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Original Article

# Pro-inflammatory effect of cystic fibrosis sputum microparticles in the murine lung

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**Abstract**

*Background:* The role of microparticles (MPs) in the inflammatory process of cystic fibrosis (CF) airways is not known. Here, we have studied the pro-inflammatory potential of CF MPs in a model of acute lung injury.
*Methods:* Swiss mice were subjected to intratracheal administration of MPs obtained from CF and primary ciliary diskinesia (PCD) patients. Histopathology, total and differential cell counts in bronchoalveolar lavage fluid were used to evaluate the inflammatory reaction in the lung. Lipopolysaccharide (LPS)-like activity of MPs was studied by Limulus amebocyte lysate assay.
*Results:* MPs obtained from acute CF patients determined peribronchial and perivascular inflammatory infiltrates similar to those elicited by LPS. This inflammation was granulocyte-dominated and higher than that determined by MPs obtained from stable CF, whereas PCD MPs caused a macrophage-dominated inflammation. While LPS-activity was not found in circulating blood MPs prepared from CF patients, it was present in MPs obtained from CF sputum and sputum CD66b$^+$ neutrophils. Finally, LPS-like activity was only detected in circulating MPs after incubation with LPS as well as in MPs obtained from LPS-stimulated neutrophils obtained from healthy donors.
*Conclusions:* These data suggest that the pro-inflammatory potential of neutrophil-derived MPs in the CF airways may be subsequent to the binding of shedded LPS.
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*Keywords:* Cystic fibrosis; Lipopolysaccharide; Lung inflammation; Neutrophils; Macrophages; Primary ciliary dyskinesia

## 1. Introduction

The hallmarks of the lung disease in cystic fibrosis (CF) patients are a persistent infection with opportunistic bacterial pathogens and an abnormal inflammatory response dominated by polymorphonuclear neutrophils (PMNs) and dysfunction in both respiratory epithelial cells and airway macrophages [1–3]. Airway colonization and infection occur with few pathogens such as *Pseudomonas aeruginosa*, *Staphylococcus aureus*, or both, even in infants at a very young age. Bacterial shedding of immunostimulatory pathogen-associated molecular patterns (PAMPs), such as cell-wall components, lipopolysaccharide (LPS), flagella and DNA, activate a brisk proinflammatory response. Due to bacterial adaptation and selection of mutants, the PMN response is not capable to clear bacteria from the CF airways

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ensuing in exaggerated apoptosis [4]. Furthermore, PMNs killed by *P. aeruginosa* release proteases that disable any neighbouring viable neutrophils [5].

Initially considered as inert cellular debris released from activated platelets in human plasma [6], microparticles (MPs) have emerged as a new concept of biomarker and biovector. MPs are small vesicles released from cells after activation by chemical (apoptotic, proinflammatory, prothombotic) or physical (shear stress) stimuli [7], they are surrounded by a lipid bilayer composed mainly of phospholipids, cholesterol and membrane-associated proteins, and are enriched with intracellular components such as proteins for signal transduction, mRNA, and miRNA [8]. MPs are heterogeneous in size (0.1–1 μm) and their composition depends on both cell origin and stimulation implicated during their generation. Thus all types of cells can theoretically release MPs at each stage of their life cycle, but the intracellular mechanism involved in MP formation are not completely elucidated [7].

MPs presence has also been documented in the bronchoalveolar lavage fluid (BALF), where MPs derived from platelets were found in pulmonary air–liquid interfaces in sedated pigs [9]. Bastarache and colleagues [10] described procoagulant MPs in BALF from acute respiratory distress (ARDS) patients, contributing with tissue factor to intra-alveolar fibrin formation, a critical pathogenic feature of acute lung injury (ALI). Further studies have demonstrated the MP role in the pathogenesis of different aspects of ALI/ARDS (for review see [11]).

In a previous study, our group for the first time was able to isolate MPs from the sputum of CF patients [12], finding that MPs in CF sputum are mainly derived from PMNs. Here, we have analyzed the pro-inflammatory potential of MPs isolated from the sputum of CF patients in the mouse lung, and compared their effects with those elicited by the administration of MPs isolated from the sputum of Primary Ciliary Dyskinesia (PCD) patients, a disease characterized by opportunistic pathogen infection of the upper and lower respiratory tract and neutrophilic inflammation [13].

## 2. Materials and methods

### 2.1. Patients

The study was approved by, and performed in accordance with, the ethical standards of institutional review boards of University of Foggia and Hospital of Cerignola on human experimentation. Written informed consent was obtained from each subject.

We enrolled 6 CF patients who consecutively had been admitted at the CF Center of the Hospital of Cerignola "G. Tatarella" for parenteral (intravenous) antibiotic therapy during acute respiratory exacerbation, and 4 stable CF patients. Exacerbation was defined as a deterioration in symptoms perceived by the patient and included an increase in cough, sputum production, dyspnoea, decline in forced expiratory volume in 1 s ($FEV_1$) compared with previous best, weight loss and fever [14]. Each patient was given a clinical score obtained from the sum of the individual parameters (0 = no symptom; 1 = moderate; 2 = severe). Serum C-reactive protein (CRP) was assessed as a marker of active inflammation [15]. CF patients were compared with 4 PCD patients.

Bacterial species in sputum specimens were identified accordingly to the North-American guidelines [16]. Sputum samples were directly spread-out in selective media, such as MacConkey agar for *P. aeruginosa*, mannitol salt agar for *S. aureus*, and BCSA for *Burkholderia cepacia* complex, and incubated at 36 ± 1 °C for a period of 18–72 h. Colonies were quantified and identified by classical (manual) phenotypical tests.

### 2.2. MP isolation from sputum

MPs were isolated as previously described by our group [12]. Briefly, spontaneous sputum was solubilized by treatment with Sputasol® (SR 0233A, Oxoid Ltd, Hampshire, UK), and then incubated in a water bath at 37 °C for 15 min until visible homogeneous. Processed sputum was centrifuged at 37 ×*g* for 3 min; the supernatant was centrifuged at 253 ×*g* for 10 min and then re-centrifuged at 253 ×*g* for 20 min to remove the cells and large debris, respectively. MP-containing supernatant was centrifuged at 14,000 ×*g* for 45 min to pellet MPs. MP pellet was subjected to two series of centrifugations at 14,000 ×*g* for 45 min. Finally, MP pellet was re-suspended in 500 μL of 0.9% saline salt solution and stored at +4 °C until total counting and usage.

To determine MP number, 10 μL of Flowcount beads (Beckman Coulter, Ireland) was added to each sample and analyzed in a flow cytometer (Beckman Coulter coulter epics XL-MCL). Sample analysis was stopped after the count of 10,000 events.

### 2.3. Isolation of CD66b-positive MPs

Positive selection of CD66b-positive MPs by EasySep (Human whole Blood CD66b positive selection kit; StemCell Technologies Inc., Vancouver, Canada) was done accordingly to the manufacturer's instructions. Briefly, recovered total sputum MPs, re-suspended in the appropriate medium, were incubated with the EasySep CD66 positive selection cocktail, a tetrameric antibody complex which recognizes CD66 and dextran, the successive step being the addition of dextran-coated magnetic nanoparticles. Magnetically labelled target cells were then separated from unlabeled unwanted cells using the EasySep magnet by pouring the unlabelled cells off.

### 2.4. Isolation of blood MPs

Peripheral blood (20 mL) from healthy donors and CF patients was collected in EDTA-treated tubes from a peripheral vein using a 21-gauge needle to minimize platelet activation and was processed for assay within 2 h [17]. After a 20 min centrifugation (270 ×*g*), platelet-rich plasma (PRP) was separated from whole blood. PRP was then centrifuged for 20 min (1500 ×*g*) to obtain platelet-free plasma (PFP). PFP (MP containing) was spun down at 14,000 ×*g* for 45 min to pellet MPs. The MP pellet was then centrifuged twice at 14,000 ×*g* for

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