Available online at www.sciencedirect.com

ScienceDirect

RANKL pretreatment plays an important role in the differentiation of pit-forming osteoclasts induced by TNF-α on murine bone marrow macrophages

Yasunori Yamashita, Takashi Ukai [*], Hirotaka Nakamura, Yasunori Yoshinaga, Hiroki Kobayashi, Yuzo Takamori, Satoshi Noguchi, Atsutoshi Yoshimura, Yoshitaka Hara

Department of Periodontology, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki University, Nagasaki, Japan

ARTICLE INFO

ABSTRACT

Background: Osteoclasts differentiated from bone marrow macrophages (BMMs) induced by TNF-α alone do not have resorbing activity. When BMMs are stimulated with receptor activator of NF-κB ligand (RANKL) before TNF-α stimulation, pit-forming osteoclasts are differentiated. However, the details of the effect of RANKL pretreatment on the pit-forming osteoclast differentiation by TNF-α have not been established. The aim of this study is to examine the condition of RANKL pretreatment for differentiation of pit-forming osteoclasts induced by TNF-α.

Murine BMMs were stimulated with various concentrations of RANKL for 24 h in the presence of M-CSF, then the medium was changed and TNF-α was added. Osteoclasts and pits formation were examined. Osteoprotegerin (OPG), decoy receptor of RANKL, was added to the culture to examine the necessity of co-existing RANKL with TNF-α on the formation of pit-forming osteoclasts. To investigate the influence of RANKL of sufficient concentration as pretreatment for pit-forming osteoclast formation by TNF-α, dose- and time-dependent changes of osteoclast formation were checked.

Results: The pit formation by osteoclasts in response to TNF-α required 10 ng/mL RANKL pretreatment. Stimulation with this concentration of RANKL led to the differentiation of mature osteoclasts in the 72 h culture. The pit formation was not inhibited by the OPG.

Conclusion: These results suggested that the concentration of RANKL pretreatment, which also alone can differentiate BMMs into osteoclasts, may be important in the differentiation of pit-forming osteoclasts by TNF-α. In addition, the effects of TNF-α after RANKL treatment might be independent of RANKL.

* *Corresponding author at*: Department of Periodontology, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki University, 1-7-1 Sakamoto, Nagasaki 852-8588, Japan. Tel.: +81 95 819 7683; fax: +81 95 819 7684.
    E-mail address: ukai@nagasaki-u.ac.jp (T. Ukai).

1. Introduction

Tumour necrosis factor-α (TNF-α) is reported to accelerate bone resorption in inflammatory bone diseases such as periodontitis and rheumatoid arthritis.[1,2] Osteoclasts are derived from monocyte-macrophage progenitor cells; they play a central role in bone resorption.[3,4] The monocyte-macrophage progenitor cells differentiate into osteoclast precursors when they are stimulated with macrophage-colony stimulating factor (M-CSF) and receptor activator of NF-κB ligand (RANKL).[5,6] With further RANKL stimulation, the osteoclast precursors become tartrate-resistant acid phosphatase (TRAP)-positive mononuclear cells.[7] Osteoclasts are generated by the fusion of mononuclear TRAP cells.[8] Activated mature osteoclasts will develop resorption activity.[9] TNF-α is reported to promote the osteoclastogenesis induced by RANKL.[10]

In general, TNF-α induces osteoclast precursor cells to become osteoclasts in the absence of RANKL. However, these osteoclasts induced by TNF-α do not have resorbing activity.[11,12] Although some studies have suggested that TNF-α induces the differentiation of pit-forming osteoclasts, their findings indicate that osteoclasts induced by TNF-α have much lower resorption activity than RANKL-induced osteoclasts.[13] Osteoclast formation and the activity of resorption are important to the promotion of bone destruction. With RANKL-pretreatment or with RANKL co-stimulation, osteoclast formation induced by TNF-α is accelerated.[10] In addition, it was reported that TNF-α coexisting with RANKL induces osteoclasts with resorption activity.[14] Thus, it is important to further elucidate the conditions in which osteoclast formation accelerates and the resorption activities produces in osteoclasts by TNF-α stimulation, as this information may have important therapeutic implications for the control of inflammatory bone diseases. However, the details of the effects of RANKL-pretreatment on the differentiation of pit-forming osteoclasts induced by TNF-α have not been established.

Osteoclasts can be differentiated from bone marrow macrophages (BMMs). In generally, 72 h stimulation of RANKL induces differentiation of mature osteoclast from BMMs.[15,16] Many studies reported that only 24 h RANKL treatment had an effect on the condition of BMMs. Osteoclast-related kinase increased in BMMs within 24 h after RANKL-stimulation.[17] Mizoguchi et al. reported that BMMs were 'cell cycle-arrested quiescent osteoclast precursors' and 'lineage-committed osteoclast precursors' after the cells were stimulated for 24 h with RANKL.[16,18] TNF-α stimulation after RANKL pretreatment promoted more osteoclast formation from BMMs compared to before RANKL treatment and simultaneous RANKL treatment.[10,19] Moreover, the acceleration was more greatly increased by 24 h than by 48 h RANKL pretreatment.[20] It was reported that in BMMs pre-cultured with a high concentration of RANKL, TNF-α alone could induce osteoclast formation with resorption activity in the absence of RANKL.[21] That study showed that RANKL pretreatment to BMMs could differentiate osteoclasts with resorbing activity by TNF-α without RANKL. However, the study used only high concentrations of RANKL, and the optimal RANKL pretreatment conditions to produce osteoclasts with resorbing activity

induced by TNF-α are thus not clear. In this study, we examined the number of osteoclasts and the resorption activity when BMMs were pretreated with various concentrations of RANKL for 24 h before TNF-α stimulation. We also evaluated the influence of these concentration of RANKL in osteoclastogenesis. Our findings help elucidate the mechanisms underlying the osteoclast formation induced by TNF-α, which may contribute to the treatment of bone diseases.

2. Material and methods

2.1. Animals

Five- to 7-week-old male CB17/Icr+/+ Jcl mice were purchased from Nihon Clea (Tokyo) and maintained in specific pathogenic-free conditions at the Biomedical Research Center for Frontier Life Sciences, Nagasaki University. The animal care and experiments proceeded according to the Guidelines for Animal Experimentation of Nagasaki University and with the approval of the Institutional Animal Care and Use Committee.

2.2. Reagents

Recombinant mouse M-CSF, recombinant mouse soluble RANKL, recombinant mouse TNF-α, and recombinant mouse osteoprotegerin (OPG) were purchased from R&D Systems (Minneapolis, MN, USA). The tartrate-resistant acid phosphatase (TRAP) kit for staining of osteoclasts was purchased from Sigma (St. Louis, MO). The Bone Resorption Assay kit 48 was purchased from PG Research (Tokyo). The TRACP & ALP Assay kit for detecting of TRAP activity in the culture was purchased from Takara Bio (Tokyo).

2.3. Preparation of bone marrow macrophages

Murine bone marrow macrophages (BMMs) were obtained as described.[22,23] Briefly, bone marrow cells were collected from mouse tibiae and femurs, and red cells were lysed. The cells were then plated at 1.5×10^7 to 2×10^7 cells in 10-cm dishes with 10 mL of alpha-minimal essential medium (αMEM) supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin, and cultured in the presence of 5 ng/mL M-CSF for 12 h. Non-adherent cells were collected and cultured with 30 ng/mL of M-CSF for 36 h. After the removal of non-adherent cells, the adherent cells were collected and used as BMMs. In all subsequent experiments, BMMs were cultured in the medium containing 30 ng/mL of M-CSF.

2.4. Osteoclast formation assays

In order to examine the influence of RANKL pretreatment on osteoclast formation stimulated with TNF-α, after BMMs (2×10^4/well) were pretreated at the designated concentration of RANKL for 24 h, the medium was changed in order to remove RANKL and the cells were cultured with/without TNF-α for various periods. In another experiment, 300 ng/mL of OPG was added with TNF-α. When the cultures were finished, the cells were stained for TRAP and the number of osteoclasts

was counted. To check effect of 300 ng/mL of OPG, BMMs (2.0×10^4/well) were cultured with 10 ng/mL of RANKL with/without OPG for 72 h in 96-well plates. To examine various concentration and period of RANKL stimulation for osteoclast formation and TRAP activity, BMMs (2.0×10^4/well) were cultured with various concentrations of RANKL for the designated period in 96-well plates. TRAP activity was measured and the cells were stained for TRAP.

2.5. TRAP staining

Cells were fixed with 4% paraformaldehyde and stained by using a TRAP kit (Sigma) for the identification of osteoclasts. TRAP-positive multinucleated cells with three or more nuclei were considered osteoclasts. The TRAP-positive cells and osteoclasts were counted under the microscope.

2.6. TRAP activity

TRAP activity was measured according to the protocol of the TRACP & ALP Assay kit (Takara Bio). Briefly, cells were solubilized by pipetting with phosphate-buffered saline (PBS) including 1% NP-40, added substrate solution and incubated at 37 °C for 60 min for the measurement of TRAP activity. The reaction was stopped with 0.5 N NaOH, and the absorbance at 405 nm was measured using a microplate reader (SH-1000 Lab, Corona, Japan).

2.7. Pit formation assay

BMMs (1×10^5/well) were seeded into a calcium phosphate (Ca-P)-coated 48-well plate (PG Research, Tokyo, Japan) used to measure the resorption activity of osteoclasts. Cells were cultured with M-CSF alone or with 10 ng/mL of TNF-α, or 10 ng/mL of RANKL. After the cells were cultured for 120 h, the pit formation status was examined. To confirm the status of osteoclast formation, cells in some well were stained for TRAP after 72 h culture. In another experiment, in order to examine the effect of RANKL pretreatment on the resorption activities of osteoclasts induced by TNF-α stimulation, after cells were stimulated with RANKL at the concentration of 0, 1 or 10 ng/mL for 24 h, the medium was changed and the cells were cultured with TNF-α with or without OPG for 96 h. To confirm the status of osteoclast formation, cells in some well were stained for TRAP after 48 h of TNF-α addition.

At the end of the culture, cells were removed with 5% sodium hypochlorite (NaClO), washed with distilled water, and dried perfectly. Pits were observed by light microscopy, and photographs were taken with a digital camera (Carl Zeiss Co., Ltd. Axio Cam HRC). The pit area per well was calculated from the photographs using Image J software (NIH, Bethesda, MD).

2.8. Statistics analysis

The statistical analyses were performed using STATMATE III software (ATMS, Tokyo). Differences among groups were assessed by a one-factor analysis of variance (ANOVA) and Tukey's test. p-Values <0.05 were considered significant.

3. Results

3.1. Osteoclast formation induced by TNF-α stimulation after RANKL pretreatment

After BMMs were stimulated with RANKL at various concentrations for 24 h, the medium was changed, TNF-α was added, and then we examined the time-dependent osteoclast formation. Almost all of the cells were TRAP-positive at 48 h after 1 or 10 ng/mL of RANKL pretreatment in the M-CSF-alone group. A few osteoclasts were observed in this group (Fig. 1A). However, there were few TRAP-positive cells following 100 pg/mL RANKL pretreatment (data not shown). In the group stimulated with 1 ng/mL TNF-α after RANKL pretreatment, only a few osteoclasts were observed (Fig. 1B). In the 48 h stimulation with 10 ng/mL TNF-α, on the other hand, the number of osteoclasts was significantly increased when BMMs were pretreated with RANKL at 100 pg/mL or more in comparison with not pretreatment. The increase was particularly higher when the 1 or 10 ng/mL RANKL pretreatment was used (Fig. 1C).

OPG was added together with TNF-α for the examination of the influence of RANKL during TNF-α stimulation. In the case of 10 ng/mL TNF-α stimulation after 10 ng/mL RANKL pretreatment, the number of osteoclasts cultured with 300 ng/mL OPG was not significantly changed compared to without OPG at 48 h (Fig. 2B). We confirmed that osteoclast formation stimulated with RANKL was completely inhibited by this concentration of OPG (Fig. 2A).

3.2. Pit formation assay

The pit formation assay was carried out using a 48-well plate coated on the bottom with Ca-P to evaluate the activity of resorption. This plate was coated with a synthetic carbonate apatite, similar to natural apatite, which can be used as an alternative to dentine discs. When BMMs were stimulated with M-CSF alone, there were no TRAP-positive cells or pits. Many osteoclasts were induced with 72 h RANKL stimulation. Pits were observed at 120 h after RANKL stimulation. TNF-α stimulation formed osteoclasts from BMMs, but they did not make pits (Fig. 3). These results were not inconsistent with previous reports.[11]

We next examined the influence of RANKL pretreatment on the resorption activities of osteoclasts induced by 10 ng/mL TNF-α. There were no pits after 96 h of TNF-α stimulation in the 1 ng/mL RANKL pretreatment, although many osteoclasts were observed after 48 h of the TNF-α stimulation (Fig. 4A). Because almost all cells seemed to be apoptotic at 96 h when the cells were stained with TRAP (data not shown), we considered that pits would be not detected if cells were cultured for longer periods. In contrast, in the 10 ng/mL RANKL pretreatment group, many osteoclasts were observed at 48 h and many pits were detected at 96 h after TNF-α stimulation (Fig. 4A). In this culture, the pit formation and area was not significantly changed compare to that without OPG, even when OPG was added together with TNF-α (Fig. 4A and B).
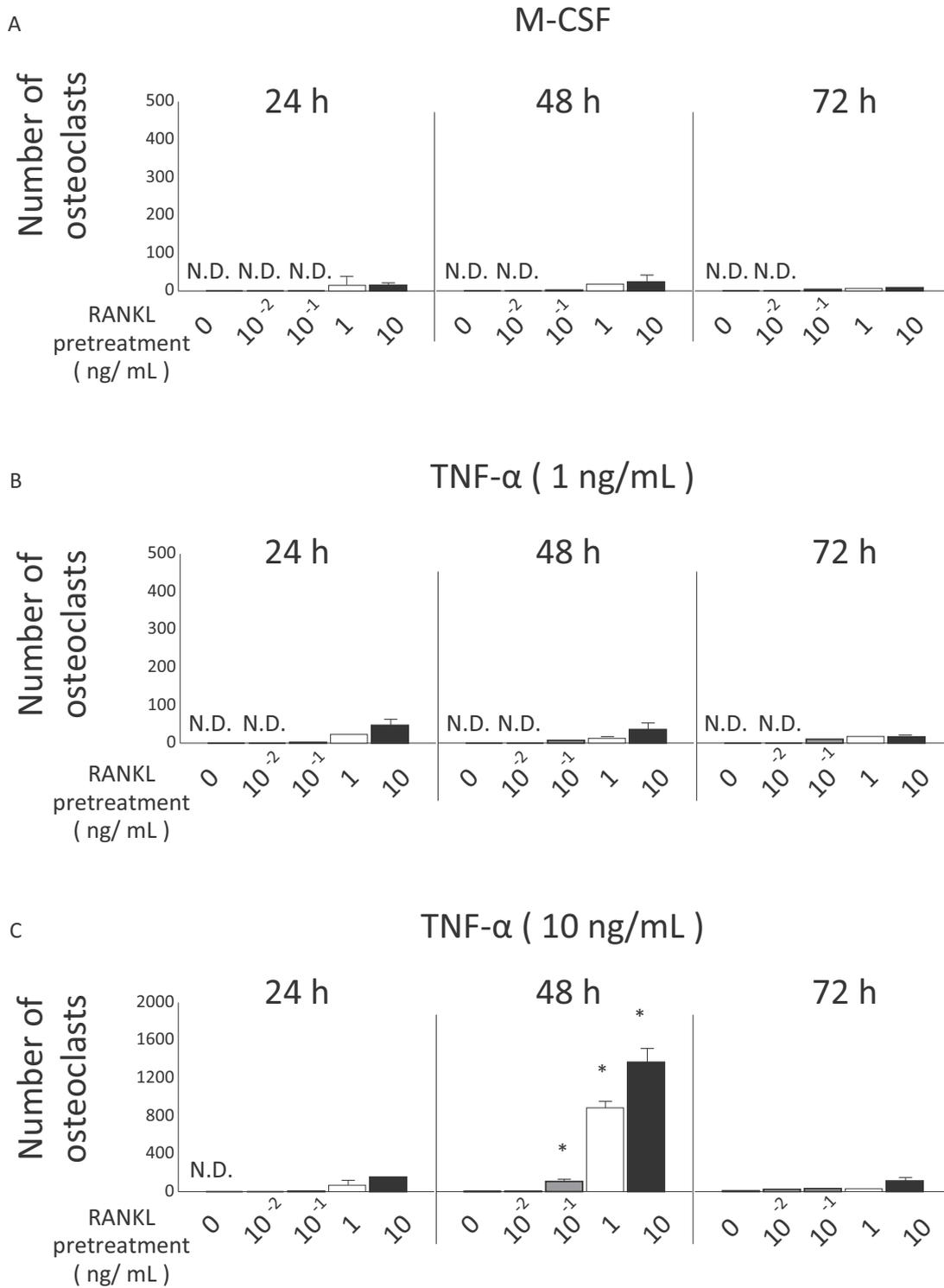
Fig. 1 – The number of osteoclasts induced by TNF-α after RANKL treatment. After mouse BMMs were treated with the designated concentration of RANKL for 24 h, they were cultured with TNF-α. After being stimulated with/without TNF-α for 24, 48 or 72 h in the presence of M-CSF, cells were stained for TRAP and the positive multinuclear cells were counted. M-CSF alone (A), and TNF-α (1 ng/mL) added (B), and TNF-α (10 ng/mL) added (C) after RANKL treatment. Bars: means ± SD. *$p < 0.001$ compared to not pretreated with RANKL. Data are representative of three independent experiments. N.D.: not detectable.
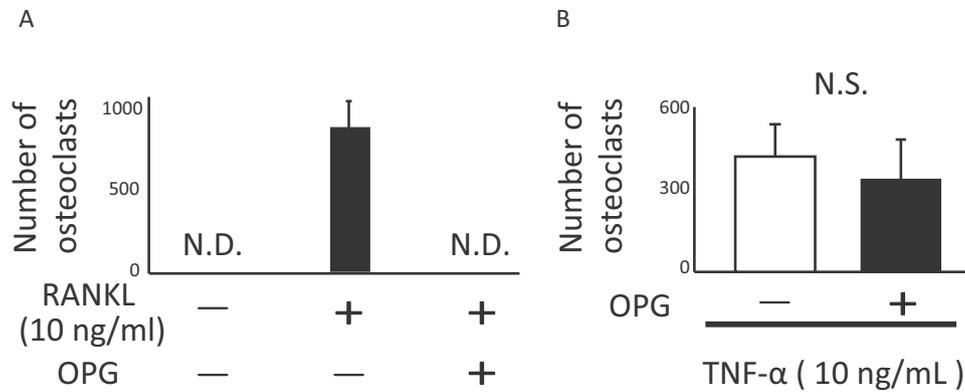
Fig. 2 – Effect of OPG on osteoclast formation induced by TNF-α after RANKL treatment. BMMs were treated with M-CSF and RANKL (10 ng/mL) with/without OPG (300 ng/mL) for 72 h (A). After BMMs were treated with 10 ng/mL of RANKL for 24 h, they were cultured with M-CSF and TNF-α (10 ng/mL) with/without OPG for 48 h (B). The cells were stained for TRAP, and the number of positive multinuclear cells was counted. Bars: means ± SD. Data are representative of three independent experiments. N.D.: not detectable. N.S.: not significant.

3.3. Time-dependent osteoclast formation induced by different of concentration of RANKL

Our present study indicated the importance of 10 ng/mL of RANKL pretreatment for TNF-α induced differentiation of pit-forming osteoclasts. Therefore, we checked the effect of this concentration of RANKL in osteoclastogenesis. In order to examine the difference of conditions of osteoclastogenesis we compared the time- and dose-dependent changes in TRAP activity and osteoclast formation from BMMs stimulated with 10 ng/mL of RANKL and other concentrations of RANKL. High TRAP activity was detected following stimulation with 1 and
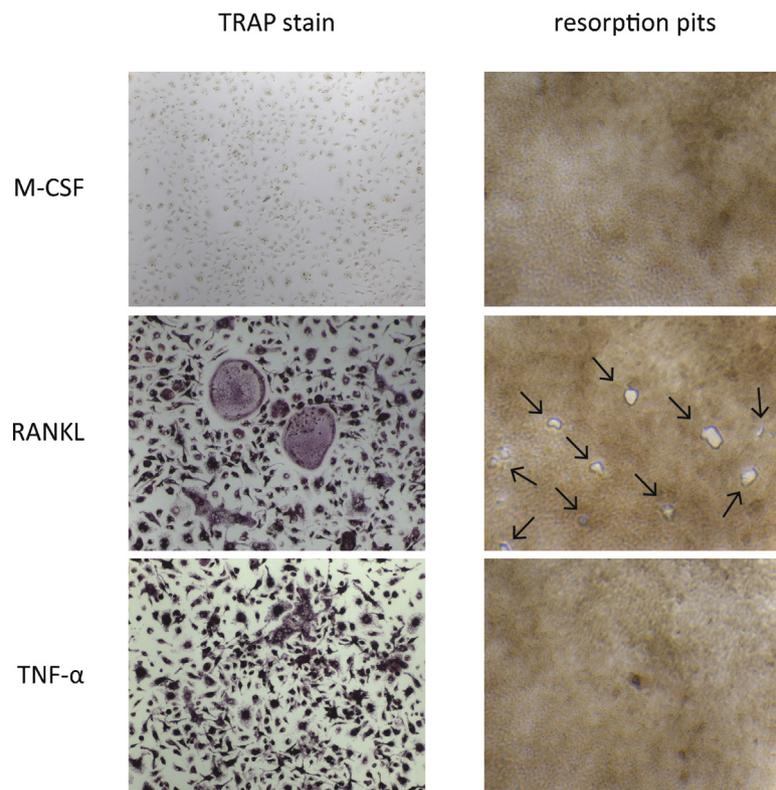
Fig. 3 – Pit formation induced by RANKL or TNF-α. BMMs were cultured with M-CSF alone, RANKL (10 ng/mL) or TNF-α (10 ng/ mL) in a calcium phosphate (Ca-P)-coated 48-well plate to measure the resorption activity of osteoclasts. After a 72-h culture, cells were stained for TRAP. For the examination of pit formation, cells were removed at 120 h and observed by microscopy. Arrowheads indicate pit-forming osteoclasts. Representative photographs of TRAP stain and resorption pits.
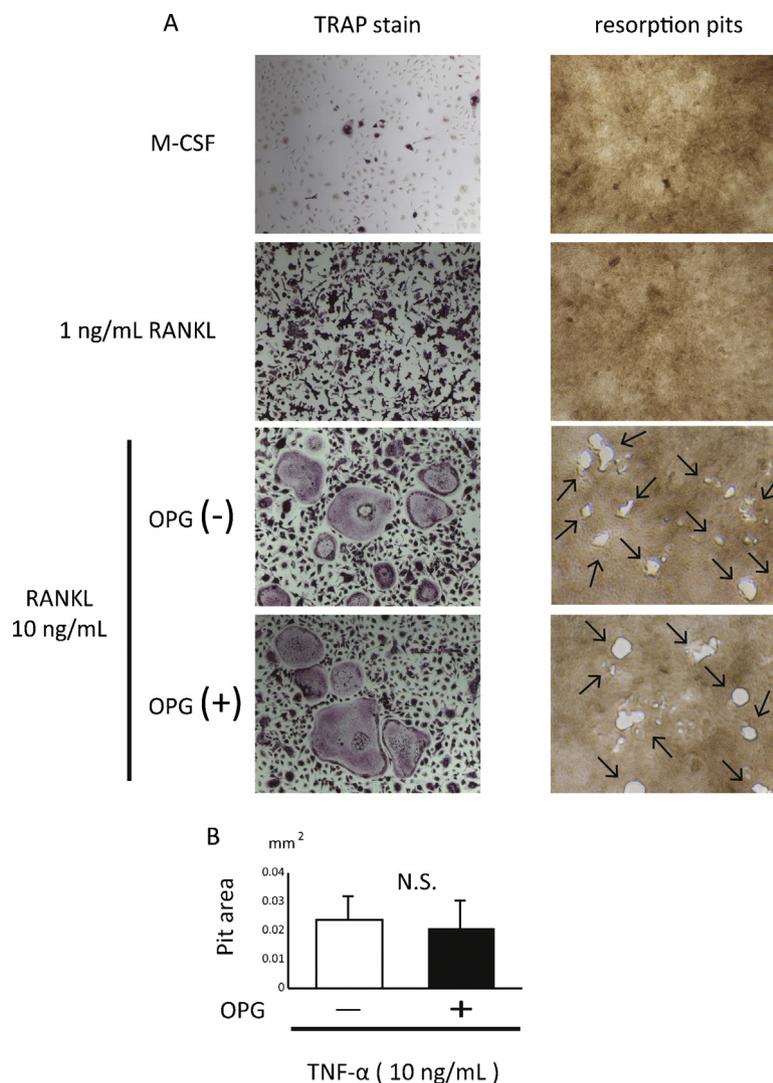
Fig. 4 – Pit formation induced by TNF-α after RANKL treatment. (A) BMMs were cultured with 10 ng/mL of TNF-α with/without OPG (300 ng/mL) after cells were treated with M-CSF (30 ng/mL) with/without RANKL (1 or 10 ng/mL) for 24 h in a Ca-P-coated 48-well plate. Cells were stained for TRAP after TNF-α stimulation for 48 h. In other wells, cells were removed and pit formation was examined after TNF-α stimulation for 96 h. Representative photographs of TRAP stain and resorption pits. (B) BMMs were cultured with 10 ng/mL of TNF-α with/without OPG for 96 h after cells were pretreated with 10 ng/mL of RANKL for 24 h in a Ca-P-coated 48-well plate. After cells were cultured, they were removed and the pit area was measured with image analyzing software. Arrows indicate resorption pits. Bars: means ± SD. Data are representative of three independent experiments. N.S.: not significant.

10 ng/mL of RANKL. However, slight activity of TRAP was observed following pretreatment with 100 pg/mL of RANKL, and little activity was observed with 10 pg/mL of RANKL (Fig. 5A). Many TRAP-positive cells were observed at 72 h and later when 1 or 10 ng/mL RANKL was used. A few TRAP positive cells were observed when 100 pg/mL of RANKL was used, but the positive cells were hardly detected following 10 pg/mL of RANKL (Fig. 5B). These data revealed that the TRAP activity could be induced in BMMs if the cells were stimulated with more than 1 ng/mL of RANKL. Many osteoclasts were differentiated following 10 ng/mL RANKL stimulation for 72 or 96 h, whereas few such cells were observed when 1 ng/mL RANKL was used (Fig. 5A).

4. Discussion

Our findings demonstrated that osteoclast formation by TNF-α was accelerated in the absence of RANKL when BMMs were pretreated for 24 h with the concentration of RANKL that could induce TRAP activity. It was also shown that pit-forming osteoclasts were differentiated with TNF-α when cells were pretreated for 24 h with a concentration of RANKL that can produce pit-forming osteoclasts within 72 h. However, when a concentration of RANKL pretreatment that is insufficient for the induction of TRAP activity and osteoclasts was used, neither the increase of osteoclasts nor the activity of resorption was induced.
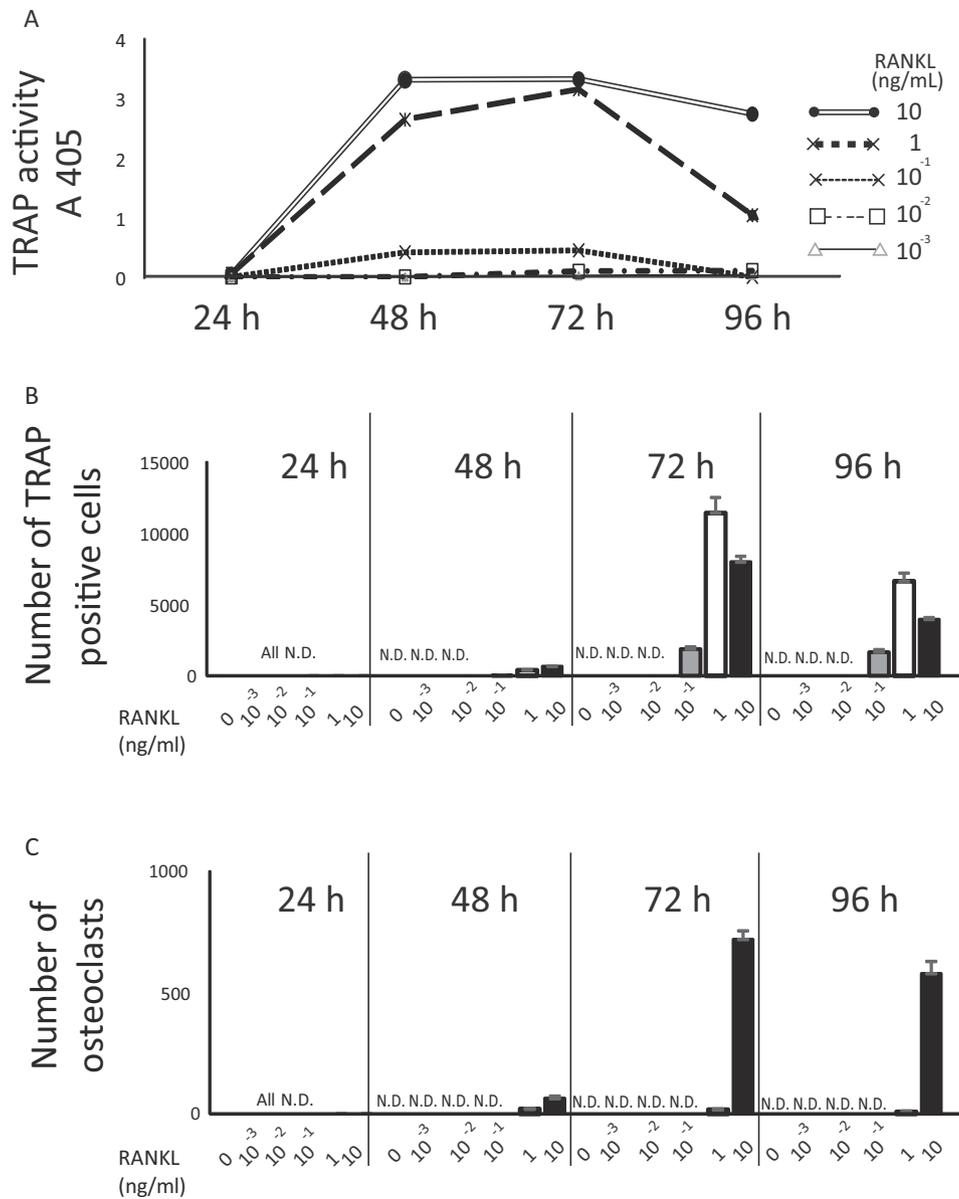
Fig. 5 – TRAP activity and osteoclast formation with RANKL stimulation. BMMs were cultured with the designated concentration of RANKL. At 24, 48, 72 and 96 h, TRAP activity was measured using a TRACP and ALP kit (A). The numbers of TRAP-positive cells (B) and TRAP-positive multinuclear cells (C) were counted following staining for TRAP. Bars: means ± SD. N.D.: not significant. Data are representative of three independent experiments.

Abbas et al. reported that osteoclast formation was promoted by 10 ng/mL TNF-α after BMMs were stimulated with 20 ng/mL of RANKL for 3 or 4 d.[24] Jules et al. noted that TNF-α alone could not directly induce BMMs to become osteoclasts, but when BMMs were stimulated with 100 ng/mL RANKL overnight, TNF-α stimulation could induce osteoclasts with resorption activity in the absence of RANKL.[21] These studies showed importance of RANKL pretreatment on acceleration of osteoclast formation and differentiation of pi-forming osteoclast induced by TNF-α. However, it is unclear what the RANKL pretreatment condition was. No studies have focused on the conditions of RANKL pretreatment, to our knowledge. In the present investigation, we found that

osteoclast formation by TNF-α can be promoted without co-existing RANKL -existence when BMMs are pre-stimulated for 24 h with a concentration of RANKL that can induce TRAP activity within 72 h. With such a concentration of RANKL pretreatment for 24 h, only a few osteoclasts were formed at 48 h after the medium was changed. In this condition, most of the other cells were TRAP-positive cells (data not shown). Hotokezaka et al. showed that TNF-α could promote osteoclast formation via the fusion of preosteoclasts.[25] In the present study, TNF-α may promote osteoclast formation by accelerating the fusion of TRAP-positive cells differentiated under the influence of only RANKL pretreatment. However, the details of the mechanism underlying the fusion of preosteoclasts are not

well understood. Further studies are necessary to fully understand these events.

Our present findings showed that pit-forming osteoclasts were differentiated by TNF-α without RANKL when cells were pretreated for 24 h with a concentration of RANKL that induces osteoclasts, although TNF-α stimulation alone did not induce resorption activity as occurred in the Kobayashi et al. study.[11] Osteoclasts with resorption activity are differentiated under stimulation with both RANKL and TNF-α.[21] It was reported that TNF-α stimulation of BMMs can promote the production of RANKL.[26] In the present study there were no significant differences in the number of pit-forming osteoclasts formed by TNF-α with or without OPG, which is a decoy receptor of RANKL. This result shows that co-exist of RANKL with TNF-α is not necessary for the induction of resorption activity. In addition, Fuller et al. reported that TNF-α promoted resorption activity in osteoclasts derived from rabbits ex vivo and in osteoclasts differentiated from BMMs stimulated with RANKL in vitro.[14] Therefore, the possibility that TNF-α promoted resorption activity of osteoclasts differentiated with RANKL pretreatment alone but not newly differentiated osteoclasts by TNF-α is considered. However, because in our current findings we observed many more pits than the number of osteoclasts differentiated with RANKL pretreatment alone in the present study, we suspect that there is another mechanism that induces the resorption activity.

Several research groups reported that osteoclasts induced by TNF-α without RANKL showed resorption activity in the presence of interleukin (IL)-1.[11,12] The signals from RANK (the receptor of RANKL) or IL-1 receptors were reported to activate the pathway of tumour necrosis factor receptor-associated factor 6 (TRAF6).[12,27] In TRAF6-deficient mice, osteoclasts were differentiated but did not have resorption activity, and the mice developed osteopetrosis.[27] Other studies revealed that the TRAF6 activity is important for the differentiation of pit-forming osteoclasts stimulated with RANKL.[28,29] TNF-α has been reported to activate TRAF2 and TRAF5 but not TRAF6,[30,31] and thus osteoclasts induced by TNF-α may not have resorbing activity. However, it was reported that pit-forming osteoclasts can be differentiated from BMMs with highly over-expressed TRAF2.[30] In one study, pit-forming osteoclasts could be formed with a high concentration of TNF-α (100 ng/mL).[14] These findings show that it is possible to differentiate pit-forming osteoclasts if the TRAF2 pathway is efficiently activated. But even if the TRAF2 pathway cannot be sufficiently activated by TNF-α stimulation alone for the induction of resorbing activity, we suspect that pit-forming osteoclasts may be differentiated if RANKL pretreatment that can activate the TRAF6 pathway exists as in the present study.

Our results confirmed that 24 h RANKL pretreatment affected osteoclast formation by TNF-α. Using an in vitro experimental system in which BMMs differentiate into osteoclasts in the presence of RANKL for 3 d (similar to our study), Mizoguchi et al. reported that BMMs treated with RANKL for 24 h became cell cycle-arrested quiescent and TRAP-negative osteoclast precursor cells.[16] They also showed that the osteoclast formation induced by RANKL was completely inhibited under the co-existence of hydroxyurea to block DNA synthesis. However, osteoclasts were differentiated even when hydroxyurea was added after 24 h RANKL

stimulation. These studies may indicate that the important part of differentiation to osteoclasts is induced during the 24 h after RANKL stimulation, even though the above-mentioned osteoclast precursor cells were TRAP-negative. In fact, in a study conducted to analyze the mRNA of the gene expression during osteoclast differentiation, the mRNA expression of TRAF6 and nuclear factor of activated T-cells, cytoplasmic 1 (NFATc1) as one of the downstream signalling molecules of TRF6 increased at 24 h after RANKL stimulation.[15] By the blocking of the binding to TRAF6 by selective TRAF6 decoy peptides at 6 h or 12 h after RANKL stimulation, osteoclast formation was inhibited.[32] These findings suggest that TRAF6 will be activated within 24 h after RANKL stimulation and that osteoclast differentiation will be promoted. Therefore, a sufficient concentration of RANKL pre-stimulation may be related to the pit-forming osteoclast formation induced by TNF-α stimulation. Mochizuki et al. reported that BMMs could not differentiate into dendritic cells after 24 h RANKL pretreatment, although BMMs are known to have the capacity to differentiate into not only osteoclasts but also dendritic cells. These cells were osteoclast committed cells in which the mRNA expression of NFATc1 as well as those of cathepsin K, αvβ3 integrin, RANK, and TRAP were already increased.[33]

At each stage in which hematopoietic stem cells differentiate into osteoclasts, the expressions of CD11b as a pan-surface marker of myeloid lineage cells and F4/80 as a mature macrophage surface marker are reported to be lower.[6,34] Although data is not shown in this study, we analyzed the expression of CD11b and F4/80 by flow cytometry when BMMs were stimulated with or without RANKL 10 ng/mL for 24 h in the presence of M-CSF. As the results, the expression of CD11b slightly decreased, and the expression of F4/80 significantly dropped. Analyzing changes of cell surface markers using comprehensive methods may be helpful for identification of a RANKL pretreatment condition that induces pit-forming osteoclasts by TNF-α.

Kostenuik et al. reported that denosumab, a fully human monoclonal antibody to RANKL for the treatment of bone diseases, inhibited bone resorption and increased bone mineral density in patients with osteoporosis or arthritis.[35] However, Stolina et al. reported that OPG had no effect on synovitis.[36] It seems reasonable to suppose that the difference in these results is due to differences in the inhibitory mechanisms, application timing, processing periods and so on. Many biologics such as anti-RANKL and anti-TNF-α have been used in treatments for rheumatoid arthritis, osteoporosis, and giant tumour cells.[37–39] These therapies seem to affect periodontal tissues. It is thus important to further clarify the mechanisms of the actions of TNF-α and RANKL against inflammatory bone diseases. Evaluating the effect of RANKL pre-stimulation for bone resorption accelerated by TNF-α will be especially useful for the effective inhibitory treatment of bone resorption when local drug therapy of anti-RANKL progresses.

In conclusion, the concentration of RANKL pretreatment, which as well alone can differentiate BMMs into osteoclasts, may also be important in the generation of pit-forming osteoclasts by TNF-α. In addition, the effects of TNF-α after RANKL pretreatment might be independent of RANKL.

REFERENCES

1. Nilsson M, Kopp S. Gingivitis and periodontitis are related to repeated high levels of circulating tumor necrosis factor-alpha in patients with rheumatoid arthritis. *J Periodontal* 2008;**79**(9):1689–96.
2. Boyce BF, Li P, Yao Z, Zhang Q, Badell IR, Schwarz EM, et al. TNF-alpha and pathologic bone resorption. *Keio J Med* 2005;**54**(3):127–31.
3. Vaananen HK, Zhao H, Mulari M, Halleen JM. The cell biology of osteoclast function. *J Cell Sci* 2000;**113**(Pt 3):377–81.
4. Teitelbaum SL. Bone resorption by osteoclasts. *Science* 2000;**289**(5484):1504–8.
5. Boyle WJ, Simonet WS, Lacey DL. Osteoclast differentiation and activation. *Nature* 2003;**423**(6937):337–42.
6. Xing L, Schwarz EM, Boyce BF. Osteoclast precursors, RANKL/RANK, and immunology. *Immunol Rev* 2005;**208**:19–29.
7. Jimi E, Akiyama S, Tsurukai T, Okahashi N, Kobayashi K, Udagawa N, et al. Osteoclast differentiation factor acts as a multifunctional regulator in murine osteoclast differentiation and function. *J Immunol* 1999;**163**(1):434–42.
8. Yagi M, Miyamoto T, Sawatani Y, Iwamoto K, Hosogane N, Fujita N, et al. DC-STAMP is essential for cell-cell fusion in osteoclasts and foreign body giant cells. *J Exp Med* 2005;**202**(3):345–51.
9. Miyamoto T. Regulators of osteoclast differentiation and cell–cell fusion. *Keio J Med* 2011;**60**(4):101–5.
10. Lam J, Takeshita S, Barker JE, Kanagawa O, Ross FP, Teitelbaum SL. TNF-alpha induces osteoclastogenesis by direct stimulation of macrophages exposed to permissive levels of RANK ligand. *J Clin Invest* 2000;**106**(12):1481–8.
11. Kobayashi K, Takahashi N, Jimi E, Udagawa N, Takami M, Kotake S, et al. Tumor necrosis factor alpha stimulates osteoclast differentiation by a mechanism independent of the ODF/RANKL–RANK interaction. *J Exp Med* 2000;**191**(2):275–86.
12. Kim N, Kadono Y, Takami M, Lee J, Lee SH, Okada F, et al. Osteoclast differentiation independent of the TRANCE–RANK–TRAF6 axis. *J Exp Med* 2005;**202**(5):589–95.
13. Azuma Y, Kaji K, Katogi R, Takeshita S, Kudo A. Tumor necrosis factor-alpha induces differentiation of and bone resorption by osteoclasts. *J Biol Chem* 2000;**275**(7):4858–64.
14. Fuller K, Murphy C, Kirstein B, Fox SW, Chambers TJ. TNFalpha potently activates osteoclasts, through a direct action independent of and strongly synergistic with RANKL. *Endocrinology* 2002;**143**(3):1108–18.
15. Takayanagi H, Kim S, Koga T, Nishina H, Isshiki M, Yoshida H, et al. Induction and activation of the transcription factor NFATc1 (NFAT2) integrate RANKL signaling in terminal differentiation of osteoclasts. *Dev Cell* 2002;**3**(6):889–901.
16. Mizoguchi T, Muto A, Udagawa N, Arai A, Yamashita T, Hosoya A, et al. Identification of cell cycle-arrested quiescent osteoclast precursors in vivo. *J Cell Biol* 2009;**184**(4):541–54.
17. Huang H, Ryu J, Ha J, Chang EJ, Kim HJ, Kim HM, et al. Osteoclast differentiation requires TAK1 and MKK6 for NFATc1 induction and NF-kappaB transactivation by RANKL. *Cell Death Differ* 2006;**13**(11):1879–91.
18. Muto A, Mizoguchi T, Udagawa N, Ito S, Kawahara I, Abiko Y, et al. Lineage-committed osteoclast precursors circulate in blood and settle down into bone. *J Bone Miner Res* 2011;**26**(12):2978–90.
19. Zhang YH, Heulsmann A, Tondravi MM, Mukherjee A, Abu-Amer Y. Tumor necrosis factor-alpha (TNF) stimulates RANKL-induced osteoclastogenesis via coupling of TNF type 1 receptor and RANK signaling pathways. *J Biol Chem* 2001;**276**(1):563–8.
20. Ochi S, Shinohara M, Sato K, Gober HJ, Koga T, Kodama T, et al. Pathological role of osteoclast costimulation in arthritis-induced bone loss. *Proc Natl Acad Sci U S A* 2007;**104**(27):11394–9.
21. Jules J, Shi Z, Liu J, Xu D, Wang S, Feng X. Receptor activator of NF-{kappa}B (RANK) cytoplasmic IVVY535-538 motif plays an essential role in tumor necrosis factor-{alpha} (TNF)-mediated osteoclastogenesis. *J Biol Chem* 2010;**285**(48):37427–35.
22. Takami M, Kim N, Rho J, Choi Y. Stimulation by toll-like receptors inhibits osteoclast differentiation. *J Immunol* 2002;**169**(3):1516–23.
23. Ozaki Y, Ukai T, Yamaguchi M, Yokoyama M, Haro ER, Yoshimoto M, et al. Locally administered T cells from mice immunized with lipopolysaccharide (LPS) accelerate LPS-induced bone resorption. *Bone* 2009;**44**(6):1169–76.
24. Abbas S, Zhang YH, Clohisy JC, Abu-Amer Y. Tumor necrosis factor-alpha inhibits pre-osteoblast differentiation through its type-1 receptor. *Cytokine* 2003;**22**(1–2):33–41.
25. Hotokezaka H, Sakai E, Ohara N, Hotokezaka Y, Gonzales C, Matsuo K, et al. Molecular analysis of RANKL-independent cell fusion of osteoclast-like cells induced by TNF-alpha, lipopolysaccharide, or peptidoglycan. *J Cell Biochem* 2007;**101**(1):122–34.
26. Kitaura H, Sands MS, Aya K, Zhou P, Hirayama T, Uthgenannt B, et al. Marrow stromal cells and osteoclast precursors differentially contribute to TNF-alpha-induced osteoclastogenesis in vivo. *J Immunol* 2004;**173**(8):4838–46.
27. Lomaga MA, Yeh WC, Sarosi I, Duncan GS, Furlonger C, Ho A, et al. TRAF6 deficiency results in osteopetrosis and defective interleukin-1, CD40, and LPS signaling. *Genes Dev* 1999;**13**(8):1015–24.
28. Yen ML, Hsu PN, Liao HJ, Lee BH, Tsai HF. TRAF-6 dependent signaling pathway is essential for TNF-related apoptosis-inducing ligand (TRAIL) induces osteoclast differentiation. *PLoS ONE* 2012;**7**(6):e38048.
29. Kadono Y, Okada F, Perchonock C, Jang HD, Lee SY, Kim N, et al. Strength of TRAF6 signalling determines osteoclastogenesis. *EMBO Rep* 2005;**6**(2):171–6.

30. Kanazawa K, Kudo A. TRAF2 is essential for TNF-alpha-induced osteoclastogenesis. *J Bone Miner Res* 2005;**20**(5):840–7.
31. Kanazawa K, Azuma Y, Nakano H, Kudo A. TRAF5 functions in both RANKL- and TNFalpha-induced osteoclastogenesis. *J Bone Miner Res* 2003;**18**(3):443–50.
32. Poblenz AT, Jacoby JJ, Singh S, Darnay BG. Inhibition of RANKL-mediated osteoclast differentiation by selective TRAF6 decoy peptides. *Biochem Biophys Res Commun* 2007;**359**(3):510–5.
33. Mochizuki A, Takami M, Kawawa T, Suzumoto R, Sasaki T, Shiba A, et al. Identification and characterization of the precursors committed to osteoclasts induced by TNF-related activation-induced cytokine/receptor activator of NF-kappa B ligand. *J Immunol* 2006;**177**(7):4360–8.
34. Takeshita S, Kaji K, Kudo A. Identification and characterization of the new osteoclast progenitor with macrophage phenotypes being able to differentiate into mature osteoclasts. *J Bone Miner Res* 2000;**15**(8):1477–88.
35. Kostenuik PJ, Nguyen HQ, McCabe J, Warmington KS, Kurahara C, Sun N, et al. Denosumab, a fully human monoclonal antibody to RANKL, inhibits bone resorption and increases BMD in knock-in mice that express chimeric (murine/human) RANKL. *J Bone Miner Res* 2009;**24**(2):182–95.
36. Stolina M, Schett G, Dwyer D, Vonderfecht S, Middleton S, Duryea D, et al. RANKL inhibition by osteoprotegerin prevents bone loss without affecting local or systemic inflammation parameters in two rat arthritis models: comparison with anti-TNFalpha or anti-IL-1 therapies. *Arthritis Res Ther* 2009;**11**(6):R187.
37. Westhovens R, Yocum D, Han J, Berman A, Strusberg I, Geusens P, et al. The safety of infliximab, combined with background treatments, among patients with rheumatoid arthritis and various comorbidities: a large, randomized, placebo-controlled trial. *Arthritis Rheum* 2006;**54**(4):1075–86.
38. Miyasaka N, Investigators CS. Clinical investigation in highly disease-affected rheumatoid arthritis patients in Japan with adalimumab applying standard and general evaluation: the CHANGE study. *Mod Rheumatol* 2008;**18**(3):252–62.
39. Hu MI, Glezerman I, Leboulleux S, Insogna K, Gucalp R, Misiorowski W, et al. Denosumab for patients with persistent or relapsed hypercalcemia of malignancy despite recent bisphosphonate treatment. *J Natl Cancer Inst* 2013;**105**(18):1417–20.