SPLUNC1 knockout enhances LPS-induced lung injury by increasing recruitment of CD11b^{+}Gr-1^{+} cells to the spleen of mice

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Abstract. Short palate, lung and nasal epithelium clone 1 (SPLUNC1) is a tissue-specific gene of nasopharyngeal tissue, and has been recognized as a potential tumor-suppressor gene in nasopharyngeal carcinoma. As a secreted protein, SPLUNC1 plays an important role in innate immunity including antimicrobial and host defense. However, the related immune cells which are regulated by SPLUNC1 remain elusive. In the present study, an acute lung injury (ALI) mouse model was established by administration of lipopolysaccharide (LPS) intraperitoneal injections to wild-type and SPLUNC1$^{-/-}$ mice (5 mg/kg). Pathologic results showed that the SPLUNC1$^{-/-}$ group appeared to have more severe pulmonary damage and infiltrated inflammatory cells compared with the WT group after LPS treatment for 24, 48, 72 and 96 h. The mRNA expression levels of interleukin-6 (IL-6), chemokine (C-C motif) ligand-2 (CCL-2), chemokine (C-C motif) ligand-3 (CCL-3) and chemokine (C-X-C motif) ligand-1 (CXCL-1) in lungs of the SPLUNC1$^{-/-}$ group were higher than these levels in lungs of the WT group at different time points after LPS injection. The percentage of splenic CD11b^{+}Gr-1^{+} myeloid-derived suppressor cells (MDSCs) in the SPLUNC1$^{-/-}$ mice was higher than this percentage in the WT mice at the time points of 72 and 96 h post LPS injection ($P<0.05$). These findings demonstrated that SPLUNC1 had a certain protective effect on the LPS-induced ALI mouse model as well as it was found to inhibit the recruitment of MDSCs to the spleen in this model.

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Abbreviations: SPLUNC1, short palate, lung and nasal epithelium clone 1; MDSCs, myeloid-derived suppressor cells; LPS, lipopolysaccharide; ALI, acute lung injury; IMCs, immature myeloid cells; DCs, dendritic cells; IHC, immunohistochemistry; GADPH, glyceraldehyde-3-phosphate dehydrogenase; WT, wild-type; NSCLC, non-small cell lung cancer; IL-6, interleukin-6; CCL-2, chemokine (C-C motif) ligand-2; CCL-3, chemokine (C-C motif) ligand-3; CXCL-1, chemokine (C-X-C motif) ligand-1

Key words: SPLUNC1, knockout mice, CD11b^{+}Gr-1^{+} cells, lipopolysaccharide, acute lung injury

Introduction

Acute lung injury (ALI) is a complicated pathogenic process with high mortality due to sepsis, pulmonary infection and shock (1). Lipopolysaccharide (LPS), a component found in the cell membrane of gram-negative bacteria, induces the production of numerous inflammatory cytokines and chemokines and has been shown as a major cause of ALI (2). Intraperitoneal administration of LPS has been widely used to model the pathogenesis of ALI and has yielded reproducible results.

Short palate, lung and nasal epithelium clone 1 (SPLUNC1) is a protein mainly secreted by large airway epithelia and submucosal glands (3,4). Accordingly, we can find this protein in the human oral cavity (5), nasal secretions (6), tracheal aspirates (7) as well as in rat lung (8), suggesting that SPLUNC1 is a highly abundant secretory product of the mammalian respiratory system. Recent studies by our group demonstrated that SPLUNC1 inhibits the overall process of Epstein Barr virus replication and is associated with nasopharyngeal carcinoma prognosis (9-12). Although SPLUNC1-deficient mice do not develop spontaneous lung disease without a bacterial insult, they do exhibit more severe impaired intrapulmonary responses than wild-type controls when infected by respiratory pathogens such as *Mycoplasma pneumoniae* (13), *Pseudomonas aeruginosa* (14) and *Klebsiella pneumoniae* (15). In addition, loss of SPLUNC1 expression predisposes mice to develop otitis media (16). Although SPLUNC1 protein has been predicted to exert host defense and has been proposed as a valuable marker in non-small cell lung cancer (NSCLC) (17), the related immune cells regulated by SPLUNC1 remain elusive.

ALI triggers innate immune responses and is associated with the induction and promotion of immune-suppressive mechanisms, such as the accumulation of myeloid-derived suppressor cells (MDSCs). MDSCs have been described as a heterogenic population of immature myeloid cells that consist of myeloid progenitors and precursors of macrophages, granulocytes and dendritic cells (DCs) and that, mainly by multiple mechanisms, suppress T cell and NK cell functions (18,19). In healthy individuals, immature myeloid cells (IMCs) can be generated in bone marrow and differentiate into mature granulocytes, macrophages or DCs. In pathological conditions such as cancer, sepsis, various infectious diseases, trauma, certain autoimmune disorders or in bone marrow transplantation, a partial block in the differentiation of IMCs into mature myeloid cells results in an expansion of this population. In mice, MDSCs are identified as cells that co-express the myeloid lineage differentiation antigen Gr-1 and CD11b (20), which make up only a small proportion (2-4%) of spleen cells and are absent from the lymph nodes (18). Recently, several researchers have demonstrated that the accumulation of MDSCs plays a vital role in LPS-induced lung injury (21). However, the molecules that are involved in the generation and expansion of MDSCs during acute inflammation are not completely understood.

In the present study, we sought to investigate the effects of SPLUNC1 on lung injury using SPLUNC1-knockout (SPLUNC1$^{-/-}$) mice, and to clarify the function of SPLUNC1-mediated recruitment of CD11b^{+}Gr-1^{+} MDSCs in the spleen of LPS-induced ALI mice.

Materials and methods

Animals. To obtain SPLUNC1-knockout mice that allowed conditional and global disruption of the SPLUNC1 gene, we used the Cre/*loxP* and flp/*FRT* recombination systems to target exons 3 of SPLUNC1. A single loxp site was inserted before exon 3 and before exon 4, and an frt-flanked PGK neo cassette was inserted between exons 3 and 4 to serve as a positive selectable marker. The targeting construct was electroporated into embryonic stem (ES) cells and the germline SPLUNC1$^{loxp\text{-}neo}$ chimeras were obtained by homologous recombination. Then, the FRT-flanked PGK neo cassette was deleted by crossing female SPLUNC1$^{loxp\text{-}neo}$ mice with transgenic Flp male mice to obtain the homozygous mice for the floxed SPLUNC1 allele (SPLUNC1$^{flox/flox}$). To generate SPLUNC1$^{-/-}$ mice, the SPLUNC1$^{flox/flox}$ mice were mated with EIIα-Cre mice (purchased from the Jackson Laboratory, Bar Harbor, ME, USA).

To produce animals for experiments, heterozygous mice were crossed to generate wild-type littermate controls. All mice used in the present study were male, 8-12 weeks old, and housed under specific pathogen-free conditions. The present study was performed in strict accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals (National Institutes of Health). Experimental protocols were approved by the Animal Ethics Committee of Central South University.

Mouse model of LPS-induced ALI. Mice were randomly divided into four groups: WT control, KO control, WT-LPS, and KO-LPS. The LPS group was intraperitoneally injected with a single dose of 5 mg/kg body weight of LPS (Sigma, Carlsbad, CA, USA) which was dissolved in saline at a concentration of 1 mg/ml. Meanwhile, the mice in the control group were intraperitoneally injected with saline alone. The mice were euthanized at 24, 48, 72 and 96 h after LPS or saline administration.

Western blot analysis. The proteins were extracted from mouse lung tissues and then separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane. The membrane was blocked using 5% non-fat milk in Tris-buffered saline containing 0.1% Tween-20 and then incubated with primary antibodies: SPLUNC1 (R&D Systems, Minneapolis, MN, USA) and GAPDH (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) at 4˚C overnight. After washing three times with TBST, the membranes were incubated with HRP-conjugated secondary antibody. Bands were detected with an electrochemiluminescence detection system.

Flow cytometric analysis. Single cell suspensions were prepared by homogenization through a sterile stainless steel mesh from the spleens. Erythrocytes were depleted using RBC lysing buffer (Sigma), and splenocytes were washed with phosphate-buffered saline (PBS). Then, the cells were stained with anti-CD11b and anti-Gr-1 antibodies (eBioscience, San Diego, CA, USA) according to the manufacturer's protocol. Fluorescence was measured using a MoFlo™ XDP High-Performance Cell Sorter and data were analyzed by Summit 5.2 software (both from Beckman Coulter, Inc., Brea, CA, USA).

RNA extraction and real-time PCR analysis. Reverse transcription reaction was performed using a Fermentas Revert Aid First Strand cDNA Synthesis kit (Fermentas, Burlington, ON, Canada), according to the manufacturer's instructions. qRT-PCR was performed using an iQ5 Multicolor Detection system (Bio-Rad, Hercules, CA, USA). The sequences of the primers were as follows: β-actin forward, 5'-TTTCCAGCCT TCCTTCTT-3' and reverse, 5'-GGTCTTTACGGATGTCA ACG-3'; IL-6 forward, 5'-CTGATGCTGGTGACAACCAC-3' and reverse, 5'-CAGAATTGCCATTGCACAAC-3'; IL-8 forward, 5'-CGTCCCTGTGACACTCAAGA-3' and reverse, 5'-GGAGCATCAGGATCCAAACA-3'; CCL-2 forward, 5'-ATGCAGTTAACGCCCCACTC-3' and reverse, 5'-ACC CATTCCTTCTTGGGGTC-3'; CCL-3 forward, 5'-GCAACC AAGTCTTCTCAGCG-3' and reverse, 5'-TTGGACCCAGG TCTCTTTGG-3'; CCL-5 forward, 5'-ACCATATGGCTCGG ACACCA-3' and reverse, 5'-GCGGTTCCTTCGAGTGA CA-3'; CXCL1 forward, 5'-TGGCTGGGATTCACCTCAAG-3' and reverse, 5'-CCGTTACTTGGGGACACCTT-3'; IL-1β forward, 5'-TGCCACCTTTTGACAGTGATG-3' and reverse, 5'-AAGGTCCACGGGAAAGACAC-3'. The expression of mRNA was assessed by evaluated threshold cycle (CT) values. The CT values were normalized with the expression levels of β-actin and the relative amount of mRNA specific to each of the target genes was calculated using the $2^{-\Delta\Delta Ct}$ method (22-25). β-actin was used as an internal control.

Histology. Lungs of the differentially treated mice were fixed with 4% paraformaldehyde, and embedded in paraffin.

Subsequently, the lung tissues were sliced into 4-μm sections, and then stained with hematoxylin and eosin to observe the inflammatory response and pathological changes using an Olympus BX51 microscope (Olympus, Tokyo, Japan). The degree of histologic lung injury was assessed based on the following criteria: neutrophil infiltration, alveolar congestion, interstitial edema, necrosis and atelectasis. The severity of injury was evaluated via a numerical rating system that ranges from 0 to 4 (score): no injury, 0; injury to 25% of the field, 1; injury to 50% of the field, 2; injury to 75% of the field, 3; and diffuse injury, 4. The mean score was then analyzed for comparison between groups.

Immunohistochemistry (IHC) and evaluation of staining. IHC was carried out using the peroxidase anti-peroxidase technique following a microwave antigen retrieval procedure. Anti-CD11b (MAB1124; 1:300), anti-Gr-1 (MAB1037; 1:300) (both from R&D Systems) was overlaid on the tissue sections and incubated overnight at 4˚C. Secondary antibody incubation (Santa Cruz Biotechnology, Inc.) was performed at room temperature for 30 min. Color reaction was developed using 3,3'-diaminobenzidine tetrachloride (DAB) chromogen solution. All slides were counterstained with hematoxylin. Positive control slides were included in every experiment in addition to the internal positive controls. The specificity of the antibody was determined with matched IgG isotype antibody as a negative control.

Immunostaining was evaluated by two investigators in a blinded manner in an effort to provide a consensus on staining patterns by light microscopy (Olympus). CD11b or Gr-1 staining was assessed according to the methods described by Hara and Okayasu (26) with minor modifications. Each case was rated according to a score that added a scale of intensity of staining to the area of staining. At least 10 high-power fields were randomly chosen, and >1,000 cells were counted for each section. The intensity of staining was graded on the following scale: 0, no staining; 1+, mild staining; 2+, moderate staining; 3+, intense staining. The area of staining was evaluated as follows: 0, no staining of cells in any microscopic fields; 1+, <30% of tissue stained positive; 2+, between 30 and 60% stained positive; 3+, >60% stained positive. The minimum score when summed (extension + intensity) was, therefore, 0, and the maximum, 6. A combined staining score (extension + intensity) of ≤2 was considered to be a negative staining (low staining); a score between 3 and 4 was considered to be a moderate staining; whereas a score between 5 and 6 was considered to be a strong staining. An optimal cut-off level was identified as follows: a staining index score of 0-2 was used to define tumors with negative expression and 3-7 indicated positive expression of these two proteins. Agreement between the two evaluators was 95%, and all scoring discrepancies were resolved by discussion between the two evaluators.

Statistical analysis. The data obtained in the present study are expressed as the mean values ± standard deviation (SD) from at least three independent experiments using triplicate samples for the individual treatments. Statistical analyses were performed using two-way ANOVA or Fisher's exact test (Prism 6; GraphPad Software, San Diego, CA, USA). A P-value of <0.05 was considered to indicate a statistically significant difference.

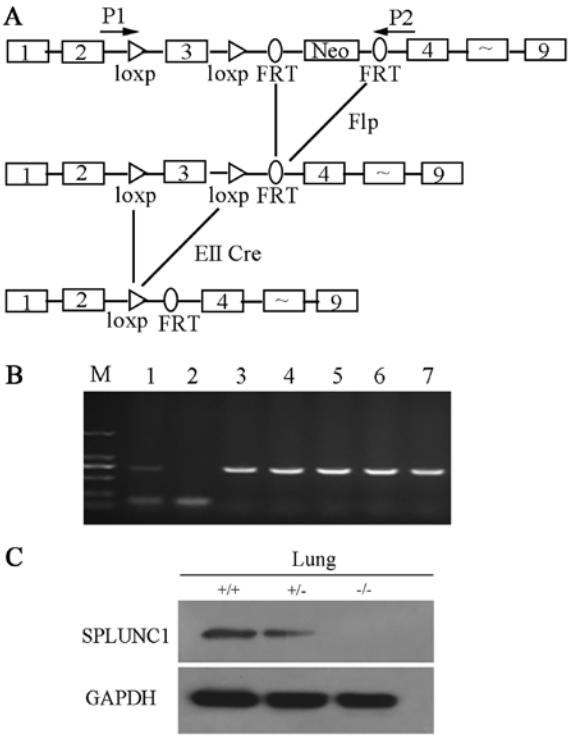

Figure 1. Identification of SPLUNC1-knockout mice. (A) Gene targeting strategy (exons are not drawn to scale). Exon 3 was flanked by LoxP sites (triangles), and a neomycin selection cassette was flanked by FRT sites (oval). (B) Genotype identification of the mice, SPLUNC1$^{+/-}$ mice (lane 1), WT mice (lanes 3-7), and SPLUNC1$^{-/-}$ mice (lane 2) by PCR with primer P1 (TGTGTGTGAACATGTGTGCTT) and primer P2 (CACAGGTGATA CAGATGGAGA). (C) Western blot analysis of SPLUNC1 expression in the lung from SPLUNC1$^{+/+}$, SPLUNC1$^{+/-}$ and SPLUNC1$^{-/-}$ mice.

Results

Generation of SPLUNC1-knockout mice. To identify the role of SPLUNC1 *in vivo*, we generated SPLUNC1-knockout mice. In this procedure, Cre/*loxp* and *Flp*/FRT recombination systems were used to target exon 3 of the mouse SPLUNC1 gene (Fig. 1A). The genomic DNA was extracted from the mouse tail and used as a template for PCR analysis with primer P1 and P2 to identify genotypes (Fig. 1B). SPLUNC1 protein expression in wild-type and SPLUNC1$^{-/-}$ mice was shown by western blot analysis (Fig. 1C). A specific SPLUNC1 band was detected in the SPLUNC1$^{+/+}$ and SPLUNC1$^{+/-}$ lung, while no SPLUNC1 protein was detected in the lung of the SPLUNC1$^{-/-}$ mice. Then, we observed that knockout of SPLUNC1 did not cause embryonic death, and there was no obvious phenotype difference between the wild-type and SPLUNC1$^{-/-}$ mice. Similar results were reported by Gally *et al* (13) that SPLUNC1$^{-/-}$ mice were viable, fertile, and largely indistinguishable from WT littermates in general appearance, body weight, locomotion and overt behavior.

Knockout of SPLUNC1 affects the distribution of CD11b^{+}Gr-1^{+} MDSCs in the spleen of LPS-induced ALI mice using flow cytometric analysis. MDSCs commonly express the markers CD11b and Gr-1 in mice; such immature myeloid cells are present in the bone marrow of healthy mice. However, they differentiate into mature myeloid cells and accumulate in the

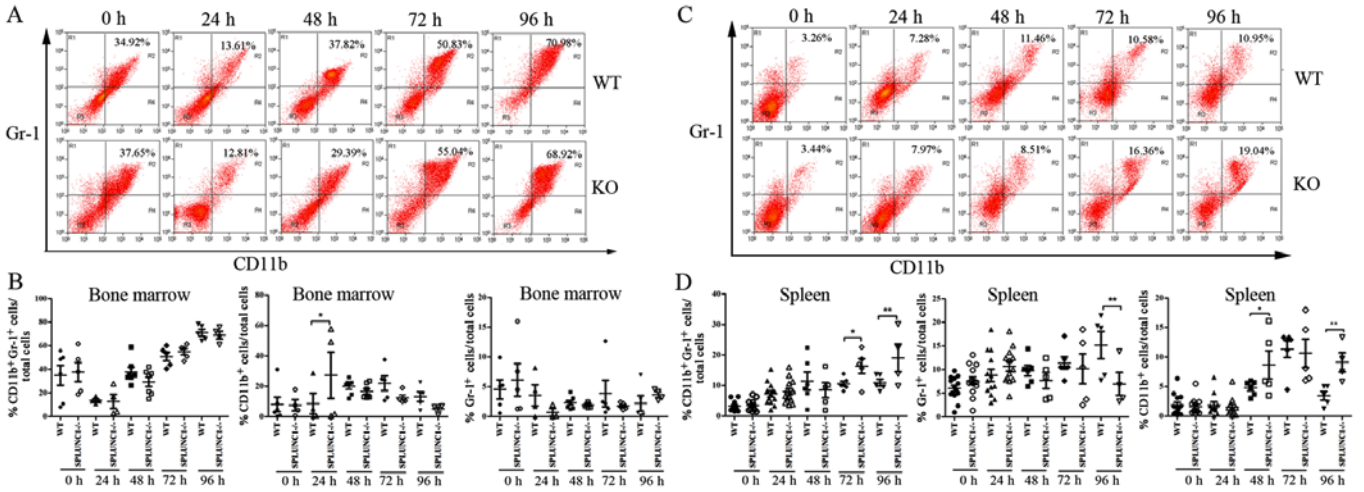

Figure 2. CD11b$^+$Gr-1$^+$ MDSCs in the bone marrow and spleen of mice after LPS injection (A) Representative dot-plots of CD11b$^+$Gr-1$^+$ cell populations from the bone marrow of SPLUNC1$^{-/-}$ and WT mice. (B) Percentages of the CD11b$^+$Gr-1$^+$ cells in mouse bone marrow, as analyzed by flow cytometry. (C) Representative dot-plots of CD11b$^+$Gr-1$^+$ cell populations from the spleen of SPLUNC1$^{-/-}$ and WT mice. (D) Percentages of the CD11b$^+$ Gr-1$^+$ cells in mouse spleen, as analyzed by flow cytometry. Data are means ± SD of at least four independent animals (n>4); *P<0.05; **P<0.01.

spleen under acute inflammation conditions. In the present study, we first measured the percentage of CD11b$^+$Gr-1$^+$ MDSCs in the bone marrow of the mice at different time points after LPS administration. The number of CD11b$^+$Gr-1$^+$ MDSCs in the WT group was reduced from 34.92±8.48 to 13.61±1.33% and in the KO group, from 37.65±8.06 to 12.81±5.92% (P<0.05; Fig. 2A) after LPS injection for 24 h, and then it rapidly recovered to the base level after injection for 48 h. Meanwhile, the number of these cells gradually increased at 72 and 96 h post LPS injection; it reached the highest level both in the WT and KO groups (70.98±2.84% in WT vs. 68.92±3.24% in KO mice at 96 h). Although the percentage of CD11b$^+$ cells in KO mice was higher than that in WT mice at 24 h after LPS induction (P<0.05; Fig. 2B), no significant differences were observed between WT and KO groups at the different time-points of 0, 24, 48, 72 and 96 h after LPS injection (P>0.05; Fig. 2B). These results indicated that knockout of SPLUNC1 had no influence on the production of MDSCs in the bone marrow of the mice with ALI.

To investigate whether SPLUNC1 affects the recruitment of MDSCs from the bone marrow to the spleen in acute inflammatory response, we next analyzed the proportion of splenetic CD11b$^+$Gr-1$^+$ MDSCs in the WT and KO groups. There was significant accumulation of CD11b$^+$Gr-1$^+$ MDSCs after LPS injection, increasing from 3.26±0.41% of all splenocytes at baseline to a peak of 11.46±2.97% by 48 h in the WT group and from 3.44±0.46% of all splenocytes at baseline to a peak of 19.04±4.53% by 96 h in the KO group (Fig. 2C). The percentage of CD11b$^+$ cells in the KO mice was higher than that in the WT mice at the time-points of 48 h (4.79±0.49% in WT mice vs. 8.67±2.38% in KO mice) and 96 h (3.44±0.74% in WT mice vs. 9.10±1.62% in KO mice) after LPS injection (P<0.05; Fig. 2D). Although the number of Gr-1$^+$ cells in the WT mice was higher than that in the KO mice 96 h (15.18±2.87% in WT mice vs. 6.97±2.41% in KO mice) post LPS injection, the percentage of CD11b$^+$Gr-1$^+$ MDSCs in the KO mice was higher than that in the WT mice at the time point of 72 h (10.58±0.79% in WT mice vs. 16.36±2.42% in KO mice) and 96 h (10.95±1.11% in WT mice vs. 19.04±4.53% in KO mice) after LPS injection (P<0.05; Fig. 2D). These data revealed that the presence of SPLUNC1 suppresses the recruitment of CD11b$^+$Gr-1$^+$ MDSCs to the spleen in LPS-induced ALI.

Expression of CD11b/Gr-1 in cells in the mouse spleen as detected by IHC. Granulocyte differentiation antigen 1 (Gr-1) is highly expressed in neutrophils and Gr-1$^+$ cells play a vital role in host defense, functioning as immunoregulators in the immune system. CD11b$^+$ cells, specifically express M-CSF receptor (CD115) or IL-4R (CD124), having immunosuppressive activity. CD11b$^+$ cells are distributed throughout the body and act as sentinels for the immune system. The frequency of CD11b$^+$ myeloid cells varies in different organs, ranging from ~48% of brain myeloid (microglia) cells, 30% of bone marrow and 6% of total nucleated cells in the gut and spleen. In mice, MDSCs are identified as cells that co-express the myeloid lineage differentiation antigens Gr-1 and CD11b, which have been shown to accumulate quickly in mouse spleen and possess immunosuppressive effect in response to LPS administration. In the present study, we aimed to investigate whether SPLUNC1 affects the recruitment of MDSCs from the bone marrow to the spleen in acute inflammation; thus, it was important to distinguish between CD11b$^+$Gr-1$^+$ cells from CD11b$^+$ and Gr-1$^+$ cells.

In the present study, we examined the protein expression of Gr-1 and CD11b in the mouse spleen in response to LPS injection. In the WT and KO groups, few Gr-1$^+$ cells were observed in the splenic tissues at 0 and 24 h after LPS injection, but a marked increase in Gr-1$^+$ cells was noted by 48 h, particularly 72 and 96 h after LPS injection (Fig. 3A). Similarly, CD11b exhibited low expression at 0 and 24 h in cells of splenic tissues after LPS injection, while 48 h post injection, the number of cells expressing CD11b gradually increased. However, statistically significant differences between the WT and KO groups were not detected by IHC at the different time points after LPS injection (Fig. 3B).

Knockout of SPLUNC1 enhances LPS-induced lung injury. To investigate the role of SPLUNC1 in acute lung inflammation,

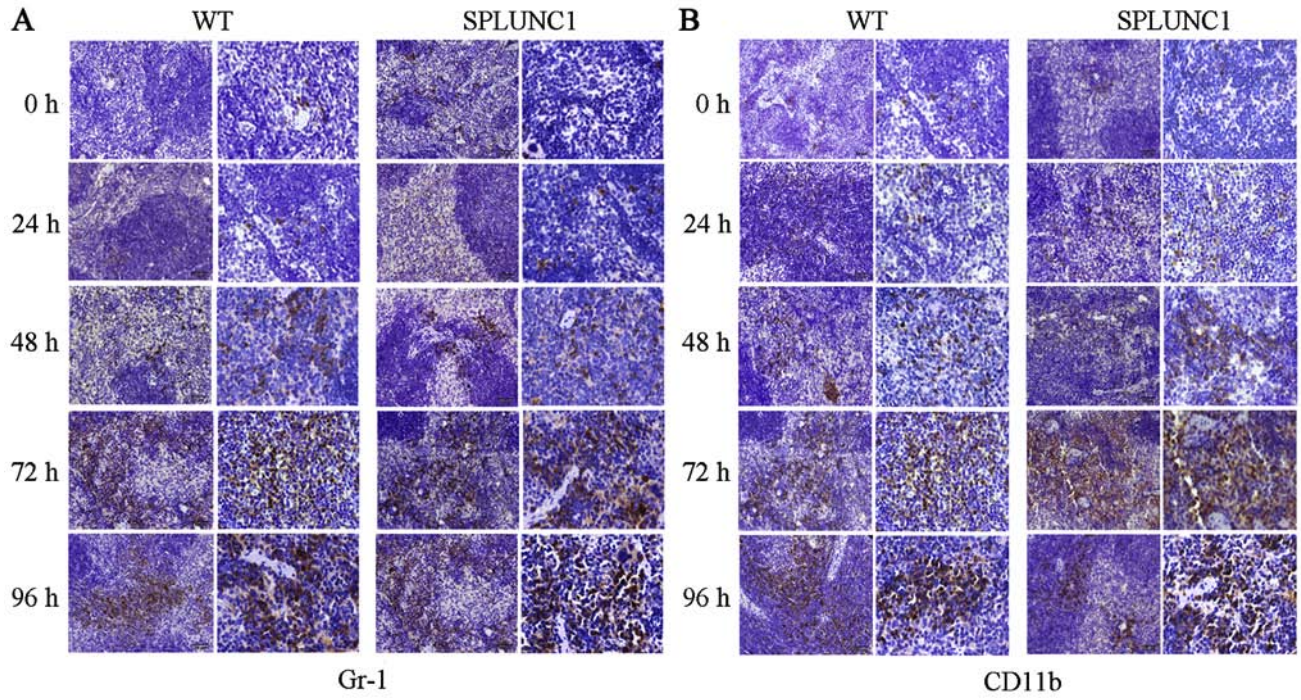

Figure 3. Immunohistochemical analysis of CD11b and Gr-1 expression in mouse spleen after LPS injection (left, x200 magnification; right, x400 magnification). (A) Gr-1 expression was detected in the mouse spleen. Few Gr-1$^+$ cells were observed in splenic tissues at 0 and 24 h after LPS injection, but a dramatic increase in Gr-1$^+$ cells was noted by 48 h, and particularly 72 and 96 h after LPS injection. (B) CD11b expression was detected in the mouse spleen, and had a similar expression trend with Gr-1.

WT and SPLUNC1$^{-/-}$ mice were treated with LPS as described in Materials and methods. The severity of lung injury was evaluated by histopathological analysis using hematoxylin and eosin (H&E) staining. In the WT and KO groups, the lungs of the mice injected with LPS showed neutrophil infiltration, thickened alveolar walls and lung congestion, while no such pathological changes were found in the control group (LPS-0 h). Meanwhile, when treated with LPS, the SPLUNC1$^{-/-}$ group appeared to have more severe pulmonary damage and infiltrated inflammatory cells compared with the WT mice after LPS treatment for 48 h (Fig. 4).

Knockout of SPLUNC1 promotes lung inflammation in response to LPS induction. Furthermore, we compared the differential expression of these cytokines in the WT and SPLUNC1$^{-/-}$ groups at different time points after LPS treatment. The mRNA expression levels of IL-6, CCL-2 and CCL-3 in the SPLUNC1$^{-/-}$ group were higher than these levels in the WT group 48 h post LPS injection. The level of CXCL-1 mRNA expression in the SPLUNC1$^{-/-}$ group was higher than the level in the WT group after LPS injection at 72 and 96 h ($P<0.05$; Fig. 5A). The SPLUNC1 expression levels were increased in the lung tissues of the WT mice after LPS injection (Fig. 5B). Taken together, these data demonstrated that SPLUNC1 deficiency upregulated ALI-related cytokines, which may promote early inflammation leading to more severe lung injury after LPS injection.

Discussion

The majority of recent studies on SPLUNC1 have attempted to explore its role in inflammation or infection, while the immune cells associated with SPLUNC1 regulation remain largely unknown. In the present study, SPLUNC1 protein was undetectable in SPLUNC1$^{-/-}$ mouse lung tissues, which confirmed gene knockout. Our daily observation found that there was no obvious phenotype difference between the wild-type and SPLUNC1$^{-/-}$ mice under normal physiological conditions, which was in accordance with a study reported by Gally *et al* (13).

SPLUNC1 plays a significant role in the host immune system with immunomodulatory (27), anti-biofilm (28) and/or chemotactic properties (29). Our *in vitro* studies have demonstrated that SPLUNC1 protein can bind to bacterial lipopolysaccharide (LPS), inhibit the growth of *P. aeruginosa* (10), regulate cell progression and apoptosis (12) and weaken the inflammatory response induced by EBV infection (11). To further explore its immune function in acute inflammation, we established an acute lung injury (ALI) mouse model by LPS intraperitoneal injection to WT and SPLUNC1-knockout mice, respectively. The symptoms of LPS-induced ALI mice closely resemble the observed pathology in humans (30); therefore, intraperitoneal injection of LPS has been widely used to study the pathogenesis of ALI.

ALI is a severe inflammatory disease that can lead to clinical syndromes including hypoxemia, pulmonary inflammation, alveolar-capillary barrier damage and multiple organ injury (2,31,32). LPS can be recognized by macrophages via their surface CD14/TLR4 receptor complexes. Activated macrophages and other immune cells then express multiple cytokines, which initiate, amplify and perpetuate the inflammatory response in ALI (33,34). As expected, the present study found that when treated with LPS, the SPLUNC1$^{-/-}$ group appeared to have more severe pulmonary damage compared

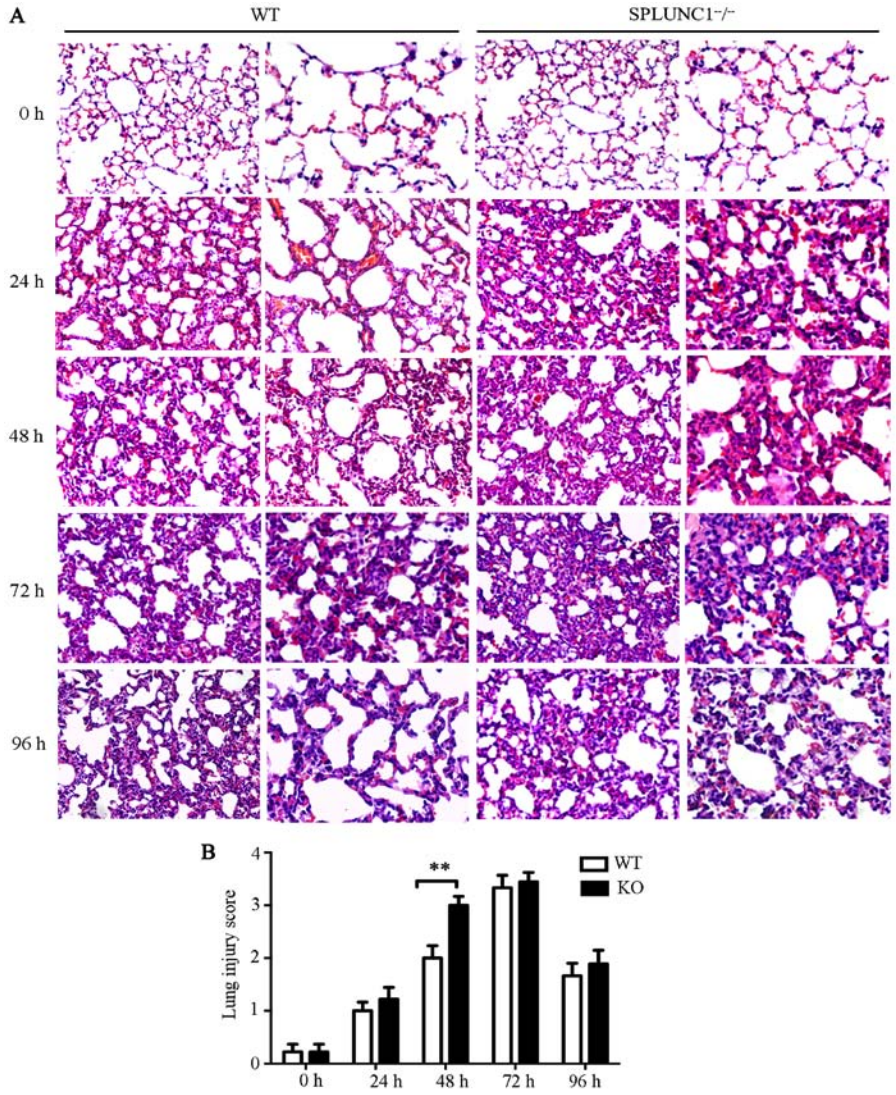

Figure 4. Knockout of SPLUNC1 enhances LPS-induced lung injury (n=5 mice/group). (A) Representative H&E-stained sections of lungs from WT and SPLUNC1$^{-/-}$ mice at different time points after LPS treatment (left, magnification, x100; right, magnification, x200). (B) Lung injury scores. All results are means ± SEM (n=5 mice/group); **P<0.01.

with the WT group after LPS treatment. The characteristic of ALI is the release of chemokines, such as CCL-2, CCL-3 and CXCL-1, which recruit macrophage and neutrophil migration from the intravascular space across the endothelium and epithelium into the airspaces (35). In the present study, we found that the mRNA expression levels of IL-6, CCL-2, CCL-3 and CXCL-1 in the SPLUNC1$^{-/-}$ group were higher than these levels in the WT group at different time points after LPS injection and the period of increase of these cytokine in the KO group was longer than that in the WT group. Moreover, immunohistochemical results indicated that the expression of SPLUNC1 in the WT group also increased after LPS injection. These results demonstrated that SPLUNC1 deficiency upregulated ALI-related cytokines, which may promote early inflammation leading to more severe lung injury after LPS injection.

There are two types of innate immune molecular proteins that play a key role in the defense of gram-negative bacteria: lipopolysaccharide-binding protein (LBP) and bactericidal/permeability increasing protein (BPI). The two structures are similar, and all can be combined with LPS. LBP presents LPS to the surface of immune cells and stimulates them to produce an immune response. However, as an antagonist of LPS, BPI can block the combination of LPS and LBP (36,37). Our previous study found that SPLUNC1 has a similar folding structure to BPI and LBP, which can bind to bacterial LPS (10). Therefore, we concluded that the homology of the BPI domain of the SPLUNC1 molecule may be one of the important molecular basis of its protective function in LPS-induced lung injury in mice.

Various studies have suggested that Gr-1^{+}/CD11b^{+} cells can play a vital role in the immune system and Gr-1^{+}CD11b^{+} MDSCs have been shown to accumulate quickly in the mouse spleen and possess immunosuppressive effect in response to LPS administration (38-41). In the present study, we found that the expression of those cells in mouse spleen was increased 48 h after LPS injection; the percentage of Gr-1^{+}CD11b^{+} MDSCs in the spleen showed an increasing production trend post LPS injection both in the WT and KO groups. In the mouse bone marrow, the number of CD11b^{+}Gr-1^{+} MDSCs declined 24 h post LPS injection, and then it recovered rapidly. Although

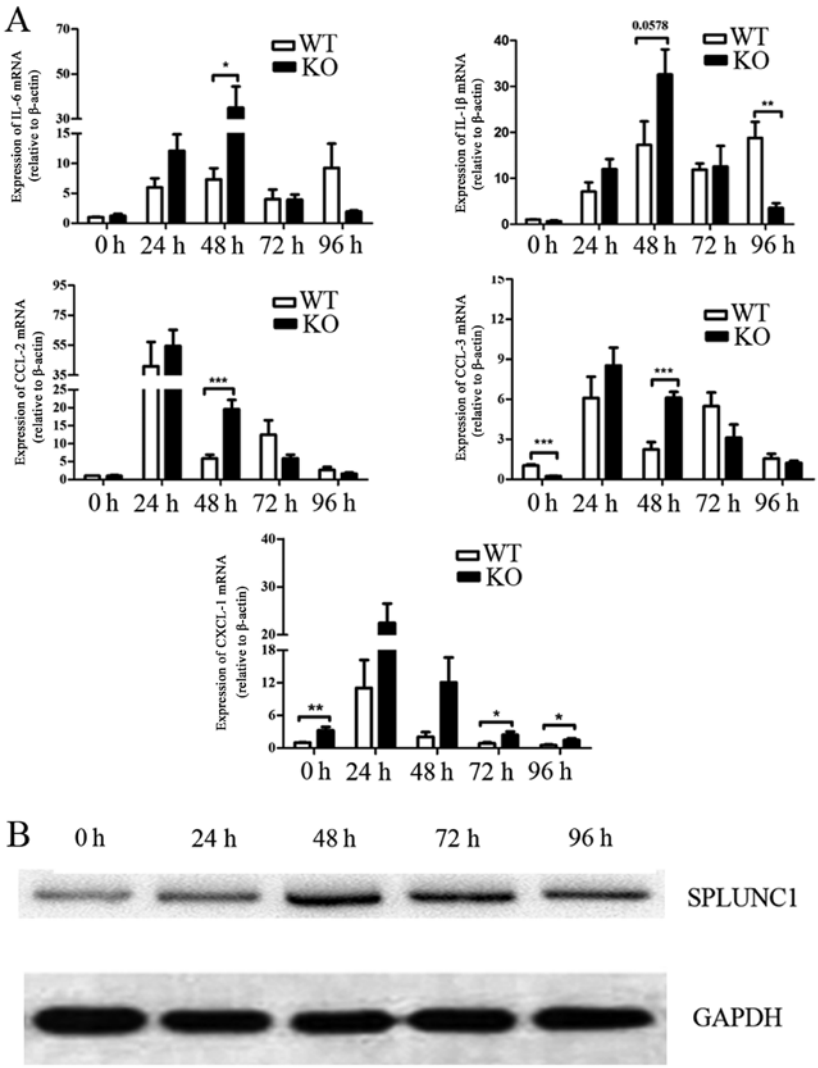

Figure 5. Knockout of SPLUNC1 promotes lung inflammation in response to LPS induction. (A) IL-6, CCL-2, CCL-3 mRNA expression in the KO group were higher than these levels in the WT group after LPS injection for 48 h, while the level of CXCL-1 mRNA expression in the KO group was higher than that level in the WT groups after LPS injection for 72 and 96 h. (B) Western blot analysis of SPLUNC1 expression in the lung tissues of WT mice at different time points after LPS treatment. All results are means ± SEM (n=5 mice/group); *P<0.05, **P<0.01, ***P<0.001 compared with the WT group at each time point.

there were no differences in mouse bone marrow between WT and KO groups after LPS injection, the percentage of splenic CD11b^{+}Gr-1^{+} MDSCs in the KO mice was significant higher than that in the WT mice at the time-points of 72 and 96 h post LPS injection. Many factors can induce MDSC activation and expansion such as macrophage colony-stimulating factor (M-CSF), IL-6 (42), vascular endothelial growth factor (VEGF) (43), prostaglandins (44) and granulocyte/macrophage colony-stimulating factor (GM-CSF) (45). It has been reported that the molecules which are produced in lung injury can be linked to the bone marrow and show significant associations with the expansion and activation of MDSCs (46,47). These results indicate that SPLUNC1 can influence the recruitment of MDSCs by upregulating the secretion of cytokines in impaired lung tissue.

In summary, based on SPLUNC1-knockout mice we established the LPS-induced ALI mouse model to study the anti-inflammatory effect of SPLUNC1. Our results found that SPLUNC1 deficiency upregulated levels of ALI-related cytokines leading to more severe lung injury after LPS injection. Moreover, we first discovered that the presence of SPLUNC1 can suppress the recruitment of CD11b^{+}Gr-1^{+} MDSCs to the spleen in LPS-induced ALI. Nevertheless, the mechanism of the CD11b^{+}Gr-1^{+} MDSC recruitment needs to be further studied.

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