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Therapeutic Effects of Human Mesenchymal Stem Cell-Derived Microvesicles in Severe Pneumonia in Mice

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Impact on clinical medicine/basic science/How research adds to our knowledge base of disease process?: Mesenchymal stem (stromal) cell-based therapy may be an innovative treatment for acute lung injury. However, some concerns remain with the long-term safety of stem cell administration. In this study, we demonstrated that microvesicles, anuclear membrane bound fragments released by human mesenchymal stem cells, were as potent as the parent stem cell in improving survival and reducing the severity of lung injury in mice following severe pneumonia, suggesting a possible alternative to using cells for stem cell-based therapy.

AT A GLANCE COMMENTARY

Scientific knowledge on the subject: We and other investigators have demonstrated that microvesicles derived from human mesenchymal stem (stromal) cells (MSC MV) reduced the severity of sterile inflammatory injury to the kidneys or lungs in part through the transfer of mRNAs for growth factors and anti-inflammatory cytokines. However, the effect of MSC MV on lung injury following an infectious etiology, such as severe bacterial pneumonia, is currently unknown.

What This Study Adds to the Fields: Using an *in vivo* model of *Escherichia coli* pneumonia in mice, administration of human MSC MV were as effective as their parent stem cells in improving survival and mitigating lung inflammation, protein permeability, and bacterial growth. The antimicrobial effect of MSC MV was in part through enhancement of monocyte phagocytosis of bacteria, which could be further increased by pre-stimulation of MSC with a toll-like receptor 3 agonist prior to the release of MV. The uptake of MSC MV through the CD44 receptor into injured human monocytes and alveolar epithelial cells was critical for their therapeutic effects. Administration of a keratinocyte growth factor neutralizing antibody abrogated the survival advantage mediated by MSC MV, suggesting a possible mechanism for the therapeutic effect.

This article has an online supplement, which is accessible from this issue's table of contents at www.atsjournals.org

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ABSTRACT

Rationale: Microvesicles are anuclear fragments of cells released from endosomal compartment or shed from surface membranes. We and other investigators demonstrated that microvesicles released by mesenchymal stem cells were as effective as the cells themselves in inflammatory injuries, such as following endotoxin-induced acute lung injury. However, the therapeutic effects of microvesicles in an infectious model of acute lung injury remain unknown.

Objectives: We investigated the effects of human mesenchymal stem cell microvesicles on lung inflammation, protein permeability, bacterial clearance and survival following severe bacterial pneumonia.

Methods: We tested the effects of microvesicles derived from human mesenchymal stem cells on *Escherichia coli* pneumonia in mice. We also studied the interactions between microvesicles and human monocytes and human alveolar epithelial type 2 cells.

Measurements and Main Results: Administration of microvesicles derived from human mesenchymal stem cells improved survival in part through keratinocyte growth factor secretion and decreased the influx of inflammatory cells, cytokines, protein, and bacteria in mice injured with bacterial pneumonia. In primary cultures of human monocytes or alveolar type 2 cells, the uptake of microvesicles was mediated by CD44 receptors, which were essential for the therapeutic effects. Microvesicles enhanced monocyte phagocytosis of bacteria, while decreasing inflammatory cytokine secretion, and increased intracellular ATP levels in injured alveolar epithelial type 2 cells. Pre-stimulation of mesenchymal stem cells with a toll-like receptor 3 agonist further enhanced the therapeutic effects of the released microvesicles.

Conclusions: Microvesicles derived from human mesenchymal stem cells were as effective as the parent stem cells in severe bacterial pneumonia.

Abstract words count: 250

Keywords: acute respiratory distress syndrome; bacterial pneumonia; mesenchymal stem cells; microvesicles

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INTRODUCTION

Bacterial pneumonia is among the primary causes of respiratory failure in critically ill patients and the leading etiology of acute respiratory distress syndrome (ARDS). Despite improvements in supportive care and appropriate antibiotic use, morbidity and mortality from ARDS remain high (1, 2). We and others reported that mesenchymal stem (stromal) cells (MSC) were effective in preclinical models of acute lung injury (ALI) from pneumonia or sepsis, due to their ability to secrete paracrine factors such as growth factors, anti-inflammatory cytokines, and anti-microbial peptides (3-11). Moreover, in an *ex vivo* perfused human lung injured with *E.coli* pneumonia, we demonstrated that the addition of MSC with antibiotics had a more potent anti-microbial effect than antibiotics alone in reducing total bacterial load (8). This was in addition to the ability of the MSC to treat the major abnormalities that underlie lung injury, including impaired alveolar fluid clearance, altered lung permeability, and dysregulated inflammation (8). However, concerns still remain whether MSC can become tumorigenic or have other side effects (12-14).

Recently, MSC have been found to release microvesicles (MV) that were as biologically active as the cells themselves. MV are anuclear plasma membrane bound fragments, 50 nm to 200 nm in size, constitutively released from multiple cell types from the endosomal compartment as exosomes or shed from the plasma membrane. Similar to the stem cells, MV derived from MSC home to the inflammatory site and transfer proteins/peptides, mRNA, microRNA, lipids, and/or organelles with reparative and anti-inflammatory properties to the injured tissue (15, 16). Camussi et al. demonstrated that the protective effects of MSC MV in acute kidney injury in mice were through the transfer of mRNA and microRNA to the injured renal epithelium, leading to a decrease in apoptosis (16-20). We recently reported that MV released by human MSC reduced the severity of

endotoxin-induced ALI in mice through the transfer of mRNA for keratinocyte growth factor (KGF) to the injured alveolus (21).

Currently, the effect of MSC MV in ALI from bacterial pneumonia is unknown. We hypothesized that administration of MSC MV was as effective as the MSC in reducing the severity of *E.coli* pneumonia, in part through increased bacterial clearance. Some of the results of these studies have been previously reported in the form of an abstract (22).

METHODS

See online supplement for detailed descriptions.

Mesenchymal Stem Cells

Human bone marrow derived MSC were obtained from a NIH repository from Texas A&M Health Science Center. Normal human lung fibroblasts (NHLF) were used as cellular controls.

Isolation of MSC-Derived Microvesicles

MV were obtained from the supernatants of MSC and NHLF using ultracentrifugation as previously described (18). MSC or NHLF MV were resuspended according to the final cell count after 48 h (10 µL per 1 x 10^6 cells). Electron microscopy, protein and mRNA levels, and Western Blot analysis for CD44 expression were performed to characterize MSC MV. In additional experiments, MSC MV were fluorescent-labelled (23) or blocked with CD44 or KGF neutralizing antibody to study MV uptake and function. Polyinosinic-polycytidylic acid (Poly (I:C)), a TLR3 agonist (24), was used to stimulate MSC before the isolation of the released MV (Poly (I:C)-MV).

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E.coli Pneumonia-Induced ALI in Mice

C57BL/6 male mice (10-12 weeks, 20-25 g) were injured with intratracheal instillation of *E.coli* K1 strain (2 or 3 x 10^6 colony forming units (CFU)), using protocols approved by the Institutional Animal Care and Use Committee at University of California San Francisco (25). Four hours later, phosphate buffered saline (PBS) as vehicle, 800,000 MSC as a positive cellular control, NHLF MV as a negative control or MSC MV were instilled intratracheal (30 or 60 µL) or injected intravenously (90 µL). In separate experiments, CD44 or KGF neutralizing antibody was given intravenously with MSC MV. Mice were euthanized at 18, 24 or 72 h, and bronchoalveolar lavage samples or lungs were collected for assessment of neutrophil counts, cytokines levels, bacterial load, protein levels and histology.

Statistics

Results are expressed as mean ± standard deviation (SD). Comparisons between two groups were made using unpaired *t*-test or Mann-Whitney. Comparisons between more than two groups were made using an analysis of variance (ANOVA) or Kruskal-Wallis test using the Bonferroni's correction for multiple-comparison testing. The *log*-rank test was used for comparing survival data at 72 h. A P value < 0.05 was considered statistically significant. All statistical analysis was performed using GraphPad Prism software (La Jolla, California, USA). N in the figure legends refers to the number of samples or mice, and are not the number of replicate experiments of the same sample or mouse.

RESULTS

Isolation and Characterization of MSC MV

The therapeutic dose chosen, 90 µL, of MSC MV was the MV released by 9 x 10^6 MSC over 48 h. The viability of MSC and NHLF prior to MV isolation was > 95% (trypan blue exclusion). Scanning electron microscopy showed that the isolation technique yielded spheroid phospholipid bilayer bound structures, measuring approximately 200 nm (Figure 1A). Protein and total RNA quantity of 90 µL MSC MV were respectively 90 ± 48 µg (n = 24) and 97 ± 90 ng (n = 7) (Figure 1B). This protein concentration falls into range doses used in previous studies following lung (26) or kidney injury (18). Western Blot analyses showed that MSC MV expressed CD44 (Figure 1C), a key receptor involved in MSC trafficking (16).

MSC MV Improved Survival in *E.coli*-Induced ALI In Part Through KGF Secretion

Intratracheal instillation of 3 x 10^6 CFU of *E.coli* resulted in significant ALI, leading to 40% survival at 72 h. Intravenous administration of 90 µL of MSC MV 4 h after the injury increased survival to 88% at 72 h, compared to mice treated with PBS or NHLF MV as negative controls (Figure 2A). MSC increased the survival rate (67% at 72 h) as well as compared to both negative control groups. No difference was found between MSC MV and MSC groups. NHLF MV at the same dose had no therapeutic effect (Figure 2A). MSC MV contained the mRNA for KGF (Figure 2B). Instillation of MSC MV or MSC increased human KGF protein levels in the injured alveolus compared with controls **(**Figure 2C**)**. Administration of MSC MV with an anti-KGF neutralizing antibody abrogated the beneficial effect on survival **(**Figure 2D).

MSC MV Decreased Lung Inflammation, Protein Permeability, and Histological Severity in *E.coli* Pneumonia

Intratracheal instillation of approximately 2 x 10^6 CFU *E.coli* produced a non lethal but severe lung injury over 24 h, characterized by influx of neutrophils, high levels of monocyte

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inflammatory protein-2 (MIP-2), increase in lung protein permeability, and development of histological evidence of ALI in the injured alveolus (Figures 3 and 4). Intravenous administration of 90 µL of MSC MV 4 h after the injury reduced the influx of white blood cells by 40%, of neutrophils by 53%, and decreased the total protein concentration by 22% in the bronchoalveolar lavage fluid at 24 h, compared to PBS treated mice (Figures 3). Bronchoalveolar lavage fluid MIP-2 levels and histological severity score were also decreased compared to mice given either PBS or NHLF MV at 18 h following injury (Figures 3B and 4). No therapeutic effect was observed with the intravenous administration of the same dose of NHLF MV (Figures 3 and 4).

In preliminary experiments, 60 µL of MSC MV given intratracheal 4 h following injury had similar beneficial effects, reducing the influx of neutrophils by 50% and the protein levels by 25% in the bronchoalveolar lavage fluid as compared to mice given PBS (Figure 3C). However, there was no significant effect of intratracheal MSC MV on MIP-2 levels. All further studies were done with intravenous administration of MSC MV to correlate with on-going clinical trials of cell-based therapy for acute lung injury.

Dose-Response Effect of MSC MV on *E.coli*-induced ALI

An initial dose response effect of intravenous MSC MV on *E.coli*-induced ALI was generated to arrive at the optimal intravenous dose of 90 µl (*see* Supplemental Figure E1).

Effect of MSC MV on *E.coli* Total Bacterial Load

Intratracheal instillation of *E.coli* resulted in a significant bacterial load of 1.2 x 10^5 CFU [8.5 x 10^3 – 9.3 x 10^5] (median [25^{th}-75^{th} percentile]) in the injured alveoli at 18 h. Intravenous administration of MSC MV significantly decreased the bacterial load in both bronchoalveolar lavage fluid and lung homogenate by 97% and 50% respectively, as compared to mice

treated with PBS (Figure 5A and 5B). MSC MV treatment also eliminated the bacteremia seen (Figure 5C). Administration of NHLF MV had no therapeutic effect.

Intratracheal MSC MV decreased the incidence of bacteremia from 100% in the PBS group to 11% in the treated mice (Figure 5E). However, although there was a reduction in the bronchoalveolar lavage fluid bacterial CFU counts by 40% with MSC MV treatment, it was not significant (Figure 5D).

CD44 Receptor Dependent Uptake of MSC MV Into Primary Cultures of Human Monocytes and Human Alveolar Epithelial Type 2 Cells

The uptake of fluorescent-labelled MSC MV was increased by 100% in human monocytes and by 150% in human alveolar epithelial type 2 cells with injury (LPS and/or cytomix, Figure 6). The impact of inflammation on the uptake of MV was confirmed using MV isolated from murine green fluorescent protein-transfected MSC on human monocytes with or without LPS exposure, allowing us to rule out any "contamination" by an excess of the fluorescent dye. Exposure to the anti-CD44 neutralizing antibody suppressed this inflammatory uptake of MSC MV, suggesting a CD44 dependent mechanism (Figure 6). A loss of the therapeutic effect of MSC MV on survival was also seen when MSC MV pre-incubated with anti-CD44 blocking antibody were administered to mice with severe *E.coli* pneumonia compared to IgG control (Figure 6D). The expression of the CD44 ligands, L-selectin and osteopontin, was increased in human monocytes and alveolar epithelial type 2 cells following inflammatory injury (*see* Figure E2).

Effect of MSC MV on Human Monocytes and Alveolar Epithelial Type 2 Cells

MSC MV treatment reduced *E.coli* bacterial CFU counts in primary cultures of human monocytes by 25% compared to PBS control (Figure 7A). This anti-microbial effect was in

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part due to an increase in percentage phagocytosis and phagocytic index of human monocytes against *E.coli* bacteria. MSC MV administration also decreased by 30% tumor necrosis factor-α (TNF-α) secretion (Figure 7B), which was associated with a numerical increase in prostaglandin E2 levels by 40% by monocytes (*P* = NS). Although the anti-microbial effect was similar between MSC MV and MSC treatment, TNF-α secretion was reduced further in the MSC group (by 65%) compared to MSC MV group (by 30%) (Figure 7B). MSC MV treatment restored intracellular ATP levels in injured human alveolar epithelial type 2 cells to control levels (Figure 7C).

Human monocytes exposed with MSC MV exhibited lower levels of mRNA for human inducible nitric oxide synthase (iNOS), a type 1 (M1, pro-inflammatory) marker, and higher levels of mRNA for transglutaminase 2 (TGM2), a type 2 (M2, anti-inflammatory) marker. However, there were no changes on other M2 markers (CD163 or CD206) by PCR or flow cytometry (*see* Figure E3).

Pre-stimulation of MSC with the Toll-Like Receptor 3 Agonist Poly (I:C) Increased the Expression of Cyclooxygenase 2 and Interleukin-10 mRNA in MSC and Human Monocytes

Human MSC expressed mRNA for TLR-3 by reverse transcription (RT)-PCR (Figure 8A). MSC pretreatment with poly (I:C) increased mRNA expression for cyclooxygenase (COX)-2 and interleukin (IL)-10 in MSC (Figure 8B and 8C), and increased the expression of COX2 in the released MV compared to standard MSC MV (Figure 8D). No detectable level of mRNA for IL-10 was found in MSC MV with and without pretreatment with Poly (I:C). More importantly, primary cultures of human monocytes exposed to Poly (I:C)-MV exhibited higher level of mRNA for COX2 and IL-10 compared to standard MSC MV (Figure 8E and 8F).

MV Released From Poly (I:C) Pretreated MSC Enhanced Human Monocytes Phagocytic Capacity Against Bacteria and Further Decreased Tumor Necrosis Factor-α and Increased Interleukin-10 Secretion

Poly (I:C)-MV treatment of human monocytes further reduced bacterial CFU counts in the culture medium by 15% compared to standard MSC MV treatment (Figure 9A and 9D). The percentage phagocytosis remained similar in both groups. However, Poly (I:C)-MV increased the phagocytic index of monocytes by 2.8 fold compared to standard MSC MV (Figure 9E and 9F). In addition, Poly (I:C)-MV treated monocytes had reduced TNF-α (by 14%) and increased IL-10 levels (by 22%) compared to standard MSC MV (Figure 9G and 9H).

***In Vivo* Therapeutic Effect of MV Released from Poly (I:C) Pretreated MSC**

Compared to standard MSC MV, intravenous administration of Poly (I:C)-MV further increased KGF secretion in bronchoalveolar lavage fluid (Figure 2C) and further reduced the bronchoalveolar lavage fluid bacterial CFU count by 74% following *E.coli* pneumonia, but had no effect on other lung inflammatory parameters (Figure 9 and *see* Figure E4).

DISCUSSION

The main findings of this work are summarized as follows: (1) Intravenous administration of MSC MV as therapy improved survival through a KGF mediated effect (Figure 2) and reduced the total bacterial load, inflammation, and lung protein permeability in the injured alveolus in mice with *E.coli* pneumonia (Figures 2-4); (2) Therapeutic effects of MSC MV were equivalent to MSC (Figures 2-4); (3) Anti-microbial effect of MSC MV was mediated in

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part through an increase in monocytes phagocytosis, which was further increased by Poly (I :C) prestimulation of MSC (Figure 5A and 9); (4) MSC MV decreased TNF-α secretion by LPS-primed human monocytes and restored intracellular ATP levels in injured human alveolar epithelial type 2 cells, suggesting immunomodulatory and metabolomic effects of MV (Figure 7); (5) TLR3 prestimulation further increased mRNA expression for COX2 and IL-10 in MSC and human monocytes exposed with Poly (I:C)-MV (Figure 8). Treatment with Poly (I:C)-MV further decreased TNF-α secretion and increased IL-10 secretion by monocytes (Figures 9G and 9H); (6) Treatment with Poly (I:C)-MV further decreased bacterial CFU counts *in vitro* and *in vivo* compared to standard MSC MV (Figure 9D and 9I); (7) And CD44 neutralizing antibody abrogated MSC MV uptake in injured human monocytes and alveolar epithelial type 2 cells (Figure 6) and suppressed the therapeutic effects of MSC MV on mice survival in *E.coli* pneumonia (Figure 6D), demonstrating the critical role of CD44 in the uptake of the MV into the injured cell for its therapeutic effect.

This is the first study demonstrating that MSC MV are as effective as MSC in improving survival in a model of *E.coli* pneumonia. We previously reported that MSC MV reduced the severity of endotoxin-induced ALI in mice (21). However, the model was not lethal and MSC MV and endotoxin were given intratracheal simultaneously (27). Here, we found that intravenous MSC MV given 4 h after bacterial inoculation were as effective as MSC in improving survival and attenuating *E.coli* bacteria-induced ALI. Others demonstrated that exosomes released by mouse MSC mitigated proinflammatory and proliferative response in a hypoxia-induced pulmonary hypertension model in mice (26). However, the pathophysiology of pulmonary hypertension is primarily sterile inflammation.

Our data suggested several potential mechanisms underlying the beneficial effects of MSC MV: (1) An increase in human KGF protein levels in the injured alveolus in part through the transfer of KGF mRNA from MSC MV; (2) An increase in alveolar and total lung

tissue bacterial clearance, through an increase in monocyte bacterial phagocytosis; (3) An immunomodulatory effect on monocytes and alveolar macrophages, suppressing cytokine-induced lung injury and lung protein permeability; (4) And a beneficial effect on alveolar epithelial type 2 cell metabolism.

In our *E.coli* pneumonia model, MSC MV administration increased KGF protein levels in the injured alveolus, in part through the expression of MV KGF mRNA as we previously demonstrated (21) (Figure 2C). Inhibition of KGF eliminated the therapeutic benefits of MSC MV, leading to higher mortality (Figure 2D). These results were consistent with previous studies demonstrating the important role of KGF in the paracrine-mediated effects of MSC such as on restoration of alveolar fluid clearance, lung permeability, and inflammation and inhibition of bacterial growth (8).

Human monocytes exposed with MSC MV exhibited lower levels of mRNA for iNOS, and higher levels of mRNA for TGM2. Although we found no differences in CD163 mRNA expression or CD206 by flow cytometry (additional M2 markers, *see* Figure E3), the results suggested that MSC MV partially changed monocytes to a more anti-inflammatory phenotype (8, 28-31), which we found *in vitro* and *in vivo* with MSC MV treatment (Figures 3, 4, 7 and 9). In our previous work, we also did not find that MSC treatment increased CD206 expression in monocytes (8).

In animal models of sepsis (9, 28, 32) and pneumonia (6, 8, 33), MSC have been shown to repolarize monocytes-macrophages from M1 to M2 phenotype characterized by high levels of IL-10, low levels of TNF-α production and increased phagocytosis. Németh et al. found prostaglandin E2 secreted by MSC was essential for reprogramming monocytes-macrophages towards a M2 phenotype (9). Since MSC MV expressed mRNA for COX2 (Figure 8D), the key enzyme in prostaglandin E2 synthesis, we speculate that the transfer of COX2 mRNA from MSC MV to monocytes, with a resultant increase in prostaglandin E2

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secretion, might be involved in shifting monocytes towards an anti-inflammatory state (34-36).

Similar to MSC, MSC MV enhanced bacterial clearance primarily by increasing monocytes phagocytosis. However, since MSC can secrete antimicrobial soluble factors (6-8), further studies investigating other mechanisms underlying bactericidal effects of MSC MV are needed.

MSC MV restored ATP levels in injured alveolar epithelial type 2 cells, suggesting a metabolic benefit. This result is in line with transcriptome and proteome profiling of MSC MV showing that MV carry key enzymes involved in metabolism such as glyceraldehyde 3-phosphate dehydrogenase and pyruvate kinase, which may be transferred to the injured tissue (36-38). Another mechanism might be through the transfer of either mitochondria (39) or mRNA for key mitochondrial genes (21, 37). Although ATP restoration may be critical for preserving key functions of alveolar epithelial type 2 cells such as fluid clearance and surfactant production, it is still unknown whether MSC MV affects the pathway involved in ATP production, such as the rate of ATP production and/or utilization. Additional studies are warranted.

Similar to MSC (40, 41), we found that CD44 was not only crucial for incorporation of MSC MV into injured human monocytes and alveolar epithelial type 2 cells, but also for their therapeutic effect in mice with pneumonia (Figure 6D). These results confirmed previous studies highlighting endocytosis and direct membrane fusion as two major ways of transferring MV cargo to recipient cells (42). Furthermore, we found that L-selectin and osteopontin, CD44 ligands, were significantly overexpressed upon inflammation (*see* Figure E2).

Recent studies indicated that TLR3 activation with Poly (I:C) can boost the immunomodulatory potential of MSC, while TLR4 activation could reduce it (24, 43-45).

Watterman et al. showed that pretreated MSC secreted higher levels of prostaglandin E2 (24). In the present sudy, Poly (I:C) pretreatment further increased expression of COX2 mRNA in not only MSC and its released MV, but also in monocytes exposed with Poly (I:C)-MV. Moreover, these monocytes displayed a more obvious M2 phenotype as seen in the decrease in TNF-α level and the increase in IL-10 level and bacteria phagocytosis (Figures 8 and 9); it is conceivable that the transfer of COX2 mRNA from Poly (I:C)-MV to monocytes caused the phenotype switch (9). More importantly, we found a 7 folds higher increase in alveolar KFG protein levels (Figure 2C), and a reduction of total alveolar bacterial load by 74% (Figure 9I) in mice treated with Poly (I:C)-MV compared to standard MSC MV. We previously found that MSC KGF secretion increased monocytes-macrophages phagocytic activity (8). Our study is the first suggesting that immunoregulatory and anti-microbial properties of MSC MV might be enhanced by MSC pre-treatment.

The current study has some limitations: (a) Ultracentrifugation may isolate a heterogeneous population of MV, including smaller exosomes. Further studies are needed to determine the contribution of each in the overall effect. (b) To compare our findings to studies using MSC in ALI, we dosed MV by total cell count, not by protein concentration. However, we found that the MV protein content was within the range used in previous studies. (c) In addition to mRNA, MV microRNA as well as lipid content may play a significant therapeutic role. (d) Finally, MSC MV may interact with other cells, such as regulatory T cells, which are involved in ALI resolution. Impact of MV on other immune cells as well as in the resolution phase of ALI will need to be studied further.

In conclusion, MV released from human bone marrow-derived MSC improved survival from *E.coli* pneumonia in mice. This was associated with enhanced phagocytosis of bacteria by human monocytes with a reduction in inflammation and increased ATP levels in

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alveolar epithelial type 2 cells. TLR3 agonist pre-treatment of MSC further increased the effects of MSC MV on monocyte's immunoregulatory and phagocytosis properties. More importantly, MSC MV were as effective as MSC as a therapeutic in ALI from bacterial pneumonia, suggesting a possible alternative to using cells given the potential limitations of any stem cell-based therapy.

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FIGURE LEGENDS

Figure 1. Characterization of Microvesicles Released from Human Mesenchymal Stem Cells. (A) Following serum starvation, human MSC constitutively release MV (see arrow), small membrane enclosed bodies from the plasma membrane; bar is 1 μm. Insert shows purified MSC MV as a homogeneous population of spheroid particles; bar is 2 μm. (B) The protein conc. of the therapeutic dose, 90 μL of MSC MV, used for the small animal studies was similar to the dose of MSC MV previously used in the literature. MSC MV contained a significant amount of total RNA. MSC MV protein and RNA contents are expressed as mean ± SD total protein (μg / 90 μL) and total RNA (ng / 90 μL) respectively. N = 24 for protein, and n = 7 for RNA content. (C) Western Blot analyses of MSC MV demonstrated significant levels of CD44, a receptor previously involved in MSC trafficking to inflammatory sites. Similar to MSC, we hypothesized that MSC MV homed to injured tissues using the CD44 receptor. Abbreviations: CD, cluster of differentiation; MV, Microvesicles; MW, molecular weight.

Figure 2. Keratinocyte Growth Factor-Mediated Effect of MSC MV on Survival Following Severe *E.coli* Pneumonia in Mice. Intravenous instillation of MSC MV improved survival in mice with severe *E.coli* pneumonia. Administration of a neutralizing KGF antibody with MSC MV abrogated this therapeutic effect. (A) Administration of MSC or MSC MV significantly increased survival over 72 h. **P* < 0.01 versus PBS, #*P* < 0.05 versus NHLF MV treated group and †*P* < 0.05 versus PBS by *log*-rank test. (B) RT-PCR demonstrated that MSC MV expressed KGF mRNA. Glyceraldehyde phosphate dehydrogenase (GAPDH) was used as an internal control to normalize loading of the RNA samples. The PCR products were 210 bp in size for KGF. (C) Intravenous administration of MSC MV increased human KGF protein levels in the bronchoalveolar lavage fluid of *E.coli*-

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bacteria injured mice at 18 h, which was not accounted for by MV intracellular KGF protein levels; lysates of 90 µL of MV yielded only 16.5 ± 5.4 pg total of KGF protein. Data are shown as mean ± SD for each condition. N = 29 for PBS, n = 12 for MSC, n = 22 for STD-MV and n = 10 for Poly (I:C)-MV, ***P < 0.01 versus (I:C)-MV group by analysis of variance (ANOVA, Bonferroni). (D) Administration of an anti-KGF neutralizing antibody with the MSC MV significantly decreased survival over 72 h compared with MSC MV + control IgG treated group or PBS. *P < 0.01 versus PBS, P < 0.01 versus MSC MV + anti-KGF antibody treated group by *log*-rank test. Abbreviations: GAPDH, glyceraldehyde phosphate dehydrogenase; KGF, keratinocyte growth factor; MSC, mesenchymal stem cells; MSC MV, Microvesicles released from mesenchymal stem cells; MV + IgG, microvesicles released from mesenchymal stem cells combined with control immunoglobulin G antibody; MV + anti-KGF Ab, microvesicles released from mesenchymal stem cells combined with an anti-KGF neutralizing antibody; NHLF MV, Microvesicles released from normal human lung fibroblast; PBS, phosphate buffered saline; SD, standard deviation.

Figure 3. Effect of MSC MV on Influx of Inflammatory Cells and on Inflammation Following *E.coli*-Bacteria Induced Acute Lung Injury in Mice. Intravenous and intratracheal administration of human MSC MV reduced the influx of inflammatory cells and lung protein permeability in the injured alveoli following *E.coli* pneumonia. (A) Intravenous administration of MSC MV decreased total white blood cell and neutrophil counts in the bronchoalveolar lavage fluid of *E.coli*-injured mice at 24 h. Data are shown as mean ± SD for each condition. N = 21 for sham, n = 20 for PBS, n = 6 for MSC, n = 22 for MSC MV, n = 5 for NHLF MV, **P < 0.01 versus PBS, ✓P < 0.05 versus NHLF MV group by analysis of variance (ANOVA, Bonferroni) for total white blood cells; *P < 0.05 versus PBS by ANOVA (Bonferroni) for neutrophil count. (B) Intravenous MSC MV reduced lung protein

permeability and MIP-2 levels in bronchoalveolar lavage fluids of mice injured with *E.coli* pneumonia at 24 and 18 h respectively. N = 17-21 for sham, n = 19-22 for PBS, n = 6-9 for MSC, n = 14-20 for MSC MV, n = 5-14 for NHLF MV, ***P < 0.01 versus PBS, ✓P < 0.05 versus NHLF MV, *P < 0.05 versus MSC for protein concentration by ANOVA (Bonferroni). (C) Intratracheal MSC MV administration decreased total white blood cells, neutrophil counts, and the protein concentration in the bronchoalveolar lavage fluid of *E.coli*-bacteria injured mice at 24 h. Data are shown as mean ± SD for each condition. N = 2-3 for sham, n = 5-20 for PBS, n = 9-14 for MSC MV, n = 5 for NHLF MV. Abbreviations: BAL, bronchoalveolar lavage; CFU, colony forming units; MIP-2, macrophage inflammatory protein-2; MSC MV, Microvesicles released from mesenchymal stem cells; NHLF MV, Microvesicles released from normal human lung fibroblast; PBS, phosphate buffered saline; SD, standard deviation; WBC, white blood cells.

Figure 4. Effect of MSC MV Administration on Lung Injury Following Severe *E.coli* Pneumonia in Mice. (A) Intravenous MSC MV or MSC significantly improved lung injury as assessed by histology. Hematoxylin and eosin stained lung sections at 18 h exhibited a reduction in neutrophil influx, edema, wall thickening and airspace congestion. (B) Lung injury as assessed by semiquantitative scoring was reduced by MSC or MSC MV treatment. Data are shown as mean ± SD for each condition. N = 4-5, *P < 0.01 versus PBS, ✓P < 0.01 versus NHLF treated group and †P < 0.01 versus MSC by Kruskal-Wallis test (Dunn). Abbreviations: MSC MV, Microvesicles released from mesenchymal stem cells; NHLF MV, Microvesicles released from normal human lung fibroblast; PBS, phosphate buffered saline; SD, standard deviation.

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Figure 5. Effect of MSC MV on Total Bacterial Load. Intravenous administration of human MSC MV reduced the total bacterial load in the alveolar space (A), lung tissue (B) and bloodstream (C) following *E.coli* pneumonia. Intratracheal administration of human MSC MV numerically reduced the total alveolar bacterial load (D) and significantly reduced the incidence of bacteremia (E). (A) Intravenous MSC MV decreased the total alveolar bacterial load in the mice injured by *E.coli* pneumonia at 18 h. Total bacterial counts were expressed as mean (CFU counts/mL) ± SD for each condition. N = 15 for PBS, n = 9 for MSC, n = 11 for MSC MV, n = 7 for NHLF, **P* < 0.05 versus PBS, ✓*P* < 0.05 versus NHLF MV group by Kruskal-Wallis test (Dunn). (B) Intravenous MSC MV decreased the total bacterial load in the lung homogenate of mice injured by *E.coli* pneumonia at 18 h. Total bacterial counts were expressed as mean (CFU counts/mL) ± SD for each condition. N = 9 for PBS, n = 8 for MSC MV, **P* < 0.05 versus PBS group by Mann-Whitney test. (C) Compared with PBS, MSC MV treated group was without bacteremia at 18 h. Total bacterial counts were expressed as individual plotted value (CFU counts/mL) with bar as median for each condition. N = 10 for both groups. (D) Intratracheal MSC MV numerically decreased total bacterial load in the injured alveolus and decreased the (E) bacteria load in the blood of mice injured by *E.coli* pneumonia at 24 h. Total bacterial counts were expressed as individual plotted value (CFU counts/mL) with bar as median for each condition. N = 5 for PBS, n = 9 for MSC MV, n = 5 for NHLF MV. Abbreviations: BAL, bronchoalveolar lavage; CFU, colony forming units; MSC MV, microvesicles released from mesenchymal stem cells; NHLF MV, microvesicles released from normal human lung fibroblast; PBS, phosphate buffered saline; SD, standard deviation; WBC, white blood cells.

Figure 6. Role of CD44 in MSC MV Uptake into Primary Cultures of Human Monocytes and Human Alveolar Epithelial Type 2 Cells. In both human monocytes and human alveolar epithelial type 2 cells, MSC MV uptake was dependent on CD44, the cell surface receptor for hyaluronic acid, following injury. (A) LPS stimulation increased the uptake of fluorescent-labelled MSC MV into monocytes, which was dependent on the CD44 receptor on the MV. Fluorescence intensity was expressed as mean (arbitrary units) ± SD for each condition. N = 352-477 cells for all groups, **P* < 0.01 versus LPS-, †*P* < 0.01 versus CD44 preincubated MSC MV and §*P* < 0.01 versus IgG preincubated MSC MV by analysis of variance (ANOVA, Bonferroni). Photomicrographs display the pattern of fluorescence levels observed in each experimental condition. Scale bar is 20 μm. (B) Using MV released by green fluorescent protein-transfected MSC, we confirmed that LPS stimulated the uptake of MSC MV. N = 269-343, **P* < 0.01 versus LPS- by student *t*-test. Photomicrographs display the pattern of fluorescence levels (green fluorescent protein) observed in both experimental conditions. Scale bar is 20 μm. (C) Stimulation by LPS with an inflammatory injury (cytomix + LPS) increased the uptake of fluorescent-labelled MSC MV into alveolar epithelial type 2 cells, which was dependent on the CD44 receptor on the MV. Fluorescence intensity was expressed as mean (arbitrary units) ± SD for each condition. N = 179-239 cells for all groups, **P* < 0.01 versus cytomix- LPS-, †*P* < 0.01 versus CD44 preincubated MSC MV and §*P* < 0.01 versus IgG preincubated MSC MV by ANOVA (Bonferroni). Photomicrographs display the pattern of fluorescence levels observed in each experimental condition. Scale bar is 20 μm. (D) Blocking the CD44 receptor on MSC MV decreased survival in mice injured with *E.coli* pneumonia treated with the MV as compared to blocking with an IgG control antibody. N = 21 for MV + IgG, n = 33 for MV+CD44ab, **P* < 0.01 versus MV + IgG by *log*-rank test. Abbreviations: Ab, antibody; ATII, human alveolar epithelial type 2 cells; AU, arbitrary units; CD44, cluster of differentiation 44; IgG,

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immunoglobulin G; LPS, lipopolysaccharide; MSC MV, Microvesicles released from mesenchymal stem cells; MV, Microvesicles; SD, standard deviation.

Figure 7. Functional Effects of MSC MV on Primary Cultures of Human Monocytes and Human Alveolar Epithelial Type 2 Cells. (A) Treatment of human blood monocytes with MSC MV for 24 h increased *Escherichia coli* bacterial clearance by 24%. Total bacterial counts are expressed as mean (% of PBS) ± SD for each condition. N = 46 for PBS, n = 12-13 for NHLF MV or MSC and n = 26 for MSC MV, **P* < 0.05 versus PBS, ***P* < 0.01 versus PBS and ✓*P* < 0.01 versus NHLF MV by analysis of variance (ANOVA, Bonferroni). (B) MSC MV decreased monocytes TNF-α secretion by 30%. TNF-α secretion levels are expressed as mean (% of PBS) ± SD for each condition. N = 21 for PBS, n = 10 for NHLF MV or MSC and n = 15 for MSC MV, ****P* < 0.01 versus PBS, *****P* < 0.01 versus PBS and ✓*P* < 0.01 versus MSC by ANOVA (Bonferroni). (C) MSC MV restored intracellular ATP levels in human alveolar epithelial type 2 cells injured with an inflammatory insult (cytomix) at 48 h. N = 11-14 per condition. Abbreviations: ATII, human alveolar epithelial type 2 cells; MSC MV, Microvesicles released from mesenchymal stem cells; NHLF MV, Microvesicles released from normal human lung fibroblast; PBS, phosphate buffered saline; SD, standard deviation; TNF-α, tumor necrosis factor -α.

Figure 8. Effect of MSC Pretreatment with Poly (I:C), a Toll-like Receptor 3 Agonist, on mRNA Expression for Cyclooxygenase 2 and Interleukin-10 in MSC and its Released MV and in Monocytes Exposed to Poly (I:C) Pretreated MSC MV. In these experiments, total RNA was extracted from either human MSC (B, C), or MSC MV (D), or human monocytes (E, F), and semiquantitative RT-PCR was performed. Top: Representative agarose gels of semiquantitative RT-PCR products from COX2 (B, D, E)

and IL-10 (C, F) mRNA amplification. Glyceraldehyde phosphate dehydrogenase (GAPDH) was used as an internal control to normalize loading of the RNA samples. The PCR products were 295 bp in size for COX2 and 500 bp for IL-10. Bottom: densitometry readings derived from the PCR gels. The band density relative to that of the GAPDH is expressed as mean ± SD. (A) Human MSC expressed TLR3. (B, C) Human MSC were either cultured with or without Poly (I:C) for 1 h, then serum starved for 48 h. TLR3 stimulation further increased the expression of COX2 and IL-10 mRNA in MSC. N = 8-22 for standard control (STD-) and Poly (I:C) stimulated MSC, $*P < 0.01$ by Student *t*-test. (D) TLR3 stimulation also further increased the mRNA expression for COX2 in the released MV, Poly (I:C)-MV. N = 10 for STD- and Poly (I:C)-MV, $*P < 0.01$ by Mann-Whitney test. (E, F) mRNA expression for COX2 and IL-10 was increased in human monocytes exposed with Poly (I:C)-MV. N = 5-8, $*P < 0.01$ by Student *t*-test. Abbreviations: COX2, cyclooxygenase type 2; GAPDH, glyceraldehyde phosphate dehydrogenase; IL-10, interleukin-10; (I:C)-MV, microvesicles released from prestimulated mesenchymal stem cells with Poly (I:C); MSC, mesenchymal stem cells; MSC MV, microvesicles released from mesenchymal stem cells; Poly (I:C), polyinosine-polycytidylic acid; RT-PCR, reverse transcription-polymerase chain reaction; SD, standard deviation; STD-MV, microvesicles released from standard mesenchymal stem cells; TLR3, toll-like receptor 3.

Figure 9. Monocytes Exposed to MV Derived from Poly (I:C) Stimulated MSC Had Increased Bacterial Phagocytosis Index and Decreased Inflammatory Cytokine Secretion. (A, B, C) Representative phagocytosis of *Escherichia coli* bacteria by human monocytes exposed with PBS (A, control), STD-MV (B) or Poly (I:C)-MV (C). Scale bar is 5 μm. (D, E, F) Human monocytes exposed to Poly (I:C)-MV exhibited a higher *E.coli* bacterial clearance capacity compared to STD-MV treatment (D). Although the

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phagocytosis rate was similar between groups (E), treatment with Poly (I:C) MV further increased the phagocytosis index for bacteria compared to treatment with STD-MV (F). Data are expressed as mean ± SD. N = 5-13 for PBS, n = 6-22 for standard MV and n = 6-19 for Poly (I:C)-MV, ***P < 0.01 versus PBS, ✓P < 0.01 versus STD-MV by analysis of variance (ANOVA, Bonferroni). (G, H) Poly (I:C)-MV further decreased TNF-α and increased IL-10 levels secretion by monocytes. N = 11-13 for PBS, n = 11-12 for STD-MV and n = 11-20 for Poly (I:C)-MV, **P < 0.01 versus PBS, ***P < 0.01 versus PBS, ✓P < 0.01 versus STD-MV by ANOVA (Bonferroni). (I) In mice injured with *E.coli* pneumonia, intravenous administration of Poly (I:C)-MV further decreased the alveolar bacterial load as compared as standard MSC MV. Total bacterial counts are expressed as mean (% of standard MSC MV) ± SD for each condition. N = 14 for standard MSC MV, and n = 8 for Poly (I:C)-MV, *P < 0.05 by Student t-test. Abbreviations: BAL, bronchoalveolar lavage; (I:C)-MV, microvesicles released from prestimulated mesenchymal stem cells with poly (I:C); IL-10, interleukin-10; MSC, mesenchymal stem cells; MSC MV, microvesicles released from mesenchymal stem cells; PBS, phosphate buffered saline; SD, standard deviation; STD-MV, microvesicles released from standard mesenchymal stem cells.

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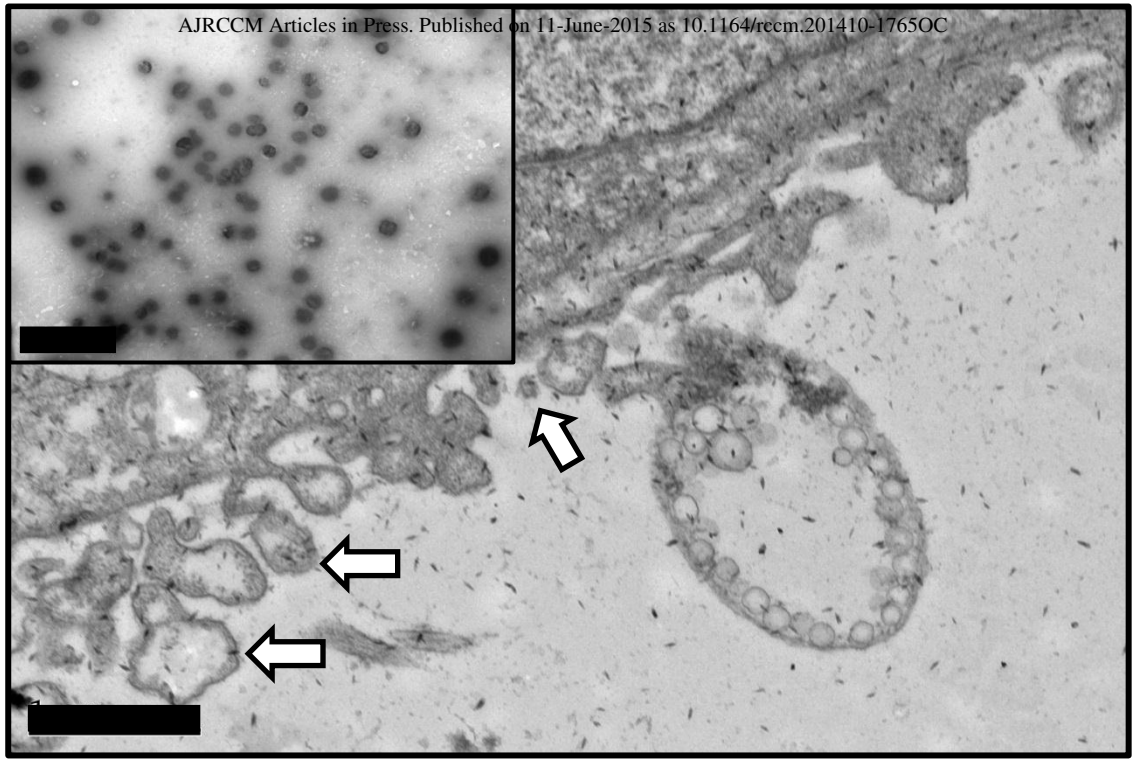

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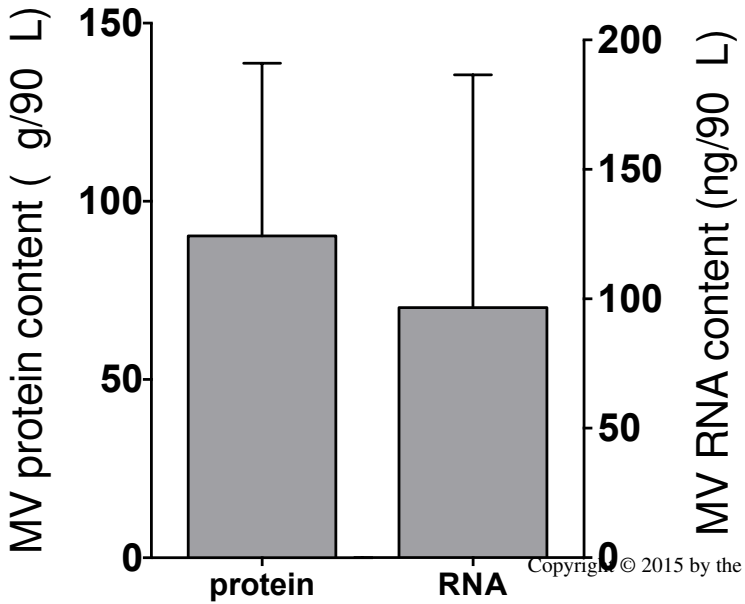

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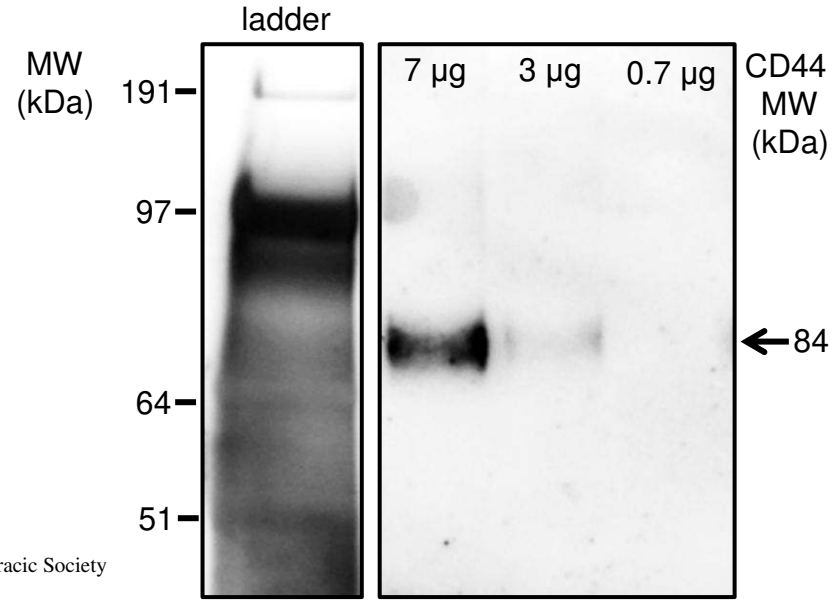

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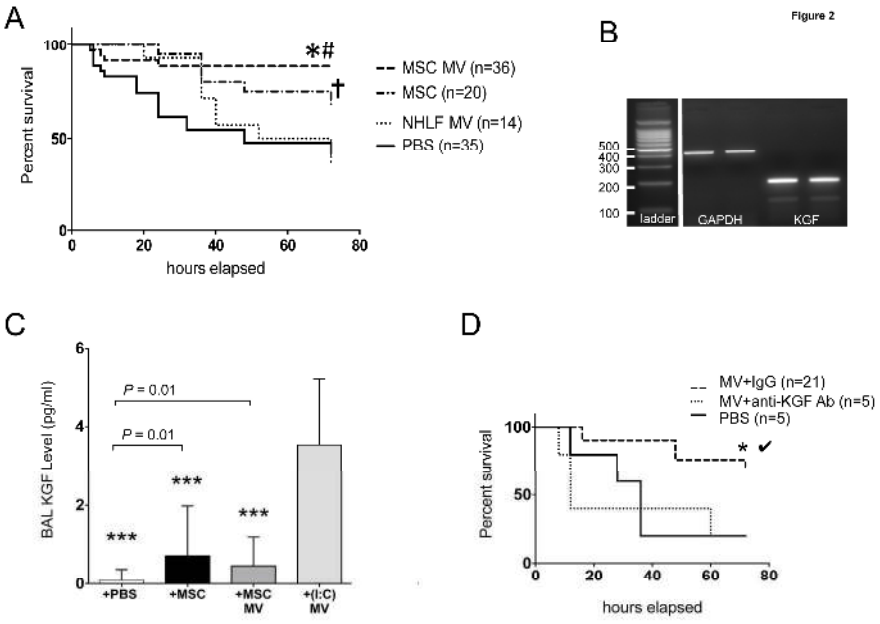

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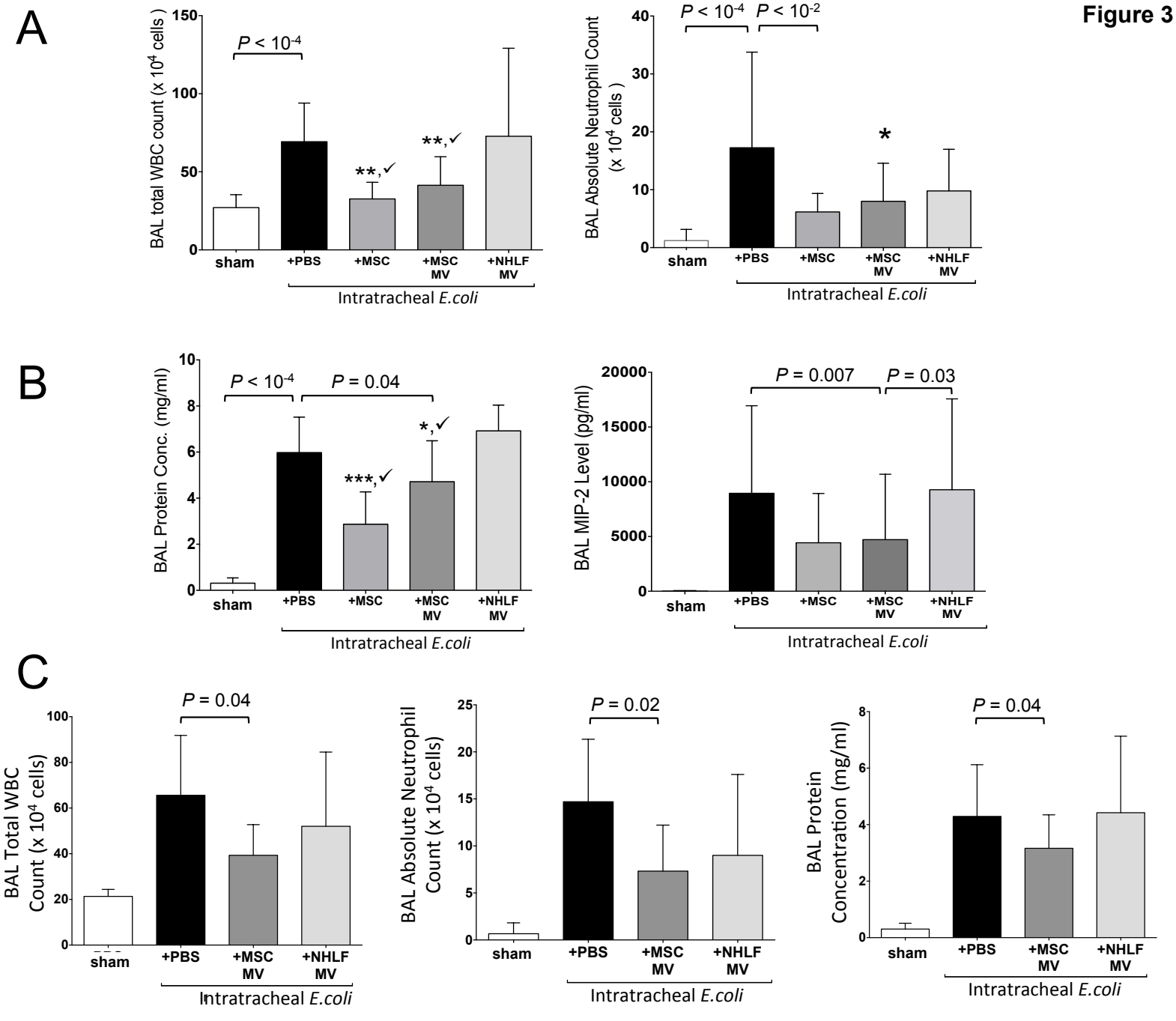
Figure 3



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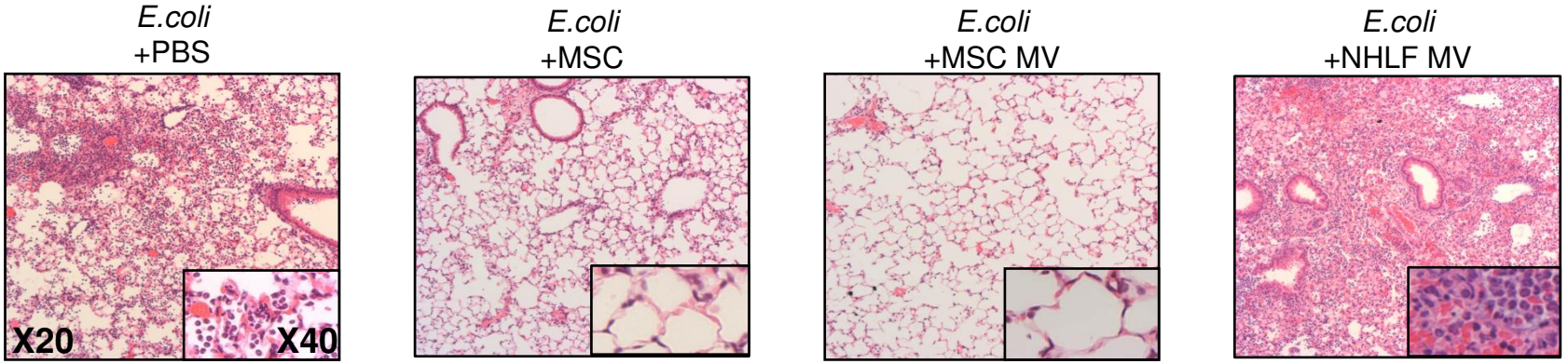

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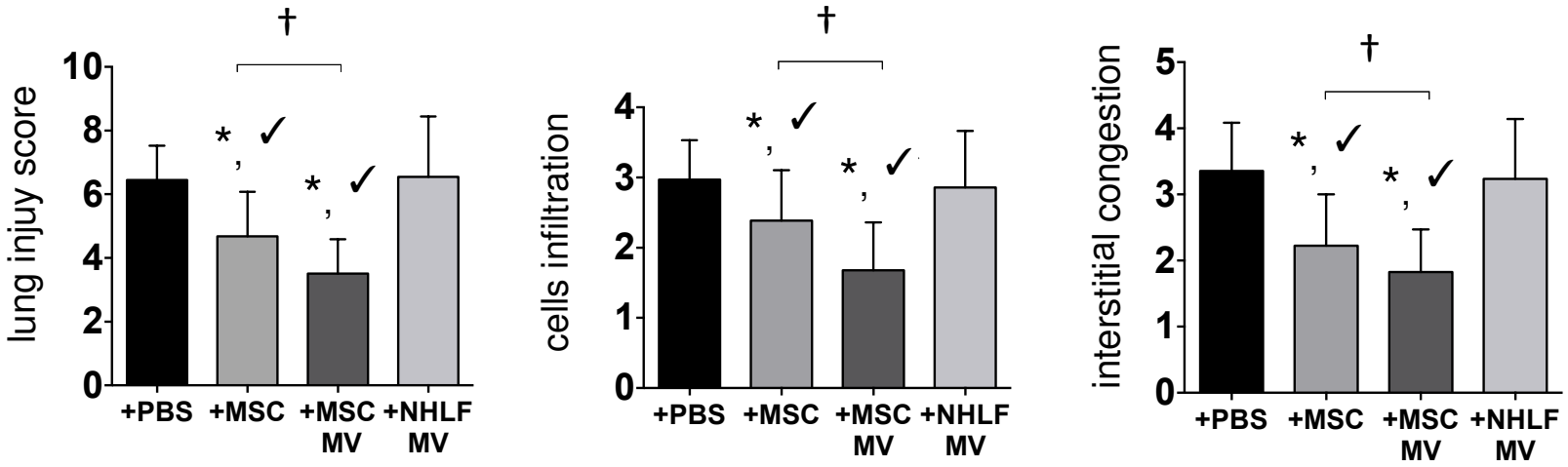

Figure 5

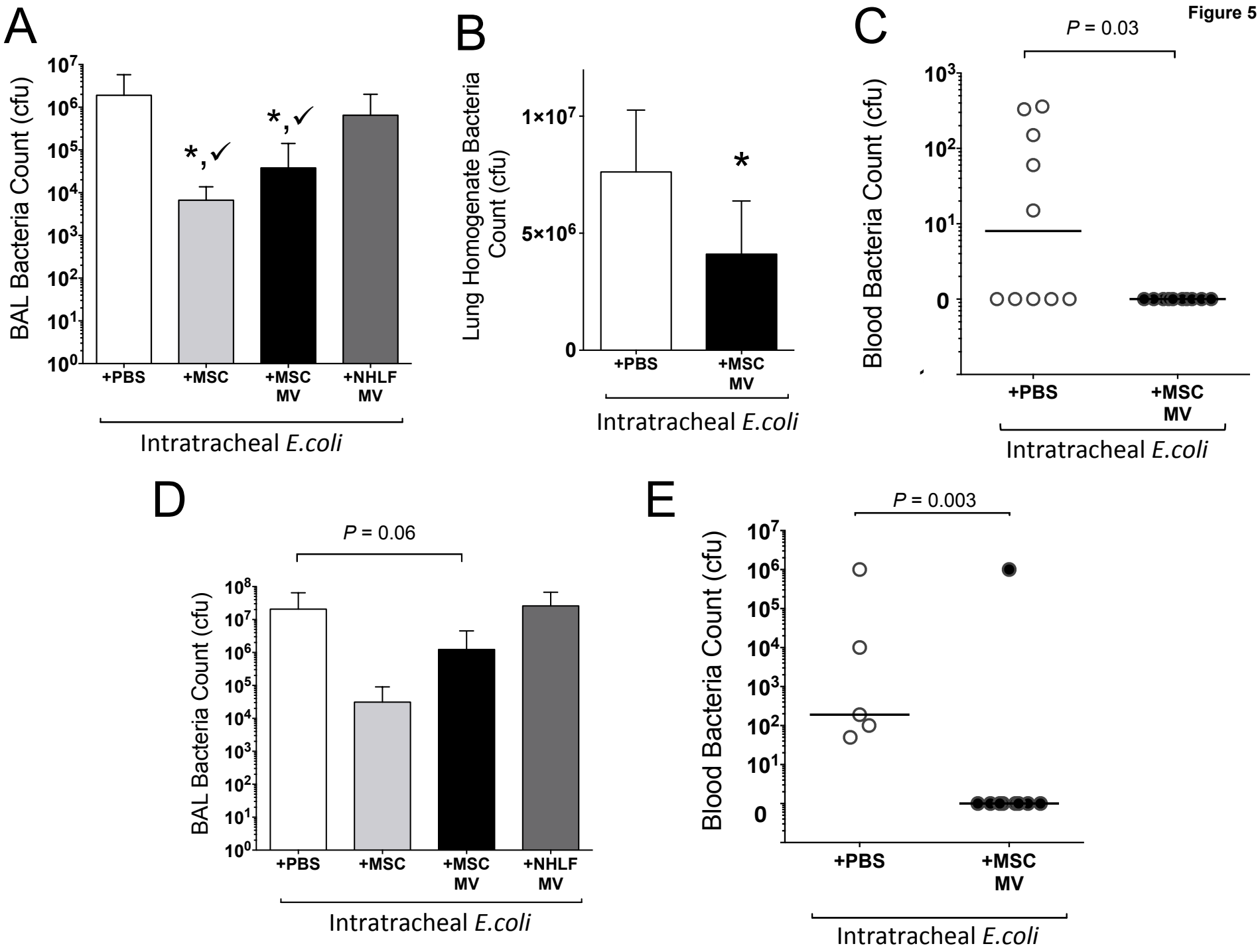

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Figure 6

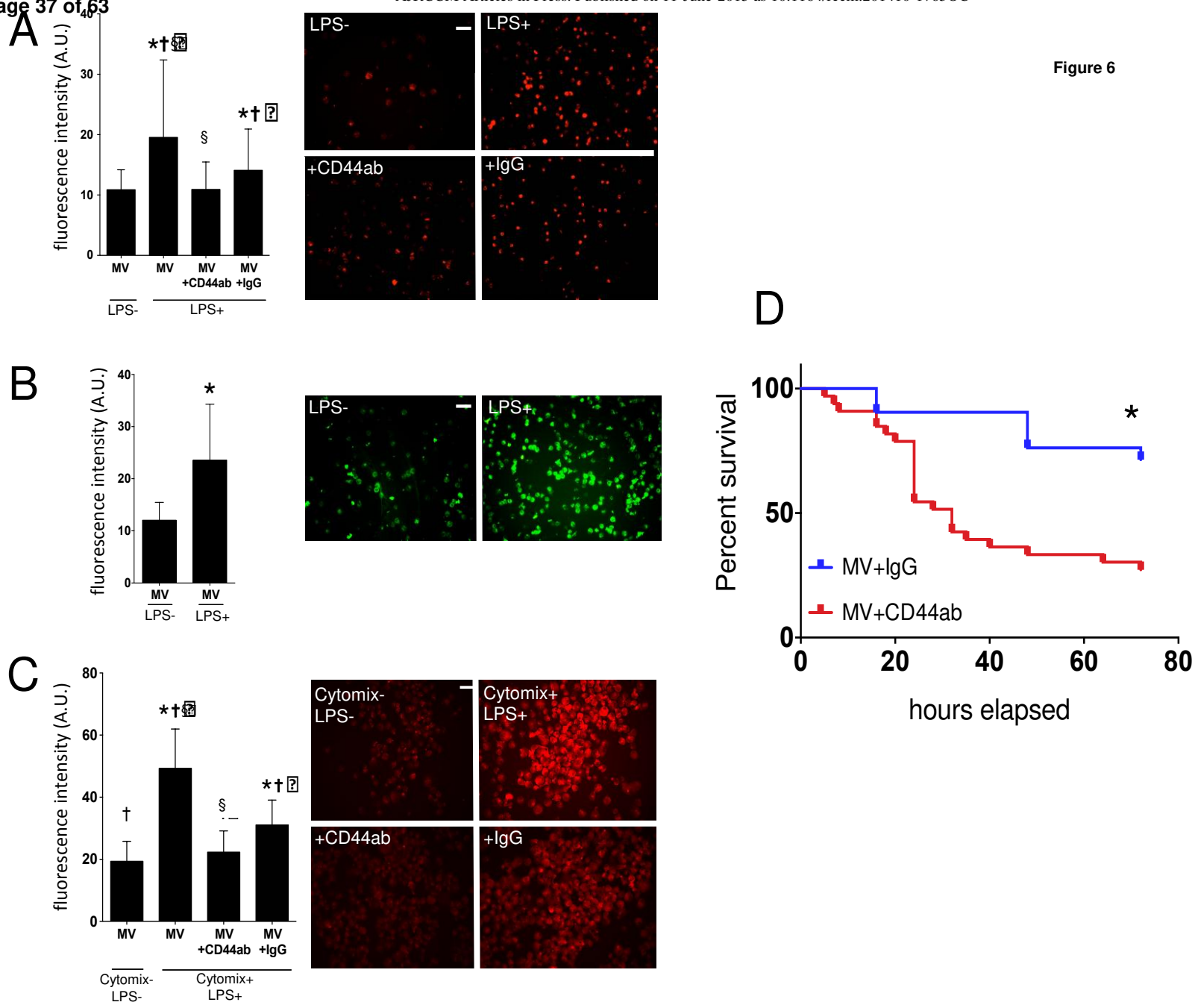
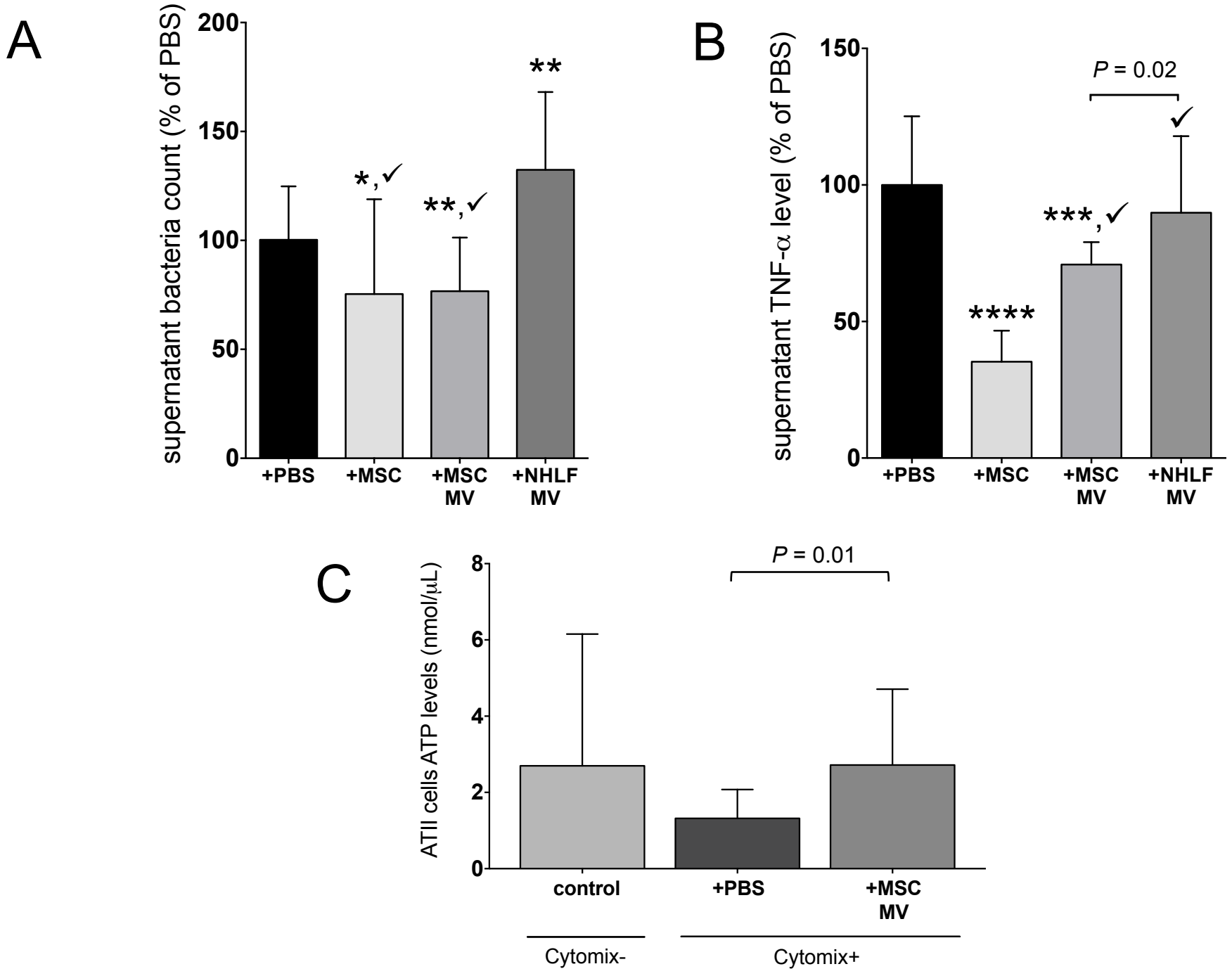


Figure 7



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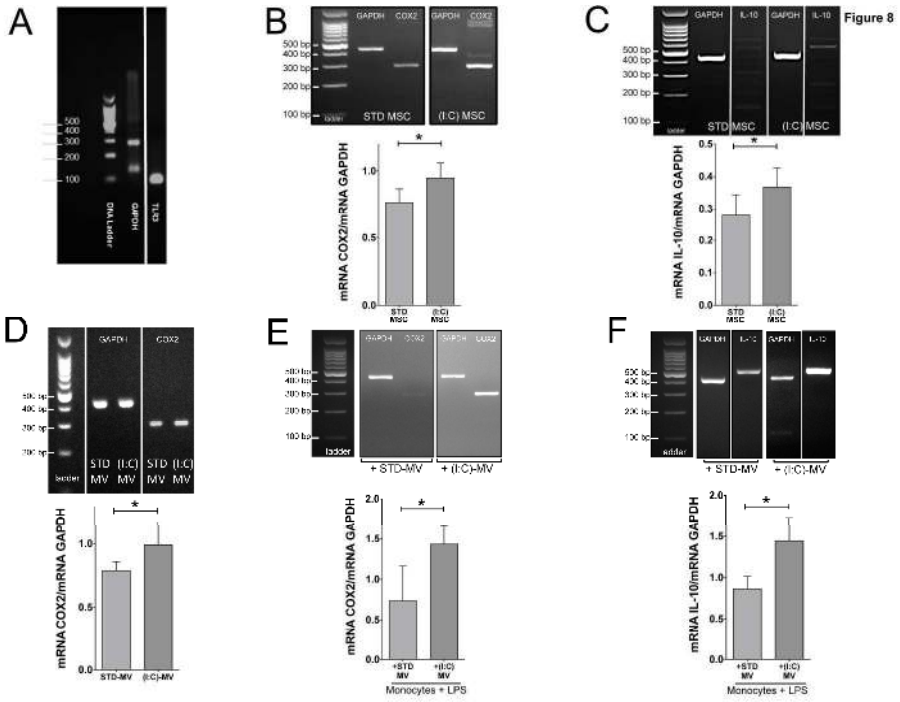

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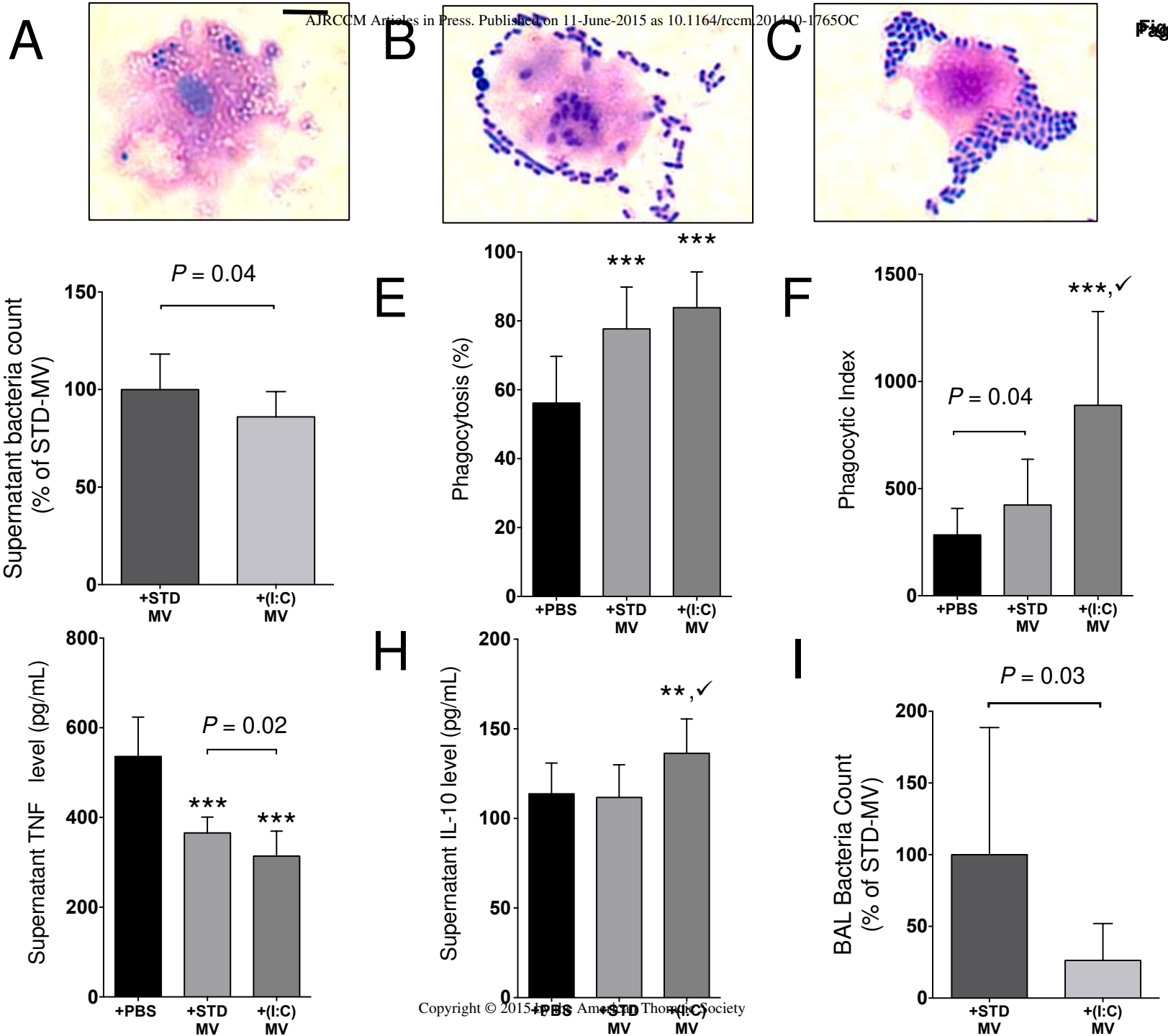

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Therapeutic Effects of Human Mesenchymal Stem Cell-Derived Microvesicles in Severe Pneumonia In Mice

Antoine Monsel, Ying-gang Zhu, Stéphane Gennai, Qi Hao, Shuling Hu, Jean-Jacques Rouby, Michelle Rosenzwajg, Michael A. Matthay, Jae W. Lee

ONLINE DATA SUPPLEMENT

MATERIALS AND METHODS

Mesenchymal Stem Cells. Human MSC were obtained from a NIH repository from Texas A&M Health Science Center. The adult stem cells fulfill the criteria for MSC as defined by the International Society of Cellular Therapy (1). MSC were cultured in α-Minimum Essential Medium without ribonucleosides or deoxyribonucleosides containing 2 mM L-glutamine and 16.5% fetal bovine serum (FBS Hyclone defined, GE Healthcare Life Sciences, Logan, Utah, USA). Cells were seeded at a density of 10,000 cells/cm^2 and used within the total passage number ≤ 8. Overall, MSC from 5 different human donors were used in the experiments. Normal adult human lung fibroblasts were used as cellular controls (Lonza, Walkersville, Maryland, USA).

Isolation of MSC-Derived Microvesicles. As previously described (2)**,** MV were obtained from the supernatants of MSC and normal human lung fibroblasts cultured over 48 h in α-Minimum Essential Medium without ribonucleosides or deoxyribonucleosides containing 2 mM L-glutamine or fibroblast basal medium deprived of fetal bovine serum and supplemented with 0.5% Bovine Serum Albumin Fraction (BSA, MP Biomedicals, LLC). To isolate the MV, the cell-free supernatants was centrifuged at 600 g for 20 minutes to remove debris and then 100,000 g (Beckman Coulter Optima L-100XP ultracentrifuge) for 1 h at 4°C. The pellet was resuspended and washed in phosphate buffered saline and submitted to a second ultracentrifugation in the same conditions. MSC or normal human lung fibroblasts MV were resuspended according to the final cell count of MSC or normal human lung fibroblasts after 48 h of serum starvation (10 µL per 1 x 10^6 cells) and stored at -80°C. The total protein content of the MSC or normal human lung fibroblasts MV was also quantified by Bradford Assay (BioRad, Hercules, CA, USA).

For uptake experiments, we labelled MSC MV by adding Cellmask stain

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(Invitrogen, Grand Island, New York, USA; final concentration: 5 μg/mL) to the supernatant. Cellmask is a red fluorescence aliphatic chromophore, which intercalates into lipid bilayers of MSC prior to the isolation of the MV. After the first ultracentrifugation cycle, MV were washed twice in phosphate buffered saline using the Amicon-2 centrifugal filter device (Millipore, Billerica, Massachusetts, USA) for removing excess chromophore and then ultracentrifuged a second time in the same conditions (3). In separate uptake experiments, MV containing GFP was isolated from the supernatant of GFP-transfected murine MSC (obtained from NIH repository from Texas A&M Health Science Center).

For CD44 neutralizing antibody experiments, standard or fluorescent labelled MSC MV were incubated with either anti-CD44 blocking antibody (BD Biosciences, San Jose, California, USA; final concentration: 1 μg/mL) or negative control IgG antibody (R&D Systems, Minneapolis, Minnesota, USA; final concentration: 1 μg/mL) for 15 minutes at 4°C.

TLR3 Priming of MSC. In order to switch MSC toward a more immunoregulatory phenotype as previously described (4), polyinosinic-polycytidylic acid (Poly (I:C),1 μg/mL, Sigma-Aldrich, St. Louis, Missouri, USA) was used as an agonist for the TLR3 expressed on MSC. Poly (I :C) was added to fresh conditioned medium (αMEM without FBS, supplemented with 0.5% Bovine Serum Albumin) and incubated for 1 h. The cells were then washed twice in phosphate buffered saline and serum starved over 48 h as previously described. Isolation of MV released from TLR3 agonist-prestimulated MSC (Poly (I :C)-MV) followed the same protocol as used for the standard MV (STD-MV).

Transmission Electron Microscopy of MSC MV. Human MSC monolayers, grown on glass coverslips, or isolated MSC MV were fixed with 3% (wt/vol) Karnovsky fixative for 1

h at 0°C. The monolayers were then postfixed for 1 h in 1% veronal buffered osmic acid and dehydrated with graded ethanols and/or propylene oxide. The cell preparations were then embedded in Epon or Araldite resins cured at 60°C. Thin sections were contrasted with saturated aqueous uranyl acetate and Reynolds lead citrate. The sections were then imaged with a JEOL 1200 EX transmission electron microscope operating at 80 kV.

Western Blot Analyses. MV lysates were first reduced and denatured with sample buffer and resolved (0.7-7 µg proteins per lane) on a 4–12% gradient Bis-Tris Plus gel (Invitrogen, Grand Island, New York, USA) using a MOPS Bolt buffer (Invitrogen, Grand Island, New York, USA) at 150 V for 45 minutes. The proteins were then transferred onto a polyvinylidene difluoride membrane and blocked with 5% BSA in Tris buffered saline with Tween-20 for 1 h. The membrane was then exposed to the primary antibody overnight at 4°C (CD44, Abcam, Cambridge, Massachusetts, USA), followed by secondary antibody conjugated to horseradish peroxidase and subjected to enhanced chemiluminescence.

RNA Isolation and RT-PCR. Total RNA was isolated from either MSC, MSC MV or human monocytes using the Qiagen RNAeasy kit (Qiagen Inc, Valencia, California, USA). After isolation, RNA samples were treated with DNase I for 60 minutes at RT. The quality of the RNA was assessed with the NanoDrop ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) according to the manufacturer's instructions. 260/280 and 260/230 nm absorbance ratios of 1.8– 2.0 indicated a pure RNA sample. Primers for human cyclooxygenase-2 (COX-2), human Interleukin-10 (IL-10), human glyceraldehyde 3-phosphate dehydrogenase (GAPDH), human inducible nitric oxide synthase (iNOS), human CD163, and human transglutaminase 2 (TGM2)

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were custom made (Integrated DNA Technology, San Diego, California, USA). The sequences were as follows: COX-2 (forward 5'-AGAAAACTGCTCAACACCGGA-3'; reverse 5'-CAAGGGAGTCGGGCAATCAT-3'), IL-10 (forward 5'-CTGTGAAAACAAGAGCAAGGC-3'; reverse 5'-GAAGCTTCTGTTGGCTCCC-3'), GAPDH (forward 5'-CCATCTTCCAGGAGCGAGAT-3'; reverse 5'-ACCTTGCCCACAGCCTTG- 3'), iNOS (forward 5'-CCAGTGACACAGGATGACCTTCAG-3´; reverse 5´-TGCCATTGTTGGTGGAGTAACG-3´), CD163 (forward 5'-ACATAGATCATGCATCTGTCATTTG-3'; reverse 5'-CATTCTCCTTGGAATCTCACTTC-3'), TGM2 (forward 5'-GGTCAACTGCAACGATGACC-3'; reverse 5'-TCGGCCCACGCTCTTAGTGC-3'). Primers for human TLR3 and human keratinocyte growth factor (KGF) were provided by a commercial kit (TLR3 and KGF QuantiTect Primer Assay, Qiagen Inc, Valencia, California, USA). RT-PCR was done using the SuperScript III One-Step RT-PCR System with Platinum Taq DNA Polymerase protocol from Invitrogen (Grand Island, New York, USA) according to manufacturer's instructions. For cDNA amplification, an initial reverse transcription step (52°C for 30 minutes) was followed by denaturing step (94°C for 2 minutes) and then by 40 cycles of denaturing (94°C for 20 seconds), annealing (60°C for 30 seconds), and extending (68°C for 30 seconds), followed by 5 minutes at 72°C for elongation. GAPDH gene amplification was used as an internal control for normalizing loading of the PCR products. The resulting amplified DNA product was run on a 1.4% agarose gel, and bands were visualized with the use of ethidium bromide.

Preparation of *E.coli* Bacteria. *E.coli* bacteria (K1 strain) were used for *in vivo* and *in vitro* experiments**.** From -80°C stored aliquots, the bacteria was thawed, washed and resuspended in sterile phosphate buffered saline. Number of colony forming units (CFU)

was calculated according to the following equation: $OD_{600\ nm}$ = 1.0 corresponds to 4 x 10^8 CFU/mL for *E.coli*.

***E.coli* Pneumonia-Induced ALI in Mice.** C57BL/6 male mice (10-12 weeks, 20-25 g, Jackson Laboratory) were used in experimental protocols approved by the Institutional Animal Care and Use Committee at University of California San Francisco. Mice were anesthetized with ketamine (90 mg/kg) and xylazine (10 mg/kg) intraperitoneal. ALI from pneumonia was then induced by the intratracheal instillation of *E.coli* K1 strain as previously described (5). After 4 h from the initiation of injury, mice were anesthetized, and phosphate buffered saline as carrier control, 800,000 MSC as a positive cellular control, normal human lung fibroblasts MV as a negative control or MSC MV (30, 60 or 90 μL) were instilled intratracheal or injected intravenous through the retro-orbital venous plexus. In separate experiments, Poly (I :C)-MV were compare to STD MV. The role of CD44 in the therapeutic effect of MV was investigated by administering MV initially preincubated with either anti-CD44 blocking antibody (BD Biosciences, San Jose, California, USA) or control IgG (R&D Systems, Minneapolis, Minnesota, USA). In order to investigate the involvement of the KGF pathway by MSC MV, 10 μg of goat anti-human KGF Ab **(**R&D Systems, Minneapolis, Minnesota, USA**)** was also administered with MSC MV. Depending on the experimental aims, mice were euthanized at 18, 24 or 72 h after *E.coli* instillation, and bronchoalveolar lavage samples or lungs were collected for assessment of neutrophil counts, cytokines, bacterial load, protein level measurements and histology. For the survival study, the mice were checked every 8 h over 72 h.

We previously showed the correlation between the degree of lung injury and the mortality rate with the intratracheal dose of *E.coli* (6). Therefore, different doses were used depending on the experimental objectives: 1) The dose of *E.coli* (3 x 10^6 CFU) was

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used for the survival study; 2) And the dose of *E.coli* (2 x 10^6 CFU) was used to study the therapeutic effect of MSC MV without significant mortality in any mice group. In preliminary experiments, MV was administered intratracheal 4 h following injury. The route of MV administration was then switched to intravenous 4 h following injury to follow the protocol of current clinical trials of cell-based therapy for ARDS.

Based on a previously generated dose response curve of the therapeutic effect of intratracheal MSC MV using 15, 30 and 60 µL in a non lethal LPS-induced ALI model (7), we carried out a dose response study by administering 30 (1X) and 60 (2X) µL of intratracheal MSC MV in the *E.coli* pneumonia model. To correlate with on-going clinical trials with MSC in ARDS and to determine if intravenous MSC MV was as efficacious as intratracheal, we switched the route of administration to intravenous. In a dose response curve with MSC MV (**Figure E1**), we found that there was a dose effect on anti-microbial activity with increasing MV doses. All subsequent studies involving the *E.coli* bacteria induced ALI model were done using the primary MSC MV dose of 90 (3X) µL, to maximize both the anti-inflammatory and anti-microbial effects.

For the experiments involving MSC administration, we chose a dose of 800,000 cells as a positive cellular control based primarily from our previous publications **(8-10)** using a similar *E.coli*-endotoxin induced ALI model or a live bacteria-induced severe sepsis model in mice. We also knew that this dose significantly reduced the mortality in a lethal *E.coli* bacteria induced pneumonia model in mice**.** We did not increase the MSC number to correlate with the MSC MV dose (90 µl) because of the potential acute elevation in pulmonary artery pressure (massive pulmonary embolism and death) with intravenous administration with higher cellular doses. In a previous study, we found that doses of 750,000 to 1,000,000 cells IV or IT correlated to 20-30 x 10^6 cells/kg for both mice and rats (11).

Measurement of Neutrophil Counts, Cytokines and Protein Levels in Bronchoalveolar Lavage Fluid. Both bronchoalveolar lavage fluid and blood samples were collected 18 or 24 h after *E.coli*-induced lung injury. Total cell count and differential were obtained using Hemavet HV950FS (Drew Scientific, Dallas, Texas, USA). Bronchoalveolar lavage samples were centrifuged at 300 g for 10 minutes, and the supernatants were collected and stored at -80°C. Macrophage inflammatory protein (MIP)-2, a mouse neutrophil chemokine, as well as human KGF were measured by ELISA (R&D Systems, Minneapolis, Minnesota, USA) in the bronchoalveolar lavage supernatants. Bronchoalveolar lavage protein concentration as a marker of lung endothelial and epithelial permeability was also measured (Pierce BCA Protein Assay Kit, Thermo Scientific, USA).

***E.coli* Quantification in Bronchoalveolar Lavage Fluid, Lung Homogenate, Blood and Supernatants of Primary Cultures of Human Monocytes.** Bacterial growth in the bronchoalveolar lavage fluid, lung homogenate, and blood following *E.coli* pneumonia in the *in vivo* experiments and culture medium of primary cultures of human monocytes exposed to *E.coli* bacteria with or without MSC MV were quantitated by counting CFU. The samples were cultured on LB agar plate (TEKnova, Hollister, California, USA) overnight at 37°C. Individuals colonies (CFU) were then counted.

Histology. Separate experiments were carried out for histological analyses of lung injury. Eighteen h following *E.coli* intratracheal instillation, lungs were excised, gently inflated with 0.5 mL of 10% formalin, followed by tracheal ligature. The lungs were then fixed in 10% Formalin and dehydrated through a serial diluted graded ethyl alcohol baths. After

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fixation, lungs were embedded in paraffin, cut into 5 μm sections, and stained with Hematoxylin & Eosin. An investigator, blinded to group assignments, analyzed the sections and determined the levels of lung injury according to semi-quantitative scoring. For each mouse, 20-30 fields of both lungs at x20 view were examined. Scoring was performed by grading as follows as previously described: infiltration or aggregation of inflammatory cells in air space or vessel wall: 1 = only wall, 2 = few cells (1–5 cells) in air space, 3 = intermediate, 4 = severe (air space congested); interstitial congestion and hyaline membrane formation: 1 = normal lung, 2 = moderate (<25% of lung section), 3 = intermediate (25–50% of lung section), 4 = severe (>50% of lung section); hemorrhage: 0 = absent, 1 = present (12).

Primary Cultures of Human Monocytes and Alveolar Epithelial Type II Cells. Monocytes were collected from whole blood of healthy donors by centrifugation over a Ficoll-paque Plus density gradient (GE healthcare Bio-Sciences Corp., Piscataway, New Jersey, USA). $CD14^+$ monocytes were isolated by positive selection using specific monoclonal antibody-coated magnetic beads and a magnetic cell sorter (Miltenyi Biotec, San Diego, California, USA) from the peripheral blood mononuclear cell monolayer. These highly purified $CD14^+$ monocytes were then suspended in RPMI (Invitrogen, Grand Island, New York, USA) and plated at a concentration of 250,000 cells/well in RPMI supplemented with 10% FBS. After 24 h, endotoxin (*E.coli*-0111 :B4 endotoxin, Sigma-Aldrich, St. Louis, Missouri, USA ; 1 μg/mL) was used for monocyte priming for 24 h prior to the experiments. This procedure routinely yielded cell populations with purity up to 90%. Monocytes viability was verified to be >95% by Trypan exclusion.

Human alveolar epithelial type 2 cells were isolated from human donor lungs declined for transplantation by the Northern California Transplant Donor Network as

previously described (13). The cells were plated on collagen I coated 24-well plates in DMEM high glucose 50%/F-12 50% mix medium containing 10% FBS and antibiotics (penicillin, streptomycin, gentamicin and amphotericin) at a concentration of 1 x 10^6 cells/well. Human alveolar epithelial type 2 viability was verified to be >95% by Trypan exclusion and the purity of the isolation was assessed by immunofluorescence staining for SP-B (Rabbit anti-sheep SP-B, Chemicon, see **Supplemental Table**). This isolation technique usually yielded alveolar epithelial type 2 cell with purity > 85-90%. Following 72 h from isolation, alveolar epithelial type 2 cells were cultured without FBS for 24 h and then exposed to endotoxin at 1 μg/mL and 50 ng/mL of cytomix, a mixture of IL-1β, TNF-α and IFN-γ (R&D Systems, Minneapolis, Minnesota, USA) often used as a surrogate for ALI pulmonary edema fluid.

Uptake of Fluorescent Labeled MSC MV. For the uptake experiments, human monocytes and human alveolar epithelial type 2 cells were exposed with pre-stained MSC MV (cellmask-fluorescent labeled MV: 30 μL/well) the same day as cell priming (endotoxin for monocytes, endotoxin plus cytomix for alveolar epithelial type 2 cells) or MV released by green fluorescent protein-transfected murine MSC. To investigate the role of CD44 in MV uptake, MSC MV were pre-incubated with anti-CD44 blocking antibody (BD Biosciences, San Jose, California, USA) or with negative control IgG (R&D Systems, Minneapolis, Minnesota, USA). After 24 h, cells were washed twice with phosphate buffered saline, cytospined on glass slides, fixed 10 minutes with 4% paraformaldehyde and mounted with fluorescent medium (Vectaschield fluorescent mounting medium, VWR, Visalia, California, USA). The cells were examined by fluorescence microscopy (Leica DM 1,000 microscope). Fluorescence intensities from images of 20 randomly selected microscopic fields of cells from each condition were

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analyzed by densitometry (ImageJ software, NIH Image).

Human Monocytes *E.coli* Bacteria Challenge and Measurement of Intracellular ATP Levels in Alveolar Type II cells Following Inflammatory Injury. Primary monocytes, primed with LPS, were exposed with either negative controls (phosphate buffered saline or normal human lung fibroblasts MV, 30 µL) or MSC MV (STD-MV, 30 µL or Poly (I :C)-MV, 30 µL) or a cellular positive control (MSC in a transwell plates, 250,000 cells in the upper chamber, 0.4-µm pore size, Costar, Corning, USA). After 24 h, monocytes were exposed to 10^7 *E.coli* bacteria (K1 strain) for 1.5 h; *E.coli* bacteria was previously opsonized with human serum (200 µL) at 37 °C for 30 minutes before monocytes exposure. Cells culture supernatants were then collected, plated on LB agar plate (TEKnova, Hollister, CA) for *E.coli* CFU count, tested for TNF-α levels, IL-10 levels, and prostaglandin E2 (PGE2) levels (R&D Systems, Minneapolis, Minnesota, USA). Cytospins of monocytes from each well were made and stained with Wright-Giemsa (Fisher Scientific, Waltham, Massachusetts, USA). The percent phagocytosis and the phagocytic index were calculated following a previously described method (9). Briefly, the number of cells that contained at least one bacterium was determined, and the percent phagocytosis was calculated: (number of cells containing bacteria/number of cells counted) x 100%. The phagocytic index (PI) was calculated as: (PI = total number of bacteria in all cells/number of cells that contained at least one bacterium) x percent phagocytosis. A total of >100 cells were counted for each experiment.

Total RNA was extracted from LPS primed human monocytes with or without MSC MV for measurement of human inducible nitric oxide synthase (iNOS), CD163, transglutaminase 2 (TGM2) and GAPDH mRNA by RT-PCR. Differential quantification of RT-PCR products was based on bands intensity analysis (Carestream Analysis Software,

Rochester, New York, USA) normalized to GAPDH. Flow cytometry was used to quantitate CD206 (BD Pharmingen) in LPS primed human monocytes with or without MSC MV. Cells were analyzed on BD FACSCalibur flow cytometry. Monocytes were gated as SSC^{high}/FSC^{high}. Samples were acquired and analyzed by using Cellquest Pro Software.

Human alveolar epithelial type 2 cells were injured with cytomix (50 ng/mL) with and without MSC MV (30 μL) or vehicle (phosphate buffered saline, 30 μL) treatment for 48 h. Intracellular ATP levels were measured in the cells lysate according to the manufacturer's instruction (ATP colorimetric assay kit, Abcam, Cambridge, Massachusetts, USA).

Immunofluorescence. For both human monocytes and human alveolar epithelial type 2 cells, cells were seeded for 24 h and cultured under specific experimental conditions (with or without inflammatory triggers) as previously described. The next day, the cell monolayer was washed twice with phosphate buffered saline, cytospined on glass slides, fixed 10 minutes with 4 % paraformaldehyde and washed 3 times in phosphate buffered saline. For osteopontin immunostaining, cells were incubated in 1% BSA, 10% normal goat serum, 0.3M glycine in 0.1% phosphate buffered saline-Tween for 1 h for blocking and permeabilization. For L-selectin and alveolar epithelial type 2 antigen immunostaining, cells were only blocked in 10% normal goat serum for 1 h. Slides were incubated with primary antibody overnight at 4°C, washed three times with phosphate buffered saline for 10 minutes and then exposed to the secondary antibody for 1 h at room temperature. Types and working concentrations used for each antibody are summarized in Supplemental Table. After washing, slides were mounted with Vectashield mounting medium. Images were obtained by Leica DM 1,000 microscope. Fluorescence

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intensities from images of 20 randomly selected microscopic fields of cells from each condition, were analyzed by densitometry (ImageJ software, NIH Image).

Impact of Poly (I :C) on Monocytes COX2 and IL-10 mRNA Expression. Human monocytes were seeded for 24 h, primed by endotoxin, then exposed with either STD-MV or Poly (I :C) stimulated MSC MV for 24 h. The cell monolayer was then washed. Total RNA extracted from the whole cell lysate was used for COX2, IL-10 and GAPDH mRNA RT-PCR. Differential quantification of RT-PCR products was based on bands intensity analysis (Carestream analysis software, Rochester, New York, USA) normalized to GAPDH band intensity in each experimental condition.

Statistical analysis. Results are expressed as mean ± Standard Deviation (SD) if the data were normally distributed and median (interquartile range (IQR)) if not. Comparisons between two groups were made using unpaired *t*-test if the data were normally distributed, Mann-Whitney test if not. Comparisons between more than two groups were made using an analysis of variance using the Bonferroni's correction for multiple-comparison testing, if the data were normally distributed. If not, a Kruskal Wallis test using Bonferroni's corrections for multiple-comparison testing was used. The *log*-rank test was used for comparing survival data at 72 h. A value of $P < 0.05$ was considered statistically significant. All statistical analysis was performed using GraphPad Prism software (La Jolla, California, USA). N refers to the number of samples or mice, and are not the number of replicate experiments of the same sample or mouse.

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SUPPLEMENTAL FIGURE LEGENDS

Supplemental Figure E1. Dose Effect of MSC MV on Influx of Inflammatory Cells, Lung Protein Permeability, and Total Bacterial Load Following *E.coli*-Induced Acute Lung Injury in Mice. Based on a previously generated dose response curve of the therapeutic effect with intratracheal MSC MV in a non lethal LPS-induced ALI model (21), we carried out a dose response study by administering 30 (1X) and 60 (2X) µL of intratracheal MSC MV. To correlate with on-going clinical trials with MSC in ARDS and to determine if intravenous MSC MV was as efficacious as intratracheal, we switched the route of administration to intravenous. We found that there was a dose effect on anti-microbial activity with increasing MV doses. Therapeutic dose effect of MSC MV was studied on alveolar influx of polymorphonuclear cells (A), lung protein permeability (B), total bacterial load within the alveolus (C) and the bloodstream (D) following *E.coli*-bacteria induced acute lung injury in mice. Data are shown as mean ± SD for each condition. N = 28-52 for PBS, n = 5-6 for MSC MV (1X), n = 8-14 for MSC MV (2X), n = 10-14 MSC MV (3X), **P* < 0.05 versus PBS, ***P*< 0.01 versus PBS, ✓*P* < 0.05 versus MSC MV (2X) by Kruskal-Wallis test (Dunn). Abbreviations: BAL, bronchoalveolar lavage; IT, intratracheal; IV, intravenous; MSC MV, Microvesicles released from mesenchymal stem cells; MSC MV (1X), 30 µL of MSC MV therapeutic dose; MSC MV (2X), 60 µL of MSC MV therapeutic dose; MSC MV (3X), 90 µL of MSC MV therapeutic dose; PBS, phosphate buffered saline; SD, standard deviation.

Supplemental Figure E2. Effect of Inflammation on L-Selectin and Osteopontin Expression in Human Monocytes and Human Alveolar Epithelial Type 2 Cells. (A) Human monocytes were isolated from peripheral blood, cultured and stimulated with LPS for 24 h. A cytospin sample of monocyte was fixed, immunostained for L-selectin and

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observed through epifluorescence microscope. Human monocyte L-selectin expression was increased after 24 h of LPS stimulation. Fluorescence intensity is expressed as mean (arbitrary units) ± SD for each condition. N = 359-540 cells for all groups, *P < 0.01 versus LPS- by Student t-test. Photomicrographs display the pattern of fluorescence levels observed in each experimental condition. Scale bar is 30 μm. (B) Human alveolar epithelial type 2 cells were isolated from human lung, cultured, stimulated by cytomix and LPS for 24 h. A cytospin sample of alveolar epithelial type 2 cells was fixed, immunostained for osteopontin and observed through epifluorescence microscope. Human alveolar epithelial type 2 cell osteopontin expression is increased after 24 h of cytomix and LPS stimulation. Fluorescence intensity is expressed as mean (arbitrary units) ± SD for each condition. N = 100-592 cells for all groups, *P < 0.01 versus cytomix-LPS- by Student t-test. Photomicrographs display the pattern of fluorescence levels observed in each experimental condition. Scale bar is 30 μm. Abbreviations: ATII, alveolar epithelial type 2 cells; AU, arbitrary units; LPS, lipopolysaccharide; MV, Microvesicles; SD, standard deviation.

Supplemental Figure E3. Effects of MSC MV on Expression of Type 1 and 2 Phenotype Markers in Primary Culture of Human Monocytes. Although we found no differences in CD163 mRNA expression (A) or CD206 by flow cytometry (B), human monocytes exposed with MSC MV exhibited lower levels of mRNA for iNOS, a M1 marker, and higher levels of mRNA for TGM2, a specific marker for M2 phenotype (A). In these experiments, human monocytes were seeded for 24 h, primed by endotoxin, then exposed with either MSC MV or PBS for 24 h. Total RNA extracted from the whole cell lysate was used for human iNOS, CD163, TGM2 and GAPDH mRNA semiquantitative RT-PCR (A). In separate experiments, human monocytes was stained with anti-human

CD206 antibody and subjected to flow cytometry (B). (A) Representative agarose gels of semiquantitative RT-PCR products from iNOS, CD163, and TGM2 mRNA amplification. Glyceraldehyde phosphate dehydrogenase (GAPDH) was used as an internal control to normalize loading of the RNA samples. The PCR products were 603 bp in size for iNOS, 295 bp for CD163, and 618 bp for TGM2. (B) Representative of flow analysis strategy for gating human monocytes exposed with either PBS or MSC MV, based on side scatter, forward scatter, and CD206 marker. Abbreviations: bp, base pair; CD, cluster of differentiation; GAPDH, glyceraldehyde phosphate dehydrogenase; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; M1, type 1 monocyte phenotype (pro-inflammatory); M2, type 2 monocyte phenotype (anti-inflammatory); MSC, mesenchymal stem cells; MSC MV, microvesicles released from mesenchymal stem cells; TGM2, transglutaminase 2.

Supplemental Figure E4. **Effect of MV Derived from Poly (I:C) Stimulated MSC on Lung Inflammation Following Severe *E.coli* Pneumonia in Mice.** In mice injured with *E.coli* pneumonia, intravenous administration of Poly (I:C)-MV did not further impact the alveolar influx of inflammatory cells (A, B), lung protein permeability (C), nor the alveolar MIP-2 protein level (D). Data are shown as mean ± SD for each condition. N = 19-20 for PBS, n = 14-22 for standard MSC MV, and n = 6-8 for Poly (I:C)-MV, $*P < 0.05$ and $***P < 0.01$ versus PBS group by analysis of variance (ANOVA, Bonferroni). Abbreviations: BAL, bronchoalveolar lavage; (I:C)-MV, microvesicles released from prestimulated mesenchymal stem cells with poly (I:C); MIP-2, macrophage inflammatory protein-2; MSC, mesenchymal stem cells; MSC MV, microvesicles released from mesenchymal stem cells; PBS, phosphate buffered saline; SD, standard deviation; STD-MV, microvesicles released from standard mesenchymal stem cells; WBC, white blood cells.

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Figure E1

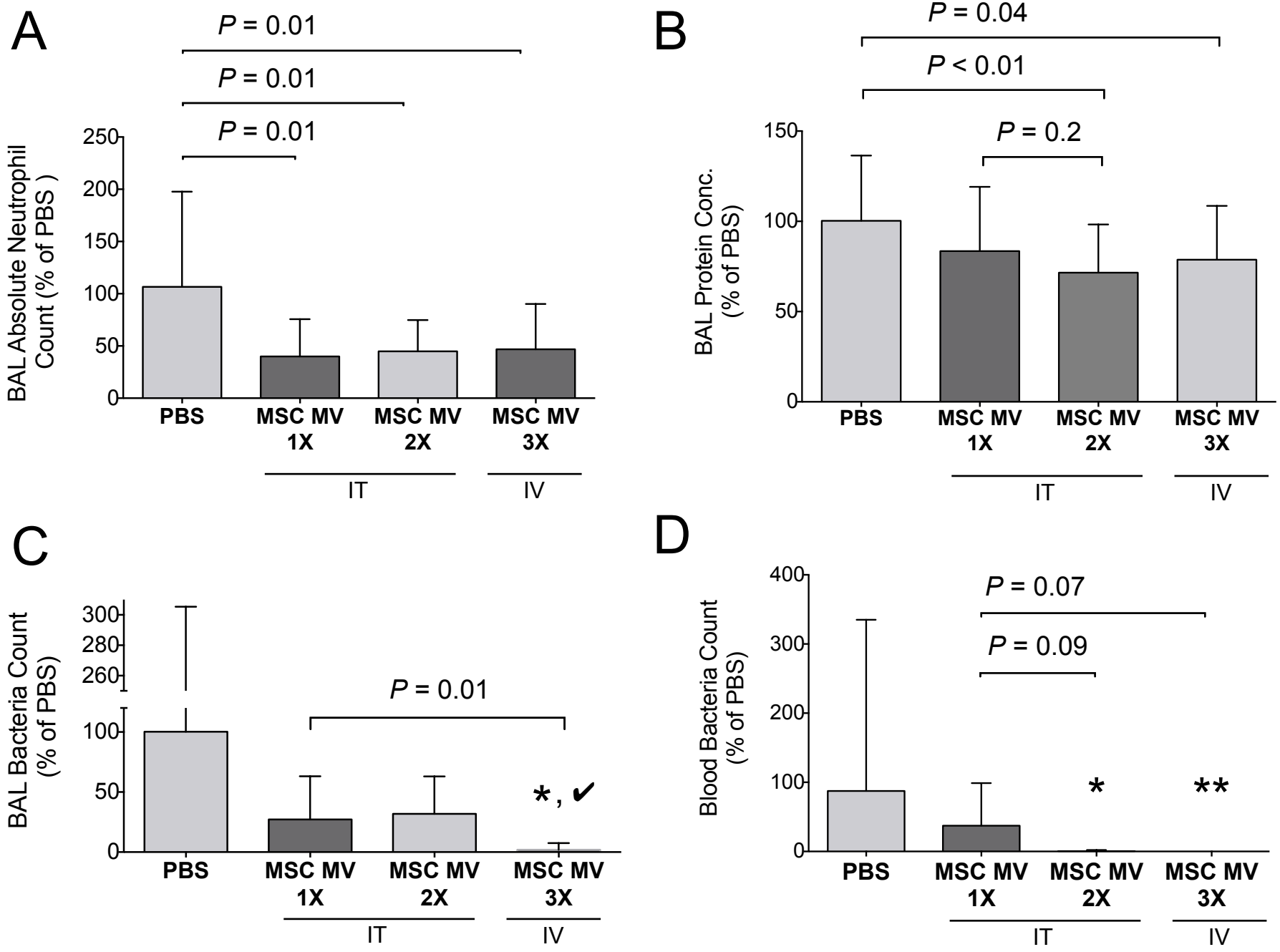

Figure E2

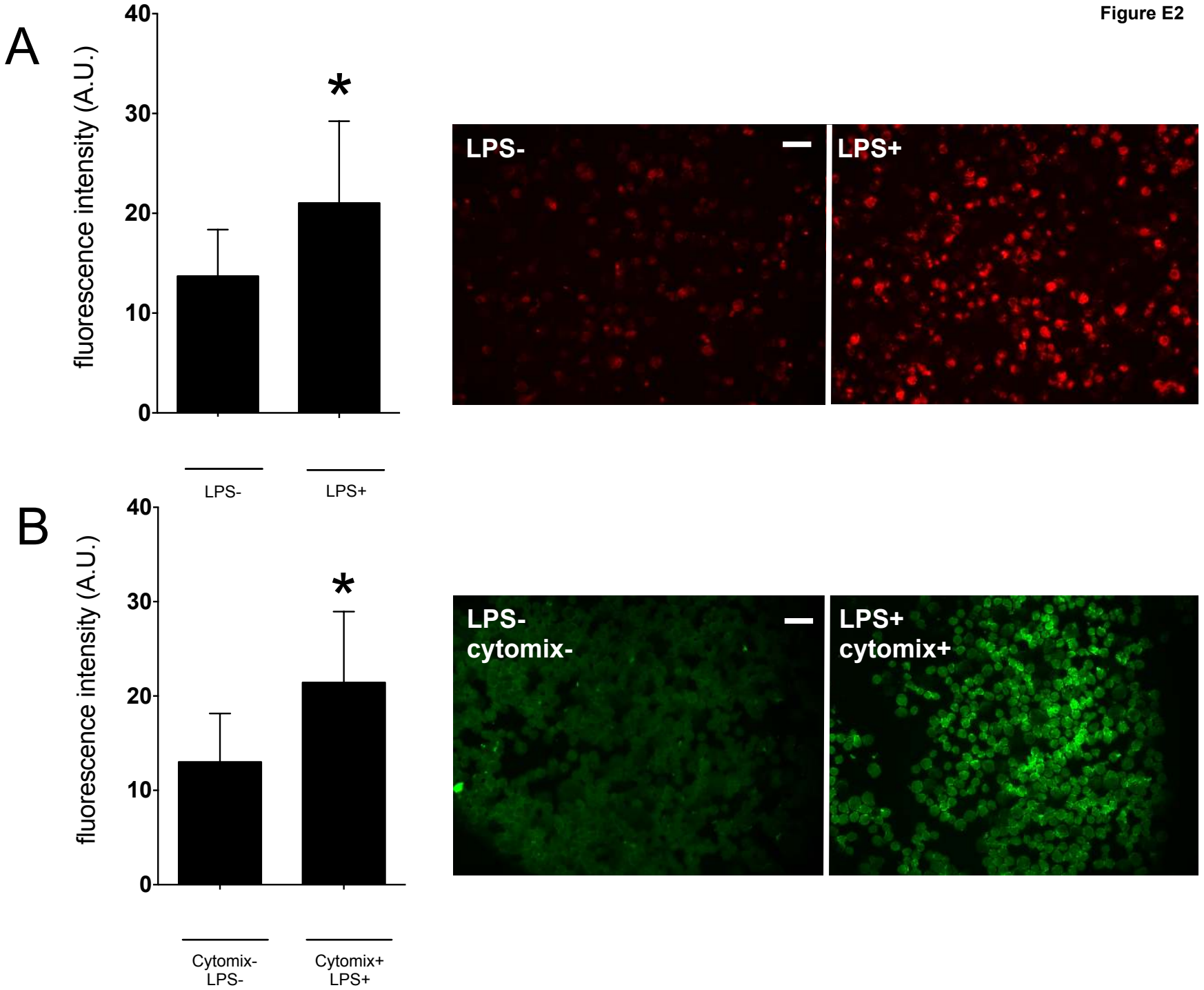

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Figure E3

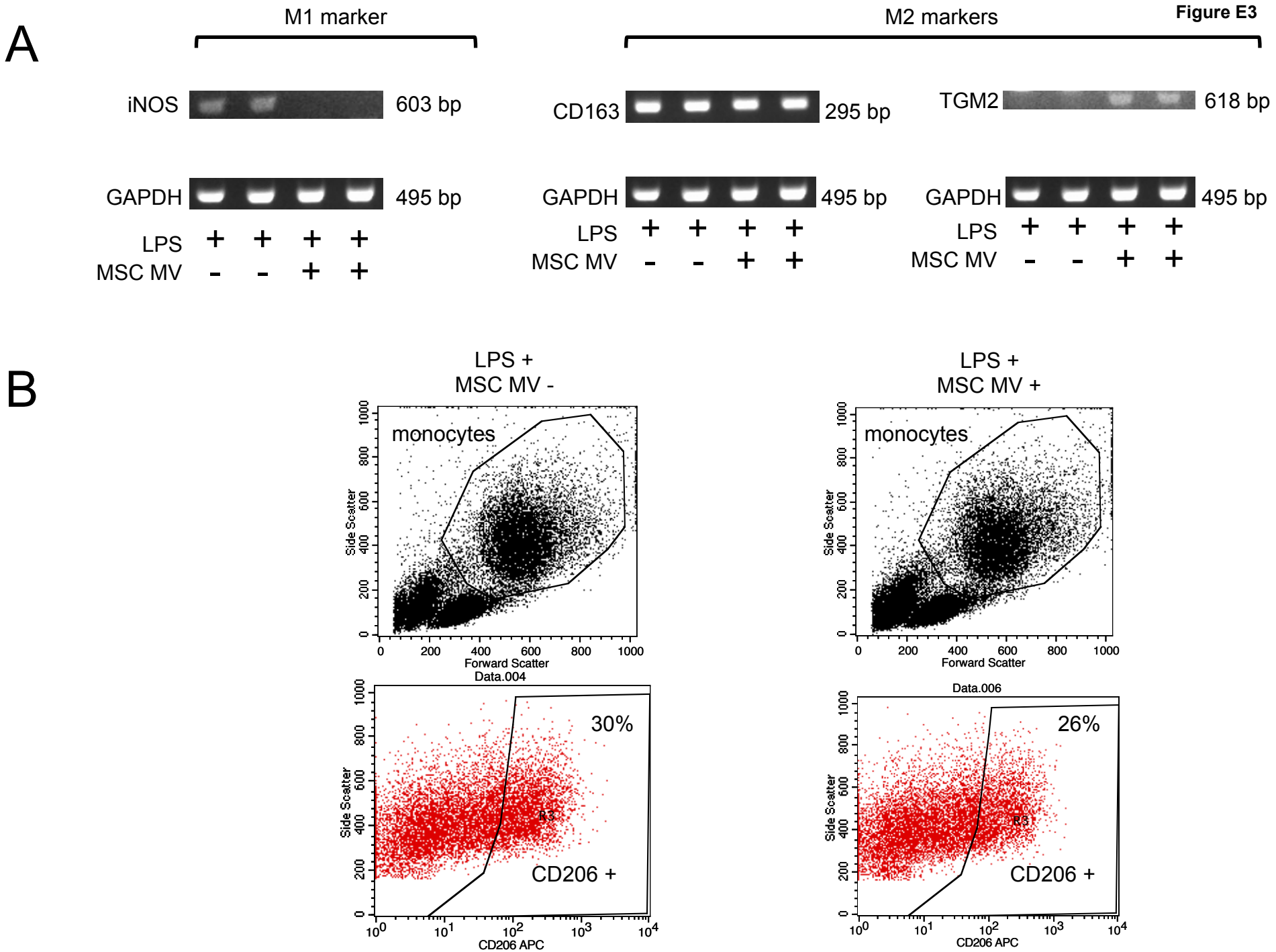
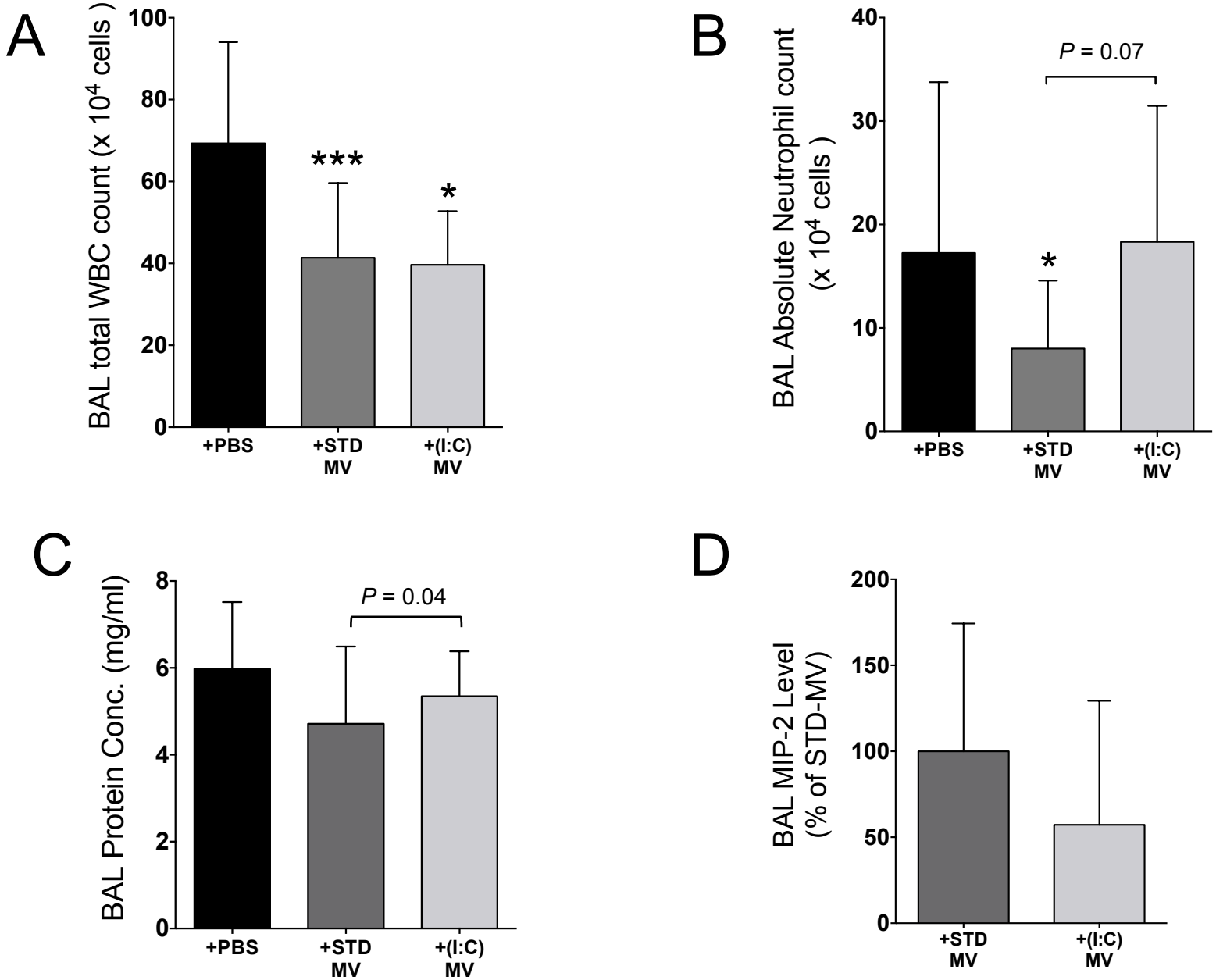


Figure E4



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Supplemental Table

Primary Antibodies						
Name	Clonality	Host	Species Reactivity	Target	Company	Working concentration
Anti-SPB	Polyclonal	Rabbit	Sheep	ATII antigen	Chemicon	1/2000
Anti-Osteopontin antibody	Polyclonal	Rabbit	Human	Osteopontin	Abcam	1/1000
Anti –L-Selectin (CD62L) clone FMC46	Monoclonal	Mouse	Human	L-Selectin	Sigma	1/200
Secondary Antibodies						
Name	Clonality	Host	Species Reactivity	Target	Company	Working concentration
Alexa Fluor 594 Goat Anti-Mouse IgG1 (ϒ1)	Polyclonal	Goat	Mouse	IgG1 (ϒ1)	LifeTechnologies	1/1000
Alexa Fluor 488 Goat Anti-Mouse IgM (µ chain)	Polyclonal	Goat	Mouse	IgM (µ)	Life Technologies	1/1000
Goat polyclonal Secondary Antibody to Rabbit IgG	Polyclonal	Goat	Rabbit	IgG	Abcam	1/1000
Goat anti-mouse IgG_{2b}	Polyclonal	Goat	Mouse	IgG ($ϒ_{2b}$)	Southern Biotech	1/200

Abbreviations: ATII = alveolar type 2 cells; Ig = immunoglobulin.