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Fas/S1P$_1$ crosstalk via NF-κB activation in osteoclasts controls subchondral bone remodeling in murine TMJ arthritis

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ABSTRACT

Enhanced turnover of subchondral trabecular bone is a hallmark of rheumatoid arthritis (RA) and it results from an imbalance between bone resorption and bone formation activities. To investigate the formation and activation of osteoclasts which mediate bone resorption, a Fas-deficient MRL/*lpr* mouse model which spontaneously develops autoimmune arthritis and exhibits decreased bone mass was studied. Various assays were performed on subchondral trabecular bone of the temporomandibular joint (TMJ) from MRL/*lpr* mice and MRL+/+ mice. Initially, greater osteoclast production was observed *in vitro* from bone marrow macrophages obtained from MRL/*lpr* mice due to enhanced phosphorylation of NF-κB, as well as Akt and MAPK, to receptor activator of nuclear factor-κB ligand (RANKL). Expression of sphingosine 1-phosphate receptor 1 (S1P$_1$) was also significantly upregulated in the condylar cartilage. S1P$_1$ was found to be required for S1P-induced migration of osteoclast precursor cells and downstream signaling via Rac1. When SN50, a synthetic NF-κB-inhibitory peptide, was applied to the MRL/*lpr* mice, subchondral trabecular bone loss was reduced and both production of osteoclastogenesis markers and sphingosine kinase (Sphk) 1/S1P$_1$ signaling were reduced. Thus, the present results suggest that Fas/S1P$_1$ signaling via activation of NF-κB in osteoclast precursor cells is a key factor in the pathogenesis of RA in the TMJ.

1. Introduction

Both dynamic changes and abnormalities in subchondral bone of the mandibular condyle have been observed in Fas-deficient MRL/*lpr* mice. In humans, enhanced turnover of subchondral trabecular bone is a hallmark of rheumatoid arthritis (RA). MRL/*lpr* mice also spontaneously develop autoimmune arthritis which is characterized by pannus formation, cell infiltration, an osteoporotic phenotype, and breakdown of bone and cartilage tissue [1–3]. Osteoclasts are multinucleated cells that exclusively resorb bone at the site of their invasion [4]. It has been shown that the commitment of osteoclast precursors to osteoclast differentiation is mediated by receptor activator of nuclear factor-κB ligand (RANKL), and RANKL is expressed on osteoblasts, CD4$^+$ T cells, and synovial cells in RA [5]. The corresponding receptor, RANK, is expressed on the surface of osteoclast precursors. To date, the molecular details regarding the formation and activation of osteoclasts in RA remain unclear.

Sphingosine kinase (Sphk) 1 and sphingosine 1-phosphate (S1P)/S1P receptor 1 (S1P$_1$) signaling enhance RANKL expression to contribute to RA pathogenesis [6–8]. In addition, S1P and its receptors regulate cell migration [9] and S1P$_1$ is necessary for the egress of osteoclast precursors from circulation into bone tissues [10]. S1P-mediated protection from death receptor-induced apoptosis has also been found to be NF-κB dependent [11]. NF-κB signaling is central to many chronic diseases, including arthritis, atherosclerosis, diabetes, and inflammatory bowel disease. Inhibition of NF-κB activation has been achieved with small peptides that cross the cell membrane and block nuclear translocation of the NF-κB complex. In particular, the peptide, SN50, has been shown to compete with NF-κB complexes for nuclear translocation [12,13].

The aim of the present study was to examine the roles of Fas/FasL signaling and S1P/S1P$_1$ signaling via NF-κB activation in osteoclast precursor cells and to elucidate the effects of SN50 on the pathogenesis of subchondral bone in the temporomandibular joint

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(TMJ) which is frequently affected in the autoimmune diseases, osteoarthritis, and RA [14,15].

2. Materials and methods

2.1. Mouse model

Female MRL/MpJ-fas*lpr* (MRL/*lpr*) mice and MRL+/+ mice were obtained from Japan SLC Laboratory (Shizuoka, Japan) and were maintained in a specific pathogen free animal facility with food and water provided *ad libitum*. At 12-week of age, the MRL/*lpr* mice were randomly divided into a negative control group (saline treatment) and three SN50 treatment groups (0.18, 1.8, and 18 μg/g body weight). Five mice per group received daily intraperitoneal (i.p.) injections for 2 weeks. This study was approved by the Animal Care and Use Committee of Tokushima University (Permit No: T27-93).

2.2. Reagents

Macrophage colony-stimulating factor (M-CSF) and recombinant RANKL were purchased from Peprotech (Le Perray-en-Yvelines, Île-de-France). Antibodies were also purchased: mouse anti-NFATc1, rabbit anti-p50, and rabbit anti-c-Fos (Santa Cruz Biotechnology, Dallas, TX); rabbit anti-phosphorylated (p)-IκBα, p-ERK, p-Akt, p-p38, NF-κB p-p65, and GAPDH (Cell Signaling Technology, Danvers, MA); mouse anti-lamin B1, mouse anti-c-Src, mouse anti-β-actin, and mouse anti-cathepsin K (Sigma-Aldrich, St. Louis, MO). The cell permeable inhibitor peptide, SN50, was purchased from Calbiochem (San Diego, CA).

2.3. Bone marrow macrophages (BMMs) and osteoclast cultures

BMMs were collected and treated to generate osteoclasts as previously described [16,17] and as described in the figure legends of the present study. In 48-well tissue culture plates, cells (1.5×10^4/well) were added to 500 μl α-MEM supplemented with 10% heat inactivated FBS with recombinant RANKL (100 ng/mL) and a 1:50 dilution of CMG14-12 supernatant that supplied an equivalent of 20 ng/ml M-CSF in the presence and absence of SN50 at various concentrations (e.g., 0.18, 1.8, and 18 μM). After 4–6 d, the cells were fixed and stained for tartrate-resistant acid phosphatase (TRAP) activity with a commercially available kit (Acid Phosphatase Kit 387-A, Sigma-Aldrich).

2.4. Micro-computed tomography (CT) analysis

Mandibular condyles were obtained from MRL+/+ and MRL/*lpr* mice and were dissected from soft tissues. After the bones were fixed overnight in 70% ethanol, they were analyzed with an LCT-200 instrument (Hitachi Aloka Medical, Ltd. Tokyo) and its analysis software.

2.5. Preparation of tissues and histologic staining

After TMJ tissues were fixed in 4% paraformaldehyde and decalcified in 14% EDTA in PBS for 20 d, the tissues were embedded in paraffin. Serial sagittal sections were subsequently cut with a HM360 microtome (Carl Zeiss, Jena) and stained with TRAP to identify osteoclasts. Immunohistochemistry was performed for histological assessments.

2.6. Immunohistochemistry

After the sections were deparaffinized and blocked in, they were incubated overnight at 4 °C with various primary antibodies diluted in PBS/0.1% bovine serum albumin. Negative controls were stained with non-immune IgGs. After being washed with PBS, the sections were incubated with the corresponding secondary antibodies for 1 h at room temperature and then mounted. Sections were examined with a BioRevoBZ-9000 microscope (KEYENCE, Osaka).

2.7. Real-time polymerase chain reaction (PCR)

Total mRNA was extracted from the mandibular condyles and gene levels were measured with a 7500 Real-time PCR System (Applied Biosystems, Foster City, CA). The primers used are listed in Supplementary Table.

2.8. Western blotting

Cells or tissues were lysed with RIPA lysis buffer supplemented with a freshly added protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific, Rockford, IL). Nuclear extracts were prepared with a NE-PER Nuclear and Cytoplasmic Extraction kit (Thermo Fisher Scientific). Standard Western blotting was performed.

2.9. Detection of actin rings and bone resorption, cell migration assays, and Rac1 small GTPase pull-down assays

The materials and methods for this experiments are provided in Supplementary Material.

2.10. Statistical analysis

Each experiment was performed, and independently repeated, at least three times for each set of conditions. The mean ± standard deviation (SD) values are presented. Student's *t*-test or one-way analysis of variance (ANOVA) with post-hoc Tukey's honest significant differences test were applied as appropriate. Statistical significance was set at $P < 0.05$.

3. Results

3.1. Osteoclastogenesis is enhanced in MRL/lpr mice in vitro

MRL+/+ and MRL/*lpr* BMMs were cultured with RANKL and M-CSF and then were stained with TRAP on day 6. The latter included a greater number of osteoclasts (Fig. 1A and B) that progressively expressed NFATc1, c-Fos, and c-Src compared with the MRL+/+ BMMs (Fig. 1C).

3.2. Signaling pathway of osteoclasts in MRL/lpr mice

Next, MRL+/+ and MRL/*lpr* bone marrow-derived osteoclast precursors were treated with RANKL. Enhanced activation of Akt, IκBα, ERK, p38, and JNK, as well as nuclear transport of NF-κB subunit p65, were observed in the MRL/*lpr* cells compared to the MRL+/+ cells (Fig. 1D and E). Thus, Fas appears to promote osteoclast differentiation by sensitizing osteoclast precursors to RANKL.

3.3. Osteoclast activity in MRL/lpr mice

To determine whether Fas modulates bone resorption, MRL+/+ and MRL/*lpr* osteoclasts were grown on dentin in the presence of RANKL and M-CSF. After 5 d, the cells were stained with FITC-phalloidin to visualize actin rings. In parallel, the osteoclasts that formed were removed and resorption pits were visualized (Fig. 1F). There was a greater number of actin rings in the MRL/*lpr*

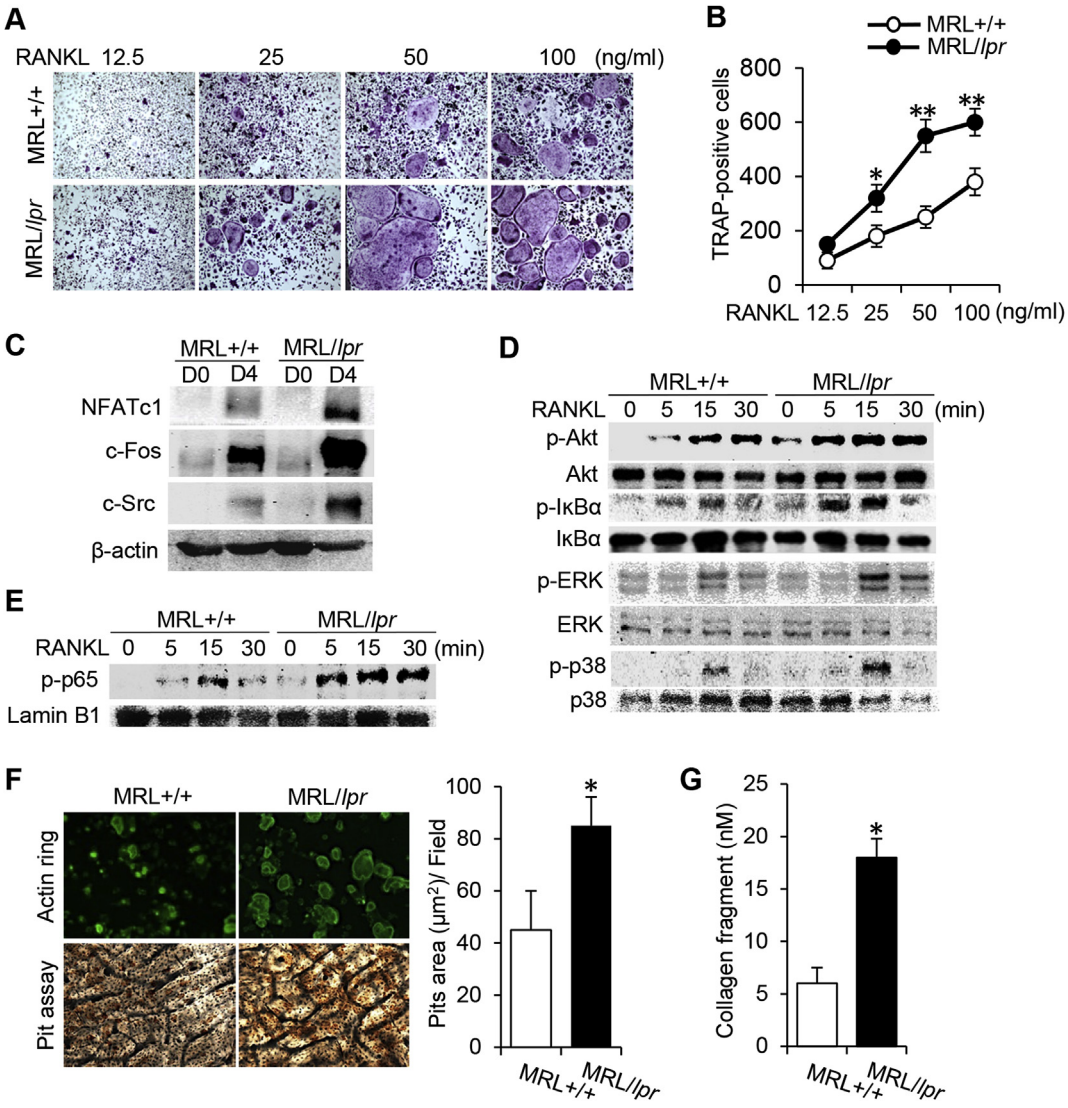

Fig. 1. Hyperactivation of osteoclasts in MRL/*lpr* mice. (A) BMMs derived from MRL+/+ and MRL/*lpr* mice were cultured with M-CSF (20 ng/mL) and increasing amounts of RANKL. (B) After 6 d, the cells were stained for TRAP activity and osteoclasts were counted. $^{*}P < 0.05$; $^{**}P < 0.01$, MRL+/+ vs. MRL/*lpr*. (C) Western blot assays detected levels of NFATc1, c-Fos, and c-Src in MRL/*lpr* BMMs that were cultured with M-CSF (10 ng/mL) for 3 d and stimulated with M-CSF (20 ng/mL) and RANKL (100 ng/mL) for an additional 4 d to generate osteoclasts. (D) A time course Western blot analysis of total and phosphorylated forms of Akt, IκBα, ERK, and p38 in MRL+/+ and MRL/*lpr* BMMs cultured with M-CSF (20 ng/mL) for 2 d and then stimulated with RANKL (100 ng/mL) for an additional 0–30 min. (E) Levels of NF-κB p65 detected in nuclear extracts from RANKL-stimulated MRL+/+ and MRL/*lpr* BMMs. Lamin B1 was detected as a loading control. (F) MRL+/+ and MRL/*lpr* BMMs were cultured on dentin with M-CSF and RANKL for 5 d. Immunofluorescent staining of actin was performed in osteoclasts obtained from MRL+/+ and MRL/*lpr* mice. The relative amount of resorbed area is graphed. $^{*}P < 0.05$. (G) MRL+/+ and MRL/*lpr* BMMs were cultured on bone slices with M-CSF (20 ng/mL) and RANKL (100 ng/mL) for 5 d. The concentration of collagen type 1 fragment (Ctx-1) in culture medium on day 5 was used to quantify resorptive activity. $^{*}P < 0.05$. The results shown are the mean ± SD from five mice in each group.

osteoclasts. Lacunae were also more abundant in the MRL/*lpr* mice which resulted in the medium containing a higher concentration of type 1 collagen fragments (Ctx-1) (Fig. 1G).

3.4. Effects of Fas deficiency on osteoclast precursor activity via NF-κB

To examine the role of NF-κB in the activation of osteoclasts, Western blot analyses of MRL+/+ and MRL/*lpr* bone marrow samples were conducted. The latter exhibited expression of phosphorylated of IκBα, an endogenous inhibitory molecule of NF-κB activation (Fig. 2A). Greater nuclear translocation of the NF-κB subunits, p50 and p65, was also detected in the MRL/*lpr* samples compared with the MRL+/+ samples (Fig. 2B). These results indicate that the defect in Fas enhances NF-κB activation in osteoclast differentiation.

3.5. A peptide inhibitor of NF-κB inhibits osteoclastogenesis in MRL/lpr mice

Next, osteoclasts from MRL+/+ and MRL/*lpr* mice were cultured with M-CSF and RANKL, and then were incubated with a cell-permeable peptide, SN50, at various concentrations. Significantly fewer TRAP-positive osteoclast cells were generated from the MRL+/+ mice compared with the MRL/*lpr* mice (Fig. 2C and D). Nuclear translocation of the p50 NF-κB subunit was also suppressed following treatment of the BMMs with RANKL stimulation from the MRL+/+ and MRL/*lpr* mice with SN50 (Fig. 2E). These results suggest that SN50 can inhibit osteoclastogenesis in MRL/*lpr* mice.

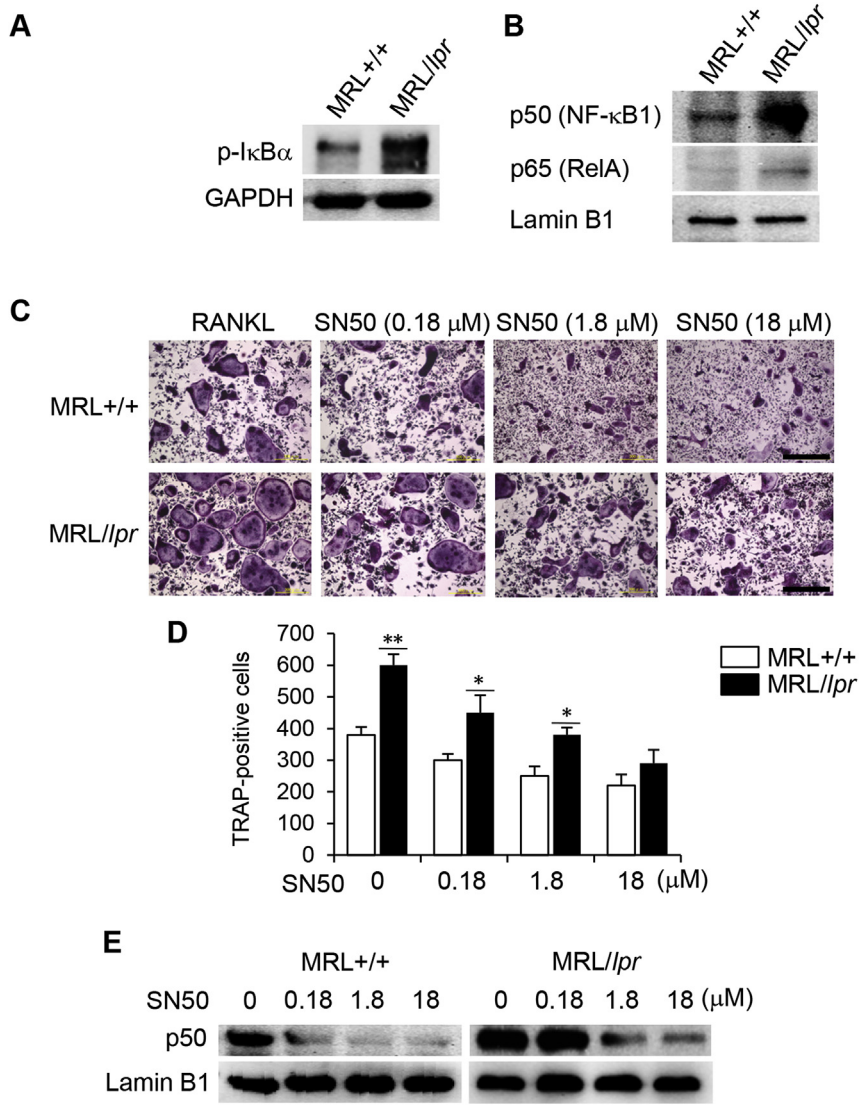

Fig. 2. SN50 peptide inhibits RANKL-induced osteoclastogenesis *in vitro*. Phosphorylation of IκBα (A) and nuclear translocation of the NF-κB subunits p50 (NF-κB1) and p65 (RelA) (B) in bone marrow samples from MRL+/+ and MRL/*lpr* mice were analyzed by Western blot. GAPDH and Lamin B1 were detected as loading controls, respectively. (C) TRAP-stained activity in BMMs derived from MRL+/+ and MRL/*lpr* mice that were cultured with M-CSF (20 ng/mL) and RANKL (100 ng/mL) or increasing concentrations of SN50 for 6 d were observed with light microscopy. (D) The number of osteoclasts in each well were counted. $^{*}P < 0.05$; $^{**}P < 0.01$. (E) Nuclear translocation of p50 in BMMs incubated with RANKL (0) versus SN50 at increasing concentrations was analyzed by Western blot. Lamin B1 was detected as a loading control.

3.6. *Effects of SN50 on subchondral trabecular bone loss*

Next, micro-CT analyses were performed (Fig. 3A). In these analyses, MRL/*lpr* mice exhibited severe subchondral trabecular bone loss compared with MRL+/+ mice. In contrast, treatment with increasing concentrations of SN50 led to attenuated subchondral trabecular bone resorption in the MRL/*lpr* mice. Subsequently, TRAP staining of osteoclast activity showed a greater number of TRAP-positive osteoclasts in the MRL/*lpr* tissues than in the MRL+/+ tissues ($p < 0.01$), thereby supporting a model in which the mandibular condyle of MRL/*lpr* mice undergoes subchondral bone loss due to increasing bone resorption. Two weeks after an injection of SN50, the number of osteoclast cells was reduced in the MRL/*lpr* mandibular condyle tissues (Fig. 3B and C). Real-time PCR analyses further showed higher expression levels of the osteoclast markers, *TRAP*, *cathepsin K*, *RANKL*, *VEGF*, and *MMP-9* and decreased expression levels after injection of SN50 in the MRL/*lpr* samples. Meanwhile, levels of *OPG* were reduced before and increased after injection of SN50 in the MRL/*lpr* samples compared with the MRL+/+ samples (Fig. 3D). This result suggests that SN50 is able to effectively inhibit osteoclast activity in MRL/*lpr* mice.

To investigate if SN50 treatment affects osteoclast marker expression, immunohistochemistry assays were performed. In mandibular condyle tissues from MRL/*lpr* mice and MRL+/+ mice, increased numbers of cells expressing VEGF, MMP-9, and RANKL were observed, while the numbers of OPG-positive cells were reduced. Thus, the MRL/*lpr* mice had an increased *RANKL*/*OPG* ratio compared to the MRL+/+ mice. In contrast, treatment of the MRL/*lpr* mice with SN50 resulted in fewer numbers of VEGF-, MMP-9-, and RANKL-positive cells and an increased number of OPG-positive cells (Fig. 3E and F). Taken together, these data indicate that SN50 is able to inhibit osteoclast activity in the condyle of MRL/*lpr* mice by regulating expression of osteoclast markers.

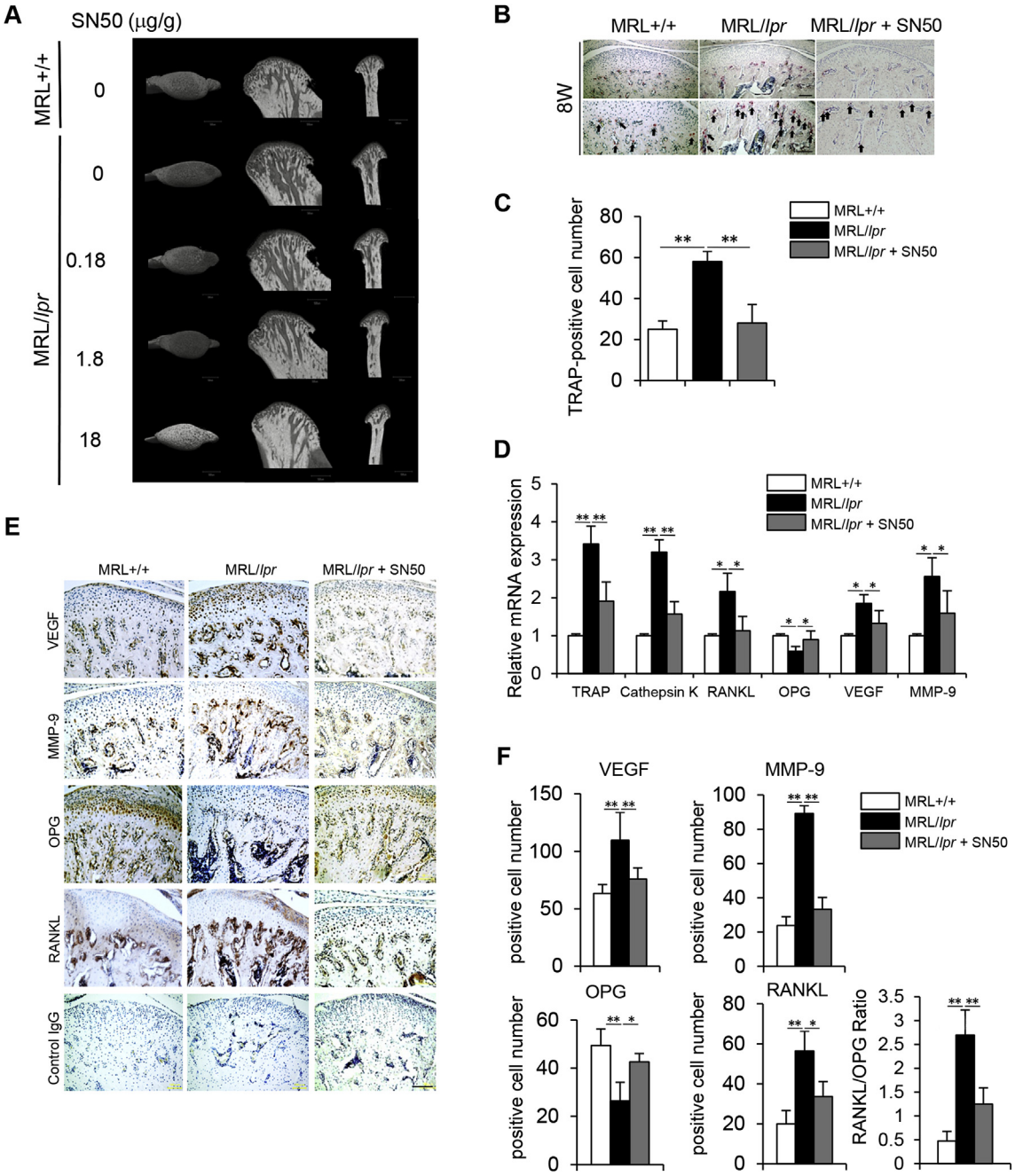

Fig. 3. Effects of SN50 on subchondral bone resorption in MRL/*lpr* mice. All panels represent data from subchondral trabecular bone of mandibular condyles collected from MRL+/+ mice and from MRL/*lpr* mice with and without SN50 treatment. (A) Prior to collecting micro-computed tomography images, intraperitoneal (i.p.) injections of saline (0) or increasing doses of SN50 were administered. Scale bar: 300 μm. (B) TRAP staining was performed and multinucleated osteoclasts are indicated with black arrows. (C) The numbers of osteoclasts were counted. (D) Real-time PCR was performed to detect mRNA levels of TRAP, cathepsin K, RANKL, OPG, VEGF, and MMP-9. (E) Immunohistochemical analyses of VEGF, MMP-9, OPG, and RANKL were performed. Staining with an isotype-matched rabbit IgG was performed as a negative control. (F) The percentage of VEGF-, MMP-9-, OPG-, and RANKL-positive cells were quantitated, as well as the RANKL/OPG ratio. $^{*}P < 0.05$; $^{**}P < 0.01$. Data are representative of five independent experiments.

3.7. *Effects of SN50 on the expression of Sphk1 and S1P$_1$*

When mRNA levels of *Sphk1* and *S1P$_{1\text{-}5}$* were detected in mandibular condyle tissues from MRL/*lpr* and MRL+/+ mice, only the levels of Sphk1 and S1P$_1$ exhibited a significant difference with higher levels detected in the MRL/*lpr* tissues (Fig. 4A). An immunoblot analysis of MRL/*lpr* and MRL+/+ mandibular condyle tissues showed similar results (Fig. 4B). When Rac1-GTP activity in the BMMs was assayed, a time-dependent increase following S1P stimulation was observed in both sets of cells (Fig. 4C). In cell migration assays, an S1P$_1$ and S1P$_3$ agonist, FTY720, as well as an S1P$_3$ antagonist, suramin, were used. MRL/*lpr* BMMs migrated in response to stimulation by S1P, while migration was inhibited following pretreatment with FTY720. However, migration was not inhibited following pretreatment with suramin (Fig. 4D). Immunohistochemical assays to detect Sphk1, S1P$_1$, and S1P$_3$ in the mandibular condyles of MRL/*lpr* and MRL+/+ mice further showed that only the expression levels of Sphk1 and S1P$_1$ were significantly higher ($p < 0.01$). Correspondingly, when the MRL/*lpr* mice were treated with SN50, a

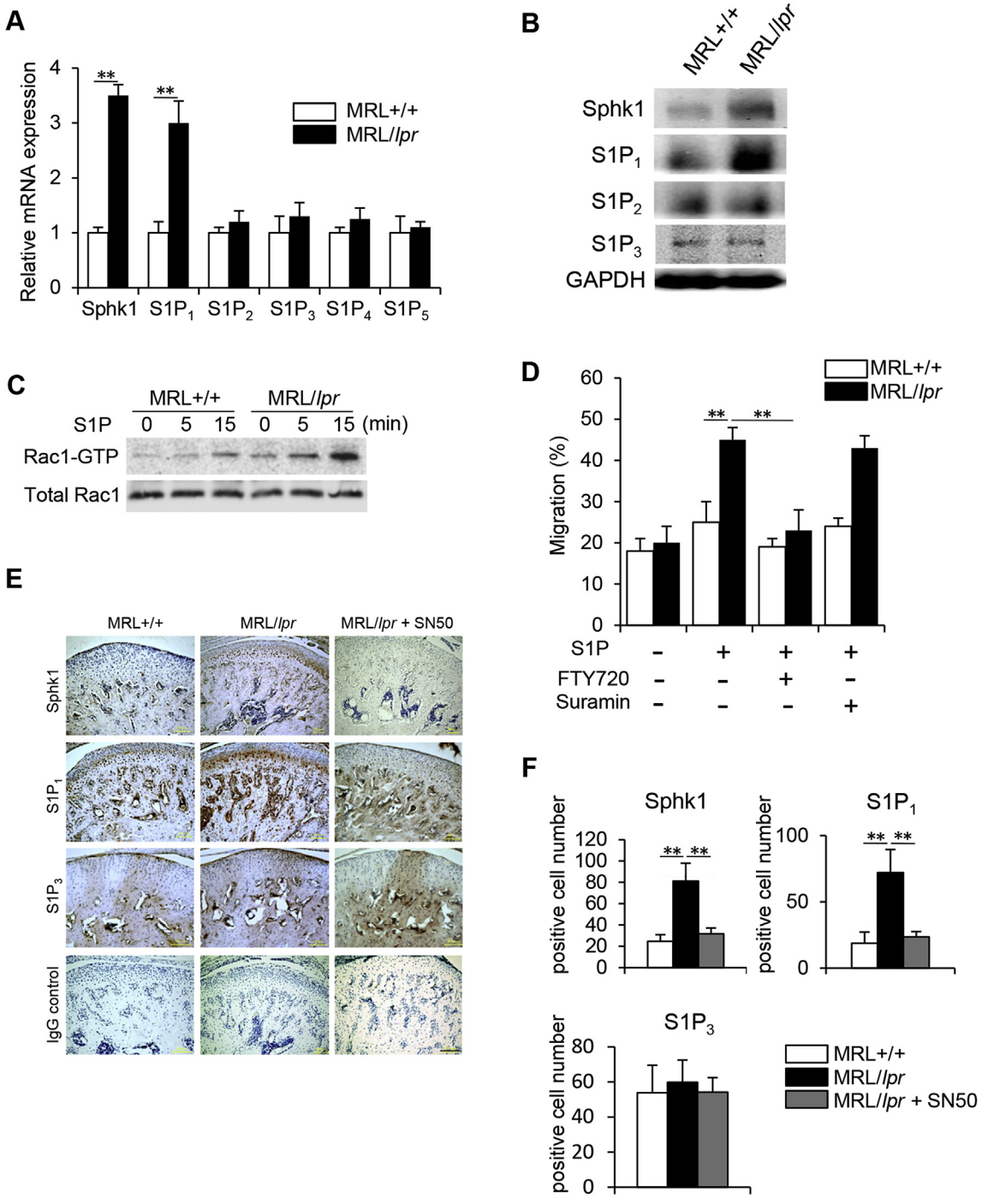

Fig. 4. Effects of SN50 on the expression of S1P receptors in the mandibular condyles of MRL/*lpr* mice. (A) Mandibular condyles were isolated from MRL+/+ and MRL/*lpr* mice and were subjected to real-time PCR analysis with primers specific for *Sphk1* and *S1P*$_{1\text{-}5}$. $^{**}P < 0.01$, MRL+/+ vs. MRL/*lpr*. (B) Extracts of mandibular condyle tissues were also subjected to Western blot to detect Sphk1 and S1P$_{1\text{-}3}$, with detection of GAPDH performed as an internal control. (C) BMMs from MRL+/+ and MRL/*lpr* mice were treated with S1P and Rac1-GTP activity was assayed at the time points indicated. (D) Percent of migrating BMMs from MRL+/+ and MRL/*lpr* mice that were pretreated for 1 h with FTY720 or suramin and then incubated with S1P are shown. $^{**}P < 0.01$. (E) Immunohistochemical analysis of the distribution of Sphk1, S1P$_1$, and S1P$_3$ in mandibular condyles from MRL+/+ mice and from MRL/*lpr* mice with and without SN50 treatment. Tissues were stained with isotype-matched rabbit IgGs as negative controls. (F) Percentages of Sphk1-, S1P$_1$-, and S1P$_3$-positive cells in mandibular condyles from MRL+/+ mice and from MRL/*lpr* mice with and without SN50 treatment were quantitated. $^{**}P < 0.01$. Data are representative of five independent experiments.

marked decrease in the number of Sphk1- and S1P$_1$-positive cells was observed, while the number of S1P$_3$-positive cells remained unchanged (Fig. 4E and F). Taken together, these data indicate that crosstalk occurs between the Fas and S1P/S1P$_1$ signaling pathways in BMMs via NF-κB.

4. Discussion

Accumulating evidence has demonstrated that defects in signaling pathways make significant contributions to autoimmune diseases [18]. MRL/*lpr* mice with a defective Fas provide a model

that spontaneously exhibits the characteristics of human RA [2,19–21]. However, the mechanism(s) mediating RA development and bone resorption in MRL/*lpr* mice remain unclear.

Previously, Fas expression was found to be upregulated during osteoclast differentiation in B6 background mice [19,22]. In the present study, the absence of Fas led to greater production of osteoclasts, enhanced signaling and translocation of p65 NF-κB in response to RANKL, greater osteoclast activity, and enhanced NF-κB activation in the MRL/*lpr* model. Thus, Fas appears to mediate an inhibitory effect on osteoclast precursor proliferation. Furthermore, when RANKL was applied to MRL/*lpr* and MRL+/+ BMMs, activation of NF-κB and MAPK were enhanced in the former. These results suggest that the Fas/FasL signaling pathway influences NF-κB and MAPK activation via RANKL signaling in osteoclast precursor cells. Furthermore, these results are consistent with a model in which binding of NF-κB to the Fas promoter is regulated by RANKL, and this system affects the differentiation and activity of osteoclasts [22,23].

An initial characterization of the increased osteoclast production in the MRL/*lpr* cultures showed that major RANKL-mediated osteoclastogenic signaling proteins, NFATc1, c-Fos, and c-Src, were expressed at higher levels. Subsequently, enhanced phosphorylation of IκBα was detected in the bone marrow of MRL/*lpr* mice, along with greater nuclear translocation of NF-κB subunits p50 and p65, compared to the MRL+/+ mice. Therefore, the Fas defect was found to accelerate RANKL-induced osteoclastogenesis via enhanced activation of NF-κB translocation in osteoclast progenitor cells.

In resting normal human peripheral blood-derived T lymphocytes, addition of the small peptide, SN50, suppresses NF-κB activity and induces apoptosis [24]. Recombinant ribosome inactivating protein B-chain-induced osteoclast differentiation has also been abolished with the addition of SN50 peptide to cultures [25]. The present data demonstrate that SN50 potentially inhibits RANKL-induced osteoclastogenesis by specifically targeting the nuclear translocation of NF-κB subunit p50 in MRL/*lpr* mice BMMs. SN50 treatment of MRL/*lpr* mice also ameliorated subchondral trabecular bone loss in the mandibular condyle, which included a reduction in the number of VEGF-, MMP-9-, and RANKL-positive cells, and an increase in the number of OPG-positive cells. It has been proposed that blocking the nuclear translocation of subunit p50 of NF-κB represents a more effective approach for targeting NF-κB during osteoclast activation and differentiation [26].

In the immune system, S1P contributes to the migration, proliferation, differentiation, survival, and cytokine production of immune cells [9,11,27]. In a recent study, interactions between S1P and $S1P_1$ were shown to play a critical role in the egress of osteoclast precursor cells from circulation into bone tissues [10]. Only expression of Sphk1 and $S1P_1$ were found to be significantly upregulated in the mandibular condyles of MRL/*lpr* mice, and S1P-$S1P_1$ interactions were perturbed by FTY720, a functional antagonist of $S1P_1$ [28,29]. In a previous study, FTY720 administration was found to protect against ovariectomy-induced bone loss due to a negative effect on osteoclast attachment to the bone matrix [10]. Treatment of MRL/*lpr* BMMs with suramin in migration assays further confirmed the role of $S1P_1$ in response to S1P.

Rho GTPases play a key role in coordinating cellular responses required for cell migration [30,31], and higher Rac1 activity was observed in MRL/*lpr* BMMs following S1P stimulation. SN50 treatment of Fas-deficient MRL/*lpr* mice also resulted in fewer Sphk1- and $S1P_1$-positive cells.

Overall, the results of the present study support a model in which crosstalk between Fas and S1P/$S1P_1$ signaling occurs in osteoclast precursors, with NF-κB having a central role, and this signaling affects the migratory behavior of these precursors to affect the pathogenesis of RA in the TMJ. Thus, targeting of the p50 or p65 subunit of NF-κB may represent an effective approach for inhibiting osteoclast activity for the treatment of RA in the TMJ.

Authors' contribution

T.I. I.H. E.T. designed and performed the experiments, I.H. A.M. T.S. A.I. analyzed data, and contributed to writing; H.M. I.H. performed the experiments and analyzed data; T.I. E.T. designed and supervised research, analyzed data, and wrote the paper.

Conflict-of-interest disclosure

The authors declare no competing financial interests.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2017.07.006.

Transparency document

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