# DAP12/TREM2 signaling affects excessive bone resorption after discontinuation of anti-RANKL antibody

Hotaka Ishizu[1,2], Tomohiro Shimizu[1], Tomoka Hasegawa[2], Norio Amizuka[2], Norimasa Iwasaki[1]
1 Department of Orthopedic Surgery, Faculty of Medicine and Graduate School of Medicine, Hokkaido University, Sapporo, Japan
2 Department of Developmental Biology of Hard Tissue, Graduate School of Dental Medicine, Hokkaido University, Sapporo, Japan
E-mail: iszhtk24@gmail.com (H, Ishizu)

**Disclosures**: H, Ishizu(N), T. T, Shimizu (N), Hasegawa (N), N, Amizuka (N), N. Iwasaki (N)

**INTRODUCTION:** Denosumab, a human monoclonal antibody against the receptor activator of nuclear factor-κB (RANK) ligand (RANKL), is a pivotal agent for managing osteoporosis. It effectively inhibits the RANK/RANKL function crucial for osteoclastogenesis, significantly increasing bone mass [1]. On the other hand, concerns about excessive bone resorption following Denosumab discontinuation have emerged, underscoring the significance of avoiding treatment interruptions [2]. It has been postulated that the disrupted osteoclastogenesis induced by Denosumab might lead to the generation of a significant osteoclast precursor population. Therefore, discontinuing the drug could prompt the accumulated osteoclast precursors to rapidly differentiate into osteoclasts, subsequently triggering excessive bone resorption. However, the precise cellular mechanism underlying this phenomenon remains elusive. Our study aimed to elucidate the spatiotemporal distribution and gene expression of osteoclasts and their precursors, as well as macrophages (MΦs), after a single RANKL antibody administration in the mouse model.

**METHODS:** Six-week-old male C57BL/6J mice (n=84) were divided into two groups: a control group (receiving vehicle injection only) and a RANKL group (intraperitoneally administered 5 mg/kg of a mouse-specific RANKL antibody procured from Oriental Yeast Co., Ltd), following the principles for animal care and research use set by Hokkaido University (approval number 20-0039). Mice from each group were euthanized at 0, 2, 4, 5, 6, and 8 weeks (n=6 for each time point). Before cardiac left ventricle perfusion with 4% paraformaldehyde diluted in 0.1 M cacodylate buffer (pH 7.4), all mice were anesthetized using an intraperitoneal injection containing a combination of medetomidine, midazolam, and butorphanol to measure body weight. Right tibiae and femora were immediately excised and immersed in the same fixative for 24 hours at 4°C. The samples underwent decalcification using 10% ethylenediamine tetraacetic acid disodium salt (EDTA-2Na) solution and stepwise dehydration in ascending ethanol solutions before embedding in paraffin. Dewaxed paraffin sections were used for histochemical assessment of tartrate-resistant acid phosphatase (TRAP), cathepsin K, osteopontin, CD206, F4/80 (EMR1/Gpf480), DNAX activating protein of 12 kDa (DAP12), and Triggering receptor expressed on myeloid cells 2 (TREM2). Micro-CT images of the right femora were acquired using a micro-CT unit (tube voltage 90 kV, CosmoScan Fx, Rigaku Corporation). Total RNA was isolated from the left tibiae and femora before perfusion. The expression levels of *Trap*, *Cathepsin K (Ctsk)*, *Siglec-15*, *Dc-stamp*, *Oc-stamp*, *Cd206*, *F4/80*, *Cd11c, Tyrobp,* and *Trem2* were determined by quantitative real-time PCR, and bulk RNA-seq were performed. All the statistical analyses were performed with one-way ANOVA followed by Tukey-Kramer multiple comparisons tests.

**RESULTS**: Histological and micro-CT imaging exhibited increased femoral metaphyseal trabeculae in mice until 5 weeks after the anti-RANKL antibody administration. However, an abrupt reduction in bone trabeculae was observed from the beginning of the 6 weeks after administration (Fig. 1). TRAP-positive osteoclasts nearly vanished at 2 weeks after administration. Subsequently, small and mononuclear TRAP-positive cells, devoid of cathepsin K immunopositivity, and TRAP-positive osteoclasts were chronologically increased. A noteworthy increase of CD206-positive M2MΦs and F4/80-reactive mature MΦs was evident within the metaphysis from 4 weeks after administration. In particular, CD206-positive multinucleated giant cells, presumed osteoclasts, demonstrated a marked surge at 6 weeks after administration (Fig. 2). Real-time PCR revealed a significantly elevated mRNA level for *Cd206* and *F4/80*, around 6-fold higher than control specimens; however, the increase in CD11c, a characteristic of M1MΦs/dendritic cells, was comparatively moderate. RNA-seq analysis conducted in 5 weeks after administration unveiled reduced expression of osteoclast-related genes such as *Dc-stamp*, *Oc-stamp*, *CtsK*, *Siglec-15*, and *Trap*, while amplified expression of *Tyrobp* (DAP12) and *Trem2* compared to the control. The population of TREM2-positive bone marrow cells continued to increase without attenuation. Further, real-time PCR verified a significantly elevated mRNA level of *Tyrobp* and *Trem2*, around 4-fold higher than the control specimens at 5 weeks after administration.

**DISCUSSION**: Our murine model demonstrated robust inhibition of osteoclast activity and increased trabecular bone by anti-RANKL antibody up to 5 weeks after administration. Conversely, 6 weeks after administration, a swift surge in TRAP-positive osteoclasts was observed, resulting in excessive bone resorption. Furthermore, small and mononuclear TRAP-positive cells emerged, accompanied by a significant rise in F4/80-reactive mature MΦs and CD206-positive M2MΦs from the 4 weeks after administration. These observations imply that administering anti-RANKL antibodies may promote the differentiation of M2MΦs, serving as osteoclast precursors, distinguished by active phagocytosis and tissue repair contributions. DAP12/TREM2 signaling is involved in phagocytosis, inflammation modulation, and promoting osteoclast differentiation and multinucleation. In our study, enhanced expression of Tyrobp and Trem2 were preceded by the increase in M2MΦ and excessive bone resorption. Therefore, DAP12/TREM2 signaling influences osteoclast precursors, enhancing their phagocytic potential upon osteoclast differentiation, likely contributing to excessive bone resorption.

**CLINICAL RELEVANCE:** The administration of anti-RANKL antibodies may promote the M2MΦs differentiation, serving as osteoclast precursors, distinguished by active phagocytosis and tissue repair contributions. DAP12/TREM2 signaling influences osteoclast precursors, enhancing their phagocytic potential upon osteoclast differentiation, likely contributing to excessive bone resorption.

**REFERENCES:** [1] Kendler et al. Adv Ther 2022,[2] Roux et al. Bone 2019

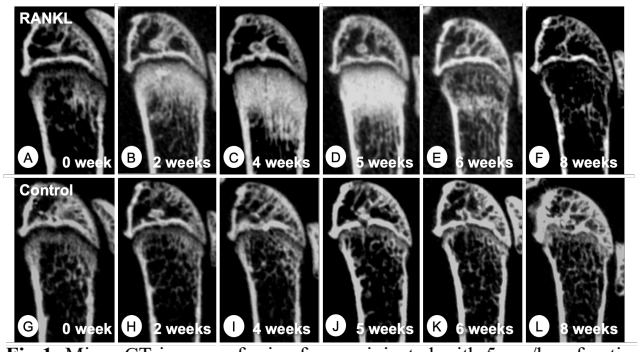


**Fig 1.** Micro-CT images of mice femora injected with 5 mg/kg of anti-RANKL antibody (A-F) or vehicle (G-L). Anti-RANKL antibodies increased bone trabeculae until 5 weeks after administration (A-D). However, the bone trabeculae decreased rapidly from 6 weeks after administration (E, F). The bone trabeculae at 8 weeks after administration (F) was less than the control (L).

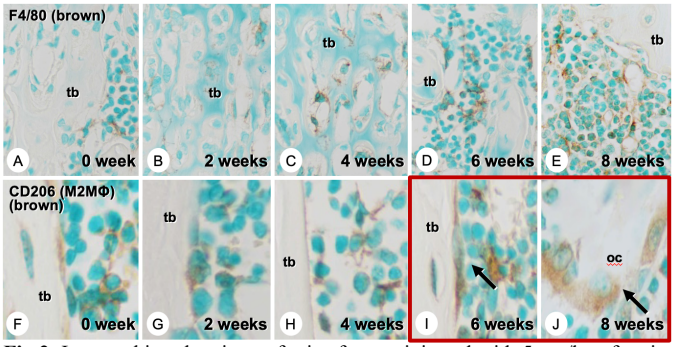


**Fig 2.** Immunohistochemistry of mice femora injected with 5 mg/kg of anti-RANKL antibody. A-E: F4/80 (brown), F-J: CD206 (brown). F4/80-positive mature MΦs and CD206-positive M2MΦs increased rapidly from 4 weeks after administration (C, H). Especially, CD206-positive multinucleated giant cells, estimated osteoclasts, increased 6 weeks after administration (I, J).