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Endocytosis of particulate matter induces cytokine production by neutrophil via Toll-like receptor 4

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ABSTRACT

Particulate matter (PM) with a median diameter < 2.5 μm, is associated with respiratory and cardiovascular diseases. We previously reported the biological effects of PM in vivo, and although neutrophils play an important role in initiating inflammation, few reports have focused on the relationship between PM inhalation and immune responses. Here, we investigated the effect of PM particle size on neutrophils, including their endocytosis activity and reactive oxygen species (ROS) production. Flow cytometry analysis indicated that 1 μm particles are readily endocytosed by neutrophils and that endocytosis is reduced at 4 °C. Inhibitors of the pleckstrin homology domain of dynamin repressed this process; however, GTPase and clathrin inhibitors did not affect endocytosis. Endocytosis by neutrophils in Toll-like receptor 4 (TLR4)- and MyD88-knockout mice was reduced compared with that in wild-type mice, indicating that TLR4 and MyD88 are important for the process. Neutrophil-mediated endocytosis caused oxidative stress, and *N*-acetylcysteine enhanced endocytosis. Expression levels of the oxidative stress markers, heme oxygenase-1 and p62 protein, were increased in an endocytosis-dependent manner. Phagocytosed neutrophils produced IL-6 and TNFα, whose production was decreased by dynamin inhibitors. We observed that infiltrated CD11b-positive cells in bronchoalveolar lavage fluid endocytose PMs. Overall, these results indicate that endocytosis and ROS production via TLR4 are important for the initiation of immune responses by neutrophils.

1. Introduction

Epidemiological studies show that exposure to particulate matter (PM) is associated with adverse health effects [1,2]. Previously, we showed that PM exposure influences second lymphoid organs such as spleen [3,4]. However, the molecular mechanism underlying the initiation of lung inflammation following splenic inflammation is unclear.

Neutrophils, localized in lymphoid organs, play an important role in the initial phase of inflammation. After inhalation of particles, neutrophils are recruited into pulmonary inflammation with production of pro-inflammatory cytokines [5,6]. Effects of particle size on macrophage responses have been reported [7,8]. These reports reveal that different immune responses occur to differently sized particles. However, reports focusing on the relationship between neutrophil responses and differently sized particles are rare.

Toll-like receptors (TLRs), expressed on neutrophils, are pattern recognition receptors that recruit MyD88 to activate signaling pathways and induce cytokine production [9]. Endocytosis also triggers signaling events. Many endocytic pathways are classified into clathrin-mediated and clathrin-independent endocytosis. Dynamin is necessary for both these pathways.

Overproduction of reactive oxygen species (ROS) leads to oxidative stress [10,11]. Heme oxygenase-1 (HO-1) has been widely studied as a model for redox-regulated gene expression. Expression of HO-1 is induced in response to chemical and physical agents, and ROS [12]. Additionally, ROS are essential second messengers in innate and

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adaptive immune cells [13,14]. The transcription factor, nuclear factor erythroid 2-related factor 2 (Nrf2), interacts with Kelch-like ECH-associated protein 1 (Keap1) [15]. Elevated ROS oxidize redox-sensitive cysteine residues on Keap1 resulting in its dissociation from Nrf2 [16]. The Keap1-Nrf2 pathway is one of the main cellular defense mechanisms against oxidative stress [17,18].

We demonstrated previously that PM2.5 exposure induced the accumulation of neutrophils in the lung [19]. Here, we investigated the effect of PM particle size on neutrophils and tested the effect of endocytosis inhibitors on cytokine production and HO-1 expression by PM-exposed neutrophils.

2. Materials and methods

2.1. Mice

Wild-type BALB/c mice were purchased from Kyudo. TLR2, TLR4, TLR9, and MyD88 knockout (KO) mice were purchased from Oriental BioService. Mice were maintained in our university's animal research center. The experimental protocols were approved by the Ethics Review Committee for Animal Experimentation and the Ethics Committee of Animal Care and Experimentation (admission number: AE13-008) at the University of Occupational and Environmental Health. Experiments were performed according to the Institutional Guidelines for Animal Experiments, and Law Number 105 and Notification Number 6 of the Japanese government. Surgeries were performed with anesthesia (50 mg/kg ketamine and 1 mg/kg medetomidine mixture), and all efforts were made to minimize suffering.

2.2. PM

Fluorescent silica particles (sicastar®-redF and sicastar®-greenF) were purchased from Corefront. Five different size particles (0.1, 0.3, 1, 3, and 10 μm) were used. Excitation and emission wave length of redF and greenF is 569 nm/585 nm and 485 nm/510 nm, respectively.

2.3. Reagents and antibodies

The peridinin chlorophyll protein complex (PerCP)-cyanine5.5 (Cy5.5)-conjugated anti-human/mouse CD11b and FITC-conjugated anti-TLR4, HO-1, β-actin (A5441), p62 antibodies were purchased from Tonbo Biosciences, Santa Cruz Biotechnology, Sigma-Aldrich, MBL, respectively. PH domain inhibitors MiTMAB (ab120466) and OctMAB (ab120467), GTPase inhibitors Dynole-34-2 (ab120463) and Iminodyn-22 (ab120461), and a clathrin inhibitor, Pitstop2, were purchased from Abcam. These inhibitors, except for MiTMAB, were dissolved in dimethyl sulfoxide and diluted with phosphate-buffered saline before administration. MiTMAB was dissolved in distilled water. NAC (Sigma-Aldrich) was dissolved in PBS and the pH adjusted to 7.5.

2.4. Intratracheal administration of particles

Red or green fluorescent silica particles were suspended in 0.9% saline for injection (Otsuka). Mice were injected intratracheally with solutions (100 μl) containing red or green fluorescent silica particles at a dose of 0.1 mg through a polyethylene tube, under 4% sevoflurane anesthesia (Wako Pure Chemical Industries) as reported previously [4]. After 24 h, the mice were killed by exsanguination under deep anesthesia by intraperitoneal pentobarbital injection.

2.5. Preparation of neutrophil and BAL cells

Infiltrated neutrophils were obtained from mice treated with an intraperitoneal injection of 2 ml 4% thioglycolate broth (Nihon Seiyaku). After 4 h, peritoneal exudate cells were harvested and suspended in RPMI 1640 medium (Nissui). Cells with high side and forward scatter regions, and CD11b-positive cells, were treated as neutrophils. BAL cells were prepared as described previously [3]. BAL cells were labeled with the Cy5.5-conjugated anti-CD11b antibody. Endocytosed silica particles were observed under a BZ-X700 fluorescence microscope (Keyence).

2.6. Confocal microscopy analysis

A confocal laser scanning microscope (LSM5 Pascal; Carl Zeiss) was used to observe fluorescence (objective lens: oil-immersion with 63× magnification and 1.4 numerical aperture). MetaMorph software was used to construct 3D images (Molecular Device, Tokyo, Japan).

2.7. Flow cytometry analysis

Neutrophils were treated with red fluorescent silica beads for 3 h with or without a 1 h pretreatment. Then, the Cy5.5-conjugated anti-CD11b antibody was added and incubated for 30 min at 4 °C. Cells were washed and CD11b-positive cells were analyzed by flow cytometry using a Sony EC800 flow cytometer. Analysis of data from the flow cytometer was carried out using FlowJo version 7.6.5 (Tree Star).

2.8. Cytokine assays

Enzyme-linked immunosorbent assay kits for mouse IL-6 and TNF-α were purchased from BioLegend. Cells (5×10^5) were treated with particles for 3 h with or without a 1 h inhibitor pretreatment. Culture supernatants were collected and assayed for cytokine levels.

2.9. Western blotting

Cells were lysed with radioimmunoprecipitation assay buffer for preparing whole-cell extracts. Equivalent amounts of protein (10 μg) were used for electrophoresis. Signal intensity was quantified using ImageJ (National Institute of Health). Expression levels of target proteins were normalized to that of β-actin.

2.10. Statistics

Results are expressed as means ± standard deviation. Statistical analyses were performed using Student's *t*-test. $p < 0.05$ was considered significant.

3. Results

3.1. Inflammation-induced neutrophils endocytosed fluorescent silica particles

To mimic neutrophilic alveolar inflammation in vitro, intraperitoneal exudative neutrophils were prepared. Endocytosis of fluorescent silica particles by these neutrophils was observed over a period of 30 min (Fig. 1A, B). There was no dramatic change in neutrophil shape, or damage and death during endocytosis. The 3D images clearly demonstrated that multiple particles were endocytosed (Fig. 1C).

3.2. Fluorescent silica particles were endocytosed in vivo

To clarify how intratracheally administered fluorescent silica particles were endocytosed in lung tissue, bronchoalveolar lavage (BAL)