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HOST RESPONSE AND INFLAMMATION

PRKCA Promotes Mitophagy through the miR-15a-5p/PDK4 Axis to Relieve Sepsis-Induced Acute Lung Injury

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ABSTRACT Acute lung injury (ALI) caused by sepsis is a common respiratory critical illness with high morbidity and mortality. Protein kinase C-alpha (PRKCA) plays a protective role in sepsis-induced ALI. However, the detailed molecular mechanism of PRKCA in ALI caused by sepsis is unclear. Animal and cell models of sepsis were established by cecal ligation and puncture (CLP)-surgery and lipopolysaccharide (LPS)/interferon-gamma (IFN-γ) treatment, respectively. Lentivirus transfection was used to overexpress PRKCA. H&E staining and lung injury in CLP-surgery mice were evaluated. Gene expression was evaluated using qPCR and Western blotting. The expression of TNF-α, IL-1β, and IL-6 was examined using qPCR and ELISA. The expression of LC3 and TOM20 was evaluated using immunofluorescence assays. Cell apoptosis was assessed using a flow cytometry assay. The bond between miR-15a-5p and PDK4 was confirmed by dual-luciferase reporter gene and RNA immunoprecipitation assays. *In vivo* and in vitro, PRKCA overexpression reduced lung injury to prompt mitophagy and inhibit the inflammatory response, ROS production, and cell apoptosis. miR-15a-5p was highly expressed in macrophages treated with LPS/IFN-γ and was negatively mediated by PRKCA. The overexpression of miR-15a-5p reduced the effects of PRKCA upregulation in macrophages. miR-15a-5p could restrain mitophagy in LPS/IFN-γ-treated macrophages by directly targeting PDK4. Furthermore, PDK4 knockdown reversed the inhibition of cell apoptosis and inflammatory factor release caused by miR-15a-5p silencing. The PRKCA/miR-15a-5p/PDK4 axis alleviated ALI caused by sepsis by promoting mitophagy and repressing anti-inflammatory response.

KEYWORDS PRKCA, miR-15a-5p, PDK4, mitophagy, acute lung injury

Sepsis is a serious inflammatory reaction; its continuous development may cause acute organ dysfunction, leading to multiple organ failures and eventually death (1). Acute lung injury (ALI) caused by sepsis is one of the most common complications of sepsis, and its main features are lung tissue edema and massive infiltration of inflammatory cells (2, 3). ALI results in severe damage to the lung tissue, and further development into irreversible pulmonary injury may lead to death (4). Therefore, seeking treatments for ALI is essential.

Protein kinase C-alpha (PRKCA) is a member of the protein kinase C family of serine-threonine-specific protein kinases (5), which engage in mediating cell growth and inflammation response (6, 7). Wu et al. (8) reported that the expression of PRKCA is abnormally low in patients with sepsis, indicating a close relationship between PRKCA and sepsis. Besides, PRKCA overexpression suppressed LPS-induced secretion of inflammatory factors, including TNF-α, IL-6, and IL-1β, thereby alleviating ALI in mice (7). However, the detailed molecular regulatory mechanism of PRKCA remains ambiguous.

MicroRNAs serve as small noncoding single-stranded endogenous RNAs that are broadly involved in the molecular regulatory processes of diseases (9, 10). miR-15a-5p plays pivotal roles in multiple diseases, such as acute myocardial infarction, endometriosis, and sepsis (11, 12). In LPS-induced injury to macrophages or ALI caused by traumatic hemorrhagic

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shock, miR-15a-5p was highly expressed (13, 14), suggesting the momentous effect of miR-15a-5p in ALI caused by sepsis. Additionally, PRKCA could negatively regulate miR-15a-5p and modulate the development of head and neck squamous cell carcinoma and renal cell carcinoma (15, 16). Thus, the PRKCA/miR-15a-5p axis may become a therapeutic target in sepsis-induced ALI, which is worth further investigation.

Pyruvate dehydrogenase kinase 4 (PDK4) is a member of the family of pyruvate dehydrogenase kinase isoenzymes. PDK4 mediates the activity of the pyruvate dehydrogenase complex through phosphorylation, thereby regulating mitochondrial pyruvate metabolism (17). PDK4 has been reported to be related to sepsis-induced ALI (18). Besides, PDK4 as an autophagy regulator participates in the process of mitophagy in Parkinson's disease (19). Autophagy is a stress-driven cellular response that establishes and maintains cell homeostasis by removing damaged proteins or organelles from the cell (20). Mitophagy is the targeted phagocytosis and destruction of the mitochondria by an autophagy device (21). As previously reported, the upregulation of mitophagy could relieve ALI (22). Therefore, we assumed that PDK4 affects sepsis-induced ALI by influencing the mitophagy process.

Considering the aforementioned information and existing putative binding sequences between miR-15a-5p and PDK4 from the TargetScan website, we hypothesized that the PRKCA/miR-15a-5p/PDK4 axis contributed to sepsis-induced ALI by affecting mitophagy. These may provide innovative ideas to treat ALI caused by sepsis.

RESULTS

Overexpression of PRKCA promoted mitophagy and alleviated lung inflammatory damage in sepsis. PRKCA was found to be lowly expressed in sepsis patients (23). To probe the role of PRKCA in sepsis, the mice were injected with lentivirus-carrying PRKCA (Lv-PRKCA) through the tail vein and subsequently were subjected to CLP surgery. First, the hematoxylin and eosin (H&E) staining results revealed severe structural destruction of the alveoli, hyperemia, and a marked increase in the infiltration of inflammatory cells in the model group. However, Lv-PRKCA infection could inhibit these effects (Fig. 1A). In mice with sepsis, the expression of PRKCA and PDK4 in the lung tissues and alveolar macrophages was decreased while miR-15a-5p expression was increased, whereas PRKCA overexpression abolished these phenomena (Fig. 1B and C and Fig. 2A to C). The lung injury score in the CLP-surgery group was significantly higher than that in the sham group. However, this effect could be reversed by PRKCA overexpression (Fig. 1D). The number of neutrophils and macrophages in the bronchoalveolar lavage fluid (BALF) samples obtained from the CLP-surgery group increased, which was partially suppressed by Lv-PRKCA infection. However, there had no significant difference in lymphocyte counts between the CLP-surgery and Lv-PRKCA infection groups (Fig. 1E). Furthermore, the myeloperoxidase (MPO) activity, lung wet-to-dry (W/D) ratio, and reactive oxygen species (ROS) level were elevated in the CLP-surgery group, while PRKCA overexpression could compromise these phenomena (Fig. 1F to H). These findings indicated that a successful sepsis model was established and that PRKCA overexpression could suppress sepsis-induced lung injury. Furthermore, PRKCA overexpression could reduce the CLP-induced increase in the secretion of inflammatory cytokines, such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6 (Fig. 1I). Considering that mitophagy is closely related to ALI caused by sepsis, we looked for the expression of PINK1, Parkin, Drp1 and the LC3II/I ratio, all of which were positively correlated with mitophagy (24). We found that CLP surgery prominently decreased the expression of PINK1, Parkin, Drp1, and the ratio of LC3II/I, whereas these suppressing effects were restored by Lv-PRKCA infection (Fig. 1J to L). These findings suggested that sepsis inhibited the mitophagy process in lung tissues. However, overexpression of PRKCA activated mitophagy. Taken together, PRKCA served as a protective factor to relieve ALI caused by sepsis by mediating the mitophagy process, the release of inflammatory cytokines, and ROS production.

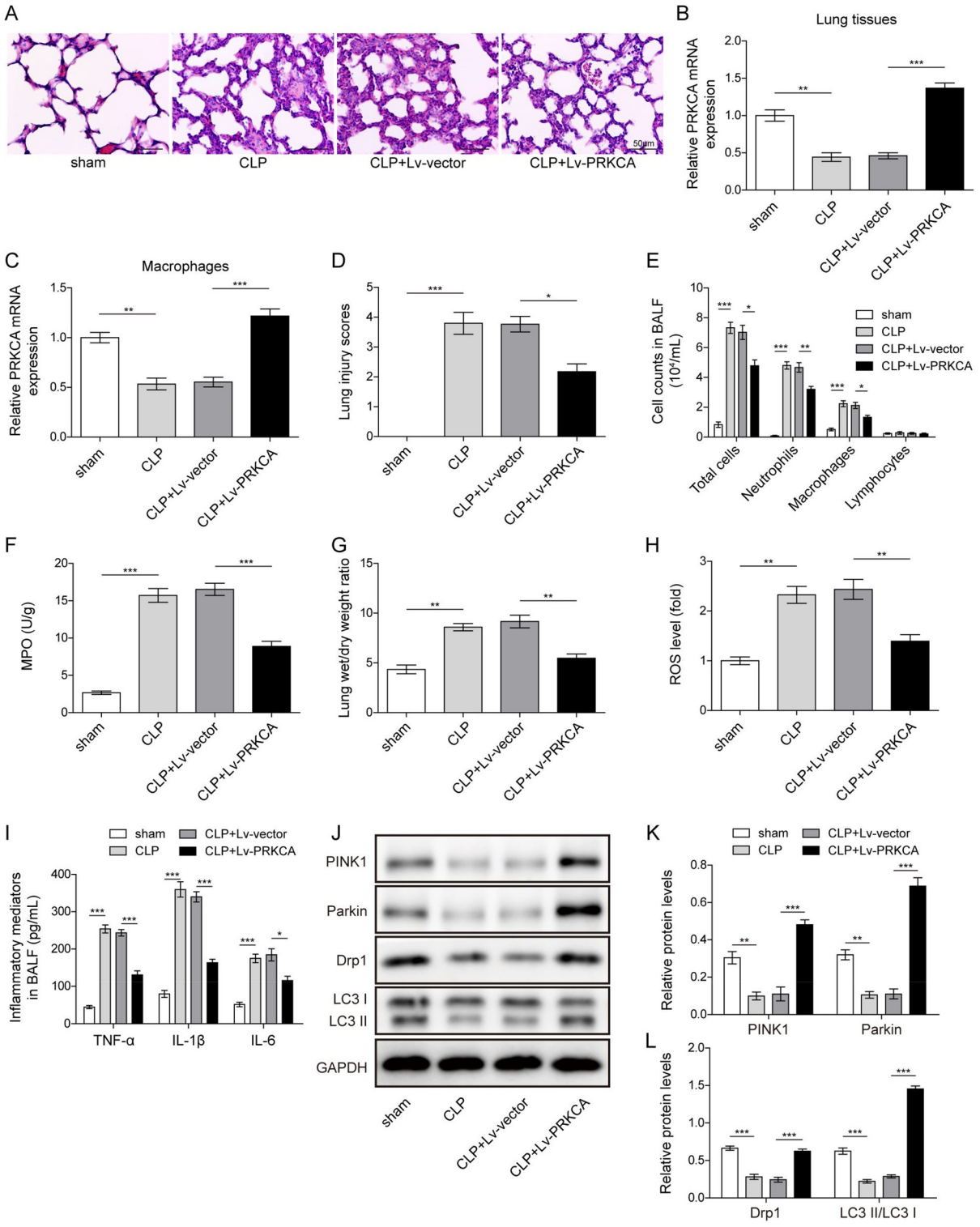

FIG 1 Overexpressing PRKCA promoted mitophagy and alleviated lung inflammatory damage in sepsis. Mice were subjected to CLP surgery with/without Lv-PRKCA injection. (A) H&E staining was applied to evaluate the structural destruction of the alveoli and inflammatory infiltration. (B and C) qPCR detected the mRNA expression of PRKCA in the lung tissues and alveolar macrophages. (D) A lung injury score was used to evaluate lung injury. (E) The Wright-Giemsa staining method was applied to examine BALF cell counts. (F) MPO detection kit was used to detect MPO activity assay. (G) Lung W/D ratio was measured through the measurement of lung weight. (H) ROS production was detected using a ROS assay kit. (I) ELISA measured the protein concentration of inflammatory factors, including TNF-α, IL-1β, and IL-6 in BALF samples. (J to L) Western blotting examined the protein expression of PINK1, Parkin, Drp1, and LC3II/I. GAPDH served as a control protein. All data were obtained from three replicate experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

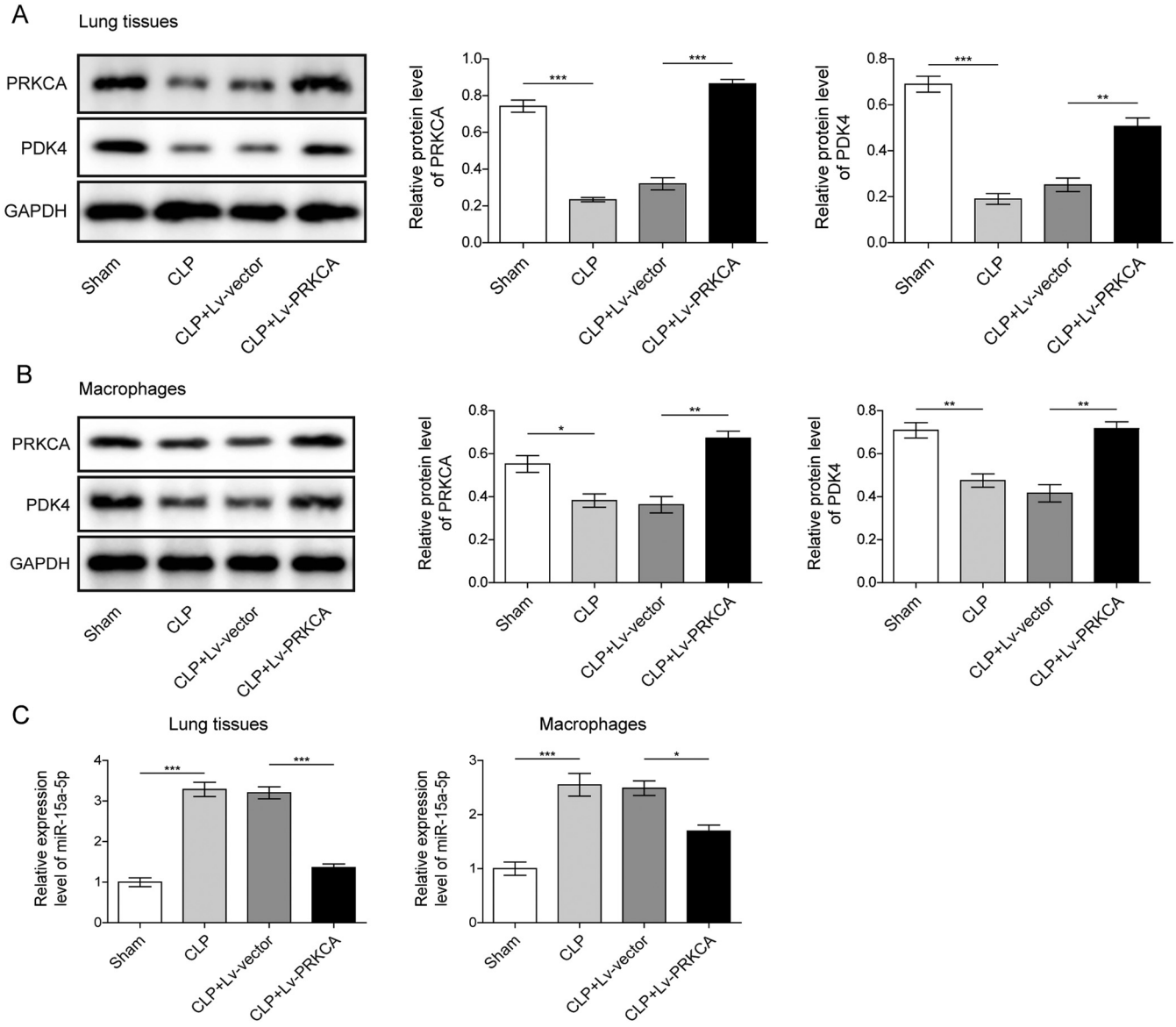

FIG 2 Overexpression of PRKCA increased PRKCA and PDK4 protein levels while decreasing miR-15a-5p expression in lung tissues and alveolar macrophages isolated from CLP-surgery mice. Mice were subjected to CLP surgery with/without Lv-PRKCA injection. (A and B) Western blotting examined the protein expression of PRKCA and PDK4 in lung tissues and alveolar macrophages. GAPDH served as a control protein. (C) qPCR examined miR-15a-5p expression in lung tissues and alveolar macrophages. All data were obtained from three replicate experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

PRKCA overexpression alleviated cell injury and apoptosis in RAW264.7 cells treated with LPS/IFN-γ. Subsequently, RAW264.7 cells were treated with LPS/IFN-γ to construct a cell model of inflammatory injury with sepsis. The expression of PRKCA was decreased with LPS/IFN-γ treatment; however, it was notably increased after Lv-PRKCA transfection (Fig. 3A). Furthermore, overexpressing of PRKCA significantly reduced the increase of inflammatory cytokines, ROS level, and cell apoptosis induced by LPS/IFN-γ treatment (Fig. 3B to E). As a result, PRKCA could protect macrophages from LPS/IFN-γ-induced injury.

PRKCA upregulation facilitated mitophagy. We investigated the expression of mitophagy-related proteins in RAW264.7 cells treated with LPS/IFN-γ to determine the mitophagy effect of PRKCA. Referring to a previous study (25), we acquired that LC3 was an important indicator of autophagy and TOM20 was an indicator of the mitochondrial outer membrane, whose colocalization could be used to assess the presence of mitophagy. We then detected the colocalization of LC3 and TOM20. As shown in Fig. 4A, LPS/IFN-γ treatment decreased the colocalization of LC3 and TOM20 and inhibited the expression of

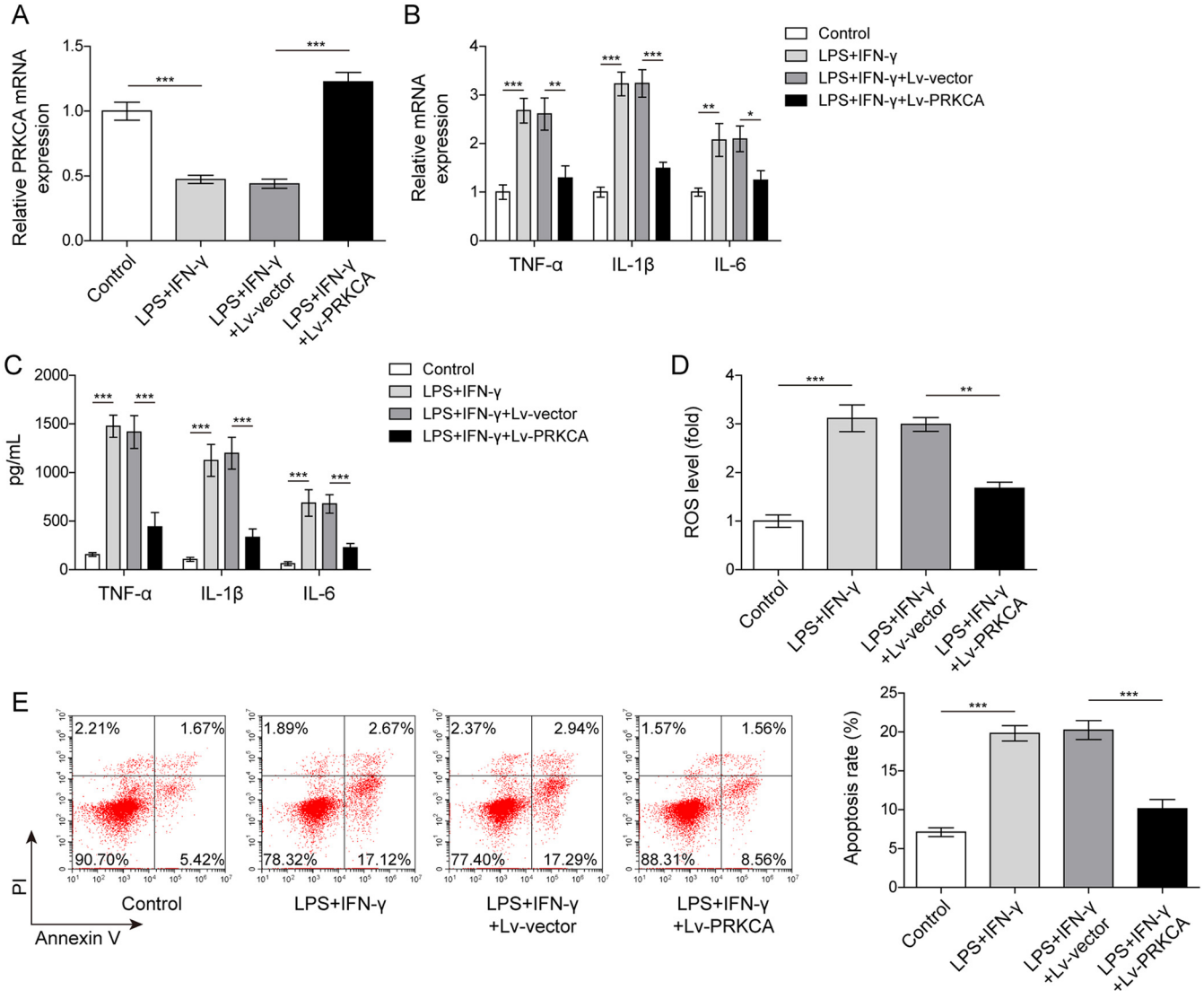

FIG 3 PRKCA alleviated cell injury and apoptosis in RAW264.7 cells treated with LPS/IFN-γ. RAW264.7 cells were transfected with Lv-PRKCA and followed LPS/IFN-γ stimulation (LPS 100 ng/mL and IFN-γ 20 ng/mL). (A) qPCR detected the mRNA expression of PRKCA. (B) qPCR examined the mRNA expression of inflammatory factors, including TNF-α, IL-1β, and IL-6. (C) ELISA was employed to measure the protein concentration of inflammatory factors, including TNF-α, IL-1β, and IL-6 in cell supernatant. (D) ROS production was detected using a ROS assay kit. (E) Flow cytometry estimated cell apoptosis. All data were obtained from three replicate experiments. *, $P < 0.05$; **, $P < 0.01$, ***, $P < 0.001$.

mitophagy-related proteins. However, overexpressing PRKCA reversed this effect (Fig. 4A to D). Notably, the expression of miR-15a-5p was elevated after LPS/IFN-γ treatment, whereas PRKCA overexpression could eliminate this phenomenon (Fig. 4E). The results suggested that PRKCA promoted mitophagy in sepsis, which might be correlated with miR-15a-5p.

PRKCA overexpression reduced LPS/IFN-γ-induced injury in RAW264.7 cells by downregulating miR-15a-5p. Given that PRKCA could mediate the expression of miR-15a-5p, we conjectured that miR-15a-15p might affect PRKCA-mediated influences on cell apoptosis and inflammatory cytokines in RAW264.7 cells. Lv-PRKCA alone or in combination with a miR-15a-5p mimic was introduced into LPS/IFN-γ-treated RAW264.7 cells. The overexpression of miR-15a-5p could remarkably compromise the suppressive influences of Lv-PRKCA on inflammatory cytokines, ROS, and cell apoptosis (Fig. 5A to D). Thus, in LPS/IFN-γ-treated RAW264.7 cells, PRKCA inhibited miR-15a-5p to decrease cell death, the release of inflammatory cytokines, and ROS generation.

miR-15a-5p targeted PDK4 and knocking it out increased mitophagy. According to the TargetScan database, there were putative binding sequences between miR-15a-5p

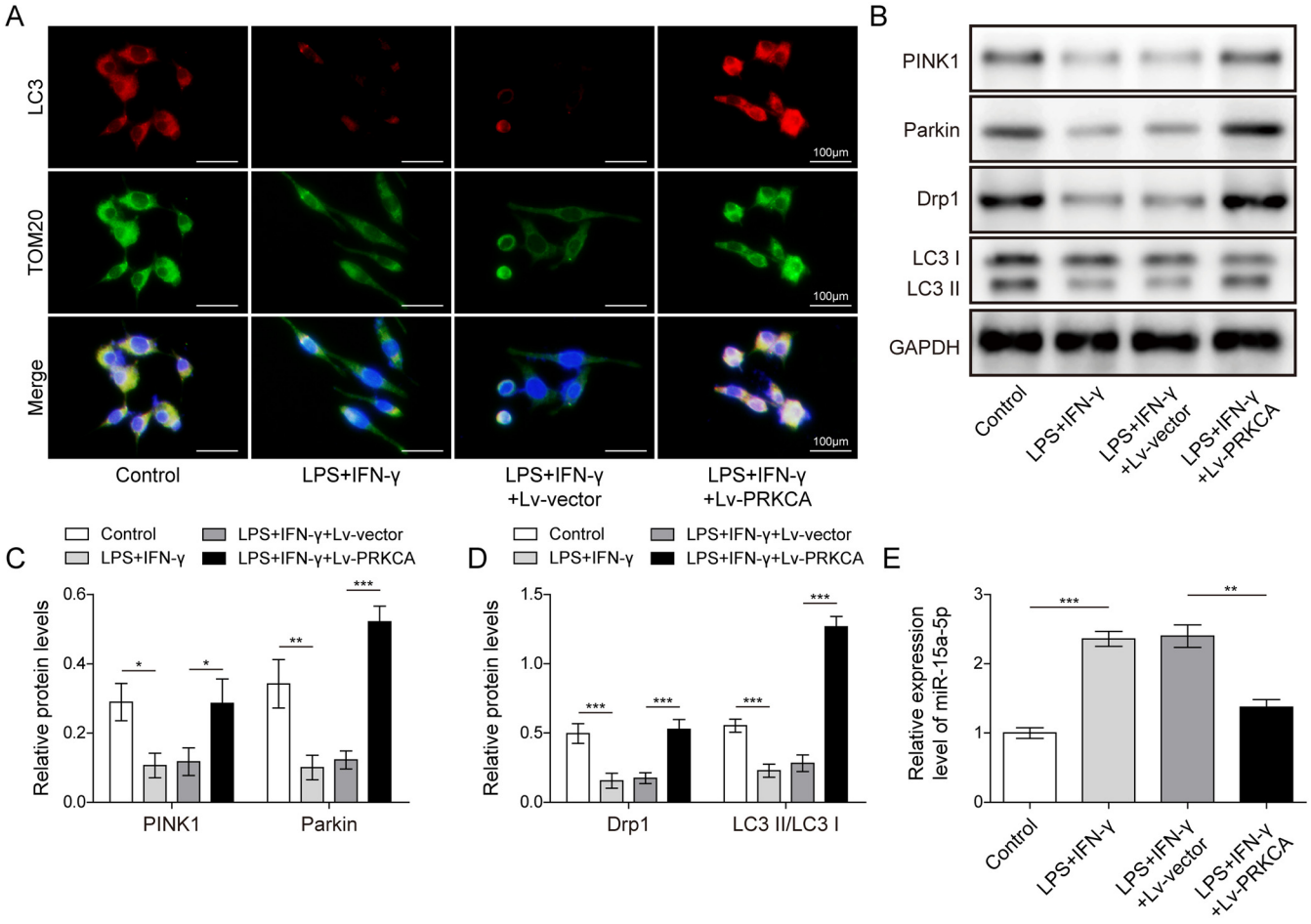

FIG 4 PRKCA upregulation facilitated mitophagy. RAW264.7 cells were transfected with Lv-PRKCA and followed LPS/IFN-γ stimulation (LPS 100 ng/mL and IFN-γ 20 ng/mL). (A) An immunofluorescence (IF) assay was employed to detect the colocalization of LC3 (red) and TOM20 (green). The yellow fluorescence represented the overlap of red and green fluorescence (scale bar, 100μm). (B to D) Western blotting examined the protein expression of PINK1, Parkin, Drp1, and LC3II/I. GAPDH served as a control protein. (E) qPCR examined miR-15a-5p expression. All data were obtained from three replicate experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

and PDK4 (Fig. 6A). Besides, the luciferase activity in PDK4-WT (wild type) was reduced by miR-15a-5p mimic. However, the PDK4-MUT (mutant type) group did not affect the luciferase activity (Fig. 6B). RNA immunoprecipitation assay (RIP) assay showed that miR-15a-5p and PDK4 were enriched in the Ago2 group, indicating that miR-15a-5p targeted PDK4 through interacting with each other (Fig. 6C). As shown in Fig. 6D, the expression of miR-15a-5p was increased by the LPS/IFN-γ induction, while the miR-15a-5p inhibitor abolished this change. Simultaneously, the mRNA expression of PDK4 was significantly reduced in LPS/IFN-γ-treated RAW264.7 cells. However, miR-15a-5p inhibition increased its expression (Fig. 6E). The protein expression variation of PDK4 was consistent with its mRNA changes (Fig. 6F to G). Regarding the function of miR-15a-5p in mitophagy, we found miR-15a-5p depletion restored the LPS/IFN-γ-induced inhibition of mitophagy (Fig. 6H to J). Collectively, miR-15a-5p inhibition promoted mitophagy in RAW264.7 cells treated with LPS/IFN-γ by upregulating PDK4.

miR-15a-5p knockdown repressed cell apoptosis and inflammatory factors by mediating PDK4. To investigate whether PDK4 was engaged in miR-15a-5p-mediated cell apoptosis and inflammatory factors, RAW264.7 cells treated with LPS/IFN-γ were transfected with miR-15a-5p inhibitor alone or in combination with sh-PDK4. As shown in Fig. 7A to B, the mRNA and protein expressions of PDK4 were prominently elevated by miR-15a-5p knockdown, whereas the PDK4 knockdown reversed these effects. Besides, the inhibitory impacts of miR-15a-5p downregulation on inflammatory cytokine secretion,

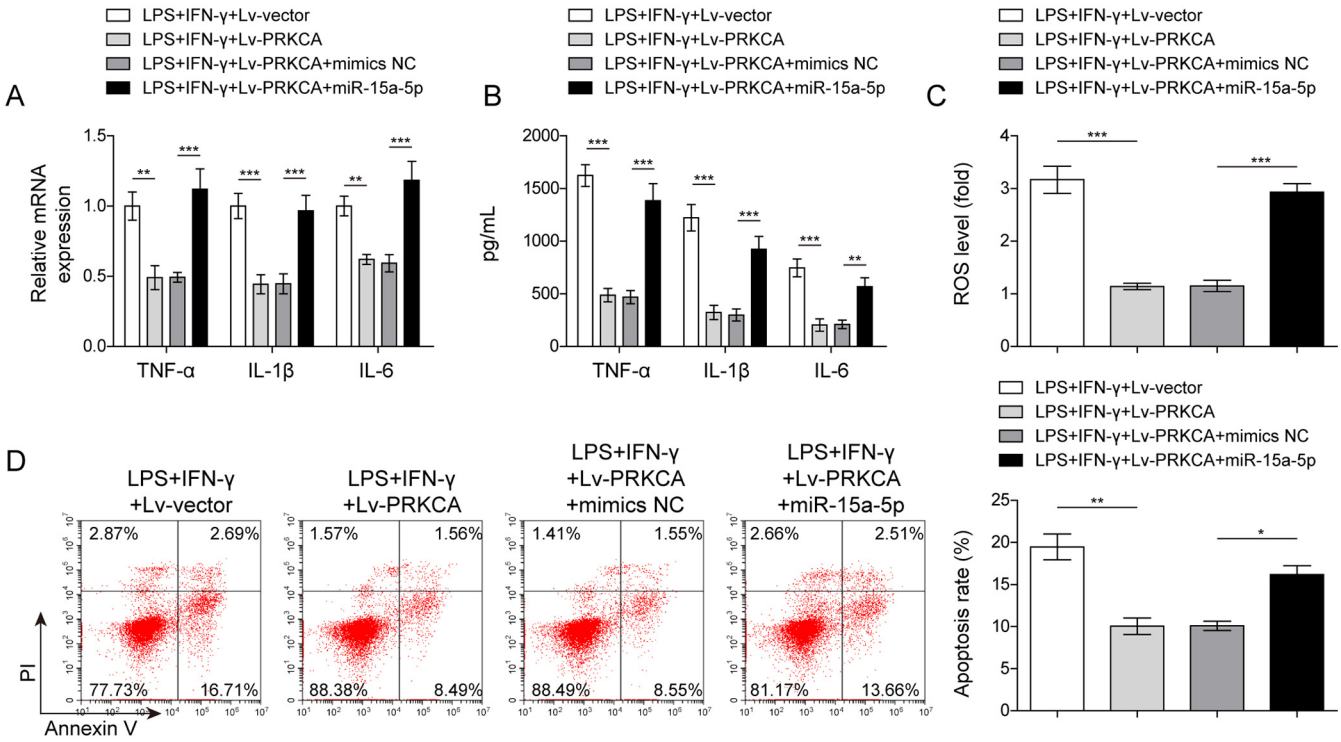

FIG 5 PRKCA overexpression reduced LPS/IFN-γ-induced injury in RAW264.7 cells by downregulating miR-15a-5p. RAW264.7 cells were transfected with Lv-PRKCA alone or together with miR-15a-5p mimic upon LPS/IFN-γ (LPS 100 ng/mL and IFN-γ 20 ng/mL). (A) qPCR examined the mRNA expression of inflammatory factors, including TNF-α, IL-1β, and IL-6. (B) ELISA was employed to measure the protein concentration of inflammatory factors, including TNF-α, IL-1β, and IL-6 in cell supernatant. (C) ROS production was detected using a ROS assay kit. (D) Flow cytometry estimated cell apoptosis. All data were obtained from three replicate experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

ROS production, and cell apoptosis rate in LPS/IFN-γ-treated RAW264.7 cells were reversed by PDK4 depletion (Fig. 7C to F). Finally, by upregulating PDK4, miR-15a-5p knockdown suppressed cell apoptosis, the release of inflammatory factors, and ROS production in RAW264.7 cells treated with LPS/IFN-γ.

DISCUSSION

ALI is an early complication of sepsis, and severe ALI can develop into acute respiratory distress syndrome, which will greatly increase the mortality of patients (26). Therefore, preventing and treating ALI is urgent. Here, we demonstrated the vital importance of the PRKCA/miR-15a-5p/PDK4 axis in ALI caused by sepsis. Appropriate interventions for the molecules in the PRKCA/miR-15a-5p/PDK4 axis could effectively promote mitophagy and reduce the release of inflammatory factors and ROS production, thereby alleviating lung injury caused by sepsis.

At present, a variety of molecules have been reported to be involved in sepsis-induced ALI. For instance, the expression of PPP1R15A was found to be downregulated in sepsis-induced ALI and Salidroside alleviated sepsis-induced ALI by increasing the expression of PPP1R15A (27). ZEB1 overexpression could promote cell apoptosis and inflammatory response in the LPS-induced sepsis cell model (28). In this study, we focused on the molecule of PRKCA in sepsis-induced ALI. PRKCA was found to be downregulated in patients with sepsis or monocytes/macrophages treated with LPS/IFN-γ/L-NAME(N(gamma)-nitro-L-arginine methyl ester) (23). Consistently, our findings validated this alteration of PRKCA in a mouse model of sepsis induced by CLP surgery and in RAW264.7 cells treated with LPS/IFN-γ. Additionally, in LPS-induced ALI, PRKCA exhibited potent anti-inflammatory properties (7). Our results were analogous to these results. In our study, PRKCA functioned as a beneficial factor in improving sepsis-induced ALI. In *in vivo* experiments, PRKCA overexpression could reduce the infiltration of inflammatory cells and the release of inflammatory factors in sepsis-induced ALI.

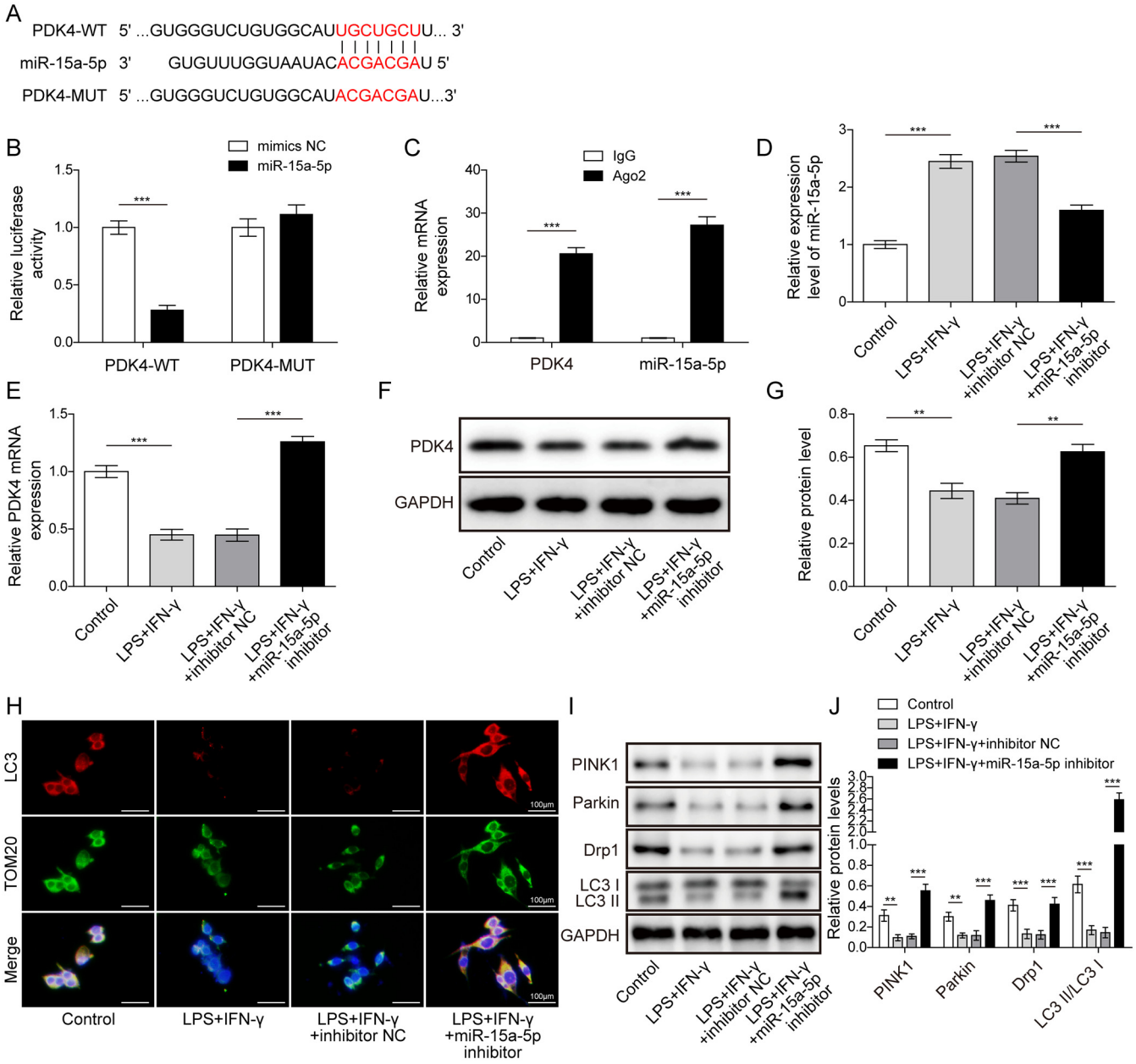

FIG 6 miR-15a-5p targeted PDK4 and knocking it out increased mitophagy. (A) The binding site between miR-15a-5p and PDK4 was predicted by the TargetScan database. (B) RAW264.7 cells were transfected with miR-15a-5p mimics together with PDK4-WT or PDK4-MUT. (C) Dual-luciferase reporter and RIP assays were employed to validate the interaction between miR-15a-5p and PDK4. Ago2 was used for immune-coprecipitation of the miRNA/mRNA complex (42, 43). IgG acted as a negative control for the RIP assay. RAW264.7 cells were transfected with miR-15a-5p inhibitor upon LPS/IFN-γ (LPS 100 ng/mL and IFN-γ 20 ng/mL) treatment. (D and E) qPCR examined the expression of miR-15a-5p and PDK4. (F and G) Western blotting examined the protein level of PDK4. (H) IF assay was employed to detect the colocalization of LC3 (red) and TOM20 (green). The yellow fluorescence represented the overlap of red and green fluorescence (scale bar, 100μm). (I and J) Western blotting examined the protein expression of PINK1, Parkin, Drp1, and LC3II/I. GAPDH served as a control protein. All data were obtained from three replicate experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Furthermore, Lv-PRKCA infection inhibited the levels of inflammatory cytokines in LPS/IFN-γ-treated RAW264.7 cells. Notably, our findings revealed that ROS production was stimulated in CLP-surgery mice and LPS/IFN-γ-treated RAW264.7 cells, whereas PRKCA overexpression abolished these effects.

Accumulating evidence has demonstrated that microRNAs play pivotal roles in the inflammatory response of diseases (29, 30). For example, the expression of miR-152-3p was upregulated in sepsis patients and LPS-treated HPAEpiC, and miR-152-3p silencing suppressed LPS-induced ALI (18). miRNA-1224-5p was reported to aggravate sepsis-ALI in mice through elevating inflammation and oxidative stress (31). miR-15a-5p was demonstrated to be related to

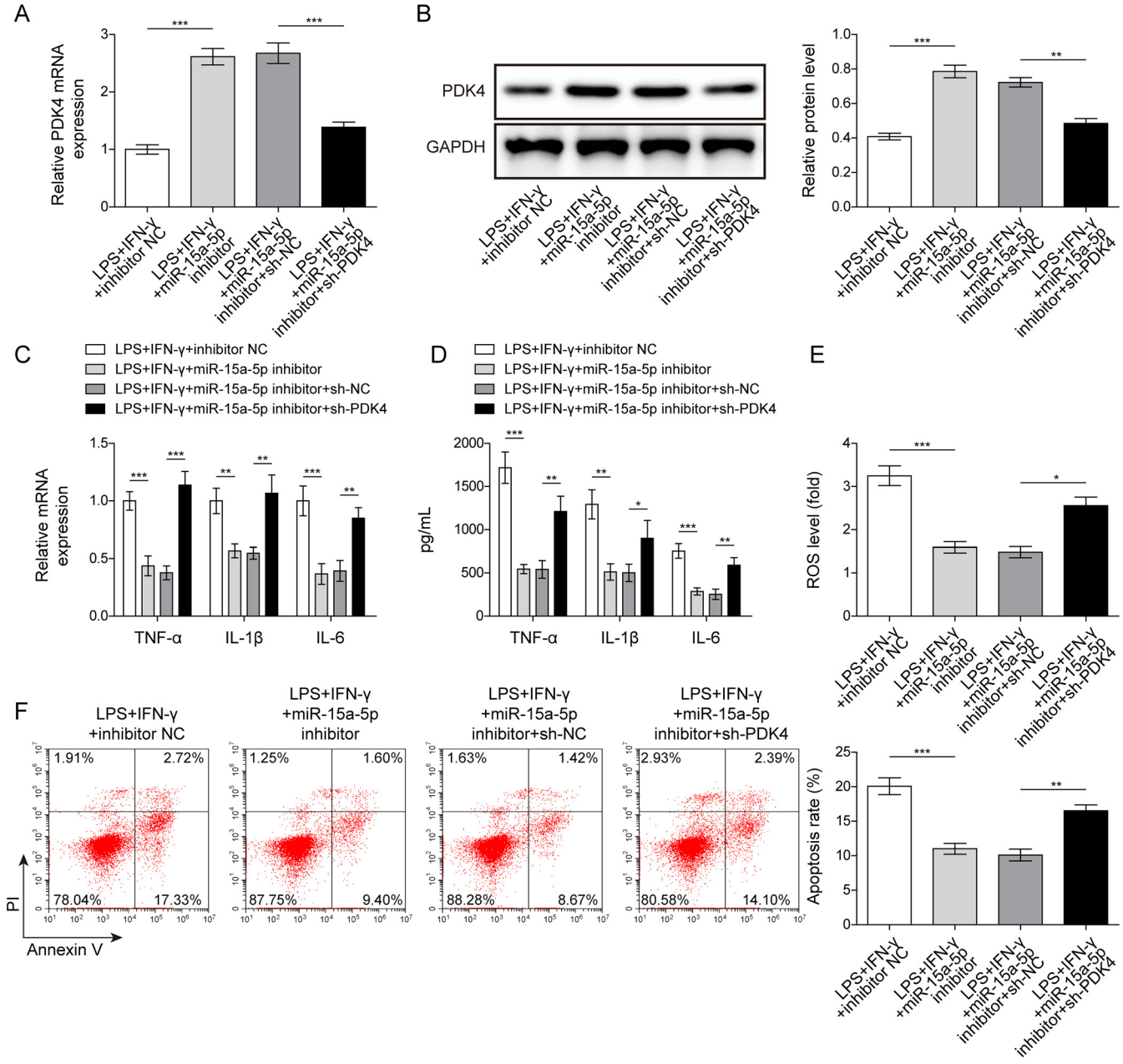

FIG 7 miR-15a-5p knockdown repressed cell apoptosis and inflammatory factors by mediating PDK4. RAW264.7 cells were transfected with miR-15a-5p inhibitor alone or together with sh-PDK4 upon LPS/IFN-γ (LPS 100 ng/mL and IFN-γ 20 ng/mL) treatment. (A) qPCR examined the mRNA expression of PDK4. (B) Western blotting examined the protein level of PDK4. GAPDH served as a control protein. (C) qPCR the mRNA expression of inflammatory factors, including TNF-α, IL-1β, and IL-6. (D) ELISA was employed to measure the protein concentration of inflammatory factors, including TNF-α, IL-1β, and IL-6 in cell supernatant. (E) ROS production was detected using a ROS assay kit. (F) Flow cytometry estimated cell apoptosis. All data were obtained from three replicate experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

proinflammation in many diseases, such as Parkinson's disease and intervertebral disc degeneration, which could promote inflammatory responses to accelerate the progression of diseases (32, 33). Certainly, its proinflammatory effect was verified in septic shock. Lou et al. discovered that miR-15a-5p levels were elevated in LPS-treated macrophages, and miR-15a-5p knockdown significantly reduced the release of inflammatory factors (13). Additionally, in lung injury induced by traumatic hemorrhagic shock (THS) or LPS, miR-15a-5p expression increased (12, 14). miR-15a-5p knockdown mitigated the injury caused by THS (14). In this study, we found a significant increase in miR-15a-5p in LPS/IFN-γ-treated RAW264.7 cells. Subsequently, the upregulation of PRKCA inhibited miR-15a-5p

expression in RAW264.7 cells. Furthermore, miR-15a-5p sufficiency offset the restriction impacts of PRKCA overexpression on cell apoptosis and the release of inflammatory factors. This indicates that miR-15a-5p was negatively mediated by PRKCA. As previously described, PRKCA could directly bind to pre-miRNA-15a in the nucleus, thereby affecting miR-15a-5p levels (15, 16). The detailed relationship between PRKCA and miR-15a-5p must be further explored.

microRNAs could combine with target gene sequences to mediate their expression (33). For instance, miR-15a-5p could directly target TNIP2 to reduce ALI caused by THS (14). In this study, we confirmed that miR-15a-5p negatively regulates the expression of PDK4 by directly interacting with PDK4. Additionally, rescue experiments showed that PDK4 knockdown counteracted miR-15a-5p inhibitor-mediated suppression of cell apoptosis and inflammatory response, indicating that PDK4 is a protective factor in ALI caused by sepsis. These results are consistent with those reported in previous studies (18, 34).

Mitophagy mediates dysfunctional mitochondria through a lysosomal clearance mechanism in pulmonary sepsis, besides the regulation of oxidative stress and inflammatory response (35, 36). It was reported that the proteins of Drp1, LC3II/I as well as PINK1/Parkin signaling pathway were implicated with the process of mitophagy (37–39). Hydrogen alleviates sepsis-induced cell damage and ALI via PINK1/Parkin-mediated promotion of mitophagy, elevating the ratio of LC3II/I and the expression of PINK1 and Parkin (40). These studies indicated that moderate mitophagy could prevent cell injury from sepsis-induced ALI by regulating the ratio of LC3II/I and the expression of Drp1, PINK1, and Parkin. In this study, we found that mitophagy was inhibited in sepsis-induced ALI and RAW264.7 cells treated with LPS/IFN-γ, as evidenced by reducing the ratio of LC3II/I and inhibiting the expression of Drp1, PINK1, and Parkin, whereas PRKCA sufficiency or miR-15a-5p inhibition activated mitophagy to protect against injury. Furthermore, miR-15a-5p targeted PDK4 and PDK4 could affect miR-15a-5p-mediated mitophagy in ALI. Our findings first put forward the PRKCA/miR-15a-5p/PDK4 axis to regulate mitophagy, which plays a crucial role in ALI caused by sepsis.

Our research first elucidated the role of the PRKCA/miR-15a-5p/PDK4 axis in ALI caused by sepsis. PRKCA overexpression promoted mitophagy and suppressed cell apoptosis, inflammatory response, and ROS production, which inhibited the development of ALI caused by sepsis to a certain extent. Furthermore, the exogenous intervention of miR-15a-5p and PDK4 could also affect LPS-induced cell injury or PRKC-mediated functions in LPS-treated cells. Therefore, we believed that targeting the molecules of PRKCA, miR-15a-5p, and PDK4 might become potential therapeutic targets in sepsis-induced ALI.

MATERIALS AND METHODS

Animals and cecal ligation and puncture operation. C57BL/6 mice (male, aged 10 to 12 weeks) were purchased from Hunan SJA Laboratory Animal Co., Ltd. (Changsha, China). Under light-dark cycle conditions, all mice could obtain food and water at liberty. According to the experimental design, 20 mice were randomly and evenly classified into the sham, CLP, CLP + Lv-vector, and CLP + Lv-PRKCA groups with five mice in each group. All experimental procedures were approved by the Children's Hospital of Soochow University. According to the experimental design, for PRKCA overexpression, each mouse was injected with 2×10^7 transducing units of Lv-PRKCA or Lv-vector (Genetop, Changsha, China) lentivirus via the caudal vein. After 5 days, the mice were subjected to CLP surgery. The sepsis mouse model was established according to previous research methods (22). Shortly, the mice were anesthetized, and the cecum was pulled out. Then, ligation was performed approximately 1 cm from the top of the cecum, and a puncture needle was used to penetrate the cecum at the distal end of the ligation, forming a cecal fistula. A small number of feces was then extruded out of the abdominal cavity. Finally, the cecum was reset to the abdominal cavity; then, the abdominal cavity was sutured. Furthermore, sham surgically operated mice were used as the controls.

Hematoxylin and eosin staining and the evaluation of the lung injury score. After fixation with formalin and embedding with paraffin, lung tissues were split into 4-μm sections. Then, the sections were dewaxed and dyestuff stained with HE. Finally, the features of the stained sections were observed using a light microscope. The lung injury score was used to evaluate lung injury, which was estimated using the degree of hemorrhage, edema, exudation, necrosis, congestion, neutrophil infiltration, and atelectasis. The scoring system used in a previous study (41) was applied as follows: 0, normal tissue; 1, injury to 25% of the field; 2, injury to 50% of the field; 3, injury to 75% of the field; and 4, diffuse injury.

Collection of bronchoalveolar lavage fluid, cell counting, and macrophage isolation. The mice were euthanized, and then, tracheal intubation was performed. BALF was collected by infusing 1 mL of ice-cold PBS into the lungs. Then, 0.1g/dL Dithiothreitol (DTT) (twice the volume of BALF) was added to the BALF samples. This process was repeated 3 times. The collected BALF samples were centrifuged, and

the supernatants were stored at −80°C for subsequent experiments. Then, the cell pellet was resuspended in PBS for differential cell counts, including total cells, neutrophils, macrophages, and lymphocytes, using the Wright–Giemsa staining method (BaSO, Guangzhou, China). Macrophage isolation from BALF was performed as described previously (22).

Assessment of myeloperoxidase activity. After the BALF collection, the upper left leaf was collected to assess the MPO activity. According to the manufacturer's manual (AmyJet Scientific, China), the absorbance was examined using a spectrophotometer (Shanghai Precision and Scientific Instrument Co. Ltd., China) at 460 nm.

Lung wet-to-dry weight ratio. The lung wet/dry weight (W/D) ratio was used to evaluate the degree of pulmonary edema. Briefly, the lungs were weighed and subsequently dried at 60°C for 48 h. The W/D ratio was calculated based on the recorded weight.

Detection of reactive oxygen species production. ROS production in the lung tissues and RAW264.7 cells with indicated treatment was evaluated using a ROS assay kit (Biosharp, Shanghai, China). Briefly, tissues and RAW264.7 cells were prepared into a single-cell suspension and then incubated with an H_2DCFH-DA probe for 30 min in the dark. Then, H_2DCFH-DA that did not enter the cells was removed. Finally, a fluorescent microplate reader was used to detect the fluorescence intensity.

RNA isolation, reverse transcription, and quantitative PCR. The total RNA was acquired from the lung tissues of mice and RAW264.7 cells treated with the TRIzol reagent (Invitrogen, CA, USA). The Prime Script Reverse Transcription reagent kit (TaKaRa, Shiga, Japan) was used to synthesize cDNA. The SYBR Premix *Ex Taq* II kit (TaKaRa) was used for qPCR. The primer sequences, PCR mix, cDNA template, and water were prepared for the reaction solution (20 μL). The reaction procedures were as follows: predenaturation at 95°C for 2 min and PCR at 94°C for 20 s, 60°C for 20 s, and 72°C for 30 s for 40 cycles.

The primer sequences were as follows PRKCA (F): 5′-AAGGCAACATGGAACTCAGG-3′; PRKCA (R): 5′-TGTCAGCAAGCATCACCTTC-3′; miR-15a-5p (F): 5′-GCCGAGTAGCAGCACATAATGG-3′; miR-15a-5p (R): 5′-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCACAAA-3′; PDK4 (F): 5′-TGAACACTCCTTCGGTGCAG-3′; PDK4 (R): 5′-TGATTGGTAACGGGGTCCAC-3′; TNF-α (F): 5′-ACATTCGAGCCAGTGAATTCGG-3′; TNF-α (R): 5′-GGCAGGTCTACTTTGGAGTCATTGC-3′; IL-1β (F): 5′-TGCCCATCAGAGGCAAGGAGGA-3′; IL-1β (R): 5′-CCTCGGCCAAGACAGGTCGC-3′; IL-6 (F): 5′-GTCACCAGCATCAGTCCCAA-3′; IL-6 (R): 5′-GGAGCCCACCAAGAACGATA-3′; glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (F): 5′-AGCCCAAGATGCCCTTCAGT-3′; GAPDH (R): 5′-CCGTGTTCCTACCCCCAATG-3′; U6 (F): 5′-CTCGCTTCGGCAGCACA-3′; U6 (R): 5′-AACGCTTCACGAATTTGCGT-3′.

All data were calculated using the $2^{-\Delta\Delta Ct}$ formula. As a reference for genes or miRNA, GAPDH, or U6 were used.

Western blotting. The total protein was extracted from the lung tissues of mice and RAW264.7 cells treated with the experimental designation RIPA buffer (Beyotime, Shanghai, China). After quantifying the protein concentration, proteins were detached on SDS-PAGE and then transferred onto polyvinylidene fluoride (PVDF) membranes. Then, the membranes were blocked with 5% skimmed milk. Subsequently, the membranes were incubated with anti-PRKCA (1:1000, Abcam, number ab11723, Cambridge, UK), anti-PINK1 (1:1000, Abcam, number ab23707), anti-Parkin (1:2000, Abcam, number ab77924), anti-Drp1 (1:1000, Abcam, number ab184247), anti-LC3II/I (1:2000, Abcam, number ab192890) and anti-GAPDH (1:2000, Abcam, number ab8245) overnight at 4°C. Then, the membranes were immersed with the secondary antibodies at room temperature for 1 h. Enhanced chemiluminescence (ECL) chemiluminescent reagent (Beyotime) was adopted to evaluate protein bands. Densitometry analysis was performed using ImageJ.

Enzyme-linked immunosorbent assay. Commercial enzyme-linked immunosorbent assay (ELISA) kits (catalog no. PT512; catalog no. PI301; catalog no. PI326, Beyotime) were used to examine the concentration of TNF-α, IL-1β, and IL-6 in the supernatants of BALF or cells following the corresponding instructions. Then, the data were recorded under an absorbance value of 450 nm.

Cell culture and transfection. RAW264.7 cells were bought from the Shanghai Institutes for Biological Sciences (Shanghai, China) and cultured in Dulbecco's Modified Eagle Medium (Thermo-Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (Gibco, MD, USA), 50 U/mL penicillin and 50 μg/mL streptomycin (Gibco, MD, USA). The cells were incubated at 37°C in a humidified chamber that contained 5% CO_2. Furthermore, RAW264.7 cells were treated with LPS (100 ng/mL)/IFN-γ (20 ng/mL) (Sigma, MO, USA) for 24 h, which aimed to establish a cell model of inflammatory injury with sepsis.

The miR-15a-5p mimic/inhibitor, PDK4 knockdown (sh-PDK4), and their negative control groups (mimic/inhibitor NC and sh-NC) were acquired from GenePharma (Shanghai, China). For *in vitro* transfection, the miR-15a-5p mimic/inhibitor, sh-PDK4, and their corresponding negative control groups were transfected into RAW264.7cells using Lipofectamine 3000 (Invitrogen) according to the manufacturer's manual. Moreover, RAW264.7 cells were infected with lentivirus particles carrying Lv-PRKCA or empty vector (GenePharma).

Flow cytometry. After treatment and transfection, RAW264.7 cells were added with FITC-annexin V and PI using the Annexin V-FITC apoptosis detection kit (Beyotime) according to the manufacturer's instructions. The number of apoptotic RAW264.7 cells was measured using a flow cytometer.

Immunofluorescence assay. IF staining was performed to evaluate the expression of LC3 and TOM20 in the mitochondria of RAW264.7 cells in the lung tissues. Briefly, after fixation with 4% paraformaldehyde and permeabilization with a solution containing 5% BSA and 0.01% Triton X-100, the cells were incubated with anti-LC3 (1:200, Novus Biologicals, CO, USA, NB100-2220), anti-TOM20 (1:200, Abcam, ab56783) antibodies overnight at 4°C. Next, the secondary antibodies were incubated. The nuclei of the cells were stained with DAPI (4′,6-diamidino-2-phenylindole). A fluorescence microscope (Olympus, Tokyo, Japan) was used to acquire images.

Dual-luciferase reporter assay. The binding sites of the 3′UTR of PDK4, which binds with miR-15a-5p, were cloned into psiChECK-2 vectors (Ke Lei Biological Technology Co., Ltd., China) to build reporter vectors for

PDK4 (WT/MUT). Additionally, miR-15a-5p mimics together with PDK4 (WT/MUT) were transfected into RAW264.7 cells using Lipofectamine 3000 (Invitrogen). After 48 h, the luciferase activity was assessed using a dual-luciferase reporter assay system (Promega, Madison, USA) according to the manufacturer's protocol.

RNA immunoprecipitation assay. According to the manufacturer's protocol, the Magna RIP RNA-Binding Protein Immunoprecipitation kit (Millipore, Bedford, MA, USA) was adopted for RIP analysis. Immunoprecipitated RNA (miR-15a-5p and PDK4) was assessed using qPCR analysis.

Statistical analysis. All data were presented as means ± standard deviation. Student's *t* test was used to compare the two groups. One-way analysis of variance, followed by Tukey's *post hoc* test, was used to compare more than two groups. When $P < 0.05$, the data are identified as having a significant difference. All statistical analyses were performed using GraphPad Prism (version 8.0).

Ethics approval and consent to participate. All experimental procedures were undertaken in accordance with the Children's Hospital of Soochow University. Informed consent was obtained from the study participants.

Data availability. All data generated or analyzed during this study are included in this article. The data sets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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