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## Therapeutic Effects of Human Mesenchymal Stem Cell Microvesicles in an *Ex Vivo* Perfused Human Lung Injured with Severe *E.coli* Pneumonia

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### Abstract

We previously reported that microvesicles (MV) released by human mesenchymal stem cells (MSC) were as effective as the cells themselves in both *Escherichia coli* (*E.coli*) LPS and live bacterial induced acute lung injury (ALI) mice models. However, it remained unclear whether the biological effect of MSC MV can be applied to human ALI. In the current study, we tested the therapeutic effects of MSC MVs in a well-established *ex vivo* perfused human model of bacterial pneumonia. Using human donor lungs not used for transplantation, we instilled *E.coli* bacteria intra-bronchially and, 1 h later, administered MSC MVs into the perfusate as therapy. After 6 h, instillation of *E.coli* bacteria caused influx of inflammatory cells which resulted in significant inflammation, lung protein permeability and pulmonary edema formation. Administration of MSC MV significantly increased alveolar fluid clearance and reduced protein permeability and numerically lowered the bacterial load in the injured alveolus. The beneficial effect on bacterial killing was more pronounced with pretreatment of MSCs with a TLR3 agonist, Poly (I:C), prior to the isolation of MVs. Isolated human alveolar macrophages had increased antimicrobial activity with MSC MV treatment *in vitro* as well. Although oxygenation and lung compliance levels were similar between injury and treatment groups, administration of MSC MVs numerically decreased median pulmonary artery pressure at 6 h. In summary, MSC MVs increased alveolar fluid clearance and reduced lung protein permeability, and pretreatment with Poly (I:C) enhanced the antimicrobial activity of MVs in an *ex vivo* perfused human lung with severe bacteria pneumonia.

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**DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST**

The authors declare no potential conflicts of interest.

## Keywords

Acute lung injury; *E.coli* pneumonia; *Ex vivo* perfused human lung; Mesenchymal stem cell; Microvesicles.

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## INTRODUCTION

Acute respiratory distress syndrome due to bacterial pneumonia and/or sepsis remains a common cause of respiratory failure in critically ill ICU patients with high mortality rates despite appropriate antibiotic use and supportive care<sup>1, 2</sup>. New therapeutic modalities are needed to improve morbidity and mortality. Multiple pre-clinical studies have demonstrated significant therapeutic potential with mesenchymal stem/stromal cells (MSC) for ALI despite limited engraftment rates of 1 ~ 5%<sup>3, 4, 5</sup>. The therapeutic potential of MSCs appeared to arise in part from the secretion of growth factors such as keratinocyte growth factors (KGF)<sup>6</sup>, anti-inflammatory products such as PGE<sub>2</sub> or Lipoxin A4<sup>7, 8</sup>, anti-permeability factors such as angiopoietin-1 (Ang1)<sup>9, 10</sup>, and antimicrobial products such as LL-37 or Lipocalin2<sup>11, 12</sup>. Based on the significant potential of MSC, several Phase I and II clinical trials focused on safety are underway (NCT02097641, NCT01902082, [Clinicaltrials.gov](https://clinicaltrials.gov))<sup>13</sup>. However, there remains some concerns with allogeneic stem cell therapy such as tumor formation and hemodynamic instability due to their physical properties.

Recently, stem cell derived therapy, such as MSC conditioned media (CM) or extracellular vesicles, have generated considerable interest due to similar therapeutic properties without the inherent challenges of using live cells. Ionoscu et al. demonstrated that MSC CM reduced lung inflammation and injury following lipopolysaccharide (LPS) induced ALI in a mouse<sup>14</sup>. Using LPS induced ALI in an *ex vivo* perfused human lung, we also found that MSC CM reduced extravascular lung water, improved lung endothelial barrier permeability and restored alveolar fluid clearance (AFC) in part by KGF secretion by MSCs<sup>6</sup>. More recently, we demonstrated that MSC derived extracellular vesicles were as biologically active as MSCs in *E.coli* LPS and bacterial induced ALI in mice in part through the transfer of mRNA and microRNA to target cells which resulted in improved resolution of pulmonary edema and increased antimicrobial activity<sup>15, 16</sup>. We and other investigators also found that pretreatment of MSCs, such as with Poly (I:C)<sup>17</sup>, carbon monoxide<sup>18</sup>, hypoxia<sup>19</sup>, etc., may be a viable method to enhance the therapeutic properties of the cells and its released MVs.

Extracellular vesicles, which are released by most cell types, can be broadly classified into exosomes, microvesicles (MV) and apoptotic bodies based largely on their size and origin. Interestingly, extracellular vesicles contain mRNA, microRNA, protein, surface receptors and potentially organelles which may be involved in their function<sup>20-22</sup>. However, whether or not stem cell derived therapy such as extracellular vesicles can be translated to large animal or even human ALI models is unknown. In the current study, we hypothesized that MSC MVs could be effective in reducing inflammation and total bacterial load in a severe pneumonia model in the *ex vivo* perfused human lung and that preconditioning MSCs with Poly (I:C) would enhance the antimicrobial properties of the released MVs.

## METHODS

### **Ex Vivo Perfused Human Lung and Measurement of Alveolar Fluid Clearance**

Either the right or left lung, from the Northern California Transplant Donor Network, was perfused slowly with DMEM without Phenol Red + 5% bovine serum albumin until a cardiac output of 0.25 L/min was reached and then ventilated (Harvard Apparatus, tidal volume 300 ml, respiratory rate = 10/min,  $FiO_2 = 21\%$ , and PEEP = 5 cmH<sub>2</sub>O) (Figure 1)<sup>23</sup>. AFC rate was then measured in the control lung lobe by the change in protein concentration of a BAL fluid over 30 min using the following equation:  $AFC (\%/h) = 2(1-C_i/C_f) \times 100$  ( $C_i$  and  $C_f$  = protein concentration at 5 and 35 min respectively). If  $0 < AFC < 10\%/h$ ,  $10^9$  CFU of *E.coli* bacteria was instilled into the lower lobe, and fresh human blood (100 ml) was added to the perfusate. One hour after injury, either 200  $\mu$ l of MSC MV (1X), 400  $\mu$ l of MSC MV (2X), 200  $\mu$ l of Toll-like receptor 3 (TLR-3) agonist polyinosinic:polycytidylic acid (Poly(I:C)) pretreated MSC MV, or 200  $\mu$ l of normal human lung fibroblasts (NHLF) MV as cellular control was administered intravenously in a random fashion. At 6 h, the AFC rate of the injured lobe was measured.

### **Isolation of Microvesicles Derived from Human Mesenchymal Stem Cells**

MVs were isolated from the conditioned medium of human bone marrow derived MSCs<sup>24</sup>, obtained from a NIH repository at Texas A&M Health Science Center, and NHLFs using ultracentrifugation. Briefly, MSCs or NHLFs were grown in a T75 flask until 90% confluency and then serum starved in  $\alpha$ MEM or FBM supplemented with 0.5% Bovine Albumin Fraction (MP Biomedical LLC). After 48 h, the conditioned medium was collected and centrifuged at 3000 rpm for 20 min to remove cellular debris and then at 100,000 g (Beckman Coulter Optima L-100XP Ultracentrifuge) to isolate the MVs for 1 h twice at 4°C. The final sediment was resuspended in PBS and stored in -80°C. Ten  $\mu$ l of the resuspension were equivalent to MVs released by 1 million MSCs or NHLFs over 48 h.

### **TLR3 Priming of MSC**

In order to switch MSC towards a more immunoregulatory phenotype, TLR3 agonist, Poly (I:C) (1  $\mu$ g/mL, Sigma Aldrich), was cultured with MSCs for 1 h<sup>17</sup>. The cells were then washed twice in PBS and serum starved over 48 h prior to isolation of MVs.

### **MSC MV Characterization**

MSC MVs were labelled with PKH26 (Sigma-Aldrich) to separate out vesicles from debris or with antibodies for CD9-FITC, control IgG1 k-FITC, CD44-FITC or control IgG2b k-FITC (eBioscience Inc. & BD Biosciences) for flow cytometry. A BD FACSAria™ Fusion Special Order (SORP) cell sorter (BD Biosciences) with 100 nm nozzle and ND filter 1 was used. The threshold was set on the SSC 200. Data were analyzed by Diva software (BD Biosciences). For fluorescence detection, we used a 586/15 band pass filter for PKH26 and 525/50 band pass filter for FITC labelled antibodies. An unstained sample was used to detect auto-fluorescence and set the photomultiplier for all the channels. Standard silica beads (Apogee Mix for Flow Cytometer, Apogee Flow Systems Ltd), with a similar refractive

index of vesicles, was used to gate the MSC MVs. MSC MVs were also characterized by scanning electron microscopy and by using NanoSight NS 300 (Malvern Instruments).

### Measurement of Lung Protein Permeability Using Evans Blue

At time T5 h, we injected 400 mg of Evans Blue (Sigma-Aldrich) into the perfusate. At time T6.5 h, 1 ml of perfusate was removed, and then two liters of PBS was used to flush the lung. Several pieces (1 – 2 gm) from both control and injured lung lobes were then collected and incubated with formamide for 3 days to release the dye. We measured the conc. of Evans blue dye from each sample to calculate lung protein permeability.

### Isolation and Bacterial Killing Effect of Human Alveolar Macrophage

Human alveolar macrophages were isolated following BAL with warm normal saline and plated with RPMI 1640 media in a transwell plate (200,000 cell/well) after washing twice with HBSS solution and stimulated with 1 µg/ml LPS with or without 90 µl of MSC MV. The next day, the cells were cultured with opsonized  $10^7$  CFU of *E.coli* bacteria for 90 min. The CFU of *E.coli* in the medium was then counted.

### Statistical Analysis

Results were expressed as the mean  $\pm$  SD or median with 25–75% percentile (interquartile range [IQR]), as appropriate. Whether or not the samples were normally distributed was determined by D'Agostino & Pearson or Shapiro-Wilk normality test. Comparisons between several groups were made using the analysis of variance (ANOVA) with Bonferroni correction if normally distributed or by Kruskal Wallis Test followed by post hoc Dunn's tests if not. We used the software GraphPad Prism.

## RESULTS

### Baseline Demographics and Clinical Data for Donor Human Lungs

The demographic and clinical data for 37 donor lungs used are listed in the Table. There appeared to be no major differences between the MV treatment groups compared with *E.coli* injury group in terms of age, sex, PaO<sub>2</sub>/FiO<sub>2</sub> (P/F) ratio, lung ischemic time and lung injury score (LIS).

### MSC MV Characterization

Scanning electron microscopy showed that the isolation technique yielded homogeneous population of spheroid particles (Supplemental Figure 1). NanoSight analyses showed MSC MV mean size was  $180 \pm 14$  nm with a concentration at  $4.7 \pm 0.2 \times 10^8$  particles per ml. For flow cytometry, we labelled MSC MV with PKH26 to quantify only membrane bound vesicles and to exclude debris. As a percentage of PKH26 labelled MSC MV, we found that  $43 \pm 8\%$  was CD44 positive. An IgG Ab was used as a control to remove background staining. For CD9, only  $0.1 \pm 0.1\%$  of PKH26 MSC MVs was labelled positively, suggesting that the majority of vesicles were MVs. However, since there is significant overlap between exosomes and MVs in terms of size, it is possible that the total number of exosome were under-represented. The MSC MV dose chosen was initially based on prior studies using

MSCs in the *ex vivo* perfused human lung and our observation that MVs were approximately 5–10x less potent than live cells<sup>23</sup>. Consequently, we administered 200 µl of MSC MVs or the MVs released by 20 million MSCs over 48 h; previous studies using the *ex vivo* human lung used 5 to 10 million MSCs as a therapeutic.

### **MSC MVs Improved Alveolar Fluid Clearance in Lungs Injured Following *E.coli* Pneumonia**

Administration of either human MSC MVs (1X or 2X) or the Poly (I:C) MSC MVs into the perfusate at 1 h following *E.coli* induced ALI significantly increased AFC at 6 h by 247% (P<0.001), 159% (P= 0.001) or 180% (P<0.001) respectively. The AFC values were also significantly elevated compared to the control lung lobe by 144% (P<0.001), 82% (P=0.008) or 97% (P<0.001) respectively, suggesting not only a restoration but an improvement. In contrast, administration of NHLF MVs had no benefit and actually significantly decreased AFC compared to the control group (P=0.002) (Figure 2A). Surprisingly, there was no dose effect with increasing MSC MV by 2X on any parameter, suggesting that there was a therapeutic ceiling to the MVs. By histology, instillation of intra-bronchial *E.coli* bacteria caused significant injury as depicted by an increase in cellularity and blood, edema and interstitial thickening which was substantially reduced with the administration of either MSC MV (1X or 2X) or Poly (I:C) MSC MVs (Figure 2B).

### **Poly (I:C) MSC MVs Decreased Bacterial Count in the Injured Alveolus**

Bacterial colony forming units (CFU) were measured in the BAL fluid following instillation of 10<sup>9</sup> *E. coli* bacteria with or without MV treatment at 6 h. Although the median values of CFU levels in the MSC MV 1X and 2X treatment groups were numerically lower than the *E.coli* injury group, only the administration of Poly (I:C) MSC MVs significantly decreased CFU counts compared to the *E.coli* injured group at 6 h by post hoc Dunn's analyses following Kruskal Wallis test (P=0.007). (Figure 3A). Administration of NHLF MVs had no significant effect. The bacterial CFU counts in perfusate were also numerically lower in the MSC MV 2X and Poly (I:C) MSC MV groups compared to *E.coli* group at 6 h but not statistically significant (Figure 3B).

When freshly isolated human alveolar macrophages were co-cultured with *E.coli* bacteria with or without LPS and/or MSC MVs, bacterial phagocytosis by alveolar macrophages significantly increased with MSC MV treatment (P=0.044) (Figure 3C).

### **MSC MVs Decreased Absolute Neutrophil Count in the Injured alveolus**

Absolute neutrophil counts in the BAL fluid following *E.coli* pneumonia were numerically lower following administration of MSC MV (1X) or Poly (I:C) MSC MV at 6 h (median 9.1 × 10<sup>6</sup> with IQR of 8.6 × 10<sup>6</sup> – 2.6 × 10<sup>7</sup> for *E.coli*, median 5.5 × 10<sup>6</sup> with IQR of 3.9 × 10<sup>6</sup> – 9.1 × 10<sup>6</sup> for + MSC MV 1X, median 7.2 × 10<sup>6</sup> with IQR of 4.7 × 10<sup>6</sup> – 9.9 × 10<sup>6</sup> for + Poly (I:C) MSC MV, N = 6 – 11), with overall P-value of 0.024 (Figure 4A). However, no pair comparisons showed statistical significance after Dunn's correction. There were also no significant differences in absolute neutrophil counts in the perfusate (data not shown).

### MSC MVs Decreased Lung Protein Permeability

Lung protein permeability was measured in all lung lobes using the extravasation of Evans Blue dye, similar to the technique used in mice (Figure 5A). Administration of MSC MVs (1X or 2X) or Poly (I:C) MSC MVs significantly reduced lung protein permeability ( $P=0.001-0.003$ ) (Figure 5B).

### Effects of MSC MVs on Pulmonary Arterial Pressure and Pulmonary Vascular Resistance

PAP was measured throughout the duration of the experiment. Although not statistically significant, administration of MSC MVs or Poly (I:C) MSC MVs lowered PAP at T6 h compared to the *E.coli* injury group (median 3.6 with IQR of 0 – 18 for *E.coli*, median 0.2 with IQR of 0 – 3.3 for + MSC MV 1X, median 0 with IQR of 0 – 1.4 for + MSC MV 2X, median 0 with IQR of 0 – 1.1 for + Poly (I:C) MSC MV, N = 6 – 11) (Figure 6A). PVR followed a similar trend (Figure 6B).

### No Effect of MSC MVs on Tracheal Pressure, Lung compliance or Oxygenation

There were no significant differences in tracheal pressure or lung compliance between control and treatment groups (Figure 7A & B). There was also no change in PaO<sub>2</sub> between groups (Figure 7C), possibly due to the short duration of the experiment and the technique utilized to measure AFC.

### Effect of MSC MV on TNF $\alpha$ Levels in the Injured Alveolus.

We measured the inflammatory cytokine, tumor necrosis factor (TNF $\alpha$ ), in the BAL fluid. Although not statistically significant, administration of human MSC MV 1X after *E.coli* pneumonia numerically reduced the levels of TNF $\alpha$  by 72% (median 5285 with IQR of 3446 – 13409 for *E.coli*, median 1454 with IQR of 681 – 8097 for + MSC MV 1X, median 5105 with IQR of 3263 – 9285 for + MSC MV 2X, median 6123 with IQR of 1766 – 9152 for + Poly (I:C) MSC MV, N = 6 – 11).

## Discussion

The main findings of these studies can be summarized as follows: (1) Intravenous administration of MSC MVs significantly increased the rate of AFC and decreased lung protein permeability and numerically lowered the CFU bacteria counts and inflammation in the injured alveolus following severe *E.coli* pneumonia in an *ex vivo* perfused human lung (Figure 2, 3 & 5); (2) Poly (I:C) pretreatment of MSCs significantly enhanced the antimicrobial property of the released MVs in the injured alveolus (Figure 3); (3) Increased antimicrobial effect of Poly (I:C) MSC MVs was associated with a numerically lower influx of inflammatory cells (i.e. neutrophils) in the injured lung (Figure 4); (4) MSC MVs and Poly (I:C) MSC MVs treatment groups had numerically lower median PAP and PVR levels at 6 h (Figure 6). However, MSC MVs and Poly (I:C) MSC MVs did not improve tracheal pressure, lung compliance or oxygenation (Figure 7); (5) Freshly isolated human alveolar macrophage had enhanced antimicrobial properties with MSC MV treatment (Figure 3); (6) And, more significantly, the pre-clinical therapeutic findings seen with MSC MVs in mice were apparent in a clinically relevant human lung injury model, especially in the effect of Poly (I:C) MSC MVs, making it a possible viable alternative to MSCs as a therapy.

Most all cell types release MVs<sup>25</sup> which can be biologically active as mediators of cell-to-cell communication or through direct effects on target cells<sup>26–28</sup>. Recently, MVs derived from MSC have been reported to exert therapeutic effects on various organ injury models<sup>29, 30</sup>. We previously found that MSC MV significantly reduced pulmonary edema in mice injured with LPS or *E.coli* pneumonia<sup>15, 16</sup> and an *ex vivo* perfused human lung model of ischemia/reperfusion<sup>31</sup>. However, the underlying mechanisms were not fully known. In the current study, both MSC MVs and Poly (I:C) MSC MVs improved AFC to a near normal level at 6 h following *E.coli* pneumonia (Figure 2). We previously demonstrated that MSC and/or MSC MVs increased AFC rate in part through enhanced KGF expression; KGF secreted from MSCs upregulated the key sodium channel ( $\alpha$ ENaC) in alveolar epithelial cells, increasing fluid absorption<sup>6</sup>.

We also found that Poly (I:C) MSC MVs enhanced bacterial killing *in vivo* and MSC MVs *in vitro* (Figure 3) and numerically reduced the influx of neutrophils (Figure 4) into the injured alveolus. Several potential mechanisms may account for these observations: 1) MSC and MSC MVs secrete or enhance the expression of antimicrobial soluble factors. Krasnodembskaya et al. found that MSCs secreted LL-37, a known antimicrobial peptide, and Gupta et al.<sup>11, 12</sup> demonstrated that MSCs enhanced survival and bacterial clearance through upregulation of Lipocalin2 by MSCs in a mice model of *E.coli* pneumonia; 2) Raffaghello et al.<sup>32</sup> demonstrated that MSCs inhibited apoptosis of neutrophils by MSC through downregulation of reactive oxygen species and Stat-3 phosphorylation; 3) And multiple groups demonstrated that MSC and MSC MVs enhanced alveolar macrophage survival via mitochondria transfer (via tunneling nanotubes) and phenotypic change from the pro-inflammatory M1 to the anti-inflammatory M2<sup>33–35</sup>. Both Nemeth et al and Mei et al.<sup>7, 36</sup> found that MSCs upregulated macrophage phagocytic activity which improved bacterial clearance in a cecal ligation and puncture model of sepsis. In addition, recent studies indicated that TLR3 activation with Poly (I:C) can enhance the immunomodulatory potential of MSC, while TLR4 activation could reduce it<sup>17</sup>. In a mice model of *E.coli* pneumonia, we found that Poly (I:C) pretreatment of MSC MV enhanced macrophage/monocyte phenotypic M2 change, as indicated by decreased inflammation and increased bacteria phagocytosis<sup>16</sup>.

MSC MVs and Poly (I:C) MSC MVs both significantly reduced lung protein permeability (Figure 5) which may be attributed to its angiopoietin-1 content, a protein with well-known anti-inflammatory, anti-permeability, and endothelial protective characteristics. Both MSCs and MSC MVs have significant levels of angiopoietin-1 mRNA. In LPS induced ALI in mice, MSC or MSC transfected with the human Ang1 gene reduced pulmonary vascular injury and the recruitment of inflammatory cells into the lung<sup>10</sup>. More recently, Tang<sup>37</sup> et al. found that Ang1 in MSC MV was important in reducing inflammation and pulmonary edema in LPS induced ALI in mice.

Our study has some limitations: (1) Lack of an intact lymphatic system 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; (3) Short duration of lung injury which limits assessment of whether the effects of MSC MVs can be sustained; (4) And limited sample size due to lack of availability of lungs for research.

Despite the limitations, the importance of the current study was in establishing the use of MVs as a possible viable alternative to using live cells in ALI. The benefits include ease of isolation and storage, avoiding dimethyl sulfoxide for preservation and the need for a bone marrow transplant facility, avoidance of using live stem cells with tumorigenic potential and which may cause hemodynamic instability with administration, and potential to modify MVs with pretreatment of the cells to enhance the therapeutic effects. However, the major obstacle remains in scaling the production of MSC MVs since the potency is approximately 5–10x less than the cells. Given that a typical MSC dose is 5 million cells/kg or 500 million cells for a 100 kg patient, future clinical trials with MSC MVs may require isolating MVs released from up to 5 billion cells per patient, which may be prohibitive. Studies are on-going to address this critical issue.

In conclusion, our study demonstrated that MSC MVs was therapeutic in a clinically relevant human model of severe *E.coli* pneumonia in lungs with baseline injury. Based on these promising results, further studies are warranted in a large animal with pneumonia and or sepsis to provide further biological rationale and additional preclinical data for possible clinical trials.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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**What is the Key Question?**

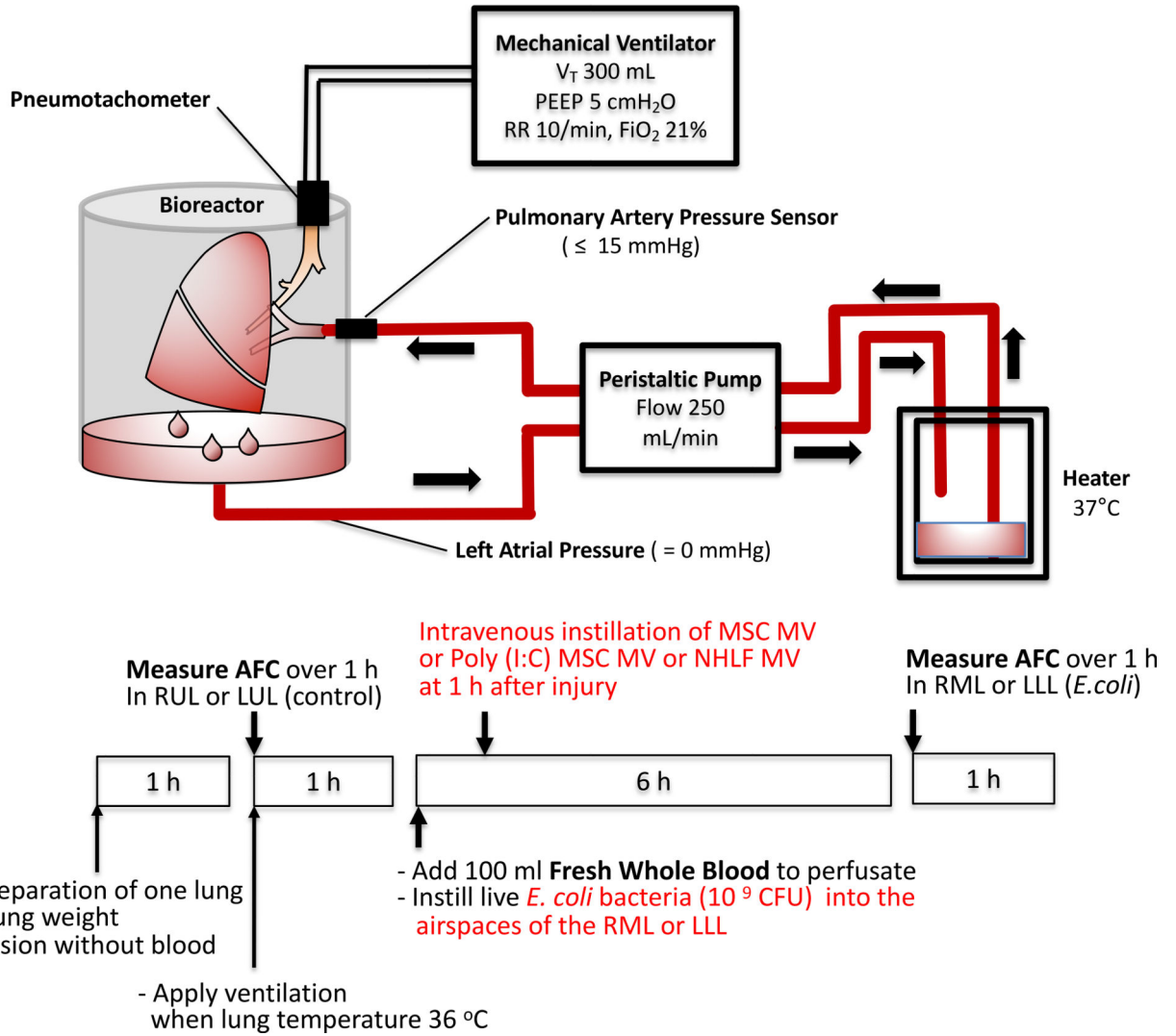
Can the therapeutic effects of MSC microvesicles, as reported in preclinical small animal models of acute lung injury, be applicable to human lung injury models?

**What is the bottom line?**

Human bone marrow derived MSC microvesicles reduced multiple indices of acute lung injury in an *ex vivo* perfused human lung injured with severe bacterial pneumonia.

**Why read on?**

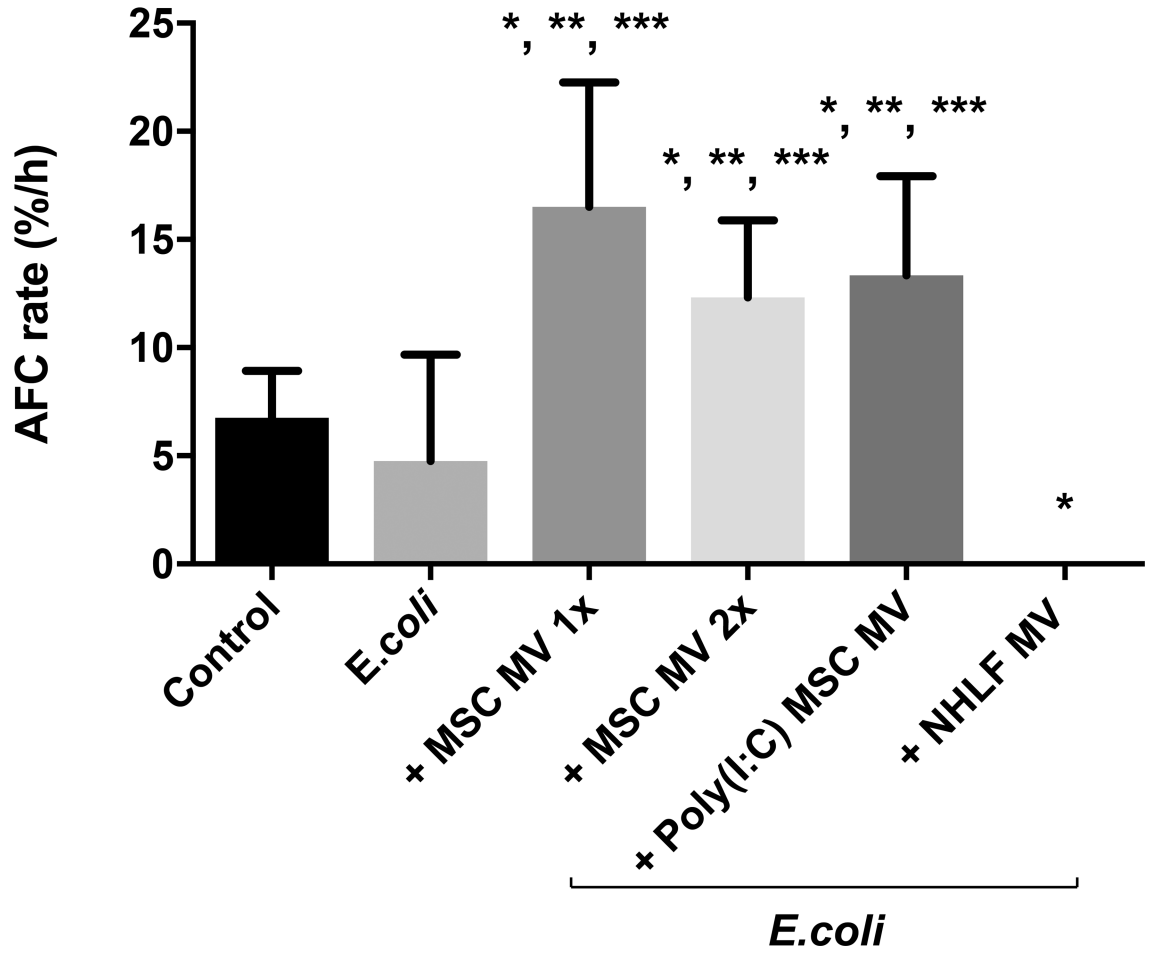
Based on promising results, further studies are warranted in a large animal model of pneumonia with or without sepsis to provide additional preclinical data for possible clinical trials.

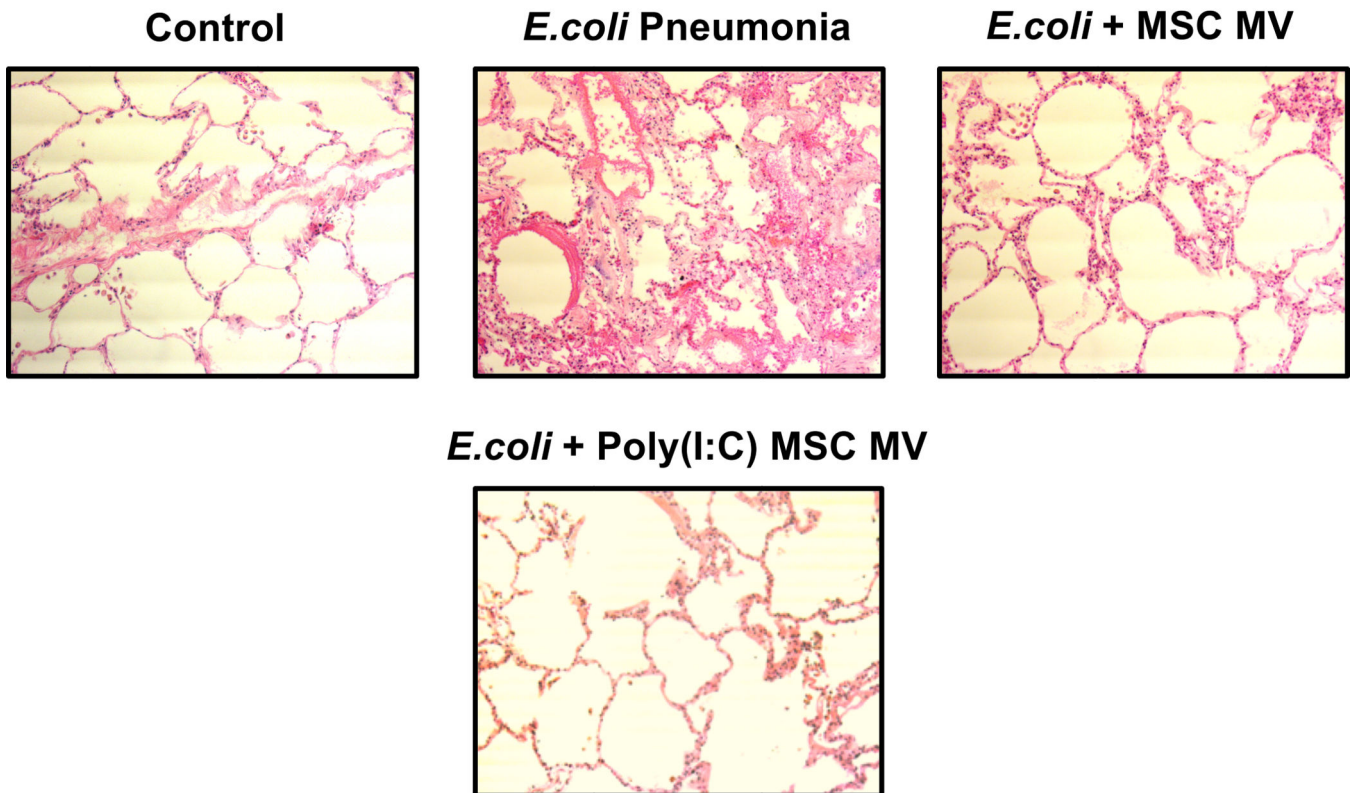


**Figure 1. Schematic of the Ex Vivo Perfused Human Lung Preparation.**

Marginal human lungs which were not used for lung transplantation were obtained from the Northern California Transplantation Donor Network. Using a large organ Harvard bioreactor, the pulmonary artery of the right or left lung was initially cannulated and gently perfused with a crystalloid solution containing 5% albumin at a rate up to 250 ml/min. Once rewarmed, the lung was ventilated using a tidal volume of 300 ml with 5 cmH<sub>2</sub>O of PEEP and a respiratory rate 10 breaths per minute in room air. AFC rate was first measured in the upper control lobe. If AFC > 0 and <10 %/h,  $10^9$  CFU *E.coli* bacteria was administered into the lower lung lobe, and 100 ml of fresh human blood was added to the perfusate. After 6 h, AFC was measured in the lower lobe as the primary endpoint. In separate experiments and randomly, 200 or 400  $\mu$ l MSC derived microvesicles (MVs), Poly (I:C) pretreated 200  $\mu$ l MSC MVs, or 200  $\mu$ l NHLF derived MVs was administered into the perfusate at 1 h.

**A**

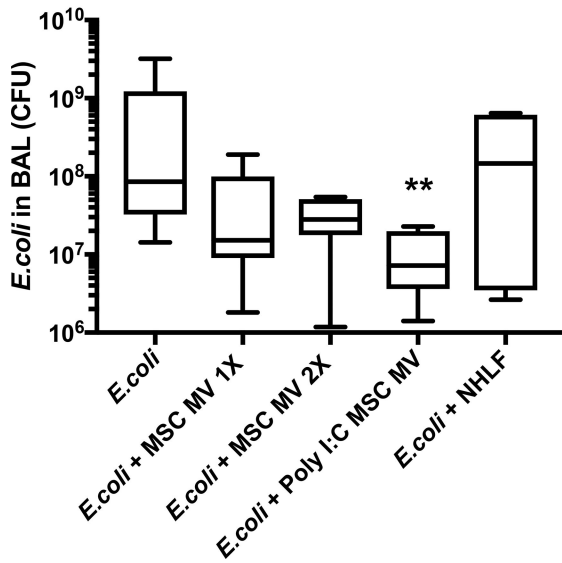


**B**

**Figure 2. Therapeutic Effect of MSC MVs on Alveolar Fluid Clearance Rate following Severe *Escherichia coli* Pneumonia in Ex Vivo Perfused Human Lung.**

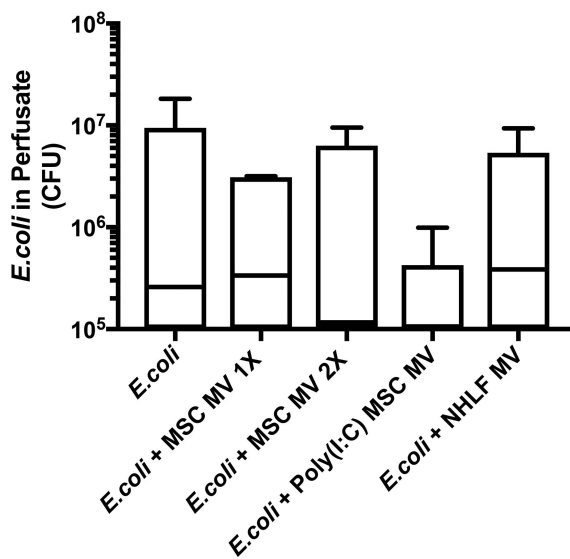
(A) Administration of MSC MVs or Poly (I:C) pretreated MSC MVs intravenously significantly increased the AFC rate in the lung lobe injured by *E.coli* pneumonia at 6 h. Administration of NHLF MVs had no beneficial effect on AFC rate. AFC was measured by the change in protein concentration of a 5% albumin instillate in the lung lobe over 1 h and expressed as mean AFC (% per h, per 150 ml of BAL fluid) with 95% confidence intervals for each condition. N = 9 for *E.coli* injured lung lobe, N = 6 for *E.coli* injured lung lobe treated with MSC MVs (200  $\mu$ l or 400  $\mu$ l) or N = 11 for 200  $\mu$ l MSC MVs pretreated Poly (I:C) or N = 5 for 200  $\mu$ l NHLF MVs. Overall P Value < 0.001, \* p is significant vs. Control, \*\* p is significant vs. *E.coli* injured, and \*\*\* p is significant vs. NHLF MVs by ANOVA (Bonferroni). P values and confidence intervals for individual pair comparisons are shown in Supplementary Table. (B) Human lungs injured with *E.coli* bacteria with and without MSC MVs 1X or Poly (I:C) pretreated MSC MVs were fixed in 10% formalin at 6 h. Sections were stained with hematoxylin and eosin. Administration of human MSC MVs 1X or Poly (I:C) pretreated MSC MVs 1 h after *E.coli* pneumonia injury reduced the level of hemorrhage, edema, and cellularity in the injured lung lobe at 6 h following *E.coli* pneumonia.

**A**

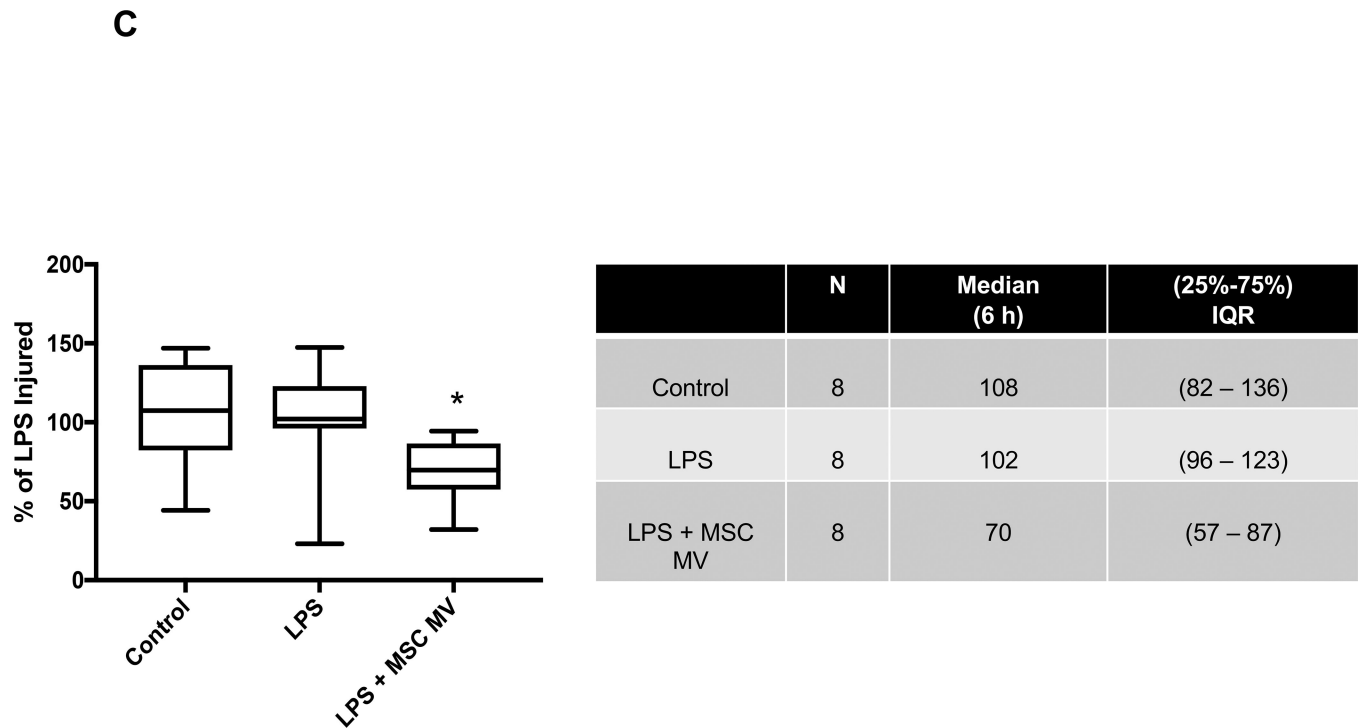


	N	Median (6 h)	(25%-75%) IQR
<i>E.coli</i>	8	$8.6 \times 10^7$	$(3.3 \times 10^7 - 1.2 \times 10^9)$
<i>E.coli</i> + MSC MV 1X	6	$1.5 \times 10^7$	$(9 \times 10^6 - 9.9 \times 10^7)$
<i>E.coli</i> + MSC MV 2X	6	$2.8 \times 10^7$	$(1.8 \times 10^7 - 5.1 \times 10^7)$
<i>E.coli</i> + Poly I:C MSC MV	11	$7.2 \times 10^6$	$(3.6 \times 10^6 - 2 \times 10^7)$
<i>E.coli</i> + NHLF MV	5	$1.5 \times 10^8$	$(3.5 \times 10^6 - 6.2 \times 10^8)$

**B**



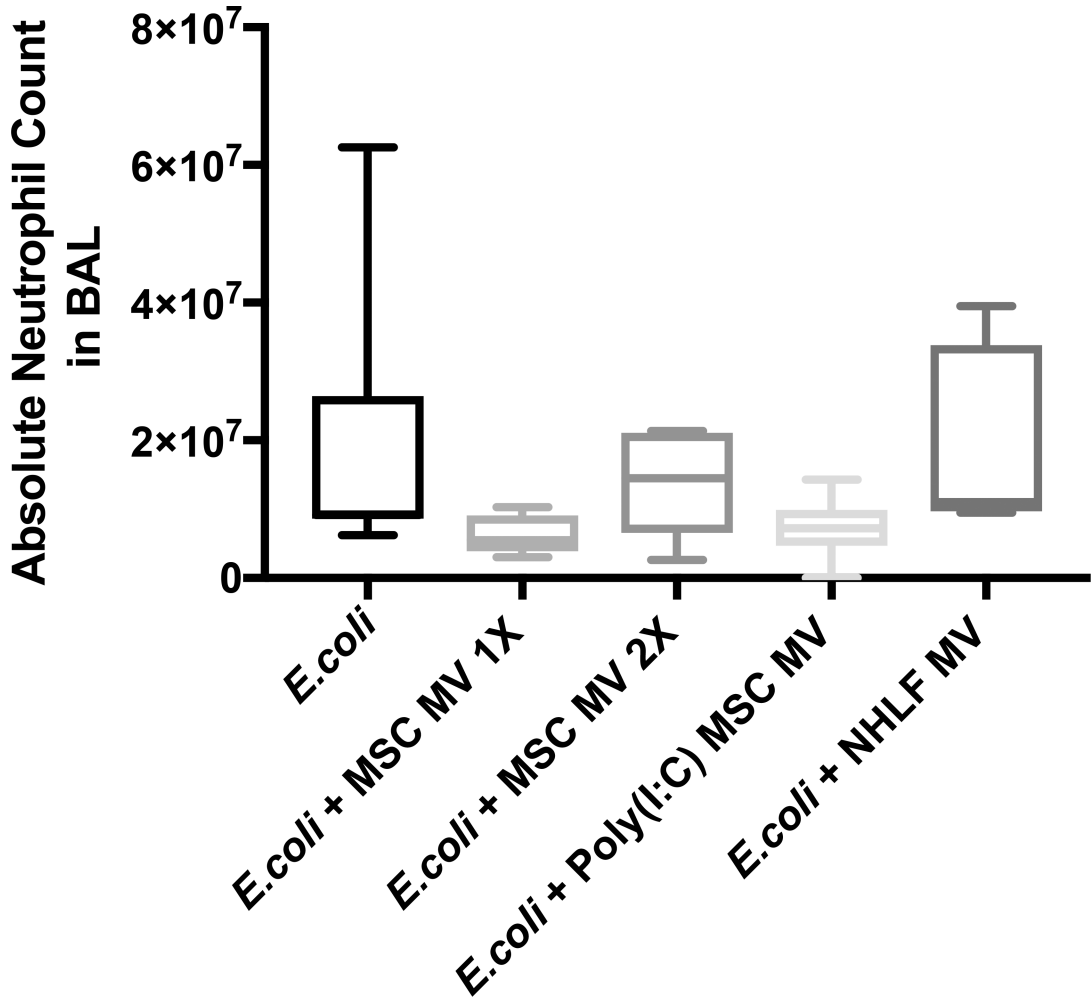
	N	Median (6 h)	(25%-75%) IQR
<i>E.coli</i>	8	258,650	(71,900 - 9,465,300)
<i>E.coli</i> + MSC MV 1X	6	335,120	(0 - 3,092,705)
<i>E.coli</i> + MSC MV 2X	6	116,320	(14,160 - 6,321,590)
<i>E.coli</i> + Poly I:C MSC MV	11	106,040	(0 - 424,800)
<i>E.coli</i> + NHLF MV	5	385,600	(0 - 5,378,760)



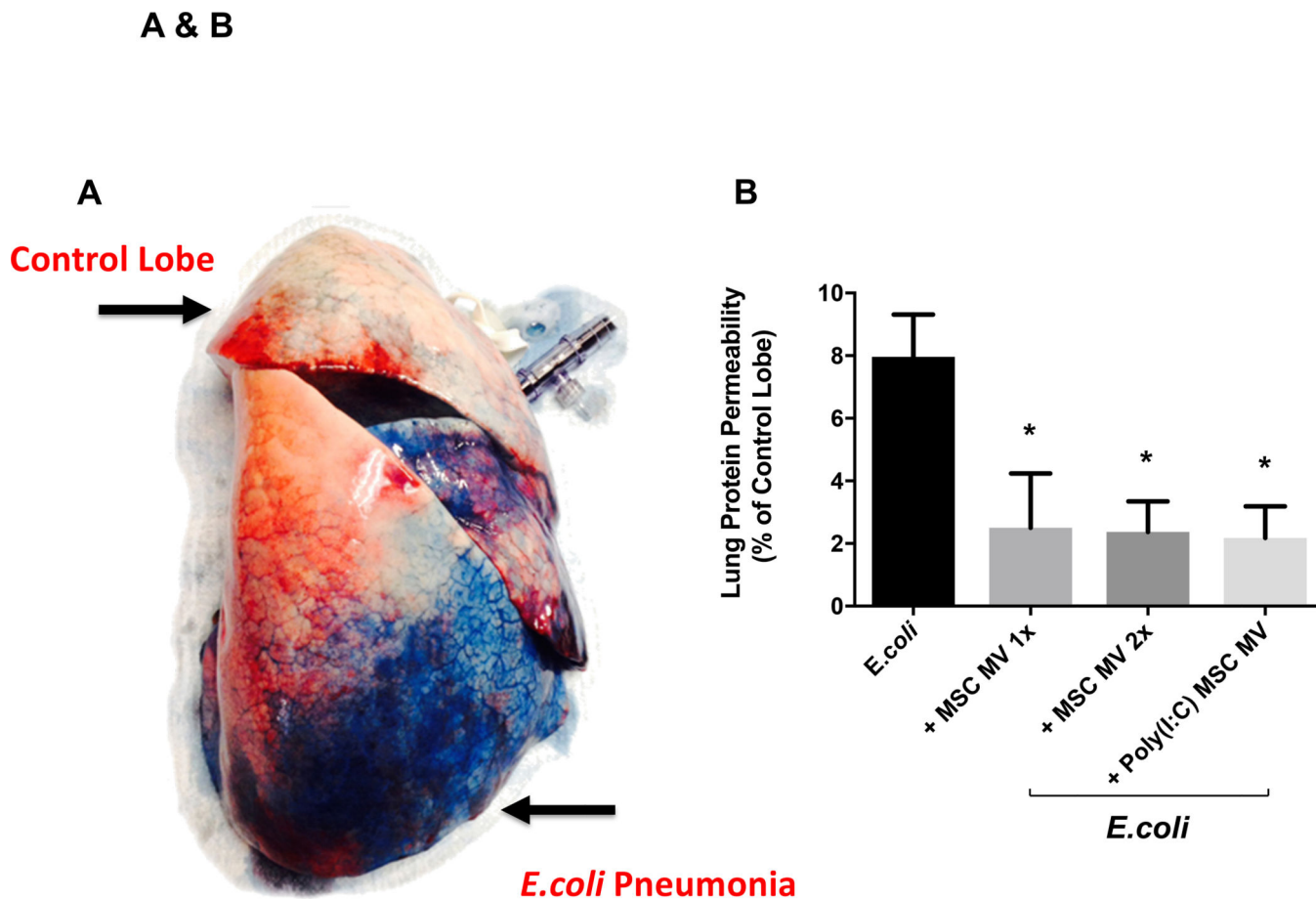
**Figure 3. Therapeutic Effect of MSC MVs on Bacterial Levels following Severe *Escherichia coli* Pneumonia in *Ex Vivo* Perfused Human Lung.**

Administration of Poly (I:C) pretreated MSC MVs decreased (A) bacterial counts in the BAL fluid at 6 h. Administration of NHLF MVs had no beneficial effect. Data is expressed in the box plot as median bacterial CFU counts with IQR for bars in the BAL fluid, Overall P value = 0.016, \*\* p is significant vs. *E.coli* injury group by Post-Hoc Dunn's Analyses Following Kruskal Wallis Test. (B) Although not statistically significant, administration of MSC MVs or Poly (I:C) pretreated MSC MVs numerically decreased bacterial CFU counts in the perfusate. Data is expressed in the graph as median bacterial CFU counts with IQR for bars in the perfusate. Overall P value = 0.624. (C) Co-incubation of MSC MVs with freshly isolated human alveolar macrophages also increased *E.coli* bacterial phagocytosis by the macrophages. Data is expressed in the graph as median bacterial CFU counts (as % of LPS control) with IQR for bars. Overall P value = 0.026, \* p is significant vs. LPS treated group by Post-Hoc Dunn's Analyses Following Kruskal Wallis test. P values and confidence intervals for individual pair comparisons are shown in Supplementary Table.



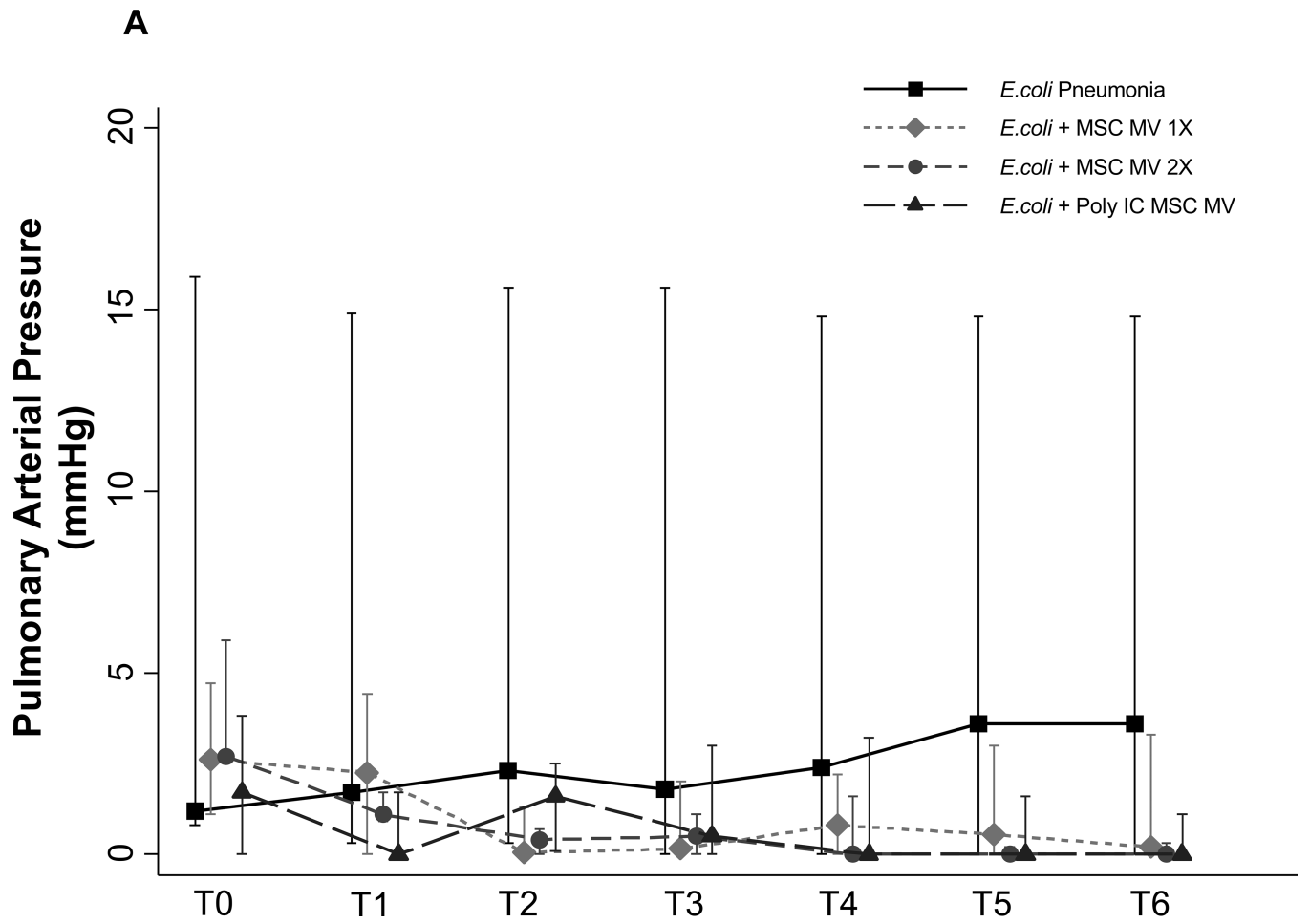


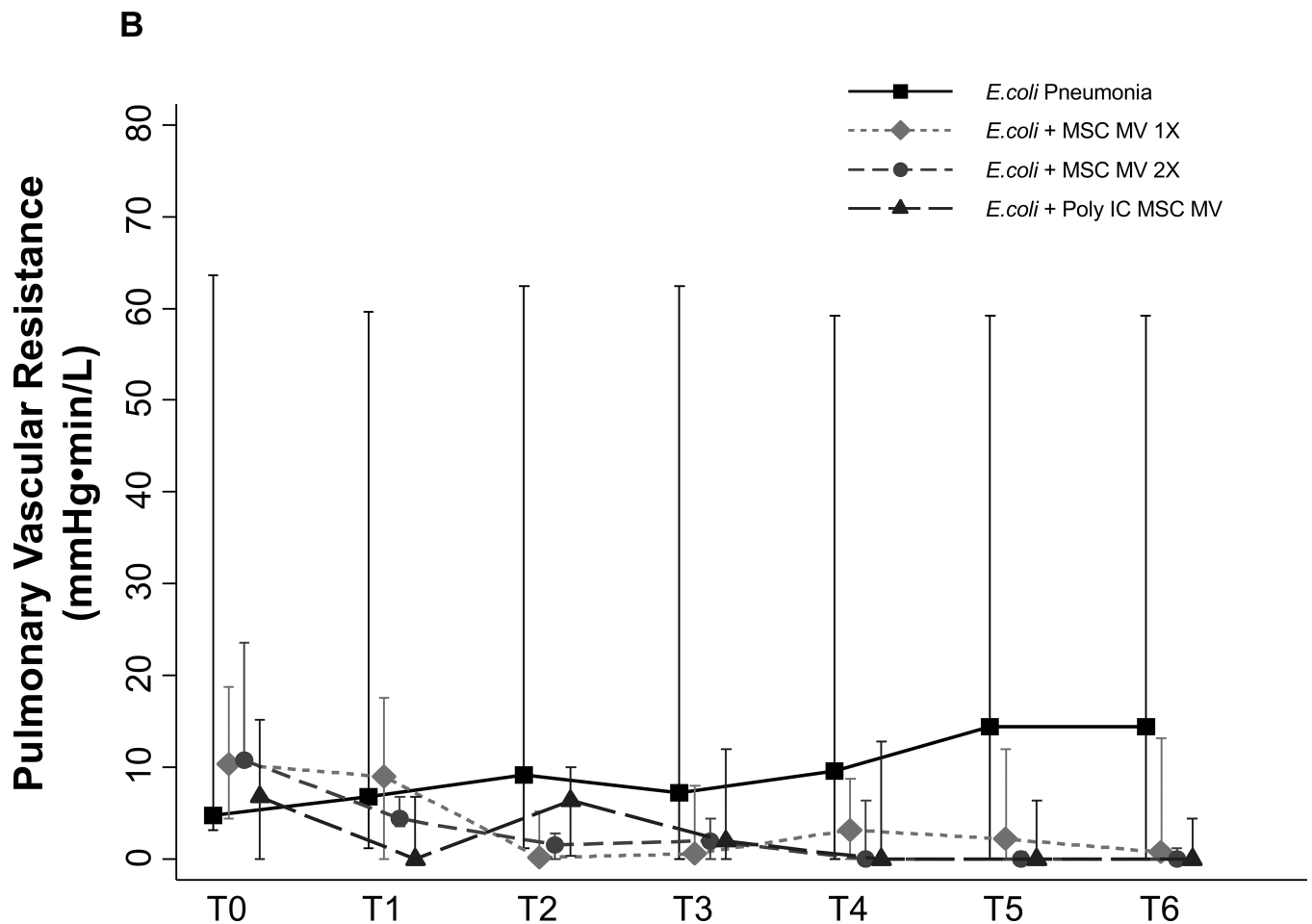
**Figure 4. Effect of MSC MVs on the Influx of Inflammatory Cells following Severe *Escherichia coli* Pneumonia in *Ex Vivo* Perfused Human Lung.** Instillation of MSC MVs numerically decreased the influx of inflammatory cells, specifically neutrophils, into the lung lobe injured by *E.coli* pneumonia at 6 h. Absolute neutrophil count is expressed as median with IQR for bars for each condition, N = 9 for *E.coli* injured lung lobe, N = 6 – 11 for *E.coli*-injured lung lobe treated with 200  $\mu$ l or 400  $\mu$ l of MSC MVs or 200  $\mu$ l of Poly (I:C) pretreated MSC MVs. Overall P value = 0.024, P values and confidence intervals for individual pair comparisons are shown in Supplementary Table.



**Figure 5. Therapeutic Effect of MSC MVs on Lung Protein Permeability following Severe *Escherichia coli* Pneumonia in *Ex Vivo* Perfused Human Lung.**

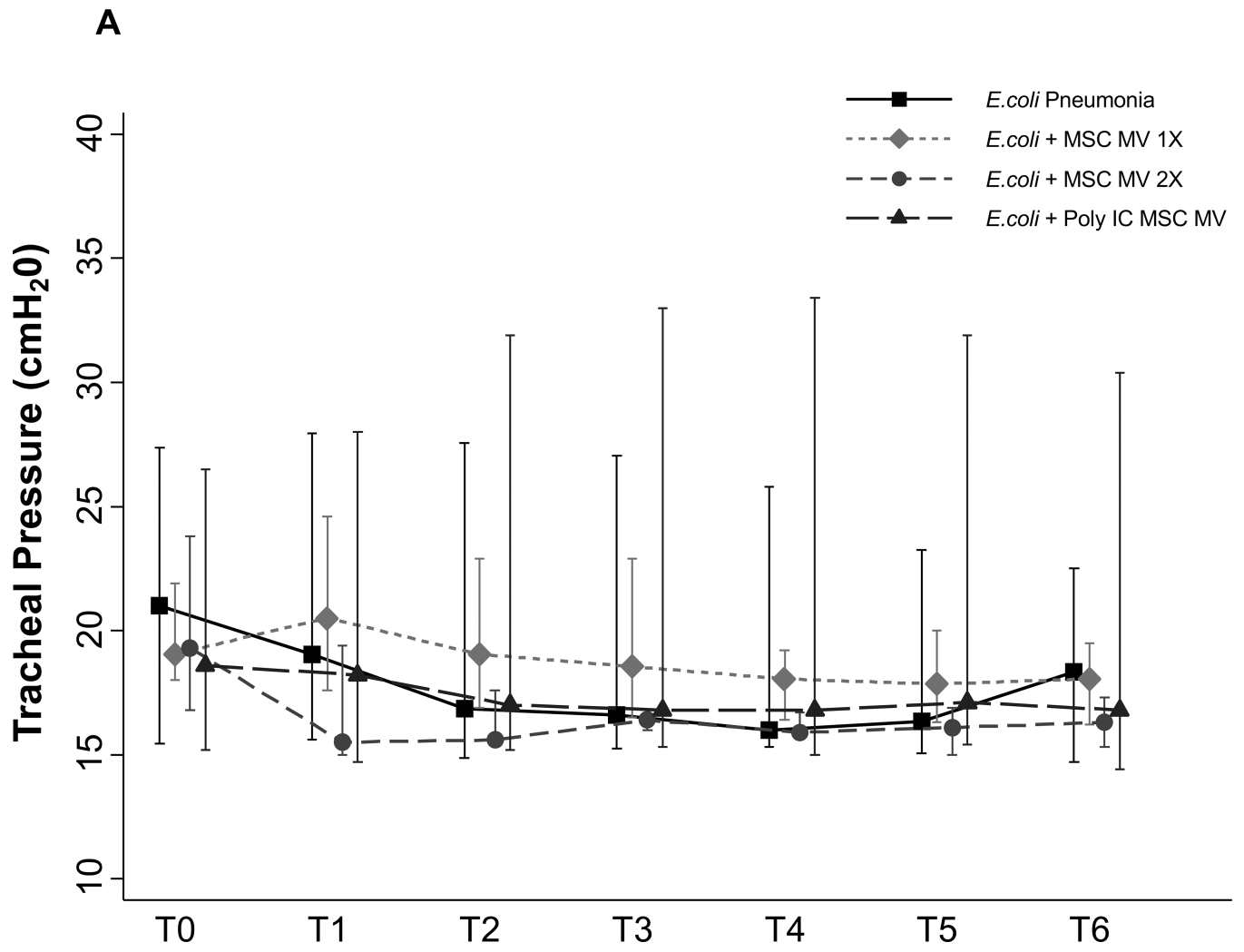
Lung protein permeability at 6 h as measured by Evan's Blue extravasation. (A) Administration of Evan's Blue intravenously demonstrated albumin protein leak into the *E.coli* bacteria injured lung lobe. (B) Compared to *E.coli* bacteria injured lung lobe, administration of MSC MVs or Poly (I:C) pretreated MSC MVs significantly reduced lung protein permeability. Data is presented as mean change in permeability with 95% confidence intervals. Overall P value = 0.001, \* P is significant vs. *E.coli* by ANOVA (Bonferroni). P values and confidence intervals for individual pair comparisons are shown in Supplementary Table.

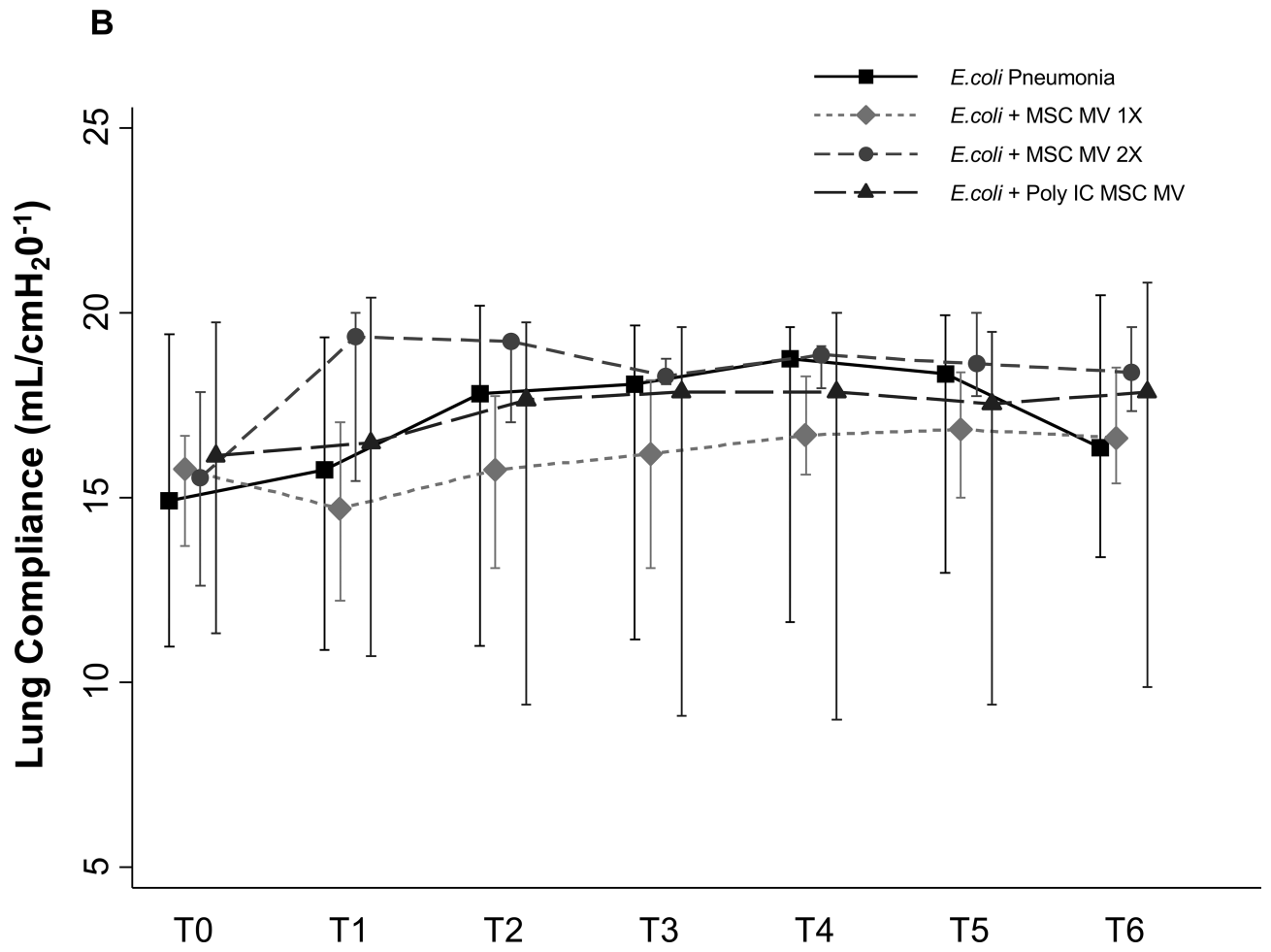


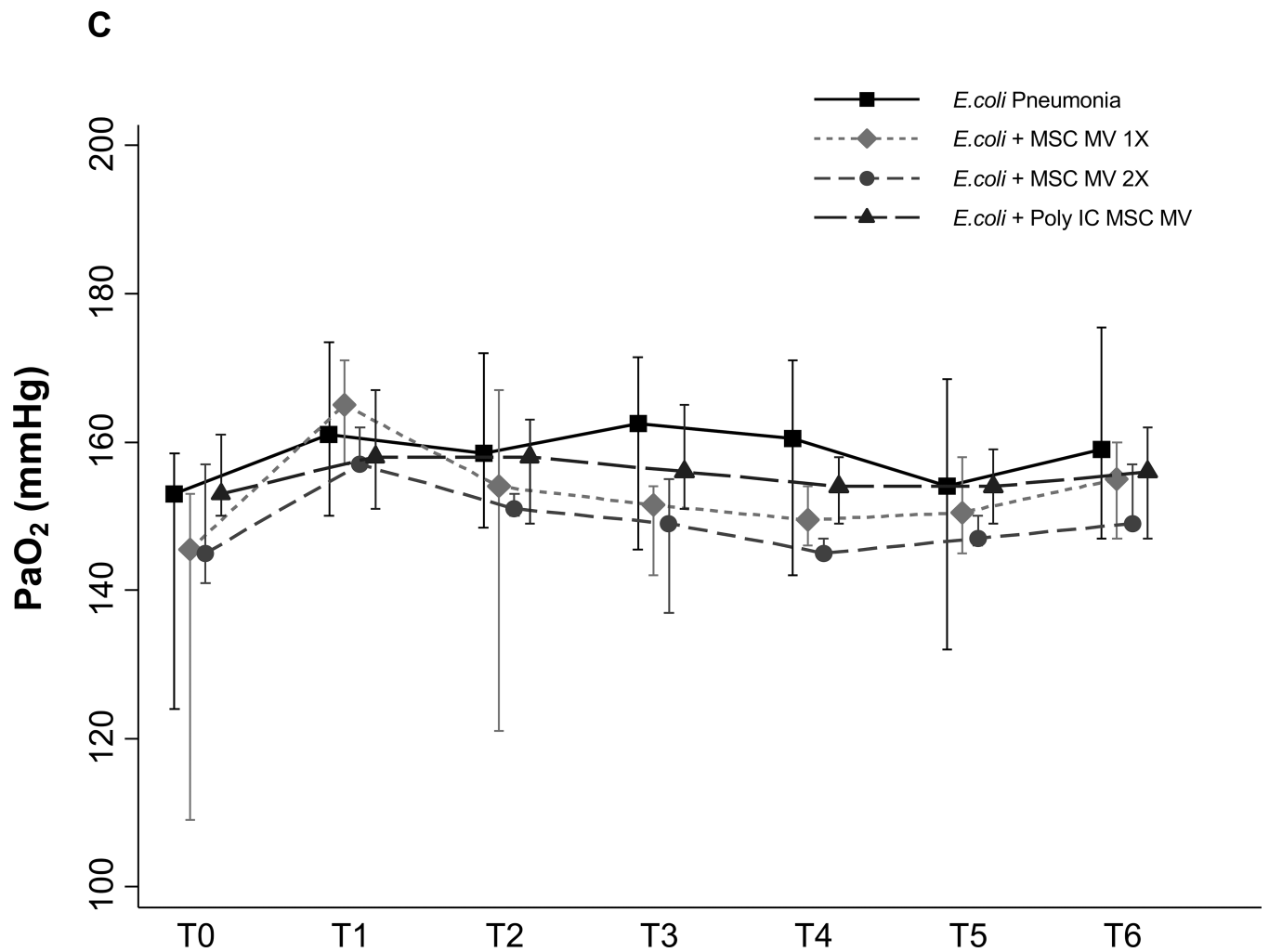


**Figure 6. Effect of MSC MVs on Pulmonary Arterial Pressure and Vascular Resistance following Severe *Escherichia coli* Pneumonia in *Ex Vivo* Perfused Human Lung.**

Administration of MSC MVs or Poly (I:C) pretreated MSC MVs numerically decreased PAP (A) or PVR (B) at T6 h compared to *E.coli* injury group. Data is presented as median PAP or PVR with IQR as bars.  $P = 0.20 - 0.66$  for each time point by Kruskal Wallis tests (A).  $P = 0.20 - 0.63$  for each time point by Kruskal Wallis tests (B).







**Figure 7. Lack of a Therapeutic Effect of MSC MVs on Lung Compliance or Oxygenation following Severe *Escherichia coli* Pneumonia in *Ex Vivo* Perfused Human Lung.**

Administration of MSC MVs or Poly (I:C) pretreated MSC MVs had no significant effect on tracheal pressure (A), lung compliance (B) or oxygenation (C) over 6 h. Data is presented as median Tracheal pressure or Lung Compliance or PaO<sub>2</sub> with IQR as bars. P = 0.56 – 0.98 for each time point by Kruskal Wallis tests (A). P = 0.56 – 0.98 for each time point by Kruskal Wallis tests (B). P = 0.10 – 0.92 for each time point by Kruskal Wallis tests (C).

**Table.**

## Clinical Data of Donor Lungs

	<i>E.coli</i> Pneumonia(N= 9)	+ MSC MV 1X(N = 6)	+ MSC MV 2X(N = 6)	+ Poly (I:C) MSC MV (N = 11)	+ NHLF MV(N = 5)
<b>Mean Age (Year)</b>	44 ± 10	54 ± 16	52 ± 21	50 ± 13	48 ± 22
<b>Male % (Number)</b>	56% (5)	83% (5)	67% (4)	73% (8)	80% (4)
<b>Donor PaO<sub>2</sub>/FiO<sub>2</sub> Ratio (mmHg)</b>	221 ± 13	112 ± 33	238 ± 44	251 ± 38	156 ± 70
<b>Ischemic Time (h:min)</b>	36:38 ± 8:40	32:36 ± 7:42	24:55 ± 11:05	29:09 ± 6:38	28:40 ± 3:26
<b>Lung Injury Score (LIS)</b>	1.4 ± 0.6	1.9 ± 0.4	1.2 ± 0.4	1.3 ± 0.4	1.9 ± 0.7

Data is presented as mean ± SD.