The Dectin 1 Agonist Curdlan Regulates Osteoclastogenesis by Inhibiting Nuclear Factor of Activated T cells Cytoplasmic 1 (NFATc1) through Syk Kinase

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Background: Dectin 1 is found on myeloid lineage cells and contains an immunoreceptor tyrosine-based activation motif from which signals are associated with bone homeostasis. Results: The dectin 1 agonist curdlan suppresses osteoclastogenesis induced by receptor activator of NF-κB ligand (RANKL). Conclusion: Curdlan regulates RANKL-induced osteoclastogenesis. Significance: Curdlan could be a potential therapeutic candidate in treating osteoclast-related diseases.

Several immune system cell surface receptors are reported to be associated with osteoclastogenesis. Dectin 1, a lectin receptor for β-glucan, is found predominantly on cells of the myeloid lineage. In this study, we examined the effect of the dectin 1 agonist curdlan on osteoclastogenesis. In mouse bone marrow cells and dectin 1-overexpressing RAW 264.7 cells (d-RAWs), curdlan suppressed receptor activator of NF-κB ligand (RANKL)-induced osteoclast differentiation, bone resorption, and actin ring formation in a dose-dependent manner. This was achieved within non-growth inhibitory concentrations at the early stage. Conversely, curdlan had no effect on macrophage colony-stimulating factor-induced differentiation. Furthermore, curdlan inhibited RANKL-induced nuclear factor of activated T cell cytoplasmic 1 (NFATc1) expression, thereby decreasing osteoclastogenesis-related marker gene expression, including tartrate-resistant acid phosphatase, osteoclast stimulatory transmembrane protein, cathepsin K, and matrix metalloproteinase 9. Curdlan inhibited RANKL-induced c-fos expression, followed by suppression of NFATc1 autoamplification, without significantly affecting the NF-κB signaling pathway. We also observed that curdlan treatment decreased Syk protein in d-RAWs. Inhibition of the dectin 1-Syk kinase pathway by Syk-specific siRNA or chemical inhibitors suppressed osteoclast formation and NFATc1 expression stimulated by RANKL. In conclusion, our results demonstrate that curdlan potentially inhibits osteoclast differentiation, especially NFATc1 expression, and that Syk kinase plays a crucial role in the transcriptional pathways. This suggests that the activation of dectin 1-Syk kinase interaction critically regulates the genes required for osteoclastogenesis.

Osteoclasts originate from hematopoietic precursors of the monocyte/macrophage lineage, which differentiate into multi-nucleated, giant cells specialized to resorb bone by fusion of mononuclear progenitors (1). Osteoclasts are unique in their ability to resorb bone and play an important role in regulating bone remodeling. Osteoclast precursors interact with osteoblasts and stromal cells to permit their differentiation into mature osteoclasts (2). Furthermore, osteoclast formation is induced in the presence of receptor activator of NF-κB ligand (RANKL), a member of the TNF superfamily expressed by osteoblasts and bone stromal cells. RANKL interacts with the osteoclast cell surface receptor RANK, which, in turn, recruits cytosolic TNF receptor-associated factors (3), prior to activation of downstream signaling pathways. These signaling pathways include ERK, p38 MAPK, NF-κB, and activator protein 1 (AP-1), c-jun, and c-fos. Finally, expression of the nuclear factor of activated T cell cytoplasmic 1 (NFATc1), a key molecule for osteoclastogenesis, is induced (4).

Pattern recognition receptors recognize molecular signatures of microbes and play an important role in initiation of immune responses to infection (5). Dectin 1 is a type II membrane receptor containing a single extracellular, C-type, lectin-like domain (6) and an immunoreceptor tyrosine-based activation motif (ITAM) (5) in the cytoplasmic tail (7). Dectin 1 is found on cells of the myeloid lineage, including monocytes, macrophages, neutrophils, dendritic cells, and a subset of T cells in mice (8). It has been shown that dectin 1 is a major receptor for the recognition of β-1,3-linked and/or β-1,6-linked glucans (β-glucans) (8, 9).

Dectin 1 is known to mediate its own signaling through its cytoplasmic tail (10), and tyrosine residues within its receptor

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2 The abbreviations used are: RANKL, receptor activator of NF-κB ligand; ITAM, immunoreceptor tyrosine-based activation motif; BMC, bone marrow cell; α-MEM, α-minimal essential medium; M-CSF, macrophage colony-stimulating factor; ANOVA, analysis of variance; TRAP, tartrate-resistant acid phosphatase; SUMO, small ubiquitin-like modifier.
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ITAM motif are phosphorylated by Src family tyrosine kinases through engagement with dectin 1. This is followed by recruitment and activation of Syk, a nonreceptor tyrosine kinase (11–13), with activation of several intracellular signaling pathways, including NF-κB, MAPK, and NFAT (14).

The β-glucans, consisting of a backbone of polymerized β-(1,3)-linked β-D-glucopyranosyl units and β-(1,6)-linked side chains, are a major cell component of fungi and are also found in plants and some bacteria (15). They are known to possess anti-infection and antitumorigenic properties by possessing the ability to activate leukocytes by stimulating their phagocytic activity and the production of reactive oxygen intermediates and inflammatory mediators, including TNF-α (16–19). However, the mechanism of β-glucan-induced activity has not been elucidated precisely because pathogen-derived components are often contaminated by β-glucan preparations. Recently, curdlan, a linear nonionic homopolymer of β-glucose with 1,3 glucosidic linkages, has been identified as a dectin 1-specific agonist and has gained attention from the pharmaceutical industry as an immunomodulatory drug delivery vehicle (20, 21).

Several cell surface receptors in the immune system are reported to be associated with osteoclastogenesis. ITAM-dependent costimulatory signals, activated by multiple immunoreceptors, are essential for the maintenance of bone homeostasis (22). It has been demonstrated that Syk modulates osteoclast function in vitro and in vivo (23–25). However, the direct effects of β-glucans on osteoclastogenesis are largely unknown. In this study, we examined the effect of curdlan, a linear homopolymer of β-glucose, on osteoclastogenesis. We also identified the precise mechanisms by which curdlan suppresses osteoclast formation in vitro.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies—Curdlan was purchased from Wako Pure Chemical Industries (Osaka, Japan) and diluted with 0.1 N NaOH. Anti-NFATc1 polyclonal antibody, anti-IκB-α polyclonal antibody, and anti-RANK polyclonal antibody were obtained from Santa Cruz Biotechnology (Santa Cruz, CA), and anti-β-actin monoclonal antibody was purchased from Sigma-Aldrich (St. Louis, MO). Anti-c-fos monoclonal antibody, anti-Syk monoclonal antibody, anti-phospho-Syk monoclonal antibody, anti-β-actin monoclonal antibody, and anti-His H3 monoclonal antibody were purchased from Cell Signaling Technology Inc. (Beverly, MA). Anti-dectin 1 polyclonal antibody was purchased from R&D Systems (Minneapolis, MN).

Cell Culture—Bone marrow cells (BMCs) were isolated from femurs and tibias of 6-week-old male ddY mice (Kyudo Co., Ltd., Saga, Japan) and maintained in α-minimum essential medium (α-MEM; Invitrogen) supplemented with 10% FCS (Sigma-Aldrich), 100 units/ml penicillin G potassium salt (Nacalai Tesque, Kyoto, Japan), and 100 μg/ml streptomycin (Wako Pure Chemical Industries). Cells (4 × 10⁶) were cultured with M-CSF (20 ng/ml, Peprotech, Rocky Hill, NJ) and RANKL (40 ng/ml, Peprotech) in the presence or absence of curdlan on 48-well plates at 37 °C in 5% CO₂ for 4 days to generate mature osteoclasts. All procedures were approved by the Animal Care and Use Committee of Kyushu Dental University.

To prepare the dectin 1 retrovirus vector, cDNA-encoded mouse dectin 1 was cloned into the pFB-Neo plasmid (Agilent Technologies, Inc., Santa Clara, CA). GP-293 packaging cells (Clontech Laboratories, Inc., Mountain View, CA) were transfected by a plasmid mixture containing dectin 1/pFB-Neo and pSV5-G (Clontech Laboratories, Inc.) with Lipofectamine LTX (Invitrogen) in accordance with the protocol of the manufacturer. The culture supernatant of GP-293 cells was harvested as a retrovirus fluid for dectin 1 transfection. RAW 264.7 cells (Riken Cell Bank, Tsukuba, Japan) were cultured with the virus vector in the presence of 8 μg/ml Polybrene for 48 h. Cells expressing dectin 1 (d-RAW) were maintained in α-MEM containing 10% FCS, penicillin G (100 units/ml), streptomycin (100 μg/ml), and G418 disulfate aqueous solution (50 mg/ml, Nacalai Tesque). For the negative control of d-RAW cells, a control virus vector without dectin 1 cDNA was used for infection (c-RAW). The expression of dectin 1 molecules on RAW 264.7 cells was examined by antibody staining using anti-mouse dectin 1 (clone RH1, BioLegend, San Diego, CA). Cells (1 × 10³ cells/well) were cultured for 7 days with RANKL (40 ng/ml) in the presence or absence of curdlan on 96-well plates at 37 °C in 5% CO₂ to generate mature osteoclasts. In some experiments, cells were treated with selective inhibitors of Syk kinase, piceatannol (Calbiochem, San Diego, CA) and BAY 61-3606 (Calbiochem), for 1 h prior to stimulation with RANKL in the presence or absence of each inhibitor. Mouse bone marrow stromal cells, ST2, were obtained from the Riken Cell Bank and maintained in α-MEM supplemented with 10% FCS, 100 units/ml penicillin G, and 100 mg/ml streptomycin at 37 °C in an atmosphere of 5% CO₂.

Evaluation of Osteoclast Differentiation—After culture, adherent cells were fixed and stained with TRAP using a leukocyte acid phosphatase kit (Sigma-Aldrich). TRAP-positive multinucleated cells containing three or more nuclei were considered to be osteoclasts and were counted using a microscope.

Assessment of Actin Ring Formation—Mature osteoclasts were prepared from BMCs by treatment with M-CSF (20 ng/ml) and RANKL (40 ng/ml) in the presence or absence of curdlan for 5 days on 8-well chamber slides (Thermo Fisher Scientific, Waltham, MA). In some experiments, d-RAWs were cultured for 8 days with RANKL (40 ng/ml) in the presence or absence of curdlan for 8 days on the chamber slides. Cells were fixed with 4% paraformaldehyde in PBS for 60 min at 4 °C, quenched with 0.2 M glycine in PBS, and permeabilized using 0.2% Triton X-100 for 10 min at room temperature. After washing in PBS, cells were incubated with Alexa Fluor 488-phalloidin (Invitrogen) for 60 min at room temperature and then washed, mounted in mounting medium containing nuclear DAPI stain (Vector Laboratories Inc., Burlingame, CA), and visualized using a BZ-9000 fluorescence microscope (Keyence Corp., Osaka, Japan). Images were captured digitally in real time and processed using BZ-II imaging software (Keyence Corp.).

Assessment of Bone Resorption—For the bone resorption assay, BMCs were cultured for 7 days with M-CSF (20 ng/ml) and RANKL (40 ng/ml) in the presence or absence of curdlan on an Osteo Assay Stripwell Plate® (Corning Inc., NY). d-RAWs were cultured for 10 days with RANKL (40 ng/ml) in
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FIGURE 1. Effect of curdlan on osteoclastogenesis in BMCs. A, mouse BMCs were incubated with M-CSF (20 ng/ml) and RANKL (40 ng/ml) in the presence or absence of curdlan (12.5–50 μg/ml). Cells were cultured for 4 days and stained for TRAP activity. Scale bars = 500 μm. Ctr, control. B, the number of osteoclasts was counted after staining for TRAP activity. Data show the number of osteoclasts from three independent samples, and error bars represent mean ± S.D. Data were analyzed by Dunnett’s test after one-way ANOVA. *, p < 0.05; **, p < 0.01 in comparison with the control without curdlan treatment. C, BMCs were incubated with M-CSF (20 ng/ml) and RANKL (40 ng/ml) in the presence or absence of curdlan (12.5–50 μg/ml) on an Osteo Assay Stripwell Plate® for 7 days. Cells were removed, and the resorption pits were visualized with light microscopy. Scale bars = 500 μm. D, the pit areas were analyzed with ImageJ software. Data show the resorption area from three independent samples, and error bars represent mean ± S.D. Data were analyzed by Dunnett’s test after one-way ANOVA. *, p < 0.05; **, p < 0.01 in comparison with the control without curdlan treatment. E, BMCs were incubated with M-CSF (20 ng/ml) and RANKL (40 ng/ml) in the presence or absence of curdlan (12.5–50 μg/ml) for 5 days. Cells were fixed and stained for F-actin. Scale bars = 500 μm.

Quantitative Real-time RT-PCR—Total RNA was isolated from cells with an RNeasy mini kit (Qiagen Inc., Valencia, CA) according to the instructions of the manufacturer. RNA was transcribed with q-Script cDNA Supermix reagents (Quanta BioSciences, Gaitherburg, MD) and amplified for 30 min at 95 °C. For real-time RT-PCR, PCR products were detected using FAST SYBR Green Master Mix (Applied Biosystems, Foster City, CA) according to the instructions of the manufacturer. Relative changes in gene expression were calculated using the comparative CT method. Total cDNA abundance between samples was normalized using primers specific to the GAPDH gene.

Immunoblot Analysis—Total protein was extracted using cell lysis buffer (Cell Signaling Technology) containing a protease inhibitor mixture (Thermo Fisher Scientific) and a phosphatase inhibitor mixture (Nacalai Tesque). Protein contents were measured using a DC protein assay kit (Bio-Rad). Total protein (20 μg/sample) was loaded and separated on a 10–20% e-PAGEI (ATTO Corp., Tokyo, Japan) and then transferred to PVDF membranes (Millipore Corp., Bedford, MA). Nonspecific binding sites were blocked for 30 min by immersing the membrane in Blocking One (Nacalai Tesque) at room temperature. Membranes were subjected to overnight incubation with the presence or absence of curdlan. To quantify resorption lacunae, cells were removed with 5% sodium hypochlorite, followed by extensive washing with distilled water and air drying. In some experiments for a pit assay, BMCs were cultured with M-CSF (20 ng/ml) and RANKL (40 ng/ml) in the presence or absence of curdlan on dentin slices (Wako Pure Chemical Industries). Cells on dentin slices were removed, and pits were stained with Mayer’s hematoxylin solution (Muto Pure Chemicals Co., Ltd., Tokyo, Japan). The absorbed areas on the discs and dentin slices were observed under a microscope and quantified using ImageJ software (National Institutes of Health, http://rsb.info.nih.gov).

The presence or absence of curdlan regulated osteoclastogenesis. TRAP-positive multi-nucleated cells were detected using ImageJ software (National Institutes of Health, http://rsb.info.nih.gov).

Quantitative Real-time RT-PCR—Total RNA was isolated from cells with an RNeasy mini kit (Qiagen Inc., Valencia, CA) according to the instructions of the manufacturer. RNA was transcribed with q-Script cDNA Supermix reagents (Quanta BioSciences, Gaitherburg, MD) and amplified for 30 min at 42 °C. For real-time RT-PCR, PCR products were detected using FAST SYBR Green Master Mix (Applied Biosystems, Foster City, CA) according to the instructions of the manufacturer: gapdh, 5′-GACGCGCCATCTTCTTGTA-3′ (forward) and 5′-CAC-ACCGACCTTCCACATTTT-3′ (reverse); dectin 1, 5′-CCTTGGGAGCCCGTACATT-3′ (forward) and 5′-GCAACCACACTTACCAAAAGC-3′ (reverse); nfatc1, 5′-ACCAACCTTTCGCACA-3′ (forward) and 5′-GTTACTGGCTTTCTTCTTCCGTTC-3′ (reverse); trap, 5′-CTGGCTGGGC CTACAA-ATCATA-3′ (forward) and 5′-GGGAG TCCTCAGATGCCAT-3′ (reverse); oc-stamp, 5′-CCGAGCCTGACATT-GAG-3′ (forward) and 5′-TCTCCTGAGTGATCTGTG- CAT-3′ (reverse); cathepsin k, 5′-TATGACCCTGCTTCCCAATAC-3′ (forward) and 5′-GCCGTCGGGCTTA TACATA-3′ (reverse); and mmp9, 5′-TATGCTGGGACGCCAGACACTAAA-3′ (forward) and 5′-TCCGCGCAAGTCCTTCA-GAGTGGT-3′ (reverse). Thermal cycling and fluorescence detection were performed using a StepOneTM real-time system (Applied Biosystems). Relative changes in gene expression were calculated using the comparative CT method. Total cDNA abundance between samples was normalized using primers specific to the GAPDH gene.

Immunoblot Analysis—Total protein was extracted using cell lysis buffer (Cell Signaling Technology) containing a protease inhibitor mixture (Thermo Fisher Scientific) and a phosphatase inhibitor mixture (Nacalai Tesque). Protein contents were measured using a DC protein assay kit (Bio-Rad). Total protein (20 μg/sample) was loaded and separated on a 10–20% e-PAGE1 (ATTO Corp., Tokyo, Japan) and then transferred to PVDF membranes (Millipore Corp., Bedford, MA). Nonspecific binding sites were blocked for 30 min by immersing the membrane in Blocking One (Nacalai Tesque) at room temperature. Membranes were subjected to overnight incubation with
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A total RNA was isolated from c-RAWs, d-RAWs, BMCs, and ST2, reverse-transcribed into cDNA, and then PCR amplification was performed using primers specific for *dectin 1* and *Gapdh*. Data show the fold changes in *dectin 1* mRNA copy number values from three independent samples. Error bars represent mean ± S.D. N.D. represents not detected. B, whole cell lysates from c-RAW, d-RAW, and BMCs were subjected to SDS-PAGE and Western blotting analyses, with the blots probed for *dectin 1*. Equivalent protein aliquots of cell lysates were also analyzed for β-actin. C, mouse BMCs were incubated overnight on culture dishes in α-MEM containing 10% FCS. After discarding adherent cells, floating cells were incubated further with M-CSF (20 ng/ml) on Petri dishes, BMCs became adherent after a 3-day culture and were used as osteoclast precursors. Osteoclast precursors were cultured further with M-CSF and RANKL (40 ng/ml) in the presence or absence of curdlan (25 μg/ml) in the presence of orabsence of curdlan (25 μg/ml) for 3 days. Cells were stained for TRAP activity. Scale bars = 500 μm. Ctr, control. D, the number of osteoclasts was counted after staining for TRAP activity. Data show the number of osteoclasts from three independent samples, and error bars represent mean ± S.D. Data were analyzed by Dunnett’s test after one-way ANOVA. ***: \( p < 0.0001 \) in comparison with control without curdlan treatment.

### FIGURE 2. Expression of *dectin 1* in c-RAWs, d-RAWs, BMCs, and ST2 cells.

A, total RNA was isolated from c-RAWs, d-RAWs, BMCs, and ST2, reverse-transcribed into cDNA, and then PCR amplification was performed using primers specific for *dectin 1* and *Gapdh*. Data show the fold changes in *dectin 1* mRNA copy number values from three independent samples. Error bars represent mean ± S.D. N.D. represents not detected. B, whole cell lysates from c-RAW, d-RAW, and BMCs were subjected to SDS-PAGE and Western blotting analyses, with the blots probed for *dectin 1*. Equivalent protein aliquots of cell lysates were also analyzed for β-actin. C, mouse BMCs were incubated overnight on culture dishes in α-MEM containing 10% FCS. After discarding adherent cells, floating cells were incubated further with M-CSF (20 ng/ml) on Petri dishes, BMCs became adherent after a 3-day culture and were used as osteoclast precursors. Osteoclast precursors were cultured further with M-CSF and RANKL (40 ng/ml) in the presence or absence of curdlan (25 μg/ml) in the presence of orabsence of curdlan (25 μg/ml) for 3 days. Cells were stained for TRAP activity. Scale bars = 500 μm. Ctr, control. D, the number of osteoclasts was counted after staining for TRAP activity. Data show the number of osteoclasts from three independent samples, and error bars represent mean ± S.D. Data were analyzed by Dunnett’s test after one-way ANOVA. ***: \( p < 0.0001 \) in comparison with control without curdlan treatment.

### FIGURE 3. Effect of curdlan on osteoclastogenesis in RAW 264.7 cells.

A, c-RAWs and d-RAWs were incubated with RANKL (40 ng/ml) in the presence or absence of curdlan (12.5–50 μg/ml). Cells were cultured for 7 days and stained for TRAP activity. Scale bars = 500 μm. Ctr, control. B, the number of osteoclasts was counted after staining for TRAP activity. Data show the number of osteoclasts from three independent samples, and error bars represent mean ± S.D. Data were analyzed by Dunnett’s test after one-way ANOVA. ***, \( p < 0.001 \); ****, \( p < 0.0001 \) in comparison with the control without curdlan treatment. C, d-RAWs were incubated with RANKL (40 ng/ml) in the presence or absence of curdlan (25 μg/ml) on an Osteo Assay Stripwell Plate for 12 days. Cells were removed and the resorption pits were visualized with light microscopy. Scale bars = 500 μm. D, the pit areas were analyzed with ImageJ software. Data show the resorption area from three independent samples, and error bars represent mean ± S.D. Data were analyzed by Student’s t test. ***, \( p < 0.001 \) in comparison with the control without curdlan treatment. E, d-RAWs were incubated with RANKL (40 ng/ml) in the presence or absence of curdlan (25 μg/ml) for 8 days. Cells were fixed and stained for F-actin. Scale bars = 500 μm.
Immunofluorescence Analysis of Syk—d-RAWs were cultured with or without curdlan (25 μg/ml) for the indicated times in 8-well chamber slides. Treated cells were fixed in 4% paraformaldehyde in PBS for 60 min at 4 °C and quenched with 0.2 M glycine in PBS. Cells were permeabilized using 0.2% Triton X-100 for 10 min at room temperature, followed by blocking with 1% BSA in PBS for 30 min. After washing in PBS, cells were incubated with anti-Syk polyclonal antibody (Santa Cruz Biotechnology, 2.0 μg/ml) overnight at 4 °C. After washing in PBS, cells were incubated with Alexa Fluor 488-conjugated anti-rabbit secondary antibody (Invitrogen) and then washed, mounted in mounting medium containing DAPI, and visualized using a BZ-9000 fluorescence microscope (Keyence Corp.). Images were captured digitally in real time and processed using BZ-II imaging software.

Silencing of Syk Expression by Specific siRNA—siRNA targeting was used to knock down Syk expression in d-RAWs. siRNA targeting mouse Syk and siRNA were purchased from Santa Cruz Biotechnology. A NEPA21 Super Electroporator (Nepa Gene Co., Ltd., Chiba, Japan) was used to deliver siRNA into cells according to the instructions of the manufacturer. In brief, 1.0 × 10⁶ cells were suspended in 100 μl of α-MEM and transfected with siRNA at a final concentration of 300 nm. Transfected cells were immediately diluted with prewarmed α-MEM and cultured in 6-well plates for 24 h before stimulation with RANKL. Specific gene knockdowns were assessed by real-time RT-PCR.

Statistical Analyses—All data were obtained from three independent experiments, and each experiment was performed in triplicate. Statistical analyses were carried out using JMP® software, version 10.0.2 (SAS Institute Inc., Cary, NC). All data were expressed as mean ± S.D. and analyzed by one-way analysis of variance (ANOVA) followed by a suitable post test (Dunnett’s or Tukey’s) or Student’s t test. p < 0.05 was considered to be statistically significant.

RESULTS

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FIGURE 4. Time course effect of curdlan on osteoclast formation in BMCs and d-RAWs. A, BMCs were incubated with M-CSF (20 ng/ml) for 3 days and cultured further with M-CSF + RANKL (40 ng/ml) in the presence or absence of curdlan (25 μg/ml) for 4 days (d). Cells were stained for TRAP activity. Scale bars = 500 μm. Ctr, control. C, d-RAWs were incubated with RANKL (40 ng/ml) in the presence or absence of curdlan (25 μg/ml). Cells were cultured for 7 days and stained for TRAP activity. Scale bars = 500 μm. The number of osteoclasts differentiated from BMCs (B) and d-RAWs (D) was counted after the staining for TRAP activity. Data show the number of osteoclasts from three independent samples, and error bars represent mean ± S.D. Data were analyzed by Dunnett’s test after one-way ANOVA. ***, p < 0.0001 in comparison with the control without curdlan treatment.
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![Diagram](https://via.placeholder.com/150)

**FIGURE 5. Time course effect of curdlan on osteoclast activity in BMCs.** A, BMCs were incubated with M-CSF (20 ng/ml) and RANKL (40 ng/ml) in the presence or absence of curdlan (25 µg/ml) on dentin slices for 7 days. Dentin slices were stained with Mayer's hematoxylin after removal of cells. The resorption pits were visualized by light microscopy. Scale bars = 200 µm. Ctr, control. B, the pit areas were analyzed with ImageJ software. Data show the resorption area from three independent samples, and error bars represent mean ± S.D. Data were analyzed by Dunnett’s test after one-way ANOVA. ***, p < 0.001** compared with the controls without curdlan treatment. C, BMCs were incubated with M-CSF (20 ng/ml) and RANKL (40 ng/ml) using RedCell® for 5 days. Mature osteoclasts were harvested and seeded on dentin slices, followed by treatment with or without curdlan (25 µg/ml) for an additional 2 days. Dentin slices were stained with Mayer’s hematoxylin after removal of cells. The resorption pits were visualized by light microscopy. Scale bars = 200 µm. D, the pit areas were analyzed with ImageJ software. Data show the resorption area from three independent samples, and error bars represent mean ± S.D. E, mature osteoclasts were stimulated by curdlan (25 µg/ml) for 1 day. Cells were fixed and stained for F-actin. Scale bars = 500 µm.

Curdlan inhibited the stimulatory effect of M-CSF and RANKL on bone resorption in a dose-dependent manner up to 50 µg/ml (Fig. 1, C and D). The actin sealing ring in osteoclasts is essential for bone resorption. Therefore, we visualized the actin cytoskeleton of differentiated BMCs by rhodamine-phalloidin staining. As shown in Fig. 1E, M-CSF and RANKL treatment induced well defined actin sealing ring formation, with a higher-intensity ring height at the cell margin. Conversely, curdlan significantly decreased the number of well defined actin rings and reduced their intensity. Curdlan had no effect on the proliferation of BMCs (data not shown).

**Curdlan-Dectin 1 Interaction Inhibits Osteoclast Formation and Function in RAW Cells**—Because the results from Fig. 1 suggested the potential relevance of curdlan in the regulation of osteoclastogenesis, we analyzed the expression of the curdlan-specific receptor detcin 1 in osteoclast precursor and stromal cells. We confirmed the expression of detcin 1 in osteoclast precursor RAW 264.7 cells and BMCs (Fig. 2, A and B). In contrast, detcin 1 gene expression was not observed in stromal ST2 cells, suggesting that the target cells of curdlan in the regulation of osteoclastogenesis are osteoclast precursor cells, not osteoblasts/stromal cells. These data were supported by the result that curdlan significantly suppressed osteoclast differentiation from osteoclast precursors derived from BMCs (Fig. 2, C and D). Therefore, the inhibitory effect of curdlan on the osteoclastogenesis of RAW 264.7 cells was evaluated in vitro. We found that curdlan had no effect on cell proliferation of c-RAWs and d-RAWs (data not shown). After 7 days of incubation with curdlan (12.5 µg/ml), there was a 58.8% decrease in the number of osteoclasts differentiated by RANKL in d-RAWs (Fig. 3, A and B). This effect of curdlan was dose-dependent, with maximum inhibition observed at 100 µg/ml (97.0% inhibition). On the other hand, curdlan slightly suppressed osteoclast forma-

**Curdlan Has No Effect on M-CSF-induced BMC Differentiation but Inhibits RANKL-induced Osteoclast Formation at an Early Stage**—Osteoclastogenesis is a multistep process that can be separated into two major events: the proliferation of BMCs and their differentiation into osteoclast precursors, induced by M-CSF, and the subsequent differentiation of osteoclast precursors into osteoclasts, induced by RANKL (29). To examine which is the most important step to induce the suppressive effect of curdlan on osteoclastogenesis, we first investigated the effect of curdlan on BMC differentiation induced by M-CSF. Curdlan had almost no effect on M-CSF-induced osteoclast formation (Fig. 4, A and B, d1–4). To examine the precise effects of curdlan on BMC differentiation into osteoclast precursors induced by M-CSF, the expression level of an M-CSF-induced osteoclast precursor marker, RANK, was assessed by Western blotting. However, curdlan had little effect on the expression of RANK induced by M-CSF (data not shown).

To determine the effect of curdlan on RANKL-induced differentiation of osteoclast precursor cells into mature osteoclasts, we examined the differentiation process of BMCs during 8-day RANKL stimulation in the presence or absence of curdlan. We observed that stimulation of curdlan strongly inhibited RANKL-induced osteoclast differentiation during the
early stage (days 4–6) of differentiation. However, curdlan had no inhibitory effect on osteoclast differentiation at later stages (days 6–8). In d-RAWs, treatment with curdlan during the early stage (days 1–3) had an almost identical suppressive effect on osteoclast differentiation induced by RANKL (Fig. 4, C and D).

**Curdlan Has Little Effect on Mature Osteoclast Bone Resorption and Actin Ring Formation**—We investigated whether curdlan inhibited osteoclast bone resorption. When BMCs were cultured on dentin slices, mature osteoclasts caused the resorption of lacunae and the formation of pits in the presence of M-CSF and RANKL. The number and area of pits on the surface of the dentin slices were decreased markedly by the addition of curdlan at the beginning of the assay (Fig. 5, A and B). However, after M-CSF and RANKL stimulation for 6 days on RedCell® (CellSeed Inc., Tokyo, Japan), mature osteoclasts formed and were then seeded on dentin slices, followed by incubation in the presence or absence of curdlan for an additional 2 days. Bone resorption pits and erosion areas showed no significant differences between the control slices and curdlan-treated slices (Fig. 5, C and D). To investigate the effect of curdlan on mature osteoclasts, we examined actin ring formation. Curdlan exerted little effect on the actin ring formation of mature osteoclast (Fig. 5E).

**Curdlan Inhibits RANKL-mediated NFATc1 Expression via Dectin 1**—NFATc1 is a transcription factor essential for RANKL-stimulated osteoclastogenesis (30). Therefore, we investigated Nfatc1 mRNA accumulation, which is related to the autoamplification of NFATc1. After 72 h of incubation with RANKL, there was a strong increase in Nfatc1 mRNA copy number compared with untreated cells. Culturing with curdlan suppressed the stimulation of Nfatc1 mRNA expression by RANKL in d-RAWs (Fig. 6A). In contrast, curdlan had almost no effect on expression levels of Nfatc1 mRNA stimulated by RANKL in c-RAWs. As shown in Fig. 6B, the level of NFATc1 protein expression was increased in d-RAWs 72 h after stimulation with RANKL, and that stimulation was down-regulated by the addition of curdlan. Similar results were obtained for M-CSF and RANKL-induced NFATc1 protein expression in BMCs (Fig. 6C).

**Curdlan Negatively Regulates the Expression of Osteoclastogenic Genes**—To further substantiate the effect of curdlan on osteoclastogenesis, we examined the expression levels of mRNAs encoding osteoclast-related genes after 72 h of RANKL stimulation in d-RAWs. Consistent with the finding for osteoclastogenesis, the expression profiles revealed that osteoclast-associated genes, including Trap (Fig. 7A), Oc-stamp (Fig. 7B), Cathepsin K (Fig. 7C), and Mmp9 (Fig. 7D), induced by RANKL, were decreased significantly by curdlan.

**Curdlan Inhibits the RANKL-mediated AP-1 Signaling Pathway via Dectin 1**—To investigate the molecular mechanism by which curdlan inhibits osteoclastogenesis, we evaluated the effect of curdlan on the activation of NF-κB and AP-1, which is required for induction of NFATc1, by Western blotting. RANKL induced NF-κB activation, which was observed by IxB-α degradation (Fig. 8A). Furthermore, the levels of c-jun phosphorylation (Fig. 8B, phospho-c-jun) and c-fos (Fig. 8C) expression were elevated by RANKL. When d-RAWs were incubated with RANKL and curdlan, the level of c-fos expression was lower than in cells treated with RANKL alone. This inhibitory effect of curdlan on RANKL-induced c-fos expression was not significant in c-RAWs. On the other hand, the activation of c-jun or IxB-α protein induced by RANKL was not affected by treatment with curdlan.

**Curdlan Inhibits NFATc1 Translocation to the Nucleus**—NFATc1, which is phosphorylated in the cell cytoplasm, is translocated to the nucleus by calcineurin-mediated dephosphorylation. Therefore, we investigated whether RANKL-induced NFATc1 translocation is suppressed
Curdlan treatment. Nuclear translocation of NFATc1 was detected in d-RAWs treated with RANKL and was observed to be down-regulated by the addition of curdlan. (Fig. 9, A and B).

Western blotting analysis revealed that NFATc1 in the nuclear fraction was increased in cells following stimulation with RANKL. When cells were incubated with both RANKL and curdlan, the level of NFATc1 protein in the nucleus was lower than in cells treated with RANKL alone (Fig. 9 C).

**Curdlan Inhibits Osteoclastogenesis via Degradation of Syk Protein**—To clarify the role of Syk on osteoclastogenesis, we examined Syk expression in d-RAWs incubated with curdlan by immunoblot analysis. Surprisingly, time-dependent Syk degradation was observed in the presence of curdlan up to 3 h, whereas curdlan transiently phosphorylated Syk protein in d-RAWs from 15–60 min (Fig. 10 A). By immunofluorescence analysis, d-RAWs, at all examined culture times, exhibited a background level of Syk that was localized primarily in the cytoplasm. Syk protein accumulation was also diminished by curdlan treatment up to 3 h (Fig. 10 B). To confirm the involvement of Syk in osteoclast formation, d-RAWs were transfected with Syk-specific siRNA and cultured with RANKL. Using Syk-specific siRNA transfected into d-RAWs, Syk mRNA was knocked down by ~50% compared with cells transfected with a nonspecific control siRNA (Fig. 10 C). The knockdown of Syk protein was also confirmed by Western blot analysis (Fig. 10 D). In d-RAWs transfected with control siRNA, RANKL-induced osteoclast formation was observed. However, in the Syk-specific siRNA-treated d-RAWs, osteoclast formation was diminished, even with RANKL treatment (Fig. 10 E and F). Furthermore, the RANKL-induced increase in NFATc1 expression was blocked in the presence of Syk siRNA (Fig. 10 G).

**Inhibition of Syk Activation Abolished RANKL-induced Osteoclastogenesis**—To further substantiate the role of curdlan-dectin 1-Syk signaling in osteoclast formation induced by RANKL, d-RAWs were pretreated with the specific inhibitor for Syk. Two reagents, piceatannol and BAY 61-3606, are commonly used to block phosphorylation of Syk (31, 32). WST-1 analysis revealed that these inhibitors had no effect on d-RAW cell growth (data not shown) in the presence or absence of RANKL. Both piceatannol and BAY 61-3606 pretreatment effectively blocked RANKL-induced osteoclast formation in a dose-dependent manner (Fig. 11, A–D). Furthermore, the increase in NFATc1 expression, induced by RANKL, was decreased by pretreatment with both inhibitors (Fig. 11 E).

**DISCUSSION**

A number of studies have documented β-glucan modulation of biological activities in macrophages (11, 33, 34). Osteoclasts are large, multinucleated cells formed by the fusion of precursor cells in the monocyte/macrophage lineage (35). There is a considerable overlap of molecules and regulatory mechanisms shared between osseous and immune systems. With the aid of
modern conditional gene targeting and transgenic technologies, the field of combined bone and immunology, known as osteoimmunology, is advancing rapidly. Previous studies have reported the existence of a specific \( /H_9252\)-glucan receptor, dectin 1, on effector cells, including macrophages (36–38). This receptor binding process is thought to be the first step in mediating the activating immunomodulatory effects of \( /H_9252\)-glucans.

Osteoclasts are derived from myeloid progenitors, which also give rise to monocytes, macrophages, and dendritic cells. The direct effects of \( /H_9252\)-glucan on osteoclastogenesis have not been investigated to date. In this study, we used curdlan, a linear \( /H_9252\)-1,3 glucan from the bacterium \( Alcaligenes\ faecalis \), as a dectin 1-specific agonist (39–42). Interestingly, osteoclast formation of BMCs, induced by M-CSF and RANKL, was decreased by addition of curdlan (Fig. 1, A and B), indicating that curdlan has a protective effects against osteoclastogenesis. This effect appears not to be due to the toxicity of curdlan because curdlan treatment did not significantly alter the proliferation of BMCs compared with M-CSF and RANKL (data not shown).

Bone resorption is a multistep process initiated by the proliferation of immature osteoclast precursors. This is followed by commitment of these cells to the osteoclast phenotype and degradation of the organic and inorganic phases of bone by mature resorptive cells. When cultured with bone or dentin, osteoclasts excavate resorptive lacunae, or pits, that are similar to the structures formed when cells degrade bone \textit{in vivo}. Furthermore, the size of the resorption lacunae formed \textit{in vitro} is used as a quantitative measure of osteoclast activity (43). In this study, we used an Osteo Assay Stripwell Plate\textsuperscript{\textregistered} coated with a calcium phosphate substrate and observed the down-regulation of the pit-forming activity of osteoclasts stimulated with M-CSF and RANKL (Fig. 1, C and D). In addition, actin cytoskeletal organization includes the ruffled membranes and the actin ring or sealing zone, which is essential for mature osteoclasts to perform bone resorption (27, 28, 44, 45). Curdlan treatment showed impaired actin cytoskeletal organization in BMCs, suggesting that curdlan is involved in the regulation of actin ring formation induced by M-CSF and RANKL (Fig. 1E).

The cell specificity of dectin 1 expression suggested that the antiosteoclastogenic effect of curdlan was mainly dependent on osteoclast precursors, not osteoblast/stromal cells (Fig. 2). We used a homogeneous clonal population of murine monocyte RAW 264.7 cells to elucidate the direct effects of RANKL and curdlan on osteoclast differentiation and function. This cell line is known to express RANK and differentiate into TRAP-positive cells when cultured with bone slices and RANKL (46). The main advantage of this system is that it does not contain any osteoblast/bone marrow stromal cells, which may also be tar-
gets of RANKL and curdlan actions. We also found that, when induced by RANKL, curdlan suppressed osteoclast formation (Fig. 3, A and B), bone resorption (Fig. 3, C and D), and actin ring formation (Fig. 3E) in RAW 264.7 cells retrovirally transduced to overexpress dectin 1 (d-RAWs) as well as BMCs. On the basis of these findings, we speculate that dectin 1 is a principal receptor responsible for the regulation of osteoclast formation and activation mediated by curdlan. In contrast to d-RAWs, c-RAWs showed less responsiveness to the inhibitory effect of curdlan on RANKL-induced osteoclast formation. These findings are consistent with reports showing that RAW 264.7 cells express low levels of endogenous dectin 1, as measured by flow cytometry (10).

M-CSF and RANKL are essential and sufficient to promote osteoclastogenesis. M-CSF can induce the proliferation of BMCs and their differentiation into osteoclast precursors, and RANKL can subsequently induce the differentiation of osteoclast precursors into mature osteoclasts (29, 35). This study revealed that curdlan had no effect on M-CSF-induced BMC proliferation and differentiation, whereas curdlan inhibited RANKL-induced differentiation of osteoclast precursors into osteoclasts at early stages (Fig. 4). These data are also supported by the finding that curdlan had little effect on the expression of RANK protein induced by M-CSF (data not shown). We also demonstrated that curdlan had no effect on bone resorption activity and actin ring formation of mature osteoclasts (Fig. 5).

Together, these results indicate that curdlan suppressed RANKL-induced osteoclastogenesis at the step of osteoclast precursor differentiation into mature osteoclasts and suggests that curdlan modulated the RANKL signaling pathway.
Previous studies have demonstrated that NFATc1 is strongly induced by RANKL and is required for terminal differentiation of osteoclasts (30, 47). We found that the level of NFATc1 expression during osteoclastogenesis was decreased by curdlan in d-RAWs and BMCs. In contrast, we did not observe a change in RANKL-induced NFATc1 expression in c-RAWs treated with curdlan (Fig. 6). These results indicate that curdlan-dectin 1 interaction impairs RANKL-induced osteoclastogenesis via suppression of NFATc1.

The inhibitory effect of curdlan on osteoclastogenesis was also confirmed by evaluating RANKL-induced mRNA expression levels of osteoclast-related genes. TRAP (48), cathepsin K (49), and MMP9 (50) are required for the bone resorptive activity of mature osteoclast, whereas osteoclast stimulatory transmembrane protein is essential for cell-cell fusion of osteoclasts (51). On the basis of promoter analyses, TRAP (30, 52, 53), cathepsin K (52, 54), and MMP9 (55) are regulated by NFATc1. In addition, a study using a specific inhibitor of NFAT revealed that osteoclast stimulatory transmembrane protein expression also requires NFATc1 in osteoclasts (51). Our results show that curdlan markedly decreased osteoclast-related gene expression regulated by NFATc1 in d-RAWs (Fig. 7). It is possible that the curdlan-dectin 1 interaction changes the RANKL-NFATc1 axis, with an ultimate decrease in osteoclast development, in addition to decreased NFATc1 production.

We also examined the mechanism by which curdlan suppressed RANKL-mediated NFATc1 expression. NF-κB is involved in the activation of immediate-early responsive genes to RANKL (56) and is important for the initial induction of NFATc1 (57). On the other hand, NFATc1 is activated and binds its own promoter. After the induction of NFATc1, the combination of an AP-1 complex containing c-fos and the continuous activation of calcium signaling allows for the sustained autoamplification of NFATc1 (57). Western blot analysis revealed that curdlan inhibited RANKL-stimulated c-fos protein expression but not activation of NF-κB (Fig. 8). These findings led us to speculate that inhibition of RANKL-stimulated NFATc1 expression by curdlan in d-RAWs, may occur because of suppression of AP-1 signaling pathway, which regulates autoamplification of NFATc1. This speculation was strongly supported by the observation that curdlan interferes with RANKL-induced nuclear translocation of NFATc1 (Fig. 9). However, curdlan itself appears to increase phosphorylation of c-jun protein in d-RAWs but not c-RAWs, and activation of c-fos by RANKL was lower in d-RAWs than c-RAWs, indicating an alternative signaling pathway in osteoclastogenesis by curdlan. We have no precise explanation for this phenomenon, and further studies are required to identify the detailed regulatory mechanisms of dectin 1 on osteoclast formation. The molecular mechanisms of the interaction between curdlan and dectin 1 are currently under investigation using dectin 1-deficient mice.

Syk has been identified to be involved in dectin 1 signaling, which is activated by engagement with β-glucan (12, 21, 58). Interestingly, treatment with curdlan increased Syk protein degradation in d-RAWs (Fig. 10, A and B), suggesting that sup-
pression of dectin 1/Syk signaling by curdlan inhibits RANKL-induced osteoclastogenesis. However, real-time RT-PCR analysis revealed that curdlan treatment did not alter mRNA expression of Syk in d-RAWs (data not shown). On the other hand, the addition of curdlan significantly increased the level of phosphorylated Syk in d-RAWs. A previous study using B cells reported that, subsequent to ITAM binding, Syk was phosphorylated on tyrosine 323, which generated a binding site for ubiquitin ligase, resulting in Syk ubiquitination and down-regulation of downstream signaling (59). From these results, the molecular mechanism for posttranslational modifications, including phosphorylation, ubiquitination, SUMOylation, acetylation, and O-glycosylation, are currently under investigation in our laboratory.

The importance of the dectin 1-Syk interaction in osteoclastogenesis was demonstrated clearly by the dramatic decrease in osteoclast formation (Fig. 10, E and F) and NFATc1 expression (Fig. 10G) induced by Syk knockdown. These findings are consistent with a previous study that reported that osteoclasts from Syk−/− precursors (obtained from bone marrow chimeras generated using Syk−/− fetal liver cells) (60) failed to differentiate normally in vitro (24).

Syk is a tyrosine kinase and a key mediator of ITAM receptor signaling (61), which is an important regulatory mechanism in osteoclast differentiation and activity (25, 62). We also found chemical inhibitors of Syk, down-regulated osteoclast formation (Fig. 11, A–D), and NFATc1 expression (Fig. 11E) induced by RANKL. These results are consistent with the premise that curdlan is a potent negative regulator of Syk signaling in osteoclasts. Taken together, the findings obtained in this study suggest that curdlan-dectin 1 binding strongly inhibits NFATc1 expression during osteoclast formation through down-regulation of Syk signaling in osteoclast progenitor cells. Therefore, one might expect that the curdlan administration could be a potential candidate for the treatment of osteoclast-related diseases such as osteoporosis.

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