Glomerular Injury Is Exacerbated in Lupus-Prone MRL/lpr Mice Treated with a Protease-Activated Receptor 2 Antagonist

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Systemic lupus erythematosus (SLE) is characterized by the production of autoantibodies, which causes multi-organ injury such as lupus nephritis. SLE is associated with hypercoagulability. Activated coagulation factors such as tissue factor and VIIa complex and factor Xa activate protease-activated receptor 2 (PAR2). PAR2 promotes cytokine production through mitogen-activated protein kinase or nuclear factor kappa B signaling, and previous reports demonstrated that inhibition of PAR2 alleviated kidney injuries such as diabetic kidney disease and renal fibrosis in animal models. However, the involvement of PAR2 in the pathogenesis of SLE remains unclear. We therefore administered a selective PAR2 peptide antagonist, FSLLRY-NH2, to SLE-prone 4-month-old MRL-*Fas*^{lpr} mice for 4 weeks. Treatment with FSLLRY-NH2 caused the significant increases in the glomerular mesangial proliferation, glomerular deposition of both immunoglobulin G and complement factor C3d, and glomerular infiltration of Mac2-positive macrophages and CD3-positive T cells, compared with MRL-*Fas*^{lpr} mice treated with saline. In addition, the treatment with the PAR2 antagonist increased renal expression levels of tumor necrosis factor-α (*Tnfa*) and monocyte chemoattractant protein 1 (*Mcp1*) mRNA. Collectively, these results suggest that inhibition of PAR2 may increase the severity of inflammation in lupus nephritis; namely, opposite to previous observations, PAR2 has anti-inflammatory properties. We propose that activation of PAR2 could serve as a potential therapeutic option for patients with SLE.

Introduction

Systemic lupus erythematosus (SLE) is characterized by the production of autoantibodies, such as anti-double-stranded DNA (dsDNA) antibody, which causes systemic inflammation and multi-organ damage (Yu et al. 2017). Inflammation is linked to dysregulation of the coagulation system (Levi and van der Poll 2010; Witkowski et al. 2016). SLE pathogenesis is associated with hypercoagulability. For example, SLE activity is associated with plasma fibrinogen and D-dimer levels (Liang et al. 2016) as well as the risk for thrombosis (Tektonidou et al. 2009; Liang et al. 2016). A previous study of a murine model demonstrated that elevated expression of coagulation tissue factor is associated with kidney injury in SLE-prone MRL/lpr mice (Yamamoto et al. 1998).

Protease-activated receptor 2 (PAR2) is one of the four members of a G protein-coupled receptor family (PAR1-4), which is activated by coagulation tissue factor and VIIa complex or Xa (Rothmeier and Ruf 2012). PAR2 increases cytokine or chemokine production through mitogen-activated protein kinase or nuclear factor kappa B signaling (Rothmeier and Ruf 2012; Vesey et al. 2013). In murine kidney disease models, genetic deletion or pharmacological inhibition of PAR2 improves diabetic kidney disease (DKD), glomerulonephritis, renal fibrosis, and cisplatin nephrotoxicity through inflammatory cytokine or pro-fibrotic mediator reduction (Moussa et al. 2007; Kumar Vr

et al. 2016; Oe et al. 2016; Hayashi et al. 2017; Watanabe et al. 2019). On the other hand, in a recent study, we demonstrated that a deficiency of PAR2 aggravates vascular endothelial growth factor inhibitor-induced glomerular injury (Oe et al. 2019). Although protective and harmful effects of PAR2 on murine kidney injury have been shown, its role in lupus nephritis is not addressed.

In this study, we examined the effect of a PAR2 antagonist FSLLRY-NH2 on the kidney function of lupus-prone MRL/lpr mice to determine the role of PAR2 in SLE pathogenesis, and thus identified the protective role of PAR2 in this disease.

Methods

Animals

SLE-prone 4-month-old MRL/MpJ-Fas^{lpr}/J (MRL/lpr) female mice and MRL/MpJ (MRL/+) female mice (controls) were purchased from CLEA Japan (Kanagawa, Japan). MRL/lpr mice are homozygous for the spontaneous mutation in the *Fas* gene, and develop systemic autoimmunity, massive lymphadenopathy, and lupus nephritis resembling human SLE (Cohen and Eisenberg 1991). PAR2 antagonist FSLLRY-NH2 (4 mg/kg/day, Peptide Institute, Osaka, Japan) or saline was continuously administered to MRL/lpr mice using the MODEL 2004 Mini-Osmotic Pump (ALZET®, USA) for 28 days. This small pump was implanted subcutaneously under anesthesia. This antagonist was chosen because its therapeutic effects on diabetic complications in murine models and the dose of administration have been established (Park et al. 2011; Kumar Vr et al. 2016). The mice were sacrificed at 5 months of age. Spot urine samples were collected before sacrifice. All experiments were conducted in compliance with the guidelines issued by Tohoku University, Japan.

Biochemical measurements of blood and urine

Plasma anti-dsDNA antibody levels were measured using an enzyme-linked immunosorbent assay (ELISA) kit (FUJIFILM Wako Shibayagi, Gunma, Japan). Urinary albumin levels were measured using the Albuwell M ELISA kit (Exocel, Philadelphia, PA, USA). Plasma creatinine levels were determined using liquid chromatography-tandem mass spectrometry (Takahashi et al. 2007).

Histological evaluation

Kidneys were fixed in 2% paraformaldehyde (PFA), embedded in paraffin, and cut into 1.5-μm-thick sections. The sections were stained with periodic acid-Schiff (PAS). The mesangial matrix score was defined as the ratio of the glomerular PAS-positive area to the glomerular tuft area. All examinations were quantified using ImageJ (National Institutes of Health, USA) (Imaruoka et al. 2019).

Immunohistochemistry

Kidneys were fixed in 2% PFA, embedded in paraffin, and cut into 2.0-μm-thick sections. Glomerular CD3 and a marker of macrophages (Mac2) were detected by heat-induced antigen retrieval using a sodium citrate buffer. Proteinase K was used to identify immunoglobulin G (IgG) and complement factor C3d. The sections were incubated overnight with the following primary antibodies at 4°C: polyclonal goat anti-mouse C3d antibody (1:2,000 dilution; R&D Systems, Minneapolis, MN, USA), monoclonal rat anti-human Mac2 antibody (1:1,000 dilution; Thermo Fisher Scientific, Waltham, MA, USA), and polyclonal rabbit anti-human CD3 antibody (ready-to-use kit; Dako, Denmark). To detect glomerular IgG, we used goat anti-mouse IgG-conjugated horseradish peroxidase (HRP; 1:1,000) (Santa Cruz Biotechnology, Dallas, TX, USA). N-histofine simple stain kits (Nichirei Biosciences, Inc., Tokyo, Japan) were used as secondary antibodies. We randomly selected 20 glomerular sections in each mouse and evaluated more than 100 glomeruli. The area positive for C3d and IgG was defined as a ratio of positive glomerular area to total glomerular area. We corrected the number of infiltrated Mac2 or CD3 cells by their glomerular area. All procedures were performed using Image J.

Quantitative real-time polymerase chain reaction (RT-PCR)

We used TRI Reagent (Molecular Research Center, Inc., Cincinnati, OH, USA) to extract RNA from the kidneys. Reverse transcription was performed using the iScript Advanced cDNA Synthesis kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. The SsoAdvanced Universal Probes Supermix kit (Bio-Rad) was used for RT-PCR. Hypoxanthine-guanine phosphoribosyltransferase (*Hprt*) was used as a reference gene. The reaction protocols were as follows: initial denaturation at 95℃ for 3 min, reaction was cycled 40 times, and each cycle comprised denaturation

Table 1. Basal characteristics of MRL+ and MRL/lpr mice in this study (n = 4-8).

	MRL/+	MRL/lpr Saline	MRL/lpr FSLLRY-NH2
BW (g)	30.1 ± 0.6	40.2 ± 0.6[a]	40.9 ± 0.8[a]
Kidney/BW (mg/g)	6.1 ± 0.1	5.3 ± 0.2	6.0 ± 0.2
Liver/BW (mg/g)	41.2 ± 2.0	47.2 ± 1.9	51.2 ± 1.5[b]
Spleen/BW (mg/g)	2.8 ± 0.3	16.3 ± 1.8[a]	16.9 ± 1.7[a]
Mesenteric LN/BW (mg/g)	N.A.	25.7 ± 2.8	23.2 ± 4.3
P-creatinine (mg/dl)	0.12 ± 0.01	0.14 ± 0.01	0.13 ± 0.01
U-Alb (μg/mg Cre)	9.6 ± 1.5	10.7 ± 3.2	31.1 ± 11.7
Plasma ds-DNA Ab (U/ml)	212.5 ± 79.3	2365.4 ± 611.2	2578.4 ± 959.1

Data are shown as mean ± SEM (standard error of the mean).
BW, body weight; LN, lymph node. P-creatinine, plasma creatinine; U-Alb, urinary albumin; Cre, creatinine; Ab, antibody; N.A., not available.
[a]P < 0.001 vs. MRL/+; [b]P < 0.01 vs. MRL/+.

at 95℃ for 5s and annealing and extension at 60℃ for 15s. We have published the primer sequences in our previous reports (Li et al. 2010; Oe et al. 2016).

Statistics analysis

Multiple groups were compared using one-way analysis of variance with the Tukey-Kramer test. JMP 12.0.0 (SAS Institute Inc., Cary, NC, USA) was used to perform all analyses. The data were presented as the mean ± standard error of the mean or box-and-whisker plots. Tests were statistically significant when P < 0.05.

Results

Renal function, urinary albumin excretion, and anti-ds DNA antibody

Table 1 shows the basal characteristics of the mice. The plasma creatinine levels were similar among MRL/+, MRL/lpr-saline, and MRL/lpr-FSLLRY-NH2 mice. The urinary albumin creatinine ratio in MRL/lpr-FSLLRY-NH2 mice (31.1 ± 11.7 μg/mg) was not significantly different compared with other mice (9.6 ± 1.5 μg/mg in MRL/+ and 10.7 ± 3.2 μg/mg in MRL/lpr-saline mice). Lymphatic tissue swelling is one of the characteristics of MRL/lpr mice
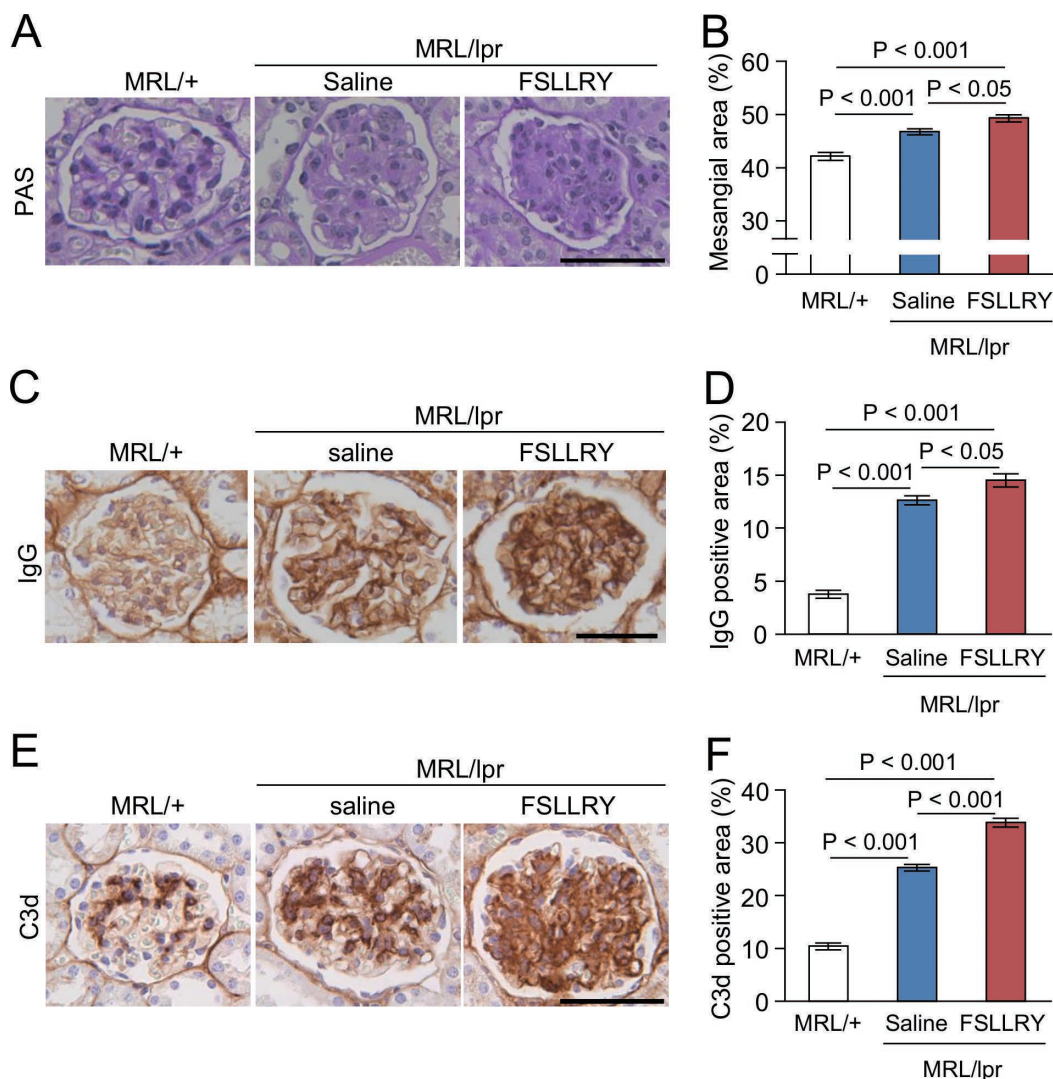

Fig. 1. PAR2 inhibition increases PAS-positive mesangial area and deposition of IgG and C3d in the glomeruli of MRL/lpr mice.
A. Representative photomicrographs of glomeruli in each group. Scale bar indicates 50 μm. B. Quantitative data of mesangial area. More than 100 glomeruli from each group were evaluated (n ≥ 5 mice each group). C. Representative photomicrographs of immunohistochemistry against IgG. Scale bar indicates 50 μm. D. The comparison of IgG positive area in the glomeruli. More than 100 glomeruli from each group were evaluated (n ≥ 5 mice each group). E. Representative photomicrographs of immunohistochemistry against C3d. Scale bar indicates 50 μm. F. The comparison of C3d positive area in the glomeruli. More than 100 glomeruli from each group were evaluated (n ≥ 5 mice each group). Data are shown as mean ± SEM (standard error of the mean).
FSLLRY, FSLLRY-NH2; PAS, periodic acid-Schiff stain.

and is associated with SLE activity (Cohen and Eisenberg 1991). We, therefore, weighed the spleens and mesenteric lymph nodes and found that FSLLRY-NH2 did not noticeably affect their weights in MRL/lpr mice. Additionally, FSLLRY-NH2 administration did not cause noticeable changes in the plasma levels of anti-dsDNA antibody in MRL/lpr mice.

Aggravation of histological injury and IgG and C3 deposition by FSLLRY-NH2

We examined whether the PAR2 antagonist affected glomerular histological injury. There was an enlargement in the PAS-positive mesangial area in MRL/lpr-saline mice compared with MRL/+ mice. FSLLRY-NH2 increased further the PAS-positive mesangial area in MRL/lpr mice (Fig. 1A, B). Increased deposition of the immune complex is an important indicator of SLE pathogenesis. Analysis of IgG and C3d deposition in the glomeruli revealed that the degree of their deposition was higher in MRL/lpr-saline mice compared with MRL/+ mice. FSLLRY-NH2 increased further both IgG and C3d deposition in MRL/lpr mice (Fig. 1C-F).

Infiltration of inflammatory cells and cytokine expression in the kidney

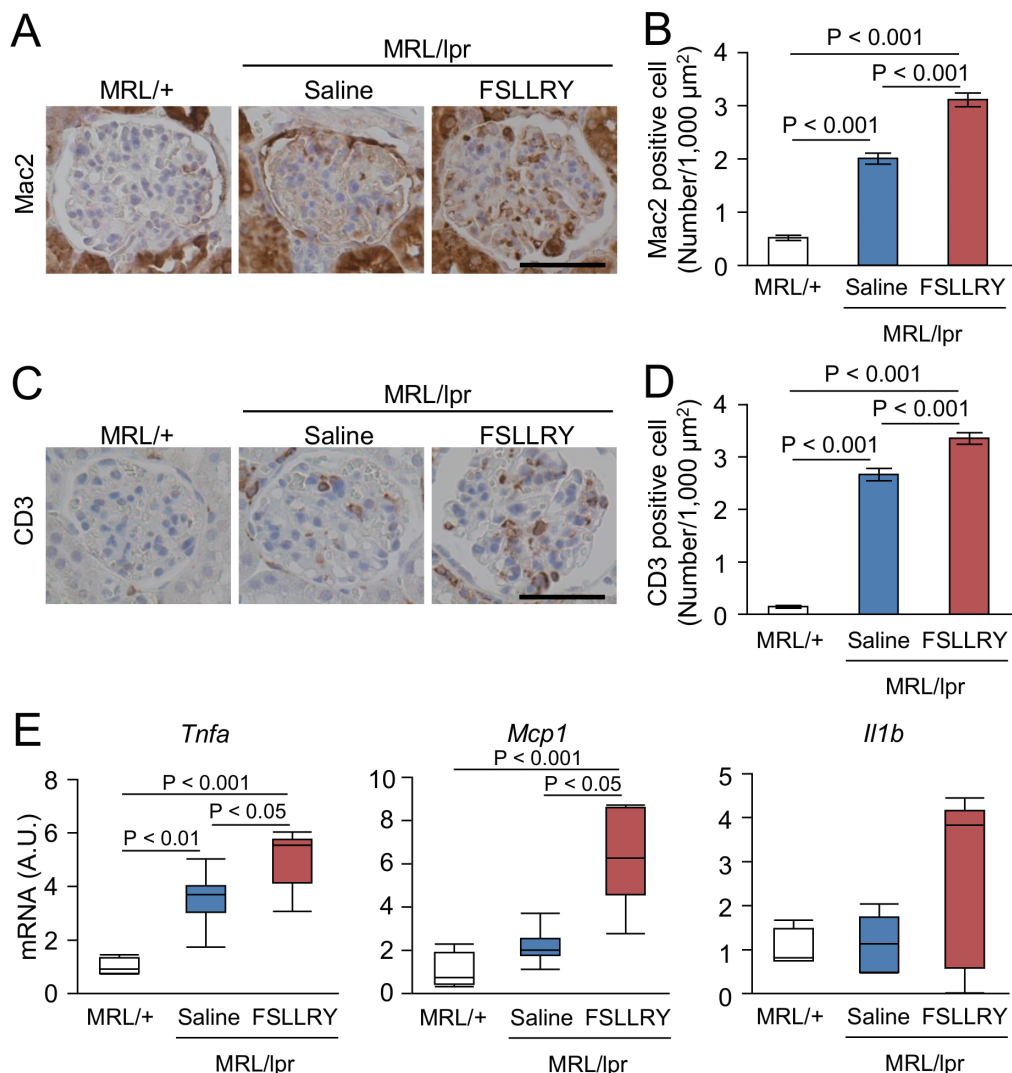To determine whether the aggravation of glomerular

Fig. 2. PAR2 inhibition exacerbates infiltration of Mac2-positive macrophages and CD3-positive cells in the glomeruli of MRL/lpr mice.
A. Representative photomicrographs of immunohistochemistry against Mac2 in the glomeruli. Scale bar indicates 50 μm. B. The number of infiltrated Mac2 positive cells each group. More than100 glomeruli from each group were evaluated (n ≥ 5 mice each group). C. Representative photomicrographs of immunohistochemistry against CD3 in the glomeruli. Scale bar indicates 50 μm. D. The number of infiltrated CD3 positive cells in each group. More than100 glomeruli from each group were evaluated (n ≥ 5 mice each group). E. Expression levels of *Tnfa*, *Mcp1*, and *Il1b* mRNA in the kidney (n = 4-7). Data are shown as mean ± SEM (standard error of the mean) or box-and-whisker plots. FSLLRY, FSLLRY-NH2; A.U., arbitrary unit.

injury caused by FSLLRY-NH2 administration was associ- ated with an increase in the severity of inflammation, we evaluated the renal infiltration of inflammatory cells and the renal expression of cytokines and chemokines. The num- bers of glomerular Mac2-positive macrophages and CD3-positive T cells were higher in the kidneys of MRL/lpr-saline mice and increased further in MRL/lpr-FSLLRY-NH2 mice (Fig. 2A-D).

We then measured the relative expression levels of tumor necrosis factor-α (*Tnfa*) and monocyte chemoattrac- tant protein 1 (*Mcp1*), and Interleukin-1β (*Il1b*) mRNA in the kidneys and found that *Tnfa* mRNA levels were higher in the kidneys of MRL/lpr-saline mice compared with MRL/+ mice. Also, *Tnfa* mRNA levels were significantly higher in MRL/lpr-FSLLRY-NH2 mice compared with MRL/+ and MRL/lpr-saline mice. *Mcp1* mRNA expression was significantly elevated in the kidneys of MRL/lpr- FSLLRY-NH2 mice compared with MRL/+ and MRL/lpr- saline mice. The change in *Il1b* mRNA expression in the kidneys of MRL/lpr-FSLLRY-NH2 mice was not significant (Fig. 2E). Our preliminary demonstration showed that FSLLRY-NH2 administration did not noticeably affect expression of cytokines and chemokines in the kidney of MRL/+ mice (data not shown), suggesting that PAR2 inhi- bition may specifically exacerbate inflammation in SLE- prone mice.

Renal expression of Par2 mRNA in SLE-prone mice

Studies using several different murine models of kid- ney injury have shown that there is an increase in the renal expression of PAR1 and PAR2 (Oe et al. 2016; Hayashi et al. 2017; Watanabe et al. 2019); thus, in this study, we mea- sured the renal expression levels of *Par1* and *Par2* mRNA. The expression levels of *Par1* mRNA were similar among MRL/+, MRL/lpr-saline, and MRL/lpr-FSLLRY-NH2 mice (Fig. 3A). By contrast, *Par2* mRNA levels were signifi-

cantly higher in the kidneys of MRL/lpr-saline mice than those in MRL/+ mice. Moreover, there was no significant difference in *Par2* mRNA levels between MRL/lpr mice- saline and MRL/lpr mice-FSLLRY-NH2 (Fig. 3B).

Discussion

Studies have shown that PAR2 is pro-inflammatory and exacerbates DKD, glomerulonephritis, renal fibrosis, and cisplatin nephrotoxicity in murine models (Moussa et al. 2007; Kumar Vr et al. 2016; Oe et al. 2016; Hayashi et al. 2017; Watanabe et al. 2019). On the basis of these find- ings, we expected that PAR2 inhibition would alleviate SLE. However, we found that FSLLRY-NH2 administra- tion exacerbated glomerular injury scores, with mesangial proliferation, IgG and C3d deposition, and renal inflamma- tion without change in the plasma level of anti-dsDNA anti- body. These findings suggest that PAR2 activation may exhibit protective effects in a murine model of SLE.

Although the mechanism of the protective effects of PAR2 in SLE remains elusive, PAR2 has anti-inflammatory effects in some inflammatory diseases. In sepsis, calprotec- tin S100A alleviates inflammation via PAR2 signaling (Sun et al. 2013), and endotoxins induce relocalization of PAR1- PAR2 complexes. PAR1 is protective in the PAR2- dependent pathway after administration of lipopolysaccha- ride (Kaneider et al. 2007). Similarly, in a murine model of experimental colitis, PAR2 agonists reduced histological injury and cytokine expression (Hansen et al. 2005). PAR2 agonists inhibited airway hyperresponsiveness and bron- choconstriction through the production of the anti-inflam- matory mediator prostaglandin E2 in a murine model of allergic inflammation (De Campo and Henry 2005). In our SLE model, we consistently found that a PAR2 antagonist increased the infiltration of inflammatory cells, such as CD3-positive T cells and macrophages, accompanied by increased expression of pro-inflammatory cytokines and
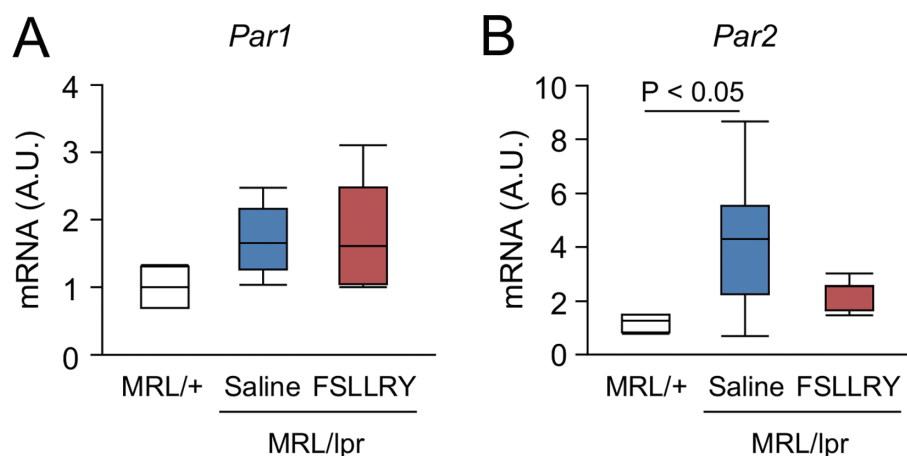

Fig. 3. Expression levels of *Par1* and *Par2* mRNA in the kidney of MRL/lpr mice.
A, B. Expression levels of *Par1* and *Par2* mRNA in the kidney. The levels of *Par2* mRNA were significantly higher in MRL/lpr mice compared with MRL/+ mice, but were not significantly changed in MRL/lpr mice treated with FSLLRY- NH2 (shown as FSLLRY). Data are shown as box-and-whisker plots (n = 4-7 each group).
A.U., arbitrary unit.

chemokines. These findings suggest that PAR2 can have both anti-inflammatory and pro-inflammatory effects in diseases. In some instances, PAR2 is anti-inflammatory and protective against glomerular injury in SLE. The factors that determine the effect of PAR2 on inflammation in different disease models are unknown, and this can be addressed in future studies.

A previous study demonstrated that administration of a cathepsin S antagonist (RO5459072) to MRL/lpr mice alleviated their systemic autoimmunity and inflammation (Tato et al. 2017). Additionally, cathepsin S exacerbated glomerular endothelial injury and albuminuria in these mice within 24 hrs. Pretreatment of MRL/lpr mice with another PAR2 antagonist (GB83) inhibited the detrimental changes by cathepsin S. In contrast, our data shows that a four-week treatment of MRL/lpr mice with FSLLRY-NH2 exacerbated kidney injury. Different experimental conditions of these two studies could explain the contrasting effects of the PAR2 antagonist on kidney injury of MRL/lpr mice. Studies of MRL/lpr mice lacking PAR2 could clarify this discrepancy.

Anticoagulation therapy is widely used for preventing venous thrombosis (Robertson et al. 2015). In addition to their anticoagulant effects, factor Xa inhibitors can block PAR2 activation (Oe et al. 2016; Bode et al. 2018). Research regarding the development of PAR2 inhibitors for suppressing inflammation is ongoing (Lim et al. 2013; Cheng et al. 2017). We need to focus on using such medications for SLE patients. However, whether PAR2 inhibitors can be administered to all patients needs to be considered.

In conclusion, PAR2 has anti-inflammatory properties, and a PAR2 antagonist exacerbates glomerular injury in lupus-prone mice. These findings suggest the diverse roles of PAR2 in kidney diseases. We propose that PAR2 activation may be beneficial for patients with SLE.

Acknowledgments

Author Contributions

R.I., Y.O., K.I., E.S., and A.S. performed experiments. R.I., Y.O., and N.T. analyzed data and co-wrote manuscript. E.S., S.Y., S.K., H.S., and S.I. interpreted the data and edited the manuscript. N.T. contributed to the conception of research.

Conflict of Interest

The authors declare no conflict of interest.

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