Biological Trace Element Research (2023) 201:220–228
https://doi.org/10.1007/s12011-022-03133-6

Subacute Cadmium Exposure Induces Necroptosis in Swine Lung via Influencing Th1/Th2 Balance

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Received: 1 November 2021 / Accepted: 23 January 2022 / Published online: 3 February 2022

Abstract

Cadmium (Cd) is a type of toxic substance, which widely exists in nature. However, the effect of Cd exposure on the toxicity of swine lungs and its underlying mechanism involved have not yet been reported. In our study, we divided swine into two groups, including a control group (C group) and Cd-exposed group. Swine in the C group were fed a basic diet, whereas swine in the Cd group were fed a 20 mg Cd/kg diet. Immunofluorescence, qRT-PCR, western blot analysis, and H&E staining were performed to detect necroptosis-related indicators. Our results found that after Cd exposure, Th1/Th2 imbalance occurred, miR-181-5p was down-regulated, TNF-α expression was increased, and the NF-κB/NLRP3 and JAK/STAT pathways and RIPK1/RIPK3/MLKL axis were activated. Furthermore, histopathological examination showed necrosis in swine lung after Cd exposure. Together, the above-mentioned results indicate that subacute Cd exposure is closely linked with necroptosis in swine lung. Our study provided evidence that Cd may act through miR-181-5p/TNF-α to induce necroptosis in swine lung. The findings of this study supplement the toxicological study of Cd and provide a reference for comparative medicine.

Keywords Cadmium · Swine · Lung · Necroptosis · Th1/Th2 balance

Introduction

Cadmium (Cd) is one of the main environmental pollution metals with biological toxicity, which dangers biological health through several exposure routes due to its high mobility and bioavailability [1]. Cd is derived from industrial waste gas, wastewater, dyes, fuels, and other non-standard use, as well as from nickel–cadmium batteries, cigarette exhaust and other waste gas, rice and other grains, vegetables [2, 3], aquatic organisms, etc., and eventually enter the food chain. Swine are common mammals that serve as main food of human beings. Cd targets multiple organs and harms human health. It has been reported that long-term exposure to Cd can cause dysplasia of the cardiovascular system in mice [4]. In male rats, Cd induces oxidative stress and apoptosis in the liver and autophagy of liver cells [5, 6]. Inflammation caused by Cd acts through the NF-κB pathway in the spleen of swine [7]. Cd induces apoptosis of porcine small intestinal epithelial cells at a dose of 30 mg/kg [8]. Moreover, Cd (50 mg/kg) can affect the activity of GSH-Px in swine blood at 6 weeks of age, thereby affecting the survival rate [9]. The lungs are important organs involved in metabolism and can be damaged by Cd accumulation in the body [10]. Straif et al. [11] showed that long-term exposure to Cd caused lung cancer, pulmonary fibrosis, chronic obstructive pulmonary disease, etc. [12]. Cd exposure induced oxidative stress and apoptosis by increasing reactive oxygen species (ROS), lipid peroxidation, and membrane damage in A549 cells [13]. Furthermore, Cd exposure enhances the virulence of *Beauveria bassiana* by damaging the immune system of insects [14]. In the coelomic epithelium cells of earthworms, Cd can damage innate immunity [15]. Cd exposure caused necroptosis, apoptosis, and autophagy through various mechanisms, such as the production of ROS and inhibition of enzyme activity [16, 17]. Cd can lead to impaired mitochondrial function of A549 cells. Abnormally increased Atg 4 can bind Bcl-2, by disassociating the Bcl-2-Beclin1 complex to cause autophagy [18]. Cd has the ability to promote

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apoptosis and necroptosis of carp lymphocytes by regulating the miR-216a-PI3K/Akt axis [19]. Cd has been reported to induce necroptosis of porcine cardiomyocytes [3], human neurons [20], and chicken hepatocytes [21].

Th1 cells (T helper 1 cell) and Th2 cells (T helper 2 cell) are the main subtypes of Th cells that differentiate from Th0 cells. Th1 cells are mainly involved in inflammatory responses and cellular immune response, while Th2 cells are mainly involved in humoral immunity by assisting B-type lymphocytes to differentiate and secrete antibodies [22]. Interleukin-1β (IL-1β), interleukin-2 (IL-2), interleukin-12 (IL-12), tumor necroptosis factor-α (TNF-α), and interferon-γ (IFN-γ) are markers of Th1. Th2 cells that secrete interleukin-4 (IL-4), interleukin-5 (IL-5), interleukin-10 (IL-10), and chemokine receptor 4 (CCR4) are the main detection indexes. Menghao et al. demonstrated that exogenous stimuli can activate the Fos/IL-8 signaling pathway in the trachea of chickens, thereby causing oxidative stress and inflammatory damage [23]. Cd, as an exogenous heavy metal stimulator, can increase IL-4 and IL-6 [24], affect the balance of Th1/Th2 [25], and eventually trigger the body's inflammatory responses. Cd exposure causes the secretion of a large number of inflammatory cytokines, which trigger necroptosis [26]. Lipopolysaccharide induces cell death by activating macrophages [27]. Influenza virus can activate PPAR-α by inducing the CYP450 product, NF-κB mediated inflammatory pathway, eventually leading to necroptosis, which significantly increases the mortality rate [28]. Sattar et al. showed that baicalin ameliorated apoptosis through the NF-κB pathway [29]. MicroRNAs (miRNAs) exist in a wide variety of organisms and play an important role in the regulation of gene expression at the post-transcriptional level. Cd has been shown to regulate the expression of miRNA [19]. miRNA directly regulates TNF-α, a marker of Th1 cells, and its mediated cell death signaling pathway [30]. In addition, Cd can induce necroptosis of carp lymphocytes by regulating inflammatory responses and glucose metabolism [31]. Furthermore, miRNA plays a regulatory role in human Th2 cell marker CCR4 [32].

Cd is involved in regulating miRNA expression, affects the Th1/Th2 balance, and causes necroptosis. Inflammation is closely related to necroptosis, but whether Cd exposure can induce inflammatory responses through miRNA regulation of the Th1/Th2 balance to induce necroptosis of lung tissue remains unclear. Therefore, we established a swine model of Cd exposure for further study. In this study, TargetScan (http://www.targetscan.org/) was applied to predict miR-181-5p binding sites in many species. Next, qRT-PCR, western blot analysis, hematoxylin and eosin (H&E) staining, and immunofluorescence techniques were used to determine the expression of miR-181-5p, Th1/Th2 cytokines, NF-κB pathway–related genes, and JAK/STAT pathway–related genes under Cd exposure conditions. The toxic effect and underlying mechanisms of Cd on swine lung were determined to provide technical reference for the research methods of Cd toxicity on swine lung.

Materials and Methods

Ethical Statement

All procedures used in this study were approved by the Northeast Agricultural University Animal Protection and Utilization Institutional Committee (SRM-111).

Animals and Treatments

Ten male weaned piglets (6 weeks old) were purchased from a local farm. After acclimating to their new surroundings for 1 week, piglets were randomly divided into two groups, including a control group (C group) and a Cd-exposed group (Cd group). Piglets were fed a standard basal diet and purified water. Piglets in the C group were fed a basic diet, and piglets in the Cd group were fed a basic diet supplemented with 20 mg/kg cadmium chloride ($CdCl_2$) [33, 34]. All experimental animals were kept under the same environmental conditions and fed three times a day with free access to water. Animals were euthanized on day 40 of the experiment, and lung tissues were collected, processed, preserved, and cryopreserved at – 80 °C.

Quantitative Real-Time PCR (qRT-PCR)

qRT-PCR was performed on a Light Cycler®480 II Detection System (Roche, Basel, Switzerland). The reactions were performed in a 10μL reaction mixture as presented in the study by Yin et al. [35]. Non-specific amplification was eliminated following software analysis of the dissociation curve. The relative expression of each mRNA was calculated. Primer sequences used are displayed in Table 1.

Western Blot Analysis

Briefly, tissues taken before were lysed in ice-cold RIPA with PMSF protease inhibitor (Beyotime, Shanghai, China). The supernatant was obtained by centrifugation and the concentration of protein was identified by using a BCA assay kit (Beyotime, Shanghai, China). From each group, an equal amount of protein was loaded and separated on 6–15% SDS-PAGE gels, transferred onto NC membrane, and blocked for 2 h at 37 °C with 5% milk-TBST. Membranes were incubated either overnight at 4 °C with primary antibodies against. The antibodies are shown in Table 2. Then, membranes were washed with TBST three times and incubated with horseradish peroxidase (HRP)–conjugated secondary antibodies

Table 1 The primers used in the present study

Gene	Forward 5' to 3'	Reverse 5' to 3'
miR-181d-5p	CGCGCGCAACATTCATTGTTGTCGGT	
U6	CTCGCTTCGGCAGCACA	
TNF-α	AGGTGATGGTGTCGAGGAGGAAG	AGACTTGTTGAGCGTGAAGCAGAC
IL-12A	AAGCCCTCCCTGGAAGAACTGG	TGGTCACCGCACGAATTCTGAAG
IL-12B	TCAGCCAAGGTTACATGCCACAAG	CAGGACACAGATGCCCATTCACTC
IL-12RB2	AAGGTCGCCACGCCTCTGAG	ATCCACTGCGGGTCCTGTCTC
IL-2	TTTACATGCCCAAGCAGGCTACAG	TAGCACTCCCTCCAGAGCTTTGAG
IL-1β	GCCAACGTGCAGTCTATGGAGTG	GGTGGAGAGCCTTCAGCATGTG
IFN-γ	CGCAAAGCCATCAGTGAACTCATC	TTTGATGCTCTCTGGCCTTGGAAC
IL-4	GCTTCGGCACATCTACAGACACC	TCTTGGCTTCATGCACAGAACAGG
IL-5	GTCTTGGAGCTGCCTACGTTAGTG	CCATCGCCTATCAGCAGAGTTCG
IL-6	ATAAGGGAAATGTCGAGGCTGTGC	GGGTGGTGGCTTTGTCTGGATTC
IL-10	GGGTGGCAGCCAGCATTAAGTC	CGCCCATCTGGTCCTTCGTTTG
CCR4	GTGGTGGTTCTGGTCCTGTTCAAG	GGTCTGAGATGGCAAGGTTGAGC
NF-κB	AGGAGCAGTGTAGTGGCCGTAC	CTCTGGATCTCGCTGTTGTGTCTG
IκK-α	TGGTGTCGCTCTTGTTGAAGTGTG	GCTGCTGTATCCGAGTGCTTGG
IκK-β	TGGTGTCGCTCTTGTTGAAGTGTG	GCTGCTGTATCCGAGTGCTTGG
NLRP3	CAGCAGATGGATAGCGGCAAGAG	CAGTGAGCAGAGACCCCAGAGG
JAK1	GCATCCATCTGGCCGTGGTAATC	GCATCCATCTGGCCGTGGTAATC
JAK2	AGCCCACCAGCGGAATTTATGC	GCATCCATCTGGCCGTGGTAATC
STAT1	TCTCTGCCCGTCGTGGTGATC	AGGTGGGTTCAGGAAGAAGGACAG
STAT2	CCCAGCCCTGTATGCCTCAAAC	GAGTCTCACCAGCAGCCTTGTTC
STAT3	GTGGAGAAGGACATCAGCGGTAAG	AGGTAGACCAGCGGAGACACAAG
STAT4	CCTGGGTGGACCATTCTGAAACTG	GTAGAGCAGACAACCGGCCTTTG
STAT6	CCACGGCAACCAAGACAACAATG	CAAAGGGCACACGGTCCATCTC
RIPK1	GGAGCATAACGAGCAGCGGAAG	CCAGAGCACGATGGCGAAGC
RIPK3	AAAGGAGGATTTGGCACGGTCTTC	CCAGCAGGAGCAGCACATAGC
MLKL	CAGTGCTGGCGTCTTGGGAAC	TCAGGGCGGTGGTTATCTCAGG
Caspase 8	GAAAAGCAAGCCTCGGGGATACTG	GAGCCGCTGCATCCAAGTCTG
β-actin	AATCCTGCGGCATCCACGAAAC	CAGCACCGTGTTGGCGTAGAG

Table 2 The antibodies used in the present study

Antibody name	Dilution ratio	Formula weight (kDa)
TNF-α	1:1000	27 kDa
NF-kappaB	1:1000	55 kDa
NLRP3	1:1000	110 kDa
JAK2	1:1000	130 kDa
STAT3	1:1000	82 kDa
RIPK1	1:1000	75 kDa
RIPK3	1:500	57 kDa
MLKL	1:500	55 kDa
Caspase 8	1:500	18 kDa
beta-actin	1:10,000	43 kDa

(1:10,000, ABclonal, Wuhan, China) for 2 h. The bands were visualized by using an ECL kit (Biosharp, Beijing, China) and scanned with a Gel imager (GE Healthcare, Shanghai, China) as presented in the study by Li et al. [36].

Immunofluorescence Analyses

Frozen lung sections were obtained for DAPI staining. TNF-α antibody (Abcam) and anti-CCR4 antibody (Abcam) were used to stain Th1 and Th2 cells in frozen lung sections, respectively. Sections were observed under a fluorescence microscope (Nikon, Japan). After the sections were dehydrated, antigens were recovered in EDTA buffer (pH8.0, ServiceBio, G1206) and sealed with BSA. Sections were incubated overnight with primary antibodies at 4 °C. After washing, a secondary antibody was added and sections were incubated at room temperature for 60 min. Nuclei were stained blue by DAPI, red by TNF-α, and green by CCR4. Finally, a fluorescence microscope (Nikon, Japan) was used for observation and image collection.

Hematoxylin and Eosin (H&E) Staining

For histological observation, light microscopy was used to observe the pathological changes that occurred in the lung of swine. Briefly, lung tissues of each group were fixed with 10% formaldehyde for 24 h and subjected to dehydration embedding. Subsequently, 4–5-μm-thick paraffin sections were cut for hematoxylin and eosin (H&E) staining and histological analyses [37].

Statistical Analysis

Results are expressed as the mean ± the standard error of the mean (SEM). *n* represents the number of individual swine as indicated in the figure legends. We performed the Shapiro–Wilk normality test in experiments that have a relatively large sample size ($n > 5$) and found that these data pass the normality test (alpha = 0.05). Data were further analyzed with two-tailed Student's and Welch's *t*-test for two-group comparisons, ANOVA for multiple comparisons. For both one-way ANOVA and two-way ANOVA, Tukey's post hoc multiple comparisons were applied as recommended by Prism. In all cases, GraphPad Prism (GraphPad Software Prism 8, San Diego, CA) was used for the calculations.

Results

Cd Exposure Influences miR-181-5p and TNF-α Expression

In many species, binding sites exist between miR-181-5p and TNF-α (http://www.targetscan.org/). To determine the effect of Cd on miR-181-5p expression in the lung, we determined the mRNA expression. We found that miR-181-5p was down-regulated in the Cd group compared with the C group. Moreover, the level of TNF-α expression that was inhibited by miR-181-5p was obviously up in the Cd group (Figs. 1 and 3C). In summary, Cd exposure influenced miR-181-5p and TNF-α expression.

Cd Exposure Disrupts the Balance Between Th1/Th2

The balance of Th1/Th2 cells plays a vital role in maintaining the normal physiological state of the body. The results

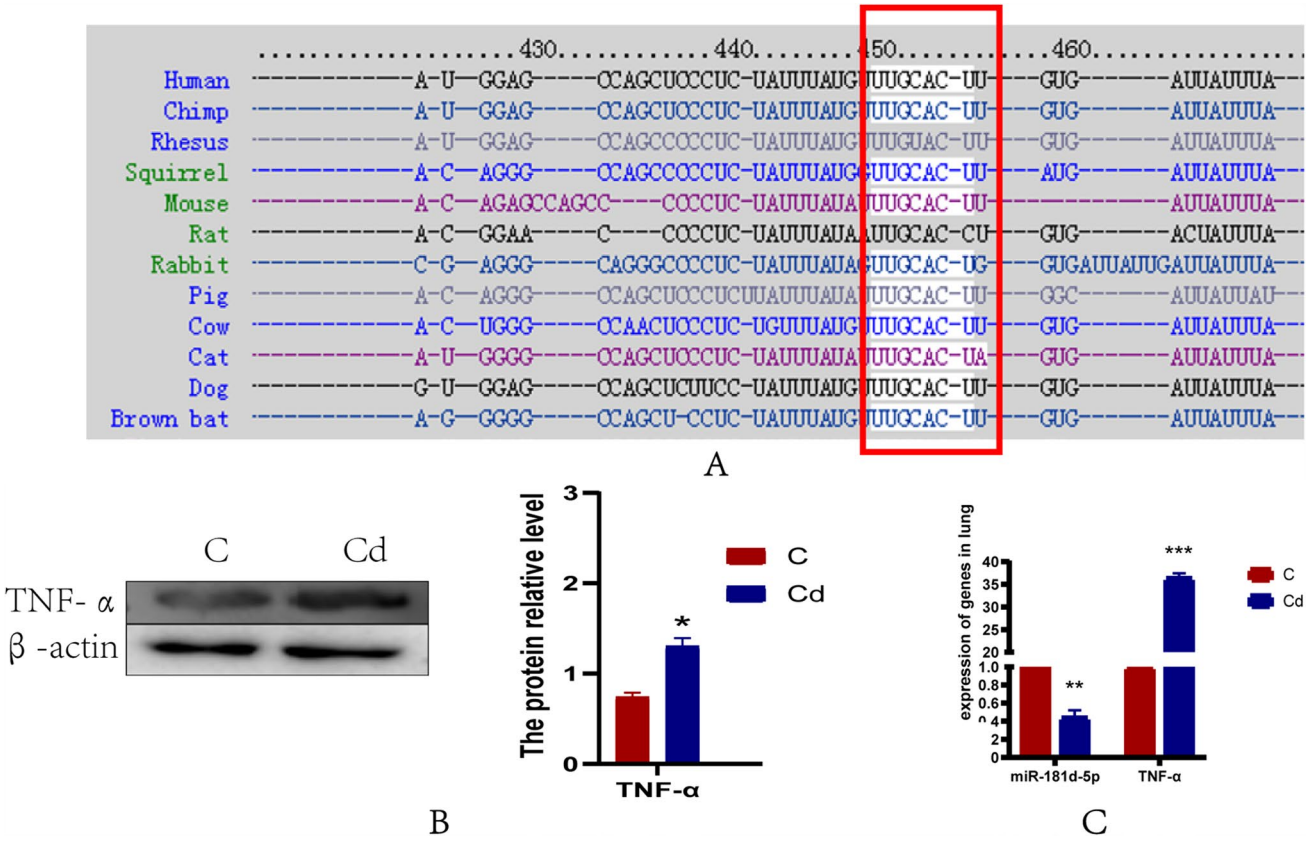

Fig. 1 **A** The predicted binding site between miR-181-5p and TNF-α. **B** and **C** Protein, mRNA expression in swine lung. Data with asterisk represent significant difference between groups ($p < 0.05$), without asterisk represent no statistically significant difference ($p > 0.05$)

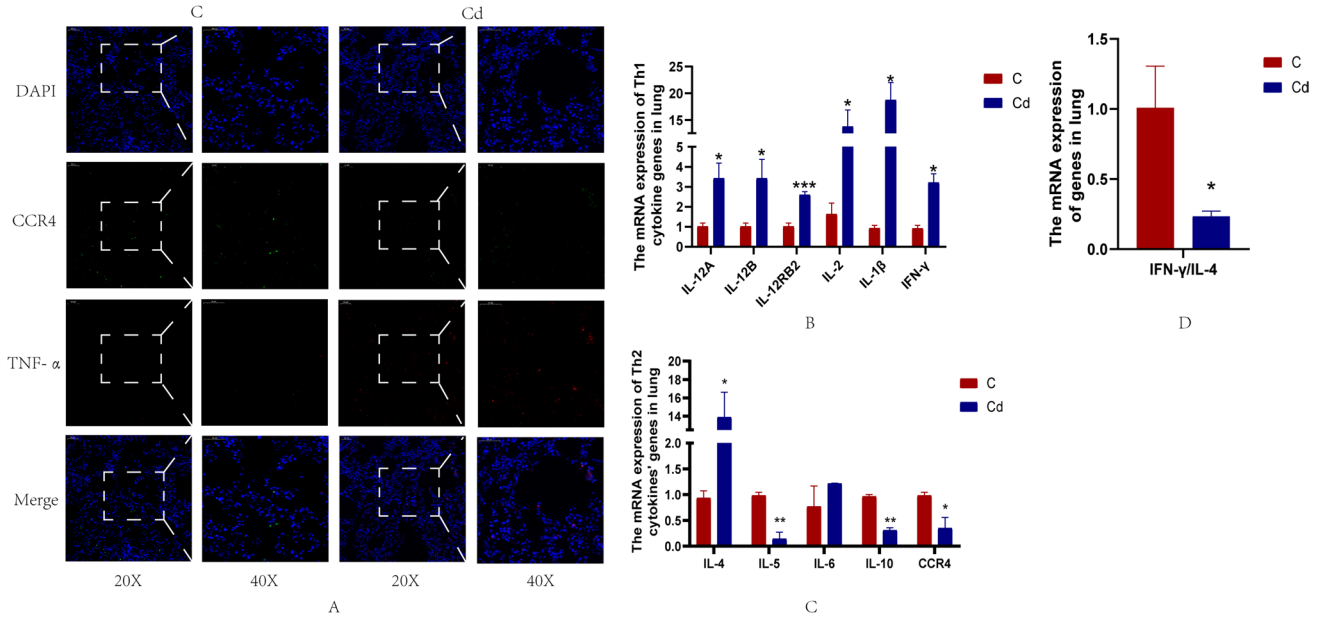

Fig. 2 **A** Immunofluorescence results. DAPI dyes the nucleus blue, CCR4 shows green, and TNF-α dyes red. **B** and **C** Relative gene expression. **D** The ratio of IFN-γ and IL-4. Data with asterisk represent significant difference between groups ($p < 0.05$), without asterisk represent no statistically significant difference ($p > 0.05$)

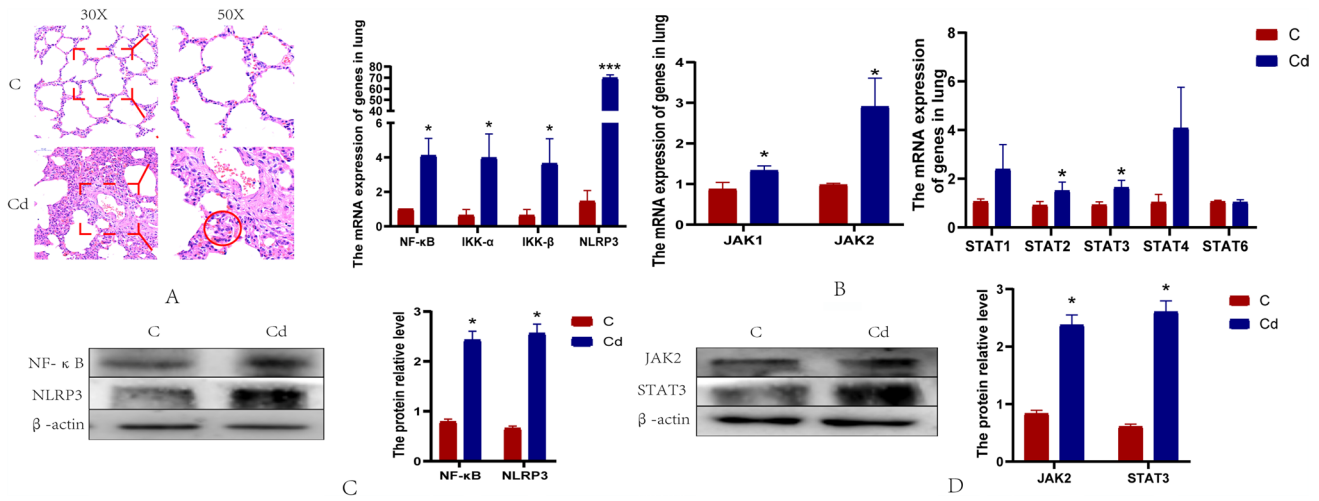

Fig. 3 **A** H&E staining under different multiple microscopes. Compared with the C groups, Cd group had significantly wider alveolar intervals. Due to the necrosis of a large number of cells in the alveolar wall, the cell nucleus is deformed, dissolved, and ruptured (the part in the red circle), and the alveolar wall has been filled with exudate from cell necrosis and disintegration, which causes the alveolar wall to thicken. **B**, **C**, and **D** NF-κB pathway, JAK/STAT pathway, and NLRP3 mRNA and protein level. Data with asterisk represent significant difference between groups ($p < 0.05$), without asterisk represent no statistically significant difference ($p > 0.05$)

of immunofluorescence analysis showed that compared with the C group, the intensity of red fluorescence (TNF-α) was increased and the intensity of green fluorescence (CCR4) was decreased in the Cd group. These findings indicated that the number of Th1 cells was significantly increased and the number of Th2 cells was down-regulated in the Cd group (Fig. 2A). Next, we determined the cytokines produced by Th1/Th2 in swine lung tissues. Related mRNA gene and protein expression is shown in Fig. 2. We found that the mRNA expression of Th1 cytokines IL-2, IL-1β, IL-12, and

IFN-γ was significantly increased in the Cd group (Fig. 2B). Furthermore, the mRNA expression of Th2 cytokines IL-5 and IL-10 was significantly decreased, the mRNA expression of CCR4 was not significantly changed, and the mRNA expression of pro-inflammatory cytokines IL-4 and IL-6 was significantly up-regulated in the Cd group (Fig. 2C). The ratio of IFN-γ and IL-4 was also changed after Cd treatment (Fig. 2D). The protein expression we detected matched the mRNA expression mentioned above (Fig. 2). The data above proved that Cd disrupted the balance between Th1 and Th2 in swine lung.

Cd Exposure Activates NF-κB and JAK/STAT Pathway

To investigate whether exposure to Cd caused immune function changed in swine lung, we detected NF-κB and JAK/STAT pathway–relative genes at the mRNA and protein level (Fig. 3). The results showed that NF-κB, IκK-α, IκK-β, JAK, and STAT were significantly increased after Cd treatment. H&E staining also affirmed that inflammation occurred in swine lung treatment by Cd (Fig. 3A). We found through these results that NF-κB and JAK/STAT pathways were activated by $CdCl_2$.

Cd Exposure Causes Necroptosis in Swine Lung Through the RIPK1/RIPK3/MLKL Axis

When inflammation pathways were enabled, changes in NLRP3 were worth testing. As expected, NLRP3 was increased after Cd exposure. Concurrently, the RIPK1/RIPK3/MLKL axis was activated, and caspase-8 was down-regulated compared with the C group (Fig. 4). Furthermore, H&E staining was used to determine if necrosis occurred in swine lung (Fig. 3). Thus, Cd exposure caused necroptosis in swine lung through the RIPK1/RIPK3/MLKL axis.

Discussion

Abundant studies have shown that exposure to Cd causes apoptosis and immune dysfunction [38]. Cd exposure negatively affected human lung function [39]. It has been

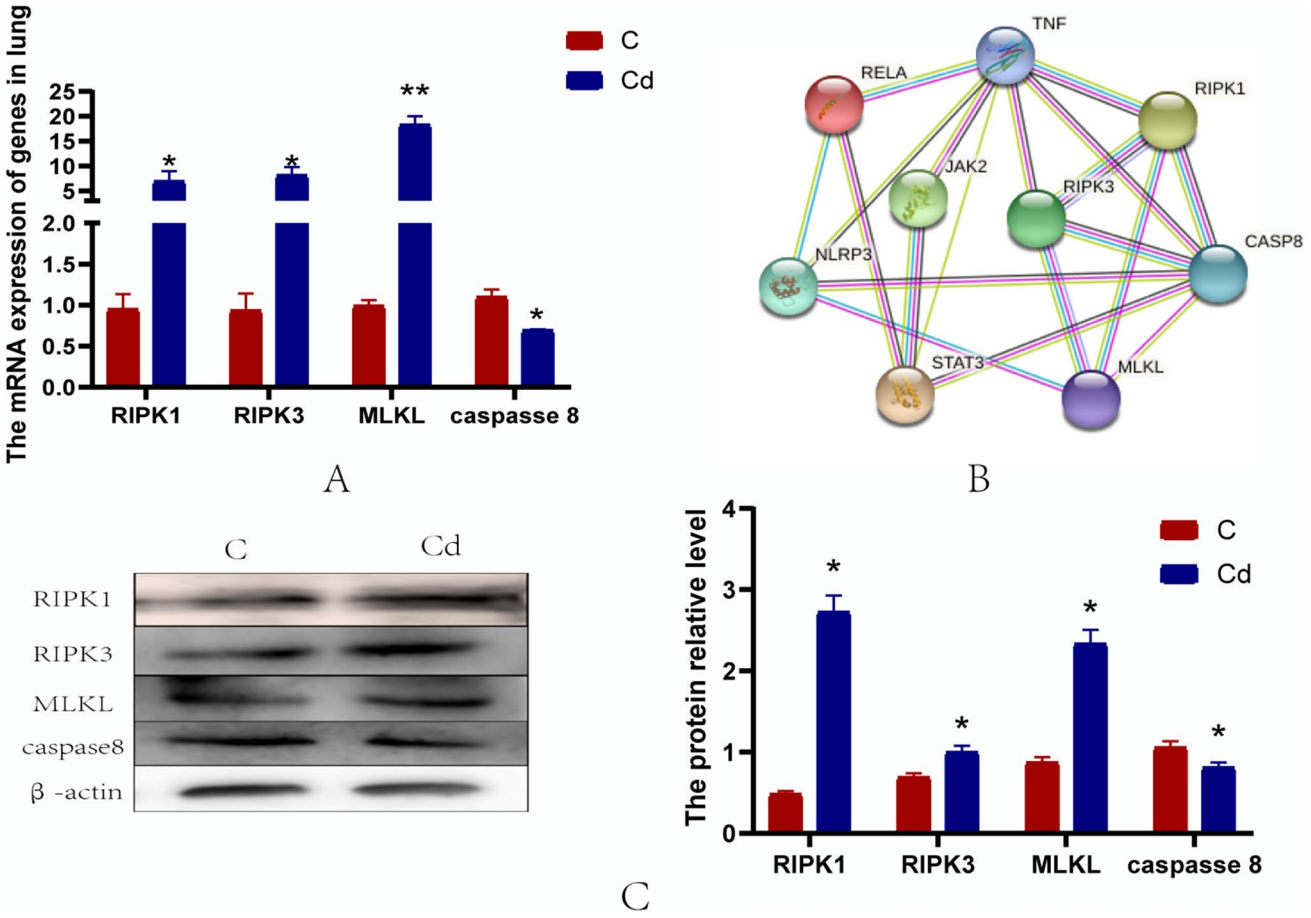

Fig. 4 **A** and **C** Relative index's mRNA and protein expression. **B** Detected proteins' relationship from string-db.org. Data with asterisk represent significant difference between groups ($p < 0.05$), without asterisk represent no statistically significant difference ($p > 0.05$)

suggested that Cd can induce necroptosis of lymphocytes in chicken peripheral blood through RIPK1/RIPK3/MLKL pathway [40]. However, the effects of Cd exposure on swine lung tissue have not yet been reported. Our study showed that necroptosis occurred in swine lung after Cd exposure (20 mg/kg). The expression of miR-181-5p in swine lungs was decreased, and the expression of Th1 cytokines IL-1β, IL-2, TNF-α, and IFN-γ was increased. The decreased expression of Th2 cytokines IL-5 and IL-10 resulted in Th1/Th2 imbalance and Th1 shift. No significant change was observed in the expression of CCR4, activation of the NF-κB and JAK/STAT pathway, and increased expression of the RIPK1/RIPK3/MLKL axis.

miRNA widely exists in organisms and plays a vital role in the regulation of gene expression at the post-transcriptional level. It has been reported that Cd can induce G2/M cell cycle arrest by up-regulating miRNA in the human body [41]. In addition, Cd induces mouse ovary apoptosis through miRNA [42]. Other's experiment showed that miR-155 could affect the immune system of carp by regulating the expression of TNF-α, IFN-γ, IL-6, and other cytokines [43]. We demonstrated the relationship between miR-181-5p and TNF-α in swine through the miRNA target gene prediction website. Bioinformatics analysis showed that the 3'UTR of TNF-α contained the binding site of miR-181-5p, which was conserved in swine, human, mice, rabbits, cattle, and other animals. The experiment has proven that miR-181-5p targets TNF-α in carp lymphocytes to regulate immune function [31]. We predicted the same miR-181-5p binding sites to be present in swine as in carp by TargetScan. In our experiment, we demonstrated that the expression of miR-181-5p decreased and the expression of Th1 cytokine TNF-α increased in swine lung cells after Cd exposure. These results, combined with TargetScan prediction results, suggested that the decrease of miR-181-5p was one of the influencing factors of increased TNF-α expression after Cd exposure. Other factors that caused an increase in TNF-α expression need to be verified by further experiments. Our findings indicated that miR-181-5p could regulate TNF-α negatively and affect the Th1/Th2 balance in swine lung cells after Cd exposure. Several experiments have shown that the expression of TNF-α is proportional to the number of Th1 cells [44]. Immunofluorescence interpretation indicated that the number of Th1 cells increased significantly after Cd treatment, thereby indicating that TNF-α could cause a Th1/Th2 imbalance and favor Th1. The balance between Th1 and Th2 can be confirmed by the expression of cytokines [45]. Therefore, we determined Th1- and Th2-related cytokines, and found that the mRNA levels of Th1 cytokines IL-1β, IL-2, IL-12B, and IFN-γ were significantly increased, and the mRNA expressions of Th2 cytokines IL-5 and IL-10 were down-regulated while the expression of IL-4 and IL-6 was up-regulated. The occurrence of a Th1/Th2 imbalance after Cd exposure was confirmed.

The Th1/Th2 balance is crucial for the body to maintain normal physiological conditions. In previous reports, it has been shown that deviation of the balance in any direction will lead to disorders of the body's immune system [46–48]. Zhang et al. [49] found a Th1/Th2 imbalance when necroptosis occurred in swine ileal tissue. IL-1β, IL-12, IL-4, IL-6, IFN-γ, and TNF-α have pro-inflammatory effects [50]. It has been reported that Th1 cytokines IL-1β and IL-2 can activate NF-κB pathways [51, 52], and that IFN-γ can activate the JAK/STAT pathways [53]. We detected the NF-κB pathway– and JAK/STAT pathway–related gene expression, and found both pathways were activated. Furthermore, the expression of NLRP3 was increased. It has been shown that TNF-α recruits RIP kinase upon activation of NF-κB, which is essential in inflammation [54, 55]. TNF-α-recruited RIPK1 and RIPK3 could interact with each other through the homotypic interaction motif RHIM-RHIM to form bad dead bodies and cause necroptosis [56]. Previous experiments have shown that Cd can induce necroptosis of human and mouse neurons and liver cells [20]. RIPK1 and RIPK3 are markers of necroptosis. The activated kinase domain-like protein of RIPK3 occurred membrane translocation, causing cell membrane damage and disruption of cell homeostasis [57, 58]. In previous studies, it has been shown that inhibition of aspartate proteolytic enzyme 8 (caspase-8) also causes necroptosis [59]. In our study, we demonstrated increased expression of necroptosis markers RIPK1, RIPK3, and MLKL, and decreased expression of caspase-8, which confirmed that Cd can induce a TNF-α surge to cause a Th1/Th2 imbalance resulting in programmed necroptosis of swine lung tissue.

In conclusion, our study showed that Cd exposure can negatively regulate TNF-α by down-regulating miR-181-5p, induce a Th1/Th2 imbalance to favor Th1, activate NF-κB and JAK/STAT pathways, recruit the necroptosis complex RIPK1-RIPK3, and cause necroptosis in swine. Furthermore, our results demonstrated that Cd induced a Th1/Th2 imbalance through miR-181-5p-induced necroptosis of swine lung tissue. In most cases, the lungs are damaged by inhalation of toxic substances, but Cd exposure through oral means can accumulate in the blood, thereby causing pulmonary toxicity as it circulates. Although Cd exposure of the respiratory system directly leads to lung injury, it has been proved that dietary Cd exposure can also cause apoptosis of lung epithelial cells through oxidative stress [31]. Therefore, we used oral Cd administration to explore whether Cd exposure could cause lung damage. Finally, our study has some limitations. This type of administration does not replicate respiratory exposure. Thus, due to the increasing accumulation of Cd in the environment, the results of this study are valuable for understanding the toxicity of Cd to the

lung and suggest that correction of the Th1/Th2 imbalance may improve the necroptosis of lung tissue after exposure to heavy metals.

Acknowledgements Supported by China Agriculture Research System of MOF and MARA. The authors extend their sincere thanks to the members of the veterinary internal medicine laboratory and Key Laboratory for Laboratory Animals at the College of Veterinary Medicine, Northeast Agricultural University.

Author Contribution Wenyue Zhang: investigation; formal analysis; writing—original draft. Xinyue Sun: draft layout, visualization. Xu Shi: software, investigation. Xue Qi: software. Hongjin Lin: conceptualization; resources; supervision; validation; writing—review and editing.

Data Availability The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Declarations

Ethical approval All other authors have read the manuscript and have agreed to submit it in its current form for consideration for publication in the Journal.

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