**Adverse mechanical ventilation and pneumococcal pneumonia induce immune and mitochondrial dysfunctions** **mitigated by mesenchymal stem cells in rabbits.**

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**Supplemental digital content 1**

**Supplementary methods**

**Primary and secondary outcomes**

The primary outcome was the 24-hour survival and secondary outcomes were bacterial clearance in the lung and spleen, venous blood gases, lung injury (macroscopic evaluation, pathology score), immune response (alveolar neutrophils count, pulmonary and plasma cytokine concentrations) and mitochondrial dysfunction (alveolar and plasma mitochondrial DNA and ATP concentrations, lung mitochondrial density, mitochondrial membrane potential in alveolar and blood immune cells).
**Mechanical ventilation model**

Under general anesthesia provided by ketamine 27 mg/Kg and xylazine 2 mg/Kg, animals were intubated by introducing a cuff tube of 2.5 mm into the trachea under visual control. The animals were placed on a heated blanket, and isotonic saline was infused. Non-invasive monitoring was used to monitor heart rate (Hewlett Packard 78353B Monitor). If death occurred earlier (by asystole), animals were autopsied within the next 2 minutes, following exsanguination. Accordingly, there was no missing data for these animals. Venous blood lactate and gases were measured just after intubation to ascertain the safety of our mechanical ventilation, and at 8 hours, 16 hours, and 24 hours (or immediately before death, when bradycardia prior to asystole occurred). Each experiment was carried out in the same room of an animal facility with a controlled temperature. For organizational reasons, all “H8” animals were intubated (H0 of the experiment) at midnight and autopsied 8 hours later (H8), and all “H24” animals were intubated at 10 am and autopsied 24 hours later.

**Experimental Pneumonia Induction**

The pneumococcal clinical strain 16089 (9V serotype, penicillin-intermediate and ceftaroline-susceptible) was used (kindly provided the Centre National de Référence des Pneumocoques, France). Bacteria were grown in 5% CO2 in brain heart infusion (BHI) broth (BioMérieux, Marcy l’Etoile, France). Before each experiment, bacteria from one frozen aliquot were cultured on agar plates and incubated for 24 hours at 37 °C. Twenty-five to 30 colonies were inoculated into 9 ml of BHI broth (BioMérieux, Marcy l’Etoile, France) for 6 hours at 37 °C, and then cultured on agar plates for 18 hours at 37 °C in an anaerobic atmosphere. This culture was diluted in isotonic saline solution to obtain a final inoculum of 8.8 log10 colony-forming units/ml in 0.5 ml of isotonic saline, according to optical density measurements in reference to a standard curve and confirmed by culture. Pneumonia was induced, by endobronchial challenge with 0.5 ml of this freshly calibrated bacterial inoculum in either spontaneous breathing or mechanical ventilation animals (1,2).

**Therapeutic delivery and monitoring**

Ceftaroline-fosamil (Pfizer, New York, USA) was reconstituted in isotonic saline solution at a final concentration of 30mg/ml and administered intramuscularly in the right thigh at a dose of 20mg/kg. Previous work showed an eradication of infection and a pharmacodynamic simulating human dose regimen with the same settings in non-ventilated infected rabbits with this same pneumococcal strain (3). The concentrations of ceftaroline in plasma were determined from iterative blood samples (just before administration and 1, 2 and 4 hours thereafter) to analyze pharmacokinetics (4). The samples were centrifuged and immediately stored at -80°C until measurement. Plasma concentrations of ceftaroline and fosamil were measured using a previously validated method (5) with some modifications. The Ultra-Performance Liquid Chromatography–Tandem Mass Spectrometry detection was used to optimize this method. After a sample preparation in SPE with a 250µL rabbit plasma sample, 10µL of recovery phase was injected into the HPLC system. The chromatographic separation was performed using an Xterra® Waters C18 column (5µm 2.1\*100mm, 25°C). The mobile phase consisted of a mixture of 2 mM ammonium formate in water and CAN (82/18, v/v) using an isocratic mode. For the MS/MS detection, the ionization used was ESI in positive mode. Mass spectra were acquired by multiple reaction monitoring, and the specific transitions selected for ceftaroline were 605.0 > 208.0 m/z.

**MSC preparation, administration, and characterization**

The umbilical cord was collected at Nancy Maternity Hospital from one mother who had signed an informed consent form in compliance with the French national legislation regarding human sample collection, manipulation, and personal data protection. The collection protocol was approved by the local ethics committee and the French ministry for research (No. DC-2014-2114). All mesenchymal stem cells were produced at clinical-grade in α-MEM culture medium (Macopharma, Mouvaux, France) enriched with 5% platelet lysate (Macopharma, Mouvaux, France) and applying good manufacturing practices (6). Briefly, the umbilical cord was immersed in an antibiotic-antifungal solution composed of gentamicin, amoxicillin, vancomycin, and amphotericin B for 1 hr. at room temperature. The cord was then cut into thin pieces which were placed in complete medium. The culture was carried out at 37 °C and in hypoxic conditions (5% of O2 and 5% of CO2). Mesenchymal stem cells were cultured until passage three and then frozen and stored in vapor phase nitrogen.

Mesenchymal stem cells were then characterized. Once 80% confluence was reached, umbilical cord-derived mesenchymal stem cells were washed with HBSS and detached by trypsinization. To examine expression levels of surface markers, 1×106 umbilical cord-tissue derived mesenchymal stem cells were labeled with anti-CD90, CD73, CD44, CD105, CD34, CD45, CD11b, CD19, and HLA-DR mAbs (Stemflow hMSC Analysis kit, Becton Dickinson, Franklin Lakes, USA). Osteogenic and adipogenic differentiation was also performed to characterize mesenchymal stem cells. Osteogenic differentiation was induced by seeding mesenchymal stem cells at a density of 3,100 cells/cm2 and maintaining them in culture for 28 days in an osteogenic induction medium (Lonza, Walkersville, USA). After 28 days, samples were fixed in 4% paraformaldehyde and then included in paraffin before staining with alizarin red. To induce adipocyte differentiation, 21,000 mesenchymal stem cells/cm2 were seeded on 24-well plates. When 100% confluence was reached, 3 induction/maintenance cycles were performed. One induction/maintenance cycle consisted in 3-day culture in induction medium (Lonza, Walkersville, USA), followed by 1 to 3 days of culture in maintenance medium (Lonza, Walkersville, USA). After 3 cycles of induction/maintenance, the cells were cultured for 7 days in complete maintenance medium (Lonza, Walkersville, USA) before staining with oil red (6).

After thawing, mesenchymal stem cells were washed once in α-Minimum Essential Medium to remove the cryoprotectant and used within 1 hour. Rabbits were intravenously infused with 3 x 106 viable mesenchymal stem cells/kg in 10 mL of isotonic saline, 4 hours after pneumonia induction.

**Material harvesting and sample collection.**

Blood samples were obtained from the venous catheter just before experiment onset (H0) and 8, and 24 hours later (or just before death when severe bradycardia occurred). Ethylenediaminetetraacetic acid anticoagulated blood was centrifuged immediately at 2,000 g for 10 minutes, and the plasma was collected and stored at -80°C until further analyses.

Twenty-four hours after pneumonia onset, the animals were euthanized by ketamine xylazine injection following euthasol overdose, and exsanguinated. However, if death occurred earlier (by asystole), animals were autopsied within the next 2 minutes, following exsanguination (2). Spontaneously breathing animals were euthanized if necessary (clinical signs of upcoming death), whereas the ventilated rabbits were already under general anesthesia. The lungs were removed via thoracotomy. Each lower pulmonary lobe was instilled twice with 2.5 mL of sterile Phosphate Buffered-Saline 1x. The bronchoalveolar lavage fluid was centrifugated at 500 g for 10 minutes to collect the cell pellet. The supernatant was centrifugated again at 3,200 g for 5 minutes to remove potential remaining cells and debris.

**Sample measurements, and lung injury measurement**

Lactate concentration was measured within 15 min. from venous plasma transported at 4°C.

Lung injury evaluation was based on a macroscopic score and microscopic examination of lungs (7). A sample measuring 1 cm3 was excised from the right lower lobe, fixed in 10% buffered formalin, and embedded in paraffin. Hematoxylin-eosin staining was applied. Each specimen was scored, in a blinded manner, using the following parameters and a scale of 0 (absent), 1 (mild), 2 (moderate), 3 (severe), and 4 (very severe): interstitial damage, vasculitis, peribronchitis, edema, thrombus formation, and pleuritis (8).

**Mitochondrial dysfunction.**

**- Mitochondrial DNA Assessment**: Mitochondrial DNA was measured in plasma and BALF (circulating cell-free mitochondrial DNA), as well as in lung and liver tissue (reflecting mitochondrial density). Quantitative polymerase chain reaction was used to measure levels of mitochondrial DNA using specific primers for NADH I (For 5' -GCC CCA ACC CTA GCT CTA AC- 3’; Rev 5'-GCT CGG AGA GCA CCA AAT AG- 3’) (2). Primers were designed using the *Oryctolagus* *cuniculus* mitochondrion complete genome NCB1 reference sequence (NC-001913.1), synthesized by Microsynth (Balgach, Switzerland), and had no significant homology with sequences from rabbit genomic DNA (Blast® site, http://blast.ncbi.nlm.nih.gov). DNA was isolated from plasma and BALF, using the QIAamp DNA Mini Kit (Qiagen, Valencia, CA, USA), with a final volume of 200 μl of DNA resuspended in elution buffer. Quantitative polymerase chain reaction was performed with one-tenth or one-hundredth dilutions of the final product, compared with a standard curve of rabbit mitochondrial DNA to quantify the amount of amplified mitochondrial DNA, and expressed as arbitrary units. Melting curves were performed to ascertain the amplification of a single amplicon. Rabbit mitochondrial DNA was isolated from peripheral blood mononuclear cells of healthy rabbits using the mitochondrial isolation kit for cultured cells from ThermoScientific (Rockford, IL, USA). In order to assess the mitochondrial density within the lung and liver parenchyma, mitochondrial DNA levels were then measured in tissue homogenates. Total cellular DNA was extracted from frozen lung and liver with the DNeasy blood and Tissue kit (Qiagen, Valencia, CA, USA). The mitochondrial DNA copy number was obtained by real-time polymerase chain reaction, and normalized to a nuclear house-keeping gene (i.e. GAPDH For 5’-ATG TTT GTG ATG GGC GTG AAC C- 3’ Rev 5’-CCC AGC ATC GAA GGT AGA GGA- 3’) expression, in order to ensure that these concentrations were not related to the number of live cells within the tissue sample. Non-infected and non-ventilated animals (H8 and H24) were considered the baseline condition for the other groups evaluated at H8 and H24, respectively. The group submitted to pneumonia and adverse mechanical ventilation (H24) and treated with isotonic saline was considered the baseline for other therapeutic groups.

**- Mitochondrial measurement in immune cells**: Blood cells were collected after red blood cell lysis (5 min), and alveolar cells were filtered through a 70-µm strainer before resuspension in Phosphate Buffered-Saline-0.5% BSA. Four mitochondrial probes (Thermo) were used to measure mitochondrial mass (Mitotracker green FM, 200 nM), mitochondrial membrane potential/active mitochondria (Mitotracker Red CMXRos, 150 nM and Tetramethylrhodamine, Methyl Ester, 100 nM), and mitochondrial reactive oxygen species production (MitoSOX, 5µM). Cells were incubated separately with these 4 probes at 37 °C, 5% CO2, during 20 min in the dark, according to the manufacturer’s instructions. The concentration of mitochondrial dye used was selected by titrating with different concentrations, and the same concentration was used throughout all the experiments. Cells were then washed with 5 ml Phosphate Buffered-Saline and centrifuged for 5 min at 500 g. Then, they were incubated with a mouse anti-rabbit CD45 (Clone L12/201) APC-Cy7 (Lynx rapid conjugaison kit) (Biorad) and mouse anti-rabbit neutrophils (RPN3/57, Biorad) Dylight® 680 (Fast conjugaison kit, Abcam) at 4°C, for 20 min in Phosphate Buffered-Saline-2%BSA in the dark. Cell viability was determined by Zombie Violet staining (Biolegend). Cells were then washed with 5 ml Phosphate Buffered-Saline, centrifuged for 5 min at 500 g and scrapped in 300 μl Phosphate Buffered-Saline 0.5% BSA. Data were acquired on a BD LSRFortessaTM cytometer and analyzed using BD FACSDIVA (BD Biosciences, San Jose, CA) and FlowJo (TreeStar, Ashland, OR) software. Blood and alveolar neutrophils were gated with SSC/FSC characteristics and identified as CD45+Neutrophils+ cells. Alveolar macrophages were gated with SSC/FSC characteristics and identified as CD45+Neutrophils- cells. Doublets and dead cells were excluded. For each mitochondrial probe, geometric median fluorescence intensity (MFI) was measured in the cells of interest, and after exclusion of dead cells. Data were expressed as a ratio of stained/unstained cells to take into account autofluorescence.

**- Electron microscopy**: Transmission electron microscopy was used to examine the mitochondria ultrastructure. Immediately after euthanasia or death, 3-mm wide lung samples were fixed for 4 hours at 4°C in 4% paraformaldehyde (Electron Microscopy grade) and 2.5% of glutaraldehyde (Electron Microscopy grade) in Sorensen phosphate buffer (0.1 M, pH 7.3). After fixation, samples were washed in Sorensen phosphate buffer. Post-fixation treatment was realized with 1% osmium tetroxide (Electron Microscopy grade) at room temperature for 1 hour. After, samples were washed in Sorensen phosphate buffer then in distilled water. Dehydration and resin impregnation of the samples were performed with a Leica EM AMW automatic microwave tissue processor. Dehydration was done by increasing degrees of aceton (50%, 70%, 100%), substitution was done by three absolute aceton : Embed-812 resin mixtures and impregnation was done by three final incubation in Embed-812 resin. The polymerization of samples was performed manually with a mixture Embed-812 : 3% BDMA in gelatin capsule maintained for 48h at 60°C. Blocks were cut on a Reichert Ultracut E ultramicrotome, and 60nm-thick sections were deposited on copper/palladium grids. After drying, sections were contrasted with uranyl acetate and lead citrate. Observations were made on a HITACHI H-7500 transmission electron microscope operating at 80kV at the Dimacell core facility (INRAe, Dijon, France). Representative image of 10 alveolar epithelial type-II cells per animal were provided with 50,000 x magnification to visualize mitochondrial localization, density, and structural changes.

**Lung transcriptome (RNA purification and sequencing analysis).**

Immediately after autopsy, lung samples were taken and conserved at -80°C in lysis buffer with 1% β-mercapto-ethanol. Total RNA was extracted using the RNA GenElute kit (Sigma), followed by a RQ1Dnase (Promega) treatment. Total RNA quality was assessed on an Agilent Bioanalyzer 2100, using RNA 6000 pico kit (Agilent Technologies). Directional RNA-Seq Libraries were constructed using the TruSeq mRNA Stranded library prep kit (Illumina), following the manufacturer’s instructions. Final library quality was assessed on an Agilent Bioanalyzer 2100, using an Agilent High Sensitivity DNA Kit. 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. Demultiplexing was done (bcl2fastq2 v2.18.12) and adapters were trimmed with Cutadapt v1.15; only reads longer than 10pb were kept. 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. Differential analyses were performed using DESeq2 v1.24.0. The RNAseq data were submitted to the Gene Expression Omnibus (accession number GSE173238).

For RNA seq analysis, genes with an adjusted p-value < 0.05 and absolute fold change > 1.5 were considered as differentially expressed genes for each comparison. Correction for multiple comparison was performed using the Benjamini-Hochberg Procedure with an False Discovery Rate at 5%. Since the rabbit genome is poorly annotated, we used Better Bunny (<http://cptweb.cpt.wayne.edu>) (9), an online functional annotation tool using data derived from public bioinformatics resources, including the NCBI and Ensembl databases, to map probes for DEGs to highly homologous human genes. Agilent rabbit microarray probe identifiers from common interesting regions of the comparisons mentioned above were input to Better Bunny, and corresponding human orthologue genes were output in Ensembl Gene (ensembl gene stable ID) format using an identity threshold >50% (10) (Supplemental digital content 3). The annotations of the candidate genes selected after the RNA-seq analyses were explored to detect significant differences in molecular pathways between conditions. Significantly enriched gene ontology terms were calculated using differentially expressed genes with Metascape ([www.metascape.org](http://www.metascape.org)) (Supplemental digital content 4).

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