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Modulation of Lipopolysaccharide-Induced Gene Transcription and Promotion of Lung Injury by Mechanical Ventilation[1]

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Mechanical ventilation (MV) with tidal volumes of 10–12 ml/kg is considered safe in the absence of acute lung injury (ALI). However, recent studies show that, when lung injury is already present, tidal volumes of this magnitude increase inflammation and injury in the lungs. We hypothesized that MV with tidal volumes of 10-ml/kg can also function as a cofactor in the initiation of ALI by modulating the transcriptional response to bacterial products. To test this hypothesis, we developed a mouse model in which MV did not independently cause inflammation or injury but augmented the inflammatory response to low-dose aspirated LPS and promoted development of ALI. We analyzed gene expression in lungs from 24 mice assigned to four different groups: control, MV only, intratracheal LPS only, and MV + LPS. There were twice as many differentially regulated genes in the MV + LPS group compared with the LPS-only group and 10 times as many differentially regulated genes compared with the MV-only group. For genes up-regulated by LPS treatment alone, the addition of MV further augmented expression. Cytokine concentrations in bronchoalveolar lavage fluid and tissue distribution of an intracellular protein, GADD45-γ, correlated with mRNA levels. We conclude that MV with conventional tidal volumes enhanced the transcriptional response to LPS and promoted development of ALI. ***The Journal of Immunology,*** **2005, 175: 3369–3376.**

Although mechanical ventilation (MV)[3] is a life-saving intervention for critically ill patients, it has also been recognized as a potential contributor to the pathogenesis of acute lung injury (ALI). MV is typically initiated with moderate tidal volumes of 8–12 ml/kg. Although this magnitude of tidal volume exceeds that typically observed at rest during spontaneous respiration, it is consistent with tidal volumes during exercise, is used routinely for prolonged periods in spinal cord injury patients (1), and has been considered safe in the absence of lung injury. Recently, however, tidal volumes of this magnitude have been associated with increased mortality as compared with smaller tidal volumes in patients with severe ALI (the acute respiratory distress syndrome (ARDS)) (2).

Whether MV using moderate tidal volumes can also function as a cofactor in the development of ALI in critically ill patients is an area of active research. In ex vivo nonperfused rat lungs, MV has been shown to augment expression of TNF-α in response to LPS (3, 4). We and others have shown that, in an in vivo rabbit model, MV with moderate tidal volumes of 10–15 ml/kg augments cytokine expression in bronchoalveolar lavage (BAL) fluid in response to different doses of systemic LPS (5, 6). Additionally, MV with moderate tidal volumes augmented inflammation following intratracheal acid instillation, suggesting that this response is not specific to stimulation with microbial products (7).

Because ALI is always associated with an excessive inflammatory response in the lungs (8, 9), it is plausible to speculate that the observed interaction between MV and LPS promotes the development of lung injury over time. Therefore, understanding the mechanism by which MV functions as an immunomodulator may facilitate development of novel therapeutic strategies for respiratory failure. We hypothesized that MV augments the transcriptional response to LPS resulting in enhanced inflammation and lung injury. To test this hypothesis, we developed a murine model of lung inflammation and injury that was dependent on concurrent low-dose LPS exposure and MV with moderate tidal volumes. Using this model we then evaluated transcriptional responses to MV, LPS, and the combination of MV and LPS using high-density oligonucleotide expression microarrays. We found that MV with a tidal volume of 10 ml/kg resulted in no significant pulmonary inflammation or injury and only modest differential gene expression compared with nonventilated controls. However, when MV was combined with LPS there was broad augmentation of gene transcription, which was associated with enhanced inflammation and the development of ALI.

Materials and Methods

LPS preparation

A stock solution of LPS derived from *Escherichia coli* serotype 0111:B4 (List Biological Laboratories) in PBS was stored in small aliquots at −20°C. Immediately before each experiment, an aliquot of LPS was thawed, sonicated, and diluted in PBS to a working concentration of 2.5 μg/ml with PBS.

Experimental protocol

The University of Washington Office of Animal Welfare approved these experiments. C57BL/6 8- to 10-wk-old male mice (Harlan) were housed for a minimum of 5 days in a modified specific pathogen-free facility before all experiments. Mice were randomly assigned to one of four

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[3] Abbreviations used in this paper: MV, mechanical ventilation; ALI, acute lung injury; BAL, bronchoalveolar lavage; PMN, polymorphonuclear leukocyte; IRF-7, IFN regulatory factor-7; GADD, growth arrest and DNA damage.

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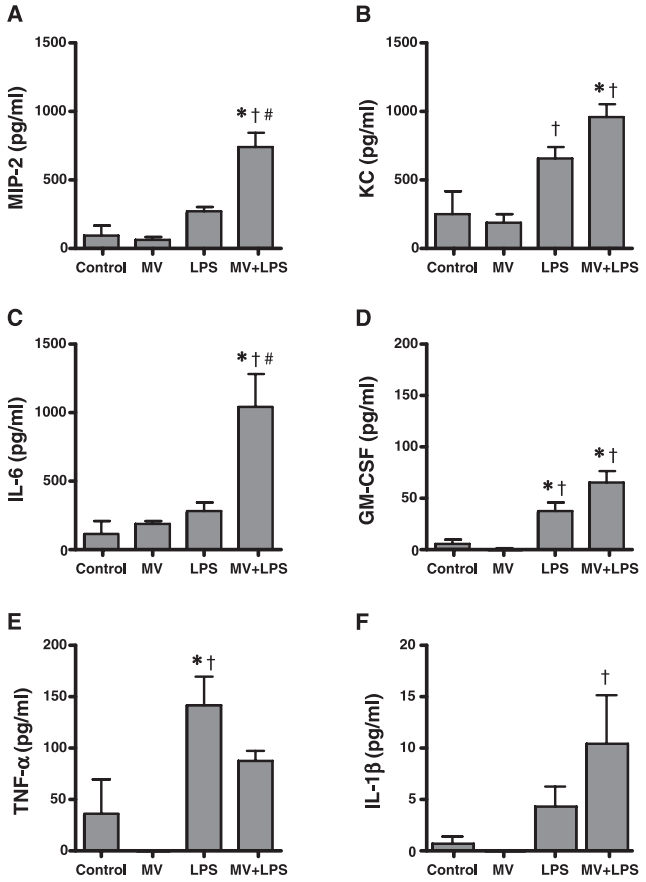

FIGURE 1. BAL fluid cytokine concentrations in the control group, MV-only group, LPS-only group, and MV + LPS group: *A*, MIP-2; *B*, KC; *C*, IL-6; *D*, GM-CSF; *E*, TNF-α; and *F*, IL-1β. $n = 6$/group; $p \leq 0.05$ compared with the control (*), the MV-only (†), and the LPS-only (#) groups.

groups: 1) PBS aspiration without MV (control); 2) PBS with MV; 3) LPS aspiration without MV (LPS), or 4) LPS with MV (MV + LPS).

Each mouse was anesthetized with 4% isoflurane and suspended by its front teeth at a 60° angle. After extending the jaw and tongue, 0.005-μg/gm body weight LPS (2 μl/gm body weight of diluted LPS) or an equal volume of pyrogen-free PBS was deposited in the oropharynx with a pipette (10). Aspiration of the liquid was visually confirmed, and the mouse returned to its cage.

Thirty minutes after aspiration, each mouse was reanesthetized with ketamine (1.3 mg) and xylazine (0.9 mg) i.p. followed by 4% isoflurane for 3 min. Mice assigned to the control or LPS groups were returned to their cages. Mice assigned to MV were positioned at a 60° angle for intubation. After the vocal cords were visualized by extending the jaw and transilluminating the larynx with an external fiber optic light source, the mice were intubated with a 20-gauge angiocatheter (BD Biosciences). The angiocatheter was connected to a 1-ml syringe with a cotton plug in the tip and a small drop of sterile saline in the syringe barrel. Tracheal insertion was confirmed by respiratory motion of the saline droplet at which point the catheter was positioned to a depth of 20 mm at the teeth and secured in place with a drop of superglue (Loctite). The hub of the angiocatheter was removed 25 mm above the catheter tip, and the mice were connected to a ventilator (MiniVent; Harvard Biosciences). Mice were ventilated with a tidal volume of 10 μl/gm body weight, a respiratory rate of 150/min, 0 cm H_2O end-expiratory pressure, and an FIO_2 of 0.21. In preliminary studies this ventilation strategy produced normal arterial blood pH values (7.36 $\pm$ 0.08, $n = 4$). MV was continued for either 4 or 6 h with anesthesia maintained by 1% isoflurane. Mixed expired CO_2 (Novametrix) and airway pressure was continuously monitored. Body temperature was monitored by a rectally inserted thermistor and regulated by a heating lamp. At the end of the MV period, the mouse was euthanized by i.p. sodium pentothal and exsanguination by cardiac puncture.

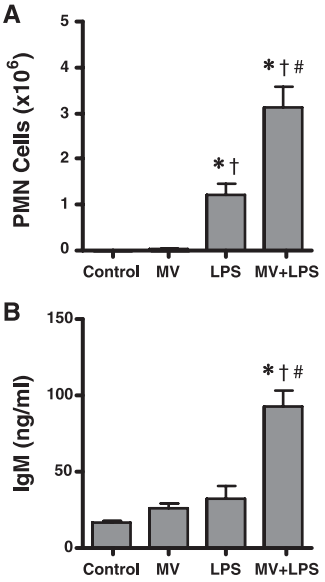

FIGURE 2. BAL fluid total PMN cell count (*A*) and IgM concentration (*B*) from the MV-only, LPS-only, and MV + LPS groups at 6 h vs the 4 h control group. $n = 6$/group; $p \leq 0.05$ compared with control (*), MV-only (†), and LPS-only (#) groups.

BAL fluid analysis

For one series of experiments, mice were euthanized after either 4 or 6 h of ventilation, and BAL of both lungs was performed using four 1-ml aliquots of PBS containing 0.6 mM EDTA. Total cell count was determined using a hemacytometer. Differential cell count was determined by cytocentrifugation and Wright-Giemsa staining. The remaining fluid was spun at 1200 $\times$ *g* for 15 min, and the supernatant was removed and stored in small aliquots at -80°C for batch protein analysis.

BAL fluid concentrations of MIP-2, mouse growth-related oncogene homologue (KC), IL-1β, IL-6, IL-10, IL-12, TNF-α, GM-CSF, and IFN-γ were determined by multiplex immunoassay (Luminex 100) using cytokine-specific bead kits (R&D Systems) according to the vendors' protocols. BAL fluid concentrations of IgM and albumin were determined by ELISA (Bethyl Laboratories).

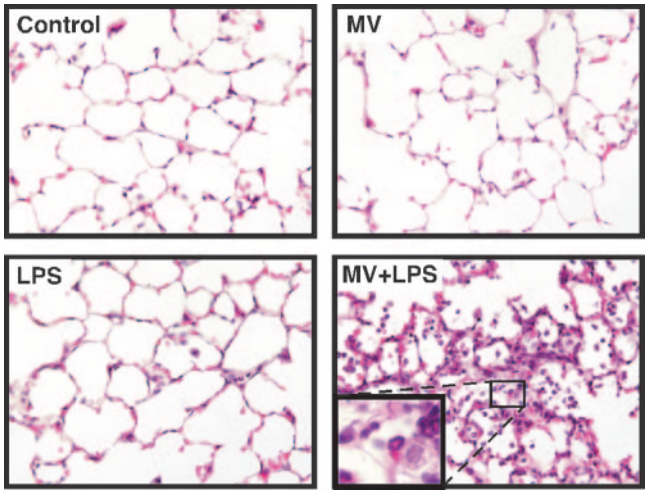

FIGURE 3. Representative histological images from the control group at 4 h and the MV-only, LPS-only, and MV + LPS groups at 6 h. The higher magnification insert for the MV + LPS histology reveals the PMN component of the cellular infiltrate as well as fibrin stranding in the alveolar space.

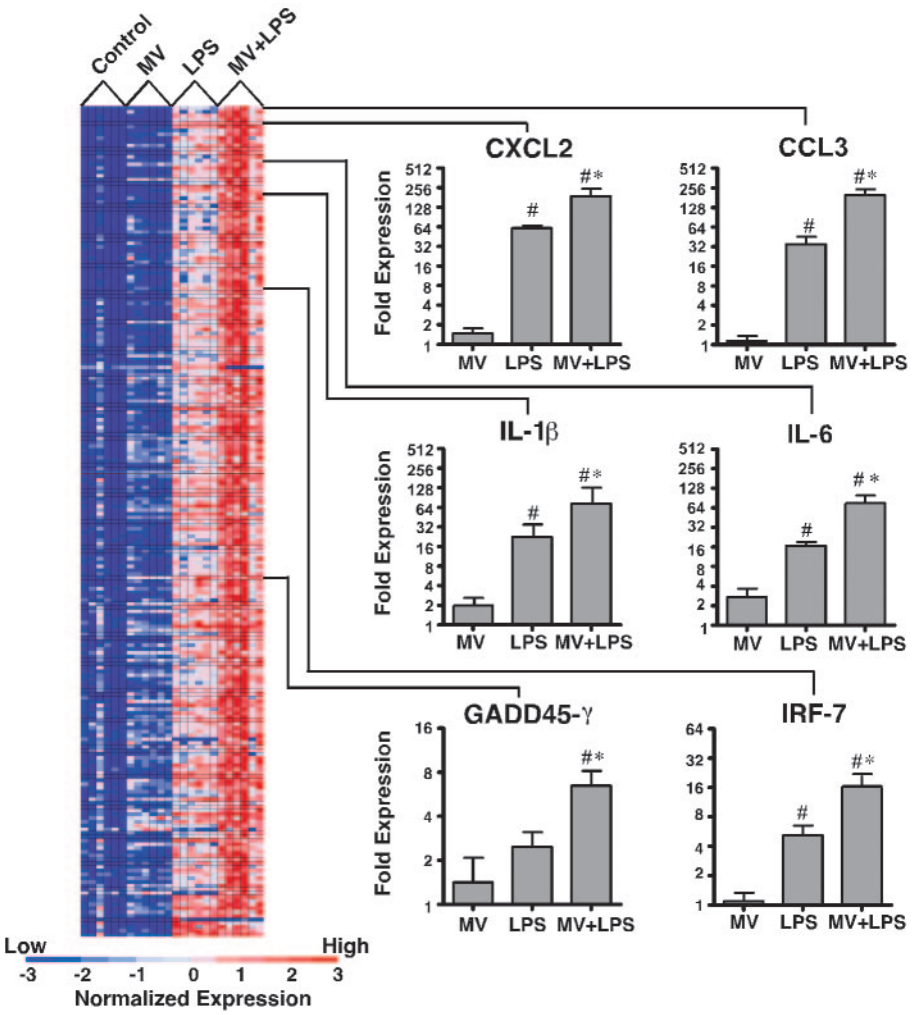

FIGURE 4. Heat map showing row normalized expression patterns across the control, MV, LPS, and MV + LPS groups for the 217 genes expressed at >3-fold above the control group in at least one of the other groups (*left side*). Independent confirmation of gene expression patterns relative to the control group and normalized to 18s RNA for selected genes by using quantitative RT-PCR (*right side*). $n = 6$/group; $p \leq 0.05$ compared with the MV-only (#) or the LPS-only (∗) groups.

Histology and immunohistochemistry

In two mice per group, the lungs were removed after 6 h and fixed at 20 cm H_2O with 4% paraformaldehyde. After fixation, the lungs were embedded in paraffin, cut into 4-μm sections, and stained with H&E. Immunohistochemistry was performed with Vectastain ABC kit (Vector Laboratories). Briefly, slides were deparaffinized by washing twice in xylene for 5 min and rehydrated by washing twice in 100% ethanol for 3 min, twice in 95% ethanol for 3 min, twice in 70% ethanol for 3 min, and twice in distilled H_2O for 5 min. Slides were rinsed twice with PBS for 5 min. Ag unmasking was performed by bringing Citrate Buffer (Vector Laboratories) to a boil and then allowing the slides to simmer for an additional 10 min. After the 10-min boil, the solution was allowed to cool for 20 min before removing the slides. After digestion, slides were rinsed three times in distilled water for 5 min each. The slides were then incubated with 1% H_2O_2 in methanol for 30 min to block endogenous peroxidases. Slides were again washed with distilled water three times for 5-min each and once with PBS for 5 min before blocking with normal goat serum (Vector Laboratories) for 60 min. Then the samples were labeled with chicken anti-human growth arrest and DNA damage 45-γ (GADD45-γ) (Chemicon International) overnight in a moist chamber at 4°C. Next, the slides were rinsed three times with PBS and labeled with rabbit anti-chicken biotinylated Ab (Vector Laboratories) at a 1/200 dilution for 30 min at room temperature. The slides were rinsed twice with PBS, labeled with the Vectastain ABC kit, incubated in a moist chamber for 30 min at room temperature, and rinsed three times with PBS. Slides were developed in a moist chamber with diaminobenzidine substrate (Sigma-Aldrich) for 8–12 min. The slides were rinsed twice with deionized H_2O and counterstained with 1% methyl green for 5 min.

Expression microarray analysis

In a second set of experiments, mice were euthanized after 4 h of ventilation. The lungs were flushed with 2 ml of iced saline via the right ventricle, and then homogenized in guanidinium isothiocyanate lysis solution with a rotor-stator homogenizer (Omni International). Total RNA was extracted using the RNeasy midi kit (Qiagen), and any remaining genomic DNA was removed with DNase I treatment (DNA-free; Ambion). RNA quality was assessed by determining the 26S/18S ratio (Agilent 2100 Bioanalyzer).

Complete details of experiment design, hybridization procedures, and raw microarray data in compliance with Minimum Information About a Microarray Experiment (MIAME) have been deposited at the Gene Expression Omnibus web site (⟨www.ncbi.nlm.nih.gov/projects/geo/⟩, query GSE 2411). Briefly, for each mouse, labeled cRNA was prepared from total RNA and hybridized to an Affymetrix GeneChip 430A oligonucleotide microarray with minor modifications from Affymetrix recommended protocols as detailed at the University of Washington's Center for Expression Arrays web site (⟨http://ra.microslu.washington.edu⟩). Microarrays were scanned using the GeneChip Scanner and image analysis was performed with Affymetrix MAS 5.0 software. Background adjustment and quantile normalization of microarray data was performed using the Robust Multi-chip Average algorithm (11). Statistically significant differential gene expression for each perturbation (MV, LPS, MV + LPS) relative to control mice was determined using significance analysis of microarrays (12) and a predetermined false discovery rate of 1%. To calculate gene expression changes relative to the control group, all data were $\log_2$-transformed. For each gene, the average of the $\log_2$-transformed results from the six control mice was subtracted from each data point within all four of the groups, and the mean value and upper and lower limits of the 95% confidence interval were calculated. The data were transformed back to the linear domain to give the average fold change in expression with 95% confidence interval for each gene within each group relative to the control group.

For confirmatory quantitative RT-PCR, first-strand cDNA was transcribed from 2 μg of total RNA using random hexamer primers, and Superscript II (Invitrogen Life Technologies). Quantitative cDNA amplification was performed using an ABI 5700 real-time PCR machine (Applied

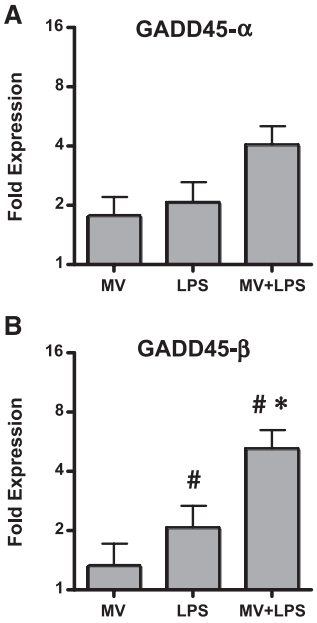

FIGURE 5. Expression of GADD45-α (*A*), and GADD45-β (*B*) in whole lung homogenate. Expression is relative to the control group normalized to 18s RNA. $n = 6$/group; $p \leq 0.05$ compared with the MV-only (#) or the LPS-only (*) groups.

Biosystems), gene-specific TaqMan primer-probe mixes (Applied Biosystems), and PCR conditions as specified by the manufacturer. All samples were run in duplicate. Target gene expression was quantified relative to 18s RNA from the same reverse transcription reaction.

Statistical analysis

Nonmicroarray data were compared among groups by ANOVA with posthoc comparisons using Tukey's HSD test. Statistical significance was assigned at $p < 0.05$. All analyses were done using JMP software (SAS). Data are presented as mean ± SEM.

Results

MV augments lung inflammation and injury in response to LPS

We measured concentrations of cytokines in BAL fluid at both the 4- and 6-h times. IL-10, IL-12, and IFN-γ were below the threshold of detection in all samples at both times. After 4 h, the combination of MV and LPS (MV + LPS group) caused increased concentrations of MIP-2 (745 ± 107 pg/ml), KC (958 ± 100 pg/ml), and IL-6 (1041 ± 243 pg/ml) compared with the other three groups ($p < 0.05$, Fig. 1). A similar trend was observed for both IL-1β and GM-CSF, although the differences between the MV + LPS group and the LPS group were not statistically significant. In contrast, mice in the LPS-only group had significant increases in the concentrations of three cytokines, KC, GM-CSF, and TNF-α, as compared with the control group. There were no differences between the MV group and the control group for any of the cytokines measured.

At 4 h, there was early lung injury in the MV + LPS group compared with the control group as indicated by total polymorphonuclear leukocyte (PMN) count in the BAL fluid ($279 \pm 67 \times 10^4$ vs $1.2 \pm 0.5 \times 10^4$, $p < 0.05$) and by permeability to IgM (39.6 ± 4.7 vs 17.1 vs 0.4 ng/ml, $p < 0.05$). PMN counts and IgM concentrations of the MV-only and the LPS-only groups were all significantly less than the MV + LPS group. The PMN count from the LPS-only group trended higher than the control group, but the difference was not statistically significant ($111 \pm 15 \times 10^4$ vs $1.2 \pm 0.5 \times 10^4$, $p > 0.05$).

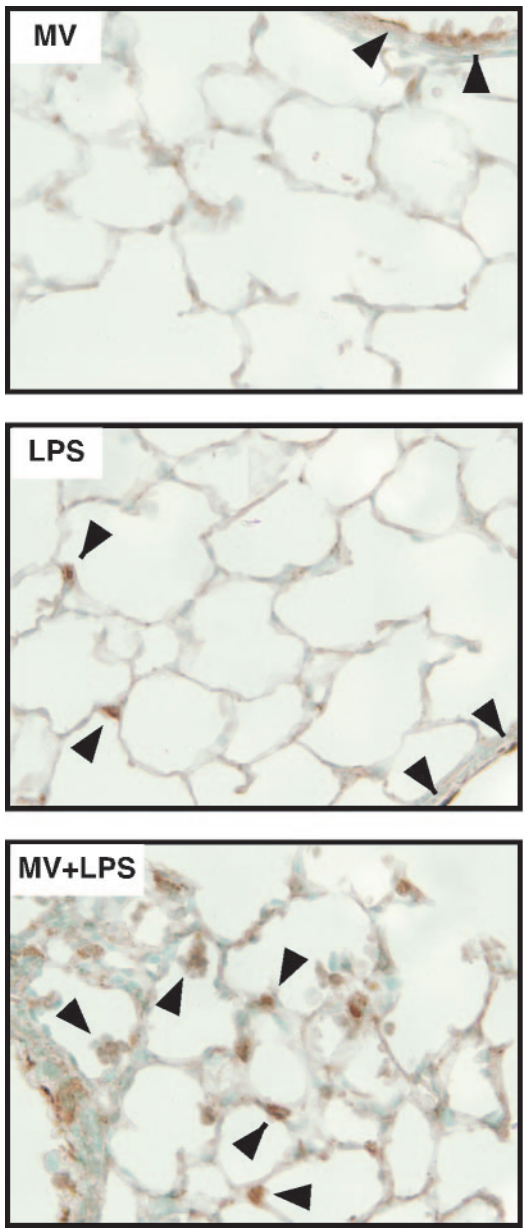

FIGURE 6. Immunohistochemistry identifying expression of GADD45-γ in animals from the MV-only, the LPS-only, and the MV + LPS groups. Arrows identify positive staining cells. All three animals have expression in airway epithelium. GADD45-γ staining is increased in the MV + LPS animal.

Inflammation and ALI were also evaluated in 18 mice divided among the MV, LPS, and MV + LPS groups ($n = 6$/group) after 6 h of MV or spontaneous respiration. These groups were compared with the previous control group. Total PMN counts in BAL fluid followed the same pattern as at 4 h except that the LPS-only group was significantly higher than the control and MV-only group (Fig. 2*A*). There were no changes within each group as compared with the 4-h time. The IgM concentrations in BAL fluid were higher for the MV-only, LPS-only, and MV + LPS groups compared with the 4-h time; however, the MV + LPS group remained the only group statistically different from Control (93.2 ± 9.9 vs 17.1 ± 0.4 ng/ml, $p < 0.05$, Fig. 2*B*). Albumin concentrations in BAL fluid had the same pattern as IgM concentration across all four groups. There was a statistically significant difference between the MV + LPS group at 6 h compared with the control

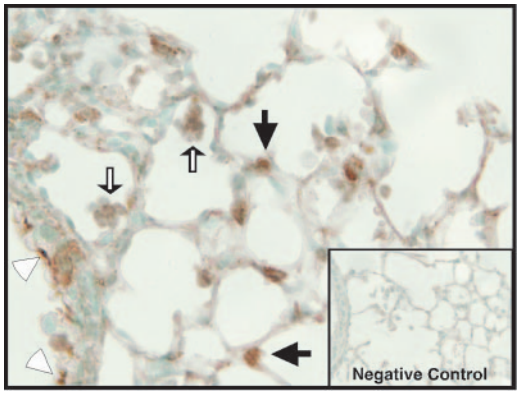

FIGURE 7. GADD45-γ staining in a lung section from an animal treated with both MV and LPS. GADD45-γ is present in airway epithelium (open arrowheads), large intra-alveolar cells (open arrows), and alveolar wall cells (solid arrows). *Inset*, A negative control in which the anti-GADD45-γ Ab was excluded.

group (122.4 ± 20.5 vs 23.5 ± 1.1 μg/ml, $p < 0.05$). Mean albumin concentration tended to be higher in the MV-only (72.7 ± 9.2 μg/ml) and LPS-only (66.3 ± 21.9 μg/ml) groups compared with the control group. Peak airway pressures in the mechanically ventilated animals were similar at baseline between the MV + LPS and MV-only groups (10.8 ± 0.4 vs 11.7 ± 1.4 cm H_2O). After 6 h, airway pressures had increased in the MV + LPS group but not in the MV-only group (14.0 ± 1.3 vs 11.8 ± 1.0 cm H_2O, $p = 0.008$), suggesting decreased compliance in the MV + LPS group but not in the MV-only group.

Lungs were prepared for histological evaluation for two animals in each of the four groups at the 6-h time. Lungs from the MV-only group were not qualitatively different from those from the control group (Fig. 3). In the LPS-only group, there was increased cellularity without edema or septal thickening. In the MV + LPS group, there was increased cellularity, septal thickening, and perivascular and peribronchial edema consistent with early lung injury.

MV augments the transcriptional response to LPS

Gene expression was assessed in six mice from each group after 4 h of MV or spontaneous breathing. Comparisons were made between the Control group and the MV, LPS, and MV + LPS groups. At a false discovery rate of 1%, a total of 6136 genes were differentially expressed in at least one of the MV, LPS, or MV + LPS groups compared with the control group. There were 324 genes up-regulated and 169 genes down-regulated in the MV group, and 1099 genes up-regulated and 1832 genes down-regulated in the LPS group. The greatest number of differentially expressed genes was in the MV + LPS group with 1976 genes up-regulated and 3753 genes down-regulated. Thus, the combination of MV with LPS stimulation resulted in twice as many differentially regulated genes as compared with the LPS-only group and over 10 times as many genes as in the MV-only group.

If only highly up-regulated genes were considered, there were 217 genes with >3-fold expression in at least one of the intervention groups compared with the control group. Within this subgroup of highly expressed genes, 98.6% had >3-fold up-regulation in the MV + LPS group as compared with 48.8% in the LPS-only group and 5.5% in the MV group. When the expression of this subgroup of genes was examined across the three interventions, there was a

Table I. *Expression pattern of genes that were also identified in a separate cross-species study of ventilator-induced (large tidal volume) lung injury (24)*

Gene Symbol	Gene ID (Entrez gene)	Control	MV	LPS	MV + LPS
Admr	11536	1.0 (0.9–1.1)	0.7 (0.6–0.8)	0.7 (0.6–0.8)	0.5 (0.5–0.6)
Aqp1	11826	1.0 (0.9–1.1)	0.8 (0.8–0.9)	0.9 (0.8–1.0)	0.5 (0.5–0.6)
Arg2	11847	1.0 (0.8–1.3)	2.0 (1.7–2.2)	3.5 (3.3–3.8)	4.6 (4.1–5.2)
Atf3	11910	1.0 (0.9–1.1)	1.5 (1.4–1.7)	2.3 (1.9–2.8)	4.9 (3.6–6.6)
Bhlhb2	20893	1.0 (0.8–1.2)	1.3 (1.2–1.5)	1.4 (1.2–1.6)	2.2 (1.8–2.8)
Btg1	12226	1.0 (0.9–1.2)	1.1 (1.1–1.2)	1.6 (1.5–1.8)	2.0 (1.9–2.2)
Ccl2 (MCP-1)	20296	1.0 (0.8–1.3)	0.8 (0.8–0.9)	2.8 (2.2–3.6)	5.4 (3.2–9.1)
Cdkn1a	12575	1.0 (0.8–1.3)	1.3 (1.2–1.5)	2.0 (1.6–2.6)	2.1 (1.9–2.2)
Cebpb	12608	1.0 (0.9–1.2)	1.5 (1.3–1.8)	1.7 (1.5–2.1)	2.7 (2.0–3.5)
Cebpd	12609	1.0 (0.9–1.2)	2.2 (1.9–2.5)	2.8 (2.6–3.1)	3.5 (3.1–3.9)
Cycs	13063	1.0 (1.0–1.1)	1.2 (1.1–1.2)	1.3 (1.2–1.3)	1.6 (1.5–1.7)
Dnaja1	15502	1.0 (0.9–1.1)	1.4 (1.3–1.6)	1.1 (1.0–1.2)	1.6 (1.4–1.8)
Egfr	13649	1.0 (0.9–1.1)	0.9 (0.9–1.0)	1.6 (1.5–1.6)	1.5 (1.4–1.7)
Eif2s1	13665	1.0 (0.9–1.2)	1.2 (1.2–1.3)	1.3 (1.2–1.4)	1.8 (1.7–2.0)
F3	14066	1.0 (0.8–1.3)	1.8 (1.5–2.1)	1.5 (1.3–1.8)	3.0 (2.2–4.1)
Gadd45α	13197	1.0 (0.9–1.2)	2.1 (1.8–2.4)	2.0 (1.7–2.3)	2.6 (2.2–3.0)
Gbp2	14469	1.0 (0.6–1.7)	1.0 (0.9–1.1)	4.6 (3.9–5.5)	7.3 (5.7–9.3)
Gch1	14528	1.0 (0.8–1.3)	1.2 (1.1–1.4)	2.5 (2.4–2.7)	3.7 (3.1–4.4)
Gclc	14629	1.0 (0.8–1.4)	1.2 (1.1–1.4)	2.4 (2.3–2.6)	2.1 (2.0–2.1)
Gja1	14609	1.0 (0.8–1.3)	1.2 (1.2–1.3)	2.3 (2.0–2.5)	2.9 (2.6–3.2)
Hspa8	15481	1.0 (0.9–1.2)	1.5 (1.3–1.6)	1.1 (1.0–1.2)	1.9 (1.8–2.1)
Ifrd1	15982	1.0 (0.9–1.1)	1.1 (1.0–1.3)	1.4 (1.3–1.6)	1.5 (1.3–1.8)
Il1b	16176	1.0 (0.7–1.6)	1.4 (1.2–1.7)	7.2 (6.2–8.3)	10.3 (7.7–13.8)
Il1r2	16178	1.0 (0.7–1.4)	3.9 (3.1–5.0)	8.9 (6.9–11.5)	13.7 (11.3–16.6)
Il6	16193	1.0 (0.9–1.2)	1.3 (1.1–1.5)	3.3 (2.6–4.3)	9.1 (5.2–15.8)
Lgals3	16854	1.0 (0.9–1.2)	1.4 (1.2–1.5)	1.2 (1.1–1.2)	1.8 (1.6–2.1)
Plaur	18793	1.0 (0.9–1.2)	1.7 (1.6–1.8)	2.0 (1.8–2.2)	3.5 (2.8–4.3)
Plcg2	234779	1.0 (0.9–1.1)	1.1 (1.0–1.1)	1.1 (1.0–1.2)	1.5 (1.3–1.6)
Ptgs2 (Cox-2)	19225	1.0 (0.9–1.1)	1.0 (0.9–1.2)	1.3 (1.2–1.4)	2.8 (1.9–4.3)
S100a9	20202	1.0 (0.6–1.8)	2.6 (2.2–3.1)	4.3 (3.6–5.2)	4.7 (4.0–5.5)
Serpine1 (PAI-1)	18787	1.0 (0.9–1.2)	2.1 (1.9–2.3)	4.0 (3.5–4.6)	7.7 (5.0–11.9)
Tcf21	21412	1.0 (0.9–1.2)	1.1 (1.0–1.2)	0.7 (0.6–0.8)	0.6 (0.4–0.8)

Table II. *Expression pattern of genes that were also identified as up-regulated by day +2 following intratracheal bleomycin instillation (27)*

Gene Symbol	Gene ID (Entrez gene)	Control	MV	LPS	MV + LPS
Ccl2 (MCP-1)	20296	1.0 (0.8–1.3)	0.8 (0.8–0.9)	2.8 (2.2–3.6)	5.4 (3.2–9.1)
Ccl7 (MCP-3)	20306	1.0 (0.8–1.3)	0.9 (0.9–1.0)	1.8 (1.5–2.1)	2.9 (1.9–4.4)
Cdkn1a (CDK11)	12575	1.0 (0.8–1.3)	1.3 (1.2–1.5)	2.0 (1.6–2.6)	2.1 (1.9–2.2)
G1p2 (ISG15)	53606	1.0 (0.7–1.4)	0.9 (0.8–1.0)	6.2 (4.9–7.9)	12.1 (9.3–15.8)
Gadd45α	13197	1.0 (0.9–1.2)	2.1 (1.8–2.4)	2.0 (1.7–2.3)	2.6 (2.2–3.0)
Ifi1	15944	1.0 (0.8–1.4)	1.0 (0.9–1.2)	3.4 (3.0–3.9)	3.8 (3.1–4.8)
Ifit1 (GARG16)	15957	1.0 (0.6–1.7)	1.1 (0.9–1.4)	9.4 (8.0–11.0)	12.1 (9.3–15.8)
Irf7	54123	1.0 (0.9–1.2)	1.1 (1.0–1.3)	3.6 (3.1–4.3)	6.9 (5.2–9.1)
Lcn2 (Lipocalin 2)	16819	1.0 (0.6–1.7)	0.9 (0.7–1.2)	2.8 (2.7–2.9)	2.7 (2.6–2.9)
Saa3	20210	1.0 (0.6–1.8)	2.3 (1.7–3.3)	15.3 (14.0–16.7)	22.8 (20.3–25.6)
Stat1	20846	1.0 (0.7–1.5)	1.0 (1.0–1.1)	1.8 (1.4–2.3)	3.3 (2.6–4.1)
Timp1	21857	1.0 (0.8–1.3)	1.0 (0.9–1.1)	2.6 (2.3–3.0)	5.9 (4.3–8.0)

pattern of increasing up-regulation comparing the LPS-only group with the MV + LPS group (Fig. 4). A number of these genes did not have well-annotated biological functions; however, when a subset of 135 genes was grouped by biological function, the largest number (53) were found to be involved in immunity/inflammation response (online supplemental data).[4] Included within this highly up-regulated group of genes were chemokines (e.g., CCL3, CXCL2), other cytokines (e.g., IL-6, IL-1β), and genes that participate in the acute transcriptional response to stress, such as components of the MAPK cascade (e.g., GADD45-γ) and transcription factors (e.g., IFN regulatory factor-7 (IRF-7)). Quantitative RT-PCR confirmed augmented expression of these genes when MV was combined with LPS exposure (Fig. 4).

We chose to further investigate GADD45 because it is a cytoplasmic protein that can be localized to specific cell types by immunohistochemistry. The GADD45 family includes three isoforms (-α, -β, and -γ), which are transcriptionally up-regulated following exposure to mitogenic stimuli such as ionizing or UV radiation (13). Both GADD45-γ and -β bind to and activate MEK kinase 4 (MEKK4) ultimately leading to phosphorylation and activation of the MAPKs p38 and JNK (14–16). Quantitative RT-PCR analysis confirmed that all three isoforms of GADD45 were up-regulated (Figs. 4 and 5) in our model. GADD45-γ, by immunohistochemistry, showed protein expression corresponding with the up-regulation in mRNA (Fig. 6). In the MV + LPS group, GADD45-γ was identified in alveolar wall cells and in large intra-alveolar cells suggesting up-regulation in both alveolar epithelium and macrophages, but not in neutrophils recruited to the air spaces (Fig. 7).

Discussion

We hypothesized that MV with a noninjurious and clinically relevant tidal volume would augment the transcriptional response to LPS, resulting in enhanced inflammation and early ALI. The main findings of this study were that: 1) MV with a tidal volume of 10 ml/kg for 6 h did not cause significant cytokine expression, PMN infiltration into the lung, or increased permeability of the alveolar-capillary barrier; 2) low dose LPS exposure by pharyngeal aspiration caused modest cytokine expression at both mRNA and protein levels and PMN migration to the alveolar space but did not cause significant lung injury; 3) MV with a clinically noninjurious tidal volume augmented lung inflammation and ALI in response to low-dose LPS exposure, and 4) this augmented response was associated with broad modulation of the acute transcriptional response in the lungs.

[4] The online version of this article contains supplemental material.

The current study significantly extends previous reports of interaction between MV and LPS (5, 6). By using intratracheal instillation of LPS instead of systemic injection, we eliminated the possibility that augmented inflammation resulted from MV-induced systemic-to-alveolar translocation of LPS or LPS-induced early response cytokines (17, 18). Additionally, we used microarray analysis to evaluate the role of transcription. We found that differential gene expression was greatest in the group that received both MV and LPS. Although alterations in the posttranscriptional mRNA half-life cannot be excluded in this in vivo model, increases in both the total number of differentially expressed genes and in the magnitude of fold change in expression for multiple genes in the MV + LPS group suggested that gene transcription was modulated by combining MV with LPS. Transcriptional modulation by MV is supported by several published studies, which show that stretching lung-derived cells in vitro activates transcription factors, which promote proinflammatory cytokine transcription (19–21). Activation of the transcription factors NF-κB and AP-1 occurs when lungs are ventilated ex vivo and in vivo with moderate to large tidal volumes (5, 22, 23).

We compared the results of our gene expression data with those from two previously published models of lung injury to identify genes that were consistently differentially expressed and, therefore, were more likely to have an important role in the pathogenesis of ALI. In one study, microarray results from ventilator-induced models of lung injury in mice, rats, and dogs and from human pulmonary endothelial cell cultures subjected to in vitro cyclical stretch were compared to identify 69 candidate genes with differential expression (64 up-regulated and 5 down-regulated) across multiple species (24). Of these 69 genes, we identified 32 genes that were differentially expressed in the same direction (29 increased, 3 decreased) in the MV + LPS group of our study (Table I). Included within this list are multiple genes involved with inflammation (CCL2, IL-1β, IL-6), regulators of transcription (activating transcription factor-3, CCAAT/enhancer binding protein-β and -δ), MAPK cascade elements (GADD45-α), and other genes associated with lung injury (F3 or tissue factor and Serpine1 or plasminogen activator inhibitor-1) (25, 26). For the majority of these genes, combining MV and LPS resulted in a larger change in expression compared with either LPS or MV alone. Using a different model of intratracheal bleomycin-induced pulmonary fibrosis, Kaminski et al. (27) evaluated the temporal pattern of gene expression in the lung. Several genes identified by these investigators were also up-regulated in our model (Table II), including GADD45 (isoform not identified), regulators of transcription (STAT-1, IRF-7), and chemokines (CCL2, CCL7). Notably,

GADD45 expression was increased in all three studies. We further examined GADD45 expression and confirmed up-regulation of all three GADD45 isoforms. Additionally, we showed that GADD45-γ protein was expressed in lung tissue. GADD45-γ was present in lung resident cells as opposed to recruited PMN cells.

The role of GADD45 up-regulation in the pathogenesis of ALI is unknown; however, one function of GADD45 is to activate MEKK4, which in turn is an upstream regulator of JNK MAPK. JNK phosphorylates and activates members of the AP-1 family of transcription factors and has been implicated in ventilator-induced lung injury (23, 28). Thus, it is a biologically plausible hypothesis that GADD45 may contribute to the pathogenesis of ALI, and further research into its role is warranted.

Although we did not directly test whether lung injury in the MV + LPS group was a consequence of altered gene expression, many of the up-regulated genes are associated with inflammation, which is thought to play a central role in the pathogenesis of ALI. Several animal models of lung injury have shown a partial protective benefit of blocking proinflammatory cytokine activity (29–32). However, in human sepsis trials, targeting individual cytokines has not reduced the observed incidence of ALI (33). Strategies directed at inhibiting the immunomodulatory role of MV may have an improved chance of reducing the development of lung injury because of a more general attenuation of inflammatory responses to infections or other inflammatory stimuli.

Several additional factors must be considered in interpreting the results of this study. MV in the MV-only and MV + LPS groups required intubation and prolonged anesthesia as compared with the spontaneously breathing mice in the control and LPS-only groups. These differences could have contributed to the augmented cytokine responses seen with MV. Our previous study in rabbits (34), which allowed for better control of these confounding factors, supports the hypothesis that MV is an independent modifier of lung response to LPS. Arterial CO_2 partial pressure was not directly measured in these animals, and, the possibility that a respiratory acidosis or alkalosis contributed to the outcome measures cannot be excluded (35, 36). However, our preliminary data showed that the chosen ventilation strategy resulted in a normal arterial pH in normal mice. Thus, the most likely explanation for our data remains that MV enhances the lung inflammatory response to LPS.

We extracted mRNA from whole lung homogenate. Because substantially more PMN cells were recruited to the lungs of animals in the MV + LPS group, some of the gene expression data may have resulted from these changes in the lung cell population. However, analysis of the transcriptionally regulated, intracellular protein GADD45-γ using immunohistochemistry indicated that resident alveolar cells participated in at least some of the observed differential gene expression (Fig. 7). A potential role for alveolar epithelial cells is supported by modulation of TNF-α-induced gene expression in A549 cells after concurrent in vitro cell stretching (37).

In conclusion, we report that a noninjurious, clinically relevant MV strategy can augment the inflammatory response to LPS and promote early ALI. This effect was associated with both an increase in the total number of differentially expressed genes and in modulation of the fold change of most genes compared with the control group. These findings suggest that MV may contribute to the development of ALI in patients with risk factors for ARDS but without pre-existing overt lung injury. Understanding the signaling pathways by which MV augments the transcriptional response to bacterial pathways is an essential first step for developing strategies to disrupt this interaction.

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Disclosures

The authors have no financial conflict of interest.

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Functional Class	Gene Name	Gene ID (Entrez Gene)	Avg. Fold Expression
Inflammatory/Immune Response (n=53)			
	bone marrow stromal cell antigen 1	12182	**3.0** (2.3-3.8)
	chemokine (C-C motif) ligand 2	20296	**5.4** (3.2-9.1)
	chemokine (C-C motif) ligand 3	20302	**22.4** (13.9-36.0)
	chemokine (C-C motif) ligand 4	20303	**6.5** (3.7-11.4)
	chemokine (C-C motif) ligand 9	20308	**4.5** (4.0-5.1)
	chemokine (C-C motif) ligand 17	20295	**3.4** (3.2-3.7)
	chemokine (C-C motif) ligand 20	20297	**5.4** (4.1-7.0)
	chemokine (C-C motif) receptor 1	12768	**6.6** (5.4-8.0)
	chemokine (C-C motif) receptor 2	12772	**3.2** (2.2-4.6)
	chemokine (C-C motif) receptor-like 2	54199	**3.8** (2.8-5.1)
	chemokine (C-X-C motif) ligand 1	14825	**11.1** (8.1-15.0)
	chemokine (C-X-C motif) ligand 2	20310	**21.4** (15.2-30.2)
	chemokine (C-X-C motif) ligand 5	20311	**12.4** (9.3-16.5)
	chemokine (C-X-C motif) ligand 10	15945	**32.2** (21.6-48.0)
	colony stimulating factor 2 (granulocyte-macrophage)	12981	**2.6** (2.0-3.5)
	colony stimulating factor 2 receptor, beta 1, low-affinity	12983	**3.9** (2.9-5.2)
	colony stimulating factor 3 receptor (granulocyte)	12986	**3.3** (2.7-4.2)
	Complement component 5, receptor 1	12273	**3.1** (1.9-5.1)
	CD14 antigen	12475	**6.4** (6.1-6.7)
	c-type lectin domain family 4, member d	17474	**10.6** (8.3-13.5)
	c-type lectin domain family 4, member e	56619	**7.3** (5.4-9.9)
	c-type lectin domain family 4, member n	56620	**3.1** (2.2-4.4)
	Epstein-Barr virus induced gene 3	50498	**3.5** (2.2-5.5)
	Fc receptor, IgE, high affinity I, gamma polypeptide	14127	**3.4** (2.8-4.2)
	Fc receptor, IgG, high affinity I	14129	**6.8** (4.8-9.7)
	Fc receptor, IgG, low affinity III	14131	**4.3** (3.6-5.3)
	guanylate nucleotide binding protein 2	14469	**7.3** (5.78-9.3)
	guanylate nucleotide binding protein 4	55932	**4.5** (3.5-5.8)
	interleukin 1 alpha	16175	**3.6** (2.5-5.3)
	interleukin 1 beta	16176	**10.3** (7.7-13.8)
	interleukin 1 family, member 9	215257	**5.2** (3.6-7.6)
	interleukin 1 receptor, type II	16178	**13.7** (11.3-16.6)
	interleukin 1 receptor antagonist	16181	**7.0** (4.4-11.1)
	interleukin 6	16193	**9.1** (5.2-15.8)
	interleukin 15	16168	**3.0** (2.2-4.3)
	interferon, alpha-inducible protein	53606	**12.1** (9.3-15.8)
	interferon inducible protein 1	15944	**3.8** (3.1-4.8)
	interferon activated gene 203	15950	**5.3** (4.3-6.4)
	interferon gamma inducible protein 47	15953	**4.3** (3.5-5.2)
	interferon-induced protein with tetratricopeptide repeats 1	15957	**12.1** (9.3-15.8)
	interferon-induced protein with tetratricopeptide repeats 2	15958	**8.9** (6.2-12.8)

Functional Class	Gene Name	Gene ID (Entrez Gene)	Avg. Fold Expression
	interferon-induced protein with tetratricopeptide repeats 3	15959	**10.0** (8.0-12.6)
	leukocyte specific transcript 1	16988	**3.1** (2.4-4.0)
	mediterranean fever	54483	**3.6** (2.5-5.2)
	myxovirus (influenza virus) resistance 1	17857	**5.8** (4.0-8.4)
	2′-5′ oligoadenylate synthetase 2	246728	**3.5** (2.8-4.4)
	2′-5′ oligoadenylate synthetase 3	246727	**5.6** (3.9-7.9)
	2′-5′ oligoadenylate synthetase-like 2	23962	**8.4** (6.9-10.1)
	paired-Ig-like receptor B	18733	**3.4** (2.6-4.3)
	phospholipase A2, group VII	27226	**3.9** (3.0-4.9)
	toll-like receptor 2	24088	**6.3** (5.0-8.1)
	triggering receptor expressed on myeloid cells 3	58218	**3.0** (2.2-4.0)
	tumor necrosis factor (ligand) superfamily, member 9	21950	**3.1** (2.8-3.4)
Apoptosis (n=4)			
	B-cell leukemia/lymphoma 2 related protein A1 (a,b,d)	12044/12045/12047	**5.1** (3.7-7.0)
	caspase 11, apoptosis- related cysteine protease	12363	**6.2** (5.2-7.5)
	interferon-induced with helicase C domain 1	71586	**4.7** (3.8-5.8)
	tumor necrosis factor, alpha- induced protein 3	21929	**3.4** (2.5-4.5)
Signal Transduction - Intracellular (n=18)			
	A kinase (PRKA) anchor protein (gravin) 12	83397	**3.6** (2.7-5.0)
	down syndrome critical region homolog 1 (human)	54720	**3.1** (2.6-3.5)
	growth arrest and DNA-damage-inducible 45 gamma	23882	**3.9** (3.6-4.3)_
	GTP binding protein (overexpressed in skeletal muscle)	14579	**3.3** (2.4-4.6)
	G protein-coupled receptor 109B	80885	**5.9** (4.6-7.5)
	G protein-coupled receptor 65	14744	**3.4** (2.7-4.3)
	G protein-coupled receptor 84	80910	**3.0** (1.9-4.6)
	interferon gamma induced GTPase	16145	**4.0** (3.2-5.0)
	lymphocyte cytosolic protein 2	16822	**4.3** (3.0-6.1)
	MARCKS-like protein	17357	**9.5** (6.1-14.8)
	Metallothionein 1	17748	**3.3** (3.1-3.5)
	Metallothionein 2	17750	**3.9** (3.7-4.2)
	mitogen activated protein kinase kinase kinase 8	26410	**3.7** (2.5-5.4)
	Pleckstrin	56193	**4.2** (3.3-5.2)
	RAB20, member RAS oncogene family	19332	**4.8** (3.8-6.0)
	src-like adaptor	20491	**3.5** (2.7-4.6)
	suppressor of cytokine signaling 3	12702	**4.6** (3.7-5.6)
	Traf2 binding protein	211550	**4.3** (3.1-6.0)

Functional Class	Gene Name	Gene ID (Entrez Gene)	Avg. Fold Expression
Signal Transduction – Cell Surface Receptor (non-immune) (n=6)			
	formyl peptide receptor, related sequence 2	14289	**5.0** (3.8-6.6)
	MAS-related GPR, member A2	235712	**6.9** (4.4-10.8)
	membrane-spanning 4-domains, subfamily A, member 6B	69774	**4.8** (3.7-6.2)
	membrane-spanning 4-domains, subfamily A, member 6C	73656	**5.1** (3.7-7.0)
	oncostatin M receptor	18414	**3.0** (2.7-3.3)
	urokinase plasminogen activator receptor	18793	**3.5** (2.8-4.3)
Transcriptional Regulation (n=17)			
	activating transcription factor 3	11910	**4.9** (3.6-6.6)
	avian reticuloendotheliosis viral (v-rel) oncogene related B	19698	**3.3** (2.6-4.2)
	B-cell leukemia/lymphoma 3	12051	**4.4** (3.3-5.7)
	CCAAT/enhancer binding protein (C/EBP), delta	12609	**3.5** (3.1-3.9)
	interferon regulatory factor 7	54123	**6.9** (5.2-9.1)
	Jun-B oncogene	16477	**3.2** (2.2-4.5)
	LPS-induced TN factor	56722	**3.4** (3.0-3.9)
	Max dimerization protein	17119	**3.1** (2.0-4.9)
	nuclear factor, interleukin 3, regulated	18030	**4.5** (3.6-5.6)
	nuclear factor of kappa light chain gene enhancer in B-cells inhibitor, alpha	18035	**3.2** (2.8-3.8)
	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, zeta	80859	**4.4** (3.0-6.4)
	sin3 associated polypeptide	60406	**3.0** (2.5-3.5)
	signal transducer and activator of transcription 1	20846	**3.3** (2.6-4.1)
	signal transducer and activator of transcription 2	20847	**3.0** (2.5-3.6)
	transcription factor EC	21426	**3.7** (2.9-4.6)
	tripartite motif protein 30	20128	**5.0** (4.2-6.0)
	v-maf musculoaponeurotic fibrosarcoma oncogene family, protein F (avian)	17133	**3.3** (2.9-3.7)
Cell Proliferation (n=4)			
	fibrinogen-like protein 2	14190	**4.0** (3.3-4.7)
	schlafen 1	20555	**10.7** (7.8-14.6)
	schlafen 2	20556	**4.2** (3.5-5.1)
	schlafen 4	20558	**20.8** (16.1-26.8)
Matrix (n=1)			
	chondroitin sulfate proteoglycan 2	13003	**6.3** (4.1-9.8)
Angiogenesis (n=3)			
	serine (or cysteine) proteinase inhibitor, clade E, member 1	18787	**7.7** (5.0-11.9)
	thrombospondin 1	21825	**3.3** (2.4-4.6)
	tumor necrosis factor, alpha- induced protein 2	21928	**3.5** (2.6-4.8)

Functional Class	Gene Name	Gene ID (Entrez Gene)	Avg. Fold Expression
Coagulation (n=3)			
	coagulation factor III	14066	**3.0** (2.2-4.1)
	coagulation factor VIII, A1 subunit	74145	**8.2** (5.5-12.1)
	tissue factor pathway inhibitor 2	21789	**3.8** (2.5-5.8)
Proteolysis (n=2)			
	a disintegrin-like and metalloprotease (reprolysin type) with thrombospondin type 1 motif, 4	240913	**3.8** (2.6-5.6)
	tissue inhibitor of metalloproteinase I	21857	**5.9** (4.3-8.0)
Metabolism (n=12)			
	arginase type II	11847	**4.6** (4.1-5.2)
	cholesterol 25-hydroxylase	12642	**8.0** (6.6-9.7)
	cytidine 5'- triphosphate synthase	51797	**3.4** (3.2-3.6)
	GTP cyclohydrolase 1	14528	**3.7** (3.1-4.4)
	glycerol kinase	14933	**4.3** (3.7-5.0)
	guanine deaminase	14544	**3.0** (2.1-4.2)
	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3	170768	**3.4** (2.7-4.3)
	superoxide dismutase 2, mitochondrial	20656	**3.3** (3.0-3.6)
	ST3 beta-galactoside alpha-2,3-sialytransferase 1	20442	**4.4** (3.4-5.5)
	transglutaminase 1, K polypeptide	21816	**3.2** (2.5-4.2)
	uridine phosphorylase 1	22271	**6.9** (5.0-9.6)
	ubiquitin specific protease 18	24110	**7.5** (5.8-9.6)
Miscellaneous (n=12)			
	adrenomedullin	11535	**6.7** (4.8-9.3)
	cytochrome b-245, beta polypeptide	13058	**3.7** (3.2-4.2)
	hemopexin	15458	**2.8** (2.5-3.1)
	interleukin 4 induced 1	14204	**3.4** (3.1-3.8)
	integrin alpha M	16409	**4.2** (2.6-6.9)
	L-selectin	20343	**6.1** (4.8-7.7)
	P-selectin	20344	**3.7** (2.7-5.1)
	serum amyloid A 3	20210	**22.8** (20.3-25.6)
	solute carrier family 15, member 3	65221	**5.8** (4.0-8.3)
	solute carrier family 26, member 4	23985	**4.5** (4.0-5.0)
	three prime repair exonuclease 1	22040	**4.0** (3.0-5.4)
	thymidylate kinase family LPS-inducible member	22169	**6.3** (4.6-8.6)

Supplemental Table 1: Selected gene expression in the MV+LPS group with 95% confidence intervals relative to mean expression in the Control group. Inluded genes are those that were upregulated ≥ 3-fold in one or more of the MV-only, LPS-only, and MV+LPS groups as compared with the Control group. Genes are grouped by broad functional classification using the gene ontology classification Mouse Genome Informatics website (http://www.informatics.jax.org/) and or published papers.