Plasticity of Myeloid Cells during Oral Barrier Wound Healing and the Development of Bisphosphonate-Related Osteonecrosis of the Jaw

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Running title: Myeloid cell plasticity in the oral barrier tissue and ONJ

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ABSTRACT

Injury to the barrier tissue initiates a rapid distribution of myeloid immune cells from bone marrow, which guide sound wound healing. Bisphosphonates, a widely used anti-bone resorptive drug with minimal systemic side effects, have been linked to an abnormal wound healing in the oral barrier tissue leading to, in some cases, osteonecrosis of the jaw (ONJ). Here we report that the development of ONJ may involve abnormal phenotypic plasticity of Ly6G+/Gr1+ myeloid cells in the oral barrier tissue undergoing tooth extraction wound healing. A bolus intravenous zoledronate (ZOL) injection to female C57BL/6 mice followed by maxillary first molar extraction resulted in the development of ONJ-like lesion during the second week of wound healing. The multiplex assay of dissociated oral barrier cells exhibited the secretion of cytokines and chemokines, which was significantly modulated in ZOL mice. Tooth extraction-induced distribution of Ly6G+/Gr1+ cells in the oral barrier tissue increased in ZOL mice at week 2. ONJ-like lesion in ZOL mice contained Ly6G+/Gr1+ cells with abnormal size and morphology, as well as different flow cytometric staining intensity. When anti-Ly6G (Gr1) antibody was intraperitoneally injected for 5 days during the second week of tooth extraction, CD11b+GR1hi cells in bone marrow and Ly6G+ cells in the oral barrier tissue were depleted and the development of ONJ-like lesion was significantly attenuated. This study suggests that local modulation of myeloid cell plasticity in the oral barrier tissue may provide the basis for pathogenesis and thus therapeutic as well as preventive strategy of ONJ.

The major barrier tissues in mammals such as skin and intestinal mucosa segregate the host and external environments and withstand the frequent mechanical injury and microbial challenge with commensal as well as pathogenic species (1,2). The highly active renewal capability of skin epidermis and intestinal epithelium affords...
rapid repopulation and recovery of barrier tissue continuity, and a repertoire of adaptive and innate immunity plays an essential role in the maintenance of barrier function and homeostatic relationship with microbes (3,4). Barrier tissues maintain resident memory T cells (5,6), innate lymphoid cells (7) and macrophages (8), which are highly specialized and capable of proliferating locally without contributions from distantly localized precursor cell populations. Infections and injuries to barrier tissues immediately activate the tissue-resident cells initiating the immune cascade.

Myeloid cells such as neutrophils are known to be the first respondent to the stressed barrier tissue and rapidly migrating into the affected tissue from the circulating peripheral blood population (9,10). Unlike the resident immune cells in the barrier tissue, which express relatively predetermined functions, the infiltrating myeloid cells possess significant plasticity and are capable of undergoing substantial, rapid and potentially reversible phenotypic and functional changes (11-14). The acquisition of plasticity plays an important role for the barrier immune system responding to various microenvironments influenced by microorganisms and frequent injury (15,16). Abnormal plasticity of immune cells has been implicated as a cause of barrier tissue diseases (17,18).

The lining mucosal membrane of the oral cavity presents a characteristic barrier tissue supporting the adaptive and innate barrier immunity. The oral environment is highly susceptible to commensal and pathogenic microbial challenge, mechanical irritation and surgical wounding (19). Tooth extraction is one of the most frequently prescribed surgical procedures, which leaves a large open wound of oral mucosa as well as a bony socket in the jawbone. Clinically, tooth extraction wound heals with minimal scarring without complications and the open wound is closed with the rapid approximation of oral mucosa (20,21). The early immune respondent to the oral wound site is circulating neutrophils that are thought to play a pivotal role in regulating innate immunity, wound healing, and inflammation resolution (22,23). However, oral barrier immunity and its functional role in responding to oral injury and infection are still largely unexplored.

Since the first report in 2003, osteonecrosis of the jaw (ONJ) related to the medications preventing osteoclastic bone resorption has been recognized as extended chronic inflammation of oral barrier tissue and alveolar bone necrosis often associated with abnormal tooth extraction wound healing (24,25). The intended target of anti-resorptive medications is the trabecular bone in the bone marrow environment of extremity and vertebral bones. Functional disturbance or differentiation of osteoclasts is achieved by bisphosphonates or humanized anti-RANKL antibody, respectively (26). Bisphosphonates are primarily affecting osteoclasts due to their strong affinity to bone minerals (27). After entering the system, bisphosphonates are rapidly adsorbed on the bone surface while the rest in circulation is excreted within 24 to 48 hours. Tooth extraction of bisphosphonate-treated patients has been shown to increase the prevalence of developing ONJ (28,29). In bisphosphonate-treated rodent models, tooth extraction associated with the development of osteonecrosis in the jawbone and prolonged chronic inflammation in oral barrier tissue (30,31).

It has been indicated that bisphosphonates can be taken up by myeloid cells such as macrophages (32,33), dendritic cells (34) and neutrophils (35,36), and modulate their viability, migration and function. This study addressed whether bisphosphonate-induced myeloid cell dysfunction might contribute to the pathological oral barrier immunity leading to the development of ONJ. Here we report that the aberrant Ly6G+/Gr1+ myeloid cell plasticity in the oral barrier tissue by intravenous injection of zoledronate (ZOL) associated with the development of ONJ-like lesion at the tooth extraction site of mouse maxilla, while bone marrow myeloid cells remained unaffected. The ONJ-like lesion was significantly attenuated by Ly6G+/Gr1+ cell depletion through intraperitoneal injection of anti-Ly6G antibody. The outcome of this study suggests that the disturbance of myeloid cell plasticity significantly disrupt the oral barrier immunity and oral wound healing. This study further suggests that local modulation of myeloid cell plasticity in the oral barrier tissue may provide the basis for ONJ pathogenesis and thus a clue for the therapeutic and preventive strategy.
RESULTS

Development of ONJ-like lesion in ZOL mice – In mice, tooth extraction triggered a course of wound healing involving wound closure of the gingival oral barrier tissue and bone remodeling in the bony socket. Mice pretreated with a bolus IV injection of ZOL showed uneventful tooth extraction wound healing at day 3. However, the tooth extraction wound exhibited delayed healing at week 2, characterized by patent oral open wound (Figure 1B) and delayed new bone formation in the extraction socket (Figure 1C). Some ZOL mice exhibited abnormal bone formation at the external surface of alveolar bone near the tooth extraction site (white arrow in Figure 1C), which appeared to persist in week 4. Histologically, a typical ONJ-like lesion in mice was composed of jawbone necrosis interfacing highly inflammatory gingival oral barrier tissue, occasionally developing pustule and fistula (Figure 1D). Epithelial hyperplasia resulted in the abnormal epithelial cell contact to the necrotic bone surface or the exposure of jawbone to the oral cavity. The area of osteonecrosis in ZOL mice significantly increased at week 2 and persisted at week 4 (Figure E). TRAP+ multinuclear osteoclasts were found on the surface of palatal bone as well as in the maxillary bone marrow (Figure 1F). In some ZOL mice, osteoclasts appeared to be detached from the bone surface and “floating” in the oral barrier connective tissue. The number of osteoclasts was transiently increased at week 2 in both control (NaCl) and ZOL mice, whereas the number of osteoclasts was relatively consistent in the jawbone bone marrow space (Figure 1G). These results were highly consistent with our previous report (31) and thus the ONJ mouse model was reproduced in this study. The effect of ZOL treatment resulting in the different tooth extraction wound healing appeared to be most significant at week 2.

The effect of ZOL on gingival oral barrier immunity during tooth extraction wound healing – After tooth extraction, gingival oral barrier tissues were harvested and subjected to cell dissociation. The dissociated oral barrier cells were analyzed by flow cytometry. On day 3, week 2 and week 4, using gating strategy to account for all CD45+ cells, oral barrier tissues were found to contain approximately 60% CD45+ cells (Figure 2A). However, the ZOL treatment increased the proportion of CD45+ cells (82%) during the active wound-healing period of week 2. It was also noted that the ZOL treatment developed a population with decreased CD45 staining intensity throughout the study period (Figure 2A). Within CD45+ cells, CD3+ T cells consisted of approximately 8% to 10% and the effect of ZOL on the T cell proportion was not observed (Figure 2B). Our dissociation protocol appeared to be suitable to isolate oral barrier cells, giving rise to relatively consistent immune cell composition in the control (NaCl) and ZOL groups.

Dissociated oral barrier cells were cultured for 3 days and the condition media were examined for the presence of cytokines and chemokines by a multiplex system (Figure 2C). The secretion of granulocyte stimulating factor (G-CSF) and keratinocyte-derived chemokine (KC) was detected in day 3 oral barrier cells of control (NaCl) and ZOL mice, whereas macrophage inflammatory proteins 1a (MIP-1a) and 1b (MIP-1b), also known as CCL3 and CCL4, respectively, were secreted more by ZOL cells. The gingival oral barrier cells of week 2 control (NaCl) mice exhibited robust increase in the secretion level of cytokines and chemokines, such as G-CSF, Eotaxin (CCL11), granulocyte-macrophage colony-stimulating factor (GM-CSF), gamma interferon (IFN-g), interleukins such as IL-6, IL-10, IL-12 and IL-13, LPS-induced CXC chemokine (LIX) and monocyte chemoattractant protein-1 (MCP-1). Strikingly, the oral barrier cells from week 2 ZOL mice had significantly impaired secretion of these cytokines and chemokines (Figure 2C). The cytokine secretion diminished by the gingival oral barrier cells of week 4 control (NaCl) mice, whereas those cells from ZOL mice maintained high levels of G-CSF, KC and macrophage inflammatory protein 2 (MIP-2) (Figure 2C).

Ly6G/Gr1+ myeloid cells in gingival oral barrier tissue were modulated by ZOL treatment – Immunohistochemical analysis revealed that polymorphonuclear (PMN) cells were positively stained with anti-Ly6G antibody in the gingival oral barrier tissue on day 3 and week 2 of tooth extraction of control (NaCl) mice (Figure 3A and 3B). Ly6G+ cells were clustered under the proliferating and migrating oral epithelium adjacent to the tooth extraction and separately at
the deep connective tissue zone. ZOL mice on day 3 showed similar infiltration of Ly6G+ cells to the gingival oral barrier tissue as control, which picked up on week 2 (Figure 3A and 3B). It was observed that Ly6G+/Gr1+ cells were found in a close proximity to the alveolar bone and osteoclasts in ZOL mice appeared to associate with immune cells including Ly6G+/Gr1+ cells (Figure 3A). Ly6G+ cells in ZOL mice included small cells with condensed nuclear structure or large disintegrated cells (Figure 3D).

The dissociated cells from gingival oral barrier tissues of week 2 control (NaCl) and ZOL mice were analyzed by flow cytometry. The back gating of control CD11b+Gr1hi cells demonstrated larger concentration of the CD11b+Gr1hi population at the lower Forward and side scatter area (gated area in the histogram) where the majority of myeloid population is expected to be seen, however, some cells can also be observed all throughout the forward and side scatter histogram. When CD11b+Gr1hi cell gate was applied to the dissociated cells of ZOL mice, the CD11b+Gr1hi cells were apparently substantially decreased or missing (Figure 3C).

The condition medium of oral barrier cells from ZOL mice induced necrosis of MLO-Y4 cells in vitro - A panel of cytokines/chemokines was shown to change expression in the dissociated oral barrier cells from ZOL mice. We tested if these modulated cytokines could influence osteonecrosis using murine osteocyte-like MLO-Y4 cells. In the initial study the majority of MLO-Y4 cells treated with HEMA acquired 7AAD and Annexin V positive staining suggesting their high sensitivity to HEMA-induced necrosis (Figure 4A); however MLO-Y4 cells were found relatively resistant to cisplatin (CDDP)-induced apoptosis.

The overnight incubation with the condition medium of oral barrier cells dissociated from ZOL mice induced a fraction of necrotic PI+/Annexin V+ MLO-Y4 cells that was almost twice as much of the control mouse oral barrier cell condition medium (Figure 4B). Furthermore, MLO-Y4 cells with the ZOL mouse oral barrier cell condition medium demonstrated enlarged MLO-Y4 cells with pyknotic nucleus, which lost the typical dendritic processes (Figure 4C). The altered morphology resembled osteocytes undergoing necrosis.

The effect of ZOL and tooth extraction wounding on bone marrow myeloid cells – It has been shown that myeloid cells in barrier tissues must be replenished from the circulating population, not from proliferating locally embedded cells. The primary therapeutic target of bisphosphonates is trabecular bone in the bone marrow of long bones and vertebral bones. Therefore, we postulated that the different response of Ly6G+/Gr1+ cells in the oral mucosa of ZOL-treated mice might be due to preconditioning in the bone marrow microenvironment. As expected, micro-CT evaluations indicated that the trabecular bone volume (bone volume over tissue volume: BV/TV) and structural integrity (connectivity density: Conn.D.) of mouse femurs after ZOL treatment (Figure 5A and 5B). Bone marrow flow through cells were harvested on day 3, week 2 and week 4 of tooth extraction and analyzed by flow cytometry. Anti-CD11b and anti-Ly6G antibodies generated various staining intensities. In the day 3 samples, CD11b+Ly6G+ cells were predominant, whereas the week 2 samples contained mostly CD11b+Ly6G+ cells (Figure 5C). Overall, the ZOL treatment moderately decreased the bone marrow CD11b+Ly6G+ myeloid cells. When the same bone marrow cells were probed for Ly6C, the CD11b+Ly6C+ cells appeared to co-localize with CD11b+Ly6G+ cells in day 3 and week 2 samples (Figure 5D). The ZOL treatment resulted in moderate increase of CD11b+Ly6C+ cells in bone marrow. The intensity of the surface staining was substantially decreased in both CD11b+Ly6G+ and CD11b+Ly6C+ populations in week 4 compared to week 2 and day 3 in NaCl and ZOL-treated mice (Figure 5C and 5D). Our data indicated that bone marrow myeloid cells appeared to be affected by oral wounding but not significantly influenced by ZOL.

Anti-Ly6G (Gr1) antibody IP injection eliminated circulating CD11b+Gr1hi cells – ZOL mice appeared to develop ONJ-like lesion in the second week of tooth extraction, when Ly6G+ cells were abundant in the gingival oral barrier tissue. In this experiment, we addressed the role of oral barrier Ly6G+ cells on the development of ONJ, the control (NaCl) and ZOL mice were treated by daily IP injections of anti-Ly6G (Gr1) antibody in the second week from day 9 to day 13 of tooth extraction (Figure 6A). The bone
marrow flow through cells harvested on week 2 (the next day of the last anti-Ly6G (Gr1) antibody injection) generated the predominant CD11b+Gr1int cell population at the expense of CD11b+Gr1hi cells (Figure 6B). This proportion was markedly contrasted with the antibody untreated and the isotype antibody-injected bone marrow samples, which showed CD11b+Gr1hi cells or CD11b+Ly6Ghi cells as the predominant population, respectively (Figure 6B). Peripheral blood samples harvested from the same animals showed that significant reduction of CD11b+Gr1hi cells in the anti-Ly6G (Gr1) antibody-treated mice, whereas the antibody untreated and the isotype antibody-injected groups identified the presence of CD11b+Gr1hi cells or CD11b+Ly6Ghi cells, respectively (Figure 6C).

Anti-Ly6G (Gr1) antibody IP injection during the second week of tooth extraction wound healing prevented the development of ONJ-like lesion in ZOL mice – When anti-Ly6G (Gr1) antibody was injected during the second week of tooth extraction, the tooth extraction wound healing was found nearly completed in not only control (NaCl) mice but more strikingly in ZOL mice (Figure 7A). The swelling of gingival oral barrier tissue was observed in ZOL mice without the antibody treatment or with the isotype control antibody injection, along with the open barrier tissue wound. These complications were not observed in all mice with the anti-Ly6G (Gr1) antibody injection (Figure 7A). The histological osteonecrosis area of ZOL mice was larger than that of control (NaCl) mice; however, the anti-Ly6G (Gr1) antibody injection appeared to attenuate the development of osteonecrosis (Figure 7B and 7C).

Histological analysis revealed that the gingival barrier tissue of control (NaCl) and ZOL mice with the anti-Ly6G (Gr1) antibody injection showed little inflammatory cell infiltration (Figure 7C). On the contrary, control (NaCl) mice with the isotype control antibody injection developed a localized inflammation at the tooth extraction socket and along the surface of the palatal bone. In addition to the localized inflammation, ZOL mice further developed a wide spread area of diffused inflammation, which interfaced with osteonecrosis lesion and epithelial hyperplasia (Figure 7C). Immunohistochemical analysis indicated that Ly6G-positive cells were disproportionately localized in the abnormal diffused inflammation area of ZOL mice. In ZOL mice with the anti-Ly6G (Gr1) antibody injection, the gingival barrier tissue was free from Ly6G-positive cells (Figure 7D). There were sporadic Ly6G-positive cells around the closing oral epithelial wound over the tooth extraction socket (data not shown).

DISCUSSION

This study reports that the abnormal Ly6G+/Gr1+ myeloid cells were present in the oral barrier tissue of ZOL mice during the second week of tooth extraction, when ONJ-like lesion was developed. The striking observation was that the deletion of those Ly6G+/Gr1+ myeloid cells clearly attenuated if not prevented the development of ONJ in ZOL mice (Figure 7). Here we propose that dysregulated myeloid cell plasticity in the oral barrier tissue may play an important role in the pathological process of ONJ.

Myeloid cells can undergo changes in the phenotype and function described as immune plasticity (37). Neutrophils are classically defined as PMN cells characterized by the high expression of Ly6G/Gr1 markers with the relatively low expression of Ly6C marker (38). However, flow cytometric analysis demonstrated the varying staining intensity of these markers providing the basis for differentiation and phenotypic plasticity. Myeloid cells expressing Gr1 marker at the intermediate intensity (Gr1int cells) have been described as myeloid-derived suppressor cells (MDSC) (39,40), which represent immature myeloid cells capable of suppressing T cell immunity (41). A recent report on subpopulations of circulating neutrophils described granulocytic MDSC with the intermediate CD11b expression (Cd11bint) (42). An evaluation of mouse splenic myeloid cells reported Ly6Ghi or Gr1hi cells to be differentiated neutrophils (43).

In the present study, bone marrow cells were suggested to undergo phenotypic change along the course of tooth extraction wound healing highlighting the CD11bintLy6Gint cell cluster on day 3 and the CD11bhiLy6Ghi (Figure 5C) or CD11bhiGr1hi cell cluster (Figure 6B) on week 2. The local injury and infection can regulate the development and differentiation of bone marrow myeloid cells through circulating cytokines and growth factors (44,45). Therefore, it is highly
conceivable that tooth extraction wounding could influence the phenotypic plasticity of bone marrow myeloid cells. It must be noted that the effect of ZOL treatment on the bone marrow myeloid cell differentiation appeared to be less significant than the tooth extraction wounding (Figure 5 and 6).

During tooth extraction wound healing, Ly6G+/Gr1+ cells appeared in the gingival oral barrier tissue as early as day 3 as previously reported (46); however, the distribution of Ly6G+ cells in the gingival oral barrier tissue was different in ZOL mice (Figure 3). Recruitment of myeloid cells to the peripheral tissue is influenced by cytokines and chemokines, such as eotaxin (47), LIX, KC and MIP-2 (48). Separately, it has been reported that bisphosphonates impair neutrophil chemotactic activity (36,49). Because the effect of ZOL on the myeloid cell population in bone marrow (Figure 5C) and peripheral blood (data not shown) was not evident, the abnormal behavior of Ly6G+/Gr1+ cells might be specific to the oral barrier tissue.

We have previously demonstrated that osteoclast functions not only for bone resorption but also as a local immune regulator (50). The treatment with nitrogen-containing bisphosphonates, but not with non-nitrogen-containing bisphosphonate, significantly upregulated the synthesis of pro-inflammatory cytokines by osteoclasts. Human γδ-T cells, when co-cultured with ZOL-pretreated osteoclasts, increased the synthesis of IFN-gamma (31). The present study suggested that the apparent osteoclastogenesis on the palatal bone surface, the increased Ly6G+/Gr1+ cells in the oral barrier tissue, the significant down-regulation of cytokine and chemokine secretion and the development of ONJ-like lesion occurred during week 2 in ZOL mice. These events may not be a merely coincidence. Although beyond the scope of this study, it is tempting to speculate that ZOL-affected osteoclasts may mediate the locally dysregulated plasticity of Ly6G+/Gr1+ myeloid cells in the oral barrier tissue, leading to abnormal oral barrier immune reactions and the development of ONJ. Mouse model of diabetes has shown a disproportionately high level of immature Gr1+myeloid cell population and the abnormal phenotype development (51,52). The increased susceptibility of bisphosphonate-treated diabetes patients to develop ONJ (24,25) may be explained, in part, by the underlining inability in myeloid cell plasticity.

We further demonstrated that the number of Ly6G+ cells in the gingival oral barrier tissue of ZOL mice was significantly increased at week 2 (Figure 3B); and, that the size and shape of Ly6G+ cells of ZOL mice were different from those of control (NaCl) mice (Figure 3A and 3B). Migration through vascular endothelial cells and the microenvironment in the local tissue can induce rapid plasticity changes in myeloid cell phenotype and function (37). It is highly conceivable that the plasticity of Ly6G+/Gr1+ cells can be significantly affected in ZOL mice.

Favot et.al., (2013) examined the reactive-oxygen species (ROS) synthesis by neutrophils harvested from oral rinse and peripheral blood of ONJ patients (49). N-formyl-methionyl-leucyl-phenylalanine (fMLP) or phorbol myristate acetate (PMA) stimulated ROS yield was not much different between neutrophils harvested from peripheral blood of mild (Stage 0-1) and advanced (Stage 2-3) bisphosphonate-related ONJ patients. However, oral neutrophils from ONJ patients, in particular with advanced stages, showed a reduction of ROS synthesis. Taken together, it may be suggested that the function of Ly6G+/Gr1+ cells may be modulated locally in the oral barrier tissue, particularly during the second week of tooth extraction wound healing.

The development of ONJ-like lesion occurred in the second week of tooth extraction in our ZOL-treated mouse model, characterized by the increased area of osteonecrosis interfacing the inflamed oral barrier tissue and abnormal oral epithelial hyperplasia (31,50) (Figure 1). The multiplex assay of cultured oral barrier cells suggested the significant reduction in secretion of cytokines and chemokines in the ZOL group at week 2 (Figure 2C), which was consistent with the previously reported microarray-based transcriptome assay of the mouse oral barrier tissue (50). The oral barrier microenvironment of ZOL mice may be less effective for wound healing and inflammatory resolution. The abnormal cytokine and chemokine repertoire of the gingival oral barrier tissue may be involved in the ONJ development. The multiplex assay demonstrated that the control oral barrier cells secreted cytokines and chemokines suggestive of increased immunity (53), which can improve barrier
immunity (54), and stimulate alternative macrophage activation (55) and epithelial cell turnover (56).

We have further investigated if the abnormal oral barrier immune factors in ZOL mice may play a role in developing osteonecrosis. The condition medium of oral barrier cells dissociated from ZOL mice induced increased necrotic reaction in murine osteocyte-like MLO-Y4 cells in vitro (Figure 4B).

It has been shown the presence of bisphosphonates in osteocyte lacunae, while osteocytes were not necrotic, suggesting that bisphosphonates may not have the direct effect for osteonecrosis (57). We have previously reported that bisphosphonate-affected osteoclasts secreted pro-inflammatory cytokines (50), which appeared to locate adjacent to the osteonecrosis area (31) (Figure 1). This study highlighted the presence of Ly6G+/Gr1+ myeloid cells in the oral barrier tissue during the tooth extraction wound healing, which may undergo local plasticity. Taken together, the pathological development of ONJ may be induced by compounded environments involving distribution of bisphosphonate and osteoclast immunity. We further propose that aberrant myeloid cell plasticity in the oral barrier tissue and abnormal oral immunity may lead to a localized trigger initiating osteonecrosis in jawbone. In conclusion, this study suggests that local modulation and possible normalization of myeloid cell plasticity in the oral barrier tissue may provide the basis for therapeutic and preventive strategy toward ONJ.

EXPERIMENTAL PROCEDURES

Ethics Statement – The UCLA Animal Research Committee reviewed and approved all experimental protocols involving animals (ARC 1997-136).

ZOL Injection and Molar Tooth Extraction in Mice – A mouse model developing ONJ-like lesion was previously reported (31). Briefly, 7-week-old female C57Bl/6J mice (the Jackson Laboratory, Bar Harbor, ME) received a bolus injection of 500 µg/kg ZOL (Reclast, Novartis, East Hanover, NJ), whereas control mice received a 0.9% NaCl vehicle injection through the retro-orbital venous plexus (Figure 1A). Ten days later, the maxillary first molar was extracted under isoflurane anesthesia using a dental explorer. Analgesic agent, carprofen (5.0 mg/kg) was subcutaneously injected immediately prior to tooth extraction and every 24 h for 48 h after tooth extraction. Mice were euthanized at the predetermined time of tooth extraction as described below. One femur bone from each mouse was harvested, fixed in 10% buffered formalin and evaluated by micro-computed tomography (micro-CT: µCT40, Scanco Medical, Basserdorf, Switzerland) at an x-ray energy level of 55 peak kV with an intensity of 145 µA. The trabecular bone morphometry was evaluated by the proprietary algorithm (Scanco Medical, Basserdorf, Switzerland). We characterized the femur structure in all animals in this experiment to ensure the reproducible and consistent effect of ZOL in compliance to the NIH’s guideline on Authentication of Key Biological and/or Chemical Resources.

Osteonecrosis Measurement at Tooth Extraction Site – Mice were euthanized by 100% CO2 inhalation on day 3, week 2 and week 4 after tooth extraction. The maxilla containing the tooth extraction wound was harvested. After standardized digital photo recording, the maxillary tissues were fixed in 10% buffered formalin and subjected to micro-CT imaging. The fixed maxillary tissues were further decalcified with 10% EDTA (pH7.0) for 7 to 10 days prior to the paraffin-embedded histological sample preparation. Serial cross sections (4 µm thick) through the frontal plane including the tooth extraction site and the contralateral first molar were stained with hematoxylin and eosin (H & E) and scanned by a high throughput imaging system (Ariol SL-50, Applied Imaging, Grand Rapids, MI). The osteonecrosis area was defined by a cluster of 4 or more non-vital osteocytes (i.e. empty osteocyte lacunae or pyknotic osteocytes). The osteonecrosis area within the palatal bone was standardized by the bone area.

Osteoclast Measurement at Tooth Extraction Site – After deparaffinization, histological sections of mouse maxilla were stained with tartrate-resistant acid phosphatase (TRAP) using a commercially available kit (Sigma-Aldrich, St. Louis, MO) at 37°C for 2–4 hours. Nuclei were stained with hematoxylin. After staining, all slides were rinsed in 1% HCl alcohol to release the background and 1% NaHCO3 solution for recovery of hematoxylin.
staining for 3–5 seconds in sequence. Osteoclasts (OC) were defined as TRAP-positive large cells with multiple nuclei (>2 nuclei) on the bone surface. The number of OC on the surface of the palatal bone and in the bone marrow was separately counted. The number of OC was standardized by the bone surface linear length. The surface length of palatal bone or bone marrow was measured using an image-processing program (Image J, National Institutes of Health, Bethesda, MD). An operator blinded to the condition performed the histological evaluation.

Flow Cytometric Analysis of Dissociated Oral Barrier Immune Cells - The gingival oral barrier tissue including the tooth extraction wound was harvested from freshly isolated mouse maxilla on day 3, week 2 and week 4 of tooth extraction and the gingiva tissues were immediately cut into 1mm³ pieces and placed into a digestion buffer containing 1mg/ml collagenase II, 10U/ml DNAse I, and 1% bovine serum albumin in DMEM and incubated for 20 minutes at 37°C on a 150 rpm shaker. After digestion, the sample was filtered through a 40µm cell strainer and centrifuged at 1500 rpm for 10 minutes at 4°C. The pellet was re-suspended in DMEM and cells counted. In this experiment, dissociated oral barrier cells from 2 mice were combined in the each group of each time point. The dissociated cells were washed and incubated with fluorescent-conjugated anti-mouse CD45 and CD3 antibodies (BioLegend, San Diego, CA). IgG2b was used isotype control. After 30 min incubation, the antibody-stained cells were washed and analyzed by flow cytometry (EPICS XL-MCL, Coulter, Miami, FL). The data were evaluated on a computer software package (FlowJo vX, Flowjo, Ashland, OR) as described above.

Mouse Osteocyte (MLO Y4) Viability with Condition Media of Oral Barrier Cells from NaCl- or ZOL-treated Mice – To test the effect of immunological factors of oral barrier cells on the pathogenesis of osteonecrosis, murine osteocyte-like cells (MLO-Y4) (58) were incubated with the condition medium of dissociated oral barrier cells from NaCl (control) or ZOL mice. MLO-Y4 cells were expanded according to the supplier’s protocol (Kerafast, Boston, MA). In the initial study, MLO-Y4 cells were incubated with known agent to induce apoptosis or necrosis: cisplatin (cis-diammine-dichloridoplatinum: CDDP) or 2-Hydroxyethyl methacrylate (HEMA), respectively. MLO-Y4 cells in 24-well plate were incubated with the condition medium for 24 hours. After photographing, cells were trypsinized and washed twice with cold cell staining buffer and resuspended in Annexin V binding buffer.
(BioLegend, San Diego, CA). Cells were then incubated with FITC-Annexin V followed by PE-Propidium Iodide (PI) or APC-7-Aminoactinomycin D (7-AAD). After incubation at RT in the dark for 15 min, Annexin V binding buffer was added to each tube and analyzed by the flow cytometry.

**Myeloid Cells in Femur Bone Marrow and Peripheral Blood** – Mice were euthanized on day 3, week 2 and week 4 of tooth extraction as described above. Peripheral blood samples were obtained by heart puncture and peripheral blood mononuclear cells were isolated by ficoll density centrifugation. Bone marrow cells were obtained from femur by flushing with PBS containing 1% FBS. Washed cells were obtained from femur by flushing with PBS containing 1% FBS. Washed cells were incubated with fluorescent-conjugated anti-mouse CD11b, Ly6G and Ly6C antibodies (BioLegend, San Diego, CA). After antibody incubation, cells were analyzed by flow cytometry and analyzed on the computer software page as described above.

**Intraperitoneal Injection of Anti-Ly6G Antibody** – To further test the role of Ly6G+/Gr1+ cells on the tooth extraction wound healing, we injected anti-Ly6G (Gr1) antibody (InVivoPlus anti-mouse Ly6G (Gr-1), Bio X Cell, West Lebanon, NH). Mice were pre-treated with ZOL (n=3) or 0.9%NaCl (n=3) followed by maxillary left first molar extraction as described above. A group of mice received daily intraperitoneal (IP) injections of anti-Ly6G antibody (300 µg per animal) from day 9 to day 13 of tooth extraction. Another group of mice (n=3 in each group) received IP injections of isotype control antibody (InVivoPlus Rat IgG2b Isotype control anti KLH, Clone LTF-2, Bio X Cell, West Lebanon, NH). All mice were euthanized on week 2 (or day 14) of tooth extraction and the maxillary tissue was harvested. The wound healing and the area of osteonecrosis were evaluated as described above. Separately, peripheral blood and bone marrow cells were evaluated by flow cytometry.

**Statistical Analysis** – The data derived from multiple samples per group were presented as the mean ± SEM. Statistical comparison was made between control (NaCl) and ZOL group using Student’s t test. *: p<0.05; **: p<0.01; ***: p<0.001 and ****: p<0.0001.

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**CONFLICT OF INTEREST** - The authors declare that they have no conflicts of interest with the contents of this article.

**AUTHOR CONTRIBUTIONS** - I.N. and A.J., in consultation with J.L., designed this study and determined the logistics of experiments. Y.S., Ka.K., Ke.K., S.P., K.M., A. H., A.J. and I.N. performed the experiment. I.N., A.J., Y.S., Ka.K., A.K. and W.H.M. analyzed and interpreted the data. I.N., A.J., Y.S., and Ka.K. drafted the manuscript. All authors agreed on the content of the manuscript. I.N. accepts responsibility for the integrity of the data analysis.

**REFERENCES**


FIGURE LEGENDS

Figure 1: Abnormal tooth extraction wound healing in ZOL-treated mice.  A: Time course schedule of mouse experiments.  In all experiments, 7-week-old female C57BL/6 mice received a bolus IV injection of ZOL (500 µg/kg) or 0.9%NaCl vehicle solution.  Ten days later, the maxillary left first molar was extracted.  This protocol has been shown to generate ONJ-like lesion at the tooth extraction area of the maxilla of ZOL-treated mice (31).  B: Tooth extraction wound (black arrow) remained open on day 3 of the surgery in both vehicle-injected control (NaCl) and ZOL-injected (ZOL) mice.  The oral barrier tissue wound was progressively closed in control mice; however, ZOL mice showed swelling and open wound (black arrow) in the oral barrier tissue at week 2 of tooth extraction.  The oral wound was healed by week 4 in both groups.  C: Reconstructed micro-CT images of day 3 samples showed the empty tooth extraction sockets (*), which were progressively filled with regenerating bone in control mice at week 2 and week 4.  ZOL mice showed delayed bone regeneration in the extraction socket.  In addition, some ZOL mice indicated the abnormal bone formation consistent with periosteal reaction (white arrow).  D: A typical ONJ-like lesion in ZOL mice was composed of osteonecrosis (Nec) of alveolar bone interfacing the inflammatory oral barrier tissue or oral exposure site (Exp).  Oral epithelium (Epi) contacted to the necrotic alveolar bone and formed pustule/fistula (P/F).  E: The area of osteonecrosis composed of 4 or more empty osteocyte lacunae was equivalent at Day 3 but increased in ZOL mice at week 2 and remained high at week 4 (Day 3 NaCl: n=7; Day 3 ZOL: n=7; Week 2 NaCl: n=7; Week 2 ZOL: n=7; Week 4 NaCl: n=7; Week 4 ZOL: n=7).  ***: p<0.001.  F: TRAP+ osteoclasts appeared on the surface of alveolar bone interfacing the oral barrier tissue (black arrowheads) near the tooth extraction site.  In ZOL mice, some TRAP+ osteoclasts (black arrow) were found detached from the bone surface.  G: The standardized osteoclast number on the palatal bone surface increased at week 2 of tooth extraction in both control (NaCl) and ZOL mice, whereas the osteoclast number in the bone marrow space remained consistent.  The osteoclast number was not different between control and ZOL mice except that in bone marrow at week 4.  (Day 3 NaCl: n=7; Day 3 ZOL: n=7; Week 2 NaCl: n=7; Week 2 ZOL: n=7; Week 4 NaCl: n=7; Week 4 ZOL: n=7).  ***: p<0.001.

Figure 2: Oral barrier immunity during tooth extraction wound healing.  Palatal gingival tissues including wound area were harvested on day 3, week 2 and week 4 after tooth extraction and cells were dissociated for flow cytometric analysis.  Dissociated cells from 2 mice of each group were combined.  The experiment was repeated at least 3 different sittings.  The representative data were presented.  A: CD45+ immune cells (red) were consistently composed of approximately 60% of the total population (blue: isotype control) of dissociated oral barrier cells in both control (NaCl) and ZOL mice.  However, the ZOL treatment increased the proportion of CD45+ cells (82%) during the active wound-healing period of week 2.  It was also noted that the ZOL treatment developed a population with decreased CD45 staining intensity throughout the study period.  B: The proportion of T cells in CD45+ cells was found undistinguishable in control (NaCl) and ZOL mice.  C: The dissociated cells were cultured and the media collected after 3 days were evaluated by the multiplex cytokine/chemokine assay.  In the control (NaCl) group, the number and the secretion levels of cytokines and chemokines were robustly increased by oral barrier cells dissociated on week 2 of tooth extraction.  By contrast, cytokine and chemokine secretion by oral barrier cells of ZOL mice was much less on week 2, while some were remained high on week 4.  Those cytokines and chemokines with less than the detection limit were not presented.

Figure 3: Different Ly6G+ myeloid cells in the wound healing oral barrier tissue of ZOL mice.  A: Immunohistology with anti-Ly6G antibody demonstrated clusters of Ly6G+ cells at the epithelial wound margin (not shown) and at the connective tissue, both of which were adjacent to the tooth extraction site on day 3.  In ZOL mice, fewer Ly6G+ cells were present in both cluster areas.  At week 2, ZOL mice exhibited more Ly6G+ cells, in particular adjacent to jawbone oral exposure and/or pustule/fistula (P/F) areas.  In some specimens, Ly6G+ cells (arrows) were found around osteoclast (Oc) and in necrotic bone.  B: The number of Ly6G+ cells in the oral barrier tissue excluding the area of obvious jawbone exposure
and P/F areas. *: p<0.05; n=4 in each group  

** Figure 4:** Murine osteocyte-like MLO-Y4 cell necrosis in vitro, induced by condition medium of oral barrier cells dissociated from ZOL mice. A: MLO-Y4 cells demonstrated the high sensitivity to HEMA-induced necrosis assessed by Annexin V/7AAD positive staining. By contrast, MLO-Y4 cells were relatively resistant to CDDP-induced apoptosis. B: MLO-Y4 cells exposed to the condition medium of ZOL mouse oral barrier cells showed the increased necrotic reaction than those exposed to the condition medium of control (NaCl) mouse oral barrier cells. C: MLO-Y4 cells exposed to the control (NaCl) mice oral barrier cell condition medium maintained the typical fibroblastic morphology with dendritic processes. The overnight incubation with the ZOL mice oral barrier cell condition medium induced enlarged cell morphology with pyknotic nucleus, which lacked dendritic processes (white arrows). The exposure to HEMA resulted in necrosis of the majority of MLO-Y4 cells.

** Figure 5:** Effect of ZOL on bone marrow myeloid cells. A: Ten days after a bolus IV injection of 500 µg/kg ZOL, the maxillary first molar was extracted and femur samples were harvested at day 3, week 2 and week 4 of tooth extraction. Reconstructed micro-CT images of the distal epiphyseal trabecular bone indicated the increased bone structure in ZOL mice. B: Micro-CT quantitative measurements indicated the significant and progressive increase of femur trabecular bone structure in ZOL mice as depicted by bone volume/tissue volume (BV/TV) and connectivity density (Conn.D). **: p<0.01; ***: p<0.001; ****: p<0.0001; n=7 in each group  

** Figure 6:** Effect of anti-Ly6G (Gr1) neutralizing antibody injection on bone marrow and peripheral blood. A: After a bolus IV injection of ZOL (500 µg/kg) or 0.9% NaCl vehicle solution and maxillary molar extraction, daily IP injections of anti-Ly6G (Gr1) neutralizing antibody (300 µg/animal/day) were performed starting from day 9 to day 13 of tooth extraction. Bone marrow flow through cells and peripheral blood samples were harvested 1 day after the last anti-Ly6G (Gr1) antibody injection or on week 2 of tooth extraction. B: Flow cytometric analysis of myeloid cell gate of bone marrow flow through cells indicated that antibody untreated and isotype control antibody injected mice presented the high concentration of CD11b+Gr1hi cells or CD11b+Ly6Ghi cells, respectively. By contrast, anti-Ly6G (Gr1) antibody injected mice contained largely CD11b+Gr1int cells with much reduced presence of CD11b+Gr1hi cells. ***: p<0.001; n=3 in each group  

** Figure 7:** Prevention of ONJ-like lesion by anti-Ly6G (Gr1) neutralizing antibody. A: Gingival swelling at the tooth extraction site (arrowheads) was exacerbated in ZOL mice, which also exhibited delayed wound closure (arrows). Mice received isotype control antibody injection showed essentially identical reactions as the antibody untreated mice. By contrast, anti-Ly6G (Gr1) antibody injections resulted in significantly attenuated gingival swelling in both control (NaCl) and ZOL mice. There was no open
wound in anti-Ly6G (Gr1) antibody-injected ZOL mice on week 2 of tooth extraction. **: The area of histological osteonecrosis in the palatal bone was increased in ZOL mice. However, anti-Ly6G (Gr1) antibody injection appeared to attenuate the development of osteonecrosis. **: p<0.01; n=6 in each group.

C: Histological analysis revealed a localized inflammatory reaction (Inf, double arrow) in gingival connective tissue (G.Co.) interfacing palatal bone of control (NaCl) mice with the isotype control antibody injection. ZOL mice with the isotype control antibody injection exhibited not only the localized inflammation but also a large area of diffused inflammatory lesion, which was associated with osteonecrosis (Nec) and epithelial hyperplasia (Epi). Anti-Ly6G (Gr1) antibody injection resulted in the complete remission of inflammatory reaction in the gingival connective tissue interfacing palatal bone of both control (NaCl) and ZOL mice. New bone formation (N.B.) in the tooth extraction socket was noted in the anti-Ly6G (Gr1) antibody injection groups. D. Immunohistochemical evaluation showed the presence of Ly6G+ cells in the diffused inflammatory zone (Inf, double arrow) of ZOL mice with isotype control antibody injection. In ZOL mice with anti-Ly6G (Gr1) antibody injection, Ly6G+ cells were not observed in the gingival connective tissue (G.Co) interfacing the palatal bone.
Figure 1

A. Timeline of experimental procedures:
- -10d
- 0d
- 3d
- 2W
- 4W

- ZOL (500µg/kg) or 0.9% NaCl IV injection
- Maxillary 1st Molar Extraction
- Tissue and Cell Collection

B. Imaging of tooth samples:
- Day 3
- Week 2
- Week 4

- NaCl
- ZOL

C. Imaging of tooth roots:
- Day 3
- Week 2
- Week 4

D. Histological section image:
- Epi, Exp, P/F, Nec

100 µm

E. Graphs of osteonecrosis area:
- Day 3
- Week 2
- Week 4

F. Images of bone marrow:
- NaCl
- ZOL

50 µm

G. Graphs of osteoclast number:
- Palatal Bone Surface
- Bone Marrow

- NaCl
- ZOL

Day 3
- Week 2
- Week 4
Figure 2

**A**

Day 3

Week 2

Week 4

**B**

**C**

Day 3

Week 2

Week 4

Protein Concentration (pg/ml)
Figure 4

A

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Annexin V

7AAD

B

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</thead>
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<tr>
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Control Medium

Untreated

Untreated

Annexin V

NaCl (control) mice

ZOL mice

(%) 6.11 11.5

NaCl (control) mice

ZOL mice

(%) 5.27 8.57

Annexin V

PI

C

Oral Barrier Cell Condition Medium

NaCl (control) mice

ZOL mice

HEMA
Figure 6

A

- ZOL (500μg/kg) Or
- 0.9% NaCl IV injection
- Maxillary 1st Molar Extraction
- Tissue and Cell Collection
- Anti-Ly6G antibody IP Injections (daily)

B

Antibody Treatment

Untreated Anti-Ly6G Isotype Control

CD11b*Gr1*Ly6G

Gr1 Ly6G

C

Antibody Treatment

Untreated Anti-Ly6G Isotype Control

CD11b*Gr1*Ly6G

Gr1 Ly6G

*** ***
Figure 7

A

Antibody Treatment

Untreated Isotype Control Anti-Ly6G

NaCl

ZOL

B

Osteonecrosis Area (%)

Isotype Control Anti-Ly6G

NaCl ZOL

C

Isotype Control Anti-Ly6G

NaCl

ZOL

D

Isotype Control Anti-Ly6G

ZOL

G.Co. Inf Nec Epi
Plasticity of Myeloid Cells during Oral Barrier Wound Healing and the Development of Bisphosphonate-Related Osteonecrosis of the Jaw
Yujie Sun, Kavaljit Kaur, Keiichi Kanayama, Kenzo Morinaga, Sai Park, Akishige Hokugo, Anna Kozlowska, William H. McBride, Jun Li, Anahid Jewett and Ichiro Nishimura

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