SUPPLEMENTAL MATERIAL

The Adjuvant Effect of Ambient Particulate Matter Is Closely Reflected by The Particulate Oxidant Potential

Ning Li,[1] Meiying Wang,[1,2] Lori A. Bramble,[3] Debra A. Schmitz,[2] James J. Schauer,[4] Constantinos Sioutas,[2,5] Jack R. Harkema,[2,3] Andre E. Nel,[1,2,§]

[1]Division of NanoMedicine, Department of Medicine, University of California, Los Angeles, CA; [2]Southern California Particle Center, University of California, Los Angeles, CA; [3]Department of Pathobiology and Diagnostic Investigation, College of Veterinary Medicine, Michigan State University, East Lansing, MI; [4]Department of Civil and Environmental Engineering at the University of Wisconsin-Madison [5]Department of Civil and Environmental Engineering, University of Southern California, Los Angeles, CA;

[§]Reprint requests: Andre E. Nel, MD, PhD, Division of NanoMedicine, Department of Medicine, University of California, 10833 Le Conte Ave., 52-175 CHS, Los Angeles, CA 90095. Phone: (310) 825-6620, fax: (310) 206-8107, e-mail: anel@mednet.ucla.edu.

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Materials and Methods

Reagents

Limulus amebocyte lysate (LAL) assay kit for endotoxin detection and endotoxin removing gel column were obtained from Lonza (Walkerville, MD) and Pierce (Rockford, IL) respectively. Ovalbumin (grade V), N-acetyl cysteine (NAC) and dithiothreitol were from Sigma-Aldrich (St. Louis, MO). Carbon black particles were purchased from Degussa (Hanau, Germany). Ketamine and xylazine were purchased from Phoenix Pharmaceutical, Inc. (St. Joseph, MO). Schuco 2000 nebulizer was from Allied Health Care Products (St Louis, MO). VectaStain ABC-AP kit was obtained from Vector Laboratories (Burlingame, CA). Cytometric Bead Array (CBA) Mouse Inflammation Kit was from BD Biosciences (San Diego, CA). FACScalibur for cytometric bead array and BD CBA Analysis Software were also from BD Bioscience (San Jose, CA).

Detection of endotoxin levels in the CAPs

Endotoxin levels in the CAPs were analyzed using the LAL assay kit according to manufacturer's instructions.

Preparation of endotoxin-free OVA

Preparation of endotoxin-free OVA was performed using endotoxin removing gel columns following manufacturer's instructions**.** After the gel column, the endotoxin level in the OVA solution (33 mg OVA/ml) was 0.1 u/ml as determined by the LAL assay.

Nasal and lung tissue preparation for morphometric analysesand immunohistochemistry

After lung lavage and removal of the right lung lobes, the left lung lobe was intratracheally perfused with 10% neutal buffered formalin at a constant pressure of 30 cm of fixative. After 1 hour, the trachea was ligated, and the inflated left lung lobe was immersed in a large volume of the same fixative for at least 24 hours. After fixation, the left lung was microdissected along the main axial airway, and two sections were than excised at the leve lof the fifth (proximal) and eleventh (distal) airway generation (Fig E1), as has been described previously in detail (Farraj et al. 2003). Five-micron thick paraffin sections were histochemically stained with hematoxylin and eosin for routine histopathologic examination or Alcian blue (pH2.5) and periodic acid Schiff's (AB/PAS) reagent for neutral and acidic mucosubstances stored in mucous goblet cells of surface epithelium lining the proximal and distal bronchiolar airways. Other paraffin sections from these same pulmonary sites were stained separately for major basic protein to identify eosinophils (further described below). The head of each mouse was also removed from the carcass immediately after death. After the lower jaw, skin and musculature were removed from the head, it was immersed in 10% neutral buffered formalin for at least 24 hours. After fixation, the heads were decalcified in 13% formic acid for 7 days and then rinsed in tap water for at least 4 hours. The nasal cavity of each mouse was transversely sectioned at three specific anatomic locations according to a modified method of Young that we have previously described (Farraj et al. 2003; Young 1981). Dental and hard palate landmarks were used to locate these tissue sections. The most proximal nasal section (T1) was taken immediately posterior to the upper incisor teeth; the middle section (T2) was taken at the level of the incisive papilla of the hard palate; the most distal nasal section (T3) was taken at the level of the second palatal ridge (Fig

E1). Tissue sections were embedded in paraffin, at a thickness of five microns, and stained like that of the lung sections, described above.

Morphometry of mucosusbstances and eosinophils in nasal and pulmonary airways

The amount of stored mucosubstances in the surface epithelium lining the maxilloturbinates in the proximal nasal section T1 and lining the proximal and distal axial airways in the lung, airway generations 5 and 11 respectively, were estimated by measuring the volume densities of AB/PAS-stained mucosubstances using computerized image analysis and standard morphometric techniques previously reported (Farraj et al. 2003; Harkema et al. 1997). The area of AB/PAS-stained mucosubstances was calculated by circumscribing the perimeter of the stained material using the Scion Image program (Scion Corporation, Frederick, MD). The length of the basal lamina underlying the surface epithelium was calculated from the contour length of the digitized image of the basal lamina. The volume of stored mucosubstances (volume density, Vs) per unit of surface area, a morphometric indicator of mucous cell metaplasia, was estimated using the method described in detail by harkemaj et al. (Harkema et al. 1987). Nasal and pulmonary tissue sections (nasal T1, left lung proximal axial airway G5, and left lung distal axial airways) were immunohistochemically stained with a polyclonal rabbit antibody directed against mouse eosinophil-specific major basic protein (MBP; generously provided by Dr. Jamie Lee of the Mayo Clinic of Scottsdale, AZ). Unstained hydrated paraffin sections were incubated with a blocking solution from Vectastain ABC-AP kit following manufacturer's instruction. The sections were then incubated with the MBP antibody followed by treatment with a secondary antibody Vectastain ABC-AP Kit anti-rabbit IgG. Some tissue sections were incubated without the secondary antibody to check for nonspecific binding. Immunoreactive MBP was

microscopically detected after treatment with Vectastain ABC-AP complex followed by Vector Red AP substrate solution. Eosinophils in the airway walls (epithelium and subepithelial interstitium) were identified by their morphologic features (ie., slightly larger than neutrophils with bilobed nuclei) and by positive immunohistochemical staining for MBP in the large cytoplasmic granules (dark red stain). Numeric cell densities for these leukocytes were determined by dividing the total number of intramural eosinophils by the length of the corresponding subepithelial basal lamina. The length of the basal lamina was determined as previously described for the determination of intraepithelial mucosubstances.

Particle chemical analysis

Fine (PM2.5, < 2.5 μm), and ultrafine (PM0.15, < 0.15 μm) particles were collected near a major in downtown Los Angeles by the Versatile Aerosol Concentration Enrichment System (VACES), as follows. The VACES uses two parallel sampling lines (concentrators) to simultaneously concentrate fine and UFP particles from a flow rate of 120 liters per minute (lpm) into a liquid impinger (BioSampler™, SKC West Inc., Fullerton, CA) at 5 lpm. Particles are injected into the BioSampler in a swirling flow pattern so that they can be collected by a combination of inertial and centrifugal forces. This inertia-based collection mechanism, coupled with the short residence time on the order of 0.2 s for particles and gases in the Biosampler precludes any inadvertent trapping of gaseous copollutants in the particulate layer. In each sampling line of the concentrator, PM2.5 and PM0.15 were concentrated from a flow of 120 lpm to a flow of 6 lpm, thereby, being enriched by a factor of 20. From the 6 lpm of concentrated flows samples, 4 lpm was drawn through the BioSampler connected to the respective minor flow, while 2 lpm passed through diffusion dryer for PM2.5 and PM0.15 only to remove excess water and dry the aerosol.

The dry concentrated aerosol flow was then split into two equal halves of 1 lpm, each diverted into a filter sampler consisting of either a 47- mm Teflon filter (2-mm pore PTFE; Gelman Science, Ann Arbor, MI) or a 47mm prebaked quartz filter (Pallflex Corp., Putnam, CT). The PTFE filters were used to determine particle mass, whereas quartz filters were used to determine the PM content of elemental and organic carbon (EC-OC), inorganic ions, and PAHs. For measurement of mass concentrations, the PTFE filters were weighed before and after each field test using a Mettler 5 Microbalance (MT 5, Mettler-Toledo Inc., Highstown, NJ), under controlled relative humidity (40–45%) and temperature (22–24 °C) conditions. At the end of each experiment, filters were stored in the control humidity and temperature room for 24 h prior to weighing to ensure removal of particle-bound water. The quartz filters were cut into two unequal parts, 1/4 and 3/4 of the total filter. The smaller piece was analyzed by means of ion chromatography to determine particle-bound sulfate and nitrate concentrations. A small area (1 cm^2) of the remaining filter (3/4) was removed to determine the EC and OC content of PM. EC and OC were determined using a thermal optical transmittance method as specified in NIOSH method 5040 (Birch ME 1996). The remaining portion from the filter above was used to determine the concentrations of PAH using procedures described elsewhere (Sheesley RJ 2003). In brief, the filters corresponding to each size range were ultrasonically extracted with dichloromethane and the PAH content of the dichloromethane extract was analyzed by high-pressure liquid chromatography fluorescence using NIST SRM 1649a as the positive control.

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References

Birch ME CR. 1996. Elemental carbon-based method for monitoring occupational exposures to particulate diesel exhaust. Aerosol Sci Technol 25:221-241.

Farraj AK, Harkema JR, Jan TR, Kaminski NE. 2003. Immune responses in the lung and local lymph node of A/J mice to intranasal sensitization and challenge with adjuvant-free ovalbumin. Toxicol Pathol 31(4):432-447.

Harkema JR, Hotchkiss JA, Griffith WC. 1997. Mucous cell metaplasia in rat nasal epithelium after a 20-month exposure to ozone: a morphometric study of epithelial differentiation. Am J Respir Cell Mol Biol 16(5):521-530.

Harkema JR, Plopper CG, Hyde DM, St George JA, Dungworth DL. 1987. Effects of an ambient level of ozone on primate nasal epithelial mucosubstances. Quantitative histochemistry. Am J Pathol 127(1):90-96.

Sheesley RJ SJ, Chowdhury Z, Cass GR, Simoneit BRT. 2003. Characterization of organic aerosols emitted from the combustion of biomass indigenous to South Asia. J Geophysical Research-Atmosphere 108:4285.

Young JT. 1981. Histopathologic examination of the rat nasal cavity. Fundam Appl Toxicol 1(4):309-312.

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Supplemental Material, Table 1. Endotoxin levels in PM collections

PM sample	Endotoxin (u/ml)
F/UF#1	0.504
UF#1	0.178
UF#2	0.201

Endotoxin level in each PM sample was determined using the LAL assay according to manufacturer's instruction.

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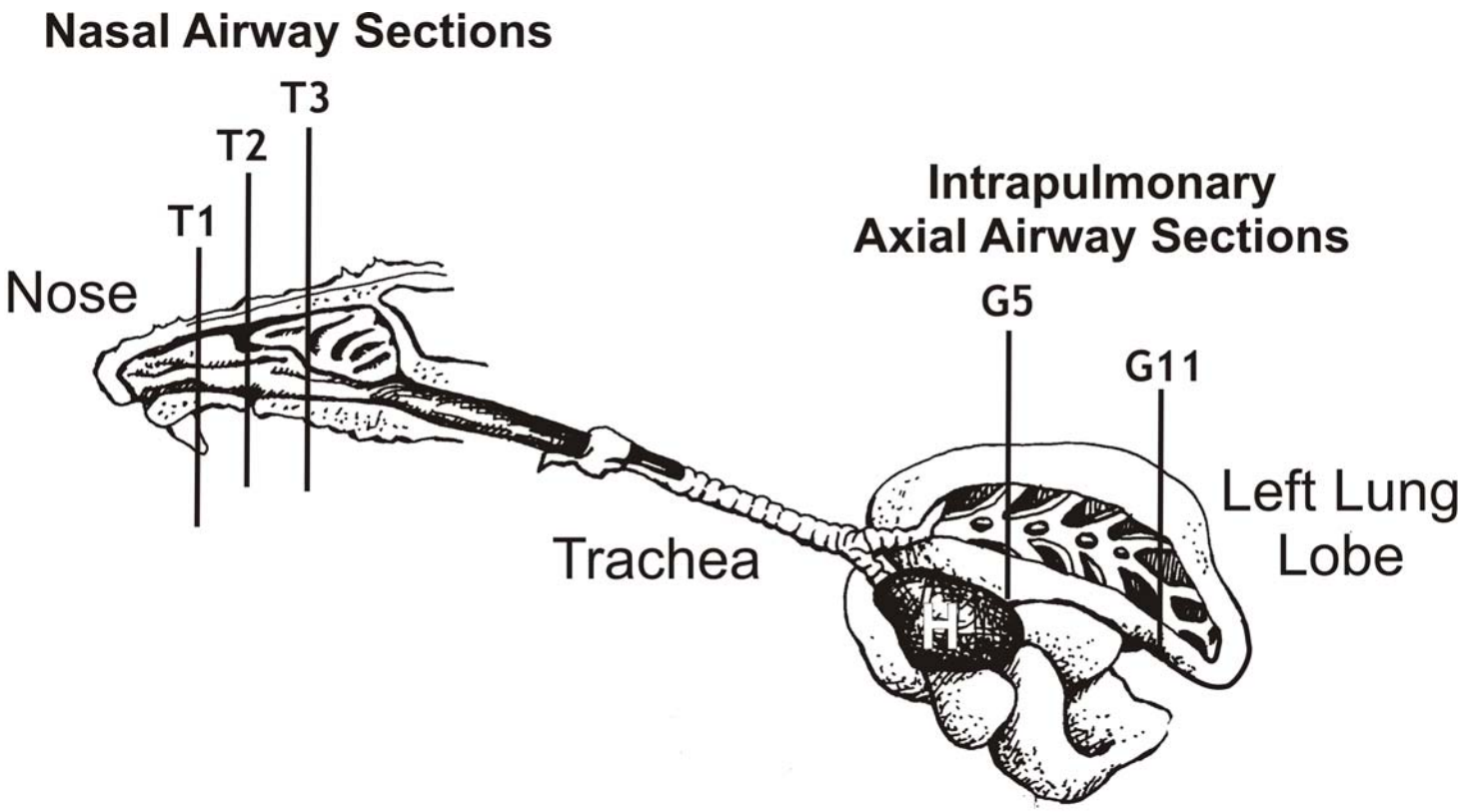
Figure Legends

Supplemental Material, Figure 1. Sites of airway tissue selection for microscopic examination. This diagram depicts the lateral wall of one nasal passage with the septum removed and the main axial airway of the left lung lobe. Transverse sections of the nose were taken from the proximal (T1), middle (T2), and distal (T3) nasal airways. Sections of the left lung were taken at the level of the proximal (generation 5, G5) and distal (generation 11, G11) axial airway. Morphometric analyses were conducted on the surface epithelium lining the maxilloturbinates in the proximal nasal airway (T1) and the proximal and distal axial airways (G5 and G11) in the left lung lobe.

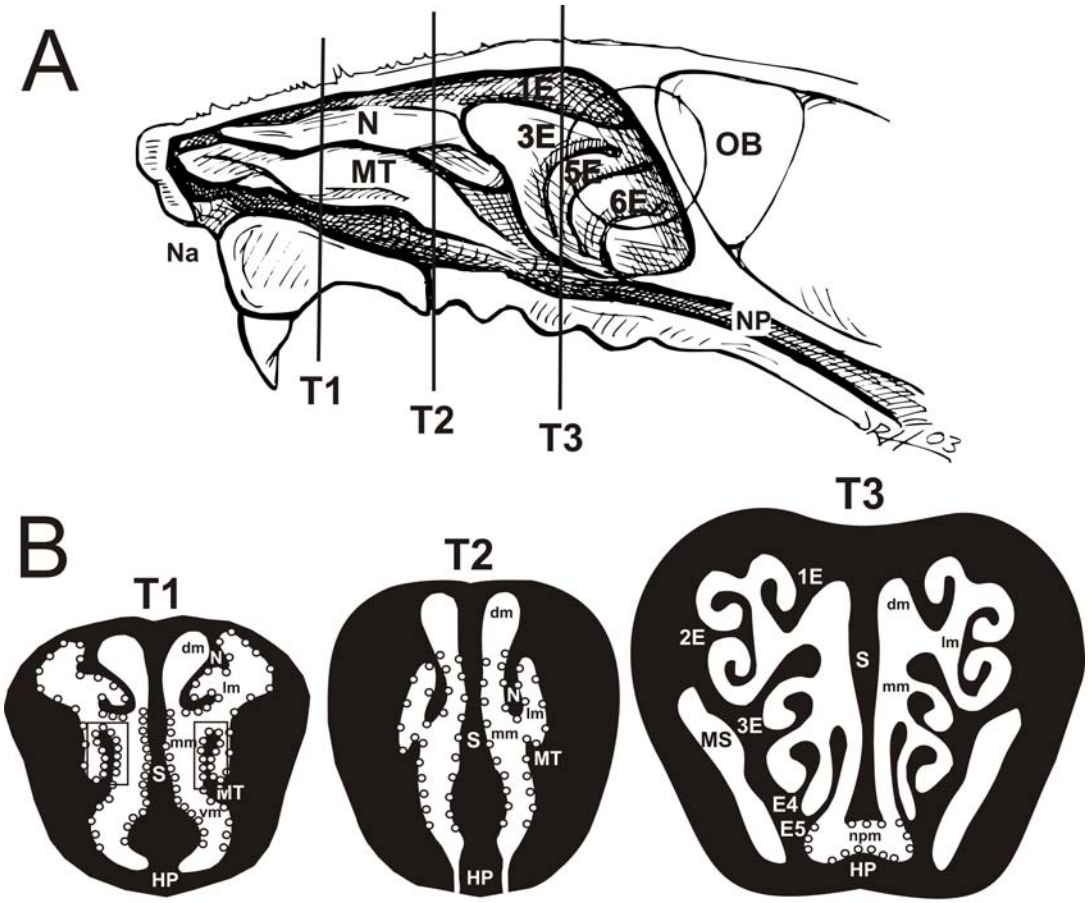
Supplemental Material, Figure 2. Intranasal distribution of OVA/UFP-induced allergic inflammation. A. Diagrammatic representation of the lateral wall of the right nasal passage of the mouse (septum removed), illustrating the location from which transverse tissue sections (T1-3) were taken for light microscopic examination and morphometric analysis. B. Anterior surface profiles of the nasal tissue sections (T1-3) with the intranasal locations of the histopathologic changes (mucosal inflammation and mucous cell metaplasia) found only in mice treated with ovalbumin plus ultrafine particles. Na, naris; N, nasoturbinate; MT, maxilloturbinate; Box around MT in T1 identifies site selected for morphometric analyses; S, septum; 1-6E, ethmoturbinates; OB, olfactory bulb of the brain; NP, nasopharynx; HP, hard palate; MS; maxillary sinus; dm, dorsomedial meatus (airway); mm, middle meatus; lm, lateral meatus; vm; ventral meatus; npm, nasopharyngeal meatus.

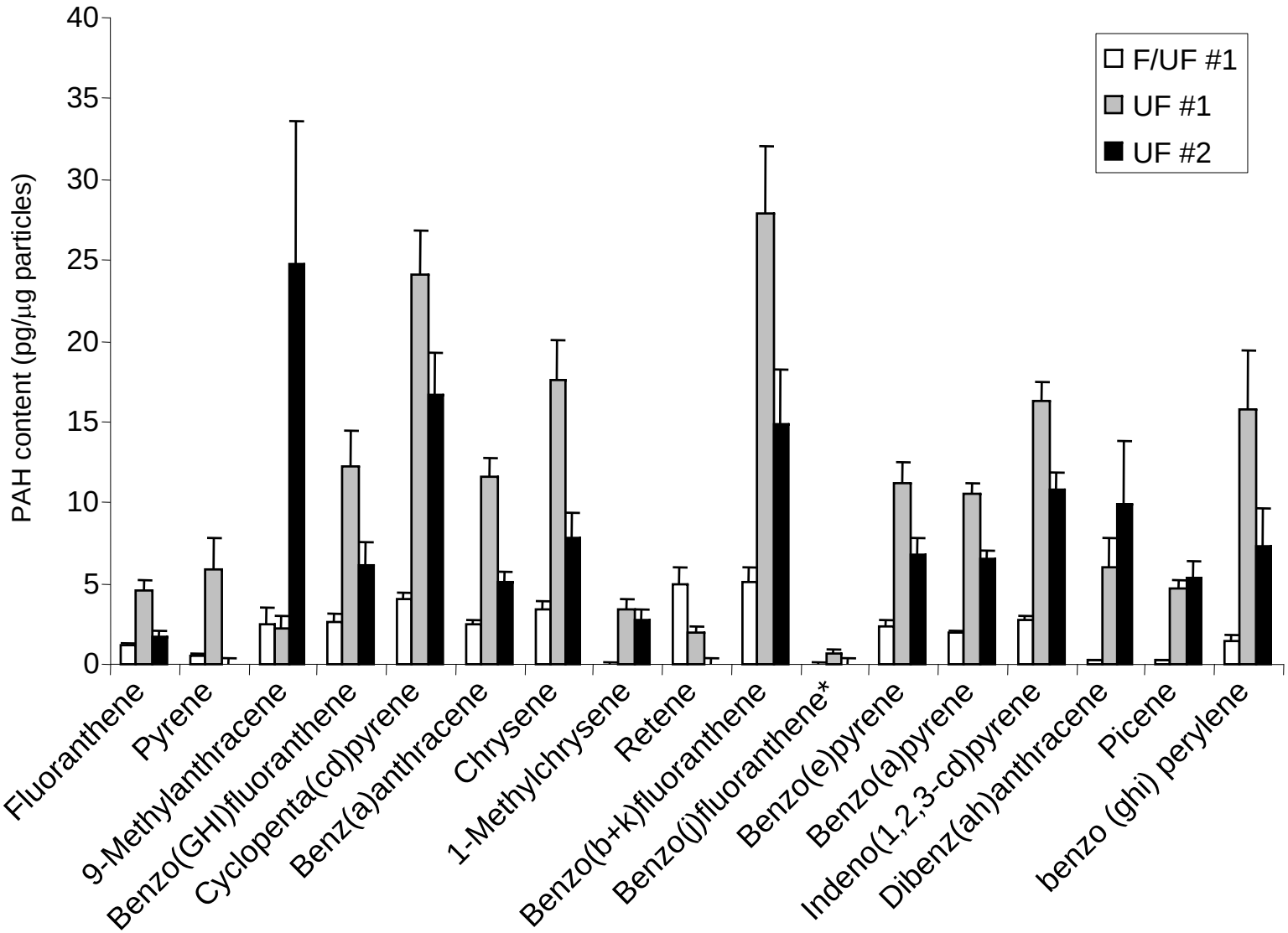
Supplemental Material, Figure 3. Analysis of PAH content in the sub-2.5 μm collection (F/UF#1) and ultrafine particles (UF#1 and UF#2). Seventeen signature PAHs representative of combustion sources were analyzed as described in the Materials and Methods in the Supplemental Material.

Supplemental Material, Figure 1



Supplemental Material, Figure 2



○ = Distribution of OVA/UF-induced inflammation and mucous cell metaplasia in the mucosa lining the nasal airways

Supplemental Material, Figure 3