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Dendritic Cell-Mediated In Vivo Bone Resorption

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Osteoclasts are resident cells of the bone that are primarily involved in the physiological and pathological remodeling of this tissue. Mature osteoclasts are multinucleated giant cells that are generated from the fusion of circulating precursors originating from the monocyte/macrophage lineage. During inflammatory bone conditions in vivo, de novo osteoclastogenesis is observed but it is currently unknown whether, besides increased osteoclast differentiation from undifferentiated precursors, other cell types can generate a multinucleated giant cell phenotype with bone resorbing activity. In this study, an animal model of calvaria-induced aseptic osteolysis was used to analyze possible bone resorption capabilities of dendritic cells (DCs). We determined by FACS analysis and confocal microscopy that injected GFP-labeled immature DCs were readily recruited to the site of osteolysis. Upon recruitment, the cathepsin K-positive multinucleated osteoclasts (5). At sites of active bone resorption, the osteoclasts form a specialized cell membrane, the ruffled border, following organization of the actin cytoskeleton in conjunction with αvβ3 integrin-mediated matrix recognition. On attachment to bone, matrix-derived signals polarize intracellular secretory vesicles that deliver H+ATPase to the plasma membrane and release proteolytic enzymes into the resorptive milieu (6). In contrast, the organic matrix, mostly composed of collagen and elastin, is proteolized by secreted cysteine and aspartic proteases, among which cathepsin K has a major role (7).

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study the osteolytic potential of DCs, we chose ultra-high m.w. poly- 
ethylene (UHMWPE)-induced osteolysis for several reasons: 1) in 
this model, the bone inflammatory response is induced by injecting 
sterile alkanes particles (UHMWPE) under the calvarial peristium 
to generate an aseptic inflammatory reaction that is easier to control 
compared with pathogen-induced osteolysis (16); 2) the resulting 
inflammatory reaction is localized to the site of injection and there- 
fore easier to quantify than a more systemic reaction; and 3) the 
inflammatory infiltrates are mostly formed by local osteoclasts, 
recruited macrophages, and DCs, with very few T and B cells 
(16). Experiments performed in Csf1r−/− mice, which are pro-
foundly deficient in osteoclasts and tissue macrophages, conclus- 
ively demonstrated the in vivo osteolytic potential of CD11c+ 
conventional DCs recruited to the sites of bone resorption.

Materials and Methods

UHMWPE-induced calvarial osteolysis

UHMWPE-induced calvarial osteolysis was performed according to 
published protocols (16). Briefly, mice in each group (C57BL/6 female 8–12 
wk old) were sedated with isoflurane gas and anesthetized with ketamine 
(20% isoflurane, 15% xylazine, and 65% saline at 0.1 ml per 20 g body 
weight). A 10-mm incision was made over the sagittal midline suture of 
the calvarium and a 1.0 × 1.0 cm area of the periosteum exposed. In sham 
control mice, the incision was closed without any further intervention, 
whereas experimental mice received 20 μl of UHMWPE particles with 
an average size of 53–75 μm (Sigma-Aldrich, St. Louis, MO). The 
particles were dispersed over the periosteum using a sterile sharp surgical 
spatula and the incision sutured. Twelve days postoperation, a microcom-
puted tomography (micro-CT) scan was performed to confirm and verify 
the development of particle-induced osteolysis. Micro-CT scans were per- 
formed on the Aloka LaTheta laboratory CT scan machine (Aloka, 
System Engineering Co., Tokyo, Japan). The x-ray voltage was set at high. For 
each skull, 162 slices were scanned with an average section-to-section 
distance of 0.15 mm and a focal distance of 0.1 mm. This was achieved using a focused beam 
front orientation. Data were analyzed using LaTheta software (Aloka, 
System Engineering, Tokyo, Japan). All experiments were conducted in 
accordance with the Institute for Animal Care and Use Committee of 
Albert Einstein College of Medicine (New York, NY).

H&E staining of mouse calvaria

Post-CT scan animals were euthanized utilizing CO2. Calvaria were excised 
and fixed in 4% parafomaldehyde in PBS (pH 7.4) and placed at 4˚C. 
After 24 h, samples were placed in 10% EDTA in PBS (pH 7.4) for 48– 
72 h at 4˚C until decalcified. The calvaria were then processed for paraffin 
embedding, sectioned to 5 μm, and stained with H&E.

In vitro bone marrow cell differentiation

Bone marrow cells were purified using the lineage-negative magnetic bead 
kit, which depletes cells expressing the following lineage Ags: CDS, B220, 
CD11b, Gr-1, CD-3, and Ter-119 (Miltenyi Biotec, Auburn, CA). Lineage 
negative (Lin−) precursors were then cultured in DMEM (Life Technolo-
gies, Rockville, MD), supplemented with 50 U/ml penicillin, 50 μg/ml streptomycin, 2 mM l-glutamine, 0.1 mM nonessential amino acids, 1 mM 
pyruvate, and 10 mM HEPES containing either GM-CSF (10 ng/ml) for 8 
to obtain immature conventional DCs or further differentiated with 
RANK-L (5 ng/ml) and M-CSF (10 ng/ml) for 8 d to obtain osteoclasts.

Intravenous injection of GFP+ DCs and FACS analysis of cells 
recovered at sites of osteolysis

In some experiments, 2 wk postsurger, mice (8–12-wk-old female C57BL/ 
6) were injected i.v. with 104 bone marrow-derived GFP+CD11c+ 
DCs prepared from C57BL/6-transgenic (Tg) (CAG-GFP)100/1 mice (The 
Jackson Laboratory, Bar Harbor, ME). The CD11c+ cells were purified 
from bone marrow cells that had been cultured in GM-CSF for 7 to 8 d, 
using CD11c+ Ab conjugated magnetic beads (Miltenyi Biotec). Cell pu-

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implanted mice (upper panels). FIGURE 2. PGK promoter-GFP or the murine CD11c prom-GFP lentivirus (1:10, were cultured in DMEM with progressively lower dilutions of either the bone marrow DCs using limiting dilution analysis. Briefly, 100,000 DCs were cultured for 24 h in StemSpan media (StemCell Technologies, Vancouver, British Columbia, Canada) supplemented with IL-3, IL-6, stem cell factor, and Flt-3 ligand cytokines. For bone-marrow transfer experiments, transduced cells were injected into the tail vein (10^5 cells/mouse) of 8- to 12-wk-old mice (C57BL/6 or Csf1r^{−/−}) that had been previously lethally irradiated (2 × 6 Gy). Full engraftment of the transplanted hematopoietic stem cells was observed 2 mo following bone marrow transplantation.

Bone marrow transplant experiments
Six to 8-wk-old male mice (Csf1r^{−/−} on an outbred C57BL/6J, C3Heb/FeJ- a/a, 129SvJ background) were killed with CO2 and the bone marrow collected by flushing femurs and tibia. Lineage negative (Lin^{-}) cells were purified as described above (Miltenyi Biotec MACS Lin− cells). Cells were transduced with either the PGK prom-GFP lentiviral vector or the mCD11c prom-GFP lentiviral vector at a concentration of 10^6 TU/ml for 10^6 cells. Transduced cells were cultured for 2 mo following bone marrow transplantation.

Characterization of GFP+ DCs in blood, bone marrow, and spleen of the transplanted mice
Spleen, blood, and bone marrow samples were harvested from each mouse 2 mo following bone marrow transplantation. Single-cell suspensions were prepared from each organ and RBCs removed by lysis. For immunostaining, 10^6 cells were incubated in staining buffer (PBS, 2% FBS) for 30 min at 4°C with 1 to 2 μg/ml of the following PE-conjugated primary Abs: CD19, CD11b, CD11c, Gr-1, CD3, and NK1.1 (BD Pharmingen, San Diego, CA).

Results
UHMWPE calvarial model
The mouse model of osteolysis was established by implanting microgram amounts of UHMWPE particles adjacent to bone in mouse calvaria as described previously (16). In sham-operated mice, the calvaria were surgically uncovered and the periosteum incised with no further intervention, whereas in the UHMWPE group, particles were deposited directly between the bone and the periosteum. Two weeks postsurgery, a strong cellular infiltrate was observed in the UHMWPE-implanted mice, and small pit-like erosions were present on the calvarial surfaces (Fig. 1a). Cellular fusion around the particles was also evident from the presence of multinucleated giant cells (mgs) exhibiting 6–12 nuclei (Fig. 1b). To quantify the extent of calvarial osteolysis, mice were analyzed with micro-CT (Fig. 1c). In mice receiving UHMWPE particle implants, a statistically significant number of GFP+ DCs were quantified by FACS as a percentage of GFP+ cells. Calculation from the titration analysis indicated 1 × 10^5 transducing viral particles per milliliter.

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significant decrease in cortical and total bone mass density was observed in the postsurgery specimens. In contrast, no significant differences were observed in the sham controls pre- and postsurgery (Fig. 1d). We therefore sought to adopt this model to gain further insight into how DCs might be involved in the bone-resorptive process.

**DCs are recruited to the site of osteolysis**

In the next set of experiments, we addressed the question of whether peripheral myeloid DCs were recruited to the tissue surrounding the area of osteolysis. Ten million CD11c<sup>+</sup> immature bone marrow-derived DCs were prepared from C57BL/6-Tg (CAG-EGFP) mice, which express the enhanced GFP (EGFP) under the control of the β-actin promoter. Cells were injected into the tail veins of UHMPE-implanted and sham-operated mice. Ninety-six hours later, the whole calvarium was observed using differential contrast imaging and fluorescent microscopy (Fig. 2a). The calvarium area adjoining the site of surgery was then excised, decalcified, and 5-μm frozen sections generated for further fluorescent microscopic analysis. Extensive cellular infiltrates were observed under light microscopy in UHMWPE-implanted mice but not in the sham control (Fig. 2b). Green fluorescent areas were observed in the frontal area of the calvaria in UHMWPE-implanted mice but not in the sham control (Fig. 2b). The calvarium area adjoining the site of surgery was then excised, decalcified, and 5-μm frozen sections generated for further fluorescent microscopic analysis. Extensive cellular infiltrates were observed under light microscopy in UHMWPE-implanted mice but not in the sham controls (Fig. 2d). Among the infiltrates, many fluorescent cells could be observed in the UHMWPE-implanted mouse (Fig. 2e, right panel, merged image of 2d and 2e shown in 2f). FACS quantification of the GFP-injected DCs recruited to the calvaria indicated that in the mice implanted with UHMWPE, 13% of the cells retrieved at the site of osteolysis were GFP<sup>+</sup> (Fig. 2c). These results demonstrate an active recruitment of DCs to the site of osteolysis.

**DCs recruited to sites of osteolysis express cathepsin K**

In the next series of experiments, we sought to determine whether conventional DCs that migrate to the site of osteolysis express cathepsin K. Among the possible markers associated with osteolytic activity, we chose cathepsin K because it is a well-characterized osteoclast marker, and its activity in bone resorption is indicated by the bone macromegalia observed in cathepsin K knockout mice (18). More importantly, CD11c<sup>+</sup> conventional DCs express low levels of cathepsin K, which can be readily upregulated in vitro following culture of DCs with M-CSF and RANK-L, indicating, at least in vitro, the bone resorption potential of DCs (Fig. 3a).

To this goal, CD11c<sup>+</sup> DCs were purified from the bone marrow of C57BL/6-Tg (CAG-EGFP) mice (Fig. 3b) and injected into syngenic non-GFP-receptor mice, which were either sham operated or implanted with UHMWPE. Confocal analysis determined that in the calvaria of sham-operated mice, essentially no recruited CD11c<sup>+</sup> GFP<sup>+</sup> cells could be observed (Fig. 3c), and the only cathepsin K<sup>+</sup> cells (red) were the resident osteoclasts (Fig. 3c). In contrast, the calvaria of mice that had been implanted with UHMWPE particles exhibited a rich infiltrate of recruited GFP<sup>+</sup> DCs (green), some of which stained positive for cathepsin K (Fig. 3d, 3e, red). In addition, some cells that were observed in contact with the bone (Fig. 3f, left panel) were positive for both GFP and cathepsin K (Fig. 3f, right panel, arrow), indicating the possibility that DCs may form bone-resorbing pits in the UHMWPE-implanted mice (Fig. 3f). To better quantify the number of CD11c<sup>+</sup>GFP<sup>+</sup> cathepsin K<sup>+</sup> cells recruited to the site of osteolysis, the calvaria of sham- or UHMWPE-implanted mice was removed. Cells retrieved from the site of osteolysis were stained for cathepsin K and analyzed by flow cytometry. UHMWPE-implanted mice showed an increased calvarial recruitment of cathepsin K<sup>+</sup> GFP<sup>+</sup> DCs as compared with sham controls (Fig. 3g).

Taken together, these data indicate that cathepsin K<sup>+</sup> GFP<sup>+</sup> DCs are readily recruited to the site of osteolysis.

**DCs recruited to the site of bone inflammatory osteolysis fuse into mgcs**

Active osteoclasts, the primary cells involved in bone erosion, are organized as mgcs that are closely connected to the erosion pits observed during inflammatory bone resorption. To determine whether DCs are recruited into the mgcs observed around the UHMWPE particles (Fig. 1b), bone marrow DCs were prepared from a C57BL/6-Tg (CAG-EGFP) female donor. These cells were then injected into male recipients that were previously surgically implanted with UHMWPE or had undergone a sham procedure. DC recruitment and their osteolytic potential were monitored by tracking GFP<sup>+</sup> cathepsin K<sup>+</sup> cells as described above (Fig. 4). In Fig. 4b, a GFP<sup>+</sup> cell can be observed in the inflammatory infiltrates, which, in a serial section, did not stain for the Y chromosome (Fig. 4c, white arrow). Thus, this cell was clearly derived from the transplanted bone marrow DCs. A similar group of inflammatory cells adjacent to the bone are shown in Fig. 4d. Transplanted bone-marrow-derived recruitment into mgcs was demonstrated by detection of nuclei missing the Y chromosome in mgcs around the UHMWPE particles (Fig. 4g). These cells contained multiple nuclei, the majority of which were positive for the Y chromosome (recipient cells), but a small number were missing the Y chromosome, indicating that they were derived from the transplanted female donor DCs (Fig. 4g, white arrows, second and fourth panels). These data support the proposition that

**FIGURE 3.** Cathepsin K-positive DCs are recruited to the site of osteolysis. a, Western blot analysis for cathepsin S, L, F, and K and TRAP of bone marrow-derived immature DCs differentiated for 8 d in GM-CSF (DC) or further differentiated with RANK-L and M-CSF for an additional week (osteoclasts). b, FACS analysis of CD11c expression of purified GFP<sup>+</sup> DCs prior to injection into GFP<sup>+</sup> recipient mice (solid line) or isotype control (dotted line). c–f, Confocal light (left panel) and fluorescent (right panel) images of calvarial sections analyzed for expression of cathepsin K<sup>+</sup> (red) and GFP<sup>+</sup> (green) DCs recruited to the site of surgery in sham (c) or UHMWPE-implanted mice (d–f). c and d, Original magnification ×63; e and f, original magnification ×100. Arrow indicates cathepsin K<sup>+</sup> GFP<sup>+</sup> DCs recruited to areas of osteolysis. g, FACS profile of GFP<sup>+</sup>cathepsin K<sup>+</sup> DCs retrieved from the calvaria of sham or UHMWPE-implanted mice. One of three experiments is shown. In each experiment, three mice for each experimental condition (sham or UHMWPE) were used.
recruited DCs can participate in the formation of giant cells found at inflammatory sites adjacent to the bone.

**DCs recruited to the inflammatory site possess bone resorption activity**

To determine in a more definitive manner whether DCs recruited to sites of bone inflammation possess osteolytic potential, we performed the UHMWPE calvarial model experiments in mice devoid of osteoclasts. To do this, we used Csf1r−/− mice, which are severely depleted of macrophages and osteoclasts and which phenotypically present with extensive osteopetrosis (9). Also, because these mice lack the CSF-1R, monocytes that might have been recruited to the inflammatory site cannot differentiate into osteoclasts. The Csf1r−/− mice are difficult to breed because the majority of newborn mice die within 1 mo. The few mice that survive are very frail, and several attempts to perform surgical experiments on these mice failed, and so chimeras were created. To create an endogenous population of GFP+ DCs, Csf1r−/− mice were lethally irradiated and transplanted with Csf1r−/− bone marrow cells that had been previously transduced with a lentiviral vector expressing GFP under the control of the CD11c promoter. The tissue specificity of the CD11c promoter was compared with the non–tissue-specific PGK promoter. As expected, transplantation of bone marrow cells with the non–tissue-specific PGK promoter controlling GFP expression gave rise to green fluorescent cells in both the lymphoid (T and B cells) and myeloid populations (macrophages, DCs, and granulocytes) (data for spleen shown in Fig. 5a). In contrast, in mice transplanted with total bone marrow cells transduced with the tissue-specific CD11c promoter, GFP expression was restricted to the myelomonocytic population in the spleen (Fig. 5a).

Two months after irradiation, chimERIC (Csf1r+/− recipient and Csf1r−/− bone marrow donor) mice were either surgically implanted with UHMWPE on their calvaria or were sham operated. Two weeks following surgery, the calvaria were removed and analyzed by CT scan for bone erosion and resorption. One hundred sixty-two scan images were overlapped as a three-dimensional image (Fig. 5b). Sham-operated chimeras or control Csf1r−/− did not display any sign of osteolysis (Fig. 5b, first and third panels). In contrast, UHMWPE-injected mice exhibited extensive osteolysis (Fig. 5b, second and fourth panels). Mice competent to produce osteoclasts, macrophages, and DCs (Fig. 5b, fourth panel), as well as mice repopulated with DCs but lacking osteoclasts and macrophages (Fig. 5b, second panel), displayed bone surface erosion (Fig. 5b), indicating the in vivo bone resorption activity of recruited DCs.

**Discussion**

Under physiological conditions, maintenance of bone mass is achieved through a delicate balance between the activity of bone-forming osteoblasts and bone-resorbing osteoclasts (19). This constant bone remodeling is necessary to support calcium homeostasis and to meet the structural needs of the musculoskeletal system. However, in certain inflammatory diseases, such as rheumatoid arthritis, this balance is lost, and osteolytic activity predominates, resulting in diffuse periarticular osteopenia and localized bone erosion (20–22). Understanding the mechanisms that determine the relative activity of osteoblasts and osteoclasts is important for the development of strategies for minimizing skeletal injury in these inflammatory diseases.

Osteoclastogenesis depends upon progenitor cells of the monocytic hematopoietic lineage. These cells differentiate into osteoclasts under the influence of M-CSF and RANK-L, derived at least in part from bone stromal cells (23, 24). DC-specific transmembrane protein and αvβ3 integrin are also part of the osteoclast differentiation pathway. DC-specific transmembrane protein controls cell fusion, which leads to mgs, whereas αvβ3 integrin is important for providing giant cells with the ability to adhere to the bone surface and to form an acidified extracellular microenvironment. Within this latter space, cathepsin K and TRAP are released to promote...
solubilization of calcium and phosphate and to digest matrix collagen and elastin within the resorbing lacunae (25).

A still-unresolved question is the source of osteoclast precursors. At the present time, we do not know how many cells are derived from the bone pool of physiological preosteoclasts versus the recruitment of peripheral circulating precursors. This question is particularly important for understanding bone resorption in inflammatory conditions. Monocytes and circulating DCs are normally recruited to inflamed tissue (24, 26, 27), where monocytes may directly differentiate into osteoclasts (26). However, DCs could play either a direct or indirect role in osteoclastogenesis (28, 29). An indirect role would involve the ability of these cells to produce cytokines, such as IL-1, IL-6, and TNF-α, that augment expression and release of TRAP and cathepsin K by resident osteoclasts (30). DCs could also promote osteoclastogenesis indirectly by stimulating T cells to express RANK-L, a major differentiation factor for osteoclast precursors (31). In contrast, DCs could potentially directly participate in bone resorption following cathepsin K release. Indeed, several in vitro studies support the notion that immature DCs can differentiate into osteoclast-like cells (14, 32, 33). Culture of DCs with naïve T cells and human periodontal pathogens indicated the in vitro ability of DCs to fuse into multinucleated cells with bone resorptive activity (14). In the current study, we addressed the osteolytic potential of DCs using an in vivo model that permits us to track recruitment of DCs to sites of bone inflammation, as well as testing knockout mice that lack macrophages and osteoclasts (9). The results demonstrated that exogenous myeloid DCs migrate to an inflammatory site of osteolysis, where they are active participants in bone resorption.

A still-open question is whether the same growth factors that control physiological osteoclastogenesis are also involved in de novo inflammatory-induced osteoclastogenesis. In vitro analysis indicates that, similarly to bona fide osteoclast precursors, immature DCs are also dependent on RANK-L and M-CSF for differentiation into mature osteoclasts (14). These data support the notion that, at least in vitro, a similar growth factor-mediated differentiation pathway is used by both precursors. However, it is also possible that additional cytokines or growth factors play a role during the in vivo process of DCs’ differentiation into osteoclasts. In fact, Langerhans cells have been shown to fuse into mcs in the rare disease of histiocytosis, which is associated with extensive focal bone erosions (34). Fusion of this subtype of DCs does not require RANK-L or M-CSF; instead it is dependent on IL-17 (34). Similarly, fusion of macrophages during inflammatory diseases does not rely on RANK-L but mostly on high levels of IL-4 (35). Thus, it is possible that conventional DC fusion into multinucleated cells with bone-resorption properties is also dependent on other cytokines and growth factors. It would be of great interest to determine whether the biological function of mature osteoclasts derived from bona fide osteoclast precursors or derived from immature DCs differs. This point is of extreme interest because the fusion of DCs and their role in bone resorption is primarily observed during inflammatory disease.

FIGURE 5. DCs induce inflammatory osteolysis in absence of endogenous macrophages and osteoclasts. A, FACS profile of T cells (CD3), B cells (CD19), granulocytes (Gr-1), macrophages (CD11b), DCs (CD11c), and NK-T cells (NK1.1) from the spleen of mice previously transduced with lentiviral vector expressing GFP under the control of a general promoter (PGK) or a tissue-specific promoter (CD11c). Mice were analyzed 2 mo after receiving the bone marrow transduced with the lentiviral vectors. Ten days prior to the FACS analysis, mice were injected with the B16-GM-CSF cells that produce endogenous GM-CSF, thus increasing the number of splenic DCs and facilitating the analysis of the CD11c promoter tissue specificity. B, Csf1r<sup>−/−</sup> recipient mice were lethally irradiated and transplanted with the bone marrow of either Csf1r<sup>−/−</sup> or Csf1r<sup>+/+</sup> mice. The procedure generates chimeras that only express CD11c<sup>+</sup> DCs (first and second panels) or DCs, macrophages, and osteoclasts (third and fourth panels), respectively. CT scan of the calvaria of chimeras mice implanted with UHMWPE or sham controls. One of two experiments is shown (in each experiment, three mice for each experimental condition [sham or UHMWPE] were used).
conditions. Intervening in the recruitment of DCs to the bone matrix or their fusion into giant cells could be part of a therapeutic approach for limiting joint injury in diseases, such as osteoarthritis and rheumatoid arthritis (36). This model could be a useful tool for the development and testing of such agents.

Disclosures

The authors have no financial conflicts of interest.

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