Iguratimod Inhibits Renal Interstitial Fibrosis in Lupus Nephritis

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Research article

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Abstract

Background: Renal interstitial fibrosis (RIF) is one of the main poor prognostic factors of lupus nephritis (LN). Here, we tested whether iguratimod could inhibit RIF in LN.

Methods: MRL/lpr mice, an animal model of lupus, were treated with iguratimod or vehicle solution. Pathological changes of kidney was evaluated blindly by the same pathologist. Renal type I collagen (COL-I), IgG, E-cadherin, fibroblast-specific protein 1 (FSP-1) were detected by immunofluorescence, immunohistochemical staining or quantitative real-time PCR. After treated with transforming growth factor β 1 (TGF- β 1) and iguratimod, E-cadherin, fibronectin, Smad2/3, p38 MAPK, p-Smad2/3 and p-p38 MAPK in HK2 cells were measured by western blotting, quantitative real-time PCR or immunofluorescence.

Results: In MRL/lpr mice, iguratimod eased the renal interstitial deposition of collagen fibers, further confirmed by decreased expression of COL-I after iguratimod treatment. Moreover, upstream pathological processes of RIF, including IgG deposition along the tubular basement membrane, infiltration of inflammatory cells into renal interstitium and tubular injury, were also prevented effectively by iguratimod treatment. Furthermore, iguratimod suppressed the expression of FSP-1 and raised the expression of E-cadherin in renal tubular epithelial cells, suggesting that iguratimod reversed the process of tubular epithelial-to-mesenchymal transition (EMT). In HK2 cells induced by TGF- β 1, co-treatment with iguratimod not only reversed cellular morphologic change, but also prevented down-regulation of E-cadherin and up-regulation of fibronectin. Finally, iguratimod blocked TGF- β 1-induced phosphorylation of Smad2/3 and p38 MAPK in HK2 cells.

Conclusions: Our results suggest that iguratimod shows an inhibitory effect on the progress of RIF in vivo and in vitro, so iguratimod may be a potential drug for the treatment of RIF in LN.

Background

Lupus nephritis (LN) is one of the major manifestations of systemic lupus erythematosus (SLE) which is a common autoimmune disease producing many kinds of autoantibodies and involving multiple organs, and the important cause of chronic kidney disease (CKD) [1]. CKD is characterized by extensive accumulation and deposition of extracellular matrix (ECM), resulting in severe glomerular sclerosis and widespread interstitial fibrosis [2, 3]. In particular, renal interstitial fibrosis (RIF) is the final common pathway of CKD [4, 5]. Glomerular lesions are specific for the disease etiology, while RIF presents almost identical pathological manifestation in all progressive CKD [5, 6]. Moreover, RIF leads to irreversible deterioration of renal function, necessitating renal replacement therapy and increased risk of death in patients. Therefore, inhibiting the progress of RIF may be an effective way to treat CKD [7, 8].

As a new antirheumatic drug, iguratimod has been proved to suppress the production of tumor necrosis factor α (TNF α), interleukin 6 (IL-6) and IL-8 in lipopolysaccharide - stimulated THP-1 cells [9], and inhibit the damage of cartilage and bone in type II collagen (COL-II) -induced arthritis in DBA/1J mice [10]. At

present, iguratimod has been recommended for the treatment of rheumatoid arthritis (RA) by Asia Pacific League of Associations for Rheumatology [11]. Recently, experimental studies have found that iguratimod may also have therapeutic effects in other diseases. Iguratimod ameliorated acute and chronic experimental autoimmune encephalomyelitis by inhibiting inflammatory cell infiltration and immune cell activation, partly through suppression of NF- κ B signaling pathway, supporting the therapeutic potential of this drug for not only acute, but also chronic multiple sclerosis [12]. In bleomycin-induced pulmonary fibrosis mice, iguratimod could significantly decrease pulmonary fibrosis via inhibition of matrix metalloproteinase 9 [13]. Iguratimod also plays a protective role in mice with dextran sulphate sodium-induced colitis via regulation of Th17/Treg cells and anti-inflammatory effects, suggesting that iguratimod may serve as a novel therapeutic drug for the treatment of inflammatory bowel disease [14].

In the MRL/lpr mouse model of lupus, iguratimod treatment not only alleviated proteinuria and acute renal pathological changes, but also restrained vascular lesions; the therapeutic effect of iguratimod on LN may be through modifying the imbalance in peripheral B cell differentiation [15]. However, whether iguratimod could inhibit RIF in LN remains unclear. Thus, the aim of this study was to evaluate the therapeutic effect of iguratimod on RIF and to elucidate its possible mechanism. In MRL/lpr mice, the present study found that iguratimod effectively blocked the progress of RIF and its upstream pathological processes, including IgG deposition along the tubular basement membrane, infiltration of inflammatory cells into renal interstitium, tubular injury and epithelial-to-mesenchymal transition (EMT). In human proximal renal tubular epithelial cells (PTECs), iguratimod remarkably reversed EMT induced by transforming growth factor β 1 (TGF- β 1), maybe via inhibition of Smad and p38 MAPK signaling pathways.

Methods

Mouse models

MRL/lpr and MRL/MpJ mice were purchased from Shanghai Slack Laboratory Animal Co. Ltd (Shanghai, China), and bred and maintained in the animal facility of the Second Affiliated Hospital of Soochow University. All animal study protocols were approved by the Ethics Committee of Soochow University (Approval No. SUDA20200225A10), Suzhou, China. Iguratimod (provided by Simcere Pharmaceutical, China) was dissolved in 1% carboxymethyl cellulose (CMC) solution at a concentration of 1.5 mg/ml. At 8 weeks of age, the MRL/lpr mice were intervened by gavage with any of the following treatments: 1.5 mg/ml iguratimod solution (0.02 ml/mg/d), 1% CMC solution (0.02 ml/mg/d). Mice were sacrificed after 16 weeks of intervention.

Serum analysis

Serum creatinine and blood urea nitrogen levels were determined by automatic biochemical analyzer (Rayto, China). The serum level of anti-double stranded DNA (anti-dsDNA) (Mlbio, China) was measured using sandwich ELISA kits, according to the manufacturer's instruction.

Cell culture

Human proximal tubular epithelial cells (HK2 cells) from Cell Bank of Chinese Academy of Sciences, were cultured in a 5% CO₂ atmosphere at 37 °C in DMEM/F-12 medium (Gibco, USA) supplemented with 10% fetal bovine serum (Gibco, USA), 100 units/ml penicillin G sodium, and 100 µg/ml streptomycin sulfate (Beyotime, China). For TGF-β1 (PeproTech, USA) alone treatment, the cells were plated at a 70% cell density, and cells were treated with TGF-β1 as indicated concentration and time. For co-treatment with TGF-β1 and iguratimod, the HK2 cells were preincubated for 2 h with iguratimod, then incubated with TGF-β1.

Quantitative real-time PCR

Total RNA was extracted from kidney tissue or HK2 cells using Trizol (Invitrogen, USA) according to the manufacturer's instructions. First strand cDNA was subsequently synthesized using the M-MuLV First Strand cDNA Synthesis Kit (Sangon Biotech, China). Quantitative real-time PCR was performed in triplicate using the SsoFast™ EvaGreen Supermix (Bio-Rad, USA) and the CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, USA). The primers used in the study are listed as follows: mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH), forward 5'-CCT CGT CCC GTA GAC AAA ATG-3' and reverse 5'-TGA GGT CAA TGA AGG GGT CGT-3' (133bps); mouse COL-1, forward 5'-GAG AGG TGA ACA AGG TCC CG-3' and reverse 5'-AAA CCT CTC TCG CCT CTT GC-3' (153bps); mouse fibroblast-specific protein 1 (FSP-1), forward 5'-GTG TCC ACC TTC CAC AAA TAC TCA-3' and reverse 5'-AAC TTC ATT GTC CCT GTT GCT G-3' (174bps); mouse E-cadherin, 5'-CGA CCG GAA GTG ACT CGA AAT-3' and reverse 5'-TCA GAA CCA CTG CCC TCG TAA T-3' (188bps); Human GAPDH, forward 5'-CTT TGG TAT CGT GGA AGG ACT C-3' and reverse 5'-GTA GAG GCA GGG ATG ATG TTC T-3' (132bps); human E-cadherin, forward 5'-AGC CCC GCC TTA TGA TTC TC-3' and reverse 5'-TTG CCC CAT TCG TTC AAG TAG-3' (132bps); human fibronectin, forward 5'-CAG CAG AGG CAT AAG GTT CGG-3' and reverse 5'-CAA AGC CTA AGC ACT GGC ACA A-3' (184bps). The GAPDH gene was used as an endogenous control to normalize for differences in the amount of total RNA in each sample. Relative gene expression changes were calculated as fold differences using $2^{-\Delta\Delta C_t}$ method.

Western blotting

Total proteins were extracted from HK2 cells with RIPA Lysis Buffer (50 mM Tris (pH 7.4), 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, sodium orthovanadate, sodium fluoride, EDTA, leupeptin) (Beyotime, China), and extraction of nuclear and cytoplasmic proteins was carried by NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific, USA). Protein concentrations were determined using the BCA Protein Assay Kit (Beyotime, China). The proteins were then separated by SDS-PAGE and electrophoretically transferred onto polyvinylidene fluoride membranes. The membranes were incubated overnight at 4 °C with rabbit polyclonal antibody specific for GAPDH and fibronectin (abcam, UK), rabbit monoclonal antibody specific for E-cadherin (abcam, UK), rabbit monoclonal antibody specific for Smad2/3, p-Smad2/3, p38 MAPK, and p-p38 MAPK (Cell Signaling Technology, USA). Detection was performed using a LumiGLO chemiluminescent substrate system (Cell Signaling Technology, USA).

Renal histopathology

The kidneys were dissected, fixed in 4% paraformaldehyde, embedded in paraffin, and stained with haematoxylin and eosin, Masson's trichrome and sirius red staining. The sections were evaluated blindly by the same pathologist, as previously described [16–18]. Tubular disease (atrophy, dilatation, casts), interstitial inflammation of renal cortex was each scored from 0 to 4 (0, absent; 1, present in < 25% of the section; 2, present in 25–50% of the section; 3, present in 50–75% of the section, and 4, present in > 75% of the section). Interstitial fibrosis was graded on a scale of 0–3 (0, < 5%; 1, 5%-10%; 2, 11%-25%; 3, > 25%).

Immunohistochemical staining

Immunohistochemical staining of kidney sections was performed by an established protocol [19]. The detection of IgG deposition was performed using horseradish peroxidase-conjugated goat anti-mouse IgG (Servicebio, China) and then DAB-chromogen (Servicebio, China). For Ki-67 and caspase-3 staining, rabbit anti-mouse polyclonal antibodies (Servicebio, China) against the antigen were used separately, followed by applying horseradish peroxidase-conjugated goat anti-rabbit IgG (Servicebio, China). The stains were scored by a renal pathologist blinded to the mice grouping, as previously described [18]. The score of Ki-67(+) cells was calculated according to the number of Ki-67(+) cells/40 × field, and the score of caspase-3(+) cells was scored from 0 to 2: 0, no significant positivity (occasional positive lymphocytes); 1, rare tubular cell staining of the section; 2, frequent tubular cell staining of the section.

Immunofluorescence staining

Indirect immunofluorescence staining was carried out according to established procedures. HK-2 cells grown on cover slips were washed with cold PBS three times and fixed in Immunol Staining Fix Solution (Beyotime, China) for 10 minutes. The cells were then extensively washed three times each for 5 minutes with Immunol Staining Scrubbing Solution (Beyotime, China). After blocking in Immunol Staining Blocking Buffer (Beyotime, China), cells were incubated at 4 °C overnight with indicated primary antibody, followed by incubation with Alexa Fluor® 488-conjugated goat antibody (abcam, UK) for 60 min at 37 °C. At last, slides were stained with DAPI Staining Solution (Beyotime, China) to visualize the cell nuclei and observed by confocal laser scanning microscopy.

Kidney sections were incubated with specific primary antibodies against COL-I, FSP-1, E-cadherin, respectively, followed by staining with Cy3-conjugated secondary antibody (Servicebio, China). The detection of IgG deposition along the tubular basement membrane was performed using Alexa Fluor® 488-conjugated goat anti-mouse IgG (Servicebio, China). Then, slides were stained with DAPI solution. Slides were viewed with a confocal laser scanning microscopy.

Statistical analysis

Data were expressed as mean means ± SEMs. Statistical analysis was performed with the GraphPad Prism 5. Comparison among groups was made using one-way ANOVA followed by Bonferroni's multiple

comparison test. Differences were considered significant at $P < 0.05$.

Results

Iguratimod inhibited the renal interstitial deposition of collagen fibers

Firstly, Masson's trichrome and sirius red staining were used to determine collagen fibers deposition which are the main components of ECM during the process of RIF [20]. Our results showed that no deposition of collagen fibers were detected in the renal interstitium of MRL/MpJ mice (the negative control), but remarkable in MRL/lpr mice (the positive control) (Fig. 1a and 1b). Remarkably, RIF was significantly alleviated in iguratimod-treated MRL/lpr mice ($P < 0.01$). Similarly, the quantitative real-time PCR results indicated that the levels of COL-I were much higher in MRL/lpr mice than MRL/MpJ mice ($P < 0.001$), whereas the mRNA levels of COL-I in iguratimod-treated MRL/lpr mice were suppressed obviously ($P < 0.001$) (Fig. 1c). Moreover, immunofluorescence was used to measure the deposition of COL-I, and compared with the positive control, the accumulation of COL-I in the renal interstitium was dramatically decreased in iguratimod-treated MRL/lpr mice (Fig. 1d). Taken together, iguratimod could effectively inhibited the progress of RIF in lupus mice.

Furthermore, as renal function deterioration signifies the substantial harmful effects of RIF [21, 22], renal function indicators including blood urea nitrogen and serum creatinine were subsequently measured. Likewise, the levels of both were decreased in the iguratimod-treated MRL/lpr mice compared with the positive control, though the differences for the latter were not statistically significant.

Iguratimod reduced immune deposition along the tubular basement membrane

SLE is characterized by the production of a large number of autoantibodies, especially anti-dsDNA antibodies, which are pivotal in the pathogenesis of LN via deposition in the mesangial, subendothelial, subepithelial, or tubulo-interstitial regions [23]. As a result, immune deposition along the tubular basement membrane is often observed in LN and suggested as a potential trigger to interstitial infiltration of inflammatory cells, tubular injury, and RIF [24]. Therefore, immunohistochemistry and immunofluorescence were used to determine the immune deposition along tubular basement membrane. There was a very small amount of IgG deposition along the tubular basement membrane in MRL/MpJ mice, whereas the staining intensity was increased evidently in MRL/lpr mice (Fig. 2a and 2b). Interestingly, iguratimod not only reduced the levels of immune deposition along the tubular basement membrane but also the serum titer of anti-dsDNA in MRL/lpr mice ($P < 0.001$) (Fig. 2c).

Iguratimod blocked the infiltration of inflammatory cells into renal interstitium

Inflammatory cells infiltrating into renal interstitium can induce apoptosis of renal tubular cells and progression of interstitial fibrosis by releasing a variety of inflammatory factors and pro-fibrosis factors, thereby leading to RIF [24–26]. In this study, the effect of iguratimod on the infiltration of inflammatory cells into renal interstitium was thus analyzed using haematoxylin and eosin staining. As shown in Fig. 3, a large number of inflammatory cells infiltrated around the renal tubules in MRL/lpr mice, while the infiltrating inflammatory cells were greatly reduced in iguratimod-treated MRL/lpr mice ($P < 0.01$).

Iguratimod alleviated the injury of renal tubules

Renal tubular injury, especially tubular atrophy, promotes the infiltration of inflammatory cells and leads to the process of tubular EMT, and finally results in the progress of RIF [23, 27, 28], so whether iguratimod can inhibit renal tubular injury was determined accordingly using Masson's trichrome staining. As shown in Fig. 4a and 4b, compared with the positive control, iguratimod obviously decreased tubular injury including tubular atrophy, dilation and casts in MRL/lpr mice ($P < 0.01$).

Next, as tubular injury was closely related to abnormal proliferation and apoptosis of tubular epithelial cells [23, 29], Ki-67 indicating cell proliferation and caspase-3 indicating apoptosis were measured using immunohistochemistry [18, 30]. Rare tubular epithelial cells were labeled by Ki-67 in MRL/MpJ mice, but a number of Ki-67 (+) cells were found in tubular area of MRL/lpr mice (Fig. 4c and 4d). In parallel, iguratimod decreased considerably the number of Ki-67 (+) cells in the tubular area of MRL/lpr mice ($P < 0.001$) (Fig. 4c and 4d). Unsurprising, the results of apoptosis measured by caspase-3 expression were consistent with those of proliferation (Fig. 4c and 4e).

Iguratimod inhibited epithelial-to-mesenchymal transition in vivo

Myofibroblasts are the main cells producing extracellular matrix in the process of RIF. Numerous studies have found that a large number of myofibroblasts are derived from tubular EMT [31, 32]. Tubular EMT is a process in which renal tubular epithelial cells change their shape from a cuboidal to a more elongated form, and is accompanied by the decreased expression of epithelial cell markers such as E-cadherin, and the increased synthesis of mesenchymal cell markers such as fibronectin, α -smooth muscle actin, and FSP-1 [33]. Therefore, expression of FSP-1 and E-cadherin in kidney were investigated using immunofluorescence and quantitative real-time PCR in this section.

As shown in Fig. 5a, a small amount of FSP-1(+) cells were detected in the renal interstitium of MRL/MpJ mice, but an increased number of tubular epithelial cells became positive for FSP-1 staining in MRL/lpr mice. Iguratimod treatment effectively inhibited the expression of FSP-1 in renal tubular epithelial cells of MRL/lpr mice (Fig. 5a). Conversely, iguratimod up-regulated the tubular expression of E-cadherin which normally reduced in MRL/lpr mice (Fig. 5b). The changes of gene level of FSP-1 and E-cadherin determined by quantitative real-time PCR were consistent with the results of immunofluorescence (Fig. 5c and 5d). These results suggested that iguratimod inhibited tubular EMT in MRL/lpr mice.

Iguratimod prevented TGF- β 1-induced EMT process in vitro

In order to confirm the inhibitory effect of iguratimod on tubular EMT, we further evaluated the role of iguratimod on tubular EMT *in vitro* with human proximal tubular epithelial cells (HK2 cells) induced by TGF- β 1 which is considered to be the strongest fibrogenic inducer [34, 35].

After treatment with 10 ng/ml TGF- β 1 for 48 h, the shape of HK2 cells became slender, and the growth of cells became chaotic (Additional file 1: Figure S1). However, co-treatment with iguratimod prevented TGF- β 1-induced morphologic changes of HK2 cells, and improved the cellular growth order.

We further tested the expression levels of E-cadherin and fibronectin in HK2 cells. Although TGF- β 1 down-regulated the expression of E-cadherin in HK2 cells, co-treatment with iguratimod significantly increased the E-cadherin expression measured by quantitative real-time PCR and western blotting (Fig. 6a and 6b). In accordance with these results, immunofluorescent staining also showed that iguratimod prevented TGF- β 1-induced down-regulation of E-cadherin (Fig. 6c). As expected, fibronectin expression in HK2 cells was up-regulated by TGF- β 1 treatment; but the co-treatment of iguratimod inhibited the rise of fibronectin expression including at gene and protein levels (Fig. 6a-c).

Iguratimod inhibited TGF- β 1-induced activation of Smad and p38 MAPK signaling pathways

Canonical Smad signaling pathway play the most important role in TGF- β 1-induced EMT [36], and p38 MAPK signaling pathway also participated in the regulation of EMT induced by TGF- β 1 [37, 38]. so we investigated whether iguratimod could inhibit the activation of Smad and p38 MAPK signaling pathways induced by TGF- β 1. As shown in Fig. 7a and 7b, western blotting results showed that co-treatment with 5 μ g/ml iguratimod significantly blocked phosphorylation of Smad2/3 and p38 MAPK induced by 10 ng/ml TGF- β 1. The levels of phospho-Smad2/3 (p-Smad2/3) and p-p38 MAPK in cytoplasm and nucleus all increased after TGF- β 1 treatment by immunofluorescent staining, but decreased after iguratimod co-treatment (Fig. 7c and 7d).

Discussion

Iguratimod, also named T-614 (3-formylamino-7-methylsulfonylamino-6-phenoxy-4H-1- benzopyran-4-one), has been considered to be a novel immunomodulator and widely used to treat RA by rheumatologists in China and Japan [39–41]. In present study, we observed the effect of iguratimod on RIF in LN. *In vivo*, iguratimod effectively inhibited RIF and its upstream pathological processes, including immune deposition along the tubular basement membrane, infiltration of inflammatory cells into renal interstitium, tubular injury and EMT; *in vitro*, iguratimod obviously reversed TGF- β 1-induced EMT process through suppressing the activation of Smad and p38 MAPK signaling pathways.

The tubulo-interstitium accounts for 90% of the total renal volume and proximal renal tubular epithelial cells are the main constituent cell type [23]. RIF is a common pathological manifestation and the ultimate result of various chronic kidney diseases. Numerous studies have shown that it is the severity of

interstitial damage, rather than glomerular injury, that is significantly associated with poor renal prognosis [33]. Therefore, the research and development of drugs for RIF is of great significance for the treatment of CKD. Yan Q et al also verified that MRL/lpr mice treated with iguratimod showed less proteinuria, less glomerular injury and vasculitis scores than vehicle-treated controls [15]. However, the effect of iguratimod on RIF in LN has not been studied. The present study found that iguratimod significantly alleviated the deposition of collagen fibers in the renal interstitium determined by Masson's trichrome and sirius red staining in MRL/lpr mice. Quantitative real-time PCR and immunofluorescence verified that the expression level of COL-I decreased evidently after iguratimod treatment, further supporting the protective role of iguratimod on inhibiting the RIF. Moreover, iguratimod treatment decreased the levels of blood urea nitrogen and serum creatinine, although the latter is not statistically significant, which may be due to the small sample size. Therefore, iguratimod could be an effective drug on inhibiting the progress of RIF in LN.

Although RIF portends poor long-term renal prognosis, the mechanisms resulting in RIF in LN has received relatively less attention to date. The deposition of immune complex in the kidney mediated by autoantibodies is the initiating factor of LN, in which anti-dsDNA plays a key role [23]. Glomerular and tubulo-interstitial expression of fibronectin is increased in mice and patients with active LN, which colocalized with IgG deposition, suggesting a pathogenic link between autoantibodies deposition and increased ECM accumulation in LN [42–44]. Immune deposition within the tubulo-interstitium is associated with increased cytokine production, immune cell infiltration, and tubular atrophy [24]. Anti-dsDNA antibodies bind to resident renal cells, including PTECs, and could induce synthesis of ECM, which promotes the progress of renal fibrosis [45]. The present study found that iguratimod showed an inhibitory effect on the deposition of IgG along the tubular basement membrane in lupus mice, which contributes to prevent the occurrence of RIF in LN. The serum titer of anti-dsDNA in MRL/lpr mice was also reduced by iguratimod, another study also found that iguratimod delayed the appearance of autoantibody and decreased autoantibody titers [15]. Therefore, we speculate that the decrease of IgG deposition along the tubular basement membrane is partly due to the reduction of serum autoantibodies in serum. whether iguratimod can inhibit the binding of autoantibodies to target antigens is not clear, which deserves further study.

Anti-dsDNA antibodies can increase secretion of TNF- α , IL-6, IL-8 and monocyte chemoattractant protein-1 in renal tubular epithelial cells, contributing to the establishment of chemotactic gradients and tubulo-interstitial infiltration of inflammatory cells [45]. Activated inflammatory cells will release a large number of pro-fibrosis factors, which could transform the resting ECM producing cells into myofibroblasts, resulting in a large number of ECM accumulation, and eventually lead to RIF [26, 46–48]. Moreover, anti-dsDNA and inflammatory factors can lead to abnormal proliferation and apoptosis of renal tubular epithelial cells [23], resulting in tubular atrophy, casts, and dilation. After tubular injury, the function of movement, migration, secretion and transformation of renal tubular epithelial cells increased significantly, and the normal tubulo-interstitial structure was destroyed [30, 49, 50]. At the same time, a large number of chemokines, inflammatory factors, pro-fibrosis factors and matrix proteins were produced [33], which further aggravated the injury of renal tubular epithelial cells and RIF. In present study, iguratimod not only

inhibited the renal interstitial infiltration of inflammatory cells, but also alleviated the tubular injury, which may be related to decreased RIF in iguratimod-treated MRL/lpr mice. The number of Ki-67 (+) cells and caspase-3 (+) cells all decreased in iguratimod-treated MRL/lpr mice, which further proved the protective role of iguratimod on renal tubules in LN, and also explained the reason for the reduction of renal tubular injury in mice treated with iguratimod.

In the process of RIF, tubular EMT is a key step [25, 51, 52]. Although a small number of renal interstitial myofibroblasts are from bone marrow, more myofibroblasts are derived by local tubular EMT during renal fibrogenesis [53]. Decreased expression of epithelial markers together with a concomitant increase in mesenchymal markers could be detected in renal biopsies from patients with LN and is associated with renal impairment, interstitial leukocyte infiltration, and interstitial fibrosis [30, 50]. In present study, iguratimod treatment decreased the FSP-1 expression and increased the E-cadherin expression in renal tubular epithelial cells of MRL/lpr mice. Moreover, in HK2 cells induced by TGF- β 1, iguratimod not only reversed morphologic changes, but also prevented down-regulation of E-cadherin and up-regulation of fibronectin. So iguratimod could effectively inhibited tubular EMT *in vivo* and *in vitro*, thus blocking the key step of RIF.

TGF- β 1 which has been identified as the key profibrogenic cytokine could regulate EMT process through multiple signaling pathways [54–56], such as Smad and p38 MAPK. Canonical Smad signaling pathway is considered to be the most important pathway in EMT and most of the TGF- β 1-induced EMT appears to be dependent on this signaling pathway [36, 57]. Thus, blocking the TGF- β 1/Smad signaling pathway may be promising for preventing RIF. In this study, iguratimod suppressed effectively TGF- β 1-induced phosphorylation of Smad2/3 and subsequent translocation into the nucleus, suggesting that iguratimod could revers TGF- β 1-induced EMT partly by inhibiting Smad signaling pathway.

Noncanonical TGF- β 1 signaling pathways also has been verified to promoting EMT process, specifically through the activation of p38 MAPK [38]. Co-treatment with iguratimod resulted in a decrease in IL-17-induced phosphorylation of p38 MAPK in the cultured fibroblast-like synoviocytes [58]. In RANKL induced RAW264.7 cells, iguratimod failed to inhibit but to markedly delay the phosphorylation of p38 MAPK [59]. However, in mesenchymal stem cells cultured with recombinant human bone morphogenetic protein-2, iguratimod exposure accelerated the activation of p-p38 MAPK [60]. These results suggest that the effect of iguratimod on p38 MAPK signaling pathway may be influenced by cell type and other co-existing inducers. In our data, iguratimod demonstrated a negative regulation on the activation of p38 MAPK signaling pathway in TGF- β 1 -induced HK2 cells, contributing to preventing the tubular EMT.

Conclusion

In conclusion, iguratimod showed an inhibitory effect on the progress of RIF in LN through reducing immune deposition along the tubular basement membrane, inhibiting the tubulo-interstitial infiltration of inflammatory cells, alleviating the tubular injury, and preventing the tubular EMT process; moreover, iguratimod effectively suppressed TGF- β 1-induced activation of Smad and p38 MAPK signaling

pathways which play an important role in the process of RIF. Therefore, iguratimod may be a potential drug on inhibiting the progress of RIF in LN.

Abbreviations

anti-dsDNA

anti-double stranded DNA; CKD:chronic kidney disease; COL-II:type II collagen; ECM:extracellular matrix; EMT:epithelial-to-mesenchymal transition; FSP-1:fibroblast-specific protein 1; GAPDH:glyceraldehyde-3-phosphate dehydrogenase; IL:interleukin; LN:lupus nephritis; p-Smad2/3:phospho-Smad2/3; PTECs:proximal renal tubular epithelial cells; RA:rheumatoid arthritis; RIF:renal interstitial fibrosis; SLE:systemic lupus erythematosus; TGF- β 1:transforming growth factor β 1; TNF:tumor necrosis factor;

Declarations

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Availability of data and materials

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

Authors' contributions

LXX, JJX, QW and YZ performed the research; LXX wrote the paper; WTL analyzed the data; CHW and JW prepared the reagents; LXX, JXF and ZCL designed the research. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All animal study protocols were approved by the Ethics Committee of Soochow University, Suzhou, China.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

Figure 1

Igaratimod inhibited the renal interstitial deposition of collagen fibers. Kidneys were collected from MRL/MpJ mice, MRL/lpr mice treated with 1% carboxymethyl cellulose solution, and MRL/lpr mice treated with iguratimod. (a) Masson's trichrome and sirius red staining were employed to observed the deposition of collagen fibers in renal interstitium. (b) Interstitial fibrosis score was evaluated blindly by the same pathologist. The expressive of renal COL-1 was detected by quantitative real-time PCR (c) and immunofluorescence staining (d), respectively. The levels of blood urea nitrogen (e) and serum creatinine (f) were also determined by automatic biochemical analyzer. The results are the mean $\pm$ SEM (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

Figure 2

Igaratimod reduced IgG deposition along the tubular basement membrane and serum levels of anti-dsDNA. Kidneys were collected from MRL/MpJ mice, MRL/lpr mice treated with 1% carboxymethyl cellulose solution, and MRL/lpr mice treated with iguratimod. IgG deposition in kidney was detected by immunohistochemical staining (a) (scale bar, 25 μ m) and immunofluorescence (b) (scale bar, 100 μ m), respectively. (c) The serum levels of anti-dsDNA were measured by sandwich enzyme-linked immunosorbent assay. The results are the mean $\pm$ SEM (**P < 0.001).

Figure 3

Igaratimod blocked the infiltration of inflammatory cells into renal interstitium. Kidneys were collected from MRL/MpJ mice, MRL/lpr mice treated with 1% carboxymethyl cellulose solution, and MRL/lpr mice treated with iguratimod. (a) The infiltration of inflammatory cells in renal interstitium was observed with haematoxylin and eosin staining. (b) Interstitial inflammation of renal cortex was evaluated blindly. Scale bar, 100 μ m. The results are the mean $\pm$ SEM (**P < 0.01, ***P < 0.001).

Figure 4

Iguratimod alleviated the injury of renal tubules. Kidneys were collected from MRL/MpJ mice, MRL/lpr mice treated with 1% carboxymethyl cellulose solution, and MRL/lpr mice treated with iguratimod. (a) Tubular injury was measured with Masson's trichrome staining (scale bar, 100 μ m). (b) The score of tubular disease (atrophy, dilatation, casts) was evaluated blindly. (c) Ki-67 and caspase-3 were detected by immunohistochemical staining (scale bar, 20 μ m). The scores of Ki-67 (+) cells (d) and caspase-3 (+) cells (e) were evaluated, respectively. The results are the mean $\pm$ SEM (* P < 0.05, ** P < 0.01, *** P < 0.001).

Figure 5

Igaratimod down-regulated the expression of FSP-1 and up-regulated the expression of E-cadherin in renal tubulo-interstitium. Kidneys were collected from MRL/MpJ mice, MRL/lpr mice treated with 1% carboxymethyl cellulose solution, and MRL/lpr mice treated with iguratimod. Immunofluorescence staining of the kidney tissue was conducted to assess the changes of FSP-1 (a) and E-cadherin (b) protein among three groups. The expression levels of renal FSP-1 (c) and E-cadherin (d) mRNA were detected by quantitative real-time PCR. Scale bar, 100 μ m. The results are the mean $\pm$ SEM (**P < 0.01, ***P < 0.001).

Figure 6

Iguratimod prevented TGF-β1-induced down-regulation of E-cadherin and up-regulation of fibronectin in HK2 cells. For TGF-β1 alone treatment, HK2 cells were treated with 10ng/ml TGF-β1 for 48h; for co-treatment with TGF-β1 and iguratimod, HK2 cells were preincubated for 2 h with iguratimod as indicated concentration, then incubated with TGF-β1. The mRNA and protein expression levels of E-cadherin and fibronectin were detected by quantitative real-time PCR (a), western blotting (b) and immunofluorescence staining (c), respectively. The ratio of TWEAK/glyceraldehyde-3-phosphate dehydrogenase (GAPDH) protein levels in MRL/MpJ mice was designated as one, and the representative band of western blotting was showed in (b). In immunofluorescence staining, the cell nuclei was stained with DAPI solution. Scale bar, 200 μm. The results are the mean ± SEM (*P < 0.05, **P < 0.01, ***P < 0.001).

Figure 7

Igaratimod inhibited TGF- β 1-induced activation of Smad and p38 MAPK signaling pathways. HK2 cells were incubated with 10ng/ml TGF- β 1 or 10ng/ml TGF- β 1 plus 5 μ g/ml iguratimod for different times, and p-Smad2/3, Smad2/3, p-p38 MAPK and p38 MAPK were detected by western blotting (a and b). Immunofluorescence staining was also used to measured p-Smad2/3 and p-p38 MAPK, and the cell nuclei was stained with DAPI solution (c and d). Scale bar, 200 μ m.

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