ANESTHESIOLOGY

Adverse Mechanical Ventilation and Pneumococcal Pneumonia Induce Immune and Mitochondrial Dysfunctions Mitigated by Mesenchymal Stem Cells in Rabbits

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EDITOR'S PERSPECTIVE

What We Already Know about This Topic

- Multiple factors, including mitochondrial dysfunction, contribute to lung injury during mechanical ventilation for pneumonia
- Mesenchymal stem cells have been shown to have potential reparative effects that may attenuate injury

What This Article Tells Us That Is New

- In a preclinical study, mesenchymal stem cell administration improved the outcome of rabbits with pneumonia and high-pressure mechanical ventilation by correcting immune and mitochondrial dysfunction
- When combined with specific antibiotic therapy, the combination was synergistic in mitigating lung inflammation

ABSTRACT

Background: Mechanical ventilation for pneumonia may contribute to lung injury due to factors that include mitochondrial dysfunction, and mesenchymal stem cells may attenuate injury. This study hypothesized that mechanical ventilation induces immune and mitochondrial dysfunction, with or without pneumococcal pneumonia, that could be mitigated by mesenchymal stem cells alone or combined with antibiotics.

Methods: Male rabbits underwent protective mechanical ventilation (8 ml/kg tidal volume, 5 cm H_2O end-expiratory pressure) or adverse mechanical ventilation (20 ml/kg tidal-volume, zero end-expiratory pressure) or were allowed to breathe spontaneously. The same settings were then repeated during pneumococcal pneumonia. Finally, infected animals during adverse mechanical ventilation received human umbilical cord–derived mesenchymal stem cells (3×10^6/kg, intravenous) and/or ceftaroline (20 mg/kg, intramuscular) or sodium chloride, 4 h after pneumococcal challenge. Twenty-four-hour survival (primary outcome), lung injury, bacterial burden, immune and mitochondrial dysfunction, and lung transcriptomes (secondary outcomes) were assessed.

Results: High-pressure adverse mechanical ventilation reduced the survival of infected animals (0%; 0 of 7) compared with spontaneous breathing (100%; 7 of 7) and protective mechanical ventilation (86%; 6 of 7; both $P < 0.001$), with higher lung pathology scores (median [interquartile ranges], 5.5 [4.5 to 7.0] *vs.* 12.6 [12.0 to 14.0]; $P = 0.046$), interleukin-8 lung concentrations (106 [54 to 316] *vs.* 804 [753 to 868] pg/g of lung; $P = 0.012$), and alveolar mitochondrial DNA release (0.33 [0.28 to 0.36] *vs.* 0.98 [0.76 to 1.21] ng/μl; $P < 0.001$) compared with infected spontaneously breathing animals. Survival (0%; 0 of 7; control group) was improved by mesenchymal stem cells (57%; 4 of 7; $P = 0.001$) or ceftaroline alone (57%; 4 of 7; $P < 0.001$) and improved even more with a combination treatment (86%; 6 of 7; $P < 0.001$). Mesenchymal stem cells reduced lung pathology score (8.5 [7.0 to 10.5] *vs.* 12.6 [12.0 to 14.0]; $P = 0.043$) and alveolar mitochondrial DNA release (0.39 (0.34 to 0.65) *vs.* 0.98 (0.76 to 1.21) ng/μl; $P = 0.025$). Mesenchymal stem cells combined with ceftaroline reduced interleukin-8 lung concentrations (665 [595 to 795] *vs.* 804 [753 to 868] pg/g of lung; $P = 0.007$) compared to ceftaroline alone.

Conclusions: In this preclinical study, mesenchymal stem cells improved the outcome of rabbits with pneumonia and high-pressure mechanical ventilation by correcting immune and mitochondrial dysfunction and when combined with the antibiotic ceftaroline was synergistic in mitigating lung inflammation.

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Community-acquired pneumonia remains the leading cause of mortality from infection, and *Streptococcus pneumoniae* is the main bacterial agent.[1] Severe pneumonia

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often requires mechanical ventilation, and high fatality rates are reported despite effective and early antibiotics, thus emphasizing the need for adjunctive therapies.[2,3] Growing evidence indicates that mitochondrial depletion and dysfunction are important events in the pathogenesis of sepsis-induced multiple organ failure and immune deficiency.[4–7] We recently demonstrated, in human alveolar epithelial cells and in healthy rabbit lungs, that cyclic stretching was likely to promote polymorphonuclear neutrophil chemotaxis and activation subsequent to the release of the so-called mitochondrial alarmins (*e.g.*, mitochondrial DNA).[8] We also showed that adverse mechanical ventilation worsened the prognosis of pneumococcal pneumonia in rabbits by causing additional lung damage (ventilator-induced lung injury) and was associated with decreased mitochondrial density within the lung and failure in mitochondrial biogenesis.[6] In addition, we observed that plasma and alveolar concentrations of cell-free mitochondrial DNA were increased in patients with acute respiratory distress syndrome (ARDS) of bacterial origin or related to SARS-CoV-2 infection.[8–10]

Because the diversity of host immune response in the setting of severe pneumonia leads to various outcomes, regardless of antibiotics and supportive care, developing personalized treatments that target relevant and critical pathophysiologic endpoints is paramount.[11,12] Accordingly, mesenchymal stem cells have been suggested as a promising adjunctive therapy because they may provide environment-dependent immunomodulatory and cell protective effects.[13] Interestingly, mesenchymal stem cells enhance alveolar epithelial and macrophage bioenergetics through mitochondrial transfer during acute lung injury.[14,15] Mesenchymal stem cells have been shown to reduce ventilator-induced lung injury in preclinical studies,[16–19] but conflicting results have been reported in preclinical in ARDS models.[20–22] Here, we hypothesized that mechanical ventilation induces immune and mitochondrial dysfunctions in rabbits with or without pneumococcal pneumonia, which can be mitigated by human umbilical cord tissue–derived mesenchymal stem cells alone or in combination with antibiotics in animals submitted to both types of injury (adverse mechanical ventilation + pneumonia).

Materials and Methods

Animals

Pathogen-free male New Zealand White rabbits (3.0 to 3.3 kg) were bred in the University of Burgundy (Dijon, France) animal facility under standard care. All experiments were performed in accordance with the local guidelines for animal experimentation. Protocols APAFIS#9858-2017051014111311v1 and APAFIS#17870-2018112915119912v1 were approved by the University Animal Care Committee (C2EA Grand Campus Dijon no. 105).

Mechanical-Ventilation Model

Under general anesthesia (27 mg/kg ketamine, 2 mg/kg xylazine), the animals were intubated under visual control,[23] put in the supine position, and connected to a volume-controlled respirator (Servo 900C, Siemens, Germany). The animals were submitted to either "adverse" mechanical ventilation (20 ml/kg tidal volume, zero end-expiratory pressure, respiratory rate of 15 breaths/min, 0.5 inspired fraction of O_2), because ventilator-induced lung injury features are obtained with such settings,[8] or "protective" mechanical ventilation (8 ml/kg tidal-volume, 5-cm H_2O positive end-expiratory pressure, respiratory rate of 35 breaths/min, 0.5 inspired fraction of O_2).[24] Only ventilated rabbits were kept anesthetized and paralyzed throughout the experiment with ketamine ($1 mg \cdot kg^{-1} \cdot h^{-1}$), midazolam ($0.2 mg \cdot kg^{-1} \cdot h^{-1}$), and cisatracurium-besilate ($0.8 mg \cdot kg^{-1} \cdot h^{-1}$; Supplemental Digital Content 1, http://links.lww.com/ALN/C752).

Experimental Pneumonia Induction

Pneumonia was induced by endobronchial challenge with 0.5 ml of a freshly calibrated bacterial inoculum (8.8 $\log_{10}$ colony-forming units/ml of the pneumococcal strain 16089; Supplemental Digital Content 1, http://links.lww.com/ALN/C752).[6,25]

Experimental Design and Therapeutic Administration

We conducted a prospective randomized animal study with three sets of experiments. Within the two first sets, the animals were allocated by random assignment into the spontaneously breathing (SB) or mechanical-ventilation groups (protective [pMV] or adverse [aMV]) in uninfected and infected animals. The rabbits were euthanized 8 or 24 h thereafter. Within the third set, including rabbits with pneumonia and adverse mechanical ventilation, the animals were randomly allocated to receive sodium chloride (control group), iv human umbilical cord tissue–derived mesenchymal stem cells (3×10^6/kg), intramuscular ceftaroline-fosamil (20 mg/kg ceftaroline), or ceftaroline + mesenchymal stem cells. Therapies were infused 4 h after inoculation, because previous work showed that significant bacteremic pneumonia had already developed by that time.[25] Ceftaroline concentrations were monitored (Supplemental Digital Content 1 and 2, http://links.lww.com/ALN/C752 and http://links.lww.com/ALN/C753).[26] Three groups were included in two different sets: SB_{H8} and SB_{H24} groups were in set 1, and the control group treated with sodium chloride were included in set 2 ($aMVP_{H24}$). Fifteen groups ($n = 7$/group) were evaluated (fig. 1).

Mesenchymal Stem Cell Preparation, Administration, and Characterization

Umbilical cord tissue–derived mesenchymal stem cells were prepared as described previously.[27] An umbilical cord was collected at Nancy Maternity Hospital from a mother who had signed an informed consent form in compliance with the French national legislation. The collection protocol was approved by the local ethics committee and the French Ministry for Research (Paris, France; no. DC-2014-2114).

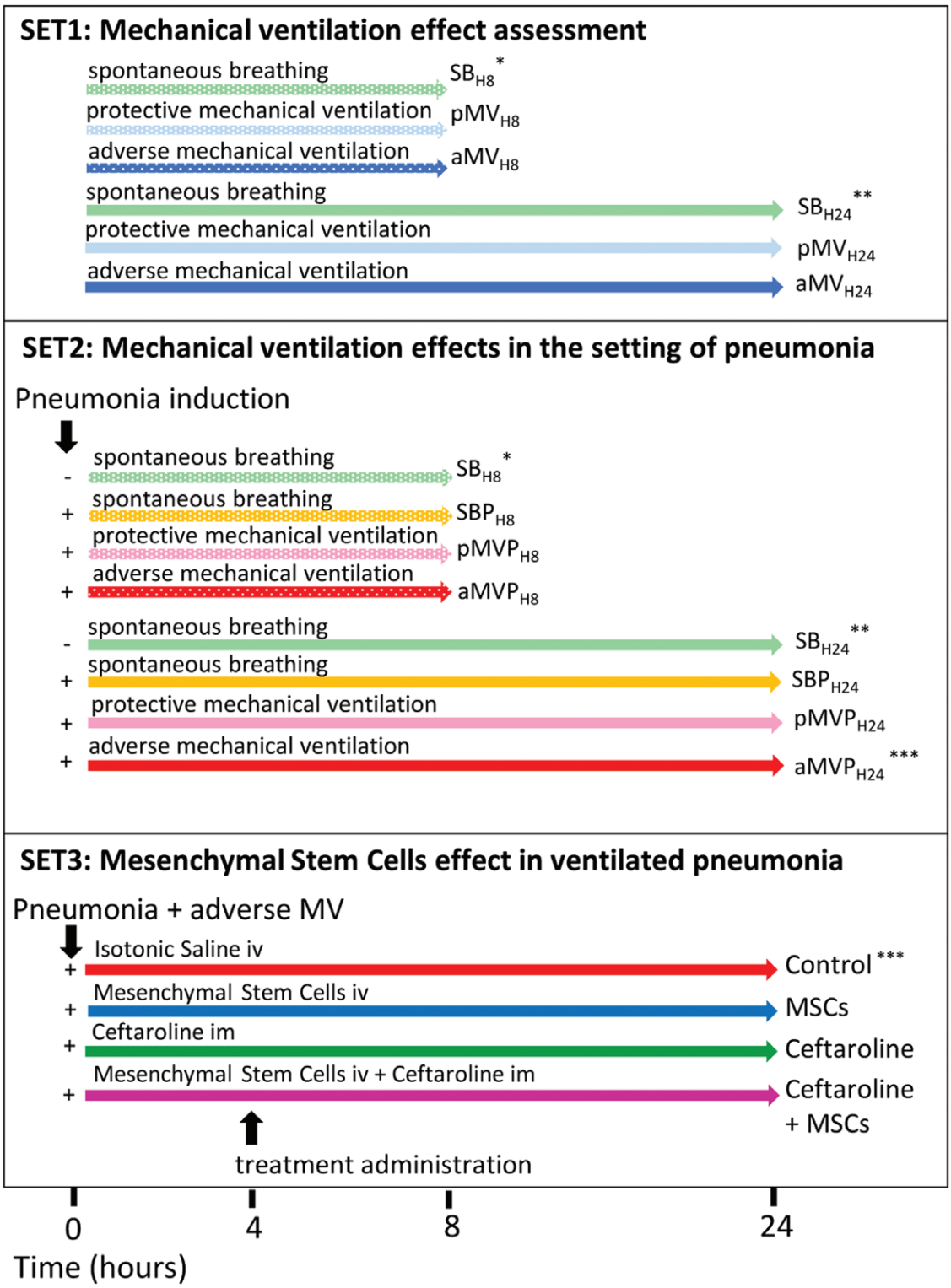

Fig. 1. Design of the three sets of experiments in rabbits. Experimental procedures were conducted in 15 groups ($n = 7$ animals/group) to assess the effect of the mechanical ventilation (*set 1*), the pneumococcal pneumonia effect and the effect of mechanical ventilation in the setting of pneumonia (*set 2*), and the effect of mesenchymal stem cells in the setting of adverse mechanical ventilation and pneumococcal pneumonia (*set 3*). *, **, *** indicate that some groups were used as comparators in two different sets. H8, euthanized after 8 h; H24, euthaniized after 24 h; im, intramuscular; iv, intravenous.

All mesenchymal stem cells were produced with clinical-grade medium, applying good manufacturing practices, and characterized (Supplemental Digital Content 1, http://links.lww.com/ALN/C752).[27] The rabbits were intravenously infused with 3×10^6 viable mesenchymal stem cells/kg in 10 ml of saline, 4 h after pneumonia induction.

Lung Injury

Lung injury evaluation was based on a macroscopic score and microscopic examination of lungs (Supplemental Digital Content 1, http://links.lww.com/ALN/C752).

Bacterial Burden

The lungs and spleen from each animal were homogenized. Bacteria were counted in a sample of this crude homogenate by plating 10-fold dilutions on sheep blood agar and incubating the plates for 24 h at 37°C. For each rabbit, the mean concentration was calculated (*e.g.*, mean concentration = Σ[organ concentration × organ weight]/Σ organ weights) and adjusted for the dilution.

Immune Response and Dysfunction

A piece of fresh lung was immediately placed into a tissue protein extraction reagent (Thermo, USA) with protease inhibitors and stored at −80°C. Total protein concentration was measured with a bicinchoninic acid protein assay (Sigma, USA) in the lung (to normalize cytokines concentrations) and in bronchoalveolar lavage fluid. Plasma and lung concentrations of interleukin-8, interleukin-1β, interleukin-10, and tumor necrosis factor-α were assessed by enzyme-linked immunosorbent assay (USCN, China).

Mitochondrial dysfunction

Mitochondrial DNA. Mitochondrial DNA was measured by quantitative polymerase chain reaction in plasma and bronchoalveolar lavage fluid (circulating cell-free mitochondrial DNA), as well as in lung and liver tissue (reflecting mitochondrial density; Supplemental Digital Content 1, http://links.lww.com/ALN/C752).[6]

Plasma and Bronchoalveolar Lavage Fluid ATP. Plasma and bronchoalveolar lavage fluid ATP concentrations were quantified using the ATP bioluminescence assay kit HS-II (Roche, Germany).

Flow Cytometry. Flow cytometry was performed using mitochondrial probes measuring mitochondrial mass, mitochondrial membrane potential, and mitochondrial radical oxygen species production in viable blood and alveolar neutrophils and alveolar macrophages. The data were acquired on a BD LSRFortessa cytometer (BD, France) and analyzed using FlowJo-v10 (USA; Supplemental Digital Content 1, http://links.lww.com/ALN/C752).

Transmission Electron Microscopy. Transmission electron microscopy was used to examine mitochondria ultrastructure from a 3-mm-wide lung sample (Supplemental Digital Content 1, http://links.lww.com/ALN/C752). Observations were made on a HITACHI H-7500 transmission electron microscope operating at 80 kV at the DImaCell core facility (INRAe/Dijon, France).

Lung Transcriptome

Total RNA was extracted using the RNA GenElute kit (Sigma), and RNA quality was assessed on an Agilent Bioanalyzer 2100. Directional RNA-sequencing libraries were constructed using the TruSeq mRNA Stranded library prep kit (Illumina). Libraries were pooled in equimolar proportions and sequenced in single-read 75-bp runs on an Illumina NextSeq500 instrument, using NextSeq 500 high-output 75 cycle kits. The reads were mapped on the rabbit genome (OryCun-v2.0 Ensembl, release 90) with TopHat2, v2.1.1 and counted using subreads featureCounts, v1.5.2. The RNA-sequencing data were submitted to the Gene Expression Omnibus (GSE173238; Supplemental Digital Content 1, http://links.lww.com/ALN/C752).

Statistical Analysis

Sample size was not calculated but based on our past experience. Except for the evaluation of the lung pathology score and alveolar neutrophils count, the analyses were not blinded. One rabbit was excluded in the pMV_{H8} group for technical reasons. The data are expressed as box-and-whisker plots. Outliers were identified with the Rout method ($Q = 1\%$), and if present, the results were reported for data including and excluding the outliers. Each judgment criteria was examined with one animal as the unit of analysis. Comparisons between multiple groups were performed using the Kruskal–Wallis analysis of variance test. To account for multiple comparisons, the *P* value was adjusted using Dunn's test. Then 8- and 24-h groups were analyzed separately, and the following comparisons were considered for set 1 (aMV *vs.* SB, pMV *vs.* SB, aMV *vs.* pMV), set 2 (SBP *vs.* SB, aMVP *vs.* pMVP, aMVP *vs.* SBP, aMVP *vs.* SB), and set 3 (mesenchymal stem cells *vs.* control, ceftaroline *vs.* control, ceftaroline + mesenchymal stem cells *vs.* ceftaroline, ceftaroline+mesenchymal stem cells *vs.* control). For repeated measures in plasma samples, the groups were compared only for the 24-h time point. Correlations were analyzed with Spearman's rank test. The cumulative probability of progression to death was compared between groups using the Kaplan–Meier method and the log-rank test. All tests were two-tailed. A *P* value lower than 0.05 was considered statistically significant. The data were analyzed with Prism software (GraphPad Prism). For RNA-sequencing analysis, the methods are explained in Supplemental Digital Content 1, http://links.lww.com/ALN/C752.

Availability of Supporting Data

All of the data are available on demand. The RNA-sequencing data are available in Supplemental Digital

Content 3 (http://links.lww.com/ALN/C754) and 4 (http://links.lww.com/ALN/C755) and have been submitted to the Gene Expression Omnibus (accession number GSE173238)

Results

Effect of Mechanical Ventilation on Lung Damage, Inflammation, and Mitochondrial Dysfunction

We sought to establish the pulmonary and systemic effects of either protective or adverse mechanical ventilation in healthy rabbits (fig. 1, set 1). Both mechanical-ventilation settings were apparently safe because all animals remained alive, without any macroscopic lung damage, while the temperature, blood pH, and lactate level remained unchanged (Supplemental Digital Content 2, fig. S1, A, B, E, and F, http://links.lww.com/ALN/C753). The aMV_{H24} regimen led to gas exchange enhancement (Supplemental Digital Content 2, fig. S1, C and D, http://links.lww.com/ALN/C753).

However, aMV_{H24} induced microscopic features of lung injury ($P = 0.077$ with outlier, $P = 0.042$ without outlier), along with increase in alveolar neutrophils count ($P < 0.001$), interleukin-8 lung concentrations ($P = 0.018$), compared to SB_{H24} (fig. 2, A to D), with no significant increase in lung tumor necrosis factor-α, interleukin-1β, and interleukin-10, alveolar total protein, and plasma interleukin-8 and interleukin-1β concentrations (fig. 2E; Supplemental Digital Content 2, fig. S2, A to G, http://links.lww.com/ALN/C753).

The aMV_{H24} regimen increased alveolar mitochondrial DNA release (aMV_{H24} *vs.* pMV_{H24}; $P = 0.042$) but not alveolar ATP (fig. 3, A and B). Mitochondrial density was statistically nonsignificantly decreased within the lung of rabbits submitted to aMV_{H24}, compared to SB_{H24} (0.6 [0.5 to 0.8] *vs.* 0.9 [0.7 to 1.5] of fold change NADH-I/Gapdh; $P = 0.231$; fig. 3C). Transmission electron microscopy images of lung tissue showed swollen and fewer mitochondria in animals under mechanical ventilation than in animals with SB (fig. 3D). Alveolar neutrophils displayed an increase in mitochondrial membrane potential in aMV_{H24} animals compared to SB_{H24} animals when assessed with tetramethylrhodamine-methyl-ester ($P = 0.003$) but not with MitoTracker Red/Green (Thermo, USA; fig. 3, E and F). Conversely, alveolar macrophages displayed a decrease in mitochondrial membrane potential in pMV_{H24} animals, compared to SB_{H24} ($P = 0.046$; Supplemental Digital Content 2, fig. S3, A and B, http://links.lww.com/ALN/C753). Mitochondrial membrane potential and reactive oxygen species in blood neutrophils, plasma mitochondrial DNA and ATP concentrations, and mitochondrial liver density remained unchanged (fig. 3, G to J; Supplemental Digital Content 2, fig. S3, C and D, http://links.lww.com/ALN/C753).

Effect of Mechanical Ventilation in the Setting of Pneumococcal Pneumonia on Outcomes, Lung Damage, Immune Response, and Mitochondrial Dysfunction

Survival was dramatically lower in infected animals submitted to aMV_{H24} (0%; 0 of 7) compared to $pMVP_{H24}$ (86%; 6 of 7; $P < 0.001$), or SBP_{H24} (100%; 7 of 7; $P < 0.001$; fig. 4A). $aMVP_{H24}$ animals also had profound alterations in gas exchange, body temperature, and lactic acidosis (fig. 4, B to D; Supplemental Digital Content 2, fig. S4, A and B, http://links.lww.com/ALN/C753) and more severe lung damage (fig. 4E; Supplemental Digital Content 2, fig. S4C, http://links.lww.com/ALN/C753). $aMVP_{H24}$ animals displayed a statistically nonsignificant higher pulmonary bacterial concentrations as compared to $pMVP_{H24}$ animals (7.1 [5.0 to 7.2] *vs.* 4.0 [3.5 to 5.7] logCFU/g of lung; $P = 0.186$), and systemic bacterial translocation was only observed when rabbits were submitted to adverse mechanical ventilation (fig. 4, F and G). $aMVP_{H24}$ led to higher a lung pathology score (5.5 [4.5 to 7.0] *vs.* 12.6 [12.0 to 14.0]; $P = 0.046$), interleukin-8 lung concentrations (106 [54 to 316] *vs.* 804 [753 to 868] pg/g of lung; $P = 0.012$), greater alveolar neutrophils count, higher tumor necrosis factor-α and lower interleukin-10 concentrations compared to SBP_{H24} (fig. 5, A to D; Supplemental Digital Content 2, fig. S5, A to E, http://links.lww.com/ALN/C753). In the systemic compartment, $aMVP_{H24}$ led to a sustained drop in leukocyte count and higher interleukin-8 concentrations when compared to SBP_{H24}, with no change in plasma interleukin-1β (fig. 5E; Supplemental Digital Content 2, fig. S5, F and G, http://links.lww.com/ALN/C753).

Moreover, $aMVP_{H24}$ had higher alveolar mitochondrial DNA concentrations, compared to SB_{H24} (0.33 [0.28 to 0.36] *vs.* 0.98 [0.76 to 1.21] ng/μl; $P < 0.001$), SBP_{H24} ($P = 0.003$), and $pMVP_{H24}$ animals ($P = 0.007$), and higher concentrations of alveolar ATP compared to SBP_{H24} ($P = 0.007$; fig. 6 A and B). Mitochondrial density was significantly lower within the lung tissue of SBP_{H24} compared to SB_{H24} animals ($P = 0.041$; fig. 6C). Accordingly, swollen mitochondria were found in the alveolar cells of infected rabbits, especially within the $aMVP_{H24}$ group (fig. 6D). Alveolar neutrophils displayed an early increase in mitochondrial membrane potential in all infected animals, especially in the aMV groups (MitoTracker Red/Green; $pMVP_{H8}$ *vs.* $aMVP_{H8;}$ $P = 0.017$; fig. 6, E and F). In contrast, alveolar macrophages exhibited decreased mitochondrial membrane potential values upon infection, regardless of mechanical ventilation (MitoTracker Red/Green; SB_{H24} *vs.* $aMVP_{H24;}$ $P = 0.031$; Supplemental Digital Content 2, fig. S6, A and B, http://links.lww.com/ALN/C753).

In the systemic compartment, infection led to a decrease in mitochondrial membrane potential, whereas aMV led to an increase in reactive oxygen species production in blood neutrophils (fig. 6, G and H; Supplemental Digital Content 2, fig. S6C, http://links.lww.com/ALN/C753).

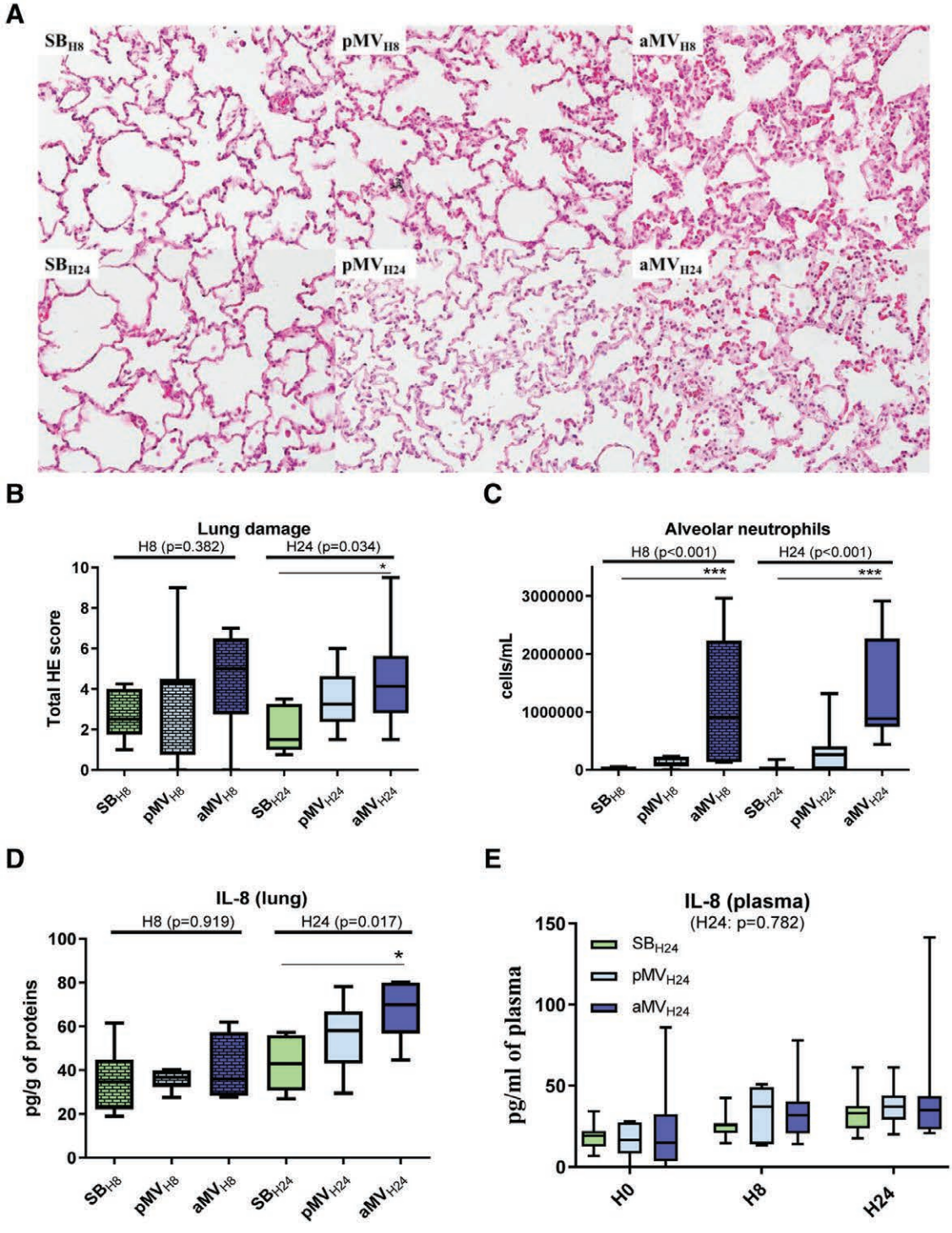

Fig. 2. Adverse mechanical ventilation causes mild lung damage and inflammation, in the pulmonary compartment. Only the adverse mechanical ventilation 24 hours (aMV_{H24}) regimen induced significant lung damage scored on hematoxylin-eosin (HE)–stained lung tissue sections (*A* and *B*), an increase in neutrophil count within the alveolar compartment (*C*), and interleukin-8 (IL-8) pulmonary concentrations at 24 h in rabbits ($n = 7$ animals/group, excepted for protective mechanical ventilation 8 hours (pMV_{H8}): $n = 6$ animals; *D*). Blood samples were obtained at baseline (H0) and at H8 and H24 to measure (*E*) plasma concentrations of interleukin-8. The data are expressed as box-and-whisker diagrams. The Kruskal–Wallis test was performed (*P* value reported for each time point in the figure), and the Dunn's *post hoc* correction for multiple comparisons was used when appropriate: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$. aMV_{H8}, adverse mechanical ventilation 8 hours; pMV_{H24}, protective mechanical ventilation 24 hours; SB_{H8}, spontaneous breathing 8 hours; SB_{H24}, spontaneous breathing 24 hours.

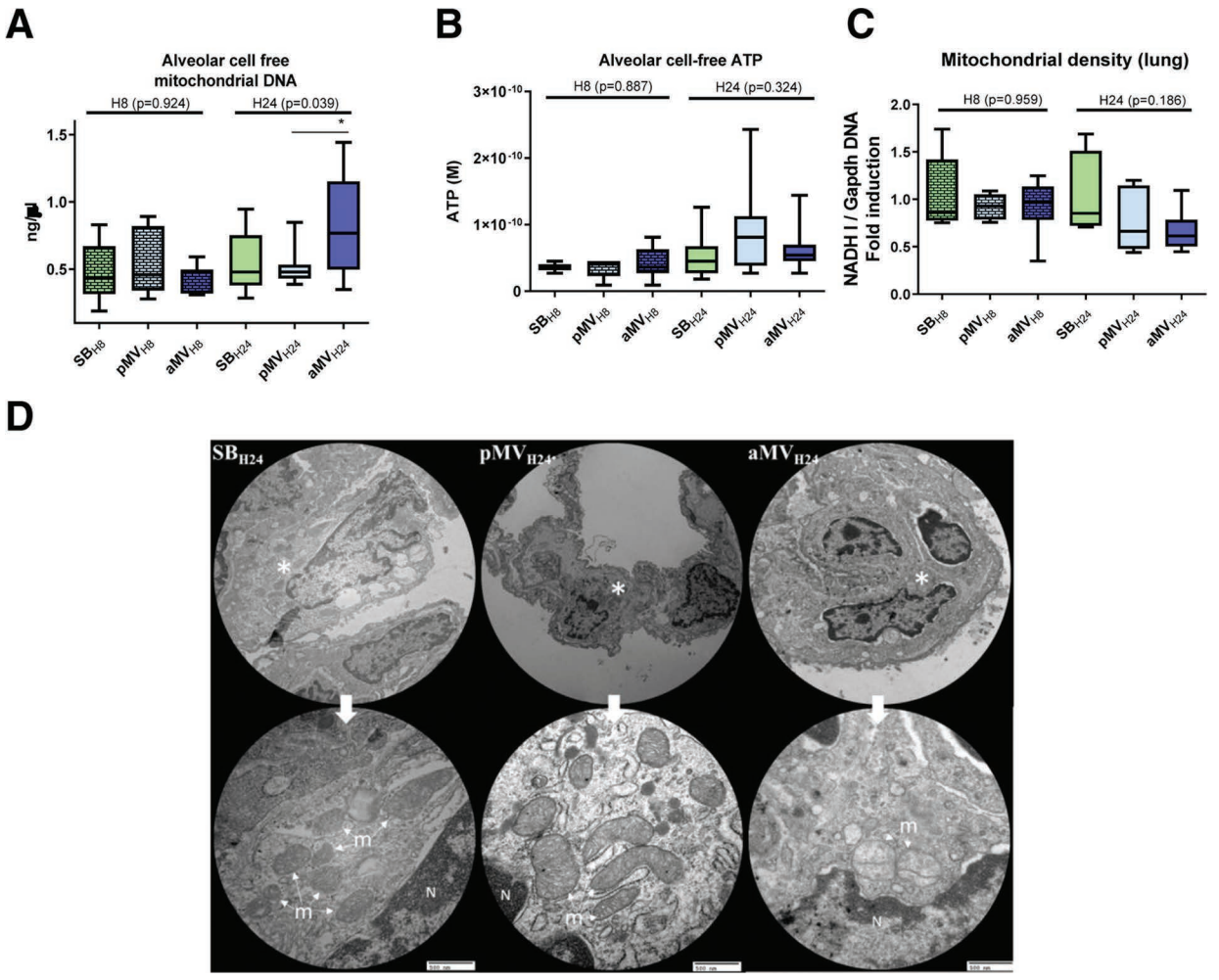

Fig. 3. Adverse mechanical ventilation causes mild mitochondrial derangements in the pulmonary compartment. (*A*, *B*) Alveolar cell-free mitochondrial DNA levels (NADH I) were significantly higher in adverse mechanical ventilation 24 hours (aMV_{H24}) as compared to protective mechanical ventilation 24 hours (pMV_{H24}) rabbits (*A*; $P = 0.077$ with one outlier in the pMV_{H24} group, $P = 0.042$ without the outlier) but not alveolar cell-free adenosine triphosphate (ATP) levels (*B*). (*C*) Mitochondrial density (NADHI/Gapdh DNA ratio) was nonsignificantly decreased in aMV_{H24} animals as compared to spontaneous breathing 24 hours (SB_{H24}) animals. (*D*) Representative transmission electron microscopy images of alveolar epithelial type II cells showed swollen mitochondria (m), with reduced density and clustered around the nucleus (N) in aMV_{H24} animals as compared to SB_{H24} animals. The *top row* shows global overviews of the alveolar epithelial type-II cells, and the *bottom row* shows ×50,000 magnification of the areas represented with *white asterisks*. (*E*, *F*) Using flow cytometry and mitochondrial fluorescent probes, we observed an increase in mitochondrial membrane potential of alveolar neutrophils in aMV_{H24} animals using MitoTracker Red/Green (Thermo, USA) ratio (*E*) and tetramethylrhodamine-methyl-ester (TMRM) median fluorescence intensity (MFI; *F*). (*G*, *H*) Mitochondrial membrane potential of blood neutrophils was measured by flow cytometry and using MitoTracker Red/Green ratio (*G*) or tetramethylrhodamine-methyl-ester median fluorescence intensity of blood neutrophils (*H*) were measured by flow cytometry. Blood samples were obtained at baseline (H0) and at H8 and H24 to measure mitochondrial alarmins. (*I*, *J*) Cell-free mitochondrial DNA (NADH I) by quantitative polymerase chain reaction (*I*) and ATP by bioluminescence assay (*J*). We used $N = 7$ animals/group, except for pMV_{H8}, for which $n = 6$ animals. The data are expressed as box-and-whisker diagrams. The Kruskal–Wallis test was performed (*P* value reported for each time point in the figure), and the Dunn's *post hoc* correction for multiple comparisons was used when appropriate: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$. aMV_{H8}, adverse mechanical ventilation 8 hours; pMV_{H8}, protective mechanical ventilation 8 hours; SB_{H8}, spontaneous breathing 8 hours; SB_{H24}, spontaneous breathing 24 hours. (*Continued*)

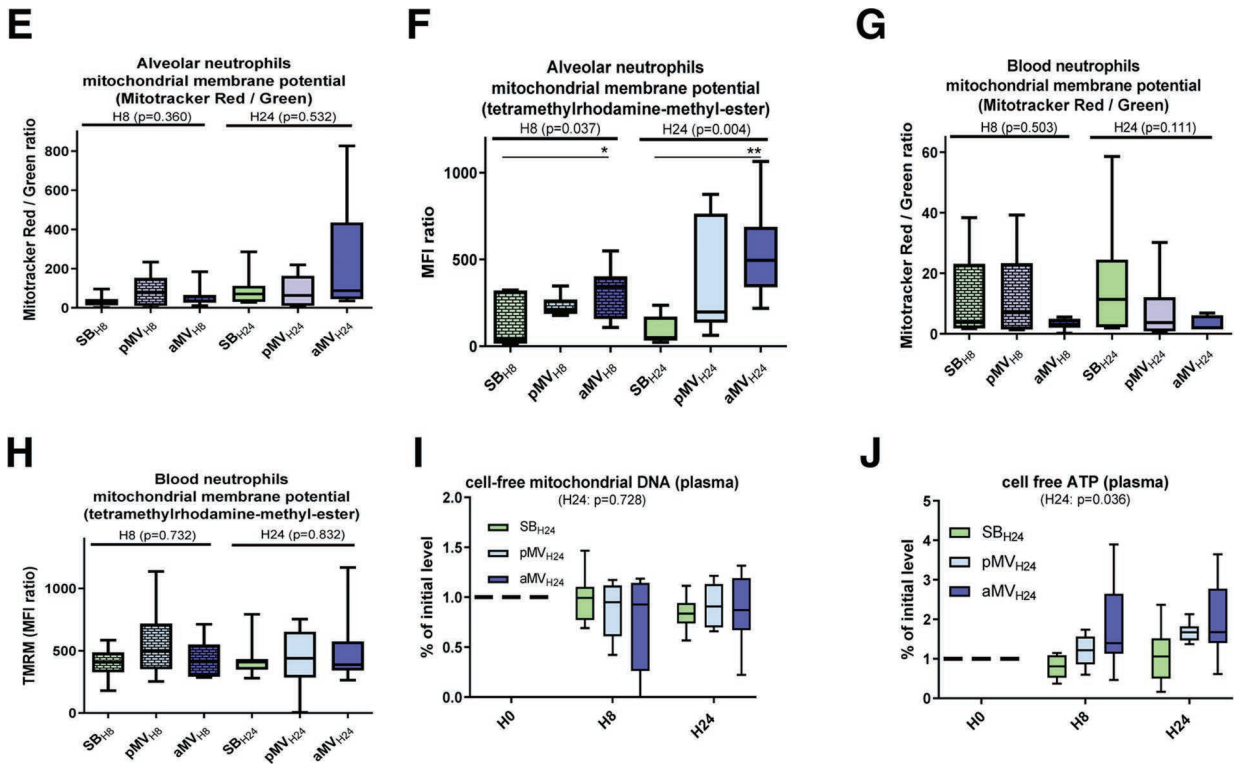

Fig. 3. (*Continued*)

In addition, the concentrations of mitochondrial alarmins dropped in the $aMVP_{H24}$ group, while they rose in the SBP_{H24} (fig. 6, I and J). Liver mitochondrial density was significantly lower in $aMVP_{H24}$ animals compared to SB_{H24} animals (Supplemental Digital Content 2, fig. S6D, http://links.lww.com/ALN/C753).

Effect of Mesenchymal Stem Cells Alone or as an Adjunctive Therapy to Ceftaroline in Animals Submitted to Adverse Mechanical Ventilation and Pneumococcal Pneumonia

Infected rabbits submitted to aMV were randomized to receive, 4 h after the bacterial challenge: sodium chloride, human umbilical cord–derived mesenchymal stem cells, ceftaroline, or both ceftaroline + mesenchymal stem cells. Plasma ceftaroline concentrations were similar in the two last groups (Supplemental Digital Content 2, fig. 7A, A and B, http://links.lww.com/ALN/C753). First, 24-h survival was dramatically improved by mesenchymal stem cells or ceftaroline administration (both 57% [4 of 7] *vs.* 0% [0 of 7] for the control group, respectively; $P = 0.001$ and $P < 0.001$) and reached its highest level when both treatments were given (86% [6 of 7] *vs.* 0% [0 of 7]; $P < 0.001$; fig. 7A). Compared to the control group, the combined therapy alleviated lactacidemia ($P = 0.023$) and reduced lung damage ($P = 0.010$; fig. 7, B and E; Supplemental Digital Content 2, fig. S8, A to C, http://links.lww.com/ALN/C753). Pulmonary ($P < 0.001$) bacterial clearance was improved (fig. 7, F and G), whereas total lung pathology score ($P = 0.007$), alveolar neutrophils count, and both pulmonary ($P = 0.017$) and plasma interleukin-8 ($P = 0.042$) concentrations were reduced (fig. 8, A to E). In addition, the alveolar release of mitochondrial DNA was decreased ($P = 0.001$) but not that of ATP (fig. 9, A and B), while the mitochondrial membrane potentials of alveolar neutrophils ($P = 0.017$) and macrophages were increased ($P = 0.041$; fig. 9, E and F; Supplemental Digital Content 2, fig. S10, A and B, http://links.lww.com/ALN/C753). In the systemic compartment, the combined therapy was associated with enhanced mitochondrial membrane potential in circulating neutrophils (tetramethylrhodamine-methyl-ester, $P = 0.012$; fig. 9, G and H) and rising levels of mitochondrial DNA and ATP (fig. 9, I and J).

The respective effects of ceftaroline and mesenchymal stem cells were also addressed. Ceftaroline alone, although the pulmonary bacterial burden was reduced ($P = 0.004$), increased tumor necrosis factor-α ($P = 0.004$) and interleukin-10 ($P = 0.001$) lung concentrations (fig. 7F; Supplemental Digital Content 2, fig. S9, A and C, http://links.lww.com/ALN/C753), without improving the mitochondrial membrane potential of alveolar or circulating neutrophils (fig. 9, E to H). By itself, mesenchymal stem cells infusion improved gas

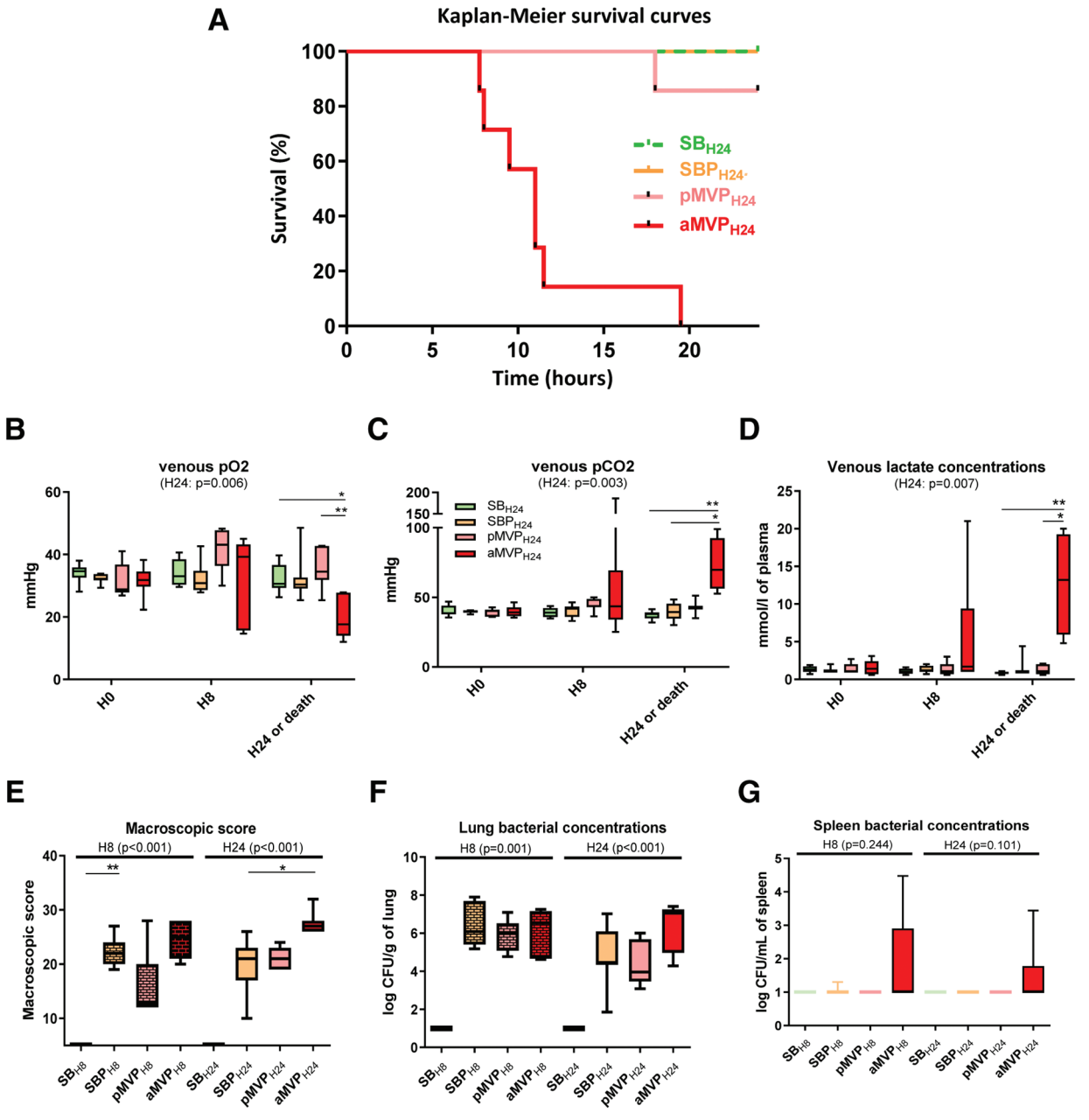

Fig. 4. Adverse mechanical ventilation worsens the prognosis of pneumococcal pneumonia. (*A*) Time-dependent probability of survival. Shown are the Kaplan–Meier survival curves from log rank tests: adverse mechanical ventilation plus pneumonia 24 hours ($aMVP_{H24}$) vs. protective mechanical ventilation plus pneumonia 24 hours ($pMVP_{H24}$; $P < 0.001$); $aMVP_{H24}$ vs. spontaneous breathing 24 hours (SB_{H24}; $P < 0.001$); $aMVP_{H24}$ vs. spontaneous breathing plus pneumonia 24 hours (SBP_{H24}; ($P < 0.001$). (*B* to *D*) The $aMVP_{H24}$ regimen led to profound changes in gas exchange: venous Po_2 (*B*) and carbon dioxide (*C*), as well as lactic acidosis (*D*) compared to $pMVP_{H24}$ or SBP_{H24} animals. (*E*, *F*) As compared to the $pMVP_{H24}$, the $aMVP_{H24}$ regimen led to a statistically nonsignificant higher lung injury according to macroscopic score calculation (*E*; 21 [19 to 23] *vs.* 27 [26 to 28]; $P = 0.080$) and bacterial lung concentrations (*F*; 4.0 [3.5 to 5.7] *vs.* 7.1 [5.0 to 7.2] log colony-forming units (CFU)/g; $P = 0.186$). (*G*) Pulmonary-to-systemic bacterial translocation was only observed in $aMVP_{H24}$ animals. The data are expressed as box-and-whisker diagrams. The Kruskal–Wallis test was performed (*P* value reported for each time point in the figure), and the Dunn's *post hoc* correction for multiple comparisons was used when appropriate: $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$. $aMVP_{H8}$, adverse mechanical ventilation plus pneumonia 8 hours; $pMVP_{H8}$, protective mechanical ventilation plus pneumonia 8 hours; SB_{H8}, spontaneous breathing 8 hours; SBP_{H8}, spontaneous breathing plus pneumonia 8 hours.

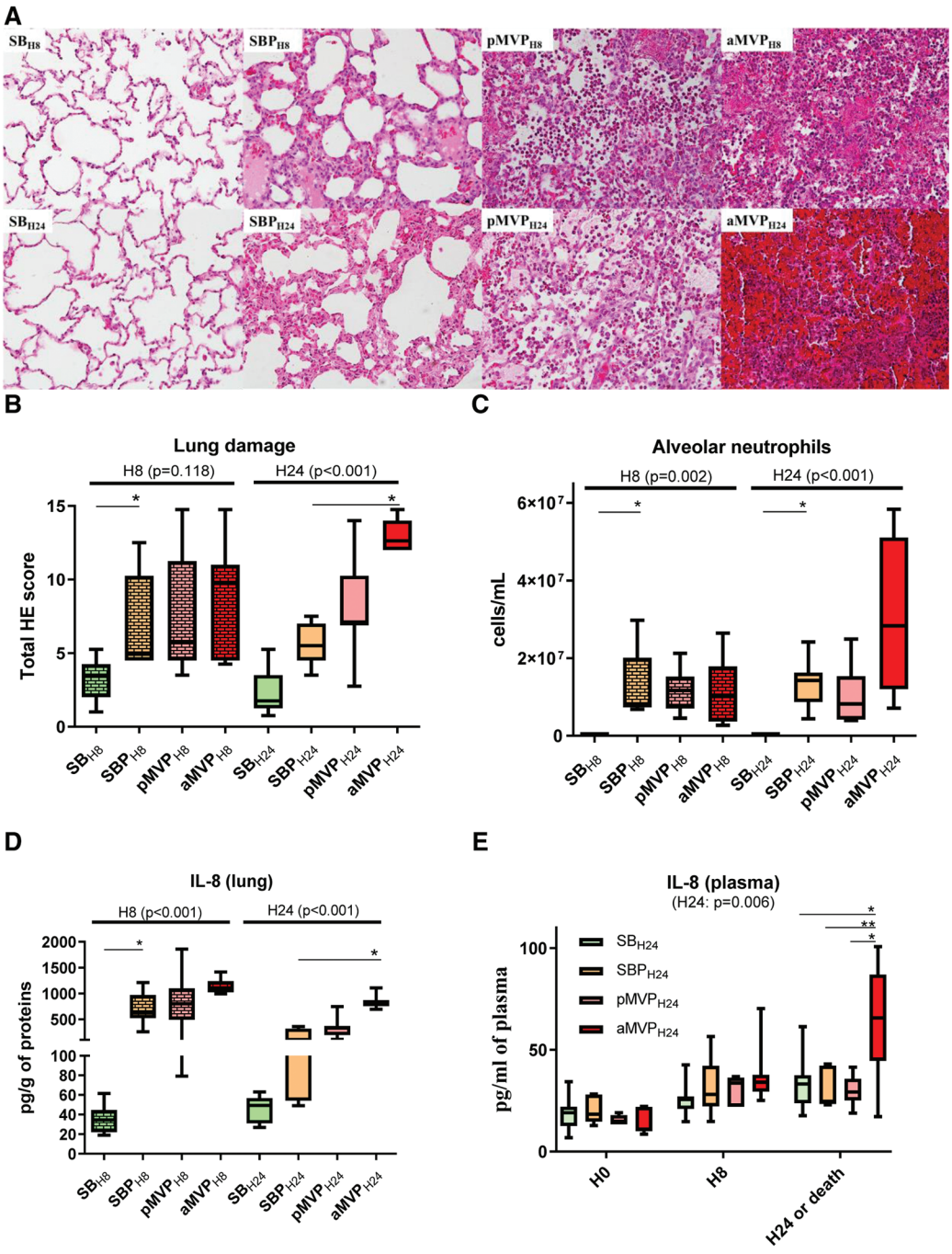

Fig. 5. Adverse mechanical ventilation was associated with an exacerbation of pulmonary and systemic inflammation during pneumococcal pneumonia. (*A*, *B*) The adverse mechanical ventilation plus pneumonia 24 hours ($aMVP_{H24}$) regimen led to significantly higher lung injury according to a pathology score (extent of inflammation scored on hematoxylin-eosin (HE)–stained tissue sections). (*C*) Polymorphonuclear cells with the alveolar compartment. (*D*) Interleukin-8 pulmonary concentrations. (*E*) Plasma concentrations. The data are expressed as box-and-whisker diagrams. The Kruskal–Wallis test was performed *P* value reported for each time point in the figure), and the Dunn's *post hoc* correction for multiple comparisons was used when appropriate: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$. aMV_{H8}, adverse mechanical ventilation 8 hours; aMV_{H24}, adverse mechanical ventilation 24 hours; $aMVPH8_{H8}$, adverse mechanical ventilation + pneumonia 8 hours; MSC, mesenchymal stem cell; pMV_{H8}, protective mechanical ventilation 8 hours; pMV_{H24}, protective mechanical ventilation 24 hours; $pMVP_{H8}$, protective mechanical ventilation plus pneumonia 8 hours; $pMVP_{H24}$, protective mechanical ventilation plus pneumonia 24 hours; SB_{H8}, spontaneous breathing 8 hours; SB_{H24}, spontaneous breathing 24 hours; SBP_{H8}, spontaneous breathing plus pneumonia 8 hours; SBP_{H24}, spontaneous breathing plus pneumonia 24 hours.

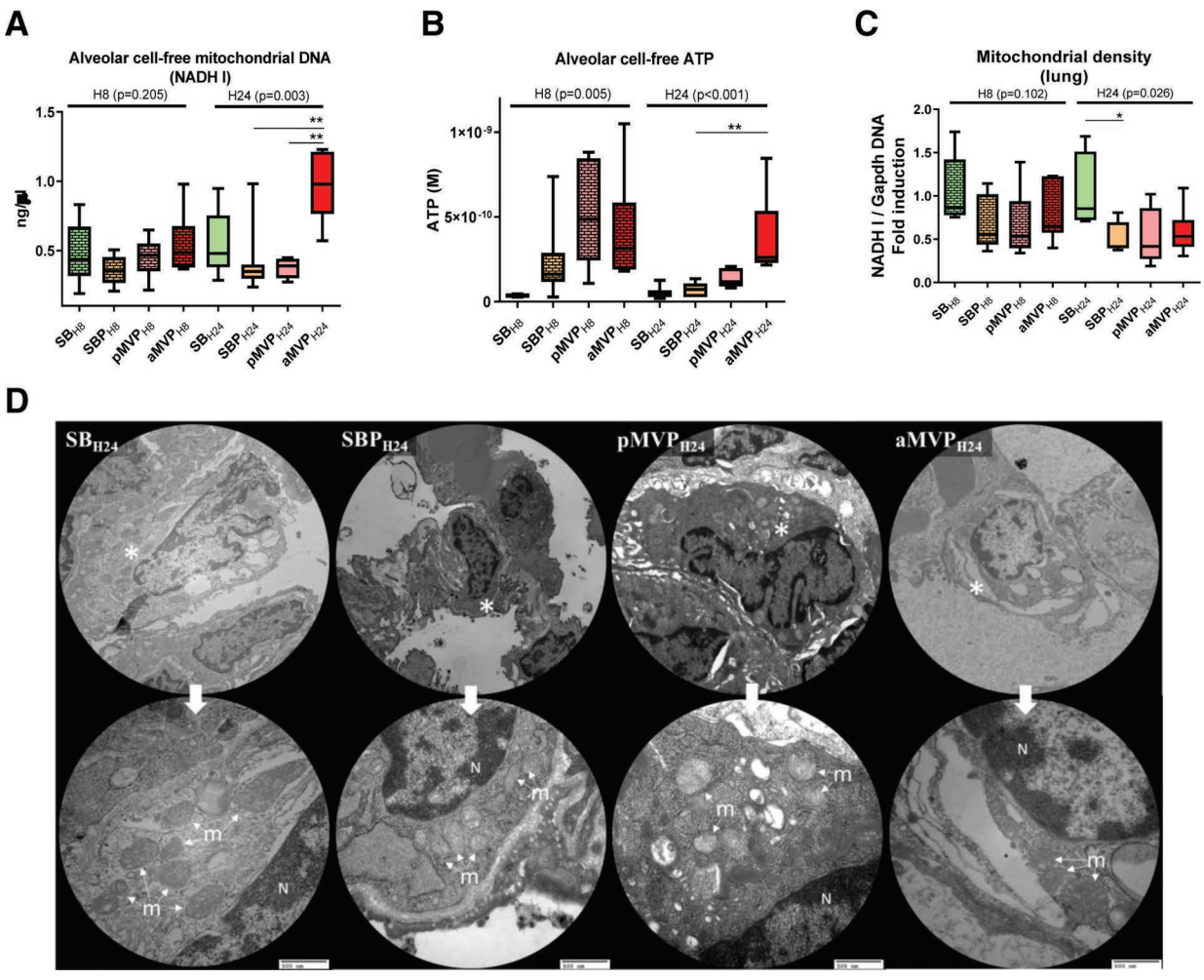

Fig. 6. In the setting of pneumococcal pneumonia, adverse mechanical ventilation is associated with mitochondrial derangements in both the pulmonary and the systemic compartments. (*A*, *B*) Compared to protective mechanical ventilation plus pneumonia 24 hours ($pMVP_{H24}$), adverse MVP_{H24} ($aMVP_{H24}$) animals exhibited larger amounts of cell-free mitochondrial DNA (*A*; NADH I, quantitative polymerase chain reaction) and cell-free adenosine triphosphate (ATP; *B*; bioluminescence assay) within the alveolar space. (*C*) Pulmonary mitochondrial DNA levels reflecting mitochondrial density (NADH I/Gapdh fold induction) were significantly lower in infected animals than in noninfected animals ($P = 0.041$). (*D*) Representative transmission electron microscopy images of alveolar epithelial type II cells of spontaneous breathing plus pneumonia 24 hours (SBP_{H24}) animals: mildly swollen mitochondria (m) with preserved density; $pMVP_{H24}$: swollen mitochondria with relatively preserved density; and $aMVP_{H24}$: swollen mitochondria with reduced density compared to the spontaneous breathing 24 hours (SB_{H24}) group. The *top row* shows global overview of the alveolar epithelial type-II cells, and the *bottom row* shows 50,000× magnification of the areas represented with *white asterisks*. (*E*, *F*) Alveolar neutrophils displayed an early and persistent increase in mitochondrial membrane potential in all infected animals, earlier in the aMVP 8 hours ($aMVP_{H8}$) than in the $pMVP_{H8}$ group. (*E*) Mitrotracker Red/Green (Thermo, USA). (*F*) Tetramethylrhodamine-methyl-ester (TMRM). (*G*, *H*) Blood neutrophils displayed a decrease in mitochondrial membrane potential in all infected animals. (*G*) Mitrotracker Red/Green ($P = 0.014$ at H24). (*H*) Tetramethylrhodamine-methyl-ester ($P = 0.166$ at H24). (*I*, *J*) Plasma cell-free mitochondrial DNA level concentrations (*I*; NADH I; quantitative polymerase chain reaction) and ATP concentrations increased continuously in SBP_{H24} animals (*J*), while they dropped in the $aMVP_{H24}$ group. The data are expressed as box-and-whisker diagrams ($n = 7$ animals/group). The Kruskal–Wallis test was performed (*P* value reported for each time point in the figure), and the Dunn's *post hoc* correction for multiple comparisons was used when appropriate. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$. aMV_{H24}, adverse mechanical ventilation 24 hours; MFI, median fluorescence intensity; N, nucleus; pMV_{H24}, protective mechanical ventilation 24 hours; SB_{H8}, spontaneous breathing 8 hours; SBP_{H8}, spontaneous breathing plus pneumonia 8 hours. (*Continued*)

exchange, reduced lung damage ($P = 0.027$), and led to a statistically nonsignificant lower lactacidemia, if compared to the control group (1.7 [0.8 to 11.7] *vs.* 13.2 [8.25 to 20.25] mmol/l; $P = 0.213$; fig. 7, B to E). In addition, mesenchymal stem cells statistically nonsignificantly reduced pulmonary bacterial concentrations by 1.4 log (5.7 [4.2 to 5.7] *vs.* 7.1 [5.0 to 7.2] logCFU/g of lung; $P = 0.908$) and prevented systemic translocation (fig. 7, F and G). Interestingly, mesenchymal stem cells alone reduced lung pathology ($P = 0.043$), without reducing pulmonary interleukin-8

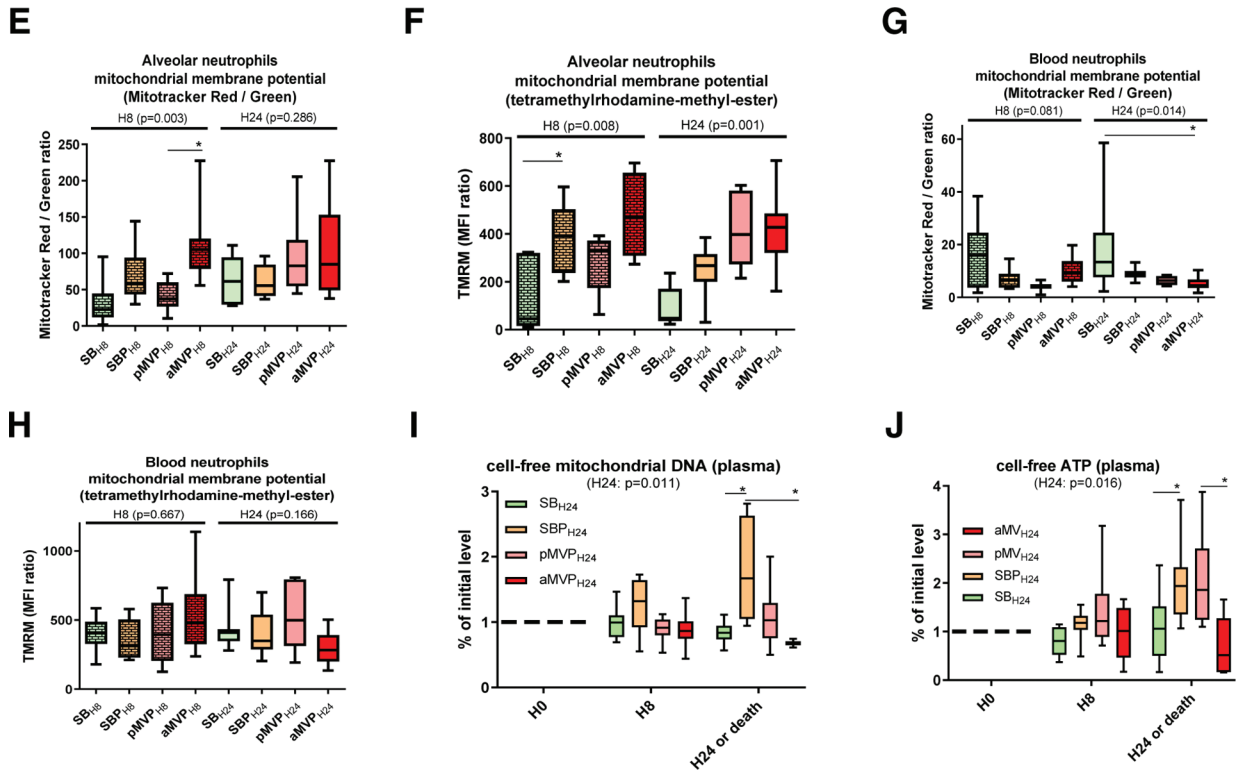

Fig. 6. (*Continued*)

and tumor necrosis factor-α and alveolar total protein concentrations (fig. 8, A, B, and D; Supplemental Digital Content 2, fig. S9E, http://links.lww.com/ALN/C753). However, the addition of mesenchymal stem cells with ceftaroline, mitigated lung concentrations of interleukin-8 (665 [595 to 795] *vs.* 804 [753 to 868] pg/g of lung; P = 0.007) and tumor necrosis factor-α (P = 0.008), as compared to ceftaroline alone (fig. 8D; Supplemental Digital Content 2, fig. S9A, http://links.lww.com/ALN/C753). The infusion of mesenchymal stem cells reduced alveolar mitochondrial DNA release (0.39 [0.34 to 0.65] *vs.* 0.98 [0.76 to 1.21] ng/μl; P = 0.025) and increased mitochondrial membrane potential in alveolar neutrophils and macrophages (fig. 9, A and E; Supplemental Digital Content 2, fig. S10A, http://links.lww.com/ALN/C753), as well as in circulating neutrophils (MitoTracker Red/Green P = 0.058 with outlier and P = 0.046 without outlier; fig. 9G). While no pulmonary mitochondrial density recovery was obtained, swollen mitochondria were no longer seen in the treated animals in contrast to controls (fig. 9D).

Finally, pulmonary gene expression was analyzed in an attempt to elucidate the beneficial effects of mesenchymal stem cells. Whereas the transcriptomic response of the lungs to both types of damage was strong (Supplemental Digital Content 2, fig. S12, http://links.lww.com/ALN/C753), it was found to be relatively low when either mesenchymal stem cells or ceftaroline was administered in $aMVP_{H24}$ animals (fig. 10, D and G). However, the mesenchymal stem cell infusion alone was associated with an upregulation of metabolic and mitochondrial pathways and a downregulation of the stress and inflammatory responses (fig. 10, B and C).

Discussion

Herein, in a rabbit model of 24-h mechanical ventilation, we showed that lung stretch worsened pneumococcal pneumonia, leading to uncontrolled infection and severe lung damage subsequent to ventilator-induced lung injury. Our findings emphasize the extent to which adverse ventilatory settings could promote profound immune and mitochondrial dysfunction, which would likely compromise the host's ability to clear bacterial pathogens, dramatically increasing the risk of death. In this context, mesenchymal stem cells administration markedly improved pneumonia outcomes through significant modulations in host response and metabolic and mitochondrial homeostasis, acting synergistically with antibiotics.

In line with previous findings, our data emphasize the importance of neutrophils in both severe pneumonia and ventilator-induced lung injury pathogenesis.[2,28] We recently demonstrated that the cyclic stretch of human alveolar epithelial cells and of healthy rabbit lungs was

likely to promote neutrophils chemotaxis and activation subsequently to the release of mitochondrial alarmins.[8] In addition, we showed that the higher the levels of cell-free mitochondrial DNA in the bronchoalveolar lavage fluid from ARDS patients, the more neutrophilic it was.[8,9] The current study provides further insights as we show that superimposed infection results in greater amounts of mitochondrial DNA and ATP in the alveolar compartment. This can be explained by the ultrastructural mitochondrial damage observed in the alveolar cells of infected animals, particularly upon mechanical ventilation.[29] In addition to *S. pneumoniae* by itself, one could assume that extracellular mitochondrial DNA stimulates interleukin-8 secretion through Toll-like receptor-9 activation, and ATP acts as a pro-inflammatory signal for neutrophils through their purinergic receptors, leading in turn to chemotaxis and activation, ultimately contributing to lung injury.[8,29,30] In line with this hypothesis, it was shown that Toll-like receptor-9 blockade could protect against ventilator-induced lung injury in a short-term mechanical-ventilation rodent model.[31]

Second, we found that neutrophils harbored mitochondria with contrasted activation levels in terms of mitochondrial membrane potential, depending on the compartment (*i.e.*, alveolar *vs.* blood). The strong mitochondrial membrane potential in alveolar neutrophils could be associated with delayed mitochondrial apoptosis, potentially contributing to their accumulation and activation of neutrophils in the lung, leading in turn to tissue injury and extrapulmonary organ failure.[32,33] In contrast,

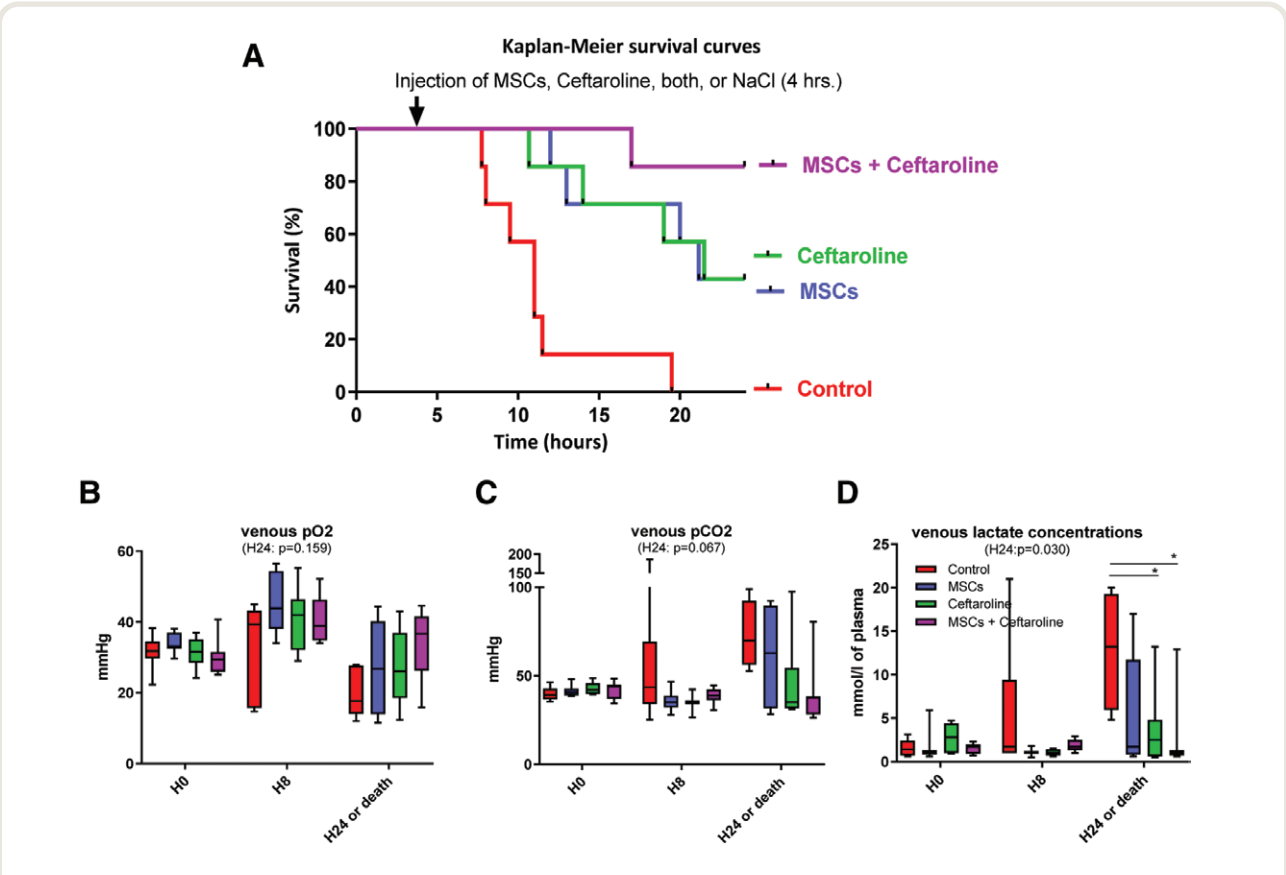

Fig. 7. Mesenchymal stem cells (MSCs) improved pneumococcal ventilated pneumonia outcome. (*A*) The 24-h survival was dramatically improved by mesenchymal stem cells (Kaplan–Meier survival curves 57% *vs.* 0%; log rank test: $P = 0.001$) or ceftaroline treatment (57% *vs.* 0%; $P < 0.001$) compared to the control group but was the higher when both treatments were combined (86% *vs.* 0%; $P < 0.001$). There were no significant differences between ceftaroline and ceftaroline + mesenchymal stem cell groups (57% *vs.* 96%; $P = 0.111$). (*B*, *C*) The combined therapy (ceftaroline + mesenchymal stem cells) nonsignificantly improved gas exchange. (*B*) Po_2. (*C*) Carbon dioxide. (*D*) Lactacidemia levels were significantly reduced by ceftaroline alone and the combined therapy but nonsignificantly by mesenchymal stem cells alone. (*E*) Lung injury according to macroscopic score calculation was significantly reduced by mesenchymal stem cells or ceftaroline alone or the combined therapy. (*F*) Lung bacteria concentrations were significantly reduced in the groups treated with ceftaroline. (*G*) Pulmonary-to-systemic bacterial translocation was abrogated in the three-therapy regimen as compared to the control group. The data are expressed as box-and-whisker diagrams ($n = 7$ at 8 hours [H8] and 24 hours [H24]). (*E* to *J*) The Kruskal–Wallis test was performed (*P* value reported for each time point in the figure), and the Dunn's *post hoc* correction for multiple comparisons was used when appropriate: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$. CFU, colony-forming units; H0, baseline. (*Continued*)

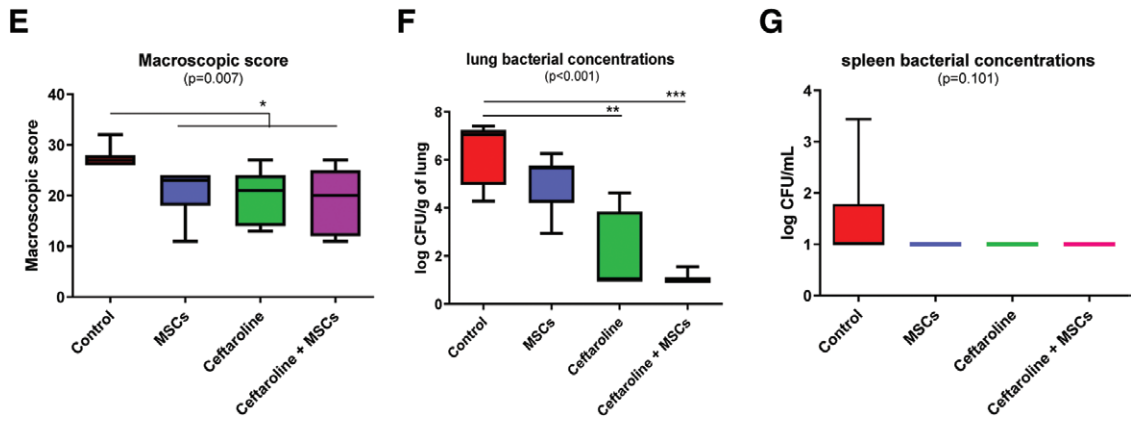

Fig. 7. (*Continued*)

the drop of mitochondrial membrane potential in circulating neutrophils might indicate increased apoptosis, which could partly explain the reported leucopenia. This finding is clinically relevant because leuconeutropenia is a strong predictor of early death during pneumococcal pneumonia.[3] Nonetheless, our data support the concept of immune response compartmentalization and the difficulty of reliably assessing host response through the analysis of circulating immune cells.[34]

Third, lung transcriptome revealed that exposure to both types of damage led to downregulation of some metabolic pathways, including lipid biosynthetic, catabolic, and fatty acid metabolic processes, potentially reflecting a metabolic switch from oxidative phosphorylation toward glycolysis. This might signal mitochondria dysfunction within the lung.

Altogether, our findings show that lung stretch and pneumococcal infection drive a hyperinflammatory state that relies on the alveolar recruitment of activated neutrophils exhibiting features of metabolic imbalance. As a result, lung injury is markedly worsened, and major disturbances in gas exchange including hypercapnia occur, thus underlining the clinical relevance of our model because increasing dead space has been consistently associated with death in ARDS patients.[35]

Finally, we assessed the effect of human cord tissue–derived mesenchymal stem cells, a promising adjunctive therapy for sepsis and ARDS.[27,36] Earlier studies mainly documented beneficial effects of mesenchymal stem cells on Gram-negative bacterial pneumonia models,[37] but little is known about the impact of mesenchymal stem cells in *S. pneumoniae* pneumonia, the most common type of bacterial pneumonia.[38] Finally, among the potential sources of mesenchymal stem cells, the fetal cord source could have greater immunomodulatory effects with less variability than bone marrow and adipose tissue and much greater availability, a critical point given the high incidence of bacterial pneumonia.[36,39,40]

The administration of both antibiotics and mesenchymal stem cells resulted in a dramatic decline in mortality along with reduced lung damage and improved gas exchange. However, the best outcome was achieved when the two treatments were administered together. Although antibiotic treatment is worthy, lower bacterial concentrations in the lung after exposure to antibiotics cannot account alone for such beneficial effects. According to previous findings, the beneficial effects of mesenchymal stem cells mainly result from their antimicrobial and anti-inflammatory properties.[5,13,27,37] In the current study, while antibiotics led to enhanced pulmonary bacterial clearance, mesenchymal stem cells themselves likely lessened pulmonary inflammation because they reduced interleukin-8 lung concentrations and subsequent alveolar recruitment of neutrophils when both therapies were given. Moreover, the rising tumor necrosis factor-α concentrations subsequent to bacterial lysis in antibiotics-treated animals were counteracted by mesenchymal stem cells.

Some previous experimental studies provide clues regarding the underlying mechanisms. The transfer of mitochondria from mesenchymal stem cells to injured epithelial cells or alveolar macrophages has been described as a likely means of reinstituting alveolar cell bioenergetics and phagocytic activity in the context of lung injury.[14,15] Accordingly, we showed herein that mesenchymal stem cells significantly reduced mitochondrial damage (lower mitochondrial DNA alveolar release) and improved mitochondrial function of immune cells (increasing mitochondrial membrane potential in alveolar and circulating neutrophils, as well as in alveolar macrophages). In addition, mesenchymal stem cells alone induce a transcriptomic response in the lungs, with an upregulation of metabolic and mitochondrial pathways. However, we should admit that our study does not allow us

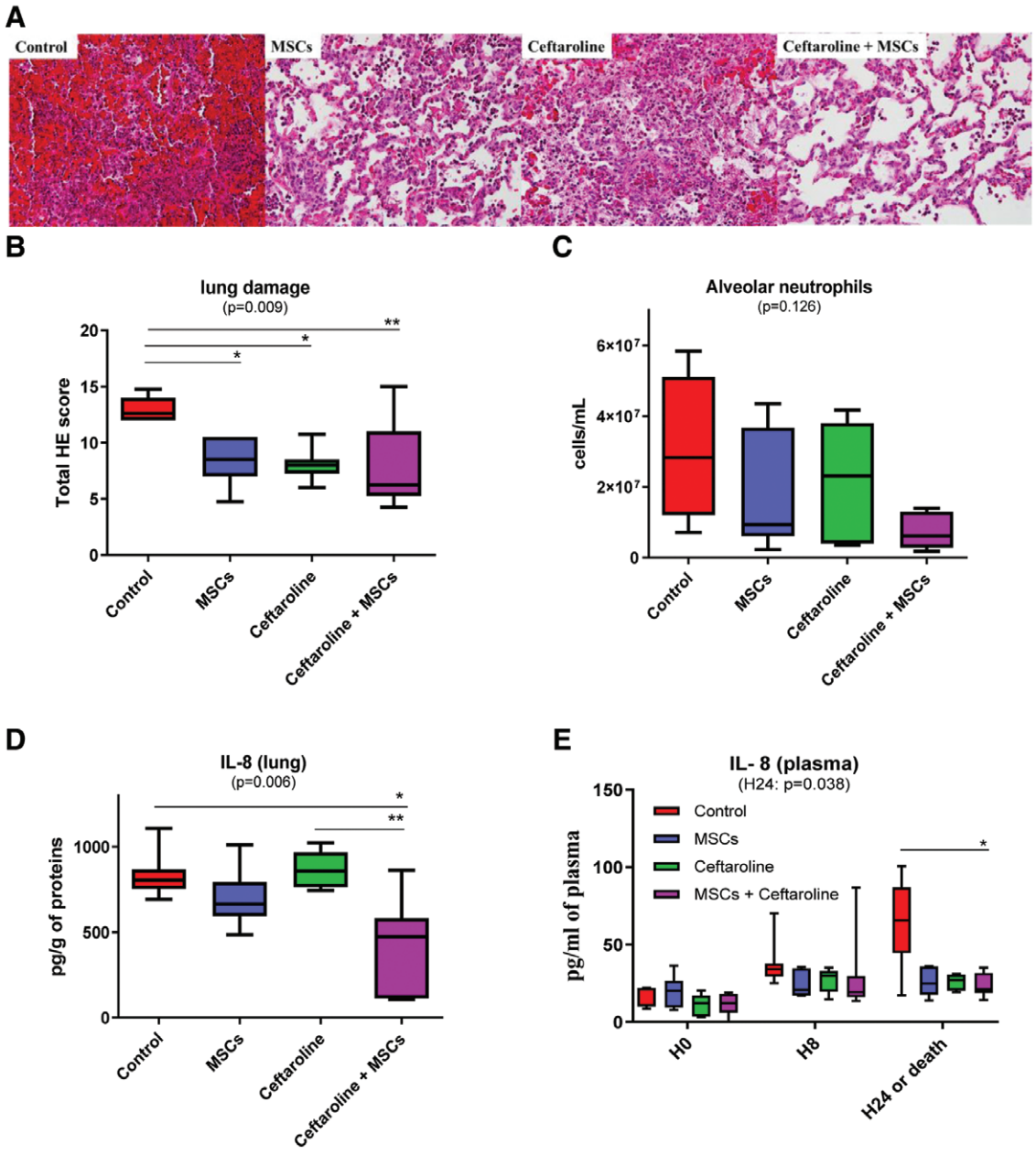

Fig. 8. Mesenchymal stem cells (MSCs) contributed to the modulation of both pulmonary and systemic inflammatory response. (*A*, *B*) As compared to the control group, mesenchymal stem cells, ceftaroline alone, or both therapies significantly improved lung injury according to a pathology score (extent of inflammation scored on hematoxylin-eosin (HE)–stained tissue sections). (*C*) Polymorphonuclear cells with the alveolar compartment. (*D*) When combined with ceftaroline, mesenchymal stem cells significantly reduced interleukin-8 (IL-8) pulmonary concentrations as compared to ceftaroline alone. (*E*) Mesenchymal stem cells alone significantly reduced interleukin-8 plasma concentrations. (*B* to *D*) The data are expressed as box-and-whisker diagrams ($n = 7$ animals). The Kruskal–Wallis test was performed (*P* value reported for each time point in the figure), and the Dunn's *post hoc* correction for multiple comparisons was used when appropriate: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$.

to decipher the mechanisms accounting for mesenchymal stem cells beneficial effects (*e.g.*, exosome-mediated process, mitochondria delivery through cell–cell contact) nor to differentiate between a lung transcriptomic effect directly related to mesenchymal stem cells or subsequent to outcome improvement.

Because pulmonary-to-systemic *S. pneumoniae* translocation was less likely in the animals treated with antibiotics

or mesenchymal stem cells, one could hypothesize that the microbicidal properties of activated circulating neutrophils cells were enhanced. The higher mitochondrial membrane potential we measured in circulating neutrophils could thus reflect an increase in oxidative phosphorylation. However, one cannot exclude a redistribution of alveolar neutrophils toward the blood flow, which could be driven by a paracrine effect of mesenchymal stem cells, because we observed concomitantly lower alveolar infiltration and higher blood leukocyte counts. Regardless, pulmonary-to-systemic neutrophil trafficking has been described in the setting of ARDS.[41] In addition, one cannot exclude those treatments prevent extrapulmonary bacterial dissemination through enhanced lung bacterial clearance and lung damage reduction.

In addition, experimental studies and clinical trials suggested that the efficiency of antibiotics could be mitigated in the setting of mechanical ventilation, supporting

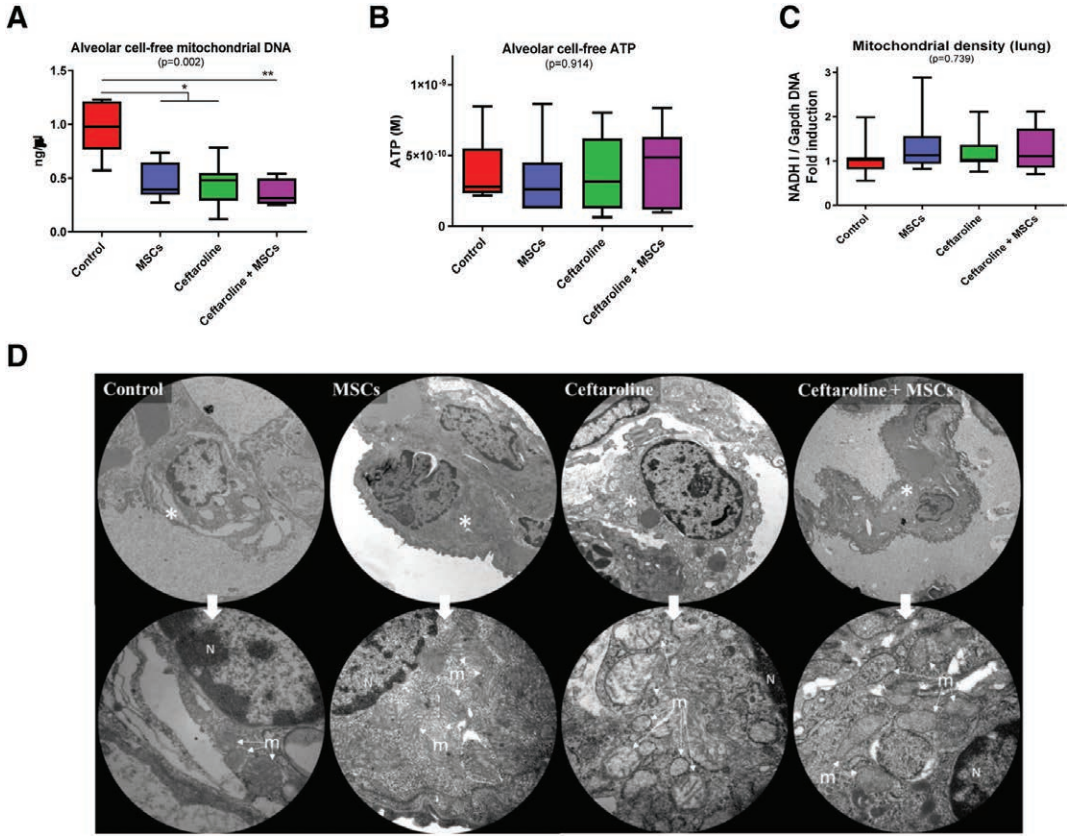

Fig. 9. Mesenchymal stem cells (MSCs) contributed to the resolution of mitochondrial derangements in pneumococcal ventilated pneumonia. (*A*, *B*) Mitochondrial alarmins were measured in bronchoalveolar lavage fluid at 8 hours (H8) or 24 hours (H24). (*A*) Cell-free mitochondrial DNA levels (NADH I) by quantitative polymerase chain reaction. (*B*) adenosine triphosphate (ATP) levels by bioluminescence assay. (*C*) Mitochondrial DNA levels were measured in the lung tissue (as a reflection of mitochondrial density). (*D*) Representative transmission electron microscopy images of alveolar epithelial type-II cells of infected animals under adverse mechanical ventilation. The control group (sodium chloride) includes swollen mitochondria (m) with reduced density; the mesenchymal stem cell group includes nonswollen mitochondria with the highest density compared to the control group; ceftaroline group includes nonswollen and swollen mitochondria with increased density; ceftaroline + mesenchymal stem cells includes nonswollen and elongated mitochondria with increased density. The *top row* shows global overview of the alveolar epithelial type-II cells, and the *bottom row* shows 50,000× magnification of the areas represented with *white asterisks*. (*E*, *F*) Mitochondrial membrane potential of alveolar immune cells was measured by flow cytometry and using mitochondrial fluorescent probes. Alveolar neutrophil mitochondrial membrane potential was assessed by MitoTracker Red/Green (*E*) or tetramethylrhodamine-methyl-ester (TMRM; *F*). (*G* to *J*) Blood samples were obtained at baseline (H0) and at H8 and H24 (or just before death, if appropriate) to measure blood neutrophil mitochondrial membrane potential (*G*, *H*) and mitochondrial alarmin plasma concentrations (*I*, *J*). (*G*) Mitrotracker Red/Green ratio (one outlier identified in the ceftaroline group; control *vs.* mesenchymal stem cells: $P = 0.058$ with and $P = 0.046$ without the outlier). (*H*) Tetramethylrhodamine-methyl-ester median fluorescence intensity. (*I*) cell-free mitochondrial DNA (NADH-I). (*J*) Cell-free ATP expressed as percentage of baseline level. The data are expressed as box-and-whisker diagrams ($n = 7$ animals). The Kruskal–Wallis test was performed (P value reported for each time point in the figure), and the Dunn's *post hoc* correction for multiple comparisons was used when appropriate: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$. N, nucleus. (*Continued*)

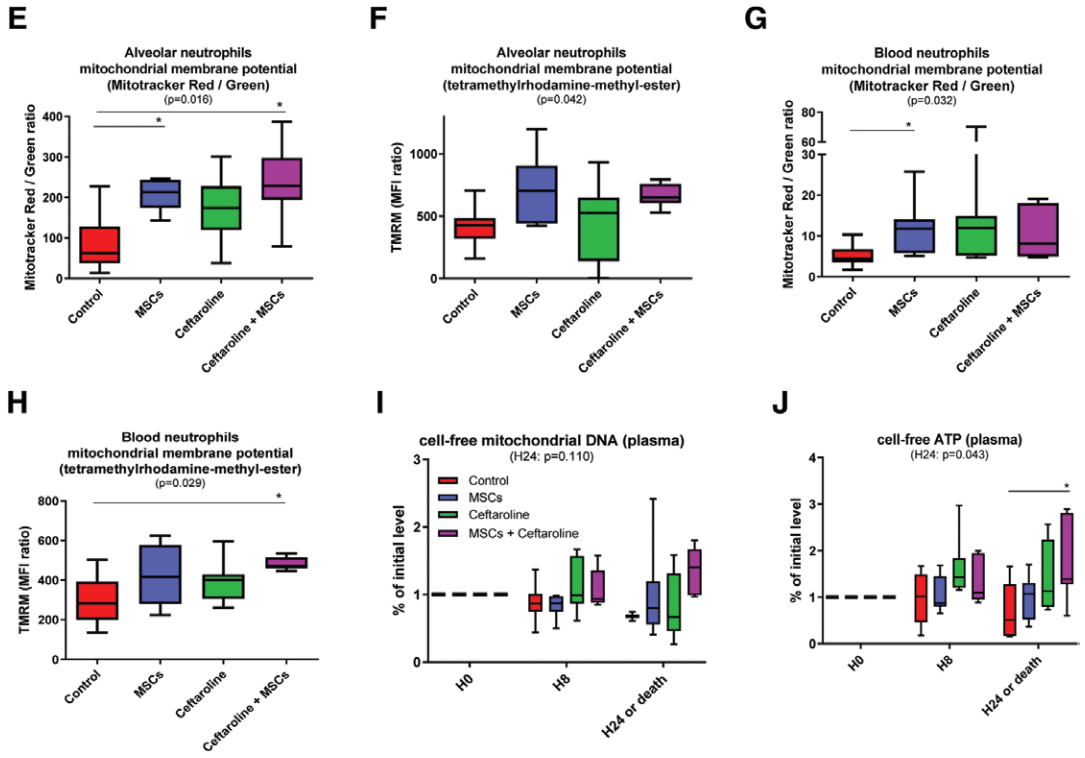

Fig. 9. (*Continued*)

the need for preclinical models.[42,43] Very few studies have tested mesenchymal stem cells in combination with antibiotics.[44,45] Notably, Alcayaga-Miranda *et al.*[44] reported that mesenchymal stem cells in a peritonitis mouse model exerted additional immunomodulatory effects, reduced organ injury, and increased bacterial clearance and survival when associated with antibiotics. Interestingly, our study showed that mesenchymal stem cells acted synergically by mitigating lung inflammation with antibiotic exposure. Finally, the comprehensive approaches used in the current study, including ultrastructural, molecular, functional, and transcriptional analysis of immune and lung cells, provide new insights into the beneficial effects of mesenchymal stem cells in the setting of ventilated pneumonia.

Several limitations should be mentioned. First, the inclusion of only male rabbits limits the extrapolation of our results. Second, the adverse mechanical-ventilation settings used here could be considered not clinically relevant. However, the current study aimed to evaluate the effects of lung overstretch on pneumonia, and it is known that lung injury is heterogeneous in ARDS patients. As a result, poorly aerated areas of the lung usually coexist with overstretched ones, even if "protective" ventilator settings are applied.[46] Moreover, a mean tidal volume (V_T) exceeding 12 ml/kg was encountered in 20% of patients kept under spontaneous ventilation despite extensive lung injury.[47] Nonetheless, we should admit that translating our findings into the clinical arena is questionable. Third, one could also argue that the severity of the pneumonia that we induced is uncertain because the Pao_2/Fio_2 ratio is not available. As a result, our model could be considered as far from human ARDS. However, diffused alveolar damages were obtained when combining infection and mechanical ventilation, a histopathological feature well correlated with the Berlin ARDS criteria.[48] Fourth, it was not possible to make conclusions about the effect of mesenchymal stem cells specifically on ventilator-induced lung injury because this treatment was not tested in ventilated animals without pneumonia. Finally, it is uncertain whether a later administration of mesenchymal stem cells would have had such a beneficial effect on the rabbit's outcome.

In conclusion, in this preclinical study, the outcome of rabbits submitted to pneumonia and adverse mechanical ventilation was improved by human cord tissue mesenchymal stem cells in addition to antibiotics, by correcting immune and mitochondrial dysfunctions, thus opening therapeutic avenues that should be tested in more clinically relevant models, especially using protective mechanical ventilation. Further research is needed to improve the understanding of the mechanisms of action of mesenchymal stem cells in the pneumonia-related lung microenvironment and to implement large-scale standardized production of

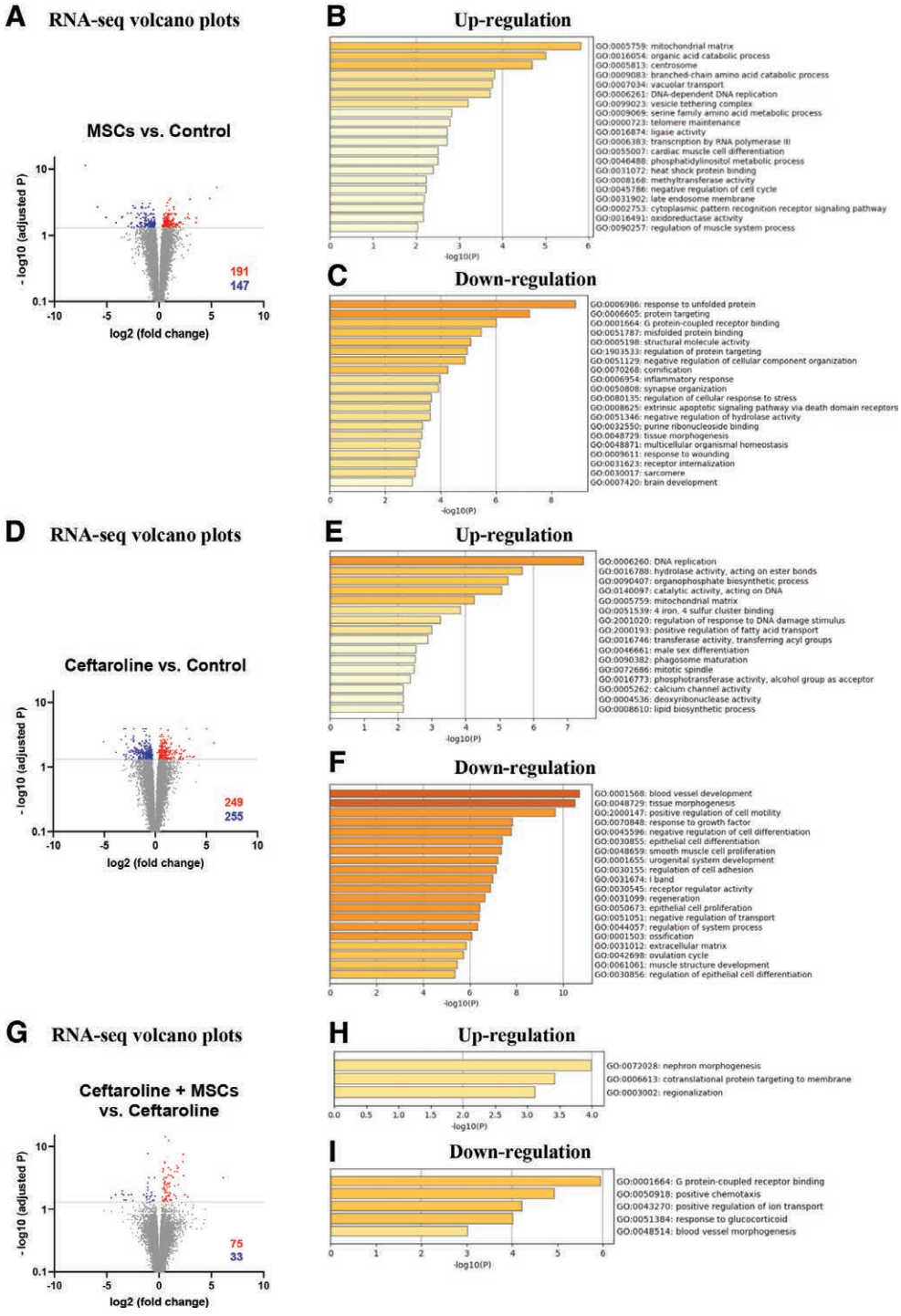

Fig. 10. Mesenchymal stem cells (MSCs) induce transcriptomic responses and upregulation of metabolic and mitochondrial pathways in the lungs of rabbits in the setting of pneumococcal pneumonia and adverse mechanical ventilation. Three comparisons were made in the setting of the two adverse conditions (*Streptococcus pneumoniae* pneumonia + adverse mechanical ventilation) to decipher the effect of the therapeutics at a transcriptomic level: comparison 1, mesenchymal stem cells *versus* control (sodium chloride; *A* to *C*); comparison 2, ceftaroline *versus* control (*D* to *F*); and comparison 3, ceftaroline + mesenchymal stem cells *versus* ceftaroline (*G* to *I*; $n = 7$ animals/group). (*A*, *D*, and *G*) The volcano plots (integrating adjusted *P* values and fold expression [$\log_2$ fold change]) depict the overall changes in gene expression. A *horizontal line* indicates the false discovery rate adjusted $P < 0.05$. *Red dots* denote overexpressed genes, and *blue dots* indicate underexpressed genes. (*B, C, E, F, H,* and *I*) Gene Ontology term analysis of upregulated (*B*, *E*, and *H*) or downregulated (*C*, *F*, and *I*) genes according to Metascape analysis (metascape.org, USA).

mesenchymal stem cells from available tissue sources, such as Wharton's jelly. Optimizing the beneficial effects of mesenchymal stem cells achieved through priming approaches is another issue that needs to be addressed.

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Competing Interests

The authors declare no competing interests.

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