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Pim-1 as a therapeutic target in human lupus nephritis

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Abstract

Objective Lupus nephritis (LN) is a major determinant of morbidity and mortality in systemic lupus erythematosus (SLE). Pim-1 regulates lymphocyte proliferation and activation. The role of Pim-1 in autoimmune disease remains unclear. Therefore, we hypothesize that Pim-1 inhibition would have therapeutic potential for LN.

Methods We first analyzed Pim-1 expression in lupus-prone NZB/W F1 mice (n=6), in human peripheral blood mononuclear cells (PBMCs) of SLE patients (n=10), and in the glomeruli of LN patients (n=8). The therapeutic effect of Pim-1 inhibitor AZD1208 was assessed in this lupus model (n=10/group). In vitro analysis was conducted to explore the mechanism of Pim-1 in mouse and human podocytes induced by anti-dsDNA antibody positive (anti-dsDNA^{+}) serum. Finally, MRL/lpr mice were used to confirm the therapeutic effects of Pim-1 inhibition in vivo (n=10/group).

Results Pim-1 upregulation was seen in renal lysates of diseased NZB/W F1 mice, peripheral blood mononuclear cells and renal biopsies from SLE patients relative to their counterparts (all $P<0.05$). Pim-1 inhibitor AZD1208 reduced proteinuria, glomerulonephritis, renal immune complex deposits and serum anti-dsDNA antibody, concomitant with suppression of NFATc1 expression and NLRP3 inflammasome activation. Moreover, in mouse and human podocytes, Pim-1 knockdown suppressed NFATc1 and NLRP3 inflammasome signaling in the presence of anti-dsDNA^{+} serum. Mechanistically, Pim-1 modulated NLRP3 inflammasome activation through intracellular Ca^{2+}. The therapeutic effect of Pim-1 blockade was replicated in MRL/lpr mice.

Conclusion These data identify Pim-1 as a critical regulator of LN pathogenesis and targeting Pim-1/NFATc1/NLRP3 pathway might be a therapy for human LN.

Keywords Lupus nephritis, Pim-1, NFATc1, NLRP3 inflammasome, podocytes

Systemic lupus erythematosus (SLE) is a prototypical autoimmune disease, characterized by generation of numerous autoantibodies. Lupus nephritis (LN) is a common and life-threatening manifestation of SLE (1). Despite improvements in therapy and outcome over the past 50 years, the rate of complete remission for severe LN is <50% (2).

The Pim family of serine/threonine kinases control cell survival, proliferation and apoptosis, consisting of Pim-1, Pim-2, and Pim-3 that are highly conserved throughout evolution (3). Despite extensively studied in tumorigenesis, the role of Pim kinases has recently been uncovered in inflammatory and autoimmune settings. Pim-1 phosphorylates RelA/p65 and activate NF-κB signaling in inflammatory conditions (4,5). Pim-1 phosphorylates the human transcription factor FOXP3 to restrict the immunosuppressive activity of human regulatory T cells (Tregs) under inflammation (6). Inhibition of Pim-1 could skew T cell differentiation towards Tregs (7) and attenuate Th17 cell differentiation (8). Pim-1 inhibition ameliorated colitis in mice via suppressing the excessive Th1- and Th17-type immune responses (7). Moreover, by associating with CD180, long form of Pim-1 kinase can transmit inflammatory into B cell survival programs, suggesting that its pharmacological inhibition may provide novel therapeutic options in autoimmune diseases

with increased B cell activity (9). However, the functional role of Pim-1 in LN remains unclear.

The calcineurin inhibitors such as cyclosporine, acting through inhibition of the nuclear factor of activated T cells (NFATc) signaling, are widely used to treat human LN (10-12). Of interest, Pim-1 kinase can enhance the expression and activity of NFATc1 (4,13). In addition, we and other researchers have shown that NLRP3 inflammasome participates in LN (14-17). Our recent publication demonstrates that NLRP3 inflammasome activation in podocytes contributes to podocyte injuries and development of proteinuria (18). This study aimed to investigate the functional role of Pim-1 in LN and its potential association with NLRP3 and NFATc1 signaling in podocytes.

Materials and Methods

Lupus models

The study protocol was approved by the Institutional Animal Care Committee of Sun Yat-sen University. The female NZB/W F1 mice (Jackson Labs, USA), female MRL/lpr mice (Shanghai SLAC Laboratory Animal Company, China) and normal female control (C57BL/6 mice, Sun Yat-sen University) were maintained in the specific pathogen-free barrier facility at the Experimental Animal Center at Sun Yat-sen University.

Treatment protocols

Female NZB/W F1 mice were orally treated with AZD1208 (15 mg/kg, Selleck Chemicals) or vehicle control [0.1% Tween-80 and 0.5% methyl cellulose (Sigma–Aldrich, St. Louis, USA) in water] (19, 20) for 12 weeks (n=10/group), starting at 22 weeks when proteinuria occurred. Mice were sacrificed at 34 weeks of age under anesthesia.

Twelve-week-old MRL/lpr mice received a selective Pim-1 inhibitor SMI-4a (60mg/kg, Selleck Chemicals) or vehicle (DMSO/PEG-400/Tween-80) twice daily according to the previous report (21). The oral gavage was administered 5 of 7 days each week for 8 weeks (n=10/group). In an independent experiment, the survival was observed until 30 weeks of age and compared between two groups (n=15/group).

Human blood samples

Written informed consent was obtained from all subjects and the study was approved by the Ethics Committee of First Affiliated Hospital, Sun Yat-sen University. All patients fulfilled the American College of Rheumatology classification criteria for SLE (22). Patients with concurrent infection, malignancy or other autoimmune diseases were excluded from the study. Disease activity was assessed using SLE Disease Activity Index (SLEDAI) (23). The female patients with active disease (SLEDAI $\geq$10) and in remission (SLEDAI$\leq$4) were included in the study. The human peripheral blood mononuclear cells (PBMCs) and

anti-dsDNA antibody positive (anti-dsDNA$^+$) serum were collected. Normal anti-dsDNA antibody negative serum from healthy volunteers was used as control.

Immunohistochemistry of human kidney specimens

Written informed consent was obtained and the study was approved by the Ethics Committee of The Fourth Affiliated Hospital of Guangxi Medical University. Renal specimens of LN patients (n=8, 4 class IV, 2 class IV+V and 2 class V LN) were obtained at Department of Nephrology, the Fourth Affiliated Hospital of Guangxi Medical University and classified using the International Society of Nephrology/Renal Pathology Society (ISN/RPS) 2003 classification of LN (24). Paraffin-embedded kidney sections (4μm) were dewaxed in xylene and rehydrated in graded ethanol solutions. Antigen retrieval was enhanced by microwaving the slides in 0.01 M citrate buffer (pH=6). Sections were incubated with primary antibody anti-Pim-1 (Abcam, USA, dilution 1:100) overnight at 4°C. The sections were then incubated with a horseradish peroxidase-conjugated secondary antibody (Beyotime, Jiangsu, China) at room temperature for 30 minutes. Antibody binding was visualized with 3,3′-diaminobenzidine tetrahydrochloride (DAB) before brief counterstaining with hematoxylin. As negative control, the primary antibody was replaced by PBS containing 1% bovine serum albumin. Non-malignant renal tissues, with normal light micrographic findings, came from surgical removal of renal cancer patients (n=8) and were used as normal control. The staining intensity of Pim-1 was scored blindly by two independent pathologists on a scale of 0 to 3 as previously described (25).

Measurements of urine protein

Urine was collected every 2 weeks. The proteinuria was determined using dipsticks (Multistix 10SG, Bayer Diagnostics) and scored on a scale of 0–4 as previously reported (15). In addition, the concentration of urine albumin was assessed by ELISA (Bethyl Laboratories Inc., USA) and urine creatinine was measured using Creatinine Colorimetric Assay Kit (Cayman chemical, USA) according to the manufacturer's instructions. The urine albumin-to-creatinine ratio (ACR) was expressed as μg/mg.

Periodic acid-Schiff staining and immunofluorescence analysis of mouse kidney

The kidneys were harvested, fixed in 10% neutral formalin and embedded in paraffin for sectioning (4μm), and stained with Periodic Acid-Schiff (PAS) in standard methods. Glomerular lesions were graded on a scale of 0–3 as previously described by two independent observers blinded to the study (15).

An immunofluorescence assay was conducted using FITC-conjugated anti-mouse IgG (Santa Cruz Biotechnology, USA) and anti-mouse complement C3 (Cedarlane, USA). The fluorescence intensity was assessed on a scale of 0–3 and analyzed as described previously (15). Images were taken with a fluorescence microscope (Olympus, Center Valley, USA).

Measurement of anti-dsDNA antibody

Serum anti-dsDNA antibody was detected by ELISA as previously described (15). The absorbance at OD450nm was determined. Normal IgG was used as negative control.

Caspase 1 activity assay

Caspases 1 activity was determined using a Caspase-Glo® 1 Inflammasome Assay (Promega, USA) according to the manufacturer's instructions. Briefly, cells were seeded in 96-well plate. After treatment, equal volume of Caspase-Glo 1 reagent was added to the culture medium, which had been equilibrated to room temperature for 1h, cells were shaken for 5 min and incubated at room temperature for 30 min. Luminescence was measured using Synergy 2 Microplate Reader (BioTek, USA).

Single cell suspension analysis by flow cytometry

Preparation of single cell suspension of glomeruli and flow cytometry analysis were performed as previously described (18). Single cell suspensions were stained for podocytes using anti-mouse CD26-PE (Biolegend), anti-nephrin-Alexa Fluor 647 (Bioss Inc.), CD45-APC-eFluor 780 (eBioscience) and anti-Pim-1 (Cell Signaling Technology). All analyses were done on a Gallios Flow Cytometer (Beckman Coulter).

Single-cell suspensions were prepared from the splenocytes, and the percentages of Th1 ($CD4^+IFN\gamma^+$) and Th17 ($CD4^+IL\text{-}17^+$) were analyzed using anti-mouse IL-17A Ab (BD Pharmingen) and an anti-IFN-γ Ab (BD Pharmingen) as described before (15).

Mouse and human podocytes

Mouse podocytes, conditionally immortalized by introducing a temperature-sensitive SV40T antigen by transfection, were purchased from Shanghai Ruilu Technology Co. (Cat. no FDCC-MSN059, Shanghai, China). In brief, cells were grown on type I collagen-coated (BD Biosciences, USA) culture plates under growth-permissive conditions at 33 ℃ in RPMI-1640 medium supplemented with 10% fetal bovine serum (Gibco, USA), 20 U/ml mouse recombinant interferon-gamma (IFN-γ, Sigma, USA) and 1% penicillin/streptomycin mixture (Sigma, USA) under 5% CO_2. For differentiation, when cell density reached 70%-80% confluence, podocytes were cultured under the non-permissive condition at 37°C in the absence of IFN-γ for 10-14 days.

Conditionally immortalized human podocytes AB8/13 were kindly provided by Dr. Moin A. Saleem (Bristol, UK) (26) and cultured in RPMI 1640 with added 10% fetal bovine serum (Gibco, USA), 1% penicillin/streptomycin (Sigma, USA) and Insulin–Transferrin–Selenium (Life Technologies, USA). The cells proliferated at 33°C and grew to 70–80% confluence, followed by differentiation at 37°C for 10–14 days.

Podocytes were incubated in the medium and stimulated with anti-dsDNA^{+} serum from SLE or control serum (5% final concentration) for 24-72h.

Pim-1 knockdown

The small interfering RNAs targeting Pim-1 (siPim-1) and non-targeting control siRNA (siNC) were synthesized by RiboBio (Guangzhou, China). All the transfection was conducted with lipofectamine 3000 (ThermoFisher Scientific) following manufacturer's instructions. Following different treatments, supernatant IL-1β was quantified by ELISA (R&D Systems, USA) according to the manufacturer's instructions. Cells were collected and subjected to Western blot analysis.

Real-time quantitative PCR

Total RNA was extracted using the Trizol reagent (Life Technologies, USA) according to the manufacturer's protocol. The complementary DNA (cDNA) was obtained with the PrimeScript RT reagent Kit (TaKaRa, Dalian, China) and subjected to real-time quantitative PCR (qPCR) analysis. GAPDH was used as an internal control. Amplification cycles were 95°C for 10 min, followed by 40 cycles at 95°C for 15s and 60°C for 1min. The Primers used for qPCR were synthesized by Shanghai Generay Biotech Co. Ltd (Generay, Shanghai, China) as follows: human Pim-1: forward 5′-GAGAAGGACCGGATTTCCGAC-3′ and reverse 5′-CAGTCCAGGAGCCTAATGACG-3′; human GAPDH: forward 5′-TGTGGG

CATCAATGGATTTGG-3′ and reverse 5′-ACACCATGTATTCCGGGTCAAT-3′; mouse Pim-1: forward 5′-TTCGGCTCGGTCTACTCTGG-3′ and reverse 5′-CAGTTCTCCCCA ATCGGAAATC-3′; mouse IFNγ: forward 5'-TACACACTGCATCTTGGCTTTG-3' and reverse 5'-CTTCCACATCTATGCCACTTGAG-3', mouse IL-17: forward 5′-GCTCCAGAA GGCCCTCAGA-3′ and reverse 5′-AGCTTTCCCTCCGCATTGA-3′ , mouse GAPDH: forward 5′-TTGTCATGGGAG TGAACGAGA-3′ and reverse 5′-CAGGCAGTTGGTGGT ACAGG-3′.

Western blot analysis

Total protein extraction was performed using M-PER Mammalian Protein Extraction Reagent (Thermo Fisher Scientific, USA) and the concentrations were determined by the BCA protein assay kit (Thermo Fisher Scientific, USA). Proteins were separated by 10% SDS polyacrylamide gel electrophoresis and then transferred to a poly vinylidene difluoride membrane (Millipore, USA). The membrane was treated with 3% bovine serum albumin (BSA) in Tris-buffered saline solution followed by incubation with primary antibodies against Pim-1 (Cell Signaling Technology, USA), NLRP3 (AdipoGen, USA), caspase-1p20 (Santa Cruz Biotechnology, USA), NFATc1(Abcam, USA), GAPDH (Cell Signaling Technology, USA). After washing three times with TBST, horseradish peroxidase-labeled secondary antibodies were used for detection. The signals on the membranes were visualized using an Enhanced Chemiluminescence Detection kit (Thermo Fisher Scientific, USA).

Intracellular Ca^{2+} analysis

Cell-permeant Fluo-3 acetoxymethylester (Fluo-3 AM) probe was purchased from Yeasen Corporation (Shanghai, China) and used for intracellular Ca^{2+} detection according to the instructions. Fluo-3 AM was dissolved in dimethyl sulfoxide (DMSO) with 20% Pluronic F127 to prevent its aggregation in Hanks balanced salt solution (HBSS, without Ca^{2+} or Mg^{2+}) and promote cellular uptake. The Fluo-3 AM solution was diluted with HBSS to prepare 4μM working solution. The suspension was incubated with water bath for 50 min at 37°C and was then centrifuged for 5 min. The resulting fluorescence intensity as the indicator of Ca^{2+} concentration was observed by flow cytometry (FACScan Flow Cytometer, Beckman-Coulter) at excitation wavelength of 488 nm and emission wavelength of 525 nm.

After siPim-1 or siNC treatment for 24h, prior to stimulation with anti-$dsDNA^{+}$ serum for 24h, cells were treated with the intracellular calcium chelator BAPTA-AM (50μM, Merck Millipore) to assess involvement of intracellular calcium in Pim-1/NLRP3 pathway.

Statistical analysis

Student t test or analysis of variance (ANOVA) was used for comparison between two or more groups. Kaplan-Meier analysis with log-rank tests was used to compare mouse survival. Data were expressed as mean ± standard deviation (SD). Statistical significance was considered at $p<0.05$. All data were processed using SPSS17.0.

Results

Pim-1 expression is enhanced in mouse and human SLE

First, we utilized a lupus model NZB/W F1 mice and compared Pim-1 expression between the pre-diseased mice (no proteinuria, 12 weeks of age), the diseased mice with severe proteinuria (3+, 34 weeks of age) and age-matched normal control. As the proteinuria developed, the expression of Pim-1 mRNA and protein was significantly elevated in the murine kidney (n=6/group, Figure 1A). In addition, Pim-1 was also upregulated in PBMCs of SLE patients with active disease, compared with those of SLE patients in remission (anti-dsDNA antibody negative) and normal PBMCs (n=10/group, Figure 1B). In LN patients, immunohistochemistry showed strong nuclear and cytoplasmic staining of Pim-1 in the glomeruli (n=8, Figure 1C). In contrast, Pim-1 is poorly expressed in the normal nephrectomy specimens (n=8).

Pim-1 inhibition attenuated lupus-like syndrome in NZB/W F1 mice

Next, we treated NZB/W F1 mice with a Pim-1 inhibitor AZD1208, which demonstrates preclinical efficacy in several cancers (19,20,27,28). The proteinuria (urine albumin-to-creatinine ratio, ACR) was remarkably delayed in AZD1208-treated animals over the 12-week observation period (Figure 2A). AZD1208-treated kidneys showed decreased severity of glomerulonephritis relative to vehicle-treated control (Figure 2B). We also observed a significant decrease in renal deposits of IgG and C3 after AZD1208 treatment (Figure 2C). Serum anti-dsDNA antibody was significantly reduced by AZD1208 (Figure 2D).

Pim-1 inhibition suppressed NFATc1 expression and NLRP3 inflammasome activation in the mouse kidney

We then investigated the potential effect of Pim-1 inhibition on NFATc1 expression and NLRP3 inflammasome activation. Pim-1 inhibition significantly reduced renal NFATc1 expression (Figure 2E). Additionally, renal expression of NLRP3 and caspase 1p20 were significantly suppressed by AZD1208 treatment (Figure 2E). The inhibitory effects of AZD1208 on caspase 1 activity was further confirmed by Caspase 1 Inflammasome Assay (Figure 2F). Consistent with these results, renal IL-1β expression was inhibited by AZD1208 (Figure 2G).

In addition, 6-week AZD1208 treatment inhibited the Th1 and Th17 response in 14-week-old MRL/lpr mice, as demonstrated by reduced Th1 and Th17 cell percentage in the spleen (Figure S-A) and decreased expression of IFNγ and IL-17 in the kidney tissue (Figure S-B).

Pim-1 expression was induced by anti-dsDNA$^+$ serum in mouse and human podocytes

By flow cytometry of single-cell suspensions of kidneys from NZB/W F1 mice, we found that Pim-1 expression was significantly decreased in podocytes from AZD1208-treated mice compared with controls (Figure 3A). Subsequently, we found that anti-dsDNA$^+$ serum from diseased NZB/WF1 mice (34 weeks of age, proteinuria 3+) induced Pim-1 expression in mouse podocytes, reaching its maximum at 24h (Figure 3B). In contrast, control serum from the pre-diseased mice had no such effect. Consistently, anti-dsDNA$^+$ serum from

treatment-naive SLE patients remarkably enhanced Pim-1 expression at 24h in human podocytes (Figure 3C).

Pim-1 knockdown hampered NFATc1 and NLRP3 inflammasome signaling in mouse and human podocytes

The silencing efficacy of Pim-1 was assessed at 48h by qPCR and Western blot and the more effective one was selected (Figure 4A and B). Results showed that Pim-1 knockdown significantly suppressed NFATc1 expression, compared with controls (Figure 4C). Furthermore, Pim-1 silencing profoundly downregulated NLRP3, caspase 1p20, caspase 1 activity and resulting IL-1β production in mouse podocytes (Figure 4C, D and E). Moreover, human podocytes were simulated with anti-dsDNA^{+} serum from human SLE in the presence or absence of siPim-1. As expected, Pim-1 knockdown suppressed autoantibody-induced NFATc1 expression and NLRP3 inflammasome activation (Figure 4F-H). These data suggest that Pim-1 modulates NFATc1 and NLRP3 signaling pathways in podocytes.

Pim-1 modulated NLRP3 inflammasome activation through intracellular Ca^{2+}

Intracellular Ca^{2+} signaling regulates NLRP3 inflammasome activation. Our results showed, compared with normal serum, anti-dsDNA^{+} serum from lupus patients induced a pronounced increase in intracellular Ca^{2+} concentration in human podocytes, which was inhibited by Pim-1 knockdown (Figure 5A). Moreover, the increased caspase 1 activity and

IL-1β production induced by anti-dsDNA^{+} serum were both attenuated by an intracellular Ca^{2+} blocker BAPTA-AM, and such an inhibitory effect was comparable to that of siPim-1 (Figure 5B-C), suggesting the Pim-1 regulates NLRP3 inflammasome via intracellular Ca^{2+}. In sum, we propose a model in which Pim-1 activates NFATc1 and NLRP3 inflammasome signaling and plays vital roles in the development of LN (Figure 5D).

Selective inhibition of Pim-1 attenuated renal disease and mortality of MRL/lpr mice

To further confirm the results above, we investigated the effect of a selective Pim-1 inhibitor SMI-4a on MRL/lpr mice. SMI-4a profoundly attenuated glomerular damage (Figure 6A). Meanwhile, SMI-4a treatment attenuated the onset of proteinuria, as compared with control (Figure 6B). Strikingly, SMI-4a significantly prolonged the survival of MRL/lpr mice (Figure 6C).

Discussion

Initial investigations have largely focused on the oncogenic role of Pim-1 in human cancers (29). In this study, we show that Pim-1 inhibition attenuated the renal damage and related immunopathology in lupus-prone NZB/W F1 mice. Mechanistic investigation showed that Pim-1 promoted LN via NFATc1 and NLRP3 signaling in podocytes. Notably, selective inhibition of Pim-1 conferred renal protection and survival benefit in another lupus model

MRL/lpr mice. Thus, our data extend the knowledge of Pim-1 to the field of autoimmune disease, providing evidence that Pim-1 is a promising therapeutic target in human LN.

A novel and important finding in this study is that Pim-1 regulates NLRP3 inflammasome in LN. Immune complex can activate NLRP3 inflammasome and induce IL-1β production from human monocytes (30). Activation of the NLRP3 inflammasome in human lupus-derived macrophages enhances production of inflammatory cytokines (31). Caspase-1, the central component of NLRP3 inflammasome, is essential for autoantibody production and renal damage in the pristine-induced lupus (32). Besides our previous reports (14-16,18), Lu and colleagues demonstrate that over-activated NLRP3 inflammasome in myeloid cells leads to severe organ damage in experimental lupus (33). It was recently reported that primary trophoblast cells transfected with Pim-1 siRNA decreased IL-1β production, when stimulated with LPS or TNF-α (34). The present study indicates that Pim-1 inhibition suppressed NLRP3/IL-1β signaling in lupus-prone mice and in vitro. Increased intracellular Ca^{2+} concentration or Ca^{2+} mobilization is critical for NLRP3 inflammasome activation (35,36). Calcium dynamics were significantly enhanced in Pim-1-overexpressing mouse transgenic hearts and depressed in Pim-1-deficient mouse hearts (37). Further investigation in the present study revealed that knockdown of Pim-1 offset the increased intracellular Ca^{2+} in anti-dsDNA^{+} serum-stimulated human podocytes, and intracellular Ca^{2+} blockade is as effective as Pim-1 silencing in neutralizing the activation of NLRP3 inflammasome. These results show that Pim-1 modulates NLRP3 inflammasome activation via intracellular Ca^{2+}.

Another valuable finding in this study is that Pim-1 promotes LN via NFATc1 induction. NFATc1 is activated in MRL/lpr T cells and helps B cells to produce autoantibodies (38). NFATc1 is activated in anti-$CD3^+$ anti-CD28-activated PBMCs from SLE patients (39). As a basic maintenance therapy for SLE, hydroxychloroquine inhibits intracellular calcium release and NFATc1 expression in SLE patients (40). Importantly, NFATc1 signaling mediates podocyte injury and loss, promoting proteinuria and eventually kidney failure (41-43). Conditional NFATc1 activation in podocytes can cause proteinuria in vivo (41). Pertinent to this study, Pim-1 interacts with NFATc1 and regulates its activity (4,13). Pim-1 can phosphorylate NFATc1 and enhance its transcriptional activity directly in a phosphorylation-dependent manner (13). However, unlike the other known NFATc kinases, Pim-1 enhances NFATc1-dependent transactivation and IL-2 production in Jurkat T cells, without any effects on the subcellular localization of NFATc1 (13). Kim and his colleagues reported that Pim-1 modulates RANKL-induced osteoclastogenesis via NFATc1 induction (4). In this study, we demonstrate that Pim-1 acts as a modulator of NFATc1 signaling. The improvement in the glomerular inflammation was at least partly attributed to the inhibition of Th1 and Th17 response. Furthermore, decreased B cell activity due to Pim-1 inhibition may account for the reduction in the titers of anti-dsDNA antibody (9).

Previous studies show that pathogenic anti-DNA autoantibodies complexed with high mobility group binding protein 1 (HMGB1) activates receptor for advanced glycation end products (RAGE) (44-46), which activates Pim-1 (47). Thus, we speculate that anti-dsDNA antibody positive serum induces Pim-1 expression via RAGE in podocytes. Taken together,

we established a pivotal role for Pim-1 in the pathogenesis of LN. We thus provide the novel evidence that targeting Pim-1 may provide a promising strategy for treating human LN.

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Author contributions

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Zhao had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. J. Zhao, R. Fu, Y. Xia and M. Li.

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Analysis and interpretation of data. J. Zhao, R. Fu, Y. Xia and M. Li.

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Figure legends

Figure 1. Increased Pim-1 expression in mouse and human SLE. (A) Expression of Pim-1 in the kidney of NZB/W F1 mice. Pim-1 RNA expression was normalized to GAPDH. Lower panel indicates representative Western blot bands. The diseased (D) mice (proteinuria 3+, 34 weeks of age) had higher Pim-1 expression compared with the pre-diseased (pre-D) mice (no proteinuria, 12 weeks of age) and age-matched normal control (N) C57BL/6 mice (n=6/group). (B) Expression of Pim-1 in human peripheral blood mononuclear cells (PBMCs). Pim-1 RNA expression was normalized to GAPDH. Lower panel indicates representative Western blot bands. Pim-1 was upregulated in PBMCs of SLE patients with active disease, compared with SLE patients in remission (anti-dsDNA antibody negative) and normal control (n=10/group). (C) Immunohistochemistry analysis of Pim-1 in the glomeruli of LN patients in comparison with that of normal nephrectomy specimens (n=8/group), (a) normal nephrectomy specimens, (b) negative control, (c-d) specimens from lupus nephritis (LN). Right panel indicates staining intensity analysis of Pim-1. Data were expressed as mean ± SD; *$p<0.05$, vs normal control.

Figure 2. Treatment with the Pim-1 inhibitor AZD1208 reduced lupus-like syndrome and suppressed NFATc1 expression and NLRP3 inflammasome activation in NZB/WF1 mice. (A) Reduced urine albumin-to-creatinine ratio (ACR, μg/mg) by AZD1208 treatment. (B) Representative Periodic Acid-Schiff (PAS) images of glomerular areas treated with vehicle or

AZD1208. Magnification: ×400. The right panel indicates staining intensity analysis. (C) Reduced renal deposition of C3 and IgG by AZD1208 treatment. The right panel indicates fluorescence intensity analysis. (D) Reduced serum anti-dsDNA antibody in AZD1208-treated mice. (E) Representative Western blot bands (one band represents one mouse kidney sample) of Pim-1, NFATc1 expression and NLRP3 inflammasome components in the mouse kidneys. (F) Reduced caspase 1 activity by AZD1208. Results are shown as fold change of control group. (G) Reduced production of renal IL-1β by AZD1208 in the mouse kidneys. Data are mean ± SD; n=10/group; *p<0.05, vs vehicle-treated control.

Figure 3. Induction of Pim-1 expression by anti-dsDNA antibody positive (anti-dsDNA$^+$) serum in mouse and human podocytes. (A) Inhibited Pim-1 expression in podocytes from AZD1208-treated mice by flow cytometry of single-cell suspensions of mouse kidney compared with control. (B) Pim-1 induction in mouse podocytes after stimulation with anti-dsDNA$^+$ serum from diseased NZB/WF1 mice (34 weeks of age, proteinuria 3+). (C) Pim-1 induction in human podocytes after stimulation with anti-dsDNA$^+$ serum from treatment-naive SLE patients. Data are mean ± SD; n=10/group; *p<0.05, vs control.

Figure 4. Pim-1 knockdown inhibited NFATc1 and NLRP3 inflammasome signaling in mouse and human podocytes. (A and B) The silencing efficacy was assessed at 48h by qPCR and Western blot and the more effective one was selected. (C) Suppressed activity of NFATc1 and NLRP3 inflammasome in mouse podocytes by Pim-1 silencing. (D) Suppressed

activity of caspase 1 in mouse podocytes by Pim-1 silencing. Results are shown as fold change of control group. (E) Reduced supernatant IL-1β production in mouse podocytes by Pim-1 silencing. (F) Suppressed activity of NFATc1 and NLRP3 inflammasome in human podocytes by Pim-1 silencing. (G) Suppressed activity of caspase 1 in human podocytes by Pim-1 silencing. Results are shown as fold change of control group. (H) Reduced supernatant IL-1β production in human podocytes by Pim-1 silencing. Data are mean ± SD from three independent experiments. siPim-1: small interfering RNAs targeting Pim-1, siNC: non-targeting control siRNA, *$p<0.05$, vs siNC.

Figure 5. Pim-1 promoted NLRP3 inflammasome activation via intracellular Ca^{2+}. (A) Pim-1 knockdown inhibited the increased intracellular Ca^{2+} concentration in human podocytes induced by human anti-dsDNA antibody positive (anti-dsDNA^{+}) serum. Results are shown as fold change of control group. (B) The increased caspase 1 activity induced by anti-dsDNA^{+} serum was attenuated by an intracellular Ca^{2+} blocker BAPTA-AM, and this inhibitory effect was comparable to that of siPim-1. Results are shown as fold change of control group. (C) The increased production of IL-1β induced by anti-dsDNA^{+} serum was attenuated by an intracellular Ca^{2+} blocker BAPTA-AM, and this inhibitory effect was comparable to that of siPim-1. (D) Proposed schematic diagram for the possible role of Pim-1 in the development of lupus nephritis. siPim-1: small interfering RNAs targeting Pim-1, siNC: non-targeting control siRNA, *$p<0.05$, vs respective controls.

Figure 6. Selective inhibition of Pim-1 attenuated renal disease and mortality of MRL/lpr mice (A) SMI-4a profoundly attenuated glomerular damage. The right panel indicates staining intensity analysis (n=10/group). (B) SMI-4a treatment attenuated the urine albumin-to-creatinine ratio (ACR, μg/mg) (n=10/group). (C) SMI-4a significantly prolonged the survival of MRL/lpr mice (n=15/group). *p<0.05, vs vehicle-treated control.

Figure S. Six-week AZD1208 treatment attenuated Th1 and Th17 response in 14-week-old MRL/lpr mice (n=6/group). (A) AZD1208 treatment inhibited Th1 ($CD4^+IFN\gamma^+$) and Th17 ($CD4^+IL\text{-}17^+$) cell percentage in the spleen of MRL/lpr mice by flow cytometric analysis. (B) The expression of IFNγ and IL-17 in the kidney tissue was decreased by AZD1208 treatment. *p<0.05, vs vehicle-treated control.