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ORIGINAL ARTICLE

Protein Kinase C Theta Inhibition Attenuates Lipopolysaccharide-Induced Acute Lung Injury through Notch Signaling Pathway *via* Suppressing Th17 Cell Response in Mice

Mengqin Li,[1] Yan Zhao,[1] Jing He,[1] Wang Deng,[1] Li Cheng,[2] Zhi Jiang,[3] and Daoxin Wang[1,4]

Abstract— Acute lung injury (ALI)/acute respiratory distress syndrome is characterized by increased pulmonary inflammation, where T helper 17 (Th17) cells play an important regulatory role. Notch signaling critically regulates Th17 differentiation and is known to be linked with proximal T cell by protein kinase C theta (PKCθ). We hypothesized that PKCθ inhibition could attenuate ALI by suppressing Th17 response *via* the Notch signaling pathway. Male C57BL/6 mice were treated with phosphate-buffered saline (PBS), lipopolysaccharide (LPS), LPS and *N*-[*N*-(3,5-difluorophenacetyl)-l-alanyl]-*S*-phenylglycine *t*-butyl ester (DAPT, a Notch signaling inhibitor), or LPS and PKCθ inhibitor (PI), and the bronchoalveolar lavage fluid (BALF), blood, and lung tissues were harvested at 48 h after the LPS challenge. CD4$^+$ T cells were treated with DAPT or PI and harvested after 72 h. PKCθ inhibition markedly attenuated pathological changes and decreased the wet to dry weight ratio of the mouse lungs. The total cell and neutrophil counts, tumor necrosis factor-α (TNF- α) in BALF, myeloperoxidase activity in lung tissue, and the leukocyte count in whole blood were markedly reduced by PKCθ inhibition. The concentration of interleukin (IL)-17 and IL-22 in BALF, and the percentage of CD4$^+$IL-17A$^+$ T cells in the lungs were significantly downregulated by PKCθ inhibition. A similar trend was observed for the expression of retinoic acid-related orphan receptor gamma t and IL-23 receptor after PKCθ inhibition accompanied with inactivation of the Notch signaling pathway *in vivo* and *in vitro*. Collectively, these data demonstrated that PKCθ inhibition protects against LPS-induced ALI by

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[1] Department of Respiratory and critical care medicine, The Second Affiliated Hospital of Chongqing Medical University, 76 Linjiang Road, Yuzhong District, Chongqing, 400010, China

[2] Physical Examination Center, The Second Affiliated Hospital of Chongqing Medical University, Chongqing, China

[3] Department of Emergency, The Affiliated Hospital of North Sichuan Medical College, Chongqing, China

[4] To whom correspondence should be addressed at Department of Respiratory and critical care medicine, The Second Affiliated Hospital of Chongqing Medical University, 76 Linjiang Road, Yuzhong District, Chongqing, 400010, China. E-mail: wangdaoxin11@163.com

suppressing the differentiation and pathogenicity of Th17, at least partially, through a Notch-dependent mechanism.

KEY WORDS: Acute lung injury; PKCθ; Th17; Notch; IL-23R; RORγt.

INTRODUCTION

Acute respiratory distress syndrome (ARDS) is a common life-threatening condition with high morbidity [1]. According to the Berlin Definition, acute lung injury (ALI), which was first described in 1967, is now included as mild ARDS [2]. T helper 17 (Th17) cells are one of the subsets of $CD4^+$ T cells and are characterized by the production of interleukin (IL)-17 (IL-17A, IL-17F), IL-22, and IL-26 [3]. Accumulating evidence has indicated that excessive recruitment and activation of neutrophils in the airways significantly contribute to the progression of ARDS [4, 5]. IL-17 plays a pro-inflammatory role in the airways by recruiting and activating neutrophils [5]. In the human respiratory tract, IL-17 and IL-22 are involved in the immunological responses to endotoxins and markedly enhance paracellular permeability [6, 7]. The increase of IL-17 production is consistent with the development of ALI and directly disrupts human alveolar epithelial barrier integrity [8, 9]. Moreover, the broad distribution of IL-17 and IL-22 receptors induces massive tissue reaction.

The transcription factor, retinoic acid-related orphan receptor-gamma t (RORγt), appears to be required for Th17 cell differentiation. Notch receptors undergo several steps before activation. Upon ligand binding, the extracellular domain of the Notch receptor is removed, which induces a conformational change that allows cleavage of the Notch protein by matrix metalloproteinase. Subsequently, the plasma membrane-embedded γ-secretase complex liberates an active Notch intracellular domain (NICD). NICD translocates to the nucleus and activates transcription thereby modulating the development of cells [10]. The Notch signaling pathway plays a pivotal role in the differentiation of Th17 cells and Notch inhibition leads to downregulation of Th17 differentiation [11–13]. Furthermore, not all Th17 cells induce tissue inflammation. Th17 cells require IL-23R in order to become pathogenic. Notch signaling pathway may be one of the key checkpoints that determine the balance between the pathogenic and non-pathogenic states of Th17 cells by regulating IL-23R [14].

Protein kinase C theta (PKCθ) facilitates Notch activation, as it augments the generation of the lamellar actin network that is required to recruit the matrix metalloproteinase to the immunological synapse [15]. Moreover, PKCθ is not necessary for the protection against pathogens, which contributes to the selective effect of immunoregulation by targeting PKCθ [16]. Furthermore, PKCθ can translocate to the immunological synapse of T cells [17]. Therefore, we hypothesized that PKCθ is an ideal target for attenuating ARDS *via* the suppression of Th17 cells.

In this study, we elucidated the role of PKCθ in a lipopolysaccharide (LPS)-induced ALI model. We found that PKCθ inhibition can attenuate ARDS by suppressing the differentiation and pathogenicity of Th17 cells *via* Notch signaling.

MATERIALS AND METHODS

Animals

All animal experiments were performed in accordance with the ethical guidelines of the National Institutes of Health on Animal Care, and the study protocol was approved by the Ethics Committee of the Second Affiliated Hospital of Chongqing Medical University. Male C57BL/6 mice, 6–8 weeks old and SPF grade, were obtained from the Department of Laboratory Animal Center of Chongqing Medical University. The mice were housed in an air-conditioned room with a 12-h dark/light cycle and granted free access to water and food.

Acute Lung Injury Model

The mice were allowed a minimum facility-acclimatization period of 7 days before being subjected to experiments. Healthy male C57BL/6 mice were randomly divided into four groups ($n = 3$): control group, LPS group, LPS+ N-[N-(3,5-difluorophenacetyl)-l-alanyl]-S-phenylglycine t-butyl ester (DAPT, a Notch signaling inhibitor) group (LPS + DAPT group), and LPS + PKCθ-inhibitor (PI) group (PI group). The mice were anesthetized through intraperitoneal administration of sodium pentobarbital (50 mg/kg) following which, 3 mg/kg LPS (Solabio, Beijing, China) in 50 μL of phosphate-buffered saline (PBS) or sterile PBS alone (control group) was instilled intratracheally. Thirty minutes before and 24 h after the LPS challenge, DAPT (40 mg/kg/day, MCE, Bridgewater, USA) was administered intraperitoneally (i.p.) to the LPS

+ DAPT group mice, and PI (a cell-permeable selective PKCθ peptide inhibitor, 0.2 mg/kg; Merck Millipore, Darmstadt, Germany) was administered i.p. to the LPS + PI group. All mice were sacrificed following the collection of whole blood, bronchoalveolar lavage fluid (BALF), and lung tissues 48 h after the LPS challenge.

Cell Culture

Single cell suspensions of mouse spleens were obtained by mechanical disruption of the tissues through a 40-μm cell strainer. Naive CD4$^+$ T cells were isolated from the prepared suspensions using magnetic-activated cell sorting separation according to the manufacturer's instructions (negative selection; Miltenyi Biotec, Bergisch Gladbach, Germany). The purified cells were pretreated at 37 °C for 30 min with 10 μmol/L DAPT (MCE, Bridgewater, NJ, USA) or 20 μmol/L PI (Merck, Darmstadt, Germany). Naive CD4$^+$ T cells were cultured in 96-well plates coated with anti-CD3 antibody (4 μg/mL, eBioscience, San Diego, CA, USA). For Th17 differentiation, cell culture medium containing anti-CD28 (2 μg/mL, eBioscience, CA, USA), IL-6 (30 ng/mL; R&D Systems, Minneapolis, MN, USA), and transforming growth factor-β (3 ng/mL, R&D Systems, Minneapolis, MN, USA) were used. After 72 h, the cells were harvested for quantitative real-time polymerase chain reaction (q-PCR) and Western blot analysis.

Lung Wet/Dry Weight Ratio

Pulmonary edema was evaluated by the wet to dry weight ratio (W/D ratio) of the lungs. After being weighed, the left lungs were dehydrated in an oven at 80 °C for 24 h. Then, the dry weight was measured to calculate the W/D ratio.

Hematoxylin and Eosin Staining and Lung Histology Evaluation

The left lung tissue was fixed in 4% paraformaldehyde for 24 h. Fixed tissues were embedded in paraffin, cut into 5-μm sections, and stained with hematoxylin and eosin (HE) [18, 19]. The lung injury score was calculated by assessing the degree of inflammatory cell infiltration, hemorrhage, interstitial and alveolar edema, and the thickness of alveolar septum in five random fields in a blinded manner, using light microscopy. A score of 0 represented no damage; 1 represented mild damage; 2 represented moderate damage; 3 represented severe damage; and 4 represented very severe histological damage [20].

Enzyme-Linked Immunosorbent Assays of IL-17, IL-22, and TNF-α Concentrations in Bronchial Alveolar Lavage Fluid and Myeloperoxidase Activity in Lung Tissue

After euthanasia by injection of a lethal dose of pentobarbital, a tracheal catheter was instilled in the mice. Sterile saline (f0.7 mL) was administered into the whole lung and extracted *via* the catheter, three times. Then, the BALF was centrifuged at 1200×*g* for 10 min at 4 °C to remove cell debris. The pellets were resuspended in 50 μL of PBS and stained with Wright–Giemsa (KeyGen Biotech Co., Nanjing, China). Total cells and neutrophils were counted with a hemocytometer in a double-blind manner. The concentrations of IL-17, IL-22 (USCN KIT INC., Wuhan, China), TNF-α (R&D Systems), and myeloperoxidase (MPO) activity of lung tissue (Colorful Gene, Wuhan, China) were measured using the appropriate enzyme-linked immunosorbent assay (ELISA) kit, according to the manufacturers' protocols.

Quantitative Real-Time Polymerase Chain Reaction

Total ribonucleic acid (RNA) was extracted from the right lower lung tissues according to the manufacturer's protocol (Invitrogen, Carlsbad, CA, USA) and subjected to reverse transcription using PrimeScript Reverse Transcription system (Takara, Japan). qPCR analysis was performed using SYBR Green (Takara, Kusatsu, Japan) [21, 22]. The relative mRNA levels of each sample were determined using NanoDrop2000 (Thermo Fisher Scientific). The primers used were as follows: 5′-CGGCATTCCAAGCTAGAGAAGGC-3′ and 5′-AAGATTCCTTGGTCGGCAGTGC-3′ for hairy and enhancer of split-1 (Hes1); 5′-GTCCAGACAGCCACTGCATTCC-3′ and 5′-TGCCGTAGAAGGTCCTCCAGTC-3′ for RORγt; and 5′-ATGTGCTCTTCAGATGGTGTCACG-3′ and 5′-AAGATTCCTTGGTCGGCAGTGC-3′ for IL-23R. The relative gene expression was calculated by the CFX manager software (Bio-Rad, Hercules, CA, USA), using the comparative Ct (△△Ct) method, with β-actin as a reference gene.

Western Blotting

Radioimmunoprecipitation assay (RIPA) buffer (Beyotime Biotechnology, Shanghai, China) was used to extract total protein from CD4$^+$ T cells and the right upper lung tissues, following the manufacturer's instructions. Briefly, cultured CD4$^+$ T cells or 100 mg homogenized lung tissues were lysed with 1000 μL RIPA buffer supplemented with protease and phosphatase inhibitors, incubated on ice for 30 min, then centrifuged at 15,000×*g* for 15 min at 4 °C. The total protein concentration in the pooled lysates was measured using a bicinchoninic acid protein assay kit (Beyotime Biotechnology, Shanghai, China). Equivalent amounts of protein samples were loaded into each well, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, electrotransferred onto nitrocellulose membranes, and blocked with 5% non-fat milk or bovine serum albumin (BSA) for 1 h. Then, the membrane was incubated with antibodies overnight at 4 °C. Thereafter, the membrane was washed three times with PBS containing Tween-20, and subsequently incubated with the secondary antibody (Abcam, Cambridge, UK, 1:8000) at 37 °C for 1 h. Later, the membrane was washed again three times. Protein bands were visualized using enhanced chemiluminescence by a UVP gel imaging system (Upland, CA, USA). The relative abundance of protein was quantified using the Quantity One Software (Bio-Rad).

For Western blot analysis, the following primary antibodies were used: anti-PKCθ (Cell Signaling Technology, Beverly, MA, USA; 1:1000), anti-pPKCθ-T538 (Cell Signaling Technology, Boston, USA, 1:1000), anti-NICD (Cell Signaling Technology, Boston, USA 1:1000), anti-Hes1 (Abcam, Cambridge, UK, 1:500), anti-RORγt (Abcam, Cambridge, UK, 1:500), and anti-IL-23R (Abcam, Cambridge, UK, 1:500).

Immunohistochemistry

Slices of left mouse lung were deparaffinized with xylene, rehydrated in an ethanol gradient, and blocked by incubating with 3% H_2O_2 at 37 °C for 15 min. Background due to non-specific binding was reduced by incubation with 1% BSA in PBS for 60 min at room temperature. Tissues were incubated with primary antibodies against RORγt (Abcam, Cambridge, UK, 1:1500) or IL-23R (Abcam, Cambridge, UK, 1:250) at 4 °C overnight. After washing, tissue sections were incubated with biotin-labeled secondary antibody at 37 °C for 30 min, and then stained with diaminobenzidine for 5 min. Samples were counterstained with hematoxylin, dehydrated in an ethanol gradient, vitrified with xylene, and sealed with neutral resins.

Flow Cytometry

Cells in lung tissues were stimulated with a cell stimulation cocktail (Biolegend, San Diego, CA, USA), composed of phorbol 12-myristate 13-acetate, ionomycin, and Brefeldin A, at 37 °C and under 5% CO_2 incubation for 5 h. Cells were then washed in PBS and surface-stained with FITC-conjugated anti-CD4 antibodies for 30 min, followed by fixation and permeabilization, after which they were intracellularly stained with APC-conjugated anti-IL-17A antibodies for 1 h. All flow cytometric measurements were conducted on a flow cytometer (Beckman Coulter, Inc., Brea, CA, USA). Results were analyzed using the Cyexpert software (Beckman Coulter, Inc.).

Statistical Analysis

Data are presented as mean ± standard deviations. Statistical analysis was performed using one-way analysis of variance with SPSS 22.0 (IBM software, Armonk, NY, USA). *Post hoc* tests (Student–Newman–Keuls and least significant difference) were performed to detect significant differences between particular groups. $P < 0.05$ was set as the threshold value for statistical significance.

RESULTS

PKCθ Inhibition Ameliorated LPS-Induced Lung Injury

LPS-induced lung injury was assessed by HE staining and the lung W/D ratio. Mice treated with LPS exhibited the typical pathological changes of ALI, including intra-alveolar and interstitial edema, hemorrhage, thickened alveolar septum, and inflammatory cell infiltration. These pathological changes were attenuated by PKCθ inhibition (Fig. 1a, b). PKCθ inhibition also reduced the lung W/D ratio (Fig. 1c).

PKCθ Inhibition Attenuated Inflammation Response and Neutrophil Infiltration in Mouse Lung

To determine whether PKCθ inhibition affects lung inflammation induced by LPS exposure, we also determined the total cell, neutrophil counts, and TNF-α in BALF; the leukocyte count in whole blood; and MPO activity of lung tissues. Administration of LPS significantly increased the total cell and neutrophils counts, TNF-α in BALF, as well as MPO activity in lung tissue, which were markedly reduced by PKCθ inhibition (Fig. 2a–d). Although the exposure to LPS decreased the leukocyte count in whole blood, the leukocyte count was further significantly reduced in mice in the PI group compared to that in the LPS group mice (Fig. 2e).

PKCθ Inhibition Suppressed the Th17 Response

To determine whether PKCθ inhibition suppressed the Th17 response, we measured the percentage of $CD4^{+}$IL-

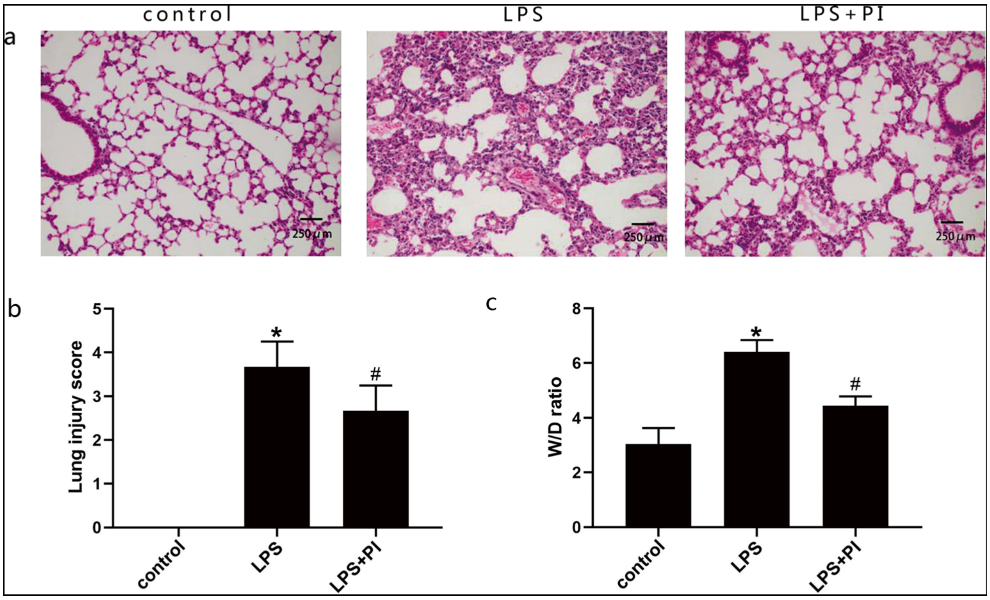

Fig. 1. Effects of PKCθ inhibition on **a** lung histological alterations (hematoxylin and eosin, × 200), **b** lung injury score, and **c** lung wet to dry weight (W/D) ratio. Data are presented as means ±S.D. (*$p < 0.05$ compared with the control group, #$p < 0.05$ compared with the LPS group).

17A$^+$ T cells in lung tissue by flow cytometry analysis and the IL-17 and IL-22 levels in BALF by ELISA. After treatment with LPS, the concentrations of IL-17 and IL-22 in BALF and the percentage of CD4$^+$IL-17A$^+$ T cells in the mouse lungs were notably elevated, which were significantly decreased by PKCθ inhibition (Fig. 3a–d).

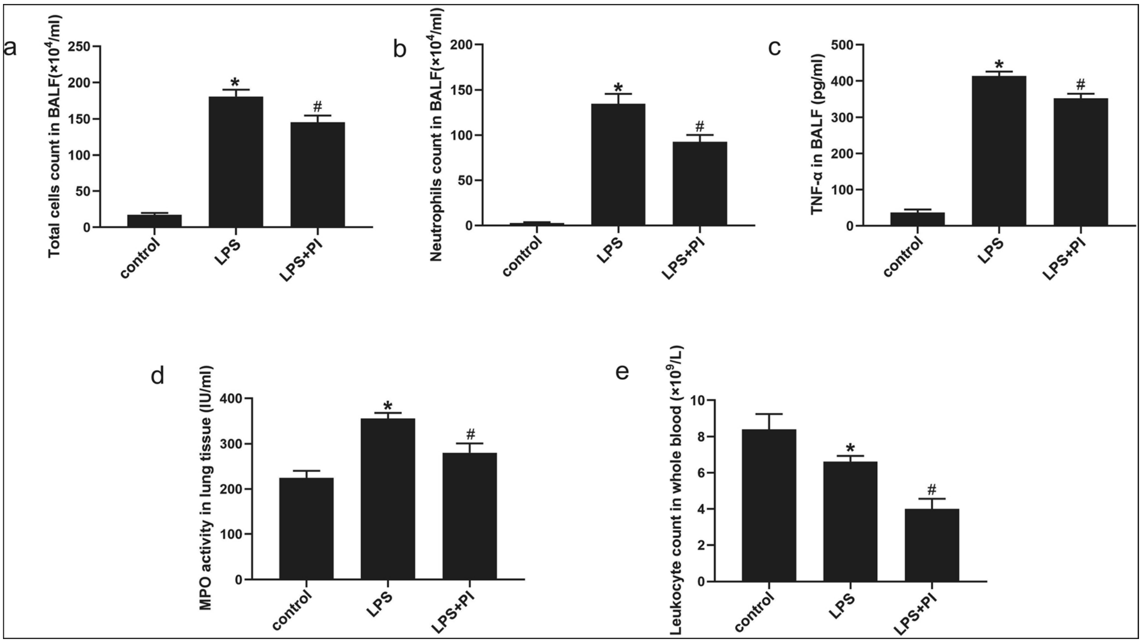

Fig. 2. The effect of a PKCθ inhibition on inflammation and neutrophil infiltration in mouse lung. **a** Total cells and **b** neutrophil counts in bronchial alveolar lavage fluid (BALF). **c** Myeloperoxidase (MPO) activity was determined by ELISA. **d** TNF-α in BALF was calculated by ELISA. **e** Leukocyte count in whole blood. Data are presented as means ±S.D. (*$p < 0.05$ compared with the control group, #$p < 0.05$ compared with the LPS group).

PKCθ Inhibition Downregulated the Protein and mRNA Levels of RORγt and IL-23R by Inhibiting the Notch Signaling Pathway in Mouse Lungs

To determine the effect of PKCθ inhibition on RORγt and IL-23R levels and its mechanism *in vivo*, Western blotting and qPCR were employed to evaluate the expression of RORγt, IL-23R, and Notch signaling pathway-related component in mouse lungs. LPS profoundly enhanced the protein and mRNA levels of RORγt and IL-23R. In contrast, PI as well as DAPT significantly decreased the protein and mRNA levels of RORγt and IL-23R (Fig. 4a–d). The protein levels of P-PKCθ-T538, NICD, and Hes1 (a downstream target of the Notch signaling pathway) in mouse lungs were analyzed by Western blot analysis to determine whether PKCθ inhibition downregulates the levels of RORγt and IL-23R *via* the Notch pathway. In addition, the mRNA levels of Hes1 were measured by qPCR. Exposure to LPS led to significant enhancement in NICD and Hes1 protein levels in mouse lungs. Both PI and DAPT could significantly decrease the NICD and Hes1 protein levels (Fig. 4b). Phosphorylation of PKCθ at T538 (P-PKCθ-T538), which is key to kinase activation, was downregulated by PI in mouse lungs (Fig. 4b). On the other hand, P-PKCθ-T538 levels remained unchanged in the presence of DAPT as compared to that of PI (Fig. 4b). Consistent with the results of Western blotting, the mRNA expression of Hes1 could be reduced by either PI or DAPT (Fig. 4e).

PKCθ Inhibition Downregulated the Protein and mRNA Levels of RORγt and IL-23R by Inhibiting the Notch Signaling Pathway in CD4$^+$ T Cells

To determine the effect of PKCθ inhibition on RORγt and IL-23R levels and its mechanism *in vitro*, Western blotting and qPCR were employed to evaluate the expression of RORγt, IL-23R, and Notch signaling pathway-related component in CD4$^+$ T cells. PI as well as DAPT significantly decreased the protein and mRNA levels of RORγt and IL-23R (Fig. 5a–c). The protein levels of P-PKCθ-T538, NICD, and Hes1 were analyzed by Western blot analysis to determine whether PKCθ inhibition downregulates the levels of RORγt and IL-23R *via* the Notch pathway. In addition, the mRNA levels of Hes1 were measured by qPCR.

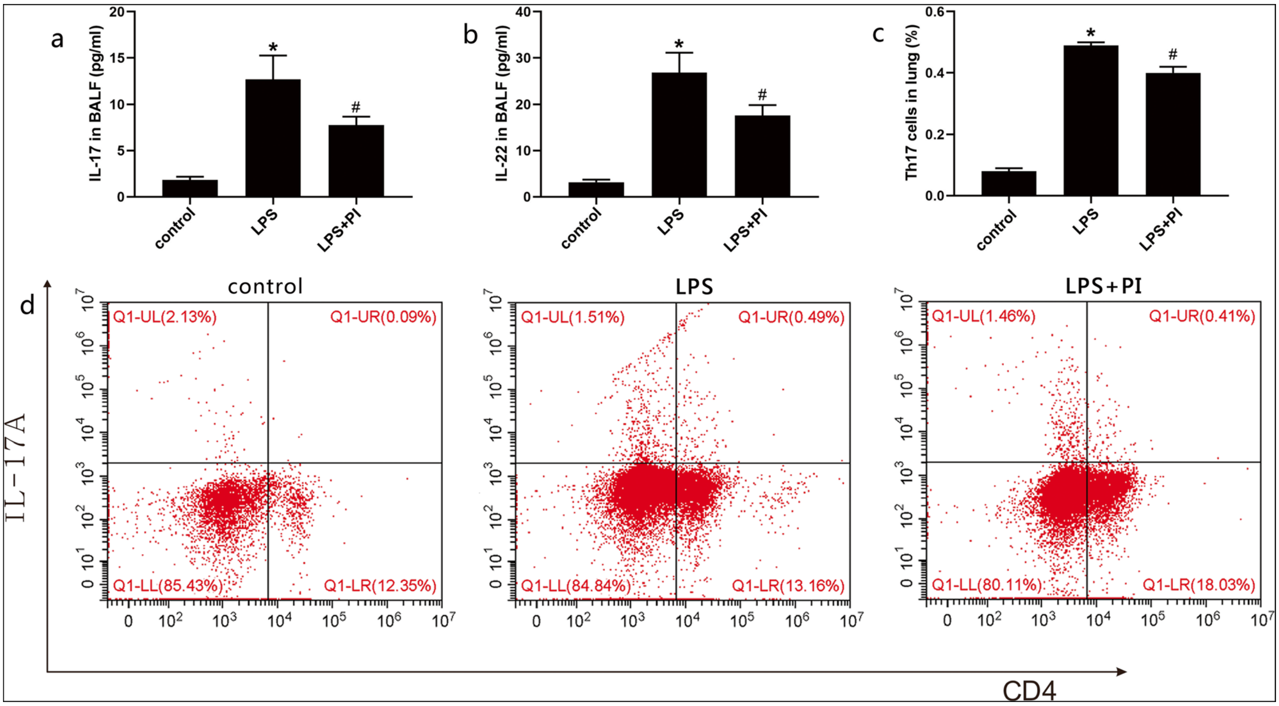

Fig. 3. PKCθ inhibition suppressed the Th17 response. The concentrations of **a** IL-17 and **b** IL-22 in bronchial alveolar lavage fluid (BALF) were measured by ELISA. The percentage of CD4$^+$IL-17A$^+$ T cells in the lungs was measured by flow cytometric analysis (**c**, **d**). Data are presented as means ± S.D. (*$p < 0.05$ compared with the control group, #$p < 0.05$ compared with the LPS group).

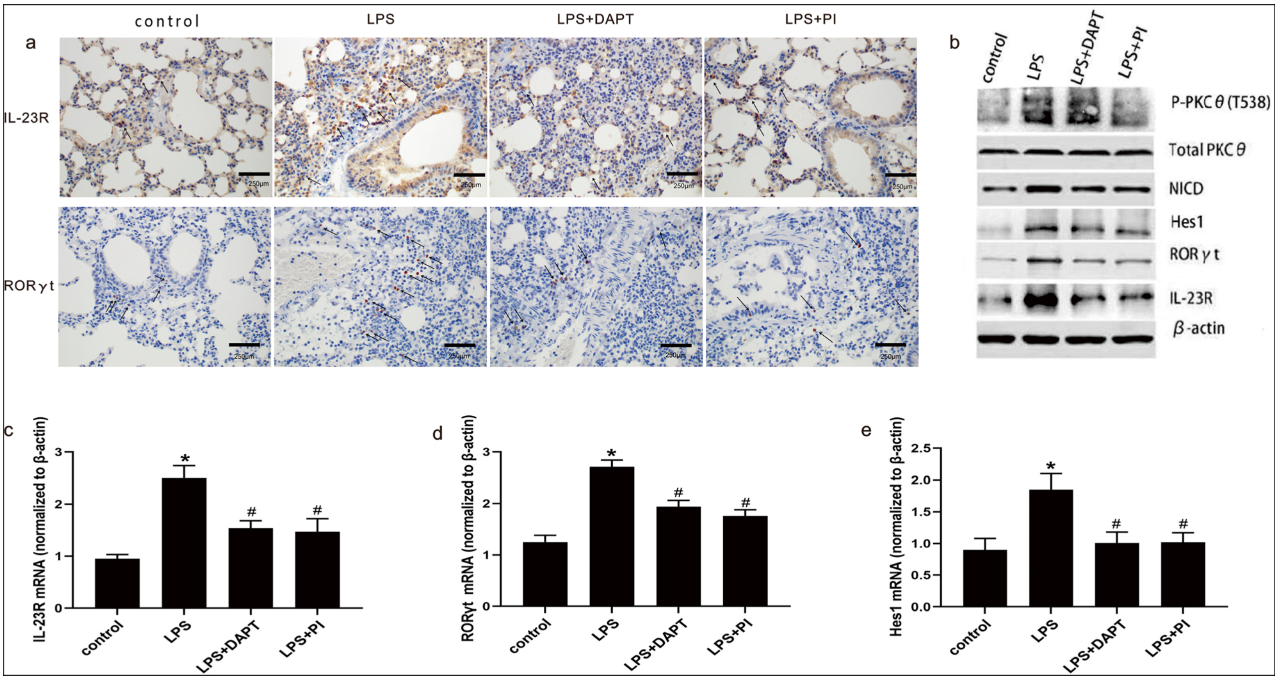

Fig. 4. PKCθ inhibition downregulated the levels of RORγt and IL-23R by inhibiting the Notch signaling pathway in mouse lungs: **a** RORγt and IL-23R in mouse lungs measured by immunohistochemistry (× 400); **b** P-PKCθ-T538, Notch signaling pathway-related component, RORγt, and IL-23R measured by Western blotting; **c** IL-23R mRNA, **d** RORγt mRNA, and **e** Hes1 mRNA measured by qPCR. Data are presented as means ± S.D. (*$p < 0.05$ compared with the control group, #$p < 0.05$ compared with the LPS group).

Both PI and DAPT significantly decreased the NICD and Hes1 protein levels (Fig. 5a). P-PKCθ-T538 was downregulated by PI but remained unchanged in the presence of DAPT (Fig. 5a). Consistent with the result of Western blotting, the mRNA expression of Hes1 could be reduced by either PI or DAPT (Fig. 5d).

DISCUSSION

In the present study, we showed that PKCθ inhibition attenuates LPS-induced ALI. PKCθ inhibition in mice decreased the infiltration of neutrophils in the lungs and TNF-α concentration in BALF. The percentage of Th17 cells in lung tissue and the IL-17 and IL-22 levels in BALF were lowered

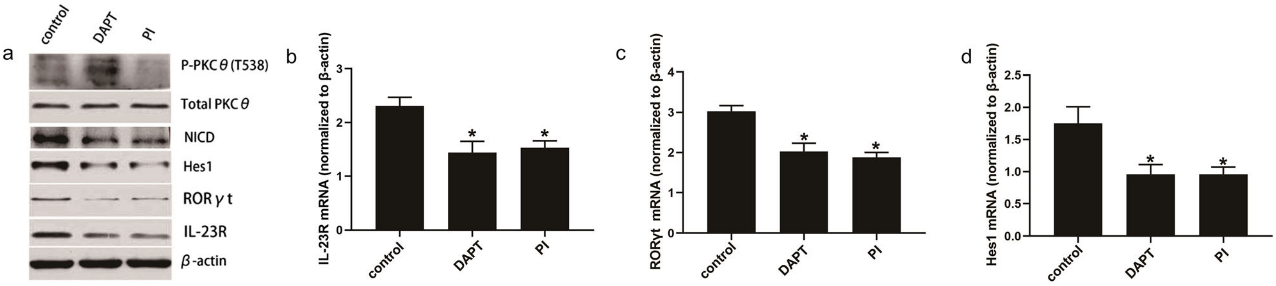

Fig. 5. PKCθ inhibition downregulated the levels of RORγt and IL-23R by inhibiting the Notch signaling pathway in CD4 + T cells: **a** P-PKCθ-T538, Notch signaling pathway-related component, RORγt, and IL-23R measured by Western blotting; **b** IL-23R mRNA; **c** RORγt mRNA; and **d** Hes1 mRNA measured by qPCR. Data are presented as means ± S.D. (*$p < 0.05$ compared with the control group).

by PKCθ inhibition. PKCθ inhibition suppressed the expression of RORγt and IL-23R, at least partially, through the Notch signaling pathway. Collectively, these data suggest that PKCθ inhibition may be valuable for the treatment of ARDS.

Inflammation plays a pivotal role in ARDS [23]. In our data, the decrease of TNF-α in BALF suggested that PKCθ inhibition significantly suppressed inflammation. Clinical studies have indicated that exaggerated recruitment and activation of neutrophils in the airways are linked to ARDS [24, 25]. In our study, blood leukocyte count was reduced in ALI mice compared to that in the control group mice, further indicating that neutrophil sequestration into the lungs is characteristic of ALI. PKCθ is required for neutrophil recruitment and adhesion; lidocaine treatment could reduce neutrophil recruitment by inhibiting PKCθ [26, 27]. Consistent with the results of these studies, we observed that PKCθ inhibition notably decreased the total cell counts, neutrophil counts in BALF, and MPO activity of lung tissues. Our data demonstrated that neutrophil infiltration in the lungs of ALI mice was notably decreased by PKCθ inhibition. However, our study primarily focused on the effects of PKCθ inhibition on Th17; thus, we did not explore further details of the mechanism by which PI affects neutrophil infiltration, such as downregulation of chemokines [5], and decreases in neutrophil deformability [28]. Future studies may be needed to establish the role of PKCθ inhibition in this regard.

Our previous study had suggested that Th17 is involved in the pathogenesis of inflammation [29]. Th17 cells play a pro-inflammatory role in neutrophilic inflammation and participate in neutrophil accumulation [30]. IL-17, a key effector molecule of Th17 cells, is a typical pro-inflammatory cytokine that induces the expression of IL-6 and TNF-α [31, 32]. Moreover, IL-17 can promote activation and recruitment of leukocytes in the lung [33, 34]. IL-22, another key effective molecule of Th17 cells has been demonstrated to play crucial roles in the development of ALI [35]. In agreement with our previous findings that suppressing Th17 can attenuate lung inflammation [29], our present study suggests that PKCθ inhibition could alleviate LPS-induced inflammation and neutrophil infiltration, which was accordant with the reduction in concentrations of IL-17 and IL-22 and percentages of Th17 in mouse lungs.

IL-23R is essential for the pathogenicity of Th17 cells [14]. According to Dawn C. Newcomb et al., in asthmatic mice, ovarian hormones upregulate IL-17A protein expression in Th17 cells through increased IL-23R, resulting in the aggravation of neutrophilic inflammation [36]. We observed that PKCθ inhibition downregulated the protein and mRNA levels of IL-23R in mouse lungs and in $CD4^+$ T cells, which is accordant with the reduction in IL-17 and IL-22 concentrations in BALF. These results suggest that PKCθ inhibition appears to suppress pathogenicity of Th17 by downregulating IL-23R expression partially through a genomic mechanism. The decrease in RBPJ may contribute to the reduction of IL-23R [14, 37]. Differentiation of Th17 cells is transcriptionally controlled by RORγt [38]. We observed that PKCθ inhibition downregulated protein and mRNA levels of RORγt in mouse lungs and $CD4^+$ T cells and was accompanied by the reduction in the percentage of Th17 in ALI mouse lungs. These data also suggest that PKCθ inhibition hampers Th17 cell differentiation by suppressing RORγt partially through a genomic mechanism, which is consistent with Kwon et al.'s finding that RORγt mRNA was significantly reduced in $PKC\theta^{-/-}$ T cells as compared to wild-type T cells [39].

Notch inhibition downregulates RORγt [10]. Recombinant signal binding protein of the immunoglobulin κJ region (RBPJ), a component of the Notch signaling, is a key driver of IL-23R [14]. Both studies suggested that the Notch signaling pathway was involved in regulating the expression of RORγt and IL-23R. PKCθ, a protein found inside T cells, is a major contributor to Notch activation. PKCθ inactivates profilin, a protein that inhibits formation of actin filaments, thereby promoting the generation of an abundant actin-rich structure that is required to recruit the Notch enzyme to the immunological synapse [15]. Our study further evaluated whether PKCθ inhibition decreases RORγt and IL-23R expression through the Notch signaling pathway. We showed that in mouse lungs, PKCθ inhibition could reduce NICD and downregulate Hes1, a Notch target gene. While phosphorylation of PKCθ at T-538 (P- PKCθ-T538) was downregulated by PI, contrarily, DAPT had no effect on the activity of PKCθ. A similar result was observed in $CD4^+$ T cells. These results indicated that PKCθ inhibition suppresses RORγt and IL-23R expression, at least in part, *via* the Notch signaling pathway.

However, the specific mechanism by which the Notch signaling pathway affects RORγt and IL-23R is not yet clear. We also could not determine the link between IL-23R and production of IL-17, IL-22 by Th17. We proved that PKCθ inhibition downregulated the expression of RORγt, but could not determine whether PKCθ inhibition affected the activity of RORγt. Further studies are necessary to define the specific and overlapping contributions of other signaling pathways that mediate the protective effects of PKCθ inhibition in ALI/ARDS.

In summary, our study provided insights into a new target for the treatment of ALI/ARDS through regulation of the immune system. We demonstrated that PKCθ inhibition can exert protective effects against pulmonary inflammation and neutrophil infiltration by suppressing the differentiation and pathogenicity of Th17 *via* the Notch signaling pathway. PKCθ inhibition may be a potential therapeutic intervention for patients with ARDS in clinical practice.

AUTHORS' CONTRIBUTION

Mengqin Li performed the experiments and wrote the manuscript. Yan Z, Jing He, Wang Deng, Li Cheng, and Zhi Jiang analyzed the data. Daoxin Wang designed the study and revised the manuscript. All the authors read and approved the final manuscript.

FUNDING

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COMPLIANCE WITH ETHICAL STANDARDS

All animal experiments were performed in accordance with the ethical guidelines of the National Institutes of Health on Animal Care, and the study protocol was approved by the Ethics Committee of the Second Affiliated Hospital of Chongqing Medical University.

Conflict of Interest. The authors declare that they have no conflict of interest.

Ethical Approval. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

REFERENCES

1. Rubenfeld GD, Caldwell E, Peabody E, Weaver J, Martin DP, Neff M, Stern EJ, Hudson LD (2005) Incidence and outcomes of acute lung injury. *The New England journal of medicine* 353 (16):1685–1693.
2. Ferguson, N.D., E. Fan, L. Camporota, M. Antonelli, A. Anzueto, R. Beale, L. Brochard, R. Brower, A. Esteban, L. Gattinoni, A. Rhodes, A.S. Slutsky, J.L. Vincent, G.D. Rubenfeld, B.T. Thompson, and V.M. Ranieri. 2012. The Berlin definition of ARDS: An expanded rationale, justification, and supplementary material. *Intensive Care Medicine* 38 (10): 1573–1582.
3. Korn, T., E. Bettelli, M. Oukka, and V.K. Kuchroo. 2009. IL-17 and Th17 cells. *Annual Review of Immunology* 27: 485–517.
4. Williams, A.E., and R.C. Chambers. 2014. The mercurial nature of neutrophils: Still an enigma in ARDS? *American Journal of Physiology. Lung Cellular and Molecular Physiology* 306 (3): L217–L230.
5. Linden, A., H. Hoshino, and M. Laan. 2000. Airway neutrophils and interleukin-17. *The European Respiratory Journal* 15 (5): 973–977.
6. Glader, P., M.E. Smith, C. Malmhall, B. Balder, M. Sjostrand, I. Qvarfordt, and A. Linden. 2010. Interleukin-17-producing T-helper cells and related cytokines in human airways exposed to endotoxin. *The European Respiratory Journal* 36 (5): 1155–1164.
7. Ramezanpour, M., S. Moraitis, J.L. Smith, P.J. Wormald, and S. Vreugde. 2016. Th17 cytokines disrupt the airway mucosal barrier in chronic rhinosinusitis. *Mediators of Inflammation* 2016: 9798206.
8. Oppeltz, R.F., M. Rani, Q. Zhang, and M.G. Schwacha. 2011. Burn-induced alterations in toll-like receptor-mediated responses by bronchoalveolar lavage cells. *Cytokine* 55 (3): 396–401.
9. Li, J.T., A.C. Melton, G. Su, D.E. Hamm, M. LaFemina, J. Howard, X. Fang, S. Bhat, K.M. Huynh, C.M. O'Kane, R.J. Ingram, R.R. Muir, D.F. McAuley, M.A. Matthay, and D. Sheppard. 2015. Unexpected role for adaptive alphabetaTh17 cells in acute respiratory distress syndrome. *Journal of Immunology (Baltimore, Md. : 1950)* 195 (1): 87–95.
10. Yu, S., C. Liu, L. Li, T. Tian, M. Wang, Y. Hu, C. Yuan, L. Zhang, C. Ji, and D. Ma. 2015. Inactivation of Notch signaling reverses the Th17/Treg imbalance in cells from patients with immune thrombocytopenia. *Laboratory Investigation; a Journal of Technical Methods and Pathology* 95 (2): 157–167.
11. Keerthivasan, S., R. Suleiman, R. Lawlor, J. Roderick, T. Bates, L. Minter, J. Anguita, I. Juncadella, B.J. Nickoloff, I.C. Le Poole, L. Miele, and B.A. Osborne. 2011. Notch signaling regulates mouse and human Th17 differentiation. *Journal of Immunology (Baltimore, Md. : 1950)* 187 (2): 692–701.
12. Mukherjee, S., M.A. Schaller, R. Neupane, S.L. Kunkel, and N.W. Lukacs. 2009. Regulation of T cell activation by Notch ligand, DLL4, promotes IL-17 production and Rorc activation. *Journal of Immunology (Baltimore, Md. : 1950)* 182 (12): 7381–7388.
13. Piggott, K., J. Deng, K. Warrington, B. Younge, J.T. Kubo, M. Desai, J.J. Goronzy, and C.M. Weyand. 2011. Blocking the NOTCH pathway inhibits vascular inflammation in large-vessel vasculitis. *Circulation* 123 (3): 309–318.
14. Meyer Zu Horste, G., C. Wu, C. Wang, L. Cong, M. Pawlak, Y. Lee, W. Elyaman, S. Xiao, A. Regev, and V.K. Kuchroo. 2016. RBPJ controls development of pathogenic Th17 cells by regulating IL-23 receptor expression. *Cell Reports* 16 (2): 392–404.
15. Britton GJ, Ambler R, Clark DJ, Hill EV, Tunbridge HM, McNally KE, Burton BR, Butterweck P, Sabatos-Peyton C, Hampton-O'Neil LA, Verkade P, Wulfing Ch C, Wraith DC (2017) PKCtheta links proximal T cell and Notch signaling through localized regulation of the actin cytoskeleton. eLife 6. https://doi.org/10.7554/eLife.20003.
16. Zhang, E.Y., K.F. Kong, and A. Altman. 2013. The yin and yang of protein kinase C-theta (PKCtheta): A novel drug target for selective immunosuppression. *Advances in Pharmacology (San Diego, Calif)* 66: 267–312.
17. Kwon, M.J., R. Wang, J. Ma, and Z. Sun. 2010. PKC-theta is a drug target for prevention of T cell-mediated autoimmunity and allograft rejection. *Endocrine, Metabolic & Immune Disorders Drug Targets* 10 (4): 367–372.
18. Zhang, Q., Y. Zhao, M. Talukder, Y. Han, C. Zhang, X.N. Li, and J.L. Li. 2019. Di(2-ethylhexyl) phthalate induced hepatotoxicity in quail (Coturnix japonica) via modulating the mitochondrial unfolded protein response and NRF2 mediated antioxidant defense. *The Science of the Total Environment* 651 (Pt 1): 885–894.

19. Zhao, Y., J.H. Fan, Y. Luo, M. Talukder, X.N. Li, Y.Z. Zuo, and J.L. Li. 2019. Di-(2-ethylhexyl) phthalate (DEHP)-induced hepatotoxicity in quail (Coturnix japonica) via suppression of the heat shock response. *Chemosphere* 228: 685–693.
20. Qi, D., J. He, D. Wang, W. Deng, Y. Zhao, Y. Ye, and L. Feng. 2014. 17beta-estradiol suppresses lipopolysaccharide-induced acute lung injury through PI3K/Akt/SGK1 mediated up-regulation of epithelial sodium channel (ENaC) in vivo and in vitro. *Respiratory Research* 15: 159.
21. Luo, Y., X.N. Li, Y. Zhao, Z.H. Du, and J.L. Li. 2019. DEHP triggers cerebral mitochondrial dysfunction and oxidative stress in quail (Coturnix japonica) via modulating mitochondrial dynamics and biogenesis and activating Nrf2-mediated defense response. *Chemosphere* 224: 626–633.
22. Wang, H., X.N. Li, P.C. Li, W. Liu, Z.H. Du, and J.L. Li. 2019. Modulation of heat-shock response is associated with Di (2-ethylhexyl) phthalate (DEHP)-induced cardiotoxicity in quail (Coturnix japonica). *Chemosphere* 214: 812–820.
23. Meduri, G.U., D. Annane, G.P. Chrousos, P.E. Marik, and S.E. Sinclair. 2009. Activation and regulation of systemic inflammation in ARDS: Rationale for prolonged glucocorticoid therapy. *Chest* 136 (6): 1631–1643.
24. Adams, J.M., C.J. Hauser, D.H. Livingston, R.F. Lavery, Z. Fekete, and E.A. Deitch. 2001. Early trauma polymorphonuclear neutrophil responses to chemokines are associated with development of sepsis, pneumonia, and organ failure. *The Journal of Trauma* 51 (3): 452–456 discussion 456-457.
25. Geerts, L., P.G. Jorens, J. Willems, M. De Ley, and H. Slegers. 2001. Natural inhibitors of neutrophil function in acute respiratory distress syndrome. *Critical Care Medicine* 29 (10): 1920–1924.
26. Bertram, A., H. Zhang, S. von Vietinghoff, C. de Pablo, H. Haller, N. Shushakova, and K. Ley. 2012. Protein kinase C-theta is required for murine neutrophil recruitment and adhesion strengthening under flow. *Journal of Immunology (Baltimore, Md. : 1950)* 188 (8): 4043–4051.
27. Berger, C., J. Rossaint, H. Van Aken, M. Westphal, K. Hahnenkamp, and A. Zarbock. 2014. Lidocaine reduces neutrophil recruitment by abolishing chemokine-induced arrest and transendothelial migration in septic patients. *Journal of Immunology (Baltimore, Md. : 1950)* 192 (1): 367–376.
28. Skoutelis, A.T., V. Kaleridis, G.M. Athanassiou, K.I. Kokkinis, Y.F. Missirlis, and H.P. Bassaris. 2000. Neutrophil deformability in patients with sepsis, septic shock, and adult respiratory distress syndrome. *Critical Care Medicine* 28 (7): 2355–2359.
29. Yan, Z., Z. Xiaoyu, S. Zhixin, Q. Di, D. Xinyu, X. Jing, H. Jing, D. Wang, Z. Xi, Z. Chunrong, and W. Daoxin. 2016. Rapamycin attenuates acute lung injury induced by LPS through inhibition of Th17 cell proliferation in mice. *Scientific Reports* 6: 20156.
30. Lo Re, S., L. Dumoutier, I. Couillin, C. Van Vyve, Y. Yakoub, F. Uwambayinema, B. Marien, S. van den Brule, J. Van Snick, C. Uyttenhove, B. Ryffel, J.C. Renauld, D. Lison, and F. Huaux. 2010. IL-17A-producing gammadelta T and Th17 lymphocytes mediate lung inflammation but not fibrosis in experimental silicosis. *Journal of Immunology (Baltimore, Md. : 1950)* 184 (11): 6367–6377.
31. Koenders, M.I., L.A. Joosten, and W.B. van den Berg. 2006. Potential new targets in arthritis therapy: Interleukin (IL)-17 and its relation to tumour necrosis factor and IL-1 in experimental arthritis. *Annals of the Rheumatic Diseases* 65 (Suppl 3): iii29–iii33.
32. Wang, L., T. Yi, M. Kortylewski, D.M. Pardoll, D. Zeng, and H. Yu. 2009. IL-17 can promote tumor growth through an IL-6-Stat3 signaling pathway. *The Journal of Experimental Medicine* 206 (7): 1457–1464.
33. Park, H., Z. Li, X.O. Yang, S.H. Chang, R. Nurieva, Y.H. Wang, Y. Wang, L. Hood, Z. Zhu, Q. Tian, and C. Dong. 2005. A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. *Nature Immunology* 6 (11): 1133–1141.
34. Miyamoto, M., O. Prause, M. Sjostrand, M. Laan, J. Lotvall, and A. Linden. 2003. Endogenous IL-17 as a mediator of neutrophil recruitment caused by endotoxin exposure in mouse airways. *Journal of Immunology (Baltimore, Md. : 1950)* 170 (9): 4665–4672.
35. Sakaguchi, R., S. Chikuma, T. Shichita, R. Morita, T. Sekiya, W. Ouyang, T. Ueda, H. Seki, H. Morisaki, and A. Yoshimura. 2016. Innate-like function of memory Th17 cells for enhancing endotoxin-induced acute lung inflammation through IL-22. *International Immunology* 28 (5): 233–243.
36. Newcomb, D.C., J.Y. Cephus, M.G. Boswell, J.M. Fahrenholz, E.W. Langley, A.S. Feldman, W. Zhou, D.E. Dulek, K. Goleniewska, K.B. Woodward, C.M. Sevin, R.G. Hamilton, J.K. Kolls, and R.S. Peebles Jr. 2015. Estrogen and progesterone decrease let-7f microRNA expression and increase IL-23/IL-23 receptor signaling and IL-17A production in patients with severe asthma. *The Journal of Allergy and Clinical Immunology* 136 (4): 1025–1034.e1011.
37. Long, Q., Q. Luo, K. Wang, A. Bates, and A.K. Shetty. 2017. Mash1-dependent Notch signaling pathway regulates GABAergic neuron-like differentiation from bone marrow-derived mesenchymal stem cells. *Aging and Disease* 8 (3): 301–313.
38. Ivanov, I.I., B.S. McKenzie, L. Zhou, C.E. Tadokoro, A. Lepelley, J.J. Lafaille, D.J. Cua, and D.R. Littman. 2006. The orphan nuclear receptor RORgammat directs the differentiation program of proinflammatory IL-17+ T helper cells. *Cell* 126 (6): 1121–1133.
39. Kwon, M.J., J. Ma, Y. Ding, R. Wang, and Z. Sun. 2012. Protein kinase C-theta promotes Th17 differentiation via upregulation of Stat3. *Journal of Immunology (Baltimore, Md. : 1950)* 188 (12): 5887–5897.