Video Article

Bronchoalveolar Lavage Exosomes in Lipopolysaccharide-induced Septic Lung Injury

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Abstract

Acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) represent a heterogeneous group of lung diseases which continues to have a high morbidity and mortality. The molecular pathogenesis of ALI is being better defined; however, because of the complex nature of the disease molecular therapies have yet to be developed. Here we use a lipopolysaccharide (LPS) induced mouse model of acute septic lung injury to delineate the role of exosomes in the inflammatory response. Using this model, we were able to show that mice that are exposed to intraperitoneal LPS secrete exosomes in Broncho-alveolar lavage (BAL) fluid from the lungs that are packaged with miRNA and cytokines which regulate inflammatory response. Further using a co-culture model system, we show that exosomes released from macrophages disrupt expression of tight junction proteins in bronchial epithelial cells. These results suggest that 1) cross talk between innate immune and structural cells through the exosomal shuttling contribute to the inflammatory response and disruption of the structural barrier and 2) targeting these miRNAs may provide a novel platform to treat ALI and ARDS.

Video Link

The video component of this article can be found at https://www.jove.com/video/57737/

Introduction

ALI and ARDS are the life-threatening forms of respiratory failure with severe hypoxemia caused by non-cardiogenic pulmonary edema which affects approximately 1 million people worldwide annually[1]. The etiology of ARDS includes direct injury to the lungs from infections or aspiration and a variety of indirect insults. Over the last decade there has been an increased understanding of the molecular pathogenesis of ARDS, however, specific targeted treatments for ARDS are yet to be developed[2,3].

Several animal models of acute lung injury have been developed which provide a bridge for translating experimental therapies to human studies[4,5]. Commonly used models include local installation of oleic acid, bacteria, LPS, and bleomycin. Other approaches include ischemia-reperfusion, cecal ligation puncture, mechanical ventilation-induced stretch injury, hyperoxia or systemic administration of bacteria and LPS[5]. These models provide a useful biological system to test clinical hypotheses and for the development of potential therapies. To simulate human ARDS, animal models should reproduce inflammation and acute injury to the epithelial and endothelial cells with defects in barrier function in the lungs.

Exosomes are membrane vesicles with 20 - 200 nm in diameter, whose molecular content contains proteins, DNA, RNA, and lipids, and facilitate inter-cellular communications in tissue microenvironment via molecular composition transfer. Exosomes are secreted by multiple types of cells, such as endothelial cells, epithelial cells, smooth muscle cells and tumor cells, and exist in human body fluid. Studies indicate that exosomes regulate cross-talk between immune cells and stromal cells during infectious and sterile inflammatory diseases, and their abnormal release appears to be regulated by various natural and experimental stimuli during physiological and pathological processes[6]. Such communication network may play an important role in the pathogenesis of lung diseases and may affect pathophysiological progression[7,8]. As 18 - 22 nucleotide non-coding RNAs, miRNAs exist in both tissue and body fluids, plasma, sera, and modulate mRNA expression at post-translational level[9,10].

Packaged miRNAs in exosomes influence differentiation and function of multiple types of cells, and excessive levels are associated with a variety of diseases, including cancer, lung diseases, obesity, diabetes, and cardiovascular disease[11,12,13,14,15,16]. Entry into the recipient cells and shuttling of exosomal miRNAs facilitate intercellular communications modifying the hemostasis of microenvironment[17,18]. Acute lung-injury is a complex processes, involving multiple cell types with extensive intercellular communications through exosomes[8]. miR-155 and miR-146a share the common transcriptional regulatory mechanism and contribute to the inflammatory response and the immune tolerance[19,20]. Recent studies indicate that both modulate inflammatory response via exosomal miRNAs shuttling between immune cells[21]. However, the molecular mechanisms underlying modulatory effects of exosomal miRNAs on alveolar response to endotoxin remain unclear, undoubtedly the potential clinical relevance and translational implication merit further investigation.

Co-culture models are being employed to define the interaction of the specific cell types in the complex environment, such as inflammation and cancer[22,23]. These platforms provide an alternative strategy to interrogate cross talk between cell types particularly for immune and structural cells.

Intra-tracheal, aerosolized, intraperitoneal or systemic administration of LPS are widely used to induce experimental lung injury[24,25,26], and have shown to induce epithelial and endothelial permeability defects. Here we use intraperitoneal LPS to induce a septic model of acute lung injury in mice. Within 24 h of intraperitoneal administration of LPS, permeability defects are induced in the lungs with recruitment of inflammatory cells. Further, we show that exosomes from BAL contain miRNA-155 and miR-146a, and exosomes from BAL fluid induce pro-inflammatory cytokines expression in recipient epithelial cells, including IL-6 and TNF-α. These data are the first to show that exosomal miRNAs are secreted in BAL in this model of acute septic lung injury.

Protocol

The overall protocol requires 2 days, including the first day of sepsis induction and isolation of BAL fluid from the animal, and the second day for exosomes isolation from the mouse BALF. All procedures have been reviewed and approved by the Institutional Animal Care and Use Committee at Atlanta VA medical center.

1. Mouse Acute Septic Lung Injury Model

1. Use 6 - 8 weeks- old male wild-type C57BL/6J mice (weight 20 - 22 g) for the animal model, and perform all procedures using aseptic techniques and instruments. Autoclave all surgical instruments, including forceps and scissors for 20 min at 121 °C.
2. Randomly distribute mice into experimental and control group. Restrain mice on a surgical board. Treat mice with *Escherichia coli* lipopolysaccharide (LPS, 15 mg/kg) by intraperitoneal route. According to the individual body weight, dilute appropriate LPS dosages to 0.1 mL PBS per mouse, then inject LPS solution using a 1 mL syringe with 27 G needle and inject an equivalent volume of PBS to control animals.
3. Keep mice in the animal BSL2 facility, which consists of clean cages at appropriate room temperature.
4. 24 h later, euthanize mice with CO_2 inhalation.
5. Select the surgical area in the center of mouse neck. Make a small incision (approximately 5 mm), and gently separate muscles for the access to the trachea using blunt forceps until the trachea is exposed.
6. Withdraw mouse BAL fluid with 10 mL sterile syringe with a 27 G needle. First, gently, insert the needle into the trachea and inject 1 mL of sterile PBS slowly into the trachea, then draw BALF into a 3 mL disposable syringe with a 27 G needle. Upon completion of the procedure, dispose of syringes and needles in biohazard sharps container.
7. Treat surgical instruments with chlorine dioxide, as described previously by Chauret *et al.*[27], and then sterilize all surgical instruments by autoclaving for 20 min at 121 °C.

2. Exosomes Isolation-serial Ultra-centrifugation and Characterization

1. Collect mouse BAL fluid in 15 mL conical tubes. To harvest alveolar cells, centrifuge samples for 10 min at 1,000 g, at 4 °C.
2. Transfer the supernatant to new 15 mL conical tubes, then centrifuge samples for 30 min at 4,000 x g at 4 °C to remove cell debris.
3. Collect the supernatant in ultracentrifuge tubes and centrifuge samples for 60 min at 10,000 x g at 4 °C to remove larger cellular particles.
4. Transfer the supernatant to new conical tubes, then filter the samples through 0.2 µm syringe-filter to remove remaining larger particles.
5. Collect samples in ultracentrifuge tubes, and balance tubes. Centrifuge samples for 2 h at 140,000 x g at 4°C to pellet the exosomes.
   Note: The size of exosomes varies 20 - 200 nm. 0.2 µm filter is used for removing larger particles that are more than 200 nm in size.
6. Discard the supernatants gently. Dissolve the pellet with 100 µL PBS or lysis solution, and transfer exosomes samples to 1.5 mL tubes, and store at -80 °C until use.
   Note: Transmission electron microscope is recommended to verify isolated exosomes.
7. Follow the standard TEM sample processing protocol[28]. Analyze exosomes samples using transmission electron microscopy, and take images, scale bar 200 nm.

3. Preparation of miRNA and Performing miRNA- q-RT-PCR

Note: miRNA-q-RT-PCR is recommended to analyze expression level of miRNAs.

1. Follow standard miRNAs isolation protocol and make sure to use an equal amount of purified RNAs in single-strand reverse transcription[24].
2. Follow the standard real-time PCR protocol to detect exosomal microRNA level in BAL fluid. Use specific real-time PCR primers against miR-155, miR-146a, and U6 SnRNA. Run PCR reaction with the quantitative PCR apparatus and normalize the expression values to internal control U6 snRNA.
   Note: Each group consists of triplicate samples[24].

4. Exosomes Labeling and Purification

1. Suspend exosomes pellet with 100 µL suspension solution in a 1.5 mL polypropylene tube, then add 0.4 µL of the exosomes dye into the other tube with 100 µL of the suspension solution.
2. Quickly add exosomes in the solution to the dye solution and mix it thoroughly by pipetting. Keep samples for 5 min at room temperature, protect from light.
3. Put one exosome spin column in a 1.5 mL elution tube. Transfer the solution mixture to the center of the exosome spin column.
4. Spin the column for 2 min at 800 x g at room temperature.

5. Discard the column and store the eluted sample at -20 °C until use.

5. Validation of Exosomes Up Taken by Recipient Cells

1. Seed 1 x 10^4 MLE 12 /cell in an 8-well chamber slide, and culture cells at 37 °C, 5% CO_2.
2. Add 10 µL fluorescent-labeled exosomes to the cells. Incubate for 1 h at 37 °C.
3. Remove culture media and wash cells with PBS, then fix cells with 1% paraformaldehyde for 10 mins at room temperature.
4. Mount the slide in mount medium with DAPI.
5. Analyze samples through a fluorescence microscope and take images, 40X magnification.

6. Verification of Interaction between Exosomes and Recipient Cells

1. Seed 2 x 10^5 MLE12/well in a 12-well culture plate containing appropriate culture medium, and culture cells at 37 °C, 5% CO_2.
2. Add 20 µg exosomes from mouse BAL fluid to MLE12 cells and set up replicates per experimental group.
3. 24 h later, wash cells with PBS and add 600 µL lysis buffer into well. Gently shake the plate and collect lysis samples in 1.5 mL tube with the pipette.
4. Follow the standard total RNA isolation protocol and cDNA reverse transcription protocol, complete the single-strand cDNA reverse transcription[29].
5. Follow the standard real-time PCR protocol to detect mRNA expression level in recipient cells[29]. Use specific real-time PCR primers against TNF-α, IL-6, and ZO-1. Run PCR reactions with the quantitative PCR apparatus and normalize the expression values to the internal control GAPDH.
   Note: Each group consists of triplicate samples.

Representative Results

To induce septic lung injury, mice were treated with intraperitoneal LPS (15 mg/kg). Within 24 h of LPS administration, neutrophilic influx was seen in the lungs, as shown in **Figure 1A**. Mouse BAL fluid was drawn, followed by isolation and purification of exosomes. Morphology of BALF exosomes was confirmed by electron transmission microscopy (**Figure 1B**).

Expression of selective pro-inflammatory miRNAs miRNA-155 and miR-146a were detected by q-RT-PCR from the purified exosomes. Expression levels of miR-155 and miR-146a were significantly increased in BAL fluid exosomes from mice that were treated with LPS, compared to control mice treated with PBS (miR-155, 1 ± 0.16 vs 11.6 ± 0.6; miR-146a, 1 ± 0.12 vs 13.5 ± 1.186) (**Figure 2**).

To define the functional effects of exosomes, mouse bronchial epithelial cells (MLE12 cells) were treated with purified exosomes extracted from BAL fluid of mice treated with LPS or PBS, respectively. First, we proved entry of BALF exosomes into recipient epithelial cells. BALF exosomes were labeled with PKH-67, and co-cultured with MLE12 cells, and then fluorescence microscope images show the uptake of exosomes by epithelial cells (**Figure 3A**). Expression of pro-inflammatory mediator, TNF-α and IL-6 were significantly increased in epithelial cells that were co-cultured with exosomes from LPS treated mice, compared to control group (TNF-α, 1 ± 0.13 vs 5.01 ± 0.22; IL-6, 1 ± 0.02 vs 1.72 ± 0.17) (**Figure 3B**). We also determined the expression of tight junction protein ZO-1 as a surrogate for epithelial integrity. Cells that were treated with exosomes from LPS treated mice showed a significantly attenuated expression of ZO-1, compared to LPS treated control mice (ZO-1, 1 ± 0.12 vs 0.66 ± 0.13) **(Figure 3C)**.

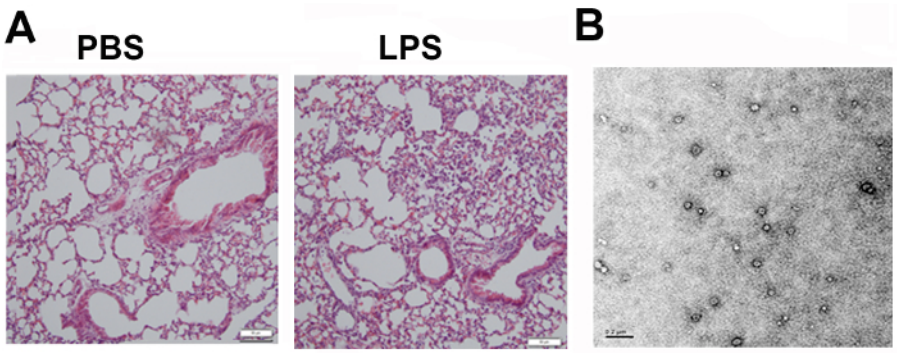

Figure 1: **Characterization of exosomes isolated from BAL fluid of LPS-treated mice.** C57BL/6J mice were treated with intraperitoneal LPS (15mg/kg) injection (N = 5), PBS was used as control (N = 5). 24 h later, mice were euthanized, and BAL fluid was performed. Representative images of mouse lung tissue treated with LPS and PBS showing acute lung injury, scale bar, 50 µm, A). Exosomes were extracted from BAL fluid. Transmission electron microscopy (TEM) analysis showing exosomes, scale bar, 200 nm, B). Please click here to view a larger version of this figure.

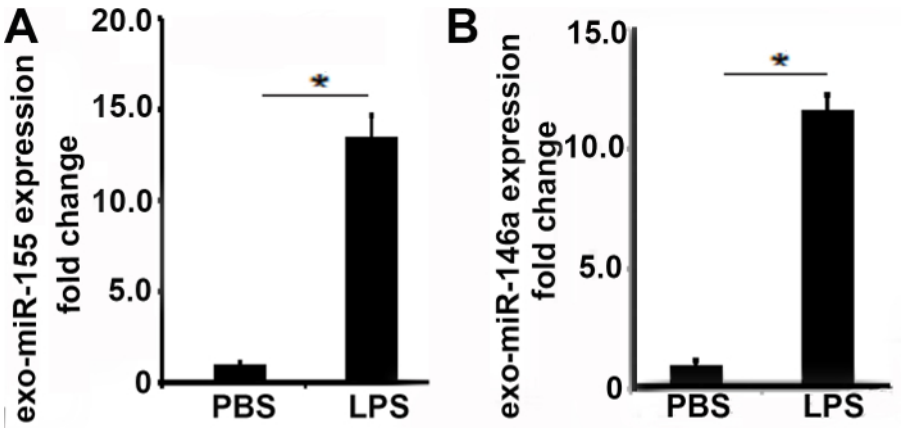

Figure 2: **Exosomal miRNAs expression in BAL fluid isolated from LPS-treated mice.** C57BL/6J mice were challenged with intraperitoneal LPS. Expression of selective microRNAs were analyzed through q-RT-PCR and normalized to U6 expression**.** A) miR-155; B) miR-146a. n = 5 mice per group. Student's test, *P <0.05. Please click here to view a larger version of this figure.

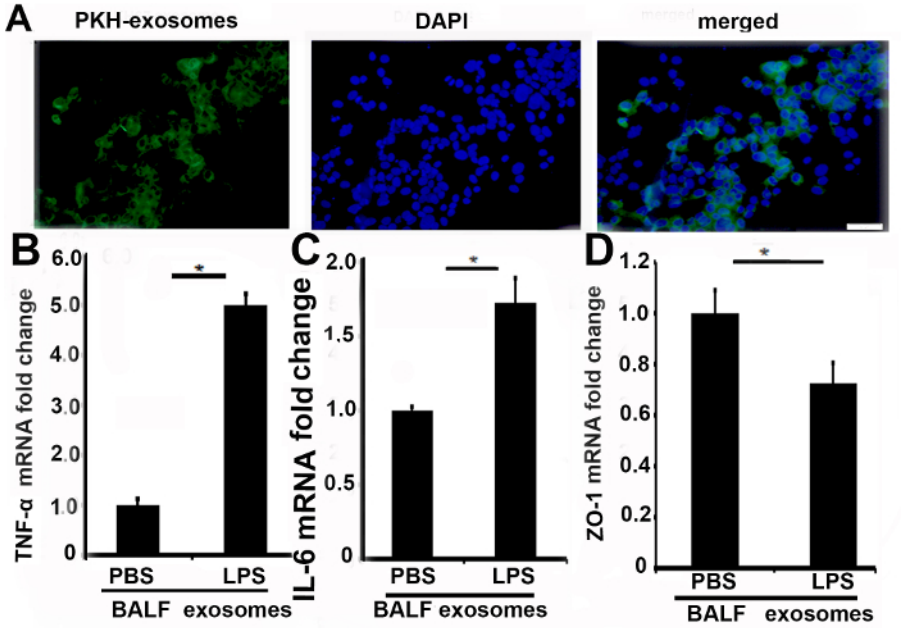

Figure 3: **Expression of inflammatory cytokines and tight junction protein in mouse epithelial cells treated with exosomes.**BAL fluid-derived exosomes were labeled with PKH-67, and fluorescence microscope showing PKH67 labeled exosomes (Green), DAPI(Blue) and merged images of show uptake of exosomes in MLE12 cells, scale bar, 50 µm, as shown in A). Mouse epithelial cells MLE12 were co-cultured with exosomes isolated from BAL fluid from mice treated with LPS or PBS. 24 h later, cells were harvested, and total RNA was isolated. Expression of TNF-α and IL-6 was measured with q-RT-PCR, normalized to GAPDH internal control. (B)TNF-α and (C) IL6; (D) ZO-1 mRNA expression was measured with q-RT-PCR. *P <0.05, Student's test. Please click here to view a larger version of this figure.

Discussion

Mouse models of diseases are commonly used to evaluate the physiological function of specific genes and to reduce the cost of experimentation[2]. The acute septic lung injury described here mimics inflammatory response seen in humans with ARDS. This model is relevant to investigate the molecular pathogenesis, development of biomarkers and to test potential new therapies[5].

Co-culture systems are relevant for *in vivo* studies in the context of investigating potential mechanisms of transfer of biological molecules such as RNA, DNA, lipids, and protein in an in vitro system[23,30]. Some of these platforms have great utility in the assessment of mechanistic understanding and development of therapies for complex diseases[22,23]. As shown here the exosomes from lavage cells (macrophages, neutrophils) can impact the integrity of structural epithelial cells through transfer of exosomes.

The pathobiological effects of exosomes are emerging as critical aspect in human diseases both from the diagnostic and the therapeutic standpoint and understanding disease pathogenesis[31,32]. To isolate exosomes from biological fluids, the most commonly-used technique includes differential centrifugation coupled with ultracentrifugation as described here[33,34]. This technique has several strengths, although is limited by the fact that it can be time-consuming and the risk of contamination with non-exosomal proteins. Novel approaches have been proposed to

overcome these limitations, such as the exosome isolation by the density gradient or the immune-affinity or the size-dependent method[35]. Exosomes are being investigated in clinical studies as biomarkers for diagnosis and prognosis, and development of targeted therapies.

Disclosures

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