ORIGINAL ARTICLE

Therapeutic Effects of Hyaluronic Acid in Bacterial Pneumonia in *Ex Vivo* Perfused Human Lungs

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Abstract

Rationale: Recent studies have demonstrated that extracellular vesicles (EVs) released during acute lung injury (ALI) were inflammatory.

Objectives: The current study was undertaken to test the role of EVs induced and released from severe *Escherichia coli* pneumonia (*E. coli* EVs) in the pathogenesis of ALI and to determine whether high-molecular-weight (HMW) hyaluronic acid (HA) administration would suppress lung injury from *E. coli* EVs or bacterial pneumonia.

Methods: *E. coli* EVs were collected from the perfusate of an *ex vivo* perfused human lung injured with intrabronchial *E. coli* bacteria for 6 hours by ultracentrifugation and then given intrabronchially or intravenously to naive human lungs. One hour later, HMW HA was instilled into the perfusate ($n = 5–6$). In separate experiments, HMW HA was given after *E. coli* bacterial pneumonia ($n = 6–10$). *In vitro* experiments were conducted to evaluate binding of EVs to HMW HA and uptake of EVs by human monocytes.

Measurements and Main Results: Administration of HMW HA ameliorated the impairment of alveolar fluid clearance, protein permeability, and acute inflammation from *E. coli* EVs or pneumonia and reduced total bacteria counts after *E. coli* pneumonia. HMW HA bound to *E. coli* EVs, inhibiting the uptake of EVs by human monocytes, an effect associated with reduced TNFα (tumor necrosis factor α) secretion. Surprisingly, HMW HA increased *E. coli* bacteria phagocytosis by monocytes.

Conclusions: EVs induced and released during severe bacterial pneumonia were inflammatory and induced ALI, and HMW HA administration was effective in inhibiting the uptake of EVs by target cells and decreasing lung injury from *E. coli* EVs or bacterial pneumonia.

Keywords: acute lung injury; extracellular vesicles; *ex vivo* perfused human lung; hyaluronic acid

Acute respiratory distress syndrome (ARDS) is a devastating clinical condition common in patients with respiratory failure in the ICU. It is associated with high mortality rates and long-term physical and psychological dysfunctions among survivors (1–3). Despite intense research into the pathophysiology, there are no specific pharmacological therapies (4).

Most eukaryotic cells release small anuclear membrane-bound vesicles into the extracellular environment in either physiological or pathophysiological conditions, often called extracellular vesicles (EVs) by the International Society for Extracellular Vesicles (5). Through their cargo containing bioactive molecules such as proteins, mRNAs, and microRNAs and their interaction with target cells, EVs are recognized as important mediators of cellular communication (6–9). The role of EVs in the pathogenesis of acute lung injury (ALI) or ARDS has generated considerable interest.

Hyaluronan or hyaluronic acid (HA) is synthesized as a high-molecular-weight (HMW) (>1,000 kD) nonsulfated glycosaminoglycan composed of repeating polymeric disaccharides of D-glucuronic acid and *N*-acetyl-D-glucosamine. In the lung, it is located mainly in the peribronchial and perialveolar space and is

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At a Glance Commentary

Scientific Knowledge on the Subject: Extracellular vesicles released by living cells may have significant inflammatory properties, contributing to lung injury during severe bacterial pneumonia.

What This Study Adds to the Field: We have discovered that high-molecular-weight hyaluronic acid may suppress inflammation by binding to extracellular vesicles released during severe bacterial pneumonia.

one of the chief components of the extracellular matrix and critical for maintaining the normal structure of the alveolar air–blood barrier and homeostasis (10). In multiple lung diseases including ALI, asthma, or chronic obstructive pulmonary disease, HA undergoes degradation by lysosomal hyaluronidases, reactive oxygen and nitrogen species, and inflammatory mediators (11). The degradation products, low-molecular-weight HA (<500 kD), can decrease endothelial cell barrier function, stimulate angiogenesis, and induce inflammation (12). Surprisingly, primarily because of its molecular size, HMW HA has the opposite properties of low-molecular-weight (LMW) HA. Therefore, many investigators have focused on the therapeutic use of exogenous administration of HMW HA in lung diseases. HA influences cell behavior through binding to various cell surface receptors such as CD44 (cluster of differentiation 44), TLR2 (toll-like receptor 2) and TLR4, HABP2 (hyaluronan-binding protein 2), or RHAMM (receptor for hyaluronan-mediated motility) (13). CD44 is commonly expressed on innate and adaptive immune cells such as macrophages, neutrophils, etc., and EVs released by these immune cells, which may be critical for trafficking.

In the current study, we initially examined the biological effects of EVs induced and released into the perfusate during *Escherichia coli* bacterial pneumonia to initiate inflammation and lung protein permeability in a naive perfused human lung. We next studied the therapeutic effects of HMW HA administration, instilled into the perfusate, on lung injury from *E. coli*–induced EVs. We hypothesized that HA may bind to CD44, commonly expressed on the surface of EVs, and prevent the uptake of EVs by target cells, ameliorating lung injury. Last, we studied the therapeutic effects of HMW HA in *E. coli* bacterial pneumonia to suppress inflammation, pulmonary edema, and, potentially, bacterial growth.

Methods

Ex Vivo Perfused Human Lung Preparation

A detailed description of the *ex vivo* perfused human lung preparation and the experimental protocols are described in the online supplement (14, 15) (Figure 1). Briefly, either the right or left human lung was slowly perfused with crystalloid solution containing 5% bovine albumin fraction V (MP Biomedicals LLC) and fresh human whole blood and ventilated. Alveolar fluid clearance (AFC) was then measured in the upper lobe. If AFC > 10%/h, 10^9 cfu of *E. coli* bacteria (K1 strain) was instilled into the middle or lower lobe. After 6 hours of perfusion, the perfusate was collected to isolate EVs (*E. coli* EVs) by ultracentrifugation. In naive human lungs, *E. coli* EVs collected from 400 ml of perfusate were given intravenously or into the middle or lower lobe intrabronchially. As therapy, 1 mg HMW HA (LifeCore, Inc.) was instilled into the perfusate 1 hour after injury. In separate experiments, marginal human lungs with AFC < 10% and injured further with 10^9 cfu of i.b. *E. coli* bacteria were treated with 1 mg HMW HA.

Statistical Analysis

Results are expressed as mean ± SD if the data were normally distributed and median with interquartile range (IQR) if not. Comparisons between two groups were made using unpaired *t* test if the data were normally distributed or Mann-Whitney test if not. Comparisons between more than two groups were made using an ANOVA using the Bonferroni's correction for multiple-comparison testing if the data were normally distributed or Dunn's test after Kruskal-Wallis analysis if not. All statistical analysis was performed using GraphPad Prism software.

Results

HMW HA Treatment Improved AFC Rate and Decreased Lung Weight Gain after Injury by *E. coli* EV Instillation

The demographic and clinical data for human lungs used for experiments with *E. coli* EVs are listed in Table 1.

Representative histological images from the middle or lower lung lobe after injury with *E. coli* EVs showed an increase in lung edema and cellularity. The lung lobes treated with intravenous (i.v.) HMW HA 1 hour after *E. coli* EV i.v. instillation showed a reduction in inflammatory cell infiltration and septal thickening. AFC significantly decreased from 17.3 ± 7.7% to 6.7 ± 4.9% or 6.1 ± 6.8% after *E. coli* EV i.b. or i.v. instillation, respectively. Administration of HMW HA significantly restored AFC rate to 18.3 ± 4.3% (Figure 2A). Total lung weight increased by 574 ± 152 g or 636 ± 65 g with *E. coli* EV i.b. or i.v. administration, respectively, after 6 hours of perfusion. Administration of HMW HA numerically decreased total lung weight gain by 14% (547 ± 120 g) compared with injury induced by i.v. *E. coli* EV, although not statistically significant.

HMW HA Reduced Inflammation Induced by *E. coli* EV Instillation

Although median neutrophil counts decreased by 46% with HMW HA administration compared with injury, there were no significant differences in total white blood cell and neutrophil counts in the BAL fluid (BALF) among the groups (6.5×10^6 median neutrophil count with IQR 2.1×10^6–17.3×10^6 for i.v. EVs, 3.6×10^6 median neutrophil count with IQR 0.0×10^6–7.9×10^6 for i.v. EVs + i.v. HA). TNFα (tumor necrosis factor α) levels in the BALF significantly increased >200× after i.b. or i.v. administration of *E. coli* EVs compared with control lung lobes. Administration of HMW HA significantly reduced the increase in TNFα levels in the BALF by >50% (4,245 ± 2,635 pg/ml for i.b. EVs or 4,465 ± 1,424 pg/ml for i.v. EVs vs. 2,067 ± 1,129 pg/ml for i.v. EVs + i.v. HA) (Figure 2B).

Instillation of i.v. *E. coli* EVs significantly increased the release of endogenous HA, predominantly LMW HA (16), into the perfusate. Administration of HMW HA

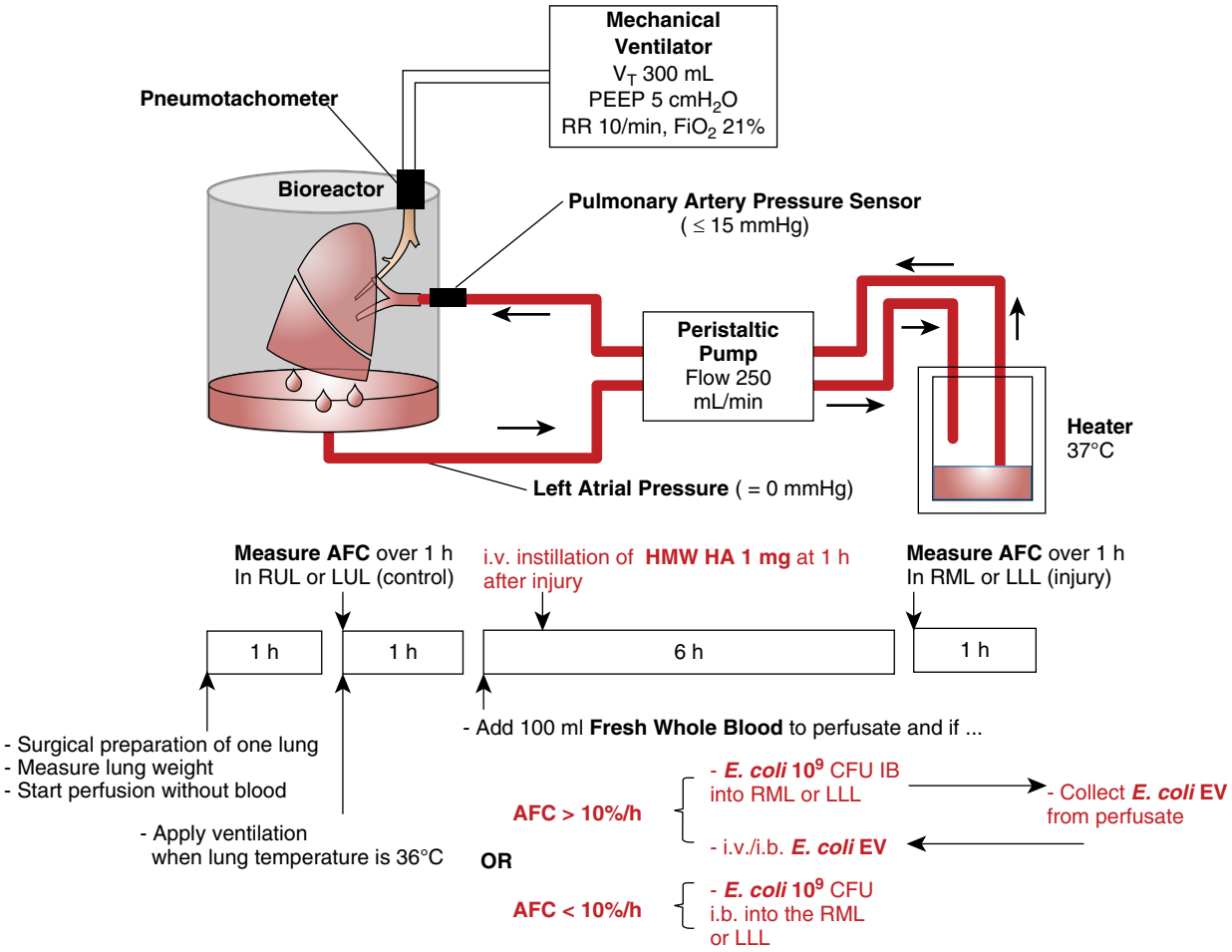

Figure 1. Schematic of the *ex vivo* perfused human lung. Human lungs rejected for clinical transplantation were perfused with Dulbecco's modified Eagle medium containing 5% albumin with 100 ml fresh human whole blood at a rate of 250 ml/min. Once rewarmed to 37°C, the lung was ventilated using a V_T of 300 ml with 5 cm H_2O of positive end-expiratory pressure and respiratory rate 10 breaths/min in room air. For lungs with alveolar fluid clearance (AFC) > 10%, 10^9 cfu of *Escherichia coli* K1 strain was instilled to the middle or lower lobe. At 6 hours, extracellular vesicles (EVs) were isolated from the perfusate (*E. coli* EVs). In separate naive human lungs with AFC > 10%/h, *E. coli* EVs collected from 400 ml of perfusate were given intrabronchially or intravenously to induce lung injury. In separate experiments with lungs with AFC < 10%, 10^9 cfu *E. coli* was instilled into the middle or lower lung lobes to induce severe bacterial pneumonia. For all treatment groups, 1 mg of HMW HA was administered through the pulmonary artery 1 hour after injury. HA = hyaluronic acid; HMW = high-molecular-weight; LLL = left lower lung lobe; LUL = left upper lung lobe; PEEP = positive end-expiratory pressure; RML = right middle lung lobe; RR = respiratory rate; RUL = right upper lung lobe.

further raised total HA concentration in the perfusate (519 ± 495 ng/ml for i.b. EVs or 714 ± 558 ng/ml for i.v. EVs vs. 1,431 ± 260 ng/ml for i.v. EVs + HA) (Figure 2B).

Surprisingly, administration of HMW HA also increased BALF levels of total HA by 42% compared with i.v. EVs; however, it was not statistically significant.

During 6 hours of perfusion, there were no differences in pulmonary artery pressure, airway pressure, or P_{O_2} levels among the groups (Figure 2C).

Table 1. Donor Demographic and Clinical Data for Experiments with *Escherichia coli* EVs and HMW HA

	Control (*n* = 17)	i.b. *E. coli* EVs (*n* = 6)	i.v. *E. coli* EVs (*n* = 5)	i.v. *E. coli* EVs + i.v. HMW HA (*n* = 6)
Age, yr	55 ± 16	65 ± 6	49 ± 16	50 ± 19
Male sex, *n* (%)	11 (65%)	2 (33%)	4 (80%)	5 (83%)
Pa_{O2}/F_{IO2}, mm Hg, median (IQR)	267 (221–308)	296 (218–339)	255 (182–271)	280 (205–306)
Cdyn, ml/cm H_2O	40 ± 12	37 ± 14	36 ± 10	49 ± 9
Murray LIS	1.5 ± 0.6	1.4 ± 0.8	1.8 ± 0.5	1.3 ± 0.5
Ischemic time, h:min	26:41 ± 8:36	22:53 ± 5:06	24:43 ± 11:20	29:36 ± 6:45

Definition of abbreviations: Cdyn = dynamic compliance; EVs = extracellular vesicles; HA = hyaluronic acid; HMW = high-molecular-weight; IQR = interquartile range; LIS = lung injury score.
Data are presented as mean ± SD unless otherwise indicated. No significant differences in Pa_{O2}/F_{IO2} ratio, lung compliance, or lung injury score prior to organ harvest or total ischemic time were observed among groups.

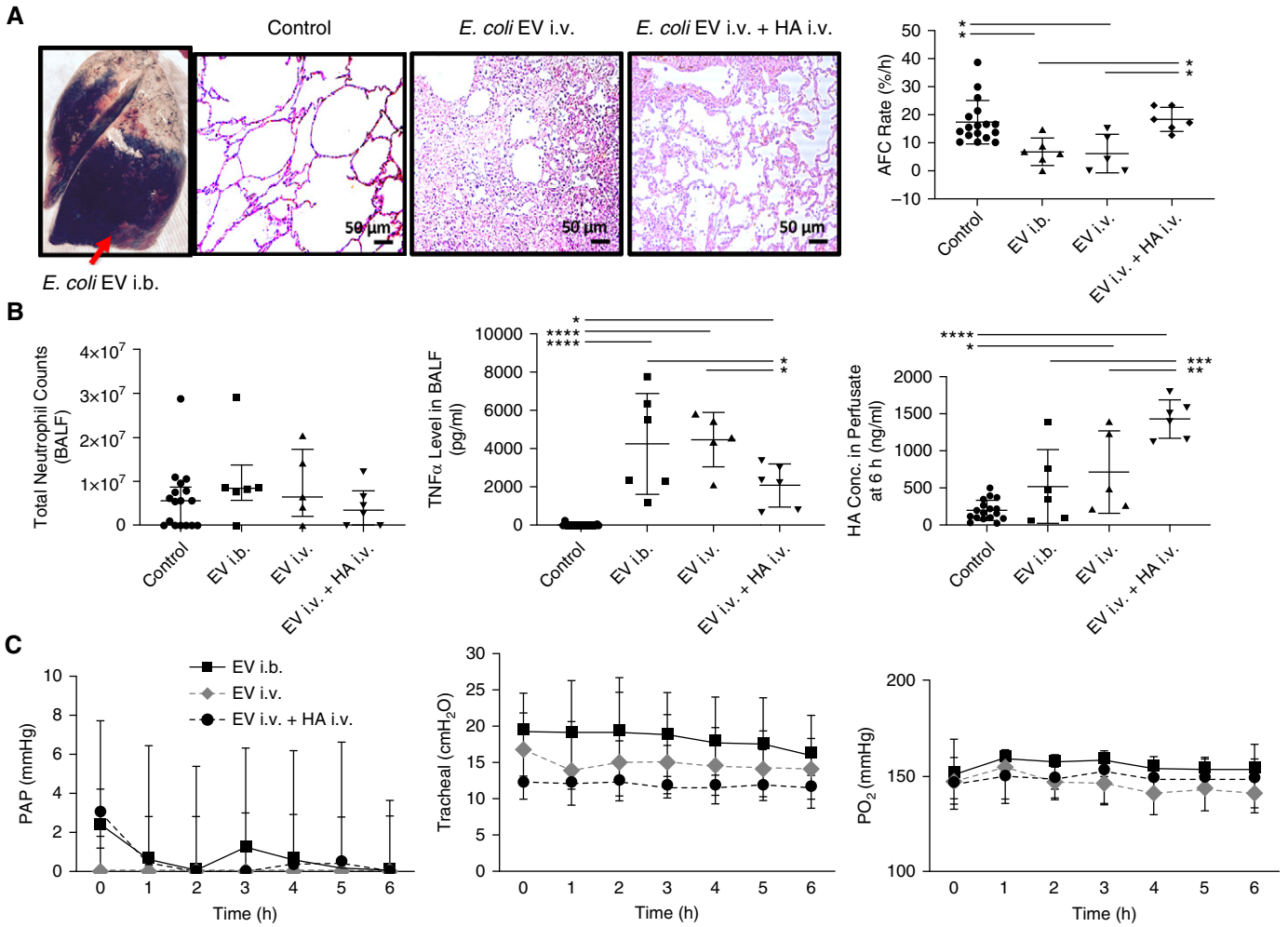

Figure 2. Effect of high-molecular-weight (HMW) hyaluronic acid (HA) on lung injury induced by *Escherichia coli* extracellular vesicle (EV) instillation. Administration of HMW HA as therapy restored alveolar fluid clearance (AFC) rate and reduced inflammation in the injured alveolus. (*A*) Gross human lung image of extravasation of Evan's blue into the injured alveolus after intrabronchial instillation of *E. coli* EVs. Representative histopathological images of lung tissue slice from control, intravenous (i.v.) *E. coli* EV, and i.v. *E. coli* EV plus i.v. HMW HA groups with hemasolin and eosin staining. Instillation of *E. coli* EVs intrabronchially or intravenously significantly reduced AFC rate, which was restored with HMW HA administration. (Data are mean ± SD, $n = 5$–6 per treatment group, and $^*P < 0.05$ by ANOVA [Bonferroni].) (*B*) Although administration of HMW HA decreased median absolute neutrophils counts, there was no statistical difference. (Data are median with interquartile range, $n = 5$–6 per treatment group.) Instillation of *E. coli* EVs intrabronchially or intravenously significantly increased levels of TNFα (tumor necrosis factor α) and total HA, presumably low-molecular-weight HA, in the injured alveolus and perfusate, respectively. Administration of HMW HA reduced TNFα in the injured alveolus and further increased total HA in the perfusate. (Data are mean ± SD, $n = 5$–6 per treatment group, $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$ by ANOVA [Bonferroni].) (*C*) There were no consistent effects on pulmonary artery pressure, airway pressure, or perfusate P_{O_2} levels over 6 hours or between individual groups. (Data are median with interquartile range, $n = 5$–6 per treatment group.) *P* values and confidence intervals are shown in Table E1 in the online supplement. BALF = BAL fluid; PAP = pulmonary artery pressure.

Characterization of *E. coli* EVs

Using electron microscopy, *E. coli* EVs appeared as spheroids between 50 and 200 nm in size (Figure 3A). The mean *E. coli* EV size was 155 nm as analyzed with NanoSight NS300 (Malvern, Inc.) (Figure 3B). About 50% of the EVs were 145–205 nm, and 25% of the EVs were 75–135 nm. Total protein concentration and RNA content of *E. coli* EVs derived from 400 ml of perfusate were 23 ± 5 μg and 2,038 ± 111 ng, respectively ($n = 4$).

By flow cytometry, 44 ± 7% and 27 ± 6% of EVs as a percentage of total PKH26-labeled EVs were CD44 positive, labeled for microvesicles, at 3 and 6 hours, respectively, and 59 ± 20% were CD9 positive, labeled for exosomes, at 6 hours ($n = 4$). At 6 hours, most EVs were released from endothelial cells (CD31+, 31 ± 15%) and platelets (CD41+, 37 ± 5%). Other cellular sources included monocytes (CD14+, 0.5 ± 0.2%), epithelial cells (CD326, 2.8 ± 0.04%), and lymphocytes (CD3+, 3.0 ± 0.7%). We were unable to detect EVs released from neutrophils using the CD66b+ Ab (Figure 3C).

HMW HA Binding to *E. coli* EVs through CD44 Expressed on the Surface of the EVs

We compared the attachment of *E. coli* EVs prelabeled with PKH26 to glass slides or glass slides precoated with HMW HA. We also confirmed the results by measuring total TNFα levels on the glass slides as a surrogate

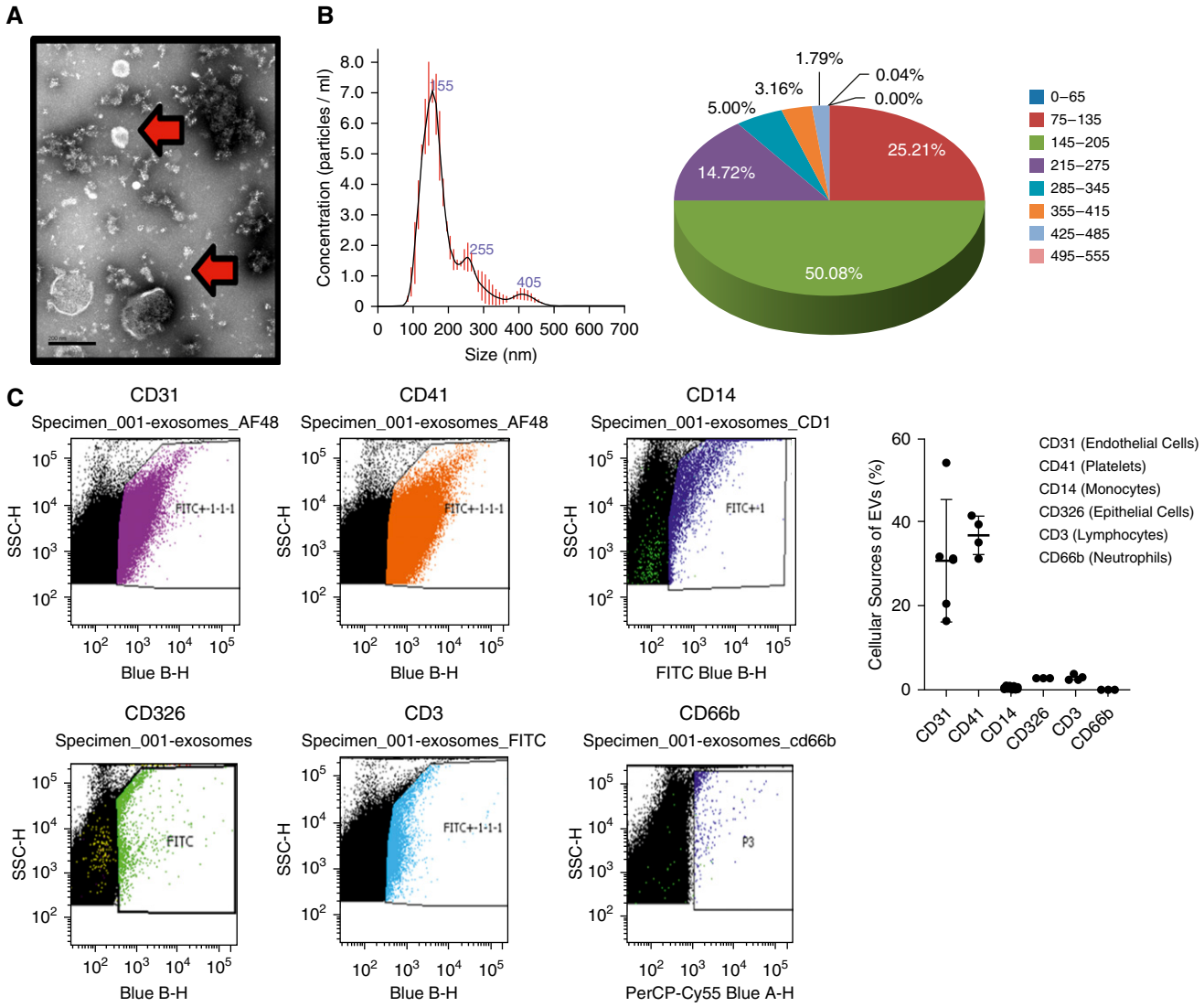

Figure 3. Characterization of extracellular vesicles (EVs) released during *Escherichia coli* pneumonia in the *ex vivo* perfused human lung. (*A*) Plasma EVs appeared as small membrane-bound vesicles by electron microscopy (scale bar, 200 nm). The arrows highlight the different sizes of EVs found. (*B*) NanoSight analyses revealed that >50% of the EVs were ~145–205 nm in size with a mean EV size of 155 nm. (*C*) By flow cytometry, EVs isolated from the perfusate 6 hours after injury were predominantly from endothelial cells and platelets. In addition, 27 ± 6% expressed CD44, a marker of microvesicles, and 59 ± 20% expressed CD9, a marker of exosomes, at 6 hours. Data are expressed as mean ± SD, $n = 3–4$. The Blue B-H axes relate to detection with a 525/50-nm bandpass filter when excited with a blue laser (488 nm). The Blue A-H axis relates to detection with a 695/40-nm bandpass filter when excited with a blue laser (488 nm). FITC = fluorescein isothiocyanate; PerCP = peridinin-chlorophyll proteins; SSC = side scatter.

marker of the intra-EV content of the cytokine. *E. coli* EVs significantly attached to HMW HA, which was inhibited with an anti-CD44 blocking antibody as compared with IgG control (Figure 4).

Binding of *E. coli* EVs to HMW HA Inhibited the Proinflammatory Effect of *E. coli* EVs

To determine if EVs transferred RNA to target cells, we initially measured mRNAs for TNFα and IL-6 in the vesicles using quantitative PCR. We found that EVs collected at 6 hours had 8× the level of TNFα and 30× the level of IL-6 mRNA compared with EVs at 0 hours (Figure 5). We then studied the uptake of EVs by human blood monocytes *ex vivo* to understand the mechanisms underlying the inflammatory effect of the vesicles. The uptake of *E. coli* EVs by monocytes progressively increased over time. However, HMW HA administration significantly reduced the uptake of *E. coli* EVs, which was associated with reduced TNFα and IL-6 levels in the medium (Figure 5).

HMW HA Administration Improved AFC and Decreased Total Lung Weight Gain after *E. coli* Pneumonia

Lungs with AFC < 10% were given i.b. *E. coli* bacteria to replicate nosocomial pneumonia in ICU patients with baseline lung injury. Their demographic, clinical data, and ischemia time for donor lungs are listed in Table 2.

By histology, i.b. instillation of *E. coli* bacteria resulted in interalveolar septal thickening, edema, and cellularity, which was largely reduced with HMW HA administration. HMW HA also

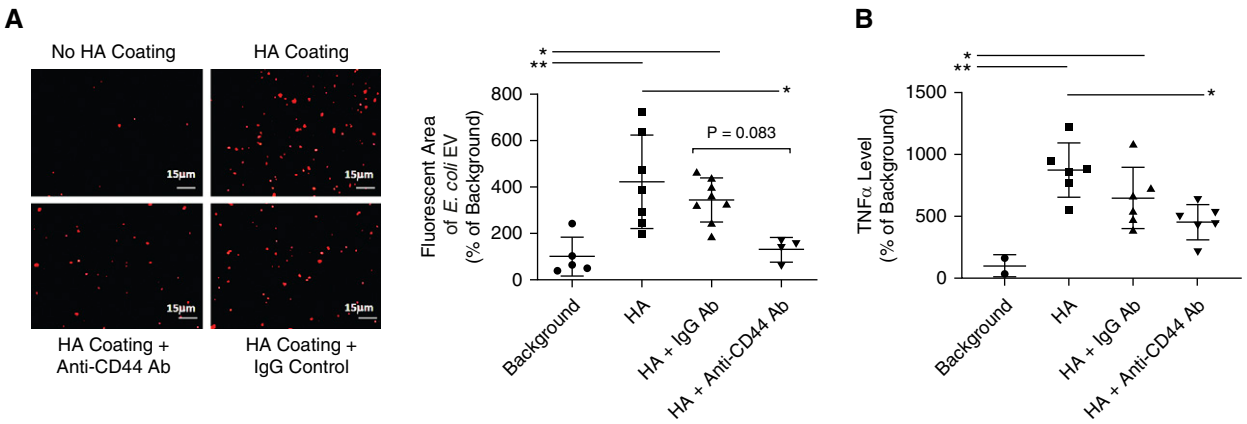

Figure 4. Binding of *Escherichia coli* extracellular vesicles (EVs) to high-molecular-weight (HMW) hyaluronic acid (HA). (*A* and *B*) PKH26-labeled *E. coli* EVs significantly bound to HMW HA plated on glass slides compared with control (scale bars, 15 μm), which was inhibited with coincubation with anti-CD44 antibody (Ab) by fluorescence (*A*) and total TNFα (tumor necrosis factor α) levels in EVs (*B*). Total TNFα levels in EVs were used as a surrogate measure of EV attachment to the HA. (Data are mean ± SD, $^*P < 0.05$ and $^{**}P < 0.01$ by ANOVA [Bonferroni], $n = 4$–8.) *P* values and confidence intervals are shown in Table E1.

significantly increased AFC rate compared with *E. coli*-injured lung lobes (4.4 ± 4.9% with *E. coli* injury alone compared with 18.6 ± 8.0% with HA administration), which was associated with reduced lung weight gain (666 g ± 140 g after *E. coli* injury compared with 532 g ± 121 g with HA administration) and decreased lung protein permeability (Figure 6A).

HMW HA Administration Improved Bacterial Clearance

We measured bacteria cfu counts in the BALF after *E. coli* bacteria instillation with or without HMW HA treatment. HMW HA administration significantly decreased total *E. coli* cfu counts (median 8.6×10^8 cfu/ml with IQR 2.1×10^7–10.0×10^8 cfu/ml after *E. coli* injury compared with 1.5×10^7 cfu/ml with IQR 3.5×10^6–7.9×10^7 cfu/ml with HA administration). To confirm the effect of HMW HA on bacterial clearance, we added HMW HA to human blood monocytes cultured *ex vivo* injured with LPS and GFP-labeled *E. coli* bacteria. HMW HA administration significantly reduced *E. coli* bacteria cfu levels in the medium and increased bacteria phagocytosis by monocytes (Figure 6B).

Effect of HMW HA in Lung Inflammation

HMW HA treatment numerically decreased TNFα levels in the BALF by 23% (median 7,865 pg/ml with IQR 4,123–24,275 for *E. coli* pneumonia vs. median 6,064 with IQR 1,473–11,131 for *E. coli* pneumonia + HMW HA). There were no significant differences in white blood cells and neutrophil counts in BALF among the groups (Figure 7A).

Although at individual time points there were statistically significant differences between groups, there appeared to be no overall effect of HMW HA administration on pulmonary arterial pressure, airway pressure, or Po_2 levels over time (Figure 7B).

Administration of HMW HA Increased Total Concentration of HA in the Injured Alveolus and Perfusate

Similar to *E. coli* EVs, *E. coli* bacteria i.b. instillation significantly increased endogenous HA concentration in both the injured alveolus and perfusate. HMW HA treatment further increased total HA concentration ~3× more in the injured alveolus or perfusate over 6 hours (Figure 8A). In the treatment group, the majority of HA in both the injured alveolus and perfusate were HMW (>1,000 kD) in size despite 6 hours of perfusion. Even in the injured group, endogenous levels of HMW HA were elevated, suggesting some potential for repair and recovery (Figure 8B).

Discussion

The main findings in this study can be summarized as follows: *1*) Instillation of EVs induced and released during *E. coli* pneumonia given intrabronchially or intravenously initiated severe lung injury in a naive *ex vivo* perfused human lung (Figure 2). *2*) Administration of HMW HA into the perfusate as therapy significantly restored AFC and reduced inflammation after ALI induced by *E. coli* EVs (Figure 2). *3*) The predominant cellular sources of *E. coli* EVs identified were from endothelial cells and platelets, although the contributions from immune cells cannot be disregarded due to the majority of EVs being exosomes (Figure 3). *4*) Binding of HMW HA to *E. coli* EVs *in vitro* inhibited the uptake of the vesicles by human blood monocytes in part through a CD44-dependent mechanism, reducing the release of TNFα and IL-6 by injured monocytes (Figures 4 and 5). *5*) Administration of HMW HA was effective in restoring AFC and reducing lung weight gain, inflammation, and protein permeability in an *ex vivo* perfused human lung injured with *E. coli* bacterial pneumonia (Figure 6). *6*) Despite administration of i.v. HMW HA, total HA levels in the BALF in *E. coli* pneumonia increased significantly, suggesting that HMW HA was drawn to the injured alveolus. Analyses of HA in the BALF and perfusate demonstrated that HA were predominantly HMW in size after 6 hours of perfusion (Figures 7 and 8). *7*) And, perhaps more significantly, HMW HA administration reduced total bacterial cfu counts in the BALF and increased the phagocytosis of *E. coli* bacteria by human monocytes (Figure 6).

To our knowledge, this is the first demonstration of the critical role of EVs in

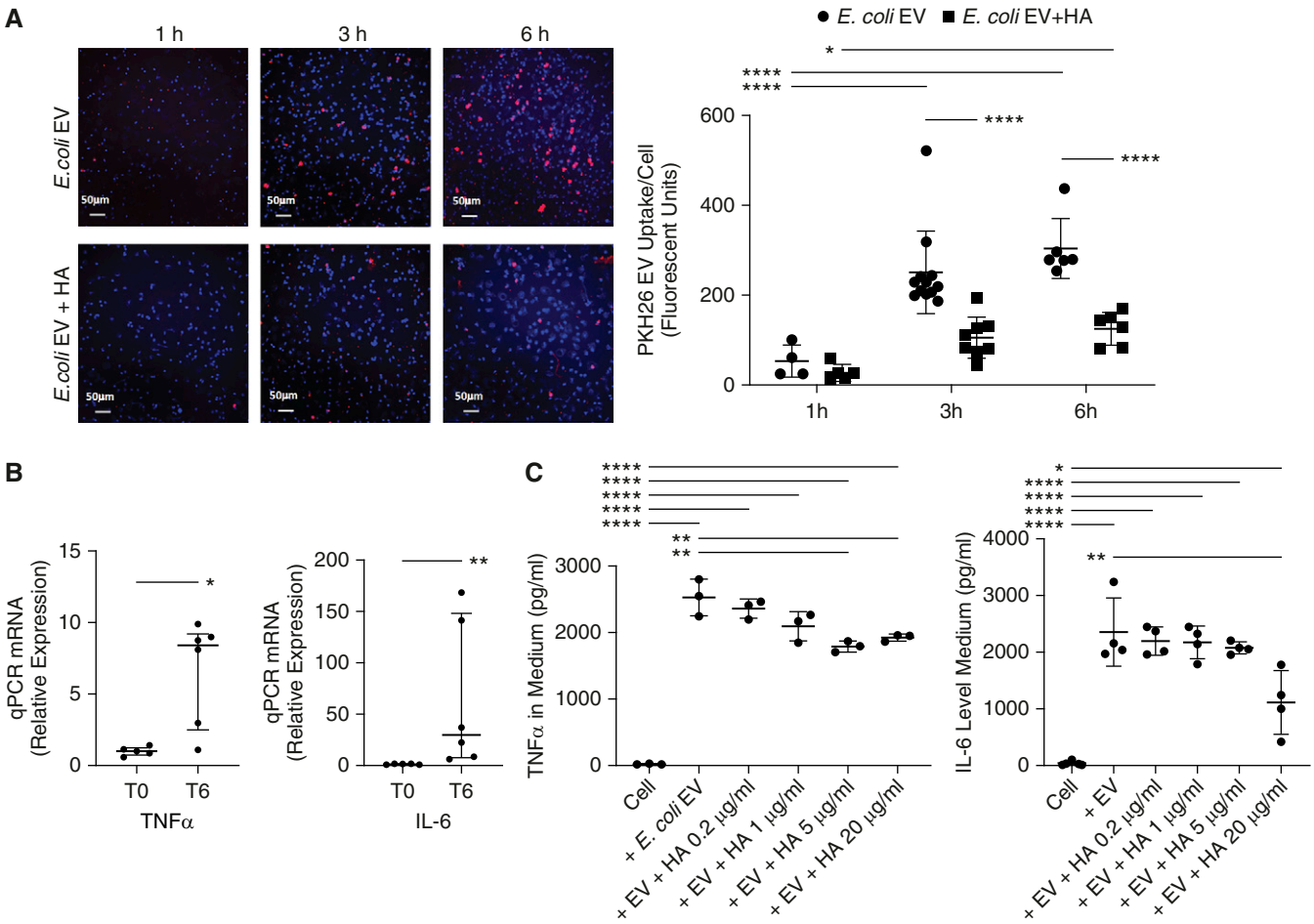

Figure 5. Update of *Escherichia coli* extracellular vesicles (EVs) by human monocytes. (*A*) The normal uptake of PKH26-labeled *E. coli* EVs by human monocytes was inhibited by coincubation with high-molecular-weight (HMW) hyaluronic acid (HA) by fluorescent microscopy as quantified by the average fluorescence intensity of PKH26 for each cell (scale bars, 50 μm). (*B*) Compared with EVs released from healthy lungs, *E. coli* EVs contained significantly higher mRNA levels of the inflammatory cytokines TNFα (tumor necrosis factor α) and IL-6 at 6 hours. (*C*) Coincubation of human monocytes with HMW HA decreased the release of TNFα and IL-6 caused by *E. coli* EVs by 29% and 53%, respectively, by human monocytes. Data are mean ± SD for PKH26 *E. coli* EV uptake and TNFα and IL-6 levels. Data are median with interquartile range for PCR data. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{****}P < 0.0001$ by ANOVA (Bonferroni) in *C*, ANOVA with Sidak's multiple comparison test in *A*, and Mann-Whitney test in *B*; $n = 3$–12. *P* values and confidence intervals are shown in Table E1. qPCR = quantitative PCR.

the inflammatory response during severe bacterial pneumonia in a human model of ALI. EVs isolated from 400 ml of perfusate, approximately one-fourth of the perfusate volume, were enough to decrease AFC and increase inflammation and pulmonary edema in a naive perfused human lung (Figure 2). The result was similar to the study by Li and colleagues that found that administration of microparticles derived from blood in LPS-induced ALI in rats if given to a naive animal either

Table 2. Donor Demographic and Clinical Data for Experiments with *Escherichia coli* Bacterial Pneumonia and HMW HA

	Control (*n* = 16)	*E. coli* Pneumonia (*n* = 10)	*E. coli* Pneumonia + i.v. HMW HA (*n* = 6)
Age, yr	45 ± 15	46 ± 12	45 ± 20
Male sex, *n* (%)	7 (41%)	5 (50%)	2 (29%)
Pa_{O2}/FI_{O2}, mm Hg, median (IQR)	188 (148–268)	178 (148–294)	193 (175–253)
Cdyn, ml/cm H_2O	40 ± 18	40 ± 19	41 ± 16
Murray LIS	1.7 ± 0.6	1.7 ± 0.7	1.8 ± 0.3
Ischemic time, h:min	32: 58 ± 4:47	33: 58 ± 5:31	31: 16 ± 3:42

Definition of abbreviations: Cdyn = dynamic compliance; HA = hyaluronic acid; HMW = high-molecular-weight; IQR = interquartile range; LIS = lung injury score. Data are presented as mean ± SD unless otherwise indicated. No significant differences in Pa_{O2}/FI_{O2} ratio, lung compliance, or lung injury score prior to organ harvest or total ischemic time were observed among groups.

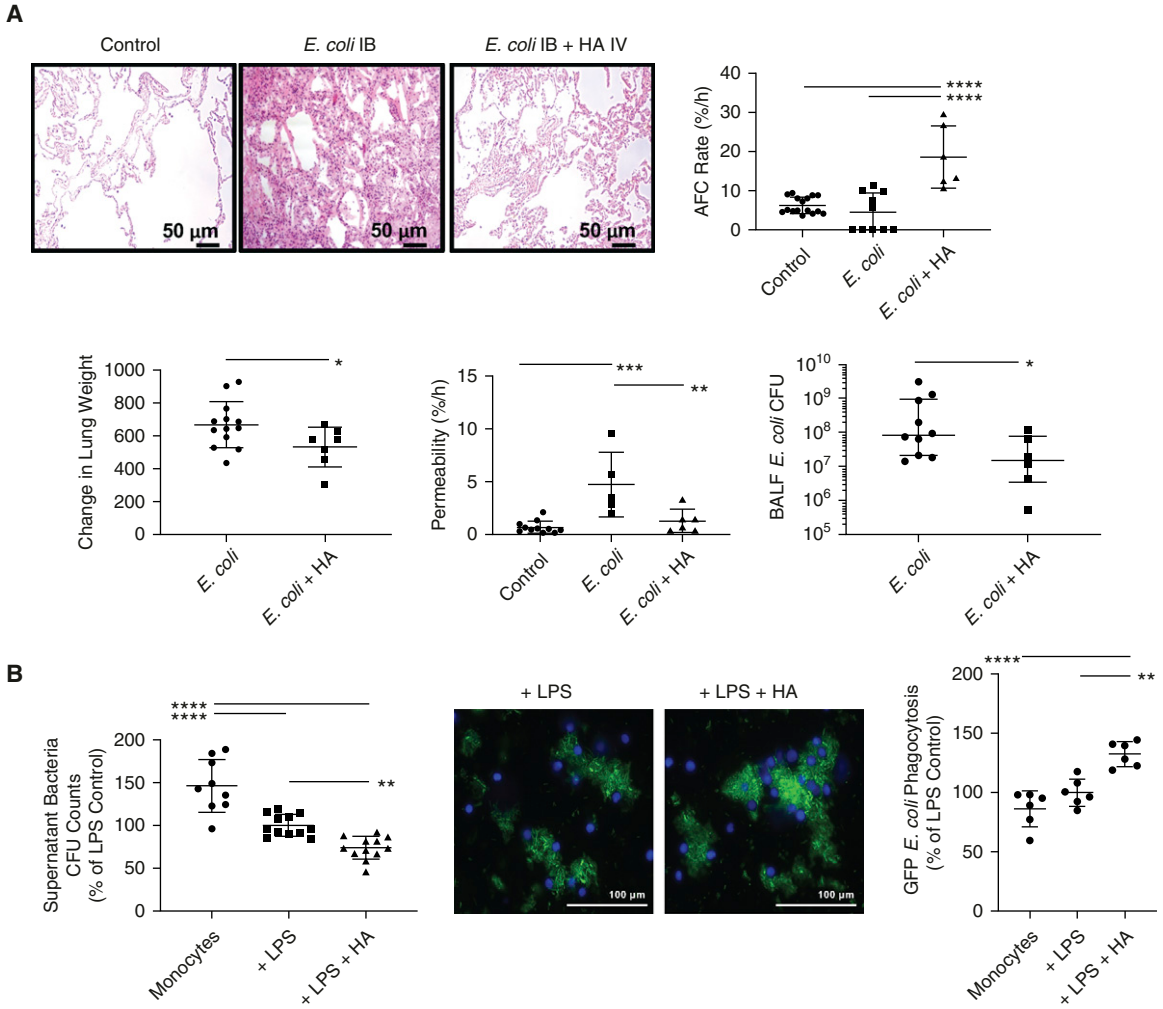

Figure 6. Therapeutic effects of high-molecular-weight hyaluronic acid (HMW HA) in *Escherichia coli* pneumonia. Intrabronchial instillation of *E. coli* bacteria caused severe lung injury, which was ameliorated with administration of HMW HA as therapy. (*A*) HMW HA reduced cellularity, interstitial thickening, and edema by hemasolin and eosin staining, restored alveolar fluid clearance (AFC) rate, and reduced lung protein permeability and pulmonary edema. HMW HA also reduced total *E. coli* cfu counts in the injured alveolus and increased the phagocytosis of bacteria by human blood monocytes *in vitro*. Data are mean ± SD for AFC rate, change in lung weight, and permeability, and data are median with interquartile range for BALF cfu counts, $n = 6$–10 per treatment group. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$ by ANOVA (Bonferroni) or Mann-Whitney test. (*B*) Coincubation of human monocytes with HMW HA decreased bacterial cfu counts in the supernatant and increased GFP-labeled *E. coli* bacteria phagocytosis as measured by fluorescence. Data are mean ± SD, $n = 5$–12. $^{**}P < 0.01$ and $^{****}P < 0.0001$ by ANOVA (Bonferroni). *P* values and confidence intervals are shown in Table E1. BALF = BAL fluid.

intrabronchially or intravenously could induce ALI (17).

EVs are a heterogeneous group and can be broadly classified as exosomes, microvesicles, and apoptotic bodies according to their size, biogenesis, and secretion mechanisms (18). Exosomes with a diameter of 20–200 nm originate from the release of intraluminal vesicles (19, 20), while microvesicles with a diameter of 200–1,000 nm are generated from outward budding and fission of the plasma membrane (21). For *E. coli* EVs, both exosomes and microvesicles were found using electron microscopy. The roles of exosomes or microvesicles or even apoptotic bodies independently in the pathogenesis of lung injury constitute new pathways that need further study. EVs can be formed and released by multiple cells under various stimuli during normal physiological or pathological conditions. In healthy humans, circulating EVs were mainly derived from platelets and, to a lesser extent, from endothelial cells (22). While in ALI, EVs derived from epithelial cells, lymphocytes, red blood cells, monocytes, or macrophages increased (23, 24). Several investigators found that EVs derived from endothelial cells were important markers of lung vascular injury in ventilator-induced lung injury, and i.v. instillation of stimulated endothelial cell–derived EVs could induce ALI (25, 26). Soni and colleagues found that alveolar macrophage-derived microvesicles played an important role in initiating ALI (23). In addition, multiple exogenous triggers can induce the release of EVs; for example, even the storage conditions of plasma like temperature or propyl gallate supplementation can lead to differences in

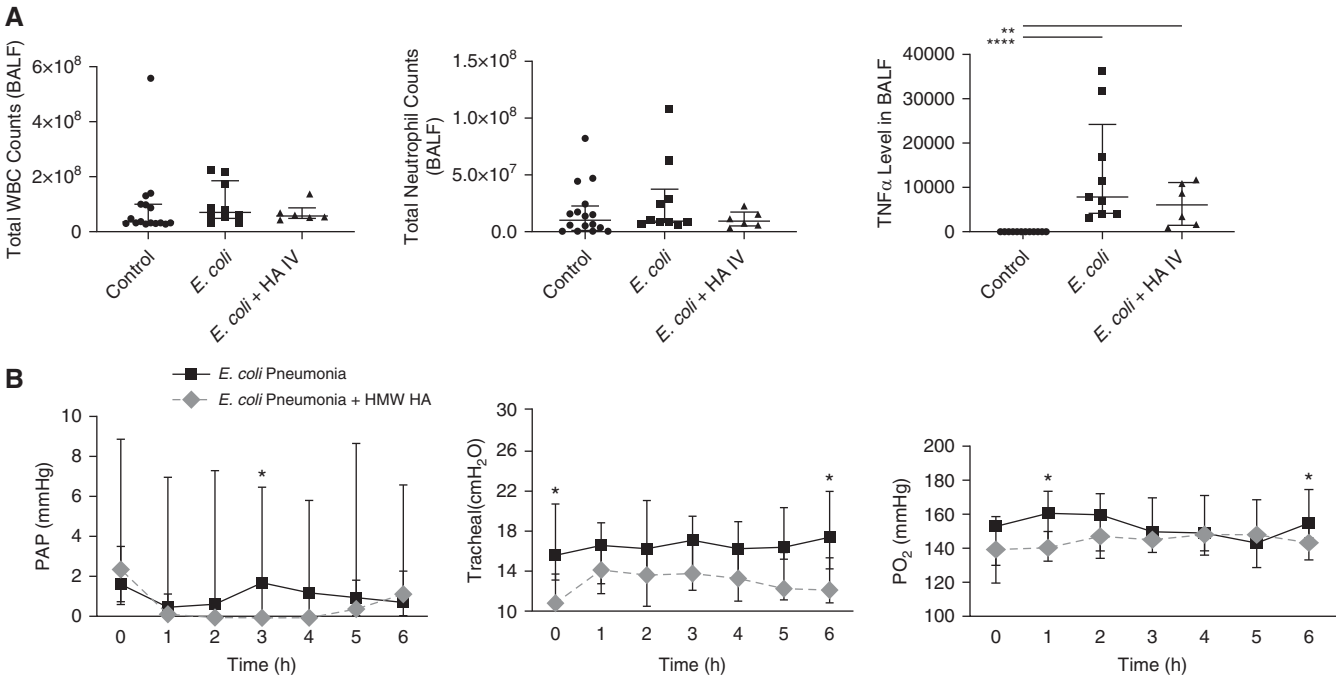

Figure 7. Effect of *Escherichia coli* pneumonia with or without high-molecular-weight hyaluronic acid (HMW HA) administration on inflammation and pulmonary arterial pressure, airway pressure, and P_{O_2} levels. (*A*) There was no significant effect of HMW HA on total white blood cell (WBC) and neutrophil counts. However, HMW HA administration reduced the median level of TNFα (tumor necrosis factor α) in the BAL fluid (BALF) by 23% induced by *E. coli* pneumonia, although it was not statistically significant. Data are median with interquartile range, $n = 6$–10 per treatment group. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{****}P < 0.0001$ by Kruskal-Wallis with Dunn's correction. *P* values and confidence intervals are shown in Table E1. (*B*) Although at individual time points there were statistically significant differences between groups, there appeared to be no overall effect of HMW HA administration on pulmonary arterial pressure, airway pressure, or P_{O_2} levels over time. Data are median with interquartile range, $n = 6$–10 per treatment group, *P* is significant versus corresponding group at each time point by Mann-Whitney test. PAP = pulmonary artery pressure.

the production of platelet-derived EVs (27, 28). In the current study, we were unable to detect significant levels of EVs derived from immune cells at 6 hours (Figure 3). However, the cellular sources of EVs often cannot be identified by flow cytometry because the EVs are commonly derived from intracellular vesicles or exosomes which contain no identifiable cellular marker.

EVs can exert their biological effect according to their interaction with target cells through possible transfer of various bioactive molecules including mRNA, microRNA, proteins, and organelles. In this study, the RNA content of the released EVs for TNFα or IL-6 increased in expression after *E. coli* pneumonia injury (Figure 5). The transfer of these mRNAs to target cells may be in part responsible for the inflammatory injury that developed with *E. coli* EVs.

HA can promote or inhibit inflammation and injury based primarily on its size. As a major component of the endothelial glycocalyx, located on the luminal side of the endothelium in all vessels (29), hyaluronan plays a critical role in limiting protein permeability (30). LMW HA activates specific HA-binding proteins to promote disruption of endothelial cell–cell contacts, while exogenous administration of HMW HA can promote vascular integrity (31). HMW HA can also exert antiinflammatory and immunosuppressive effects, while LMW HA can stimulate gene expression and synthesis of proinflammatory cytokines (32). Therefore, potential therapeutic application of HMW HA for sterile ALI has attracted considerable interest. Previous publications demonstrated that HMW HA administration enhanced endothelial cell barrier properties, reducing protein permeability and the influx of immune cells in LPS or ventilator-induced lung injury (33, 34). Singleton and colleagues found that intravenous administration of HMW HA 4 hours after LPS-induced ALI improved endothelial permeability (33) via CD44, sphingosine 1 phosphate, Akt and Rac signaling (35). In the current study, we also found that instillation of HMW HA into the perfusate ameliorated lung injury induced by *E. coli* EVs in an *ex vivo* perfused human lung and inhibited *E. coli* EV–induced inflammation in cultured human blood monocytes (Figures 2 and 5).

However, the mechanisms underlying the therapeutic effects remain largely unknown. Most of the focus in the literature has been on the repair of damaged vascular endothelium by HMW HA (33). In the current study, we hypothesized that binding of HMW HA to inflammatory EVs, thus inhibiting the uptake of EVs by target cells, may contribute to its protective effect. The biological rationale was based on binding of HMW HA to CD44, a major transmembrane glycoprotein commonly expressed on immune cells and its released EVs. In a previous study, we found that CD44 expressed on stem cell microvesicles was critical for the uptake of the microvesicles by target cells and its therapeutic effect (15, 36). In the current study, we found that binding of *E. coli* EVs by HMW HA, which was dependent in

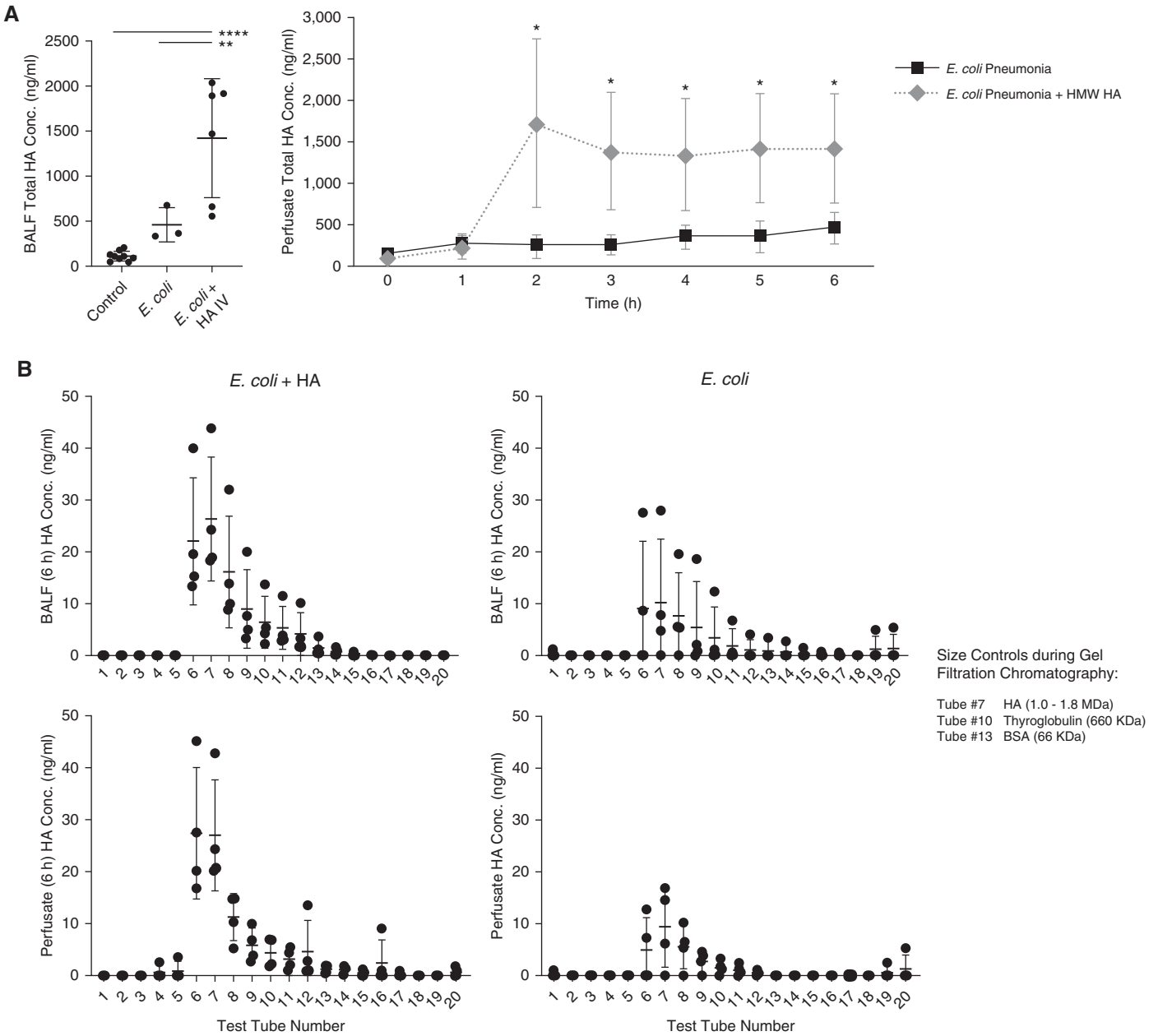

Figure 8. Effect of *Escherichia coli* pneumonia with or without high-molecular-weight hyaluronic acid (HMW HA) administration on total HA concentration in the injured alveolus and perfusate. (*A*) Administration of HMW HA dramatically increased total HA levels in both the injured alveolus and perfusate over time. Data are mean ± SD, $n = 3$–6. For BALF HA levels, $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{****}P < 0.0001$ by ANOVA (Bonferroni). For perfusate serial data, P is significant versus corresponding group at each specific time point by Student's t test. P values and confidence intervals are shown in Table E1. (*B*) In the treatment group, the majority of HA in both the injured alveolus and perfusate were HMW (>1,000 kD) in size despite 6 hours of perfusion. Even in the injured group, endogenous levels of HMW HA were elevated, suggesting some attempt at repair and recovery, although lower when compared with the treatment group. Size distribution of HA was separated out by gel filtration chromatography (Figure E1), and the concentration measured by ELISA. Data are mean ± SD, $n = 3$. BALF = BAL fluid; BSA = bovine serum albumin; Conc. = concentration.

part on CD44, inhibited the uptake of EVs by human blood monocytes and reduced inflammation induced by *E. coli* EVs (Figures 4 and 5). Endogenous LMW HA fragments may also interact with CD44, but the effect it induces in target cells may be different than those caused by HMW HA. Studies are ongoing to determine if the differential effect of LMW HA from HMW HA is due to receptor avidity (13) or clustering (37) or due to inhibition of internalization of the EVs into the target cells.

The protective effects of HMW HA in *E. coli* EV–induced lung injury raised the possibility for the therapeutic use of HMW HA in severe pneumonia and/or sepsis, the

most common causes of ARDS. To address this hypothesis, we instilled *E. coli* bacteria into marginal human lungs with an AFC < 10% to induce a severe bacterial pneumonia. Similar to the effect of HA on injury induced by *E. coli* EVs, administration of HMW HA restored and improved AFC and decreased lung protein permeability. Unexpectedly, we found that the administration of HMW HA also decreased total bacterial cfu levels in the injured alveolus (Figure 6). There are several possible explanations for the antimicrobial effect of HMW HA: *1*) As shown in the current study, HMW HA may enhance bacteria phagocytosis by immune cells, possibly through activation of CD44, which is involved in phagocytosis (38, 39). *2*) HMW HA may interfere with bacterial adhesion to a cellular substrate (40), as adhesion to oral and airway cells is necessary for the first step of microbial colonization and pathogenesis. Currently, several new biomaterials composed with HMW HA are undergoing clinical investigation to prevent the formation of biofilms and the adhesion of bacteria (41, 42). *3*) HMW HA appears to have bacteriostatic properties (43, 44). *4*) And last, the beneficial effect of HA on endothelial barrier properties may prevent translocation of bacteria (45).

There are some limitations to the current study: *1*) lack of an intact lymphatic system in our *ex vivo* perfused human lung for lung interstitial fluid clearance, which may be relevant if AFC is measured over longer periods; *2*) lack of other immune organs such as the spleen or liver which may participate in injury and/or repair and is required for HA metabolism (46); *3*) a short duration of lung injury, which limits assessment of whether the effects of HA can be sustained; *4*) and lack of study on the contributions of EVs released from bacteria itself on ALI.

In conclusion, EVs isolated from human lungs with *E. coli* bacterial pneumonia induced ALI in naive human lungs, emphasizing the potential pathogenetic importance of EVs, generated in a localized pneumonia, for compounding ALI and suggesting that studies of EVs in patients at risk or with ARDS are needed to understand the pathogenesis of ARDS more completely. And for the first time in a human model of ARDS, exogenous administration of HMW HA was found to bind to these inflammatory EVs, inhibiting uptake by target cells and decreasing inflammation and injury and enhancing the antimicrobial activity of immune cells. Based on these new findings, further investigations of HMW HA are warranted for the treatment of ARDS and/or sepsis. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

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