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Netrin1 Is Highly Expressed And Required In Inflammatory Infiltrates In Wear Particle-Induced Osteolysis

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Abstract

Objective—Netrin-1 is a chemorepulsant and matrix protein expressed during and required for osteoclast differentiation, which also plays a role in inflammation by preventing macrophage egress. Because wear particle-induced osteolysis requires osteoclast-mediated destruction of bone, we hypothesized that blockade of Netrin-1 or Unc5b, a receptor for Netrin-1, may diminish this pathological condition.

Methods—6–8-wk-old C57BL/6 mice had 3mg of ultrahigh-molecular-weight-polyethylene particles (UHMWPE) implanted over the calvaria and then received 10µg of monoclonal antibodies for Netrin-1 or its receptors, Unc5b and DCC injected IP on a weekly basis. After 2 weeks, microCT and histology analysis were performed. Netrin-1 expression was analyzed in

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DISCLOSURES:

A.M. and B.N.C. have filed a patent on use of adenosine A_{2A}R agonists to prevent prosthesis loosening (pending). A.M. B.N.C., B.R., K.J.M., have filled a patent on the use of Antibodies against Netrin-1 for the treatment of bone diseases. T.W., E.P. S.G. Z.D. C.L. do not have any disclosures. B.N.C. holds patents numbers 5,932,558; 6,020,321; 6,555,545; 7,795,427; adenosine A₁R and A_{2B}R antagonists to treat fatty liver (pending); adenosine A_{2A}R agonists to prevent prosthesis loosening (pending). B.N.C. is a consultant for Bristol-Myers Squibb, AstraZeneca, Novartis, CanFite Biopharmaceuticals, Cypress Laboratories, Regeneron (Westat, DSMB), Endocyte, Protalex, Allos, Inc., Savient, Gismo Therapeutics, Antares Pharmaceutical, Medivector, King Pharmaceutical, Celizome, Tap Pharmaceuticals, Prometheus Laboratories, Sepracor, Amgen, Combinatorx, Kyowa Hakka, Hoffman-LaRoche and Avidimer Therapeutics. BNC has stock in CanFite Biopharmaceuticlas.

Author contribution:

BC and KM have designed the experiments, wrote and revised the manuscript. AM designed the experiments and has been the primary person responsible for carrying out all experimental procedures and writing the manuscript. TW helped on surgery and animal treatments and revised the manuscript. BR performed the immunohistochemistries and revised the manuscript. EP and SG carried out human subjects experiments and revised the manuscript. Z.D. and C.L performed undercalcified bone preparation, sectioning, staining and scanning.

human tissue obtained following primary prosthesis implantation or after prosthesis revision for peri-implant osteolysis and aseptic implant loosening.

Results—Weekly injection of anti-Netrin-1 or anti-Unc5b-antibodies significantly reduced particle-induced bone pitting in calvaria-exposed to wear particles (46 ± 4 and $49\pm3\%$ of control bone pitting respectively, $p<0.001$) but anti-DCC-antibody did not affect inflammatory osteolysis ($80\pm7\%$ of control bone pitting, $p=ns$). Anti-Netrin-1 or anti-Unc5b, but not anti-DCC, antibody-treatment markedly reduced the inflammatory infiltrate and the number of TRAP-positive osteoclasts (7 ± 1 , 4 ± 1 and 14 ± 1 cells/high power field (hpf) respectively vs. 12 ± 1 cells/hpf for control, $p<0.001$), with no significant changes in Alkaline Phosphatase-positive osteoblasts on bone forming surfaces in any antibody-treated-group. Netrin-1 immunostaining colocalized with CD68 staining for macrophages. The peri-implant tissues of patients undergoing prosthesis revision surgery showed an increase in Netrin-1 expression whereas there was little Netrin-1 expression in soft tissues removed at the time of primary joint replacement.

Conclusion—These results demonstrate a unique role for Netrin-1 in osteoclast biology and inflammation and may be a novel target for prevention/treatment of inflammatory osteolysis.

Keywords

Netrin-1; Unc5b; antibodies; inflammation; osteolysis

INTRODUCTION

Total hip and knee replacements are successful surgical interventions with success rates close to 90% at 10–25 years after surgery in terms of reducing pain, improving function, and enhancing quality of life in patients.[1] However, despite improvements in surgical techniques, material and implant designs, nearly 40,000 hip arthroplasties have to be revised each year in the US[1], and it is expected that the rates of revision will increase by 137% for total hip and 601% for total knee revisions over the next 25 years.[2] Peri-prosthetic inflammatory bone destruction due to wear particles is the most common cause of prosthesis failure requiring revision.[3]

Wear particles consist of debris shed from joint prostheses, whether polymeric, metallic or ceramic, and these particles are phagocytosed by macrophages and other local cells, which become activated.[4, 5] Further recruitment of both macrophages and osteoclasts (bone-resorbing cells) to the site[6, 7] leads to up-regulation of pathways leading to bone destruction, and down-regulation of bone formation.[8, 9] To date, no biological approaches to limiting the chronic inflammatory reaction associated with wear particles have proved clinically successful.[10]

Netrin-1, a secreted laminin-related protein, is a member of the axonal guidance protein family that inhibits migration of monocytes, neutrophils and lymphocytes by activation of its receptors A2BR (adenosine A2B receptor) and Unc5.[11–13] Acting through Unc5b receptor, Netrin-1 reduces renal ischemia–reperfusion injury and its associated renal inflammation.[14] Netrin-1 is expressed on vascular endothelium, where it is regulated by infection and inflammatory cytokines, and inhibits inflammatory cell migration into tissues

and its down-regulation at the onset of sepsis/inflammation may facilitate leukocyte recruitment[11]. Netrin-1 also promotes chronic inflammation in atherogenesis and diet-induced obesity; tissue macrophages in atherosclerotic plaques and obese adipose tissue increase their expression of Netrin-1,[15, 16] which promotes macrophage retention and survival[17] further increasing chronic inflammation. Recently we have demonstrated that Netrin-1 is an autocrine and paracrine regulator of osteoclast differentiation.[18] In osteoclast precursors, the binding of Netrin-1 to its receptor Unc5b, but not to its alternative receptor DCC, triggers the signaling cascade involved in the activation of the small GTPase RhoA by a mechanism that has not previously been observed in osteoclast precursors. Activation of RhoA via LARG and RGMa leads to the cytoskeletal rearrangements required for osteoclast fusion and differentiation.[18]

Here we tested the hypothesis that blockade of Netrin-1 or Unc5b might be a novel approach to the prevention of osteoclast-mediated bone resorption and inflammation at the site of prosthesis shedding of wear particles.

METHODS

Wear particle preparation

Ultrahigh molecular weight polyethylene particles (UHMWPE) particles, a gift of P. H. Wooley (Via Christi Regional Medical Center), had a mean particle size (equivalent circle diameter) of $1.74 \pm 1.43 \mu\text{m}$ (range 0.05–11.06), with more than 34% of the particles smaller than $1 \mu\text{m}$. For decontamination from endotoxins, the particles were washed twice in 70% ethanol for 24 h at room temperature. The particles were washed in phosphate-buffered saline (PBS) and dried in a desiccator.

Surgical procedure

All protocols followed internationally recognized guidelines and were approved by the NYU SoM Institutional Animal Care and Use Committee. Osteolysis was induced by implantation of UHMWPE particles over the mouse calvaria, as we have previously described.[19] Briefly, 6–8-wk-old male C57BL/6 mice were anesthetized by intraperitoneal injection of 100 mg/kg ketamine and 10 mg/kg xylazine, and a 1-cm midline sagittal incision was made over the calvaria anterior to the line connecting both ears as previously described.[19] 5 mice received no particles (sham, untreated mice) and the incision was closed without any further intervention. The remaining animals (n=40) received 3mg of dried UHMWPE particles. Of these animals, 10 mice received intraperitoneally (IP) 100 μl of 0.9% saline (control) and the rest received 10 μg (in a final volume of 100 μl) of mouse monoclonal Netrin-1, Unc5b or DCC antibodies (n=10 each), beginning immediately before surgery and continuing on a weekly basis until sacrifice (2 weeks later). Water and food were given *ad libitum* until sacrifice. Animals were sacrificed after 14 days in a CO₂ chamber and the calvaria were removed, fixed, and prepared for microCT, histological staining, and cytokine measurements.

Histomorphometry

See online supplementary text.

Micro-X-Ray Computed Tomography (micro-CT) analysis

After sacrifice, 5 calvaria per treatment group were fixed in 70% ethanol and prepared for high-resolution microCT as previously described to perform qualitative and quantitative analyses of resorbing areas in murine calvarial bone.[19] Analyses were performed in the NYU College of Dentistry microCT core with a Skyscan 1172 microCT (Bruker, WI, USA) using the previously described parameters[20]: 60kV, 167uA, 9.7 micron pixel size, 2000×1332 matrix, 0.3 degree rotation steps, 6 averages, movement correction of 10, 0.5mm Al filter, 2 segments scanned per sample (56 minutes/segment). Images were reconstructed using the Skyscan NRECON software (histogram range 0–0.065, beam hardening correction of 35, Gaussian smoothing (factor 1), ring artifact correction of 7). For quantitative analysis of UHMWPE particle-induced osteolysis, a square-shaped region of interest across the parietal bone of approximately 4 mm right and left of the midline suture of the skull was placed in one of the 2D-reconstructed slices, as described previously,[19, 21] and a Matlab software application was utilized to analyze calvarial bone resorption, as previously described.[19]

Histological studies

Calvariae were fixed in 4% paraformaldehyde for 48h, followed by decalcification in 10% EDTA for four weeks and paraffin embedding (n=5 per treatment). Sections (5 µm) were cut and H&E staining was performed. Inflammatory infiltration in midsagittal suture areas was quantified from 5 images per animal using Sigma Scan Pro Image 5.0.0 software.

TRAP staining was carried out in a TRAP buffer (0.1M acetate buffer, 0.3M Sodium Tartrate 10mg/ml Naphtol AS-MX phosphate, 0.1% Triton X-100, 0.3mg/ml Fast Red Violet LB (Sigma-Aldrich, MO, USA)). After deparaffinization and acetate buffer incubation, samples were incubated in TRAP buffer for 30 minutes and counterstained with Fast Green.

Immunohistochemistry analysis for Netrin-1, Unc5b, DCC, Alkaline Phosphatase (ALP), Cathepsin K and CD68 were carried out as previously described[19] (See online supplementary text).

Images were observed in a Leica microscope equipped with SlidePath Digital Image Hub Version 3.0 software, or under light microscope (Nikon) equipped with Nis Elements F3.0 SP7 software.

Netrin-1 expression in implant biopsies

Netrin-1 immunostaining was performed in human tissue obtained from donors following primary prosthesis implantation or after prosthesis revision for peri-implant osteolysis and aseptic implant loosening (Hospital for Special Surgery, New York NY, USA).[22] Tissue was associated with reactions to orthopedic implant wear debris. Specimens of soft tissue and bone were collected from regions of bone resorption during joint revision surgery. The specimens were fixed in freshly prepared 4% paraformaldehyde, followed by demineralization with 14% EDTA, processed and embedded in paraffin and 5 µm sections were prepared. Sections of bone and soft tissue taken at the time of primary hip implant were obtained from fixed, paraffin-embedded tissue. Paraffin embedded sections (n=5 each)

were deparaffinized and after rehydration, Netrin-1 immunostaining was performed following the protocol described above. TRAP staining was also performed as described above. All procedures were approved by the Institutional Review Boards of the Hospital for Special Surgery and NYU School of Medicine.

Measurement of inflammatory cytokines

See online supplementary text.

Statistical analysis

Statistical significance for differences between groups was determined by use of one-way ANOVA and Bonferroni post-hoc test or Student's *t* test, as appropriate. All statistics were calculated using GraphPad® software (GraphPad, San Diego, CA, USA).

RESULTS

Netrin-1 is highly expressed in human periarticular soft tissue removed during revision

We have recently reported that during osteoclast maturation, expression of Netrin-1 and its receptor Unc5b is increased in osteoclast precursors, and required for osteoclast differentiation.[18] In parallel, activated tissue macrophages were reported to induce Netrin-1 expression in atherosclerotic plaques and diet-induced obesity[15, 17] where Netrin-1 contributes to inflammation.

To determine if these findings are relevant to orthopaedic wear-particle-induced osteolysis in humans, we studied the expression of Netrin-1 in peri-implant tissues retrieved from patients undergoing revision surgery for peri-implant osteolysis and aseptic implant loosening. As seen in Figure 1A there is little expression of Netrin-1 in cells populating the soft tissues removed at the time of primary joint replacement but high levels of Netrin-1 expression in the matrix and the cells populating the peri-implant tissues from patients undergoing revision surgery. TRAP staining revealed the presence of osteoclasts in patches on the bone surfaces in both specimens from initial joint replacement and revision implant loosening samples.

Netrin-1 and Unc5b are induced in the inflammatory infiltrate after wear particle exposure in mice

The results described above suggested that Netrin-1 might play a critical role in inflammatory osteolysis and contribute to the progression of inflammatory osteolysis. To test this hypothesis we employed a model of orthopaedic wear particle-induced osteolysis[19] in which UHMWPE particles are placed under the periosteum over the calvaria. In sham-operated animals we observed negligible levels of Netrin-1-, Unc5b- and DCC-positive cells in the bone and soft tissue at the surgical site (Figure 1B). In contrast, in the calvaria of mice exposed to UHMWPE particles there was a marked increase in Netrin-1 and Unc5b expression (118 ± 3 Netrin-1 positive cells/hpf (high power field) and 60 ± 3 Unc5b positive cells/hpf vs. 8 ± 1 Netrin-1 positive cells/hpf and 20 ± 2 Unc5b positive cells/hpf for Sham-operated, $p < 0.001$, $n = 5$), primarily in the cells of the inflammatory infiltrate which was composed almost entirely of macrophages, as previously described.[19] In addition, we

observed a modest increase in DCC-positive cells (59 ± 2 DCC positive cells/hpf vs. 45 ± 2 positive cells/hpf for Sham, $p < 0.001$, $n = 5$) in the inflammatory infiltrate of mice exposed to UHMWPE particles (Figure 1B).

Netrin-1 and Unc5b are required for wear particle-induced osteolysis

Since Netrin-1 is highly expressed in inflammatory infiltrates in mice and in peri-implant joint tissue in humans, we determined if targeting Netrin-1 or its receptors blocked progression of or reversed inflammatory osteolysis *in vivo*. 10 μ g murine monoclonal anti-Netrin-1, anti-Unc5b or anti-DCC antibodies were injected IP on a weekly basis in the murine model of inflammatory osteolysis. Weekly injections of anti-Netrin-1 or anti-Unc5b antibodies significantly reduced the area of particle-induced bone pitting in calvaria exposed to UHMWPE (46 ± 4 and $49 \pm 3\%$ of control bone pitting, respectively, $p < 0.001$, $n = 5$, Figure 2A) but anti-DCC receptor antibody did not affect UHMWPE-induced pitting and resorption ($80 \pm 7\%$ bone pitting, $p = \text{ns}$ vs control, Figure 2A). MicroCT also revealed a significant increase in bone volume (BV) and bone volume/total volume ratio (BV/TV) in both anti-Netrin-1 and -Unc5b treated mice (Figure 2A, Table 1).

MicroCT results were confirmed by histomorphometry (see online supplementary text).

We also observed a marked reduction in the inflammatory infiltrate in calvaria from mice treated with anti-Netrin-1 and -Unc5b antibodies, whereas anti-DCC antibody treatment did not affect the inflammatory infiltrate ($83 \pm 2\%$ reduction for anti-Netrin-1, $81 \pm 1\%$ reduction for anti-Unc5b and $2 \pm 2\%$ reduction for anti-DCC, $p < 0.001$ and $p = \text{ns}$ respectively, $n = 5$) (Figure 2B). Anti-Netrin-1 and anti-Unc5b treatment, but not anti-DCC treatment, markedly reduced the number of TRAP-positive osteoclasts in affected bone (7 ± 1 TRAP-positive cells/hpf for anti-Netrin-1, 4 ± 1 TRAP-positive cells/hpf for anti-Unc5b and 14 ± 1 TRAP-positive cells/hpf for anti-DCC compared to 12 ± 2 TRAP-positive cells/hpf for control, $p < 0.001$ and $p = \text{ns}$ respectively, $n = 5$) (Figure 2B).

Inflammatory cytokines also contribute to inflammatory osteolysis and anti-Netrin-1 and -Unc5b, but not -DCC, antibody treatment abrogated wear particle-induced increases in IL-1 β and TNF α (see online supplementary text).

To further confirm that Netrin-1 and Unc5b are key factors in bone resorption *in vivo*, immunohistologic examination was performed. When osteoclasts were identified by immunohistologic staining for Cathepsin K, similar results as those observed for TRAP staining were found (Figure 3). Treatment with anti-Netrin-1 or anti-Unc5b antibody markedly reduced the number of Cathepsin K positive cells, whereas treatment with anti-DCC antibody did not significantly change the number of Cathepsin K-positive cells (10 ± 2 cells/hpf for anti-Netrin-1, 9 ± 1 cells/hpf for anti-Unc5b and 19 ± 3 cells/hpf for anti-DCC compared to 24 ± 2 cells/hpf for control, $p < 0.001$ and $p = \text{ns}$ respectively, $n = 5$) (Figure 3). In contrast, there were no significant changes in Alkaline Phosphatase positive osteoblasts on bone forming surfaces in any antibody-treated group compared to control (8 ± 1 cells/hpf for anti-Netrin-1, 10 ± 1 cells/hpf for anti-Unc5b and 10 ± 2 cells/hpf for anti-DCC compared to 9 ± 2 cells/hpf for control, $p = \text{ns}$, $n = 5$) (Figure 4).

As previously described,[18] the only cells that express Netrin-1 in bone are osteoclasts and osteoclast precursors. This is confirmed *in vivo* as Netrin-1 stain co-localized with CD68 immunostaining (Supplemental Figure 2). Both anti-Netrin-1 and -Unc5b treatments markedly reduced the number of CD68-positive macrophages and, consequently, the immunostaining for Netrin-1, whereas anti-DCC treatment did not change the number of CD68-positive macrophages when compared to control (6 ± 1 cells/hpf for anti-Netrin-1, 8 ± 2 cells/hpf for anti-Unc5b and 24 ± 3 cells/hpf for anti-DCC compared to 29 ± 4 cells/hpf for control, $p<0.001$ and $p=ns$ respectively, $n=5$) (Supplemental Figure 2).

Because of their effect on osteoclast differentiation we tested the effect of monoclonal anti-Netrin-1 and -Unc5b antibodies on bone density and found that treatment with these antibodies directly increased bone density in mice (see online supplementary text and Table 2).

DISCUSSION

Here we report that Netrin-1 is highly expressed by macrophages at sites of wear particle-induced osteolysis in the inflamed peri-implant soft tissue from patients undergoing implant revision and in macrophages and osteoclasts in a murine model of wear particle-induced bone destruction. Moreover, *in vivo* blockade of Netrin-1 and its receptor Unc5b by murine monoclonal antibodies prevents wear particle-induced bone destruction. These results indicate that Netrin-1 plays a critical role in inflammatory osteolysis.

Multiple studies have shown that orthopaedic wear products are potent inducers of proinflammatory mediators, including IL-1 β and TNF α , and other soluble stimuli for osteoclast formation and bone resorption.[19] Here we demonstrated that treatment with monoclonal antibodies in both *ex vivo* cultures of calvaria exposed to wear particles, and in the RAW264.7 cell line, diminished both IL-1 β and TNF α secretion. Macrophages represent a major component of the cellular infiltrate associated with wear particle-induced inflammation. Based on the findings in atherosclerotic plaques it is reasonable to hypothesize that Netrin-1 may contribute to the recruitment and retention of macrophages at the site of wear particle deposition and that signaling by Netrin-1 may further contribute to the osteolytic process via enhancement of osteoclast differentiation and activity. Prevention of wear particle-induced bone destruction by blockade of Netrin-1 and Unc5b by murine monoclonal antibodies indicates that Netrin-1 is acting in both an autocrine and paracrine fashion to promote bone destruction, as described *in vitro*.[18] The demonstration that blockade of DCC had no effect on bone destruction provides strong evidence that the effects of the anti-Netrin-1 and anti-Unc5b antibodies on osteolysis are specific, since DCC is also expressed by osteoclasts and macrophages.[18]

Because of its chemorepulsant activities, administration of Netrin-1 has been described as an anti-inflammatory treatment in a number of settings, including inflammatory bowel disease, pancreatitis, peritonitis and pulmonary inflammation.[11, 12, 14, 23–29] Neuronal guidance proteins present in peripheral tissues contribute to the local control of leukocyte migration and inflammation.[30, 31] Netrin-1 administration was found to direct leukocyte traffic during acute inflammation in peripheral organs,[23, 27] to reduce local injury and

inflammatory responses,[27, 31] and to stimulate resolution mechanisms and production of resolvins.[32] In accordance, Netrin-1 administration regulates inflammation and infiltration of monocytes and ameliorates ischemia reperfusion-induced kidney injury,[14, 33] by inducing overexpression of macrophage a M2-like phenotype and suppression of expression of M1 markers,[34] and regulates the inflammatory response of neutrophils and macrophages, and suppresses ischemic acute kidney injury by inhibiting COX-2-mediated PGE2 and thromboxane A2 production.[33] Netrin-1 is also generated within the intestinal epithelium to dampen neutrophil recruitment in acute colitis and may have a beneficial function in chronic models of intestinal inflammation.[35]

However, the anti-inflammatory effects of Netrin-1 do not accord with the demonstration that Netrin-1 plays a critical role in promoting atherogenesis by inhibiting macrophage emigration from plaque,[15, 17] and other findings that identify Netrin-1 as a macrophage retention signal in adipose tissue of obese mice that promotes chronic inflammation and insulin resistance. Recently, the molecular mechanism linking plaque hypoxia and macrophage infiltration has been demonstrated in mouse and human plaques.[17] It involves hypoxia-inducible factor 1 α -mediated up-regulation of Netrin-1 and Unc5b in human and mouse atherosclerotic plaques and subsequent macrophage chemostasis and protection from apoptosis.[17]

Nonetheless, the observation that there is marked overexpression of Netrin-1 in cells at sites of inflammatory bone destruction both in mice and in humans suggests that Netrin-1 plays an important role in inflammatory osteolysis. Our *in vitro* data suggest that this may be in part attributable to the effects of Netrin-1 on enhancing osteoclast differentiation, attachment and adhesion to the bone surfaces, which are known to play a role in bone resorption.[36] Moreover, histomorphometry analysis in long bones treated with monoclonal antibodies for two weeks, suggested a trend to increased trabecular bone formation with changes in BV/TV and BMD and a decrease in osteoclast number. Although the change in bone formation detected by the calcein/declomycin double-labeling technique was not significant there was a tendency to increased bone formation. In some prior studies Netrin-1 was reported to mediate its effects on myeloid cells and inflammation by a mechanism that involves adenosine A_{2B} receptors.[13, 23, 27, 37, 38] Nonetheless, it is unlikely that adenosine A_{2B} receptors are involved in the effects of Netrin-1 on osteoclast differentiation and function since He and colleagues recently reported that stimulation of adenosine A_{2B} receptors inhibits osteoclast differentiation.[39] Thus, Netrin-1 most likely plays a direct role in inflammatory bone destruction by increasing or permitting osteoclast differentiation and function.

In conclusion, our results indicate that Netrin-1 is an autocrine factor produced by osteoclast precursors that enhances osteoclast differentiation and function. The demonstration of high levels of Netrin-1 expression in cells at sites of inflammatory bone resorption, furthermore suggests a contributory role in the enhanced bone resorption associated with inflammatory processes. Thus, Netrin-1 may be a novel therapeutic target for the reduction of osteoclast-mediated bone resorption and other forms of inflammatory bone destruction.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Netrin-1 is highly expressed in inflammatory infiltrates in wear particle-induced osteolysis

A) The figures show representative images for H&E, Netrin-1 immunostaining and TRAP staining in human tissue biopsies from implant revision and primary implants (n=5 each). B) The figures show representative images for Netrin-1, Unc5b and DCC immunostaining in a murine UHMWPE particle-induced osteolysis model (n=5 mice per group). Images were taken at 100X and 400X magnification. Scale bar indicates 50 and 100 μ m. ***p<0.001, compared to untreated (Sham) (ANOVA).

Figure 2. Blocking Netrin-1 using monoclonal antibody injections decreases bone pitting, inflammation and osteoclasts in mouse calvariae after exposure to wear particles

A) The figures show representative microCT images of calvaria of mice treated with UHMWPE wear particles combined with anti-IgG (Control), anti-Netrin-1, anti-Unc5b or anti-DCC blocking antibodies at 10 μ g (n=5 mice per group). B) Calvariae were stained with hematoxylin & eosin to determine the presence of inflammation on the outer bone surface. The area of inflammatory infiltrate was quantified and expressed as a percentage of the area of the control particle-exposed mice (n = 5 mice per group). Shown are representative images for TRAP staining for osteoclasts in mice calvariae and the mean ($\pm$ SEM, n=5 mice per group) number of osteoclasts/hpf. Images were taken at 400X magnification. Scale bar

indicates 50 μm . Data were expressed as mean $\pm$ SEM (n=5 per group). * $p<0.05$, ** $p<0.001$, *** $p<0.001$ compared to control (ANOVA).

Figure 3. Immunohistochemistry for markers of osteoclasts

Calvaria were processed and immunohistologic staining carried out. Shown are representative H&E sections of calvariae (from $n=5$ mice per group) stained for Cathepsin K (green). Nuclei are shown in blue (Dapi). Quantification of the number of positive cells/hpf was done. Data are means $\pm$ SEM ($n=5$ mice per group). All images were taken at the same magnification 200X and 400X. Scale bar indicates 50 μ m. *** $p<0.001$ (ANOVA).

Figure 4. Immunohistochemistry for markers of osteoblasts

Calvariae were processed and immunohistologic staining carried out. Shown are representative representative H&E sections of calvaria (from n=5 mice per group) stained for Alkaline Phosphatase (green). Nuclei are shown in blue (Dapi). Quantification of the number of positive cellst/hpf was done. Data are means±SEM (n=5 mice per group). All images were taken at the same magnification 200X and 400X. Scale bar indicates 50 μm. ***p<0.001 (ANOVA).

Table 1
microCT analysis of mouse calvariae after treatment

Digital morphometric analysis of microCT images from wild-type mice treated with saline or murine monoclonal antibodies against Netrin-1, Unc5b or DCC. Data are reported as means±SEM.

Treatment	Bone volume (BV) (mm ³)	Total volume (TV) (mm ³)	BV/TV	Bone mineral density (BMD) (mg)
Particulate + saline	1.98 ± 0.02	1.37 ± 0.01	0.17 ± 0.003	809.99 ± 1.20
Particulate + anti-Netrin-1	2.31 ± 0.02 ***	1.48 ± 0.06	0.19 ± 0.004 *	819.94 ± 2.06 **
Particulate + anti-Unc5b	2.28 ± 0.04 ***	1.46 ± 0.05	0.18 ± 0.002 *	819.39 ± 1.39 **
Particulate + anti-DCC	1.95 ± 0.08	1.43 ± 0.03	0.17 ± 0.002	811.43 ± 1.40

* $P < 0.05$,

** $P < 0.01$,

*** $P < 0.005$, compared to saline (ANOVA).