Pathologic Mechanical Stress and Endotoxin Exposure Increases Lung Endothelial Microparticle Shedding

Eleftheria Letsiou¹, Saad Sammani¹, Wei Zhang^{2,3}, Tong Zhou⁴, Hector Quijada⁴, Liliana Moreno-Vinasco¹, Steven M. Dudek¹, and Joe G. N. Garcia⁴

¹Division of Pulmonary, Critical Care, Sleep, and Allergy, Department of Medicine, ²Department of Pediatrics, and ³Institute of Human Genetics, University of Illinois, Chicago, Illinois; and ⁴Department of Medicine, University of Arizona, Tucson, Arizona

Abstract

Acute lung injury (ALI) results from infectious challenges and from pathologic lung distention produced by excessive tidal volume delivered during mechanical ventilation (ventilator-induced lung injury [VILI]) and is characterized by extensive alveolar and vascular dysfunction. Identification of novel ALI therapies is hampered by the lack of effective ALI/VILI biomarkers. We explored endothelial cell (EC)-derived microparticles (EMPs) (0.1–1 μ m) as potentially important markers and potential mediators of lung vascular injury in preclinical models of ALI and VILI. We characterized EMPs (annexin V and CD31 immunoreactivity) produced from human lung ECs exposed to physiologic or pathologic mechanical stress (5 or 18% cyclic stretch [CS]) or to endotoxin (LPS). EC exposure to 18% CS or to LPS resulted in increased EMP shedding compared with static cells ($\sim$ 4-fold and $\sim$ 2.5-fold increases, respectively). Proteomic analysis revealed unique 18% CS-derived ($n = 10$) and LPS-derived EMP proteins ($n = 43$). VILI-challenged mice (40 ml/kg, 4 h) exhibited increased plasma and bronchoalveolar lavage CD62E (E-selectin)-positive MPs compared with control mice. Finally, mice receiving intratracheal instillation of 18% CS-derived EMPs displayed significant lung inflammation and injury. These findings indicate that ALI/VILI-producing stimuli induce

significant shedding of distinct EMP populations that may serve as potential ALI biomarkers and contribute to the severity of lung injury.

Keywords: microparticles; ventilator-induced lung injury; cyclic stretch; LPS; proteomics

Clinical Relevance

Acute lung injury (ALI) results from infectious challenges and pathologic lung distention produced by excessive tidal volume delivered during mechanical ventilation (ventilator-induced lung injury [VILI]) and is characterized by extensive alveolar and vascular dysfunction. Identification of novel ALI therapies is hampered by the lack of effective ALI/VILI biomarkers. We explored endothelial cell (EC)-derived microparticles (EMPs) (0.1–1 μ m) as potentially important markers and potential mediators of lung vascular injury in preclinical models of ALI and VILI. Our findings indicate that ALI/VILI-producing stimuli induce significant shedding of distinct EMP populations that may serve as potential ALI biomarkers and contribute to the severity of lung injury.

Acute lung injury (ALI), and its more severe form acute respiratory distress syndrome (ARDS), develop in response to major insults such as sepsis, trauma, pneumonia, and gastric content aspiration (1). Although mechanical ventilation (MV) remains the main supportive treatment for patients with

ALI, it is well recognized that MV may cause or worsen lung injury, a syndrome known as ventilator-induced lung injury (VILI) (2). ALI and VILI potentially develop via distinct pathogenetic mechanisms but share similar characteristics, such as increased release of inflammatory

mediators and disruption of the integrity of alveolar and vascular endothelial barriers, events associated with profound physiologic impairment (1, 2). Given the excessively high mortality of ALI (> 30%), there is considerable interest in identifying the underlying pathogenetic mechanisms

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Correspondence and requests for reprints should be addressed to Joe G. N. Garcia, M.D., Arizona Health Sciences Center, University of Arizona, Drachman Hall, Room B-207, 1295 North Martin Avenue, Tucson, AZ 85721. E-mail: skipgarcia@email.arizona.edu

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and novel biological markers to further the development of therapeutic ALI/VILI interventions (3).

Although there is a paucity of ALI biomarkers, there is increasing recognition that circulating microparticles (MPs) shed by activated or apoptotic cells are potential inflammatory mediators and biomarkers in specific disease states (4, 5). MPs are defined as submicron membrane vesicles (0.1–1 μm) displaying exposed phosphatidylserine moieties and encargoed with proteins, lipids, and nucleic acids that reflect cellular origin and the MP-triggering process (5, 6). These vesicles are formed during membrane blebbing through a regulated process requiring calcium release and cytoskeletal rearrangement (6). Originally considered to be only artifactual, MPs are now recognized as important biological messengers delivering antigens, lipids, messenger RNA, microRNA, and receptors to downstream targets (4, 7, 8). MPs derived from endothelial cells (EMPs) are produced after challenge with cytokines, endotoxin (LPS), thrombin, and other inflammatory agonists and have emerged as markers of endothelial cell (EC) dysfunction (9–12). Released EMPs impair vascular function by initiating coagulation (13), attenuating NO production from ECs (9, 14), stimulating the release of inflammatory cytokines, and disrupting EC barrier integrity (14, 15). EMP shedding occurs at very low levels under normal conditions but is markedly increased in plasma during pathologic states, including vascular diseases (11, 16–18), with unique disease-specific alterations in MP composition (19, 20).

Recent reports indicate that MPs bearing tissue factor are present in edema fluid from patients with ARDS (21) and that EMPs derived from plasminogen activator inhibitor-challenged ECs may contribute to ALI development via increased cytokine production and neutrophil recruitment (15). Recently, we demonstrated that the sphingosine 1-phosphate receptor, S1PR3, is a novel ALI biomarker released into circulation within biologically active microvesicles (22). Although these studies suggest an important role of MPs in ALI pathogenesis, the role of lung EMPs as potential ALI/VILI markers and mediators has not been fully addressed.

In this study, we sought to determine if ALI/VILI-relevant stimuli induce release of distinct lung EC MP populations by characterizing the number and proteome of

MPs derived after EC exposure to mechanical stretch or LPS *in vitro*. We further explored the release of MPs during VILI and studied the effects of mechanical stretch-derived EMPs on lung function in mice. A portion of these results have been previously reported in abstract form (23, 24).

Materials and Methods

Detailed descriptions of the methods are provided in the online supplement.

EMP Isolation, Characterization, and Quantification

Human pulmonary artery ECs (Lonza, Walkersville, MD) were exposed to either low or high magnitude (5 or 18% cyclic stretch [CS]) (15 cycles/min, 4 or 24 h) mechanical CS. Cells grown on bioflex plates were kept in static condition (control) in the same incubator. ECs were also exposed to LPS (1 $\mu\text{g}/\text{ml}$) for 24 hours. After CS or LPS exposure, EC culture media was differentially centrifuged, and the final supernatant (free of dead cells, large debris, and apoptotic bodies) was ultracentrifuged at $21,000 \times g$ for 70 minutes (Beckman-L8M; Beckman Coulter, Pasadena, CA) to isolate EMPs. The size and shape of EMPs were examined by transmission electron microscopy (TEM). MP samples were further analyzed by flow cytometry using an LSR Fortessa (Becton-Dickinson, Franklin Lakes, NJ). MPs were double-stained for annexin V-FITC and CD31-PE (eBioscience, San Diego, CA). For quantification of MPs, a known amount of countbright absolute counting beads (Molecular Probes, Grant Island, NY) was assayed in parallel. Results were expressed as the number of EMPs (double-positive events for annexin V and CD31, $< 1 \mu\text{m}$ in size)/ μl .

Nano Liquid Chromatography–Tandem Mass Spectrometry Analysis of EMPs

Stimulus-specific MP populations were isolated, and samples were processed by nano-scale liquid chromatography–tandem mass spectrometry (Orbitrap Velos Pro). All tandem mass spectrometry samples were analyzed using Mascot (Matrix Science, London, UK). Scaffold (Proteome Software Inc., Portland, OR) was used to validate tandem mass spectrometry–based peptide and protein identifications.

Gene Ontology, Pathway, and Network Analyses

For these analyses, proteins were analyzed using the corresponding gene name. Genes were assigned functional annotations based on the Gene Ontology (GO) (25) and Kyoto Encyclopedia of Genes and Genomes (KEGG) (26) databases using the (DAVID) tool (27). The Reactome FI (functional interaction) Cytoscape (28) plugin was used to demonstrate functional interactions among identified and linker genes.

VILI Model and Analysis of Plasma/BAL MPs

All animal experiments were approved by the Animal Care and Use Committee at the University of Illinois at Chicago. Male C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME), age 8 to 10 weeks, were subjected to high tidal volume MV (VILI) (tidal volume: 40 ml/kg, 65 breaths/min; PEEP: 0 cm H_2O). Spontaneously breathing (SB) mice served as controls. After 4 hours, blood and BAL were collected, plasma/BAL MPs were quantified after staining with annexin V-FITC or CD62E-PE (BD Biosciences, San Jose, CA), and plasma MPs were analyzed by immunoblotting for VE-cadherin (Santa Cruz Biotech, Santa Cruz, CA).

EMP Function

ECs were exposed *in vitro* to EMPs or LPS (1 $\mu\text{g}/\text{ml}$) for the indicated time. Cell lysates were processed for immunoblotting analysis. For *in vivo* studies, EMPs were generated from 9×10^6 ECs, isolated, and resuspended in 40 μl PBS, and sterile PBS (control group), control EMPs (ctr-EMPs, derived from static EC), or 18% CS-derived EMPs were intratracheally instilled in mice. After 18 hours, BAL and lung tissues were collected, with quantification of BAL cell counts (total and differential), BAL protein levels, and lung tissue myeloperoxidase (MPO) activity.

Statistical Analysis

Differences between groups were analyzed using Student's *t* test or one-way ANOVA (Bonferroni post test). $P < 0.05$ was considered statistically significant.

Results

Characterization of EMPs Shed after Exposure to CS or LPS

We exposed human ECs to CS or LPS as described in MATERIALS AND METHODS.

Figure 1A demonstrates that pathologic 18% CS induces actin microfilament reorientation in ECs as previously described (29). ECs exposed to physiologic 5% CS, a level of mechanical stretch that mirrors normal tidal breathing, exhibit similar patterns to 18% (data not shown). Inflammatory agonists, such as LPS, are potent inducers of actin rearrangement (30), with substantial loss of EC barrier integrity reflected by prolonged reductions in transendothelial resistance (TER) in ECs grown on microelectrodes (Figure 1B). Electron microscopic examination of centrifuged media from 18% CS-, and LPS-challenged ECs identified small particles consistent with EMPs (a range of MP size from 100 to 800 nm was observed for all conditions). Figure 1C depicts TEM pictures of single MPs derived under different conditions, and Figure E1A in the online supplement (TEM picture of an 18% CS-derived EMP sample) demonstrates the heterogeneity in MP size (0.1–0.8 μm) in these samples. After isolation, EMPs were characterized by flow cytometry. To define the MP gate (upper limit of forward scatter), latex beads of known size (0.8 and 1 μm) were used (Figure 1D). Figures 1E, 1F, and 1G depict representative scattergrams for the 18% CS-derived EMP population. Flow cytometry analysis revealed that > 95% of isolated gated particles (21,000 $\times$ g) were < 1 μm (Figure 1E). EMPs were double stained positively for annexin V, a known marker of MPs (annexin V binds to MP phosphatidylserine), and CD31 (Figure 1F). EMPs also were double stained for IgG isotype-PE and annexin V-FITC in the absence of Ca^{2+} (incubation with EDTA) to determine the background fluorescence of EMPs (negligible) (Figure 1G). Only events that were double positive for annexin V and CD31 and < 1 μm were classified as EMPs and counted. A known amount of countbright beads (7 μm) was assessed in parallel to determine the absolute number of EMPs (Figure E1B).

Quantification of Endothelial MP Shedding by CS or LPS

The production of EMPs was quantified in ECs in the basal state and after exposure to CS or LPS. Flow cytometric data demonstrated that 4-hour exposure to 5% CS or 18% CS did not increase EMP levels significantly (Figure 2A). ECs exposed to 5% CS for 24 hours induced a 2-fold

increase in number of EMPs but did not achieve statistical significance ($P = 0.54$, Bonferroni *post hoc* test). In contrast, 24 hours of 18% CS produced significant increases in EMPs (~ 4 -fold) compared with untreated ECs ($P < 0.001$) and ~ 2 -fold compared with 5% CS ($P = 0.0013$). LPS also induced EMP release (~ 2.5 -fold compared with controls; $P = 0.037$). EMP lysates isolated at 24 hours were analyzed in parallel by immunoblotting for CD31. Figure 2B depicts highly increased CD31 expression in EMP lysates after 24 hours of exposure to 18% CS or LPS in comparison to control EMP lysates. Neither flow cytometry analysis (data not shown) nor immunoblotting detected soluble CD31 immunoreactivity (Figure 2B) in EMP-free

media supernatants. To explore whether the shedding of EMPs after CS is due to apoptosis or cell death, cell cultures exposed to 5% or 18% CS were analyzed by flow cytometry using the annexin V/propidium iodide (PI) assay. There were no significant differences in the percentages of necrotic (PI staining) and apoptotic (annexin V/PI staining) cells for any of the conditions (static, 5% CS, or 18% CS) (data not shown).

ECs Are Activated by Exposure to EMPs Released by 18% CS

We next investigated the temporal effects of EC exposure to basal (static or control) or to 5% CS- or 18% CS-derived EMPs on mitogen-activated protein kinase (MAPK)

Figure 1. Characterization of endothelial microparticles (MPs) shed after exposure to cyclic stretch (CS) or LPS. (A) F-actin staining of static or 18% CS human endothelial cells (ECs). Representative images of multiple independent experiments are shown. (B) Transendothelial electrical resistance measurements of ECs challenged with LPS (1 $\mu\text{g}/\text{mL}$). Data are presented as mean $\pm$ SEM ($n = 6$ per condition). (C) Transmission electron micrographs of single endothelial MPs (EMPs) isolated from the media of static ECs or ECs exposed to 18% CS (24 h) or LPS (24 h). Pictures were taken under different magnifications. Scale bars are 100 to 200 nm as shown (100 nm for control EMP (ctr-EMP) and 200 nm for CS and LPS EMP). (D) Latex beads of 0.8 and 1 μm were used to define the MP gate (gate events < 1 μm ; MP gate: R1). (E) Representative scattergram from flow cytometric analysis of an unstained 18% CS-derived EMP population with 95% of the total events within the MP gate. (F) Double-positive events (< 1 μm) for annexin V-FITC and CD31-PE were defined EMPs and counted for each sample. (G) Double staining of EMPs for isotype-PE and annexin V-FITC in the presence of EDTA.

activation and signaling. Western blot analysis detected activation of the ERK1/2 pathway (after 60 min challenge) in ECs exposed to EMPs derived from 18% CS-treated ECs but not EMPs from unchallenged ECs (Figure 3). Similar to static-derived EMPs, 5% CS-derived EMPs failed to activate ECs (data not shown). LPS, a known ERK activator, served as a positive control in these experiments.

Protein Composition of Stimulus-Specific EMPs

Using a mass spectrometry proteomic approach, we compared the protein composition of four different EMP populations (control, 5% CS-, 18% CS-, and LPS-derived EMPs). A total of 206 EMP proteins were identified across the four populations (Figure 4A), with 81 proteins (39%) shared by all EMP populations (see Table E1 for a list of proteins). Bioinformatic analysis of GO enrichment was performed to investigate potential mechanisms of EMP shedding and function of the shared proteins present within EMPs independent of stimulus. Figure 4B depicts the top enriched categories of molecular function in which the shared EMP proteins are predicted to participate. GO cellular component (GO-CC) categories for the shared EMP proteins include plasma membrane (41.9%), cytosol (28.3%), vesicle (27.2%), cytoskeleton (28.3%), membrane-bound vesicle (25.9%), and cell surface (14.8%). The entire list of enriched GO-MF, GO-CC, and GO-biological function (GO-BP) proteins is provided in Excel file 1 in the online supplement.

Among the list of the shared proteins, we identified the barrier-regulatory linker moesin as a protein of interest (31). This ERM family member and cytoskeleton-associated protein was consistently present within 18% CS-derived EMPs (present in 5 out of 5 separate mass spectrometry analyses) but not in control EMPs (2 out of 5 analyses). These observations led to further investigation of moesin expression in EMP lysates by Western blot analysis. Consistent with mass spectrometry analysis results, Figure E6A depicts increased moesin expression in 18% CS-derived EMP lysates compared with control EMPs.

Proteomic analyses also revealed multiple proteins that were uniquely present in the specific MP populations, with seven proteins unique to control EMPs, 10 proteins unique to 18% CS-derived EMPs,

43 proteins unique to LPS-derived EMPs, and nine proteins shared between 18% CS-derived and LPS-derived EMPs (Figure 4A; Table E1). KEGG analysis of these populations highlights the top enriched pathways that are detailed in Figure E2. Figure 4C lists proteins identified in individual EMP populations for each enriched KEGG pathway (proteins shared among all populations are not included). Detailed KEGG pathway analysis and complete lists of proteins per category for the CS and LPS-EMP proteomes are provided in the Figure E2 and in Excel file 2 in the online supplement. GO analysis (BP) was performed for the specific EMP populations (Figure E3), and full lists of GO (BP) are provided in Excel file 3 in the online supplement.

We next performed additional analyses of the EMPs generated by EC exposure to 18% CS. Figure 5A depicts the unique proteins identified in this population and lists 18% CS-derived EMP proteins previously reported to be associated with ALI/VILI pathophysiology. To identify associations of 18% CS-derived EMPs with pathways not included in the KEGG database, we performed analysis using the Pubmatrix and Pubmed databases. All identified 18% CS-derived EMP proteins were manually analyzed using these databases, with associations for selected proteins listed in Figure 5A. For the “leukocyte transendothelial migration” pathway, a known KEGG category, proteins were identified using the DAVID database. In addition, the 139 proteins identified in 18% CS-derived EMPs were analyzed using the Genetic Association Database (GAD), and multiple associations with cardiovascular, inflammatory, and lung diseases were found (Figure 5B).

Upon release into extracellular space, MPs interact with cells to alter downstream effects through mechanisms that are not fully understood. The exploration of the interactions between the EMP proteins and linker proteins expressed by the target cells could provide important insight into the possible mechanisms that mediate MP-cell communication. We explored the possible targets of EMP proteins using gene network analysis and identified several potential linker targets that appear unique to the EMP-generating stimulus. For example, the linker proteins for 5% CS, HRAS (GTPase HRas protein), and CAV1 (caveolin 1) (Figure E4A), were selected as linker

proteins based on “betweenness centrality,” a measure of the relative importance of proteins in a network. For 18% CS, potential linkers for the secreted EMP proteins were integrin b1, Src kinase, paxillin, and ubiquitin C (Figure 5C and Figure E4B). Finally, tyrosine kinase Fyn and epidermal growth factor receptor were the most important linker targets for LPS-derived EMPs (Figure E4C). Figures E4A, E4B, and E4C display all interacting proteins.

In Vivo Identification of MPs in a Murine Model of VILI

To extend the *in vitro* EMP data generated, we analyzed MPs in plasma and BAL derived from mice exposed to a well-characterized murine model of high tidal volume (40 ml/kg) MV-induced lung injury. VILI-exposed mice displayed significantly increased BAL total protein levels and BAL cell counts compared with untreated controls (Figures 6A and 6B). MPs from VILI-exposed and SB mice were isolated from plasma and analyzed. As depicted in Figure 6C and Figure E5A, well-defined annexin V-positive plasma MP populations ($< 1 \mu\text{m}$) free of platelet contamination were isolated, and the specificity of annexin V staining was confirmed by parallel staining of MPs with annexin V-FITC in the presence of EDTA (Figure E5B). The number of plasma annexin V-positive MPs was increased by 82% after VILI exposure compared with SB mice (Figure 6D). Using Western blot analysis, expression of specific proteins of interest VE-cadherin (an endothelial marker) (Figure 6E) and moesin (Figure E6B) were found to be increased in MP lysates from VILI-challenged mice compared with controls. Analysis of plasma supernatants from SB and VILI mice after MP isolation (“MP and platelet”-free plasma) for VE-cadherin expression failed to detect VE-cadherin in any sample (Figure E7). To confirm the increase of EMPs during VILI, the number of CD62E (E-selectin)-positive MPs was determined in platelet-free plasma. MPs positive for CD62E were increased 1.38-fold ($P = 0.0497$) in platelet-free plasma from VILI-challenged mice compared with SB mice (Figure 6F). We next isolated MPs from BAL and evaluated for CD62E-positive particles to determine the level of EMPs in the alveolar space. Figure 6G depicts a representative scattergram of CD62E(+)

Figure 2. Effects of CS and LPS on EMP media release. Confluent ECs were subjected to 5% CS or 18% CS for 4 and 24 hours or to LPS (1 μ g/ml, 24 h). (A) Isolated EMPs were analyzed and quantified by flow cytometry. The bar graphs represent the mean $\pm$ SD of the EMP number for the different experimental conditions. ^{*} $P < 0.05$ compared with control (24 h). [#] $P < 0.05$, 5% CS versus 18% CS (24 h) (Bonferroni *post hoc* test). (B) MP lysates from untreated ECS (ctr-EMPs), CS (CS-EMPs), and LPS (LPS-EMPs)-treated ECs and supernatants were analyzed for CD31 by immunoblotting. Results are representative of three or four independent experiments.

MPs detected in BAL from VILI mice. CD62E(+) BAL MPs were increased by 1.87-fold in VILI-challenged mice compared with SB mice ($P = 0.035$) (Figure 6H).

18% CS-Derived EMPs Induce Lung Injury

We next explored the capacity for EMPs to directly affect lung function by isolating MPs from human pulmonary ECs (static or 18% CS) and instilling these EMPs intratracheally into mice. Figure 7 demonstrates the effects of pathologic mechanical stretch-derived EMPs on lung injury *in vivo*, with mice receiving intratracheal 18% CS-derived EMP instillation displaying significantly increased cell counts (macrophages, neutrophils, and red blood cells) in the alveolar compartment compared with PBS (Figure 7A). Differential cell counting indicates increased accumulation of neutrophils (Figure 7B) and red blood cells (data not shown) after 18% CS-derived EMP challenge. Neutrophil activation was confirmed by lung tissue analysis of MPO activity (Figure 7C) in mice receiving 18% CS-derived EMPs compared with PBS-treated mice. Mice challenged with 18% CS-derived EMPs exhibited significant greater BAL cell counts and MPO activity compared with mice receiving ctr-EMPs. Finally, a significant increase in BAL protein levels (Figure 7D) was observed in mice receiving 18% CS-derived EMPs compared with PBS-treated mice ($P = 0.01$, Bonferroni's test). ctr-EMPs produced small increases in all these ALI indices that

were not significant compared with the PBS group. These results suggest that 18% CS-derived EMPs are potential mediators of lung injury pathophysiology.

Discussion

MP shedding into the extracellular space results from cell activation- or apoptosis-mediated increases in intracellular calcium and increased bleb formation. Because MPs represent enrichment of low abundance proteins that are difficult to detect in cell lysates or other biological fluids, the extracellular release of these vesicles has led to recent interest as novel biomarkers in diverse pathologies including ALI (32). Our current study strongly supports a role for endothelial-derived MPs, or EMPs, as markers of endothelial dysfunction in ALI/VILI and bioactive mediators with the

capacity to modulate lung function. Recognizing that a variety of microvesicles exist that are distinguished by size, density, composition, and mechanism of formation (4), we used sequential centrifugation to successfully isolate MP populations less than 1 μ m in size and free of detached cells, apoptotic bodies, or platelets ($> 2\text{--}4\text{ }\mu\text{m}$) and exosomes (40–100 nm). Our results demonstrate that *in vitro* (lung ECs) or *in vivo* (murine lung) exposure to recognized ALI stimuli, such as pathologic CS and LPS results in increased EMP shedding. Proteomic analysis of stimuli-specific EMP populations identified proteins both common to all EMPs studied as well as proteins unique to the ALI stimulus used to increase EMP shedding. The EMP populations generated each exhibited comparable size distribution and shape, as defined by TEM, suggesting similar mechanisms of MP formation. The

Figure 3. Effect of EMPs on human EC signaling. ECs were incubated with EMPs isolated from untreated or 18% CS-treated cells. Cell lysates were analyzed by Western blot for phospho-ERK. (A) Representative blots are shown for p-ERK 1/2, total ERK, and GAPDH. (B) Densitometry of p-ERK was normalized to GAPDH. ^{*} $P < 0.05$ compared with untreated cells (Bonferroni *post hoc* test). Western blots are representative of three independent experiments.

Figure 4. (A) Venn diagram of proteins identified in control (static), 5% and 18% CS-derived, and LPS-derived EMPs. Proteins were identified in each EMP population by LC/MS/MS (control EMPs, 5% CS-derived EMPs, 18% CS-derived EMPs, and LPS-derived EMPs). The total number of individual proteins identified in each subpopulation is shown. (B) Gene ontology annotation analysis for shared proteins. The DAVID database was used to highlight the top enriched molecular function categories (GO-MF) of the 81 proteins that were shared among all EMP populations. (C) KEGG pathway analysis. Proteins that are uniquely identified compared with shared proteins are reported for enriched KEGG pathways. Proteins common to all EMP populations are not included.

structure and the size heterogeneity of the MPs generated from lung ECs are similar to MPs reported for human umbilical vein ECs (33), Jurkat cells (34), and thymocytes (35).

Despite the significant association of MPs with inflammatory diseases, few studies have addressed MP participation in the pathogenesis of ALI/VILI or as markers of lung injury. Bastarache and colleagues reported that ARDS edema fluid contained higher procoagulant (tissue factor-enriched) alveolar MPs compared with a control group of patients with hydrostatic pulmonary edema (21). Indirect evaluation of EMPs in ALI pathogenesis have provided evidence that MPs derived from plasminogen activator inhibitor

1-treated ECs promote endothelial dysfunction and lung injury via cytokine release, neutrophil recruitment, and MPO release (14, 15). Although a recent study reported increased MP generation in cultured lung ECs exposed to 18% CS (36), to date there has not been a careful assessment of the influence of excessive mechanical stress in generation of EMPs, its effects on their composition, and their potential functional role in lung injury. Because increased mechanical stress is essential to the development of VILI and is a well-known trigger of multiple signaling pathways that increase cytokine production, leukocyte infiltration, and alveolar and vascular barrier disruption, we used previously well characterized *in vitro*

and *in vivo* models of excessive mechanical stress (37, 38) to assess the influence of mechanical stress on EMP formation. Our prior *in vitro* studies demonstrated that 5% CS (mirroring normal tidal breathing) and 18% CS (pathologic mechanical stress) produce comparable patterns of actin rearrangement, a process required for bleb formation and active MP shedding (39, 40). However, our prior work also indicated that the magnitude of mechanical stress directly determines markedly distinct EC gene expression profiles and vascular barrier-regulatory properties (29, 37). Specifically, 18% CS primes ECs for enhanced barrier disruption after thrombin, whereas 5% CS enhances EC barrier recovery (29, 37). Consistent with these observations, our current results indicate that 5% CS and 18% CS have distinct effects on the magnitude of EMP generation and on the protein composition of CS-generated EMPs. ECs subjected to pathologic 18% CS exhibited an approximately 4-fold increase in EMP shedding. Similarly, the gram-negative bacteria cell wall component, LPS, another well-characterized ALI stimulus, produces actin cytoskeleton rearrangement and EC dysfunction (30) and significantly increased EMP release (~2.5-fold increase), consistent with prior reports (41). To more fully translate our *in vitro* findings to a preclinical murine model of VILI, we assessed MP levels in mice exposed to high tidal volume MV. Our novel data demonstrate that VILI significantly increases the release of annexin V-positive MPs into circulation. Specific endothelial markers CD62E (by flow cytometry) and VE-cadherin (by Western blot) demonstrate that circulating EMP levels are significantly increased in VILI mice. Moreover, analysis of BAL samples from these mice indicates that CD62E(+) EMPs are released into the alveolar space after VILI, consistent with a recent report that EMPs can be detected in BAL from patients with ARDS but not in control individuals (42). These findings indicate that ALI stimuli result in significant shedding of endothelial MPs, suggesting EMPs as markers of endothelial dysfunction in ALI/VILI.

Given that EMP release is induced in multiple diseases, quantification of MP levels alone is unlikely to be sufficient as a predictive biomarker for specific pathologies. Therefore, analysis of the composition of specific MP populations is necessary for improved characterization

Figure 5. Proteins identified in 18% CS-EMPs. (A) Unique proteins identified in 18% CS–derived EMPs and other selected 18% CS–derived EMP proteins that are associated with mechanotransduction/VILI, ALI/ARDS, endothelial barrier function (Pubmatrix database), and leukocyte transendothelial migration (KEGG pathway database). (B) Association of 18% CS–derived EMP proteins with diseases. Shown are disease processes associated with proteins detected in 18% CS–derived EMPs according to the Genetic Association Database (GAD). (C) Gene network analysis of 18% CS–derived EMP proteins. A gene network based on “betweenness centrality,” a statistic measure of a node’s centrality in a network (i.e., its relative importance). Circle: An input protein unique under 18% CS relative to the control. Diamond: A linker gene used for inferring a network, provided by the Reactome database. The size and color of a protein represents its relative importance (green: low importance; orange/yellow: intermediate importance; red: high importance). The most important nodes are labeled.

of unique molecules (proteins, lipids, or microRNAs) present in MPs under specific pathophysiologic conditions. In this study, we used a mass spectrometry approach to analyze the proteome of differential MP populations generated *in vitro* by lung ECs activated by ALI-relevant stimuli. The goals for analyzing the EMP proteome were (1) exploration of potential

mechanisms for shedding and function of the specific EMP populations and (2) investigation of specific proteins carried by EMPs as potential ALI/VILI biomarkers and mediators of lung injury. The proteomic analysis revealed 81 common proteins shared among the different EMP populations (Figure 4A). These shared proteins include the

endothelial marker PECAM (CD31), the lipid raft structural protein caveolin, “plasma membrane–related” integrins, membrane-binding annexins, multiple cytoskeleton-associated proteins (actin, myosin, vinculin, filamin, and cofilin-1), heat shock proteins, and a variety of other enzymes. These findings are similar to those previously reported for MPs

Figure 6. *In vivo* detection of MPs in VILI-exposed mice. (A and B) VILI model. (A) Bronchoalveolar lavage (BAL) total protein levels in VILI-challenged (Vt, 40 ml/kg, 4 h) and spontaneously breathing (SB) mice. (B) BAL total cell counts in VILI-challenged and SB mice. (C) Representative flow cytometry scattergram of annexin V(+) MPs isolated from plasma of VILI-challenged mice. (D) Quantification of annexin V(+) MPs isolated from plasma of VILI or SB mice. (E). Representative Western blot for VE-cadherin expression in MP lysates isolated from plasma of SB and VILI mice. (F) Quantification of CD62E(+) MPs in platelet-free plasma of VILI or SB mice. (G) Representative flow cytometry scattergram of CD62E(+) MPs isolated from BAL of VILI-challenged mice. (H) Quantification of CD62E(+) MPs isolated from BAL of VILI or SB mice ($n = 8$ for A and B; $n = 3$ [blood from three mice was used for each MP population/sample] for D and E; $n = 3-6$ for F and H). * $P < 0.05$ compared with SB mice (Student's *t* test).

derived from human umbilical vein ECs, monocytes, and plasma (43–47). GO annotation analysis of the shared proteins provided further insights into the possible

molecular functions of MPs (Figure 4B). Among the top enriched molecular function categories, GO analysis revealed that MPs contain proteins that are

important for the structural integrity of the vesicle and cytoskeletal-associated proteins, observations consistent with literature describing MPs as membrane vesicles that contain organized cytoskeleton (48). In addition, EMPs isolated in our study contain proteins that could interact with integrins or other cell surface linkers, with calcium-binding proteins, and with unfolded proteins. This last group suggests that one cellular function of MPs may be for disposal of accumulated unfolded proteins. Our proteomic analyses identified moesin as a component of the studied EMPs. Moesin is a member of the ERM family of proteins (ezrin, radixin, and moesin) and functions as an actin-binding linker, regulating cellular processes that require membrane cytoskeletal reorganization (49). For example, we recently demonstrated a critical role for ERM proteins in human pulmonary EC cytoskeletal rearrangement and barrier regulation that revealed differential effects of individual ERM proteins on these properties, with moesin promoting increased EC permeability (31, 50). Our current data indicate that moesin is markedly enriched in pathologic 18% CS-derived EMPs compared with control EMPs as assessed by Western blot, a finding confirmed in plasma MPs of VILI-challenged mice, indicating a possible role as a novel marker of vascular injury and potential ALI mediator.

Mass spectrometry proteomic analysis also revealed unique 18% CS-derived ($n = 10$) and LPS-derived EMP proteins ($n = 43$). A significant number of 5% CS-derived EMP proteins (~70%) and 18% CS-derived EMP proteins (~58%) were shared with EMPs generated from static-exposed ECs. Nineteen proteins were unique to 18% CS when compared with 5% CS and static EMPs and included antioxidants (SOD1, glutathione S-transferase p1, and PRDX1,4) and cytoskeletal linkers (ACTN1, TPM3, CKAP4, and PLS3). Multiple 18% CS-derived EMP proteins are involved in neutrophil transmigration, regulation of the actin cytoskeleton, and EC barrier function, cellular processes known to be fundamental for ARDS/VILI pathogenesis. UCHL1, a deubiquitinating enzyme prominent in 18% CS-derived EMPs, has been recently found to be involved in VILI (38). In addition, pathologic stretch-derived EMPs carry several proteins that have

Figure 7. EMPs induce ALI. Inflammatory indices obtained from C57BL/6J mice 18 hours after intratracheal instillation of PBS or EMPs obtained from static ECs or ECs exposed to 18% CS. EMPs were generated from 9×10^6 ECs, isolated, and resuspended in sterile PBS. Depicted are (A) BAL total cell counts (macrophages, neutrophils, and red blood cells). (B) BAL neutrophil counts. (C) Myeloperoxidase (MPO) activity was measured as marker of neutrophil infiltration/activation in lung tissues. (D) BAL total protein levels. *P* values as indicated (Bonferroni *post hoc* test) ($n = 4-7$ per condition).

been associated with cardiovascular, inflammatory, and lung diseases, as determined using the GAD. For example, glutathione S-transferase p1, one of the unique proteins identified only in 18% CS-derived EMPs, was found to be associated with human ALI/ARDS by GAD analysis. Among the most important potential protein targets for 18% CS-derived EMPs, we identified several focal adhesion-related proteins (ITGB1, paxillin, Src kinase) and ubiquitin C, which could serve to activate cell responses such as protein degradation and endocytosis (Pubmed Gene database). Compared with CS-generated MP proteins, LPS-EMPs exhibited less overlap with control EMPs (~45%). Forty-three proteins were unique to LPS-derived EMPs, indicating profound effects of LPS on EMP composition. EMP proteins unique to LPS challenge included ICAM-1, an adhesion protein known to be up-regulated under inflammatory conditions such as LPS (51), and C1K19, a cytokeratin linked to

ALI with increased BAL levels in patients with ARDS (52).

To gain additional insights into the possible pathways relevant to specific EMP populations, we performed KEGG pathway analysis and observed potential similarities and differences between EMP populations (Figure 4C). Given that a large number of proteins were shared among the EMPs, the enriched categories were very similar among the different conditions, but Ctr-EMPs exhibited the least significant enrichment in most of the categories (Figure E2). In addition, for the same KEGG pathway, we identified certain proteins that were differentially expressed among the individual EMP populations (Figure 4C). Together, these data support selective protein packaging into EMPs after cellular activation and suggest that further characterization of 18% CS- or LPS-derived EMP proteins may help identify new biomarkers for ALI/VILI.

Having characterized the levels and the composition of EMPs, we explored the

potential bioactive properties of MPs that may contribute to ARDS severity and progression, as recently suggested in reviews of the topic (32, 53). Circulating MPs bind cellular targets, transferring their encargoed components to trigger signaling cascades or activate the coagulation cascade via externalized phosphatidylserines or tissue factor residing on the MP surface (8). Our proteomic analysis suggests that MPs may be involved in several ALI/VILI relevant pathways. Given the distinct characteristics of each EMP population (levels and composition), we speculated that they may induce differential responses. Our data demonstrate that endothelial-derived MPs generated under pathologic mechanical stress induce a rapid and selective MAPK pathway signaling in lung ECs, consistent with previous reports of apoptosis- or LPS-derived MPs (41, 54). EMPs generated under static or 5% CS failed to activate the MAPK signaling pathway, suggesting that the unique characteristics of 18% CS-derived EMPs are responsible for the cellular responses they induce. In addition, *in vivo* experiments produced strong evidence supporting a role for EMPs in lung injury. Because VILI increased the number of EMPs present in the alveolar space, we explored the effects of 18% CS-derived EMPs instilled intratracheally into the lungs. Instillation of these EMPs into the airspaces resulted in increased activation and migration of neutrophils into the alveolar space and increased permeability of the alveolar-capillary barrier. Our data are supported by reports from other groups that PAI-1-derived EMPs, or stored red blood cell MPs, induce neutrophil activation and lung injury when injected into mice (14, 15, 55). In another recent study, microvesicles derived from human mesenchymal stem cells were found to be protective against LPS-induced lung injury (56), supporting the hypothesis that both the number and the composition of MPs determine their effects on lung function.

Our study has some important limitations. First, the number of proteins reported per EMP population was small, and the proteomic approach used was not quantitative. To increase confidence in the accuracy of our approach, we elected to use very stringent “protein identification” criteria (further details are provided in the online supplement), which excluded

a large number of low-confidence proteins from the lists reported here. Although a quantitative proteomic approach could provide important insights into the relative expression levels of common MP proteins, our goal in the current study was to provide an initial differential proteomic characterization of these EMPs using a rapid and technically feasible approach given the very low concentration of protein within these particles. Second, MPs isolated from mouse plasma were not double stained for annexin V and CD31, as we did for *in vitro* EMPs. Therefore, these isolated plasma MPs were not solely of endothelial origin because annexin-V-positive MPs are also generated by other cells. Technical limitations precluded double staining of plasma MPs. The amount of plasma MPs obtained was too low to allow for double staining even when samples from multiple mice were combined. In addition, we observed that mouse plasma MPs exhibit a much higher fluorescent background (after staining with isotypes) than the EMPs isolated from cultured ECs *in vitro*, and as a result double staining of MPs would likely produce artificial positive events. Given these technical issues, we elected to stain for CD62E (E-selectin) as a specific marker for endothelial activation (57) to confirm increased levels of EMPs in the mouse plasma and BAL samples. The third

major limitation of our study is that we did not control for the absolute number of MPs in the functional assays but instead chose to control for the number of cells that produced the MPs. This approach does not allow us to differentiate completely between the effects of MP number and MP composition. However, we have selected this approach because we believe it is a more pathophysiologically relevant model for evaluating the potential functional effects of MPs generated by ALI-relevant stimuli. Our experiments were performed to mimic the human pathophysiologic condition in which a similar number of cells will shed MPs under normal or diseased states and MP level and composition combine to determine their downstream effects. Future studies will be pursued to determine the specific functional effects of individual MP composition. It is also important to acknowledge that our *in vivo* model is not fully representative of the pathophysiology of human disease because we used *in vitro*-derived EMPs instilled into the airway to induce lung injury in mice. Our overall hypothesis is that during high tidal volume MV, lung endothelium is exposed to pathologic stretch that triggers the release of a distinct population of endothelial MPs into the circulation. These shed

EMPs can transmigrate into the alveolar space due to the disruption of the alveolocapillary membrane, which characterizes VILI to further potentiate lung injury. Future mechanistic studies are required to more precisely characterize this hypothesis.

In summary, *in vitro* studies using human lung ECs and *in vivo* studies using a preclinical model of VILI indicate that ARDS/VILI is characterized by endothelial MP formation and shedding into the circulation. The magnitude and composition of shed MPs are altered in a stimulus-specific manner, suggesting EMPs as potential markers of lung vascular injury in ARDS. These studies support the hypothesis that pathologic stretch-derived MPs contribute to ALI pathophysiology and severity. Although further investigation is required to define EMP involvement in ARDS/VILI pathobiology, these studies support the notion that MPs represent a novel diagnostic, prognostic, and therapeutic tool in ARDS and VILI potentially linked to disease pathogenesis, severity and outcome. ■

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