Supplemental Materials

Molecular Biology of the Cell

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Extended Experimental Procedures

Animals

Mice deficient for TLR4 and MyD88 were used at backcross eight on C57BL/6 genetic background. All mice, including wild type control C57BL/6, were from the Beijing Biocytogen Co.,Ltd. (Beijing, China). For experiments, adult mice (eight-week-old, weighing 22±3g) were kept in ventilated cages. The animals were cared for in accordance with the guidelines of the Experimental Animal Center, West China Hospital, Sichuan University. All animals had access to water and food ad libitum before and after exposure.

Cigarette smoke exposure and experimental treatment

Mice were placed in a plexiglass chamber (volume of 17L) covered by a disposable filter. The smoke produced by cigarette (Harmful components and tar reduction in cigarette, Sichuan key laboratory, Chengdu, China) burning was introduced at a rate of 25 ml/min into the chamber with continuous airflow generated by a mechanical ventilator (Heidolph PD 5101). The air flow had no influence on the chamber temperature (<0.1°C variation). The animals received smoke of five cigarettes (Harmful components and tar reduction in cigarette) per exposure, 4 exposures a day with 30 min smoke-free intervals during 3 days.

Under cigarette smoke exposure, mice (n = 5~7 in each group) received an intravenous injection of 1.0 or 3.0 mg/kg of TAK-242, 200 μg/kg human recombinant HMGB1 (rHMGB1; Sigma-Aldrich, St. Louis, MO) or vehicle (PBS) via the tail vein

per day. Additionally, mice (n = 5~7 in each group) were intravenous administered neutralizing chicken anti-HMGB1 polyclonal antibody (10 mg/kg; Shino-Test, Tokyo, Japan), normal chicken IgY (10 mg/ kg; Sigma-Aldrich), or ethyl pyruvate (20 mg/kg; Sigma-Aldrich), which is an inhibitor of HMGB1 release, under cigarette smoke exposure.

Bronchoalveolar lavage

After the last cigarette smoke exposure, mice were sacrificed and BAL was performed under sodium pentobarbital anesthesia. After taking both lungs and bronchotracheal trees together, a silicon tube was introduced to the trachea of mice. Both lungs were lavaged with 1ml PBS to obtain cells in the alveolar space and airways. Total cell was counted by hemacytometer. Differential cell counts were performed on cytospin preparations (Cytospin 3, Thermo-Shandon) after May-Grunwald/Giemsa staining. Differential cell counts were made on 200 cells using standard morphological criteria.

Mouse tracheal epithelial (MTE) cells stimulation in vitro

Primary mouse tracheal epithelial (MTE) cells were obtained from naïve TLR4-deficient mice, MyD88-deficient mice and C57BL/6 mice as described [31]. In brief, cells from the tracheobronchial were isolated ,allow cell to differentiate for 10-14 days, and then cultured D-media containing 2% Nuserum and retinoic acid.. CS condensate was prepared as described before [28,32]. MTE cells were plated in 96-well microculture plates (at 10^5 cells/well) and stimulated with CS condensate (20 μg/ml) for 24 h. To examine the effects of HMGB1 on MTE cells secretion of inflammatory cytokines, the isolated cells were pre-incubated (2 h at 37℃) with

anti-TLR4 monoclonal antibody (InvivoGen, San Diego, CA, USA, 20 mg/ml) and isotype control antibody, and then were stimulated with human HMGB1 (Sigma-Aldrich Inc, 0, 50 or 100 ng/ml) for 48 h. After stimulation, the culture supernatant and cells were collected.

Cell Viability Assay

Cell viability after exposure to cigarette smoke was determined by MTT assay. Briefly, MTE cells were initially plated in collagen-coated 96-well microplates at a density of 5×105 cells/mL (100 μL per well). MTE cells were stimulated by CS medium for 24 h, the cells were incubated in 0.5 mg/mL MTT dissolved in RPMI1640 for 1.5 h at 37°C. The generated formazan crystals were dissolved in 200 μL dimethyl sulfoxide (DMSO) and absorbance was measured at 570 nm. Experiments were performed in triplicate and were repeated 4–6 times. Viability was calculated as percentage compared to non-treated control cells.

Quantitative real-time PCR

Total RNA was extracted from tissue preserved in RNAlater or cells harvested after culture using RNAprep pure Tissue Kit (TIANGEN, Beijing, China). Approximately 1 μg RNA was reversely transcribed to cDNA by iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA). Quantitative SYBR green-based real-time PCR analysis of 84 genes related to TLR-mediated signal transduction was performed using the Toll-Like Receptor Signaling Pathway RT2 Profiler TM PCR Array (SABiosciences, Qiagen) in an ABI Prism 7000 System (Applied Biosystems, Foster City, CA, USA). Relative expression of the target genes were estimated by the 2-ΔCT

method using ribosomal protein L13a (RPL13A) as a housekeeping gene. Samples were analyzed in triplicate, and experiments were repeated at least three times. For the genes showing at least a 4-fold difference in gene expression between test and control groups we performed a gene set enrichment analysis using the tool GOrilla6. This tool uses the Gene Ontology database to obtain sets of genes which are significantly enriched in specific molecular functions, biological processes and cellular components. To confirm the results obtained from the PCR array, a number of significantly over-expressed genes were tested separately by quantitative reverse transcription PCR using primers purchased from Bioneer (Shanghai, China).

Determination of proinflammatory cytokine and HMGB1

The BAL and interstitial fluid, and the culture supernatant samples were analyzed using the MILLIPLEX Mouse Cytokine/Chemokine panel (Millipore Corporation, Billerica, MA). The kit can detect the following 6 cytokines (IL-1β, IL-6, TNF-α,IL-8, IFN-γ and MCP-1) according to the manufacturer's instructions. The samples were analyzed using Luminex laser-based fluorescent analytical test instrumentation (Luminex 200, Austin, TX). Cytokine concentrations were determined from standard curves prepared on each plate and expressed as picogram per milliliter (pg/ml). The levels of HMGB1 in the BAL fluid and the culture supernatant were measured using an HMGB1 sandwich ELISA kit (Shino-Test) according to the manufacturer's protocol.

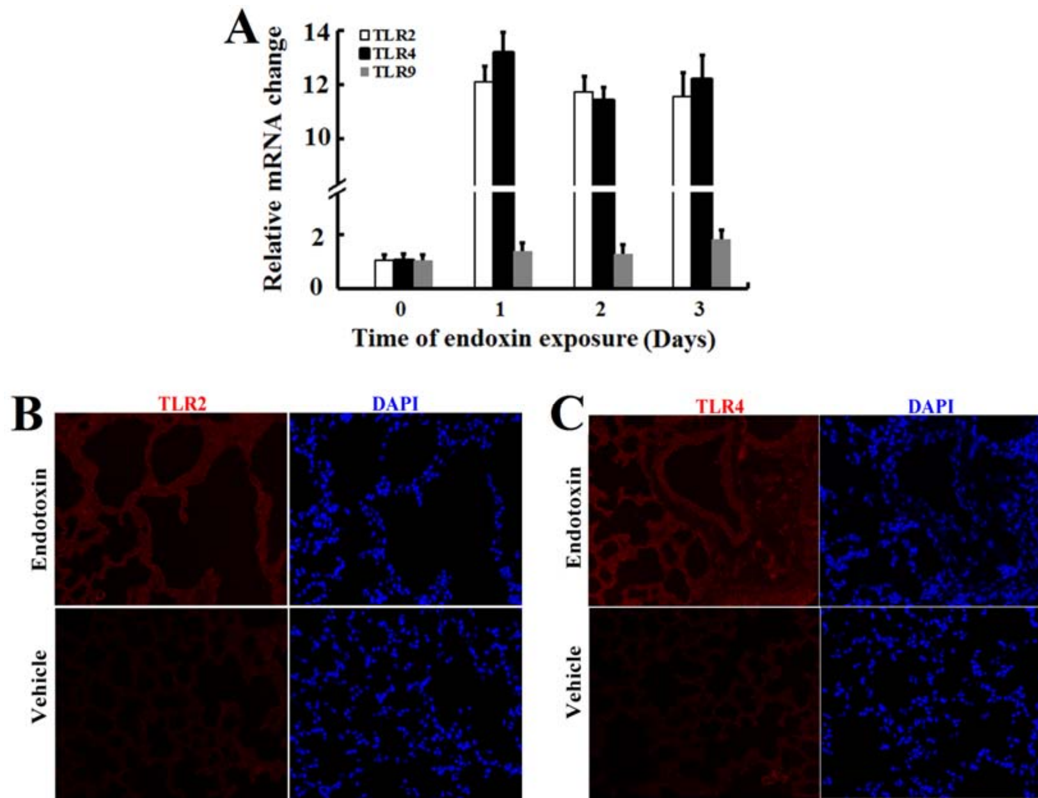
Western blot analysis

The cytoplasmic and nuclear protein extracts from the lungs tissues or

tracheobronchial epithelial cells were resolved on 10% SDS-PAGE gels followed by electrophoretic transfer to nitrocellulose membranes. Then immunoblot analysis was performed with specific primary antibodies at a 1:500 dilution, and the secondary antibody conjugated with horseradish peroxidase at a 1:1,000 dilution. Membranes were visualized with substrate from ECL-PLUS Western Blotting Detection kit (Amersham Pharmacia Biotech) in the Molecular Imager Gel Doc XR System (Bio-Rad). Anti-TLR2, Anti-TLR4, Anti-TLR9 and anti-HMGB1 antibodies were purchased from Santa Cruz Biotechnology (CA, USA). Anti-JNK, anti-phospho JNK, anti-p38, anti-phospho p38, IκBα, anti-phospho IκBα, and anti-Actin monoclonal antibodies were purchased from Abcam (Cambridge, UK).

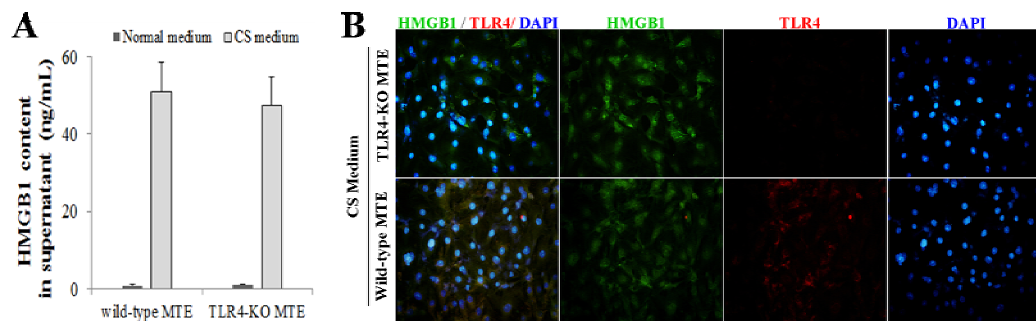
Immunofluorescence microscopy

Immunofluorescence was performed to detect TLR2, TLR4, TLR9, HMGB1, TNF-α and IL-1β in lung tissue and cultured epithelial cells. Briefly, Lung tissue was fixed in 4% buffered formalin and block endogenous peroxidase activity. Cells were fixed with 4% paraformaldehyde, permeabilized with 0.5% Triton X-100, and blocked with 10% goat serum. Sections or cells were then incubated for 1 h with primary antibodies (1:100-500; Santa Cruz or Abcam) in 5% goat serum and subsequently stained for 1 h with a FITC/RBITC-conjugated secondary antibody (1: 500; Cell Signaling or Invitrogen). The images were captured by fluorescence microscopy (Nikon, Japan), and analysis was performed using Advanced Spot software (Diagnostic Instruments, Sterling Heights, MI).



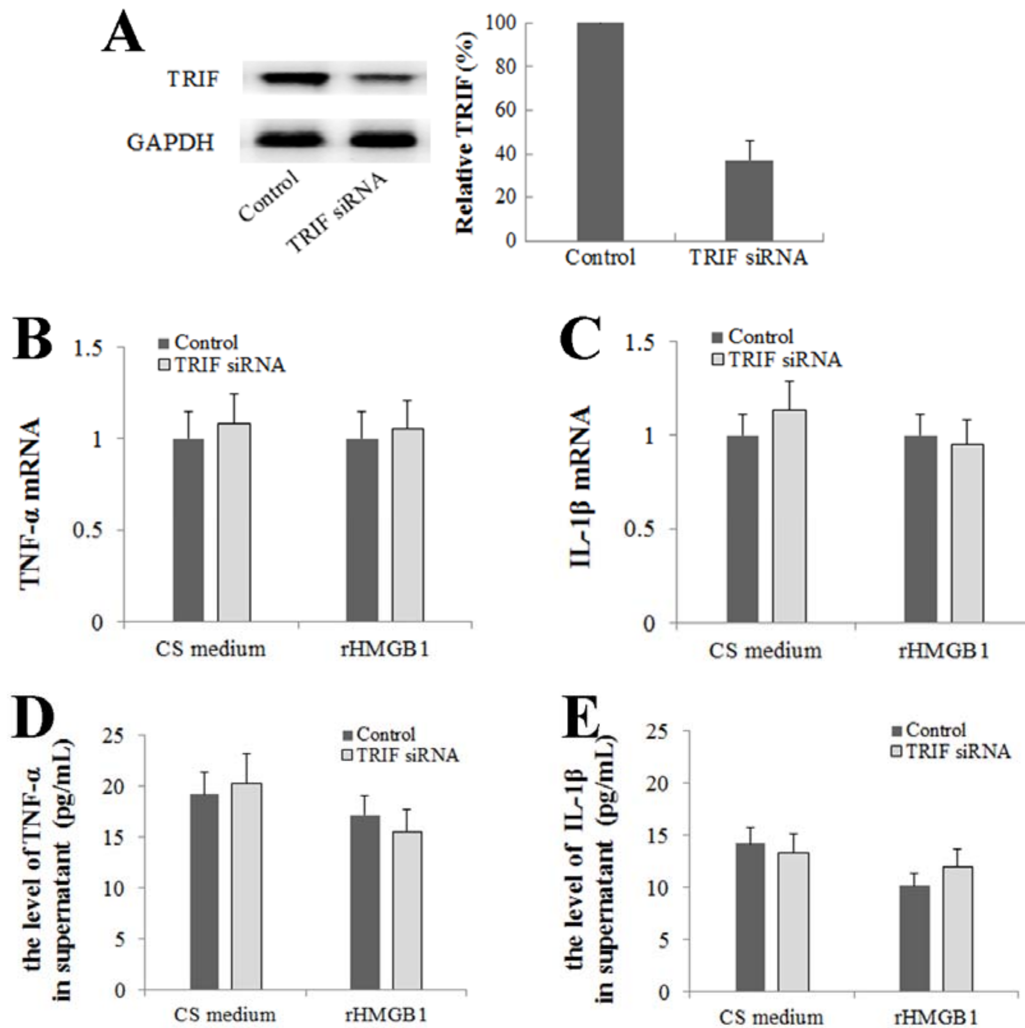
Supplementary Fig1: Endotoxin increased expression of TLR2 and TLR4 in lungs

Endotoxin was perfused to tracheas of mice by 5 EUs per animal, the expression of TLR2, TLR4 and TLR9 was determined by real-time PCR and immunofluorescence analysis in lungs after exposed 0, 1, 2 and 3 day). Real-time PCR revealed Endotoxin exposure increased the expression of TLR2 and TLR4, and peaked at 1 day (A). Immunofluorescence study also observed high expression of TLR2 (B) and TLR4 (C) in lungs after exposed 1 days.



Supplementary Fig2: cigarette smoke does not require TLR4 on MTE cells to induce HMGB1

MTE cells, harvested from wild-type mice and TLR4-KO mice, were stimulated by CS medium for 24 h. HMGB1 content in supernatant and HMGB1 translocation were determined by ELISA (A) and immunoflurescence (B), respectively. The results are displayed as the means ± SD. from three independent experiments.* P<0.05.



Supplementary Fig3: cigarette smoke and HMGB1 induces TNF-α and IL-1β in TRIF-independent maner

MTE cells were treated with control or TRIF siRNA (A), and then were stimulated with CS medium or human rHMGB1 (100 ng/ml) for 24 h. After stimulation, cells and the culture supernatant were collected. TNF-α and IL-1β mRNA expression levels were determined by quantitative RT-PCR (B, C) and the concentration of TNF-α and IL-1β in supernatant were determined by multiplex cytokine assay (D, E). All of the treatments in this figure were carried out in triplicates, and the results are displayed as the means ± SD.