Relation between respiratory mechanics, inflammation, and survival in experimental mechanical ventilation

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Online Data Supplement

Materials and Methods

The study was approved by the Institutional Animal Care and Use Committee at Massachusetts General Hospital, and conducted in conformity with the National Institutes of Health guidelines.

Animal preparation and experimental design

C57BL/6 adult mice were anesthetized with intraperitoneal ketamine (120 mg/kg) and fentanyl (0.05 mg/kg). Following tracheotomy, the animals were connected to a small animal ventilator (MiniVent 845, Harvard Apparatus, Holliston, Massachusetts, USA) and mechanically ventilated with tidal volume (Vt)=8 ml/kg, respiratory rate (RR)=160/min, positive end-expiratory pressure (PEEP)=2 cmH_2O. The carotid artery was cannulated to continuously monitor blood pressure and assess the depth of anesthesia during the course of the study. Muscle relaxant (vecuronium 1.5-2 mg/kg/hr) and heparin (4 U/kg/hr) in Lactated Ringer's with 5% dextrose solution were administered via the arterial catheter (8-10 μl/g/hr). When the systolic blood pressure rose above 120 mmHg, a continuous infusion of ketamine (~30 mg/kg/hr) was started via an intraperitoneal catheter. The animal was covered with plastic wrap to prevent heat and fluid loss. Body temperature was monitored and maintained between 36.5-37.5 ºC with an infrared heating lamp and a heating pad (DC Temperature Controller, FHC Inc., Bowdoin, Maine, USA). After the surgical preparation, the mice were disconnected from the MiniVent and connected to a computer-controlled ventilator (FlexiVent, Scireq, Montreal, Canada). Mice were assigned to two main groups according to ventilator settings: the low tidal volume ("protective") group (Vt=6 ml/kg, RR=180/min, n=10) and the injurious group. This latter group was composed of two subgroups: *inj-15* subgroup (Vt=15 ml/kg, RR=80/min, n=7) and *inj-20* subgroup (Vt=20 ml/kg, RR=52/min, n=5). All groups had FiO_2=50%, PEEP=2 cmH_2O,

inspiratory-to-expiratory time ratio=1:2. Standardization of lung volume history was performed with 2 recruitment maneuvers (RMs) after the animals were connected to the FlexiVent. Each RM consisted of a 5-second (s) ramp increase of airway pressure to 30 cmH_2O followed by a 1-s plateau. One RM was performed every 5 minutes during the experiment in each group to recruit the atelectatic areas in the lung. Frequency and pressure of the maneuver were based on studies showing that these settings maintained respiratory mechanics stable in mice ventilated for 2 to 6 hours with tidal volume and PEEP similar to ours (E1, E2). Ventilation was continued for 16 hours or until the mouse expired. Mice who did not survive the experiment had blood pressure lower than 30 mmHg for 5 minutes and loss of heart beat pulsatility on the arterial pressure waveform when they were terminated.

Four mice in the protective group and two in the injurious group expired within the first 3 hours of the assigned ventilation and were excluded from the study because, at the tidal volumes employed, death within this time window is not expected to result from VILI (Figure 1B of (E3)). In most cases, this early mortality was associated with bleeding from the carotid cannulation site or with blood loss during the surgical preparation.

In addition, 7 mice underwent the same surgical preparation but, instead of being connected to the FlexiVent, they were sacrificed and their lungs excised for biochemical analysis. These mice served as sham controls.

In vivo measurements

Respiratory input impedance was measured at zero PEEP every hour during the ventilation protocol using the Broad Band Forced Oscillation Technique with FlexiVent (E4). This system uses mutually prime frequency waveforms between 1 and 20.5 Hz delivered by the ventilator piston to obtain the respiratory impedance data. The measurement consisted of 4

subsequent identical perturbations lasting 3 seconds each separated by 15 seconds of mechanical ventilation at the assigned settings. These measurements followed the recruitment maneuver by 2 minutes (Figure E1). Tissue elastance was calculated by fitting the constant phase model to the respiratory impedance (E5). Elastance was measured as the average of the values of the 4 perturbations. Inspiratory capacity was measured as the volume delivered during the RM starting at zero PEEP (E6). Pressure-volume curves were generated every hour using a ramp-style pressure-driven perturbation (6-s ascent to airway pressure of 30 cmH_2O followed by 6-s discent). Hysteresis was determined as the area between the inspiratory and expiratory limbs of the pressure-volume curve. An upper pressure limit of 30 cmH_2O was chosen both because considered to correspond to total lung capacity (E7) and because it corresponded to the pressure of the recruitment maneuver, which the curve replaced at hourly intervals. A flow chart of the sequence of these measurements is shown in Figure E1. Elastance, inspiratory capacity and hysteresis were normalized by the first value of each subject measured after standardization of lung volume history (baseline).

Lung strain was calculated as (E8): (Vt+ PEEP volume)/FRC

Weighted lung strain was calculated as (E8):

((Vt + PEEP volume)/FRC * Tinsp + (PEEP volume/FRC) * Texp) / (Tinsp + Texp)

PEEP volume was estimated as the product of respiratory compliance measured from the Flexivent by the PEEP measured in the individual animal. Functional Residual Capacity (FRC) was estimated from physiological studies in healthy animals of the same species, as described in (E8). Weighted lung strain represents the weighted average of the strain applied to the lung during inspiration and expiration, where the weights are the inspiratory (Tinsp) and expiratory (Texp) times.

At the end of the study, arterial blood gas samples were collected in 13 animals (protective n=7, injurious n=6) from the carotid arterial cannula.

Ex vivo measurements

Protein levels of interleukin-1β (IL-1β) and interleukin-6 (IL-6) in the lung were measured by enzyme-linked immunosorbent assay (protective n=10, injurious n=7) according to the manufacturer's instruction (DouSet, R&D systems, Minneapolis, Minnesota, USA). Assays were performed in duplicate. Tissue homogenate was diluted to 1:3 and 1:10 for the IL-1β and IL-6 assays, respectively. Results are expressed as pg/ml for IL-1β and IL-6. The mRNA levels of IL-1β, IL-6, and macrophage inflammatory protein-2 (MIP-2) were measured by quantitative real-time reverse transcription-polymerase chain reaction, (q)RT-PCR, in lung tissue. (q)RT-PCR was performed in duplicate as described previously (E9). Changes in relative gene expression normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were determined using the relative cycle threshold number method. The protective group served as reference for calculation of the fold change of the mRNA levels. The sequences of the oligonucleotide primers (E10, E11) are listed in Table E1 of the online supplement. The right upper lobe was placed in an oven at 80°C to dry for 2 days to measure the wet-to-dry ratio.

Human data

We compared plasma levels of IL-6 on day 3 between the lower and higher tidal volume groups for the patients who did or did not survive to hospital discharge from the ARMA study (E12). Data were provided by the Biologic Specimen and Data Repository Information Coordinating Center (BioLINCC), National Heart, Lung and Blood Institute (NHLBI), National Institutes of Health, Bethesda, Maryland, USA. This manuscript was prepared using ARDSNET Research Materials obtained from the NHLBI BioLINCC and does not necessarily reflect the

opinion or views of the ARDSNET or the NHLBI. A query was placed with the Institutional Review Board of Massachusetts General Hospital, which determined that use of that data as reported in this manuscript does not meet the definition of human subjects research.

Statistical Analysis

Changes in elastance, inspiratory capacity and hysteresis between baseline ($elastance_0$, $insp\ capacity_0$, $hysteresis_0$) and final values and differences between protective and injurious ventilatory strategy were analyzed with two-way repeated measures ANOVA after testing for normality and homoscedasticity. Pairwise comparisons were performed with the Holm-Sidak method. Hourly values of elastance, inspiratory capacity and hysteresis were fit to a mixed-random and fixed-effects longitudinal model as dependent variables in separate analyses (E13) in order to assess the temporal behavior of these variables irrespective of differences in the timing of the final value (defined as the last hourly value before the animal expired or at 16 hours). We used backward elimination with a $P<0.05$ cut-off, beginning with five fixed predictor terms (group, time in hours (linear and quadratic) and the interaction of group with linear and quadratic time) and three random terms (intercept, linear time and their correlation). We analyzed residuals of the model to ensure that they conformed to normality and homoscedasticity assumptions of the tests. We performed separate analyses for the two-group contrast of protective versus injurious ventilation groups and for the three-group contrast of protective, *inj-15*, and *inj-20* subgroups. Goodness of fit of the model to the actual data was tested both for the fixed and for the mixed (fixed plus random) model predicted values (SAS Version 9.4, SAS Institute Inc., Cary, North Carolina, USA).

ELISA and (q)RT-PCR data were logarithmically transformed (E14) and groups were compared with one-way ANOVA. Pairwise comparisons were performed with the Holm-Sidak method.

Blood pressure was compared between the protective and injurious groups with two-way repeated measures ANOVA. Lung strain, blood gases and human data were compared between groups with Student t–test for unpaired data or, if the Shapiro-Wilk normality test or the equal variance test failed, with the Mann-Whitney-Wilcoxon Rank Sum Test. The wet-to-dry ratio was analyzed with Kruskal-Wallis test. Pairwise comparisons were performed with Dunn's method. A P value <0.05 was considered statistically significant. Data are presented as mean ± SEM.

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FIGURE LEGENDS

Figure E1. Flow chart of the sequence of respiratory mechanics measurements during one hour of mechanical ventilation. This pattern was repeated until 16 hours or the animal expired. RM: recruitment maneuver; FOT: forced oscillation technique; PV: pressure-volume curve. RM was repeated every 5 minutes throughout the study. Time axis is not to scale.

Figure E2. Blood pressure of the two groups. Circle: protective; triangle: injurious groups. Data are mean ± SEM.

Figure E3. (A) IL-1β protein and mRNA levels showed strong correlation (r=0.94, $P<0.001$). (B) IL-6 protein and mRNA levels were also correlated (r=0.59, $P<0.05$). Empty symbols: survivors; filled symbols: non-survivors; circle: protective; upward triangle: *inj-15*; downward triangle: *inj-20*.

Figure E4. MIP-2 mRNA level at the end of the study. $^{\dagger}P<0.01$ versus sham. Square: sham; empty symbols: survivors (surv); filled symbols: non-survivors; circle: protective (prot); upward triangle: injurious (*inj*)-*15*; downward triangle: *inj-20*; line: mean.

Table E1: Oligonucleotide primer sequences for (q)RT-PCR.

Gene	Primer Sequence
Mouse GAPDH	**Forward: 5'- AACTTTGGCATTGTGGAAGG -3'** **Reverse: 5'- GGATGCAGGGATGATGTTCT -3'**
Mouse IL-1β (E10)	**Forward: 5'- GCCCATCCTCTGTGACTCAT -3'** **Reverse: 5'- AGGCCACAGGTATTTTGTCG -3'**
Mouse IL-6 (E6)	**Forward: 5'- AGTTGCCTTCTTGGGACTGA -3'** **Reverse: 5'- TCCACGATTTCCCAGAGAAC -3'**
Mouse MIP-2 (E14)	**Forward: 5'- CCGCTGTTGTGGCCAGTGAACTGCG -3'** **Reverse: 5'- TTAGCCTTGCCTTTGTTCAGTAT -3'**

Figure E1

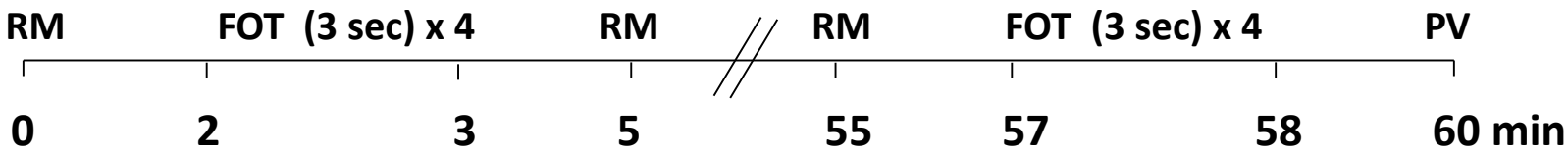


Figure E2

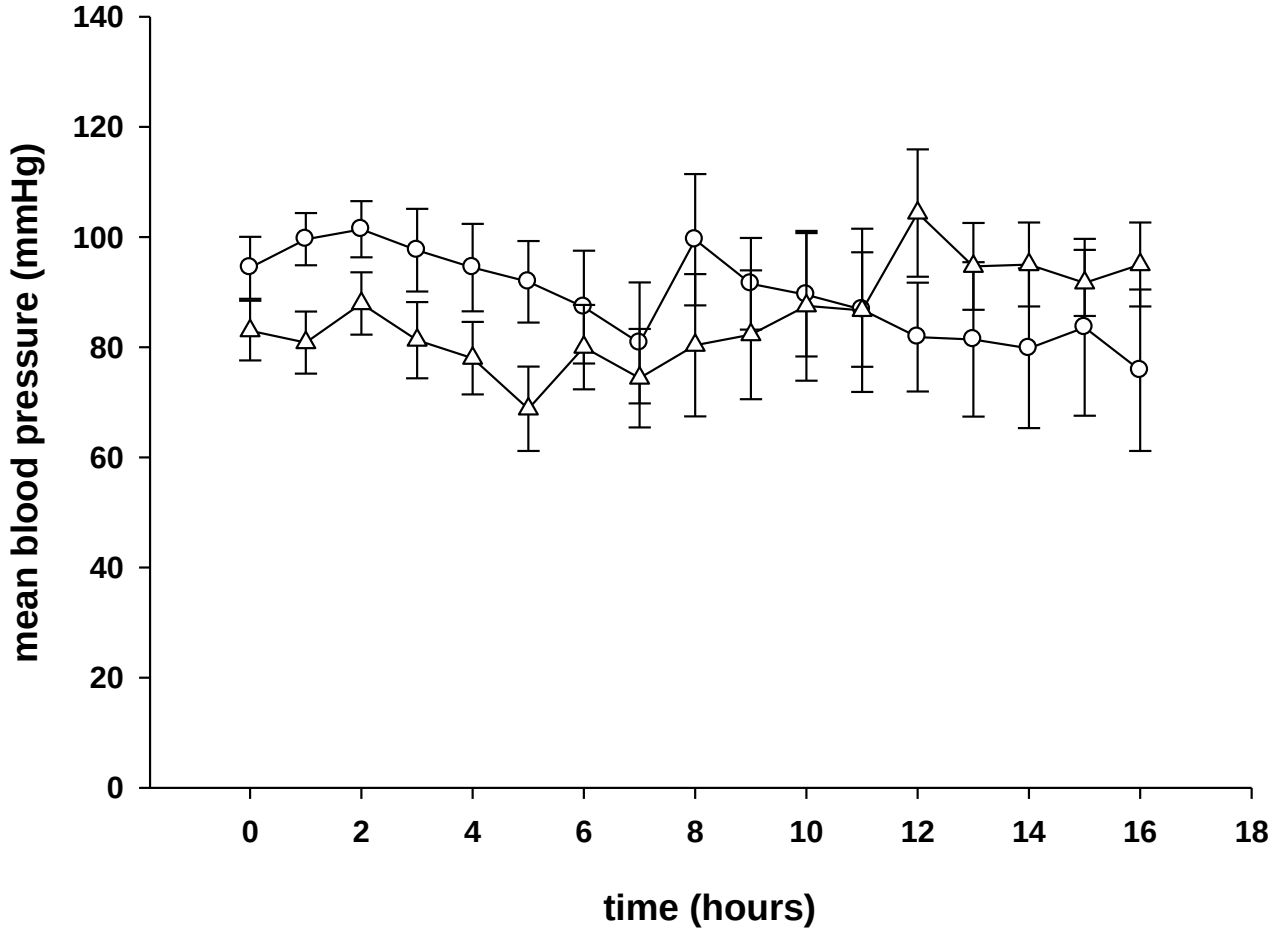

mean blood pressure (mmHg)
140
120
100
80
60
40
20
0
0
2
4
6
8
10
12
14
16
18
time (hours)

Figure E3

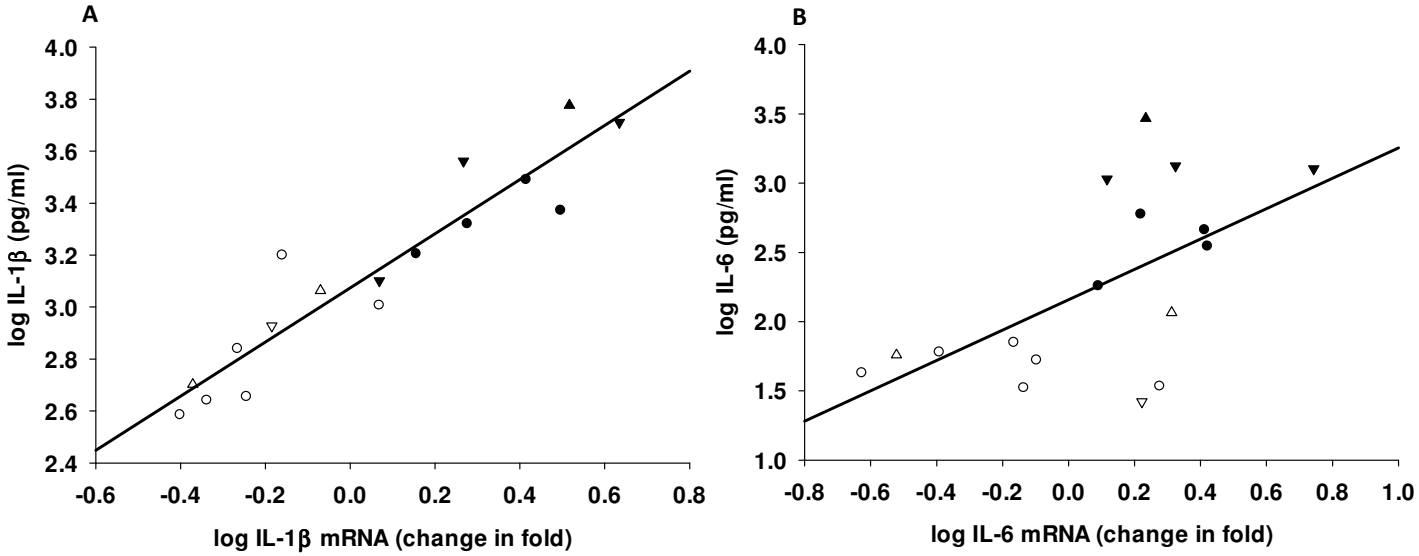
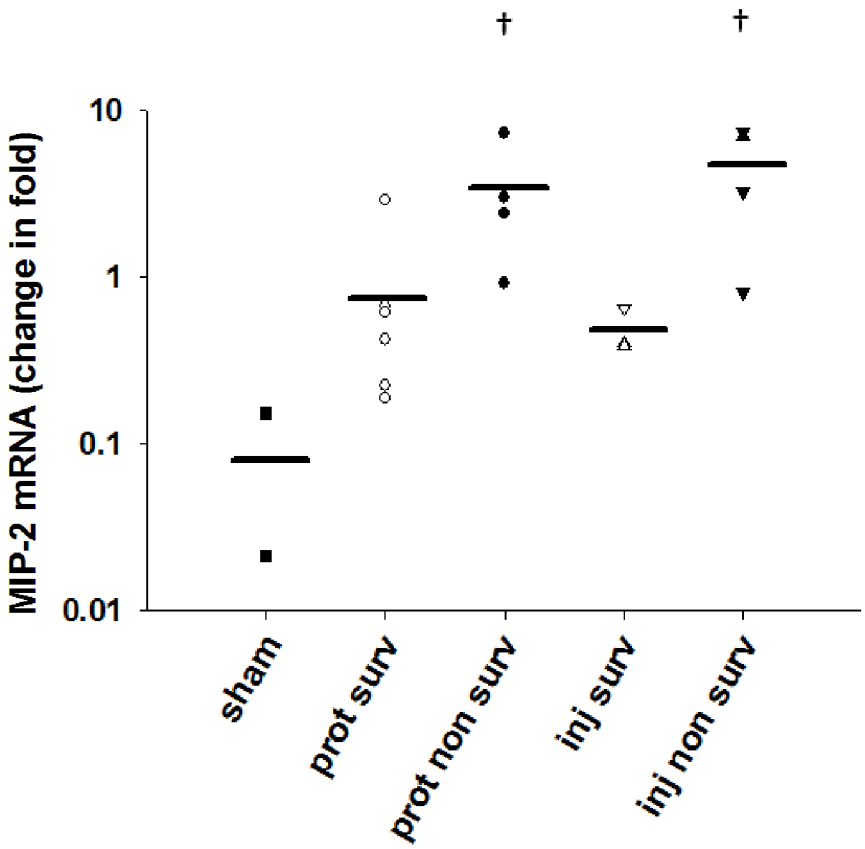

A
log IL-1β (pg/ml)
4.0
3.8
3.6
3.4
3.2
3.0
2.8
2.6
2.4
-0.6
-0.4
-0.2
0.0
0.2
0.4
0.6
0.8
log IL-1β mRNA (change in fold)
B
log IL-6 (pg/ml)
3.5
3.0
2.5
2.0
1.5
1.0
-0.8
-0.6
-0.4
-0.2
0.0
0.2
0.4
0.6
0.8
1.0
log IL-6 mRNA (change in fold)

Figure E4


MIP-2 mRNA (change in fold)
10
1
0.1
0.01
†
†
sham
prot surv
prot non surv
inj surv
inj non surv