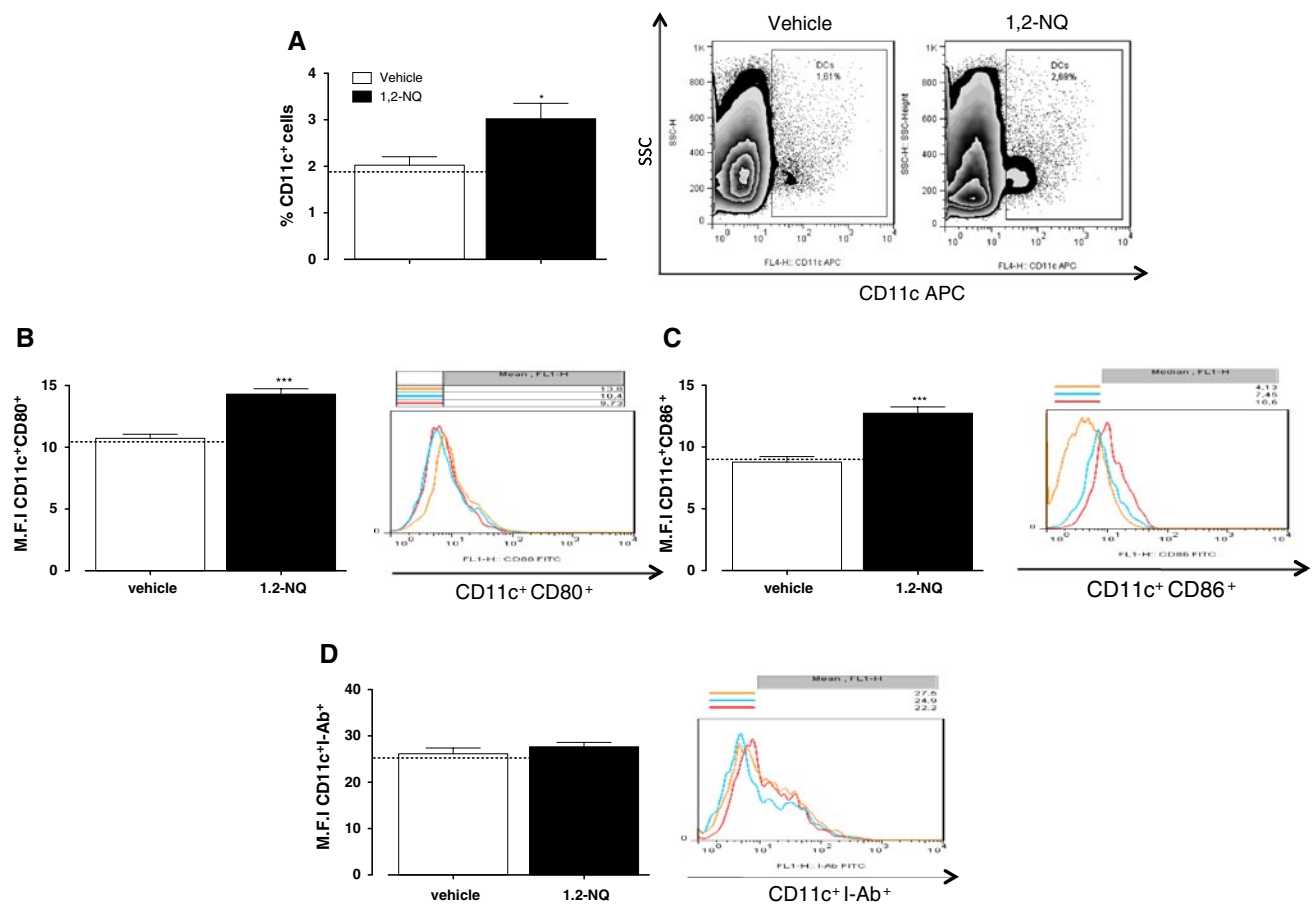


Fig. 6 Effects of 1,2-NQ on CD11c⁺ presentation and expression of co-stimulatory molecules in spleen cell suspension from neonate mice. **a** illustrates CD11c⁺ cell population (SSC or percentage value) and mean fluorescence intensity (MFI) of CD11c⁺CD80⁺ (**b**), CD11c⁺CD86⁺ (**c**) and CD11c⁺I-Ab⁺ cells (**d**). Data are expressed

as mean $\pm$ SEM for n animals ($n = 6-7$ and $n = 6-7$; for vehicle and 1,2-NQ, respectively). * $P < 0.05$ and *** $P < 0.001$ compared with the respective control group (vehicle). *Right panels* illustrate the respective histograms. *Dashed lines* indicate splenocyte MFI obtained from naïve mice

In order to investigate whether the postnatal contact with 1,2-NQ could induce exacerbation of allergic asthma via, at least in part, increased production of IL-17 in T CD4⁺ cells, we made use of isolated mice spleen cells, since that cDC subset is highly expressed in this organ. In the mice lung, beside DC subsets, there are heterogeneous cell population, thus aggravating the ability to prepare pure populations of lung APCs (Maldonado-López and Moser, 2001; Inoue et al. 2009a,b; 2010). In keeping with this, the present study shows that the short exposure of neonate mice to 1,2-NQ *per se* did not lead to CD4⁺ T cell amplification or CD4⁺/IL-17 increased population in the splenocytes; however, it seems that 1,2-NQ may act as a nonspecific priming agent to these cells, which under re-stimulation with the nonspecific lymphocyte activator agents PMA and ionomycin resulted in markedly increased levels of both Th17-associated IL-17 and Th1-associated IFN- γ . Besides lacking significant effects on CD4⁺ T cell population in the splenocytes during the postnatal period, mice exposed to

1,2-NQ at neonatal period exhibited a marked neutrophilia by adulthood; however, additional antigen stimuli did not exacerbate neutrophil counts (Supplemental Table S1A and B), as clearly seen for the eosinophilia in either the airways or bone marrow. Also, the exposure to 1,2-NQ during the neonatal period did not change Th2 CD4⁺ cell population in mice splenocytes; however, the re-stimulation with OVA, but not with PMA and ionomycin, led to a marked increase in the Th2-associated cytokine IL-4, thus suggesting that DCs are activated and prone to polarize the T cell response. Together, these findings support the concept that early contact with 1,2-NQ acts as a CD4⁺ T cells-sensitizing/priming agent, after which a further Th2-derived stimulus can lead to the induction of CD4⁺ lymphocyte maturation and differentiation of CD4⁺/IFN- γ , CD4⁺/IL-4 and CD4⁺/IL-17, thus inducing a mixed Th1/Th2 and Th17 response, which as a whole contributes to increased susceptibility to the allergic inflammatory response. In agreement with this, Inoue et al. (2009b) showed that exposure

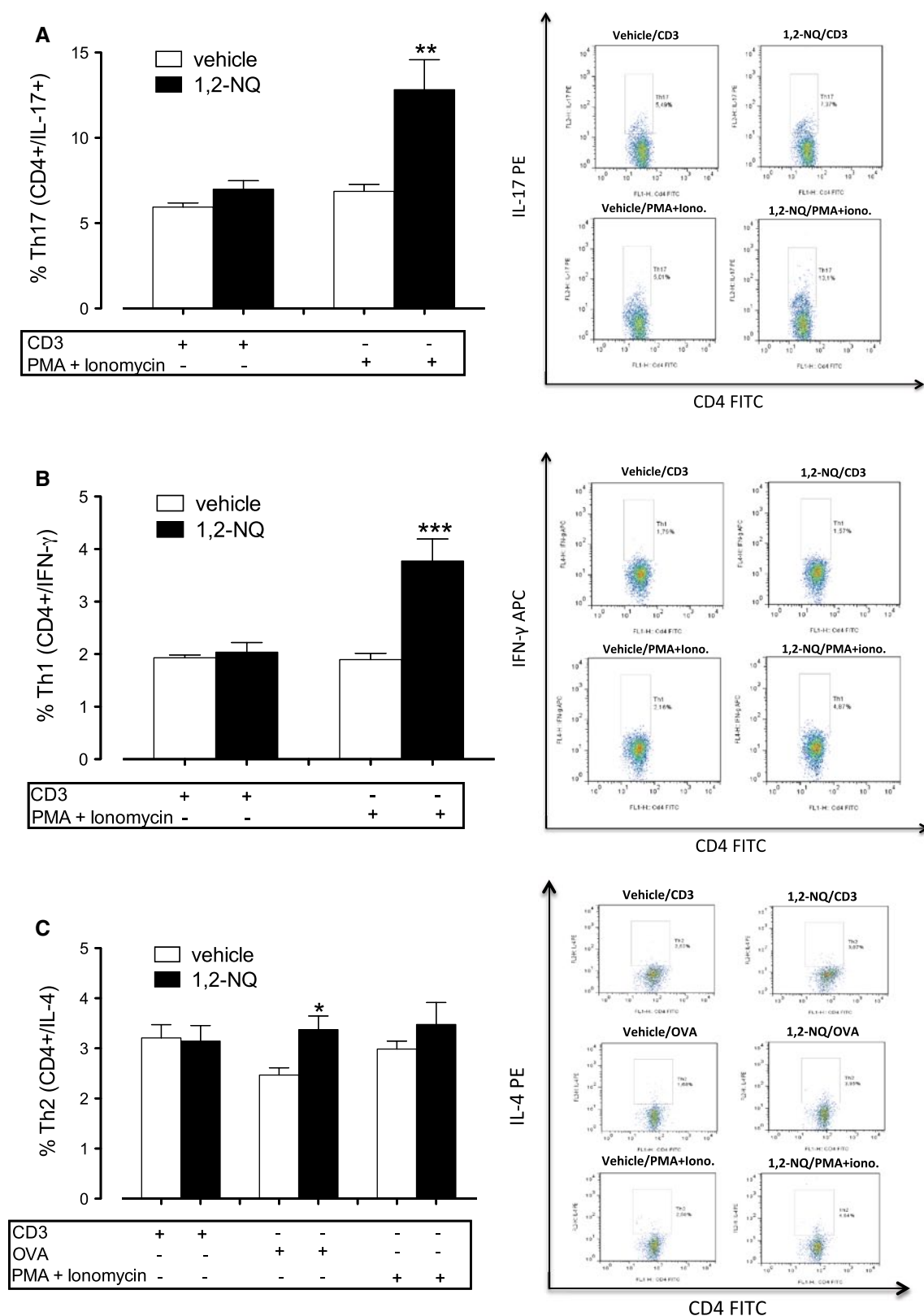


Fig. 7 Effects of postnatal exposure to 1,2-NQ on T lymphocyte subsets (as cell population percentage values and scattergrams). Splenocytes were stimulated in vitro with CD3, PMA + ionomycin or OVA 24 h after last 1,2-NQ (or vehicle) exposure. **a:** Th17 CD4⁺/IL-17⁺

phenotype, **b:** Th1 CD4⁺/IFN-γ⁺ phenotype, **c:** Th2 CD4⁺/IL-4⁺ phenotype. Data are expressed as mean ± SEM for *n* animals (*n* = 7 and *n* = 7; for vehicle, 1,2-NQ, respectively). **P* < 0.05, ***P* < 0.01 and ****P* < 0.001 compared with the respective control group (vehicle)

of adult mice to DEP failed to activate CD4⁺ T cells in spleen-derived DCs, although a prominent cell increase was observed after OVA challenge. Also, Lee et al. (2002) showed that CD4⁺ T spleen cells from adult BALB/c mice immunized with *keyhole limpet hemocyanin* exhibit high expression of IL-4 and IgE following hydroquinone treatment in vitro, as well as markedly increased concentrations of IgE and IL-4.

In parallel with Th2-associated cytokine IL-4 in splenocytes from mice treated with 1,2-NQ and stimulated with OVA in vitro, we showed a significant production of IL-13 and concomitantly eosinophilia in the airways of mice exposed to 1,2-NQ during the neonatal phase and later challenged with antigen in vivo, thus reinforcing the relevance of the Th2 subset cells in our model. Noteworthy, a marked concentration of IL-10 in the airways of mice exposed to 1,2-NQ during adulthood was seen in our study, which may contribute to the weak inflammatory response observed in these animals. Inoue et al. (2007a) showed that the i.t. injection of allergic adult mice with 1,2-NQ also evoked eosinophilia, increased leukocyte influx and high levels of IL-4 and IL-5 in the airways.

As well known, DC modulation is one of the mechanisms involved in PM-induced Th2 polarization (Provoost et al. 2012). Indeed, the development of either Th1 or Th2 immunity is highly dependent on DCs and its co-stimulatory molecules CD80/86. Most of the tissues contain immature DCs, which can be directly activated and matured by pathogens or indirectly by inflammatory cytokines. Once mature, DCs act as one of the most potent antigen-presenting cells to prime naïve T cells against antigens (Guermonprez et al. 2002). Since activation of naïve T cells and primed T cells is distinctly influenced by the availability of co-stimulatory molecules, we have furthered this research to find out whether DCs provided co-stimulatory molecules are recruited in the shift of Th-Th2 effector responses upon early contact with 1,2-NQ, and whether differences would appear for co-stimulatory molecules after exposure to 1,2-NQ in the absence (priming phase of naïve T cells) and presence of antigen (primed cells switch to effector T cells).

Here, we found that CD80/86-CD11c⁺ co-stimulation is required by DCs from neonate mouse splenocytes for efficient 1,2-NQ priming. Furthermore, increased percentage of CD11c⁺ expression is also seen in splenocytes of mice exposed to 1,2-NQ at neonatal period and later to antigen. These results corroborate the antigen-induced exacerbated eosinophilia in both the airways and bone marrow of mice exposed to 1,2-NQ at neonatal period. Independently of concomitant eosinophilia in the mice airway, the early exposure to 1,2-NQ *per se* induced marked up-regulation of two cell surface CD11c⁺ populations (high and low gate) when compared to vehicle- and antigen-treated mice.

Supporting these data, others showed that DEP activates DCs and leads to high levels of Th2 cytokines, possibly via T CD4⁺ cell activation in mice spleen (splenocytes), thus reinforcing the role of extra-thoracic lymphoid organs as the main target for ambient pollutants (Porter et al. 2007; Inoue et al. 2009a, b, 2010; Braun et al. 2010).

It is well known that chronic and acute lung diseases are mainly characterized by leukocyte influx (eosinophilia) in the airways together with elevated concentrations of inflammatory mediators, such as histamine and cysteinyl leukotrienes (cys-LTs), being the latter lipid mediators involved in smooth muscle constriction and inflammation. The biological functions of LTs are regulated by widely expressed receptors, including mast cells and granulocytes (Lei et al. 2013). Increased serum concentration of LTE₄ has been positively associated with asthmatic schoolchildren living in areas with high concentration of particulate air pollution (Delfino et al. 2009). Interestingly, in our study, an increased production of LTB₄ was measured in the BAL of mice exposed to 1,2-NQ at neonatal period, but not at adulthood. After further contact with antigen, LTB₄ levels in these animals were significantly increased as compared to its allergic matched group, and this increase was parallel to augmented humoral response (IgE) and IL-13 production, known as critical cytokine for eosinophilic inflammation, and possibly released from mast cells (Grunig et al. 1998).

Upon sensitization, mast cells are activated through FcεRI induced by allergen inhalation challenge. This can lead to mast cells' release of stored inflammatory mediators (e.g., histamine) along with serine proteases and products capable of generating newly formed mediators (e.g., LTs, IL-13), which in turn may act on airway smooth muscle leading to AHR or bronchoconstriction.

AHR refers to increased sensitivity of asthmatics to non-specific inhaled stimuli, such as pollution. In fact, increasing ambient pollution has been associated with worsening asthma signs and symptoms (Matsumoto et al. 2006; Gehring et al. 2010). However, exposure to 1,2-NQ either at neonatal period or during adulthood evoked a partial degranulation of mast cell population in the lung parenchyma, without affecting cell population. While further exposure to antigen led to complete degranulation of mast cells in the airways of mice exposed to 1,2-NQ as adult, the antigen challenge in mice exposed to 1,2-NQ during neonatal period still partially increased mast cell degranulation. From these results, we can speculate that mast cell contents (or population) in the airways do not play an important role in the increased susceptibility of mice exposed to 1,2-NQ during the neonatal period to allergic asthma; however, whether 1,2-NQ can directly stimulate mast cells is unknown.

Mast cells express different receptor types, including tachykinin NK1, a receptor for substance P (Krumins and

Broomfield 1992). We showed that DEP- and 1,2-NQ-induced inflammation in the rat airways is greatly inhibited by NK1 receptor antagonist, thus indicating a neurogenic-mediated mechanism (Teles et al. 2010). Consistent with this, Kikuno et al. (2006) showed that 1,2-NQ causes guinea pig trachea contraction mediated by a TRP V1 receptor (which is abundantly expressed on C nerve fibers). Pulmonary neutrophil recruitment and bronchial reactivity in rats exposed to formaldehyde, another ambient pollutant, are modulated by a complex mechanism involving mast cells and neuropeptides (Lino dos Santos Franco et al. 2006). We can, thus, infer that 1,2-NQ promotes substance P release by direct stimulation of C nerve fibers, and as a result, substance P acts on its NK1 receptors on mast cells, among other cell types.

It is arguable that a close relationship between AHR, inflammation (and markers) and lung function exists, as these factors vary according to the inflammatory process. In keeping with this, variation in AHR has been shown among mice and humans and, even, between mice strains. It can also be affected by other factors rather than only local airway inflammation, such as smooth muscle responses, neuronal regulation, local concentration of mediators, mast cell distribution and hereditary factors (Ewart et al. 1996; Becker et al. 2011). Mast cells are generally less abundant in the mouse airways than in the trachea or large bronchi, and thus, the animals may exhibit either low or high reactivity to MCh in the naïve state (De Sanctis et al. 1999; Noguchi et al. 2005; see review Lei et al. 2013).

We showed here that MCh challenge caused a marked and dose-dependent increase in Penh in allergic mice, indicating the presence of AHR, as seen in asthmatics. Neither neonate nor adult mice exposure to 1,2-NQ increased *per se* MCh-induced AHR (Penh) as compared to its respective vehicle or allergic group. When Penh was evaluated as AUC, a reduced trend for MCh-induced AHR was noticed in mice exposed to 1,2-NQ at neonatal period and challenged with antigen. Our results indicate that exacerbation of eosinophilic influx in mice airways exposed to 1,2-NQ at neonatal period occurs independently of AHR. Supporting our findings, Rosi et al. (1999) suggest that eosinophilia in BAL can be independently regulated by, but closely inter-related to, AHR. Moreover, controversial studies show that both mast cells do not contribute to induce AHR (Foresi et al. 1997; Becker et al. 2011) or support the concept that they are necessary (Tsai et al. 2005).

Our findings provide evidence that the short exposure of mice to ambient pollutant 1,2-NQ during early life (post-natal development), and to a lesser extent later (during adulthood), increases the susceptibility to allergic pulmonary inflammation, without affecting body weight, a common side effect of chemical exposure during perinatal and postnatal development. The increase in CD11c⁺ population

and DC co-stimulatory molecules CD80 and CD86 herein suggests that the underlying mechanism by which 1,2-NQ promotes allergic inflammatory exacerbation is through the activation of APC cells, which leads to cell presentation amplification, Th2 polarization and increased pulmonary production of cytokines, LTB₄ and humoral response (IgE). Thus, both CD80 and CD86 co-stimulatory molecules may act as potential targets in exacerbated pulmonary allergic diseases related to the ambient pollutant 1,2-NQ. These findings may be useful for future investigations focused on the increased pulmonary illnesses observed in children living in heavily polluted areas.

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Conflict of interest None.

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