Effects of Age on the Synergistic Interactions between Lipopolysaccharide and Mechanical Ventilation in Mice

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ONLINE DATA SUPPLEMENT

MATERIALS AND METHODS

Animal Protocol

The Animal Research Committee of the Veterans Affairs Puget Sound Health Care System approved all of the experiments. Male or female 14 day and 15 week-old C57BL/6J mice were purchased from Jackson Laboratories (Bar Harbor, ME), and housed in an SPF animal facility until the day of the experiment. Twenty-one day and 16 week old mice were divided into four different treatment groups: 1) no treatment, 2) aerosolized LPS with spontaneous ventilation, 3) mechanical ventilation, or 4) aerosolized LPS combined with mechanical ventilation. The duration of the experiments to collect whole lung mRNA for expression array analysis were 2 hr, and the duration of the experiments to measure inflammation and injury were 3 hr. Spontaneous breathing mice were allowed free access to food and water in their cages, breathing room air for the duration of the experimental protocol. C57BL/6J-*Ticam1*^{Lps2}/J (TRIF-/-) mice were bred in the vivarium of the VA Puget Sound Medical Center Animal Facility and 21 day old mice were treated with 1) aerosolized LPS with spontaneous ventilation or 2) aerosolized LPS combined with mechanical ventilation for 3 hr.

Reagents

A stock solution of lipopolysaccharide (LPS) derived from *Escherichia coli* serotype 0111:B4 (List Biological Laboratories, Campbell, CA) was diluted in phosphate buffered saline (PBS) to 1 mg/mL and stored at -20°C. On each experiment day, LPS was thawed and 2 mg were diluted in 20 mL PBS (0.1 mg/mL). Multiplex bead-based immunoassays for TNF-α, MIP-2, KC, MCP-1, IL-

12p70, IL-6, IL-1β, IFNγ, IL-10, IL-2, IL-13, IL-17, VEGF and GM-CSF were performed with murine specific antibodies (R&D Systems, Minneapolis, MN) and an automated analysis system (Luminex Corp, Austin, TX). Total protein was measured using the BCA Protein Assay Kit (Pierce, Rockford, IL), and the ELISA for IgM was performed using the Mouse IgM Quantitation Kit (Bethyl Laboratories, Montgomery, TX). Quantitative PCR was performed with the High Capacity cDNA reverse transcription kit (#4374966) and primers to Bcl2, S100a9, and Tlr4 from ABI, Foster City, CA.

Murine Model of Ventilator Induced Lung Injury

LPS aerosol treatment

Mice were placed in individual wire mesh cages in a sealed plexiglass aerosol chamber and exposed to an aerosol of LPS (20 mL of 0.1 mg/mL) for 30 min., which was generated by twin jet nebulizers. An airflow rate of 25 - 30 mL/min was maintained in the chamber (1). The experimental protocol began immediately after completion of the aerosol treatment. Mice that were treated with LPS alone were recovered and returned to their cages with free access to food and water for the duration of the experimental protocol. Mice that were treated with LPS+MV were anesthetized, intubated and mechanically ventilated immediately after exposure to aerosolized LPS.

Mechanical Ventilation

Mechanically ventilated mice were anesthetized with an intraperitoneal injection of ketamine (0.075 mg/g) and xylazine (0.015 mg/g) mixed in Ringer's Lactate, and

then orotracheally intubated by direct laryngoscopy with 22 gauge (mice 6 – 7.7 g) or 20 gauge (mice > 7.7 g) catheters (Terumo Surflo IV Catheter, Elkton, MD). In the 2 hr studies, the mice were anesthetized with 4% isoflurane. Maintenance anesthesia was provided using intramuscular injections of the ketamine/xylazine mixture (0.05/0.01 mg/g). Maintenance anesthesia was provided for the mice that were mechanically ventilated for 2 hr using 2% isoflurane nebulized into the ventilator circuit. The depth of sedation was assessed by heart rate (HR), blood pressure (BP), and response to paw pinch. Mice were paralyzed with intraperitoneal (ip) pancuronium 0.1 mg/g if there was ventilator dyssynchrony, no response to paw pinch, and HR and BP were within 10% of baseline. Mice were warmed with a heating lamp to maintain rectal temperature between 37 - 38ºC. Mice were mechanically ventilated for 2 or 3 hr with FiO_2 = 0.3, 2 cm H_2O positive end expiratory pressure (PEEP), V_T = 15 mL/kg, and rate = 80 breaths/min providing a normal minute ventilation (22). A lung recruitment maneuver with 5 cm H_2O PEEP for thirty seconds was completed every 30 min for mice that were mechanically ventilated for 3 hr (23).

Physiological Monitoring

Temperature (Mon-a-therm, Nellcor, Pleasanton, CA), airway pressure, and heart rate (ECG) (Animal BioAmp ML136, PowerLab 16/30, ADInstruments, Colorado Springs, CO) were measured continuously in all mechanically ventilated mice. Non-invasive blood pressure was measured every thirty minutes for the first hour and then hourly (NIBP Controller ML125, PowerLab 16/30, ADInstruments, Colorado Springs, CO).

Sample Processing

After 3 hr, the animals were euthanized, exsanguinated, and the trachea was cannulated with a 20 g catheter (Terumo Surflo IV Catheter, Elkton, MD). The lungs of adult mice (average lung weight 150 mg) were lavaged with an initial aliquot of 1.2 mL PBS/EDTA, followed by three separate aliquots of 1.0 mL. The lungs of juvenile mice (average weight 75 mg) were lavaged with 0.6 mL PBS/EDTA, followed by 0.5 mL three times. The lavage fluid was recovered by gentle suction and placed on ice, and immediately processed for total and differential cell counts. The remainder of the lavage fluid was spun at 200 x g for 30 minutes, and the supernatant was removed aseptically and stored in individual aliquots at -70°C.

After the lavage procedure, the lungs were placed in ice-cold sterile water with Complete, Mini Protease Inhibitor Cocktail (#11 836 153 001, Roche Applied Science, Indianapolis, IN), and homogenized with a hand-held homogenizer. The whole lung homogenate was vigorously mixed in a buffer solution containing DNase I (Sigma), RNase (Sigma), and 0.5% Triton-X-100, 150 mM NaCl, 15 mM Tris, 1 mM CaCl, and 1mM MgCl, pH 7.40, incubated for 30 minutes at 4°C, and then spun at 10,000 g for 20 minutes. The supernatants were stored at -80°C for later cytokine measurements.

Histology

The excised lungs were fixed by inflation with 4% paraformaldehyde at a transpulmonary pressure of 15 cm H_2O for 24 hr, then placed in 70% ethanol and

embedded in paraffin. Lung sections were stained with hematoxylin and eosin for light microscopy.

RNA Analysis

To measure early changes in gene expression, additional mice were euthanized after 2 hr of mechanical ventilation, and the lungs were immediately removed and placed into RNA*later* (Ambion, Austin, TX) for at least 24 hr. Total lung mRNA was purified using the Stratgene Absolutely RNA kit (Ambion). The RNA was concentrated using potassium acetate precipitation and reconstituted in RNase free water. The quality of the RNA was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc, Santa Clara, CA), and samples with an RNA integrity index of greater than 7.0 were reverse transcribed, labeled and hybridized on the Affymetrix mouse 430 2.0 expression array chip at the University of Washington Array Center.

Measurements

An aliquot of the BAL fluid was processed immediately to assess cell viability with trypan blue (Sigma-Aldrich, St. Louis, MO). Then the cells were stained with crystal violet (Sigma-Aldrich) and total cell counts were performed on a hemocytometer. Differential cell counts were performed on cytospin preparations stained with modified Wright-Giemsa stain (Diff-Quick, American Scientific Products, McGaw Park, IL).

The total protein in the BAL fluid was measured using the bicinchoninic acid method (BCA assay: Pierce Co., Rockford IL), and IgM concentrations in the BAL fluid were measured with a murine specific immunoassay (Bethyl Laboratories, Inc,

Montgomery, TX). Cytokines and growth factors were measured in lung homogenates using N = 10 samples per experimental group using 1:1 and 1:5 dilutions. Each result was evaluated for inclusion in the statistical analysis according to the following paradigm: 1) at the low end and the middle of the standard curve the 1:1 result was included if there was > 20% difference in the 1:1 and 1:5 dilution; 2) at the high end of the standard curve, the 1:5 result was included if there was > 20% difference in the 1:1 and 1:5 dilution; 3) for values that were above or below the limit of the standard curve a value of +/- 5% of the assay detection limit was applied.

Statistical Analysis

In order to determine 1) the effect of each treatment in the juvenile and adult mice, 2) whether there were synergistic interactions between LPS combined with mechanical ventilation (LPS+MV) in each age group, and 3) whether age modified the responses to each treatment, we used regression analysis with a 3-factorial model that included age, mechanical ventilation (MV), and LPS using PMN, total protein, IgM, and cytokines as the outcome variables (SPSS 16.0.1, SPSS Inc, Chicago, IL). Synergistic interactions between LPS and mechanical ventilation were identified when the difference between untreated mice and mice treated with LPS+MV was significantly different than the sum of the effects of mechanical ventilation and LPS alone. Age was determined to modify the effects of treatment (MV, LPS or LPS+MV) when the measured outcome in the juvenile mice was significantly different from the adult mice. Model residuals were examined graphically to check for normality of the data. Dependent variables with non-normal

distributed data were $\log_{10}$ transformed to achieve a closer approximation of a normal distribution.

In order to determine the effect of age and treatment with MV and LPS+MV on the physiology of the mice we used 2-way ANOVA with the post-hoc Bonferroni adjustment for $p < 0.01$ (Prism 4.0, GraphPad Software, Inc, La Jolla, CA) to compare temperature, respiratory rate, heart rate, and systolic blood pressure in the juvenile and adult mice at time = 0, 0.5, 1, 2, and 3 hr. In order to determine the effect of age and treatment with MV and LPS+MV on cardio-respiratory physiology we used one-way ANOVA with the post-hoc Bonferroni adjustment for $p < 0.01$ to compare normally distributed data (pH) and the Kruskal-Wallis test with Dunn's multiple comparisons on non-normally distributed data ($PmvCO_2$, $SmvO_2$, and lactate).

Microarray Data Analysis

The raw gene expression array data were normalized using the Robust Multichip Average algorithm (2). In order to determine the variability of gene expression in the individual samples, a Principal Component Analysis (PCA) was performed (MeV 4.0) (3, 4). The transcriptional response was determined using Bayesian t-tests with a false discovery rate (FDR) < 0.1% using the Benjamini & Hochberg method (5). In order to identify genes likely to be biologically significant for the acquisition of synergistic responses to LPS combined with mechanical ventilation, we first used 2-way ANOVA (adjusted p-value 0.01) to identify genes with an interaction effect between age and treatment (MeV 4.0) (3). K-median consensus clustering (KMC) using the Pearson's correlation with a maximum of 50

iterations was used on the genes with a significant interaction effect between age and treatment to identify major expression patterns across the experimental conditions (MeV 4.0) (3). Functional annotation and functional clustering was used to explore enriched biological modules in the KMC clusters of interest using the Database for Annotation, Visualization and Integration Discovery (DAVID). Multiple hypothesis testing was addressed by applying a false discovery rate (FDR) cutoff of < 5% (6).

Interaction networks were created from gene members of KMC clusters of interest using Ingenuity System's software and knowledge base (7). This knowledge base has been manually curated from >200,000 full-text, peer-reviewed scientific articles encompassing approximately 10,000 human, 8000 mouse, and 5000 rat genes. A comprehensive molecular network of direct and indirect interactions between the gene products of these mammalian orthologues has been developed, and serves as the basis for creating smaller networks from user provided gene lists. For each cluster, an interaction network, or interactome, was built around genes with the highest connectivity (seeds) using an iterative algorithm that systematically connects additional nodes to the initial seed. Details of the IPA program and database can be found at (http://www.ingenuity.com/products/pathways_analysis.html).

Quantitative PCR

In order to confirm the expression array results, quantitative realtime PCR was used to measure gene expression of candidate genes Cd14, Tlr4, S100a9, Bcl2, Fibp, Fn1, and Smad4. The RNA was reverse-transcribed to cDNA using High

Capacity cDNA reverse transcription kit (#4374966, ABI, Foster City, CA), and realtime PCR was performed on each sample in triplicate using Assay-on-demand probe and primer set for mouse Cd14 (Mm00438094_g1, ABI), Tlr4 (Mm00445274_m1, ABI), S100a9 (Mm00656925_m1, ABI), Bcl2 (Mm02528810_s1, ABI), Fibp (Mm00517555_m1, ABI), Fn1 (, ABI), and Smad4 (, ABI) and TaqMan master mix (ABI). Analysis of the amplification was performed using the delta-delta threshold cycle (ddCt) method with 18s rRNA (Hs99999901_s1, ABI) as the endogenous control.

RESULTS

Effect of LPS and MV on Protein and Gene Expression in the Lungs.

In order to determine whether gene expression in whole lung homogenates after 2 hr of treatment with LPS and mechanical ventilation corresponded with protein expression in whole lung homogenates after 3 hr of treatment, the gene expression array data were mined for the cytokines and growth factors that were measured by multiplex ELISA. The patterns of gene and protein expression of IL-6, KC/CXCL1, MIP-2/CXCL2, MCP-1/CCL2, and GM-CSF were very similar in the lungs of adult and juvenile mice, whereas the pattern of IL-1β and TNFα gene and protein expression was similar in the lungs of juvenile but not adult mice (Figures E1 – E4).

In order to confirm the expression array results for candidate genes (Table 1) in juvenile and adult mice treated with LPS+MV, quantitative realtime PCR was performed. For each gene, the average of the log2-transformed results of the adult

mice treated with LPS+MV was subtracted from each data point, and the mean value and upper and lower 95% confidence interval were calculated. The data were transformed back to the linear domain, and the data are shown as the average fold change in expression relative to the untreated, spontaneously breathing adult mice. In six out of the seven candidate genes measured by realtime PCR (Cd14, Tlr4, Bcl2, Fibp, Fn1, and Smad4) the pattern of gene expression was similar as measured by the expression array chips and qRT-PCR. These data confirm that the expression of the Cd14 gene increased more in adult mice treated with LPS+MV than in juveniles (Figure E5). These data also show that Bcl2 and Smad4 increased in the juvenile as compared with adult mice treated with LPS+MV (Figure E6 and E7).

Physiology.

Juvenile mice had lower temperatures than adult mice at time = 0 (‡ $p < 0.01$), but by time = 30 min there were no differences in temperature between any age or treatment group (Figure E8A). The juvenile mice had higher respiratory rates than adult mice at 1 hr (Figure E8B, ★ $p < 0.05$). Juvenile mice treated with MV also had higher respiratory rates than adult mice treated with MV at 2 hr (‡ $p < 0.01$). The juvenile mice treated with LPS+MV had higher peak inspiratory pressures at 3 hr as compared with all other groups of mice. The juvenile mice had higher heart rates than adults at time = 0 (Figure E8C, † $p < 0.001$), but there were no significant differences between any age or treatment groups for all other time points. There were no significant differences in blood pressure between any age or treatment group at any time points during the experiments (Figure E8C). At the end of each

experiment terminal blood gases were obtained by direct right ventricular cardiac puncture. Juvenile mice treated with MV and LPS+MV had lower pH in mixed venous blood as compared with adult mice treated with MV (Figure E8D, $p < 0.01$), whereas there were no significant differences between any other age or treatment groups. The juvenile mice treated with LPS+MV had higher $PmvCO_2$ as compared with adult mice treated with MV and LPS+MV (Figure E8D, $p < 0.05$). Juvenile mice treated with MV had lower mixed venous oxygen saturations ($SmvO_2$) as compared with adult mice treated with MV and LPS+MV (Figure E8E, $p < 0.01$ and $p < 0.05$ respectively). Juvenile mice treated with MV had higher lactates than adult mice treated with MV (Figure E8E, $p < 0.01$), but lactate did not differ between any other age or treatment groups.

Figures.

Figure E1.

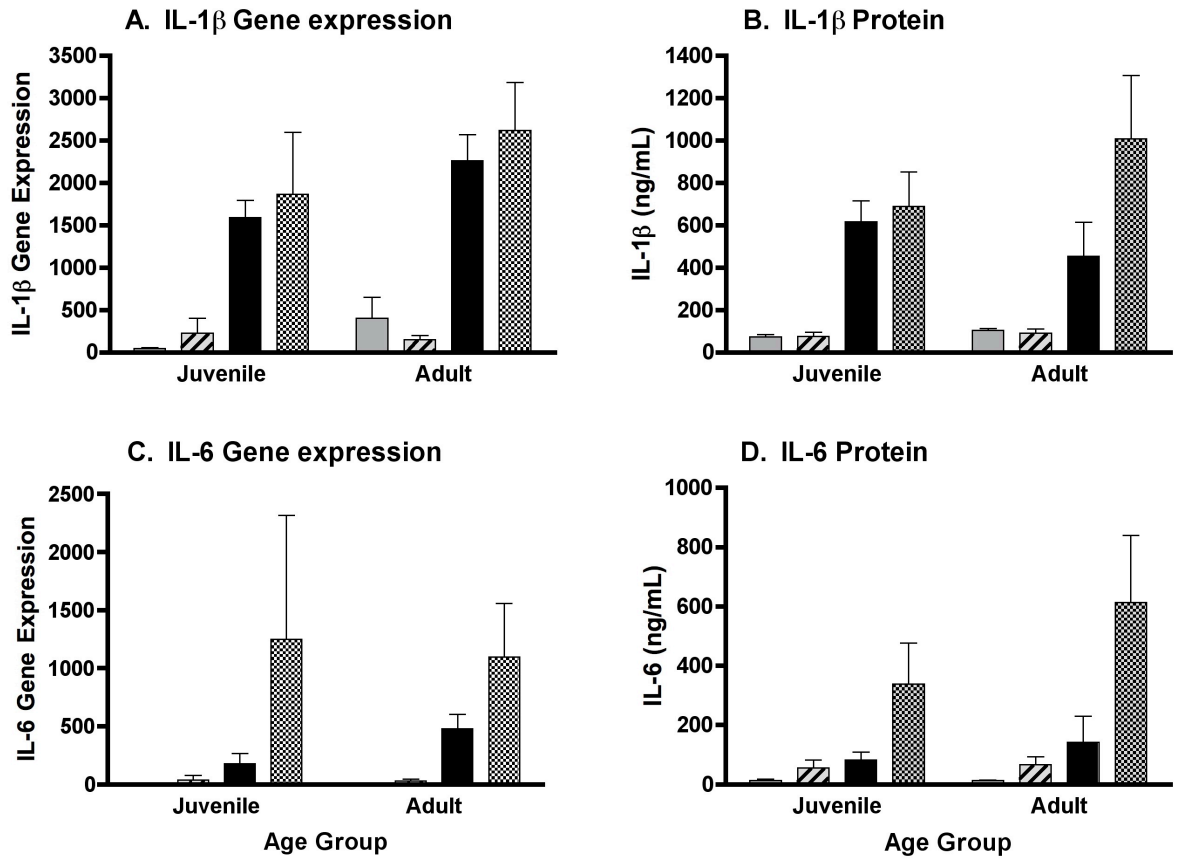

Figure E1. Effect of LPS and MV on gene and protein expression in the lungs. (A) IL-1β gene, (B) IL-1β protein, (C) IL-6 gene, and (D) IL-6 protein expression were measured in the lungs of adult and juvenile mice treated with MV, LPS, and LPS+MV. The gene expression was measured after two hr of treatment, and the protein expression was measured after 3 hr of treatment.

Figure E2.

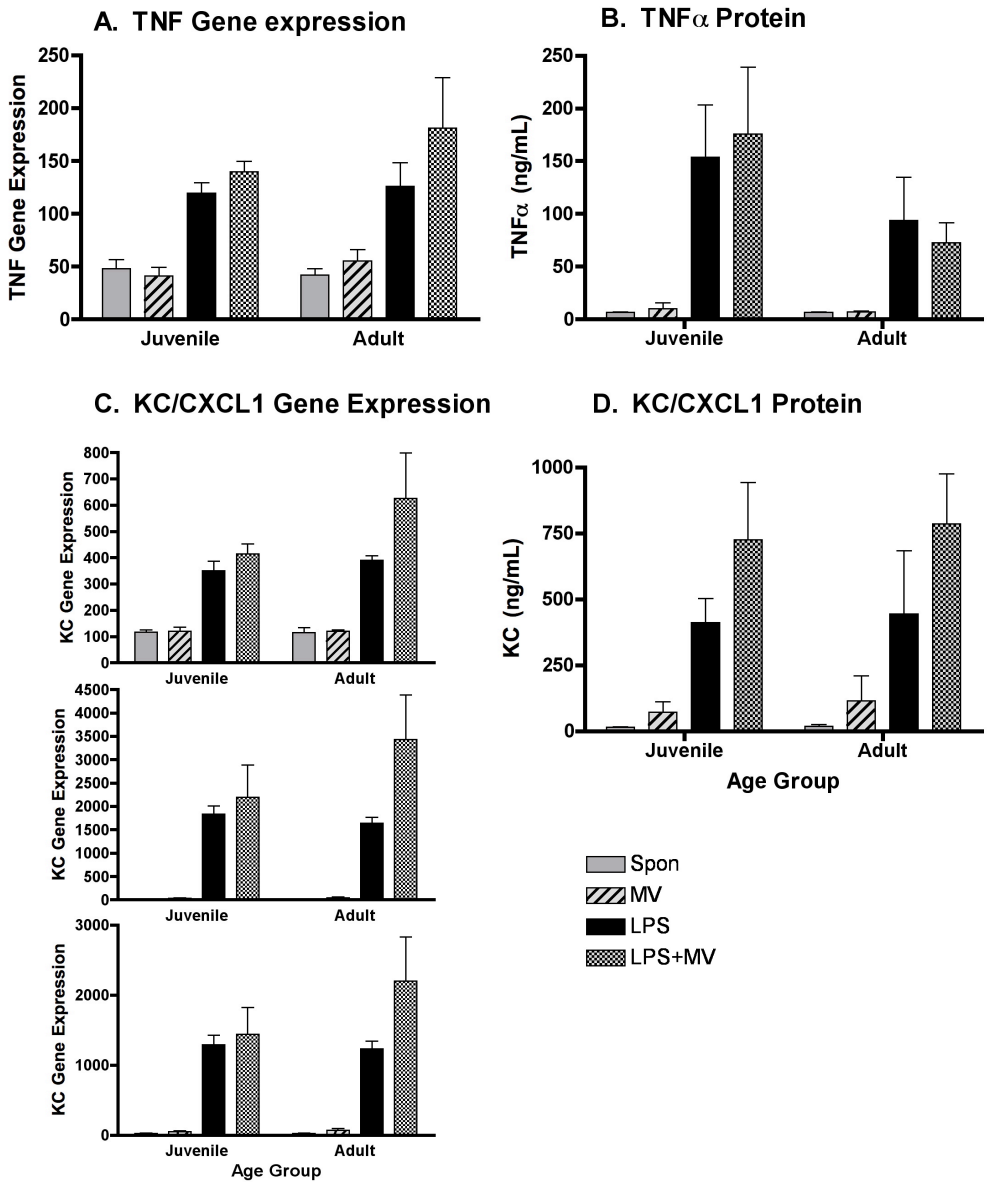

Figure E2. Effect of LPS and MV on gene and protein expression in the lungs. (A) TNFα gene, (B) TNFα protein, (C) three different probes for KC/CXCL1 gene, and (D) KC/CXCL1 protein expression were measured in the lungs of adult and juvenile mice treated with MV, LPS, and LPS+MV. The gene expression was measured after two hr of treatment, and the protein expression was measured after 3 hr of treatment.

Figure E3.

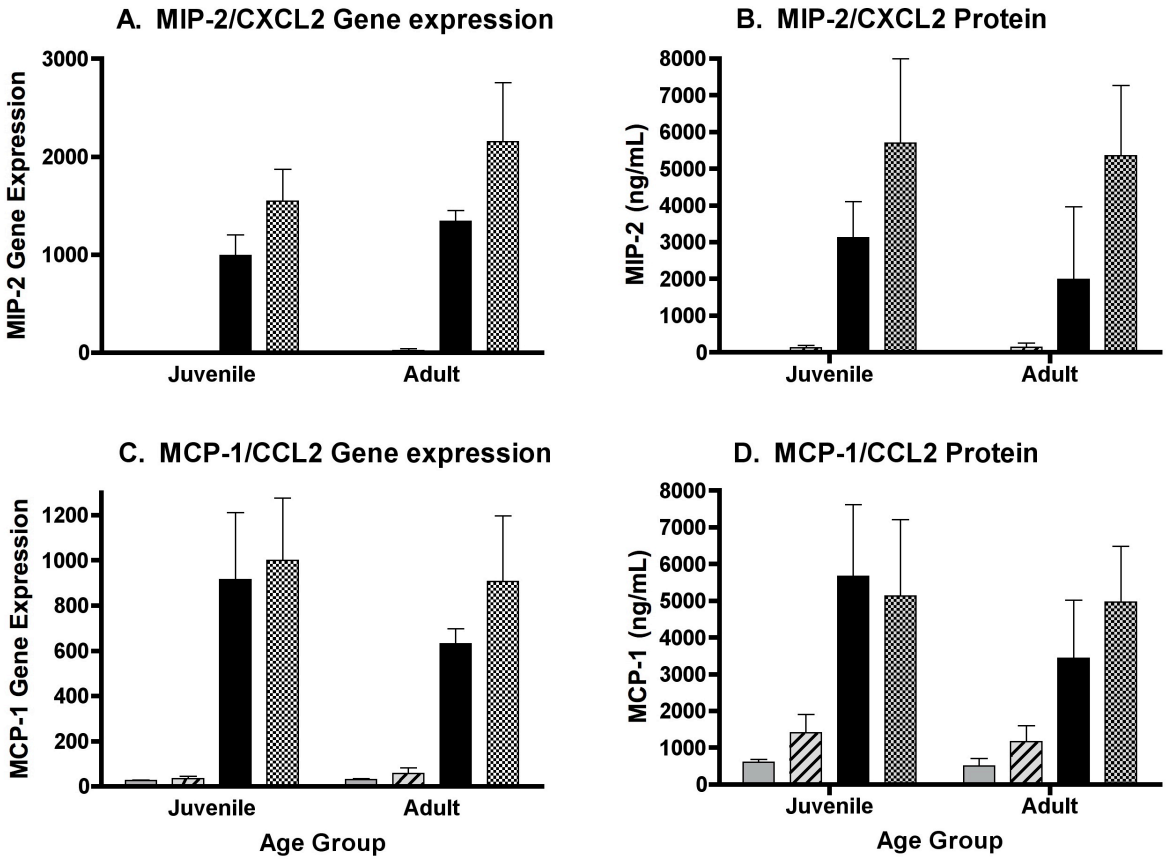

Figure E3. Effect of LPS and MV on gene and protein expression in the lungs. (A) MIP-2 gene, (B) MIP-2 protein, (C) MCP-1/CCL2 gene, and (D) MCP-1/CCL2 protein expression were measured in the lungs of adult and juvenile mice treated with MV, LPS, and LPS+MV. The gene expression was measured after two hr of treatment, and the protein expression was measured after 3 hr of treatment.

Figure E4.

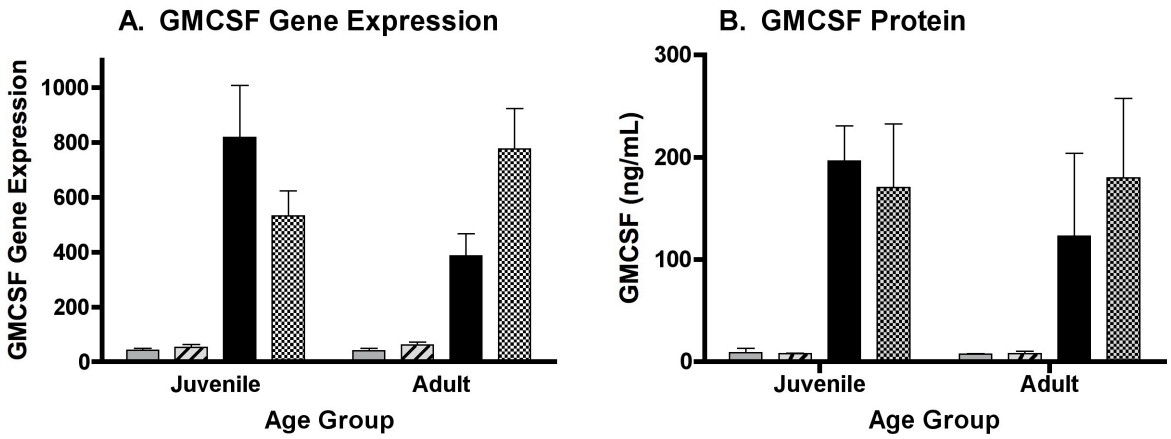

Figure E4. Effect of LPS and MV on GMCSF gene and protein expression in the lungs. (A) GMCSF/Csf2 gene and (B) GMCSF protein expression were measured in the lungs of adult and juvenile mice treated with MV, LPS, and LPS+MV. The gene expression was measured after two hr of treatment, and the protein expression was measured after 3 hr of treatment.

Figure E5.

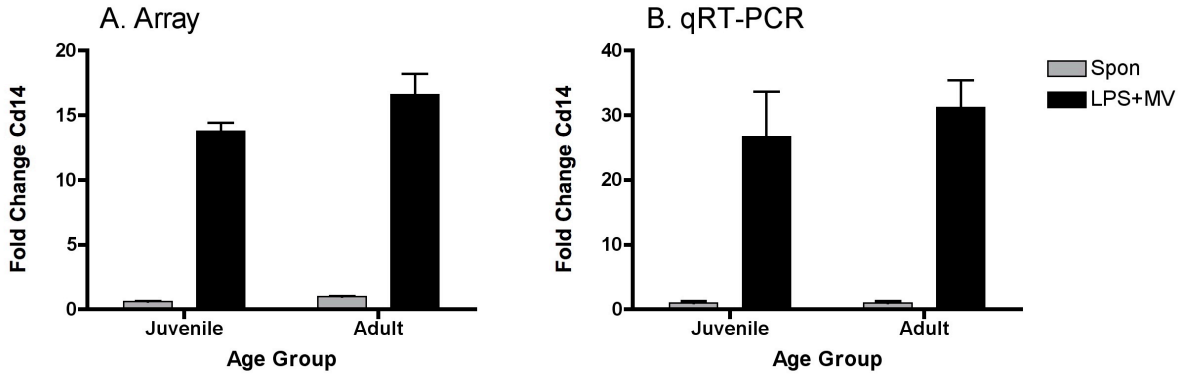

Figure E5. Effect of age and treatment with LPS combined with MV on Cd14 gene expression in the lungs. Cd14 gene expression was measured by (A) the Affymetrix 430 2.0 expression array chip and (B) qRT-PCR in the lungs of juvenile and adult mice treated with LPS+MV. The data are shown as fold change relative to untreated, spontaneously breathing adult mice.

Figure E6.

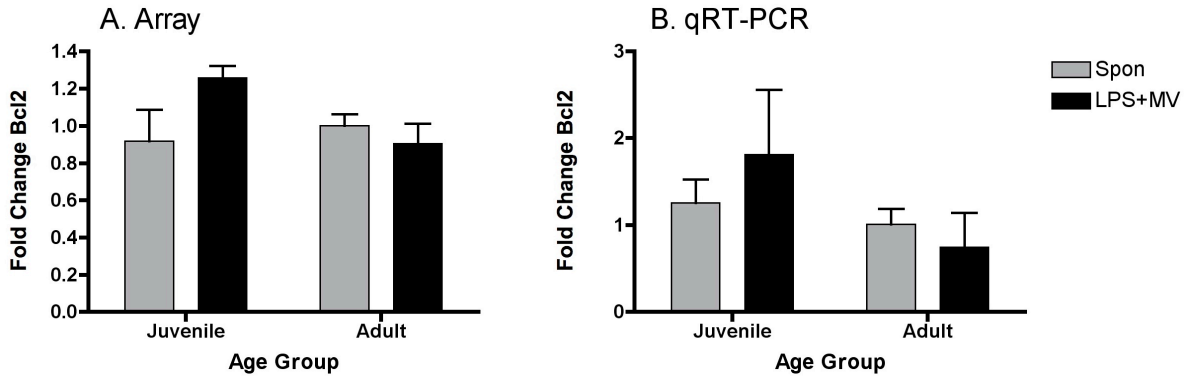

Figure E6. Effect of age and treatment with LPS combined with MV on Bcl2 gene expression in the lungs. Bcl2 gene expression was measured by (A) the Affymetrix 430 2.0 expression array chip and (B) qRT-PCR in the lungs of juvenile and adult mice treated with LPS+MV. The data are shown as fold change relative to untreated, spontaneously breathing adult mice.

Figure E7.

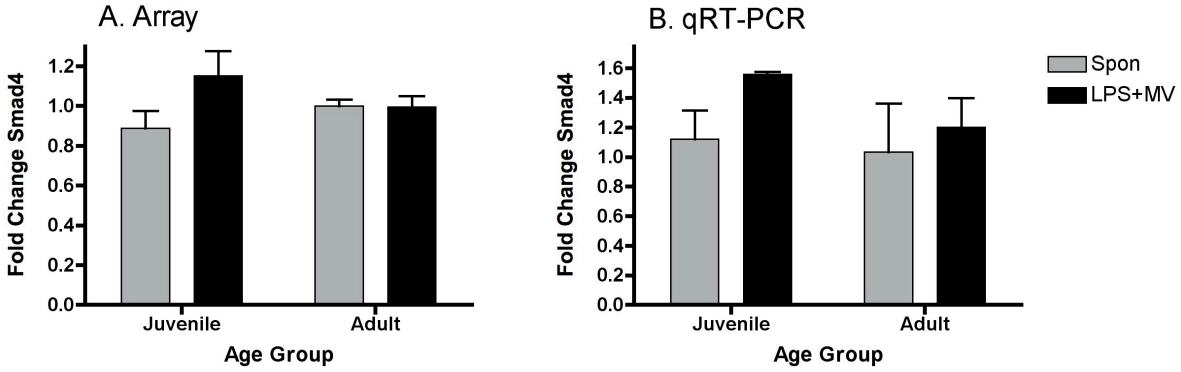

Figure E7. Effect of age and treatment with LPS combined with MV on Smad4 gene expression in the lungs. Smad4 gene expression was measured by (A) the Affymetrix 430 2.0 expression array chip and (B) qRT-PCR in the lungs of juvenile and adult mice treated with LPS+MV. The data are shown as fold change relative to untreated, spontaneously breathing adult mice.

Figure E8. Effect of MV and LPS+MV on physiology.

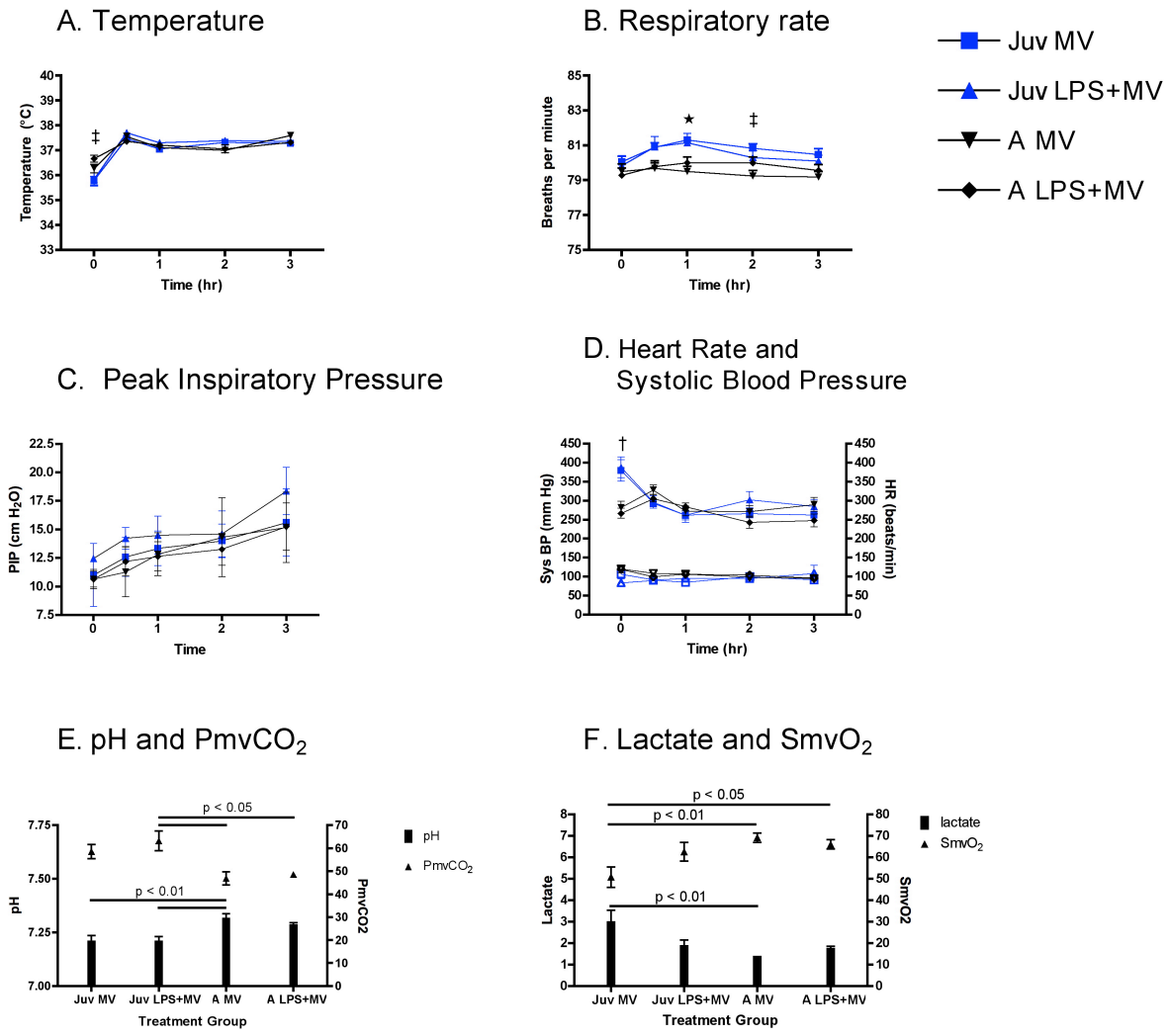

Figure E8. Effects of mechanical ventilation (MV) and LPS combined with MV (LPS+MV) on temperature (A), respiratory rate (B), peak inspiratory pressure (C) heart rate (closed symbols) and systolic blood pressure (open symbols) (D), pH and the partial pressure of carbon dioxide in mixed venous blood (PmvCO2) (E), and lactate and the saturation of oxygen in mixed venous blood (SmvO2) (F) on adult and juvenile mice.

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