Effects of Macrophage Depletion and Transplantation on Bisphosphonate-related Osteonecrosis of the Jaw-like Lesions in Mice

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Abstract

Background: Bisphosphonate-related osteonecrosis of the jaw (BRONJ) is a potentially intractable disease with no definitive pathophysiology and treatment and prevention strategies. Cell-based therapy is one of the useful methods for resolving intractable diseases. This study aimed to investigate whether time-selective depletion and transplantation of macrophages worsens and ameliorates, respectively, BRONJ-like lesions in mice.

Methods: A murine model of high-prevalence BRONJ-like lesions in combination with zoledronate/chemotherapeutic drug administration and tooth extraction was created according to our previous studies. Daily submucosal administration of clodronate-loaded liposomes and systemic transplantation of cultured M2 macrophages induced by macrophage colony-stimulation factor and interleukin-4 and interleukin-10 were performed immediately after tooth extraction. Spleens, femora, tibiae, and maxillae were dissected 2 weeks after extraction to evaluate BRONJ-like lesions and systemic conditions by micro-computed tomography analysis, followed by histomorphometric and immunofluorescent analyses, and serum was assessed with ELISA.

Results: Depletion of macrophages significantly decreased the numbers of local and systemic macrophages, which markedly worsened osseous healing with increased necrotic bone and empty lacunae and soft tissue healing with decreased collagen production and increased infiltration of polymorphonuclear cells. Interestingly, depletion of macrophages significantly shifted macrophage polarization to M1 macrophages by increased M1 macrophages and decreased M2 macrophages. On the other hand, transplantation of M2 macrophages significantly increased the number of local macrophages, but not systemic macrophages, resulting in amelioration and/or cure of early-stage BRONJ-like lesions by promoting osseous and soft tissue healing, with increased distributions of blood and lymphatic vessels and shifting macrophage polarization to M2 macrophages in the connective tissue of the early stages of BRONJ-like lesions.

Conclusions: These data demonstrated that polarization shifting of macrophages is one of the essential factors for development or healing of BRONJ. Cell-based therapy using M2 macrophages could become a useful treatment and/or prevention strategy for BRONJ if safe procedures are established.

Background

Bisphosphonate-related osteonecrosis of the jaw (BRONJ), which was first documented in 2003 by Marx [1], is considered a rare but potentially severe intractable diseases. The prevalence of BRONJ in cancer patients receiving zoledronic acid and in osteoporosis patients taking oral bisphosphonates ranges from 0 to 18% and 0.02 to 0.05%, respectively, which indicates that cancer patients receiving zoledronic acid are obviously at high risk for the development of BRONJ [2]. Moreover, chemotherapeutic drugs, used for the treatment of cancers, are among the high-risk factors for developing BRONJ in combination with bisphosphonate therapy [3, 4]. BRONJ has been demonstrated to worsen both quality of life (QoL) [5] and...
oral health-related QoL [6] due to soft and hard tissue defects in the oral and maxillofacial regions. Therefore, elucidation of the pathophysiology and pathology and the development of prevention/treatment strategies are imperative. However, the definitive pathophysiology and pathology of BRONJ remain unclear based on current basic and clinical evidence. Consequently, effective treatment and prevention strategies for BRONJ have not been established.

Immune cells including neutrophils, dendritic cells, and macrophages are key players in resolving inflammatory sites and promoting wound healing. Debris and numerous bacteria invade into wounds from immediately after invasive surgeries such as tooth extraction and dentoalveolar surgery. Macrophages, which are among the crucial immune cells contributing to innate and acquired immune responses, clean wounds by phagocytosing debris and bacteria and play an important role as antigen-presenting cells, as are dendritic cells. Recently, the potential involvement of macrophages in BRONJ has been reported from basic and clinical research studies. Our previous in vivo studies have demonstrated that the distribution of the pan-macrophage marker F4/80+ cells was significantly changed in BRONJ-like lesions [7–9]. The ratio of macrophage populations was significantly altered in murine BRONJ-like lesions and in BRONJ in humans. Polarization shifting has also been demonstrated to occur between M1 and M2 macrophages within the lesions [10, 11]. M1 and M2 macrophages are mainly classified as pro-inflammatory or classical, and anti-inflammatory or alternatively activated macrophages, respectively [12, 13]. M1 macrophages predominantly produce pro-inflammatory cytokines and chemokines, whereas M2 macrophages predominantly produce anti-inflammatory cytokines, chemokines and other growth factors [14]. Therefore, both types of macrophages are thought to be absolutely required for normal wound healing and amelioration of BRONJ-like lesions. Based on these basic and clinical scientific data, we hypothesized that supply inhibition of macrophages and increased distribution of M2 macrophages into tooth extraction wounds during wound healing processes are imperative for the deterioration and amelioration, respectively, of BRONJ lesions in association with changing macrophage polarization.

The aims of this study were: 1) to investigate the effects of temporary macrophage depletion on wound healing of tooth extraction sockets: and 2) to elucidate the effects of M2 macrophage transplantation on soft and hard tissue healing of tooth extraction sockets using a murine model of early-stage BRONJ-like lesions.

**Methods**

**Animals, drugs, and cytokines**

A total of 57 female C57BL/6J mice (39 8-week-old and 18 10-week-old mice) were used (CLEA Japan Inc., Osaka, Japan). Sample numbers in each experiment was decided according our previous studies [8, 15]. The mice were allowed access to water and a standard diet ad libitum in a temperature-controlled room. Zoledronic acid (Zol; Novartis, Stein, Switzerland) and cyclophosphamide (CY, C3797; Sigma-Aldrich, St. Louis, MO, USA) were purchased to create a murine model of high-prevalence BRONJ-like lesions with tooth extractions. Clodronate and control liposomes (Liposoma BV, Amsterdam, The
Netherlands) were obtained to temporarily deplete systemic macrophages. Macrophage-colony stimulating factor (M-CSF) (Peprotech, Rocky Hill, NJ, USA), IL-10 (Bio-Techne, Minneapolis, MN, USA), and IL-4 (Bio-Techne) were obtained to prepare murine M2 macrophages. Tooth extraction and cell transplantation were performed under systemic anesthesia (33 mg/kg of ketamine and 6.6 mg/kg of xylazine mixture). All mice were euthanized with carbon dioxide. All animal experiments were performed according to the ARRIVE Guidelines (https://arriveguidelines.org/resources/author-checklists).

**Creation of a murine model of high-prevalence BRONJ-like lesions**

A murine model of high-prevalence BRONJ-like lesions was created, as previously described [15–17]. Briefly, 11 8-week-old female C57BL/6J mice were used. CY/Zol combination administration was continued for 5 weeks. Zol was subcutaneously administered twice a week at a total dosage of 0.1 mg/kg, whereas 150 mg/kg of CY were injected intraperitoneally twice and once a week before and after tooth extraction, respectively. Saline and phosphate-buffered saline were used as vehicle controls (VCs). Both maxillary first molars were carefully extracted under higher magnification with a dental explorer not to fracture the molar roots. Euthanasia was performed 2 weeks after tooth extraction (n = 4/group) (Fig. 1A). Compromised osseous and soft tissue healing of tooth extraction 2 weeks after tooth extraction was designated as early-stage BRONJ-like lesions based on our previous studies [8]. One mouse was excluded in this study due to root fractures. Maxillae, tibiae and sera were collected just after euthanasia, with higher magnification intraoral photos.

All animal experiments except for cell transplantation (MS) were performed by one operator (RK) to avoid potential confounding.

**Time-selective depletion of macrophages**

Eighteen 12-week-old female C57BL/6J mice were used. To temporarily deplete systemic macrophages, clodronate-loaded liposomes were used. All mice received CY/Zol combination administration for 5 weeks to create BRONJ-like lesions according to the above-mentioned regime, and they were randomly divided into 2 groups. Buccal submucosal injection of clodronate-loaded liposomes at a dosage of 5 µL/g around the right side of tooth extraction sockets was performed daily for 2 weeks just after tooth extraction, according to previous studies [18, 19]. An injection of phosphate-buffered saline (PBS)-loaded liposomes into the remaining mice was used as a control (n = 7/group) (Fig. 1B). Four mice were excluded in this study due to 2 root fractures and 2 deaths caused by macrophage depletion. Euthanasia was performed 2 weeks after tooth extraction. Maxillae, tibiae, femora, spleens and sera were collected just after euthanasia, when intraoral photos of the tooth extraction sockets were also taken.

**Transplantation of M2 macrophages**

Twenty-eight 10-week-old female C57BL/6J mice were used. Twenty tibiae and femora were collected from 10 8-week-old female C57BL/6J mice. Bone marrow cells were collected with flushing just after harvesting long bones at euthanasia, and cultured for 24 hours at a density of $1.0 \times 10^6$ cells/mL after
the elimination of red blood cells (Gibco ACK lysing buffer; A10492, Thermo Fisher Scientific Inc., Waltham, MA, USA) in D-MEM with 10% fetal bovine serum and 10 U/mL of penicillin (Invitrogen, Carlsbad, CA, USA) and 100 µg/mL of streptomycin (Invitrogen). Suspended cells were collected with centrifugation, then plated and cultured at a density of $2.5 \times 10^5$ cells/cm$^2$ in the media with 50 ng/mL of M-SCF (Peprotech) for 3 days to induce macrophages. Furthermore, M2 macrophages were cultured in the presence of interleukin (IL)-4 (100 µg/mL; Bio-Techne), IL-10 (100 µg/mL; Bio-Techne) and M-SCF (50 ng/mL; Peprotech) for 24 hours to induce M2 macrophages as previously described [20]. Sixteen mice were used. The prepared M2 macrophages were systemically transplanted into 9 randomly selected CY/Zol-treated mice at 3 weeks just after tooth extraction via the left external jugular vein. Four mice were excluded in this study due to 2 root fractures and 2 deaths caused by cell transplantation. The mice were euthanized 2 weeks after tooth extraction and cell transplantation with continuing CY/Zol combination administration. Saline injection via the left external jugular vein was used as a control (n = 7/group) (Fig. 1C). Maxillae, tibiae, femora, spleens, and sera were collected just after euthanasia when intraoral photos of the tooth extraction sockets were also taken.

**Micro-computed tomography (microCT) analysis**

Thirty-eight left maxillae and tibiae were fixed with 10% neutral buffered formalin for 24 hours after euthanasia. MicroCT (R_mCT2; Rigaku Co. Ltd., Tokyo, Japan; 20-µm voxels, 90-kV tube voltage) and TRI/3D-Bon software (Ratoc System Engineering, Tokyo, Japan) were used for sample scans and bone structural analysis, respectively. The regions of interest (ROIs) for tooth extraction sockets and tibiae were defined as the regions combining one mesial root and two distal roots constructed of the outer bone wall from the alveolar ridge to the root apex, and the tibial metaphysis regions from 200 to 2,200 µm below the growth plate, respectively. Bone volume of tooth extraction sockets and tibiae (BV/TV), trabecular number (Tb.N), trabecular thickness (Tb.Th), trabecular separation (Tb.Sp), and bone mineral density (BMD) were used as the evaluation parameters according to the guidelines for microCT analyses [21].

**Hematoxylin and eosin (H&E), tartrate-resistant acid phosphatase (TRAP), and trichrome staining**

Thirty-eight right maxillae and femora were fixed in 10% neutral formalin at 4°C for 24 hours after dissection, demineralized with ethylenediaminetetraacetic acid (pH = 7.3, FUJIFILM Wako Pure Chemical Co., Osaka, Japan) at 4°C for 21 days, and 5-µm-thick sections were prepared following paraffin embedding. Thirty-eight spleens were also fixed in 10% neutral formalin, embedded in paraffin, and prepared as 5-µm-thick sections. Maxillary sections underwent H&E staining, TRAP staining, and trichrome staining to evaluate soft and hard tissue wound healing, osteoclasts and collagen production, and infiltration of polymorphonuclear cells, respectively. H&E staining and TRAP staining were performed for femora to evaluate bone area beneath the growth plate and osteoclasts on the bone surface, respectively. H&E staining of spleen sections was also performed, with the standard manufacturer's protocols. TRAP staining was conducted (Naphthol ASMX phosphatase disodium salt, N5000; Fast Red Violet, Sigma F3381; Sigma Aldrich) according to the standard staining protocol and counterstained with hematoxylin. Stained sections were photomicrographed with a light microscope under higher
magnification (Axio Scope A1; Zeiss, Carl Zeiss, Gottingen, Germany). The acquired images were automatically merged to create whole images of tooth extraction sockets, femora, and spleens. These merged images were then used for histomorphometric analysis with Zen2 software (Zeiss) and ImageJ [version 1.47; National Institutes of Health (NIH), Bethesda, MD, USA; https://imagej.nih.gov/ij/].

**Immunofluorescence staining**

To visualize macrophages in the soft tissue of tooth extraction sockets and splenic tissues, immunofluorescence staining was carried out. Thirty-eight right maxillary paraffin-embedded sections underwent antigen retrieval following dehydration.

Serial maxillary sections were prepared and incubated with goat F(ab) anti-mouse IgG H&L polyclonal antibody (ab6668; Abcam, Cambridge, MA, USA; 1:20) for 1 hour to block endogenous mouse IgG in the tissue. An antibody cocktail of rat anti-mouse F4/80 monoclonal antibody (ab16911; Abcam; 1:25) and mouse anti-human CD38 monoclonal antibody (sc-374650; Santa Cruz, Dallas, TX, USA; 1:50) was applied to the first section with incubation for 12 hours as a primary antibody to visualize M1 macrophages. An antibody cocktail of rat anti-mouse F4/80 monoclonal antibody (ab16911; Abcam; 1:25) and mouse anti-rat CD163 monoclonal antibody (sc-58965; Santa Cruz; 1:50) was also incubated for 12 hours as a primary antibody to detect M2 macrophages, as previously described. A cocktail of Alexa Fluor 546 goat anti-rat IgG (Invitrogen; 1:200) and FITC-conjugated goat anti-mouse IgG H&L antibody (ab6785; Abcam; 1:100) was applied for 1 hour at room temperature as a secondary antibody. Stained sections were mounted with VECTASHELD Antifade Mounting Medium with DAPI (H-1200; Vector Laboratories, Burlingame, CA, USA), visualized and photomicrographed by an immunofluorescence microscope (Zeiss) and semi-automatically analyzed with Zen2 software (Zeiss) and ImageJ (NIH).

**Evaluation of serum TRAP isoform 5b (TRAcP5b) with the Enzyme-linked immuno sorbent assay (ELISA)**

To confirm and/or investigate the effects of drug administration, depletion of macrophages and transplantation of M2 macrophages on serum TRAcP5b, ELISA was performed using the MouseTRAP™ Assay (TRAcP5b ELISA) (DS-SBTR103; Immunodiagnostic Systems Ltd., Boldon, Tyne and Wear, UK). Cardiac puncture and centrifugation were conducted for the collection of fresh serum just after euthanasia, then kept at −80°C just before use (n = 38). A microplate reader at an absorbance of 405 nm (MultiSkan FC Advance, Thermo Scientific) was used for the measurement of serum TRAcP5b levels. Mean values with duplicate data were used as measurement values in this study.

**Assessment of femora and spleens**

To investigate the effects of CY/Zol combination administration, systemic depletion of macrophages, and transplantation of M2 macrophages on femora and spleens, the following parameters were evaluated using H&E staining, TRAP staining, or immunofluorescence staining: 1) femoral bone area at 200 to 2,200 µm below the growth plate (areas of interest: AOIs) [BA/TA (mm²)]; 2) osteoclast number on the femoral bone surface in the AOIs [N.Oc/BS (#/mm)]; 3) total cell area occupied by total tissue area in
the spleens [cell area (%)]; and; 4) macrophage distribution in the splenic cell areas [F4/80+ macrophages (#/mm²)]. Histomorphometric analysis was carried out using merged images with Zen2 software (Zeiss) and ImageJ (NIH).

**Evaluation of gross wound healing of tooth extraction sockets**

To assess wound areas and perimeters of the tooth extraction sockets, intraoral photos were taken just after dissection of 38 maxillae. Wound area and perimeters were semiautomatically measured with the image software and ImageJ (NIH). The average values of the right and left sides of the wounds were used in this study.

**Evaluation of soft and hard tissue wound healing of tooth extraction sockets**

To evaluate soft and hard tissue healing of tooth extraction sockets, quantitative analyses were performed using H&E-, TRAP-, and trichrome-stained images, and immunofluorescence images of sagittal tooth extraction sockets. From the H&E- and TRAP-stained images, the AOI for the evaluation of hard tissue wound healing was also defined as the area surrounded by the line connecting mesial, interradicular and distal alveolar ridges and the curved line parallel to the line within 50 µm of the outer line of the laminar dura in bone tissue of tooth extraction sockets [h-AOI]. The following evaluation parameters for osseous healing were used to evaluate healing: 1) the ratio of total bone area including the living and necrotic bones to h-AOI [BA/TA (%)]; 2) the ratio of bone area with normal osteocytes in lacunae [living bone area (%)]; 3) the number of osteocytes in the living bone area [osteocyte numbers (#/mm²)]; 4) the ratio of bone area with more than 10 empty or pyknotic lacunae to total bone area [necrotic bone area (%)]; 5) the number of empty or pyknotic lacunae in the h-AOI [number of empty lacunae (#/mm²)]; and 8) osteoclast number per bone surface in the h-AOI [N.Oc/BS (#/mm)].

On the other hand, the AOI for the assessment of soft tissue wound healing was defined as the area surrounded by the line drawing the border between the epithelial layer and the connective tissue, and the line connecting the mesial, interradicular and distal alveolar ridges [s-AOI]. The following evaluation parameters were used to assess soft tissue wound healing of tooth extraction sockets: 1) thickness of epithelium (mm); 2) length of the rete ridge (mm); 3) stratum corneum (mm); 4) stratum granulosum (mm); 5) stratum spinosum (mm); 6) stratum basale (mm); 7) the ratio of collagen fibers in the s-AOI [collagen (%)]; 8) the number of polymorphonuclear cells within 100 µm away from bone surface of the extraction sockets [PMN infiltration (#/mm²)]; 9) the number of F4/80+ cells in the s-AOIs [F4/80+ macrophages (#/mm²)]; 10) the number of CD38+ cells in the s-AOIs [CD38+ macrophages (#/mm²)]; 11) the number of CD163+ cells in the s-AOIs [CD163+ macrophages (#/mm²)], and 12) the ratio of M1 to M2 macrophages (M1/M2 ratio).

**Statistical analysis**
Random allocation of mice in each study was performed using the allocation tool RandoMice [22]. All statistical analyses were performed in a blinded fashion. The Shapiro-Wilk test was used to test normality. Student’s t-test and the Mann-Whitney U test were used for parametric and nonparametric data, respectively. The statistical software Systat 13.2 (Systat Software, Chicago, IL, USA) was used for statistical analysis. All data are presented as means ± SEM. A p-value < 0.05 was deemed significant.

Results

Creation of a mouse model of high-prevalence BRONJ-like lesions

CY/Zol combination administration significantly induced bone exposure with open wounds compared to VC (VC, 0% at socket and individual levels, respectively; CY/Zol, 75% and 90% at socket and individual levels, respectively) (Fig. S1a; p = 0.014). CY/Zol resulted in deterioration of osseous healing by significantly decreasing living bone and osteocyte density and by significantly increasing necrotic bone and empty lacunae compared to VC (Fig. S1a; living bone area, p = 0.029; osteocyte number, p < 0.001; necrotic bone area, p = 0.003; empty lacunae, p = 0.003). CY/Zol significantly suppressed osteoclast number per bone surface are compared to VC (Fig. S1a; p = 0.001). Moreover, CY/Zol negatively affected soft tissue healing in the connective tissue of tooth extraction sockets by significantly suppressing collagen production, with increased infiltration of polymorphonuclear cells compared with VC (Fig. S1a; collagen, p = 0.015; PMN infiltration, p = 0.018). CY/Zol significantly decreased the number of F4/80+ macrophages and inhibited formation of both blood and lymphatic vessels compared to VC (Fig. S1a; macrophages, p = 0.035; BVs, p = 0.034; LVs, p = 0.002). Overall, it was confirmed that BRONJ-like lesions developed following CY/Zol combination administration, as in our previous study [16, 17]. BRONJ-like lesions with open wounds have been demonstrated to be sustained for 4 weeks after tooth extraction in our murine model [16].

Effects of time-selective depletion of macrophages on gross and osseous healing of tooth extraction sockets in early-stage BRONJ-like lesions

Administration of clodronate-loaded liposomes for 2 weeks via buccal submucosa in the oral cavity significantly decreased cell-occupied areas and the number of macrophages in the spleen compared to administration of PBS-loaded liposomes (Fig. S2a; cell occupied area, p < 0.001; F4/80+ macrophages, p < 0.001), which confirmed that clodronate-loaded liposomes significantly suppressed systemic macrophages, even though the administration route was submucosal injection, not intraperitoneal injection. Moreover, administration of clodronate-loaded liposomes did not change the serum TRAcP5b level (Fig. S2a; p = 0.655) and tibial bone architecture (Fig. S2b; BV/TV, p = 0.451; Tb.N, p = 0.982; Tb.Th, p = 0.228; Tb.Sp, p = 0.416; BMD, p = 0.812) compared to administration of PBS-loaded liposomes, although the number of osteoclasts on the femoral bone surface was significantly decreased by administration of clodronate-loaded liposomes (Fig. S2c; p = 0.001).
On examination of intra-oral photos and quantitative analysis, administration of clodronate-loaded liposomes appeared to worsen gross wound healing of tooth extraction sockets (Fig. 2a). Indeed, open wound area and perimeter were significantly decreased by administration of clodronate-loaded liposomes (Fig. 2a; open wound area, \( p < 0.001 \); open wound perimeter, \( p < 0.001 \)). On microCT analysis, administration of clodronate-loaded liposomes significantly decreased BV/TV and Tb.N, whereas its administration significantly increased Tb.Sp and BMD compared to administration of PBS-loaded liposome (Fig. 2b; BV/TV, \( p < 0.001 \); Tb.N, \( p < 0.001 \); Tb.Th, \( p = 0.487 \); Tb.Sp, \( p = 0.001 \); BMD, \( p = 0.002 \)). Administration of clodronate-loaded liposome significantly decreased BA/TA with decreased living bone and osteocyte density and increased necrotic bone and empty lacunae density compared to administration of PBS-loaded liposomes (Fig. 3a; BA/TA, \( p = 0.007 \); living bone area, \( p = 0.048 \); osteocyte numbers, \( p = 0.038 \); necrotic bone area, \( p = 0.048 \); empty lacunae, \( p = 0.017 \)), although the number of osteoclasts on bone tissue of tooth extraction sockets was significantly decreased by administration of clodronate-loaded liposomes (Fig. 3b; \( p = 0.001 \)). Therefore, administration of clodronate-loaded liposomes significantly worsened gross and osseous healing of BRONJ-like lesions.

**Effects of time-selective depletion of macrophages on soft tissue healing of tooth extraction sockets of early-stage BRONJ-like lesions**

Soft tissue healing was also examined. Administration of clodronate-loaded liposomes significantly induced abnormal epithelial conditions with decreased thicknesses of epithelium, stratum corneum, and stratum granulosum compared to administration of PBS-loaded liposomes, whereas no differences in changes of rete ridge length and of thicknesses of stratum spinosum and stratum basale were noted between groups (Fig. 4a; epithelial thickness, \( p = 0.004 \); rete ridge length, \( p = 0.091 \); stratum corneum, \( p = 0.011 \); stratum granulosum, \( p < 0.001 \); stratum spinosum, \( p = 0.083 \), stratum basale, \( p = 0.002 \)). Collagen production in the connective tissue and infiltration of polymorphonuclear cells near extraction sockets were significantly decreased and increased, respectively, by administration of clodronate-loaded liposomes (Fig. 4b; collagen, \( p < 0.001 \); PMN infiltration, \( p < 0.001 \)). Next, the distribution of macrophages in the connective tissue of lesions was also quantitatively measured to investigate the influence of macrophage depletion on BRONJ-like lesions. Administration of clodronate-loaded liposomes significantly decreased the number of F4/80+ macrophages with an increase in the number of F4/80+CD38+ macrophages and a decrease in the number of F4/80+CD163+ macrophages, which resulted in a significant increase in the M1/M2 ratio (Fig. 4c; F4/80+ macrophages, \( p = 0.029 \); F4/80+CD38+ macrophages, \( p = 0.020 \); F4/80+CD163+ macrophages, \( p = 0.026 \); M1/M2 ratio, \( p = 0.022 \)). Therefore, administration of clodronate-loaded liposomes resulted in significant soft tissue deterioration.

**Effects of M2 macrophage transplantation on gross and osseous wound healing in early-stage BRONJ-like lesions**

Transplantation of M2 macrophages did not change the cell-occupied area and the distribution of F4/80+ macrophages in the spleen (Fig. S3a; cell occupied area, \( p = 0.821 \); F4/80+ macrophages, \( p = 0.682 \), the
serum level of TRAcP5b (Fig. S3a; \( p = 0.700 \)), tibial bone architecture (Fig. S3b; BV/TV, \( p = 0.461 \); Tb.N, \( p = 0.065 \); Tb.Th, \( p = 0.782 \); Tb.Sp, \( p = 0.044 \); BMD, \( p = 0.410 \)), and femoral osteoclast numbers (Fig. S3c; \( p = 0.101 \)) compared to the control group. Transplantation of M2 macrophages significantly reduced open wound area and perimeter compared to the control group (Fig. 5a; open wound area, \( p < 0.001 \); open wound perimeter, \( p < 0.001 \)). On microCT analysis, transplantation of M2 macrophages significantly increased BV/TV, with increased Tb.N and decreased Tb.Sp and Tb.BMD compared to the control group (Fig. 5b; BV/TV, \( p < 0.001 \); Tb.N, \( p < 0.001 \); Tb.Th, \( p = 0.636 \); Tb.Sp, \( p = 0.001 \); BMD, \( p = 0.032 \)). On histological analysis, transplantation of M2 macrophages significantly increased BA/TA by increasing living bone with a decreased number of osteocytes and by decreasing necrotic bone with a decreased number of empty lacunae compared to the control group (Fig. 6a; BA/TA, \( p = 0.008 \); living bone area, \( p = 0.001 \); osteocyte numbers, \( p = 0.008 \); necrotic bone area, \( p < 0.001 \); empty lacunae, \( p < 0.001 \)). However, transplantation of M2 macrophages did not change the number of osteoclasts on the bone surface of the lesions compared to the control group (Fig. 6b; \( p = 0.596 \)).

**Effects of M2 macrophage transplantation on soft tissue healing in the early-stage BRONJ-like lesions**

Finally, soft tissue healing of lesions was assessed. Transplantation of M2 macrophages significantly increased thicknesses of the stratum corneum and granulosum compared to the control group, whereas no differences in the changes of epithelium thickness, rete ridge length, and thicknesses of stratum spinosum and stratum basale were observed between groups (Fig. 7a; epithelial thickness, \( p = 0.113 \); rete ridge length, \( p = 0.134 \); stratum corneum, \( p = 0.001 \); stratum granulosum, \( p < 0.001 \); stratum spinosum, \( p = 0.774 \); stratum basale, \( p = 0.088 \)). Transplantation of M2 macrophages significantly increased collagen production and decreased PMN infiltration compared to the control group (Fig. 7b; collagen, \( p < 0.001 \); PMN infiltration, \( p < 0.001 \)). On quantitative analyses using immunofluorescence staining, transplantation of M2 macrophages significantly increased the number of F4/80\(^+\) macrophages in the connective tissue of the lesions compared to the control group (Fig. 7c; F4/80\(^+\) macrophages, \( p = 0.001 \)). Interestingly, transplantation of M2 macrophages significantly decreased the number of F4/80\(^+\)CD38\(^+\) macrophages and increased the number of F4/80\(^+\)CD163\(^+\) macrophages, which resulted in a significant decrease in the M1/M2 ratio (Fig. 7c; F4/80\(^+\)CD38\(^+\) macrophages, \( p < 0.001 \); F4/80\(^+\)CD163\(^+\) macrophages, \( p = 0.001 \); M1/M2 ratio, \( p = 0.007 \)). Moreover, transplantation of M2 macrophages significantly increased the number of blood and lymphatic endothelial cells compared to the control group (Fig. 7c; CD31\(^+\) BVs, \( p = 0.001 \); LYVE-1\(^+\) LVs, \( p < 0.001 \)).

**Discussion**

In this study, we demonstrated that time-selective depletion of macrophages worsened early-stage BRONJ-like lesions by causing deterioration of osseous healing, with an increase in necrotic bone, and by causing deterioration of soft tissue healing, with decreases in production of collagen fibers and infiltration of polymorphonuclear cells. It was shown that time-selective depletion of macrophages
significantly decreased the number of F4/80+ macrophages and shifted macrophage polarization by increasing F4/80+CD38+ M1 macrophages and decreasing F4/80+CD163+ M2 macrophages in the connective tissue of early-stage BRONJ-like lesions. On the other hand, we demonstrated that systemic transplantation of cultured M2 macrophages significantly ameliorated and/or healed early-stage BRONJ-like lesions by promoting osseous healing with a decrease in necrotic bone and by enhancing soft tissue healing by increased distribution of CD31+ vascular and LYVE-1+ lymphatic endothelial cells in the connective tissue. It was also demonstrated that transplantation of M2 macrophages significantly shifted macrophage polarization from F4/80+CD38+ M1 macrophages to F4/80+CD163+ M2 macrophages in the connective tissue of early-stage BRONJ-like lesions.

Clodronate-loaded liposomes have been well used in basic science research investigating the distributions and functions of systemic and/or local macrophages. Clodronate-loaded liposomes have been shown to induce osteoclast apoptosis by inhibiting the mitochondrial ADP/ATP translocase via intracellular formation of adenosine 5’-(b,g-dichloromethylene) triphosphate (AppCCl2p) [23, 24]. Clodronate is also recognized as a foreign body by macrophage, which encapsulate it into a liposome, resulting in macrophage apoptosis [25]. It has been reported that one subcutaneous injection of clodronate-loaded liposomes to limb hind footpads started the depletion of medullary macrophages in popliteal lymph nodes 24 hours after administration [26], which shows that locally injected clodronate-loaded liposomes are effective in tissues and/or organs close to injection sites. Thus, in the present study, submucosal administration of clodronate-loaded liposomes was used, since stronger effects on wound healing of early-stage BRONJ-like lesions on the injection side compared to the non-injection side were considered. As expected, the open wound and perimeter were significantly greater in injection sites compared to non-injection sites (data not shown), which strongly suggests that submucosal injection of clodronate-loaded liposomes has local effects on gross wound healing in this study, as well as the above-mentioned previous study [26]. Thus, injection sites (right sides of the maxilla) were used to evaluate the details of osseous and soft tissue healing of early-stage BRONJ-like lesions. Moreover, non-injection sites (left sides of the maxilla) were used to assess bone architecture in the extraction sockets, since gross wound healing of BRONJ-like lesions was significantly worsened in non-injection sites compared to BRONJ-like lesions no affected by clodronate-loaded liposomes.

It has been reported that apoptosis of macrophages in the spleen, parathymic lymph nodes, and liver was induced 4 days after 2 intraperitoneal injections of clodronate-loaded liposomes in rats by circulation of clodronate-loaded liposomes via blood flow [27], although no effects of subcutaneous injection of clodronate-loaded liposomes on splenic macrophages with their depletion in lymph nodes by circulation of the drug via lymph flow were observed in a murine study [26]. However, in present study, submucosal administration of clodronate-loaded liposomes also had systemic effects, even though the administration route was local. Therefore, clodronate-loaded liposomes reach the spleen via blood flow, resulting in the depletion of splenic macrophages.
Recently, polarization shifting of macrophages from M2 to M1 induced by deficiency of G-protein-coupled receptor interacting protein 1 in macrophages has been demonstrated to significantly worsen bone formation of injured tibiae in mice [28], which was partially in accordance with our findings regarding osseous healing of tooth extraction sockets, although the target sites were different (tibiae vs. maxillae). On the other hand, a recent study demonstrated that intraperitoneal administration and local injection into extraction sockets of clodronate-loaded liposomes had no effects on BV/TV, without alteration of N.Oc/BS in the extraction sockets 14 days after daily administration in non-treated wild-type mice [19], which was different from those of our current study. Even though submucosal administration of clodronate-loaded liposomes had systemic effects in the present study, different administration routes (local plus intraperitoneal administration [19] vs. submucosal administration in the present study), administration dosage [2 µL of local injection and 10 µL/g (days 0 to 6) and 6 µL/g (days 7 to 13) by intraperitoneal injection [19] vs. 5 µL/g (days 0 to 13) by submucosal injection in the present study), and systemic conditions (non-treatment vs. CY/Zol treatment in the present study) could have caused the clear distinction between the previous studies and our current study. Overall, deterioration of the osseous condition with increased necrotic bone and decreased living bone in early-stage BRONJ-like lesions is thought to be induced by significant suppression of bone remodeling with decreased N.Oc/BS in the present study.

There have been no studies investigating the effects of clodronate-loaded liposomes on soft tissue wound healing in the connective tissue of extraction sockets. More severely abnormal epithelial healing with expanded open wounds and reduced epithelial thickness due to thinner stratum corneum and stratum granulosum in the current study were partially in accordance with the previous in vitro study demonstrating toxicity of Zol on epithelial cells by suppressing proliferation and migration and by increasing cell apoptosis [29, 30], although epithelial hyperplasia has also been observed in Zol-treated mice with tooth extraction in another study [31]. Deterioration of soft tissue healing induced by clodronate-loaded liposomes became evident with reduced collagen production and more severe infiltration of PMNs in early-stage of BRONJ-like lesions. A clinical study reported that macrophage polarization shifted to M1 macrophages according to the severity of BRONJ stages [11]. Another study also reported that M1 polarization occurred in BRONJ-lesions in mice and humans [10]. Our previous and current animal studies indicated a significant decrease in F4/80+ macrophages in early-stage BRONJ-like lesions compared to those in VC-treated mice [8, 9]. Therefore, a significant decrease in F4/80+ macrophages induced by clodronate-loaded liposomes and polarization shifting of macrophages from M2 to M1 macrophages in the connective tissue are linked to the deterioration of early-stage BRONJ-like lesions, although the mechanisms why polarization shifting occurred by administration of clodronate-loaded liposomes are unclear. Tissue-resident macrophages derived from yolk sac macrophages [32, 33], recruited limited monocyte-derived macrophages from bone marrow [34], and transdifferentiation of other subsets of macrophages or cells into M1 macrophages [35] may contribute to polarization shifting of macrophages. Further animal studies are needed to elucidate the mechanisms.
On the other hand, the effects of cultured M2 macrophage transplantation were completely opposite to those induced by macrophage depletion, which resulted in cure and/or amelioration of early-stage BRONJ-like lesions in mice. Several researchers have tried cell transplantation using mesenchymal stem cells [36], adipose derived stem cells [37], adipose tissue-derived stromal vascular fraction cells [17], and quality and quantity-controlled peripheral blood mononuclear cells [15] to reduce BRONJ lesions in animals and humans. However, there are no reports of transplantation of macrophages into BRONJ-like lesions, although macrophage-based therapy has been expected to become one of the useful treatment strategies in regenerative medicine [38]. Namely, this is the first report demonstrating our hypothesis that systemic transplantation of M2 macrophages cures and/or ameliorates BRONJ-like lesions in mice.

Macrophages, which are categorized as yolk sac-derived tissue-resident macrophages, monocyte-derived tissue-resident macrophages, macrophages recruited from bone marrow monocytes, and memory macrophages, play important roles in innate and acquired immune responses [39]. Moreover, it has been reported that there are several subsets of macrophages, including M1, M2a, M2b, M2c, M2d, M3, and Mox and Mhem macrophages in mice or humans [40], although the hierarchy of macrophages is not fully understood in soft or hard tissues in the oral cavity. In the present study, the number of osteoclasts on the bone surface did not change with transplantation of M2 macrophages, whereas bone formation of tooth extraction sockets increased significantly, which suggests that there are key cells related to bone formation rather than osteoclasts. A recent study using singe cell-RNA seq has demonstrated that M2 macrophages were found and associated with heterotopic bone formation, which was inhibited by administration of clodronate-loaded liposomes [41]. M2 macrophages were also found in fractured callus [41]. Therefore, these findings strongly support our data on the enhancement of bone formation in BRONJ-like lesions by transplantation of M2 macrophages in the present study.

The result that accumulation of M2 macrophages in connective tissue promoted soft tissue healing of BRONJ-like lesions in the present study was almost in accordance with data on the enhancement of collagen production and suppression of PMN infiltration in our previous studies by transplantation of adipose tissue-derived stromal vascular fraction cells and quality and quantity-controlled peripheral blood mononuclear cells [15, 17]. In the present study, M-CSF was used to induce macrophages from bone marrow cells, IL-4 was used to induce M2a macrophages, and IL-10 was used to enhance the phenotype of M2 macrophages, according to the previous study [20]. M2a macrophages have been shown to produce collagen precursors and factors stimulating fibroblasts [42], resulting in promotion of extracellular matrix (ECM) in the wounds, contributing to enhancement of collagen production in the connective tissue of BRONJ-like lesions.

It has been reported that spatial distributions of monocyte-derived macrophages during angiogenesis occur in various tissues, since they produce ECM-modifying proteins such as MMP-9 for capillary sprouting [43, 44]. M1 and M2a macrophages predominantly secrete vascular endothelial cell growth factor (VEGF)-A and platelet-derived growth factor (PDGF)-BB, which are imperative for angiogenesis [44, 45]. A subset of monocytes, MACs, resembling M2 macrophages has been demonstrated to differentiate into vascular endothelial cells in murine ischemia models [46, 47]. Moreover, macrophages have been
reported to upregulate the secretion of VEGF-C, leading to lymphangiogenesis [48, 49]. Therefore, macrophage accumulation and polarization shifting to M2 macrophages in the connective tissue of BRONJ-like lesions by transplantation of M2 macrophages may contribute to upregulation of both angiogenesis and lymphangiogenesis by increases in the production of VEGF-A, PDGF-BB and/or VEGF-C, resulting in amelioration and/or cure of early-stage BRONJ-like lesions. Promotion of production of anti-inflammatory cytokines, chemokines and growth factors by polarization shifting to M2 macrophages in the connective tissue may also contribute to enhanced soft tissue healing of BRONJ-like lesions.

**Conclusions**

Collectively, within the limitations of this study due to its being an animal study, polarization shifting of macrophages is one of the essential factors for developing BRONJ-like lesions, due to deterioration of BRONJ-like lesions by polarization shifting to M1 macrophages using administration of clodronate-loaded liposomes and amelioration of BRONJ-like lesions by polarization shifting using transplantation of M2 macrophages. Transplantation of M2 macrophages could become a useful treatment or prevention strategy for BRONJ in humans if safe transplantation procedures are established, since transplantation of M2 macrophages had no effects on the systemic conditions of splenic macrophages and bone architecture of long bones.

**Abbreviations**

BRONJ, bisphosphonate-related osteonecrosis of the jaw; QoL, quality of life; Zol, zoledronic acid; CY, cyclophosphamide; M-CSF, macrophage-colony stimulating factor; IL-10, interleukin-10; IL-4, interleukin-4; VC, vehicle control; PBS, phosphate-buffered saline; MicroCT, micro-computed tomography; ROI, region of interest; BV/TV, Bone volume per tissue volume; Tb.N, trabecular number; Tb.Th, trabecular thickness; Tb.Sp, trabecular separation; BMD, bone mineral density; H&E, Hematoxylin and eosin; TRAP, tartrate-resistant acid phosphatase; TRAcP5b, serum TRAP isoform 5b; ELISA, enzyme-linked immuno sorbent assay; BA/TA, bone area per tissue area; N.Oc/BS, number of osteoclasts per bone surface; AOI, area of interest; PMN cells, polymorphonuclear cells.

**Declarations**

**Ethics approval and consent to participate**

Animal care and experimental procedures were performed in accordance with the Guidelines for Animal Experimentation of Nagasaki University, with approval from the Ethics Committee for Animal Research (Approval number: 1708241404-5).

**Consent for publication**

Not applicable
Availability of data and material

Not applicable

Competing interests

The authors declare that they have no competing interests.

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Author’s contributions

SK contributed to the conceptualization, supervision and project administration. SK, HK\textsuperscript{2}, HH and KN were responsible for the funding acquisition together. RK, SK, MS, HK\textsuperscript{1} and FA completed the methodology and investigation. RN, SK and HK\textsuperscript{1} fulfilled validation and formal analysis. RN, SK, MS and FA finished data curation. SK and TS revised the paper. All authors read and approved the final manuscript (HK\textsuperscript{1}: Haruka Kaneko, HK\textsuperscript{2}: Hiroe Kakehashi).

Acknowledgments

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References


Figures
Figure 1

Experimental schedule. **a** Protocol for the creation of a high-prevalence murine model of BRONJ-like lesions. **b** Depletion regimen for murine BRONJ-like lesions. **c** Transplantation regimen for M2 macrophages to murine BRONJ-like lesions. Euthanasia at 2 weeks after tooth extraction.
Figure 2

Effects of macrophage depletion on gross healing and bone architecture of extraction sockets. 

**a** Representative intra-oral photo images, occlusal views. The areas surrounded by red arrows indicate open wounds. M2 and M3 point to the second and third molars, respectively. Quantitative analyses using intra-oral photo images of open wound area (mm²) and perimeter in Del vs. Ctrl. 

**b** Representative micro computed tomography (CT) images of extraction sockets. Area surrounded by red dots indicates
extraction sockets. Quantitative analyses using microCT images of bone volume/tissue volume [BV/TV (%)], trabecular number [Tb.N (1/mm)], trabecular thickness [Tb.Th (mm)], trabecular separation [Tb.Sp (mm)], and bone mineral density [BMD (mg/cc)] in Ctrl. vs. Del. Values are means ± SEM (error bars). **p < 0.01, ***p < 0.001.

**Fig. 3**

![Image of Figure 3](image)

**Figure 3**
Effects of macrophage depletion on osseous healing of extraction sockets. **a** Representative hematoxylin & eosin (H&E) staining of extraction sockets. Red and black bars indicate 200 µm and 100 µm, respectively. Quantitative analyses using H&E staining of bone area/tissue area [BA/TA (%)], living bone [living bone area (%)], osteocyte numbers (#/mm$^2$), necrotic bone [necrotic bone area (%)], and number of empty lacunae [empty lacunae (#/mm$^2$)] in Ctrl. vs. Del. **b** Representative images of TRAP staining in the tooth extraction sockets. Black bar indicates 100 µm. Quantitative analysis using TRAP staining of the number of osteoclasts on the bone surface [N.Oc/BS (#/mm)] in Ctrl. vs. Del. Values are means ± SEM (error bars). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. 
Figure 4

Effects of macrophage depletion on soft tissue healing of extraction sockets. **a** Quantitative analyses using H&E staining of epithelial thickness (mm), rete ridge length (mm), stratum corneum (mm), stratum granulosum (mm), stratum spinosum (mm), and stratum basale (mm) in Ctrl. vs. Del. **b** Representative trichrome staining of tooth extraction sockets. Quantitative analyses using trichrome staining of production of collagen fibers [collagen (%)] and infiltration of polymorphonuclear cells [PMN infiltration...
Ctrl. vs. Del. c Representative immunofluorescence images of connective tissue of extraction sockets. White bars indicate 100 µm. White, yellow, and aqua blue arrows indicate F4/80+ macrophages, F4/80+CD38+ macrophages, and F4/80+CD163+ macrophages, respectively. Quantitative analyses using immunofluorescence images of F4/80+ macrophages (#/mm²), F4/80+CD38+ macrophages (#/mm²), F4/80+CD163+ macrophages (#/mm²), and the M1/M2 ratio in Ctrl. vs. Del. Values show means ± SEM (error bars). *p < 0.05, ***p < 0.001.
Figure 5

Effects of M2 macrophage transplantation on gross healing and bone architecture of extraction sockets. 

a Representative intra-oral photo images, occlusal views. Area surrounded by red arrows indicate open wounds. M2 and M3 point to second and third molars, respectively. Quantitative analyses using intra-oral photo images of open wound area (mm$^2$) and perimeter in Ctrl. vs. Test. 

b Representative micro computed tomography (CT) images of extraction sockets. Area surrounded by red dots indicates extraction sockets. Quantitative analyses using microCT images of bone volume/tissue volume [BV/TV (%)], trabecular number [Tb.N (1/mm)], trabecular thickness [Tb.Th (mm)], trabecular separation [Tb.Sp (mm)], and bone mineral density [BMD (mg/cc)] in Ctrl. vs. Test. Values are means ± SEM (error bars). *$p$ < 0.05, **$p$ < 0.01, ***$p$ < 0.001.
Figure 6

Effects of M2 macrophage transplantation on osseous healing of extraction sockets. a Representative hematoxylin & eosin (H&E) staining of tooth extraction sockets. Red and black bars indicate 200 μm and 100 μm, respectively. Quantitative analyses using H&E staining of bone area/tissue area (BA/TA (%)), living bone [living bone area (%)], osteocyte numbers (#/mm²), necrotic bone [necrotic bone area (%)], and number of empty lacunae [empty lacunae (#/mm²)] in Ctrl. vs. Test. Representative images of TRAP
staining in the tooth extraction sockets. Black bar indicates 100 µm. Quantitative analysis using TRAP staining of number of osteoclasts on the bone surface [N.Oc/BS (#/mm)] in Ctrl. vs. Test. Values are means ± SEM (error bars). **p < 0.01, ***p < 0.001.

Figure 7
Effects of M2 macrophage transplantation on soft tissue healing of extraction sockets. **a** Quantitative analyses using H&E staining of epithelial thickness (mm), rete ridge length (mm), stratum corneum (mm), stratum granulose (mm), stratum spinosum (mm), and stratum basale (mm) in Test. vs. Ctrl. **b** Representative trichrome staining of tooth extraction sockets. Quantitative analyses using trichrome staining of production of collagen fibers [collagen (%)] and infiltration of polymorphonuclear cells [PMN infiltration (#/mm$^2$)] in Test. vs. Ctrl. **c** Representative immunofluorescence images of connective tissue of tooth extraction sockets. White bars indicate 100 µm. White, yellow, aqua blue, red and greenish yellow arrows indicate F4/80$^+$ macrophages, F4/80$^+$CD38$^+$ macrophages, F4/80$^+$CD163$^+$ macrophages, CD31$^+$ vascular endothelial cells, and LYVE-1$^+$ lymphatic endothelial cells, respectively. Quantitative analyses using immunofluorescence images of F4/80$^+$ macrophages (#/mm$^2$), F4/80$^+$CD38$^+$ macrophages (#/mm$^2$), F4/80$^+$CD163$^+$ macrophages (#/mm$^2$), the M1/M2 ratio, CD31$^+$ vascular endothelial cells [CD31$^+$ BVs (#/mm$^2$)], and LYVE-1$^+$ lymphatic endothelial cells [LYVE-1$^+$ LVs (#/mm$^2$)] in Test. vs. Ctrl. Values show means ± SEM (error bars). **$p$ < 0.01, ***$p$ < 0.001.

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