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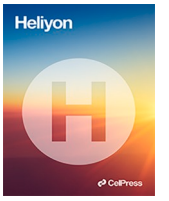

Syndecan-4 is the key proteoglycan involved in mediating sepsis-associated lung injury

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ABSTRACT

Vascular endothelial cell dysfunction involving syndecan (SDC) proteoglycans contributes to acute sepsis-associated lung injury (ALI), but the exact SDC isoform involved is unclear. We aimed to clarify which SDCs are involved in ALI. A relevant gene expression dataset (GSE5883) was analysed for differentially expressed genes (DEGs) between lipopolysaccharide (LPS)-treated and control lung endothelial cells and for SDC isoform expression. Bioinformatic analyses to predict DEG function were conducted using R language, Gene Ontology, and the Kyoto Encyclopedia of Genes and Genomes. SDC2 and SDC4 expression profiles were examined under inflammatory conditions in human lung vascular endothelial cell and mouse sepsis-associated ALI models. Transcription factors regulating SDC2/4 were predicted to indirectly assess SDC involvement in septic inflammation. Of the DEGs, 224 and 102 genes were up- and downregulated, respectively. Functional analysis indicated that DEGs were involved in modulating receptor ligand and signalling receptor activator activities, cytokine receptor binding, responses to LPS and molecules of bacterial origin, regulation of cell adhesion, tumour necrosis factor signalling, and other functions. DEGs were also enriched for cytoplasmic ribonucleoprotein granules, transcription regulator complexes, and membrane raft cellular components. *SDC4* gene expression was 4.5-fold higher in the LPS group than in the control group, while *SDC2* levels were similar in both groups. SDC4 mRNA and protein expression was markedly upregulated in response to inflammatory injury, and SDC4 downregulation severely exacerbated inflammatory responses in both *in vivo* and *in vitro* models. Overall, our data demonstrate that SDC4, rather than SDC2, is involved in LPS-induced sepsis-associated ALI.

1. Introduction

The pathogenesis of acute lung injury/acute respiratory distress syndrome (ALI/ARDS) leads to disturbances in lung homoeostasis due to virus- or bacteria-induced damage to the capillary endothelium or alveolar epithelium. This process leads to increased tissue permeability and tissue inflammation. Indirect ALI, mainly induced by sepsis and trauma, causes changes in microvascular endothelial cell structure and function and involves an extensive acute inflammatory response. Furthermore, indirect ALI accounts for high mortality in clinical settings [1]. The endothelium at the vascular alveolar septum comprises more than a single nucleated layer and

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influences various functions, including angiogenesis, blood clotting, inflammation, and the expulsion of numerous molecules, including proteins [2]. In a normal state, the endothelium positively influences the innate immune system to eliminate microbial invaders. In contrast, endothelial dysfunction can lead to a direct increase in vessel permeability, causing fluid leakage and oedema. Furthermore, such disruption interrupts inflammatory processes initiated by neutrophil granulocytes due to its influence on vascular wall adhesion molecules and intracellular signal transduction, which dysregulates inflammatory cascades [3]. Endothelial cells are important in the inhibition of innate immune responses to protect against overreaction. Endothelial dysfunction is associated with neutrophil infiltration, adhesion molecule expression, and activation of intracellular signalling pathways [3–5].

Endothelial cells rely heavily on membrane surface syndecans (SDCs), which are membrane-attached proteoglycans, to repel an initial pyemic "hit". Four SDC isoforms have been detected in humans to date; however, only two of them, SDC2 and SDC4, are expressed on vascular endothelial cells. SDCs are membrane proteoglycans with HS chains attached to their ectodomains, SDC stimulation can induce specific HS sulfation and dimerization, and SDCs interact with numerous ligands involved throughout inflammatory responses to mediate endothelial cell activation levels [6–8]. Various stimuli, including cytokines, heparinases, and histamines, can accelerate glycocalyx degradation [9] and trigger inflammation by interacting with heparin sulphate (HS)-binding molecules circulating in the blood. Hence, elucidation of the specific function of SDCs in the glycocalyx and development of therapeutic strategies to overcome sepsis-associated ALI are urgent priorities. SDC4 is ubiquitous in many cell types and participates in various inflammatory responses. For example, Godmann et al. found that SDC4 can modulate inflammatory arthritis through the formation of a complex with interleukin 1 (IL-1) [10]. Brioudes et al. reported that SDC4 can be expressed in islets and regulated by IL-1b. In the lungs, CXCL8 and SDC4 can form a glycosaminoglycan-binding complex that induces inflammation [11]. Furthermore, SDC4 is involved in various inflammatory conditions, including rheumatoid arthritis [12], colitis [13], and cardiac inflammation [14]. Most studies of SDC2 have primarily focused on angiogenesis, tumorigenesis, and metastasis. In the lungs, some studies have investigated the roles of SDC2 in lung adenocarcinoma [7] and cancer [15]; however, there has been little focus on the investigation of ALI. SDC2 and SDC4 are structurally and evolutionarily similar and likely have overlapping functions [16,17]; therefore, evaluation of the involvement of SDC2 and SDC4 in the process of ALI is warranted.

In the present study, we applied bioinformatics methods to screen for differentially expressed genes (DEGs) between human lung microvascular endothelial cells treated with lipopolysaccharide (LPS) and controls. A total of 326 DEGs were identified, the majority of which were upregulated, and DEGs were enriched in the TNF signalling and cytokine receptor interaction pathways. Importantly, SDC4 expression was upregulated severalfold in response to LPS, whereas SDC2 maintained a comparably stable profile over time. To verify these results experimentally, we established LPS-induced *in vivo* sepsis-associated ALI and endothelial injury *in vitro* models. Examination of SDC2 and SDC4 mRNA and protein expression levels in cell lysates and lung tissue generated results consistent with those of the bioinformatics analysis. Furthermore, the involvement of SDC2 and SDC4 in the inflammatory process was indirectly assessed by predicting transcription factors (TFs) that regulate their expression, as well as using SDC4 knockdown experiments. Our results showed that SDC4, but not SDC2, was regulated by nuclear factor kappa beta (NF-κβ) and that SDC4 knockdown led to markedly worse inflammatory injury. Overall, our data indicate that SDC4, rather than SDC2, is involved in sepsis-associated AL1; however, the exact underlying mechanism and signalling pathway require further study.

2. Materials and methods

2.1. Bioinformatics analysis and prediction

To predict and determine which isoform of SDC is involved in endothelial damage on endotoxin attack, we searched the Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo) with the key words "LPS" and "endothelial cells," and an appropriate dataset, "GSE5883" (generated on the GPL570 platform with an Affymetrix Human Genome U133 Plus 2.0 Array), was retrieved. The study included 24 endothelial cell samples pretreated with LPS at 4, 8, and 24 h.

An acute inflammatory response of <6 h is the longest that can induce variation in SDC expression (our unpublished data); therefore, GSE5883 4 h data were pooled and analysed using R-package (version 4.1.2). Sample data were extracted, and a data matrix was constructed using the ggplot package in R studio (version 4.04). After normalization, a two-dimensional (2D) principal components analysis (PCA) was conducted to determine the primary sources of variability in the data. A gene expression matrix was then constructed, and DEGs were identified based on threshold values, $p < 0.01$ and log-(fold change) $FC > 1.5$. Analytical tools, including DAVID (https://david.ncifcrf.gov/), Gene Ontology (GO; http://geneontology.org/), Kyoto Encyclopedia of Genes and Genomes (KEGG), and STRING (https://cn.string-db.org/, version 11.5), were used to predict DEG functions. DEGs were also analysed using Cytoscape software. Signalling pathways enriched for DEGs predicted using KEGG were visualized using R studio. SDC expression data in the GSE5883 dataset were searched using R language, and the results were analysed using the *t*-test in R studio. Molecules associated with SDC2 and SDC4 were also screened.

2.2. Experimental verification of SDC isoforms

2.2.1. Model construction

For verification of the predictions generated by bioinformatics analyses, *in vivo* and *in vitro* sepsis-associated inflammatory injury models were constructed. LPS was chosen as the stimulatory factor because it has a similar ability to TNFα and IL-1B to induce inflammation; the volume selected was based on our pilot study. Ethical approval was granted by the laboratory animal ethics committee of JXMC (JUMC2021-124; July 7th, 2021).

2.2.1.1. In vitro model. Experimental cells were immortalized human lung vascular endothelial cells obtained from Meisen Chinese Tissue Culture Collections. Cells were cultured using standard techniques in specific medium and treated with phosphate-buffered saline (PBS; control) or LPS (0–10 μg/ml) from *Escherichia coli* O55:B5 for 0, 6, 12, and 24 h.

2.2.1.2. In vivo model. An *in vivo* injury model was established by intraperitoneal injection of LPS (20 μg/g) into male C57BL/6 mice at 6, 12, and 24 h, followed by collection of 0.5 ml blood samples from each mouse from the eye angular vein under anaesthesia with 0.3% pentobarbital sodium (50–60 mg/kg). Furthermore, alveolar lavage was performed, and BAL fluid was collected, followed by excision of both lungs. After necessary operations were conducted, all animals were euthanized by carbon dioxide asphyxiation (flow rate, 5 L/min, with 50% volume displacement/min) in a chamber for approximately 20 min. Haematoxylin and eosin (H&E) staining and immunofluorescence examination of SDC2 and SDC4 expression in mouse lungs were conducted at different time points. The overall protein concentration in bronchoalveolar lavage (BAL) samples was also determined.

2.2.2. ELISAs and immunofluorescence assays

Cell culture supernatant, murine serum, and BAL samples were collected and centrifuged. The inflammatory biomarkers TNFα and IL-6 were analysed using precoated antibody ELISA kits (RD Systems; Cat No. MAB2063-SP, DTA00D) following the manufacturer's exact instructions. For determination of which SDC isoforms were expressed in murine lung and localization of SDC expression, lung samples collected from control and LPS (20 μg/g) group C57BL/6 mice were harvested after 24 h and embedded in 4% paraformaldehyde. The sections were then prepared and double stained with *anti*-SDC4 (1:500, Abcam, ab286154) and anti-CD3 (1:500, Abcam ab222783) primary antibodies. Subsequently, a fluorescent-conjugated secondary antibody was added, and samples were examined by microscopy to determine the distribution of fluorescent signals.

2.2.3. Quantitative real-time polymerase chain reaction (RT–qPCR)

Cells were harvested after two washes with PBS, and RNA was extracted using TRIzol. The total RNA concentration was determined using a Nanodrop instrument (Thermo Fisher Scientific, USA). RT–qPCR experiments were conducted using a commercial RNA reverse transcription kit (PrimeScript^{RT} Master Mix [Real Time], RR036A) and a qPCR kit (TB Green Premix DimerEraser [Perfect Real Time], RR091A) (TaKaRa Biomedical Technology [Beijing] Co., Ltd.) following the manufacturer's instructions. In brief, up to 1 μg of total RNA was reverse-transcribed to cDNA. Reactions were conducted in a 20 μl volume containing Master Mix and 2 μl of cDNA. The reaction parameters were 40 cycles of (1) 95 °C, 1 min; (2) 95 °C, 5 s; and (3) 60 °C, 1 min. *SDC2* and *SDC4* gene expression levels were calculated relative to those of the housekeeping gene, *GAPDH*. In addition to primers for amplification of *IL-6*, *IL-1β*, and vascular cell adhesion model 1 (*VCAM-1*), primers for *SDC2* and *SDC4* were (1) *SDC2*, F 5′-CTAGCAGCCGTCATTGC-3′, R 5′-TCCAAGGTCG-TAGCTTCC-3′ and (2) *SDC4*, F 5′-CGACGACGAAGATGCTG-3′, R 5′-CACCAAGGGCTCAATCAC-3'.

2.2.4. Western blotting

For determination of SDC2/4 protein expression levels *in vitro*, control and LPS group cells were seeded in 6-well plates. Cell lysates were prepared using RIPA lysis buffer with protease inhibitors. After determination of the overall protein concentrations using a bicinchoninic acid protein assay, 50 μg lysate samples were loaded per lane and separated by 12.5% sodium dodecyl gel electrophoresis for approximately 1 h. Proteins were subsequently transferred to membranes for 2 h and blocked in milk plus 5% bovine serum albumin (1 h). Primary antibodies were rabbit anti-SDC2 (1:1000, Abcam, ab205884) and rabbit anti-SDC4 (1:1000, Abcam, ab74139). Mouse anti-tubulin was used as an endogenous control. Membranes were incubated with primary antibodies overnight in a 4 °C refrigerator and then washed three times for 10 min, followed by the addition of horseradish peroxidase-conjugated anti-rabbit secondary antibody (1:10000, Abcam, ab288151) and incubation at room temperature (1 h). Finally, specific protein bands were visualized using an enhanced bioluminescence instrument (Bio-Rad, USA) under ECL reagent. Each experiment was conducted in triplicate.

2.2.5. SDC4 knockdown

Small interfering RNA (siRNA) targeting specific *SDC4* sequences was commercially designed by Tsingke (Shanghai, China); nontargeting siRNA served as an isotype control. SDC4 siRNAs (10 pmol) and 3 μl of Lipofectamine RNAiMAX (Invitrogen) were combined and used for transfection at 37 °C for 6 h. Subsequently, the culture medium was changed to complete medium for the remainder of the experiment. Knockdown was confirmed by RT–qPCR 24 h after transfection. The pHBAAV-TIE-ZsGreen vector (Hanheng Company) was used to create a construct containing the Mir30-*m*-Sdc4 sequence targeting a specific *SDC4* sequence in the endothelium to knock down its mRNA expression (Mir30-*m*-Sdc4 sequence: AAGGTATATTGCTGTTGACAGTGAGCGGATTCGAGA-GACAGAGGTCATTAGTGAAGCCACAGATGTAATGACCTCTGTCTCTCGAATCTGCCTACTGCCTCG). After RT–qPCR amplification and virus packaging, the HBAAV2/VEC-TIE-Mir30-*m*-Sdc4-ZsGreen virus was generated at titres of $\geq 1.1 \times 10^{12}$ vg/mL. In the experimental group, 65 μl of virus stock was injected transtracheally 30 days before model construction.

2.3. Prediction of TFs that regulate SDC2 and SDC4

For exploration of the functional roles of SDC2/4, TFs predicted to regulate the syndecans were identified using the GSEABase and clusterProfiler packages in R studio based on the GSE5883 dataset to indirectly clarify their potential involvement in inflammatory pathways. For SDC4, prediction was based on 87 DEGs, including all TFs in the DEG dataset identified using http://www.gsea-msigdb.

org/gsea/msigdb/collections.jspC3, which incorporates human TF and miRNA data. TFs predicted to regulate SDC2 were deduced from all 17,690 genes detected on the gene chip due to the lack of significant changes in its expression levels in response to LPS.

3. Results

3.1. Identification of DEGs

From the GSE5883 expression profiling dataset, eight samples (n = 4 each for the control and LPS treatment groups) were analysed to identify DEGs (threshold, $\log_2$ FC > 1.5; p < 0.01). After standardization, a total of 326 DEGs were identified from 17,690 probes. Sample data were extracted, and a data matrix was constructed (Fig. 1b). DEG expression profiles were clustered and presented as 2D PCA plots, which showed a clear separation between the control and LPS-treated samples; the first principal component could explain 19.6% of the variation in the transcriptomic profiles (Fig. 1a). Interestingly, of the 326 DEGs detected (Fig. 1c), most (224 genes) were upregulated upon LPS stimulation (Fig. 1d). DEGs are presented in Supplementary file S1.

3.2. GO and KEGG analysis of DEGs

For functional analysis of the 326 DEGs, GO enrichment (http://geneont ology.org/) and KEGG signalling pathway analyses were conducted. The analysed GO terms included those in the biological process, cellular component, and molecular function categories.

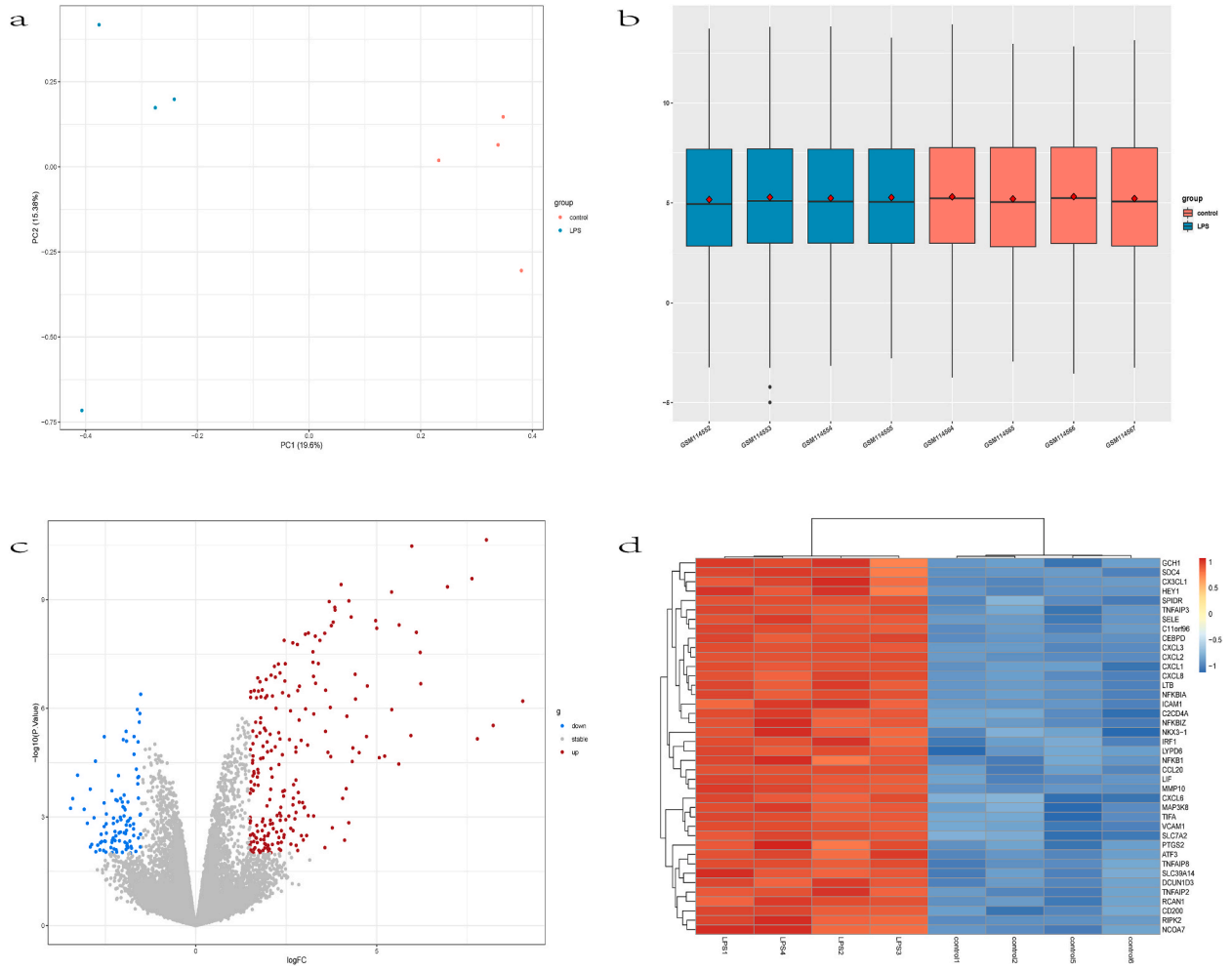

Fig. 1. Results of bioinformatics analyses. a) Two-dimensional (2D) principal component analysis (PCA) plot including data from eight samples. Blue and red dots represent the lipopolysaccharide (LPS)-treated and control samples, respectively. b) General expression matrix. c) Volcano plot showing differentially expressed genes (DEGs). Red and blue dots represent up- and downregulated genes, respectively. d) Heatmap of the top 40 DEGs. Red and blue colours represent up- and downregulated genes, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

The top five significantly enriched GO terms for each category are listed according to gene count and p value (Fig. 2a). Genes in the category GO:0000785 had the lowest p value, but gene numbers included in the top five terms had the lowest values. Genes were enriched in terms including intracellular membrane-bound organelles (270 genes), nucleus (195 genes), nuclear lumen (135 genes), and nucleoplasm (130 genes). In the molecular function category, 64 DEGs were significantly enriched in the categories binding (GO:0005488) and protein binding (GO:0005515). Other categories enriched for DEGs were cytokine and transcription regulator activities (76 and 26 genes, respectively). Regarding biological processes, the most significantly enriched for DEGs was regulation of metabolic processes, which included 217 DEGs. DEGs were also enriched in the cellular response to cytokine stimuli and response to organic substances categories.

DEGs were then analysed using the online KEGG tool (http://www.genome.jp/kegg), and the top 20 enriched pathways were visualized using R-studio (Fig. 2b). The first and second most significantly enriched pathways were TNF signalling and cytokine–cytokine receptor interaction pathways, with >30 genes significantly enriched ($p < 0.00001$). Other pathways enriched for DEGs were NF-κβ, IL-17, and nucleotide-binding oligomerization domain-like (NOD-like) receptor signalling. Protein–protein interaction (PPI) cluster analysis of DEGs using Cytoscape identified three clusters with moderate confidence (0.4) (Supplementary file S2).

3.3. SDC gene expression

Bioinformatics analysis indicated that the identified DEGs were enriched in inflammatory reactions, and LPS stimulation was established to induce considerable inflammation and inflammatory cascade signalling in endothelial cells; however, little is known

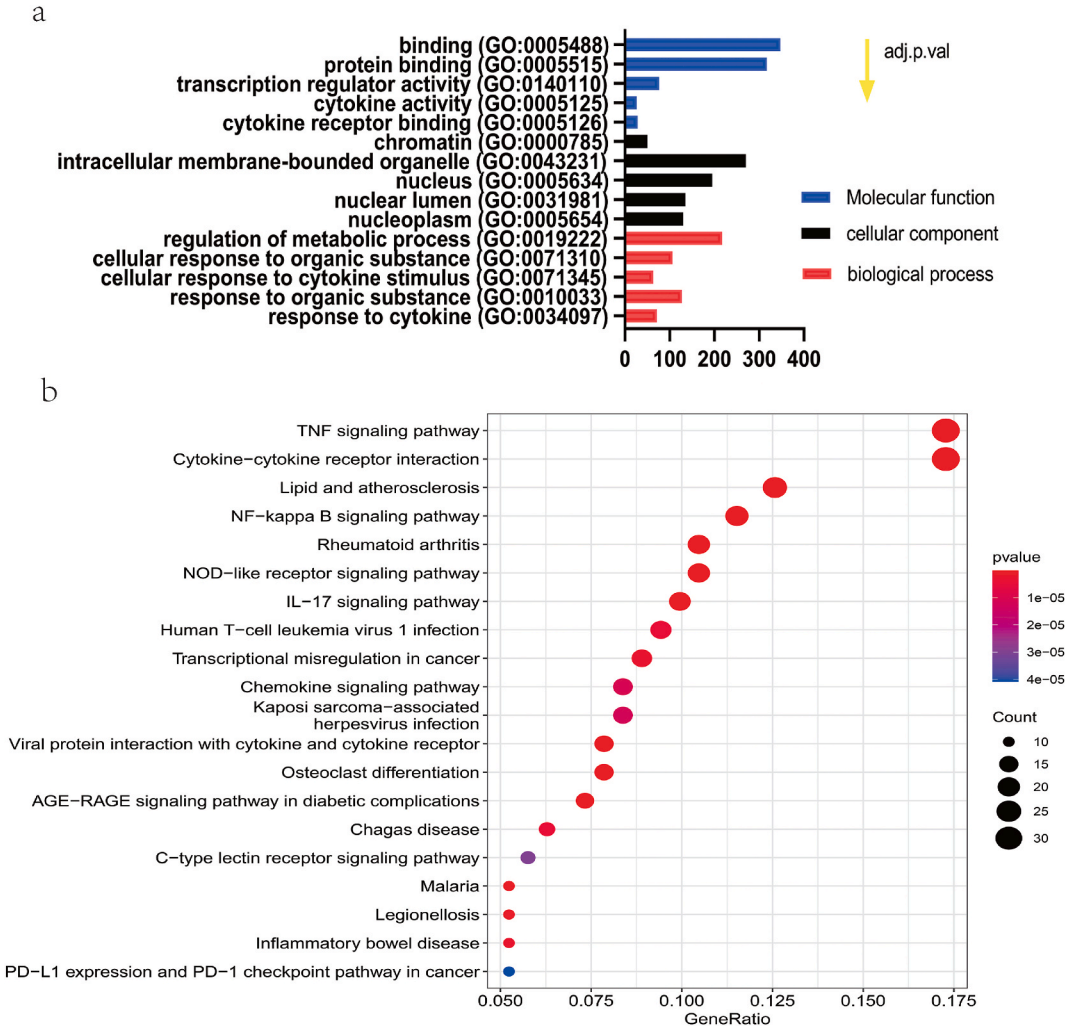

Fig. 2. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses of screened DEGs. GO enrichment analysis was conducted using three categories: (1) molecular function, (2) cellular components, and (3) biological processes. a) GO terms listed according to adjusted p value and gene count. The top 20 most significantly enriched terms are indicated by round bubbles. b) Rank determined according to p value and signalling pathways shared by DEGs.

about the role of SDCs and which SDC isoform participates in those reactions. Therefore, we quantified SDC expression to determine which isoform can translocate through lung vascular endothelial cell membranes to function in this context. *SDC1* and *SDC3* both showed mean expression levels of 5–6 (Supplementary file S3a), while *SDC2* and *SDC4* were expressed at 500–1500 times higher levels. Moreover, LPS treatment stimulated a 4.5-fold increase in *SDC4* expression relative to that of the control group ($p < 0.001$) (Fig. 3a), while there was no significant change in *SDC2* levels (Fig. 3b). Cytoscape PPI network analysis demonstrated that molecules closely associated with *SDC4* were also detected in Cluster 1 (Supplementary file S3b), including the relevant candidates chemokine ligand 5 (CCL5), C-X-C chemokine ligand motif (CXCL8 and 10), and IL-1B.

3.4. Verification of SDC expression

3.4.1. Establishment of cell and animal inflammatory injury models

H&E staining of mouse lung samples from the mice treated with LPS directly demonstrated the extent of tissue injury (Supplementary file S4). The inflammatory indicators TNFα and IL-6 both significantly increased in a dose-dependent manner after stimulation by LPS, particularly at LPS doses of 20 and 30 μg/g. BAL analysis generated similar results (Fig. 4a–d).

In the *in vitro* inflammatory model, cells were treated with physiologically meaningful doses of LPS (range, 0.5–10 μg/ml). At 6 h after LPS administration, a cellular inflammatory response was induced, as indicated by IL-6 upregulation in the supernatant (Fig. 4e and f).

3.4.2. Expression of SDCs in experimental models

SDCs can be expressed in cells other than endothelial cells; hence, the highly complex nature of lung tissue, which contains neutrophils and mononuclear macrophages that may also express these molecules in response to stimulation, makes it challenging to analyse SDC levels. To identify the subcellular location of SDC2/4 in the lungs and whether they colocalize with the lung microvasculature, we conducted double staining with CD31 (an endothelial cell marker). As shown in Fig. 5, treatment with LPS (1 μg/ml LPS for 6 h) led to marked increases in SDC4 at both the mRNA and protein expression levels compared to those of the control group (incubated with PBS) *in vitro* ($p < 0.05$). In contrast, SDC2 showed no significant change in expression level (Fig. 5a–c, the original western blots see supplementary file S5). In *in vivo* experiments, SDC2 and SDC4 were both detected in murine lungs, regardless of LPS stimulation; however, only SDC4 colocalized with CD31 in the lung microvasculature (Fig. 5d and e).

3.4.3. TFs predicted to regulate SDC2 and SDC4

TFs predicted to regulate SDC2 and SDC4 using R studio based on the pooled DEGs ($p < 0.05$) are presented in Table 1. The TFs predicted to regulate SDC4 were NF-κβ, NF-κβ65, and c-REL. NF-κβ and NF-κβ 65 belong to the NF-κβ inflammatory signalling pathway, which mediates the transcription of an array of downstream inflammatory biomolecules. C-REL, encoded by the REL gene, is also an NF-κβ subunit that facilitates apoptosis, inflammation, immune responses, and oncogenic processes involving immune cells. TFs predicted to regulate SDC2 included zinc finger proteins (ZNF512 and ZNF512B) and F10. According to the GeneCards website (https://www.genecards.org/), F10 is vitamin K-dependent coagulation factor X of the blood coagulation cascade, while ZNF512 and ZNF512B are proteins containing four putative zinc finger motifs involved in the regulation of hair colour phenotype and appendicular lean mass (Supplementary file S6).

3.4.4. Regulation of acute inflammation by SDC4

As shown in Fig. 6, SDC4 knockdown *in vitro* led to a clear increase in the expression of three key inflammatory cytokines, IL-6, IL-1β, and VCAM-1, which are involved in endothelial injury (Fig. 6a–c), while as the knockdown of SDC2 has not this effect (Supplementary file S7). Overexpression of these cytokines induced by LPS *in vivo* along with expression of HBAAV2/VEC-TIE-Mir30-*m*-Sdc4-ZsGreen led to a marked exacerbation of lung injury, as determined by H&E staining of pathological sections (Fig. 6d–f). These results show critical injury of lung tissue in the knockdown group relative to the LPS group, including more thickening of the alveolar vascular barrier, increased inflammatory cells, and hyaline membrane formation.

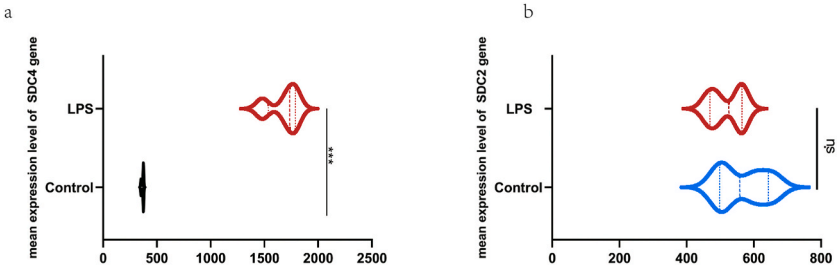

Fig. 3. Sydecan (SDC) levels among DEGs. a) Compared to the control, lipopolysaccharide (LPS) pretreatment induced very high *SDC4* expression. b) SDC2 expression was comparable in the LPS pretreatment and control groups. n.s., not significant; ***$p < 0.001$.

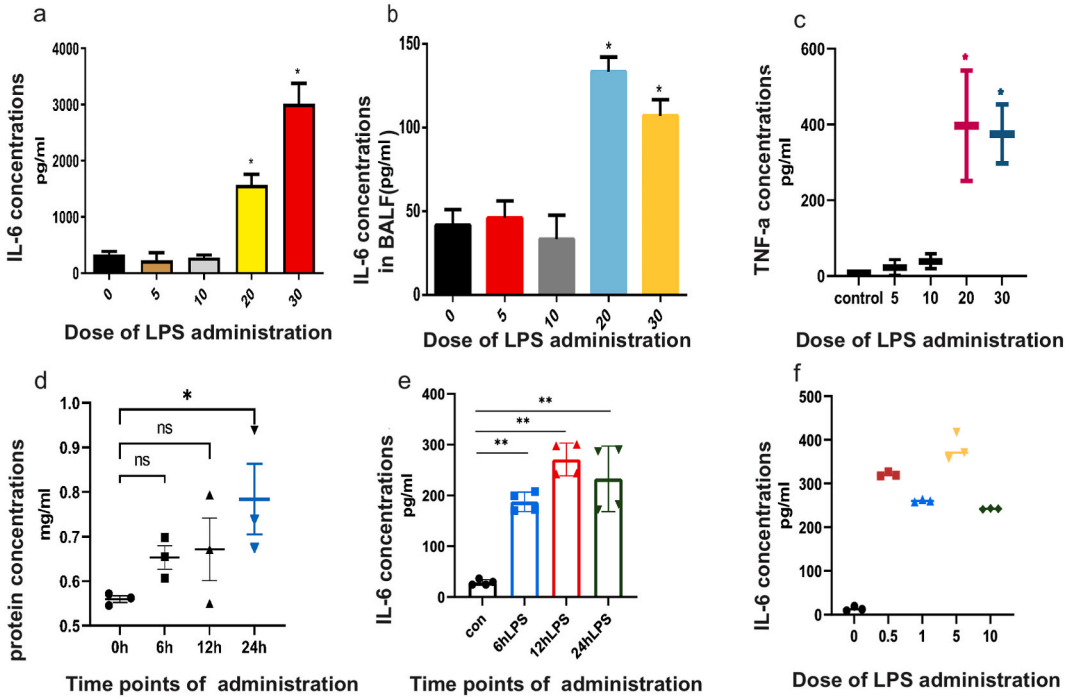

Fig. 4. Inflammatory marker expression in endothelial cell and C57BL/6 mouse experimental models. a) Interleukin 6 (IL-6) concentration in lung homogenates from mice administered different doses of LPS. b) IL-6 concentration in mouse bronchiolar lavage fluid (BALF). c) Tumour necrosis factor alpha (TNFα) concentration in lung homogenates from mice. d) Overall protein concentration in BALF. e, f) IL-6 concentrations in cell supernatants after administration of LPS at various time points and at different doses. n.s., not significant; ***$p < 0.001$.

4. Discussion

It is well known that the microvascular alveolar barrier serves as the primary defensive line for the development of sepsis associated ALI, and the function of syndecans located in the inner layer of endothelium is of great importance to function the interaction with ligands, transduction of signalling and the transmigration of neutrophils. In the present study, the two SDC isoforms of the lung microvascular endothelial cells, including SDC2 and 4, were found to express under LPS-induced septic inflammation. Among them, SDC4 is the only isoform playing a vital importance in modulating the inflammatory response under this situation.

ALI or ARDS is critically ill in clinical settings and is predominantly characterized by diffuse endothelial injury, coagulation imbalance, and hyperpermeability [18]. Particularly, endothelial cell dysfunction is the leading cause of development of ARDS, because the syndecan are the main mediators to interact with inflamed blood and transduce the inflammation from blood to lungs. With the increasing number of studies shedding light on the endothelial protection field, the glycocalyx and SCDs have attracted more and more attention due to their structural and functional roles. Such as the earlier report by Hui [19], the ectodomain of endothelial SCDs is degraded and shed under acute inflammatory conditions, a process that facilitates the downstream inflammatory cascade; hence, levels of specific syndecans can serve as biomarkers in many inflammatory diseases. Furthermore, in the coronavirus pandemic in 2019, the severity of pulmonary inflammation was related to the degree of glycocalyx shedding in both infected children [20] and older adults [21]. So, the definition of SDCs on endothelium in this process is scientifically valuable. The microarray chip analysis and transcriptome sequencing technology are well-used methods for researchers to screen target genes, however, it's hard to isolate endothelial cells from any type of live organism. In present analysis, dataset was obtained from GEO database in PubMed, the GSE5883 was found suitable only as it comprises gene-chip data from lung microvascular endothelial cells during the short phase of inflammation (4 h) after LPS treatment in spite of dozens of collections were screened. As illustrated in GSE5883, there was other analysis data form different time-points which showed no differentially expressed genes by terms of syndecan family members at all, this is consistent with results in our pilot study that SDC4 is temporarily expressed under inflammation. At last, the SDC4 was found quantitatively overexpressed at 4 h's exposure except for SDC1, SDC2 and SDC3.

Among the 17690 genes analysed, the mRNA expression levels of SDC1–4 were assessed and compared with those of the control group, but only SDC2 and SDC4 showed an absolute quantitative increase in gene expression of >100-fold, while SDC1 and SDC3 did not (Supplementary file S3). Surprisingly, a significant 4.5-fold change in SDC4 levels was detected, whereas SDC2 levels did not differ significantly in response to LPS, emphasizing the important role of SDC4 in the ALI pathway. Moreover, in PPI construction, molecules significantly associated with SDC4 included CCL5, CXCL 8, CXCL 10 and IL-1B. Among these molecules, CCL5 (P13501), CXCL10 (P10145), and CXCL8 (P10145) are chemotactic factors characterized by the ability to bind their respective receptors to induce the migration of inflammatory or immune cells to sites of infection (UniProt) and are all involved in cellular responses to organic cyclic

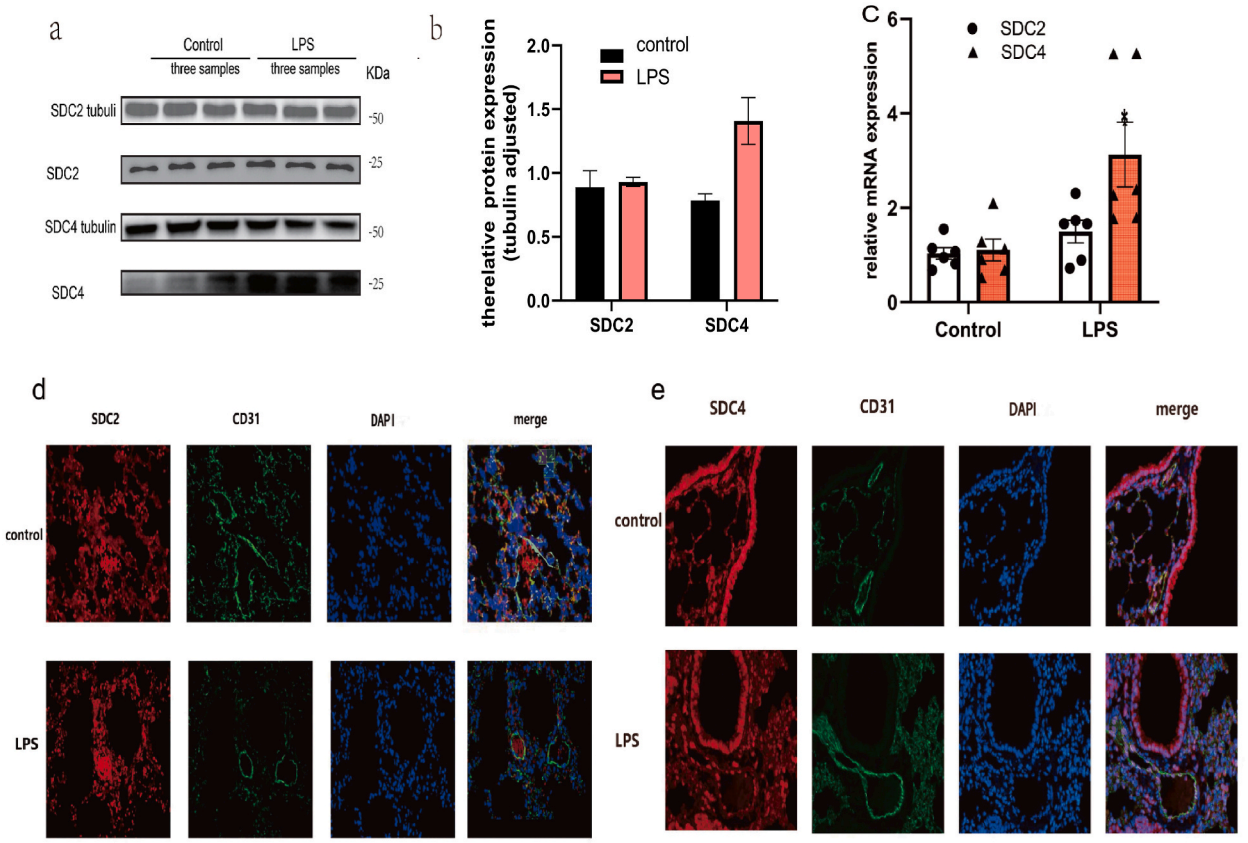

Fig. 5. *In vivo* and *in vitro* SDC2/4 expression in response to inflammation. a) SDC2/4 protein expression relative to that of tubulin following administration of 1 μg/ml LPS for 6 h; each group was measured in triplicate. b) Statistical analysis of the results presented in a. c) *SDC2* and *SDC4* mRNA expression under LPS induction *in vitro*. d, e) SDC2/4 expression *in vivo* in lungs subjected to inflammatory stimulation, as determined by immunofluorescence staining, including costaining of SDC2/4 with CD31 *$p < 0.05$.

Table 1
Transcription factors (TF) predicted to regulate *SDC2* and *SDC4* using R Studio.

Target	Predicted TF	*P*-value
SDC4	NFKAPPAB	2.05E−20
	CREL	5.14E-14
	NFKAPPAB65	1.38E-13
SDC2	ZNF512	1.41E-12
	ZNF512B	4.34E-15
	F10	1.25E-11

compounds and the inflammatory response. IL-1B (P01584) is a potent proinflammatory cytokine. Just like some reviews indicated that SDCs can bind to CCL5 [22], CXCL10 [23], IL-1B [11], and CXCL8 [24] in various inflammatory contexts to modulate intracellular signal transduction or function as coreceptors to facilitate downstream pathways. Other than above mentioned, the enrichment of GO and KEGG of DEGs at the 4 h time point indicated that the strongest enrichment are the TNF and cytokine receptor signalling pathways, which is consistent with the following experimental findings. For example, molecules (GO:0005488) or proteins (GO:0005515) were statistically bind to DEGs including SDC4. Enriched biological processes also included regulation of metabolic processes and cellular responses to cytokine stimuli and organic substances. Similarly, Xia and colleagues conducted integrated bioinformatics analysis to identify key genes involved in sepsis, and found 619 overlapping genes, using different criteria from those applied in our study [22], and reached similar conclusions to ours, namely, that DEGs were involved in protein binding and TNF signalling pathway. Apart from that, transcription factor of SDC4 is also predicted to be regulated by NF-κβ and its subfamily members. For this result, there is consistent evidence from previous studies in the fields of inflammation and cancer that NF-κβ can regulate SDC4 transcription [25,26]. All in all, SDC4, as one of the DEGs, connected to intracellular signal transduction and inflammatory molecule transcription in endothelial cells in response to LPS stimulation.

Literature mining also confirmed that endothelial cells and SDC4 has long been seen as promising therapeutic targets in dealing

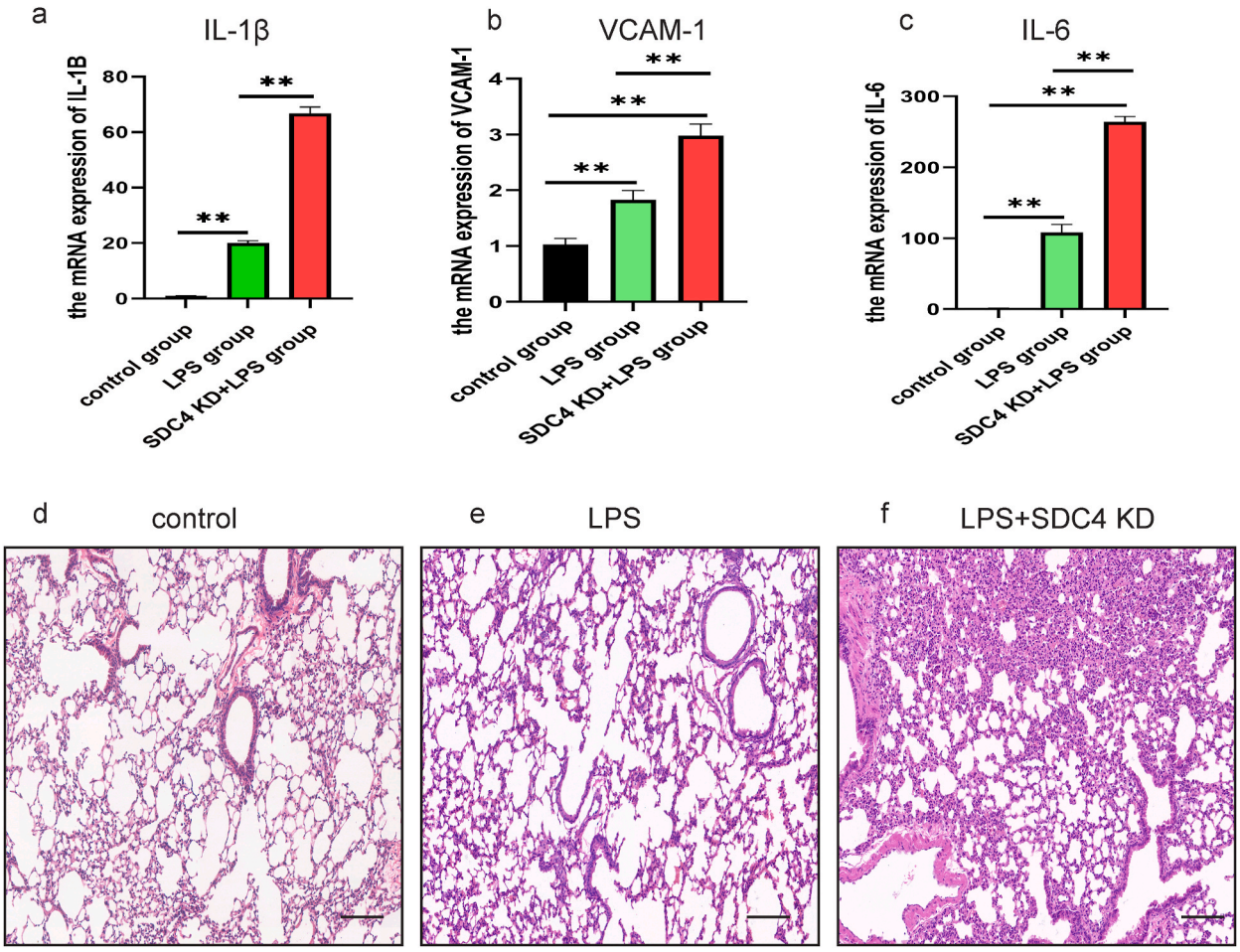

Fig. 6. The inflammatory phenotypes of SDC4 knockdown models *in vivo* and *in vitro*. a–c) Expression of three key inflammatory mediators in inflamed endothelium induced by LPS. d–f) Histological presentation of haematoxylin and eosin-stained pathological sections from mouse lungs subjected to inflammation. Scale bar 100μm. **p < 0.01.

with various inflammatory diseases. According to Schmidt et al. the thickness of the glycocalyx in the lungs is crucial [3] to determine the outcome of pulmonary inflammation because of its multifunction. Villalba et al. reported [27] the integrity of glycocalyx is of importance for the progression of an existed sepsis. As this event is concerned, it's hypothesised that SDCs might take the most responsibility because SDC4 is predicted to be the key functional syndecan in present bioinformatics analysis. Moreover, due to chondroitin sulphate and HS chains in glycosaminoglycan, SDC4 has been comprehensively studied in the context of osteoarthritis [10,28,29], cancer and other diseases [30]. For inflammatory injury, thu and colleagues [31] reported that SDC3 and SDC4 were both upregulated in response to inflammatory stimuli; however, they used human umbilical vein endothelial cells for their experiments, which are not relevant to studies of the lung. Furthermore, Santoso et al. [32] found that SDC4 can modulate the Keap1-Nrf2 antioxidant pathway in the lung, leading to reduced inflammation when administered systemically, indirectly illustrating the interaction between SDC4 and lung microvascular endothelial cells. Moreover, some research has found that SDC4 may substitute for SDC1 in ALI. Apart from that, SDC4 has been proved its superiority in delivering several types of viruses including AAV9 [33] and SARS-CoV-2 [34]. As for the mechanism of regulation of lung injury, SDC4 deserves the verification in basal experiments field.

With the construction of *in vivo* and *in vitro* model, the expression of SDC4 was levelled either by immunofluorescence, mRNA or western blotting methods. Compared to SDC2, there was remarkable elevation of SDC4 expression, which is consistent to the result of previous bioinformatic analysis. To our knowledge, this is the first experimental confirmation of SDC4's involvement on ALI/ARDS ground, this conclusion would be a forthcoming target to thoroughly examine the mechanism of sepsis associated ALI although the properties of SDC4 was partly verified with the application of siRNA technique. In syndecan family, SDC4 is the only glycosaminoglycan having the capacity to inhibit the pulmonary inflammation.

This study has several limitations. First, only one GSE dataset was analysed, which may be insufficient; however, the examples and matrix distribution were perfect, and our conclusions are consistent with those of previous studies. Second, TF regulation analysis was limited to bioinformatics prediction, and further verification using dual luciferase assays is required to support our findings. Third, based on our results, the peak inflammatory state occurred 24 h after LPS induction, while SDC4 was expressed in a relatively early

phase (6 h). The function of SDC4 in later phases of induction was not analysed in this study, and further analysis is warranted in forthcoming studies. Finally, *in vivo* SDC4 protein expression levels in the endothelium could not be determined due to technical limitations.

5. Conclusion

Using bioinformatic analysis and laboratory experiments, we identified SDC4 from a GEO dataset and found that it is the only SDC involved in inflammatory regulation in this context. Moreover, SDC4 involvement was verified to be protective in lung inflammatory injury.

Author contribution statement

Zhipeng Zhu: Conceived and designed the experiments; Analysed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Xiaoyan Ling, Junran Xie: Performed the experiments.

Hongmei Zhou: Contributed reagents, materials, analysis tools or data.

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Data availability statement

Data included in article/supplementary material/referenced in article and will be made available upon request.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e18600.

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