Microarray profiling of lung long non-coding RNAs and mRNAs in lipopolysaccharide-induced acute lung injury mouse model

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Research Article

Long non-coding RNAs (IncRNAs) are involved in various biological processes as well as many respiratory diseases, while the role of IncRNAs in acute lung injury (ALI) remains unclear. The present study aimed to profile the expression of lung IncRNAs and mRNAs in lipopolysaccharide (LPS)-induced ALI mouse model. C57BL/6 mice were exposed to LPS or phosphate-buffered saline for 24 h, and IncRNAs and mRNAs were profiled by Arraystar mouse LncRNA Array V3.0. Bioinformatics analysis gene ontology including (GO) and pathway analysis and cell study in vitro was used to investigate potential mechanisms. Based on the microarray results, 2632 IncRNAs and 2352 mRNAs were differentially expressed between ALI and control mice. The microarray results were confirmed by the quantitative real-time PCR (qRT-PCR) results of ten randomized selected IncRNAs. GO analysis showed that the altered mRNAs were mainly related to the processes of immune system, immune response and defense response. Pathway analysis suggests that tumor necrosis factor (TNF) signaling pathway, NOD-like receptor pathway, and cytokine–cytokine receptor interaction may be involved in ALI. LncRNA-mRNA co-expression network analysis indicated that one individual IncRNA may interact with several mRNAs, and one individual mRNA may also interact with several IncRNAs. Small interfering RNA (siRNA) for ENSMUST00000170214.1, - ENSMUST00000016031.13 significantly inhibited LPS-induced TNF-α and interleukin (IL)-1β production in murine RAW264.7 macrophages. Our results found significant changes of IncRNAs and mRNAs in the lungs of LPS-induced ALI mouse model, and intervention targeting IncRNAs may attenuate LPS-induced inflammation, which may help to elucidate the role of IncRNAs in the pathogenesis and treatment of ALI.

Introduction

Acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) are a sequence of lung injuries arising from a wide variety of stimulus, followed by uncontrolled inflammation, which frequently result in multiple organ dysfunction with high mortality [1]. It was estimated that the incidence of ALI is approximately 200000 with an overall mortality rate of 40% in the U.S.A. from an epidemiologic study [2]. Although significant progress has been achieved on the treatment of ALI/ARDS with mechanical ventilation or other drugs to inhibit the excessive inflammation [3,4], the prognosis of ALI/ARDS is still not optimistic. Evidence from a recent systematic review suggests that even after 2010, the overall mortality rates of ARDS in hospital, Intensive Care Unit, 28/30 days, and 60 days were 45, 38, 30, and 32%, respectively [5]. Since
the pathogenesis of ALI has not been fully elucidated, finding a novel therapeutic target for ALI/ARDS is imperatively needed.

Long non-coding RNAs (lncRNAs) are a new class of non-coding RNAs that play a role in regulating gene transcription, protein expression and epigenetic regulation at various levels. They play important roles in various diseases, including cancers, rheumatic diseases, cardiac and infectious diseases [6–10]. Our previous study observed significant changes of lncRNAs expression profiles in cigarette-smoke exposed mouse lung and revealed a potential role of lncRNAs in the pathogenesis of cigarette smoke-associated airway inflammatory disorders [11]. Huang et al. [12] also reported that the expression profiles of lncRNA were changed in the blood of pneumonia patients, suggesting that lncRNAs and their target genes may be closely associated with the progression of pneumonia. Dysregulated lncRNAs were also observed in the peripheral blood of patients with eosinophilic asthma, suggesting that lncRNAs may take part in the immune regulation of eosinophilic asthma [13]. All these studies reveal a potential role of lncRNAs in respiratory disorders. However, the potential role of lncRNAs in ALI has not been fully reported, the present study aimed to profile both lncRNAs and mRNAs in the lung of ALI mice, and tried to discuss their roles in ALI through bioinformatics analysis and cell study in vitro.

Materials and methods

Animal preparation

The study protocol was reviewed and approved by the hospital Animal Ethics Committee (2017095A). The C57BL/6 mice were prepared based on the Animal Research: Reporting of In Vivo Experiment guidelines. Ten specific pathogen-free male C57BL/6 mice (8–10 weeks, 24–26 g) were purchased and were randomly divided into two groups: control group and lipopolysaccharide (LPS)-stimulated group, each group contained five mice (Dashuo Biological Technology Co, Ltd, Chengdu, China).

ALI model preparation

After anesthetizing with Sevoflurane Inhalation Anesthetic (Hengrui Medicine, Jiangsu, China), two group male C57BL/6 mice were challenged with phosphate-buffered saline (Life Technologies, Grand Island, NY, U.S.A.) or LPS (Escherichia coli O111:B4, Sigma–Aldrich, St Louis, MO, U.S.A.) through intratracheal spray using a MicroSprayer™ (PennCentury, Philadelphia, PA) [14], respectively. After 24 h, the mice were killed through intraperitoneal injection of sodium pentobarbital (100 mg/kg), followed by exsanguination from the abdominal aorta to collect lung tissue sample.

Mouse histology

The left lung was fixed with 4% formaldehyde, and embedded by paraffin, then, Hematoxylin and Eosin (HE) stains on slices of lung tissue were used to observe the pathological changes of lung (Sigma–Aldrich, St. Louis, MO, U.S.A.), and lung injury score was assessed following the official standard of American Thoracic Society [15].

RNA isolation

The total RNA from mouse lung tissues was extracted and purified using TRIzol reagent (Invitrogen, Carlsbad). The quantitation and quality of RNA and RNA integrity was evaluated by standard method as previously described [11].

Microarray analysis

First, mRNA was purified from 1 mg of total RNA, each sample was amplified and transcribed into fluorescent cRNAs utilizing random primers (Arraystar Flash RNA Labeling Kit, Arraystar). Then, the cRNAs were hybridized on to the mouse LncRNA Microarray 3.0 (Arraystar). The arrays were scanned (Agilent Scanner, G2505C), and array images were analyzed (Agilent Feature Extraction Software, version 11.0.1.1). Data normalization and subsequent processing were carried out with the GeneSpring GX v12.1 software package (Agilent Technologies, Santa Clara, CA, U.S.A.).

A volcano plot filtering was used to identify differentially expressed IncRNAs and mRNAs, with the threshold defined as fold-change > 2.0 (Student's t test P<0.05). Distinguishable lncRNA expression profile between case and control mice was showed by Hierarchical clustering. All the microarray hybridization and analysis was performed by KangChen Biotech (Shanghai, China).
Validation of microarray results
To validate the reliability of microarray results, five up-regulated and five down-regulated lncRNAs were randomly selected to be examined by further quantitative real-time PCR (qRT-PCR), primers of the ten selected lncRNAs were listed in Supplementary Table S1.

Briefly, RNA was extracted and cDNA was synthesized using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, U.S.A.). qRT-PCR was carried out by the CFX96 real-time PCR detection system using SsoFast EvaGreen Supermix (Bio-Rad, Hercules, CA, U.S.A.). Each lncRNA was quantitated by standard curve and all data were normalized to GAPDH gene expression. Student’s t test was used to examine the differences of lncRNA expression between ALI and controls mice (SPSS Inc., Chicago, IL, U.S.A., version of 22.0). Natural logarithm was used to analyze the relationship between fold changes of qRT-PCR and microarray analysis, a two-sided \( P < 0.05 \) was set as significant.

Bioinformatics analysis
Gene Ontology (GO) analysis was applied to calculate the functions of differentially expressed genes, including biological processes, molecular functions and cellular components. Signal pathway analysis was used to map genes to Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways. Fisher’s exact tests were used for the statistical analyses.

LncRNA–mRNA co-expression network analysis
The correlation between differentially expressed mRNAs and lncRNAs was evaluated by coding-non-coding gene co-expression network (CNC network) analysis. The lncRNA–mRNA pairs were identified by Pearson’s correlation coefficients (PCC) of no less than 0.9 [11]. The figure of lncRNA–mRNA co-expression network was generated by Cytoscape software (The Cytoscape Consortium, San Diego, CA, U.S.A.).

Cis- and trans-regulated gene analysis
Cis-acting lncRNAs regulate the expression of genes that are positioned in the vicinity of their transcription sites, whereas trans-acting lncRNAs modulate the expression of genes being at independent loci [16]. The gene locations for different lncRNAs on the chromosome were determined, then the co-expressed genes \( (r > 0.85 \) or \( r < -0.85, P < 0.01) \) located within the 300 kbp windows upstream and downstream of the differentially expressed lncRNAs were identified as the potential ‘cis-regulated mRNAs.’ ‘trans-regulated mRNAs’ were potentially coding genes of trans-regulated protein as co-expressed with dysregulated lncRNAs and beyond 100 kb in genomic distance from them. To analyze functions of the potential ‘cis-regulated mRNAs’ or ‘trans-regulated mRNAs,’ GO enrichment and pathway analysis was also performed.

Cell culture and transfection
Murine RAW264.7 macrophages were purchased from Geneseed Biotechnology Co., Ltd (Guangzhou, China). Murine RAW264.7 macrophages were cultured in high-glucose DMEM (Gibco, Invitrogen Life Technologies Corporation, NY, U.S.A.) supplemented with 10% fetal bovine serum (ExCell Bio, Shanghai, China) in a humidified atmosphere with 5% CO2 at 37°C. Then, cells were plated in a six-well plate for further experiments.

Cells transfection with small interfering RNA
The small interfering RNA (siRNA) duplexes for ENSMUST00000170214.1 (sequence: GCAGCAGAAGTCACTTATA) and ENSMUST00000016031.13 (sequence: AAAGAACAGGGAGCTTCAA) were commercially synthesized by Invitrogen (Cat.No. 11668019). According to the manufacturer’s instructions, RAW264.7 cells were transfected at 70% confluence with siRNA-ENSMUST00000170214.1, -ENSMUST00000016031.13 and siRNA-scrambled by Lipofectamine 2000. Twenty-four hours after transfection, RAW264.7 cells were exposed to LPS (100 ng/ml) (Sigma Chemical Co, St. Louis, MO, U.S.A.) for 24 h, and then the culture supernatants were collected for further measurement.

Enzyme-linked immunosorbent assay measurement
The concentrations of tumor necrosis factor (TNF)-\( \alpha \) and interleukin (IL)-1\( \beta \) in the culture supernatants were detected by enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minnesota, MN, U.S.A.) following the manufacturer’s instructions.
Figure 1. Mouse histologic changes
The histologic changes were performed with HE-staining in LPS-treated mice (A), PBS-treated mice (B) and lung injury score was evaluated based on these slices (C). **\( P < 0.05 \).

**Statistical analysis**
All data were presented as mean ± S.D., independent t test was used to determine the difference between two groups. And \( P < 0.05 \) was considered to be statistically significant. Statistical analyses were carried out with SPSS software, version 18.0 (SPSS, Inc., Chicago, IL, U.S.A.).

**Results**

**Establishment of ALI mouse model**
After LPS stimulation, significant inflammation was observed in the lung of ALI mouse. HE-staining showed that LPS dramatically increased the leukocyte-infiltration in mouse lungs (Figure 1A, B), ALI score increased significantly in LPS-challenged mice (\( P < 0.05 \)) (Figure 1C), suggesting successful establishment of ALI mouse model.

**LncRNA and expression profile and validation**
A total of 2632 differentially expressed lncRNAs were identified in the lung tissue of LPS-exposed mice, with 1214 of them up-regulated and the rest 1418 down-regulated (fold-change > 2.0; \( P < 0.05 \), Figure 2A). LncRNAs uc007pnu.1 (fold-change: 75.22, \( P = 1.46 \times 10^{-7} \)) and ENSMUST00000144634 (fold-change: 41.80, \( P = 7.33 \times 10^{-7} \)) were the most up- and down-regulated lncRNAs, respectively. Table 1 listed the top 20 differentially expressed lncRNAs identified by microarray analysis.

**Validation of lncRNAs microarray results**
The expression of ten selected lncRNAs was validated by qRT-PCR (Figure 3A), and the results of microarray and qRT-PCR were compared in Figure 3B. Correlation analysis showed that the results of microarray were positively correlated with qRT-PCR (\( r = 0.9274, P < 0.001 \), Figure 3C), suggesting the reliability of the microarray data.
Figure 2. Heat maps showing the distinct lncRNA (A) and mRNA (B) expression profiles between LPS-stimulated mice and control mice.

Hierarchical clustering of significantly \( P < 0.05 \), >2-fold change) regulated lncRNAs (A) and mRNAs (B) are shown as heat maps. Expression values are presented with different colors ranging from green to red, indicating low relative expression to high relative expression, respectively \( (n=5 \) for control group and LPS-stimulated group, respectively).

Table 1 The detailed information of the top ten up-regulated and top ten down-regulated lncRNAs

| Probe name         | Regulation | Seqname | Gene Symbol | RNA length | chrom | Fold change | \( \text{P-value} \) |
|--------------------|------------|---------|-------------|------------|--------|-------------|------------------|
| ASMM10P051398      | Up         | uc007pnu.1 | AK045681    | 2769       | chr13  | 75.2210375  | 1.46509E-07      |
| ASMM10P010513      | Up         | uc007pik.1 | AK145614    | 1723       | chr13  | 48.3250172  | 8.4448E-09       |
| ASMM10P014251      | Up         | AK149396  | AK149396    | 3559       | chr16  | 44.5581719  | 1.9159E-09       |
| ASMM10P020427      | Up         | ENSMUST00000147219 | Lcn2 | 1374 | chr2 | 37.7155939 | 3.4948E-06 |
| ASMM10P052114      | Up         | uc007rru.1 | AK089519    | 1193       | chr13  | 32.6411577  | 7.77233E-07      |
| ASMM10P021290      | Up         | uc007cok.2 | Lemd1       | 5292       | chr1   | 26.5422785  | 7.67235E-06      |
| ASMM10P052634      | Up         | NR045616  | 1700011B04Rik | 648 | chr13 | 24.7399888 | 7.0118E-08 |
| ASMM10P051617      | Up         | uc007qai.1 | AK161362    | 3212       | chr13  | 23.6750817  | 2.4655E-08       |
| ASMM10P037088      | Up         | AK148588  | AK148588    | 933        | chr7   | 18.8202011  | 3.6855E-09       |
| ASMM10P020061      | Up         | ENSMUST00000138796 | BC100530 | 510 | chr16 | 18.8158229 | 1.10227E-05 |
| ASMM10P020347      | Down       | ENSMUST00000144634 | Gm13373 | 655 | chr2 | 41.8033669 | 7.32501E-07 |
| ASMM10P035100      | Down       | AK047865  | AK047865    | 3562       | chr10  | 30.2058144  | 1.00927E-06      |
| ASMM10P035091      | Down       | AK081905  | AK081905    | 2277       | chr10  | 28.5792577  | 2.242E-10        |
| ASMM10UP345        | Down       | uc.428+   | uc.428      | 239        | chr18  | 27.4789604  | 1.16684E-08      |
| ASMM10P035102      | Down       | AK048117  | AK048117    | 3421       | chr10  | 26.2808204  | 1.99338E-07      |
| ASMM10P035838      | Down       | AK132971  | AK132971    | 2765       | chr10  | 20.667653   | 1.31455E-06      |
| ASMM10P013477      | Down       | ENSMUST00000159177 | Fer16 | 2776 | chr15 | 20.188906   | 0.000124486 |
| ASMM10P011787      | Down       | uc007cs.1 | AK047145    | 690        | chr14  | 18.4579932  | 2.32328E-08      |
| ASMM10P011784      | Down       | AK06017   | AK06017     | 653        | chr14  | 17.9018036  | 2.76482E-08      |
| ASMM10P036217      | Down       | uc012fmg.1 | A230057D06Rik | 1713 | chr7 | 15.5120843 | 2.12844E-06 |

These are top ten up-regulated and top ten down-regulated lncRNAs between LPS-exposed mice and controls.
Figure 3. Comparison between microarray data and qRT-PCR results

qRT-PCR was performed to test the differentially expressed IncRNAs between controls and LPS-stimulated mice (A); the fold change of each IncRNA between LPS-stimulated mice and controls was tested with microarray and qRT-PCR, respectively (B). The correlation between microarray and qRT-PCR was performed with natural logarithms of these different fold changes (C). *: \( P < 0.05 \), \( r \): standard correlation coefficient \((n=5\) for control group and LPS-stimulated group, respectively). Abbreviations: CON, control group; LPS, lipopolysaccharide group.

Table 2 The detailed information of the top ten up-regulated and top ten down-regulated mRNAs

| Probe name       | Regulation | Seqname     | Gene Symbol | RNA length | chrom | Fold change | P-value |
|------------------|------------|-------------|-------------|------------|--------|-------------|---------|
| ASMM10P005163    | Up         | NM_016960   | Ccl20       | 852        | chr1   | 554.0964063| 9.12E-11 |
| ASMM10P005162    | Up         | NM_001159738| Ccl20       | 849        | chr1   | 135.0928322| 3.4132E-09|
| ASMM10P028947    | Up         | NM_011016   | Orm2        | 774        | chr4   | 102.971434  | 1.09916E-06|
| ASMM10P013206    | Up         | NM_030720   | Gpr84       | 1611       | chr15  | 94.7282259 | 4.85E-11 |
| ASMM10P011036    | Up         | NM_053113   | Ear11       | 722        | chr14  | 89.7366424 | 9E-13   |
| ASMM10P028944    | Up         | NM_008768   | Orm1        | 788        | chr14  | 87.3439735 | 7.2301E-08|
| ASMM10P030638    | Up         | NM_008599   | Cxcl9       | 2905       | chr5   | 80.7283366 | 1.52587E-06|
| ASMM10P006833    | Up         | NM_009140   | Cxcl2       | 1083       | chr5   | 74.3332726 | 2.07961E-07|
| ASMM10P043399    | Up         | NM_008694   | Ngp         | 1176       | chr9   | 66.9952274 | 6.28066E-06|
| ASMM10P014372    | Up         | NM_025288   | Sfr6a3      | 412        | chr16  | 61.2734967 | 9.99142E-06|
| ASMM10P036911    | Down       | NM_00101488| Gag1l       | 3924       | chr7   | 207.5899744| 5.13E-11 |
| ASMM10P021970    | Down       | NM_00101272| Wtde16      | 1129       | chr6   | 84.7721375 | 7.29101E-06|
| ASMM10P024917    | Down       | NM_007529   | Bcan        | 3267       | chr3   | 50.9586882 | 7.62062E-07|
| ASMM10P033396    | Down       | NM_144943   | C207        | 1530       | chr6   | 33.2684171 | 4.743E-10 |
| ASMM10P024918    | Down       | NM_001109758| Bcan        | 2885       | chr6   | 32.2586958 | 1.01766E-05|
| ASMM10P047527    | Down       | NM_01099774| Krtap17-1   | 783        | chr11  | 31.9873817 | 2.1869E-09|
| ASMM10P054528    | Down       | NM_009605   | Adipoq      | 1233       | chr16  | 31.1071707 | 3.4967E-06|
| ASMM10P024907    | Down       | NM_030707   | Forls       | 1991       | chr6   | 26.480804  | 4.524E-10 |
| ASMM10P034922    | Down       | NM_001109749| Chnn14      | 5262       | chr6   | 24.5078928 | 3.757E-10 |
| ASMM10P025605    | Down       | NM_025285   | Stmn2       | 1904       | chr5   | 22.3629809 | 2.58718E-05|

There are top ten up-regulated and top ten down-regulated mRNAs between LPS-exposed mice and controls.

mRNA expression profile

A total of 1108 up-regulated and 1244 down-regulated mRNAs were identified in the lungs of LPS-exposed mice (fold-change > 2.0; \( P < 0.05 \)) (Figure 2B). NM_016960 (fold change: 554.10, \( P = 9.12E-11 \)) and NM_001101488 (fold change: 207.59, \( P = 5.13E-11 \)) were the most up- and down-regulated mRNAs, respectively. Table 2 summarized the top 20 differentially expressed mRNAs identified by microarray analysis.

GO analysis

GO analysis showed that the top three enriched biological processes of up-regulated genes were: immune system process (GO:002376), immune response (0006955), and defense response (0006952), the top three enriched cellular components of up-regulated genes were extracellular space (GO:0005615), extracellular region (GO:0005576) and extracellular region part (GO:0044421), and the top three enriched molecular function of up-regulated genes were protein binding (GO:0005515), receptor binding (GO:0005102), and binding (GO:0005488) (Figure 4A).
Figure 4. Biological functions of up-expressed and down-expressed mRNAs
The most significantly up-regulated mRNAs (A) and down-regulated mRNAs (B) involved in biological process, cellular component and molecular function were identified by GO analysis.
Figure 5. Pathway analysis for up-regulated and down-regulated mRNAs

The most significant pathways related to the up-regulated genes (left) and down-regulated genes (right) were achieved by KEGG pathway analysis.

![Pathway analysis for up-regulated and down-regulated mRNAs](image)

For down-regulated genes, the most significant enriched biological processes were muscle system process (GO:0003012), single-organism process (GO:0044699), and single-multicellular organism process (GO:0044707). The most significant enriched cellular components were cell periphery (GO:0071944), plasma membrane (GO:0005886), and extracellular region (GO:0005576). And the most enriched molecular functions were protein binding (GO:0005515), binding (GO:0005488), and calcium ion binding (GO:0005509) (Figure 4B).

**KEGG pathway analysis**

KEGG signal pathway analysis found that the main pathways of up-regulated genes are TNF signaling pathway, NOD-like receptor pathway, and cytokine–cytokine receptor interaction (Figure 5), while down-regulated transcripts in LPS-treated lung tissues are associated with dilated cardiomyopathy, hypertrophic cardiomyopathy and cGMP-PKG signaling pathway (Figure 5).

**CNC network analysis**

CNC network analysis showed all the differentially expressed lncRNAs which has a PCC value more than 0.9 with their related mRNAs. Those ten lncRNAs validated by qRT-PCR were marked as red and green. Part of results was presented in Figure 6, and the full CNC network analysis was presented in Supplementary Figure S1. Many lncRNAs were co-expressed with multiple mRNAs and lncRNAs.
Table 3 The top three GO function terms for cis- and trans-regulated gene analysis

| Genes                  | GO terms                          | Contents                                                      |
|------------------------|----------------------------------|---------------------------------------------------------------|
| cis-regulated genes    | Biological processes             | chemokine-mediated signaling pathway; positive regulation of leukocyte chemotaxis; inflammatory response |
|                        | Cellular components              | specific granule; tertiary granule; tertiary granule membrane |
|                        | Molecular function               | cytokine activity; chemokine activity; chemokine receptor binding |
| trans-regulated genes  | Biological processes             | response to molecule of bacterial origin; inflammatory response; calcium-mediated signaling |
|                        | Cellular components              | specific granule; specific granule membrane; tertiary granule membrane |
|                        | Molecular function               | cytokine activity; chemokine activity; chemokine receptor binding |

Figure 7. Pathway analysis of cis- and trans-regulated genes
(A) Pathway analysis of cis-regulated gene. (B) Pathway analysis of trans-regulated gene.

The cis- and trans-regulated genes analysis

The GO analysis of cis- and trans-regulated genes was summarized in Table 3. The pathway analysis showed that cytokines and inflammatory response, spinal cord injury, IL-1 signaling pathway, focal adhesion and IL-3 signaling pathway etc were the joint pathways for cis- and trans-regulated genes (Figure 7).

TNF-α and IL-1β measurement

In the present study, we chose ENSMUST00000170214.1 and ENSMUST00000016031.13 for further functional research. When compared with control group, siRNA for ENSMUST00000170214.1 or ENSMUST00000016031.13 significantly decreased the concentrations of TNF-α and IL-1β in the culture supernatants of murine RAW264.7 macrophages after stimulation of LPS for 24 h (Figure 8).

Discussion

Recent studies on transcriptome microarray or sequencing have characterized the unique function of thousands of differentially expressed lncRNAs in the development and progression of various disorders [6–10]. However, studies regarding the role of lncRNAs in ALI are still limited. In the current study, we investigated the lncRNA expression profiles in the lung tissue of LPS-challenged mice, 2632 differentially expressed lncRNAs and 2352 mRNAs were identified via microarrays. The microarray results of randomly selected lncRNAs were further validated by qRT-PCR analysis, and the data from qRT-PCR analysis matched well with those from microarray assay. In addition, study in vitro suggests that lncRNAs may play a role in the treatment of ALI through the regulation of inflammation. Our study indicated that there may be a potential role of lncRNAs in the pathogenesis and therapy of ALI.
Figure 8. Effect of siRNAs for lncRNAs on LPS-induced cytokines production in murine RAW264.7 macrophages

(A) siRNA for ENSMUST00000170214.1 significantly decreased the concentrations of TNF-α in the culture supernatants of murine RAW264.7 macrophages after stimulation of LPS for 24 h. (B) siRNA for ENSMUST00000016031.13 significantly decreased the concentrations of IL-1β in the culture supernatants of murine RAW264.7 macrophages after stimulation of LPS for 24 h. *P<0.05, **P<0.01.

Previous studies have confirmed that lncRNAs participate in various physiologic or pathologic processes at different levels, including chromatin remodeling, regulation of gene transcription, protein expression and epigenetic regulation [17]. Accordingly, GO analyses were performed to determine the potential roles of the differentially expressed genes. Our data revealed that the up-regulated mRNAs are mainly involved in immune system process, immune response and defense response. It has been widely accepted that immune system and host defense were involved in the onset of ALI/ARDS, the immune system contains diverse cell types that coordinate responses to infection [18]. LncRNAs may play key roles in epigenetic and transcriptional regulation, and have shown great potential as key regulatory molecules of immune cell gene expression programs in response to microbial-derived clues [19]. Previous studies showed that lncRNAs regulate the LPS-stimulated inflammatory response in human monocytes, and lncRNAs would be important regulators of human innate immune response [20]. Our data strongly supported these previous studies, indicating lncRNAs-mediated immune defense mechanism participates in the LPS-induced ALI model and may offer clues for early intervention of ALI through lncRNAs.

KEGG analysis showed most up-regulated genes were related to TNF signaling pathway and NOD-like receptor. TNF families have been found to provoke the release of downstream inflammatory cytokines, thus further mediating the innate immune response and inflammatory process in ALI [21]. Recent study reported that lncRNA-HOTAIR increase the release of TNF-α in the cardiomyocytes of LPS-induced sepsis mice by activating NF-κB through the phosphorylation of NF-κB p65 subunit, suggesting that lncRNA may plays a role in ALI through the regulation of TNF-α [22]. Growing studies revealed that both (NOD-) like receptor protein 9b and (NOD-) like receptor protein 3 play a role in the regulation of ALI [23,24], and these findings supported our bioinformatics results.

Based on current evidence, most of the lncRNAs may function locally to activate or suppress the expression of their neighboring or overlapping gene [25]. Thus, the potential cis- or trans-target genes for different expressed lncRNAs were predicted to investigate the possible significance of these lncRNAs in the lung response to LPS stimulus. And GO analysis found that cytokines, chemokines, and inflammatory response are involved in the pathogenesis of ALI, which were confirmed by previous publications [26,27]. Signal pathway analysis found that both cis- and trans-regulated genes are involved in pathways of cytokines and inflammatory response, IL-1 signaling pathway, focal adhesion, and IL-3 signaling pathway, based on previous findings, these pathways are critical for development of ALI [26,28,29], suggesting dysregulated lncRNAs may play a role in ALI/ARDS through such signal pathways enriched by cis- or trans-target genes.

Moreover, based on CNC network analysis, we found that many lncRNAs were significantly related to the expression of multiple protein-coding genes, and different lncRNAs were differentially correlated with the same genes.
Notably, uc009lwr.1 was positively, while ENSMUST00000146390 and NR_033299 were negatively related to CXCL-1 and CXCL-2. These two chemokines have been found to be involved in the pathogenesis of ALI. CXCL-1 is released by the activated neutrophils, and then mediates neutrophilic airway inflammation [30]. CXCL-2 is secreted by monocytes, and trigger downstream inflammatory response [31]. Herein, our date implied that these IncRNAs might play different roles in the immune responses during ALI. Specifically, uc009lwr.1 would promote the onset of inflammatory responses, while ENSMUST00000146390 and NR_033299 would play protective role against ALI-related inflammation. These findings supported the results of GO and KEGG pathway analyses, indicating the critical role of innate immune response in LPS-induced inflammatory response. CNC analysis suggests most IncRNAs were co-expressed with multiple mRNAs and IncRNAs, indicating that multiple trans-regulative mechanisms were involved. Further studies should be performed to explore the underlying mechanisms of these differentially expressed IncRNAs.

To further confirm the role of IncRNAs in ALI, we performed a study in vitro to investigate whether interventions targeting on IncRNAs plays a role in the protection of ALI, and we used siRNAs on two PCR-verified IncRNAs, ENSMUST00000170214.1 and ENSMUST0000016031.13, and we observed that the interfering of ENSMUST00000170214.1 and ENSMUST0000016031.13 decreased LPS-induced TNF-α and IL-1β production, considering the important role of TNF-α and IL-1β in ALI [32,33], we proposed that therapy targeting IncRNAs may provide novel direction for the treatment of ALI/ARDS. And more studies in vivo and clinical research should be performed to investigate therapeutic potential of IncRNAs in ALI/ARDS.

Taken together, thousands of IncRNAs and mRNAs in the lung were differentially expressed after LPS treatment. Bioinformatics analyses revealed that different IncRNAs would exhibit diverse potential functions which were related to differentially expressed genes. Study in vitro suggests the intervention on IncRNAs may attenuate inflammation in ALI/ARDS. However, further studies need to be implemented to investigate the molecular mechanisms and biological functions of IncRNAs and determine whether they can serve as novel therapeutic targets in ALI.

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Competing Interests
The authors declare that there are no competing interests associated with the manuscript.

Author Contribution
J.W., Y.-C.S., Z.-N.C., Z.-C.Y., K.L., and D.-J.L. designed the study, performed the experiment, carried out data analysis, interpreted and drafted the manuscript. J.W., Y.-C.S., Z.-C.Y., H.W., and F.-Q.W. contributed to study design, data collection, analysis, and critical revision of this manuscript. K.L. and D.L. revised the final version of manuscript, and is responsible for fielding correspondence. All authors approved the final version of the manuscript.

Abbreviations
ALI, acute lung injury; ARDS, acute respiratory distress syndrome; CNC network, coding-non-coding gene co-expression network; GO, gene ontology; HE, Hematoxylin and Eosin; IL, interleukin; KEGG, Kyoto Encyclopedia of Genes and Genomes; IncRNA, long non-coding RNA; LPS, lipopolysaccharide; qRT-PCR, quantitative real-time PCR; siRNA, small interfering RNA; TNF, tumor necrosis factor.

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