Surfactant protein A /D-CD14 is Associated with Phagocytosis of Nanomaterials and Cytokine Production by Alveolar Macrophages

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Abstract

Background: Alveolar macrophages are responsible for clearance of airborne dust and pathogens. How they recognize and phagocytose a variety of engineered nanomaterials (ENMs) with different properties is an important issue for safety assessment of ENMs. Surfactant-associated proteins, specifically existing in the pulmonary surfactant, are important opsonins for phagocytosis of airborne microorganisms. The purposes of the current study are to understand whether opsonization of ENMs by surfactant-associated proteins promotes phagocytosis of ENMs and cytokine production and to find out a common pathway for ENMs with different properties.

Results: 4 ENMs including MWCNT-7, TiO_2 , SiO_2 , and fullerene C60, each with different shape, size, chemical composition and surface reactivity, were chosen for the study. Short-term pulmonary exposure of MWCNT-7, TiO_2 , SiO_2 , and C60 induced inflammation in the rat lung, and most of the administered ENMs were phagocytosed by alveolar macrophages. The ENMs were phagocytosed by isolated primary alveolar macrophages (PAMs) *in vitro*, which was enhanced by the rat bronchioalveolar lavage fluid (BALF), suggesting that proteins in BALF were associated with the phagocytosis. Further analysis of the 4 ENMs-bound proteins by LC/MS indicated that surfactant-associated proteins A and D (SP-A, SP-D) were common binding proteins for all the 4 ENMs. Like BALF, SP-A, but not SP-D, enhanced TNF- α production in the MWCNT-7-treated PAMs; both SP-A and SP-D increased IL- β production in the TiO_2 - or SiO_2 -stimulated PAMs; while SP-A and SP-D enhanced IL-6 production in the C60-stimulated PAMs. Knockdown of CD14, a receptor for SP-A/D, significantly reduced the cytokine production and ENMs phagocytosis.

Conclusions: These results indicate that SP-A/D can opsonize all the studied ENMs to enhance phagocytosis of the ENMs by alveolar macrophages through CD14, suggesting that SP-A/D-CD14 is a common pathway mediating phagocytosis of ENMs. Cytokine production induced by ENMs, however, is dependent on what an ENM is phagocytosed. Our results are helpful for the understanding of clearance of ENMs by alveolar macrophages and mechanisms of different ENMs-induced lung toxicity.

Background

The respiratory tract is the major exposure route for airborne dusts and pathogens. Alveolar macrophages (AMs) reside in the airway and alveoli, account for 95% of leukocytes in the lower respiratory tract [1], and function mainly in host defense and alveolar homeostasis. Phagocytosis by AMs is a major mechanism for clearance of dusts and microorganisms often encountered in the lung [2]. AMs have many types of pattern-recognition receptors (PRRs) on the plasma membrane, and recognize a variety of pathogen-associated molecular patterns (PAMPs) in microorganisms [3, 4]. AMs also express Fc receptors and complement receptors for the Fc portions of IgG antibodies and complements that specifically bind IgG or complements-coated pathogens [5, 6]. The process of coating pathogens to promote phagocytosis is called opsonization. The direct binding of PRRs to their corresponding PAMPs and the binding of Fc receptors and complement receptors to opsonized pathogens induce a number of

responses in AMs, including production of cytokines, inflammatory mediators and microbicidal enzymes, and mobilization of cytoskeleton leading to phagocytosis, cell migration and granule exocytosis. Secreted cytokines and inflammatory mediators then exert their regulatory functions of inflammation and immune responses [7, 8]. AMs are also involved in the clearance of apoptotic and necrotic cells and the subsequent regression of pulmonary inflammation [9].

With advance in nanotechnology, engineered nanomaterials (ENMs) are increasingly being developed. This may lead to increased respiratory exposure of ENMs during their production, consumption and disposal. Numerous studies in animals indicate that respiratory exposed ENMs are taken up and cleared by AMs [10, 11]. ENMs have little antigenicity and do not possess molecular structural regions like PAMPs in microbes. How do AMs recognize different ENMs with different size, chemical composition, physical morphology and surface reactivity? It is widely accepted that ENMs are bound with proteins from various biological fluids, forming tiers of proteins coating the surface of ENMs, and in turn, the so-called protein corona may promote phagocytosis through interaction of bound complements or IgG with complement receptors and Fc receptors [12, 13]. Properties of ENMs can modulate the composition of the protein corona [14]. Such studies on the interaction between ENMs and proteins are mainly based on the serum/plasma. In the lung, the pulmonary surfactant is a complex mixture composed of more than 90% lipids and 5–10% proteins, covers all the inner face of the alveoli, and reduces surface tension at the air-water interface in the alveoli to prevent alveolar collapse at end-expiration [15]. Surfactant-associated proteins (SP-A, SP-B, SP-C and SP-D) differ from one another in their synthesis, oligomerization, and function [16]. SP-B and SP-C are hydrophobic and reduce the surface tension of the distal lung, while SP-A and SP-D are more hydrophilic and have important roles in the regulation of innate immune responses [17]. SP-A and SP-D can bind to many different microbial pathogens and act as opsonins to enhance phagocytosis of microbial pathogens by AMs [18, 19].

In the current study, 4 kinds of ENMs (fullerene C60, TiO₂, SiO₂ and MWCNT) with different size, shape, surface reactivity and chemical composition were chosen for analysis of their binding proteins from rat bronchoalveolar lavage fluid (BALF). SP-A and SP-D were identified as common binding proteins for all the 4 ENMs, and enhanced phagocytosis of the ENMs and cytokine production by AMs in vitro. Also, the enhancing effects were CD14-dependent. Our results are helpful for understanding of ENMs-induced toxicity and for production of safe ENMs.

Results

Most of the intratracheally exposed nanomaterials were phagocytosed by alveolar macrophages

For comparison of toxic effects in the lung, 4 kinds of ENMs with different shape, chemical composition, size and surface property were chosen for this study. MWCNT-7 is a carbon-based nanotube with a hydrophobic surface; TiO₂ is a rod-like particle with a hydrophilic surface; SiO₂ and C60 are round

nanoparticles with a hydrophilic and hydrophobic surface, respectively. They formed aggregates in the suspensions with different size. Characterizations concerning their shape, chemical composition and size distribution are shown in Figure S1, S2 and S3.

Short-term administered MWCNT-7, TiO_2 , SiO_2 , and C60 suspensions to the rat lung induced pulmonary inflammation to a different extent. As shown in the left panel of Fig. 1, MWCNT-7 elicited a strong lung inflammation, with accumulation of immune cells and thickening of the alveolar epithelium (Fig. 1A); while TiO_2 and SiO_2 (Fig. 1B & C) had little effect on the alveolar epithelium, although particle-burden alveolar macrophages were often observed. Similar to MWCNT-7, C60 caused a strong lung inflammation (Fig. 1D).

Light microscopic and electron microscopic observation revealed that most of the administered ENM aggregates were found within alveolar macrophages (Fig. 1E, F, G and H corresponding to MWCNT-7, TiO_2 , SiO_2 , and C60, respectively), indicating that phagocytosis by alveolar macrophages is a main mechanism for clearance of the invading particles.

BALF enhanced the uptake of nanomaterials by alveolar macrophages

Previous studies have demonstrated that ENMs are bound with proteins in many types of biological fluids, forming tiers of proteins surrounding the nanomaterials. This so-called protein corona affects biological behaviors of the nanomaterials, including phagocytosis by macrophages [20]. For understanding whether secretory fluid in the respiratory tract and alveoli influences phagocytosis of the studied 4 ENMs by alveolar macrophages, we first prepared BALF and primary alveolar macrophages (PAMs). Immunofluorescence staining indicated that more than 95% of isolated cells were positive for CD68, a macrophage marker (Figure S4). The 4 ENMs were then exposed in vitro to the isolated PAMs in the absence or presence of BALF, and the rates of ENMs-burden PAMs were compared under polarized microscopy. The results indicated that addition of BALF increased the uptakes of MWCNT-7, TiO_2 , and SiO_2 by PAMs (Fig. 2A, B & C), compared with those without addition of BALF (Fig. 2D, E & F). The rates of PAMs with burden-particles in the absence of BALF were 31.7%, 22.7% and 18.2% for MWCNT-7, TiO_2 , and SiO_2 , respectively, significantly lower than those in the presence of BALF (61.0%, 43.3% and 53.7%, Fig. 2I). Because of C60 was not brightening under the polarized microscope, its phagocytosis could not be observed (Fig. 2G & H). These results suggested that proteins in the BALF were likely to bind to the ENMs to promote the phagocytosis by PAMs.

SP-A, SP-D and SP-B shared common proteins bound to ENMs

For understanding which proteins from BALF were bound to each of the 4 ENMs, the binding proteins were dissociated, and then subjected for SDS-PAGE and LC-MS. SDS-PAGE and silver staining revealed that kinds and abundance of the binding proteins were different between the 4 ENMs (Fig. 3A). Further

analysis with LC-MS showed that total 892 proteins in the BALF were checked out, with 332 proteins being common bound proteins for all the 4 ENMs (Fig. 3B). Scrutinizing top 50 abundant proteins indicating that albumin, complements, immunoglobulins, apolipoproteins and other serum-derived proteins had the highest ratio, and respiratory tract- or lung-derived proteins and other proteins of unknown origin were also common (Table S1).

The pulmonary surfactant is a complex mixture containing 4 surfactant-associated proteins (SP-A, SP-B, SP-C and SP-D). Since surfactant-associated proteins are specific proteins in the lung, we paid special attention in the relationship of surfactant-associated proteins and phagocytosis of ENMs by alveolar macrophages in this study. As shown in Fig. 3C, SP-A, SP-D and SP-B were found in the all 4 ENMs-binding protein spectra, ranking at the 184, 48 and 112 places in the abundance of MWCNT-7-binding proteins, 523, 112 and 182 for TiO₂, 185, 65 and 94 for SiO₂, and 109, 17 and 99 for C60 (data not shown).

SP-A and SP-D enhanced nanomaterials-cytokine production

For further clarifying whether surfactant-associated proteins affect macrophage activation, TNF- α , IL-1 β and IL-6, the main pro-inflammatory cytokines produced upon macrophage activation, were used as judging parameters. Preliminary experiments showed that SP-B had little effects on production of cytokines (data not shown), therefore, we focused on the effects of SP-A and SP-D in the subsequent experiments. Pre-addition of 1 μ g/ml human recombinant SP-A or SP-D to the serum-culture medium of PAMs significantly increased TNF- α expression in the MWCNT-7-treated PAMs at the mRNA level, while an increase in IL-1 β and IL-6 mRNA expression was not found (Fig. 4A). Similar results were also observed by addition of BALF (Fig. 4A). ELISA examination of the culture media indicated that SP-A, SP-D and BALF enhanced MWCNT-7-induced TNF- α secretion (Fig. 4E). In the presence of SP-A, SP-D or BALF, IL-1 β expression, but not the TNF- α nor IL-6, was especially elevated in the TiO₂- or SiO₂-stimulated PAMs at both the mRNA and protein levels (Fig. 4B, C, F and G). C60 specifically stimulated IL-6 production, which was promoted by addition of SP-A, SP-D or BALF (Fig. 4D and H). In brief, like BALF, SP-A and SP-D enhanced ENMs-induced cytokine production of PAMs, although the inflammatory cytokines differed from one another by the stimulation of different ENMs.

Knockdown of CD14 expression in alveolar macrophages reduced cytokine production and ENMs uptake

It has been reported that LRP1, CD14, and SIRP α are potential receptors for SP-A and SP-D [21–23]. Thus, we tried to find out if SP-A/D-enhanced the cytokine production was mediated by these receptors at first. The expressions of these receptors in PAMs were knocked down by siRNAs. The genes-specific siRNAs obviously decreased the mRNA and protein expressions of LRP1, CD14, and SIRP α (Figure S5). Knockdown of CD14, but not LRP1 and SIRP α , significantly reduced the TNF- α expression enhanced by SP-A in the MWCNT-7-stimulated PAMs (Fig. 5A and E), indicating that the SP-A/CD14 axis was involved

in MWCNT-7 induced TNF- α production. Contrary to SP-A, the SP-D-enhanced TNF- α expression were increased by CD14 silencing (Fig. 5A). TNF- α expression was elevated by knockdown of LRP1 or SIRP α (Fig. 5A), suggesting that LRP1 and SIRP α induce inhibitory signaling in the TNF- α production. Similarly, qPCR revealed that knockdown of CD14 decreased the IL-1 β mRNA expression which was enhanced by SP-A or SP-D in the TiO₂- or SiO₂-stimulated PAMs (Fig. 5B & C). The declined IL-1 β protein secretion by CD14 silencing was confirmed by ELISA (Fig. 5F & G). In the SP-A-promoted IL-6 expression of the C60-stimulated PAMs, silencing of CD14 and SIRP α had a remarkable downregulation effect (Fig. 5D & H). Taken together, these results indicated that SP-A/D-CD14 axis was a common pathway for ENMs-induced cytokine production, although the ENMs are different from each other in their properties.

Next, we investigated whether the SP-A/D-CD14 axis is associated with phagocytosis of the ENMs by PAMs. Compared with the control, knockdown of CD14 reduced the phagocytosis rate in the MWCNT-7-stimulated PAMs (Fig. 6A, B & K) in the presence of SP-A (65.3% vs 12.9%), while the SP-A/D-enhanced phagocytosis of TiO₂ or SiO₂ was decreased by CD14 silencing (Fig. 6C, D, E, F, G, H, I, & J). As shown in Fig. 6K, phagocytosis rates in the presence of SP-A and SP-D were 47.3% and 66.1% for TiO₂, 46.0% and 55.5% for SiO₂ respectively, decreased to 19.5% and 16.6%, 16.7% and 11.2% after CD14 silencing. As mentioned above, phagocytosis of C60 could not be evaluated under the polarized microscopy.

Discussion

As a major type of innate immune cells, macrophages exert a variety of actions both in the innate and the adaptive immunity. AMs reside in the low respiratory tract and alveoli, clear airborne dusts and microbial pathogens often encountered in the lung by phagocytosis and release pro-inflammatory cytokines. Unlike microorganisms composed of biological macromolecules, most kinds of ENMs are usually formed by substances with relatively simple chemical composition, which lack immunogenicity and are difficult to stimulate adaptive immune responses. Therefore, the immune responses against ENMs mainly mediated by innate immune cells such as macrophages and by soluble molecules such as cytokines. It is plausible that ENMs are opsonized and then phagocytosed by AMs in the lung.

The deep lung is composed of approximately 3 million alveolar sacs, forming a broad surface area through which oxygen and carbon dioxide are exchanged in the blood of alveolar capillaries [21]. SP-A and SP-D are lung-specific proteins existing in the pulmonary surfactant. Except for their functions in the homeostasis of the pulmonary surfactant [24, 25], they are also involved in the host defense against various pulmonary pathogens, such as respiratory syncytial virus, mycobacterium tuberculosis, bacteria, viruses and fungi [26, 27]. Structurally, SP-A and SP-D belong to the collectin family with a C-terminal carbohydrate recognition domain (CRD) and an N-terminal collagen like domain,[28] and act as opsonins by interaction via the CRD with various microorganisms and their derived components to enhance phagocytic function of AMs through CD14, Toll-like receptors and other receptors expressing on the surface of AMs [29, 30].

In the current study, we identified SP-A/D-CD14 as a common pathway that mediated cytokine production and/or phagocytosis by PAMs in all the studied ENMs (summarized in the Figure S6). Obviously, in the low respiratory tract and alveoli, ENMs are coated by SP-A and/or SP-D, and in turn, interact with CD14 on the surface of AMs to enhance phagocytosis of the ENMs. This opsonization effect of SP-A/D are often found in the defense of microbial invasion, as mentioned above. Consistent with our observations, SP-A and SP-D are found in the corona of PEG-, PLGA-, or Lipid-modified nanoparticles [31] and SP-A increases cellular binding and uptake of nanoparticles with modification of different polymers by alveolar macrophages [32]. These observations, combined with our current results, indicate that opsonization by SP-A and/or SP-D is an important defense mechanism both in elimination of invading microbes and in the clearance of inhaled ENMs and other dusts. Of course, plasma-derived opsonins such as IgG antibodies and complements may also be involved in the clearance of ENMs in the lung, since they are found to abundantly bind to MWCNT-7, TiO₂, SiO₂ and C60. Another finding in the current study is that different ENMs induce different cytokine production, i.e., increased TNF- α by MWCNT-7, elevated IL-1 β by TiO₂, SiO₂, and enhanced IL-6 expression by C60. The difference in the induction of these cytokines, probably, and other cytokines, contributes to the difference and extent of the pulmonary inflammation by the ENMs. It is likely that expression profile is ENM-specific, although the SP-A/D-CD14 axis enhanced phagocytosis is the same. Detailed molecular mechanisms for the ENM-specific cytokine induction need further investigation. Also, it should be noted that without BALF, SP-A, or SP-D, AMs are still able to phagocytose the studied ENMs (Fig. 2 and Fig. 6), suggesting that AMs have additional mechanisms for the recognition and phagocytosis of the ENMs other than opsonization.

Conclusions

SP-A/D can opsonize all the studied ENMs to enhance phagocytosis of the ENMs by alveolar macrophages through CD14, suggesting that SP-A/D-CD14 is a common pathway mediating phagocytosis of ENMs. Cytokine production induced by the ENMs, however, is dependent on what an ENM is phagocytosed. Our results are helpful for the understanding of clearance of ENMs by alveolar macrophages and mechanisms of different ENMs-induced lung toxicity. Further studies, for example, roles of CD14 in the ENMs-induced lung inflammation and molecular mechanisms for the ENM-specific cytokine induction, are required for safety assessment of ENMs.

Materials And Methods

Preparation of Nanomaterial Suspensions

MWCNTs (MWCNT-7) were obtained from Mitsui Chemicals Co., Ltd. Tokyo, Japan; TiO₂ (rutile type, with a mean primary size of 20 nm) was provided by Japan Cosmetic Association, Tokyo, Japan; SiO₂ (with a primary size of 10–20 nm) was purchased from Sigma-Aldrich, USA; and Fullerene C60 (with a mean primary size of 1 nm) was provided by Frontier Carbon Corporation, Japan. 10 mg of these 4 types of ENMs was suspended in saline containing 0.5% (w/v) Pluronic® F-68 (a non-ionic detergent from Sigma-

Aldrich, USA) to a final concentration of 500 µg/ml. The MWCNT-7 suspension was homogenized four times, with 1 minute each time, using the Polytron PT1600E bench-top homogenizer (Kinematica, Switzerland) at a speed of 3000 rpm. The prepared four nanomaterial suspensions were sonicated at 600W for 30 minutes using the JY92-2 sonicator (Scientz Co., Ltd, Ningbo, China), 5 minutes for 6 times with 2 minute-interval rests. To ensure the dispersion and suspension of the nanomaterials, the suspensions were further sonicated for 5 minutes for 4 times just before use. Characterization of the suspended 4 nanomaterials, including shape, size distribution and element analysis, was shown in the Figure S1, S2 and S3.

Animals

Eight weeks-old female wild-type Sprague-Dawley (SD) rats were obtained from and housed in the Animal Center of Anhui Medical University, and received Oriental MF basal diet and water *ad libitum*. The animal experiment protocols were approved by the Institutional Animal Care and Use Committee.

Intratracheal spraying of nanomaterial suspensions

Twenty-five female SD rats were divided in to 5 groups and 0.5 ml of the suspensions were intratracheally sprayed with 0.5 ml of the vehicle, 500 µg/ml MWCNT-7, TiO₂, SiO₂, or C60 suspensions, using an intratracheal aerosolizer (series IA-1B, Penn-century, Philadelphia, USA), as previously described [33], 2 times per week for 2 weeks. The total amount of the administered nanomaterials was 1 mg per rat. Three days after the last spraying, the animals were sacrificed under isoflurane anesthesia, and the lung was excised, and then fixed in 4% paraformaldehyde solution in phosphate-buffered saline (PBS) adjusted to pH 7.3 and processed for light microscopic examination and transmission electron microscopy (TEM) or scanning electron microscopy.

Light microscopy and electron microscopy

Hematoxylin-eosin (HE) stained sections of the lung tissues treated with the 4 ENM suspensions were used to observe lung inflammation and localization of the nanomaterials. For transmission electron microscopic (TEM) observation of TiO₂, SiO₂ and C60, paraffin blocks were deparaffinized and small pieces of the lung tissues were embedded in epon resin and processed for nanomaterial observation using the JEM-2100 transmission electron microscope (JEOL Co. Ltd, Tokyo, Japan). Since MWCNT-7 are hard to be cut by the electronic microtome, scanning electron microscopy (SEM) was used to observe the MWCNT-7-treated lung tissues. Briefly, the HE-stained slides of the lung tissues were immersed in xylene for 3 days to remove the cover glass, immersed in 100% ethanol for 10 min to remove the xylene, and then air-dried for 2 hours at room temperature. The slides were then coated with platinum for observation using Model S-4700 Field Emission SEM (Hitachi High Technologies Corporation, Tokyo, Japan) at 5–10 kV.

Preparation of BALF and isolation of rat Alveolar Macrophages

Eight weeks-old female wild-type SD rats were sacrificed under anesthesia with intraperitoneally injected sodium pentobarbital, the lung was excised under aseptic conditions and injected with 5 ml of saline through the trachea. After gently shaking the lung, the fluid in the lung was taken out. Repeat the washing steps for another 2 times. The collected fluid was centrifuged at 1800g for 5min at 4°C, and the supernatant was BALF, concentrated with a concentrator tube (Millipore), and then stored at -80°C for later use.

The cell pellet was resuspended in RPMI 1640 medium containing 10% fetal bovine serum (Gibco, USA), seeded in a six-well plate and cultured at 37°C for 90 minutes. The cells were washed with PBS three times to remove red blood cells, other cells and cell debris, and the remaining adherent cells were stained with immunofluorescence for CD68, a macrophage marker, to confirm their identity. Briefly, the adherent cells were fixed in 4% paraformaldehyde and treated with 0.2% Triton X-100 containing 10% fetal bovine serum (Gibco)/1% bovine serum albumin in PBS at room temperature for 15 minutes, and then incubated with rabbit anti-CD68 (1:50 dilution, Bioss, Beijing, China) overnight at 4°C and added with Cy3 labelled anti-rabbit IgG (1:100 dilution, Proteintech, Wuhan, China). After washing, the cells were counter-staining with DAPI (Sigma-Aldrich). Images were captured with a fluorescence microscope (ZEISS LSM880 + Airyscan, Germany). As shown in Figure S4, more than 95% of the adherent cells were positive for CD68. About 5×10^5 - 10^6 alveolar macrophages per rat were isolated.

Binding of nanomaterials to BALF proteins, SDS-PAGE and LC-MS

1.6 ml of the four ENM suspensions (equal to 800 µg of each ENM) were incubated with 1ml of the 20-fold concentrated rat BALF at 37°C in a shaker at 200 rpm for 4–6 hours, and then centrifuged at 20000×g for 30 minutes to separate the nanomaterials from the supernatants. The precipitates were re-suspended in PBS, and the suspensions were centrifuged 20000×g for 10 minutes and the supernatants were discarded. The washing steps were repeated for another 2 times. The final ENM precipitates with their bound proteins were resuspended in 50 µl of sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) buffer (10% glycerol, 62.5 mM Tris-HCl [pH 6.8], 2% 2-mercaptoethanol, 2% SDS), heated for 5 minutes at 95°C, and then centrifuged at 20000×g for 30 minutes to dissociate the bound proteins from the nanomaterial precipitates.

After protein quantification with BCA (BestBio, Shanghai, China), 10 µl of the final suspensions was subjected for SDS-PAGE and the gels was silver-stained with a silver staining kit (BestBio). 20 µl of the remaining SDS-PAGE buffer-dissociated protein solution was added DTT to a final concentration of 100 mM and boiled 5 minutes. The samples were subjected for Liquid Chromatography Method with Tandem Mass Spectrometry (LC-MS) analysis (Shanghai Applied Protein Technology Company, Ltd, Shanghai, China).

Exposure of ENMs to primary alveolar macrophages in vitro

1×10⁶ rat primary alveolar macrophages (PAMs) were cultured at 37°C overnight in a 6-well plate in RPMI 1640 culture medium containing 10% fetal bovine serum (Gibco), washed with PBS for 3 times, further cultured in the X-VIVO™ serum-free medium (Lonza, Belgium) containing 1 µg/ml of recombinant human SP-A or SP-D, or 10% concentrated BALF for 1 hours, and the cells were treated with 1 µg/ml MWCNT-7, TiO₂, SiO₂, or C60 and cultured for 12 hours. The cells were harvested for RNA isolation and quantified polymerase chain reaction (qPCR) analysis of cytokine expression, and the culture supernatants were collected for ELISA.

qPCR and ELISA analyses of IL-1β, IL-6 and TNF-α production

Total RNA in rat PAMs was extracted with Trizol (Magen, Guangzhou, China) according to the manufacturer's instruction. 500 ng of the RNA samples was reverse transcribed using the HiScript®RT SuperMix kit (Vazyme Biotech, Nanjing, China), and qPCR analyses of IL-1β, IL-6 and TNF-α were then performed using the AceQqPCR SYBR Green Master Mix (Vazyme). GAPDH was used as an internal reference, and the relative expression of each gene was analyzed by 2^{-ΔΔCT} method. The detailed sequences of the primer pairs (forward/backward) are described as follows: CAGCAGCATCTCGACAAGAG/CATCATCCCACGAGTCACAG for IL-1β; AGTTGCCTTCTTGGGACTGA/TCCAAGATCTCCCTGAGAACA for IL-6; ACTCCCAGAAAGCAAGCAA/CGAGCAGGAATGAGAAGAGG for TNF-α; and GACATGCCGCCTGGAGAAAC/AGCCCAGGATGCCCTTTAGT for GAPDH. ELISA detection of IL-1β, IL-6, and TNF-α in the supernatants of rat primary alveolar macrophage culture was performed with the rat ELISA kits (MLBio, Shanghai, China), as described by the manufacturer.

Analysis of ENM phagocytosis by polarizing microscopy

1×10⁶ rat PAMs were seeded in a 6-cm culture dish pre-placed a circle microscope cover glass (NEST, China) and cultured in the X-VIVO™ serum-free medium (Lonza) containing 1 µg/ml of recombinant human SP-A or SP-D, or 10% concentrated BALF for 1 hours. The cells were treated with 1 µg/ml MWCNT-7, TiO₂, SiO₂, or C60 and continuously cultured for 12 hours. The cells in the cover glass were fixed in 4% paraformaldehyde and stained with HE. The stained cover glass was observed under the ECLIPSE polarizing microscope (LV100NPOL, Nikon, Japan). Total number of cells and number of the cells with brightening phagocytosed ENMs under dark background in each 20× sight field was counted, and 5 sight fields of each cover glass were randomly selected. Finally, phagocytosis rates were calculated by number of the cells with brightening phagocytosed ENMs/total number of cells.

Knockdown of LRP1, CD14 and SIRPα with siRNAs and its influence on cytokine production and phagocytosis

Small interfering RNAs (siRNA) were used to knock-down the expression of potential receptors of SP-A/D, including LDL receptor related protein 1 (LRP1), CD14 and signal regulatory protein alpha (SIRPα). 3 pairs of siRNAs for each of the genes were provided by GenePharma Co. Ltd., Shanghai, China, and 1 pair of

siRNAs with the best silencing efficacy determined by preliminary experiments were chosen for further use. The siRNA sequences are as follow: GCUAAACUCGCUCAAUCUATT/UAGAUUGAGCGAGUU- UAGCTT for CD14; CCAUCAAAACGGGCAUUCAUTT/AUGAAUGCCCGUUUGAU- GGTT for LRP1; and GCUCUAUGUACUCGCCAAATT/UUUGGCGAGUACAUAAG- AGCTT for SIRPα.

Briefly, 1×10^6 rat PAMs were seeded in each well of a 6-well plate and cultured at 37°C overnight. Negative control RNA or siRNAs for LRP1, CD14 and SIRPα were transfected into the cells using Lipofectamine 2000 (Thermo Fisher, USA). 6 hours later, the culture media were changed with the X-VIVO™ serum-free medium (Lonza) containing 1 µg/ml of recombinant human SP-A or SP-D, or 10% concentrated BALF, and the cells were treated with 1 µg/ml MWCNT-7, TiO₂, SiO₂, or C60 and continuously cultured for 12 hours. The cells were harvested for RNA isolation, qPCR analysis of silencing efficacy and cytokine expression, and western blotting; the culture supernatants were collected for ELISA.

The silencing efficacy for LRP1, CD14 and SIRPα was analyzed by qPCR, as described above, and confirmed by western blotting. The specific primers are CCAGGA- ACTTTGGCTTTGCTC/ACCGATGGACAACCTTTCAGG for CD14; CCAATTGTGC- ATTTTTCAG/GAATCAGGGGCATAGGTGAA for LRP1; and GTGTCTGTTGCT- GCTGGAGA/GCATCTTCTGGGGTGACATT for SIRPα. The expression of LRP1, CD14 and SIRPα at protein level was detected by western blotting. The cells were lysed in RIPA buffer (150mM NaCl, 50mM Tris pH 7.4, 1% sodium deoxycholate, 0.1% SDS, 1% Triton X-100, and 1mM PMSF) for 5 minutes and centrifuged at 4°C, 12000 rpm for 20min. After protein quantification with BCA kit (Bestbio), aliquots of the supernatants (20 µg protein) were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), transferred to a PVDF membrane (Millipore, Boston, USA). After blocking in 5% nonfat milk, the PVDF membranes were incubated with primary antibodies (anti-GAPDH from Peprotech, USA, 1:500 dilution, as an internal control; anti-CD14 from Bioss, Beijing, China, 1:500 dilution; anti-SIRPα from Cell Signaling Technology, Danvers, USA, 1:1000 dilution; anti-LRP1 from Abcam, USA, 1:10000 dilution) at 4°C overnight. After three washings, the PVDF membrane was incubated with peroxidase conjugated anti-mouse or anti-rabbit secondary antibodies (1:10,000) for 60 minutes. The protein was visualized with ECL (Thermo Fisher) detection solution in the GEL Imaging System (Tanon, Shanghai, China).

Influence of knockdown of LRP1, CD14 or SIRPα on IL-1β, IL-6 and TNF-α production was analyzed by qPCR and ELISA, and its effect on ENMs phagocytosis was assessed by the polarized microscopy, as described above.

Statistical Analysis

Statistical analysis was performed using SSPS17 software. The statistical significance was analyzed using two tailed Student's t test. A p value of < 0.05 was considered to be significant.

Abbreviations

ENMs, engineered nanomaterials; SP-A/D, surfactant-associated proteins A/D; AMs, alveolar macrophages; PAMs, primary alveolar macrophages; PRRs, pattern-recognition receptors; PAMPs, pathogen-associated molecular patterns; BALF, bronchoalveolar lavage fluid; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; LC-MS, Liquid Chromatography Method with Tandem Mass Spectrometry.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

Available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

QQW, QW, ZZh, JF, and LQ were responsible for the experiments; QQW and QW analyzed the data; QW wrote the initial manuscript, and DBA and JX revised the manuscript; HTs, DZh and JX designed the study and provided the funding.

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Figures

Figure 1

Lung inflammation and Phagocytosis of the ENMs by alveolar macrophages Female SD rats were intratracheally sprayed with 0.5 ml of 500 $\mu\text{g}/\text{ml}$ MWCNT-7, TiO_2 , SiO_2 , or C60 suspensions. Lung inflammation in the HE-stained slides was examined under light microscopy (the left panel), and phagocytosis of the ENMs were observed under electron microscopy (the right panel). A & E, B & F, C & G, and D & H were images treatment with MWCNT-7, TiO_2 , SiO_2 , or C60, respectively.

Figure 2

Influence of BALF on phagocytosis of the ENMs by primary alveolar macrophages Primary alveolar macrophages were in vitro exposed to 4 types of ENMs (MWCNT-7, TiO₂, SiO₂, and C60) in the absence (A, B, C and G) or presence (D, E, F and H) of BALF, and phagocytosed ENMs were examined by polarized microscopy under 40×manification. A & D, B & E, C & F, and G & H were imagines treatment with MWCNT-7, TiO₂, SiO₂, or C60, respectively. Arrows indicate the alveolar macrophages with phagocytosed ENMs; bars=50 μm. Because C60 was not brightening under polarized microscopy, only percentages of alveolar macrophages with burden-MWCNT-7, TiO₂ and SiO₂ were calculated (I).

Figure 3

Analysis of the ENMs-bound proteins 800 µg of MWCNT-7, TiO₂, SiO₂, or C60 was incubated with 1ml of the 20-fold concentrated rat BALF at 37°C, and the bound proteins were disassociated for SDS-PAGE and LC/MS. A, imagine of SDS-PAGE; B, Venn diagram showing the distribution of the binding proteins; and C, abundance of SP-A, SP-D and SP-B in the bound proteins of each types of the ENMs.

Figure 4

Effects of SP-A and SP-D on cytokine production Rat primary alveolar macrophages cultured in the X-VIVO™ serum-free medium containing 1 μ g/ml of recombinant human SP-A or SP-D, or 10% concentrated BALF were treated with 1 μ g/ml MWCNT-7, TiO₂, SiO₂, or C60 and cultured for 12 hours. The RNAs isolated from the treated cells were used for qPCR analysis of TNF- α , IL-1 β and IL-6 (A, B, C & D), and the collected culture media were used for ELISA detection of TNF- α , IL-1 β and IL-6 (E, F, G & H).

The treatments with MWCNT-7, TiO₂, SiO₂, and C60 were indicated in A & E, B & F, C & G, and D & H for MWCNT-7, TiO₂, SiO₂, and C60, respectively. *, **, *** and **** are expressed as a p value less than 0.05, 0.01, 0.001 and 0.0001, respectively, compared with the negative control (NC).

Figure 5

Influences of LRP1, CD14 or SIRPα Knockdown on cytokine production Expression of LRP1, CD14 or SIRPα in rat primary alveolar macrophages were knocked down (k/d) by the gene-specific siRNA and were

treated with 1 $\mu\text{g/ml}$ MWCNT-7, TiO_2 , SiO_2 , or C60 in the X-VIVO^{TM} serum-free medium containing 1 $\mu\text{g/ml}$ of recombinant human SP-A or SP-D, or 10% concentrated BALF for 12 hours. The cells were harvested for RNA isolation and qPCR analysis (A, B, C & D) and the culture supernatants were collected for ELISA (E, F, G & H). A & E indicate detection of TNF- α in the MWCNT-treated cells; B & F and C & G for detection of IL-1 β in the TiO_2 - or SiO_2 -treated cells; and D & H for detection of IL-6 in the C60 -treated cells. *, ** and *** are expressed as a p value less than 0.05, 0.01 and 0.001, respectively, compared with the negative control (NC).

Figure 6

effects of CD14 Knockdown on phagocytosis of MWCNT-7, TiO_2 and SiO_2 Phagocytosis between primary alveolar macrophages (A, C, E, G & I) and CD14-silenced primary alveolar macrophages (B, D, F, H & J) was compared under polarized microscopy. A & B indicate the treatment with MWCNT-7 in the presence of SP-A; C & D for the treatment with TiO_2 in the presence of SP-A; E & F for the treatment with TiO_2 in the presence of SP-D; G & H for the treatment with SiO_2 in the presence of SP-A; and I & J for the treatment with SiO_2 in the presence of SP-D. K indicates percentages of alveolar macrophages with burden-MWCNT-7, TiO_2 and SiO_2 . Arrows indicate the alveolar macrophages with phagocytosed ENMs; bars=50 μm .

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