Implant Wear Induces Inflammation, but Not Osteoclastic Bone Resorption, in RANK$^{-/-}$ Mice

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ABSTRACT: Signaling of RANK (receptor activator of nuclear factor kappa B) through its ligand RANKL appears critical in osteolysis associated with aseptic loosening (AL). The purpose of this study was to investigate the role of RANK in a murine osteolysis model developed in RANK knockout (RANK$^{-/-}$) mice. Ultra high molecular weight polyethylene (UHMWPE) debris was introduced into established air pouches on RANK$^{-/-}$ mice, followed by implantation of calvaria bone from syngeneic littermates. Wild type C57BL/6 (RANK$^{+/+}$) mice injected with either UHMWPE or saline alone were included in this study. Pouch tissues were collected 14 days after UHMWPE inoculation for molecular and histology analysis. Results showed that UHMWPE stimulation induced strong pouch tissue inflammation in RANK$^{-/-}$ mice, as manifested by inflammatory cellular infiltration, pouch tissue proliferation, and increased gene expression of IL-1β, TNFα, and RANKL. However, the UHMWPE-induced inflammation in RANK$^{-/-}$ mice was not associated with the osteoclastic bone resorption observed in RANK$^{+/+}$ mice. In RANK$^{+/+}$ mice subjected to UHMWPE stimulation, a large number of TRAP^{+} cells were found on the implanted bone surface, where active osteoclastic bone resorption was observed. No TRAP^{+} cells were found in UHMWPE-containing pouch tissues of RANK$^{-/-}$ mice. Consistent with the lack of osteoclastic activity shown by TRAP staining, no significant UHMWPE particle-induced bone resorption was found in RANK$^{-/-}$ mice. A well preserved bone collagen content (Van Gieson staining) and normal plateau surface contour [microcomputed tomography (μCT)] of implanted bone was observed in RANK$^{-/-}$ mice subjected to UHMWPE stimulation. In conclusion, this study provides the evidence that UHMWPE particles induce strong inflammatory responses, but not associated with osteoclastic bone resorption in RANK$^{-/-}$ mice. This indicates that RANK signaling is essential for UHMWPE particle-induced osteoclastic bone resorption, but does not participate in UHMWPE particle-induced inflammatory response. © 2006 Orthopaedic Research Society. Published by Wiley Periodicals, Inc. J Orthop Res 24:1575–1586, 2006

Keywords: RANK; RANKL; wear debris; osteolysis; osteoclastogenesis

INTRODUCTION

Aseptic loosening (AL) is characterized by the development of an inflammatory membrane at the bone–implant interface, leading to bone resorption and loss of prosthesis fixation.[1] Enhanced osteoclastogenesis (formation of mature osteoclasts from their precursor stem cells) is a hallmark of osteoporotic complications, including the bone loss associated with AL.[2] Two important regulators of osteoclastogenesis have been recently identified.[3,4] Receptor activator of nuclear factor kappa B (RANK, TNFR11B) is a novel type I transmembrane receptor of the TNF receptor superfamily (TNFRSF) that was originally identified using a dendritic cell cDNA library.[5] RANK is ubiquitously expressed in human tissues, but is particularly apparent on the cell surface of osteoclasts and their precursors.[4] Its ligand, RANKL, also known as ODF/TRANCE/OPGL, is a TNF-related cytokine that is produced by marrow stromal cells and osteoblasts.[6] The availability of a soluble form of recombinant human RANKL, comprising the extracellular domain of the protein (sRANKL), has allowed the in vitro generation of large numbers of bone resorbing osteoclasts from hemopoietic precursors in the absence of stromal/osteoblast cells.[7] An emerging concept is that RANK represents a common mediator for inflammatory osteoclastogenesis.[8–10] Li et al.[8] reported that osteoclastogenesis was not observed

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in RANK gene knockout ($RANK^{-/-}$) mice following IL-1 treatment, illustrated by the complete absence of $TRAP^+$ and cathepsin K^+ osteoclasts in calvaria. In addition, the failure of $RANK^{-/-}$ mice to mount a significant osteoclastic response in the presence of experimental inflammatory arthritis further supports the concept that RANK plays a gate-keeping role in controlling osteoclastogenesis,[10] and mice lacking RANKL have defects in osteoclastogenesis that leads to severe osteopetrosis.[10] However, there is controversy as to whether TNFα can stimulate myeloid osteoclastogenesis by a RANK/RANKL independent mechanism.[11,12] Sabokbar et al.[13] found that the addition of TNFα to macrophages isolated from human loosening periprosthetic tissues induced the formation of $TRAP^+$ multinucleated osteoclasts capable of extensive lacunar resorption. TNFα-induced osteoclastogenesis cannot be blocked by the treatment with either osteoprotegerin (OPG) or RANK:Fc IgG (which inhibit RANKL-induced osteoclastogenesis), suggesting this process is distinct from the RANK/RANKL signaling pathway. These data are interesting because one favored hypothesis concerning the pathogenesis of AL is that wear particles generated from the prosthesis stimulate phagocytosis by macrophages with the subsequent release of proinflammatory cytokines.[14–16] Ultra high molecular weight polyethylene (UHMWPE) has been widely used in total joint prosthesis, and UHMWPE components removed at revision surgery usually show evidence of wear.[17,18] Using our established mouse osteolysis model, we have demonstrated that UHMWPE particles provoke significant inflammation, osteoclastogenesis, and bone resorption.[19,20] The purpose of this study was to investigate the role of RANK in UHMWPE particle-induced inflammatory osteolysis by the use of RANK knockout ($RANK^{-/-}$) mice.

MATERIALS AND METHODS

Ultra High Molecular Weight Polyethylene (UHMWPE) Particles

UHMWPE particles (the generous gift of Dr. John Cuckler from University of Alabama, Birmingham, AL) were used to stimulate inflammatory reactions. The size and distribution of UHMWPE particles was evaluated using a Coulter particle counter and by scanning electron microscopy, as described elsewhere.[21] The UHMWPE particles had a mean particle diameter of 2.6 μm, with a range from <0.6 μm to 21 μm. The particles were washed in 70% ethanol solution to remove endotoxin and heat sterilized, and the absence of endotoxin was confirmed using the Limulus assay (Endosafe; Charles Rivers, Charlestown, SC). Particles were suspended in sterile PBS and stored at 4°C until use.

Mouse Osteolysis Model

Institutional approval was obtained for all animal procedures. The use of the bone implanted inflammatory air pouch as a model of debris-induced osteolysis has been described previously.[19] Air pouches were established by subcutaneous injection of sterile air on the back of C57BL/6 mice of either $RANK^{+/+}$ or $RANK^{-/-}$ phenotype (the generous gift of Amgen Inc., Seattle, WA). Each experimental group consisted of 10 mice. Six days later, mice were anesthetized by the intraperitoneal injection of pentobarbital (50 mg/kg; Fisher Scientific, Pittsburgh, PA). A 0.5-cm incision overlying the air pouch was made and a section of calvaria bone from a genetically identical donor mouse (approximately 0.4×0.25 cm) was inserted into the pouch. The pouch layers and the skin incision were then closed using 4-0 Prolene sutures. On the following day, pouches were injected with 0.5 ml saline containing 1% UHMWPE particles. Pouches injected with saline alone were used as negative controls in $RANK^{+/+}$ mice. Mice were sacrificed in a carbon dioxide chamber 2 weeks after bone implantation. The pouch membranes containing implanted bone were harvested for histological and molecular analyses.

Histological Assessment

Pouch tissues were fixed in 10% formalin, decalcified in 10% EDTA, and paraffin embedded. Paraffin sections (6 μm) were stained with hematoxylin & eosin (H&E) and the microscopic digital images photographed and analyzed as described previously.[19,22] Modified naphthol AS-D chloroacetate esterase staining was used to detect monocytes/macrophages. Osteoclast formation was determined by tartrate-resistant acid phosphatase (TRAP, EC3.1.3.2) staining in paraffin tissue sections (6 μm) using a commercial kit (Sigma, St. Louis, MO), as described previously.[23] The presence of dark purple staining granules in the cytoplasm was considered as the specific criterion for $TRAP^+$ cells. Positive TRAP localization was quantified by pixel area count and reported as a percentage of the of total implanted bone area in the porch tissue. Bone collagen depletion was determined by Van Gieson stain and quantified by image analysis[19,24] using the ImagePro software package (MediaCybernetics, Silver Spring, MD, USA).

Immunohistological Staining for TNFα, IL-1β, CD68, and RANKL

Paraffin tissue sections were deparaffinized, and rinsed sections were immersed into antigen retrieval buffer (BioGenex, San Ramon, CA) with microwave heating

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(power 60% for 2 min, followed by power 30% for 5 min), followed by cooling to room temperature. Sections were blocked with 1.5% normal goat serum for 1 h, and incubated with goat anti-mouse TNFα, IL-1β, CD68, and RANKL antibodies (2 μg/ml, Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C overnight. After rinsing, sections were incubated for 30 min with biotin-conjugated secondary antibody (Vector Laboratories, Burlingame, CA). Avidin biotin enzyme reagent was then applied onto sections for 30 min. Color was formed by adding 3.3′-diaminobezidine tetrahydrochloride (DAB), with counterstaining using Mayer's hematoxylin. In negative control sections, the primary antibody was omitted or an irrelevant antibody applied at the same concentration as the primary antibody. Digital images of representative fields of view were captured and analyzed using the ImagePro software package.

Enzyme Linked Immunosorbent Assay (ELISA)

ELISA assays were performed to examine the protein expression of IL-1β and TNFα in the homogenates of pouch membrane using commercial kits (R&D, Minneapolis, MN) following manufacturer's instructions.

Gene Level of RANKL and Cathepsin K (CPK)

Total RNA from pouch homogenates was reverse transcribed to cDNA as described elsewhere.[22] Real time quantitative PCR was carried out according to the manufacturer's instruction (Perkin Elmer-Applied Biosystems, Foster City, CA). Primers used in this study have been described elsewhere.[20] To standardize the target gene level with respect to variability in RNA and cDNA concentrations, the housekeeping gene GADPH was used as an internal control. To determine the relative level of gene expression, the comparative C_T (threshold cycle) method with arithmetic formulas was used. Subtracting the C_T of the housekeeping gene from the C_T of target gene yields the ΔC_T in each group (control and experimental groups), which was entered into the equation $2^{-\Delta CT}$ and calculated for the exponential amplification of PCR. The gene activity in control group (PBS) was arbitrarily assigned to 1 to serve as reference. The expression of the target gene from experimental groups therefore represents the fold-difference expression relative to the reference gene.

Microcomputed Tomography (MicroCT) Image Acquisition

Mice were scanned 2 weeks after bone implantation (right before termination of the experiment) using an RS-9 In Vivo MicroCT Scanner (General Electric Systems, London, Ontario). The scan was performed at a resolution of 0.093 mm (cubic voxel length) using the manufacturer's defined settings. After data acquisition, two-dimensional projection data was reconstructed into three-dimensional volumes, which were then reoriented in the appropriate dimensions to place the data sets in consistent orientation for image analysis.

Statistical Analysis

Statistical analysis among groups was performed using the ANOVA test, with the Schafer formula for post hoc multiple comparisons, using the SPSS software package (version 7.5; SPSS Inc., Chicago, IL). Data were expressed as mean $\pm$ standard error of the mean. A p-value of less than 0.05 was considered as significant difference.

RESULTS

UHMWPE Particle-Induced Tissue Inflammation in RANK$^{+/+}$ and RANK$^{-/-}$ Mice

UHMWPE particle induced a similar level of pouch tissue inflammation in RANK$^{-/-}$ mice when compared with RANK$^{+/+}$ mice. As seen in Figure 1A, gross pathology verified that pouches injected with UHMWPE particle developed a pronounced inflammatory response in both RANK$^{+/+}$ and RANK$^{-/-}$ mice, as compared with control pouches that received saline injection. H&E staining (Fig. 1B) revealed that UHMWPE particle stimulation significantly increased both the membrane thickness and the number of infiltrating cells in pouches, as compared to saline controls. Quantitative image analysis of histological sections revealed that both cellular infiltration ($7{,}878 \pm 1{,}531$ mm^2) and membrane thickness (0.132 ± 0.04 mm) in RANK$^{-/-}$ mice were significantly increased, as compared with saline control mice ($4{,}789 \pm 1{,}065$ mm^2 and 0.089 ± 0.03 mm, respectively; $p < 0.05$), but showed no difference compared with RANK$^{+/+}$ mice. There was no difference with respect to any UHMWPE particle-induced inflammation parameters between RANK$^{-/-}$ mice and RANK$^{+/+}$ mice (Fig. 2).

We investigated whether UHMWPE particles can induce macrophage infiltration in pouch tissues in RANK$^{-/-}$ mice. As shown in Figure 3B, nonspecific esterase (NSE) stained cells were significantly increased in UHMWPE stimulated pouch tissues from both RANK$^{-/-}$ and RANK$^{+/+}$ mice. These NSE$^+$ cells were particularly concentrated in regions of tissue with marked particle accumulation. The local macrophage accumulation was confirmed by immunohistochemical staining using anti-CD68, a specific cell surface marker for activated macrophages (Fig. 3A). These data indicated that the UHMWPE particle-induced

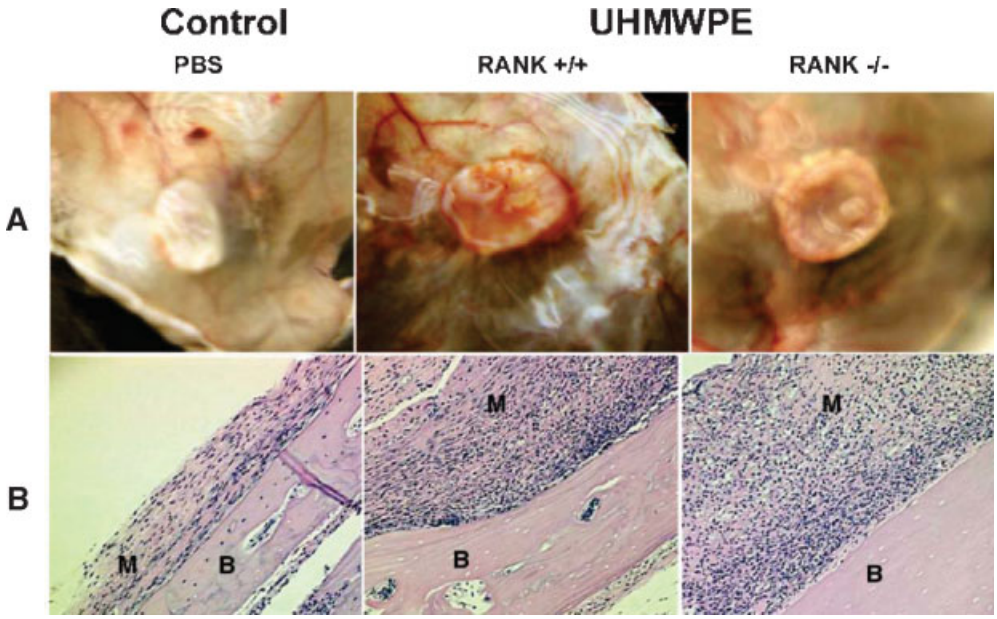

Figure 1. Representative pouch tissues histology of both macroscopic (A) and microscopic (B) appearance. All the main micrographs (B) are tissue sections stained with hematoxylin and eosin (H&E). (Original magnification, ×100.) B, implanted bone; M, pouch membrane. Note the significant inflammatory cellular infiltration and proliferated pouch membranes in UHMWPE containing pouches of both $RANK^{+/+}$ and $RANK^{-/-}$ mice.

recruitment of macrophages was not impaired in $RANK^{-/-}$ mice.

UHMWPE Particle-Stimulated IL-1β and TNFα Production in Pouch Tissues from $RANK^{-/-}$ Mice

UHMWPE particle-stimulated tissue inflammation was invariably accompanied by local expression of proinflammatory cytokines, such as IL-1β and TNFα. As seen in Figure 4A, immunostaining revealed that increased staining of both IL-1β and TNFα occurred in UHMWPE stimulated pouches from $RANK^{-/-}$ mice, compared with saline controls. Staining for these cytokines in UHMWPE stimulated pouches was predominantly located within the cytoplasm of inflammatory cell aggregates. There was no significant difference with respect to the staining intensity of either IL-1β or TNFα between $RANK^{-/-}$ and $RANK^{+/+}$ mice. The protein levels of IL-1β and TNFα was also measured in the supernatants of pouch homogenates using the ELISA assay (Fig. 4B), and a similar protein expression profile was observed. These data verify that, similar to $RANK^{+/+}$ mice, a significant increase of proinflammatory cytokine production can be induced by UHMWPE particle stimulation in $RANK^{-/-}$ mice.

UHMWPE Particle Did Not Induce Osteoclastic Bone Resorption in $RANK^{-/-}$ Mice

Immunohistochemical staining was used to investigate RANKL protein expression in $RANK^{-/-}$ mice. As depicted in Figure 3, intense RANKL staining was observed in UHMWPE stimulated pouches from both $RANK^{-/-}$ and $RANK^{+/+}$ mice, compared with levels seen in saline controls. RANKL staining was predominantly seen at the membrane interface with implanted bone, and in the cytoplasm of inflammatory cells that were forming debris associated aggregates. Similar to the immunohistochemical findings, real time RT-PCR assay (Fig. 5) showed that UHMWPE particles significantly increased RANKL gene expression in pouch tissues of both $RANK^{+/+}$ mice (5.2-fold) and $RANK^{-/-}$ mice (3.9-fold), as compared with saline control ($p < 0.05$). There is no statistical difference of RANKL gene level between $RANK^{-/-}$ mice and $RANK^{+/+}$ mice.

Although UHMWPE particle increased RANKL gene production in $RANK^{-/-}$ mice, no UHMWPE particle-stimulated osteoclastogenesis and bone resorption changes were observed in $RANK^{-/-}$ mice. As shown in Figure 5B, UHMWPE particle stimulated CPK gene expression in $RANK^{+/+}$ mice

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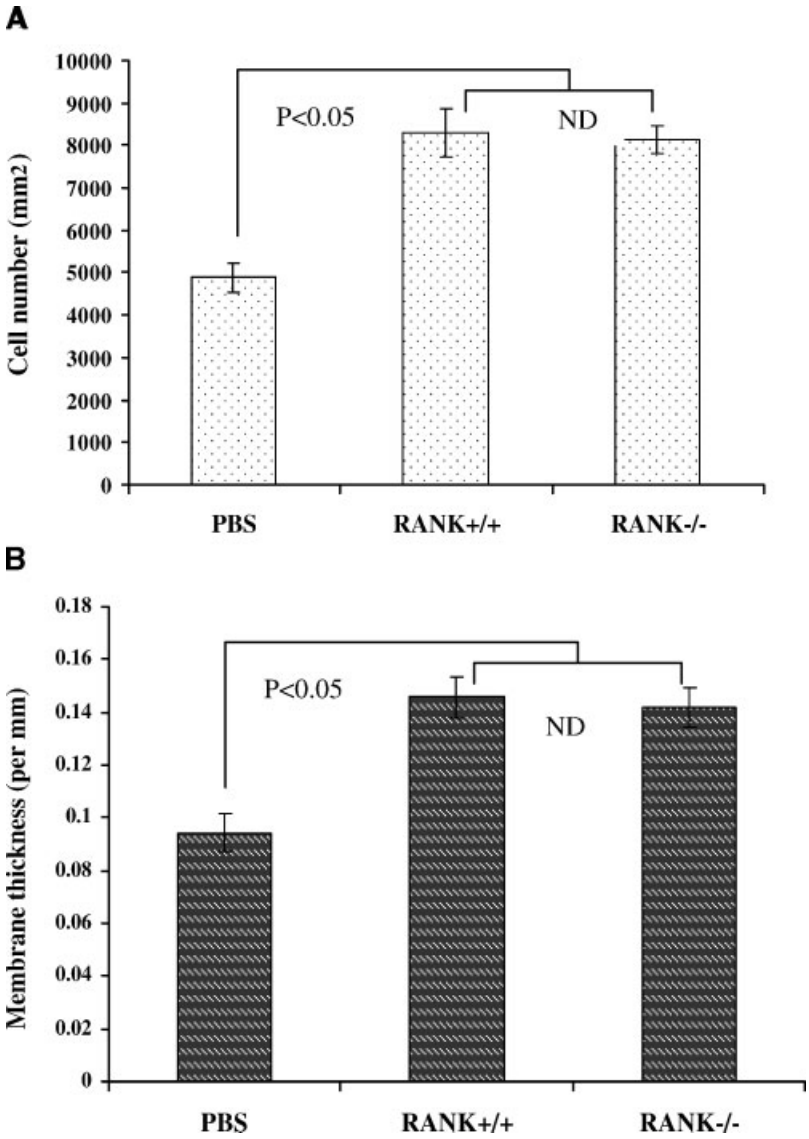

Figure 2. Quantitative histological assessment of UHMWPE-stimulated total cell counts (A) and pouch membrane thickness (B). Minimum of three separate sections per specimen were evaluated in a blinded fashion using the ImagePro software. Data expressed as mean ± SD for total 30 mice (10 mice per group). $p < 0.05$; ND, no statistical difference.

(4.1-fold). Interestingly, CPK gene expression was significantly decreased in RANK$^{-/-}$ mice, and represents only 25% of total CPK level, as compared with saline controls, or 10% of corresponding RANK$^{+/+}$ mice ($p < 0.05$). Constitutive expression of a housekeeping gene, GAPDH, was essentially constant with a relative standard error of ±3% for all groups in the presence or absence of UHMWPE particle stimulation (data not shown).

Histochemical TRAP staining was used to address whether UHMWPE particle can induce osteoclast formation in RANK$^{-/-}$ mice. As shown in Figure 6A, a discrete focus of TRAP staining was observed at the interface between implanted bone and pouch membrane in pouches with saline injection alone. The bone morphology remained intact, and no resorption lacunae were observed. Intensified TRAP staining was found in pouches following UHMWPE stimulation, and extended into the adjoining tissues in RANK$^{+/+}$ mice. As depicted in Figure 6B, a significant ($p < 0.05$) increase in TRAP^{+} cells (6.5-fold) was observed in UHMWPE-containing pouches of RANK$^{+/+}$ mice, compared to saline controls. Regions where TRAP^{+}

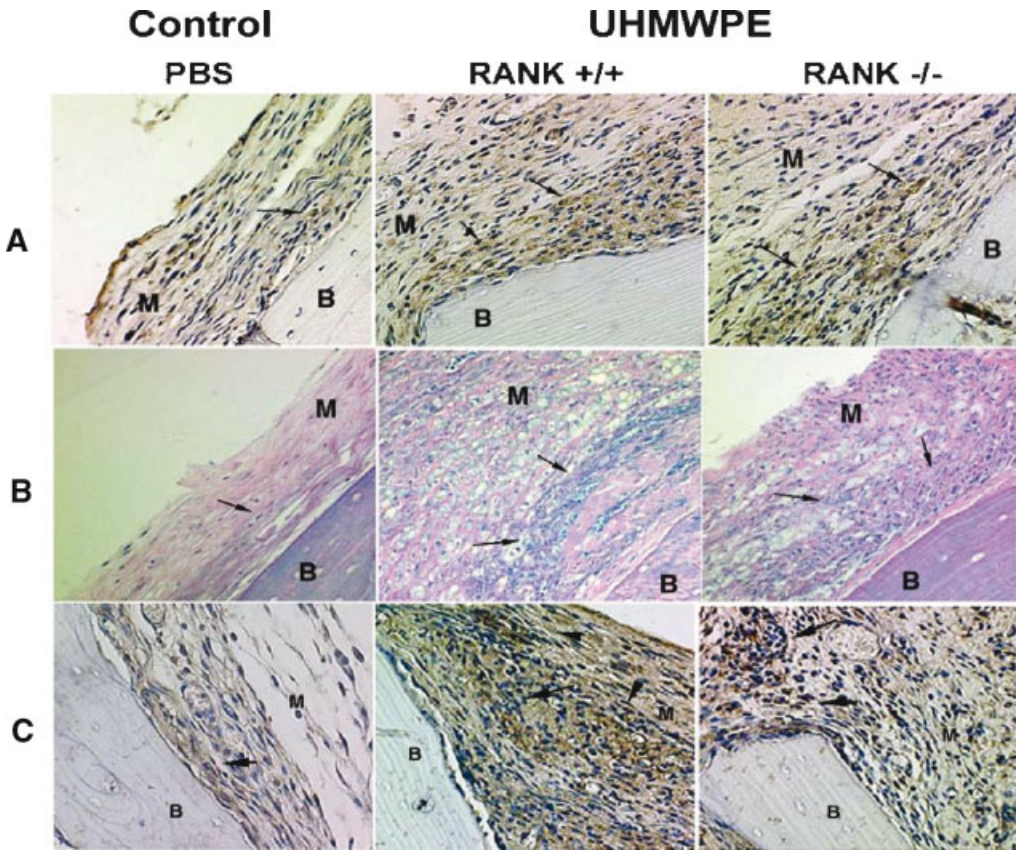

Figure 3. Macrophage staining and immunohistological staining of CD68 and RANKL in pouch membrane. The number of infiltrating macrophages in pouch membrane was evaluated by both immunostaining of CD 68, a macrophage cell marker (A) and nonspecific esterase (NSE) histological stain (B). Immunohistological staining of RANKL in pouch membrane was shown in (C). B, implanted bone; M, pouch membrane. Arrows indicate positive staining. (Original magnification, $\times 200$).

cells localized were often pitted, suggesting active osteoclastic bone resorption. However, no TRAP staining was observed in any specimens from $RANK^{-/-}$ mice, suggesting that osteoclastogenesis is completely blocked in the absence of a functional RANK gene.

Van Gieson stain was performed to quantify the bone collagen contents of implanted bone in pouch tissues, and representative images of van Gieson stained sections are shown in Figure 7A. UHMWPE particle stimulation dramatically increased the loss of bone collagen content at the bone surface in close contact with the inflammatory pouch membranes, in comparison to the bone collagen changes in sections from control (saline-stimulated) pouches. Quantitative image analysis indicated that the bone collagen content of implanted bone was well preserved in $RANK^{-/-}$ mice (Fig. 7B, $p < 0.05$).

As shown in Figure 8, the μCT imaging analysis of implanted bones showed a significant alteration of plateau surface contour in pouches with UHMWPE particle stimulation in $RANK^{+/+}$ mice, as compared to control (saline-stimulated) pouches, suggesting the progressive implanted bone degradation. However, UHMWPE particle stimulation did not induce any visible alteration of plateau surface contour in pouches from $RANK^{-/-}$ mice.

DISCUSSION

$RANK^{-/-}$ mice develop severe osteopetrosis characterized by the accumulation of newly synthesized bone and defective bone resorption.[8,9] Hematopoesis in these mice is unaffected, with the exception that osteoclasts are absent throughout the skeleton.[8,9] Our study revealed that UHMWPE stimulation induced strong pouch

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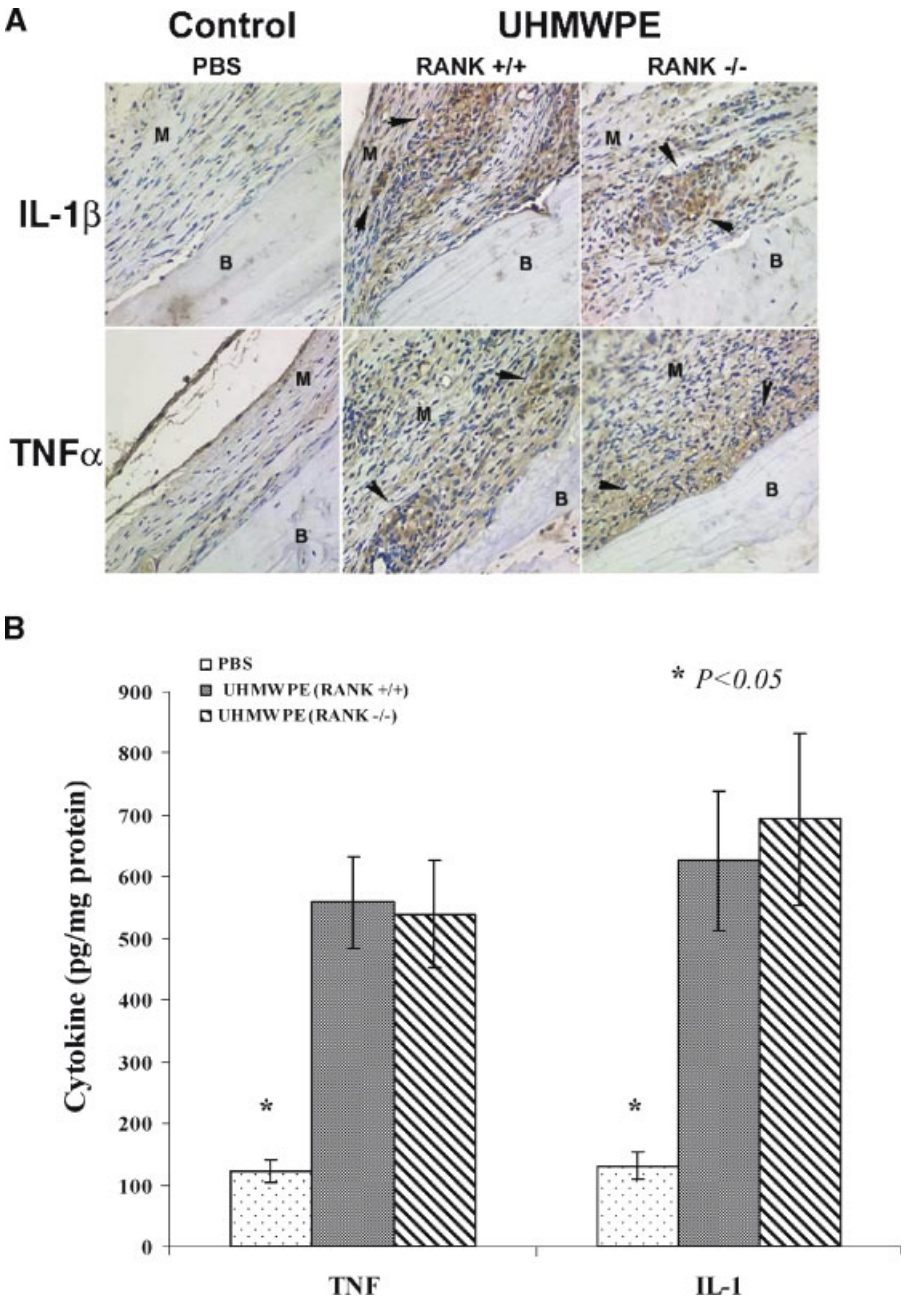

Figure 4. Expression of IL-1β and TNFα in pouch membranes. (A) Immunohistochemical staining of IL-1β and TNFα in pouch membranes. B, implanted bone; M, pouch membrane. Arrowheads indicate positive staining. (Original magnification, ×200.) (B) The protein level of IL-1β and TNFα in the supernatants of the pouch membrane homogenates was measured by ELISA. Data expressed as mean ± SD for total 30 mice (10 mice per group). *$p < 0.05$.

tissue inflammation in RANK$^{-/-}$ mice, as manifested by inflammatory cellular infiltration, pouch tissue proliferation, and increased gene expression of IL-1β, TNFα, and RANKL. However, the UHMWPE-induced inflammation in RANK$^{-/-}$ mice was not associated with the osteoclastic bone resorption observed in RANK$^{+/+}$ mice, as evidenced by the lack of TRAP^{+} cells, remarkable lower CPK gene production, and well preserved bone morphology and bone collagen contents of implanted bone in pouch tissues. Our data indicates that RANK signaling is essential for

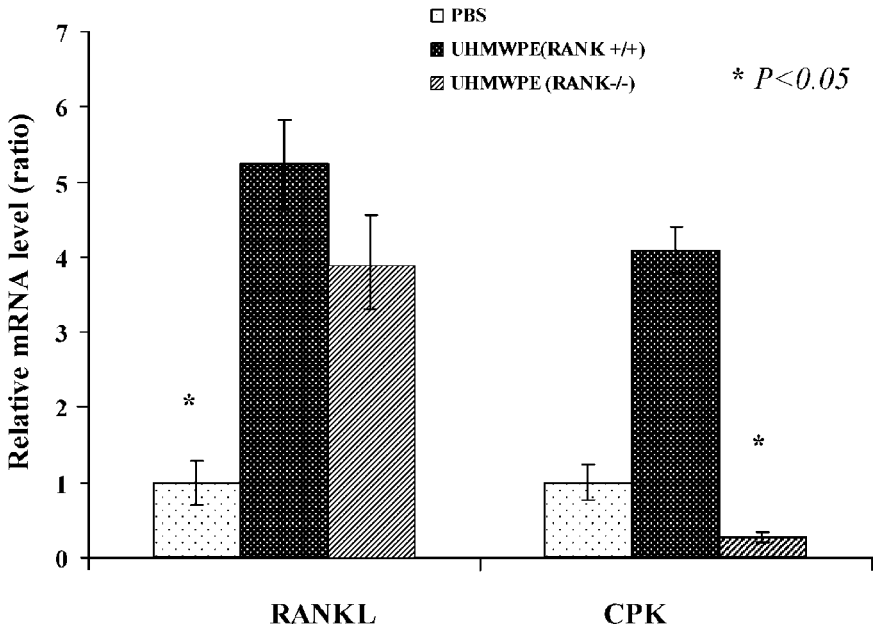

Figure 5. Real time RT-PCR analysis of mRNA gene copies of RANKL and cathepsin K (CPK) in mouse pouch tissues. Pouch membranes were harvested 14 days after bone implantation. Total RNA prepared from pouch membranes were reverse transcribed to cDNA for RT-PCR analysis. Values are mean ± SD for total 30 mice (10 mice per group). *$p < 0.05$.

UHMWPE particle-stimulated inflammatory osteoclastogenesis, and may play a critical role in the AL development.

In 1999, Dougal et al.[9] generated RANK$^{-/-}$ mice, and demonstrated that RANK expression was not required for the commitment, differentiation, and functional maturation of macrophages and dendritic cells from their myeloid precursors. These macrophages are functionally intact and normally distributed in the tissues of RANK$^{-/-}$ animals. These findings can explain why UHMWPE particles can induce similar tissue inflammation and macrophage infiltration responses in pouch tissues of RANK$^{-/-}$ mice, when compared with RANK$^{+/+}$ mice.

RANK is the sole osteoclast receptor for RANKL in vivo.[8] Our previous in vivo study[19,20] demonstrated that UHMWPE particle stimulation significantly increased RANKL production, and a positive association was found between RANKL gene expression and pouch tissue inflammation, as well as the gene level of IL-1β and TNFα (which tend to be co-produced at sites of local inflammation). Abnormally higher levels of RANKL (mRNA and protein) were predominantly associated with cells containing wear particles, and may significantly contribute to the UHMWPE particle-induced osteoclastogenesis.[25] The data from this study illustrate that UHMWPE particles induce similar pouch tissue inflammation, and increased gene expression of RANKL, TNFα, and IL-1β in RANK$^{-/-}$ mice. CPK is a protease known to be a cellular marker for mature osteoclasts.[26] We found that CPK gene expression was significantly decreased in RANK$^{-/-}$ mice, and represents only 25% of total CPK level observed in saline controls. A recent study[27] reported that RANKL stimulates CPK mRNA expression in a dose- and time-dependent fashion, indicating that activation of RANK signaling is critical in the regulation of CPK gene expression. Most importantly, the UHMWPE particle-induced osteoclastic bone resorption developed in RANK$^{+/+}$ mice, was not observed in RANK$^{-/-}$ mice. These findings support the concept that RANK is the sole receptor of RANKL, and is essential for the development of UHMWPE particle-induced osteoclastic bone resorption. Our findings are in agreement with a recent report by Schwarz et al.,[28] where treatment with antagonist RANK:Fc IgG successfully prevented titanium (Ti) debris-induced inflammatory osteolysis in a mouse calvaria model, yielding results that were statistically equivalent to data obtained with Ti-treated RANK$^{-/-}$ mice.[29]

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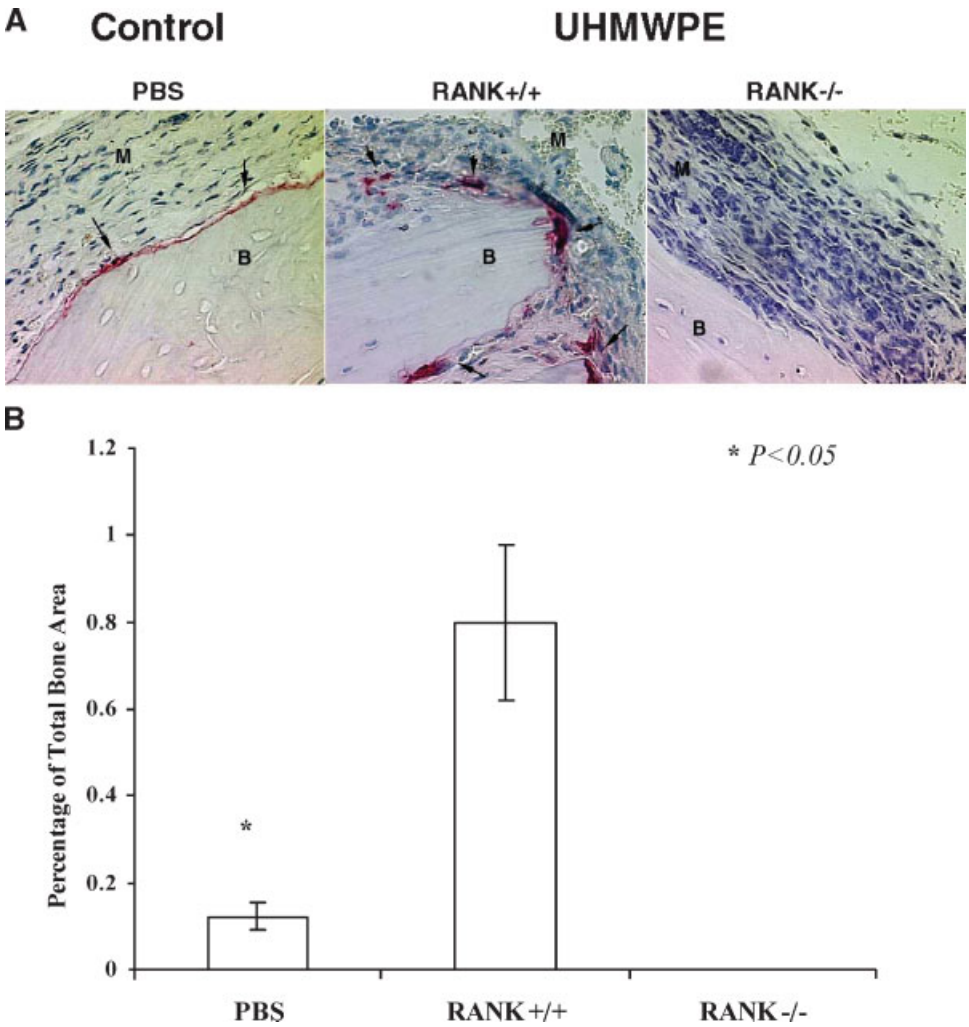

Figure 6. TRAP^{+} cells are absent in RANK$^{-/-}$ mice. (A) The representative TRAP staining in paraffin tissue sections (original magnification, ×200). TRAP was stained dark red and is indicated by arrows. B, implanted bone; M, pouch membrane. (B) TRAP^{+} cells were quantified by Image Analysis software as described in Materials and Methods. The value represents TRAP^{+} cells location quantified as percentage of total implanted bone area. Values are mean ± SD for total 30 mice (10 mice per group). *$p < 0.05$.

A major controversy has arisen due to disagreement in findings regarding the role of TNFα in the induction of osteoclastogenesis in the absence of RANK signaling. Azuma et al.[11] were the first to report that TNFα can directly induce murine osteoclast precursors to differentiate to functional osteoclasts in the presence of M-CSF. Similar data were provided by Kobayashi et al.[12] In both these studies, bone marrow cells (containing osteoclast precursors and bone marrow stromal cells) were cultured in the presence of M-CSF to expand the population of osteoclast progenitors prior to purifying the cells for use in experiments with TNFα. Interpretation of their results was questioned, because previous studies have shown the importance of RANKL/RANK signaling for osteoclastogenesis in RANK$^{-/-}$ mice.[6,8] Lam et al.[30] demonstrated that when pure populations of mononuclear phagocytes were isolated directly from murine bone marrow by immunopurification, these cells failed to differentiate into osteoclasts in the presence of M-CSF and TNFα even at superphysiological concentrations. These data suggested that TNFα can only induce osteoclastogenesis in precursor cells previously exposed to RANKL. They proceeded to demonstrate that low concentrations of TNFα (500 pg/ml) were able to synergize with low concentrations of RANKL (1 ng/ml) in the induction

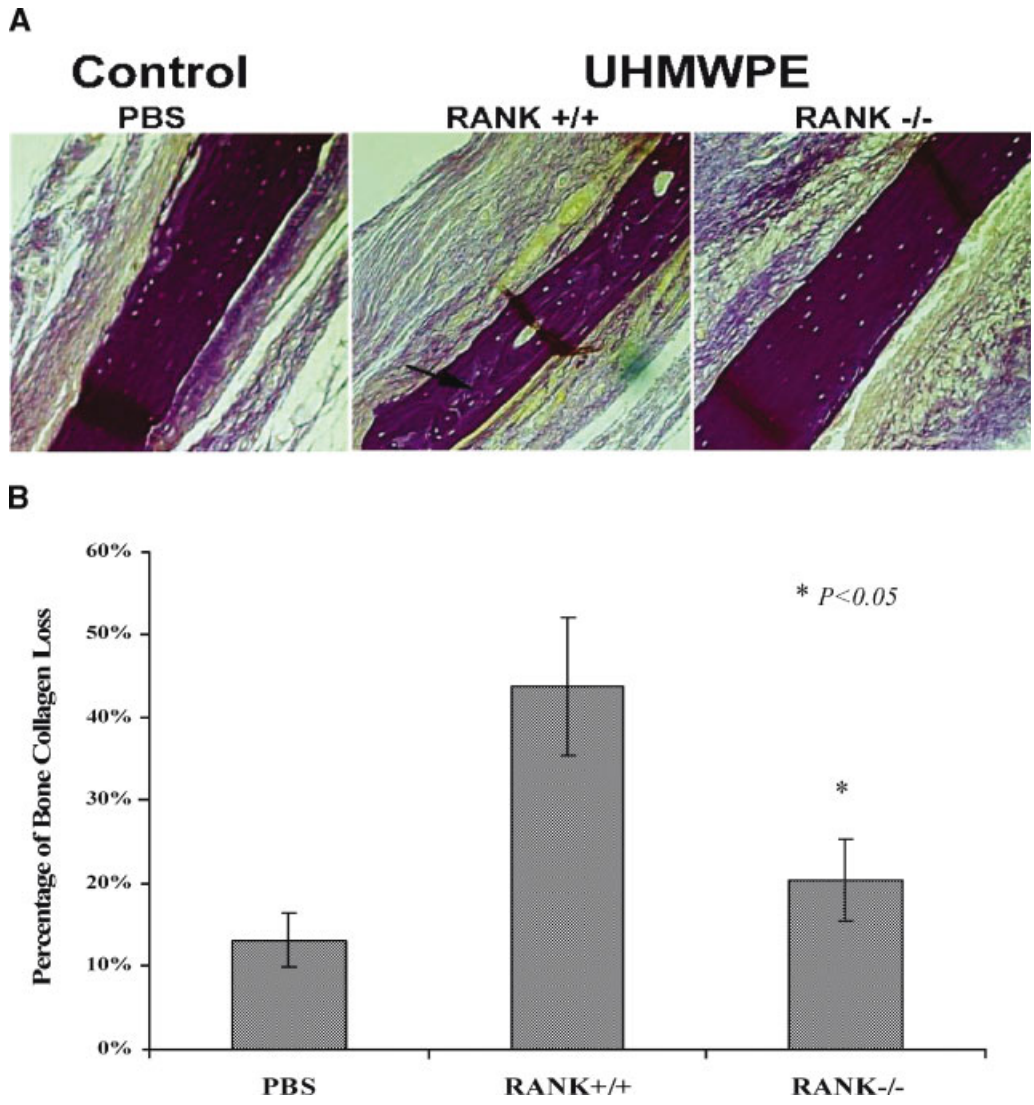

Figure 7. Collagen loss of implanted bones was illustrated by van Gieson staining. (A) Van Gieson stain was performed to evaluate bone collagen content (dark red coloration). Diminished coloration is indicated by arrow (original magnification, $\times 200$). (B) Collagen content of implanted bone was quantified by Image Analysis software as described in Materials and Methods. The value represents percentage of bone collagen loss. Values are mean $\pm$ SD for total 30 mice (10 mice per group). $^*p < 0.05$.

of osteoclastogenesis. Since this concentration of RANKL was 10-fold below the concentration of RANKL previously shown to induce osteoclastogenesis in osteoclast precursors, they concluded that TNFα was able to act directly on the osteoclast precursors in the presence of basal, nonstimulated levels of RANKL to induce osteoclastogenesis. Evidence for this was also provided by Zhang et al.[31] who demonstrated that TNFα stimulation enhanced osteoclastogenesis by RANKL pre-treated murine osteoclast precursors. This effect was abrogated when they utilized cells from TNFR-1 deficient mice. Overall, the exact molecular mechanism of TNFα signaling in osteoclastogenesis is unclear. Our data suggests that the roles of TNFα and RANKL in osteoclastogenesis exhibit a considerable overlap in the signaling pathways.[31] Data presented here demonstrates that UHMWPE particle-induced osteoclastogenesis is controlled by RANKL/RANK signaling. At the level of the osteoclast, the diverse humoral and cytokine signaling regulating bone remodeling

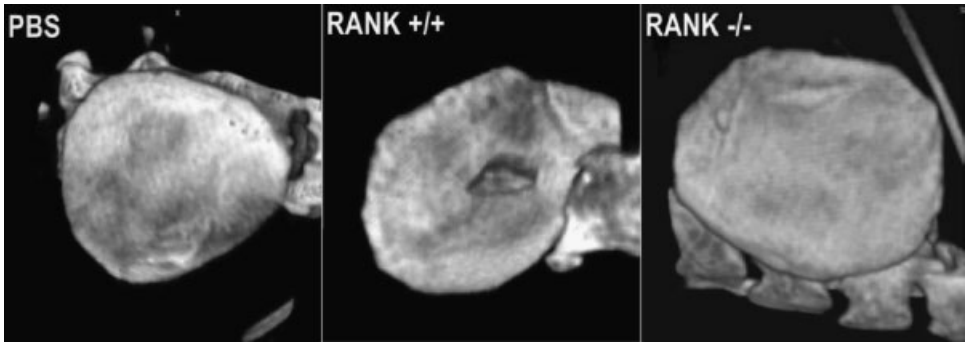

Figure 8. Evaluation of implanted bone resorption by microCT analysis. A representative segmented series of high-resolution microCT images of mouse pouch tissue with implanted bone. Localized bone resorption was noticed in RANK$^{+/+}$ mice with UHMWPE particle injection. Total six mice were scanned (two mice per group).

ultimately regulates the activation of RANK on the osteoclast/osteoclast precursor cell surface. The precise signal transduction pathways that emanate from this receptor should define the key intracellular processes that control osteoclastogenesis.

In summary, our study provides solid evidence that RANK/RANKL signaling is essential for UHMWPE particle-induced inflammatory osteoclastogenesis, and may play a critical role in the AL development. These data provide a biological rationale for the RANK-targeted treatment strategy; especially at the early stage of wear debris-induced inflammatory responses.

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