Pin1-Targeted Therapy for Systemic Lupus Erythematosus

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Abstract

**Objective**—Systemic lupus erythematosus (SLE) is a debilitating autoimmune disease affecting multiple organs in the body, but therapeutic options are still very limited and often come with adverse effects. Increasing evidence has underlined an important role of the Toll-like receptor 7 (TLR-7)/TLR-9/interleukin-1 receptor-associated kinase 1 (IRAK-1)/interferon regulatory factor 7 (IRF-7) pathway in the development and progression of SLE. Notably, the prolyl isomerase Pin1 is an essential regulator of IRAK-1 in TLR-7/TLR-9 signaling, but its role in SLE is unknown. We undertook this study to determine whether Pin1 is activated and plays any role in the development and treatment of SLE.

**Methods**—Activation of Pin1 and TLR-7/TLR-9/IRAK-1/IRF-7 signaling was determined in various cell types among peripheral blood mononuclear cells from healthy controls and SLE patients. The effects of Pin1 and TLR signaling on SLE development were determined using validated Pin1 short hairpin RNA (shRNA), Pin1 genetic knockout, and the small-molecule Pin1 inhibitor all-trans-retinoic acid (ATRA) in immune cells and in several strains of lupus-prone mice.

**Results**—We found abnormal activation of Pin1 and its downstream targets IRAK-1 and IRF-7 in SLE patients. Furthermore, inhibition of Pin1 using either validated Pin1 shRNA or ATRA blocked TLR-7—induced activation of IRAK-1 and IRF-7 in SLE patient—derived immune cells. Moreover, in multiple lupus-prone animals, both Pin1 knockout and ATRA strikingly attenuated the expression of autoimmunity, including skin lesions, lymphadenopathy, splenomegaly, and other immune-related features.
glomerulonephritis, proteinuria, and production of anti—double-stranded DNA antibodies and CD4–CD8— T cells, and also prolonged overall survival in MRL/lpr and B6.lpr mice.

**Conclusion**—Pin1 plays a critical role in the development of SLE, and Pin1-targeted therapy offers a promising new strategy for treating SLE.

Systemic lupus erythematosus (SLE) is a debilitating autoimmune disease characterized by chronic inflammation and extensive dysregulation of the immune system and damage to multiple organs in the body (1). The pathogenesis of SLE has been attributed to many factors—genetic, environmental, hormonal, epigenetic, or immunoregulatory. SLE leads to disorder of the immune system and to generation of autoantibodies, immune complexes, autoreactive or inflammatory T cells, or inflammatory cytokines that could initiate and intensify inflammation and damage to various vital organs, such as the kidney, skin, lung, brain, and heart (1).

First-line therapies prescribed for SLE patients include nonsteroidal antiinflammatory drugs, antimalarial agents, glucocorticoids, or immunosuppressive drugs including cyclophosphamide (2), azathioprine (3), methotrexate (4), and mycophenolate mofetil (5), all of which may cause significant side effects (6). Targeted therapies against SLE have been explored, but only 1 new drug (belimumab) with moderate efficacy has been approved in the last 50 years (7–9). Given the limited therapeutic choices for SLE patients and their adverse side effects, there is an urgent need to develop novel targeted therapies for the disease.

Notably, recent advances in the understanding of SLE immunopathogenesis have suggested an effective anti-SLE approach of targeting Toll-like receptor 7 (TLR-7) and TLR-9 signaling (10–12), because recognition of self nucleic acids by TLR-7 and TLR-9 on B cells and plasmacytoid dendritic cells is an important step in the pathogenesis of SLE (11). Moreover, TLR-7/TLR-9 signaling has recently been demonstrated to be under tight regulation and controlled by Pin1, a unique prolyl isomerase governing proline-based cis-trans conformational change of its substrates (13). After TLR-7/TLR-9 stimulation, Pin1 is activated and then in turn interacts with interleukin-1 receptor–associated kinase 1 (IRAK-1) and also dissociates IRAK-1 from the receptor complex, resulting in nuclear translocation of interferon regulatory factor 7 (IRF-7) to induce type I interferons (IFNs) (13). Consequently, Pin1-deficient cells and mice failed to mount TLR-mediated, IFN-dependent immune responses (13). These intriguing mechanistic links suggested that targeting activation of TLR-7/TLR-9/IRAK-1/IRF-7 signaling by Pin1 inhibition might represent a promising therapeutic approach for SLE, but the role of Pin1 in SLE is unknown.

Pin1, a unique and conserved peptidylprolyl-cis-trans-isomerase (PPIase), recognizes the motif consisting of phospho-Ser or phospho-Thr residues preceding Pro (pSer/Thr-Pro) (14–16). Using its WW domain, Pin1 binds to specific pSer/Thr-Pro motif(s), thus targeting Pin1 close to its substrates, where its PPIase domain catalyzes cis-trans isomerization of certain pSer/Thr-Pro motifs (17,18) in a subset of proteins pivotal to a variety of physiologic events and diseases (14–16). In addition to cancer and neurodegenerative diseases, Pin1 plays a pivotal role in the regulation of the immune response and related disease (14,19,20). For example, it binds to and isomerizes the phosphorylated p65 subunit of NF-κB and prevents NF-κB from binding to and thereby being inhibited by the endogenous inhibitory protein.
IκB, which leads to increased NF-κB activity (21). Moreover, Pin1 has an important role in asthma development and in the response to microbial infection (22–24). During asthma, Pin1 is activated by dephosphorylation and functions as an essential component of the RNP complex, which is responsible for granulocyte—macrophage colony-stimulating factor messenger RNA stabilization, cytokine secretion, and eosinophil survival (22). These results all suggest a potential therapeutic role of Pin1 inhibition in immune diseases.

Development of Pin1 inhibitors has been challenging due to Pin1’s unique property of recognizing substrate containing the pSer/Thr-Pro motif. Most Pin1 inhibitors have been shown to lack potency or specificity or have been unable to penetrate cells (25,26); this changed with our discovery that all-trans-retinoic acid (ATRA) is a Pin1 inhibitor (27). Use of ATRA for acute promyelocytic leukemia is considered the first example of modern targeted cancer therapy (28–30), but its drug target had remained elusive (31,32). Unexpectedly, ATRA was discovered to be a Pin1 inhibitor from a high-throughput screening. ATRA was proved to be a potent submicromolar Pin1 inhibitor that specifically binds to, inhibits, and ultimately degrades active Pin1 selectively without cross-reacting with other isomerase members (27).

In the present study, we investigated the efficacy of Pin1-targeted therapy for treating SLE. Activated Pin1 was prevalent in SLE patients, particularly in monocytes. Genetic ablation using validated short hairpin RNA (shRNA) or gene knockout or pharmacologic inhibition of Pin1 using ATRA effectively turned off TLR-7/TLR-9/IRAK-1/IRF-7 signaling in human samples and cultured cells and drastically reversed or prevented a wide range of expression of SLE phenotypes in several lupus-prone animal strains. Our study offers a novel avenue for developing Pin1-targeted therapy for SLE.

**MATERIALS AND METHODS**

**Human studies**

Patients who fulfilled at least 4 of the 11 criteria for the classification of SLE as set forth by the American College of Rheumatology in 1982 (33) and revised in 1997 (34) were enrolled in the study, as were healthy individuals. The Beth Israel Deaconess Medical Center Institutional Review Board (IRB) approved the study protocol (IRB protocol no. 2006P-298). Informed consent was obtained from all study subjects. Disease activity for each patient was calculated using the SLE Disease Activity Index (35).

**Animal studies**

Female MRL/MpJ-*Fas*<sup>lpr</sup>/J (MRL/lpr), B6.MRL-*Fas*<sup>lpr</sup>/J (B6.*lpr*), C57BL/6-Ii17atm1Bcgen/J (coex-pressing interleukin-17 [IL-17] and green fluorescent protein [GFP]), and (NZB × NZW)F1/J mouse strains were purchased from The Jackson Laboratory. The Pin1<sup>−/−</sup>-B6.*lpr* genetic background was confirmed by genotyping of Pin1 and *Fas*<sup>lpr</sup> using poly-merase chain reaction. For the experiment with MRL/lpr mice, placebo or 5 mg 21-day ATRA-releasing pellets (Innovative Research of America) was implanted in the backs of these mice. Urea and serum samples were collected weekly for MRL/lpr mice and monthly for Pin1<sup>−/−</sup>-B6.*lpr* and (NZB × NZW)F1/J mice, followed by various
examinations. All experiments were performed according to a protocol approved by the Beth Israel Deaconess Medical Center Institutional Animal Care and Use Committee.

**Cell culture and reagents**

THP-1 cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and cultured at 37°C in a humidified incubator containing 5% CO₂. R848 and CpG were purchased from Sigma. Antibodies against various proteins were obtained from several sources. Mouse monoclonal antibodies (mAb) included mAb against Pin1 (previously described [36]), against α-tubulin and β-actin (Sigma), and against cyclin D1 (Santa Cruz Biotechnology). Rabbit antibodies included those against Pin1 phosphorylated at Ser^{16} (Cell Signaling Technology), IRAK-1 (Millipore), and IRF-7 (Abcam). Antibodies against Pin1 phosphorylated at Ser^{71} were previously described (37). A periodic acid—Schiff staining kit was purchased from Sigma. Enzyme-linked immunosorbent assay kits for IgG anti—double-stranded DNA (anti-dsDNA) and IL-17 were from eBioscience. All assays were performed according to the manufacturers’ instructions. Samples of human peripheral blood mononuclear cells (PBMCs) were from the Division of Rheumatology at Beth Israel Deaconess Medical Center, and sample collection was approved by the Beth Israel Deaconess Medical Center IRB.

**Pin1 enzymatic assay**

Pin1 PPIase enzymatic activity was assayed according to a previously described method (17).

**Immunoblotting**

PBMCs or THP-1 cells, which received treatments with different doses of ATRA or shRNA knockdown, were lysed for 30 minutes at 4°C in lysis buffer (50 mM HEPES, pH 7.4, 150 mM NaCl, 1% Triton X-100, and 10% glycerol) with freshly added phosphatase and protease inhibitors consisting of 100 μM (2-aminoethyl)benzenesulfonyl fluoride, 80 nM aprotinin, 5 μM bestatin, 1.5 μM E-64 protease inhibitor, 2 μM leupeptin, 1 μM pepstatin A, 2 mM imidazole, 1 mM sodium fluoride, 1 mM sodium molybdate, 1 mM sodium orthovanadate, and 4 mM sodium tartrate dihydrate. After centrifugation at 13,000g for 10 minutes, one-tenth of the supernatant was stored as input, and the remainder was incubated for 12 hours with M2 Flag agarose (Sigma). After brief centrifugation, immunoprecipitates were collected, extensively washed twice with the aforementioned lysis buffer, suspended in 2x sodium dodecyl sulfate (SDS) sample buffer (100 mM Tris HCl, pH 6.8, 4% SDS, 5% β-mercaptoethanol, 20% glycerol, and 0.1% bromphenol blue), boiled for 10 minutes, and subjected to immunoblotting analysis.

Equal amounts of protein were resolved on 15% SDS—polyacrylamide gels. After electrophoresis, gel was transferred to nitrocellulose membranes using a semidy transfer cell. The transblotted membrane was washed twice with Tris buffered saline containing 0.1% Tween 20 (TBST). After blocking with TBST containing 5% bovine serum albumin (BSA) for 1 hour, the membrane was incubated overnight at 48°C with the appropriate primary antibody (diluted 1:1,000) in TBST containing 2% BSA. After incubation with the primary antibody, the membrane was washed 3 times with TBST for a total of 30 minutes.
followed by incubation with horseradish peroxidase—conjugated goat anti-rabbit or antimouse IgG (diluted 1:2,500) for 1 hour at room temperature. After extensive washing 3 times with TBST for a total of 30 minutes, the immunoblots were visualized by enhanced chemiluminescence.

**Immunostaining**

PBMCs, THP-1 cells, or mouse renal tissue was washed with phosphate buffered saline (PBS) and fixed with 4% paraformaldehyde at room temperature for 20 minutes, followed by permeabilization and blocking with PBS containing 0.1% Triton X-100 and 5% FBS for 1 hour. After another wash with PBS, immunostaining was performed by incubating the cells overnight at 4°C with the appropriate primary antibody (diluted 1:100). Primary antibodies were diluted in PBS containing 0.1% Triton X-100, 0.2% BSA, 0.5 mM phenylmethylsulfonyl fluoride, and 1 mM dithiothreitol. After washing with PBS, secondary Alexa Fluor 488—conjugated goat anti-mouse antibodies or Alexa Fluor 564—conjugated goat anti-rabbit antibodies (diluted 1:200; Invitrogen) were added for 2 hours at room temperature. Samples were nuclear counterstained with DAPI, mounted, and visualized with an LSM510 confocal imaging system (Zeiss).

**Single-cell isolation and T cell culture**

Spleens were excised, and single-cell suspensions were obtained. Naive CD4+ T cells were purified using a mouse CD4+CD62L+ T Cell Isolation Kit II according to the instructions of the manufacturer (Miltenyi Biotec). Purified naive T cells were stimulated with plate-bound goat anti-hamster antibodies, soluble anti-CD3 (0.25 μg/ml, 145-2C11; BioLegend), and anti-CD28 (0.5 μg/ml, 37.51; BioLegend). In addition, stimulation with the following was used for each polarized condition: for Th17 cells, IL-6 (20 ng/ml; R&D Systems), transforming growth factor β (TGFβ1) (3 ng/ml; R&D Systems), anti—IL-4 (10 μg/ml, C17.8; BioLegend), and anti-IFN γ (10 μg/ml, XMG1.2; BioLegend); for Treg cells, IL-2 (20 ng/ml; R&D Systems), TGFβ1 (3 ng/ml), anti—IL-4 (10 μg/ml), and anti-IFN γ (10 ng/ml).

**Flow cytometry**

Using a Zombie Aqua Fixable Viability Kit (BioLegend), staining was performed for eliminating dead cells. For Th17 cell detection, surface staining on ice was performed for 20–30 minutes. For Treg cell detection, after surface staining, FoxP3 was stained using a Mouse Regulatory T cell staining kit according to the instructions of the manufacturer (eBioscience). For flow cytometry analysis, the antibodies anti-CD4 (GK1.5) and anti-CD25 (PC61) were purchased from BioLegend, and the antibodies anti-CD3α (17A2) and anti-FoxP3 (FJK-16s) were purchased from eBioscience. All flow cytometry data were acquired on an LSRII flow cytometer (BD Biosciences) and analyzed with FlowJo software (Tree Star).

**Statistical analysis**

Experiments were performed at least 3 times, and the number of repeats was increased according to effect size or sample variation. We estimated the sample size considering the
variation and mean of the samples. No statistical method was used to predetermine sample size. No animals or samples were excluded from any analysis. Animals were randomly assigned to groups for in vivo studies; no formal randomization method was applied when assigning animals for treatment. Group allocation and outcome assessment (including for animal studies) were not performed in a blinded manner. Significance of the differences in disease-free survival among the cohorts was determined using the log rank (Mantel-Cox) test. All other data are presented as the mean ± SD, with significant differences determined using Student’s 2-tailed t-test or two-way analysis of variance. *P* values less than 0.05 were considered significant.

**RESULTS**

**Activated Pin1 and TLR-7/TLR-9/IRAK-1/IRF-7 signaling in monocytes from SLE patients**

Growing evidence suggests a central role of TLR-7/TLR-9 signaling in autoimmune diseases (10–12). Notably, we have previously identified an essential role of Pin1 in promoting TLR-7/TLR-9—initiated immune responses via the IRAK-1/IRF-7 axis, with Pin1 inhibition blocking IRAK-1 activation and IRF-7 nuclear translocation as well as IFNα signaling in response to TLR-7/TLR-9 (13). These results suggest that Pin1 inhibition might represent a potential therapeutic strategy for treating TLR-dependent SLE.

To examine this possibility, we first examined whether Pin1 is abnormally activated in SLE patients. It has been shown that Pin1 catalytic activity and substrate-binding activity are inhibited by phosphorylation at the Ser71 residue (37) or the Ser16 residue (38), the first of which prevents Pin1 substrates from entering into the catalytic pocket, and the second of which sequesters Pin1 in the cytoplasm. Thus, we examined Pin1 Ser71 and Ser16 phosphorylation status in PBMCs from SLE patients and normal subjects (see Supplementary Table 1, available on the Arthritis & Rheumatology web site at http://onlinelibrary.wiley.com/doi/10.1002/art.39741/abstract). Levels of Pin1 phosphorylation at Ser71 and/or Ser16 were significantly lower in samples from SLE patients, although total Pin1 expression remained unchanged, indicating that Pin1 is active in SLE (Figures 1A–C). These results were further confirmed by comparing Pin1 enzymatic activity in PBMCs isolated from SLE patients and normal controls using an in vitro PPIase assay (Figure 1F). Of note, the extent of the increase in Pin1 enzymatic activity was similar to that observed in human asthma (22). Furthermore, as shown previously (13), stimulation with the TLR-7 ligand R848 or with the TLR-9 ligand CpG significantly increased Pin1 activity (Figure 1G), which was potently suppressed by ATRA (Figure 1H) as expected because ATRA is a Pin1 inhibitor (27). Moreover, consistent with the ability of Pin1 to activate IRAK-1 (13), elevated IRAK-1 activity in SLE patients was also detected by increasing IRAK-1 phosphorylation (Figures 1A and D). Although the expression of total IRF-7 was not different between samples from SLE patients and those from normal controls (Figures 1A and E), nuclear translocation of IRF-7 was observed in SLE patients (Figures 1I and J), indicating that IRF-7 is activated in SLE patients. These results indicate that Pin1/IRAK-1/IRF-7 signaling is activated in PBMCs in SLE patients.
To identify the particular cell type(s) with active TLR-7/TLR-9 signaling in SLE pathology, we performed cell sorting to isolate various cell types from SLE PBMCs, including monocytes, B cells, and CD4+ and CD8+ T cells (Figures 1I–L), followed by examination of Pin1 phosphorylation at Ser\textsuperscript{71} and Ser\textsuperscript{16} and IRF-7 localization. IRF-7 nuclear localization was revealed mostly in monocytes and partially in CD4+ T cells (Figures 1I and J), while Pin1 phosphorylation at Ser\textsuperscript{71} and Ser\textsuperscript{16} had vanished in all types of cells from SLE patients (Figures 1I–L) (see Supplementary Figures 1A and B, http://onlinelibrary.wiley.com/doi/10.1002/art.39741/abstract). However, we did not observe IRF-7 translocation in B cells and CD8+ T cells (Figures 1K and L) (see Supplementary Figures 1C and D, http://onlinelibrary.wiley.com/doi/10.1002/art.39741/abstract). Taken together, these results suggest that the TLR-7/TLR-9/Pin1/IRAK-1/IRF-7 pathway is active in monocytes from SLE patients.

**Pin1/IRAK-1/IRF-7 axis suppressed in vitro by treatment with ATRA or knockdown of Pin1**

Given the identification of ATRA as a potent inhibitor specifically targeting active Pin1 that is not phosphorylated at Ser\textsuperscript{71} (37), which was prevalent in SLE patients (Figures 1A and B), we examined whether ATRA could inhibit TLR-7/TLR-9/Pin1/IRAK-1/IRF-7 signaling in a monocyte cell line, THP-1. THP-1 cells were treated with various concentrations of ATRA for 72 hours, and cell lysate was collected and subjected to Western blotting for detecting Pin1 expression. As shown in Figure 2A, ATRA at 5 μM effectively induced Pin1 degradation, suggesting that ATRA might inhibit Pin1-dependent IRAK-1/IRF-7 signaling. To examine this possibility, we stimulated THP-1 cells with the TLR-7 ligand R848 in the absence or presence of ATRA or in the absence or presence of Pin1 knockdown using a validated Pin1 shRNA (27). Indeed, either ATRA or Pin1 knockdown inhibited the ability of R848 to activate IRAK-1 in a dose-dependent manner (Figure 2B). Moreover, either ATRA or Pin1 knockdown significantly suppressed R848-induced IRF-7 transactivation, as shown by the luciferase reporter assay (Figure 2C), and also blocked R848-induced nuclear translocation of IRF-7, as shown by immunostaining (Figure 2D). Thus, pharmacologic or genetic inhibition of Pin1 potently blocks TLR-7/TLR-9/Pin1/IRAK-1/IRF-7 signaling in vitro.

**Expression of SLE phenotypes in lupus-prone mice effectively suppressed by treatment with ATRA**

Given that pharmacologic or genetic inhibition of Pin1 effectively blocked activation of the TLR-7/TLR-9 pathway in samples from SLE patients or in a monocyte cell line, we next tested whether inhibition of Pin1 could prevent TLR ligand—induced inflammation in mice. We applied imiquimod, a specific TLR-7 ligand known to induce inflammation of the skin (39), to the skin of wild-type (Pin1\textsuperscript{++}) mice or Pin1-knockout (Pin1\textsuperscript{−/−}) mice for 3 days and examined the skin tissue. Interestingly, imiquimod-induced skin inflammation was significantly attenuated in Pin1\textsuperscript{−/−} mice but not in Pin1\textsuperscript{++} mice (see Supplementary Figure 2, http://onlinelibrary.wiley.com/doi/10.1002/art.39741/abstract), suggesting that genetic Pin1 depletion might serve as an approach to attenuate a TLR-stimulated immune response such as SLE in mice.

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To further confirm this idea, we examined whether ATRA, a small chemical inhibitor (27), could affect Th17 or Treg cell populations, because both of them are essential SLE phenotypes (1,40,41). Naive T cells isolated from the splenocytes of C57BL/6-Il17atm1Bcgen/J (IL-17/GFP– coexpressing) mice were Th17 cell—or Treg cell—polarized in the presence or absence of ATRA. As shown in Supplementary Figure 3 (http://onlinelibrary.wiley.com/doi/10.1002/art.39741/abstract), ATRA significantly reduced differentiation of IL-17—producing Th17 cells but had only a marginal effect on Treg cell differentiation. These results show that pharmacologic depletion of Pin1 by ATRA attenuates the SLE phenotype ex vivo (Supplementary Figure 3).

These results encouraged us to examine the effects of ATRA (27) on an SLE-prone mouse strain, (NZB × NZW)F1/J, which spontaneously produces high levels of antinuclear antibodies and proteinuria as well as progressive immune complex glomerulonephritis (42). Eight-week-old female mice were implanted with 5 mg 21-day ATRA-releasing pellets or placebo, and urea samples were collected monthly. The pharmacologic effects of ATRA on the mice were validated by the detection of degradation of Pin1 and its substrate, cyclin D1 (see Supplementary Figure 4A, http://onlinelibrary.wiley.com/doi/10.1002/art.39741/abstract). Treatment with ATRA led to a trend toward reductions in IgG renal deposition (Supplementary Figure 4B), IgG anti-dsDNA (Supplementary Figure 4C), IL-17 (Supplementary Figure 4D), and proteinuria (Supplementary Figure 4E). The lifespan of (NZB × NZW)F1/J mice was also moderately extended by ATRA from 8 months to 11 months (Supplementary Figure 4F). Thus, ATRA not only inhibits Pin1 and its downstream target, but also attenuates expression of SLE phenotypes in lupus-prone (NZB × NZW)F1/J mice.

To confirm the above findings, we implanted slow-releasing ATRA pellets in mice of another SLE-prone strain, MRL/lpr (43,44). The MRL/lpr strain is FasL deficient and presents SLE-like manifestations including skin lesions, lymphadenopathy, splenomegaly, glomerulonephritis, and proteinuria as well as overproduction of anti-dsDNA antibodies, CD4–CD8— T cells, and many cyto-kines, and these mice have an average survival of 120 days (43,44). Eight-week-old female MRL/lpr mice were implanted with 5 mg 21-day ATRA-releasing pellets or placebo, and urea, blood samples, and survival were assayed or recorded weekly. As expected, compared with treatment with placebo, treatment with ATRA reduced levels of total Pin1 and activated IRAK-1 in MRL/lpr mice (Figure 3D). Phosphorylation of Pin1 at Ser71 and Ser16 also decreased with ATRA treatment, probably due to reduction of total Pin1 (Figure 3D). More importantly, MRL/lpr mice that received placebo rapidly developed apparent SLE manifestations at 14 weeks (Figure 3A), including malar rash, skin lesions, and lymphadenopathy. However, these SLE characteristics were thoroughly suppressed in mice implanted with ATRA pellets (Figure 3A).

Keratosis, indicated by hyperproliferative skin, was seen in lesion sections from placebo-implanted mice but significantly reduced in those from ATRA-implanted mice (Figure 3E). Enlarged spleens and lymph nodes found in the placebo group were not observed in ATRA-treated MRL/lpr mice (Figures 3B and C). Consistent with the above results, although renal size remained unaltered (Figures 3B and C), deposition of IgG, C3, and monocyte/macrophages (CD68) in the glomerulus was largely reduced in ATRA-treated mice (Figure
Levels of IgG anti-dsDNA, IL-17, proteinuria, and CD4–CD8— T cells were all reduced in MRL/lpr mice implanted with ATRA pellets (Figures 3G–J). Most importantly, separation of the survival curves indicated the ability of ATRA to prolong the lifespan of MRL/lpr mice from 17 months to 23 months (Figure 3K). Taken together, these findings indicate that ATRA has potent activity to reverse substantial SLE phenotypes, presumably via ablating Pin1.

**Expression of SLE phenotypes in lupus-prone mice effectively prevented by Pin1 knockout**

The above results demonstrate the dramatic inhibitory effects of the Pin1 inhibitor ATRA on expression of spontaneous SLE phenotypes. Given that ATRA is known to have other cellular targets such as retinoic acid receptors (RARs) (45,46), it is critical to use Pin1 genetic deletion to further confirm the significance of Pin1 in the pathogenesis of SLE. To this end, we crossed Pin1−/− mice with lupus-prone B6.lpr mice (43,44), both on the C57BL/6j background. The B6.lpr strain carries the same FasL deficiency as the MRL/lpr strain, on the FasL receptor. Thus, this strain is known to develop a similar disease phenotype, although the onset and severity are slower and milder than those of the MRL/lpr strain. The average survival of B6.lpr mice is 284 days (43). Each pair of Pin1+/−-B6.lpr and Pin1−/−-B6.lpr mice was from the same litter and mother to exclude unnecessary experimental factors.

Five pairs of B6.lpr mice were selected, with their genotype verified by confirming the presence or absence of Pin1 (Figure 4D), and, as expected, IRAK-1 activation was blocked in Pin1−/− mice (Figure 4D). Remarkably, Pin1 knockout in B6.lpr mice prevented the spontaneously developed exterior SLE phenotypes (Figure 4A), including malar rash, skin lesions, and lymphadenopathy, as well as the internal phenotypes, including splenomegaly (Figures 4B and C), lymphadenopathy (Figures 4B and C), and keratosis (Figure 4E). Furthermore, Pin1 knockout effectively suppressed glomerulonephritis in B6.lpr mice, as indicated by deposition of IgG, C3, and monocyte/macrophages (CD68) in the renal glomerulus (Figure 4F), although the size of the kidney was not affected (Figures 4B and C). Moreover, Pin1 knockout also significantly reduced other SLE indicators, including serum levels of IgG anti-dsDNA (Figure 4G) and IL-17 (Figure 4H) as well as levels of proteinuria (Figure 4I) and CD4–CD8— T cells (Figure 4J). Finally, Pin1 knockout significantly doubled lifespan from 7 months to 16 months (Figure 4K). Thus, Pin1 knockout alone is sufficient to prevent almost all SLE phenotypes and extend the lifespan of SLE-prone B6.lpr mice, establishing Pin1 as a key mediator of SLE progression. The results with Pin1 knockout paralleled perfectly those with ATRA treatment, which supports the hypothesis that the in vivo effectiveness of ATRA against SLE is through the ablation of Pin1.

**DISCUSSION**

It has been difficult to find a cure for SLE due to its clinical heterogeneity. Current treatments thus far cannot provide a curative strategy largely due to adverse effects and the lack of effective targeted therapy. In the present study, we discovered that Pin1-targeted therapy via genetic knockdown or knockout or pharmacologic inhibitor (ATRA) was effective at reversing or preventing numerous SLE symptoms including skin lesions,
lymphadenopathy, splenomegaly, glomerulonephritis, and proteinuria as well as overproduction of IgG anti-dsDNA antibodies, CD4−CD8− T cells, and cytokines, and that it eventually improved overall survival. Mechanistically, Pin1 inhibition led to inactivation of TLR-7/TLR-9/IRAK-1/IRF-7 signaling, which is a crucial pathway for SLE pathology.

It is worth noting that a recent study indicated improvements in some organs but worsening in others in MRL/lpr mice [47]. One of the most important differences between our experiments and those reported by Liao et al [47] involves the administration of ATRA. In our experiments, we implanted pellets releasing 10 mg ATRA subcutaneously over 21 days [27], which has been reportedly shown to maintain the serum concentration of ATRA constant at 0.6 μM [32,48]. This is because we have shown that ATRA is a submicromolar Pin1 inhibitor that can inhibit and degrade Pin1 at ~0.5−1.0 μM, as reported [27], while Liao et al administered ATRA orally at 6 mg/kg body weight per day.

It has been well documented that oral ATRA is quickly metabolized in the liver, with a very short half-life both in mice and in humans. For example, as reported [49], following oral dosing of mice with 10 mg/kg, tissue ATRA levels reach a maximum within 30−120 minutes, then decline after 3 hours in an exponential manner with half-life values of 25−68 minutes. Similarly, the half-life of ATRA in humans is 45 minutes [50]. With such a short half-life, it is impossible to have ATRA concentrations sufficient to keep Pin1 function disabled at all times, as we have previously shown [27]. However, at these low concentrations, ATRA can still activate its receptors (RARs or retinoid X receptors [RXRs]) because their affinity is low nanomolar.

Whether worsening phenotypes in some tissues observed by Liao et al could be due to activation of RARs or RXRs is not known. However, it is also worth noting that there was a significant therapeutic response to ATRA in 2 patients with steroid-resistant SLE [51]. SLE patients received ATRA treatment for 6 months and achieved complete remission, with reduced proteinuria and anti-dsDNA antibody levels. Disease did not recur, and neither patient experienced any adverse effects during > 1 year of follow-up. Moreover, ATRA was previously used to treat mice of 2 representative SLE-prone strains, MRL/lpr [52] and (NZB × NZW)F1/J [53], and it significantly inhibited a number of SLE symptoms, although the mechanisms of inhibition were not determined. ATRA-treated MRL/lpr mice had reductions in SLE manifestations, including lymphadenopathy, splenomegaly, proteinuria, glomerulonephritis, and cytokine overproduction [52]. ATRA-treated (NZB × NZW)F1/J mice also had reductions in SLE manifestations, including glomerulonephritis and cytokine overproduction, and had an increased rate of survival [53].

It remains to be determined why the effect of ATRA treatment seems to be weaker in (NZB × NZW)F1/J mice than in MRL/lpr mice in our experiments, but it may be because lupus-like pathogenesis differs between these strains [54]. Nevertheless, these studies in humans and mice provide proof of the concept that ATRA is a promising therapeutic tool for SLE, although the molecular targets underlying its therapeutic efficacy were previously unknown.

We recently found that ATRA targets and degrades active Pin1 selectively to exert potent anticancer activity against acute promyelocytic leukemia and breast cancer [27]. Our current
results further demonstrate that ATRA inhibits and degrades activated Pin1 and thereby interrupts TLR-7/TLR-9/Pin1/IRAK-1/IRF-7 signaling, which can explain its efficacy in treating SLE. Given that ATRA has a short half-life of 45 minutes in humans (50,55,56), these results underscore the importance of developing ATRA or Pin1-targeted ATRA derivatives with a longer half-life for treating SLE.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

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**References**


Figure 1.
Pin1 and Toll-like receptor 7 (TLR-7)/TLR-9/interleukin-1 receptor—associated kinase 1 (IRAK-1)/interferon regulatory factor 7 (IRF-7) signaling are activated in monocytes from patients with systemic lupus erythematosus (SLE). A–E, Activation of Pin1, IRAK-1, and IRF-7 in peripheral blood mononuclear cells (PBMCs) from SLE patients. PBMCs were isolated from 8 SLE patients and 5 age-matched controls (Ctrl) and subjected to immunoblotting with various antibodies (A), followed by quantification using ImageJ software (B–E). Dephosphorylation of Pin1 at Ser\textsuperscript{71} and Ser\textsuperscript{16} indicates active Pin1, while IRAK-1 phosphorylation indicates active IRAK-1. F, Pin1 enzymatic activity in PBMCs from SLE patients and normal controls, determined by the peptidylprolyl-cis-trans-isomerase (PPIase) assay. G and H, All-trans-retinoic acid (ATRA) inhibition of R848- or CpG-induced Pin1 activation. Cells were treated with R848, CpG, or vehicle control in the absence (G) or presence (H) of ATRA, followed by the PPIase assay. I–L, Cellular localization of IRF-7, Pin1 phosphorylated at Ser\textsuperscript{71}, Pin1 phosphorylated at Ser\textsuperscript{16}, and total Pin1 in isolated monocytes (I), CD4+ T cells (J), CD8+ T cells (K), and B cells (L) from SLE patients. Cells were isolated from SLE patients and controls and subjected to immunostaining with various antibodies. Original magnification × 400. In B–E, values are the mean ± SD. ** =P < 0.01; *** =P < 0.001 by Student’s t-test.
Figure 2.
Treatment with ATRA or Pin1 knockdown suppresses the Pin1/IRAK-1/IRF-7 axis in vitro. 
A, ATRA reduces Pin1 protein levels in a dose-dependent manner. Human monocytic THP-1 cells were treated with different concentrations of ATRA, followed by immunoblotting to detect Pin1 levels. 
B, ATRA or Pin1 short hairpin RNA (shRNA) suppresses R848-stimulated IRAK-1 activation. THP-1 cells were treated with different concentrations of R848 in the absence or presence of ATRA or Pin1 shRNA (shPin1), followed by detection of IRAK-1 using immunoblotting. IRAK-1 phosphorylation indicates IRAK-1 activation. 
C and D, ATRA or Pin1 shRNA represses R848-stimulated IRF-7 transactivation. THP-1 cells were treated with R848 in the absence or presence of ATRA or Pin1 shRNA, followed by detection of IRF-7 promoter luciferase activity with Renilla luciferase activity as an internal control (n = 3 independent runs of IRF-7 promoter luciferase assay) (C) or followed by detection of IRF-7 subcellular localization using immunostaining (D). Original magnification × 400. In C, values are the mean ± SD. ** = P < 0.01 by Student’s t-test. PBS = phosphate buffered saline (see Figure 1 for other definitions). 
Color figure can be viewed in the online issue, which is available at http://onlinelibrary.wiley.com/journal/doi/10.1002/art.39741/abstract.
Treatment with ATRA effectively suppresses expression of SLE phenotypes in SLE-prone MRL/MpJ-Fas<sup>+/−</sup> (MRL/lpr) mice. Female MRL/lpr mice were implanted with slow-releasing ATRA (5 mg over 21 days) or placebo pellets at age 8 weeks, and expression of various SLE phenotypes was examined. A. ATRA treatment inhibits exterior SLE phenotypes in MRL/lpr mice, including malar rash (left), skin lesions (middle), and lymphadenopathy (right). B, C, and E, ATRA treatment inhibits internal SLE phenotypes in MRL/lpr mice, including splenomegaly (B and C, left), lymphadenopathy (B and C, middle), and keratosis (E), without affecting kidney size (B and C, right). D, ATRA treatment reduces expression of total Pin1, Pin1 phosphorylated at Ser<sup>71</sup>, Pin1 phosphorylated at Ser<sup>16</sup>, and activated IRAK-1 in MRL/lpr mice, as detected by immunoblotting. Samples were spleens shown in B. F, ATRA treatment inhibits glomerulonephritis in MRL/lpr mice, including deposition of IgG, C3, and monocyte/macrophages (CD68) in the renal glomerulus, as shown by immunostaining. Original magnification 3 400. G and H, ATRA treatment reduces production of blood IgG anti-double-stranded DNA (anti-dsDNA) (G) and interleukin-17 (IL-17) (H) in MRL/lpr mice. I and J, ATRA treatment reduces proteinuria (I) and production of CD4–CD8<sup>−</sup> T cells (J) in MRL/lpr mice. K, ATRA treatment increases overall survival of MRL/lpr mice. Values in C and G–J are the mean ± SD (n 54 mice per group). * = P < 0.05; ** = P < 0.01; *** = P < 0.001; **** = P < 0.0001.
0.001 by Student’s t-test, two-way analysis of variance, or log rank (Mantel-Cox) test. PAS = periodic acid—Schiff (see Figure 1 for other definitions).
Figure 4.
Pin1 knockout effectively prevents expression of SLE phenotypes in SLE-prone B6.MRL-Fas\(^{lpr}\) (B6.lpr) mice. Five pairs of female Pin1\(^{+/+}\)-B6.lpr and Pin1\(^{-/-}\)-B6.lpr mice were selected and examined for expression of various SLE phenotypes. Each pair was from the same litter and mother to exclude unnecessary experimental factors. A, Pin1 knockout prevents exterior SLE phenotypes of B6.lpr mice, including malar rash (left), skin lesions (middle), and lymphadenopathy (right). B, C, and E, Pin1 knockout prevents internal SLE phenotypes of B6.lpr mice, including splenomegaly (B and C, left), lymphadenopathy (B and C, middle), and keratosis (E), without affecting kidney size (B and C, right). D, Pin1 knockout in B6.lpr mice is confirmed by immunoblotting. IRAK-1 activation is prevented in Pin1\(^{-/-}\)-B6.lpr mice. Samples were spleens shown in B. F, Pin1 knockout prevents glomerulonephritis in B6.lpr mice, including deposition of IgG, C3, and monocyte/macrophages (CD68) in the renal glomerulus, as shown by immunostaining. Original magnification × 400. G and H, Pin1 knockout prevents production of blood IgG anti—double-stranded DNA (anti-dsDNA) (G) and interleukin-17 (IL-17) (H) in B6.lpr mice. I and J, Pin1 knockout prevents proteinuria (I) and production of CD4–CD8— T cells (J) in B6.lpr mice. K, Pin1 knockout increases overall survival of B6.lpr mice. Values in C and G–J are the mean ± SD (n = 5 mice per group). * = P < 0.05; ** = P < 0.01 by Student’s t-test, two-way analysis of variance, or log rank (Mantel-Cox) test. PAS = periodic acid-Schiff (see Figure 1 for other definitions).