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Corresponding Author:	Hideki Kitaura Tohoku Daigaku Sendai, Miyagi JAPAN
Corresponding Author's Institution:	Tohoku Daigaku
Corresponding Author E-Mail:	hkitaura@m.tohoku.ac.jp;marahleh.aseel@hotmail.com
Order of Authors:	Hideki Kitaura Aseel Marahleh Masahiko Ishida Kazuhiro Shima Akiko Kishikawa Saika Ogawa Wei-Ren Shen Jiawei Qi Fumitoshi Ohori Takahiro Noguchi Yasuhiko Nara Itaru Mizoguchi
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TITLE:

Effect of Anti-c-fms Antibody on Osteoclast Formation and Proliferation of Osteoclast Precursor In Vitro

AUTHORS AND AFFILIATIONS:

Aseel Marahleh, Hideki Kitaura, Masahiko Ishida, Kazuhiro Shima, Akiko Kishikawa, Saika Ogawa, Wei-Ren Shen, Jiawei Qi, Fumitoshi Ohori, Takahiro Noguchi, Yasuhiko Nara, Itaru Mizoguchi

Division of Orthodontics and Dentofacial Orthopedics, Department of Translational Medicine, Tohoku University Graduate School of Dentistry, Sendai, Japan

Corresponding Author:

Hideki Kitaura

hkitaura@m.tohoku.ac.jp

Tel.: +81-22-717-8374

Email Addresses of Co-authors:

Aseel Marahleh marahleh.aseel.mahmoud.t6@dc.tohoku.ac.jp

Masahiko Ishida isimasd4@dent.tohoku.ac.jp

Kazuhiro Shima k-shima@dent.tohoku.ac.jp

Akiko Kishikawa kishikawa@dent.tohoku.ac.jp

Saika Ogawa saika.ogawa.a4@tohoku.ac.jp

Wei-Ren Shen shen.wei.ren.t5@dc.tohoku.ac.jp

Jiawei Qi qi.jiawei.p8@dc.tohoku.ac.jp

Fumitoshi Ohori fumitoshi.ohori.t3@dc.tohoku.ac.jp

Takahiro Noguchi takahiro.noguchi.r4@dc.tohoku.ac.jp

Yasuhiko Nara yasuhiko.nara.q6@dc.tohoku.ac.jp

Itaru Mizoguchi mizo@hoku-iryo-u.ac.jp

KEYWORDS:

osteoclast, resorption, bone marrow macrophage, M-CSF, RANKL, TNF- α , anti-c-fms antibody

SUMMARY:

This protocol includes dissection of murine long bones and bone marrow isolation, to generate bone marrow macrophages and produce osteoclasts using macrophage colony stimulating factor (M-CSF) and receptor activator of nuclear factor Kappa-B ligand (RANKL) or tumor necrosis factor alpha (TNF- α) and their arrest by anti-c-fms antibody, M-CSF receptor.

ABSTRACT:

Bone remodeling is a complex process and it involves periods of deposition and resorption. Bone resorption is a process by which bone is broken down by osteoclasts in response to different stimuli. Osteoclast precursors differentiate into multinuclear osteoclasts in response to macrophage colony stimulating factor (M-CSF) and receptor activator of nuclear factor Kappa-B ligand (RANKL). Under pathologic conditions, the cytokine profile is different and

involves a mixture of inflammatory cytokines. Tumor necrosis factor alpha (TNF- α) is one of the most important cytokines as it is found in large amounts in areas involved with inflammatory osteolysis. The purpose of this protocol is to provide a method by which murine bone marrow is isolated to generate osteoclasts through induction with M-CSF and either RANKL or TNF- α which will be subsequently inhibited by increasing doses of anti-c-fms antibody, the receptor for M-CSF. This experiment highlights the therapeutic value of anti-c-fms antibody in diseases of inflammatory bone resorption.

INTRODUCTION:

Osteoclasts are highly specialized cells, and they differentiate from hematopoietic stem cells through the fusion of multiple osteoclast precursors. They are essential for healthy bone remodeling and contribute to pathologic bone resorption associated with inflammatory osteolytic diseases such as rheumatoid arthritis and periodontal disease¹.

Osteoclastogenesis and osteoclast function are mediated by two key factors; macrophage colony stimulating factor (M-CSF) and receptor activator of nuclear factor kappa-B ligand (RANKL). Both M-CSF and RANKL are important for osteoclast differentiation². Another factor which has been shown to induce osteoclast formation from bone marrow macrophages *in vitro* is tumor necrosis factor alpha (TNF- α)³⁻⁵. TNF- α mediated osteoclast formation has been shown to be critical for osteolysis in destructive bone diseases such as rheumatoid arthritis⁶, periodontal disease⁷, and postmenopausal osteoporosis⁸.

C-fms is the membrane receptor of M-CSF and mediates its action. The role of c-fms is well documented in the literature as it was demonstrated that the administration of an antibody against c-fms (anti-c-fms antibody) completely arrested osteoclastogenesis in an arthritis model as well as in TNF- α induced bone erosions while osteoclastogenesis distant from the inflamed joint was still robust⁹. Administration of anti-c-fms antibody inhibited osteoclast formation and bone resorption induced by lipopolysaccharide (LPS) in mouse calvariae¹⁰ as well as in LPS-induced periodontitis model¹¹. Moreover, anti-c-fms antibody inhibited mechanical stress-induced root resorption during orthodontic tooth movement¹², as well as orthodontic tooth movement and bone resorption associated with orthodontic tooth movement¹³.

M-CSF has been reported to have other functions which are essential to the host immune response. The absence of M-CSF in op/op mice with bacterial pneumonia lead to the increase of bacterial burden and bacterial dissemination to the liver with hepatic necrosis¹⁴. Therefore, M-CSF is important for immunological response in the protection from infection.

This study demonstrates the effect of anti-c-fms antibody on M-CSF and RANKL or TNF- α induced osteoclast formation in vitro and M-CSF induced proliferation of osteoclast precursors. This protocol demonstrates the isolation of murine bone marrow cells from long bones, and the steps to generate bone marrow macrophages (BMM) which are regarded as osteoclast precursors and to induce the differentiation of BMM into multinuclear osteoclasts by two methods; either RANKL or TNF- α . The protocol also compares the arrest of osteoclastogenesis

in both methods using anti-c-fms antibody.

The process by which bone marrow is extracted and subsequently used to generate osteoclast precursors is reliable in producing large quantities of pure osteoclast cultures which can be used in multiple downstream applications such as drug testing. The use of either RANKL or TNF- α to induce osteoclastogenesis in this protocol distinguishes osteoclastogenesis as a physiologic process (RANKL) from osteoclastogenesis as a pathologic process (TNF- α), which in turn provides two alternative procedures to guide the decision of which one is superior in terms of the reagents to be used in downstream applications. This protocol also provides a method by which we can determine a suitable concentration of anti-c-fms antibody that can be safely used for later studies involved in bone resorption and osteolytic diseases.

PROTOCOL:

All animal procedures and animal care were performed according to Tohoku University rules and regulations.

1. Murine Hindlimb Dissection and Handling

1.1. Before dissection, fill a plate with approximately 50 mL of α -MEM and place it on ice. This will serve as a collection container until the dissection of all bones is complete. For this and subsequent experiments, use α -MEM containing 10% fetal bovine serum (FBS), 100 IU/mL penicillin G, and 100 μ g/mL streptomycin.

1.2. Euthanize C57Bl/6J mouse by inhalation of an overdose of 5% isoflurane.

1.3. Fix the mouse in a supine position using pins pierced through the palms and feet and spray thoroughly with 70% ethanol.

1.4. Make a skin incision using sterile surgical scissors and tweezers at the level of the hip and extend it down to the feet exposing the muscles.

1.5. Cut the tendon attaching the quadriceps muscle and the tendon attaching the hamstring muscle to the bone. Peel away the muscles exposing both hip and knee joints. Locating these tendons will ease freeing both muscles from their attachments without leaving soft tissue tags. Do not cut into the bone.

1.6. Position the scissors between the head of the femur and the joint space and cut into it freeing the femur but keeping the bone intact. This will enable detachment of the bones without the risk of exposing the bone marrow.

1.7. Similarly cut away the muscles attaching to the tibia. Cut the tibia free from its attachment at the ankle joint keeping the bone intact to avoid contamination.

1.8. Scrape any remaining soft tissue from the femur and tibia using the scissors blades and kimwipe and place it in a plate with α -MEM placed on ice.

2. Murine Hindlimb Bone Marrow Isolation

2.1. Fill a 30 G needle syringe with α -MEM.

2.2. Disconnect the tibia and femur away from the knee joint.

2.3. Cut the ends of the long bone from both sides using scissors.

2.4. Hold the bone from the shaft using tweezers, insert the needle into the marrow space of the bone and slowly flush the content of the marrow into a 6 cm culture dish. The bone will appear white, indicating the extraction of all bone marrow content. Repeat for all bones.

2.5. Strain the bone marrow extract using a 40 μ m nylon cell strainer into a 50 mL conical centrifuge tube and centrifuge at 300 x *g* for 5 min.

2.6. Discard the supernatant, wash with 5 mL of α -MEM, and centrifuge at 300 x *g* for 5 min. Repeat wash for a total of 2 times.

2.7. Resuspend the pellet in 10 mL of α -MEM and perform a cell count using a hemocytometer as follows:

2.7.1. Make a 1:1 dilution of cell suspension by adding 10 μ L of cell suspension to 10 μ L of 0.4% trypan blue in a 1.5 mL tube and pipette once.

2.7.2. Cover the hemocytometer chamber with a slide cover and transfer the dye suspension to the hemocytometer. It is important not to overfill or underfill the chamber and allow the suspension to fill the chamber by capillary action.

2.7.3. Place the hemocytometer on a microscope stage. Using 10X objective, focus on the center square. Count all cells bound by 3 lines. To make sure that cells are not counted twice, count the upper and right corners of each square. Dead cells will appear dark blue, these are excluded from the count.

2.8. Depending on the downstream application, seed the cells into an appropriate culture vessel. For this particular experiment, follow section 3.

3. Generation of BMM

3.1. Seed 1×10^7 cells/10 mL in a 10 cm culture dish including M-CSF. The final concentration of M-CSF is 100 ng/mL of culture medium. Incubate the culture at 37 °C, 5% CO₂ for 3 days.

3.2. After 3 days, remove the culture medium, wash the cells vigorously with 10 mL of phosphate buffered saline (PBS) twice to remove non-adherent cells, add 5 mL of room temperature 0.02% trypsin-EDTA in PBS and incubate at 37 °C, 5% CO₂ for 5 min.

3.3. Detach the cells by thorough pipetting. Make sure that the cells have been detached by observing the culture under a microscope (the cells should appear rounded and floating in media). If the cells remain attached, repeat pipetting thoroughly to detach them. When the cells have been detached, add 5 mL of α -MEM to inactivate the reaction.

3.4. Collect the cells into a 50 mL conical tube and centrifuge at 300 x *g* for 5 min. Discard the supernatant, and wash with 5 mL of α -MEM.

3.5. Centrifuge at 300 x *g* for 5 min and resuspend the pellet in 10 mL of α -MEM.

3.6. Perform a cell count as previously described in step 2.7.1.

3.7. Seed 1x10⁶ cells/10 mL in a 10 cm culture dish including M-CSF. The final concentration of M-CSF is 100 ng/mL of culture medium. Incubate the culture at 37 °C, 5% CO₂ for 3 days.

4. Generation of Osteoclasts from BMM as Osteoclast Precursors

4.1. After 3 days, harvest the attached cells which represent BMM as osteoclast precursors, following steps 3.2-3.7.

4.2. Seed BMM at 5x10⁴ cells/200 μ L of α -MEM in a 96 well plate. Add RANKL for a final concentration of 50 ng/mL or TNF- α for a final concentration of 100 ng/mL. Add M-CSF for a final concentration of 100 ng/mL to each well.

4.3. Add anti-c-fms antibody (final concentrations of 0, 1, 10, 100, 1000 ng/mL) to each well.

4.4. Incubate at 37 °C, 5% CO₂ for 4 days. Change media every other day for 4 days.

5. Tartrate-resistant Acid Phosphatase (TRAP) Stain

5.1. After 4 days of incubation, gently aspirate the culture medium of each well. Wash each well once with 200 μ L of room temperature 1x PBS.

5.2. Add 200 μ L of 10% formalin to each well for fixation and incubate for 1 h at room temperature. Aspirate the formalin and wash each well 3 times with deionized water.

5.3. Add 200 μ L of 0.2% Triton X-100 in PBS to each well for permeabilization and incubate for 1 h at room temperature. Aspirate the Triton X-100 and wash each well 3 times with

deionized water.

5.4. Add 200 μ L of TRAP stain solution to each well. Visualize the stain under the microscope. It will take from 10-30 min for the stain to develop properly.

5.5. Aspirate the stain and wash each well with deionized water 3 times, and air dry. Keep at room temperature to use in further observation.

6. Proliferation Assay

6.1. Seed BMM as osteoclast precursors at 5×10^3 cells/200 μ L of α -MEM in a 96 well plate. Add M-CSF for a final concentration of 100 ng/mL to each well.

6.2. Add anti-c-fms antibody (final concentrations of 0, 1, 10, 100, 1000 ng/mL) and incubate at 37 $^{\circ}$ C, 5% CO₂ for 3 days without medium change.

6.3. After 3 days, wash the cells with 1x PBS. Add 100 μ L of α -MEM to each well.

6.4. Add 10 μ L of cell counting kit solution and incubate at 37 $^{\circ}$ C, 5% CO₂ for 2 h and determine the absorbance of each well at 450 nm using a microplate reader.

REPRESENTATIVE RESULTS:

The purpose of this protocol is to evaluate the effect of anti-c-fms antibody on osteoclast formation in the presence of RANKL or TNF- α , and to determine the effect of M-CSF on osteoclast precursors proliferation. In this protocol, we have provided a reliable process by which large quantities of pure osteoclast cultures are generated. We have also provided a way to test anti-c-fms antibody suitable concentration for inhibiting osteoclast formation under different culture conditions.

The osteoclast formation in M-CSF+RANKL culture with anti-c-fms antibody is shown in **Figure 1**. At 1, 10 ng/mL anti-c-fms antibody, there was no significant decrease in the number of osteoclasts compared to control (0 ng/mL). While at 100, 1000 ng anti-c-fms antibody, there was a significant decrease in the number of osteoclasts compared to control.

The osteoclast formation in M-CSF+TNF- α culture with anti-c-fms antibody is shown in **Figure 2**. At 1 ng/mL anti-c-fms antibody, there was no significant decrease in the number of osteoclasts compared to control (0 ng/mL). While at 10, 100, 1000 ng/mL anti-c-fms antibody, there was a significant decrease in the number of osteoclasts compared to control.

Osteoclast precursors culture with M-CSF+anti-c-fms antibody is shown in **Figure 3**. At 1, 10, 100 ng/mL, there was no significant decrease in the number of osteoclast precursors compared to control (0 ng/mL), while at 1000 ng/mL, there was a significant decrease in the number of osteoclast precursors compared to control. This indicates that a concentration as high as 1000

ng/mL is needed to inhibit proliferation of osteoclast precursors significantly.

These results provide information about the robust nature of RANKL in producing osteoclasts while a concentration of 50 ng/mL of RANKL was used, and a concentration of 100 ng/mL of TNF- α was needed to obtain the same number of osteoclasts. Both of these methods are suitable for osteoclast generation but the decision of which is superior depends on the downstream application in the context of the reagents to be used later. As the results indicate, using RANKL is not the equivalent of using TNF- α to generate osteoclasts.

In this protocol, we used anti-c-fms antibody to inhibit osteoclastogenesis, and by looking at the results, we find that a concentration of 100 ng/mL of anti-c-fms antibody was needed to produce significant decrease in osteoclast number in RANKL wells. A concentration of 1000 ng/mL of anti-c-fms antibody was needed to inhibit osteoclast precursor proliferation in MCSF proliferation assay, while a concentration of only 10 ng/mL of anti-c-fms antibody was needed to produce significant decrease in osteoclast number in TNF- α wells.

FIGURE AND TABLE LEGENDS:

Figure 1: Effect of anti-c-fms antibody on RANKL-induced osteoclast formation. Microscopic observation of osteoclast precursors that were treated with M-CSF, RANKL and various dose of anti-c-fms antibody (0, 1, 10, 100 and 1000 ng/mL) for 4 days. Cells were fixed and stained with TRAP staining. Number of TRAP-positive cells cultured with M-CSF, RANKL and various concentrations of anti-c-fms antibody (0, 1, 10, 100 and 1000 ng/mL) for 4 days. Cells were fixed and stained with TRAP staining. The number of TRAP-positive cells was assessed. Results were expressed as mean \pm S.D. of four cultures. Statistical differences were detected by using Scheffe's tests ($n = 4$; $*p < 0.01$). Scale bars = 200 μ m.

Figure 2: Effect of anti-c-fms antibody on TNF- α -induced osteoclast formation. Microscopic observation of osteoclast precursors that were treated with M-CSF, TNF- α and various dose of anti-c-fms antibody (0, 1, 10, 100 and 1000 ng/mL) for 4 days. Cells were fixed and stained with TRAP staining. Number of TRAP-positive cells cultured with M-CSF, TNF- α and various concentrations of anti-c-fms antibody (0, 1, 10, 100 and 1000 ng/mL) for 4 days. Cells were fixed and stained with TRAP staining. The number of TRAP-positive cells was assessed. Results were expressed as mean \pm S.D. of four cultures. Statistical differences were detected by using Scheffe's tests ($n = 4$; $*p < 0.01$). Scale bars = 200 μ m.

Figure 3: Effect of anti-c-fms antibody on proliferation of osteoclast precursor. Cell viability of osteoclast precursors that were treated with M-CSF and various dose of anti-c-fms antibody (0, 1, 10, 100 and 1000 ng/mL) for 3 days. Cell counting kit-8 was used to measure viability. Data are presented as a percentage to compare the relative activity of the wells containing M-CSF+anti-c-fms versus the wells containing M-CSF alone and expressed as mean \pm S.D. of four cultures. Statistical differences were detected by using Scheffe's tests ($n = 4$; $*p < 0.01$).

DISCUSSION:

In this study, we investigated the effect of anti-c-fms antibody on RANKL-induced osteoclast formation, TNF- α -induced osteoclast formation and M-CSF-induced osteoclast precursor proliferation. We found that the effective amount of anti-c-fms antibody for the inhibition of osteoclastogenesis among RANKL-induced osteoclast formation, TNF- α induced osteoclast formation and M-CSF-induced proliferation of osteoclast precursors is different.

RANKL mediates osteoclastogenesis in the presence of M-CSF *in vitro*, and RANK-null mice experience severe osteopetrosis due to the failure of osteoclasts to develop¹⁵, thus RANKL is an obligatory cytokine for osteoclastogenesis and has an important regulatory role in bone homeostasis. Therefore, if RANKL-induced osteoclast formation can be controlled, the decrease in bone mass can be controlled. However, if excessive suppression occurs, bone homeostasis will not be maintained. In this experiment, we show that anti-c-fms antibody needs to be at high concentration 100 ng/mL to be able to decrease osteoclast formation induced by RANKL.

TNF- α mediated osteoclast formation has been shown to be critical for osteolysis in destructive bone diseases such as rheumatoid arthritis⁶, periodontal disease⁷, and postmenopausal osteoporosis⁸ and here we show that anti-c-fms antibody can easily inhibit osteoclast formation mediated by TNF- α at a low concentration of 10 ng/mL. The result suggests that a low concentration of anti-c-fms antibody could be of therapeutic value to decrease the process of osteoclastogenesis in pathologic conditions but would have minimal effect on osteoclastogenesis in normal conditions.

M-CSF is a key factor for osteoclast formation as the op/op mice which lack *Csf1* the gene coding for M-CSF show an osteopetrotic phenotype due to complete lack of osteoclasts¹⁶. M-CSF also promotes the survival of osteoclast precursors². M-CSF levels are increased in the serum of patients with rheumatoid arthritis who have severe ankylosing spondylitis¹⁷ and in the synovial fluid around loose joint prosthesis¹⁸. TNF- α increases the number of osteoclast precursors *in vivo* as a result of inducing M-CSF gene expression in stromal cells⁹. In the light of these data we can conclude that M-CSF is an important mediator of inflammation due to the impact of TNF- α on stromal cells which causes an increase in M-CSF production from these cells.

It has been reported that M-CSF plays an important role in host immune response such as antimicrobial functions of both lung and liver mononuclear phagocytes during pneumonia¹⁴. Therefore, if excessive suppression of M-CSF occurs, there is a possibility that the immune response will decrease. In this experiment, we showed that a very high concentration of anti-c-fms antibody (1000 ng/mL) was needed to inhibit osteoclast precursors survival and proliferation mediated by M-CSF. These results taken side by side suggest that anti-c-fms antibody may work as a therapeutic to arrest osteoclastogenesis in TNF- α induced osteolytic diseases at low concentration, at the same time this concentration will spare osteoclast formation induced by RANKL and M-CSF which will allow for bone remodeling to occur.

Critical steps in the protocol have to be performed carefully to ensure the success of the experiment. In the beginning of the protocol while dissecting, it is important to avoid cutting into the bone to avoid contamination of the bone marrow. In the process of extracting bone marrow, to ensure that all bone marrow have been extracted, the flushing procedure should be repeated per needed until all bone marrow have been extracted, which will provide a high yield of cells. This protocol can be utilized in multiple downstream applications, such as reagent testing, and it also provides a way by which the concentration of anti-c-fms antibody can be tested avoiding false interpretation of the results. As we showed, the concentration of anti-c-fms antibody which was needed to inhibit osteoclast formation is not equal among the methods by which osteoclasts were obtained (RANKL or TNF- α), thus any results obtained with regard to the concentration of anti-c-fms have to be carefully examined in future experiments.

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DISCLOSURES:

The authors have nothing to disclose.

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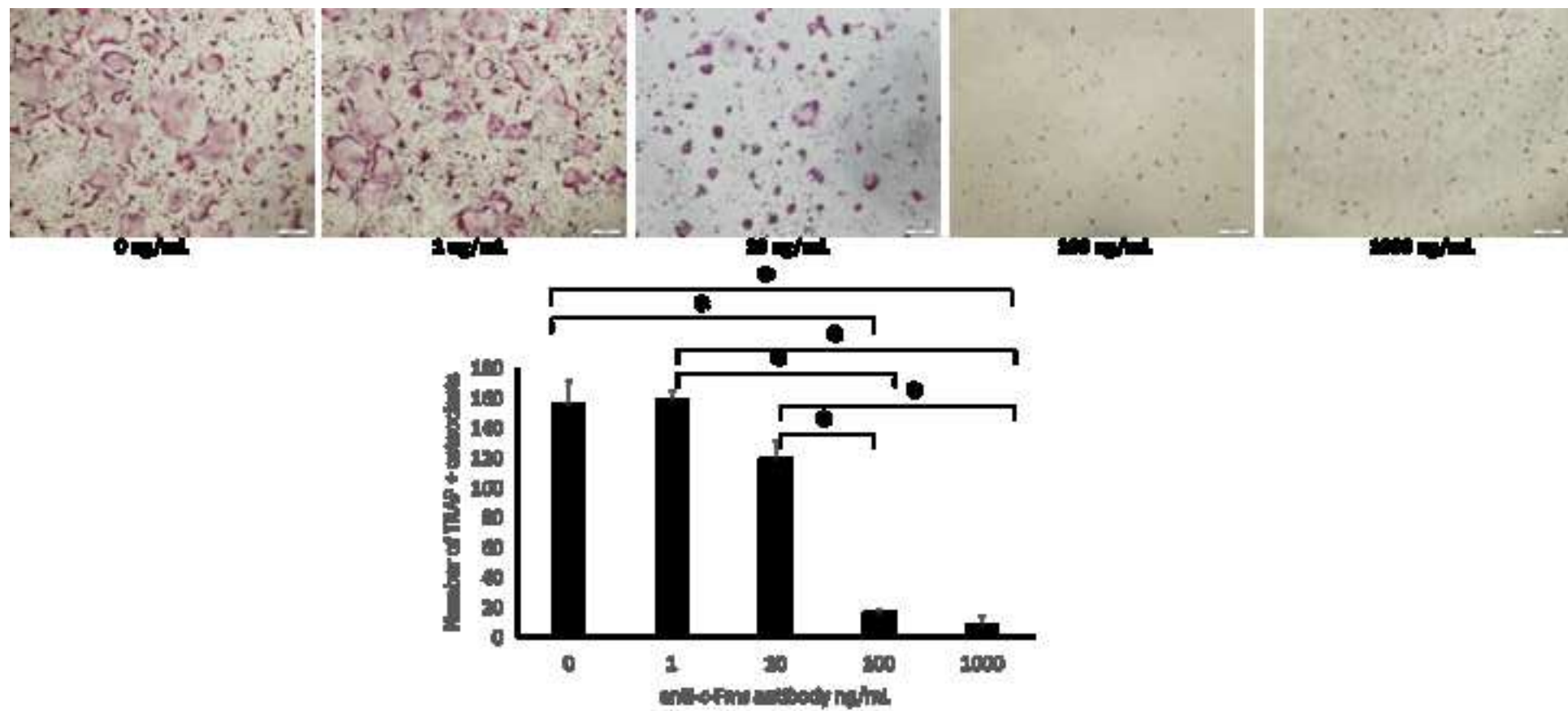


Figure 2

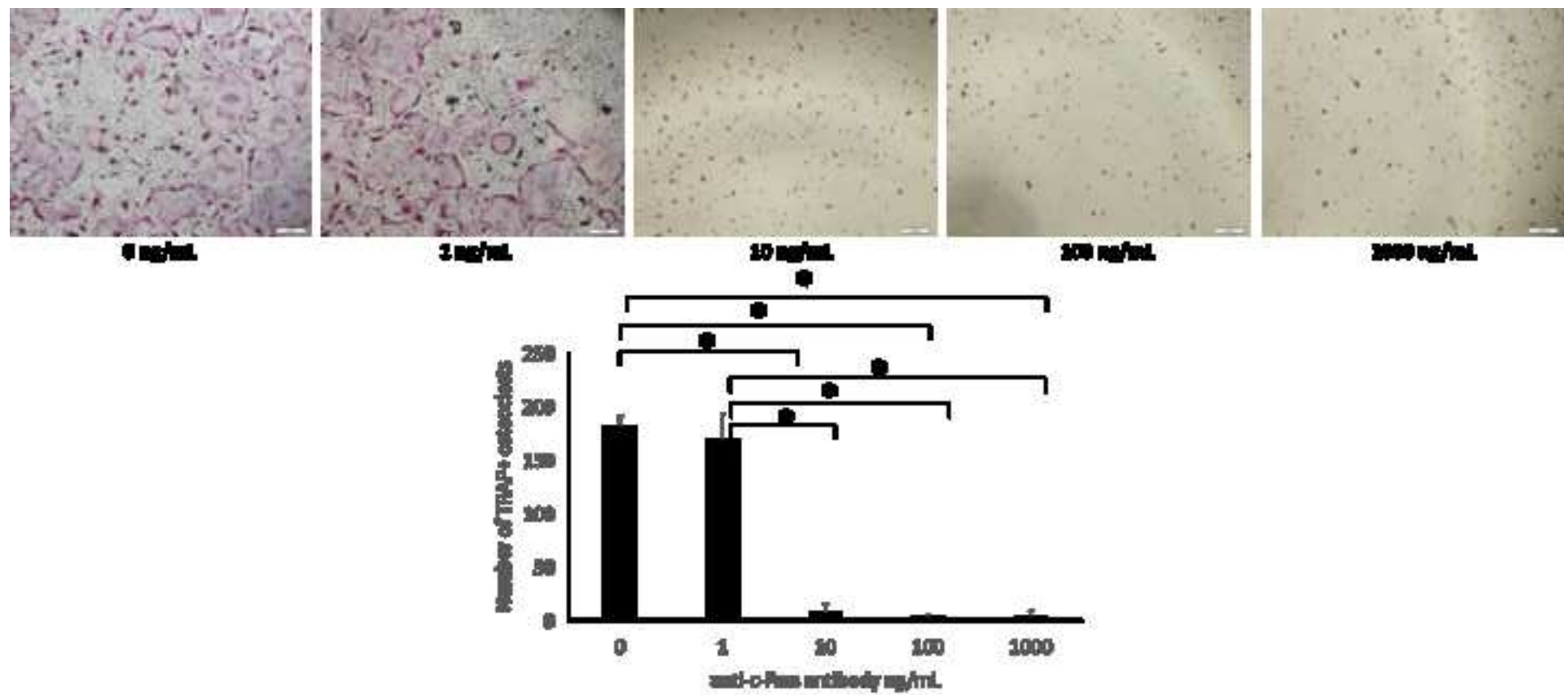
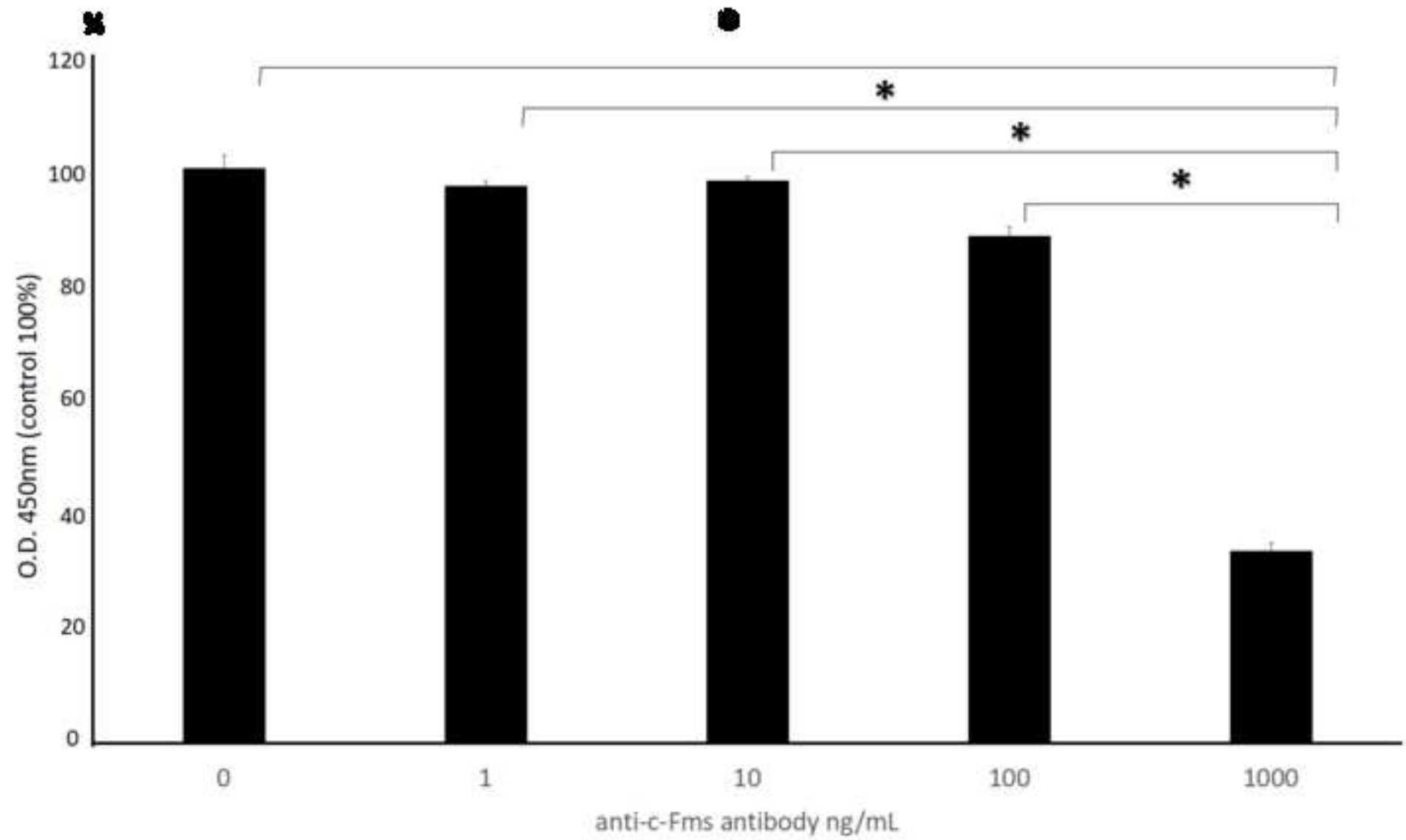


Figure 3



Name of Material/ Equipment		Company	Catalog Number
Anti-c-Fms antibody			
TNF- α			
RANKL		PEPROTECH	315-11
M-CSF			
α -MEM		Wako	
Fetal Bovine Serum		Biowest	s1820-500
Culture dish		Corning	
96-well plate		Thermofischer Scientific	
Cell counting kit-8		Dojindo, Kumamoto, Japan	
Microplate reader		Sunrise REMOTE; Tekan Japan, Kawasaki, Japan	
Cell strainer		Corning	
Centrifuge tube		Corning	
Triton X-100		Wako	

Comments/Description

AFS98, a rat monoclonal, antimurine, c-Fms antibody (IgG2a)

Recombinant murine TNF- α prepared in our laboratory. TNF- α cDNA fragment cloned by RT-PCR and cloned into a pGEX-6P-I (Amersham Biosciences, Piscataway, NJ) to generate a GST-fusion protein. GST-TNF- α was expressed in Escherichia coli BL21 cells (Stratagene, La Jolla, CA). The cells were lysed under nondenaturing conditions and GST-TNF- α was purified over a glutathione-Sepharose column. GST was cleaved off by PreScission Protease (Amersham Biosciences) by manufacturer's directions and was removed by a glutathione-Sepharose column

Recombinant Murine sRANK Ligand, Source: E.coli

Recombinant human M-CSF. 1/10 vol of CMG14–12 cell line culture supernatant at 5×10^6 cells in a 10-cm suspension culture dish.

with L-Glutamine and phenol red

Fetal Bovine Serum French Origin

100 mm x 20 mm style dish

Nunc Delta surface

40 μ m Nylon

50 mL CentriStar cap

Polyoxyethylene (10) Octyphenyl ether



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CORRESPONDING AUTHOR

Name:	Hideki Kitaura	
Department:	Division of Orthodontics and Dentofacial Orthopedics, Department of Translational Medicine	
Institution:	Tohoku University Graduate School of Dentistry, Sendai, Japan	
Title:	Associate Professor Ph.D. D.D.S	
Signature:		Date: 9/12/2018

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27 November 2018

Dear Editor of Journal of Visualized Experiments:

Thank you very much for your decision letter, dated November 23, 2018, regarding manuscript, JoVE59089 "Effect of anti-c-fms antibody on osteoclast formation and proliferation of osteoclast precursor *in vitro*". The following are our answers to the editor's comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

We reviewed the manuscript and corrected any grammar or spelling issues.

2. The Summary is over the 50 word limit.

We adjusted the summary to the 50 word limit.

3. Please use h, min, s for time units.

We changed the text as necessary.

4. Step 1.2: Please write this step in the imperative tense. Please specify the dose of isoflurane. Is anesthesia or euthanasia performed? Please specify.

We made necessary changes and included isoflurane dose.

5. 3.1: How much M-CSF is added? Is 100ng/mL the concentration of M-CSF? Or does it mean adding 100 ng of M-CSF per 1 mL of cell suspension?

We made the necessary changes to the text and specified the doses and amounts.

The final concentration of M-CSF is 100 ng/mL of culture medium.

6. 3.7: How much M-CSF is added?

We added the amount of M-CSF in the text.

7. 4.2: How much RANKL or TNF- α is added?

We added the amount of RANKL and TNF- α in the text.

8. 6.1: How much M-CSF is added?

We added the amount of M-CSF in the text.