Time Dependent Innate Immune Responses to Lipopolysaccharide in Acute and Sub-Chronic Lung Injury via NLRP3/Nrf2 Pathway

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Research Article

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Abstract

Acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) are significance clinical syndromes of acute respiratory failure with high rate of mortality. Nucleotide-binding oligomerization domain (NOD)-like receptor containing pyrin domain 3 (NLRP3) and nuclear factor E2-related factor 2 (Nrf2) have been reported to be associated in signaling pathway of ALI. However, its role in lipopolysaccharide (LPS)-induced lung injury model remains controversial. The aim of this study was to explore whether LPS triggered multiple immune cascades at different stage of lung injury, and track the changes of NLRP3/Nrf2 pathway in this process. In this study, the lung tissues were collected from 4h (acute phase) to 144 h (sub-chronic phase) after LPS (4 mg/kg) administrated by intratracheal injection on C57/B6 mice. Obvious histopathological damages with neutrophils infiltration were photographed in LPS-induce lung injury model, and peaked at 48h. After LPS stimulation, the levels of inflammatory mediators such as Interleukin (IL)-1β, CXCL1/KC, macrophage inflammatory protein 2 (MIP-2), tumor necrosis factor (TNF)-a were markedly increased in bronchoalveolar lavage fluid (BALF) and serum at peaked at 4h. Moreover, $CD4^+$ T cells upregulated and peaked at 4h, as well as increased the mRNA level and protein expression of Ror-γt in lung. LPS promoted toll like receptor-4 (TLR-4), oxidative stress, thus activated the NLRP3/Nrf2 signal and pyroptosis. Collectively, this study disclosed that how LPS triggered multiple inflammatory molecules and T cells subpopulation at different time points, which might be closely involved with the irregular redox status, NLRP3/Nrf2 pathways and pyroptotic cell death.

Background

Acute respiratory distress syndrome (ARDS) is categorized as minor disease according to the berlin definition [1] . But they also recognized acute lung injury (ALI) as a major severe respiratory failure in worldwide, which leads to high morbidity and mortality in ill patients due to increased vascular permeability, alveolar-capillary membrane dysfunction, flooding of protein-rich fluid, alveolar hemorrhage, and fibrin deposition [2, 3]. Recent reports had documented that lung injury develops due to accumulation of excessive amount of immune cells including macrophages and neutrophils in the infected area following the exposure to the foreign particles such as lipopolysaceride (LPS), that are key factors in the pathogenesis of ALI/ARDS [4, 5].

LPS is a part of the outer membrane of gram-negative bacteria that binds to a signal-transducing integral membrane protein such as TLR-4, thus leads to secrete pro-inflammatory cytokines via mediating various signaling pathway [6]. Except macrophage, previous studies demonstrated that TLRs are also expressed on T cells. It has been suggested that LPS may directly affect T cells activity [7, 8]. T cells play an important role in immune system. Functioning role of $CD4^+$ T cells, neutrophils and macrophages can significantly influence to secrete pro-inflammatory cytokines such as Interleukin (IL)-1β, CXCL1/KC, macrophage inflammatory protein 2 (MIP-2), tumor necrosis factor-a (TNF-a) [9, 10]. Recent study expanded that activated $CD4^+$ T cells are involved in the ALI disease and regulates cytotoxic T lymphocyte antigen 4 (CTLA4) following the treatment with LPS [11]. Asides from $CD4^+$ T cells, their

transcription factor such as RORγt trigger the clonal expansion of pro-inflammatory Th17 cells [12]. Gathered evidence suggested that the activation of Th17 immune responses augments the inflammatory response in various inflammation and autoimmune disorders [13]. The interplay between T cells and lung injury mediated dysfunction has been poorly established. Therefore, more in-depth, systemic and time dependent studies on the $CD4^+$ T cells population as well as the involved molecular mechanisms are still needed to be elucidated.

Nuclear factor E2-related factor 2 (Nrf2) and the nucleotide-binding oligomerization domain (NOD)-like receptor containing pyrin domain 3 (NLRP3) inflammasome are both regulated in inflammatory conditions due to generation of reactive oxygen species (ROS) [14]. Previous studies had showed that Nrf2 plays negative role in acute and chronic inflammatory diseases [15, 16]. During cellular stress conditions, Nrf2 is released from Keap1 (Kelch-like ECH-associated protein 1) by proteasomal pathway and the accumulated Nrf2 is translocated into the nucleus to initiate gene transcription of HO-1 (heme oxygenase 1), NQO1 (NAD(P)H dehydrogenase [quinone] 1) etc to show anti-inflammatory role [17]. Inflammasome is a multi-complex of protein that is constructed by inactive NLRP3, the adaptor protein ASC (apoptosis-associated speck-like protein containing a CARD), and caspase-1. Later, activated caspase-1 leads to mature IL-1b and IL-18 from premature condition. Importantly, these are the considerable markers for pyroptosis [18, 19]. It is also documented that activation of Nrf2 is essential to express NLRP3 inflammasome by ASC oligomerazation [20]. Several reports suggested that Nrf2 deficiency aid to mitigates NLRP3 expression in acute and chronic inflammatory diseases and these results demonstrate an unexpected pro-inflammatory role of Nrf2 [15, 16]. Thus, pro-inflammatory role of Nrf2 still is a conflicting and undefined issue. To date, the information on the immunomodulatory and pro-inflammatory potential of LPS in NLRP3/Nrf2 pathway has not been extensively investigated *in vivo* models.

In this study, we set up acute and sub chronic ALI models by treating mice with an intratracheally injection of 4 mg/kg LPS. Comprehensively, the present study showed the changes of the potential inflammatory molecules and immune-related pathways in the lung injury model at different time points. Moreover, we have determined the inflammatory cellular profiles in BALF, blood serum, and $CD4^+$ T cells, macrophages, neutrophils differentiation in lung tissue, and redox status, along with expressions of Nrf2/NLRP3 signaling pathways and pyroptosis in this lung injury model.

Methods

Mouse model of intratracheal injection of LPS and experimental design

In the present study thirty male C57/B6 mice, 6–7 weeks old and weighing 20–25 g were purchased from SLAC Laboratory Animal (Certificate No: SCXK2017-0016, Shanghai, China). All the animal experimental protocols were reviewed and approved by the Animal Care and Ethics Committee of Zhejiang University. All efforts were made to minimize their suffering. Mice were kept on standard day/night (12 h light/12 h darkness cycle, 45–55% relative humidity and temperature 23–25 ºC) and pathogen free conditions. The

experimental timeline is shown in Fig. 1. 30 male mice were randomly divided into 6 groups, each group set as 6 time points after LPS administration (0, 4, 24, 48, 96 and 144 h). All mice were anesthetized with 4% chloral hydrate (Sigma-Aldrich, USA) via intraperitoneally (i.p.). Later, LPS (4 mg/kg body weight, Escherichia coli, 0111:B4, Sigma Aldrich, St. Louis, United States) was injected intratracheally into the mice using a microsyringe. After administration of LPS, blood serum, bronchoalveolar lavage fluid (BALF), lung tissues were collected at different time points.

Histopathology Examination

For histological analysis, lung tissue were fixed with 10 % neutral formalin for 48 -72 h at room temperature, embedded in paraffin, and sectioned to 4 μm thicknesses. After deparaffinization and rehydration, the sections were stained with hematoxylin and eosin solution (Jiancheng, Nanjin, China), and then observed by light microscopy (Olympus BX51, Tokyo, Japan). The histopathology result was then determined according to the degree of lung inflammation.

Determination of cytokine in BALF and serum by enzyme-linked immunosorbent assay (ELISA)

To evaluate the production of inflammatory cytokines, BALF and serum were collected and centrifuged at 1000 × g for 8 min at 4°C for biochemical analysis such as interleukin (IL)-1β (DY401), CXCL1/KC (DY453), MIP-2 (DY452), TNF-a (DY410) (R&D system, Minneapolis city, USA) by using ELISA kit following the manufacturer's protocol. The levels of IL-1β, CXCL2-KC, MIP-2 and TNF-a were finally expressed as pg/ml.

Flow cytometry analysis

Single-cell suspensions were prepared from left lung of the mice by cutting into small fragments and digested for 45 min at 37 °C with type I collagenase (3 mg/ml, sigma, USA)and DNase I (30 μg/ml) in RPMI 1640 medium(10040, Corning, USA). Digested lungs were mechanically disrupted by passage through a sterile strainer (100μm, Falcon, BD Biosciences) using the flat portion of a plunger from a 3-ml syringe followed by an additional 40μm strainer (Falcon, BD Biosciences). Red blood cells were lysed with 150mM NH_4Cl, 10mM $KHCO_3$ and 0.1mM EDTA. After washed with phosphate buffered solution (PBS) for two times, cells were stained with Zombie Aqua™ Fixable Viability Kit (Biolegend, San Diego, CA, 423101) for 15-30 min in the dark at room temperature (RT) and washed one time with cell staining buffer. After isolated live cells, we used BUV395 Rat Anti-Mouse CD45 (564279), PerCP-Cy™5.5 Hamster Anti-Mouse CD3e (551163), FITC Rat Anti-Mouse CD4 (557307), APC-Cy™7 Rat Anti-Mouse CD8a (557654), PE-Cy™7 Rat Anti-Mouse Ly-6G (560601), PE Rat Anti-Mouse F4/80 (565410) and BV650 Rat Anti-CD11b (563402) antibodies incubated at 4 °C for 30 min, respectively. All antibodies were purchased from BD Biosciences (New Jersey, USA). The samples were evaluated by CytoFLEX LX flow cytometry analyzer (Beckman Coulter, Inc. 250S. Kraemer Boulevard Brea, CA 92821, USA). The results were analyzed by CytExpert Version 2.4 software (Beckman Coulter, Inc. 250S. Kraemer Boulevard Brea, CA 92821, USA).

Total RNA isolation and real-time PCR analysis

RNA from mouse lung tissue was isolated using RNAiso plus (9109, takara Bio INC, Kusatsu, Shiga, Japan) and reverse transcribed. Quantitative PCR was performed on the Bio-Rad C1000 real-time PCR system using SYBR green Master Mix reagent (Bio-Rad). PCR amplification reactions were: 95 °C for 30 s, and subjected to 40 cycles of 95 °C for 3 s and 62 °C for 30 s. Primers were designed by using primer bank website. The data were evaluated using the $2^{-\Delta\Delta Ct}$ formula for relative quantitation and normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The sequences of the primers for mouse gene expression are listed below (forward and reverse):

Table 1 The sequence of primers

Primers name	Forword primer	Reverse primer
TLR-4	CGCTTTCACCTCTGCCTTCACTACAG	ACACTACCACAATAACCTTCCGGCTC
RorgT	TCCAGACAGCCACTGCATTC	GTGCGCTGCCGTAGAAGGT
GAPDH	GGCTGTTGTCATACTTCTCATGG	GGAGCGAGATCCCTCCAAAAT

Western Blot Analysis

The total proteins were extracted from lung tissue by cold RIPA (Radio Immunoprecipitation Assay) lysis buffer (including 1 % protease inhibitor cocktail (Roche), 2 % PMSF (Sigma) and 1x PhosSTOP (Roche) and centrifuged at 10000 × g at 4 °C for 10 minutes. Protein concentration was evaluated by the Bio-rad reagent (Bio-Rad Inc., California, USA). Later, 5× loading buffer (Beyotime Inc., Nantong, China) was added to protein sample and then denatured at 100 °C for 5 min. 12 % sodium dodecyl sulfate-polyacrylamide gel Electrophoresis (SDS-PAGE) was used to separate the proteins. Equivalent loading of the gel was determined by quantitation of protein as well as by re-probing the membranes for GAPDH detection. Separated proteins were electroblotted onto PVDF (Polyvinylidene difluoride) membrane and blocked for 1 h at room temperature with tris-buffered saline (TBS) containing 5% BSA. The membranes were then probed with primary antibodies: TLR-4 (1:1000) (ab13867 Abcam, Massachusetts, USA) ; Anti-NLRP3 (1:1000) (AG-20B-0014, Adipogen Life Sciences, San diego, USA); antiprocaspase-1/cleaved caspase-1 (1:1000) (ab179515, Abcam, Massachusetts, USA) ; pNrf2(1:1000) (db523, Digbio, Hangzhou, China) ; Anti-Nrf2 antibody (1:1000) (ab137550, Abcam, Massachusetts, USA); Anti-Keap1 antibody ((1:1000) (ab119403, Abcam, Massachusetts, USA); Anti-GSDMD antibody (1:1000)(ab155233, Abcam, Massachusetts, USA); cleaved GSDMD (1:1000)(Asp 276, Cell Signaling Technology, Danvers, Massachusetts, USA) ; ROR□t (1:1000) (ab207082, Abcam, Massachusetts, USA); anti-caspase-8 (1:1000) (ab227430, Abcam, Massachusetts, USA); Anti-Asc (1:1000) (sc-514559, Santa cruz biotechnology, Dallas, TX, USA) and anti-GAPDH (1:5000) (db106, Digbio, Hanzhou, China) at room temperature for overnight. After washing, incubated with HRP conjugated secondary antibodies (1:5000) (IRDye 800CW goat anti-rabbit; IRDye 680CW goat anti-mouse (LI-COR Biosciences, Cambridge, UK) for 1 h 30 min. Membranes were washed by 1X TBST from three times, 5 min for each times. The images were captured

using the oddessy clx infrared laser dual colors imagine analysis system (LI153 COR, Inc., Lincoln, Nebraska, USA). The density of each protein band on the membrane is reported as the densitometric ratio between the protein of interest and GAPDH.

Intracellular ROS measurement

ROS generation from lung tissues were estimated by a method described Socci DJ et al., with slight modifications [21]. Homogenate of tissue samples were prepared with ice-cold 40 mM Tris–HCl buffer (pH 7.4), 100 µl of samples solution were mixed with tris-HCl buffer and 5 µM of dichlorodihydrofluorescein diacetate (DCFH-DA Sigma Aldrich, St. Louis, United States),). Later, mixture was incubated for 15 min in 37 °C. ROS scavenger N158 acetyl-l-cysteine (NAC) (3 mM, HY-B0215, MedChemExpress, Shanghai, China) was used as a negative control and hydrogen peroxide H_2O_2 (100 µM, E004-1-1, Reactive oxygen species Assay Kit, Jiancheng Biotechnology, Nanjing, China) was used as positive control. 100 µM of DCFH-DA was added for 30 min. DCF fluorescence intensities of the samples were detected by Varioskan Flash microplate reader (Thermo Scientific, Vantaa, Finland) at excitation and emission wavelengths of 485 and 535 nm, respectively.

Results

LPSinducestime-dependent histopathological injuries in lung

Infiltration of neutrophils in lung tissues is one the most important pathological characteristics of LPS induce ALI. As shown in Fig. 2, LPS challenge mice had injury in the lung tissues at 4, 24, 48, 96 and 144 h as compared with 0 h and the highest lung injury was recorded from 24 h to 96 h and then trended to reduce at 144 h. Additionally, LPS treatment also resulted in the highest number of neutrophils infiltrations after 4 h. From 4 h to 96 h, following the administration of LPS, the features of lung injury, described above, became more evident. On noted, there is significant recovery of histological injury was observed at 144 h.

LPS up-regulates pro-inflammatory cytokines in serum and BALF

Accumulated evidenced suggested that macrophages, neutrophils and T cells are classically considered to be the major sources of IL-1β, CXCL1/KC, MIP-2, TNF-a . To study the effect of LPS on pro-inflammatory cytokines expression, blood serum and BALF were collected from C57BL/6 mice at 0, 4, 24, 48, 96, 144 h after intratracheal injection of LPS. As shown in Fig. 3a to 3h the secretion of IL-1β, CXCL1/KC, MIP-2, TNF-a were increased and reached to peak at 4 h and 24 h (3 to 5 folds) and then trended to decrease during the period of 96 h to144 h after induction of LPS. The results demonstrated that LPS-induced up-regulation of pro-inflammatory cytokines, indicating the role of LPS in inducing pro-inflammatory mediators IL-1β, CXCL1/KC, MIP-2, TNF-a secretion in C57BL/6 mice. Noted, upon normal conditions (0 h time point), above mentioned cytokines are not expressed constitutively. They are produced after exposure of macrophages and neutrophils to LPS.

LPS activates immune cells in lung

$CD4^+$ T cells are important mediators of inflammatory diseases. Previous study also suggested that CD4 ($CD4^+$) T cells influences in the regulation of TNF-a secretion by alveolar macrophages [22]. Besides, function of T helper cell-associated cytokines can polarize, recruit and activate macrophages during the inflammatory periods [9]. Our result showed that after the treatment of LPS, $CD4^+$ T cells were raised by 19.97%, 6.68%, 13.91%, 19.46% and 11.52%, respectively at 4 h, 24 h, 48 h and 96 h and 144 h, whereas 4 h to 96 h showed the significant difference. The percentage of macrophages and neutrophils at 0 h were 24.89 % and 4.57 %, respectively, and LPS treatment caused to activate macrophages and neutrophils by 34.97 % and 58.02 % at 4 h, 19.76 % and 73.7 % at 24 h, 23.97 % and 42.36 % at 48 h, 19.29 % and 51.6 % at 96 h and 29.27 % and 0.26 % at 144 h (Fig. 4). Accumulated results have confirmed activation of $CD4^+$ T cells, macrophages and neutrophils may contribute to the acute lung injury via increasing pro-inflammatory cytokines response following LPS stimulation.

LPS stimulatesretineic-acid-receptor-related orphan nuclear receptor gamma (RORt) expression in lung

It is reported that RORγT is a critical transcription factors for Th17 cells expansion and contributes to show the inflammatory role [23]. The expression of RORγT was enhanced by LPS in protein (Fig. 5a) and mRNA levels (Fig. 5c). As shown in Fig.5b, RORγT expression was significantly increased in LPS-4 h to 144 h groups and overexpression of RORγT reached at 24 h and 96 h after LPS administration. Subsequently, the up-regulation of RORγT gradually decreased over 144 h. These data suggest that LPS influence RORγT at transcription and translation level, and these phenomena is evidenced in a time dependent manner.

LPS induces time-dependent NLRP3 inflammasome activation in lung

Next, we determined the changes in NLRP3 and caspase-1 activation in different time points in lung tissue after LPS treatment. The expression of NLRP3, ASC and cleaved caspase-1 product p10/12 fragment markedly increased in a time dependent manner from 4 h to 96 h after administration of LPS, but the expression of NLRP3 and cleaved caspase-1 were high at 24 h and 48 h after intratracheal injection of LPS (Fig. 6). These data suggested that a time-dependent change of inflammasome activity occurred after the LPS administration.

LPS induces time-dependent oxidative stress in C57BL/6 mice

We next examined the production ROS as an indicator of oxidative stress using a H_2DCFDA fluorescence probe. As shown in Fig. 7 the level of intracellular ROS production in lung tissue was significantly increased after LPS treatment at 4 h and 96 h whereas the highest level was recorded at 48 h and showed the decreasing trend at 144 h. These data suggested that ROS took a crucial part to hinder the normal architectures in a time dependent manner after LPS induction.

LPS activates Nrf2 pathway and TLR-4 receptor in lung

Nrf2 serves as an important mediator to regulate ROS-dependent inflammasome activation in ALI model. To observe Nrf2 pathway activation, we first assayed for expression of the pNrf2, tNrf2, keap-1 and TLR-4 by Western blotting (Fig. 8a). LPS induced TLR-4 and Keap-1 expression in a time-dependent manner, with marked induction at 24 h, 48 h and 96 h (Fig.8b and 8d). Similarly, the mRNA expression of TLR-4 was noted in the same time point (Fig. 8c) following the induction of LPS. However, LPS caused the phosphorylation of Nrf2 in accordance with the Keap-1, suggests the Nrf2 upregulation was independent of Keap-1. In Figure 8e, the Nrf2 level was significantly increased in LPS-4 h, LPS-24 h and LPS-48 h groups. Taken together, these results indicate that TLR signaling can indeed activate the Nrf2 pathway.

LPS induces the pyroptosis in lung

In pyroptosis, IL-1β secretion and caspase-1 activation are few of the imminent characteristics. IL-1β secretion (Figure 3a and 3e), as well as caspase-1 (Fig.6c), were activated in a time dependent manner after LPS-treatment. It is reported that during the inhibition of TGF b-Activated Kinase 1(TAK1) , caspase-8 promotes the cleavage of both gasdermin D (GSDMD) and gasdermin E (GSDME) in murine macrophages, thus leads to pyroptotic cell death [24]. In this study we evaluated caspase -8; pyroptosis markers gesdermin D and cleaved GSDMD expression by western blotting following LPS treatment for 0, 4, 24, 48, 96, 144 h (Fig.9a). Western blot assay results demonstrated that LPS upregulated the protein levels of caspase-8 and cleaved GSDMD in a time-dependent manner (Fig. 9b and 9c). Although, this study found that cleaved caspase-8 and cleaved GSDMD peaked at 48 h and exhibited significant difference at 4h to 96 h, when compared to 0 h. These results indicate that LPS plays an important role in pyroptotic cells death by upregulating the level of cleaved caspase-8 and cleaved GSDMD.

Discussion

This study demonstrated a time dependent process in LPS-stimulated innate immunity. After LPS intratracheal administration, mice were sacrificed at 0, 4, 24, 48, 96, and 144 h. Here, we observed not only macrophages and neutrophils, but $CD4^+$ T cells differentiations were also significantly augmented in time dependent manners. Besides, NLRP3 inflammasome activation, subsequent release of pro-inflammatory cytokines and pyroptotic cell death on lung tissue were evidenced as a probable consequence of Nrf2/NLRP3 signaling.

The differentiation of $CD4^+$ T cells and $CD8^+$ T cells have gain considerable attention to evaluate the immune system [25]. Although many studies report the role of $CD4^+$ T cells in various infectious diseases but still remains as a controversial issue in respiratory syndrome. Hu W et al., demonstrated that arsenic decreases the $CD4^+$ T cells numbers, which could possibly result in arsenic related immune responses and release pro-inflammatory markers such as TNF-a [26]. On the other hand, some studies have reported that LPS-induced TNF-α secretion in macrophages is mitigated when $CD4^+$ T cells are depleted [22, 27]. Thus, it is very urgent to reveal the role of T cells to facilitate neutrophils and macrophages migration at different stage of ALI, perhaps in diminishing inflammation in ALI. It is well-documented that LPS challenge increases the population of $CD4^+$ T lymphocytes in the inflammatory infiltration during acute

lung injury model [28]. Additionally, it is also reported that $CD4^+$ T cells are involved in the phenotypic transformation of macrophages and neutrophils [9]. The regulation of Th1, Th2, Th17 and Treg cells, play important and specific roles in the initiation of immune responses by interacting with other cells and associated in several inflammatory immune-mediated disorders, mostly sub-chronic and chronic inflammatory disorders [29]. Moreover, stimulating these cells can trigger the release of TGF-β , IL-6, IL-1β, MIP-2, and also activate their transcriptional factor transcription factors T-bet, GATA-3, RORgt and Forkhead box protein P3 (FOXP3) [30, 31]. Recent evidence exhibited stimulation of TNF-a triggers the expression of neutrophil-attracting chemokines such as CXCL1, CXCL2 [32]. Our current study dictated that LPS-induces activation of $CD4^+$ T cells from 4h to 144 h time courses, and all pro-inflammatory cytokines are aggravated the lung after LPS administration from 4h (acute phase) to 96 h (subacute phase). In the meantime, the protein expression and mRNA levels of RORgt and TLR-4 strikingly increased compared with 0 h. Moreover, activation of macrophages and neutrophils played a very important role in ALI to release the mature IL-1β by means of histone process through interaction of these two cells [33]. Sustained LPS exposure may exhaust the immunological regulation between the $CD4^+$ T cells subpopulations, macrophages and neutrophils activation in inflamed area that provides a deeper understanding to optimize the timing to study the therapeutic approaches in future experiments with LPS-induced immune-abnormalities in lung injury model.

Growing evidences suggested that inflammasome complex is activated by various danger signals, including those from both endogenous and exogenous sources [34]. Oxidative stress, a condition resulting from the failure of antioxidant systems, has been well known to play important roles to produce ROS and inflammasome activation [35]. Nrf2 is a pivotal transcription factor that regulates intracellular redox balance through activation of antioxidant genes [36]. Therefore, we primarily assumed that Nrf2 deletion would promote inflammasome formation via regulating a large battery of genes that reduce intracellular redox homeostasis. Surprisingly, our present study showed that LPS-stimulated Nrf2 activation may be involved in NLRP3 expression along with cleaved caspase-1 activation via aggravation the level of ROS at time dependent manner. Therefore, NLRP3/Nrf2 pathway might be associated in LPS-induced lung tissue of both acute and sub-chronic conditions. Recently, it has been also reported that Nrf2 activation may contribute to the ASC speck formation [20]. It is thus conceivable that Nrf2-signaling could promote IL-1b responses by sensing loss of organelle integrity similar to that shown for the NLRP3 inflammasome [37, 38]. But the potential implications of Nrf2 pathway in the crosstalk regulation of imbalanced immune responses are still under elucidation.

Besides being induced via death receptor ligation, caspase-8-mediated apoptosis can be engaged by inflammasome components. Previously, protease regulatory subunit 8 homolog (SUG1 protein)-mediated signaling results in enhanced ubiquitination and mediates Fas associated via death domain (FADD) dependent caspase-8 expression by NLRC4 [39]. Additionally, presence of caspase-1 and -11 inflammasomes can involve in pyroptotic cell death in macrophages [40] and absence of cas-1 promote to apoptotic cell death [41]. It is noteworthy that LPS reportedly induces NLRP3 inflammasome and activates pyroptosis [42]. However, macrophages and neutrophils are regarded as important participators

in this process [43]. In addition, neutrophil extracellular traps (NETs) may play a contributory role to ALI by promoting pyroptosis of alveolar macrophages and systemic inflammation [44]. Herein, we found that LPS-triggered caspase-8 activation was markedly enhanced in the presence of caspase-1 expression at the similar time points from 4 h to 96 h. This suggests that time dependent enhancement of caspase-8 has sensitive reaction(s) to the pyroptotic cascade. Additionally, during LPS-induced ALI, the time courses of pyroptotic marker cleaved GSDMD-D expression were similar to the cleaved caspase-8 expression in ALI model. Thus, it confirmed that caspase-8 participate in the process of pyroptosis-associated lung injury from 4 h to 96 h.

Conclusion

In conclusion, our present study demonstrated that both acute and sub-chronic intratracheally administration of LPS enhances multiple inflammatory responses with raise the percentage of macrophages and neutrophils along with $CD4^+$ T cells from 4 h to 96 h. Accumulated results have suggested that aberrantly function of $CD4^+$ cells can exert profound effects on neutrophils and macrophage activation and associate to intervene of lung injury through regulation of pro-inflammatory cytokines at acute and sub-chronic stage. Moreover, disturbance of redox balance, and Nrf2/NLRP3 signaling pathway might also be involved in LPS-induced immune responses via activating pyroptosis. The predominant mechanism of LPS induces acute lung injury were summarized in Fig.10. Therefore, epigenetic program of $CD4^+$ T cells and Nrf2/NLRP3 signaling pathway may provide a novel therapeutic option for treatment of acute and sub-chronic lung disease.

Abbreviations

ALI: Acute lung injury; NLRP3: Nucleotide-binding oligomerization domain (NOD)-like receptor containing pyrin domain 3; Nrf2: nuclear factor E2-related factor 2; IL: Interleukin; MIP 2: macrophage inflammatory protein 2; TLR-4: Toll like receptor-4; BALF: roncho alveolar lavage fluid; Keap-1: Kelch-like ECH-associated protein 1; CTLA4: cytotoxic T lymphocyte antigen 4; HO-1: heme oxygenase 1; NQO-1: NAD(P)H dehydrogenase [quinone] 1; RORgt: Retineic-acid-receptor-related orphan nuclear receptor gamma; GSDMD: Gasdermin D.

Declarations

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Availability of data and materials

The datasets analyzed during the current study are available from the corresponding author on reasonable request.

Author Contributions

DR, ZLJ, LN participated in most experiments, interpreted the results and wrote the manuscript. LYJ and RMN contributed the preparation of animal model and collection of tissue specimens and interpreted many experiments. HZQ, CXW, WXF, ZXY contributed the tissue harvesting. XXL and THF conceived all the experiments and revised the manuscript.

Ethics approval and consent to participate

The study was approved by the Ethics Committee of the Zhejiang university, and the experiments were performed in accordance with the National Institutes of Health Guidelines for the Use of Laboratory Animals.

Consent for publication

All authors read and approved the final version of the manuscript.

Competing interests

The authors declare they have no competing interests.

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Figures

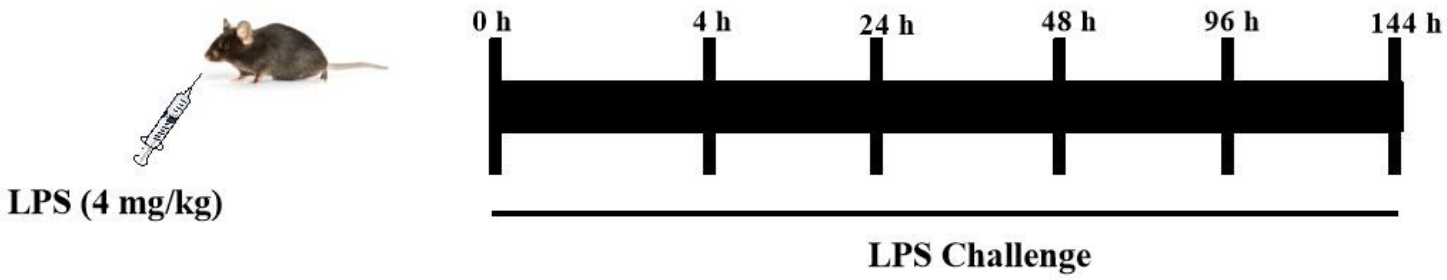

Figure 1

Schematic representation of experimental design for the LPS-induced lung injury model. LPS was injected by intratracheally at a dose of 4 mg/kg. After 0 h, 4 h, 24 h, 48 h, 96 h, 144 h of LPS injection, all mice were sacrificed for the biological experiments.

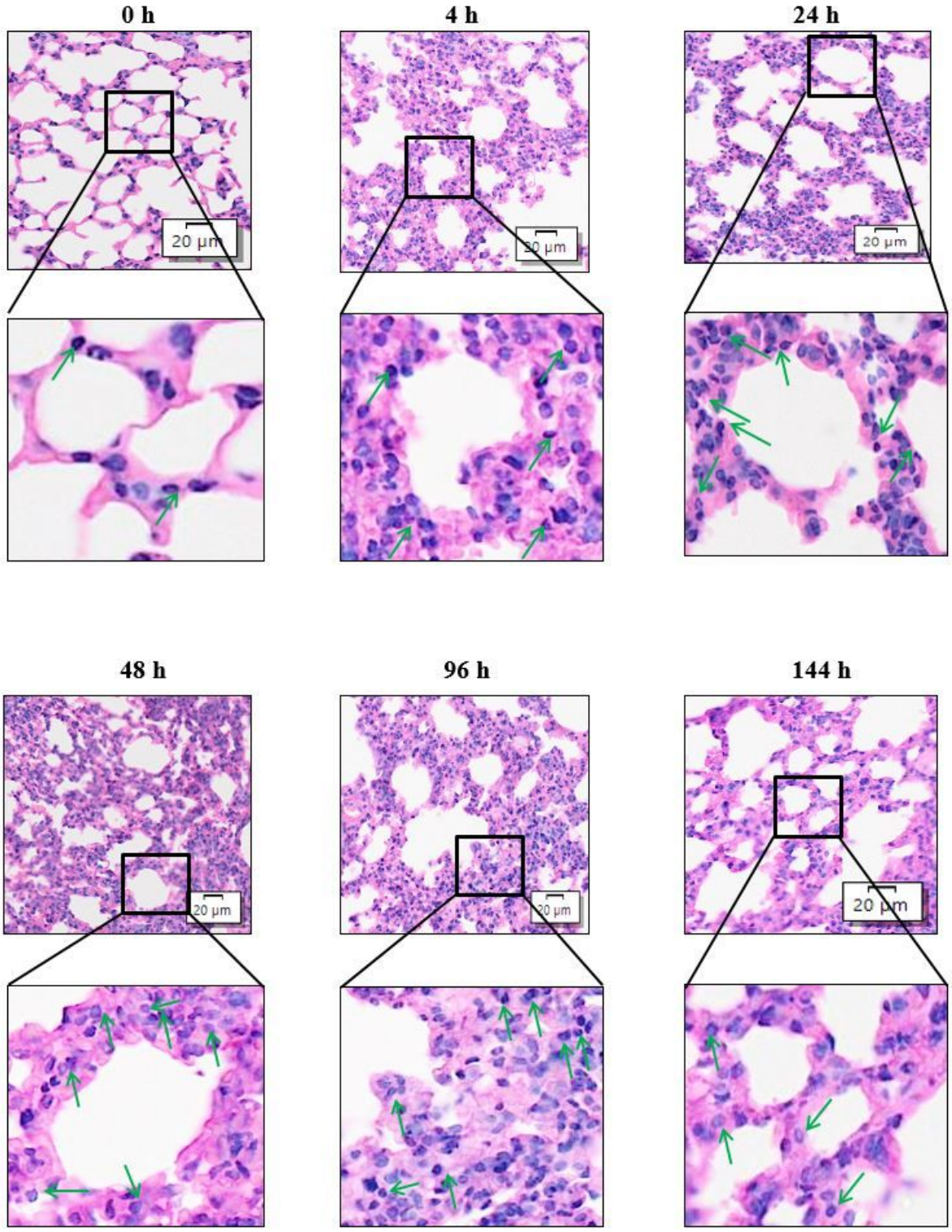

Figure 2

Time dependent effects of LPS on mouse lung. C57BL/6 mice were treated with 4 mg/kg LPS intratracheally for 0 h, 4 h, 24 h, 48 h, 96 h, 144 h. Histological structures right lung lobes were fixed stained with H&E staining for LPS groups at different time points after LPS exposure (original magnification: x20). The results showed accumulation of pulmonary neutrophils in LPS induces lung tissue.

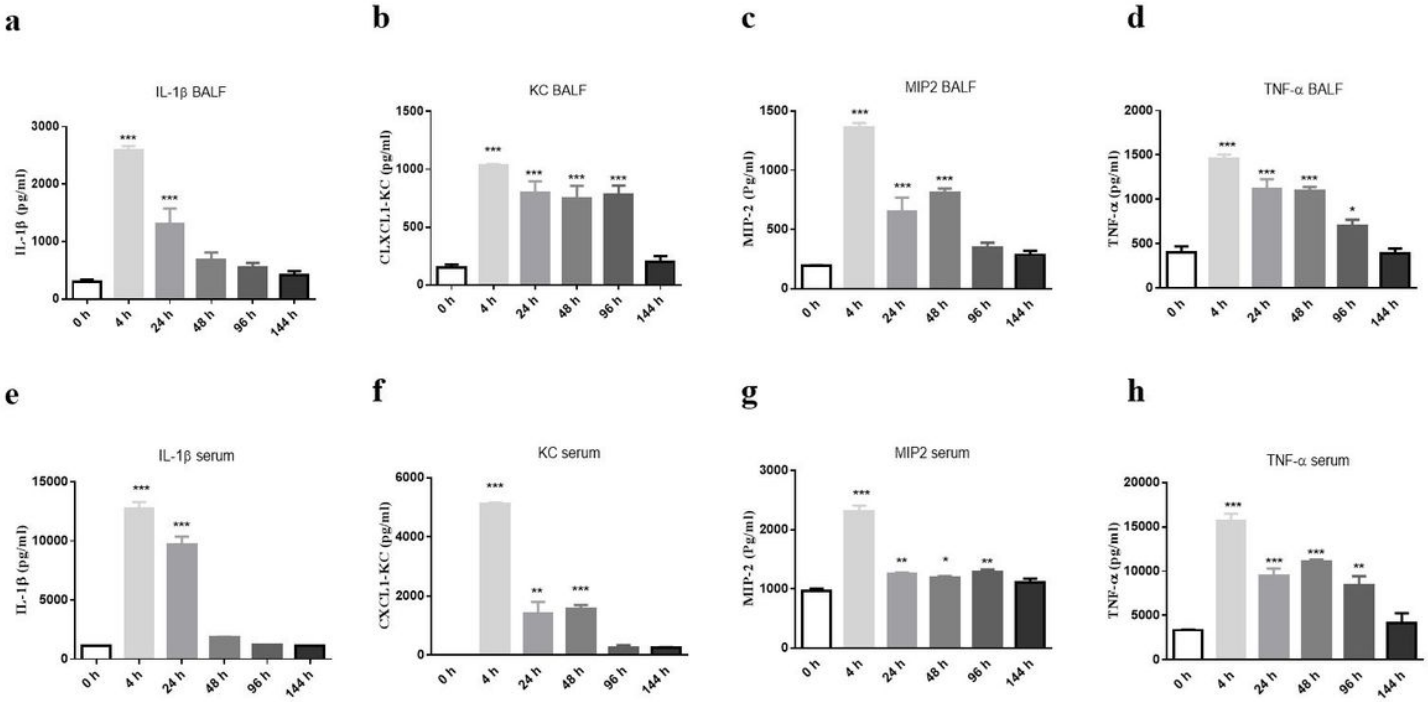

Figure 3

Up-regulation of proinflammatory cytokines in both BALF and serum of LPS-exposed mice. C57BL/6 mice were treated with 4 mg/kg LPS intratracheally for 0 h, 4 h, 24 h, 48 h, 96 h, 144 h. The cytokines levels in BALF (a-d) and serum (e-h) were measured by ELISA and shown as IL-1β (a and e) CXCL1/KC (b and f) MIP-2 (c and g) TNF-α(d and h) accordingly. Results were expressed as mean ± SEM, n = 4. *p < .05, **p < .01, ***p < .001 versus 0 h.

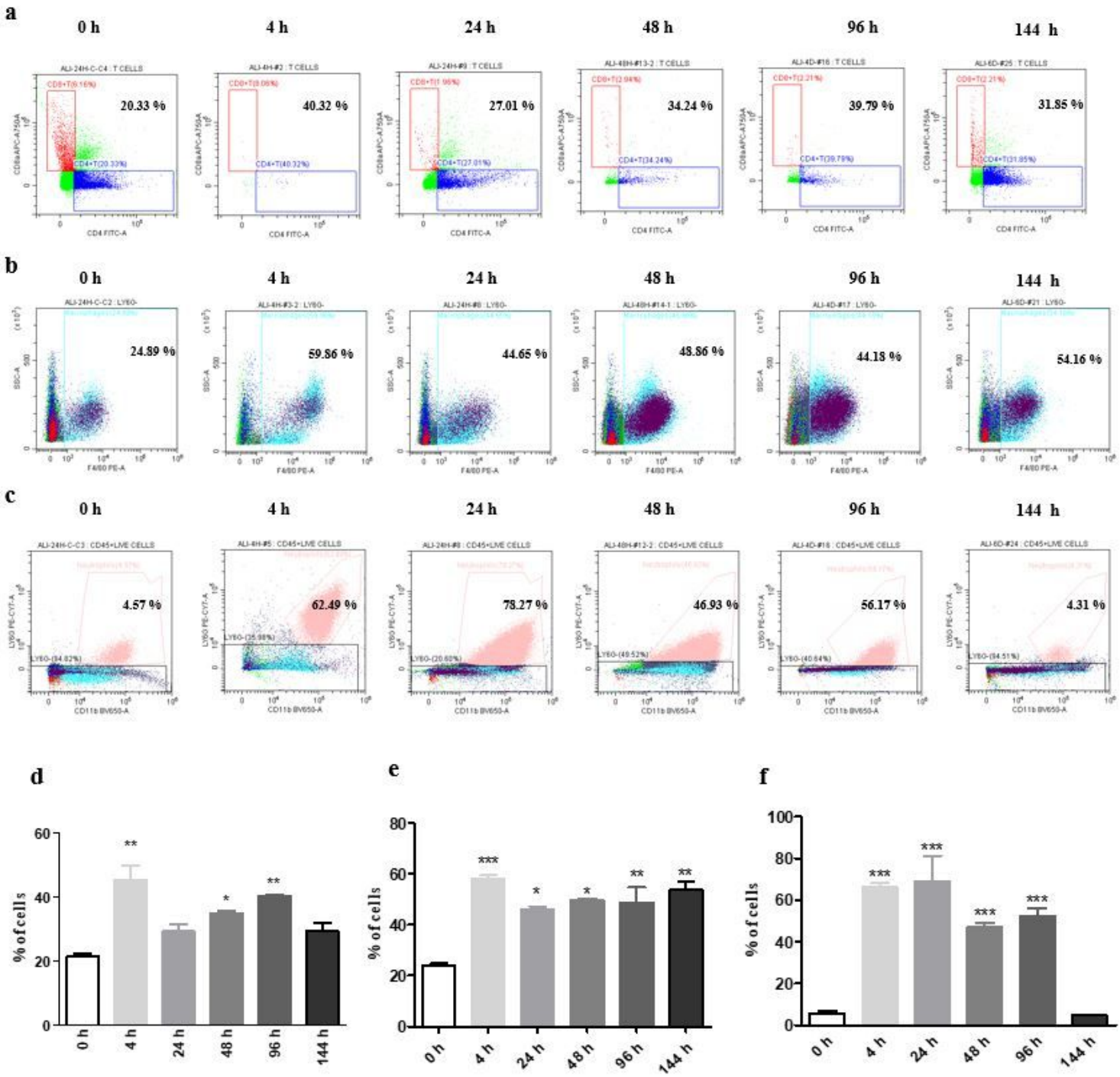

Figure 4

Effects of LPS exposure on the CD4+ T-cell differentiation, as well as the macrophages and neutrophils accumulation in the mouse lung C57BL/6 mice were treated with LPS intratracheally for 0 h, 4 h, 24 h, 48 h, 96 h, 144 (a, b, and c) The percentage of CD4+ T cells, macrophages and neutrophils in lung were calculated by flow cytometry. CD4+ T cells, macrophages and neutrophils were presented in bar diagram (d,e,f). Results were expressed as mean ± SEM (n = 4). *p < .05, **p < .01, ***p < .001 versus 0 h.

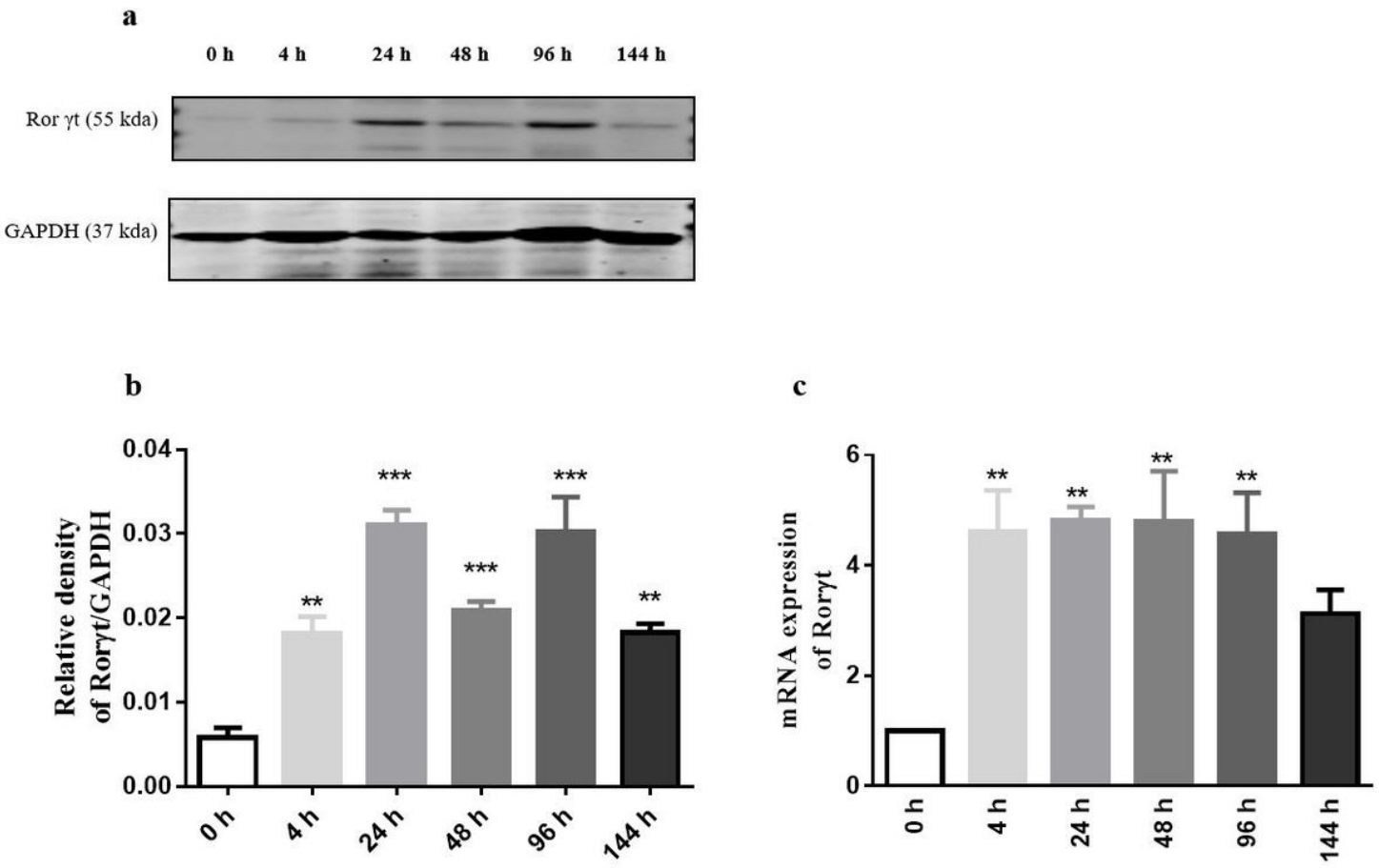

Figure 5

Effects of LPS on the Ror γt protein expression as well as mRNA level in the mouse lung. C57BL/6 mice were treated with LPS intratracheally for 0 h, 4 h, 24 h, 48 h, 96 h, 144 h. The extracted proteins of Ror γt (a) in lung tissue was assessed by western blotting using corresponding antibody (upper panel) and relative expression levels of the proteins were determined by densitometric analysis (lower panel) (b). GAPDH was used as the loading control. The mRNA levels of T-cell lineage-specific master transcription factors (Ror-γt) (c). Results were expressed as mean ± SEM (n = 4). *p < .05, **p < .01, ***p < .001 versus 0 h.

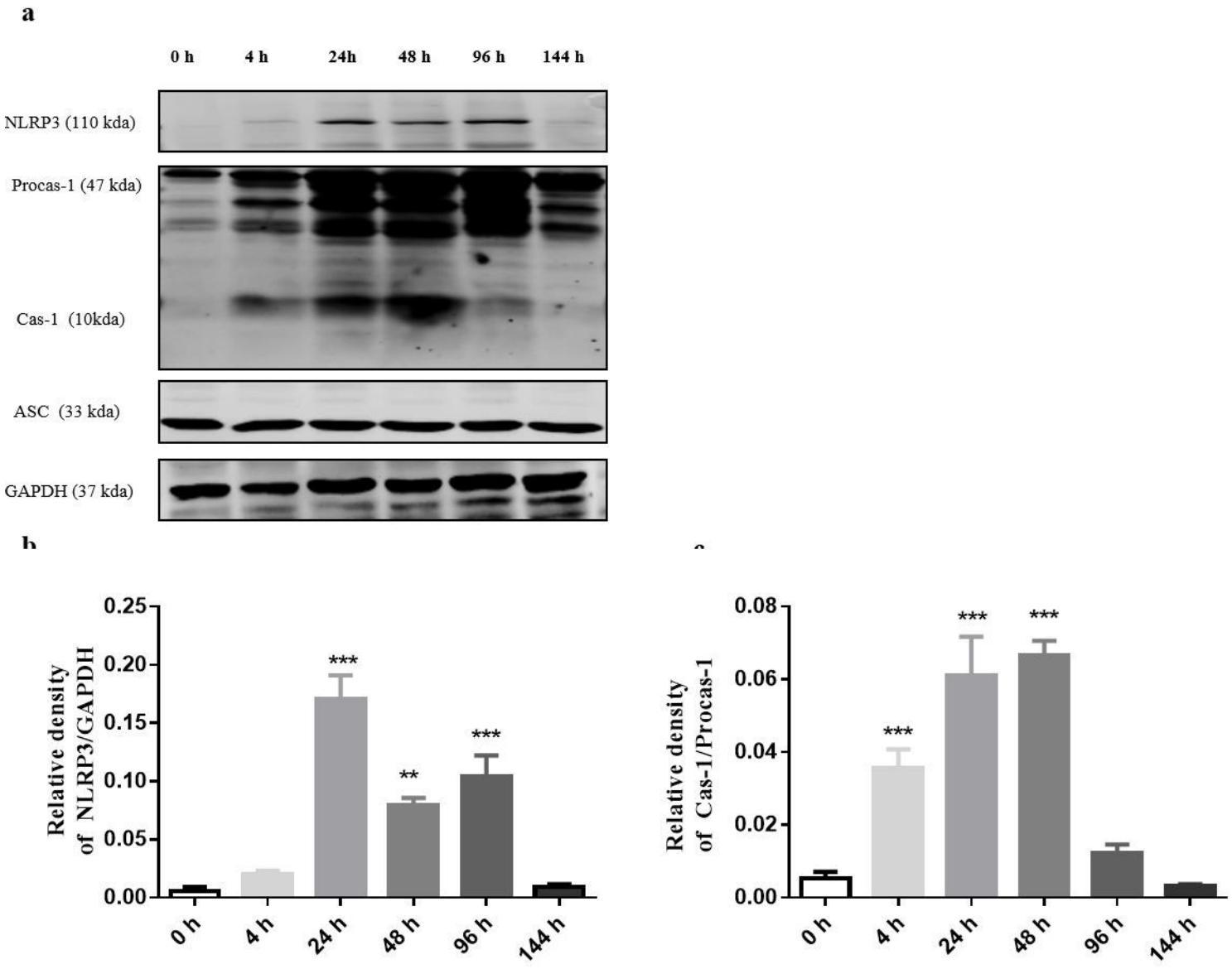

Figure 6

Effects of LPS increases the expression of NLRP3 inflammasome markers in mice lung. C57BL/6 mice were treated with LPS intratracheally for 0 h, 4 h, 24 h, 48 h, 96 h, 144 h. Time-dependent changes in the protein level of NLRP3, ASC and pro-caspase and cleaved caspase-1 in lung tissues from mice injected with LPS (4 mg/kg) at different time points (0 h, 4 h, 24 h, 48 h, 96 h, 144 h). Protein expressions of NLRP3, ASC, procaspase-1/cleaved caspase-1 and GAPDH (internal control) (a) were determined by Western blot analysis using corresponding antibodies (upper panel). Relative expression levels of the proteins were determined by densitometric analysis (lower panel) (b and c). One-way ANOVA was used to analyze significant differences in the protein level of NLRP3, Prcaspase-1 and cleaved caspase-1 at each time points between control verse LPS groups. Data are shown as mean ± SEM, n=4. *p < .05, ** p < .01, ***p < .001 versus 0 h.

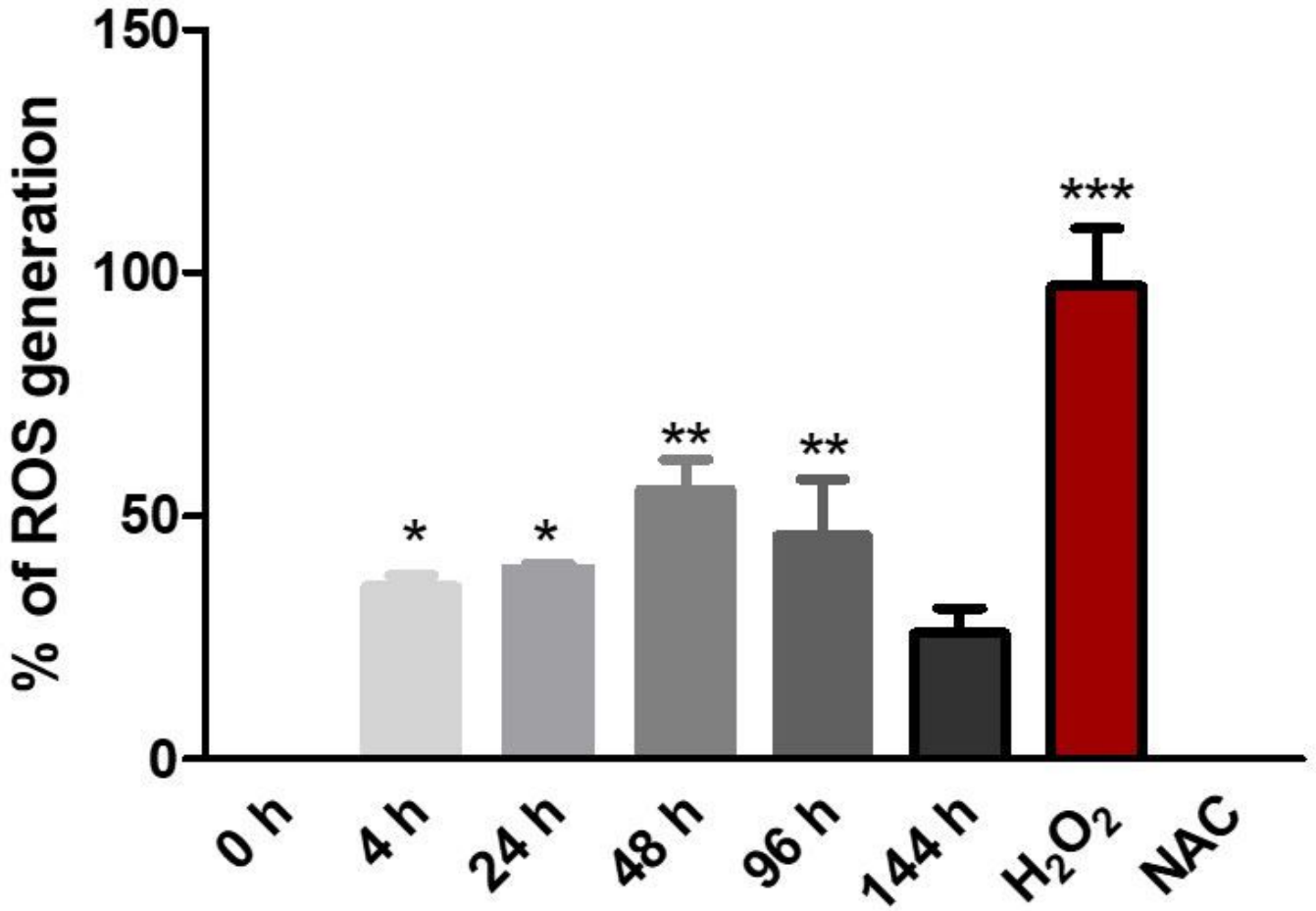

Figure 7

LPS induces ROS Production in lung tissue. C57BL/6 mice were treated with LPS intratracheally for 0 h, 4 h, 24 h, 48 h, 96 h, 144 h. LPS increased ROS production in lung tissue at different time points of LPS group. Data are presented as mean ± S.E.M, n = 4. *p < .05, **p < .01, ***p < .001 versus 0 h.

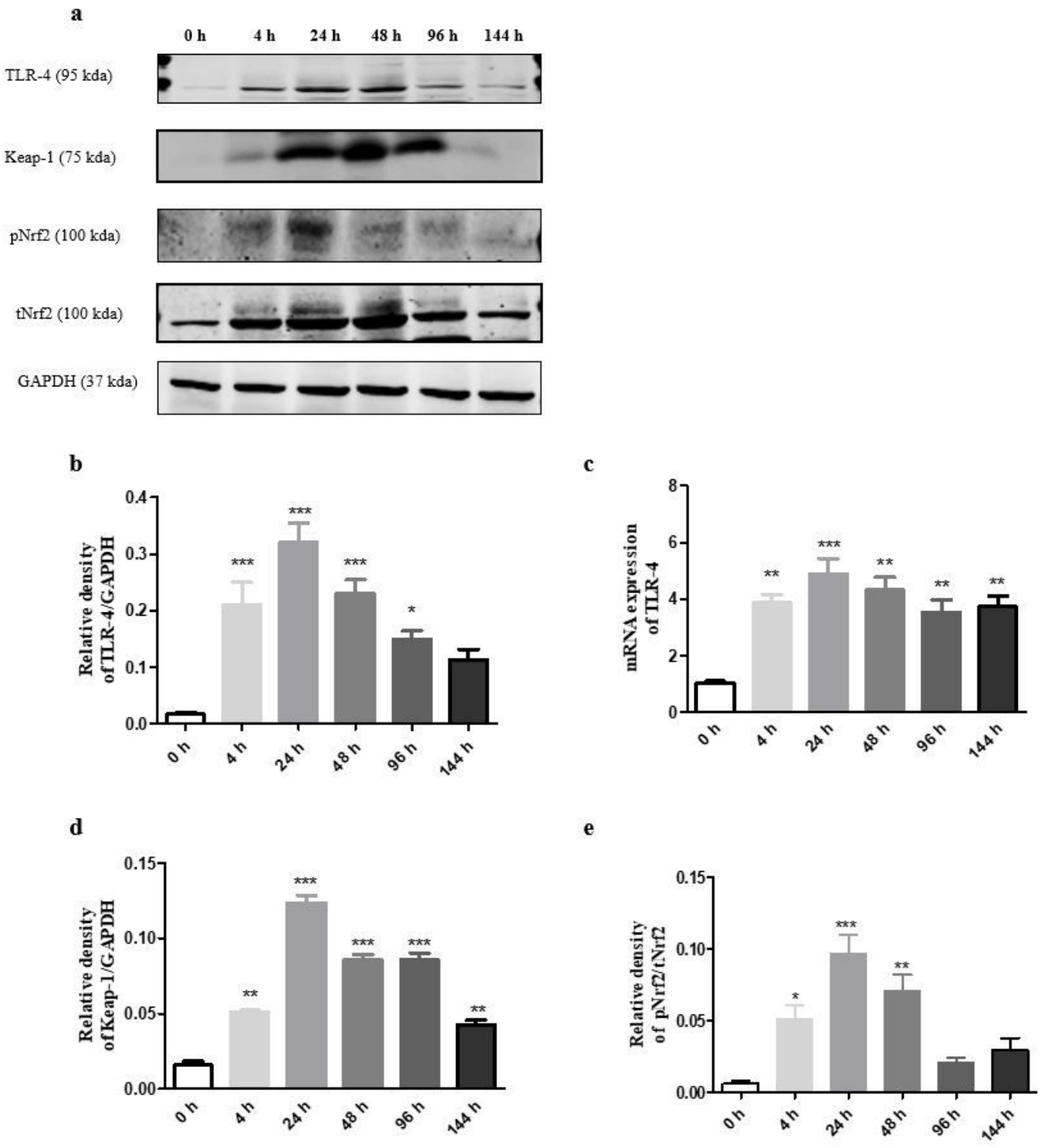

Figure 8

Effects of LPS on TLR-4/Nrf2 pathway in the mouse lung. C57BL/6 mice were treated with LPS intratracheally for 0 h, 4 h, 24 h, 48 h, 96 h, 144 h. Expressions of TLR-4, Keap-1 and pNrf2 and t Nrf2 in the lung of mice were assessed by western blotting and the band densities were quantified. GAPDH was blotted as the loading control. Protein expression of TLR-4, Keap-1, pNrf2, t Nrf2 and GAPDH were determined by Western blot analysis using corresponding antibodies (upper panel) (a). Relative

expression levels of the proteins were determined by densitometric analysis (lower panel) (b, d and E). The mRNA levels of TLR-4 in mice lung were shown as (c). Results were expressed as mean ± SD (n = 4). *p < .05, **p < .01, ***p < .001 versus 0 h group.

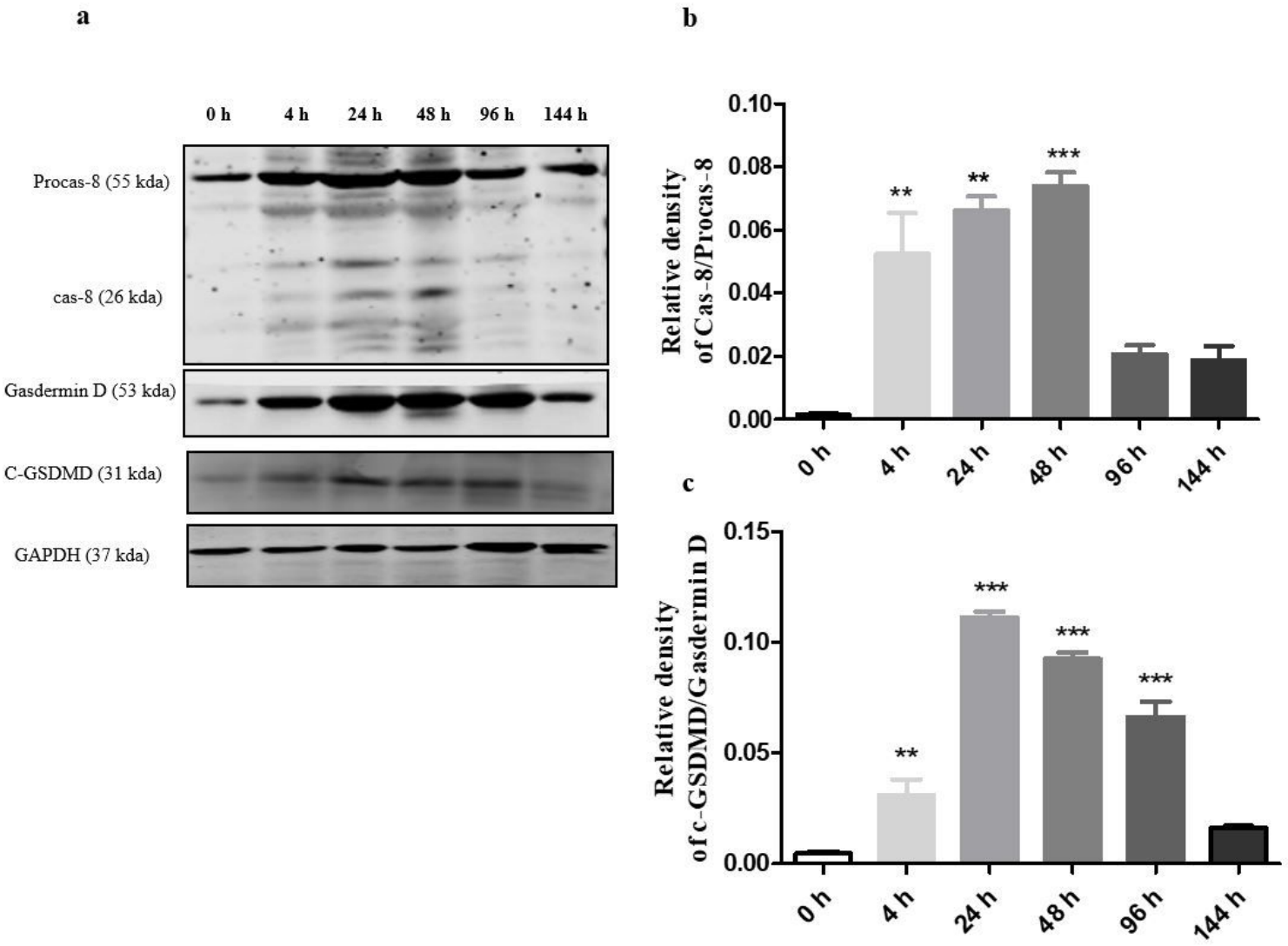

Figure 9

Pyroptosis in LPS-induced lung injury. C57BL/6 mice were treated with LPS intratracheally for 0 h, 4 h, 24 h, 48 h, 96 h, 144 h. The extracted proteins of lung tissue were subjected to western blot. Expression of procaspase-8, cleaved caspase-8, gesdermin D and cleaved GSDMD (left panel) in the lung tissue were assessed by western blotting (a) and quantified (right panel) (b). GAPDH was used as the loading control. Results were expressed as mean ± SEM (n = 4). *p < .05, **p < .01, ***p < .001 versus 0 h group.

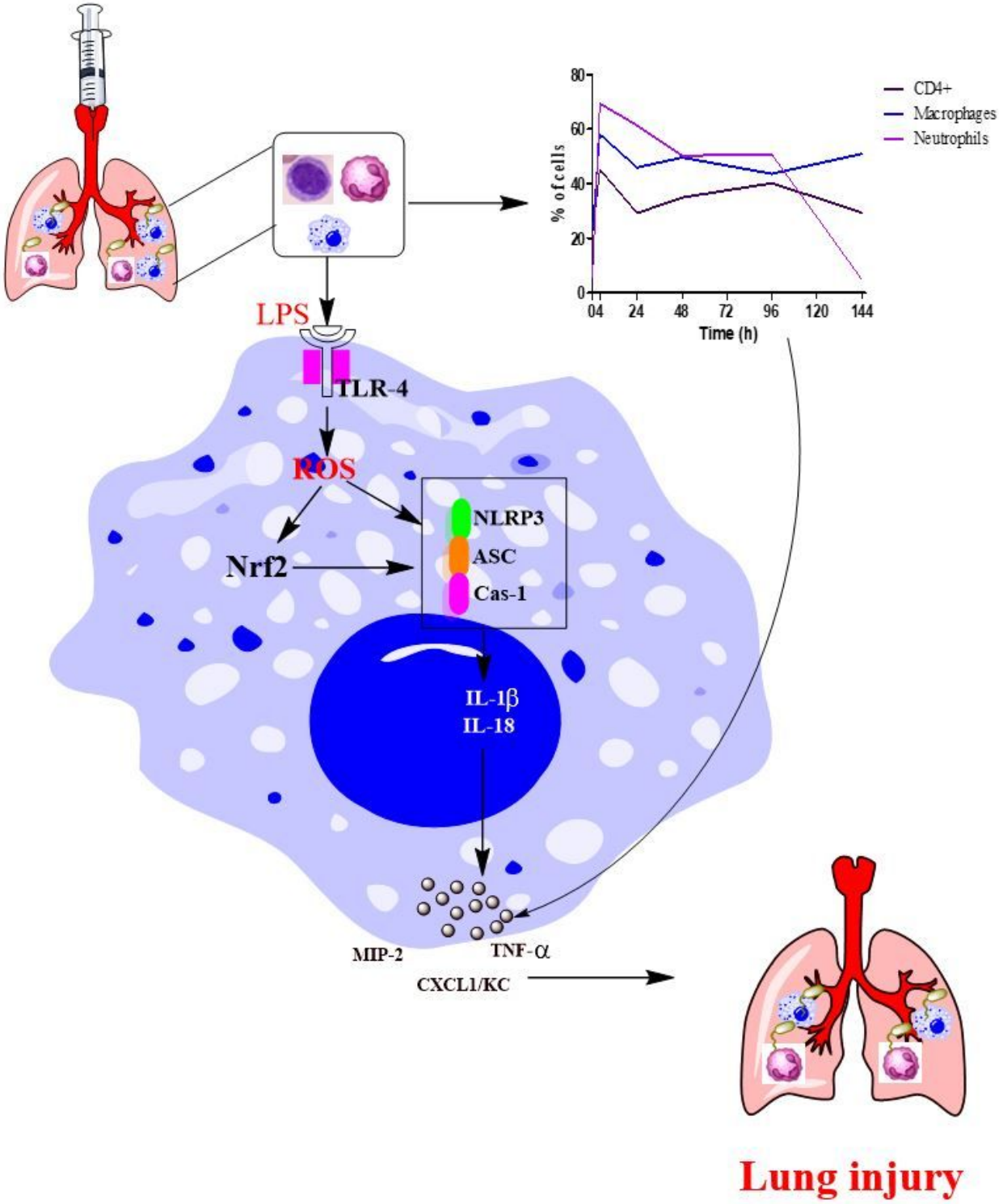

Figure 10

Predominant mechanism of LPS induces acute lung injury. The entry of LPS in lung tissue via binding to TLR-4, causes stimulate of the inflammatory condition in the lungs. LPS also generates oxidative stress and subsequent activation of the NLRP3 inflammasome activation both of are controlled by Nrf2 and leads to the release of large amounts of cytokines and chemokines such as interleukin (IL)-1β, CXCL1/KC,

MIP-2, TNF-α, driving the recruitment of immune cells in the inflamed area of lungs as a time dependent manner. The recruitment of immune cells promotes cytokines storm and regulates acute lung injury (ALI).