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Orthopaedic Wear Particle-induced Bone Loss and Exogenous Macrophage Infiltration is mitigated by Local Infusion of NF-κB Decoy Oligodeoxynucleotide

Tzuhua Lin[1], **Jukka Pajarinen**[1], **Akira Nabeshima**[1], **Luis A. Córdova**[1,3], **Florence Loi**[1], **Emmanuel Gibon**[1,4], **Laura Lu**[1], **Karthik Nathan**[1], **Eemeli Jämsen**[1], **Zhenyu Yao**[1], and **Stuart B. Goodman**[1,2]

[1]Department of Orthopaedic Surgery, Stanford University, Stanford, CA, USA

[2]Department of Bioengineering, Stanford University, Stanford, CA, USA

[3]Department of Oral and Maxillofacial Surgery, Faculty of Dentistry, University of Chile—CONICYT, Santiago, Chile

[4]Biomechanics and bone & joint Biomaterials laboratory - UMR CNRS 7052, School of Medicine - Paris7 University, Paris, France

Abstract

Excessive production of wear particles from total joint replacements (TJRs) induces chronic inflammation, macrophage infiltration, and consequent bone loss (periprosthetic osteolysis). This inflammation and bone remodeling are critically regulated by the transcription factor NF-κB. We previously demonstrated that inhibition of NF-κB signaling by using the decoy oligodeoxynucleotide (ODN) mitigates polyethylene wear particle-induced bone loss using in vitro and in vivo models. However, the mechanisms of NF-κB decoy ODN action, and in particular its impact on systemic macrophage recruitment, remain unknown. In the current study, this systemic macrophage infiltration was examined in our established murine femoral continuous particle infusion model. RAW264.7 murine macrophages expressing a luciferase reporter gene were injected into the systemic circulation. Quantification of bioluminescence showed that NF-κB decoy ODN reduced the homing of these reporter macrophages into the distal femurs exposed to continuous particle delivery. Particle-induced reduction in bone mineral density at the distal diaphysis of the femur was also mitigated by infusion of decoy ODN. Histological staining showed that the decoy ODN infusion decreased osteoclast and macrophage numbers, but had no significant effects on osteoblasts. Local infusion of NF-κB decoy ODN reduced systemic macrophage infiltration and mitigated particle-induced bone loss, thus providing a potential strategy to treat periprosthetic osteolysis.

Please address correspondence to: Stuart B. Goodman MD PhD, Department of Orthopaedic Surgery, Stanford University School of Medicine, 450 Broadway Street, Redwood City, CA, USA, 94063, Phone: 01-650-721-7662, FAX: 01-650-721-3470, goodbone@stanford.edu.

Keywords

wear particles; macrophage infiltration; NF-κB decoy oligodeoxynucleotide; periprosthetic osteolysis

Introduction

Periprosthetic osteolysis is an inflammatory bone disease caused mainly by the generation of excessive wear debris from total joint replacements (TJRs). The wear particles initiate an inflammatory response, which leads to further recruitment of immune cells including macrophages. The disease may progress into chronic inflammation with continuous particle release and subsequently leads to loss of bone that supports the implant[1]. Revision surgery, which is associated with higher complication rates, is required in patients with progressive osteolysis and implant loosening. Although the demand of TJR is significantly increasing in the aging society as well as younger patients with advanced arthritis, an efficacious non-surgical treatment for periprosthetic osteolysis remains unavailable.

During the wear particle induced chronic inflammation, particles are recognized and phagocytosed by macrophages, which induces the production of various pro-inflammatory cytokines and chemokines. Chemokines including MCP1 further recruit macrophages to infiltrate the local area, which exacerbate the inflammation and ultimately induce osteoclastic bone resorption. This cascade is importantly mediated by the transcriptional factor NF-κB, a master regulator of inflammation and bone remodeling. Activation of the NF-κB pathway can be induced directly by particle recognition or indirectly by paracrine or autocrine pro-inflammatory cytokine signaling during the pathogenesis of periprosthetic osteolysis. Therefore, inhibition of the NF-κB pathway is a putative target to mitigate particle-induced bone loss[2].

We previously demonstrated that inhibition of the NF-κB pathway using a decoy oligodeoxynucleotide (ODN) mitigated particle-induced inflammation and bone loss using in vitro[3,4] and in vivo models[5,6]. However, the biological mechanisms underlying this phenomenon, especially the direct effect of NF-κB decoy ODN on systemic macrophage recruitment, remain unclear. As continued macrophage recruitment is crucial for perpetuation of the inflammatory response and adverse bone remodeling processes, the direct impact of NF-κB decoy ODN on macrophage recruitment was studied. Murine RAW264.7 macrophages stably expressing the luciferase reporter gene were injected into the systemic circulation to examine systemic macrophage trafficking to the murine distal femur undergoing continuous ultra-high molecular weight polyethylene (UHMWPE) particle infusion. The therapeutic effect of NF-κB decoy ODN on UHMWPE particle-induced inflammation and bone loss was also examined.

Materials and Methods

Decoy oligodeoxynucleotide

The NF-κB decoy ODN sequences used are 5’-CCTTGAAGGGATTTCCCTCC-3’ and 3’-GGAACTTCCCTAAAGGGAGG-5’. Scrambled ODN sequences used as negative controls

are 5'-TTGCCGTACCTGACTTAGCC-3' and 3'-AACGGCATGGACTGAATCGG-3'[13]. The ODNs were synthesized by Integrated DNA Technologies (IDT, Coralville, IA, USA) in HPLC grade.

Ultra-High Molecular Weight PolyEthylene particles

Conventional UHMWPE particles were a gift from Dr. Timothy Wright (Hospital for Special Surgery, New York) and obtained from knee joint simulator tests and isolated according to an established protocol[14]. Frozen aliquots of the particles containing serum were lyophilized for 4–7 days. The dried material was digested in 5 M sodium hydroxide at 60°C for 1h, and ultrasonicated for 10 min. The digested particle suspension was centrifuged through a 5% sucrose gradient at 40K rpm at 10°C for 3 h. The collected particles at the surface of the sucrose solution were incubated at 80°C for 1 h and centrifuged again through an isopropanol gradient (0.96 and 0.90 g/cm^3) at 40K rpm at 10°C for 1 h. The purified particles at the interface between the two layers of isopropanol were harvested and the isopropanol was evaporated from the particle mixture. After lyophilization until dry, particles were re-suspended in 95% ethanol, which was evaporated completely. The particles tested negative for endotoxin using a Limulus Amebocyte Lysate Kit (BioWhittaker, Walkersville, MD). The mean diameter of the particles was 0.48 ± 0.10 μm (mean ± SE, averaged from 125 scanned particles ranged from 0.26μm to 0.81μm) measured by electron microscopy.

Continuous femoral infusion murine model

Male athymic nude mice (Charles River), 10–12 weeks of age were used for the experiments (10–12 mice per group). The animal protocol was approved by the institutional ethics committee. Institutional guidelines for the care and use of laboratory animals were observed in all aspects of this project. Alzet mini-osmotic pumps (Model 2006) were connected to vinyl tubing and a hollow titanium rod placed in the distal femur through the intercondylar region. The pumps were filled with various combinations of UHMWPE particles (15mg/ml), decoy ODN (50μM), and/or LPS (1 μg/ml) with the following experimental groups defined: 1) negative control, 2) particle infusion alone, 3) particle infusion with decoy ODN, 4) particle infusion with scrambled ODN, 5) particle infusion with lipopolysaccharide (LPS), 6) particle infusion with LPS and decoy ODN, and 7) particle infusion with LPS and scrambled ODN. The pumps were in place for 21 days. The mice were euthanized at 3 weeks post-operation.

In Vivo Imaging System (IVIS)

The murine macrophage reporter cell line RAW 264.7 (ATCC® TIB-71™) expressing firefly luciferase and GFP were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS) and an antibiotic/antimycotic solution (100 units of penicillin, 100 mg of streptomycin, and 0.25 mg of Amphotericin B per ml; Hyclone, Thermo Scientific). The reporter cells (5×10^5 resuspended in Hank's balanced salt solution) were injected into the lateral tail vein of the operated mice one week after implantation of the pump and titanium rod. IVIS-100 (Perkin Elmer) was used for in vivo bioluminescence imaging at day 2, 4, 6, 8, 10, and 12 post-injection. Luciferase substrate D-luciferin was administrated by intra-peritoneal injection (150mg/kg) 5mins before imaging.

The data was analyzed by Living Image Software (Perkin Elmer), and presented in the ratio of bioluminescence (recorded as p/s/cm^2/sr) in the local region of interest at distal femur to the total murine body.

Micro-Computational Tomography (μCT)

The mice underwent μCT scans at Day 0 (before surgery) and Day 21 (after sacrifice and removal of the titanium rod) using a TriFoil CT120 μCT scanner with 50μm resolution (TriFoil Imaging, Chatworth, CA). The region of interest (ROI) at the diaphysis site (4mm × 4mm × 3mm) was defined from the end of the metaphysis and proceeding proximally. The threshold bone mineral density (BMD, data presented in unit of mg/ml) was quantified by GEMS MicroView (threshold: 700 HU).

Tissue processing and Histology

The femurs (9–11 samples per group) from operated and non-operated sides were excised after μCT scanning. The rod was removed to allow for thin sectioning and immunohistochemical analysis. Femurs were fixed in 4% paraformaldehyde overnight, and decalcified in 0.5M ethylenediamine tetra acetic acid (EDTA, pH 7.9) for 2 weeks. The specimens were embedded in optimal cutting temperature (OCT) compounds and the ROI between 2–3mm from the distal end of femur was cut into 10μm transverse sections for subsequent histological staining.

Immunohistochemistry and cell counting

Macrophages were detected by immunofluorescence staining using FITC-conjugated anti-F4/80 antibody (1:50, AbD Serotec, Raleigh, NC). Corresponding rat IgG2b-PE antibodies were used as isotype control. The samples were sectioned by using Leica CM3050S Cryostat. The slides were mounted with ProLong Gold Antifade Mount with DAPI (Life Technologies, Grand Island, NY). The slides were assigned with random numbers and F4/80+ cells were counted blindly under a fluorescence microscope (Axio Observer 3.1, Zeiss, Oberkochen, Germany) in 3 randomly selected fields of view. Osteoblasts and osteoclasts were determined as previously described[15]. In brief, osteoblasts were identified by anti-alkaline phosphatase (ALP) antibody (R&D, Minneapolis, MN) with the use of Avidin-biotin complex (Vector Laboratories) immunohistochemistry. The positively-stained cells were counted blindly and normalized by total lengths of periosteum and endosteum quantified by Image J software. Osteoclast-like cells were determined by leukocyte tartrate resistant acid phosphatase (TRAP) staining kit (Sigma Aldrich) with multi-nucleated cells located on the bone perimeter within the resorption lacunae. The stained-positive cells were counted manually and normalized by total bone area quantified by Image J (software, National Institutes of Health, USA).

Statistical analysis

One-way ANOVA with Tukey’s post-hoc test was performed to statistically analyze the μCT data. Kruskal-Wallis with Dunn’s multiple correction test was performed to analyze the results of immunohistochemical staining. The statistical analysis was conducted using Prism

7 (GraphPad Software, San Diego, CA). Data are reported as mean ± standard deviation. $P<0.05$ was chosen as the threshold of significance.

Results

NF-κB decoy ODN mitigates exogenous macrophage infiltration stimulated by infusion of PE particles with LPS

The bioluminescence signals, which represent the exogenous macrophage distribution, were quantified and shown as the ratio of signal from the local ROI at the distal femur (purple circle) to the total body (red circle) (Fig.1a). Compared to the vehicle control, infusion of PE particles at the distal femur enhanced the infiltration of RAW264.7 reporter macrophages that were injected in the tail vein. Local infusion of NF-κB decoy ODN with the PE particles significantly reduced the ratio (and thus macrophage infiltration) at a later time point (Day 12, $p<0.05$, Fig.1b) compared to PE particle alone group. However, no significant difference between decoy ODN and scrambled ODN-treated groups was observed in PE particle alone groups (Fig.1b). Infusion of PE particle with LPS further increased macrophage infiltration at the distal femur compared to the PE particle alone group. The decoy ODN, but not the scrambled ODN, significantly reduced macrophage infiltration induced by particles plus LPS infusion (Day 12, $p<0.05$, Fig.1c).

NF-κB decoy ODN mitigates the decrease in bone mineral density resulting from infusion of PE particles alone or with LPS

The ROI for CT image analysis was defined based on our previous studies, where particle-induced osteolysis was observed[6,16] (Fig.2b, see **Materials and Methods** for detail). Bone mineral density in the ROI was significantly reduced in PE particles alone (510.4±30.79mg/ml) or PE plus LPS (515.2±33.27mg/ml) infusion groups, compared to the vehicle control (554.2±23.99mg/ml). The decoy ODN mitigated this bone loss effects in particle (565.1±31.06mg/ml) and particle plus LPS (547.6±32.3mg/ml) groups (Fig.2a). The protective effects were not observed in scrambled ODN-treated groups.

NF-κB decoy ODN decreased the number of activated osteoclasts, but had no significant effect on the number of osteoblasts

The osteoblast and osteoclast numbers were examined by staining for ALP+ and TRAP+ cells, respectively (Fig.3a). Particle infusion decreased osteoblast ($p<0.01$) and increased osteoclast ($p<0.005$) numbers (Fig.3b & c). Particle plus LPS infusion had no significant effects on the number of osteoblasts, however osteoclast numbers were increased compared to controls ($p<0.005$). The decoy ODN treatment reduced the number of osteoclasts induced by particle or particle plus LPS infusion, but had no significant beneficial effect on osteoblast numbers (Fig.3b & c).

NF-κB decoy ODN reduced PE particle- and LPS infusion-associated macrophage infiltration

The infiltrated exogenous and endogenous macrophages were quantified on histological sections by staining the F4/80+ cells (Fig.4a). Consistent with the IVIS imaging results (Fig. 1), particles ($p<0.005$) or particle plus LPS ($p<0.005$) infusion significantly increased

macrophage infiltration, while decoy ODN treatment suppressed the number of infiltrating macrophages in both the particle and particle plus LPS groups (Fig.4b).

Discussion

Our findings have provided further clarification concerning systemic macrophage recruitment in the presence of an inflammatory response induced by continuous infusion of wear particles. Rather than a single bolus of particles, as is seen in other models such as the calvarial model, our in vivo model simulates the clinical scenario of periprosthetic osteolysis more closely by utilizing a continuous infusion of particles over several weeks. Using this continuous infusion model, local infusion of NF-κB decoy ODN reduced wear particle-induced macrophage infiltration, decreased the number of osteoclasts and mitigated subsequent osteolysis.

Although local infusion of NF-κB decoy ODN suppressed macrophage infiltration compared to the treatment without ODN, surprisingly, the cell infiltration due to particles plus NF-κB decoy ODN and particles plus scrambled ODN showed no significant difference. It has been demonstrated previously that only the decoy ODN used in the current study specifically binds to activated NF-κB transcriptional factor, and reduces their binding to genomic targeted sequences[17,18]. Previous in vitro studies also showed that the scrambled ODN had no significant effects on NF-κB activity and downstream target gene expressions[3,4]. Therefore, it is unlikely that the scrambled ODN interfered with NF-κB activity in the current in vivo murine model. The potential off-target effects of the scrambled ODN could be associated with the CpG motif that only exists in the scrambled ODN. The CpG motifs on the synthetic ODN are a well-known Toll-like receptor ligand that can trigger a cellular response[19]. In addition, the sequence-specific cellular uptake processes could also determine the cell type that is affected by the ODNs[20]. Therefore, the different sequences between the decoy and scrambled ODNs could potentially change the subsequent targeted cell types and the affected cellular pathways. Overall, other in vivo murine models[5,6] have demonstrated the therapeutic potential of NF-κB decoy ODN against particle-induced bone loss compared to the vehicle controls. Furthermore, it should be noted that in the presence of particles together with LPS, NF-κB decoy ODN was more effective in reducing macrophage infiltration compared to scrambled ODN.

We also show that systemic delivery of a luciferase reporter RAW264.7 macrophage cell line is an efficient strategy to monitor macrophage trafficking to a local inflammatory site in vivo. Local infiltration of the reporter cells in the murine femurs with particle infusion was further validated previously by staining GFP-positive cells[16]. However, the application of reporter cells may be limited to a relatively short time period (<3 weeks) due to cell engraftment to the lymph nodes and spine region. An alternative approach using primary murine macrophages transduced with the reporter gene can be applied to the experiments with a longer time frame[21]. Considering the extra time and effort necessary to prepare primary reporter macrophages, the RAW264.7 cell-based reporter system remains an efficient and cost-effective option to track macrophage migration in vivo.

Modulation of macrophage infiltration is a crucial step to mitigate particle-induced bone loss. We recently demonstrated that local release of 7ND, a mutant decoy of CCL2, efficiently suppresses macrophage recruitment to murine femurs with particle infusion[16]. The therapeutic effects of these two pharmaceutical strategies may not be directly comparable due to different drug delivery models (release from layer-by-layer coating versus local pump infusion) and the experimental conditions (3 weeks versus 4 weeks of particle exposure). Nevertheless, these translational models and treatments both demonstrated the crucial role of continued macrophage recruitment in the pathogenesis of particle-induced bone loss.

Our previous studies using the continuous femoral particle infusion model showed that infusion of wear particles had no significant effect on osteoblast number[6,16]. However, decreased osteoblast number by particle infusion alone was observed at the distal femur in the current model. Although a transient inflammatory signal is crucial for successful bone regeneration[9,10,22,23], continuous inflammatory stimuli have been shown to impair osteoblastic differentiation, and is dependent on the dose, exposure period, and cell differentiation status[24]. The difference in the osteoblast numbers could be due to the particle exposure time (3 weeks versus 4 weeks), the increased number of infiltrated systemic macrophages, or the biological difference between endogenous macrophages and RAW264.7 cell line.

Conclusion

Local infusion of NF-κB decoy ODN was shown to be effective in reducing systemic macrophage infiltration and mitigating polyethylene wear particle-associated bone loss in the murine continuous femoral particle infusion model. Local infusion of NF-κB decoy ODN may be a potential translational strategy for the treatment of wear particle-associated periprosthetic osteolysis

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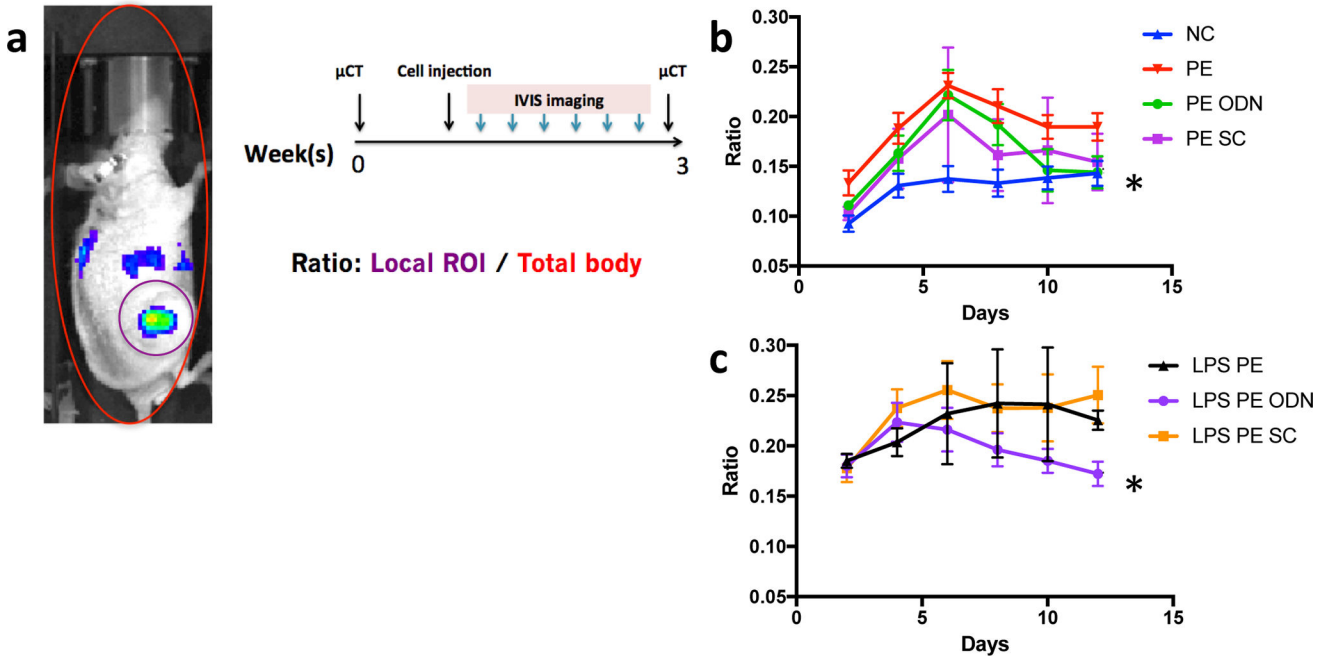

Figure 1. NF-κB decoy ODN reduced exogenous macrophage infiltration in the murine femur during continuous polyethylene (PE) wear particle infusion

(a) Timeline of continuous femoral infusion murine model and the quantification of IVIS imaging. The bioluminescence at the ROI was normalized to the signal from total mouse body. **(b & c)** Quantification of exogenous macrophage infiltration by the IVIS system. *p< . 05, PE: UHMWPE particles; NC: negative control; SC: scrambled ODN.

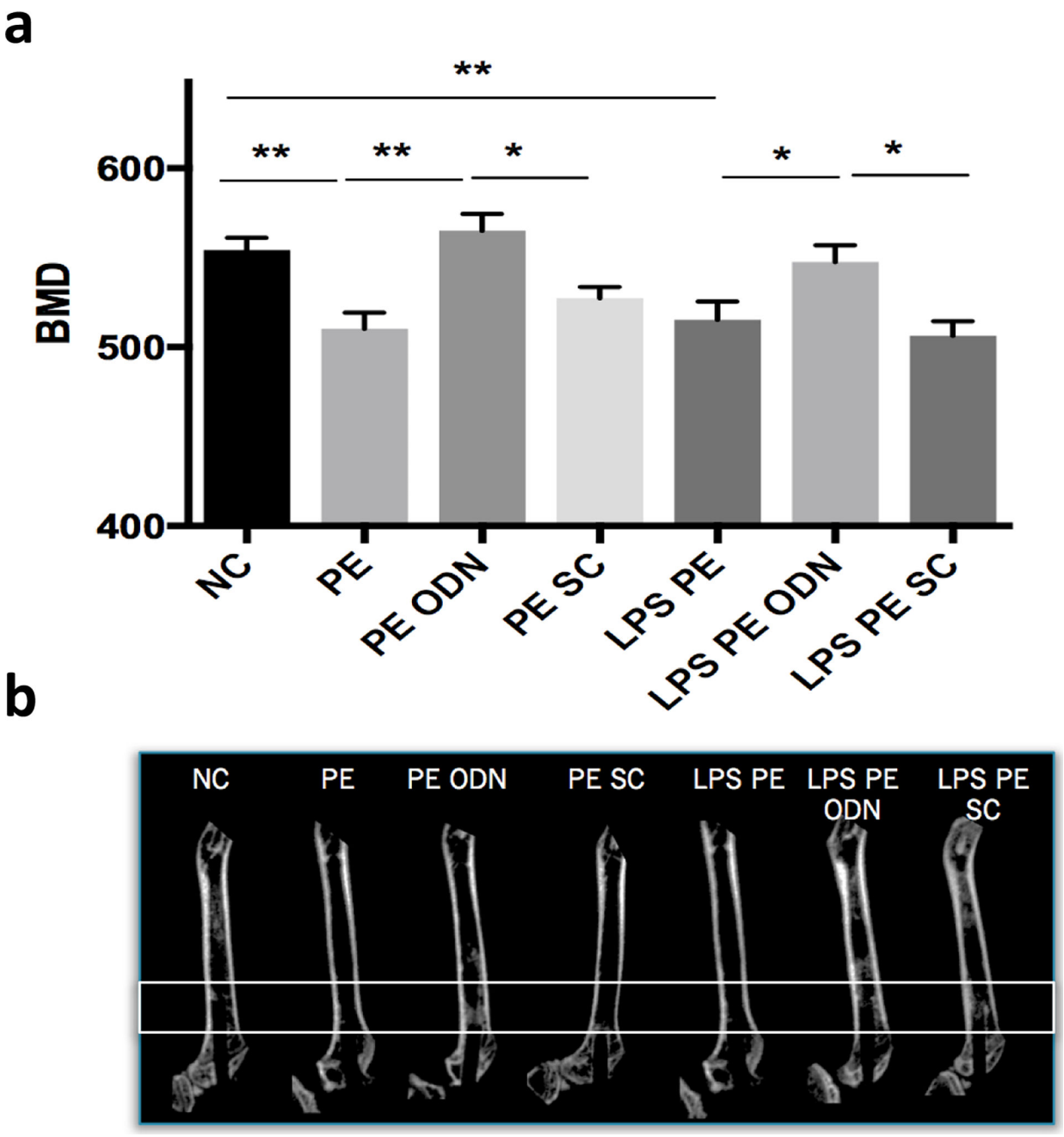

Figure 2. NF-κB decoy ODN mitigated bone loss in the murine femur during continuous polyethylene (PE) wear particle infusion

(a) The Bone Mineral Density (above 700 HU) in the region of interest (ROI, indicated by white rectangle in **b**) was quantified by MicroView. *p< .05, ** p< .01,

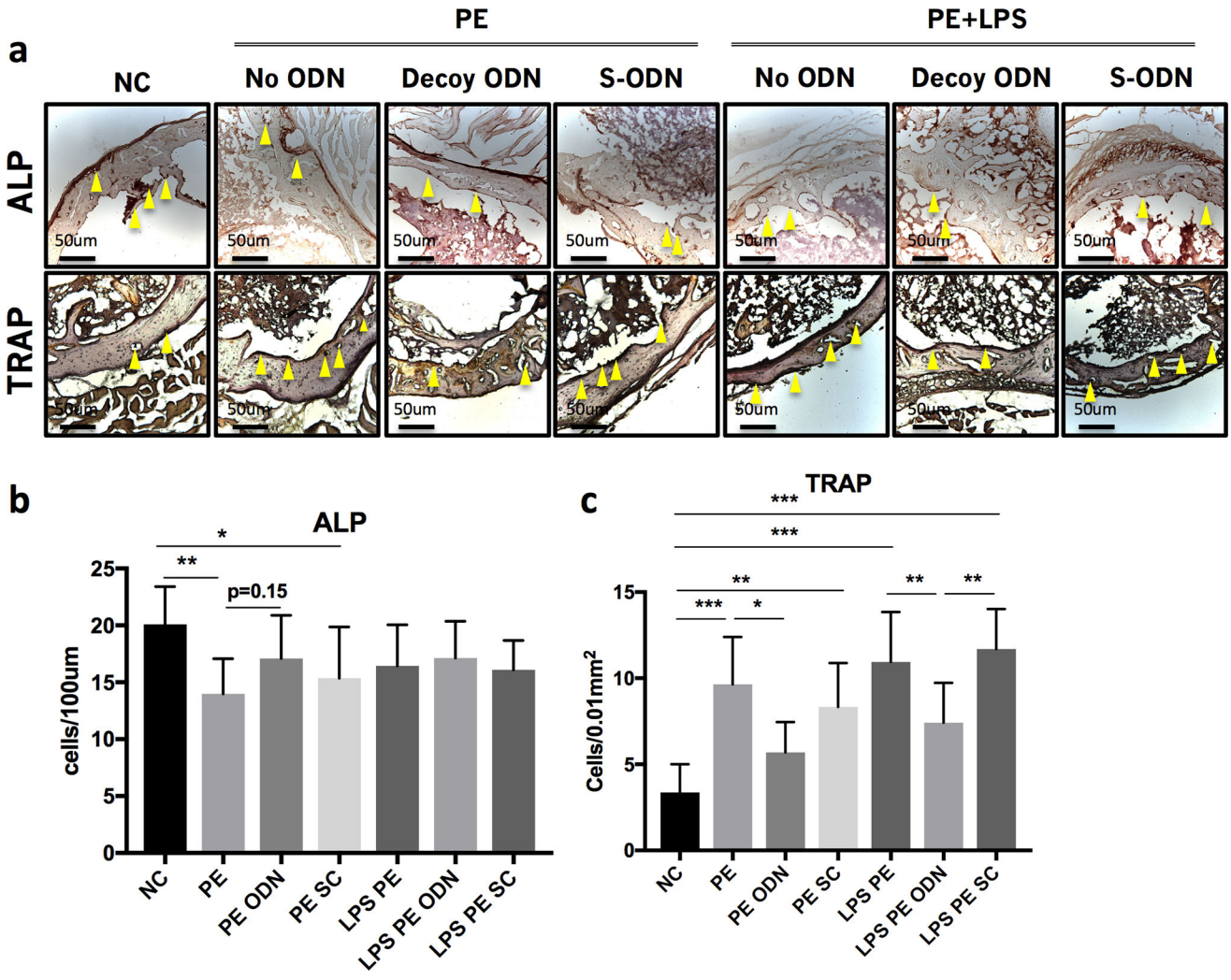

Figure 3. NF-κB decoy ODN reduced osteolysis in part by decreasing bone resorption by osteoclasts

(**a**) Immunohistochemical stainings for ALP and TRAP. (**b**) NF-κB decoy ODN treatment had no statistically significant effects on osteoblastic activity (ALP positive cells), but (**c**) reduced osteoclast activation (TRAP positive cells) in the distal femur in response to UHMWPE particles with or without LPS. PE: UHMWPE particles; NC: negative control; SC: scrambled ODN. *p< .05, ** p< .01, ***p<0.005.

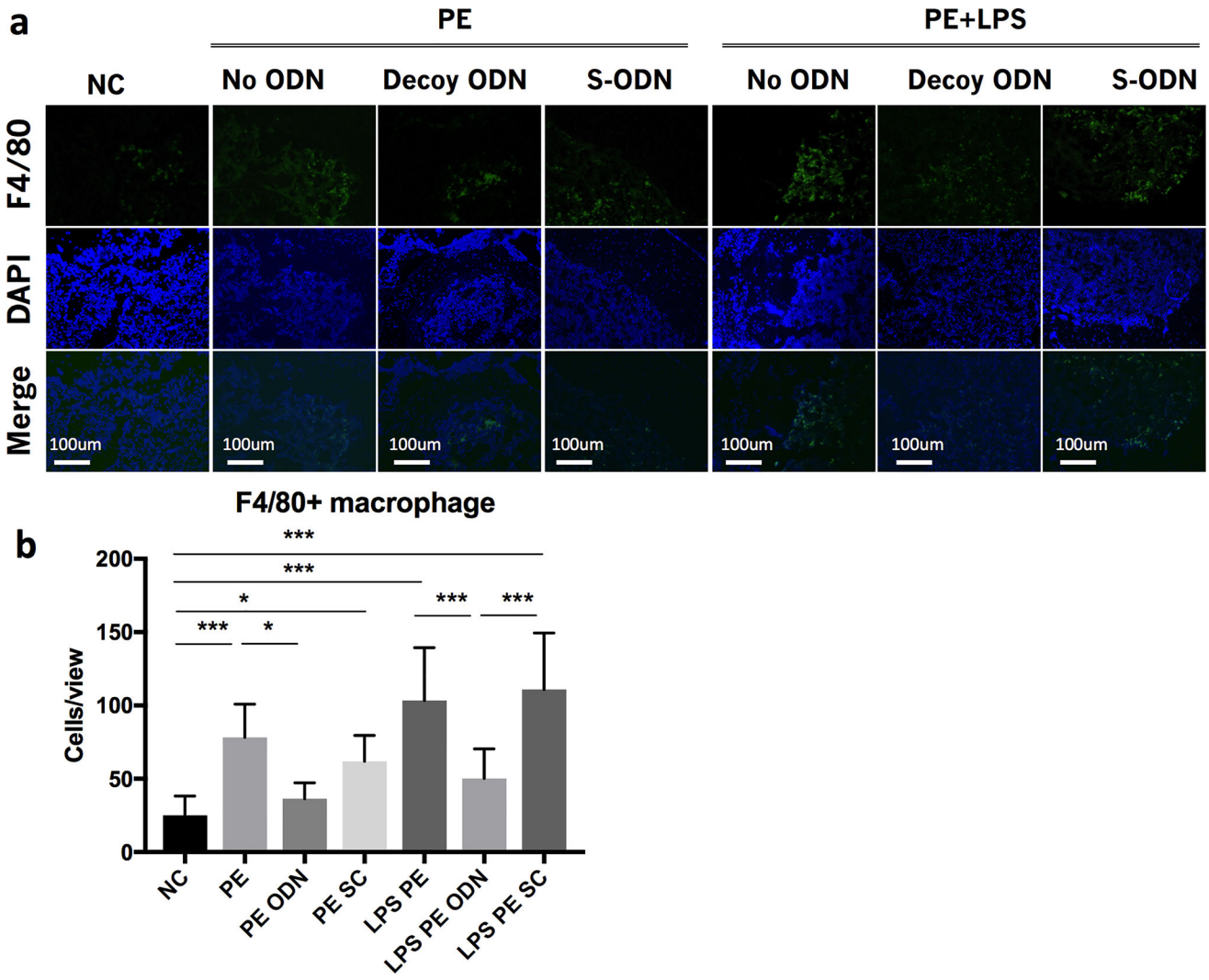

Figure. 4. NF-κB decoy ODN treatment reduced macrophage infiltration in the distal femur in response to UHMWPE particles with or without LPS

(**a**) F4/80 (FITC) and Cellular nucleus (DAPI) images were captured with fluorescence. (**b**) F4/80 positive cell numbers per image view were counted manually in 3 randomly selected views. PE: UHMWPE particles; NC: negative control; S-ODN: scrambled ODN. *p< .05, *** p<.005