Fingerprint of Lung Fluid Ultrafine Particles, a Novel Marker of Acute Lung Inflammation

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UFP D_{50} of 23.7 nm for resting BALF (p < 0.0001). This UFP profile was highly reproducible and independent of the intensity or duration of the inflammatory trigger. It returned to baseline after resolution of the inflammation. Neither total body irradiation nor induction of acute cough induced this fingerprint.

Conclusions: The UFP fingerprint in the BALF of resting and inflamed lungs can serve as a binary biomarker of healthy and acutely inflamed lungs. This marker can be used as a novel readout for the onset of inflammatory lung diseases and for complete lung recovery from different insults.

Key Words
Nanotechnology · Ultrafine particles · Lung inflammation · Animal model · Biomarkers

Abstract
Background: Acute lung inflammation can be monitored by various biochemical readouts of bronchoalveolar lavage fluid (BALF). Objective: To analyze the BALF content of ultrafine particles (UFP; <100 nm) as an inflammatory biomarker in early diagnosis of acute and chronic lung diseases. Methods: Mice were exposed to different stress conditions and inflammatory insults (acute lipopolysaccharide inhalation, tobacco smoke and lethal dose of total body irradiation, i.e. 950 rad). After centrifugation, the cellular pellet was assessed while cytokines and ultrafine particles were measured in the soluble fraction of the BALF. Results: A characteristic UFP distribution with a D_{50} (i.e. the dimension of the 50th UFP percentile) was shared by all tested mouse strains in the BALF of resting lungs. All tested inflammatory insults similarly shifted this size distribution, resulting in a unique UFP fingerprint with an averaged D_{50} of 58.6 nm, compared with the mean UFP D_{50} of 23.7 nm for resting BALF (p < 0.0001). This UFP profile was highly reproducible and independent of the intensity or duration of the inflammatory trigger. It returned to baseline after resolution of the inflammation. Neither total body irradiation nor induction of acute cough induced this fingerprint. Conclusions: The UFP fingerprint in the BALF of resting and inflamed lungs can serve as a binary biomarker of healthy and acutely inflamed lungs. This marker can be used as a novel readout for the onset of inflammatory lung diseases and for complete lung recovery from different insults.

Introduction

Acute and chronic lung inflammatory diseases are very common pathologies whose morbidity and mortality are increasing globally, placing chronic obstructive pulmonary disease (COPD), lower respiratory infections and lung cancer among the 5 most common causes of death worldwide [1]. The association between these dis-
eases and the inhalation of noxious substances, such as cigarette smoke, environmental and occupational particles and different infectious agents, is well known [2, 3]. New technologies for monitoring lung diseases by specific biomarkers of lung inflammation may offer significant benefits for early diagnosis and for better understanding of the pathogenesis, management and progression of these lethal diseases that impose a heavy burden on health resources.

Inhaled particulates are known to have an important role in the induction and progression of respiratory diseases [2]. Particulates are a mixture of solid and liquid small airborne particles of different origins. Particulate matter (PM) is a portion of air pollution that is made up of small particles and liquid droplets containing metals, organic chemicals, acids, and soil or dust particles. The terminology that has been used for characterization of indoor and outdoor particle mass concentrations includes the PM<sub>10</sub>, PM<sub>2.5</sub> and the PM<sub>0.1</sub> fractions. PM<sub>10</sub> (coarse particles) are particles with aerodynamic diameters smaller than 10 μm, PM<sub>2.5</sub> (fine particles) are smaller than 2.5 μm, and PM<sub>0.1</sub> (ultrafine particles, UFP) are the fraction of ambient particulates smaller than 0.1 μm [4]. Multiple studies have shown an association between airborne PM exposure and an increase in morbidity and mortality from cardiovascular and respiratory disease [3, 5]. These studies indicated that PM exposure results in the induction of oxidative stress and inflammation that lead to pulmonary anatomical and physiological remodeling [2, 6]. In general, the UFP content consists of a mixture of endogenous and inhaled particles in the 1- to 100-nm size range, which can be found and measured in different milieux (exhaled air, body fluids, organs, etc.). These nanoparticles should not be mistaken with the man-made engineered particles, which are mostly uniform in size and are largely used in experimental studies in the form of exogenous UFP. Numerous studies have been performed to assess the potential toxic effects of these engineered nanoparticles in a variety of lung cell types both in vitro and in animal models and humans, where they were shown to cause inflammation, fibrosis, oxidative stress and genotoxicity leading to cancer [7, 8]. In contrast, the properties of bronchoalveolar lavage (BAL) UFP, which include endogenous and exogenous particles within differently inflamed lungs and, in particular, their variation with different disease states and lung quiescence, remain unexplored. We therefore monitored the properties of UFP in lung fluid of mice and assessed the possibility that, once better defined during both homeostatic and different stress conditions, these properties can serve as potential biomarkers of differently inflamed lungs. In this study we aim to define the distribution of nanosize particles in lung fluid as a biomarker for lung inflammation.

**Materials and Methods**

**Animals**

C57 BL/6, C3H and Balb/c wild-type mice (8–12 weeks old) were housed in a specific pathogen-free animal facility at the Weizmann Institute of Science. The animals had free access to food and water, and were maintained at a 12-hour light/12-hour dark cycle. All experimental procedures were approved by the Animal Ethics Committee of the Weizmann Institute of Science, Rehovot, Israel (approval ID: 03890612-2).

**Exposure to Lipopolysaccharide and Tobacco Smoke in vivo**

Mice were exposed to lipopolysaccharide (LPS) via inhalation as described elsewhere [9]. They were placed in a 20 × 20 × 25 cm plexiglass chamber, where they were unrestrained, conscious and spontaneously breathing. The chamber was connected to a nebulizer (Aeroneb Pro-X Nebuliser system, Aerogen Ltd., Ireland) on one side, and a vacuum system on the opposite side. Seven milliliters of 0.5 mg/ml LPS (extracted from Escherichia coli serotype O111:B4; Sigma L-2630) dissolved in phosphate-buffered saline (PBS) were aerosolized into the chamber. Control mice were given aerosolized vehicle (PBS). Complete aerosolization of LPS or vehicle was achieved at 15 min, and the mice were maintained in the chamber for another 15 min until complete disappearance of the aerosolized mist. For cigarette smoke exposure, mice were exposed to tobacco smoke in a whole-body chamber (Teague TE-10 Exposure System; Teague Enterprises, Davis, Calif., USA) containing either air alone or air supplemented with 200 mg/m<sup>3</sup> cigarette smoke derived from standard research cigarettes (3R4F; University of Kentucky Tobacco Research and Development Center, Lexington, Ky., USA). Daily exposure periods lasted 4 h, with one rest interval of 30 min after the first 2 h. Mice were exposed for either 3 days or for 2 weeks (6 days per week).

**Irradiation and Cough Induction**

Mice were placed in an acrylic container and irradiated with a single lethal total body irradiation of 950 rad. For cough induction mice were exposed to a single intranasally introduced aliquot of capsaicin (100 μmol/l in 50 μl).

**Bronchoalveolar Lavage**

Twenty-four hours after the last exposure, mice were euthanized with an overdose of pentobarbital, and BAL fluid (BALF) was collected, as described previously [10]. BALF was centrifuged (5 min, 1,400 rpm) and the cell pellet was separated from the supernatant. BALF cell-free supernatants were stored at ~80°C until cytokine concentration and UFP measurements. A total cell count was performed in a Burker chamber, and cytospin preparations were stained with May-Grünwald-Giemsa. Lavage cells were incubated with fluorescent antibodies according to the manufacturers’ recommendations (CD45 Pacific Blue and Ly-6G APC). After 20 min of incubation at 4°C, the samples were washed twice in flow cytometry buffer.
UFP Measurements

UFP size distribution was measured in BALF cell-free supernatant using nanoparticle tracking analysis (NTA). NTA measurements were performed by a NanoSight LM20 instrument (NanoSight, Amesbury, UK), consisting of a conventional optical microscope, a Marlin-charged coupled device camera, and an LM20 unit (sample unit) with a laser light source. The samples were injected into the LM20 unit (approx. 300 μl) with a 1-ml sterile syringe. The capturing settings (shutter and gain) and analyzing settings were manually set according to the manufacturer’s protocol. The NanoSight LM20 recorded 60-second sample videos which were then analyzed with the NTA 2.3 analytical software. NTA provides direct and real-time visualization, sizing and counting of particulate materials between 10 nm and 2 μm in liquid suspension. The technique works on a particle-by-particle basis, relating the degree of movement under brownian motion to the sphere equivalent hydrodynamic diameter particle size and producing high-resolution particle size distributions [11, 12]. Since particles were measured in a polydispersed biological fluid, in which the determination of concentrations is less precise, we focused our analysis on the size distribution of particles in the range of 10–100 nm, since this determinant was independent of the absolute particle concentrations. Data from the software are given as particle size distribution curves in which the percentile is the independent variable, and the size of the particles is the dependent variable. D₅₀ is the value of the particle diameter at 50% in the cumulative distribution, and it represents the size in which 50% of the particles in the sample are smaller/larger than it.

Statistical Analysis

The average particle percentiles (up to 100 nm) for each group were stored into 33-nm bins (0–33, 34–66, 67–100), and their distribution was compared using a χ² contingency test. The neutrophil counts and the cytokine concentrations for each group were compared using the nonparametric Mann-Whitney test. A p < 0.05 was considered significant.

Results

UFP Content Can Be Measured in BALF and Their Size Is Highly Reproducible in Different Mouse Strains

BALF analysis of UFP size distribution in C57 BL/6 mice revealed highly reproducible UFP size distribution curves between different mice (n = 6, the experiment was performed twice; fig. 1a; p = 0.156). Furthermore, a similar UFP distribution (i.e. the UFP fingerprint) was observed in different mouse strains, such as Balb/c and C3H (n = 4, the experiment was repeated twice; fig. 1b; p = 0.170). These results taken together suggest that the UFP fingerprint within the BALF of intact lungs is highly reproducible and strain independent.
Acute LPS Inhalation Results in a Transient Shift of the UFP Fingerprint in the BALF

We next examined whether acute lung inflammation affects the conserved UFP fingerprint we have identified. To address this question we induced massive acute lung inflammation by LPS inhalation. Massive elevation of neutrophils was recorded in the BAL of the LPS-treated group compared to the PBS-treated group 24 h after the exposure (288,297 vs. 353, respectively; p = 0.007; fig. 2a), as well as a considerable elevation in the inflammatory cytokines, keratinocyte chemoattractant and interleukin 6 (288 and 171.8 vs. 2.3 and 3.07, respectively; p = 0.028; b). UFP size distribution curves show a significant shift towards larger particle sizes in the LPS-exposed mice; p < 0.01 (fig. 2c). D 50  of LPS-exposed BALF is higher than in PBS controls; p = 0.015 (see text).
We next wished to elucidate whether this dramatic shift in UFP size distribution is reversible, i.e. whether it remains within the BALF of mice fully recovered from the acute LPS challenge. Notably, 1 week after the LPS challenge we could already see a full resolution of the inflammation (fig. 3a) together with a total return of the UFP fingerprint to the normal pattern characteristic of naïve lungs (fig. 3b). These results suggest that the shifted UFP fingerprint in acutely inflamed lungs is transient and fully reversible once the inflammatory stimulus is removed and inflammation resolved.

**UFP Fingerprint after 3–14 Days of Smoke Exposure Induces a Similar Shift in the UFP Fingerprint Reminiscent of LPS Inflammation**

Lung exposure to tobacco smoking is an inflammatory trigger that can generate progressive injury in the C57 BL/6 background [10, 13]. We therefore tested whether variable daily exposure of this strain to high levels of tobacco smoke would also change the BALF UFP distribution. A 2-week exposure to tobacco smoke resulted in massive smoke loading into the vast majority of the murine alveolar macrophages (fig. 4) but only mild inflammation, as indicated by a moderate elevation in neutrophil count in the absence of classical inflammatory cytokines (fig. 5a and data not shown). Interestingly, although considerably weaker than LPS inflammation, this smoke exposure was sufficient to shift the UFP size distribution curve to that observed after a single exposure to high-dose LPS (fig. 5b). This observation suggests that both weak and strong levels of lung inflammation are associated with considerable UFP fingerprint alteration in the BALF of inflamed lungs. Surprisingly, measurement of neutrophil accumulation in the BALF after a 3-day smoke exposure revealed a comparable degree of inflammation, along with a UFP fingerprint similar to that observed after a 2-week smoke exposure (fig. 5c, d). Collectively, these results suggest that the inflammatory state of the lung BAL and not the type of the inflammatory stimuli results in a conserved characteristic UFP size distribution, thus implying that this UFP fingerprint can serve as a very sensitive marker of an ongoing lung inflammation.

**Whole Body Irradiation Does Not Induce Lung Inflammation and Does Not Change UFP Distribution in BALF**

Since neither the degree of inflammation nor the type of the inflammatory insult were found to change the UFP fingerprint, we hypothesized that the change in UFP size distribution results in mechanical changes in lung permeability rather than in biochemical inflammatory changes. To address this possibility we challenged the lungs with a strong irradiation protocol known to result in lung structural injury. Mice were irradiated with a single total body irradiation of 950 rad, which induces inflammatory lung

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Fig. 3. UFP size distribution returns to quiescence as the inflammation resolves. Animals (n = 5, experiment was repeated twice) were exposed to LPS inhalation (7 ml of 0.5 mg/ml) or PBS. BALF was harvested and analyzed 24 h or 1 week (LPS-exposed mice only) later. a The neutrophil counts returned to baseline 1 week after the inflammatory insult. b The UFP size distribution curve returned to a normal pattern as of PBS-treated mice.
injury mostly 4–12 weeks after exposure [14]. Twenty-four hours after this irradiation, subendothelial and perivascular edema takes place as well as injury to the alveolar type II cell and early release of surfactant, all of which impair alveolar surface activity [15]. Although severe, this irradiation did not result in lung inflammation 24 h after irradiation, as reflected by the absence of infiltrating BAL neutrophils and the very low basal levels of proinflammatory cytokines recovered in the BALF (fig. 6a and data not shown). Under these noninflammatory conditions, in spite of its severe injury-inducing effects, lung irradiation did not result in any noticeable shift in the UFP fingerprint of the intact resting control lungs (fig. 6b). Thus, acute lung inflammation can be considered as being mandatory for the UFP fingerprint alteration observed by us in both LPS- and smoke-exposed lungs.

**The BALF UFP Fingerprint Can Also Be Altered by Increased Cough**

Another explanation for the UFP shift could be secondary mechanical changes in minute ventilation and cough reflex that are associated with the primary inflammatory states developed in either LPS- or smoke-exposed lungs. To test that possibility, we induced noninflammatory cough by intranasal instillation of capsaicin, the pungent ingredient of 'hot' chili peppers [16], and followed the BALF UFP fingerprint 1 h thereafter, prior to the onset of inflammation. Interestingly, capsaicin treatment induced a slight but significant alteration in the UFP fingerprint toward larger particles compared to naïve mice (fig. 7; p = 0.0075), though it was lower than the effect of LPS, our standard inflammatory trigger. This observation supports the notion that exacerbated cough, on its own,
even before the onset of acute inflammation, is one of several factors underlying the UFP fingerprint shift induced by an acute inflammatory insult.

**Discussion**

There is an increasing need for new biomarkers which can elucidate the pathogenesis, explain the progression, direct the management and predict the prognosis of patients with various pulmonary diseases. Our study provides a novel potential biomarker for acute lung inflammation and lung recovery from inflammation based on measurements of particles smaller than 100 nm in the BALF of mice experiencing different lung inflammatory conditions.

Inhaled UFP are heterogeneous in terms of type and intensity of the hazard they pose following deposition in the lungs. They cause an inflammatory process and may contribute to the pathogenesis of several inflammatory lung diseases (such as asthma, COPD, idiopathic pulmonary fibrosis, sarcoidosis) as well as worsen an established...
disease state. So far, most studies focused upon the harmful effect of man-made airborne UFP on the lung after their administration and did not investigate the complete UFP content in the lungs prior to and following lung injury and during defined inflammatory conditions. Our findings indicate, for the first time, that UFP in lung fluid are measurable and have a reproducible fingerprint of particle size distribution as represented in a percentile-to-size curve. To support our results, we looked at the UFP fingerprint in different mouse strains and obtained similar curves, suggesting that this fingerprint is conserved between resting lungs and may thus serve as a marker for lung inflammation.

We used 3 models of lung inflammation in order to test whether the UFP fingerprint can serve as a new reporter of lung inflammation: acute LPS inhalation, acute smoke exposure (3 consecutive days) and subacute smoke exposure (2 weeks). All 3 inflammatory models resulted in a major shift in the UFP fingerprint curve towards significantly larger particles. Interestingly, the magnitude of the acute inflammation or the nature of the inflammatory insult did not alter this dramatic shift in UFP size distribution, since both a strong proinflammatory stimulus such as a singular LPS exposure and a weak stimulus such as a daily smoke exposure resulted in nearly identical UFP size distributions. This binary pattern of UFP size distribution suggests that although the UFP size within the BALF is highly divergent, it is equally sensitive to multiple inflammatory states and independent of the cytokine or cellular content of the lung BALF. Moreover, this unique...
size distribution fingerprint demonstrated a full return to a normal steady state fingerprint, when lung quiescence was regained. As such it can serve as a sensitive marker for the full resolution of lung injury or inflammation, and conversely, it may indicate that lungs have not reached complete recovery from inflammatory damage even when the BAL of these lungs appears apparently healthy by conventional determination of BALF cytokines or BAL leukocyte content.

In spite of the utility of this readout, several experimental limitations of this investigational tool must be considered. Firstly, the measurement of polydispersed biological fluid influences the NTA results since the fluid could contain vesicles and protein aggregates, which are not eliminated by the centrifugation. Secondly, this readout is associated with an inevitable background signal which requires standardized handling of all samples, and the use of serum supplement-free medium (i.e. PBS) since serum supplement often contains particles that may increase the background signal. A third weakness of the system is the complexity of measuring exact concentrations of the UFP in the polydispersed biological sample: for this reason, our results are focused mainly on size distribution which was found to be conserved among different samples and inflammatory conditions. Lastly, this new readout is binary and does not correlate with the degree of inflammation, which makes it less useful for the investigation of kinetics and severity of disease. This disadvantage can be utilized however to confirm full resolution of lungs from parenchymal and airway disorders since the return to normal UFP distribution requires full resolution of acute lung injury.

In vivo studies showed that labeled nanoparticles are eliminated from the lungs in two ways: one is by translocation beyond the epithelial barrier into the interstitium and eventually into the blood circulation, and the other is by the clearance pathway up the airway tree [17]. Assuming that the latter mechanism could explain the alteration of the lung particle distribution, we imitated an excessive cough condition by the administration of capsaicin, a strong cough inducer, but could show only a partial effect on the distribution of the particles. Thus, coughing on its own, with minimal inflammatory outcomes, can also shift the UFP size distribution towards larger particle sizes. Taken together, our results suggest that while the UFP fingerprint is highly conserved in different acute inflammatory conditions, a fraction of the inflammation-dependent changes in UFP size distribution can be attributed to coughing.

The inflammation-induced UFP size distribution shift we observed can be explained by lung uptake of smaller-sized particles which leaves the larger nanoparticles in the BALF and thereby predisposes the size distribution of the UFP towards the larger size range. Consistent with this hypothesis, human studies which exposed healthy and COPD patients to inhaled nanoparticles showed that the deposition of particles increases with the severity of the disease [18], suggesting that penetration or translocation of these minute particles through the respiratory epithelium into the lung parenchyma increases when the alveolar barrier is inflamed. Consistent with those results, in vitro studies showed that a pharmacological decrease in the tight junctional resistance of mouse alveolar epithelial cell monolayers causes a drastic increase in the translocation of engineered nanoparticles across the epithelial barrier [19] and that smaller nanoparticles cross the rat alveolar epithelial monolayer 3 times faster than larger ones [20], observations which can explain the shift towards larger particles in our inflammatory models. If so, our results also predict that any inflammatory imbalance of the lung epithelial layer, regardless of its cause and magnitude, results in a similar absorption of small UFP in the lung. Conversely, a full recovery of the lung epithelium from acute inflammatory stress and injury will result in the return of a normal UFP size distribution characteristic of intact healthy pulmonary tissue.

We cannot, however, exclude the possibility that the shift in UFP size towards the larger size range was also due to an increase in the absolute amount of larger particles from an endogenous origin that had been induced by acute lung inflammation. For example, large endogenous UFP in lung fluids, such as exosomes, may be secreted by various respiratory epithelial or immune cells such as alveolar macrophages in particular in response to inflammatory stimuli. Exosomes are vesicles in the size range of 50–200 nm within the large UFP size range as determined by us. These vesicles can be secreted by a wide range of cell types [21] and were shown to exhibit a variety of biological activities both in immunostimulatory and immunosuppressive domains [22, 23]. Consistent with our hypothesis, one of the stimuli used by us, LPS, was shown to induce exosome secretion by monocyte-derived dendritic cells [12, 24]. It is therefore possible that alveolar macrophages and epithelial cell-derived exosomes also account for a portion of the large UFP we measured in the BALF of our acutely inflamed lung models. It is also possible that the smoke-induced shift of the UFP fingerprint towards larger particles could reflect a small contribution of inhaled smoke particles remaining in the BALF, i.e.
particles which were not taken up by lung epithelial cells and macrophages. Interestingly, the shift in UFP size observed after 3 and 14 days of smoke exposure was essentially identical even though the cumulative amount of inhaled smoke particles was likely much higher in the longer exposure group. These results collectively suggest that the BALF UFP changes are primarily due to the inflammatory process rather than to mere changes in composition of free smoke particles in the BALF.

Taken together, our results are consistent with a dual effect of inflammation on UFP content and distribution in BALF. Different inflammatory stimuli can trigger the release of exosomes and exosome-derived nanoparticles from various epithelial cells and their associated alveolar macrophages. Moreover, inflammation increases epithelial permeability, allowing the preferential absorption of the smaller-sized particles (exogenous as well as endogenous) from the BAL into the lung parenchyma, with the larger particles remaining in the BALF. Apart from their size distribution, the biochemical composition of these penetrating or remaining particles awaits elucidation as it may provide essential insights into the end target of the absorbed particles, and what types of cells release the endogenous particles in either resting or inflamed lungs.

In conclusion, our study suggests that the UFP fingerprint of lungs can serve as a sensitive marker of acute lung inflammatory insults of multiple origins. Future studies should be aimed to compare the UFP profile in human lung fluid samples of patients with different degrees of inflammatory lung diseases, such as COPD and asthma. It would be interesting to find out if in humans the severity of a given lung disease will similarly not change the apparently universal UFP fingerprint of acutely inflamed lungs. These future studies will help substantiate whether different UFP fingerprints may indeed serve as sensitive reporters for both acute and ongoing chronic inflammation on the one hand, as well as for the full recovery of lungs from previous exposure to smoke and other environmental irritants on the other.

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Financial Disclosure and Conflicts of Interest

None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this paper.

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