Identification of key immune genes for sepsis-induced ARDS based on bioinformatics analysis

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ABSTRACT

Regarding the extremely high mortality caused by sepsis-induced acute respiratory distress syndrome (ARDS), it is urgent to develop new biomarkers of sepsis-induced ARDS for treatment. Here, 532 differential expression genes (DEGs) related to sepsis and 433 DEGs related to sepsis-induced ARDS were screened in the GSE32707 dataset. Compared with sepsis samples, sepsis ARDS samples showed a higher infiltration of activated memory CD4 T cells and naive B cells, but a relatively lower infiltration of CD8 T cells. The pink and green modules which are significantly associated with sepsis-induced ARDS were then screened through co-expression network analysis. Differentially up-regulated GYPE and aberrantly down-regulated HSPB1, were subsequently found in the pink module of ARDS. CD81 and RPL22, two differentially low-expressed genes peculiar to ARDS, were identified in the green module. The function of CD81 was verified at the cellular level, and it was found that the up-regulation of CD81 in A549 could alleviate the LPS-induced injury of A549 cells. More importantly, the overexpressed CD81 can also increase the content of CD4\textsuperscript{+} CD25\textsuperscript{+} Foxp3 \textsuperscript{+} Treg in Jurkat cells, and after the co-culture of overexpressed CD81 Jurkat cells with LPS treatment A549 cells, the LPS-induced lung epithelial cell damage can be improved. Overall, four new plasma biomarker candidates were found in sepsis-induced ARDS, and we verified that CD81 may play critical roles in the biological and immunological processes of sepsis-induced ARDS.

1 Introduction

Sepsis is an infection of organ dysfunction [1]. There were about 48.9 million sepsis patients and 1.1 million sepsis-related deaths worldwide in 2017, accounting for 19.7% of all deaths in the globe. In the same year, WHO resolved to consider sepsis as a global health priority [2]. Acute respiratory distress syndrome (ARDS) is a common complication of sepsis patients in hospitals’ intensive care unit [3]. It usually occurs after pulmonary or extrapulmonary infections [4], resulting in the damage of lung epithelial and endothelial cells, increasing the permeability of alveolar-capillary membrane, and causing severe pulmonary edema and hypoxia as well as ventilation difficulties [5]. Clinical studies have shown that sepsis-related ARDS is more severely than non-sepsis-related ARDS, because the lung injury caused by sepsis-related ARDS is more difficult to recover, and the mortality of it is relatively higher [6]. Therefore, the identification of key molecules in sepsis-related ARDS is important for the better treatment of sepsis.

Although the drug intervention of ARDS has been studied in recent years, no effective biological target has been found for the treatment of ARDS [7]. Nowadays, potential interest in the biomarker-targeted therapy for ARDS is emerging. Previous studies have shown that specific markers may play essential roles in the detection and prevention of sepsis-induced ARDS. Cyclin B1 (CCNB1), cyclin B2 (CCNB2), DNA topoisomerase II Alpha (TOP2A) and transcription factor fork head box protein M1 (FOXM1) have already been identified as new targets for the gene therapy of ARDS [8]. Circulating PI3 is an effective clinical marker for monitoring the early development of ARDS. Increased MUC1 level is
a good predictor of ARDS in patients with sepsis [9,10]. Sepsis-induced ARDS is an inflammatory process regulated by multiple inflammatory factors [11]. As part of the adaptive immunity, T lymphocytes are affected in sepsis-induced ARDS and play important roles in limiting the tissue damage induced by immune inflammation [12,13].

Therefore, the mRNA expression profiles of patients with sepsis and sepsis-related ARDS were analyzed from the database downloaded from GEO, using differential expression analysis, immune infiltration analysis and functional enrichment analysis. A co-expression network was constructed based on all these analyses, and then the key genes in sepsis-related ARDS were screened. Furthermore, the function of CD81 was verified in a cell model. This study aims to provide effective biological information for discovering key immune genes in sepsis-induced ARDS.

2 Materials and methods

2.1 Data and patients

The GSE32707 dataset was downloaded from the GEO database, and it contains 144 whole blood samples and can be divided into four groups: sepsis group (sepsis), sepsis with ARDS (sepsis-induced ARDS), systemic inflammatory response syndrome (SIRS), and control group. Blood samples were collected from patients on admission and 7 days after admission. SIRS samples were removed from the analysis.

2.2 Data preprocessing and analysis of differential expression genes (DEGs) and immune infiltration

Limma was used to analyze the DEGs of sepsis (sepsis_0d) vs control (untreated) and sepsis_ARDS (sepsis-induced ARDS) vs control (untreated) (| logFC | > 1, pval < 0.05). Then, the two differentially expressed genes were intersected. CIBERSORT has advantages in terms of noise, unknown mixture content and closely related cell types, and can characterize cell composition through gene expression profile of complex tissues. As a result, CIBERSORT was used in this study to evaluate the difference of immune infiltration between the sepsis group and the sepsis-induced ARDS group.

2.3 Screening of the co-expression module

Immune-related genes were obtained from innateDB, and the genes expressed in GSE32707 data sets were screened for analysis. WGCNA was applied to analyze the co-expression modules related to immune cell content in sepsis-induced ARDS. All the samples were clustered and the outlier samples were excluded. Then, the co-expression network was built using the step-by-step network construction method. By analyzing the correlation between modules and phenotypes, the co-expression blocks significantly related to phenotypes were identified. The correlation between meme block and the contents of ARDS, naïve B cells, CD8 T cells and activated memory CD4 T cell was analyzed.

2.4 Functional enrichment analysis and gene recognition of important module genes

GO and KEGG enrichment analysis were performed on the selected co-expression modules using clusterProfiler. Genes in the coexpression module were intersected with the differential genes specific to ARDS, and the differential expression of these genes in control group, sepsis group, and sepsis ARDS group was analyzed, respectively.

2.5 Cell culture and experimental grouping

A549 cells (Human lung epithelial cells) and Jurkat cells (human T lymphocyte cells) were purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA) website and cultured according to the manufacturer’s instructions. The cultured cells were divided into the following groups: overexpression CD81 group (oe-CD81 group): transfected with cDNA lentivirus plasmid, including pBLV-CMV-IRES-ZsGreenCD81; the empty carrier control group (oe-NC group): transfected with empty carrier cDNA lentivirus plasmid (Shanghai Hanbio, China). The lentivirus supernatant was obtained by transfecting 293 T cells...
with Lipofectamine 2000 (Invitrogen, Carlsbad, USA). Then, A549 or Jurkat cells were infected with lentivirus supernatant to obtain CD81-overexpressing cells. A549 cells were treated with 10 μg/ml LPS (Sigma, Aldrich, USA). Jurkat cells were stimulated with CD3 and CD28 antibodies.

A549 cells were stimulated with LPS and co-cultured with Jurkat cells at a ratio of 5:1, as previously described [14]. The detailed grouping is as follows: co-culture + LPS + oe-NC group: LPS-induced A549 cells were co-cultured with oe-NC Jurkat cells; co-culture+LPS+oe-CD81 group: LPS-induced A549 cells were co-cultured with oe-CD81 Jurkat cells; co-culture+LPS+oe-CD81+ Transwell insert group: LPS-induced injured A549 cells were co-cultured with oe-CD81 Jurkat cells, and the two cells were separated by Transwell insertion; co-culture+LPS+oe-CD81+ anti-CD81 group: CD81 blocking antibody was added into the co-culture system.

2.6 Flow cytometry analysis

As specified, the CD81 was overexpressed using lentivirus in Jurkat cells, and the cells including the control cells were stained with antibodies (CD4-PE-Cyanine5, CD25-PE, and Foxp3-FITCmAb) for 30 min. After being washed twice, the samples were detected using FACS Canto II flow cytometry (BD Biosciences, USA), and the data was analyzed in FlowJo [7].

2.7 Apoptosis assay

LPS-treated Oe-CD81 group or oe-NC group A549 cells were digested with trypsin, washed with PBS, and then resuscitated with a binding buffer. Next, 5 μl Annexin V-FITC and 5 μl Lodide (PI) were added into the cell suspension and incubated with flow cytometry for 15 min. Finally, the apoptosis rate of cells was calculated by FACS Calibur (BD Biosciences).

2.8 Cell migration assay

The migration of LPS-stimulated oe-CD81 group or oe-NC group A549 cells was evaluated by wound healing and Transwell assay. Before the wound healing experiment, the cells were inoculated in the ibidi wound healing petri dish. When the cells formed a 100% confluent monolayer, the protective tape at the bottom of the culture dish was removed. Images were captured under a microscope at 0 h and 24 h. During the determination of Transwell, the cells were inoculated in 200 μl serum-free medium containing 10 μg/mL LPS or the same amount of PBS, and 600 μl complete medium containing 10% FBS was added to the bottom chamber. The unmigrated cells were gently brushed off with cotton swabs after 24 h. The migrated cells were fixed by 4% paraformaldehyde into the lower chamber, stained with crystal violet (Beyotime, Biotechnology, Shanghai, China), then observed and counted under a microscope.

2.9 qRT-PCR

The total RNA of the cells was extracted using TRIzol reagent (Thermo Fisher Scientific) and 1000 ng RNA was used as a template to synthesize the first strand cDNA. Amplification was performed using the IQTM SYBR Green Supermix kit (Bio-Rad, Berkeley, CA, USA) [7]. The relative expression of CD81 was then calculated by 2^−ΔΔCT method and GAPDH was set as inner reference. The primers sequences were as follows: GAPDH F, 5’- GGAGCGAGATCCTCCAAAT-3’, and R, 5’- GGCTTGTGATCATACTTCATGG -3’; CD81 F, 5’- GCGCCCAACACCTTCTATGTA-3’, and R, 5’- GCGCCCAACACCTTCTATGTA-3’.

2.10 Western blot

The total cell protein was extracted with RIPA lysate (Beyotime), then the protein concentration was determined by BCA kit (Beyotime), and 40 μg sample was added into the sample well. The protein was separated by SDS-PAGE, transferred to the membrane, and blocked with 5% skim milk [11]. Western blotting was performed on the first anti-CD81 antibody and GAPDH antibody, and then they were incubated with HRP-conjugated second antibody. Finally, the blots were detected using enhanced chemiluminescence and detected with FujifilmLAS-3000 imaging system to display protein bands.
2.11 ELISA

The proinflammatory cytokines (interleukin-6 (IL-6), interleukin-1 β (IL-1 β) and tumor necrosis factor-α (TNF-α)) in the supernatant of LPS treated oe-CD81 group or oe-NC group A549 cells were detected using ELISA kit (BD Biosciences) according to the manufacturer’s instructions [7].

2.12 Statistical analysis

GraphPad Prism 6.0 Software (La Jolla, CA, USA) was used for statistical analysis, and t test was used to analyze the difference between two groups. \( P < 0.05 \) indicated that the difference is significant.

3 Results

3.1 Identification of DEGs in sepsis

Firstly, differential expression analysis was performed on the data in the dataset. 532 DEGs in sepsis vs control group were identified through Limma differential expression analysis, including 334 up-regulated genes and 198 down-regulated genes (Figure 1a). And there were 433 DEGs in sepsis_ARDS vs control group, including 122 up-regulated genes and 311 down-regulated genes (Figure 1b). Through Wayne diagram analysis, 251 genes in sepsis were found in common with the control group, and the remaining 182 genes were specific to sepsis-induced ARDS (Figure 1c).

3.2 Immune cell infiltration of sepsis-induced ARDS group

To investigate the difference of immune infiltration between the sepsis group and the sepsis-induced ARDS group, CIBERSORT was used to evaluate the distribution of 22 kinds of immune cells between the two groups (Figure 2a). There were significant differences in the infiltration of naive B cells, CD8 T cells and activated memory CD4 T cells between the two groups. Compared with the samples with sepsis, the infiltration of activated memory CD4 T cells and naive B cells was higher in the samples with sepsis-induced ARDS, while that of CD8 T cells was relatively lower (Figure 2b). The correlation analysis of 22 immune cells showed that the proportion of Neutrophils was negatively correlated with resting mast cells, and Eosinophils had the highest positive correlation with activated mast cells (Figure 2c).

3.3 Construction of weighted co-expression network and identification of key modules

A total of 4677 immune-related genes were obtained from innateDB, among which 3853 were expressed in GSE32707 data sets. All samples were clustered by WGCNA, and five interest group samples were excluded (Figure 3a,3b). According to the approximate scale-free topology criterion, the soft threshold = 6 was set to define the adjacency matrix (Figure 3c,d). Then the system cluster tree was

*Figure 1. Identification of DEGs in sepsis.*

(a) Heat-map of difference expression genes (DEGs) between sepsis vs control. (b) Heat-map of DEGs between sepsis-induced ARDS vs control by Limma. (c) Venn diagram of differential genes in the sepsis and sepsis-induced ARDS groups.
Figure 2. Immune cell infiltration between the sepsis and sepsis-induced ARDS groups.
(a) The distribution of 22 immune cells in the sepsis and sepsis-induced ARDS groups was assessed using CIBERSORT. (b) Immune infiltration difference between the sepsis and sepsis-induced ARDS samples (the sepsis group was marked as blue, and the sepsis-induced ARDS group was marked as red). (c) Correlation analysis of 22 types of immune cells. (The color purple represents negative correlation, while red represents positive correlation. The more relevant, the darker the color.)
constructed based on the matrix, and 16 modules were obtained (Figure 3e). Correlation analysis between the gene module and ARDS, naive B cells, CD8 T cells, activated memory CD4 T cells showed that the green module (containing 294 genes) was significantly negatively correlated with ARDS, but positively correlated with CD8 T cells. The pink module (containing 198 genes) was positively correlated with ARDS, but negatively correlated with activated memory CD4 T cells (figure 3f).

### 3.4 Module functional enrichment analysis and identification of key genes

ClusterProfiler was used to analyze the enrichment of the green and pink modules by GO and KEGG, respectively. The results showed that the green modules were mainly enriched in terms of T cell activation, external side of plasma membrane, immune receptor activity as well as pathway of immune receptor activity cytokine–cytokine receptor interaction, and T cell receptor signaling pathway (Figure 4a,b). The pink module was mainly enriched in terms of response to hypoxia, proteasome complex and pathway of Huntington disease, and Epstein–Barr virus infection (Figure 4c,d). The green module and the pink module were intersected with the 182 differential genes specific to sepsis-induced ARDS, respectively. It was also found that CD81 and RPL22 in the green module were specific to sepsis-induced ARDS, and both of them were less expressed compared with the control group. (Figure 4e,f). In the pink module, GYPE and HSPB1 were unique to ARDS, in which the expression of GYPE was up-regulated and the expression of HSPB1 was down-regulated (Figure 4g,h). And then, ROC curves were drawn to further evaluate the diagnostic value of key genes, as shown in Supplementary Figure 1A-D.

### 3.5 CD81 alleviated the LPS-induced injury of lung epithelial cells

The injury of alveolar epithelial cells and capillary endothelial cells is the main cause of ARDS [15].
Figure 4. Module functional enrichment analysis and identification of key genes. (a-b) Enrichment analysis of green modules using GO and KEGG. (c-d) Enrichment analysis of pink modules using GO and KEGG. (e-h) Differential expression analysis of CD81, RPL22, GYPE, and HSPB1 in the control group, sepsis group and sepsis-induced ARDS group.
Therefore, LPS was used to stimulate A549 cells to induce the injury of lung epithelial cells. CD81 has the function of regulating cell adhesion, migration, proliferation, and differentiation. Cell morphological changes and signal transduction. It is involved in diverse biological reactions and has a certain regulatory effect on the maturation of T cells and B cells. Therefore, CD81 was selected for in-depth study in this study. In this study, the cells were stimulated with LPS for 6, 12, 24 and 36 h, and the expression of CD81 was detected by qRT-PCR at each of the above time point. Cell proliferation was detected by CCK-8. Cell apoptosis was analyzed using flow cytometry.

In this study, LPS was used to stimulate A549 cells to induce the injury of lung epithelial cells. The cells were stimulated with LPS for 6, 12, 24 and 48 h, and the expression of CD81 at each of the above time point was detected. Compared with the control group, the expression level of CD81 in LPS treatment group decreased with the extensions of treatment time, and reached the lowest value at 24 h, and the level have no further reduce at 48 h (Figure 5a). Therefore, A549 cells induced by LPS for 24 h were selected as the standard for subsequent experimental cell treatment. Transfection of CD81 into A549 cells upregulated the level of CD81 (Figure 5b). From the result of CCK-8 experiment, it was found that CD81 upregulation alleviates the viability of LPS-induced A549 cell injury [16] (Figure 5c). Meanwhile, it was shown by the apoptosis analysis that CD81 overexpression reduced the apoptosis rate in LPS-stimulated A549 cells. (Figure 5d). Those results indicated that CD81 could alleviate the injury of LPS-induced lung epithelial cells.

### 3.6 CD81 Overexpression accelerated the cell migration of LPS-treated A549 cells

The structural and functional recovery of alveolar epithelial cells in ARDS is closely related to the enhancement of cell migration and intercellular junction [3]. LPS was used to stimulate cells, and then wound healing and Transwell cross-hole
experiment were performed to detect cell migration. The results revealed that CD81 overexpression could accelerate the wound healing of A549 cells (Figure 6a). And Transwell assay indicated that CD81 overexpression promoted the migration of A549 cells (Figure 6b).

### 3.7 CD81 overexpression contributed to the effect of Tregs on the LPS-induced injury of lung epithelial cells

The differentiation of Th17/Treg plays an important role in the development of ARDS. Th17 can release multiple inflammatory cytokines to mediate acute inflammatory responses. Treg, as an important type of immunosuppressive cell, can also be activated in ARDS. In this study, flow cytometry was used to detect the proportion of CD4⁺ CD25⁺ Foxp3⁺ Treg cells in CD81 overexpressed Jurkat cells. The secretion levels of IL-6, IL-1β, TNF-α and other inflammatory cytokines in each group were detected by ELISA, and the proliferation of A549 cells in the co-culture system of Jurkat cells and LPS-treated A549 cells was further detected using Ki67 staining.

Compared with oe-NC-treated Jurkat cells, the proportion of CD4⁺ CD25⁺ Foxp3⁺ Treg in oe-CD81 Jurkat cells was significantly increased when detected with flow cytometry (Figure 7a). The mRNA expression of T cell expression marker, Foxp3, was also quantified by qRT-PCR. Compared with the oe-NC Jurkat cells, the expression of Foxp3 in oe-CD81 Jurkat cells was significantly increased (Figure 7b). One of the important pathological features of ARDS is cascade inflammation [3]. Based on the important role of T cells in inflammation, ELISA was used to detect the contents of IL-6, IL-1β and TNF-α in oe-CD81 Jurkat cells. As shown in Figure 7c, CD81 overexpression could significantly reduce the levels of IL-6, IL-1β, and TNF-α in LPS-stimulated Jurkat cells. In LPS-induced injury of A549 cells, the co-culture with oe-CD81 Jurkat cells significantly improved the proliferation activity of A549 cells, which was consistent with Ki67. When using Transwell insert to separate the two types of cells, the proliferation of A549 cells was not disturbed. Then, we used CD81 blocking antibody to neutralizing the effect of CD81. After CD81-blocking antibody were added to the co-culture system, the proliferation rate of A549 cells was observably reduced (Figure 7d). Therefore, it can be concluded that CD81 overexpression can increase the content of Treg, which can improve the LPS-induced injury on lung epithelial cells when cocultured with it.

### 4 Discussion

The latest reports suggested that biomarkers may be the key to personalized medication for septicemia, because it allows patients to receive tailored treatment based on their unique characteristics [17]. The rapid development of bioinformatics has brought many novel analytical tools, providing technical support for biomarker identification. These tools have been widely used in the study of septicemia, playing important roles in the study of diseases at the molecular level through pathway analysis, statistical analysis, and visual processing. Furthermore, the use of these tools means a lot in
screening large amounts of molecular and clinical data through data mining [18]. Host biomarkers that can be used as laboratory diagnostic markers are usually proteins or nucleic acids that are easy to measure [19]. Protein biomarkers in ARDS are most often studied in the blood or in the air cavity of the lungs [20]. Hence, in the current study, whole blood samples were obtained from GSE32707 chip data, and 532 sepsis-related DEGs as well as 433 DEGs related to sepsis-induced ARDS were screened.

The inflammatory cell infiltration of macrophages, neutrophils, dendritic cells (DC), and T cells is an important cause of severe immune disorders in the pro-inflammatory phase of sepsis [21]. Therefore, our study used CIBERSORT to detect the differences in the immune infiltration between the sepsis group and the sepsis-induced ARDS group. As a result, compared to the sepsis samples, the sepsis ARDS samples showed a higher infiltration of activated memory CD4 T cells and naive B cells and a lower infiltration of CD8 T cells.

Figure 7. CD81 overexpression contributed to the effect of Tregs on LPS-induced injury of lung epithelial cells.
(a) The proportion of CD4+ CD25+ Foxp3+ Tregs was detected by flow cytometry after oe-CD81 treatment. (b) qRT-PCR quantified the mRNA expression of Foxp3, a marker for T cell expression. (c) ELISA was used to detect the secretion levels of IL-6, IL-1β and TNF-α in oe-CD81-transfected Jurkat cells. (d) The proliferation of A549 cells in the co-culture system of LPS-treated Jurkat cells and A549 cells were detected with Ki67 staining.
As a system-wide phenomenon, septicemia requires a systematic approach to identify pathways [22]. Functional module analysis has been widely used in the research field of many diseases [23]. We constructed a gene co-expression network by WGCNA and screened two modules significantly related to the phenotype of ARDS. Among them, the green module has a significantly negative correlation with ARDS, but a significantly positive correlation with CD8 T cells. There was a significantly positive correlation between the pink module and ARDS, but a significantly negative correlation between the pink module and activated memory CD4 T cells. Differentially up-regulated GYPE and abnormally down-regulated HSPB1 were found in ARDS in the pink module. Previous studies have revealed that HSPB1 can inhibit the occurrence of sepsis in mice [24]. It is prompted that HSPB1 plays an important role in sepsis. Two differentially low-expressed genes specific to ARDS, CD81, and RPL22, were identified in the green module. Here, an in vitro experiment was designed in order to verify the expression and function of CD81 in sepsis-induced ARDS. The results showed that the expression of CD81 was down-regulated after A549 cells were stimulated by LPS. The overexpressed CD81 remitted the LPS-induced proliferation inhibition of lung epithelial cells and facilitated the migration of LPS-treated A548 cells, indicating that CD81 played an essential role in ARDS.

CD81 and RPL22 played direct or indirect roles in regulating the function of T cells. CD81 has been reported to be associated with CD4 and CD8 on T cells and also been considered to provide co-stimulatory signals with CD3 [25]. Rpl 22 has already been found to selectively inhibit the development of alpha Beta T cells by activating p53-dependent checkpoints [26]. Based on these studies, the effect of CD81 on T cells was verified, and it was confirmed that CD81 overexpression can increase the content of Treg and promote the effect of Treg on the injury and proliferation of LPS-induced lung epithelial cells.

5 Conclusion

In summary, CD81, RPL22, GYPE, and HSPB1 were screened and they are significantly associated with the immunity in sepsis-induced ARDS. Among the four genes, we confirmed that upregulation of CD81 could promote the proliferation and migration in LPS-induced lung epithelial cells, and up-regulate the content of CD4+ CD25+ Foxp3+ Tregs in in Jurkat cells. And when the CD81 overexpressing Jurkat cells were co-cultured with LPS-treatment lung epithelial cells, the LPS-induced injury and proliferation can be improved. These findings may provide new insights for the discovery of new diagnostic and therapeutic targets for ARDS.

Disclosure statement

No potential conflict of interest was reported by the author(s).

Funding

This work was supported by the [Traditional Chinese Medical Science and Technology Plan of Zhejiang Province 2020ZB105, 2021ZA076]; [Medical Health Science and Technology Project of Zhejiang Provincial Health Commission 2021KY226].

Authors’ contribution

Ye Chen: Conceptualization, Formal analysis, Methodology, Draft preparation. Chenhui Qiu: Investigation, Data curation, Validation, Visualization. Wanru Cai: Methodology, Supervision; Reviewing and Editing.

Data availability statement

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request (https://www.ncbi.nlm.nih.gov/geo/).

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