Bortezomib treatment prevents glomerulosclerosis associated with lupus nephritis in a murine model through suppressive effects on the immune and renin-angiotensin systems

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Corresponding Author: Kazuhisa Nozawa, MD.PhD
Juntendo University, School of Medicine
Tokyo, JAPAN

Corresponding Author Secondary Information:

Corresponding Author's Institution: Juntendo University, School of Medicine

Corresponding Author's Secondary Institution:

First Author: Yuko Matsuki-Muramoto

First Author Secondary Information:

Order of Authors: Yuko Matsuki-Muramoto
Kazuhisa Nozawa, MD.PhD
Kaori Uomori
Iwao Sekigawa
Yoshinari Takasaki

Order of Authors Secondary Information:

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Results. Bortezomib reduced the serum anti-dsDNA antibody titers and the proteinuria levels. It prevented inflammatory cell infiltrations into and the deposition of immunoglobulin G within the glomeruli. Bortezomib reduced the interferon-, interleukin (IL)-4, and IL-10 levels in the serum and the ribonucleic acid expression levels for these cytokines within the PBMCs. Bortezomib prevented type I collagen synthesis by downregulating TGF- and AT1R expression in the glomeruli.

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Bortezomib treatment prevents glomerulosclerosis associated with lupus nephritis in a murine model through suppressive effects on the immune and renin-angiotensin systems

Yuko Matsuki-Muramoto*, Kazuhisa Nozawa*, Kaori Uomori*, Iwao Sekigawa†#, and Yoshinari Takasaki*

*Department of Rheumatology, Juntendo University School of Medicine, 2-1-1 Hongo, Bunkyo, Tokyo 113-8421, Japan.

†Department of Internal Medicine and Rheumatology, Juntendo University Urayasu Hospital, 2-1-1 Tomioka, Urayasu, Chiba 279-0021, Japan.

#Institute for Environment and Gender Specific Medicine, Juntendo University Graduate School of Medicine, Chiba, 2-1-1 Tomioka, Urayasu, Chiba 279-0021, Japan.

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Corresponding author: Kazuhisa Nozawa, MD, PhD

Department of Rheumatology, Faculty of Medicine, Juntendo University, 2-1-1 Hongo, Bunkyo, Tokyo 113-8421, Japan.

Telephone number: 81-3-5802-1067

Fax number: 81-3-5800-4893

Email address: k-nozawa@juntendo.ac.jp

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Abstract

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Methods. Bortezomib was administered subcutaneously every 3 days to NZB/W F1 mice, and the serum anti-double stranded (ds) deoxyribonucleic acid (DNA) antibody titers and proteinuria levels were measured. The kidneys and peripheral blood mononuclear cells (PBMCs) were examined histologically or used for real-time quantitative reverse transcription-polymerase chain reaction analysis after 18 weeks of treatment. Serum cytokine and anti-dsDNA antibody levels were measured using flow cytometry and enzyme-linked immunoassays every 3 weeks. Transforming growth factor (TGF)-β, angiotensin II type 1 receptor (AT1R), and type I collagen expression levels in the glomeruli were evaluated using immunohistochemistry.

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Conclusions. Bortezomib exerts multiple immunosuppressive effects and thus ameliorates LN. Furthermore, bortezomib can prevent glomerulosclerosis formation in NZB/W F1 mice through suppressive effects on the renin-angiotensin system.
**Introduction**

Systemic lupus erythematosus (SLE) is an autoimmune disease with multi-organ involvement. Its course is unpredictable and its manifestations are varied. Lupus nephritis (LN) affects almost half of the patients with SLE, and is one of the main causes of morbidity and mortality in SLE. The treatment of LN poses a serious clinical challenge, particularly in adolescent and young patients. Although combination therapy comprising steroids and immunosuppressants that include cyclophosphamide, azathioprine, and mycophenolate mofetil, is well established for the treatment of LN, it is often not effective. Hence, establishing new therapies for LN is necessary.

LN was classified in 2003 by the International Society of Nephrology and the Renal Pathology Society [1], as described next. Classes I and II are used to describe mesangial involvement only, where class I is used for mesangial immune deposits without mesangial hypercellularity, and class II is used for mesangial immune deposits with mesangial hypercellularity. Class III is used to describe focal glomerulonephritis that involves 50% of the total number of glomeruli, and it includes subdivisions for active and sclerotic lesions. Class IV is used to depict diffuse glomerulonephritis that involves 50% of the total number of glomeruli, with either segmental (class IV-S) or global (class IV-G) involvement, and it also includes subdivisions for active and sclerotic lesions. Class V is used to describe membranous LN. Class VI is used to describe advanced sclerosing lesions, and it may represent advanced stages of chronic class III, class IV, or class V LN. Glomerulosclerosis is defined as an increase in extracellular matrix (ECM) and/or other material that obliterates the glomerular capillaries and solidifies all or part of the glomerular tuft. Moreover, glomerulosclerosis is associated with glomerular fibrosis and increased levels of ECM proteins, which are typically represented as type I collagen, in the capillary tufts [2]. Glomerulosclerosis and glomerular fibrosis are irreversible.
end points that are not amenable to treatment. Autoimmune-mediated renal damage gives rise to active glomerulonephritis lesions and these change from active lesions to chronic lesions that ultimately lead to glomerulosclerosis. The factors underlying the development of glomerulosclerosis are diverse and it can progress independent of the autoimmune-mediated inflammatory response.

Inflammatory cells infiltrate the glomeruli, and the glomerular mesangial cells play an important role in the development of LN. The glomerular mesangial cells are thought to provide structural support and to form a functional unit for the glomerular tuft, which together with the adjacent glomerular capillary endothelial cells and podocytes, regulate glomerular filtration. The mesangial cells are the major contributors to the ECM protein synthesis that constitutes the mesangium, and they are important in the maintenance of homeostasis within the mesangial matrix. They are also major targets of a number of glomerular diseases, including IgA nephropathy and diabetic nephropathy. As LN progresses, the mesangial cells proliferate and produce excessive amounts of ECM proteins, such as type I collagen, which is mainly mediated by transforming growth factor (TGF)-β stimulation, and this leads to glomerulofibrosis subsequently resulting in glomerulosclerosis [3]. Therefore, preventing both the autoimmune-mediated renal inflammatory and the glomerular fibrotic processes would be an ideal treatment strategy for LN.

Bortezomib is a selective inhibitor of the 26S proteasome that is approved for the treatment of relapsed multiple myeloma [4]. Proteasomes are multi-enzymatic protein complexes that are crucial in cell homeostasis. Their functions include the degradation of unfolded or misfolded proteins, the control of the cell cycle, the regulation of gene expression, and the activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB).
Consequently, bortezomib is involved in inhibiting NF-κB, modulating tumor microenvironments, cytokine expression, and stromal cell interactions [5,6]. Proteasome inhibition can also lead to apoptosis via the activation of the unfolded protein response, which is a cellular stress response associated with the endoplasmic reticulum that is pronounced in cells with high levels of immunoglobulin (Ig) synthesis [7].

NZB/W F1 mice spontaneously develop nephritis that resembles human LN. In NZB/W F1 mice, the nephritis usually begins with proteinuria at the age of 5–7 months and its progress causes the death of the animals at a mean age of 8–9 months [7]. Proteasome inhibition by bortezomib ameliorates nephritis in NZB/W F1 mice by depleting short- and long-lived plasma cells [8], preventing type I interferon (IFN) activation [9], and by preserving the glomerular and tubulointerstitial architecture [7]. Recently, bortezomib has been reported to be effective in patients with SLE who have persistent disease activity that is accompanied by LN [10]. However, the precise mechanisms underlying the amelioration of LN by bortezomib have not yet been fully elucidated. Therefore, we undertook the present study to further clarify the mechanisms underlying the amelioration of nephritis following treatment with bortezomib in lupus-prone mice. This study had a particular focus on its suppressive effects on the immune response and its effects on glomerulosclerosis formation in LN.

Materials and methods

Animals and treatment

NZB/W F1 mice spontaneously develop a disease at the age of 24–30 weeks that closely resembles human SLE, and this is accompanied by proliferative nephritis that is very similar to human proliferative LN [9]. Female NZB/W F1 mice were purchased from Sankyo Laboratories
(Tokyo, Japan) and they were housed in the animal facilities at Juntendo University. All of the mice were aged between 10 and 12 weeks at the start of the experiments, and they were kept under specific pathogen-free conditions during the experiments. All of the animal experiments were approved by Juntendo University’s Animal Experiment Committee.

Millennium Pharmaceuticals, Inc., The Takeda Oncology Company (Cambridge, MA, USA) kindly provided the bortezomib. The NZB/W F1 mice ($n = 18$) were administered bortezomib (0.75 mg/kg) subcutaneously every 3 days from the age of 22 weeks until the age of 40 weeks. The control animals ($n = 18$) were administered phosphate-buffered saline (PBS) instead of bortezomib. Serum and urine samples were collected every 3 weeks during treatment. The mice were sacrificed 18 weeks after the treatment began when they were aged 40 weeks, and their spleens and kidneys were collected for the subsequent analyses.

Proteinuria measurements

The proteinuria levels were assessed every 3 weeks and they were graded from 0 to 5 based on the urine albumin levels, as previously described with slight modifications [11]: grade 0: < 37 mg/100 mL, grade 1: ≥ 37 mg/100 mL, grade 2: ≥ 74 mg/mL, grade 3: ≥ 111 mg/100 mL, grade 4: ≥ 333 mg/100 mL, and grade 5: ≥ 1000 mg/100 mL.

Enzyme-linked immunosorbent assay

Serum samples were collected every 3 weeks from the bortezomib-treated and control mice, and the anti-double stranded (ds) deoxyribonucleic acid (DNA) antibody levels were measured using enzyme-linked immunosorbent assay plates (MESACUP DNA-II TEST; Medical & Biological Laboratories Co. Ltd., Tokyo, Japan) in accordance with the manufacturer’s instructions.
Histochemical analysis

Paraffin sections of the kidneys from the mice were deparaffinized, rehydrated, and washed with water as previously reported [12]. They were used for immunofluorescence staining and for hematoxylin and eosin (H&E) staining. For the immunohistochemical analyses, frozen sections were fixed with 4% paraformaldehyde. Some sections were incubated for 60 min at 37°C with a rabbit anti-mouse type I collagen antibody (Abcam plc, Cambridge, UK) at a 1:100 dilution in PBS. Other sections were incubated overnight at 4°C with a rat anti-mouse cluster of differentiation (CD)138 antibody (Biolegend Inc., San Diego, CA, USA) at a 1:200 dilution in PBS or they were incubated for 30 min at 37°C with a rabbit anti-mouse TGF-β antibody (Santa Cruz Biotechnology Inc., Dallas, TX, USA) at a 1:100 dilution in PBS. After washing with PBS, the bound antibodies were visualized using the iVIEW™ Universal DAB detection Kit (Ventana Medical Systems, Inc., Tucson, AZ, USA) in accordance with the manufacturer’s instructions. The immunofluorescence staining technique involved incubating the sections overnight at 4°C with a rabbit anti-angiotensin II type 1 receptor (AT1R) antibody (Santa Cruz Biotechnology Inc., Dallas, TX, USA) at a 1:100 dilution. After washing, the bound antibodies were labeled with an Alexa 488 conjugated anti-rabbit IgG antibody (Thermo Fisher Scientific, Waltham, MA, USA) that facilitates immunofluorescence detection in tissues. Other sections were directly stained with an anti-rabbit IgG antibody (Thermo Fisher Scientific, Waltham, MA, USA) for the detection of glomerular IgG deposits, or they were stained with H&E.

Real-time quantitative reverse transcription-polymerase chain reaction

Ribonucleic acid (RNA) was purified from splenic peripheral blood mononuclear cells (PBMCs)
and renal samples using the RNeasy Mini Kit (Qiagen, Venlo, Limburg, Netherlands) in accordance with the manufacturer’s instructions. The real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was performed as previously described [12].

The primers used were as follows: mouse IFN-γ (GenBank accession no. NM_008337.3):

5-CGGCACAGTCATTGAAAGCCTA-3 (forward) and 5-GTGCTGATGGCCCTGATTGTC-3 (reverse), mouse interleukin (IL)-4 (GenBank accession no. NM_021283.2):

5-ACGGAGATGGATGGCCTC-3 (forward) and
5-AAGCACCTTGGAAGCCCTACAGA-3 (reverse), mouse IL-10 (GenBank accession no. NM_010548.2):

5-GTTGCTGATGGCCTGATTGTC-3 (reverse), mouse IL-6 (GenBank accession no. NM_031168.1):

5-GATAAGGCTTGGCAACCCAAGTAA-3 (reverse), mouse CXC chemokine ligand 13 (CXCL13) (GenBank accession no. NM_018866.2):

5-GACAGAGCCACATGCTCCTA-3 (forward) and
5-GTTGCTGATGGCCTGATTGTC-3 (reverse), mouse β-actin:

5-GAACTCCACCTCCAGGCAGAA-3 (forward) and 5-GTTGCTGATGGCCTGATTGTC-3 (reverse), mouse glyceraldehyde 3-phosphate dehydrogenase (GAPDH):

5-CGCCGTAAAGACCTCTATGCCAAC-3 (forward) and
5-ATGGAGCCACCGATCCACA-3 (reverse), and mouse glyceraldehyde 3-phosphate dehydrogenase (GAPDH):

5-CGCCGTAAAGACCTCTATGCCAAC-3 (forward) and
5-ATGGAGCCACCGATCCACA-3 (reverse). The qRT-PCR was performed using a 10 µL sample volume with 500 ng of complimentary DNA in a SYBR Premix Ex Taqkit (Takara Bio Inc., Shiga, Japan). The amplification cycles consisted of 95°C for 5 s as the first step (1 cycle), 95°C for 5 s and 60°C for 30 s as the second step (40 cycles), and 95°C for 5 s, 60°C for 30 s, and 95°C for 15 s as the third step (1 cycle), in accordance with the manufacturer’s protocol (Takara Bio Inc., Shiga, Japan). To quantify the expression levels of the transcripts, sample
loading was monitored and transcript expression was normalized according to the expression of
the β-actin or GAPDH transcripts.

Serum cytokine levels
The levels of the cytokines in the serum samples were measured using the BD Cytometric Bead
Array (CBA) Mouse Th1/Th2/Th17 Cytokine Kit (BD Biosciences, San Jose, CA, USA), which
simultaneously detects mouse cytokines, including IL-4, IL-6, IFN-γ, and IL-10. The assays were
performed in accordance with the manufacturer’s instructions. Briefly, beads coated with 7
specific capture antibodies were mixed together. Subsequently, 50 μL of the mixed beads, 50 μL
of the unknown serum sample or the standard dilution, and 50 μL of the phycoerythrin (PE)
detection reagent were added consecutively to each assay tube, and they were incubated for 2 h
at room temperature in the dark. The samples were washed with 1 mL of the wash buffer at 200g
for 5 min and they were centrifuged, the supernatants were discarded, and the bead pellets were
resuspended in 300 μL buffer. The samples were evaluated using a BD FACSVerse™ flow
cytometer (BD Biosciences, San Jose, CA, USA), and they were analyzed using FCAP Array™
Software (BD Biosciences, San Jose, CA, USA). The concentrations of the individual cytokines
were indicated by their fluorescent intensities. Each cytokine standard was serially diluted to
facilitate the construction of the calibration curves, which enabled the determination of the
protein concentrations within the test samples.

Statistical analysis
A paired t-test and a non-parametric Mann-Whitney U test were used to compare the study
groups. P values of < 0.05 were considered statistically significant.
Results

Bortezomib suppresses glomerulonephritis in NZB/W F1 mice

NZB/W F1 mice spontaneously develop glomerulonephritis at 24–30 weeks of age, which resembles human LN, and this is characterized by the production of anti-dsDNA antibodies, proteinuria, and IgG deposits in the glomeruli. To elucidate the precise mechanisms underlying the therapeutic effect of bortezomib on LN, we administered bortezomib to NZB/W F1 mice for 18 weeks from the age of 22 weeks. The control mice started to produce anti-dsDNA antibodies 3 weeks after the mock treatment began when they were 25 weeks old, and the levels of these antibodies continued to increase until the end of the experiment, which was 18 weeks after the treatment began (Fig. 1a). Bortezomib treatment suppressed the production of anti-dsDNA antibodies compared with that seen in the control mice (Fig 1a). In addition to the anti-dsDNA antibodies, we evaluated the levels of proteinuria, which is the major symptom of glomerulonephritis. The control mice began to develop proteinuria at the age of 25 weeks, which was 3 weeks after the mock treatment began, and it peaked at 15–18 weeks after the treatment was initiated, when the mice were 37–40 weeks old (Fig. 1b). Compared with the control mice, the proteinuria levels were suppressed in the bortezomib-treated mice (Fig. 1b).

Next, we histologically analyzed the renal tissues from bortezomib-treated or control NZB/W F1 mice to evaluate the LN amelioration. H&E stained renal tissues from the control mice showed glomerular hypertrophy, increased levels of mesangial proliferation, and progressive infiltration by mononuclear cells (Fig. 2), which are indicative of glomerulonephritis.
The histological analysis of the renal tissues from the bortezomib-treated mice showed the amelioration of these manifestations of glomerulonephritis (Fig. 2). The renal tissues from the control mice showed high levels of glomerular IgG deposits, which is one of the other major histological characteristics of LN (Fig. 2), and in the bortezomib-treated mice the glomerular IgG deposits were diminished (Fig. 2).

Bortezomib suppresses cytokine production in NZB/W F1 mice

We investigated changes in the expression of IFN-γ, IL-4, TGF-β, IL-6, and IL-10 following bortezomib treatment. Compared with the PBMCs from the control mice, the levels of expression of IFN-γ, IL-4, and IL-10 RNA derived from the PBMCs were suppressed in the bortezomib-treated mice (Fig. 3). Compared with the PBMCs from the control mice, the level of expression of IL-6 RNA was not significantly higher in the bortezomib-treated mice (Fig. 3).

The changes in the serum levels of the cytokines that were evaluated using cytometric bead arrays, reflected the changes at the RNA level in the PBMCs, but the differences between the study groups in relation to the levels of IFN-γ and IL-10 in the serum did not reach statistical significance ($p = 0.06$ and $p = 0.07$, respectively) (Fig. 4). The serum level of IL-6 showed a statistically significant difference between the control mice and the bortezomib-treated mice, although the difference between the control mice and the bortezomib-treated mice in relation to the level of RNA expression for IL-6 within the PBMCs was not statistically significant.

Bortezomib prevents aberrant plasma cell infiltration into the glomeruli

To investigate whether bortezomib attenuates plasma cell infiltration, we evaluated CD138+ plasma cells in the glomeruli. Marked infiltrations of CD138+ plasma cells were found within the
glomeruli of the control mice (Fig. 5a), and the accumulations of CD138+ plasma cells were less prominent in the glomeruli of the bortezomib-treated mice (Fig. 5a).

CXCL13 is a chemokine known as B cells chemoattractant. We evaluated the expression of CXCL13 in the renal tissues from NZB/W F1 mice, and we found that the expression of CXCL13 was significantly suppressed in the bortezomib-treated mice compared with the control mice (Fig. 5b).

Bortezomib suppresses the glomerulosclerosis associated with nephritis in NZB/W F1 mice

Numerous reports suggest that renin-angiotensin system (RAS) plays an important role in the progression of glomerulonephritis independent of immunological response [13]. Therefore, we assessed the expression of TGF-β, AT1R and collagen type I, which are involves in RAS, in the glomeruli of the control mice and in the glomeruli of the mice treated with bortezomib to investigate its effects on renal fibrotic processes. TGF-β was strongly expressed in the glomeruli of the control mice and this was suppressed by bortezomib treatment (Fig. 6a). The expression of AT1R was also strongly expressed in the control mice, particularly at the surfaces of the glomeruli, (Fig. 6b) and its expression was suppressed in the bortezomib-treated mice (Fig. 6b). To further confirm the suppressive effect of bortezomib on the development of renal fibrosis, we assessed the glomerular synthesis of type I collagen, which is a major component of the ECM [14]. Bortezomib-treated mice showed a marked reduction in the synthesis of type I collagen compared with the control mice (Fig. 6).

Discussion
The treatment of LN in patients with SLE poses a serious clinical challenge, especially in patients in whom treatment is not effective. Despite aggressive immunosuppressive therapy that includes cyclophosphamide, azathioprine, and mycophenolate mofetil, which are administered in combination with high-dose glucocorticoids, the LN remissions are often brief or they may not occur in SLE. Although new treatments, for example, belimumab, rituximab, and abatacept, have been tested on SLE patients with LN [15], other treatments are awaited that will offer novel therapeutic strategies for LN.

Bortezomib, which is a selective inhibitor of the 26S proteasome, has been approved for the treatment of relapsed multiple myeloma, which is a plasma cell neoplasm. Neubert et al. reported that bortezomib treatment reduced anti-dsDNA antibody production, proteinuria levels, and kidney damage in NZB/W F1 mice by depleting the plasma cells that can produce anti-dsDNA antibodies [8]. Moreover, the beneficial effect of bortezomib treatment in SLE patients was demonstrated recently [10]. Subsequent reports that have described the mechanisms that may underlie the potentially beneficial effects of bortezomib in the treatment of LN, have suggested that bortezomib contributes to the amelioration of LN by inhibiting type I IFN and by depleting B or plasma cells in NZB/W F1 mice [9,16].

Our study showed that CD138⁺ plasma cells massively infiltrate the glomeruli in NZB/W F1 mice, and that this infiltration was prevented by bortezomib. Plasma cells accumulate and survive in the inflamed kidneys of NZB/W F1 mice and they could produce autoantibodies locally, which would enhance the local concentrations of autoantibodies and immune complexes [17,18]. Furthermore, it has been reported that the infiltrating plasma cells play a major role in the deposition of immune complexes of the renal tissues of NZB/W F1 mice [19]. Our results indicate that bortezomib treatment may affect the autoreactive B cells or plasma cells that are...
localized within the kidney, which would ameliorate LN. Moreover, we have confirmed that compared with control mice, the level of RNA expression of IFN-α, a type I IFN, derived from the PBMCs, was reduced in NZB/W F1 mice treated with bortezomib (data not shown). These results support those from previous studies in relation to the mechanisms underlying the beneficial effects of bortezomib treatment in LN [9,16]. A new finding from the current study was that bortezomib suppresses chemokine, namely, CXCL13, production in renal tissues. CXCL13 is mainly produced by the dendritic cells within the lymphoid tissues and it is important in the context of autoimmunity, because B cell trafficking and the development of the lymphatic organs are predominantly influenced by CXCL13 [20]. Moreover, Ishikawa et al. reported an increase in the expression of CXCL13 in kidneys from NZB/W F1 mice with nephritis [21]. Our data suggests that bortezomib can prevent the infiltration of abnormal plasma cells into the renal tissues by suppressing CXCL13 production. As CXCL13 is mainly produced by dendritic cells, we assumed that bortezomib affected the dendritic cells that were localized within the kidney and that it suppressed the production of the chemokine from the cells that were present in the areas of glomerulonephritis in the NZB/W F1 mice. Therefore, bortezomib may prevent the aberrant migration of B cells into the glomeruli in NZB/W F1 mice subsequently resulting in impediment to progress differentiation from B cells to plasma cells, thereby ameliorating LN.

In addition to its impacts on type I IFN and on the B cells/plasma cells, we showed that compared with the control mice, the levels of IFN-γ, IL-4, and IL-10 were suppressed in NZB/W F1 mice that had been treated with bortezomib, and that this was observed in the serum and in the RNA of PBMCs. Dysregulation of the cytokine synthesis underlies progressive glomerulonephritis. Cytokines such as IFN-γ, IL-4, TGF- β , IL-6, and IL-10 have been reported
to be involved in the development of LN [22, 23]. Aberrations in T lymphocyte function, particularly in relation to the T helper cell subsets, are highly relevant to the development of LN. In this context, the important T helper cell subsets include Th1, Th2, Th17, and Treg [24].

Bortezomib has been shown to suppress the immune functions of CD 4+ T cells [25]. Furthermore, high levels of IFN-γ have been implicated in disease progression in lupus-prone mice, because IFN-γ-treated animals show an accelerated development of fatal immune complex glomerulonephritis [26]. These findings suggest that the Th1/Th2 balance and the IFN-γ level are critical for the development of autoimmune diseases. On the other hand, Nakajima et al. reported that anti-IL-4 monoclonal antibody treatment was effective at preventing the onset of LN in NZB/W F1 mice [27]. Shimizu et al. also reported that the Th2 response favored the development of membranous LN lesions in lupus-prone mice [28]. Hence, these reports indicate that both the Th1 and Th2 responses contribute to the pathogenesis of the autoimmunity in LN.

Furthermore, IL-10 has been reported to correlate positively with anti-dsDNA antibody and proteinuria levels in NZB/W F1 mice [29]. Therefore, multiple T helper cell subsets, cytokines, and chemokines underlie the development of LN in NZB/W F1 mice. The data from the present study indicate that bortezomib suppressed cytokine and chemokine production, including that of CXCL13, which is mainly produced by the dendritic cells. Furthermore, we showed that the serum level of IL-6 showed a statistically significant difference between the control mice and the bortezomib-treated mice, although the difference between the control mice and the bortezomib-treated mice in relation to the level of RNA expression for IL-6 within the PBMCs was not statistically significant. These data suggest that bortezomib also exerts immunosuppressive effects in relation to IL-6 production by cells other than the PBMCs, for example, the mesangial cells, because its immunosuppressive effect in relation to IL-6
production was only seen in the serum samples. Taken together, we consider that bortezomib broadly suppresses the immunological functions and that it ameliorates LN through multiple mechanisms.

Renal fibrosis, which ultimately causes glomerulosclerosis, is characterized by the formation of excessive amounts of fibrous connective tissue in the tissues’ interstitial spaces, which compromises kidney function and results in eventual tubular atrophy and organ failure. The destructive pathology of glomerulosclerosis represents the end stage of many different kidney disorders, including those associated with diabetes, hypertension, and autoimmune glomerulonephritis [30]. TGF-β has been proposed as the primary mediator of renal fibrosis and eventual organ failure [31]. TGF-β has been reported to stimulate the synthesis of type I collagen, which is a major component of the ECM in renal fibrosis [32]. Suppressing TGF-β expression in NZB/W F1 mice reduces the level of glomerulosclerosis [33]. In this study, we showed that bortezomib treatment prevents the aberrant glomerular expression of TGF-β and type 1 collagen synthesis in NZW/B F1 mice. Furthermore, we found that bortezomib treatment suppressed AT1R expression within the glomeruli of NZB/W F1 mice. Renin-angiotensin system activity is higher in the glomeruli of experimental models of glomerulonephritis, and an increase in angiotensin II production induces oxidative stress and TGF-β expression, which leads to progressive glomerular injury [13]. Interestingly, an AT1R blocker has been reported to improve proteinuria and the pathologic alterations associated with glomerulonephritis, including glomerulosclerosis [34]. Our data indicate that bortezomib prevents the development of LN by suppressing both the immune response and the fibrotic processes mediated by RAS in NZB/W F1 mice.

Taken together, the data from the current study suggest that bortezomib is an effective
therapeutic reagent for LN through multiple mechanisms. In particular, the suppressive effects of bortezomib on glomerulosclerosis and on the formation of the fibrosis associated with glomerulonephritis may be beneficial in the treatment of refractory SLE patients who have LN, because these SLE patients are at risk of developing glomerulosclerosis and/or glomerular fibrosis. A comprehensive understanding of the effects of bortezomib will undoubtedly help identify novel therapeutic strategies, particularly for refractory SLE patients who have LN.
Conflict of interest: None

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Figure legends

**Fig. 1** Bortezomib treatment ameliorates lupus nephritis in NZB/W F1 mice. (A) Compared with the control mice (black line), the bortezomib-treated mice (dotted line) showed reduced levels of proteinuria. *Indicates statistical significance at \( p < 0.05 \). (B) Compared with the control mice (black line), the bortezomib-treated mice (dotted line) showed reduced levels anti-ds deoxyribonucleic acid antibody production. *Indicates statistical significance at \( p < 0.05 \). DNA deoxyribonucleic acid.

**Fig. 2** Bortezomib treatment ameliorates autoimmune nephritis in NZB/W F1 mice. Hematoxylin and eosin staining (upper panels) of renal samples taken from bortezomib-treated and untreated NZB/W F1 mice after 18 weeks showed swelling and the infiltration of inflammatory cells in the glomeruli of the control mice (indicated by the arrow) that was prevented by bortezomib treatment (indicated by the arrow head). Immunofluorescence staining for immunoglobulin (Ig)G (lower panels) showed intensely immunofluorescent IgG deposits in the glomeruli (indicated by the arrow) of the control mice. The glomerular IgG deposits were less evident in the mice treated with bortezomib (indicated by the arrow head). H&E hematoxylin and eosin, Ig immunoglobulin.

**Fig. 3** Bortezomib treatment alters cytokine gene expression in the peripheral blood mononuclear cells (PBMCs) from NZB/W F1 mice. The levels of expression of the genes for the cytokines interferon (IFN)-\( \gamma \), interleukin (IL)-4, IL-10, and IL-6 were evaluated in PBMCs derived from splenocytes using the real-time quantitative reverse transcription-polymerase chain reaction. Compared with the control mice, the bortezomib-treated mice showed significant reductions in
the levels of IFN-γ, IL-4, and IL-10. The bars indicate the standard deviations. *Indicates statistical significance at \( p < 0.05 \). IFN interferon, IL interleukin.

**Fig. 4** Bortezomib treatment alters the serum cytokine levels in NZB/W F1 mice. The serum levels of the cytokines interferon (IFN)-γ, interleukin (IL)-4, IL-10, and IL-6 were evaluated using cytometric bead array assays. Bortezomib-treated mice showed significantly lower serum IL-4 levels compared with the control mice. Reductions in the serum levels of IFN-γ and IL-10 were also observed, but these reductions were not statistically significant \( (p = 0.06 \) and \( p = 0.07 \), respectively). The bars indicate the standard deviations. *Indicates statistical significance at \( p < 0.05 \). IFN interferon, IL interleukin.

**Fig. 5** Bortezomib prevents the infiltration of the glomeruli by plasma cells by suppressing CXCL13 expression in NZB/W F1 mice. (A) Renal sections stained using an anti-cluster of differentiation (CD)138 antibody showed massive accumulations of CD138+ plasma cells within the glomeruli in the control mice (left panel, indicated by the arrow). The accumulation of CD138+ plasma cells was prevented by the bortezomib treatment (right panel, indicated by the arrow head). (B) The levels of CXCL13 gene expression were evaluated in the renal tissues of NZB/W F1 mice using the real-time quantitative reverse transcription-polymerase chain reaction. Compared with the control mice, CXCL13 gene expression was significantly reduced in the bortezomib-treated mice. The bars indicate the standard deviations. *Indicates statistical significance at \( p < 0.05 \). CD cluster of differentiation, CXCL13 chemokine CXC ligand 13.

**Fig. 6** Bortezomib prevents glomerular fibrosis by inhibiting the expression of transforming
growth factor (TGF)-β that is mediated by the angiotensin II type 1 receptor (AT1R) in NZB/W F1 mice. (A) Glomerular TGF-β expression was evaluated by immunohistochemistry using an anti-TGF-β antibody. High levels of TGF-β expression were observed in the glomeruli of the control mice. Bortezomib treatment markedly reduced the glomerular TGF-β expression. (B) Glomerular AT1R expression was evaluated by indirect immunofluorescence staining using an anti-AT1R antibody. High levels of AT1R expression were observed at the surface of glomeruli in the control mice. Bortezomib treatment markedly reduced the glomerular AT1R expression. (C) Representative images of the immunohistochemical staining show the glomerular synthesis of type I collagen, which is a hallmark of glomerular fibrosis in NZB/W F1 mice. Intensely stained deposits of type I collagen were observed in the glomeruli of the control mice. Bortezomib treatment markedly reduced the glomerular deposits of type I collagen.

TGF-β transforming growth factor-β, AT1R angiotensin II type 1 receptor.
Figure 3

- **IFN-γ**
  - Real time normalized IFN-γ (ng/ml)
  - Bortezomib (+) vs Bortezomib (-)
  - Statistically significant difference

- **IL-4**
  - Real time normalized IL-4 expression
  - Bortezomib (+) vs Bortezomib (-)
  - Statistically significant difference

- **IL-10**
  - Real time normalized IL-10 expression
  - Bortezomib (+) vs Bortezomib (-)
  - Statistically significant difference

- **IL-6**
  - Real time normalized IL-6 expression
  - Bortezomib (+) vs Bortezomib (-)
Figure 6

A

Bortezomib (-)  
Bortezomib (+)  

TGF-β  

Bortezomib (-)  
Bortezomib (+)  

B

AT1R  

Bortezomib (-)  
Bortezomib (+)  

C

Collagen type I  

Bortezomib (-)  
Bortezomib (+)  

Scale: 50μm
Certification Form — to be submitted with the manuscript

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