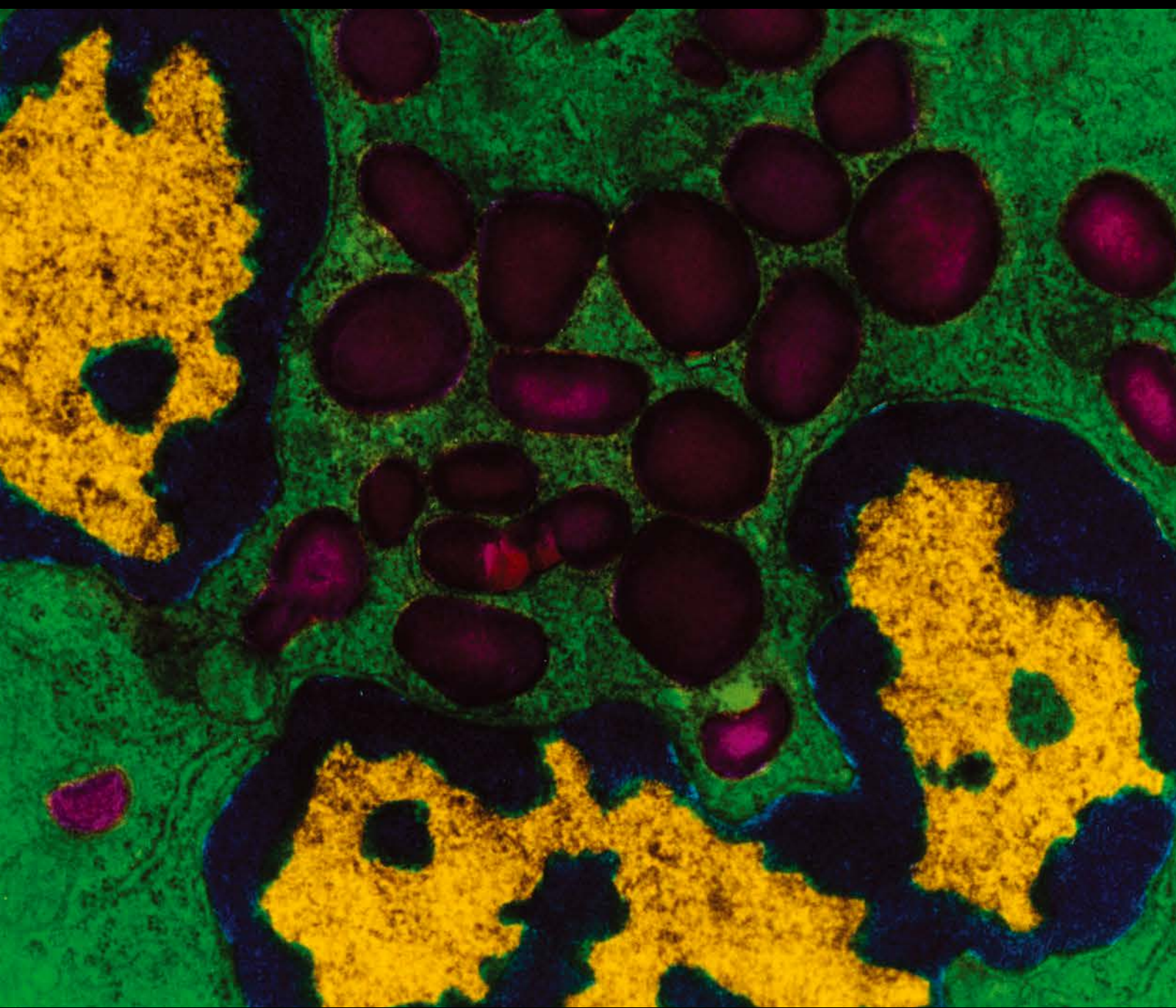
Mediators of Inflammation

Immunotherapeutic Targeting in Autoimmune Diseases

Guest Editors: Nona Janikashvili, Maxime Samson, Eli Magen, and Tinatin Chikovani



Immunotherapeutic Targeting in Autoimmune Diseases

Mediators of Inflammation

Immunotherapeutic Targeting in Autoimmune Diseases

Guest Editors: Nona Janikashvili, Maxime Samson, Eli Magen, and Tinatin Chikovani

Editorial Board

Contents

Hindawi Publishing Corporation
Mediators of Inflammation
Volume 2016, Article ID 1432702, 2 pages
http://dx.doi.org/10.1155/2016/1432702

Editorial

Immunotherapeutic Targeting in Autoimmune Diseases

Nona Janikashvili,[1] Maxime Samson,[2] Eli Magen,[3] and Tinatin Chikovani[1]

[1]*Tbilisi State Medical University, Tbilisi, Georgia*
[2]*University Hospital of Dijon, Université Bourgogne Franche-Comté, Dijon, France*
[3]*Barzilai University Medical Center, Ben-Gurion University of the Negev, Ashkelon, Israel*

Correspondence should be addressed to Nona Janikashvili; njanikashvili@tsmu.edu

Received 1 September 2016; Accepted 1 September 2016

The induction and maintenance of immune tolerance represent major therapeutic goals in autoimmunity. Current strategies for controlling autoimmune disorders are based on the administration of immunosuppressive drugs leading to severe infections or resulting in patient relapse following drug withdrawal. More targeted approaches are therefore needed in this context. The administration of monoclonal antibodies against specific inflammatory mediators has been tested as a more refined strategy. Such monoclonal antibodies have highlighted potential in preclinical and clinical applications. Nevertheless, as they target global immune activation pathways, the impairment of regulatory immune responses has also been demonstrated. Therefore, the sustained clinical responses upon their long-term administration are still under debate. Immunosuppressive/regulatory immune cell-based therapy is a relatively recent approach with promising potential. To date, however, the implementation of autologous immunosuppressive cells in the clinic has been limited by their peculiarly low frequency in patients with inflammatory conditions. Strategies aimed at promoting their expansion and enhancing their suppressive function may also open new therapeutic options.

In this special issue, authors addressed topics related to the immune targets of autoimmune processes and seek to identify novel strategies for the therapeutic intervention of cellular, molecular, and genomic instabilities in various autoimmune disorders.

In their case report, A. Pozdzik et al. evaluated the circulating B cell subtypes including plasmablasts ($CD3^-CD19^+CD20^-IgD^-CD27^{high}CD38^{high}$) and memory ($CD3^-CD19^+CD20^+IgD^-CD27^+CD38^-$) and naive ($CD3^-CD19^+CD20^+IgD^+CD27^-CD38^{low}$) B cells in a patient with anti-phospholipase A2 receptor 1 autoantibody (PLA2R1 Ab) related membranous nephropathy (MN) during 4 years of follow-up after rituximab therapy (RTX). The authors suggested that circulating plasmablasts could be a new cellular biomarker of residual autoimmunity in PLA2R1 related MN and will support the rapid assessment of RTX response in clinical practice.

In the line of exploring autoreactive B cell functions, Q. Pan et al. investigated the association between IgG4 autoantibody and complement abnormalities in systemic lupus erythematosus (SLE). The experimental subjects included 72 newly diagnosed and untreated SLE patients, 67 rheumatoid arthritis (RA) patients, and 41 healthy donors, who served as control subjects. The authors concluded that the IgG4 autoantibody (antinuclear IgG4) may dampen the inflammatory response in SLE by competitively binding to autoantigens to form nonpathogenic ICs that result from the low affinity of IgG4 for both the Fcγ receptor and the C1 complement molecule, thus maybe providing a novel therapeutic target for SLE.

The complex mechanism of RA involves numerous cell types: lymphocytes, monocytes, and fibroblast-like synoviocytes (FLSs), which, upon activation, produce high levels of proinflammatory cytokines, such as IL-6, TNF-α, and IL-1β. Of these proinflammatory mediators, IL-6 plays a crucial role as it triggers the hepatic acute-phase response and augments joint inflammation and bone erosion in RA. Inhibition of IL-6 signaling significantly improves autoimmune arthritis in experimental animal models and in RA patients. X. Luo et al. identified Krüppel-like factor 4 (KLF4) to be a TNF-α-induced transcription factor that is higher expressed in synovial tissues and fibroblast-like synoviocytes from RA

patients than from osteoarthritis patients. These investigators established the notion that KLF4 is localized in the nuclei of RA FLSs and regulates the expression of IL-6 through both direct promoter activation and interaction with NF-κB.

Y. Liu and G. Sun explored the antiarthritic effect of *Periploca forrestii* saponin (PFS) and its derived Periplocin in adjuvant-induced arthritis in rats. The study suggests the antiarthritic activity of PFS and Periplocin via modulation of the key proinflammatory cytokines IL-6, Th1 (IFN-γ), Th2 (TGF-β1 and IL-13), and Th17 (IL-22) and transcription factor T-bet, GATA3, and C-Jun genes.

M. Ciechomska and S. O'Reilly further summarized the latest information about the potential therapeutic application of epigenetic modifications in targeting immune abnormalities of rheumatic diseases. By covering extensive volume of works and discussing major categories of epigenetic layers and players, these authors highlighted the relevance of the epigenome targeting treatment. While such drugs have already being applied in cancer and cardiovascular diseases, M. Ciechomska and S. O'Reilly addressed the evident emergence of such treatments for autoimmune rheumatic diseases. In another review article, R. Sujashvili overviewed the advantages of extracellular ubiquitin as a new tool for targeted therapy for immune mediated disorders of various etiologies.

In the context of acute inflammation, some enzymes acquire robust proinflammatory activities. A research paper by G. Zhou et al. addressed the role of a lipid-signaling enzyme phospholipase D2 (PLD2) in the pathogenesis of inflammatory bowel diseases (IBD). The authors reported on the high expression of PLD2 in peripheral blood cells and in inflamed mucosa of patients with active IBD. Among various proinflammatory cytokines establishing IBD pathogenic microenvironment, TNF-α markedly supported the upregulated expressions of PLD2. Inhibition of PLD2 was associated with the amelioration of the intestinal colitis via promoting neutrophil migration through CXCR2 upregulation. Collectively, these data identified PLD2 as a new therapeutic target for the management of IBD.

This special issue encompasses fundamental and translational data aimed at identifying novel immunotherapeutic targets in autoimmune diseases. We believe that these original research articles and review papers will stimulate the continuing efforts to improve current therapies of patients with such diseases.

Acknowledgments

We thank the authors for their valuable contributions to this special issue.

Nona Janikashvili
Maxime Samson
Eli Magen
Tinatin Chikovani

Hindawi Publishing Corporation
Mediators of Inflammation
Volume 2016, Article ID 7941684, 11 pages
http://dx.doi.org/10.1155/2016/7941684

Research Article

Periploca forrestii Saponin Ameliorates Murine CFA-Induced Arthritis by Suppressing Cytokine Production

Yingqin Liu,[1] Minghui Li,[1] Qiuhong He,[1] Xinping Yang,[2] Fang Ruan,[2] and Guangchen Sun[2]

[1]*Biotechnology College, Guilin Medical University, No. 109 North 2nd Huan Cheng Road, Guilin, Guangxi 541004, China*
[2]*Pharmaceutical College, Guilin Medical University, No. 109 North 2nd Huan Cheng Road, Guilin, Guangxi 541004, China*

Correspondence should be addressed to Guangchen Sun; sungc@glmc.edu.cn

Received 21 May 2016; Accepted 7 August 2016

Academic Editor: Nona Janikashvili

Periploca forrestii Schltr. has been used as a Chinese folk medicine due to its versatile pharmacological effects such as promoting wounds and rheumatoid arthritis. However, the antiarthritic activity of *Periploca forrestii* saponin (PFS) and its active compound Periplocin has still not been demonstrated. Here, we evaluated the antiarthritic effects of PFS in adjuvant-induced arthritis (AIA) rats by intragastric administration at a dose of 50 mg/kg. The anti-inflammatory activities of Periplocin were also examined in LPS-induced AIA splenocytes and synoviocytes. PFS significantly ameliorated joint swelling; inhibited bone erosion in joints; lowered levels of IL-6 and TGF-β1 in AIA rat splenocyte; and reduced joint protein expression levels of phospho-STAT3 and IKKα. Using LPS-induced AIA splenocytes, we demonstrate that Periplocin suppressed the key proinflammatory cytokines levels of IL-6, IFN-γ, TGF-β1, and IL-13 and IL-22 and transcription factor levels of T-bet, GATA3, and C-Jun genes. Periplocin also suppressed LPS-induced cytokine secretion from synoviocytes. Our study highlights the antiarthritic activity of PFS and its derived Periplocin and the underlying mechanisms. These results provide a strong rationale for further testing and validation of the use of *Periploca forrestii* Schltr. as an alternative modality for the treatment of RA.

1. Introduction

Rheumatoid arthritis (RA), which afflicts about 1% of the world's population, is a systemic, chronic, autoimmune inflammatory disease that preferentially attacks the synovial lining of the joints, destroys local articular structures, and further affects related tissues and organ systems [1, 2]. Untreated RA leads to deformities and disability [3]. Therefore, therapy to joint inflammation and cartilage destruction is a logical strategy for preventing the progression of RA [4, 5].

A major contributor to joint inflammation in RA and antigen-induced arthritis (AIA) is proinflammatory cytokines. IFN-γ, IL-6, and IL-22 are found to be elevated during the development of arthritis, and inhibition of TNF and IL-6 represents successful treatments of RA [6, 7]. Conversely, anti-inflammatory cytokines (e.g., IL-10, TGF-β, and IL-13) may dampen arthritis [3].

Despite this clear link between inflammation and increased bone turnover in RA and the existence of several therapeutical options, their efficacy on inflammation and bone treatment seem to be uncoupled, with some drugs suppressing inflammation but failing to protect bone [8, 9] and others halting bone destruction but with no effect on controlling inflammation [10].

The dry root or whole vine of *Periploca forrestii* Schltr. of Asclepiadaceae is effective in clinical prescription for promoting blood flow and eliminating wind effect cardiac antitumor and anti-inflammation, widely used in the treatment of rheumatoid diseases [11, 12]. Periploca forrestii mainly contain cardiac glycosides, flavonoids, C21 steroid saponins and other triterpenoid ingredients. Saponins are the characteristic components and also the main active ingredients of *Periploca forrestii*. Periplocin is one of cardiac glycosides extracted from *Periploca forrestii*, several studies have addressed the various heart conditions [11–14]. Recent studies also suggest that Periplocin extracted from cortex periplocae can inhibit cell growth in colon cancer cells, lung cancer cells, and hepatocellular carcinoma cells [15–17].

Our aim in the herein study was to test the effect of PFS treatment in the gene expression of inflammatory cytokines

and on the overall synovial tissue joint structure in a rat AIA model, as a further argument to its possible efficacy in RA treatment. In this work we also report that Periplocin significantly decreases cytokines in AIA splenocytes and normal synoviocytes.

2. Materials and Methods

2.1. Reagents and Animals. Periplocin was purchased from State General Administration of the People's Republic of China for Quality Supervision and Inspection and Quarantine. p-Stat3, IKKα, and p-I-κBα were purchased from Santa Clauz. Female Sprague Dawley rats (6–8 weeks old) with a mean weight of 150–180 g were obtained from Laboratory Animal Center of Guilin Medical University, China. Rats were housed in an appropriate environment with an air-filtering system. All experimental procedures were approved by the Research Ethics Committee of Guilin Medical University, China.

2.2. Collection of Periploca forrestii and Saponin Extract Process. Periploca forrestii were collected from Guangxi province, China, and identified by Dr. Sun from Guilin Medical University. The powder obtained by mincing dried roots was extracted for 2 h with 70% ethanol. The extract was reextracted twice following the same procedure and then filtered. The filtrate was partitioned with petroleum ether (1 : 1) and then butanol. The butanol solvent was evaporated in a vacuum evaporator to yield the butanol fraction.

The final extract was concentrated and dried to obtain PFS. To study the antiarthritic activity of PFS, the butanol extract was dissolved in water and arthritic Sprague Dawley rats were fed at the dose of 50 mg/kg body weight (normalized from the amount of human dose) by using the regimen described below.

2.3. Induction of Arthritis. Female Sprague Dawley rats were randomly divided into the following three groups: normal group = normal rats; AIA control group = CFA-induced arthritis rats; FPS group = PFS 50 mg/kg treated AIA rats. Chronic arthritis was induced by five-point injection of total of 0.5 ml of Freund's complete adjuvant (CFA, Sigma Aldrich) containing 1 mg of heat killed *Mycobacterium tuberculosis* in 1 mL of mineral oil into the back skin of the Sprague Dawley rat intradermally. Two weeks later, arthritis was induced by subplantar administration of 0.1 ml of CFA into both hind paws of all the rats, respectively. Before 7 days of adjuvant injection in paws, immunized groups were orally given with 50 mg/kg/day until the day before tissue harvesting, 28th day. Control group were given water. The thickness of the paw was measured before induction using a dial thickness gauge (Peacock Japan) and after treatment.

2.4. Histology, and Immunohistochemistry. The hind paws were harvested from rats on day 28 after CFA immunization. Each paw was fixed in 4% paraformaldehyde overnight and then demineralized with 10% ethylenediaminetetraacetic acid (EDTA) for one month before embedding in paraffin. Paraffin-embedded tissue was serially sectioned at 5 μm distances serially using a microtome and mounted on microscope slides. Then the sections were stained either with toluidine blue and fast green or with safranin-O (safranin-O stain is taken up by the intact cartilage), Tartrate Resistant Acid Phosphatase (TRAP), or Matrix Metalloproteinase 11 (MMP-11). Histopathological changes in the joints like synovial hyperplasia, pannus formation, and cartilage erosion and bone erosion were observed under a microscope using the Spot Imaging Software, and Representative photos were taken with an Olympus microscope at 100x magnification.

2.5. Western Blotting. Frozen synovial tissue (whole joints including synovium, adjacent tissues, and bones) was weighed and broken into pieces on dry ice. Paw lysates were homogenized in Radio Immunoprecipitation Assay (RIPA) lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100, and 1% deoxycholate) supplemented with 15 mM sodium fluoride, 1 mM sodium vanadate, 2 mM sodium pyrophosphate, 1 mM sodium glycerophosphate, 2 mM imidazole, 100 mg/ml phenylmethylsulfonyl fluoride and proteinase inhibitor cocktail (Sigma Aldrich), and sonicated in the lysis buffer, cleared by centrifugation at 12,000 g for 10 minutes at 4°C. The protein samples were isolated from the inflamed paw tissue and quantified using Bradford assay and kept at −80°C until use. The protein samples (25 μg/well) were electrophoresed by using 10% sodium dodecyl sulfate- (SDS-) polyacrylamide gel electrophoresis (PAGE) and transferred to polyvinylidene fluoride membrane. Immunoblot analysis was carried out using rabbit anti-rat polyclonal antibody (1 : 1000) as primary antibody and Horseradish Peroxidase (HRP) conjugated goat anti-rabbit IgG (1 : 5000) as secondary antibodies, respectively. The proteins were detected using enhanced chemiluminescence (ECL). The band intensity was measured densitometrically using Image J software. β-Actin was used to normalize the target and the relative expressions were stated as fold ratio.

2.6. Preparation of Splenocytes and Their Restimulation with LPS for Cytokine Testing. Sprague Dawley rats were injected 100 μl CFA (1 μg/ml) under the right plantar inflammation intradermally; two weeks later, splenocytes were isolated. After erythrocytes were removed from spleen cell suspensions using red cell removal buffer 0.16 M Tris-NH_4Cl solution, splenocytes in the suspensions were adjusted to a cell density of 2×10^6 cells/ml. These spleen cells were placed in a 96-well plate at 37°C in Dulbecco minimum essential medium (DMEM) supplemented with 5% fetal bovine serum (FBS), 2 mM L-glutamine, 100 units/ml penicillin G sodium, and 100 μg/ml streptomycin sulfate. Splenocytes (100 μl) were added to each of the wells of a 96-well plate in the presence or absence of 100 μl of final concentrations of 2 μg/ml periplogenin and lipopolysaccharide (LPS) (1 μg/ml). The cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 for 3, 6, 12, and 24 h.

2.7. Synoviocyte Culture. Synovial tissues were obtained from the knee joints of normal Sprague Dawley rats. The synovial tissues were minced and stirred with type IV collagenase (Sigma Aldrich Co., USA) in serum-free DMEM/F12

Table 1: Primers for target genes.

Gene name	Primers	bp	Cycle	(°C)
IL-6	F: GCAAGAGACTTCCAGCCAGTT R: CATCATCGCTGTTCATACAATCA	229	35	54
TGF-β1	F: CTCAACACCTGCACAGCTCC R: ACGATCATGTTGGACAACTGCT	349	35	56
IFN-γ	F: GGAACTGGCAAAAGGACGGT R: GGGTTGTTCACCTCGAACT	209	35	55
T-bet	F: TCCTGTCTCCAGCCGTTTCT R: CGCTCACTGCTCGGAACTC	122	35	55
GATA3	F: CCCCATTACCACCTATCCGC R: CTCCGTTAGCGTTCCTCCTC	356	35	55
C-Jun	F: ATGACTGCAAAGATGGAAACG R; TATTCTGGCTATGCAGTTCAG	376	35	50.4
IL-22	F: GTTCCGAGGAGTCAAAGCCAA R: ACCTCCTGCATGTAAGGCTG	117	35	60
IL-13	F: CACAAGACCAGAAGACTTCCCT R: ACATCCGAGGCCTTTTGGTT	172	35	58
β-Actin	F: GGAGATTACTGCCCTGGCTCCTA R: GACTCATCGTACTCCTGCTTGCTG	150	35	58.2

medium at 37°C for 2 h in an incubator shaker. The synovial tissue lysate was then filtered through a 40 μm nylon mesh, washed extensively, and seeded in 12-well microplates. The cells were cultured in DMEM/F12 supplemented with 10% FBS and benzylpenicillin potassium (100 units/ml) at 37°C/5% CO_2.

2.8. Quantitative Expression for Target Genes. The total RNA of spleen or cell was isolated using TRIzol reagent according to the manufacture's instruction (TRIzol, Invitrogen). The purity and yield of RNA were assessed by measuring the absorbance of RNA solution at 260 nm and 280 nm. The RNA product sizes were estimated relative to 100 bp DNA ladder. cDNA was synthesized from 1 μg of RNA with 2x Tak PCR Master Mix RT mix (Aidlab Biotechnologies) on the final volume of 20 μL according to manufacturer's guidelines. The specific forward and reverse primers were used for target genes expression (Table 1). Beta-actin was used to normalize differences in RNA isolation, RNA degradation, and the efficiencies of the reverse transcription. The relative expression of target genes was stated as fold ratio using Smart View Method.

2.9. Statistical Analysis. All experimental results are expressed as mean (SD) of several independent experiments. Multiple comparisons of data were carried out by analysis of variance (ANOVA) with Dunnett's test. p values of less than 5% were regarded as significant.

3. Results

In the present study, we investigated the in vivo efficacy of PFS in AIA rats. Each group of Sprague Dawley rats was fed daily with PFS starting before CFA immunization and then continued for 35 days. The control rats received water orally. Rats immunized with 100 μg CFA began to develop arthritis in the first week. Then the initial manifestation of arthritis was erythema and swelling of ankle joints, followed by the inflammation of the metatarsal and interphalangeal joints. Disease progression can be evaluated by measuring paw swelling thickness, which is an indicator of the degree of inflammation. In order to evaluate the antiarthritic efficacy of PFS, the paw thickness changes were quantified using Peacock thickness meter. PFS significantly ameliorated paw swelling. At the end of the experiment, more significant reductions of paw thickness were observed in groups treated with PFS (50 mg/kg) (Figures 1(a) and 1(b)).

Effects of PFS on joint histopathological changes of experimental animals were examined by safranin-O, toluidine blue, TRAP, and MMP-11 staining. Representative microphotographs were presented for normal, control, and PFS-treated group. Normal animals had shown no detectable abnormalities; for the control arthritic rats; the knee joints had moderate evidence of articular cartilage damage with pannus formation. The formation of pannus is a result of overgrowth of the synoviocytes and the observed accumulation of inflammatory cells that led to deformed cartilage and bone. The synovial membrane and capsule were both markedly thickened as a result of pannus formation and inflammatory cell infiltration (Figure 1(c)). In control group, the chronic inflammation destroyed the joint lining, including extensive proteoglycan depletion cartilage and other nearby supporting structures, such as bone (Figure 1(d)). At some locations in AIA joints osteoclasts resorbed the subchondral bone plate up to the noncalcified cartilage (Figure 1(e)). The infiltration of MMP-11 was found in hyperplastic synovial linings with sloughing of synoviocytes into the joint space (Figure 1(f)). In addition, these changes were ameliorated

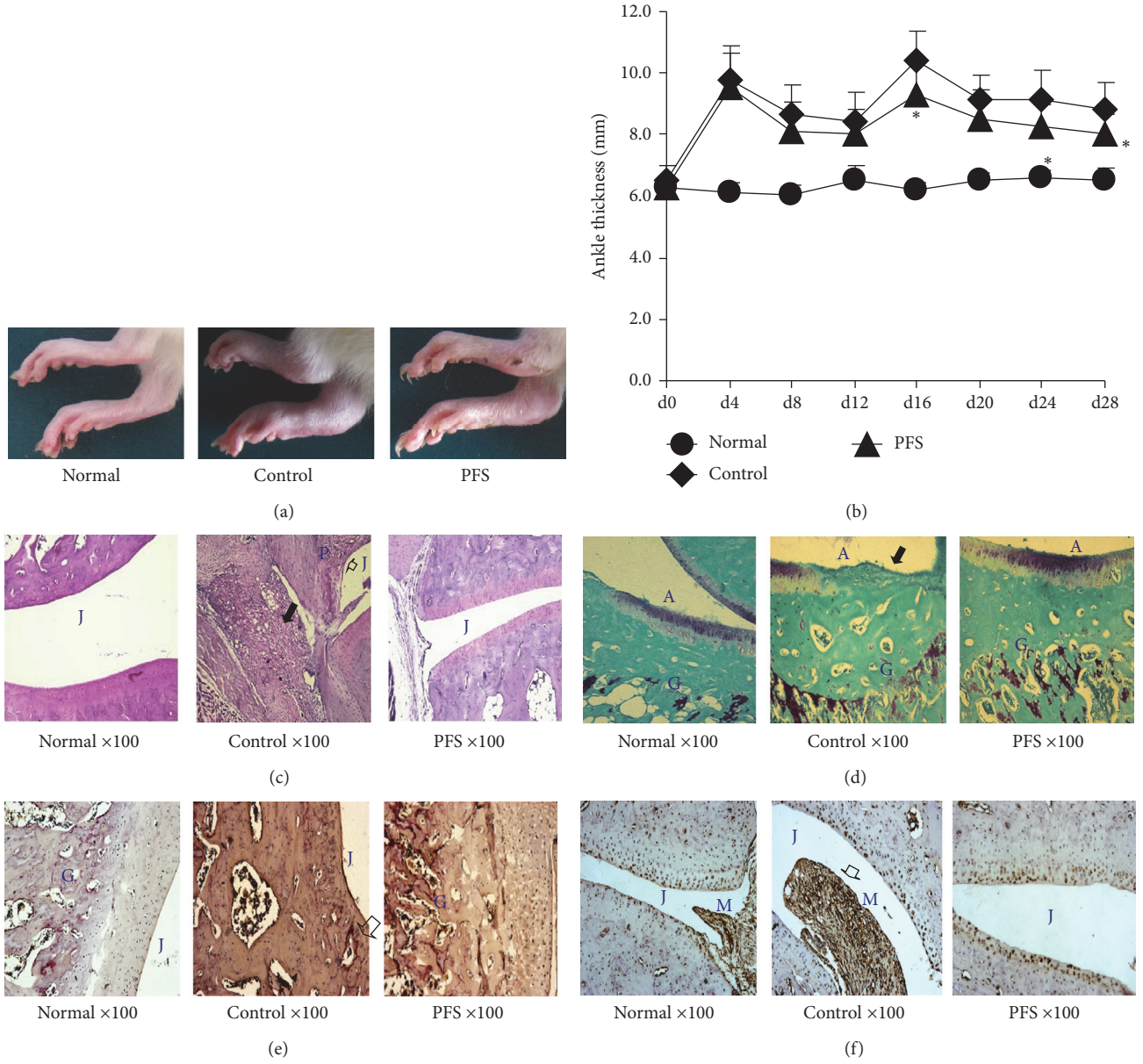


Figure 1: PFS prophylactically suppress inflammation in CFA-induced arthritis in rats. AIA was induced as detailed in Methods. Rats were randomly divided into 3 treatment groups: normal, control, and PFS. PFS were administered orally until the day before tissue harvesting 28th day. (a) Representative photographs of days 21–27 paws from the 3 treatment groups. (b) Change in ankle thickness. The changes in paw thickness were measured every four days. The results are expressed as the mean ± standard error ($n = 7$–9). Statistical values conducted on days 0–28 for changes in paw thickness compared with control were as follows: $^{*}p < 0.05$ compared with the control group, Dunnett's test. ((c)–(f)) Representative photographs of knee joint tissues stained with safranin-O-fast green, toluidine blue-fast green, TRAP, or MMP-11 (magnification, 100x). Note that the intense synovial inflammatory infiltration ((c) black arrowhead), pannus formation ((c) arrowheads), cartilage destruction, extensive proteoglycan depletion ((d) black arrowhead), and MMP-11 infiltration ((f) arrowhead) were significantly reduced in the joints of PFS-treated rats compared with the respective control rats. A: articular cartilage; G: growth plate; J: joint space; M: synovial membrane; P: pannus formation.

in PFS-treated animals. These results show that PFS have antiarthritic activity.

We performed in vivo assay of CFA-induced cytokine production by rat splenocytes to clarify that whether PFS treatment regulates cytokine production. Cytokine levels were measured in the spleen tissues to obtain insight into the mechanisms of beneficial PFS-mediated effects. In Figure 2, the reverse-transcription polymerase chain reaction (RT-PCR) results indicated the presence of relatively small amounts of IL-6 and TGF-β1 mRNA in the spleens of healthy rats. However, the concentrations of these transcripts exhibited a substantial increase in the joints of the rats with

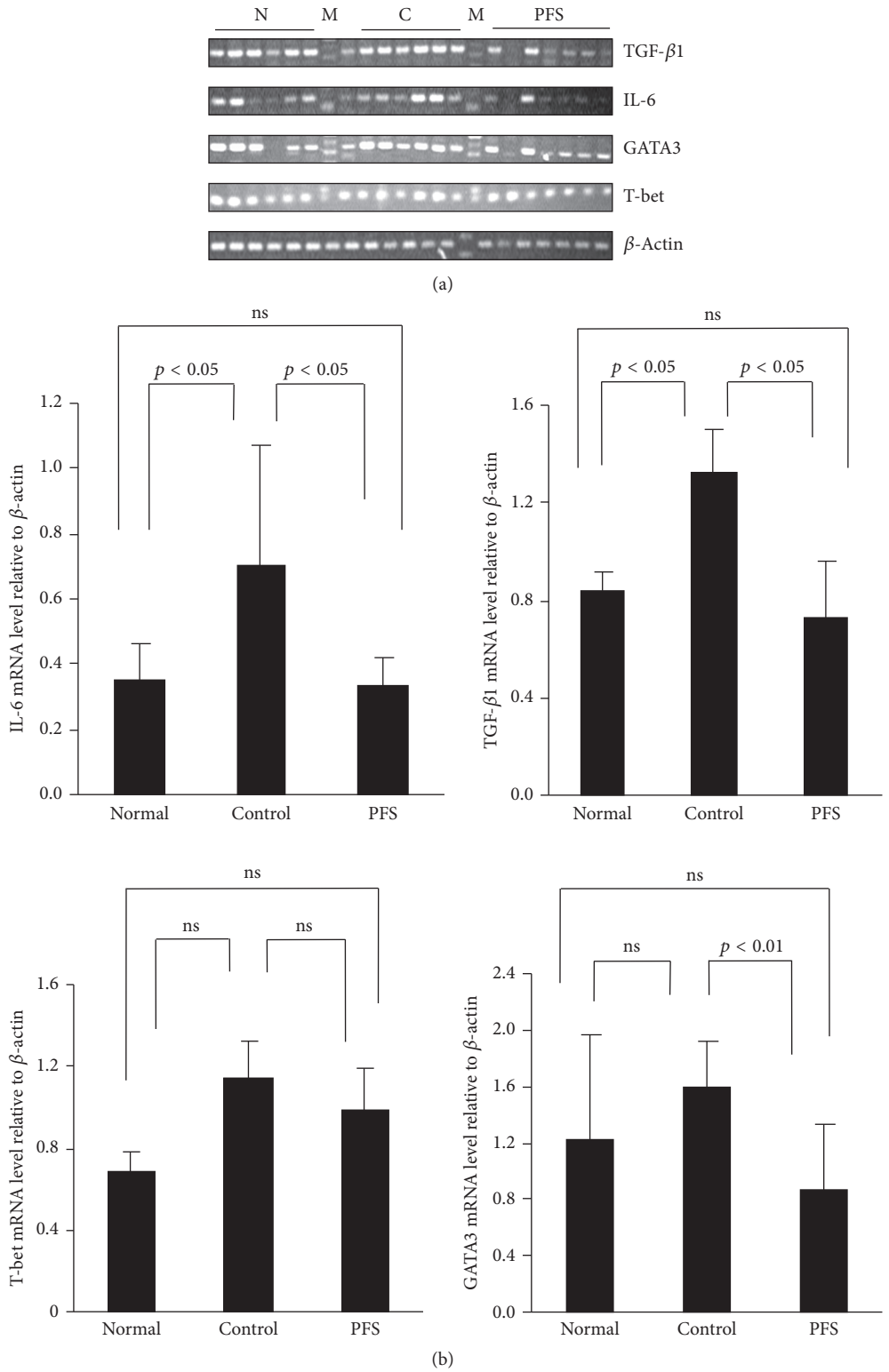


Figure 2: PFS suppressed the expression of inflammatory cytokines. AIA was induced as detailed in Methods. Arthritic rats were treated with 50 mg/kg PFS for 35 days. Day 28: splenocytes were harvested for measuring cytokine expression by quantitative RT-PCR. (a) RT-PCR analysis of inflammatory cytokine genes and related transcription factors and (b) relative amounts of each cytokine gene level were determined by densitometric analysis. Dunnett's test. N: normal; C: control; M: marker. ns: no significant difference.

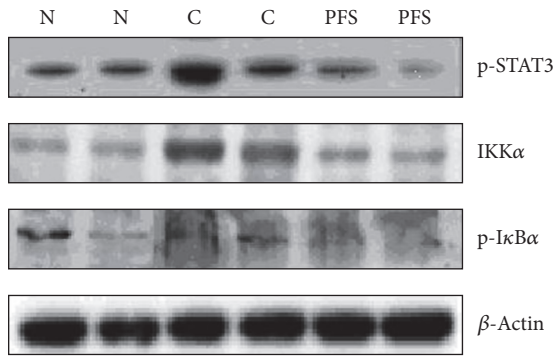


Figure 3: Effect of PFS treatment on protein expression levels of p-STAT3, IKKα, and p-IκBα protein expression levels in the AIA rat model. AIA was induced as described in Methods. Arthritic rats were treated with 50 mg/kg PFS for 35 days. In day 28 off-target organs paws were processed and protein lysates were probed for expression of p-STAT3 and related NF-κB family members (IKKα and p-IκBα) by Western blotting. N: normal; C: control.

AIA. There was a significant decrease ($p < 0.05$) in the expression of TGF-β1 and IL-6 mRNA in PFS-treated rats compared with their respective controls. However, the decline in the T-bet mRNA level in PFS-treated rats was not significant. These results suggested that PFS regulated systematic immunological response by simultaneously reducing IL-6 and TGF-β1 production and transcription factors at spleen in rats with RA.

As STAT3 has crucial roles in inflammation, the regulatory effects of PFS on the inflammatory response in the AIA rats were examined. p-STAT3 protein expression was elevated in the AIA rats compared with that of the normal group (Figure 3). Notably, PFS administration at 50 mg/kg significantly reduced p-STAT3 and IKKα protein expression in the RA rat model.

LPS is a major component of the cell wall of Gram-negative bacteria and is a well-known potent inducer of inflammation and inflammatory bone loss [18]. We prepared splenocytes from Freund's complete adjuvant Sprague Dawley rats. LPS-induced production of proinflammatory factors IL-6, Th1 (IFN-γ), Th2 (TGF-β1 and IL-13), and Th17 (IL-22) and transcription factors T-bet, GATA3, and C-Jun mRNA were increased in splenocytes harvested. However, the mRNA levels were reduced in a time-dependent manner in Periplocin-treated splenocytes (Figure 4).

LPS can stimulate FLS to secrete MMPs, and this induction is regulated at the transcriptional and translational levels [19]. Effect of different concentrations of mRNA expression of IL-6, TGF-β1, and C-Jun in LPS-induced synoviocytes was examined. In the blank group, there was weak mRNA expression of IL-6 and TGF-β1, while the expression of cytokines was significantly increased in the LPS group. As the dose of Periplocin gradually increased from 0.5 μg/ml to 2 μg/ml, mRNA expression of IL-6 and TGF-β1 was reduced in a concentration-dependent manner. While C-Jun level was reduced in LPS-induced synoviocytes, however, increased C-Jun level was found in a concentration-dependent manner (Figure 5).

4. Discussion

AIA is characterized by a rapid onset and progression to articular inflammation. Usually the disease is severe and leads to permanent joint malformations, including ankylosis. Symmetric joint involvement, lymphocyte infiltration, cartilage degradation, synovial hyperplasia, and T cell dependence are shared features with human RA [20, 21]. Subdermal injection of FCA at multiple sites around the tibiotarsal joint of female Sprague Dawley rats caused a localised inflammatory reaction to develop in 24 h. A striking feature was the presence of synovial thickening with foci of cartilage erosion and a prominent bone destruction around the ankle joint.

Many potent antiarthritic drugs are available for the management of arthritis. However, their prolonged use is associated with severe adverse effects and they are ineffective in a proportion of patients. In addition, they are expensive. However, their prolonged use is associated with severe adverse effects. Accordingly, an increasing number of patients with RA and other diseases in developed countries are using natural products and other complementary and alternative medicine (CAM) approaches for their healthcare needs [22–26].

A variety of herbs belonging to traditional Chinese medicine have been used in China for centuries for the treatment of rheumatic diseases, including RA. Many studies suggest that traditional herbal resources benefit the management of inflammatory arthritis and may therefore benefit RA. Many Chinese herbs recorded effective in the therapy of RA have been analyzed and the related mechanisms have been further detected [27].

Periploca forrestii shows several medicinal prophylactic effects in traditional Chinese folk medicine; because several plant saponins were recently found having therapeutic effects in different models of autoimmune diseases, such as arthritis [28, 29], we suggest that PFS may have clinically beneficial effects on autoimmune diseases by their induction of immunosuppression during chronic administration. Therefore, the present study was conducted to evaluate the anti-inflammatory and antiarthritic activities of PFS based

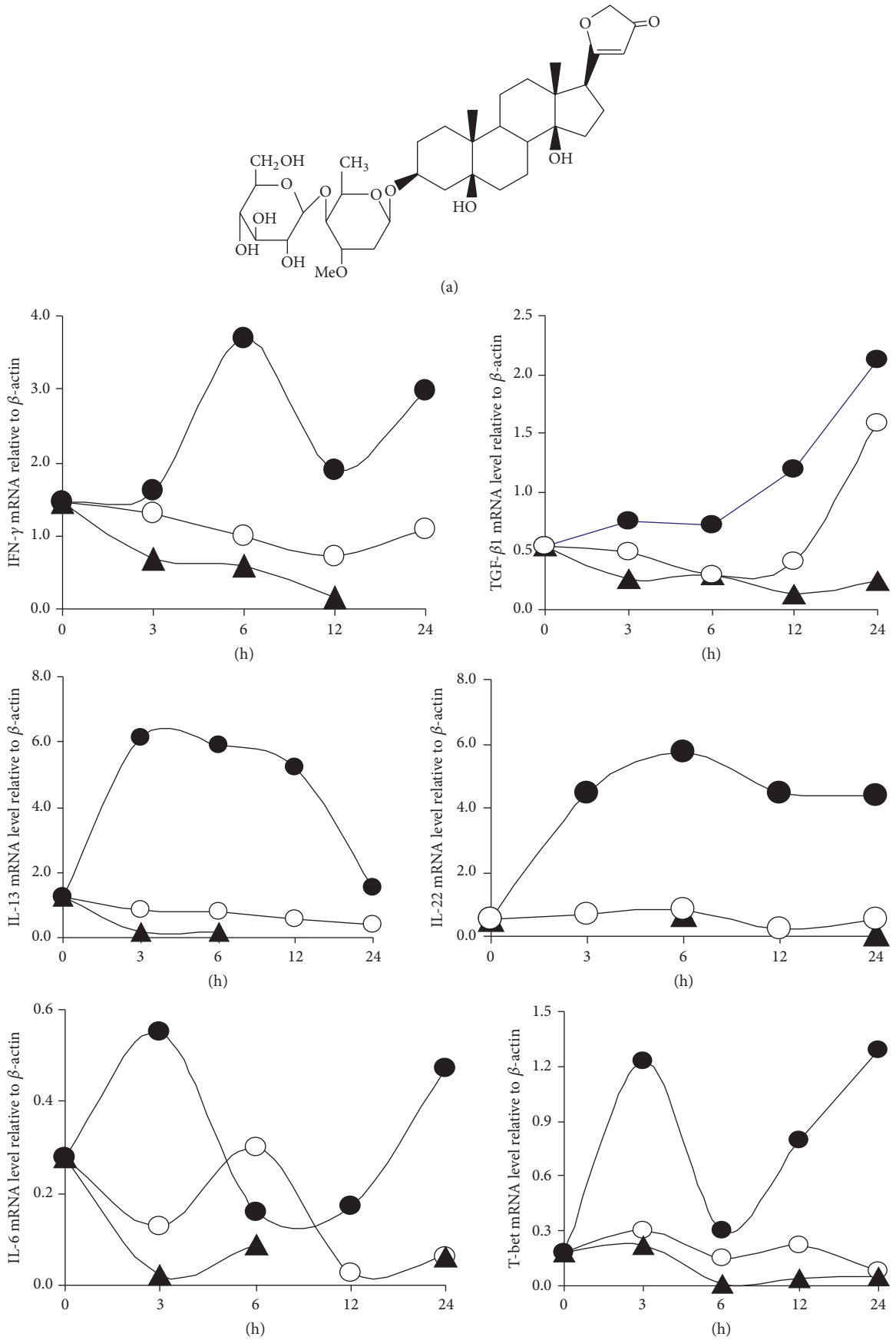
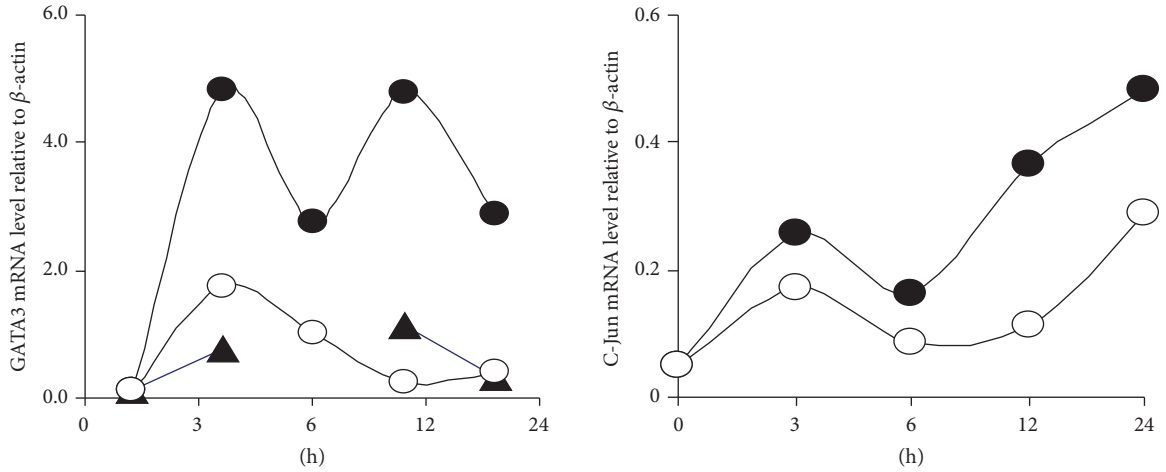


Figure 4: Continued.



(b)

Figure 4: Effect of Periplocin on proinflammatory cytokine expression in splenocytes from Freund's complete adjuvant Sprague Dawley rats. Splenocytes were prepared as described under Methods. Splenocytes were cultured in the presence or absence of Periplocin or LPS for indicated time. The total mRNA was prepared for RT-PCR analysis. (a) Chemical structure of Periplocin, (b) RT-PCR analysis of inflammatory cytokine genes and related transcription factors, and relative amounts of each cytokine gene level were determined by densitometric analysis. L: LPS; P: Periplocin; ●: LPS only; ○: LPS + P; ▲: P only.

on an experimental model of autoimmune arthritis (AIA model).

In this study, PFS alleviated the clinical outcomes, synovial hyperplasia inflammatory cells infiltration, and cartilage destruction in CFA rats. It revealed that PFS inhibited inflammatory cytokines and the transcriptional activity by suppressing STAT3 signaling. The splenocytes of CFA-immunized Sprague Dawley rats treated with PFS showed a significantly reduced expression of TGF-β1 and IL-6 and further GATA3, but a slight effect on T-bet. The relative lack of effect on T-bet suggested that PFS had a major effect on the activity of the differentiation of naive T cells into Th2 cells, but not Th1 cells. To address whether PFS modulated the NF-κB signaling pathway, we attempted to analyze the expression of NF-κB signaling transduction proteins in the absence or presence of PFS. We showed that CFA induced phosphorylation of STAT3 and that PFS inhibited the effect. Furthermore, it has been shown that IL-6 can induce the phosphorylation of STAT3 in certain cell lines [30]. In this regard, it is likely that the reduced paw p-STAT3 observed by Western blotting in our study might be attributable both to a direct effect of PFS on STAT3 and to an effect via reduced IL-6 expression. These data indicate that PFS might improve AIA by modulating STAT3-regulated genes and thus affects various biological events. The precise mechanisms involved remain to be tested.

As no studies have been conducted to evaluate the efficacy of PFS for the treatment of RA, it is difficult to perform advanced mechanistic and specificity of action studies using a crude plant extract, which possesses multiple components. It is rationale for examining the mechanism of action of Periplocin derived from the plant *Periploca forrestii* Schltr., which then could be extrapolated to that of the natural PFS. Therefore, the present study was conducted to evaluate the anti-inflammatory and antiarthritic activities of PFS active ingredient Periplocin on LPS-induced AIA splenocytes and on LPS-induced synoviocytes. Most of the studies on Periplocin are based on its activity on circulatory system and tumor disease. Our study has unraveled the immunological basis of the antiarthritic property of this naturally occurring plant compound and most of the mechanistic attributes of Periplocin are similar to those of PFS.

Because LPS stimulated many TGF-β1 and IL-6 production by splenocytes (Figure 5), we considered whether the TGF-β1 and IL-6-inhibiting activity of PFS might correlate with immunoregulation activity. We found that Periplocin derived from PFS had relatively high immunoregulation activity. Indeed, it has been demonstrated that Periplocin reduces the levels of RA factors IL-6, Th2 cytokines (TGF-β1 and IL-13), Th1 (IFN-γ and IL-33), and Th17 (IL-22) and inhibits the expression of GATA3, T-bet, and C-Jun in LPS-induced splenocytes. Similar results were also found in LPS-induced synoviocytes by suppressing the IL-6 and TGF-β1 expression; Periplocin has thus a broad spectrum of targets, modulating not only immune cytokines but also relative transcription factors.

Cytokines are directly implicated in many of the immune processes that are associated with the pathogenesis of rheumatoid arthritis. There are several studies comparing circulating levels of cytokine; they often show discrepancy in their results. Similar to our AIA, serum IL-6 levels are substantially increased in RA with significant circadian variations corresponding to the circadian rhythm of symptoms in RA [31]. Studies addressing the role of TGF-β and IL-10 in experimental arthritis have shown variable results. Significantly elevated TGF-β1 levels have been reported in serum of RA patients [32]. Anti-TGF-β and anti-TGF-βRI antibodies injection inhibits chronic synovial inflammation in rats with streptococcal cell wall-induced arthritis [33] and

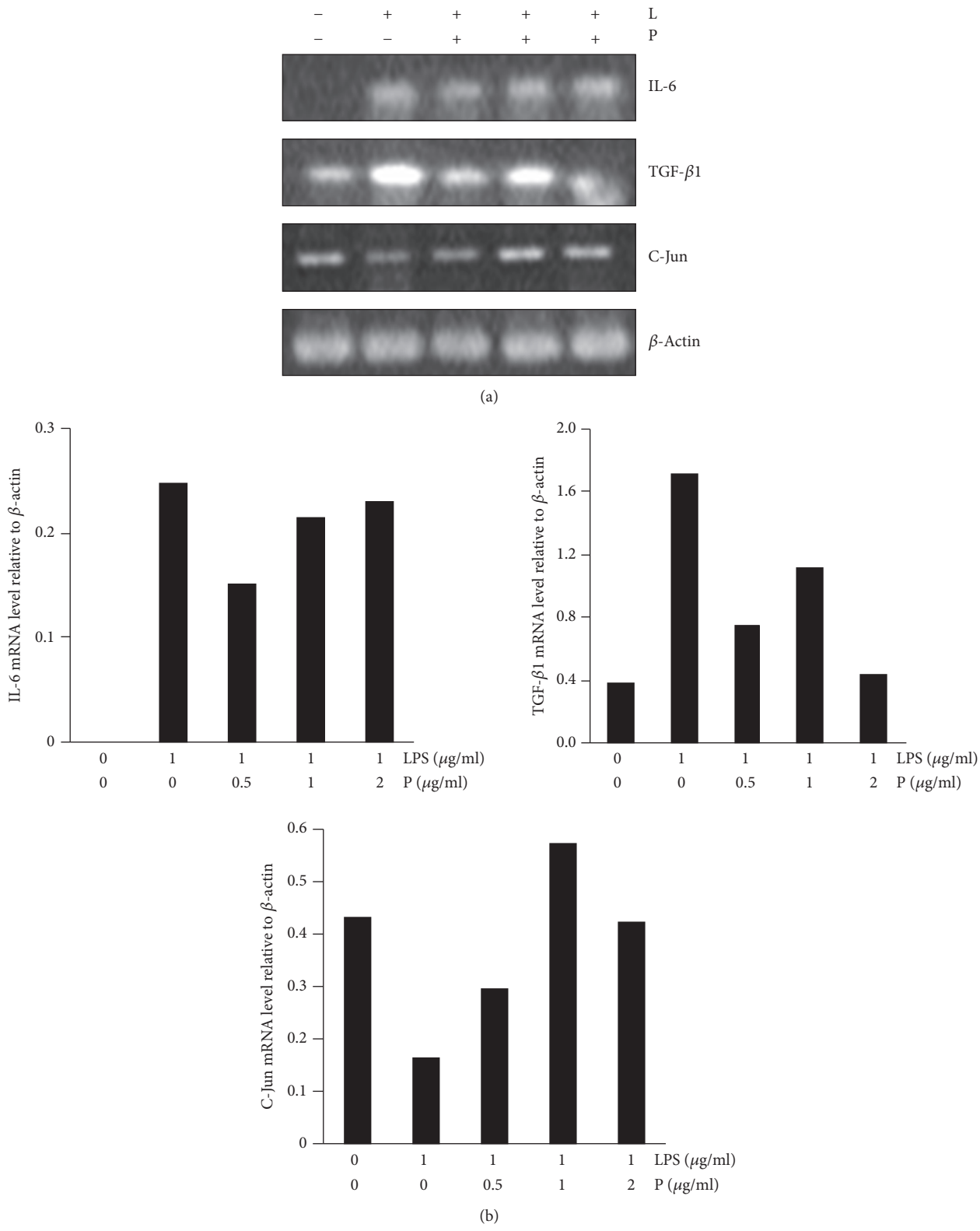


Figure 5: Effect of Periplocin on proinflammatory cytokine expression in synoviocytes from normal Sprague Dawley rats. Synoviocytes were prepared as detailed in Methods. Synoviocytes were incubated with the indicated concentrations of Periplocin for 24 h in the presence or absence of LPS (1 μg/mL) stimulation. After 24 h, the mRNA was measured by RT-PCR and β-actin was used as the internal controls, respectively. Representative gels stained for RT-PCR products of IL-6 and TGF-β mRNA expression of synoviocytes in AA model rats treated with Periplocin. (a) RT-PCR analysis of inflammatory cytokine genes and related transcription factors and (b) relative amounts of each cytokine gene level were determined by densitometric analysis. Steady-state expression of β-actin was used to control equal loading of the PCR product onto gels. The histogram shows the mRNA levels. B: blank; L: LPS; P: Periplocin.

in antigen- and collagen-induced arthritis mice [34]. In this study, the splenocytes of CFA-immunized Sprague Dawley rats lead to increased TGF-β1 levels in vivo and LPS induce higher level TGF-β1 in CFA-immunized Sprague Dawley rats splenocytes in vitro and LPS added to normal synoviocytes causes higher amount of TGF-β1. Increased levels of IL10 in serum were found in patients with RA [35]. In addition to losing anti-inflammatory functions, IL-10 can acquire proinflammatory functions. Evidence exists that interleukin- (IL-) 10 family cytokines may be involved in the pathogenesis of RA [36]. Increased serum levels of many cytokines were indeed found in other rheumatic diseases: notably psoriatic arthritis (IL-6, IL-7, IL-10, and IFN-γ, TGF-β, or TNF-α) suggesting that such rises may reflect inflammation rather than being disease specific [37–40].

Taken together, CFA-immunized Sprague Dawley rats are characteristic of a rapid onset of inflammation and higher values for common RA parameters, as shown by splenocyte cytokine analysis, the most accurate prognostic marker of human RA. This model is very useful for assessing the efficacy of various arthritis treatment protocols. Therefore, PFS and Periplocin can prophylactically treat autoimmune arthritis not only by controlling the systemic autoimmune responses but also by controlling local inflammation and bone destruction of the joints.

Competing Interests

The authors declare that there are no competing interests regarding the publication of this paper.

Acknowledgments

This work is supported by National Natural Science Foundation of China (Grants nos. 81260462 and 81460474) and Guangxi Natural Science Foundation China (Grant no. AA139104). Guangchen Sun is the grant recipient.

References

[1] G. S. Firestein, "Evolving concepts of rheumatoid arthritis," *Nature*, vol. 423, no. 6937, pp. 356–361, 2003.

[2] G. Weissmann, "The pathogenesis of rheumatoid arthritis," *Bulletin of the NYU Hospital for Joint Diseases*, vol. 64, no. 1-2, pp. 12–15, 2006.

[3] S. M. Nanjundaiah, B. Astry, and K. D. Moudgil, "Mediators of inflammation-induced bone damage in arthritis and their control by herbal products," *Evidence-Based Complementary and Alternative Medicine*, vol. 2013, Article ID 518094, 20 pages, 2013.

[4] R. F. van Vollenhoven, "Treatment of rheumatoid arthritis: state of the art," *Nature Reviews Rheumatology*, vol. 5, no. 10, pp. 531–541, 2009.

[5] P. Emery, "Treatment of rheumatoid arthritis," *British Medical Journal*, vol. 332, no. 7534, pp. 152–155, 2006.

[6] B. Astry, E. Harberts, and K. D. Moudgil, "A cytokine-centric view of the pathogenesis and treatment of autoimmune arthritis," *Journal of Interferon and Cytokine Research*, vol. 31, no. 12, pp. 927–940, 2011.

[7] P. P. Take and J. R. Kaleen, "Advances in rheumatology: new targeted therapeutics," *Arthritis Research & Therapy*, vol. 13, supplement 1, p. S5, 2011.

[8] J. E. Fonseca, H. Canhão, N. J. Tavares, M. Cruz, J. Branco, and M. V. Queiroz, "Persistent low grade synovitis without erosive progression in magnetic resonance imaging of rheumatoid arthritis patients treated with infliximab over 1 year," *Clinical Rheumatology*, vol. 28, no. 10, pp. 1213–1216, 2009.

[9] L. A. Joosten, M. M. Helsen, T. Saxne, F. A. van De Loo, D. Heinegard, and W. B. van Den Berg, "IL-1 alpha beta blockade prevents cartilage and bone destruction in murine type II collagen-induced arthritis, whereas TNF-alpha blockade only ameliorates joint inflammation," *Journal of Immunology*, vol. 163, no. 9, pp. 5049–5055, 1999.

[10] J. S. Smolen, C. Han, M. Bala et al., "Evidence of radiographic benefit of treatment with infliximab plus methotrexate in rheumatoid arthritis patients who had no clinical improvement: a detailed subanalysis of data from the anti-tumor necrosis factor trial in rheumatoid arthritis with concomitant therapy study," *Arthritis and Rheumatism*, vol. 52, no. 4, pp. 1020–1030, 2005.

[11] J. Wang, C. Wang, J. Chen, J. Du, D. Qiu, and M. Qiu, "Chemical constituents of Periploca forrestii and their cytotoxicity activity," *Zhongguo Zhongyao Zazhi*, vol. 34, no. 24, pp. 3214–3216, 2009.

[12] R. Xu, J. Du, L. Deng et al., "A new cardiac glycoside from Periploca forrestii," *Zhongguo Zhongyao Zazhi*, vol. 37, no. 15, pp. 2286–2288, 2012.

[13] H. Itokawa, J. P. Xu, and K. Takeya, "Studies on chemical constituents of antitumor fraction from *Periploca sepium* BGE. I," *Chemical & Pharmaceutical Bulletin*, vol. 35, no. 11, pp. 4524–4529, 1987.

[14] H. Itokawa, J. Xu, and K. Takeya, "Studies on chemical constituents of antitumor fraction from *Periploca sepium*. V. Structures of new pregnane glycosides, periplocosides J, K, F and O," *Chemical and Pharmaceutical Bulletin*, vol. 36, no. 11, pp. 4441–4446, 1988.

[15] L. Zhao, B. Shan, Y. Du, M. Wang, L. Liu, and F.-Z. Ren, "Periplocin from Cortex periplocae inhibits cell growth and down-regulates survivin and c-myc expression in colon cancer in vitro and in vivo via β-catenin/TCF signaling," *Oncology Reports*, vol. 24, no. 2, pp. 375–383, 2010.

[16] Z. J. Lu, Y. Zhou, Q. Song et al., "Periplocin inhibits growth of lung cancer in vitro and in vivo by blocking AKT/ERK signaling pathways," *Cellular Physiology and Biochemistry*, vol. 26, no. 4-5, pp. 609–618, 2010.

[17] C.-F. Cheng, I.-H. Lu, H.-W. Tseng et al., "Antitumor effect of periplocin in TRAIL-resistant human hepatocellular carcinoma cells through downregulation of IAPs," *Evidence-Based Complementary and Alternative Medicine*, vol. 2013, Article ID 958025, 11 pages, 2013.

[18] M. Ishida, H. Kitaura, K. Kimura et al., "Muramyl dipeptide enhances lipopolysaccharide-induced osteoclast formation and bone resorption through increased RANKL expression in stromal cells," *Journal of Immunology Research*, vol. 2015, Article ID 132765, 12 pages, 2015.

[19] K. Kessenbrock, V. Plaks, and Z. Werb, "Matrix metalloproteinases: regulators of the tumor microenvironment," *Cell*, vol. 141, no. 1, pp. 52–67, 2010.

[20] B. Joe, M. M. Griffiths, E. F. Remmers, and R. L. Wilder, "Animal models of rheumatoid arthritis and related inflammation," *Current Rheumatology Reports*, vol. 1, no. 2, pp. 139–148, 1999.

[21] L. Bevaart, M. J. Vervoordeldonk, and P. P. Tak, "Evaluation of therapeutic targets in animal models of arthritis: how does it relate to rheumatoid arthritis?" *Arthritis and Rheumatism*, vol. 62, no. 8, pp. 2192–2205, 2010.

[22] P. M. Barnes, B. Bloom, and R. L. Nahin, "Complementary and alternative medicine use among adults and children: United States. 2007," *National Health Statistics Reports*, vol. 12, pp. 1–23, 2008.

[23] U. Härtel and E. Volger, "Use and acceptance of classical natural and alternative medicine in Germany—findings of a representative population-based survey," *Forschende Komplementarmedizin und Klassische Naturheilkunde*, vol. 11, no. 6, pp. 327–334, 2004.

[24] K. J. Hunt, H. F. Coelho, B. Wider et al., "Complementary and alternative medicine use in England: results from a national survey," *International Journal of Clinical Practice*, vol. 64, no. 11, pp. 1496–1502, 2010.

[25] M. H. Goldrosen and S. E. Straus, "Complementary and alternative medicine: assessing the evidence for immunological benefits," *Nature Reviews Immunology*, vol. 4, no. 11, pp. 912–921, 2004.

[26] K. D. Moudgil and B. M. Berman, "Traditional Chinese medicine: potential for clinical treatment of rheumatoid arthritis," *Expert Review of Clinical Immunology*, vol. 10, no. 7, pp. 819–822, 2014.

[27] P. Zhang, J. Li, Y. Han, X. W. Yu, and L. Qin, "Traditional Chinese medicine in the treatment of rheumatoid arthritis: a general review," *Rheumatology International*, vol. 30, no. 6, pp. 713–718, 2010.

[28] G. Li, D. Liu, Y. Zhang et al., "Celastrol inhibits lipopolysaccharide-stimulated rheumatoid fibroblast-like synoviocyte invasion through suppression of TLR4/NF-κB-mediated matrix metalloproteinase-9 expression," *PLoS ONE*, vol. 8, no. 7, Article ID e68905, 2013.

[29] R. Cascão, B. Vidal, I. P. Lopes et al., "Decrease of CD68 synovial macrophages in celastrol treated arthritic rats," *PLoS ONE*, vol. 10, no. 12, Article ID e0142448, 2015.

[30] M. A. Ortiz, C. Diaz-Torné, M. V. Hernández et al., "IL-6 blockade reverses the abnormal STAT activation of peripheral blood leukocytes from rheumatoid arthritis patients," *Clinical Immunology*, vol. 158, no. 2, pp. 174–182, 2015.

[31] N. G. Arvidson, B. Gudbjornsson, L. Elfman, A.-C. Ryden, T. H. Totterman, and R. Hallgren, "Circadian rhythm of serum interleukin-6 in rheumatoid arthritis," *Annals of the Rheumatic Diseases*, vol. 53, no. 8, pp. 521–524, 1994.

[32] E. Gonzalo-Gil, G. Criado, B. Santiago, J. Dotor, J. L. Pablos, and M. Galindo, "Transforming Growth Factor (TGF)-β signalling is increased in rheumatoid synovium but TGF-β blockade does not modify experimental arthritis," *Clinical & Experimental Immunology*, vol. 174, no. 2, pp. 245–255, 2013.

[33] S. M. Wahl, J. B. Allen, G. L. Costa, H. L. Wong, and J. R. Dasch, "Reversal of acute and chronic synovial inflammation by anti-transforming growth factor beta," *Journal of Experimental Medicine*, vol. 177, no. 1, pp. 225–230, 1993.

[34] M. Sakuma, K. Hatsushika, K. Koyama et al., "TGF-β type I receptor kinase inhibitor down-regulates rheumatoid synoviocytes and prevents the arthritis induced by type II collagen antibody," *International Immunology*, vol. 19, no. 2, pp. 117–126, 2007.

[35] J. J. Cush, J. B. Splawski, R. Thomas et al., "Elevated interleukin-10 levels in patients with rheumatoid arthritis," *Arthritis and Rheumatism*, vol. 38, no. 1, pp. 96–104, 1995.

[36] T. T. Antoniv and L. B. Ivashkiv, "Dysregulation of interleukin-10-dependent gene expression in rheumatoid arthritis synovial macrophages," *Arthritis and Rheumatism*, vol. 54, no. 9, pp. 2711–2721, 2006.

[37] O. Elkayam, I. Yaron, I. Shirazi, M. Yaron, and D. Caspi, "Serum levels of IL-10, IL-6, IL-1ra, and sIL-2R in patients with psoriatic arthritis," *Rheumatology International*, vol. 19, no. 3, pp. 101–105, 2000.

[38] S. E. Jacob, M. Nassiri, F. A. Kerdel, and V. Vincek, "Simultaneous measurement of multiple Th1 and Th2 serum cytokines in psoriasis and correlation with disease severity," *Mediators of Inflammation*, vol. 12, no. 5, pp. 309–313, 2003.

[39] O. Arican, M. Aral, S. Sasmaz, and P. Ciragil, "Serum levels of TNF-α, IFN-γ, IL-6, IL-8, IL-12, IL-17, and IL-18 in patients with active psoriasis and correlation with disease severity," *Mediators of Inflammation*, vol. 2005, no. 5, pp. 273–279, 2005.

[40] A. Burska, M. Boissinot, and F. Ponchel, "Cytokines as biomarkers in rheumatoid arthritis," *Mediators of Inflammation*, vol. 2014, Article ID 545493, 24 pages, 2014.

Hindawi Publishing Corporation
Mediators of Inflammation
Volume 2016, Article ID 2543070, 14 pages
http://dx.doi.org/10.1155/2016/2543070

Research Article

Blockade of PLD2 Ameliorates Intestinal Mucosal Inflammation of Inflammatory Bowel Disease

Guangxi Zhou, Lin Yu, Wenjing Yang, Wei Wu, Leilei Fang, and Zhanju Liu

Department of Gastroenterology, The Shanghai Tenth People's Hospital of Tongji University, Shanghai 200072, China

Correspondence should be addressed to Zhanju Liu; liuzhanju88@126.com

Received 11 May 2016; Revised 15 August 2016; Accepted 21 August 2016

Academic Editor: Nona Janikashvili

Background. Inflammatory bowel diseases (IBD), including Crohn's disease (CD) and ulcerative colitis (UC), are chronically remittent and progressive inflammatory disorders. Phospholipase D2 (PLD2) is reported to be involved in the pathogenesis of several inflammatory diseases. However, the exact role of PLD2 in IBD is obscure. *Methods*. PLD2 expression was determined in peripheral blood cells and inflamed mucosa from patients with IBD by qRT-PCR. Colonic biopsies were also obtained from CD patients before and after infliximab (IFX) treatment to examine PLD2 expression. PLD2 selective inhibitor (CAY10594) was administrated daily by oral gavage in DSS-induced colitis mice. Bone marrow neutrophils from colitis mice were harvested to examine the migration using Transwell plate. *Results*. PLD2 was found to be significantly increased in peripheral blood cells and inflamed mucosa in patients with active IBD. Treatment with IFX could significantly decrease PLD2 expression in intestinal mucosa in patients with CD. Moreover, blockade of PLD2 with CAY10594 could markedly ameliorate DSS-induced colitis in mice and promote neutrophil migration. *Conclusions*. PLD2 plays a critical role in the pathogenesis of IBD. Blockade of PLD2 may serve as a new therapeutic approach for treatment of IBD.

1. Introduction

Inflammatory bowel diseases (IBD), comprising of Crohn's disease (CD) and ulcerative colitis (UC), are chronically remittent and progressive inflammatory disorders occurring in gastrointestinal tract [1–3], characterized by intestinal mucosal inflammation culminating in abdominal pain, recurrent diarrhea, blood stools, and loss of body weight [4, 5]. Although the underlying etiology and pathology of IBD remain elusive, accumulating evidence has demonstrated that the pathogenesis of IBD is the result of abnormal immune response to intestinal microbiota in genetically susceptible individuals [6–8]. During acute inflammation, large numbers of leukocytes infiltrate into inflamed mucosa of IBD, and these leukocytes, as well as intestinal stromal and epithelial cells, produce large amounts of proinflammatory cytokines, which further contribute to inflammatory damage in gut mucosa [9]. $CD4^+$ T cells, an important component of adaptive immune system, have been reported to play a critical role in the pathogenesis of IBD. Studies have implicated that T helper (Th) 1-related cytokines, such as interferon-(IFN-) γ and tumor necrosis factor (TNF-) α, Th17 associated cytokines, such as interleukin- (IL-) 17A, IL-6, and IL-23, and Th2 related cytokines such as IL-4, IL-5, and IL-13 are involved in the induction and development of IBD [10]. Neutrophils, the most abundant leukocyte population, are also indispensable in protecting the host from the invading of microbial pathogens [11]. They are endowed with the several capacities of antimicrobial functions, such as phagocytosis, formation of neutrophil extracellular trap (NET), degranulation, and release of reactive oxygen species (ROS) [12]. Moreover, neutrophils can also facilitate epithelial repairing and mucosal healing by releasing cytokines and chemokines necessary for the resolution of mucosal inflammation [12, 13].

Phospholipase D2 (PLD2), a lipid-signaling enzyme, is a member of PLD family and can catalyze the hydrolysis of phosphatidylcholine (PC) to produce the signaling molecule phosphatidic acid (PA) [14]. PLD2 and PA can interact with several types of phosphatases, kinases, proteins and phospholipases, and further mediate multiple cellular functions, including activation, proliferation, apoptosis, and migration [14–17]. Evidence has shown that PLD2 participates in

the pathogenesis of several diseases, such as pathological angiogenesis, sepsis, asthma, Alzheimer's disease, and cancer [18–21]. It has been found to be involved in the enhancement of basal permeability through regulating cytoskeleton reorganization and occludin expression [22]. Inhibition of PLD2 could promote colon cancer cell apoptosis through downregulating PI3K-AKT signaling pathway and play a protective role in colonic cancer [23]. Moreover, PLD2 also induces the aggravation and drives mortality in sepsis by inhibiting the formation of neutrophil extracellular trap and reducing the expression of CXCR2 [18]. These data suggest that PLD2 was involved in the development of inflammation and colorectal cancer; however, whether PLD2 participates in the pathogenesis of IBD remains unknown.

In this study, we found that PLD2 was highly expressed in peripheral blood cells and inflamed mucosa in patients with active IBD. Stimulation with TNF-α could markedly enhance PLD2 expression in neutrophils, and treatment with IFX could reverse the increased expression of PLD2. Moreover, inhibition of PLD2 with CAY10694 could significantly ameliorate DSS-induced intestinal colitis in mice. Inhibition of PLD2 could also promote neutrophil migration through upregulating CXCR2 expression. Therefore, our data suggest that blockade of PLD2 ameliorates intestinal mucosal inflammation and that PLD2 could be a therapeutic target for treatment of IBD.

2. Materials and Methods

2.1. Patients. All peripheral blood and colonic biopsies samples were collected from IBD patients from July 2014 to October 2015 at the Department of Gastroenterology, the Shanghai Tenth People's Hospital of Tongji University (Shanghai, China). Peripheral blood samples were obtained from patients with active CD (A-CD, $n = 25$), patients with CD in remission (R-CD, $n = 19$), patients with active UC (A-UC, $n = 20$), patients with UC in remission (R-UC, $n = 21$), and healthy controls ($n = 28$). Colonic biopsy samples were collected from patients with A-CD ($n = 21$), R-CD ($n = 27$), A-UC ($n = 26$), R-UC ($n = 26$), and HC ($n = 18$) during colonoscopy. The final diagnoses for CD or UC were based on clinical characteristics, radiological and endoscopic examination, and histological findings (see Supplementary Table 1 in Supplementary Material available online at http://dx.doi.org/10.1155/2016/2543070) [24]. International standard criteria such as Crohn's disease activity index (CDAI) and Mayo scores were used to assess the disease severity in patients with CD and UC, respectively [25, 26]. This study was approved by the Institutional Review Board for Clinical Research of the Shanghai Tenth People's Hospital of Tongji University. Written informed consent was also obtained from all subjects before study.

2.2. Anti-TNF mAb Treatment in Patients with Active CD. Seventeen patients were diagnosed as active CD according to a CDAI score ≥ 150 points and treated with anti-TNF mAb (5 mg/kg, infliximab (IFX); Cilag AG, Schaffhausen, Switzerland) at weeks 0, 2, and 6 as described previously [27]. All patients were monitored weekly during the follow-up examination, and colonic biopsies were collected at weeks 0 and 12 after the first infusion. The efficacy of IFX treatment was assessed according to CDAI and mucosal healing by endoscopy as described previously [27]. Clinical remission was defined as a CDAI score of <150 points, and clinical response as a decrease of CDAI score ≥ 70 points at the evaluation time point in comparison with the baseline index.

2.3. Mucosal Biopsy Culture In Vitro. Colonic biopsies were obtained from patients with A-CD ($n = 17$) during endoscopic examination and cultured *ex vivo* (2 biopsy samples/well) in 1 mL RPMI 1640 medium in the presence of IFX or control human IgG (HIg) (both at 50 μg/mL) at 37°C in 5% CO2 humidified air for 24 h. RNA was then extracted from cultured mucosal tissues and used to the examination of PLD2 by qRT-PCR.

2.4. Mice. Specific pathogen-free C57BL/6 mice were purchased from the Shanghai SLAC Laboratory Animal Co. Ltd. (Shanghai, China). Mice were raised under specific pathogen-free conditions with filtered air and allowed free access to sterile water and autoclaved food. Mice used in experiment were at 8–10 weeks of age and 20–25 g of weight. Animal studies were reviewed and approved by the Institutional Animal Care and Use Committee of Tongji University.

2.5. Establishment of DSS-Induced Colitis Model in Mice. DSS-induced colitis model was established using a method described previously [28]. Briefly, two groups of C57BL/6 (10 mice per group) were given 2.5% DSS in the drinking water for continuous seven days, and at 8th day, all the mice were given sterile water for another three days. One group of mice was administered with PLD2 selective inhibitor (CAY10694, Santa Cruz Biotechnology, 4 mg/kg) daily by oral gavage, and another group of mice were administered with PBS as controls. Other two groups of mice (10 mice per group) were given sterile water for ten continuous days as negative controls. During the observation of 10 days, characteristics of acute colitis were observed daily, including diarrhea, bloody stools, body weight, and survival rates. At the 10th day, all the mice were sacrificed; colonic tissues were obtained from mice. A small part of colon (0.5 cm) was fixed in 10% paraformaldehyde overnight used for H&E staining, and another small part of colon (0.5–1.0 cm) was used for RNA extraction and qRT-PCR analysis. Furthermore, bone marrow cells of mice were also isolated after red blood cell lysis. Neutrophils were then isolated from bone marrow of mice using neutrophil isolation kit (Miltenyi Biotec, order: 130097658) and used for flow cytometry analysis and migration capacity analysis by Transwell plates (MultiScreen, 5 μm) *in vitro*.

2.6. Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR). Total RNA was extracted from the fresh-frozen biopsies or mouse colonic tissues, and the quantity and quality were assessed using a NanoVue spectrophotometer (GE Healthcare, Piscataway, NJ, USA), with a 260/280 ratio between 1.8 and 2.0. The complementary DNA (cDNA) was

Table 1: The primers using in qRT-PCR analysis.

Gene	Species	DNA sequence (sense 5′-3′)	DNA sequence (antisense 5′-3′)
PLD2	Human	CAGATGGAGTCCGATGAGGTG	CCGCTGGTATATCTTTCGGTG
IL-17A	Mouse	TTTAACTCCCTTGGCGCAAAA	CTTTCCCTCCGCATTGACAC
IFN-γ	Mouse	ATGAACGCTACACACTGCATC	CCATCCTTTTGCCAGTTCCTC
IL-1β	Mouse	TTCAGGCAGGCAGTATCACTC	GAAGGTCCACGGGAAAGACAC
IL-10	Mouse	GCTCTTACTGACTGGCATGAG	CGCAGCTCTAGGAGCATGTG
CXCR2	Mouse	TGTCTGGGCTGCATCTAAAGT	AGGTAACCTCCTTCACGTATGAG
GRK2	Mouse	AGCCCTTGGTGGAGTTCTAC	CCCCTCGGAGGTTCTGACA
PLD2	Mouse	GTGGTGGGCACCGAAAGATAC	CATGCGTCAAGCGAACAGAA
GAPDH	Human	CTGGGCTACACTGAGCACC	AAGTGGTCGTTGAGGGCAATG
GAPDH	Mouse	AGGTCGGTGTGAACGGATTTG	GGGGTCGTTGATGGCAACA

synthesized with 5x All-In-One RT MasterMix (abm) according to the manufacturer's instructions. Reverse transcription-PCR reactions were performed using the following conditions: 25°C for 10 min and 42°C for 15 min, followed by 85°C for 5 min. The synthesized cDNA was stored at −20°C. qRT-PCR was performed using SYBR green methodology according to the following conditions: 95°C for 1 min, followed by 40 cycles at 95°C for 15 s and 60°C for 30 s with 40 cycles. All samples for qRT-PCR analysis were performed in triplicate wells. All the primers were synthesized from ShengGong BioTeck (Shanghai, China) and GAPDH was used as the endogenous reference gene (Table 1). The relative levels of target gene expression were calculated as a ratio relative to the GAPDH reference. qRT-PCR analysis was carried out using the $2^{-\Delta\Delta Ct}$ method [29].

2.7. Immunohistochemistry. Immunohistochemistry was performed on 5-μm-thick sections from fresh-frozen biopsies from IBD patients and healthy controls. Sections were air-dried overnight, fixed in acetone for 10 min, and rinsed in phosphate-buffered saline (PBS) for 5 min. After incubation with EnVision FLEX Peroxidase-Blocking Reagent for 10 min, these sections were incubated with rabbit anti-human PLD2 polyclonal antibody (Abcam, dilution 1 : 100) at 4°C overnight. After washing in PBS, the sections were incubated for 60 min with HRP-conjugated goat anti-rabbit or goat anti-mouse IgG (dilution 1 : 400) at room temperature. The colour reaction was developed with 3,3′-diaminobenzidine and the sections were counterstained with haematoxylin. As negative controls, sections were treated with PBS instead of primary antibody. To determine the proportion of positive cells, five fields of intestinal mucosa were selected randomly at high power (×400) [29].

2.8. Statistical Analysis. Data were expressed as mean ± SEM and analyzed using SPSS statistics version 14.0 (SPSS, Chicago, IL, USA). Statistical comparisons were performed using an unpaired two-tailed Student's t-test or one-way analysis of variance (ANOVA). Paired t-test was performed to analyze the statistics before and after IFX treatment. $^{*}p < 0.05$ was considered statistically significant, $^{**}p < 0.01$ was considered obviously statistically significant, and $^{***}p < 0.001$ was considered very obviously statistically significant.

3. Results

3.1. PLD2 Is Highly Expressed in Peripheral Blood Cells and Inflamed Mucosa in Patients with Active IBD. Previous work has demonstrated that PLD2 participates in the pathogenesis of sepsis and chronic asthma [18, 21]; we hypothesized that PLD2 may also involve the induction and development of IBD. Thus, peripheral blood and inflamed mucosa were collected from patients with active IBD and healthy controls, and we found that PLD2 expression was significantly increased in peripheral blood cells and inflamed mucosa in A-CD and A-UC patients compared with healthy controls. However, there was no significant difference between patients with R-CD or R-UC and healthy controls. No statistical difference was observed between CD and UC groups (Figures 1(a) and 1(b)). Furthermore, we compared PLD2 expression in inflamed and unaffected mucosa from the same IBD patients and found that PLD2 expression was markedly more increased in inflamed mucosa than that in unaffected controls (Figures 1(c) and 1(d)). Immunohistochemistry staining showed that a percentage of PLD2 positive cells were significantly increased in lamina propria in inflamed mucosa from patients with CD or UC compared with healthy controls (Figure 1(e)).

To determine phenotypic expression of PLD2 in different subsets of cells, we isolated neutrophils, $CD4^+$ T cells, $CD8^+$ T cell, $CD14^+$ monocytes, and $CD20^+$ B cells from healthy donors, and expression of PLD2 was analyzed by qRT-PCR. Figure 2(a) shows that PLD2 was mainly expressed in neutrophils. Moreover, we analyzed percentage and absolute numbers of neutrophils in peripheral blood from patients with IBD and observed that percentage and absolute numbers of neutrophils were significantly increased (Supplementary Figure 1). CD66b was a specific marker expressed in neutrophils, and immunohistochemical staining revealed that $CD66b^+$ cells were markedly increased in inflamed mucosa from patients with IBD compared with those in HC (Supplementary Figure 2). We then isolated neutrophils from peripheral blood of patients with IBD and found that PLD2 mRNA expression was highly increased in peripheral neutrophils from IBD patients compared with HC (Figure 2(b)). Therefore, our data indicate that PLD2 is highly expressed in peripheral neutrophils and inflamed mucosa of IBD and that it may play an important role in the pathogenesis of IBD.

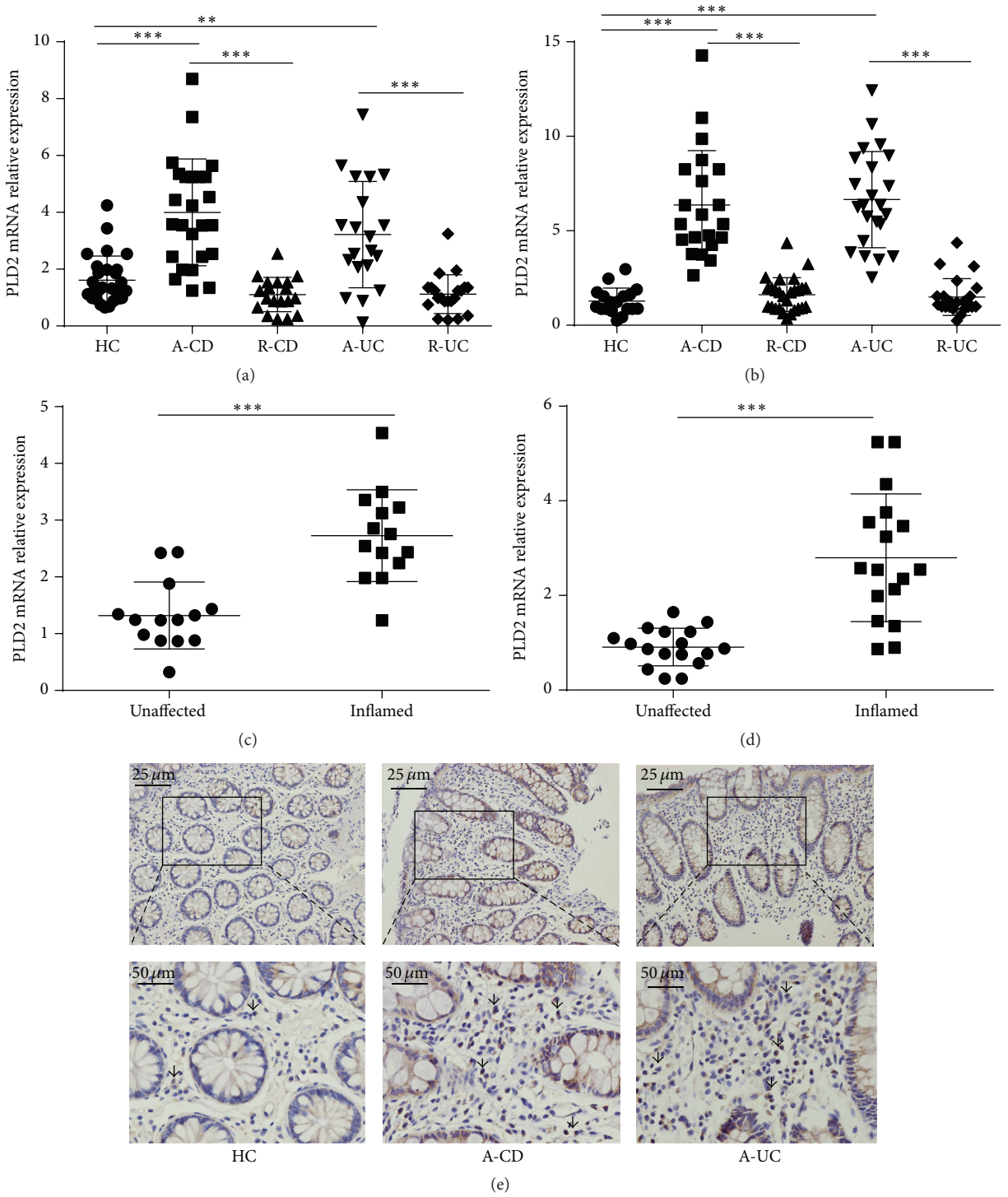


Figure 1: PLD2 is highly expressed in patients with active IBD. (a) Peripheral blood samples were collected from patients with active CD (A-CD, $n = 25$), patients with CD in remission (R-CD, $n = 19$), patients with active UC (A-UC, $n = 20$), patients with UC in remission (R-UC, $n = 21$), and healthy controls ($n = 28$). Expression of PLD2 mRNA was detected by qRT-PCR. (b) Colonic biopsies were collected from patients with A-CD ($n = 21$), R-CD ($n = 27$), A-UC ($n = 26$), R-UC ($n = 26$), and HC ($n = 18$). Expression of PLD2 mRNA was examined by qRT-PCR. Gene expression was normalized to GAPDH in each group. $^{**}p < 0.01$ and $^{***}p < 0.001$ versus HC. ((c) and (d)) Expression of PLD2 mRNA in inflamed and healthy intestinal mucosa from the same patients with A-CD ((c) $n = 14$) and A-UC ((d) $n = 17$) was examined by qRT-PCR. Gene expression was normalized to GAPDH in each group. $^{**}p < 0.01$ and $^{***}p < 0.001$ versus unaffected mucosa. (e) Representative images of immunohistochemical staining of PLD2 in inflamed colon from patients with A-CD, A-UC, and normal colonic mucosa from HC. Original magnification ×200 (top) and original magnification ×400 (bottom).

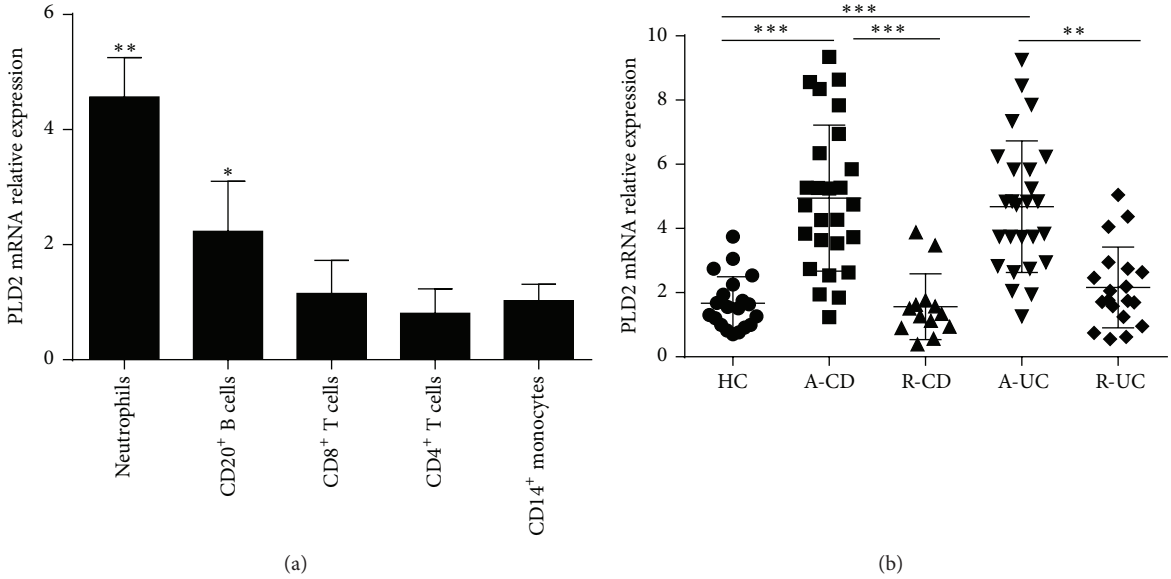


Figure 2: PLD2 is mainly expressed in neutrophils. (a) Expression of PLD2 in different subsets of immune cells. Peripheral neutrophils, $CD20^+$ B cells, $CD8^+$ T cells, $CD4^+$ T cells, and $CD14^+$ monocytes (1×10^6) were isolated from healthy donors ($n = 10$), and expression of PLD2 was detected by qRT-PCR. $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ versus $CD14^+$ monocytes. (b) Neutrophils were isolated from peripheral blood of patients with A-CD ($n = 26$), R-CD ($n = 23$), A-UC ($n = 26$), R-UC ($n = 19$), and HC ($n = 20$). Expression of PLD2 mRNA was examined by qRT-PCR. $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ versus HC. Gene expression was normalized to GAPDH in each group.

3.2. TNF-α Markedly Upregulates PLD2 Expression. After confirming that PLD2 was increased in inflamed mucosa in patients with active IBD, we then investigated the mechanisms involved in increased expression of PLD2. Since accumulating evidences have reported that a variety of cytokines (e.g., TNF-α, IL-6, IL-17A, IFN-γ, and IL-1β) participate in the pathogenesis of IBD [29–32], we investigated whether these proinflammatory cytokines participated in the upregulation of PLD2 expression. Peripheral blood neutrophils were isolated from healthy donors and stimulated with IL-6, IL-17A, TNF-α, LPS, IFN-γ, and IL-1β, respectively, for 4 h *in vitro*. As shown in Figure 3(a), TNF-α greatly enhanced PLD2 expression in human peripheral neutrophils, and LPS and IL-1β could modestly enhance PLD2 expression. Furthermore, neutrophils were stimulated with TNF-α at different concentrations for different time points, and we found that TNF-α could upregulate PLD2 expression in a dose- and time-dependent manner (Figures 3(b) and 3(c)). Previous work has confirmed that anti-TNF-α treatment could ameliorate intestinal mucosal inflammation in patients with active IBD [33, 34]. Therefore, we collected colonic biopsies from patients with CD before and after treatment. Patients with active CD were also treated with IFX at weeks 0, 2, and 6 as described previously [27], and colonic biopsies were obtained from 17 patients who achieved clinical remission. PLD2 expression was found to be markedly decreased after IFX treatment (Figure 4(a)), while there was no difference in PLD2 expression in intestinal mucosa from CD patients who failed in IFX therapy. We further investigated whether IFX treatment could decrease PLD2 expression in colonic mucosa *ex vivo*. Freshly inflamed colonic tissues were collected from patients with A-CD and A-UC and cultured *in vitro* under stimulation with either IFX or control human IgG (HIg) for 24 h. As shown in Figures 4(b) and 4(c), expression of PLD2 was also markedly downregulated after IFX treatment compared with controls. Taken together, these data indicate that expression of PLD2 is increased in patients with active IBD, and anti-TNF treatment could downregulate PLD2 expression in intestinal mucosa.

3.3. Blockade of PLD2 Ameliorates DSS-Induced Colitis in Mice. To further investigate the potential role of PLD2 in the pathogenesis of intestinal mucosal inflammation, acute colitis was induced in C57BL/6 mice by 2.5% DSS as described in Materials and Methods. PLD2 selective inhibitor CAY10594 (4 mg/Kg) was then administrated daily by oral gavage as indicated [18], and PBS as medium was also administrated daily as controls. Another two groups of mice were administrated CAY10694 or PBS daily as mentioned above without DSS exposure to serve as negative controls. Clinical characteristics of colitis, such as diarrhea, bloody stools, body weight, and survival rate, were observed daily. On day 10, all the mice were sacrificed, and colonic tissues and bone marrow cell were obtained for further study. As shown in Supplementary Figures 3(A) and 3(B), expression of PLD2 was observed to be markedly increased in inflamed mucosa and bone marrow-derived neutrophils in mice with DSS-induced acute colitis. Interestingly, administration of CAY10594 markedly ameliorated intestinal mucosal inflammation, characterized by higher survival rates, slighter decrease of body weight, less or even no bloody stools, and lower levels of pathological scores compared with controls

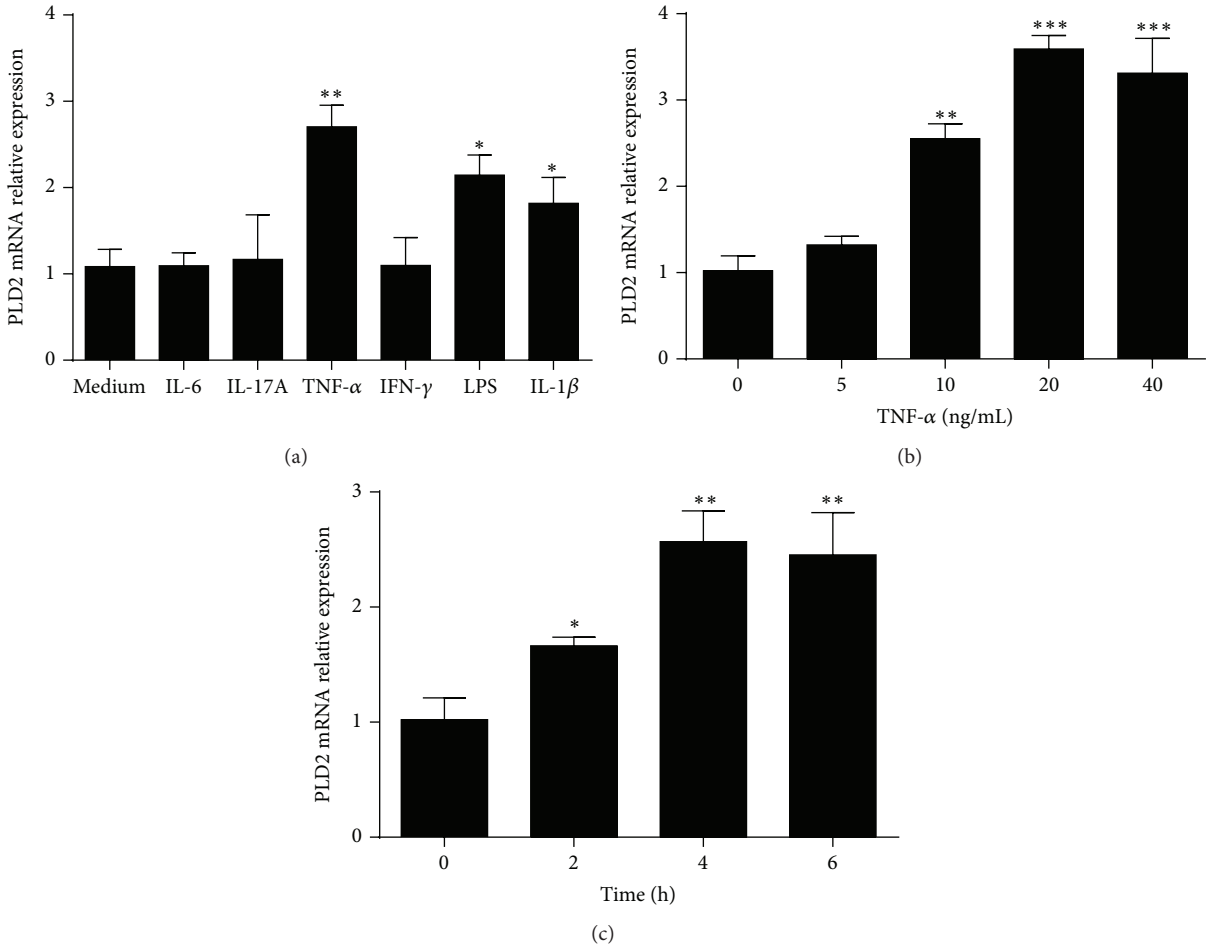


FIGURE 3: TNF-α upregulates PLD2 expression in neutrophils. (a) Neutrophils isolated from healthy donors ($n = 10$) were stimulated with IL-6 (10 ng/mL), IL-17A (10 ng/mL), TNF-α (10 ng/mL), IFN-γ (10 ng/mL), LPS (10 ng/mL), and IL-1β (10 ng/mL), respectively, for 4 h. Expression of PLD2 mRNA was detected by qRT-PCR. $^{*}p < 0.05$, $^{**}p < 0.001$, and $^{***}p < 0.01$ versus medium alone. (b) Neutrophils isolated from healthy donors ($n = 10$) were stimulated *in vitro* with different concentrations of TNF-α as indicated for 4 h; expression of PLD2 was detected by qRT-PCR. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ versus that under culture in medium alone. (c) Neutrophils isolated from healthy donors ($n = 10$) were stimulated with TNF-α (10 ng/mL) for 2, 4, and 6 h, and expression of PLD2 was detected by qRT-PCR. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ versus that at the beginning of stimulation. Gene expression was normalized to GAPDH in each group.

(Figure 5). RNA was extracted from colonic tissue to detect cytokine expression, and expression of proinflammatory cytokines, such as TNF-α, IL-6, IL-23, and IL-1β, was found to be markedly decreased after blockade of PLD2 in DSS-induced colitis, whereas anti-inflammatory cytokine (e.g., IL-10) was significantly increased after inhibition of PLD2 (Figures 6(a)–6(f)). Moreover, fresh colonic samples were also obtained and cultured *in vitro* for 24 h; the supernatants were collected to detect cytokines by ELISA. As shown in Figures 6(g)–6(j), the levels of proinflammatory cytokines (e.g., IL-17A, TNF-α, and IL-1β) were found to be decreased, while anti-inflammatory cytokine (e.g., IL-10) was found to be increased after blockade of PLD2, suggesting that blockade of PLD2 could ameliorate intestinal mucosal inflammation.

3.4. Blockade of PLD2 Improves Neutrophil Migration. To determine how PLD2 regulated intestinal mucosal inflammation, we isolated bone marrow cells from mice with DSS-induced colitis. As shown in Figures 7(a) and 7(b), the percentage of neutrophils in bone marrow was found to be significantly decreased after blockade of PLD2 in DSS-induced colitis mice, indicating that PLD2 was involved in the capacity of neutrophils migration. Previous data has shown that CXCR2, a chemokine receptor, is mainly expressed in neutrophils and mediates neutrophil mobilization to peripheral blood and inflammatory sites, which is regulated by G protein-coupled receptor kinase (GRK2) [35, 36]. We isolated neutrophils from bone marrow of mice with DSS-induced acute colitis and found that expression of CXCR2 was significantly increased in inflamed colon of DSS-induced colitis mice after blockade of PLD2 and that GRK2 expression was markedly decreased after PLD2 inhibition compared with WT controls (Figures 7(c) and 7(d)). To further determine the role of PLD2, we isolated neutrophils from bone marrow of wild-type mice with DSS-induced colitis and analyzed neutrophil migration in Transwell plate

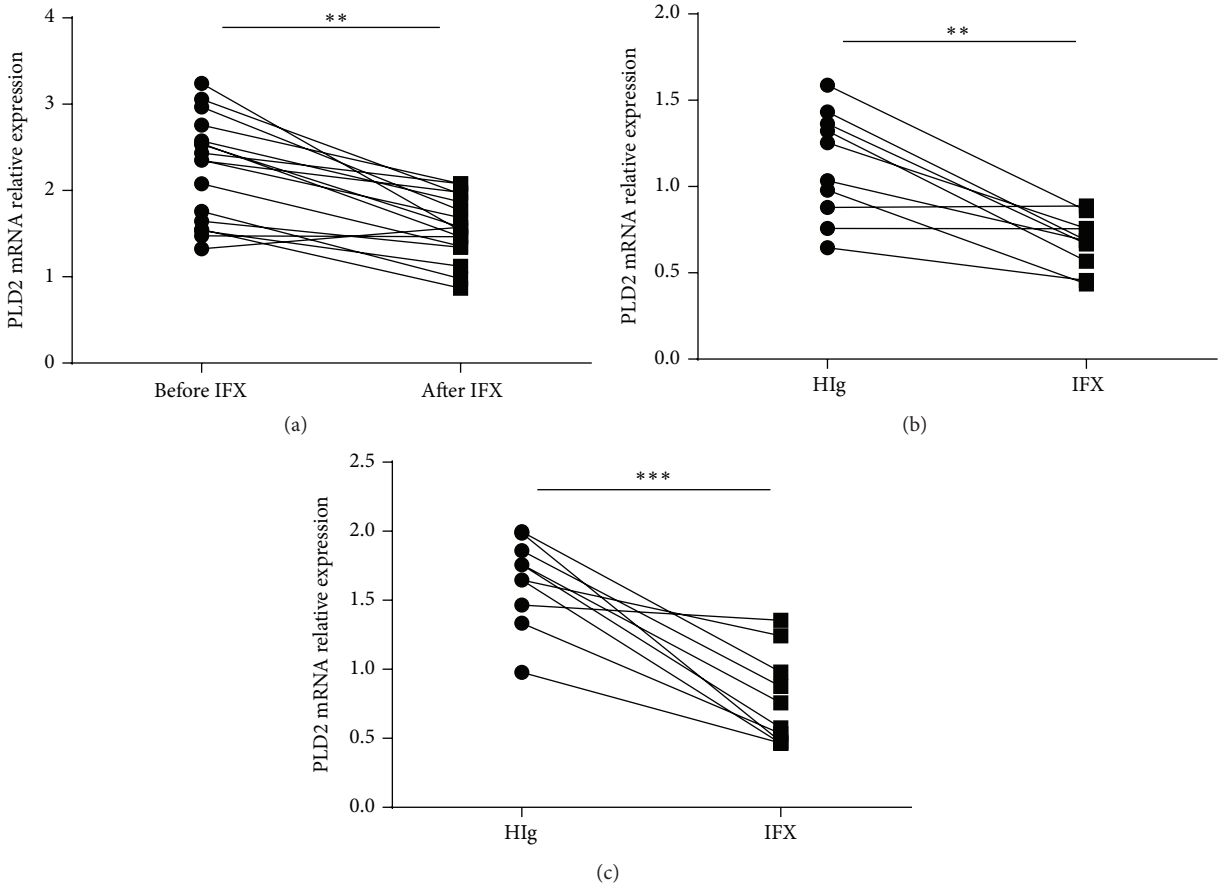


Figure 4: Anti-TNF therapy downregulates PLD2 expression. (a) Patients with A-CD ($n = 17$) were treated with anti-TNF mAb (IFX, 5 mg/kg) as indicated. Intestinal mucosal biopsies were collected before and at week 12 after the first infusion. Expression of PLD2 mRNA in intestinal mucosa was detected by qRT-PCR. $^{**}p < 0.01$ versus that before IFX treatment. ((b) and (c)) Fresh colonic biopsies were harvested from inflamed mucosa in patients with active CD ((b) $n = 10$) or active UC ((c) $n = 10$) and cultured *in vitro* with IFX or control human IgG (HIg) (50 μg/mL) for 24 h. Expression of PLD2 mRNA was detected by qRT-PCR. $^{**}p < 0.01$ and $^{***}p < 0.001$ versus controls.

in vitro. After inhibition of PLD2 *in vitro*, the capacity of neutrophil migration was significantly upregulated (Figure 7(e)). Taken together, these data suggest that inhibition of PLD2 may enhance neutrophil migration capacity, promote its mobilization to inflammatory intestinal mucosa, and further ameliorate mucosal inflammation.

4. Discussion

The main characteristics of IBD are multiple inflammatory responses associated with mucosal damage, increased epithelial permeability, bacterial invasion, and massive recruitment of neutrophils [8]. Neutrophils are one of major immune cells involved in the pathogenesis of IBD. In this study, we demonstrated that PLD2 was mainly expressed in neutrophils and that TNF-α could upregulate PLD2 expression. Importantly, blockade of PLD2 markedly ameliorated intestinal mucosal inflammation through enhancing neutrophils migration. Therefore, our data suggest that PLD2 plays an important role in the pathogenesis of IBD and that blockade of PLD2 may be a new therapeutic target for the management of IBD.

Neutrophils, critical components of innate immune system, are the first line of cellular defense extravasating into inflammatory tissue in response to invading of pathogens and functions as essential mediators in many diseases [37]. Patients with congenital neutrophil disorders are more susceptible to severe infection of bacteria and fungi, suggesting that neutrophils are indispensable in protecting the host from microbes. In patients with chronic granulomatous disease, neutrophils are defective in the production of ROS due to the dysfunction of NADPH oxidase, which culminates in the recurrent infection and life-span shortening and further underscores the importance of neutrophils [38]. In mouse sepsis models, when mice lack some antimicrobial capacity (e.g., degranulation), mice die quickly after the exposure to sepsis [39]. These studies highlight the indispensable role of neutrophils in antimicrobial defense in acute inflammation. However, what does the role of neutrophils in intestinal inflammation of IBD? In DSS-induced mouse colitis, intestinal inflammation has been observed to be exacerbated after the depletion of neutrophils by administration of anti-Gr1 antibodies, indicating a beneficial role of neutrophils in colitis [40–42]. Moreover, neutrophils have been shown to promote

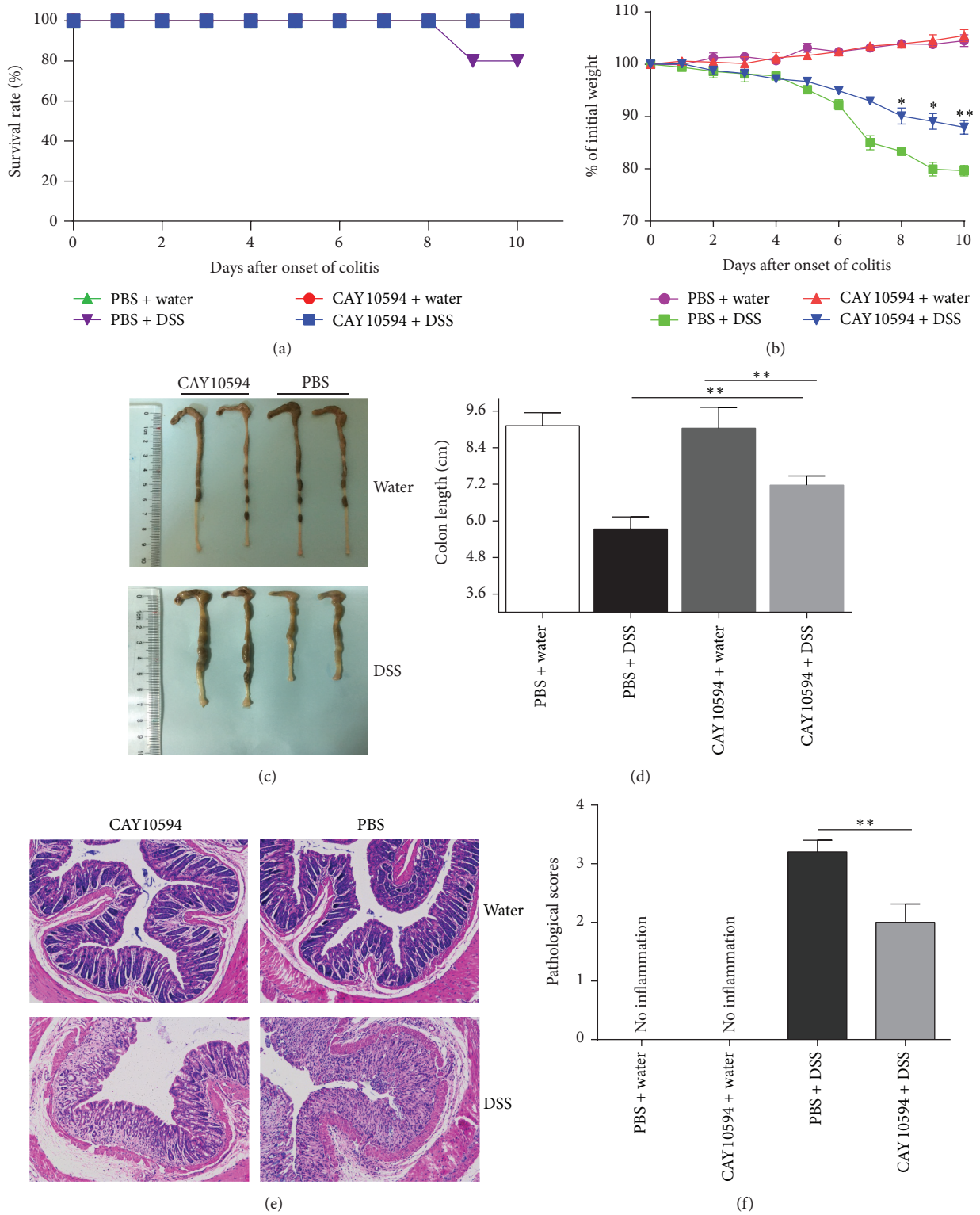


Figure 5: Blockade of PLD2 alleviates DSS-induced colitis in mice. DSS-induced colitis in C57BL/6 mice was induced as indicated. Two groups of DSS-exposed mice ($n = 10$) were treated with PLD2 inhibitor (CAY10594, 4 mg/Kg) or PBS as controls daily by oral gavage. Two groups of none DSS-exposed mice ($n = 10$) were also treated with CAY10594 or PBS as negative controls. (a) The survival rates of mice after DSS exposure over 10 days. (b) The changes of body weight were observed and expressed as a percentage of initial body weight at the start of experiments during 10-day observation. (c) Gross morphology of colonic tissues on day 10 after DSS induction. (d) The statistical length of colons in different groups. $^{*}p < 0.05$ and $^{**}p < 0.01$ versus controls. (e) Representative H&E staining images of colonic sections (×200). (f) The changes in pathological scores from colonic sections were calculated as indicated. $^{*}p < 0.05$ and $^{**}p < 0.01$ versus controls.

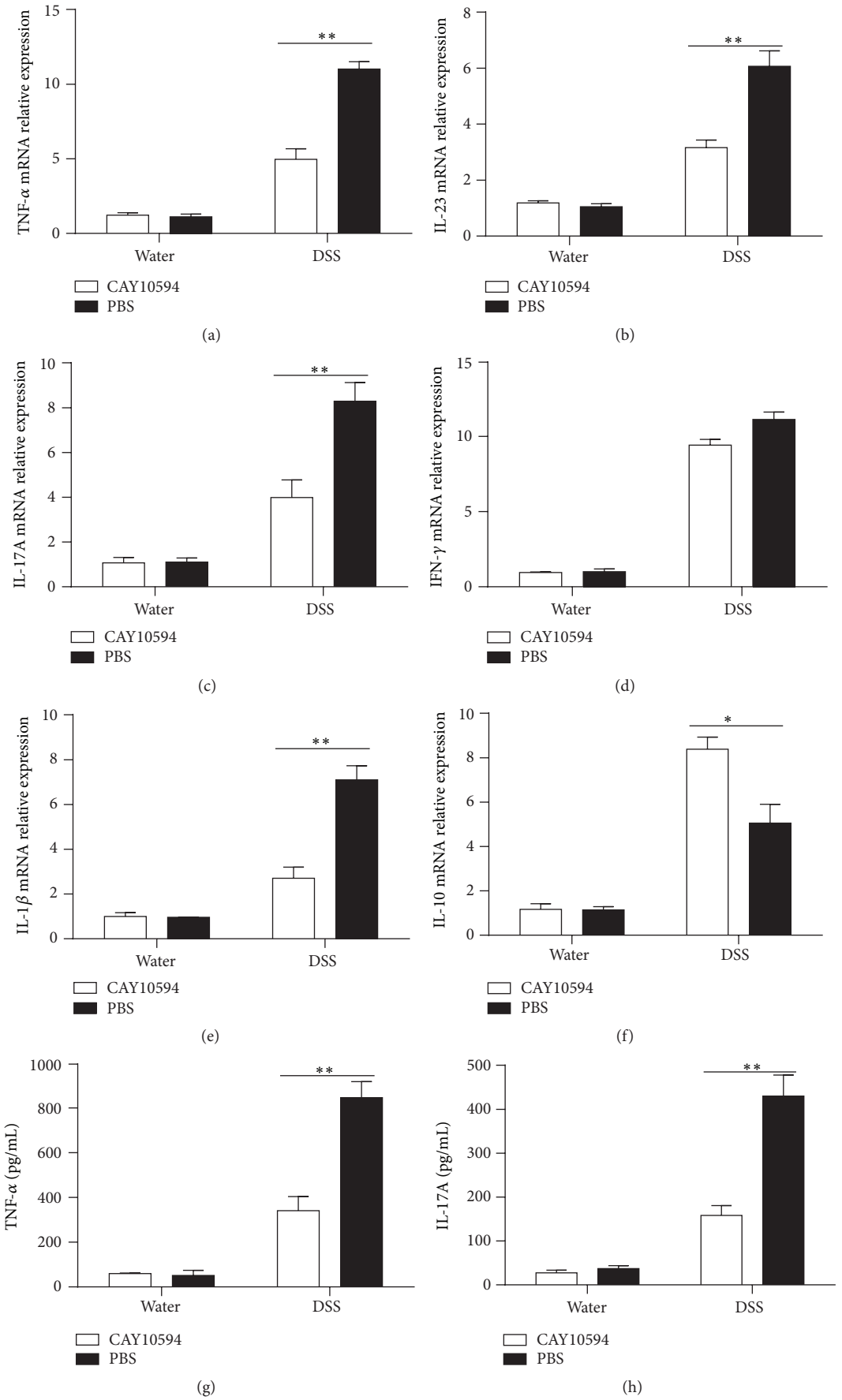


Figure 6: Continued.

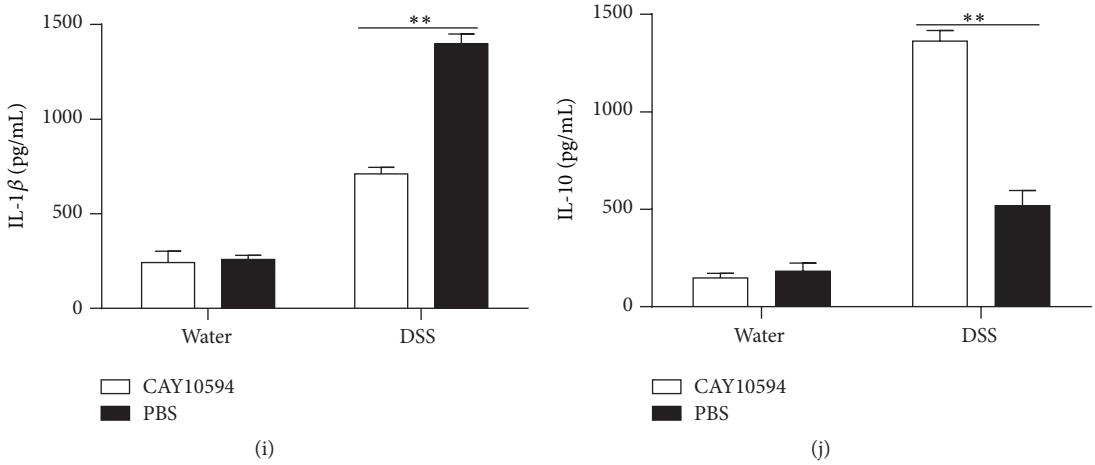


Figure 6: Cytokines profiles in colonic tissues from DSS-induced colitis mice after PLD2 inhibition. ((a)–(f)) Colonic tissues were obtained from mice on day 10 after DSS-induced colitis, and total RNA was extracted to detect mRNA levels of various cytokines by qRT-PCR. $^{*}p < 0.05$ and $^{**}p < 0.01$ versus controls. ((g)–(j)) Colonic tissues (0.01 g/sample) from mice on day 10 after DSS-induced colitis were cultured *ex vivo* at 37°C for 24 h; the supernatants were then collected for detection of TNF-α, IL-17A, IL-1β, and IL-10 by ELISA. $^{*}p < 0.05$ and $^{**}p < 0.01$ versus controls.

the production of several growth factors (e.g., vascular endothelial growth factor), proresolution lipid (e.g., lipoxins, resolvins, and protectins), and anti-inflammatory molecule (e.g., lipoxin A4), contributing to resolution of mucosal inflammation [11]. Interestingly, treatment with lipoxin A4 has been found to ameliorate DSS-induced intestinal inflammation [43]. Furthermore, previous study has confirmed that neutrophils can promote epithelial repairing and mucosal healing through the production of IL-22 [44]. In the current study, our data also demonstrated that percentage and numbers of neutrophils were significantly increased in peripheral blood and inflamed mucosa from patients with IBD, further proving the critical role of neutrophils in the pathogenesis of IBD.

PLD2 is an enzyme that catalyzes the conversion of membrane PC to choline and PA. PLD2 is expressed in nearly all types of leukocytes and has been associated with phagocytosis, degranulation, microbial killing, and leukocyte maturation [20]. Di Fulvio and Gomez-Cambronero found that the expression of PLD2 increased during neutrophil granulocytic differentiation [45]. In another study of sepsis [18], Lee et al. found that PLD2 deficiency not only increases survival but also decreases vital organ damage during experimental sepsis. Production of proinflammatory cytokines (e.g., TNF, IL-17, and IL-23) and cellular apoptosis in kidney and liver are markedly decreased in PLD2$^{-/-}$ mice. PLD2$^{-/-}$ neutrophils significantly protect wild-type mice against sepsis-induced death through the increased formation of NET [18]. In our study, we demonstrated that PLD2 expression was highly increased in peripheral blood and inflamed mucosa of IBD patients and was mainly expressed in neutrophils. Moreover, inhibition of PLD2 ameliorated intestinal mucosal inflammation and reduced the mortality after exposure to DSS. Therefore, PLD2 plays an important role in mucosal inflammation of IBD.

Chemotaxis is one important feature of neutrophils in regulating inflammation [46]. Impairment of chemotaxis has been reported in a wide variety of diseases associated with increased susceptibility to infection. In patients with chronic kidney disease (CKD), FGF23, an endocrine hormone that can regulates phosphorus homeostasis, inhibits the activation and recruitment of neutrophils, and FGF23 neutralization in mouse CKD models restore leukocyte recruitment and host defense [47]. Evidences have been proven that MAPK p38 signaling pathway is required for the recruitment of neutrophils to sites of inflammation and that aberrant p38 signaling in neutrophils aggravates the inflammation in acute lung injury and life-threatening acute respiratory distress syndrome [48]. Moreover, other studies have confirmed that the interaction between neutrophil transepithelial migration and epithelial-expressed ICAM-1 can regulate epithelial homeostasis and promote intestinal mucosal wound healing [49]. These data suggest that neutrophil migration plays a beneficial role in the protection of inflammation. In this study, we found that blockade of PLD2 with selective inhibitor could ameliorate DSS-induced colitis in mice and promote neutrophil mobilization from the bone marrow into intestinal mucosa. CXCR2, a chemokine receptor expressed in neutrophils, mediates neutrophils mobilization from the bone marrow and peripheral blood to the sites of inflammation under the stimulation of its ligand (CXCL1 and CXCL2). In a study of sepsis, IL-33 can reverse the decreased expression of CXCR2 in neutrophils through the inhibition of GRK2 [35, 36]. During viral infection, IFN can suppress CXCR2-mediated neutrophil recruitment into the sensory ganglia [50]. In experimental sepsis model, PLD2 deficiency protects wild-type mice against sepsis-induced death by enhancing neutrophil recruitment. A CXCR2-selective antagonist (SB225002) abolishes the protection conferred by PLD2 deficiency during experimental sepsis, suggesting that enhanced CXCR2 expression in neutrophils promotes

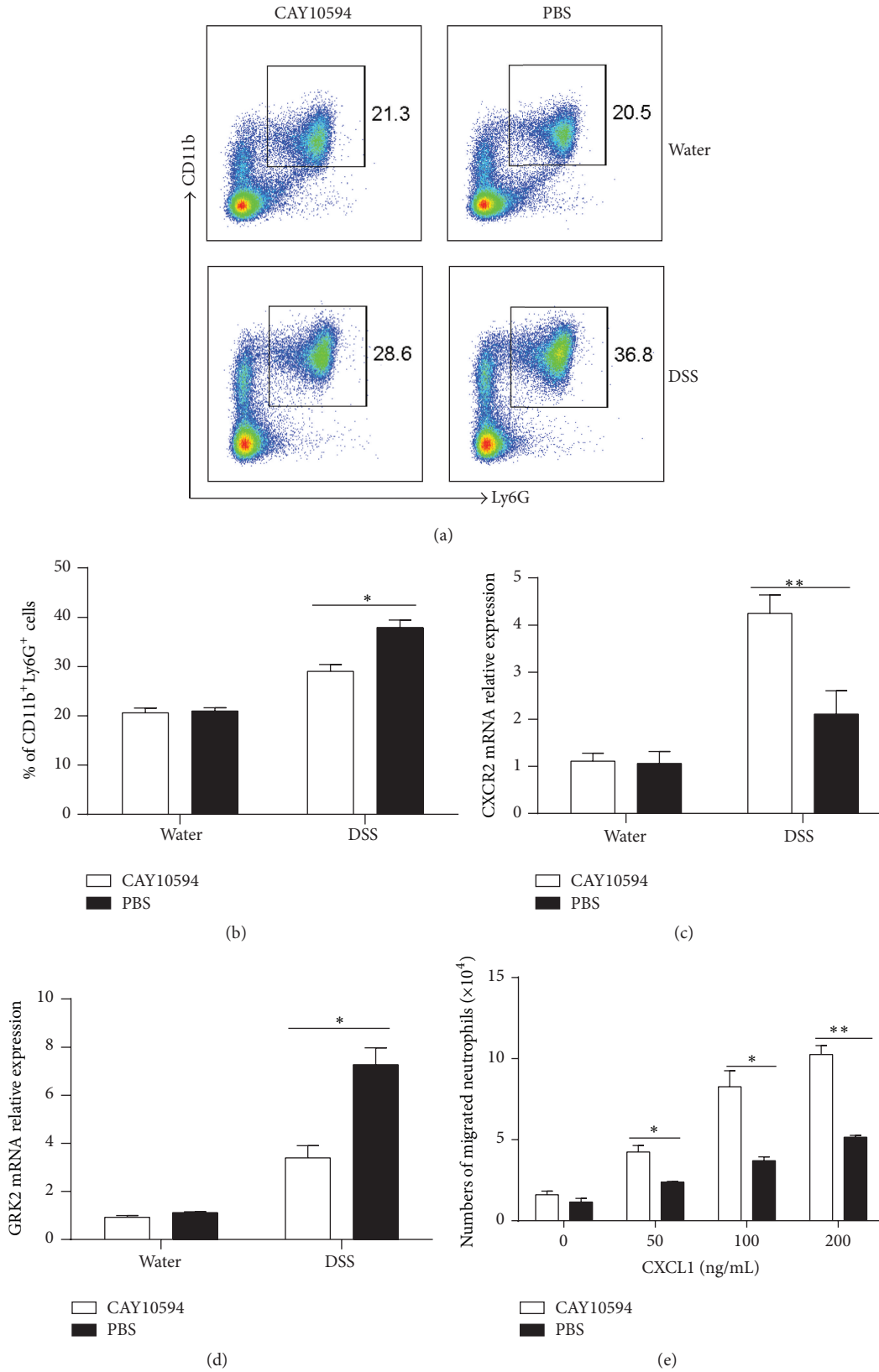


Figure 7: Inhibition of PLD2 promotes neutrophil migration. (a) Bone marrow cells were isolated from DSS-induced mice on day 10, and expression of Ly6G and CD11b was analyzed by flow cytometry. (b) Percentages of $Ly6G^+CD11b^+$ neutrophils were calculated. $^*p < 0.05$ and $^{**}p < 0.01$ versus controls. ((c) and (d)) Neutrophils were isolated from the bone marrow of mice; expression of CXCR2 (c) and GRK2 (d) was examined by qRT-PCR. $^*p < 0.05$ and $^{**}p < 0.01$ versus controls. (e) Neutrophils were isolated from the bone marrow of mice on day 10 after DSS-induced colitis and stimulated *in vitro* with PLD2 inhibitor (CAY10594, 10 μm) for 30 min; neutrophil migration was then analyzed by a Transwell plate (5 μm) under the stimulation with CXCL1 (0, 50, 100, 200 ng/mL) for 30 min. $^*p < 0.05$ and $^{**}p < 0.01$ versus controls.

survival in PLD2$^{-/-}$ mice [18]. In our study, we found that blockade of PLD2 could promote neutrophil recruitment through upregulating CXCR2 in a GRK2-dependent manner. However, we also found that PLD2 expression was increased in peripheral blood and inflamed mucosa of IBD patients, the mechanisms whereby increased infiltration of neutrophils may be also attributed to other molecules (e.g., TRPC6, IL-18, IL-8, Lewis X, and MAPK p38) at different stages of intestinal mucosal inflammation [51–54]. Moreover, when neutrophils are recruited into inflamed mucosa, they may be engaged in complex bidirectional interactions with epithelium, macrophages, dendritic cells, natural killer cells, and T lymphocytes, which further modulate neutrophil migration at advanced stage of intestinal inflammation [11]. Since these molecules and immune cells are involved in intestinal inflammatory response, regulation of neutrophil migration is a complicate network, and the increased numbers of neutrophils in peripheral blood and inflamed mucosa are the result of mutual modulation of these molecules and immune cells. In our study, PLD2 blockade was the only variable *in vitro*, which could account for the fact that increased neutrophil migration was the result of PLD2 blockade. Therefore, we concluded that blockade of PLD2 improves neutrophil migration.

In summary, our data indicate that PLD2 is a novel indispensable regulator in the pathogenesis of IBD by inhibiting neutrophil migration through downregulating CXCR2 expression. Blockade of PLD2 could ameliorate intestinal mucosal inflammation induced by DSS in mice. Our study has shed a new light on elucidating the role of PLD2 in the pathogenesis of IBD, and blockade of PLD2 may be a new therapeutic target for the management of IBD.

Competing Interests

The authors do not have any competing interests.

Authors' Contributions

Guangxi Zhou, Lin Yu, Wenjing Yang, Wei Wu, and Zhanju Liu conceived and designed the experiments; Guangxi Zhou, Lin Yu, Wenjing Yang, and Leilei Fang performed the experiments; Guangxi Zhou, Lin Yu, Wenjing Yang, and Leilei Fang analyzed the data; Guangxi Zhou, Lin Yu, Wenjing Yang, and Wei Wu contributed materials/analysis tools; Guangxi Zhou, Lin Yu, Wenjing Yang, and Zhanju Liu wrote the paper.

Acknowledgments

This work was supported by grants from the National Natural Science Foundation of China (81270470, 81470822, and 81630017) and Shanghai Science and Technology Commission (14401970100).

References

[1] Y. Guo, G. Zhou, C. He, W. Yang, Z. He, and Z. Liu, "Serum levels of lipopolysaccharide and 1,3- β-D-glucan refer to the severity in patients with Crohn's disease," *Mediators of Inflammation*, vol. 2015, Article ID 843089, 9 pages, 2015.

[2] A. Kaser, S. Zeissig, and R. S. Blumberg, "Inflammatory bowel disease," *Annual Review of Immunology*, vol. 28, pp. 573–621, 2010.

[3] A. Michielan and R. D'Incà, "Intestinal permeability in inflammatory bowel disease: pathogenesis, clinical evaluation, and therapy of leaky gut," *Mediators of Inflammation*, vol. 2015, Article ID 628157, 10 pages, 2015.

[4] Z. Zeng, Z. Zhu, Y. Yang et al., "Incidence and clinical characteristics of inflammatory bowel disease in a developed region of Guangdong Province, China: a prospective population-based study," *Journal of Gastroenterology and Hepatology*, vol. 28, no. 7, pp. 1148–1153, 2013.

[5] D. C. Baumgart and W. J. Sandborn, "Crohn's disease," *The Lancet*, vol. 380, no. 9853, pp. 1590–1605, 2012.

[6] M. Sun, C. He, Y. Cong, and Z. Liu, "Regulatory immune cells in regulation of intestinal inflammatory response to microbiota," *Mucosal Immunology*, vol. 8, no. 5, pp. 969–978, 2015.

[7] S. C. Ng, W. Tang, R. W. Leong et al., "Environmental risk factors in inflammatory bowel disease: a population-based case-control study in Asia-Pacific," *Gut*, vol. 64, no. 7, pp. 1063–1071, 2015.

[8] K. J. Maloy and F. Powrie, "Intestinal homeostasis and its breakdown in inflammatory bowel disease," *Nature*, vol. 474, no. 7351, pp. 298–306, 2011.

[9] M. Z. Cader and A. Kaser, "Recent advances in inflammatory bowel disease: mucosal immune cells in intestinal inflammation," *Gut*, vol. 62, no. 11, pp. 1653–1664, 2013.

[10] R. J. Xavier and D. K. Podolsky, "Unravelling the pathogenesis of inflammatory bowel disease," *Nature*, vol. 448, no. 7152, pp. 427–434, 2007.

[11] A. Mantovani, M. A. Cassatella, C. Costantini et al., "Neutrophils in the activation and regulation of innate and adaptive immunity," *Nature Reviews. Immunology*, vol. 11, no. 8, pp. 519–531, 2011.

[12] B. M. Fournier and C. A. Parkos, "The role of neutrophils during intestinal inflammation," *Mucosal Immunology*, vol. 5, no. 4, pp. 354–366, 2012.

[13] K. L. Mumy and B. A. McCormick, "The role of neutrophils in the event of intestinal inflammation," *Current Opinion in Pharmacology*, vol. 9, no. 6, pp. 697–701, 2009.

[14] J.-H. Jang, C. S. Lee, D. Hwang, and S. H. Ryu, "Understanding of the roles of phospholipase D and phosphatidic acid through their binding partners," *Progress in Lipid Research*, vol. 51, no. 2, pp. 71–81, 2012.

[15] M. Mahankali, H.-J. Peng, K. M. Henkels, M. C. Dinauer, and J. Gomez-Cambronero, "Phospholipase D2 (PLD2) is a guanine nucleotide exchange factor (GEF) for the GTPase Rac2," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 108, no. 49, pp. 19617–19622, 2011.

[16] W. C. Hwang, M. K. Kim, J. H. Song, K.-Y. Choi, and D. S. Min, "Inhibition of phospholipase D2 induces autophagy in colorectal cancer cells," *Experimental & Molecular Medicine*, vol. 46, no. 12, article e124, 2014.

[17] W. H. Ali, Q. Chen, K. E. Delgiorno et al., "Deficiencies of the lipid-signaling enzymes phospholipase D1 and D2 alter cytoskeletal organization, macrophage phagocytosis, and cytokine-stimulated neutrophil recruitment," *PLoS ONE*, vol. 8, no. 1, Article ID e55325, 2013.

[18] S. K. Lee, S. D. Kim, M. Kook et al., "Phospholipase D2 drives mortality in sepsis by inhibiting neutrophil extracellular trap formation and down-regulating CXCR2," *The Journal of Experimental Medicine*, vol. 212, no. 9, pp. 1381–1390, 2015.

[19] J. Ghim, C. Chelakkot, Y.-S. Bae, P.-G. Suh, and S. H. Ryu, "Accumulating insights into the role of phospholipase D2 in human diseases," *Advances in Biological Regulation*, vol. 61, pp. 42–46, 2016.

[20] J. Gomez-Cambronero, M. Di Fulvio, and K. Knapek, "Understanding phospholipase D (PLD) using leukocytes: PLD involvement in cell adhesion and chemotaxis," *Journal of Leukocyte Biology*, vol. 82, no. 2, pp. 272–281, 2007.

[21] L. Zhu, W. Zou, C. Yu, J. Lin, and X. He, "Human recombinant PLD2 can repress p65 activity of guinea pigs of chronic asthma in vivo," *Cellular & Molecular Immunology*, vol. 3, no. 4, pp. 307–310, 2006.

[22] C. Zeiller, S. Mebarek, R. Jaafar et al., "Phospholipase D2 regulates endothelial permeability through cytoskeleton reorganization and occludin downregulation," *Biochimica et Biophysica Acta—Molecular Cell Research*, vol. 1793, no. 7, pp. 1236–1249, 2009.

[23] M. Liu, Z. Fu, X. Wu, K. Du, S. Zhang, and L. Zeng, "Inhibition of phospholipaseD2 increases hypoxia-induced human colon cancer cell apoptosis through inactivating of the PI3K/AKT signaling pathway," *Tumor Biology*, vol. 37, no. 5, pp. 6155–6168, 2016.

[24] S. Nikolaus and S. Schreiber, "Diagnostics of inflammatory bowel disease," *Gastroenterology*, vol. 133, no. 5, pp. 1670–1689, 2007.

[25] E. S. Yüksel, S. Ipek, F. Topal et al., "Assessment of presence and grade of activity in ileal Crohn's disease," *The Turkish Journal of Gastroenterology*, vol. 25, no. 3, pp. 264–270, 2014.

[26] T. Tahara, T. Shibata, M. Okubo et al., "Telomere length in non-neoplastic colonic mucosa in ulcerative colitis (UC) and its relationship to the severe clinical phenotypes," *Clinical and Experimental Medicine*, vol. 15, no. 3, pp. 327–332, 2015.

[27] J. Su, T. Chen, X.-Y. Ji et al., "IL-25 downregulates Th1/Th17 immune response in an IL-10-dependent manner in inflammatory bowel disease," *Inflammatory Bowel Diseases*, vol. 19, no. 4, pp. 720–728, 2013.

[28] C. O. Elson, R. B. Sartor, G. S. Tennyson, and R. H. Riddell, "Experimental models of inflammatory bowel disease," *Gastroenterology*, vol. 109, no. 4, pp. 1344–1367, 1995.

[29] C. Liu, X. Xia, W. Wu et al., "Anti-tumour necrosis factor therapy enhances mucosal healing through down-regulation of interleukin-21 expression and T helper type 17 cell infiltration in Crohn's disease," *Clinical and Experimental Immunology*, vol. 173, no. 1, pp. 102–111, 2013.

[30] Z. Liu, L. Yang, Y. Cui et al., "IL-21 enhances NK cell activation and cytolytic activity and induces Th17 cell differentiation in inflammatory bowel disease," *Inflammatory Bowel Diseases*, vol. 15, no. 8, pp. 1133–1144, 2009.

[31] Z. Liu, P. K. Yadav, X. Xu et al., "The increased expression of IL-23 in inflammatory bowel disease promotes intraepithelial and lamina propria lymphocyte inflammatory responses and cytotoxicity," *Journal of Leukocyte Biology*, vol. 89, no. 4, pp. 597–606, 2011.

[32] Z. Liu, B.-S. Feng, S.-B. Yang, X. Chen, J. Su, and P.-C. Yang, "Interleukin (IL)-23 suppresses IL-10 in inflammatory bowel disease," *The Journal of Biological Chemistry*, vol. 287, no. 5, pp. 3591–3597, 2012.

[33] Z. Li, S. Vermeire, D. Bullens et al., "Anti-tumor necrosis factor therapy restores peripheral blood b-cell subsets and CD40 expression in inflammatory bowel diseases," *Inflammatory Bowel Diseases*, vol. 21, no. 12, pp. 2787–2796, 2015.

[34] L. Yu, X. Yang, L. Xia et al., "Infliximab preferentially induces clinical remission and mucosal healing in short course Crohn's disease with luminal lesions through balancing abnormal immune response in gut mucosa," *Mediators of Inflammation*, vol. 2015, Article ID 793764, 9 pages, 2015.

[35] J. C. Alves-Filho, F. Snego, F. O. Souto et al., "Interleukin-33 attenuates sepsis by enhancing neutrophil influx to the site of infection," *Nature Medicine*, vol. 16, no. 6, pp. 708–712, 2010.

[36] J. C. Alves-Filho, A. Freitas, F. O. Souto et al., "Regulation of chemokine receptor by Toll-like receptor 2 is critical to neutrophil migration and resistance to polymicrobial sepsis," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 106, no. 10, pp. 4018–4023, 2009.

[37] H. R. Jones, C. T. Robb, M. Perretti, and A. G. Rossi, "The role of neutrophils in inflammation resolution," *Seminars in Immunology*, vol. 28, no. 2, pp. 137–145, 2016.

[38] S. M. Holland, "Chronic granulomatous disease," *Hematology/Oncology Clinics of North America*, vol. 27, no. 1, pp. 89–99, 2013.

[39] E. L. Chiswick, J. R. Mella, J. Bernardo, and D. G. Remick, "Acute-phase deaths from murine polymicrobial Sepsis are characterized by innate immune suppression rather than exhaustion," *Journal of Immunology*, vol. 195, no. 8, pp. 3793–3802, 2015.

[40] A. A. Kühl, H. Kakirman, M. Janotta et al., "Aggravation of different types of experimental colitis by depletion or adhesion blockade of neutrophils," *Gastroenterology*, vol. 133, no. 6, pp. 1882–1892, 2007.

[41] Y. Nemoto, T. Kanai, S. Tohda et al., "Negative feedback regulation of colitogenic CD4+ T cells by increased granulopoiesis," *Inflammatory Bowel Diseases*, vol. 14, no. 11, pp. 1491–1503, 2008.

[42] R. Zhang, S. Ito, N. Nishio, Z. Cheng, H. Suzuki, and K.-I. Isobe, "Up-regulation of Gr1+CD11b+ population in spleen of dextran sulfate sodium administered mice works to repair colitis," *Inflammation & Allergy—Drug Targets*, vol. 10, no. 1, pp. 39–46, 2011.

[43] S. Fiorucci, J. L. Wallace, A. Mencarelli et al., "A β-oxidation-resistant lipoxin A4 analog treats hapten-induced colitis by attenuating inflammation and immune dysfunction," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 101, no. 44, pp. 15736–15741, 2004.

[44] C. L. Zindl, J.-F. Lai, Y. K. Lee et al., "IL-22-producing neutrophils contribute to antimicrobial defense and restitution of colonic epithelial integrity during colitis," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 110, no. 31, pp. 12768–12773, 2013.

[45] M. Di Fulvio and J. Gomez-Cambronero, "Phospholipase D (PLD) gene expression in human neutrophils and HL-60 differentiation," *Journal of Leukocyte Biology*, vol. 77, no. 6, pp. 999–1007, 2005.

[46] P. H. Leliefeld, C. M. Wessels, L. P. Leenen, L. Koenderman, and J. Pillay, "The role of neutrophils in immune dysfunction during severe inflammation," *Critical Care*, vol. 20, article 73, 2016.

[47] J. Rossaint, J. Oehmichen, H. Van Aken et al., "FGF23 signaling impairs neutrophil recruitment and host defense during CKD," *Journal of Clinical Investigation*, vol. 126, no. 3, pp. 962–974, 2016.

[48] A. Ittner, H. Block, C. A. Reichel et al., "Regulation of PTEN activity by p38δ-PKD1 signaling in neutrophils confers inflammatory responses in the lung," *The Journal of Experimental Medicine*, vol. 209, no. 12, pp. 2229–2246, 2012.

[49] R. Sumagin, J. C. Brazil, P. Nava et al., "Neutrophil interactions with epithelial-expressed ICAM-1 enhances intestinal mucosal

wound healing," *Mucosal Immunology*, vol. 9, no. 5, pp. 1151–1162, 2016.

[50] A. T. Stock, J. M. Smith, and F. R. Carbone, "Type I IFN suppresses Cxcr2 driven neutrophil recruitment into the sensory ganglia during viral infection," *Journal of Experimental Medicine*, vol. 211, no. 5, pp. 751–759, 2014.

[51] T. K. Lapointe and A. G. Buret, "Interleukin-18 facilitates neutrophil transmigration via myosin light chain kinase-dependent disruption of occludin, without altering epithelial permeability," *American Journal of Physiology-Gastrointestinal and Liver Physiology*, vol. 302, no. 3, pp. G343–G351, 2012.

[52] J. C. Brazil, R. Sumagin, R. D. Cummings, N. A. Louis, and C. A. Parkos, "Targeting of neutrophil Lewis X blocks transepithelial migration and increases phagocytosis and degranulation," *The American Journal of Pathology*, vol. 186, no. 2, pp. 297–311, 2016.

[53] O. Lindemann, D. Umlauf, S. Frank et al., "TRPC6 regulates CXCR2-mediated chemotaxis of murine neutrophils," *The Journal of Immunology*, vol. 190, no. 11, pp. 5496–5505, 2013.

[54] X. Liu, B. Ma, A. B. Malik et al., "Bidirectional regulation of neutrophil migration by mitogen-activated protein kinases," *Nature Immunology*, vol. 13, no. 5, pp. 457–464, 2012.

Hindawi Publishing Corporation
Mediators of Inflammation
Volume 2016, Article ID 4190390, 6 pages
http://dx.doi.org/10.1155/2016/4190390

Review Article

Advantages of Extracellular Ubiquitin in Modulation of Immune Responses

Rusudan Sujashvili

Department of Biophysics, I.Beritashvili Center of Experimental Biomedicine, 14 Gotua, 0160 Tbilisi, Georgia

Correspondence should be addressed to Rusudan Sujashvili; sujaruss@gmail.com

Received 10 May 2016; Revised 18 July 2016; Accepted 4 August 2016

Academic Editor: Nona Janikashvili

T and B lymphocytes play a central role in protecting the human body from infectious pathogens but occasionally they can escape immune tolerance, become activated, and induce autoimmune diseases. All deregulated cellular processes are associated with improper functioning of the ubiquitin-proteasome system (UPS) in eukaryotic cells. The role of ubiquitin in regulation of immune responses and in autoimmune diseases is only beginning to emerge. Ubiquitin is found in intra- and extracellular fluids and is involved in regulation of numerous cellular processes. Extracellular ubiquitin ascribed a role in lymphocyte differentiation. It regulates differentiation and maturation of hematopoietic cell lines. Ubiquitination is involved in initiation, propagation, and termination of immune responses. Disrupted ubiquitination can lead to autoimmunity. Recent observations showed that it can suppress immune response and prevent inflammation. Exogenous ubiquitin may provide good potential as a new tool for targeted therapy for immune mediated disorders of various etiologies.

1. Introduction

Synthesis and degradation of proteins is constant process in all cells. Protein content is regulated by the cycle of its permanent turnover and is crucial for cell survival. Derangements of protein metabolism lead to the development of severe diseases and mainly are caused by diverse mechanisms. Different factors can cause ubiquitin system dysfunction and contribute to the accumulation of damaged proteins. UPS plays an important role in regulation of cellular processes via degradation of damaged proteins and control of protein quantity in the eukaryotic cell. Almost all cellular processes including antigen processing, immune response and inflammation, and modulation of cell surface receptors are under control of UPS [1]. It is suggested that extracellular ubiquitin is an immune modulator affecting T and B lymphocytes [2]. Extracellular ubiquitin has important implications to investigation of pathways involved in regulation of immune system. Ubiquitin appears to be a modulator of innate and adaptive immune responses. Ubiquitination regulates initiation, propagation, and termination of immune responses.

2. UPS

Ubiquitin is a small regulatory highly conserved protein and occurs in all eukaryotic cells. The attachment of ubiquitin to the ε-amine lysine residues of target proteins in course of mono- and polyubiquitination requires a series of ATP-dependent enzymatic steps by ubiquitin activating enzyme (E1), ubiquitin conjugating enzyme (E2), and ubiquitin ligase (E3). The C-terminal Gly76 residue of ubiquitin is a key residue that functions in diverse chemistry of ubiquitin reactions. Ubiquitin can be conjugated to itself via one of the seven lysines sequenced in ubiquitin molecule (K6, K11, K27, K29, K33, K48, and K63) by forming covalent isopeptide bond. Cell contains numerous E3 enzymes that recognize particular target proteins by structural features and thus account for substrate selectivity [3]. Ubiquitination can regulate the activity and location of target proteins by means of monoubiquitination or alternate multiubiquitination. E3 ligases are represented as members of two different families. Protein C-terminus domains found in E3, homologous to the E6-AP carboxyl terminus (HECT), act as monomers and

ubiquitin is initially transferred to a cysteine residue of a ligase before it is transferred to substrate. The E3 containing zinc cations binding motif (RING/U-box protein) family members function as a scaffold that binds to both an E2 bound to ubiquitin and a substrate. Proteasomes recognize polyubiquitin tetramers linked through lysin-48 as a signal to deubiquitinate and destroy the substrate. Finely, free and reusable ubiquitin and amino acids are released by the action of a large variety of ubiquitin C-terminal hydrolases and ATP-ases [4, 5]. Recently a new type of polyubiquitin linear chain has been identified in which the C-terminal glycine of ubiquitin is conjugated to the α-amino group of the amino-terminal methionine of another ubiquitin [6]. UPS is the major intracellular degradation pathway and the defects in clearance of cellular proteins can lead to autoimmunity.

3. K Linkages Involved in Immune Modulation

Polyubiquitin chains formed via various linkages carry distinct structural and functional information. Ubiquitin chains linked by different lysine residues define the location and character of protein processing. K48 ubiquitin chains target substrates to the proteasome for degradation. In contrast, Lys63- or K6-linked chains perform nonproteolytic functions in at least four pathways: DNA damage repair, endocytosis, cellular signaling, intracellular trafficking, and ribosomal biogenesis [7, 8]. Polyubiquitin chains linked through K63 and K48 participate in innate immune responses via activation of pattern recognition receptor, proteins expressed by the cells of innate immune system (PRRs) that will result in activation of nuclear factor kappa-B (NF-kB) and the following induction of cytokines: tumor necrosis factor (TNF) and interleukin-1 (IL-1) [9]. K33-linked polyubiquitination of T cell receptors (TCR-Z) functionally modifies the receptor phosphorylation and protein binding in a proteolysis independent manner. K11 linkage mediates proteasomal degradation in mitosis, cell cycle regulation, membrane protein trafficking, and TNF signaling [10]. Alternatively, monoubiquitination can lead to export and translocation of proteins into the cytoplasm. Linear polyubiquitin chains are involved in termination of TNF-induced cell death. Mutated ubiquitin-like-domain-containing complex (SHARPIN), component of linear ubiquitin chain assembly complex (LUBAK), causes immune system disorders and multiorgan inflammation. SHARPIN deficiency in mice causes activation of inhibitor of nuclear complex kappa-B kinase (IKK) and NF-kB in B cells, macrophages, and embryonic fibroblasts. It leads to rapid cell death upon TNF-alpha stimulation via caspase-8 dependent manner [11]. In eukaryotic cells several ubiquitin binding proteins (UBPs) are determined to be essential for immune signaling pathways [12, 13].

4. E3 Ligases and Deubiquitinases (DUBs) as Potential Targets for Immune Therapy

Ubiquitin adding (i.e., ubiquitylation, a reversible in vivo covalent modification of target proteins) affects antigen processing by antigen-presenting cells and upgrades immunological tolerance by modification signaling components to move the balance away from activation and toward anergy. Decreased expression of E3 ligases induces autoimmunity by the loss of immune tolerance and the absence of epitopes against the immune system [14]. Dysfunction of E3 ligases, which catalyze the final step of ubiquitin attachment, can lead to indiscriminate T cell activation and loss of tolerance to self-antigens [15]. The induction of anergy in T cells is an active process that is dependent on new protein synthesis and is associated with the increased expression of E3 ubiquitin ligases Cbl-b, Itch, and GRAIL and other negative regulators of TCR signaling [16–23].

T cell activation is subject to tight regulation to avoid inappropriate responses to self-antigens. In mice lacking both Itch and Cbl-b E3 ubiquitin protein ligases that cooperate in K33-TCR-Z linkage spontaneously develop systemic autoimmunity characterized by splenomegaly, lymphocyte infiltration of lungs, liver, kidneys, and heart correlating with increased TCR-Z phosphorylation and accumulation of cytokines in serum [24]. In the absence of Cbl-b, T cells are hyperproliferative and are able to be fully activated even without CD28 costimulation [25–28]. As a consequence, Cbl-b total body knockout mice develop spontaneous autoimmunity resulting in infiltration of T and B lymphocytes in different organs and parenchymal damage [27, 29, 30]. Moreover, the lack of Cbl-b prevents T cell tolerance induction in vivo [2]. Besides Cbl-b, other E3 ligases, such as the HECT-type E3 ligase Itch and the gene related to anergy in lymphocytes RING-type E3 ligase (GRAIL), have been identified as critical regulators of T cell activation and tolerance. As in the case of Cbl-b, Itch and GRAIL deficient T cells hyperrespond to TCR stimulation [31–33] and removal of either of these E3 ligases causes T cells resistant to anergy induction, both in vitro and in vivo [34].

Itch negatively regulates T cell growth factor production and proliferation. Itch attaches ubiquitin to substrate proteins. Ubiquitination of the T cell receptor mediates its downregulation [35]. Human Itch deficiency results in a complex phenotype that affects physical growth, craniofacial morphology, muscle development, and immune function. The consequences of Itch deficiency in humans appear to be similar to those in $Itch^{-/-}$ mice. This is probably a result of immune deregulation in patients with autoimmune disease [36–38].

Genetic deficiency in the pellino E3 ubiquitin protein ligase 1 (Peli1) causes hyperactivation of T cells and increased T cells resistance to suppression by regulatory T cells. Peli1-deficient mice spontaneously developed autoimmunity characterized by multiorgan inflammation and autoantibody production. Peli1 is identified as a critical factor in the maintenance of peripheral T cell tolerance [39].

DUBs the enzymes that disassemble ubiquitin chains and remove ubiquitin from ubiquitin protein conjugates are central to the UPS. DUBs participate in termination of immune responses which is as important as initiation of the process. Ubiquitin carboxyl-terminal hydrolase 15 (USP15) is a negative regulator of T cell activation. DUB USP15 deficiency promotes T cell activation [40]. Conditional knockout of DUB TNFAIP3-tumor necrosis factor, alpha-induced protein

3 (A20) developed autoimmunity. A20 has been reported as a disease susceptibility gene for human inflammatory and autoimmune pathology, including Rheumatoid Arthritis (RA) and Juvenile Idiopathic Arthritis, Systemic Lupus Erythematosus (SLE), Inflammatory Bowel Disease (IBD), celiac disease, psoriasis, type 1 diabetes, Sjogren's syndrome, coronary artery disease, rheumatic heart disease, and systemic sclerosis. As ubiquitin is highly conserved protein it lacks immunogenicity, but ubiquitinated proteins accumulated in cells are highly immunogenic in autoimmune patients [41–43]. The diversity of E3 ligases and DUBs involved in controlling autoimmunity makes these proteins potential targets for immune therapy.

5. Extracellular Ubiquitin Is a Promising Regulator of Immune System

Extracellular ubiquitin is found at nanomolar concentrations in human plasma and serum. It is detectable in cerebrospinal fluid (CSF) and bronchoalveolar lavage fluid (BALF). Elevation of plasma ubiquitin levels is found during hairy cell leukemia (HCL), allergic, autoimmune infections, and other disorders [44, 45].

Release mechanisms of ubiquitin into extracellular fluids are not known. Source of extracellular ubiquitin might be the passive release from cells during physiological processes, like apoptosis and necrosis, but some authors report on extraction of ubiquitin from normal cells [46, 47]. Up to date many aspects of extracellular ubiquitin activity remain unclear, specially concerning its possible pathways and the role in various cellular processes involved in immune responses [48].

Injected proteins can be used as authentic tests of the action of endogenous proteins. Extracellular ubiquitin is easily available for protein modifications. It has been used in numerous experiments for elucidation of the role and pathways of extracellular and exogenous ubiquitin. Initially, in vivo injected extracellular ubiquitin was identified as an inducer of both T and B cell differentiation markers on precursors of mouse lymphoid cells. Ubiquitin is capable of inducing the functional differentiation of granulocytes [49]. Extracellular ubiquitin secreted by activated T-lymphocytes was shown to inhibit cytotoxic activity of platelets [50]. Later it was studied as a modulator of hematopoietic progenitor cells. Ubiquitin secreted from hairy cells had an inhibitory effect on the growth of normal hematopoietic progenitor cells [51].

Microinjected extracellular ubiquitin incorporated into hematopoietic cells mediates their growth suppression and apoptosis through proteasome-dependent degradation of selective cellular proteins such as signal transducer and activator of transcription 3 (STAT3) in IL-6-dependent human T-lymphoma cell line (KT-3 cells) [52]. STAT3 is the major mediator of glycoprotein 130 (gp130) subunit of cytokine receptor signal. Inside the cell gp130 is shown to be modified by K63 polyubiquitin chains and directed to lysosome for degradation which is essential for termination of IL-6 signaling [53]. IL-6 mainly functions in hematopoietic and lymphoid cell systems. It is originally identified as a B cell differentiation factor that induces final maturation of B cells. It also acts on T cells and hematopoietic progenitor cells [54].

STAT3 and cytokines are main targets for ubiquitin in process of regulation of hematopoietic cells proliferative activity. Moreover, forkhead box protein 3 is regulated by the E3 ubiquitin protein ligases Itch and Cbl-b and induces regulatory activity of T cells [55]. Itch E3 ubiquitin protein ligase plays a role in lymphoid cell differentiation and regulation of immune responses. Mutation of this protein causes multisystem autoimmune disease [56]. Cbl-b functions as a negative regulator of T cells. It is involved in the regulation of peripheral tolerance and anergy of T cells. Ex vivo generated human monocyte-derived suppressive cells (HuMoSCs) were suggested as inhibitors of effector T-lymphocytes and promoters for expansion of immunosuppressive forkhead box protein 3-positive $CD8^+$ regulatory T-lymphocytes. Therefore, they are supposed to be an efficient therapeutic tool to prevent graft-versus-host disease (GvHD) transplant rejection and autoimmune diseases. Interaction of HuMoSCs with T cells is dependent on STAT3 and cytokine activity [57]. Therefore, these data prove the significance of ubiquitination in the abovementioned processes. One can speculate that extracellular ubiquitin might play a significant role in process of HuMoSCs interaction with T Reg cells. Study of extracellular ubiquitin effect on HuMoSCs seems to be an attractive goal for farther investigation of molecular pathways that might play pivotal role in GvHD transplant rejection and autoimmune diseases therapy.

Extracellular ubiquitin was identified as an endogenous agonist of CXC chemokine receptor type 4 (CXCR4) [58]. CXCR4 is a 7-transmembrane G protein-coupled receptor that is expressed by a variety of cells, including peripheral blood lymphocytes, monocytes, thymocytes, and pre-B cells. CXCR4 serves as a receptor for T cell tropic human immunodeficiency virus type 1 (HIV-1) strains. Extracellular ubiquitin binds CXCR4 receptor on monocyte, B cell, and T cell surfaces and induces calcium ions influx into the cells. Affinity of extracellular ubiquitin to B- and monocyte cell surface is higher than to T cell's surface [59]. CXCR4 agonist properties of extracellular ubiquitin indicate its possible role in leukocyte differentiation and in normal and pathological hematogenesis [2]. Extracellular ubiquitin changes the ratios of the heterogeneous population of bone marrow and peripheral blood [60]. In vivo injected exogenous ubiquitin inhibits mitotic activity of bone marrow cells by about 53% in intact rats [61]. Recent observations showed that extracellular ubiquitin can suppress immune response and prevent inflammation [62]. Ubiquitin has been suggested as a promising anti-inflammatory protein therapeutic. Ubiquitin is involved in regulation of immunodepression in critically ill patients. TNF-alpha is associated with increased risk of sepsis in critically ill patients [59]. In vivo induced ubiquitin reduced lipopolysaccharide (LPS) stimulated TNF-α production of whole blood and peripheral blood mononuclear cells (PBMNCs). Extracellular ubiquitin incorporated into monocytic cells restores endogenous ubiquitin pool and provides ubiquitin protein ligase system with additional substrates to maintain intracellular protein turnover during immunological responses [62]. Peptide fragment corresponding to the

ubiquitin (50–59) sequence possessed the immunosuppressive activity comparable with that of ubiquitin [63, 64].

Ubiquitination of proteins is essential for proper course of normal and pathological cellular processes. However, we are far from fully understanding the multiple pathways of cellular and extracellular ubiquitin involved in regulation and deregulation of numerous cellular activities involved in immune responses. Investigation of the role of extracellular ubiquitin in modulation of immune responses by means of exogenous ubiquitin seems to be informative for this purpose.

6. Conclusion

Regulated protein turnover by the UPS is essential for the survival of eukaryotic cells. Alterations in the UPS are demonstrated to be correlated with a variety of human pathologies including autoimmunity, immunodeficiency, hematopoietic, and malignant [48]. The finding that proliferating cells are more sensitive to defects in protein degradation suggests that further emphasis on UPS could provide new therapeutic tools to target disorders in hemato- and lymphogenesis. Extracellular ubiquitin is considered as a disease biomarker, as numerous diseases are associated with increased concentrations of ubiquitin in body fluids [2, 44, 45]. Modification of proteins by ubiquitin may impact their visibility by the immune system; this should highlight the therapeutic potential of manipulating extracellular ubiquitin in autoimmune diseases. Further investigation of extracellular ubiquitin might reveal new pathways involved in development of autoimmunity and other immunological diseases and open new strategies to targeted therapy for immune mediated disorders of various etiologies.

Competing Interests

The author declares that there are no competing interests regarding the publication of this article.

References

[1] K. E. Krueger and S. Srivastava, "Posttranslational protein modifications: current implications for cancer detection, prevention, and therapeutics," *Molecular and Cellular Proteomics*, vol. 5, no. 10, pp. 1799–1810, 2006.

[2] M. Majetschak, "Extracellular ubiquitin: immune modulator and endogenous opponent of damage-associated molecular pattern molecules," *Journal of Leukocyte Biology*, vol. 89, no. 2, pp. 205–219, 2011.

[3] A. Ciechanover, "The ubiquitin-proteasome pathway: on protein death and cell life," *The EMBO Journal*, vol. 17, no. 24, pp. 7151–7160, 1998.

[4] A. Hershko, A. Ciechanover, and A. Varshavsky, "The ubiquitin system," *Nature Medicine*, vol. 6, no. 10, pp. 1073–1081, 2000.

[5] T. A. M. Groothuis, N. P. Dantuma, J. Neefjes, and F. A. Salomons, "Ubiquitin crosstalk connecting cellular processes," *Cell Division*, vol. 1, article 21, 2006.

[6] K. Iwai and F. Tokunaga, "Linear polyubiquitination: a new regulator of NF-κB activation," *EMBO Reports*, vol. 10, no. 7, pp. 706–713, 2009.

[7] J. R. Morris and E. Solomon, "induces the formation of conjugated ubiquitin structures, dependent on K6 of ubiquitin in cells during DNA replication and repair," *Human Molecular Genetics*, vol. 13, no. 8, pp. 807–817, 2004.

[8] M. A. Nakasone, N. Livnat-Levanon, M. H. Glickman, R. E. Cohen, and D. Fushman, "Mixed-linkage ubiquitin chains send mixed messages," *Structure*, vol. 21, no. 5, pp. 727–740, 2013.

[9] J. Zinngrebe, A. Montinaro, N. Peltzer, and H. Walczak, "Ubiquitin in the immune system," *EMBO Reports*, vol. 15, no. 1, pp. 28–45, 2014.

[10] K. E. Wickliffe, A. Williamson, H.-J. Meyer, A. Kelly, and M. Rape, "K11-linked ubiquitin chains as novel regulators of cell division," *Trends in Cell Biology*, vol. 21, no. 11, pp. 656–663, 2011.

[11] F. Ikeda, Y. L. Deribe, S. S. Skånland et al., "SHARPIN forms a linear ubiquitin ligase complex regulating NF-κB activity and apoptosis," *Nature*, vol. 471, no. 7340, pp. 637–641, 2011.

[12] I. Dikic, S. Wakatsuki, and K. J. Walters, "Ubiquitin-binding domains from structures to functions," *Nature Reviews Molecular Cell Biology*, vol. 10, no. 10, pp. 659–671, 2009.

[13] J. H. Hurley, S. Lee, and G. Prag, "Ubiquitin-binding domains," *Biochemical Journal*, vol. 399, no. 3, pp. 361–372, 2006.

[14] V. G. Bhoj and Z. J. Chen, "Ubiquitylation in innate and adaptive immunity," *Nature*, vol. 458, no. 7237, pp. 430–437, 2009.

[15] L. E. Matesic, D. C. Haines, N. G. Copeland, and N. A. Jenkins, "*Itch* genetically interacts with *Notch1* in a mouse autoimmune disease model," *Human Molecular Genetics*, vol. 15, no. 24, pp. 3485–3497, 2006.

[16] V. Heissmeyer, F. Macián, S.-H. Im et al., "Calcineurin imposes T cell unresponsiveness through targeted proteolysis of signaling proteins," *Nature Immunology*, vol. 5, no. 3, pp. 255–265, 2004.

[17] F. Macián, F. García-Cózar, S.-H. Im, H. F. Horton, M. C. Byrne, and A. Rao, "Transcriptional mechanisms underlying lymphocyte tolerance," *Cell*, vol. 109, no. 6, pp. 719–731, 2002.

[18] S. Bandyopadhyay, M. Duré, M. Paroder, N. Soto-Nieves, I. Puga, and F. Macián, "Interleukin 2 gene transcription is regulated by Ikaros-induced changes in histone acetylation in anergic T cells," *Blood*, vol. 109, no. 7, pp. 2878–2886, 2007.

[19] J. E. Harris, K. D. Bishop, N. E. Phillips et al., "Early growth response gene-2, a zinc-finger transcription factor, is required for full induction of clonal anergy in $CD4^+$ T cells," *The Journal of Immunology*, vol. 173, no. 12, pp. 7331–7338, 2004.

[20] C. G. King, T. Kobayashi, P. J. Cejas et al., "TRAF6 is a T cell-intrinsic negative regulator required for the maintenance of immune homeostasis," *Nature Medicine*, vol. 12, no. 9, pp. 1088–1092, 2006.

[21] I. Puga, A. Rao, and F. Macian, "Targeted cleavage of signaling proteins by caspase 3 inhibits T cell receptor signaling in anergic T cells," *Immunity*, vol. 29, no. 2, pp. 193–204, 2008.

[22] M. Safford, S. Collins, M. A. Lutz et al., "Egr-2 and Egr-3 are negative regulators of T cell activation," *Nature Immunology*, vol. 6, pp. 472–480, 2005.

[23] R. M. Thomas, N. Chunder, C. Chen, S. E. Umetsu, S. Winandy, and A. D. Wells, "Ikaros enforces the costimulatory requirement for IL2 gene expression and is required for anergy induction in $CD4^+$ T lymphocytes," *Journal of Immunology*, vol. 179, no. 11, pp. 7305–7315, 2007.

[24] H. Huang, M. S. Jeon, L. Liao et al., "K33-linked polyubiquitination of T cell receptor-ζ regulates proteolysis-independent T cell signaling," *Immunity*, vol. 33, no. 1, pp. 60–70, 2010.

[25] P. S. Bilodeau, J. L. Urbanowski, S. C. Winistorfer, and R. C. Piper, "The Vps27p–Hse1p complex binds ubiquitin and mediates endosomal protein sorting," *Nature Cell Biology*, vol. 4, no. 7, pp. 534–539, 2002.

[26] Q. Lu, L. W. Hope, M. Brasch, C. Reinhard, and S. N. Cohen, "TSG101 interaction with HRS mediates endosomal trafficking and receptor down-regulation," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 100, no. 13, pp. 7626–7631, 2003.

[27] K. Bachmaier, C. Krawczyk, I. Kozieradzki et al., "Negative regulation of lymphocyte activation and autoimmunity by the molecular adaptor Cbl-b," *Nature*, vol. 403, no. 6766, pp. 211–216, 2000.

[28] Y. J. Chiang, H. K. Kole, K. Brown et al., "Cbl-b regulates the CD28 dependence of T-cell activation," *Nature*, vol. 403, no. 6766, pp. 216–220, 2000.

[29] M.-S. Jeon, A. Atfield, K. Venuprasad et al., "Essential role of the E3 ubiquitin ligase Cbl-b in T cell anergy induction," *Immunity*, vol. 21, no. 2, pp. 167–177, 2004.

[30] M. A. Gronski, J. M. Boulter, D. Moskophidis et al., "TCR affinity and negative regulation limit autoimmunity," *Nature Medicine*, vol. 10, no. 11, pp. 1234–1239, 2004.

[31] D. Fang, C. Elly, B. Gao et al., "Dysregulation of T lymphocyte function in itchy mice: a role for Itch in TH2 differentiation," *Nature Immunology*, vol. 3, no. 3, pp. 281–287, 2002.

[32] R. I. Nurieva, S. Zheng, W. Jin et al., "The E3 ubiquitin ligase GRAIL regulates T cell tolerance and regulatory T cell function by mediating T cell receptor-CD3 degradation," *Immunity*, vol. 32, no. 5, pp. 670–680, 2010.

[33] N. Anandasabapathy, G. S. Ford, D. Bloom et al., "GRAIL: an E3 ubiquitin ligase that inhibits cytokine gene transcription is expressed in anergic $CD4^+$ T cells," *Immunity*, vol. 18, no. 4, pp. 535–547, 2003.

[34] K. Venuprasad, C. Elly, M. Gao et al., "Convergence of itch-induced ubiquitination with MEKK1-JNK signaling in Th2 tolerance and airway inflammation," *Journal of Clinical Investigation*, vol. 116, no. 4, pp. 1117–1126, 2006.

[35] D. L. Mueller, "E3 ubiquitin ligases as T cell anergy factors," *Nature Immunology*, vol. 5, no. 9, pp. 883–890, 2004.

[36] K. L. Lorick, Y. Yang, J. P. Jensen, K. Iwai, and A. M. Weissman, *Current Protocols in Cell Biology*, Studies of the Ubiquitin Proteasome System, chapter 15, unit 15.9, John Wiley & sons, Hoboken, NJ, USA, 2006.

[37] A.-L. A. Katzenstein and J. L. Myers, "Nonspecific interstitial pneumonia and the other idiopathic interstitial pneumonias: classification and diagnostic criteria," *American Journal of Surgical Pathology*, vol. 24, no. 1, pp. 1–3, 2000.

[38] B. W. Kinder, H. R. Collard, L. Koth et al., "Idiopathic nonspecific interstitial pneumonia: lung manifestation of undifferentiated connective tissue disease?" *American Journal of Respiratory and Critical Care Medicine*, vol. 176, no. 7, pp. 691–697, 2007.

[39] Ch. Mikyoung, J. Wei, Ch. Jae-Hoon et al., "The ubiquitin ligase Peli1 negatively regulates T cell activation and prevents autoimmunity," *Nature Immunology*, vol. 12, pp. 1002–1009, 2011.

[40] Q. Zou, J. Jin, H. Hu et al., "USP15 stabilizes MDM2 to mediate cancer-cell survival and inhibit antitumor T cell responses," *Nature Immunology*, vol. 15, no. 6, pp. 562–570, 2014.

[41] F. Martin and V. M. Dixit, "A20 edits ubiquitin and autoimmune paradigms," *Nature Genetics*, vol. 43, no. 9, pp. 822–823, 2011.

[42] L. Catrysse, L. Vereecke, R. Beyaert, and G. van Loo, "A20 in inflammation and autoimmunity," *Trends in Immunology*, vol. 35, no. 1, pp. 22–31, 2014.

[43] S. Muller, J.-P. Briand, and M. H. V. Van Regenmortel, "Presence of antibodies to ubiquitin during the autoimmune response associated with systemic lupus erythematosus," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 85, no. 21, pp. 8176–8180, 1988.

[44] G. P. Wang, K. Iqbal, G. Bucht, B. Winblad, H. M. Wisniewski, and I. Grundke-Iqbal, "Alzheimer's disease: paired helical filament immunoreactivity in cerebrospinal fluid," *Acta Neuropathologica*, vol. 82, no. 1, pp. 6–12, 1991.

[45] K. Takada, H. Nasu, N. Hibi et al., "Serum concentrations of free ubiquitin and multiubiquitin chains," *Clinical Chemistry*, vol. 43, no. 7, pp. 1188–1195, 1997.

[46] S. U. Sixt and B. Dahlmann, "Extracellular, circulating proteasomes and ubiquitin—incidence and relevance," *Biochimica et Biophysica Acta—Molecular Basis of Disease*, vol. 1782, no. 12, pp. 817–823, 2008.

[47] S. I. Buschow, J. M. P. Liefhebber, R. Wubbolts, and W. Stoorvogel, "Exosomes contain ubiquitinated proteins," *Blood Cells, Molecules, and Diseases*, vol. 35, no. 3, pp. 398–403, 2005.

[48] D. Popovic, D. Vucic, and I. Dikic, "Ubiquitination in disease pathogenesis and treatment," *Nature medicine*, vol. 20, no. 11, pp. 1242–1253, 2014.

[49] G. Goldstein, M. Scheid, U. Hammerling, D. H. Schlesinger, H. D. Niall, and E. A. Boyse, "Isolation of a polypeptide that has lymphocyte differentiating properties and is probably represented universally in living cells," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 72, no. 1, pp. 11–15, 1975.

[50] V. Pancré, R. J. Pierce, F. Fournier et al., "Effect of ubiquitin on platelet functions: possible identity with platelet activity suppressive lymphokine (PASL)," *European Journal of Immunology*, vol. 21, no. 11, pp. 2735–2741, 1991.

[51] H. Shibayama, T. Machii, Y. Tokumine et al., "Establishment of a new cell line from a patient with hairy cell leukemia-Japanese variant," *Leukemia and Lymphoma*, vol. 25, no. 3-4, pp. 373–380, 1997.

[52] H. Daino, I. Matsumura, K. Takada et al., "Induction of apoptosis by extracellular ubiquitin in human hematopoietic cells: possible involvement of STAT3 degradation by proteasome pathway in interleukin 6-dependent hematopoietic cells," *Blood*, vol. 95, no. 8, pp. 2577–2585, 2000.

[53] Y. Tanaka, N. Tanaka, Y. Saeki et al., "c-Cbl-dependent monoubiquitination and lysosomal degradation of gp130," *Molecular and Cellular Biology*, vol. 28, no. 15, pp. 4805–4818, 2008.

[54] T. Kishimoto, S. Akira, M. Narazaki, and T. Taga, "Interleukin-6 family of cytokines and Gp130," *Blood*, vol. 86, no. 4, pp. 1243–1254, 1995.

[55] F. Sellebjerg, M. Krakauer, M. Khademi, T. Olsson, and P. S. Sørensen, "FOXP3, CBLB and ITCH gene expression and cytotoxic T lymphocyte antigen 4 expression on $CD4^+CD25$high T cells in multiple sclerosis," *Clinical and Experimental Immunology*, vol. 170, no. 2, pp. 149–155, 2012.

[56] N. J. Lohr, J. P. Molleston, K. A. Strauss et al., "Human ITCH E3 ubiquitin ligase deficiency causes syndromic multisystem autoimmune disease," *The American Journal of Human Genetics*, vol. 86, no. 3, pp. 447–453, 2010.

[57] N. Janikashvili, M. Trad, A. Gautheron et al., "Human monocyte-derived suppressor cells control graft-versus-host disease by inducing regulatory forkhead box protein 3-positive $CD8^+$ T lymphocytes," *Journal of Allergy and Clinical Immunology*, vol. 135, no. 6, pp. 1614–1624, 2015.

[58] V. Saini, A. Marchese, and M. Majetschak, "CXC chemokine receptor 4 is a cell surface receptor for extracellular ubiquitin," *Journal of Biological Chemistry*, vol. 285, no. 20, pp. 15566–15576, 2010.

[59] M. Majetschak, U. Krehmeier, M. Bardenheuer et al., "Extracellular ubiquitin inhibits the TNF-α response to endotoxin in peripheral blood mononuclear cells and regulates endotoxin hyporesponsiveness in critical illness," *Blood*, vol. 101, no. 5, pp. 1882–1890, 2003.

[60] H. Daino, H. Shibayama, T. Machii, and T. Kitani, "Extracellular ubiquitin regulates the growth of human hematopoietic cells," *Biochemical and Biophysical Research Communications*, vol. 223, no. 2, pp. 226–228, 1996.

[61] R. Sujashvili, I. Ioramashvili, N. Gvinadze, and K. Aptsiauri, "Inhibition of proliferative activity of bone marrow cells by extracellular ubiquitin," *Proceedings of the Georgian National Academy of Science, Biomedical Series*, vol. 40, no. 5-6, pp. 265–270, 2014.

[62] M. Majetschak, N. Ponelies, and T. Hirsch, "Targeting the monocytic ubiquitin system with extracellular ubiquitin," *Immunology and Cell Biology*, vol. 84, no. 1, pp. 59–65, 2006.

[63] Z. Szewczuk, P. Stefanowicz, A. Wilczyński et al., "Immunosuppressory activity of ubiquitin fragments containing retro-RGD sequence," *Biopolymers*, vol. 74, no. 5, pp. 352–362, 2004.

[64] Ł. Jaremko, M. Jaremko, P. Pasikowski et al., "The immunosuppressive activity and solution structures of ubiquitin fragments," *Biopolymers*, vol. 91, no. 6, pp. 423–431, 2009.

Hindawi Publishing Corporation
Mediators of Inflammation
Volume 2016, Article ID 2196986, 7 pages
http://dx.doi.org/10.1155/2016/2196986

Research Article

Association between IgG4 Autoantibody and Complement Abnormalities in Systemic Lupus Erythematosus

Qingjun Pan, Linjie Guo, Jing Wu, Jun Cai, Huanjin Liao, Qiaofen Lan, Yanxia Peng, Yiming He, and Hua-feng Liu

Institute of Nephrology, Affiliated Hospital of Guangdong Medical University, Zhanjiang, Guangdong 524001, China

Correspondence should be addressed to Hua-feng Liu; hf-liu@263.net

Received 1 April 2016; Accepted 13 July 2016

Academic Editor: Tinatin Chikovani

In order to investigate the association between IgG4 autoantibody and complement abnormalities in systemic lupus erythematosus (SLE), 72 newly diagnosed SLE patients, 67 rheumatoid arthritis (RA) patients, and 41 healthy normals were employed. Serum levels of antinuclear IgG4 and IgG4-specific IgM-rheumatoid factor (RF) were measured, and the correlations between serum levels of antinuclear IgG4 and several clinical parameters were analyzed. Also, the levels of IgG subclasses, C1q, and C3 deposition in lupus nephritis (LN) were detected. The results showed that serum levels of antinuclear IgG4 were higher in SLE patients relative to healthy normals ($P < 0.01$). Serum levels of antinuclear IgG4 in SLE patients were positively correlated with serum levels of total IgG4, albumin, and C3 ($r = 0.61$, $P < 0.05$; $r = 0.40$, $P < 0.05$; and $r = 0.54$, $P < 0.05$, resp.) and negatively correlated with 24-hour urinary protein ($r = 0.49$, $P < 0.05$). Serum levels of IgG4-specific IgM-RF were higher in RA patients than in SLE patients ($P < 0.001$). Also, the ratio of the deposition score for IgG4/(IgG1 + IgG2 + IgG3 + IgG4) was negatively correlated with the score for C1q and C3 deposition in LN ($r = 0.34$, $P < 0.05$; $r = 0.51$, $P < 0.01$, resp.). In summary, the IgG4 autoantibody may dampen the inflammatory response in SLE, thus maybe providing a novel therapeutic target for SLE.

1. Introduction

Systemic lupus erythematosus (SLE) is a systemic disease that can affect multiple organs, such as lupus nephritis (LN) resulting from autoantibody and complement-fixing immune complexes (ICs) deposition [1]. Also, complement abnormalities in SLE [2] and the function of complement molecules (C3, C1q, etc.) in the processing of ICs are well recognized [3]. However, molecular mechanisms that can affect complement consumption by specific autoantibody in SLE still need to be further investigated.

Human IgG is the main component of serum immunoglobulin, which can be divided into four subclasses: IgG1 (60–70%), IgG2 (15–20%), IgG3 (5–10%), and IgG4 (4–6%) [4, 5]. The development of IgG4-related disease (IgG4-RD) has directed the attention of autoimmune disease research to the smallest of the subclasses—IgG4. Compared with the other subclasses of IgG, the serum levels of IgG4 are low (4–8%) and IgG4 is negatively charged in the physiological environment. Additionally, the length and sequence of the amino acids in the hinge region of IgG4, located between the Fab and the C terminal of the two heavy chains (CH_2 and CH_3), determine the ability of IgG4 to bind to C1q and Fcγ receptors, and this region in IgG4 is significantly different from the hinge regions in the other IgG subclasses, resulting in a lower binding ability. Thus, IgG4 cannot stimulate the classical pathway of complement activation, even though the binding ability of IgG4 toward targeted antigen is the same as those of the other subclasses of IgG [6–9]. It can be speculated that IgG4 may have a protective effect in SLE, which may occur through the inhibition of autoantigen-mediated complement consumption.

In this study, serum levels of anti-nuclear IgG4 and IgG4-specific IgM-rheumatoid factor (IgM-RF) were detected, and the correlations between the serum levels of anti-nuclear IgG4 and several clinical parameters of SLE patients were analyzed. Additionally, the levels of IgG subclass, C1q, and C3 deposition in the kidney were detected by immunofluorescence staining to investigate the association between

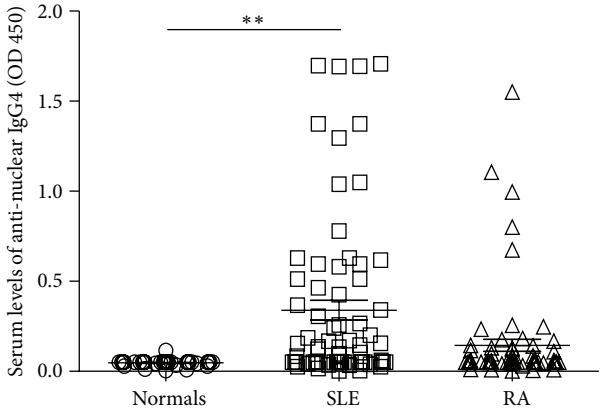


Figure 1: Comparison of serum levels of anti-nuclear IgG4 in various groups. Detection of serum levels of anti-nuclear IgG4 in the serum of normals and SLE and RA patients was conducted using a molecular biology kit as described in Section 2 (normal = 41, SLE = 72, and RA = 67; $^{**}P < 0.01$).

IgG4 autoantibody (anti-nuclear IgG4) and complement abnormalities in SLE.

2. Materials and Methods

2.1. Human Subjects. Seventy-two newly diagnosed and untreated SLE patients (10 males and 62 females with an average age of 28, ranging from 11 to 66) and 67 RA patients (11 males and 56 females with an average age of 48 ranging from 17 to 86) were selected from the Kidney Disease Department of Internal Medicine, Affiliated Hospital of Guangdong Medical University, from March 2013 to December 2014. Forty-one healthy normals (6 males and 35 females with an average age of 24, ranging from 22 to 49) were included as normal control subjects. All SLE patients were diagnosed according to the 1997 revised criteria of the American College of Rheumatology (ACR). Lupus nephritis (LN) was diagnosed in accordance with the ISN/RPS 2003 classification of LN, and rheumatoid arthritis (RA) was diagnosed in accordance with the 2009 criteria of the ACR/European League Against Rheumatism (EULAR). To eliminate the influence of IgG4-RD on IgG4 in SLE, patients who had IgG4-RD were excluded from the study. The clinical data and laboratory results of the SLE patients were collected. Written informed consent was obtained, and the protocol of this study was approved by the Medical Ethics Committee of the Affiliated Hospital of Guangdong Medical University.

2.2. Antibodies and Reagents. Antibodies and reagents were obtained as follows: mouse anti-human IgG4 antibody (AbD Serotec Company, UK), HRP-labeled rabbit anti-human IgG4 antibody (Boster Company, Wuhan, China), ANA Screen Test System (ZEUS ELISA™ Kit, ZEUS Scientific, Inc., USA), Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific Company, USA), mouse anti-human IgG1, IgG2, IgG3, and IgG4-FITC (Sigma, USA), rabbit anti-human C1q and goat anti-human C3 (MBL, Japan), and Multiskan MK3 microplate reader (Thermo Fisher Scientific Company, USA).

2.3. Detection of Serum Levels of IgG4 and Anti-Nuclear IgG4. Serum from patients and healthy normals was collected and stored at 80°C before testing. Serum IgG subclass levels were measured using the immunonephelometric assay [10, 11] with a molecular biology kit (Siemens, Germany, N Latex IgG4 and N Supplementary Reagent/Precipitation) and an automatic protein analyzer (Siemens BN ProSpec-I, Germany) according to the manufacturer's instructions. To measure anti-nuclear IgG4, a commercial anti-nuclear IgG ELISA Kit was modified to use anti-human IgG4-HRP instead of anti-human IgG-HRP as the secondary antibody. The Excel software was used to calculate the relative concentration of anti-nuclear IgG4 according to the optical density (OD) value measured at 450 nm.

2.4. Detection of Serum Levels of Albumin, C3, Scr, SUA, and 24-Hour Urinary Protein. Serum levels were measured in the clinical laboratory at our hospital as follows: albumin, Scr, and SUA were detected using an automatic biochemistry analyzer (Roche Cobas® 8000); 24-hour urinary protein was detected using an automatic biochemistry analyzer (Olympus AU2700); and serum C3 was detected by rate nephelometry using a Beckman Coulter IMMAGE 800.

2.5. Detection of IgG4-Specific IgM-RF in the Serum of SLE Patients by ELISA. After obtaining plasma from SLE patients, the anti-human IgG4 affinity column was manufactured using an established method [12] by conjugation of mouse anti-human IgG4 antibodies to agarose gel. The IgG4 was purified from the serum of SLE patients using the affinity column and quantified using the Pierce BCA Protein Assay Kit as per the manufacturer's instructions and then measured using a microplate reader at 570 nm. The isolated IgG4 was then coated onto plates in 0.05 M carbonate-bicarbonate coating buffer (pH 9.6) overnight at 4°C. IgG4-specific IgM-RF was then detected in the serum of SLE patients using an established ELISA method with anti-human IgM-HRP as the secondary antibody.

2.6. Detection of Subclasses of IgG Deposition in LN Renal Tissue by Direct Immunofluorescence Staining. One hundred forty-one cases, including 46 newly diagnosed and 65 treated SLE patients, were diagnosed with LN by renal biopsy. Kidney tissue specimens were divided and processed for immunofluorescence. Fragments from each biopsy were snap-frozen and cut using a Leica-CM1510-freezing microtome into 5 μm sections, which were then stained with diluted mouse anti-human IgG1, IgG2, IgG3, IgG4, C3, or C1q-FITC using an established direct IF staining method [13–15]. Immunofluorescence sections were examined using an Olympus BX60 microscope. Immunofluorescence staining for IgG subclasses (IgG1, IgG2, IgG3, and IgG4) and for complement proteins (C3, C1q) was evaluated according to the fluorescence intensity, scored on a scale of 0–4+ as follows: 0, negative; 0.5, weak; 1, one plus; 2, two plus; 3, three plus; and 4, four plus. All slides

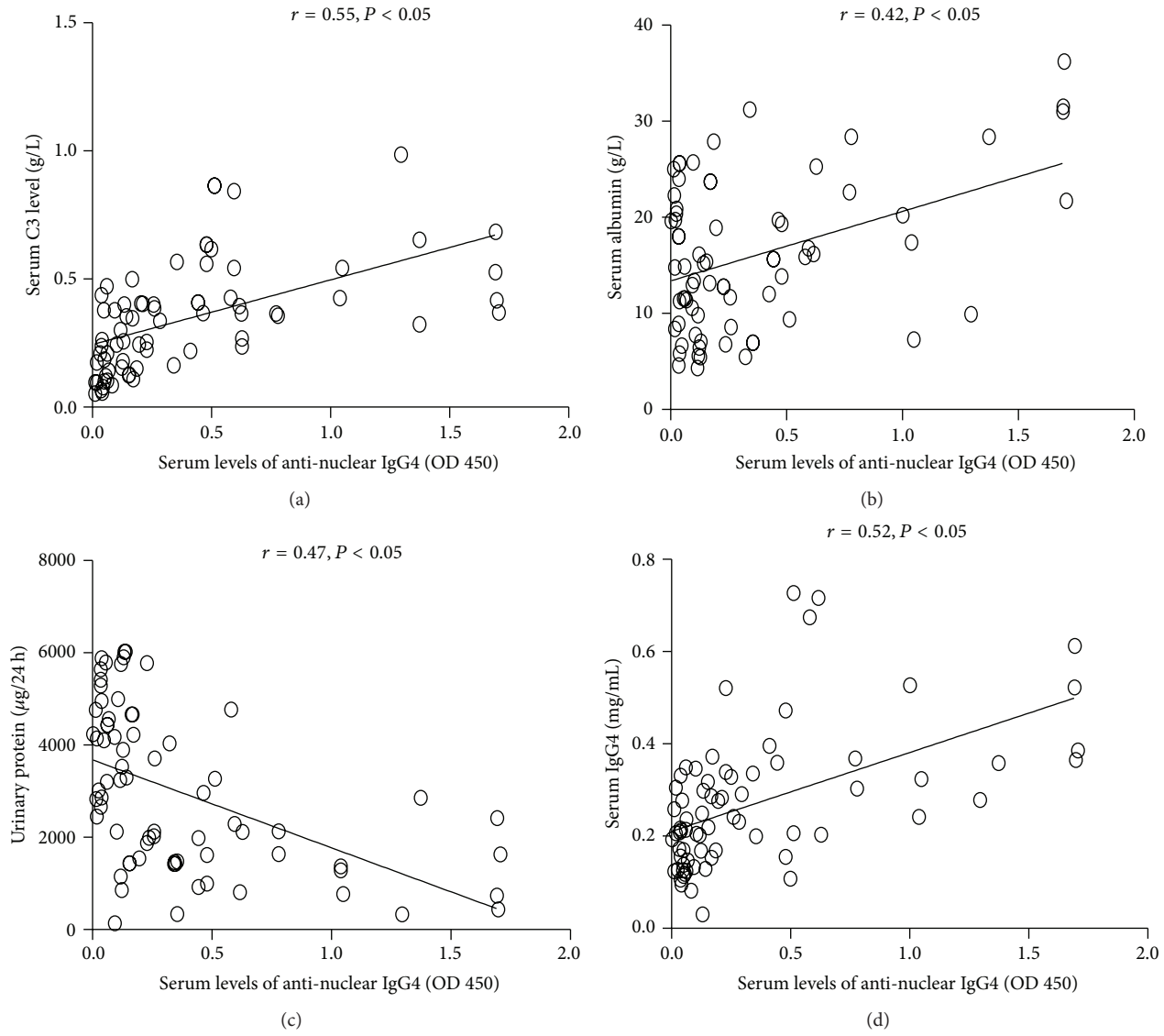


Figure 2: The correlations between serum levels of anti-nuclear IgG4 in SLE patients and clinical parameters. Detection of serum levels of IgG4 and anti-nuclear IgG4 was conducted by ELISA as described in Section 2. Serum levels of albumin, C3, Scr, SUA, and 24-hour urinary protein were analyzed using a commercial kit as described in Section 2.2. The correlations between serum levels of anti-nuclear IgG4 and serum levels of C3, albumin, total IgG4, and 24-hour urinary protein were analyzed using Spearman's rank correlation coefficient. The P value was considered statistically significant if it was less than 0.05. ($n = 72$, newly diagnosed SLE patients).

were blindly examined by two pathologists, and ambiguous samples were reviewed to achieve a consensus.

2.7. Statistical Analysis. All statistical tests were performed using SPSS version 16 (SPSS, Inc., Chicago, IL, USA) and Prism 5 software (GraphPad Software, Inc., San Diego, CA, USA). All data are expressed as the mean ± standard deviation (SD). Two-group comparisons were carried out using our independent sample t-test. Multiple-group comparisons were performed using ANOVA, followed by the Bonferroni or Dunnett post hoc tests. The correlations between different parameters were analyzed using Spearman's rank correlation coefficient. The P value was considered statistically significant if it was less than 0.05.

3. Results

3.1. Serum Levels of Anti-Nuclear IgG4 in SLE Patients. The serum levels of anti-nuclear IgG4 were significantly higher in SLE patients than in healthy normals ($P < 0.01$). However, there was no significant difference between the SLE patients and RA patients ($P > 0.05$) (Figure 1).

3.2. Correlations between Serum Levels of Anti-Nuclear IgG4 and Clinical Parameters in SLE Patients. Serum levels of anti-nuclear IgG4 in SLE patients were positively correlated with serum levels of C3 and albumin ($r = 0.55$, $P < 0.05$; $r = 0.42$, $P < 0.05$) (Figures 2(a) and 2(b)), negatively associated with 24-hour urinary protein ($r = 0.47$, $P < 0.05$)

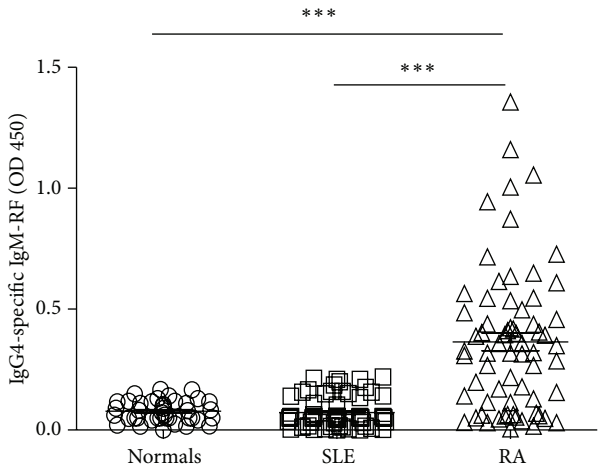


Figure 3: Comparison of serum levels of IgG4-specific IgM-RF in various groups. Detection of IgG4-specific IgM-RF in the serum of normals and SLE and RA patients was conducted by ELISA as described in Section 2 (normal = 41, SLE = 72, and RA = 67; $^{***}P < 0.001$).

(Figure 2(c)), and positively correlated with serum levels of total IgG4 ($r = 0.52$, $P < 0.05$) (Figure 2(d)). However, there was no significant difference between the serum levels of anti-nuclear IgG4 and the levels of Scr or SUA ($P > 0.05$).

3.3. IgG4-Specific IgM-RF in the Serum of SLE Patients. Serum levels of IgG4-specific IgM-RF in RA patients were significantly higher than in SLE patients and healthy normals ($P < 0.001$, $P < 0.001$), but there was no significant difference between the SLE patients and the healthy normals ($P > 0.05$) (Figure 3).

3.4. Levels of IgG Subclass Deposition and C1q and C3 Deposition in Lupus Nephritis. The immunofluorescence staining results indicated that deposition of IgG subclass, C1q, and C3 was common (Figure 4(a)), and the ratio of deposition score for IgG4/(IgG1 + IgG2 + IgG3 + IgG4) was negatively correlated with the scores for C1q and C3 deposition in LN ($r = 0.34$, $P < 0.05$; $r = 0.51$, $P < 0.01$, resp.) (Figure 4(b)).

4. Discussion

In SLE, autoreactive B cells produce various autoantibodies against autoantigens. Anti-nuclear antibody is a disease activity marker in SLE [16] and in lupus nephritis [17], with a positivity rate of 95% to 100% at disease onset [18].

There is still controversy regarding serum levels of IgG4 in SLE, and the serum levels of anti-nuclear IgG4 have not been reported. Morland et al., Lin et al., and Sun et al. reported that serum levels of IgG4 in SLE patients had a declining trend compared to those of normal subjects [19–21]. Additionally, Kuroki et al. reported that there was no significant difference in the serum levels of IgG4 between LN patients and controls [22]. Zhang et al. reported that there was no significant difference in IgG4 or IgG4/IgG between SLE and normal subjects [23]. However, Yu reported that, compared with normal subjects, the serum levels of IgG4 were significantly higher in SLE patients who were also more likely to exhibit interstitial pneumonia and pancreatic involvement [24]; however, interstitial pneumonia and autoimmune pancreatitis have been associated with IgG4-RD, where patients show elevated serum IgG4 concentrations [25, 26]. Thus, it is unclear whether the increase in the serum levels of IgG4 in SLE patients is due to IgG4-RD. These reports suggest that there may be no significant differences in the serum levels of IgG4 when comparing SLE patients to control subjects because of variations in the detection methods or because of variability within the subject groups in this investigation (such as individual patient differences within the SLE group or SLE patients who also have LN). This suggests that the nature of IgG4 involvement in the progression of SLE remains unclear. Thus, detection of anti-nuclear IgG4, rather than total IgG4, may be a more effective clinical guideline. Our results showed that the serum levels of anti-nuclear IgG4 were significantly higher in SLE patients than in control subjects, and there were associations between the serum levels of anti-nuclear IgG4 and the clinical parameters examined. These results indicate that anti-nuclear IgG4 may play a protective role in the pathogenesis of SLE.

Because denatured IgG (including IgG4) exposes CH_2, the Fc fragment of IgG is recognized by rheumatoid factor (RF), and the resulting formation of RF-IgG immune complexes (ICs) continues to activate complement, leading to tissue and organ damage [27]. One study indicated that approximately 26.7% of SLE patients were RF-positive, with mainly IgM-RF [28]. However, the results of our study found that the serum levels of IgG4-specific IgM-RF in SLE patients were not significantly different from those of normal subjects, though higher serum levels of IgG4-specific IgM-RF may be related to lower levels of anti-nuclear IgG4 in RA patients.

Complement abnormalities [2] and the roles of complement molecules in the processing of ICs are well recognized [3]. It is generally believed that the ICs that activate complement factors show more pathological significance, even though the induction of inflammation by ICs may be complement-dependent or complement-independent. Muso et al. found that the levels of this type of IC, which were closely associated with clinical and serological disease activities, were significantly higher in SLE patients compared to controls [29], and some experimental models have shown a role for complement in the induction of inflammatory injury [30, 31]. ICs that contain IgG4 have a limited ability to induce immune responses because of their low affinity for both Fcγ receptors and the C1 complement molecules [32–35]. van der Zee et al. reported that the IgG4 antibody of phospholipase A (PLA), found in the blood of beekeepers, effectively inhibited complement activation by IgG1 antibodies primarily by inhibiting the binding of C1q to IgG1 in mixed IgG1- and IgG4-containing ICs, thus reducing injury caused by immune inflammation [36]. Another study showed that IgG4 binds to the Fc portions of IgG1, IgG2, and IgG3 and blocks the Fc-mediated effector functions of IgG1 and IgG3 complexes (e.g., IgG1-class ICs, which mediate tissue damage) and may assist in the clearance of ICs by forming larger complexes

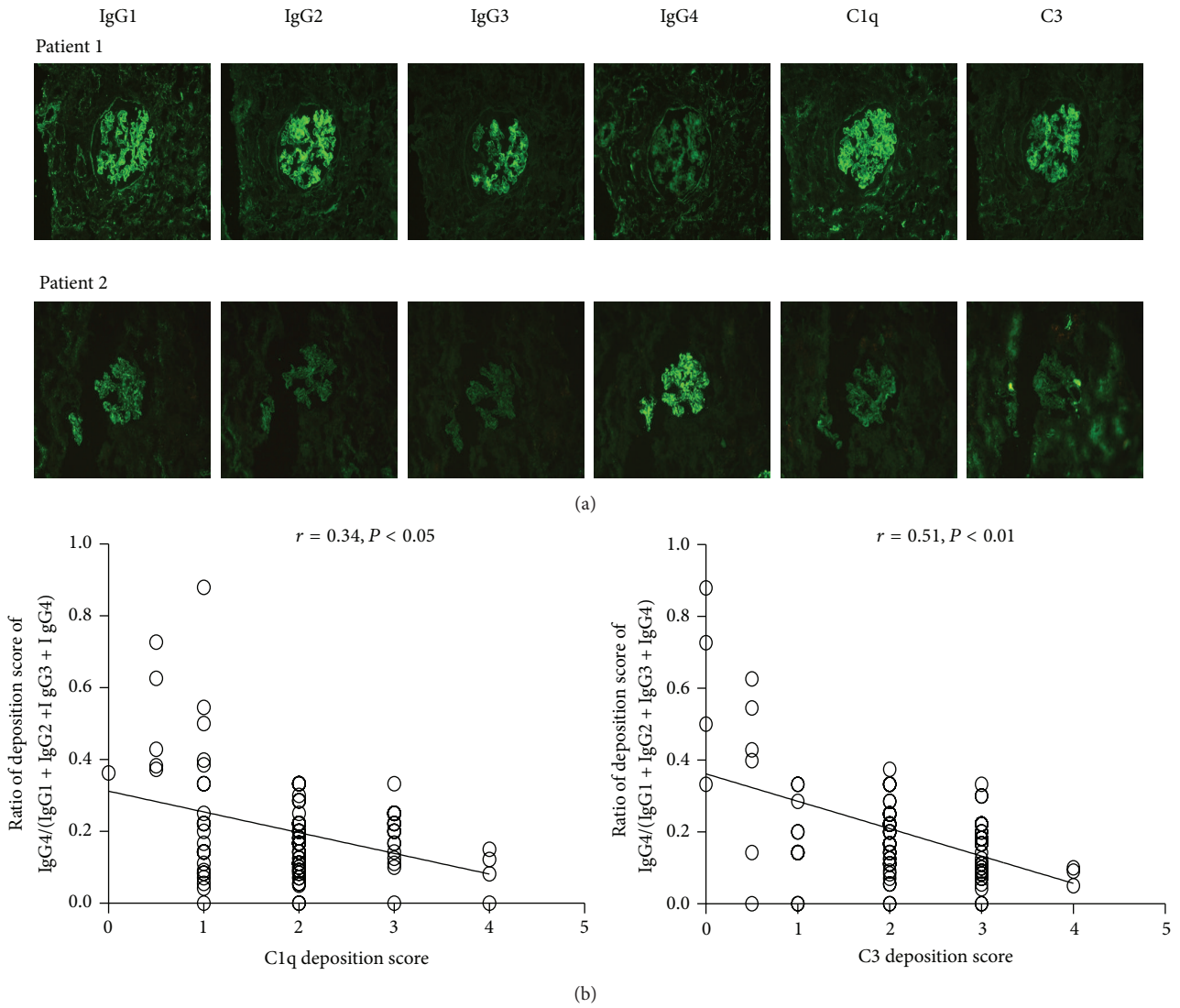


Figure 4: Levels of IgG subclass, C1q, and C3 deposition in lupus nephritis. (a) IgG subclass, C1q, and C3 deposition in LN renal tissue were detected by direct immunofluorescence staining as described in Section 2. (b) The correlations between the ratio of deposition score for IgG4/(IgG1 + IgG2 + IgG3 + IgG4) and the score for C1q and C3 deposition were analyzed using Spearman's rank correlation coefficient. The P value was considered statistically significant if it was less than 0.05 (n = 141, including 46 newly diagnosed LN patients and 65 LN patients treated with prednisone plus cyclophosphamide or azathioprine or hydroxychloroquine or mycophenolate mofetil).

that are more effectively cleared, resulting in termination of the inflammatory process [37]. Therefore, it is possible that IgG4 autoantibody (anti-nuclear IgG4) carries out an anti-inflammatory function instead of driving the disease process.

5. Conclusion

This study firstly investigated the association between IgG4 autoantibody (anti-nuclear IgG4) and complement abnormalities in SLE and found that the IgG4 autoantibody (anti-nuclear IgG4) may dampen the inflammatory response in SLE by competitively binding to autoantigens to form nonpathogenic ICs that result from the low affinity of IgG4 for both the Fcγ receptor and the C1 complement molecule, thus maybe providing a novel therapeutic target for SLE.

Abbreviations

SLE:	Systemic lupus erythematosus
RA:	Rheumatoid arthritis
ELISA:	Enzyme-linked immune sorbent assay
PBMC:	Peripheral blood mononuclear cell
ICs:	Immune complexes
IgG4-RD:	IgG4-related disease
ACR:	American College of Rheumatology
EULAR:	European League Against Rheumatism
PLA:	Phospholipase A

RF: Rheumatoid factor
LN: Lupus nephritis
Ig: Immunoglobulin.

Competing Interests

The authors declare that there is no conflict of interests.

Authors' Contributions

Qingjun Pan, Linjie Guo, and Jing Wu are equal contributors to this paper.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (81471530) and Zhanjiang City Science and Technology Project (2015A01026).

References

[1] A. Rahman and D. A. Isenberg, "Systemic lupus erythematosus," *The New England Journal of Medicine*, vol. 358, no. 9, pp. 929–939, 2008.

[2] M. C. Pickering and M. J. Walport, "Links between complement abnormalities and systemic lupus erythematosus," *Rheumatology*, vol. 39, no. 2, pp. 133–141, 2000.

[3] M. J. Walport and K. A. Davies, "Complement and immune complexes," *Research in Immunology*, vol. 147, no. 2, pp. 103–109, 1996.

[4] F. Shakib, *The Human IgG Subclasses: Molecular Analysis of Structure, Function and Regulation*, Elsevier, New York, NY, USA, 2013.

[5] R. R. Rich, T. A. Fleisher, W. T. Shearer et al., *Clinical Immunology: Principles and Practice*, Elsevier Health Sciences, 2012.

[6] A. M. Davies, T. Rispens, P. Ooijevaar-De Heer et al., "Structural determinants of unique properties of human IgG4-Fc," *Journal of Molecular Biology*, vol. 426, no. 3, pp. 630–644, 2014.

[7] A. Nirula, S. M. Glaser, S. L. Kalled, and F. R. Taylora, "What is IgG4? A review of the biology of a unique immunoglobulin subtype," *Current Opinion in Rheumatology*, vol. 23, no. 1, pp. 119–124, 2011.

[8] R. C. Aalberse, S. O. Stapel, J. Schuurman, and T. Rispens, "Immunoglobulin G4: an odd antibody," *Clinical and Experimental Allergy*, vol. 39, no. 4, pp. 469–477, 2009.

[9] H. Liu and K. May, "Disulfide bond structures of IgG molecules: structural variations, chemical modifications and possible impacts to stability and biological function," *mAbs*, vol. 4, no. 1, pp. 17–23, 2012.

[10] A. Vlug, E. J. Nieuwenhuys, R. V. W. van Eijk, H. G. M. Geertzen, and A. J. Van Houte, "Nephelometric measurements of human IgG subclasses and their reference ranges," *Annales de Biologie Clinique*, vol. 52, no. 7-8, pp. 561–567, 1994.

[11] M. Pressac, F. Allouche, R. Circaud, and P. Aymard, "Evaluation of human IgG subclass assays on Beckman array," *Annals of Clinical Biochemistry*, vol. 32, no. 3, pp. 281–288, 1995.

[12] B. V. Ayyar, S. Arora, C. Murphy, and R. O'Kennedy, "Affinity chromatography as a tool for antibody purification," *Methods*, vol. 56, no. 2, pp. 116–129, 2012.

[13] T. Doi, M. Mayumi, K. Kanatsu, F. Suehiro, and Y. Hamashima, "Distribution of IgG subclasses in membranous nephropathy," *Clinical and Experimental Immunology*, vol. 58, no. 1, pp. 57–62, 1984.

[14] H. Imai, K. Hamai, A. Komatsuda, H. Ohtani, and A. B. Miura, "IgG subclasses in patients with membranoproliferative glomerulonephritis, membranous nephropathy, and lupus nephritis," *Kidney International*, vol. 51, no. 1, pp. 270–276, 1997.

[15] Y. S. Song, K.-W. Min, J. H. Kim, G.-H. Kim, and M. H. Park, "Differential diagnosis of lupus and primary membranous nephropathies by IgG subclass analysis," *Clinical Journal of the American Society of Nephrology*, vol. 7, no. 12, pp. 1947–1955, 2012.

[16] D. A. Isenberg, J. J. Manson, M. R. Ehrenstein, and A. Rahman, "Fifty years of anti-ds DNA antibodies: are we approaching journey's end?" *Rheumatology*, vol. 46, no. 7, pp. 1052–1056, 2007.

[17] M. Sui, Q. Lin, Z. Xu et al., "Simultaneous positivity for anti-DNA, anti-nucleosome and anti-histone antibodies is a marker for more severe lupus nephritis," *Journal of Clinical Immunology*, vol. 33, no. 2, pp. 378–387, 2013.

[18] M. R. Arbuckle, M. T. McClain, M. V. Rubertone et al., "Development of autoantibodies before the clinical onset of systemic lupus erythematosus," *The New England Journal of Medicine*, vol. 349, no. 16, pp. 1526–1533, 2003.

[19] C. Morland, J. Michael, and D. Adu, "IgG subclasses of PEG precipitable IgG in systemic lupus erythematosus sera," *Clinical Nephrology*, vol. 31, no. 4, pp. 204–209, 1989.

[20] G.-G. Lin and J.-M. Li, "IgG subclass serum levels in systemic lupus erythematosus patients," *Clinical Rheumatology*, vol. 28, no. 11, pp. 1315–1318, 2009.

[21] W. Sun, R.-F. Gao, Y. Chen, Y.-Y. Su, and L.-L. Dong, "Measurement of serum IgG4 levels by an established ELISA system and its clinical applications in autoimmune diseases," *Journal of Huazhong University of Science and Technology [Medical Sciences]*, vol. 33, no. 4, pp. 611–614, 2013.

[22] A. Kuroki, T. Shibata, H. Honda, D. Totsuka, K. Kobayashi, and T. Sugisaki, "Glomerular and serum IgG subclasses in diffuse proliferative lupus nephritis, and idiopathic membranous nephropathy," *Internal Medicine*, vol. 41, no. 11, pp. 936–942, 2002.

[23] H. Zhang, P. Li, D. Wu et al., "Serum IgG subclasses in autoimmune diseases," *Medicine*, vol. 94, no. 2, p. e387, 2015.

[24] C. X. Yu, *Preliminary Study on the Relationship between IgG and Systemic Lupus Erythematosus*, North Sichuan Medical College, Nanchong, China, 2013 (Chinese).

[25] J. H. Stone, Y. Zen, and V. Deshpande, "IgG4-related disease," *The New England Journal of Medicine*, vol. 366, no. 6, pp. 539–551, 2012.

[26] T. Kamisawa, Y. Zen, S. Pillai, and J. H. Stone, "IgG4-related disease," *The Lancet*, vol. 385, no. 9976, pp. 1460–1471, 2015.

[27] S. Duquerroy, E. A. Stura, S. Bressanelli et al., "Crystal structure of a human autoimmune complex between IgM rheumatoid factor RF61 and IgG1 Fc reveals a novel epitope and evidence for affinity maturation," *Journal of Molecular Biology*, vol. 368, no. 5, pp. 1321–1331, 2007.

[28] T. P. Cui, J. L. Xie, L. K. Yu et al., "Anti-CCP/RA33/AKA/RF autoantibodies in rheumatoid arthritis: a clinical diagnosis," *Shanghai Journal of Medical Laboratory Sciences*, vol. 18, no. 4, pp. 221–222, 2003 (Chinese).

[29] E. Muso, M. Yashiro, Y. Ito, H. Yoshida, and S. Sasayama, "Correlations of C1q- and C3d-bearing circulating immune complexes with immunopathological disease activity in lupus nephritis patients," *Nihon Jinzo Gakkai Shi*, vol. 36, no. 4, pp. 345–354, 1994.

[30] U. E. Höpken, B. Lu, N. P. Gerard, and C. Gerard, "Impaired inflammatory responses in the reverse arthus reaction through genetic deletion of the C5a receptor," *Journal of Experimental Medicine*, vol. 186, no. 5, pp. 749–756, 1997.

[31] T. Heller, J. E. Gessner, R. E. Schmidt, A. Klos, W. Bautsch, and J. Köhl, "Cutting edge: Fc receptor type I for IgG on macrophages and complement mediate the inflammatory response in immune complex peritonitis," *Journal of Immunology*, vol. 162, no. 10, pp. 5657–5661, 1999.

[32] S. M. Canfield and S. L. Morrison, "The binding affinity of human IgG for its high affinity Fc receptor is determined by multiple amino acids in the CH2 domain and is modulated by the hinge region," *The Journal of Experimental Medicine*, vol. 173, no. 6, pp. 1483–1491, 1991.

[33] M.-H. Tao, S. M. Canfield, and S. L. Morrison, "The differential ability of human IgG1 and IgG4 to activate complement is determined by the COOH-terminal sequence of the CH2 domain," *Journal of Experimental Medicine*, vol. 173, no. 4, pp. 1025–1028, 1991.

[34] M.-H. Tao, R. I. F. Smith, and S. L. Morrison, "Structural features of human immunoglobulin G that determine isotype-specific differences in complement activation," *Journal of Experimental Medicine*, vol. 178, no. 2, pp. 661–667, 1993.

[35] E. Della-Torre, M. Lanzillotta, and C. Doglioni, "Immunology of IgG4-related disease," *Clinical and Experimental Immunology*, vol. 181, no. 2, pp. 191–206, 2015.

[36] J. S. van der Zee, P. van Swieten, and R. C. Aalberse, "Inhibition of complement activation by IgG4 antibodies," *Clinical and Experimental Immunology*, vol. 64, no. 2, pp. 415–422, 1986.

[37] M. van der Neut Kolfschoten, J. Schuurman, M. Losen et al., "Anti-inflammatory activity of human IgG4 antibodies by dynamic Fab arm exchange," *Science*, vol. 317, no. 5844, pp. 1554–1557, 2007.

Hindawi Publishing Corporation
Mediators of Inflammation
Volume 2016, Article ID 9607946, 11 pages
http://dx.doi.org/10.1155/2016/9607946

Review Article

Epigenetic Modulation as a Therapeutic Prospect for Treatment of Autoimmune Rheumatic Diseases

Marzena Ciechomska[1] and Steven O'Reilly[2]

[1]*National Institute of Geriatrics Rheumatology and Rehabilitation, Department of Pathophysiology and Immunology, Warsaw, Poland*
[2]*Faculty of Health and Life Sciences, Northumbria University, Newcastle upon Tyne, UK*

Correspondence should be addressed to Marzena Ciechomska; m.m.ciechomska@gmail.com

Received 20 May 2016; Accepted 12 July 2016

Academic Editor: Nona Janikashvili

Systemic inflammatory rheumatic diseases are considered as autoimmune diseases, meaning that the balance between recognition of pathogens and avoidance of self-attack is impaired and the immune system attacks and destroys its own healthy tissue. Treatment with conventional Disease Modifying Antirheumatic Drugs (DMARDs) and/or Nonsteroidal Anti-Inflammatory Drugs (NSAIDs) is often associated with various adverse reactions due to unspecific and toxic properties of those drugs. Although biologic drugs have largely improved the outcome in many patients, such drugs still pose significant problems and fail to provide a solution to all patients. Therefore, development of more effective treatments and improvements in early diagnosis of rheumatic diseases are badly needed in order to increase patient's functioning and quality of life. The reversible nature of epigenetic mechanisms offers a new class of drugs that modulate the immune system and inflammation. In fact, epigenetic drugs are already in use in some types of cancer or cardiovascular diseases. Therefore, epigenetic-based therapeutics that control autoimmunity and chronic inflammatory process have broad implications for the pathogenesis, diagnosis, and management of rheumatic diseases. This review summarises the latest information about potential therapeutic application of epigenetic modification in targeting immune abnormalities and inflammation of rheumatic diseases.

1. Autoimmune Rheumatic Diseases

Systemic autoimmune rheumatic diseases are characterised by pain and chronic joint inflammation. There are more than 200 different conditions that are labelled as rheumatic diseases including rheumatic arthritis, systemic sclerosis, systemic lupus erythematosus, psoriatic arthritis, ankylosing spondylitis, and Sjogren syndrome. Moreover, autoimmune rheumatic diseases share many common features, which makes them difficult to differentiate within the group. Indeed, up to 50 percent of patients with autoimmune rheumatic diseases cannot be easily categorised with a specific disorder in the first 12 months of follow-up [1].

One of the major characteristics of rheumatic diseases is chronic inflammation and autoimmunity, which consequently leads to tissue destruction and reduces patients' mobility. Immune cells play a key role in inflammation due to involvement in initiation and maintenance of the chronic inflammatory stages. In particular, circulating monocytes that may differentiate towards macrophages or dendritic cells are able to produce proinflammatory cytokines including interleukin-1 (IL-1), IL-6, IL-8, and tumour necrosis factor-α (TNF-α) [2]. Monocytes are also responsible for the production of inflammatory mediators including reactive oxygen species (ROS) and cyclooxygenase-2 (COX-2) [3]. COX-2 is a key enzyme in prostaglandins biosynthesis driving the inflammatory response. Monocytes can produce chemokines which attract T and B cells for the secretion of proinflammatory cytokines. Activated B cells are able to present autoantigens and produce autoantibodies maintaining the inflammatory process leading to tissue destruction. The presence of autoantibodies is a hallmark of autoimmune

rheumatic diseases [4]. Also, a subset of helper CD4+ T cells, Th17, is reported to be involved in rheumatic pathogenesis [5]. Th17 cells are characterised by production of IL-17. This interleukin is a potent proinflammatory cytokine that amplifies ongoing inflammation by induction of TNF-α, IL-1β, and IL-6 in macrophages as well as of other cell types such as keratinocytes, fibroblasts, and synoviocytes. Fibroblast-like synoviocytes (FLS) located inside joints in the synovium also play a key role in pathogenesis of rheumatic diseases due to their production of proinflammatory cytokines, adhesion molecules, and matrix proteases contributing to cartilage destruction. Rheumatoid FLS develop a unique autoaggressive phenotype that increases invasiveness into the extracellular matrix, promotes inflammatory cell recruitment, and elevates production of COX-2. NSAIDs are widely used anti-inflammatory agents that act through the inhibition of the COX enzymes. Although COX-inhibitors lead to reduced synthesis of prostaglandins at the site of inflammation, suppression of gastrointestinal or renal prostaglandins synthesis is associated with mechanism-based toxicities. This limits the usefulness of these otherwise potent drugs. In addition, COX-2 inhibitors have been found to increase the risk of myocardial infarction. Thus, finding new agents which will specifically block inflammation may provide therapeutic opportunities in immune-mediated rheumatic diseases.

2. Overview on Epigenome-Influencing Drugs

Epigenetics is defined as reversible and heritable changes in gene function without alteration of the underlying DNA sequence itself [6]. Epigenetic mechanisms are sensitive to external stimuli, bridging the gap between environmental and genetic factors. In particular, monozygotic (MZ) twins do not show complete concordance for many complex diseases. MZ discordance rates for autoimmune diseases are 20–80 percent, indicating a substantial role of epigenetic factors in the development of these disorders [7, 8]. Indeed, it has been reported that epigenetic mechanisms mediate development of chronic inflammation by modulating the expression of proinflammatory cytokines including TNF-α, IL-6, and IL-1 and induction of COX-2 and transcription factor NF-κB. These molecules are constitutively produced by a variety of immune cells under chronic inflammatory conditions, which consequently leads to the development of many diseases including cancer, cardiovascular diseases, or autoimmune rheumatic disorders.

3. Noncoding RNA

Three main epigenetic mechanisms have been described including noncoding RNA species, DNA methylation, and histone modification. The first group of noncoding RNAs includes microRNA (miRNA) and long noncoding RNA (lncRNA). MicroRNAs (miRNAs) are endogenous, single-stranded RNAs of 19–25 nucleotides in length which can negatively regulate gene expression on posttranscriptional level. In particular, miRNA can hybridise to 3–8 nucleotides within 3′-untranslated region (3′UTR) of target messenger RNA (mRNA) referred to as "seed sequence" [9]. The formation of such miRNA-mRNA duplexes leads to mRNA degradation or translational repression. miRNAs have been studied extensively due to their role in regulation of almost every cellular process. It is known that miRNAs can act as a fine-tuner of gene expression and can negatively regulate approximately 30 percent of human protein-coding genes [10]. In addition, miRNAs are attractive as potential biomarkers. Some of miRNAs have been already tested in preclinical studies that aimed to treat cancer including lung, prostate, or leukemia [11]. Interestingly, randomised, phase IIa, double blind clinical trial (test number NCT01200420) has been conducted to treat hepatitis C virus (HCV) using locked nucleic acid inhibitor of miRNA-122 (Table 1). miRNA-122 is crucial for viral replication in hepatocytes; thus the reintroduction of miRNA-122 inhibitor significantly reduced virus replication [12, 13]. Recent phase I clinical trial has also tested the drug called MRX34. MRX34 is a double-stranded miRNA-34 encapsulated in liposomal nanoparticles. miRNA-34a represses the expression of more than 20 oncogenes which results in inhibition cancer cell viability, stemness, metastasis, or chemoresistance. Thus, MRX34 is widely tested in solid tumours and hematological malignancies [9]. Many studies have also shown the role of lncRNA in diverse cellular processes. lncRNAs are non-protein-coding transcripts longer than 200 nucleotides regulating gene expression. However, the exact functional roles and mechanisms of lncRNAs are still unclear.

Table 1: FDA-approved epigenetic drugs.

Drug	Epigenetic effect	Clinical trial
Miravirsen	Neutralisation of miRNA-122	Phase II [13]
MRX34	Ectopic expression of miRNA-34	Phase I [9]
Azacitidine (Vidaza)	DNA methyltransferase inhibitor	Phase III [15]
Decitabine (Dacogen)	DNA methyltransferase inhibitor	Phase III [17]
Vorinostat (Zolinza)	Pan-HDAC inhibitor	Phase II [24]

4. DNA Methylation

Another mechanism of epigenetic changes is DNA methylation induced by a highly conserved family of DNA methyltransferases (DNMTs). DNMT1 is the most abundant DNA methyltransferase in mammalian cells [14]. The insertion of methyl group to cytosine at the carbon 5 position leads to structural changes in chromatin and is mostly associated with gene silencing. In humans, methylation mainly occurs when cytosine is followed by guanine and is linked with phosphate called CpG islands. Approximately 1 percent of the genome consists of CpG islands [11]. Also, it is reported that roughly 60–70 percent of human genes are linked to promoter CpG islands which suggests that methylation of CpG island is an important regulatory mechanism of gene expression [12]. It has been shown that vitamin B12 rich diet (B vitamins acted as methyl donors) in agouti mouse model prevented from development of inflammation mediated diabetes and

cancer. In contrast, mice which did not receive vitamin B were predisposed for these diseases [14]. Therefore, methylation plays a key role in physiological conditions and the alteration in DNA methylation signature can have impact on disease development. One of the Food and Drug Administration-(FDA-) approved drugs inhibiting DNA methylation is 5′-azacytidine (commercial name Vidaza). This drug has been already used in phase III randomised, controlled trial to treat myelodysplastic syndrome and leukemia [15]. Similar inhibitory effect on DNA methyltransferases has the 5-aza-2′-deoxycytidine or 5′-AZA (known under commercial name Decitabine), which is used to treat many types of cancer and the myelodysplastic syndrome [16, 17]. DNA hydroxymethylation is also epigenetic modification mediated by Ten-Eleven Translocation (TET) family proteins which were discovered relatively recently [18]. TET enzymes are dioxygenases capable of oxidizing the methyl group of 5-methylcytosine (5mC) and converting 5mC into 5-hydroxymethylcytosine (5hmC), which results in DNA demethylation. It has been shown that the increased expression of TET1–TET3 enzymes in monocytes and TET2 in T cells leads to aberrant global DNA hydroxymethylation of early RA patients [19]. Interestingly, treatment with methotrexate partially reduces the DNA hydroxymethylation level. Indirect TET inhibition induced by AGI-5198 compound leads to growth suppression and promotes differentiation of glioma cells [20]. Similarly, HMS-101 inhibitor limits the growth of acute myeloid leukemia cells suggesting potential therapeutic application of TET inhibitors in cancer and also in rheumatic diseases [21].

5. Histone Modification

Another epigenetic phenomenon is histone modification. This modification alters the electrostatic charge of the histones resulting in conformational changes in protein binding sites and facilitating or blocking DNA accessibility. Histone modifications can be mostly represented by acetylation, methylation, phosphorylation, ubiquitination, ribosylation, citrullination, biotinylation, and sumoylation of histone N-terminal tail domains and also core domains [22]. It is believed that the histone acetylation is usually associated with increased binding of transcription factors to nucleosomal DNA and facilitates transcription initiation, whereas histone methylation can either activate or repress gene expression. Acetylation removes the positive charge on the histones and reduces the interaction between histones and negatively charged phosphate groups on DNA [23]. Therefore, the condensed heterochromatin is transformed into a more relaxed euchromatin that is associated with greater levels of gene transcription. Vorinostat (also known as suberanilohydroxamic acid, SAHA) has been shown to bind to the active site of histone deacetylases (HDACs). HDACs catalyse the removal of acetyl group from lysine residue. Inhibition of HDACs by Vorinostat leads to accumulation of hyperacetylated histones. This drug was the first histone deacetylase inhibitor to be approved by FDA (2006) to treat cutaneous T cell lymphoma with substantial response rates over 30 percent in patients [24]. Unlike in the cancer field, there is still no epigenetics-based drug on the market to treat rheumatic disorders. Finding new agents is greatly needed, because the economic burden of rheumatic diseases is substantial. Their cost is estimated at more than 200 billion Euros per year in Europe and they are the most expensive of all diseases for the European health care systems [25]. This review highlights the impact of chronic inflammation and immune disability on globally disturbed DNA methylation pattern, aberrant histone modification' profile, and divergent miRNA signature that is observed in autoimmune rheumatic diseases. However, it is a chicken or egg dilemma and it needs to be further investigated to find out whether inflammation triggers epigenetic changes or epigenetic alteration drives inflammation. Epigenome-influencing drugs may have future impact on diagnosis and/or therapeutics of rheumatic diseases. Epigenetic mechanisms, which modify immune cells and fibroblasts in rheumatic diseases, are depicted in Figure 1.

6. Rheumatoid Arthritis

Rheumatoid arthritis (RA) is the most common inflammatory joint disease which affects approximately 1% of the population worldwide with unknown etiology. RA is a chronic autoimmune inflammatory condition which is characterised by an influx of inflammatory cells from the blood stream into the synovial membrane or synovial fluid. Such influx of immune cells producing inflammatory cytokines results in progressive erosion of articular cartilage. Phagocytes, B cells, and T cells are the most prominent cells in the rheumatoid synovium. Macrophages along with granulocytes are an important source of proinflammatory cytokines, chemokines, and reactive oxygen species (ROS) that accompany inflammatory processes [4]. On the other hand, antigen-specific B cells are involved in autoantigens presentation to T cells and in production of autoantibodies, which mediates in joint destruction. In addition, the presence of ectopic follicular structures in chronically inflamed tissues resembling germinal centres provides strong evidence of ongoing immune reactions [26]. Recent studies have indicated that miRNA plays a critical role in pathogenesis of RA. Raj and Mufti showed that miRNA-346 regulates TNF-α synthesis (one of the major proinflammatory cytokines involved in the pathogenesis of RA) in LPS stimulated synovial fibroblasts [15]. The level of miRNA-146a is significantly upregulated in CD4+ T cell subset and positively correlates with TNF-α concentration in RA patients [16, 17]. Also, the level of miRNA-150 is elevated in IL-17 producing T cells [27]. In contrast, Zhang et al. reported that miRNA-23b inhibits IL-17-associated autoimmune inflammation by targeting TGF-β binding protein 2 (TAB2) and TAB3 [18]. Emerging evidence revealed that lncRNAs have various expression in autoimmune diseases. It has been shown that adalimumab (anti-TNF-α antibody) and tocilizumab (anti-IL-6R antibody) treated RA patients have differential expression of 85 lncRNAs in CD14+ monocytes [28]. Similarly, Song et al. have shown elevated expression level of lncRNAs called Hotair in PBMC and serum exosome, suggesting that lncRNAs could be used as potential biomarkers for diagnosing RA [29], while overexpression of Hotair by introduction of lentiviral construct results in decreased expression of MMP-2 and

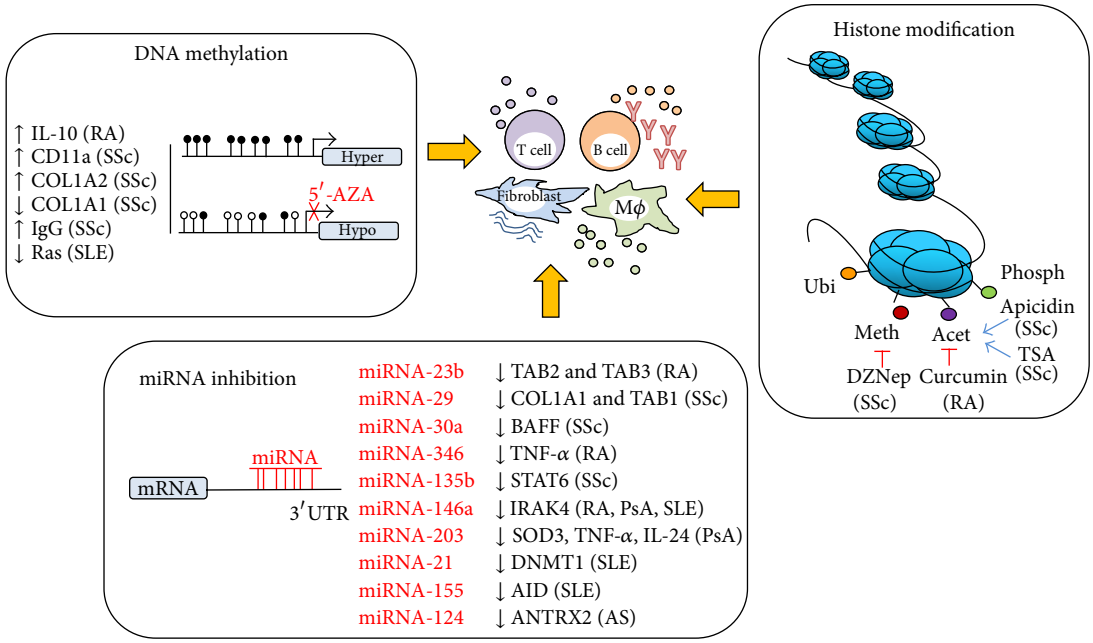


Figure 1: Epigenetic agents modulating immune response in rheumatic diseases including RA, SSc, SLE, AS, and PsA. Schematic of the epigenetic modulations represented by DNA methylation, histone modification, and RNA interference influencing immune cells (B cells, T cells, and monocytes) and fibroblasts. *DNA methylation* refers to covalent addition of a methyl group to the 5-position of the cytosine ring, which can be inhibited by 5′-AZA. 5′-AZA induces DNA hypomethylation and drives differential gene expression. *Histone modifications* are reversible and site-specific histone alterations including acetylation (Acet), methylation (Meth), phosphorylation (Phosph), or ubiquitination (Ubi). Histone methylation or acetylation can be either activated by apicidin and TSA or inhibited by DZNep and curcumin. *miRNA inhibition* is a formation of miRNA-mRNA duplexes in the position of 3′UTR. This leads gene silencing (genes in black) by specific miRNAs (in red).

MMP-13 in FLS from RA patients. DNA methylation pattern is also impaired in RA affecting immune-related genes and consequently influencing immune responses. In particular, global DNA hypomethylation is observed in peripheral blood mononuclear cells (PBMCs) derived from RA patients [30]. It has been shown that hypomethylated promoter region of chemokine CXCL12 leads to increased MMPs expression and joint destruction in RA patients [31]. Similarly, the methylation levels of IL-6 promoter in PBMCs was significantly lower in RA patients than those in controls [32]. In contrast, gene coding for dual specific phosphate 22 (DUSP22) is hypermethylated in T cells of RA and in Sjogren's syndrome patients [33, 34]. DUSP22 is a tyrosine phosphatase which negatively regulates the IL-6 transcription factor STAT3. It is known that IL-6 plays a pivotal role in chronic inflammation in autoimmune diseases [35]. Another group has identified that the promoter of anti-inflammatory cytokine IL-10 is hypermethylated in four different regions of CpG site [36]. The level of IL-10 which is mainly produced by monocytes and T reg cells is reduced in RA. It has been shown that PBMCs treated with 5′-AZA have increased production of IL-10. This suggests that specific demethylation of IL-10 promoter induced by 5′-AZA can prevent development of RA by induction of anti-inflammatory IL-10 and suppression of immune responses. It has been also found that alteration in histone modification can contribute to RA development. The balance of histone acetylase (HAT) versus HDAC is strongly shifted towards chronic histone hyperacetylation in RA patients [37]. This consequently leads to proinflammatory genes expression including IL-6 and IL-8. Indeed, the level of histone H3 acetylation in the IL-6 promoter is significantly elevated in RA synoviocytes resulting in enhanced IL-6 secretion and joint destruction [38]. Surprisingly, treatment with curcumin abrogated H3 acetylation and reduced IL-6 secretion which suggests that epigenetic mechanisms are implicated in targeting RA pathogenesis.

7. Systemic Sclerosis

Systemic sclerosis (SSc) is an autoimmune connective tissue disease characterised by autoimmunity, vascular abnormalities, and fibrosis via accumulation of extracellular matrix (ECM) proteins. Uncontrolled fibrosis progression often results in dysfunction of the affected organs and consequently leads to premature death in SSc patients [39]. The role of miRNA has also been demonstrated in SSc pathogenesis. It has been shown that the family of miRNA-29 plays a pivotal role in SSc skin fibrosis by targeting collagen expression [19, 20]. In addition, we have shown that SSc fibroblasts are able to reverse fibrotic phenotype following miRNA-29 transfection. We found that miRNA-29 can modulate its novel target gene, TAB1, and that it downregulates tissue inhibitor of metalloproteinases-1 (TIMP-1) expression as a result of TAB1 degradation [21]. Similarly, transfection of miRNA-30a-3p in IFN-γ-activated SSc fibroblasts decreases synthesis of B cell-activating factor (BAFF) [40]. BAFF plays a central

role in the survival and homeostasis of B cells and plasma cells. Autoreactive B cells are strongly dependent on BAFF presence and the increased level of BAFF correlates with high autoantibody titers and with disease activity in SSc. Therefore, miRNA-30 targeting BAFF expression could be used in SSc therapy. In addition, 5′-AZA-treated SSc T cell has shown increased CD11a expression, whereas 5′-AZA-treated SSc T cells cocultured with either B cells or fibroblasts resulted in increased production of IgG or COL1A2, respectively [41]. These data suggest that demethylation of CD11a regulatory elements and subsequent CD11a overexpression in T cells may mediate immunological abnormalities and fibrotic processes in SSc. Therapies which reduce CD11a due to specific DNA methylation are needed in SSc. On the contrary, our latest results have shown that 5′-AZA-treated fibroblasts decreased expression of collagen and upregulated the miRNA-135b expression level. miRNA-135b targets STAT6 and attenuates the IL-13-induced collagen expression. This indicates that specific targeting DNA methylation may represent a novel therapeutic approach for the treatment of SSc.

Another hallmark of SSc is perivascular infiltration of immune cells, mainly monocytes, which are the first immune cells to infiltrate the SSc skin. The results from our group demonstrated that circulating monocytes from SSc patients contribute to the imbalance between TIMP-1 and MMPs and to increased profibrotic IL-6 production upon TLR8 agonist stimulation (ssRNA) [42–44]. Interestingly, we have also shown that epigenetic modification induced by DZNep (histone methyltransferases) or apicidin (inhibitor of histone acetylases) in SSc monocytes can modulate TIMP-1 expression and subsequently fibroblasts transdifferentiation [44]. Another study has shown that global H4 but not H3 acetylation of SSc B cells was positively correlated with disease activity and that the expression of HDAC2 protein was negatively correlated with skin thickness [45]. This clearly indicates that epigenetic alteration plays an important role in the pathogenesis of SSc.

8. Psoriatic Arthritis

Psoriatic Arthritis (PsA) is a chronic inflammatory skin disease with unknown etiology. The interactions between genetics and the environmental factors in PsA are still not well defined. The disease is characterised by abnormal proliferation and differentiation of keratinocytes. In addition, infiltration of immune cells which secrete high level of various immune-regulated inflammatory cytokines and chemokines is observed in PsA. Recently, imbalance in epigenetic networks has been indicated to be an important element in psoriasis development. Several studies have shown that differentially expressed miRNAs levels play a role in psoriasis pathogenesis. In particular, it has been reported that miRNA-203 expression is downregulated in psoriatic lesion. Based on bioinformatic analysis, miRNA-203 targets gene suppressors of cytokine signalling 3 (SOC3). SOC3 is involved in negative regulation of the IL-6 transcription factor STAT3. Furthermore, miRNA-203 directly targets TNF-α and proinflammatory IL-24 in primary keratinocytes [46]. Another group identified that miRNA-146a is also dysregulated in psoriatic lesions. miRNA-146a targets the TNF receptor-associated factor 6 (TRAF6) and the IL-1 receptor-associated kinase 1 (IRAK1). Activation of IRAK1 triggers the production of TNF-α, IL-6, IL-8, and IL-1β. Xia et al. also found that the increased level of miRNA-146a is positively correlated with the Psoriasis Symptom Inventory (PSI) score [47]. In contrast, the addition of anti-TNF-α blocking antibody reduced the level of miRNA-146a in patients' serum. These data suggest that overexpression of miRNA-203 and miRNA-146a may be useful in repression of the immune-mediated inflammation process and may provide potential therapeutic strategy in psoriasis pathogenesis. Another study reported that the DNA methylation pattern is changed in psoriatic skin in comparison to normal tissue. They showed strong correlation between S100 Calcium Binding Protein A9 (S100A9) and DNA methylation signature of psoriasis patient samples fallowing phototherapy [48]. S100A9 is a calcium binding protein which plays a prominent role in regulation of inflammatory processes and immune response. Also, Gervin et al. have demonstrated that monozygotic twins (MT) have a different methylation pattern between an unaffected twin and a twin suffering for PsA [8]. They showed the differences in DNA methylation pattern of proinflammatory TNF-α ligand 11 also known as the receptor activator of nuclear factor kappa-B ligand (RANKL) in MZ twins. Moreover, DNA methylation signature of the arachidonate 5-lipoxygenase-activating protein (ALOX5AP) gene is altered in psoriatic MZ twin. ALOX5AP is involved in catalysis of arachidonic acid regulating inflammation via leukotrienes production. Another study has shown that 50% of CpG islands in the promoter region of p16 gene are hypermethylated in psoriatic epidermis and correlated with diseases activity [49]. p16 is an antiapoptotic protein that supports the concept of an abnormal mechanism of hyperproliferative skin diseases. Abnormal expression of HATs and HDACs regulating gene expression has been also observed in PsA. Indeed, Ham et al. have shown that the promoter region of HDAC-6 is hypermethylated in naive CD4+ T cells in patients. Furthermore, the level of HDAC-1 in skin samples and PBMCs from PsA patients is increased compared to healthy subjects [50, 51]. These findings implicate that novel therapy for PsA should be also supplemented with agents altering the abnormal histone modification pattern.

9. Systemic Lupus Erythematosus

The etiology of Systemic Lupus Erythematosus (SLE) remains to be elucidated; however it is an autoimmune disorder with clear links to the innate and adaptive immune systems. Environmental triggers may initiate the disease on a genetic susceptibility background. SLE is a multiorgan disorder in which there are autoantibodies to DNA that are not only diagnostic of the disease but also pivotal in disease pathogenesis.

It was as early as 1990 that methylation abnormalities were first described in SLE T cells. The most convincing evidence comes from the fact that procainamide and 5′-AZA (both hypomethylating drugs) treated CD4+ T cells cause a lupus-like disease in mice [52]. This suggests a critical role of hypomethylation in T cells in mediating SLE [52]. It was

suggested that decreased Ras signalling is involved also in DNA hypomethylation in T cells [53]. Other studies have suggested that growth arrest and DNA damage-induced gene 45α (GADD45α) are associated with DNA hypomethylation in SLE [54]. In further support of this it was demonstrated that increased oxidative stress in T cell in SLE may alter the expression of various proteins but also force downregulation of DNMT1 expression and thus hypomethylation [55]. Indeed, adoptive transfer of T cells modified by oxidative stress into syngeneic mice resulted in lupus-like disease with reduced methylation [56]. A study found that isolated T cells from SLE patients were globally hypomethylated. The genes methylated include CD11a and CD70 [57]. CD11a of course forms lymphocyte function-associated antigen 1 and would be important in immune responses. The X chromosome in women with lupus is hypomethylated suggesting a reason for the preponderance in females [58]. A large scale genomewide DNA methylation study in isolated CD4+ T cells from lupus patients found 236 hypomethylated CG sites. Enrichment of genes associated with apoptosis was found. A further study of DNA methylation in isolated T cells found that a large amount of interferon regulated genes is methylated differently. The authors suggest that there is an epigenetic alteration of interferon genes which explains the interferon response in SLE [59]. Similarly, it has been found that interferon regulated genes in CD4+ T cells of SLE patients that had quiescent disease are hypomethylated, which suggests that they are poised "to trigger" [60]. Recently, a study of SLE patients has demonstrated that altered DNA methylation pattern of interferon genes is associated with production of autoantibodies characterising SLE [61]. As well as T cells playing a critical role, B cells have also been described to be involved in SLE pathogenesis. Indeed, hypomethylation of SLE B cells has been described and blocking IL-6 with a monoclonal antibody restores B cell methylation levels. These data suggest that IL-6 is driving B cell alterations [62]. Interestingly, miRNA-155 targets Activation Induced Cytidine Deaminase (AICDA) which is critical in B cell development. It has been demonstrated that AICDA is dysregulated in SLE. miRNA-29b appears to be overexpressed in SLE CD4+ T cells and indirectly regulates hypomethylation by targeting DNMT1. DMNT1 is an enzyme which is important in DNA methylation [63]. The important negative regulators of TLR signalling are miRNA-146a and miRNA-29 which are dysregulated in SLE [64, 65]. It was also further shown that miRNA-21 additionally targets DNMT1 in SLE CD4+ T cells [66]. Interestingly, the authors demonstrated that targets of miRNA-146 included the interferon regulatory factor 5 and STAT1, a downstream target of IFN activation, and these are indeed dysregulated in SLE. A great study demonstrated that miRNA-3148 affects the stability and regulation of TLR7 [67]. TLR7 is the receptor for RNA and this is clearly important in SLE and is highly expressed on dendritic cells and may link RNA, TLR, and miRNAs together. In T cells miRNA-31 has also been found to be dysregulated in T cells and this dysregulation is associated with the impaired production of IL-2, a critical T cell growth factor [68]. It is clear that a multitude of miRNAs are dysregulated in SLE; therefore cell-free miRNAs have recently emerged as noninvasive biomarkers [69]. miRNA-146 and miRNA-155 have been found to be a possible biomarker in SLE derived from urinary sediment [69]. Abnormal histone modifications have also been described in SLE. It has been reported that in SLE patients there is a global histone hypoacetylation due to downregulation of Ezh2 enzyme. Ezh2 is involved in histone methylation [70]. The epigenetic modifying enzyme Ezh2 has been also found to be downregulated in the SLE T cells and this is one of the enzymes that methylate the histones [70]. Ezh2 has been also shown to be regulating the expression of the transcription factor STAT5 to epigenetically repress the immunoglobulin K chain complex, critical in B cell lineages [71]. Using the lupus-prone mouse model, it was found in the isolated T cells from this model that the HDACs were dysregulated suggestive of the mechanism of altered histone methylation [72]. Histone H3 trimethylation has also been described to be altered in SLE [73]. The major histone modifications which are implicated in SLE include methylation and acetylation and both are reversible. It has been shown in the lupus mouse model that introduction of TSA (a broad spectrum HDAC inhibitor) reduced IL-6 level and proteinuria [74]. CD70 is also elevated on CD4+ T cells from SLE patients and associated with higher dimethylated H3 lysine 4 in these patients [75].

10. Sjogren's Syndrome

Sjogren's syndrome (SS) is an autoimmune disorder that affects the lacrimal and salivary glands, causing hypofunction which leads to dry eyes and dry mouth (xerostomia). There are a large prominent lymphocytic infiltrate in the salivary glands and also specific autoantibodies in the disease too. Patients with SS have a 20–40-fold increased risk of developing lymphoma. There are both associations with the innate and adaptive immune systems. Because the innate immune system has been heavily implicated in disease pathogenesis many studies have focussed on this system. One of the first miRNAs that has been shown to be dysregulated is miRNA-146a [76]. miRNA-146a has been found to be elevated in PBMCs of SS patients; however, the precise cell type of the PBMCs has not been confirmed [76, 77]. More importantly, Pauley et al. have shown that enhanced miRNA-16a levels in monocytes lead to increased phagocytosis in functional assays. This could represent a mechanism to help restore the altered phagocytosis seen in the disease. A follow-up study confirmed the increased miRNA-146a levels and the decreased target gene IRAK1 levels in PBMCs [78]. Using the minor salivary gland and whole miRNA arrays, a number of differentially regulated miRNAs in salivary glands from SS patients was found; however, their targets and the functional consequences of the differentially expressed miRNAs are still unknown [79]. A very interesting recent study showed that the SS related antigen B promotes global miRNA processing [80].

It has been recently shown that salivary gland epithelial cells are globally hypomethylated compared to controls [81]. A very interesting observation was that the global gland epithelial cells hypomethylation may be attributed to B cells as treatment with the B cell depleting antibody rituximab

had more methylation. In isolated T cells from SS patients it has been found that they have lower FoxP3 expression levels, both mRNA and protein levels, and that this is associated with hypermethylation of the promoter region [82]. This observation could explain the reduced number of T reg cells in SS. A recent genomewide methylation study in isolated CD4+ T helper cells identified a multitude of genes that are differentially regulated in SS. The interferon pathways genes STAT1, IFI44L, and USP18 are all hypomethylated [83].

11. Ankylosing Spondylitis

Ankylosing spondylitis (AS) is a chronic and common inflammatory rheumatic disease characterised by new bone formation, ankyloses, and inflammation of the hips and spine. miRNA-16 and miRNA-221 are aberrantly expressed in T cells [84]. A functional SNP variant in miRNA-196a was found to be associated with Behcet's disease but not AS [85]. A recent study in AS demonstrated that miRNA-124 is elevated in peripheral blood cell of AS patients. miRNA-124 targets Anthrax Toxin Receptor 2 (ANTXR2) which is associated with risk of AS development [86]. Interestingly, inhibition of ANTXR2 by miR-mimics *in vitro* caused autophagy and subsequently protects T cells from apoptosis conferring advantage. Niu et al. have looked at common polymorphism in miRNA-146a associated with AS. However, no polymorphism was associated [87]. In AS, T cells are proposed to play a role; however, their precise role is unclear. It was shown that FoxP3 positive T cells are elevated in the inflamed joint in AS and that the FoxP3 locus is demethylated. This suggests that epigenetic mechanisms control FoxP3 expression [88]. Recently, in serum it was found that there were higher levels of SOCS1 methylation as compared to healthy controls and higher levels of SOCS methylation associated with higher IL-6 levels [89]. SOCS1 is the negative regulator of STAT1 signalling which is initiated after IL-6 stimulation; thus a reduction in the negative regulator of STAT1 would lead to unperturbed STAT1 signalling.

Reduced HDAC and HAT activities have been described in AS in PBMCs compared to controls [90]. The functional relevance of this is unknown because the balance between these two enzymes was not different. Furthermore, after anti-TNF-α therapy HAT activity increased in AS patients, with a clear increase in the HAT/HDAC balance [91]. Only one manuscript has demonstrated the altered histone methylation in CD4+ T cells in AS. In particular, it has been shown that specific AS SNP genotype has an altered histone modification profile and possibly alters binding to important transcription factors critical in the disease [92].

12. Giant Cell Arteritis

Giant Cell Arteritis (GCA) is a systemic autoimmune disease primarily affecting the elderly. It is characterised by inflammation of the large- and medium-sized arteries. GCA typically affects the temporal arteries. One of the most devastating features of the disease can be acute visual loss and patients can be present with ischaemic complications of the disease. One study found 853 hypomethylated genes in temporal arteries from GCA patients compared to controls. Many of these hypomethylated genes were associated with both Th1 and Th17 cells [93]. DNA methylation was also found to be altered in nuclear factor of activated T cells (NFAT), which was confirmed to be altered by immunohistochemistry [93]. NFAT is a critical factor mediating production of proinflammatory cytokines including IL-23. Thus, methylation regulation of NFAT may be crucial in driving the activation of Th17 cells in GCA. Only one report of dysregulated miRNA has been published in GCA and this study found that miRNA-21 was dysregulated in GCA temporal biopsies [94]. miRNA-21 was overexpressed in the biopsies and this appears to be tissue specific, as the use of PBMCs derived from the same donors when compared across groups demonstrated no difference. These data suggest that the increase of miRNA-21 level is tissue specific [94]. To this day only, a handful of studies have looked at the epigenome in GCA and this is a rich area for research. Epigenetics could underpin the variable clinical course of the disease.

13. Conclusions

It is now clear that in all the autoimmune rheumatic diseases there are various epigenetic aberrations (Figure 1). Each specific disease is likely to have its own epigenetic signature; for example, RA appears to be hypomethylated whereas in SSc the fibroblasts, at least, appear hypermethylated. Thus, different approaches to treatment will be warranted. It is likely that in the hypermethylated state in SSc the use of decitabine may be useful but in RA where the fibroblasts are already hypermethylated this would exacerbate the situation. Histone modifications are also likely to differ in different diseases and any drugs that target specific histone modifications must be used with knowledge of the precise histone modifications occurring in that particular setting. This will be critical in the treatment regime. Noncoding RNAs like miRNAs are now emerging as excellent druggable targets; however, issues regarding their stability and targeting *in vivo* still remain unclear. How do we get the right miR-mimic or antagomiR to the precise tissue? This is an active area of research and it is already bearing fruit with the use of aptamers. Epigenetic therapies are now coming to the fore and the use of the first miRNA therapy in HCV appears to be a success.

Competing Interests

There are no competing interests.

Authors' Contributions

Marzena Ciechomska and Steven O'Reilly wrote the paper. Marzena Ciechomska takes the responsibility for the integrity of the paper.

Acknowledgments

This work was supported by 2015/16/S/NZ6/00041 grant from the National Science Centre (Poland) (Marzena Ciechomska).

References

[1] G. S. Alarcon, G. V. Williams, J. Z. Singer et al., "Early undifferentiated connective tissue disease. I. Early clinical manifestation in a large cohort of patients with undifferentiated connective tissue diseases compared with cohorts of well established connective tissue disease," *The Journal of Rheumatology*, vol. 18, no. 9, pp. 1332–1339, 1991.

[2] G. Arango Duque and A. Descoteaux, "Macrophage cytokines: involvement in immunity and infectious diseases," *Frontiers in Immunology*, vol. 5, article 491, 2014.

[3] Y. Lu and L. M. Wahl, "Oxidative stress augments the production of matrix metalloproteinase-1, cyclooxygenase-2, and prostaglandin E2 through enhancement of NF-κB activity in lipopolysaccharide-activated human primary monocytes," *Journal of Immunology*, vol. 175, no. 8, pp. 5423–5429, 2005.

[4] A. Aggarwal, "Role of autoantibody testing," *Best Practice & Research Clinical Rheumatology*, vol. 28, no. 6, pp. 907–920, 2014.

[5] N. Qu, M. Xu, I. Mizoguchi et al., "Pivotal roles of T-helper 17-related cytokines, IL-17, IL-22, and IL-23, in inflammatory diseases," *Clinical and Developmental Immunology*, vol. 2013, Article ID 968549, 13 pages, 2013.

[6] A. P. Feinberg, "Phenotypic plasticity and the epigenetics of human disease," *Nature*, vol. 447, no. 7143, pp. 433–440, 2007.

[7] M. Wahren-Herlenius and T. Dörner, "Immunopathogenic mechanisms of systemic autoimmune disease," *The Lancet*, vol. 382, no. 9894, pp. 819–831, 2013.

[8] K. Gervin, M. D. Vigeland, M. Mattingsdal et al., "DNA methylation and gene expression changes in monozygotic twins discordant for psoriasis: identification of epigenetically dysregulated genes," *PLoS Genetics*, vol. 8, no. 1, Article ID e1002454, 2012.

[9] B. D. Adams, C. Parsons, and F. J. Slack, "The tumor-suppressive and potential therapeutic functions of miR-34a in epithelial carcinomas," *Expert Opinion on Therapeutic Targets*, 2015.

[10] J. Lu and A. G. Clark, "Impact of microRNA regulation on variation in human gene expression," *Genome Research*, vol. 22, no. 7, pp. 1243–1254, 2012.

[11] X.-M. Hua, J. Wang, D.-M. Qian et al., "DNA methylation level of promoter region of activating transcription factor 5 in glioma," *Journal of Zhejiang University Science B*, vol. 16, no. 9, pp. 757–762, 2015.

[12] M. Weber, I. Hellmann, M. B. Stadler et al., "Distribution, silencing potential and evolutionary impact of promoter DNA methylation in the human genome," *Nature Genetics*, vol. 39, no. 4, pp. 457–466, 2007.

[13] H. L. A. Janssen, H. W. Reesink, E. J. Lawitz et al., "Treatment of HCV infection by targeting microRNA," *The New England Journal of Medicine*, vol. 368, no. 18, pp. 1685–1694, 2013.

[14] D. C. Dolinoy, "The agouti mouse model: an epigenetic biosensor for nutritional and environmental alterations on the fetal epigenome," *Nutrition Reviews*, vol. 66, supplement 1, pp. S7–S11, 2008.

[15] K. Raj and G. J. Mufti, "Azacytidine (Vidaza^5) in the treatment of myelodysplastic syndromes," *Therapeutics and Clinical Risk Management*, vol. 2, no. 4, pp. 377–388, 2006.

[16] D. Wu, X. Du, J. Jin et al., "Decitabine for treatment of myelodysplastic syndromes in Chinese patients: an open-label, phase-3b study," *Advances in Therapy*, vol. 32, no. 11, pp. 1140–1159, 2015.

[17] M. Lübbert, S. Suciu, A. Hagemeijer et al., "Decitabine improves progression-free survival in older high-risk MDS patients with multiple autosomal monosomies: results of a subgroup analysis of the randomized phase III study 06011 of the EORTC Leukemia Cooperative Group and German MDS Study Group," *Annals of Hematology*, vol. 95, no. 2, pp. 191–199, 2016.

[18] H. Zhang, X. Zhang, E. Clark, M. Mulcahey, S. Huang, and Y. G. Shi, "TET1 is a DNA-binding protein that modulates DNA methylation and gene transcription via hydroxylation of 5-methylcytosine," *Cell Research*, vol. 20, no. 12, pp. 1390–1393, 2010.

[19] M. C. de Andres, E. Perez-Pampin, M. Calaza et al., "Assessment of global DNA methylation in peripheral blood cell subpopulations of early rheumatoid arthritis before and after methotrexate," *Arthritis Research and Therapy*, vol. 17, no. 1, article 233, 2015.

[20] D. Rohle, J. Popovici-Muller, N. Palaskas et al., "An inhibitor of mutant IDH1 delays growth and promotes differentiation of glioma cells," *Science*, vol. 340, no. 6132, pp. 626–630, 2013.

[21] A. Chaturvedi, M. M. Araujo Cruz, N. Jyotsana et al., "Mutant IDH1 promotes leukemogenesis in vivo and can be specifically targeted in human AML," *Blood*, vol. 122, no. 16, pp. 2877–2887, 2013.

[22] E. L. Mersfelder and M. R. Parthun, "The tale beyond the tail: histone core domain modifications and the regulation of chromatin structure," *Nucleic Acids Research*, vol. 34, no. 9, pp. 2653–2662, 2006.

[23] A. J. Bannister and T. Kouzarides, "Regulation of chromatin by histone modifications," *Cell Research*, vol. 21, no. 3, pp. 381–395, 2011.

[24] M. B. Wozniak, R. Villuendas, J. R. Bischoff et al., "Vorinostat interferes with the signaling transduction pathway of T-cell receptor and synergizes with phosphoinositide-3 kinase inhibitors in cutaneous T-cell lymphoma," *Haematologica*, vol. 95, no. 4, pp. 613–621, 2010.

[25] "*Horizon 2020 Framework Programme EULAR's position and recommendations*," http://www.eular.org/myUploadData/files/EU_Horizon_2020_EULAR_position_paper.pdf.

[26] F. Humby, M. Bombardieri, A. Manzo et al., "Ectopic lymphoid structures support ongoing production of class-switched autoantibodies in rheumatoid synovium," *PLoS Medicine*, vol. 6, article e1, 2009.

[27] T. Niimoto, T. Nakasa, M. Ishikawa et al., "MicroRNA-146a expresses in interleukin-17 producing T cells in rheumatoid arthritis patients," *BMC Musculoskeletal Disorders*, vol. 11, article 209, 2010.

[28] N. Müller, F. Döring, M. Klapper et al., "Interleukin-6 and Tumour Necrosis Factor-α differentially regulate lincRNA transcripts in cells of the innate immune system in vivo in human subjects with rheumatoid arthritis," *Cytokine*, vol. 68, no. 1, pp. 65–68, 2014.

[29] J. Song, D. Kim, J. Han, Y. Kim, M. Lee, and E.-J. Jin, "PBMC and exosome-derived Hotair is a critical regulator and potent marker for rheumatoid arthritis," *Clinical and Experimental Medicine*, vol. 15, no. 1, pp. 121–126, 2014.

[30] C.-C. Liu, T.-J. Fang, T.-T. Ou et al., "Global DNA methylation, DNMT1, and MBD2 in patients with rheumatoid arthritis," *Immunology Letters*, vol. 135, no. 1-2, pp. 96–99, 2011.

[31] E. Karouzakis, Y. Rengel, A. Jüngel et al., "DNA methylation regulates the expression of CXCL12 in rheumatoid arthritis synovial fibroblasts," *Genes and Immunity*, vol. 12, no. 8, pp. 643–652, 2011.

[32] K. Ishida, T. Kobayashi, S. Ito et al., "Interleukin-6 gene promoter methylation in rheumatoid arthritis and chronic periodontitis," *Journal of Periodontology*, vol. 83, no. 7, pp. 917–925, 2012.

[33] J. R. Glossop, R. D. Emes, N. B. Nixon et al., "Genome-wide DNA methylation profiling in rheumatoid arthritis identifies disease-associated methylation changes that are distinct to individual T- and B-lymphocyte populations," *Epigenetics*, vol. 9, no. 9, pp. 1228–1237, 2014.

[34] N. Altorok, P. Coit, T. Hughes et al., "Genome-wide DNA methylation patterns in naive $CD4^+$ t cells from patients with primary Sjögren's syndrome," *Arthritis and Rheumatology*, vol. 66, no. 3, pp. 731–739, 2014.

[35] S. O'Reilly, R. Cant, M. Ciechomska, and J. M. van Laar, "Interleukin-6: a new therapeutic target in systemic sclerosis?" *Clinical & Translational Immunology*, vol. 2, no. 4, p. e4, 2013.

[36] L.-H. Fu, C.-L. Ma, B. Cong, S.-J. Li, H.-Y. Chen, and J.-G. Zhang, "Hypomethylation of proximal CpG motif of interleukin-10 promoter regulates its expression in human rheumatoid arthritis," *Acta Pharmacologica Sinica*, vol. 32, no. 11, pp. 1373–1380, 2011.

[37] L. C. Huber, M. Brock, H. Hemmatazad et al., "Histone deacetylase/acetylase activity in total synovial tissue derived from rheumatoid arthritis and osteoarthritis patients," *Arthritis and Rheumatism*, vol. 56, no. 4, pp. 1087–1093, 2007.

[38] T. T. Wada, Y. Araki, K. Sato et al., "Aberrant histone acetylation contributes to elevated interleukin-6 production in rheumatoid arthritis synovial fibroblasts," *Biochemical and Biophysical Research Communications*, vol. 444, no. 4, pp. 682–686, 2014.

[39] M. R. York, T. Nagai, A. J. Mangini, R. Lemaire, J. M. Van Seventer, and R. Lafyatis, "A macrophage marker, siglec-1, is increased on circulating monocytes in patients with systemic sclerosis and induced by type I interferons and toll-like receptor agonists," *Arthritis and Rheumatism*, vol. 56, no. 3, pp. 1010–1020, 2007.

[40] G. Alsaleh, A. François, L. Philippe et al., "MiR-30a-3p negatively regulates BAFF synthesis in systemic sclerosis and rheumatoid arthritis fibroblasts," *PLoS ONE*, vol. 9, no. 10, Article ID e111266, 2014.

[41] Y. Wang, Y. Shu, Y. Xiao et al., "Hypomethylation and overexpression of ITGAL (CD11a) in $CD4^+$ T cells in systemic sclerosis," *Clinical Epigenetics*, vol. 6, no. 1, article 25, 2014.

[42] M. Ciechomska, C. A. Huigens, T. Hügle et al., "Toll-like receptor-mediated, enhanced production of profibrotic TIMP-1 in monocytes from patients with systemic sclerosis: role of serum factors," *Annals of the Rheumatic Diseases*, vol. 72, no. 8, pp. 1382–1389, 2013.

[43] S. O'Reilly, R. Cant, M. Ciechomska et al., "Serum amyloid A induces interleukin-6 in dermal fibroblasts via Toll-like receptor 2, interleukin-1 receptor-associated kinase 4 and nuclear factor-κB," *Immunology*, vol. 143, no. 3, pp. 331–340, 2014.

[44] M. Ciechomska, S. O'Reilly, S. Przyborski, F. Oakley, K. Bogunia-Kubik, and J. M. van Laar, "Histone demethylation and toll-like receptor 8-dependent cross-talk in monocytes promotes transdifferentiation of fibroblasts in systemic sclerosis via fra-2," *Arthritis & Rheumatology*, vol. 68, no. 6, pp. 1493–1504, 2016.

[45] Y. Wang, Y. Yang, Y. Luo et al., "Aberrant histone modification in peripheral blood B cells from patients with systemic sclerosis," *Clinical Immunology*, vol. 149, no. 1, pp. 46–54, 2013.

[46] M. N. Primo, R. O. Bak, B. Schibler, and J. G. Mikkelsen, "Regulation of pro-inflammatory cytokines TNFα and IL24 by microRNA-203 in primary keratinocytes," *Cytokine*, vol. 60, no. 3, pp. 741–748, 2012.

[47] P. Xia, X. Fang, Z.-H. Zhang et al., "Dysregulation of miRNA146a versus IRAK1 induces IL-17 persistence in the psoriatic skin lesions," *Immunology Letters*, vol. 148, no. 2, pp. 151–162, 2012.

[48] X. Gu, E. Nylander, P. J. Coates, R. Fahraeus, and K. Nylander, "Correlation between reversal of DNA methylation and clinical symptoms in psoriatic epidermis following narrow-band UVB phototherapy," *Journal of Investigative Dermatology*, vol. 135, no. 8, pp. 2077–2083, 2015.

[49] M. Chen, Z.-Q. Chen, P.-G. Cui et al., "The methylation pattern of p16INK4a gene promoter in psoriatic epidermis and its clinical significance," *British Journal of Dermatology*, vol. 158, no. 5, pp. 987–993, 2008.

[50] L. E. Tovar-Castillo, J. C. Cancino-Díaz, F. García-Vázquez et al., "Under-expression of VHL and over-expression of HDAC-1, HIF-1α, LL-37, and IAP-2 in affected skin biopsies of patients with psoriasis," *International Journal of Dermatology*, vol. 46, no. 3, pp. 239–246, 2007.

[51] P. Zhang, Y. Su, M. Zhao, W. Huang, and Q. Lu, "Abnormal histone modifications in PBMCs from patients with psoriasis vulgaris," *European Journal of Dermatology*, vol. 21, no. 4, pp. 552–557, 2011.

[52] J. Quddus, K. J. Johnson, J. Gavalchin et al., "Treating activated CD4+ T cells with either of two distinct DNA methyltransferase inhibitors, 5-azacytidine or procainamide, is sufficient to cause a lupus-like disease in syngeneic mice," *Journal of Clinical Investigation*, vol. 92, no. 1, pp. 38–53, 1993.

[53] C. Deng, M. J. Kaplan, J. Yang et al., "Decreased ras-mitogen-activated protein kinase signaling may cause DNA hypomethylation in T lymphocytes from lupus patients," *Arthritis and Rheumatism*, vol. 44, no. 2, pp. 397–407, 2001.

[54] Y. Li, M. Zhao, H. Yin et al., "Overexpression of the growth arrest and DNA damage-induced 45α gene contributes to autoimmunity by promoting DNA demethylation in lupus T cells," *Arthritis and Rheumatism*, vol. 62, no. 5, pp. 1438–1447, 2010.

[55] Y. Li, G. Gorelik, F. M. Strickland, and B. C. Richardson, "Oxidative stress, T cell dna methylation, and lupus," *Arthritis and Rheumatology*, vol. 66, no. 6, pp. 1574–1582, 2014.

[56] F. M. Strickland, Y. Li, K. Johnson, Z. Sun, and B. C. Richardson, "$CD4^+$ T cells epigenetically modified by oxidative stress cause lupus-like autoimmunity in mice," *Journal of Autoimmunity*, vol. 62, pp. 75–80, 2015.

[57] Q. Lu, M. Kaplan, D. Ray et al., "Demethylation of ITGAL (CD11a) regulatory sequences in systemic lupus erythematosus," *Arthritis and Rheumatism*, vol. 46, no. 5, pp. 1282–1291, 2002.

[58] Q. Lu, A. Wu, L. Tesmer, D. Ray, N. Yousif, and B. Richardson, "Demethylation of CD40LG on the inactive X in T cells from women with lupus," *The Journal of Immunology*, vol. 179, no. 9, pp. 6352–6358, 2007.

[59] P. Coit, M. Jeffries, N. Altorok et al., "Genome-wide DNA methylation study suggests epigenetic accessibility andtranscriptional poising of interferon-regulated genes in naïve CD4+ T cellsfrom lupus patients," *Journal of Autoimmunity*, vol. 43, pp. 78–84, 2013.

[60] D. M. Absher, X. Li, L. L. Waite et al., "Genome-wide DNA methylation analysis of systemic lupus erythematosus reveals

persistent hypomethylation of interferon genes and compositional changes to CD4+ T-cell populations," *PLoS Genetics*, vol. 9, no. 8, article e1003678, 2013.

[61] S. A. Chung, J. Nititham, E. Elboudwarej et al., "Genome-wide assessment of differential DNA methylation associated with autoantibody production in systemic lupus erythematosus," *PLoS ONE*, vol. 10, no. 7, Article ID e0129813, 2015.

[62] T. Fali, C. Le Dantec, Y. Thabet et al., "DNA methylation modulates HRES1/p28 expression in B cells from patients with lupus," *Autoimmunity*, vol. 47, no. 4, pp. 265–271, 2014.

[63] H. Qin, X. Zhu, J. Liang et al., "MicroRNA-29b contributes to DNA hypomethylation of $CD4^+$ T cells in systemic lupus erythematosus by indirectly targeting DNA methyltransferase 1," *Journal of Dermatological Science*, vol. 69, no. 1, pp. 61–67, 2013.

[64] Y. Tang, X. Luo, H. Cui et al., "MicroRNA-146a contributes to abnormal activation of the type I interferon pathway in human lupus by targeting the key signaling proteins," *Arthritis and Rheumatism*, vol. 60, no. 4, pp. 1065–1075, 2009.

[65] Y. Hong, J. Wu, J. Zhao et al., "miR-29b and miR-29c are involved in toll-like receptor control of glucocorticoid-induced apoptosis in human plasmacytoid dendritic cells," *PLoS ONE*, vol. 8, no. 7, article e69926, 2013.

[66] W. Pan, S. Zhu, M. Yuan et al., "MicroRNA-21 and microRNA-148a contribute to DNA hypomethylation in lupus CD4+ T cells by directly and indirectly targeting DNA methyltransferase 1," *The Journal of Immunology*, vol. 184, no. 12, pp. 6773–6781, 2010.

[67] Y. Deng, J. Zhao, D. Sakurai et al., "MicroRNA-3148 modulates allelic expression of toll-like receptor 7 variant associated with systemic lupus erythematosus," *PLoS Genetics*, vol. 9, no. 2, Article ID e1003336, 2013.

[68] W. Fan, D. Liang, Y. Tang et al., "Identification of microRNA-31 as a novel regulator contributing to impaired interleukin-2 production in T cells from patients with systemic lupus erythematosus," *Arthritis & Rheumatism*, vol. 64, no. 11, pp. 3715–3725, 2012.

[69] G. Wang, L.-S. Tam, B. C.-H. Kwan et al., "Expression of miR-146a and miR-155 in the urinary sediment of systemic lupus erythematosus," *Clinical Rheumatology*, vol. 31, no. 3, pp. 435–440, 2012.

[70] N. Hu, X. Qiu, Y. Luo et al., "Abnormal histone modification patterns in lupus $CD4^+$ T cells," *The Journal of Rheumatology*, vol. 35, no. 5, pp. 804–810, 2008.

[71] M. Mandal, S. E. Powers, M. Maienschein-Cline et al., "Epigenetic repression of the Igk locus by STAT5-mediated recruitment of the histone methyltransferase Ezh2," *Nature Immunology*, vol. 12, no. 12, pp. 1212–1220, 2011.

[72] H. Long, W. Huang, H. Yin, S. Zhao, M. Zhao, and Q. Lu, "Abnormal expression pattern of histone demethylases in CD4 + T cells of MRL/lpr lupus-like mice," *Lupus*, vol. 18, no. 14, pp. 1327–1328, 2009.

[73] Q. Zhang, H. long, J. Liao et al., "Inhibited expression of hematopoietic progenitor kinase 1 associated with loss of jumonji domain containing 3 promoter binding contributes to autoimmunity in systemic lupus erythematosus," *Journal of Autoimmunity*, vol. 37, no. 3, pp. 180–189, 2011.

[74] N. Mishra, C. M. Reilly, D. R. Brown, P. Ruiz, and G. S. Gilkeson, "Histone deacetylase inhibitors modulate renal disease in the MRL-lpr/lpr mouse," *Journal of Clinical Investigation*, vol. 111, no. 4, pp. 539–552, 2003.

[75] Y. Zhou, X. Qiu, Y. Luo et al., "Histone modifications and methyl-CpG-binding domain protein levels at the TNFSF7 (CD70) promoter in SLE CD4+ T cells," *Lupus*, vol. 20, no. 13, pp. 1365–1371, 2011.

[76] K. M. Pauley, C. M. Stewart, A. E. Gauna et al., "Altered miR-146a expression in Sjögren's syndrome and its functional role in innate immunity," *European Journal of Immunology*, vol. 41, no. 7, pp. 2029–2039, 2011.

[77] H. Shi, L.-Y. Zheng, P. Zhang, and C.-Q. Yu, "miR-146a and miR-155 expression in PBMCs from patients with Sjögren's syndrome," *Journal of Oral Pathology and Medicine*, vol. 43, no. 10, pp. 792–797, 2014.

[78] E. Zilahi, T. Tarr, G. Papp, Z. Griger, S. Sipka, and M. Zeher, "Increased microRNA-146a/b, TRAF6 gene and decreased IRAK1 gene expressions in the peripheral mononuclear cells of patients with Sjögren's syndrome," *Immunology Letters*, vol. 141, no. 2, pp. 165–168, 2012.

[79] M. Tandon, A. Gallo, S.-I. Jang, G. G. Illei, and I. Alevizos, "Deep sequencing of short RNAs reveals novel microRNAs in minor salivary glands of patients with Sjögren's syndrome," *Oral Diseases*, vol. 18, no. 2, pp. 127–131, 2012.

[80] C. Liang, K. Xiong, K. E. Szulwach et al., "Sjögren syndrome antigen B (SSB)/La promotes global microRNA expression by binding microRNA precursors through stem-loop recognition," *Journal of Biological Chemistry*, vol. 288, no. 1, pp. 723–736, 2013.

[81] Y. Thabet, C. Le Dantec, I. Ghedira et al., "Epigenetic dysregulation in salivary glands from patients with primary Sjögren's syndrome may be ascribed to infiltrating B cells," *Journal of Autoimmunity*, vol. 41, pp. 175–181, 2013.

[82] X. Yu, G. Liang, H. Yin et al., "DNA hypermethylation leads to lower FOXP3 expression in CD4+ T cells of patients with primary Sjögren's syndrome," *Clinical Immunology*, vol. 148, no. 2, pp. 254–257, 2013.

[83] N. Altorok, P. Coit, T. Hughes et al., "Genome-wide DNA methylation patterns in naive cd4+ t cells from patients with primary sjögren's syndrome," *Arthritis and Rheumatology*, vol. 66, no. 3, pp. 731–739, 2014.

[84] N.-S. Lai, H.-C. Yu, H.-C. Chen, C.-L. Yu, H.-B. Huang, and M.-C. Lu, "Aberrant expression of microRNAs in T cells from patients with ankylosing spondylitis contributes to the immunopathogenesis," *Clinical & Experimental Immunology*, vol. 173, no. 1, pp. 47–57, 2013.

[85] J. Qi, S. Hou, Q. Zhang et al., "A functional variant of pre-miRNA-196a2 confers risk for Behcet's disease but not for Vogt-Koyanagi-Harada syndrome or AAU in ankylosing spondylitis," *Human Genetics*, vol. 132, no. 12, pp. 1395–1404, 2013.

[86] Y. Xia, K. Chen, M.-H. Zhang et al., "MicroRNA-124 involves in ankylosing spondylitis by targeting ANTXR2," *Modern Rheumatology*, vol. 25, no. 5, pp. 784–789, 2015.

[87] Z. Niu, J. Wang, H. Zou, C. Yang, W. Huang, and L. Jin, "Common MIR146A polymorphisms in Chinese ankylosing spondylitis subjects and controls," *PLoS ONE*, vol. 10, no. 9, Article ID e0137770, 2015.

[88] H. Appel, P. Wu, R. Scheer et al., "Synovial and peripheral blood CD4+FoxP3+ T cells in spondyloarthritis," *The Journal of Rheumatology*, vol. 38, no. 11, pp. 2445–2451, 2011.

[89] N.-S. Lai, J.-L. Chou, G. C. W. Chen, S.-Q. Liu, M.-C. Lu, and M. W. Y. Chan, "Association between cytokines and methylation of SOCS-1 in serum of patients with ankylosing spondylitis," *Molecular Biology Reports*, vol. 41, no. 6, pp. 3773–3780, 2014.

[90] E. Toussirot, W. Abbas, K. A. Khan et al., "Imbalance between HAT and HDAC activities in the PBMCs of patients with ankylosing spondylitis or rheumatoid arthritis and influence of

HDAC inhibitors on TNF alpha production," *PLoS ONE*, vol. 8, no. 8, Article ID e70939, 2013.

[91] É. Toussirot, D. Wendling, and G. Herbein, "Biological treatments given in patients with rheumatoid arthritis or ankylosing spondylitis modify HAT/HDAC (histone acetyltransferase/histone deacetylase) balance," *Joint Bone Spine*, vol. 81, no. 6, pp. 544–545, 2014.

[92] A. R. Roberts, M. Vecellio, L. Chen et al., "An ankylosing spondylitis-associated genetic variant in the *IL23R-IL12RB2* intergenic region modulates enhancer activity and is associated with increased Th1-cell differentiation," *Annals of the Rheumatic Diseases*, 2016.

[93] P. Coit, L. B. De Lott, B. Nan, V. M. Elner, and A. H. Sawalha, "DNA methylation analysis of the temporal artery microenvironment in giant cell arteritis," *Annals of the Rheumatic Diseases*, vol. 75, no. 6, pp. 1196–1202, 2016.

[94] S. Croci, A. Zerbini, L. Boiardi et al., "MicroRNA markers of inflammation and remodelling in temporal arteries from patients with giant cell arteritis," *Annals of the Rheumatic Diseases*, vol. 75, no. 8, pp. 1527–1533, 2016.

Hindawi Publishing Corporation
Mediators of Inflammation
Volume 2016, Article ID 7651024, 10 pages
http://dx.doi.org/10.1155/2016/7651024

Research Article

Circulating ($CD3^-CD19^+CD20^-IgD^-CD27^{high}CD38^{high}$) Plasmablasts: A Promising Cellular Biomarker for Immune Activity for Anti-PLA2R1 Related Membranous Nephropathy?

Agnieszka Pozdzik,[1,2] Ingrid Beukinga,[3] Chunyan Gu-Trantien,[4] Karen Willard-Gallo,[4] Joëlle Nortier,[1,2] and Olivier Pradier[3]

[1] *Department of Nephrology, Dialysis and Renal Transplantation, Cliniques Universitaires de Bruxelles (CUB), Erasme Hospital, 1070 Brussels, Belgium*
[2] *Unit of Experimental Nephrology, Department of Biochemistry, Faculty of Medicine, Université Libre de Bruxelles (ULB), 1070 Brussels, Belgium*
[3] *Department of Hematology, Cliniques Universitaires de Bruxelles (CUB), Erasme Hospital, 1070 Brussels, Belgium*
[4] *Unit of Molecular Immunology, Institute Jules Bordet, Université Libre de Bruxelles (ULB), 1000 Brussels, Belgium*

Correspondence should be addressed to Agnieszka Pozdzik; agapozdzik@gmail.com

Received 11 January 2016; Revised 25 April 2016; Accepted 8 May 2016

Academic Editor: Maxime Samson

Membranous nephropathy (MN) is a kidney specific autoimmune disease mainly mediated by anti-phospholipase A2 receptor 1 autoantibody (PLA2R1 Ab). The adequate assessment of chimeric anti-CD20 monoclonal antibody, rituximab (RTX), efficacy is still needed to improve clinical outcome of patient with MN. We evaluated the modification of plasmablasts ($CD3^-CD19^+CD20^-IgD^-CD27^{high}CD38^{high}$), a useful biomarker of RTX response in other autoimmune diseases, and memory ($CD3^-CD19^+CD20^+IgD^-CD27^+CD38^-$) and naive ($CD3^-CD19^+CD20^+IgD^+CD27^-CD38^{low}$) B cells by fluorescence-activated cell sorter analysis in PLA2R1 related MN in one patient during the 4 years of follow-up after RTX. RTX induced complete disappearance of $CD19^+$ B cells, plasmablasts, and memory B cells as soon as day 15. Despite severe $CD19^+$ lymphopenia, plasmablasts and memory B cells reemerged early before naive B cells (days 45, 90, and 120, resp.). During the follow-up, plasmablasts decreased more rapidly than memory B cells but still remained elevated as compared to day 0 of RTX. Concomitantly, anti-PLA2R1 Ab increased progressively. Our single case report suggests that, besides monitoring of serum anti-PLA2R1 Ab level, enumeration of circulating plasmablasts and memory B cells represents an attractive and complementary tool to assess immunological activity and efficacy of RTX induced B cells depletion in anti-PLA2R1 Ab related MN.

1. Introduction

Primary membranous nephropathy (MN) is the most common cause of nephrotic syndrome (NS) in Caucasian adults [1]. Recent discovery of autoantibodies (Ab) which recognize specific antigen expressed by podocytes, mainly a soluble receptor of phospholipase A2 (PLA2R1), has greatly improved our understanding of MN physiopathology [2–4]. The anti-PLA2R1 antibody, predominantly the IgG4 subclass, has been reported in sera of nearly 80% of adult MN patients [2, 5, 6]. Accurate cellular immune mechanism(s) involving controlling the synthesis of anti-PLA2R1 Ab still remain(s) unknown [7].

However, the discovery of anti-PLA2R1 Ab highlighted the underestimated role of humoral immunity in MN [9, 10]. Indeed, activated B cells may contribute to the disease progression, not only as effector cells, a precursor of short- and long-lived plasma cells (main cells secreting autoantibodies), but also as regulatory cells of immune response capable of activating T cells [8, 11]. A fraction of plasmablasts ($CD3^-CD19^+CD20^-IgD^-CD27^{high}CD38^{high}$), the intermediate cells between activated B cells and short-lived plasma

cells, migrate from secondary lymphoid organs to the bone marrow where they become long-lived plasma cells within the survival niches, a special microenvironment [12]. Plasmablasts produce important cytokines, synthetize antibodies, and act as antigen-presenting cells in inflammatory microenvironment, exhibiting so far underestimated roles in immune regulation [13]. Recently, circulating plasmablasts have been recognized as an early biomarker of immunological activity in autoimmune diseases [14–16].

Reassessment of the pathophysiological involvement of B cells encouraged clinical interest for chimeric anti-CD20 monoclonal antibody (rituximab; RTX) as a more selective treatment modality for PLA2R1 related MN [17–19]. Indeed, RTX is less toxic than actual recommended standard protocols based on corticosteroids and nonspecific immunosuppressants with heavy long-term side effects [20–22]. Series of observational short-term studies have reported the safety and efficacy of RTX alone or in association with other immunosuppressive drugs or plasma exchange in primary as well as in high-risk patients with MN refractory to conventional treatment [17, 23–27]. Discrepancy in dose and treatment duration of RTX, concomitant use of other immunosuppressive drugs, and time of retreatment [9, 10, 28, 29] remain and, unfortunately, relapses and resistance to RTX of anti-PLA2R1 related MN still occur. Indeed, improvement of the clinical outcome of MN is required. Therefore, monitoring of circulating plasmablasts represents an attractive approach to evaluate autoimmune activity and to optimize immunosuppressive therapy in this disease.

To our knowledge, there are no data reporting the time-course of circulating plasmablasts following RTX administration and their relation with circulating anti-PLA2R1 Ab in MN. In this context, we studied the circulating B cells subpopulations by fluorescence-activated cell sorter analysis (FACS) in a single PLA2R1 related MN patient. We looked principally for circulating plasmablasts, memory and naïve B cells, $IgG4^+$ B cells, and T regulatory (Treg) cells and we related them to the serum anti-PLA2R1 Ab as well as to proteinuria and glomerular filtration rate (GFR), the current robust kidney clinical endpoints.

2. Materials and Methods

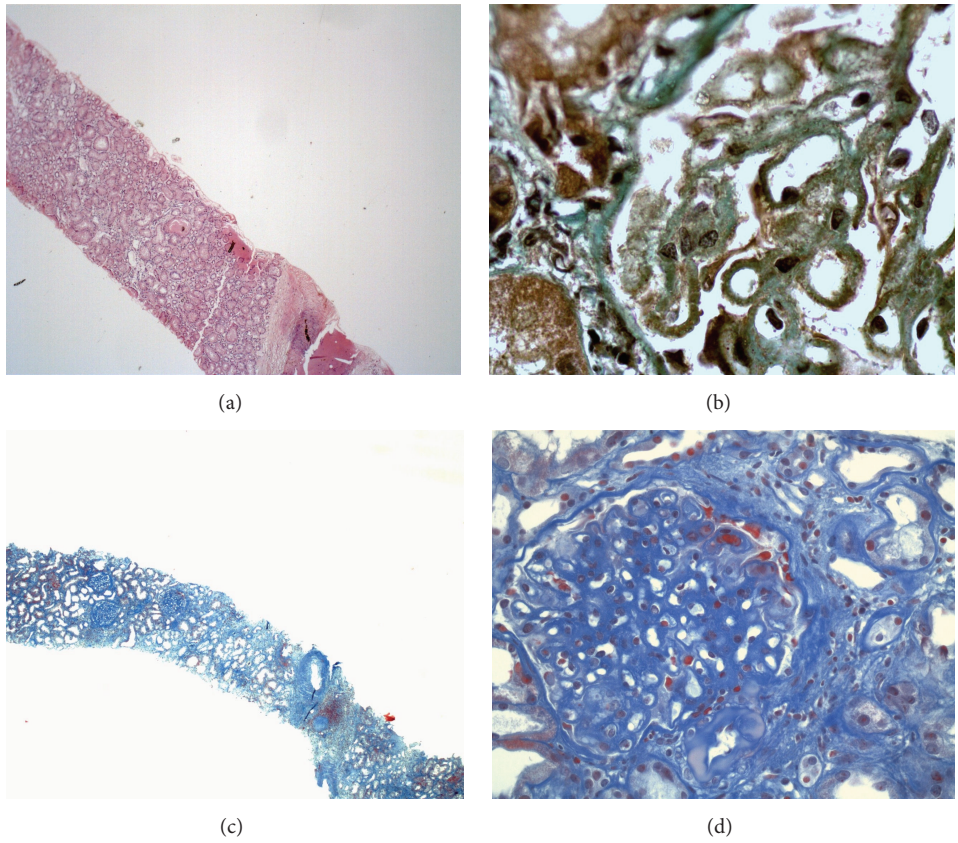
A 48-year-old man presented with nephrotic syndrome and a normal renal function in 1999. Optical and electron microscopy analyses of kidney tissue biopsy were performed in another hospital at that time and showed glomerular lesions typical for membranous nephropathy (Figures 1(a) and 1(b)). Screenings for secondary immunological causes including antinuclear antibody, rheumatoid factor, anti-neutrophil cytoplasmic antibody, and serology for virus hepatitis B and C were negative. Complement level was normal. Secondary drug-induced MN was evoked considering chronic use of nonsteroidal anti-inflammatory drug. Nephroprotection was started.

2.1. Assessment of Peripheral T and B Cells Subpopulations, $IgG4^+$ B Cells, and Plasmablasts. In our case, we performed a peripheral blood analysis during a 5-year follow-up under three different immunosuppressive therapies: firstly under MPD in association with TRL, secondly under MMF alone, and finally under RTX. We started to study only $CD3^+CD4^+/CD3^+CD8^+$ T cells ratio and $CD19^+$ B cells under MPD + TRL and MMF by standardized routine protocol. Concomitantly, we develloped the analysis of B cells subpopulations. Briefly, whole blood samples (5 mL EDTA anticoagulated) were received at the hematology laboratory and WBC count was performed on UniCel DxH™ 800 hematology analyzers from Beckman Coulter within 4 hours. In the flow cytometry laboratory, 2 mL blood was washed three times with PBS-1% BSA to remove plasma. The white and red cells pellet was suspended vol/vol in PBS-1% BSA and 100 μL was stained with 20 μL monoclonal antibody combinations: IgD-FITC (DakoCytomation, Heverlee, Belgium), CD27-PE, CD10-ECD, CD5-PE-Cy5.5, CD19 PE-Cy7, CD23-APC-AF700, CD38-APC-AF750, CD20 Pacific blue, and CD45 Krome Orange (all from Beckman Coulter). T, NK, and monocyte markers were added in the same ratio to negatively purify the B cell populations. Staining was performed during 15 min in the dark and the cell suspension was washed once with PBS-1% BSA. The B and plasma cells fluorescence was acquired with a 10-color Beckman Coulter Navios flow cytometer driven by CXP-Navios software. At least 4000 CD19 positive cells were acquired. Analysis was performed using Kaluza 1.2 Software (Beckman Coulter). Mature B cells were defined as $CD19^-CD20^-CD45^{bright}$ positive cells, naive B cells as IgD^+, and $CD38^{dim}$ (resting naive), $CD38^+$ (activated naive), and memory cells as IgD^- with $CD38^{dim}$ or $CD38^+$. Transitional B cells were isolated as $CD38^{bright}$, $CD20^+$, $CD19^+$ IgD^+, and $CD27^-$ cells and finally plasmablasts were $CD45^+$ but not bright, $CD19^+$, $CD20^-$, $CD38^{bright}$, $CD27^+$, and IgD^-. Circulating B cells' absolute values were calculated in double platform using absolute lymphocyte count from the hematological analyzer associated with the B cell population percentages.

2.2. Detection of Circulating Anti-PLA2R1 Antibodies. Circulating antibodies against PLA2R1 were assessed by commercially available cell based assay using indirect immunofluorescence using human embryonic kidney (HEK-293) cells transfected with human PLA2R1 (Euroimmun, Lubeck, Netherlands) [30] and by commercially available PLA2R1 ELISA kit (Biognost, Heule, Belgium) according to the manufacturer's instructions as reported by others [6, 30, 31].

2.3. Analysis of Renal Biopsy Specimens. The specimens from both kidney biopsies were prepared for light and immunofluorescence microscopy by standard technique as previously described [32].

2.4. Detection of PLA2R1 Antigen in Paraffin Embedded Kidney Biopsy. Both kidney biopsies were assessed for localization of PLA2R1 in glomerular deposits. We used rabbit affinity purified specific anti-PLA2R1 antibodies (Atlas Antibodies, AB, Stockholm, Sweden) followed by goat FITC-conjugated anti rabbit Fab IgG as previously reported [33].



(a) (b) (c) (d)

Figure 1: Representative pictures of histopathological analysis performed on formalin-fixed and paraffin embedded kidney tissue section obtained from a first (a-b) and a second kidney punction-biopsy (c-d) performed in patient with membranous nephropathy. (a) Haematoxylin eosin, Jone's silver (b), and Masson Trichrome (c-d) standard stainings. (a) Absence of tubulointerstitial involvement and (b) marked thickening of glomerular basement membrane with stubby spikes-like projections corresponding to stage 2 of membranous nephropathy. (c) Tubular atrophy (tubular dilatation and flattening of epithelium), sparse interstitial inflammation, and fibrosis. (d) Marked thickening of glomerular basement membrane with double contours. Original magnifications: (a) ×40, (b) ×1000 (kindly provided by Dr. Selda Aydin, Pathology Department, St. Luc Hospital, UCL, Brussels, Belgium), (c) ×40, and (d) ×400 (kindly provided by Dr. Michel Depierreux, Pathology Department, Erasme Hospital, CUB, ULB, Brussels, Belgium).

2.5. Detection of Macrophages, T and B Cells. Only the second kidney biopsy was available for immunophenotyping of intrarenal macrophages, T and B cells. We performed immunohistochemistry using the following anti-human primary antibodies: CD68 (macrophages), CD8 ($CD8^+$ T cells subpopulation), CD4 ($CD4^+$ T cells subpopulation), and CD20 (mature B cells) as described previously [32].

2.6. Detection of Intrarenal Follicular Dendritic Cells. Only second kidney biopsy was available for immunofluorescence staining that was adapted from Gu-Trantien et al. [34]. We used the following primary antibodies: CD20 (ab9475, 1/50), CXCL13 (AF801, 1/200), and CD21 (ab75985, 1/500) and donkey anti-mouse (ab98767, 1/200), anti-goat (ab98514, 1/200), and anti-rabbit (ab98491, 1/200) secondary antibodies. Except for CXCL13 obtained from R&D System (Abingdon, UK), all were provided from Abcam (Cambridge, UK). Briefly, after paraffin removal with xylene, mounted sections were pretreated (citrate 10 mM, pH 6.0, at 95°C to 99°C for 30 minutes), blocked with 1% BSA for 30 min, and incubated with primary antibodies (at 4°C, moist chamber, overnight). After washing, secondary antibodies were applied for 2 h at room temperature. Slides were mounted in the ProLong Gold Antifade Mountant with DAPI (P36941, Life Technologies, California, USA) and images were acquired with a Zeiss LSM 710 confocal microscope.

3. Results

In December 2005, because of uncontrolled nephrotic proteinuria [urinary proteins (UPr) 11.0 g/24 h] and renal function decline, cyclophosphamide and methylprednisolone (MPD) were given according to Ponticelli revised protocol. Only partial remission was obtained (UPr 3.0 g/24 h) (Figure 2(a)). In June 2007, considering relapse of NS (UPr 11.0 g/24 h), cyclosporine A and MPD (275 mg and 12 mg daily, resp.) were started but, due to drop in proteinuria, remained below the nephrotic range. At the time of admission in our nephrology clinic in November 2007, we noticed uncontrolled NS (lower limbs edema, uncontrolled arterial

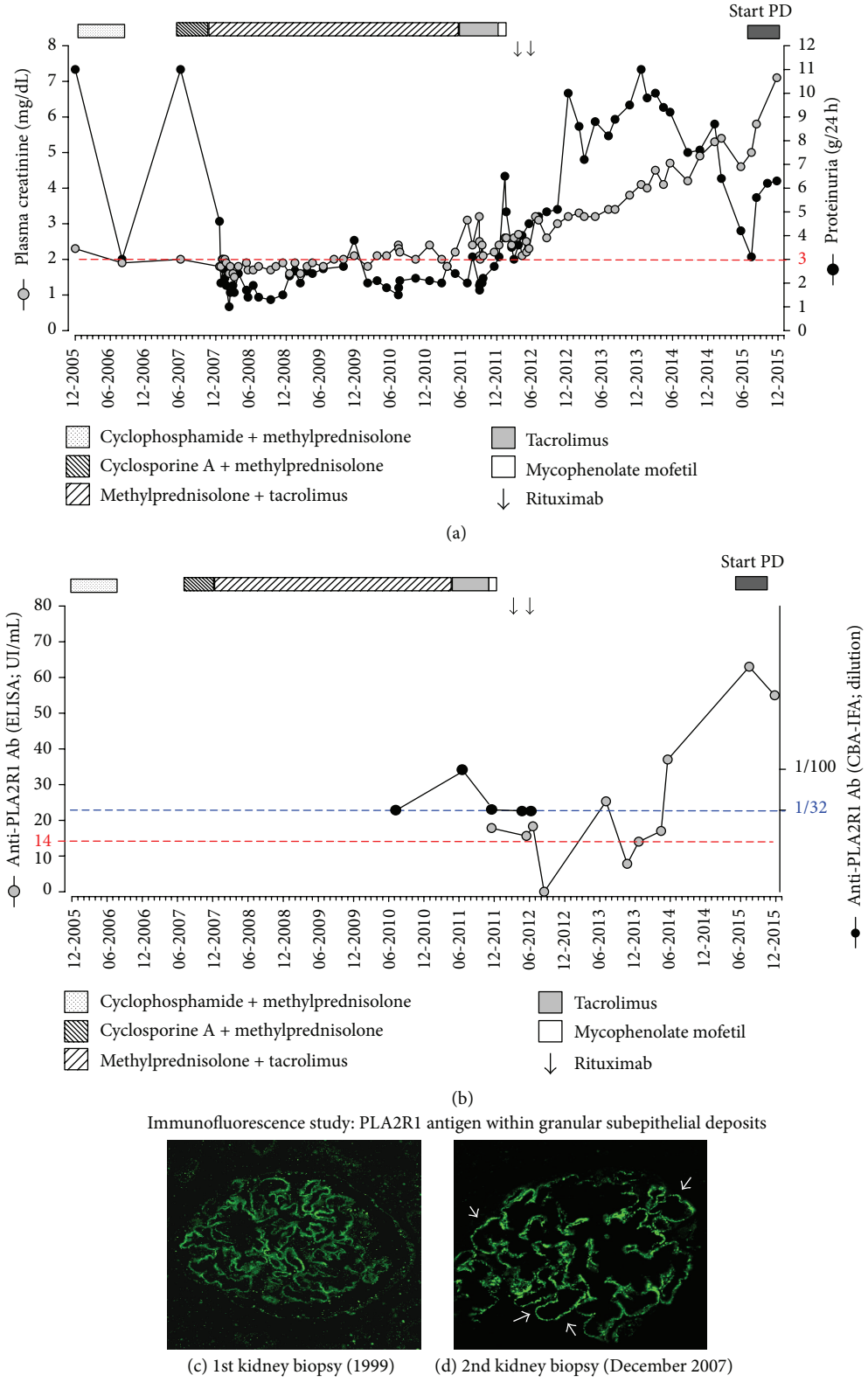


Figure 2: Time-course of renal function parameters (a) plasma creatinine and 24 h-proteinuria levels under immunosuppressive therapy and (b) values of serum anti-PLA2R1 antibody levels. (c and d) Representative pictures of immunofluorescence of phospholipase A2 receptor expression within the glomerulus performed on formalin-fixed and paraffin embedded kidney tissue section obtained from a first (c) and a second (d) kidney biopsy in patient with membranous nephropathy. Confocal microscopic analysis of a paraffin kidney biopsy specimen revealed the presence of PLA2R1 in subepithelial deposits along glomerular capillary loops (white arrows), ×400 magnification. Kindly performed by Hanna Debiec INSERM U702, Hôpital Tenon, Paris, France.

hypertension, and dyslipidemia). In December 2007, proteinuria remained nephrotic (UPr 4.0 g/24 h). In order to exclude the lesions of focal segmental glomerulosclerosis (FSGS) secondary to chronic proteinuria, kidney biopsy was performed and showed only advanced stage of MN without FSGS, mild tubular atrophy, and renal interstitial fibrosis associated with mononuclear cells interstitial infiltration (Figures 1(c) and 1(d)). Then, we switched immunosuppression to the association of tacrolimus (TRL) with MPD because of cyclosporine A adverse effects, mainly important gingival hypertrophy. In July 2011, considering acute kidney injury (stage 3) secondary to a viral gastroenteritis (Figure 2(a)), we removed sartans because of uncontrolled hyperkaliemia and reduced MPD (2 mg daily) because of long-term corticoids adverse effects (partial rupture of left biceps). As renal function declined progressively, we started mycophenolate mofetil (1500 mg daily) in March 2012.

During early pretransplant, we retrospectively screened both kidney biopsies taken in 1999 and in 2007 by immunofluorescence (Figures 2(c) and 2(d)). The presence of several granular PLA2R1 antigens within extramembranous deposits was demonstrated in only one but well-preserved glomerulus in tissue provided from a first kidney biopsy (1999). Kidney biopsies contained ineffaceable fingerprints of anti-PLA2R1 autoimmunity also in several glomeruli provided from second biopsy (after 7 years of well-conducted immunosuppression) and confirmed primary MN.

Unfortunately, the serum was unavailable at the moment of MN diagnosis. Available serum samples from 2007 to 2010 were negative for anti-PLA2R1 Ab (sensitive technique, cell based assay using indirect immunofluorescence (CBA-IFA), a reference technique for diagnosis of MN [6, 31, 35, 36]). In 2012, after removal of immunosuppression, we detected low levels of circulating anti-PLA2R1 Ab as measured by CBA-IFA and by enzyme-linked immunoabsorbent assay (Figure 2(b)). Our long-term data reinforce the importance of methodological screening of renal tissues and repeated serum assessment for PLA2R1 staining within extramembranous deposits and for circulating anti-PLA2R1 Ab, respectively, in the management of IMN.

In May 2012, we gave RTX (two doses of 375 mg/m^2 every 2 weeks, off label use, low doses as proposed previously) according to promising data form Cravedi et al. [37] and MPD (4 mg daily, to prevent anti CD20-antibody immunization) in PLA2R1 related MN in an attempt to control NS and CKD progression. Despite targeted CD19$^+$ cells, lymphopenia and proteinuria remained in the nephrotic range and the renal function deteriorated progressively (PrU 6.0 g/24 h, PCr 3.5 mg/dL). We withdrew immunosuppression and addressed the patient to the predialysis unit. For the moment, he is doing well on peritoneal dialysis and he is on the waiting list for kidney transplantation. However, as the level of circulating anti-PLA2R1 Ab remains high (66 UI/mL, normal range 0–20 UI/mL), RTX will be administrated in order to reduce the levels of Ab.

The number of CD19$^+$ B cells was not affected by MPD + TRL but was lower than normal range under MMF. After one dose of RTX, CD19$^+$ B cells decreased rapidly (<5 cells/μL) and were fully cleared at day 30 (data not shown).

All subpopulations of circulating B cells: (CD3$^-$CD19$^+$CD20$^+$IgD$^-$CD27$^+$CD38$^-$) memory B cells, plasmablasts (CD3$^-$CD19$^+$CD20$^-$IgD$^-$CD27^{high}CD38^{high}), and naïve B cells (CD3$^-$CD19$^+$CD20$^+$IgD$^+$CD27$^-$CD38^{low}) (Figure 3(a)) were affected. Plasmablasts, memory B cells were cleared at day 15 and naive B cells at day 45. Despite persisting CD19$^+$ lymphopenia, as soon as 45 days after first RTX injection, memory B cells, plasmablasts, and naive B cells repopulated peripheral blood (Figures 3(b)–3(d)). After a transient pick (14 months), the number of circulating plasmablasts decreased progressively. However, the number of memory B cells remained elevated.

Because anti-PLA2R1 antibody is related mainly to IgG4 subclass, we investigated circulating IgG4$^+$ B cells. These B cells disappeared after RTX and their reappearance followed kinetics of circulating plasmablasts. Moreover, circulating anti-PLA2R1 antibodies followed the increase in circulating plasmablasts, memory B cells, and IgG4$^+$ B cells (Figures 3(b)–3(e)).

Peripheral CD4$^+$CD25^{high}CD127^{dim}FoxP3$^+$ regulatory T cells (Treg) were also affected by RTX (data not shown). The peripheral Treg cells progressively increased after RTX administration.

Furthermore, we performed immunohistochemical stainings of renal tissue from the second kidney biopsy to characterize intrarenal inflammatory cells. Several CD8$^+$, CD4$^+$, and CD68$^+$ cells infiltrated interstitial areas diffusely, reflecting renal interstitium infiltration by T and B cells as well as the presence of monocytes/macrophages, respectively (Figures 4(a)–4(d)). Several CD20$^+$ cells formed rather patchy, focal infiltrate in peritubular areas. Interestingly, one of the three small B cell intrarenal follicles detected on the biopsy sample contains some CD21$^+$ follicular dendritic cells (FDCs) (Figures 4(e) and 4(f)) indicating germinal center (GC) corresponding to tertiary lymphoid organ (TLO), despite absence of the B cells attracting chemokine CXCL13 within the GC.

4. Discussion

To our knowledge, this is the first report of long-term modification in circulating B cell subtypes including plasmablasts (CD3$^-$CD19$^+$CD20$^-$IgD$^-$CD27^{high}CD38^{high}) and memory (CD3$^-$CD19$^+$CD20$^+$IgD$^-$CD27$^+$CD38$^-$) and naive (CD3$^-$CD19$^+$CD20$^+$IgD$^+$CD27$^-$CD38^{low}) B cells in relation to serum level of anti-PLA2R1 Ab, proteinuria, and GFR.

We suggest that circulating CD3$^-$CD19$^+$CD20$^-$IgD$^-$CD27^{high}CD38^{high} plasmablasts could be a new cellular biomarker of residual autoimmunity in PLA2R1 related MN. We argue our suggestion considering (1) the serum anti-PLA2R1 antibody, a proposed biomarker of humoral activity in this disease, (2) the evidence of marked CD20$^+$ B cells infiltration, and (3) the formation of tertiary lymphoid organ by CD21$^+$ follicular dendritic cells (FDCs) indicating germinal center (GC) activities in our case.

However, we fully recognize the following limitations of our study: firstly, this being a one-case study; secondly, the late stage of disease; and thirdly, the possibility that

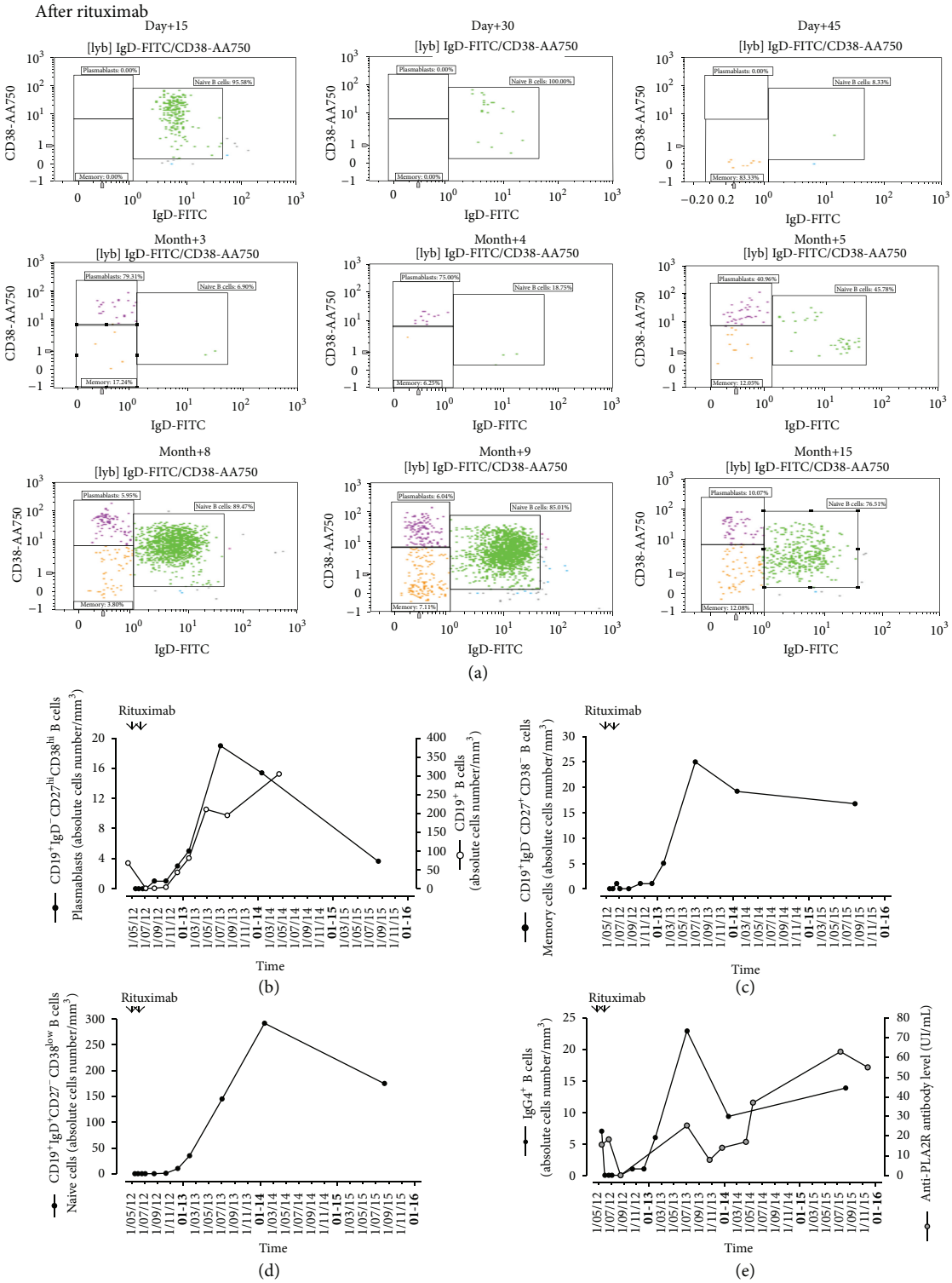


Figure 3: Distribution of the B lymphocyte subpopulations after B cells depleting therapy with rituximab as evaluated by fluorescence-activated cell sorter (FACS) analysis of peripheral blood mononuclear cells (PBMC). (a) Dot-plots representing the expression of IgD (FITC) and CD38 (AA750) monitored in our patient with corticosteroid-resistant PLA2R1 related membranous nephropathy during 15 months after first rituximab injection. Graphs representing the kinetics of absolute cells number per mm^3 of (b) plasmablasts ($CD3^-CD19^+CD20^-IgD^-CD27^{high}CD38^{high}$), (c) memory B cells ($CD3^-CD19^+CD20^+IgD^-CD27^+CD38^-$), and (d) naïve ($CD3^-CD19^+CD20^+IgD^+CD27^-CD38^{low}$) B cells. (e) $IgG4^+$ B cells (absolute cells number per mm^3) and anti-PLA2R1 antibody assessed in serum expressed in IU/mL.

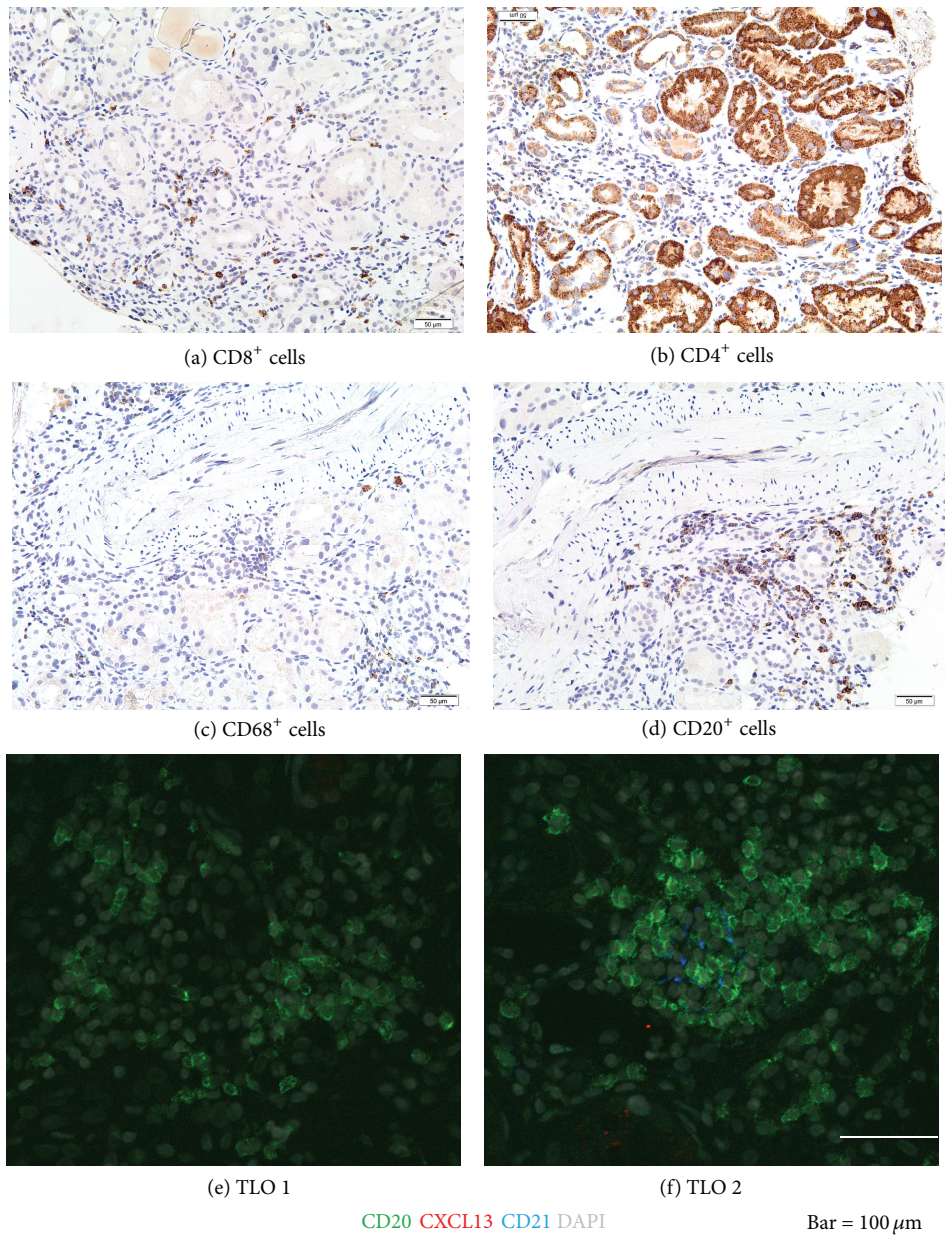


FIGURE 4: Representative pictures of intrarenal inflammatory cells identified by immunoperoxidase and double immunofluorescence stainings in tissue samples provided from second kidney biopsy. Kidney tissue sections were probed with antibodies against the following antigens: CD8 (cytotoxic T cells), CD4 (helper/suppressor T cells), CD68 (monocytes/macrophages), and CD20 (B cells). One of the two small B cell follicles, which corresponded to tertiary lymphoid organs contained CD21+ follicular dendritic cells indicating the presence of germinal center activities despite the absence of the B cell attracting chemokine, CXCL13 within the germinal center. Original magnifications: (a–d) ×200 and (e-f) ×1000.

baseline number of plasmablasts before RTX could have been influenced by previous immunosuppression.

We hypothesized that previous immunosuppressive drugs blocked anti-PLA2R1 Ab synthesis but were unable to clear it away from glomerular deposits and/or to remove intrarenal TLO hypothetical site of B cells activation. In our case, after short disappearance induced by RTX, serum PLA2R1 Ab became positive, probably secondary to immunosuppression withdrawal, and correlated with progressive increase in PCr and proteinuria. Failure of RTX in our patient could be explained at least by the following reasons: (1) low levels of anti-PLA2R1 Ab (slightly higher than the normal range) as RTX has been reported to preserve residual renal function in patients, especially in those with high levels; (2) development of glomerulosclerosis, which is always possible with such a long history of active MN and which was recognized previously to be associated with poorer outcome under RTX therapy [38] or/and secondary tubulointerstitial lesion considering the many years of calcineurin inhibitors use; (3) the possibility that

AKI episode could also be involved as cytokines and/or chemokines from injured tubular renal cells, which are able to induce autoimmune B cells response [39]. Indeed, we found marked intrarenal infiltration by $CD8^+$ and $CD4^+$ cells, which has been associated with pejorative evolution of MN [40]. In both human and experimental models of MN, the Th2-polarization of $CD4^+$ T cells stimulated intrarenal autoreactive $CD20^+$ B cells to generate IgG4 [41]. We observed several $CD20^+$ B cells that exclusively infiltrated the tubulointerstitium, but not the glomerular tuft. However, no relation has been found between the response to RTX and $CD20^+$ cells in the kidney biopsy in MN [42].

In our case, RTX induced a complete depletion of all peripheral B cells subpopulations (naive, transitional, memory B cells, and plasmablasts) but circulating plasmablasts appeared first despite of achieved $CD19^+$ depletion and were followed by an increase in serum anti-PLA2R1. Recent evidence suggests that plasmablasts produce cytokines, synthetize antibodies, and act as antigen-presenting cells especially in chronically inflamed organ [12]. Increase in percentage of IgD^-CD27^{high} plasmablasts has been associated with high anti-ds DNA antibody levels relapse in SLE patient [43]. Circulating plasmablast counts have been proposed as a useful biomarker of residual autoimmunity assessing response to treatment and determining the appropriate time to retreatment independently of IgG4 serum concentration in the IgG4-related disease [44]. Tissue plasmablasts have been recognized as a pivotal predictor of clinical outcome in patients receiving RTX for refractory rheumatoid arthritis [45]. However, RTX induced B cell deletion promotes a suitable microenvironment for maturation and survival of autoimmune long-lived plasma cells in the spleen [46]. Accordingly to Cohen et al. [47], higher doses or longer duration of RTX could be necessary to control circulating plasmablasts in MN. Despite the fact that origin and localization of B cells producing anti-PLA2R1 Ab remain unknown, previously proposed titration of RTX to circulating $CD20^+$ or $CD19^+$ B cells needs to be revised in MN as not measuring diversity of B cells subpopulations [48]. Indeed, level of plasmablasts, rather than the criterion of complete depletion of peripheral $CD20^+$ or $CD19^+$ B cells after RTX administration, predicted the RTX success in rheumatoid arthritis [49]. It could be speculated that circulating plasmablasts originate from activated intrarenal B cells within remaining TLO considering that tissue B cells are difficult to deplete by RTX [16], but this hypothesis needs to be proven. Clearly, further clinical long-term follow-up studies of MN patients under immunosuppression are indispensable to investigate the usefulness of B cells subsets monitoring to improve the assessment of autoimmunity in MN. We need the responses to 2 questions: (1) Are the circulating plasmablasts a more specific marker of residual autoimmunity in MN? (2) Is resistance to immunosuppression related to the persistence of long-lived plasma cells within intrarenal TLO?

In conclusion, measurement of circulating ($CD3^-CD19^+CD20^-IgD^-CD27^{high}CD38^{high}$) plasmablasts rapidly assesses RTX response and appears as a promising cellular biomarker to improve RTX therapy in clinical practice. We suggest that besides monitoring of serum anti-PLA2R1 Ab level, enumeration of plasmablasts and memory B cells represents an attractive and complementary tool to assess autoimmune activity and/or efficacy of RTX induced B cells depletion in anti-PLA2R1 Ab related MN.

Abbreviations

CKD:	Chronic Kidney Disease
PCr:	Plasma creatinine
anti-PLA2R1 Ab:	Anti-phospholipase A2 receptor type 1 antibody
MN:	Membranous nephropathy
NS:	Nephrotic syndrome
ESKD:	End Stage Kidney Disease
TLO:	Tertiary lymphoid organ.

Competing Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contributions

Agnieszka Pozdzik and Ingrid Beukinga contributed equally to this work.

Acknowledgments

The authors are grateful to Hanna Debiec (Ph.D.) and Pierre Ronco (M.D., Ph.D.) (INSERM U702, Hôpital Tenon, Paris, France) for immunostainings of PLA2R1 antigen and expert opinion in clinical management of this case. The authors thank L. Bienfait (M.D.), M. Depierreux (M.D.), and S. Rorive (M.D., Ph.D.) (Pathology Department, CUB, Erasme Hospital, Brussels, Belgium), S. Aydin (M.D., Ph.D.) (Pathology Department, UCL, Saint Luc, Brussels, Belgium), and C. Husson (B.S.) (Experimental Nephrology Unit, Department of Biochemistry, Faculty of Medicine, ULB, Brussels, Belgium) for their substantial technical help.

References

[1] C. Ponticelli and R. J. Glassock, "Glomerular diseases: membranous nephropathy—a modern view," *Clinical Journal of the American Society of Nephrology*, vol. 9, no. 3, pp. 609–616, 2014.

[2] L. H. Beck Jr., R. G. B. Bonegio, G. Lambeau et al., "M-type phospholipase A2 receptor as target antigen in idiopathic membranous nephropathy," *The New England Journal of Medicine*, vol. 361, no. 1, pp. 11–21, 2009.

[3] H. Debiec and P. Ronco, "Immunopathogenesis of membranous nephropathy: an update," *Seminars in Immunopathology*, vol. 36, no. 4, pp. 381–397, 2014.

[4] A. A. Pozdzik, H. Debiec, I. Brochériou et al., "Anti-NEP and anti-PLA2R antibodies in membranous nephropathy: an update," *Revue Medicale de Bruxelles*, vol. 36, no. 3, pp. 166–171, 2015.

[5] L.-H. Noël, P. Aucouturier, R. C. Monteiro, J.-L. Preud'homme, and P. Lesavre, "Glomerular and serum immunoglobulin G subclasses in membranous nephropathy and anti-glomerular

basement membrane nephritis," *Clinical Immunology and Immunopathology*, vol. 46, no. 2, pp. 186–194, 1988.

[6] J. M. Hofstra, H. Debiec, C. D. Short et al., "Antiphospholipase A2 receptor antibody titer and subclass in idiopathic membranous nephropathy," *Journal of the American Society of Nephrology*, vol. 23, no. 10, pp. 1735–1743, 2012.

[7] P. Ronco and H. Debiec, "Membranous nephropathy: a fairy tale for immunopathologists, nephrologists and patients," *Molecular Immunology*, vol. 68, no. 1, pp. 57–62, 2015.

[8] M. Mahévas, P. Patin, F. Huetz et al., "B cell depletion in immune thrombocytopenia reveals splenic long-lived plasma cells," *Journal of Clinical Investigation*, vol. 123, no. 1, pp. 432–442, 2013.

[9] A. Howman, T. L. Chapman, M. M. Langdon et al., "Immunosuppression for progressive membranous nephropathy: a UK randomised controlled trial," *The Lancet*, vol. 381, no. 9868, pp. 744–751, 2013.

[10] J. M. Hofstra, F. C. Fervenza, and J. F. M. Wetzels, "Treatment of idiopathic membranous nephropathy," *Nature Reviews Nephrology*, vol. 9, no. 8, pp. 443–458, 2013.

[11] R. Stasi, G. Del Poeta, E. Stipa et al., "Response to B-cell-depleting therapy with rituximab reverts the abnormalities of T-cell subsets in patients with idiopathic thrombocytopenic purpura," *Blood*, vol. 110, no. 8, pp. 2924–2930, 2007.

[12] K. Roth, L. Oehme, S. Zehentmeier, Y. Zhang, R. Niesner, and A. E. Hauser, "Tracking plasma cell differentiation and survival," *Cytometry Part A*, vol. 85, no. 1, pp. 15–24, 2014.

[13] B. Tiburzy, U. Kulkarni, A. E. Hauser, M. Abram, and R. A. Manz, "Plasma cells in immunopathology: concepts and therapeutic strategies," *Seminars in Immunopathology*, vol. 36, no. 3, pp. 277–288, 2014.

[14] J. H. Anolik, J. Barnard, A. Cappione et al., "Rituximab improves peripheral B cell abnormalities in human systemic lupus erythematosus," *Arthritis & Rheumatism*, vol. 50, no. 11, pp. 3580–3590, 2004.

[15] D. Saadoun, M. Rosenzwajg, D. Landau, J. C. Piette, D. Klatzmann, and P. Cacoub, "Restoration of peripheral immune homeostasis after rituximab in mixed cryoglobulinemia vasculitis," *Blood*, vol. 111, no. 11, pp. 5334–5341, 2008.

[16] H. E. Mei, D. Frölich, C. Giesecke et al., "Steady-state generation of mucosal IgA^+ plasmablasts is not abrogated by B-cell depletion therapy with rituximab," *Blood*, vol. 116, no. 24, pp. 5181–5190, 2010.

[17] P. Ruggenenti, C. Chiurchiu, V. Brusegan et al., "Rituximab in idiopathic membranous nephropathy: a one-year prospective study," *Journal of the American Society of Nephrology*, vol. 14, no. 7, pp. 1851–1857, 2003.

[18] P. Ruggenenti, P. Cravedi, and G. Remuzzi, "Rituximab for membranous nephropathy and immune disease: less might be enough," *Nature Clinical Practice Nephrology*, vol. 5, no. 2, pp. 76–77, 2009.

[19] H. Sugiura, T. Takei, M. Itabashi et al., "Effect of single-dose rituximab on primary glomerular diseases," *Nephron—Clinical Practice*, vol. 117, no. 2, pp. c98–c105, 2011.

[20] D. Santoro, V. Pellicanò, L. Visconti, G. Trifirò, V. Cernaro, and M. Buemi, "Monoclonal antibodies for renal diseases: current concepts and ongoing treatments," *Expert Opinion on Biological Therapy*, vol. 15, no. 8, pp. 1119–1143, 2015.

[21] T. H. Tran, G. J. Hughes, C. Greenfeld, and J. T. Pham, "Overview of current and alternative therapies for idiopathic membranous nephropathy," *Pharmacotherapy*, vol. 35, no. 4, pp. 396–411, 2015.

[22] A. S. Bomback, V. K. Derebail, J. G. McGregor, A. V. Kshirsagar, R. J. Falk, and P. H. Nachman, "Rituximab therapy for membranous nephropathy: a systematic review," *Clinical Journal of the American Society of Nephrology*, vol. 4, no. 4, pp. 734–744, 2009.

[23] P. A. Michel, K. Dahan, P. Y. Ancel et al., "Rituximab treatment for membranous nephropathy: a French clinical and serological retrospective study of 28 patients," *Nephron Extra*, vol. 1, no. 1, pp. 251–261, 2011.

[24] E. Hoxha, S. Harendza, H. Pinnschmidt, U. Panzer, and R. A. K. Stahl, "M-type phospholipase A2 receptor autoantibodies and renal function in patients with primary membranous nephropathy," *Clinical Journal of the American Society of Nephrology*, vol. 9, no. 11, pp. 1883–1890, 2014.

[25] G. Bandak, B. A. Jones, J. Li, J. Yee, and K. Umanath, "Rituximab for the treatment of refractory simultaneous anti-glomerular basement membrane (anti-GBM) and membranous nephropathy," *Clinical Kidney Journal*, vol. 7, no. 1, pp. 53–56, 2014.

[26] J. Müller-Deile, L. Schiffer, M. Hiss, H. Haller, and M. Schiffer, "A new rescue regimen with plasma exchange and rituximab in high-risk membranous glomerulonephritis," *European Journal of Clinical Investigation*, vol. 45, no. 12, pp. 1260–1269, 2015.

[27] Q. Reynaud, M. Killian, A. Robles et al., "Review of the current use of rituximab during 4 years in a French university hospital," *La Revue de Médecine Interne*, vol. 36, no. 12, pp. 800–812, 2015.

[28] C. Ponticelli, "What is the role of rituximab in idiopathic membranous nephropathy?" *Expert Review of Clinical Immunology*, vol. 9, no. 1, pp. 13–16, 2013.

[29] P. Cravedi, G. Remuzzi, and P. Ruggenenti, "Rituximab in primary membranous nephropathy: first-line therapy, why not?" *Nephron Clinical Practice*, vol. 128, no. 3-4, pp. 261–269, 2014.

[30] E. Hoxha, S. Harendza, G. Zahner et al., "An immunofluorescence test for phospholipase-A2-receptor antibodies and its clinical usefulness in patients with membranous glomerulonephritis," *Nephrology Dialysis Transplantation*, vol. 26, no. 8, pp. 2526–2532, 2011.

[31] A. Behnert, M. J. Fritzler, B. Teng et al., "An anti-phospholipase A_2 receptor quantitative immunoassay and epitope analysis in membranous nephropathy reveals different antigenic domains of the receptor," *PLoS ONE*, vol. 8, no. 4, Article ID e61669, 2013.

[32] A. A. Pozdzik, A. Berton, H. H. Schmeiser et al., "Aristolochic acid nephropathy revisited: a place for innate and adaptive immunity?" *Histopathology*, vol. 56, no. 4, pp. 449–463, 2010.

[33] H. Debiec and P. Ronco, "PLA2R autoantibodies and PLA2R glomerular deposits in membranous nephropathy," *The New England Journal of Medicine*, vol. 364, no. 7, pp. 689–690, 2011.

[34] C. Gu-Trantien, S. Loi, S. Garaud et al., "$CD4^+$ follicular helper T cell infiltration predicts breast cancer survival," *The Journal of Clinical Investigation*, vol. 123, no. 7, pp. 2873–2892, 2013.

[35] C. Dähnrich, L. Komorowski, C. Probst et al., "Development of a standardized ELISA for the determination of autoantibodies against human M-type phospholipase A2 receptor in primary membranous nephropathy," *Clinica Chimica Acta*, vol. 421, pp. 213–218, 2013.

[36] A. Behnert, M. Schiffer, J. Müller-Deile, L. H. Beck, M. Mahler, and M. J. Fritzler, "Antiphospholipase A_2 receptor autoantibodies: a comparison of three different immunoassays for the diagnosis of idiopathic membranous nephropathy," *Journal of Immunology Research*, vol. 2014, Article ID 143274, 5 pages, 2014.

[37] P. Cravedi, M. C. Sghirlanzoni, M. Marasà, A. Salerno, G. Remuzzi, and P. Ruggenenti, "Efficacy and safety of rituximab

second-line therapy for membranous nephropathy: a prospective, matched-cohort study," *American Journal of Nephrology*, vol. 33, no. 5, pp. 461–468, 2011.

[38] M. Busch, C. Rüster, C. Schinköthe, J. Gerth, and G. Wolf, "Rituximab for the second-and third-line therapy of idiopathic membranous nephropathy: a prospective single center study using a new treatment strategy," *Clinical Nephrology*, vol. 80, no. 2, pp. 105–113, 2013.

[39] G. R. Kinsey and M. D. Okusa, "Expanding role of T cells in acute kidney injury," *Current Opinion in Nephrology and Hypertension*, vol. 23, no. 1, pp. 9–16, 2014.

[40] Q. Wu, K. Jinde, M. Nishina et al., "Analysis of prognostic predictors in idiopathic membranous nephropathy," *American Journal of Kidney Diseases*, vol. 37, no. 2, pp. 380–387, 2001.

[41] A. Kuroki, M. Iyoda, T. Shibata, and T. Sugisaki, "Th2 cytokines increase and stimulate B cells to produce IgG4 in idiopathic membranous nephropathy," *Kidney International*, vol. 68, no. 1, pp. 302–310, 2005.

[42] F. C. Fervenza, F. G. Cosio, S. B. Erickson et al., "Rituximab treatment of idiopathic membranous nephropathy," *Kidney International*, vol. 73, no. 1, pp. 117–125, 2008.

[43] M. N. Lazarus, T. Turner-Stokes, K.-M. Chavele, D. A. Isenberg, and M. R. Ehrenstein, "B-cell numbers and phenotype at clinical relapse following rituximab therapy differ in SLE patients according to anti-dsDNA antibody levels," *Rheumatology*, vol. 51, no. 7, pp. 1208–1215, 2012.

[44] Z. S. Wallace, H. Mattoo, M. Carruthers et al., "Plasmablasts as a biomarker for IgG4-related disease, independent of serum IgG4 concentrations," *Annals of the Rheumatic Diseases*, vol. 74, no. 1, pp. 190–195, 2015.

[45] Y. K. O. Teng, E. W. N. Levarht, M. Hashemi et al., "Immunohistochemical analysis as a means to predict responsiveness to rituximab treatment," *Arthritis and Rheumatism*, vol. 56, no. 12, pp. 3909–3918, 2007.

[46] M. Mahévas, M. Michel, B. Vingert et al., "Emergence of long-lived autoreactive plasma cells in the spleen of primary warm auto-immune hemolytic anemia patients treated with rituximab," *Journal of Autoimmunity*, vol. 62, pp. 22–30, 2015.

[47] C. D. Cohen, N. Calvaresi, S. Armelloni et al., "CD20-positive infiltrates in human membranous glomerulonephritis," *Journal of Nephrology*, vol. 18, no. 3, pp. 328–333, 2005.

[48] P. Cravedi, P. Ruggenenti, M. C. Sghirlanzoni, and G. Remuzzi, "Titrating rituximab to circulating B cells to optimize lymphocytolytic therapy in idiopathic membranous nephropathy," *Clinical Journal of the American Society of Nephrology*, vol. 2, no. 5, pp. 932–937, 2007.

[49] H.-P. Brezinschek, F. Rainer, K. Brickmann, and W. B. Graninger, "B lymphocyte-typing for prediction of clinical response to rituximab," *Arthritis Research & Therapy*, vol. 14, no. 4, article R161, 2012.

Hindawi Publishing Corporation
Mediators of Inflammation
Volume 2016, Article ID 1062586, 13 pages
http://dx.doi.org/10.1155/2016/1062586

Research Article

Krüppel-Like Factor 4 Is a Regulator of Proinflammatory Signaling in Fibroblast-Like Synoviocytes through Increased IL-6 Expression

Xinjing Luo,[1,2] Jie Chen,[1] Jianwei Ruan,[3] Yongfeng Chen,[1] Xuanrong Mo,[1] Jiangwen Xie,[4] and Guoju Lv[4]

[1]*Department of Basic Medical Sciences, School of Medicine, Taizhou University, Taizhou, Zhejiang 318000, China*
[2]*Institute of Tumor Research, School of Medicine, Taizhou University, Taizhou 318000, China*
[3]*Department of Orthopedics and Sports Medicine, Taizhou Municipal Hospital, Taizhou 318000, China*
[4]*Department of Cardiology, Yingzhou District Second People's Hospital, Ningbo 315000, China*

Correspondence should be addressed to Xinjing Luo; luoxjing@126.com

Received 12 February 2016; Revised 9 May 2016; Accepted 23 May 2016

Academic Editor: Nona Janikashvili

Human fibroblast-like synoviocytes play a vital role in joint synovial inflammation in rheumatoid arthritis (RA). Proinflammatory cytokines induce fibroblast-like synoviocyte activation and dysfunction. The inflammatory mediator Krüppel-like factor 4 is upregulated during inflammation and plays an important role in endothelial and macrophage activation during inflammation. However, the role of Krüppel-like factor 4 in fibroblast-like synoviocyte activation and RA inflammation remains to be defined. In this study, we identify the notion that Krüppel-like factor 4 is higher expressed in synovial tissues and fibroblast-like synoviocytes from RA patients than those from osteoarthritis patients. *In vitro*, the expression of Krüppel-like factor 4 in RA fibroblast-like synoviocytes is induced by proinflammatory cytokine tumor necrosis factor-α. Overexpression of Krüppel-like factor 4 in RA fibroblast-like synoviocytes robustly induced interleukin-6 production in the presence or absence of tumor necrosis factor-α. Conversely, knockdown of Krüppel-like factor 4 markedly attenuated interleukin-6 production in the presence or absence of tumor necrosis factor-α. Krüppel-like factor 4 not only can bind to and activate the interleukin-6 promoter, but also may interact directly with nuclear factor-kappa B. These results suggest that Krüppel-like factor 4 may act as a transcription factor mediating the activation of fibroblast-like synoviocytes in RA by inducing interleukin-6 expression in response to tumor necrosis factor-α.

1. Introduction

The autoimmune disease rheumatoid arthritis (RA) is characterized by persistent synovial inflammation and progressive joint destruction. Several cell types have been implicated in its pathogenesis, including lymphocytes, monocytes, and fibroblast-like synoviocytes (FLSs) [1–3]. FLSs, resident mesenchymal cells in the joint synovium, respond to proinflammatory stimuli including tumor necrosis factor-α (TNF-α) and interleukin-1 (IL-1) and exhibit features of inflammatory cells contributing to the pathogenesis of RA [1, 4]. Once activated, RA FLSs produce several types of cytokines and chemokines, including interleukin-6 (IL-6), interleukin-8 (IL-8), and macrophage inflammatory protein-1 (MIP-1) [5–7]. Of these proinflammatory mediators, IL-6 is an acute-phase inflammatory cytokine that plays a crucial role in joint inflammation and augments bone erosion in RA [8]. Blockade of IL-6 signal transduction represents a potentially useful therapeutic strategy to ameliorate RA inflammation [9, 10]. Given the importance of IL-6 in RA inflammation, identification of the molecular mechanisms regulating its expressions is of considerable importance. Induction of IL-6 by proinflammatory stimuli has been characterized and appears to occur mainly at the level of transcription. Several transcription factors have been implicated in this process [11]. The transcription factor nuclear factor-kappa B (NF-κB) is activated in response to proinflammatory stimuli in RA FLSs and induces IL-6 gene expression [12].

Krüppel-like factors (KLFs) are a subclass of transcription factors that share homology with the *Drosophila* gene Krüppel [13]. These proteins contain zinc finger domains, located at the C-terminus, which bind to either CACCC elements or GC-boxes. The N terminus of these proteins mediates activation or repression of transcription and other protein-protein interactions [14]. Krüppel-like factor 4 (KLF4) is a member of the KLF family which was first found in the epithelial lining of the gut and skin and is involved in terminal differentiation and growth of epithelial cells [15, 16]. KLF4 has also been found to regulate stem cell function, cell survival, proliferation, and differentiation [17, 18]. KLF4 was recently implicated in the inflammation mediated by macrophages and endothelial cells [14, 19, 20] and is reported to be induced by several inflammatory stimuli and to play an important role in the production of inflammatory mediators. For example, in macrophages, KLF4 expression is upregulated in response to interferon-γ (IFN-γ), lipopolysaccharide (LPS), and TNF-α and interacts with the NF-κB family member p65 to cooperatively activate the promoter of inducible nitric oxide synthase (iNOS) [14]. High-mobility group box 1 (HMGB1), an important late inflammatory cytokine during inflammation, participates in the pathogenesis of systemic inflammation and local inflammations, including sepsis and rheumatoid arthritis [21, 22]. It has been demonstrated that HMGB1 interacts with toll-like receptor-2 (TLR-2) and toll-like receptor-4 (TLR-4) to promote the activation of NF-κB and mitogen-activated protein kinases (MAPK), leading to production of proinflammatory cytokines in macrophage and endothelial cells [23–25]. Recently, KLF4 binds to the promoter of HMGB1 and promotes its expression, translocation, and release in RAW 264.7 macrophages in response to LPS stimulation [26]. However, KLF4 may also perform anti-inflammatory functions. In human umbilical vein endothelial cells (HUVECs), KLF4 induced anti-inflammatory and antithrombotic factors, including endothelial nitric oxide synthase (eNOS) and thrombomodulin (TM), and inhibited expression of TNF-α-induced vascular cell adhesion molecule-1 (VCAM1) and tissue factor (TF) [19]. Therefore, the role of KLF4 in inflammation appears to be pleiotropic and its proinflammatory or anti-inflammatory functions are cell-type dependent. However, the role of KLF4 in FLS activation and RA inflammation remains to be defined.

In this study, we examined whether KLF4 is expressed in synovial tissue and in FLSs isolated from RA patients and whether the proinflammatory cytokine TNF-α can induce expression of KLF4. We also assessed the effect of KLF4 on expression of the proinflammatory cytokine IL-6 in RA FLSs and the mechanism by which KLF4 regulates IL-6 gene expression.

2. Methods

2.1. Tissue Preparation and Cell Culture. Human synovial tissue samples were obtained from RA patients and osteoarthritis (OA) patients at joint replacement surgery. OA patients were enrolled in this study as a control. All RA patients fulfilled the American College of Rheumatology 1987 criteria for RA [27]. The criteria are as follows: morning stiffness in and around joints lasting at least 1 hour before maximal improvement; soft tissue swelling of 3 or more joint areas; swelling of the proximal interphalangeal, metacarpophalangeal, or wrist joints; symmetric swelling; rheumatoid nodules; the presence of rheumatoid factor; and radiographic erosion and/or periarticular osteopenia in hand and/or wrist joints. Rheumatoid arthritis is defined by the presence of 4 or more criteria. We included RA patients with a disease duration of at least six months. Patients were allowed to use certain disease-modifying antirheumatic drugs (DMARD) and nonsteroidal anti-inflammatory drugs (NSAID). Patients who received immunosuppressive agents and biological agent such as TNF-α inhibitor were excluded. This study was approved by the Institutional Ethics Committee of Taizhou University, and informed consent was provided from all participants. FLSs were isolated as previously described [28] and cultured in Dulbecco's modified Eagle's medium (Life Technologies, Carlsbad, California, USA) containing 100 IU/mL penicillin and 100 μg/mL streptomycin, supplemented with 10% fetal bovine serum (Life Technologies) at 37°C, in 5% CO_2, and at 95% humidity. Cells at passages 4 to 8 were used in the following experiments.

2.2. Immunohistochemistry. Expression of KLF4 in human synovium was assessed by immunohistochemistry assay as previously described [29]. Synovial tissue obtained from RA patients was fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 24 h and then imbedded in paraffin and sliced into 5 μm sections. The sections were deparaffinized in toluene and rehydrated in a gradient of alcohols. Antigen retrieval of synovium was achieved by incubation at 100°C for 5 min and peroxidase quenching by treatment with 3% hydrogen peroxide in methanol for 10 min. The sections were further blocked with 2% normal goat serum for 1 h and then incubated with rabbit anti-human KLF4 antibody (1 : 100, Abcam, Cambridge, MA, USA) at 4°C overnight. Antibody binding was detected by incubation with a peroxidase-conjugated goat anti-rabbit IgG (1 : 500, Santa Cruz, CA, USA) at room temperature (RT) for 1 h. Staining was developed using diaminobenzidine (DAB) reagent (Boster, Wuhan, China), and counterstaining was performed with hematoxylin.

2.3. Immunocytochemistry. Cells, cultured on chamber slides, were fixed in 4% formaldehyde for 30 min at room temperature (RT). Slides were blocked with 2% bovine serum albumin (BSA) for 1 h at RT and then incubated with rabbit anti-human KLF4 (1 : 200, Abcam) overnight at 4°C, followed by Cy3-conjugated goat anti-rabbit IgG (1 : 100, Santa Cruz) for 2 h. Nuclei were stained with Hoechst 33342, and staining was visualized using fluorescence microscopy (Olympus, Tokyo, Japan).

2.4. Generation of Expression Constructs. Expression plasmids for KLF4 and NF-κB were generated by reverse-transcription PCR (RT-PCR) and cloned into pcDNA3.1. All the constructs were confirmed by sequencing (commercially by Invitrogen) (data not shown). Transfection of RA FLSs was performed by following the manufacturer's

instructions (Lipofectamine™ 2000, Invitrogen, Carlsbad, California, USA).

2.5. KLF4 Knockdown Experiments. Short interfering RNA (siRNA) against human KLF4 (sense: 5′-GCA GCU UCA CCU AUC CGA UTT-3′) and scrambled siRNA (ScRNA) (sense: 5′-UUC UCC GAA CGU GUC ACG UTT-3′) were designed and synthesized by GenePharma (Shanghai, China). 50 nM of KLF4 siRNA or scrambled siRNA was incubated with Lipofectamine RNAiMAX (Invitrogen) for 5 min at RT before addition to the FLSs. After 24 h or 48 h, transfected cells were harvested and KLF4 knockdown and target gene expression were assessed by PCR and western blotting.

2.6. Quantitative Real-Time PCR. Total RNA was isolated from cultured cells using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. RNA (1 μg) was reverse-transcribed using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA). Real-time PCR was performed as previously described [30]. The following primers were used: KLF4 primers, 5′-GCA ATA TAA GCA TAA AAG ATC ACC T-3′ (sense) and 5′-AAC CAA GAC TCA CCA AGC ACC-3′ (antisense); IL-6, 5′-CCT CCA GAA CAG ATT TGA GAG TAG T-3′ (sense) and 5′-GGG TCA GGG GTG GTT ATT GC-3′ (antisense); GAPDH, 5′-CGC TGA GTA CGT CGT GGA GTC-3′ (sense) and 5′-GCT GAT GAT CTT GAG GCT GTT GTC-3′ (antisense). Real-time fluorescence detection was performed on a ABI Prism 7700 sequence detection system (Applied Biosystems, Foster City, CA). Reactions were carried out by mixing 10 μL of power SYBR green PCR master mix (Applied Biosystems), cDNA (100 ng), and forward and reverse primers (0.2 μM each) in a final PCR reaction volume of 20 μL. The amplification parameters were as follows: 95°C for 3 min (1 cycle), 95°C for 15 s, and 60°C for 60 s (40 cycles). The fluorescent signal was plotted versus cycle number, and the cycle threshold (Ct) was determined. Product specificity was confirmed by melting curve analysis. The slope of the standard curve was used to determine the amplification efficiency. Samples were analyzed in duplicate, using housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an endogenous control, and the results were quantified using the comparative Ct method [31]. The relative quantity of each sample was normalized by subtracting the Ct of GAPDH to the target gene ([Δ]Ct = Ct of target gene − Ct of GAPDH). The relative quantity of target gene in a test sample was determined by comparing normalized target quantity in a test sample to normalized target quantity in the reference sample (−[Δ][Δ]Ct). Values were expressed using the following formula: $2^{-[\Delta][\Delta]Ct}$.

2.7. Western Blot. Cultured cells were lysed in radioimmune precipitation assay buffer [50 mM Tris-HCl (pH 7.4), 1% Triton X-100, 0.5% sodium deoxycholate, 150 mM sodium chloride, and 1% Nonidet P-40] supplemented with the complete protease inhibitor cocktail (Sigma) for 40 min at 4°C. The homogenate was centrifuged for 15 min, and then the supernatant was collected and the protein concentration of the fractions was estimated by a standard Bradford assay. 20–30 μg of each protein sample was reduced and denatured by boiling in 2x sodium dodecyl sulfate (SDS) sample buffer [100 mM Tris-HCl (pH 6.8), 200 mM dithiothreitol (DTT), 4% SDS, 20% glycerol, and 0.1% bromophenol blue]. The proteins were separated on 10% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) and wet-transferred onto polyvinylidene fluoride (PVDF) membrane (Millipore, MA, USA) at 380 mA for 60 min. Membranes were blocked in blocking buffer (5% skim milk, 0.2% Tween-20 in Tris-buffered saline) for 4 h at RT and incubated with the primary antibody [rabbit anti-KLF4 polyclonal antibody (1 : 2000, Abcam); rabbit anti-NF-κB-p65 polyclonal antibody (1 : 2000, Cell Signaling Technology, MA, USA); rabbit anti-tubulin polyclonal antibody (1 : 1000, Cell Signaling Technology); rabbit anti-histone 3 (H3) monoclonal antibody (1 : 1000, Cell Signaling Technology); rabbit anti-GAPDH monoclonal antibody (1 : 1000, Cell Signaling Technology)] overnight at 4°C with gentle agitation. After three washes of ten minutes each in 1x PBS-Tween-20 (1x PBST), the membranes were incubated with goat anti-rabbit horseradish peroxidase conjugate (1 : 5000, Santa Cruz, CA, USA) for 1 h at RT. The membranes were rinsed again in 1x PBST. Immunoreactive bands were developed by enhanced chemiluminescence reagent (Millipore) and bands of interest were scanned and quantified with Band Leader software (Shanghai, China).

2.8. Luciferase Assay and Reporter Construct. The IL-6 promoter construct was generated by PCR amplification of the IL-6 promoter from human genomic DNA using the following primers: 5′-CGC CTC GAG TGG ATG TAT GCT CCC GAC TT-3′ (forward) and 5′-CGC AAG CTT GCT ACA GAC ATC CCC AGT CTC-3′ (reverse). The fragment was cloned into the pGL3 vector, and the construct was confirmed by sequencing. For the luciferase reporter assay, RA FLSs were seeded in 24-well culture plates and 24 h later transfected using Lipofectamine 2000 transfection reagent according to the manufacturer's recommendations (Invitrogen). Each transfection was performed with 500 ng of pGL3-IL-6 promoter reporter construct, with or without transcription factor plasmid, and 20 ng of pRL-null control plasmid, in triplicate. Transfected cells were treated with TNF-α (Sigma) for the indicated time. After cell lysis, supernatants were collected and luciferase activity was detected using the Promega dual luciferase reporter assay system according to the manufacturer's instructions (Promega, Wisconsin, USA).

2.9. Electrophoretic Mobility Shift Assay (EMSA). EMSA was performed using nuclear extract from RA FLSs using a LightShift Chemiluminescent EMSA Kit according to the manufacturer's instructions (Pierce Biotechnology, Rockford, USA). Biotin-labeled DNA probes for the KLF4 binding sites at positions −109 to −90 bp and −132 to −102 bp of the IL-6 promoter were generated as previously described [32]. Briefly, nuclear extract (5 μg) was incubated with biotin-labeled DNA probes or unlabeled cold probe at RT for 20 min. The reaction mixtures were electrophoresed on 6% polyacrylamide gels and wet-transferred onto a nylon membrane (Millipore, MA, USA). After transfer, the protein-bound

probe and free probe were immobilized using a UV cross-linker. The membranes were blocked and then incubated with streptavidin-horseradish peroxidase conjugate solution. After washing with wash buffer supplied by the manufacturer, the membrane was incubated in substrate equilibration buffer supplied by the manufacturer for 5 min, and immunoreactive bands were developed by enhanced chemiluminescence reagent (Millipore). For supershift assays, the nuclear extract was preincubated with rabbit anti-KLF4 antibody (Abcam) at 4°C for 1 h before biotin-labeled probes were added to the binding reaction mixture.

2.10. Coimmunoprecipitation (Co-IP). Immunoprecipitation (IP) was performed using nuclear extract from RA FLSs as previously described [30]. Nuclear extracts (50 μg) of RA FLSs were incubated with antibodies directed towards NF-κB-p65 (1 μg, Cell Signaling Technology), KLF4 (1 μg, Abcam), and rabbit IgG (1 μg) in IP buffer (50 mM Tris-HCl (pH 7.6), 150 mM sodium chloride, 1% Nonidet P-40, and 0.5% sodium deoxycholate) at 4°C overnight. 10 μL of Sepharose G beads (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) was rinsed with IP buffer [50 mM Tris-HCl (pH 7.6), 50 mM sodium chloride, 0.1% Nonidet P-40, and 0.05% sodium deoxycholate] and mixed with the antibody-incubated supernatant at RT for 4 h with gentle shaking. Then, the mixture was centrifuged at 4°C and 890 g for 3 min to collect the beads. The beads were washed in IP buffer (50 mM Tris-HCl (pH 7.6), 0.1% Nonidet P-40, and 0.05% sodium deoxycholate) and then mixed with 2x SDS loading buffer and boiled for 5 min. The supernatant was collected by centrifugation and loaded onto the gel for immunoblotting (as previously described).

2.11. Enzyme-Linked Immunosorbent Assay (ELISA). RA FLSs were incubated with TNF-α (Sigma) for the indicated times. Supernatants were harvested and IL-6 concentrations were determined using sandwich ELISA, following the manufacturer's instructions (eBioscience). Absorbance at 450 nm was measured with a microplate reader (Bio-Rad, USA). A standard curve was generated by plotting absorbance versus log recombinant human IL-6 concentration. IL-6 was quantitated from a standard curve including known amounts of recombinant human IL-6. All data were normalized by cell number.

2.12. Statistical Analysis. Data was expressed as mean ± SEM. Unpaired Student's t-test was used for comparison between two groups. One-way analysis of variance (ANOVA) followed by Tukey-Kramer *post hoc* test was used for multiple comparisons. A P value of <0.05 was considered to represent statistical significance.

3. Results

3.1. KLF4 Expression in the Human Synovial Tissues of RA Patients. We used real-time PCR and western blotting to investigate whether KLF4 is expressed in the synovial tissues from patients with RA and OA. KLF4 expression was found in the synovial tissues from patients with both RA and OA, and KLF4 expression was higher in RA than in OA (Figures 1(a) and 1(b)). Immunohistochemistry revealed that KLF4-positive cells in the synovial tissues from RA patients were found predominantly in the synovial lining (Figure 1(c)).

To investigate whether this pattern of KLF4 expression is reflected in cultured primary FLSs isolated from the synovial tissues from patients with RA and OA, we harvested total mRNA and protein from these cells. RT-PCR and western blotting verified that KLF4 is expressed in cultured FLSs from patients with both RA and OA, and KLF4 expression was higher in RA than in OA (Figures 1(d) and 1(e)). Immunofluorescence revealed that KLF4 was localized in the nuclei of RA FLSs (Figure 1(f)).

3.2. TNF-α Induces KLF4 and IL-6 Gene Expression in RA FLSs. Previous studies have demonstrated that KLF4 is induced by proinflammatory stimuli in several cell types including macrophages and endothelial cells [14, 19]. However, the influence of proinflammatory stimuli on its expression in RA FLSs remains unclear. To determine whether KLF4 can be induced in human RA FLSs under inflammatory conditions, cells were treated with different doses of TNF-α for various periods of time. We found that the level of KLF4 mRNA and protein rapidly increased in RA FLSs incubated with TNF-α (20 ng/mL) within 24 h (Figures 2(a) and 2(b)). The expression of KLF4 mRNA and protein was peaked after 3 h and 16 h incubation with TNF-α, respectively. TNF-α elevated expression of both KLF4 mRNA and protein in a concentration-dependent manner. Maximal expression was observed in cells incubated with 20 to 40 ng/mL TNF-α (Figures 2(c) and 2(d)).

FLS activation and inflammation in RA are associated with upregulation of several proinflammatory cytokines. IL-6 is reported to be the key proinflammatory cytokine produced by FLSs in the RA synovium [33]. Thus, we analyzed the influence of TNF-α on IL-6 expression in FLSs. As shown in Figure 2(e), IL-6 mRNA levels in FLSs were significantly increased after incubation with TNF-α for 1 to 6 h and peaked at 3 h. Similarly, levels of IL-6 protein also increased significantly after incubation with TNF-α for 3 to 24 h and peaked at 24 h (Figure 2(f)). The increase in the level of IL-6 corresponded well to the increase in the level of KLF4 in RA FLSs induced by TNF-α, suggesting a relationship between these two proteins.

3.3. Overexpression of KLF4 Increases IL-6 Expression in RA FLSs. Because KLF4 is induced by TNF-α and TNF-α-induced KLF4 expression corresponded to TNF-α-induced IL-6 expression in RA FLSs, we considered the possibility that KLF4 may regulate IL-6 expression.

In order to investigate the effect of KLF4 on expression of IL-6, RA FLSs were transfected with a pcDNA3.1-KLF4 construct. Overexpression of KLF4 induced significant increases in cellular content of both IL-6 mRNA and protein in the absence of TNF-α (Figure 3). This effect was potently enhanced after TNF-α stimulation. These data indicate that KLF4 overexpression enhances expression of IL-6 in RA FLSs.

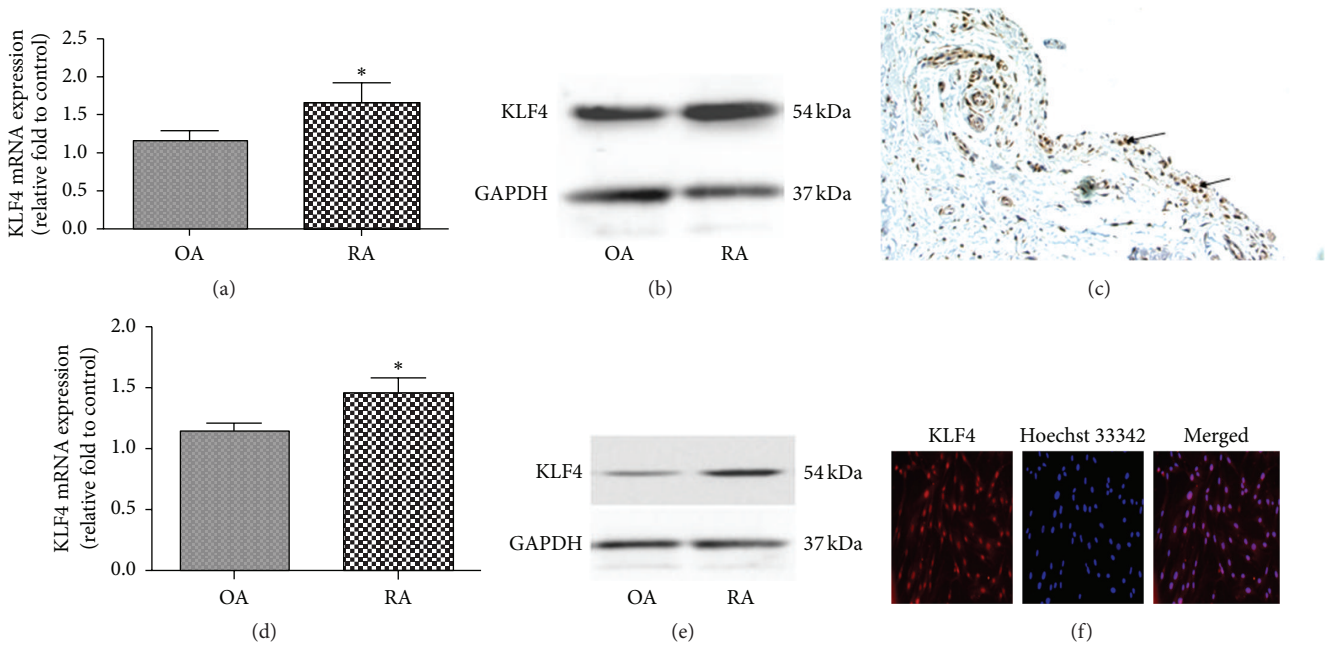


Figure 1: KLF4 expression in synovial tissue and FLSs from RA patients. Real-time PCR (a) and western blot (b) were performed to assess KLF4 expression in synovial tissues from patients with RA ($n = 9$) and OA ($n = 4$). GAPDH as an endogenous control. $^{*}P < 0.05$ in comparison to OA group. (c) KLF4 expression in synovial tissues from patients with RA was assessed by immunohistochemistry. Arrows indicate KLF4-positive cells (200x magnification). Real-time PCR (d) and western blot (e) were performed to assess KLF4 expression in isolated FLSs from patients with RA ($n = 9$) and OA ($n = 4$). GAPDH as an endogenous control. $^{*}P < 0.05$ in comparison to OA group. (f) KLF4 expression in isolated FLSs from patients with RA was assessed by immunofluorescence. Cell nuclei were visualized by Hoechst 33342 staining (100x magnification).

3.4. Knockdown of KLF4 Decreases Expression of IL-6 in RA FLSs. To further assess the role of KLF4 in regulating IL-6 expression, siRNA was used to knock down KLF4 expression in RA FLSs. The level of IL-6 mRNA and protein was significantly lower in siRNA-transfected cells than in ScRNA-transfected cells, both in the presence and in the absence of TNF-α ($P < 0.05$; Figure 4). Taken together, these results suggest that KLF4 can regulate IL-6 expression in RA FLSs.

3.5. KLF4 Regulates IL-6 Promoter Activity in RA FLSs. To understand the mechanism by which KLF4 regulates IL-6 gene expression in RA FLSs, we investigated activation of the IL-6 promoter using a luciferase assay. As shown in Figure 5(a), KLF4 transactivated the IL-6 promoter in RA FLSs. IL-6 promoter activity was significantly higher in cells transfected with the KLF4 expression plasmid than in cells transfected with empty vector, both in the presence and in the absence of TNF-α stimulation ($P < 0.05$).

To determine whether KLF4 can bind the potential KLF4 binding element on the IL-6 promoter, EMSA experiments were performed using a biotinylated IL-6 probe for two segments of the IL-6 promoter (−109 to −90 bp and −132 to −102 bp), which are hypothesized to be important for KLF4 binding to the IL-6 promoter [32]. As shown in Figures 5(b) and 5(c), KLF4 binds to these two elements, and the addition of antibody against KLF4 led to a supershift of the band. The specificity of KLF4 for the probe sequence was confirmed using mutant oligonucleotides and cold probes. DNA-protein binding was significantly increased after TNF-α stimulation for 1 or 2 h (Figures 5(d) and 5(e)). Taken together, these results indicate that KLF4 interacts directly with the IL-6 promoter to regulate IL-6 expression.

3.6. KLF4 Potentially Interacts with NF-κB to Induce the IL-6 Promoter. In response to inflammatory stimuli, the transcription factor NF-κB has been demonstrated to induce IL-6 in RA FLSs [11]. The KLF4 binding sites on the IL-6 promoter are located in close proximity to the NF-κB binding site. Therefore, we hypothesized that KLF4 and NF-κB may interact directly. First, we investigated whether KLF4 could enhance induction of IL-6 expression by NF-κB-p65. We found that KLF4 overexpression augmented the induction of the IL-6 promoter by NF-κB (Figure 6(a)). Next, we assessed whether KLF4 mediates its effects *via* regulation of either NF-κB expression or nuclear translocation. KLF4 overexpression did not increase expression of p65 or its nuclear accumulation in response to TNF-α (Figure 6(b)). Finally, we used coimmunoprecipitation studies to investigate whether KLF4 and NF-κB directly interact. Nuclear extracts were probed with antibodies directed towards NF-κB-p65 or KLF4, and we found that KLF4 was present in the complexes immunoprecipitated by the anti-NF-κB-p65 antibody (Figure 6(c)). Conversely, NF-κB-p65 was present in complexes precipitated by the anti-KLF4 antibody. Taken together, our findings suggest that KLF4 interacts physically with NF-κB to cooperatively induce the IL-6 promoter.

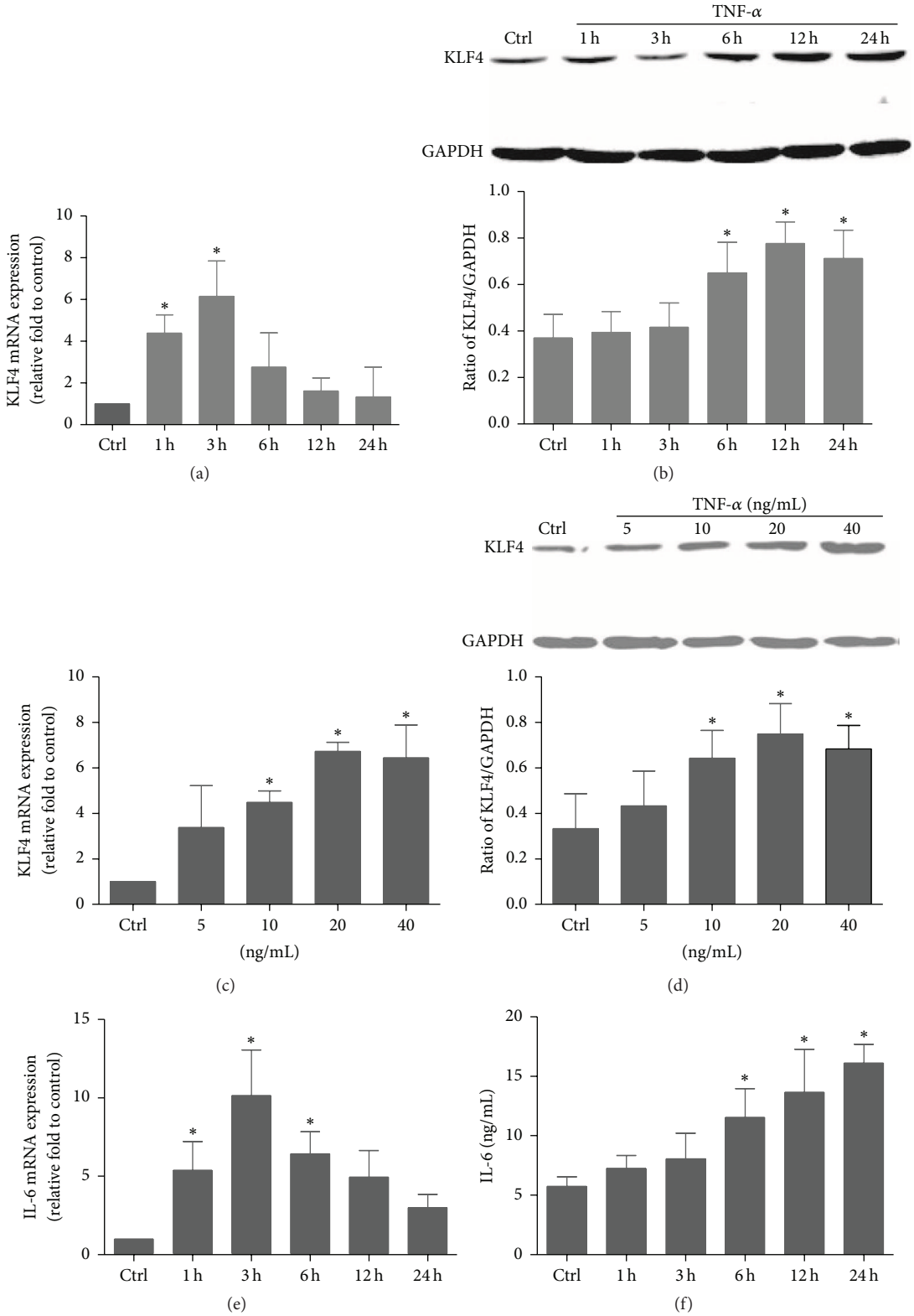


Figure 2: Expression of KLF4 and IL-6 in RA FLSs in response to TNF-α. KLF4 is induced by TNF-α in a time- and dose-dependent fashion. RA FLSs were incubated with 20 ng/mL TNF-α for the indicated time periods, and expression of KLF4 was assessed by real-time PCR (a) and western blotting (b). RA FLSs were incubated with TNF-α at different doses, and expression of KLF4 was assessed by real-time PCR after 3 h (c) and western blotting after 12 h (d). The lower panels in (b) and (d) indicate the ratio of KLF4/GAPDH protein. Time course of RA FLS IL-6 expression in response to 20 ng/mL TNF-α was assessed by real-time PCR (e) and ELISA (f). GAPDH as an endogenous control. Data were expressed as means ± SEM ($n = 3$); $^*P < 0.05$ in comparison to the untreated cells (Ctrl).

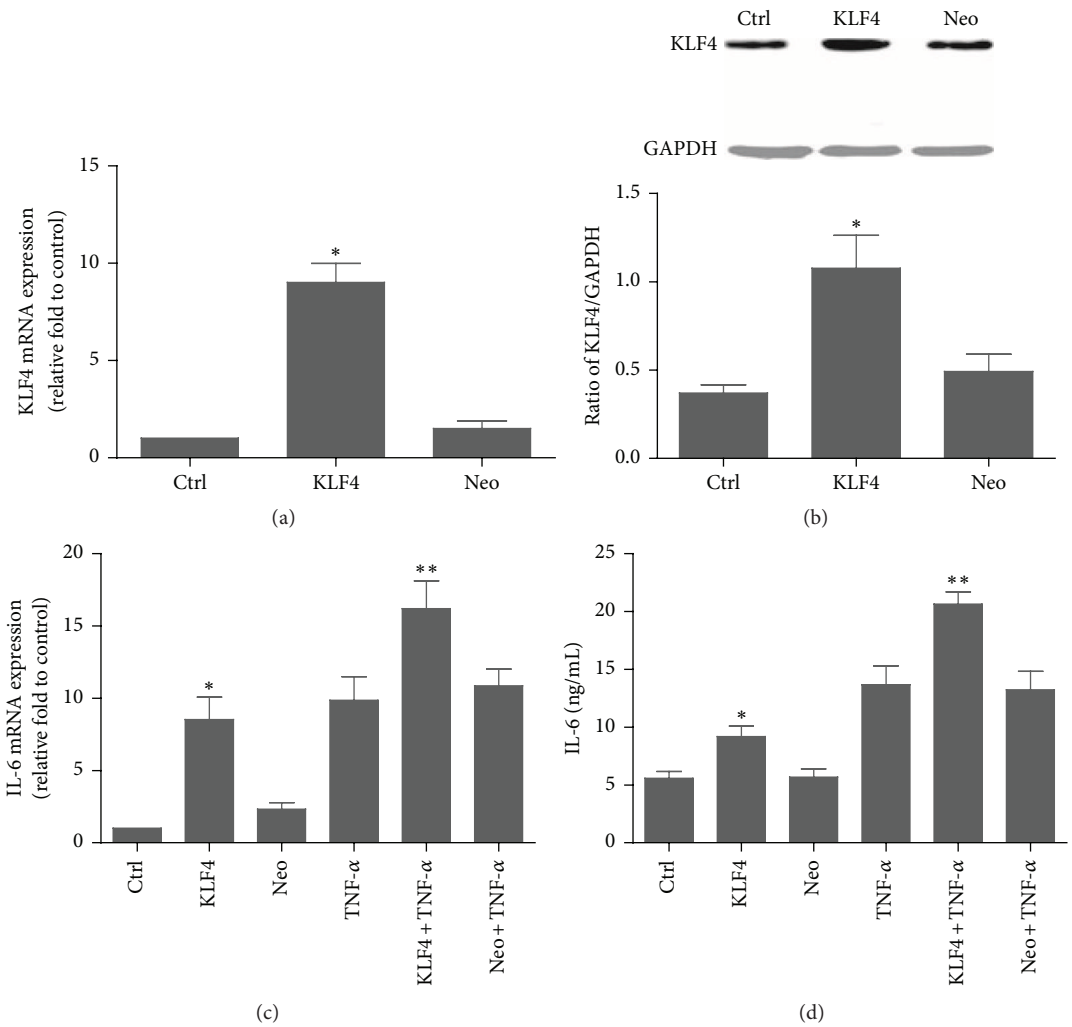


Figure 3: KLF4 overexpression induces IL-6 expression in RA FLSs. RA FLSs were transiently transfected with pcDNA3.1-KLF4 (KLF4) or the pcDNA3.1 empty vector (Neo) and then incubated with TNF-α (20 ng/mL). Expression of KLF4 was assessed after 3 h by real-time PCR (a) and after 12 h by western blotting (b). Expression of IL-6 was assessed after 3 h by real-time PCR (c) and ELISA (d). GAPDH as an endogenous control. Data were expressed as mean ± SEM ($n = 3$); $^{*}P < 0.05$ in comparison to the untreated cells (Ctrl); $^{**}P < 0.05$ in comparison to the TNF-α group.

4. Discussion

FLSs play a crucial role in joint inflammation and destruction in RA, mainly through production of proinflammatory cytokines. Several signaling pathways and transcription factors have been implicated in FLS activation and inflammation associated with RA [11, 34]. Recently, KLF4, a zinc finger-containing transcription factor, was reported to be involved in endothelial and macrophage-mediated inflammation [14, 19]. KLF4 was initially found in the epithelial lining of the gut and skin and was considered to be an epithelium-specific transcription factor that is involved in normal development of epithelia and carcinogenesis [35]. KLF4 was also recently reported to be expressed in the monocyte/macrophage lineage [14]. KLF4 regulates not only monocyte commitment and differentiation [36, 37], but also macrophage activation. KLF4 overexpression in J774a macrophages induced the macrophage activation marker iNOS and reduced expression of plasminogen activator inhibitor-1 [14]. Conversely, KLF4 knockdown inhibited iNOS induction by IFN-γ and/or LPS, whereas it led to enhanced responsiveness to TGF-β1 and Smad3 signaling [14]. In the RAW246.7 macrophage cell line, KLF4 has been shown to induce expression, translocation, and release of the late proinflammatory cytokine HMGB1 in response to LPS [26]. KLF4 expression has been demonstrated in microglial cells, the resident macrophages of the central nervous system, and it is significantly induced by LPS [30]. KLF4 knockdown in microglial cells reduced expression of TNF-α, MCP-1, IL-6, iNOS, and cyclooxygenase-2 (Cox-2) after induction by LPS [30]. Similar to mouse cells, KLF4 is markedly induced by proinflammatory cytokines including IFN-γ, LPS, or TNF-α and mediates proinflammatory signaling in human macrophages [14]. Several *in vitro* and *in vivo* studies [19, 20] demonstrated that human and mouse endothelial cells from arterial and venous blood vessels also

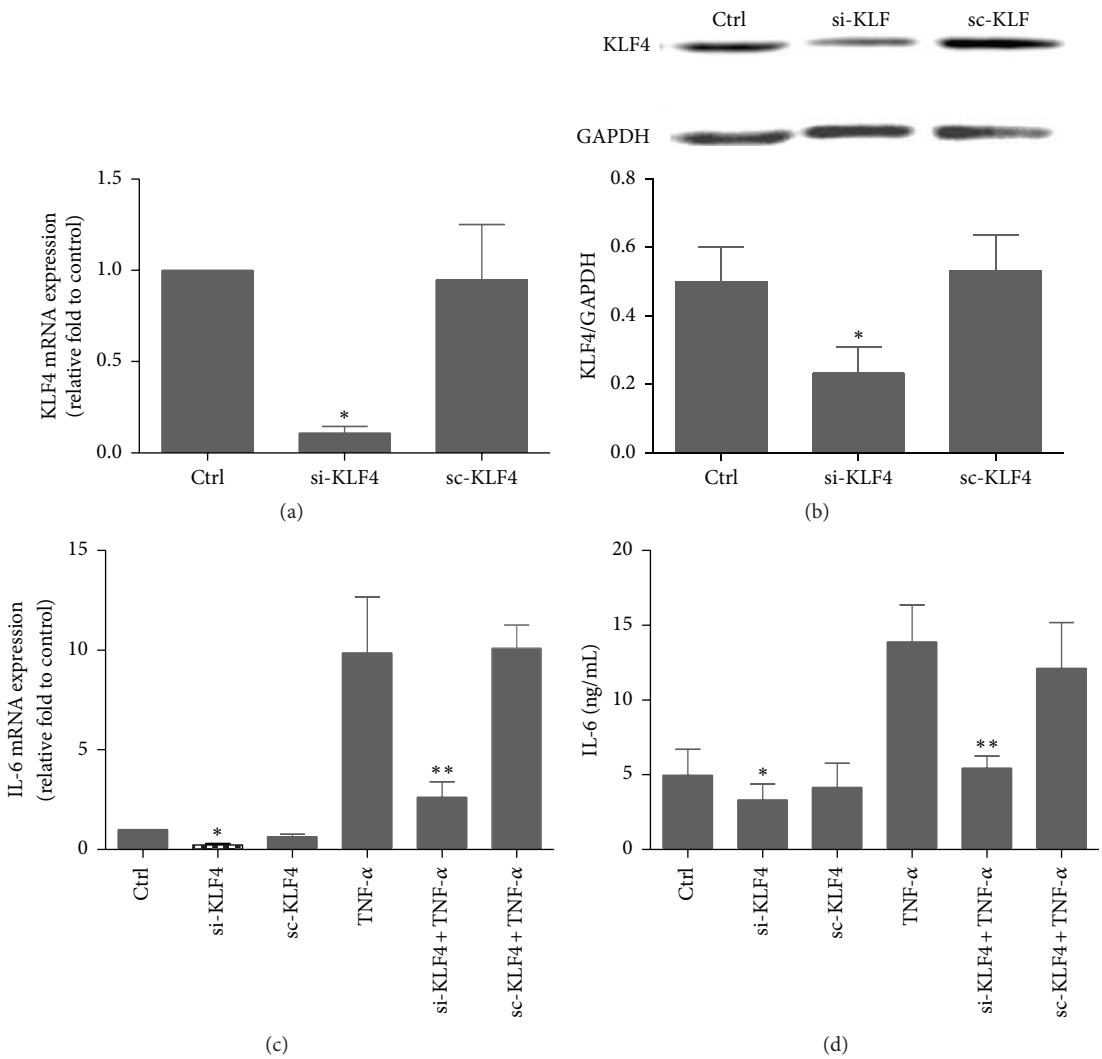


Figure 4: Effect of KLF4 knockdown on IL-6 expression in RA FLSs. RA FLSs were transiently transfected with KLF4 siRNA oligonucleotide (si-KLF4) or KLF4 scrambled siRNA (sc-KLF4) for 48 h and then incubated with TNF-α (20 ng/mL). KLF4 inhibition was assessed after 3 h by real-time PCR (a) and after 12 h by western blot (b). IL-6 expression was assessed by real-time PCR (c) and ELISA (d). GAPDH as an endogenous control. Data were expressed as means ± SEM ($n = 3$); $^{*}P < 0.05$ in comparison to the untreated cells (Ctrl); $^{**}P < 0.05$ in comparison to the TNF-α group.

expressed KLF4, and KLF4 was markedly induced by shear stress and inflammatory cytokines including TNF-α, IL-Iβ, and interferon-γ. KLF4 has been found to suppress inflammation by downregulating MCP-1, VCAM, and TM and upregulating eNOS and TF in endothelial cells [19]. Recent studies showed that KLF4 was highly expressed in thymocytes and mature T cells, and KLF4 directly binds to the promoter of IL-17 and positively regulates its expression [38]. The wide influence of KLF4 on inflammatory cells spurred our interest in exploring the relationship between KLF4 and RA. In this study, we established that KLF4 was expressed in synovial tissue and cultured FLSs isolated from both RA patients and OA patients, and KLF4 expression was higher in synovial tissue from RA patients than those from OA patients. We also found that the proinflammatory cytokine TNF-α induced KLF4 expression in RA FLSs in a time- and dose-dependent manner. Immunostaining revealed that KLF4 is mainly localized to the nucleus, in accordance with previous reports that found KLFs to be associated with specific regions of the nucleus, potentially with nucleoli [30, 39]. This data suggests that KLF4 is a transcription factor localized in nuclear regions of active transcription and regulates target gene expression in RA FLSs. Our results suggest a possible role for KLF4 in TNF-α-mediated inflammation in RA FLSs. While our studies have demonstrated that KLF4 expression was induced by TNF-α in RA FLSs in a dose-dependent manner, the relation between KLF4 expression in FLSs and TNF-α level *in vivo* has not been elucidated. Further investigations will also be needed to determine whether medications, including DMARGD or TNF inhibitors, affect KLF4 expression in RA FLSs.

Several cytokines and chemokines contribute to the progression and maintenance of inflammation in RA. Of these,

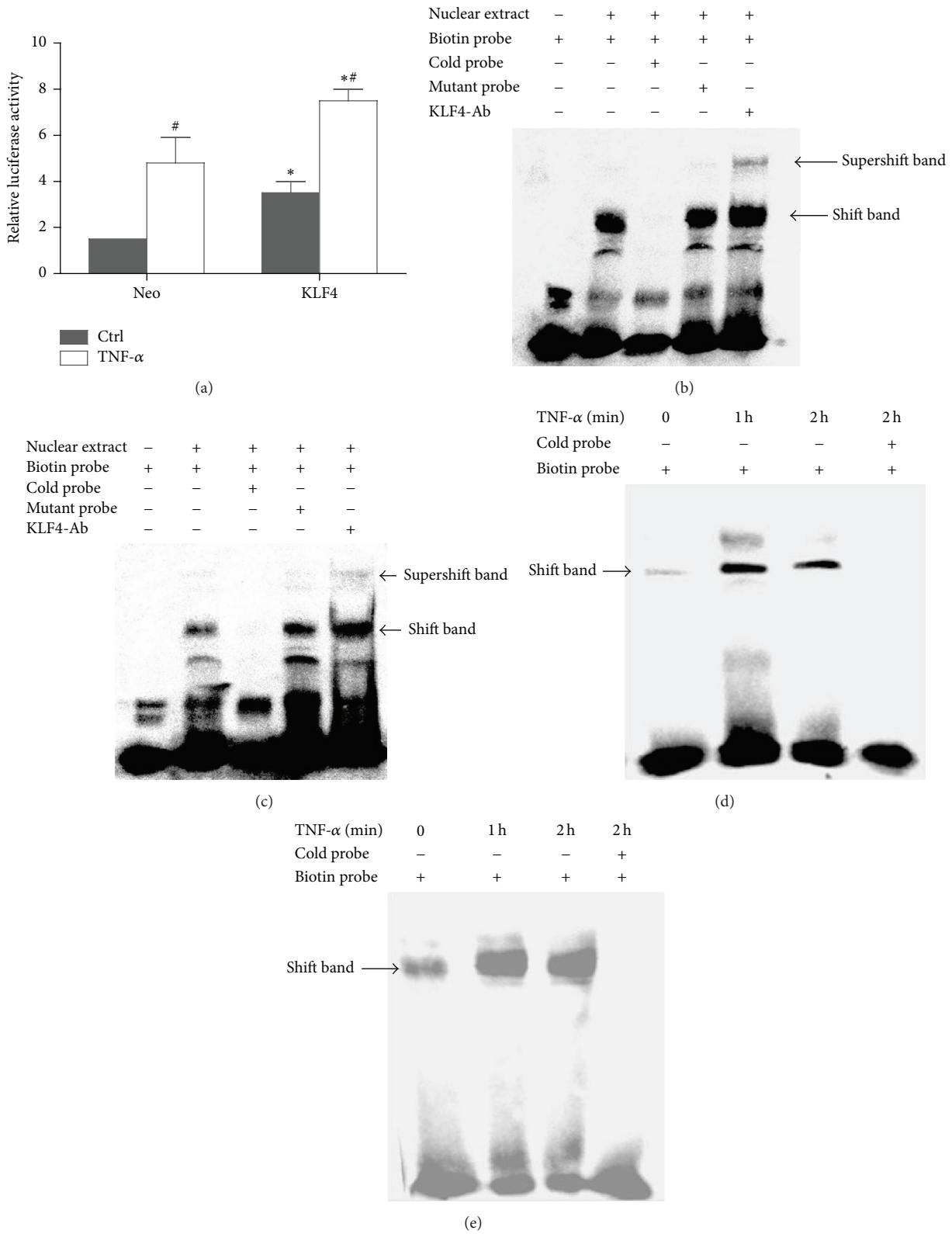


Figure 5: KLF4 regulates IL-6 promoter activity in RA FLSs. KLF4 transactivates the IL-6 promoter. RA FLSs were cotransfected in triplicate with the indicated IL-6 promoter luciferase plasmids, pcDNA3.1-KLF4 expression plasmid (KLF4), or pcDNA3.1 control vector (Neo) for 48 h and then incubated with TNF-α (20 ng/mL) for 12 h. Transcriptional activity was detected by the Dual Luciferase Assay (a). Data were expressed as means ± SEM ($n = 3$). $^{*}P < 0.05$ in comparison to the vector control group (Neo). $^{\#}P < 0.05$ in comparison to untreated group (Ctrl). KLF4 binds the IL-6 promoter. EMSA was performed with nuclear extracts using a biotinylated probe of the −109 to −90 bp site (b) and −132 to −102 bp site (below) in the IL-6 promoter (c). TNF-α augmented binding of KLF4 to the IL-6 promoter. EMSA was performed with nuclear extracts from cells incubated with TNF-α for 1 or 2 h. The previously described biotinylated probes (−109 to −90 bp and −132 to −102 bp) were used ((d) and (e), resp.).

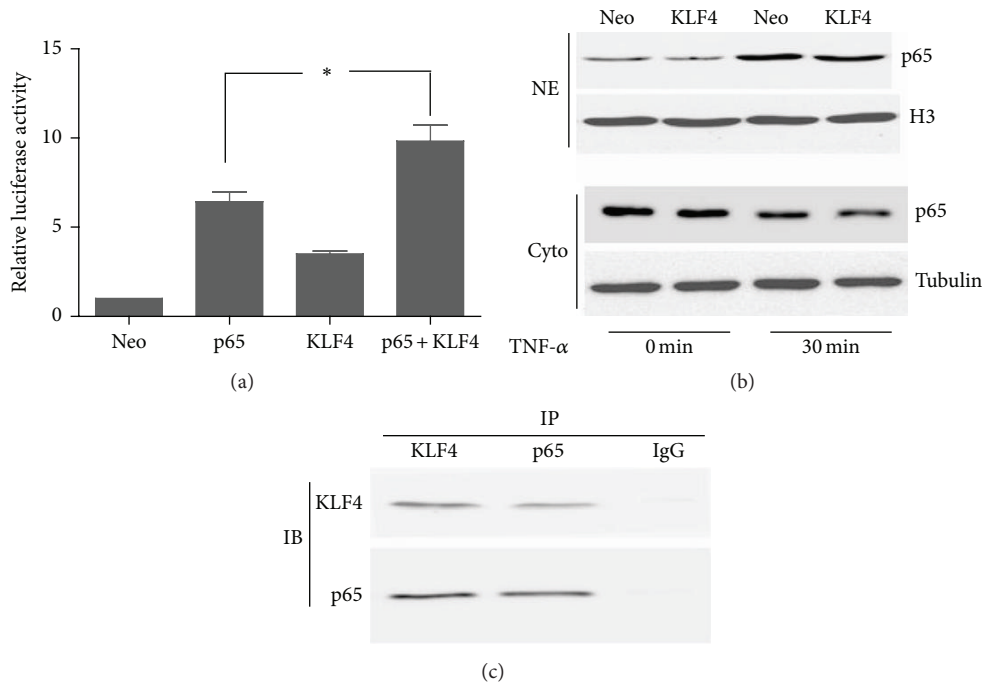


Figure 6: KLF4 and NF-κB-p65 cooperatively induce the IL-6 promoter. KLF4 and NF-κB-p65 cooperatively induce IL-6 promoter activity. RA FLSs were cotransfected with the indicated IL-6 promoter luciferase plasmids, pcDNA3.1-NF-κB-p65 expression plasmid (p65), pcDNA3.1-KLF4 expression plasmid (KLF4), or the pcDNA3.1 empty vector (Neo). IL-6 promoter activity was measured by Dual Luciferase Assay (a). Data were expressed as means ± SEM ($n = 3$). $^*P < 0.05$ in comparison to the p65 group. KLF4 does not affect either expression or nuclear translocation of NF-κB-p65. RA FLSs were transfected in triplicate with pcDNA3.1-KLF4 expression plasmid (KLF4) or pcDNA3.1 empty vector (Neo) and then incubated with TNF-α (20 ng/mL) for 30 min. Expression of NF-κB-p65 (p65) in the cytoplasm and nucleus was detected by western blot. Cyto: cytoplasmic extracts; NE: nuclear extracts. Histone 3 (H3) and Tubulin served as controls for nuclear and cytoplasmic proteins. KLF4 associates with NF-κB-p65. Nuclear extracts from RA FLSs were immunoprecipitated (IP) with anti-KLF4, anti-NF-κB-p65 (p65), or anti-IgG antibodies, followed by immunoblotting (IB) with the indicated antibodies (c).

FLS-derived IL-6 is considered to be crucially important and is therefore a target of RA drug development [12, 40]. IL-6 is a pleiotropic cytokine with a variety of biological activities including B- and T-cell activation, induction of autoantibody and peripheral blood platelet production, and release of acute-phase proteins [33]. In fact, increased levels of IL-6 have been found in the serum and synovial fluid of RA patients, and IL-6 levels were closely associated with disease activity [41–44]. IL-6 may both amplify the effects of TNF-α and IL-1 and stimulate the production of rheumatoid factors, acute-phase reactants, tissue-degrading enzymes, and other proinflammatory mediators [45, 46]. IL-6 has been well documented to play an indispensable role in RA inflammation, as blocking the IL-6 receptor can alleviate symptoms of RA [9, 10, 47]. In addition to macrophages and dendritic cells, human FLSs produce IL-6 and are considered to be a main source of IL-6 in the RA synovium [33]. IL-6 is spontaneously produced by cultured FLSs, and its expression is markedly upregulated by TNF-α [4]. Although TNF-α is a potent inducer of IL-6 production in human FLSs, the mechanism by which TNF-α induces IL-6 expression in FLSs remains unclear. Transcription factor NF-κB was also reported to participate in regulation of IL-6 expression. Binding sites for NF-κB have been identified in the promoter region of the IL-6 gene, and, under certain conditions, NF-κB has been reported to activate transcription of the IL-6 gene and expression of IL-6 in RA FLSs [11, 12, 34].

Recently, the transcription factor KLF4 was reported to be essential for the regulation of inflammatory mediator gene expression in the primordial mesenchymal cell lineage, thus controlling generation of lymphocytes [18, 38], macrophages [14, 26, 35], and endothelial cells [19, 20]. Using bioinformatics analysis, we found that the IL-6 promoter contains KLF4 binding sites (CACCC). Importantly, we found that TNF-α-induced expression of KLF4 corresponded to IL-6 expression in RA FLSs. Therefore, we hypothesized that KLF4 might regulate IL-6 expression in RA FLSs. In this study, we demonstrated that KLF4 is essential for IL-6 production in RA FLSs. By experimentally overexpressing KLF4 or using KLF4 siRNA to downregulate KLF4 expression in RA FLSs, we found that expression of both IL-6 mRNA and protein was significantly upregulated after KLF4 overexpression and downregulated after KLF4 knockdown in the presence or absence of TNF-α. Our results indicate that KLF4 can promote IL-6 expression in RA FLSs, even under inflammatory conditions, suggesting that it may play a vital role in RA inflammation. Consistent with these proinflammatory effects, it has been reported that KLF4 can

induce expression of IL-6 in microglia and dendritic cells [32]. However, in endothelial cells, KLF4 has been reported to suppress expression of IL-6 [19]. These observations indicate that the effect of KLF4 on the regulation of IL-6 expression varies with cell type; however, our findings indicate that, in FLSs, KLF4 regulates IL-6 expression, and this activity is likely relevant to the induction of IL-6 observed in RA.

KLF4 has previously been demonstrated to bind KLF4 DNA binding sites in the promoters of iNOS, TNF-α, IL-10, MCP-1, HMGB1, and IL-6 and to regulate expression of these inflammatory mediators [14, 26, 32, 35]. KLF4 has been reported to activate the iNOS promoter in macrophages by binding KLF4 binding sites at positions −95 and −212 bp [14]. KLF4 regulates HMGB1 expression in RAW264.7 macrophages by binding the KLF4 binding element in the HMGB1 promoter [26]. In dendritic cell lines, KLF4 activates expression of IL-6 primarily through binding to a proximal CACCC site in the IL-6 promoter [32]. In this study, we also demonstrated that KLF4 regulates transcription of IL-6 in RA FLSs by binding to two KLF4 binding elements in regions −109 to approximately −90 bp and −132 to approximately −102 bp from the transcriptional start site of the IL-6 promoter, and binding activity was enhanced after TNF-α stimulation. These results suggest that KLF4 regulates expression of IL-6 in RA FLSs by directly binding the IL-6 promoter.

The role of NF-κB in the regulation of TNF-α-induced IL-6 expression in FLSs is well established. Interestingly, the KLF4 binding sites in the IL-6 promoter are close to NF-κB binding site [32]. Kaushik et al. previously demonstrated that KLF4 interacts with pNF-κB to cooperatively induce transcription of the iNOS and Cox-2 genes in microglial cells [30]. We hypothesized that KLF4 might exert its effects through direct interaction with other proteins such as NF-κB in RA FLSs. Our results demonstrate that KLF4 interacts directly with NF-κB to induce the IL-6 promoter in RA FLSs. We also considered the possibility that KLF4 exerts its effects through promoting expression or nuclear translocation of NF-κB. However, we found that KLF4 overexpression did not affect either the expression or the nuclear accumulation of NF-κB in either the presence or the absence of TNF-α. In addition, we did not find evidence that KLF4 expression and KLF4-NF-κB binding in RA FLSs were altered by increased NF-κB expression (data not shown). Therefore, our data suggest that KLF4 is a binding partner of NF-κB and coregulates IL-6 expression in FLSs from RA patients. Of interest, contrary to our studies, KLF4 inhibited TNF-α-induced expression of the vascular cell adhesion molecule-1 (VCAM1) through blocking the binding of NF-κB to VCAM1 promoter in cultured endothelial cells [48]. These differences may lie in the different types of cells used for the experiments. In addition, whether interaction of KLF4 with additional factors influences the regulation of IL-6 expression in RA FLSs needs further investigation. It has been shown that the interaction of HMGB1 and TLR4 leads to the activation of NF-κB [23, 24]. It is possible that the HMGB1-TLR4 pathway induces KLF4 expression, which may subsequently result in enhanced activation of NF-κB in FLSs.

5. Conclusions

In summary, we established KLF4 to be a TNF-α-induced transcription factor that regulates expression of the key proinflammatory cytokine IL-6 in RA FLSs through both direct promoter activation and interaction with NF-κB. These results indicate that KLF4, which is induced by proinflammatory stimuli, can regulate FLSs activation and RA-associated inflammation. Studies on other target inflammatory mediator genes regulated by KLF4 are currently underway.

Competing Interests

The authors declare that there are no competing interests regarding the publication of this paper.

Authors' Contributions

Xinjing Luo and Jie Chen conceived of the study, participated in the design, and drafted the paper. Jianwei Ruan participated in the design, performed the experiments, and helped to draft the paper. Xuanrong Mo and Yongfeng Chen performed the experiment and revised the paper. Jiangwen Xie and Guoju Lv analyzed and interpreted the data and helped to revise the paper.

Acknowledgments

This research was supported by the National Natural Science Foundation of China (no. 81373139), the Zhejiang Provincial Natural Science Foundation of China (Y16H100004), the Zhejiang Provincial Public Welfare Technology Application Research Project (no. 2015C37122), the Zhejiang Provincial Medical Scientific Research Foundation (no. 2015103108), and the Taizhou University Scientific Research Foundation (no. 2015QN017).

References

[1] C. Ritchlin, "Fibroblast biology. Effector signals released by the synovial fibroblast in arthritis," *Arthritis Research*, vol. 2, no. 5, pp. 356–360, 2000.

[2] J. J. Goronzy and C. M. Weyand, "Developments in the scientific understanding of rheumatoid arthritis," *Arthritis Research and Therapy*, vol. 11, article 249, 2009.

[3] G. R. Burmester, B. Stuhlmüller, G. Keyszer, and R. W. Kinne, "Mononuclear phagocytes and rheumatoid synovitis: mastermind or workhorse in arthritis?" *Arthritis and Rheumatism*, vol. 40, no. 1, pp. 5–18, 1997.

[4] B. Bartok and G. S. Firestein, "Fibroblast-like synoviocytes: key effector cells in rheumatoid arthritis," *Immunological Reviews*, vol. 233, no. 1, pp. 233–255, 2010.

[5] T. Nanki, K. Nagasaka, K. Hayashida, Y. Saita, and N. Miyasaka, "Chemokines regulate IL-6 and IL-8 production by fibroblast-like synoviocytes from patients with rheumatoid arthritis," *The Journal of Immunology*, vol. 167, no. 9, pp. 5381–5385, 2001.

[6] X. Luo, X. Zuo, Y. Zhou et al., "Extracellular heat shock protein 70 inhibits tumour necrosis factor-α induced proinflammatory mediator production in fibroblast-like synoviocytes," *Arthritis Research and Therapy*, vol. 10, article R41, 2008.

[7] P. Loetscher, B. Dewald, M. Baggiolini, and M. Seitz, "Monocyte chemoattractant protein 1 and interleukin 8 production by rheumatoid synoviocytes. Effects of anti-rheumatic drugs," *Cytokine*, vol. 6, no. 2, pp. 162–170, 1994.

[8] P. K. K. Wong, I. K. Campbell, P. J. Egan, M. Ernst, and I. P. Wicks, "The role of the interleukin-6 family of cytokines in inflammatory arthritis and bone turnover," *Arthritis and Rheumatism*, vol. 48, no. 5, pp. 1177–1189, 2003.

[9] H. Nakahara and N. Nishimoto, "Anti-interleukin-6 receptor antibody therapy in rheumatic diseases," *Endocrine, Metabolic and Immune Disorders—Drug Targets*, vol. 6, no. 4, pp. 373–381, 2006.

[10] N. Nishimoto, T. Kishimoto, and K. Yoshizaki, "Anti-interleukin 6 receptor antibody treatment in rheumatic disease," *Annals of the Rheumatic Diseases*, vol. 59, no. 1, pp. i21–i27, 2000.

[11] K. Miyazawa, A. Mori, K. Yamamoto, and H. Okudaira, "Constitutive transcription of the human interleukin-6 gene by rheumatoid synoviocytes: spontaneous activation of NF-κB and CBF1," *The American Journal of Pathology*, vol. 152, no. 3, pp. 793–803, 1998.

[12] C. Georganas, H. Liu, H. Perlman, A. Hoffmann, B. Thimmapaya, and R. M. Pope, "Regulation of IL-6 and IL-8 expression in rheumatoid arthritis synovial fibroblasts: the dominant role for NF-κB but not C/EBPβ or c-Jun," *Journal of Immunology*, vol. 165, no. 12, pp. 7199–7206, 2000.

[13] J. M. Shields, R. J. Christy, and V. W. Yang, "Identification and characterization of a gene encoding a gut-enriched Kruppel-like factor expressed during growth arrest," *The Journal of Biological Chemistry*, vol. 271, no. 33, pp. 20009–20017, 1996.

[14] M. W. Feinberg, Z. Cao, A. K. Wara, M. A. Lebedeva, S. SenBanerjee, and M. K. Jain, "Kruppel-like factor 4 is a mediator of proinflammatory signaling in macrophages," *The Journal of Biological Chemistry*, vol. 280, no. 46, pp. 38247–38258, 2005.

[15] J. P. Katz, N. Perreault, B. G. Goldstein et al., "The zinc-finger transcription factor Klf4 is required for terminal differentiation of goblet cells in the colon," *Development*, vol. 129, no. 11, pp. 2619–2628, 2002.

[16] J. A. Segre, C. Bauer, and E. Fuchs, "Klf4 is a transcription factor required for establishing the barrier function of the skin," *Nature Genetics*, vol. 22, no. 4, pp. 356–360, 1999.

[17] K. Takahashi and S. Yamanaka, "Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors," *Cell*, vol. 126, no. 4, pp. 663–676, 2006.

[18] Z. Cao, X. Sun, B. Icli, A. K. Wara, and M. W. Feinberg, "Role of Krüppel-like factors in leukocyte development, function, and disease," *Blood*, vol. 116, no. 22, pp. 4404–4414, 2010.

[19] A. Hamik, Z. Lin, A. Kumar et al., "Kruppel-like factor 4 regulates endothelial inflammation," *The Journal of Biological Chemistry*, vol. 282, no. 18, pp. 13769–13779, 2007.

[20] B. Shen, R. S. Smith Jr., Y.-T. Hsu, L. Chao, and J. Chao, "Kruppel-like factor 4 is a novel mediator of kallistatin in inhibiting endothelial inflammation via increased endothelial nitric-oxide synthase expression," *The Journal of Biological Chemistry*, vol. 284, no. 51, pp. 35471–35478, 2009.

[21] A. Tsung, S. Tohme, and T. R. Billiar, "High-mobility group box-1 in sterile inflammation," *Journal of Internal Medicine*, vol. 276, no. 5, pp. 425–443, 2014.

[22] Z.-W. He, Y.-H. Qin, Z.-W. Wang, Y. Chen, Q. Shen, and S.-M. Dai, "HMGB1 acts in synergy with lipopolysaccharide in activating rheumatoid synovial fibroblasts via p38 MAPK and NF- B signaling pathways," *Mediators of Inflammation*, vol. 2013, Article ID 596716, 10 pages, 2013.

[23] J. A. Nogueira-Machadoa and C. M. de Oliveira Volpea, "HMGB-1 as a target for inflammation controlling," *Recent Patents on Endocrine, Metabolic and Immune Drug Discovery*, vol. 6, no. 3, pp. 201–209, 2012.

[24] M. Yu, H. Wang, A. Ding et al., "HMGB1 signals through toll-like receptor (TLR) 4 and TLR2," *Shock*, vol. 26, no. 2, pp. 174–179, 2006.

[25] J. S. Park, D. Svetkauskaite, Q. He et al., "Involvement of toll-like receptors 2 and 4 in cellular activation by high mobility group box 1 protein," *The Journal of Biological Chemistry*, vol. 279, no. 9, pp. 7370–7377, 2004.

[26] J. Liu, Y. Liu, H. Zhang, G. Chen, K. Wang, and X. Xiao, "KLF4 promotes the expression, translocation, and release of HMGB1 in RAW264.7 macrophages in response to LPS," *Shock*, vol. 30, no. 3, pp. 260–266, 2008.

[27] F. C. Arnett, S. M. Edworthy, D. A. Bloch et al., "The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis," *Arthritis & Rheumatism*, vol. 31, no. 3, pp. 315–324, 1988.

[28] X. Luo, X. Zuo, B. Zhang et al., "Release of heat shock protein 70 and the effects of extracellular heat shock protein 70 on the production of IL-10 in fibroblast-like synoviocytes," *Cell Stress and Chaperones*, vol. 13, no. 3, pp. 365–373, 2008.

[29] X. Luo, X. Zuo, X. Mo, Y. Zhou, and X. Xiao, "Treatment with recombinant Hsp72 suppresses collagen-induced arthritis in mice," *Inflammation*, vol. 34, no. 5, pp. 432–439, 2011.

[30] D. K. Kaushik, M. Gupta, S. Das, and A. Basu, "Krüppel-like factor 4, a novel transcription factor regulates microglial activation and subsequent neuroinflammation," *Journal of Neuroinflammation*, vol. 7, article 68, 2010.

[31] K. J. Livak and T. D. Schmittgen, "Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C_T}$ method," *Methods*, vol. 25, no. 4, pp. 402–408, 2001.

[32] J. M. Rosenzweig, J. D. Glenn, P. A. Calabresi, and K. A. Whartenby, "KLF4 modulates expression of IL-6 in dendritic cells via both promoter activation and epigenetic modification," *The Journal of Biological Chemistry*, vol. 288, no. 33, pp. 23868–23874, 2013.

[33] K. Miyazawa, A. Mori, H. Miyata, M. Akahane, Y. Ajisawa, and H. Okudaira, "Regulation of interleukin-1β-induced interleukin-6 gene expression in human fibroblast-like synoviocytes by p38 mitogen-activated protein kinase," *The Journal of Biological Chemistry*, vol. 273, no. 38, pp. 24832–24838, 1998.

[34] L. Neff, M. Zeisel, J. Sibilia, M. Schöller-Guinard, J.-P. Klein, and D. Wachsmann, "NF-κB and the MAP kinases/AP-1 pathways are both involved in interleukin-6 and interleukin-8 expression in fibroblast-like synoviocytes stimulated by protein I/II, a modulin from oral streptococci," *Cellular Microbiology*, vol. 3, no. 10, pp. 703–712, 2001.

[35] J. Liu, H. Zhang, Y. Liu et al., "KLF4 regulates the expression of interleukin-10 in RAW264.7 macrophages," *Biochemical and Biophysical Research Communications*, vol. 362, no. 3, pp. 575–581, 2007.

[36] M. W. Feinberg, A. K. Wara, Z. Cao et al., "The Kruppel-like factor KLF4 is a critical regulator of monocyte differentiation," *EMBO Journal*, vol. 26, no. 18, pp. 4138–4148, 2007.

[37] J. K. Alder, R. W. Georgantas III, R. L. Hildreth et al., "Kruppel-like factor 4 is essential for inflammatory monocyte differentiation in vivo," *Journal of Immunology*, vol. 180, no. 8, pp. 5645–5652, 2008.

[38] J. An, S. Golech, J. Klaewsongkram et al., "Krüppel-like factor 4 (KLF4) directly regulates proliferation in thymocyte development and IL-17 expression during Th17 differentiation," *The FASEB Journal*, vol. 25, no. 10, pp. 3634–3645, 2011.

[39] L. Medugno, F. Florio, E. Cesaro et al., "Differential expression and cellular localization of ZNF224 and ZNF255, two isoforms of the Krüppel-like zinc-finger protein family," *Gene*, vol. 403, no. 1-2, pp. 125–131, 2007.

[40] N. Nishimoto, "Interleukin-6 in rheumatoid arthritis," *Current Opinion in Rheumatology*, vol. 18, no. 3, pp. 277–281, 2006.

[41] T. Matsumoto, T. Tsurumoto, and H. Shindo, "Interleukin-6 levels in synovial fluids of patients with rheumatoid arthritis correlated with the infiltration of inflammatory cells in synovial membrane," *Rheumatology International*, vol. 26, no. 12, pp. 1096–1100, 2006.

[42] A. Al-Awadhi, S. Olusi, N. Al-Zaid, and K. Prabha, "Serum concentrations of interleukin 6, osteocalcin, intact parathyroid hormone, and markers of bone resorption in patients with rheumatoid arthritis," *Journal of Rheumatology*, vol. 26, no. 6, pp. 1250–1256, 1999.

[43] S. Kotake, K. Sato, K. J. Kim et al., "Interleukin-6 and soluble interleukin-6 receptors in the synovial fluids from rheumatoid arthritis patients are responsible for osteoclast-like cell formation," *Journal of Bone and Mineral Research*, vol. 11, no. 1, pp. 88–95, 1996.

[44] R. Madhok, A. Crilly, J. Watson, and H. A. Capell, "Serum interleukin 6 levels in rheumatoid arthritis: correlations with clinical and laboratory indices of disease activity," *Annals of the Rheumatic Diseases*, vol. 52, no. 3, pp. 232–234, 1993.

[45] W. P. Arend and J.-M. Dayer, "Cytokines and cytokine inhibitors or antagonists in rheumatoid arthritis," *Arthritis and Rheumatism*, vol. 33, no. 3, pp. 305–315, 1990.

[46] M. Odeh, "New insights into the pathogenesis and treatment of rheumatoid arthritis," *Clinical Immunology and Immunopathology*, vol. 83, no. 2, pp. 103–116, 1997.

[47] E. Choy, "Interleukin 6 receptor as a target for the treatment of rheumatoid arthritis," *Annals of the Rheumatic Diseases*, vol. 62, supplement 2, pp. ii68–ii69, 2003.

[48] T. Yoshida, M. Yamashita, C. Horimai, and M. Hayashi, "Deletion of Krüppel-like factor 4 in endothelial and hematopoietic cells enhances neointimal formation following vascular injury," *Journal of the American Heart Association*, vol. 3, no. 1, Article ID e000622, 2014.