Mediators of Inflammation

Cytokines, Immunity, and Inflammation

Lead Guest Editor: Matilde Otero-Losada
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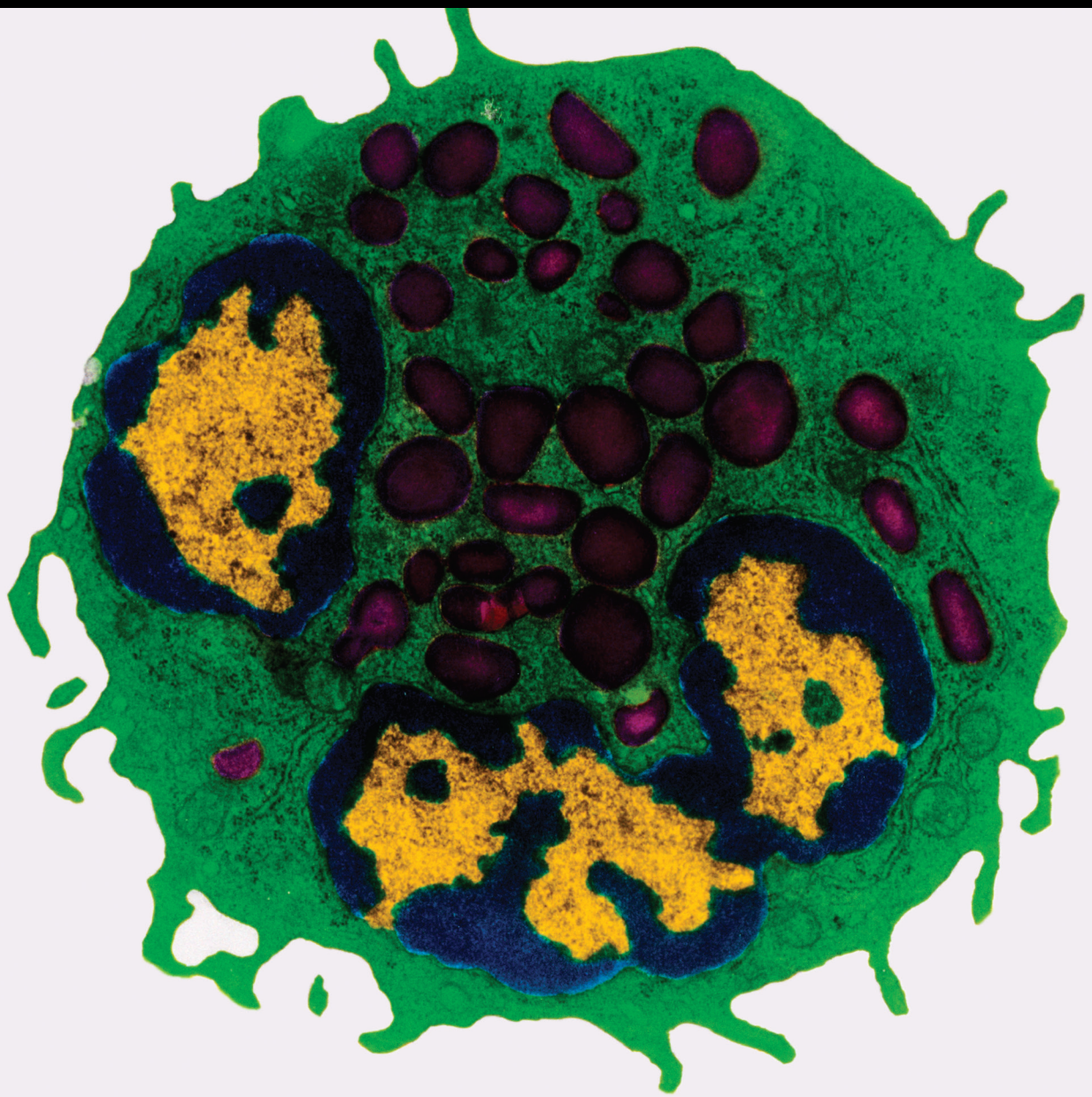

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Research Article

IFN-α-2b Reduces Postoperative Arthrofibrosis in Rats by Inhibiting Fibroblast Proliferation and Migration through STAT1/p21 Signaling Pathway

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Objective. To investigate the effect of IFN-α-2b in preventing postoperative arthrofibrosis in rats, its antiproliferation effect on fibroblasts in vitro, and its molecular mechanism. *Methods*. The rat model of arthrofibrosis was established and treated with different concentrations of drugs. Knee specimens were collected for histological and immunohistochemical staining to observe the effect of IFN-α-2b on arthrofibrosis in rats. The biological information was further mined according to the database data, and the possible regulatory mechanism of IFN-α-2b on fibroblasts was analyzed. The inhibitory effect of IFN-α-2b on fibroblast proliferation and migration in vitro was detected by cell counting kit-8 (CCK-8), immunofluorescence analysis, cell cycle test, EdU assay, wound healing test, and Transwell method, and the analysis results were verified by Western blotting method. *Results*. The test results of rat knee joint specimens showed that IFN-α-2b significantly inhibited the degree of fibrosis after knee joint surgery, the number of fibroblasts in the operation area was less than that of the control group, and the expression of collagen and proliferation-related proteins decreased. In vitro experimental results show that IFN-α-2b can inhibit the proliferation and migration of fibroblasts. According to the results of database analysis, it is suggested that the STAT1/P21 pathway may be involved, and it has been verified and confirmed by Western blotting and other related methods. *Conclusion*. IFN-α-2b can reduce surgery-induced arthrofibrosis by inhibiting fibroblast proliferation and migration, which may be related to the regulation of STAT1/p21 signaling pathway.

1. Introduction

Arthrofibrosis is a common complication in joints after trauma and surgery, which is characterized by the production of excessive fibrous scar tissue in joints [1–3]. Previous studies have shown that arthrofibrosis is related to the excessive proliferation of fibroblasts in the surgical area. The excessive proliferated fibroblasts will migrate to the area after surgery, secreting excessive extracellular matrix (ECM) and collagen deposition, which eventually leads to arthrofibrosis [4, 5]. As the overhyperplasia of fibrosis tissue causes pain and limits the normal range of joint activity, it can seriously affect the patient's postoperative life [6, 7].

In recent years, many strategies have been adopted, such as all-round movements of the knee joint after anesthesia, arthroscopic cleaning of joint cavity, and local drug treatment to reduce the formation of postoperative arthrofibrosis [8–10]. Other studies have reported that the best treatment for arthrofibrosis is early identification and intervention [11]. Therefore, the early application of drugs to prevent arthrofibrosis deserves attention. But, although some achievements have been made in the research of locally applied drugs, there are still considerable limitations before

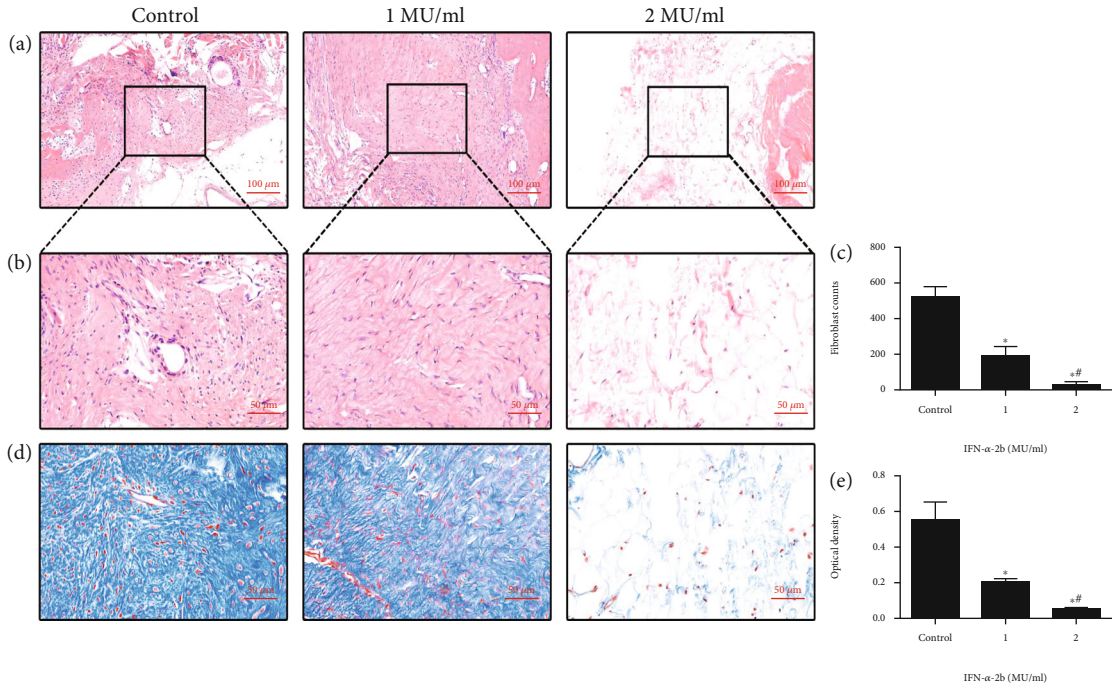


Figure 1: Histological evaluation of joint fibrosis in different groups. (a, b) HE staining showed fibroblasts in each group of joint fibrotic tissue; (c) the number of fibroblasts decreased with the increase of IFN-α-2b concentration; (d) Masson's staining showed the content of collagen in knee tissues of each group. (e) The results of optical density analysis suggest that IFN-α-2b can effectively reduce the production of collagen. *Compared with the control group. #Comparison between the two groups of IFN-α-2b medications, $P < 0.05$ ($n = 6$).

clinical trials due to the side effects of the above drugs or the effect of the route of administration.

IFN-α-2b is a kind of cytokine with many biological activities such as antiviral, antitumor, antiproliferation, and immunomodulation. It has good drug tolerance in the body, even in high-dose application [12]. Previous studies have shown that IFN-α-2b can treat some fibroproliferative diseases (such as hypertrophic scar and keloid) [13]. In addition, it also plays an active preventive role in scar formation after glaucoma filtration surgery and scar formation after cleft palate surgery [14–16]. It can not only inhibit the proliferation of fibroblasts but also reduce the formation of collagen, thus preventing the formation of fibrosis. However, there is no research on IFN-α-2b for arthrofibrosis in the existing literature. Based on the above research background, we choose IFN-α-2b on rats for the prevention of postoperative arthrofibrosis and the treatment of fibroblasts in vitro in order to explore its effect of preventing knee arthrofibrosis and the possible mechanism involved.

2. Materials and Methods

2.1. Reagent. The reagent used is recombinant human interferon alpha 2b (IFN-α-2b), purity > 95%, originated from GenScript Biotechnology Co., Ltd. (Nanjing, China).

2.2. Animals. The animal research in this experiment was approved by the Animal Research Committee of Yangzhou University. All rats received strict care. A total of 36 SD male rats weighing about 300 g were selected and randomly divided into 3 groups (12 in each group).

2.3. Establishment of Arthrofibrosis Model. After the rats were successfully anesthetized, the knee joint was opened through the medial approach of the patella, and the medial and lateral femoral condyles were fully exposed. The cortical bone of about 4*4 mm was excised until the cancellous bone was exposed, and the articular cartilage was intact; then, the wound was covered with saline or IFN-α-2b gauze for 10 minutes. After hemostasis, the gauze was removed to suture the joint capsule and skin. After the operation, antibiotics were applied for 3 consecutive days (intramuscularly, 50 mg/kg), and saline or corresponding concentration of IFN-α-2b 50 μl was injected locally in the operation knee joint 3 times a week [17].

2.4. Histological Analysis. The experiment ended 4 weeks after surgery. Six rats were randomly selected from each group, and knee joint specimens were collected for histological analysis. The knee joint specimens were fixed with 4% paraformaldehyde for 48 hours, fully decalcified in ethylenediamine tetra acetic acid (EDTA), and embedded in paraffin. 4 μm serial sections were deparaffinized to water, and hematoxylin-eosin (HE) staining was performed to detect the degree of fibrosis and the number of fibroblasts, and Masson's trichrome staining was used to observe the collagen production. The collagen was stained with picric acid-Sirius red, and the type I collagen and type III collagen were observed and distinguished under a polarized light

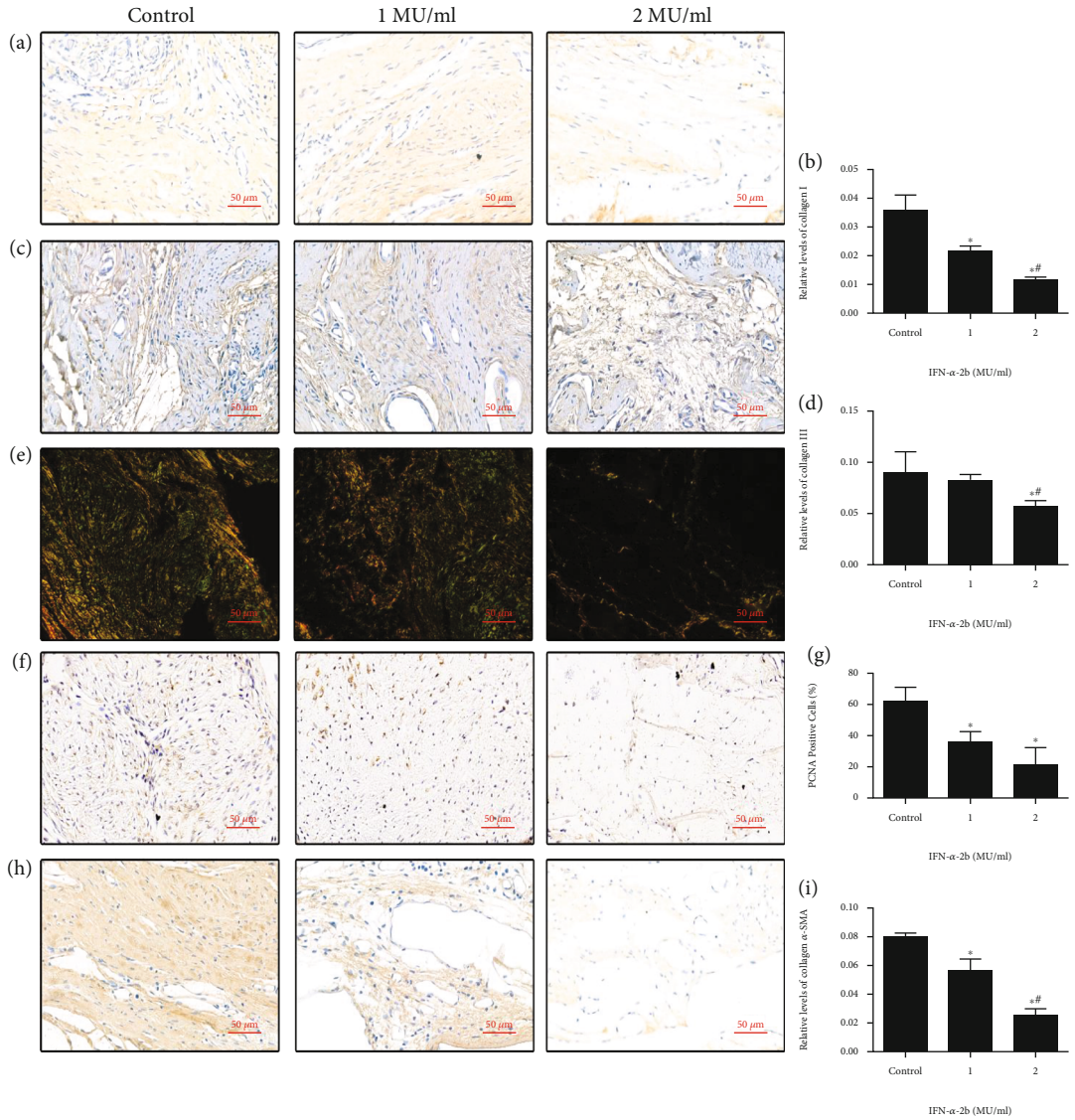
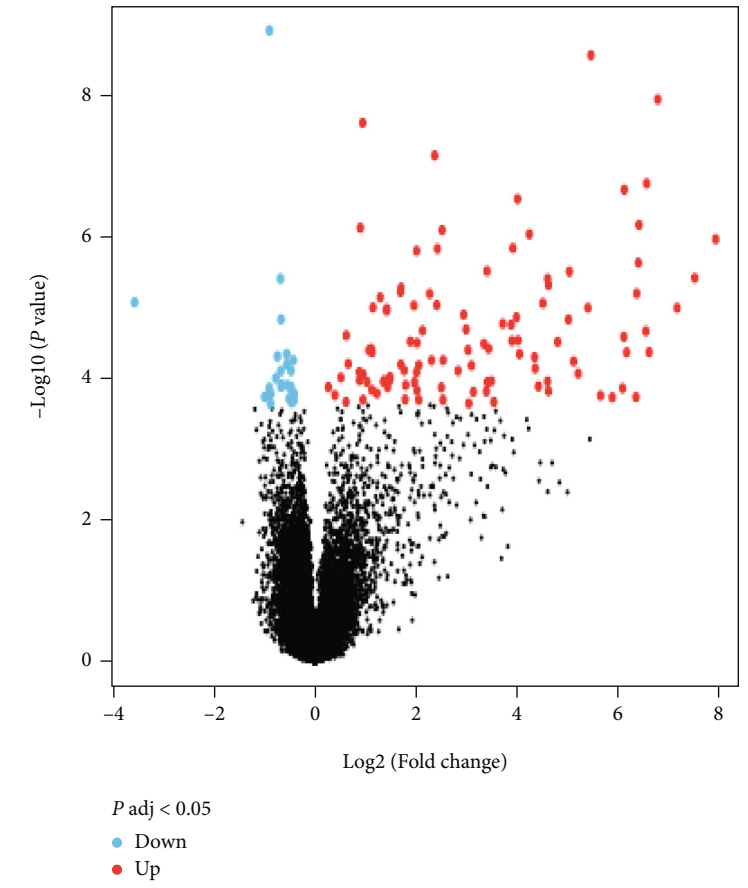


Figure 2: Immunohistochemical staining images of joint fibrosis in different groups (×400 magnification). (a, c) The results of immunohistochemical staining of type I collagen and type III collagen in joint fibrotic tissue. (b, d) The positive expression of collagen immunohistochemical staining in the IFN-α-2b group was significantly reduced. IFN-α-2b effectively inhibits the formation of collagen I and collagen III in fibrotic tissues. (e) The result of Sirius red staining was visualized by polarized light microscope, in which type I collagen was yellow and type III collagen was green. The image shows that collagen fibers decrease with increasing drug concentration. (f, h) Immunohistochemical staining image of PCNA and α-SMA. (g, i) The IFN-α-2b medication group can significantly reduce the expression of PCNA and α-SMA. *Compared with the control group. #Comparison between the two groups of IFN-α-2b medications, $P < 0.05$ ($n = 6$).

microscope; the optical density value of the stained image and the cell count analysis was detected by ImageJ software.

2.5. Immunohistochemical Staining. The sections were deparaffinized and rehydrated. Sodium citrate buffer was used to activate antigenicity, 3% H_2O_2 inhibited peroxidase activity, and the sections were washed with PBS solution for 3 time. Then, the primary antibodies (anti-PCNA, anti-collagen I, and anti-α-SMA) were incubated overnight at 4°C. The sections were incubated with anti-mouse IgG for 30 minutes at room temperature, and the DAB kit was used to detect antibody binding. Finally, hematoxylin counter-staining was carried out, and the results were observed and photographed under an optical microscope.

2.6. Dataset Selection and DEG Identification. The gene expression dataset GSE38652 was obtained from the GEO database (https://www.ncbi.nlm.nih.gov/geo/). The screening basis included (a) IFN-α-2b as the processing factor, (b) fibroblasts as the intervention object, and (c) the organism as Homo sapiens. GSE38652 is based on the GPL10558 (Illumina HumanHT-12 V4.0 expression beadchip) platform, and all data can be obtained online for free. Use GEO2R (https://www.ncbi.nlm.nih.gov/geo/geo2r/)



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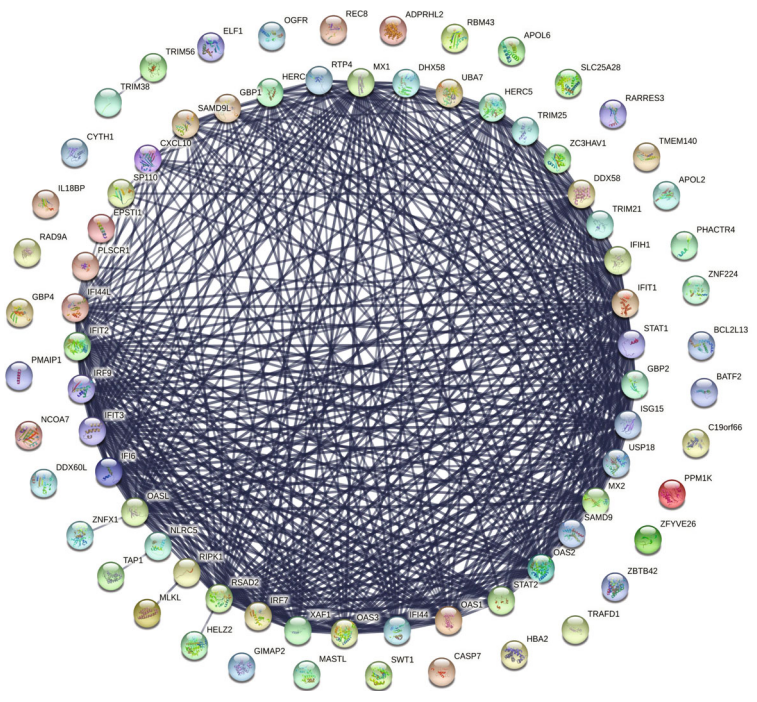

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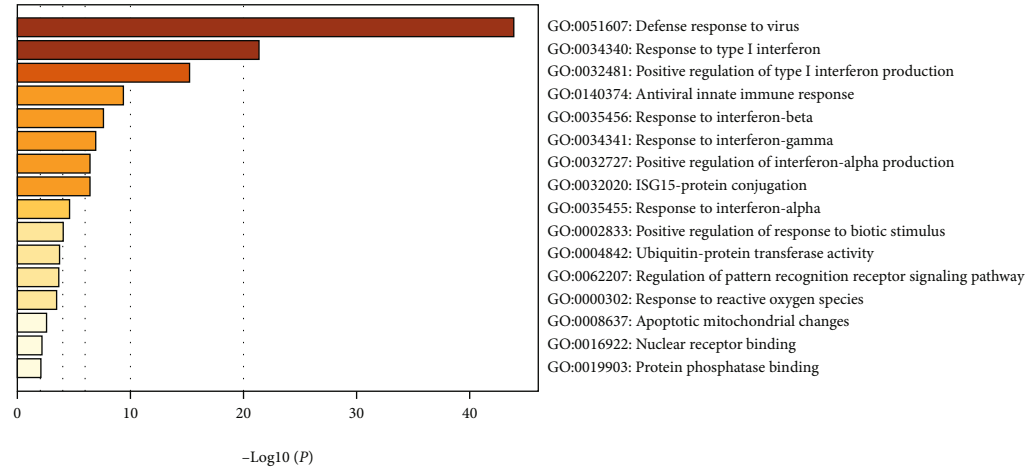

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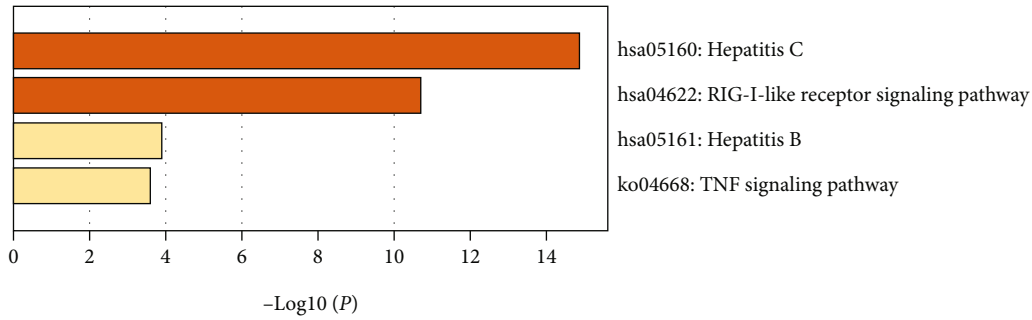

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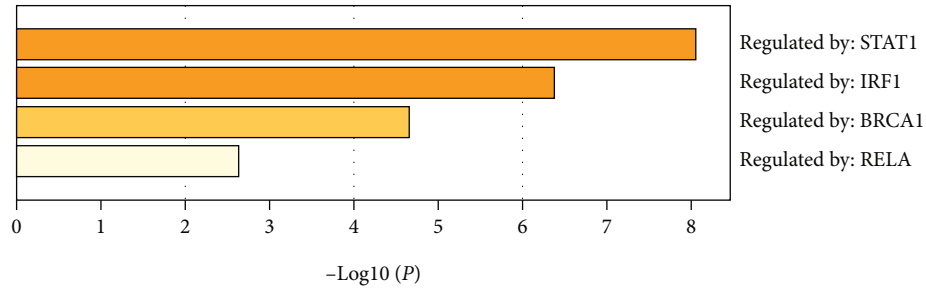

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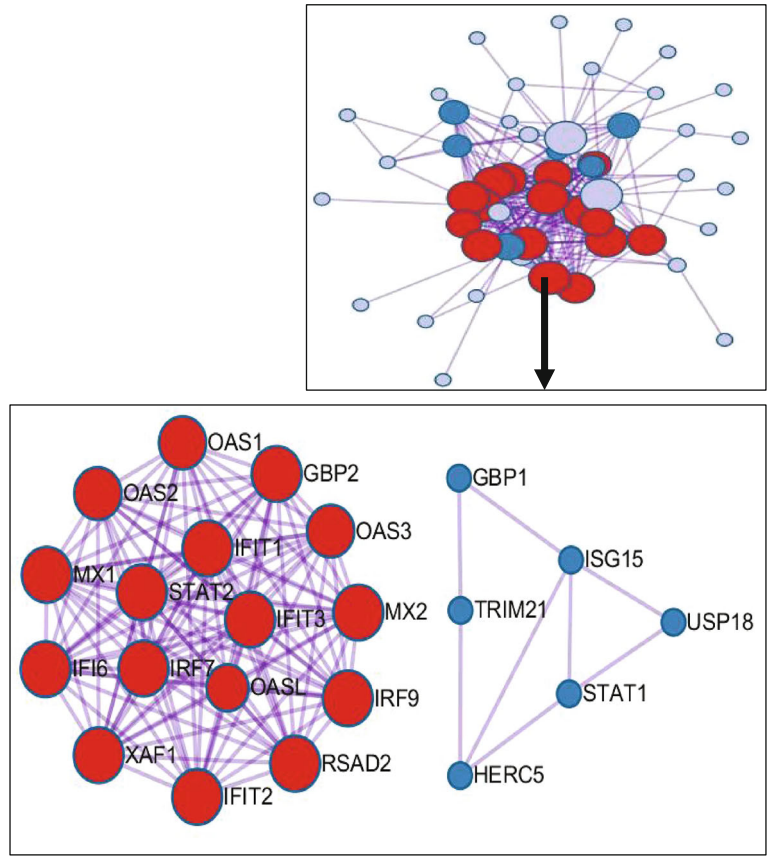


(f)

Figure 3: Dataset selection and DEG enrichment analysis. (a) The volcano map of the differentially expressed genes of IFN-α-2b acting on fibroblasts in the dataset GSE38652. (b) Based on the STRING database, the PPI interaction network diagram showing significant DEGs with an absolute value of logFc ≥ 1. (c–f) Enrichment analysis results based on Metascape. (c) GO enrichment analysis results; (d) KEGG pathway enrichment analysis; (e) TRRUST transcriptional regulatory network analysis results; (f) differential gene interaction relationship PPI network diagram and hub genes.

online software to analyze raw data and identify differentially expressed genes (DEGs). $P < 0.05$ and $|\log FC| \geq 1$ were used as cut-off criteria to obtain DEGs.

2.7. Enrichment Analyses of DEGs and PPI Network. The protein-protein interaction (PPI) network was established by STRING database (http://string-db.org) [18]. In this research, the interaction with high confidence > 0.7 was statistically significant. In order to explore more biological information related to DEGs and obtain more comprehensive gene and protein function, we conducted Gene Ontology (GO) enrichment analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis through Metascape (http://metascape.org) [19]. GO enrichment analysis includes biological processes (BP), cell component (CC), and molecular function (MF). In addition, we also performed TRRUST transcriptional regulatory network analysis [20].

2.8. Fibroblast Culture and IFN-α-2b Treatments. The human fibroblast cell line was offered by Jenino Biotech Co., Ltd. (Guangzhou, China) and then cultured in a medium containing 15% fetal bovine serum (FBS; Gemini, USA) and 1% streptomycin/penicillin (Beyotime, Shanghai, China), at 37°C with 5% CO_2. Select fibroblasts between 3 and 5 generations for subsequent experiments. Fibroblasts were treated with IFN-α-2b in four concentration groups (0, 1000, 5000, and 10000 IU/ml) [17]. In the mechanism research group, we pretreated fibroblasts with 50 μM fludarabine (a specific Stat1 inhibitor) for 2 hours and then changed to a medium containing different concentrations of IFN-α-2b to continue incubating [21].

2.9. Cell Viability Assay. Firstly, 100 μl cell suspension was planted in 96-well culture plate. When the cell density reached 60%-70%, different concentrations of IFN-α-2b were added to treat fibroblasts. After drug stimulation, 10 μl CCK-8 (Dojindo, Tokyo, Japan) solution was added and then cultured at 37°C for 2 hours. Finally, the absorbance at 450 nm was measured.

2.10. Cell Cycle Analysis. Fibroblasts were treated with IFN-α-2b of 5,000 U/ml for 48 hours and then operated according to the instructions of Cell Cycle Testing Kit (Beyotime, Shanghai, China). Cells were collected, centrifuged at 2,000 r/min for 5 minutes, washed with precooled PBS, fixed

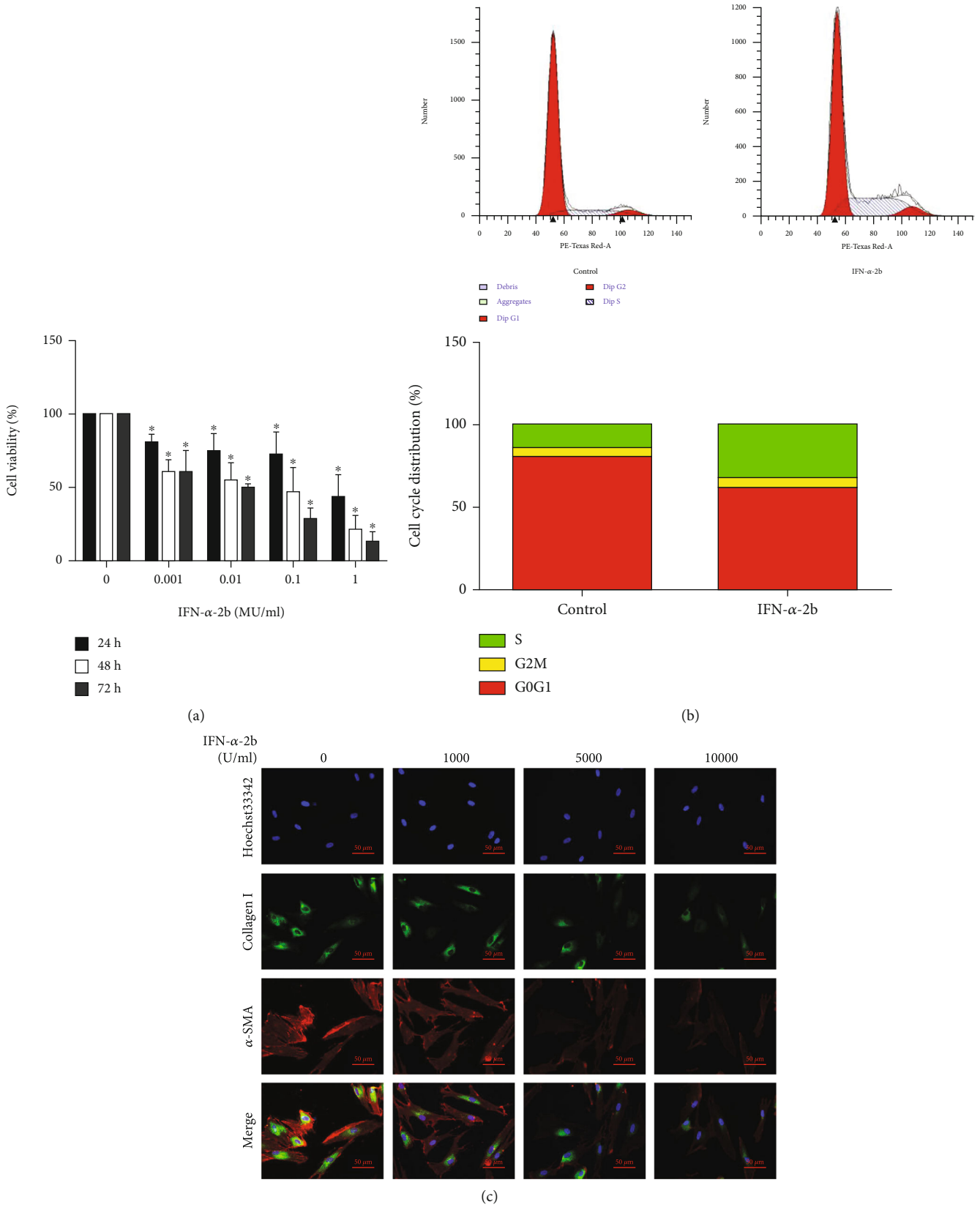
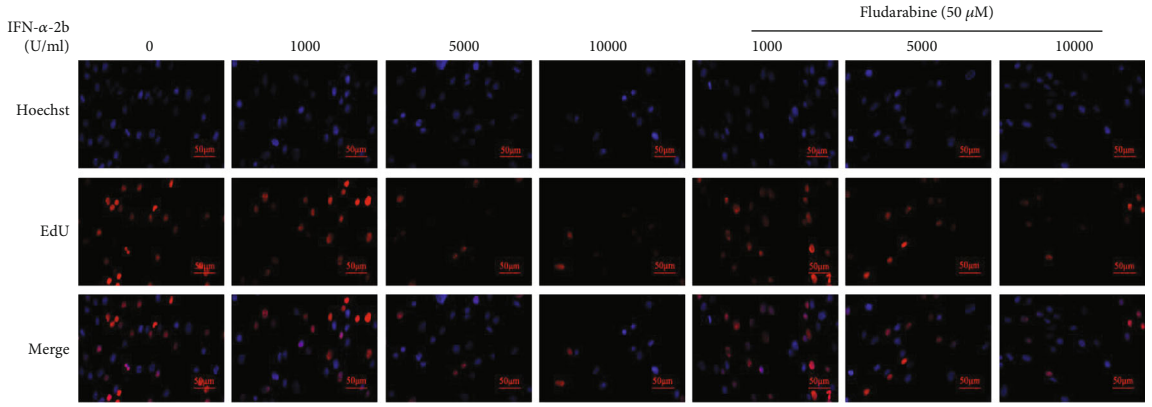


FIGURE 4: IFN-α-2b inhibited fibroblast proliferation and extracellular matrix secretion. (a) CCK-8 assay showed that IFN-α-2b inhibited fibroblast activity in a concentration- and time-dependent manner. (b) Flow cytometry analysis of cell cycle distribution showed that fibroblasts were arrested in the S phase after 48 hours of IFN-α-2b treatment. (c) The expression images of α-SMA and collagen I in cells treated with IFN-α-2b for 48 hours were observed under fluorescence microscope.



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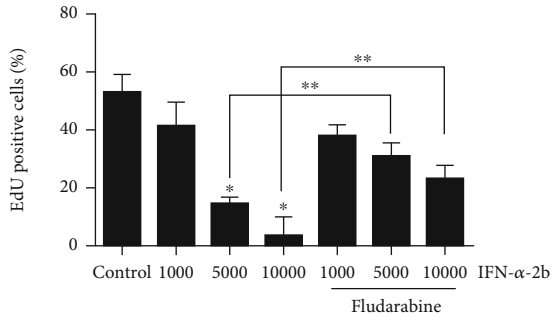

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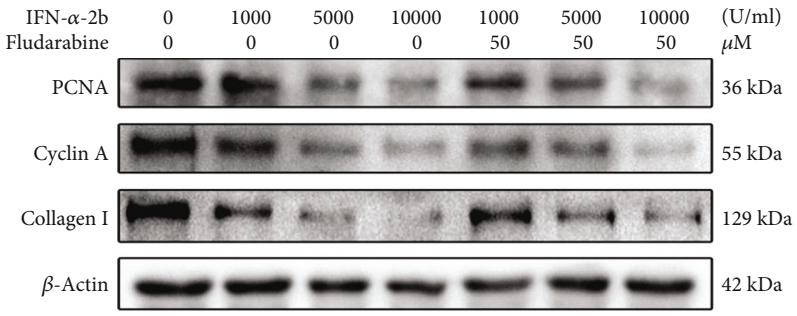

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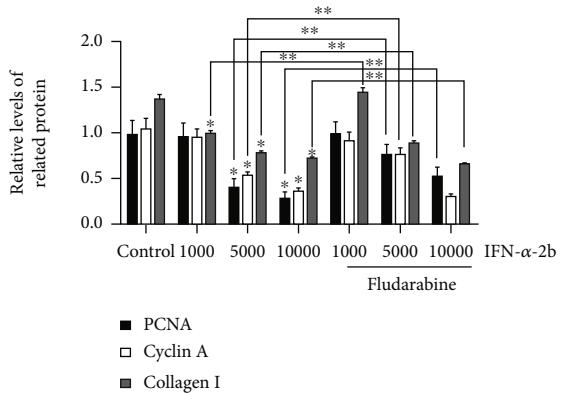

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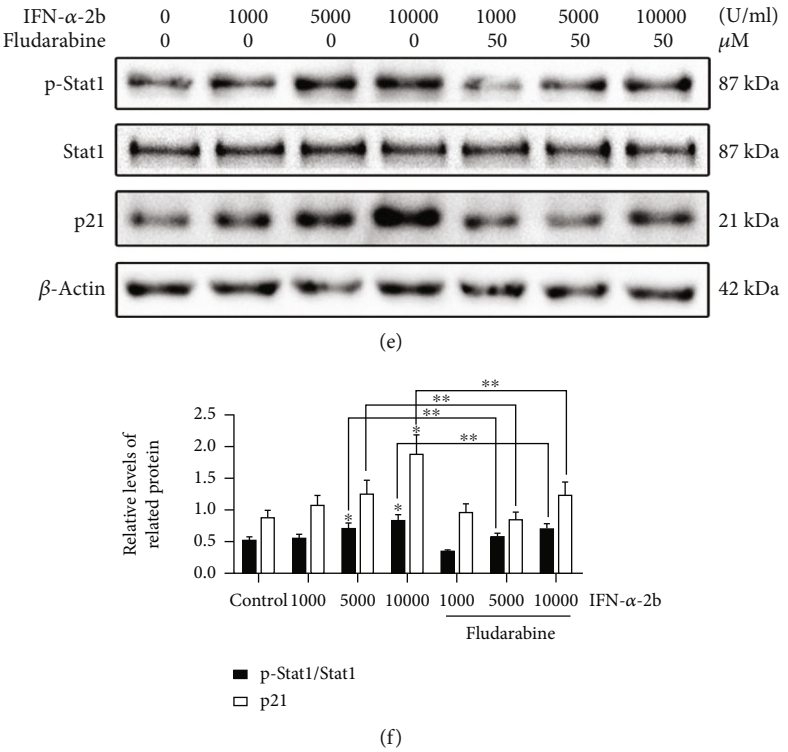


Figure 5: The inhibitory effect of IFN-α-2b on the proliferation of fibroblasts by STAT1/P21 signaling pathway. (a) After treating fibroblasts with IFN-α-2b or combined with fludarabine, perform EdU staining to analyze the result image. The cells shown in red are marked as positive. (b) The results of the EdU incorporation test showed that as the concentration of IFN-α-2b increased, the percentage of positive cells decreased significantly. This trend was partially reversed by fludarabine. (c, e) Western blotting shows the increase of PCNA, cyclin A, and collagen I and predicts the expression levels of related pathway proteins. (d, f) The analysis results showed that IFN-α-2b significantly reduced the relative expression levels of PCNA, cyclin A, and collagen I in a concentration-dependent manner, while increasing the relative expression levels of P-STAT1 and P21. And after fludarabine application, the trend of action was partially reversed. The histogram shows the results of three repeated detections of the gray value of the Western blot band. *Compared with the control group. **Compared between the two groups, $P < 0.05$ ($n = 3$).

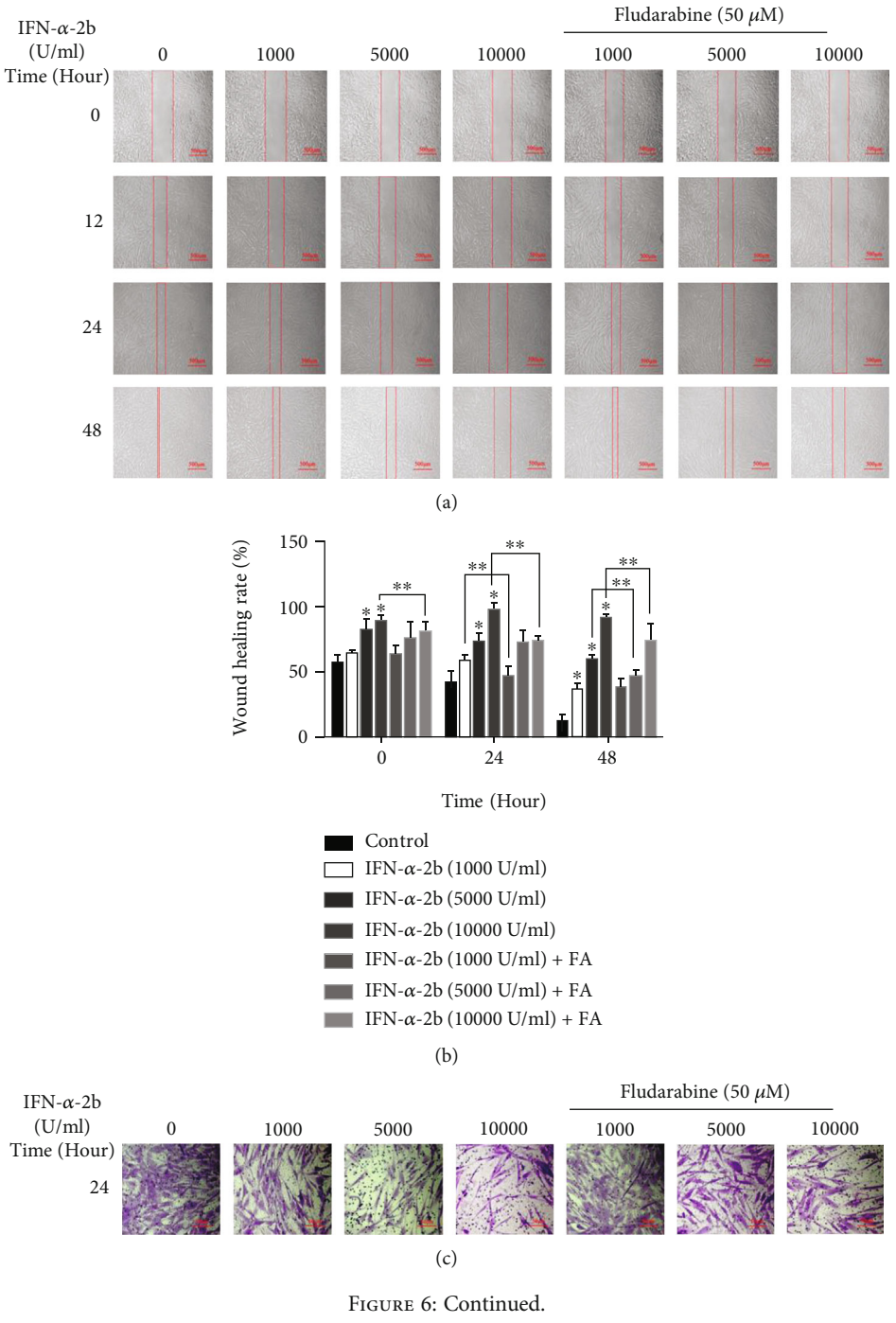
with 70% absolute ethanol, and fixed overnight at 4°C. After centrifugation again, cells were resuspended with 500 μl propidium iodide staining solution prepared in advance, incubated at room temperature for 30 minutes in the dark, and then, flow cytometry was performed. Finally, the distribution of cells in different periods was calculated by ModFit LT software.

2.11. Cell Immunofluorescence Imaging Analysis. Fibroblasts were fixed in 4% paraformaldehyde for 20 minutes, infiltrated with 0.5% Triton X-100 for 10 minutes, and blocked with 5% goat serum at room temperature for 30 minutes. Then, the primary antibody mixture (type I collagen and α-SMA) was dripped onto the cell slide and incubated overnight at 4°C. After PBST cleaning, the secondary antibody was incubated in the dark at room temperature for 1 hour. After cleaning, DAPI was stained in the dark for 10 minutes. Finally, the images were collected under the fluorescence microscope (Zeiss, Germany).

2.12. EdU Incorporation Assay. Cell-Light KFluor555 EdU Kit (KeyGEN, Nanjing, China) was used to detect the proliferation of fibroblasts. Operate according to the instructions. First, plant fibroblasts on glass cell slides in a 6-well plate at a cell density of 1×10^5/well. The cells were cultured at 37°C overnight, and different concentrations of interferon-α-2b were added to the culture medium for 48 hours. After supplementing with 10 μM EdU and incubating for 2 h, the cells were fixed with 4% paraformaldehyde for 15 minutes, and then infiltrate with 0.5% Triton X-100 for 20 minutes. Finally, stain with Hoechst 33342, incubate in the dark at room temperature for 10 min, and observe the positive staining under an upright fluorescence microscope.

2.13. Cell Migration Assay. Wound healing test and Transwell migration test were used to evaluate cell migration behavior. In short, put a sterilized culture-inserts in the center of each hole of the 12 well culture plate, add 70 ml of cell suspension in each interval of the culture-inserts, place it in the cell incubator until the cells are completely fused at the bottom, and then gently remove the culture-inserts. Wash with PBS for 3 times, and then, serum-free medium and detection reagents were added; observe and collect images at different time points (0, 12, 24, and 48 h).

Polycarbonate membrane Transwell filter was selected for Transwell assay (Corning Company, New York, USA). Firstly, 600 μl complete culture medium was added into each well of the 24-well plate, and then, 100 μl of serum-free



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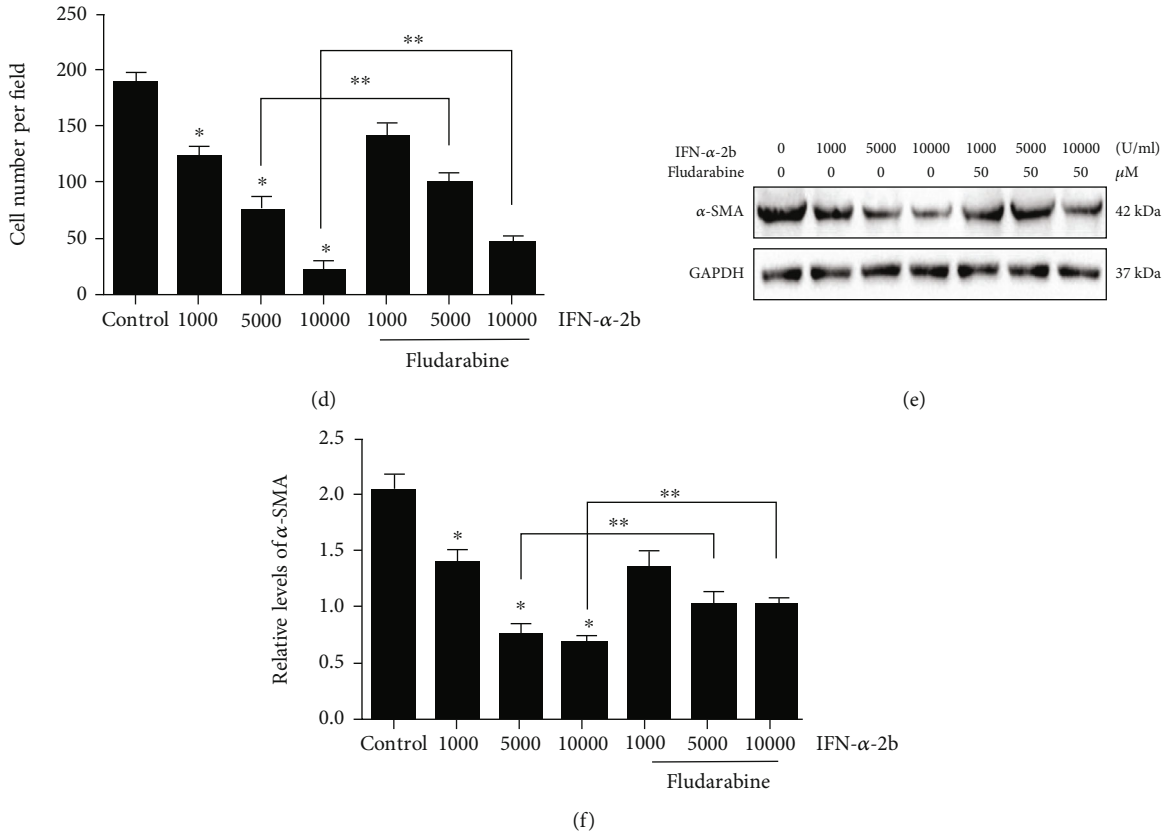

Figure 6: IFN-α-2b inhibits the migration of fibroblasts by STAT1/P21 signaling pathway. (a, b) In vitro wound healing experiments showed that the IFN-α-2b treatment group significantly inhibited the migration rate of fibroblasts. (c, d) Compared with the control group, the number of cells passing through the cell bottom membrane in the IFN-α-2b treatment group was significantly reduced. And it is concentration-dependent. (e, f) Western blotting shows that the IFN-α-2b treatment group can reduce the relative expression level of α-SMA, while the fludarabine and IFN-α-2b coapplication groups can partially reverse the above-mentioned inhibitory trend of IFN-α-2b. Each sample randomly selects 10 microscope fields and conducts 3 independent experiments. *Compared with the control group. **Compared between the two groups, $P < 0.05$ ($n = 3$).

fibroblast suspension (5×10^4 cells) was transferred to the upper layer of the Transwell chamber and treated with different reagents. After cultured in the cell incubator for 24 hours, the chamber was lightly washed with PBS, fixed with 4% paraformaldehyde, stained with crystal violet, and gently wiped the upper cells of the chamber with a cotton swab. Finally, the images were collected under Zeiss inverted microscope. The wound healing rate and migrating transmembrane cells were calculated by ImageJ software.

2.14. Western Blotting Analysis. According to the instructions of RIPA Lysis Solution (Beyotime, Shanghai, China), the total proteins of different groups of fibroblasts were extracted. The same amount of total protein (60 μg/lane) was electrophoresed on 10% or 12% SDS-PAGE and then transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA) at low temperature. Block with 5% skimmed milk or 3% bovine serum albumin (BSA) at room temperature for 2 hours, incubate the primary antibody overnight at 4°C, and then incubate the secondary antibody at room temperature for 2 hours. Finally, the enhanced chemiluminescence detection kit (ECL Plus kit, Beyotime) was used to detect protein bands.

The mouse monoclonal antibodies PCNA (#2586), rabbit monoclonal antibodies cyclin A (#67955), horseradish peroxidase-conjugated goat anti-mouse (#7056), and goat anti-rabbit (#7074) antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). The rabbit monoclonal antibodies STAT1 (ab109320) and phospho-STAT1 (ab109461) were offered by Abcam (Cambridge, UK). The mouse monoclonal α-SMA (67735-1-Ig), rabbit polyclonal antibody P21 (10355-1-AP), collagen type I (14695-1-AP), β-actin (20536-1-AP), and GAPDH (10494-1-AP) were offered by Proteintech Group (Wuhan, China).

2.15. Statistical Analysis. All data in this study were analyzed by SPSS 19.0 statistical software. The data were expressed as mean ± standard deviation. Data were analyzed by one-way analysis of variance (ANOVA), followed by Tukey's test for comparison between groups. $P < 0.05$ was considered statistically significant.

3. Results

3.1. IFN-α-2b Improves Postoperative Arthrofibrosis in Rats. According to the HE staining results, the number of scar

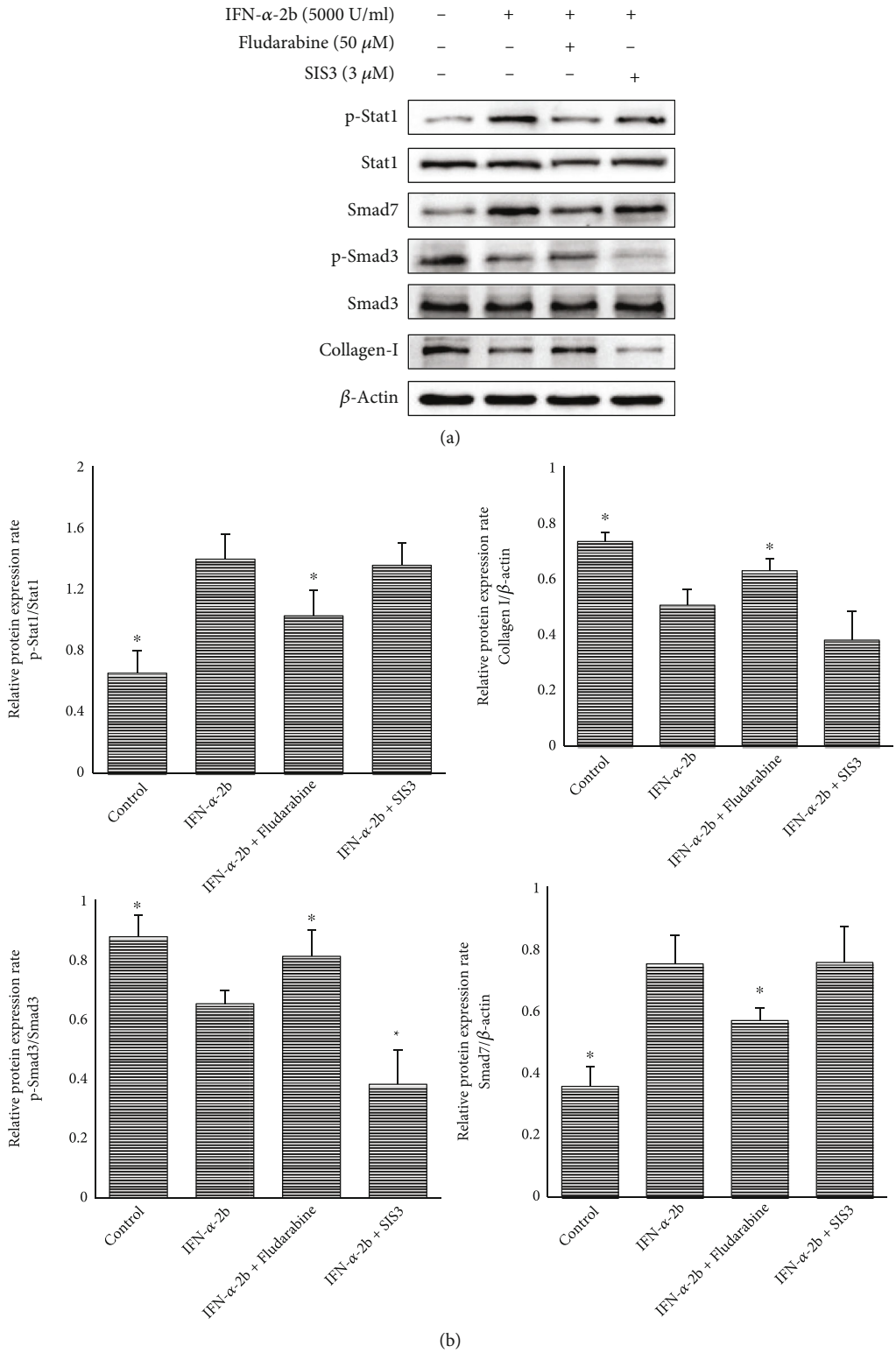

Figure 7: Crosstalk between STAT1/P21 and TGFβ/Smad signaling pathways. (a) Western blotting showed the expression levels of p-Stat1, Smad7, p-Smad3, and collagen I in different groups. (b) The analysis results showed that IFN-α-2b significantly reduced the relative expression levels of p-Smad3 and collagen I, while increasing the relative expression levels of p-STAT1 and Smad7. Within the fludarabine prestimulation group, the effect trend was partially reversed. Compared with the IFN-α-2b group, the change trend of p-Smad3 and collagen I was enhanced in the SIS3 prestimulation group, but only the former had statistical significance, while p-STAT1 and Smad7 had no significant change. The histogram shows the results of three repeated detections of the gray value of the Western blot band. *Compared with the IFN-α-2b group, $P < 0.05$ ($n = 3$).

fibroblasts and dense fibrous tissue around the knee operation area in the IFN-α-2b group decreased, the tissue structure was sparse, and the degree of fibrosis was improved (Figures 1(a) and 1(b)); the number of fibroblasts decreased with the increase of IFN-α-2b concentration (Figure 1(c)). Masson's staining was used to evaluate the degree of collagen synthesis in arthrofibrosis after local administration of IFN-α-2b. The staining results showed that collagen synthesis in arthrofibrosis decreased after application of IFN-α-2b, especially in the high-dose application group (Figure 1(d)); the results of optical density analysis also suggest that IFN-α-2b can effectively reduce the production of collagen (Figure 1(e)).

Immunohistochemical staining showed that the expression of type I collagen in the IFN-α-2b treatment group was lower than that in the normal saline application group (Figures 2(a) and 2(b)). The same was true for the expression of type III collagen (Figures 2(c) and 2(d)). Sirius red staining showed that type I collagen fibers (red or yellow) and type III collagen fibers (green) decreased in the IFN-α-2b treatment group (Figure 2(e)). Similarly, immunohistochemical staining of proliferation- and migration-related proteins PCNA (Figure 2(f)) and α-SMA (Figure 2(h)) showed that the expression of the IFN-α-2b-treated group was significantly lower than that of the control group (Figures 2(g) and 2(i)). In conclusion, the above in vivo experimental results suggest that IFN-α-2b has the potential to improve arthrofibrosis in rats.

3.2. DEG Acquisition and PPI Network Relationship, GO, KEGG, and TRRUST Enrichment Analyses. The volcano map shows the distribution of 28,553 expressed genes (Figure 3(a)). Among them, according to $|\log FC| \geq 1$ and $P < 0.05$ as the cut-off criteria, 78 DEGs were extracted, and the interaction relationship is shown in Figure 3(b). In order to obtain more biological information, we used the online database (Metascape) to perform GO and KEGG enrichment analyses. DEGs are divided into three functional groups: biological processes (BP), molecular functions (MF), and cellular components (CC). GO analysis shows that changes in biological processes are significantly enriched in defense response to virus, negative regulation of viral process, response to type I interferon, positive regulation of type I interferon production, and other immune response and transcriptional regulation. As for cellular components, DEGs are abundant in the perinuclear region of cytoplasm. Changes in molecular functions are mainly concentrated in 2′-5′-oligoadenylate synthetase activity, RNA helicase activity, nuclearoside-triphosphatase activity, ubiquitin-protein transferase activity, protein homodimerization activity, ubiquitin-like protein ligase binding, nuclear receptor binding, and protein phosphatase binding aspect (Figure 3(c)). KEGG pathway analysis showed that DEGs play a key role in hepatitis C/B, RIG-I-like receptor signaling pathway, and TNF signaling pathway (Figure 3(d)). The TRRUST database can provide information on how to regulate these interactions, and analysis suggests that DEG transcriptional regulation is mainly enriched in genes such as STAT1, IRF1, BRCA1, and RELA (Figure 3(e)). The protein-protein interaction (PPI) network results showed 22 hub genes and 125 connections (Figure 3(f)), including the STAT1 gene. Through the above analysis, we speculate that the role played by IFN-α-2b on fibroblasts is mainly regulated by STAT1, and P21, as a downstream molecule of STAT1, can regulate cell proliferation. Therefore, we verified the STAT1/P21 pathway in vitro.

3.3. IFN-α-2b Inhibits Fibroblast Proliferation. CCK-8 assay showed that the activity of fibroblasts decreased with the increase of IFN-α-2b concentration and the prolongation of treatment time. The results showed that IFN-α-2b inhibited the activity of fibroblasts in a time- and concentration-dependent manner (Figure 4(a)). Flow cytometry analysis showed that the proportion of IFN-α-2b-treated cells increased significantly in the S phase, suggesting that the cell cycle may be blocked in the S phase (Figure 4(b)). Fluorescence microscopy analysis showed that with the increase of IFN-α-2b concentration, the fluorescence intensity of type I collagen and α-SMA gradually decreased, suggesting that IFN-α-2b reduces the formation of extracellular matrix (Figure 4(c)).

The analysis of EdU experimental results showed that as the concentration of IFN-α-2b increased, EdU-positive fibroblasts decreased, the statistical results showed a significant difference, and the fludarabine group partially reversed the trend (Figures 5(a) and 5(b)). In addition, Western blot results showed that the expression levels of proliferation-related proteins PCNA, cyclin A, and type I collagen also decreased with the increase of IFN-α-2b concentration (Figures 5(c) and 5(d)), while STAT1 and P21 were activated with the application of IFN-α-2b. This is because the expression levels of p-STAT1/STAT1 and P21 gradually increased (Figures 5(e) and 5(f)). With the addition of fludarabine, the above phenomenon reversed the blocking expression trend to a certain extent.

3.4. IFN-α-2b Inhibits Fibroblast Migration. After treatment with IFN-α-2b, the migration of fibroblasts was inhibited. Compared with the control group, the wound healing rate of the IFN-α-2b group decreased in a dose-dependent manner (Figures 6(a) and 6(b)). The results of Transwell migration experiment showed that the concentration of IFN-α-2b increased. As the concentration of IFN-α-2b increases, the number of cells on the bottom membrane of the migration chamber gradually decreases (Figures 6(c) and 6(d)). Western blot showed that the expression level of α-SMA in the IFN-α-2b application group showed a downward trend, and the fludarabine application group also saw a partial reversal of the inhibition trend (Figures 6(e) and 6(f)). In summary, the above experimental results show that IFN-α-2b can inhibit the proliferation and migration of fibroblasts, and the STAT1/P21 signaling pathway may be involved in the regulation.

3.5. Crosstalk between STAT1/P21 and TGFβ/Smad Signaling Pathways. Our team before published an article on IFN-α-2b treatment of epidural postoperative adhesion, which showed that IFN-α-2b can play a role by inhibiting TGFβ/Smad signal pathway [17]. The relevant signaling pathways

involved in this manuscript are experimentally verified by us after screening the network database. The results showed that IFN-α-2b could inhibit proliferation and fibrosis after knee surgery through STAT1/P21 pathway. For these two signaling pathways in the study of the relationship, we conducted the experiment verification. Here, we added Smad3-specific inhibitor (SIS3) and STAT1-specific inhibitor fludarabine for prestimulation [21, 22], and the results showed that STAT1 phosphorylation was activated by IFN-α-2b application and Smad7 expression was increased (which inhibited Smad3 phosphorylation). In addition, Smad3 phosphorylation and type I collagen expression were significantly decreased, which could be reversed by fludarabine pretreatment. The activation of p-STAT1 and the expression of Samd7 decreased, the phosphorylation of Smad3 increased, and the expression of type I collagen increased. However, the phosphorylation level of Smad3 was significantly reduced in the group treated with SIS3 in advance, although the expression of type 1 collagen also showed a downward trend, but there was no statistical difference, and the activated expression levels of Smad7 and p-STAT1 did not change significantly (Figures 7(a) and 7(b)). According to the experimental results, we inferred that there is crosstalk between STAT1/P21 and TGFβ/Smad signaling pathways, which may be due to the phosphorylation and activation of STAT1, which stimulates the increase of Smad7 and then plays a role in inhibiting the activation of Smad3.

4. Discussion

As mentioned earlier, excessive proliferation of fibroblasts and excessive secretion of extracellular matrix (ECM) in the surgical area can lead to fibrosis [10], while collagen is one of the important components of extracellular matrix, which can promote cell proliferation and migration [23]. Fibroblasts are transformed into myofibroblasts during fibrosis, resulting in excessive synthesis and deposition of extracellular matrix proteins [24, 25]. The expression of α-SMA is considered to be a specific marker of myofibroblasts [26]. Clinical studies have reported that the expression of α-SMA increased significantly in joint fibrosis specimens [27], and the high expression of α-SMA is also closely related to the proliferation and migration of fibroblasts [28, 29]. In this study, both Masson's staining and Sirius red staining showed a decrease in collagen deposition in the IFN-α-2b treatment group. The results of in vitro experiments also suggested that the expression of collagen I and α-SMA decreased in the IFN-α-2b-treated group.

A number of studies have reported that IFN-α-2b can activate STAT1, resulting in the phosphorylation of STAT1 [30, 31]. STAT1 is a member of STAT protein family. In response to cytokines and growth factors, STAT family members are phosphorylated by receptor-related kinases and then form homodimers or heterodimers, which are transferred to the nucleus as transcriptional activators. It has been reported that the mice, struck off the STAT1 gene, are more likely to have chemically induced lung and liver fibrosis [32]. In the skin model, the expression of collagen and α-SMA in the granulation tissue around the wound of mice with fibroblast STAT1 gene defects increased, and the perivascular fibrosis increased significantly. These results indicated that STAT1 plays a role in tissue repair [33]. As a key element of IFN-α-2b signal transduction, the function of STAT1 is mainly determined by its phosphorylation state. As an active form of STAT1, p-STAT1 has been shown to inhibit tumor growth by regulating cell cycles [34]. As a regulator downstream of STAT1, p21 is the first identified cyclin-dependent kinase inhibitor (CKI) protein member. P21 can bind to several compounds of cyclins and cyclin-dependent kinases (CDK), such as cyclin A/CDK2, cyclin E/CDK2, cyclin D1/CDK4, and cyclin D2/CDK4 [35, 36]. The increased expression of p21 can reduce the expression of cyclin, thus inducing cell cycle stagnation and inhibiting cell proliferation [37], which is consistent with our experimental results. In this study, with the increase of IFN-α-2b concentration, the expression of p-stat1 and p21 increased, while cell proliferation and migration were inhibited. The results of the pretreatment group with STAT1-specific inhibitor (fludarabine) partially reversed this trend. This means that STAT1/p21signaling pathway is involved in the antiproliferation and migration inhibition of IFN-α-2b. In this study, we explored the relationship between STAT1/P21 and TGFβ/Smad signaling pathways. After all, the TGFβ/Smad signaling pathway plays a crucial role in the formation of fibrosis. The results suggest that there is crosstalk between the two signal pathways; that is, the activation of Stat1 can promote the increase of the expression of Smad7, thus preventing the expression of Smad3, which is similar to some previous studies [38, 39].

During our experiment, there were no delayed wound healing, epidermal necrosis, surgical incision infection, and death. However, we did not explore the application of larger dose and time, nor did we screen the optimal application concentration of drugs, which is also the deficiency of this experiment. In addition, the formation mechanism of fibrosis is very complex. In addition to the proliferation and migration of fibroblasts, inflammatory response is also involved in the formation of arthrofibrosis [40]. Interestingly, STAT1 acts as a transcription factor in regulating pro-inflammatory and anti-inflammatory responses, making STAT1 an attractive anti-inflammatory target [41]. However, these were not measured in this study. We will continue to refine these explorations in future research. We are looking forward to more comprehensive and in-depth research and application.

5. Conclusion

In conclusion, this study firstly verified that IFN-α-2b can reduce surgery-induced arthrofibrosis by inhibiting fibroblast proliferation and migration, which may be related to the regulation of STAT1/p21 signaling pathway. Our study provides a new therapeutic target for intervention in arthrofibrosis.

Abbreviations

STAT1: Signal transducer and activator of transcription 1
α-SMA: α-Smooth muscle actin

ECM: Extracellular matrix
PCNA: Proliferating cell nuclear antigen
DEGs: Differentially expressed genes
IFN-α-2b: Interferon-alpha-2b
PPI: Protein-protein interaction
HE: Hematoxylin-eosin.

Data Availability

The datasets supporting the conclusions of this article are included within the article.

Ethical Approval

This experimental study was approved by the Ethics Committee and Research Committee of Jiangsu Subei People's Hospital. The animal experiment was approved by the Animal Ethics Committee of Yangzhou University, and all the rats received humanitarian care.

Conflicts of Interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work; there is no professional or other personal interest of any nature or kind in any product, service, and/or company that could be construed as influencing the position presented in, or the review of, the manuscript entitled.

Authors' Contributions

Zhendong Liu and Zhehao Fan equally contributed to the paper.

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References

[1] V. A. Cheuy, J. R. H. Foran, R. J. Paxton, M. J. Bade, J. A. Zeni, and J. E. Stevens-Lapsley, "Arthrofibrosis associated with total knee arthroplasty," *The Journal of Arthroplasty*, vol. 32, no. 8, pp. 2604–2611, 2017.

[2] T. L. Sanders, H. M. Kremers, A. J. Bryan, W. K. Kremers, M. J. Stuart, and A. J. Krych, "Procedural intervention for arthrofibrosis after ACL reconstruction: trends over two decades," *Knee Surgery, Sports Traumatology, Arthroscopy*, vol. 25, no. 2, pp. 532–537, 2017.

[3] P. S. Vezeridis, D. P. Goel, A. A. Shah, S. Y. Sung, and J. J. P. Warner, "Postarthroscopic arthrofibrosis of the shoulder," *Sports Medicine and Arthroscopy Review*, vol. 18, no. 3, pp. 198–206, 2010.

[4] B. Bayram, A. K. Limberg, C. G. Salib et al., "Molecular pathology of human knee arthrofibrosis defined by RNA sequencing," *Genomics*, vol. 112, no. 4, pp. 2703–2712, 2020.

[5] X. Li, H. Chen, Y. Sun et al., "Hydroxycamptothecin prevents intraarticular scar adhesion by activating the PERK signal pathway," *European Journal of Pharmacology*, vol. 810, pp. 36–43, 2017.

[6] K. M. Usher, S. Zhu, G. Mavropalias, J. A. Carrino, J. Zhao, and J. Xu, "Pathological mechanisms and therapeutic outlooks for arthrofibrosis," *Bone Research*, vol. 7, no. 1, p. 9, 2019.

[7] J. Manrique, M. M. Gomez, and J. Parvizi, "Stiffness after total knee arthroplasty," *The Journal of Knee Surgery*, vol. 28, no. 2, pp. 119–126, 2015.

[8] R. Thompson, D. Novikov, Z. Cizmic et al., "Arthrofibrosis after total knee arthroplasty: pathophysiology, diagnosis, and management," *The Orthopedic Clinics of North America*, vol. 50, no. 3, pp. 269–279, 2019.

[9] X. Li, L. Yan, J. Wang et al., "Comparison of the effects of mitomycin C and 10-hydroxycamptothecin on an experimental intraarticular adhesion model in rabbits," *European Journal of Pharmacology*, vol. 703, no. 1-3, pp. 42–45, 2013.

[10] Y. Liu, Z. Zhang, L. Yan et al., "Everolimus reduces postoperative arthrofibrosis in rabbits by inducing autophagy-mediated fibroblast apoptosis by PI3K/Akt/mTOR signaling pathway," *Biochemical and Biophysical Research Communications*, vol. 533, no. 1, pp. 1–8, 2020.

[11] S. Ekhtiari, N. S. Horner, D. de SA et al., "Arthrofibrosis after ACL reconstruction is best treated in a step-wise approach with early recognition and intervention: a systematic review," *Knee Surgery, Sports Traumatology, Arthroscopy*, vol. 25, no. 12, pp. 3929–3937, 2017.

[12] L. Prata, A. Folgado, J. Maia-Seco, A. Carvalho, E. Mendonça, and A. Mâncio-Santos, "Human alfa-2b in rabbit's eyes," *Ophtalmologie*, vol. 4, no. 4, pp. 382–384, 1990.

[13] J. H. Lee, S. E. Kim, and A. Y. Lee, "Effects of interferon-α2b on keloid treatment with triamcinolone acetonide intralesional injection," *International Journal of Dermatology*, vol. 47, no. 2, pp. 183–186, 2008.

[14] Y.-F. Gong, X.-M. Zhang, F. Liu et al., "Inhibitory effect of recombinant human endostatin on the proliferation of hypertrophic scar fibroblasts in a rabbit ear model," *European Journal of Pharmacology*, vol. 791, pp. 647–654, 2016.

[15] M. Yin, H. Li, Y. Zhang, H. Dai, F. Luo, and Z. Pan, "Interferon alpha-2b eye drops prevent recurrence of pterygium after the bare sclera technique: a single-center, sequential, and controlled study," *Cornea*, vol. 38, no. 10, pp. 1239–1244, 2019.

[16] A. M. H. Cornelissen, J. W. Von den Hoff, J. C. Maltha, and A. M. Kuijpers-Jagtman, "Effects of interferons on proliferation and collagen synthesis of rat palatal wound fibroblasts," *Archives of Oral Biology*, vol. 44, no. 7, pp. 541–547, 1999.

[17] Z. Liu, H. Chen, Z. Fan et al., "IFN-α-2b inhibits the proliferation and migration of fibroblasts via the TGFβ/Smad signaling pathway to reduce postoperative epidural fibrosis," *Journal of Interferon & Cytokine Research*, vol. 41, no. 8, pp. 271–282, 2021.

[18] D. Szklarczyk, A. L. Gable, K. C. Nastou et al., "The STRING database in 2021: customizable protein-protein networks, and functional characterization of user-uploaded gene/measurement sets," *Nucleic Acids Research*, vol. 49, no. D1, pp. D605–D612, 2021.

[19] Y. Zhou, B. Zhou, L. Pache et al., "Metascape provides a biologist-oriented resource for the analysis of systems-level datasets," *Nature Communications*, vol. 10, no. 1, p. 1523, 2019.

[20] H. Han, J. W. Cho, S. Lee et al., "TRRUST v2: an expanded reference database of human and mouse transcriptional regulatory interactions," *Nucleic Acids Research*, vol. 46, no. D1, pp. D380–D386, 2018.

[21] K. Lee, S. H. Um, D. K. Rhee, and S. Pyo, "Interferon-alpha inhibits adipogenesis via regulation of JAK/STAT1 signaling," *Biochimica et Biophysica Acta (BBA) - General Subjects*, vol. 1860, no. 11, pp. 2416–2427, 2016.

[22] M. Jinnin, H. Ihn, and K. Tamaki, "Characterization of SIS3, a novel specific inhibitor of Smad3, and its effect on transforming growth factor-β1-induced extracellular matrix expression," *Molecular Pharmacology*, vol. 69, no. 2, pp. 597–607, 2006.

[23] D. Naci and F. Aoudjit, "Alpha2beta1 integrin promotes T cell survival and migration through the concomitant activation of ERK/Mcl-1 and p38 MAPK pathways," *Cellular Signalling*, vol. 26, no. 9, pp. 2008–2015, 2014.

[24] B. Hinz and D. Lagares, "Evasion of apoptosis by myofibroblasts: a hallmark of fibrotic diseases," *Nature Reviews Rheumatology*, vol. 16, no. 1, pp. 11–31, 2020.

[25] R. Weiskirchen, S. Weiskirchen, and F. Tacke, "Organ and tissue fibrosis: molecular signals, cellular mechanisms and translational implications," *Molecular Aspects of Medicine*, vol. 65, pp. 2–15, 2019.

[26] F. N. Unterhauser, U. Bosch, J. Zeichen, and A. Weiler, "Alpha-smooth muscle actin containing contractile fibroblastic cells in human knee arthrofibrosis tissue," *Archives of Orthopaedic and Trauma Surgery*, vol. 124, no. 9, pp. 585–591, 2004.

[27] H. O. Mayr, F. F. Fassbender, W. C. Prall, F. Haasters, A. Bernstein, and A. Stoehr, "Immunohistochemical examination in arthrofibrosis of the knee joint," *Archives of Orthopaedic and Trauma Surgery*, vol. 139, no. 3, pp. 383–391, 2019.

[28] A. Sugiyama, Y. Hirano, M. Okada, and H. Yamawaki, "Endostatin stimulates proliferation and migration of myofibroblasts isolated from myocardial infarction model rats," *International Journal of Molecular Sciences*, vol. 19, no. 3, p. 741, 2018.

[29] K. Masada, S. Miyagawa, Y. Sakai, A. Harada, T. Kanaya, and Y. Sawa, "Synthetic prostacyclin agonist attenuates pressure-overloaded cardiac fibrosis by inhibiting FMT," *Molecular Therapy - Methods & Clinical Development*, vol. 19, pp. 210–219, 2020.

[30] G. B. Lesinski, S. V. Kondadasula, T. Crespin et al., "Multiparametric flow cytometric analysis of inter-patient variation in STAT1 phosphorylation following interferon alfa immunotherapy," *Journal of the National Cancer Institute*, vol. 96, no. 17, pp. 1331–1342, 2004.

[31] B. Xu, B. Tang, and J. Wei, "Role of STAT1 in the resistance of HBV to IFN-α," *Experimental and Therapeutic Medicine*, vol. 21, no. 6, p. 550, 2021.

[32] D. M. Walters, A. Antao-Menezes, J. L. Ingram et al., "Susceptibility of signal transducer and activator of transcription-1-deficient mice to pulmonary fibrogenesis," *The American Journal of Pathology*, vol. 167, no. 5, pp. 1221–1229, 2005.

[33] S. C. Medley, B. H. Rathnakar, C. Georgescu, J. D. Wren, and L. E. Olson, "Fibroblast-specific Stat1 deletion enhances the myofibroblast phenotype during tissue repair," *Wound Repair and Regeneration*, vol. 28, no. 4, pp. 448–459, 2020.

[34] K. Meissl, S. Macho-Maschler, M. Müller, and B. Strobl, "The good and the bad faces of STAT1 in solid tumours," *Cytokine*, vol. 89, pp. 12–20, 2017.

[35] Y. E. Chin, M. Kitagawa, W. C. Su, Z. H. You, Y. Iwamoto, and X. Y. Fu, "Cell growth arrest and induction of cyclin-dependent kinase inhibitor p21 WAF1/CIP1 mediated by STAT1," *Science*, vol. 272, no. 5262, pp. 719–722, 1996.

[36] A. Karimian, Y. Ahmadi, and B. Yousefi, "Multiple functions of p21 in cell cycle, apoptosis and transcriptional regulation after DNA damage," *DNA Repair*, vol. 42, pp. 63–71, 2016.

[37] N. Prasad, A. Sabarwal, U. C. S. Yadav, and R. P. Singh, "Lupeol induces S-phase arrest and mitochondria-mediated apoptosis in cervical cancer cells," *Journal of Biosciences*, vol. 43, no. 2, pp. 249–261, 2018.

[38] Y. Ishida, T. Kondo, T. Takayasu, Y. Iwakura, and N. Mukaida, "The essential involvement of cross-talk between IFN-gamma and TGF-beta in the skin wound-healing process," *Journal of Immunology*, vol. 172, no. 3, pp. 1848–1855, 2004.

[39] C. Reardon and D. M. McKay, "TGF-beta suppresses IFN-gamma-STAT1-dependent gene transcription by enhancing STAT1-PIAS1 interactions in epithelia but not monocytes/macrophages," *Journal of Immunology*, vol. 178, no. 7, pp. 4284–4295, 2007.

[40] A. K. Limberg, M. E. Tibbo, C. G. Salib et al., "Reduction of arthrofibrosis utilizing a collagen membrane drug-eluting scaffold with celecoxib and subcutaneous injections with ketotifen," *Journal of Orthopaedic Research*, vol. 38, no. 11, pp. 2474–2483, 2020.

[41] C. Y. Loh, A. Arya, A. F. Naema, W. F. Wong, G. Sethi, and C. Y. Looi, "Signal transducer and activator of transcription (STATs) proteins in cancer and inflammation: functions and therapeutic implication," *Frontiers in Oncology*, vol. 9, p. 48, 2019.

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Research Article

Expression of Thymic Stromal Lymphopoietin in Immune-Related Dermatoses

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Thymic stromal lymphopoietin (TSLP), long known to be involved in Th2 response, is also implicated in multiple inflammatory dermatoses and cancers. The purpose of this study was to improve our understanding of the expression of TSLP in the skin of those dermatoses. Lesional specimens of representative immune-related dermatoses, including lichen planus (LP), discoid lupus erythematosus (DLE), eczema, bullous pemphigoid (BP), psoriasis vulgaris (PsV), sarcoidosis, and mycosis fungoides (MF), were retrospectively collected and analyzed by immunohistochemistry. Morphologically, TSLP was extensively expressed in the epidermis of each dermatosis, but the expression was weak in specimens of DLE. In a semiquantitative analysis, TSLP was significantly expressed in the epidermis in LP, BP, eczema, PsV, sarcoidosis, and MF. TSLP expression was higher in the stratum spinosum in LP, eczema, BP, PsV, and MF and higher in the stratum basale in sarcoidosis and PsV. Moreover, we found positive TSLP staining in the dermal infiltrating inflammatory cells of BP, PsV, and sarcoidosis. Our observation of TSLP in different inflammatory dermatoses might provide a novel understanding of TSLP in the mechanism of diseases with distinctly different immune response patterns and suggest a potential novel therapeutic target of those diseases.

1. Introduction

Thymic stromal lymphopoietin (TSLP), a type of epithelial-derived cytokine, was originally identified as a factor promoting B cell proliferation and development [1, 2]. Subsequently, TSLP was found to induce dendritic cells (DCs) or directly act on innate lymphoid cells and CD4+ T cells and to promote the immune responses of helper T type (Th) 2 cells [2, 3]. Furthermore, previous studies have demonstrated that TSLP promotes Th17 cell differentiation under Th2 polarizing conditions [4] and acts on neutrophils to enhance *S. aureus* killing in the innate immune response [5]. Hematopoietic progenitor cells, basophils, eosinophils, mast cells, monocytes/macrophages, and platelets also express TSLP receptors (TSLPR) and thus could be activated by TSLP [3, 6].

Initially, research about TSLP mainly focused on allergic disorders, such as allergic asthma, atopic dermatitis (AD), allergic rhinitis, eosinophilic esophagitis, and food allergies [2, 7, 8]. It was gradually revealed that TSLP is extensively involved in autoimmune diseases and cancers [3]. Moreover, expression of TSLP was found to participate in many inflammatory dermatoses, such as psoriasis vulgaris (PsV) [9, 10], bullous pemphigoid (BP) [11], and alopecia areata [12]. Those diseases distinctly differ in clinical manifestations, pathology, and immune response patterns.

Combining clinical and histopathologic phenotypes with immunology and molecular genetics, noncommunicable inflammatory dermatoses could be classified into six immune response patterns [13]. PsV is a classic psoriatic pattern dermatosis and regards as a Th1/Th17-induced inflammatory disease [13, 14]. Eczema is a common

inflammatory dermatosis with widely diverse aetiologies and is considered one of the type 2 immune diseases. BP, the most common autoimmune blister disease, is mainly induced by pathogenic autoantibodies and the Th2 response. TSLP expression was elevated in sera and lesions of patients with PsV and BP [9–11, 15]. But the previous studies have not revealed the specific site of skin lesions that TSLP expressed in those dermatoses. Lichen planus (LP), as a typical lichenoid pattern dermatosis, is primarily induced by cytotoxic $CD8^+$ T cells attacking the keratinocytes. Previous research has reported that TSLP expression is elevated in the oral epithelium and sera of patients with oral LP [16, 17]. Sarcoidosis is a typical noninfectious granulomatous pattern disease, characterized by granulomas and fibrosis. Previous studies have shown that the TSLP level in the bronchoalveolar lavage fluid of pulmonary sarcoidosis is not higher than that of normal controls [18]. Thus far, no studies have investigated TSLP expressed in LP and cutaneous sarcoidosis. In conclusion, the involvement of TSLP in multiple dermatoses with different features is notable.

To better understand the exact location and pattern of TSLP expression in the skin of patients suffering from different dermatoses, we analyzed by immunohistochemistry retrospective samples of representative immune-related dermatoses, including LP, discoid lupus erythematosus (DLE), PsV, BP, eczema, sarcoidosis, and mycosis fungoides (MF).

2. Materials and Methods

2.1. Specimen Selection. The dermatopathology database of the Department of Dermatology at Peking Union Medical College Hospital (Beijing) was queried to identify samples consistent with LP, LE, eczema, BP, PsV, sarcoidosis, and MF. Healthy controls (HC) for stains were recruited from normal surrounding skin in patients with nevi, cysts, or seborrheic keratosis. This study was performed in line with the principles of the Declaration of Helsinki (as revised in 2013). Approval was granted by the Ethical Review Committee of Peking Union Medical College Hospital (ZS-1735). Potential participants who had received systematic glucocorticoids, immunosuppressants, biologics, or long-term topical treatment were excluded from the study. The criteria for specimen selection are shown in Table 1.

2.2. Immunohistochemistry. Paraffin sections were deparaffinized and blocked with 3% hydrogen peroxide for 15 min, then incubated with goat serum solution for 1 h at room temperature. Next, samples were incubated with rabbit anti-human TSLP (ProteinTech Group, Rosemont, IL, USA) overnight at 4°C, followed by incubation with HRP-conjugated AffiniPure goat anti-rabbit IgG (ProteinTech Group, Rosemont, IL, USA). The specimens were then stained with DAB (Solarbio, Beijing, China), followed by washing and counterstaining with hematoxylin (Solarbio, Beijing, China).

2.3. Specimen Grading. For quantitative image analysis, the slides were scanned using a NanoZoomer 2.0-RS (Hamamatsu, Japan), and five visual fields were selected randomly for each slide. Each section was graded semiquantitatively on a scale of 0–3 (0: negative, 1: mild, 2: moderate, and 3: strong) blindly assessed by two dermatologists in five randomly selected visual fields at high (400X) magnification of scanned sections. The section whose average grade is greater than one was considered a positive case. The gradient of the stratum basale and the stratum spinosum was mathematically calculated by subtracting the stratum basale score from that of the stratum spinosum.

2.4. Statistical Analysis. The grades of TSLP expression in the stratum basale and the stratum spinosum were presented as means ± standard deviations. A one-way ANOVA and Dunnett's test (the HC group was considered the control group) were used to determine the significance of grade. One-group Student's t-test was used to determine the significance of the gradient of the stratum basale and the stratum spinosum. A one-way ANOVA was used to assess general differences among each dermatosis group, and Bonferroni adjustment was used to assess differences between each dermatosis group pair. The grade correlation of TSLP expression between the stratum basale and the stratum spinosum was evaluated using the Pearson correlation test. Statistical significance was assigned at $p < 0.05$. SAS 9.4 (SAS Institute, Cary, NC, USA) was used for statistical analyses. The Prism 7 software (GraphPad Software, Inc, La Jolla, CA, USA) was used to generate statistical graphs.

3. Results

3.1. TSLP Was Expressed in the Epidermis of Inflammatory Dermatoses. A total of 76 specimens, composed of LP ($n = 10$), DLE ($n = 10$), eczema ($n = 10$), BP ($n = 10$), PsV ($n = 11$), sarcoidosis ($n = 10$), MF ($n = 10$), and HC ($n = 5$), were collected for our investigation (Table 2). Morphologically, TSLP was distinctly expressed in the epidermis of LP, BP, eczema, PsV, sarcoidosis, and MF specimens, while in the epidermis with DLE, TSLP-positive keratinocytes could also be observed focally, not as widespread as other dermatoses in our investigation. There were a fewer lightly stained positive cells in the epidermis of HC (Figure 1).

Positive rates of TSLP expression in the epidermis in each group are presented in Table 3, and mean grades of TSLP expression in the stratum basale and the stratum spinosum are presented in Figure 2. Compared to that in HC, TSLP was significantly expressed semiquantitatively in the stratum basale of the epidermis with PsV ($p = 0.002$) and sarcoidosis ($p = 0.025$) specimens and not significantly expressed in LP ($p = 0.102$), DLE ($p = 0.360$), eczema ($p = 0.253$), BP ($p = 0.145$), and MF ($p = 0.436$) (Figure 2(a)). Similarly, TSLP was significantly expressed in the stratum spinosum of the epidermis in LP ($p = 0.002$), BP ($p = 0.031$), PsV ($p = 0.002$), eczema ($p = 0.041$), and MF ($p = 0.046$) specimens and not significantly expressed in DLE ($p = 0.217$) and sarcoidosis ($p = 0.309$) (Figure 2(b)). In the epidermis of DLE, the semiquantitative score of TSLP expression was not significant.

Table 1: Inclusion criteria for specimens used in retrospective analysis.

Disease	Inclusion criteria
LP	Typical clinical manifestation + wedge-shaped hypergranulosis, dense band-like lymphocytic infiltrate with an absence of eosinophils or parakeratosis
DLE	Clinical suspicion + hyperkeratosis, vacuolar interface change, thickened basement membrane with patchy superficial and deep lymphocytic infiltrate
Eczema	Clinical suspicion + spongiosis with superficial perivascular inflammatory infiltrate, and without clear aetiology, not atopic dermatitis or contact dermatitis
BP	Clinical suspicion + subepidermal blister with direct or indirect immunofluorescence and/or autoantibodies
PsV	Clinical suspicion + confluent parakeratosis with regular acanthosis
Sarcoidosis	Clinical suspicion + "naked" granulomas with no/minimal lymphocytes surrounding granuloma, rule tuberculosis and leprosy out
MF	Clinical suspicion + epidermotropic lymphocytes, intraepidermal collections of lymphocytes, atypical lymphocytes, typical immunophenotype with clonal TCR rearrangement

LP: lichen planus; DLE: discoid lupus erythematosus; BP: bullous pemphigoid; PsV: psoriasis vulgaris; MF: mycosis fungoides.

Table 2: List of participants for specimens used in a retrospective analysis.

Group	Number of cases	Age, years (mean (range))	Sex	
			Male	Female
LP	10	48.8 (29-65)	3	7
DLE	10	41.5 (24-67)	5	5
Eczema	10	50.2 (27-74)	5	5
BP	10	70.4 (51-87)	5	5
PsV	11	49.7 (26-73)	6	5
Sarcoidosis	10	55.5 (30-76)	3	7
MF	10	38.4 (20-67)	6	4
HC	5	40.8 (28-56)	2	3

Age was record at the time of samples collection. LP: lichen planus; DLE: discoid lupus erythematosus; BP: bullous pemphigoid; PsV: psoriasis vulgaris; MF: mycosis fungoides; HC: healthy control.

To further investigate the diversity of TSLP expression among these dermatoses, we compared the grade between every two groups. There were no significant differences in TSLP expression in both the stratum spinosum and the stratum basale grades among these dermatosis groups, which means TSLP expression did not significantly differ between each disease group overall.

3.2. The Differences of TSLP Expression between the Stratum Basale and the Stratum Spinosum. Morphologically, there were no apparent differences in TSLP stain intensity between the stratum basale and the stratum spinosum. However, mean semiquantitative grades in the stratum spinosum were significantly higher than those in the stratum basale in LP ($p = 0.022$) specimens and lower than those in the stratum basale in sarcoidosis ($p = 0.019$) specimens. The differences of TSLP expression between the stratum basale and the stratum spinosum in other dermatoses were not significant (Figure 2(c), DLE $p = 0.584$, eczema $p = 0.153$, BP $p = 0.146$, PsV $p = 0.777$, and MF $p = 0.173$).

Moreover, to further investigate the gradients of the stratum basale and the stratum spinosum expression among dermatoses, we compared it between every two groups. The gradients of the stratum basale and the stratum spinosum expression of TSLP in sarcoidosis were different from LP ($p = 0.009$), eczema ($p = 0.030$), BP ($p = 0.027$), and MF ($p = 0.023$), respectively. The differences of gradients between other dermatoses were not significant (Figure 2(c)).

3.3. The Correlations of TSLP Expression between the Stratum Basale and the Stratum Spinosum. Generally, there was a significantly positive correlation in TSLP expression between the stratum spinosum and the stratum basale in all specimens ($r = 0.713$, $p < 0.001$). Similar correlations could also be found in LP ($r = 0.654$, $p = 0.040$), DLE ($r = 0.673$, $p = 0.033$), eczema ($r = 0.851$, $p = 0.002$), BP ($r = 0.904$, $p < 0.001$), PsV ($r = 0.618$, $p = 0.043$), and sarcoidosis ($r = 0.886$, $p < 0.001$) specimens, whereas in MF ($r = 0.305$, $p = 0.391$) and HC ($r = -0.25$, $p = 0.685$) specimens, this correlation was not significant (Table 4).

3.4. TSLP Was Also Expressed in the Dermis of BP, PsV, and Sarcoidosis. Finally, we found positive TSLP staining in the dermal infiltrating inflammatory cells in BP, PsV, and sarcoidosis. In BP, TSLP-positive cells scattered among

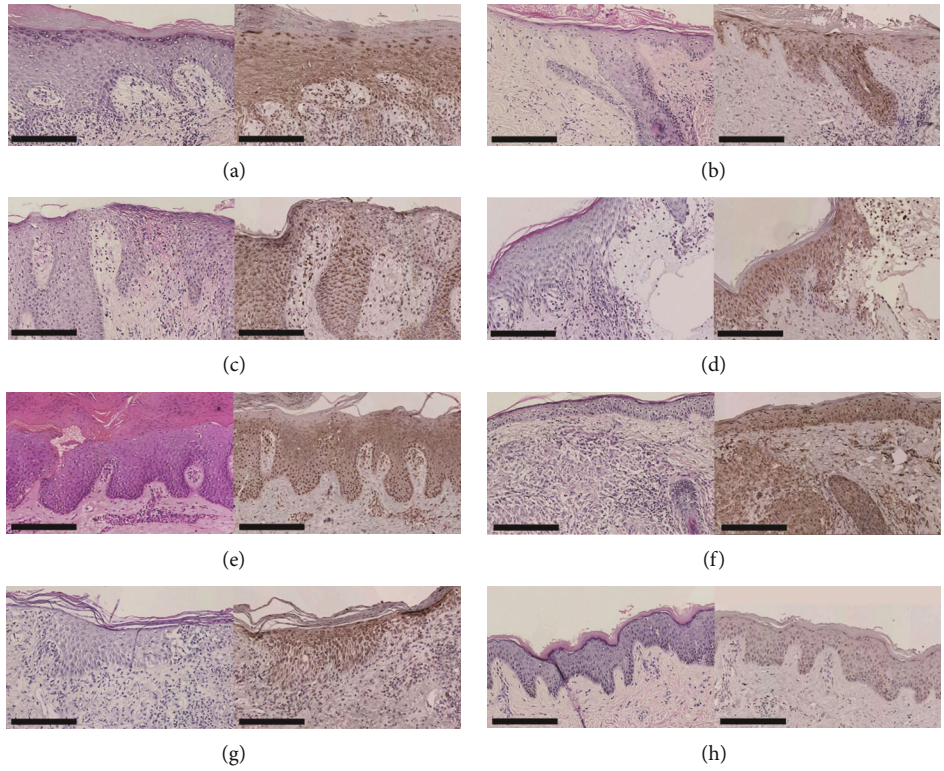

Figure 1: TSLP was expressed in the epidermis of all skin samples: (a) LP, (b) DLE, (c) eczema, (d) BP, (e) PsV, (f) sarcoidosis, (g) MF, and (h) HC. Original magnification: ×200, black scale bar = 200 μm.

Table 3: Positive rates of thymic stromal lymphopoietin expression in the epidermis in each group.

Group	Positive rates of TSLP in the stratum basale	Positive rates of TSLP in the stratum spinosum
Total	61.84%	67.11%
LP	70%	60%
DLE	60%	50%
Eczema	50%	70%
BP	70%	80%
PsV	100%	100%
Sarcoidosis	70%	60%
MF	40%	80%
HC	0	0

LP: lichen planus; DLE: discoid lupus erythematosus; BP: bullous pemphigoid; PsV: psoriasis vulgaris; MF: mycosis fungoides; HC: healthy control.

abundant inflammatory cells (Figures 3(a) and 3(b)). In PsV, TSLP was expressed in the dermal superficial perivascular inflammatory cells and endothelial cells (Figures 3(c) and 3(d)). In sarcoidosis, TSLP was expressed in epithelioid cells in "naked" granulomas (Figures 3(e) and 3(f)). Regardless of the extent of inflammatory cell infiltrating into the dermis, we did not observe predominant TSLP expression in other dermatoses specimens.

4. Discussion

In our study, we investigated the lesional expression of TSLP in representative dermatoses with different immune patterns. Morphologically, we found TSLP expressed in each dermatosis to some extent. Using semiquantitative analysis, except for DLE, TSLP was expressed in the stratum basale or the stratum spinosum in those dermatoses, and the difference between each dermatosis was not generally significant. Besides, TSLP expression in the stratum spinosum is significantly higher than that in the stratum basale of LP specimens and significantly lower than that in sarcoidosis specimens. Furthermore, the correlations of TSLP expression between the stratum basale and the stratum spinosum were significant, except for MF and HC. In addition, we found that TSLP was also expressed in the dermal infiltrating inflammation cells in BP, PsV, and sarcoidosis.

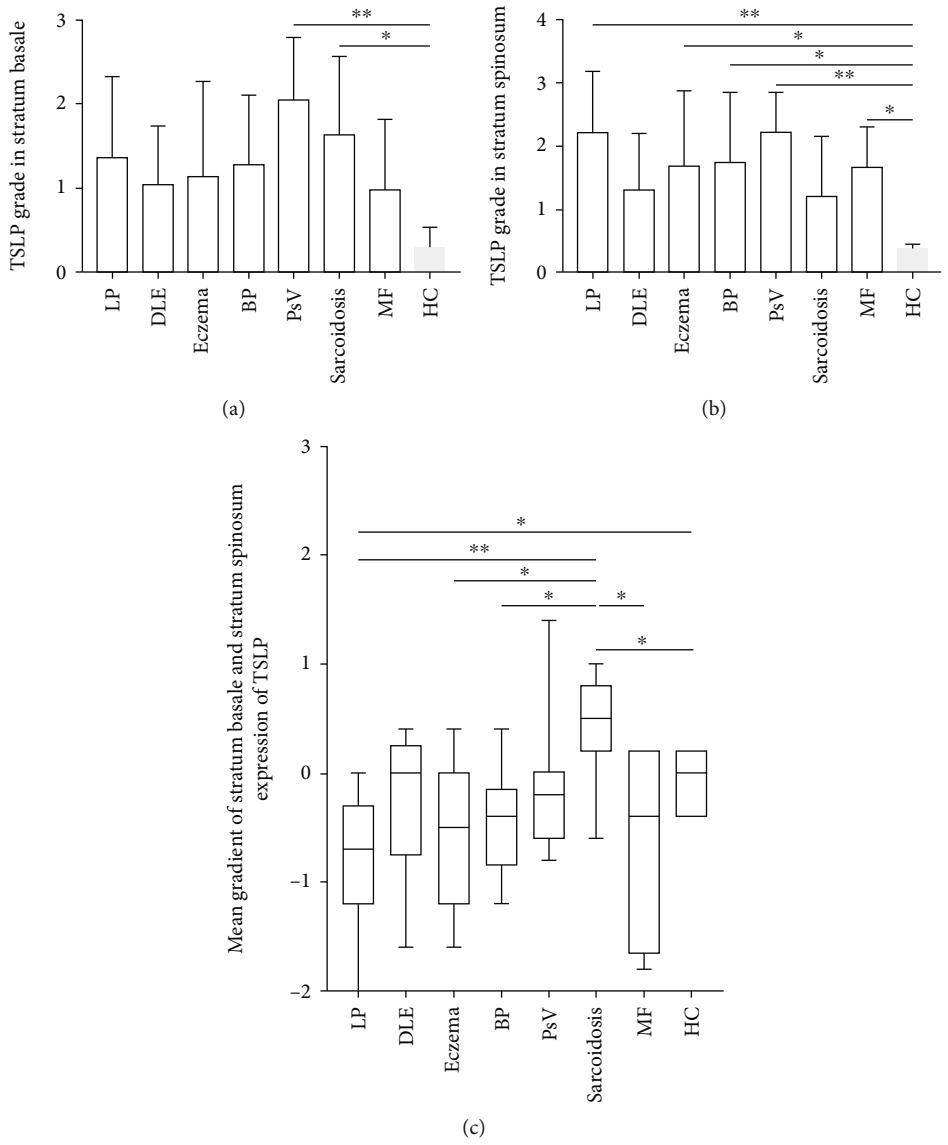

Figure 2: TSLP was significantly expressed in the epidermis in LP, BP, eczema, PsV, sarcoidosis, and MF and nonsignificantly expressed in DLE, in a semiquantitative analysis. (a) TSLP was significantly expressed in the stratum basale of the epidermis with PsV and sarcoidosis specimens. (b) TSLP was significantly expressed in the stratum spinosum of LP, eczema, BP, PsV, and MF. (c) TSLP expression in the stratum basale was significantly higher than that in the stratum spinosum in sarcoidosis specimens and was significantly lower in the stratum spinosum in LP specimens, but not significant in other dermatoses. The mean gradient of stratum basale and stratum spinosum expression of TSLP in sarcoidosis was different from in LP, eczema, BP, and MF, respectively. The score of each section was graded semiqualitatively on a scale of 0–3 (0: negative, 1: mild, 2: moderate, and 3: strong) blindly by two dermatologists in five randomly visual fields at high power field (HPF, ×400 magnification) of scanned sections. The gradient of the stratum basale and the stratum spinosum was mathematically calculated by subtracting the stratum basale score from stratum spinosum. p values were determined by the one-group Student t-test. Significant differences are noted between the groups: n.s.: not significantly different; $^{*}p < 0.05$; $^{**}p < 0.01$.

Collectively, our data showed that the expression of TSLP is elevated in the skin of inflammatory dermatosis and may be involved in their pathogenesis.

TSLP is a classic Th2-related cytokine and can induce DC-promoted naïve T cell differentiating to Th2 cells [19]. It could also directly activate $CD4^{+}$ T cells, mast cells, basophils, and eosinophils to promote Th2 immune response [3, 19]. In our study, we found elevated TSLP expression in the epidermis of eczema, BP, and MF, all of which are typical Th2-dominant dermatoses [13, 20]. This result is consistent with previous studies and confirms the relationship of TSLP and Th2 immune response [15, 21–25].

PsV is a Th1/Th17-induced inflammatory disease [13, 14]. However, in our study, we found that TSLP expression was also elevated in the epidermis of PsV. Similar to our finding, previous research demonstrated that the levels of TSLP were elevated in lesions and serum of PsV patients and paralleled to the severity of disease [9, 26, 27]. However, in lesions of a murine model and PsV patients, the TSLP levels rose after being treated with topical vitamin D3

Table 4: Pearson's correlation between stratum spinosum and stratum basale thymic stromal lymphopoietin expression.

Group	Correlation coefficient	p value
Total	0.713	<0.001
LP	0.654	0.040
DLE	0.673	0.033
Eczema	0.852	0.002
BP	0.904	<0.001
PsV	0.618	0.043
Sarcoidosis	0.886	<0.001
MF	0.305	0.391
HC	-0.250	0.685

LP: lichen planus; DLE: discoid lupus erythematosus; BP: bullous pemphigoid; PsV: psoriasis vulgaris; MF: mycosis fungoides; HC: healthy control.

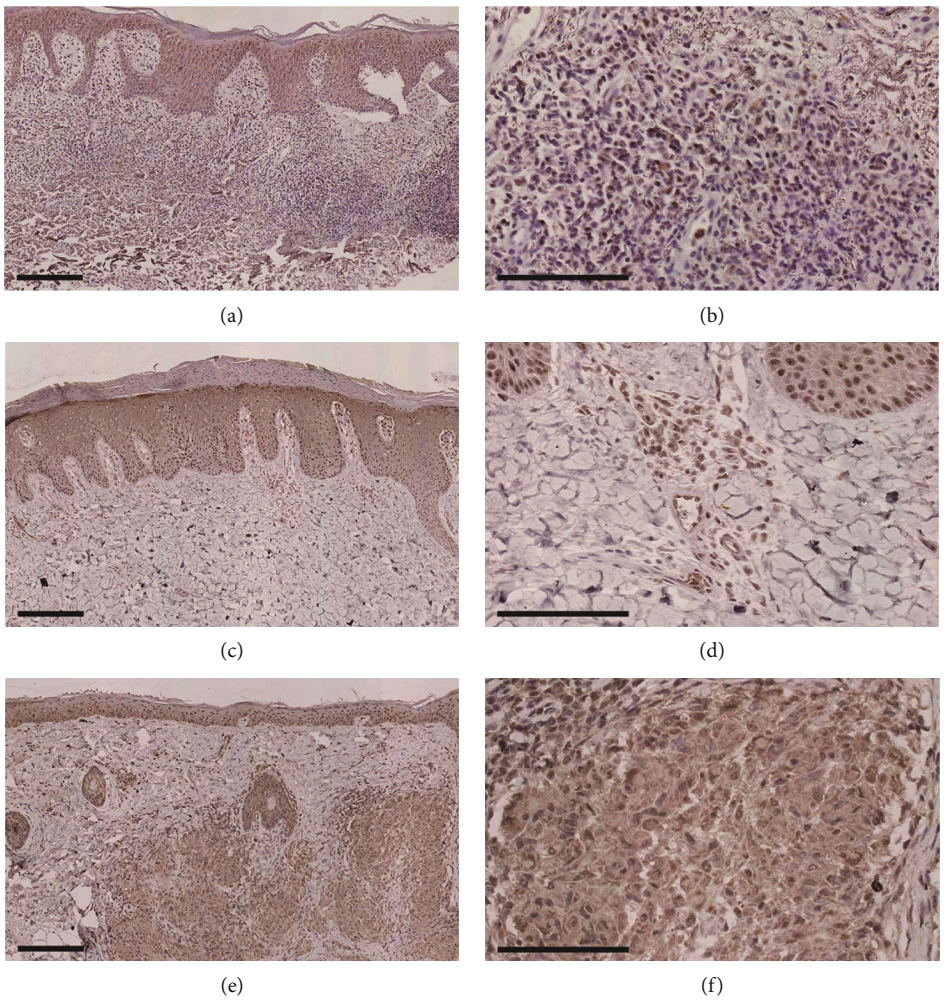

Figure 3: TSLP expressed in infiltrating inflammatory cells in the dermis in BP, PsV, and sarcoidosis. TSLP expressed in the superficial dermis inflammatory cells in BP in (a) and (b), in the superficial perivascular inflammatory cells in PsV in (c) and (d), and in the epithelioid cells in sarcoidosis in (e) and (f). Original magnification: ×100 in (a), (c), and (e), black scale bar = 200 μm; ×400 in (b), (d), and (f), black scale bar = 100 μm.

analogs calcipotriol, and it was conducted that calcipotriol could induce TSLP expression, suppress Th1/Th17, and enhance Th2 [28, 29]. It may be due to complex reasons that TSLP plays quite opposite roles in PsV. On the one hand, TSLP is a pleiotropic cytokine that can promote two different groups of DCs, one inducing expression of interferon-γ, IL-17A, and IL-22, and the other expressing IL-4, IL-5, IL-9, IL-13, and IL-10 [30]. TSLP was also found to have different immunomodulatory functions in different inflammatory environments [9]. Moreover, some investigators

demonstrated that the activity of TSLP was heavily dependent on the TSLP receptors rather than TSLP [27]. Thus, TSLP may also promote the Th1/Th17 immune response in PsV to some extent. On the other hand, those variable results may be caused by heterogeneity of PsV. PsV patients could be divided into two clusters; in one of the clusters, Th2-related gene, including TSLP, was inducted significantly higher in lesions of patients than those present in atopic dermatitis [31]. Therefore, TSLP may promote PsV through Th-2 immune response.

In this study, TSLP was significantly expressed in the epidermis of LP. A similar observation has been documented by Valdebran et al. [24]. Other available reports referred to the relationship between oral LP and TSLP. Sun et al. [16] suggested that TSLP is involved in oral LP by activating the $CD8^+$ T cell. Other researches imply that TSLP and other Th2-related chemokines were involved in the pathogenesis [17, 32], which does not support the opinion expressed in a previous study that LP is a Th1 immune response [13]. Similar to PsV, multiple functions of TSLP and heterogeneity of LP might account for the results.

Growing evidence shows that Th1, Th2, and Th17 are associated with the activity and severity of systematic lupus erythematosus (SLE) [33]. Correlation between the TSLP signal pathway and SLE was identified in a previous study [34]. Previous study demonstrated that expression of TSLP in sera and lesions did not significantly increase in SLE patients, compared to HC [35]. Likewise, Soumelis et al. [36] also failed to find TSLP expression increasing in lesions of disseminated lupus erythematosus patient. In our experiment, TSLP-positive keratinocytes could be observed focally in the epidermis of DLE morphologically, but mean grades of TSLP expression in the epidermis did not significantly increase as assessed by the semiquantitative analysis.

Thus far, no studies about the relationship between cutaneous sarcoidosis and TSLP have been reported yet, although one study about idiopathic pulmonary fibrosis showed that TSLP level in the bronchoalveolar lavage fluid of pulmonary sarcoidosis was not higher than that of normal controls [18]. In our study, we found TSLP was significantly expressed in the epidermis of sarcoidosis and the dermal epithelioid cells in "naked" granulomas, which implies that TSLP may be secreted by both keratinocytes and epithelioid cells in cutaneous sarcoidosis. Moreover, it may also account for the high level of TSLP in the stratum basale.

Regarding BP and PsV, the dermal infiltrating inflammatory cells were also positive for TSLP staining, which indicates that TSLP was expressed not only by keratinocytes but also by the infiltrating inflammatory cells in BP and PsV. This observation has not been reported previously, and the specific mechanism is still unknown. Nevertheless, TSLP was also expressed by mast cells, CD163+ macrophage cells, and endothelial cells in other immune-related dermatoses [35, 37, 38]. In addition, TSLP expression in the dermis might be related to a higher expression of TSLP in the stratum basale than in the stratum spinosum in BP and PsV specimens, although the differences were not significant.

Although our study showed no differences of TSLP expression between the stratum basale and the stratum spinosum morphologically, TSLP expression is significantly more intense in the stratum spinosum in LP, which is not consistent with a previous report [24]. By reviewing the figures in other studies, we found that the difference in TSLP expression between the stratum basale and the stratum spinosum was not obvious either [15, 17, 36]. There are two reasons for these discrepancies, namely, a small sample size in both studies and the multiple functions of TSLP. More comprehensive studies with a larger sample size are needed to explain the difference further. The specific expression pattern might reflect the resource of TSLP in the dermatoses. For instance, LP is primarily induced by cytotoxic $CD8^+$ T cells attacking the basal keratinocytes, but TSLP is expressed predominantly higher in the spinous keratinocytes. It may imply that the spinous cells play more important roles in pathogenesis of LP than previously thought, whereas in sarcoidosis, TSLP in the skin may come from the dermis or circulation. Moreover, if TSLP was produced from spinous keratinocytes in those dermatoses, it would be valuable to prevent TSLP generation topically. Recently, topical agents were found to reduce TSLP secretion in the epidermis [39, 40]. There is some prospect of lowering TSLP expression in the epidermis, especially the spinous keratinocytes, to improve inflammatory dermatoses.

Finally, this study has potential limitations. As mentioned above, the sample size of this research is small in some extent, whereas our study attempts to conduct a preliminary study and our intention is to raise a concern about TSLP and its effect in cutaneous diseases with different patterns of immune response. We would like to enlarge the sample size in the further study of specific dermatosis. Furthermore, we only detected the levels of TSLP in skin specimens of patients. Some immune-related dermatoses, such as PsV, BP and sarcoidosis, were not only skin diseases but also systemic disorders. It might be meaningful to detect the levels of TSLP in the sera or other samples and to compare the results with those in skin lesions. Moreover, TSLP interacts with IL-33 and other cytokines and mediates a Th2 immune response so that it is valuable to detect other cytokines and compare their expressions [41].

5. Conclusions

As a whole, our study found that the expression of TSLP was elevated in different immune response dermatoses. This finding adds to the accumulating evidence of the importance of TSLP in each inflammatory disease. It remains to be elucidated if TSLP plays a role in some inflammatory diseases, such as DLE and sarcoidosis. Tezepelumab, the first-in-class anti-TSLP monoclonal antibody, was approved as a biological agent for the treatment of severe asthma [42]. A randomized phase 2a clinical trial reported that patients of atopic dermatitis have improved under the treatment of tezepelumab [43]. It is necessary to investigate TSLP in those inflammatory diseases further, in order to discover a potential novel therapeutic target.

Data Availability

The dataset included in this paper is available from the corresponding author on reasonable request and with appropriate additional ethical approvals, when necessary.

Conflicts of Interest

The authors have no conflict of interest to declare.

Acknowledgments

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References

[1] S. L. Friend, S. Hosier, A. Nelson, D. Foxworthe, D. E. Williams, and A. Farr, "A thymic stromal cell line supports in vitro development of surface IgM+ B cells and produces a novel growth factor affecting B and T lineage cells," *Experimental Hematology*, vol. 22, no. 3, pp. 321–328, 1994.

[2] C. A. Akdis, P. D. Arkwright, M. C. Brüggen et al., "Type 2 immunity in the skin and lungs," *Allergy*, vol. 75, no. 7, pp. 1582–1605, 2020.

[3] G. Varricchi, A. Pecoraro, G. Marone et al., "Thymic stromal lymphopoietin isoforms, inflammatory disorders, and cancer," *Frontiers in Immunology*, vol. 9, p. 1595, 2018.

[4] Y. Liang, B. Yu, J. Chen et al., "Thymic stromal lymphopoietin epigenetically upregulates Fc receptor γ subunit- related receptors on antigen-presenting cells and induces T_H2/T_H17 polarization through dectin-2," *The Journal of Allergy and Clinical Immunology*, vol. 144, no. 4, pp. 1025–1035.e7, 2019.

[5] E. E. West, R. Spolski, M. Kazemian, Z. X. Yu, C. Kemper, and W. J. Leonard, "A TSLP-complement axis mediates neutrophil killing of methicillin-resistantStaphylococcus aureus," *Science Immunology*, vol. 1, no. 5, article eaaf8471, 2016.

[6] G. M. Gauvreau, R. Sehmi, C. S. Ambrose, and J. M. Griffiths, "Thymic stromal lymphopoietin: its role and potential as a therapeutic target in asthma," *Expert Opinion on Therapeutic Targets*, vol. 24, no. 8, pp. 777–792, 2020.

[7] J. F. Lai, L. J. Thompson, and S. F. Ziegler, "TSLP drives acute T_H2-cell differentiation in lungs," *The Journal of Allergy and Clinical Immunology*, vol. 146, no. 6, pp. 1406–1418.e7, 2020.

[8] K. Tsilingiri, G. Fornasa, and M. Rescigno, "Thymic stromal lymphopoietin: to cut a long story short," *Cellular and Molecular Gastroenterology and Hepatology*, vol. 3, no. 2, pp. 174–182, 2017.

[9] E. Volpe, L. Pattarini, C. Martinez-Cingolani et al., "Thymic stromal lymphopoietin links keratinocytes and dendritic cell-derived IL-23 in patients with psoriasis," *The Journal of Allergy and Clinical Immunology*, vol. 134, no. 2, pp. 373–381.e4, 2014.

[10] O. Suwarsa, H. P. Dharmadji, E. Sutedja et al., "Skin tissue expression and serum level of thymic stromal lymphopoietin in patients with psoriasis vulgaris," *Dermatology Reports*, vol. 11, no. 1, p. 8006, 2019.

[11] S. Z. Li, X. X. Jin, X. L. Ge, Y. G. Zuo, and H. Z. Jin, "Thymic stromal lymphopoietin is implicated in the pathogenesis of bullous pemphigoid by dendritic cells," *Journal of Immunology Research*, vol. 2020, Article ID 4594630, 10 pages, 2020.

[12] S. H. Wang and Y. G. Zuo, "Thymic stromal lymphopoietin in cutaneous immune-mediated diseases," *Frontiers in Immunology*, vol. 12, article 698522, 2021.

[13] K. Eyerich and S. Eyerich, "Immune response patterns in non-communicable inflammatory skin diseases," *Journal of the European Academy of Dermatology and Venereology*, vol. 32, no. 5, pp. 692–703, 2018.

[14] M. Carretero, S. Guerrero-Aspizua, N. Illera et al., "Differential features between chronic skin inflammatory diseases revealed in skin-humanized psoriasis and atopic dermatitis mouse models," *The Journal of Investigative Dermatology*, vol. 136, no. 1, pp. 136–145, 2016.

[15] V. Kumari, K. Timm, A. A. Kühl, G. Heine, and M. Worm, "Impact of systemic alitretinoin treatment on skin barrier gene and protein expression in patients with chronic hand eczema," *The British Journal of Dermatology*, vol. 175, no. 6, pp. 1243–1250, 2016.

[16] M. Sun, W. Tan, S. Liu et al., "In situ expression and serum level of thymic stromal lymphopoietin in oral lichen planus," *Journal of Oral Pathology & Medicine*, vol. 43, no. 10, pp. 740–745, 2014.

[17] M. Yamauchi, M. Moriyama, J. N. Hayashida et al., "Myeloid dendritic cells stimulated by thymic stromal lymphopoietin promote Th2 immune responses and the pathogenesis of oral lichen planus," *PLoS One*, vol. 12, no. 3, article e0173017, 2017.

[18] J. U. Lee, H. S. Chang, H. J. Lee et al., "Upregulation of interleukin-33 and thymic stromal lymphopoietin levels in the lungs of idiopathic pulmonary fibrosis," *BMC Pulmonary Medicine*, vol. 17, no. 1, p. 39, 2017.

[19] I. Cvitas, A. Galichet, S. C. Ling, E. J. Müller, and E. Marti, "Toll-like receptor-ligand induced thymic stromal lymphopoietin expression in primary equine keratinocytes," *Veterinary Dermatology*, vol. 31, no. 2, pp. 154–162, 2020.

[20] K. Fujii, "New therapies and immunological findings in cutaneous T-cell lymphoma," *Frontiers in Oncology*, vol. 8, p. 198, 2018.

[21] Y. Zhang, B. J. Hwang, Z. Liu et al., "BP180 dysfunction triggers spontaneous skin inflammation in mice," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 115, no. 25, pp. 6434–6439, 2018.

[22] M. Sakamoto, T. Miyagaki, H. Kamijo et al., "Serum vascular endothelial growth factor A levels reflect itch severity in mycosis fungoides and Sézary syndrome," *The Journal of Dermatology*, vol. 45, no. 1, pp. 95–99, 2018.

[23] M. Tuzova, J. Richmond, D. Wolpowitz et al., "CCR4+T cell recruitment to the skin in mycosis fungoides: potential contributions by thymic stromal lymphopoietin and interleukin-16," *Leukemia & Lymphoma*, vol. 56, no. 2, pp. 440–449, 2015.

[24] M. Valdebran, E. H. Kowalski, D. Kneiber et al., "Epidermal expression of eotaxins and thymic stromal lymphopoietin in eosinophil rich dermatoses," *Archives of Dermatological Research*, vol. 311, no. 9, pp. 705–710, 2019.

[25] T. Shigeno, M. Katakuse, T. Fujita, Y. Mukoyama, and H. Watanabe, "Phthalate ester-induced thymic stromal lymphopoietin mediates allergic dermatitis in mice," *Immunology*, vol. 128, 1 Part 2, pp. e849–e857, 2009.

[26] M. I. El-Ghareeb, A. Helmy, S. Al Kazzaz, and H. Samir, "Serum TSLP is a potential biomarker of psoriasis vulgaris activity," *Psoriasis: Targets and Therapy*, vol. 9, pp. 59–63, 2019.

[27] E. Guttman-Yassky, M. A. Lowes, J. Fuentes-Duculan et al., "Major differences in inflammatory dendritic cells and their products distinguish atopic dermatitis from psoriasis," *The Journal of Allergy and Clinical Immunology*, vol. 119, no. 5, pp. 1210–1217, 2007.

[28] E. Sato-Deguchi, S. Imafuku, B. Chou, K. Ishii, K. Hiromatsu, and J. Nakayama, "Topical vitamin D3 analogues induce thymic stromal lymphopoietin and cathelicidin in psoriatic skin lesions," *The British Journal of Dermatology*, vol. 167, no. 1, pp. 77–84, 2012.

[29] M. Li, P. Hener, Z. Zhang, S. Kato, D. Metzger, and P. Chambon, "Topical vitamin D3 and low-calcemic analogs induce thymic stromal lymphopoietin in mouse keratinocytes and trigger an atopic dermatitis," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 103, no. 31, pp. 11736–11741, 2006.

[30] M. Omori-Miyake, H. Watarai, K. Sato, S. F. Ziegler, and J. Yagi, "An accumulation of two populations of dendritic cells in skin-draining lymph nodes in response to the expression of thymic stromal lymphopoietin in the skin," *Cellular Immunology*, vol. 353, article 104116, 116 pages, 2020.

[31] J. Chen, C. Li, H. Li et al., "Identification of a TH2-high psoriasis cluster based on skin biomarker analysis in a Chinese psoriasis population," *Journal of the European Academy of Dermatology and Venereology*, vol. 35, no. 1, pp. 150–158, 2021.

[32] Z. R. Zhang, L. Y. Chen, H. Y. Qi, and S. H. Sun, "Expression and clinical significance of periostin in oral lichen planus," *Experimental and Therapeutic Medicine*, vol. 15, no. 6, pp. 5141–5147, 2018.

[33] F. Muhammad Yusoff, K. K. Wong, and N. Mohd Redzwan, "Th1, Th2, and Th17 cytokines in systemic lupus erythematosus," *Autoimmunity*, vol. 53, no. 1, pp. 8–20, 2020.

[34] A. E. Gorji, Z. Roudbari, A. Alizadeh, and B. Sadeghi, "Investigation of systemic lupus erythematosus (SLE) with integrating transcriptomics and genome wide association information," *Gene*, vol. 706, pp. 181–187, 2019.

[35] M. E. Truchetet, B. Demoures, J. Eduardo Guimaraes et al., "Platelets induce thymic stromal lymphopoietin production by endothelial cells: contribution to fibrosis in human systemic sclerosis," *Arthritis & Rheumatology*, vol. 68, no. 11, pp. 2784–2794, 2016.

[36] V. Soumelis, P. A. Reche, H. Kanzler et al., "Human epithelial cells trigger dendritic cell-mediated allergic inflammation by producing TSLP," *Nature Immunology*, vol. 3, no. 7, pp. 673–680, 2002.

[37] A. Usategui, G. Criado, E. Izquierdo et al., "A profibrotic role for thymic stromal lymphopoietin in systemic sclerosis," *Annals of the Rheumatic Diseases*, vol. 72, no. 12, pp. 2018–2023, 2013.

[38] R. B. Christmann, A. Mathes, A. J. Affandi et al., "Thymic stromal lymphopoietin is up-regulated in the skin of patients with systemic sclerosis and induces profibrotic genes and intracellular signaling that overlap with those induced by interleukin-13 and transforming growth factor β," *Arthritis and Rheumatism*, vol. 65, no. 5, pp. 1335–1346, 2013.

[39] J. Fitoussi, S. Virassamynaïk, S. Callejon et al., "Inhibition of thymic stromal lymphopoietin production to improve pruritus and quality of life in infants and children with atopic dermatitis," *Journal of Cosmetic Dermatology*, vol. 19, no. 8, pp. 2061–2069, 2020.

[40] J. K. Lee, J. K. Seok, I. Cho et al., "Topical application of celastrol alleviates atopic dermatitis symptoms mediated through the regulation of thymic stromal lymphopoietin and group 2 innate lymphoid cells," *Journal of Toxicology and Environmental Health. Part A*, vol. 84, no. 22, pp. 922–931, 2021.

[41] O. Kacem, W. Kaabachi, I. B. Dhifallah, A. Hamzaoui, and K. Hamzaoui, "Elevated expression of TSLP and IL-33 in Behçet's disease skin lesions: IL-37 alleviate inflammatory effect of TSLP," *Clinical Immunology*, vol. 192, pp. 14–19, 2018.

[42] M. G. Matera, P. Rogliani, L. Calzetta, and M. Cazzola, "TSLP inhibitors for asthma: current status and future prospects," *Drugs*, vol. 80, no. 5, pp. 449–458, 2020.

[43] E. L. Simpson, J. R. Parnes, D. She et al., "Tezepelumab, an anti-thymic stromal lymphopoietin monoclonal antibody, in the treatment of moderate to severe atopic dermatitis: a randomized phase 2a clinical trial," *Journal of the American Academy of Dermatology*, vol. 80, no. 4, pp. 1013–1021, 2019.

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Research Article

mTOR Modulates the Endoplasmic Reticulum Stress-Induced CD4$^+$ T Cell Apoptosis Mediated by ROS in Septic Immunosuppression

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Introduction. When sepsis attacks the body, the excessive reactive oxygen species (ROS) production can result to endoplasmic reticulum stress (ERS) and eventually cause lymphocyte apoptosis. The mammalian target of rapamycin (mTOR) is essential for regulating lymphocyte apoptosis; we hypothesized that it mediates CD4$^+$ T cell apoptosis during ROS-related ERS. *Method*. We, respectively, used ROS and ERS blockers to intervene septic mice and then detected ERS protein expression levels to verify the relationship between them. Additionally, we constructed T cell-specific mTOR and TSC1 gene knockout mice to determine the role of mTOR in ROS-mediated, ERS-induced CD4$^+$ T cell apoptosis. *Results*. Blocking ROS significantly suppressed the CD4$^+$ T cell apoptosis associated with the reduction in ERS, as revealed by lower levels of GRP78 and CHOP. ERS rapidly induced mTOR activation, leading to the induction of CD4$^+$ T cell apoptosis. However, mTOR knockout mice displayed reduced expression of apoptotic proteins and less ER vesiculation and expansion than what was observed in the wild-type sepsis controls. *Conclusion*. By working to alleviate ROS-mediated, ERS-induced CD4$^+$ T cell apoptosis, the mTOR pathway is vital for CD4$^+$ T cell survival in sepsis mouse model.

1. Introduction

An epidemiologic survey in 2020 reported that although the average worldwide death rate from sepsis is around 15%–30%, it is even higher in some underdeveloped regions [1]. Sepsis causes countless deaths and imposes huge economic burdens on countries [2]. Over the years, research has determined that immunosuppression is one of the major reasons for high mortality in patients with sepsis [3, 4]. Abnormal immune cell death is an important cause of immunosuppression in sepsis, and excessive apoptosis of lymphocytes is one such type of immune cell death [5, 6].

In recent years, researchers have discovered a new type of apoptosis caused by endoplasmic reticulum stress (ERS), and this type of apoptosis has been named the third apoptotic pathway. In pathological conditions such as infection, sepsis, and hypoxia, cell energy metabolism disorders induce reactive oxygen species (ROS) production, which renders the endoplasmic reticulum (ER) unable to correctly synthesize proteins [7]. Large amounts of unfolded or misfolded proteins accumulate, and this leads to ERS. When ERS persists and cell homeostasis cannot be recovered, cell apoptosis will occur [8]. Therefore, preventing ERS may relieve the inflammatory state and damage to tissues and organs [9, 10].

As essential immune system components, reduced numbers of CD4$^+$ T lymphocytes will further aggravate immune deficiency in the body. Reduced CD4$^+$ T cell absolute number is an independent risk factor that influences the outcome of sepsis patients [11]. Mammalian target of rapamycin (mTOR) is a serine/threonine protein kinase, which played

significant role in regulating various cellular metabolic activities including cell energy metabolism and protein synthesis. One important role that it plays is in regulating cell autophagy and apoptosis. One important role that it plays is in regulating cell autophagy and apoptosis. In addition, mTOR complex 1 (mTORC1) responds to low ATP levels or a hypoxic unstable intracellular environment. Tuberous sclerosis complex 1 (TSC1) functions as a GTPase-activating protein to activate mTORC1 [12]. We have previously confirmed that mTOR is involved in the immunosuppression of sepsis and that the apoptosis of $CD4^+$ T cells can be attenuated by mTOR intervention [13, 14]. However, the region regulating $CD4^+$ T cell apoptosis upstream of mTOR has yet to be studied, and this knowledge gap in the mTOR pathway awaits elucidation. Interestingly, emerging literature has indicated that the mTOR signaling acts downstream of the ER and mediates ERS-induced apoptosis of pulmonary vascular endothelial cells [15]. We speculate that mTOR might also function as import regulator during the process of $CD4^+$ T cell apoptosis caused by ERS in sepsis.

We hypothesized that mTOR is involved in the regulation of $CD4^+$ T cell apoptosis triggered by ROS-related ERS. The cecal ligation and puncture (CLP) model is referred to be the "gold standard" for the induction of polymicrobial sepsis in experimental settings to research the fundamental mechanisms of sepsis. Besides, the cytokine profile of the CLP model is similar to that seen in human sepsis where there is increased lymphocyte apoptosis [16]. To test this hypothesis, we constructed an mTOR knockout mouse sepsis model to study the role of mTOR. We used this model to investigate whether mTOR could be pharmacologically used to regulate $CD4^+$ T cell apoptosis thereby enhancing the immunity and improving the prognosis of patients with sepsis.

2. Materials and Methods

2.1. Mice. Male C57BL/6N mice were housed in a pathogen-free animal house with comfortable environment (room temperature $25 \pm 1°C$, 12 h day/night cycle). We used 16-18 g healthy male C57BL/6N mice (aged 4-5 weeks) to establish the sepsis model. Lck-cre mice were generated by crossing TSC1loxp/loxp and mTORloxp/loxp mice with mice expressing Cre recombinase. Lck-cre mTORloxp/loxp (lck-mTOR) and Lck-cre TSC1loxp/loxp (lck TSC1) mice obtained, Lck-cre-negative mTOR loxp/loxp mice were their corresponding controls. Lck-mTOR mice were used in the Lck-mTOR+CLP group ($n = 6$). The Lck-TSC1 mice were used in the Lck-TSC1+CLP group ($n = 6$). 30 lck-mTOR mice ($n = 6$ per group) were allocated to each group: WT, CLP, CLP+NAC, CLP+CIRP-Ab, and CLP+4-PBA group.

2.2. Sepsis Model. We establish a midgrade sepsis model according to the protocol as previously described [16].

2.3. Drug Administration. In treatment groups, CIRP-Ab (C23. 8 mg/kg BW. GenScript: Nanjing, China) was administered by intravenous tail injection after 2 h of the CLP operation. N-Acetyl-L-cysteine (NAC, 150 mg/kg BW, Beyotime: Shanghai, China) and 4-phenylbutyric acid (4-PBA. 40 mg/kg BW, TargetMol, USA) were administered intraperitoneally 60 min before surgical procedure. Besides, DMSO (10% DMSO, 4 ml/kg BW) was administered intraperitoneally to mice in the control group. After 18 h of the treatment, all mice were killed, and spleens were collected immediately. The experiments were carried out under strict guidance and principles according to the guidelines of PUMCH Clinical Laboratory. JS-1170 was the approval number for the study from the Ethics Committee at Peking Union Medical College Hospital (Beijing, China).

2.4. Spleen Tissue Single-Cell Suspension. The harvested spleens were minced, and the homogenate was then passed through a 40 μm cell strainer. The obtained cells were lysed in red blood cell lysis buffer, followed by being transferred to 15 ml centrifuge tubes. To make single-cell suspension, samples were centrifuged at 1500 rpm for 10 min and resuspended in 5 ml of PBS.

2.5. Lymphocyte Purification. Mouse spleen lymphocytes were purified using the lymphocyte separation kit (Solarbio, Beijing, China.). Briefly, 5 ml of lymphocyte separation medium was gently added to 5 ml spleen tissue single-cell suspension; after centrifugation at 800g for 20 minutes, lymphocytes were transferred into a new 15 ml centrifuge tube. Following washing with 10 ml washing solution and centrifugation at 250g for 10 min, the separated spleen lymphocytes were suspended with PBS.

2.6. Sorting $CD4^+$ T Cells. $CD4^+$ T cells were isolated from lymphocytes by negative magnetic bead sorting using magnetic microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). Briefly, we stained splenocytes with biotin-conjugated anti-CD4 antibody for 30 minutes and then washed with PBS for 5 min 3 times; we next incubated the cells for 15 minutes with magnetic streptavidin. Afterward, we used the $CD4^+$ T cells for apoptosis array, ROS detection, RT-PCR, western blotting, and transmission electron microscopy.

2.7. Western Blot. $CD4^+$ T cells lysate was prepared by lysing in RIPA buffer containing protease inhibitors. Protein concentrations were estimated (BCA protein assay kit). After that, the protein samples were loaded onto each lane by SDS-PAGE for electrophoresis and transferred to 0.45 μM PVDF membrane (Millipore, MA, USA). Next, PVDF membranes were blocked with 5% skimmed milk in TBS-Tween for 60 min, followed by incubation with antibody at 4°C for 12-16 h. The following primary antibodies are as follows: anti-mTOR (Cat#AF6308, dilution rate 1 : 1000), anti-P-mTOR (Cat#AF3308, 1 : 1000), anti-P-p70s6k (Cat#AF3228, 1 : 1000), anti-p70s6k (Cat#AF6226, 1 : 1000), anti-4EBP (Cat#AF6432, 1 : 1000), anti-caspase-3 (Cat#AF6311, 1 : 1000), anti-Bcl-2 (Cat#AF6139, 1 : 1000), anti-Bax (Cat#AF0120, 1 : 1000), anti-GRP78 (Cat#AF5366, 1 : 1000), anti-CIRP (Cat#DF2643, 1 : 1000), anti-CHOP (Cat#DF6025, 1 : 1000), anti-CIRP (Cat#AF5366, 1 : 1000), and anti-actin-β (Cat#AF7018, 1 : 3000) were purchased from Affinity Biosciences (Jiangsu, China). Anti-P-4EBP

(anti-eIF4EBP1, ab27792) was from Abcam (CA, USA). After washing with TBS-Tween 3 times, the membranes were incubated with a goat anti-rabbit IgG antibody (1 : 5000, Affinity Biosciences) at 25°C for 1 h, and the chemiluminescence signals were detected using an electrochemiluminescence detection system. Band densities were quantified by the *ImageJ software*.

2.8. Apoptosis Array. Cell apoptosis array was carried out following the protocol of the apoptosis detection kit (BD Pharmingen, USA). Cells were resuspended at 1×10 cells/ml in binding buffer and incubated with Annexin V-FITC and PI for 15 minutes in the dark before analyzing cells on a flow cytometer. FCS files were analyzed using the FlowJo software.

2.9. Intracellular ROS Detection. The level of ROS in $CD4^+$ T cells was evaluated according to the protocol of ROS detection kit (Biyuntian, Shanghai, China). DCFH-DA and $CD4^+$ T cells were mixed and incubated at 37°C for 20 minutes. ROS levels were then measured by flow cytometry.

2.10. RNA Extract and RT-PCR. $CD4^+$ T cells were collected and RNA were harvested in TRIzol reagent (Tiangen, Beijing, China). RNA was then reverse transcribed using a PrimeScript RT reagent kit. qPCR was performed with SYBR Premix EX Taq™ II in ABI 7500 real-time PCR system (Applied Biosystems, USA). Primers are as follows: Bim sense: 5′-GAGATACGGATTGCACAGGA-3′, Bim antisense: 5′ -TCAGCCTCGCGGTAATCATT-3′; β-actin sense: 5′-ACTGGGACGACATGGAGAAG-3′, β-actin antisense: 5′-GGGGTGTTGAAGGTCTCAAA-3′. Data analysis used the 2-$\Delta\Delta$Ct method and normalized to the housekeeping β-actin gene.

2.11. Conventional Reverse Transcriptase Polymerase Chain Reaction. To detect mTOR and TSC1 expression levels, cDNA was amplified through a 32-cycle PCR: 95°C for 30 sec, followed by 32 cycles of 55°C for 30 sec and 30 sec at 72°C. Agarose gel electrophoresis method (1.5% agarose gel) was conducted to evaluated PCR products. The size of the PCR products was checked by 2 kb DNA Ladder (3427Q, Takara, Japan).

2.12. Transmission Electron Microscopy. $CD4^+$ T cells were fixed with 2.5% glutaraldehyde and stored at 4°C. Next, the samples were fixed with osmic acid at 4°C for 3 h after washing with phosphate buffer. They were then dehydrated stepwise in increasing concentrations of ethanol, infiltrated in Spurr resin overnight, embedded in Spurr resin, and cured in a 70°C oven for 24 h. Finally, thin sections (90 nm) were made and stained with uranyl acetate and lead citrate; the sections were afterward viewed with a transmission electron microscope. Representative images from randomly selected fields under the microscope are shown.

2.13. Statistical Analysis. The number of mice used for the experiments comprised at least six mice per treatment. All data in this study were derived from 3 or more independent experiments. The data are presented as the means ± SD. The results were analyzed using the GraphPad Prism 8.0 software. One-way ANOVA with Tukey's post hoc or Dunnett's post hoc analysis was done to test multiple comparisons. Kaplan-Meier with log rank test was used for survival analysis. In the case of western blot, apoptosis, and electron microscope, one representative set of data is shown. P values < 0.05 were considered statistically significant. $^*P \le 0.05$, $^{**}P \le 0.01$, $^{***}P \le 0.001$, and $^{****}P \le 0.0001$.

3. Results

3.1. Genotype Identification of Conditional Gene Knockout Mouse Used in CLP Model and Survival Observation. To determine the role of mTOR in ERS-induced $CD4^+$ T cell apoptosis, we constructed a sepsis model with T cell-specific mTOR and TSC1 gene knockout mice. The genotypes of the experimental mice were confirmed by RT-PCR (Figure 1). The expression of mTOR mRNA was significantly decreased in lck-mTOR $CD4^+$ T cells, while the expression of TSC1 mRNA was significantly decreased in lck-TSC1 $CD4^+$ T cells. RT-PCR results confirmed the establishment of T cell-specific mTOR/TSCI-KO gene knockout mouse. Survival rates between groups were also analyzed. Compared to the CLP group, the LCK-TSC1+CLP group showed higher mortality, whereas the Lck-mTOR+CLP group had significantly lower mortality (Figure 2).

3.2. Endoplasmic Reticulum Stress-Induced Apoptosis Leads to $CD4^+$ T Cell Depletion in Sepsis. By constructing a mouse sepsis model, we next investigated the relationship between sepsis, ERS, and $CD4^+$ T apoptosis. In comparison to the WT group, the CLP group exhibited statistically higher GRP78 and CHOP expression levels (Figure 3(b)). Electron microscopy also showed that significant ERS manifestations, such as dilatation and vesiculation of the ER structures, had occurred in the CLP group (Figure 4(b)). Furthermore, western blots showed that Bax, caspase-3, and BIM expression levels were upregulated in the CLP group, while BCL-2 was downregulated (Figure 3(c) and Figure 5(b)). As showed in the flow cytometry results, compared with other groups, the CLP group has higher apoptosis rate of $CD4^+$ T cells (Figure 4(b)).

The relationship between ERS and $CD4^+$ T cell apoptosis was studied using 4-PBA since it blocks ERS. Compared with the CLP group, GRP78 and CHOP expression levels were reduced in the CLP+4-PBA group (Figure 3(b)), and less serious ERS was observed by electron microscopy (Figure 4(b)). Compared with other groups, the CLP+4-PBA group displayed decreased p-mTOR, p-p70s6k, p-4EBP1, and CHOP expression and apoptosis-related proteins such as BIM, Bax/Bak, and caspase-3 and an upregulated Bcl-2 expression (Figures 3(a)–3(c)). The flow cytometry results showed that in the CLP+4-PBA group, fewer apoptotic $CD4^+$ T cells were found than in the other groups (Figure 4(a)). These results indicate that sepsis induces ERS and then causes $CD4^+$ T cell apoptosis, and that ERS activates mTOR and blocking ERS can alleviate $CD4^+$ T cell apoptosis.

3.3. mTOR Is Involved in ERS-Induced $CD4^+$ T Cell Apoptosis. The p-mTOR expression levels were found to have decreased significantly in the $CD4^+$ T cells from the

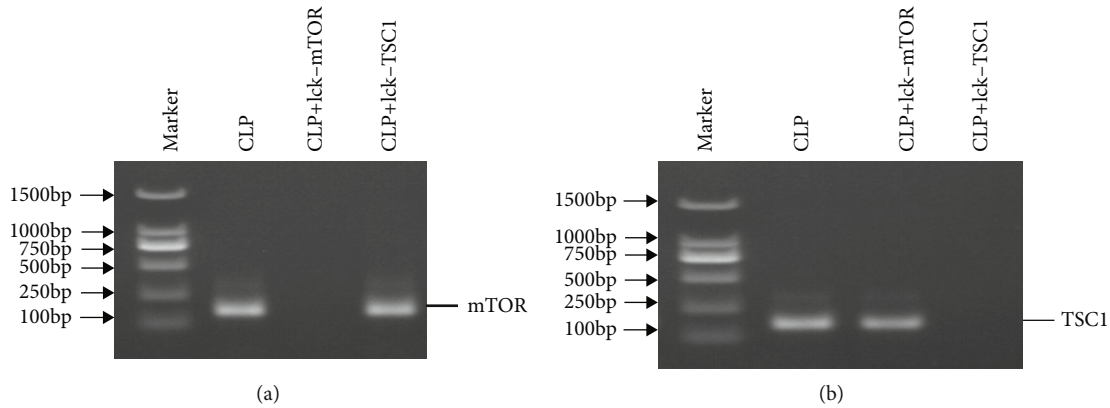

Figure 1: mTOR and TSC1 mRNA expression in $CD4^+$ T cells in CLP, lck-mTOR, and lck-TSC1 mice. Agarose gel electrophoresis of colony PCR (a) mTOR and (b) TSC1 genotyping.

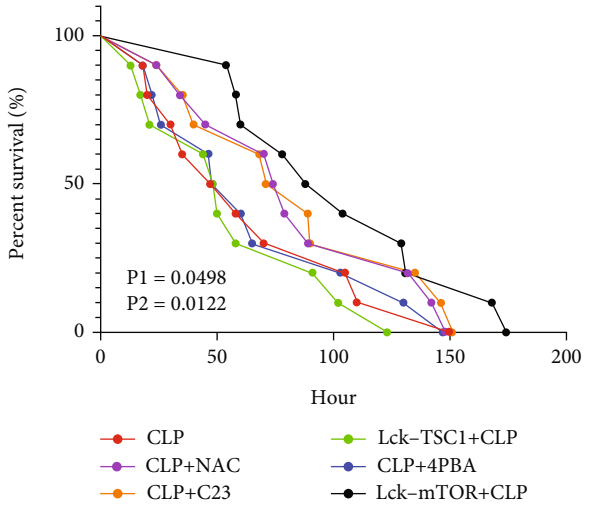

Figure 2: Survival curves. Survival rates between CLP, CLP+NAC, CLP+C23, CLP+4-PBA, Lck-TSC1+CLP, and Lck-mTOR+CLP groups. During the first 18 hours after CLP operation, the survival conditions were regularly monitored every 2 hours and then observed every 6 hours. Median survival time of CLP was 64 h, and LCK-TSC1+CLP group was 56 h, while Lck-mTOR +CLP had a significantly prolonged median survival time of 104 h (Kaplan-Meier with log rank test). $n = 10$-14 mice per group. x -axis: survival time; y-axis: survival rate. P1 = LCK-TSC1+CLP vs. Lck-mTOR+CLP; P2 = CLP vs. Lck-mTOR+CLP. NAC = N-acetyl-L-cysteine; 4-PBA = sodium phenylbutyrate; C23 = CIRP-Ab; CIRP = cold-inducible RNA-binding protein.

lck-mTOR mice and increased in the lck-TSC1 mice. In contrast, in the lck-mTOR+CLP and lck-TSC1+CLP groups, GRP78 and CHOP levels did not differ significantly (Figures 6(a) and 6(b)). We next examined what role mTOR plays in $CD4^+$ T cell apoptosis. We found that the expression levels of Bax, caspase-3, and BIM were downregulated, and the expression level of Bcl-2 increased.

In our study, we found that Bax, caspase-3, and BIM expression levels decreased, whereas Bcl-2 expression increased, and the $CD4^+$ T cell apoptosis rate decreased significantly in the lck-mTOR sepsis mice. However, Bax, caspase-3, and BIM were upregulated, the expression levels of Bax, caspase-3, and BIM were upregulated, the BCL-2 expression level was downregulated, and the $CD4^+$ T cell apoptosis rate significantly increased in the lck-TSC1 sepsis mice (Figure 5(b) and Figure 6(c)). The above results suggest that ERS activates mTOR, thereby causing apoptosis, and that blocking mTOR can alleviate ERS-related $CD4^+$ T apoptosis.

3.4. The Role Played by ROS in ERS, mTOR Expression Regulation, and $CD4^+$ T Cell Apoptosis. We focus on the effects of ROS on ERS and $CD4^+$ T cell apoptosis in the mouse sepsis model by using NAC to block ROS. Mice were first injected intraperitoneally with NAC 1 h before surgery. Thereafter, the effects of NAC on ERS and apoptosis in splenic $CD4^+$ T cells from the mice were examined postsurgery. In comparison with the CLP group, the CLP+CIRP-Ab group had a lower ROS level (Figure 7(b)) and lower CHOP, GRP78, P-mTOR, P-4EBP, and p-p70s6k expression levels (Figures 6(a) and 6(b)). Furthermore, in the CLP+CIRP-Ab group, BIM expression was significantly downregulated, Bcl-2 was upregulated, and Bax and caspase-3 expression levels were downregulated (Figure 5(b) and Figure 6(c)). CHOP and GRP78 expression was decreased, and they also significantly differed from some of the other groups (Figure 6(b)). Furthermore, electron microscopy results showed fewer signs of ERS within the $CD4^+$ T cells from the CIRP-Ab+CLP mouse group compared with those from the CLP group (Figure 7(c)); a lower percentage of apoptotic $CD4^+$ T cells was also recorded in the former group (Figure 7(b)).

CIRP, a damage-associated molecular pattern (DAMP) molecule, is an important upstream molecule of ROS [17]. To further explore the role played by ROS, we blocked CIRP expression in mice using a CIRP-Ab and examined its effects on ROS level, ERS, and apoptosis. The ROS level of the CLP +CIRP-Ab group was lower than that of the CLP group (Figure 7(b)) and lower CHOP, GRP78, P-mTOR, P-4EBP, and p-p70s6k expression levels (Figures 6(a) and 6(b)). Furthermore, in the CLP+CIRP-Ab group, BIM expression was decreased, Bcl-2 was increased, Bax and caspase-3 were downregulated (Figure 4(b) and Figure 6(c)), and CHOP and GRP78 expression was downregulated and significantly

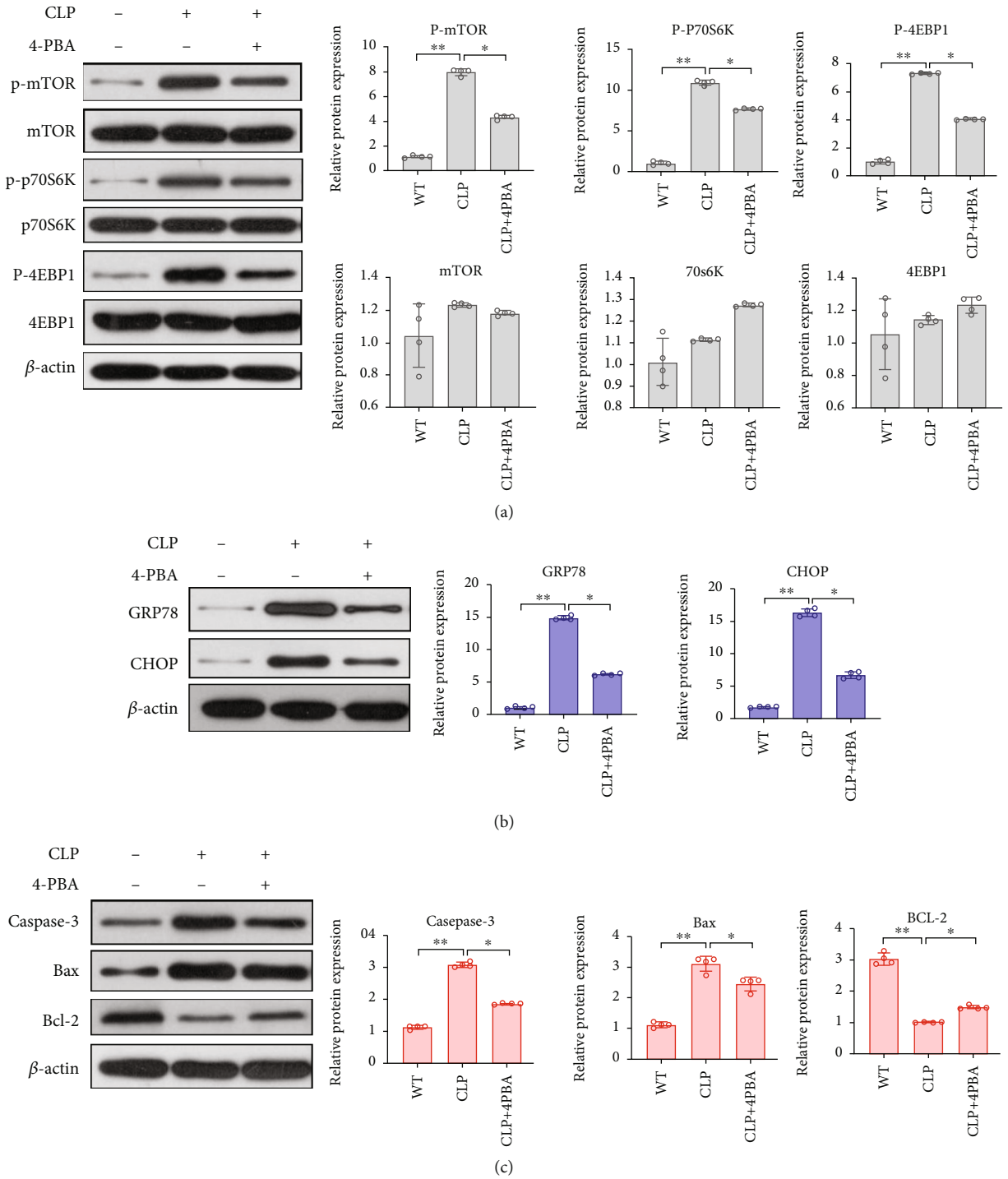

FIGURE 3: Expression levels of mTOR pathway proteins, ERS-associated proteins, and apoptosis-associated proteins in WT, CLP, and CLP +4-PBA mouse groups. After purifying the $CD4^+$ T cells from mouse spleen lymphocytes, whole cell lysates were assessed for the protein expression of (a) patterns of mTOR pathway proteins, including mTOR, P-mTOR, downstream effectors p70s6k, p-p70s6k, 4EBP, and P-4EBP; (b) ERS-associated proteins, including GRP78 and CHOP; (c) apoptosis-associated proteins, including caspase-3, Bax, and Bcl-2. The protein expression was detected by immunoblotting. Data are mean ± SD. $n = 4$ biologically independent experiments (one-way ANOVA Tukey's post hoc test). $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

different (Figure 6(b)). Furthermore, the electron microscopy results showed fewer signs of ERS within the $CD4^+$ T cells from the CIRP-Ab+CLP mouse group compared with those from the CLP group (Figure 7(c)); a lower level of apoptotic $CD4^+$ T cells was also recorded in the former group (Figure 7(b)).

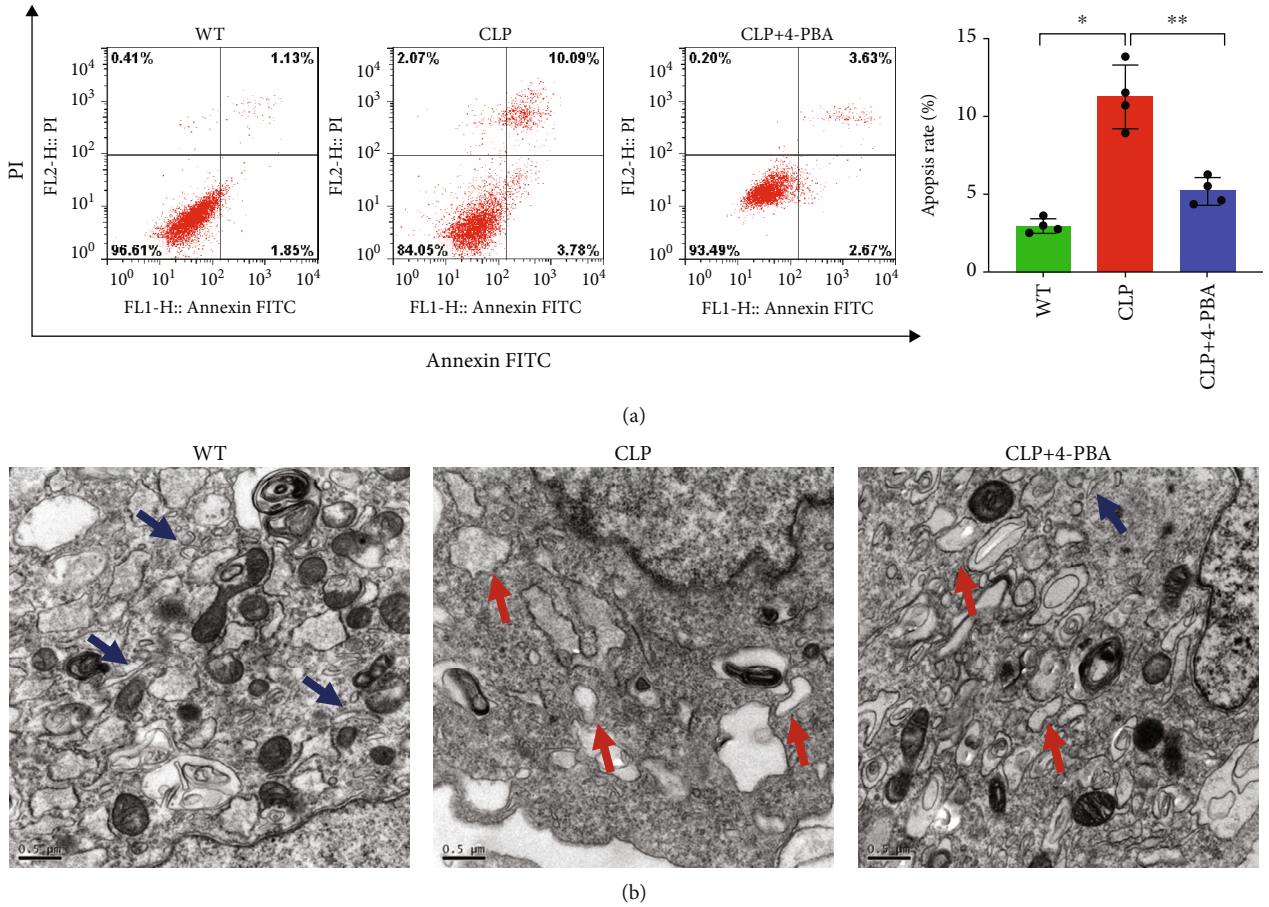

Figure 4: ERS-induced apoptosis leads to $CD4^+$ T cell apoptosis in CLP mice. Apoptosis in $CD4^+$ T cells from WT, CLP, and CLP+4-PBA mice was assessed by flow cytometry analysis. Apoptosis ratios are the early apoptosis percentage plus the late apoptosis percentage (a). *x*-axis: V-FITC; *y*-axis: PI. Left panel: gating strategy for apoptosis cells; right panel: percentage of apoptotic cells in the left panel. Graphs show means ± SD, four to six mice per group. Representative images of ER in $CD4^+$ T cells from WT, CLP, and CLP+4-PBA mice, as observed by electron microscopy (b). Data are mean ± SD, $n = 4$ (one-way ANOVA Dunnett's post hoc test). Blue arrows represent normal-sized ER. Red arrows represent dilation and vesiculation of the ER. Scale bars = 0.5 μm. FITC: fluorescein isothiocyanate; PI: propidium iodide.

4. Discussion

Sepsis is a type of life-threatening organ dysfunction feature based on early-onset inflammatory storm and late-onset immunosuppression [18]. Despite the advancement of many medical technologies such as early 6-hour sepsis bundle therapy, immunotherapy, and advanced organ function support, the prognosis for sepsis patients is far from ideal. One reason for this is unrelieved immunosuppression in those with deteriorating infections [5]. Studies by Inoue et al. found that excessive T lymphocyte apoptosis is closely associated with immunosuppression and is apparently linked with the prognosis for sepsis [19, 20]. The present study, together with our previous research on the abnormal apoptosis mechanism in $CD4^+$ T cells, revealed the complete process underlying sepsis-related CIRP–ROS-ERS–mTOR–$CD4^+$ T cell apoptosis. This is the first time that mTOR proved to be involved in the $CD4^+$ T cell apoptosis caused by ROS-mediated ERS in sepsis. We found that inhibiting mTOR reduced the high apoptotic rate of $CD4^+$ T cells in septic host and ultimately improved the prognosis for these mice.

The ER is a vital organelle involved in protein synthesis and secretion. It is also responsible for protein translation, processing, and modification. When sepsis, infection, and other diseases attacked the body, there is a mass of unfolded or misfolded proteins accumulated in cells. This accumulation disrupts homeostasis in the ER and causes ERS [21]. Moreover, there are many diseases associated with ERS, including sepsis, cancer, and diabetes [22–24]. The use of drugs or gene therapy strategies to inhibit ERS has successfully improved the pathological characteristics of such stress, thereby imparting therapeutic effects on the target organs [10, 24–26]. Therefore, lymphocyte ERS is expected to serve as a new therapeutic target to improve immune function in the sepsis patient. ROS accumulation is a sign of cell energy metabolism disorders. Under cell stress, hypoxia, and other oxidative stimuli, intracellular ROS production greatly increases. Excessive ROS can cause cell damage, cell metabolic disorders, even cell apoptosis [27]. Many recent studies have shown that when the cell energy transfer process is impeded, and the accumulation of excessive ROS will also seriously affect the cell's protein synthesis function; this situation inevitably causes ERS, further leading to cell death

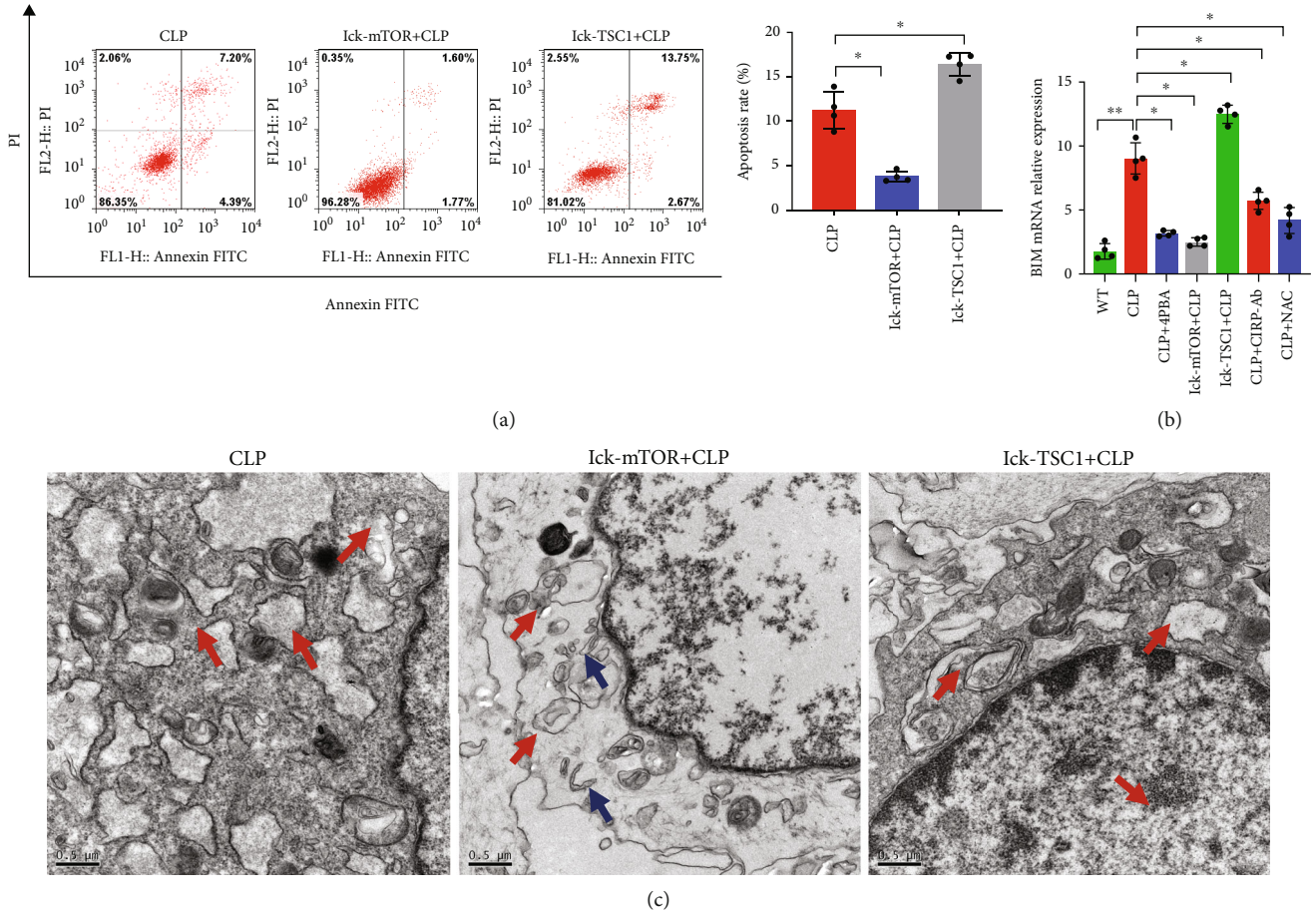

Figure 5: Role played by mTOR in ERS and $CD4^+$ T cell apoptosis. $CD4^+$ T cell apoptosis percentage was assessed by flow cytometry (a). Left panel: gating strategy for apoptosis cells; right panel: percentage of apoptotic cells in the left panel. Graphs show means ± SD, four mice per group. mRNA expression level of BIM in $CD4^+$ T cells was analyzed by RT-PCR (b). Representative images of ER in $CD4^+$ T cells from CLP, LCK-TSC1+CLP, and Lck-mTOR+CLP mice, as observed by electron microscopy (c). Data are mean ± SD, $n = 4$, analyzed using one-way ANOVA. Blue arrows represent the normal-sized ER. Red arrows represent dilation of the ER. Scale bars = 0.5 μm.

[28, 29]. Therefore, ROS and ERS are causal with respect to each other, and they ultimately affect the cell death process [30]. In this study, to verify the role of ROS and ERS in the apoptosis of septic $CD4^+$ T cells, NAC and 4-PBA were used to block ROS and ERS, respectively, and $CD4^+$ T cell apoptosis decreased in both groups.

When sepsis happened, there are two pathways that lead to systemic inflammation and oxidative stress: endogenous DAMPs and exogenous PAMPs (pathogen-associated molecular patterns). DAMPs are proinflammatory substances released upon host cell damage. As effective activators of body immune reaction that initiate and maintain damaged inflammatory responses, DAMPs can cause systemic inflammation, organ dysfunction, and even death [31]. CIRP, a newly discovered DAMP, recognized among different kinds of cells (e.g., T cells, B cells, and macrophages), is an important upstream molecular of ROS [17]. By blocking CIRP, we observed that ROS production and ERS occurrence were reduced, $CD4^+$ T cell apoptosis decreased, and the survival rates of the septic mice improved. From these results, we conclude that ROS and ERS are involved in septic $CD4^+$ T cell apoptosis and that ROS can cause ERS. The occurrence of ERS further mediates the damaging effect of ROS on $CD4^+$ T cells and causes $CD4^+$ T cell apoptosis.

mTOR is a sensitive and conserved cellular energy sensor. It is activated by various hormones and growth factors and has a key function in regulating cell metabolism, protein synthesis, growth, and differentiation [32]. Studies have highlighted mTOR's role in the energy metabolism of cells [33, 34]. When sepsis occurs, the body is in an inflammatory state caused by severe hypoxia and stress. Oxygen utilization and energy metabolism in the body's cells are both affected, and ROS production increases. ROS accumulation affects the translation, synthesis, and processing of proteins, leading to the occurrence of ERS and, ultimately, cell apoptosis [30]. In the body's fierce anti-infection process, lymphocytes are undoubtedly the first line of defense to bear the brunt of the infection, as evidenced by the apoptosis of large numbers of lymphocytes and the subsequent immunosuppression. The flow cytometry results from the present study are consistent with this. Sepsis therefore causes lymphocyte apoptosis and immunosuppression, and ROS accumulation aggravates the lymphocyte energy metabolism disorder. As

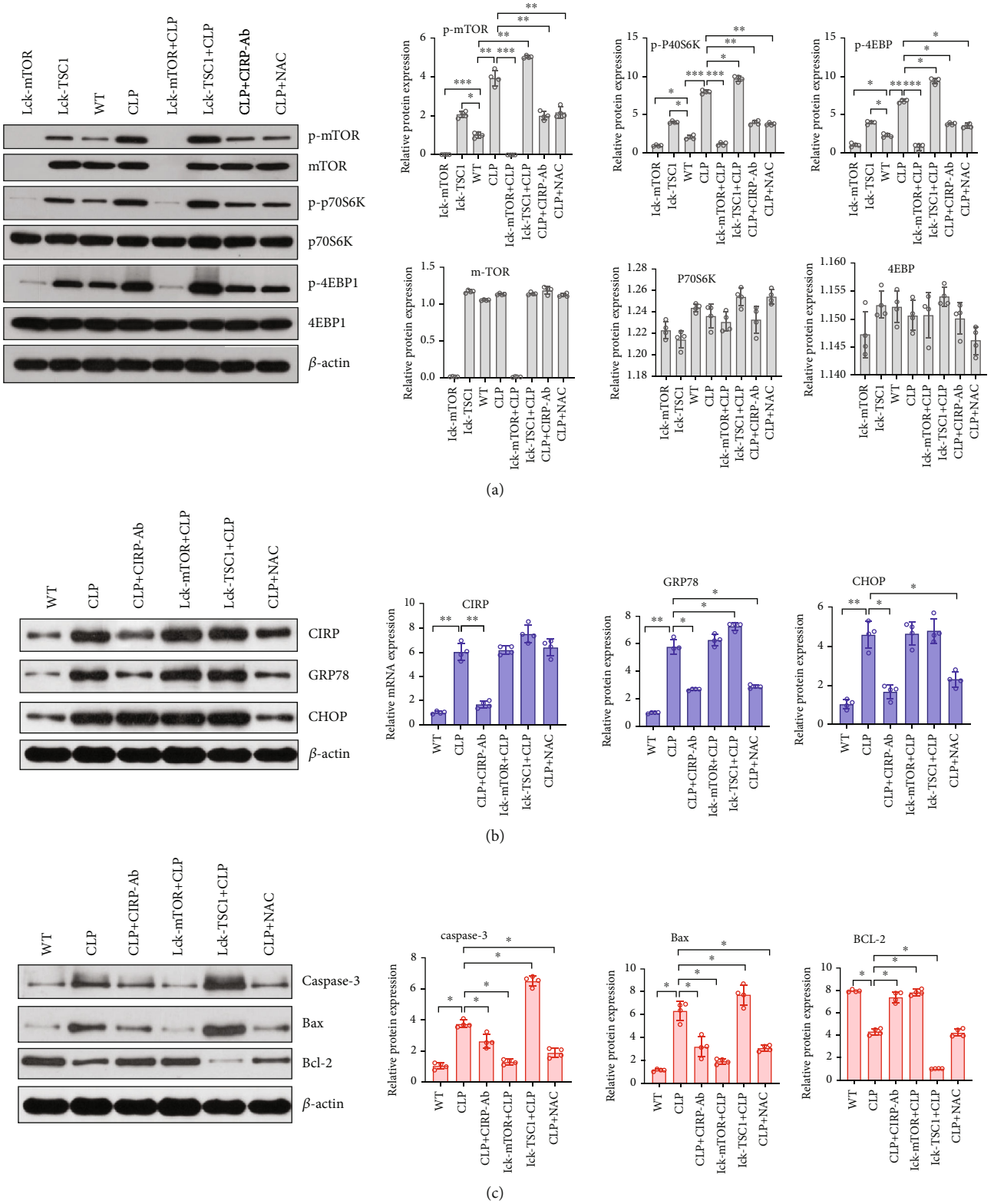

Figure 6: $CD4^+$ T cells were assessed for the expression of mTOR pathway-associated proteins, ERS-associated proteins, and apoptosis-associated proteins in WT, LCK-mTOR, LCK-TSC1, CLP, CLP+CIRP-Ab, CLP+NAC, LCK-TSC1+CLP, and Lck-mTOR+CLP mouse groups. Data are mean ± SD. Number of mice per group = 4 (one-way ANOVA Tukey's post hoc test). $CD4^+$ T cells were purified from CIRP-Ab-treated mice, NAC-treated mice, TSC1 knockout mice, and mTOR mouse spleen lymphocytes. Total proteins were western blotted to identify the expression patterns of mTOR pathway proteins (P-mTOR, mTOR, p70s6k, p-p70s6k, P-4EBP, and 4EBP) (a); CIRP and ERS-associated proteins (GRP78 and CHOP) (b); and apoptosis-related proteins (caspase-3, Bax, and Bcl-2) (c).

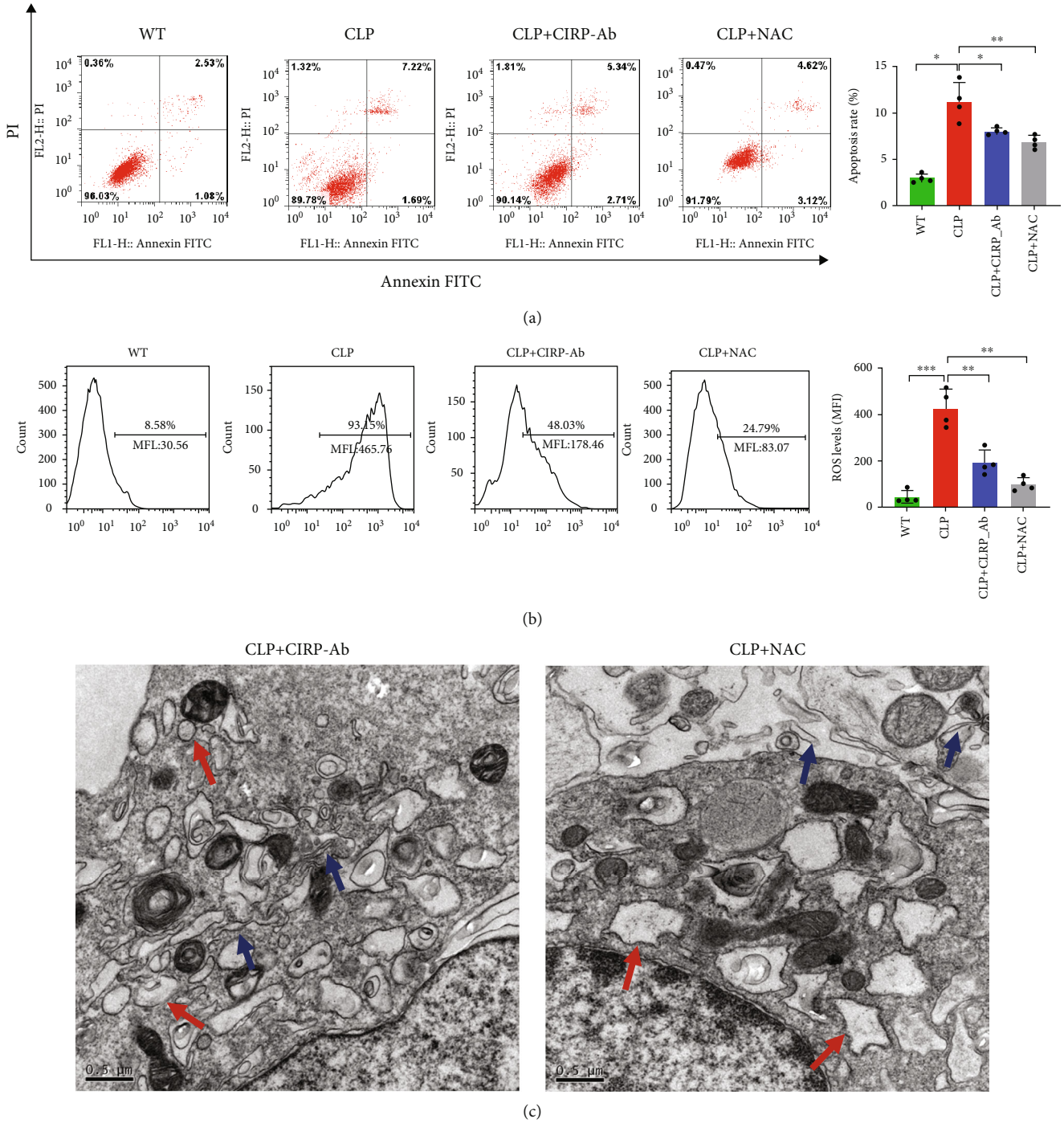

Figure 7: Role played by ROS in ERS and $CD4^+$ T cell apoptosis. The apoptosis percentage of $CD4^+$ T cell from WT, CLP, CLP+CIRP-Ab, and CLP+NAC mice was flow cytometrically determined (a). Left panel: gating strategy for apoptosis cells; right panel: percentage of apoptotic cells in the left panel. Data represent the mean ± SD. Graphs show means ± SD, four mice per group. $CD4^+$ T cells were treated with CM-H2DCFDA for 45 min prior to ROS analysis by flow cytometry (b). Representative images of ER in $CD4^+$ T cells from CLP, CLP+CIRP-Ab, and CLP+NAC mice, as observed by electron microscopy (c). Data are mean ± SD of 4 independent experiments (one-way ANOVA Tukey's post hoc test). Blue arrows indicate normal-sized ER. Red arrows indicate dilatation and vesiculation of the ER. Magnification, ×20,000; scale bars = 0.5 μm.

a crucial part of energy metabolism regulation, what role does mTOR play in this process? To answer this question, we explored the role of mTOR in the ERS-related $CD4^+$ T cell apoptosis mediated by ROS. We explored the role of mTOR by constructing septic models with T cell-specific mTOR/TSCI-KO gene knockout mice. Our results showed that $CD4^+$ T cell apoptosis in the Lck-mTOR+CLP group decreased, while $CD4^+$ T cell numbers increased. Significantly longer survival time was observed in mice that were treated with Lck-mTOR+CLP. This indicates that mTOR is activated by ERS to affect $CD4^+$ T cell apoptosis during sepsis.

Our results showed that when ERS occurred, mTOR is activated, as was revealed by two main results. (1) The inhibition of ERS by 4-PBA downregulated mTOR expression in parallel with decreased $CD4^+$ T cell apoptosis. (2) The occurrence of ERS was unaffected when mTOR and TSC1 (the inhibitor in the mTOR signaling pathway) genes were knocked out. Consistently, the study by Kato et al. showed that ERS rapidly activated mTORC1 in a mouse model of renal tubular injury, and mTORC1 is rapidly activated with the treatment of ERS inducers thapsigargin and tunicamycin [10]. However, some researchers believe that activating mTOR induces ERS. For example, Ozcan et al. reported that the loss of TSC1 or TSC2 (the inhibitor of mTOR) and the subsequent activation of mTORC1 led to ERS, thereby making the cells more susceptible to apoptosis and death [35]. The reasons for the inconsistencies between the present study and others may be related to the different disease models and different stimulus methods used to induce ERS. For example, in their article, Ozcan et al. pointed out that ERS was provoked by drug stimulation on isolated cells and that this may differ from the real situation in vivo. In contrast, our mouse model of sepsis was used to induce ERS in a manner as close as possible to the pathophysiological process of severe infection. The ERS in our study was more serious ERS and led to cell apoptosis and ultimately death in the host. The flow cytometry and survival curves confirmed the lymphocyte apoptosis and the timing of host death. Relevant studies have also shown that ERS upregulates the expression of mTORC1 and that the cytotoxicity of ERS is significantly related to mTORC1 activation [10, 36]. Therefore, our results suggest that mTOR is involved in regulating ERS-induced apoptosis, although the specific mechanisms involved require further exploration.

Herein, we found that mTOR is involved in ROS-related energy metabolism disorders. Through the intervention of mTOR, the stress and damaged state of $CD4^+$ T cells in sepsis were improved, $CD4^+$ T cell apoptosis was reduced, and the prognosis of the host was improved. These results provide a possible treatment avenue for improving the immune status of patients with sepsis. However, some aspects of our study require further investigation. First, when ERS occurs, there are three known signaling pathways that sense and relieve the occurrence of ERS; namely, IRE1, PERK, and ATF6 sensory pathways. In future research, we will focus on the precise sensing signal pathways to investigate how mTOR perceives and regulates ERS-related $CD4^+$ T cell apoptosis. Second, further study is warranted to determine the exact mechanism of the mTOR pathway leading to $CD4^+$ T cell apoptosis, including the detection of more specific downstream cytokine levels.

5. Conclusions

As far as we know, the upstream mechanisms by which mTOR regulates lymphocyte apoptosis in sepsis have not been elucidated. We explored the role of mTOR in $CD4^+$ T cell apoptosis induced by ERS. We found that ROS accumulation in sepsis led to ERS occurrence and that the mTOR pathway operating downstream of ERS induced $CD4^+$ T cell apoptosis. By inhibiting mTOR, $CD4^+$ T cell apoptosis was reduced, and the prognosis of the septic mice was improved. This indicates that mTOR participates in and regulates ROS-mediated ERS-related $CD4^+$ T cell apoptosis in sepsis, raising the possibility of mTOR becoming a new targeted treatment strategy for alleviating $CD4^+$ T cell apoptosis and improving the immune status of those experiencing sepsis.

Data Availability

Data are available on request.

Conflicts of Interest

The authors declare no conflict of interest.

Authors' Contributions

Na Cui was responsible for conceptualization; Hao Wang and Jianwei Chen were responsible for methodology; Hao Wang and Jianwei Chen were responsible for validation; Guangxu Bai was responsible for formal analysis; Wen Han was responsible for investigation; Guo Ran was responsible for resources; Guangxu Bai was responsible for data curation; Jianwei Chen was responsible for writing—original draft preparation; Hao Wang was responsible for writing—review and editing; Wen Han was responsible for visualization; Na Cui was responsible for supervision; Na Cui was responsible for project administration; Hao Wang was responsible for funding acquisition. Each of the authors has reviewed and approved this manuscript. Hao Wang and Jianwei Chen contributed equally to this work and share first authorship.

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References

[1] K. E. Rudd, S. C. Johnson, K. M. Agesa et al., "Global, regional, and national sepsis incidence and mortality, 1990-2017: analysis for the Global Burden of Disease Study," *Lancet*, vol. 395, no. 10219, pp. 200–211, 2020.

[2] R. Markwart, H. Saito, T. Harder et al., "Epidemiology and burden of sepsis acquired in hospitals and intensive care units: a systematic review and meta-analysis," *Intensive Care Medicine*, vol. 46, no. 8, pp. 1536–1551, 2020.

[3] F. Venet and G. Monneret, "Advances in the understanding and treatment of sepsis-induced immunosuppression," *Nature Reviews. Nephrology*, vol. 14, no. 2, pp. 121–137, 2018.

[4] M. J. Delano and P. A. Ward, "The immune system's role in sepsis progression, resolution, and long-term outcome," *Immunological Reviews*, vol. 274, no. 1, pp. 330–353, 2016.

[5] R. S. Hotchkiss, G. Monneret, and D. Payen, "Sepsis-induced immunosuppression: from cellular dysfunctions to

immunotherapy," *Nature Reviews. Immunology*, vol. 13, no. 12, pp. 862–874, 2013.

[6] C. Bhan, P. Dipankar, P. Chakraborty, and P. P. Sarangi, "Role of cellular events in the pathophysiology of sepsis," *Inflammation Research*, vol. 65, no. 11, pp. 853–868, 2016.

[7] S. S. Cao and R. J. Kaufman, "Endoplasmic reticulum stress and oxidative stress in cell fate decision and human disease," *Antioxidants & Redox Signaling*, vol. 21, no. 3, pp. 396–413, 2014.

[8] H. Puthalakath, L. A. O'Reilly, P. Gunn et al., "ER stress triggers apoptosis by activating BH3-only protein Bim," *Cell*, vol. 129, no. 7, pp. 1337–1349, 2007.

[9] Q. Chen, J. Thompson, Y. Hu, and E. J. Lesnefsky, "Chronic metformin treatment decreases cardiac injury during ischemia-reperfusion by attenuating endoplasmic reticulum stress with improved mitochondrial function," *Aging (Albany NY)*, vol. 13, no. 6, pp. 7828–7845, 2021.

[10] H. Kato, S. Nakajima, Y. Saito, S. Takahashi, R. Katoh, and M. Kitamura, "mTORC1 serves ER stress-triggered apoptosis via selective activation of the IRE1-JNK pathway," *Cell Death and Differentiation*, vol. 19, no. 2, pp. 310–320, 2012.

[11] W. Cheng, H. Wang, J. Zhang et al., "Lymphocyte subset counts as diagnostic and prognostic markers for carbapenem-resistant Enterobacteriaceae (CRE) infection in critically ill patients," *International Journal of Infectious Diseases*, vol. 96, pp. 315–322, 2020.

[12] D. R. Myers, B. Wheeler, and J. P. Roose, "mTOR and other effector kinase signals that impact T cell function and activity," *Immunological Reviews*, vol. 291, no. 1, pp. 134–153, 2019.

[13] H. Wang, G. Bai, N. Cui, W. Han, and Y. Long, "T-cell-specific mTOR deletion in mice ameliorated CD4(+) T-cell survival in lethal sepsis induced by severe invasive candidiasis," *Virulence*, vol. 10, no. 1, pp. 892–901, 2019.

[14] G. Bai, H. Wang, W. Han, and N. Cui, "T-bet expression mediated by the mTOR pathway influences CD4(+) T cell count in mice with lethal Candida sepsis," *Frontiers in Microbiology*, vol. 11, p. 835, 2020.

[15] F. Zhang, M. Brenner, W. L. Yang, and P. Wang, "A cold-inducible RNA-binding protein (CIRP)-derived peptide attenuates inflammation and organ injury in septic mice," *Scientific Reports*, vol. 8, no. 1, p. 3052, 2018.

[16] D. Rittirsch, M. S. Huber-Lang, M. A. Flierl, and P. A. Ward, "Immunodesign of experimental sepsis by cecal ligation and puncture," *Nature Protocols*, vol. 4, no. 1, pp. 31–36, 2009.

[17] M. Aziz, M. Brenner, and P. Wang, "Extracellular CIRP (eCIRP) and inflammation," *Journal of Leukocyte Biology*, vol. 106, no. 1, pp. 133–146, 2019.

[18] M. Singer, C. S. Deutschman, C. W. Seymour et al., "The third international consensus definitions for sepsis and septic shock (Sepsis-3)," *JAMA*, vol. 315, no. 8, pp. 801–810, 2016.

[19] S. Inoue, K. Suzuki-Utsunomiya, Y. Okada et al., "Reduction of immunocompetent T cells followed by prolonged lymphopenia in severe sepsis in the elderly," *Critical Care Medicine*, vol. 41, no. 3, pp. 810–819, 2013.

[20] J. C. Andreu-Ballester, C. Tormo-Calandín, C. Garcia-Ballesteros et al., "Association of $\gamma\delta$ T cells with disease severity and mortality in septic patients," *Clinical and Vaccine Immunology*, vol. 20, no. 5, pp. 738–746, 2013.

[21] D. S. Schwarz and M. D. Blower, "The endoplasmic reticulum: structure, function and response to cellular signaling," *Cellular and Molecular Life Sciences*, vol. 73, no. 1, pp. 79–94, 2016.

[22] S. A. Oakes and F. R. Papa, "The role of endoplasmic reticulum stress in human pathology," *Annual Review of Pathology*, vol. 10, no. 1, pp. 173–194, 2015.

[23] W. Rozpedek, D. Pytel, B. Mucha, H. Leszczynska, J. A. Diehl, and I. Majsterek, "The role of the PERK/eIF2α/ATF4/CHOP signaling pathway in tumor progression during endoplasmic reticulum stress," *Current Molecular Medicine*, vol. 16, no. 6, pp. 533–544, 2016.

[24] L. Ni, C. Yuan, and X. Wu, "Endoplasmic reticulum stress in diabetic nephrology: regulation, pathological role, and therapeutic potential," *Oxidative Medicine and Cellular Longevity*, vol. 2021, Article ID 7277966, 11 pages, 2021.

[25] L. Wang, Y. Liu, X. Zhang et al., "Endoplasmic reticulum stress and the unfolded protein response in cerebral ischemia/reperfusion injury," *Frontiers in Cellular Neuroscience*, vol. 16, 2022.

[26] B. Zhang, Y. Liu, J. S. Zhang et al., "Cortistatin protects myocardium from endoplasmic reticulum stress induced apoptosis during sepsis," *Molecular and Cellular Endocrinology*, vol. 406, pp. 40–48, 2015.

[27] M. Redza-Dutordoir and D. A. Averill-Bates, "Activation of apoptosis signalling pathways by reactive oxygen species," *Biochimica et Biophysica Acta*, vol. 1863, no. 12, pp. 2977–2992, 2016.

[28] G. Li, C. Scull, L. Ozcan, and I. Tabas, "NADPH oxidase links endoplasmic reticulum stress, oxidative stress, and PKR activation to induce apoptosis," *The Journal of Cell Biology*, vol. 191, no. 6, pp. 1113–1125, 2010.

[29] E. Pedruzzi, C. Guichard, V. Ollivier et al., "NAD(P)H oxidase Nox-4 mediates 7-ketocholesterol-induced endoplasmic reticulum stress and apoptosis in human aortic smooth muscle cells," *Molecular and Cellular Biology*, vol. 24, no. 24, pp. 10703–10717, 2004.

[30] J. Lee and C. H. Song, "Effect of reactive oxygen species on the endoplasmic reticulum and mitochondria during intracellular pathogen infection of mammalian cells," *Antioxidants*, vol. 10, no. 6, article 872, 2021.

[31] N. L. Denning, M. Aziz, S. D. Gurien, and P. Wang, "DAMPs and NETs in sepsis," *Frontiers in Immunology*, vol. 10, p. 2536, 2019.

[32] M. Laplante and D. M. Sabatini, "mTOR signaling in growth control and disease," *Cell*, vol. 149, no. 2, pp. 274–293, 2012.

[33] D. Cota, K. Proulx, K. A. Smith et al., "Hypothalamic mTOR signaling regulates food intake," *Science*, vol. 312, no. 5775, pp. 927–930, 2006.

[34] C. Folgueira, S. Barja-Fernandez, L. Prado et al., "Pharmacological inhibition of cannabinoid receptor 1 stimulates gastric release of nesfatin-1 via the mTOR pathway," *World Journal of Gastroenterology*, vol. 23, no. 35, pp. 6403–6411, 2017.

[35] U. Ozcan, L. Ozcan, E. Yilmaz et al., "Loss of the tuberous sclerosis complex tumor suppressors triggers the unfolded protein response to regulate insulin signaling and apoptosis," *Molecular Cell*, vol. 29, no. 5, pp. 541–551, 2008.

[36] S. Nakajima, N. Hiramatsu, K. Hayakawa et al., "Selective abrogation of BiP/GRP78 blunts activation of NF-κB through the ATF6 branch of the UPR: involvement of C/EBPβ and mTOR-dependent dephosphorylation of Akt," *Molecular and Cellular Biology*, vol. 31, no. 8, pp. 1710–1718, 2011.

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Research Article

MFG-E8 Knockout Aggravated Nonalcoholic Steatohepatitis by Promoting the Activation of TLR4/NF-κB Signaling in Mice

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Nonalcoholic steatohepatitis (NASH) is the common liver disease characterized by hepatic steatosis, inflammation, and fibrosis; there are no approved drugs to treat this disease because of incomplete understanding of pathophysiological mechanisms of NASH. Milk fat globule-epidermal growth factor-factor 8 (MFG-E8), a multifunctional glycoprotein, has shown anti-inflammation and antifibrosis. Here, MFG-E8 was shown to play a key role in NASH progression. Using methionine and choline deficient (MCD) diet-fed mice, we found MFG-E8 knockout exacerbated hepatic damage and steatosis as indicated by increased plasma transaminases activities and hepatic histopathologic change, higher hepatic triglycerides (TGs), and lipid accumulation. Moreover, liver fibrosis and inflammation elicited by MCD were aggravated in MFG-E8 knockout mice. Mechanistically, MFG-E8 knockout facilitated activation of hepatic toll-like receptor 4 (TLR4)/nuclear factor kappa B (NF-κB) signaling pathway in MCD-fed mice. *In vitro* experiment, the TLR4 specific antagonist TAK-242 rescued palmitic acid- (PA-) primed lipid formation and inflammation in MFG-E8 knockout primary murine hepatocytes. These findings indicated that MFG-E8 is involved in the progression of NASH and the possible mechanism by which MFG-E8 knockout exacerbated NASH in mice is associated with activation of the TLR4/NF-κB signaling pathway.

1. Introduction

Nonalcoholic fatty liver disease (NAFLD) is one of the most prevalent chronic liver diseases, occurring in 25%-30% of the general population [1, 2]. NAFLD is defined as a clinicopathological syndrome, ranging from simple nonalcoholic fatty liver to nonalcoholic steatohepatitis (NASH) [3, 4]. As an inflection point for the deterioration of NAFLD, NASH can lead to the development of liver cirrhosis, liver failure, and hepatocellular carcinoma, which has emerged as the main cause of liver-related mortality and liver transplantation. NASH is characterized by hepatic steatosis, inflammation, hepatocytes ballooning, and variable degrees of fibrosis [5]. However, its molecular mechanisms are still not fully understood, and no effective control measures are available. Therefore, there is an urgent need to explore the pathophysiological mechanisms of NASH development.

Accumulating evidence has shown that excessive lipid accumulation-induced production of proinflammatory mediators and innate immune cell activation play the pivotal roles in the progression of NASH. Due to the secretion of inflammatory mediators such as chemokines, the innate immune cell macrophages and neutrophils are recruited into the liver and activated by damage-associated molecular

patterns (DAMPs) released from injured hepatocytes, leading to aggravation of hepatic steatosis and fibrosis [6, 7]. As an important innate immune pattern recognition receptor (PRR), toll-like receptor 4 (TLR4) has been found to be upregulated in both NAFLD patients and animal models and is counted for the progression of hepatic steatosis, inflammation, and fibrosis [8–10]. It is well known that nuclear factor kappa B (NF-κB), a key downstream molecule of TLR4 signal pathway, plays an important role for the transformation from simple steatosis to steatohepatitis [11, 12]. In the canonical pathway, NF-κB proteins are bound and inhibited by IκB proteins. DAMPs, metabolites such as free fat acid, and LPS trigger TLR4 signal to phosphorylate IL-1 receptor-associated kinases (IRAKs). Sequentially, the IKKβ protein is activated, which phosphorylates IκB protein, resulting in IκB ubiquitination and degradation to release NF-κB proteins. The freeing NF-κB proteins such as p65 are activated by phosphorylation modification and translocated to the nucleus in which, as the key transcription factors, they induce these target genes expression such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and IL-6 [13, 14]. Therefore, targeting TLR4/NF-κB signaling may be the underlying mechanisms and key therapeutic strategies of NASH development.

Milk fat globule-epidermal growth factor-factor 8 (MFG-E8), a secreted glycoprotein with two EGF-like domains, contains an RGD motif that is able to bridge phosphatidylserine on apoptotic cells and integrin $\alpha v\beta 3$ or $\alpha v\beta 5$ in phagocytes to accelerate phagocytosis of apoptotic cells, resulting in the inhibition of inflammatory responses [15–17]. It has been shown that MFG-E8 protects against liver fibrosis in mice by interfering with the action of transforming growth factor-β1 (TGF-β1) [18]. A recent study has found that MFG-E8 is highly expressed in human hepatocellular carcinoma (HCC) tissues and positively regulates HCC progression, and anti-MFG-E8 antibodies could effectively inhibit HCC progression and metastasis [19]. In addition, it has been demonstrated that serum MFG-E8 can be feasibly served as a diagnostic, and prognostic biomarker for HCC and hepatic MFG-E8 prevents from the development of hepatic steatosis and inflammation [20, 21]. Furthermore, it has been reported that MFG-E8 could attenuate the release of proinflammatory cytokines from immune cells by inhibiting TLR4 and NF-κB pathways [22, 23] and is a key regulator of neutrophil infiltration in acute lung injury [24, 25]. However, the potential roles and mechanisms of MFG-E8 in the pathogenesis of NASH need to further be elucidated. Therefore, in the present study, we investigated the impact of MFG-E8 deficiency on the development of MCD-induced NASH model in mice and explored its potential mechanisms.

2. Materials and Methods

2.1. Chemicals and Reagents. The kits for alanine aminotransferase (ALT), aspartate aminotransferase (AST), and triglyceride (TG) were supplied by Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Enzyme-linked immunosorbent assay (ELISA) kits for TNF-α and IL-1β were purchased from Bender MedSystems (Vienna, Austria). PE rat anti-mouse Ly6G was obtained from BD Biosciences (New Jersey, USA). Neutrophils and F4/80 antibodies were obtained from Thermo Scientific (Rockford, IL, USA). Rabbit anti-mouse TLR4 antibody, rabbit anti-mouse phospho-IRAK1, phospho-p38, phosphor-IKKβ, IKKβ, phospho-IκBα, IκBα, phospho-p65, p38, p65, β-actin, Lamin B1, and GAPDH were purchased from Abcam (Cambridge, UK). PE rat anti-mouse F4/80, FITC rat anti-mouse CD11b, and FITC rat anti-mouse CD45 antibodies were from Biolegend, Inc. (San Diego, USA). TLR4-specific antagonist TAK-242 was from MedChemExpress LLC (Shanghai, China). Palmitic acid (PA) was from Sigma-Aldrich (St. Louis, USA).

2.2. Animals and Animal Experiments. Male C57BL/6J (WT) mice (6–8 weeks, 20-25 g) were supplied by the Experimental Animal Center of Chongqing Medical University (Chongqing, China). MFG-E8 knockout (KO) mice were donated by Professor Tianpen Cui at Wuhan No. 1 Hospital affiliated to Tongji Medical College of Huazhong University of Science and Technology. The experimental animals were maintained in a specific standard laboratory condition (20-25°C, 55 ± 5% humidity and a circle of 12 h light/dark) and were fed regularly and watered ad libitum. All mice were acclimatized for at least 1 week prior to use. The experiments involving mice were performed in accordance with the guidelines of the Animal Care and Use Committee of Chongqing Medical University.

Mice were randomly divided into four groups ($n = 6$ in each group): CD-WT group, CD-KO group, MCD-WT group, and MCD-KO group. Mice in both MCD-WT and MCD-KO groups were fed MCD diet (purchased by Trophic Animal Feed High-tech Co., Ltd. Jiangsu, China) for 5 weeks to induce NASH. During the same period, mice in the other two groups were fed standard chow diet (CD) (purchased from Trophic Animal Feed High-tech Co., Ltd. Jiangsu, China). At the end of 5 weeks, all mice were sacrificed under anesthesia via sevoflurane, blood samples were collected from the retroorbital sinus, and liver tissues were collected for next analysis.

2.3. Cell Culture and Treatment. Liver tissues were perfused with Hank's balanced salt solution (HBS), followed by Dulbecco's modified Eagle's medium (DMEM) with 0.05% IV collagenases at 37°C. Primary murine hepatocytes were collected by centrifugation at 50 g for 2 min, then were seeded in coated collagen type I cultural plates with DMEM supplemented with 10% fetal bovine serum (FBS).

The primary murine hepatocytes from wild type or MFG-E8 knockout mice were stimulated with palmitic acid (PA) (0.5 mM) with or without TLR4 specific antagonist TAK-242 (10 nM) for 24 h. The supernatant was collected for cytokines assay; the cells were fixed by 75% ethanol and stained with Oil Red O solution. In other experiment, the cells were lysed with chloroform-methanol solution for intracellular TG measurement.

2.4. Biochemical Analysis. Blood samples were collected from mice and centrifuged to obtain serum. Liver samples were

homogenized using a tissue homogenizer, and the supernatant was obtained by centrifugation. The activities of ALT and AST in serum and triglyceride (TG) in liver were measured using commercial assay kits according to the manufacturer's instructions.

2.5. Histological Analysis. Liver tissues were fixed in 4% paraformaldehyde, embedded in paraffin, and sliced at 5 μm thickness. Subsequently, tissue sections were stained with hematoxylin and eosin (HE) staining, Masson's Trichrome staining, or Sirius Red staining and evaluated using light microscopy (Olympus, Tokyo, Japan). Steatosis, inflammation, and hepatocyte ballooning in HE staining of the liver were scored according to the NAS (NAFLD activity score).

2.6. Oil Red O Staining. Liver tissues were embedded in optimum cutting temperature (OCT) compound, sectioned at 15 μm thickness, and fixed in 75% ethanol. Then, frozen sections were stained with Oil Red O solution and counterstained nuclear with hematoxylin.

2.7. Immunofluorescence of Macrophages and Neutrophils. Macrophages and neutrophils in the liver were visualized by immunofluorescence. Briefly, frozen sections (8 μm) were incubated with primary antibody FITC rat anti-mouse F4/80 or PE rat anti-mouse Ly6G at 37°C in the dark for 1 h. Then, actin filaments were then labeled with ActinRed 555 or ActinGreen 488 (Thermo scientific, Rockford, USA) at 37°C for 1 h. Finally, sections were counterstained nuclear with 4,6-diamino-2-phenyl indole (DAPI) and analyzed by fluorescence microscopy (Olympus, Tokyo, Japan).

2.8. Flow Cytometry. Liver samples were grounded and digested with 0.05% IV collagenases at 37°C which were filtered and centrifuged at 50 g for 5 min for the supernatant. Then, the precipitate was obtained by centrifuging the supernatant at 500 g for 5 min and was resuspended by phosphate buffer. Next, liver nonparenchymal cells (NPC) in the precipitates were incubated with labeled CD45, F4/80, Ly6G and CD11b antibodies in the condition of 4°C and darkness. The infiltration of macrophages ($CD11b^+F4/80^+$) and neutrophils ($CD45^+Ly6G^+$) was detected by flow cytometry.

2.9. Enzyme-Linked Immunosorbent Assay (ELISA). The levels of TNF-α and IL-1β in the liver and supernatants were measured by the ELISA kits following the manufacturer's protocols.

2.10. Reverse Transcription-Quantitative Polymerase Chain Reaction (RT-qPCR). Briefly, 100 mg of liver tissue and 1 mL Trizol reagent (Invitrogen, CA, USA) were homogenized using a homogenizer. The lysed liver sample was incubated for 5 min to permit the nucleoprotein dissociation and added 200 μL chloroform to mix, then securely cap the tube to incubate for 2 min. The sample was centrifuged at 4°C, 12000 g for 15 min, and the mixing contents were transferred 600 μL of the colorless, upper aqueous phase containing the RNA to a new RNase-free tube. The RNA mixture was added an equal volume of 70% ethanol to vortex. After that, the supernatant was carefully discarded, and the precipitation was dried at room temperature, pipetting 200 μL of RNase-free water to dissolve the pellet, and the total RNA solution was prepared after mixing well.

The complementary DNA (cDNA) was synthesized by the PrimeScript RT kit (Takara, Dalian, China). Quantitative real-time PCR was performed using the SYBR Green real-time PCR amplification kit (Takara, Dalian, China) following the manufacturer's protocol. The relative expression levels of all mRNAs were normalized to GAPDH expression. The primer sequences were listed as Table 1.

2.11. Western Blotting. The whole cell lysate, cytoplasmic, and nuclear soluble proteins from mouse liver tissues were separated by the RIPA lysis and Subcellular Protein Fractionation Kits (Thermo Fisher Scientific, Waltham, USA) according to the instructions. In brief, precisely weighed 100 mg of liver and 1000 μL of newly prepared RIPA lysis buffer or cytoplasmic extraction buffer (CEB) were placed into prechilled tube on ice, homogenizing fully on ice. The supernatants (whole cell lysate or cytoplasmic extract) were transferred into clean prechilled tube to use. The pellet in CEB was added 225 μL nuclear extraction buffer (NEB) containing protease inhibitors to vortex for 15 sec and incubate at 4°C for 30 min with gentle mixing. Then, the tube was centrifuged at 4°C, 5000 g for 5 min, and the supernatant (soluble nuclear extract) was collected. The protein concentrations were detected using the BCA assay kit.

Subsequently, proteins were subjected to electrophoresis through polyacrylamide-sodium dodecyl sulfate (SDS-PAGE) gel and transferred to polyvinylidene fluoride (PVDF) membrane. The membranes were then blocked with 5% bovine serum albumin (BSA) solution at room temperature for 1 h. Afterward, the membranes were incubated overnight at 4°C with appropriately diluted primary antibodies, followed by incubation with horseradish peroxidase-(HRP-) conjugated secondary antibodies for 1 h at room temperature. Eventually, antibody binding was displayed using an ECL chemiluminescent system and analyzed by Image Lab software.

2.12. Statistical Analysis. All data in this study were expressed as mean ± standard deviation (SD). Student's t-test was used to compare the difference between the two groups. One-way analysis of variance (ANOVA) followed by the Tukey *post hoc* test was used for multiple comparisons. P value < 0.05 was considered statistically significant.

3. Results

3.1. MFG-E8 Knockout Increased Serum ALT and AST Activities in NASH Mice. Serum ALT and AST activities are the crucial biochemical indicators for the evaluation of liver function. As shown in Figure 1, serum ALT and AST activities in the MCD-WT group were significantly higher than those in the CD-WT group ($P < 0.01$). Compared with the MCD-WT group, MFG-E8 knockout markedly increased serum ALT and AST activities ($P < 0.01$). In

Table 1: The primers of RT-qPCR.

Target gene	Forward primer	Reverse primer
TNF-α	5′-CAGGCGGTGCCTATGTCTC-3′	5′-CGATCACCCCGAAGTTCAGTAG-3′
IL-1β	5′-GAAATGCCACCTTTTGACAGTG-3′	5′-TGGATGCTCTCATCAGGACAG-3′
IL-6	5′-CTGCAAGAGACTTCCATCCAG-3′	5′-AGTGGTATAGACAGGTCTGTTGG-3′
ICAM-1	5′-GTGATGCTCAGGTATCCATCCA-3′	5′-CACAGTTCTCAAAGCACAGCG-3′
CCL2	5′-TAAAAACCTGGATCGGAACCAAA-3′	5′-GCATTAGCTTCAGATTTACGGGT-3′
CXCL2	5′-CCAACCACCAGGCTACAGG-3′	5′-GCGTCACACTCAAGCTCTG-3′
TGF-β	5′-CCACCTGCAAGACCATCGAC-3′	5′-CTGGCGAGCCTTAGTTTGGAC-3′
GAPDH	5′-TGACCTCAACTACATGGTCTACA-3′	5′-CTTCCCATTCTCGGCCTTG-3′

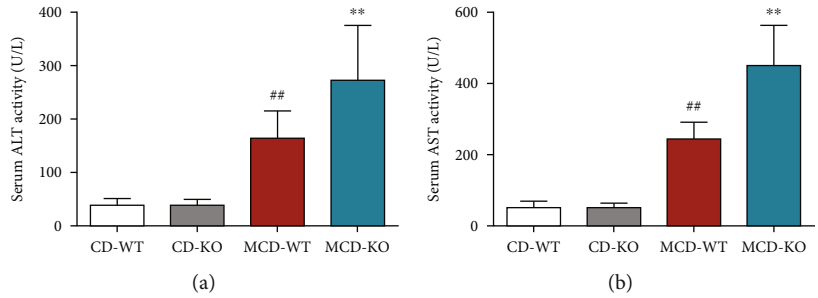

Figure 1: Effect of MFG-E8 knockout on serum ALT and AST activities in mice. Serum ALT (a) and AST (b) activities were measured after 5 weeks of feeding MCD diet or standard chew diet (CD) in wildtype (WT) or MFG-E8 knockout (KO). Data were expressed as mean ± SD, $n = 6$, $^{\#\#}P < 0.01$, compared with CD-WT group; $^{**}P < 0.01$, compared with MCD-WT group.

addition, there was no significant difference between the CD-WT and CD-KO groups, indicating that MFG-E8 knockout did not affect the liver function in mice.

3.2. MFG-E8 Knockout Aggravated MCD-Induced Hepatic Damage in Mice. To further confirm the effect of MFG-E8 on NASH, the histopathological changes of liver tissues were evaluated by HE staining and NAS scoring. As shown in Figure 2(a), there were not obvious pathological changes in both the CD-WT and CD-KO groups. In contrast, the apparent and diffuse hepatic steatosis with lobular inflammatory foci, as well as some ballooned hepatocytes were observed in the liver of the MCD-WT group, which were further aggravated in MFG-E8 knockout mice fed with MCD. Likewise, NAS scores showed that MFG-E8 knockout mice developed more severe hepatic pathological damage than wildtype mice after MCD diet for 5 weeks (Figure 2(b)).

3.3. MFG-E8 Knockout Deteriorated Hepatic Steatosis in NASH Mice. To assess the effect of MFG-E8 on lipid droplet formation, Oil Red O staining and TG content measurement were performed. MCD diet induced a marked lipid deposition and fat vacuole accumulation in hepatocytes, which are typical histological features of steatosis. However, MFG-E8 knockout significantly deteriorated the size and number of hepatic lipid droplets (Figure 3(a)). Meanwhile, as shown in Figure 3(b), the Oil Red O staining positive area was significantly higher in the liver of MFG-E8 knockout mice compared to wildtype group ($P < 0.01$). Similarly, MFG-E8 knockout mice showed remarkably higher hepatic TG contents than control mice in MCD diet ($P < 0.01$) (Figure 3(c)).

3.4. MFG-E8 Knockout Exacerbated MCD-Induced Liver Fibrosis in Mice. NASH is closely associated with liver fibrosis progressive. Thus, the extent of liver fibrosis was determined by Masson's Trichrome staining and Sirius Red staining. Compared with chow diet mice (CD-WT), MCD diet mice (MCD-WT) showed more significant liver fibrosis, which was drastically exacerbated by MFG-E8 knockout, as demonstrated by Masson's Trichrome stain (Figures 4(a) and 4(b), blue indicates collagen fibers) and Sirius Red stain (Figures 4(c) and 4(d), red indicates collagen fibers).

3.5. MFG-E8 Knockout Promoted Infiltration of Hepatic Macrophages and Neutrophils in NASH Mice. The infiltration of macrophages and neutrophils into the liver is one of the most crucial events in NASH development. Immunofluorescence staining analysis showed that compared to the MCD-WT group, MFG-E8 knockout mice exhibited augmented infiltration of $F4/80^+$ macrophages and $Ly6G^+$ neutrophils into the liver (Figures 5(a)–5(d)). Furthermore, as expected, flow cytometry analysis experiments showed a similar result that hepatic inflammatory cell ($CD11b^+F4/80^+$ macrophages and $CD45^+Ly6G^+$ neutrophils) numbers were markedly elevated in MCD diet-fed MFG-E8 knockout mice compared with MCD-WT group (Figures 5(e) and 5(f)).

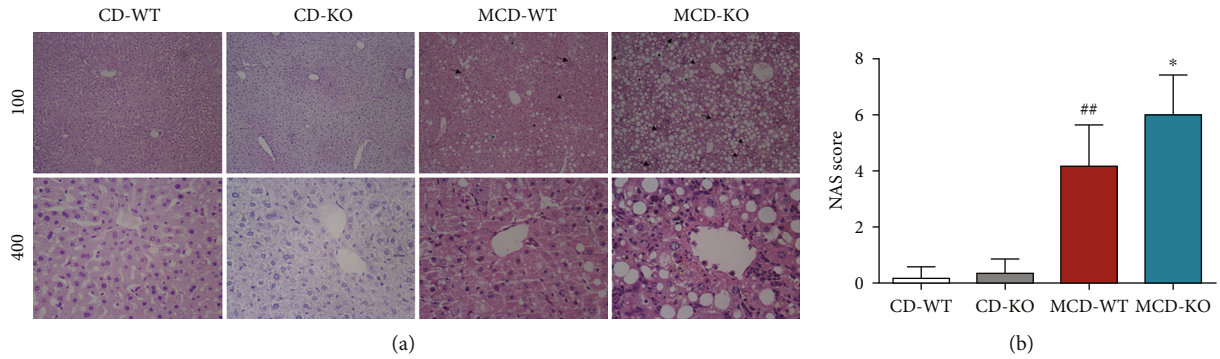

Figure 2: Effect of MFG-E8 knockout on liver injury induced by MCD in mice. (a) The liver pathological changes were determined by HE staining and observed under a microscope. (b) The NAS scores were analyzed. Arrow indicated inflammatory foci, arrowhead indicated hepatocyte ballooning, and asterisk indicated hepatocyte steatosis. Data were expressed as mean ± SD, $n = 6$, $^{\#\#}P < 0.01$ compared with CD-WT group; $^{*}P < 0.05$ compared with MCD-WT group.

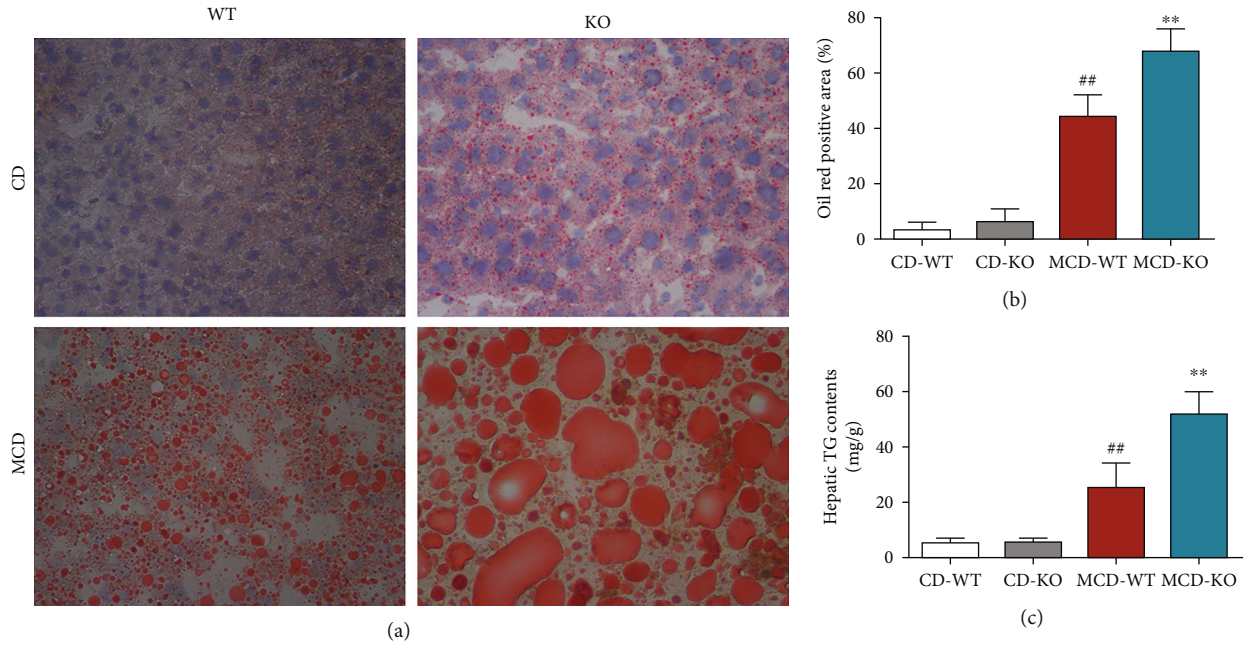

Figure 3: Effect of MFG-E8 knockout on MCD-induced hepatic lipid accumulation in mice. (a) Lipid accumulation in frozen sections of liver tissue was determined by Oil Red O staining (200x). (b) The Oil Red O staining positive area was measured and quantified. (c) The content of TG in the liver was assayed. Data were expressed as mean ± SD, $n = 6$, $^{\#\#}P < 0.01$ compared with CD-WT group; $^{**}P < 0.01$, compared with MCD-WT group.

3.6. MFG-E8 Knockout Enhanced the Production of Inflammatory Mediators in the Liver of NASH Mice. Further, the expression of proinflammatory mediators in the liver of NASH mice was analyzed by ELISA and RT-qPCR. As shown in Figures 6(a) and 6(b), after MCD diet for 5 weeks, the protein levels of hepatic TNF-α and IL-1β in MFG-E8 knockout mice were higher than that in wildtype mice (P<0.01). Consistently, the mRNA levels of inflammatory mediators TNF-α and IL-1β, as well as IL-6, ICAM, CCL2, CXCL2, and TGF-β, which were indicated as progressive inflammatory response and liver fibrosis, were significantly elevated in the liver of MFG-E8 knockout mice compared with the MCD-WT group (Figure 6(c)).

3.7. MFG-E8 Knockout Facilitated MCD-Induced Activation of TLR4/NF-κB Signaling in the Liver of Mice. To further explore the potential mechanism by which MFG-E8 knockout aggravated NASH progression, Western blotting was used to detect the activation of TLR4/NF-κB signaling pathway. The results showed that the levels of TLR4, p-IRAK1, p-p38, and p-p65 proteins in the liver of the MCD-WT group were significantly higher than those of CD-WT group, but the total p38 and p65 protein levels were not significant. However, MFG-E8 knockout obviously upregulated the levels of hepatic TLR4, p-IRAK1, p-p38, and p-p65, indicating that MFG-E8 knockout enhanced MCD-induced TLR4 signal activation

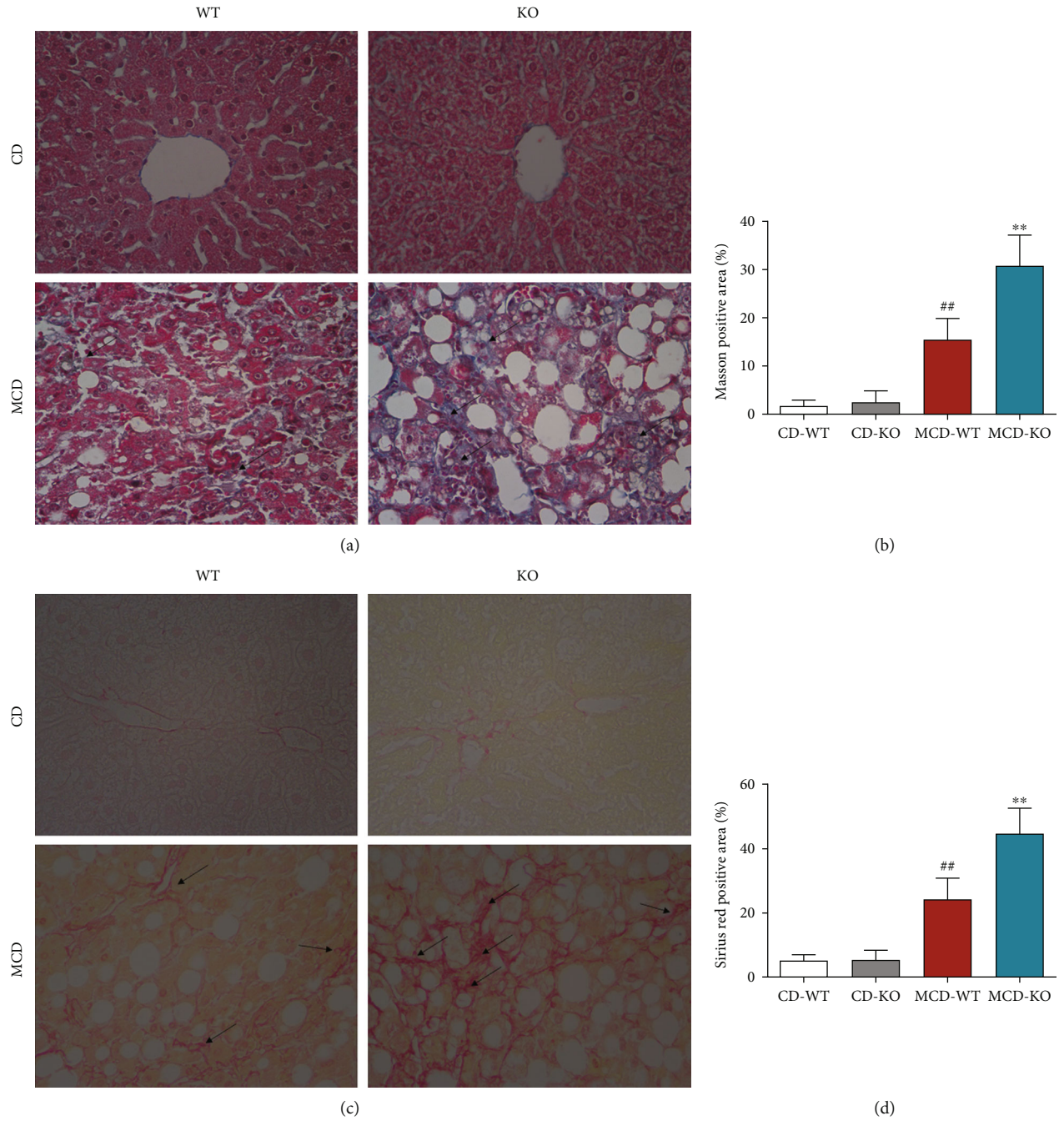

Figure 4: Effect of MFG-E8 knockout on MCD-induced liver fibrosis in mice. The liver section was performed by Masson's Trichrome staining and Sirius Red staining to assess the degree of liver fibrosis. (a) Images of Masson's Trichrome staining (200x). (b) The positive area of Masson's Trichrome staining. (c) Images of Sirius Red staining (200x). (d) The positive area of Sirius Red staining. Arrow indicated hepatic fibrosis changes. Data were expressed as mean ± SD, $n = 6$, $^{\#\#}P < 0.01$ compared with CD-WT group; $^{**}P < 0.01$, compared with MCD-WT group.

(Figures 7(a) and 7(b)). Accordingly, compared to MCD-WT group, MFG-E8 knockout significantly increased MCD-induced NF-κB activation, as supported by enhancing the phosphorylation of IKKβ and IκBα, as well as increased IκBα degradation. Moreover, the analysis of p65 in the subcellular localization indicated that freeing p65 was sharply translocated from cytoplasm into nucleus in the liver of MFG-E8 knockout mice compared with WT group fed by MCD (Figures 7(c) and 7(d)).

3.8. TLR4 Antagonist Rescued MFG-E8 Knockout-Enhanced TGs Synthesis and Proinflammatory Cytokine Production in Primary Hepatocytes Stimulated by PA. To evaluate whether TLR4 mediated MFG-E8 knockout-aggravated NASH phenotype in MCD-fed mice, the primary hepatocytes separated from WT or MFG-E8 knockout mice were stimulated by PA with or without TLR4-specific antagonist TAK-242. In parallel with these results from *in vivo* animal experiment, MFG-E8 knockout hepatocytes showed

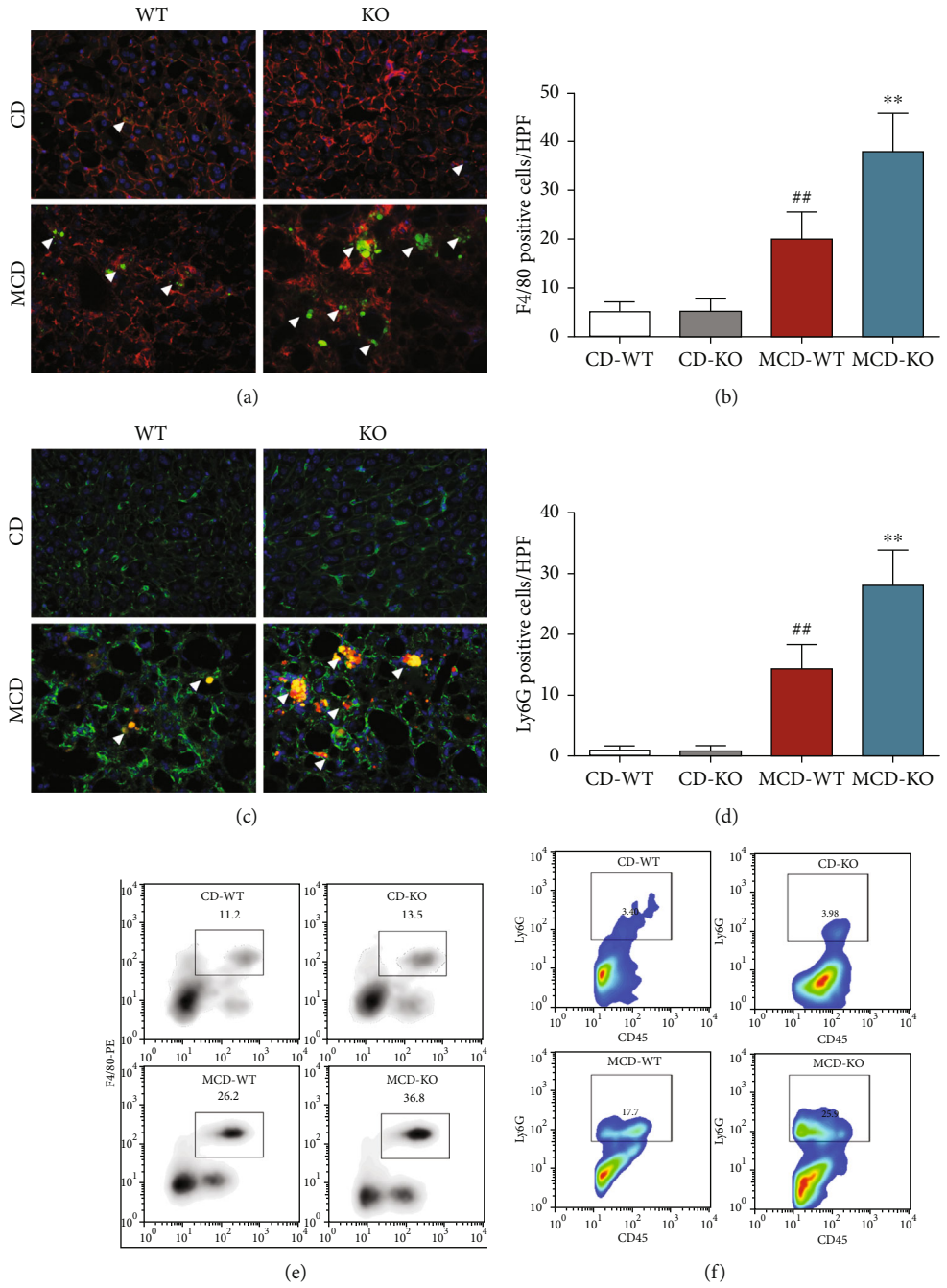

Figure 5: Effect of MFG-E8 knockout on hepatic macrophage and neutrophil infiltration in NASH mice. Hepatic macrophages and neutrophils were determined by immunofluorescence and flow cytometry using specific macrophage marker antibody F4/80 and neutrophil marker antibody Ly6G. (a) Representative immunofluorescence images of hepatic macrophages (200x), F4/80 positive cells were labeled with Green; F-actin was labeled with ActinRed 555. (b) F4/80 positive cells in high power field (HPF) were quantified. (c) Representative immunofluorescence images of hepatic neutrophils (200x), Ly6G positive cells were labeled with Red; F-actin was labeled with ActinGreen 488. (d) Ly6G positive cells in HPF were quantified. White arrowhead in the images indicated macrophage or neutrophil. (e) The $CD11b^+F4/80^+$ cells indicated as macrophages in representative flow cytometry analysis of hepatic nonparenchymal cells. (f) The $CD45^+Ly6G^+$ cells indicated as neutrophils in representative flow cytometry analysis of hepatic nonparenchymal cells. Data were expressed as mean ± SD, $n = 6$, $^{\#\#}P < 0.01$ compared with CD-WT group; $^{**}P < 0.01$, compared with MCD-WT group.

higher lipid droplet formation and TG synthesis, as well as massive inflammatory cytokines TNF-α and IL-1β production compared with wildtype hepatocytes in response to PA stimulation. However, TLR4-specific antagonist TAK-242 significantly reverted MFG-E8 knockout-aggravated NASH phenotype, as indicated by decreased lipid droplet formation and TG synthesis, and weakened inflammatory cytokine production (Figure 8), suggesting that TLR4 might participate in the function of MFG-E8 on modulating NASH progression.

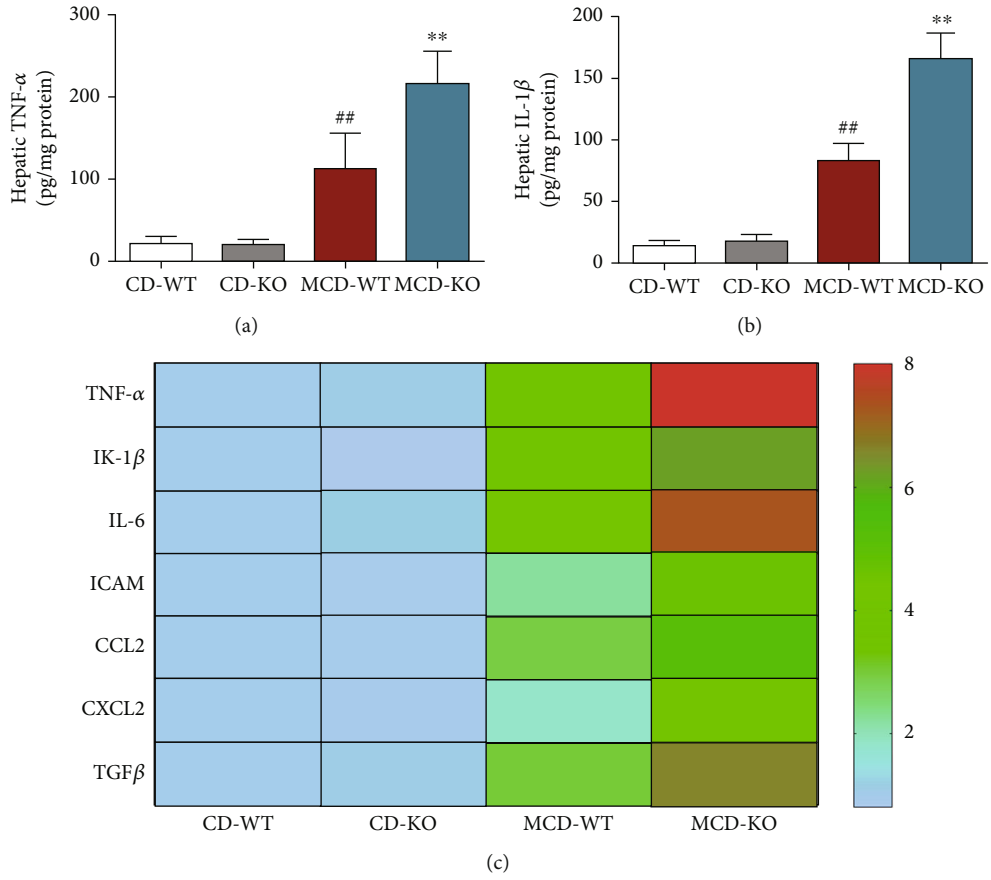

Figure 6: Effect of MFG-E8 knockout on hepatic inflammatory mediators in NASH mice. (a) TNF-α and (b) IL-1β protein levels in the liver were detected by ELISA. (c) The mRNA levels of hepatic TNF-α, IL-1β, IL-6, ICAM, CCL2, CXCL2, and TGF-β were measured by RT-qPCR. Data were expressed as mean ± SD, $n = 6$, $^{\#\#}P < 0.01$ compared with CD-WT group; $^{**}P < 0.01$, compared with MCD-WT group.

4. Discussion

NASH, a potentially progressive subtype of NAFLD that results in hepatocirrhosis and liver cancer, is closely associated with the metabolic syndrome and responsible for considerable economic burden globally [26, 27]. In the current study, we demonstrated that MFG-E8 plays an important role in the development of NASH. Our results found that MFG-E8 knockout significantly increased serum ALT and AST activities, exacerbated the histopathological liver injury as well as hepatic lipid accumulation, and promoted hepatic inflammatory responses and fibrosis in mice induced by MCD diet.

Liver fibrosis is a main histopathological feature of progressive NASH, exposing patients to a significant risk for cirrhosis and hepatocellular carcinoma. Previous several studies have suggested that MFG-E8 might be involved in the pathogenesis of fibrosis in various organs and tissues. For example, the expression of MFG-E8 was significantly downregulated in the sclerotic skin lesions in systemic sclerosis patients with skin fibrosis, in the smooth muscle cells surrounding the fibrotic respiratory tracts of asthma patients, and in the cirrhotic livers [18, 28, 29]. As indicated, MFG-E8 KO mice developed severe pulmonary fibrosis and skin fibrosis upon intratracheal bleomycin administration [28, 30]. Similarly, our results showed that MFG-E8 knockout mice exhibited more severe hepatic fibrosis compared to the MCD-WT group.

Mounting evidence has revealed that the imbalance lipid metabolism is the main etiology of hepatic steatosis and fibrosis. Excessive lipid accumulation in the liver induces metabolic stress and causes lipotoxicity, resulting in liver parenchymal cell death. The hepatocyte-death-released DAMPs activate innate immune signaling by PPRs, which trigger sustained inflammatory cascade and further worsen metabolic disorders and, finally, drive NASH progression. Thus, dissection of lipid metabolic disorder and excessive innate immune reaction is important for exploring the underlying mechanisms or identifying novel therapeutic targets of NASH development [31–33]. In this study, MFG-E8 knockout deteriorated hepatic steatosis in NASH mice, indicating that MFG-E8 may lower hepatic lipid production through a direct or indirect molecular mechanism. However, a previous study has showed that MFG-E8 could promote fatty acid uptake and cause obesity in mice by inducing the translocation of CD36 and FATP1 into cell surface. This data seems be controversial with our present results. However, this report showed that MFG-E8 mainly affects adipocytes but not hepatocytes in the absorption of fatty acid from blood [34]. In addition, in our experiment, the MCD diet but

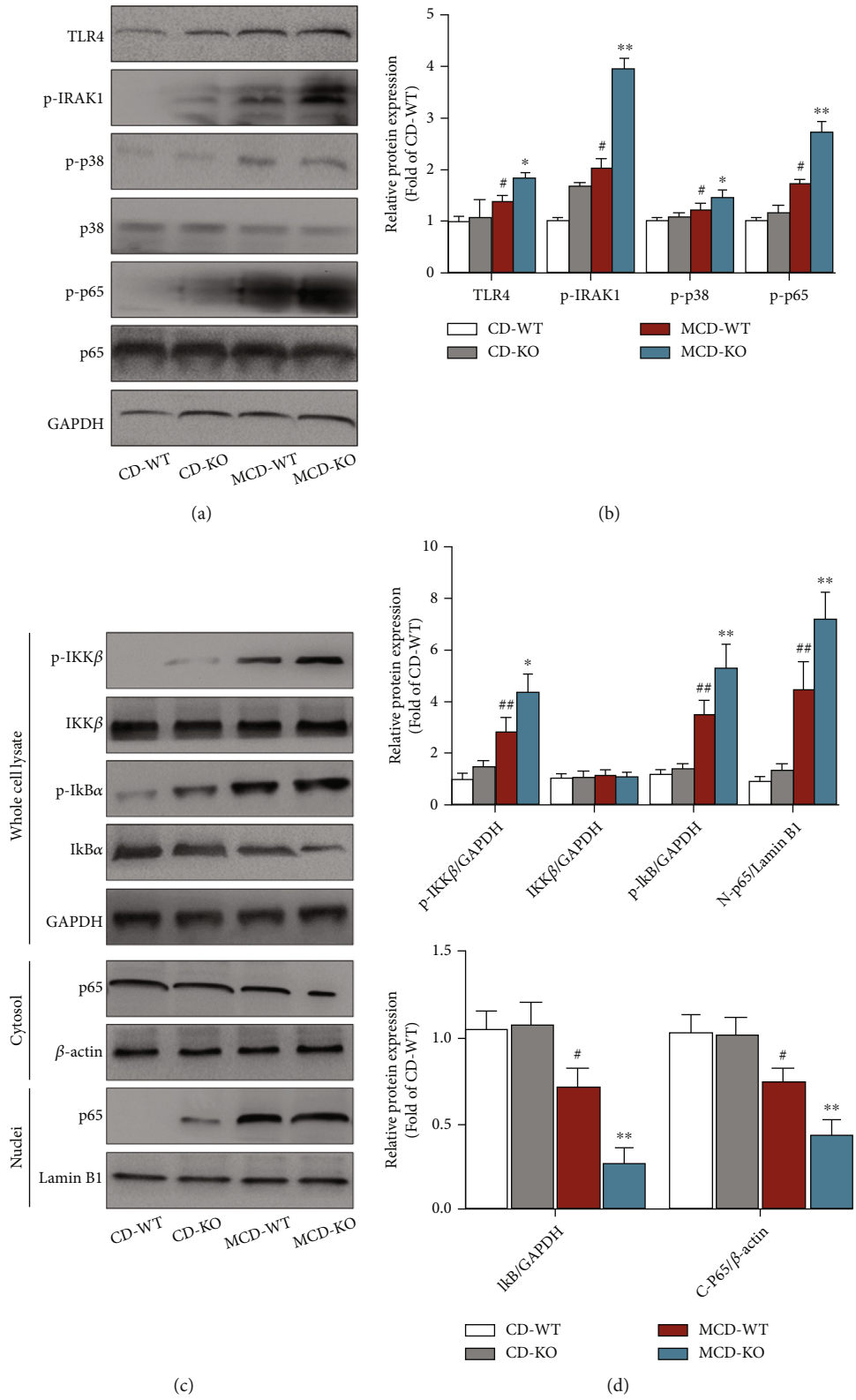

Figure 7: Effect of MFG-E8 knockout on TLR4/NF-κB signaling pathway in the liver of NASH mice. The indicated proteins in whole cell lysates, cytoplasmic extraction, and nuclear extraction from the liver tissues were detected by Western blotting, respectively. Representative Western blotting (a) and quantification (b) of TLR4, p-IRAK1, p-p38, p38, p-p65, p65, and GAPDH protein levels in the liver. Representative Western blotting (c) and quantification (d) of p-IKKβ, IKKβ, p-IκBα, IκBα, and GAPDH in the whole cell lysates, p65, and β-actin in the cytoplasmic extraction, as well as p65 and Lamin B1 in the nuclear extraction from liver tissues. Data were expressed as mean ± SD, $n = 3$, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared with CD-WT group, $^{*}P < 0.05$, $^{**}P < 0.01$, compared with MCD-WT group.

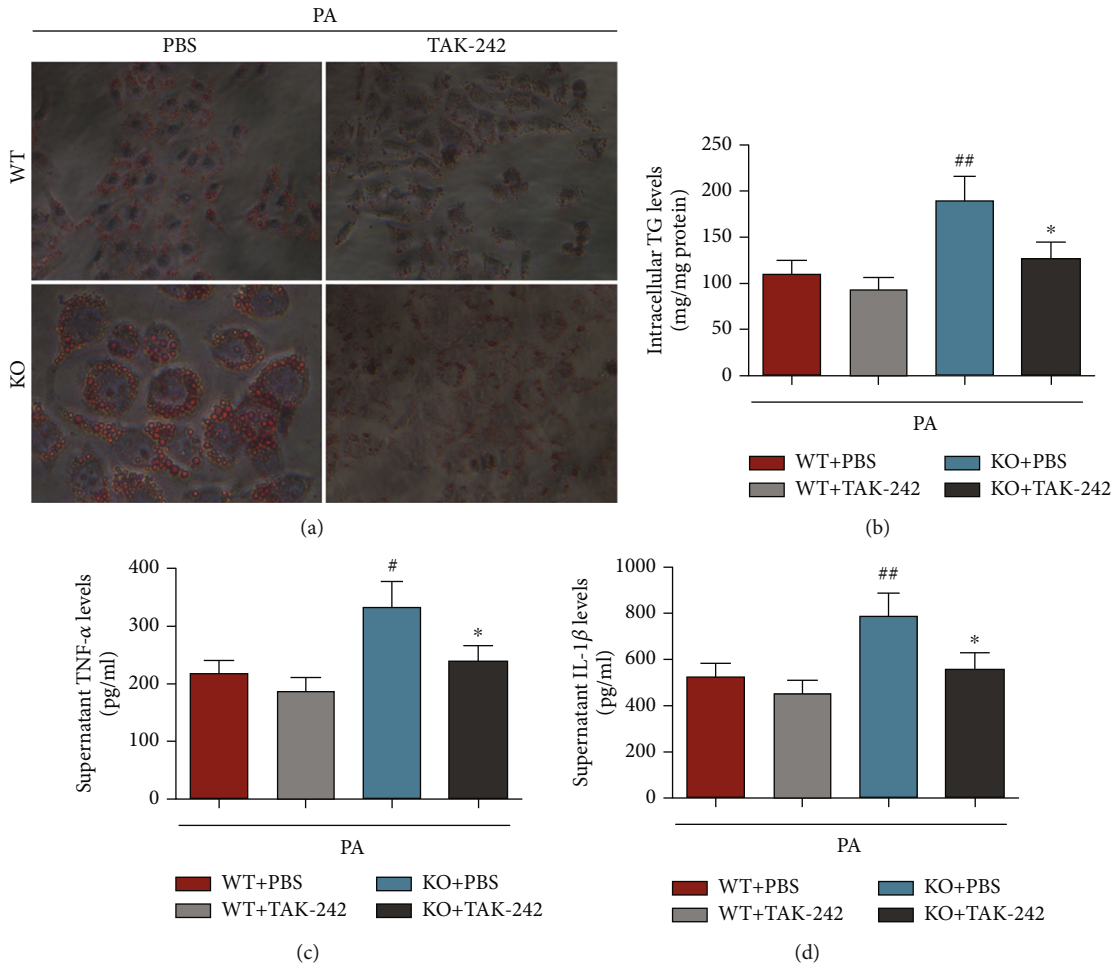

Figure 8: TLR4 antagonist rescued MFG-E8 knockout-enhanced TG synthesis and proinflammatory cytokine production in primary hepatocytes stimulated by palmitic acid (PA). The primary hepatocytes separated from WT or MFG-E8 knockout mice were stimulated by PA (0.5 mM) with or without TLR4 specific antagonist TAK-242 (10 nM) for 24 h, the lipid droplet was evaluated by Oil Red O staining (a), TG content was assayed by a commercial kit (b), and TNF-α (c) and IL-1β (d) protein levels in the supernatants were measured by ELISA. Data were expressed as mean ± SD, $n = 6$, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared with WT + TAK-242 group, $^{*}P < 0.05$, compared with KO + PBS group.

not high fatty diet- (HFD-) induced NASH model was used. The two NASH models have obvious different phenotype and pathogenesis. In the MCD model, lack of methionine and choline in diet interrupts the VLDL assembly, which leads to decreased TG secretion, resulting in hepatic lipid accumulation [35]. In fact, lipid metabolic disorder is involved in an imbalance between hepatic lipid input and output. Recently, Zhang et al. reported that MFG-E8 improved hepatic steatosis and inflammation through inhibiting apoptosis signal-regulating kinase 1 (ASK1) and mitogen-activated protein kinase (MAPK) signaling in hepatocytes [21]. Considering that ASK1 and MAPKs are downstream molecules of TLR4 signal pathway, and the lipid output but not its input is declined in MCD-induced NASH, we speculate that MFG-E8 does not directly regulate lipid metabolism but might block inflammatory cascade-worsened metabolic disorders by inhibiting innate immune TLR4 signal.

It is acknowledged that metabolic inflammation is tightly regulated by innate immune signal. Hepatic macrophages and neutrophils have been identified as the main innate immune cells in NAFLD [36, 37]. Infiltrating macrophages and neutrophils secrete proinflammatory cytokines and chemokines that promote the progression of liver inflammation and fibrosis and aggravate hepatic steatosis [38–40]. Established data suggested that MFG-E8 could inhibit the production of proinflammatory mediators and alleviate macrophage and neutrophil infiltration [23, 41–43]. Consistently, in the present study, by immunofluorescence staining and flow cytometry, we found that MFG-E8 knockout exhibited more severe hepatic macrophages and neutrophil infiltration in the liver of NASH mice. In addition, the RT-qPCR and ELISA analysis also indicated that MFG-E8 knockout upregulated the expression of inflammatory mediators.

TLR4 and NF-κB play a critical role in the innate immune inflammatory responses and are closely related to the production of inflammatory mediators and cellular damage. It has been well demonstrated that activation of TLR4/NF-κB signaling pathway aggravates inflammatory responses and promotes NASH progression [10, 44–46].

We previously reported that mice fed by an MCD diet exhibited severe inflammation and liver injury through upregulating the expression of proinflammatory cytokines and chemokines, which coincided with activation of TLR4/NF-κB signaling pathway in the liver [14]. Additionally, inhibition of TLR4 or NF-κB activation has been shown to exert the beneficial therapeutic role in several NASH mouse models [47, 48]. In other inflammatory models, MFG-E8 is also indicated to be effective for attenuating inflammatory response through inhibiting the activation of TLR4/NF-κB pathway [22, 23]. Notably, several previous studies have shown that the expression of MFG-E8 is downregulated by activation of TLR signal *in vitro* and *in vivo*, indicating that there might be negative feedback mutual interaction between TLR signal and MFG-E8 [49, 50]. In the current study, overactivated TLR4/NF-κB signaling pathway, as well as the elevated levels of inflammatory mediators, was observed in the MCD-KO group, suggesting that the effect of MFG-E8 in NASH might be mediated by TLR4/NF-κB signal pathway. Furthermore, using a primary hepatocyte model, we found that specific inhibiting of TLR4 could effectively rescue MFG-E8 knockout-aggravated NASH phenotype. Thus, our data suggested that MFG-E8 knockout promoted hepatic steatosis, inflammation, and fibrosis in MCD-induced NASH, which might be by activation of TLR4/NF-κB signaling pathway.

5. Conclusion

In conclusion, we confirmed that MFG-E8 knockout exacerbated the development of NASH, and the underlying mechanism may be related to the activation of TLR4/NF-κB signaling pathway, which led to hepatic inflammatory cell infiltration and proinflammatory mediator production. Considering the role of MFG-E8 knockout in promoting liver inflammation and fibrosis, it is reasonable to expect that targeting MFG-E8 may be a promising strategy for improving NASH outcome.

Data Availability

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

Ethical Approval

All animal experiments were in line with the Guide for the Animal Care and Use Committee of Chongqing Medical University.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

All the authors contributed to the manuscript and reviewed and approved it as presented here. XRY, JYW, and XG conceived and designed the experiments. JH, HD, YLY, JL, and SWW performed the experiments. PPG and JXY analyzed the data. JH, YLY, JYW, and XG collected the literature and wrote the manuscript. HD, JH, JYW, and XG revised the manuscript. All authors have read and agreed to the published version of the manuscript. All authors read and approved the final manuscript. Jun Hu and Hui Du contributed equally to this work.

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References

[1] Z. M. Younossi, A. B. Koenig, D. Abdelatif, Y. Fazel, L. Henry, and M. Wymer, "Global epidemiology of nonalcoholic fatty liver disease-meta-analytic assessment of prevalence, incidence, and outcomes," *Hepatology*, vol. 64, no. 1, pp. 73–84, 2016.

[2] F. Zhou, J. Zhou, W. Wang et al., "Unexpected rapid increase in the burden of NAFLD in China from 2008 to 2018: a systematic review and meta-analysis," *Hepatology*, vol. 70, no. 4, pp. 1119–1133, 2019.

[3] L. Calzadilla Bertot and L. A. Adams, "The natural course of non-alcoholic fatty liver disease," *International Journal of Molecular Sciences*, vol. 17, no. 5, article 774, 2016.

[4] M. E. Rinella, "Nonalcoholic fatty liver disease," *JAMA*, vol. 313, no. 22, pp. 2263–2273, 2015.

[5] A. Wree, L. Broderick, A. Canbay, H. M. Hoffman, and A. E. Feldstein, "From NAFLD to NASH to cirrhosis–new insights into disease mechanisms," *Nature Reviews. Gastroenterology & Hepatology*, vol. 10, no. 11, pp. 627–636, 2013.

[6] G. C. Farrell, F. Haczeyni, and S. Chitturi, "Pathogenesis of NASH: how metabolic complications of overnutrition favour lipotoxicity and pro-inflammatory fatty liver disease," *Advances in Experimental Medicine and Biology*, vol. 1061, pp. 19–44, 2018.

[7] M. Nati, D. Haddad, A. L. Birkenfeld, C. A. Koch, T. Chavakis, and A. Chatzigeorgiou, "The role of immune cells in metabolism-related liver inflammation and development of non-alcoholic steatohepatitis (NASH)," *Reviews in Endocrine & Metabolic Disorders*, vol. 17, no. 1, pp. 29–39, 2016.

[8] K. Miura, E. Seki, H. Ohnishi, and D. A. Brenner, "Role of toll-like receptors and their downstream molecules in the development of nonalcoholic fatty liver disease," *Gastroenterology Research and Practice*, vol. 2010, Article ID 362847, 9 pages, 2010.

[9] S. Kiziltas, P. Ata, Y. Colak et al., "TLR4 gene polymorphism in patients with nonalcoholic fatty liver disease in comparison to healthy controls," *Metabolic Syndrome and Related Disorders*, vol. 12, no. 3, pp. 165–170, 2014.

[10] B. Liu, X. Deng, Q. Jiang et al., "Scoparone alleviates inflammation, apoptosis and fibrosis of non-alcoholic steatohepatitis by suppressing the TLR4/NF-κB signaling pathway in mice," *International Immunopharmacology*, vol. 75, article 105797, 2019.

[11] J. Yu, C. Zhu, X. Wang et al., "Hepatocyte TLR4 triggers inter-hepatocyte Jagged1/notch signaling to determine NASH-induced fibrosis," *Science Translational Medicine*, vol. 13, no. 599, 2021.

[12] D. Ye, F. Y. Li, K. S. Lam et al., "Toll-like receptor-4 mediates obesity-induced non-alcoholic steatohepatitis through activation of X-box binding protein-1 in mice," *Gut*, vol. 61, no. 7, pp. 1058–1067, 2012.

[13] C. A. Rivera, P. Adegboyega, N. van Rooijen, A. Tagalicud, M. Allman, and M. Wallace, "Toll-like receptor-4 signaling and Kupffer cells play pivotal roles in the pathogenesis of non-alcoholic steatohepatitis," *Journal of Hepatology*, vol. 47, no. 4, pp. 571–579, 2007.

[14] J. Liu, Y. Yuan, X. Gong et al., "Baicalin and its nanoliposomes ameliorates nonalcoholic fatty liver disease via suppression of TLR4 signaling cascade in mice," *International Immunopharmacology*, vol. 80, article 106208, 2020.

[15] K. Asano, M. Miwa, K. Miwa et al., "Masking of phosphatidylserine inhibits apoptotic cell engulfment and induces autoantibody production in mice," *The Journal of Experimental Medicine*, vol. 200, no. 4, pp. 459–467, 2004.

[16] R. Hanayama, M. Tanaka, K. Miwa, A. Shinohara, A. Iwamatsu, and S. Nagata, "Identification of a factor that links apoptotic cells to phagocytes," *Nature*, vol. 417, no. 6885, pp. 182–187, 2002.

[17] M. Jinushi, M. Sato, A. Kanamoto et al., "Milk fat globule epidermal growth factor-8 blockade triggers tumor destruction through coordinated cell-autonomous and immune-mediated mechanisms," *The Journal of Experimental Medicine*, vol. 206, no. 6, pp. 1317–1326, 2009.

[18] S. Y. An, Y. J. Jang, H. J. Lim et al., "Milk fat globule-EGF factor 8, secreted by mesenchymal stem cells, protects against liver fibrosis in mice," *Gastroenterology*, vol. 152, no. 5, pp. 1174–1186, 2017.

[19] D. S. Ko, S. H. Kim, J. Y. Park et al., "Milk fat globule-EGF factor 8 contributes to progression of hepatocellular carcinoma," *Cancers*, vol. 12, no. 2, p. 403, 2020.

[20] T. Shimagaki, S. Yoshio, H. Kawai et al., "Serum milk fat globule-EGF factor 8 (MFG-E8) as a diagnostic and prognostic biomarker in patients with hepatocellular carcinoma," *Scientific Reports*, vol. 9, no. 1, p. 15788, 2019.

[21] L. Zhang, R. Tian, X. Yao et al., "Milk fat globule-epidermal growth factor-factor 8 improves hepatic steatosis and inflammation," *Hepatology*, vol. 73, no. 2, pp. 586–605, 2021.

[22] M. Aziz, A. Jacob, A. Matsuda et al., "Pre-treatment of recombinant mouse MFG-E8 downregulates LPS-induced TNF-α production in macrophages via STAT3-mediated SOCS3 activation," *PLoS One*, vol. 6, no. 11, article e27685, 2011.

[23] R. Wu, W. W. Chaung, M. Zhou et al., "Milk fat globule EGF factor 8 attenuates sepsis-induced apoptosis and organ injury in alcohol-intoxicated rats," *Alcoholism, Clinical and Experimental Research*, vol. 34, no. 9, pp. 1625–1633, 2010.

[24] M. Aziz, A. Matsuda, W. L. Yang, A. Jacob, and P. Wang, "Milk fat globule-epidermal growth factor-factor 8 attenuates neutrophil infiltration in acute lung injury via modulation of CXCR2," *Journal of Immunology*, vol. 189, no. 1, pp. 393–402, 2012.

[25] M. Aziz, W. L. Yang, L. M. Corbo, W. W. Chaung, S. Matsuo, and P. Wang, "MFG-E8 inhibits neutrophil migration through $\alpha v\beta 3$-integrin-dependent MAP kinase activation," *International Journal of Molecular Medicine*, vol. 36, no. 1, pp. 18–28, 2015.

[26] J. C. Cohen, J. D. Horton, and H. H. Hobbs, "Human fatty liver disease: old questions and new insights," *Science*, vol. 332, no. 6037, pp. 1519–1523, 2011.

[27] S. W. Wong and W. K. Chan, "Epidemiology of non-alcoholic fatty liver disease in Asia," *Indian Journal of Gastroenterology*, vol. 39, no. 1, pp. 1–8, 2020.

[28] C. Fujiwara, A. Uehara, A. Sekiguchi et al., "Suppressive regulation by MFG-E8 of latent transforming growth factor β–induced fibrosis via binding to αv integrin: significance in the pathogenesis of fibrosis in systemic sclerosis," *Arthritis & Rheumatology*, vol. 71, no. 2, pp. 302–314, 2019.

[29] M. Kudo, S. M. Khalifeh Soltani, S. A. Sakuma et al., "Mfge8 suppresses airway hyperresponsiveness in asthma by regulating smooth muscle contraction," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 110, no. 2, pp. 660–665, 2013.

[30] K. Atabai, S. Jame, N. Azhar et al., "Mfge8 diminishes the severity of tissue fibrosis in mice by binding and targeting collagen for uptake by macrophages," *The Journal of Clinical Investigation*, vol. 119, no. 12, pp. 3713–3722, 2009.

[31] S. L. Friedman, B. A. Neuschwander-Tetri, M. Rinella, and A. J. Sanyal, "Mechanisms of NAFLD development and therapeutic strategies," *Nature Medicine*, vol. 24, no. 7, pp. 908–922, 2018.

[32] J. Cai, X. J. Zhang, and H. Li, "The role of innate immune cells in nonalcoholic steatohepatitis," *Hepatology*, vol. 70, no. 3, pp. 1026–1037, 2019.

[33] Z. Chen, Y. Yu, J. Cai, and H. Li, "Emerging molecular targets for treatment of nonalcoholic fatty liver disease," *Trends in Endocrinology and Metabolism: TEM*, vol. 30, no. 12, pp. 903–914, 2019.

[34] A. Khalifeh-Soltani, W. McKleroy, S. Sakuma et al., "Mfge8 promotes obesity by mediating the uptake of dietary fats and serum fatty acids," *Nature Medicine*, vol. 20, no. 2, pp. 175–183, 2014.

[35] M. A. Febbraio, S. Reibe, S. Shalapour, G. J. Ooi, M. J. Watt, and M. Karin, "Preclinical models for studying NASH-driven HCC: how useful are they?," *Cell Metabolism*, vol. 29, no. 1, pp. 18–26, 2019.

[36] G. Baffy, "Kupffer cells in non-alcoholic fatty liver disease: the emerging view," *Journal of Hepatology*, vol. 51, no. 1, pp. 212–223, 2009.

[37] R. Xu, H. Huang, Z. Zhang, and F. S. Wang, "The role of neutrophils in the development of liver diseases," *Cellular & Molecular Immunology*, vol. 11, no. 3, pp. 224–231, 2014.

[38] F. Tacke and H. W. Zimmermann, "Macrophage heterogeneity in liver injury and fibrosis," *Journal of Hepatology*, vol. 60, no. 5, pp. 1090–1096, 2014.

[39] F. Marra and F. Tacke, "Roles for chemokines in liver disease," *Gastroenterology*, vol. 147, no. 3, pp. 577–594.e1, 2014.

[40] N. Beraza, Y. Malato, S. Vander Borght et al., "Pharmacological IKK2 inhibition blocks liver steatosis and initiation of non-alcoholic steatohepatitis," *Gut*, vol. 57, no. 5, pp. 655–663, 2008.

[41] W. L. Yang, A. Sharma, F. Zhang et al., "Milk fat globule epidermal growth factor-factor 8-derived peptide attenuates organ injury and improves survival in sepsis," *Critical Care*, vol. 19, no. 1, p. 375, 2015.

[42] M. Miksa, D. Amin, R. Wu et al., "Maturation-induced down-regulation of MFG-E8 impairs apoptotic cell clearance and enhances endotoxin response," *International Journal of Molecular Medicine*, vol. 22, no. 6, pp. 743–748, 2008.

[43] T. Cui, M. Miksa, R. Wu et al., "Milk fat globule epidermal growth factor 8 attenuates acute lung injury in mice after intestinal ischemia and reperfusion," *American Journal of Respiratory and Critical Care Medicine*, vol. 181, no. 3, pp. 238–246, 2010.

[44] A. Alisi, N. Panera, and V. Nobili, "Toll-like receptor 4: a starting point for proinflammatory signals in fatty liver disease," *Hepatology*, vol. 51, no. 2, pp. 714-715, 2010.

[45] F. Bessone, M. V. Razori, and M. G. Roma, "Molecular pathways of nonalcoholic fatty liver disease development and progression," *Cellular and Molecular Life Sciences: CMLS*, vol. 76, no. 1, pp. 99–128, 2019.

[46] T. W. Jung, C. Kang, J. Goh et al., "WISP1 promotes non-alcoholic fatty liver disease and skeletal muscle insulin resistance via TLR4/JNK signaling," *Journal of Cellular Physiology*, vol. 233, no. 8, pp. 6077–6087, 2018.

[47] F. Marra, "Nuclear factor-kappaB inhibition and non-alcoholic steatohepatitis: inflammation as a target for therapy," *Gut*, vol. 57, no. 5, pp. 570–572, 2008.

[48] J. Liu, Z. J. Zhuang, D. X. Bian et al., "Toll-like receptor-4 signalling in the progression of non-alcoholic fatty liver disease induced by high-fat and high-fructose diet in mice," *Clinical and Experimental Pharmacology & Physiology*, vol. 41, no. 7, pp. 482–488, 2014.

[49] M. Jinushi, Y. Nakazaki, M. Dougan, D. R. Carrasco, M. Mihm, and G. Dranoff, "MFG-E8-mediated uptake of apoptotic cells by APCs links the pro- and antiinflammatory activities of GM-CSF," *The Journal of Clinical Investigation*, vol. 117, no. 7, pp. 1902–1913, 2007.

[50] H. Komura, M. Miksa, R. Wu, S. M. Goyert, and P. Wang, "Milk fat globule epidermal growth factor-factor VIII is down-regulated in sepsis via the lipopolysaccharide-CD14 pathway," *Journal of Immunology*, vol. 182, no. 1, pp. 581–587, 2009.

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Review Article

A Mechanistic Insight into the Pathogenic Role of Interleukin 17A in Systemic Autoimmune Diseases

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Interleukin 17A (IL-17A) has been put forward as a strong ally in our fight against invading pathogens across exposed epithelial surfaces by serving an antimicrobial immunosurveillance role in these tissues to protect the barrier integrity. Amongst other mechanisms that prevent tissue injury mediated by potential microbial threats and promote restoration of epithelial homeostasis, IL-17A attracts effector cells to the site of inflammation and support the host response by driving the development of ectopic lymphoid structures. Accumulating evidence now underscores an integral role of IL-17A in driving the pathophysiology and clinical manifestations in three potentially life-threatening autoimmune diseases, namely, systemic lupus erythematosus, Sjögren's syndrome, and systemic sclerosis. Available studies provide convincing evidence that the abundance of IL-17A in target tissues and its prime source, which is T helper 17 cells (Th17) and double negative T cells (DNT), is not an innocent bystander but in fact seems to be prerequisite for organ pathology. In this regard, IL-17A has been directly implicated in critical steps of autoimmunity. This review reports on the synergistic interactions of IL-17A with other critical determinants such as B cells, neutrophils, stromal cells, and the vasculature that promote the characteristic immunopathology of these autoimmune diseases. The summary of observations provided by this review may have empowering implications for IL-17A-based strategies to prevent clinical manifestations in a broad spectrum of autoimmune conditions.

1. Introduction

Interleukin 17A (IL-17A) represents a pleiotropic cytokine that has gained attention as signature cytokine of $CD4^+$ T helper 17 (Th17) cells and has been put forward as critical determinant of psoriasis, a chronic relapsing T cell-mediated inflammatory disorder of the skin, and rheumatic musculoskeletal diseases like psoriatic arthritis and peripheral and axial spondylarthritis. In the last decade, a plethora of effective biological disease-modifying antirheumatic drugs (bDMARD) targeting the IL-23/IL-17A pathway has been developed, consisting of monoclonal antibodies against the common p40 subunit of IL-23/IL-12 and anti-IL-17A and the IL-23 p19 unit. Emerging data now indicates that IL-17A exert a wide range of functions that may be responsible for the development or exacerbation of systemic autoimmune diseases. Given the available data thus far, targeting IL-17A may be considered a novel strategy to prevent clinical manifestations in a broad spectrum of autoimmune conditions. In this review, we will focus on the pathophysiologic role of IL-17A in three major systemic autoimmune diseases including systemic lupus erythematosus (SLE), Sjögren syndrome (SS), and systemic sclerosis (SSC). For the sake of space restrictions, we will not address the role of IL-17A in other autoimmune diseases or rheumatic musculoskeletal diseases.

2. IL-17A: An Introduction to Its Immunological Functions

IL-17A is a front runner in the IL-17 family that comprises the six homologues IL-17A to IL-17F. IL-17A shares the

greatest homology with IL-17F, where IL-17A and IL-17F represent the best studied members of the IL-17-family [1, 2]. Like other proinflammatory cytokines such as TNF, IL-17A displays AU rich repeats in the 3′untranslated region of its messenger RNA (mRNA). IL-17A has been originally identified as a T cell hybridoma-derived molecule CTLA-8 and shares a 57% amino acid sequence homology with a putative protein that was found in the T cell tropic γ-herpesvirus *saimiri* [3, 4]. Amongst the five canonical IL-17 receptors, named IL-17RA to RE, IL-17A signals as a homodimer IL-17A/A via the heterodimeric IL-17RA/RC receptor complex, which may also be engaged by the homodimer IL-17F/F (IL-17F) and the IL-17A/IL-17F heterodimer (IL-17A/F) [1] (see Figure 1). Of note, CD93 has recently been identified as receptor for IL-17D in group 3 innate lymphoid cells [5]. In terms of the hierarchy of activity, the most potent ligand of the IL-17RA/IL-17RC receptor complex is IL-17A/A, followed by IL-17A/F and IL-17F/F. Where the IL-17RA subunit is ubiquitously expressed, IL-17RC expression is restricted to nonhematopoietic epithelials and mesenchymal cell types [2]. Recently, IL-17RD has been suggested to represent an alternative receptor for IL-17A signaling, particularly in mouse and human keratinocytes [6]. The IL-17RA/RC receptor complex is expressed by various cells including fibroblasts, macrophages, epithelial cells, endothelial cells, and astrocytes. Although IL-17RA has been detected on T cells, both primary cells and cell lines, exposure to IL-17A fails to induce the expression of the canonical IL-17 target genes in the absence of the RC receptor unit [7]. The main producers of IL-17A and IL-17F are Th17, as reflected by their signature cytokines, CD4/CD8 double-negative TCRαß$^+$ T cells (DNT; see later) and to a lesser degree cytotoxic CD8$^+$ T cells (mucosal-associated invariant (MAIT) T cells, gamma-delta ($\gamma\delta$) T cells, and innate lymphoid cells like group 3 innate lymphoid cells (ILC3)) [2, 8].

In the last three decades, the immunological functions of IL-17A in the context of different clinical settings have been increasingly elucidated. A large pile of evidence alludes to IL-17A as first defence to preserve the barrier integrity of epithelial organs including skin and respiratory and gastrointestinal tracts to fight against invading pathogens, particularly extracellular bacteria and fungi. Clinical data from human models of defective IL-17A signaling, resulting from genetic, therapeutic, or viral causes, have underpinned its crucial role for the antimicrobial immunity surveillance across exposed surfaces [9–11]. Amongst the primary mechanisms of IL-17A to maintain epithelial barrier integrity is stimulating the production of antimicrobial peptides such as β-defensins and S100A8 (Calgranulin A) and S100A9 (Calgranulin B) that are together expressed as the heterodimer calprotectin. Further, IL-17A may prevent colonic injury and restore intestinal epithelial homeostasis by triggering the expression of tissue plasminogen activator (tPA), with subsequent activation of TGF-β-mediated anti-inflammatory pathways [12]. In the skin, IL-17A has been implicated in physiological wound repair by inducing the expression of regenerating islet-derived protein 3-alpha (REG3A) that promotes proliferation in keratinocytes [13]. Second, IL-17A attracts effector cells to the site of inflammation to help eliminate potential threats and assist in the repair of tissue damage. Illustratively, the IL-17A/G-CSF axis has been involved in regulation of bone marrow granulopoiesis and neutrophil recruitment to the inflammatory site. In this respect, counterregulatory mechanisms involving CXCR2 expression on neutrophils, CXCL5, and commensal bacteria are instated to keep neutrophil homeostasis in check [14]. Third, IL-17A in synergy with IL-13 (in a CXCL13-dependent manner) may support the host response to intracellular pathogens by driving the development of ectopic lymphoid structures, composed of highly organized T cell and B cell zones that emerge during infections with intracellular pathogens such as pneumocystis jiroveci and mycobacterium or in response to inflammatory stimuli [15, 16]. Several of these immune properties of IL-17A have been recapitulated in autoinflammatory disorders and more recently cancer, and on top of that, new features have been uncovered [17, 18]. As for the latter, IL-17A assists in shaping the tumor microenvironment by dampening tumor-specific immune responses involving proangiogenic signals, progressive loss of antitumor Th1 immunity, and suppression of T cell immune surveillance [19, 20]. In addition, IL-17A has been shown to promote protumorigenic factors like proliferation capacity, immune cell infiltration, resistance to chemotoxicity, and migratory and invasive properties [19–26].

3. IL-17A Signaling Pathway

At first sight, IL-17A represents a proinflammatory cytokine that appears to have only modest properties *in vitro* as compared to other cytokines such as TNF and IL-6. Thus, IL-17A signaling initiates a cascade of events that results in transcriptional regulation of a variety of inflammatory RNAs with the release of their corresponding proteins that are dominated by a set of signature genes comprising IL-6, granulocyte colony-stimulating factor (G-CSF), chemokines such as CXCL1-2, CCL20, lipocalin-2 (Lnc2), metalloproteinases, and beta defensins [27, 28]. Adapter molecule Act1 encoded by the gene TRAF3IP2 (also known as CIKS) mediates the downstream signaling of IL-17A and is therefore essential for its transcriptional and posttranslational mechanisms [28]. Act1, containing different TNF receptor-associated factor (TRAF) binding motifs, constitutes a multifunctional platform for various TRAFs, which context-dependently may be recruited to trigger different downstream pathways. Thus, the IL-17R/Act1 complex recruits the adaptor molecule TRAF6 as intermediate component in the signaling cascade, which subsequently results in TGF-β-activated kinase (TAK) 1 phosphorylation and feedforward activation of the canonical NFkB pathway but also the MAPK pathways p38, ERK, or JNK [2, 29]. IL-17A constitutes a weak activator of the NFkB signaling pathway, but instead, IL-17A signaling may activate other downstream targets including transcription factors CCAAT enhancer-binding protein (C/EBP-β), AP-1 complex, and IκBζ (encoded by NFKBIZ) [30]. Act1 can also recruit and interact with TRAF2 and TRAF5 and the splicing regulatory factor SF2 (ASF) to form a complex in order to prolong the stability of inflammatory

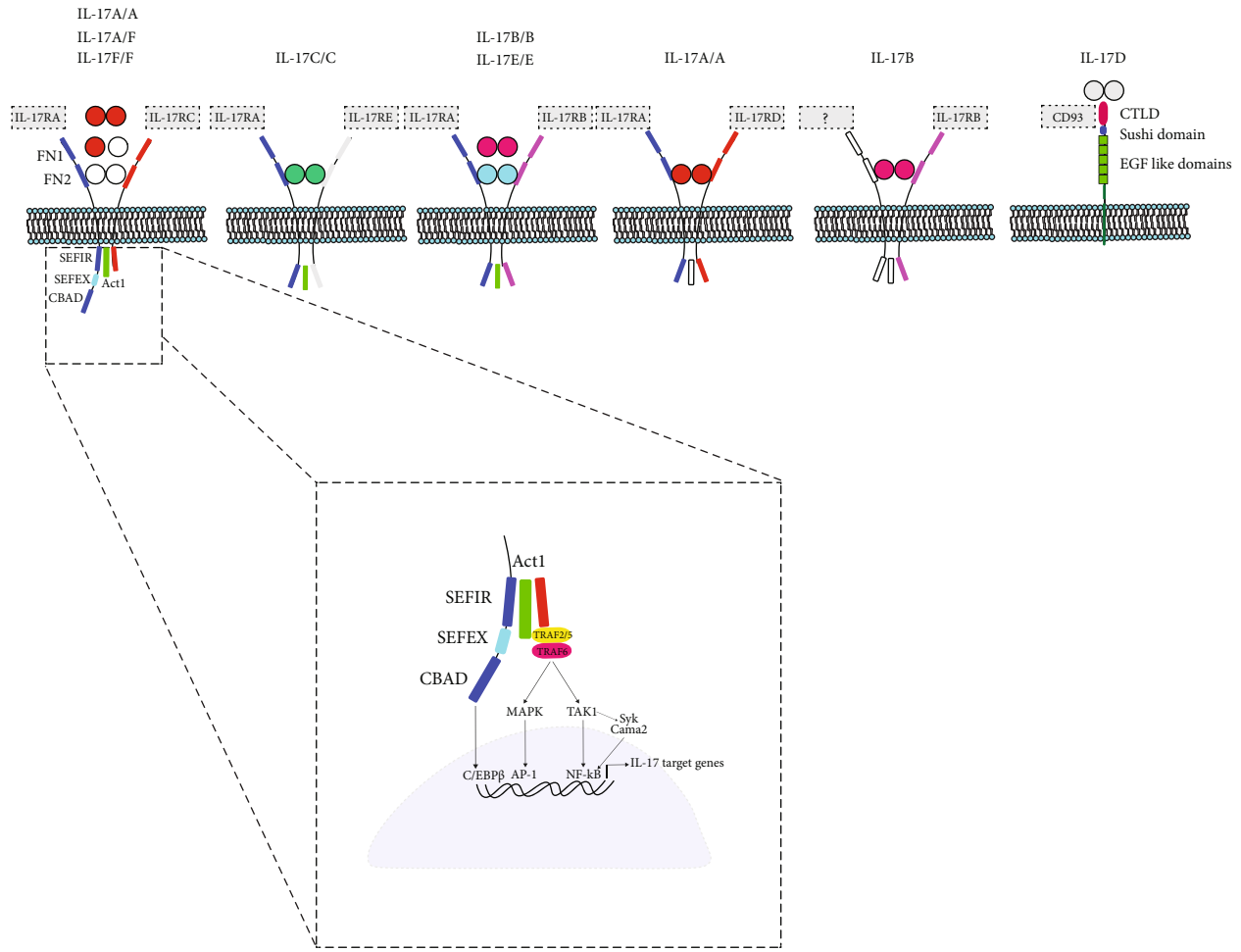

Figure 1: Schematic representation of IL-17 family of cytokines and receptors. IL-17A is the front runner in the IL-17 family of cytokines, comprising six homologues IL-17A to IL-17F in total. The IL-17 receptor family consists of five canonical IL-17 receptors, named IL-17RA to RE; CD93 has recently been identified as receptor for IL-17D. SEFIR: similar expression of fibroblast growth factor and IL-17Rs; SEFEX: SEFIR extension; CBAD: C/EBPβ activation domain.

mRNAs like CXCL1 mRNA (see further) [31]. Other TRAFs like TRAF4 may engage in antagonizing Act1-mediated induction of IL-17A-related inflammatory genes by competing with TRAF6 [32]. Further, Act-1 may suppress IL-23/IL-6-induced STAT3 inhibition as a negative regulator in T and B cells [33].

Engagement of IL-17R with Act1 is mediated via interaction between a "similar expression to fibroblast growth factor genes/IL17R" (SEFIR) domain in the cytoplasmic tail, which is a conserved region amongst all IL-17Rs, and the SEFIR domain present on Act1. The IL-17 target genes are enriched for DNA binding sites of the transcription factors (transcriptional regulatory elements) in their proximal promoter regions [27].

Other functions of Act1 include the E3 ubiquitin ligase activity towards TRAFs that may control their fate and activity in IL-17A signaling, as illustrated by the K63-linked polyubiquitination of TRAF6 mediated by TAK-1 [2, 34, 35]. Lysine-124 residue of TRAF6 has been implicated in Act1-mediated ubiquitination of TRAF6 and TRAF6's ability to mediate IL-17-induced activation of NFkB [36]. A counter-regulatory mechanism is represented by the ubiquitin-specific peptidase 25, a deubiquitinating protease that reverses the modification of TRAF6 and thus decreases TRAF6 assembly to Act-1 by remodeling the K63 polyubiquitin.

In addition, Act1 has been found to suppress pathways mediated by CD40 and BAFF, both members of the TNF receptor (TNFR) superfamily, which play critical roles in B cell survival and differentiation. Thus, Act1 knockout mice develop lymphadenopathy and splenomegaly, hypergammaglobulinemia, and autoantibody formation, where Act1-deficient B cells exhibit stronger IkappaB (IkB) phosphorylation, NFkB2 signaling, and activation of JNK, ERK, and p38 pathways [37]. A similar phenotype was observed in IL-17RA knockout mice on a C57BL/6 *lpr* background [38].

Posttranscriptional regulation that may prolong or shorten RNA stability is another important feature of IL-17A signaling, a capacity that is mediated via mRNA-binding proteins that are seemingly at the crossroads of host

immune response to microbial inflammation and the development of autoimmune disorders. RNA-binding proteins (RBPs), like AT-rich interactive domain-containing protein 5A (arid5a), act on the adenylate/uridylate- (AU-) rich elements (ARE) of the 3′untranslated region (3′UTR) for stabilizing various inflammatory mRNA genes and have specific target genes. Thus, Arid5a has been found to stabilize the IL-6 gene and not that of other cytokines like TNF, via competition with the ribonuclease (RNAse) Regnase I (see further) on the same region in 3′UTR of IL-6 [39, 40]. In addition, Arid5a promotes the stability of the mRNA of CXCL1 and CXCL5 amongst others. Other RBPs that can be recruited to the IL-17R/Act 1 complex via TRAF2 and TRAF5 include DEADbox helicase 3X-linked (DD3X3) and Hu-antigen R (HuR). The latter competes with RNA decay factor splicing factor 2SF2. Through selective stabilization of STAT3, Arid5a may skew differentiation of $CD4^+$ T cells to the Th17 subset [41].

Noticeably, there are counterregulatory mechanism to constrain IL-17-induced inflammation. Regnase-1, also known as zinc finger CCCH-type containing 12A (ZC3H12A) or monocyte chemoattractant protein I-inducing protein (MCP1P1), represents an immune response modifier with RNase activity. Regnase-1 is a cytoplasm localized protein with a CCCH-type zinc finger motif that can be induced through toll-like receptor (TLR) signaling [42]. The RNase activity is mediated via a putative amino-terminal nuclease domain that sets off the decay of a set of inflammatory genes like IL-6, IL-12p40, and calcitonin receptor gene via their 3′UTR [42]. Mice lacking Regnase-1 display a phenotype of autoimmunity that resembles systemic lupus erythematosus (SLE) in humans, including antinuclear antibodies, anti-double-stranded DNA (dsDNA), autoantibodies, hyperglobulinemia, anemia, plasma cell infiltration in lung interstitial tissue, and splenomegaly and lymphadenopathy [42]. A similar phenotype has been found in the lupus-prone Sanroque mice, where a key role for another RING-type ubiquitin ligase protein with a CCCH-type zinc-finger domain Roquin has been established in repressing autoimmunity [43]. Recently, Regnase-1 has been identified as negative regulator of antitumor activity of $CD8^+$ T cells and thereby suppressing their accumulation and mitochondrial fitness by targeting BATF (rheostat) [44]. A recent study has pointed to IL-17-mediated Act1/DDX3X interaction that controls stability of Regnase-1 [45].

Negative inhibitors of IL-17A signaling involve the deubiquitinase zinc-finger protein A20, a key player in the negative feedback regulation of NF-κB pathway, which is mediated via the CEBP beta activity domain (CBAD) [46]. Other negative regulators of IL-17A signaling includes noncoding RNA miR-23B that targets TAB2 and TAB3 and miR30a that induces degradation of Act1 [47, 48]. In certain conditions of inflammation, IL-17A may team up with other proinflammatory mediators like epidermal growth factor, FGF2, and Notch1 [18, 49, 50]. Further, synergistic activities of TNF and IL-17A, involving transcription factors CUX1 and IκBζ (NFKBIZ), have been described in stromal-resident fibroblast-like synoviocytes, resulting in secretion of IL-6 and CXCL8 and neutrophil recruitment [51].

4. The Role of IL-17A in Systemic Lupus Erythematosus

SLE represents a heterogeneous, multicompartment autoimmune disease that may involve the skin, lymphatic network, musculoskeletal system, and internal organs like kidney, lungs, and central nervous system (CNS) [52]. Next to clinical manifestations, SLE features various serological abnormalities including autoantibody formation, hypergammaglobulinemia, hypocomplementemia, and autoimmune-mediated cytopenias. Prototypically, the antinuclear antibodies consist of (1) anti-nucleosome autoantibodies that are directed at DNA, histones, or DNA-histone complex, (2) cytoplasmic proteins, (3) RNA, or (4) U1-small nuclear ribonucleoprotein complex like U1-70 [53]. SLE is associated with long-term morbidity, coexistential disorders like cardiovascular disease, and an increased risk of death.

To date, several studies in young and adult SLE patients have reported on the association of increased serum IL-17A levels or frequencies of IL-17A expressing T cells with disease severity, particularly in those with CNS involvement [52, 54–61]. In this regard, good interpretation of these studies has been flawed by the limited numbers of patients, heterogeneity of disease manifestations, and a large proportion of study subjects on various treatment strategies. Preclinical studies that have hinted at involvement of IL-17A in SLE pathology indicate that the absence of IL-17 in experimental lupus models is associated with inhibition of autoantibody formation targeting DNA, RNP, and chromatin and, even more striking, lupus nephritis [62, 63].

A closer look at the T cell compartment, which exhibits various anomalies in cytokine production and cellular functions in active SLE, may offer insights into the mechanisms underlying these associations. Thus, patients with active SLE display a marked expansion of IL-17A-expressing T cell subpopulations comprising $CD4^+$ Th17 cells and DNTs [57, 64, 65]. Normally, DNTs take up 1% to 2% of the total T cell pool in peripheral blood and lymph nodes (LN) of healthy donors [66]. These IL-$17A^+$ subpopulations are antigen specific, given that tetramer studies have identified RORγt^+ IL-17A-producing T cells that are specific for U1-70 in humans and lupus-prone mice [67]. Similar to Th17 cells, DNTs express RORγt, the master regulator of the Th17 lineage, and IL-23R [57, 64]. In lupus-prone mice, IL-23 has been identified as an important driver of DNT expansion and IL-17A production [68]. Phenotypically, peripheral DNTs exhibit extraordinary migratory and tissue invasive properties, which may result in severe organ pathology in various inflammatory settings like ischemic stroke and spondyloartropathy [57, 69, 64, 70, 71]. Their origin is not completely understood; however, earlier studies have suggested that DNTs originate from the thymus and spleen. Recently, splenic marginal zone macrophages (MZMs) have been implicated as regulators of DNT development. Upon experimental depletion of MZMs, the compartment of autoreactive $CD8^+$ T cells expand and lose their CD8 expression to adopt the DNT phenotype including loss of regulatory properties, enhanced migratory potential, IL-17A-producing

potency, hyperproliferative state, and a narrowed TCR repertoire [72]. Another regulator of DNT development that has been suggested is Act1 [33]. The expansion of splenic DNTs instigates hallmark symptoms of SLE in lupus-prone mice including the emergence of germinal centers (GC), generation of anti-double-stranded DNA (dsDNA) autoantibodies, and inflamed kidney characterized by infiltration of DNTs [72].

Apart from the peripheral blood compartment, IL-17A-expressing T cells have been detected in various SLE-related target tissues. Thus, IL-17A-producing cells particularly DNTs have been shown to invade inflamed kidneys of lupus nephritis patients (see Figure 2) [64]. In more detail, infiltrating IL-17A-expressing T cells gather in close proximity to blood, which has been shown in vascular beds of skin and lungs [57]. In pediatric SLE patients with pulmonary involvement, these IL-17A$^+$ T cells have been postulated to exert direct adverse effects on airway smooth muscle remodeling worsening small airway obstruction [59, 73]. Their presence seems to be a prerequisite for organ pathology SLE. Thus, regression of DNT presence in the lupus-prone B6/lpr mice, as mediated by IL-23R deficiency, was associated with mitigation of lymphoid hyperplasia and suppression of the development of lupus nephritis [74].

Based on the available evidence from preclinical mouse models, the contribution of IL-17A to the disease pathology in SLE appears to be reflected by four different mechanisms.

First, IL-17A mediates the recruitment of effector cells like neutrophils, IL-17A$^+$-expressing T cell subsets, and CCR6$^+$ B cells into SLE target tissues and GCs [75]. Second, IL-17A may represent a driving force behind autoimmunity [76]. In the BXD2 mouse model that recapitulates many SLE features like enhanced activation-induced cytidine deaminase (AICDA) activity, autoantibody generation, circulating immune complexes, and progressive glomerulonephritis, IL-17A has been shown to induce and stabilize autoreactive GC formation via B cell retention within GCs and increased CXCL12/CXCR4-mediated interactions between B cells and T cells resulting in AICDA upregulation and autoantibody generation [77–79]. Further, IL-17A may promote class switching to IgG2a and IgG3, plasma cell development, and MHC class II expression on B cells, whereas DNT derived from patients with SLE have been found to directly promote cationic IgG antibodies against DNA in coculture [78–80].

Third, IL-17A may play a role in enhanced vascular-immune interactions. Thus, endothelial activation upon exposure to IL-17A derived from PBMC of patients with active SLE promotes the adherence of Jurkat cells to vascular endothelium, which is mediated by augmented endothelial expression of E-cadherin, ICAM-1, and VCAM-1 [57]. The last mechanism refers to the T cell-neutrophil interaction as partners in crime. The release of neutrophil extracellular trap (NET) formation has been implicated in the pathogenesis and organ injury in SLE, which is driven by increased REDD1/autophagy axis [81]. Interestingly, depositions of NET in actively inflamed skin and kidney have been found to colocalize with bioactive tissue factor and IL-17A that in a synergistic manner may promote fibrotic activity in the stromal cell compartment [81]. In Fc gamma receptor IIb-(Fcgr2b-) deficient mice that develop fatal lupus pathology, IL-17A/Act1 signaling has been shown to adversely affect the course of glomerulonephritis by promoting the recruitment of immune cells in particular neutrophils and NET deposition in inflamed kidneys [79].

The external and intrinsic factors that enable Th17 and DNT subpopulations to successfully invade target tissues and promote SLE pathogenesis are ample. Aside from the inflammatory microenvironmental milieu that is enriched with chemoattractants like CCL20, endothelium-derived CD95 expression has been shown to promote infiltration of IL-17A-expressing T cells into the perivascular space in a PI3K- and calcium signaling-dependent manner [82]. The chemokine receptor CCR6, a nonpromiscuous receptor with as sole ligand C-C motif chemokine ligand 20 (CCL20), has been found to a play a key role in the trafficking of Th17 cells to the inflamed kidney in experimental lupus nephritis [83]. In this respect, the serine/threonine kinase calcium/calmodulin-dependent kinase IV may promote CCR6 expression in IL-17A-expressing T cells, as well as CCL20 secretion that recruits other CCR6$^+$ T cells through a positive feedback mechanism that may propagate tissue inflammation and accelerate glomerular injury in the inflamed kidney [60]. In children with lupus nephritis, enhanced migratory activities of IL-17A-expressing T cell subsets have been linked to enhanced Akt signaling [61]. Moreover, expansion of IL-17A-expressing T cells has been ascribed to heightened intrinsic activity of the nonreceptor phosphatase (PTP) protein tyrosine phosphatase SH2 domain-containing PTP (SHP2) in humans and mice [84]. Using adoptive transfer studies, fate reporter mice, and mouse models of lupus nephritis, kidney-infiltrating Th17 cells have been found to display very limited spontaneous plasticity, where Th17 cells usually show high degree of plasticity to transdifferentiate into other T cell phenotypes upon inflammatory stimuli [85]. Last, SLE may be associated with functional impairment of CD147 (basigin), an extracellular matrix metalloproteinase inducer (EMMPRIN), which may act as a brake on the disproportional expansion of Th17 cells [86].

Enhanced activity of IL-17A-expressing T cell subsets has been ascribed to increased ROCK activity [87, 88]. In addition, SLE T cells exhibit augmented expression of signaling lymphocyte activation molecules (SLAMs). Particularly, expression of SLAMF6 and 3 has been associated with superior costimulatory activity *in vitro*, as compared to CD3/CD28 [89, 90]. Inversely, SLAMF1 ligation in cocultures of B and T cells may reduce IL-17A and IL-21 production [91]. Another mechanism involves synergistic activity between enhanced recruitment of RORγt to the IL-17A promoter and CD28-induced nuclear abundance of the transcription factor nuclear factor of activated T cells (NFAT) [92]. The latter can be dampened by dipyridamole, a recently recognized specific inhibitor of calcineurin–NFAT interactions [93]. Further, upregulated expression of ubiquitin-specific protease 17 (USP17) in CD4$^+$ T cells from SLE patients has been found to prolong RORγt-dependent IL-17A transcription by increasing the stability of RORγt

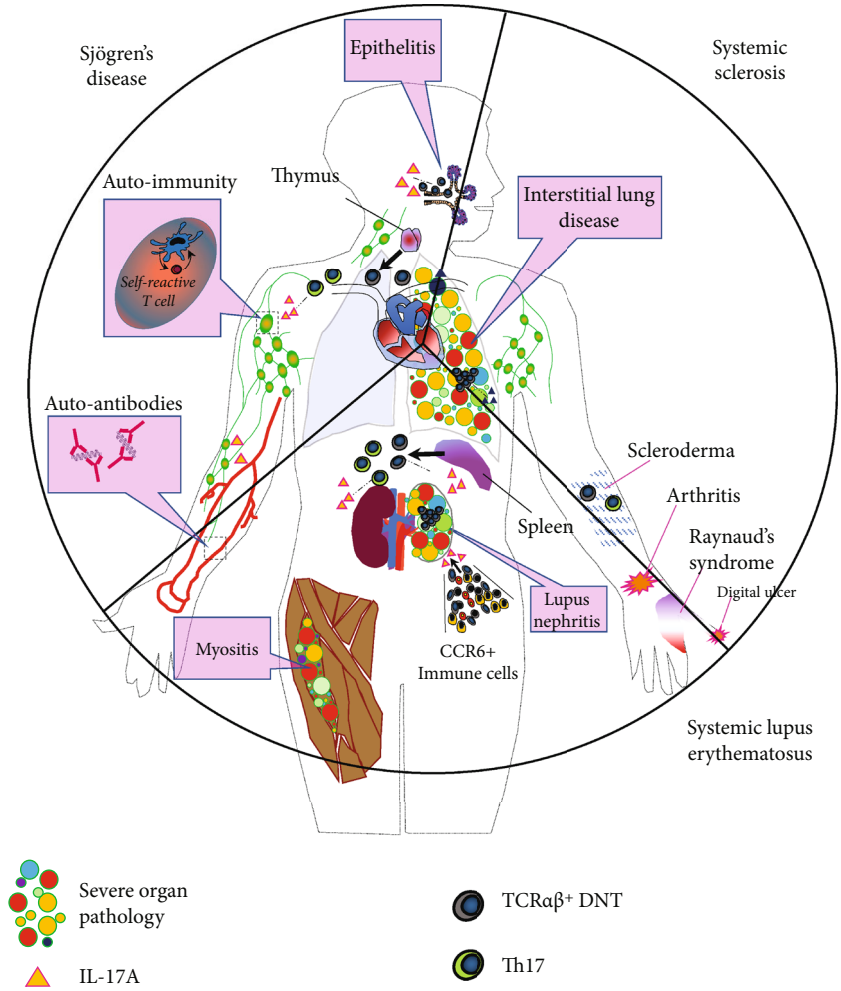

Figure 2: Schematic representation of the contributive role of IL-17A in the pathogenesis of three major systemic autoimmune diseases. Growing evidence suggests that the abundance of IL-17A and its prime source, i.e., Th17 cells and DNTs, in the target tissues may deteriorate clinical and immunological patterns in any of these autoimmune disorders by promoting (1) autoimmunity, immune cell recruitment, and vascular-immune interactions in SLE, (2) induction of autoantigen expression and undermining of endothelial integrity/barrier function in SS, and (3) fibrogenesis (indirect mechanism) in myofibroblast precursors and vasculopathy in SSC. Th17 and DNTs, originating from the spleen and thymus, display excellent properties to infiltrate disease-associated organs, which in concert with tissue-derived factors ensure coordinated temporal-spatial distribution as well as activation of IL-17A-expressing T cells within lymphoid and nonlymphoid tissues.

and preventing it from proteosomal degradation [94]. Another modifier that has been identified to augment IL-17A production is transcription factor friend leukemia integration 1 (Fli-1) that regulates the expression of numerous cytokines and chemokines [95]. Last, epigenetic mechanisms that have been implicated in SLE pathogenesis involve cAMP response modulator (CREM)α that mediates demethylation of IL-17A promoter and trans-repression of the IL-2 gene, resulting in enrichment of effector memory T cell phenotypes [65]. In juvenile onset lupus, CREMα has been recognized to drive increased IL-17A expression and reduced IL-2 production in $CD4^+$ T cells [96].

5. The Role of IL-17A in Sjögren's Syndrome

The concept for the extent to which IL-17A is involved in the pathogenesis of SS is less developed as compared to SLE, given the restricted amount of clinical and experimental data. SS presents itself primarily with sicca syndrome and exocrine gland dysfunction that results from lymphocytic infiltration into lacrimal and salivary glands (SG). In addition, SS features a constellation of clinical and serological signs, consisting of autoantibody formation, hypergammaglobulinemia, fatigue, arthritis, cutaneous manifestations, and increased risk for malignant lymphomas [97]. The prototypical autoantibodies target Ro/SSa (two subunits, 52 kDa and 60 kDa) and La/SSb antigens and may be detected up to ~5 years before diagnosis [98, 99]. Ro52 constitutes an E3 ligase that belongs to the tripartite motif family and has been implicated in the transcriptional regulation of proinflammatory cytokines like IL-17A given its RING-dependent E3 ligase activity [100]. Also, Ro52 regulates several members of the interferon regulatory factor (IRF) family like IRF3 that suppress IL-17A and IL-23R expression by holding off

RORγt from accessing corresponding DNA-binding sites/enhancer regions [101, 102]. In a Ro52 reporter (Rho-deficient) mouse strain, where the Ro52 locus is replaced by GFP, tissue-specific enrichment of Ro52 protein expression is detectable in lymphoid tissues, including spleen, LNs, and thymus, which corresponds with the clinical picture of SS [100]. Ro60 represents a RNA-binding protein that has been implicated in environmental stress, and its loss has been associated with photosensitivity and cutaneous lesions in SLE [103].

Several exploratory studies in SS patients and preclinical mouse models have reported on the association between IL-17A and SS pathology, including increased IL-17A levels in serum and in target tissue SGs and the lacrimal system [104–110]. Moreover, histological examination of SGs of patients, suffering from SS, reveals a lymphocytic infiltration, the majority of which are IL-17A-expressing $CD4^+$ T cells and to lesser degree $CD8^+$ T cells (see Figure 2) [111].

Additional confirmation for IL-17A involvement in SS comes from observations in the Ro52-null mice that develop SS-like manifestations comprising dermatitis, autoantibody formation, hypergammaglobulinemia, lymphadenopathy, splenomegaly, and kidney pathology characterized by proteinuria with mesangium and intraglomerular immunoglobulin depositions [100]. Tissue inflammation and the overactive immune system in these mice display an "IL-17A signature," as attested by hyperproliferating LN and spleen cells that spontaneously secrete IL-17A (and related cytokines), which together with a substantial enrichment for IL-17A-expressing T cells in the $CD4^+$ and $CD4^-$ compartments could be jacked up by T cell activation. Conversely, abrogation of the IL-23/IL-17A axis in these Rho52-null mice restores a substantial part of the SS-related pathology [100]. Of note, the effects of IL-17 ablation on SS-like manifestations appear to be more prominent in female animals, suggesting sexual dimorphism [110]. Other lines of evidence that implicates the IL-17A pathway in the pathogenesis of SS derives from adenovirus-mediated delivery studies (IL-17A overexpression) and genetically engineered mouse models (IL-17A entrapment). Thus, adenovirus-induced IL-17A overexpression in SGs of non-susceptible C57Bl/6 gives rise to pathognomonic signs of SS that include decreased saliva production, lymphocytic infiltration in SGs, and positive ANA test with a fine nuclear speckled pattern [112]. Inversely, IL-17A entrapment through a fusion protein that combines IL-17R and a Fc portion (IL-17R:Fc) results in amelioration of the clinical and immunological pattern in established mouse models for SS [109, 113, 114].

Based on currently available data, the contribution of IL-17A to the pathophysiology of SS is reflected by three different mechanisms. First, IL-17A may induce the expression of autoantigens, characteristically that of glandular tissue kallikreins (KLK) which belong to the large KLK family of serine proteases. Thus, glandular KLK13, which is found to be enriched in striated duct cells of SGs [115], shows enhanced expression in SS-like IQI/Jic mice and acts as proliferative stimulus for splenic T cells [116]. Moreover, SG-derived KLK13 and KLK1 exhibit cross-reactivity with autoantibodies in serum of IQI/Jic mice [116]. In a similar manner, KLK1b22 has been found to be upregulated in the SGs of SS-like ERdj5 knockout mice [117]. Noticeably, proteomic analysis of glandular tissues in SS-like Aec1/Aec2 mice that underwent ultrasound-guided adenoviral-mediated IL-17R:Fc gene therapy of the SGs reveals that IL-17A entrapment is associated with reduced expression of KLK1b22 [118].

Second, IL-17A has been implicated in the impairment of the epithelial tight junction (TJ) integrity and barrier function of SGs. In more detail, IL-17A appears to mediate SG tissue damage and salivary dysfunction in NOD and Aec1/Aec2 mice by targeting Claudin-4 and zonula occludens I, both functional and structural components that are crucial to TJs [110, 119].

Third, IL-17A may promote an inflammatory environment within target tissues like SGs through IL-6 expression [111] that may facilitate mononuclear recruitment and infiltration [115] and invigorate the Th17 differentiation program.

DNTs, as in SLE, may play a contributive role in the pathogenesis of SS, given their expansion in the peripheral blood and SG compartments of SS patients (see Figure 2) [70]. Of note, mast cells are considered a potential source for IL-17A, as their numbers, in parallel to IL-17A expression, shrink in SS patients in response to anti-CD20 therapy [120].

Tissue-derived factors that may regulate IL-17A expression in $CD4^-$ and $CD4^+$ T cell compartments include IL-27. Thus, IL-27 display an inhibitory effect on IL-17A secretion in PBMC cultures of SS patients and not RA patients or age-matched healthy donors [121]. Further, induction of experimental sialadenitis in IL-27receptor subunit alpha knockout mice aggravates the formation of ectopic lymphoid structures in SGs, as compared to wild-type mice, a finding that can be restored by IL-17A neutralization [121]. Other local mediators that may control IL-17 expression are the lysophosphatidic acid receptor signaling pathway and retinoic RORα that together with RORγt and IκBζ may promote IL-17A transcription and Th17 differentiation [122, 123].

6. The Role of IL-17A in Systemic Sclerosis

SSC is a multiorgan connective tissue disease that is characterized by high morbidity and mortality related to organ complications like lung fibrosis and pulmonary arterial hypertension [124]. Approximately 1 in 10,000 people appears to be affected globally.

The triad of pathologic changes that defines SSC comprises autoimmunity, vasculopathy, and fibrosis of skin and internal organs. Fibrosis, a hallmark of more advanced SSC disease stages, results from excess deposition of extracellular matrix (ECM) and differentiation of mesenchymal stromal cells like fibroblasts and endothelial cells into myofibroblasts, a key determinant of end-stage SSC pathology [125, 126]. The fibrosis stage in SSC is preceded by an edematous phase that is characterized by mononuclear cell infiltrates in the dermis, comprising plasma cells, IL-13-

producing $CD8^+$ T cells, and Th17 [127, 128], and progressive failure of the locoregional blood and lymphatic vasculature [124]. Consecutively, these events may result in lymphedema with ensuing elastin degeneration, hyperplasia/hypertrophy of adipose tissue, and increase of collagen fibres and fibrous deposits that causes hardening of the skin.

Several association studies in humans and experimental mouse models have hinted at the contributive role of the IL-17A pathway in the pathogenesis of SSC, showcasing increased IL-17A levels (or IL-23 as partner in crime) in serum and affected skin, as well as increased frequencies of $IL\text{-}17A^+$-producing T cells(see Figure 2) [126, 129–133, 125, 134]. In the SSC skin, IL-17A-expressing T cells have been detected throughout the skin, both superficial and deep layers and in close proximity of αSMA-positive myofibroblasts [134]. In these studies, enhanced IL-17A production has been repeatedly associated with early stages of SSC [131, 135]. Outside the skin, augmented IL-17A expression is detectable in lymphocytes, derived from peripheral blood and bronchoalveolar lavage of SSC patients. Involvement of the IL-17A pathway in SSC has been further suggested by a large case-control study involving three different European populations, where polymophisms in the chemokine receptor CCR6 gene have been associated with increased susceptibility to SSC, particularly in patients with an anti-topoisomerase (Scl-70) autoantibody profile [136].

The implication of IL-17A in the pathophysiology of SSC has been fueled by several findings associated with disease progression. The mechanism that has gained increasing attention involves the impact of IL-17A on the fibroblast phenotype and its transition to myofibroblasts. According to findings in two established SSC-like mouse models, the contributive role of IL-17A in the progression of skin fibrosis is reflected by enhanced leucocyte recruitment and a driving force behind the expression of the profibrotic mediators transforming growth factor (TGF) β and connective tissue growth factor (CTGF) in the skin [125]. In addition, these authors and others reveal the capacity of IL-17A to stimulate collagen production in cultured mouse and human skin fibroblasts [125, 126]. However, the concept that IL-17A directly mediates fibrogenesis in the SSC dermal fibroblast has become controversial after conflicting data from more recent work. These studies demonstrate a stimulatory effect on the proliferating response in SSC and control skin fibroblasts, but fail to show enhanced collagen synthesis whether or not in the presence of TGFβ [135, 137, 132, 138, 139]. The discrepancy in results between these studies may be explained by different experimental settings, as well as distinct methods of fibroblast isolation and culture.

Second, IL-17A may assist in shaping the inflammatory milieu within target tissues, through enhancement of immune cell recruitment, tissue migration, and vascular immune interactions via cytokines (e.g., MCP-1, IL-6, and IL-8), chemokine networks (CCL20-CCR6, CXCL12-CXCR4), endothelial adhesion molecules, and metalloproteinases [137, 134, 130, 132, 135]. Synergistic activity of IL-17A and TGFβ in terms of inducing the expression of these inflammatory cues has been reported [138]. A third way involves the negative impact of IL-17A on SSC-associated vasculopathy. In this regard, IL-17A has been shown to exert adverse effects on dermal vascular smooth muscle cells (DVSMCs) that may promote vascular wall fibrosis and microangiopathy [140]. Analogue to pathological changes of the blood vasculature in the skin, progressive loss of lymphatic vessels (rarefaction) has been acknowledged, particularly in the advanced stages of SSC [141]. Besides mechanisms that involve anti-endothelial cell autoantibodies and dysregulated expression of vascular growth factors, IL-17A and TNF are amongst the candidate cytokines that may (potentially in concert) negatively affect lymphatic neovascularization [142, 143].

Like the aforementioned disorders, SSC has been associated with increased numbers of DNTs in peripheral blood, where particularly the Vα and Vβ repertoires seem narrowed in diversity, as compared to $CD4^+$ and $CD8^+$ T cells [144, 145]. Moreover, these T cell subsets with restricted usage of TCR Vβ genes tend to oligoclonally expand in the SSC skin (rather than in the peripheral blood compartment), suggesting an autoantigen-driven process, and this phenomenon ceases in later stages of the disease [146, 147]. Additional anomalies in the peripheral T cell pool that may promote IL-17A expression involve the emergence of $FoxP3^+IL17^+$ T cells with reduced suppressive capacity and higher RORC expression in the regulatory T cell compartment, which may point to increased Treg-to-Th17 transition [148].

Local tissue-derived factors that may promote IL-17A-expressing T cells involve the inducible T cell costimulator-(ICOS-) ICOSL axis. Thus, SS patients, particularly in early stages of SSC, show higher levels of sICOS in serum and increased ICOSL expression in lesional skin, where ICOS costimulation induces the expression of IFNγ and IL-17A as well as profibrogenic cytokines (IL-4) from $CD4^+$ T cells [149].

7. Conclusive Remarks

In conclusion, this review presents evidence of the ability of IL-17A to drive the development and exacerbation of clinical manifestations in three major autoimmune diseases. Available studies provide convincing evidence that the abundance of IL-17A and its prime source, i.e., Th17 cells and DNTs in the target tissues, is not an innocent bystander but in fact seems to be prerequisite for organ pathology. In support, IL-17A has been directly implicated in critical steps of autoimmunity comprising the emergence and stabilization of autoreactive GC formation, AICDA upregulation, class switching to IgG2a and IgG3, and autoantibody generation. In addition, this review reports on the synergistic interactions of IL-17A with other critical determinants such as B cells, neutrophils, stromal cells, and the vasculature that promote the characteristic immunopathology of these autoimmune diseases. The summary of observations provided by this review may have empowering implications for IL-17A-based strategies to prevent clinical manifestations in a broad spectrum of autoimmune conditions.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

References

[1] S. L. Gaffen, "Structure and signalling in the IL-17 receptor family," *Nature Reviews Immunology*, vol. 9, no. 8, pp. 556–567, 2009.

[2] X. Li, R. Bechara, J. Zhao, M. J. McGeachy, and S. L. Gaffen, "IL-17 receptor-based signaling and implications for disease," *Nature Immunology*, vol. 20, pp. 1594–1602, 2019.

[3] E. Rouvier, M. F. Luciani, M. G. Mattei, F. Denizot, and P. Golstein, "CTLA-8, cloned from an activated T cell, bearing AU-rich messenger RNA instability sequences, and homologous to a herpesvirus saimiri gene," *The Journal of Immunology*, vol. 150, pp. 5445–5456, 1993.

[4] Z. Yao, W. C. Fanslow, M. F. Seldin et al., "Herpesvirus Saimiri encodes a new cytokine, IL-17, which binds to a novel cytokine receptor," *Immunity*, vol. 3, pp. 811–821, 1995.

[5] J. Huang, H. Y. Lee, X. Zhao et al., "Interleukin-17D regulates group 3 innate lymphoid cell function through its receptor CD93," *Immunity*, vol. 54, no. 4, pp. 673–686, 2021.

[6] Y. Su, J. Huang, X. Zhao et al., "Interleukin-17 receptor D constitutes an alternative receptor for interleukin-17A important in psoriasis-like skin inflammation," *Science immunology*, vol. 4, 2019.

[7] M. J. Lindemann, Z. Hu, M. Benczik, K. D. Liu, and S. L. Gaffen, "Differential regulation of the IL-17 receptor by gammac cytokines: inhibitory signaling by the phosphatidylinositol 3-kinase pathway," *Journal of Biological Chemistry*, vol. 283, pp. 14100–14108, 2008.

[8] Y. Cai, X. Shen, C. Ding et al., "Pivotal role of dermal IL-17-producing gammadelta T cells in skin inflammation," *Immunity*, vol. 35, pp. 596–610, 2011.

[9] J. M. Brenchley, M. Paiardini, K. S. Knox et al., "Differential Th17 CD4 T-cell depletion in pathogenic and nonpathogenic lentiviral infections," *Blood*, vol. 112, pp. 2826–2835, 2008.

[10] J. D. Milner, J. M. Brenchley, A. Laurence et al., "Impaired T (H)17 cell differentiation in subjects with autosomal dominant hyper-IgE syndrome," *Nature*, vol. 452, pp. 773–776, 2008.

[11] A. Deodhar, P. J. Mease, I. B. McInnes et al., "Long-term safety of secukinumab in patients with moderate-to-severe plaque psoriasis, psoriatic arthritis, and ankylosing spondylitis: integrated pooled clinical trial and post-marketing surveillance data," *Arthritis research & therapy*, vol. 21, p. 111, 2019.

[12] G. E. Kaiko, F. Chen, C. W. Lai et al., "PAI-1 augments mucosal damage in colitis," *Science translational medicine*, vol. 11, 2019.

[13] Y. Lai, D. Li, C. Li et al., "The antimicrobial protein REG3A regulates keratinocyte proliferation and differentiation after skin injury," *Immunity*, vol. 37, pp. 74–84, 2012.

[14] J. Mei, Y. Liu, N. Dai et al., "Cxcr2 and Cxcl5 regulate the IL-17/G-CSF axis and neutrophil homeostasis in mice," *The Journal of clinical investigation*, vol. 122, pp. 974–986, 2012.

[15] Z. Z. Wang, J. Song, H. Wang et al., "Stromal cells and B cells orchestrate ectopic lymphoid tissue formation in nasal polyps," *Allergy*, 2020.

[16] T. Eddens, W. Elsegeiny, M. L. Garcia-Hernadez et al., "_Pneumocystis_ -Driven Inducible Bronchus-Associated Lymphoid Tissue Formation Requires Th2 and Th17 Immunity," *Cell reports*, vol. 18, no. 13, pp. 3078–3090, 2017.

[17] C. Liu, R. Liu, B. Wang et al., "Blocking IL-17A enhances tumor response to anti-PD-1 immunotherapy in microsatellite stable colorectal cancer," *Journal for immunotherapy of cancer*, vol. 9, 2021.

[18] X. Chen, G. Cai, C. Liu et al., "IL-17R-EGFR axis links wound healing to tumorigenesis in Lrig 1(+) stem cells," *Journal of Experimental Medicine*, vol. 216, pp. 195–214, 2019.

[19] B. Dawod, J. Liu, S. Gebremeskel et al., "Myeloid-derived suppressor cell depletion therapy targets IL-17A-expressing mammary carcinomas," *Scientific reports*, vol. 10, p. 13343, 2020.

[20] Y. F. Ma, C. Chen, D. Li et al., "Targeting of interleukin (IL)-17A inhibits PDL1 expression in tumor cells and induces anticancer immunity in an estrogen receptor-negative murine model of breast cancer," *Oncotarget*, vol. 8, pp. 7614–7624, 2017.

[21] L. Benevides, D. M. da Fonseca, P. B. Donate et al., "IL17 promotes mammary tumor progression by changing the behavior of tumor cells and eliciting tumorigenic neutrophils recruitment," *Cancer research*, vol. 75, pp. 3788–3799, 2015.

[22] D. He, H. Li, N. Yusuf et al., "IL-17 promotes tumor development through the induction of tumor promoting microenvironments at tumor sites and myeloid-derived suppressor cells," *The Journal of Immunology*, vol. 184, pp. 2281–2288, 2010.

[23] L. H. Huang, B. H. Zinselmeyer, C. H. Chang et al., "Interleukin-17 drives interstitial entrapment of tissue lipoproteins in experimental psoriasis," *Cell Metabolism*, 2018.

[24] S. Cochaud, J. Giustiniani, C. Thomas et al., "IL-17A is produced by breast cancer TILs and promotes chemoresistance and proliferation through ERK1/2," *Scientific reports*, vol. 3, p. 3456, 2013.

[25] S. Wu, K. J. Rhee, E. Albesiano et al., "A human colonic commensal promotes colon tumorigenesis via activation of T helper type 17 T cell responses," *Nature medicine*, vol. 15, pp. 1016–1022, 2009.

[26] W. J. Chae, T. F. Gibson, D. Zelterman, L. Hao, O. Henegariu, and A. L. Bothwell, "Ablation of IL-17A abrogates progression of spontaneous intestinal tumorigenesis," *Proceedings of the National Academy of Sciences*, vol. 107, pp. 5540–5544, 2010.

[27] F. Shen, Z. Hu, J. Goswami, and S. L. Gaffen, "Identification of common transcriptional regulatory elements in interleukin-17 target genes," *Journal of Biological Chemistry*, vol. 281, pp. 24138–24148, 2006.

[28] Y. Qian, C. Liu, J. Hartupee et al., "The adaptor Act 1 is required for interleukin 17-dependent signaling associated with autoimmune and inflammatory disease," *Nature immunology*, vol. 8, pp. 247–256, 2007.

[29] R. Schwandner, K. Yamaguchi, and Z. Cao, "Requirement of tumor necrosis factor receptor-associated factor (TRAF)6 in interleukin 17 signal transduction," *The Journal of experimental medicine*, vol. 191, pp. 1233–1240, 2000.

[30] M. J. McGeachy, D. J. Cua, and S. L. Gaffen, "The IL-17 family of cytokines in health and disease," *Immunity*, vol. 50, pp. 892–906, 2019.

[31] D. Sun, M. Novotny, K. Bulek, C. Liu, X. Li, and T. Hamilton, "Treatment with IL-17 prolongs the half-life of chemokine CXCL1 mRNA via the adaptor TRAF5 and the splicing-regulatory factor SF2 (ASF)," *Nature immunology*, vol. 12, pp. 853–860, 2011.

[32] J. A. Zepp, C. Liu, W. Qian et al., "Cutting edge: TNF receptor-associated factor 4 restricts IL-17-mediated pathology and signaling processes," *The Journal of Immunology*, vol. 189, pp. 33–37, 2012.

[33] C. J. Zhang, C. Wang, M. Jiang et al., "Act 1 is a negative regulator in T and B cells via direct inhibition of STAT3," *Nature immunology*, vol. 9, p. 2745, 2018.

[34] J. Talreja and L. Samavati, "K63-linked polyubiquitination on TRAF6 regulates LPS-mediated MAPK activation, cytokine production, and bacterial clearance in toll-like receptor 7/8 primed murine macrophages," *Frontiers in immunology*, vol. 9, p. 279, 2018.

[35] C. Garcia-Barcena, N. Osinalde, J. Ramirez, and U. Mayor, "How to inactivate human ubiquitin E3 ligases by mutation," *Frontiers in Cell and Developmental Biology*, vol. 8, p. 39, 2020.

[36] C. Liu, W. Qian, Y. Qian et al., "Act1, a U-box E3 ubiquitin ligase for IL-17 signaling," *Science Signaling*, vol. 2, p. ra63, 2009.

[37] Y. Qian, J. Qin, G. Cui et al., "Act1, a negative regulator in CD40- and BAFF-mediated B cell survival," *Immunity*, vol. 21, pp. 575–587, 2004.

[38] O. B. J. Corneth, F. Schaper, F. Luk et al., "Lack of IL-17 receptor A signaling aggravates lymphoproliferation in C57BL/6 lpr mice," *Scientific Reports*, vol. 9, p. 4032, 2019.

[39] K. Masuda, B. Ripley, R. Nishimura et al., "Arid 5a controls IL-6 mRNA stability, which contributes to elevation of IL-6 level in vivo," *Proceedings of the National Academy of Sciences*, vol. 110, pp. 9409–9414, 2013.

[40] K. Masuda, A. Kimura, H. Hanieh et al., "Aryl hydrocarbon receptor negatively regulates LPS-induced IL-6 production through suppression of histamine production in macrophages," *International Immunology*, vol. 23, pp. 637–645, 2011.

[41] K. Masuda, B. Ripley, K. K. Nyati et al., "Arid5a regulates naive CD4+ T cell fate through selective stabilization of Stat3 mRNA," *Journal of Experimental Medicine*, vol. 213, pp. 605–619, 2016.

[42] K. Matsushita, O. Takeuchi, D. M. Standley et al., "Zc3h12a is an RNase essential for controlling immune responses by regulating mRNA decay," *Nature*, vol. 458, pp. 1185–1190, 2009.

[43] C. G. Vinuesa, M. C. Cook, C. Angelucci et al., "A RING-type ubiquitin ligase family member required to repress follicular helper T cells and autoimmunity," *Nature*, vol. 435, pp. 452–458, 2005.

[44] J. Wei, L. Long, W. Zheng et al., "Targeting REGNASE-1 programs long-lived effector T cells for cancer therapy," *Nature*, vol. 576, pp. 471–476, 2019.

[45] D. Somma, P. Mastrovito, M. Grieco et al., "CIKS/DDX3X interaction controls the stability of the Zc3h12a mRNA induced by IL-17," *The Journal of Immunology*, vol. 194, pp. 3286–3294, 2015.

[46] A. V. Garg, M. Ahmed, A. N. Vallejo, A. Ma, and S. L. Gaffen, "The deubiquitinase A20 mediates feedback inhibition of interleukin-17 receptor signaling," *Science Signaling*, vol. 6, p. ra44, 2013.

[47] Q. Wan, Z. Zhou, S. Ding, and J. He, "The miR-30a negatively regulates IL-17-mediated signal transduction by targeting Traf3ip2," *Journal of Interferon & Cytokine Research*, vol. 35, pp. 917–923, 2015.

[48] S. Zhu, W. Pan, X. Song et al., "The microRNA miR-23b suppresses IL-17-associated autoimmune inflammation by targeting TAB2, TAB3 and IKK-alpha," *Nature medicine*, vol. 18, pp. 1077–1086, 2012.

[49] X. Shao, S. Chen, D. Yang et al., "FGF2 cooperates with IL-17 to promote autoimmune inflammation," *Scientific Reports*, vol. 7, p. 7024, 2017.

[50] C. Wang, C. J. Zhang, B. N. Martin et al., "IL-17 induced NOTCH1 activation in oligodendrocyte progenitor cells enhances proliferation and inflammatory gene expression," *Nature Communications*, vol. 8, p. 15508, 2017.

[51] K. Slowikowski, H. N. Nguyen, E. H. Noss et al., "CUX1 and IkappaBzeta (NFKBIZ) mediate the synergistic inflammatory response to TNF and IL-17A in stromal fibroblasts," *Proceedings of the National Academy of Sciences*, vol. 117, pp. 5532–5541, 2020.

[52] G. C. Tsokos, "Systemic lupus erythematosus," *New England Journal of Medicine*, vol. 365, pp. 2110–2121, 2011.

[53] D. S. Pisetsky and P. E. Lipsky, "New insights into the role of antinuclear antibodies in systemic lupus erythematosus," *Nature Reviews Rheumatology*, vol. 16, pp. 565–579, 2020.

[54] F. B. Vincent, M. Northcott, A. Hoi, F. Mackay, and E. F. Morand, "Clinical associations of serum interleukin-17 in systemic lupus erythematosus," *Arthritis Research & Therapy*, vol. 15, p. R97, 2013.

[55] C. K. Wong, C. Y. Ho, E. K. Li, and C. W. Lam, "Elevation of proinflammatory cytokine (IL-18, IL-17, IL-12) and Th2 cytokine (IL-4) concentrations in patients with systemic lupus erythematosus," *Lupus*, vol. 9, pp. 589–593, 2000.

[56] R. M. Talaat, S. F. Mohamed, I. H. Bassyouni, and A. A. Raouf, "Th1/Th2/Th17/Treg cytokine imbalance in systemic lupus erythematosus (SLE) patients: correlation with disease activity," *Cytokine*, vol. 72, pp. 146–153, 2015.

[57] J. Yang, Y. Chu, X. Yang et al., "Th17 and natural Treg cell population dynamics in systemic lupus erythematosus," *Arthritis & Rheumatism*, vol. 60, pp. 1472–1483, 2009.

[58] M. Edelbauer, S. Kshirsagar, M. Riedl et al., "Activity of childhood lupus nephritis is linked to altered T cell and cytokine homeostasis," *Journal of Clinical Immunology*, vol. 32, pp. 477–487, 2012.

[59] A. Hammad, E. Osman, Y. Mosaad, and M. Wahba, "Serum interleukin-17 in Egyptian children with systemic lupus erythematosus: is it related to pulmonary affection?," *Lupus*, vol. 26, pp. 388–395, 2017.

[60] T. Koga, K. Otomo, M. Mizui et al., "Calcium/calmodulin-dependent kinase IV facilitates the recruitment of interleukin-17-producing cells to target organs through the CCR6/CCL20 axis in Th17 cell-driven inflammatory diseases," *Arthritis & Rheumatology*, vol. 68, pp. 1981–1988, 2016.

[61] S. Kshirsagar, M. Riedl, H. Billing et al., "Akt-dependent enhanced migratory capacity of Th17 cells from children with lupus nephritis," *The Journal of Immunology*, vol. 193, pp. 4895–4903, 2014.

[62] G. Amarilyo, E. V. Lourenco, F. D. Shi, and A. La Cava, "IL-17 promotes murine lupus," *The Journal of Immunology*, vol. 193, pp. 540–543, 2014.

[63] S. A. Summers, D. Odobasic, M. B. Khouri et al., "Endogenous interleukin (IL)-17A promotes pristane-induced systemic autoimmunity and lupus nephritis induced by pristane," *Clinical and Experimental Immunology*, vol. 176, pp. 341–350, 2014.

[64] J. C. Crispin, M. Oukka, G. Bayliss et al., "Expanded double negative T cells in patients with systemic lupus erythematosus produce IL-17 and infiltrate the kidneys," *The Journal of Immunology*, vol. 181, pp. 8761–8766, 2008.

[65] C. M. Hedrich, J. C. Crispin, T. Rauen et al., "cAMP response element modulator alpha controls IL2 and IL17A expression during CD4 lineage commitment and subset distribution in lupus," *Proceedings of the National Academy of Sciences*, vol. 109, pp. 16606–16611, 2012.

[66] K. Fischer, S. Voelkl, J. Heymann et al., "Isolation and characterization of human antigen-specific TCR alpha beta+ CD4(-)CD8- double-negative regulatory T cells," *Blood*, vol. 105, pp. 2828–2835, 2005.

[67] N. H. Kattah, E. W. Newell, J. A. Jarrell et al., "Tetramers reveal IL-17-secreting CD4+ T cells that are specific for U1-70 in lupus and mixed connective tissue disease," *Proceedings of the National Academy of Sciences*, vol. 112, pp. 3044–3049, 2015.

[68] H. Dai, F. He, G. C. Tsokos, and V. C. Kyttaris, "IL-23 limits the production of IL-2 and promotes autoimmunity in lupus," *The Journal of Immunology*, vol. 199, pp. 903–910, 2017.

[69] H. Meng, H. Zhao, X. Cao et al., "Double-negative T cells remarkably promote neuroinflammation after ischemic stroke," *Proceedings of the National Academy of Sciences*, vol. 116, pp. 5558–5563, 2019.

[70] A. Alunno, O. Bistoni, E. Bartoloni et al., "IL-17-producing CD4-CD8- T cells are expanded in the peripheral blood, infiltrate salivary glands and are resistant to corticosteroids in patients with primary Sjogren's syndrome," *Annals of the Rheumatic Diseases*, vol. 72, pp. 286–292, 2013.

[71] J. P. Sherlock, B. Joyce-Shaikh, S. P. Turner et al., "IL-23 induces spondyloarthropathy by acting on ROR-gammat+ CD3+CD4-CD8- entheseal resident T cells," *Nature Medicine*, vol. 18, pp. 1069–1076, 2012.

[72] H. Li, I. E. Adamopoulos, V. R. Moulton et al., "Systemic lupus erythematosus favors the generation of IL-17 producing double negative T cells," *Nature communications*, vol. 11, p. 2859, 2020.

[73] Y. Chang, L. Al-Alwan, P. A. Risse et al., "Th17-associated cytokines promote human airway smooth muscle cell proliferation," *The FASEB Journal*, vol. 26, pp. 5152–5160, 2012.

[74] V. C. Kyttaris, Z. Zhang, V. K. Kuchroo, M. Oukka, and G. C. Tsokos, "Cutting edge: IL-23 receptor deficiency prevents the development of lupus nephritis in C57BL/6-lpr/lpr mice," *The Journal of Immunology*, vol. 184, pp. 4605–4609, 2010.

[75] A. Y. S. Lee, J. L. Bannan, M. J. Adams, and H. Korner, "Expression of CCR6 on B cells in systemic lupus erythematosus patients," *Clinical rheumatology*, vol. 36, pp. 1453–1456, 2017.

[76] Z. Wen, L. Xu, W. Xu, Z. Yin, X. Gao, and S. Xiong, "Interleukin-17 expression positively correlates with disease severity of lupus nephritis by increasing anti-double-stranded DNA antibody production in a lupus model induced by activated lymphocyte derived DNA," *PLoS One*, vol. 8, p. e58161, 2013.

[77] H. C. Hsu, P. Yang, J. Wang et al., "Interleukin 17-producing T helper cells and interleukin 17 orchestrate autoreactive germinal center development in autoimmune BXD2 mice," *Nature immunology*, vol. 9, pp. 166–175, 2008.

[78] M. Mitsdoerffer, Y. Lee, A. Jager et al., "Proinflammatory T helper type 17 cells are effective B-cell helpers," *Proceedings of the National Academy of Sciences*, vol. 107, pp. 14292–14297, 2010.

[79] P. Pisitkun, H. L. Ha, H. Wang et al., "Interleukin-17 cytokines are critical in development of fatal lupus glomerulonephritis," *Immunity*, vol. 37, pp. 1104–1115, 2012.

[80] S. Shivakumar, G. C. Tsokos, and S. K. Datta, "T cell receptor alpha/beta expressing double-negative (CD4-/CD8-) and CD4+ T helper cells in humans augment the production of pathogenic anti-DNA autoantibodies associated with lupus nephritis," *The Journal of Immunology*, vol. 143, pp. 103–112, 1989.

[81] E. Frangou, A. Chrysanthopoulou, A. Mitsios et al., "REDD1/autophagy pathway promotes thromboinflammation and fibrosis in human systemic lupus erythematosus (SLE) through NETs decorated with tissue factor (TF) and interleukin-17A (IL-17A)," *Annals of the Rheumatic Diseases*, vol. 78, pp. 238–248, 2019.

[82] A. Poissonnier, D. Sanseau, M. Le Gallo et al., "CD95-mediated calcium signaling promotes T helper 17 trafficking to inflamed organs in lupus-prone mice," *Immunity*, vol. 45, pp. 209–223, 2016.

[83] J. E. Turner, H. J. Paust, O. M. Steinmetz et al., "CCR6 recruits regulatory T cells and Th17 cells to the kidney in glomerulonephritis," *Journal of the American Society of Nephrology*, vol. 21, pp. 974–985, 2010.

[84] J. Wang, M. Mizui, L. F. Zeng et al., "Inhibition of SHP2 ameliorates the pathogenesis of systemic lupus erythematosus," *The Journal of clinical investigation*, vol. 126, pp. 2077–2092, 2016.

[85] C. F. Krebs, J. E. Turner, H. J. Paust et al., "Plasticity of Th17 cells in autoimmune kidney diseases," *The Journal of Immunology*, vol. 197, pp. 449–457, 2016.

[86] K. Maeda, T. Kosugi, W. Sato et al., "CD147/basigin limits lupus nephritis and Th17 cell differentiation in mice by inhibiting the interleukin-6/STAT-3 pathway," *Arthritis & Rheumatology*, vol. 67, pp. 2185–2195, 2015.

[87] C. Rozo, Y. Chinenov, R. K. Maharaj et al., "Targeting the RhoA-ROCK pathway to reverse T-cell dysfunction in SLE," *Annals of the rheumatic diseases*, vol. 76, pp. 740–747, 2017.

[88] J. Isgro, S. Gupta, E. Jacek et al., "Enhanced rho-associated protein kinase activation in patients with systemic lupus erythematosus," *Arthritis & Rheumatism*, vol. 65, pp. 1592–1602, 2013.

[89] M. Chatterjee, T. Rauen, K. Kis-Toth et al., "Increased expression of SLAM receptors SLAMF3 and SLAMF6 in systemic lupus erythematosus T lymphocytes promotes Th17 differentiation," *The Journal of Immunology*, vol. 188, pp. 1206–1212, 2012.

[90] P. A. Valdez, H. Wang, D. Seshasayee et al., "NTB-A, a new activating receptor in T cells that regulates autoimmune disease," *Journal of Biological Chemistry*, vol. 279, pp. 18662–18669, 2004.

[91] M. P. Karampetsou, D. Comte, A. Suarez-Fueyo et al., "Signaling lymphocytic activation molecule family member 1

engagement inhibits T cell-B cell interaction and diminishes interleukin-6 production and plasmablast differentiation in systemic lupus erythematosus," *Arthritis & Rheumatology*, vol. 71, pp. 99–108, 2019.

[92] M. Chatterjee, C. M. Hedrich, T. Rauen, C. Ioannidis, C. Terhorst, and G. C. Tsokos, "CD3-T cell receptor co-stimulation through SLAMF3 and SLAMF6 receptors enhances RORgammat recruitment to the IL17A promoter in human T lymphocytes," *Journal of Biological Chemistry*, vol. 287, pp. 38168–38177, 2012.

[93] V. C. Kyttaris, Z. Zhang, O. Kampagianni, and G. C. Tsokos, "Calcium signaling in systemic lupus erythematosus T cells: a treatment target," *Arthritis & Rheumatism*, vol. 63, pp. 2058–2066, 2011.

[94] L. Han, J. Yang, X. Wang et al., "The E3 deubiquitinase USP17 is a positive regulator of retinoic acid-related orphan nuclear receptor gammat (RORgammat) in Th17 cells," *Journal of Biological Chemistry*, vol. 289, pp. 25546–25555, 2014.

[95] S. Sato, X. K. Zhang, J. Temmoku et al., "Ets family transcription factor Fli-1 promotes leukocyte recruitment and production of IL-17A in the MRL/Lpr mouse model of lupus nephritis," *Cells*, vol. 9, 2020.

[96] S. R. Hofmann, K. Mabert, F. Kapplusch et al., "cAMP response element modulator alpha induces dual specificity protein phosphatase 4 to promote effector T cells in juvenile-onset lupus," *Journal of Biological Chemistry*, vol. 203, pp. 2807–2816, 2019.

[97] M. Voulgarelis, U. G. Dafni, D. A. Isenberg, and H. M. Moutsopoulos, "Malignant lymphoma in primary Sjogren's syndrome: a multicenter, retrospective, clinical study by the European Concerted Action on Sjogren's Syndrome," *Arthritis & Rheumatism: Official Journal of the American College of Rheumatology*, vol. 42, pp. 1765–1772, 1999.

[98] K. Didier, L. Bolko, D. Giusti et al., "Autoantibodies associated with connective tissue diseases: what meaning for clinicians?," *Frontiers in immunology*, vol. 9, p. 541, 2018.

[99] E. Theander, R. Jonsson, B. Sjostrom, K. Brokstad, P. Olsson, and G. Henriksson, "Prediction of Sjogren's syndrome years before diagnosis and identification of patients with early onset and severe disease course by autoantibody profiling," *Arthritis & Rheumatology*, vol. 67, pp. 2427–2436, 2015.

[100] A. Espinosa, V. Dardalhon, S. Brauner et al., "Loss of the lupus autoantigen Ro52/Trim21 induces tissue inflammation and systemic autoimmunity by disregulating the IL-23-Th17 pathway," *Journal of experimental medicine*, vol. 206, pp. 1661–1671, 2009.

[101] L. Ysebrant de Lendonck, S. Tonon, M. Nguyen et al., "Interferon regulatory factor 3 controls interleukin-17 expression in CD8 T lymphocytes," *Proceedings of the National Academy of Sciences*, vol. 110, pp. E3189–E3197, 2013.

[102] J. Yan, S. P. Pandey, B. J. Barnes, J. R. Turner, and C. Abraham, "T cell-intrinsic IRF5 regulates T cell signaling, migration, and differentiation and promotes intestinal inflammation," *Cell reports*, vol. 31, article 107820, 2020.

[103] D. Xue, H. Shi, J. D. Smith et al., "A lupus-like syndrome develops in mice lacking the Ro 60-kDa protein, a major lupus autoantigen," *Proceedings of the National Academy of Sciences*, vol. 100, pp. 7503–7508, 2003.

[104] G. E. Katsifis, S. Rekka, N. M. Moutsopoulos, S. Pillemer, and S. M. Wahl, "Systemic and local interleukin-17 and linked cytokines associated with Sjogren's syndrome immunopathogenesis," *The American journal of pathology*, vol. 175, pp. 1167–1177, 2009.

[105] Y. Sun, Y. Wang, S. Chen et al., "Expression of Gαq Is Decreased in Lymphocytes from Primary Sjögren's Syndrome Patients and Related to Increased IL-17A Expression," *Journal of Immunology Research*, vol. 2018, 7 pages, 2018.

[106] Y. Fei, W. Zhang, D. Lin et al., "Clinical parameter and Th17 related to lymphocytes infiltrating degree of labial salivary gland in primary Sjogren's syndrome," *Clinical rheumatology*, vol. 33, pp. 523–529, 2014.

[107] M. Fusconi, I. Musy, D. Valente et al., "Immunohistochemical detection of IL-17 and IL-23 improves the identification of patients with a possible diagnosis of Sjogren's syndrome," *Pathology-Research and Practice*, vol. 216, article 153137, 2020.

[108] R. Liu, C. Gao, H. Chen, Y. Li, Y. Jin, and H. Qi, "Analysis of Th17-associated cytokines and clinical correlations in patients with dry eye disease," *PLoS One*, vol. 12, article e0173301, 2017.

[109] C. Q. Nguyen, M. H. Hu, Y. Li, C. Stewart, and A. B. Peck, "Salivary gland tissue expression of interleukin-23 and interleukin-17 in Sjogren's syndrome: findings in humans and mice," *Arthritis & Rheumatism: Official Journal of the American College of Rheumatology*, vol. 58, pp. 734–743, 2008.

[110] A. Voigt, L. Esfandiary, A. Wanchoo et al., "Sexual dimorphic function of IL-17 in salivary gland dysfunction of the C57BL/6.NOD-Aec1Aec2 model of Sjogren's syndrome," *Scientific reports*, vol. 6, p. 38717, 2016.

[111] A. Sakai, Y. Sugawara, T. Kuroishi, T. Sasano, and S. Sugawara, "Identification of IL-18 and Th17 cells in salivary glands of patients with Sjogren's syndrome, and amplification of IL-17-mediated secretion of inflammatory cytokines from salivary gland cells by IL-18," *The Journal of Immunology*, vol. 181, pp. 2898–2906, 2008.

[112] C. Q. Nguyen, H. Yin, B. H. Lee, W. C. Carcamo, J. A. Chiorini, and A. B. Peck, "Pathogenic effect of interleukin-17A in induction of Sjogren's syndrome-like disease using adenovirus-mediated gene transfer," *Arthritis research & therapy*, vol. 12, p. R220, 2010.

[113] C. Q. Nguyen, H. Yin, B. H. Lee, J. A. Chiorini, and A. B. Peck, "IL17: potential therapeutic target in Sjogren's syndrome using adenovirus-mediated gene transfer," *Laboratory investigation*, vol. 91, pp. 54–62, 2011.

[114] X. Lin, K. Rui, J. Deng et al., "Th17 cells play a critical role in the development of experimental Sjogren's syndrome," *Annals of the rheumatic diseases*, vol. 74, pp. 1302–1310, 2015.

[115] M. Uddin, M. Polley-Mandal, and O. U. Beg, "Kallikrein-like prorenin-converting enzymes in inbred hypertensive mice," *Biochemical and biophysical research communications*, vol. 304, pp. 724–728, 2003.

[116] K. Takada, M. Takiguchi, A. Konno, and M. Inaba, "Autoimmunity against a tissue kallikrein in IQI/Jic mice: a model for Sjogren's syndrome," *Journal of Biological Chemistry*, vol. 280, pp. 3982–3988, 2005.

[117] P. Moustardas, N. Yamada-Fowler, E. Apostolou, A. G. Tzioufas, M. V. Turkina, and G. Spyrou, "Deregulation of the kallikrein protease family in the salivary glands of the Sjogren's syndrome ERdj5 knockout mouse model," *Frontiers in immunology*, vol. 12, article 693911, 2021.

[118] C. Wu, Z. Wang, L. Zourelias, H. Thakker, and M. J. Passineau, "IL-17 sequestration via salivary gland gene therapy in a mouse model of Sjogren's syndrome suppresses disease-associated expression of the putative autoantigen Klk1b22," *Arthritis research & therapy*, vol. 17, p. 198, 2015.

[119] L. W. Zhang, X. Cong, Y. Zhang et al., "Interleukin-17 impairs salivary tight junction integrity in Sjogren's syndrome," *Journal of dental research*, vol. 95, pp. 784–792, 2016.

[120] F. Ciccia, G. Guggino, A. Rizzo et al., "Rituximab modulates IL-17 expression in the salivary glands of patients with primary Sjogren's syndrome," *Rheumatology (Oxford).*, vol. 53, pp. 1313–1320, 2014.

[121] D. Lucchesi, R. Coleby, E. Pontarini et al., "Impaired Interleukin-27–Mediated Control of CD 4+ T Cell Function Impact on Ectopic Lymphoid Structure Formation in Patients With Sjögren's Syndrome," *Arthritis & Rheumatology*, vol. 72, no. 9, pp. 1559–1570, 2020.

[122] X. Weng, Y. Liu, S. Cui, and B. Cheng, "The role of RORalpha in salivary gland lesions in patients with primary Sjogren's syndrome," *Arthritis Research & Therapy*, vol. 20, p. 205, 2018.

[123] E. Park, D. Kim, S. M. Lee, and H. S. Jun, "Inhibition of lysophosphatidic acid receptor ameliorates Sjogren's syndrome in NOD mice," *Oncotarget*, vol. 8, pp. 27240–27251, 2017.

[124] C. P. Denton and D. Khanna, "Systemic sclerosis," *Lancet*, vol. 390, pp. 1685–1699, 2017.

[125] Y. Okamoto, M. Hasegawa, T. Matsushita et al., "Potential roles of interleukin-17A in the development of skin fibrosis in mice," *Arthritis & Rheumatism*, vol. 64, pp. 3726–3735, 2012.

[126] X. Yang, J. Yang, X. Xing, L. Wan, and M. Li, "Increased frequency of Th17 cells in systemic sclerosis is related to disease activity and collagen overproduction," *Arthritis research & therapy*, vol. 16, p. R4, 2014.

[127] S. Cascio, T. A. Medsger Jr., W. F. Hawse et al., "14-3-3z sequesters cytosolic T-bet, upregulating IL-13 levels in TC2 and CD8(+) lymphocytes from patients with scleroderma," *Journal of Allergy and Clinical Immunology*, vol. 142, pp. 109–119, 2018.

[128] R. S. G. Goncalves, M. C. Pereira, A. T. Dantas et al., "IL-17 and related cytokines involved in systemic sclerosis: perspectives," *Autoimmunity*, vol. 51, pp. 1–9, 2018.

[129] W. Xu, L. Su, P. Qing et al., "Elevated levels of TL1A are associated with disease activity in patients with systemic sclerosis," *Clinical Rheumatology*, vol. 36, pp. 1317–1324, 2017.

[130] X. Xing, J. Yang, X. Yang et al., "IL-17A induces endothelial inflammation in systemic sclerosis via the ERK signaling pathway," *PLoS One*, vol. 8, article e85032, 2013.

[131] M. Murata, M. Fujimoto, T. Matsushita et al., "Clinical association of serum interleukin-17 levels in systemic sclerosis: is systemic sclerosis a Th17 disease?," *Journal of dermatological science*, vol. 50, pp. 240–242, 2008.

[132] T. Nakashima, M. Jinnin, K. Yamane et al., "Impaired IL-17 signaling pathway contributes to the increased collagen expression in scleroderma fibroblasts," *The Journal of Immunology*, vol. 188, pp. 3573–3583, 2012.

[133] K. Komura, M. Fujimoto, M. Hasegawa et al., "Increased serum interleukin 23 in patients with systemic sclerosis," *The Journal of Rheumatology*, vol. 35, pp. 120–125, 2008.

[134] M. E. Truchetet, N. C. Brembilla, E. Montanari et al., "Interleukin-17A+ cell counts are increased in systemic sclerosis skin and their number is inversely correlated with the extent of skin involvement," *Arthritis & Rheumatism*, vol. 65, pp. 1347–1356, 2013.

[135] K. Kurasawa, K. Hirose, H. Sano et al., "Increased interleukin-17 production in patients with systemic sclerosis," *Arthritis & Rheumatism*, vol. 43, pp. 2455–2463, 2000.

[136] E. Koumakis, M. Bouaziz, P. Dieude et al., "A regulatory variant in CCR6 is associated with susceptibility to antitopoisomerase-positive systemic sclerosis," *Arthritis & Rheumatism*, vol. 65, pp. 3202–3208, 2013.

[137] N. C. Brembilla, E. Montanari, M. E. Truchetet, E. Raschi, P. Meroni, and C. Chizzolini, "Th17 cells favor inflammatory responses while inhibiting type I collagen deposition by dermal fibroblasts: differential effects in healthy and systemic sclerosis fibroblasts," *Arthritis research & therapy*, vol. 15, p. R151, 2013.

[138] A. M. Dufour, M. Alvarez, B. Russo, and C. Chizzolini, "Interleukin-6 and type-I collagen production by systemic sclerosis fibroblasts are differentially regulated by interleukin-17A in the presence of transforming growth factor-beta 1," *Frontiers in Immunology*, vol. 9, p. 1865, 2018.

[139] A. M. Dufour, J. Borowczyk-Michalowska, M. Alvarez et al., "IL-17A dissociates inflammation from fibrogenesis in systemic sclerosis," *Journal of Investigative Dermatology*, vol. 140, pp. 103–112, 2020.

[140] M. Liu, J. Yang, X. Xing, X. Cui, and M. Li, "Interleukin-17A promotes functional activation of systemic sclerosis patient-derived dermal vascular smooth muscle cells by extracellular-regulated protein kinases signalling pathway," *Arthritis research & therapy*, vol. 16, p. 4223, 2014.

[141] A. Akhmetshina, J. Beer, K. Zwerina et al., "Decreased lymphatic vessel counts in patients with systemic sclerosis: association with fingertip ulcers," *Arthritis research & therapy*, vol. 62, pp. 1513–1522, 2010.

[142] R. Bisoendial, F. Tabet, P. P. Tak et al., "Apolipoprotein A-I limits the negative effect of tumor necrosis factor on lymphangiogenesis," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 35, pp. 2443–2450, 2015.

[143] H. J. Park, C. M. Yuk, K. Shin, and S. H. Lee, "Interleukin-17A negatively regulates lymphangiogenesis in T helper 17 cell-mediated inflammation," *Mucosal Immunology*, vol. 11, pp. 590–600, 2018.

[144] T. Sumida, A. Sakamoto, H. Murata et al., "Selective reduction of T cells bearing invariant V alpha 24J alpha Q antigen receptor in patients with systemic sclerosis," *The Journal of experimental medicine*, vol. 182, pp. 1163–1168, 1995.

[145] A. Sakamoto, T. Sumida, T. Maeda et al., "T cell receptor V beta repertoire of double-negative alpha/beta T cells in patients with systemic sclerosis," *Arthritis & Rheumatism: Official Journal of the American College of Rheumatology*, vol. 35, pp. 944–948, 1992.

[146] K. P. Tiev, J. Abriol, M. C. Burland et al., "T cell repertoire in patients with stable scleroderma," *Clinical & Experimental Immunology*, vol. 139, pp. 348–354, 2005.

[147] R. De Palma, F. Del Galdo, S. Lupoli, P. Altucci, G. Abbate, and G. Valentini, "Peripheral T lymphocytes from patients with early systemic sclerosis co-cultured with autologous fibroblasts undergo an oligoclonal expansion similar to that occurring in the skin," *Clinical & Experimental Immunology*, vol. 144, pp. 169–176, 2006.

[148] X. Liu, N. Gao, M. Li et al., "Elevated levels of CD4(+)CD25(+)FoxP3(+) T cells in systemic sclerosis patients contribute to the secretion of IL-17 and immunosuppression dysfunction," *PLoS One*, vol. 8, p. e64531, 2013.

[149] M. Hasegawa, M. Fujimoto, T. Matsushita, Y. Hamaguchi, and K. Takehara, "Augmented ICOS expression in patients with early diffuse cutaneous systemic sclerosis," *Rheumatology (Oxford)*, vol. 52, pp. 242–251, 2013.

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Research Article

Interleukin-19 Aggravates Pulmonary Fibrosis via Activating Fibroblast through TGF-β/Smad Pathway

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Background. Idiopathic pulmonary fibrosis (IPF) is a progressive and fatal interstitial pneumonia disease with no cure. Communication between injured cells is triggered and maintained by a complicated network of cytokines and their receptors. IL-19 is supported by increasing evidences for a deleterious role in respiratory diseases. However, its potential role in lung fibrosis has never been explored. *Methods*. Bioinformatic, immunohistochemistry and western blot analysis were used to assess the expression of IL-19 in human and mouse fibrosis lung tissues. CCK-8, transwell and flow cytometry assay were utilized to analyze the effect of IL-19 on biological behaviors of lung fibroblasts. Histopathology was used to elucidate profibrotic effect of IL-19 in vivo. *Results*. IL-19 was upregulated in fibrosis lung tissues. IL-19 promoted lung fibroblasts proliferation and invasion, inhibited cell apoptosis, and induced differentiation of fibroblasts to the myofibroblast phenotype, which could be revised by LY2109761, a TGF-β/Smad signaling pathway inhibitor. Furthermore, we found that IL-19 aggravated lung fibrosis in murine bleomycin-induced lung fibrosis. *Conclusions*. Our results imply the profibrotic role for IL-19 through direct effects on lung fibroblasts and the potential of targeting IL-19 for therapeutic intervention in pulmonary fibrosis.

1. Introduction

Idiopathic pulmonary fibrosis (IPF) is an age-related and progressive disease with no cure. Incidence of IPF has risen over time. It is reported to 2.8–18/100000 people per year in Europe and North America while 0.5–4.2/100000 in Asia and South America [1]. Mortality of IPF is very high, with a median survival time of 2–4 years from diagnosis [2]. IPF is a predominant type of interstitial lung disease (ILD), characterized by chronic inflammation and interstitial fibrosis. Genetic susceptibility, environmental risk factors and exposures can cause repetitive local microinjuries to the lung tissue and the vascular system, which can trigger a cascade of inflammatory responses and fibrosis [3]. Chronic inflammation is considered as a common hallmark of fibrosis diseases [4]. Injured intrinsic and immune cells contribute to the sustainment of chronic inflammation and augment extracellular matrix (ECM) generation through releasing widespread inflammatory cytokines and growth factors [5].

IL-19, a member of the IL-10 cytokine family, is generated by immune cells, epithelial cells, and vascular structural cells [6]. The function of IL-19 is confusing and often contradictory depending on the organization and disease [7]. IL-19 plays multiple roles in several human diseases and their animal models, such as cardiovascular disease [8], inflammatory bowel disease [9], psoriasis [10], rheumatoid arthritis [11], acute kidney injury [12], and breast cancer [13]. In respiratory diseases, IL-19, which is reported involved in inflammatory responses and causing pulmonary injury by activating lung epithelial cells [14], is positively associated with the progression of asthma [15] and chronic obstructive pulmonary disease (COPD) [16]. The immunoregulatory cytokine IL-19 holds promise as new treatment and prevention [17]. Targeting the IL-19 signaling might

be a new target for therapeutic intervention in chronic asthma [15]. However, the impact of IL-19 on the development of IPF has never been explored.

Here, we investigated IL-19 expression in human and mouse lung fibrosis tissues and the effect of IL-19 on lung fibroblasts as well as the possible mechanism. Then, we focused on role of IL-19 on wild-type mice and bleomycin(BLM)-induced pulmonary fibrosis mouse models. Overall, our study highlights the role of IL-19 on pulmonary fibrosis in vitro and vivo and proposes a new insight for future research and provides a promising management strategy for treating pulmonary fibrosis.

2. Materials and Methods

2.1. Bioinformatics Analysis. Gene expression profiles for two datasets (GSE77326, GSE2051) were obtained from the Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo/). The mRNA samples were obtained from GSE2051, comprising of 11 lung tissues of patients with IPF and 13 normal lung tissues samples, and GSE77326, comprising of 6 bleomycin instilled mouse lung tissues and 6 Sham group mouse lung tissues. Samples were subjected to gene expression profiling to determine the different expression profiles between IPF and normal lung tissues. The data sets were processed using the GeoR2 software.

To identify significantly dysregulated biological pathways of IL-19 in IPF, the GSEA was performed by GSEA 4.0 (http://www.gsea-msigdb.org/gsea/index.jsp) under functional annotations of the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (https://www.genome.jp/kegg/). All the genes in each dataset were submitted to cluster profiler, with the permutation number and the minimum gene set size set as 10000 and 10, respectively. The significance level was set as $FDR < 0.05$.

2.2. Primary Mouse Lung Fibroblasts Isolation and Cell Culture. Primary mouse lung fibroblasts were prepared as follows [18]: (1) lung tissues obtained from 6–8 weeks old C57BL/6 mice were perfused with 10–20 ml PBS into the right ventricle until the lungs were blood flushed and had a white appearance. (2) The tissue was cut into very small pieces using surgical scissors and then incubated with 0.5 ml of collagenase I (final concentration, 1000 U/ml) (Biofroxx) at 37°C for 30 min. (3) Centrifuge at 1000 g for 5 min, discard the supernatant. (4) Then add 500 μl 0.25% trypsin-EDTA to digest tissues at 37°C for 10 min. (5) After centrifuge, plate the suspension into a 100 mm cell culture dish and adherent cells for 1 hour at 37°C, then discard the supernatant.

The human embryo lung fibroblast cell (HELF) was purchased from iCell Bioscience (Shanghai, China). All cells were cultured in DMEM (11995-065, Gibco) supplemented with 10% fetal bovine serum (FBS, 10099141, Gibco) and 1% penicillin-streptomycin (SV30010, Hyclone) in an atmosphere at 37°C and 5% CO_2. Human and mouse IL-19 and TGF-β1 were purchased from GenScript (Nanjing, China). LY2109761 was obtained from Selleck Chemicals (Houston, USA), solubilized in dimethyl sulfoxide (DMSO).

2.3. CCK-8 Assay. The abilities of cell proliferation were assessed by CCK-8 assay (HY-K0301, MCE). HELF and primary mouse lung fibroblasts were placed into plates and cultivated in incubator for 0 h, 24 h, 48 h, and 72 h. Then cells were interacted with CCK-8 solution at 37°C for 2–4 h, and the absorbance at 450 nm was measured.

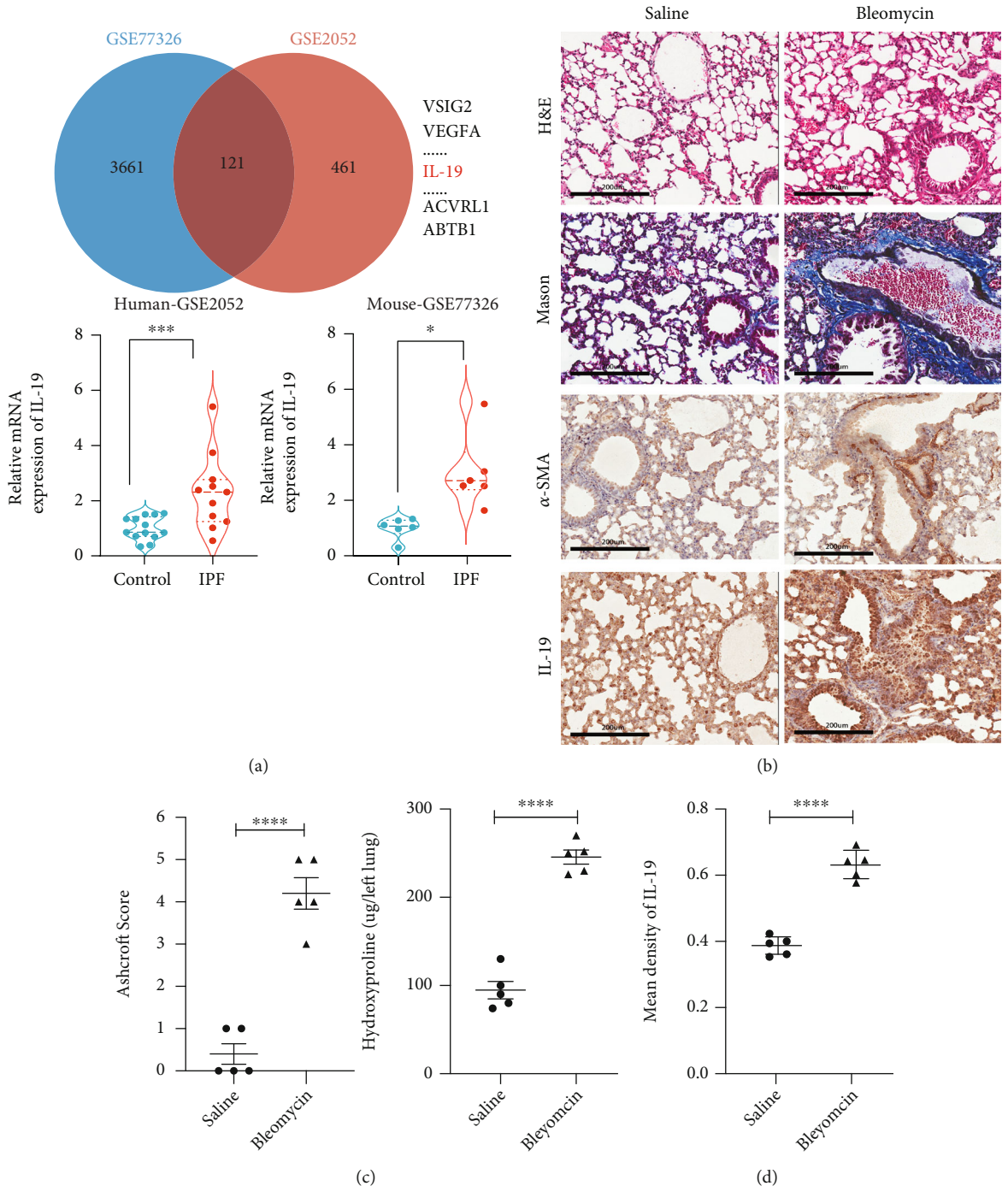
2.4. Transwell Assay. The abilities of cell invasion were evaluated by transwell assay. Cells were added to the upper chambers (Corning, USA) and incubated with stimulation for 72 h while the lower chambers were incubated with DMEM medium containing 10% FBS. Fixed cells migrating into the lower chamber with methanol and stained with crystal violet (C0121, Beyotime). After washed by PBS, cells migrating through the membrane were stained and counted by microscopy (Nikon).

2.5. Apoptosis Assay. Apoptosis was determined by Annexin V-FITC staining and analyzed by flow cytometry. The apoptosis of fibroblasts were evaluated at baseline and after treatment with H_2O_2 and IL-19. The Annexin V-FITC/PI apoptosis detection kit (A211-02, Vazyme) was used to evaluate the ratio of apoptotic fibroblasts, and the apoptosis was assessed by FACS Calibur Flow cytometer.

2.6. Western Blot Assay. Tissues and cells were lysed with RIPA buffer containing protease inhibitors. Load and separate equal amounts of proteins on SDS-PAGE gels. Transfer the proteins to a PVDF membrane after electrophoresis, block membrane in 5% skimmed milk and incubate overnight with the primary antibody (IL-19 (ab154187, Abcam), α-SMA (ab7817, Abcam), Collagen I (ab260043, Abcam), TGF-β1 (ab215715, Abcam), Smad2/3 (ab202445, Abcam), pSmad3 (ab52903, Abcam), and GAPDH (60004-1-Ig, Proteintech)). Wash the membrane three times with 0.1% tween phosphate buffer solution (PBST) for 10 minutes each time and then incubate the membrane with goat anti-mouse or anti-rabbit for one hour at room temperature. Protein bands were analyzed by ImageJ software. The relative grey values of the target protein to the GAPDH bands were calculated to determine the change in protein expression.

2.7. Immunofluorescence. Cells were fixed with 4% paraformaldehyde for 15 minutes, permeabilized with Triton X-100 (1139ML100, Biofroxx) for 20 minutes, blocked with 1% BSA (4240GR100, Biofroxx) for 30 minutes, and incubated overnight with specific primary antibody. Thereafter, probe cells with conjugated goat anti-mouse IgG ($H + L$) (SA00013-1, Proteintech) or rabbit IgG ($H + L$) (SA00013-2, Proteintech) at room temperature in the dark for one hour. Counterstain cell nuclei with DAPI (C1002, Beyotime). Then observe cells under a fluorescent microscope.

2.8. Induction of Pulmonary Fibrosis In Vivo. Male C57BL/6 mice weighing 18–22 g were divided into 4 groups: (a) saline group ($n = 6$); (b) BLM ($n = 8$) group; (c) IL-19 ($n = 8$) group; (d) $BLM + IL - 19$ group ($n = 10$). On day 0, mice were anaesthetized by Avertin (125 mg/kg, i.p.). After sterilizing the neck using betadine, make a 1 cm midline incision with sterile scissors and insert the microinjector into the



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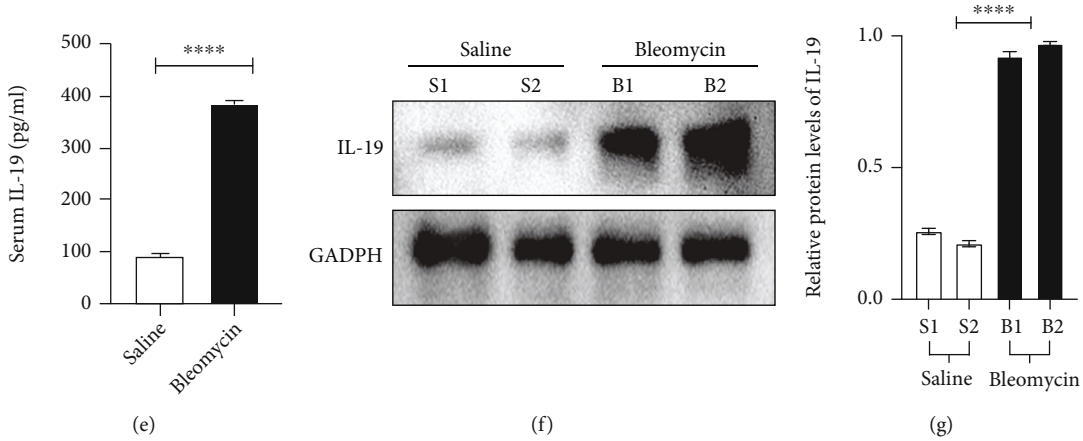


Figure 1: Expression of IL-19 is upregulated in human and mouse lung fibrosis tissues. (a) The gene expression profiles of two independent datasets (GSE77326, GSE2051) were used to assess the differential gene expression in lung fibrosis tissues. 121 gene expressions were higher both in human and mouse lung fibrosis tissues than controls, and mRNA expression of IL-19 in IPF was significantly higher than that in control. (b) Representative images (taken at ×20 magnification) of H&E, Masson's trichrome, and immunohistochemical staining of IL-19 and α-SMA in BLM-induced murine lung fibrosis tissues and controls. Scale bars, 200 μm. (c) Quantification of the degree of pulmonary fibrosis according to the modified Ashcroft scale and pulmonary hydroxyproline content. (d) Amount of IL-19 was quantified using automated image analysis of the IL-19 staining. (e) Serum IL-19 expression was analyzed by ELISA in the BLM-induced mice and controls. (f) IL-19 protein expression in lung fibrosis was analyzed by western blot, and (g) relative protein levels were quantified. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$.

exposed trachea, inject the saline/bleomycin (2.5 mg/kg, HY-17565A, MCE)/IL-19 (200 ng/kg, Z03113, GenScript) during a single inspiration. After withdrawing the needle, close the incision with suture clips. Place the animal on warmer pads to allow recovery [19]. The peripheral bloods were collected on day 21 to determine the levels of IL-19 by Mouse IL-19 ELISA KIT (SEKM-0020, Solarbio). The lung tissues were collected for histopathological examination, hydroxyproline analysis [20] and western blot analysis.

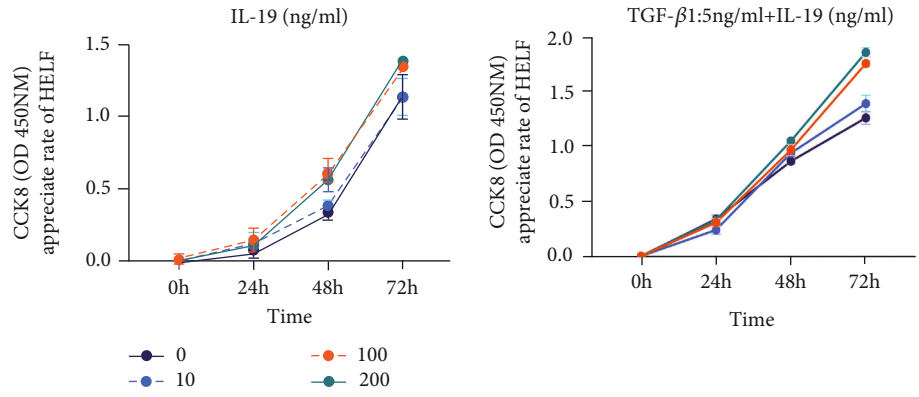
2.9. Histopathology and Immunohistochemistry. The lung lobes were fixed in 4% buffered paraformaldehyde and immobilized in paraffin for H&E or Masson trichrome staining. The severity of fibrosis in lung tissues was semi-quantitatively assessed by Ashcroft scoring system [21]. Histological changes are assessed on the basis of alveolar wall thickening, inflammatory disorder, and the extent of collagen deposition. Immunohistochemistry was performed by incubating tissue sections with primary antibodies (IL-19 and α-SMA) as described in previous protocol [22].

2.10. Statistical Analysis. Data are reported as the *mean* ± *SEM*. Difference between two groups comparing experimental groups was analyzed using Student's *t*-test, while more than two groups comparison using one-way analysis of variance (ANOVA). Analysis of data was conducted by GraphPad Prism 8 software. $P < 0.05$ was considered as statistically significant difference.

3. Results

3.1. IL-19 Is Upregulated in Human and Mouse Lung Fibrosis Tissues. Bioinformatics analysis of IPF patients and BLM-induced pulmonary fibrosis mice were initially conducted to explore the differential gene expression in lung fibrosis tissues. Data obtained from tissue specimens of IPF patients and mice from GEO database (GSE77326, GSE2051) were investigated. It was found that the lung fibrosis tissues exhibited higher mRNA expressions of IL-19 compared with the normal lung tissues in both human and mice (Figure 1(a)). Following in vivo, we tested whether IL-19 was also upregulated in the well-established BLM-induced murine fibrosis models. C57BL/6 mouse lungs were acquired following the intratracheal injection of either saline or BLM (2.5 mg/kg) on day 21 [23]. We performed histological examination of lung tissue, including H&E staining, Masson's trichrome staining and IL-19 immunohistochemical staining (Figure 1(b)). The degree of lung fibrosis was quantified on the basis of modified ashcroft scale and lung hydroxyproline content (Figure 1(c)). The results demonstrated that BLM-induced thickening of the major tracheal wall and higher collagen contents in interstitial tissues, indicating the successful model construction. IL-19 immunohistochemical staining revealed the higher IL-19 expressions in lung fibrosis tissues (Figure 1(d)), as well as alpha smooth muscle Actin (α-SMA). Besides, we found BLM-induced mice had elevated levels of IL-19 in peripheral blood compared with control groups by ELISA (Figure 1(e)). The western blot analysis of lung tissues showed increased expressions of IL-19 in BLM-induced mice when compared with controls (Figures 1(f) and 1(g)).

3.2. IL-19 Is a Profibrotic Cytokine by Activating Fibroblast in Lung. To clarify whether IL-19 is pro fibrotic or antifibrotic in the lung, we first explored the effect of IL-19 on the biological behavior of lung fibroblasts. Cell proliferation and migration abilities were analyzed in HELF and primary mouse lung fibroblasts. We evaluated the proliferation abilities of lung fibroblasts exposed to IL-19 by CCK8 assay, while TGF-β1 was



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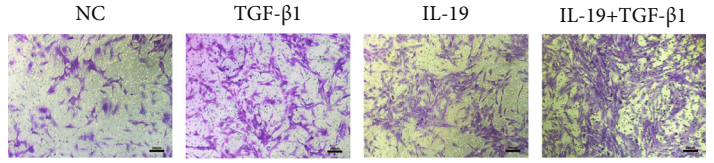

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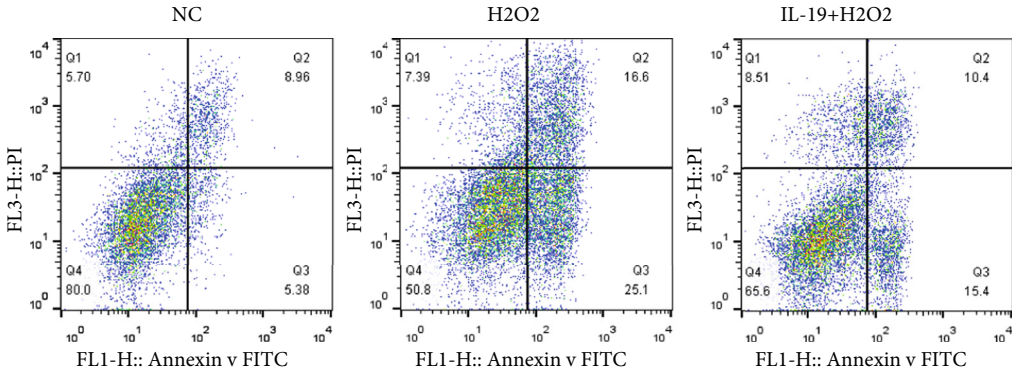

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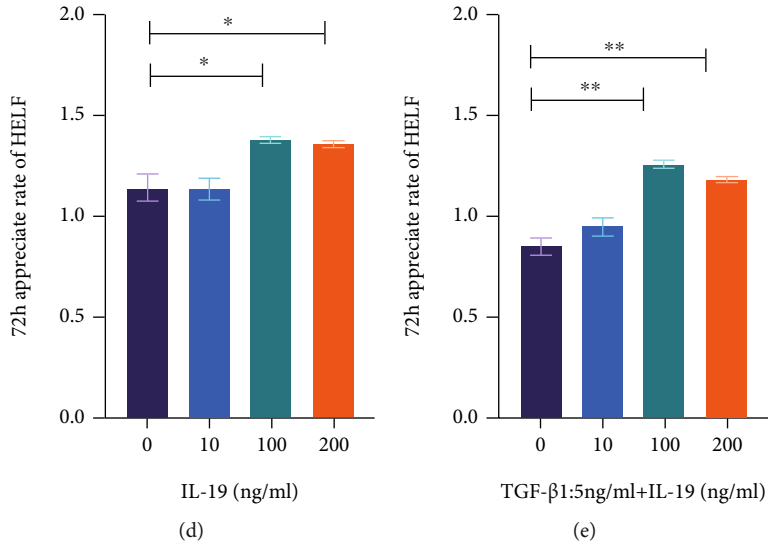

(d) (e)

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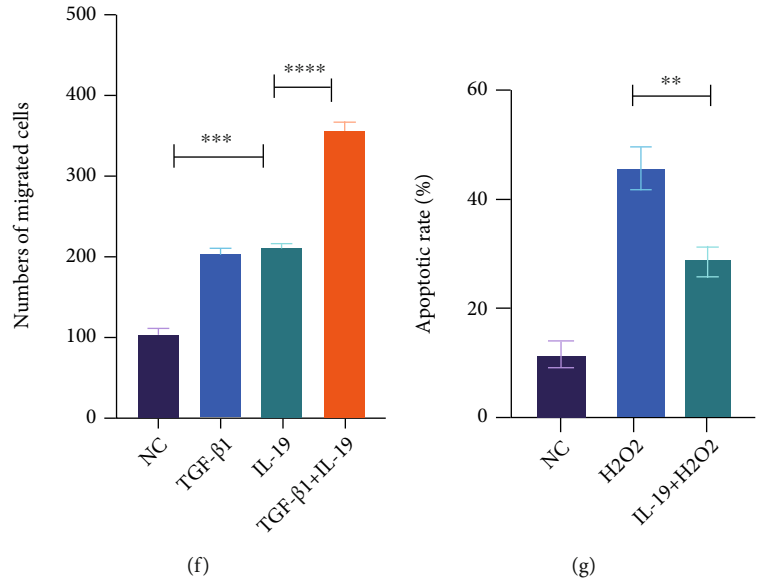


Figure 2: IL-19 promotes proliferation and invasion, while represses apoptosis of lung fibroblasts. (a) Lung fibroblasts appreciate rates were assessed by CCK-8 assay at 0 h, 24 h, 48 h, and 72 h with different IL-19 concentration of 0, 10, 100, and 200 ng/ml, and combination TGF-β1 (5 ng/ml) of different IL-19 concentration (0, 10, 100 and 200 ng/ml), and 72 h cell appreciate rates were analyzed (d and e). (b) Lung fibroblasts were, respectively, treated with TGF-β1 (5 ng/ml), IL-19 (100 ng/ml), IL-19 + TGF-β1 ($5\,ng/ml + 100\,ng/ml$), cell invasions were evaluated by transwell assay, and numbers of migrated cells were quantified (f). (c) The degrees of apoptosis were assessed by flow cytometry using propidium iodide and Annexin V staining, and apoptosis rates were calculated (g). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$.

used to be the positive control (Figure 2(a)). Cells proliferation rates were calculated at 0 h, 24 h, 48 h, and 72 h with different IL-19 concentrations of 0, 10, 100, and 200 ng/ml. We found cells growth were significantly promoted after exposure at the concentration of 100 ng/ml for 72 h (Figure 2(d)), while combination of TGF-β1 (5 ng/ml) and IL-19 (100 ng/ml) showing a higher appreciated rate, which provided the optimal concentration and time for further studies (Figure 2(e)). The transwell assay indicated that cells invasion abilities increased after IL-19 treatment and further enhanced along with TGF-β1 (Figures 2(b) and 2(f)). Furthermore, flow cytometry analysis of FITC/PI showed that IL-19 suppressed the apoptosis rate induced by H_2O_2 in lung fibroblasts (Figures 2(c) and 2(g)).

In addition, epithelial-mesenchymal transition (EMT) and fibrotic markers were analyzed in lung fibroblasts. We incubated lung fibroblasts with IL-19 (100 ng/ml, 72 hours) with or without TGF-β1 (5 ng/ml), then detecting protein expression of α-SMA and Col-1 by western blot. As seen in Figures 3(a) and 3(b), expression levels of α-SMA and Col-1 were elevated after IL-19 or TGF-β1 stimulation, and further elevated when combined IL-19 with TGF-β1. Immunofluorescence staining analysis also demonstrated the increase of α-SMA and Col-1 by IL-19 stimulation. We observed the highest intensity of fluorescence staining of α-SMA and Col-1 in IL-19 + TGF-β1 stimulation groups (Figure 3(c)).

3.3. IL-19 Promotes Lung Fibrosis through TGF-β/Smad Cascade. TGF-β/Smad signaling has a central role in the development of pulmonary fibrosis that drives activation of myofibroblasts (MFs), excessive production of ECM, and inhibition of ECM degradation [24]. TGF-β1 phosphorylates Smad2/3 and regulates target gene expression [25]. Above results showed the effect of IL-19 on biological behaviors of fibroblast could be enhanced along with TGF-β1, implying a potential interactive relationship between them. To identify significantly dysregulated biological pathways of IL-19 in IPF lung tissues, we performed bioinformatics analysis of GSEA under functional annotations of the KEGG database. KEGG functional enrichment analysis showed that TGF-β/Smad pathway enriched in human and mouse lung fibrosis and the expression of IL-19 in IPF was positively correlated with the profibrosis critical TGF-β/Smad signaling pathway (Figure 4(a)). Accordingly, to investigate the impact of IL-19 on TGF-β/Smad signaling pathway, we treated fibroblasts with increased concentration of IL-19 (0, 10, 100, and 200 ng/ml) and measured TGF-β1, Smad2/3 and phospho-Smad3 (pSmad3) expressions by western blot. The results showed the elevated expression levels of TGF-β1, pSmad3/Smad2/3 along with IL-19 concentration growth, demonstrating the activation of TGF-β/Smad cascade pathway induced by IL-19 (Figures 4(b) and 4(d)).

LY2109761 is a TGF-β type I/II receptor kinase inhibitor that suppressing the phosphorylation of Smad2 and Smad3. To confirm that IL-19 could active TGF-β/Smad signaling pathway, we examined the rescued impact of LY2109761 on IL-19-stimulated lung fibroblasts. We treated fibroblasts with concentrations of 0.5–10 μM of LY2109761 for 72 h. The proteomic analyses of TGF-β1, Smad2/3, and pSmad3 showed that the treatment of LY2109761 effectively blocked TGF-β/Smad cascade (Figures 4(c) and 4(e)). We further determined the effect of LY2109761 on the biological behaviors of IL-19-stimulated lung fibroblasts. Cells were treated

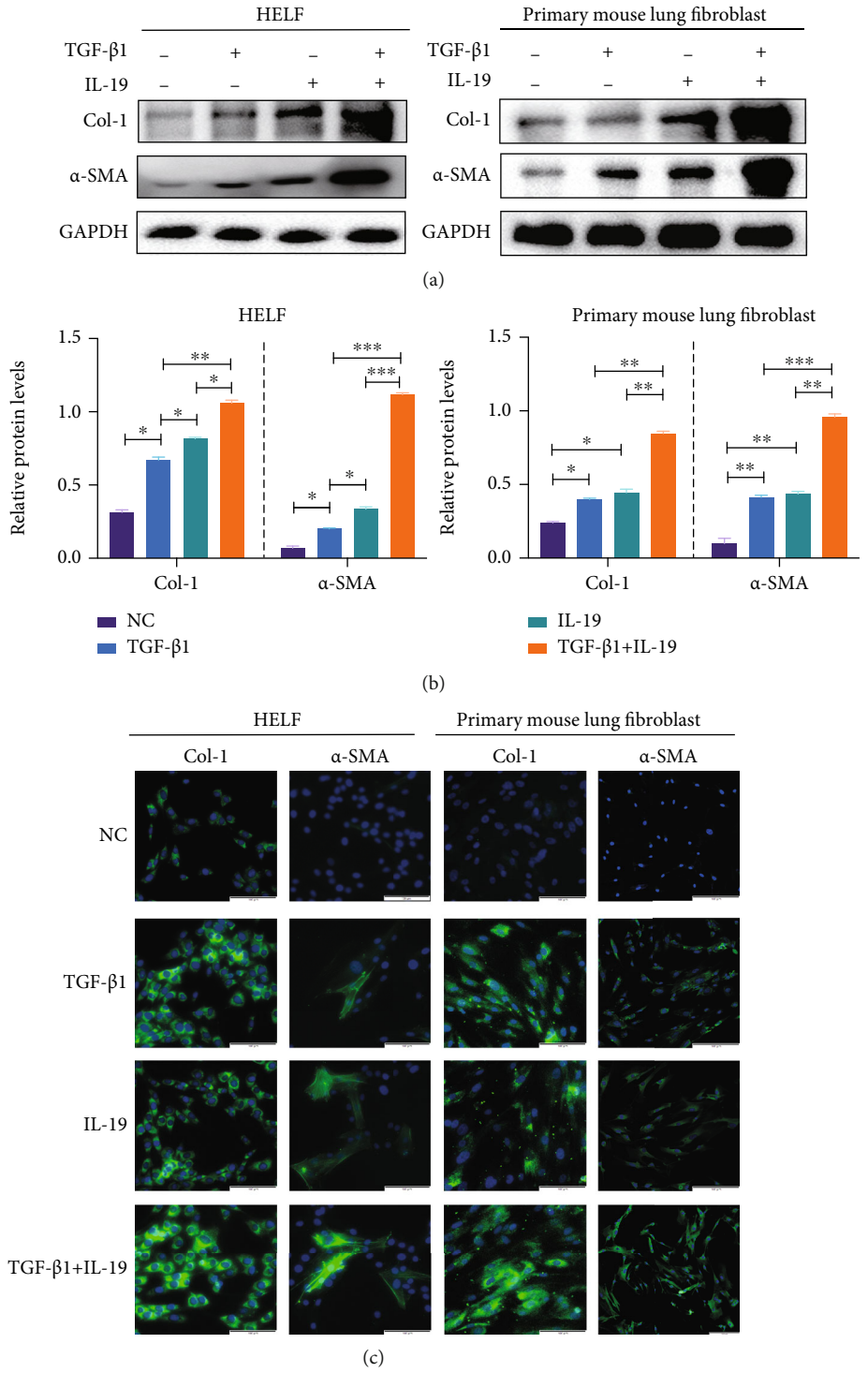
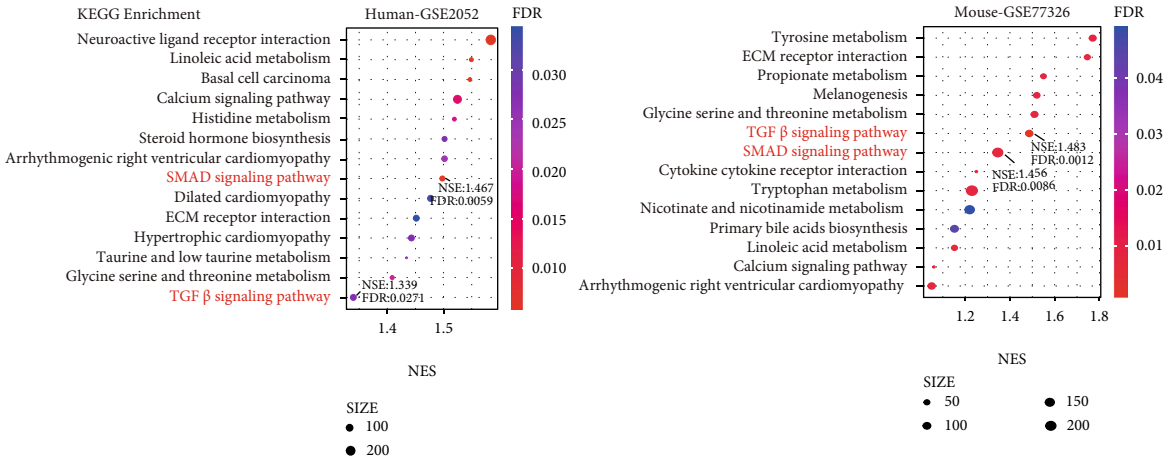
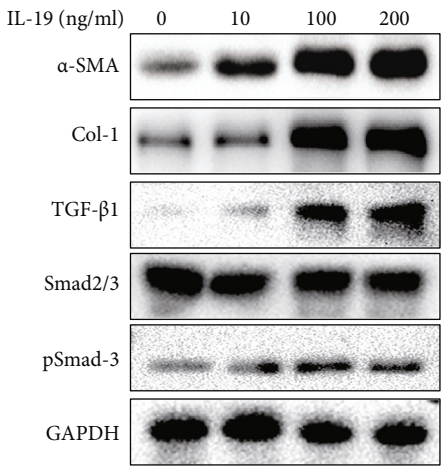


Figure 3: IL-19 promotes differentiation and collagen synthesis of lung fibroblasts. Proteomic analyses of Col-1 and α-SMA in HELF and primary mouse lung fibroblast treated by IL-19 (100 ng/ml) with or without TGF-β1 (5 ng/ml) by western blot (a), quantitative relative protein levels (b), and immunofluorescence staining (c). NC: negative control. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$.

with IL-19 (100 ng/ml) with or without LY2109761 (10 μM) for 72 h. Cell viabilities were determined by the CCK8 assay. Figures 4(f) and 4(g) showed that the proliferation promotion effect of IL-19 on fibroblasts could be significantly inhibited by LY2109761. The transwell analysis showed that LY2109761 significantly suppressed the promotion of migration induced by IL-19 on lung fibroblasts (Figure 4(h)). In addition, LY2109761 inhibited secretion of α-SMA and Col-1 proteins in a concentration-dependent manner in IL-19-stimulated lung fibroblasts (Figures 4(c) and 4(e)), indicating LY2109761 suppressed the differentiation and collagen synthesis induced by IL-19 in lung fibroblasts.



(a)



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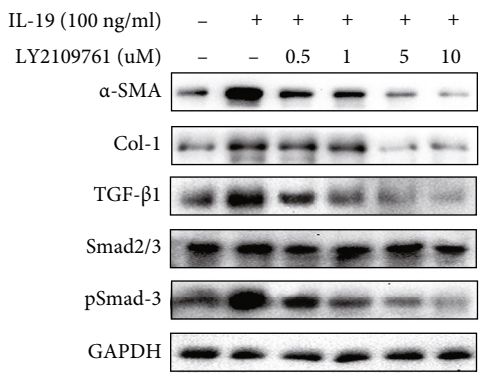

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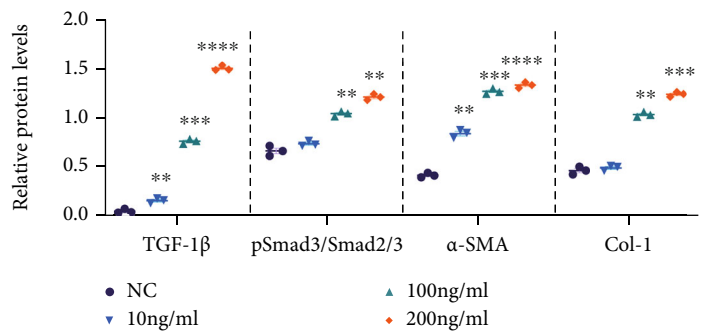

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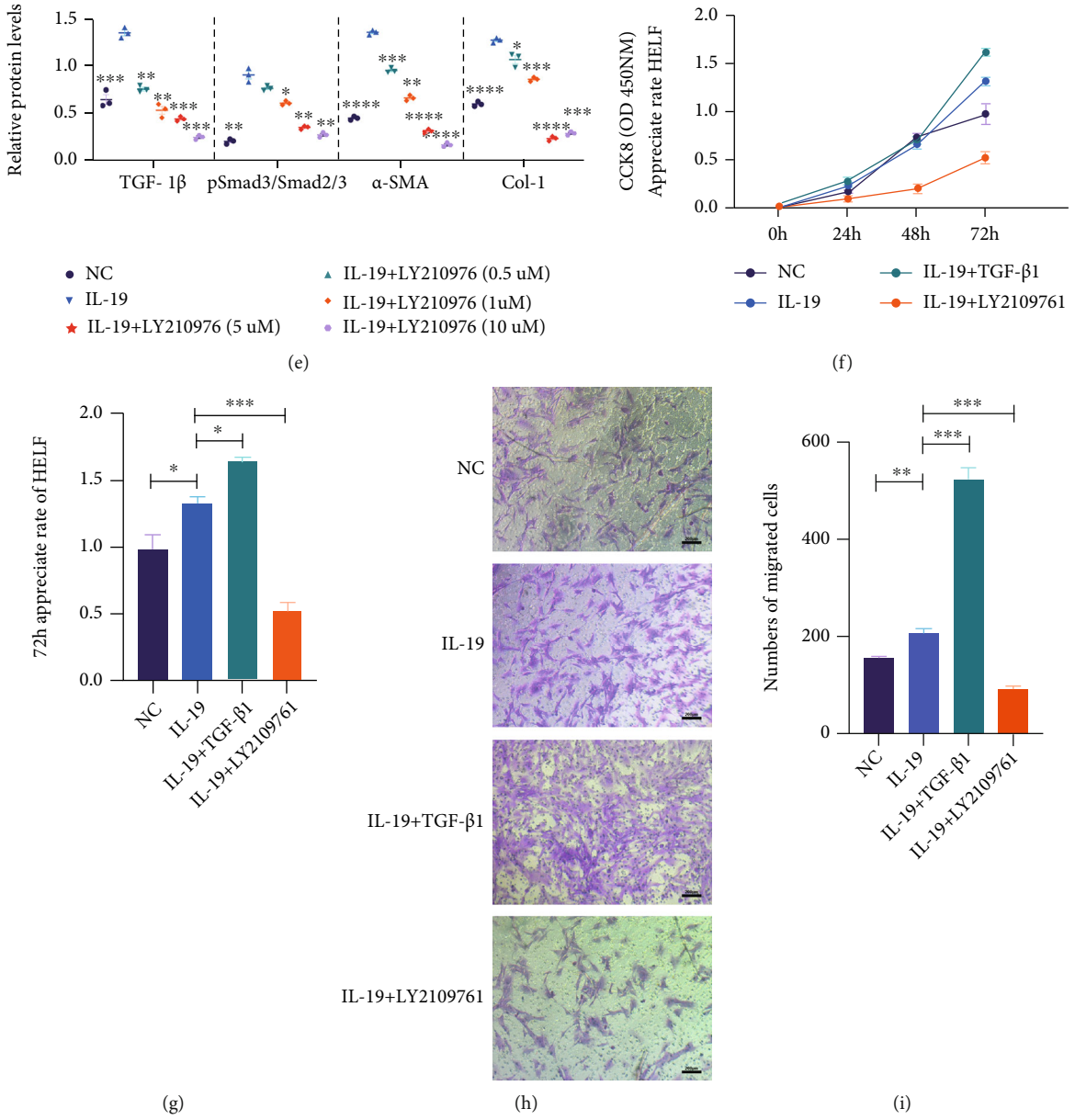

Figure 4: TGF-β1 blocker treatment attenuates expression and function of profibrotic markers in IL-19-stimulated lung fibroblasts. (a) The bioinformatics KEGG functional enrichment analysis of IL-19 in IPF patients and BLM-induced pulmonary fibrosis mouse lung tissues. (b) the western blots were used to analysis the expression of TGF-β1, Smad2/3, p-Smad3, Col-1, and α-SMA in lung fibroblasts treated with increased IL-19 concentration of 0, 10, 100, and 200 ng/ml, and (d) relative protein levels were quantified. Data were compared with NC group. (c) IL-19-stimulated lung fibroblasts treated with LY2109761 at concentration of 0.5–10 μM for 72 h, and (e) relative protein levels were quantified. Data were compared with IL-19 group. (f) Lung fibroblasts were treated with IL-19 (100 ng/ml) or $IL-19\,(100\,ng/ml)+TGF-\beta1\,(5\,ng/ml)$ or IL-19 $(100\,ng/ml)+LY2109761\,(10\,\mu M)$, cell appreciate rates were assessed by CCK-8 assay at 0 h, 24 h, 48 h, and 72 h, and (g) 72 h cell appreciate rates were analyzed. (h) Cell invasions were analyzed by transwell assay and (i) numbers of migrated cells were quantified. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$.

Our results indicated that TGF-β/Smad signaling was activated in IL-19-stimulated lung fibroblasts and profibrotic effect of IL-19 on lung fibroblasts could be inhibited if we blocked TGF-β/Smad signaling pathway.

3.4. IL-19 Aggravates Lung Fibrosis In Vivo. To determine the effect of IL-19 in progression of pulmonary fibrosis in vivo, we, respectively, administered a single dose of IL-19 (200 ng/kg) or BLM (2.5 mg/kg) or combination of these two treatments to wild-type C57BL/6 mice by intratracheal route. The lung tissues were collected on day 21. As shown in Figure 5(a), IL-19 treatment induced disruption of lung structure and collagen deposition in wild-type mice, while the extent of lung fibrosis (Figure 5(b)), lung hydroxyproline

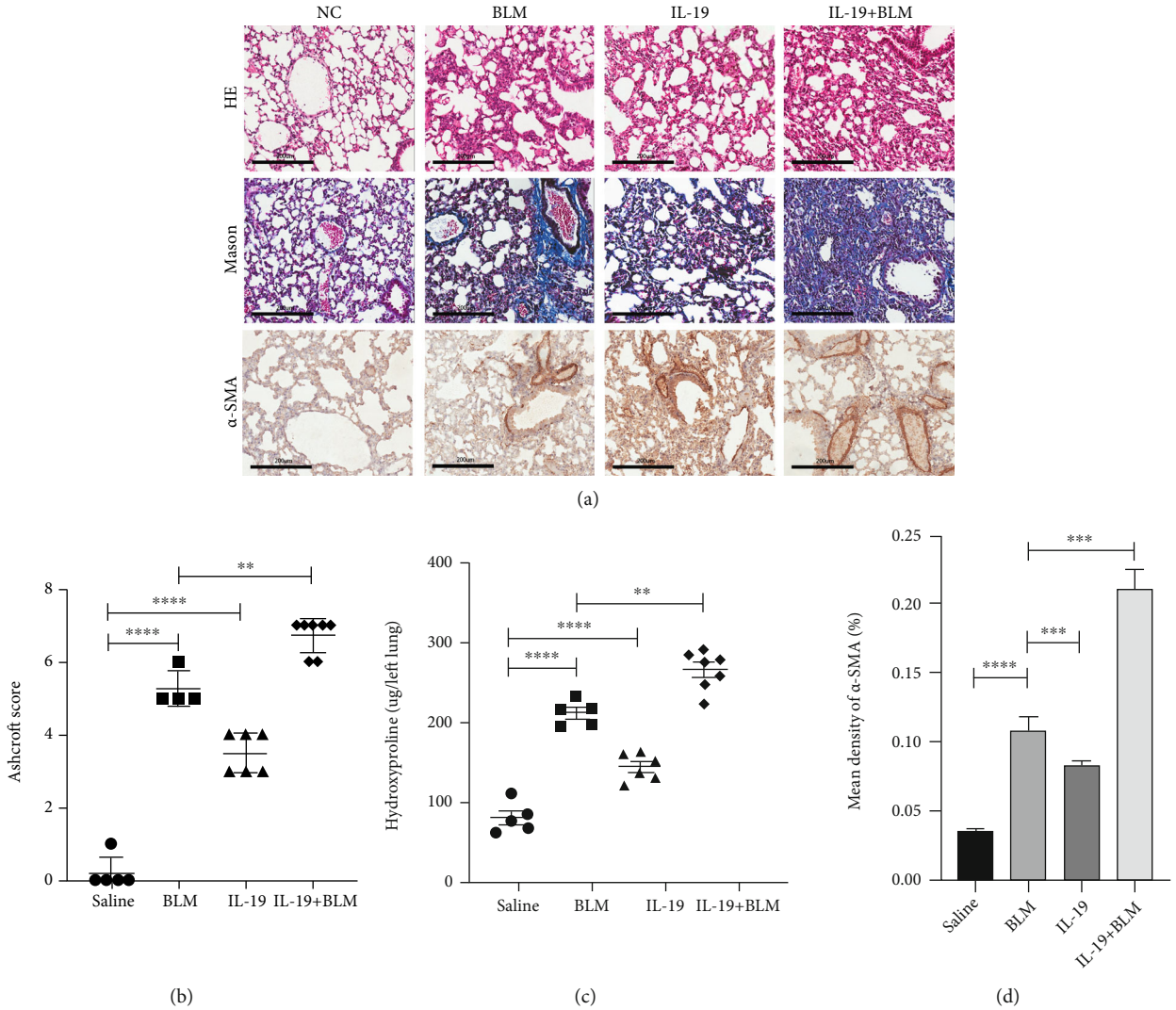

Figure 5: IL-19 aggravated lung fibrosis in wild-type and murine bleomycin fibrosis models. Schematic showing the induction of pulmonary fibrosis of lung collagen visualized by histopathological analysis after IL-19 and BLM challenge. After treatment with a single dose of BLM (2.5 mg/kg) or IL-19 (200 ng/kg) and combination of IL-19 and BLM, C57BL/6 mouse lungs were isolated and subjected for histopathology. (a) Representative pictures (20×) of H&E-stained, Masson's trichrome-stained sections and immunohistochemistry for α-SMA in lung sections. Effect of IL-19 on mice lung tissues were reflected by changes in the Ashcroft histology score (b) and lung hydroxyproline content (c). (d) Table represents the semiquantitative evaluation of protein expression (α-SMA) in specified treatment groups. In b–c, each symbol represents an individual mouse, a total of 5 to 7 mice per group. Scale bars, 200 μm. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$.

content (Figure 5(c)), and the expression of α-SMA (Figure 5(d)) were also higher than that in control groups. Furthermore, IL-19 treatment exacerbated BLM-induced abnormal lung changes with increased inflammatory cell infiltration and collagen deposition. Collectively, these studies demonstrated that IL-19 treatment could promote and aggravate the lung fibrosis progression induced by BLM in vivo.

4. Discussion

IL-19, as a new inflammatory factor in the regulation of the immune system, is related to the progression of many diseases, including autoimmune diseases, inflammation diseases and cancer [11, 26]. The debates on IL-19 being proinflammatory or anti-inflammatory factor have not yet been settled. The function of IL-19 in the inflammatory response depends on the cell type and disease model. The expression of IL-19 is reported increased in asthma and COPD patients, and shows the positive correlation with the progression of these diseases [16]. Bronchial epithelial cells from asthma and COPD patients express large amounts of IL-19, which is involved in allergic airway inflammation through the activation of group 2 innate lymphocytes (ILC2, 15] and induction of Th2-dominant immune response disorder [27]. Accordingly, we speculated IL-19 expression in respiratory diseases might be deleterious. However, none of studies explore the role of IL-19 in the etiopathogenesis of pulmonary fibrosis.

Usual interstitial pneumonia (UIP) is the histopathological marker of IPF, characterized by the conversion of

fibroblasts to MFs, which are responsible for the production of ECM and excessive collagen deposition in the lung [28]. Although two drugs, pirfenidone and nidanib, have been approved for the therapies of IPF [29], the response to antifibrotic therapy exhibits significant heterogeneity, making it difficult for individual prognosis. The pathophysiology of IPF is the subject of ongoing research. As our understanding of cytokine-mediated regulation of fibrosis continues to increase, novel approaches are likely to promote development of fibrotic diseases treatment. The TGF-β superfamily of ligands [30] are well-known drivers of fibrosis, and IL-1–IL-17A–TGFβ axis [31] and the type 2 cytokine (IL-4 and IL-13) response [32] have been considered as critical roles in the progression of fibrosis. Our study firstly demonstrates the profibrotic role for IL-19 through direct effects on lung fibroblasts through TGF-β/Smad pathway. We find IL-19 is upregulated in IPF patients' lung tissues and BLM-induced murine fibrosis models, and the stimulation of lung fibroblasts by IL-19 induces its proliferation and invasion, inhibits apoptosis and promotes its differentiation to myofibroblast phenotype, which can be revised by LY2109761, a TGF-β/Smad signaling pathway inhibitor. In vivo study, we determine that IL-19 aggravates lung fibrosis in wild-type mice and BLM-induced pulmonary fibrosis models.

IL-19, IL-20, IL-22, IL-24, and IL-26 are categorized in the IL-20 subfamily, the organ-specific effects of these cytokines are attributed to variation of their receptor heterodimers between tissues. IL-19, IL-20, and IL-24 target a specific two types of receptor complexes: IL-20RA/IL-20RB which predominantly localized in the lung, skin, testis, ovary, and placenta, leading to duplication of their target cell profiles and biological functions [6]. The three receptor subunits are expressed by resident effector cells of target organs, including keratinocytes [33], synovial fibroblasts [34], osteoclasts [35], vascular smooth muscle cells [8], and intestinal [36] and airway epithelial cells [15], and not on cells traditionally associated with the immune system [35]. MFs are the key effector cells of fibrosis diseases, characterized by α-SMA positive and lack of epithelial or endothelial markers [37]. IL-20 has been found to activate quiescent hepatic stellate cells (HSCs) and promote the proliferation, migration, and production of inflammatory cytokines, and deposition of ECM from activated HSCs. Anti-IL-20 receptor monoclonal antibody shows the protective effects on CCl4-induced liver injury mouse models. IL-20RA$^{-/-}$ mice are resistant to CCl4-induced liver fibrosis [38]. Consistently, our data support the mechanism of fibroblasts activation by IL-19 that lung fibroblasts are converted to MFs, implying that targeting at IL-19 may be a potential treatment strategy for lung fibrosis.

Chronic inflammation is a common hallmark of fibrosis disease [39]. Immune cells and injured intrinsic cells of the affected organ release large amounts of inflammatory cytokines and growth factors to maintain chronic inflammation, promote MFs proliferation, and enhance ECM production [3]. The expression of IL-19 is initially detected in immune cells, including in monocytes, macrophages, and B cells [40]. Airway epithelial cells [16], synovial fibroblasts [34], keratinocytes [33], and vascular smooth muscle cells (VSMCs) [8] are subsequently confirmed to express IL-19. Our study demonstrates the activation of lung fibroblast induced by IL-19, however, whether immune response dysfunction involved in this progression is currently unclear. Moreover, chronic dysregulation of type II alveolar epithelial cells (AEC2s) is thought to be central of pathological mechanisms of fibrogenesis in IPF, most of epithelial cells in IPF lungs are abnormally activated and produce mediators to promote the amplification of myofibroblasts [41, 42]. Whether epithelial cells could produce IL-19 to participate the progression and pathogenesis of lung fibrosis is worthy of further study.

5. Conclusions

In conclusion, our study firstly highlights the deleterious role of IL-19 on development of pulmonary fibrosis by modulating fibroblasts through TGF-β/Smad pathway and reinforces its promise as a new therapeutic target for intervention in pulmonary fibrosis.

Data Availability

The data used to support the findings of this study are included within the article.

Conflicts of Interest

The authors declare no conflict of interest regarding the publication of this paper.

Acknowledgments

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References

[1] J. Hutchinson, A. Fogarty, R. Hubbard, and T. McKeever, "Global incidence and mortality of idiopathic pulmonary fibrosis: a systematic review," *The European Respiratory Journal*, vol. 46, no. 3, pp. 795–806, 2015.

[2] B. Ley, H. R. Collard, and T. E. King Jr., "Clinical course and prediction of survival in idiopathic pulmonary fibrosis," *American Journal of Respiratory and Critical Care Medicine*, vol. 183, no. 4, pp. 431–440, 2011.

[3] L. Richeldi, H. R. Collard, and M. G. Jones, "Idiopathic pulmonary fibrosis," *Lancet*, vol. 389, no. 10082, pp. 1941–1952, 2017.

[4] S. A. Eming, T. A. Wynn, and P. Martin, "Inflammation and metabolism in tissue repair and regeneration," *Science*, vol. 356, no. 6342, pp. 1026–1030, 2017.

[5] P. Spagnolo, J. A. Kropski, M. G. Jones et al., "Idiopathic pulmonary fibrosis: disease mechanisms and drug development," *Pharmacology & Therapeutics*, vol. 222, p. 107798, 2021.

[6] S. Rutz, X. Wang, and W. Ouyang, "The IL-20 subfamily of cytokines–from host defence to tissue homeostasis," *Nature Reviews Immunology*, vol. 14, no. 12, pp. 783–795, 2014.

[7] G. Gallagher, "Interleukin-19: multiple roles in immune regulation and disease," *Cytokine & Growth Factor Reviews*, vol. 21, no. 5, pp. 345–352, 2010.

[8] M. V. Autieri, "IL-19 and other IL-20 family member cytokines in vascular inflammatory diseases," *Frontiers in Immunology*, vol. 9, p. 700, 2018.

[9] Y. Fujimoto, K. Aono, and Y. T. Azuma, "The clarified role of interleukin-19 in the inflammatory bowel disease and hypersensitivity: insights from animal models and humans," *The Journal of Veterinary Medical Science*, vol. 81, no. 8, pp. 1067–1073, 2019.

[10] K. Kingo, R. Mössner, T. Traks et al., "Further association analysis of chr 6q22-24 suggests a role of _IL-20RA_ polymorphisms in psoriasis," *Journal of Dermatological Science*, vol. 57, no. 1, pp. 71–73, 2010.

[11] Y. H. Hsu, P. P. Hsieh, and M. S. Chang, "Interleukin-19 blockade attenuates collagen-induced arthritis in rats," *Rheumatology*, vol. 51, no. 3, pp. 434–442, 2012.

[12] Y. H. Hsu, H. H. Li, J. M. Sung, W. T. Chen, Y. C. Hou, and M. S. Chang, "Interleukin-19 mediates tissue damage in murine ischemic acute kidney injury," *PLoS One*, vol. 8, no. 2, article e56028, 2013.

[13] C. H. Hsing, H. H. Li, Y. H. Hsu et al., "The distribution of interleukin-19 in healthy and neoplastic tissue," *Cytokine*, vol. 44, no. 2, pp. 221–228, 2008.

[14] S. G. Smith, A. Gugilla, M. Mukherjee et al., "Thymic stromal lymphopoietin and IL-33 modulate migration of hematopoietic progenitor cells in patients with allergic asthma," *The Journal of Allergy and Clinical Immunology*, vol. 135, no. 6, pp. 1594–1602, 2015.

[15] Y. H. Weng, W. Y. Chen, Y. L. Lin, J. Y. Wang, and M. S. Chang, "Blocking IL-19 signaling ameliorates allergen-induced airway inflammation," *Frontiers in Immunology*, vol. 10, p. 968, 2019.

[16] B. Rong, Y. Liu, M. Li, T. Fu, W. Gao, and H. Liu, "Correlation of serum levels of HIF-1α and IL-19 with the disease progression of COPD: a retrospective study," *International Journal of Chronic Obstructive Pulmonary Disease*, vol. Volume 13, pp. 3791–3803, 2018.

[17] Y. Fujimoto, N. Kuramoto, M. Yoneyama, and Y. T. Azuma, "Interleukin-19 as an Immunoregulatory cytokine," *Current Molecular Pharmacology*, vol. 14, no. 2, pp. 191–199, 2021.

[18] B. L. Edelman and E. F. Redente, "Isolation and Characterization of Mouse Fibroblasts," *Methods in Molecular Biology*, vol. 1809, pp. 59–67, 2018.

[19] T. Liu, F. G. De Los Santos, and S. H. Phan, "The bleomycin model of pulmonary fibrosis," *Methods in Molecular Biology*, vol. 1627, pp. 27–42, 2017.

[20] T. H. Sisson, M. Mendez, K. Choi et al., "Targeted injury of type II alveolar epithelial cells induces pulmonary fibrosis," *American Journal of Respiratory and Critical Care Medicine*, vol. 181, no. 3, pp. 254–263, 2010.

[21] R. H. Hübner, W. Gitter, N. E. El Mokhtari et al., "Standardized quantification of pulmonary fibrosis in histological samples," *Biotechniques*, vol. 44, no. 4, pp. 507–517, 2008.

[22] Y. Liu, B. Liu, G. Q. Zhang, J. F. Zou, M. L. Zou, and Z. S. Cheng, "Calpain inhibition attenuates bleomycin-induced pulmonary fibrosis via switching the development of epithelial-mesenchymal transition," *Naunyn-Schmiedeberg's Archives of Pharmacology*, vol. 391, no. 7, pp. 695–704, 2018.

[23] R. Carrington, S. Jordan, S. C. Pitchford, and C. P. Page, "Use of animal models in IPF research," *Pulmonary Pharmacology & Therapeutics*, vol. 51, pp. 73–78, 2018.

[24] X. M. Meng, D. J. Nikolic-Paterson, and H. Y. Lan, "TGF-β: the master regulator of fibrosis," *Nature Reviews. Nephrology*, vol. 12, no. 6, pp. 325–338, 2016.

[25] K. K. Kim, D. Sheppard, H. A. Chapman, and H. A. Chapman, "TGF-β1 signaling and tissue fibrosis," *Cold Spring Harbor Perspectives in Biology*, vol. 10, no. 4, 2018.

[26] T. Alanärä, K. Karstila, T. Moilanen, O. Silvennoinen, and P. Isomäki, "Expression of IL-10 family cytokines in rheumatoid arthritis: elevated levels of IL-19 in the joints," *Scandinavian Journal of Rheumatology*, vol. 39, no. 2, pp. 118–126, 2010.

[27] T. Leigh, R. G. Scalia, and M. V. Autieri, "Resolution of inflammation in immune and nonimmune cells by interleukin-19," *American Journal of Physiology. Cell Physiology*, vol. 319, no. 3, pp. C457–c464, 2020.

[28] P. J. Wolters, H. R. Collard, and K. D. Jones, "Pathogenesis of idiopathic pulmonary fibrosis," *Annual Review of Pathology*, vol. 9, no. 1, pp. 157–179, 2014.

[29] P. Rogliani, L. Calzetta, F. Cavalli, M. G. Matera, and M. Cazzola, "Pirfenidone, nintedanib and _N_ -acetylcysteine for the treatment of idiopathic pulmonary fibrosis: A systematic review and meta-analysis," *Pulmonary Pharmacology & Therapeutics*, vol. 40, pp. 95–103, 2016.

[30] D. Chanda, E. Otoupalova, S. R. Smith, T. Volckaert, S. P. De Langhe, and V. J. Thannickal, "Developmental pathways in the pathogenesis of lung fibrosis," *Molecular Aspects of Medicine*, vol. 65, pp. 56–69, 2019.

[31] M. S. Wilson, S. K. Madala, T. R. Ramalingam et al., "Bleomycin and IL-1beta-mediated pulmonary fibrosis is IL-17A dependent," *The Journal of Experimental Medicine*, vol. 207, no. 3, pp. 535–552, 2010.

[32] R. L. Gieseck 3rd, M. S. Wilson, and T. A. Wynn, "Type 2 immunity in tissue repair and fibrosis," *Nature Reviews Immunology*, vol. 18, no. 1, pp. 62–76, 2018.

[33] S. M. Sa, P. A. Valdez, J. Wu et al., "The effects of IL-20 subfamily cytokines on reconstituted human epidermis suggest potential roles in cutaneous innate defense and pathogenic adaptive immunity in psoriasis," *Journal of Immunology*, vol. 178, no. 4, pp. 2229–2240, 2007.

[34] N. Sakurai, T. Kuroiwa, H. Ikeuchi et al., "Expression of IL-19 and its receptors in RA: potential role for synovial hyperplasia formation," *Rheumatology*, vol. 47, no. 6, pp. 815–820, 2008.

[35] T. W. Kragstrup, T. Andersen, L. D. Heftdal et al., "The IL-20 cytokine family in rheumatoid arthritis and Spondyloarthritis," *Frontiers in Immunology*, vol. 9, p. 2226, 2018.

[36] J. H. Niess, P. Hruz, and T. Kaymak, "The interleukin-20 cytokines in intestinal diseases," *Frontiers in Immunology*, vol. 9, p. 1373, 2018.

[37] N. C. Henderson, F. Rieder, and T. A. Wynn, "Fibrosis: from mechanisms to medicines," *Nature*, vol. 587, no. 7835, pp. 555–566, 2020.

[38] Y. S. Chiu, C. C. Wei, Y. J. Lin, Y. H. Hsu, and M. S. Chang, "IL-20 and IL-20R1 antibodies protect against liver fibrosis," *Hepatology*, vol. 60, no. 3, pp. 1003–1014, 2014.

[39] M. Mack, "Inflammation and fibrosis," *Matrix Biology*, vol. 68-69, pp. 106–121, 2018.

[40] W. Ouyang, S. Rutz, N. K. Crellin, P. A. Valdez, and S. G. Hymowitz, "Regulation and functions of the IL-10 family of cytokines in inflammation and disease," *Annual Review of Immunology*, vol. 29, no. 1, pp. 71–109, 2011.

[41] T. J. Desai, D. G. Brownfield, and M. A. Krasnow, "Alveolar progenitor and stem cells in lung development, renewal and cancer," *Nature*, vol. 507, no. 7491, pp. 190–194, 2014.

[42] J. R. Rock, C. E. Barkauskas, M. J. Cronce et al., "Multiple stromal populations contribute to pulmonary fibrosis without evidence for epithelial to mesenchymal transition," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 108, no. 52, pp. E1475–E1483, 2011.

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Research Article

Glibenclamide Alleviates LPS-Induced Acute Lung Injury through NLRP3 Inflammasome Signaling Pathway

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Glibenclamide displays an anti-inflammatory response in various pulmonary diseases, but its exact role in lipopolysaccharide- (LPS-) induced acute lung injury (ALI) or acute respiratory distress syndrome (ARDS) remains unknown. Herein, we aimed to explore the effect of glibenclamide in vivo and in vitro on the development of LPS-induced ALI in a mouse model. LPS stimulation resulted in increases in lung injury score, wet/dry ratio, and capillary permeability in lungs, as well as in total protein concentration, inflammatory cells, and inflammatory cytokines including IL-1β, IL-18 in bronchoalveolar lavage fluid (BALF), and lung tissues, whereas glibenclamide treatment reduced these changes. Meanwhile, the increased proteins of NLRP3 and Caspase-1/p20 after LPS instillation in lungs were downregulated by glibenclamide. Similarly, in vitro experiments also found that glibenclamide administration inhibited the LPS-induced upregulations in cytokine secretions of IL-1β and IL-18, as well as in the expression of components in NLRP3 inflammasome in mouse peritoneal macrophages. Of note, glibenclamide had no effect on the secretion of TNF-α in vivo nor in vitro, implicating that its anti-inflammatory effect is relatively specific to NLRP3 inflammasome. In conclusion, glibenclamide alleviates the development of LPS-induced ALI in a mouse model via inhibiting the NLRP3/Caspase-1/IL-1β signaling pathway, which might provide a new strategy for the treatment of LPS-induced ALI.

1. Introduction

Acute lung injury (ALI) or acute respiratory distress syndrome (ARDS), a common disease in intensive care unit (ICU), is the consequence of biased inflammatory response to various causes including sepsis, trauma, and ventilation [1–3]. Although supportive treatment and intensive care are developing, there have been no clinically effective pharmacologic therapies and the prognosis of ALI/ARDS remains poor with a high morbidity and mortality [1, 3]. Thus, to further explore the underlying mechanisms and the potential treatment approaches for ALI/ARDS is necessary and urgent.

NLRP3, belonging to a family of NLRs, is one of the immediate responses of the innate immune system. It is

postulated that a two-step mechanism is required for the full activation of the NLRP3 inflammasome [4]. The first is a priming step that is initiated by pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs), resulting in the upregulations of pro-IL-1β, pro-IL-18, and the components of the inflammasome. The second is an activation step which is the assembly of these components into the inflammasome structure and then to produce mature proinflammatory interleukins. Recent research has described the function of the NLRP3 inflammasome in various pulmonary diseases including respiratory infections, chronic obstructive pulmonary disease, and asthma [5, 6]. Currently, the role of NLRP3 inflammasome in the development of multiple types of ALI is also reported, and the main pathogenic mechanisms include the following: (1) increased permeability of alveolar epithelial and barrier dysfunction; (2) overproduction of cytokines including IL-6, IL-1β, and TNF-α; and (3) involvement of tissue remodeling and pulmonary fibrosis in the late stage of ALI [7–9]. These results imply that the NLRP3 inflammasome participates in the pathogenesis of ALI/ARDS.

Glibenclamide, besides as a kind of hypoglycemic drug, displays an anti-inflammatory role in many diseases from respiratory, urinary, heart, and central nervous systems [10]. Given the fact of ALI/ARDS considered as an inflammatory disorder and the anti-inflammatory activity of glibenclamide, hence, glibenclamide might be against the development of ALI/ARDS in theory. In fact, a large number of studies have indicated that glibenclamide involves in the regulation of inflammation in different animal models of ALI, including oleic acid-, ozone-, radiation-, hemorrhagic shock-, and ventilator-induced ALI [11–14]. However, no such studies are reported concerning the role of glibenclamide in LPS-induced ALI. LPS, a major constituent of the outer membrane of Gram-negative bacteria, acts as one of the common causes in the pathogenesis of sepsis, septic shock, and sepsis-related ALI/ARDS [15]. In addition, animal model of LPS-induced ALI is widely used as a clinically relevant model of Gram-negative bacteria-related ALI/ARDS [16–18]. Therefore, it would be of great clinical significance to explore whether glibenclamide can ameliorate LPS-induced ALI.

Taken together, we proposed the hypothesis that glibenclamide has a protective effect on LPS-induced ALI/ARDS, which might be associated with its inhibition of NLRP3 inflammasome signaling pathway. Thus, we herein attempted to confirm this hypothesis via in vivo and in vitro experiments in a mouse model of LPS-induced ALI and for the first time found that glibenclamide alleviated the development of LPS-induced ALI in a mouse model via inhibiting the NLRP3/Caspase-1/IL-1β signaling pathway, which might provide a new strategy for the treatment of LPS-induced ALI.

2. Materials and Methods

2.1. Animals and Study Design. Male C57BL/6 mice (6-8 weeks) were purchased from the Shanghai SLAC Laboratory Animal Co., Ltd. (Shanghai, China). All experimental procedures were conducted in accordance with the ethics committee of the animal laboratory of Zhejiang University. Mice were divided into four groups ($n = 6$/group): control (Con) group, glibenclamide (Gly) group, LPS group, and LPS+glibenclamide (LPS+Gly) group. Glibenclamide (Sigma-Aldrich, St. Louis, MO, USA) was diluted in DMSO for 100 mg/ml concentration according to the instruction manual. Glibenclamide was given intraperitoneally for 3 days before LPS administration, whereas DMSO was used as vehicle. LPS (Sigma-Aldrich, St. Louis, MO, USA) was injected into the trachea of mice with a microsyringe to establish ALI model, while PBS was used as vehicle. After intratracheal instillation, mice were kept vertical for at least 1 min to ensure the distribution of the PBS or LPS in the lungs. Twenty-four hours later after LPS administration, the mice were sacrificed for experiments. Bronchoalveolar lavage fluid (BALF) was collected with PBS via a tracheal catheter as described in our previous study [19]. After centrifugation, the supernatant and cells were separated for further experiments. The lung tissues were collected for further analysis.

2.2. Lung Histology and Immunohistochemistry Analysis. The lung tissues fixed in 4% paraformaldehyde were embedded in paraffin and then sliced at a thickness of 4 μm for hematoxylin and eosin (H&E) staining. The histology scoring system was used to evaluate lung injury [11]. Four pathological parameters were scored as previously described: (1) alveolar congestion, (2) hemorrhage, (3) leukocyte infiltration, and (4) thickness of alveolar wall/hyaline membrane formation. Each category was graded using a 4-pointscale: 0: minimal damage, 1: mild damage, 2: moderate damage, and 3: maximal damage. The total histology score was expressed as the sum of the score for all parameters. Three slides of each mouse were prepared for evaluation.

Immunohistochemistry (IHC) was performed to determine the protein expression of NLRP3. The paraffin sections were pretreated at 62°C for 30 min, then dewaxed in xylene, hydrated, and washed. Hydrogen peroxide solution was used to inhibit the endogenous peroxidase. The sections were incubated overnight at 4°C with anti-NLRP3 antibody (Abclonal, Wuhan, China) (1 : 100). Then, membranes were washed thoroughly with phosphate-buffered saline solution. The secondary antibodies (Tuling, Hangzhou, China) were added and incubated at 37°C for 30 min. Diaminobenzidine was added, and the sections were counterstained with hematoxylin to visualize the reaction products. All the sections were semi-quantitatively analyzed by the ImageJ software. The integrated IOD/area (density mean) was measured by evaluating the staining in images at ×200 magnification. Three slides of each mouse were prepared for evaluation.

2.3. Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR). Total RNA was extracted from lung tissues or cells using Trizol (Thermo Fisher Scientific). cDNA was synthesized using a cDNA synthesis kit (Takara, Dalian, Liaoning, China) following the manufacturer's instructions. For mRNA detection, β-actin was used as the reference housekeeping gene. Real-time PCR was conducted using SYBR

Green (TaKaRa) with an Applied Biosystems 7500 real-time PCR system (Thermo Fisher Scientific). The primer sequences used are shown as follows: mouse NLRP3 sense primer: 5′-TCACAACTCGCCCAAGGAGGAA-3′ and mouse NLRP3 antisense primer: 5′-AAGAGACCACG GCAGAAGCTAG-3′ and mouse β-actin sense primer: 5′-GGCTGTATTCCCCTCCATCG-3′ and mouse β-actin antisense primer: 5′-CCAGTTGGTAACAATGCCATGT-3′.

2.4. Western Blot Analysis. Cell or tissue lysate was resuspended in 5× SDS loading buffer, subsequently incubated at 100°C for 5 min and centrifuged at 12,000 × g for 10 min. Protein concentrations were detected using a BCA Protein Assay Kit (Thermo Fisher Scientific). A total of 20 μg of protein from the tissue or cell lysate was separated by SDS-PAGE gel (Thermo Fisher Scientific) and then transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore). The membrane was blocked using 5% nonfat milk for 2 h at room temperature and then incubated with appropriate primary antibodies: anti-NLRP3 (Abclonal, Wuhan, China) (1 : 1,000) and anti-Caspase-1 (Abclonal, Wuhan, China) (1 : 1,000) in blocking buffer overnight at 4°C. Anti-β-actin (HuaBio, Shanghai, China) (1 : 2,000) was used as a loading control. After washing three times with PBST, the membranes were incubated with HRP-conjugated secondary antibodies for 1.5 h at room temperature. The bands were detected using an ECL kit (MultiSciences, Hangzhou, Zhejiang, China).

2.5. ELISA Assays. The levels of IL-1β, IL-18, and TNF-α concentrations in lung tissues, BALF, and cell supernatant were analyzed using ELISA Kit (BioLegend, San Diego, CA, USA), according to the manufacturer's protocol.

2.6. Mouse Lung Wet/Dry Ratio Assay. Twenty-four hours after intratracheal instillation of LPS, mice were killed and the lobes of the right lungs were excised after removal of excess blood and then weighed to obtain the "wet" weight. Subsequently, the lungs were dried in an oven at 60°C for 72 h for "dry" weight.

2.7. The Measurement of BALF. Total cell number of BALF was counted, and total protein concentration in BALF was determined using a BCA assay Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. The inflammatory cells in BALF were analyzed with a Cytoflex machine (Beckman Coulter), and the following fluorescence-conjugated antibodies were used for the experiment: PE-conjugated anti-mouse CD11b (BioLegend, San Diego, CA, USA), FITC-conjugated anti-mouse F4/80 (BioLegend, San Diego, CA, USA), and BV650-conjugated anti-mouse LY-6G (Invitrogen, Carlsbad, CA, USA).

2.8. Mouse Alveolar-Capillary Leakage Assay. Twenty-four hours after LPS administration, the mice were injected with 20 mg/kg Evans blue solution by the tail vein. Two hours later, the mice were exsanguinated through the heart with syringe. Then, the lungs were removed and placed in 100 mg/ml formamide (Sigma-Aldrich, St. Louis, MO, USA). The tissues were incubated at 60°C for 24 h, and the absorbance of formamide was measured at 620 nm.

2.9. Isolation and Purification of Mouse Peritoneal Macrophages. C57BL/6 mice at 8-week-old were i.p. injected with 2 ml of 3% sterile thioglycolate medium (BD Biosciences, Sparks, MD), and peritoneal macrophages (PMs) were extracted three days later. To isolate and purify the PMs, each mouse was euthanized with 40 mg/kg pentobarbital sodium and soaked in 75% ethanol for 3 min. The outer layer of the peritoneum was incised with scissors; 15 ml RPMI 1640 was injected intraperitoneally into mice with a 20 ml syringe. The intraperitoneal fluid was collected into the tube with a 20 ml syringe after gently massaging the peritoneum and centrifuged at 4°C for 250 × g for 5 min. The supernatant was discarded, and the sediment was suspended in RPMI 1640 medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. The cells were then added to 12-well cell culture plates as needed to obtain a density of 5×10^6 cells/well and cultured for 2 h at 37°C in 5% CO2. Then, nonadherent cells were removed by gentle washing with PBS three times. The isolated macrophages were prepared for experiments in vitro.

2.10. Cell Proliferation Assay. Cell Counting Kit-8 kits (CCK-8, TransGen, Beijing, China) were used to evaluate PM proliferation. PMs were plated into 96-well cell culture plates at a density of 1×10^4 cells/well for 24 h at 37°C and then treated with different concentrations of glibenclamide (0~200 μM) for 24 h. The viability was assayed at 24 h by using a Cell Counting Kit-8 assay (TransGen, Beijing, China).

2.11. Inflammasome Activation Assays. PMs were seeded at 5×10^6/ml in 12 well-cell culture plates. The overnight medium was replaced on the following day, and cells were primed with 2 μg/ml LPS for 6 h. Then, medium was added with glibenclamide (50 μM) or DMSO (1 : 1,000) for another 6 h. Cells were finally stimulated with inflammasome activators: 2 mM adenosine triphosphate (Sigma-Aldrich, St. Louis, MO, USA) for 1 h. Supernatant was removed and analyzed using ELISA kits according to the manufacturer's instructions. Cells were collected for Western blot analysis.

2.12. Statistical Analysis. Statistical analysis was carried out using Graphpad Prism. The data were expressed as mean ± SD. The unpaired Student t-test was used for comparisons between two groups. Differences were considered significant at $P < 0.05$.

3. Results

3.1. Glibenclamide Attenuates LPS-Induced Lung Injury. After intratracheal instillation of LPS, mice showed greater diffuse alveolar damage, thickened alveolar wall, hemorrhage, and more inflammatory cell infiltration, whereas pretreatment with glibenclamide alleviated these pathological

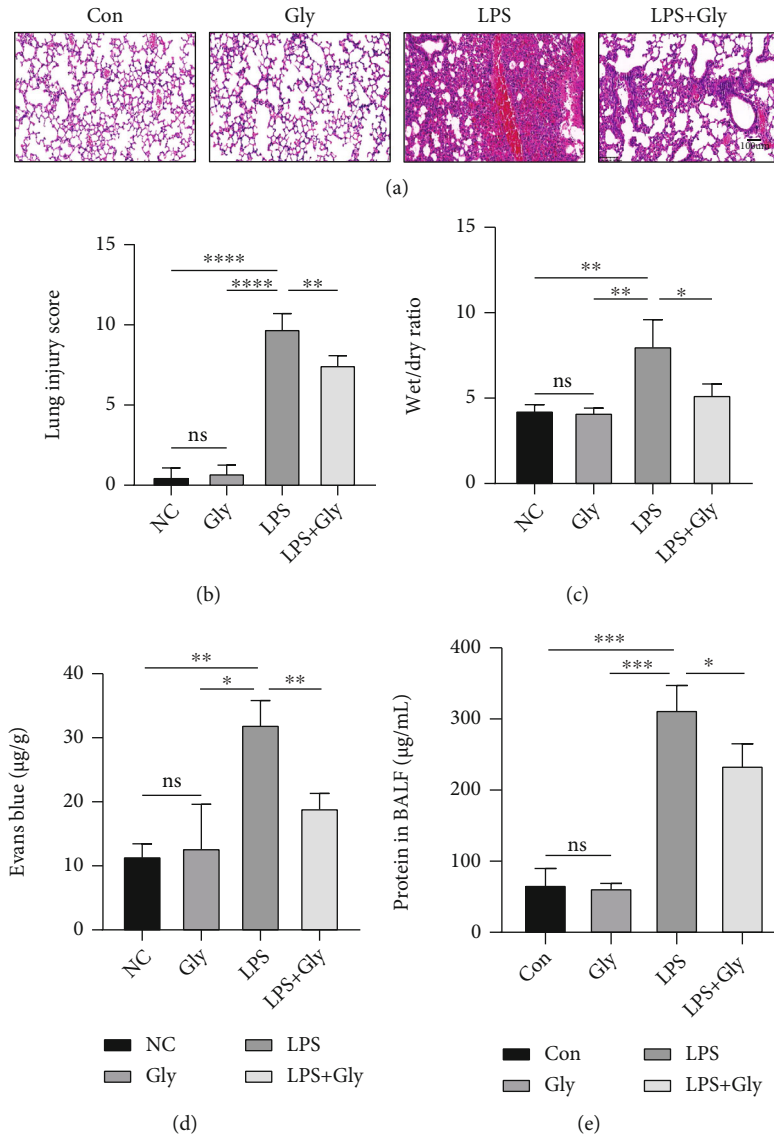

Figure 1: The effect of glibenclamide on the pathological injury in LPS-induced ALI. (a) The pathological alternations in lung tissues were evaluated with HE staining ($n = 4$). (b) Lung injury scores in four groups. (c) Wet/dry ratio in four groups ($n = 4$). (d) Alveolar-capillary leakage in four groups ($n = 3$). (e) The total protein concentration in BALF ($n = 3$). Scale bars, 100 μm. Data are representative of three independent experiments (mean and SD). ns: not significant. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ (unpaired Student's t-test).

changes (Figure 1(a)). Correspondingly, glibenclamide treatment reduced the LPS-induced increases in inflammation score, the wet/dry ratio, alveolar-capillary leakage of lungs, and the concentration of total protein in BALF (Figures 1(b)–1(e)). These results suggested that glibenclamide attenuates the LPS-induced lung injury.

3.2. Glibenclamide Decreases LPS-Induced Lung Inflammation. In comparison with the LPS group, pretreatment with glibenclamide significantly reduced the total cell number and the percentage of neutrophils and macrophages in BALF (Figures 2(a)–2(c)). The levels of proinflammatory cytokines including IL-1β and IL-18 were also markedly downregulated in both BALF (Figures 2(d) and 2(e)) and lung tissues (Figures 2(g) and 2(h)). Surprisingly, glibenclamide treatment did not affect the LPS-mediated increase in the production of TNF-α either in BALF (Figure 2(f)) or in lung homogenates (Figure 2(i)).

3.3. Glibenclamide Suppresses the Expression of NLRP3 and Caspase-1 Activity. Based on the fact that glibenclamide can reduce the downstream products of NLRP3 signaling way, we supposed that glibenclamide could directly suppress the activation of NLRP3 inflammasome. Accordingly, we detected the expression of NLRP3, one of the main components of inflammasome, and Caspase-1/P20, a biologically active form of Caspase-1. Compared with the control group, the mRNA and protein levels of NLRP3 were obviously increased in the LPS group (Figures 3(a)–3(e)). Similarly, protein level of Caspase-1/P20 was also upregulated by LPS

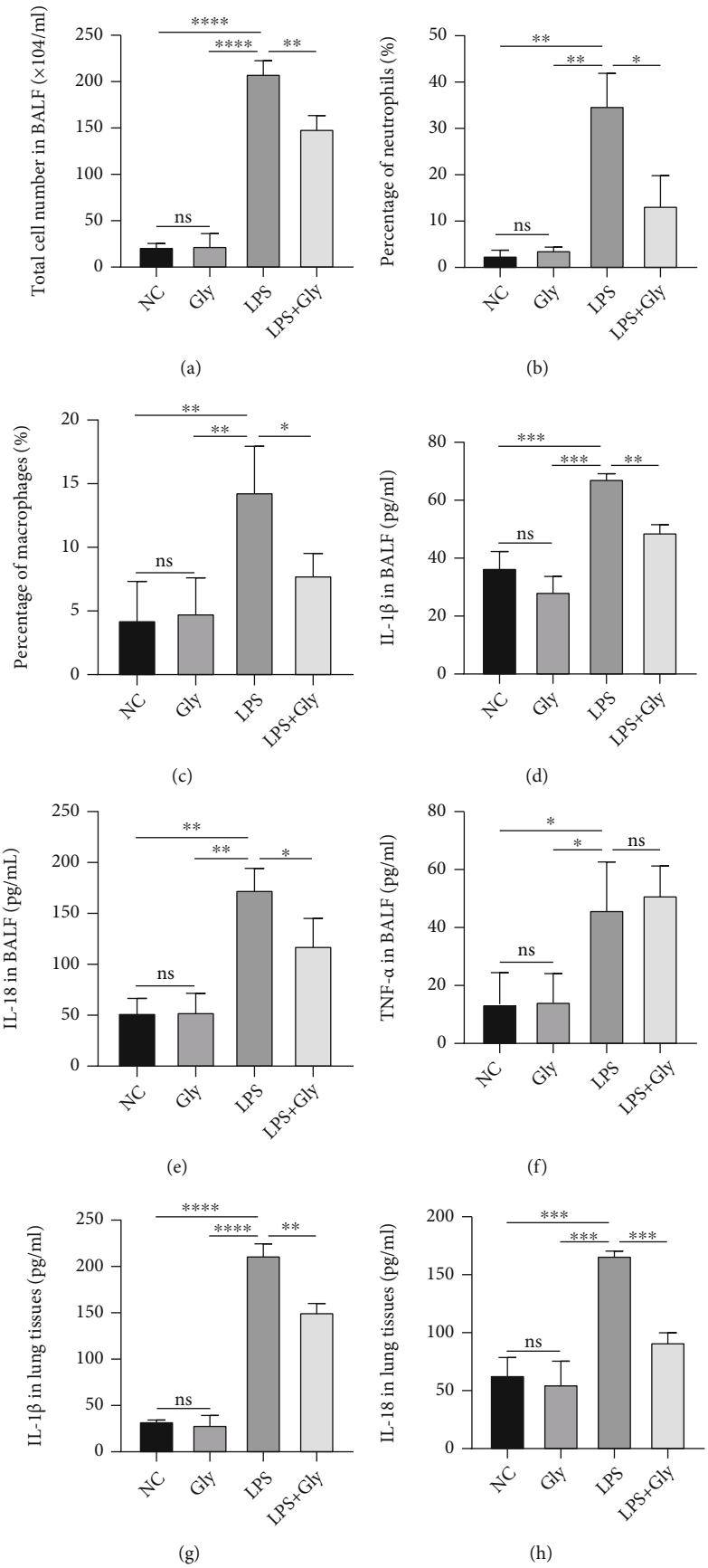

Figure 2: Continued.

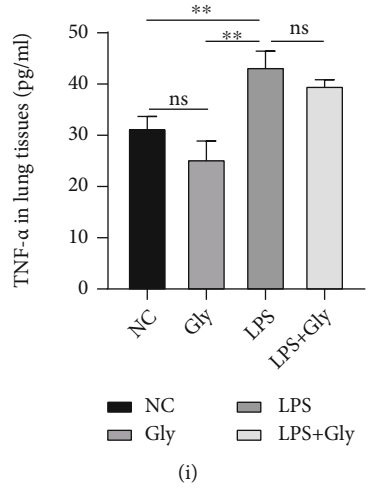

Figure 2: The effect of glibenclamide on the LPS-stimulated inflammatory response in lungs. (a) The total cell number counts in BALF ($n = 3$). (b, c) The percentage of neutrophils and macrophages is presented ($n = 3$). (d–i) The content of IL-1β, IL-18, and TNF-α in (d–f) BALF ($n = 3$) and (g–i) lung tissues ($n = 3$) was measured by ELISA. Data are representative of three independent experiments (mean and SD). ns: not significant. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$ (unpaired Student's t-test).

stimulation (Figures 3(b) and 3(c)). However, the elevated expressions of NLRP3 and Caspase-1/P20 in the LPS group were inhibited by glibenclamide treatment (Figures 3(a)–3(e)).

3.4. Glibenclamide Exerts Anti-Inflammatory Effect In Vitro. Since the protective function of glibenclamide on LPS-induced ALI had been verified in vivo, then we further confirmed its effect with an in vitro model. Macrophages are key orchestrators of the inflammatory and repair responses in the lung [20]. In vitro inflammatory model established by LPS stimulation of macrophages including macrophage cell lines [21] or primary macrophages [22] can simulate the inflammatory process in vivo and is often used in the study of the mechanism of anti-inflammatory drugs [21, 22]. Herein, mouse peritoneal macrophages (PMs) were used as the in vitro cell model. First, we measured the in vitro cytotoxicity of glibenclamide to PMs. With the increased concentration, the cell viability decreased by approximately 36.9% at 100 μM glibenclamide compared with the control (Figure 4(a)). Consequently, 50 μM was recommended as the experimental dosage as glibenclamide at this concentration had no obvious cytotoxicity. PMs were first primed with LPS, then pretreated with glibenclamide, and lastly stimulated with the NLRP3 stimulus ATP. LPS stimulation promoted the expressions of NLRP3 mRNA and protein in PMs, and these upregulations were inhibited by glibenclamide treatment (Figures 4(b)–4(f)). Likewise, glibenclamide suppressed the activation of Caspase-1 (Figures 4(c) and 4(d)) and the release of IL-1β and IL-18 enhanced by LPS administration (Figures 4(g) and 4(h)). In contrast, the LPS-induced increase in expression of TNF-α was still not decreased after glibenclamide treatment (Figure 4(i)), which was consistent with the in vivo results (Figures 2(f) and 2(i)).

4. Discussion

In the current study, we revealed a previously unrecognized protective role of glibenclamide against LPS-induced acute lung injury. Glibenclamide could improve the pathological injury of lungs and attenuate pulmonary inflammation in a mouse model of LPS-induced ALI. Mechanistically, this protective effect is related to downregulations in the expression and activation of NLRP3/Caspase-1/IL-1β signaling pathway in vivo and in vitro. In addition, the inhibition in the inflammatory response by glibenclamide is partly specific to target NLRP3 inflammasome as it has no effect on the production/secretion of other inflammatory cytokines like TNF-α.

It is well-known that ALI/ARDS is characterized by sustained inflammation, excessive oxidative stress, and loss of alveolar-capillary membrane integrity, leading to increased lung microvascular permeability, alveolar edema, and leukocyte extravasations [1–3]. And so far, there have been considerable interventions described in the publications to prevent ALI/ARDS [23–25]. Among them, the anti-inflammatory property of glibenclamide in ALI/ARDS has been increasingly concerned and validated effectively in various animal models of ALI, including oleic acid-, ozone-, radiation-, hemorrhagic shock-, and ventilator-induced ALI [11–14]. In our study, administration of glibenclamide inhibits LPS-stimulated lung edema, vascular hyperpermeability damage, and inflammatory cell infiltration. These results indicate that in addition to the various models of ALI reported in previous studies [11–14], glibenclamide also exerts a protective role in the current model of LPS-induced ALI.

Based on our previous research, the mechanisms of glibenclamide underlying its anti-inflammatory role are summarized as follows [10]: (1) inhibiting the activation of NLRP3/IL-1β signaling, (2) downregulating the

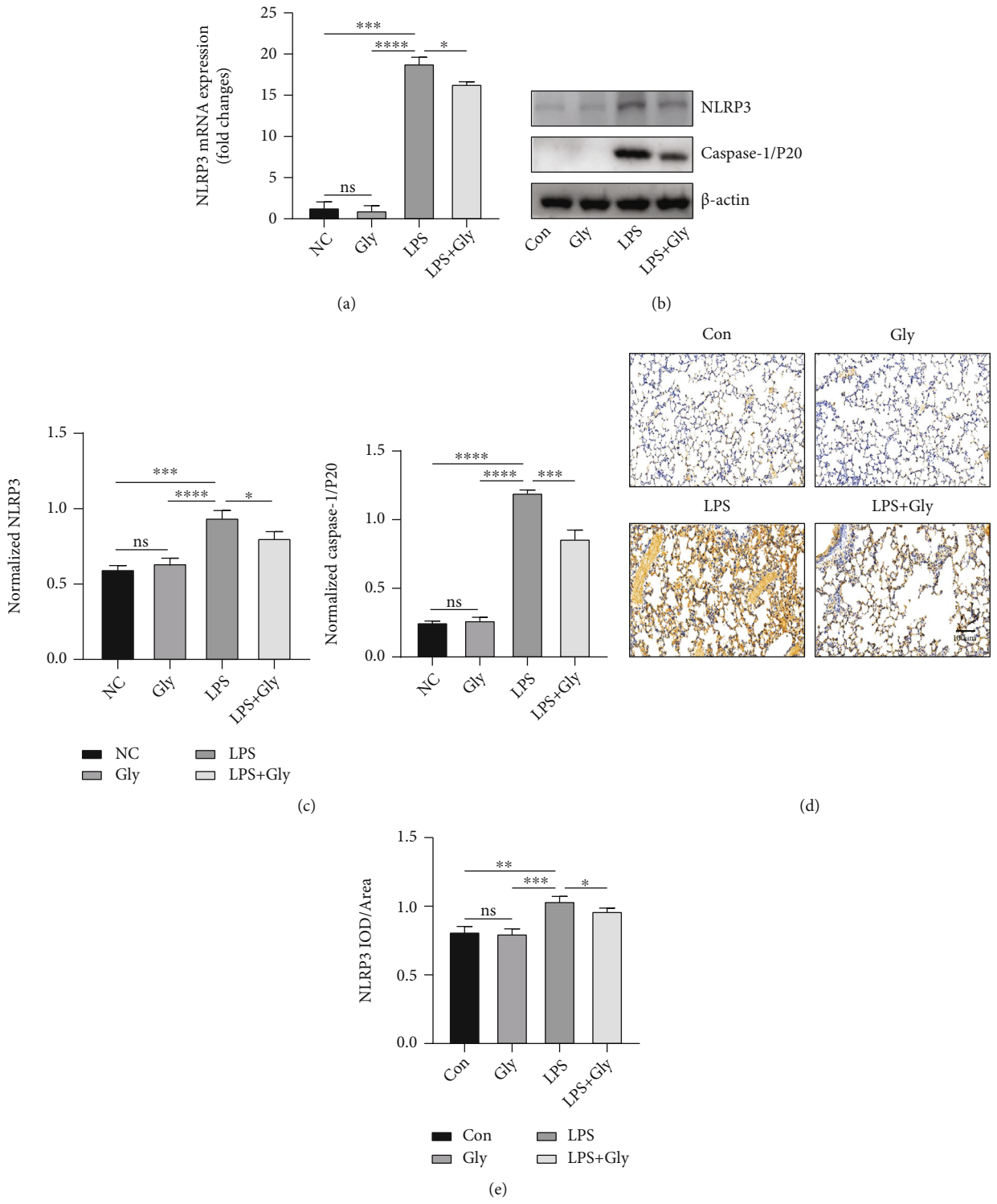

Figure 3: The inhibition role of glibenclamide in the NLRP3/Caspase-1/IL-1β signaling pathway induced by LPS. (a) Relative NLRP3 mRNA expression in lungs was measured by real-time PCR ($n = 3$). (b, c) Representative images of Western blot of NLRP3 and Caspase-1/p20 in the lungs and quantitative analysis ($n = 3$). (d, e) Representative images of immunohistochemical staining of NLRP3 in the lungs and quantitative analysis ($n = 3$). Scale bars, 100 μm. Data are representative of three independent experiments (mean and SD). ns: not significant. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$ (unpaired Student's t-test).

generation of reactive oxygen species, and (3) suppressing the migration of inflammatory cells like neutrophils and eosinophil. In the current research, the LPS-induced increases in levels of proinflammatory cytokines such as IL-1β and IL-18 and inflammatory cells like neutrophils and macrophages were decreased remarkably by

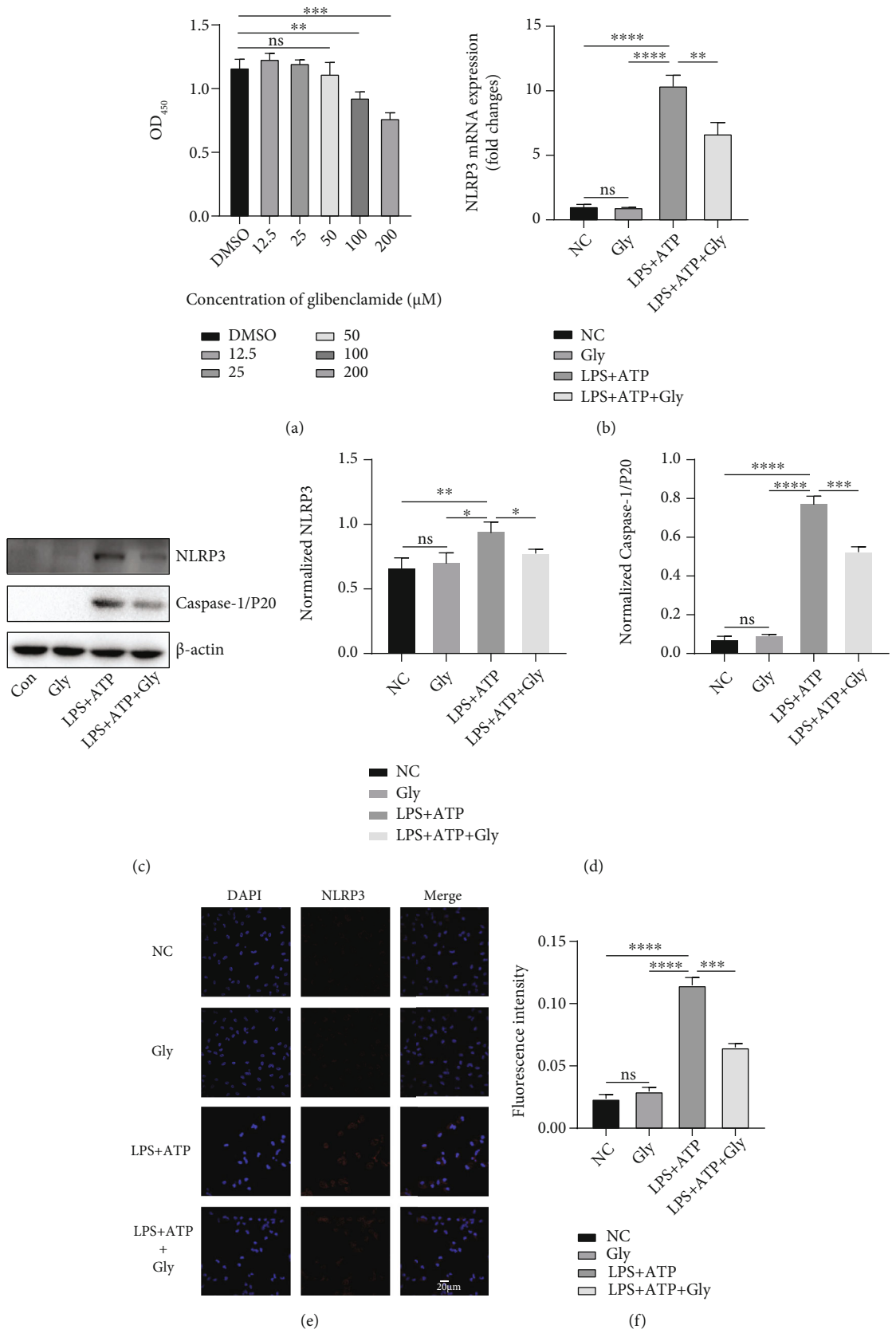

Figure 4: Continued.

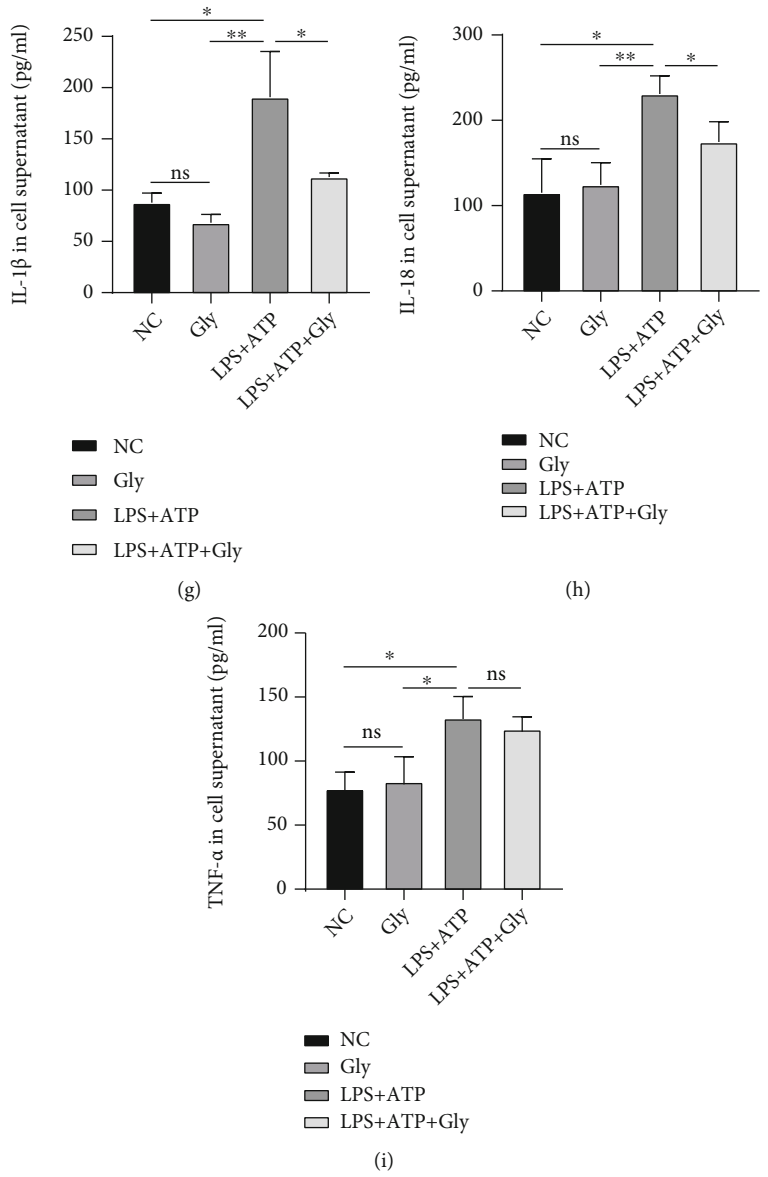

Figure 4: A protective role of glibenclamide in vitro inflammatory model. (a) CCK-8 assay was used to determine glibenclamide cytotoxicity ($n = 3$). (b) Relative NLRP3 mRNA expression in PMs was measured by real-time PCR ($n = 3$). (c, d) Representative images of Western blot of NLRP3 and Caspase-1/p20 in PMs and quantitative analysis ($n = 3$). (e) Representative immunofluorescence staining of NLRP3 in PMs. (f) Calculated percentage of NLRP3-positive nuclei ($n = 3$). (g–i) The level of IL-1β, IL-18, and TNF-α in cell supernatant was measured by ELISA ($n = 3$). Data are representative of three independent experiments (mean and SD). Scale bars, 20 μm. ns: not significant. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$ (unpaired Student's t-test).

glibenclamide in BALF and lung tissues. As IL-1β and IL-18 were known as the indicator of NLRP3 inflammasome induction [26–28], we hypothesized that the protective response of glibenclamide might be associated with its inhibition of NLRP3 inflammation. Indeed, a large number of studies have found that NLRP3 inflammasome plays an important role in ALI [14, 29]. Consistently, NLRP3/Caspase-1/IL-1β signaling was activated in vivo and in vitro after LPS or LPS plus ATP treatment, while the activation of NLRP3 inflammasome was inhibited by glibenclamide in our study. Thus, we confirmed that glibenclamide exerts its anti-inflammatory effect mainly by blocking NLRP3 signaling pathway.

Moreover, we noticed that the secretion of TNF-α, considered as an inflammasome-unrelated cytokine, was not impaired by glibenclamide. Consistent with previous studies [30, 31], our result found that glibenclamide did not affect LPS-stimulated TNF-α production, ruling out a more general anti-inflammatory effect by glibenclamide. These results suggest that the anti-inflammatory effect of glibenclamide is specifically related to NLRP3 inflammasome signaling pathway in LPS-induced ALI, though it could inhibit Th2 cytokines in ovalbumin-induced mouse model of asthma in our previous study [32].

Some limitations also exist in the current research. First, we did not determine the role of glibenclamide on

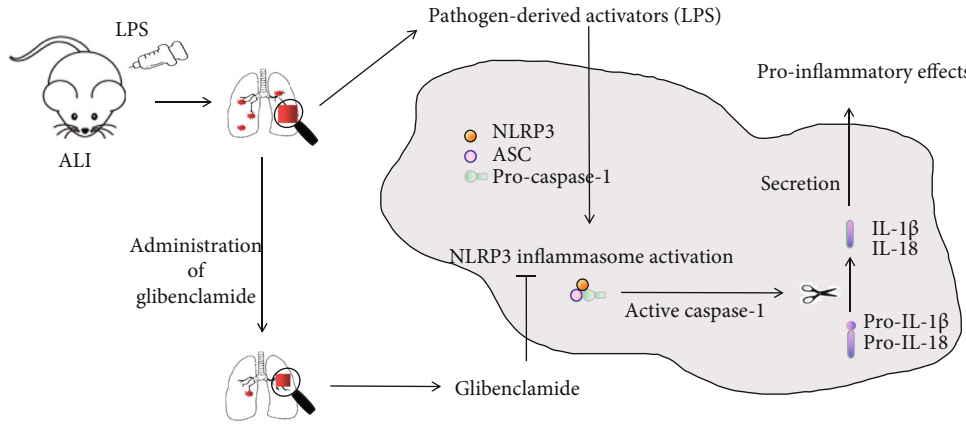

Figure 5: Schematic diagram of the role of glibenclamide on NLRP3 inflammasome in the pathogenesis of LPS-induced ALI. Glibenclamide blocked the activation of NLRP3 inflammasome induced by LPS and thus reduced the release of proinflammatory cytokines and then attenuated the lung injury consequently.

other inflammasome complexes such as NLRP1, NLRC4, and AMI2. Second, we did not establish the model of LPS-induced ALI in NLRP3-/-mice, so the extent to which glibenclamide blocked the NLRP3 inflammasome has not been better clarified. Third, TNF-α is the product of activation of multiple inflammatory signaling pathways including the generic mitogen-activated protein kinases (MAPK) signaling pathway [33], the Janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathway [34], and Hedgehog pathway [35]. Although the secretion of TNF-α was similar regardless of glibenclamide treatment, we did not observe whether glibenclamide has a role in the activation of these aforementioned multiple inflammatory signaling pathways. Thus, the evidence for the idea that the anti-inflammatory effect of glibenclamide is relatively specific to NLRP3 inflammasome seems not so solid. Last, we did not measure the blood glucose concentration in mice in the current study. Although the mouse model in the current study is different from the previous one which was ovalbumin-induced allergic asthma, we used the same therapeutic dose as before as 40 μmol/kg for the treatment of LPS-induced ALI/ARDS and we did not make a fast pretreatment prior to glibenclamide administration as our previous study shown [32]. According to our previous work, we observed that the blood glucose concentration of mice was not affected at this concentration without a fast pretreatment prior to glibenclamide administration [32]; thus, we consider that the dose of glibenclamide at 40 μmol/kg has no effect on blood glucose of mice in this study.

5. Conclusion

We demonstrated that glibenclamide alleviates LPS-induced ALI injury via an inhibition of inflammatory response, which is attributed to the suppression of NLRP3 inflammasome (Figure 5). Taken together, our results provide evidence that glibenclamide might be a promising candidate for the adjuvant therapy for LPS-induced ALI.

Data Availability

All data of this study are available from the first author Yang G or the correspoinding author Zhang G if needed.

Conflicts of Interest

There was no competing interest to declare.

Authors' Contributions

Jie Yang, Jiawen Yang, and Xiaofang Huang designed the study, performed the majority of the experiments, analyzed the data, and wrote the manuscript; Huiqing Xiu, Songjie Bai, and Jiahui Li performed experiments and analyzed the data; Zhijian Cai provided expert technical assistance, clinical advice, and critical discussion of work; Zhanghui Chen, Shufang Zhang, and Gensheng Zhang designed the study, supervised the project, and revised the manuscript. Jie Yang, Jiawen Yang, and Xiaofang Huang contributed equally to this work.

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References

[1] J. Villar, D. Sulemanji, and R. M. Kacmarek, "The acute respiratory distress syndrome: incidence and mortality, has it changed?," *Current Opinion in Critical Care*, vol. 20, no. 1, pp. 3–9, 2014.

[2] B. T. Thompson, R. C. Chambers, and K. D. Liu, "Acute respiratory distress syndrome," *The New England Journal of Medicine*, vol. 377, no. 6, pp. 562–572, 2017.

[3] V. Fanelli and V. M. Ranieri, "Mechanisms and clinical consequences of acute lung injury," *Annals of the American Thoracic Society*, vol. 12, Supplement 1, pp. S3–S8, 2015.

[4] F. Bauernfeind, A. Ablasser, E. Bartok et al., "Inflammasomes: current understanding and open questions," *Cellular and Molecular Life Sciences*, vol. 68, no. 5, pp. 765–783, 2011.

[5] D. De Nardo, C. M. De Nardo, and E. Latz, "New insights into mechanisms controlling the NLRP3 inflammasome and its role in lung disease," *The American Journal of Pathology*, vol. 184, no. 1, pp. 42–54, 2014.

[6] O. Gross, C. J. Thomas, G. Guarda, and J. Tschopp, "The inflammasome: an integrated view," *Immunological Reviews*, vol. 243, no. 1, pp. 136–151, 2011.

[7] M. Xiang, X. Shi, Y. Li et al., "Hemorrhagic shock activation of NLRP3 inflammasome in lung endothelial cells," *Journal of immunology*, vol. 187, no. 9, pp. 4809–4817, 2011.

[8] N. Kolliputi, R. S. Shaik, and A. B. Waxman, "The inflammasome mediates hyperoxia-induced alveolar cell permeability," *Journal of immunology*, vol. 184, no. 10, pp. 5819–5826, 2010.

[9] N. Kolliputi, L. Galam, P. T. Parthasarathy, S. M. Tipparaju, and R. F. Lockey, "NALP-3 inflammasome silencing attenuates ceramide-induced transepithelial permeability," *Journal of Cellular Physiology*, vol. 227, no. 9, pp. 3310–3316, 2012.

[10] G. Zhang, X. Lin, S. Zhang, H. Xiu, C. Pan, and W. Cui, "A protective role of glibenclamide in inflammation-associated injury," *Mediators of Inflammation*, vol. 2017, Article ID 3578702, 2017.

[11] H. Chen, Y. Ding, W. Chen, Y. Feng, and G. Shi, "Glibenclamide alleviates inflammation in oleic acid model of acute lung injury through NLRP3 inflammasome signaling pathway," *Drug Design, Development and Therapy*, vol. 13, pp. 1545–1554, 2019.

[12] H. Shao, L. Huang, S. Duan et al., "Glyburide attenuates ozone-induced pulmonary inflammation and injury by blocking the NLRP3 inflammasome," *Environmental Toxicology*, vol. 35, no. 8, pp. 831–839, 2020.

[13] P. Xia, K. Cao, X. Hu et al., "KATP channel blocker glibenclamide prevents radiation-induced lung injury and inhibits radiation-induced apoptosis of vascular endothelial cells by increased ca (2+) influx and subsequent PKC activation," *Radiation Research*, vol. 193, no. 2, pp. 171–185, 2020.

[14] M. T. Kuipers, H. Aslami, J. R. Janczy et al., "Ventilator-induced lung injury is mediated by the NLRP3 inflammasome," *Anesthesiology*, vol. 116, no. 5, pp. 1104–1115, 2012.

[15] G. D. Rubenfeld, E. Caldwell, E. Peabody et al., "Incidence and outcomes of acute lung injury," *The New England Journal of Medicine*, vol. 353, no. 16, pp. 1685–1693, 2005.

[16] U. Seydel, A. Ulmer, S. Uhlig, and E. T. Rietschel, "Lipopolysaccharide a membrane-forming and inflammation-inducing bacterial macromolecule," *Membrane Structure in Disease and Drug Therapy*, pp. 217–252, 2000.

[17] N. Chaudhuri, M. K. Whyte, and I. Sabroe, "Reducing the toll of inflammatory lung disease," *Chest*, vol. 131, no. 5, pp. 1550–1556, 2007.

[18] H. M. Wang, M. Bodenstein, and K. Markstaller, "Overview of the pathology of three widely used animal models of acute lung injury," *European Surgical Research*, vol. 40, no. 4, pp. 305–316, 2008.

[19] G. Zhang, P. Wang, Z. Qiu et al., "Distant lymph nodes serve as pools of Th1 cells induced by neonatal BCG vaccination for the prevention of asthma in mice," *Allergy*, vol. 68, no. 3, pp. 330–338, 2013.

[20] L. K. Johnston, C. R. Rims, S. E. Gill, J. K. Mcguire, and A. M. Manicone, "Pulmonary macrophage subpopulations in the induction and resolution of acute lung injury," *American Journal of Respiratory Cell and Molecular Biology*, vol. 47, no. 4, pp. 417–426, 2012.

[21] Y. Li, X. Chen, H. Zhang et al., "4-Octyl itaconate alleviates lipopolysaccharide-induced acute lung injury in mice by inhibiting oxidative stress and inflammation," *Drug Design, Development and Therapy*, vol. 14, pp. 5547–5558, 2020.

[22] X. Ge, Z. Feng, T. Xu et al., "A novel imidazopyridine derivative, X22, attenuates sepsis-induced lung and liver injury by inhibiting the inflammatory response in vitro and in vivo," *Drug Design, Development and Therapy*, vol. 10, pp. 1947–1959, 2016.

[23] W. C. Huang, C. L. Lai, Y. T. Liang, H. C. Hung, H. C. Liu, and C. J. Liou, "Phloretin attenuates LPS-induced acute lung injury in mice via modulation of the NF-kappa B and MAPK pathways," *International Immunopharmacology*, vol. 40, pp. 98–105, 2016.

[24] J. S. Chang, H. J. Lin, J. S. Deng, W. T. Wu, S. S. Huang, and G. J. Huang, "Preventive effects of velvet antler (Cervus elaphus) against lipopolysaccharide-induced acute lung injury in mice by inhibiting MAPK/NF-kappa B activation and inducing AMPK/Nrf 2 pathways," *Evidence-based Complementary and Alternative Medicine*, vol. 2018, Article ID 2870503, 2018.

[25] H. Lv, Q. Liu, Z. Wen, H. Feng, X. Deng, and X. Ci, "Xanthohumol ameliorates lipopolysaccharide (LPS)-induced acute lung injury via induction of AMPK/GSK3beta-Nrf 2 signal axis," *Redox Biology*, vol. 12, pp. 311–324, 2017.

[26] T. D. Kanneganti, M. Lamkanfi, Y. G. Kim et al., "Pannexin-1-mediated recognition of bacterial molecules activates the cryopyrin inflammasome independent of Toll-like receptor signaling," *Immunity*, vol. 26, no. 4, pp. 433–443, 2007.

[27] Q. Zhang, D. Wu, Y. Yang, T. Liu, and H. Liu, "Dexmedetomidine alleviates hyperoxia-induced acute lung injury via inhibiting NLRP3 inflammasome activation," *Cellular Physiology and Biochemistry*, vol. 42, no. 5, pp. 1907–1919, 2017.

[28] Y. P. Luo, L. Jiang, K. Kang et al., "Hemin inhibits NLRP3 inflammasome activation in sepsis-induced acute lung injury, involving heme oxygenase-1," *International Immunopharmacology*, vol. 20, no. 1, pp. 24–32, 2014.

[29] J. Wu, Z. Yan, D. E. Schwartz, J. Yu, A. B. Malik, and G. Hu, "Activation of NLRP3 inflammasome in alveolar macrophages contributes to mechanical stretch-induced lung inflammation and injury," *Journal of immunology*, vol. 190, no. 7, pp. 3590–3599, 2013.

[30] M. Lamkanfi, J. L. Mueller, A. C. Vitari et al., "Glyburide inhibits the Cryopyrin/Nalp 3 inflammasome," *The Journal of Cell Biology*, vol. 187, no. 1, pp. 61–70, 2009.

[31] G. C. Koh, T. A. Weehuizen, K. Breitbach et al., "Glyburide reduces bacterial dissemination in a mouse model of melioidosis," *PLoS Neglected Tropical Diseases*, vol. 7, no. 10, p. e2500, 2013.

[32] W. Cui, S. Zhang, Z. Cai et al., "The antidiabetic agent glibenclamide protects airway hyperresponsiveness and inflammation in mice," *Inflammation*, vol. 38, no. 2, pp. 835–845, 2015.

[33] M. Li, J. Ye, G. Zhao et al., "Gas6 attenuates lipopolysaccharide-induced TNF-α expression and apoptosis in H9C2 cells through NF-κB and MAPK inhibition via the Axl/PI3K/Akt pathway," *International Journal of Molecular Medicine*, vol. 44, no. 3, pp. 982–994, 2019.

[34] S. F. Ahmad, M. A. Ansari, K. M. Zoheir et al., "Regulation of TNF-alpha and NF-kappa B activation through the JAK/STAT signaling pathway downstream of histamine 4 receptor in a rat model of LPS-induced joint inflammation," *Immunobiology*, vol. 220, no. 7, pp. 889–898, 2015.

[35] H. Zhen, L. Zhao, Z. Ling, L. Kuo, X. Xue, and J. Feng, "Wip1 regulates blood-brain barrier function and neuro-inflammation induced by lipopolysaccharide via the sonic hedgehog signaling signaling pathway," *Molecular Immunology*, vol. 93, pp. 31–37, 2018.

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Review Article

The Role of Cytokines in Nephrotic Syndrome

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Idiopathic nephrotic syndrome (INS) is an important primary glomerular disease characterized by severe proteinuria. Evidence supports a role for T cell dysfunction in the pathogenesis of INS. Glucocorticoids are the primary therapy for INS; however, steroid-resistant NS (SRNS) patients are at a higher risk of drug-induced side effects and harbor poor prognosis. Although the exact mechanism of the resistance is unknown, the imbalances of T helper subtype 1 (Th1), Th2, and regulatory T cells (Tregs) and their cytokines may be involved in the pathogenesis of glucocorticoid responsiveness. Up to now, no confirmed biomarkers have been able to predict SRNS; however, a panel of cytokines may predict responsiveness and identify SRNS patients. Thus, the introduction of distinctive cytokines as novel biomarkers of SRNS enables both preventions of drug-related toxicity and earlier switch to more effective therapies. This review highlights the impacts of T cell population imbalances and their downstream cytokines on response to glucocorticoid responsiveness state in INS.

1. Introduction

Idiopathic nephrotic syndrome (INS) is a clinical definition, described by extreme proteinuria due to podocyte injury and foot process effacement. Focal segmental glomerulosclerosis (FSGS) and minimal change disease (MCD) are the two most important light microscopic pictures of this glomerular disease and the most common causes of INS in both adults and pediatrics. Despite the current lack of knowledge in a comprehensive understanding of the disease mechanism, the response to glucocorticoids and/or other immunosuppressant agents indicates the primary involvement of the immune system. The current observations are in favor of the association of T regulatory (Treg), T helper subtype 1 (Th1), and T helper subtype 2 (Th2) imbalances and their related cytokines in the pathogenesis of INS [1, 2].

The activation of the inflammation cascades is heterogeneous and diverse in FSGS or MCD. A sequential production of proinflammatory cytokines leads to a systemic inflammatory response initiated with the synthesis of IL-1 and TNF-α (TNF), which, in turn, escalates the generation of IL-6. The production of cytokines stimulates the formation of acute-phase proteins such as haptoglobin, haemopexin, or C-reactive protein (CRP), suPAR (soluble urokinase-type plasminogen activator receptor), α-1 antitrypsin, and fibrinogen in the liver. Alpha-1 antitrypsin and fibrinogen are more sensitive to IL-6 stimulation while others are generally synthesized in response to IL-1 [3]. Different studies have reported the plausible connection between cytokine production and proteinuria in INS [4, 5]. However, conflicting results have been obtained when the serum levels of the major cytokines and acute-phase proteins are measured in patients with NS [6–8].

Glucocorticoids are the standard initial pharmacological regimen in INS, which block the production of cytokines in both immune and nonimmune cells effectively and result in

remissions in approximately 85-90% of pediatric cases. However, individuals exhibit different degrees of glucocorticoid responsiveness and variable patterns of relapses [9]. Glucocorticoids represent a key index of outcomes, and drug-resistant patients pose a challenge to clinicians. Furthermore, glucocorticoid dependency is observed in about 40–50% of the responders who are at high risk of therapy-associated unwanted effects [10]. Indeed, no clinical test is available to predict steroid resistance and/or dependence.

The response to glucocorticoids has been considered as the key variable in long-term outcomes of FSGS and MCD patients [11]. The potential effects of glucocorticoids highlight the possible role of cytokines in determining the drug response. SRNS patients without podocyte genetic defects may also respond to other immunosuppressive agents, such as cyclosporine, tacrolimus, and mycophenolate.

2. Factors Involved in SRNS

The impacts of epigenetic, pharmacogenetic, and genetic factors on the pathogenesis of SRNS have been comprehensively reviewed previously [12–15]. In the presence of podocyte cytoskeletal-related mutations, glucocorticoids are ineffective at restoring normal podocyte function. About 30% of SRNS patients have mutations in one of the podocyte-expressed genes. Circulating factors, such as serum urokinase-type plasminogen activator receptor or cardiotrophin-like cytokine 1, are another proposed pathogenic mechanism [16]. Cytokines are reported to modulate the glucocorticoid responses in NS [17–19]. In the following sections, we provide reported articles linking imbalanced T cell populations and their dysregulated cytokines to SRNS.

3. Cytokines Affect the Responses to Glucocorticoid Therapy

Because of the controversial reports regarding the cytokine patterns of Th1/Th2, subtypes, and glucocorticoid response, studies are aimed at introducing these possible biomarkers [20, 21]. In the following sections, we focus on the impacts of T cell population imbalance and its downstream cytokines on SRNS.

3.1. T Cell Population Imbalance in SRNS. Despite conflicting evidence, the imbalance between Th1, Th2, and Treg cells has been associated with the incidence of SRNS. If glucocorticoids mediate alterations in T cells' population and their cytokine profile, then steroid-sensitive NS (SSNS) and SRNS patients should have differences in their T cell populations. It has been demonstrated that Th1/Treg and Th2/Treg ratios are higher in SRNS compared to SSNS patients and healthy individuals, while Th1/Th2 ratios are similar among the groups. A higher ratio of Treg in comparison with Th1 and Th2 is connected with glucocorticoid sensitivity, while the reverse ratio is associated with SRNS [22]. Guimarães et al. made a study on a group of children with INS (steroid-sensitive (16 boys/9 girls) and steroid-resistant 8/6) and 10 healthy controls. They observed downregulated levels of adhesion molecules (integrin, CD18) and higher levels (48%) of Treg ($TCD4^+CTLA\text{-}4^+$ $FoxP3^+$) in the steroid-sensitive group [23]. NS patients who are more prone to relapse or do not respond to glucocorticoids show an immunological switching from Th2 to Th1 [24]. In line with these findings, serum cytokines shift toward the Th1 pattern in FSGS patients [24]. Additionally, in a study on a group of INS children (29 SSNS and 14 SRNS children, aged between 2 and 19 years), higher levels of Th1 cytokines (e.g., IL-2) have been found in their serum and urine samples, whereas elevated Th2-related cytokine (i.e., IL-4) generation was associated with long-term remission [5]. However, both glucocorticoid sensitive and resistant patients show similar levels of Th1- and Th2-associated cytokines; these differences might be due to different lymphocyte stimuli [4]. Stachowski and coworkers also reported similar results and concluded that the $CD4^+$ T cell-related cytokine pattern and the distribution of particular T cell subsets, including suppressor-effector ($CD45RA^+CD8^+$), suppressor-inducer ($CD45RA^+CD4^+$), and memory cells ($CD45RO^+CD4^+$), might predict the patients' sensitivity to glucocorticoids at the onset of NS [25]. The importance of the Th1/Th2 balance has been confirmed by increased levels of Th1 cytokines (including IL-2, soluble IL-2 receptor (sIL-2R), and IFN-γ) in SSNS patients during relapse [26]. Hence, assessing the balance of Th1/Th2 could be valuable in predicting glucocorticoid responsiveness.

Effective glucocorticoid therapy has been shown to restore the functional balance of the Th-17/Treg population in MCD patients [27]. Moreover, primary glucocorticoid therapy has reduced $CD8^+$T, Th2, and $CD4^+$ Th1 cells in NS patients. Accordingly, glucocorticoid therapy effectively diminishes $CD8^+$T, Th2, and $CD4^+$ Th1 cells in new-onset pediatric NS cases [28].

Response to glucocorticoid therapy in children with NS is influenced by the levels of IL-13 and TNF-β (lymphotoxin-alpha). Elevated levels of TNF-β are observed in SRNS patients after treatment while SSNS cases developed higher levels of IL-13. Increased levels of IL-13 may be in connection with TNF-β downregulation in SSNS patients since the latter is suppressed via Th2 cytokines [29]. Interaction between TNF receptor and soluble lymphotoxin-alpha promotes inflammatory responses. T cell deviation towards the Th2 population in NS patients might also be linked to the overproduction of IL-13. These findings propose that Th1-dominant patients might develop glucocorticoid-resistance, while increased IL-13 and Th2 phenotypes are in favor of a satisfactory outcome, and glucocorticoid responsiveness. Therefore, alteration in Th1 and Th2 populations and subsequent changes in IL-13/TNF-β cytokines balance substantially affect NS pathophysiology in children [29].

3.2. T Cell Resistance to Glucocorticoids. Particular mediators influence T cell resistance to glucocorticoids. For example, IL-2 and IL-4 promote lymphocyte glucocorticoid resistance during an in vitro study [30]. In addition, nuclear factor-κB (NF-κB) and transcription factor activator protein-1 (AP-1) are pivotal mediators of proinflammatory cytokine generation and have been found to interfere with glucocorticoid functions on T cells [31]. In this context, the glucocorticoid

receptor α (GRα) suppresses AP-1 activity via direct protein-protein interaction with a c-Jun subunit of the AP-1 family [32]. Interestingly, it is documented that AP-1 modulates the structure of basal chromatin and increases the accessibility of GR and its binding to proinflammatory genes [33]. Hence, it appears that the interactions between AP-1 and glucocorticoids are far more complicated.

3.2.1. NF-κB Signaling. NF-κB is a transcription factor that regulates the transcription of genes participating in inflammation. Sun and colleagues reported that the overexpression of NF-κB in the juvenile Sprague-Dawley rat model of nephrotic syndrome induces the expression of inflammatory cytokines (IL-1 and IL-6), increases blood urea nitrogen and creatinine levels, and exacerbates renal injury [34]. NF-κB as a member of the Rel family contains two subunits (p50 and p65) [35]. The binding of NF-κB to the endogenous IκB family proteins makes it inactive. The release of NF-κB from IκB occurs upon antigenic stimulation and subsequent phosphorylation of IκB via IκB kinases α and β. SRNS patients have a lower level of NF-κB p65 subunit in the whole-cell lysates, prepared from the peripheral mononuclear blood cells (PMBC) compared to glucocorticoid-sensitive cases [36]. Both lower levels of NF-κB p65 and GRα are connected with poor glucocorticoid responses in some patients with INS. This difference is more prominent in those experiencing relapses [36]. However, both SSNS and SRNS patients express similar levels of the p50 subunit. The translocation of the NF-κB p50 subunit into the nucleus is essential for the interaction of NF-κB with glucocorticoids, and the absence of such translocation impairs the ability of GRs to inhibit immune functions and NF-κB transcriptional activity, inducing glucocorticoid resistance [31, 32].

The expression of IL-2 is also increased during the relapse of both SSNS and SRNS patients in comparison with controls. These results indicated alterations in the T cell populations between untreated SRNS and SSNS patients. The upregulation of IL-2 and down-regulation of NF-κB p65 subunits are possible mechanisms of glucocorticoid resistance in NS [37]. It has been reported that three mechanisms are involved in this process. First, the absence of required protein-protein interactions, especially among GRα and p65 subunits. Second, disturbances in nuclear export of NF-κB dimers, and third plunged affinity of NF-κB for the glucocorticoid-stimulated leucine zipper that acts as an inhibitor of NF-κB nuclear translocation [37]. In SRNS patients, steroid-based treatment might fail by enhancing NF-κB function, which would worsen disease by elevating transcription of inflammatory cytokines [38].

4. Cytokines in SRNS

The prevalence of relapses in NS has been associated with the serum levels of particular cytokines (Table 1). Some researchers have attempted to identify urinary, plasma, and salivary cytokine-based biomarkers for SRNS in children [39–41]. Both SSNS and SRNS patients have shown suppressed levels of IL-5, IL-7, IL-13, IFN-γ, and TNF after glucocorticoid administration. Furthermore, SRNS patients have been shown to have higher levels of MIP-1β, IL-17A, IL-5, and INF-γ in comparison with SSNS cases in pre- and posttreatment specimens. Agrawal et al. studied the plasma profile of cytokines in children [SSNS ($n = 26$) and SRNS ($n = 14$)] aged between 18 months and 18 years before and after (7 weeks) treatment with glucocorticoids. Using a bead-based fluorescence assay, the profiling of 27 cytokines was evaluated on a Luminex Technology platform (Waltham, MA). Different levels of 13 plasma cytokines were observed between SSNS versus SRNS before therapy. Three cytokines (IL-7, IL-9, and MCP-1) exhibited ROC (receiver operating characteristic) values of 0.846, 0.64 sensitivity, and 0.84 specificity and could differentiate children with SRNS from those with SSNS at the disease onset. Furthermore, their results detected significant reductions in cytokine levels (e.g., IFN-γ, TNF, IL-5, IL-7, and IL-13) in response to glucocorticoid treatment in SSNS compared to SRNS patients. The authors proposed that glucocorticoid therapy decreases cytokine production by $CD4^+$ Th1 cells, Th2 cells, and $CD8^+$ cells in children with new-onset NS [28] (Figure 1).

Increased IL-8 concentration has been associated with relapses in NS [42] and antibodies against IL-8 could neutralize the ability of mononuclear cells to trigger albuminuria in the Wistar rat model [43]. Moreover, surged amount of IL-1β, IL-6, and IL-8 has been observed in INS relapses compared to healthy controls or remission in children [44]. In addition, IL-4, IL-6, and TNF polymorphisms have been in connection with glucocorticoid responsiveness in INS children [45]. The activation of TGF-β1 has been reported in SRNS cases, which further develop chronic kidney disease (CKD). FSGS patients have shown higher levels of urinary TGF-β1 compared to MCD patients. However, urinary TGF-β1 has not been validated as a glucocorticoid responsiveness biomarker [19]. Elevated serum levels of IL-6, haptoglobin, and haemopexin are also independent markers of glucocorticoid resistance in FSGS and MCD patients [3].

T cell expressing inflammatory cytokines, plasma macrophage migration inhibitory factor (MIF), and urinary MCP-1 are increased during persistent proteinuria in pediatric SRNS [41]. The role of glomerular macrophages and the underlying mechanism of macrophage-related glucocorticoid resistance have not been clarified. The substantial connection between urinary MCP-1 and IL-6 or interferon-inducible protein-10 (IP-10) suggests that the MCP-1-stimulated macrophages can generate IL-6 or IP-10 after recruitment to the glomeruli, which might then lead to tissue damage and enrollment of other immune cells [46, 47].

4.1. MIF. MIF has been considered as a suitable marker for glucocorticoid responsiveness among 48 evaluated cytokines. According to cytokine analysis, the increased plasma concentrations of MIF (cutoff concentration of MIF > 501 pg/ml) at diagnosis could identify NS children at high risk of glucocorticoid resistance. Low levels (MIF mean concentration 124.5 pg/ml in healthy controls vs. 466.1 pg/ml in INS patients) of this cytokine could also successfully discriminate INS patients from controls [48]. MIF displays proinflammatory activities as a result of interactions with T cells

Table 1: Summary of articles evaluated the levels of cytokines in clinical samples of patients with SRNS.

		Participants	Age	Sample	Cytokines	Results	Ref.
1	Children	SS: 26 SR: 14	SS: 5.7 ± 0.7 SR: 9.5 ± 1.0	Plasma	A panel of 27 cytokines	In children with new-onset NS, glucocorticoid therapy decreases the levels of plasma cytokines secreted by $CD8^+$, $CD4^+$ TH1, and TH2 cells. Moreover, MCP-1, IL-9, and L-7 could predict SRNS prior to glucocorticoid therapy at disease presentation.	[28]
2	Children	C: 20 SS: 19 SR: 7	C:11.9 ± 2.5SS: 8.8 ± 3.9SR: 9.9 ± 5.5	Saliva	IL-1β, IL-4, IL-6, IL-8, and IFN-γ	The studied cytokines were not able to discriminate SRNS from SSNS or the relapse from remission states.	[40]
3	Children	SS: 29 SD: 24 SR: 9	SS: 4.3 (2–11) SD: 3.2 (1–13) SR: 8.5 (2–17)	Plasma	48 cytokines	Plasma levels of MIF can identify cases at high risk of SRNS. A cutoff MIF concentration of more than 501 pg/ml could discriminate SRNS cases with 71.4% specificity and 85.7% sensitivity.	[48]
4	Children	Remission: 9 SS: 6 SR: 4	5.9 (3.0–14.4)	PBMCs	18551 genes	In the SSNS group, the gene expression profile was enriched in genes relating to TGF-β1 signaling, IL-4 and IL-6, targets of FoxP3 in T lymphocytes, p53 signaling, and the cell cycle.	[21]
5	Children	C: 12 SR:12 SS: 12	C: 12.9 ± 4.3 SR: 12.3 ± 5.5 SS: 14.1 ± 4.7	Kidney and urine	TGF-1, ICAM-1	TGF-1 in urine could differentiate between MCD and FSGS; however, it was not a biomarker of steroid responsiveness.	[19]
6	Children	C: 15 NS: 42	—	Serum	IL-13, TNF-β	A different Th2/Th1 reaction demonstrated by an imbalance of IL-13/TNF-β could play a pathophysiologic role in NS. SRNS and SSNS cases had, respectively, a higher serum TNF-β and IL-13 level after glucocorticoid therapy than that before treatment.	[29]
7	Children	C: 10 SR: 20 SS: 34	SR: 11.3 (4–17) SS: 10.5 (4–16)	Plasma	IL-20, IL-4R, IL-6ST, JUN, MPL, MYC, SP1 and SRC, SOCS1-5	In SRNS cases, levels of IL-20, IL-6, SOCS5, and SOCS3 were elevated after 6 weeks of treatment with steroids compared to control and SSNS groups. Increased expressions of SOCS3 and SOCS5 mRNAs may predict early resistance to steroids.	[56]
8	Children	C: 5 SR: 8 SS: 8	—	$CD4^+$ T cell	IFN-c, NF-κB, AP-1	There were significant increase in IL-2 expression and decrease in the p65 subunit of NF-κB in T cells from SRNS patients.	[37]
9	Adults	SRNS/FSGS: 96	22 ± 13	Serum, immortalized thermosensitive human podocytes (clone AB8/13)	TNF-α pathway genes	In podocytes of FSGS patients, activation of TNF-α pathway genes happens.	[54]
10	Adults	SS: 50 SR: 27	C: 50 SS: 39.8 ± 19.2 SR: 45.3 ± 18.2		IL-6, IL-1, TNF, IFN-γ, CRP, suPAR, haemopexin, haptoglobin	Increased levels of IL-6, haemopexin, and haptoglobin are only associated with steroid resistance in a certain group of patients. In other cases, steroid resistance is clearly unrelated to an activated inflammatory response. Multivariate analyses indicated that the levels of these 3 inflammatory factors are independent predictors of SR.	[3]

C: control; SD: steroid dependent; SS: steroid sensitive, PBMCs: peripheral blood mononuclear cells; SRNS/FSGS: steroid-resistant nephrotic syndrome/Focal segmental glomerulosclerosis; MCD: minimal change disease; TNF-α: tumor necrosis factor-α; IFN-γ: interferon-gamma; NF-κB: nuclear factor-κB; AP-1: activating protein-1; IL-6: interleukin-6; suPAR: soluble urokinase-type plasminogen activator receptor; CRP: C-reactive protein.

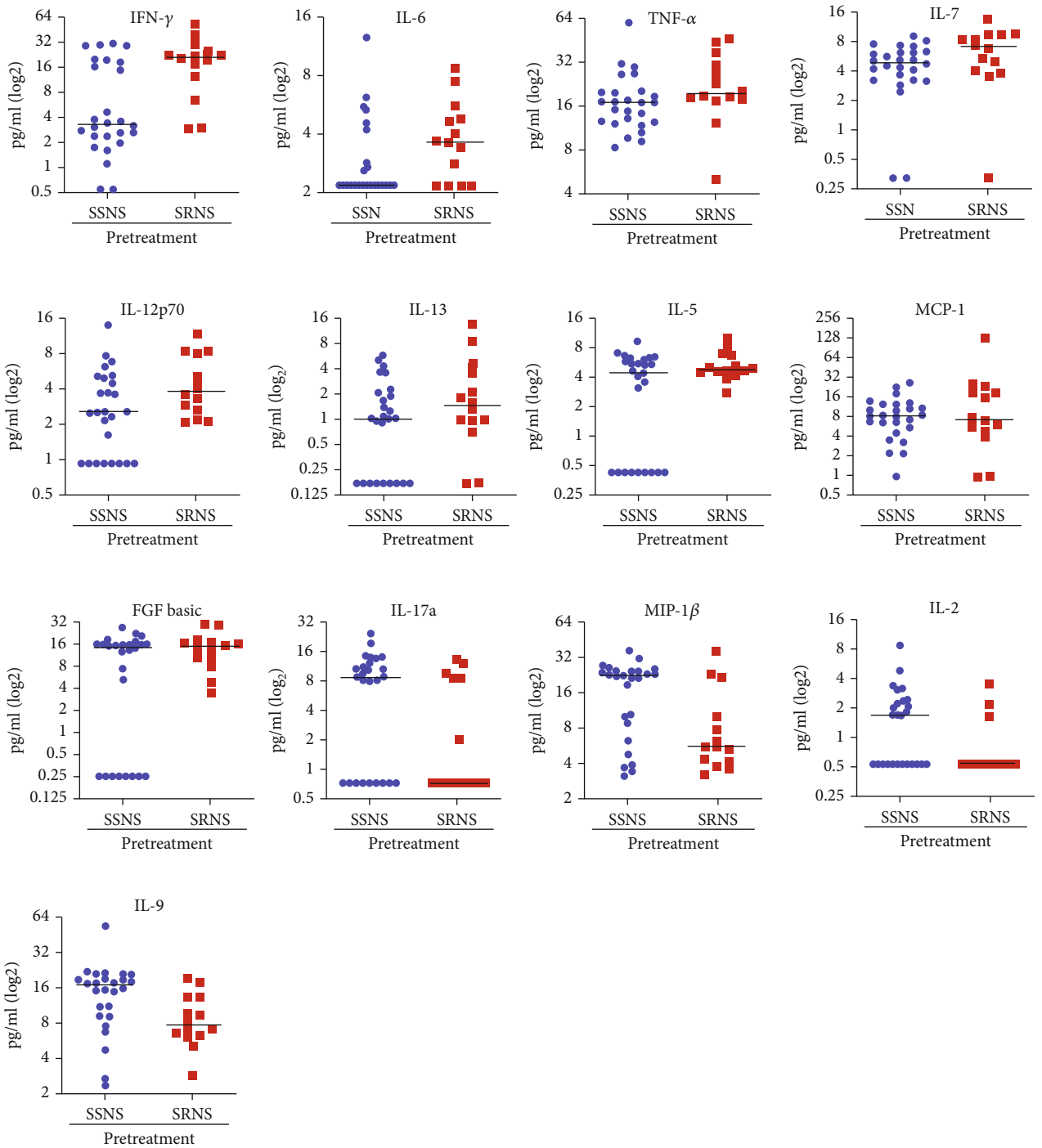

Figure 1: Cytokines can identify the SRNS cases before therapy. Different levels of 13 plasma cytokines were observed between SSNS versus SRNS before therapy, three of which (MCP-1, IL-9, and L-7) had values to discriminate SRNS from SSNS prior to glucocorticoid therapy with ROC value = 0.84, 0.64 sensitivity, and 0.84 specificity. FGF: fibroblast growth factor; MCP-1: monocyte chemoattractant protein-1; MIP-1β: macrophage inflammatory protein-1β; SSNS: steroid-sensitive nephrotic syndrome; SRNS: steroid-resistant nephrotic syndrome; TNF: tumor necrosis factor; ROC: receiver operating characteristic. Adapted from Ref. [28] with permission. The reference [28] article is available under the Creative Commons CC-BY-NC-ND license.

and macrophages. Glucocorticoids decrease the formation of inflammatory mediators; however, they accelerate MIF release from T cells and macrophages [49]. Then, MIF counterregulates the suppressor effects of glucocorticoids on proinflammatory cytokines [50]. Although the underlying mechanisms are not completely known, it has been postulated that MIF interferes with the function of glucocorticoid under an inflammatory condition mediated by NF-κB-dependent manner. Glucocorticoids prevent the NF-κB activation through the induction of IκBα synthesis, whereas MIF

enhances the translocation of NF-κB to the nucleus [51]. Furthermore, MIF potently induces the extracellular signal-regulated kinase- (ERK-) 1 and ERK-2 pathways, which in turn, activate the intracellular isoform of phospholipase A2 (PLA2) and lead to the liberation of arachidonic acid [52]. Glucocorticoids are recognized blockers of PLA2 stimulation, and this impact is countered by MIF. Additionally, to suppress the transcription of proinflammatory genes, glucocorticoids can raise the degradation of these mRNAs; moreover, this has been revealed to be linked to the inhibitory effect of MIF on glucocorticoids. Although there are inadequate data to elucidate the proinflammatory functions of MIF entirely, the mentioned mechanisms could describe its impact on glucocorticoid-related immunosuppression [48, 53].

4.2. TNF. In kidney glomeruli of patients with FSGS/SRNS, activation of the TNF pathway was observed [54]. TNF is an inflammatory cytokine produced by infiltrating/circulating macrophages and monocytes. The proposed TNF mechanisms of action includes (1) leukocyte recruitment to the glomerular damage site, (2) stimulation of growth factors and cytokines, and (3) generation of oxygen radicals. Consequently, glomerular endothelial damage, apoptosis, and albumin permeability could be the result of those TNF-mediated adverse effects [55]. The intrinsic activation of the TNF signaling pathway leads to podocyte damage that can be reversed by the TNF blockader [54].

4.3. Suppressors of Cytokine Signaling. Suppressors of Cytokine Signaling (SOCS) prohibit Signal Transducer and Activator of Transcription (STAT) phosphorylation via blocking Janus Kinases (JaKs), and the effects of glucocorticoids on the JaK/STAT signaling cascade in children with SRNS and SSNS have been investigated. Accordingly, IL-6, IL-20, SOCS3, and SOCS5 were significantly higher in plasma samples of SRNS patients in comparison with SSNS cases. Moreover, the authors suggested the potential role of SOCS3 and SOCS5 mRNA levels as predicting factors of glucocorticoid resistance in patients with NS [56]. Furthermore, substantial lower methylation of one region of the SOCS3 promoter was observed in SRNS participants versus SSNS and normal controls [56, 57].

4.4. Other Cytokines. The activation of T lymphocytes and release of IFN-γ, IL-4, and IL-2 have been seen in SSNS children with relapse [7]. The plasma level of IL-8 has significantly been in connection with IL-4 and IL-13 in all stages of SSNS in children. Likewise, during the active phase, increased levels of IL-13, IL-4, TNF, and IgE were significantly seen in pediatric SSNS compared to patients in remission and controls [58]. It is deemed that a type-2 cytokine production succeeds in children with active SSNS, and this kind of immune response is closely correlated with the expression of IL-18 [6]. Moreover, serum levels of IL-18 are associated with both IL-4 and IL-13 in pediatric SSNS patients [59]. However, it is also reported that increased levels of IL-18 after therapy can be involved in the SRNS development [60].

5. Treatment

The goal of SRNS therapy is inducing complete remission; however, even partial remission may have clinical benefits. For cases with nongenetic-based SRNS, treatment with calcineurin inhibitors (tacrolimus and ciclosporin) is the standard of care therapy and 70% of them attain a partial or complete remission. The renin-angiotensin inhibitors as antihypertensive and antiproteinuric are quintessential for decreasing proteinuria [61]. Proinflammatory cytokines derived from immune cells promote the formation of angiotensin II (Ang II) both systemically and locally. Production of angiotensinogen by inflammatory cytokines is suggested as a key mechanism for the development of Ang II-dependent high blood pressure [62]. Nonresponding patients to calcineurin inhibitors or immunosuppressives are at risk for ESRD [61].

Epigenetic modification by targeting histone deacetylases (HDACs) are a promising therapeutic approach in NS. Histone deacetylase inhibitors (HDACi) play an important role in treating CKD due to their anti-fibrotic, anti-inflammatory, and immunosuppressive activities. HDACi inhibits HDACs, remodels the structure of proteins in transcription factor complexes, and causes modifications in gene transcription by removing the acetyl groups from the lysine amino acid on histone. Thus, HDACi enhances chromatin condensation and exerts a repressor effect on transcription. It is a promising intervention for targeting glomerular sclerosis and fibrosis as important pathologic features of fibrosis and CKD progression both in FSGS and INS patients. Moreover, evidence from various research has demonstrated an irregular expression of HDACs involved in renal fibrosis and glomerulosclerosis which are common pathological features of NS [63]. A combination of HDACi, vorinostat with an ACE inhibitor benazepril in an animal model of nephropathy could significantly reduce proteinuria and kidney injury via modulating different signaling cascades such as NF-κB, IL-1, TGF-β, MAPK, and apoptosis machinery [66].

6. Conclusion

Alterations in cytokine patterns in INS may contribute to proteinuria and glomerular injury and influence therapeutic interventions. Thus, the identification of distinct cytokines as novel biomarkers of SRNS at the early diagnosis can benefit patients by both enabling the prevention of glucocorticoid toxicity and directing to earlier switch to more effective therapeutic options. Understanding the molecular mechanisms involved in SRNS and the development of molecular-based diagnosis and predictive biomarkers would have a significant value in the management of SRNS patients in years to come.

Data Availability

No original data were used in this study.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

SZV and MA developed the idea. EA and YRS reviewed the literature and prepared the first draft. MA and SZV revised the manuscript. All authors have read and approved the final manuscript.

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References

[1] J. J. Candelier and H. K. Lorenzo, "Idiopathic nephrotic syndrome and serum permeability factors: a molecular jigsaw puzzle," *Cell and Tissue Research*, vol. 379, no. 2, pp. 231–243, 2020.

[2] E. T. McCarthy, M. Sharma, and V. J. Savin, "Circulating permeability factors in idiopathic nephrotic syndrome and focal segmental glomerulosclerosis," *Clinical Journal of the American Society of Nephrology*, vol. 5, no. 11, pp. 2115–2121, 2010.

[3] N. Roca, C. Martinez, E. Jatem, A. Madrid, M. Lopez, and A. Segarra, "Activation of the acute inflammatory phase response in idiopathic nephrotic syndrome: association with clinicopathological phenotypes and with response to corticosteroids," *Clinical Kidney Journal*, vol. 14, no. 4, pp. 1207–1215, 2021.

[4] A. P. Carlotti, P. B. Franco, L. L. Elias et al., "Glucocorticoid receptors, in vitro steroid sensitivity, and cytokine secretion in idiopathic nephrotic syndrome," *Kidney International*, vol. 65, no. 2, pp. 403–408, 2004.

[5] V. Daniel, Y. Trautmann, M. Konrad, A. Nayir, and K. Schärer, "T-Lymphocyte populations, cytokines and other growth factors in serum and urine of children with idiopathic nephrotic syndrome," *Clinical Nephrology*, vol. 47, no. 5, pp. 289–297, 1997.

[6] N. Printza, F. Papachristou, V. Tzimouli, A. Taparkou, and F. Kanakoudi-Tsakalidou, "IL-18 is correlated with type-2 immune response in children with steroid sensitive nephrotic syndrome," *Cytokine*, vol. 44, no. 2, pp. 262–268, 2008.

[7] T. J. Neuhaus, M. Wadhwa, R. Callard, and T. M. Barratt, "Increased IL-2, IL-4 and interferon-gamma (IFN-gamma) in steroid-sensitive nephrotic syndrome," *Clinical and Experimental Immunology*, vol. 100, no. 3, pp. 475–479, 1995.

[8] T. Kanai, H. Shiraishi, T. Yamagata et al., "Th2 cells predominate in idiopathic steroid-sensitive nephrotic syndrome," *Clinical and Experimental Nephrology*, vol. 14, no. 6, pp. 578–583, 2010.

[9] R. J. Hogg, R. J. Portman, D. Milliner, K. V. Lemley, A. Eddy, and J. Ingelfinger, "Evaluation and management of proteinuria and nephrotic syndrome in children: recommendations from a pediatric nephrology panel established at the National Kidney Foundation conference on proteinuria, albuminuria, risk, assessment, detection, and elimination (PARADE)," *Pediatrics*, vol. 105, no. 6, pp. 1242–1249, 2000.

[10] D. Hahn, E. M. Hodson, N. S. Willis, and J. C. Craig, "Corticosteroid therapy for nephrotic syndrome in children," *The Cochrane Database of Systematic Reviews*, vol. 18, no. 3, 2015.

[11] M. Nakayama, R. Katafuchi, T. Yanase, K. Ikeda, H. Tanaka, and S. Fujimi, "Steroid responsiveness and frequency of relapse in adult-onset minimal change nephrotic syndrome," *American Journal of Kidney Diseases*, vol. 39, no. 3, pp. 503–512, 2002.

[12] S. M. Hejazian, S. Zununi Vahed, H. Moghaddas Sani et al., "Steroid-resistant nephrotic syndrome: pharmacogenetics and epigenetic points and views," *Expert Review of Clinical Pharmacology*, vol. 13, no. 2, pp. 147–156, 2020.

[13] S. M. Hosseiniyan Khatibi, M. Ardalan, S. Abediazar, and S. Zununi Vahed, "The impact of steroids on the injured podocytes in nephrotic syndrome," *The Journal of Steroid Biochemistry and Molecular Biology*, vol. 196, article 105490, 2020.

[14] Y. Rahbar Saadat, S. M. Hejazian, Z. Nariman-Saleh-Fam et al., "Glucocorticoid receptors and their upstream epigenetic regulators in adults with steroid-resistant nephrotic syndrome," *BioFactors*, vol. 46, no. 6, pp. 995–1005, 2020.

[15] S. Z. Vahed, H. M. Sani, S. Rajabzadeh et al., "The importance of genetic study in steroid-resistant nephrotic syndrome," *Journal of Renal Injury Prevention*, vol. 8, no. 4, pp. 271–282, 2019.

[16] A. Widiasta, K. Wahyudi, H. Nugrahapraja, Y. Sribudiani, and D. Rachmadi, "The unique difference between serum level of soluble urokinase plasminogen activator receptor (suPAR) in steroid-resistant nephrotic syndrome children treated with an alkylating agent and calcineurin inhibitors," *Journal of Comprehensive Pediatrics*, vol. 12, no. 2, article e109912, 2021.

[17] Y. S. Youn, H. H. Lim, and J. H. Lee, "The clinical characteristics of steroid responsive nephrotic syndrome of children according to the serum immunoglobulin E levels and cytokines," *Yonsei Medical Journal*, vol. 53, no. 4, pp. 715–722, 2012.

[18] H.-K. Yap, W. Cheung, B. Murugasu, S.-K. Sim, C.-C. Seah, and S. C. Jordan, "Th1 and Th2 cytokine mRNA profiles in childhood nephrotic syndrome: evidence for increased IL-13 mRNA expression in relapse," *Journal of the American Society of Nephrology*, vol. 10, no. 3, pp. 529–537, 1999.

[19] R. P. Woroniecki, I. F. Shatat, K. Supe, Z. Du, and F. J. Kaskel, "Urinary cytokines and steroid responsiveness in idiopathic nephrotic syndrome of childhood," *American Journal of Nephrology*, vol. 28, no. 1, pp. 83–90, 2007.

[20] H. Stone, B. Magella, and M. R. Bennett, "The search for biomarkers to aid in diagnosis, differentiation, and prognosis of childhood idiopathic nephrotic syndrome," *Frontiers in Pediatrics*, vol. 7, p. 404, 2019.

[21] H. G. Kang, H. Seo, J. H. Lim et al., "Markers of disease and steroid responsiveness in paediatric idiopathic nephrotic syndrome: whole-transcriptome sequencing of peripheral blood mononuclear cells," *The Journal of International Medical Research*, vol. 45, no. 3, pp. 948–963, 2017.

[22] A. Jaiswal, N. Prasad, V. Agarwal et al., "Regulatory and effector T cells changes in remission and resistant state of childhood nephrotic syndrome," *Indian Journal of Nephrology*, vol. 24, no. 6, pp. 349–355, 2014.

[23] F. T. L. Guimarães, R. N. Ferreira, G. E. A. Brito-Melo et al., "Pediatric patients with steroid-sensitive nephrotic syndrome have higher expression of T regulatory lymphocytes in comparison to steroid-resistant disease," *Frontiers in Pediatrics*, vol. 7, p. 114, 2019.

[24] N. Futrakul, P. Butthep, S. Patumraj, and P. Futrakul, "Glomerular endothelial dysfunction and altered cytokines in severe nephrosis," *Nephron*, vol. 86, no. 2, p. 199, 2000.

[25] J. Stachowski, C. Barth, J. Michałkiewicz et al., "Th1/Th2 balance and CD45-positive T cell subsets in primary nephrotic syndrome," *Pediatric Nephrology*, vol. 14, no. 8, pp. 779–785, 2000.

[26] G. Lama, I. Luongo, G. Tirino, A. Borriello, C. Carangio, and M. E. Salsano, "T-lymphocyte populations and cytokines in childhood nephrotic syndrome," *American Journal of Kidney Diseases*, vol. 39, no. 5, pp. 958–965, 2002.

[27] L. L. Liu, Y. Qin, J. F. Cai et al., "Th17/Treg imbalance in adult patients with minimal change nephrotic syndrome," *Clinical Immunology*, vol. 139, no. 3, pp. 314–320, 2011.

[28] S. Agrawal, M. E. Brier, B. A. Kerlin et al., "Plasma cytokine profiling to predict steroid resistance in pediatric nephrotic syndrome," *Kidney International Reports*, vol. 6, no. 3, pp. 785–795, 2021.

[29] Y. L. Tain, T. Y. Chen, and K. D. Yang, "Implications of serum TNF-beta and IL-13 in the treatment response of childhood nephrotic syndrome," *Cytokine*, vol. 21, no. 3, pp. 155–159, 2003.

[30] J. C. Kam, S. J. Szefler, W. Surs, E. R. Sher, and D. Y. Leung, "Combination IL-2 and IL-4 reduces glucocorticoid receptor-binding affinity and T cell response to glucocorticoids," *Journal of Immunology*, vol. 151, no. 7, pp. 3460–3466, 1993.

[31] L. Panda, A. Gheware, R. Rehman et al., "Linoleic acid metabolite leads to steroid resistant asthma features partially through NF-κB," *Scientific Reports*, vol. 7, no. 1, p. 9565, 2017.

[32] K. A. Smoak and J. A. Cidlowski, "Mechanisms of glucocorticoid receptor signaling during inflammation," *Mechanisms of Ageing and Development*, vol. 125, no. 10-11, pp. 697–706, 2004.

[33] S. C. Biddie, S. John, P. J. Sabo et al., "Transcription factor AP1 potentiates chromatin accessibility and glucocorticoid receptor binding," *Molecular Cell*, vol. 43, no. 1, pp. 145–155, 2011.

[34] X. M. Sun, X. H. Sun, Z. Y. Li, C. J. Yuan, and J. Wu, "Expression of NF-κB in juvenile rats with nephrotic syndrome and its effects on inflammatory changes and renal injury," *European Review for Medical and Pharmacological Sciences*, vol. 23, no. 9, pp. 4010–4016, 2019.

[35] A. Oeckinghaus and S. Ghosh, "The NF-kappaB family of transcription factors and its regulation," *Cold Spring Harbor Perspectives in Biology*, vol. 1, no. 4, article a000034, 2009.

[36] K. Szilagyi, L. Podracka, N. E. Franke, J. Mojzis, and L. Mirossay, "A new link between steroid resistance, glucocorticoid receptor and nuclear factor kappa B p65 in idiopathic nephrotic syndrome," *Neuro Endocrinology Letters*, vol. 30, no. 5, pp. 629–636, 2009.

[37] D. H. Aviles, V. Matti Vehaskari, J. Manning, A. C. Ochoa, and A. H. Zea, "Decreased expression of T-cell NF-kappaB p65 subunit in steroid-resistant nephrotic syndrome," *Kidney International*, vol. 66, no. 1, pp. 60–67, 2004.

[38] C. Dawson, A. Dhanda, B. Conway-Campbell, A. Dimambro, S. Lightman, and C. Dayan, "NFκB and glucocorticoid receptor activity in steroid resistance," *Journal of Receptor and Signal Transduction Research*, vol. 32, no. 1, pp. 29–35, 2012.

[39] F. T. L. Guimarães, G. Melo, T. M. Cordeiro et al., "T-Lymphocyte-expressing inflammatory cytokines underlie persistence of proteinuria in children with idiopathic nephrotic syndrome," *Jornal de Pediatria*, vol. 94, no. 5, pp. 546–553, 2018.

[40] D. Polak, Y. Borovitz, D. Clyman-Levy et al., "Salivary cytokines in children with nephrotic syndrome versus healthy children: a comparative study," *Journal of Clinical Medicine*, vol. 9, no. 9, p. 2691, 2020.

[41] Y. Matsumoto, Y. Ikezumi, T. Kondo et al., "Urinary monocyte chemotactic protein 1 as a predictive marker of steroid responsiveness in children with idiopathic nephrotic syndrome," *Fujita Medical Journal*, vol. 4, no. 1, pp. 17–22, 2018.

[42] E. H. Garin, D. K. Blanchard, K. Matsushima, and J. Y. Djeu, "IL-8 production by peripheral blood mononuclear cells in nephrotic patients," *Kidney International*, vol. 45, no. 5, pp. 1311–1317, 1994.

[43] E. H. Garin, P. Laflam, and L. Chandler, "Anti-interleukin 8 antibody abolishes effects of lipoid nephrosis cytokine," *Pediatric Nephrology*, vol. 12, no. 5, pp. 381–385, 1998.

[44] A. A. Al-Eisa, M. Al Rushood, and R. J. Al-Attiyah, "Urinary excretion of IL-1β, IL-6 and IL-8 cytokines during relapse and remission of idiopathic nephrotic syndrome," *Journal of Inflammation Research*, vol. 10, pp. 1–5, 2017.

[45] T. Jafar, S. Agrawal, A. A. Mahdi, R. K. Sharma, S. Awasthi, and G. G. Agarwal, "Cytokine gene polymorphism in idiopathic nephrotic syndrome children," *Indian Journal of Clinical Biochemistry*, vol. 26, no. 3, pp. 296–302, 2011.

[46] G. D. Han, K. Suzuki, H. Koike et al., "IFN-inducible protein-10 plays a pivotal role in maintaining slit-diaphragm function by regulating podocyte cell-cycle balance," *Journal of the American Society of Nephrology*, vol. 17, no. 2, pp. 442–453, 2006.

[47] R. O'Leary, H. Penrose, K. Miyata, and R. Satou, "Macrophage-derived IL-6 contributes to ANG II-mediated angiotensinogen stimulation in renal proximal tubular cells," *American Journal of Physiology*, vol. 310, no. 10, pp. F1000–F1007, 2016.

[48] E. Cuzzoni, R. Franca, S. De Iudicibus et al., "MIF plasma level as a possible tool to predict steroid responsiveness in children with idiopathic nephrotic syndrome," *European Journal of Clinical Pharmacology*, vol. 75, no. 12, pp. 1675–1683, 2019.

[49] T. Calandra and T. Roger, "Macrophage migration inhibitory factor: a regulator of innate immunity," *Nature Reviews. Immunology*, vol. 3, no. 10, pp. 791–800, 2003.

[50] T. Calandra, J. Bernhagen, C. N. Metz et al., "MIF as a glucocorticoid-induced modulator of cytokine production," *Nature*, vol. 377, no. 6544, pp. 68–71, 1995.

[51] J. M. Daun and J. G. Cannon, "Macrophage migration inhibitory factor antagonizes hydrocortisone-induced increases in cytosolic IkappaBalpha," *American Journal of Physiology*, vol. 279, no. 3, pp. R1043–R1049, 2000.

[52] R. A. Mitchell, C. N. Metz, T. Peng, and R. Bucala, "Sustained mitogen-activated protein kinase (MAPK) and cytoplasmic phospholipase A2 activation by macrophage migration inhibitory factor (MIF). Regulatory role in cell proliferation and glucocorticoid action," *The Journal of Biological Chemistry*, vol. 274, no. 25, pp. 18100–18106, 1999.

[53] T. Roger, A. L. Chanson, M. Knaup-Reymond, and T. Calandra, "Macrophage migration inhibitory factor promotes innate immune responses by suppressing glucocorticoid-induced expression of mitogen-activated protein kinase phosphatase-1," *European Journal of Immunology*, vol. 35, no. 12, pp. 3405–3413, 2005.

[54] C. F. Chung, T. Kitzler, N. Kachurina et al., "Intrinsic tumor necrosis factor-α pathway is activated in a subset of patients with focal segmental glomerulosclerosis," *PLoS One*, vol. 14, no. 5, article e0216426, 2019.

[55] A. Ortiz, C. Bustos, J. Alonso et al., "Involvement of tumor necrosis factor-alpha in the pathogenesis of experimental and human glomerulonephritis," *Advances in Nephrology from the Necker Hospital*, vol. 24, pp. 53–77, 1995.

[56] D. Ostalska-Nowicka, M. Smiech, M. Jaroniec et al., "SOCS3 and SOCS5 mRNA expressions may predict initial steroid response in nephrotic syndrome children," *Folia Histochemica et Cytobiologica*, vol. 49, no. 4, pp. 719–728, 2011.

[57] K. Zaorska, P. Zawierucha, D. Ostalska-Nowicka, and M. Nowicki, "SOCS3 is epigenetically up-regulated in steroid resistant nephrotic children," *Acta Biochimica Polonica*, vol. 63, no. 1, pp. 131–138, 2016.

[58] D. M. Youssef, R. M. Elbehidy, A. S. El-Shal, and L. M. Sherief, "T helper 1 and T helper 2 cytokines in atopic children with steroid-sensitive nephrotic syndrome," *Iranian Journal of Kidney Diseases*, vol. 9, no. 4, pp. 298–305, 2015.

[59] S. A. Shalaby, H. M. Al-Edressi, S. A. El-Tarhouny, M. Fath El-Bab, and M. A. Zolaly, "Type 1/type 2 cytokine serum levels and role of interleukin-18 in children with steroid-sensitive nephrotic syndrome," *Arab Journal of Nephrology and Transplantation*, vol. 6, no. 2, pp. 83–88, 2013.

[60] H. K. Jiang, G. Luo, and H. Jiang, "Interleukin-18 expression in peripheral blood mononuclear cells in children with steroid-resistant nephrotic syndrome," *Chinese Journal of Contemporary Pediatrics*, vol. 11, no. 5, pp. 337–340, 2009.

[61] K. Tullus, H. Webb, and A. Bagga, "Management of steroid-resistant nephrotic syndrome in children and adolescents," *The Lancet Child & Adolescent Health*, vol. 2, no. 12, pp. 880–890, 2018.

[62] R. Satou, H. Penrose, and L. G. Navar, "Inflammation as a regulator of the renin-angiotensin system and blood pressure," *Current Hypertension Reports*, vol. 20, no. 12, p. 100, 2018.

[63] R. Varghese and A. Majumdar, "Current therapies in nephrotic syndrome: HDAC inhibitors, an emerging therapy for kidney diseases," *Current Research in Biotechnology*, vol. 3, pp. 182–194, 2021.

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Research Article

The Colombian Strain of *Trypanosoma cruzi* Induces a Proinflammatory Profile, Neuronal Death, and Collagen Deposition in the Intestine of C57BL/6 Mice Both during the Acute and Early Chronic Phase

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The objective of this study was to evaluate the histopathological changes caused by infection with the Colombian strain of *Trypanosoma cruzi (T. cruzi)* in the acute and chronic experimental phases. C57Bl/6 mice were infected with 1000 trypomastigote forms of the Colombian strain of *T. cruzi*. After 30 days (acute phase) and 90 days (early chronic phase) of infection, the animals were euthanized, and the colon was collected and divided into two parts: proximal and distal. The distal portion was used for histopathological analysis, whereas the proximal portion was used for quantification of pro- and anti-inflammatory cytokines. In addition, the weight of the animals and parasitemia were assessed. The infection induced gradual weight loss in the animals. In addition, the infection induced an increase in interferon gamma (IFNγ) and tumor necrosis factor-alpha (TNF-α) in the intestine in the acute phase, in which this increase continued until the early chronic phase. The same was observed in relation to the presence of intestinal inflammatory infiltrates. In relation to interleukin (IL)-10, there was an increase only in the early chronic phase. The Colombian strain infection was also able to induce neuronal loss in the myenteric plexus and deposition of the collagen fibers during the acute phase. The Colombian strain of *T. cruzi* is capable of causing histopathological changes in the intestine of infected mice, especially in inducing neuronal destructions. Thus, this strain can also be used to study the intestinal form of Chagas disease in experimental models.

1. Introduction

More than 100 years after the discovery of the etiologic agent of Chagas disease (CD), *Trypanosoma cruzi*, the disease still has a great socioeconomic impact. This disease is estimated to cost approximately $627 million per year for global public health [1]. In addition, due mainly to the loss of productivity and premature death of those infected, approximately 1.2

billion dollars are spent annually worldwide [2]. The main pathological manifestations of CD include the heart, digestive system, and chagasic megacolon, which accounts for 10–20% of cases that evolve the digestive forms [3]. Little is known about the mechanisms involved in progression, and because of this, experimental models are used to assist in the search for answers.

The strain of *T. cruzi* most commonly used in experimental models of mouse [4, 5], rat [6, 7], or dog [8] that mimic the intestinal form of CD is the Y strain. Thus, most of the findings related to intestinal histopathological and immunological changes due to *T. cruzi* infection are related to this strain. Mice of the C57BL/6 strain infected with the Y strain, for example, show changes in the width of the colon and thickness of the muscle layer and an increase in the inflammatory infiltrate in the intestine, as well as tissue parasitism, myositis, ganglionitis, and periganglionitis during the acute experimental phase [9]. During the chronic phase, there is an increase in the deposition of collagen fibers in the intestine, which is associated with fibrosis in the organ [10]. The same process has also been reported in the human chagasic megacolon [11]. In addition, infection by this strain leads to neuronal decrease in experimental models in both the acute and chronic phases [5, 12], which is also a milestone in the progression of the megacolon in humans [13, 14].

There is a diversity of strains of *T. cruzi* that have different biological behaviors, mainly related to molecular biology, tissue tropism, and the form of the developed DC [15, 16]. While infection in experimental models with strains such as Y [5, 10] and MORC-1 [17] causes intestinal neuronal destruction, strains such as Ninoa, Queretaro [18], and Brazil [19], which induce intestinal changes, have not been evaluated for the number of neurons in the intestinal plexuses.

Furthermore, the Colombian strain is widely used for studies based on experimental Chagas heart disease all because of the myotropism, mainly cardiac and skeletal, that this strain has [20, 21]. The presence of the parasite and intestinal changes has been reported, although less frequently, in infections with this strain [4, 22]. However, in these studies, the relationship between intestinal neuronal number, cytokine behavior, and fibrose deposition has not been evaluated in mice. Thus, the objective of this study was to assess whether the Colombian strain is related to immunopathological changes and neuronal destruction in the intestine, both during the acute and early chronic phases of experimental infection.

2. Material and Methods

2.1. Animals, Infection, and Euthanasia. The study was approved by the Ethics Committee on the Use of Animals of the Federal University of Goiás (protocol number: 051/19). Thus, all conditions of handling, maintenance, and euthanasia of the animals were followed as indicated.

The animals used in this study were bred and donated by the Bioterium of the Institute of Tropical Pathology and Public Health of the Federal University of Goias. Male C57Bl/6 mice (22–27 g) were infected, subcutaneously, or not with 1000 trypomastigote forms of *T. cruzi* Colombian strain obtained from BALB/c mice at the peak of parasitemia. From the day of infection, the animals were followed for 30 days ($n = 5$) and 90 days ($n = 4$) during acute and early chronic phases, respectively. Control animals without infection were also followed for 30 days ($n = 5$) or 90 days ($n = 5$). At the time of euthanasia (cervical dislocation after confirmation of the anesthetic status, induced by 50 mg/kg of xylazine hydrochloride intraperitoneally), the final portion of the colon was collected, and the proximal portion was used for the measurement of cytokines, whereas the distal portion was used for histopathological analysis.

2.2. Parasitemia and Animal Weight. Parasitemia of infected mice was performed at 3-day intervals until the total disappearance of blood trypomastigotes. For this, 5 μL of blood was collected from the tail vein of the animals and then placed on a slide and cover slip. Then, 50 random fields were evaluated under an ordinary light microscope to count the circulating trypomastigotes. The weight of the animals was collected on the day of infection (0 day) and on the subsequent days for euthanasia (30 and 90 days).

2.3. Histopathological Evaluations. The distal part of the colon of the animals was washed with ×1 phosphate buffered saline (PBS), transferred to a filter paper, and fixed within 48 h with 4% paraformaldehyde. The fixed material was then processed according to a previous study [18].

For analysis of the inflammatory infiltrate, three serial cuts (100 μm apart) were stained with hematoxylin-eosin. Then, 10 photos of each cut (final = 30 photos), under ×400 magnification, were captured using a common light microscope attached to the camera. First, the intensity of the inflammatory infiltrate was established qualitatively in the submucosa and muscle, following the classification of 1 for mild, 2 for moderate, and 3 for accentuated. After this classification, the average of the 30 photos was obtained and classified according to the following score: 0–0.3, normal; 0.4–1.0, discrete; 1.1–2.0, moderate; and 2.1–3.0, accentuated [4].

Slides stained with Giemsa stain were used to quantify the intestinal nerve ganglia. Four serial slices, with 100 μm between each slice, were evaluated under standard optical microscopy at ×400 magnification. The nerve ganglia of the entire fragment were counted for each serial cut, and the mean was obtained. The slides were scanned using a common printer to measure each fragment. Using the ImageJ software, the average of the four cuts was normalized to 1 cm, and the result was obtained as the number of ganglia/cm of the intestine.

Slides stained with picrosirius and hematoxylin were used for morphometric evaluation of the deposition of the connective tissue in the mucosa, submucosa, and intestinal muscle layers. For each intestinal fragment, 20 fields were analyzed at ×400 magnification following the methodology established in a previous study [18]. The results are expressed as the percentage of the collagen/animal.

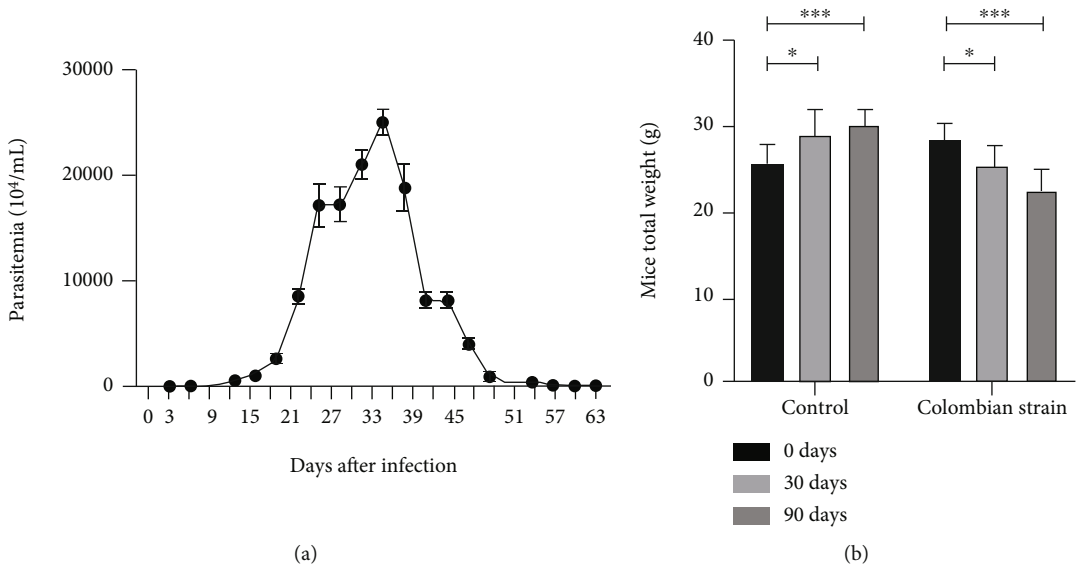

Figure 1: Blood parasitemia (a) and weight (b) differences between the acute and chronic phases of *T. cruzi* Colombian strain infected C57Bl/6 mice. Two-way ANOVA test. *Significant statistical differences at $p < 0.05$.

2.4. Immunological Evaluations. The colon proximal fragment (approximately 1 cm) was transferred to an Eppendorf tube containing ×1 phosphate buffered saline solution and Complete™ protease inhibitor (Sigma, USA). The fragments were then homogenized in a homogenizer (DREMEL, EUA). The homogenates obtained were centrifuged at 12000 × g for 30 min, and the supernatants were stored at -80°C for quantification of cytokines and total proteins. The quantification of interferon gamma (IFN-γ) (R&D Systems), tumor necrosis factor-alpha (TNF-α) (R&D Systems), and interleukin-(IL-) 10 (BD OptEIA™) was performed on homogenates of the proximal portion of the colon using an immunoenzymatic assay (ELISA) according to the manufacturers' instructions. Tetramethylbenzidine (TMB) (3,3,5,5-tetramethylbenzidine) was used for the colorimetric reaction, and the optical density was measured using a microplate reader (Bio-Rad 2550 READER EIA, USA). To normalize the concentration of cytokines, they were used as total proteins of the modern intestinal homogenate in a nanodrop (Thermo Fisher Scientific, USA). The results are expressed in pg/mg.

2.5. Statistical Analysis. Statistical analyses were performed using the GraphPad Prism 8.0.1 (Graphpad Software, USA). The normality of the distribution of the quantitative variables was verified using the Shapiro-Wilk test. For comparison of the two groups, the Mann–Whitney test for data with nonnormal distribution was used. Results such as animal weight were analyzed using a two-way analysis of variance (ANOVA) test. For correlation, the Spearman's test was used. The results were considered statistically significant at $p < 0.05$.

3. Results

The count of circulating parasites showed slow transit parasitemia (Figure 1(a)). It started at 9 days after infection and declined completely on the 60th day. A peak was observed on the 33rd day after infection. At 30 days of infection (Figure 1(b)), there was a significant reduction in weight when compared to the day of the inoculum ($p = 0.0192$), which continued progressively until 90 days ($p < 0.0001$). Animals without infection gradually gained weight (0 days compared to 30 days, $p = 0.0205$, and 90 days, $p = 0.0002$).

To analyze the effects during the acute and early chronic infection, histological evaluations were performed. Regarding the presence of the inflammatory infiltrate (Figure 2(a)), during the acute phase, 100% of the animals showed moderate inflammatory infiltrate (1.30–2.06) (Figure 3(a)). During the early chronic phase, 50% of the animals are characterized by mild inflammatory infiltrate in the intestine (0.75-1) and the other half as moderate (1.15–1.46) (Figure 3(b)). Although there was a reduction, there was no difference between the experimental times ($p = 0.1905$) (Figure 2(a)). Although the quantification of amastigote nests is not performed, only one amastigote nest was found in a mouse during the acute stage of infection (Figure 3(c)).

Regarding the myenteric ganglion nerve, the acute phase was a determinant of structure reduction, which was demonstrated by a significant decrease when compared to the respective uninfected group ($p = 0.0079$) (Figure 2(b)). The same was observed when comparing the early chronic phase with its respective control ($p = 0.0159$). However, 90 days of infection were not enough to continue with the destruction of the ganglia when compared to that found in the acute phase ($p = 0.9048$). In the control group, preserved myenteric plexus architecture was observed (Figure 3(d)), both in the acute and chronic phases. Disorganization and intrusion of inflammatory cells close to the neurons of this structure were observed (Figures 3(e) and 3(f), respectively).

The collagen deposition process started in the acute phase and continued until the early chronic phase, which is

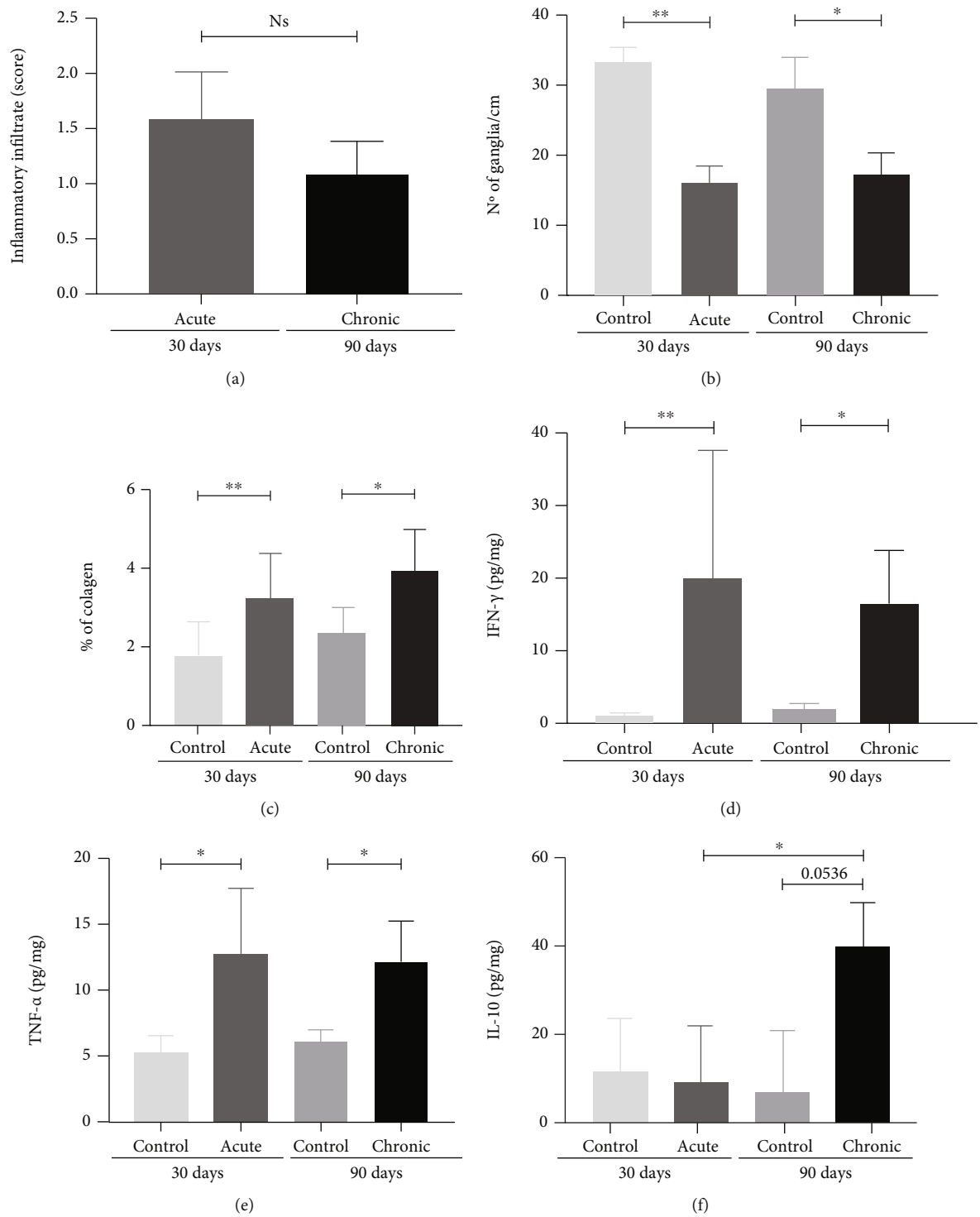

Figure 2: Intestinal immune and histopathological differences between the acute and chronic phases of *T. cruzi* Colombian strain infected C57Bl/6 mice. (a) Intensity of the intestinal inflammatory infiltrate. (b) Number of intestinal nerve ganglia. (c) Percentage of intestinal collagen deposition. Quantification of intestinal levels of (d) IFN-γ, (e) TNF-α, and (f) IL-10. Mann–Whitney test. *Significant statistical differences at $p < 0.05$.

demonstrated by the difference between the respective controls ($p = 0.0303$ and $p = 0.0242$, respectively) (Figure 2(c)). However, 90 days of infection were no longer sufficient to increase the collagen deposition compared to 30 days ($p = 0.3524$). Although uninfected mice had little intestinal collagen (Figure 3(g)), there was a great predominance of collagen fiber deposition in the intestinal submucosal layer in the infected mice at the two experimental times (Figures 3(h) and 3(i)). However, deposition in the mucosa and muscles was also observed.

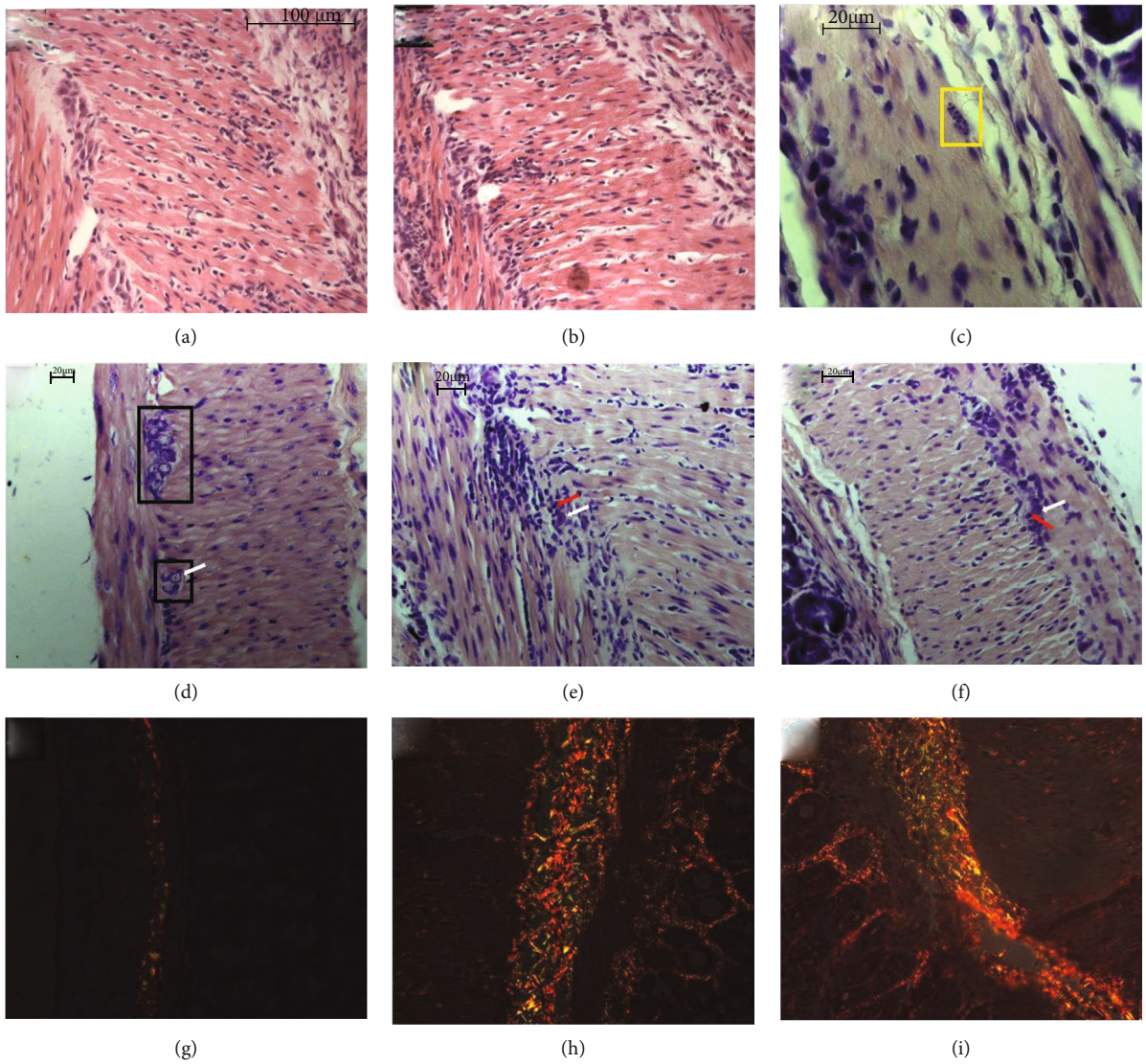

Figure 3: Intestinal photomicrographs of the intestinal histopathological differences between the noninfected, acute, and early chronic phases of *T. cruzi* Colombian strain infected C57Bl/6 mice. Intestinal inflammatory infiltrate (HE): (a) acute phase and (b) early chronic phase. (c) Intestinal amastigote nest in the acute phase highlighted by yellow lines (GIEMSA). Intestinal nerve ganglia (GIEMSA): (d) the two intestinal nerve ganglia of the myenteric plexus of uninfected mice highlighted using black lines (30 days); nervous ganglion without borderline of infected mice in the (e) acute and (f) early chronic phase with neuron (white arrow) and inflammatory cells (red arrow) remarkably close. Intestinal collagen deposition (Picrosirius): (g) noninfected, (h) acute, and (i) early chronic phase of infection.

Cytokines were used during both time points to analyze the intestinal immunological response. IFN-γ (Figure 2(d)) and TNF-α (Figure 2(e)), proinflammatory cytokines, were upregulated during the acute phase when compared with the respective controls ($p = 0.0317$ and $p = 0.0079$, respectively). In addition, the levels of TNF-α and IFN-γ remained high during the early chronic phase, without difference with acute levels ($p > 0.9999$ and $p = 0.7857$, respectively), but with differences compared to the respective control ($p = 0.0357$ and $p = 0.0357$, respectively). IL-10 (Figure 2(f)), an anti-inflammatory cytokine, was upregulated only during the early chronic phase when compared to the acute phase ($p = 0.0357$) and showed a tendency with the respective control ($p = 0.0536$), which suggests an attempt to control the immune response.

After observing that both in the acute and early chronic phases, there were histological changes (increased inflammatory infiltrate, neuronal destruction, and collagen deposition) and the maintenance of a proinflammatory profile (IFN-γ and TNF-α) with an attempt to regulate (IL-10), and the next objective was to evaluate the relationship between these factors after 90 days of infection. Thus, it was observed that the increase in IFN-γ (Figure 4(a)) and TNF-α (Figure 4(b)) demonstrated a significant and negative correlation with the decrease in the nerve ganglia in the myenteric plexus ($r = -0.7626$ and $p = 0.0002$ and $r = -0.6594$ and $p = 0.0029$, respectively). However, IL-10 (Figure 4(c)) did not correlate with this decrease ($r = -0.03296$ and $p = 0.8967$). In addition, only the increase in IFN-γ (Figure 4(d)) correlated significantly and positively with the increase in intestinal

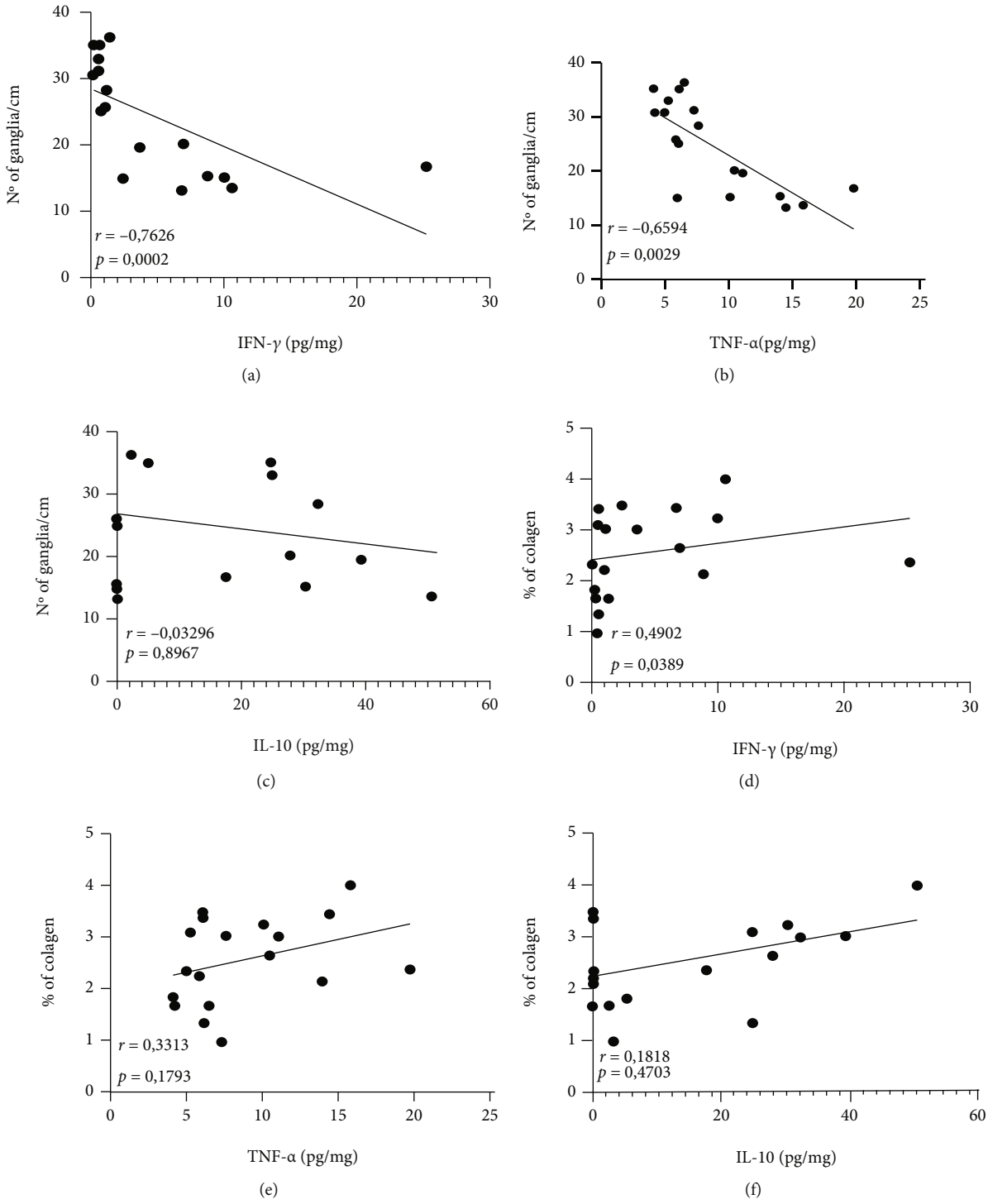

Figure 4: Correlations between the amount of nerve ganglia in the myenteric plexus with levels of intestinal (a) IFN-γ, (b) TNF-α, and (c) IL-10. Correlations between the percentage of intestinal collagen deposition with levels of intestinal (d) IFN-γ, (e) TNF-α, and (f) IL-10. Data obtained from uninfected animals in the acute phase and in the early chronic phase. Correlations were performed using the Spearman test. Significant statistical differences at $p < 0.05$.

collagen deposition ($r = 0.4902$ and $p = 0.0389$), while TNF-α (Figure 4(e)) and IL-10 (Figure 4(f)) showed no significant correlation ($r = 0.3313$ and $p = 0.1793$ and $r = 0.1818$ and $p = 0.4703$, respectively).

4. Discussion

The focus of this study was to evaluate whether the Colombian strain was capable of inducing immunopathological

changes in the intestines of C57Bl/6 mice during the acute and early chronic experimental phases. Our results show that during the acute stage, there are intestinal changes, such as increased inflammatory infiltrate, neuronal destruction, and collagen deposition, along with the maintenance of the inflammatory process with proinflammatory cytokines until the early chronic phase.

A participant in DTU I and representative of biodema III, the Colombian strain, is defined by its low proliferative capacity, maximum peak close to 30 days, and myotropism [22, 23]. In addition, *T. cruzi* infection is characterized by excessive weight loss in experimental models, mainly in mice. These findings corroborate those of our model. This weight loss may be related to the inflammatory process, especially the presence of circulating proinflammatory cytokines such as TNF-α and IFN-γ, which may be associated with cachexia in experimental models [24, 25].

Infection with the Colombian strain and other strains of *T. cruzi* induces the appearance of the inflammatory intestinal infiltrate [4, 22, 26, 27], which also corroborates the findings of our model. However, the intensity of infiltration in the intestine may vary depending on the strain and inoculum concentration used in the infection [4, 9, 28]. It has been reported that infection by strain Y, for example, does not maintain the inflammatory process until the chronic phase, unlike what is observed in the Colombian strain [4]. This suggests that infection with the Colombian strain is more intense and stays longer.

Phenotypically, the inflammatory infiltrate in the human chagasic megacolon presents a great number of mononuclear cells, especially $CD3^+$ lymphocytes [29]. In addition, eosinophils, mast cells, macrophages ($CD68^+$), natural killer cells ($CD57^+$), and cytotoxic T lymphocytes ($TIA\text{-}1^+$) have also been reported in the organs of these individuals [30, 31]. The presence of these cells and the maintenance of the inflammatory process are associated with neuronal destruction, intestinal remodeling, and progression of chagasic megacolon and megaesophagus [32]. In addition, cells present in the enteric nervous system, such as the enteric glial cells, have also been associated with the progression of CD [31]. However, the role of these cells needs to be better understood. From this diverse cellular microenvironment, proinflammatory and regulatory cytokines, and microbicide components, such as nitric oxide (NO) and reactive oxygen species (ROS) can be produced by different cell types and induce neuronal death [32]. Although the characterization of these cells was not carried out in our study, it was demonstrated that the inflammatory infiltrate and proinflammatory cytokines persisted during the acute to the chronic phase, and this was correlated with neuronal destruction.

In addition, the maintenance of the intensity of the intestinal inflammatory infiltrate found in our study, which may be related to the production of proinflammatory cytokines (IFN-γ and TNF-α) were also maintained until the early chronic phase. IFN-γ is one of the cytokines most closely involved in resistance to *T. cruzi* infection [33–35], participating in the inhibition of intracellular replication of parasites [35], activation and maintenance of the T helper (Th) 1 response profile, and production of antibodies [36]. The inhibition of this cytokine in infected mice, for example, influences the increase in parasitemia, decreased survival, and decreased NO production [34]. Synergistically to IFN-γ, TNF-α activates macrophages with a microbicidal profile and results in the destruction of intracellular forms of the parasite; thus, it acts in the control of infection [37].

In the case of IL-10, a regulatory cytokine, only during the early chronic phase, there was an increase in the intestine. Differential production of IL-10 is one of the parameters that allow the differentiation of strains after infection [38]. A study that used a clone of the Colombian strain, Col cl1.7, demonstrated that this strain induced greater production of IL-10 compared to the infection established by strain Y in monocytes in vitro [38]. In an experimental model of the chronic phase, it has also been demonstrated that the Y strain does not induce changes in the production of this cytokine in the intestine of animals [10]. Thus, it is suggested that the intestinal increase observed in our study is related to a compensatory mechanism for controlling tissue damage due to the intense inflammatory process induced by the Colombian strain, which has also been suggested in experimental chagasic heart disease [20].

However, the intense inflammatory process established in the intestine is also related to tissue damage, especially the neuronal destruction [12, 39]. Arantes et al. (2004), using C57Bl/6 knockout mice for iNOS and IFN-γ infected with 100 blood trypomastigote forms of strain Y, demonstrated that the absence of NO was a determinant for neuronal survival after 10 days of infection. The failure to induce NO production via IFN-γ prevented denervation via oxidative stress in an experimental acute phase model [12]. This finding may explain the negative correlation between TNF-α and IFN-γ and the amount of the nerve ganglia in the myenteric plexus found in our study. Thus, the more the proinflammatory cytokines, the more is the NO, and the fewer are the neurons in the colon.

In addition to the participation of TNF-α, IFN-γ, and NO, other mechanisms related to the neuronal destruction in CD have been proposed. Substance *P*, a neuropeptide, has been shown to be increased in dilated portions of patients with chagasic megacolon, and this increase has been shown to be related to the induction/maintenance of intestinal inflammation and leukocyte chemotaxis, which may be related to neuronal damage [40, 41]. In addition, proteases produced by mast cells, such as tryptase, are correlated with neuronal death in patients with chagasic megacolon, mainly by decreasing immunoreactive PAR2 neurons [42]. Our group demonstrated that type 2 bone morphogenetic proteins are correlated with neuronal destruction and with the maintenance of the intestinal proinflammatory profile in an acute-phase experimental model infected with the Y strain [10].

Consequently, the positive correlation of IFN-γ with collagen deposition may also be related, since fibrosis proceeds the destruction of tissue. Contrary to what has been previously found for Y strain, which only included an increase in the connective tissue during the chronic phase of experimental infection [5, 10], our study demonstrated that the process of the collagen fiber deposition begins even in the acute phase of infection with the Colombian strain. What

can also be related to the connected events of establishment of the inflammatory process with production of proinflammatory cytokines, destruction of neurons, and deposition of collagen fibers.

From these results, it is clear that the Colombian strain can also be used in experimental models to study the intestinal form of CD. The results of this study contribute to the understanding of the mechanisms related to the formation and progression of Chagas megacolon.

Data Availability

All the data used to support the findings of this study are included within the article, figures, and references.

Ethical Approval

All procedures involving animals were in compliance with the Ethics Committee on the Use of Animals of the Federal University of Goiás, and ethical approval was granted by the same committee (protocol number: 051/19).

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

José Rodrigues do Carmo Neto and Arthur Wilson Florêncio da Costa contributed equally to this paper.

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References

[1] B. Y. Lee, K. M. Bacon, M. E. Bottazzi, and P. J. Hotez, "Global economic burden of Chagas disease: a computational simulation model," *The Lancet Infectious Diseases*, vol. 13, no. 4, pp. 342–348, 2013.

[2] L. Conteh, T. Engels, and D. H. Molyneux, "Socioeconomic aspects of neglected tropical diseases," *Lancet*, vol. 375, no. 9710, pp. 239–247, 2010.

[3] J. D. Stanaway and G. Roth, "The burden of Chagas disease estimates and challenges," *Global Heart*, vol. 10, no. 3, pp. 139–144, 2015.

[4] M. Wesley, A. Moraes, A. . C. Rosa et al., "Correlation of parasite burden, kdna integration, autoreactive antibodies, and cytokine pattern in the pathophysiology of chagas disease," *Frontiers in Microbiology*, vol. 10, p. 1856, 2019.

[5] C. F. Campos, S. D. Cangussú, A. L. C. Duz et al., "Enteric neuronal damage, intramuscular denervation and smooth muscle phenotype changes as mechanisms of Chagasic megacolon: evidence from a long-term murine model of Trypanosoma cruzi infection," *PLoS One*, vol. 11, no. 4, pp. 1–18, 2016.

[6] E. R. S. Camargos, D. J. Franco, C. M. M. G. Garcia et al., "Infection with different Trypanosoma cruzi populations in rats: myocarditis, cardiac sympathetic denervation, and involvement of digestive organs," *The American Journal of Tropical Medicine and Hygiene*, vol. 62, no. 5, pp. 604–612, 2000.

[7] C. F. Brustolin Aleixo, F. N. Ferraz, P. F. Massini et al., "Beneficial immunomodulatory and neuro digestive effect in Trypanosoma cruzi infection after Lycopodium clavatum 13c treatment," *Microbial Pathogenesis*, vol. 112, pp. 1–4, 2017.

[8] N. C. Nogueira-Paiva, K. S. da Fonseca, P. M. A. de Vieira et al., "Myenteric plexus is differentially affected by infection with distinct Trypanosoma cruzi strains in beagle dogs," *Memórias do Instituto Oswaldo Cruz*, vol. 109, no. 1, pp. 51–60, 2014.

[9] B. P. Vazquez, T. P. Vazquez, C. B. Miguel et al., "Inflammatory responses and intestinal injury development during acute Trypanosoma cruzi infection are associated with the parasite load," *Parasites and Vectors*, vol. 8, no. 1, p. 206, 2015.

[10] J. R. do Carmo Neto, M. Vinicius da Silva, Y. L. L. Braga et al., "Correlation between intestinal BMP2, IFNγ, and neural death in experimental infection with Trypanosoma cruzi," *PLoS One*, vol. 16, no. 2, article e0246692, 2021.

[11] S. W. Pinheiro, A. M. . O. Rua, R. M. Etchebehere et al., "Morphometric study of the fibrosis and mast cell count in the circular colon musculature of chronic Chagas patients with and without megacolon," *Revista da Sociedade Brasileira de Medicina Tropical*, vol. 36, no. 4, pp. 461–466, 2003.

[12] R. M. E. Arantes, H. H. F. Marche, M. T. Bahia, F. Q. Cunha, M. A. Rossi, and J. S. Silva, "Interferon-γ-Induced Nitric Oxide Causes Intrinsic Intestinal Denervation in Trypanosoma cruzi -Infected Mice," *The American Journal of Pathology*, vol. 164, no. 4, pp. 1361–1368, 2004.

[13] S. J. Adad, D. C. Andrade, E. R. Lopes, and E. Chapadeiro, "Pathological anatomy of chagasic megaesophagus," *Revista do Instituto de Medicina Tropical de São Paulo*, vol. 33, no. 6, pp. 443–450, 1991.

[14] S. J. Adad, C. G. Cançado, R. M. Etchebehere et al., "Neuron count reevaluation in the myenteric plexus of chagasic megacolon after morphometric neuron analysis," *Virchows Archiv*, vol. 438, no. 3, pp. 254–258, 2001.

[15] B. Zingales, M. A. Miles, D. A. Campbell et al., "The revised Trypanosoma cruzi subspecific nomenclature: Rationale, epidemiological relevance and research applications," *Infection, Genetics and Evolution*, vol. 12, no. 2, pp. 240–253, 2012.

[16] B. Zingales, "Trypanosoma cruzi genetic diversity: Something new for something known about Chagas disease manifestations, serodiagnosis and drug sensitivity," *Acta Tropica*, vol. 184, pp. 38–52, 2018.

[17] L. C. Caetano, S. Zucoloto, L. M. Kawasse, M. P. A. Toldo, and J. C. Do Prado, "Does cyclophosphamide play a protective role against neuronal loss in Chronic T. cruzi infection?," *Digestive Diseases and Sciences*, vol. 53, no. 11, pp. 2929–2934, 2008.

[18] B. Espinoza, N. Solorzano-Domínguez, A. Vizcaino-Castillo, I. Martínez, A. L. Elias-López, and J. A. Rodríguez-Martínez, "Gastrointestinal infection with Mexican TcI Trypanosoma cruzi strains: different degrees of colonization and diverse immune responses," *International Journal of Biological Sciences*, vol. 7, no. 9, pp. 1357–1370, 2011.

[19] J. Guarner, J. Bartlett, S. R. Zaki, D. G. Colley, M. J. Grijalva, and M. R. Powell, "Mouse model for Chagas disease: Immunohistochemical distribution of different stages of Trypanosoma cruzi in tissues throughout infection," *The American Journal of Tropical Medicine and Hygiene*, vol. 65, no. 2, pp. 152–158, 2001.

[20] J. Reis Machado, M. V. Silva, D. C. Borges et al., "Immunopathological aspects of experimental trypanosoma cruzi reinfections," *BioMed Research International*, vol. 2014, Article ID 648715, 9 pages, 2014.

[21] M. V. Da Silva, V. L. De Almeida, W. D. De Oliveira et al., "Upregulation of Cardiac IL-10 and Downregulation of IFN-γ in Balb/c IL-4−/− in Acute Chagasic Myocarditis due to Colombian Strain of Trypanosoma cruzi," *Mediators of Inflammation*, vol. 2018, Article ID 3421897, 9 pages, 2018.

[22] E. Camandaroba, T. S. Thé, D. H. Pessina, and S. G. Andrade, "Trypanosoma cruzi: clones isolated from the Colombian strain, reproduce the parental strain characteristics, with ubiquitous histotropism," *International Journal of Experimental Pathology*, vol. 87, no. 3, pp. 209–217, 2006.

[23] R. Devera, O. Fernandes, and J. R. Coura, "Should Trypanosoma cruzi be called "cruzi" complex? A review of the parasite diversity and the potential of selecting population after in vitro culturing and mice infection," *Memórias do Instituto Oswaldo Cruz*, vol. 98, no. 1, pp. 1–12, 2003.

[24] M. J. Tisdale, "Biology of cachexia," *Journal of the National Cancer Institute*, vol. 89, no. 23, pp. 1763–1773, 1997.

[25] J. A. Hatter, Y. M. Kouche, S. J. Melchor et al., "Toxoplasma gondii infection triggers chronic cachexia and sustained commensal dysbiosis in mice," *PLoS One*, vol. 13, no. 10, p. e0204895, 2018.

[26] R. C. Melo and Z. Brener, "Tissue tropism of different Trypanosoma cruzi strains," *The Journal of Parasitology*, vol. 64, no. 3, pp. 475–482, 1978.

[27] J. A. Diego, P. Penin, J. Del Rey, R. Mayer, and C. Gamallo, "A comparative pathological study of three strains of Trypanosoma cruzi in an experimental model," *Histology and Histopathology*, vol. 6, pp. 199–206, 1991, https://europepmc.org/article/med/1802119.

[28] D. C. Borges, N. M. Araújo, C. R. Cardoso, and J. E. Lazo Chica, "Different parasite inocula determine the modulation of the immune response and outcome of experimental Trypanosoma cruzi infection," *Immunology*, vol. 138, no. 2, pp. 145–156, 2013.

[29] E. C. Oliveira, M. A. R. Freitas, A. Brehmer, and A. B. Da Silveira, "Immunological challenges to the development of Chagasic mega syndromes," *International Journal of Cell Science & Molecular Biology*, vol. 3, article 555623, 2018.

[30] A. B. M. da Silveira, S. J. Adad, R. Correa-Oliveira, J. B. Furness, and D. D'Avila Reis, "Morphometric study of eosinophils, mast cells, macrophages and fibrosis in the colon of chronic chagasic patients with and without megacolon," *Parasitology*, vol. 134, no. 6, pp. 789–796, 2007.

[31] A. B. M. da Silveira, E. M. Lemos, S. J. Adad, R. Correa-Oliveira, J. B. Furness, and D. D'Avila Reis, "Megacolon in Chagas disease: a study of inflammatory cells, enteric nerves, and glial cells," *Human Pathology*, vol. 38, no. 8, pp. 1256–1264, 2007.

[32] J. R. do Carmo Neto, Y. L. L. Braga, A. W. F. da Costa et al., "Biomarkers and Their Possible Functions in the Intestinal Microenvironment of Chagasic Megacolon: An Overview of the (Neuro)inflammatory Process," *Journal of Immunology Research*, vol. 2021, 17 pages, 2021.

[33] J. S. Silva, P. J. Morrissey, K. H. Grabstein, K. M. Mohler, D. Anderson, and S. G. Reed, "Interleukin 10 and interferon gamma regulation of experimental trypanosoma cruzi infection," *The Journal of Experimental Medicine*, vol. 175, no. 1, pp. 169–174, 1992.

[34] J. S. Silva, G. N. R. Vespa, M. A. G. Cardoso, J. C. S. Aliberti, and F. Q. Cunha, "Tumor necrosis factor alpha mediates resistance to Trypanosoma cruzi infection in mice by inducing nitric oxide production in infected gamma interferon-activated macrophages," *Infection and Immunity*, vol. 63, no. 12, pp. 4862–4867, 1995.

[35] F. Cardillo, J. C. Voltarelli, S. G. Reed, and J. S. Silva, "Regulation of Trypanosoma cruzi infection in mice by gamma interferon and interleukin 10: role of NK cells," *Infection and Immunity*, vol. 64, no. 1, pp. 128–134, 1996.

[36] F. Cardillo, R. T. De Pinho, P. R. Z. Antas, and J. Mengel, "Immunity and immune modulation in Trypanosoma cruzi infection," *Pathogens and Disease*, vol. 73, no. 9, 2015.

[37] E. C. Santos Lima, I. Garcia, M. H. Vicentelli, P. Vassalli, and P. Minoprio, "Evidence for a protective role of tumor necrosis factor in the acute phase of Trypanosoma cruzi infection in mice," *Infection and Immunity*, vol. 65, no. 2, pp. 457–465, 1997.

[38] L. M. D. Magalhães, A. Viana, E. Chiari, L. M. C. Galvão, K. J. Gollob, and W. O. Dutra, "Differential activation of human monocytes and lymphocytes by distinct strains of Trypanosoma cruzi," *PLoS Neglected Tropical Diseases*, vol. 9, no. 7, p. e0003816, 2015.

[39] C. M. . Almeida-Leite, L. M. . C. Galvão, L. C. C. Afonso, F. . Q. Cunha, and R. M. E. Arantes, "Interferon-γ induced nitric oxide mediates in vitro neuronal damage by Trypanosoma cruzi -infected macrophages," *Neurobiology of Disease*, vol. 25, no. 1, pp. 170–178, 2007.

[40] A. B. M. da Silveira, D. D'Avila Reis, E. C. de Oliveira et al., "Neurochemical coding of the enteric nervous system in chagasic patients with megacolon," *Digestive Diseases and Sciences*, vol. 52, no. 10, pp. 2877–2883, 2007.

[41] A. B. M. da Silveira, M. A. R. Freitas, E. C. de Oliveira et al., "Substance P and NK1 receptor expression in the enteric nervous system is related to the development of chagasic megacolon," *Transactions of the Royal Society of Tropical Medicine and Hygiene*, vol. 102, no. 11, pp. 1154–1156, 2008.

[42] P. R. Martins, R. D. Nascimento, A. T. dos Santos, E. C. de Oliveira, P. M. Martinelli, and D. d'Avila Reis, "Mast cellnerve interaction in the colon of Trypanosoma cruzi-infected individuals with chagasic megacolon," *Parasitology Research*, vol. 117, no. 4, pp. 1147–1158, 2018.

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Research Article

Polymorphisms within Genes Coding for IL-17A and F and Their Receptor as Clinical Hallmarks in Ankylosing Spondylitis

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IL-17A and IL-17F together with their coreceptor (IL-17RA/RC) were reported to play a significant role in the pathogenesis of spondyloarthritis. The group of axial spondyloarthritis comprises ankylosing spondylitis (AS), a rheumatic disease characterized by chronic inflammation of the joints in the spine. This study is aimed at investigating *IL-17A*, *IL-17F*, *IL-17RA*, and *IL-17RC* polymorphisms as potential biomarkers of disease susceptibility, clinical parameters, and anti-TNF treatment outcome in a cohort of Polish ankylosing spondylitis patients. In total, 328 subjects, including 138 AS patients and 190 healthy volunteers, participated in the study. Genotyping of *IL-17A* rs2275913 (G/A), *IL-17F* rs763780 (A/G), *IL-17RA* rs4819554 (A/G), and *IL-17RC* rs708567 (G/A) was performed on real-time PCR instrument using LightSNiP assays. No significant differences were revealed in genotype and allele distribution between patients and controls despite the association of the *IL-17RC* rs708567 *AA* homozygosity with the earlier onset of the disease. Moreover, some relationships between *IL-17F* rs763780 and *IL-17RA* rs4819554 polymorphisms with clinical parameters related to the disease activity and anti-TNF treatment outcome were observed. *IL-17F* rs763780 G allele was found to be associated with high disease activity and BASDAI after 6 months and poor response to the treatment while higher VAS values were more common among *IL-17RA* rs4819554 G variant carriers. In conclusion, the *IL-17F* rs763780 polymorphism should be considered as a promising biomarker of disease activity and anti-TNF treatment outcome. The *IL-17RA* rs48419554 G allele may serve as a potential marker of disease severity in Polish AS patients.

1. Introduction

Ankylosing spondylitis (AS) is characterized by visible radiographic changes within the spine or sacroiliac joints. The axial spondyloarthritis (axSpA) group comprises AS, radiographic axial spondyloarthritis, and a nonradiographic (nr-axSpA) form of the disease [1]. AS patients suffer from inflammatory back pain and morning stiffness. Symptoms can also involve enthesitis and peripheral arthritis manifestations. The disease affects mostly men (ratio men to women is 2 to 1), those under thirty years of age, and with a strong genetic association linked to HLA-B27 [2]. Prevalence differs

between geographical regions and ethnicity, reaching 0.23% in the general European population [3] and roughly 0.083% in Polish people [4].

The standard pharmacological treatment against AS involves tumour necrosis factor-alpha (TNF-α) inhibitor (anti-TNF) dosage after the primary failure of nonsteroidal anti-inflammatory (NSAIDs) administration. The long-term anti-TNF approach has positive effects on patient's functional outcome, lessens disease activity, and reduces radiographic progression [5].

The IL-17 family consists of six cytokines: IL-17A, IL-17B, IL-17C, IL-17D, IL-17E (IL-25), and IL-17F. Those proteins transmit signals through defined heterodimeric transmembrane receptors (IL-17R). IL-17A, IL-17F, and IL-17A/F heterodimer act via the IL-17RA/RC receptor complex. IL-17E triggers responses through IL-17RA/RB, and IL-17C induces the IL-17RA/RE heterodimer. For other proteins, the heterotrimeric receptor compound has not been fully elucidated. IL-17A and IL-17F have a high degree of homology, and both are secreted by Th17 cells, $\gamma\delta$ T cells, innate lymphoid cells, cytotoxic T cells, and natural killer T (NKT) cells [6].

IL-17 was reported to have a crucial role in the immunopathogenesis of spondyloarthritis [7]. Elevated levels of IL-17 in serum have been observed in ankylosing spondylitis patients [8]. Besides, associations between IL-17 level and the Bath Ankylosing Spondylitis Disease Activity Index (BASDAI) [9, 10] have been described.

However, our knowledge of *IL-17* gene polymorphisms in AS is still limited. Thus, based on previous research focused on rheumatoid arthritis [11] and osteoarthritis [12], we hypothesized that *IL-17A* rs2275913 and *IL-17* rs763780 might influence AS susceptibility. The targeted single nucleotide polymorphisms were selected based on the available literature, especially on Caucasians, as well as our preliminary experiment on rheumatoid arthritis patients [13]. We also decided to investigate polymorphisms of IL-17 receptors *IL-17RA* and *IL-17RC*. *IL-17RA* rs4819554 was previously linked with response to etanercept in psoriatic arthritis [14], while *IL-17RC rs708567* was associated with lupus arthritis [15] and was described in Tunisians with rheumatoid arthritis [16]. Moreover, the newly performed study considered the *IL-17RA* polymorphism as an AS risk factor [17]. To the best of our knowledge, no investigations have been conducted to assess the association between *IL-17RA* and *IL-17RC* polymorphisms and rheumatic diseases in the Polish population.

This study examines the *IL-17A* rs2275913, *IL-17F* rs763780, *IL-17RA* rs4819554, and *IL-17RC* rs708567 genetic variants as potential biomarkers of disease susceptibility, clinical parameters, and anti-TNF treatment outcome in a cohort of Polish AS patients.

2. Materials and Methods

2.1. Patients and Controls. One hundred thirty-eight AS patients and one hundred ninety controls were involved in the study. AS patients were recruited from the Department of Rheumatology and Internal Medicine, Wroclaw Medical University, Poland, and from the Department of Rheumatology and Connective Tissue Diseases, Jan Biziel University Hospital No. 2 in Bydgoszcz, Poland. All the participants diagnosed with AS were Caucasians over 18 years of age, and 74% (102/138) were male. Included criteria comprise a resistance to treatment with at least two nonsteroidal anti-rheumatic drugs (NSAIDs), high disease activity before starting biological treatment, initialization of anti-TNF therapy at the time of the research, and complete medical history. Subjects with the coexistence of acute or chronic disorders besides AS, other autoimmune diseases, malignancies, or current infections, during pregnancy and breastfeeding, as well as with insufficient clinical records, and an unwillingness or inability to cooperate were excluded from the study.

AS patients were diagnosed according to the 1984 modified New York Criteria [18].

Data such as gender, age, disease onset, disease duration, body mass index (BMI), presence of HLA-B27, C-reactive protein (CRP) level, pain visual analogue scale (VAS, range: 0-100 mm), and Bath Ankylosing Spondylitis Disease Activity Index (BASDAI, range: 0-10) were collected from patients.

90.4% of patients were HLA-B27 positive, and most of them (76.7%) had the axial form of AS. Drug administration comprised MTX in 27.5% of cases, corticosteroids (20.3%) of subjects and NSAIDs have been taken by 71.3% of patients.

Bath Ankylosing Spondylitis Disease Activity Index (BASDAI) was used to calculate disease activity, which was considered to be high (BASDAI ≥ 4), moderate ($3 \leq$ BASDAI < 4), or low (BASDAI < 3). The clinical outcome was assessed after 3 and 6 months of anti-TNF treatment. Significant improvement after therapy was defined as a reduction of BASDAI (ΔBASDAI ≥ 2.0), good outcome as ΔBASDAI ≥ 2.0 and BASDAI < 3.0 at the endpoint, moderate response as ΔBASDAI ≥ 2.0 and BASDAI ≥ 3.0 at the endpoint, and no improvement as ΔBASDAI < 2.0 [19].

The patient's demographic and clinical characteristics are described in Table 1. The data are presented as median with range (minimum to maximum).

The control group was enrolled from the healthy volunteers, 63 females (33%) and 127 (67%) males, from the Regional Centre of Transfusion Medicine and Blood Bank in Wroclaw without a personal history of rheumatic diseases.

Informed consent was obtained from all participants involved in the study. The research was approved by the Wroclaw Medical University Ethics Committee (identification code KB-625/2016).

2.2. SNP Selection and Genotyping. Tested genetic variants were selected based on analysis of previous publications and search results from NCBI Database of Short Genetic Variations (dbSNP) and SNPinfo Web Server [20]. Minor allele frequency (MAF) in EUR was above 5% (1000 Genomes Project) [21].

In total, four single nucleotide polymorphisms (SNPs) were chosen for analysis: *IL-17A* rs2275913 (G/A) and *IL-17F* rs763780 (A/G) located on chromosome 6, *IL-17RA* rs4819554 (A/G) located on chromosome 22, and *IL-17RC* rs708567 (G/A) located on chromosome 3. Two of them,

Table 1: Clinical characteristics of the study cohort.

Characteristic	*N*	Median (range)
Age (years)	138	43.5 (22-75)
Disease duration (years)	135	10 (0-48)
Disease onset (years)	135	33 (6-56)
BMI	113	25.32 (18.61-40.31)
CRP before treatment (mg/l)	108	16.83 (0.3-561)
CRP at 3 months (mg/l)	79	5.75 (0.2-175)*
CRP at 6 months (mg/l)	72	5.495 (0.2-204.3)*
VAS before treatment (mm)	132	80 (45-100)
VAS at 3 months (mm)	138	30 (0-80)*
VAS at 6 months (mm)	131	20 (0-100)*
BASDAI before treatment	138	8 (4.05-10)
BASDAI at 3 months	138	3.2 (0.7-6.7)*
BASDAI at 6 months	132	2.25 (0.2-9.75)*
Treatment (anti-TNF drug)	*N* = 138	*n* (%)
Adalimumab		63 (45.6%)
Etanercept		44 (31.9%)
Certolizumab		17 (12.3%)
Golimumab		12 (8.69%)
Infliximab		2 (1.45%)

N: number of patients with clinical information; BMI: body mass index; CRP: C-reactive protein; MTX: methotrexate; NSAIDs: nonsteroidal anti-inflammatory drugs; BASDAI: Bath Ankylosing Spondylitis Disease Activity Index; VAS: visual analogue scale; HLA-B27: human leukocyte antigen B27. *$p < 0.001$; p value comparing the clinical variables between baseline and after 3 or 6 months of treatment.

IL-17F rs763780 and *IL-17RC* rs708567 are missense variants in exon 3 (His161Arg) and exon 4 (Ser111Leu), respectively. *IL-17A* rs2275913 and *IL-17RA* rs4819554 are substitutions within gene promoter regions with a predicted transcription factor binding site (TFBS).

Whole blood samples were collected in EDTA tubes (BD Vacutainer® Blood Collection Tubes). Genomic DNA was isolated from peripheral blood using QIAamp DNA Blood Midi/Maxi Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. The genotyping of selected SNPs: *IL-17A* rs2275913 (G/A), *IL-17F* rs763780 (A/G), *IL-17RA* rs4819554 (A/G), and *IL-17RC* rs708567 (G/A) was performed using LightSNiP assays (TIB MOLBIOL, Berlin, Germany) on the LightCycler 480 Real-Time PCR Instrument (Roche Diagnostics, Basel, Switzerland).

2.3. Statistical Analysis. The genotype frequencies were tested for the Hardy-Weinberg equilibrium (HWE). Potential differences in allele and genotype distributions between the patient and control groups were calculated using Fisher's exact test. Continuous variables were tested for normal distribution by the Shapiro–Wilk test. Quantitative variables that were normally distributed were presented as mean ± SEM, while medians with interquartile ranges (IQRs) were calculated for nonnormally distributed variables. The unpaired two-sample Wilcoxon test (for nonparametric data) or unpaired two-sample *t*-test (for normally distributed data) were performed to identify associations within genetic variants and clinical parameters. Fisher's exact test was also applied to detect relationships between genotypes and categorical variables such as disease activity or treatment outcome. A p value lower than 0.05 ($p < 0.05$) was considered statistically significant. All statistical analysis was performed using R Software (http://www.r-project.org) and GraphPad Prism 7 for Windows.

Table 2: The distribution of *IL-17* genotypes and alleles in AS patients and the control group.

	Patients	Controls
IL-17A rs2275913	*N* = 138	*N* = 190
G	174 (63.0%)	234 (61.6%)
A	102 (37.0%)	146 (38.4%)
GG	50 (36.2%)	69 (36.3%)
GA	74 (53.6%)	96 (50.5%)
AA	14 (10.1%)	25 (13.2%)
IL-17F rs763780	*N* = 138	*N* = 189
A	265 (96.0%)	359 (95.0%)
G	11 (4.0%)	19 (5.0%)
AA	127 (92.0%)	170 (89.9%)
AG	11 (7.97%)	19 (10.1%)
GG	0 (0%)	0 (0%)
IL-17RA rs4819554	*N* = 138	*N* = 190
A	215 (77.9%)	311 (81.8%)
G	61 (22.1%)	69 (18.2%)
AA	83 (60.1%)	126 (66.3%)
AG	49 (35.5%)	59 (31.1%)
GG	6 (4.35%)	5 (2.63%)
IL-17RC rs708567	*N* = 138	*N* = 189
A	150 (54.3%)	205 (54.2%)
G	126 (45.7%)	173 (45.8%)
AA	41 (29.7%)	47 (24.9%)
AG	68 (49.3%)	111 (58.7%)
GG	29 (21.0%)	31 (16.4%)

3. Results

3.1. Distribution of IL-17A, IL-17F, IL-17RA, and IL-17RC Alleles and Genotypes in Patients and Controls. The distribution of genotypes and alleles of *IL-17A* rs2275913, *IL-17F* rs763780, *IL-17RA* rs4819554, and *IL-17RC* rs708567 did not differ between AS patients and healthy individuals (Table 2). Also, no significant gender-dependent differences were detected between patients and healthy subjects (not shown). Please note that none of the patients or controls were homozygous for the *IL-17F* rs763780 G allele. Thus, in the further analyses, *AA* homozygotes were being compared with *AG* genotype reflecting also the *G* allele carriers.

On the other hand, the significant association between disease onset and genotype frequency was observed for *IL-17RC* rs708567 SNP. Patients with *AA* genotype had a lower age of disease onset (29.39 ± 1.405) than those with *G* allele (33.43 ± 1.001) (*AA* vs. *AG*+*GG*, $p = 0.022$; *AA* vs. *AG*, $p =$

0.015). However, disease duration was not found to be affected by any of the analysed SNPs.

3.2. Associations between IL-17A, IL-17F, IL-17RA, and IL-17RC Genotypes and Clinical Parameters. The potential associations between *IL-17A*, *IL-17F*, *IL-17RA*, and *IL-17RC* genotypes and CRP level, VAS, and BASDAI values were analysed.

During anti-TNF therapy, these major clinical parameters were decreased. CRP level was significantly lower after 3 and 6 months compared to baseline ($p < 0.0001$). Also, VAS and BASDAI were reduced after 3 and 6 months related to baseline, as well as after 6 months in comparison to 3 months of therapy ($p < 0.0001$) (Table 1). A significant improvement in clinical parameters was achieved after administration of anti-TNF agents.

Higher VAS values at the baseline were found in *IL-17A* rs2275913 *GG* (*GG* vs. *GA*+*AA*, $p = 0.005$; *GG* vs. *GA*, $p = 0.006$) and *IL-17F* rs763780 *AG* (*AA* vs. *AG*, $p = 0.027$) genotype carriers (Table 3(a)).

The *IL-17RA* rs4819554 G allele was found to be more common among patients who presented with higher VAS and BASDAI values after anti-TNF treatment induction.

Patients possessing the *IL-17RA* rs4819554 G allele had higher VAS values after 3 months of anti-TNF therapy (*AA* vs. *AG*+*GG*, $p = 0.002$). This result was also observed after 6 months of treatment (*AA* vs. *AG*+*GG*, $p = 0.002$).

Besides, *IL-17RA* rs4819554 G patients demonstrated greater BASDAI values at 6 months of therapy than *AA* homozygotes (*AA* vs. *AG*+*GG*, $p = 0.046$) (Table 3(b)). The *IL-17RA* results concerning VAS at 3 and 6 months and BASDAI at 6 months were confirmed by the overdominant model (*AA*+*GG* vs. *AG*, $p = 0.008$, $p = 0.006$, and $p = 0.045$, respectively). Additionally, a significant relationship with an absolute BASDAI change (ΔBASDAI 0-3 m.) (*AA*+*GG* vs. *AG*, $p = 0.027$) was noted. Tendencies were observed regarding BASDAI score at 3 months (*AA* vs. *AG*+*GG*, $p = 0.066$; AA+GG vs. AG, $p = 0.064$) and absolute BASDAI change (ΔBASDAI 0-6 m.) (*AA*+*GG* vs. *AG*, $p = 0.057$).

The BASDAI parameter at 6 months was also higher in the group of AS individuals bearing *IL-17F* rs763780 *AG* genotype (*G* allele) (*AA* vs. *AG*, $p = 0.035$) in comparison to *AA* carriers (Table 3(a)).

As for CRP levels, *IL-17RC* rs708567 and *IL-17A* rs2275913 polymorphisms were identified as significantly associated with CRP level after 3 months of TNF inhibitor administration. At that time point, *IL-17A* rs2275913 *GG* was more frequently observed among patients with an elevated level of CRP (>10 mg/l) (*GG* vs. *GA*+*AA*, $p = 0.022$, OR = 3, and 95%CI = 1.237-7.046), and *IL-17RC* rs708567 G patients showed a significantly higher CRP level as compared to the *AA* patients (*AA* vs. *AG*+*GG*, $p = 0.043$; *AA* vs. *AG*, $p = 0.018$) (Table 3(b)).

No other significant differences between clinical parameters of AS patients and their *IL-17* genotype distribution were detected.

3.3. Effect of IL-17F Polymorphisms on the Disease Activity and Anti-TNF Treatment Outcome. Before the anti-TNF administration, all patients were characterized with high disease activity (BASDAI > 4). After 3 months of therapy, 25.4% (35/138), 44.9% (62/138), and 29.7% (41/138) of subjects had a high, moderate, and low disease activity, respectively. After 6 months, only 3.03% (4/132) and 1.52% (2/132) were described with high and moderate disease activity, respectively. The remaining 95.5% (126/132) of patients presented low disease activity. After 3 months of anti-TNF treatment, 97.8% (135/138) achieved a good or moderate outcome, and 2.2% (3/128) were nonresponders. Similarly, after 6 months, 3% (4/132) of patients did not respond positively to treatment.

Out of *IL-17A*, *IL-17F*, *IL-17RA*, and *IL-17RC* polymorphisms studied, a significant association concerning disease activity was detected for the *IL-17F* rs763780 variant. AS patients homozygous for the *A* allele more likely presented low or moderate disease activity (BASDAI < 4) after 6 months of treatment than heterozygotes (*AA* vs. *AG*, $p = 0.035$, OR = 13.22, and 95%CI = 1.82-87.84). The same genotype was significantly more common among subjects with a good or moderate response to TNF inhibitor therapy (*AA* vs. *AG*, $p = 0.035$, OR = 13.22, and 95%CI = 1.82-87.84).

The other studied *IL-17A* rs2275913, *IL-17RA* rs4819554, and *IL-17RC* rs708567 genetic variants were not found to significantly differ among AS patients in respect to disease activity and biological agent treatment outcome.

4. Discussion

In the present study, patients with ankylosing spondylitis and controls were genotyped for the *IL-17A*, *IL-17F*, *IL-17RA*, and *IL-17RC* polymorphisms to assess whether their genetic variants may be associated with susceptibility to the disease, clinical parameters, and anti-TNF treatment outcome in our Polish population.

Comparison made between our patients and controls did not show any significant association with predisposition to the disease as in both groups, similar distributions of alleles and genotypes of all SNPs studied were observed. However, disease onset was found to be affected by the *IL-17RC* rs708567 SNP and the presence of *AA* homozygosity prevailed in patients that had a lower age of disease onset than those with *G* allele.

Among currently analysed genetic variants, *IL-17A* rs2275913 and *IL-17F* rs763780 have been previously extensively studied for associations with various rheumatic disorders.

In our current study, neither *IL-17A* rs2275913 nor *IL-17F* rs763780 was found to be associated with AS risk. Nevertheless, association with AS susceptibility and *IL-17A* rs2275913 in Chinese [22] and *IL-17F* rs763780 in Turkish [23] populations has been reported. Erkol et al. did not find the relationship between *IL-17A* rs2275913 and AS susceptibility in Turkish patients [23]. More recently, Rocha Loures et al. reported rs2275913 *A* variant and rs763780 G allele as risk factors for AS, spondyloarthritis (SpA), and psoriatic arthritis (PsA) in Brazilian patients [24].

As for the associations of *IL-17* polymorphisms with other diseases, many previous studies focused on

Table 3: The distribution of (a) *IL-17A* and *IL-17F* and (b) *IL-17RA* and *IL-17R* genotypes with respect to clinical parameters of ankylosing spondylitis patients.

(a)

	IL-17A rs2275913						*IL-17F* rs763780			
	GG		*GA*		*AA*		*AA*		*AG*	
	N	Median (IQR)	*N*	Median (IQR)	*N*	Median (IQR)	*N*	Median (IQR)	*N*	Median (IQR)
CRP 0 m.	43	13.99 (7.06-47.09)	54	18 (6.983-38.86)	11	15.3 (7.85-33)	97	18.33 (7.825-39.27)	11	9.38 (5-16.73)
CRP 3 m.	30	10.11 (1.39-21.12)	40	5.1 (1.325-10.08)	9	2.6 (0.95-7.9)	71	6.2 (1.52-13.55)	8	1.3 (0.375-11.84)
CRP 6 m.	25	7.06 (0.8-12.67)	39	5.41 (0.9-15.6)	8	3.35 (0.9-9.758)	64	5.71 (1.55-13.22)	8	1.1 (0.35-19.76)
VAS 0 m.	48	87 (80-90)[(a)]	70	80 (70-90)	14	80 (70-90)	122	80 (72.5-90)[(b)]	10	88.5 (84.5-92.5)
VAS 3 m.	50	35 (24.5-40)	74	30 (29.5-40)	14	30 (25-30)	127	30 (27-40)	11	30 (22-35)
VAS 6 m.	47	20 (10-29)	71	20 (15-29)	13	20 (16-23.5)	120	20 (14.25-25)	11	20 (11-30)
BASDAI 0 m.	50	7.95 (7-9)	74	7.95 (6.775-8.6)	14	8.3 (6.375-9)	127	8 (6.8-8.8)	11	8.6 (7-9)
BASDAI 3 m.	50	3.275 (2.744-4.1)	74	3.2 (2.65-3.94)	14	3 (2.8-3.063)	127	3.2 (2.75-4)	11	3 (2.725-3.3)
BASDAI 6 m.	48	2.35 (1.925-2.8)	71	2.25 (2-2.7)	13	2 (2-2.825)	121	2.15 (2-2.7)[(c)]	11	2.5 (2.3-2.9)

(b)

	IL-17RA rs4819554						*IL-17RC* rs708567					
	AA		*AG*		*GG*		*AA*		*AG*		*GG*	
	N	Median (IQR)	*N*	Median (IQR)	*N*	Median (IQR)	*N*	Median (IQR)	*N*	Median (IQR)	*N*	Median (IQR)
CRP 0 m.	63	16.93 (6.9-39.11)	41	18.46 (7.825-42.09)	4	9.62 (9.248-25.34)	32	14.8 (6.928-28.73)	54	18.4 (8.29-45.73)	22	14.12 (7.17-33.51)
CRP 3 m.	46	5.745 (1.3-13.78)	31	6.54 (1-12.27)	2	5.51 (1.52-9.5)	26	2.15 (0.675-10.53)[(g)]	40	9.46 (2.55-14.79)	13	3 (0.9-9.555)
CRP 6 m	44	6.49 (0.75-18.33)	26	4.655 (2.05-12.07)	2	5 (3.8-6.2)	23	3.6 (0.7-12.88)	34	6.06 (1.35-13.37)	15	4.2 (2-15.94)
VAS 0 m.	80	80.5 (80-90)	46	80 (70-90)	6	84.5 (68.25-91.25)	39	80 (70-90)	65	81 (79-90)	28	84 (76-90)
VAS 3 m.	83	30 (23-40)[(d)]	49	35 (30-40)	6	38 (25-46.25)	41	30 (25-38.5)	68	30 (26.5.5-40)	29	35 (30-40)
VAS 6 m.	81	20 (10-23.5)[(e)]	45	20 (20-30)	5	21 (15-35)	40	20 (11.25-26.5)	62	20 (15-25)	29	20 (10-30)
BASDAI 0 m.	83	8 (7-8.8)	49	7.9 (6.6-8.95)	6	8.213 (6.675-9.25)	41	7.8 (6.1-8.6)	68	7.95 (7-8.8)	29	8.2 (7.4-9)
BASDAI 3 m.	83	3.1 (2.55-3.7)	49	3.325 (3-4)	6	3.3 (1.975-4.2)	41	3 (2.175-3.95)	68	3.2 (3-4)	29	3.2 (2.5-3.9)
BASDAI 6 m.	81	2.1 (1.925-2.6)[(f)]	46	2.375 (2-2.8)	5	2.3 (1.45-2.9)	40	2.2 (2-2.5)	63	2.25 (2-2.8)	29	2.3 (1.95-2.8)

[(a)]*GG* vs. *GA+AA*, $p = 0.005$; *GG* vs. *GA*, $p = 0.006$; *GA* vs. *GG+AA*, $p = 0.016$; [(b)]*AA* vs. *AG*, $p = 0.027$; [(c)]*AA* vs. *AG*, $p = 0.035$; [(d)]*AA* vs. *AG+GG*, $p = 0.002$; *AA* vs. *AG*, $p = 0.004$; *AA+GG* vs. *AG*, $p = 0.008$; [(e)]*AA* vs. *AG+GG*, $p = 0.002$; *AA* vs. *AG*, $p = 0.004$; *AA+GG* vs. *AG*, $p = 0.006$; (f) *AA* vs. *AG+GG*, $p = 0.046$; *AA* vs. *AG*, $p = 0.040$; *AA+GG* vs. *AG*, $p = 0.045$; [(g)]*AA* vs. *AG+GG*, $p = 0.043$; *AA* vs. *AG*, $p = 0.018$; *AA+GG* vs. *AG*, $p = 0.011$. *N*: number of patients in groups; IQR: interquartile range; CRP: C-reactive protein; VAS: visual analogue scale; DAS28: disease activity score 28; BASDAI: Bath Ankylosing Spondylitis Disease Activity Index; 0 m.: at the baseline; 3 m.: after 3 months of therapy; 6 m.: after 6 months of treatment.

osteoarthritis (OA) and rheumatoid arthritis (RA). Results of analysis performed on an Asian OA group suggested that the *IL-17A* rs2275913 *A* allele [25–27] and *IL-17F* rs763780 G variant [26] increased susceptibility to knee OA. In Caucasians, no association between *IL-17A* rs2275913 polymorphism and risk of hip or knee OA was found [28], but the *IL-17F* rs763780 G allele had a significant impact on the risk of the hip [28] and knee OA [29]. *IL-17A* rs2275913 *GA* [29], *IL-17F* rs763780 *AA* [28], and *IL-17A-F* G-A haplotype [30] seem to play a rather protective role in the knee, hip, or hip and knee OA, respectively. Further meta-analysis performed by Lu et al. highlighted higher susceptibility to OA in patients with *IL-17A* rs2275913 *A* allele and *IL-17F* rs763780 G allele among Asians, as well as with *IL-17F* rs763780 *G* genetic variant in a Caucasian cohort [12].

Interestingly, papers concerning the role of *IL-17A* rs2275913 and *IL-17F* rs763780 in RA are inconsistent. *IL-17A* rs2275913 *GG* genotype [31, 32] and *G* allele [33] have been found to increase susceptibility to RA, whereas Shen et al. described *AA* genotype as being linked to lower RA risk [34]. Other studies showed no significant correlations between *IL-17A* rs2275913 variant and prevalence to develop RA in Polish [13, 35], Turkish [36], Brazilian [37], Tunisian [16, 38], Algerian [39], Mexican [40], and Egyptian [41] patients. Growing evidence suggests that *IL-17F* rs763780 G is associated with susceptibility to the disease [13, 33, 38]. However, many studies did not confirm this polymorphism as an RA risk factor [35–37, 39, 41, 42]. Recent meta-analysis findings led to the identification of *IL-17A GG* and *IL-17F AG* genotypes as more frequently distributed among RA patients [11].

With regard to *IL-17RA* and *IL-17RC* polymorphisms, the present analysis did not show differences in genotype and allele distribution between patients and controls. This observation confirms previous results for *IL-17RC* among RA patients [16] but stays in contrast with *IL-17RA* developments in AS Spanish cohorts [17]. *IL-17RA* rs48419554 was also identified as a risk factor for psoriasis [43, 44]. However, other *IL-17RA* SNPs were not found to be associated with PsA [45].

Our current results also show that *IL7RC* rs708567 G variant has an effect on disease onset and is more frequently detected among patients that developed the diseases approximately 4 years later than *AA* homozygotes. To the best of our knowledge, no one has studied *IL-17RC* rs708567 in AS so far. This genetic variant and its homozygosity were also described to affect arthritis among systemic lupus erythematosus Bulgarian patients [46]. On the other hand, in a study conducted by Dhaouadi et al., *IL7RC A* allele of this polymorphism tended to show higher DAS28 in RA subjects [16]. However, the functional consequence of *IL-17RC* rs708567 polymorphism remains unknown.

In the present study, some interesting results were described regarding the *IL-17F* rs763780 SNP and our cohort of patients with AS and unfavourable effect of the *IL-17F* rs763780 G allele.

Likewise, the *IL-17F* rs763780 *G* allele was observed by Paradowska-Gorycka et al. to be positively correlated with the number of tender joints, as well as to tend to reach insignificantly higher values of DAS28-CRP and health assessment questionnaire (HAQ) score [42].

According to earlier findings established in Turkey, the *IL-17F* rs763780 *GG* genotype was prone to greater BASFI scores and *AG* variant to higher CRP level [23]. As suggested, the evidence we found points to an association between this polymorphism and disease activity in AS patients. Our results show that the *AG* genotype is significantly correlated with higher, both VAS values before treatment and BASDAI score after 6 months. Also, we link *AA* genotype with moderate/low disease activity and good/moderate response to treatment after 6 months. This concurs well with results obtained by Prieto-Peréz et al., who observed that rs763780 can predict response to adalimumab at 6 months, in psoriasis [47].

Of note, our previous analysis showed the association of the *IL-17F* rs763780 G allele with higher IL-17F secretion [48]. Also, Braga et al. observed this association between the *IL-17F* rs763780 *G* allele and increased IL-17F serum levels in Brazilian AS patients and controls [49]. These results suggest that alleles or genotypes associated with higher IL-17F production may play an unfavourable role.

Recently, the novel insight into functional consequences of the *IL-17F* polymorphism was described by Nisar et al. The change at position 161 (His to Arg) is located in the C-terminal end of IL-17, which interacts with IL-17RA. This substitution resulted in more favourable conformation, enhanced stability of the trimeric IL-17A/F/IL-17RA complex. The stronger binding may induce the proinflammatory effect and influence the severity of RA [50].

One of our previous studies performed on RA patients found that *IL-17A* rs2275913 *GG* homozygous females were characterized with the most active disease after 3 months and poor response to anti-TNF therapy [13]. On the other hand, de la Peña et al. reported that *A* allele carriers were predicted to present more severe RA and needed more than three DMARDs to control the disease [51].

It has been reported that the *IL-17A* rs2275913 polymorphism located in the promoter region can regulate gene transcription and stimulate IL-17 cytokine secretion (-197A allele) [52].

Current analysis demonstrates a significant correlation between the *IL-17A GG* genotype and higher VAS values before starting therapy in AS patients. The same genotype more frequently characterized patients with elevated CRP after 3 months. We did not find any significant correlation between *IL-17A* variants and response to the therapy. However, it was reported in the literature that rs2275913 was associated with response to anti-TNF therapy among patients with inflammatory bowel disease [53].

Our results are also in line with the findings of Vidal-Castiñeira et al. concerning significantly higher BASDAI scores in AS patients carrying the *IL-17RA* rs4819554 G allele. Moreover, our study reveals associations between the *IL-17A G* allele and greater VAS after 3 and 6 months of anti-TNF treatment. These correlations are worth mentioning because they indicate an impact of *G* variant, located in the gene promoter, on AS severity. This region was also noted to affect the response to anti-TNF therapy outcome in psoriasis [54].

The *IL-17RA* rs4819554 variant is encoded within the promotor region and may have a functional effect by modulating the gene transcription. This SNP was found to be in linkage disequilibrium with rs4819553 and rs4819958. Those polymorphisms are predicted to be related to transcription factor binding sites (TFBSs) belonging to the Ikaros (IK) family. These are involved in Th17 cell differentiation [54].

In *G* allele carriers, the increase of Th17 cytokines could promote the pathogenic mechanism via IL-23/Th17 pathway [54]. It could explain the higher VAS values and BASDAI scores that we observed.

These findings shed some light on common genetic variants in *IL-17A*, *IL-17F*, and *IL-17RA* genes. The investigated polymorphisms can affect biological activity of the protein and thus influence immunological features like a response to etanercept [14].

There is still considerable controversy surrounding *IL-17* SNP relationships and AS development. Therefore, our results need to be interpreted with caution. In fact, population diversity and treatment approach may explain the differences between studies. Although the advantage of our methodology is homogeneity of the Polish population, we are aware that the main limitation of our study is the relatively limited number of cases included in the analysis. Therefore, further data collection from AS patients is required to confirm these observations.

Other interesting genetic variants within *IL-17F* include rs11465553 [35] and rs2397084 [33, 35, 36, 38, 39, 42], which were investigated in RA. In a Polish cohort, the rs2397084 polymorphism was correlated with longer disease duration [42], whereas in Tunisians, it was associated with disease severity [38]. Additionally, rs2397084 [29] and rs1889570 [28, 30] were studied in osteoarthritis patients. Regarding the *IL-17A* gene, rs3804513 was associated with radiographic progression in early RA [55]. Other *IL-17A* polymorphisms were studied in a Chinese population. *IL-17A* rs4711998 and rs8193037 were not associated with RA, whereas rs3819024, rs3819025, and rs8193036 were correlated with the risk of RA [34]. These polymorphisms may be of interest for further study on ankylosing spondylitis.

5. Conclusions

The analysis shows that *IL-17* polymorphisms are associated with clinical parameters in Polish patients with ankylosing spondylitis and have influence on AS severity and potential course of the disease and may be biomarkers of response to anti-TNF drugs in Polish patients. The *IL-17F* rs763780 polymorphism should be considered as a candidate biomarker of disease activity and anti-TNF treatment outcome. The *IL-17RA* rs48419554 G allele may serve as a potential marker of disease severity.

Data Availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Conflicts of Interest

The authors declare that there is no conflict of interest.

Authors' Contributions

Conceptualization was contributed by K.B.-K. and J.W.; methodology was done by J.W. and K.B.-K.; formal analysis was carried out by J.W. and K.B.-K.; investigation was done by J.W. and K.B.-K.; resources were contributed by J.S., K.K., B.B., M.Ch.-M., S.J., and K.B.-K.; data curation was performed by J.W., J.S., K.K., B.B., M.Ch.-M., and S.J.; writing—original draft preparation was contributed by J.W. and K.B.-K.; writing—review and editing was contributed by J.S., K.K., B.B., M.Ch.-M., S.J., and K.B.-K.; supervision was contributed by J.S., S.J., and K.B.-K.; project administration was done by K.B.-K.; funding acquisition was contributed by K.B.-K. All authors have read and agreed to the published version of the manuscript.

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References

[1] S. P. Raychaudhuri and A. Deodhar, "The classification and diagnostic criteria of ankylosing spondylitis," *Journal of Autoimmunity*, vol. 48–49, pp. 128–133, 2014.

[2] J. Sieper and D. Poddubnyy, "Axial spondyloarthritis," in *The Lancet*, pp. 73–84, Lancet Publishing Group, 2017.

[3] L. E. Dean, G. T. Jones, A. G. Macdonald, C. Downham, R. D. Sturrock, and G. J. Macfarlane, "Global prevalence of ankylosing spondylitis," *Rheumatol (United Kingdom)*, vol. 53, pp. 650–657, 2014.

[4] M. Tłustochowicz, M. Brzozowska, W. Wierzba et al., "Prevalence of axial spondyloarthritis in Poland," *Rheumatology International*, vol. 40, pp. 323–330, 2020.

[5] M. S. Osman and W. P. Maksymowych, "An update on the use of tumor necrosis factor alpha inhibitors in the treatment of ankylosing spondylitis," *Expert Review of Clinical Immunology*, vol. 13, no. 2, pp. 125–131, 2017.

[6] L. Monin and S. L. Gaffen, "Interleukin 17 family cytokines: signaling mechanisms, biological activities, and therapeutic implications," *Cold Spring Harbor Perspectives in Biology*, vol. 10, 2018.

[7] H. Tsukazaki and T. Kaito, "The role of the IL-23/IL-17 pathway in the pathogenesis of spondyloarthritis," *International Journal of Molecular Sciences*, vol. 21, pp. 1–19, 2020.

[8] Y. Mei, F. Pan, J. Gao et al., "Increased serum IL-17 and IL-23 in the patient with ankylosing spondylitis," *Clinical Rheumatology*, vol. 30, pp. 269–273, 2011.

[9] W.-S. Chen, Y.-S. Chang, K.-C. Lin et al., "Association of serum interleukin-17 and interleukin-23 levels with disease activity in Chinese patients with ankylosing spondylitis," *Journal of the Chinese Medical Association*, vol. 75, pp. 303–308, 2012.

[10] L. Xueyi, C. Lina, W. Zhenbiao, H. Qing, L. Qiang, and P. Zhu, "Levels of circulating Th17 cells and regulatory T cells in ankylosing spondylitis patients with an inadequate response to antiTNF-α therapy," *Journal of Clinical Immunology*, vol. 33, pp. 151–161, 2013.

[11] I. Agonia, J. Couras, A. Cunha, A. J. Andrade, J. Macedo, and B. Sousa-Pinto, "IL-17, IL-21 and IL-22 polymorphisms in rheumatoid arthritis: A systematic review and _meta_ -analysis," *Cytokine*, vol. 125, p. 154813, 2020.

[12] F. Lu, P. Liu, Q. Zhang, W. Wang, and W. Guo, "Association between the polymorphism of IL-17A and IL-17F gene with knee osteoarthritis risk: a meta-analysis based on case-control studies," *Journal of Orthopaedic Surgery and Research*, vol. 14, 2019.

[13] K. Bogunia-Kubik, J. Świerkot, A. Malak et al., "IL-17A, IL-17F and IL-23R gene polymorphisms in polish patients with rheumatoid arthritis," *Archivum Immunologiae et Therapiae Experimentalis (Warsz)*, vol. 63, pp. 215–221, 2015.

[14] G. Murdaca, S. Negrini, O. Magnani, E. Penza, M. Pellecchio, and F. Puppo, "Impact of pharmacogenomics upon the therapeutic response to etanercept in psoriasis and psoriatic arthritis," *Expert Opinion on Drug Safety*, vol. 16, pp. 1173–1179, 2017.

[15] M. Hristova, Z. Kamenarska, G. Dzhebir et al., "The role of IL-17 rs2275913, IL-17RC rs708567 and TGFB1 rs1800469 SNPs and IL-17A serum levels in patients with lupus nephritis," *Rheumatology International*, 2021.

[16] T. Dhaouadi, M. Chahbi, Y. Haouami et al., "IL-17A, IL-17RC polymorphisms and IL17 plasma levels in Tunisian patients with rheumatoid arthritis," *PLoS One*, vol. 13, 2018.

[17] J. R. Vidal-Castiñeira, A. López-Vázquez, R. Diaz-Peña et al., "A single nucleotide polymorphism in the Il17ra promoter is associated with functional severity of ankylosing spondylitis," *PLoS One*, vol. 11, 2016.

[18] S. van der Linden, H. A. Valkenburg, and A. Cats, "Evaluation of diagnostic criteria for ankylosing spondylitis. A proposal for modification of the New York criteria," *Arthritis and Rheumatism*, vol. 27, pp. 361–368, 1984.

[19] J. Zochling and J. Braun, "Assessment of ankylosing spondylitis," *Clinical and Experimental Rheumatology*, vol. 23, pp. S133–S141, 2005.

[20] Z. Xu and J. A. Taylor, "SNPinfo: integrating GWAS and candidate gene information into functional SNP selection for genetic association studies," *Nucleic Acids Research*, vol. 37, pp. W600–W605, 2009.

[21] A. Auton, G. R. Abecasis, D. M. Altshuler, R. M. Durbin, D. R. Bentley, and A. Chakravarti, "A global reference for human genetic variation," *Nature*, vol. 526, no. 7571, pp. 68–74.

[22] B. Yang, Y. Xu, X. Liu, Z. Huang, and L. Wang, "IL-23R and IL-17A polymorphisms correlate with susceptibility of ankylosing spondylitis in a Southwest Chinese population," *Oncotarget*, vol. 8, no. 41, pp. 70310–70316, 2017.

[23] E. Erkol Inal, O. Görükmez, S. Eroğlu, Ö. Sağ, and G. Solak, "Associations between polymorphisms of IL-17F and IL-17A genes with disease activity and clinical outcome of ankylosing spondylitis," *Acta Reumatológica Portuguesa*, vol. 2016, 2016.

[24] M. A. Rocha Loures, L. C. Macedo, D. M. Reis et al., "Influence of TNF and IL17 gene polymorphisms on the spondyloarthritis immunopathogenesis, regardless of HLA-B27, in a Brazilian population," *Mediators of Inflammation*, vol. 2018, 2018.

[25] L. Han, H. S. Lee, J. H. Yoon et al., "Association of IL-17A and IL-17F single nucleotide polymorphisms with susceptibility to osteoarthritis in a Korean population," *Gene*, vol. 533, pp. 119–122, 2014.

[26] Y. Bai, S. Gao, Y. Liu, S. Jin, H. Zhang, and K. Su, "Correlation between Interleukin-17 gene polymorphism and osteoarthritis susceptibility in Han Chinese population," *BMC Medical Genetics*, vol. 20, 2019.

[27] L. Jiang, X. Zhou, Y. Xiong, J. Bao, K. Xu, and L. Wu, "Association between interleukin-17A/F single nucleotide polymorphisms and susceptibility to osteoarthritis in a Chinese population," *Medicine*, vol. 98, 2019.

[28] G. Vrgoc, J. Vrbanec, R. K. Eftedal et al., "Interleukin-17 and Toll-like receptor 10 genetic polymorphisms and susceptibility to large joint osteoarthritis," *Journal of Orthopaedic Research*, vol. 36, pp. 1684–1693, 2018.

[29] H. H. Bafrani, M. Ahmadi, D. Jahantigh, and M. Karimian, "Association analysis of the common varieties of IL17A and IL17F genes with the risk of knee osteoarthritis," *Journal of Cellular Biochemistry*, vol. 120, pp. 18020–18030, 2019.

[30] R. K. Eftedal, G. Vrgoc, Z. Jotanovic, and Z. Dembic, "Alternative interleukin 17A/F locus haplotypes are associated with increased risk to hip and knee osteoarthritis," *Journal of Orthopaedic Research*, vol. 37, pp. 1972–1978, 2019.

[31] I. I. F. G. da Silva, H. D. Angelo, E. Rushansky, M. H. Mariano, M. de Mascena Diniz Maia, and P. R. E. de Souza, "Interleukin (IL)-23 Receptor, IL-17A and IL-17F gene polymorphisms in Brazilian patients with rheumatoid arthritis," *Archivum Immunologiae et Therapiae Experimentalis*, vol. 65, pp. 537–543, 2017.

[32] G. B. N. Nordang, M. K. Viken, J. E. Hollis-Moffatt et al., "Association analysis of the interleukin 17A gene in Caucasian rheumatoid arthritis patients from Norway and New Zealand," *Rheumatology*, vol. 48, pp. 367–370, 2009.

[33] A. Amin, N. Sheikh, M. Mukhtar et al., "Association of interleukin-17 gene polymorphisms with the onset of rheumatoid arthritis," *Immunobiology*, vol. 226, p. 152045, 2021.

[34] L. Shen, H. Zhang, T. Yan, G. Zhou, and R. Liu, "Association between interleukin 17A polymorphisms and susceptibility to rheumatoid arthritis in a Chinese population," *Gene*, vol. 566, pp. 18–22, 2015.

[35] A. Pawlik, D. Kotrych, D. Malinowski, V. Dziedziejko, M. Czerewaty, and K. Safranow, "IL17A and IL17F gene polymorphisms in patients with rheumatoid arthritis," *BMC Musculoskeletal Disorders*, vol. 17, pp. 1–6, 2016.

[36] E. E. İnal, O. Görükmez, Ü. Dündar et al., "The influence of polymorphisms of interleukin-17A and -17F genes on susceptibility and activity of rheumatoid arthritis," *Genetic Testing and Molecular Biomarkers*, vol. 19, pp. 461–464, 2015.

[37] C. N. Carvalho, R. F. do Carmo, A. L. P. Duarte, A. A. T. Carvalho, J. C. Leão, and L. A. Gueiros, "IL-17A and IL-17F polymorphisms in rheumatoid arthritis and Sjögren's syndrome," *Clinical Oral Investigations*, vol. 20, pp. 495–502, 2016.

[38] O. S. Marwa, T. Kalthoum, K. Wajih, and H. Kamel, "Association of IL17A and IL17F genes with rheumatoid arthritis disease and the impact of genetic polymorphisms on response to treatment," *Immunology Letters*, vol. 183, pp. 24–36, 2017.

[39] S. Louahchi, I. Allam, L. Berkani, A. Boucharef, A. Abdesemed, and N. Khaldoun, "Association study of single nucleotide polymorphisms of IL23R and IL17 in rheumatoid arthritis in the Algerian population," *Acta Reum Port.*, vol. 41, pp. 151–157, 2016.

[40] I. Montúfar-Robles, R. E. Barbosa-Cobos, I. Alemán-Ávila, and J. Ramírez-Bello, "IL-17A haplotype confers susceptibility to systemic lupus erythematosus but not to rheumatoid arthritis in Mexican patients," *International Journal of Rheumatic Diseases*, vol. 22, pp. 473–479, 2019.

[41] F. M. Elfasakhany, M. A. Eldamarawi, and A. E. Khalil, "Association between interleukin-17 gene polymorphism and rheumatoid arthritis among Egyptians," *Meta Gene.*, vol. 16, pp. 226–229, 2018.

[42] A. Paradowska-Gorycka, E. Wojtecka-Lukasik, J. Trefler, B. Wojciechowska, J. K. Lacki, and S. Maslinski, "Association between IL-17F gene polymorphisms and susceptibility to and severity of rheumatoid arthritis (RA)," *Scandinavian Journal of Immunology*, vol. 72, pp. 134–141, 2010.

[43] D. Sabry, N. Aboraia, and M. Samir, "A potential association between psoriasin to rs4819554 of IL-17RA gene polymorphism in psoriasis Egyptian patients," *Archives of Dermatological Research*, vol. 312, pp. 273–281, 2020.

[44] A. Batalla, E. Coto, L. González-Lara et al., "Association between single nucleotide polymorphisms IL17RA rs4819554 and IL17E rs79877597 and psoriasis in a Spanish cohort," *Journal of Dermatological Science*, vol. 80, pp. 111–115, 2015.

[45] M. G. Catanoso, L. Boiardi, P. Macchioni et al., "IL-23A, IL-23R, IL-17A and IL-17R polymorphisms in different psoriatic arthritis clinical manifestations in the northern Italian population," *Rheumatology International*, vol. 33, pp. 1165–1176, 2013.

[46] Z. Kamenarska, M. Hristova, and G. Dzhebir, "Association of IL-17RC rs708567 with systemic lupus erythematosus," *Madridge Journal of Dermatology & Research*, vol. 65, pp. 65–67, 2018.

[47] R. Prieto-Pérez, G. Solano-López, T. Cabaleiro et al., "The polymorphism rs763780 in the IL-17F gene is associated with response to biological drugs in patients with psoriasis," *Pharmacogenomics*, vol. 16, pp. 1723–1731, 2015.

[48] M. Kamińska, W. Niepiekło-Miniewska, A. Nieszporek, K. Gębura, and K. Bogunia-Kubik, "Poster Presentations," *HLA*, vol. 91, no. 5, pp. 354–474, 2018.

[49] M. Braga, F. F. Lara-Armi, J. S. F. Neves et al., "Influence of IL10 (rs1800896) polymorphism and TNF-α, IL-10, IL-17A, and IL-17F serum levels in ankylosing spondylitis," *Frontiers in Immunology*, vol. 12, 2021.

[50] H. Nisar, U. Pasha, M. U. Mirza et al., "Impact of IL-17F 7488T/C functional polymorphism on progressive rheumatoid arthritis: novel insight from the molecular dynamic simulations," *Immunological Investigations*, vol. 50, pp. 416–426, 2021.

[51] M. G. de la Peña, R. M. Cruz, E. G. Guerrero, A. G. López, G. P. Molina, and N. E. H. González, "Polymorphism rs2275913 of Interleukin-17A is related to more intensive therapy with disease-modifying anti rheumatic drugs in Mexican patients with rheumatoid arthritis," *Acta Reum Port*, vol. 42, pp. 155–161, 2017.

[52] J. L. Espinoza, A. Takami, K. Nakata et al., "A genetic variant in the IL-17 promoter is functionally associated with acute graft-versus-host disease after unrelated bone marrow transplantation," *PLoS One*, vol. 6, 2011.

[53] S Bank, P. S. Andersen, J. Burisch et al., "Associations between functional polymorphisms in the NFκB signaling pathway and response to anti-TNF treatment in Danish patients with inflammatory bowel disease," *The Pharmacogenomics Journal*, vol. 14, pp. 526–534, 2014.

[54] A. Batalla, E. Coto, J. Gómez et al., "IL17RA gene variants and anti-TNF response among psoriasis patients," *The Pharmacogenomics Journal*, vol. 18, pp. 76–80, 2018.

[55] T. Furuya, M. Hakoda, N. Ichikawa et al., "Associations between HLA-DRB1, RANK, RANKL, OPG, and IL-17 genotypes and disease severity phenotypes in Japanese patients with early rheumatoid arthritis," *Clinical Rheumatology*, vol. 26, pp. 2137–2141, 2007.

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Review Article

Interleukin-1 Links Autoimmune and Autoinflammatory Pathophysiology in Mixed-Pattern Psoriasis

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Autoinflammatory and autoimmune diseases are characterized by an oversensitive immune system with loss of the physiological endogenous regulation, involving multifactorial self-reactive pathological mechanisms of mono- or polygenic nature. Failure in regulatory mechanisms triggers a complex network of dynamic relationships between innate and adaptive immunity, leading to coexistent autoinflammatory and autoimmune processes. Sustained exposure to a trigger or a genetic alteration at the level of the receptors of the natural immune system may lead to abnormal activation of the innate immune system, adaptive system activation, loss of self-tolerance, and systemic inflammation. The IL-1 family members critically activate and regulate innate and adaptive immune responses' diversity and plasticity in autoimmune and/or autoinflammatory conditions. The IL-23/IL-17 axis is key in the communication between innate immunity (IL-23-producing myeloid cells) and adaptive immunity (Th17- and IL-17-expressing CD8+ T cells). In psoriasis, these cytokines are decisive to the different clinical presentations, whether as plaque psoriasis (psoriasis vulgaris), generalized pustular psoriasis (pustular psoriasis), or mixed forms. These forms reflect a gradient between autoimmune pathophysiology with predominant adaptive immune response and autoinflammatory pathophysiology with predominant innate immune response.

1. Introduction

Autoinflammatory and autoimmune diseases are characterized by immune system hyperactivity, typically featuring an against-self pathological process. They are systemic diseases and mono- or polygenic. The innate immune system directly causes tissue inflammation in autoinflammatory diseases. An adaptive immune dysregulation—against self—is found in autoimmune diseases. Both combined are present in mixed autoinflammatory-autoimmune pattern diseases (Figure 1).

The former characterization of autoinflammatory diseases, the more recent mixed form presentation (autoinflammatory-autoimmune), and the changing contribution of underlying autoinflammatory processes to autoimmunity pathways further complicated understanding the pathophysiological processes [1].

The immune system responds to independent pathways, either exogenous (bacteria) or endogenous (injured tissue), yet with crosscommunication.

The clinical diversity of immune diseases may result from the variable expression of autoinflammatory and autoimmune factors in disease production, establishing a continuous spectrum of mixed patterns [2]. Connecting molecules or molecular aggregates between them are critical

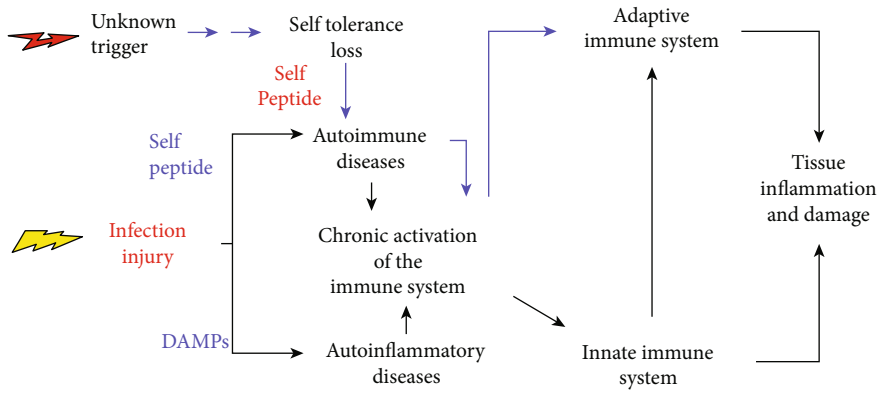

Figure 1: Simplified representation of the relative contribution of autoimmune, autoinflammatory, and mixed-pattern forms to tissue damage. DAMPs: damage-associated molecular patterns.

to understanding autoinflammatory-autoimmune disease interactions. Members of the IL-1 family and the inflammasome are key components in crosscommunication between these diseases with mixed components. Mutations in genes related to the inflammasome have been associated with autoinflammation. This multiprotein complex has been associated with organ-specific autoimmunity since a wide spectrum of endogenous danger signals can activate inflammasome products, including IL-1β, triggering adaptive immunity pathways [3].

Genetic predisposition involves many loci encoding key immune pathway molecules. These genes are under epigenetic control, influenced by several environmental factors in susceptible individuals. Interleukin-1 seems a critical link between autoinflammatory and autoimmune diseases involving innate and adaptive mechanisms.

Trends for classifying and providing a theoretical framework for all immune diseases that include autoinflammatory and autoimmune nature still hold. However, deeper comprehension of immune-mediated pathologies is required before postulating a unified explicative model.

In this review, we examine current knowledge on the inflammatory role of the IL-1 cytokine family, their association with the inflammasome in autoinflammatory and autoimmune disorder regulation, and the underlying implication of innate and adaptive immunity in diseases with a mixed pathogenic pattern, with particular focus on psoriasis [4].

2. The IL-1 Family

Eleven members of the IL-1 family participate in natural immunity and contribute to acute and chronic inflammation. The clinical severity results from the balance between the proinflammatory and anti-inflammatory IL-1 family members in some forms of rheumatic disease [5]. The IL-1β is the best-characterized member of the IL-1 family cytokines and potent inflammation mediator in the immune-inflammatory response. The IL-1 family—IL-1α; IL-1β; IL-18; IL-33; IL-36α, β, and γ; IL-37; and IL-38—includes regulatory factors that modify the intensity of the inflammatory response like decoy receptors, receptor antagonists, and inflammatory pathway signaling inhibitors. The identification of multiple negative regulation pathways of the IL-1/IL-1R family highlighted the need for tight control of the IL-1 family repertoire [6]. The pathophysiological role of IL-1β is well established in autoinflammatory diseases, and IL-1β and IL-18 are critically associated with severity in various autoimmune and chronic inflammatory pathologies [7].

Inflammasomes comprise a multimolecular complex of specialized intracellular sensors. Mutations in inflammasome-related genes have been associated with autoinflammation and autoimmunity. A broad spectrum of endogenous danger signals can activate inflammasome products, including IL-1β, triggering adaptive immunity pathways [3].

The pathophysiological transition results from the imbalance between the proinflammatory activities of IL-1 cytokines and their control mechanisms.

The IL-1 family members (Table 1) play a key role in innate and adaptive immunity and in the pathogenesis of autoimmune and autoinflammatory diseases. Members of the IL-1R-like receptor family include signaling molecules and negative regulators.

3. Psoriasis: A Leading Case in Mixed-Pattern Psoriasis Diseases

Psoriasis is considered a systemic chronic inflammatory disease with an immunogenetic basis that can be triggered extrinsically or intrinsically [8, 9]. The disease is characterized by critical interactions between components of the adaptive and the innate immune systems [10, 11].

In recent years, remarkable progress has been made in our understanding of the critical immune pathways involved in psoriasis. Genetic studies have shown that susceptibility to psoriasis involves components of both the adaptive and innate immune systems. Activation of both arms of the immune system is implicated in psoriasis of the skin, while autoimmune adaptive pathogenic immune responses predominate in chronic plaque psoriasis and innate autoinflammatory pathogenic responses dominate in pustular forms of psoriasis, and other clinical subtypes span a spectrum between plaque and pustular psoriasis. This makes psoriasis a mixed autoimmune and autoinflammatory disease, where

Table 1: IL-1 family of ligands and receptors.

IL-1 family	Specific receptor	Coreceptor	Function
IL-1α, IL-1β	IL-1R1	IL-1RAP	Proinflammatory
IL-1β	IL-1R2	IL-1RAP	Anti-inflammatory
IL-1Ra	IL-1R1	Not available	Anti-inflammatory
IL-18	IL-18R1	IL-18RAP	Proinflammatory
IL-33	IL-1RL1	IL-1RAP	Proinflammatory
IL-36α, β, γ	IL-1RL2	IL-1RAP	Proinflammatory
IL-36Ra	IL-1RL2	IL-1RAP	Anti-inflammatory
IL-37	IL-18R1	SIGIRR	Anti-inflammatory
IL-38	IL-1RL2	IL-1RAPL2	Anti-inflammatory

the balance between the two responses determines the clinical presentation [12].

The relative expression of inflammatory mediators, influenced by different IL-1 family members, determines different subclinical patterns along a wide transition spectrum that extends from one type of psoriasis in which the autoimmune component dominates to another in which the autoinflammatory component dominates. Plaque psoriasis presents a typical adaptive immune response, with immune synapsis in secondary lymphoid organs and adaptive leukocyte-effector inflammatory functions in the skin. In contrast, generalized pustular psoriasis is characterized by enhanced chemotaxis-mediated phagocyte infiltration and phagocyte effector functions [13]. The IL-23/IL-17 axis in psoriasis highlighted the strong interaction between cells of the innate immune system (represented by IL-23-producing myeloid antigen-presenting cells) with cells of the adaptive immune system (represented by Th17- and IL-17-expressing cytotoxic CD8+ T cells). The balance between IL-36 and IL-17 partially influences the clinical expression profile (psoriasis vulgaris vs. psoriasis pustulosa) [14] (Figure 2).

3.1. Autoimmune Processes in Psoriasis. Autoimmune signature in psoriasis seems driven by local and systemic Th17 patterns, expressing IL-17A, IL-22, and IFN-γ [10] (Figure 3). Chronic-stimulated dendritic cells sustain activation and differentiation of lesional Th17 cells primarily through secretion of IL-23 [15].

Both HLA restriction and T cell peptide specificity are determined by the T cell receptor repertoire. Antigenic stimulation triggers T cells' activation and clonal expansion. In the absence of foreign antigens, clonal T cell expansion likely suggests autoimmunity in inflammatory diseases [16].

Psoriasis seems to be driven by locally prevailing antigens [17]. Environmental factors are mostly rated, including stress, smoking, drugs, and infections [18]. Activated clonal T cells exert disease inflammatory process in combination with locally inflammatory leucocytes. In the last years, putative autoantigens like cathelicidin LL-37, melanocytic ADAMTSL5, lipid antigen PLA2G4D, and keratin 17 have been identified in psoriasis [19–22].

3.2. Crosstalk between Adaptive and Innate Immunity in Psoriasis. Complex crosstalk between the innate and adaptive immune systems in psoriasis adds up to antigen-specific exacerbation of inflammation in psoriasis. The accumulating circumstantial evidence suggests that in patients with stable and mild psoriasis, adaptive immunity is likely more prevalent, while innate immunity might contribute more to the active and severe disease, systemic involvement, and comorbid conditions [23] (Figure 3). The coexistence of comorbidities like atherosclerosis in severe psoriasis has been interpreted as a systemic inflammatory reaction to the innate local inflammation in affected tissues [24]. The involved factors are not psoriasis-specific, though they magnify the overall inflammatory burden in patients with severe psoriasis.

The studies addressing the interplay between IL-17- and IL-36-driven inflammation might help understand how certain mediators influence the psoriasis spectrum by shifting innate or adaptive immunity [25]. All IL-36 isoforms (IL-36α, β, and γ) are members of the IL-1 family and are expressed in psoriatic skin [26]. They bind to a specific receptor (IL-1RL2), triggering the transcription of several inflammatory mediators through NF-κB activation.

The IL-36 seems to be associated with the clinical manifestation of specific psoriatic phenotypes. The skin in psoriasis vulgaris differs significantly from that in pustular psoriasis, representing opposite ends of the psoriasis spectrum. The balance between IL-36 and IL-17 might contribute to differential clinical symptoms between the vulgaris and pustulosa forms, in line with the response to given therapies [27].

The IL-23/IL-17 axis is key as it comprises innate immunity (IL-23-producing myeloid cells) and adaptive immunity (Th17- and IL-17-expressing CD8+ T cells). Understanding psoriasis might help shed light on such relationships.

3.3. On How IL-1 and IL-8 Participate in Th1 and Th17 Activation by IL-12/23. Some cytokines, including IL-1, induce IL-17 release in human T lymphocytes. Their ability to promote Th17 cells depends not only on the induction of IL-23, IL-6, and TGF-β by dendritic cells but also on directly or indirectly activating the inflammasome and inducing IL-1β.

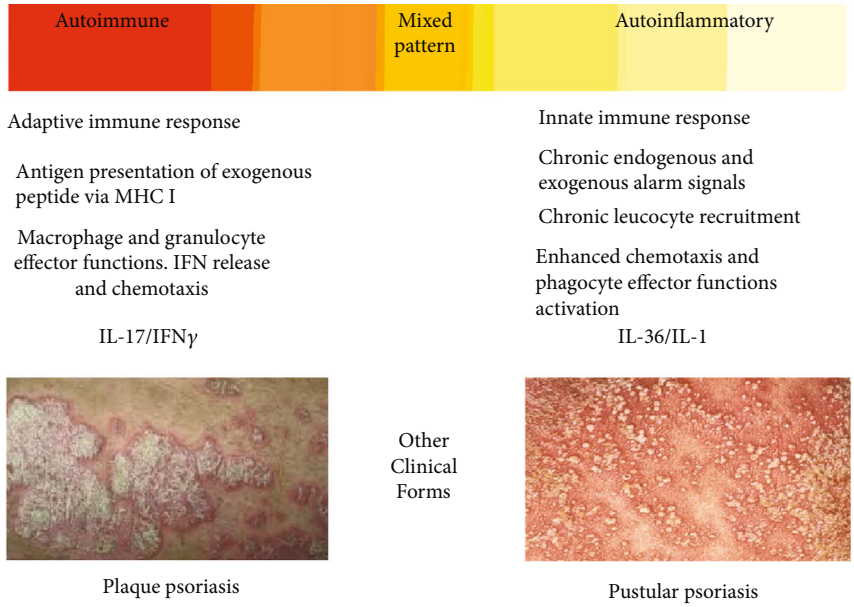

Figure 2: Autoimmune vs. autoinflammatory responses in psoriasis. Complicate interactions between the innate and the adaptive immune systems characterize the pathophysiology of psoriasis. Once settled, the relative contribution of inflammatory and regulatory mediators of adaptive and innate immunity determines the clinical manifestation towards chronic stable vs. highly inflammatory and/or pustular psoriasis. Plaque psoriasis (psoriasis vulgaris) and generalized pustular psoriasis (psoriasis pustulosa) represent autoimmune (IL-17A/IFN-γ secretion profile) and autoinflammatory (IL-36/IL-1 secretion profile) response patterns, respectively.

IL-12 and IL-23 are extremely important to induce Th1 and Th17, respectively, and their production mediated by antigen-presenting cells is distinctively regulated. Innate immunity-derived stimuli regulate IL-12 and IL-23 production, influencing the induced T lymphocyte phenotype. IL-23 promotes IL-23R expression in myeloid cells and induces proinflammatory TNF-α and IL-1β cytokine production. Further, IL-23 promotes CD4+ precursors' differentiation to the Th17 effector in the absence of IFN-γ and IL-4 [28].

IL-1 promotes lymphocytes' growth and differentiation. The differential expression of IL1R1 in CD4 T lymphocyte subtypes confers different effector functions.

Th17 cells' response to IL-12 and sustained exposure to IL-23 promote Th17 change to the Th1 phenotype [29], indicating the strong environmental influence [30]. Th17-derived Th1 cells are called "nonclassic Th1" and express CD161 and IL1R1 [31]. IL-1β and IL-23 combination promotes T cells' production with the Th17 and Th1 phenotype in CD4+ CD161+ and CD4+ CD161- cell fractions. This suggests that Th1 cells respond to IL-1β and that CD4+ CD161+ clones in inflamed tissue are able to produce IFN-gamma and express IL1R1 mRNA [32].

IL-8 (CXCL8) participates in the pathophysiology of psoriasis recruiting neutrophils and other inflammatory leukocytes. In fact, IL-8 highly expresses in plaque psoriasis and, up to tenfold, in pustular psoriasis [25].

IL-36, highly expressed in plaque psoriasis, acts on keratinocytes and myeloid dendritic cells [33] and is a potent inducer of the neutrophil CXCL1 and IL-8 chemotactic cytokines. Infiltrating neutrophils play a fundamental role in psoriatic plaque, amplifying the IL-36-mediated autoinflammatory loop in psoriasis [13].

4. Association of Inflammasomes with Innate and Adaptive Immunity

Inflammasomes are tripartite complexes comprising a cytoplasmic sensor, an adapter known as ASC, and procaspase-1. Inflammasomes are defined by their cytoplasmic sensor, which includes AIM2, Pyrin, NLRP1, NLRP3, and NLRC4 and belongs to the NOD2-like receptor family. Sensors' diversity and specificity allow inflammasomes to respond to a wide range of either extrinsic (microbial molecules) or intrinsic (danger signals) stimuli.

The NLRP3 inflammasome is the prototypical and best-characterized inflammasome, and its activation has been sequenced [34]. A first signal, priming, provided by microbial molecules like lipopolysaccharide induces NLRP3 and pro-IL1β expression in an NF-κB-dependent fashion. Microbial molecules like toxins or danger signals like monosodium urate offer the second signal and trigger multimerization to make up an inflammasome (Figure 4).

The NLRP3 assembles to ASCs, leading to caspase-1 activation, which induces proteolytic maturation of IL-1β and IL-18 and Gasdermin D cleavage. The next pore formation of Gasdermin D in the cell membrane induces pyroptosis, a fast proinflammatory cell death [35]. Pyroptosis associated with the release of IL-1β, IL-18, and alarmins contributes to danger signal propagation beyond the damaged or infected cell, recruiting mono- and polymorphonuclear phagocytes (Figure 3). Oligomeric particles may be released from the inflammasome to further amplify the inflammatory response after phagocytosis by surrounding macrophages.

In intact phagocytes, IL-1β secretion can occur independently from pyroptosis. Autophagy regulates the

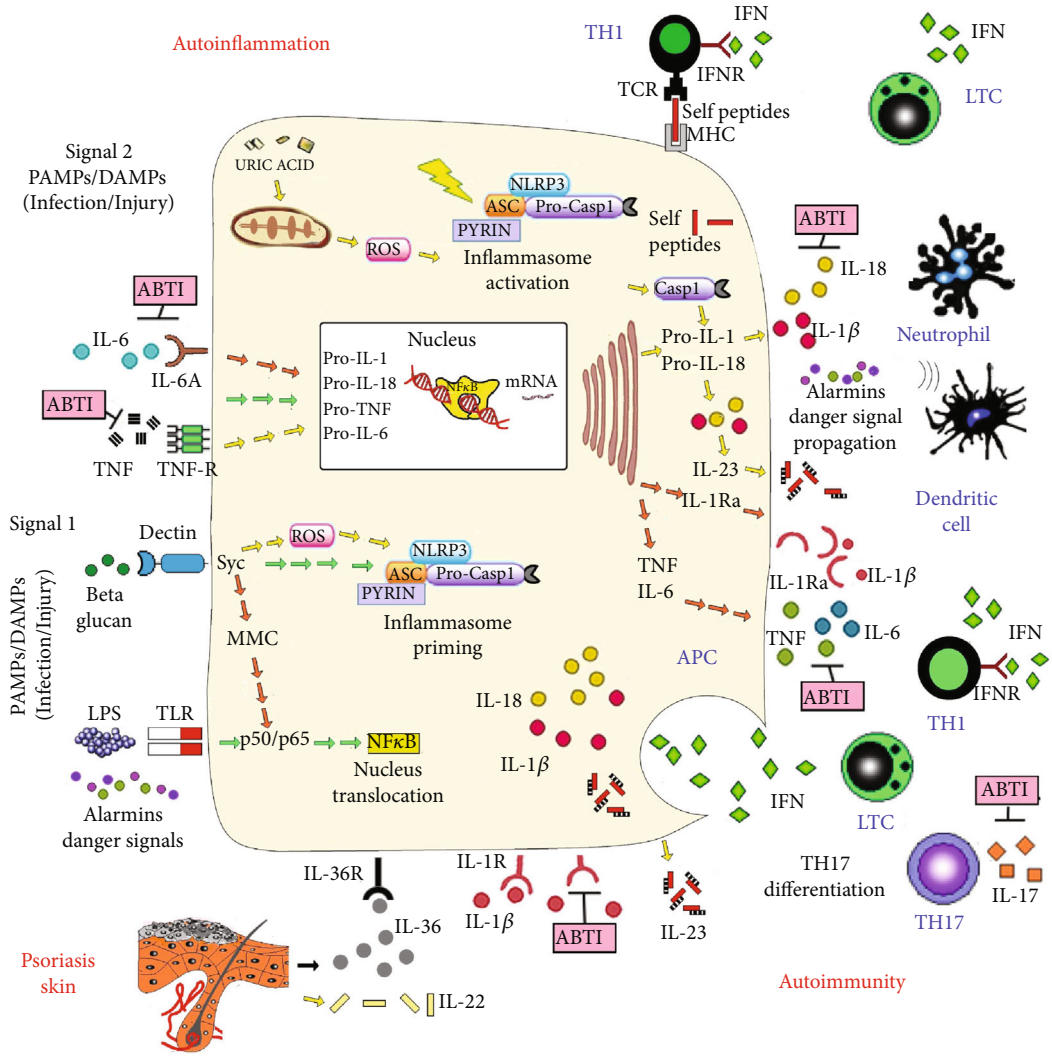

Figure 3: Schematic representation of inflammasome signaling mechanisms in mixed-pattern inflammatory diseases. Inflammatory agents and pathogens trigger the canonical inflammasome pathway. PAMPs and DAMPs are detected by specific innate immune sensors, leading to oligomerization and inflammasome assembly. The therapeutic targets in autoinflammatory diseases are as follows: signal 1 inflammasome activation: surface pattern recognition receptors like Toll-like receptors (TLR) and pathogen-associated molecular patterns stimulate the production of molecules like NF-κB and activate inflammasome assembly through downstream immunologic processes; signal 2 inflammasome activation: crystals in gout, heat-shock proteins, and damaged tissue as in burns, another pathogen- and damage-associated molecular pattern, activate inflammasome assembly through reactive oxygen species (ROS) production and downstream immunologic processes. Certain mediators influence the spectrum of psoriasis, shifting to innate or adaptive immune processes. The interplay between IL-17- and IL-36-driven inflammation seems involved in innate-adaptive immune balance. Inflammasome-induced hyperactive dendritic cells (DC) trigger enhanced T cell responses, preserving antigen and autoantigen presentation and contextualizing T helper cell responses through IL-1β, IL-18, and IL-23 secretion. These cytokines trigger Th1/Th17 responses. IL-18 amplifies IFN-γ production by Th1 cells and reinforces Th1 differentiation, while IL-1β promotes Th17 polarization and IL-17 secretion, causing a mixed autoinflammatory-autoimmune pathology. The image shows the potential sites for antibody-based therapeutic intervention (ABTI). ASC: apoptosis-associated speck protein; ER: endoplasmic reticulum; IFNAR: interferon-associated receptor; IFN: interferon; IL-1: interleukin-1; IL-1 R: IL-1 receptor; IL-1Ra: IL-1 receptor antagonist; IL-6: interleukin 6; IL-6R: IL-6 receptor; IL-18: interleukin-18; JAK: Janus kinase; NLRP3: NOD-like receptor P3; ROS: reactive oxygen species; TLR: Toll-like receptor; TNF: tumor necrosis factor; TNF-R: TNF receptor; ASC: apoptosis-associated speck-like protein containing a CARD; CARD: caspase recruitment domain; DAMP: damage-associated molecular pattern; LPS: lipopolysaccharide; NLR: NOD-like receptor; NOD: nucleotide-binding oligomerization domain; PAMP: pathogen-associated molecular pattern; PYD: Pyrin domain; MMC: multimolecular complex; HMGB1: high-mobility group box 1; TCR: T cell receptor.

inflammasome-processed cytokines, which induce IL-17. Autophagy intersects with the inflammasome-dependent generation of IL-1β and IL-18 at different stages. Autophagosomes can remove endogenous inflammasome-activating stimuli, including mitochondrial DNA, ROS, damaged lysosomes, pro-IL-1β, and inflammasome components as well. Autophagy inhibits IL-23 secretion due to its effects on IL-1β [36].

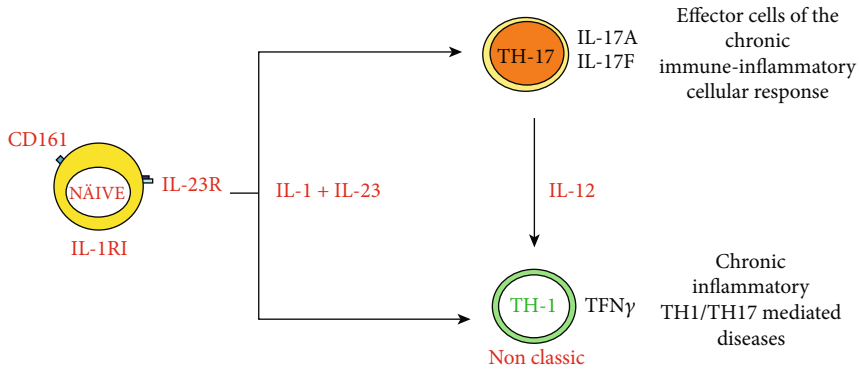

Figure 4: IL-1 and IL23 induce CD4 CD161 precursors' differentiation to classical Th-17 and Th-1 cells in the presence of IL-12. IL-1: interleukin-1; IL-23: interleukin-23; IL-12: interleukin-12; Th: T-helper cells.

Involvement in a variety of pathophysiological conditions poses inflammasomes as interesting antibody-based therapeutic intervention targets (Figure 3). From a pathogenetic perspective, they are characterized by chronic activation of the immune system, causing tissue inflammation in genetically predisposed individuals. However, damage-specific effectors are different. In autoinflammatory diseases, the innate immune system directly causes tissue inflammation, while in autoimmune disorders, the innate immune system activates the adaptive immune system, ultimately responsible for the inflammatory process [37].

Some diseases have a mixed autoimmune-autoinflammatory root [38]. Inflammasome dysregulation is associated with autoinflammatory and autoimmune diseases like familial Mediterranean fever, rheumatoid arthritis, psoriasis, and systemic lupus erythematosus [4, 39]. Some immune-inflammatory diseases may reflect a variable expression in the pathogenetic autoinflammatory and autoimmune factors [40].

In an explanatory attempt, Polly Matzinger put forward the danger signal theory. This proposes that the immune system does not so much discriminate between endogenous and exogenous signals but increases responses to danger signals, regardless if they are exogenous pathogenic bacteria or endogenous damaged tissues [41]. However, the hazard model does not adequately explain the exquisite specificity of adaptive immune responses in autoimmune diseases. Recent advances in genetic and molecular studies allow converging to a united classification for all immunological diseases in a theoretical framework. Psoriasis, ankylosing spondylitis, Behcet's syndrome, uveitis, and other diseases show a mixed pattern.

Inflammasome-hyperactivated dendritic cells elicit enhanced T cell responses. They preserve their antigen-presenting function and contextualize T-helper cell responses through IL-1β and IL-18 secretion. These cytokines drive Th1/Th17 responses in particular. The IL-18 amplifies IFN-γ production by Th1 cells, while IL-1β promotes Th17 polarization and IL-17 secretion [42] (Figure 3).

Inflammasome-dependent IL-1β-driven Th17 responses are essential for host defense against infections by fungi like Candida albicans. The C-type lectins Dectin-1 are involved in host defense mechanisms against fungal infection, driving inflammatory and adaptive immune responses. Dectin-1 is a type-C lectin receptor that detects β-glucans [43]. This leads to Syk-dependent NF-κB activation and NLRP3 inflammasome assembly, while Th17 responses yield immune protection against the pathogen [44]. Notably, Dectin-1 signaling also triggers IL-1β production through a noncanonical caspase-8 inflammasome [45].

The divergent roles of IL-1β and IL-18 in adaptive immunity setup have drawn much attention to inflammasomes as adjuvants to vaccines. The Th1-mediated humoral responses, cytotoxic T cell/Th1/Th17 immunity, and immune memory can be manipulated using inflammasome-activating ligands [46]. Type I interferons inhibit pro-IL-1 synthesis, promote IL-18 maturation, and, combined with inflammasomes' activation, might aid in modeling protective Th1 responses [47]. The vaccine adjuvant chitosan is a cationic polysaccharide that induces type I IFN production, NLRP3 inflammasome activation, and intense Th1 responses. More studies are needed to better understand the role of inflammasomes in pathological and protective immunity.

Apoptosis and pyroptosis are two well-studied cell death patterns, traditionally believed as unrelated. Emerging evidence shows their extensive interrelation as converging pathways, activating the same cell death effector, the pore-forming protein Gasdermin D [48].

Pyroptosis is the inflammatory cell death triggered by intracellular detection of signs of damage or pathogens [49]. Pyroptotic cells show swelling, fragmented genetic material, membrane pore formation, plasma membrane rupture, and release of inflammatory mediators and cytoplasmic content to the extracellular space [50]. Lipopolysaccharide, a hallmark of the gram-negative bacterial cell wall, is a prototypical trigger of immune cell pyroptosis. Pyroptosis starts with the innate TLR4 activation step. This induces NF-κB activation and translocation to the nucleus to boost gene transcription for precursors pro-IL-1β, pro-IL-18, and pro-caspases and intracellular Nod-like receptors' transcription. In psoriatic lesions, Dectin-1 upregulation seems under the control of psoriasis-associated cytokines, while its role in the biology of skin inflammation and infection is to be explored [51].

The second signal induces oligomerization of intracellular complexes called inflammasomes [52], which facilitate

pro-IL-1β and procaspase-1 maturation into their active forms. While IL-1β is released and induces a proinflammatory state, caspase-1 breaks down the cytoplasmic gasdermin D, forming pores in the membrane and triggering cell death through cytoplasmic components' leakage.

Autophagy is a self-degrading process required to restore cell homeostasis when menacing factors are detected [53]. This ubiquitous lysosomal degradation mechanism removes damaged proteins and organelles, contributes to antigen presentation to the cell surface, protects against genome instability, and prevents tissue damage. Autophagy is of physiological relevance, helping defend against damaging stress while leading to pathology when in excess or defect [54].

As an essential homeostatic mechanism, autophagy is upregulated in response to environmental and pharmacological triggers. It has a very important role in cancer, neurodegeneration, diabetes, and liver and autoimmune diseases. Molecular elements that lead to this type of cell death also collaborate in the stress response.

In the immune system, autophagy serves as a source of peptides for antigen presentation [55], provides a mechanism for the absorption and degradation of intracellular pathogens, and is a key regulator of inflammatory cytokines. It is also involved in regulating inflammasome activation and helping remove inflammasome components and endogenous activators [56] and plays a role in determining IL-1β fate in autophagosomes. Present understanding suggests that autophagy is a critical regulator of inflammasome activation and IL-1 family cytokines' release [57].

5. Inflammasome-Induced IL-1 Promotes IL-17-Mediated Responses

An inflammasome is a multiprotein complex that contributes to defense against pathogens and repair during inflammatory processes while producing inflammatory diseases under aberrant chronic conditions. Inflammasome assembly triggers caspases' activation, setting off inflammatory cytokines, including IL-1 activation.

The finding of IL-17 and IL-17-secreting T cells has improved our understanding of the T cell role in autoimmune and other inflammatory diseases. The Th1 cells were first considered key pathogenic T cells in many autoimmune diseases. However, mice deficient in IFN-γ or IL-12 signaling had exacerbated symptoms in certain autoimmune diseases [58].

Dendritic cells associated with inflammasome hyperactivity boost T lymphocyte activity (Th1/Th17) through increased IL-1β, IL-18, and IL-23 release. IL-18 amplifies Th1 cells' IFN-γ production and enhances Th1 differentiation while IL-1β promotes Th17 polarization and IL-17 release, triggering a pathological autoinflammatory and autoimmune profile.

Besides, IL-1 and IL-23 (Figure 4) can induce and activate human Th1/Th17 cell differentiation. IL-1 can induce cells of the innate immune system to produce IL-6, which stimulates naïve T cell differentiation to Th17 [59].

6. Conclusions

From a pathogenic perspective, most autoinflammatory and autoimmune diseases share a chronic aberrant immune system activation, which leads to tissue inflammation and damage of varying magnitude in genetically predisposed individuals. IL-1 has grown into a complex, multifaceted family of cytokines with complex regulatory mechanisms and diverse functions in health and disease.

IL-1 and inflammasome are strongly associated with adaptive and autoimmune disorders. The role of the inflammasome-associated IL-1 cytokines' family in shaping adaptive immune responses is now well-established regarding the differentiation of Th17 cells and promoting effector functions of Th1 cells and CD8 T cells. In addition, cell lysis triggers inflammasome activation, releasing additional DAMPs and self-antigens, linking autoinflammation and autoimmunity. The contribution of IL-1 and associated molecules to inflammasome regulation needs exploration to improve our understanding of inflammatory diseases.

The relevance of the IL1-related cytokines has outreached classic immunopathology and is a critical bridge to understanding mixed-pattern diseases.

Novel therapeutic intervention strategies may be anticipated after deepening our understanding of inflammatory disorders and the molecular pathways of autoinflammation, autoimmunity, and immune homeostasis regulation.

Abbreviations

HLAs: Human leukocyte antigens
IL-: Interleukin
Th17: T helper lymphocyte
CD8+: Cytotoxic T cells
IFN-γ: Interferon gamma
NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells
ASC: Apoptosis-associated protein
AIM: Absent in melanoma
NLRP: NLR family pyrin domain containing
NOD: Binding oligomerization domain
ROS: Reactive oxygen species
TLR4: Toll like-4 immune receptor.

Data Availability

The data supporting this review are from previously reported studies, which have been cited.

Conflicts of Interest

The authors declare no conflicts of interest.

Authors' Contributions

Rodolfo Kölliker Frers and Tamara Kobiec shared authorship.

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References

[1] D. E. Smilek and E. W. St Clair, "Solving the puzzle of autoimmunity: critical questions," *F1000Prime Reports*, vol. 7, p. 17, 2015.

[2] D. McGonagle and M. F. McDermott, "A proposed classification of the immunological diseases," *PLoS Medicine*, vol. 3, no. 8, article e297, 2006.

[3] G. Lopalco, L. Cantarini, A. Vitale et al., "Interleukin-1 as a common denominator from autoinflammatory to autoimmune disorders: premises, perils, and perspectives," *Mediators of Inflammation*, vol. 2015, Article ID 194864, 21 pages, 2015.

[4] S. Tartey and T. D. Kanneganti, "Inflammasomes in the pathophysiology of autoinflammatory syndromes," *Journal of Leukocyte Biology*, vol. 107, no. 3, pp. 379–391, 2020.

[5] C. A. Dinarello, "The IL-1 family of cytokines and receptors in rheumatic diseases," *Nature Reviews Rheumatology*, vol. 15, no. 10, pp. 612–632, 2019.

[6] C. Garlanda, F. Riva, E. Bonavita, and A. Mantovani, "Negative regulatory receptors of the IL-1 family," *Seminars in Immunology*, vol. 25, no. 6, pp. 408–415, 2013.

[7] A. Mantovani, C. A. Dinarello, M. Molgora, and C. Garlanda, "Interleukin-1 and Related Cytokines in the Regulation of Inflammation and Immunity," *Immunity*, vol. 50, no. 4, pp. 778–795, 2019.

[8] W. H. Boehncke and W. Sterry, "Psoriasis–a systemic inflammatory disorder: clinic, pathogenesis and therapeutic perspectives," *Journal der Deutschen Dermatologischen Gesellschaft*, vol. 7, no. 11, pp. 946–952, 2009.

[9] K. Schäkel, M. P. Schön, and K. Ghoreschi, "Pathogenese der psoriasis vulgaris [pathogenesis of psoriasis]," *Der Hautarzt*, vol. 67, no. 6, pp. 422–431, 2016.

[10] W. H. Boehncke and M. P. Schön, "Psoriasis," *Lancet*, vol. 386, no. 9997, pp. 983–994, 2015.

[11] J. C. Prinz, "Human leukocyte antigen-class I alleles and the autoreactive T cell response in psoriasis pathogenesis," *Frontiers in Immunology*, vol. 9, p. 954, 2018.

[12] Y. Liang, M. K. Sarkar, L. C. Tsoi, and J. E. Gudjonsson, "Psoriasis: a mixed autoimmune and autoinflammatory disease," *Current Opinion in Immunology*, vol. 49, pp. 1–8, 2017.

[13] A. M. Foster, J. Baliwag, C. S. Chen et al., "IL-36 promotes myeloid cell infiltration, activation, and inflammatory activity in skin," *Journal of Immunology*, vol. 192, no. 12, pp. 6053–6061, 2014.

[14] B. Germán, R. Wei, P. Hener et al., "Disrupting the IL-36 and IL-23/IL-17 loop underlies the efficacy of calcipotriol and corticosteroid therapy for psoriasis," *JCI Insight*, vol. 4, no. 2, article e123390, 2019.

[15] M. P. Schön and L. Erpenbeck, "The interleukin-23/interleukin-17 axis links adaptive and innate immunity in psoriasis," *Frontiers in Immunology*, vol. 9, p. 1323, 2018.

[16] D. J. Woodsworth and M. Castellarin, "Holt review sequence analysis of T-cell repertoires in health and disease," *Genome medicine*, vol. 5, no. 10, p. 98, 2013.

[17] S. M. Kim, L. Bhonsle, P. Besgen et al., "Analysis of the paired TCR α- and β-chains of single human T cells," *PLoS One*, vol. 7, no. 5, article e37338, 2012.

[18] M. Roszkiewicz, K. Dopytalska, E. Szymańska, A. Jakimiuk, and I. Walecka, "Environmental risk factors and epigenetic alternations in psoriasis," *Annals of Agricultural and Environmental Medicine*, vol. 27, no. 3, pp. 335–342, 2020.

[19] H. Valdimarsson, R. H. Thorleifsdottir, S. L. Sigurdardottir, J. E. Gudjonsson, and A. Johnston, "Psoriasis–as an autoimmune disease caused by molecular mimicry," *Trends in Immunology*, vol. 30, no. 10, pp. 494–501, 2009.

[20] J. M. Kahlenberg and M. J. Kaplan, "Little peptide, big effects: the role of LL-37 in inflammation and autoimmune disease," *Journal of Immunology*, vol. 191, no. 10, pp. 4895–4901, 2013.

[21] R. Lande, J. Gregorio, V. Facchinetti et al., "Plasmacytoid dendritic cells sense self-DNA coupled with antimicrobial peptide," *Nature*, vol. 449, no. 7162, pp. 564–569, 2007.

[22] L. L. ten Bergen, A. Petrovic, A. K. Aarebrot, and S. Appel, "Current knowledge on autoantigens and autoantibodies in psoriasis," *Scandinavian Journal of Immunology*, vol. 92, no. 4, article e12945, 2020.

[23] P. M. Brunner, E. Guttman-Yassky, and D. Y. Leung, "The immunology of atopic dermatitis and its reversibility with broad-spectrum and targeted therapies," *The Journal of Allergy and Clinical Immunology*, vol. 139, no. 4S, pp. S65–S76, 2017.

[24] E. Christophers and P. C. M. van de Kerkhof, "Severity, heterogeneity and systemic inflammation in psoriasis," *Journal of the European Academy of Dermatology and Venereology*, vol. 33, no. 4, pp. 643–647, 2019.

[25] A. Johnston, X. Xing, L. Wolterink et al., "IL-1 and IL-36 are dominant cytokines in generalized pustular psoriasis," *The Journal of Allergy and Clinical Immunology*, vol. 140, no. 1, pp. 109–120, 2017.

[26] P. Muhr, J. Zeitvogel, I. Heitland, T. Werfel, and M. Wittmann, "Expression of interleukin (IL)-1 family members upon stimulation with IL-17 differs in keratinocytes derived from patients with psoriasis and healthy donors," *The British Journal of Dermatology*, vol. 165, no. 1, pp. 189–193, 2011.

[27] D. Wilsmann-Theis, L. M. Schnell, V. Ralser-Isselstein et al., "Successful treatment with interleukin-17A antagonists of generalized pustular psoriasis in patients without IL36RN mutations," *The Journal of Dermatology*, vol. 45, no. 7, pp. 850–854, 2018.

[28] L. E. Harrington, R. D. Hatton, P. R. Mangan et al., "Interleukin 17-producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages," *Nature Immunology*, vol. 6, pp. 1123–1132, 2005.

[29] Y. K. Lee, H. Turner, C. L. Maynard et al., "Late developmental plasticity in the T helper 17 lineage," *Immunity*, vol. 30, no. 1, pp. 92–107, 2009.

[30] K. Nistala, S. Adams, H. Cambrook et al., "Th17 plasticity in human autoimmune arthritis is driven by the inflammatory environment," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 107, no. 33, pp. 14751–14756, 2010.

[31] L. Cosmi, R. de Palma, V. Santarlasci et al., "Human interleukin 17-producing cells originate from a CD161+CD4+ T cell precursor," *The Journal of Experimental Medicine*, vol. 205, no. 8, pp. 1903–1916, 2008.

[32] L. Cosmi, R. Cimaz, L. Maggi et al., "Evidence of the transient nature of the Th17 phenotype of CD4+CD161+ T cells in the

synovial fluid of patients with juvenile idiopathic arthritis," *Arthritis and Rheumatism*, vol. 63, no. 8, pp. 2504–2515, 2011.

[33] A. Johnston, X. Xing, A. M. Guzman et al., "IL-1F5, -F6, -F8, and -F9: a novel IL-1 family signaling system that is active in psoriasis and promotes keratinocyte antimicrobial peptide expression," *Journal of Immunology*, vol. 186, no. 4, pp. 2613–2622, 2011.

[34] A. Baroja-Mazo, F. Martín-Sánchez, A. I. Gomez et al., "The NLRP3 inflammasome is released as a particulate danger signal that amplifies the inflammatory response," *Nature Immunology*, vol. 15, no. 8, pp. 738–748, 2014.

[35] S. B. Kovacs and E. A. Miao, "Gasdermins: effectors of pyroptosis," *Trends in Cell Biology*, vol. 27, pp. 673–684, 2017.

[36] V. A. Rathinam and K. A. Fitzgerald, "Inflammasome complexes: emerging mechanisms and effector functions," *Cell*, vol. 165, no. 4, pp. 792–800, 2016.

[37] C. Dostert, K. Ludigs, and G. Guarda, "Innate and adaptive effects of inflammasomes on T cell responses," *Current Opinion in Immunology*, vol. 25, no. 3, pp. 59–365, 2013.

[38] S. Savic, A. Mistry, A. G. Wilson et al., "Autoimmune-autoinflammatory rheumatoid arthritis overlaps: a rare but potentially important subgroup of diseases," *RMD Open*, vol. 3, no. 2, article e000550, 2017.

[39] P. J. Shaw, M. F. McDermott, and T. D. Kanneganti, "Inflammasomes and autoimmunity," *Trends in Molecular Medicine*, vol. 17, no. 2, pp. 57–64, 2011.

[40] C. M. Hedrich, "Shaping the spectrum - from autoinflammation to autoimmunity," *Clinical Immunology*, vol. 165, pp. 21–28, 2016.

[41] A. Broggi and F. Granucci, "Microbe- and danger-induced inflammation," *Molecular Immunology*, vol. 63, no. 2, pp. 127–133, 2015.

[42] C. L. Evavold and J. C. Kagan, "How inflammasomes inform adaptive immunity," *Journal of Molecular Biology*, vol. 2018, no. 430, pp. 217–237, 2018.

[43] K. Tone, M. H. T. Stappers, J. A. Willment, and G. D. Brown, "C-type lectin receptors of the Dectin-1 cluster: physiological roles and involvement in disease," *European Journal of Immunology*, vol. 49, no. 12, pp. 2127–2133, 2019.

[44] R. V. Luckheeram, R. Zhou, A. D. Verma, and B. Xia, "$CD4^+$T cells: differentiation and functions," *Clinical & Developmental Immunology*, vol. 2012, article 925135, pp. 1–12, 2012.

[45] R. Uchiyama and H. Tsutsui, "Caspases as the key effectors of inflammatory responses against bacterial infection," *Archivum Immunologiae et Therapiae Experimentalis (Warsz)*, vol. 63, no. 1, pp. 1–13, 2015.

[46] M. G. Netea, J. Domínguez-Andrés, L. B. Barreiro et al., "Defining trained immunity and its role in health and disease," *Nature Reviews. Immunology*, vol. 20, no. 6, pp. 375–388, 2020.

[47] J. Deng, X. Q. Yu, and P. H. Wang, "Inflammasome activation and Th17 responses," *Molecular Immunology*, vol. 107, pp. 142–164, 2019.

[48] K. W. Chen, B. Demarco, and P. Broz, "Beyond inflammasomes: emerging function of gasdermins during apoptosis and NETosis," *EMBO Journal*, vol. 39, no. 2, article e103397, 2020.

[49] D. Frank and J. E. Vince, "Pyroptosis versus necroptosis: similarities, differences, and crosstalk," *Cell Death and Differentiation*, vol. 26, no. 1, pp. 99–114, 2019.

[50] T. Bergsbaken, S. L. Fink, and B. T. Cookson, "Pyroptosis: host cell death," *Nature Reviews. Microbiology*, vol. 7, no. 2, pp. 99–109, 2009.

[51] H. D. de Koning, D. Rodijk-Olthuis, I. M. van Vlijmen-Willems et al., "A comprehensive analysis of pattern recognition receptors in normal and inflamed human epidermis: upregulation of dectin-1 in psoriasis," *The Journal of Investigative Dermatology*, vol. 130, no. 11, pp. 2611–2620, 2010.

[52] N. Kelley, D. Jeltema, Y. Duan, and Y. He, "The NLRP3 inflammasome: an overview of mechanisms of activation and regulation," *International Journal of Molecular Sciences*, vol. 20, no. 13, p. 3328, 2019.

[53] Y. Ohsumi, "Yoshinori Ohsumi: autophagy from beginning to end. Interview by Caitlin Sedwick," *The Journal of Cell Biology*, vol. 197, no. 2, pp. 164-165, 2012.

[54] W. Lin and G. Xu, "Autophagy: a role in the apoptosis, survival, inflammation, and development of the retina," *Ophthalmic Research*, vol. 61, no. 2, pp. 65–72, 2019.

[55] J. Valečka, C. R. Almeida, B. Su, P. Pierre, and E. Gatti, "Autophagy and MHC-restricted antigen presentation," *Molecular Immunology*, vol. 99, pp. 163–170, 2018.

[56] S. A. Jones, K. H. Mills, and J. Harris, "Autophagy and inflammatory diseases," *Immunology and Cell Biology*, vol. 91, no. 3, pp. 250–258, 2013.

[57] J. Harris, T. Lang, J. P. W. Thomas, M. B. Sukkar, N. R. Nabar, and J. H. Kehrl, "Autophagy and inflammasomes," *Molecular Immunology*, vol. 86, pp. 10–15, 2017.

[58] D. J. Cua, J. Sherlock, Y. Chen et al., "Interleukin-23 rather than interleukin-12 is the critical cytokine for autoimmune inflammation of the brain," *Nature*, vol. 421, no. 6924, pp. 744–748, 2003.

[59] K. H. Mills, L. S. Dungan, S. A. Jones, and J. Harris, "The role of inflammasome-derived IL-1 in driving IL-17 responses," *Journal of Leukocyte Biology*, vol. 93, no. 4, pp. 489–497, 2013.

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Research Article

The Health Hazards of Volcanoes: First Evidence of Neuroinflammation in the Hippocampus of Mice Exposed to Active Volcanic Surroundings

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Neuroinflammation is a process related to the onset of neurodegenerative diseases; one of the hallmarks of this process is microglial reactivation and the secretion by these cells of proinflammatory cytokines such as TNF*α*. Numerous studies report the relationship between neuroinflammatory processes and exposure to anthropogenic air pollutants, but few refer to natural pollutants. Volcanoes are highly inhabited natural sources of environmental pollution that induce changes in the nervous system, such as reactive astrogliosis or the blood-brain barrier breakdown in exposed individuals; however, no neuroinflammatory event has been yet defined. To this purpose, we studied resting microglia, reactive microglia, and TNF*α* production in the brains of mice chronically exposed to an active volcanic environment on the island of São Miguel (Azores, Portugal). For the first time, we demonstrate a proliferation of microglial cells and an increase in reactive microglia, as well an increase in TNF*α* secretion, in the central nervous system of individuals exposed to volcanogenic pollutants.

1. Introduction

The role of microglial cells in neuroinflammatory events currently represents one of the main research areas in neurobiology due to the potential therapeutic application. Microglia is a population of resident immune cells in the central nervous system (CNS) being a front-line defence against a threat to the nervous tissue [1, 2]. Although these cells are present throughout the nervous system, they predominate in the grey matter [3], being abundant in areas such as the hippocampus, olfactory telencephalon, basal ganglia, and the substantia nigra [4]. Normally, in the mature health brain, microglial cells are found in their resting form, exhibiting a rounded cell body, which generally remains fixed, and long and highly branched prolongations. These ramifications undergo cycles of formation and retraction that give the cells pronounced motility, thus enabling the monitoring of the cellular neighbourhood [5], safeguarding the homeostasis of the nervous system, and clearing the parenchyma of accumulated metabolic products and debris from deteriorated tissues. In addition, microglial cells show another peculiarity: upon an immune stimulus or CNS damage, they are rapidly activated undergoing a dramatic morphological transformation and exhibiting a set of surface molecules [6–8] including CD68, a transmembrane protein on both lysosomal [9] and plasma membrane [10] which is present on monocytes and macrophages, acting as a modulator of the immune response [8]. Furthermore, in response to damage, reactive microglia secrete a wide range of trophic factors and cytokines that can act in either beneficial or detrimental

ways on the surrounding cells [11–15]. Microglia activation is the hallmark of neuroinflammation.

Multiple neuroinflammatory processes are regulated by these cytokines [16]. Tumour necrosis factor alpha (TNFα), one of the best characterised proinflammatory cytokines, plays both a homeostatic and pathologic role in the CNS [17]. In the healthy nervous system, TNFα is involved in processes such as synaptic plasticity [18, 19] or learning and memory [20, 21]. However, in face of damage or threat to the nervous system, some glial cells, mostly astrocytes and microglia, and certain neuronal populations can produce this cytokine in large quantities. This released is considered a key component of neuroinflammation [22] that leads to a wide range of double-edged sword responses: it has a protective role at acute levels but can contribute to tissue damage and trigger disease when it is a sustained response over time [23]. There are several studies in the literature linking chronic neuroinflammation to neuronal death [23–27] and thus to neurodegenerative diseases such as Alzheimer's disease [28–34], Parkinson's disease [35–37], or multiple sclerosis [38–41].

The occurrence of neuroinflammatory processes as well as neurodegenerative diseases in relation to chronic exposure to environmental pollution has been extensively studied [42–52]. However, all the literature refers to anthropogenic pollution, and little is known about the effect of natural pollution on health, even though there are large natural sources of pollution, such as volcanoes, which can cause health problems.

Volcanoes are attractive for human settlements due to the fertility of their soils and their touristic interest [53–55], but they are also dangerous due to the geochemical processes that take place during both eruptive and noneruptive periods. Considering that volcanoes are a major source of natural pollution, with emissions of certain gases comparable to anthropogenic emissions [56], and that an estimated 44 million people live within 10 km of an active volcano [57], it is very interesting to study the effect of such exposure on health.

The island of São Miguel (Azores archipelago, Portugal) has three active volcanoes: Sete Cidades, Fogo, and Furnas. The latter, considered one of the most active in the archipelago due to its very marked volcanic activity, exhibits different hydrothermal manifestations such as strong ground degassing, thermal and cold CO_2 springs, and fumarolic fields. Although it is estimated that Furnas volcano emits 1000 tons of CO_2 per day [58], the village of Furnas, with about 1700 inhabitants, is located inside the volcano crater. Numerous studies have shown that people chronically exposed to such volcanic manifestations can develop chronic bronchitis and other respiratory diseases [55, 59] and certain types of cancer such as lip, oral cavity, or pharyngeal cancer [60]. However, the respiratory system is not the only one that reacts to exposure to such a hostile environment; changes in the CNS have already been reported, such as the accumulation of inorganic mercury in different areas of the brain parenchyma [61], which suggests a breakdown in the blood-brain barrier, as well as astrocyte reactivity and dysfunction in important areas of the brain such as the hippocampus [62].

Since, as mentioned above, the literature focuses on neuroinflammation as one of the main events following long-term exposure to air pollutants and as a trigger for future neurodegenerative diseases, our work is aimed at detecting a neuroinflammatory response in individuals chronically exposed to volcanic pollutants by studying microglia (resting and reactive form) and the proinflammatory cytokine TNFα.

2. Material and Methods

2.1. Study Areas and Animal Capture. Two groups of feral mice, *Mus musculus*, were captured alive in two different areas of the island of São Miguel: the village of Furnas and Rabo de Peixe. The Furnas village, built on the degassing soil of the crater of the homonymous volcano, presents important manifestations of volcanic activity such as soil degassing. This phenomenon is responsible for the release of hazardous gases such as radon (^{222}Rn), hydrogen sulphide (H_2S), and carbon dioxide (CO_2) among others, as well as volatile metals into the atmosphere [63–65]. On the other hand, Rabo de Peixe village, used as a control site, is located 20 km from the exposed area and shows no evidence of active volcanism or sources of anthropogenic contamination. In addition, this area is placed near the coast, presenting a high air renewal rate.

The selection of *Mus musculus* as a surrogate species is due to several important reasons: on the one hand, the fact that it shares habitat with humans, being sometimes captured even inside inhabited houses, both in the volcanically active area and in the reference area. On the other hand, different authors have reported the robustness of research using feral specimens in the evaluation of the effects of contaminant exposure on individuals, compared to laboratory studies, since the latter may present discrepancies with reality in terms of diet, animal behaviour, and even the mixture of contaminants [66, 67].

Mus musculus individuals (Furnas, $N = 5$ and Rabo de Peixe, $N = 5$) were captured by trapping at different points in the study areas and transferred to the laboratory in the shortest possible time for processing. To avoid any animal distress, mice were anaesthetised with isofluorane until an optimal level of anaesthesia was reached and then transcardially perfused with phosphate buffered saline followed by 4% paraformaldehyde solution (PFA). After perfusion, the animals were necropsied by surgical extraction of the brain, which was fixed by immersion in 4% PFA overnight at 4°C. Sex, body weight, and age were recorded for each individual; the latter was estimated using the dry weight of the crystalline lens according to the methodology of Quere and Vincent [68]. Individuals weighing less than 10 g were discarded for this study.

Experimental procedures were approved by the Ethics Committee of the University of Azores (REF: 10/2020). All procedures were performed conformed with the recommendations of the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (ETS 123), directive 2010/63EU and Portuguese law decree (DL 113/2013).

2.2. Tissue Processing and Immunofluorescence Assay. After overnight fixation in 4% PFA, the brains were processed for paraffin embedding, and once included, serial sagittal 4 μm thickness sections were cut using a microtome (Microm HM

340E). The slides were dewaxed using xylol and hydrated in decreasing concentration of ethanol until PBS 0.1 M, and after hydration, the immunofluorescence assay was performed as follows. Briefly, brain sections containing the hippocampus were subjected to heat-induced epitope retrieval and blocked with 10% BSA for 90 minutes at room temperature. Then, samples were immunolabeled at 4°C overnight using the following primary antibodies at 1 : 100 dilution: anti-Iba1 (GTX101495, Genetex), anti-CD68 (GTX37743, Genetex), and anti-TNFα (GTX110520, Genetex). The next day, the slides were washed and incubated with the secondary antibody (SAB4600310, Sigma Aldrich Co.) diluted at 1 : 500 during 3 hours at room temperature. Then, sections were washed repeatedly and covered with Vectashield medium (Vector Laboratories, Burlingame CA) containing DAPI to counterstain nuclei.

2.3. Quantitative Analysis. Confocal images of the hippocampus were taken using Zeiss confocal scanning microscope (LSM 800), and the immunofluorescence assessment was carried out following the methodology reported by Navarro et al. [61]. Altogether, from each individual, three coronal sections every 150 μm were taken and analysed keeping constant pinhole, contrast, and brightness. From each section, photographs were obtained at 20x magnification, every 0.5 μm z-step and assembled in an orthogonal projection through the Zen Blue software.

The region of interest (ROI) of our experiments was a specific hippocampal formation (Figure 1), the dentate gyrus. In this brain circumvolution, two subareas were analysed: the polymorphic layer (PL) and the granular layer (GL). The total number of immunopositive cells per μm^2 in both subareas was counted and expressed in cells/mm^2 using the Image J software. For this count, three different researchers performed blindly these quantifications and the results were averaged.

2.4. Statistical Analysis. Data regarding the density of $Iba1^+$ and $CD68^+$ cells in mouse dentate gyrus from both study locations was compared using Student's t-test, and a p value of less than 0.05 was considered as statistically significant. The software Graph Pad Prism (Graph Pad Software Inc., La Jolla, CA, USA) was used to conduct all the statistical analysis.

3. Results

All samples used in this study correspond to male individuals. No statistical differences were found between both study groups in age (Furnas: 204 ± 9 days old and Rabo de Peixe: 213 ± 13 days old; $p = 0.193$, Student's t-test) and weight (Furnas: 13.55 ± 2.42 g and Rabo de Peixe: 14.10 ± 0.90 g; $p = 0.728$, Student's t-test).

3.1. Iba1 Expression Is Increased in Individuals Chronically Exposed to Volcanic Environments. Microglial cells were confirmed in this study by staining with the anti-Iba1 antibody (ionizing calcium-binding adaptor molecule 1) in the dentate gyrus from Furnas and Rabo de Peixe mice. It is a protein that consistently is expressed on all microglial subtypes.

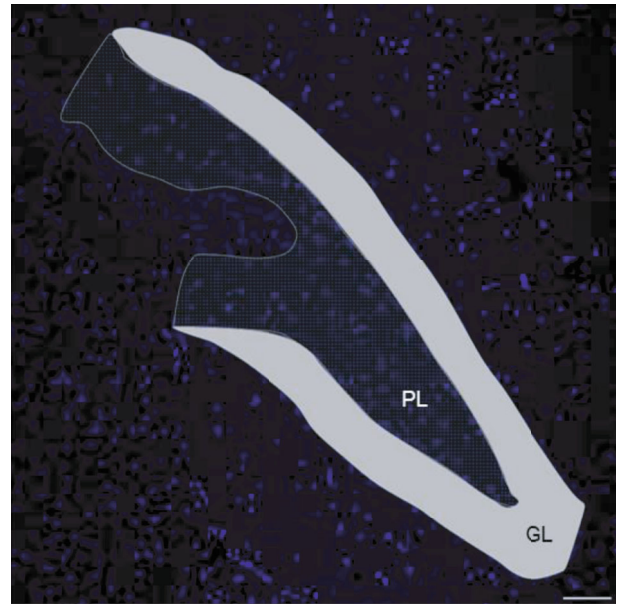


Figure 1: Region of interest (ROI) for the different analyses. The hippocampal dentate gyrus is divided in two areas: granular layer (GL) and polymorphic layer (PL). Scale bar: 50 μm.

Qualitative comparison from immunofluorescence analysis of Iba1 revealed that the staining pattern for Iba1 was much higher in samples from mice living in the Furnas region, both in the polymorphic layer and in the granular layer of the dentate gyrus (Figure 2).

The number of Iba1 positive cells in the immunofluorescence assay was quantified in each layer of the dentate gyrus: granular and polymorphic layer, from individuals captured in the two study areas. An increase in the number of these cells was observed in those animals chronically exposed to volcanogenic pollutants compared to individuals from Rabo de Peixe, in both the granular layer (448.71 ± 33.41 cells/mm^2 vs. 258.45 ± 9.42 cells/mm^2; $^{***}p > 0.001$) and the polymorphic layer (535.67 ± 31.47 vs. 341.84 ± 13.08; $^{***}p < 0.001$) (Figure 3).

3.2. Expression of CD68, a Marker of Active Microglia. CD68 (macrosialin in mice) is one of the most helpful and descriptive markers of microglial function. This protein is localised to the lysosomal membrane of microglia and is upregulated in active phagocytic cells [69]. It is, therefore, a marker of microglial activation with phagocytic activity.

Immunofluorescence evaluation of $CD68^+$ positive cells in both layers of the hippocampal dentate gyrus revealed that chronic exposure to an active volcanic environment induces the increment of these cells in the assessed tissue (Figure 4). Likewise, an important $CD68^+$ immunofluorescent staining was observed in the choroid plexus and the area surrounding it in individuals from Furnas village; such staining was less evident in rodents from Rabo de Peixe.

3.3. Immunofluorescence and Localisation of TNFα in the Dentate Gyrus of the Hippocampus of Exposed Mice. TNFα was used as a proinflammatory marker. TNFα expression

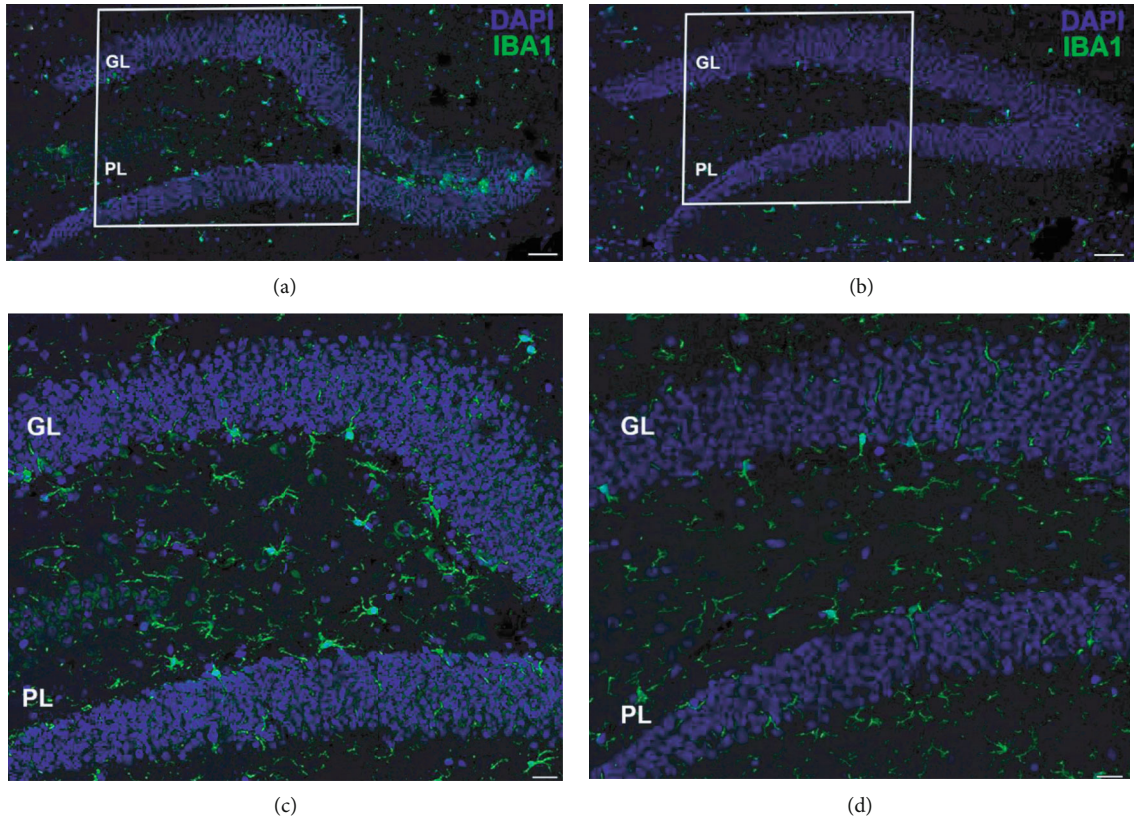


Figure 2: Expression of the microglial marker Iba1 in the dentate gyrus of mice from (a, b) Furnas and (c, d) Rabo de Peixe. Scale bar: 50 μm. Magnification of a section of the total microglia in the dentate gyrus of (c) Furnas and (d) Rabo de Peixe. GL: granular layer; PL: polymorphic layer. Scale bar: 20 μm.

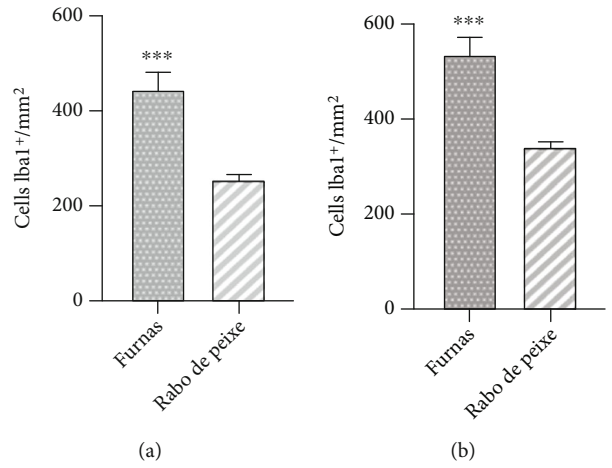


Figure 3: Quantification of Iba1$^+$ cells/mm^2 in both dentate gyrus layers: (a) granular and (b) polymorphic layers of mice from the two study locations. Data reported in the bar graph is represented as mean ± SEM. The statistical analysis was performed using Student's t -test, $^{***}p < 0.001$.

was evaluated in mouse dentate gyrus cells from both locations by immunofluorescence staining. The immunoreactivity is localised in the intracellular spaces around the nucleus of neurons in both polymorphic and granular layers of the dentate gyrus of mice chronically exposed to a volcanic environment. In contrast, no reaction was detected in the perikaryon of the dentate gyrus neurons of the Rabo de Peixe samples.

In the samples from the animals inhabiting Furnas, the immunoreactive cells are found in the subgranular zone (SGZ) located on the inner surface of the granule cell layer. These cells could be compatible with neural stem cells

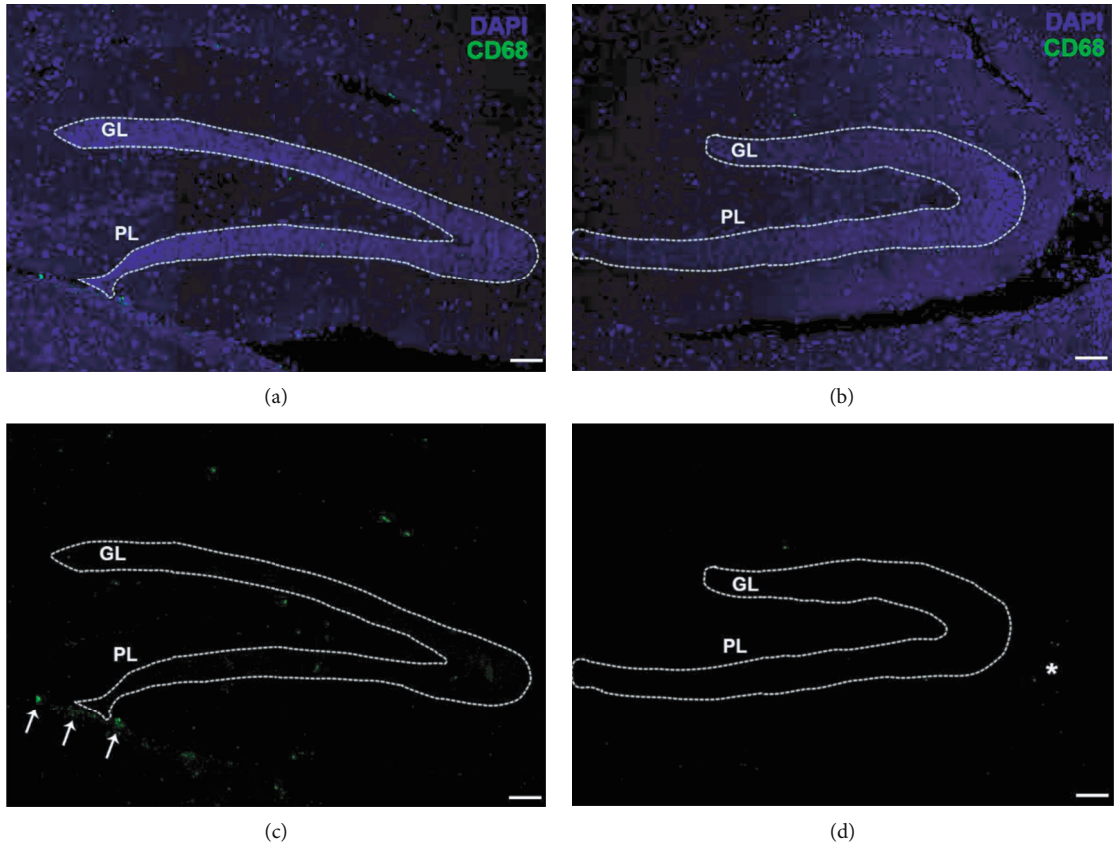


Figure 4: Analysis of active microglia in GL and PL of mice chronically exposed to (a, b) volcanogenic pollutants and from the (c, d) control site. CD68 (green) is expressed in both dentate gyrus layers only in those animals inhabiting (b) active volcanic environments; no immunofluorescence signal is observed in the dentate gyrus of mice from (d) Rabo de Peixe. Note a higher number of $CD68^+$ cells in the surrounding area of the choroid plexus in Furnas' mice (white arrows) were compared to a few cells observed in the vicinity of the plexus in control site mice (asterisk). GL: granular layer; PL: polymorphic layer. Scale bar: 50 μm.

(Figure 5). As for the polymorphic layer, in the image, we can observe a minor marking, consistent with the mossy cells of this layer.

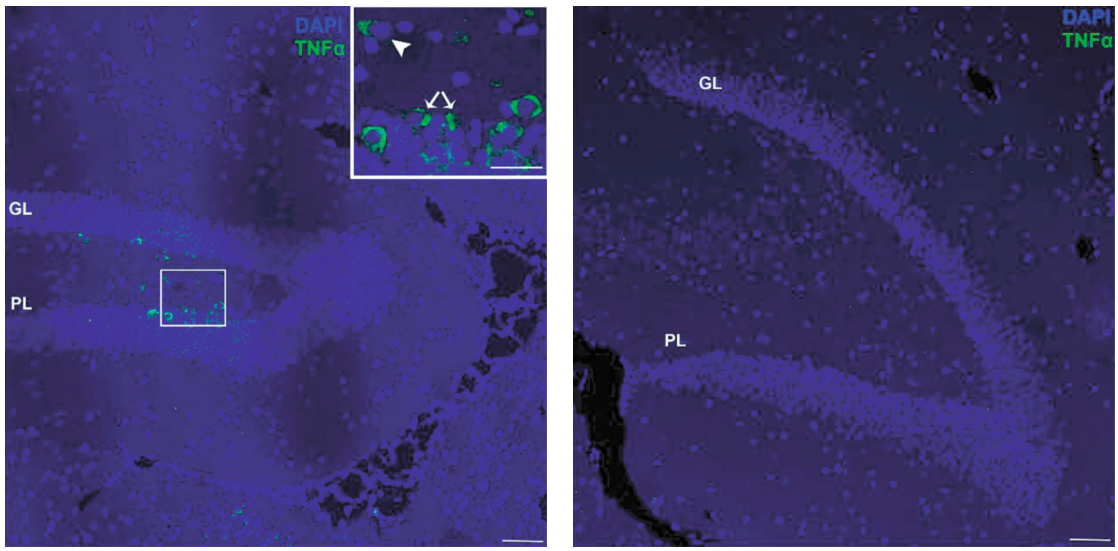
4. Discussion

Air pollution is a major public health concern due to the large number of studies that have linked long-term exposure to various health effects. Although some studies have shown a link between chronic exposure to anthropogenic pollution and effects on the nervous system, only a few have focused on studying these effects regarding volcanic pollution [61, 62]. The Azores archipelago is a volcanic area with several manifestations of active volcanism, making it an ideal place to study environmental health problems [70]. Previous research has shown that volcanic areas are associated with an increased incidence of a wide range of diseases [55, 59, 60, 71–74]. However, very little is known about the consequences on the nervous system of people inhabiting volcanic environments.

Both volcanoes and geothermal areas are associated with emissions of a variety of gases that classically include carbon dioxide (CO_2), sulphur dioxide (SO_2), hydrogen chloride (HCl), hydrogen fluoride (HF), hydrogen sulphide (H_2S), carbon monoxide (CO), radon (Rn), and some heavy metals such as lead and mercury, among others [56]. Therefore, volcanoes are considered an important source of pollutants, including air pollutants, that can damage the health of individuals living in these natural spaces.

Air pollution is a prevalent proinflammatory stimulus for the CNS, which until a few decades ago was not known to be involved as a risk factor for neurodegenerative diseases [45, 49]. For this reason, the rationale of this work has been to relate volcanic contamination to proinflammatory events in the CNS individuals chronically exposed to volcanic contamination. For this purpose, we have studied microglial cells.

As mentioned above, microglia are immunoregulatory cells that play an important role in the healthy and diseased CNS. They help maintain the homeostasis of the brain environment under normal conditions but show a strong reaction in response to adverse conditions, becoming activated microglia and adopting an amoeboid phenotype. These microglia proliferate and migrate to the site of injury or damage, where they perform a protective function, removing cellular debris [75–77]. On the other hand, overactivation of microglia, with excess production of inflammatory mediators, can have neurotoxic consequences. Whether microglial



(a) (b)

Figure 5: Immunofluorescence assay of TNFα in the dentate gyrus of rodents inhabiting (a) Furnas village and (b) Rabo de Peixe. Accumulation of TNFα is evident inside some cells located in the subgranular zone, compatible with neural stem cells (inset, white arrows) and in the hilum, compatible with mossy cells (inset, arrowhead). GL: granular layer; PL: polymorphic layer. Scale bar: 50 μm.

function in neurodegenerative diseases is beneficial but insufficient or whether microglia are only effective in the early stages of the disease but become detrimental in later stages is still unknown.

The intense reaction of microglia is collectively termed "microgliosis." As revealed by Li and Zhang [78], this may exist at least three sources for microgliosis in the adult CNS: local proliferation of reactive microglia, infiltration of blood-derived cells, and mobilization of latent progenitors within the CNS. Each or all of these sources may play a role in microgliosis in different pathological conditions. Alterations in microglia functionality are therefore implicated in brain neurodegeneration.

Our results show several proinflammatory events in the dentate gyrus of animals chronically exposed to an active volcanic environment. These events are the proliferation of the microglial population, the presence of activated microglial cells with phagocytic activity, and intracellular accumulation of TNFα. The dentate gyrus is a very relevant area of the hippocampal formation, not only because it has been described as highly sensitive to oxidative stress [79], but because it is where the adult neurogenesis takes place [80].

Proinflammatory mediators produced in epithelial and olfactory tissue as a result of chronic exposure to volcanic pollutants can induce systemic inflammation and reach the brain parenchyma through the breakdown of the blood-brain barrier. This inflammation is accompanied by the production of different proinflammatory cytokines, such as IL1β, IL6, or TNFα, for which brain vessel endothelial cells exhibit constitutive and induced receptors. Endothelial cytokine-receptor binding activates endothelial cells thereby disrupting the blood-brain barrier. Our study focused on the proinflammatory cytokine TNFα revealed its increase in mice from Furnas. Camarinho et al. [71] also observed its overproduction in the respiratory tissue of mice living in the same location as our study (Furnas village). It is not unreasonable to think that this same cytokine could be present in the CNS from two sources: either by entry from the systemic circulation or by being produced in the CNS itself. Within the central nervous system, microglia, astrocytes, and neurons are major sources of TNFα [81–86]. In fact, we have detected immunoreactivity in cells located in the subgranular zone of the dentate gyrus in chronically exposed animals. These cells, whose location and size are compatible with neural stem cells (NSCs), must have received a proinflammatory signal of environmental origin that led them to activate the NFkb transcription machinery, which regulates numerous genes, including those coding for proinflammatory cytokines [87–90]. Therefore, the presence of cytokines in the extracellular milieu may be a stimulus for these cells to initiate TNFα production and thus enter a proinflammatory loop. It is important to note that this staining was not observed in individuals living in Rabo de Peixe, our control population.

As demonstrated by Widera et al. [91], following CNS injury, TNFα plays a critical role in the development of pathology and inflammation, as well as activating NSC proliferation, triggering a neuroprotective mechanism. In this regard, Pluchino et al. [92] demonstrated that during CNS inflammation, NSCs were able to secrete neuroprotective cytokines. Neuroinflammation may be beneficial as a tissue protector process; however, if this is sustained over the time can lead to a chronic neuroinflammation cycle essential for the pathogenesis and progression of neurodegenerative diseases, such as Alzheimer's disease, Parkinson's disease, Huntington's disease, and multiple sclerosis [93, 94]. In addition, a chronic neuroinflammation condition contributes to both cognitive impairment [95] and memory formation,

disrupting the acquisition and impairing the consolidation/reconsolidation process [96, 97].

The proliferation of microglia, quantified by the marker Iba1, and its morphological change towards a phagocytic form, in which CD68 expression increases, as observed in the dentate gyrus of exposed animals compared to those captured in Rabo de Peixe, suggest that an innate immune response of the CNS to volcanic contamination is taking place. This type of response by microglial cells is consistent with that reported by numerous papers focusing on anthropogenic pollution [43, 45, 98–101]. Importantly, microglial activation is necessary to repair the injured microenvironment by removing cellular debris. However, as a consequence of this activation, these microglial cells can damage living neurons through their phagocytic capacity or by releasing cytokines [102].

Moreover, in addition to the existence of $CD68^+$ cells in the hippocampal dentate gyrus, immunofluorescence has also been observed in the ventricles and areas adjacent to them. This finding indicates that systemic macrophage infiltration of the brain parenchyma may be occurring, preceded by a loss of BBB integrity. Again, it is important to note that, in mice captured in Rabo de Peixe, no immunofluorescence signal was observed in the vicinity of the ventricles. This agrees with the results obtained by Navarro-Sempere et al. [61] in which they reported accumulations of heavy metals, such as mercury, in different areas of the brain, supporting the premise that the aetiology of mercury toxicity in the brain is the breakdown of the blood-brain barrier.

On the other hand, according to our previous research data [62] regarding the long-term exposure of animals to volcanic contaminants, not only the microglial cells have undergone changes but also differences in astrocytes were recorded between the studied populations: Mice from Furnas showed reactive astrogliosis, marked by an increase in GFAP (glial fibrillary acidic protein) and morphological transformation, as well as astrocyte dysfunction, with lower expression of the enzyme glutamine synthetase, when compared to individuals from Rabo de Peixe. Such events already indicated a possible proinflammatory response of the CNS to exposure to volcanic pollutants.

In this context, our previous studies and the present work have provided evidence for the existence of different inflammatory events in the brains of mice living in active volcanic environments, raising awareness about possible neurological health hazards in individuals inhabiting volcanically active areas. However, it should be noted that this neuroinflammatory process may not have a detrimental effect, as neuroinflammation may be playing a beneficial role.

Data Availability

The data used to support the findings of this study can be found in the manuscript itself.

Ethical Approval

The study was conducted according to the guidelines of the Declaration of Helsinki, and all the experimental procedures were approved by the University of the Azores (10/2020) and by the Ethics Committee of the University of Alicante (UA-2020-10-30). All procedures were performed conformed with the recommendations of the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (ETS 123), directive 2010/63EU and Portuguese law decree (DL 113/2013).

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

García, M. and Segovia, Y. contributed equally to this work.

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References

[1] L. Fetler and S. Amigorena, "NEUROSCIENCE: Brain under surveillance: the microglia patrol," *Science*, vol. 309, no. 5733, pp. 392-393, 2005.

[2] B. Li, C. Dasgupta, L. Huang, X. Meng, and L. Zhang, "MiRNA-210 induces microglial activation and regulates microglia-mediated neuroinflammation in neonatal hypoxic-ischemic encephalopathy," *Cellular & Molecular Immunology*, vol. 17, no. 9, pp. 976–991, 2020.

[3] A. Sierra, F. de Castro, J. del Río-Hortega, J. Rafael Iglesias-Rozas, M. Garrosa, and H. Kettenmann, "The "Big-Bang" for modern glial biology: translation and comments on Pío Del Río-Hortega 1919 series of papers on microglia," *Glia*, vol. 64, no. 11, pp. 1801–1840, 2016.

[4] L. J. Lawson, V. H. Perry, P. Dri, and S. Gordon, "Heterogeneity in the distribution and morphology of microglia in the normal adult mouse brain," *Neuroscience*, vol. 39, no. 1, pp. 151–170, 1990.

[5] H. Kettenmann, U.-K. Hanisch, M. Noda, and A. Verkhratsky, "Physiology of microglia," *Physiological Reviews*, vol. 91, no. 2, pp. 461–553, 2011.

[6] R. B. Rock, G. Gekker, S. Hu et al., "Role of microglia in central nervous system infections," *Clinical Microbiology Reviews*, vol. 17, no. 4, pp. 942–964, 2004.

[7] B. P. Cho, D. Y. Song, S. Sugama et al., "Pathological dynamics of activated microglia following medial forebrain bundle transection," *Glia*, vol. 53, no. 1, pp. 92–102, 2006.

[8] D. E. Korzhevskii and O. V. Kirik, "Brain microglia and microglial markers," *Neuroscience and Behavioral Physiology*, vol. 46, no. 3, pp. 284–290, 2016.

[9] M. R. Parwaresch, H. J. Radzun, H. Kreipe, M. L. Hansmann, and J. Barth, "Monocyte/macrophage-reactive monoclonal antibody Ki-M6 recognizes an intracytoplasmic antigen," *The American Journal of Pathology*, vol. 125, no. 1, pp. 141–151, 1986.

[10] M. J. Smith and G. L. E. Koch, "Differential expression of murine macrophage surface glycoprotein antigens in

intracellular membranes," *Journal of Cell Science*, vol. 87, no. 1, pp. 113–119, 1987.

[11] K. Nakajima and S. Kohsaka, "Microglia: activation and their significance in the central nervous system," *Journal of Biochemistry*, vol. 130, no. 2, pp. 169–175, 2001.

[12] G. C. Brown and A. Vilalta, "How microglia kill neurons," *Brain Research*, vol. 1628, no. Part B, pp. 288–297, 2015.

[13] G. T. Norris and J. Kipnis, "Immune cells and CNS physiology: microglia and beyond," *The Journal of Experimental Medicine*, vol. 216, no. 1, pp. 60–70, 2019.

[14] S. Marinelli, B. Basilico, M. C. Marrone, and D. Ragozzino, "Microglia-neuron crosstalk: signaling mechanism and control of synaptic transmission," *Seminars in Cell & Developmental Biology*, vol. 94, pp. 138–151, 2019.

[15] I. D. Vainchtein and A. V. Molofsky, "Astrocytes and microglia: In sickness and in health," *Trends in Neurosciences*, vol. 43, no. 3, pp. 144–154, 2020.

[16] R. Sivangala and G. Sumanlatha, "Cytokines that mediate and regulate immune responses," in *Innovative Immunology*, pp. 1–26, New Jersy, Austing Publishing Group, 2015.

[17] S. L. Montgomery and W. J. Bowers, "Tumor necrosis factor-alpha and the roles it plays in homeostatic and degenerative processes within the central nervous system," *Journal of Neuroimmune Pharmacology*, vol. 7, no. 1, pp. 42–59, 2012.

[18] E. C. Beattie, D. Stellwagen, W. Morishita et al., "Control of synaptic strength by glial TNFalpha," *Science*, vol. 295, no. 5563, pp. 2282–2285, 2002.

[19] M. Kaneko, D. Stellwagen, R. C. Malenka, and M. P. Stryker, "Tumor Necrosis Factor-α Mediates One Component of Competitive, Experience- Dependent Plasticity in Developing Visual Cortex," *Neuron*, vol. 58, no. 5, pp. 673–680, 2008.

[20] B. T. Baune, F. Wiede, A. Braun, J. Golledge, V. Arolt, and H. Koerner, "Cognitive dysfunction in mice deficient for TNF- and its receptors," *American Journal of Medical Genetics Part B: Neuropsychiatric Genetics*, vol. 147B, no. 7, pp. 1056–1064, 2008.

[21] C. Beste, B. T. Baune, M. Falkenstein, and C. Konrad, "Variations in the TNF-α gene (TNF-α -308G→A) affect attention and action selection mechanisms in a dissociated fashion," *Journal of Neurophysiology*, vol. 104, no. 5, pp. 2523–2531, 2010.

[22] G. Olmos and J. Lladó, "Tumor necrosis factor alpha: a link between neuroinflammation and excitotoxicity," *Mediators of Inflammation*, vol. 2014, 12 pages, 2014.

[23] M. Lyman, D. G. Lloyd, X. Ji, M. P. Vizcaychipi, and D. Ma, "Neuroinflammation: the role and consequences," *Neuroscience Research*, vol. 79, no. 1, pp. 1–12, 2014.

[24] N. G. Bazan, A. Halabi, and M. Ertel, Eds.N. A. Petasis, "Neuroinflammation," in *Basic Neurochemistry*, N. G. Bazan, A. Halabi, M. Ertel, D. L. Price, , , and , Eds., pp. 610–620, Academic Press, New York, 2012.

[25] S. Cheng, J. Hou, C. Zhang et al., "Minocycline reduces neuroinflammation but does not ameliorate neuron loss in a mouse model of neurodegeneration," *Scientific Reports*, vol. 5, no. 2015, pp. 1–14, 2015.

[26] K. S. Kirkley, K. A. Popichak, S. L. Hammond, C. Davies, L. Hunt, and R. B. Tjalkens, "Genetic suppression of IKK2/NF-κB in astrocytes inhibits neuroinflammation and reduces neuronal loss in the MPTP-Probenecid model of Parkinson's disease," *Neurobiology of Disease*, vol. 127, no. 2018, pp. 193–209, 2019.

[27] Q. Wang, E. A. Oyarzabal, S. Song, B. Wilson, J. H. Santos, and J. S. Hong, "Locus coeruleus neurons are most sensitive to chronic neuroinflammation-induced neurodegeneration," *Brain, Behavior, and Immunity*, vol. 87, no. 9, pp. 359–368, 2020.

[28] R. M. Ransohoff, "How neuroinflammation contributes to neurodegeneration," *Science*, vol. 353, no. 6301, pp. 777–783, 2016.

[29] M. T. Heneka, M. J. Carson, J. KhouryEl et al., "Neuroinflammation in Alzheimer's disease," *The Lancet Neurology*, vol. 14, no. 4, pp. 388–405, 2015.

[30] V. Calsolaro and P. Edison, "Neuroinflammation in Alzheimer's disease: current evidence and future directions," *Alzheimer's & Dementia*, vol. 12, no. 6, pp. 719–732, 2016.

[31] A. Nazem, R. Sankowski, M. Bacher, and Y. Al-Abed, "Rodent models of neuroinflammation for Alzheimer's disease," *Journal of Neuroinflammation*, vol. 12, no. 1, pp. 1–15, 2015.

[32] A. Ardura-Fabregat, E. W. G. M. Boddeke, A. Boza-Serrano et al., "Targeting neuroinflammation to treat Alzheimer's disease," *CNS Drugs*, vol. 31, no. 12, pp. 1057–1082, 2017.

[33] Y. Seung-Hoon, "Cellular and molecular mediators of neuroinflammation in Alzheimer disease," *International Neurourology Journal*, vol. 23, Suppl 2, pp. S54–S62, 2019.

[34] H. Hampel, F. Caraci, A. C. Cuello et al., "A path toward precision medicine for neuroinflammatory mechanisms in Alzheimer's disease," *Frontiers in Immunology*, vol. 11, 2020.

[35] S. Vivekanantham, S. Shah, R. Dewji, A. Dewji, C. Khatri, and R. Ologunde, "Neuroinflammation in Parkinson's disease: role in neurodegeneration and tissue repair," *The International Journal of Neuroscience*, vol. 125, no. 10, pp. 717–725, 2015.

[36] G. Gelders, V. Baekelandt, and A. Van der Perren, "Linking neuroinflammation and neurodegeneration in Parkinson's disease," *Journal of Immunology Research*, vol. 2018, 12 pages, 2018.

[37] Z. Liu, A. W. Qiu, Y. Huang et al., "IL-17A exacerbates neuroinflammation and neurodegeneration by activating microglia in rodent models of Parkinson's disease," *Brain, Behavior, and Immunity*, vol. 81, pp. 630–645, 2019.

[38] V. Pegoretti, W. Baron, J. D. Laman, and U. L. M. Eisel, "Selective modulation of TNF-TNFRs signaling: insights for multiple sclerosis treatment," *Frontiers in Immunology*, vol. 9, pp. 1–8, 2018.

[39] C. M. Ribeiro, S. R. Oliveira, D. F. Alfieri et al., "Tumor necrosis factor alpha (TNF-α) and its soluble receptors are associated with disability, disability progression and clinical forms of multiple sclerosis," *Inflammation Research*, vol. 68, no. 12, pp. 1049–1059, 2019.

[40] C. A. Wagner, P. J. Roqué, and J. M. Goverman, "Pathogenic T cell cytokines in multiple sclerosis," *The Journal of Experimental Medicine*, vol. 217, no. 1, pp. 1–10, 2020.

[41] K. El-Salem, A. H. Al-Mistarehi, H. Khalil, A. Al-Sharman, and A. Yassin, "Serum tumor necrosis factor-alpha levels correlate with cognitive function scales scores in multiple sclerosis patients," *Multiple Sclerosis and Related Disorders*, vol. 47, article 102621, 2021.

[42] P. V. Moulton and W. Yang, "Air Pollution, Oxidative Stress, and Alzheimer's Disease," *Journal of Environmental and Public Health*, vol. 2012, 9 pages, 2012.

[43] L. Calderón-Garcidueñas, A. C. Solt, C. Henríquez-Roldán et al., "Long-term air pollution exposure is associated with neuroinflammation, an altered innate immune response, disruption of the blood-brain barrier, ultrafine particulate deposition, and accumulation of amyloid β-42 and α-synuclein in children and young adults," *Toxicologic Pathology*, vol. 36, no. 2, pp. 289–310, 2008.

[44] J. Wang, T. Ma, D. Ma et al., "The impact of air pollution on neurodegenerative diseases," *Therapeutic Drug Monitoring*, vol. 43, no. 1, pp. 69–78, 2021.

[45] M. L. Block and L. Calderón-Garcidueñas, "Air pollution: mechanisms of neuroinflammation and CNS disease," *Trends in Neurosciences*, vol. 32, no. 9, pp. 506–516, 2009.

[46] L. Calderón-Garcidueñas, B. Azzarelli, H. Acuna et al., "Air pollution and brain damage," *Toxicologic Pathology*, vol. 30, no. 3, pp. 373–389, 2002.

[47] S. Levesque, M. J. Surace, J. McDonald, and M. L. Block, "Air pollution and the brain: subchronic diesel exhaust exposure causes neuroinflammation and elevates early markers of neurodegenerative disease," *Journal of Neuroinflammation*, vol. 8, no. 1, pp. 1–10, 2011.

[48] R. L. Jayaraj, E. A. Rodriguez, Y. Wang, and M. L. Block, "Outdoor ambient air pollution and neurodegenerative diseases: the neuroinflammation hypothesis," *Current Environmental Health Reports*, vol. 4, no. 2, pp. 166–179, 2017.

[49] L. Calderón-Garcidueñas, E. Leray, P. Heydarpour, R. Torres-Jardón, and J. Reis, "Air pollution, a rising environmental risk factor for cognition, neuroinflammation and neurodegeneration: the clinical impact on children and beyond," *Revue Neurologique*, vol. 172, no. 1, pp. 69–80, 2016.

[50] L. Calderón-Garcidueñas, M. Kavanaugh, M. Block et al., "Neuroinflammation, hyperphosphorylated tau, diffuse amyloid plaques, and down-regulation of the cellular prion protein in air pollution exposed children and young adults," *Journal of Alzheimer's Disease*, vol. 28, no. 1, pp. 93–107, 2012.

[51] R. Peters, N. Ee, J. Peters, A. Booth, I. Mudway, and K. J. Anstey, "Air pollution and dementia: a systematic review," *Journal of Alzheimer's Disease*, vol. 70, no. s1, pp. S145–S163, 2019.

[52] L. G. Costa, T. B. Cole, K. Dao, Y. C. Chang, J. Coburn, and J. M. Garrick, "Effects of air pollution on the nervous system and its possible role in neurodevelopmental and neurodegenerative disorders," *Pharmacology & Therapeutics*, vol. 210, article 107523, 2020.

[53] I. Kelman and T. A. Mather, "Living with volcanoes: The sustainable livelihoods approach for volcano-related opportunities," *Journal of Volcanology and Geothermal Research*, vol. 172, no. 3–4, pp. 189–198, 2008.

[54] H. Sigurdsson, B. Houghton, S. McNutt, H. Rymer, and J. Stix, *Encyclopedia of Volcanoes*, Elsevier, Amsterdam, 2015.

[55] D. Linhares, P. V. Garcia, F. Viveiros, T. Ferreira, and A. . S. Rodrigues, "Air pollution by hydrothermal volcanism and human pulmonary function," *BioMed Research International*, vol. 2015, Article ID 326794, 9 pages, 2015.

[56] A. Hansell and C. Oppenheimer, "Health hazards from volcanic gases: a systematic literature review," *Archives of Environmental Health*, vol. 59, no. 12, pp. 628–639, 2004.

[57] K. Holmberg and C. Small, "People and volcanoes: how many and how Closc?," in *Fall Meeting of the American Geophysical Union*, American Geophysical Union, San Francisco, 2015.

[58] F. Viveiros, C. Cardellini, T. Ferreira, S. Caliro, G. Chiodini, and C. Silva, "Soil CO2 emissions at Furnas volcano, São Miguel Island, Azores archipelago: volcano monitoring perspectives, geomorphologic studies, and land use planning application," vol. 115, no. B12, pp. 1–17, 2010.

[59] A. Amaral, C. Cabral, C. Guedes, and A. Rodrigues, "Apoptosis, metallothionein, and bioavailable metals in domestic mice (Mus musculus L.) from a human-inhabited volcanic area," *Ecotoxicology*, vol. 16, no. 6, pp. 475–482, 2007.

[60] A. Amaral, V. Rodrigues, J. Oliveira et al., "Chronic exposure to volcanic environments and cancer incidence in the Azores," *Science of The Total Environment*, vol. 367, no. 1, pp. 123–128, 2006.

[61] A. Navarro-Sempere, Y. Segovia, A. S. Rodrigues, P. V. Garcia, R. Camarinho, and M. García, "First record on mercury accumulation in mice brain living in active volcanic environments: a cytochemical approach," *Environmental Geochemistry and Health*, vol. 43, no. 1, pp. 171–183, 2021.

[62] A. Navarro, M. García, A. S. Rodrigues, P. V. Garcia, R. Camarinho, and Y. Segovia, "Reactive astrogliosis in the dentate gyrus of mice exposed to active volcanic environments," *Journal of Toxicology and Environmental Health, Part A*, vol. 84, pp. 213–226, 2021.

[63] E. Bagnato, M. Barra, C. Cardellini, G. Chiodini, F. Parello, and M. Sprovieri, "First combined flux chamber survey of mercury and CO2 emissions from soil diffuse degassing at Solfatara of Pozzuoli crater, Campi Flegrei (Italy): mapping and quantification of gas release," *Journal of Volcanology and Geothermal Research*, vol. 289, pp. 26–40, 2014.

[64] E. Bagnato, F. Viveiros, J. E. Pacheco, F. D'Agostino, C. Silva, and V. Zanon, "Hg and CO2 emissions from soil diffuse degassing and fumaroles at Furnas volcano (São Miguel Island, Azores): gas flux and thermal energy output," *Journal of Geochemical Exploration*, vol. 190, pp. 39–57, 2018.

[65] M. Durand, C. Florkowski, P. George, T. Walmsley, P. Weinstein, and J. Cole, "Elevated trace element output in urine following acute volcanic gas exposure," *Journal of Volcanology and Geothermal Research*, vol. 134, no. 1–2, pp. 139–148, 2004.

[66] D. Forsyth, "Extrapolation of laboratory tests to field populationsitle," *Ecotoxicology of wild mammals; Shore*, F. Richard and B. Rattner, Eds., pp. 577–634, New York: Wiley, 2001.

[67] K. Tersago, W. de Coen, J. Scheirs et al., "Immunotoxicology in wood mice along a heavy metal pollution gradient," *Environmental Pollution*, vol. 132, no. 3, pp. 385–394, 2004.

[68] J. Quere and J. Vincent, "Determination de l'âge chez le mulot gris (Apodemus sylvaticus L., 1758) par la pesee des cristallins," *Mammalia*, vol. 53, no. 2, 1989.

[69] E. Zotova, V. Bharambe, M. Cheaveau et al., "Inflammatory components in human Alzheimer's disease and after active amyloid-B_{42} immunization," *Brain*, vol. 136, no. 9, pp. 2677–2696, 2013.

[70] D. Linhares, P. Garcia, and A. Rodrigues, "Trace elements in volcanic environments and human health effects," in *Trace Elements in the Environment-New Approaches and Recent Advances*, M. Murillo-Tovar, H. Saldarriaga-Noreña, and A. Saeid, Eds., pp. 2–21, Intechopen, 2020.

[71] R. Camarinho, P. V. Garcia, H. Choi, and A. S. Rodrigues, "Overproduction of TNF-*A* and lung structural remodelling due to chronic exposure to volcanogenic air pollution," *Chemosphere*, vol. 222, pp. 227–234, 2019.

[72] D. P. S. Linhares, P. V. Garcia, C. Silva et al., "DNA damage in oral epithelial cells of individuals chronically exposed to indoor radon (222Rn) in a hydrothermal area," *Environmental Geochemistry and Health*, vol. 40, no. 5, pp. 1713–1724, 2018.

[73] A. F. Ferreira, P. V. Garcia, R. Camarinho, and A. dos Santos Rodrigues, "Volcanogenic pollution and testicular damage in wild mice," *Chemosphere*, vol. 132, pp. 135–141, 2015.

[74] R. Camarinho, P. V. Garcia, and A. S. Rodrigues, "Chronic exposure to volcanogenic air pollution as cause of lung injury," *Environmental Pollution*, vol. 181, pp. 24–30, 2013.

[75] J. Brockhaus, T. Möller, and H. Kettenmann, "Phagocytozing ameboid microglial cells studied in a mouse corpus callosum slice preparation," *Glia*, vol. 16, no. 1, pp. 81–90, 1996.

[76] M. A. Petersen and M. E. Dailey, "Diverse microglial motility behaviors during clearance of dead cells in hippocampal slices," *Glia*, vol. 46, no. 2, pp. 195–206, 2004.

[77] R. Kölliker-frers, L. Udovin, M. Otero-losada et al., "Neuroinflammation: An Integrating Overview of Reactive-Neuroimmune Cell Interactions in Health and Disease," *Mediators of Inflammation*, vol. 2021, 20 pages, 2021.

[78] T. Li and S. Zhang, "Microgliosis in the injured Brain," *The Neuroscientist*, vol. 22, no. 2, pp. 165–170, 2016.

[79] N. Uysal, K. Tugyan, I. Aksu et al., "Age-related changes in apoptosis in rat hippocampus induced by oxidative stress," *Biotechnic & Histochemistry*, vol. 87, no. 2, pp. 98–104, 2012.

[80] P. S. Eriksson, E. Perfilieva, T. Björk-Eriksson et al., "Neurogenesis in the adult human hippocampus," *Nature Medicine*, vol. 4, no. 11, pp. 1313–1317, 1998.

[81] A. P. Lieberman, P. M. Pitha, H. S. Shin, and M. L. Shin, "Production of tumor necrosis factor and other cytokines by astrocytes stimulated with lipopolysaccharide or a neurotropic virus," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 86, no. 16, pp. 6348–6352, 1989.

[82] X. S. Liu, Z. H. Luo, Z. C. Yang, W. H. Huang, and A. Li, "The significance of changes in serum tumour necrosis factor (TNF) activity in severely burned patients," *Burns*, vol. 20, no. 1, pp. 40–44, 1994.

[83] M. C. Morganti-Kossman, P. M. Lenzlinger, V. Hans et al., "Production of cytokines following brain injury: beneficial and deleterious for the damaged tissue," *Molecular Psychiatry*, vol. 2, no. 2, pp. 133–136, 1997.

[84] L. T. Lau and A. C. H. Yu, "Astrocytes produce and release interleukin-1, interleukin-6, tumor necrosis factor alpha and interferon-gamma following traumatic and metabolic injury," *Journal of Neurotrauma*, vol. 18, no. 3, pp. 351–359, 2001.

[85] U. K. Hanisch, "Microglia as a source and target of cytokines," *Glia*, vol. 40, no. 2, pp. 140–155, 2002.

[86] H. Klintworth, G. Garden, and Z. Xia, "Rotenone and paraquat do not directly activate microglia or induce inflammatory cytokine release," *Neuroscience Letters*, vol. 462, no. 1, pp. 1–5, 2009.

[87] B. Baumann, J. Seufert, F. Jakob et al., "Activation of NF-*K*B signalling and TNF*α*-expression in THP-1 macrophages by TiAlV- and polyethylene-wear particles," *Journal of Orthopaedic Research*, vol. 23, no. 6, pp. 1241–1248, 2005.

[88] Y. Ben-Neriah and M. Karin, "Inflammation meets cancer, with NF-*κ*B as the matchmaker," *Nature Immunology*, vol. 12, no. 8, pp. 715–723, 2011.

[89] S. T. Smale, "Hierarchies of NF-*κ*B target-gene regulation," *Nature Immunology*, vol. 12, no. 8, pp. 689–694, 2011.

[90] M. S. Hayden and S. Ghosh, "NF-*κ*B, the first quarter-century: remarkable progress and outstanding questions," *Genes & Development*, vol. 26, no. 3, pp. 203–234, 2012.

[91] D. Widera, I. Mikenberg, B. Kaltschmidt, and C. Kaltschmidt, "Potential role of NF-*κ*B in adult neural stem cells: the underrated steersman?," *International Journal of Developmental Neuroscience*, vol. 24, no. 2–3, pp. 91–102, 2006.

[92] S. Pluchino, L. Zanotti, B. Rossi et al., "Neurosphere-derived multipotent precursors promote neuroprotection by an immunomodulatory mechanism," *Nature*, vol. 436, no. 7048, pp. 266–271, 2005.

[93] H. M. Gao and J. S. Hong, "Why neurodegenerative diseases are progressive: uncontrolled inflammation drives disease progression," *Trends in Immunology*, vol. 29, no. 8, pp. 357–365, 2008.

[94] H. B. Stolp and K. M. Dziegielewska, "Review: role of developmental inflammation and blood-brain barrier dysfunction in neurodevelopmental and neurodegenerative diseases," *Neuropathology and Applied Neurobiology*, vol. 35, no. 2, pp. 132–146, 2009.

[95] A. Kumar, "Editorial: Neuroinflammation and cognition," *Frontiers in Aging Neuroscience*, vol. 10, pp. 1–3, 2018.

[96] R. A. Kohman and J. S. Rhodes, "Neurogenesis, inflammation and behavior," *Brain, Behavior, and Immunity*, vol. 27, no. 1, pp. 22–32, 2013.

[97] R. Yirmiya and I. Goshen, "Immune modulation of learning, memory, neural plasticity and neurogenesis," *Brain, Behavior, and Immunity*, vol. 25, no. 2, pp. 181–213, 2011.

[98] T. B. Cole, J. Coburn, K. Dao et al., "Sex and genetic differences in the effects of acute diesel exhaust exposure on inflammation and oxidative stress in mouse brain," *Toxicology*, vol. 374, no. 374, pp. 1–9, 2016.

[99] K. J. Bai, K. J. Chuang, C. L. Chen et al., "Microglial activation and inflammation caused by traffic-related particulate matter," *Chemico-Biological Interactions*, vol. 311, p. 108762, 2019.

[100] H. Cheng, D. A. Davis, S. Hasheminassab, C. Sioutas, T. E. Morgan, and C. E. Finch, "Urban traffic-derived nanoparticulate matter reduces neurite outgrowth via TNF*α* in vitro," *Journal of Neuroinflammation*, vol. 13, no. 1, pp. 1–11, 2016.

[101] T. E. Morgan, D. A. Davis, N. Iwata et al., "Glutamatergic neurons in rodent models respond to nanoscale particulate urban air pollutants in vivo and in vitro," *Environmental Health Perspectives*, vol. 119, no. 7, pp. 1003–1009, 2011.

[102] C. Luo, R. Koyama, and Y. Ikegaya, "Microglia engulf viable newborn cells in the epileptic dentate gyrus," *Glia*, vol. 64, no. 9, pp. 1508–1517, 2016.

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Review Article

Key Factor Regulating Inflammatory Microenvironment, Metastasis, and Resistance in Breast Cancer: Interleukin-1 Signaling

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Breast cancer is one of the top-ranked cancers for incidence and mortality worldwide. The biggest challenges in breast cancer treatment are metastasis and drug resistance, for which work on molecular evaluation, mechanism studies, and screening of therapeutic targets is ongoing. Factors that lead to inflammatory infiltration and immune system suppression in the tumor microenvironment are potential therapeutic targets. Interleukin-1 is known as a proinflammatory and immunostimulatory cytokine, which plays important roles in inflammatory diseases. Recent studies have shown that interleukin-1 cytokines drive the formation and maintenance of an inflammatory/immunosuppressive microenvironment through complex intercellular signal crosstalk and tight intracellular signal transduction, which were found to be potentially involved in the mechanism of metastasis and drug resistance of breast cancer. Some preclinical and clinical treatments or interventions to block the interleukin-1/interleukin-1 receptor system and its up- and downstream signaling cascades have also been proven effective. This study provides an overview of IL-1-mediated signal communication in breast cancer and discusses the potential of IL-1 as a therapeutic target especially for metastatic breast cancer and combination therapy and current problems, aiming at enlightening new ideas in the study of inflammatory cytokines and immune networks in the tumor microenvironment.

1. Introduction

The history of interleukin-1 (IL-1) dates back to the early 1940s, from the identification of the fever-inducing activity of "soluble factors" produced by endotoxin-stimulated leukocytes, to the discovery of inflammasomes and clinical benefits of anti-IL-1β therapy, encompassing the entire field of inflammatory cytokines, Toll-like receptors (TLRs), and innate immune responses [1]. IL-1 includes two agonists, IL-1α and IL-1β, which trigger signals via binding to IL-1 receptor 1 (IL-1R1) and recruitment of an accessory peptide chain [2]. The reason for having two IL-1 agonists may lie in the difference in robustness or specific functions between them [3]. Subsequently, the IL-1 receptor antagonist (IL-1Ra) was discovered, which specifically blocks IL-1R1 [4]. IL-1, together with several other sequentially discovered structurally related members, constitutes the IL-1 family. To date, there are 11 members of the IL-1 family [5]. These cytokines have pleiotropic functions, including regulating innate and adaptive immune responses; participating in the physiological regulation of homeostatic processes and host defense against pathogens, injury, and environmental

stresses; and directly affecting transcription of mRNA [6, 7]. Antagonists in the IL-1 family cytokines and inhibitors in the IL-1 receptor family that function as membrane-bound or soluble decoy receptors have an important role in the biological activity of IL-1 and the negative regulation of inflammation induced by IL-1 [8].

IL-1 is a proinflammatory cytokine that affects cellular and organ inflammatory reactions, immune responses, and homeostatic regulation at low concentrations, and has long been known to be important in oncogenesis, invasion, metastasis, and tumor host interactions [9, 10]. IL-1 blockers applied to some autoimmune and inflammatory diseases are currently being tested in preclinical and human clinical experiments for tumor therapy [11]. The role and regulatory mechanisms of IL-1 signaling has been extensively studied in a variety of infectious diseases, inflammation, and inflammation-related cancers, such as colon cancer, liver cancer, gastric cancer, cervical carcinoma, and lymphoma, but is still not well understood in breast cancer (BC) [12–14]. Although BC has not been recognized as an inflammation-related cancer except inflammatory breast cancer (IBC) [15], it is well known that inflammation is a fundamental feature of the tumor microenvironment (TME) [16]. TME infiltrated a large number of immune and inflammatory mediators, including abundant IL-1 cytokines derived from immune or tumor cells. These mediators were thought to be key regulators of TME [17, 18]. Studies in recent years have also confirmed the important role of IL-1 in the BC microenvironment. However, the role of IL-1 signaling in the BC microenvironment is controversial, despite most studies showing its tumor promoting effects [19]. The dual functions in tumorigenesis, both pro- and antitumorigenic, largely depend on the source of the cytokines, levels present in TME, tissues and organs involved, inflammatory context, and stage of the cancer [20].

No matter which subtype of BC, it shows different degrees of inflammatory status in cancer progression, which is a common denominator, and thus can provide a generally applicable therapeutic idea. Whether IL-1 is the originator of the protumor inflammatory microenvironment in BC remains unclear. Questions on the regulatory mechanisms of IL-1 signaling; the crosstalk network between different cells and between different intracellular signal transductions, by which IL-1 signaling and its regulation affect aspects of inflammation, immunity, metastasis, and drug resistance in BC microenvironment; and the usability of IL-1 signaling blockade in terms of clinical treatment in BC may lead us to discover a novel biomarker or effective therapeutic target.

2. Breast Cancer and Targeted Therapy

In the last decade, the global incidence of BC has shown an increasing trend with no significant reduction in mortality [21, 22]. Patients with early, locally advanced, and locally recurrent BC are considered to have a higher chance of cure. But nearly 12% of patients diagnosed with BC will eventually develop into metastatic disease, which received palliative treatment only trying to relieve symptoms, prolong survival, and maintain quality of life [23–25].

Systematic treatment of patients with nonmetastatic BC is determined by subtypes: hormone receptor-positive (HR +) patients receive endocrine therapy, and a few of them receive chemotherapy at the same time; human epidermal growth factor receptor 2-positive (HER2+) patients receive HER2-targeted antibody or small molecule inhibitor combined chemotherapy; due to the high heterogeneity, invasiveness, and lack of treatment options, chemotherapy is still the standard treatment for triple negative breast cancer (TNBC). Local treatment of nonmetastatic BC includes surgical resection and postoperative radiotherapy [26, 27]. In recent years, the progress of chemotherapy, endocrine therapy, immunotherapy, new targeted therapy, and combination therapy has significantly improved the clinical outcomes and prognosis of BC, and made the prospect of long-term disease control of metastatic BC more and more realistic [28–31]. However, acquired tumor resistance is the major reason limiting the treatment effect [32]. Therefore, great efforts have been devoted in recent years to evaluate the molecular characteristics of metastasis and elucidate the mechanisms of drug resistance in BC in order to find novel molecular targets and therapeutic strategies [33–35].

Targeted therapies locate and inhibit tumor-related pathways, such as phosphoinositide 3-kinase (PI3K)/V-akt murine thymoma viral oncogene homolog (AKT)/mammalian/mechanistic target of rapamycin (mTOR), rapidly accelerated fibrosarcoma (RAF), mitogen-activated protein kinase (MAPK), histone deacetylase (HDAC), cyclin-dependent kinases (CDK), and poly(ADP-ribose) polymerase (PARP), by molecules binding to extracellular receptors, such as trastuzumab against HER2 and bevacizumab against vascular endothelial growth factor (VEGF), or by cytoplasmic blocking of small molecules, which is mainly aimed at the tumor [36–38]. Targeted therapies hold good promise in cancer treatment. But targeted therapies for BC face the challenges of diminishing returns, increasing costs of cancer care, and risk of overtreatment [39]. TME is associated with proliferation, angiogenesis, metastasis, apoptosis inhibition, immune system suppression, and drug resistance in BC [40]. Due to the recognition that the cancer stroma is the protagonist of cancer progression and the fact that TME is much more genetically stable than cancer, the development of dual anticancer strategies that target both cancer cells and TME will undoubtedly become the focus of current and future research, which may also be the key to the treatment success of BC with genetic and phenotypic heterogeneity [41].

3. Breast Cancer Microenvironment

In addition to tumor cells, the BC microenvironment also contains a large number of other distinct cell types collectively referred to as stromal cells, including vascular endothelial cells (VECs), cancer-associated fibroblasts (CAFs), mesenchymal stem cells (MSCs), and immune cells such as tumor-associated macrophages (TAMs), myeloid-derived suppressor cells (MDSCs), T lymphocytes, B lymphocytes, as well as myoepithelial cells and adipocytes. Besides, several noncellular components, including extracellular matrix (ECM), exosomes, soluble cytokines or signaling molecules,

and occasionally the blood and lymphatic vascular network, have been identified. The physical characteristics of TME, such as hypoxia, acidity, inflammation, and high interstitial fluid pressure, also differ from those of normal tissues [42]. TME is now recognized as a hallmark of cancer biology. Infiltration of TAMs, tumor-associated neutrophils (TANs), MDSCs, T regulatory cells (Tregs), T helper IL-17-producing cells (Th17s), metastasis-associated macrophages (MAMs), and CAFs enables immune escape, tumor growth, angiogenesis, metastasis, and treatment resistance in multiple tumors, including BC [43–45]. Moreover, the ECM, ECM proteins, chemokines, cytokines, growth factors, and the physical state of TME can all influence the behavior and treatment response of solid tumor in complex ways and predict clinical outcomes [35, 46, 47].

The relevance of inflammation to cancer has been demonstrated. Normally, proinflammatory and anti-inflammatory signals are maintained in a state of balance called inflammatory homeostasis (central). However, dysregulation and prolonged maintenance of inflammation lead to chronic inflammation or immunosuppression and may contribute to the development of several diseases, including cancer [48]. Tumor itself and tumor-triggered inflammation can also promote malignant progression and cause immunosuppression through the recruitment and activation of inflammatory cells [49]. Complement system and kinins, vasoactive amines, cytokines, and hormones are considered important inflammatory mediators in the BC microenvironment [50]. Various overexpressed inflammatory mediators exert their biological effects locally or at a distance through the systemic circulation to influence progression, metastasis, and treatment outcome of BC by establishing a supportive immune or inflammatory microenvironment [51]. Compared with other cancers, the role of the immune and inflammatory systems in the development of human BC remains poorly understood. As the prototypical inflammatory cytokine upstream of the cytokine cascade, the role of IL-1 in tumor initiation and progression and tumor-associated inflammation is of sufficient interest [10]. The availability of IL-1R1 conditional demolded mouse models has made it possible to dissect the role of IL-1/IL-1R1 signaling in different cell types in TME [18].

4. IL-1 Single-Nucleotide Polymorphisms and Breast Cancer Risk

Genetic variation is an important inducer of cancer, and single-nucleotide polymorphisms (SNPs) are one of the most common heritable variations in the human genome. There has been an attempt to explore the association between IL-1 SNPs and cancer risk. 144 different SNPs have been described in the IL-1 β gene [52]. At base pairs in these transcription sites, base transitions between C and T have been widely reported [53, 54]. Recent studies have shown that IL-1β SNPs rs1143634, rs1143627, rs1143623, and rs10490571 were suggested to be associated with BC risk, while the results of the association of rs16944 with BC risk were inconsistent [55–62]. More studies on IL-1 SNPs and their functions affecting the balance of IL-1 protein may help to identify patients at risk and the severity of the disease and may provide additional therapeutic options in some groups of patients.

5. IL-1 Signaling

IL-1 signaling under the title of the article refers to intercellular crosstalk and intracellular signal transduction driven by the IL-1α/β-IL-1R system. IL-1 is derived from dendritic cells (DCs), monocytes, macrophages, mast cells, neutrophils, B cells, T cells, endothelial cells, epithelial cells, dying cells, and tumor cells [5]. As the initial member of the IL-1 family, IL-1 has been recognized as a key immune and inflammatory mediator with important roles in tumorigenesis, invasion, metastasis, and tumor host interactions by mediating chronic inflammation, tumor angiogenesis, activation of the IL-17 pathway, induction of MDSCs, recruitment of macrophages, and skewing and suppression of antitumor immunity [9, 10].

The potent proinflammatory effects of IL-1 follow three major steps: cellular expression, membrane receptor binding, and intracellular signal transduction. IL-1α and IL-1β are translated into 31 kDa precursor forms (pro-IL-1α and pro-IL-1β), cleavage of which generates 17 kDa mature forms (IL-1α and IL-1β). Unlike pro-IL-1β, pro-IL-1α has a functional nuclear localization signal in the N-terminal domain [63, 64]. Thus, both forms of IL-1α are biologically active and have dual functions, i.e., binding to IL-1R1 to exert damage-associated molecular patterns (DAMPs) or "alarming" function, or directly regulating transcription of genes [20, 65]. It was found that HS-1-associated protein X (HAX) 1, a protein associated with mitochondria, endoplasmic reticulum, and nuclear membrane, can bind to pro-IL-1α and promote its nuclear localization. Pro-IL-1α interacts with histone acetyltransferases P300, p300/CBP-associated factor (PCAF), and general control nonrepressible 5 (GCN5) in the nucleus and regulates gene expression independently of IL-1R. Pro-IL-1α is also posttranslationally modified, including myristoylation at Lys82, phosphorylation at Ser90, and glycosylation at D64. Myristoylation and glycosylation are associated with the membrane-bound form of IL-1α. But the functions of these modifications are largely unknown [63]. The production of IL-1α requires intracellular or extracellular proteases (calpain II, caspase-1, chymotrypsin, elastase, and granzyme B) [63, 64]. The necessity of this proteolytic cleavage may manifest in the enhanced biological potency of pro-IL-1α cleaved by inflammatory proteases [66]. IL-1α is constitutively expressed in epithelial, endothelial, and stromal cells and can be upregulated in hematopoietic and nonhematopoietic cells by a variety of stimuli, including Toll-like receptor (TLR) agonists, inflammatory cytokines, oxidative stress, fatty acid-induced mitochondrial uncoupling, and hormones [63]. IL-1α promoter lacks typical TATA and CAAT box regulatory regions but contains binding sites for activator protein-1 (AP1) and nuclear factor kappa B (NF-κB) transcription factors, which are upregulated during inflammatory stimulation [63].

As a key proinflammatory cytokine, IL-1β is mainly expressed in innate immune cells [18]. Different from IL-1α, IL-1β is only active as a mature, secreted molecule, with tightly regulated processes of production and secretion. IL-1β requires a "dual signal" process to become activated. Signal 1 events represent the transcription and translation of pro-IL-β induced through activation of TLR, tumor necrosis factor (TNF), IL-1R, AP1, or NF-κB. Signal 2 is an activation step dependent on the inflammasome complex, which consists of a sensing molecule NOD-like receptor (NLR)/AIM2-like receptor (ALR), an adaptor molecule apoptosis-associated speck-like protein (ASC), and an activation and recruitment domain of the caspase. The inflammasome platform recruits and activates caspase-1/11, which cleaves the N-terminal 116 amino acids of the pro-IL-1β polypeptide to convert it into mature IL-1β [63, 67–71]. NLR protein families mostly have a variable N-terminal domain and a C-terminal leucine-rich repeat (LRR) domain. This family is further divided into NLRP or NLRC receptors based on the presence of an N-terminal pyrin domain (PYD) or caspase activation and recruitment domain (CARD). Among them, NLRP1 (NOD-like receptor family PYD domain-containing protein 1), NLRP3, and NLRC4 (NOD-like receptor family CARD-containing protein 4) are able to induce the formation of an inflammasome, serving as platforms for activating caspase-1 [72]. However, inflammasome-independent processing of IL-1β has also been demonstrated in caspase-1/11-deficient mice, and neutrophil proteases including elastase, proteinase-3, granzyme A, and cathepsin G are able to extracellularly convert pro-IL-1β into active mature protein [65]. Since IL-1α and IL-1β lack a signal peptide, they are not secreted via the conventional endoplasmic reticulum/Golgi pathway but via an unconventional protein secretion pathway [73]. This mode of secretion may involve exocytosis of secretory lysosomes, cytolysis, multivesicular body formation, microvesicle shedding, and direct efflux during hypertonic cell death, and cleavage of IL-1β is thought to be necessary for this mode [65, 67].

The IL-1 receptor family comprises 10 members, simply named IL-1R1~IL-1R10 [65]. The extracellular Ig domains of the receptors share the same structure with the intracellular Toll-like/IL-1R (TIR) domain [8]. IL-1α and IL-1β bind to the extracellular Ig domain of IL-1R1. Ligand-induced conformational changes recruit the nonbinding accessory chain IL-1RAcP to form a heterotrimeric complex [8]. The trimeric IL-1R complex recruits myeloid differentiation primary response gene 88 (MyD88) via its C-terminal TIR domains. MyD88 oligomerizes via its death domain (DD) and TIR domain, and it interacts with interleukin-1 receptor-associated kinase 4 (IRAK4) to form the myddosome complex, which serves as a platform to phosphorylate IRAK4, IRAK2, and IRAK1. Alterations in the recruitment and oligomerization of TNF receptor-associated factor 6 (TRAF6) and other signaling intermediates then occur, which participate in the activation of NF-κB, MAPK, p38, Janus kinase, extracellular signal-regulated kinase (ERK) and signal transducer and activator of transcription 3 (STAT3) to initiate the transcription of inflammatory cytokines [5, 71, 74]. The synthesis, secretion, activated signal transduction, and subsequent role of IL-1 signaling are shown in Figure 1. The targeted genes of IL-1 include IL-1α and IL-1β themselves, as well as other inflammatory factors such as IL-6, IL-8, monocyte chemotactic protein 1 (MCP-1)/C-C chemokine ligand 2 (CCL2), and cyclooxygenase-2 (COX-2) [68, 75, 76]. IL-1R1 can also bind to IL-1Ra, which does not produce a signal due to its lack of an IL-1 receptor accessory protein interaction domain, thereby acting as a competitive binding factor to inhibit proinflammatory signaling [66]. IL-1R2 is a membrane-bound or released form of a decoy receptor with an extracellular region similar to that of IL-1R1. But it has a short cytoplasmic domain unable to generate a signal, acting as a molecular trap to block signal generation [5, 10, 77]. IL-1R2 is the key negative regulator of the IL-1 signaling, acting intracellularly, on the cell surface, and extracellularly to inhibit maturation of IL-1α/β, sequester their active form, or hinder the assembly of signaling complexes [77]. Soluble receptors (sIL-1R1, sIL-1R2, and sIL-1RAcP) present in the circulation can also sequester IL-1 and reduce signal production [78].

6. Role of IL-1 Signaling in Breast Cancer Microenvironment

As shown in Figures 2 and 3, there is a complex intercellular and intracellular crosstalk mediated by IL-1 signaling in the breast cancer microenvironment, which may contribute to its role in tumor-associated inflammation, immunosuppression during tumor development, metastasis leading to recurrence, and acquired drug resistance.

6.1. Formation and Maintenance of Inflammatory Microenvironment. It has been demonstrated that inflammation in cancer is driven by IL-1β. "IL-1 signature" is found in patients with HER2- BC [79]. Primary BC cells secrete high levels of the chemokines RANTES/CCL5, CCL2, and granulocyte-colony stimulating factor (G-CSF) that recruit and activate monocytes and instruct them to secrete high levels of IL-1β and IL-8. This interaction also promotes the secretion of high levels of matrix metalloproteinase-1 (MMP-1), MMP-2, and MMP-10, ultimately creating a chronic inflammatory microenvironment that supports malignant progression and invasiveness [80]. One of the Th2 inflammatory pathways favoring tumor protection in BC relies on the secretion of IL-1β from primary BC induced by T cell cytokines and thymic stromal lymphopoietin (TSLP). Furthermore, IL-1β produced by myeloid cells is involved in the activation of inflammasomes by BC cell-derived factors. Breast cancer cell membrane-associated transforming growth factor-beta (TGF-β) is required for IL-1β production by DCs. IL-1-dependent transcriptional signaling has also been shown in the blood of patients with metastatic BC [81]. BC cell-derived IL-1α also induces expression of TSLP from tumor infiltrating myeloid cells, and TSLP, in turn, induces expression of B cell lymphoma-2 (Bcl-2) in tumor cells, promotes tumor cell survival, and skews the TME toward Th2 inflammation, sustaining lung metastatic survival [82]. Inflammasomes are one of the key regulators of IL-1 production. BC cells induce release of IL-1β from myeloid and T cells via activation of the NLRP3

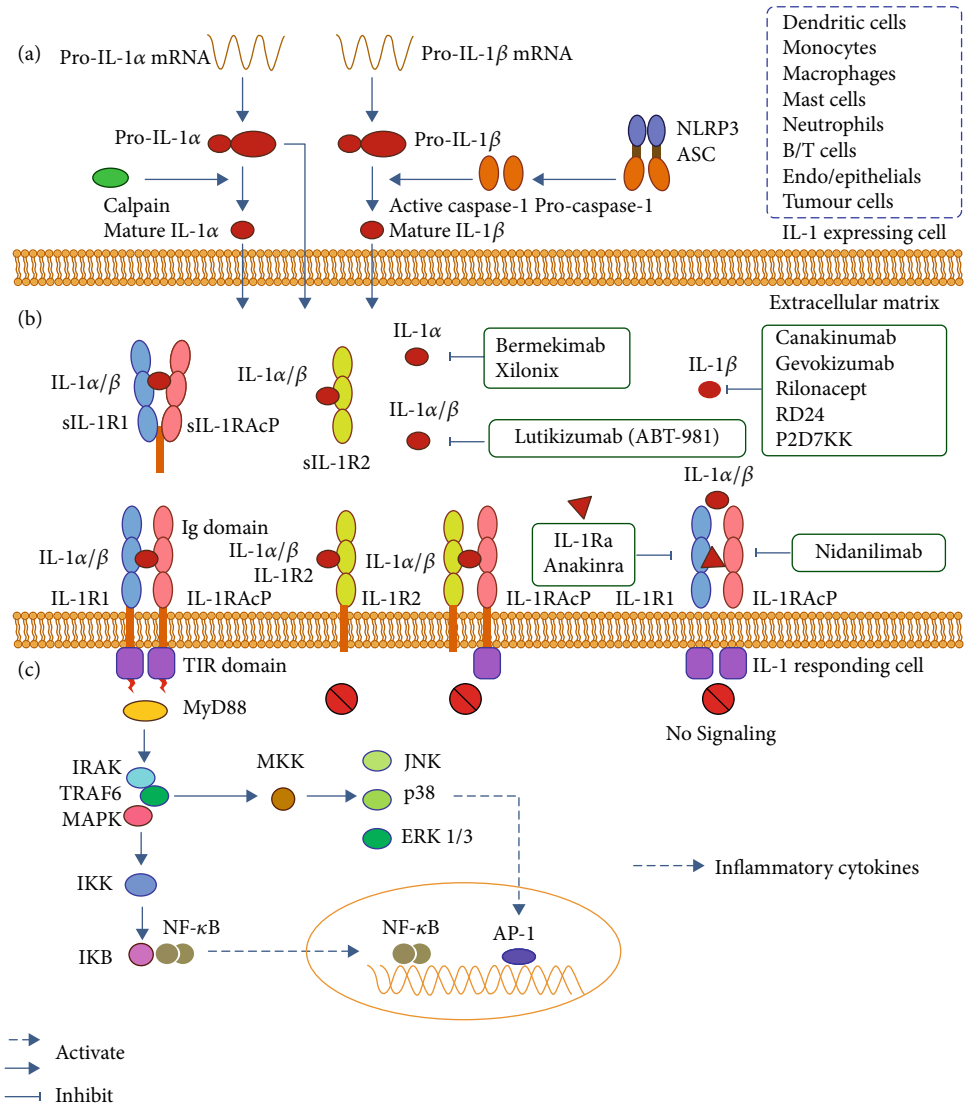

Figure 1: Typical IL-1 signaling. (a) Synthesis and secretion of IL-1α and IL-1β. (b) Several natural or recombinant biologics block IL-1 and its process of binding to membrane receptors. (c) IL-1 activated intracellular signaling. Abbreviations: IL—interleukin; IL-1R—IL-1 receptor; IL-1Ra—IL-1 receptor antagonist; IL-1RAcP—IL-1 receptor accessory protein; Ig—immunoglobulin; TIR—Toll- and IL-1R-like; NLRP3—NOD-like receptor family PYD domain-containing protein 3; ASC—apoptosis-associated speck-like protein; MyD88—myeloid differentiation primary response gene 88; IRAK—interleukin-1 receptor-associated kinase; TRAF6—TNF receptor-associated factor 6; MKK—mitogen-activated protein kinase kinase; JNK—c-Jun N-terminal kinase; ERK—extracellular signal-regulated kinase; MAPK—mitogen-activated protein kinase; IKB—inhibitor kappa B; IKK—inhibitor kappa B kinase; NF-κB—nuclear factor kappa B; AP1—activator protein-1.

inflammasome, and IL-1β activates the transcription factors aryl hydrocarbon receptor (AhR) and retinoid-related orphan nuclear receptor gamma t (RORγt) to induce IL-22 production in memory cluster determinant 4-positive (CD4+) T cells to promote tumor growth [83]. Activation of inflammasomes as well as increased level of IL-1β at the primary and metastatic sites promote the infiltration of myeloid cells such as MDSCs and TAMs into the TME [84].

Transactivation p73β (TAp73β) has been shown to directly activate the positive transcription of caspase-1 and upregulate the expression of pro-IL-1β mRNA and IL-1β protein, and thus may be important for the regulation of the inflammasomes and inflammation in tumor [85]. In addition, soluble CD44 (sCD44) antigen derived from the TNBC cell membrane triggers the production of macrophage-derived IL-1β, regulates the inflammatory TME, and promotes the growth of primary tumor [86]. However, the role of inflammation in HER2-induced tumorigenesis remains controversial. New studies have found that in HER2+ BC, overexpression of HER2 induces the expression and secretion of IL-1α, triggers the activation of other signal sequences including IL-6, and stimulates the NF-κB and STAT3 pathways to generate and maintain cancer stem cells (CSCs) and chronic inflammation to promote cancer initiation and progression [87]. In addition, the BC microenvironment in the context of obesity is associated with the increase of tumor infiltrating myeloid cells, which have

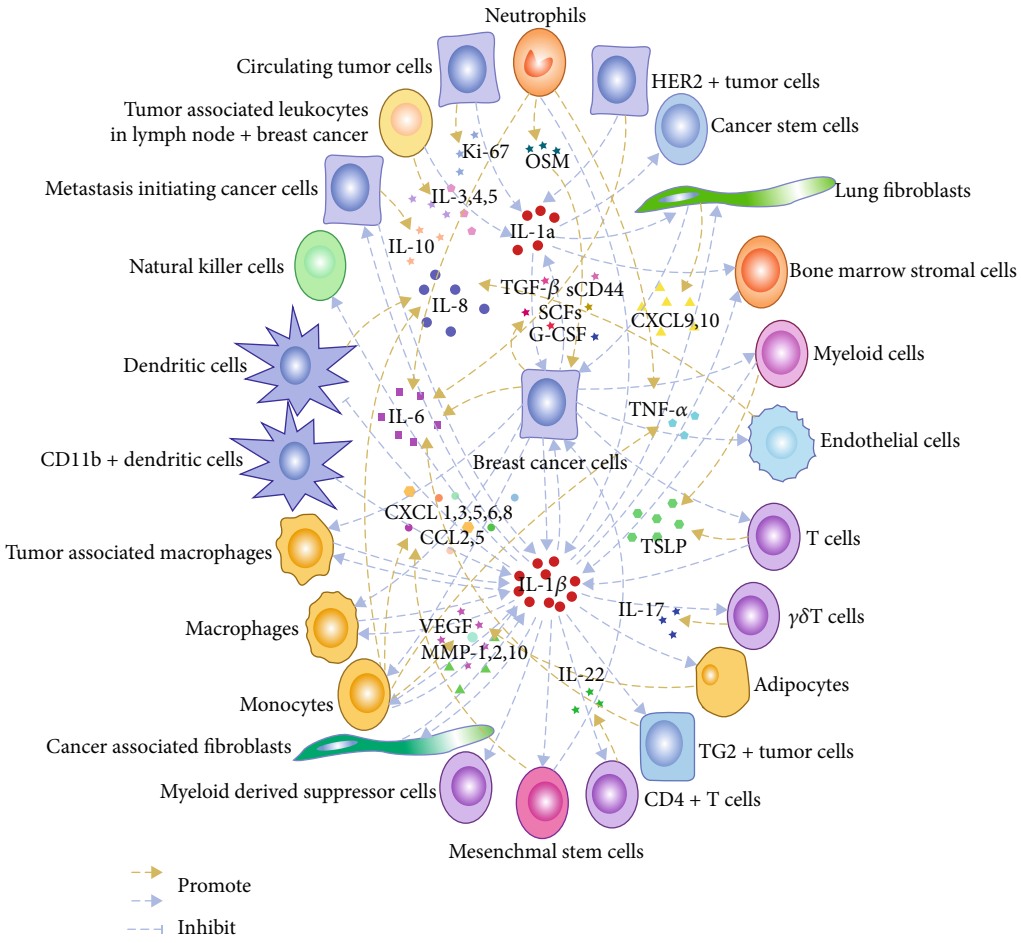

Figure 2: IL-1 signaling-mediated intercellular and intracellular crosstalk in a breast cancer microenvironment. Blue arrows indicate the source and fate of IL-1. Yellow arrows indicate the network of reciprocal influences among different cytokines engaged by IL-1. The source of IL-1 in the breast cancer microenvironment is extensive. Its directions mainly include various immune or inflammatory cells, where it acts to recruit cells and promote differentiation and secretion. Abbreviations: IL—interleukin; MMP—matrix metalloproteinase; TSLP—thymic stromal lymphopoietin; TGF-β—transforming growth factor-beta; TNF-α—tumor necrosis factor-alpha; sCD4—soluble cluster determinant 4; OSM—oncostatin M; SCFs—stem cell factors; G-CSF—granulocyte-colony stimulating factor; VEGF—vascular endothelial growth factor; CXCL—(C-X-C motif) ligand; CCL—C-C chemokine ligand.

an activated NLRC4 inflammasome and IL-1β, which drive disease progression through activation of c-Jun N-terminal kinase (JNK)-mediated expression of VEGFA and angiogenesis in adipocytes [88]. The level of chronic inflammation usually also means a higher risk of recurrence of BC after primary treatment [87, 89].

6.2. Involvement in Tumor Immunosuppression/Escape. The systemic inflammatory cascade is orchestrated through a CCL2–macrophage–IL-1β–$\gamma\delta$T cell–IL-17–immunosuppressive neutrophil axis in BC. CCL2 recruits C-C chemokine receptor 2-positive (CCR2+) monocytes from the bone marrow to elsewhere in the body and induces their differentiation into macrophages, promoting the expression of IL-1 derived from TAMs. $\gamma\delta$T cells are subsequently induced to expand and produce IL-17, promoting the systemic expansion of immunosuppressive neutrophils and formation of metastasis [90]. Whereas IL-1β deficiency leads to low levels of CCL2, hinders recruitment of monocytes and, together with low levels of CSF-1, inhibits differentiation of monocytes into macrophages and results in a relatively high proportion of CD11b+ DCs, whose secretion of IL-12 supports antitumor immunity [91]. In addition, upregulation of IL-1R8 in mammary epithelial cell transformation and primary BC decreased IL-1-dependent activation of NF-κB and proinflammatory cytokine production, inhibited activation of NK cells, and promoted M2-like polarization of macrophages, resulting in impaired innate immune sensing and T cell rejection of the TME [92]. Proinflammatory cytokines expressed by primary breast tumors activate an IL-1β-dependent innate immune response in innate immune cells infiltrating the microenvironment of distant metastasis-initiating cancer cells (MICs), which may prevent the development of secondary disease and, conversely, primary tumor resection may prompt recurrence [93]. Another study also showed that the expression of IL-1β by MICs in BC was significantly associated with longer relapse-free survival and overall survival, while the lack expression of IL-1β

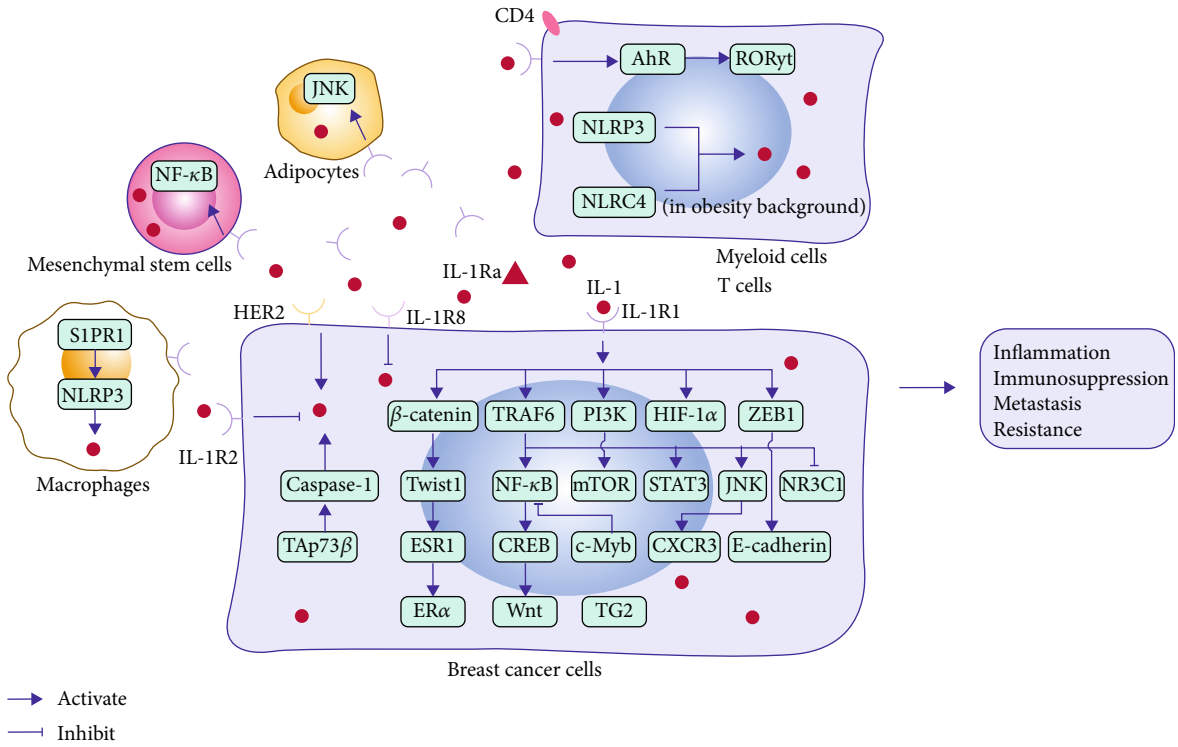

Figure 3: IL-1 signaling-mediated intracellular signal transduction and activation in a breast cancer microenvironment. Abbreviations: IL—interleukin; IL-1R—IL-1 receptor; IL-1Ra—IL-1 receptor antagonist; HER2—human epidermal growth factor receptor 2; ERα—estrogen receptor alpha; PI3K—phosphoinositide 3-kinase; mTOR—mammalian/mechanistic target of rapamycin; NF-κB—nuclear factor kappa B; STAT3—signal transducer and activator of transcription 3; AhR—hydrocarbon receptor; RORγt—retinoid-related orphan nuclear receptor gamma t; CD4—cluster determinant 4; JNK—c-Jun N-terminal kinase; CXCR3—C-X-C chemokine receptor type 3; ZEB1—zinc-finger E-box binding protein 1; HIF-1α—hypoxia-inducible transcription factor-1 alpha; Wnt—wingless and int-1; CREB—cyclic AMP response-element binding protein; TG2—transglutaminase 2; TRAF6—TNF receptor-associated factor 6; NLRP3—NOD-like receptor family PYD domain-containing protein 3; NLRC4—NOD-like receptor family CARD-containing protein 4.

by MICs are associated with the worst prognosis, and may contribute to tumor immune escape [94]. Thus, IL-β secreted by BC cells at the primary and metastatic sites may have a positive effect on tumor immune escape and metastasis suppression, whereas IL-β secreted by immune cells infiltrating the TME exerts a detrimental effect. These new evidences suggest a new line of thinking to link the immunosuppressive/escape microenvironment of BC with IL-1 and thus design tumor suppressive approaches.

6.3. Promotion of Metastasis. The infiltration of IL-1β inflammatory factors can directly promote the metastasis of BC. In TNBC, the increase of IL-1β directly affects the invasiveness of tumor cells [95]. IL-1β is both transmission supportive and colonization inhibitory. At the metastatic site, IL-1β maintains the systemic environment disseminated MICs in an active differentiated state of zinc-finger E-box binding protein 1 (ZEB1), preventing MICs from producing highly proliferative progeny with active E-cadherin [93]. In addition, the cytokine network composed of IL-1 together with other cytokines has a complex role in metastasis of BC cells. IL-6, oncostatin M (OSM), and IL-1β are correlative in expression. OSM induces phosphorylation of STAT3, and IL-1β promotes phosphorylation of p65 to synergistically induce IL-6 secretion of ER-MDA-MB-231 cells, promoting the onset of acute and chronic inflammation and metastasis [96]. Evaluation of serum samples from BC patients showed significant positive correlations between levels of IL-1β and (C-X-C motif) ligand 8 (CXCL8), and between levels of IL-1β and sCD200 in controls. Serum levels of sCD200, CXCL8, IL-1β, and CRP were significantly higher in early and advanced BC patients compared to controls [97]. Human IL-1β induces expression and secretion of stem cell factors (SCFs) in MCF-7 human epithelial BC cells in a manner dependent on the PI3K/mTOR pathway and hypoxia-inducible transcription factor-1alpha (HIF-1α) accumulation/activation [98]. IL-1β confers stem-cell-like ability of tumor cells to enhance their metastatic potential. However, another study showed that IL-1β increased migration of MDA-MB-231 cells, accumulation of HIF-1α, upregulation of CXCR1, and expression of CXCL8 and NF-κB under hypoxia. But inhibition of HIF-1α had no effect on IL-1β-migration of induced hypoxic cells and could not reduce expression of NF-κB and CXCL8. The NF-κB/CXCL8 pathway in a hypoxic microenvironment may play a compensatory role in the IL-1β-induced migration of MDA-MB-231 cells [99].

Several studies have shown that a complex interplay between MSCs and BC cells is closely related to the metastatic potential of BC cells. Compared with normal and other

subtypes of BC, the highest level of BRCA1-IRIS (hereafter IRIS) expression was observed in TNBC, the cellular necrotic/hypoxic/inflammatory centre of IRIS overexpressing (IRISOE) tumors or the vicinity formed an invasive niche, and IL-1β secreted by IRISOE-TNBC cells recruited and activated bone marrow MSCs to secrete CXCL1. CXCL1 enabled IRISOE-TNBC cells to secrete higher levels of CCL2 and VEGF, which recruit and activate TAMs and endothelial cells (ECs), and induce these cells to secrete S100A8/9 and IL-8, respectively. This interaction contributes to the generation of the metastatic precursor of IRISOE-TNBC [100]. Invasive BC cells (MDA-MB-231 cells) activate NF-κB signaling in MSCs by secreting IL-1β, inducing and increasing the production of the same chemokines (CXCL1, 3, 5, 6, 8, and CCL2, 5, etc.) as metastatic ER- BC [101]. Cocultured TNBC cells and MSCs/CAFs in the presence or stimulation of TNF-α or IL-1β showed increased expression of the prometastatic chemokines CXCL8, CCL2, and CCL5, enhanced angiogenesis, migration and invasion of cancer cells, and a significantly enhanced prometastatic phenotype in TME and tumor cells themselves. Among them, CXCL8 plays a key mediating role [102]. Umbilical cord-derived mesenchymal stem cells (UC-MSCs) were cocultured with breast or ovarian cancer cells, and the switched inflammatory UC-MSCs had no obvious effect on the proliferation or apoptosis of the two cancer models, but IL-1β produced in an autocrine manner promoted stem-cell-like properties of cancer cells, initiating the formation of a prestem niche [103].

There is a causal relationship between the inflammatory microenvironment and metastasis. Research found that loss of p53, a key regulator of prometastatic neutrophils, induced secretion of Wingless and int-1 (Wnt) ligands from cancer cells, which stimulated TAMs to produce IL-1β, which drives systemic inflammation. Pharmacologically and genetically blocking the secretion of Wnt reverses IL-1β expression by macrophages and subsequent neutrophilic inflammation, leading to reduced metastasis formation [104]. High expression of the transcription factor c-Myb was found to repress the expression of a set of inflammatory signature genes in BC, including *Ccl2*, *Cxcl1*, *cxcl2*, *cxcl6*, *Cxcl16*, *Icam1*, *Il1a*, *Tnfrsf9*, *Lcn2*, and *Ikbke*, which were denoted as c-Myb-inflammatory signature [105]. It was subsequently found that c-Myb reduced autocrine signal transduction of the NF-κB pathway in BC and the ability of BC cells to migrate and cross the endothelial barrier through inhibition of the expression of IL-1α. Overexpression of IL-1α as well as the addition of recombinant protein of IL-1α activated NF-κB signaling and restored the expression of inflammatory signature genes that were suppressed by c-Myb [53]. Mouse models of BC reflect that periodontal inflammation (PI) and the resulting IL-1β promote the expression of CCL5, CXCL12, CCL2, and CXCL5, which in turn recruit MDSCs and macrophages, ultimately creating a premetastatic niche at the site of inflammation [106].

Tumor lymphangiogenesis is associated with metastasis, but the exact mechanism remains unclear. The novel study identified that sphingosine 1-phosphate receptor 1 (S1PR1) signaling in macrophages promoted lymphangiogenesis via NLRP3-dependent IL-1β secretion in mouse mammary tumors infiltrated with $CD11b^{hi}CD206^{+}$ TAMs. And since IL-1β is involved in tumor pathological rather than physiological lymphangiogenesis, the side effects of targeting IL-1β to block tumor lymphangiogenesis may be limited [107]. Macrophage-derived caspase-1-dependent IL-1β plays an important role in BC cell lymphatic endothelial cell adhesion and migration across endothelial cell barriers [108]. Tumor-associated leukocytes isolated from lymph node+ BC patients secreted 2- to 5-fold more cytokines than lymph node- patients, with the most increased cytokines being thymus and activation-regulated chemokine (TARC/CCL17), IGF-1, IL-3, TNF-β, IL-5, G-CSF, IL-4, and IL-1α. These cytokines promote epithelial mesenchymal transition (EMT) and BC lymph node metastasis by upregulating TGF-β and vimentin, downregulating E-cadherin, and activating epidermal growth factor receptor (EGFR) (Tyr845) and NF-κB/p65 (ser276) signaling [109]. Circulating tumor cells (CTCs) are precursors to the formation of metastatic lesions and, therefore, are also prognostic markers of poor survival in patients with early-stage BC before the initiation of systemic adjuvant therapy and after adjuvant chemotherapy. Studies have found that IL-1α is a marker of tumor cells released into the circulation rather than into the lymphatic system [110]. Neutrophils can assist the formation of a precancerous metastatic niche in distant organs of BC due to activated neutrophils escorting CTCs, facilitating the adhesion of CTCs and ECs, and most CTC-associated leukocytes are N2-like neutrophils. Ki-67 expression was higher in disseminated tumor cells derived from CTC-neutrophil clusters compared with independent CTCs. In contrast, CTC-associated neutrophils frequently expressed TNF-α, OSM, IL-1β, and IL-6, which matched their receptors on the corresponding CTCs [111].

Studies have confirmed the importance of IL-1 signaling in the promotion of BC bone metastasis. Using a clinically relevant humanized mouse model of BC bone metastasis, altered expression of IL-1β, IL-1R1, S100A4, cathepsin K (CTSK), secreted phosphoprotein 1 (SPP1), and receptor activator of NF-κB (RANK) in BC cells as they progress from primary tumor to bone metastasis was demonstrated, and these molecules can be used to predict future bone recurrence in BC patients [112]. This model established that the presence and active function of IL-1β had an impact on the occurrence of bone metastases. In-depth studies have shown that bone marrow-derived IL-1β stimulates bone colonization of BC cells by inducing NF-κB/cyclic AMP response-element binding protein- (CREB-) Wnt signaling and colony formation of CSCs [113]. Furthermore, IL-1β produced endogenously by BC cells in primary sites promotes EMT, invasion, migration, and bone colonization. Upon arrival in the bone environment, contact between tumor cells and osteoblasts or myeloid cells increases the secretion of IL-1β by all three of these cell types. High concentrations of IL-1 β cause increased proliferation of the bone metastatic niche and bone resorption by osteoclasts, stimulating disseminated tumor cells to grow into overt metastases [114]. Additionally, IL-1 is also a differential regulator associated with pain of metastatic cancer in bone [115]. Bone marrow dissemination of BC cells is an early

event, but cells can become latently dormant for years before the development of bone metastases [114]. Treatment of bone metastases is not effective, and IL-1 signaling inhibitors may become new adjuvants to inhibit colonization of disseminated cells to metastases.

The lung is also a common metastatic site for BC. IL-1α and IL-1β secreted by metastatic BC cells induce the production of CXCL9 and CXCL10 by lung fibroblasts through the NF-κB signaling pathway. A small subset of BC cells specifically expressing CXCR3 exhibited tumor-initiating ability when cotransplanted with fibroblasts, driving JNK signaling, increasing expression of IL-1α/β, forming a supportive metastatic niche, and promoting lung metastatic tumor growth [116]. The inflammasome/IL-1 pathway is an important mechanism in the development of BC lung metastasis, as confirmed by the significant reduction of lung metastasis in inflammasome or caspase-1-deficient mice, and may be related to IL-1β-induced expression of CCL2 in macrophages and tumor cells [84]. In vitro invasion assay confirmed that irradiation targeting D2A1 tumor and its microenvironment increased the levels of plasma IL-1β, promoted the infiltration of tumor cells and the development of lung metastasis and increased the activity of MMP-2 and MMP9 [117]. Conversely, genetic studies utilizing the mouse mammary tumor virus polyoma middle tumor (MMTV-PyMT) mouse model revealed that IL-1α-mediated IL-1R1 signaling inhibits the proliferation, growth, and lung metastasis of BC cells at early stages of tumorigenesis [118]. Therefore, the role of IL-1α-mediated IL-1 signaling in BC lung metastasis may be biphasic depending on the stage and context of tumor development.

6.4. Involvement in Tumor Resistance. BC is a HR-driven cancer, so many patients are treated with therapies that lower hormone levels or directly block HR, but most will eventually develop therapeutic resistance. A recent study proposed that IL-1 may provide a conserved basal gene expression pattern in HR+ BC cells that mimic HR- BC cells. Sequestome-1 (SQSTM1/p62) is a differentially expressed gene induced by IL-1 in HR+ and HR- BC cells and is required for survival of HR- cells, playing a role in acquired HR-independent survival and therapeutic resistance. P62 binds to and polyubiquitinates TRAF6, leading to transactivation of NF-κB, forming a positive feedback loop inducing production of IL-1β and activation of signaling. P62 may also be involved in the crosstalk between IL-1 and glucocorticoid signaling by inhibiting NR3C1, which encodes a glucocorticoid nuclear receptor that suppresses inflammatory gene expression [52]. In addition, IL-1 was found to mediate the inhibition of estrogen receptor α (ERα) and progesterone receptor (PR) induced by bone marrow stromal cells in ERα+/PR+ BC cells, the upregulation of p62/SQSTM1 and autophagy, and the p62-LC3 interaction. Thus, IL-1β may depend on the function of p62 and autophagy to confer a viable ERα-/PR- molecular phenotype in ERα+/PR+ BC cells, and this may underlie endocrine resistance [119]. In HER2+ BC, HER2 induced expression of IL-1α and IL-6, which then increased drug-resistance-related CSCs in primary tumor, while blocking IL-1 signaling increased the efficacy of chemotherapy when combined with cisplatin and paclitaxel [87].

In the cell model of BC cells (6D cells) with EMT induced by IL-1β through the activation of the IL-1β/IL-1R1/β-catenin pathway, upregulation of Twist1 resulted in methylation of the ESR1 gene promoter, which significantly reduced the level of ERα and increased the resistance to tamoxifen [120]. After IL-1β-highly responsive clone (6D cells) from noninvasive MCF-7 BC cells were stimulated by IL-1β, the expression of CDKN1A/p21, TP63, small-fiber neuropathy (SFN), and especially BIRC3, was upregulated, which made BC cells resistant to doxorubicin [121]. The IL-1β/IL-1R1/β-catenin signaling pathway can also upregulate the expression of tumor protein 63 (TP63) isoform ΔNp63α, which in turn leads to increased expression of EGFR and phosphatase 1D (Wip1) and decreased DNA damage sensors and ataxia telangiectasia mutated (ATM). This is involved in the enhancement of the cisplatin resistance of BC cells [122]. Furthermore, IL-1β induces IL-6 production by transglutaminase 2- (TG2-) expressing MCF-7 cells through NF-κB-, PI3K-, and JNK-dependent mechanisms, ultimately increasing the stem-cell-like phenotype of cancer cells associated with drug resistance [123].

7. Targeting IL-1 Signaling for Breast Cancer Treatment

7.1. Direct Blockade of IL-1 Signaling. There are currently four known IL-1 blocking biologics: anakinra, canakinumab, gevokizumab, and rilonacept. Anakinra, the recombinant form of the human IL-1Ra, acts by competitively preventing the binding of IL-1α and IL-1β to IL-1R1 [124]. Canakinumab is a human monoclonal antibody (mAb) specific for IL-1β [125]. Gevokizumab is a recombinant humanized allosteric monoclonal antibody that negatively regulates IL-1β signaling through an allosteric mechanism [78]. Rilonacept (ril on'a sept), an approved recombinant fusion protein comprising the extracellular portion of human IL-1R1 and IL-1RAcP fused to the Fc portion of human IgG1, binds to and inactivates IL-1, acting as an "IL-1 trap" [126]. Anakinra and canakinumab are currently approved for the treatment of rheumatoid arthritis, familial Mediterranean fever (FMF), cryopyrin-associated periodic syndrome, Still's disease, and gouty arthritis, while gevokizumab does not currently have a specific indication [69, 127, 128]. Among them, canakinumab has been widely used in clinical experiments for lung cancer [129–131]. The protein formulation, i.e., the solution of IL-1Ra (kineret), may have ultra-long-term stability for 10 years and has clinical applications in metastatic BC (NCT01802970) [11, 132]. In addition, there are several other mAbs against two cytokines or their receptors, respectively, such as lutikizumab (ABT-981), a double variable domain Ig that binds to and inhibits both IL-1α and IL-1β [133]; anti-IL-1α Xilonix [134]; Bermekimab, a true human mAb targeting IL-1α cloned directly from human B cells (Epstein-Barr virus immortalized) isolated from humans with endogenous anti-IL-1α antibodies [135]; two IL-1β neutralizing antibodies, RD24 and P2D7KK [136]; and Nidanilimab, an entire humanized

mAb against IL-1RAcP. Most of these antibodies are being used in clinical cancer therapy to block IL-1 signaling [11]. These blockers are shown in Figure 1.

Anakinra is an ideal treatment option with a short half-life for patients who have undergone chemotherapy, and it is increasingly used as an adjuvant therapy to reduce inflammation in metastatic cancer [79, 137]. In humanized mouse models of BC bone metastasis, anakinra treatment reduced the number of mice that developed metastases in human bone implants from 57.14% to 0% [112]. Anti-IL-1β treatment reduced hindlimb bone metastasis in the spontaneous MDA-MB-231 BH mouse model [113]. Knockout of IL-1R1 (IL-1R1$^{-/-}$), anakinra or canakinumab reduced bone metastasis and the number of tumor cells shed into the circulation [114]. Blocking IL-1R with IL-1Ra inhibited the invasiveness of Hs578t and MDA-MB231 TNBC cells and the development of bone metastasis [95], and it inhibited tumor growth while reducing the accumulation of myeloid cells [84]. Treatment with anti-IL-1β antibody attenuates production of IL-6, the stem-like phenotype, and tumor growth and metastasis in TG2+ BC cells [123]. The use of anakinra *in vivo* reduced the production of IL-22 and tumor growth in BC [83]. Anakinra or TGF-β neutralizing antibody treatment significantly decreased the production of IL-13, IL-4, IL-17, and TSLP; increased the production of NF or interferon-gamma (IFNγ); and suppressed growth of BC [81]. Secretion of IL-1β by IRISOE-TNBC cells within the invasive niche initiates a bidirectional effect with MSCs. Anakinra could break these bidirectional interactions; inhibit generation of MSCs, tumor recruitment, and secretion of CXCL1 *in vivo*; and enhance the efficacy of chemotherapy on IRISOE-TNBC, especially on metastasis [100]. Neither prophylactic nor therapeutic administration of anakinra significantly inhibited the growth of MDA-MB-231-IV tumors in bone and reduced the number of mice that developed bone metastases and subcutaneous tumor volume [138]. Anti-IL-1R1 antibody and anakinra treatment inhibits the growth of E0771 tumor in DIO mice [88]. These evidences revealed that anakinra can modulate the BC microenvironment by blocking IL-1 signaling, reducing tumor growth and metastasis. Unfortunately, there have been few studies on rilonacept and gevokizumab and other biologics used to block IL-1 for the treatment of BC except for the human antibody scFv 12H7, one specially prepared specific binder of IL-1RAcP with high affinity, which had growth inhibitory activity against TNBC cells *in vitro* and *in vivo* [139].

Anakinra provides an optimal treatment. The short half-life of subcutaneously injected anakinra is a distinct advantage, allowing oncologists to stop anakinra treatment at the first sign of infection. This is something that cannot be achieved with persistent antibodies, such as canakinumab [79]. But the results of experiments using anakinra alone may limit understanding of the pleiotropic role of IL-1 in BC, as it is not clear how much of its efficacy is due to blocking IL-1α and how much is due to blocking IL-1β [140]. Because chemotherapy often leads to myelosuppression, and IL-1 blockade therapy can also suppress peripheral blood neutrophils, the risk of infection may be increased when using IL-1 blockade therapy alone or when using anakinra in combination with standard chemotherapy regimens. Therefore, the precise timing and dosage of IL-1 blockade should be determined before application to cancer patients. In this context, modulation of cancer-cell-induced production of IL-1 might be a better option [86].

7.2. Blockade of Up- and Downstream Regulatory Signals of IL-1. Blocking IL-1 in tumors has now expanded immensely. Primary mammary tumor growth and lung metastasis were significantly reduced in NLRP3 knockout mice and caspase-1 knockout mice designed to reduce mature IL-1 production [84]. Tumor growth was significantly reduced in caspase-1/11$^{-/-}$ and NLRC4$^{-/-}$ diet-induced obese mice [88]. TGF-β neutralizing antibody treatment was able to decrease production of IL-1β in humanized mouse tumors [81]. Antibody-mediated neutralization of sCD44 abrogated production of IL-1β in macrophages, modulated the tumor inflammatory microenvironment, and inhibited primary tumor growth [86]. IL-1R8 deficiency in the transgenic mouse model of BC (MMTV-neu/IL-1R8$^{-/-}$) delayed tumorigenesis and reduced tumor burden and metastasis [92]. miRNAs are noncoding microRNAs that negatively regulate gene expression, and play important roles in self-renewal, growth, and metastasis of BC cells [141, 142]. miR-146a-5p can downregulate expression of IRAK1 by directly binding to its 3′-untranslated region and inhibit proliferation and invasion of BC cells [143]. NF-κB inhibitor Bay11-7085 reduced basal levels of IL-1β and invasiveness of TNBC cells [95]. Furthermore, since IL-1-induced p62 mediated survival and HR treatment resistance of BC cells, the p62 targeting drug verteporfin (visudyne®) was cytotoxic to HR- BC cell lines [52]. These results illustrate that targeting NLRP3, NLRC4, caspase-1, TGF-β, sCD44, IL-1R8, IRAK1, and NF-κB, which affect production and activation of IL-1, and IL-1-mediated downstream signaling p62, are also effective ways to modulate IL-1 signaling in the BC microenvironment.

7.3. Combination Therapy. It has been mentioned before that anti-IL-1 or anakinra may decrease the resistance of BC and improve the efficacy of chemotherapy. Furthermore, it was shown that the median duration of treatment with anakinra in combination with one of the standard chemotherapeutic agents (albumin-bound paclitaxel, eribulin, or capecitabine) for BC was 4 months in 11 women with metastatic HER2- BC. Gene expression of IL-1β, IL-1R1, IL-1R2, and IL-1RAcP, the five members of the TLR family and the IL-1 signaling kinases MyD88 and spleen tyrosine kinase (SYK) were decreased during two weeks of daily anakinra and during the pilot trial. Conversely, the expression of some NK cell and cytotoxic T cell genes that favor immune-mediated tumor destruction was increased [79]. It is suggested that chemotherapy combined with anakinra treatment may also have the effect of restoring antitumor immunity. In addition, anakinra may also enhance the effects of other treatment modalities, such as immunotherapy. Treatment of wild type mice with 4T1 tumors first with anti-IL-1β antibody and then with antiprogrammed cell death protein 1 (PD-1) antibody resulted in a therapeutic outcome that differed from the partial growth inhibition

Table 1: Treatments or interventions targeting or affecting IL-1 signaling in breast cancer.

Treatment/intervention	Targets	*In vivo*/*in vitro*	Models	Findings	References
Anakinra; anti-TGFβ	IL-1R1	*In vivo*	Hs578T; NOD/SCID/β_2m$^{-/-}$; patients with HER2 metastatic BC (NCT01802970)	Prevented tumor progression and production of IL-13 in humanized mouse model; downregulated specific components of the systemic inflammatory signature observed in patients with metastatic BC and rescued cytotoxic programs thought to be critical for antitumor activity	[81]
Anti-IL-1R; anakinra	IL-1 signaling	*In vivo*	4T1; E0771; BALB/c; C57BL/6	Reduced tumor progression and production of IL-22$^+$ cells	[83]
IL1Ra; caspase-1 inhibitor; Ac-YVAD-cmk; anti-IL-1β; anticaspase-1; caspase-1$^{-/-}$; NLRP3$^{-/-}$	IL-1 signaling	*In vivo* and *in vitro*	EO771; PyT8; MDA-MB-231; C57BL/6J; NSG; MMTV-PyMT	Reduced tumor growth and lung metastasis accompanied by decreased myeloid cell accumulation	[84]
Anti-CD44	CD44	*In vitro*	MDA-MB231; MDA-MB-468; MCF-7; MCF-10A; 4T1-Luc; THP-1; human serum samples; BALB/c	Abrogated IL1β production in macrophages and inhibited growth of primary tumors	[86]
IRAK1 inhibitor synergized with either cisplatin or paclitaxel	IL-1α signaling	*In vivo*	FVB/N	Reduction of CSCs and improvement of the chemotherapy efficacy	[87]
Anti-IL-1R1; anakinra; caspase 1/11$^{-/-}$; NLRP3$^{-/-}$; NLRC4$^{-/-}$	NLRC4/ IL-1β	*In vivo*	Py8119; E0771; C57BL/6N	Reduced tumor growth except NLRP3$^{-/-}$ mice	[88]
Anti-IL-1β, anti-PD-1	IL-1β, PD-1	*In vivo*	4T1; BALB/c	Anti-IL-1β Abs and anti-PD-1 Abs have a synergistic antitumor activity	[91]
IL-1R8$^{-/-}$	IL-1R8	*In vivo* and *in vitro*	HB4a; HB4a-C5.2; NKL; THP-1; MMTV-neu	Reduced tumor growth and metastasis	[92]
IL-1Ra; Bay; Zerumbone	NF-κB signaling pathway	*In vitro*	Hs578T; MDA-MB231	Inhibition of IL-1β expression and cell invasiveness	[95]
Anakinra	IL-1R1	*In vivo*	MSCs; IRISOE cell lines; SCID	Decreased recruitment of mouse MSCs into IRISOE-TNBC tumors and their activation to produce and secrete CXCL1	[100]
IL-1Ra	IL-1R1	*In vitro*	MDA-MB-231 and UC-MSCs coculturing system	Blocked prostemness effects of UC-MSCs on cancer cells	[103]
Anakinra	IL-1R1	*In vivo* and *in vitro*	T47D; MCF-7; BB3RC32; BB2RC08; BB6RC37	Reduced bone metastasis	[112]
Sulfasalazine; KG-501	NF-κB; CREB	*In vitro*	MCF-7; MDA-MB-231_BH	Inhibited Wnt-dependent CSC colony formation in the bone environment	[113]
Anti-IL-1β	IL-1β	*In vivo*	NSG	Reduced tumor formation; increased trabecular bone volume	
IL-1Ra; canakinumab	IL-1β signaling	*In vivo*	MDA-MB-231; E0771; NOD/SCID; BALB/c nude	Reduced spontaneous metastasis to human bone	[114]
Caspase-1 inhibitor	Caspase-1	*In vitro*	MDA-MB-231	Abrogated level of transmigration of MDA-MB-231 cells through both blood and lymphatic endothelial cell barriers	
Verteporfin; siRNA-silenced p62	SQSTM1/ p62	*In vitro*	MCF-7; MDA-MB-231	Cytotoxic for HR- cell lines	[52]
IRAK1/4 inhibitor; BAY11-7082; SP600125; and LY294002	NF-κB, JNK, PI3K	*In vitro*	MCF-7 (ATCCHTB-22); MCF-7_TG2	Inhibited expression of IL-6 from IL-1β-stimulated TG2-overexpressing MCF-7_TG2 BC cells	[123]

Table 1: Continued.

Treatment/intervention	Targets	*In vivo*/*in vitro*	Models	Findings	References
Anakinra	IL-1R1	*In vivo*	MDA-MB-231-IV or MCF-7; BALB/c	Reduced growth of tumors in bone and the number of mice that developed bone metastases	[138]
scFv 12H7	IL-1RAcP	*In vivo* and *in vitro*	Patients; MDA-MB-231; HCC-70	Increased expression of IL-1RAcP in both TNBC cell lines and TNBC patient cohort; scFv 12H7 inhibited tumor growth via inhibiting IL-1-activated-NF-κB pathway in TNBC cells	[139]
miR-146a-5p	IRAK1	*In vitro*	MDA-MB-453; MCF-7	Repressive effects on the proliferation and invasion behavior of BC cells by targeting IRAK1	[143]
CAR-T therapy and IL-37	IL-1, IL-33			Inhibited inflammation and toxicity generated in tumor CAR-T therapy	[144]

Notes. $^{-/-}$Symbols indicate that the gene has been knocked out. Abbreviations: CSC—cancer stem cell; MSC—mesenchymal stem cell; NLRP3—NOD-like receptor family pyrin domain domain-containing protein 3; CREB—cyclic AMP response-element binding protein; IRAK1—interleukin-1 receptor-associated kinase 1; TG2—transglutaminase 2; CAR-T—chimeric antigen receptor T cell.

resulting from each antibody alone in that, instead, the tumor progression was completely abrogated [91]. Chimeric antigen receptor- (CAR-) T cells are genetically modified T cells with potential to target the TME and treat solid tumors. Toxicities arising in tumor CAR-T therapy, IL-1-mediated inflammation, and IL-1-induced IL-33-mediated anaphylaxis could be suppressed by the anti-inflammatory cytokine IL-37, thereby contributing to the amelioration of adverse effects of CAR-T therapy [144]. Anakinra can reduce the inflammation and immunosuppression caused by IL-1, contributing to the enhanced antitumor activity. More information on these treatments or interventions is shown in Table 1.

The combination with mAbs is a great focus of future development for IL-1-related therapies, both with immune checkpoint inhibitors, and with other molecularly targeted antibody classes of drugs. As tumor-hyperactivated IL-1 signaling is also responsible for the failure of targeted therapies, targeted therapies using monoclonal antibodies in combination with IL-1 blockade might have improved efficacy [145]. There are a number of recent studies that support this inference. For example, inhibition of IL-1R1 reduced the resistance of metastatic colorectal cancer (mCRC) to cetuximab (a monoclonal antibody-targeting EGFR) [146]. IL-1α induced a T cell-dependent antitumor immune response increasing the antitumor efficacy of cetuximab against head and neck squamous cell carcinoma (HNSCC) [147]. In a phase II study, the good activity and manageable safety profile of fluorouracil (5-FU) in combination with bevacizumab and anakinra were demonstrated in mCRC patients who did not respond to chemotherapy and antiangiogenic therapy [137]. But such studies are lacking in BC.

As mentioned earlier, fatal systemic inflammation is a drawback of this therapy. IL-1R1 blockade in combination with chemotherapy may also increase toxicity [81]. Therefore, whether anakinra is a safe adjuvant to chemotherapy and other treatments remains to be demonstrated.

8. Conclusions and Perspectives

As a key regulatory inflammatory cytokine, IL-1 is produced in response to not only the stimulus of cell damage, necrosis or environmental stress but also the demand of certain tumors, including BC, and in turn it activates downstream and surrounding inflammatory signals that act to recruit, promote inflammation, induce immunosuppression, promote metastasis, and participate in drug resistance, thus providing a favorable environment for tumor survival.

IL-1-mediated inflammatory signaling participates in immunosuppression and immune escape through the production and maintenance of an inflammatory microenvironment, which is conducive to the progress of BC. Therefore, blocking of abnormal IL-1 signaling caused by a tumor can be used as an immunotherapy or adjuvant immunotherapy to reduce inflammation/immunosuppression and enhance antitumor immunity [148]. In the context of BC, the dysregulated expression of genes, transcription factors, inflammatory cytokines, chemokines, and signaling pathway proteins that depend on or involve in the regulation of the production, secretion, and function of IL-1 signaling molecules, as well as the IL-1-mediated crosstalk between tumor cells and tumor infiltrating immune cells plays an important role in determining the prometastatic potential and therapeutic resistance. To date, the role of IL-1 signaling in tumors has been controversial, in part due to differences in cancer contexts, pleiotropic effects of IL-1, and distinct functions of the two IL-1 cytokines. Most of what is currently known about the role of IL-1 signaling in tumors comes from studying the function of individual recombinant or extracellular forms of IL-1 cytokines, actually leading to an inability to determine their relevance to the function of cells as well as established malignant cells, either from cancer patients or from transplantable mouse models [118].

The levels of IL-1 in combination with other cytokines or IL-1α/β alone in TME or serum in BC patients are correlated

with treatment outcome and are likely to be predictive of poorer outcome. The clinical implications of biomarkers that can classify cases in need of action versus those that are best addressed individually are gaining traction [149, 150]. Currently, only a minority of molecules form part of routine molecular diagnosis of BC, and microenvironment-derived biomarkers are potential additions to existing panels of predictive and prognostic markers [35]. Interestingly, a study conducted in 2017-2018 explored the relationship between cognitive function, severity of depressive symptoms, and IL-1 expression in patients with BC treated with systemic anticancer therapy. The protein expression levels of IL-1α and IL-1β in patients after chemotherapy were significantly lower, and the severity of depressive symptoms was also lower than that before chemotherapy [151]. We need a more detailed understanding of how different types of cells interact in the microenvironment and how IL-1 signaling promotes or suppresses tumors to better use immune cells and IL-1 as targets and biomarkers for BC therapy.

Among the related therapeutic strategies, the results of several *in vivo* and *in vitro* experiments demonstrated the potential of IL-1 as a therapeutic target for metastatic BC. Anakinra is the most widely used FDA approved biological agent for cancer-related inflammation, which targets the BC microenvironment by directly blocking IL-1 signaling, reducing tumor growth and metastasis, and enhancing chemotherapy efficacy. But this therapeutic approach may interfere with the IL-1-mediated innate immunity *in vivo*, which is the biggest limitation. Compared with several other blockers, anakinra mimics the natural mode of IL-1 blockade with more direct apparent effects and representativeness, which may have contributed to the lower cost-effectiveness and use of other blockers in clinical studies. On the other hand, none of the primary aims of various related studies were to investigate the antitumor effects of IL-1 blockade on a targeted basis. We therefore believe that focusing on the antitumor effects of the blockade of IL-1 signaling in future studies is warranted. There are also other candidate targeting strategies in terms of IL-1 signaling blockade, such as IL-1RAcP, IL-1R2/8, inflammasome/caspase-1, IRAK, and NF-κB pathways. At present, BC has a high recurrence rate and rapid disease progression after monotherapy; thus, combination therapy has become a hallmark of BC treatment. Drug combinations using different mechanisms are able to reduce the likelihood of cancer cells developing drug resistance while reducing the therapeutic dose and toxicity of monotherapies [152].

Targeting the tumor microenvironment often requires innovative drug delivery systems, such as nanoformulations, to achieve drug accumulation at the tumor site. How to link nanomedicine to tumor delivery of anti-IL-1 drugs is also a question to be addressed in the future [153]. Altogether, further understanding of the mechanisms by which IL-1 signaling regulates inflammation, immunity, metastasis, and drug resistance in the BC microenvironment, and finding novel targets that are closely related to tumor development and whose blockade does not later have a devastating impact on the role of IL-1 signaling in innate immunity will provide new perspectives for therapeutic strategies in BC, especially in metastatic BC.

Conflicts of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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References

[1] C. A. Dinarello, "IL-1: discoveries, controversies and future directions," *European Journal of Immunology*, vol. 40, pp. 599–606, 2010.

[2] J. K. Fields, S. Günther, and E. J. Sundberg, "Structural basis of IL-1 family cytokine signaling," *Frontiers in Immunology*, vol. 10, p. 1412, 2019.

[3] A. Mantovani, C. A. Dinarello, M. Molgora, and C. Garlanda, "Interleukin-1 and related cytokines in the regulation of inflammation and immunity," *Immunity*, vol. 50, pp. 778–795, 2019.

[4] C. H. Hannum, C. J. Wilcox, W. P. Arend et al., "Interleukin-1 receptor antagonist activity of a human interleukin-1 inhibitor," *Nature*, vol. 343, pp. 336–340, 1990.

[5] J. E. Sims and D. E. Smith, "The IL-1 family: regulators of immunity," *Nature Reviews Immunology*, vol. 10, pp. 89–102, 2010.

[6] I. Striz, "Cytokines of the IL-1 family: recognized targets in chronic inflammation underrated in organ transplantations," *Clinical Science*, vol. 131, pp. 2241–2256, 2017.

[7] F. L. van de Veerdonk and M. G. Netea, "New insights in the immunobiology of IL-1 family members," *Frontiers in Immunology*, vol. 4, p. 167, 2013.

[8] D. Boraschi, P. Italiani, S. Weil, and M. U. Martin, "The family of the interleukin-1 receptors," *Immunological Reviews*, vol. 281, pp. 197–232, 2018.

[9] R. N. Apte, S. Dotan, M. Elkabets et al., "The involvement of IL-1 in tumorigenesis, tumor invasiveness, metastasis and tumor-host interactions," *Cancer Metastasis Reviews*, vol. 25, pp. 387–408, 2006.

[10] A. Mantovani, I. Barajon, and C. Garlanda, "IL-1 and IL-1 regulatory pathways in cancer progression and therapy," *Immunological Reviews*, vol. 281, pp. 57–61, 2018.

[11] A. Litmanovich, K. Khazim, and I. Cohen, "The role of interleukin-1 in the pathogenesis of cancer and its potential as a therapeutic target in clinical practice," *Oncology and therapy*, vol. 6, pp. 109–127, 2018.

[12] J. J. McDonald, D. C. McMillan, and B. J. A. Laird, "Targeting IL-1α in cancer cachexia: a narrative review," *Current Opinion in Supportive and Palliative Care*, vol. 12, pp. 453–459, 2018.

[13] J. Mora and A. Weigert, "IL-1 family cytokines in cancer immunity - a matter of life and death," *Biological Chemistry*, vol. 397, pp. 1125–1134, 2016.

[14] O. S. Dmitrieva, I. P. Shilovskiy, M. R. Khaitov, and S. I. Grivennikov, "Interleukins 1 and 6 as main mediators of inflammation and cancer," *The Biochemist*, vol. 81, pp. 80–90, 2016.

[15] A. Valeta-Magara, A. Gadi, V. Volta et al., "Inflammatory breast cancer promotes development of M2 tumor-associated macrophages and cancer mesenchymal cells through a complex chemokine network," *Cancer Research*, vol. 79, pp. 3360–3371, 2019.

[16] K. Taniguchi and M. Karin, "NF-κB, inflammation, immunity and cancer: coming of age," *Nature Reviews Immunology*, vol. 18, pp. 309–324, 2018.

[17] C. Belli, D. Trapani, G. Viale et al., "Targeting the microenvironment in solid tumors," *Cancer Treatment Reviews*, vol. 65, pp. 22–32, 2018.

[18] W. Zhang, N. Borcherding, and R. Kolb, "IL-1 signaling in tumor microenvironment," *Advances in Experimental Medicine and Biology*, vol. 1240, pp. 1–23, 2020.

[19] I. Martínez-Reza, L. Díaz, D. Barrera et al., "Calcitriol inhibits the proliferation of triple-negative breast cancer cells through a mechanism involving the proinflammatory cytokines IL-1β and TNF-α," *Journal of Immunology Research*, vol. 2019, Article ID 6384278, 11 pages, 2019.

[20] K. J. Baker, A. Houston, and E. Brint, "IL-1 family members in cancer; two sides to every story," *Frontiers in Immunology*, vol. 10, p. 1197, 2019.

[21] A. Ahmad, "Breast cancer statistics: recent trends," *Advances in Experimental Medicine and Biology*, vol. 1152, pp. 1–7, 2019.

[22] J. Huang, P. S. Chan, V. Lok et al., "Global incidence and mortality of breast cancer: a trend analysis," *Aging (Albany NY)*, vol. 13, pp. 5748–5803, 2021.

[23] O. Peart, "Metastatic Breast Cancer," *Radiologic technology*, vol. 88, no. 5, pp. 519m–539m, 2017.

[24] B. Wörmann, "Breast cancer: basics, screening, diagnostics and treatment," *Medizinische Monatsschrift für Pharmazeuten*, vol. 40, pp. 55–64, 2017.

[25] C. D. Savci-Heijink, H. Halfwerk, G. K. Hooijer, H. M. Horlings, J. Wesseling, and M. J. van de Vijver, "Retrospective analysis of metastatic behaviour of breast cancer subtypes," *Breast Cancer Research and Treatment*, vol. 150, pp. 547–557, 2015.

[26] A. G. Waks and E. P. Winer, "Breast cancer treatment: a review," *Journal of the American Medical Association*, vol. 321, pp. 288–300, 2019.

[27] K. J. Ruddy and P. A. Ganz, "Treatment of nonmetastatic breast cancer," *Journal of the American Medical Association*, vol. 321, pp. 1716-1717, 2019.

[28] N. Harbeck and M. Gnant, "Breast cancer," *Lancet*, vol. 389, pp. 1134–1150, 2017.

[29] S. Nagini, "Breast cancer: current molecular therapeutic targets and new players," *Anti-Cancer Agents in Medicinal Chemistry*, vol. 17, pp. 152–163, 2017.

[30] O. Peart, "Breast intervention and breast cancer treatment options," *Radiologic Technology*, vol. 86, pp. 535M–558M, 2015, quiz 559–62.

[31] F. A. Fisusi and E. O. Akala, "Drug combinations in breast cancer therapy," *Pharmaceutical nanotechnology*, vol. 7, pp. 3–23, 2019.

[32] M. Fahad Ullah, "Breast cancer: current perspectives on the disease status," *Advances in Experimental Medicine and Biology*, vol. 1152, pp. 51–64, 2019.

[33] M. Nedeljković and A. Damjanović, "Mechanisms of chemotherapy resistance in triple-negative breast cancer—how we can rise to the challenge," *Cell*, vol. 8, p. 957, 2019.

[34] J. J. Wang, K. F. Lei, and F. Han, "Tumor microenvironment: recent advances in various cancer treatments," *European Review for Medical and Pharmacological Sciences*, vol. 22, pp. 3855–3864, 2018.

[35] S. Mittal, N. J. Brown, and I. Holen, "The breast tumor microenvironment: role in cancer development, progression and response to therapy," *Expert Review of Molecular Diagnostics*, vol. 18, pp. 227–243, 2018.

[36] T. G. Odle, "Precision medicine in breast cancer," *Radiologic technology*, vol. 88, no. 4, pp. 401m–421m, 2017.

[37] H. Rachdi, A. Mokrani, R. Batti, M. Ayadi, N. Chraiet, and A. Mezlini, "Target therapy for metastatic breast cancer," *La Tunisie Médicale*, vol. 96, pp. 465–471, 2018.

[38] G. Gu, D. Dustin, and S. A. Fuqua, "Targeted therapy for breast cancer and molecular mechanisms of resistance to treatment," *Current Opinion in Pharmacology*, vol. 31, pp. 97–103, 2016.

[39] L. The, "Breast cancer targeted therapy: successes and challenges," *Lancet*, vol. 389, p. 2350, 2017.

[40] K. G. Deepak, R. Vempati, G. P. Nagaraju et al., "Tumor microenvironment: challenges and opportunities in targeting metastasis of triple negative breast cancer," *Pharmacological research*, vol. 153, article 104683, 2020.

[41] M. Truffi, S. Mazzucchelli, A. Bonizzi et al., "Nano-strategies to target breast cancer-associated fibroblasts: rearranging the tumor microenvironment to achieve antitumor efficacy," *International Journal of Molecular Sciences*, vol. 20, p. 1263, 2019.

[42] Q. Q. Liu, F. Peng, and J. P. Chen, "The role of exosomal microRNAs in the tumor microenvironment of breast cancer," *International Journal of Molecular Sciences*, vol. 20, p. 3884, 2019.

[43] J. Plava, M. Cihova, M. Burikova, M. Matuskova, L. Kucerova, and S. Miklikova, "Recent advances in understanding tumor stroma-mediated chemoresistance in breast cancer," *Molecular Cancer*, vol. 18, p. 67, 2019.

[44] D. Bauer, N. Redmon, E. Mazzio, and K. F. Soliman, "Apigenin inhibits TNFα/IL-1α-induced CCL2 release through IKBK-epsilon signaling in MDA-MB-231 human breast cancer cells," *PLoS One*, vol. 12, article e0175558, 2017.

[45] D. T. Chu, T. N. T. Phuong, N. L. B. Tien et al., "The effects of adipocytes on the regulation of breast cancer in the tumor microenvironment: an update," *Cells*, vol. 8, p. 857, 2019.

[46] T. Wu and Y. Dai, "Tumor microenvironment and therapeutic response," *Cancer Letters*, vol. 387, pp. 61–68, 2017.

[47] E. Hirata and E. Sahai, "Tumor microenvironment and differential responses to therapy," *Cold Spring Harbor Perspectives in Medicine*, vol. 7, p. a026781, 2017.

[48] N. J. Brady, P. Chuntova, and K. L. Schwertfeger, "Macrophages: regulators of the inflammatory microenvironment during mammary gland development and breast cancer," *Mediators of Inflammation*, vol. 2016, Article ID 4549676, 13 pages, 2016.

[49] N. Singh, D. Baby, J. P. Rajguru, P. B. Patil, S. S. Thakkannavar, and V. B. Pujari, "Inflammation and cancer," *Annals of African Medicine*, vol. 18, pp. 121–126, 2019.

[50] M. D. Allen and L. J. Jones, "The role of inflammation in progression of breast cancer: friend or foe? (review)," *International Journal of Oncology*, vol. 47, pp. 797–805, 2015.

[51] S. K. Deshmukh, S. K. Srivastava, T. Poosarla et al., "Inflammation, immunosuppressive microenvironment and breast cancer: opportunities for cancer prevention and therapy," *Annals of translational medicine*, vol. 7, p. 593, 2019.

[52] A. F. Nawas, M. Kanchwala, S. E. Thomas-Jardin et al., "IL-1-conferred gene expression pattern in ERα(+) BCa and AR(+) PCa cells is intrinsic to ERα(-) BCa and AR(-) PCa cells and promotes cell survival," *BMC Cancer*, vol. 20, p. 46, 2020.

[53] M. Dúcka, M. Kučeríková, F. Trčka et al., "c-Myb interferes with inflammatory IL1α-NF-κB pathway in breast cancer cells," *Neoplasia*, vol. 23, pp. 326–336, 2021.

[54] H. Li, N. Duan, Q. Zhang, and Y. Shao, "IL1A & IL1B genetic polymorphisms are risk factors for thyroid cancer in a Chinese Han population," *International Immunopharmacology*, vol. 76, article 105869, 2019.

[55] H. Ji, L. Lu, J. Huang et al., "IL1A polymorphisms is a risk factor for colorectal cancer in Chinese Han population: a case control study," *BMC Cancer*, vol. 19, p. 181, 2019.

[56] Y. Wang, X. Chu, and H. Wang, "Note of clarification regarding data on the association between the interleukin-1β-511C>T polymorphism and breast cancer risk," *Breast Cancer Research and Treatment*, vol. 155, pp. 419–421, 2016.

[57] Y. Wang, Y. Wang, and L. Li, "Note of clarification regarding data about the association between the interleukin-1β-31T>C polymorphism and breast cancer risk," *Breast Cancer Research and Treatment*, vol. 155, pp. 415–417, 2016.

[58] B. Wang and F. Yuan, "The association between interleukin-1β gene rs1143634 polymorphism and the risk of breast cancer," *Cytokine*, vol. 113, pp. 475-476, 2019.

[59] S. Pooja, S. Rajender, and R. Konwar, "Response to commentary article: "The association between interleukin-1β gene rs1143634 polymorphism and the risk of breast cancer by Bei Wang, Fenlai Yuan"," *Cytokine*, vol. 113, p. 477, 2019.

[60] N. Eras, F. T. Daloglu, T. Çolak, M. Guler, and E. Akbas, "The correlation between IL-1β-C31T gene polymorphism and susceptibility to breast cancer," *Journal of breast cancer*, vol. 22, pp. 210–218, 2019.

[61] J. Wang, Y. Shi, G. Wang, S. Dong, D. Yang, and X. Zuo, "The association between interleukin-1 polymorphisms and their protein expression in Chinese Han patients with breast cancer," *Molecular Genetics & Genomic Medicine*, vol. 7, article e804, 2019.

[62] L. N. Al-Eitan, B. H. Al-Ahmad, and F. A. Almomani, "The association of IL-1 and HRAS gene polymorphisms with breast cancer susceptibility in a Jordanian population of arab descent: a genotype-phenotype study," *Cancers*, vol. 12, 2020.

[63] A. Malik and T. D. Kanneganti, "Function and regulation of IL-1α in inflammatory diseases and cancer," *Immunological Reviews*, vol. 281, pp. 124–137, 2018.

[64] M. J. Bou-Dargham, Z. I. Khamis, A. B. Cognetta, and Q. A. Sang, "The role of interleukin-1 in inflammatory and malignant human skin diseases and the rationale for targeting interleukin-1 alpha," *Medicinal Research Reviews*, vol. 37, pp. 180–216, 2017.

[65] L. A. Borthwick, "The IL-1 cytokine family and its role in inflammation and fibrosis in the lung," *Seminars in Immunopathology*, vol. 38, pp. 517–534, 2016.

[66] K. Khazim, E. E. Azulay, B. Kristal, and I. Cohen, "Interleukin 1 gene polymorphism and susceptibility to disease," *Immunological Reviews*, vol. 281, pp. 40–56, 2018.

[67] V. Ziaee, L. Youssefian, M. Faghankhani et al., "Homozygous IL1RN mutation in siblings with deficiency of interleukin-1 receptor antagonist (DIRA)," *Journal of Clinical Immunology*, vol. 40, pp. 637–642, 2020.

[68] N. Toplak, Š. Blazina, and T. Avčin, "The role of IL-1 inhibition in systemic juvenile idiopathic arthritis: current status and future perspectives," *Drug Design, Development and Therapy*, vol. 12, pp. 1633–1643, 2018.

[69] B. Afsar, A. Covic, A. Ortiz, R. E. Afsar, and M. Kanbay, "The future of IL-1 targeting in kidney disease," *Drugs*, vol. 78, pp. 1073–1083, 2018.

[70] M. Kantono and B. Guo, "Inflammasomes and cancer: the dynamic role of the inflammasome in tumor development," *Frontiers in Immunology*, vol. 8, p. 1132, 2017.

[71] K. D. Mayer-Barber and B. Yan, "Clash of the cytokine titans: counter-regulation of interleukin-1 and type I interferon-mediated inflammatory responses," *Cellular & Molecular Immunology*, vol. 14, pp. 22–35, 2017.

[72] A. Malik and T. D. Kanneganti, "Inflammasome activation and assembly at a glance," *Journal of Cell Science*, vol. 130, pp. 3955–3963, 2017.

[73] C. A. Dinarello, "Overview of the IL-1 family in innate inflammation and acquired immunity," *Immunological Reviews*, vol. 281, pp. 8–27, 2018.

[74] H. J. Anders, "Of inflammasomes and alarmins: IL-1β and IL-1α in kidney disease," *Journal of the American Society of Nephrology*, vol. 27, pp. 2564–2575, 2016.

[75] J. W. Singer, A. Fleischman, S. Al-Fayoumi, J. O. Mascarenhas, Q. Yu, and A. Agarwal, "Inhibition of interleukin-1 receptor-associated kinase 1 (IRAK1) as a therapeutic strategy," *Oncotarget*, vol. 9, pp. 33416–33439, 2018.

[76] S. A. Scarneo, P. F. Hughes, K. W. Yang et al., "A highly selective inhibitor of interleukin-1 receptor-associated kinases 1/4 (IRAK-1/4) delineates the distinct signaling roles of IRAK-1/4 and the TAK1 kinase," *The Journal of Biological Chemistry*, vol. 295, pp. 1565–1574, 2020.

[77] T. Schlüter, C. Schelmbauer, K. Karram, and I. A. Mufazalov, "Regulation of IL-1 signaling by the decoy receptor IL-1R2," *Journal of Molecular Medicine*, vol. 96, pp. 983–992, 2018.

[78] H. Issafras, J. A. Corbin, I. D. Goldfine, and M. K. Roell, "Detailed mechanistic analysis of gevokizumab, an allosteric anti-IL-1β antibody with differential receptor-modulating properties," *The Journal of Pharmacology and Experimental Therapeutics*, vol. 348, pp. 202–215, 2014.

[79] C. A. Dinarello, "An interleukin-1 signature in breast cancer treated with interleukin-1 receptor blockade: implications for treating cytokine release syndrome of checkpoint inhibitors," *Cancer Research*, vol. 78, pp. 5200–5202, 2018.

[80] N. A. Espinoza-Sánchez, G. K. Chimal-Ramírez, A. Mantilla, and E. M. Fuentes-Pananá, "IL-1β, IL-8, and matrix metalloproteinases-1, -2, and -10 are enriched upon monocyte-breast cancer cell cocultivation in a Matrigel-based three-dimensional system," *Frontiers in Immunology*, vol. 8, p. 205, 2017.

[81] T. C. Wu, K. Xu, J. Martinek et al., "IL1 receptor antagonist controls transcriptional signature of inflammation in patients with metastatic breast cancer," *Cancer Research*, vol. 78, pp. 5243–5258, 2018.

[82] E. L. Kuan and S. F. Ziegler, "A tumor-myeloid cell axis, mediated via the cytokines IL-1α and TSLP, promotes the progression of breast cancer," *Nature Immunology*, vol. 19, pp. 366–374, 2018.

[83] C. Voigt, P. May, A. Gottschlich et al., "Cancer cells induce interleukin-22 production from memory CD4(+) T cells via interleukin-1 to promote tumor growth," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 114, pp. 12994–12999, 2017.

[84] B. Guo, S. Fu, J. Zhang, B. Liu, and Z. Li, "Targeting inflammasome/IL-1 pathways for cancer immunotherapy," *Scientific Reports*, vol. 6, article 36107, 2016.

[85] P. Vikhreva, V. Petrova, T. Gokbulut et al., "TAp73 upregulates IL-1β in cancer cells: potential biomarker in lung and breast cancer?," *Biochemical and Biophysical Research Communications*, vol. 482, pp. 498–505, 2017.

[86] J. H. Jang, D. H. Kim, J. M. Lim et al., "Breast cancer cell-derived soluble CD44 promotes tumor progression by triggering macrophage IL1β production," *Cancer Research*, vol. 80, pp. 1342–1356, 2020.

[87] S. Liu, J. S. Lee, C. Jie et al., "HER2 overexpression triggers an IL1α proinflammatory circuit to drive tumorigenesis and promote chemotherapy resistance," *Cancer Research*, vol. 78, pp. 2040–2051, 2018.

[88] R. Kolb, L. Phan, N. Borcherding et al., "Obesity-associated NLRC4 inflammasome activation drives breast cancer progression," *Nature Communications*, vol. 7, p. 13007, 2016.

[89] Y. Wen, M. Wang, J. Yang et al., "A comparison of fentanyl and flurbiprofen axetil on serum VEGF-C, TNF-α, and IL-1β concentrations in women undergoing surgery for breast cancer," *Pain Practice*, vol. 15, pp. 530–537, 2015.

[90] K. Kersten, S. B. Coffelt, M. Hoogstraat et al., "Mammary tumor-derived CCL2 enhances pro-metastatic systemic inflammation through upregulation of IL1β in tumor-associated macrophages," *Oncoimmunology*, vol. 6, article e1334744, 2017.

[91] I. Kaplanov, Y. Carmi, R. Kornetsky et al., "Blocking IL-1β reverses the immunosuppression in mouse breast cancer and synergizes with anti-PD-1 for tumor abrogation," *Proceedings of the National Academy of Sciences*, vol. 116, pp. 1361–1369, 2019.

[92] L. F. Campesato, A. P. M. Silva, L. Cordeiro et al., "High IL-1R8 expression in breast tumors promotes tumor growth and contributes to impaired antitumor immunity," *Oncotarget*, vol. 8, pp. 49470–49483, 2017.

[93] Z. Castaño, B. P. San Juan, A. Spiegel et al., "IL-1β inflammatory response driven by primary breast cancer prevents metastasis-initiating cell colonization," *Nature Cell Biology*, vol. 20, pp. 1084–1097, 2018.

[94] B. Fernandez-Garcia, N. Eiro, M. A. Miranda et al., "Prognostic significance of inflammatory factors expression by stroma from breast carcinomas," *Carcinogenesis*, vol. 37, pp. 768–776, 2016.

[95] M. Jeon, J. Han, S. J. Nam, J. E. Lee, and S. Kim, "Elevated IL-1β expression induces invasiveness of triple negative breast cancer cells and is suppressed by zerumbone," *Chemico-Biological Interactions*, vol. 258, pp. 126–133, 2016.

[96] K. Tawara, H. Scott, J. Emathinger et al., "HIGH expression of OSM and IL-6 are associated with decreased breast cancer survival: synergistic induction of IL-6 secretion by OSM and IL-1β," *Oncotarget*, vol. 10, pp. 2068–2085, 2019.

[97] B. Celik, A. D. Yalcin, G. E. Genc, T. Bulut, S. Kuloglu Genc, and S. Gumuslu, "CXCL8, IL-1β and sCD200 are pro-inflammatory cytokines and their levels increase in the circulation of breast carcinoma patients," *Biomedical reports*, vol. 5, pp. 259–263, 2016.

[98] R. W. Wyszynski, B. F. Gibbs, L. Varani, D. Iannotta, and V. V. Sumbayev, "Interleukin-1 beta induces the expression and production of stem cell factor by epithelial cells: crucial involvement of the PI-3K/mTOR pathway and HIF-1 transcription complex," *Cellular & molecular immunology*, vol. 13, pp. 47–56, 2016.

[99] I. Filippi, F. Carraro, and A. Naldini, "Interleukin-1β affects MDAMB231 breast cancer cell migration under hypoxia: role of HIF-1α and NFκB transcription factors," *Mediators of Inflammation*, vol. 2015, Article ID 789414, 10 pages, 2015.

[100] D. Ryan, A. Sinha, D. Bogan, J. Davies, J. Koziol, and W. M. ElShamy, "A niche that triggers aggressiveness within BRCA1-IRIS overexpressing triple negative tumors is supported by reciprocal interactions with the microenvironment," *Oncotarget*, vol. 8, pp. 103182–103206, 2017.

[101] P. Escobar, C. Bouclier, J. Serret et al., "IL-1β produced by aggressive breast cancer cells is one of the factors that dictate their interactions with mesenchymal stem cells through chemokine production," *Oncotarget*, vol. 6, pp. 29034–29047, 2015.

[102] Y. Liubomirski, S. Lerrer, T. Meshel et al., "Tumor-stroma-inflammation networks promote pro-metastatic chemokines and aggressiveness characteristics in triple-negative breast cancer," *Frontiers in Immunology*, vol. 10, p. 757, 2019.

[103] X. Luo, S. Huang, N. He et al., "Inflammatory human umbilical cord-derived mesenchymal stem cells promote stem cell-like characteristics of cancer cells in an IL-1β-dependent manner," *BioMed Research International*, vol. 2018, Article ID 7096707, 12 pages, 2018.

[104] M. D. Wellenstein, S. B. Coffelt, D. E. M. Duits et al., "Loss of p 53 triggers WNT-dependent systemic inflammation to drive breast cancer metastasis," *Nature*, vol. 572, pp. 538–542, 2019.

[105] L. Knopfová, E. Biglieri, N. Volodko et al., "Transcription factor c-Myb inhibits breast cancer lung metastasis by suppression of tumor cell seeding," *Oncogene*, vol. 37, pp. 1020–1030, 2018.

[106] R. Cheng, S. Billet, C. Liu et al., "Periodontal inflammation recruits distant metastatic breast cancer cells by increasing myeloid-derived suppressor cells," *Oncogene*, vol. 39, pp. 1543–1556, 2020.

[107] B. Weichand, R. Popp, S. Dziumbla et al., "S1PR1 on tumor-associated macrophages promotes lymphangiogenesis and metastasis via NLRP3/IL-1β," *The Journal of Experimental Medicine*, vol. 214, pp. 2695–2713, 2017.

[108] S. J. Storr, S. Safuan, N. Ahmad, M. El-Refaee, A. M. Jackson, and S. G. Martin, "Macrophage-derived interleukin-1beta promotes human breast cancer cell migration and lymphatic adhesion in vitro," *Cancer Immunology, Immunotherapy*, vol. 66, pp. 1287–1294, 2017.

[109] E. A. Elghonaimy, S. A. Ibrahim, A. Youns et al., "Secretome of tumor-associated leukocytes augment epithelial-mesenchymal transition in positive lymph node breast cancer patients via activation of EGFR/Tyr 845 and NF-κB/p 65 signaling pathway," *Tumour Biology*, vol. 37, pp. 12441–12453, 2016.

[110] T. Vilsmaier, B. Rack, A. König et al., "Influence of circulating tumour cells on production of IL-1α, IL-1β and IL-12 in sera of

patients with primary diagnosis of breast cancer before treatment," *Anticancer Research*, vol. 36, pp. 5227–5236, 2016.

[111] W. Zhang, Y. Shen, H. Huang et al., "A Rosetta Stone for breast cancer: prognostic value and dynamic regulation of neutrophil in tumor microenvironment," *Frontiers in Immunology*, vol. 11, p. 1779, 2020.

[112] D. Lefley, F. Howard, F. Arshad et al., "Development of clinically relevant in vivo metastasis models using human bone discs and breast cancer patient-derived xenografts," *Breast Cancer Research*, vol. 21, p. 130, 2019.

[113] R. Eyre, D. G. Alférez, A. Santiago-Gómez et al., "Microenvironmental IL1β promotes breast cancer metastatic colonisation in the bone via activation of Wnt signalling," *Nature Communications*, vol. 10, p. 5016, 2019.

[114] C. Tulotta, D. V. Lefley, K. Freeman et al., "Endogenous production of IL1B by breast cancer cells drives metastasis and colonization of the bone microenvironment," *Clinical Cancer Research*, vol. 25, pp. 2769–2782, 2019.

[115] R. G. Ungard, K. Linher-Melville, M. G. Nashed, M. Sharma, J. Wen, and G. Singh, "xCT knockdown in human breast cancer cells delays onset of cancer-induced bone pain," *Molecular Pain*, vol. 15, article 1744806918822185, 2019.

[116] M. Pein, J. Insua-Rodríguez, T. Hongu et al., "Metastasis-initiating cells induce and exploit a fibroblast niche to fuel malignant colonization of the lungs," *Nature Communications*, vol. 11, p. 1494, 2020.

[117] G. Bouchard, H. Therriault, R. Bujold, C. Saucier, and B. Paquette, "Induction of interleukin-1β by mouse mammary tumor irradiation promotes triple negative breast cancer cells invasion and metastasis development," *International Journal of Radiation Biology*, vol. 93, pp. 507–516, 2017.

[118] M. Dagenais, J. Dupaul-Chicoine, T. Douglas, C. Champagne, A. Morizot, and M. Saleh, "The interleukin (IL)-1R1 pathway is a critical negative regulator of PyMT-mediated mammary tumorigenesis and pulmonary metastasis," *Oncoimmunology*, vol. 6, article e1287247, 2017.

[119] A. F. Nawas, R. Mistry, S. Narayanan et al., "IL-1 induces p62/SQSTM1 and autophagy in ERα(+)/PR(+) BCa cell lines concomitant with ERα and PR repression, conferring an ERα(-)/PR(-) BCa-like phenotype," *Journal of cellular biochemistry*, vol. 120, no. 2, pp. 1477–1491, 2019.

[120] A. M. Jiménez-Garduño, M. G. Mendoza-Rodríguez, D. Urrutia-Cabrera et al., "IL-1β induced methylation of the estrogen receptor ERα gene correlates with EMT and chemoresistance in breast cancer cells," *Biochemical and Biophysical Research Communications*, vol. 490, pp. 780–785, 2017.

[121] M. Mendoza-Rodríguez, H. Arévalo Romero, E. M. Fuentes-Pananá, J. T. Ayala-Sumuano, and I. Meza, "IL-1β induces up-regulation of BIRC3, a gene involved in chemoresistance to doxorubicin in breast cancer cells," *Cancer Letters*, vol. 390, pp. 39–44, 2017.

[122] M. G. Mendoza-Rodríguez, J. T. Ayala-Sumuano, L. García-Morales, H. Zamudio-Meza, E. A. Pérez-Yepez, and I. Meza, "IL-1β inflammatory cytokine-induced TP63 isoform ΔNP63α signaling cascade contributes to cisplatin resistance in human breast cancer cells," *International Journal of Molecular Sciences*, vol. 20, p. 270, 2019.

[123] K. Oh, O. Y. Lee, Y. Park, M. W. Seo, and D. S. Lee, "IL-1β induces IL-6 production and increases invasiveness and estrogen-independent growth in a TG2-dependent manner in human breast cancer cells," *BMC Cancer*, vol. 16, p. 724, 2016.

[124] S. J. Vastert, Y. Jamilloux, P. Quartier et al., "Anakinra in children and adults with Still's disease," *Rheumatology*, vol. 58, pp. vi9–vi22, 2019.

[125] H. Ozdogan and S. Ugurlu, "Canakinumab for the treatment of familial Mediterranean fever," *Expert Review of Clinical Immunology*, vol. 13, pp. 393–404, 2017.

[126] M. F. McDermott, "Rilonacept in the treatment of chronic inflammatory disorders," *Drugs Today (Barc)*, vol. 45, no. 6, pp. 423–430, 2009.

[127] A. Bettiol, E. Silvestri, G. Di Scala et al., "The right place of interleukin-1 inhibitors in the treatment of Behçet's syndrome: a systematic review," *Rheumatology International*, vol. 39, pp. 971–990, 2019.

[128] G. Cavalli and C. A. Dinarello, "Anakinra therapy for non-cancer inflammatory diseases," *Frontiers in Pharmacology*, vol. 9, p. 1157, 2018.

[129] P. M. Ridker, J. G. Mac Fadyen, T. Thuren, B. M. Everett, P. Libby, and R. J. Glynn, "Effect of interleukin-1β inhibition with canakinumab on incident lung cancer in patients with atherosclerosis: exploratory results from a randomised, double-blind, placebo-controlled trial," *Lancet*, vol. 390, pp. 1833–1842, 2017.

[130] K. M. Schenk, J. E. Reuss, K. Choquette, and A. I. Spira, "A review of canakinumab and its therapeutic potential for non-small cell lung cancer," *Anti-Cancer Drugs*, vol. 30, pp. 879–885, 2019.

[131] A. Dhorepatil, S. Ball, R. K. Ghosh, M. Kondapaneni, and C. J. Lavie, "Canakinumab: promises and future in cardiometabolic diseases and malignancy," *The American Journal of Medicine*, vol. 132, pp. 312–324, 2019.

[132] Y. Peng and X. Wang, "Kineret protein solution survives ten years," *Journal of Pharmaceutical and Biomedical Analysis*, vol. 160, pp. 383–385, 2018.

[133] R. M. Fleischmann, H. Bliddal, F. J. Blanco et al., "A phase II trial of lutikizumab, an anti-interleukin-1α/β dual variable domain immunoglobulin, in knee osteoarthritis patients with synovitis," *Arthritis & Rhematology*, vol. 71, pp. 1056–1069, 2019.

[134] C. A. Dinarello, "Treatment of inflammatory diseases with IL-1 blockade," *Current otorhinolaryngology reports*, vol. 6, pp. 1–14, 2018.

[135] R. Kurzrock, T. Hickish, L. Wyrwicz et al., "Interleukin-1 receptor antagonist levels predict favorable outcome after bermekimab, a first-in-class true human interleukin-1α antibody, in a phase III randomized study of advanced colorectal cancer," *Oncoimmunology*, vol. 8, article 1551651, 2019.

[136] C. F. Li, T. C. Chan, C. I. Wang et al., "RSF1 requires CEBP/β and hSNF2H to promote IL-1β-mediated angiogenesis: the clinical and therapeutic relevance of RSF1 overexpression and amplification in myxofibrosarcomas," *Angiogenesis*, vol. 24, pp. 533–548, 2021.

[137] N. Isambert, A. Hervieu, C. Rébé et al., "Fluorouracil and bevacizumab plus anakinra for patients with metastatic colorectal cancer refractory to standard therapies (IRAFU): a single-arm phase 2 study," *Oncoimmunology*, vol. 7, article e1474319, 2018.

[138] I. Holen, D. V. Lefley, S. E. Francis et al., "IL-1 drives breast cancer growth and bone metastasis in vivo," *Oncotarget*, vol. 7, pp. 75571–75584, 2016.

[139] P. Zheng, Y. Zhang, B. Zhang, Y. Wang, Y. Wang, and L. Yang, "Synthetic human monoclonal antibody targets hIL1 receptor accessory protein chain with therapeutic potential in triple-negative breast cancer," *Biomedicine & Pharmacotherapy*, vol. 107, pp. 1064–1073, 2018.

[140] C. A. Dinarello and J. W. van der Meer, "Treating inflammation by blocking interleukin-1 in humans," *Seminars in Immunology*, vol. 25, pp. 469–484, 2013.

[141] Y. Zhang, B. Xu, and X. P. Zhang, "Effects of miRNAs on functions of breast cancer stem cells and treatment of breast cancer," *Oncotargets and Therapy*, vol. 11, pp. 4263–4270, 2018.

[142] H. Akbulut, Y. E. Ersoy, E. Coskunpinar et al., "The role of miRNAs as a predictor of multicentricity in breast cancer," *Molecular Biology Reports*, vol. 46, pp. 1787–1796, 2019.

[143] J. P. Long, L. F. Dong, F. F. Chen, and Y. F. Fan, "miR-146a-5p targets interleukin-1 receptor-associated kinase 1 to inhibit the growth, migration, and invasion of breast cancer cells," *Oncology letters*, vol. 17, pp. 1573–1580, 2019.

[144] A. Caraffa, C. E. Gallenga, S. K. Kritas, G. Ronconi, P. Di Emidio, and P. Conti, "CAR-T cell therapy causes inflammation by IL-1 which activates inflammatory cytokine mast cells: anti-inflammatory role of IL-37," *Journal of Biological Regulators and Homeostatic Agents*, vol. 33, pp. 1981–1985, 2019.

[145] V. Gelfo, D. Romaniello, M. Mazzeschi et al., "Roles of IL-1 in cancer: from tumor progression to resistance to targeted therapies," *International Journal of Molecular Sciences*, vol. 21, p. 6009, 2020.

[146] V. Gelfo, M. Mazzeschi, G. Grilli et al., "A novel role for the interleukin-1 receptor axis in resistance to anti-EGFR therapy," *Cancers*, vol. 10, p. 355, 2018.

[147] M. Espinosa-Cotton, S. N. Rodman Iii, K. A. Ross et al., "Interleukin-1 alpha increases anti-tumor efficacy of cetuximab in head and neck squamous cell carcinoma," *Journal for Immunotherapy of Cancer*, vol. 7, p. 79, 2019.

[148] E. Voronov and R. N. Apte, "Targeting the tumor microenvironment by intervention in interleukin-1 biology," *Current Pharmaceutical Design*, vol. 23, pp. 4893–4905, 2017.

[149] C. Woolston, "Breast cancer," *Nature*, vol. 527, p. S101, 2015.

[150] J. Shi, F. Liu, and Y. Song, "Progress: targeted therapy, immunotherapy, and new chemotherapy strategies in advanced triple-negative breast cancer," *Cancer Management and Research*, vol. 12, pp. 9375–9387, 2020.

[151] J. Jasionowska, M. Talarowska, E. Kalinka et al., "Interleukin 1 level, cognitive performance, and severity of depressive symptoms in patients treated with systemic anticancer therapy: a prospective study," *Croatian Medical Journal*, vol. 60, pp. 166–173, 2019.

[152] M. Z. Aumeeruddy and M. F. Mahomoodally, "Combating breast cancer using combination therapy with 3 phytochemicals: piperine, sulforaphane, and thymoquinone," *Cancer*, vol. 125, pp. 1600–1611, 2019.

[153] M. Du, Y. Ouyang, F. Meng et al., "Polymer-lipid hybrid nanoparticles: a novel drug delivery system for enhancing the activity of psoralen against breast cancer," *International Journal of Pharmaceutics*, vol. 561, pp. 274–282, 2019.

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Research Article

Evaluation of the Therapeutic Effects of the Hydroethanolic Extract of *Portulaca oleracea* on Surgical-Induced Peritoneal Adhesion

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Objective. Peritoneal adhesion (PA) is an abnormal connective tissue that usually occurs between tissues adjacent to damaged organs during processes such as surgery. In this study, the anti-inflammatory and antioxidant effects of *Portulaca oleracea* (PO) were investigated against postoperative-induced peritoneal adhesion. *Methods*. Thirty healthy male Wistar rats (220 ± 20 g, 6-8 weeks) were randomly divided into four groups: (1) normal, (2) control (induced peritoneal adhesion), and (3) and (4) PO extracts (induced peritoneal adhesion and received 100 or 300 mg/kg/day of PO extract for seven days). Finally, macroscopic and microscopic examinations were performed using different scoring systems and immunoassays in the peritoneal lavage fluid. *Results*. We found that the levels of adhesion scores and interleukin- (IL-) 1β, IL-6, IL-10, tumour necrosis factor- (TNF-) α, transforming growth factor- (TGF-) β_1, vascular endothelial growth factor (VEGF), and malondialdehyde (MDA) were increased in the control group. However, PO extract (100 and 300 mg/kg) notably reduced inflammatory (IL-1β, IL-6, and TNF-α), fibrosis (TGF-β_1), angiogenesis (VEGF), and oxidative (MDA) factors, while increased anti-inflammatory cytokine IL-10, antioxidant factor glutathione (GSH), compared to the control group. *Conclusion*. Oral administration of PO improved postoperational-induced PA by alleviating the oxidative factors, fibrosis, inflammatory cytokines, angiogenesis biomarkers, and stimulating antioxidative factors. Hence, PO can be considered a potential herbal medicine to manage postoperative PA. However, further clinical studies are required to approve the effectiveness of PO.

1. Introduction

Abnormal connective fibrous tissues join in the surgical area and cause adhesions between the organs and nearby tissues at nonanatomic locations [1]. Notably, infertility, reintervention, abdominal pain, and intestinal occlusion occur following peritoneal adhesions (PA). The progression rate of PA has related to some risk factors such as surgical trauma, genetic factors, presence of infection, and peritoneal contamination during the surgical operation [2]. In particular, peritonitis is considered one of the main reasons for PA progression mentioned in animal and cellular studies. Furthermore, the duration of operation and type of surgical approach directly are related to the PA formation [3]. In this regard, the open procedure (laparotomy) has been more frequently associated with PA than the laparoscopic approach [4]. Also, in the United States in 1998, the economic burden of adhesions was estimated at around 1437.1 million dollars per year [5]. Some studies indicated the reduction of tissue plasminogen activator (tPA)/plasminogen activator inhibitor-1 (PAI-1) ratio [6], increase of transforming growth factor-beta-1 (TGF-β1), tumour necrosis factor-alpha (TNF-α) [6], interleukin- (IL-) 6 [7], vascular endothelial growth factor (VEGF) [8] and cyclooxygenase (COX), and inhibition of proteolytic enzymes (e.g., matrix metalloproteases (MMPs)) [9], caused to PA development [10]. In addition, further research also proved that upregulation of inducible nitric oxide synthase (iNOS) [9], stress oxidative markers, and myeloperoxidase (MPO) promoted PA progression and development [10]. These alterations are considered underlying factors for the generation of collagen type-1 and PA development [11]. As primary cells involving PA development, phagocytic and secretory activities of macrophages are increased after five days from surgical procedure and injury by rolling the immune system. Indeed, the surface of the injured area was renewed by macrophages that provide new mesothelial layers with the help of fibroblast cells, usually three to five days after surgical injury [12]. The formation and development of the fibrins were inhibited by chemical agents such as glucocorticosteroids, calcium channel blockers, nonsteroidal anti-inflammatory drugs (NSAIDs), antibiotics, histamine antagonists, and fibrinolytic agents [13]. However, they have no enough effectiveness and efficacy for the prevention or treatment of PA.

The therapeutic advantages of herbal medicine were considered in some research because of its availability, possible efficacy, and safety [14]. *Portulaca oleracea* L. (PO) is a warm-climate herbaceous, namely, "purslane" in the USA and Australia, a famous rig in Egypt, pigweed in England, pourpier in France, Ma-Chi-Xian in China, and Qurfeh in Iran [15, 16]. Also, it is the main source of phosphorus, calcium selenium, manganese, iron [17], and omega-3 fatty acids that promote immune function [16, 18]. There are several reports of benefits of the PO plant, including attenuating effects on cancers, coronary artery disease, hypertension, and inflammatory and autoimmune disorders [16, 19]. Experimentally, it has been shown that the plant has several active constituents, including monoterpenes (portulosides A and B), diterpenes (portulene), β-amyrin type triterpenoids, and vitamin A [20]). Besides, it contains α-tocopherol, ascorbic acid, B-complex vitamins (niacin, pyridoxine, and riboflavin) [21], and amino acids (leucine, lysine, phenylalanine, methionine, isoleucine, proline, cysteine, valine, threonine, and tyrosine) [16, 22]. Moreover, further *in vivo* and *in vitro* studies represented neuroprotective [18], antidiabetic [23], antioxidant [24], anticancer [25], antiulcerogenic [26], and hepatoprotective [15] effects of PO. However, there is no study evaluating the effectiveness of the oral administration of PO on preventing surgical-induced peritoneal adhesion. Therefore, in the present study, we investigated the protective effects of PO against the surgical-induced peritoneal adhesion in a rat model.

2. Materials and Methods

2.1. Drugs and Chemicals. Ethanol was prepared from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Normal saline was purchased from the Samen® pharmacy factory (Iran). Ketamine and xylazine were obtained from ChemiDaru Company (Iran). Enzyme-linked immunosorbent assay (ELISA) kits, including VEGF, IL-1β, IL-6, TGF-β, IL-10, and TNF-α, were purchased from IBL International® Company (Switzerland), and malondialdehyde (MDA), nitric oxide (NO), and glutathione (GSH) kits were prepared from ZellBio Company (Germany).

2.1.1. Plant Material and Preparation of the Extract. PO (herbarium No. 12-1615-240) was prepared from Mashhad, Khorasan Razavi Province, Iran, in Jan 2020. First, the aerial parts of the plant were freshly prepared then washed and dried in the shadow (25°C). After the complete drying of the plant, 100 g of the plant was powdered using a mill. Next, the extract was prepared by the maceration method using 800 ml of 70% *v*/*v* ethanol/water solution for the next 72 h. Afterwards, the obtained liquid extract was concentrated using a rotary evaporator at 40°C, which yields a solid powder (20% *w*/*w* of dried powder) [27–29]. This powder is stored in the freezer at -20°C until experimenting. Finally, the extract was dissolved in normal saline containing 5% *v*/*v* Tween 80 [30].

2.1.2. Liquid Chromatography-Mass Spectrometry (LC-MS) Apparatus. The LC-MS analysis was performed using an AB SCIEX QTRAP (Shimadzu) liquid chromatography coupled with a triple quadrupole mass spectrometer. Liquid chromatography separation was performed on a Supelco C18 (15 mm × 2.1 mm × 3 μm) column. The analysis was done at a flow rate of 0.2 ml/min. The binary mobile phase consisted of A: 0.1% formic acid in water and B: 0.1% formic acid in acetonitrile. The gradient analysis started with 10% of B, isocratic conditions were maintained for 10 min, gradually turned to 30% B over 20 min, gradually increased to 80% B over 30 min, and held at 80% B for 10 min, and the system was turned to the initial condition of 10% B in 5 min. Finally, the system was reequilibrated over 5 min. The mass spectra were acquired in a range of 100 to 1700 within the 80-minute scan time. The positive electrospray ionisation (ESI) mode was applied for the mass spectrometer. Mass feature

Table 1: Scoring system for peritoneal adhesion according to the Nair et al. criteria [53].

Grade	Description of adhesive bands
0	The complete absence of adhesions
1	Only one band of adhesions among the viscera or between one viscera and the abdominal wall
2	Two bands: among viscera's or from viscera to abdominal wall
3	More than two bands: among viscera or from viscera to the abdominal wall or all intestine making a mass without adhesion to the abdominal wall
4	Viscera adhered directly to the abdominal wall, independent of the number and the extension of adhesion bands

extraction of the acquired LC-MS data and maximum detection of peaks was done using the *MZ*mine analysis software package, version 2.3.

2.2. Experimental Animals and Ethical Statements. The investigation was performed with male Wistar rats weighing 200-250 g obtained from the animal laboratory of Faculty of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran. The animals were preserved in the institute animal house at 24 ± 1°C with a 12/12 h light-dark cycle for one week before and through the experiment with free access to water and food. All animal procedures were carried out in compliance with the National Institutes of Health's Guide for the Use and Care of Laboratory Animals guidelines of institutional guidelines. Moreover, the National Institute for Medical Research Development ethical committee approved all procedures involving animals based on the policies of animal experiments and care (Ethical approval code: 971254, Approval date: 2018–07–01, Approval ID: IR.NIMAD.REC.1397.084).

2.3. Model Induction and Grouping and Interventions. To generate abdominal adhesions, the sterilised gauze was used to induce peritoneal abrasion and adhesion. First, all rats were anaesthetised with intraperitoneal (i.p.) injection of 100 mg/kg ketamine, 10 mg/kg xylazine, and 3 mg/kg acepromazine, and then, rats' abdominal hair was carefully shaved and then washed with 70% *v*/*v* ethanol [30]. Next, the sterilised gauze was repeatedly contacted to the peritoneum wall until the appearance of the faded purple spot on the peritoneum. Then, the peritoneal tissue was sutured with nonabsorbable sutures No. 4.0. After that, this area was disinfected with a few drops of chloramphenicol [31–33]. At the end of the surgery, the rats received cefazolin 300 mg/kg (i.m.) to prevent wound infection [32, 33] and then were transferred to the recovery room for grouping and interventions for seven days. The surgery procedure was prolonged to a maximum of ten min. It should be noted that the gavage of groups was performed on the first day after the surgery.

Thirty healthy male Wistar rats (220 ± 20 g, 6-8 weeks) were randomly divided into four groups, as described below: group one, normal (six rats without surgical procedures); group two, control (eight rats induced peritoneal adhesion and gavaged with the vehicle of PO extract for seven consecutive days); group three (low dose, eight rats induced peritoneal adhesion and received 100 mg/kg/day of PO extract for seven consecutive days); group four (high dose, eight rats induced peritoneal adhesion and received 300 mg/kg/day of PO extract for seven consecutive days). The doses of PO were selected according to the preliminary evaluation.

2.4. Assessment of Adhesion Grade. The rats' laparotomy was done on the 8th day after the surgery. The peritoneal adhesion grades were scored via the two scoring systems (Tables 1 and 2) with two independent researchers blinded on the procedure and the grouping.

2.5. Histological Evaluation. In this study, paraffin-embedded histological sections were stained by Masson's trichrome staining to assess the extent and distribution of fibrosis in rats' peritoneal tissue as described by the manufacturer (Sigma-Aldrich) [34, 35]. In addition, to prepare the peritoneal tissue sample, after removing formalin and washing with distilled water three times, the tissues were transferred to different concentrations of alcohol (50-100% *v*/*v*) for some minutes. Tissue sections were observed with magnifications of 4x, 20x, and 40x using a Nikon E-1000 microscope (Japan) under bright-field optics and photomicrographed using Easy Image 1 (Bergström Instrument AB, Sweden).

2.6. Total Protein Measurement Method. The Bradford protein assay was performed to quantify the total protein concentration in a sample [36]. For this reason, first, the Coomassie Brilliant Blue G-250 dye (10 mg) was dissolved in 50 ml ethanol 96%. Then, phosphoric acid 85% (10 ml) was added and the volume of the solution was increased to 100 ml. Next, bovine serum albumin (4 mg/ml) solution was prepared as a standard curve. Then, after sample pouring (20 μl), a Bradford reagent (200 μl) was added to the 96-well microplate. Finally, the light absorption was read at 595 nm with a microplate reader after 5 minutes.

2.7. Evaluation of Inflammatory, Angiogenesis, and Fibrosis Biomarkers. According to the manufacturer's instruction, as indices of inflammation, the levels of TNF-α, IL-1β, and IL-6 were measured in peritoneal lavage fluid using ELISA kits [37, 38]. In addition, the levels of VEGF, as an angiogenesis marker, TGF-β, as a fibrosis factor, and IL-10, as an anti-inflammatory and suppressive cytokine, were also measured in the peritoneal fluid sample by ELISA kits according to the manufacturer's instruction [31, 39].

2.8. Measurement of Oxidant and Antioxidant Parameters. The levels of malondialdehyde (MDA), as an oxidant marker, and glutathione (GSH), as an antioxidant marker, were measured in the peritoneal fluid using commercially available biochemistry kits [31, 38, 40].

Table 2: Scoring system for peritoneal adhesion according to Adhesion Scoring Scheme [44, 53].

Grade	Description of adhesive bands
0	Absence of adhesions
1	A thin layer adhesion
2	More than a thin layer adhesion
3	Thick adhesive tissue attached to the surgical site
4	Thick adhesive tissue attached to different areas of the abdomen
5	Thick adhesive tissue containing blood vessels or too much adhesive tissue or organ adhesive tissue

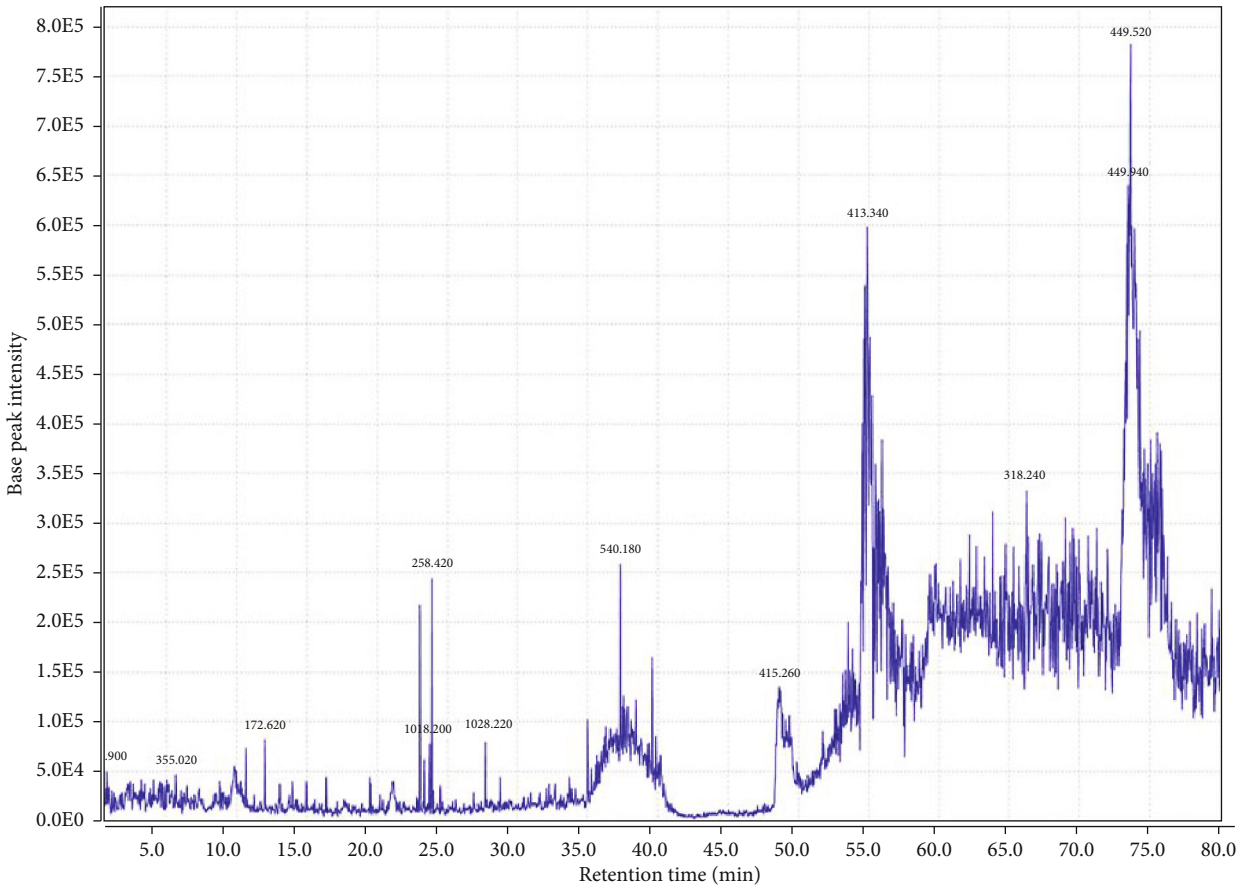
Table 3: Peak assignment of metabolites in the hydroethanol extract of PO using LC-MS in the positive mode.

Peak No.	Compound identification	t_R (min)	M + H (m/z)	Ref.
1	Portulacanone D	26.9	299.76	[25]
2	Noradrenaline	37.0	170.7	[54]
3	Dopa	15.0	198.12	[55]
4	Oleraceins A	62.5	504.66	[55]
5	Oleraceins B	9.5	533.76	[55]
6	Oleraceins C	64.1	666.06	[55]
7	Oleraceins D	13.1	696.84	[55]
8	Adenosine	19.8	268.8	[55]
9	(3R)-3,5-Bis(3-methoxy-4-hydroxyphenyl)-2,3-dihydro-2(1H)-pyridinone	89.3	342.36	[56]
10	Aurantiamide acetate	36.4	445.8	[57]
11	Cyclo(L-tyrosinyl-L-tyrosinyl)	67.7	327.24	[57]
12	Portuloside A	72.2	332.22	[58]
13	Portulene	66.3	337.02	[15]
14	Lupeol	66.5	427.5	[15]
15	(3S)-3-O-(β-D-Glucopyranosyl)-3,7-dimethylocta-1,6-dien-3-ol	67.8	318.12	[59]
16	Friedelane	54.9	413.34	[60]
17	Quercetin	39.4	303.18	[61]
18	Myricetin	55.1	318.24	[61]
19	Genistin	65.4	433.20	[62]
20	Indole-3-carboxylic acid	77.8	162.90	[25]
21	Palmitic acid	62.2	256.14	[63]
22	Stearic acid	37.8	285.18	[63]
23	Caffeic acid	65.8	181.08	[64]
24	Riboflavin	35.0	376.62	[21]
25	Vitamin C	28.5	177.00	[21]
26	α-Tocopherol	67.1	431.22	[63]
27	Hesperidin	76.8	611.58	[65]
28	Portulacerebroside A	64.6	843.18	[60]
29	β-Sitosterol	48.7	415.32	[15]
30	β-Carotene	37.5	538.74	[63]

2.9. Statistical Analysis. Data were analysed using GraphPad Prism software (version 6.01) and represented as mean ± SD and median ± interquartile range (IQR), according to the nature of parametric or nonparametric data, respectively. A one-way analysis of variance (ANOVA) was performed with Tukey's Kramer multiple comparison posttest for parametric data. However, for the nonparametric data (adhesion scores), a Kruskal-Wallis' test was performed with Dunn's multiple comparison post hoc test. P values (P) when lower than 0.05 were considered statistically significant [28, 39, 40].

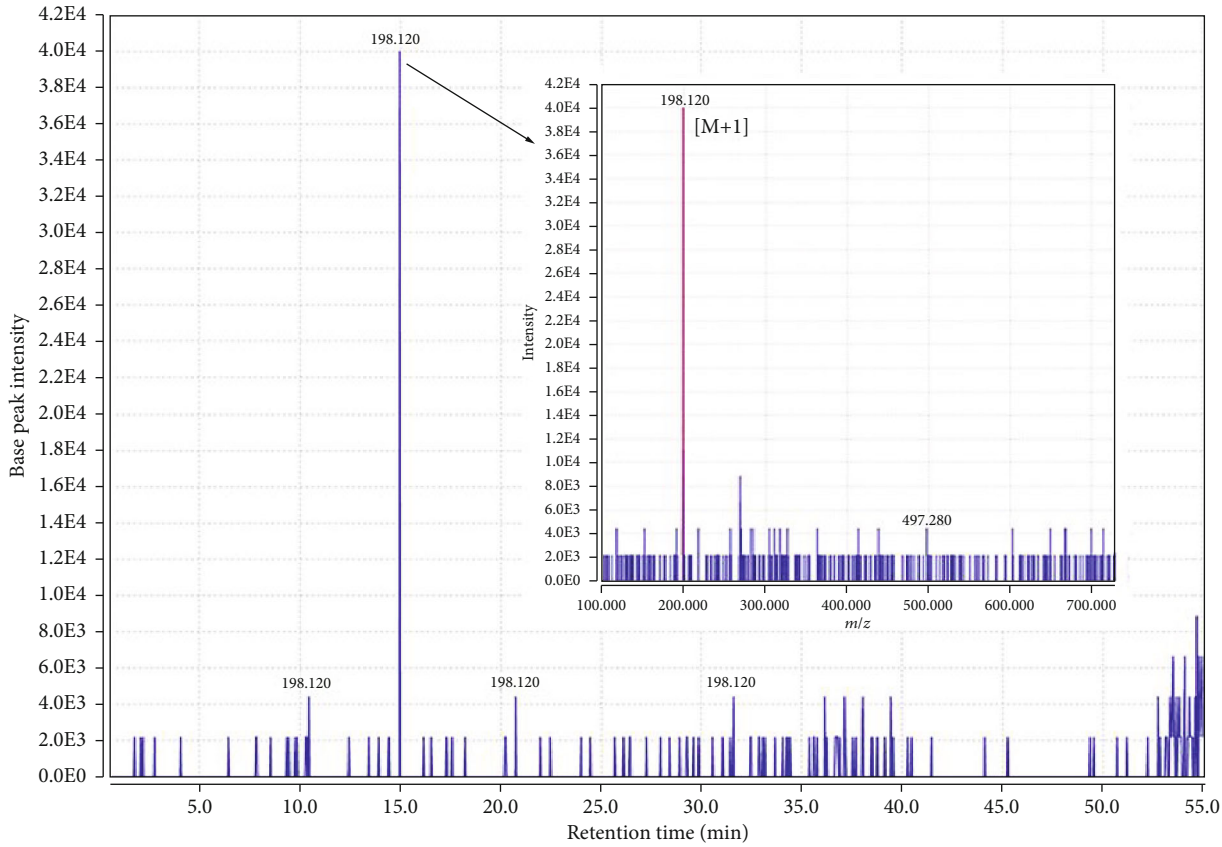
3. Results

3.1. LC-MS Analysis of PO Extract. In total, 30 compounds were identified in the hydroethanolic extract of the aerial parts of PO, mainly including alkaloids, flavonoids,



(a)

Figure 1: Continued.



(b)

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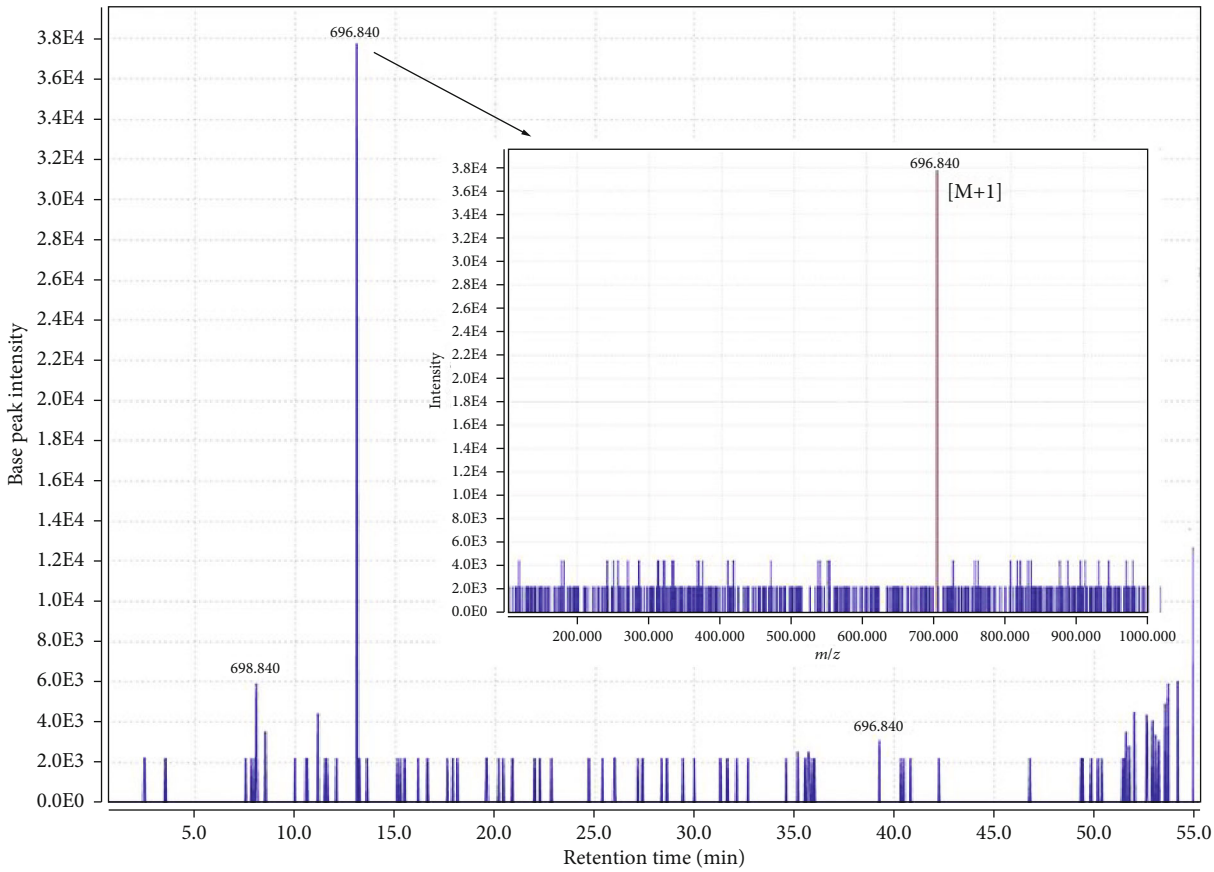
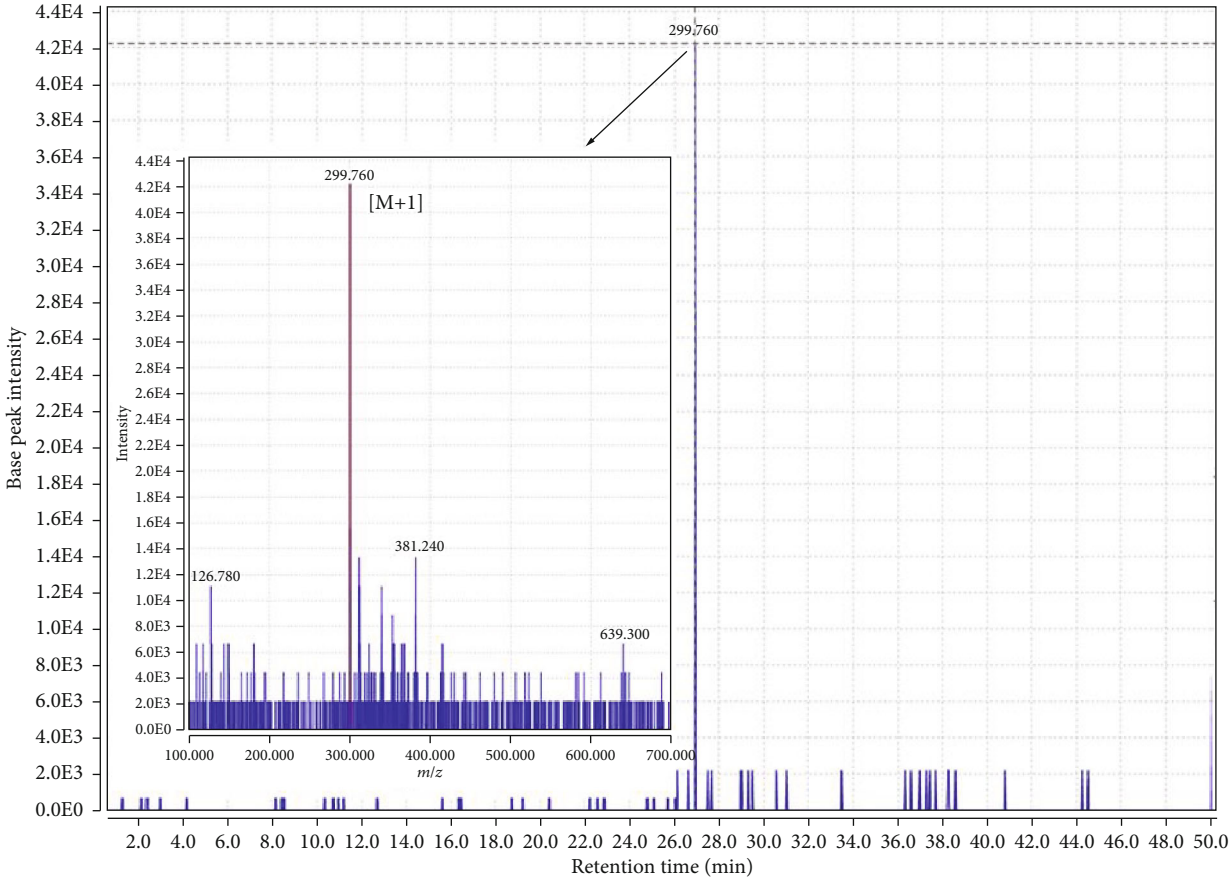


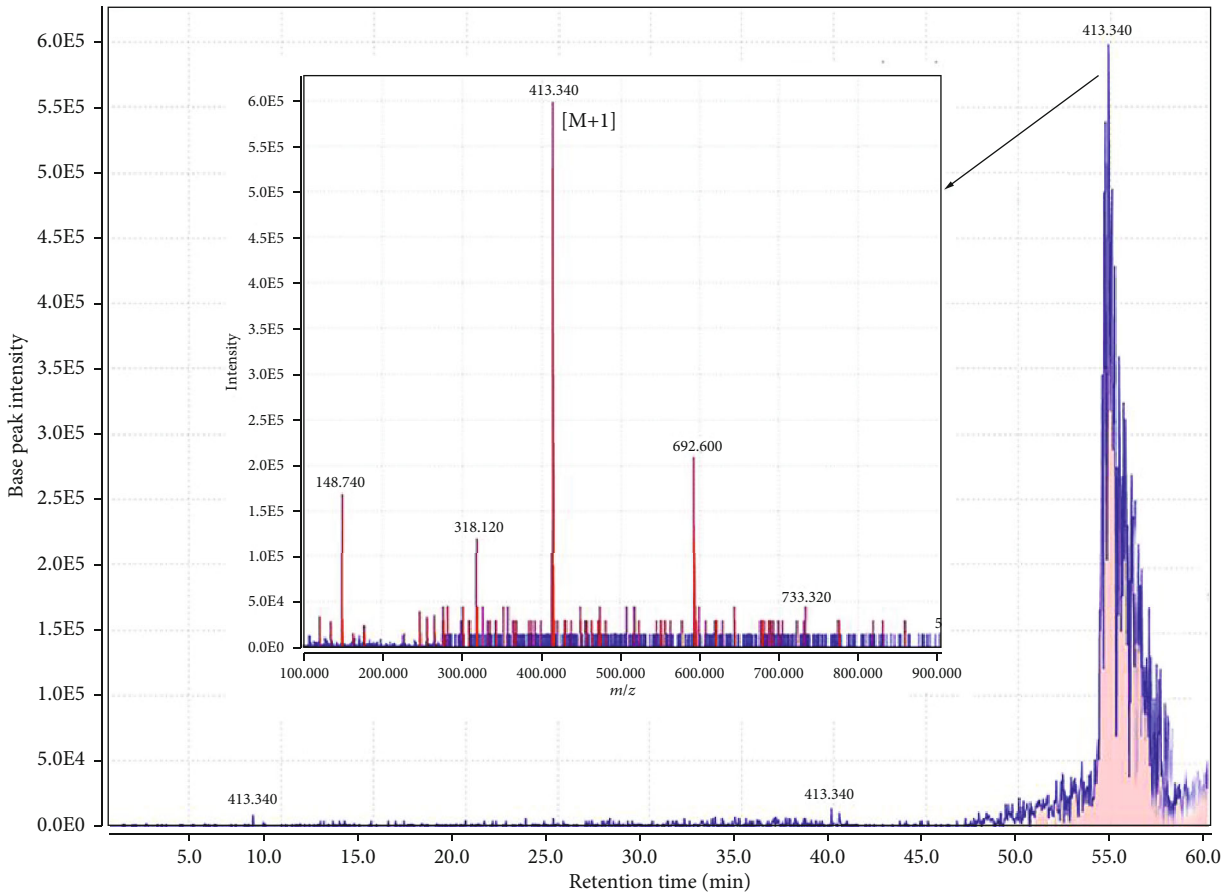
(c)

Figure 1: Continued.



(d)

Figure 1: Continued.



(e)

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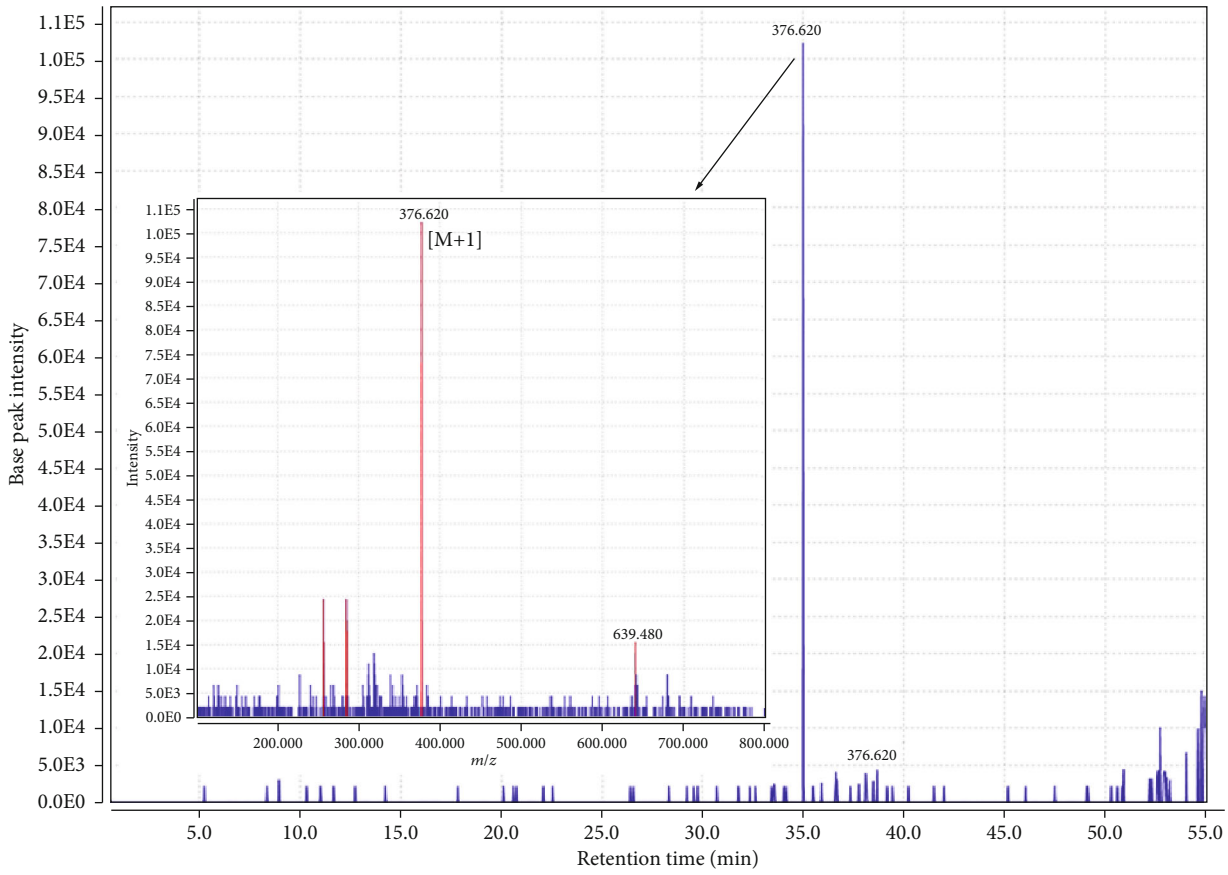
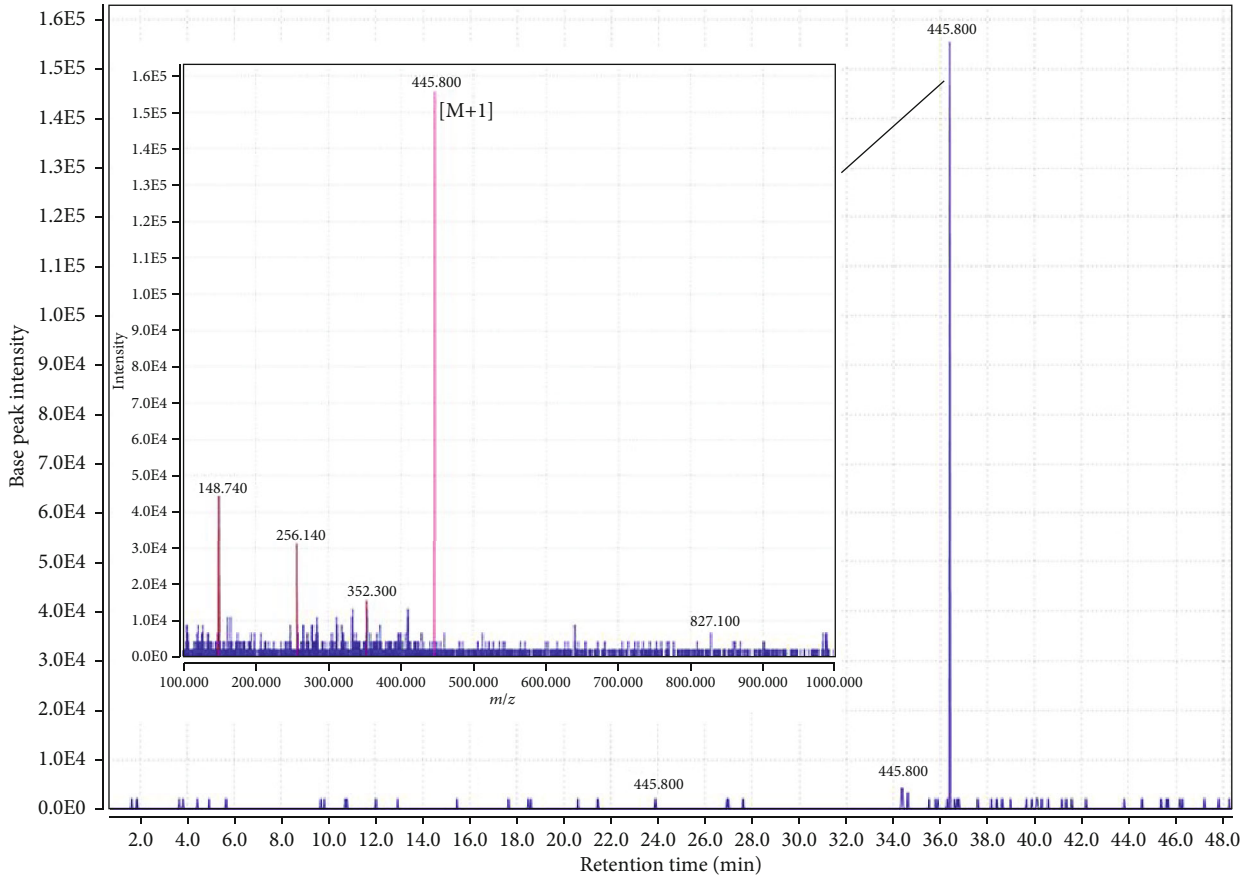


(f)

Figure 1: Continued.



(g)

Figure 1: (a) The total ion chromatogram of *Portulaca oleracea* extract; (b) chromatogram of dopa and corresponding mass adduct, [M + 1], at *m*/*z* 198.120; (c) chromatogram of oleraceins D and corresponding mass adduct, [M + 1], at *m*/*z* 696.84; (d) chromatogram of portulacanone D and corresponding mass adduct, [M + 1], at *m*/*z* 299.76; (e) chromatogram of friedelane and corresponding mass adduct, [M + 1], at *m*/*z* 413.34; (f) chromatogram of riboflavin and corresponding mass adduct, [M + 1], at *m*/*z* 376.62; (g) chromatogram of aurantiamide acetate and corresponding mass adduct, [M + 1], at *m*/*z* 445.8.

terpenoids, and vitamins. Data concerning the identification of the compounds are represented in Table 3. The total ion chromatogram of PO extract is shown in Figure 1(a). In addition, the MS spectral data were compared with the reported compounds in some previous literature. Examples of extracted ion chromatograms from the total ion chromatogram and its related mass are represented in Figures 1(b)–1(g). Alkaloids are one of the important chemicals found in PO, including dopa, noradrenalin, and oleraceins A, B, C, and D (cyclodopa alkaloids). Moreover, PO contains monoterpenes (portulosides A), diterpenes (portulene), ascorbic acid, α-tocopherol, and riboflavin.

3.2. The Effects of PO Extract on Peritoneal Adhesion (PA) Scoring. A macroscopic evaluation of PA scores was performed at the end of the experiment (Figure 2). We found that the PA scores were significantly increased in the control group compared to the standard group ($P < 0.01$ for both scoring systems, Figures 3(a) and 3(b)). Conversely, both doses of PO (100 and 300 mg/kg) markedly abolished the

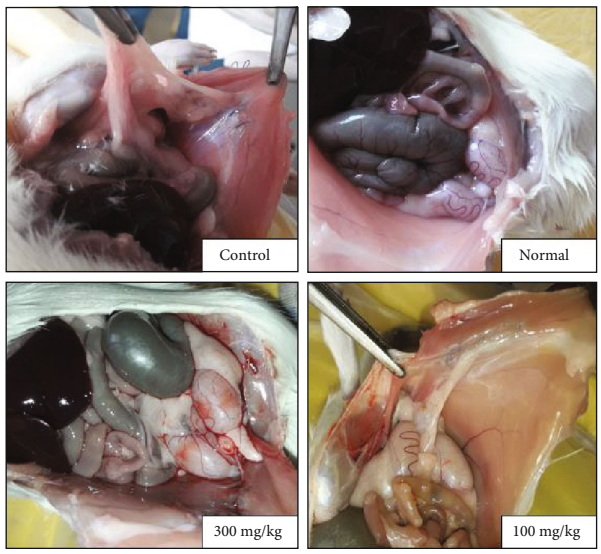


Figure 2: Macroscopic evaluation of PA bands in normal, control, and PO at doses of 100 and 300 mg/kg groups.

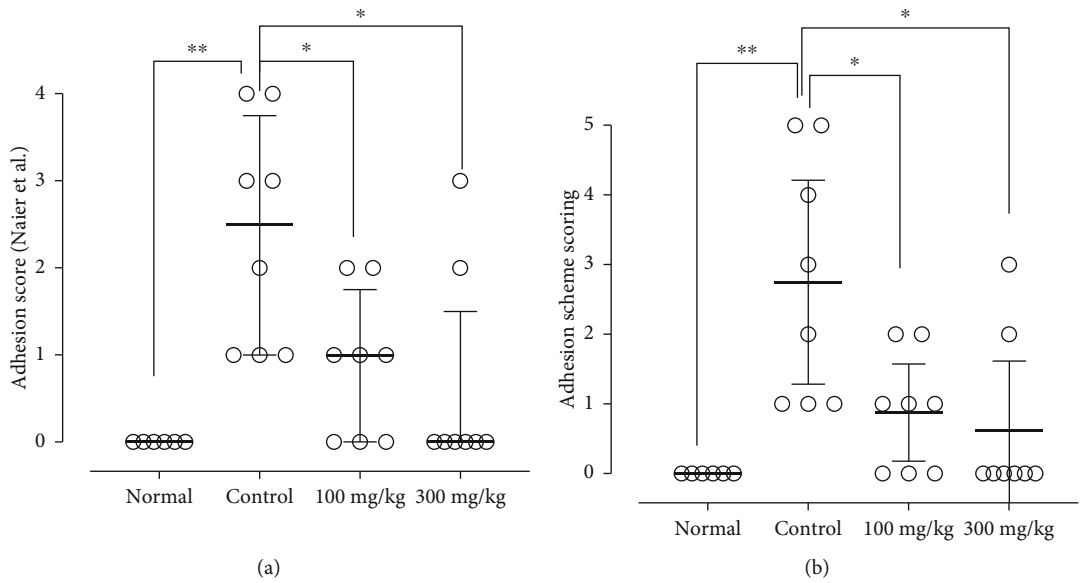


Figure 3: The effects of different doses of PO on adhesion scores evaluated by Naier et al. (a) and Adhesion Scoring Scheme (b) scoring systems; data were presented as median ± interquartile range, IQR ($n = 8$ for all groups except normal as 6). $^{*}P < 0.05$ and $^{**}P < 0.01$.

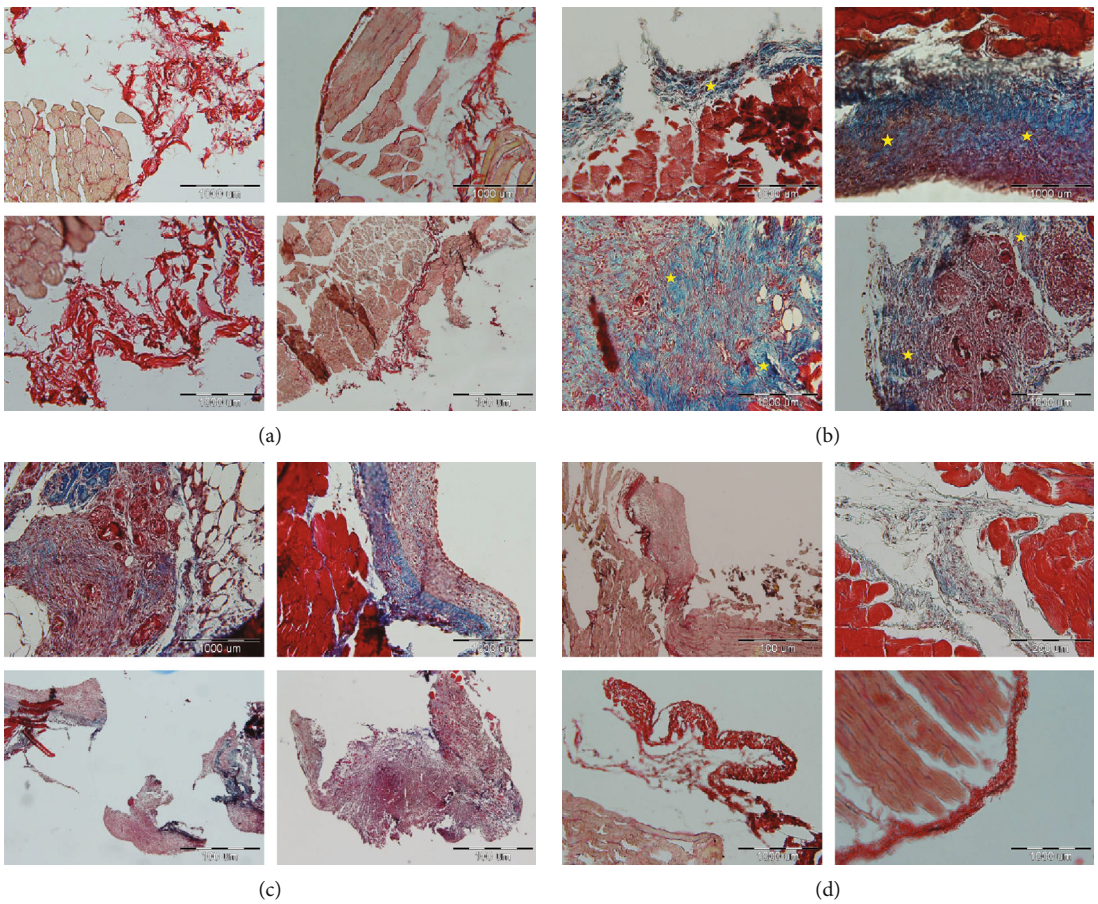


Figure 4: The effects of different doses of PO on adhesion formation and collagen deposition by histopathological evaluation using Masson's trichrome staining; blue colour intensities (marked with yellow stars) represent fibrosis and collagen deposition. Pathological imaging; (a) normal group, (b) control group, (c) PO at the dose of 100 mg/kg, and (d) PO at the dose of 300 mg/kg. The highest rate of tissue fibrosis (blue colour) and collagen deposition was observed in the control group (b) compared to the normal group (a), which had the lowest fibrosis rate. Conversely, the blue colour intensities as a marker of fibrosis and collagen deposition in both doses of the extract groups (100 and 300 mg/kg) were significantly decreased compared to the control group (c, d).

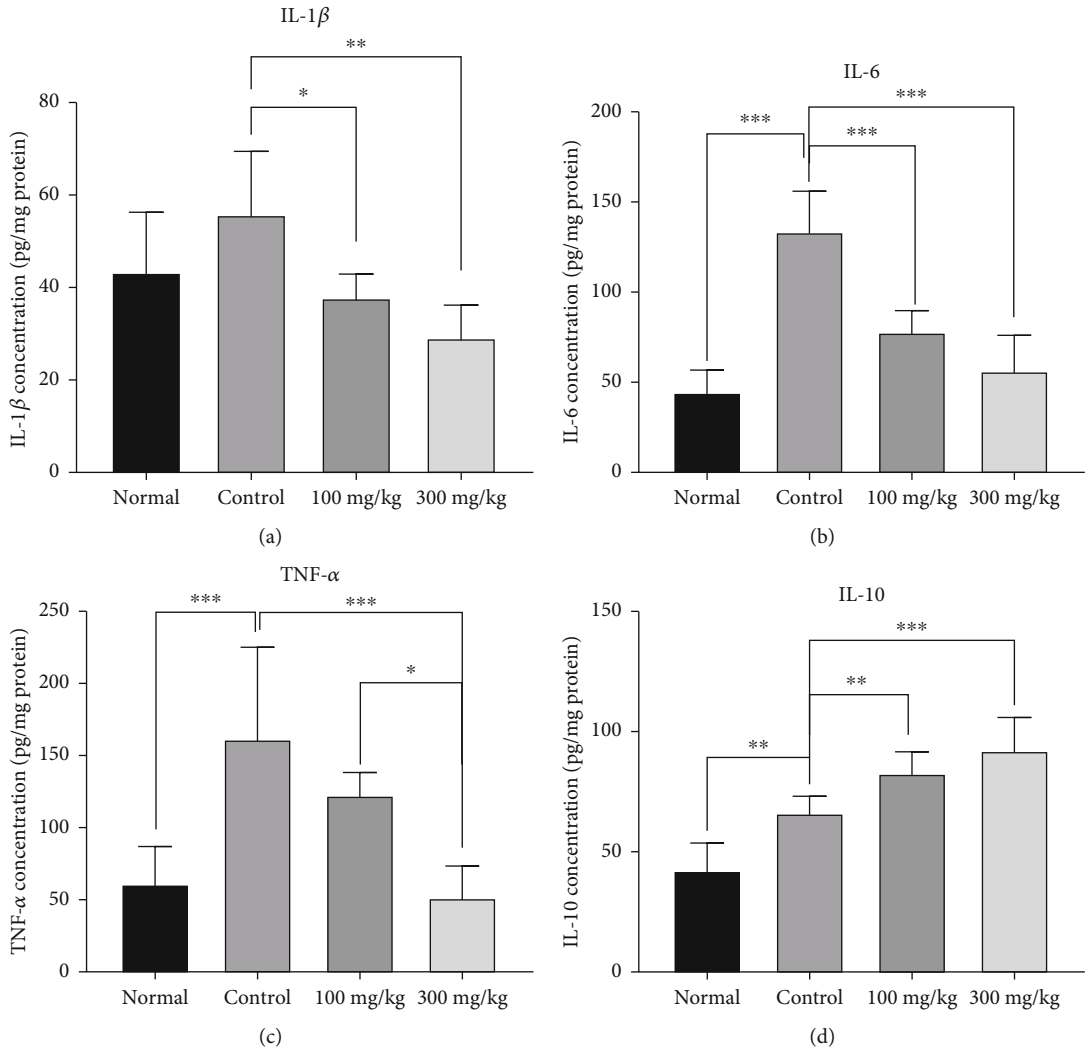


Figure 5: The effects of different doses of PO on the peritoneal lavage levels of (a) IL-1β, (b) IL-6, (c) TNF-α, and (d) IL-10; data were presented as mean ± SD ($n = 8$ for all groups except normal as 6). $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

PA scores compared to the control group ($P < 0.05$ for both cases and scoring systems).

3.3. The Effects of PO Extract on Histopathological Alteration of Peritoneal Fibrosis. The histopathological study was performed using Masson's trichrome staining to evaluate the rate of peritoneal fibrosis. As shown in Figure 4, the highest rate of tissue fibrosis (blue colour) and collagen deposition was observed in the control group (Figure 4(b)) compared to the normal group (Figure 4(a)), which had the lowest fibrosis rate. In contrast, the blue colour intensities as a marker of fibrosis and collagen deposition in both doses of the extract groups (100 and 300 mg/kg) were significantly decreased compared to the control group (Figures 4(c) and 4(d)).

3.4. The Effects of PO Extract on Inflammatory and Anti-Inflammatory Biomarkers. Our results indicated that the levels of inflammatory mediators, including IL-6 ($P < 0.001$, Figure 5(b)) and TNF-α ($P < 0.001$, Figure 5(c)), and anti-inflammatory cytokine IL-10 ($P < 0.01$, Figure 5(d)) were significantly increased in the control group compared to the normal group. However, the level of IL-1β was greater than the normal group, but this increment was not statistically significant (Figure 5(a)). Administration of PO for seven consecutive days notably reduced the levels of IL-1β (100 mg/kg, $P < 0.05$, and 300 mg/kg, $P < 0.001$, Figure 5(a)), IL-6 ($P < 0.001$ for 100 and 300 mg/kg, Figure 5(b)), and TNF-α (300 mg/kg, $P < 0.001$, Figure 5(c)) and significantly elevated the level of IL-10 (100 mg/kg, $P < 0.01$, and 300 mg/kg, $P < 0.001$, Figure 5(d)) in the peritoneal lavage fluid, compared to the control group.

3.5. The Effects of PO Extract on Fibrosis and Angiogenesis Parameters. Following the PA, the levels of TGF-β1 as a fibrotic factor and VEGF as an angiogenesis factor were significantly enhanced in the control group compared to the normal group ($P < 0.001$ for both cases, Figures 6(a) and 6(b)). In contrast, the use of 300 mg/kg/day PO significantly mitigated the levels of TGF-β1 and VEGF compared to the control group ($P < 0.001$ for both cases, Figures 6(a) and 6(b)). However, 100 mg/kg/day of PO could significantly attenuate the level of TGF-β1 compared to the control group

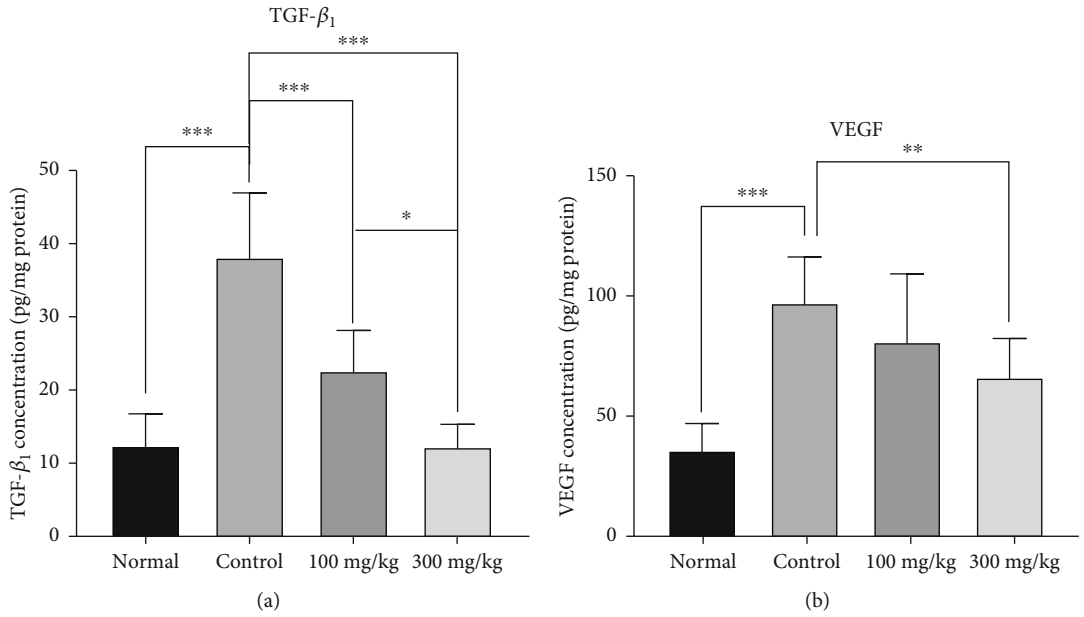


Figure 6: The effects of different doses of PO on the peritoneal lavage levels of (a) TGF-β and (b) VEGF; data were presented as mean ± SD ($n = 8$ for all groups except normal as 6). $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

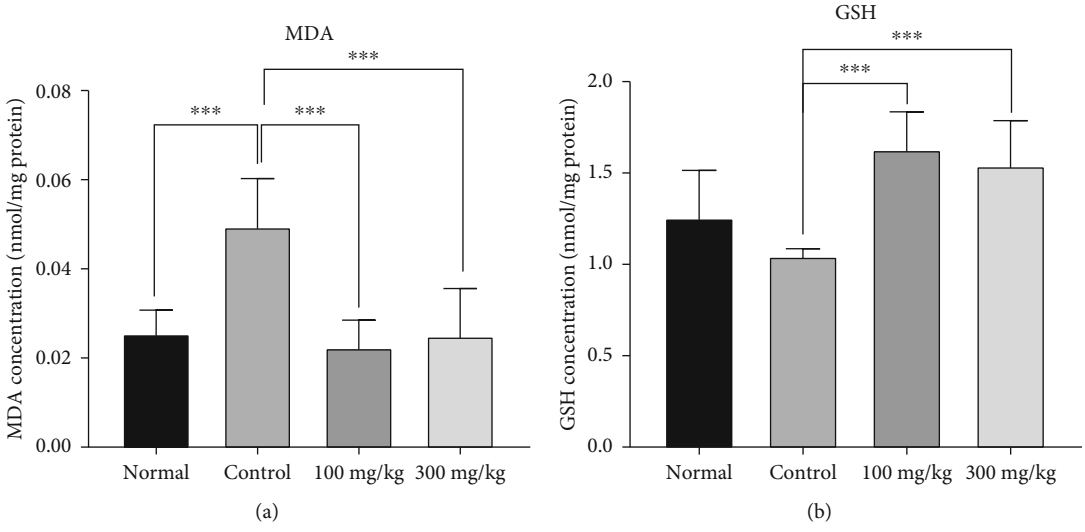


Figure 7: The effects of different doses of PO on the peritoneal lavage levels of (a) MDA and (b) GSH; data were presented as mean ± SD ($n = 8$ for all groups except normal as 6). $^{**}P < 0.01$ and $^{***}P < 0.001$.

($P < 0.001$, Figure 6(a)). Moreover, the results indicated that the suppressive effect of 300 mg/kg/day PO on TGF-β1 level was more than 100 mg/kg/day PO ($P < 0.05$, Figure 6(a)).

3.6. The Effects of PO Extract on Oxidant and Antioxidant Levels. The results revealed that the MDA level was significantly elevated in the control group compared to the normal group ($P < 0.001$, Figure 7(a)). Nevertheless, 100 and 300 mg/kg/day PO administration remarkably abolished the MDA levels compared to the normal group ($P < 0.001$ for both cases, Figure 7(a)). Following the PA induction, we observed that the level of GSH slightly decreased in the control group in comparison to the normal group (Figure 7(b)). However, administration of PO (100 and 300 mg/kg/day) considerably augmented the GSH levels in peritoneal lavage fluid compared to the control group ($P < 0.001$ for both cases, Figure 7(b)).

4. Discussion

To the best of our knowledge, this is the first study evaluating the effects of oral administration of PO extract on PA in a rat model. As a result, we found that PO at both doses significantly reduced the adhesion formation score by lowering the inflammatory cytokines (IL-1β, IL-6, and TNF-α), increasing the anti-inflammatory cytokine IL-10, and

suppressing TGF-β1 and VEGF as fibrotic and angiogenesis factors, respectively. Moreover, PO extract regulated the imbalanced oxidant/antioxidant markers by lowering MDA level as a marker of lipid peroxidation and enhancing GSH level as an antioxidant system's reservoir.

The peritoneum is a thin and delicate membrane covering the abdominal cavity and protects and structures internal organs. Some pathologic processes, including ischemia, haemorrhage, endometriosis, infections, trauma, and surgical procedures, cause PA generation in the overwhelming majority of patients [12, 41]. Nevertheless, its effects in everlasting fibrinous adhesions are influenced by the integrity of the fibrinolytic system [42]. Several investigations showed that inflammatory cytokines, including TNF-α, IL-6, and IL-1β, are the primary reasons for PA generation [30, 43, 44]. In this regard, our investigation also revealed that the levels of the inflammatory cytokines (TNF-α, IL-6, and IL-1β) were meaningfully increased in the control group following postoperational-induced PA. In contrast, PO at both doses markedly abolished these inflammatory markers. In line with our findings, it has been reported that administration of PO (200 and 400 mg/kg/day for four weeks) significantly abrogated systemic oxidative stress (MDA) and inflammatory markers (TNF-α and IL-6) in streptozotocin-induced diabetic rats [45].

Transforming growth factor-β (TGF-β), as a pleiotropic cytokine, modulates the immune system activity of T cells and regulates inflammation [30, 46]. VEGF is the essential angiogenesis activator. It also induced leakage and proliferating in endothelial cells to the adhesion site and generated new blood vessels [47, 48]. The Cahill et al. investigation results revealed that suppressing the VEGF via VEGF monoclonal antibody (bevacizumab) mitigates PA formation in mice [49]. In fact, both VEGF and TGF-β1 expression levels are increased during the PA process [47, 48, 50]. Our present study demonstrated that VEGF and TGF-β1 levels were significantly elevated in the control group following the postoperational-induced PA compared to the normal group accordingly. In immunohistological research, treatment of mice with PO (300 mg/kg/day, p.o., for ten weeks) revealed that it could reduce the expression levels of advanced glycation end products (AGE), TGF-β1, and intercellular adhesion molecule- (ICAM-) 1 in diabetic nephropathy through suppression of renal fibrosis and inflammation in diabetic *db*/*db* mice [50]. Similarly, PO extract (300 mg/kg/day, p.o., for ten weeks) reduced vascular-related adhesion molecules such as endothelial vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), and E-selectin in diabetic *db*/*db* mice [51]. Moreover, we also exhibited that the PO extract (100 and 200 mg/kg, p.o.) inhibited lung inflammation by downregulating TNF-α, IL-6, IL-β, TGF-β, and PGE_2 levels and upregulating the expression level of IL-10 [29]. In addition, it was found that PO abolished the lung wet/dry ratio that was an index of oedema and improved the levels of MDA, MPO, WBC, thiol group formation, and CAT and SOD activities compared with the LPS group [29].

One of the essential parameters of PA generation is oxidative stress [52]. Hence, in the current study, we investigated the levels of MDA and GSH as oxidant/antioxidant markers. Our data emphasised that PO meaningfully reduced the level of MDA and enhanced the GSH level following the postoperational-induced PA. Furthermore, it has been reported that the PO extract could elevate the SOD and CAT levels, while downregulating the MDA level in lipopolysaccharide- (LPS-) induced acute lung injury rats [37]. In this context, another study indicated that the PO extract improves SOD and GSH levels and prevents MDA and IL-6 levels in the STZ-induced diabetic rats [45]. Thus, these studies can support our findings on the antioxidant activity of PO that led to the reduction of adhesion formation.

In conclusion, our investigation represented that oral administration of PO improved postoperational-induced PA via alleviating the oxidative factors, fibrosis, inflammatory cytokines, angiogenesis biomarkers, and stimulating antioxidative factors. Hence, PO can be considered a potential herbal medicine to manage postoperative PA. However, further clinical studies are required to approve the effectiveness of PO.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon reasonable request.

Ethical Approval

All procedures involving animals were approved by the ethical committee based on the guidelines of animal experiments in the National Institute for Medical Research Development (Ethical approval code: 971254, Approval date: 2018–07–01, Approval ID: IR.NIMAD.REC.1397.084).

Consent

All persons gave their informed consent before their inclusion in the study.

Conflicts of Interest

The authors declare that there is no conflict of interest.

Authors' Contributions

Ali Jaafari and Vafa Baradaran Rahimi shared the first coauthorship.

Acknowledgments

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References

[1] K. M. Braun and M. P. Diamond, "The biology of adhesion formation in the peritoneal cavity," *Seminars in Pediatric Surgery*, vol. 23, no. 6, pp. 336–343, 2014.

[2] H. Toh, M. Torisu, H. Shimura et al., "*In Vitro* Analysis of Peritoneal Adhesions in Peritonitis," *The Journal of Surgical Research*, vol. 61, no. 1, pp. 250–255, 1996.

[3] A. Moreno, J. L. Aguayo, G. Zambudio, P. Ramirez, M. Canteras, and P. Parrilla, "Influence of abdominal incision on the formation of postoperative peritoneal adhesions: an experimental study in rats," *The European Journal of Surgery*, vol. 162, no. 3, pp. 181–185, 1996.

[4] A. Tittel, E. Schippers, K. H. Treutner et al., "Laparoscopy versus laparotomy. An animal experiment study comparing adhesion formation in the dog," *Langenbecks Archiv fur Chirurgie*, vol. 379, no. 2, pp. 95–98, 1994.

[5] N. F. Ray, W. G. Denton, M. Thamer, S. C. Henderson, and S. Perry, "Abdominal adhesiolysis: inpatient care and expenditures in the United States in 1994," *Journal of the American College of Surgeons*, vol. 186, no. 1, pp. 1–9, 1998.

[6] M.-L. Ivarsson, M. P. Diamond, P. Falk, and L. Holmdahl, "Plasminogen activator/plasminogen activator inhibitor-1 and cytokine modulation by the PROACT™ system," *Fertility and Sterility*, vol. 79, no. 4, pp. 987–992, 2003.

[7] D. R. Ambler, N. M. Fletcher, M. P. Diamond, and G. M. Saed, "Effects of hypoxia on the expression of inflammatory markers IL-6 and TNF-a in human normal peritoneal and adhesion fibroblasts," *Systems Biology in Reproductive Medicine*, vol. 58, no. 6, pp. 324–329, 2012.

[8] V. I. Shavell, G. M. Saed, and M. P. Diamond, "Review: cellular metabolism: contribution to postoperative adhesion development," *Reproductive Sciences*, vol. 16, no. 7, pp. 627–634, 2009.

[9] Z. Alpay, G. M. Saed, and M. P. Diamond, "Female infertility and free radicals: potential role in adhesions and Endometriosis," *The Journal of the Society for Gynecologic Investigation: JSGI*, vol. 13, no. 6, pp. 390–398, 2006.

[10] G. M. Saed, A. AL-Hendy, S. A. Salama, and M. P. Diamond, "Adenovirus-mediated expression of cyclooxygenase-2 antisense reverse abnormal genetic profile of human adhesion fibroblasts," *Fertility and Sterility*, vol. 89, no. 5, pp. 1455–1460, 2008.

[11] N. M. Fletcher, Z. L. Jiang, M. P. Diamond, H. M. Abu-Soud, and G. M. Saed, "Hypoxia-generated superoxide induces the development of the adhesion phenotype," *Free Radical Biology and Medicine*, vol. 45, no. 4, pp. 530–536, 2008.

[12] G. Saed and M. Diamond, "Molecular characterization of postoperative adhesions: the adhesion phenotype," *The Journal of the American Association of Gynecologic Laparoscopists*, vol. 11, no. 3, pp. 307–314, 2004.

[13] P. R. Koninckx, V. Gomel, A. Ussia, and L. Adamyan, "Role of the peritoneal cavity in the prevention of postoperative adhesions, pain, and fatigue," *Fertility and Sterility*, vol. 106, no. 5, pp. 998–1010, 2016.

[14] F. Nourbakhsh, M. I. Read, G. E. Barreto, and A. Sahebkar, "Boosting the autophagy-lysosomal pathway by phytochemicals: a potential therapeutic strategy against Alzheimer's disease," *IUBMB life*, vol. 72, no. 11, pp. 2360–2281, 2020.

[15] E. S. Elkhayat, S. R. Ibrahim, and M. A. Aziz, "Portulene, a new diterpene fromPortulaca oleraceaL," *Journal of Asian Natural Products Research*, vol. 10, no. 11, pp. 1039–1043, 2008.

[16] V. B. Rahimi, F. Ajam, H. Rakhshandeh, and V. R. Askari, "A pharmacological review on *Portulaca oleracea* L.: focusing on anti-inflammatory, anti- oxidant, immuno-modulatory and antitumor activities," *Journal of Pharmacopuncture*, vol. 22, no. 1, pp. 7–15, 2019.

[17] A. I. Mohamed and A. S. Hussein, "Chemical composition of purslane (Portulaca oleracea)," *Plant Foods for Human Nutrition*, vol. 45, no. 1, pp. 1–9, 1994.

[18] A. E. Abdel Moneim, "The neuroprotective effects of purslane (Portulaca oleracea) on rotenone-induced biochemical changes and apoptosis in brain of rat," *CNS & Neurological Disorders Drug Targets*, vol. 12, no. 6, pp. 830–841, 2013.

[19] A. P. Simopoulos, D. X. Tan, L. C. Manchester, and R. J. Reiter, "Purslane: a plant source of omega-3 fatty acids and melatonin," *Journal of Pineal Research*, vol. 39, no. 3, pp. 331-332, 2005.

[20] H. L. Xin, Y. F. Xu, Y. H. Hou et al., "Two novel triterpenoids fromPortulaca oleraceaL," *Helvetica Chimica Acta*, vol. 91, no. 11, pp. 2075–2080, 2008.

[21] M. K. Uddin, A. S. Juraimi, M. S. Hossain, M. A. Nahar, M. E. Ali, and M. M. Rahman, "Purslane weed (Portulaca oleracea): a prospective plant source of nutrition, omega-3 fatty acid, and antioxidant attributes," *Scientific World Journal*, vol. 2014, article 951019, pp. 1–6, 2014.

[22] U. R. Palaniswamy, B. B. Bible, and R. J. Mcavoy, "Effect of nitrate: ammonium nitrogen ratio on oxalate levels of purslane," in *Trends in New Crops and New Uses*, J. Janick and A. Whipkey, Eds., ASHS Press, Alexandria, VA, 2002.

[23] M. I. El-Sayed, "Effects of _Portulaca oleracea_ L. seeds in treatment of type-2 diabetes mellitus patients as adjunctive and alternative therapy," *Journal of Ethnopharmacology*, vol. 137, no. 1, pp. 643–651, 2011.

[24] B. Chen, H. Zhou, W. Zhao, W. Zhou, Q. Yuan, and G. Yang, "Effects of aqueous extract of Portulaca oleracea L. on oxidative stress and liver, spleen leptin, PARα and FAS mRNA expression in high-fat diet induced mice," *Molecular Biology Reports*, vol. 39, no. 8, pp. 7981–7988, 2012.

[25] J. Yan, L. R. Sun, Z. Y. Zhou et al., "Homoisoflavonoids from the medicinal plant *Portulaca oleracea*," *Phytochemistry*, vol. 80, pp. 37–41, 2012.

[26] G. Karimi, H. Hosseinzadeh, and N. Ettehad, "Evaluation of the gastric antiulcerogenic effects of Portulaca oleracea L. extracts in mice," *Phytotherapy Research*, vol. 18, no. 6, pp. 484–487, 2004.

[27] V. R. Askari, S. A. Rezaee, K. Abnous, M. Iranshahi, and M. H. Boskabady, "The influence of hydro-ethanolic extract of *Portulaca oleracea* L. on Th_1/Th_2 balance in isolated human lymphocytes," *Journal of Ethnopharmacology*, vol. 194, pp. 1112–1121, 2016.

[28] V. Baradaran Rahimi, V. R. Askari, and S. H. Mousavi, "Ellagic acid dose and time-dependently abrogates d-galactose-induced animal model of aging: investigating the role of PPAR-γ," *Life Sciences*, vol. 232, article 116595, 2019.

[29] V. Baradaran Rahimi, S. H. Mousavi, S. Haghighi, S. Soheili-FAR, and V. R. Askari, "Cytotoxicity and apoptogenic properties of the standardized extract of *Portulaca oleracea* on glioblastoma multiforme cancer cell line (U-87): a mechanistic study," *EXCLI Journal*, vol. 18, pp. 165–186, 2019.

[30] Y. Roohbakhsh, V. Baradaran Rahimi, S. Silakhori et al., "Evaluation of the effects of peritoneal lavage with Rosmarinus officinalis extract against the prevention of postsurgical-induced peritoneal adhesion," *Planta Medica*, vol. 86, no. 6, pp. 405–414, 2020.

[31] M. Ghadiri, V. Baradaran Rahimi, E. Moradi et al., "Standardised pomegranate peel extract lavage prevents postoperative peritoneal adhesion by regulating TGF-β and VEGF levels," *Inflammopharmacology*, vol. 29, no. 3, pp. 855–868, 2021.

[32] P. Liang, W. Shan, and Z. Zuo, "Perioperative use of cefazolin ameliorates postoperative cognitive dysfunction but induces gut inflammation in mice," *Journal of Neuroinflammation*, vol. 15, no. 1, p. 235, 2018.

[33] M. D. Sarap, K. S. Scher, and C. W. Jones, "Anaerobic coverage for wound prophylaxis: Comparison of cefazolin and cefoxitin," *American Journal of Surgery*, vol. 151, no. 2, pp. 213–215, 1986.

[34] C. Dees, A. Akhmetshina, P. Zerr et al., "Platelet-derived serotonin links vascular disease and tissue fibrosis," *The Journal of Experimental Medicine*, vol. 208, no. 5, pp. 961–972, 2011.

[35] M. Hu, Y. Zhang, X. Guo et al., "Perturbed ovarian and uterine glucocorticoid receptor signaling accompanies the balanced regulation of mitochondrial function and NFκB-mediated inflammation under conditions of hyperandrogenism and insulin resistance," *Life Sciences*, vol. 232, p. 116681, 2019.

[36] M. M. Bradford, "A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding," *Analytical Biochemistry*, vol. 72, no. 1-2, pp. 248–254, 1976.

[37] V. Baradaran Rahimi, H. Rakhshandeh, F. Raucci et al., "Anti-inflammatory and anti-oxidant activity of Portulaca oleracea extract on LPS-induced rat lung injury," *Molecules*, vol. 24, no. 1, p. 139, 2019.

[38] R. Shirazinia, A. A. Golabchifar, V. B. Rahimi et al., "Protective effect of Opuntia dillenii haw fruit against lead acetate-induced hepatotoxicity: in vitro and in vivo studies," *Evidence-based Complementary and Alternative Medicine*, vol. 2021, Article ID 6698345, 13 pages, 2021.

[39] V. R. Askari, V. Baradaran Rahimi, S. A. Tabatabaee, and R. Shafiee-Nick, "Combination of imipramine, a sphingomyelinase inhibitor, and β-caryophyllene improve their therapeutic effects on experimental autoimmune encephalomyelitis (EAE)," *International Immunopharmacology*, vol. 77, article 105923, 2019.

[40] V. B. Rahimi, V. R. Askari, and S. H. Mousavi, "Ellagic acid reveals promising anti-aging effects against d-galactose-induced aging on human neuroblastoma cell line, SH-SY5Y: a mechanistic study," *Biomedicine & Pharmacotherapy*, vol. 108, pp. 1712–1724, 2018.

[41] R. M. Kamel, "Prevention of postoperative peritoneal adhesions," *European Journal of Obstetrics, Gynecology, and Reproductive Biology*, vol. 150, no. 2, pp. 111–118, 2010.

[42] C. Ren, L. Zhu, and J. C. Sun, "Creation of an animal model for post-operative adhesion prevention," *Zhongguo Yi Xue Ke Xue Yuan Xue Bao. Acta Academiae Medicinae Sinicae*, vol. 34, no. 2, pp. 109–115, 2012.

[43] Z. Alpay, G. M. Saed, and M. P. Diamond, "Postoperative adhesions: from formation to prevention," *Seminars in Reproductive Medicine*, vol. 26, no. 4, pp. 313–321, 2008.

[44] V. B. Rahimi, R. Shirazinia, N. Fereydouni et al., "Comparison of honey and dextrose solution on post-operative peritoneal adhesion in rat model," *Biomedicine & Pharmacotherapy*, vol. 92, pp. 849–855, 2017.

[45] S. Samarghandian, A. Borji, and T. Farkhondeh, "Attenuation of oxidative stress and inflammation by Portulaca oleracea in streptozotocin-induced diabetic rats," *Journal of Evidence-Based Complementary & Alternative Medicine*, vol. 22, no. 4, pp. 562–566, 2017.

[46] P. A. Lucas, D. J. Warejcka, H. E. Young, and B. Y. Lee, "Formation of Abdominal Adhesions Is Inhibited by Antibodies to Transforming Growth Factor-β1," *The Journal of Surgical Research*, vol. 65, no. 2, pp. 135–138, 1996.

[47] E. T. Condon, R. A. Cahill, D. B. O'Malley, N. J. Aherne, and H. P. Redmond, "Evaluation of postoperative peritoneal adhesion formation following perioperative nicotine administration," *The Journal of Surgical Research*, vol. 140, no. 1, pp. 135–138, 2007.

[48] A. P. Laddha and Y. A. Kulkarni, "VEGF and FGF-2: promising targets for the treatment of respiratory disorders," *Respiratory Medicine*, vol. 156, pp. 33–46, 2019.

[49] R. A. Cahill, J. H. Wang, S. Soohkai, and H. P. Redmond, "Mast cells facilitate local VEGF release as an early event in the pathogenesis of postoperative peritoneal adhesions," *Surgery*, vol. 140, no. 1, pp. 108–112, 2006.

[50] A. S. Lee, Y. J. Lee, S. M. Lee et al., "An aqueous extract of Portulaca oleraceaAmeliorates diabetic nephropathy through suppression of renal fibrosis and inflammation in diabetic db/db mice," *The American Journal of Chinese Medicine*, vol. 40, no. 3, pp. 495–510, 2012.

[51] A. S. Lee, Y. J. Lee, S. M. Lee et al., "*Portulaca oleracea* ameliorates diabetic vascular inflammation and endothelial dysfunction in db/db mice," *Evidence-based Complementary and Alternative Medicine*, vol. 2012, Article ID 741824, 9 pages, 2012.

[52] V. Belebecha, R. Casagrande, M. R. Urbano et al., "Effect of the platelet-rich plasma covering of polypropylene mesh on oxidative stress, inflammation, and adhesions," *International Urogynecology Journal*, vol. 31, no. 1, pp. 139–147, 2020.

[53] S. K. Nair, I. K. Bhat, and A. L. Aurora, "Role of proteolytic enzyme in the prevention of postoperative intraperitoneal adhesions," *Archives of Surgery*, vol. 108, no. 6, pp. 849–853, 1974.

[54] J. Chen, Y. P. Shi, and J. Y. Liu, "Determination of noradrenaline and dopamine in Chinese herbal extracts from *Portulaca oleracea* L. by high-performance liquid chromatography," *Journal of Chromatography. A*, vol. 1003, no. 1-2, pp. 127–132, 2003.

[55] L. Xiang, D. Xing, W. Wang, R. Wang, Y. Ding, and L. Du, "Alkaloids from *Portulaca oleracea* L.," *Phytochemistry*, vol. 66, no. 21, pp. 2595–2601, 2005.

[56] J. L. Tian, X. Liang, P. Y. Gao et al., "Two new alkaloids from Portulaca oleracea and their cytotoxic activities," *Journal of Asian Natural Products Research*, vol. 16, no. 3, pp. 259–264, 2014.

[57] X. Liang, J. Tian, L. Li et al., "Rapid determination of eight bioactive alkaloids in *Portulaca oleracea* L. by the optimal microwave extraction combined with positive -negative conversion multiple reaction monitor (+/−MRM) technology," *Talanta*, vol. 120, pp. 167–172, 2014.

[58] N. Sakai, K. Inada, M. Okamoto, Y. Shizuri, and Y. Fukuyama, "Portuloside A, a monoterpene glucoside, from _Portulaca oleracea_," *Phytochemistry*, vol. 42, no. 6, pp. 1625–1628, 1996.

[59] Y. Seo, J. Shin, H. J. Cha et al., "A new monoterpene glucoside from Portulaca oleracea," *Bulletin-Korean Chemical Society*, vol. 24, pp. 1475–1477, 2003.

[60] H.-L. Xin, Y.-F. Xu, Y.-H. Hou et al., "Two novel triterpenoids from *Portulaca oleracea* L," *Helvetica Chimica Acta*, vol. 91, no. 11, pp. 2075–2080, 2008.

[61] X. Xu, L. Yu, and G. Chen, "Determination of flavonoids in _Portulaca oleracea_ L. by capillary electrophoresis with

electrochemical detection," *Journal of Pharmaceutical and Biomedical Analysis*, vol. 41, no. 2, pp. 493–499, 2006.

[62] H. Zhu, Y. Wang, Y. Liu, Y. Xia, and T. Tang, "Analysis of flavonoids in Portulaca oleracea L. by UV–Vis spectrophotometry with comparative study on different extraction technologies," *Food Analytical Methods*, vol. 3, no. 2, pp. 90–97, 2010.

[63] U. R. Palaniswamy, R. J. Mcavoy, and B. B. Bible, "Stage of harvest and polyunsaturated essential fatty acid concentrations in purslane (Portulaca oleraceae) leaves," *Journal of Agricultural and Food Chemistry*, vol. 49, no. 7, pp. 3490–3493, 2001.

[64] Z. Yang, C. Liu, L. Xiang, and Y. Zheng, "Phenolic alkaloids as a new class of antioxidants in Portulaca oleracea," *Phytotherapy Research*, vol. 23, no. 7, pp. 1032–1035, 2009.

[65] Z. Cheng, D. Wang, W. Zhang et al., "LC determination and pharmacokinetic study of the main phenolic components of Portulaca oleracea L. extract in rat plasma after oral administration," *Natural Product Research*, vol. 26, no. 23, pp. 2247–2250, 2012.

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Research Article

Posttreatment Downregulation of Type III Interferons in Patients with Acute Brucellosis

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There is a limited number of clinical studies on interferon (IFN) levels in human brucellosis. The novel group of interferons, type III interferons, which consists of four IFN-λ (lambda) molecules called IFN-λ1 or interleukin-29 (IL-29), IFN-λ2 or IL-28A, IFN-λ3 or IL-28B, and IFN-λ4, is not fully known. This study is one of the first studies of IL-28A and IL-29 levels in brucellosis cases at the end of their treatment course. A total of 33 acute brucellosis patients were included in this study. We considered changes in the levels of IL-28A and IL-29 in cases with acute brucellosis before and after treatment with standard therapy that referred to the Ayatollah Rohani Hospital in Babol, northern Iran. Of 33 included patients, 22 (66.6%) were males, and 11 (33.4%) were females. The range of patients' age was 49.21 ± 17.70 years. Serum IL-29 and IL-28A (acute form: 56.4 ± 30.32 pg/mL and 48.73 ± 27.72 pg/mL, respectively, and posttreatment: 40.15 ± 20.30 pg/mL and 38.79 ± 22.66 pg/mL, respectively) levels were elevated significantly in acute brucellosis than after treatment ($p < 0.05$). These findings indicate that considering biomarker levels in brucellosis patients may indicate the chronicity of infection. In conclusion, we suggest that IL-29 and IL-28A levels may be valuable biomarkers for follow-up patients with brucellosis.

1. Introduction

Brucellosis is a zoonotic bacterial disease caused by one of the various species of the *Brucella* spp. [1–3]. Although approximately half-million new brucellosis cases are reported worldwide, the actual incidence rate has been much more significant [4, 5]. Even though the gold standard for diagnosing this disease is leukocyte culture, this test has a high false-negative rate. Moreover, its cost and a 10-day delay before confirmation restrict its use as a standard diagnostic test in acute brucellosis [6]. As a result, clinicians chiefly rely on other laboratory tests to evaluate patients with brucellosis, such as agglutination test, white blood cell (WBC) counts, platelet (PLT) counts, liver function tests, erythrocyte sedimentation rate (ESR), and C-reactive protein (CRP). Nevertheless, the diagnosis of brucellosis remains a challenge in most cases [6].

Although rare, brucellosis infection has a chronic disease course that may continue to trouble patients for years. No approved human antibrucellosis vaccine is currently available [7]. So far, brucellosis studies have been focused primarily on epidemiological investigations, and the immune response against these bacteria was somehow neglected [8]. After the entry of this pathogen, several immunogenic changes are prominent in the host body. For example, interferon-gamma (IFN-γ) and tumor necrosis factor-alpha (TNF-α) levels are elevated as naïve T cells differentiate to $CD4^+$ helper T cells type 1 (Th_1). On the other hand, interleukin-4 (IL-4) levels are increased as naïve T cells give rise to $CD4^+$ helper T cells type 2 (Th_2) [6].

Moreover, transforming growth factor-beta (TGF-β) levels are also raised due to the surge in the regulatory T cell (Treg) population [6]. Several studies have shown that interferon-gamma serum concentration may be recognized as an essential factor in chronic brucellosis [8]. It is vital to note that the association between other types of cytokines, such as type III interferons or interferon-lambda (IFN-λ), and the clinical course of brucellosis, including response to treatment, is not yet fully understood. Nevertheless, some studies recognized that bacterial pathogens might activate IFN-λ1 or interleukin-29 (IL-29), IFN-λ2 or IL-28A, IFN-λ3, or IL-28B [9, 10].

Therefore, this study was conducted to evaluate the levels of IL-28A and IL-29 in patients with brucellosis, both pretreatment and posttreatment.

2. Materials and Methods

2.1. Patients. In this case-control study at the Ayatollah Rohani Hospital in Babol, northern Iran, 33 pretreatment and posttreatment acute brucellosis patients were included. Inclusion criteria were defined as receiving a clinical diagnosis of acute brucellosis (clinical presentation time: acute form ($\leq$2 months) based on the symptoms, compatible clinical findings, standard tube agglutination (STA) test titer $\geq 1:160$, and the presence of 2-mercaptoethanol (2ME) agglutination $\geq 1:80$. The control group consisted of the same 33 patients who had undergone a complete course of treatment with gentamicin 5 mg/kg/day IM for seven days plus doxycycline 100 mg tablet BID for 45 days [11]. Then, the patients were included in the control group and considered treated when no clinical manifestations related to brucellosis and compatible paraclinical findings, i.e., the STA test titer $< 1:160$ and 2ME agglutination $< 1:80$, were present [12]. The exclusion criteria were pregnancy, age < 18 years, and other chronic infectious or immunodeficiency diseases. Informed consent was obtained from all study participants.

2.2. Determination of Cytokine Levels. Blood samples were collected in an ethylenediamine tetraacetic acid- (EDTA-) containing tube. One blood sample (5 mL) was obtained from all patients before and after treatment. The blood samples were centrifuged at 400 g for 35 minutes, and collected sera were stored at −80°C for further analysis. IL-28A and IL-29 levels in all serum samples were measured using a commercial enzyme-linked immunosorbent assay (ELISA) kit (eBioscience Inc., San Diego, CA, USA) according to the manufacturer's guidelines.

2.3. Statistical Analysis. All data are expressed as the mean ± standard deviation (SD). Statistical analysis was performed using the SPSS software version 16.0 (IBM, Chicago, IL, USA). The difference between groups was analyzed using the paired *t*-test, and the relationship between variables was evaluated using Spearman's rank correlation test. A *p* value of ≤ 0.05 was defined as statistically significant.

3. Results

Of 33 included patients, 22 (66.6%) were males, and 11 (33.4%) were females. The range of patients' age was 49.21 ± 17.70 years.

Serum levels of IL-28A and IL-29 in the study groups (before and after the treatment) are illustrated in Figure 1. Serum IL-28A levels were 48.73 ± 27.72 pg/mL and 38.79 ± 22.66 pg/mL pre- and posttreatment, respectively ($p = 0.038$) (Figure 1). Also, IL-29 serum levels were 56.45 ± 30.32 pg/mL and 40.15 ± 20.30 pg/mL pre- and posttreatment, respectively ($p = 0.026$) (Figure 1). Also, it is noteworthy that IL-29 levels both before and after treatment were more than IL-28A levels.

4. Discussion

In this case-control study, the IL-28A and IL-29 levels of 33 patients with confirmed acute brucellosis were measured both pre- and posttreatment. It was observed that the levels of these biomarkers were significantly decreased after the eradication of the disease.

Several studies revealed the crucial role of type III IFNs for resistance to viral infections, principally by induction of the antiviral state. Furthermore, some studies revealed that type III IFNs play a significant role in inhibiting virus replication by mediating and expressing interferon-regulated genes (IRGs) [13]. Subsequent studies have also shown an inhibiting role of interferon in replicating Zika virus (ZIKV), influenza A and B viruses, coronavirus, and respiratory syncytial virus (RSV) [14, 15]. Ank et al. concluded that interferon-lambda plays a crucial role in the innate immune response through activating the macrophages and dendritic cells against human herpesvirus type 1 (HHV-1) [16]. Another study demonstrated dengue virus (DENV) replication inhibition through interferon-regulating gene expression [17].

The role of IL-29 in bacterial and parasitic infections and its increased expression in these diseases have been proven before. Also, the critical role of interferon-lambda has been highlighted in the acquired immune response in previous studies. This cytokine initiates the received immune response through its effect on antigen-presenting cells. Moreover, type III IFNs suppress the immune response through regulatory T cells (Tregs), promoting the acquired immune response [18]. As shown in previous studies, the human immune system's defense against brucellosis depends on cellular immunity, which mainly affects antigen-presenting cells (APC), such as macrophages, dendritic cells, and $CD4^+$ and $CD8^+$ T cells. Other defense cells, such as natural killer (NK) cells and other T lymphocytes, also play a crucial role in cellular immunity against *Brucella* [19]. As a result, the Th_1-dependent immune response is dominant at the onset of *Brucella* infection and is critical in eradicating the disease. Therefore, this issue fully justifies the current study results, particularly the higher levels of IL-29 in patients with acute brucellosis before treatment than after receiving the standard treatment [19]. Figure 2 illustrates a summary of the immune reactions of the host's body and the vital role of different cytokines, such as IFN-γ, TNF-α, and IFN-λ, in the eradication of *Brucella* spp.

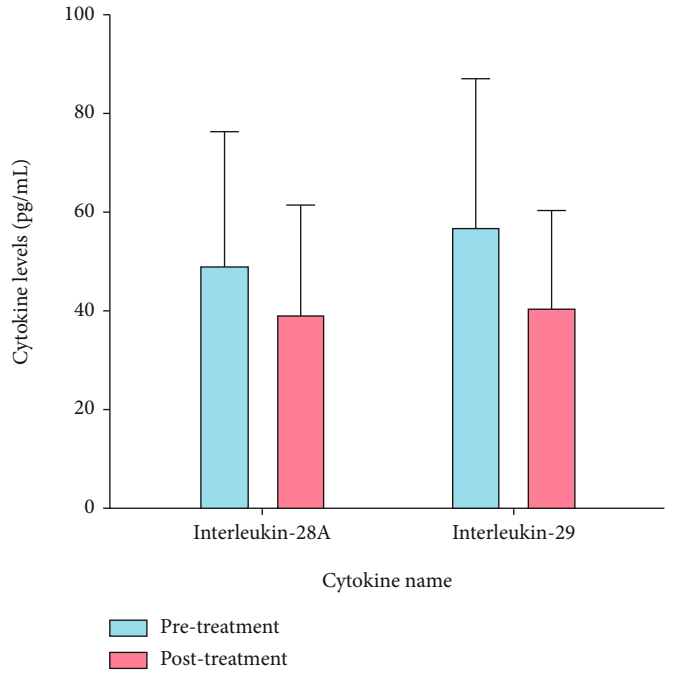

FIGURE 1: Serum levels of interleukin-28A and interleukin-29 among patients with brucellosis before and after treatment. All data are presented as the mean ± SD. ∗ indicates $p < 0.05$.

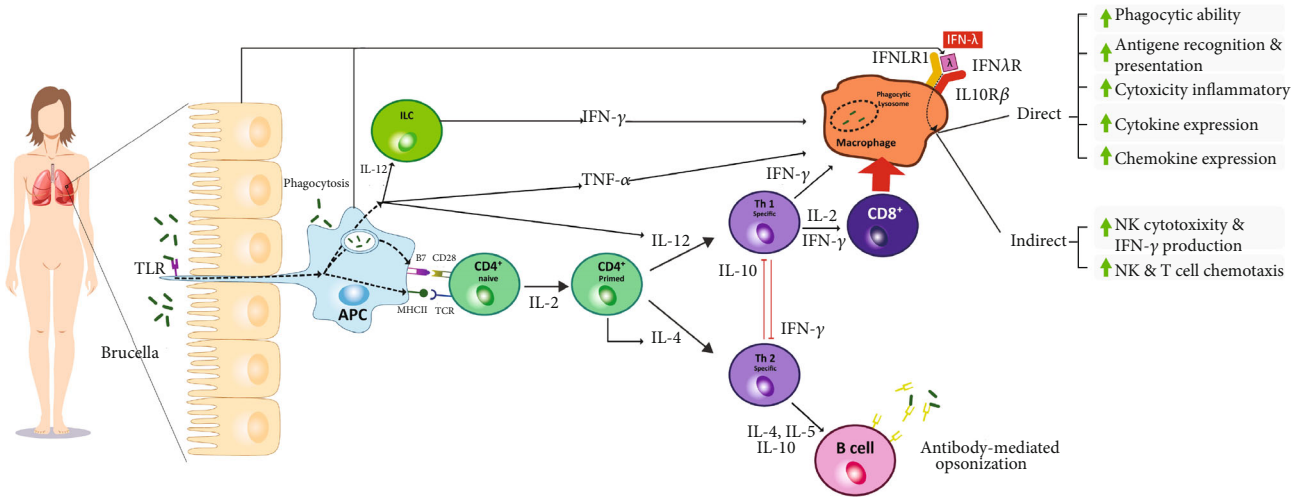

FIGURE 2: Interactions of *Brucella* spp. with the immune system. After the activation of antigen-presenting cells (APCs) with *Brucella* antigen via the Toll-like receptor (TLR) signaling pathway, a cascade of events leads to the priming of $CD4^+$ T cells to helper T cells type 1 (Th_1) and type 2 (Th_2). Th_1 cells secrete various cytokines, such as TNF-α and IFN-γ, which activate and enhance the anti-Brucella mechanisms of macrophages and activate $CD8^+$ T cells, which boost the immune responses of macrophages even further. Moreover, APCs can trigger Th_2 activation, which switches on B lymphocytes and the humoral immunity, facilitating the opsonization and faster eradication of the pathogen from the host's body. It is noteworthy that Th_1 and Th_2 cells can inhibit either pathway via secreting cytokines, such as IFN-γ and IL-10, respectively. Furthermore, macrophages can be stimulated by secreting another cytokine called type III interferons or interferon-λ by APCs or epithelial cells. These activated macrophages exert their immunomodulatory effects through two different pathways: direct and indirect. In the direct pathway, chemokine and inflammatory cytokine expression, antigen recognition and presentation, and macrophages' cytotoxicity are elevated. Through the indirect pathway, these cells can enhance natural killer (NK) and T cell chemotaxis and NK cell cytotoxicity and elevate the production and release rate of IFN-γ, which in turn, via activating the Th_1 pathway, helps better and faster eradication of *Brucella spp.* Abbreviations: TLR: Toll-like receptor; ILC: innate lymphocyte cells; IL-12: interleukin-12; APC: antigen-presenting cells; B7: cluster of differentiation 80/86; MHC II: major histocompatibility complex type 2; CD28: cluster of differentiation 28; TCR: T cell receptor; IFN-γ: interferon-gamma; TNF-α: tumor necrosis factor-alpha; IL-4: interleukin-4; Th_1: helper T cell type 1; Th_2: helper T cell type 2; IL-2: interleukin-2; IL-10: interleukin-10; IL-5: interleukin-5; IFN-λ: interferon-λ; IFNLR1: interferon-lambda receptor 1; IL10Rβ: interleukin-10 receptor beta; IFNλR: interferon-lambda receptor; NK cell: natural killer cell.

The present study showed a significant decrease in IL-28-A and IL-29 levels after treatment with the standard antibiotic regimen, i.e., gentamicin 5 mg/kg/day IV for seven days plus doxycycline 100 mg tablet BID for 45 days. Regarding the IL-28A levels, it seems that cross-linking of IL-28A with type I interferons (IFNs) and subsequent innate and acquired immune responses against brucellosis may be the cause. For IL-29, this reduction can be attributed to its vital role in acquired immunity, eradicating brucellosis using antibiotic treatment, and reduced inflammation [19]. IFNs initiate an innate immune response after contact with pathogens. The immune response and immune mediators and the subsequent inflammation are expected to decline following the control and eradication of infection. It is important to note that some standard diagnostic tests, such as STA, Coombs Wright, and 2ME, may remain positive even at high titers for up to two years after treatment [20, 21]. Thus, such tests' application was not justified for follow-up patients. Practically, most patients are generally followed up with their symptoms [22].

The current study results revealed a significant reduction in serum IL-28A and IL-29 levels after treatment, making these biomarkers a valuable indicator for monitoring the patients. While the effects of IL-28A on the acquired immunity are not significant, its functions are chiefly exerted via innate immunity, with no effect on increasing or decreasing immunoglobulins (Ig) [22]. On the other hand, some previous studies have shown a positive correlation of IL-29 with serological tests that indicate its impact on acquired immunity and Ig production. However, it is essential to note that cellular immunity plays a more crucial role in eliminating brucellosis [22].

The primary limitation of this was our small sample size. It is recommended to reperform such studies in a larger sample size. Also, another limitation of the current study was the sole evaluation of interferon-lambda. It is suggested that future researchers assess the levels of other vital cytokines, such as interferon-alpha (IFN-α) and interferon-gamma (IFN-γ), tumor necrosis factor-alpha (TNF-α), transforming growth factor-beta (TGF-β), interleukin-2 (IL-2), and interleukin-12 (IL-12). Furthermore, performing molecular analysis, i.e., PCR test, would be of interest. Moreover, future studies with a prolonged follow-up assessing these IFNs in different periods could help confirm the role of IFN-γ as a reliable biomarker in chronic brucellosis.

5. Conclusion

This study's findings confirmed previous studies on bacterial infections and validated the pivotal role of IFN-λ during acute brucellosis via strengthening innate and promoting acquired immunities. Therefore, the significant reduction in serum levels of IL-28A and IL-29 in patients with brucellosis after a standard treatment regimen may promise the emergence of valuable biomarkers in patient follow-up.

Data Availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Ethical Approval

This study protocol was approved by the ethics committee of Babol University of Medical Sciences (IR.MUBABOL.HRI.REC.1397.212).

Conflicts of Interest

All authors declare no conflict of interest.

Authors' Contributions

MS designed the research study, collected data, and analyzed data. OGK collected data and helped with manuscript writing. MMA collected data and helped with manuscript writing. MSHZ collected data and helped with manuscript writing. AH worked on visualization and software. MB collected data and contributed substantial revisions to the content of all sections of the manuscript. SE designed the research study and contributed substantial revisions to all sections of the manuscript.

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References

[1] M. Mohammadnia-Afrouzi and S. Ebrahimpour, "Assessment of TGF-β and IL10 levels in human brucellosis," *Current Issues in Pharmacy and Medical Sciences*, vol. 31, no. 1, pp. 22–24, 2018.

[2] Y. Wang, C. Xu, S. Zhang, Z. Wang, Y. Zhu, and J. Yuan, "Temporal trends analysis of human brucellosis incidence in mainland China from 2004 to 2018," *Scientific Reports*, vol. 8, no. 1, p. 15901, 2018.

[3] G. Pappas, N. Akritidis, M. Bosilkovski, and E. Tsianos, "Medical progress brucellosis," *The New England journal of medicine*, vol. 352, no. 22, pp. 2325–2336, 2005.

[4] M. R. Hasanjani Roushan and S. Ebrahimpour, "Human brucellosis: an overview," *Caspian journal of internal medicine*, vol. 6, no. 1, pp. 46-47, 2015, https://pubmed.ncbi.nlm.nih.gov/26221498.

[5] M. R. Hasanjani Roushan, M. Bayani, S. Soleimaniamiri, M. Mohammadnia-Afrouzi, H. R. Nouri, and S. Ebrahimpour, "Evaluation of CD4+CD25+ FoxP3+ regulatory T cells during treatment of patients with brucellosis," *Journal of Biological Regulators and Homeostatic Agents*, vol. 30, no. 3, pp. 675–682, 2016, http://www.ncbi.nlm.nih.gov/pubmed/27655484.

[6] G. Xu, P. Zhang, R. Dang et al., "Dynamic changes of Th1 cytokines and the clinical significance of the IFN-γ/TNF- ratio in acute brucellosis," *Mediators of Inflammation*, vol. 2019, 10 pages, 2019, https://www.hindawi.com/journals/mi/2019/5869257/.

[7] S. D. Perkins, S. J. Smither, and H. S. Atkins, "Towards a Brucella vaccine for humans," *FEMS Microbiology Reviews*, vol. 34, no. 3, pp. 379–394, 2010.

[8] H. A. N. Li-hong, L. I. U. Ri-hong, and L. I. U. Z.-g. CB, "High level IFN-γ may be one of the characteristics of chronic brucellosis," *Chinese Journal of Zoonoses*, vol. 33, no. 8, pp. 716–719, 2017, http://www.rsghb.cn/EN/abstract/abstract23948.shtml.

[9] T. S. Cohen and D. Parker, "Microbial pathogenesis and type III interferons," *Cytokine and Growth Factor Reviews*, vol. 29, pp. 45–51, 2016.

[10] H. E. Rich, C. C. McCourt, W. Q. Zheng et al., "Interferon lambda inhibits bacterial uptake during influenza superinfection," in *Infection and Immunity*, A. J. Bäumler, Ed., vol. 87, no. 5, 2019.

[11] M. R. Hasanjani Roushan, M. Mohraz, M. Hajiahmadi, A. Ramzani, and A. A. Valayati, "Efficacy of gentamicin plus doxycycline versus streptomycin plus doxycycline in the treatment of brucellosis in humans," *Clinical Infectious Diseases*, vol. 42, no. 8, pp. 1075–1080, 2006.

[12] M. R. Hasanjani Roushan and S. Ebrahimpour, "Human brucellosis: an overview," *International Journal of Infectious Diseases*, vol. 6, no. 1, pp. 46-47, 2015, http://caspjim.com/article-1-447-en.html.

[13] K. Pervolaraki, S. Rastgou Talemi, D. Albrecht et al., "Differential induction of interferon stimulated genes between type I and type III interferons is independent of interferon receptor abundance," *PLoS Pathogens*, vol. 14, no. 11, article e1007420, 2018.

[14] S. V. Kotenko, A. Rivera, D. Parker, and J. E. Durbin, "Type III IFNs: beyond antiviral protection," *Seminars in Immunology*, vol. 43, article 101303, 2019.

[15] Y. J. Jeon, J. H. Lim, S. An et al., "Type III interferons are critical host factors that determine susceptibility to influenza A viral infection in allergic nasal mucosa," *Clinical and Experimental Allergy*, vol. 48, no. 3, pp. 253–265, 2018.

[16] N. Ank, H. West, and S. R. Paludan, "IFN-λ: novel antiviral cytokines," *Journal of Interferon and Cytokine Research*, vol. 26, no. 6, pp. 373–379, 2006.

[17] H. K. Palma-Ocampo, J. C. Flores-Alonso, V. Vallejo-Ruiz et al., "Interferon lambda inhibits dengue virus replication in epithelial cells," *Virology Journal*, vol. 12, no. 1, p. 150, 2015.

[18] I. Misumi and J. K. Whitmire, "IFN-λ exerts opposing effects on T cell responses depending on the chronicity of the virus infection," *The Journal of Immunology*, vol. 192, no. 8, pp. 3596–3606, 2014.

[19] K. Witte, E. Witte, R. Sabat, and K. Wolk, "IL-28A, IL-28B, and IL-29: promising cytokines with type I interferon-like properties," *Cytokine and Growth Factor Reviews*, vol. 21, no. 4, pp. 237–251, 2010.

[20] M. R. H. Roushan, M. J. S. Amiri, A. Laly, A. Mostafazadeh, and A. Bijani, "Follow-up standard agglutination and 2-mercaptoethanol tests in 175 clinically cured cases of human brucellosis," *International Journal of Infectious Diseases*, vol. 14, no. 3, pp. e250–e253, 2010.

[21] G. F. Araj, "Update on laboratory diagnosis of human brucellosis," *International Journal of Antimicrobial Agents*, vol. 36, Supplement 1, pp. S12–S17, 2010.

[22] A. Dolganiuc, K. Kodys, C. Marshall et al., "Type III interferons, IL-28 and IL-29, are increased in chronic HCV infection and induce myeloid dendritic cell-mediated FoxP3+ regulatory T cells," *PLoS one*, vol. 10, no. 10, article e44915, 2012.

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Research Article

Effect of Lipopolysaccharide and TNFα on Neuronal Ascorbic Acid Uptake

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Vitamin C (ascorbic acid: AA) uptake in neurons occurs via the sodium-dependent vitamin C transporter-2 (SVCT2), which is highly expressed in the central nervous system (CNS). During chronic neuroinflammation or infection, CNS levels of lipopolysaccharide (LPS) and LPS-induced tumor necrosis factor-α (TNFα) are increased. Elevated levels of LPS and TNFα have been associated with neurodegenerative diseases together with reduced levels of AA. However, little is known about the impacts of LPS and TNFα on neuronal AA uptake. The objective of this study was to examine the effect of LPS and TNFα on SVCT2 expression and function using *in vitro* and *in vivo* approaches. Treatment of SH-SY5Y cells with either LPS or TNFα inhibited AA uptake. This reduced uptake was associated with a significant decrease in SVCT2 protein and mRNA levels. *In vivo* exposure to LPS or TNFα also decreased SVCT2 protein and mRNA levels in mouse brains. Both LPS and TNFα decreased *SLC23A2* promoter activity. Further, the inhibitory effect of LPS on a minimal *SLC23A2* promoter was attenuated when either the binding site for the transcription factor Sp1 was mutated or cells were treated with the NF-κB inhibitor, celastrol. We conclude that inflammatory signals suppress AA uptake by impairing *SLC23A2* transcription through opposing regulation of Sp1 and NF-κB factors.

1. Introduction

Vitamin C (ascorbic acid: AA) is an essential micronutrient for cellular function, growth, and development, serving as a cofactor for an array of biological reactions and as a pleiotropic intracellular antioxidant [1, 2]. AA also serves as a first-line antioxidant defense to neutralize reactive oxygen species (ROS) by promoting the regeneration of endogenous antioxidants [3]. Brain tissue is susceptible to free radical damage and oxidative stress, since the brain is the most metabolically active organ in the body, and for this reason, the brain contains the highest concentration of vitamin C [3]. Accumulation of vitamin C in the brain cells occurs by a two-step mechanism, first by absorption across the choroid plexus and second by concentration into neurons and glia [4, 5]. The human sodium-dependent vitamin C transporter-2 (hSVCT2, the product of the *SLC23A2* gene) controls these steps [4, 5]; knockout of murine SVCT2 results in undetectable levels of AA in the mouse brain [6].

Deficiencies of vitamin C could play a major role in brain dysfunction and neurodegeneration. Plasma vitamin C levels are found to be significantly lower in patients with neurodegenerative diseases [3, 7–10]. For instance, in Alzheimer's disease (AD), reduced vitamin C levels may accelerate amyloid-beta (Aβ) accumulation and cognitive impairment [3, 7, 8, 11]. Reciprocally, restoration of vitamin C levels

and maintaining its homeostasis appear to safeguard against cognitive decline and the progression of AD neuropathology [12]. Therefore, studies aimed at understanding the underlying molecular mechanisms that control vitamin C homeostasis in the CNS may prove essential for developing strategies to counteract conditions of disease-enhanced oxidative stress through optimization of vitamin C homeostasis.

Neuroinflammation plays a pivotal role in the pathophysiology of many neurodegenerative diseases [13–16]. Chronic neuroinflammation and systemic bacterial infection lead to increased levels of proinflammatory cytokines like TNFα, IL-6, and IL-1β [17–20]. Lipopolysaccharide (LPS) is a cell wall-derived endotoxin of most gram-negative bacteria that is capable of inducing a strong neuroinflammatory response [21, 22]. Recent studies have shown that LPS and bacterial components are associated with plaques in postmortem AD brains [21, 23]. In addition, LPS is present in a septic patient's blood plasma [24–26], where it is assumed to play an important role in systemic inflammatory response syndrome, and also, some evidence indicates that sepsis is associated with lower blood vitamin C levels [27–29]. LPS is known to affect the function and expression of certain neuronal transporters [30–32]; however, the effect of LPS and subsequently induced TNFα on AA uptake and SVCT2 expression has been overlooked. Therefore, we studied the impact of LPS and TNFα exposure on neuronal AA uptake using both *in vitro* (SH-SY5Y cells) and *in vivo* (mouse) models.

2. Materials and Methods

2.1. Materials. The ^{14}C-AA (specific activity 2.8 mCi/mmol; radiochemical purity > 98%) used in vitamin uptake analysis was acquired from PerkinElmer, Inc. (Boston, MA). LPS (*E. coli* 0111:B4) was purchased from Millipore Sigma (St. Louis, MO). Human TNFα was bought from Invitrogen (Carlsbad, CA), and murine TNFα was from PeproTech, Inc. (Rocky Hill, NJ). Antibodies were obtained from the following sources: anti-β-actin antibodies (ThermoFisher Scientific, Huntington Beach, CA), anti-NF-κB p65 and anti-IKK$\alpha\beta$ antibodies (Abcam, Cambridge, MA), and anti-laminin antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). LI-COR (Lincoln, NE) IRDyes 800CW and 680LT goat anti-mouse and anti-rabbit secondary antibodies were used for western blot. Celastrol was ordered from InvivoGen, Inc. (San Diego, CA). Services provided by Integrated DNA Technologies (San Diego, CA) were used to synthesize oligonucleotide primers (Table 1). All other molecular biology-grade chemicals, reagents, and materials used in this study were from commercial sources.

2.2. Culturing of SH-SY5Y Cells and AA Uptake Analysis. Human-derived neuroblastoma SH-SY5Y cells (ATCC, Manassas, VA) were given DMEM-F12 medium (ATCC) (with 20% fetal bovine serum (FBS) and penicillin-streptomycin added) and cultured in a temperature-controlled CO_2 incubator at 37°C. In assays treating SH-SY5Y cells with LPS or TNFα, overnight serum starvation was followed by exposure either with LPS (20 μg/ml) or TNFα (20 ng/ml) or LPS plus celastrol (100 nM) in DMEM-F12 (with 0.5% FBS and no antibiotics added). SH-SY5Y cells were subjected to pretreatment with celastrol (100 nM) for 5 h before LPS treatment. After 48 h of LPS or TNFα or LPS plus celastrol treatment, ^{14}C-AA uptake was performed *in vitro* [33, 34]. Briefly, SH-SY5Y cells were incubated (30 min) with ^{14}C-AA (0.1 μCi) in Krebs-Ringer (KR) buffer at 37°C in a water bath, and then, sample lysates were prepared for radioactivity determination using a liquid scintillation counter [33, 34].

2.3. Animal Studies. For *in vivo* experiments, adult male C57BL/6 mice aged 8-12 weeks (Jackson Laboratory, Bar Harbor, ME) were administered either a single injection of LPS (5 mg/kg body weight; 100 μl of PBS) [33, 35] or TNFα (15 μg/mouse; 100 μl of PBS) [34, 36] or vehicle alone (100 μl of PBS) intraperitoneally (IP). After 72 h, mouse brains were removed immediately after euthanization and protein and RNA extracted. The animal protocol gained approval from the Institutional Animal Care and Use Committee (IACUC), Veteran Administration Medical Center, Long Beach, CA, and University of California, Irvine, CA.

2.4. Real-Time PCR (RT-qPCR) Analysis. Total RNA was prepared from SH-SY5Y cells and mouse brain exposed to either LPS or TNFα or LPS plus celastrol and their respective controls using the TRIzol reagent (Life Technologies). One microgram of total RNA sample was reverse-transcribed (RT) to cDNA using the i-Script cDNA synthesis kit (Bio-Rad, CA). RT-qPCR analysis was then performed using the cDNA and gene-specific primers (Table 1) in a CFX96 Real-Time iCycler (Bio-Rad) [33, 34]. Simultaneously amplified β-actin acted as a base for comparison to normalize the relative expression of different mRNAs, which were quantified using a relative relationship method (Bio-Rad) [33, 34].

2.5. Heterogeneous Nuclear RNA (hnRNA) Analysis. Table 1 shows the gene-specific hnRNA primers for *Slc23a2* that were utilized in RT-qPCR analysis of total RNA prepared using LPS- or TNFα-treated mouse brain samples and parallel controls [37]. DNase I (Invitrogen) was added to the RNA samples for digestion before they were reverse-transcribed using i-Script cDNA synthesis kit reagents (Bio-Rad). β-Actin was again used to normalize RT-qPCR data, which was calculated as described above.

2.6. Transfection and Promoter Assays. SH-SY5Y cells were cultured on twelve-well plates (Corning) and cotransfected using Lipofectamine 2000 (3 μl/well; Invitrogen) with full-length, minimal, or mutant *SLC23A2* promoter constructs (3 μg plasmid DNA/well) [38, 39] and *Renilla* luciferase-thymidine kinase (pRL-TK, 100 ng/well; Promega). Cells were left to incubate for 24 h before being treated with either LPS or TNFα or LPS plus celastrol for an additional 48 h. Then, the samples were processed following the Promega Dual-Luciferase Reporter Assay System. In short, each sample was lysed using passive lysis buffer (Promega), and a luminometer detected both the *firefly* and *Renilla* luciferase activities sequentially [38, 39].

Table 1: Oligonucleotide primer combinations used to amplify coding region of the respective genes by RT-qPCR.

Gene name	Forward and reverse primers (5′-3′)
Real-time PCR primers	
hSVCT2	TCTTTGTGCTTGGATTTTCGAT; ACGTTCAACACTTGATCGATTC
hRFVT2	CCCTGGTCCAGACCCTA; ACACCCATGGCCAGGA
hSp1	CCATACCCCTTAACCCCG; GAATTTTCACTAATGTTTCCCACC
hβ-actin	CATCCTGCGTCTGGACCT; TAATGTCACGCACGATTTCC
mSVCT2	AACGGCAGAGCTGTTGGA; GAAAATCGTCAGCATGGCAA
mNLRP3	ATTACCCGCCCGAGAAAGG; TCGCAGCAAAGATCCACACAG
mTNFα	CATCTTCTCAAAATTCGAGTGACAA; TGGGAGTAGACAAGGTACAACCC
mβ-actin	ATCCTCTTCCTCCCTGGA; TTCATGGATGCCACAGGA
hnRNA primers	
mSVCT2	ACTCTTGTCCATGGCTCTGG; GGGCAAAATCTTCGTTGGGT
mβ-actin	AGATGACCCAGGTCAGTATC; GAGCAGAAACTGCAAAGAT

2.7. Western Blotting. SH-SY5Y cells and mouse brain total protein were prepared by homogenization in RIPA (Radioimmunoprecipitation Assay) Buffer (Sigma) with 1X protease inhibitor cocktail (Roche, Nutley, NJ). The nuclear and cytosolic fractions from LPS- or LPS plus celastrol-treated SH-SY5Y cells were obtained using the NE-PER nuclear and cytoplasmic extraction kit (ThermoFisher Scientific). Total protein (60 μg) was separated using 4-12% NuPAGE Bis-Tris protein gels (Invitrogen) and transferred to a PVDF membrane. After protein transfer, the membrane was blocked for 10 min at room temperature in LI-COR Odyssey Blocking Buffer and then probed with previously characterized primary SVCT2 antibodies (1 : 500 dilution) [40], anti-IKK$\alpha\beta$ antibodies (1 : 1000 dilution; Abcam), anti-NF-$\kappa\beta$ p65 antibodies (1 : 1000 dilution; Abcam), anti-laminin antibodies (1 : 300 dilution; Santa Cruz Biotechnology), and anti-β-actin mouse monoclonal antibody (1 : 3000 dilution; ThermoFisher Scientific) used. The respective secondary antibodies (anti-rabbit IRDye-800 and anti-mouse IRDye-680, LI-COR Biosciences) were used in 1 : 30,000 dilutions [33, 34, 40]. Odyssey Infrared imaging system (LI-COR Biosciences) software was used to quantify the densitometry of specific band signal intensities normalized against β-actin.

2.8. Statistical Analyses. Carrier-mediated AA uptake analysis data from these investigations are presented as the means ± SE of at least 3 to 4 separate investigations with multiple determinations and represent a percentage relative to simultaneously performed untreated controls. RT-qPCR, western blot, and promoter assays were determined from at least 3 different batches of cells or 3 pairs of mouse samples. Student's t-test with $P < 0.05$ set as statistically significant was chosen to perform statistical analysis.

3. Results

3.1. Effect of LPS on hSVCT2 Function In Vitro. Recent studies have shown detectable levels of LPS in the AD brain [21, 23]. LPS is a potent inflammatory stimulator, which can affect the neuronal transport of many different substrates [30–32]. To assess the effect of LPS on AA uptake, we measured hSVCT2 mRNA levels by RT-qPCR after exposure of cells to various concentrations of LPS (10-50 μg/ml for 48 h). Data showed a concentration-dependent decrease in hSVCT2 mRNA expression relative to untreated control SH-SY5Y cells (Figure 1). To address the specificity of the LPS effect, we also determined mRNA levels of the brain-specific human riboflavin transporter-2 (hRFVT2) [41]. There was no significant change in hRFVT2 mRNA in SH-SY5Y cells treated with LPS (20 μg) compared to untreated cells (100 ± 11 and 117 ± 20 for control and LPS treatment, respectively).

An LPS treatment paradigm of 20 μg/ml for 48 h also caused a significant ($P < 0.001$) inhibition of AA uptake (Figure 2(a)), coupled with a significant ($P < 0.05$) decrease in hSVCT2 protein expression (Figure 2(b)). The action of LPS was then interrogated at the level of the *SLC23A2* promoter, by monitoring promoter activity of a luciferase reporter construct (pGL3-*SLC23A2*). This reporter construct was transiently transfected into SH-SY5Y cells, and then, cells were treated (24 h after transfection) with LPS (20 μg/ml for 48 h) before *firefly* luciferase activity was determined. LPS treatment caused significantly ($P < 0.01$) reduced *SLC23A2* promoter activity when compared to untreated SH-SY5Y cells (Figure 2(c)). These data suggest that LPS decreases hSVCT2 function via transcriptional regulation.

3.2. Effect of LPS on SVCT2 Function In Vivo. Next, we examined whether similar effects occurred *in vivo*. LPS (5 mg/kg body weight; single dose [33, 35]) was administered intraperitoneally to wild-type (WT) mice, and responses were compared with vehicle (PBS)-injected controls. To monitor inflammation, the expression of nucleotide-binding, oligomerization domain- (NOD-) like receptor family, pyrin domain containing 3 (NLRP3) was examined. NLRP3 mRNA levels were found to be significantly ($P < 0.05$) increased in LPS-administered brain samples 72 h after injection (Figure 2(d)). These data demonstrate activation of an inflammatory marker following LPS administration in mouse brain samples. As expected, TNFα mRNA expression was also significantly ($P < 0.0001$) increased in LPS-administered mouse brain compared to controls (100 ± 19

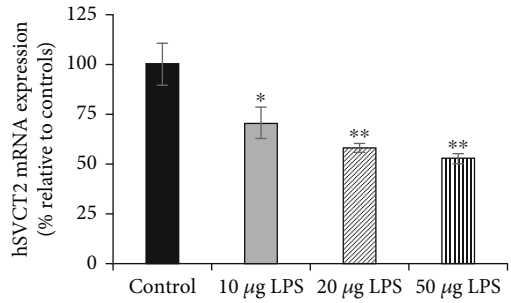

Figure 1: Effect of different concentrations of LPS on hSVCT2 mRNA expression in SH-SY5Y cells. SH-SY5Y cells were serum-deprived overnight and exposed to different concentrations of LPS (10, 20, and 50 μg/ml). After 48 h, total RNA was prepared to carry out RT-qPCR. Data are means ± SE of at least 6 separate determinations utilizing multiple batches of SH-SY5Y cells. $^{**}P < 0.01$; $^{*}P < 0.05$.

and 351 ± 33 for control and LPS-administered mouse brains, respectively). Levels of mSVCT2 protein, mRNA, and heterogeneous nuclear RNA (hnRNA) were then determined in control and LPS-injected animals. Results showed that the expression levels of mSVCT2 protein, mRNA, and hnRNA were all markedly reduced in LPS-injected mouse brain samples versus controls (Figures 2(e)–2(g)). The latter represents the initial products of gene transcription, reflecting the rate of transcription of a given gene [37]. Collectively, these findings suggest that LPS also decreases the mSVCT2 functional expression *in vivo*, and this occurs via a transcriptional mechanism.

3.3. Effect of TNFα on SVCT2 Function. Elevated levels of proinflammatory cytokines such as TNFα, IL-6, and IL-1β in the brain and blood are linked to neuroinflammation and systemic bacterial infection [17–20]. TNFα is upregulated in AD brain samples and in the blood of patients infected with bacteria [18, 20]. Still, there is little evidence to describe the effect of TNFα on SVCT2 expression and function in neuronal systems. Treatment of SH-SY5Y cells with TNFα (20 ng/ml) significantly ($P < 0.001$) inhibited AA uptake (Figure 3(a)). This inhibition in uptake was again accompanied by marked decreases in the hSVCT2 protein (Figure 3(b)) and mRNA (Figure 3(c)) expression levels, as well as a significant reduction in *SLC23A2* promoter activity (Figure 3(d)). To assess responses to TNFα *in vivo*, mice were injected intraperitoneally with TNFα (15 μg/mouse) [34, 36], followed by evaluation of mSVCT2 protein, mRNA, and hnRNA expression levels in mouse brain after 72 h. Results showed a significant ($P < 0.05$ for all) decrease in mSVCT2 protein, mRNA, and hnRNA in TNFα-administrated mouse brain samples compared to control mouse brain samples (Figures 3(e)–3(g)). Together, these results suggest that the TNFα-mediated decrease in SVCT2 functional expression also occurs via transcriptional mechanism(s).

3.4. Role of the Transcription Factor Sp1 in the Effect of LPS on Neuronal AA Uptake. As shown above, the full-length *SLC23A2* promoter activity is inhibited by LPS in SH-SY5Y cells (Figure 2(c)). To investigate the molecular basis for this effect in greater depth, we tested whether the LPS inhibitory effect was also apparent on specific regions of the promoter. First, we assessed LPS action on a *SLC23A2* minimal promoter reporter construct (-97 bp to +102 bp; Figure 4(a)) transiently expressed in SH-SY5Y cells. The *SLC23A2* minimal (WT) promoter activity was significantly ($P < 0.001$) inhibited following LPS treatment compared with controls (Figure 4(b)). The minimal promoter region contains one Sp1-binding and two KLF-binding sites. It has been previously established that both transcription factors, Sp1 and KLF, are necessary to drive the basal transcriptional activity of the *SLC23A2* promoter [39, 42]. Therefore, we tested the role of mutations at these sites on the inhibitory LPS effect. Mutant minimal *SLC23A2* promoter constructs were transiently transfected into SH-SY5Y cells. After 24 h of transfection, cells were exposed to LPS for 48 h. Mutational ablation of either KLF-binding site (KLF1 or KLF2) had no effect on the inhibitory action of LPS (Figure 4(b)). In contrast, mutational ablation of the Sp1-binding site led to a loss of the LPS inhibitory effect on the *SLC23A2* promoter activity (Figure 4(b)). Based on this result, we examined the effect on Sp1 protein and mRNA expression in SH-SY5Y cells after exposure to LPS. LPS treatment resulted in significantly ($P < 0.05$ for protein and $P < 0.001$ for mRNA) decreased human Sp1 protein and mRNA levels compared with untreated SH-SY5Y cells (Figures 4(c) and 4(d)). These data suggest that the transcription factor Sp1 mediates the LPS-induced inhibition of neuronal AA uptake.

3.5. NF-κB Signaling Regulates the Inhibitory Effect of LPS. The NF-κB inflammatory signaling pathway is a part of the regulatory mechanism that mediates the action of LPS on gene expression [43–45]. LPS activates the NF-κB pathway in SH-SY5Y cells by driving nuclear translocation of NF-κB and promoting degradation of IKK$\alpha\beta$ in the cytoplasm (Figures 5(a) and 5(b)). Both these actions were blocked by celastrol, which can act as a NF-κB inhibitor (Figures 5(a) and 5(b)). As Sp1 and NF-κB are often involved in coordinated regulation of gene expression [46–48], we examined whether NF-κB was engaged by LPS to repress hSVCT2 expression. The addition of celastrol to inhibit NF-κB action markedly reversed the effect of LPS-induced inhibition on AA uptake (Figure 5(c)). Celastrol markedly increased the hSVCT2 protein, mRNA expression levels, and *SLC23A2* promoter activity (Figures 5(d)–5(f)). Collectively, these data support the concept that Sp1 and NF-κB signaling pathway coordinate to regulate *SLC23A2* promoter activity in neuronal cells, where NF-κB is activated (Figures 5(a) and 5(b)) and Sp1 is inhibited (Figure 4) by elevated LPS.

4. Discussion

The highest concentration of vitamin C is found in the brain, and its levels can be markedly lower in the plasma of patients with neurodegenerative disease [3, 9, 10]. Expression levels of SVCT2 are also markedly lower in human and mouse brain tissue with AD pathology (unpublished observations), which

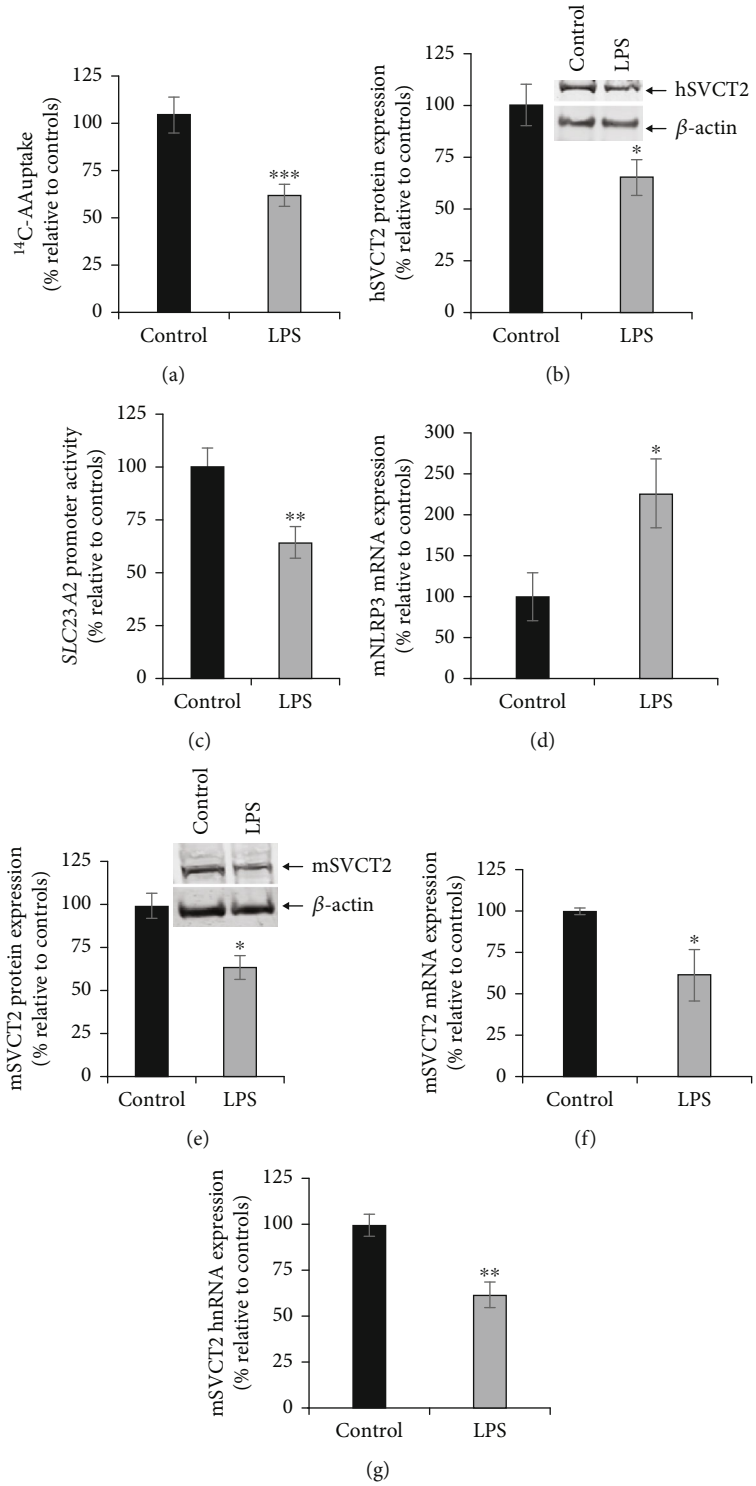

Figure 2: Effect of exposure of SH-SY5Y cells and mouse brain with LPS on different aspects of SVCT2 functional expression. (a) SH-SY5Y cells were serum-deprived overnight and exposed to LPS (20 μg/ml). After 48 h, AA uptake was determined. (b) SH-SY5Y cells were exposed to LPS for 48 h, and the protein was prepared to perform western blot analysis to determine the hSVCT2 protein expression levels. (c) *SLC23A2* full-length promoter activity was determined in LPS-treated SH-SY5Y cells. (d, f) Total RNA isolated from LPS-administered and control mouse brain were used to determine the mSVCT2 and NLRP3 mRNA expression levels by RT-qPCR. (e) Protein samples prepared from mouse brain tissue of LPS (5 mg/kg body weight; 72 h) exposed and controls were subjected to western blotting to determine mSVCT2 protein expression levels. (g) Total RNA prepared from LPS-injected and control mouse brain were subjected to RT-qPCR to determine the mSVCT2 hnRNA expression levels. Values are means ± SE of at least 3-5 independent investigations utilizing multiple batches of SH-SY5Y cells or at least 3-5 pairs of mice. $^{***}P < 0.001$; $^{**}P < 0.01$; $^{*}P < 0.05$.

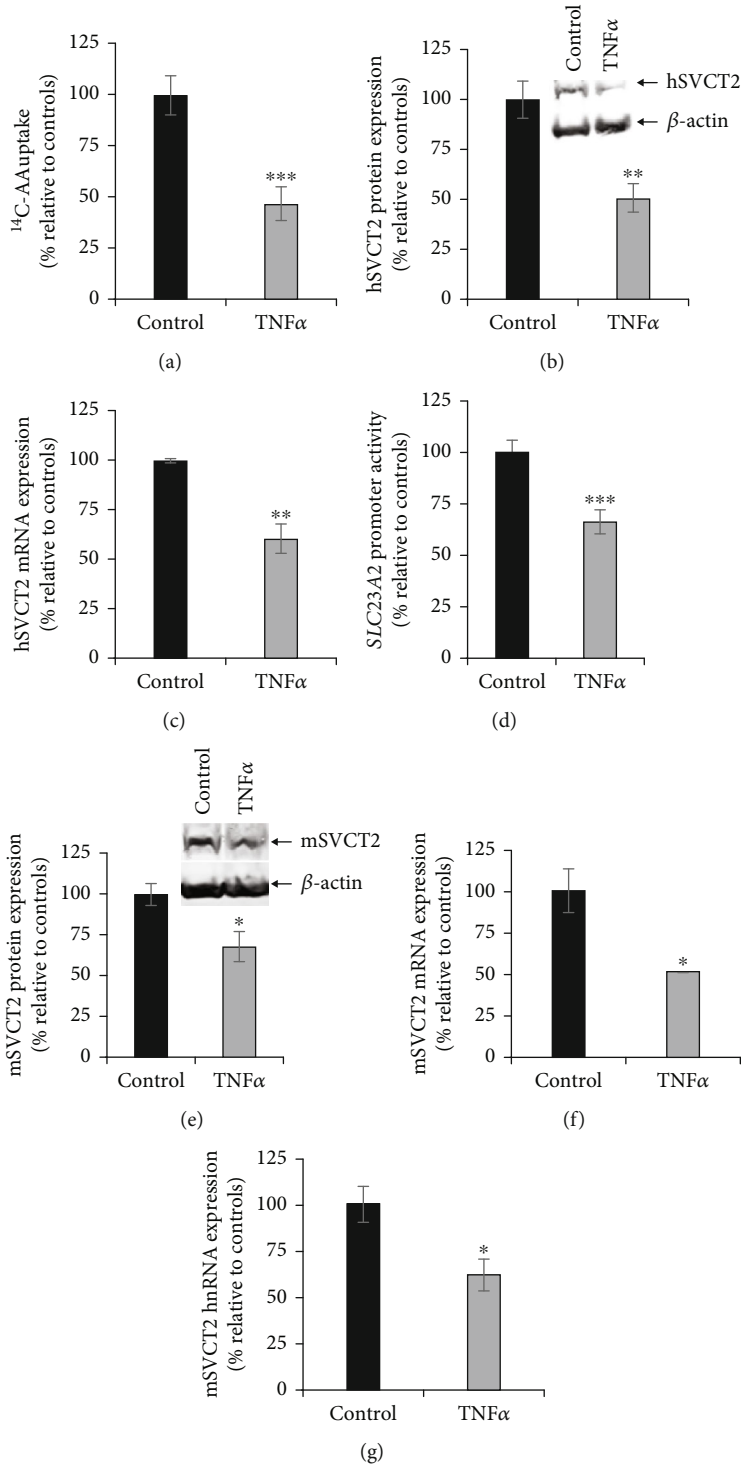

Figure 3: Effect of exposure of SH-SY5Y cells and mouse brain with TNFα on different aspects of SVCT2 functional expression. (a) SH-SY5Y cells were serum-deprived overnight and exposed to TNFα (20 ng/ml). After 48 h, AA uptake was performed. (b) SH-SY5Y cells were pretreated with TNFα for 48 h, and the total protein was prepared and used to perform western blotting to determine hSVCT2 protein expression levels. (c) Total RNA isolated from TNFα pretreated SH-SY5Y cells was utilized to determine the level of hSVCT2 mRNA expression by RT-qPCR. (d) *SLC23A2* full-length promoter activity was determined in TNFα pretreated SH-SY5Y cells. (e) Protein samples from mouse brain tissue of TNFα (15 μg/mouse; 72 h) exposed and control mice were isolated to perform western analysis to determine mSVCT2 protein expression levels. (f) Total RNA isolated from TNFα-injected and control mouse brain was used to determine the mSVCT2 mRNA expression by RT-qPCR. (g) Total RNA samples prepared from TNFα-administered and control mouse brain were subjected to determination of the mSVCT2 hnRNA expression levels by RT-qPCR. Values are means ± SE of at least 3-5 separate investigations using different batches of SH-SY5Y cells or at least 3-4 pairs of mice. $^{***}P < 0.001$; $^{**}P < 0.01$; $^{*}P < 0.05$.

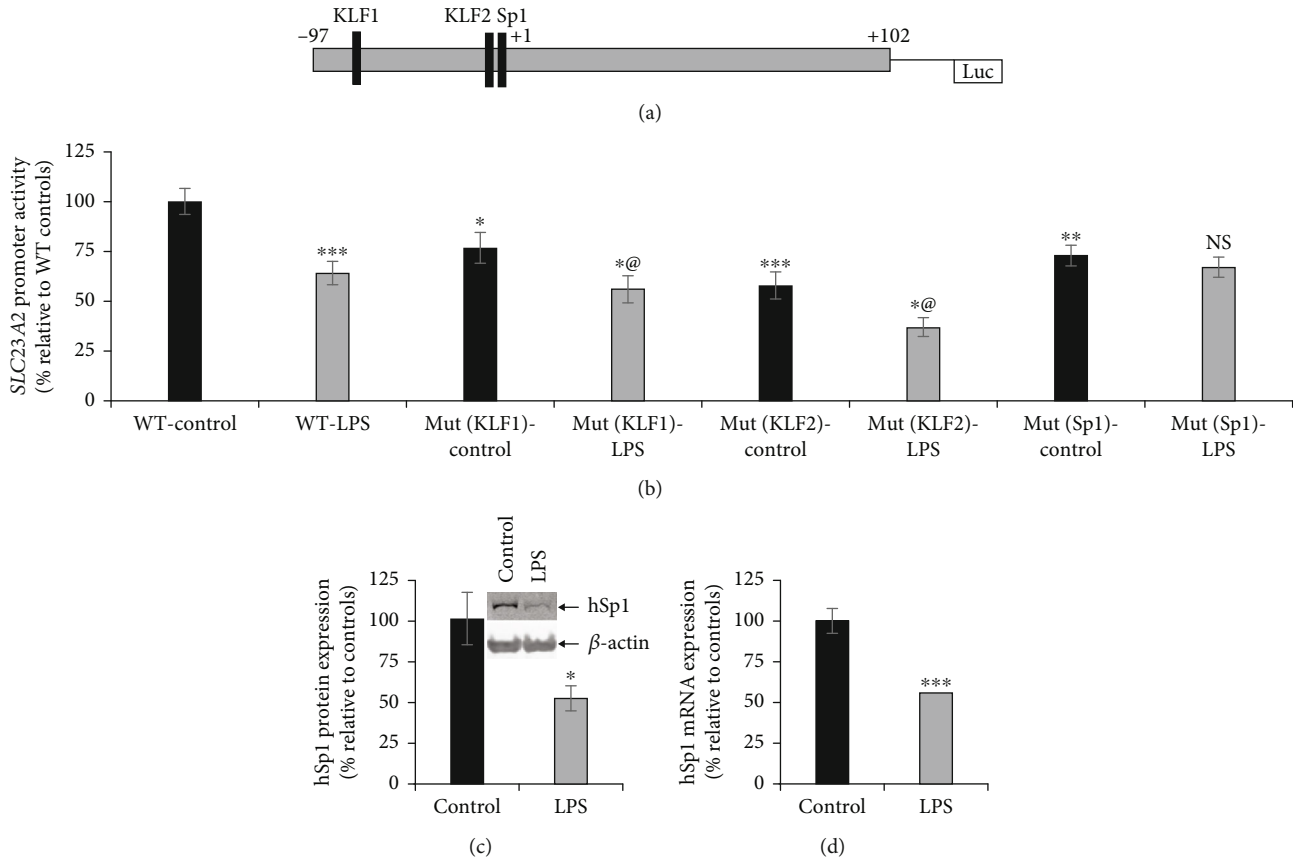

Figure 4: Effect of pretreating SH-SY5Y cells with LPS on the activity of *SLC23A2* minimal (WT) and mutant promoter constructs. (a) Schematic depiction of *SLC23A2* minimal promoter and the locations of Sp1- and KLF-binding sites. (b) *SLC23A2* minimal (WT), Sp1- and KLF-binding sites mutated promoter construct activities were determined in LPS-exposed and control SH-SY5Y cells (KLF1 designates the site 5′ of the second site named KLF2). Western blot analysis was done to determine the expression levels of Sp1 protein (c), and RT-qPCR was done to determine the level of Sp1 mRNA (d) in LPS-exposed cells. @: cells exposed to LPS treatment significantly decreased compared to KLF1 or KLF2 controls. NS: not significant. Values are means ± SE of at least 4 separate experiments. $^{***}P < 0.001$; $^{**}P < 0.01$; $^{*}P < 0.05$.

is a possible explanation for this deficit. Recent studies have shown that LPS and other bacterial products are associated with amyloid-beta (Aβ) plaques in AD brains [21, 23], suggesting that abnormal buildup of these bacterial components may be an additional factor to trigger chronic neuroinflammation during the disease course. Brain vitamin C dyshomeostasis induced by inflammation may therefore serve as a mechanism linking inflammation to exacerbated disease phenotypes. LPS and TNFα both affect neuronal gene function and expression [30–32, 49, 50]. Here, we investigated their roles in regulating AA uptake and SVCT2 expression in neuronal systems.

Both *in vitro* and *in vivo* assays suggest that the lower levels of SVCT2 functional expression observed upon LPS exposure in neuronal systems are mediated through transcriptional regulation of the *SLC23A2* gene. Substantial evidence shows that Sp1 drives the basal activity of *SLC23A2* promoter in different cellular systems [33, 39, 42] and has also been implicated in transporter regulation in inflammatory conditions [51]. In our investigation, we have used a *SLC23A2* minimal promoter construct expressed in neuronal cells [38, 39] to demonstrate that Sp1 mutation attenuated the LPS-induced decrease in *firefly* luciferase activity (Figure 4). LPS also markedly decreased the Sp1 protein and mRNA expression, signifying that LPS degrades Sp1 and thus reduces *SLC23A2* activity [52].

Sp transcription factors often interact with NF-κB signals mediated at the same DNA binding sites [46–48]. NF-κB is a pleiotropic regulator of many genes responsible for host defense, inflammatory response, and apoptosis [53–56]. The observation that celastrol reversed the inhibitory action of LPS and nuclear translocation of NF-κB implies a convergent regulation of hSVCT2 promoter activity by these dual and often dueling transcription factors. Further work will be needed to resolve the details of how these factors may exert their opposing influences on hSVCT2 promoter activity, possibly even via the very same DNA binding sites. It is worth mentioning that NF-κB is a redox-sensitive transcription factor and regulates SVCT2 mRNA expression in response to redox-state unsteadiness [57], and also, nitric oxide (NO) regulates SVCT2 expression via the NF-κB signaling pathway [58].

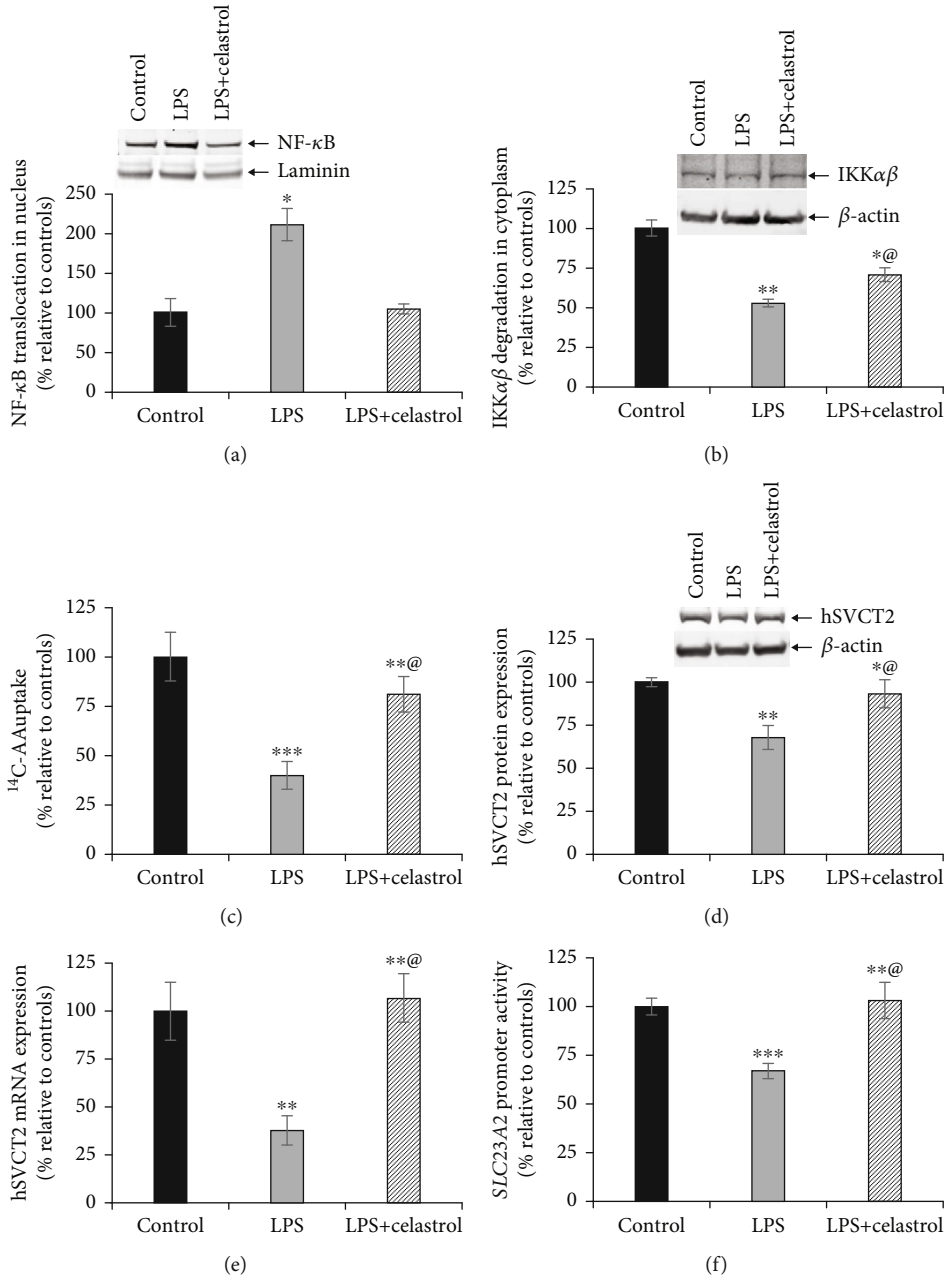

Figure 5: NF-κB signaling pathway plays a role in mediating the LPS-induced inhibitory effect on SVCT2 functional expression. SH-SY5Y cells were pretreated with celastrol (100 nM) for 5 h before LPS treatment; then 48 h later, the NF-κB expression in the nucleus (a), IKK$\alpha\beta$ expression in the cytoplasm (b), carrier-mediated AA uptake (c), hSVCT2 protein expression (d), hSVCT2 mRNA expression (e), and *SLC23A2* promoter activity (f) were determined. @: cells exposed to LPS plus celastrol treatment significantly recovered versus LPS alone-treated cells. Values are means ± SE of at least 3 separate investigations. $^{***}P < 0.001$; $^{**}P < 0.01$; $^{*}P < 0.05$.

In summary, our findings suggest that LPS and TNFα downregulate the functional expression of SVCT2, the major vitamin C transporter in the brain. These actions may contribute to the low levels of AA observed during neuroinflammatory insults.

Data Availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Conflicts of Interest

No potential conflict of interest was reported by the authors.

Authors' Contributions

Conceptualization was performed by V.S.S., M.K., and J.S.M.; formal analysis was performed by T.T. and V.S.S.; investigation and writing (review and editing) were performed by T.T., A.A., M.K., J.S.M., and V.S.S.; writing (original draft

preparation) was performed by T.T., A.A., J.S.M., and V.S.S.; and visualization was performed by T.T., A.A., and V.S.S. All authors have read and approved the manuscript.

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References

[1] L. Packer and J. Fuchs, *Vitamin C in Health and Disease*, Marcel Dekker Inc, New York, NY, USA, 1997.

[2] A. C. Carr and B. Frei, "Toward a new recommended dietary allowance for vitamin C based on antioxidant and health effects in humans," *The American Journal of Clinical Nutrition*, vol. 69, no. 6, pp. 1086–1107, 1999.

[3] F. Monacelli, E. Acquarone, C. Giannotti, R. Borghi, and A. Nencioni, "Vitamin C, Aging and Alzheimer's disease," *Nutrients*, vol. 9, no. 7, p. 670, 2017.

[4] M. E. Rice, "Ascorbate regulation and its neuroprotective role in the brain," *Trends in Neurosciences*, vol. 23, no. 5, pp. 209–216, 2000.

[5] M. Bürzle, Y. Suzuki, D. Ackermann et al., "The sodium-dependent ascorbic acid transporter family SLC23," *Molecular Aspects of Medicine*, vol. 34, no. 2-3, pp. 436–454, 2013.

[6] S. Sotiriou, S. Gispert, J. Cheng et al., "Ascorbic-acid transporter Slc23a1 is essential for vitamin C transport into the brain and for perinatal survival," *Nature Medicine*, vol. 8, no. 5, pp. 514–517, 2002.

[7] K. Murakami, N. Murata, Y. Ozawa et al., "Vitamin C restores behavioral deficits and amyloid-β oligomerization without affecting plaque formation in a mouse model of Alzheimer's disease," *Journal of Alzheimer's Disease*, vol. 26, no. 1, pp. 7–18, 2011.

[8] S. Dixit, A. Bernardo, J. M. Walker et al., "Vitamin C deficiency in the brain impairs cognition, increases amyloid accumulation and deposition, and oxidative stress in APP/PSEN1 and normally aging mice," *ACS Chemical Neuroscience*, vol. 6, no. 4, pp. 570–581, 2015.

[9] K. E. Charlton, T. L. Rabinowitz, L. N. Geffen, and M. A. Dhansay, "Lowered plasma vitamin C, but not vitamin E, concentrations in dementia patients," *The Journal of Nutrition, Health & Aging*, vol. 8, no. 2, pp. 99–107, 2004.

[10] P. Rinaldi, M. C. Polidori, A. Metastasio et al., "Plasma antioxidants are similarly depleted in mild cognitive impairment and in Alzheimer's disease," *Neurobiology of Aging*, vol. 24, no. 7, pp. 915–919, 2003.

[11] F. E. Harrison, A. H. Hosseini, M. P. McDonald, and J. M. May, "Vitamin C reduces spatial learning deficits in middle-aged and very old APP/PSEN1 transgenic and wild-type mice," *Pharmacology, Biochemistry, and Behavior*, vol. 93, no. 4, pp. 443–450, 2009.

[12] F. E. Harrison, "A critical review of vitamin C for the prevention of age-related cognitive decline and Alzheimer's disease," *Journal of Alzheimer's Disease*, vol. 29, no. 4, pp. 711–726, 2012.

[13] F. Zhang, J. Liu, and J. S. Shi, "Anti-inflammatory activities of resveratrol in the brain: role of resveratrol in microglial activation," *European Journal of Pharmacology*, vol. 636, no. 1-3, pp. 1–7, 2010.

[14] Y. N. Huang, C. C. Lai, C. T. Chiu, J. J. Lin, and J. Y. Wang, "L-ascorbate attenuates the endotoxin-induced production of inflammatory mediators by inhibiting MAPK activation and NF-κB translocation in cortical neurons/glia Cocultures," *PLoS One*, vol. 9, no. 7, article e97276, 2014.

[15] S. H. Kim, M. Y. Noh, H. J. Kim et al., "A therapeutic strategy for Alzheimer's disease focused on immune-inflammatory modulation," *Dementia and neurocognitive disorders*, vol. 18, no. 2, pp. 33–46, 2019.

[16] M. Sochocka, E. S. Koutsouraki, K. Gasiorowski, and J. Leszek, "Vascular oxidative stress and mitochondrial failure in the pathobiology of Alzheimer's disease: a new approach to therapy," *CNS & Neurological Disorders Drug Targets*, vol. 12, no. 6, pp. 870–881, 2013.

[17] J. Bauer, S. Strauss, U. Schreiter-Gasser et al., "Interleukin-6 and alpha-2-macroglobulin indicate an acute-phase state in Alzheimer's disease cortices," *FEBS Letters*, vol. 285, no. 1, pp. 111–114, 1991.

[18] L. Wen, C. Chiu, Y. Huang, C. Chang, and J. Wang, "Rapid glia expression and release of proinflammatory cytokines in experimental _Klebsiella pneumoniae_ meningoencephalitis," *Experimental Neurology*, vol. 205, no. 1, pp. 270–278, 2007.

[19] F. Alasmari, C. R. Ashby Jr., F. S. Hall, Y. Sari, and A. K. Tiwari, "Modulation of the ATP-binding cassette B1 transporter by neuro-inflammatory cytokines: role in the pathogenesis of Alzheimer's disease," *Frontiers in Pharmacology*, vol. 9, p. 658, 2018.

[20] P. Grammas and R. Ovase, "Inflammatory factors are elevated in brain microvessels in Alzheimer's disease," *Neurobiology of Aging*, vol. 22, no. 6, pp. 837–842, 2001.

[21] X. Zhan, B. Stamova, L. W. Jin, C. DeCarli, B. Phinney, and F. R. Sharp, "Gram-negative bacterial molecules associate with Alzheimer disease pathology," *Neurology*, vol. 87, no. 22, pp. 2324–2332, 2016.

[22] B. Li, Y. He, J. Ma et al., "Mild cognitive impairment has similar alterations as Alzheimer's disease in gut microbiota," *Alzheimer's & Dementia*, vol. 15, no. 10, pp. 1357–1366, 2019.

[23] Y. Zhao, V. Jaber, and W. J. Lukiw, "Secretory products of the human GI tract microbiome and their potential impact on Alzheimer's disease (AD): detection of lipopolysaccharide (LPS) in AD hippocampus," *Frontiers in Cellular and Infection Microbiology*, vol. 7, p. 318, 2017.

[24] G. Cepinskas and J. X. Wilson, "Inflammatory response in microvascular endothelium in sepsis: role of oxidants," *Journal of Clinical Biochemistry and Nutrition*, vol. 42, no. 3, pp. 175–184, 2008.

[25] S. M. Opal, "The clinical relevance of endotoxin in human sepsis: a critical analysis," *Journal of Endotoxin Research*, vol. 8, no. 6, pp. 473–476, 2002.

[26] C. Venet, F. Zeni, A. Viallon et al., "Endotoxaemia in patients with severe sepsis or septic shock," *Intensive Care Medicine*, vol. 26, no. 5, pp. 538–544, 2000.

[27] J. Armour, K. Tyml, D. Lidington, and J. X. Wilson, "Ascorbate prevents microvascular dysfunction in the skeletal muscle of the septic rat," *Journal of Applied Physiology (1985)*, vol. 90, no. 3, pp. 795–803, 2001.

[28] C. J. Schorah, C. Downing, A. Piripitsi et al., "Total vitamin C, ascorbic acid, and dehydroascorbic acid concentrations in plasma of critically ill patients," *The American Journal of Clinical Nutrition*, vol. 63, no. 5, pp. 760–765, 1996.

[29] F. Wu, K. Tyml, and J. X. Wilson, "Ascorbate inhibits iNOS expression in endotoxin- and IFN gamma-stimulated rat

skeletal muscle endothelial cells," *FEBS Letters*, vol. 520, no. 1-3, pp. 122–126, 2002.

[30] M. Ogura, N. Nakamichi, K. Takano et al., "Functional expression of a glutamine transporter responsive to down-regulation by lipopolysaccharide through reduced promoter activity in cultured rat neocortical astrocytes," *Journal of Neuroscience Research*, vol. 83, no. 8, pp. 1447–1460, 2006.

[31] P. T. Hsueh, H. H. Wang, C. L. Liu, W. F. Ni, Y. L. Chen, and J. K. Liu, "Expression of cerebral serotonin related to anxiety-like behaviors in C57BL/6 offspring induced by repeated subcutaneous prenatal exposure to low-dose lipopolysaccharide," *PLoS One*, vol. 12, no. 6, article e0179970, 2017.

[32] G. Wittmann, P. Mohácsik, M. Y. Balkhi, B. Gereben, and R. M. Lechan, "Endotoxin-induced inflammation down-regulates L-type amino acid transporter 1 (LAT1) expression at the blood-brain barrier of male rats and mice," *Fluids Barriers CNS*, vol. 12, no. 1, p. 21, 2015.

[33] V. S. Subramanian, S. Sabui, H. Moradi, J. S. Marchant, and H. M. Said, "Inhibition of intestinal ascorbic acid uptake by lipopolysaccharide is mediated via transcriptional mechanisms," *Biochimica et Biophysica Acta - Biomembranes*, vol. 1860, no. 2, pp. 556–565, 2018.

[34] V. S. Subramanian, S. Sabui, G. A. Subramenium, J. S. Marchant, and H. M. Said, "Tumor necrosis factor alpha reduces intestinal vitamin C uptake: a role for NF-κB-mediated signaling," *American Journal of Physiology. Gastrointestinal and Liver Physiology*, vol. 315, no. 2, pp. G241–g248, 2018.

[35] Y. Qin, Y. Liu, W. Hao et al., "Stimulation of TLR4 attenuates Alzheimer's disease-related symptoms and pathology in tau-transgenic mice," *Journal of Immunology*, vol. 197, no. 8, pp. 3281–3292, 2016.

[36] P. Arda-Pirincci, F. Oztay, B. B. Bayrak, R. Yanardag, and S. Bolkent, "Teduglutide, a glucagon-like peptide 2 analogue: a novel protective agent with anti-apoptotic and anti-oxidant properties in mice with lung injury," *Peptides*, vol. 38, no. 2, pp. 238–247, 2012.

[37] C. J. Elferink and J. J. Reiners Jr., "Quantitative RT-PCR on CYP1A1 heterogeneous nuclear RNA: a surrogate for the in vitro transcription run-on assay," *BioTechniques*, vol. 20, no. 3, pp. 470–477, 1996.

[38] J. C. Reidling, V. S. Subramanian, T. Dahhan, M. Sadat, and H. M. Said, "Mechanisms and regulation of vitamin C uptake: studies of the hSVCT systems in human liver epithelial cells," *American Journal of Physiology. Gastrointestinal and Liver Physiology*, vol. 295, no. 6, pp. G1217–G1227, 2008.

[39] J. C. Reidling and S. A. Rubin, "Promoter analysis of the human ascorbic acid transporters SVCT1 and 2: mechanisms of adaptive regulation in liver epithelial cells," *The Journal of Nutritional Biochemistry*, vol. 22, no. 4, pp. 344–350, 2011.

[40] C. W. Heskett, T. Teafatiller, C. Hennessey et al., "Enteropathogenic Escherichia coli infection inhibits intestinal ascorbic acid uptake via dysregulation of its transporter expression," *Digestive Diseases and Sciences*, vol. 66, no. 7, pp. 2250–2260, 2020.

[41] A. R. Foley, M. P. Menezes, A. Pandraud et al., "Treatable childhood neuronopathy caused by mutations in riboflavin transporter RFVT2," *Brain*, vol. 137, no. 1, pp. 44–56, 2014.

[42] H. Qiao and J. M. May, "Regulation of the human ascorbate transporter SVCT2 exon 1b gene by zinc- finger transcription factors," *Free Radical Biology & Medicine*, vol. 50, no. 9, pp. 1196–1209, 2011.

[43] P. Alexandrov, Y. Zhai, W. Li, and W. Lukiw, "Lipopolysaccharide-stimulated, NF-kB-, miRNA-146a- and miRNA-155-mediated molecular-genetic communication between the human gastrointestinal tract microbiome and the brain," *Folia Neuropathologica*, vol. 57, no. 3, pp. 211–219, 2019.

[44] C. Yao, N. Purwanti, M. R. Karabasil et al., "Potential down-regulation of salivary gland AQP5 by LPS via cross-coupling of NF-κB and p-c-Jun/c-Fos," *The American Journal of Pathology*, vol. 177, no. 2, pp. 724–734, 2010.

[45] W. Pan, C. Yu, H. Hsuchou, and A. J. Kastin, "The role of cerebral vascular NFkappaB in LPS-induced inflammation: differential regulation of efflux transporter and transporting cytokine receptors," *Cellular Physiology and Biochemistry*, vol. 25, no. 6, pp. 623–630, 2010.

[46] X. Mao, A. M. Moerman, and S. W. Barger, "Neuronal kappa B-binding factors consist of Sp1-related proteins. Functional implications for autoregulation of N-methyl-D-aspartate receptor-1 expression," *Journal of Biological Chemistry*, vol. 277, no. 47, pp. 44911–44919, 2002.

[47] F. Hirano, H. Tanaka, Y. Hirano et al., "Functional interference of Sp1 and NF-kappaB through the same DNA binding site," *Molecular and Cellular Biology*, vol. 18, no. 3, pp. 1266–1274, 1998.

[48] A. Liu, P. W. Hoffman, W. Lu, and G. Bai, "NF-κB site interacts with Sp factors and up-regulates the NR1 promoter during neuronal differentiation," *The Journal of Biological Chemistry*, vol. 279, no. 17, pp. 17449–17458, 2004.

[49] D. Stellwagen, E. C. Beattie, J. Y. Seo, and R. C. Malenka, "Differential regulation of AMPA receptor and GABA receptor trafficking by tumor necrosis factor-alpha," *The Journal of Neuroscience*, vol. 25, no. 12, pp. 3219–3228, 2005.

[50] M. A. Motagally, M. K. Lukewich, S. P. Chisholm, S. Neshat, and A. E. Lomax, "Tumour necrosis factor alpha activates nuclear factor kappaB signalling to reduce N-type voltage-gated Ca^{2+} current in postganglionic sympathetic neurons," *The Journal of Physiology*, vol. 587, no. 11, pp. 2623–2634, 2009.

[51] R. Kekuda, P. Saha, and U. Sundaram, "Role of Sp1 and HNF1 transcription factors in SGLT1 regulation during chronic intestinal inflammation," *American Journal of Physiology. Gastrointestinal and Liver Physiology*, vol. 294, no. 6, pp. G1354–G1361, 2008.

[52] X. Ye and S. F. Liu, "Lipopolysaccharide down-regulates Sp1 binding activity by promoting Sp1 protein dephosphorylation and degradation," *The Journal of Biological Chemistry*, vol. 277, no. 35, pp. 31863–31870, 2002.

[53] P. A. Baeuerle and T. Henkel, "Function and activation of NF-kappaB in the immune system," *Annual Review of Immunology*, vol. 12, no. 1, pp. 141–179, 1994.

[54] N. Li and M. Karin, "Is NF-κB the sensor of oxidative stress?," *The FASEB Journal*, vol. 13, no. 10, pp. 1137–1143, 1999.

[55] H. L. Pahl, "Activators and target genes of Rel/NF-κB transcription factors," *Oncogene*, vol. 18, no. 49, pp. 6853–6866, 1999.

[56] Y. Yamamoto and R. B. Gaynor, "Therapeutic potential of inhibition of the NF-kappaB pathway in the treatment of inflammation and cancer," *The Journal of Clinical Investigation*, vol. 107, no. 2, pp. 135–142, 2001.

[57] I. Savini, A. Rossi, M. V. Catani, R. Ceci, and L. Avigliano, "Redox regulation of vitamin C transporter SVCT2 in C2C12 myotubes," *Biochemical and Biophysical Research Communications*, vol. 361, no. 2, pp. 385–390, 2007.

[58] C. C. Portugal, T. G. da Encarnação, R. Socodato et al., "Nitric oxide modulates sodium vitamin C transporter 2 (SVCT-2) protein expression via protein kinase G (PKG) and nuclear factor-κB (NF-κB)," *The Journal of Biological Chemistry*, vol. 287, no. 6, pp. 3860–3872, 2012.

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Review Article

Neuroinflammation: An Integrating Overview of Reactive-Neuroimmune Cell Interactions in Health and Disease

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The concept of central nervous system (CNS) inflammation has evolved over the last decades. Neuroinflammation is the response of reactive CNS components to altered homeostasis, regardless of the cause to be endogenous or exogenous. Neurological diseases, whether traumatic, neoplastic, ischemic, metabolic, toxic, infectious, autoimmune, developmental, or degenerative, involve direct and indirect immune-related neuroinflammation. Brain infiltrates of the innate and adaptive immune system cells appear in response to an infective or otherwise noxious agent and produce inflammatory mediators. Mediators of inflammation include local and recruited cells and signals. Processes derived from extrinsic and intrinsic CNS diseases also elicit the CNS inflammatory response. A deeper understanding of immune-related inflammation in health and disease is necessary to find potential therapeutic targets for preventing or reducing CNS damage. This review is aimed at discussing the innate and adaptive immune system functions and their roles in regulating brain cell responses in disease and homeostasis maintenance.

1. Introduction

The cardinal signs of acute inflammatory diseases involve cellular and molecular events, typically self-limiting, unlike autoimmune and neurodegenerative lesions, which are due to the failure in chronic inflammation resolution. Unresolved inflammatory conditions typically lack the proinflammatory to proresolving phase switch. This implies sustained recruitment and persistence of inflammatory cells at the site of inflammation because of lacking apoptosis and dead cell clearance, macrophages not switching to an anti-inflammatory/regenerative phenotype, no way out for the effector cells, and partial tissue regeneration.

Some of these unsuccessful resolution phase scenarios appear common to acute and chronic diseases.

Both in chronic inflammation with unsuccessful resolution and acute inflammation with a self-limited resolution, making sense of the interaction interlayer between parenchyma cells and immune cells is key to understanding the inflammation-repair process.

Both the immune and central nervous (CNS) systems produce and use immune factors and neuroendocrine mediators. Immune cells and mediators play a regulatory role in the CNS, participating in neurodevelopmental synaptic plasticity and removal and synaptic plasticity in adulthood. Far-distance talk of immune cells with the CNS allows the

immune system to engage the body in fighting infection by pathogenic microorganisms and the nervous system to regulate immunity.

Cross-talk between the immune, nervous, and endocrine systems involves a great variety of mediators, including cytokines, neurotransmitters, and hormones.

The nervous system lays out functional connections with the immune system directly innervating the lymphoid system with adrenergic, peptidergic, and catecholaminergic fibers and via receptors for neuropeptides (substance P (SP), somatostatin, and vasointestinal peptide, (VIP)) and neurotransmitters (noradrenaline, acetylcholine, enkephalin, and endorphin) on immune cells. These mediators can modulate the synthesis and release of cytokines, including the chemokines, chemotactic cytokines. Chemokines are low molecular weight cytokines that recruit secondary proinflammatory leukocytes and might act as central neuromodulators [1]. Neuropeptides and neurotransmitters reach the immune cells by nerve-terminal nearby diffusion (nonsynaptic transmission) and bloodstream circulation. Hormonal receptors in lymphoid tissues allow the neuroendocrine mediators to interact with the immune system. In the last years, the consistent characterization of receptors and hormones in lymphoid tissues has brought out interesting information on the cross-talk between the immune and neuroendocrine systems and the involved mechanisms.

Brain tissue is a particular target of immune-inflammatory reactions. In the past, the CNS was believed immune-privileged, v.g., not prone to undergo strong inflammation, and lacking lymphatic drainage. Accrued evidence on neuroimmune interactions has questioned the historical idea of the brain, isolated by the blood-brain barrier (BBB), immune-privileged. This intrinsic characteristic of the CNS is conferred by constitutive and reactive components including the BBB, microglial cells, astrocytes, oligodendrocytes, and infiltrating myeloid and lymphoid cells. Astrocytes appear to protect the CNS from T cell-mediated neuroinflammation [2]. This review offers an update on the key inflammatory mediators and the role of inflammatory cells in infectious and noninfectious conditions on neuroinflammation. We discuss the relationship between neuroinflammatory processes, hypoxia, and oxidative stress and how innate and adaptive immunity shape up an integrative network to regulate immunological processes, affecting brain homeostasis.

2. Neuroinflammatory Diseases

The BBB-derived immune privilege of the brain is, at least, questionable by now. Central nervous system cells are reactive to peripheral inflammatory factors, and peripheral immune cells can infiltrate the brain. In encephalitis, meningitis, encephalopathy, hypoxia, and other conditions, the inflammatory response of brain cells evidences neurological involvement. Neurologic manifestations of infective (parasite, virus, bacteria, and fungi) and not mutually exclusive, noninfective agents (traumatic, neurodegenerative, and autoimmune) result in morbidity and mortality. The best treatment for these neurologic complications, with varying degrees of recovery and sequelae, is yet unclear.

Inflammation is emerging as a pivotal mechanism common to different neuropathological conditions [3–10].

2.1. Neuroinfectious Diseases. Innate immunity offers a rapid response to infections, often called the first line of host defense, enhancing adaptive immune responses. During neuroinflammatory infections, specific types of innate immune molecular and cell pathways seem activated. Their functional effectiveness to limit brain injury spread is crucial.

Neurologic dysfunction with acute alteration in mental status due to inflammation is a hallmark of CNS infections by neurotropic pathogens [11]. Postinfectious neurologic dysfunction has been attributed to irreversible damage caused by pathogens on their own [12–15].

Neurologic involvement and manifestations were reported in some parasitic infections, v.g., Chagas disease, toxoplasmosis, human African and American trypanosomiasis, echinococcosis, cysticercosis, leishmaniasis, onchocerciasis, schistosomiasis, food-borne trematodiasis, dracunculiasis, filariasis, and soil-transmitted helminthiasis [16].

Chagas disease is associated with brain atrophy independent from structural cardiac disease related to cardiomyopathy. Brain atrophy, rather than multiple infarcts, may represent the main anatomical substrate of cognitive impairment in Chagas' disease [17].

An important determinant of brain inflammation is the delicate balance between proinflammatory and counterinflammatory mediators. In mouse models of human African trypanosomiasis, proinflammatory mediators like the tumor necrosis factor (TNF-α), interferon-gamma (IFN-γ), and CXC ligand 10 (CXCL10) have been crucial to parasite CNS invasion. The administration of IL-10, a prototypical counterinflammatory molecule, reduces the CNS parasite burden, the severity of the neuroinflammatory response, and the clinical symptoms associated [18].

Viral infections associated, or not, with acquired immunodeficiency like dengue, rabies, infections by Epstein Barr virus (EBV), herpes papillomavirus (HPV), human immunodeficiency virus (HIV), and others could cause neurological complications.

Human immunodeficiency viruses infect the CNS during primary infection and persist in resident macrophages, leading to low-grade chronic inflammation. Various CNS viral infection-mediated inflammations take place in perivascular inflammatory infiltrates of the CNS parenchyma [19].

Malignant and nonmalignant tumors are rare. Based on serologic findings and literature, the pathogenetic mechanism of this rare intracranial tumor is believed a chronic reactive response to EBV infection [20].

Bacterial infections of the CNS can cause meningitis, granulomatous infections like tuberculosis, syphilis, spirochete infections, and others, cerebral and epidural abscesses, and bacterial exotoxin-related diseases like diphtheria, tetanus, and botulism, affecting the CNS.

Certain mycoses can affect the brain causing neuroinflammation and neurodegeneration [5, 7, 8] or toxicity [21]. Coccidioidal meningitis (CM) often affects immunocompromised people [22]. Cerebral aspergillosis is a highly fatal infection [23], and mucormycosis is an opportunistic fungal

infection with a poor prognosis among generalized fungal infections that promote brain degeneration.

The past years have established a key role for infectious pathogens in certain neurological autoimmune-associated diseases. Certain systemic and organ-specific autoimmune diseases, rheumatic mainly, can cause neuroinflammation. Fibromyalgia [4], destructive joint diseases [6], and systemic lupus erythematosus [3] are a few examples. Neurodegeneration studies suggest that peripheral infection might be related to onset and progression of age-related neurodegeneration [24]. Aged patients appear more vulnerable to infection-related cognitive changes associated with Alzheimer's disease (AD). This may occur from typical infectious challenges like respiratory tract infections, although some specific viral, bacterial, and fungal pathogens have been associated with disease development as well. To date, whether these microorganisms are directly related to AD progression or are opportunistic pathogens colonizing dementia patients and exacerbating the preexisting ongoing inflammation [8, 25] is unclear.

Neuroinflammation with altered synaptic plasticity following perinatal infectious-inflammatory challenges is of concern. The effects of congenital infection on neural cell proliferation and survival, axonal damage, and myelination have been studied in different experimental settings [26]. Microglia, as an antigen-presenting cell (APC), exerts a special role during neuroinflammation-associated injury to the immature brain [27]. However, microglial activation also participates in the immunoregulation-triggering response, although the evidence suggests that the microglia critically influences brain plasticity in the healthy developing brain [28].

2.2. Not Pathogen-Associated CNS Diseases. Neurodegenerative diseases progressively affect cognitive and motor functions and interfere with daily tasks' performance. Advances in genetics and animal models are showing an unexpected role of the immune system in the pathogenesis and onset of diseases. The role of cytokines, growth factors, and immune signaling pathways in disease pathogenesis is still being examined [29].

Traumatic brain injury (TBI) elicits a robust immune response within hours and days [30]. Peripheral immune cell infiltration to the damaged tissue with activation of brain resident astrocytes and microglia has been observed in patients and TBI animal models. Regulatory T cell-reduced neuroinflammation, T lymphocyte brain infiltration, reactive astrogliosis, interferon-γ gene expression, and transient motor deficits have been observed in an acute TBI murine model [31].

Postmortem brain and cerebrospinal fluid of Parkinson's disease (PD) patients had a high concentration of proinflammatory cytokines, indicating ongoing neuroinflammation beyond pathology. Inflammation might lead to oxidative stress promoting dopaminergic neuron degeneration [9]. Several studies have reported inflammation and immune responses as determinant factors in disease progression, responsible for pathogenic processes in familial and sporadic PD onset [10]. One study reported activated microglia in the substantia nigra (SN) and putamen of patients diagnosed with PD [32]. In 2005, another study suggested microglia-mediated inflammation presenting at an early stage of parkinsonism [33]. Several authors suggested pathogenic mutations in the α-synuclein (SNCA) gene and the leucine-rich repeat kinase 2 (LRRK2). Alpha-synuclein accumulation, a major stimulant of microglial activation, participates in PD progression [34–36]. Both central and peripheral inflammation is responsible for the sustained progression of PD. Degeneration of dopaminergic neurons occurs with the infiltration of T cells and activation of microglia, with increased production of inflammatory cytokines and chemokines due to pathological SNCA accumulation [34, 37, 38].

In addition, the CNS is an autoimmune disease target. Multiple sclerosis (MS) is one of the most ravaging disorders, presenting with spontaneous onset, remitting-relapsing periods sometimes, and a progressive disease pattern in genetically predisposed hosts. Experimental autoimmune encephalomyelitis (EAE) is the traditional animal model for MS. However, despite its similarities with MS, most treatments for EAE have failed in translation to humans. Adaptive and innate, systemic, and resident in the CNS immune components contribute to neurodegenerative and neurobehavioral disorders' progression as found in animal models and correlated with human studies.

Environmental triggers affecting the CNS during the prenatal and postnatal periods trigger microglia activation and astrogliosis, upregulate proinflammatory cytokines, and are critically associated with neuroinflammation [39, 40]. It is not only a hallmark of infections but secondary to not-infective insults as well, like cerebral hypoxia-ischemia [41]. Noteworthily, inflammatory brain glial cells appear pivotal in regulating synaptic structure and function. Synaptic physiology and pathophysiology studies suggest that the immune system dynamically affects neurodevelopmental synapse organization [42, 43].

Though seldom exposed to harmful agents, brain tissue has limited restorative ability to repair damaged cells. The expanding molecular biology findings offer increasing insights into immune glial system interactions, including innate and adaptive immune molecules and receptors mediating tissue injury and repair [44].

3. Key Components of the Neuroinflammatory Process

Newly evolving neuropathology evidence offers proper interpretations of a plethora of diverse disorders. Microglia response, infiltrating immune cells, generation of oxidative stress species, and proinflammatory cytokines offer a common background to neuroinflammatory and neuroimmune responses.

Neuroinflammation is often harmful yet contributes to normal brain development [42] and homeostasis and is actually necessary for brain plasticity during critical developmental periods [45]. Perpetuating inflammatory processes lead to progressive chronic inflammatory conditions, mainly derived from autoimmune or neurodegenerative disorders. Adaptive

immune-mediated neuroinflammation is a frontier grey zone between injury and healing in chronic diseases in particular.

In homeostasis, the neuronal function requires glial cells and BBB integrity. Accumulating evidence suggests that neuroinflammation targeting glial cells is implicated in neurodegenerative disorders [46].

3.1. Inflammatory Mediators in the CNS

3.1.1. Cytokines and Chemokines at the Neuroinflammation Border. Chemokines and cytokines are bioactive proteins and peptides involved in feedback activation of protein signaling cascades. Peripheral macrophages and lymphocytes and central astrocytes and microglia produce and release cytokines and chemokines. These are necessary for neuronal metabolism, immune surveillance, leukocyte trafficking, and uptake of other inflammatory mediators. They participate in neurodevelopment and synaptic transmission and are the main inducers of neuroinflammation. Cytokines and chemokines bind to specific membrane receptors at the extracellular ligand-binding region, activating the intracellular region which triggers signal transmission to the nucleus [47].

Cytokines and chemokines are neuroprotective and neuroinflammatory, and their dysregulation is decisive for neuroinflammation, neurodegeneration, and demyelination in the central and peripheral nervous systems [48].

(1) Chemokines. Chemokines comprise two categories based on their expression. One of them, constitutively expressed, is responsible for the maintenance of homeostasis, surveillance, and immune system monitoring. The other one, inducible by inflammation following damage, amplifies the innate and adaptive immune system responses.

Chemokines act via chemokine-unspecific G protein-coupled receptors (GPCR). They can attract or activate immune cells and affect neuronal activity and survival [49]. They may induce neuronal death directly, activating neuronal chemokine receptors, or indirectly, activating microglial killing mechanisms. Some chemokines are neuroprotective and act as pro- or anti-inflammatory mediators [48]. One of the most important neuroinflammatory chemokines is the monocyte chemoattractant protein-1 (MCP-1), also known as C-C motif ligand 2 (CCL2) or C-X3-C motif ligand 1 (CX3CL1). It regulates the migration of monocytes, T lymphocytes, and "natural killer" cells towards the affected area. In its soluble form, MCP-1 participates in the interaction between neurons and other inflammatory cells.

The MCP-1 acts via the CCR2 receptor and is expressed in neurons and glial cells. The astrocytes are the major source of MCP-1 after neuronal damage or infection. It plays an important role in neuroinflammation linked to various diseases involving neuronal degeneration. Neuronal MCP-1/CCL2 induction during mild impairment of oxidative metabolism caused by microglial recruitment/activation exacerbated neurodegeneration in thiamine deficiency- (TD-) induced neuronal death. Knockout mice lacking CCL2 were resistant to TD-induced neuronal death, suggesting that CCL2 mediated microglial recruitment and neurodegeneration in this model [50]. However, several studies show that suppressing MCP-1 may be beneficial, reducing inflammation in some diseases. In patients with complications associated with inflammatory processes, a high blood level of CCL2 contributes to ischemic cerebrovascular disease and myocardium infarct. Brain overexpression of CCL2 aggravates ischemic injury [51], while CCL2 deficiency confers neuroprotection against permanent carotid artery obliteration [52]. Mice lacking CCR2 showed reduced cerebral edema, infarct size, and BBB disruption and decreased leukocyte, monocytes, and neutrophil infiltration. They also had decreased expression of a variety of proinflammatory cytokines (IL-1β, TNF-α, and IFN-γ) and endothelial cell adhesion molecules preventing leukocyte-endothelial cell interaction during reperfusion [53, 54]. Interestingly, MCP-1-deficient mice showed reduced neuroinflammatory responses and increased peripheral inflammatory responses to peripheral endotoxin insult [55]. In the hypoxia-ischemia model, CCR2 knockout mice had impeded transendothelial diapedesis in response to CCL2, showing that CCR2 was required for stem cell migration to promote CNS regeneration via CCL2 chemotaxis [56]. Likewise, CCL2 protected cultures of human neurons and astrocytes from glutamate toxicity and HIV-transactivator of transcription- (HIV-tat-) induced apoptosis [57]. Rat dorsal hippocampal neurons in culture treated with kainic acid (KA) showed increased CCL2 and macrophage inflammatory protein-2 (MIP-2) levels, both inducers of basic fibroblast growth factor (bFGF) and astrocyte activation. Astrocytes stimulated with CCL2 facilitated bFGF-dependent neuronal cell differentiation and induced H19-7 neurons' survival in vitro, suggesting a supporting trophic role for chemokine-activated astrocytes [58]. Astrocytes produce chemokines in response to proinflammatory cytokines like IL-1β and TNF-α and synthesize MCP-1 via nuclear factor-kappa B (NF-κB) [55]. Primary astrocytes treated with lipopolysaccharide (LPS) and interleukin- (IL-) 1β were responsible for the exacerbated cytokine response observed in vivo in the absence of CCL2 postinjury. Evidence of CCL2-induced inhibition of IL-6 and TNF-α produced by astrocytes following IL-1β stimulation suggests a novel CCL2 immunomodulatory role in acute neuroinflammation [59].

Other chemokines like CXCL9, CXCL10, and CXCL11 and their receptor (CXCR3) are crucially involved in AD and MS. They are implicated in the Th1-type response in various diseases. Their expression is induced by IFN-γ, the most typical Th1 cytokine associated with tissue T cell infiltration [60]. Accordingly, MCP-1 might have a dual neuroinflammatory or neuroprotective role in neurodegenerative diseases, depending on the neuroinflammatory milieu.

(2) Cytokines. The small proteins known as cytokines are signaling molecules released in response to a variety of stimuli under physiological and pathological conditions. Present in up to picomolar concentrations, they regulate inflammation and the duration of the immune response and modulate cellular activities like growth, survival, and differentiation. The large and diversified group of pro- or anti-inflammatory cytokines comprises different families based on their

structural homology and that of their receptors [48]. The main proinflammatory cytokines are TNF-α, IL-1β, and IL-6 interleukins and IFNs. Anti-inflammatory cytokines are IL-10 and IL-4, among others. Cytokines act as neuromodulators and regulate neurodevelopment, neuroinflammation, and synaptic transmission. They are crucial to brain immunity comaintaining immune surveillance, facilitating leukocyte traffic, and recruiting other inflammatory factors. The role of cytokines in neurodegenerative diseases is complicated by their dual roles in neuroprotection and neurodegeneration [48].

To illustrate, IL-6 has dual roles in brain injury and disease. It is essential in regulating inflammation, balancing between pro- and anti-inflammatory responses, and participating in neurodegenerative and neuroprotective processes [61]. The peripheral nervous system and the CNS, v.g., neurons, microglia, and astrocytes, in particular, show IL-6 [60]. In neuroinflammatory processes, IL-6 promotes astrogliosis and microglial activation. During reactive astrogliosis, IL-6 acts as a neurotrophin, promoting neuronal survival in response to neuronal damage. A high level of IL-6 has been associated with brain disease [61]. Interleukin-6 is upregulated upon neuroinflammation as observed after CNS infection or injury, viral meningitis, experimental encephalitis, and acute viral infections. In all these conditions, its cerebrospinal fluid (CSF) level rises in patients [62]. Other examples of high IL-6 level conditions are mouse experimental cerebral malaria [63], TBI [64], and advanced stages of patients with HIV infection [65]. Conversely, studies in IL-6 knockout mice show a compromised inflammatory response, increased oxidative stress, impaired neuroglial activation, decreased lymphocyte recruitment, and a slower rate of recovery and healing [61]. In physiological conditions, TNF-α participates in homeostasis regulation, synaptic plasticity, learning and memory, and sleep/wake cycles. However, a high TNF-α level is related to neuroinflammation and neurodegenerative diseases [66]. Its major source is the microglia, along with astrocytes and neurons during neuroinflammation [66]. Together with the interferon-gamma protein (IFN-γ), TNF-α is proinflammatory during acute brain inflammation and is immunosuppressive upon chronicity [67].

Interferon-gamma (IFN-γ) is a multifunctional cytokine that participates in inflammation onset and consolidation, in innate and adaptive immune responses, induced in many cell types, including neurons [68]. It is a potent inducer of TNF-α gene expression in microglia, having complementary roles during neuroinflammation [66]. TNF-α induces neurotoxicity by high glutamate production, leading to neuronal excitotoxicity and death [69]. Inactivating IL-1? and TNF-α with neutralizing antibodies reduced neuronal death in SK-N-SH cells, a neuroblastoma cell line induced by the West Nile virus [70]. Deleting the TNF-α gene reduces neurodegeneration in Sandhoff disease (SD), a lysosomal storage disorder [71]. However, TNF-α receptor-1-deficient mice showed severe experimental autoimmune neuritis suggesting an anti-inflammatory role for TNF-α at least in this model [72]. Two surface receptors, TNFR1 and TNFR2, recognize TNF-α. They differ in their expression, signaling cascade transduction, and TNF-α binding affinity [73]. Downregulating TNFR1 reduced JNK activation and attenuated neuroinflammation, neurovascular damage, and brain injury in the LPS-sensitized hypoxic-ischemia mouse model [69]. Upregulating TNFR2 protected neurons from excitotoxicity and promoted neuronal survival, activating the PI3K/NF-κB signaling pathway in a glutamate-induced cell death model [74]. Different receptor-related signaling pathways account for TNF-α dual effects [75, 76].

IL-1β is a very potent signaling molecule of the family of pleiotropic cytokines, expressed at low levels usually, but induced rapidly in response to local or peripheral insults. It coordinates the host defense response to pathogens and injury, not surprisingly, not only systemically but in the CNS as well. Upon injury or in brain disease, IL-1β presence has been correlated with effects on neurons and nonneuronal cells [77]. It is also involved in neuroinflammation, fever, appetite, learning, and memory [78]. It is synthesized by macrophages, microglia, astrocytes, T and B lymphocytes, or neutrophils, among others [77]. Binding to the IL-1R receptor induces the production of other inflammatory cytokines like IL-6 and TNF-α, and the increase in the PLA2, COX-2, and iNOS enzymes which produce arachidonic acid, prostaglandins, and NO, respectively [79, 80]. Studies in IL-1R1 receptor-deficient mice found decreased activation of microglia and astrocytes and of IL-6 and COX-2 production in brain injury, indicating the key role of IL-1β [79, 81]. Interleukin-1β was rapidly induced in experimental stroke, while a low IL-1β level protected from ischemic injury and neuronal loss, reducing infarct volume [79, 82]. Multiple sclerosis patients had high IL-1β levels in CSF and demyelinated lesions [83]. Oppositely, IL-1β induced the production of fibroblast growth factor-2 (FGF-2), transforming growth factor-β1 (TGF-β1), and nerve growth factor (NGF), promoting neurite growth in vitro [84]. Taken together, IL-1β appears important in the initiation and development of the inflammatory cascade and in neuronal survival in a variety of neurodegenerative diseases.

Neuropoietic cytokines are a group of immune mediators that participate in normal brain development, promoting neural precursors' proliferation, fate determination and differentiation, neuronal and glia migration, cell survival, and activity-dependent changes in synaptic function. Inflammation during development may cause widespread injury, interfering with the normal balance in cytokine signaling and developmental processes, or increase neurological vulnerability later in life [85].

3.1.2. The Role of the Complement Cascade in Neuroinflammation. The complement system comprises around 30 proteins, nearly 5% of total whey protein and a low proportion of membrane proteins. It participates in the recognition, trafficking, and elimination of pathogens and any unfamiliar material to the host as a powerful arm of the innate immune system. In normal conditions, its components do not pass through the blood-brain barrier. Glial cells and neurons produce complement components, largely in response to neural damage or inflammatory signals [86]. The complement cascade is also expressed during

physiological development when neuron-derived complement proteins tag synapses for pruning by microglial cells [87, 88]. Astrocytes and microglia are the largest producers of complement elements in both normal and pathological conditions. The microglia expresses high complement receptor levels, crucial at inducing phagocytosis of complement-labeled structures, regulating cytokine signals and chemotaxis. Astrocytes, oligodendrocytes, and neurons express high levels of the C3 complement fraction and other members of the complement cascade [89, 90]. The complement system is implicated in several neurological disorders. Complement mRNAs have been found in the cerebral cortex and the hippocampus in man. The postmortem examination of samples from patients with AD showed an increased level of these mRNAs in pyramidal neurons. This finding along with reactive oxygen species and proteases portrays a local inflammatory nest compatible with neuronal dysfunction and cognitive decline [91–93]. Products of the activation cascades are generated in human AD, MS, Huntington's disease, Parkinson's disease, spinal cord injury, TBI, and cerebral ischemia [94–102]. In addition, $C3^{-/-}$ mice showed reduced brain edema in intracerebral hemorrhage [102]. Complement overactivation, associated with glial activation and the release of proinflammatory compounds, appears implicated in synaptic loss concomitant with aging physiological, cognitive decline, and brain diseases [103]. The complement system role in the pathology of neurodegenerative diseases opens new avenues for understanding its involvement in neuroinflammatory processes and as a promising target for future therapeutic strategies.

3.2. Inflammatory Cells in the CNS. Neurons and glial cells produce cytokines either constitutively or by induction in appropriate culture media.

Glial cells, unlike neurons, are not excitable and comprise the microglia and the macroglia (astrocytes, oligodendrocytes, and ependymal cells). Some of them are involved in the isolation, support, and supply of substances to maintain neuronal metabolism. The microglia are considered brain resident macrophages able to migrate to the inflammatory foci. Glial cells release cytokines, which establish functional connections with each other and with neurons. Upon inflammatory stimuli, they can participate in the pathogenesis of neurological diseases.

3.2.1. Microglia. Microglia, the resident immune cells of the CNS, are derived from yolk sac macrophages arising during the first wave of primitive hematopoiesis and populating the developing CNS via the bloodstream once embryonic circulation is established [104]. Central nervous system glia and a mononuclear phagocyte are involved in physiologic processes, inflammatory and immune responses, and in the pathogenesis of several CNS disorders [46]. These cells share innate immunological functions with other mononuclear phagocytes like monocytes, macrophages, and dendritic cells, mostly related to phenotypic characteristics and lineage-related immunological properties, including the ability to secrete cytokines common to immune antigen-presenting cells, described over two decades ago [105].

Surveillant microglia cells contribute to maintaining CNS homeostasis [106]. In response to inflammation challenge, microglia promptly becomes ameboid and upregulates cell surface receptors involved in innate immune responses, proinflammatory type (classical or M1 activation). This is because they have pattern recognition receptors (PRRs) like the toll-like receptors (TLR), the nucleotide-binding oligomerization domain-like (NOD) receptors, receptors for advanced glycation end products (RAGE), scavenger receptors (CD36, CD91), phagocytic receptors like the CR3 and CR4, and triggering receptor expressed on myeloid cells (TREM). These receptors are involved in the innate immune response, increasing the expression of various cytokines, chemokines, surface receptors, and metabolic enzymes [107]. The microglia can take on an anti-inflammatory profile (M2 microglia), promoting healing, tissue regeneration, and angiogenesis. The M2 microglia has been subdivided into different M2 subtypes depending on the expression of specific markers and secreted cytokines and chemokines [82, 108].

Microglia is crucial in restricting neuroinflammation. In osteopetrotic (op/op) mice, defective in producing functional colony-stimulating factor (M-CSF), a decrease in the number of tissue macrophages and microglial cells led to neuropathology exacerbation [109, 110]. All the same, resident glial cells can turn into aggressive effectors, attacking healthy neurons by phagocytosis, or secreting factors on their own, or in coordination with infiltrated immune cells [111]. This rich repertoire of responses may account for the dichotomic microglia reactivity in promoting neuronal survival or degeneration.

The presence of activated microglia in nearly every neurological insult leads to possibly oversimplifying in vitro study design. Assuming that activated microglia and associated inflammatory responses are harmful to the brain should be cautious [112]. The reactive response of the microglia might be interpreted mostly as beneficial. The regulatory control of neuroinflammation is normally imposed, and interfering with homeostatic regulations may be detrimental. Unfortunately, the way to reaching a healthy balance and its modulation under psychological distress and neurological diseases is still unclear [107].

3.2.2. Astrocytes. Astrocytes have been traditionally considered supportive cells for neurons, responsible for brain homeostasis and neuronal functions. They are the largest cell population in the CNS, even compared with neurons [107]. Astrocytes give metabolic support to the neuron, generate neurovascular coupling, and control BBB permeability. They are essential in recapturing several neurotransmitters, K^+ damping, and other functions. They express a wide variety of cytokine receptors like the PRRs, contributing to brain immunity [113]. The expression kinetics indicates that chemokines contribute to amplifying the inflammatory reaction or that astrocytes can promote recruitment and proliferation of regulatory T cells (Tregs) via the anti-inflammatory cytokine transforming growth factor β (TGF-β) and chemokine CXCL12 (stromal cell-derived factor-1 (SDF-1)) [107]. Astrocytes secreting other anti-inflammatory cytokines like IL-10 might exert important immunoregulatory functions

in the CNS, reducing microglia and astrocytes' presenting capacity and interfering with antigen-specific T lymphocyte proliferation.

Activated T lymphocytes (Th1 and Th17) in the secondary lymphatic organs cross the BBB and are locally reactivated upon surface antigen recognition on the antigen-presenting cells. They secrete cytokines that stimulate microglia and astrocytes, increasing cell recruitment in a variety of neurological disorders. Astrocytes act as a source of cell surface receptor/ligands and cytokines to modulate both innate and adaptive immune cell system in the neuropathy, and the way around, immune cells regulate astrocyte activity [114, 115].

Microglia and astrocytes play an active dual role in brain inflammatory diseases. Not only can they boost immune responses and promote neurodegeneration but can also protect and restrict CNS inflammation. What factors or scenarios determine whether a beneficial or detrimental response follows remains a matter of research.

3.2.3. Oligodendrocytes. The oligodendrocytes are glial cells that start myelinization, allow electric potential propagation, and give metabolic support to neurons. From an immunological point of view, oligodendrocytes were classically thought of as inert and merely representing bystander victims of immune responses. This view has now changed in the light of accumulating evidence that oligodendrocytes actively produce a wide range of immune-regulatory factors and express the corresponding receptors [115].

Neuroinflammatory responses can be deleterious for cell survival, leading to irreversible and extensive brain damage, if long-sustained in particular. Oligodendrocytes are the main target of the immunoinflammatory response in the CNS. This occurs due to deleterious cytokines released by infiltrating macrophages and microglia, T lymphocyte cytotoxicity, or antibodies triggering antibody-mediated cytotoxicity (antibody-dependent cellular cytotoxicity).

Oligodendrocytes produce immune mediators that modulate microglia activity in response to stress. Chemoattractants like CXCL10, CCL2, CXCR2, and CCL3, CXCR2 expressed on oligodendroglia, in particular, have been implicated in the pathogenesis of neuroinflammatory demyelinating diseases and in amplifying the migration, proliferation, and myelin production by the oligodendroglia [116]. Oligodendrocytes express receptors to IL-4, IL-6, IL-10, IL-12, and other cytokines and markers like the CD200 during inflammation and infection, suggesting that they recruit microglia to damaged tissues [115]. A wide range of proinflammatory cytokines, including IL 1, 2, and 3, IFN α, β, and γ, TNF-α, and lymphotoxin, released by microglia, have been detected in demyelinating pathologies like MS, suggesting that microglial activity and oligodendrocyte damage may be associated [116, 117]. In vitro stimulation with IFN-γ induced MHC-I expression, making them susceptible to death caused by CD8+ T cells (often called cytotoxic T lymphocytes) [118]. Likewise, oligodendrocytes express both IL-18 and IL-18R receptors during the active MS period. The large amount of IFN-γ observed in these circumstances adds to oligodendrocyte damage [119]. Human oligodendrocytes are susceptible to MHC class I restricted CD8+ T cell mediated cytotoxicity in vitro [120, 121], to non-MHC restricted cytotoxicity mediated by $\gamma\delta$ T cells [122], and to cytokine-activated natural killer (NK) cells [123]. The cytotoxic activity of killer (K) cells in enriched cultures of bovine oligodendrocytes (BOL) was investigated in MS. Human K cells mediated cytotoxicity to primary cultures of BOL, where the antibody-dependent cell-mediated cytotoxicity (ADCC) to BOL was mediated by large granular lymphocytes [124].

Oligodendrocytes play a central role in the pathogenesis of a wide spectrum of neurological disorders encompassing various neurodegenerative diseases, besides the classical demyelinating disorders. The interaction between oligodendrocytes and other glial cells like microglia offers an insight into the neuroinflammatory dynamics in different neurological conditions. More studies are needed on the communication between microglia and oligodendrocytes. The outcome will help to develop new approaches to treat disorders with myelin damage associated with innate immune activation, promoting repair and reducing inflammation in the CNS. This is summarized in Figure 1.

3.3. CNS Immune-Mediated Inflammation, Hypoxia, and Oxidative Stress Crossovers. The CNS is sensitive to peripheral inflammatory events and peripheral immune cell and cytokine infiltration. Unfortunately, unsuccessful repair leads to lasting cellular damage. Any insult to the CNS involves immune-mediated inflammation-hypoxia and oxidative stress. Often, there is a massive epithelial cell loss and interstitial fibroblast proliferation with an extracellular collagenous matrix deposition known as fibrosis because of a failure in repairing injured parenchyma cells [125]. This interpretation is not conclusive. Fibroblast expansion is intrinsic to damage due to tissue-resident macrophage activation and macrophage-like cell influx rather than parenchyma repairing attempt by macrophages. Whether fibrosis benefits or aggravates damage is not clear [126]. Clarifying this issue applies to whether the intervention should point against fibrosis development (fibroblasts' expansion and collagen deposition) or not if the repair strategy avoids axonal loss and brain damage.

Functional recovery after hypoxic brain damage poses a complex scenario. Hypoxia impairs gene expression and downregulates transcription and translation mechanisms and gene activation as the hypoxia-inducible factor (HIF1-α) and its target molecules [127]. Hypoxia triggers two main molecular and cell cascades. One leads to hypoxia-damaged cell removal via ubiquitination, peroxisome, and caspase pathway activation, resulting in apoptosis or necrosis, the latter encompassing proinflammatory effects [128, 129]. The other is compensatory, reducing cell loss via multiple mechanisms, including DNA repair, preserving homeostasis [130].

Eventually, the loss and salvage of cells impact brain development, neuronal wiring, and neuron-glia interactions. Whatever further negative impact comes up during development will reinforce the sequel of damage, aggravating neurological deficits and ensuing neurological disorders.

Inflammation and hypoxia are inextricably linked. Nuclear factor kappa B (NF-κB) regulates the HIF1-α system

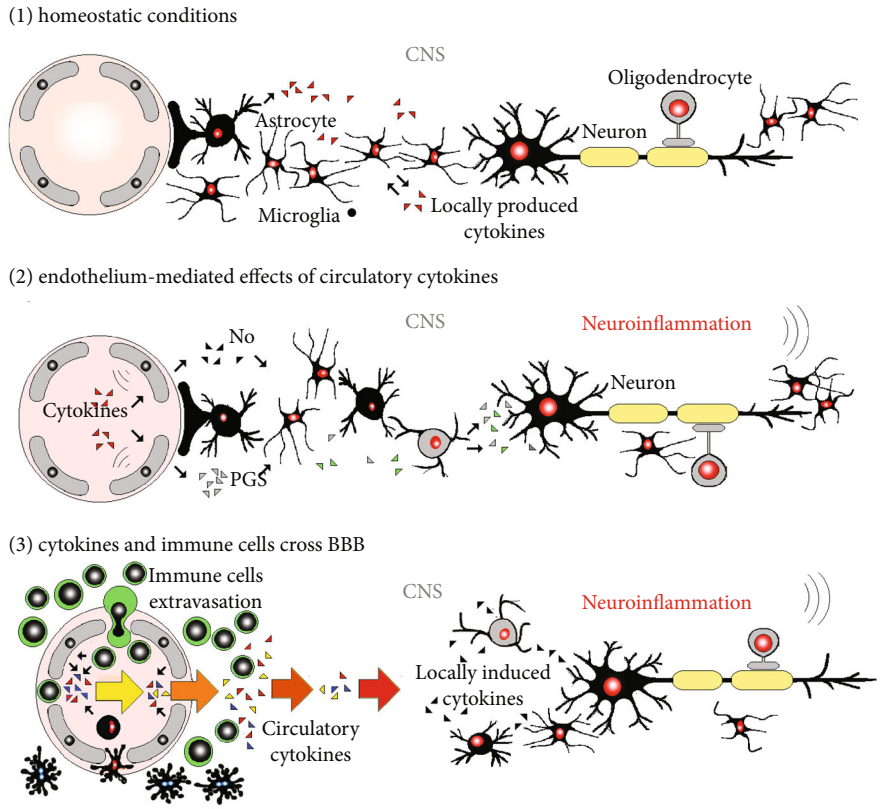

Figure 1: Essential ways cytokines affect the brain. (1) De novo synthesis of cytokines in the CNS in homeostatic conditions is clear. (2) Peripheral cytokines can induce brain cytokine synthesis. Also, cytokines can act centrally via endothelial cells. Cytokine-endothelial cell interaction triggers the release of second messengers like nitric oxide (NO) and prostaglandins (PGS) with central effects. Hence, the signal mediated by a cytokine as IL-1β can be transduced from the periphery without crossing the BBB. (3) Systemic administration of IL-1β and TNF-α to experimental animals decreases BBB selectivity. Cytokines induce glial stimulation.

[131]. The concept of hypoxia leading to inflammation has gained general acceptance after studies on the hypoxia signaling pathway. Mountain sickness increases the circulating level of proinflammatory cytokines and vascular leakage, triggering pulmonary or cerebral edema [129, 132–134].

Hypoxia signaling and the NF-κB family of transcription factors regulate inflammation and orchestrate immune responses to guarantee tissue homeostasis [135]. The interaction of the NF-κB family with the HIF pathway links inflammation with hypoxia. The NF-κB-independent ATIA- (anti-TNF-α-induced apoptosis-) thioredoxin 2 (TRX2) axis inhibits TNF-α- and hypoxia-induced apoptosis irrespective of NF-κB through TRX2-mediated elimination of excess reactive oxygen species (ROS) (Figure 2) [136].

Ischemia-reperfusion activates NF-κB in epithelial cells, releasing proinflammatory tumor necrosis factor α (TNF-α) while attenuating apoptotic hypoxia-activated pathways [137, 138].

One study identified an NF-κB-independent ATIA-thioredoxin 2 axis that inhibits TNF-α- and hypoxia-induced apoptosis, eliminating ROS directly [139]. Currently, the paradigm for inhibition of TNF-α-induced apoptosis points to NF-κB, which inhibits caspases and prevents sustained JNK activation [73]. Besides, the antiapoptotic effect of NF-κB has been associated with excessive ROS elimination.

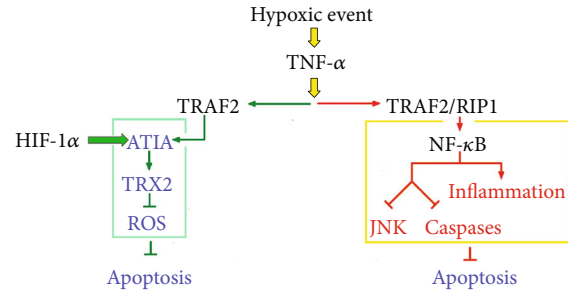

Figure 2: Schematic representation of ATIA as the proposed inflammation and hypoxia crossover.

The evidence poses a novel paradigm for apoptosis inhibition by TNF-α and other death signals, controlling ROS accumulation. The pleiotropic inflammatory cytokine TNF-α regulates immune responses, inflammation, proliferation, and cell death (apoptosis and necrosis) and regulates apoptosis binding to its membrane receptor 1 (TNF-R1).

Upon TNF-α stimulation, TNF-R1 trimer recruits multiple adaptors like TRAF2, TRAF5, RIP1, cIAP1, and cIAP2 and other modulators or regulators like Miz1 and the linear ubiquitin chain assemble complex [140–143].

Cells of the adaptive and innate immune systems in the brain parenchyma and meningeal space are relevant in both brain health and disease.

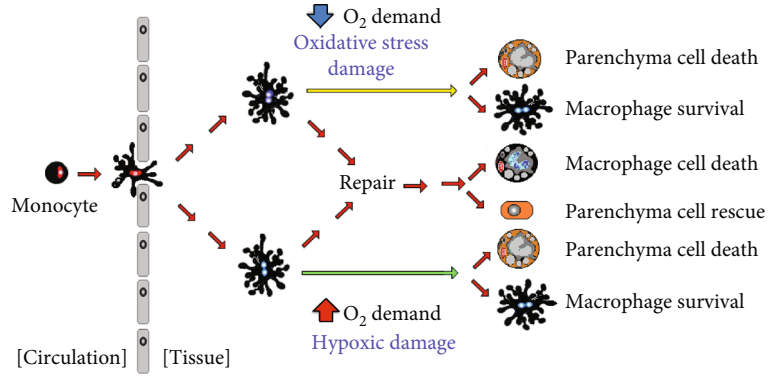

Figure 3: Schematic representation of parenchyma and macrophage cell fate in hypoxia and oxidative stress-inflammation environment.

The ATIA-TRX2 axis inhibits apoptosis induced by both TNF-α and a low oxygen level, eliminating excessive ROS in mitochondria. This rescues parenchyma cells from undergoing apoptosis. The activity of ATIA may be a key regulator in carcinogenesis because tumor cells often take advantage of normal tissue under hypoxic conditions.

3.4. Autophagy-Associated Inflammation in the CNS. Autophagy plays an important role in both innate and adaptive immune responses [144]. This lysosome-dependent catabolic process serving to the turnover of proteins and organelles is crucial in the inflammatory response and cell survival. Immune and inflammatory signals induce autophagy in macrophages through TLRs, among others [145]. Nevertheless, the physiological role of autophagy and its signaling mechanisms in microglia remains poorly understood [145]. Autophagy-related genes (Atg) in microglia are largely suppressed after TLR4 activation by lipopolysaccharide (LPS), inversely as the LPS-mediated stimulation in macrophages [145].

Microglial cells are activated during various phases of tissue repair in certain CNS pathologies. Spinal cord injury-(SCI-) associated anoxemia has a key pathogenic effect, resulting in tissue damage. Besides, HIF-1α protects against apoptosis and necrosis under ischemic and anoxic conditions, upregulating the expression of downstream target genes in brain stroke. Both HIF-1α expression and autophagic cell death were described in microglial cells during brain damage [146]. Autophagy suppression with decreased cell viability and increased inflammatory cytokines were reported associated with HIF-1α inhibition or HIF-1α silencing [146]. If confirmed, HIF-1α may lead to minor autophagic cell death of microglial cells associated with hypoxia-mediated inflammation and may provide a novel therapeutic approach for SCI diseases with deleterious microglial cell activation.

Certain bacteria and pathogenic viruses are implicated in neurodegenerative processes, oxidative stress, decreased autophagy, synaptopathy, and neuronal death [147]. However, how infections influence neurological disease progression is still controversial. Mitochondrial antiviral signaling (MAVS) protein has an important role in antiviral immunity and autoimmunity. However, the pathophysiological role of this signaling pathway, especially in the brain, remains elusive [148]. Autophagy regulated MAVS signaling activity in mouse embryonic fibroblasts (MEFs) [149]. In addition, defective autophagy was associated with neurodegenerative disease development [150–152]. Also, MAVS signaling was involved in microglial activation in vivo [148]. Inflammation is concurrent with autophagic activation, and autophagy inhibition in microglial cells strengthens MAVS-mediated inflammation [148]. This accounts for a regulation of MAVS-dependent microglial activation in the CNS, where autophagy has a key role in microglia-driven inflammatory brain diseases.

MicroRNAs (miRNAs) have a role in regulating immune cell development and modulating innate and adaptive immune responses [153]. Abnormal autophagy occurs during infectious and autoimmune diseases associated with certain miRNAs as novel and potent modulators of autophagic activity [154]. The deficiency of miRNA 223 has been found to reduce CNS inflammation, demyelination, and the clinical symptoms of experimental autoimmune encephalomyelitis (EAE) and increased resting microglia and autophagy [154] found that. Taken together, targeting autophagic proteins may be considered as a potential therapeutic strategy in neuroinflammation-associated diseases [144].

3.5. Neuroinflammation and Natural Immunity. The innate immune response is the first line of defense after tissue injury, hypoxia, or metabolic stress. Activation of innate immunity in response to tissue injury is crucial to homeostasis restoration and wound healing [155, 156].

A balanced oxygen environment is imperative for survival, while away from the balance point, it may be harmful (Figure 3). Both oxygen deficit and excess are detrimental to parenchymal cells and favor macrophage influx [157–159].

Following an insult, cell fate depends on the balance between cell damage and repair, along with oxygen level restoration.

During hypoxia-driven inflammatory damage and oxidative stress-associated inflammatory injury, cell rescue is possible, and parenchyma cells survive. In both scenarios, the immune system orchestrates immune reactive CNS components to restore homeostasis, maximizing parenchyma survival. Provided oxygen level normalizes by homeostatic immune-mediated compensatory mechanisms, parenchyma

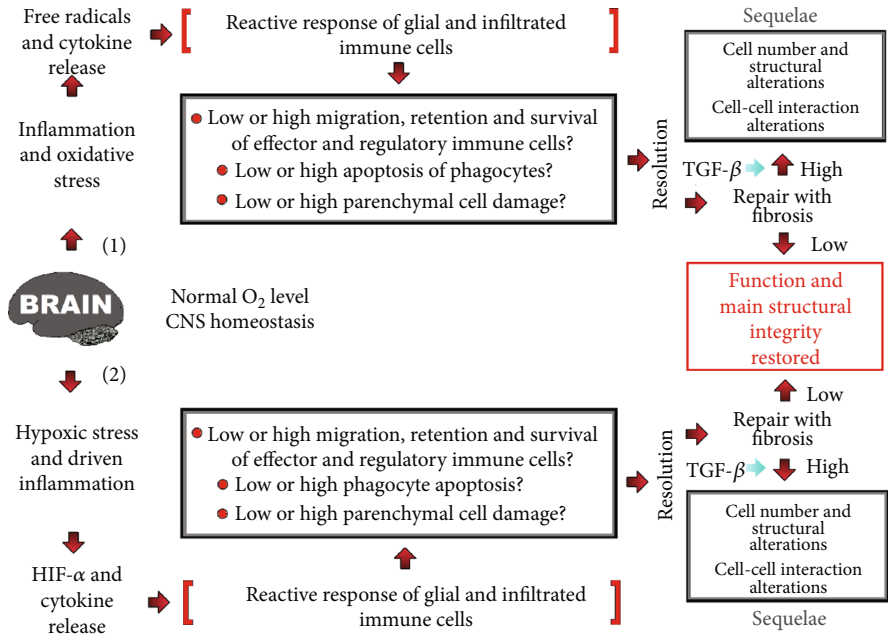

Figure 4: Hypoxic and nonhypoxic inflammation and neuroimmune interactions involved in the prohemostatic response in the CNS. Brain parenchyma, the functional tissue, comprises neurons and glia cells. Brain damage or trauma often leads to cognitive deterioration and/or motor disability with parenchyma structural alterations and eventual cell death. Triggering (1) nonhypoxic and (2) hypoxic reactive inflammation might subserve functional postinjury recovery. Oxidative stress by a high oxygen level induces a compensatory antioxidant response to cut out damage progression. At the other end, hypoxia (hypoxic stress) by a low oxygen level upregulates pathways involved in boosting the oxygen supply. In any case, a fault in oxygen homeostasis draws inflammation with immune cell infiltrates and resident glial cells to restore homeostasis. Light-blue arrow: regulation; red arrow: stimulation.

cells may successfully recover, and the infiltrated macrophages die.

Fully restoring altered homeostasis is not possible in the innate autoimmune response, and inflammation perpetuates [160]. Studies on the interlinkages between hypoxia, tissue alarm signals, neoangiogenesis, and reactive tissue repair mechanisms have allowed identifying early immune response molecules. These are the TLRs, inflammatory cytokines, and putative danger signals, among others, that trigger, sustain, and end the homeostatic response. Janeway's "recognition of microbial nonself" hypothesis explains the activation of an immune response to infection or injury [161]. The "danger model" postulates alternative mechanisms for inducing an appropriate immune response unless there is evidence of tissue injury, termed as "alarm" signals [162]. Innate receptors, like C-type lectins and TLR, seem involved in neuroinflammation and might play a crucial role in the pathogenesis of EAE, an MS animal model [155, 156, 163, 164].

Growing evidence shows that macrophages have various functions in the CNS. Understanding the mechanisms governing the reparative and pathological properties of activated macrophages is at the forefront of neuroscience research. Both macrophage-mediated repair and macrophage-mediated injury occur. Two innate immune receptor subtypes participate in developmental processes and neurological diseases. Danger-associated molecular signals released from dying cells in the injured spinal cord appear to activate different subtypes of macrophage pattern recognition receptors, including TLRs and fungal C-type lectin receptors (e.g., dectin-1) causing neuroprotection or neurotoxicity [165].

Oxidative stress and hypoxic stress trigger divergent pathways to restore homeostasis, resulting in survival or death according to the cell type. Hypoxia often amplifies inflammation and has a prosurvival effect on neutrophils, monocytes, and eosinophils. Complete restoring of oxygen homeostasis ensues macrophage apoptosis and wound healing (Figure 4).

3.6. Neuroinflammation and Adaptive Immunity. Adaptive immunity makes use of immunological memory to recognize specific pathogens, adding up to the innate immunity response, overall achieving an amplified response. Adaptive immunity is typically initiated after innate immune cells like dendritic cells, macrophages, or microglia via their pattern recognition receptors (PRRs) recognize broad specificities of pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs). These are associated with microbial pathogens, cellular stress, or cell components of damaged tissues [166]. In addition, adaptive immunity includes a plethora of effector T cells (Th1, Th17, Th3, Th2, and T regulatory), effector B cells, and antibodies that, in turn, infiltrate the brain during neuroinflammation. Macrophages can act during innate and adaptive immune responses.

During brain hypoxia, the NF-κB pathway is amplified, upregulating TLRs, which enhance antimicrobial factor

production and stimulate phagocytosis, leukocyte recruitment, and adaptive immunity. Besides, HIF-1α increases, influencing adaptive immunity. Patients with a rheumatic disease showed HIF-1α-deficient lymphocytes and high serum levels of anti-double-stranded DNA antibodies and rheumatoid factor [167].

Brain hypoxia depends on signaling mediated by T cell HIF-1α receptors [168]. In vivo and in vitro experiments have suggested that immune responses mediated by T cells and HIF-1α are key downregulators in vascular inflammation and remodeling tissue, contributing to vascular remodeling modulation together with B lymphocytes [169]. The deletion of HIF-1α in T cells impairs differentiation of CD4+ Th17-producing cells in vitro and in MOG/CFA-induced EAE [168]. In a brain hypoxia-ischemia (H/I) mouse model, the expression of TIM-3 (a member of the T cell immunoglobulin that downregulates the TH1-dependent immune response) increases in activated microglia and astrocytes (brain resident immune cells) depending on HIF-1α [170]. Blocking of TIM-3 reduces infarct size, neuronal death, edema formation, and neutrophil infiltration in H/I mice [170]. Other studies suggest that HIF-1α modulates T cell differentiation towards a Th17 cytokine-secreting phenotype. A decrease in HIF-1α resulted in reduced Th17 but enhanced T regulatory cell differentiation, protecting mice from autoimmune neuroinflammation [171]. Others reported that HIF-1α induced FoxP3+ Tregs during inflammation [172]. Likewise, in the EAE model, CD4+ cells decreased, and the CD4+CD25+FoxP3 Treg subset increased in the spinal cord of EAE mice exposed to chronic mild hypoxia compared with normoxic counterparts [173]. The increase in Trx-1 contributes to reducing Treg sensitivity to oxidative stress. Along with inflammatory stimuli, especially TNF-α, this dynamic negative feedback promotes Tregs in the inflammatory milieu to prevent a sustained or excessive immune response [145, 174]. Inflammatory mediators like cytokines dependent on the Th1, Th17 lymphocyte subpopulation [175], NO, or free radicals [176, 177] have been observed during clinical relapse phases in MS. Conversely, suppressive cellular activity by Th2, Th3, and Tr1 cells, in particular, has been reported during remission periods [178]. The increase in CD4+ and CD8+ T cells found in mouse models of AD [179] suggests an important contribution of T cells to disease pathogenesis [180]. Depleting Tregs enhanced T cell infiltration and reactive astrogliosis in a model of TBI, suggesting tissue damage modulation by Tregs following injury [181].

The evidence of the role of innate and adaptive immunity in neuroinflammation is conclusive. The key events triggering the pathology or charting the chronology of the early changes upon disease is yet to be clarified, even considering the vast literature available. The infiltration of immune cells, T cells, in particular, prompts further examining the role of adaptive immunity [179].

4. Fibrotic Reaction to Inflammation

The regulation of fibrotic processes in the CNS is little known. After an inflammatory response, the fibrotic reaction ensues the increase in extracellular matrix components. Different chronic inflammatory diseases with MS-resembling traits like psoriasis or rheumatoid arthritis present severe and intermittent progression with phases of acute exacerbation and remission. They show an influx of inflammatory cells (macrophages, granulocytes, and T cells) and increased expression of proinflammatory mediators, including those locally released by parenchyma cells. These diseases may nevertheless differ in their pathogenesis [182].

Eventually, inflammation rests, but massive fibrosis prevents fully restoring tissue integrity. Even three decades after identifying the master cytokine in immune regulation and fibrosis, we find it hard to ascribe only one role to TGF-β [183, 184].

Regulatory T cells (Tregs) release TGF, a potent cytokine that downregulates immune responses and is involved in tissue-specific repair and homeostasis [185].

Most responses to brain injury involve reactive gliosis, resident astrocyte hypertrophy, and neuron cell loss with fibrosis. Fibrosis engages fibrocytes and macrophages derived from the bone marrow. Fibrocytes and activated macrophage type 2- (M2-) microglia cells may act as profibrotic in the CNS as well [186].

The glial scar is a structural formation of reactive glia around a damaged area. Traditionally viewed as a hindrance to axon regeneration, beneficial functions of the glial scar have been recently reported. Discrepancies have been discussed on the functional heterogeneity of the glial scar cells, astrocytes, NG2 glia, and microglia (Figure 5). The NG2 glia regulates brain innate immunity via the TGF-β2/TGFBR2 axis [187]. After TBI, ischemic stroke, and neurodegenerative diseases, including MS, newly proliferated reactive astrocytes are observed. The NG2 glia and microglia round the severely damaged area or lesion core. This core presents perivascular-derived fibroblasts, pericytes, ependymal cells, and phagocytic macrophages. Previous studies have sometimes referred to the entire CNS lesion as the glial scar, leading to discrepancies. Different glial cells are associated with the lesion or fibrotic lesion core, rich in extracellular matrix proteins, inhibiting axonal growth and remyelination. Yet, some glial cell types counteract, and others regulate scar formation [188].

Immune neuroinflammation involves complex neural and immune cell interactions, regulating the balance between neural tissue repair and scar formation. Reactive microglia (RM) differentiation leads to microglial subpopulations, like macrophage differentiation pathways (inflammatory type M1 and anti-inflammatory type M2). M1 microglia induces A1 reactive astrocyte (RA), derived from a common precursor astrocyte (nervous stem cell abv-NSC-), which under certain signals differentiates to astrocyte cell phenotypes A1 (A1 astroglia are neurotoxic) and A2 (A2 astroglia are neuroprotective). The A1 astrocytes secrete a toxin that kills oligodendrocytes (OD). The A2 astrocytes promote axonal growth. The M2 microglia induces NG2 (neuron-glial antigen 2, also called oligodendrocyte precursor cells) glia differentiation to oligodendrocytes. In addition, NG2 glia regulates brain innate immunity via the TGF-β2/TGFB-R2 axis.

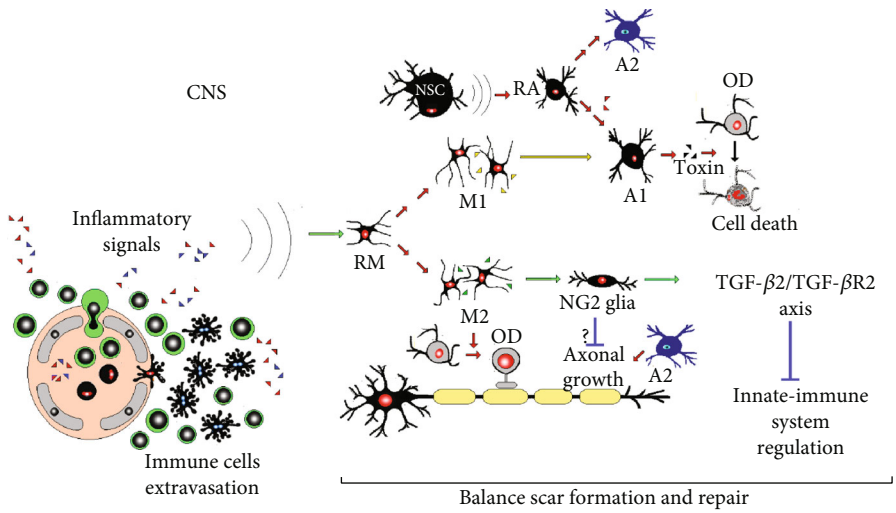

Figure 5: Complexity of brain cell interactions in scar formation and repair.

5. Conclusion

Classifying neuroinflammatory and neuroimmune reactions as beneficial or detrimental is an oversimplification. There is a myriad of interactions between diverse brain cell types and the triggered signaling cascades in different disorders. Various families of cytokines and cytokine receptors, cell-specific distribution, growth factors, and chemokines influence the apoptotic or survival pathways of neurons and the degree of inflammatory processes in the CNS. Even with the growing knowledge of neuroinflammation in health and disease, a deep comprehension of the underlying mechanisms in neuropathology remains limited.

Hypoxia interacts with inflammation at the molecular, cellular, and clinical levels. The immune system reacts to restore homeostasis in two crucial scenarios. One of them is hypoxic stress, causing cells to upregulate pathways involved in increasing oxygen supply. The other one is oxidative stress, causing cells to upregulate antioxidant pathways. Provided that oxygen homeostasis is achieved, epidermal cells survive, and inflammatory leucocytes die. Targeting oxygen-sensing mechanisms and hypoxia signaling pathways might aid in reducing inflammation. Oxidative stress and inflammation underlie most neurological disorders, whether neurodegenerative, autoimmune, traumatic, neoplastic, ischemic, metabolic, toxic, infectious, or other. All of them show direct and indirect immune-related neuroinflammation.

Targeting hypoxia-dependent signaling pathways might help to attenuate organ failure, reducing hypoxia-driven inflammation. Chronic and/or sustained inflammation and hypoxia lead to the survival of macrophages, which further releases oxidative and inflammatory mediators [189, 190].

Inflammatory conditions like meningeal infiltrations, meningoencephalitis with perivascular infiltrates, reactive gliosis, and inflammatory-necrotic lesions showed central immune interactions in different homeostatic alterations.

Regardless of the infective nature, or not, of the central insult, the immune-mediated neuroinflammation orchestrates the response of reactive CNS components to altered homeostasis. Unsuccessful restoration leads to disease, sometimes perpetuating neuroinflammation, and damage. Whether fibrogenesis should be disrupted or not is crucial to understand the pathogenesis and how to go ahead.

There is still a road to walk before a deep insight into underlying factors in pathogenesis allows for designing better treatments.

Abbreviations

AD:	Alzheimer's disease
ADCC:	Antibody-dependent cell-mediated cytotoxicity
APC:	Antigen-presenting cell
Atg:	Autophagy-related gene
ATIA:	Anti-TNF-α-induced apoptosis
BBB:	Blood-brain barrier
bFGF:	Basic fibroblast growth factor
BOL:	Bovine oligodendrocytes
$C3^{-/-}$:	Complement C3 deficient
CCL2:	C-C motif ligand 2
CCL3:	C-C motif ligand 3
CCR2:	Chemokine receptor 2
CD:	Cluster of differentiation
CD200:	Cluster of differentiation 200
CD4+Th17:	CD4+ T helper 17 cells
CD8+ T:	Cytotoxic T lymphocytes
ClAP1:	Cellular inhibitor of apoptosis protein-1
CLAP2:	Cellular inhibitor of apoptosis protein-2
CM:	Coccidioidal meningitis
CNS:	Central nervous system
COX2:	Cyclooxygenase 2
CCR:	C-C motif chemokine receptor
CSF:	Cerebrospinal fluid
CX3CL1:	C-X3-C motif ligand 1
CXC:	Cysteine X cysteine
CXCL10:	C-X-C motif chemokine ligand 10

CXCL11: C-X-C motif chemokine ligand 11
CXCL12: C-X-C motif chemokine ligand 12
CXCL9: C-X-C motif chemokine ligand 9
CXCR3: C-X-C motif chemokine receptor 3
DAMP: Damage-associated molecular pattern
DNA: Deoxyribonucleic acid
EAE: Experimental autoimmune encephalomyelitis
EBV: Epstein Barr virus
FGF-2: Fibroblast growth factor-2
FoxP3: Forkhead box P3
GPCR: G protein-coupled receptors
H/I: Hypoxia-ischemia
H19-7: Imprinted maternally expressed transcript (nonprotein coding)
HIF1-α: Hypoxia-inducible factor-1-alpha
HIV: Human immunodeficiency virus
HIV-tat: Human immunodeficiency virus transactivator of transcription
HPV: Herpes papillomavirus
IFN-γ: Interferon-gamma
IL-10: Interleukin-10
IL-18: Interleukin-18
IL-1R1: Interleukin-1 receptor-1
IL-1β: Interleukin-1 beta
IL-4: Interleukin-4
IL-6: Interleukin-6
INOS: Inducible nitric oxide synthase
JNK: c-Jun N-terminal kinase
K: Killer
K^+: Potassium
KA: Kainic acid
LPS: Lipopolysaccharide
LRRK2: Leucine-rich repeat kinase 2
M1: Macrophage type 1 (proinflammatory)
M2: Macrophage type 2 (anti-inflammatory)
MAVS: Mitochondrial antiviral signaling
MCP-1: Monocyte chemoattractant protein-1
M-CSF: Macrophage colony-stimulating factor
MEF: Mouse embryonic fibroblast
MHC-I: Major histocompatibility complex-I
MIP-2: Macrophage inflammatory protein-2
miRNA: MicroRNA
Miz1: Myc-interacting zinc-finger protein 1
MOG/CFA: Myelin oligodendrocyte glycoprotein/ complete Freund's adjuvant
MS: Multiple sclerosis
NF-κB: Nuclear factor kappa B
NG2: Neuron-glial antigen 2
NGF: Nerve growth factor
NK: Natural killer
NO: Nitric oxide
NOD: Nucleotide-binding oligomerization domain-containing protein
OD: Oligodendrocyte
op/op: Osteopetrotic
PAMP: Pathogen-associated molecular pattern
PD: Parkinson's disease
PGS: Prostaglandins
PI3K: Phosphatidylinositol 3-kinase
PLA2: Phospholipase A2
PRR: Pattern recognition receptor
RA: Reactive astrocyte
RAGE: Receptor for advanced glycation end products
RIP1: Receptor-interacting serine/threonine-protein kinase 1
RM: Reactive microglia
ROS: Reactive oxygen species
SCI: Spinal cord injury
SDF-1: Stromal cell-derived factor-1
SK-N-SH: A neuroblastoma cell line displaying epithelial morphology, growing in adherent culture
SN: Substantia nigra
SNCA: Synuclein gene-α
SP: Substance P
T cells: A type of lymphocyte
TBI: Traumatic brain injury
TD: Thiamine deficiency
TGF-β: Transforming growth factor-β
TGF-β2/TGFB-R2 axis: Transforming growth factor, beta receptor 2
Th1: T helper 1 cells
Th17: T helper 17 cells
TIM-3: T cell immunoglobulin and mucin-domain containing-3
TLR4: Toll-like receptor 4
TNF: Tumor necrosis factor
TNF-R1: Tumor necrosis factor, receptor 1
TNF-R2: Tumor necrosis factor, receptor 2
TRAF2: Tumor necrosis factor receptor-associated factor 2
TRAF5: Tumor necrosis factor receptor-associated factor 5
Treg: Regulatory T cells
TREM: Triggering receptor expressed on myeloid cells
TRX2: Thioredoxin 2
VIP: Vasointestinal peptide
$\gamma\delta$ T cells: Gamma delta T cells.

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The funding body had no role in writing this manuscript.

Conflicts of Interest

The authors declare that they have no competing interests.

Authors' Contributions

RKF and LU contributed in design and writing of the original draft; MOL contributed in supervision, revision, and editing—grammar, style, and language; TK, MIH, JP, and GR contributed in revision; FC contributed in funding acquisition and revision. Rodolfo Kölliker-Frers and Lucas Udovin contributed equally to this work.

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References

[1] R. J. Miller, W. Rostene, E. Apartis et al., "Chemokine action in the nervous system," *Journal of Neuroscience*, vol. 28, no. 46, pp. 11792–11795, 2008.

[2] U. Gimsa, N. A. Mitchison, and M. C. Brunner-Weinzierl, "Immune privilege as an intrinsic CNS property: astrocytes protect the CNS against T-cell-mediated neuroinflammation," *Mediators of Inflammation*, vol. 2013, Article ID 320519, 11 pages, 2013.

[3] H. Fernandez, A. Cevallos, R. Jimbo Sotomayor, F. Naranjo-Saltos, D. Mera Orces, and E. Basantes, "Mental disorders in systemic lupus erythematosus: a cohort study," *Rheumatology International*, vol. 39, no. 10, pp. 1689–1695, 2019.

[4] R. B. Abd-Ellatief, H. K. Mohamed, and H. I. Kotb, "Reactive astrogliosis in an experimental model of fibromyalgia: effect of dexmedetomidine," *Cells, Tissues, Organs*, vol. 205, no. 2, pp. 105–119, 2018.

[5] R. Alonso, A. M. Fernández-Fernández, D. Pisa, and L. Carrasco, "Multiple sclerosis and mixed microbial infections. Direct identification of fungi and bacteria in nervous tissue," *Neurobiology of Disease*, vol. 117, pp. 42–61, 2018.

[6] M. Oono, Y. Fujita, N. Uchida et al., "Rheumatoid meningitis developed in patient with stable rheumatoid arthritis and myasthenia gravis-detailed analysis of intracranial inflammation using flow cytometry," *Journal of Neuroinflammation*, vol. 15, no. 1, p. 151, 2018.

[7] R. Alonso, D. Pisa, A. M. Fernández-Fernández, A. Rábano, and L. Carrasco, "Fungal infection in neural tissue of patients with amyotrophic lateral sclerosis," *Neurobiology of Disease*, vol. 108, pp. 249–260, 2017.

[8] R. M. McManus and M. T. Heneka, "Role of neuroinflammation in neurodegeneration: new insights," *Alzheimer's Research & Therapy*, vol. 9, no. 1, p. 14, 2017.

[9] M. Tiwari, "The role of serratiopeptidase in the resolution of inflammation," *Asian Journal of Pharmaceutical Sciences*, vol. 12, no. 3, pp. 209–215, 2017.

[10] M. Deleidi and T. Gasser, "The role of inflammation in sporadic and familial Parkinson's disease," *Cellular and Molecular Life Sciences*, vol. 70, no. 22, pp. 4259–4273, 2013.

[11] T. P. Johnson and A. Nath, "Neurological syndromes driven by postinfectious processes or unrecognized persistent infections," *Current Opinion in Neurology*, vol. 31, no. 3, pp. 318–324, 2018.

[12] Y. Wu, X. Xu, Z. Chen et al., "Nervous system involvement after infection with COVID-19 and other coronaviruses," *Brain, Behavior, and Immunity*, vol. 87, no. 20, pp. 18–22, 2020.

[13] P. S. Sahu and E. Ter, "Interactions between neurotropic pathogens, neuroinflammatory pathways, and autophagic neural cell death," *Neuroimmunology and Neuroinflammation*, vol. 5, no. 1, p. 2, 2018.

[14] G. W. Koutsouras, R. L. Ramos, and L. R. Martinez, "Role of microglia in fungal infections of the central nervous system," *Virulence*, vol. 8, no. 6, pp. 705–718, 2017.

[15] D. Wang, "Astroviruses – emerging neurotropic pathogens," *Journal of the Pediatric Infectious Diseases Society*, vol. 5, no. 2, pp. 188-189, 2016.

[16] A. N. Peón, Y. Ledesma-Soto, J. E. Olguín, M. Bautista-Donis, E. Sciutto, and L. I. Terrazas, "Helminth products potently modulate experimental autoimmune encephalomyelitis by downregulating neuroinflammation and promoting a suppressive microenvironment," *Mediators of Inflammation*, vol. 2017, Article ID 8494572, 16 pages, 2017.

[17] J. Oliveira-Filho, R. M. Vieira-de-Melo, P. S. O. Reis et al., "Chagas disease is independently associated with brain atrophy," *Journal of Neurology*, vol. 256, no. 8, pp. 1363–1365, 2009.

[18] J. Rodgers, I. Steiner, and P. G. E. Kennedy, "Generation of neuroinflammation in human African trypanosomiasis," *Neurology - Neuroimmunology Neuroinflammations*, vol. 6, no. 6, article e610, 2019.

[19] M. Bonnan, B. Barroso, S. Demasles, E. Krim, R. Marasescu, and M. Miquel, "Compartmentalized intrathecal immunoglobulin synthesis during HIV infection – A model of chronic CNS inflammation?," *Journal of Neuroimmunology*, vol. 285, pp. 41–52, 2015.

[20] Y. Lin, T. M. Yang, J. W. Lin, M. Z. Song, and T. C. Lee, "Posterior fossa intracranial inflammatory pseudotumor: a case report and literature review," *Surgical Neurology*, vol. 72, no. 6, pp. 712–716, 2009.

[21] P. W. French, R. Ludowyke, and G. J. Guillemin, "Fungal neurotoxins and sporadic amyotrophic lateral sclerosis," *Neurotoxicity Research*, vol. 35, no. 4, pp. 969–980, 2019.

[22] R. Lang, W. Stokes, J. Lemaire, A. Johnson, and J. Conly, "A case report of Coccidioides posadasii meningoencephalitis in an immunocompetent host," *BMC Infectious Diseases*, vol. 19, no. 1, p. 722, 2019.

[23] T. K. Kourkoumpetis, A. Desalermos, M. Muhammed, and E. Mylonakis, "Central nervous system aspergillosis: a series of 14 cases from a general hospital and review of 123 cases from the literature," *Medicine*, vol. 91, no. 6, pp. 328–336, 2012.

[24] A. M. M. Orellana, A. R. Vasconcelos, J. A. Leite et al., "Age-related neuroinflammation and changes in AKT-GSK-3β and WNT/β-catenin signaling in rat hippocampus," *Aging*, vol. 7, no. 12, pp. 1094–1108, 2015.

[25] S. Castro-Gomez, J. Binder, and M. T. Heneka, "Neuroinflammation as motor of Alzheimer's disease," *Nervenarzt*, vol. 90, no. 9, pp. 898–906, 2019.

[26] B. Fleiss and P. Gressens, "Tertiary mechanisms of brain damage: a new hope for treatment of cerebral palsy?," *Lancet Neurology*, vol. 11, no. 6, pp. 556–566, 2012.

[27] W. C. Pierre, P. L. P. Smith, I. Londono, S. Chemtob, C. Mallard, and G. A. Lodygensky, "Neonatal microglia: the

cornerstone of brain fate," *Brain, Behavior, and Immunity*, vol. 59, pp. 333–345, 2017.

[28] R. C. Paolicelli, G. Bolasco, F. Pagani et al., "Synaptic pruning by microglia is necessary for normal brain development," *Science*, vol. 333, no. 6048, pp. 1456–1458, 2011.

[29] N. Hammond, A. B. Munkacsi, and S. L. Sturley, "The complexity of a monogenic neurodegenerative disease: More than two decades of therapeutic driven research into Niemann-Pick type C disease," *Biochimica et Biophysica Acta - Molecular and Cell Biology of Lipids*, vol. 1864, no. 8, pp. 1109–1123, 2019.

[30] N. Plesnila, "The immune system in traumatic brain injury," *Current Opinion in Pharmacology*, vol. 26, pp. 110–117, 2016.

[31] T. J. Krämer, N. Hack, T. J. Brühl et al., "Depletion of regulatory T cells increases T cell brain infiltration, reactive astrogliosis, and interferon-γ gene expression in acute experimental traumatic brain injury," *Journal of Neuroinflammation*, vol. 16, no. 1, p. 163, 2019.

[32] S. Iannaccone, C. Cerami, M. Alessio et al., "_In vivo_ microglia activation in very early dementia with Lewy bodies, comparison with Parkinson's disease," *Parkinsonism & Related Disorders*, vol. 19, no. 1, pp. 47–52, 2013.

[33] Y. Ouchi, E. Yoshikawa, Y. Sekine et al., "Microglial activation and dopamine terminal loss in early Parkinson's disease," *Annals of Neurology*, vol. 57, no. 2, pp. 168–175, 2005.

[34] M. B. Harms, D. Neumann, B. A. Benitez et al., "Parkinson disease is not associated with *C9ORF72* repeat expansions," *Neurobiology of Aging*, vol. 34, no. 5, pp. 1519.e1–1519.e2, 2013.

[35] F. Gillardon, R. Schmid, and H. Draheim, "Parkinson's disease-linked leucine-rich repeat kinase 2(R1441G) mutation increases proinflammatory cytokine release from activated primary microglial cells and resultant neurotoxicity," *Neuroscience*, vol. 208, pp. 41–48, 2012.

[36] M. S. Moehle, P. J. Webber, T. Tse et al., "LRRK2 inhibition attenuates microglial inflammatory responses," *The Journal of Neuroscience*, vol. 32, no. 5, pp. 1602–1611, 2012.

[37] X. Su and H. J. Federoff, "Immune responses in Parkinson's disease: interplay between central and peripheral immune systems," *Biomedical Research*, vol. 2014, article 275178, pp. 1–9, 2014.

[38] M. L. Block, L. Zecca, and J. S. Hong, "Microglia-mediated neurotoxicity: uncovering the molecular mechanisms," *Nature Reviews Neuroscience*, vol. 8, no. 1, pp. 57–69, 2007.

[39] A. A. Baburamani, V. G. Supramaniam, H. Hagberg, and C. Mallard, "Microglia toxicity in preterm brain injury," *Reproductive Toxicology*, vol. 48, pp. 106–112, 2014.

[40] V. G. Supramaniam, R. Vontell, L. Srinivasan, J. Wyatt-Ashmead, H. Hagberg, and M. Rutherford, "Microglia activation in the extremely preterm human brain," *Pediatric Research*, vol. 73, no. 3, pp. 301–309, 2013.

[41] H. Maj, C. Mallard, and H. Hagberg, "Inflammatory gene profiling in the developing mouse brain after hypoxia-ischemia," *Journal of Cerebral Blood Flow and Metabolism*, vol. 24, no. 12, pp. 1333–1351, 2004.

[42] A. Mottahedin, M. Ardalan, T. Chumak, I. Riebe, J. Ek, and C. Mallard, "Effect of neuroinflammation on synaptic organization and function in the developing brain: implications for neurodevelopmental and neurodegenerative disorders," *Frontiers in Cellular Neuroscience*, vol. 11, p. 190, 2017.

[43] M. M. Riccomagno and A. L. Kolodkin, "Sculpting neural circuits by Axon and dendrite pruning," *Annual Review of Cell and Developmental Biology*, vol. 31, no. 1, pp. 779–805, 2015.

[44] L. M. Healy, M. Yaqubi, S. Ludwin, and J. P. Antel, "Species differences in immune-mediated CNS tissue injury and repair: a (neuro) inflammatory topic," *Glia*, vol. 68, no. 4, pp. 811–829, 2020.

[45] L. D. S. Chagas, P. Trindade, A. L. T. Gomes et al., "Rapid plasticity of intact axons following a lesion to the visual pathways during early brain development is triggered by microglial activation," *Experimental Neurology*, vol. 311, pp. 148–161, 2019.

[46] R. M. Ransohoff and J. El Khoury, "Microglia in health and disease," *Cold Spring Harbor Perspectives in Biology*, vol. 8, no. 1, article a020560, 2016.

[47] C. H. Heldin, B. Lu, R. Evans, and J. S. Gutkind, "Signals and receptors," *Cold Spring Harbor Perspectives in Biology*, vol. 8, no. 4, article a005900, 2016.

[48] G. Ramesh, A. G. MacLean, and M. T. Philipp, "Cytokines and chemokines at the crossroads of neuroinflammation, neurodegeneration, and neuropathic pain," *Mediators of Inflammation*, vol. 2013, Article ID 480739, 20 pages, 2013.

[49] G. Conductier, N. Blondeau, A. Guyon, J. L. Nahon, and C. Rovere, "The role of monocyte chemoattractant protein MCP1/CCL2 in neuroinflammatory diseases," *Journal of Neuroimmunology*, vol. 224, no. 1-2, pp. 93–100, 2010.

[50] G. Yang, Y. Meng, W. Li et al., "Neuronal MCP-1 mediates microglia recruitment and neurodegeneration induced by the mild impairment of oxidative metabolism," *Brain Pathology*, vol. 21, no. 3, pp. 279–297, 2011.

[51] L. W. Chen, L. Egan, Z. W. Li, F. R. Greten, M. F. Kagnoff, and M. Karin, "The two faces of IKK and NF-κB inhibition: prevention of systemic inflammation but increased local injury following intestinal ischemia- reperfusion," *Nature Medicine*, vol. 9, no. 5, pp. 575–581, 2003.

[52] P. M. Hughes, P. R. Allegrini, M. Rudin, V. H. Perry, A. K. Mir, and C. Wiessner, "Monocyte chemoattractant protein-1 deficiency is protective in a murine stroke model," *Journal of Cerebral Blood Flow and Metabolism*, vol. 22, no. 3, pp. 308–317, 2002.

[53] O. B. Dimitrijevic, S. M. Stamatovic, R. F. Keep, and A. V. Andjelkovic, "Absence of the chemokine receptor CCR2 protects against cerebral ischemia/reperfusion injury in mice," *Stroke*, vol. 38, no. 4, pp. 1345–1353, 2007.

[54] G. Tang, D. N. Charo, R. Wang, I. F. Charo, and L. Messina, "CCR2$^{-/-}$ knockout mice revascularize normally in response to severe hindlimb ischemia," *Journal of Vascular Surgery*, vol. 40, no. 4, pp. 786–795, 2004.

[55] W. L. Thompson, W. J. Karpus, and L. J. Van Eldik, "MCP-1-deficient mice show reduced neuroinflammatory responses and increased peripheral inflammatory responses to peripheral endotoxin insult," *Journal of Neuroinflammation*, vol. 5, no. 1, p. 35, 2008.

[56] R. H. Andres, R. Choi, A. V. Pendharkar et al., "The CCR2/CCL2 interaction mediates the transendothelial recruitment of intravascularly delivered neural stem cells to the ischemic brain," *Stroke*, vol. 42, no. 10, pp. 2923–2931, 2011.

[57] E. A. Eugenin, T. G. D'Aversa, L. Lopez, T. M. Calderon, and J. W. Berman, "MCP-1 (CCL2) protects human neurons and

astrocytes from NMDA or HIV-tat-induced apoptosis," *Journal of Neurochemistry*, vol. 85, no. 5, pp. 1299–1311, 2003.

[58] A. N. Kalehua, J. E. Nagel, L. M. Whelchel et al., "Monocyte chemoattractant protein-1 and macrophage inflammatory protein-2 are involved in both excitotoxin-induced neurodegeneration and regeneration," *Experimental Cell Research*, vol. 297, no. 1, pp. 197–211, 2004.

[59] B. D. Semple, T. Frugier, and M. C. Morganti-Kossmann, "CCL2 modulates cytokine production in cultured mouse astrocytes," *Journal of Neuroinflammation*, vol. 7, no. 1, p. 67, 2010.

[60] O. M. Koper, J. Kaminska, K. Sawicki, and H. Kemona, "CXCL9, CXCL10, CXCL11, and their receptor (CXCR3) in neuroinflammation and neurodegeneration," *Advances in Clinical and Experimental Medicine*, vol. 27, no. 6, pp. 849–856, 2018.

[61] M. Erta, A. Quintana, and J. Hidalgo, "Interleukin-6, a major cytokine in the central nervous system," *International Journal of Biological Sciences*, vol. 8, no. 9, pp. 1254–1266, 2012.

[62] K. Frei, T. P. Leist, A. Meager et al., "Production of B cell stimulatory factor-2 and interferon gamma in the central nervous system during viral meningitis and encephalitis. Evaluation in a murine model infection and in patients," *The Journal of Experimental Medicine*, vol. 168, no. 1, pp. 449–453, 1988.

[63] G. E. Grau, K. Frei, P. F. Piguet et al., "Interleukin 6 production in experimental cerebral malaria: modulation by anticytokine antibodies and possible role in hypergammaglobulinemia," *The Journal of Experimental Medicine*, vol. 172, no. 5, pp. 1505–1508, 1990.

[64] T. Kossmann, V. H. Hans, H. G. Imhof et al., "Intrathecal and serum interleukin-6 and the acute-phase response in patients with severe traumatic brain injuries," *Shock*, vol. 4, no. 5, pp. 311–317, 1995.

[65] S. R. Pagliusi, M. Schachner, P. H. Seeburg, and B. D. Shivers, "The adhesion molecule on glia (AMOG) is widely expressed by astrocytes in developing and adult mouse brain," *The European Journal of Neuroscience*, vol. 2, no. 5, pp. 471–480, 1990.

[66] G. Olmos and J. Llado, "Tumor necrosis factor alpha: a link between neuroinflammation and excitotoxicity," *Mediators of Inflammation*, vol. 2014, Article ID 861231, 12 pages, 2014.

[67] S. M. Lucas, N. J. Rothwell, and R. M. Gibson, "The role of inflammation in CNS injury and disease," *British Journal of Pharmacology*, vol. 147, Supplement 1, pp. S232–S240, 2006.

[68] E. Lee, S. Chanamara, D. Pleasure, and A. M. Soulika, "IFN-γ signaling in the central nervous system controls the course of experimental autoimmune encephalomyelitis independently of the localization and composition of inflammatory foci," *Journal of Neuroinflammation*, vol. 9, no. 1, 2012.

[69] L. Wang, Y. Chang, S. Y. Chen et al., "TNFR1-JNK signaling is the shared pathway of neuroinflammation and neurovascular damage after LPS-sensitized hypoxic-ischemic injury in the immature brain," *Journal of Neuroinflammation*, vol. 11, no. 1, p. 215, 2014.

[70] M. Kumar, S. Verma, and V. R. Nerurkar, "Pro-inflammatory cytokines derived from West Nile virus (WNV)-infected SK-N-SH cells mediate neuroinflammatory markers and neuronal death," *Journal of Neuroinflammation*, vol. 7, no. 1, p. 73, 2010.

[71] H. Abo-ouf, A. W. M. Hooper, E. J. White, H. J. Janse van Rensburg, B. L. Trigatti, and S. A. Igdoura, "Deletion of tumor necrosis factor-α ameliorates neurodegeneration in Sandhoff disease mice," *Human Molecular Genetics*, vol. 22, no. 19, pp. 3960–3975, 2013.

[72] M. O. Lu, R. S. Duan, H. C. Quezada et al., "Aggravation of experimental autoimmune neuritis in TNF-α receptor 1 deficient mice," *Journal of Neuroimmunology*, vol. 186, no. 1-2, pp. 19–26, 2007.

[73] H. Wajant and D. Siegmund, "TNFR1 and TNFR2 in the control of the life and death balance of macrophages," *Frontiers in Cell and Development Biology*, vol. 7, p. 91, 2019.

[74] L. Marchetti, M. Klein, K. Schlett, K. Pfizenmaier, and U. L. M. Eisel, "Tumor Necrosis Factor (TNF)-mediated Neuroprotection against Glutamate-induced Excitotoxicity Is Enhanced by _N_ -Methyl-D-aspartate Receptor Activation:," *The Journal of Biological Chemistry*, vol. 279, no. 31, pp. 32869–32881, 2004.

[75] V. Baud and K. Karin, "Signal transduction by tumor necrosis factor and its relatives," *Trends in Cell Biology*, vol. 11, no. 9, pp. 372–377, 2001.

[76] E. Shohami, I. Ginis, and J. M. Hallenbeck, "Dual role of tumor necrosis factor alpha in brain injury," *Cytokine & Growth Factor Reviews*, vol. 10, no. 2, pp. 119–130, 1999.

[77] A. Basu, J. K. Krady, and S. W. Levison, "Interleukin-1: a master regulator of neuroinflammation," *Journal of Neuroscience Research*, vol. 78, no. 2, pp. 151–156, 2004.

[78] N. J. Rothwell and G. N. Luheshi, "Interleukin 1 in the brain: biology, pathology and therapeutic target," *Trends in Neurosciences*, vol. 23, no. 12, pp. 618–625, 2000.

[79] A. Basu, J. K. Krady, M. O'Malley, S. D. Styren, S. T. DeKosky, and S. W. Levison, "The type 1 interleukin-1 receptor is essential for the efficient activation of microglia and the induction of multiple proinflammatory mediators in response to brain injury," *The Journal of Neuroscience*, vol. 22, no. 14, pp. 6071–6082, 2002.

[80] N. Degousee, E. Stefanski, T. F. Lindsay et al., "p38 MAPK Regulates Group IIa Phospholipase A_2Expression in Interleukin-1β-stimulated Rat Neonatal Cardiomyocytes," *The Journal of Biological Chemistry*, vol. 276, no. 47, pp. 43842–43849, 2001.

[81] H. W. Lin, A. Basu, C. Druckman, M. Cicchese, J. K. Krady, and S. W. Levison, "Astrogliosis is d. elayed in type 1 interleukin-1 receptor-null mice following a penetrating brain injury," *Journal of Neuroinflammation*, vol. 3, no. 1, p. 15, 2006.

[82] H. Boutin, R. A. LeFeuvre, R. Horai, M. Asano, Y. Iwakura, and N. J. Rothwell, "Role of IL-1alpha and IL-1beta in ischemic brain damage," *The Journal of Neuroscience*, vol. 21, no. 15, pp. 5528–5534, 2001.

[83] S. Rossi, V. Studer, C. Motta et al., "Cerebrospinal fluid detection of interleukin-1β in phase of remission predicts disease progression in multiple sclerosis," *Journal of Neuroinflammation*, vol. 11, no. 1, p. 32, 2014.

[84] F. Boato, D. Hechler, K. Rosenberger et al., "Interleukin-1 beta and neurotrophin-3 synergistically promote neurite growth in vitro," *Journal of Neuroinflammation*, vol. 8, no. 1, p. 183, 2011.

[85] H. B. Stolp, "Neuropoietic cytokines in normal brain development and neurodevelopmental disorders," *Molecular and Cellular Neurosciences*, vol. 53, pp. 63–68, 2013.

[86] H. H. Andersen, K. B. Johnsen, and T. Moos, "Iron deposits in the chronically inflamed central nervous system and contributes to neurodegeneration," *Cellular and Molecular Life Sciences*, vol. 71, no. 9, pp. 1607–1622, 2014.

[87] J. Presumey, A. R. Bialas, and M. C. Carroll, "Complement system in neural synapse elimination in development and disease," *Advances in Immunology*, vol. 135, pp. 53–79, 2017.

[88] A. R. Bialas and B. Stevens, "TGF-β signaling regulates neuronal C1q expression and developmental synaptic refinement," *Nature Neuroscience*, vol. 16, no. 12, pp. 1773–1782, 2013.

[89] C. Luchena, J. Zuazo-Ibarra, E. Alberdi, C. Matute, and E. Capetillo-Zarate, "Contribution of neurons and glial cells to complement-mediated synapse removal during development, aging and in Alzheimer's disease," *Mediators of Inflammation*, vol. 2018, Article ID 2530414, 12 pages, 2018.

[90] R. Veerhuis, H. M. Nielsen, and A. J. Tenner, "Complement in the brain," *Molecular Immunology*, vol. 48, no. 14, pp. 1592–1603, 2011.

[91] A. J. Tenner, "Complement in Alzheimer's disease: opportunities for modulating protective and pathogenic events," *Neurobiology of Aging*, vol. 22, no. 6, pp. 849–861, 2001.

[92] Y. Shen, R. Li, E. G. McGeer, and P. L. McGeer, "Neuronal expression of mRNAs for complement proteins of the classical pathway in Alzheimer brain," *Brain Research*, vol. 769, no. 2, pp. 391–395, 1997.

[93] B. J. Cummings, C. J. Pike, R. Shankle, and C. W. Cotman, "β-amyloid deposition and other measures of neuropathology predict cognitive status in Alzheimer's disease," *Neurobiology of Aging*, vol. 17, no. 6, pp. 921–933, 1996.

[94] A. Alawieh, A. Elvington, and S. Tomlinson, "Complement in the homeostatic and ischemic brain," *Frontiers in Immunology*, vol. 6, p. 417, 2015.

[95] S. L. Peterson and A. J. Anderson, "Complement and spinal cord injury: traditional and non-traditional aspects of complement cascade function in the injured spinal cord microenvironment," *Experimental Neurology*, vol. 258, pp. 35–47, 2014.

[96] P. Proitsi, M. K. Lupton, F. Dudbridge et al., "Alzheimer's disease and age-related macular degeneration have different genetic models for complement gene variation," *Neurobiology of Aging*, vol. 33, no. 8, pp. 1843.e9–1843.e17, 2012.

[97] M. W. Ma, J. Wang, Q. Zhang et al., "NADPH oxidase in brain injury and neurodegenerative disorders," *Molecular Neurodegeneration*, vol. 12, no. 1, p. 7, 2017.

[98] B. M. Bellander, S. K. Singhrao, M. Ohlsson, P. Mattsson, and M. Svensson, "Complement activation in the human brain after traumatic head injury," *Journal of Neurotrauma*, vol. 18, no. 12, pp. 1295–1311, 2001.

[99] S. E. Stoltzner, T. J. Grenfell, C. Mori et al., "Temporal Accrual of Complement Proteins in Amyloid Plaques in Down's Syndrome with Alzheimer's Disease," *The American Journal of Pathology*, vol. 156, no. 2, pp. 489–499, 2000.

[100] S. K. Singhrao, J. W. Neal, B. P. Morgan, and P. Gasque, "Increased Complement Biosynthesis By Microglia and Complement Activation on Neurons in Huntington's Disease," *Experimental Neurology*, vol. 159, no. 2, pp. 362–376, 1999.

[101] T. Yamada, P. L. McGeer, and E. G. McGeer, "Lewy bodies in Parkinson's disease are recognized by antibodies to complement proteins," *Acta Neuropathologica*, vol. 84, no. 1, pp. 100–104, 1992.

[102] G. Li, R. M. Fan, J. L. Chen et al., "Neuroprotective effects of argatroban and C5a receptor antagonist (PMX53) following intracerebral haemorrhage," *Clinical and Experimental Immunology*, vol. 175, no. 2, pp. 285–295, 2014.

[103] A. H. Stephan, B. A. Barres, and B. Stevens, "The complement system: an unexpected role in synaptic pruning during development and disease," *Annual Review of Neuroscience*, vol. 35, no. 1, pp. 369–389, 2012.

[104] F. Ginhoux, S. Lim, G. Hoeffel, D. Low, and T. Huber, "Origin and differentiation of microglia," *Frontiers in Cellular Neuroscience*, vol. 7, 45 pages, 2013.

[105] N. A. Flaris, T. L. Densmore, M. C. Molleston, and W. F. Hickey, "Characterization of microglia and macrophages in the central nervous system of rats: definition of the differential expression of molecules using standard and novel monoclonal antibodies in normal CNS and in four models of parenchymal reaction," *Glia*, vol. 7, no. 1, pp. 34–40, 1993.

[106] D. Gomez-Nicola and V. H. Perry, "Microglial dynamics and role in the healthy and diseased brain: a paradigm of functional plasticity," *The Neuroscientist*, vol. 21, no. 2, pp. 169–184, 2015.

[107] L. Tian, L. Ma, T. Kaarela, and Z. Li, "Neuroimmune crosstalk in the central nervous system and its significance for neurological diseases," *Journal of Neuroinflammation*, vol. 9, no. 1, p. 155, 2012.

[108] Y. Nakagawa and K. Chiba, "Role of microglial m1/m2 polarization in relapse and remission of psychiatric disorders and diseases," *Pharmaceuticals (Basel)*, vol. 7, no. 12, pp. 1028–1048, 2014.

[109] V. Pons and S. Rivest, "New therapeutic avenues of mCSF for brain diseases and injuries," *Frontiers in Cellular Neuroscience*, vol. 12, p. 499, 2018.

[110] A. Sasaki, H. Yokoo, M. Naito, C. Kaizu, L. D. Shultz, and Y. Nakazato, "Effects of macrophage-colony-stimulating factor deficiency on the maturation of microglia and brain macrophages and on their expression of scavenger receptor," *Neuropathology*, vol. 20, no. 2, pp. 134–142, 2000.

[111] J. D. Cherry, J. A. Olschowka, and M. O'Banion, "Neuroinflammation and M2 microglia: the good, the bad, and the inflamed," *Journal of Neuroinflammation*, vol. 11, no. 1, p. 98, 2014.

[112] M. J. Carson, J. Cameron Thrash, and B. Walter, "The cellular response in neuroinflammation: the role of leukocytes, microglia and astrocytes in neuronal death and survival," *Clinical Neuroscience Research*, vol. 6, no. 5, pp. 237–245, 2006.

[113] M. Pekny, M. Pekna, A. Messing et al., "Astrocytes: a central element in neurological diseases," *Acta Neuropathologica*, vol. 131, no. 3, pp. 323–345, 2016.

[114] R. A. Kölliker Frers, *Inmunología, Inmunopatogenia y Fundamentos Clínico-Terapéuticos*, Buenos Aires, Corpus Libros Médicos y Científicos, 1st edition, 2016.

[115] L. Peferoen, M. Kipp, P. van der Valk, J. M. van Noort, and S. Amor, "Oligodendrocyte-microglia cross-talk in the central nervous system," *Immunology*, vol. 141, no. 3, pp. 302–313, 2014.

[116] K. M. Omari, G. R. John, S. C. Sealfon, and C. S. Raine, "CXC chemokine receptors on human oligodendrocytes: implications for multiple sclerosis," *Brain*, vol. 128, no. 5, pp. 1003–1015, 2005.

[117] E. N. Benveniste, "Role of macrophages/microglia in multiple sclerosis and experimental allergic encephalomyelitis," *Journal of Molecular Medicine*, vol. 75, no. 3, pp. 165–173, 1997.

[118] P. T. Massa, K. Ozato, and D. E. McFarlin, "Cell type-specific regulation of major histocompatibility complex (MHC) class I gene expression in astrocytes, oligodendrocytes, and neurons," *Glia*, vol. 8, no. 3, pp. 201–207, 1993.

[119] B. Cannella and C. S. Raine, "Multiple sclerosis: cytokine receptors on oligodendrocytes predict innate regulation," *Annals of Neurology*, vol. 55, no. 1, pp. 46–57, 2004.

[120] C. G. Bien, J. Bauer, T. L. Deckwerth et al., "Destruction of neurons by cytotoxic T cells: a new pathogenic mechanism in rasmussen's encephalitis," *Annals of Neurology*, vol. 51, no. 3, pp. 311–318, 2002.

[121] A. Jurewicz, W. E. Biddison, and J. P. Antel, "MHC class I-restricted lysis of human oligodendrocytes by myelin basic protein peptide-specific CD8 T lymphocytes," *Journal of Immunology*, vol. 160, no. 6, pp. 3056–3059, 1998.

[122] M. S. Freedman, T. C. Ruijs, L. K. Selin, and J. P. Antel, "Peripheral blood gamma-delta T cells lyse fresh human brain-derived oligodendrocytes," *Annals of Neurology*, vol. 30, no. 6, pp. 794–800, 1991.

[123] R. H. Morse, R. Séguin, E. L. McCrea, and J. P. Antel, "NK cell-mediated lysis of autologous human oligodendrocytes," *Journal of Neuroimmunology*, vol. 116, no. 1, pp. 107–115, 2001.

[124] J. Satoh, S. U. Kim, and L. F. Kastrukoff, "Cytolysis of oligodendrocytes is mediated by killer (K) cells but not by natural killer (NK) cells," *Journal of Neuroimmunology*, vol. 31, no. 3, pp. 199–210, 1991.

[125] C. Zhang, L. Li, K. Feng, D. Fan, W. Xue, and J. Lu, "'Repair' Treg cells in tissue injury," *Cellular Physiology and Biochemistry*, vol. 43, no. 6, pp. 2155–2169, 2017.

[126] Y. Zhou, J. Jin, M. Feng, and D. Zhu, "Wnt signaling in inflammation in tissue repair and regeneration," *Current Protein & Peptide Science*, vol. 20, no. 8, pp. 829–843, 2019.

[127] H. Cui, W. Han, L. Yang, and Y. Chang, "Expression of hypoxia-inducible factor 1 alpha and oligodendrocyte lineage gene-1 in cultured brain slices after oxygen-glucose deprivation," *Neural Regeneration Research*, vol. 8, no. 4, pp. 328–337, 2013.

[128] C. T. Taylor and S. P. Colgan, "Regulation of immunity and inflammation by hypoxia in immunological niches," *Nature Reviews Immunology*, vol. 17, no. 12, pp. 774–785, 2017.

[129] H. K. Eltzschig and P. Carmeliet, "Hypoxia and inflammation," *The New England Journal of Medicine*, vol. 364, pp. 656–665, 2011.

[130] R. Chen, U. H. Lai, L. Zhu, A. Singh, M. Ahmed, and N. R. Forsyth, "Reactive oxygen species formation in the brain at different oxygen levels: the role of hypoxia inducible factors," *Frontiers in Cell and Development Biology*, vol. 6, p. 132, 2018.

[131] D. Bandarra, J. Biddlestone, S. Mudie, H. A. Müller, and S. Rocha, "HIF-1α restricts NF-κB-dependent gene expression to control innate immunity signals," *Disease Models & Mechanisms*, vol. 8, pp. 169–181, 2015.

[132] M. P. W. Grocott, D. S. Martin, D. Z. H. Levett, R. McMorrow, J. Windsor, and H. E. Montgomery, "Arterial blood gases and oxygen content in climbers on Mount Everest," *The New England Journal of Medicine*, vol. 360, pp. 140–149, 2009.

[133] G. L. Semenza, "Life with oxygen," *Science*, vol. 318, pp. 62–64, 2007.

[134] G. Hartmann, M. Tschöp, R. Fischer et al., "High altitude increases circulating interleukin-6, interleukin-1 receptor antagonist and c-reactive protein," *Cytokine*, vol. 12, no. 3, pp. 246–252, 2000.

[135] C. T. Taylor, "Interdependent roles for hypoxia inducible factor and nuclear factor-κB in hypoxic inflammation," *The Journal of Physiology*, vol. 586, pp. 4055–4059, 2008.

[136] A. Lin, "ATIA: a link between inflammation and hypoxia," *Molecular Cell*, vol. 42, no. 5, pp. 557-558, 2011.

[137] E. P. Cummins, E. Berra, K. M. Comerford et al., "Prolyl hydroxylase-1 negatively regulates IkappaB kinase-beta, giving insight into hypoxia-induced NFκB activity," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 103, pp. 18154–18159, 2006.

[138] D. Chen, Y. Ding, B. Schröppel et al., "Differential chemokine and chemokine receptor gene induction by ischemia, alloantigen, and gene transfer in cardiac grafts," *American Journal of Transplantation*, vol. 3, no. 10, pp. 1216–1229, 2003.

[139] S. Choksi, Y. Lin, Y. Pobezinskaya et al., "A HIF-1 target, ATIA, protects cells from apoptosis by modulating the mitochondrial thioredoxin, TRX2," *Molecular Cell*, vol. 42, no. 5, pp. 597–609, 2011.

[140] C. H. Emmerich, A. C. Schmukle, T. L. Haas et al., "The linear ubiquitin chain assembly complex forms part of the TNF-R1 signalling complex and is required for effective TNF-induced gene induction and prevents TNF-induced apoptosis," *Advances in Experimental Medicine and Biology*, vol. 691, pp. 115–126, 2011.

[141] Z. Liu, S. Wang, H. Zhou, Y. Yang, Y. Zhang, and M. Zhang, "Na+/H+ exchanger mediates TNF-alpha-induced hepatocyte apoptosis via the calpain-dependent degradation of Bcl-xL," *Journal of Gastroenterology and Hepatology*, vol. 24, no. 5, pp. 879–885, 2009.

[142] U. Schwertassek, Y. Balmer, M. Gutscher et al., "Selective redox regulation of cytokine receptor signaling by extracellular thioredoxin-1," *The EMBO Journal*, vol. 26, pp. 3086–3097, 2007.

[143] L. Y. Liu, M. E. Bates, N. N. Jarjour, W. W. Busse, P. J. Bertics, and E. A. Kelly, "Generation of Th1 and Th2 chemokines by human eosinophils: evidence for a critical role of TNF-α," *Journal of Immunology*, vol. 179, no. 7, pp. 4840–4848, 2007.

[144] M. L. Guo, K. Liao, P. Periyasamy et al., "Cocaine-mediated microglial activation involves the ER stress-autophagy axis," *Autophagy*, vol. 11, no. 7, pp. 995–1009, 2015.

[145] G. R. Lee, "The balance of Th17 versus Treg cells in autoimmunity," *International Journal of Molecular Sciences*, vol. 19, no. 3, p. 730, 2018.

[146] X. Wang, J. Ma, Q. Fu et al., "Role of hypoxia-inducible factor1α in autophagic cell death in microglial cells induced by hypoxia," *Molecular Medicine Reports*, vol. 15, no. 4, pp. 2097–2105, 2017.

[147] M. Z. Alam, Q. Alam, M. A. Kamal et al., "Infectious agents and neurodegenerative diseases: exploring the links," *Current Topics in Medicinal Chemistry*, vol. 17, no. 12, pp. 1390–1399, 2017.

[148] J. Cheng, Y. Liao, L. Xiao et al., "Autophagy regulates MAVS signaling activation in a phosphorylation-dependent manner in microglia," *Cell Death and Differentiation*, vol. 24, no. 2, pp. 276–287, 2017.

[149] M. C. Tal, M. Sasai, H. K. Lee, B. Yordy, G. S. Shadel, and A. Iwasaki, "Absence of autophagy results in reactive oxygen species-dependent amplification of RLR signaling," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 106, pp. 2770–2775, 2009.

[150] L. G. Friedman, M. L. Lachenmayer, J. Wang et al., "Disrupted autophagy leads to dopaminergic axon and dendrite degeneration and promotes presynaptic accumulation of α-Synuclein and LRRK2 in the brain," *The Journal of Neuroscience*, vol. 32, no. 22, pp. 7585–7593, 2012.

[151] T. Hara, K. Nakamura, M. Matsui et al., "Suppression of basal autophagy in neural cells causes neurodegenerative disease in mice," *Nature*, vol. 441, pp. 885–889, 2006.

[152] M. Komatsu, S. Waguri, T. Chiba et al., "Loss of autophagy in the central nervous system causes neurodegeneration in mice," *Nature*, vol. 441, pp. 880–884, 2006.

[153] H. F. Lodish, B. Zhou, G. Liu, and C. Z. Chen, "Micromanagement of the immune system by microRNAs," *Nature Reviews Immunology*, vol. 8, no. 2, pp. 120–130, 2008.

[154] Y. Li, D. Zhou, Y. Ren et al., "Mir223 restrains autophagy and promotes CNS inflammation by targeting ATG16L1," *Autophagy*, vol. 15, no. 3, pp. 478–492, 2019.

[155] M. N'diaye, S. Brauner, S. Flytzani et al., "C-type lectin receptors Mcl and Mincle control development of multiple sclerosis-like neuroinflammation," *The Journal of Clinical Investigation*, vol. 130, no. 2, pp. 838–852, 2020.

[156] B. Mannini, G. Vecchi, A. Labrador-Garrido et al., "Differential interactome and innate immune response activation of two structurally distinct misfolded protein oligomers," *ACS Chemical Neuroscience*, vol. 10, no. 8, pp. 3464–3478, 2019.

[157] A. H. Nissim Ben Efraim, R. Eliashar, and F. Levi-Schaffer, "Hypoxia modulates human eosinophil function," *Clinical and Molecular Allergy*, vol. 8, p. 10, 2010.

[158] J. Roiniotis, H. Dinh, P. Masendycz et al., "Hypoxia prolongs monocyte/macrophage survival and enhanced glycolysis is associated with their maturation under aerobic conditions," *Journal of Immunology*, vol. 182, no. 12, pp. 7974–7981, 2009.

[159] S. R. Walmsley, C. Print, N. Farahi et al., "Hypoxia-induced neutrophil survival is mediated by HIF-1α-dependent NF-κB activity," *The Journal of Experimental Medicine*, vol. 201, pp. 105–115, 2005.

[160] C. Pacheco-Tena and S. A. González-Chávez, "The danger model approach to the pathogenesis of the rheumatic diseases," *Journal of Immunology Research*, vol. 2015, Article ID 506089, 23 pages, 2015.

[161] R. Medzhitov and C. A. Janeway Jr., "Decoding the patterns of self and nonself by the innate immune system," *Science*, vol. 296, no. 5566, pp. 298–300, 2002.

[162] P. Matzinger, "The danger model: a renewed sense of self," *Science*, vol. 296, no. 5566, pp. 301–305, 2002.

[163] A. Webers, M. T. Heneka, and P. A. Gleeson, "The role of innate immune responses and neuroinflammation in amyloid accumulation and progression of Alzheimer's disease," *Immunology and Cell Biology*, vol. 98, no. 1, pp. 28–42, 2020.

[164] Y. Shi, X. Zhao, Z. Wang, Y. Bao, and C. Li, "Novel Ca^{2+}-independent C-type lectin involved in immune defense of the razor clam Sinonovacula constricta," *Fish & Shellfish Immunology*, vol. 84, pp. 502–508, 2019.

[165] J. C. Gensel, Y. Wang, Z. Guan et al., "Toll-like receptors and dectin-1, a C-type lectin receptor, trigger divergent functions in CNS macrophages. Toll-like receptors and dectin-1, a C-type lectin receptor, trigger divergent functions in CNS macrophages," *The Journal of Neuroscience*, vol. 35, no. 27, pp. 9966–9976, 2015.

[166] G. Murugaiyan, A. Pires da Cunha, A. K. Ajay et al., "MicroRNA-21 promotes Th17 differentiation and mediates experimental autoimmune encephalomyelitis," *The Journal of Clinical Investigation*, vol. 125, no. 3, pp. 1069–1080, 2015.

[167] M. Sitkovsky and D. Lukashev, "Regulation of immune cells by local-tissue oxygen tension: HIF1α and adenosine receptors," *Nature Reviews Immunology*, vol. 5, pp. 712–721, 2005.

[168] E. N. McNamee, D. K. Johnson, D. Homann, and E. T. Clambey, "Hypoxia and hypoxia-inducible factors as regulators of T cell development, differentiation, and function," *Immunologic Research*, vol. 55, no. 1-3, pp. 58–70, 2013.

[169] S. Tomita, Y. Kihira, M. Imanishi et al., "Pathophysiological response to hypoxia - from the molecular mechanisms of malady to drug discovery: inflammatory responses of hypoxia-inducible factor 1α (HIF-1α) in T cells observed in development of vascular remodeling," *Journal of Pharmacological Sciences*, vol. 115, no. 4, pp. 433–439, 2011.

[170] H. S. Koh, C. Y. Chang, S. B. Jeon et al., "The HIF-1/glial TIM-3 axis controls inflammation-associated brain damage under hypoxia," *Nature Communications*, vol. 6, no. 1, article 6340, 2015.

[171] L. Z. Shi, R. Wang, G. Huang et al., "HIF1α-dependent glycolytic pathway orchestrates a metabolic checkpoint for the differentiation of TH17 and Treg cells," *The Journal of Experimental Medicine*, vol. 208, no. 7, pp. 1367–1376, 2011.

[172] E. T. Clambey, E. N. McNamee, J. A. Westrich et al., "Hypoxia-inducible factor-1 alpha-dependent induction of FoxP3 drives regulatory T-cell abundance and function during inflammatory hypoxia of the mucosa," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 109, no. 41, pp. E2784–E2793, 2012.

[173] N. Esen, V. Katyshev, Z. Serkin, S. Katysheva, and P. Dore-Duffy, "Endogenous adaptation to low oxygen modulates T-cell regulatory pathways in EAE," *Journal of Neuroinflammation*, vol. 13, p. 13, 2016.

[174] D. Mougiakakos, C. C. Johansson, R. Jitschin, M. Bottcher, and R. Kiessling, "Increased thioredoxin-1 production in human naturally occurring regulatory T cells confers enhanced tolerance to oxidative stress," *Blood*, vol. 117, pp. 857–886, 2011.

[175] G. Arellano, E. Acuña, L. I. Reyes et al., "Th1 and Th17 cells and associated cytokines discriminate among clinically isolated syndrome and multiple sclerosis phenotypes," *Frontiers in Immunology*, vol. 8, p. 753, 2017.

[176] B. Adamczyk and M. Adamczyk-Sowa, "New insights into the role of oxidative stress mechanisms in the pathophysiology and treatment of multiple sclerosis," *Oxidative Medicine and Cellular Longevity*, vol. 2016, Article ID 1973834, 18 pages, 2016.

[177] J. van Horssen, M. E. Witte, G. Schreibelt, and H. E. de Vries, "Radical changes in multiple sclerosis pathogenesis," *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease*, vol. 1812, no. 2, pp. 141–150, 2011.

[178] G. K. Vasileiadis, E. Dardiotis, A. Mavropoulos et al., "Regulatory B and T lymphocytes in multiple sclerosis: friends or foes?," *Auto Immun Highlights*, vol. 9, no. 1, p. 9, 2018.

[179] C. N. Chasaide and M. A. Lynch, "The role of the immune system in driving neuroinflammation," *Brain and Neuroscience Advances*, vol. 4, article 2398212819901082, 2020.

[180] R. M. McManus, K. H. G. Mills, and M. A. Lynch, "T cells—protective or pathogenic in Alzheimer's disease?," *Journal of Neuroimmune Pharmacology*, vol. 10, pp. 547–560, 2015.

[181] T. J. Krämer, N. Hack, T. J. Brühl et al., "Depletion of regulatory T cells increases T cell brain infiltration, reactive astrogliosis, and interferon-gamma gene expression in acute experimental traumatic brain injury," *Journal of Neuroinflammation*, vol. 16, no. 1, p. 163, 2019.

[182] M. Diebold, S. Müller, T. Derfuss, and B. F. Décard, "A case of concomitant psoriasis and multiple sclerosis: secukinumab and rituximab exert dichotomous effects in two autoimmune conditions," *Multiple Sclerosis and Related Disorders*, vol. 31, pp. 38–40, 2019.

[183] R. S. Muraoka-Cook, N. Dumont, and C. L. Arteaga, "Dual role of transforming growth factor B in mammary tumorigenesis and metastatic progression," *Clinical Cancer Research*, vol. 11, pp. 937s–943s, 2005.

[184] A. B. Roberts and L. M. Wakefield, "The two faces of transforming growth factor in carcinogenesis," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 100, no. 15, pp. 8621–8623, 2003.

[185] M. Ito, K. Komai, T. Nakamura, T. Srirat, and A. Yoshimura, "Tissue regulatory T cells and neural repair," *International Immunology*, vol. 31, no. 6, pp. 361–369, 2019.

[186] A. Aldrich and T. Kielian, "Central nervous system fibrosis is associated with fibrocyte-like infiltrates," *The American Journal of Pathology*, vol. 179, no. 6, pp. 2952–2962, 2011.

[187] S. S. Zhang, Q. Q. Wang, Q. Q. Yang et al., "NG2 glia regulate brain innate immunity via TGF-β2/TGFBR2 axis," *BMC Medicine*, vol. 17, p. 204, 2019.

[188] K. L. Adams and V. Gallo, "The diversity and disparity of the glial scar," *Nature Neuroscience*, vol. 21, no. 1, pp. 9–15, 2018.

[189] J. Liu, K. Nolte, G. Brook et al., "Post-stroke treatment with argon attenuated brain injury, reduced brain inflammation and enhanced M2 microglia/macrophage polarization: a randomized controlled animal study," *Critical Care*, vol. 23, no. 1, p. 198, 2019.

[190] A. Kumar, J. P. Barrett, D. M. Alvarez-Croda, B. A. Stoica, A. Faden, and D. Loane, "NOX2 drives M1-like microglial/-macrophage activation and neurodegeneration following experimental traumatic brain injury," *Brain, Behavior, and Immunity*, vol. 58, pp. 291–309, 2016.