Therapeutic Effects of Hyaluronic Acid in Bacterial Pneumonia

In the *Ex Vivo* Perfused Human Lungs

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ONLINE DATA SUPPLEMENT

Online Supplementary Material & Methods

Ex vivo Perfused Human Lung Preparation

An *ex vivo* perfused human lung preparation established by our research group was used in the current study[1, 2] (**Fig. 1**). Human lungs which were not used for clinical transplantation were obtained from the Northern California Transplant Donor Network. Within 48 h of cold ischemia time, either the right or left lung was perfused with Dulbecco's Modified Eagle Medium (DMEM) solution containing 5% Bovine albumin fraction V (MP biomedical LLC, CA) with 100 ml fresh human whole blood at a cardiac output of 0.25 L/min. After gentle reperfusion and rewarming to 37°C, the lung was ventilated with a tidal volume of 300 ml with 5 cmH_2O of PEEP and respiratory rate of 10 breaths per minute in room air. Alveolar fluid clearance (AFC) was then measured in the right or left upper lobe. If AFC > 10%/h suggesting lungs with an intact alveolar epithelium and endothelium, 10^9 CFU of *E. coli* bacteria K1 strain was instilled into the middle or lower lobe. After 6 h of perfusion, the remaining perfusate was collected to isolate the EVs (*E.coli* EV) by ultracentrifugation. To test the biological activity of the EVs, *E.coli* EVs collected from 400 ml of perfusate was given into the middle or lower lobe intra-bronchially (IB) or intravenously (IV) through the pulmonary artery to naïve human lungs. As therapy, 1 mg HMW HA (Sodium Hyaluronate with MW 850 to 1000 KDa, LifeCore Inc., MN) was administered IV, into the perfusate, 1 h after injury (N = 5-6 lung per group). In separate experiments, marginal human lungs with AFC < 10% and injured further with 10^9 CFU of *E.coli* bacteria IB was treated with 1 mg HMW HA IV 1 h following injury (N = 6-10 lungs per group).

Pulmonary artery pressure (PAP), airway pressure and lung temperature were continuously monitored. Perfusate pH, PO_2, and PCO_2 were measured every h from time (T)0 to T6 h (OptiMedical, GA). Lungs weight at T0 before and at T6 h after injury with or without therapy were recorded. The absolute total number of white blood cells and neutrophils in the bronchoalveolar lavage fluid (BALF) were measured using Hemavet 950FS (Drew Scientific Inc., FL).

The AFC rate for the upper control and injured lobes were measured at the beginning and end of the experiment independently by the change in protein concentration of an instillate containing 5% albumin (125

ml) over 1 h. AFC was calculated using the equation as used in our previous experiments: AFC(%/h)=(1-Ci/Cf) × 100 (Ci = protein concentration at time 0 and Cf = protein concentration after 1 h)[1, 3]. The concentration of HA and TNFα in BALF or perfusate was measured by commercialized sandwich enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Inc., MN).

Lung Protein Permeability

For lungs with AFC < 10% and injured with *E.coli* bacteria, lung protein permeability was measured in both the control and injured lung lobes as previously done[4]. Four hundred mg of Evans Blue (Sigma-Aldrich, MO) was administered into the perfusate at T5 h. At the end of the experiment or T6.5 h, 1 ml of the perfusate was collected and combined with 9 ml of formamide. After flushing the lung with 2 L of PBS to remove remaining blood, multiple lung pieces (1 – 2 gm each) from both the control and injured lobes were dissected out and incubated with 10 ml formamide at 55℃ for 72 h. Evans blue was quantified by measuring the optical density of the formamide extract at 620 nm. Absorbance was compared with a standard curve for each sample to calculate lung protein permeability.

Isolation of *E.coli* EV

To isolate *E.coli* EV from the perfusate, the perfusate collected after 6 h of injury was centrifuged at 2000 x g for 10 min, followed by 10,000 x g for 30 min to remove cells and cellular debris. The final supernatant was then ultracentrifuged (Beckman Coulter Optima L-100XP 19 Ultracentrifuge) at 100,000 × g at 4°C for 1 h. The pellet was resuspended and washed in PBS and then submitted to a second ultracentrifugation with the same condition[5, 6]. After ultracentrifugation, *E.coli* EV was resuspended with PBS (10 μl of PBS per 1 ml of perfusate) and stored at -80℃.

Protein and RNA content in *E.coli* EV

E.coli EV collected from 400 ml of perfusate was combined with NP40 cell lysis buffer (Thermo Fisher Scientific, CA) supplemented with protease inhibitor cocktail 1 (RPI Corp., IL) to extract total protein. The

concentration of total protein was measured by micro BCA protein assay kit (Thermo Fisher Scientific). Total RNA was separated from *E.coli* EV using RNeasyMini Kit (QIAGEN Sciences, Germany), and the RNA content was quantified using NanoDrop ND1000 Spectrophotometer (Thermo Fisher Scientific).

Fluorescent Labeling and Analysis of *E.coli* EV with Flow Cytometry

Although all carrier solutions were filtered using 0.22 μm membrane filter, large amount of background events could be detected by flow cytometry. These events had similar forward scatter (FSC) and side scatter (SSC) distribution as the majority of *E.coli* EVs. We initially tried to discriminate EVs from background using standard silica beads (ApogeeMix for Flow Cytometer, England), which have similar refractive index as real EVs and come in a variety of sizes from 110 to 1300 nm. However, the beads were indistinguishable from background events from D-PBS. To gate just through FSC and SSC, we then prelabeled *E.coli* EV with PKH26. An unstained sample was used to detect auto-fluorescence and set the photomultiplier for all the channels. The total number of *E.coli* EV collected in 20 seconds was calculated as total events collected x percentage of PKH26 positive events. And the percentage of certain target protein such as surface markers was equal to protein positive events divided by the total number of EV.

E.coli EVs were labeled with PKH26 (PKH26 Red Fluorescent Cell Linker Mini Kit, Sigma-Aldrich, MO), a widely used amphiphilic lipid dye according to the manufacturer's protocol. *E.coli* EVs isolated from 1 ml of perfusate were resuspended with 500 µl Diluent C, which was then combined with the same volume of Diluent C containing 2 µl PKH26. After 5 minutes of incubation in room temperature, 1 ml D-PBS containing 1% BSA were added to prevent further staining followed by a washing step with D-PBS. For flow cytometry, 1 ml of perfusate derived *E.coli* EVs were resuspended with 100 µl Staining buffer (BD Biosciences, CA) containing 5 µl CD9-FITC (eBioscience Inc., CA), 20 µl CD44-FITC (BD Biosciences), 5 µl CD3-Alexa Fluor 488 (Thermo Fisher Scientific), 20 µl CD14-FITC (BD Biosciences), 5 µl CD41-Alexa Fluor 488 (BioLegend, CA), 5 µl CD326-Alexa Fluor 488 (BioLegend), 5 µl CD66b-PerCP-Cy5.5 (BD Biosciences) or CD31-Alexa Fluor 488 (BioLegend), and then incubated for 30 minutes at room temperature. The *E.coli* EVs were washed once with D-PBS containing 1% BSA.

The fluorescence expression of stained *E.coli* EVs was detected by a BD FACSAria™ Fusion Special Order (SORP) cell sorter (BD Biosciences) with 100 nm nozzle and ND filter 1. The threshold was set on the SSC 200. For fluorescence detection, we used a 586/15 band pass filter for PKH26, 525/50 band pass filter for CD9-FITC, CD44-FITC, CD3-Alexa Fluor 488, CD14-FITC, CD41-Alexa Fluor 488, CD326-Alexa Fluor 488 and CD31-Alexa Fluor 488, and 695/40 band pass filter for CD66b-PerCP-Cy5.5. Samples loaded were acquired for 20 second to be analyzed after the event rate was stable. Collected data were analyzed by Diva software (BD Biosciences).

Binding of Extracellular Vesicles to High Molecular Weight Hyaluronic Acid

Glass slides were coated with 10 mg/ml HMW HA overnight and dried in room temperature as previously described[7]. *E.coli* EV labelled with PKH26 was added onto the glass slide with or without HA coating. Two h later, the slides were washed with PBS to remove unattached EVs. The slides were studied under fluorescence microscope (Axioskop, Zeiss, Germany), and the area of fluorescence were analyzed using Image J software. To help quantify EVs binding to HA, total protein from the slides was extracted, and the content of TNFα was quantified using ELISA. In separate experiments, to determine key receptors involved in the binding of EVs to HA, EVs were pretreated with anti-CD44 antibody (BD Biosciences) or IgG1 control (BD Biosciences).

Uptake of Fluorescent Labeled *E.coli* EV by Human Blood Monocytes

Human blood monocytes were collected from 10 ml of whole blood from healthy donor and diluted with 10 ml D-PBS and slowly transferred onto 15 ml Ficoll-Paque™ Plus (BD Biosciences) gradient to avoid disturbing the interphase. After spinning down at 400 g for 30 min, the middle layer containing mononuclear cells was collected and transferred into a new tube and washed twice with Roswell Park Memorial Institute 1640 (RPMI) medium + 1% L-glutamine. The cells were then resuspended using RPMI 1640 + 10% FBS and placed in 24 well plate (2.5×10^5 cells in 500 µl) at 37℃ in 5% CO_2 incubator overnight. After 24 h, > 90% of the remaining cells were CD14+.

To detect the uptake of *E.coli* EV by monocytes, *E.coli* EV pre-labeled with PKH26 from 0.5 ml of perfusate was added into the culture media per well. One h later, HMW HA was added to the culture media at a concentration of 0.2, 1, 5 or 20 μg/ml. Following 1-3 h of incubation, the monocytes were washed twice with PBS and then spun down on glass slides. The cells were mounted and stained with mounting media with DAPI (Vectashield Mounting Medium with DAPI, Vector Laboratories, CA). The cells were examined by fluorescence microscopy (Nikon Eclipss 80i, Nikon, Japan). The average fluorescent intensity for each cell was compared among groups. In addition, after 6 h of incubation, culture media was collected for the measurement of TNFα and IL-6 levels to assess the level of inflammation induced by *E.coli* EVs.

qPCR

High capacity RNA-to-cDNA kit (Thermo Fisher Scientific) was used to generate single stranded cDNA from equal amount of purified RNAs. Subsequent quantitative polymerase chain reaction (qPCR) was performed in a StepOnePlus real-time PCR system (Applied Biosystems, CA) using TaqMan fast universal PCR master mix (Thermo Fisher Scientific) and the corresponding human primers of IL-6 (Hs00174131_m1), TNFα (Hs00174128_m1) and GAPDH (Hs03929097_g1) (Applied Biosystems, CA). Reactions were performed in triplicates and threshold cycle values were normalized to GAPDH gene expression. The specificity of the products was determined by melting curve analysis. The relative expression of target genes to GAPDH was calculated by $\Delta C(t)$ formula.

Measurement of Phagocytosis of *E.coli* Bacteria by Human Monocytes

Primary cultures of human monocytes were stimulated with 1 μg/ml LPS (*Escherichia coli* O111:B4, Sigma Aldrich) with or without 25 μg/ml HMW HA for 24 h. Then 10^7 CFU of *E.coli* bacteria, previously incubated with human plasma, was added. After 90 minutes of incubation at 37℃, the supernatant was collected, seeded on LB agar plates and kept at 55℃ overnight (Teknova Inc., CA). Colony forming unit (CFU) levels was measured the next day. To corroborate the CFU data, primary cultures of human monocytes were stimulated with LPS and GFP labelled *E.coli* bacteria (ATCC® 25922 GFP™). Intracellular *E.coli* bacteria or

level of phagocytosis was measured by both immunofluorescence and microplate spectrophotometer following lysis of the monocytes.

Gel Filtration Column Chromatography

To characterize the size and concentration of the HA in the perfusate and BAL, a 15 cm gel filtration or size exclusion chromatography column was set up using Sepharose 6B100 agarose beads (Sigma-Aldrich). Twenty ml of PBS was used as the elution buffer and collected in glass tubes, 1 ml per tube, by gravity into 20 test tubes. Size standards were generated using both HA standards (1.5 M or 1010 – 1800 KDa, Lifecore) and proteins (mouse thyroglobulin, 660 KDa, and bovine serum albumin, 66 KDa, Sigma-Aldrich). The test tube at which each standard had the highest peak elution was identified by BCA assay or HA ELISA (R&D Systems). The elution of HMW HA (1.5 M or 1010 – 1800 KDa) peaked early at test tube 7. Elution of mouse thyroglobulin peaked at test tube 10, and elution of BSA peaked at test tube 13 (**Supplemental Figure E1**). For the samples, 0.5 ml of BALF or perfusate was placed onto the Sepharose column prior to the elution buffer, 20 ml of PBS. The test tube with the peak elution and the concentration of the samples were identified and measured using HA ELISA (R&D Systems).

References

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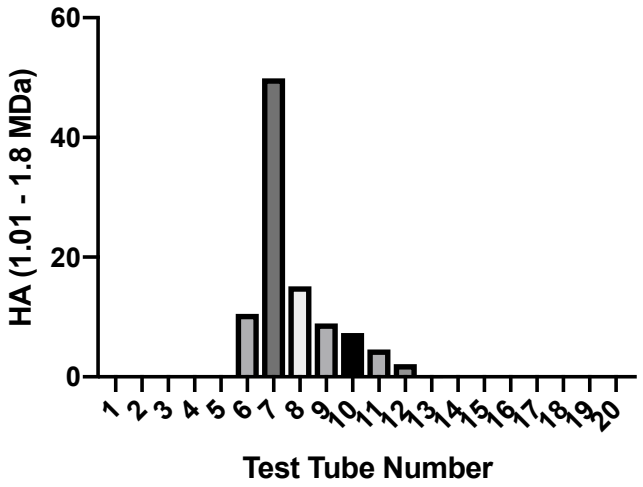
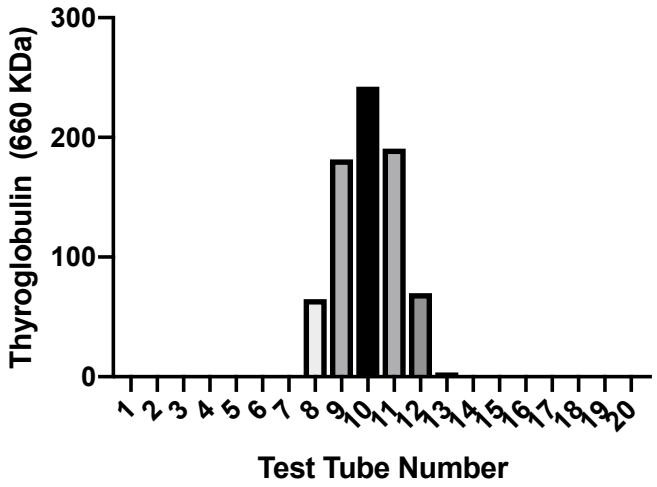
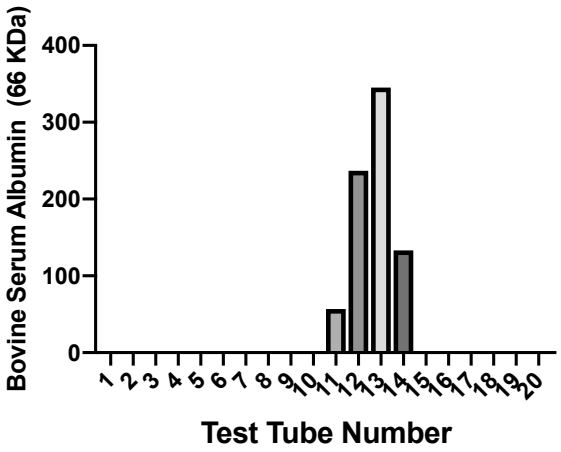
Supplemental Figure E1







Peak Elution Time for Each Size Control during Gel Filtration Chromatography:

Tube #7 HA (1.0 - 1.8 MDa)
Tube #10 Thyroglobulin (660 KDa)
Tube #13 BSA (66 KDa)

Supplementary Table E1

Figure 2A. AFC

				***Mean difference (vs.* Control)**		***Mean difference (vs. E.coli EV IB)***		***Mean difference (vs. E.coli EV IV)***		***Overall P-value***
Group	**N**	**Mean**	**SD**	**95% CI**	**P-value***	**95% CI**	**P-value***	**95% CI**	**P-value***	0.0012
Control	17	17.3	7.7	(reference)		-		-		
E.coli EV IB	6	6.7	4.9	1.6 to 19.6	0.014	(reference)		-		
E.coli EV IV	5	6.1	6.8	1.5 to 20.9	0.016	-10.9 to 12.1	>0.9999	(reference)		
E.coli EV IV + HA IV	6	18.3	4.3	-10.0 to 8.0	>0.9999	-22.6 to -0.7	0.033	-23.7 to -0.7	0.032	

*P Values were given by ANOVA test followed by Bonferroni adjustment. For the mean differences of the pair group comparisons, 95% confidence interval (CI) were calculated by unpaired t-tests.

Figure 2B. TNFα in BALF

				***Mean difference (vs.* Control)**		***Mean difference (vs. E.coli EV IB)***		***Mean difference (vs. E.coli EV IV)***		***Overall P-value***
Group	**N**	**Mean**	**SD**	**95% CI**	**P-value***	**95% CI**	**P-value***	**95% CI**	**P-value***	<0.0001
Control	17	19	53	(reference)						
E.coli EV IB	6	4245	2635	-5944 to -2507	<0.0001	(reference)				
E.coli EV IV	5	4465	1424	-6287 to -2605	<0.0001	-2412 to 1971	>0.9999	(reference)		
E.coli EV IV + HA IV	6	2067	1129	-3766 to -329	0.013	88 to 4267	0.037	207 to 4590	0.026	

*P Values were given by ANOVA test followed by Bonferroni adjustment. For the mean differences of the pair group comparisons, 95% confidence interval (CI) were calculated by unpaired t-tests.

Figure 2B. Total HA Conc. in Perfusate

				Mean difference (vs. **Control)**		*Mean difference (vs. E.coli EV IB)*		*Mean difference (vs. E.coli EV IV)*		*Overall P-value*
Group	**N**	**Mean**	**SD**	**95% CI**	**P-value***	**95% CI**	**P-value***	**95% CI**	**P-value***	<0.0001
Control	17	197	137	(reference)						
E.coli EV IB	6	519	495	-754 to 110	0.264	(reference)				
E.coli EV IV	5	714	558	-980 to -54	0.022	-746 to 356	>0.9999	(reference)		
E.coli EV IV + HA IV	6	1431	260	-1667 to -803	<0.0001	-1438 to -388	0.0002	-1269 to -167	0.0055	

*P Values were given by ANOVA test followed by Bonferroni adjustment. For the mean differences of the pair group comparisons, 95% confidence interval (CI) were calculated by unpaired t-tests.

Figure 4A. Fluorescence Area of *E.coli* EV (% of Background)

				Mean difference (vs. **Control)**		*Mean difference (vs. HA)*		*Mean difference (vs. HA + IgG Control)*		*Overall P-value*
Group	**N**	**Mean**	**SD**	**95% CI**	**P-value***	**95% CI**	**P-value***	**95% CI**	**P-value***	0.0008
Control	5	100	83	(reference)						
HA	7	422	200	-545 to -99	0.0025	(reference)				
HA + IgG Ab	8	344	95	-461 to -27	0.022	-119 to 275	>0.9999	(reference)		
HA + Anti-CD44 Ab	4	129	54	-285 to 226	>0.9999	54 to 531	0.011	-18 to 448	0.083	

*P Values were given by ANOVA test followed by Bonferroni adjustment. For the mean differences of the pair group comparisons, 95% confidence interval (CI) were calculated by unpaired t-tests.

Figure 4B. TNFα Level (% of Background)

				Mean difference (vs. Control)		*Mean difference (vs. HA)*		*Mean difference (vs. HA + IgG Control)*		*Overall P-value*
Group	**N**	**Mean**	**SD**	**95% CI**	**P-value***	**95% CI**	**P-value***	**95% CI**	**P-value***	0.0011
Control	3	100	91	(reference)						
HA	6	873	219	-1268 to -277	0.0015	(reference)				
HA + IgG Ab	6	650	246	-1045 to -54	0.0251	-128 to 573	0.4421	(reference)		
HA + Anti-CD44 Ab	6	454	142	-850 to 141	0.2819	68 to 769	0.0147	-155 to 546	0.6788	

*P Values were given by ANOVA test followed by Bonferroni adjustment. For the mean differences of the pair group comparisons, 95% confidence interval (CI) were calculated by unpaired t-tests.

Figure 5C. TNFα Level in Medium (pg/ml)

				Mean difference (vs. Control)		*Mean difference (vs. EV)*		*Overall P-value*
Group	**N**	**Mean**	**SD**	**95% CI**	**P-value***	**95% CI**	**P-value***	<0.0001
Monocytes	3	17	5	(reference)				
+ EV	3	2529	278	-2991 to -2032	<0.0001	(reference)		
+ EV + HA 0.2 μg/ml	3	2358	141	-2820 to -1861	<0.0001	-309 to 650	>0.9999	
+ EV + HA 1 μg/ml	3	2091	220	-2553 to -1594	<0.0001	-41 to 917	0.0895	
+ EV + HA 5 μg/ml	3	1788	81	-2250 to -1292	<0.0001	261 to 1220	0.0016	
+ EV + HA 20 μg/ml	3	1922	53	-2384 to -1425	<0.0001	127 to 1086	0.0089	

*P Values were given by ANOVA test followed by Bonferroni adjustment. For the mean differences of the pair group comparisons, 95% confidence interval (CI) were calculated by unpaired t-tests.

Figure 5C. IL-6 Level in Medium (pg/ml)

				Mean difference (vs. **Control)**		*Mean difference (vs. EV)*		***Overall P-value***
Group	**N**	**Mean**	**SD**	**95% CI**	**P-value***	**95% CI**	**P-value***	<0.0001
Monocytes	3	37	51	(reference)				
+ EV	3	2354	600	-3315 to -1319	<0.0001	(reference)		
+ EV + HA 0.2 μg/ml	3	2195	247	-3156 to -1160	<0.0001	-765 to 1083	>0.9999	
+ EV + HA 1 μg/ml	3	2176	286	-3137 to -1141	<0.0001	-746 to 1102	>0.9999	
+ EV + HA 5 μg/ml	3	2076	102	-3036 to -1040	<0.0001	-646 to 1202	>0.9999	
+ EV + HA 20 μg/ml	3	1113	562	-2074 to -78	0.0281	317 to 2165	0.004	

*P Values were given by ANOVA test followed by Bonferroni adjustment. For the mean differences of the pair group comparisons, 95% confidence interval (CI) were calculated by unpaired t-tests.

Figure 6A. AFC

				Mean difference (vs. **Control)**		*Mean difference (vs. E.coli Pneumonia)*		***Overall P-value***
Group	**N**	**Mean**	**SD**	**95% CI**	**P-value***	**95% CI**	**P-value***	<0.0001
Control	16	6.2	2.1	(reference)		-		
E.coli Pneumonia	10	4.4	4.9	-2.9 to 6.4	>0.9999	(reference)		
E.coli + HMW HA	6	18.6	8.0	-18.0 to -6.9	<0.0001	-20.1 to -8.2	<0.0001	

*P Values were given by ANOVA test followed by Bonferroni adjustment. For the mean differences of the pair group comparisons, 95% confidence interval (CI) were calculated by unpaired t-tests.

Figure 6A. Permeability (%/h)

				***Mean difference (vs.* Control)**		***Mean difference (vs. E.coli Pneumonia)***		***Overall P-value***
Group	**N**	**Mean**	**SD**	**95% CI**	**P-value***	**95% CI**	**P-value***	0.0005
Control	11	0.7	0.6	(reference)		-		
E.coli Pneumonia	5	4.7	3.1	-6.3 to -1.8	0.0004	(reference)		
E.coli + HMW HA	6	1.3	1.1	-2.7 to 1.5	>0.9999	1.0 to 6.0	0.0054	

*P Values were given by ANOVA test followed by Bonferroni adjustment. For the mean differences of the pair group comparisons, 95% confidence interval (CI) were calculated by unpaired t-tests.

Figure 6B. Supernatant *E.coli* Bacteria CFU Levels (% of LPS Control)

				***Mean difference (vs.* Control)**		***Mean difference (vs. + LPS)***		***Overall P-value***
Group	**N**	**Mean**	**SD**	**95% CI**	**P-value***	**95% CI**	**P-value***	<0.0001
Monocytes	9	146	31	(reference)		-		
+ LPS	12	100	13	24 to 68	<0.0001	(reference)		
+ LPS + HMW HA	12	74	13	51 to 94	<0.0001	6 to 46	0.0072	

*P Values were given by ANOVA test followed by Bonferroni adjustment. For the mean differences of the pair group comparisons, 95% confidence interval (CI) were calculated by unpaired t-tests.

Figure 6B. GFP *E.coli* Phagocytosis (% of LPS Control)

				***Mean difference (vs.* Control)**		***Mean difference (vs. + LPS)***		***Overall P-value***
Group	**N**	**Mean**	**SD**	**95% CI**	**P-value***	**95% CI**	**P-value***	<0.0001
Monocytes	6	86	15	(reference)		-		
+ LPS	6	100	12	-33 to 6	0.2406	(reference)		
+ LPS + HMW HA	6	132	11	-66 to -26	<0.0001	-52 to -13	0.0015	

*P Values were given by ANOVA test followed by Bonferroni adjustment. For the mean differences of the pair group comparisons, 95% confidence interval (CI) were calculated by unpaired t-tests.

Figure 8A. BALF Total HA Conc. (ng/ml)

				***Mean difference (vs.* Control)**		***Mean difference (vs. + E.coli Pneumonia)***		***Overall P-value***
Group	**N**	**Mean**	**SD**	**95% CI**	**P-value***	**95% CI**	**P-value***	<0.0001
Control	9	111	55	(reference)		-		
E.coli Pneumonia	3	459	190	-1046 to 351	0.6006	(reference)		
E.coli + HMW HA	6	1421	659	-1862 to -758	<0.0001	-1703 to -222	0.0097	

*P Values were given by ANOVA test followed by Bonferroni adjustment. For the mean differences of the pair group comparisons, 95% confidence interval (CI) were calculated by unpaired t-tests.