

High-mobility group box 1 accelerates lipopolysaccharide-induced lung fibroblast proliferation *in vitro*: involvement of the NF- κ B signaling pathway

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The mechanism underlying lipopolysaccharide (LPS)-induced aberrant proliferation of lung fibroblasts in Gram-negative bacilli-associated pulmonary fibrosis is unknown. High-mobility group box 1 (HMGB1) is a ubiquitous nuclear protein that is released from the nuclei of lung fibroblasts after LPS stimulation. It can exacerbate LPS-induced inflammation and hasten cell proliferation. Thus, this study investigated the effects of LPS- and/or HMGB1-stimulating murine lung fibroblasts on gene expression using various assays *in vitro*. Thiazolyl-diphenyl-tetrazolium bromide (MTT) assay data showed that either LPS or HMGB1 could induce lung fibroblast proliferation. Endogenous HMGB1 secreted from lung fibroblasts was detected by enzyme-linked immunosorbent assay (ELISA) 48 h after LPS stimulation. Pretreatment with an anti-HMGB1 antibody inhibited the proliferative effects of LPS on lung fibroblasts. DNA microarray data showed that the NF- κ B signaling genes were upregulated in cells after stimulated with LPS, HMGB1, or both. Secretion of matrix metalloproteinase (MMP)-2 and MMP-9, and tissue inhibitor of metalloproteinase 2 (TIMP-2) was significantly upregulated after treatment with LPS, HMGB1, or their combination. However, an NF- κ B inhibitor was able to downregulate levels of these proteins. In addition, levels of Toll-like receptor 4 (TLR4), Toll-like receptor 2 (TLR2), and receptors for advanced glycation end products (RAGE) mRNA and proteins were also upregulated in these cells after LPS treatment and further upregulated by LPS plus HMGB1. In conclusion, the data from the current study demonstrate that LPS-induced lung fibroblast secretion of endogenous HMGB1 can augment the proliferative effects of LPS and, therefore, may play a key role in exacerbation of pulmonary fibrosis. The underlying molecular mechanisms are related to the activation of the TLR4/NF- κ B signaling pathway and its downstream targets.

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Pulmonary fibrosis is characterized by abnormal activation and proliferation of lung fibroblasts.¹ Lipopolysaccharide (LPS), a prominent component of endotoxins from Gram-negative bacilli, is an important etiologic factor of organ fibrosis.² Our previous study showed that LPS stimulated lung fibroblasts to undergo aberrant proliferation and secretion of collagen, the effects that exacerbate pulmonary fibrosis.^{3–6} Aberrant proliferation of lung fibroblasts plays an important role in LPS-induced fibrogenesis during early-stage pulmonary fibrosis. However, the molecular mechanism underlying this induction of fibrogenesis is not well understood.

High-mobility group box 1 (HMGB1) is a ubiquitous nuclear protein^{7–12} that acts as a crucial factor in acute lung

injury progression^{13,14} and pulmonary fibrosis.¹⁵ HMGB1 can be released actively or passively from various cells in response to stimulation of LPS or endogenous proinflammatory cytokines.^{16–19} HMGB1 can induce proliferation and migration of human gingival fibroblasts *in vitro*.¹⁸ Endogenous HMGB1 has been shown to augment the LPS-induced inflammatory responses in peritoneal macrophages⁷ and synovial fibroblasts,⁸ and to enhance LPS-induced proliferation of human gingival fibroblasts.²⁰ HMGB1 involvement in fibroblast proliferation suggests that it may contribute to pulmonary fibrosis. However, the mechanism by which LPS promotes proliferation of lung fibroblasts is unclear. That is, it is not known whether LPS promotes lung fibroblast

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proliferation only indirectly through LPS-induced secretion of HMGB1, which then promotes the proliferation, or whether LPS has an additional effect on proliferation, as has been observed in other types of fibroblasts *in vitro*.²⁰

Thus, in this study, we first compared lung fibroblast proliferation in culture following stimulation with LPS alone, HMGB1 alone, or the two in combination to probe the role of HMGB1 in this process. We then assessed the effects of the endogenous HMGB1 secretion from lung fibroblasts after LPS stimulation on cell proliferation. In parallel, we administered an anti-HMGB1 antibody to block HMGB1 activity during LPS-induced lung fibroblast proliferation. We then performed a cDNA microarray analysis to identify which genes were differentially expressed genes across the three treatment conditions. Finally, we compared the expression of NF- κ B pathway genes (eg, matrix metalloproteinase (MMP)-2 and -9, tissue inhibitor of metalloproteinase 2 (TIMP-2), and the LPS- and HMGB1-related receptors Toll-like receptor 4 (TLR4), Toll-like receptor 2 (TLR2), and receptors for advanced glycation end products (RAGE)) in LPS- vs HMGB1-induced cell proliferation.

MATERIALS AND METHODS

Cell Lines and Culture

Mouse lung fibroblasts (MIC-CELL-0040) were obtained from Wuhan Pricells Biotechnology & Medicine (Wuhan, China) and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, 100 IU/ml penicillin G, and 100 IU/ml streptomycin at 37 °C in a humidified 5% CO₂ incubator (all from Invitrogen/Labotect, Gottingen, Germany). The growth medium was refreshed every 3 days. When the culture reached 80% confluence, adherent cells were detached by exposure to 0.25% trypsin for 5 min, and cells were seeded into six-well plates at 1:5–10 dilutions, depending on the requirements of the particular experiment.

Cell Treatment

Cells were seeded into each of treatment groups and treated with LPS (10, 50, or 100 ng/ml), HMGB1 (0.01, 0.1, or 1.0 μ g/ml), or both (10 ng/ml LPS+0.01 μ g/ml HMGB1, 50 ng/ml LPS+0.1 μ g/ml HMGB1, or 100 ng/ml LPS+1 μ g/ml HMGB1) for 0, 12, 24, and 48 h. Some cultures were pretreated with the NF- κ B inhibitor pyrrolidine dithiocarbamate (PDTC, 30 μ M, Sigma-Aldrich, St Louis, MO, USA). LPS (O55:B5 *Escherichia coli*) and recombinant human HMGB1 (rhHMGB1) were purchased from Sigma-Aldrich. All dilutions were prepared in 0.25 ml of sterile phosphate-buffered saline (PBS). PBS was used as a negative control. Recombinant human RAGE Fc chimera (RAGE-Fc) was purchased from R&D Systems (Minneapolis, MN, USA). A chicken anti-mouse IgY (Abcam, UK) was used as a negative control for all antibody experiments. Normal Ig was used as a reference in place of HMGB1 antibody in the control group and LPS group. Following a 1-h pretreatment with 4 μ g/ml of an anti-

HMGB1, anti-TLR2, or anti-TLR4 neutralizing antibody (all from Shino-Test, Tokyo, Japan), cells were stimulated with LPS (100 ng/ml) or exposed to PBS for 48 h.

Methyl Thiazolyl-Diphenyl-Tetrazolium Bromide (MTT) Cell Viability Assay

MTT assays were used to detect lung fibroblast viability. Cells were grown and prepared at a concentration of 3.5×10^4 /l, and 100- μ l aliquots of cells were plated in a 96-well plates containing 10% FCS-DMEM and allowed to incubate overnight in accordance with the specified group treatment. After 36-h treatments, the medium was changed to fresh DMEM (100 μ l per well). Then, 10 μ l of MTT solution (5 mg/ml in PBS) was added to each well, and the cells were incubated for an additional 4 h at 37 °C in 5% CO₂. The colored formazan product was then dissolved in 100 μ l of dimethyl sulfoxide (DMSO), and the optical density (OD) of each well was read at 490 nm.

Enzyme-Linked Immunosorbent Assay (ELISA)

Secretion of HMGB1, MMP-2, MMP-9, and TIMP-2 proteins was detected by ELISA. After a 48-h LPS stimulation (or sham control), supernatant fractions of cell culture were collected. HMGB1 concentration was detected with commercially available ELISA kits (HMGB1, Shino-Test Corporation;

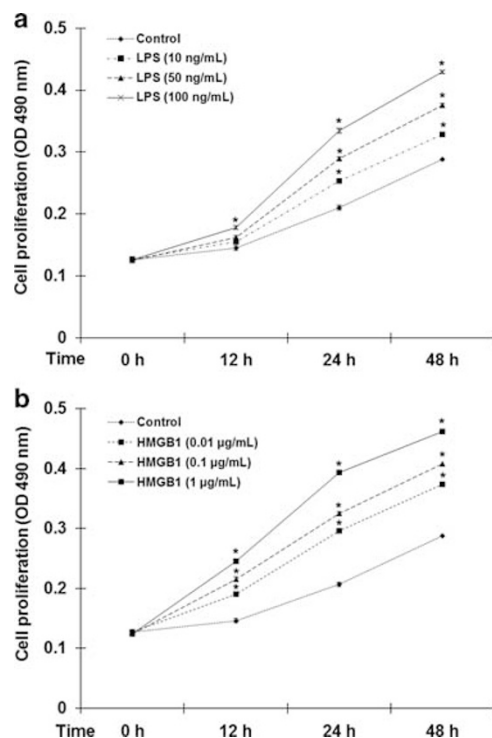


Figure 1 LPS and HMGB1 induce lung fibroblast proliferation. Increased cell proliferation compared with PBS-exposed control cells, as determined by MTT assay (OD_{490 nm}), following LPS (a) or HMGB1 (b) exposure for 12, 24, and 48 h. Mean values from triplicate experiments are shown with s.d. **P*<0.001 vs control cells at the same time point.

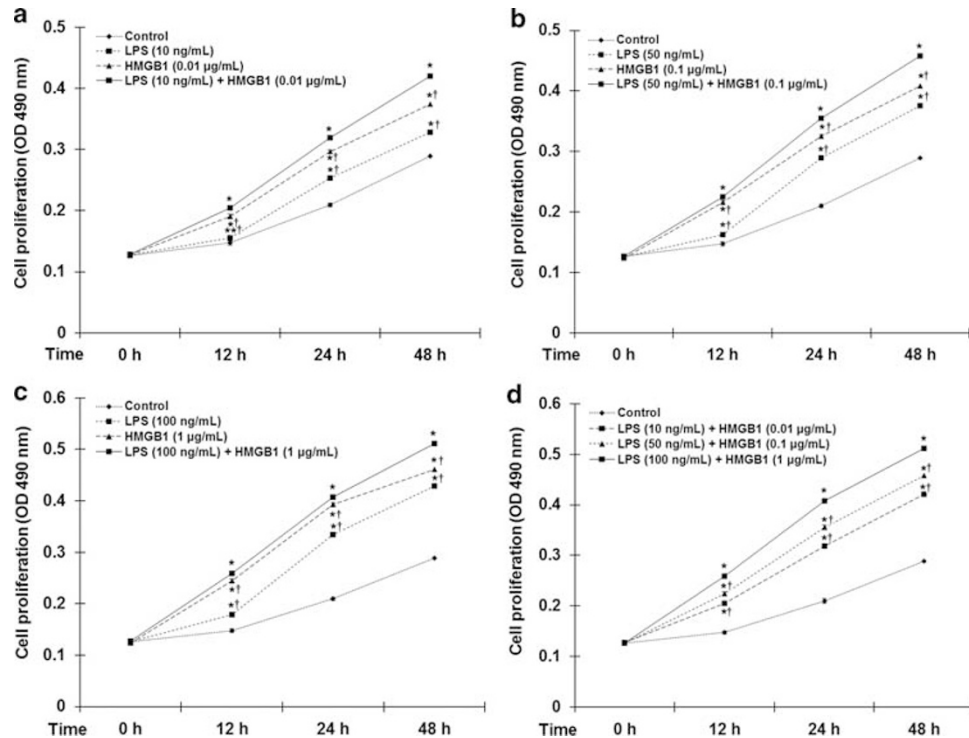


Figure 2 HMGB1 enhances LPS-induced proliferative effect in lung fibroblasts. MTT assay data (OD_{490 nm}) showing the proproliferative effects of 12, 24, and 48-h exposure of lung fibroblasts to low (a), medium (b), and high (c) doses of LPS alone, HMGB1 alone, or both, compared with control cells exposed to only PBS. (d) Dose-dependent additive effects of HMGB1 and LPS on proliferation. * $P < 0.001$, ** $P < 0.01$ vs control cells (a–d); † $P < 0.001$ vs LPS+HMGB1-stimulated cells at the same time point (a–c); † $P < 0.001$ vs LPS (100 ng/ml)+HMGB1 (1 µg/ml)-stimulated cells at the same time point (d). Mean values of triplicate experiments are shown with s.d. error bars.

MMP-2 and MMP-9, R&D Systems) according to the manufacturers' instructions as described previously.²¹ For detection of TIMP-2, a mouse anti-TIMP-2 antibody (Abcam, Cambridge, MA, USA) was used as the primary antibody and all other steps were performed in accordance with standard ELISA methods.

cDNA Microarray Analysis

Lung fibroblasts were stimulated for 24 h with LPS (100 ng/ml), HMGB1 (1 µg/ml), or both (100 ng/ml LPS plus 1 µg/ml HMGB1), and PBS-treated cells served as a nonstimulated control. Total cellular RNA was isolated from cells using the Trizol reagent (Invitrogen) and purified with a total RNA prep kit (Illumina, San Diego, CA, USA). RNA quality was checked using a Bioanalyzer 2200 (Agilent, Santa Clara, CA, USA) and stored at -80°C until use. The RNA samples with an RNA integrity number >7.0 were used for cDNA microarray analysis. In brief, cDNA was prepared using One-Cycle Target Labeling and Control Reagents (Illumina) and cRNA was then created with a GeneChip IVT labeling kit (Illumina) and these biotin-labeled, fragmented (B200 nt) cRNA probes were hybridized to GEX-HBY arrays for 16 h at 45°C (mouse WG-6 expression BeadChips; Illumina). All arrays were washed, stained, and read by an Illumina BeadChip Reader. The fluorescence signal was excited at

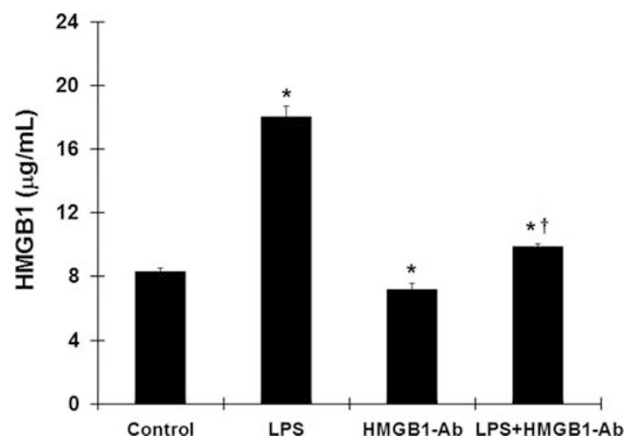


Figure 3 LPS induces HMGB1 secretion in lung fibroblasts. ELISAs of supernatant samples from cells exposed to LPS (final concentration of 100 ng/ml) or sham, stimulation for 48 h following a 1-h pretreatment with anti-HMGB1 antibody (4 µg/ml) or IgY-negative control antibody (control and LPS groups). Mean values from triplicate experiments are shown with s.d. error bars. * $P < 0.001$ vs sham-stimulated controls. † $P < 0.001$ vs LPS alone group.

570 nm, and data were collected via a confocal scanner. Data were analyzed using Illumina BeadStudio data analysis software. Three replicates of each sample were pooled together. Differentially expressed genes were identified based

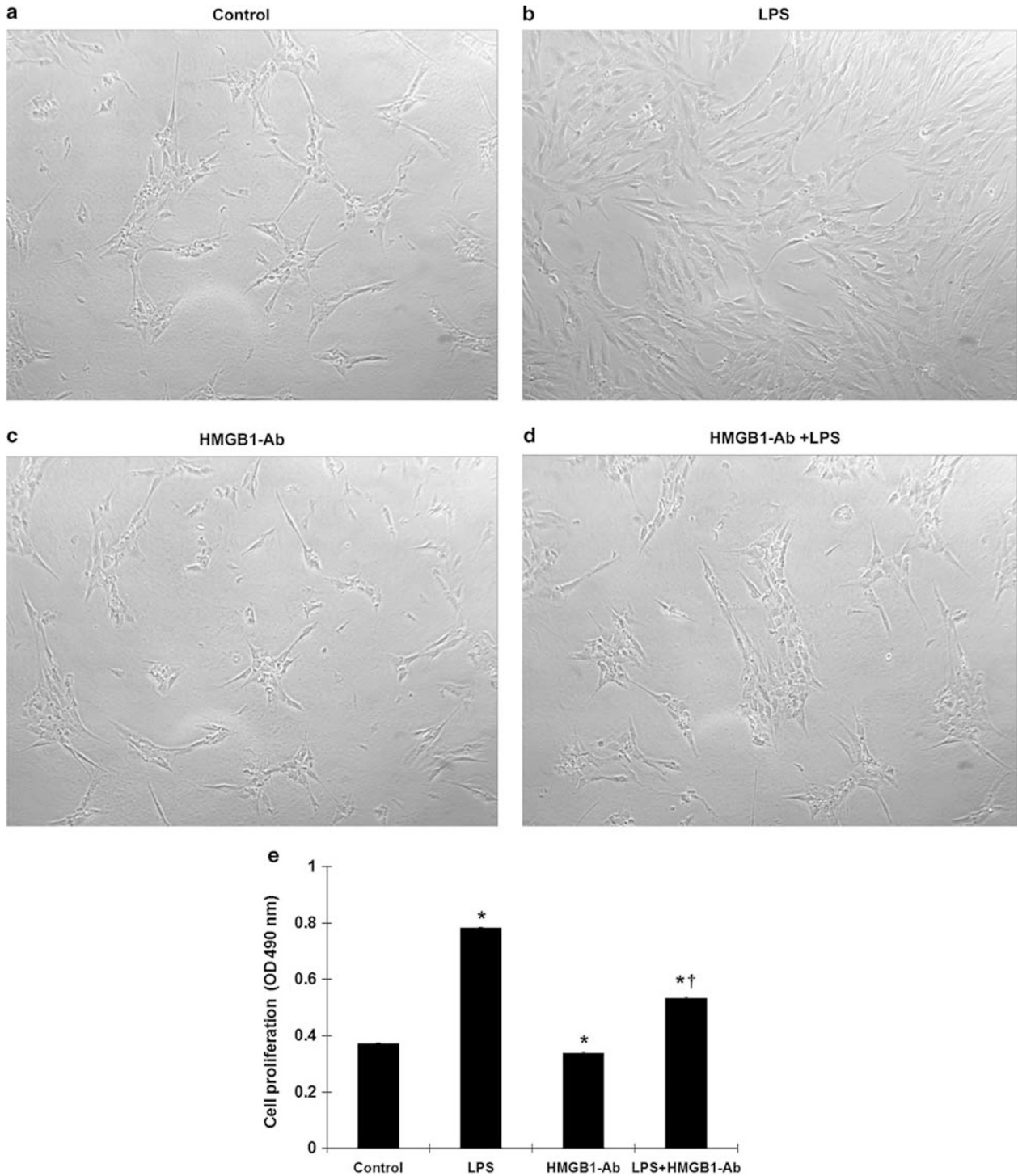


Figure 4 HMGB1 neutralization inhibits LPS-induced lung fibroblast proliferation. (a–d) Images of cultured cells following stimulation with or without LPS (final concentration, 100 ng/ml) for 48 h following 1-h pretreatment with anti-HMGB1 antibody (4 μg/ml) or IgY-negative control antibody (control and LPS groups) (×100). (e) MTT assay (OD_{490 nm}) proliferation data of cells following the aforementioned treatments. Mean values from triplicate experiments are shown with s.d. error bars. **P* < 0.001 vs control cells; †*P* < 0.001 vs LPS alone.

on a random variance model (RVM) *t*-test and false discovery rate (FDR) analysis, as described previously.⁵

After the statistical and FDR analyses, we selected differentially expressed genes using the criterion of a fold

change >2 and a FDR threshold of <0.05. Expression of some of these genes was validated by qRT-PCR, and a high correlation was observed between our qPCR and microarray data. The main functions of genes were determined by GO

Table 1 Numbers of differentially expressed genes following specified treatments in lung fibroblasts

Effect	LPS vs Con.	HMGB1 vs Con.	LPS+ HMGB1 vs Con.	LPS+ HMGB1 vs LPS	LPS+ HMGB1 vs HMGB1
Upregulated, no. of genes	2231	1707	860	621	1484
Downregulated, no. of genes	764	580	740	1826	518

Con, control; HMGB1, high-mobility group box 1 protein; LPS, lipopolysaccharide.

analysis as described previously.^{22–24} We used Fisher's exact test and χ^2 test results to classify the GO categories, and FDRs were calculated to correct *P*-values. Pathway analysis was used to identify the significantly changed pathways of the differential genes according to KEGG, Biocarta, and Reatome.^{25–27}

Protein Extraction and Western Blot

Protein expression was assessed with western blots. Briefly, cells were collected and lysed with an RIPA lysis buffer on ice for 10–15 min. Supernatants were collected after centrifugation and protein concentration was quantified using the bicinchoninic acid method. The protein samples were then resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinyl-fluoride membranes. The membranes were incubated for 16 h at 4 °C with primary rabbit antibodies (anti-NF- κ B p65 (1:1000), anti- β -actin (1:1500), anti-H3 (1:800), anti-TLR2 (1:1000), anti-TLR4 (1:1000), or anti-RAGE (1:1000); first three from Cell Signaling Technology (Danvers, MA, USA) and latter three from Abcam, UK). The membranes were then incubated for 1 h at the room temperature with a goat anti-rabbit secondary antibody at a dilution of 1:10 000 and the protein bands were detected using an ECL Plus kit (GE Healthcare, Pittsburgh, PA, USA).

RNA Isolation and qRT-PCR

Total RNA from cells was isolated with Trizol reagent and an RNeasy kit (both from Invitrogen) and reverse transcribed into cDNA with the M-MLV polymerase (Thermo Fisher Scientific, Waltham, MA, USA). qPCR was performed in a 7300 Real-Time PCR System (Applied Biosystems, Warrington, UK) in accordance with the manufacturer's instructions with the following sequence-specific primers: GAPDH, 5'-ATCACTGCCACCCAGAAG-3' and 5'-TCCACGACGGA CACATTG-3'; TLR-2, 5'-CTTCCAGGTCTTCAGTCTTC-3' and 5'-TGATTGCGGACACATCTC-3'; TLR-4, 5'-GCCGTT GGTGTATCTTTG-3' and 5'-GCTGTTTGCTCAGGATTC -3'; and RAGE, 5'-GAAAGCCCTCCTGTGCAGCATC-3' and 5'-GGCACCATTCTCTGGCATCTC-3'. All samples were run in triplicate and relative gene expression was determined according to the $2^{-\Delta\Delta C_t}$ method. The housekeeping gene *GAPDH* served as the normalization standard.

Statistical Analysis

Each experiment was conducted independently and repeated at least three times with similar results. The data are reported as mean \pm s.d. The SPSS statistical software version 12.0 (SPSS, Chicago, IL, USA) was used for mean value comparisons of single-factor multiple samples. The homogeneity of variance within the data sets was analyzed with a one-factor analysis of variance (ANOVA) least squares difference test, for parametric data, or a Kruskal–Wallis rank-sum test, for nonparametric data. In all cases, *P*-values < 0.05 were considered statistically significant.

RESULTS

Proliferative Effects of LPS, HMGB1, and LPS Plus HMGB1 on Lung Fibroblasts

As shown in Figure 1a and b, MTT data demonstrated that, relative to the nonstimulation control, LPS (10, 50, and 100 ng/ml) and HMGB1 (0.01, 0.10, and 1 μ g/ml) each had a significant positive effect on cell viability at 12, 24, and 48 h. The effects were both time dependent and dose dependent. Lung fibroblasts treated with both agents (10 ng/ml LPS + 0.01 μ g/ml HMGB1, 50 ng/ml LPS + 0.1 μ g/ml HMGB1, and 100 ng/ml LPS + 1 μ g/ml HMGB1) showed greater induction of cell viability than either LPS- or HMGB1-treated cells (Figure 2a–c). The effects were both time dependent and dose dependent (Figure 2d).

LPS Stimulation Induces Lung Fibroblast Secretion of HMGB1 That Can Be Attenuated by an Anti-HMGB1-Neutralizing Antibody

As shown in Figure 3, ELISA data demonstrated that stimulation of lung fibroblasts with LPS (100 ng/ml) for 48 h resulted in increased levels of HMGB1 in cell culture supernatants, relative to levels in nonstimulated control cells. Pretreatment with an anti-HMGB1-neutralizing antibody (4 μ g/ml) for 1 h attenuated LPS induction of HMGB1 expression, relative to levels observed when cells were pretreated with negative control antibody (chicken anti-mouse IgY). In addition, the MTT assay data showed that LPS (100 ng/ml) stimulation for 48 h increased lung fibroblast viability, and that pretreatment with the anti-HMGB1 antibody (4 μ g/ml) for 1 h inhibited the proliferative effect of LPS (Figure 4a–e).

Table 2 Ten most overrepresented GO terms in each GO category associated with genes responsive to LPS, or HMGB1, or LPS plus HMGB1 stimulation

Class	Overrepresented GO terms								
	LPS+HMGB1 vs blank			LPS vs blank			HMGB1 vs blank		
	Term	P	Benj	Term	P	Benj	Term	P	Benj
Biological process	CC	6.40E-17	2.30E-13	CC	1.10E-11	3.20E-08	CC	3.70E-05	9.40E-02
	CC process	2.20E-12	2.20E-09	CC process	6.30E-09	9.20E-06			
	Cell div	1.00E-11	6.80E-09	M phase	2.80E-07	2.80E-04			
	M phase	5.60E-11	2.80E-08	CC phase	8.00E-07	5.90E-04			
	CC phase	6.90E-10	2.80E-07	Cell div	3.40E-06	2.00E-03			
	DNA metab	1.00E-09	3.40E-07	Mitotic CC	6.50E-06	3.20E-03			
	Nuclear div	5.10E-09	1.50E-06	M phase of mitotic CC	7.40E-06	3.10E-03			
	Mitosis	5.10E-09	1.50E-06	Response to DNA damage	1.40E-05	5.00E-03			
	M phase of mitotic CC	7.70E-09	2.00E-06	Nuclear div	3.10E-05	1.00E-02			
	Mitotic CC	1.00E-08	2.30E-06	Mitosis	3.10E-05	1.00E-02			
Cellular component	Chrom	1.60E-12	5.10E-10	Chrom	2.20E-10	1.00E-07	Synapse	5.50E-05	2.30E-02
	Chrom part	3.50E-10	5.50E-08	Chrom part	4.50E-09	1.00E-06	Cytoskeleton	5.60E-05	1.20E-02
	Chrom, cntr	1.20E-08	1.20E-06	Chrom, cntr	1.50E-07	2.20E-05	Cell junction	1.20E-04	1.60E-02
	Condensed Chrom	1.70E-06	1.30E-04	kntchr	4.10E-07	4.70E-05	Plasma mb part	2.00E-04	2.10E-02
	i-c non-mb-bound org	2.10E-06	1.30E-04	Condensed chrom	1.00E-06	9.30E-05	Plasma mb	3.00E-04	2.50E-02
	Non-mb-bound org	2.10E-06	1.30E-04	Condensed chrom, cntr	6.20E-06	4.70E-04	Non-mb-bound org	1.10E-03	7.40E-02
	Condensed chrom, cntr	2.50E-04	1.30E-02	Condensed chrom kntchr	2.60E-05	1.70E-03	i-c Non-mb-bound org	1.10E-03	7.40E-02
	kntchr	3.20E-04	1.40E-02	Non-mb-bound org	1.00E-03	5.70E-02	Sarcolemma	1.40E-03	8.20E-02
	Spindle	9.70E-04	3.70E-02	i-c non-mb-bound org	1.00E-03	5.70E-02	Cell surface	2.00E-03	9.80E-02
	Molecular	DNA bnd	9.40E-07	5.40E-04					
Adenyl ribo-nt bnd		4.00E-04	1.10E-01						
ATP bnd		4.90E-04	8.90E-02						
KEGG	DNA repl	8.80E-06	1.20E-03	CC	5.60E-04	9.00E-02	dlt d crdmpy	3.00E-04	4.70E-02
	CC	3.90E-05	2.60E-03	SCLC	1.20E-03	9.30E-02	hrtr crdmpy	1.20E-03	9.50E-02
	Homo recomb	1.30E-03	5.60E-02						
	pwycancer	2.40E-03	7.60E-02						

Benj, Benjamini; bnd, binding; CC, cell cycle; chr, chromosome; cntr, centromeric region; crdmpy, cardiomyopathy; div, division; dlt d, dilated; homo recomb, homologous recombination; hrtr, hypertrophic; i-c, intracellular; kntchr, kinetochore; mb, membrane; metab, metabolic process; nt, nucleotide; org, organelle; pwycancer, pathways in; repl, replication; SCLC, small-cell lung cancer.

The NF-κB Pathway Implicated in Proliferative Effects of HMGB1 and LPS

The DNA microarray analysis results profiling differentially expressed genes (cutoff value of >1.2 fold change) after

stimulation with LPS (100 ng/ml), HMGB1 (1 μg/ml), or both (100 ng/ml LPS plus 1 μg/ml HMGB1) are reported in Tables 1 and 2. We found that 2231, 1707, and 860 genes were upregulated relative to control levels in the LPS, HMGB1, and

Table 3 Up- and down-regulated genes in LPS- and/or HMGB1-treated lung fibroblasts

	LPS vs Con.	HMGB1 vs Con.	LPS+HMGB1 vs Con.	LPS+HMGB1 vs LPS	LPS+HMGB1 vs HMGB1	
Upregulated genes	<i>LOC100042970</i>	<i>B130049M08Rik</i>	<i>2610101N10Rik</i>	<i>Pim2</i>	<i>Pnmt</i>	
	<i>Accn4</i>	<i>V2r12</i>	<i>Nusap1</i>	<i>Picalm</i>	<i>Mmp19</i>	
	<i>5430420C16Rik</i>	<i>LOC331177</i>	<i>A530095A18Rik</i>	<i>Bcl9</i>	<i>4933407P14Rik</i>	
	<i>LOC100039693</i>	<i>Rnps1</i>	<i>Csf3</i>	<i>Ptgs1</i>	<i>Slc2a9</i>	
	<i>Bcl2l14</i>	<i>Syt11</i>	<i>Slc11a2</i>	<i>LOC211870</i>	<i>Vsx1</i>	
	<i>Tob2</i>	<i>Atg4c</i>	<i>LOC100042970</i>	<i>Zbtb33</i>	<i>Arhgap6</i>	
	<i>D930020N02Rik</i>	<i>Dsg1b</i>	<i>Spin</i>	<i>Flg2</i>	<i>Olf1r1143</i>	
	<i>Khlh4</i>	<i>Olf1r1031</i>	<i>B4galnt3</i>	<i>col11a2</i>	<i>Tm4sf20</i>	
	<i>Glce</i>	<i>C130023109Rik</i>	<i>Setd8</i>	<i>LOC100044736</i>	<i>Dsg1b</i>	
	<i>Krtap5-4</i>	<i>4732426B21Rik</i>	<i>Vrk1</i>	<i>2210409F01Rik</i>	<i>BC050777</i>	
	Downregulated genes	<i>Gpr84</i>	<i>Reln</i>	<i>Sspo</i>	<i>C230096C10Rik</i>	<i>Map3k10</i>
		<i>1700021K14Rik</i>	<i>Clp1</i>	<i>1810049H19Rik</i>	<i>Ercc8</i>	<i>Gm1008</i>
		<i>1200009O22Rik</i>	<i>EG634650</i>	<i>A830009P14Rik</i>	<i>4933411B03Rik</i>	<i>Jph2</i>
<i>Aoah</i>		<i>Use1</i>	<i>Slc2a9</i>	<i>Slc37a4</i>	<i>Art5</i>	
<i>Sspo</i>		<i>Jph2</i>	<i>Matn1</i>	<i>Khlh4</i>	<i>Pim2</i>	
<i>Atp1b1</i>		<i>Glrx3</i>	<i>41523</i>	<i>D930014E17Rik</i>	<i>Gsta2</i>	
<i>Bmp5</i>		<i>Slc16a6</i>	<i>Olfm1</i>	<i>LOC385707</i>	<i>LOC669458</i>	
<i>Ptgs1</i>		<i>Sspo</i>	<i>Sfmbt2</i>	<i>Oas3</i>	<i>Lsm14b</i>	
<i>1700016D06Rik</i>		<i>Mep1b</i>	<i>1700081D17Rik</i>	<i>Tdrd1</i>	<i>D930008O12Rik</i>	
<i>LOC435565</i>		<i>Tnip2</i>	<i>Fgf1</i>	<i>Slc2a9</i>	<i>6330548G22Rik</i>	

LPS+HMGB1 groups, respectively. In addition, 764, 580, and 740 genes, respectively, were downregulated relative to controls in these groups. We then verified some of the results using qRT-PCR (data not shown). The most significantly up- and down-regulated genes after LPS plus HMGB1 treatment are shown in Table 3. Interestingly, as shown in Figure 5, a number of genes encoding proteins related to a variety of signaling pathways or cell proliferation and growth (according to KEGG pathway analysis) were upregulated in the LPS +HMGB1-stimulated cells, including constituents of the NF- κ B signaling pathway and of the transforming growth factor- β (TGF- β) and phosphatidylinositol 3-kinase (PI3K)–Akt signaling pathways.

We further confirmed the role of the NF- κ B signaling pathway in mediation of LPS, HMGB1, or LPS+HMGB1-stimulated cell proliferation (Figure 6a–e). The data showed that the NF- κ B signaling pathway had an additive effect on HMGB1- and/or LPS-induced lung fibroblast proliferation. To further verify posttreatment activation of the NF- κ B pathway, we assessed cytosolic and nuclear levels of the NF- κ B p65 protein (Figure 6f and g). Both LPS and HMGB1 decreased cytosolic levels of NF- κ B p65, and increased nuclear levels, suggesting that the treatments promoted translocation of NF- κ B p65 to the nucleus. Furthermore, application of an HMGB1-neutralizing antibody attenuated LPS-induced

nuclear NF- κ B p65 levels ($P < 0.05$), indicating that HMGB1 was involved in LPS-induced NF- κ B activation.

LPS- and/or HMGB1-Stimulated Secretion of MMP-2, MMP-9, and TIMP-2 in Lung Fibroblasts

Examination of cellular supernatant levels of MMP-2, MMP-9, and TIMP-2 revealed that, compared with the control group, the LPS and HMGB1 treatments each stimulated secretion of these proteins (all $P < 0.05$). The combined LPS+HMGB1 treatment elevated MMP-2, MMP-9, and TIMP-2 levels significantly more than the LPS-alone treatment (all $P < 0.05$). Meanwhile, the HMGB1-neutralizing antibody inhibited LPS-induced secretion of MMP-2, MMP-9, and TIMP-2, indicating that HMGB1 was involved in the LPS-mediated induction of the secretion of these molecules (Figure 7a–c).

NF- κ B Pathway Mediated LPS- and HMGB1-Stimulated Secretion of MMP-2, MMP-9, and TIMP-2 in Lung Fibroblasts

Cytosolic NF- κ B p65 protein levels were significantly higher in cells treated with the NF- κ B inhibitor PDTC (30 μ M) only and in cells treated with LPS+HMGB1 (100 ng/ml LPS and 1 μ g/ml HMGB1) and PDTC than in the LPS+HMGB1 group not exposed to PDTC ($P < 0.05$), whereas nuclear NF- κ B p65

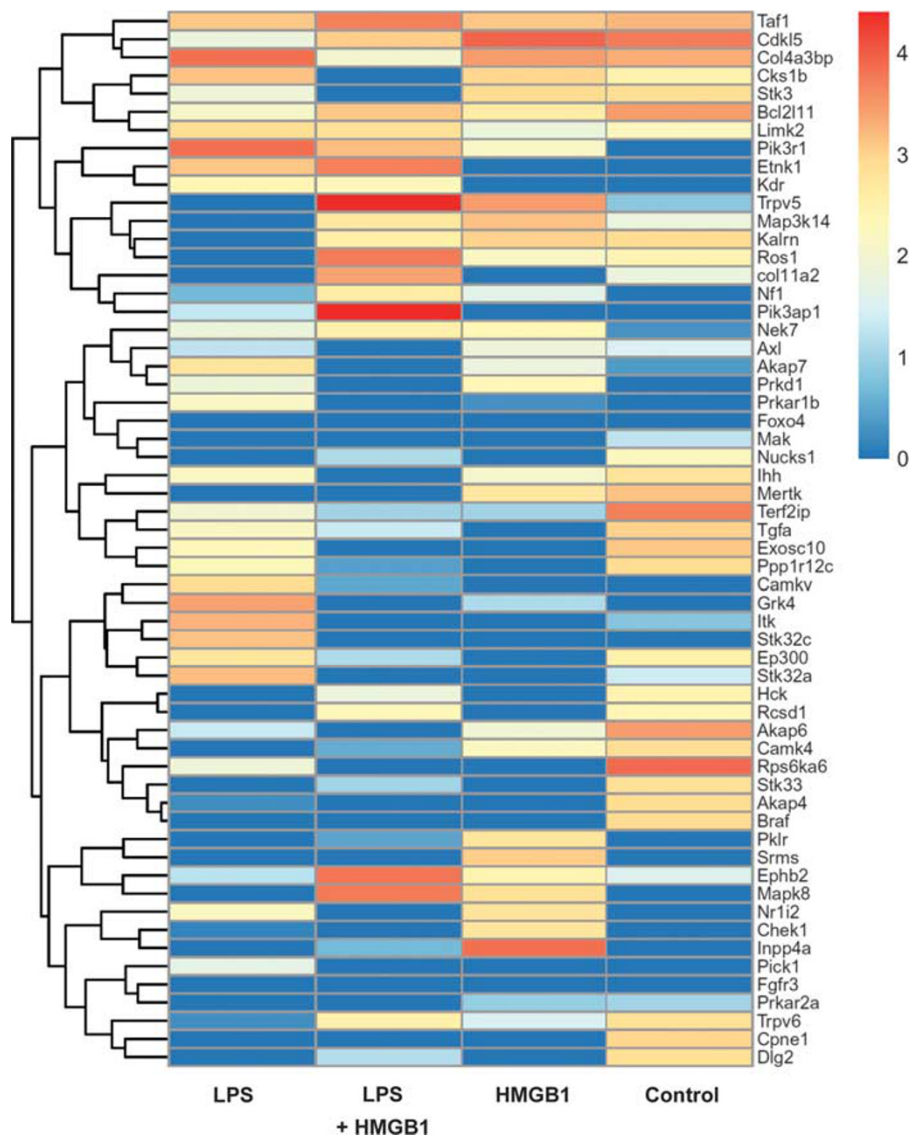


Figure 5 Heat map representation of candidate genes identified as being involved in lung fibroblast proliferation. From left to right: lane 1 is LPS-treated cells, lane 2 is LPS+HMGB1-treated cells, lane 3 is HMGB1-treated cells, and lane 4 is untreated controls. Red nodes represent the key regulatory genes involved in LPS- and/or HMGB1-stimulated fibroblast proliferation. Similarities are indicated with matching node color.

protein levels were significantly lower in the PDTC-exposed groups ($P < 0.05$ vs LPS+HMGB1; Figure 7d and e). MMP-2, MMP-9, and TIMP-1 levels were significantly increased in cells treated with LPS+HMGB1, but significantly decreased after PDTC exposure (all $P < 0.05$ vs LPS+HMGB1; Figure 7f–h), pointing to the NF- κ B pathway as a potential mediator of LPS and HMGB1 effects on secretion of MMP-2, MMP-9, and TIMP-1.

LPS and/or HMGB1 Upregulated Expression of TLR4, TLR2, and RAGE in Lung Fibroblasts

The relative levels of *Tlr2*, *Rage*, and, especially, *Tlr4* mRNA were upregulated after LPS treatment (all $P < 0.05$ vs untreated controls) and further upregulated by the LPS+HMGB1

treatment (all $P < 0.05$ vs LPS+HMGB1). The HMGB1-neutralizing antibody attenuated these increases ($P < 0.05$; Figure 8a). The western blot data were consistent with the qRT-PCR data (Figure 8b).

TLR4-Mediated LPS- and HMGB1-Induced Activation of NF- κ B Signaling

Pretreating cells with anti-TLR4 antibody before LPS +HMGB1 treatment inhibited LPS- and HMGB-stimulated NF- κ B activation in lung fibroblasts ($P < 0.05$; Figure 8c and d). Pretreatments with anti-TLR2 antibody or RAGE-Fc did not produce as robust inhibition as that observed with the anti-TLR4 antibody pretreatment, suggesting that LPS- and

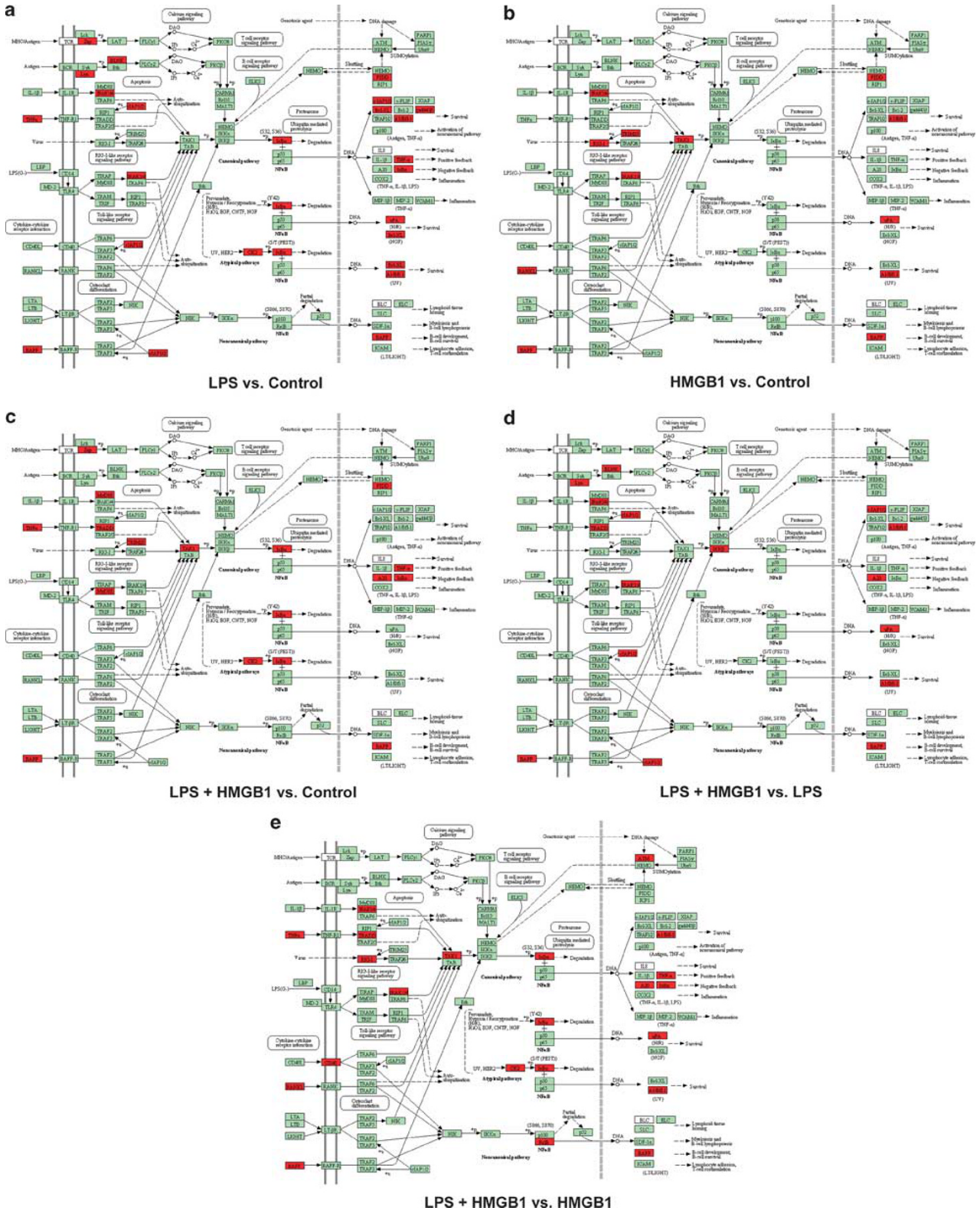


Figure 6 NF-κB pathway regulation of HMGB1 and LPS effects on lung fibroblasts. DNA microarray and KEGG pathway enrichment analysis of lung fibroblasts treated for 24 h with LPS alone (100 ng/ml), HMGB1 alone (1 μg/ml), or both (100 ng/ml LPS and 1 μg/ml HMGB1) indicated that the treatments stimulated intracellular signal transduction pathways. (a–e) Differentially expressed NF-κB pathway-related genes in lung fibroblasts following each treatment. Green color indicates no change, and red color indicates upregulation compared with control cells. (f) Cytosolic NF-κB p65 protein levels following LPS alone, HMGB1 alone, or a combination of both treatments in lung fibroblasts. (g) Nuclear NF-κB p65 protein levels following LPS alone, HMGB1 alone, or a combination of both treatments in lung fibroblasts. **P*<0.05 vs controls. [†]*P*<0.05 vs LPS-alone group.

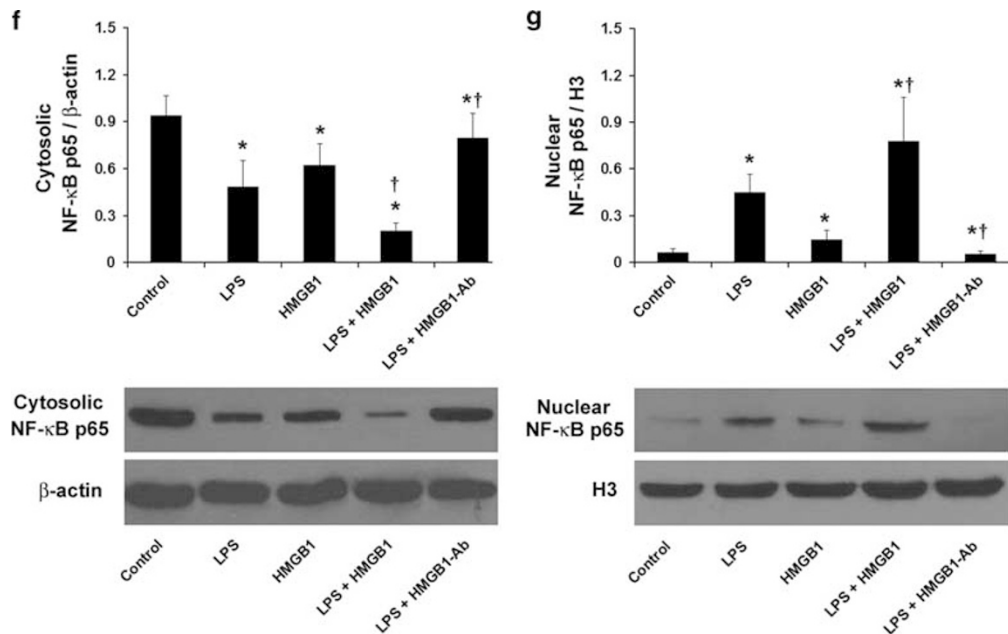


Figure 6 Continued.

HMGB1-induced activation of the NF- κ B signaling pathway is mediated through the TLR4.

DISCUSSION

Our current study demonstrated that LPS and HMGB1, individually or in combination, were able to induce lung fibroblast proliferation in a dose- and time-dependent manner, and that HMGB1 treatment results in cell proliferation that exceeds that seen with LPS. This study also showed, for the first time, that LPS can induce lung fibroblasts to secrete HMGB1 *in vitro*. In addition, the DNA microarray data showed that fibroblasts stimulated with LPS, HMGB1, or LPS+HMGB1 exhibited pronounced changes in the expression of genes encoding proteins in the NF- κ B, TGF- β , and PI3K-Akt signaling pathways. The same three treatments were able to induce TLR4 expression, and anti-TLR4 antibody blocked the activating effects of LPS+HMGB1 on NF- κ B signaling. The present results suggest that targeting these pathways, especially TLR4/NF- κ B, could potentially attenuate pulmonary fibrosis.

Our results related to LPS- and HMGB1-induced lung fibroblast proliferation are consistent with several previous studies.^{3,5,28} Our finding that HMGB1 can enhance LPS-induced lung fibroblast proliferation is novel, yet consistent with findings of cooperative effects in other types of cells, including synovial fibroblasts, macrophages, and human monocytes.²⁹⁻³¹ For example, Wahamaa *et al*³² showed that HMGB1 increased LPS-induced secretion of cytokines, such as tumor necrosis factor- α (TNF- α), interleukin (IL)-6, and IL-8, in synovial fibroblasts. HMGB1 was also shown to enhance the proinflammatory activity of LPS in activated macrophages by promoting the phosphorylation of p38

mitogen-activated protein kinase.²⁹ In addition HMGB1 binding to LPS has been reported to facilitate LPS cellular transformation to a CD14+ profile that enhances LPS-mediated TNF- α production in human monocytes.³⁰ The present study fits with these prior data, and also provides new clues about the mechanisms involved, as described below.

LPS has been reported previously to induce endogenous HMGB1 secretion, directly or indirectly, from various cell types (macrophages, myocardial fibroblasts, and gingival fibroblasts).^{20,31,33} Our data showed, for the first time, that LPS was able to induce lung fibroblasts to secrete HMGB1 and that anti-HMGB1-neutralizing antibody blocked the effects of LPS on lung fibroblast proliferation, suggesting that LPS effects on lung fibroblast proliferation are mediated, at least in large part, through HMGB1. Furthermore, these results indicate that endogenous HMGB1 can collude with LPS in stimulating lung fibroblast proliferation and thereby may function to exacerbate pulmonary fibrosis.

Mechanistically, we found that HMGB1 stimulation produces strong changes in the expression of NF- κ B signal transduction pathway components and nuclear translocation of NF- κ B p65. These data are consistent with the findings of Wu *et al*³¹ showing that LPS can induce HMGB1 relocation and release through activation of the NF- κ B signal transduction pathway in murine macrophage-like RAW264.7 cells. The NF- κ B signaling was important in HMGB1-induced synovial fibroblast migration and the release of inflammatory mediators³⁴ and associated with abnormal proliferation of lung or cardiac fibroblasts.^{35,36} However, before this study, there was no direct evidence demonstrating whether activation of the NF- κ B signaling was involved in LPS-induced secretion of endogenous HMGB1 from lung fibroblasts or

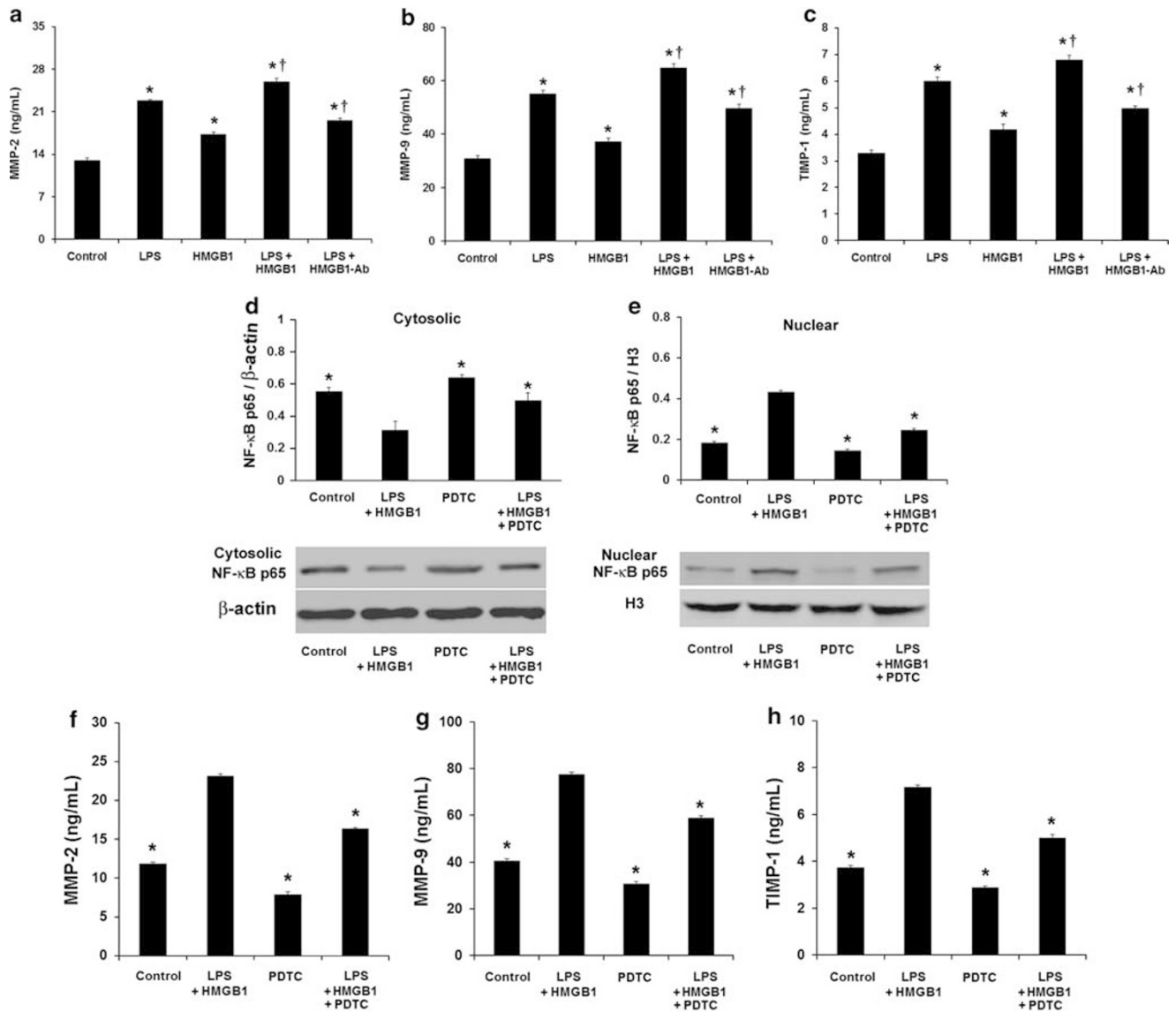


Figure 7 NF- κ B pathway involvement in LPS- and HMGB1-mediated induction of MMP-2, MMP-9, and TIMP-2 secretion. (a–c) Supernatant levels of MMP-2, MMP-9, and TIMP-2 proteins after 48-h LPS and/or HMGB1 treatment of lung fibroblasts with or without a 30-min anti-HMGB1 antibody pretreatment. Mean values from triplicate ELISA experiments are shown with s.d. error bars. * $P < 0.05$ vs controls. † $P < 0.05$ vs LPS-alone group. (d and e) Cytosolic and nuclear NF- κ B p65 protein levels, assessed by western blot, of lung fibroblasts treated for 48 h with LPS and HMGB1 with or without a 30-min pretreatment with the NF- κ B inhibitor PDTC. The fold changes from triplicate experiments are shown with s.d. error bars. * $P < 0.05$ vs LPS+HMGB1 group. (f–h) Supernatant levels of MMP-2, MMP-9, and TIMP-2 proteins, as determined by ELISAs, for the same treatment groups as in (d) and (e). Mean values from triplicate ELISA experiments are shown with s.d. error bars. * $P < 0.05$ vs LPS+HMGB1 group.

that NF- κ B signaling was crucial for LPS- and/or HMGB1-induced lung fibroblast proliferation. The presently observed changes in expression of the NF- κ B pathway constituents in LPS-, HMGB1-, and LPS+HMGB1-stimulated cells suggest that abnormal activation of the NF- κ B pathway may play a role in LPS-induced secretion of endogenous HMGB1 and lung fibroblast proliferation. Furthermore, the TLR4 protein, whose expression was also regulated by these treatments, has been associated previously with the development and progression of pulmonary fibrosis.^{37,38} The presently

observed changes in MMP-2, MMP-9, and TIMP-2 further confirm the importance of the NF- κ B pathway in mediating the effects of LPS and HMGB1 on lung fibroblast proliferation.

In conclusion, the current study demonstrated that LPS and HMGB1, individually or in combination, induce lung fibroblast proliferation and provided insights into the molecular events underlying these effects. Our *in vitro* fibroblast proliferation findings should be confirmed in *in vivo* lung fibrosis studies. In particular, the importance of

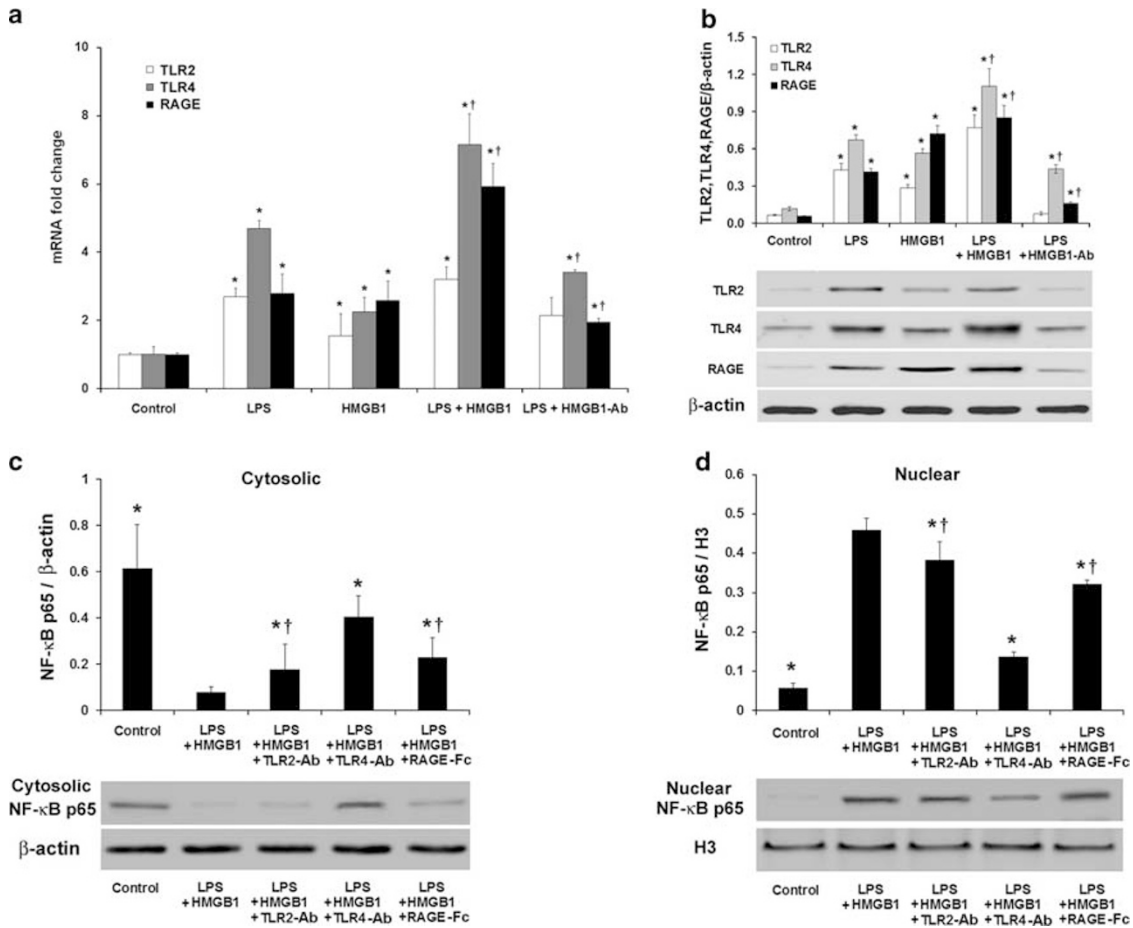


Figure 8 LPS and/or HMGB1 treatment-mediated expression of TLR2, TLR4, and RAGE and activation of NF-κB. **(a and b)** Levels of TLR2, TLR4, and RAGE mRNAs and proteins, assessed by qRT-PCR and western blots, respectively, after 48-h LPS and/or HMGB1 treatment of lung fibroblasts with or without a 1-h anti-HMGB1 antibody pretreatment. Fold change values from triplicate experiments are shown with s.d. error bars. **P* < 0.05 vs controls. †*P* < 0.05 vs LPS-alone group. **(c and d)** Cytosolic and nuclear NF-κB p65 protein levels, as assayed by western blot, following 1-h pretreatment with TLR2, TLR4, and RAGE antibody treatments and 48-h LPS and HMGB1 stimulation. Fold changes from triplicate experiments are shown with s.d. error bars. **P* < 0.05 vs LPS+HMGB1 group. †*P* < 0.05 vs LPS+HMGB1+TLR4-Ab group.

the proteins implicated in the regulation of fibroblast proliferation/fibrosis in this study should be examined further.

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DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

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