Biochemical and Biophysical Research Communications 460 (2015) 320–326

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

IL-33 inhibits RANKL-induced osteoclast formation through the regulation of Blimp-1 and IRF-8 expression

Hiroyasu Kiyomiya [a, b], Wataru Ariyoshi [a], Toshinori Okinaga [a], Takeshi Kaneuji [c], Sho Mitsugi [b], Takuma Sakurai [a, b], Manabu Habu [b], Izumi Yoshioka [c], Kazuhiro Tominaga [b], Tatsuji Nishihara [a, *]

[a] Division of Infections and Molecular Biology, Department of Health Promotion, Kyushu Dental University, 2-6-1 Manazuru, Kokurakita-ku, Kitakyushu, Fukuoka 803-8580, Japan
[b] Division of Oral and Maxillofacial Surgery, Department of Science of Physical Functions, Kyushu Dental University, 2-6-1 Manazuru, Kokurakita-ku, Kitakyushu, Fukuoka 803-8580, Japan
[c] Division of Oral Medicine, Department of Science of Physical Functions, Kyushu Dental University, 2-6-1 Manazuru, Kokurakita-ku, Kitakyushu, Fukuoka 803-8580, Japan

ARTICLE INFO

Article history:
Received 23 February 2015
Available online 18 March 2015

Keywords:
Osteoclastogenesis
IL-33
Blimp-1
IRF-8

ABSTRACT

Interleukin (IL)-33 is a recently discovered proinflammatory cytokine that belongs to the IL-1 family. Several studies have reported that IL-33 inhibits osteoclast differentiation. However, the mechanism of IL-33 regulation of osteoclastogenesis remains unclear. In the present study, we examined the effect of IL-33 on osteoclast formation *in vitro*. IL-33 suppressed osteoclast formation in both mouse bone marrow cells and monocyte/macrophage cell line RAW264.7 cells induced by receptor activator of NF-κB ligand (RANKL) and/or macrophage stimulating factor (M-CSF). IL-33 also inhibited the expression of RANKL-induced nuclear factor of activated T-cell cytoplasmic 1 (NFATc1), thereby decreasing the expression of osteoclastogenesis-related marker genes, including *Cathepsin K*, *Osteoclast stimulatory transmembrane protein* (*Oc-stamp*) and *Tartrate-resistant acid phosphatase* (*Trap*). Blockage of IL-33-ST2 binding suppressed the IL-33-mediated inhibition of NFATc1. RANKL-induced B-lymphocyte-induced maturation protein-1 (Blimp-1) expression was also suppressed by IL-33, which was followed by the stimulation of anti-osteoclastic genes such as interferon regulatory factor-8 (IRF-8). These results suggest that IL-33-ST2 interactions down-regulate both RANKL-induced NFATc1 activation and osteoclast differentiation via the regulation of Blimp-1 and IRF-8 expression.

1. Introduction

Bone remodeling is highly regulated by osteoblastic bone formation and osteoclastic bone resorption. These processes are strictly coupled in healthy bone by factors that include growth factors, hormones, and mechanical loading. Disorders of bone remodeling cause diseases such as rheumatoid arthritis, osteoporosis and osteopetrosis, as well as inflammatory bone resorption [1].

Osteoclast precursors interact with osteoblasts and stromal cells to permit their differentiation into mature osteoclasts [2]. Receptor activator of NF-κB ligand (RANKL) is expressed by osteoblasts/bone stromal cells. RANKL binding to the receptor RANK leads to the recruitment of intracellular tumor necrosis factor (TNF)-receptor-associated factor 6, and in turn, to the activation of the intercellular signaling pathway associated with nuclear factor of kappa B (NF-κB), mitogen-activated protein kinases, c-jun, and c-fos [3–5]. Finally, the interaction of RANKL with RANK induces the expression of the nuclear factor of activated T-cell cytoplasmic 1 (NFATc1), the master regulator for osteoclastogenesis [6].

IL-33 is a recently discovered member of the IL-1 family of cytokines [7]. Full-length IL-33 is the bioactive form and it is released in the extracellular space in response to cell damage or mechanical injury. IL-33-mediated signaling involved its interaction with a heterodimeric receptor comprising ST2 and IL-1R accessory protein, leading to the activation of extracellular signal-regulated kinase 1/2 (Erk1/2), p38, c-jun N- terminal kinase (JNK), and NF-κB [8] and subsequently to the production of cytokines such as IL-1β,

* Corresponding author. Fax: +81 93 581 4984.
E-mail address: tatsujin@kyu-dent.ac.jp (T. Nishihara).

http://dx.doi.org/10.1016/j.bbrc.2015.03.033
0006-291X/

Interferon-γ (INF-γ), and TNF-α, which play a role in inflammatory diseases such as asthma, atopic dermatitis, rheumatoid arthritis, and anaphylactic shock [9].

IL-33 is expressed by bone-forming osteoblasts [10]. Moreover, recent studies reported that IL-33 was expressed in bone tissue, where it plays an important role in bone remodeling by effectively blocking osteoclastogenesis [10,11]; however, the mechanisms underlying the effects of IL-33 on osteoclast formation and function are largely unknown. In the present study, we used an *in vitro* culture system to investigate the molecular mechanisms by which IL-33 regulates osteoclastogenesis.

2. Materials and methods

2.1. Reagents and antibodies

Recombinant human IL-33 was obtained from R&D systems (Minneapolis, MN, USA). Recombinant human soluble RANKL and recombinant human macrophage colony stimulating factor (M-CSF) was purchased from Peprotech (Rocky Hill, NJ, USA). Anti-NFATc1 polyclonal antibodies and anti-Blimp-1 monoclonal antibody were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-β-actin monoclonal antibody was purchased from Sigma-Aldrich (St. Louis, MO, USA). Anti-IFN-regulatory-factor-8 (IRF-8), and anti-histone H3 monoclonal antibodies were purchased from Cell Signaling Technology Inc. (Beverly, MA, USA).

2.2. 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 cultured in the presence of M-CSF (20 ng/mL) for 3 days. For differentiation into mature osteoclasts, osteoclast precursors (2.5×10^5 cells/well) were cultured with M-CSF (20 ng/mL) and RANKL (40 ng/mL) in the presence or absence of IL-33 on 24-well plate for 5 days. All procedures were approved by the Animal Care and Use Committee of Kyushu Dental University. The murine monocyte/macrophage cell line RAW264.7 was obtained from Riken Cell Bank (Tsukuba, Japan) and maintained in α-minimal essential medium (α-MEM; Gibco, Grand Island, NY, USA) containing 10% fetal calf serum (FCS), penicillin G (100 units/mL), and streptomycin (100 ∝ g/mL). The cells were maintained at 37 °C in an atmosphere of 5% CO_2. Cells (1×10^3 cells/well) were cultured for 6 days with RANKL (40 ng/mL) in the presence or absence of IL-33 on 96-well plate to generate mature osteoclasts.

2.3. Evaluation of osteoclast differentiation

After culture, adherent cells were fixed and stained with tartrate-resistant acid phosphatase (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.

2.4. Quantitative real-time RT-PCR

RAW264.7 cells (2×10^5 cells/well) were cultured for 48 h with RANKL (40 ng/mL) in the presence or absence of IL-33 (50 ng/mL). In some experiments, the cells were pre-treated with Rat IgG (37.5 μg/mL; R&D Systems) or with an anti-ST2 monoclonal antibody (37.5 μg/mL; R&D Systems) for 1 h prior to their stimulation with RANKL and IL-33. Total RNA was isolated from cells with an

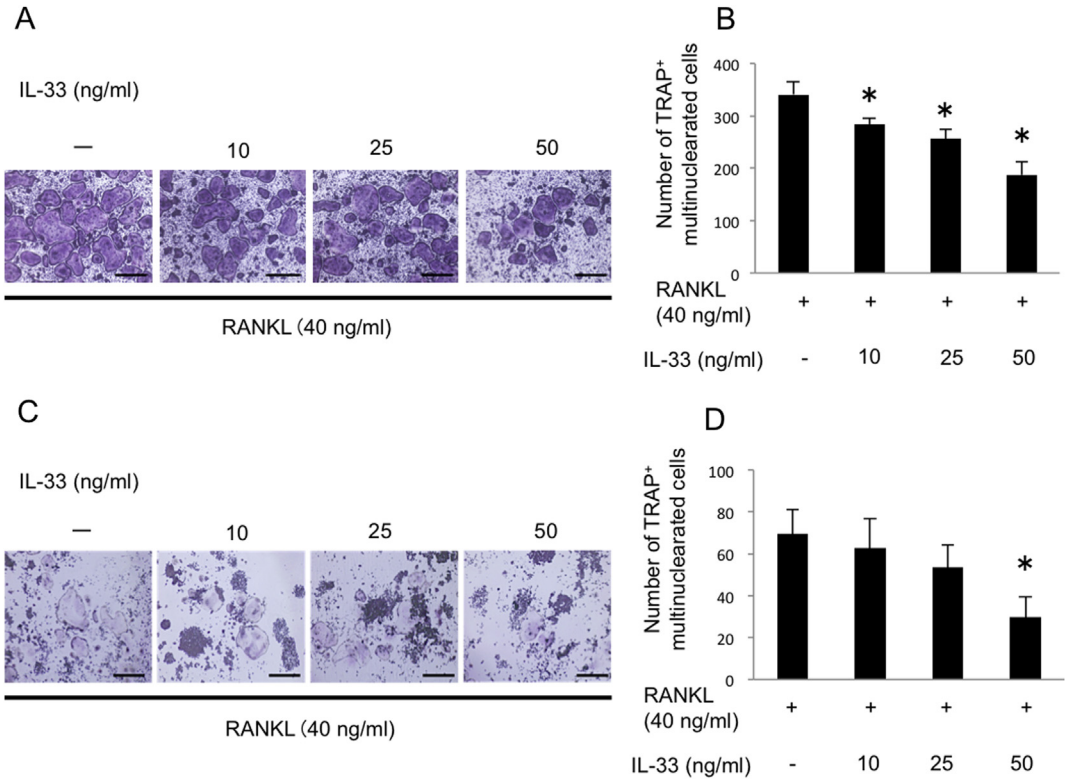

Fig. 1. Effect of IL-33 on osteoclast formation in BMCs and RAW264.7 cells. (A) BMCs were incubated with M-CSF and RANKL in the presence or absence of IL-33 for 5 days and then stained for TRAP activity. Scale bars show 500 μm. (B) Numbers of TRAP positive multinucleated cells were counted. (C) RAW264.7 cells were cultured with RANKL in the presence or absence of IL-33 for 6 days and then stained for TRAP activity. Scale bars show 500 μm. (D) Numbers of TRAP positive multinucleated cells were counted. Data are expressed as the mean ± SD of triplicate cultures. Student's *t*-test, $^*p < 0.05$ compared with the control without IL-33 treatment.

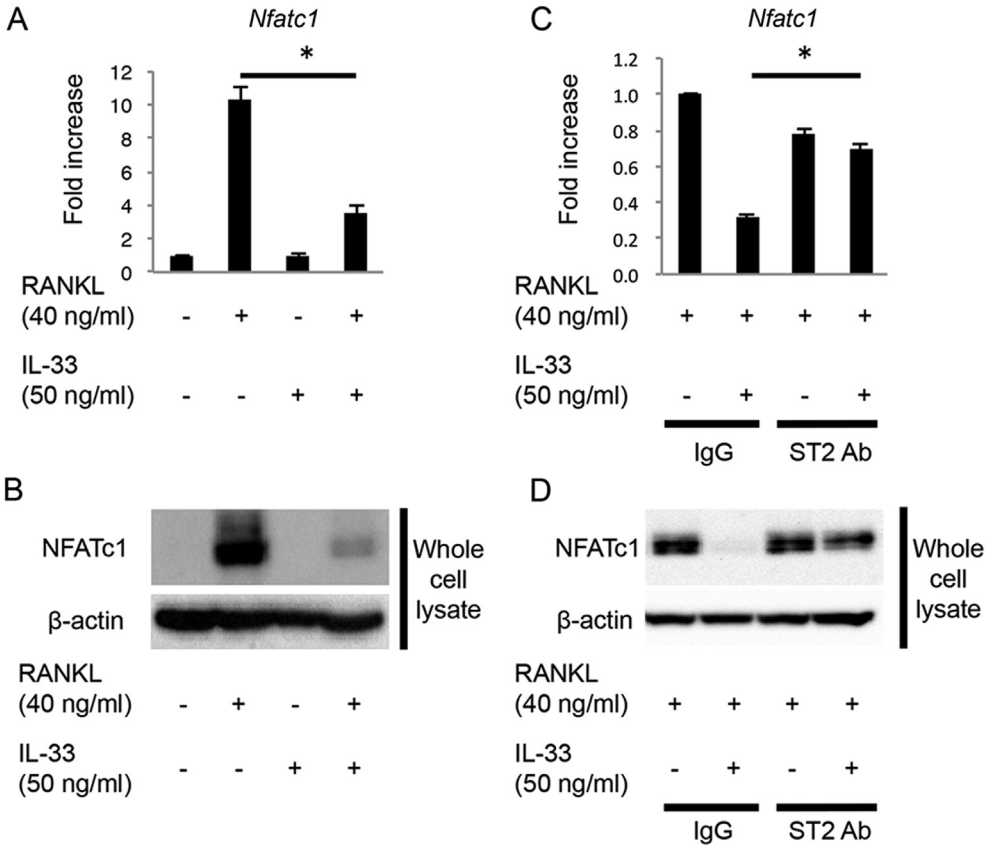

Fig. 2. Effect of IL-33 on RANKL-stimulated NFATc1 expression in RAW264.7 cells. RAW264.7 cells were stimulated with RANKL in the presence or absence of IL-33 for 48 h (real-time RT-PCR) or 72 h (Western Blotting). In neutralizing experiments, RAW264.7 cells were treated with a ST2 antibody for 1 h prior to stimulate with IL-33. (A), (C) The mRNA level of *Nfatc1* was measured by real-time RT-PCR. Data are expressed as the mean ± SD of triplicate cultures. Student's *t*-test, $^{*}p < 0.05$ compared with RANKL treatment. (B), (D) Whole cell lysates were subjected to SDS-PAGE and Western blot analyses, with the blots probed for NFATc1. Equivalent amounts of protein in the cell lysates were determined by measuring β-actin levels.

RNeasy mini kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. RNA was transcribed with q-script cDNA supermix reagents (Quanta BioSciences, Gaithersburg, MD, USA). For real-time RT-PCR, the products were detected using the FAST SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA) using the following primer sequences: *Gapdh*, 5′-GACGGCCGCATCTTCTTGA-3′ (forward) and 5′-CACACCGACCTT-CACCATTTT- 3′ (reverse); *Nfatc1*, 5′-ACCACCTTTCCGCAACCA-3′ (forward) and 5′-GGTACTGG- CTTCTCTTCCGTTTC-3′ (reverse); *Trap*, 5′-CTGCTGGGCCTACAAATCATA-3′ (forward) and 5′-GGGAGTCCT-CAGATCCATAGT-3′ (reverse); *Oc-stamp*, 5′-CCGCA- GCCTGA-CATTTGAG-3′ (forward) and 5′-TCTCCTGAGTGATCGTGTGCAT-3′ (reverse); *Cathepsin k*, 5′-TATGACCACTGCCTTCCAATAC- 3′ (forward) and 5′-GCC- GTGGCGTTATACATACA-3′ (reverse); *Blimp-1*, 5′-TTCTTGTGTGGTATTGTCGGG- ACTT-3′ (forward) and 5′-TTGGGGA-CACTCTTTGGGTAGAGTT-3′ (reverse); *Irf-8*, 5′-GGGCTGATCTGG-GAAAATGA-3′ (forward) and 5′-CACCTCCTGATTGTAATCC- TGCTT-3′ (reverse). Thermal cycling and fluorescence detection were performed using a StepOne 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.

2.5. Western blot analysis

RAW264.7 cells (3×10^6 cells/well) were cultured with RANKL (40 ng/mL) in the presence or absence of IL-33 (50 ng/mL) for 48 or 72 h. In some experiments, the cells were pre-treated with Rat IgG (37.5 μg/m) or an anti-ST2 monoclonal antibody (37.5 μg/mL) for 1 h prior to their stimulation with RANKL and IL-33. Total protein was extracted using cell lysis buffer (Cell Signaling Technology Inc.) containing a protease inhibitor mixture (Thermo Fisher Scientific, Waltham, MA, USA) and a phosphatase inhibitor mixture (Nacalai Tesque Inc., Kyoto, Japan). Protein content was measured using a DC protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA). Equivalent amounts of protein were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). Non-specific binding sites were blocked for 30 min by immersing the membrane in Blocking One solution (Nacalai Tesque Inc.) at room temperature. The membranes were then incubated with diluted primary antibodies overnight 4 °C, followed by horseradish-peroxidase (HRP)-conjugated secondary antibodies for 60 min at room temperature. HRP-conjugated anti-mouse and anti-rabbit IgG (GE Healthcare, Little Chalfont, UK) or HRP-conjugated anti-rat IgG (Santa Cruz Biotechnology) antibodies were used as secondary antibodies. The membranes were washed and then treated with the ECL reagent (GE Healthcare) or Chemi-Lumi One Super (Nacalai Tesque Inc.). The resulting chemiluminescence detected digitally with GelDoc XR Plus (Bio-Rad Laboratories).

2.6. Nuclear translocation of NFATc1

RAW264.7 cells (3×10^6 cells/well) were cultured with RANKL (40 ng/mL) in the presence or absence of IL-33 (50 ng/mL) for 72 h. The cell pellets were treated with NE-PER nuclear and cytoplasmic extraction reagents (Thermo Scientific) according to the manufacturer's instructions. Cell fractions were subjected to SDS-PAGE and immunoblotted with an antibody against NFATc1. In other

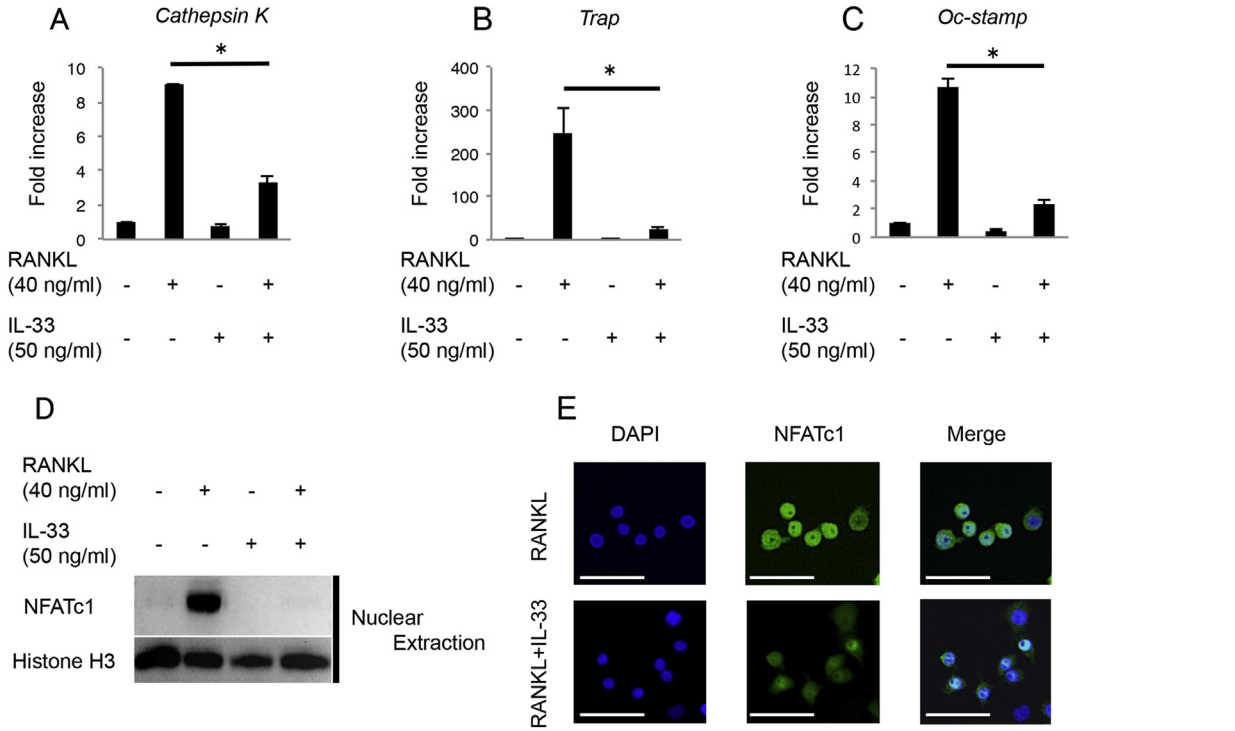

Fig. 3. Effect of IL-33 on the RANKL-induced expression of osteoclastogenic genes and NFATc1 translocation in RAW264.7 cells. The cells were stimulated with RANKL in the presence or absence of IL-33 for 48 h. The mRNA levels of *Cathepsin K* (A), *Trap* (B), and *Oc-stamp* (C) were measured by real-time RT-PCR. Data are expressed as the mean ± SD of triplicate cultures. Student's *t*-test, $^{*}p < 0.05$ compared with RANKL treatment. (D) RAW264.7 cells were stimulated with RANKL in the presence or absence of IL-33 for 72 h. Nuclear fractions were prepared and analyzed by Western blotting, probing the blots for NFATc1. Equivalent amounts of protein in the nuclear fractions were determined by measuring histone H3 levels. (E) RAW264.7 cells were stimulated with RANKL in the presence or absence of IL-33 for 12 h. The cells were fixed, permeabilized, and stained for NFATc1 (green) and nuclei (blue). Scale bars show 50 μm.

experiments, RAW264.7 cells (1×10^4 cells/well) were cultured with RANKL (40 ng/mL) in the presence or absence of IL-33 (50 ng/mL) for 12 h on 4-well chamber slides (Thermo Scientific) and then fixed in 4% paraformaldehyde in phosphate-buffered saline (pH 7.2; PBS) for 60 min at 4 °C, followed by quenching with 0.2 M glycine in PBS. The cells were permeabilized using 0.2% Triton X-100 for 10 min at room temperature, blocked with 1% bovine serum albumin in PBS for 30 min, and then incubated with an anti-NFATc1 polyclonal antibody overnight at 4 °C. After a wash in PBS, the cells were incubated with an Alexa Fluor 488-conjugated anti-mouse secondary antibody (Invitrogen, Carlsbad, CA, USA), washed again, mounted in mounting medium containing 4′, 6-diamino-2-phenylidole (DAPI), and visualized using a BZ-9000 fluorescence microscope (Keyence, Osaka, Japan). Images were captured digitally in real time and processed using BZ-II imaging software.

2.7. Statistical analysis

All data were obtained from three independent experiments, and each experiment was performed in triplicate. Statistical differences were determined using an unpaired Student's *t*-test with Bonferroni correction for multiple comparisons. All data are expressed as the mean ± standard deviation.

3. Results

3.1. IL-33 suppresses osteoclast formation in BMCs and RAW264.7 cells

To examine the effects of IL-33 on osteoclast differentiation, TRAP-positive multinucleated cells were counted as an indicator of osteoclast number. IL-33 inhibited the differentiation of BMCs into osteoclast-like cells mediated by M-CSF and RANKL. As shown in Fig. 1A and B, the effect of IL-33 was dose-dependent, with maximum inhibition observed at a concentration of 50 ng/mL (45.1 % inhibition). IL-33 also inhibited RANKL-induced differentiation of RAW264.7 cells into osteoclasts in a dose-dependent manner, with significant inhibition obtained at a concentration of 50 ng/mL (57.4% inhibition, Fig. 1C and D).

3.2. RANKL-stimulated expression of NFATc1 is inhibited by IL-33

In the intracellular signaling pathway of RANKL, NFATc1 is essential for osteoclast differentiation and is considered to be the master regulator for osteoclastogenesis. Therefore, we examined the effect of IL-33 on RANKL-induced NFATc1 expression by real-time RT-PCR and Western blotting. IL-33 significantly down-regulated the expression of RANKL-induced *Nfatc1* mRNA (Fig. 2A) and completely inhibited the expression of NFATc1 protein (Fig. 2B).

3.3. The IL-33 signaling pathway regulates NFATc1 expression via the ST2 receptor

To investigate the role of ST2 as an IL-33 receptor in the down-regulation of RANKL-induced NFATc1 expression, RAW264.7 cells were pre-treated with ST2 neutralizing antibody for 1 h prior to their stimulation with RANKL and IL-33. PCR and Western blot analysis showed that pre-treatment with the neutralizing antibody effectively prevented the IL-33-induced down-regulation of NFATc1 (Fig. 2C and D).

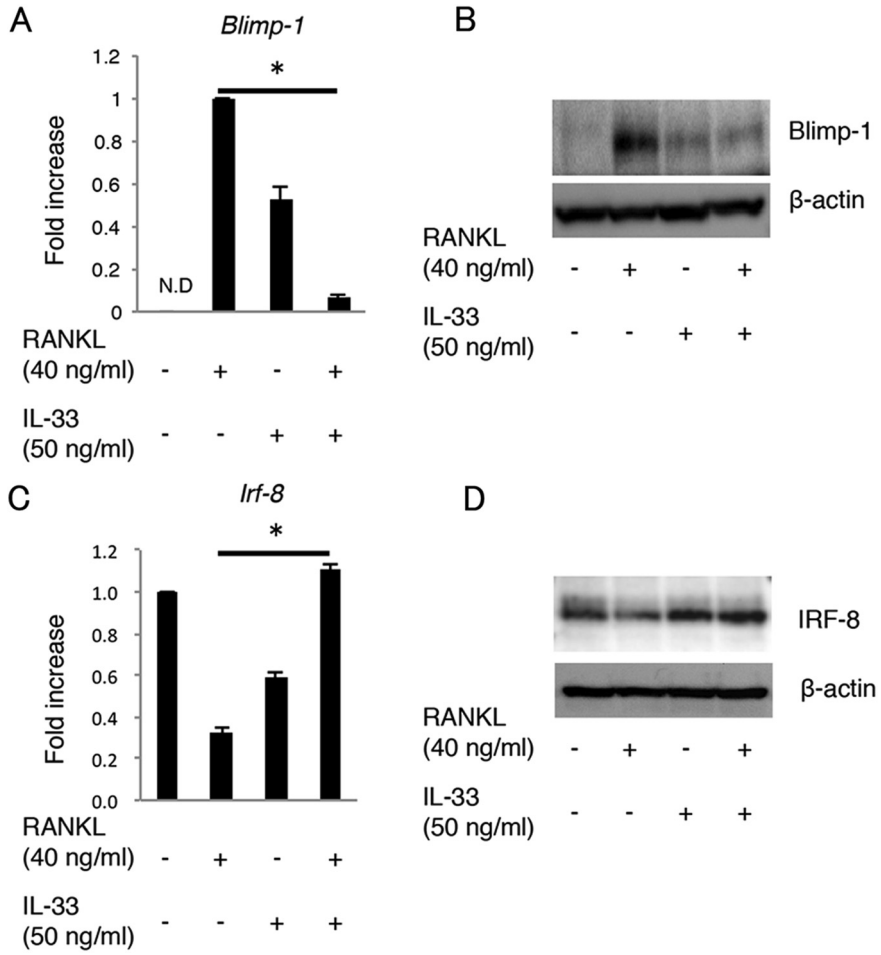

Fig. 4. Effect of IL-33 on Blimp-1 and IRF-8 expression in RAW264.7 cells induced by RANKL. The cells were stimulated with RANKL in the presence or absence of IL-33 for 48 h. The mRNA levels of *Blimp-1* (A) and *Irf-8* (C) were measured by real-time RT-PCR. Data are expressed as the mean ± SD of triplicate cultures. Student's *t*-test, $^{*}p < 0.05$ compared with RANKL treatment. Whole cell lysates were subjected to SDS-PAGE and Western blotting, with the blots probed for Blimp-1 (B) and IRF-8 (D). Equivalent amounts of protein in the cell lysates were determined by measuring β-actin levels.

3.4. IL-33 inhibits RANKL-induced osteoclast-related gene expression

The effect of IL-33 on osteoclast differentiation was investigated by measuring the expression levels of mRNAs encoding osteoclast-related genes in RAW264.7 cells after 48 h of RANKL stimulation. Consistent with the results of TRAP staining, IL-33 caused a significant decrease in the expression of osteoclast-related genes, such as *Cathepsin K* (Fig. 3A), *Trap* (Fig. 3B), *and Oc-stamp* (Fig. 3C) in RANKL-stimulated RAW264.7 cells.

3.5. IL-33 suppresses nuclear translocation of RANKL-induced NFATc1

NFATc1 is translocated from the cytoplasm to the nucleus by calcineurin-mediated dephosphorylation. Therefore, we investigated whether IL-33 altered RANKL-induced NFATc1 translocation. Nuclear translocation of NFATc1 was detected in RAW264.7 cells stimulated with RANKL. When the cells were incubated with both RANKL and IL-33, the level of NFATc1 protein in the nucleus was lower than that in cells treated with RANKL alone (Fig. 3D). Immunofluorescence analysis also revealed that nuclear translocation of NFATc1 in RAW264.7 cells induced by RANKL was decreased by IL-33 treatment (Fig. 3E).

3.6. IL-33 reduced Blimp-1 and recovered IRF-8 induced by RANKL at the gene expression level

We then analyzed the expression of Blimp-1, which is the transcriptional repressor of anti-osteoclastogenic genes and of IRF-8, the negative regulator of osteoclast differentiation. Our results showed that the RANKL-induced Blimp-1 expression was significantly suppressed by IL-33 (Fig. 4A and B). On the other hand, the down-regulation of IRF-8 induced by RANKL was recovered by the addition of IL-33 (Fig. 4C and D).

4. Discussion

Osteoclasts are large multinucleated cells formed by the fusion of precursor cells in the monocyte-macrophage lineage [12]. Their differentiation is dependent on growth factors, cytokines, and hormones. IL-33 is an alarmin cytokine and acts on a variety of cells, such as Th2 lymphocytes, mast cells, and macrophages [13,14]. Several studies reported that IL-33 was expressed by differentiated osteoblasts [15] and inhibited osteoclast formation [11,16,17].

Osteoclast formation of BMCs induced by M-CSF and RANKL was inhibited by the addition of IL-33 (Fig. 1A and B), indicating that IL-33 had a suppressive effect on osteoclastogenesis. We also found that IL-33 suppressed osteoclast formation in RAW264.7 cell

as well as BMCs when induced by RANKL (Fig. 1C and D). These findings suggest that IL-33 induces osteoclast differentiation through signaling pathways in the osteoclast precursor cells. NFATc1 is strongly induced by RANKL and is required for the terminal differentiation of osteoclasts [6,18]. The level of NFATc1 expression in RAW264.7 cells during osteoclastogenesis was suppressed by IL-33 (Fig. 2A and B), consistent with the down-regulation of osteoclast formation by IL-33 via the suppression of NFATc1.

Another important finding of our study is that the IL-33 receptor ST2 is required for the inhibition of RANKL-stimulated NFATc1 expression mediated by IL-33. The binding of IL-33 to ST2 is known to be involved in the onset of a variety of biological activities. A monoclonal anti-ST2 antibody is routinely used in IL-33-related blocking experiments [8]. As shown in Fig. 2C and D, pre-treatment with this antibody remarkably inhibited the effect of IL-33 on the down-regulation of NFATc1 in RAW264.7 cells. On the basis of these findings, we drew the connection of ST2 on RAW264.7 cells and IL-33 concerning the regulation of osteoclast formation and activation.

The inhibitory effect of IL-33 on osteoclastogenesis was confirmed by evaluating RANKL-induced expression of mRNAs of osteoclast-related genes. Cathepsin K [19] and TRAP [20] are related to the bone resorptive function of mature osteoclasts, and osteoclast stimulatory transmembrane protein (OC-STAMP) is associated with cell–cell fusion of osteoclasts [21]. It was demonstrated that *Cathepsin K*, *Trap* and *Oc-stamp* mRNA levels are regulated by NFATc1. Our results show that IL-33 notably decreased osteoclast-related gene expression regulated by *Nfatc1* in RAW264.7 cells (Fig. 3A, B and C). These results suggest that IL-33 modified the RANKL-NFATc1 signaling pathway, leading to a decrease in osteoclast formation.

During osteoclastogenesis, RANKL stimulation is found to induce the nuclear translocation of NFATc1. Furthermore, NFATc1 binds to its own promoter, which results in the robust induction of NFATc1. This autoamplification process of NAFTc1 is an important mechanism in osteoclastogenesis [22]. NFATc1 induction is also dependent on the activation of both the NF-κB [23] and the AP-1 complex containing c-fos [22]. However, IL-33 had no inhibitory effect on the NF-κB and AP-1 activation induced by RANKL (data not shown). So, we next confirmed the effect of IL-33 on NFATc1 nuclear translocation induced by RANKL and clarified that IL-33 interferes with RANKL-induced nuclear translocation of NFATc1 (Fig. 3D and E). Taken together, we conclude IL-33 suppresses RANKL-induced NFATc1 nuclear translocation through NF-κB- or AP-1-independent pathways during the differentiation of osteoclast precursors into mature osteoclasts. Further studies are needed to identify the other signaling molecules that are needed for osteoclastogenesis mediated by IL-33-ST2 interaction.

Recent studies reported that, during osteoclastogenesis, NFATc1 activity is negatively regulated by other transcription factors such as IRF-8, B cell lymphoma 6 (Bcl6), and v-Maf musculoaponeurotic fibrosarcoma oncogene family member protein B (MafB) [24–26]. Blimp-1 is a transcriptional repressor that plays crucial roles in the differentiation and/or function of numerous cell types, including macrophages and lymphocytes [27]. Furthermore, a genome wide screening of RANKL-inducible transcription factors revealed that Blimp-1 functions as a transcriptional repressor of *Irf-8*, *Bcl6*, and *Mafb* during osteoclastogenesis [24,25,28,29]. Interestingly, we found that the RANKL-induced Blimp-1 expression was down-regulated by IL-33 (Fig. 4A and B), while the suppression of IRF-8 expression mediated by RANKL was inhibited by IL-33 (Fig. 4C and D). IRF-8 is specifically expressed in immune cells, including macrophages [30]. IRF-8 binds to NFATc1 in osteoclast precursors and suppresses its transcriptional activity, resulting in the down-regulation of NFATc1 autoamplification and the expression of NFATc1 target osteoclast marker genes [24]. Taken together, these findings suggest that the inhibitory effect of IL-33 on RANKL-induced osteoclastogenesis involves modification of the Blimp-1-IRF-8 molecular axis.

In conclusion, we demonstrated that IL-33-ST2 interaction regulated Blimp-1 and IRF-8 expression and inhibited the activation of NFATc1 during RANKL-induced osteoclast formation. Although IL-33 strongly suppressed NFATc1 expression, it had only a moderate inhibitory effect on osteoclastogenesis induced by RANKL. Further elucidation of the molecular mechanisms of IL-33 in osteoclastogenesis will provide additional knowledge for homeostatic to prevent excessive bone resorption in both physiological and inflammatory conditions.

Conflict of interest

None.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.03.033.

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