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TLR9-engineered photoresponsive biomimetic nanodecoys for targeted NETs clearance to mitigate periodontitis-associated bone resorption

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Periodontitis is a widespread and multifactorial inflammatory disease, that leads to the progressive destruction of periodontal tissues and resorption of alveolar bone. The treatment of periodontitis is complicated by complex interactions between the host and oral microbiota, as well as difficulties in obtaining satisfactory treatment effects. Although neutrophil extracellular traps (NETs) participate in the process of disease development induced by periodontitis, their persistent accumulation aggravates inflammatory injury and prevents regenerative processes. Therefore, on the basis of the identification of Toll-like receptor 9 (TLR9) as an important receptor for NETs recognition, a biomimetic and photoresponsive nanodecoy system was designed for the enhanced targeting and efficient degradation of NETs. Taking advantage of the inherent DNA-binding property of TLR9 and incorporating DNase I, this nanodecoy system was designed to drive effective NETs clearance. Under near-infrared radiation, the nanodecoys demonstrate photothermal properties to enhance the clearance of NETs and create an environment conducive to bone regeneration. Moreover, this design inhibits osteoclast overactivation and promotes osteoblast-mediated mineralization to modulate the bone remodeling process. These results demonstrate a promising targeted treatment strategy for periodontitis, which includes the targeting and clearance of NETs and inducing bone regeneration.

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INTRODUCTION

Periodontitis is a chronic inflammatory disease driven by host-microbial interactions, leading to alveolar bone resorption and tooth loss. Neutrophils serve as the first line of defense in the immune system,¹ but their prolonged activation and impaired clearance contribute to tissue damage. When plaque microorganisms and their products invade periodontal tissues, neutrophils are rapidly recruited to the site and exert their immune effects through multiple mechanisms,² which help prevent further invasion by pathogens. Neutrophils eliminate pathogens through phagocytosis, releasing large amounts of proinflammatory factors and reactive oxygen species into local tissues via respiratory bursts.³ A key bactericidal mechanism is the formation of neutrophil extracellular traps (NETs), which ensnare pathogens but also exacerbate inflammation if not adequately removed.⁴ These structures can entrap large numbers of pathogens and kill them efficiently because of the high local concentration of antimicrobial enzymes.⁵ However, while neutrophils aid in pathogen clearance, the products they release may damage host tissues. In periodontitis, the lifespan of neutrophils is prolonged, and the reduced degradation rate of NETs enhances local inflammation and has more severe consequences.⁶

NETs degradation relies on extracellular DNases and macrophage uptake.⁷ Dysregulated NETs clearance is linked to chronic

periodontitis, and restoring degradation capacity after treatment reduces disease severity.⁸ Therefore, promoting NETs removal holds promise as a therapeutic strategy.⁹ While exogenous DNase can degrade NETs, its clinical utility is limited by short half-life and lack of targeting.¹⁰ Nanomaterial-based systems have been explored to improve NETs clearance, but challenges remain in terms of specificity and efficiency.^{11–13}

Here, we develop a targeted, photoresponsive nanodecoy system for NETs degradation and periodontal tissue repair. The system consists of black phosphorus quantum dots (BPQDs) loaded with DNase I and encapsulated within Toll-like receptor 9 (TLR9)-overexpressing cell membranes. Our research revealed that TLR9 mediates NETs-DNA recognition. On the basis of this principle, the TLR9-overexpressing membranes mimic natural biological recognition, providing nanodecoys that specifically target NETs. This design enables the release of dual-responsive DNase I under near-infrared (NIR) irradiation and NETs exposure.¹⁴ This approach aims to locally resolve NETs-mediated inflammation and support periodontal tissue regeneration in periodontitis. This is the first study to engineer TLR9-overexpressing cell membranes into a biomimetic nanodecoy that simultaneously achieves NETs targeting, NIR-controlled DNase I release, and local photothermal effects, thereby offering a novel multifunctional strategy for periodontitis therapy.

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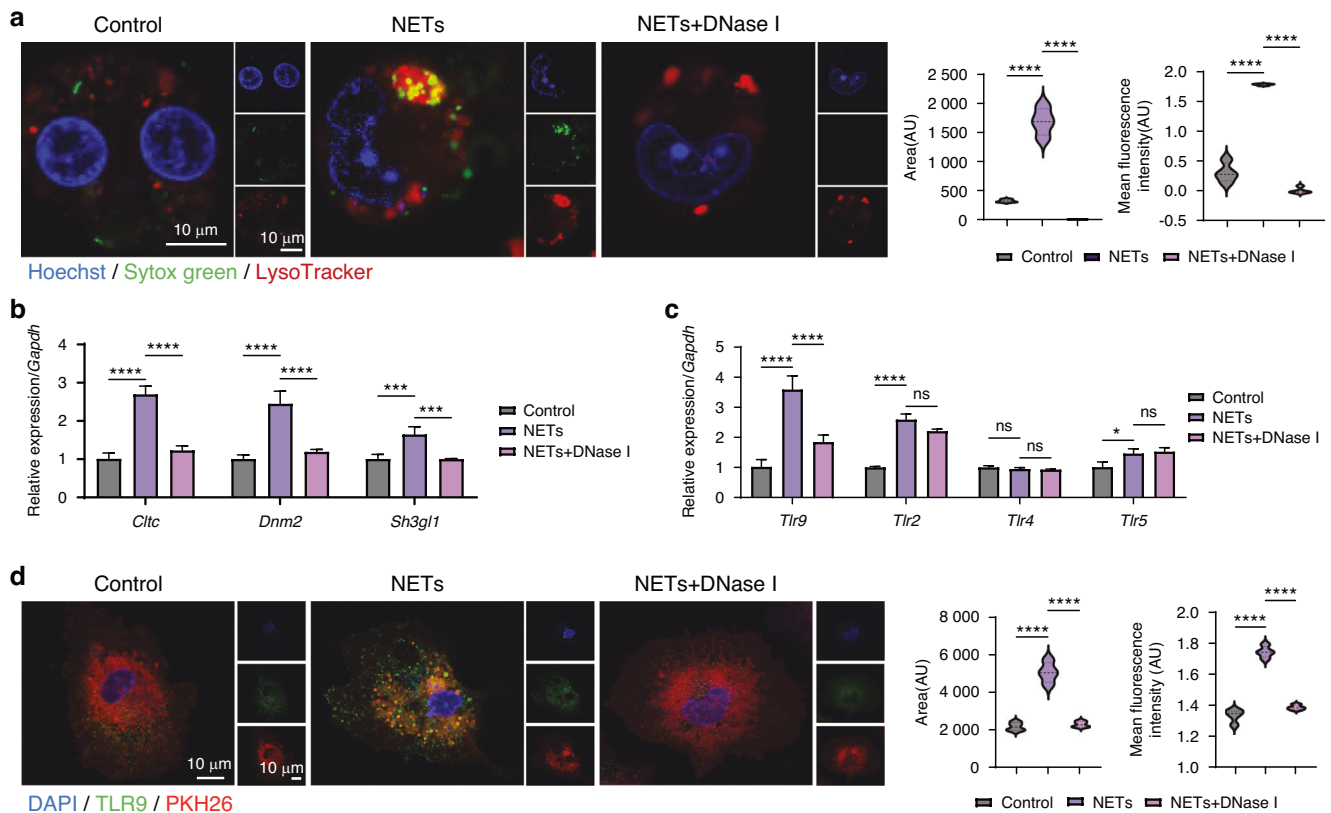


Fig. 1 TLR9 mediates NETs recognition. **a** Immunofluorescence colocalization staining of NETs and lysosomes in osteoclast precursor cells and quantitative analysis of NETs (green fluorescence)-positive areas and intensities ($n = 4$). **b** RT-qPCR analysis of endocytosis-related genes in osteoclast precursor cells ($n = 3$). **c** RT-qPCR analysis of osteoclast precursor cells ($n = 3$). **d** Immunofluorescence staining of TLR9 in osteoclasts and quantitative analysis ($n = 4$). The data are presented as the mean $\pm$ SD. Statistical significance was determined by one-way ANOVA with Tukey's test: ns, $P \geq 0.05$; * $P < 0.05$; *** $P < 0.001$; **** $P < 0.0001$. All experiments were independently repeated at least three times

RESULTS

Osteoclastic endocytosis of NETs

NETs release from neutrophils was induced by *P. gingivalis* (*P. g*) ultrasonic extract, after which NETs were cocultured with osteoclast precursor cells. Immunofluorescence staining revealed that NETs (green fluorescence) were endocytosed by osteoclast precursor cells and colocalized with lysosomes (red fluorescence); osteoclast precursor cell nuclei were labeled with blue fluorescence (Fig. 1a). However, no NETs were detected in the cells when the NETs were pretreated with 30 μ g/mL DNase I. Similar results were obtained by reverse transcription quantitative PCR (RT-qPCR). In the presence of NETs, the expression of the endocytosis-related genes *Cltc*, *Dnm2*, and *Sh3gl1*, which are involved in clathrin mediated endocytosis, was upregulated in osteoclast precursor cells, whereas this effect was reversed by DNase I treatment (Fig. 1b). To explore the functional association between TLR9 and endocytic proteins, immunofluorescence staining was performed. The colocalization of TLR9 and clathrin was observed in the NETs group (Fig. S1). Clathrin was observed to surround TLR9, forming a cage-like structure, indicating that TLR9-mediated endosome formation may depend on the clathrin-mediated pathway. These findings suggest that TLR9 may cooperate with the clathrin machinery during NETs internalization, although the precise molecular linkage remains to be defined. Clathrin-mediated endocytosis requires specific membrane receptors to recognize ligands for activation. In addition to their DNA scaffold, histones, and various enzymes, NETs may also contain virulence factors and antigens from the ultrasonic extract of *P. g*.

TLRs play crucial roles in immune recognition through the identification of both exogenous pathogens and endogenous

danger signals. NETs contain not only exogenous stimuli but also endogenous components. Considering that osteoclasts, which are differentiated from monocytes, have immune recognition capabilities, we further examined the expression of *Tlr2*, *Tlr4*, *Tlr5* (pathogen-sensing), and *Tlr9* (DNA-sensing) in osteoclasts induced by RAW264.7 cells cocultured with NETs using RT-qPCR. Compared with those in the control group, only the expression of *Tlr9*, *Tlr2*, and *Tlr5* was upregulated in the NETs group, and only the expression of *Tlr9* decreased in the DNase I-treated group (Fig. 1c).

TLR9 mediates NETs recognition and osteoclast differentiation. These findings suggest that TLR9 may be a critical receptor through which osteoclasts recognize NETs, with NETs-DNA acting as a critical ligand. This hypothesis was supported by immunofluorescence staining, which revealed high TLR9 expression on the cell membrane of osteoclasts in the NETs group. Furthermore, with the membrane dye PKH26 used to label the plasma membrane, internalized membrane regions visible as red fluorescence in the cytoplasm indicated endocytosis. The colocalization of red and green fluorescence in the cytoplasm suggested that NETs promote endocytosis and that TLR9 is highly expressed within these endocytic structures. In contrast, in the NETs + DNase I group, TLR9 expression was similar to that in the control group, and TLR9 was localized mainly in the cytoplasm (Fig. 1d). These observations indicate a potential association between TLR9 and NETs endocytosis, although direct evidence of TLR9 trafficking from the membrane to endosomes remains to be established.

To clarify the role of TLR9 in mediating the recognition of NETs by osteoclasts and its regulatory effects on osteoclast

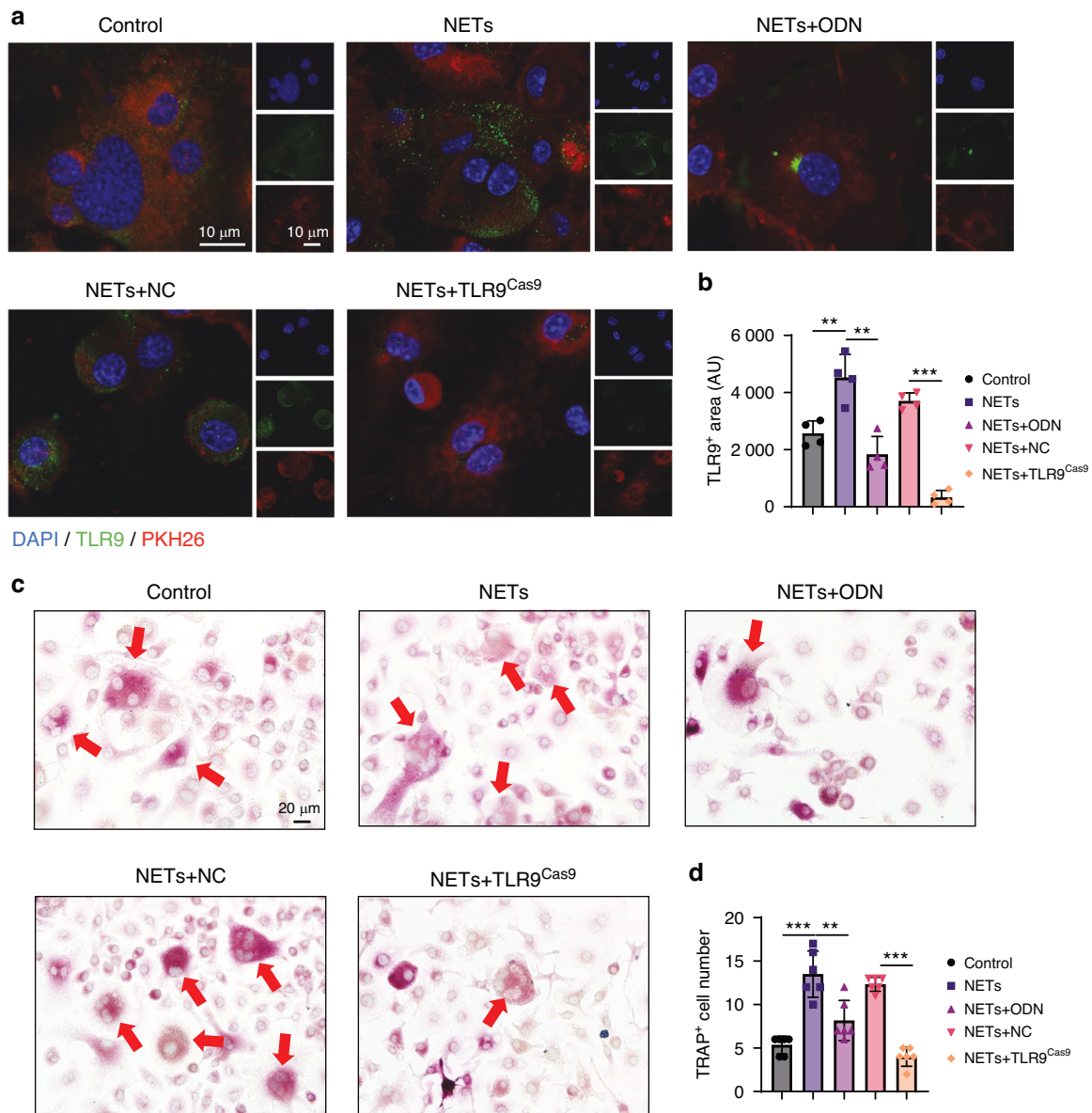


Fig. 2 TLR9 mediates osteoclast differentiation. **a** Immunofluorescence staining of TLR9 in osteoclasts ($n = 4$). **b** Quantitative analysis of immunofluorescence staining of TLR9 ($n = 4$). **c** TRAP staining of osteoclasts ($n = 6$). **d** Quantitative analysis ($n = 6$). The data are presented as the mean $\pm$ SD. Statistical significance was determined by one-way ANOVA with Tukey's test: ** $P < 0.01$; *** $P < 0.001$. All experiments were independently repeated at least three times

differentiation, we used both pharmacological and genetic inhibition approaches. The TLR9 inhibitor ODN TTAGGG, an inhibitory oligonucleotide that suppresses TLR9 expression upon binding, was used to investigate the relationship between TLR9 and NETs. Additionally, TLR9 knockout was achieved using lentivirus mediated delivery of Cas9 (TLR9^{Cas9}), followed by coculture with NETs to further validate our hypothesis. Immunofluorescence staining confirmed that TLR9 expression was effectively suppressed in both the ODN group and the TLR9^{Cas9} group, even in the presence of NETs, which demonstrated the efficacy of both interventions (Fig. 2a, b). In contrast, TLR9 expression in the negative control virus (NC) group remained unaffected.

The differentiation and maturation of osteoclasts in each group were evaluated by tartrate-resistant acid phosphatase (TRAP) staining, RT-qPCR, and western blotting. TRAP staining confirmed that NETs promoted osteoclast maturation, whereas TLR9 inhibition reversed this effect (Fig. 2c, d). The RT-qPCR results further

indicated that the expression of *Ctsk* and *Acp5* was significantly increased in the NETs group. Both ODN inhibition and TLR9 knockout reduced the expression of these genes. The suppression of *Ctsk* and *Acp5* expression was most pronounced in the TLR9^{Cas9} group, even in the presence of NETs (Fig. S2a). Western blotting analysis corroborated these findings, demonstrating decreased CTSK protein levels in the ODN and TLR9^{Cas9} groups (Fig. S2b). These results demonstrate that TLR9 serves as an important receptor for NETs recognition by osteoclasts. NETs are endocytosed into osteoclasts in a TLR9-dependent manner, thereby promoting osteoclast differentiation.

Preparation and characterization of TLR9 nanodecoys

On the basis of our findings, engineered TLR9 nanodecoys (ND^{TLR9}) were designed to simultaneously target NETs clearance and alveolar bone regeneration. Specifically, by mimicking the mechanism through which osteoclasts recognize NETs via TLR9, we developed an ND^{TLR9} capable of targeting NETs and releasing

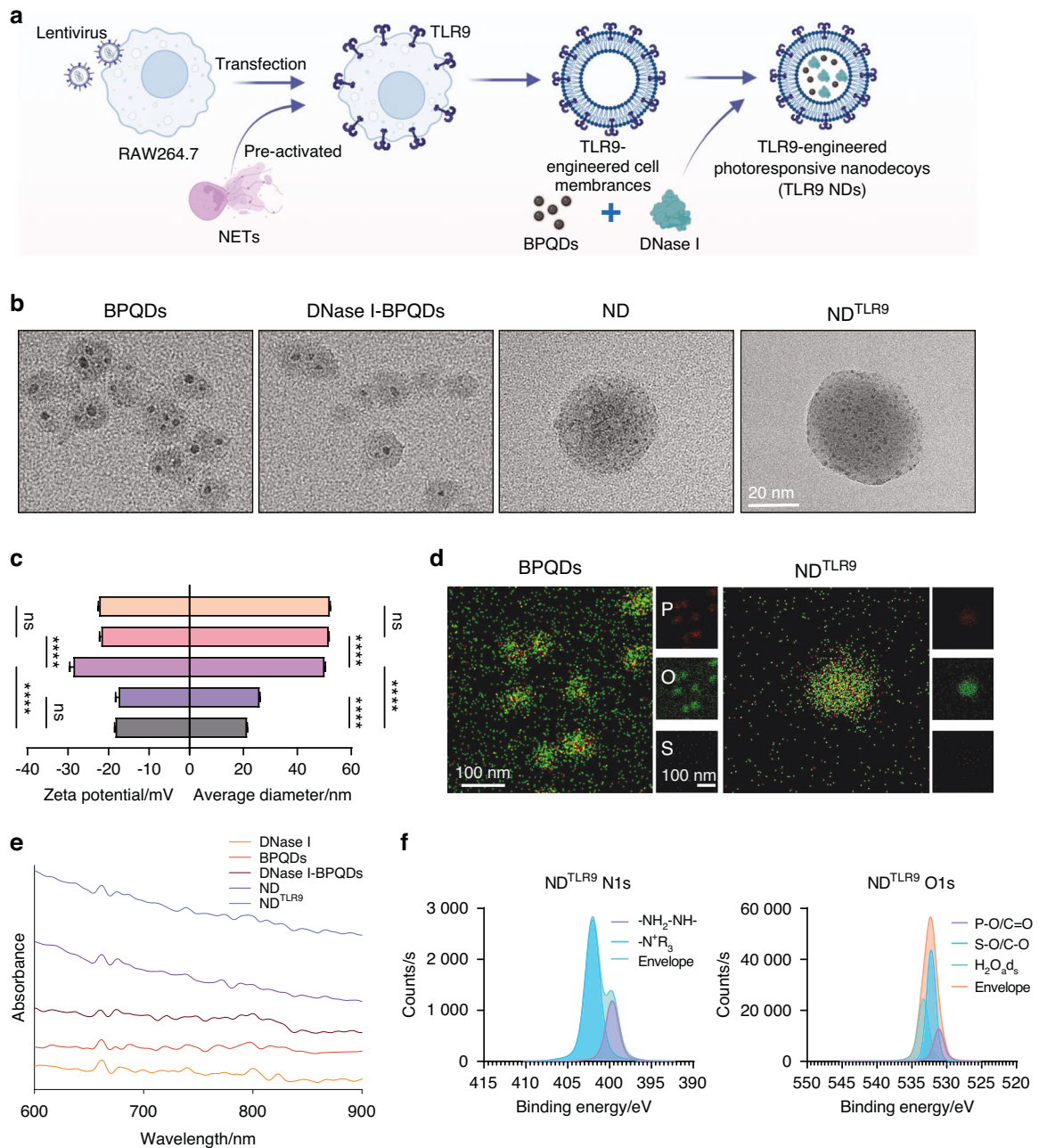


Fig. 3 Characterization of TLR9 nanodecoys. **a** Schematic illustration of the preparation process for TLR9 nanodecoys. **b** TEM images. **c** Particle size and zeta potential ($n = 3$). **d** Element mapping. **e** UV-Vis-NIR spectrum. **f** High-resolution N1s and O1s spectra of ND^{TLR9}. The data are shown as the mean $\pm$ SD. Statistical significance was determined by one-way ANOVA with Tukey's test: ns, $P \geq 0.05$; **** $P < 0.0001$. All experiments were independently repeated at least three times

DNase I upon NIR irradiation. Furthermore, in the subsequent stages, the use of NIR radiation along with the biodegradation products of BPQDs enhances the osteogenic differentiation of BMSCs, promoting the regeneration of periodontal bone tissue.

Considering the homology of RAW264.7 cells with osteoclasts, as well as their ease of culture and great expansion ability, the RAW264.7 cell line was selected as the cellular membrane source for the nanodecoys. RAW264.7 cells were transfected with lentivirus to overexpress TLR9. Western blotting analysis confirmed successful TLR9 overexpression (Fig. S3). Subsequent coculture with NETs further induced the membrane localization of TLR9, after which the cell membranes were harvested. BPQDs and DNase I were self-assembled into DNase I-BPQDs complexes via ultrasonication. Under ultrasonic conditions, the engineered

TLR9 cell membranes and DNase I-BPQDs were integrated through charge interactions to form ND^{TLR9} (Fig. 3a). The loading capacity and encapsulation efficiency of DNase I were 82.7% and 61.3%, respectively (Fig. S4a). Transmission electron microscopy (TEM) was employed to characterize the morphologies of BPQDs, DNase I-BPQDs, ND (untreated cell membranes assembled with DNase I-BPQDs), and ND^{TLR9}. These results indicated that conjugation with DNase I did not significantly change the morphology of the BPQDs. In contrast, after membrane coating, a clear core-shell structure was observed (Fig. 3b). Dynamic light scattering (DLS) measurements revealed narrow size distributions for both ND and ND^{TLR9}, indicating high uniformity of DNase I-BPQDs, cell membrane fragments (CM), ND, and ND^{TLR9} (Fig. S4b). Notably, a marked increase in hydrodynamic size was detected after cell

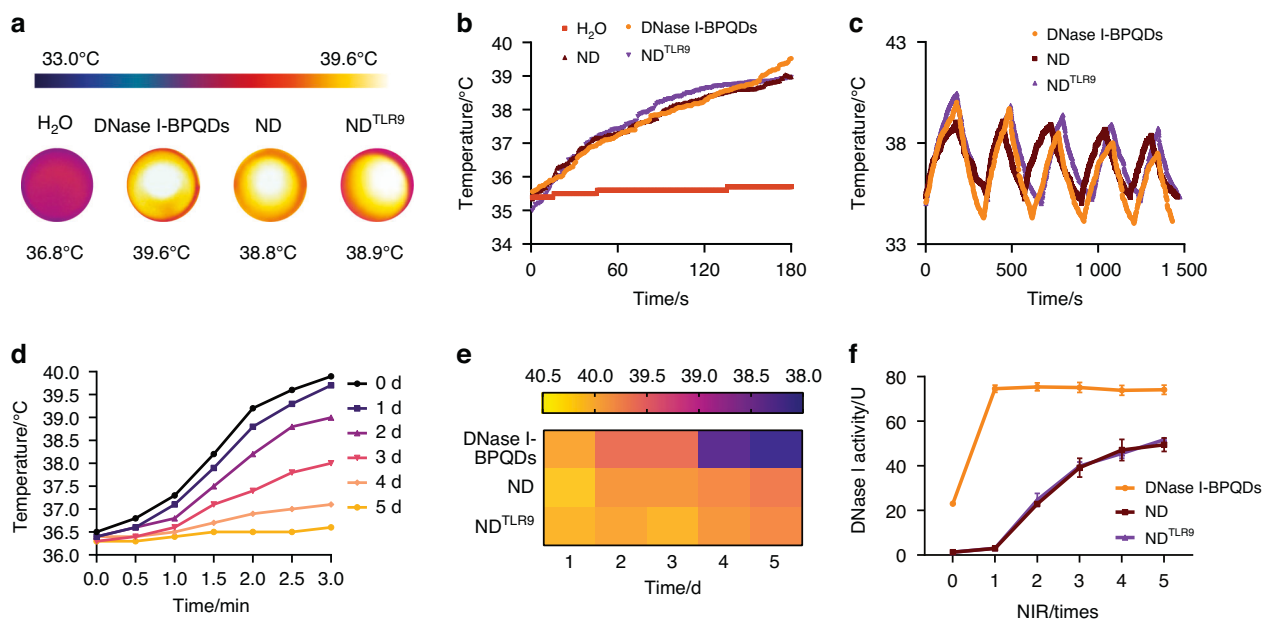


Fig. 4 Photothermal effects of TLR9 nanodecoys. **a** Thermal image of DNase I-BPQDs, ND and ND^{TLR9} under NIR irradiation ($n=4$). **b** Representative real-time thermal temperature profiles under NIR irradiation (808 nm, 1.5 W/cm², 3 min). **c** Temperature increase and cooling curves during five NIR light irradiation cycles ($n=3$). **d** Real-time thermal temperature profiles of DNase I-BPQDs under NIR irradiation for 5 days ($n=3$). **e** Thermal stability after NIR irradiation for 3 min per day over 5 days ($n=3$). **f** DNase I activity released from DNase I-BPQDs, ND and ND^{TLR9} under 5 times repeated NIR irradiation ($n=3$). The data are shown as the mean $\pm$ SD. All experiments were independently repeated at least three times

membrane coating. Zeta potential analysis revealed a slight increase after the binding of DNase I to the BPQDs, whereas an obvious decrease occurred after the cell membrane encapsulation of the DNase I-BPQDs. The difference in zeta potential between the constructed ND^{TLR9} and bare cell membranes may be attributed to partial insertion of DNase I-BPQDs into the membranes (Fig. 3c). Elemental mapping analysis further confirmed the presence of phosphorus (P) in ND^{TLR9} (Fig. 3d), providing additional evidence of successful core-shell assembly and the fabrication of ND^{TLR9}.

The influence of DNase I modification on BPQDs was evaluated using ultraviolet–visible–near-infrared (UV–Vis–NIR) absorption spectroscopy (Fig. 3e). No significant difference in absorption at 808 nm was observed between ND^{TLR9} and pure BPQDs, indicating that the conjugation of proteins and cell membranes did not alter the optical absorption properties. X-ray photoelectron spectroscopy (XPS) analysis of ND^{TLR9} and CM revealed characteristic peaks of carbon (C), nitrogen (N), oxygen (O), sulfur (S), and phosphorus (P) (Fig. S5a). High-resolution spectra of N1s and O1s in ND^{TLR9} are presented in Fig. 3f. Compared with the high-resolution XPS spectrum of the CM alone (Fig. S5b), the C1s spectrum of ND^{TLR9} showed a peak at 288.86 eV, which is attributed to an increased proportion of C=O bonds. Similarly, the N1s spectrum exhibited a notable increase in the intensity of -N⁺R₃ bonds at 402.02 eV. These changes suggested the successful insertion of DNase I into the surface shell of the nanodecoys. An increase in the -N⁺R₃ content increased the positive surface charge of ND^{TLR9}, which was expected to improve its ability to target negatively charged DNA in NETs. These findings were consistent with the results of the zeta potential measurements. Furthermore, the O1s spectrum showed a peak at 533.33 eV assigned to adsorbed water (H₂O_{ad}), indicating significantly improved hydrophilicity of ND^{TLR9} and confirming the enhanced biocompatibility of the nanodecoys.

Photothermal effect of ND^{TLR9}

The *in vitro* photothermal performance of the nanodecoys was evaluated under NIR laser irradiation, and the temperature

changes were monitored using an infrared thermal camera (Fig. 4a). The results indicated that both ND and ND^{TLR9} rapidly increased in temperature under NIR irradiation, and the difference between them was not significant (Fig. 4b). Under the same conditions, the temperature of the DNase I-BPQDs reached 39.6 °C, which was slightly higher than that of ND and ND^{TLR9}. This difference may be attributed to the partial oxidation of BPQDs during synthesis, which can compromise their photothermal efficacy. Considering the requirements of further *in vitro* and *in vivo* applications as well as the potential influence of temperature on cells, we chose 1.5 W/cm² for laser irradiation *in vitro*. Furthermore, both ND and ND^{TLR9} demonstrated excellent photothermal stability over five consecutive laser on/off cycles, with no significant loss of photothermal conversion efficiency (Fig. 4c). In contrast, the photothermal performance of the DNase I-BPQDs gradually decreased over repeated cycles, which is consistent with the tendency of the BPQDs to degrade in aqueous and oxygen-containing environments. To further evaluate the photothermal stability, the samples were incubated at 37 °C under 5% CO₂ for 5 days. While DNase I-BPQDs maintained a relatively effective photothermal response for only 3 days (Fig. 4d), both ND and ND^{TLR9} still reached temperatures of nearly 39 °C after 3 min of NIR irradiation on Day 5 (Fig. 4e). The detection of DNase I activity released from the nanodecoys after five rounds of NIR irradiation confirmed that enzymatic activity was positively correlated with the number of NIR exposures (Fig. 4f). These results demonstrated that ND^{TLR9} had great photothermal stability and controlled DNase I release capability, supporting its potential for repeated activation during photothermal therapeutic applications.

Favorable biosafety of ND^{TLR9} *in vitro*

To evaluate the biosafety of ND^{TLR9}, a concentration of 50 µg/mL (equivalent to 30 µg/mL of DNase I) was selected for *in vitro* experiments on the basis of the results of the cell counting kit-8 (CCK-8) assay (Fig. S6a). Notably, 1.5 W/cm² NIR irradiation had no adverse effect on cell proliferation. However, compared with both

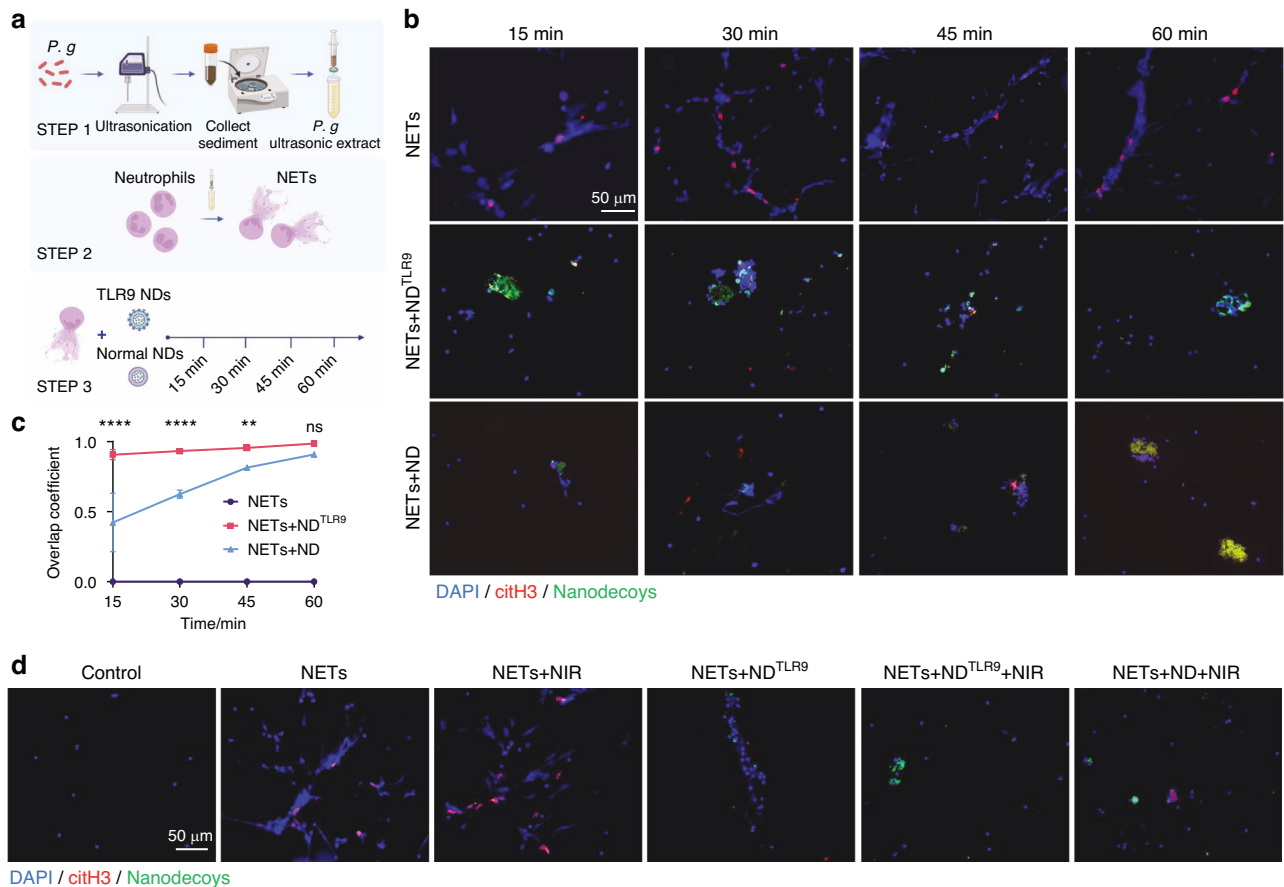


Fig. 5 NETs targeting and clearance effect of ND^{TLR9}. **a** Schematic illustration of the NETs-targeting ability and clearance effect of TLR9 nanodecoys. **b** Immunofluorescence staining of NETs cocultured with ND or ND^{TLR9} (*n* = 5). **c** Quantitative colocalization analysis of citH3 and ND or ND^{TLR9} (*n* = 5). **d** Immunofluorescence staining of NETs cocultured with NIR, ND^{TLR9}, ND^{TLR9} + NIR, or ND + NIR (*n* = 5). The data are shown as the mean ± SD. Statistical significance was determined by one-way ANOVA with Tukey's test: ns, *P* ≥ 0.05; ***P* < 0.01; *****P* < 0.0001. All experiments were independently repeated at least three times

ND and ND^{TLR9}, the DNase I-BPQDs exhibited stronger cytotoxicity (Fig. S6b). RT-qPCR analysis after coculture with RAW264.7-derived macrophages further revealed that DNase I-BPQDs significantly upregulated the expression of the *Tnfa* and *Il-6* genes, whereas neither ND nor ND^{TLR9} increased the expression of these gene expression (Fig. S6c). These findings were consistent with those of the apoptosis assays performed on both bone marrow mesenchymal stem cells (BMSCs) and RAW264.7-derived macrophages (Fig. S6d, e). Using an in vitro coculture system, we confirmed that ND^{TLR9} was specifically taken up by RAW264.7-derived macrophages (Fig. S6f). These results demonstrated that ND^{TLR9} had favorable biocompatibility in vitro and mitigated the cytotoxicity associated with direct exposure of cells to bare BPQDs. Additionally, the excess nanodecoys were cleared by macrophages during circulation, thereby minimizing potential adverse effects on other cells in tissues.

ND^{TLR9} targeting and clearance of NETs

The ability of ND^{TLR9} to target NETs was evaluated using fluorescence colocalization assays. *P. g* ultrasonic extract was used to stimulate neutrophils to release NETs. The NETs were then cocultured with either ND@PKH67 or ND^{TLR9}@PKH67 for 15–60 min (Fig. 5a). The NETs showed a typical filamentous DNA structure and were colocalized with citrullinated histone H3 (citH3). After 15 min of coculture, strong colocalization of citH3 and ND^{TLR9}@PKH67 was observed (Fig. 5b). As the coculture time increased, the citH3⁺ area gradually decreased, and the DNA

structure of NETs became less distinct (Fig. S7a). In contrast, ND@PKH67 showed noticeable colocalization with citH3 only after 45 to 60 min (Fig. 5c). These results indicated that ND^{TLR9} exhibited superior targeting efficiency toward NETs. Moreover, even without NIR irradiation, the DNase I on the surface of the nanodecoys may also have the ability to clear NETs.

The degradation of NETs by nanodecoys was further evaluated after NIR irradiation. No significant structural changes in NETs or a reduction in the citH3⁺ area were observed with NIR treatment alone (NETs + NIR group). In the ND^{TLR9} group without irradiation (NETs + ND^{TLR9} group), the citH3⁺ area was notably reduced, although some NETs-DNA aggregation remained visible. In comparison, the NETs + ND^{TLR9} + NIR group showed an obvious reduction in NETs, with no clear NETs-DNA structure detected. The NETs + ND + NIR group also exhibited considerable NETs-DNA degradation, yet the citH3⁺ area remained significantly larger than that in the NETs + ND^{TLR9} + NIR group (Fig. 5d and S8a, b). Subsequent quantitative analysis using a NETs-DNA ELISA kit corroborated these findings (Fig. S7b). These results demonstrated that NIR-released DNase I from ND^{TLR9} effectively cleared NETs, highlighting the potential of ND^{TLR9} for targeted NETs degradation under photothermal stimulation.

ND^{TLR9} inhibits osteoclast differentiation caused by NETs and promotes the osteogenic effect of BMSCs

As indicated in our previous studies, NETs can promote the differentiation (Fig. S9a, b) and maturation of osteoclasts (Fig. S9c).

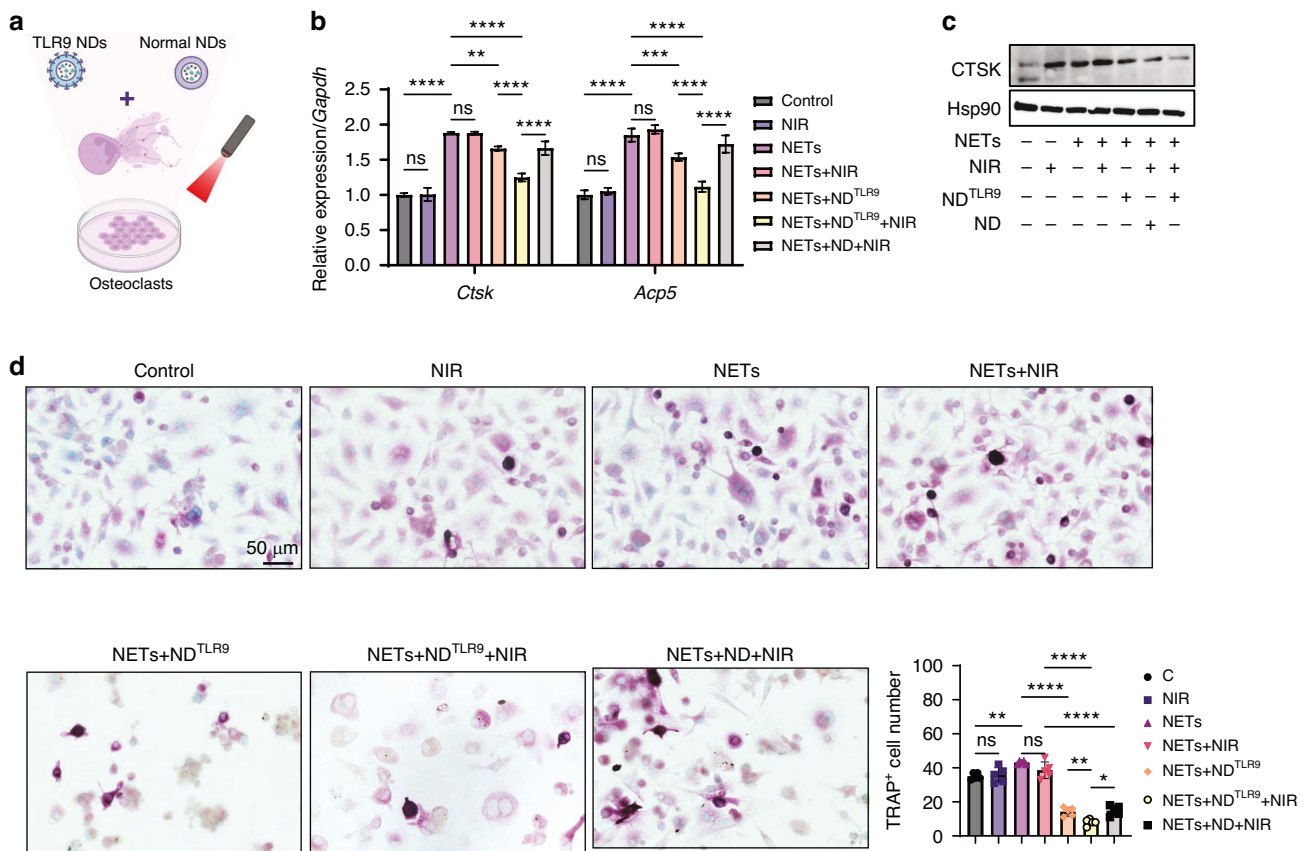


Fig. 6 ND^{TLR9} inhibits osteoclast differentiation caused by NETs. **a** Schematic illustration of the cell culture protocol. **b** RT-qPCR analysis of osteoclasts ($n = 3$). **c** Western blotting analysis of CTSK protein expression in osteoclasts ($n = 3$). **d** TRAP staining of osteoclasts and corresponding quantitative analysis ($n = 5$). The data are shown as the mean $\pm$ SD. Statistical significance was determined by one-way ANOVA with Tukey's test: ns, $P \geq 0.05$; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$. All experiments were independently repeated at least three times

To further evaluate the therapeutic potential of ND^{TLR9}, we cocultured NETs and osteoclasts with either ND or ND^{TLR9} (Fig. 6a). RT-qPCR after 5 days of culture revealed that NIR irradiation alone (NIR group) did not affect the expression of osteoclast-related genes. Similarly, in the presence of NETs, NIR irradiation did not suppress the expression of *Ctsk* or *Acp5* (NETs+NIR group). In contrast, both the NETs+ND^{TLR9} and NETs+ND+NIR groups showed inhibitory effects on osteoclast related gene expression. The most significant gene suppression was observed in the NETs+ND^{TLR9}+NIR group, whose osteoclast-related gene expression levels were markedly lower than those in the NETs+ND^{TLR9} or NETs+ND+NIR groups (Fig. 6b). These findings were validated by western blotting after 7 days and TRAP staining after 9 days (Fig. 6c, d).

We further investigated the effect of ND^{TLR9} on the osteogenic differentiation of BMSCs using RT-qPCR, alkaline phosphatase (ALP), and alizarin red S (ARS) staining. Under NIR irradiation, the 5-day RT-qPCR results revealed upregulated expression of osteogenic markers (NIR group). NETs significantly suppressed osteogenic gene expression, but this effect could not be reversed by NIR irradiation alone. Although *Spp1* expression was significantly different between the NETs+NIR and NETs groups, the expression of the other genes did not significantly differ. Osteogenic gene expression in the NETs+ND^{TLR9} and NETs+ND+NIR groups was partially rescued compared with that in the NETs group, and the rescue effect was most significant in the NETs+ND^{TLR9}+NIR group (Fig. 7a). Similar trends were observed for ALP staining on Day 7 and ARS staining ON Day 21 (Fig. 7b–d). A schematic

illustration of how ND^{TLR9} mitigates osteoclast activation and promotes osteogenesis through NETs clearance is presented in Fig. S10.

Given the strong inhibitory effect of NETs on BMSC osteogenesis, it is reasonable to hypothesize that the clearance of NETs may restore osteogenic function. The best osteogenic effect was achieved in the NETs+ND^{TLR9}+NIR group, which can be attributed to the combined benefits of NETs degradation, NIR-induced osteogenesis, a photothermal effect on osteogenesis and the release of phosphate ions from BQDs degradation. In this study, NIR primarily triggered DNase I release from the nanodecoys, whereas its photothermal effect played only a minor role in NETs clearance and bone protection. The robust release of DNase I is a powerful drug for clearing NETs and plays a critical role in promoting osteogenesis. In conclusion, ND^{TLR9} plus NIR irradiation mitigates osteoclast differentiation and promotes osteogenesis through multiple mechanisms, providing strong evidence for its application in vivo.

ND^{TLR9} exhibits biosafety and photothermal effects in vivo. The establishment of the periodontitis model is illustrated in Fig. 8a. ND^{TLR9} was locally administered to the periodontitis region, which was then subjected to NIR irradiation at 1.0 W/cm² for 5 s (PD+ND^{TLR9}+NIR group). Local temperature changes were monitored daily using an infrared thermal camera (Fig. 8b). Quantitative analysis revealed that neither NIR irradiation alone (PD+NIR group) nor local application of ND^{TLR9} alone (PD+ND^{TLR9} group) induced noticeable temperature changes, whereas the combination of ND^{TLR9} and NIR irradiation led to a significant

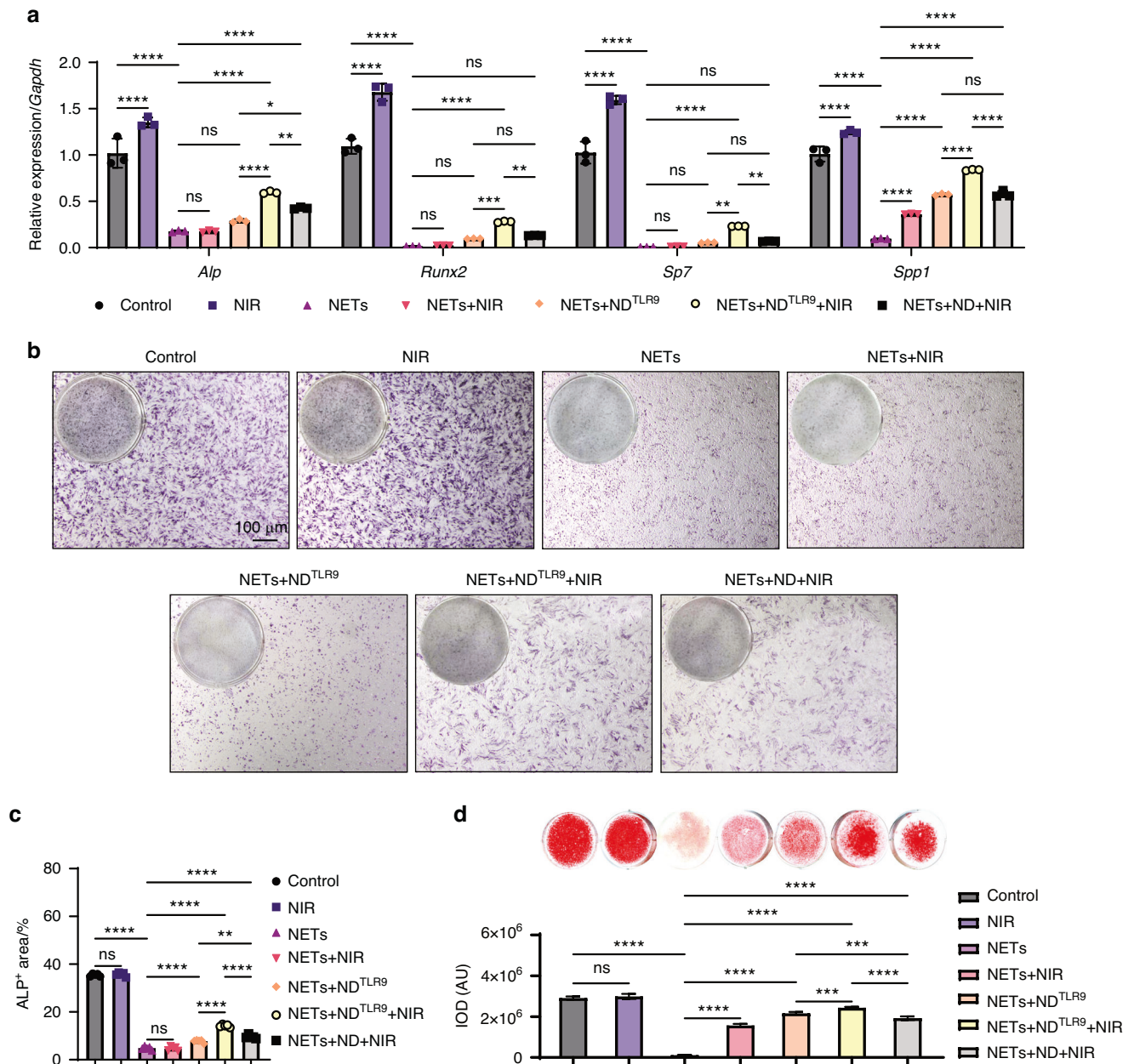


Fig. 7 ND^{TLR9} promotes the osteogenic effect of BMSCs. **a** RT-qPCR analysis of osteogenic gene expression in BMSCs ($n = 3$). **b** ALP staining and **c** quantitative analysis of BMSCs ($n = 5$). **d** Alizarin red staining and quantitative analysis of BMSCs ($n = 5$). The data are shown as mean $\pm$ SD. Statistical significance was determined by one-way ANOVA with Tukey's test: ns, $P \geq 0.05$; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$. All experiments were independently repeated at least three times

temperature increase, reaching up to 40 °C. With repeated irradiation over several days, the PD + ND^{TLR9} + NIR group maintained the photothermal effect for 4 days. However, further NIR irradiation did not cause additional temperature increase (Fig. 8c). ND^{TLR9} was gradually degraded under NIR exposure in vivo, indicating that ND^{TLR9} does not present a long-term burden on the body.

To further evaluate the biological safety of ND^{TLR9}, organ sections from all groups were subjected to hematoxylin and eosin (H&E) staining, and no obvious abnormalities were observed (Fig. S11). Additionally, the total phosphorus content in organ tissues was measured. The results indicated that the phosphorus level in the kidneys was greater in the PD + ND^{TLR9} + NIR group than in the other groups (Fig. S12).

To further clarify the metabolic pathway involved, we measured phosphorus levels in urine and feces at 7 days post-treatment (Fig. S13a, b). Compared with the other groups, the PD + ND^{TLR9} + NIR group had significantly greater urinary phosphorus excretion and higher fecal phosphorus levels on Day 7, indicating that the increase in renal phosphorus is transient and eliminated via both renal and intestinal routes. Nevertheless, no significant abnormalities were detected in routine blood tests, and no notable differences were observed in the blood levels of P or calcium (Ca) among the groups (Fig. S14). Since the metabolism of these two ions is often interrelated, and given the short duration and dosage of ND^{TLR9} application in vivo, it can be concluded that the biosafety of ND^{TLR9} is controllable.

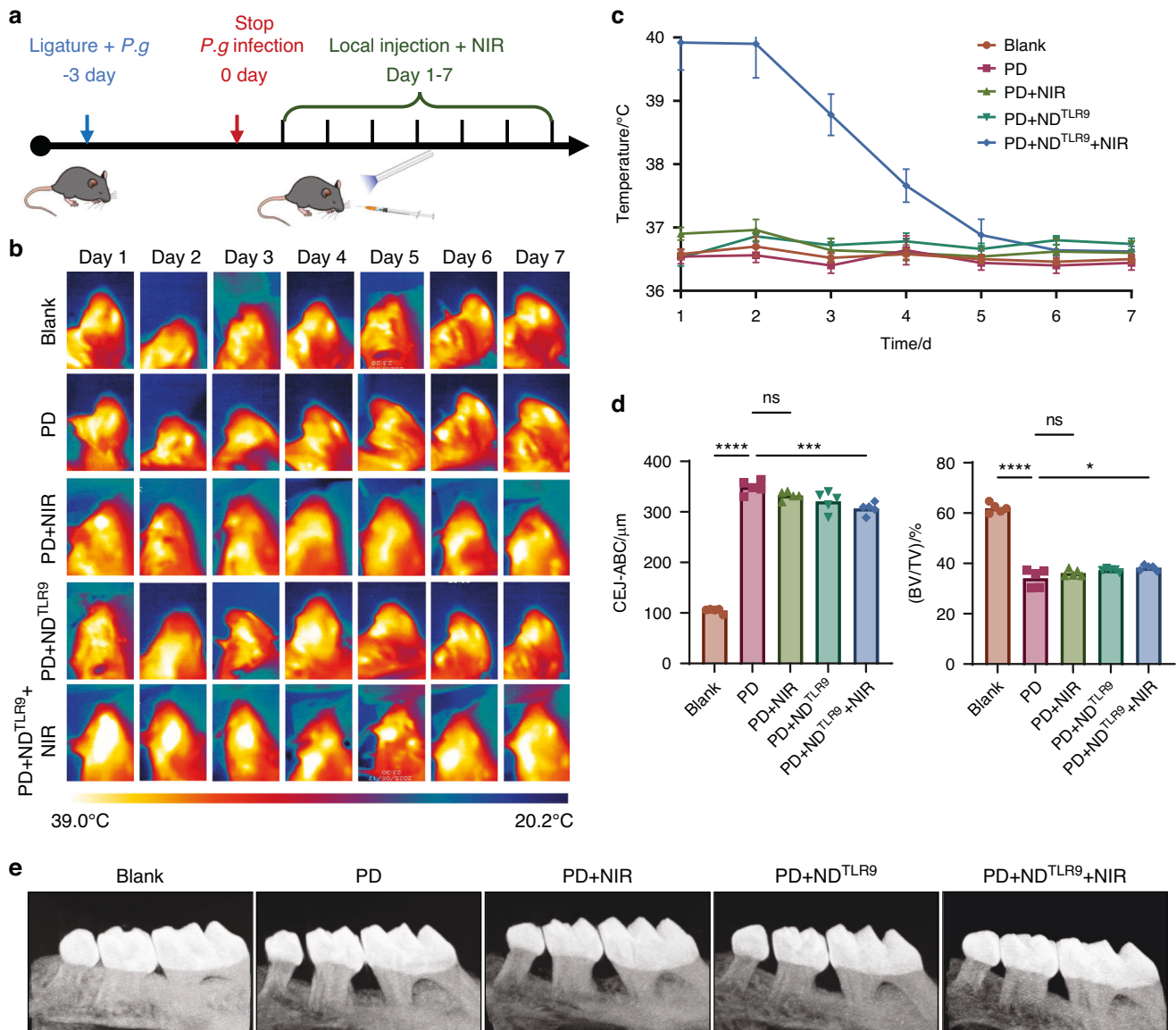


Fig. 8 The in vivo photothermal effect of alleviates experimental periodontitis in mice. **a** Schematic illustration of the experimental process in vivo. **b** Thermal images of different groups under NIR irradiation (808 nm, 1.0 W/cm², 5 s). **c** Representative real-time thermal temperature under NIR irradiation for 7 days ($n = 5$). **d** Quantitative measurement of the CEJ-ABC distance and BV/TV measurements of alveolar bone ($n = 5$). **e** 3D images of the second maxillary molar area obtained via micro-CT ($n = 5$). The data are shown as the mean $\pm$ SD. Statistical significance was determined by one-way ANOVA with Tukey's test: ns, $P \geq 0.05$; * $P < 0.05$; *** $P < 0.001$; **** $P < 0.0001$. All experiments were independently repeated at least three times

ND^{TLR9} alleviates experimental periodontitis in mice by inhibiting alveolar bone resorption and promoting bone remodeling. Compared with the blank group, the periodontitis (PD) group exhibited a significantly greater bone loss distance and lower BV/TV. Simple NIR irradiation did not significantly affect bone loss. Bone resorption was mitigated in both the PD + ND^{TLR9} and PD + ND^{TLR9} + NIR groups. The most significant therapeutic effect was observed in the PD + ND^{TLR9} + NIR group (Fig. 8d, e and S15a). H&E staining revealed inflammatory cell infiltration in both the gingival epithelium and mesenchyme in the PD and PD + NIR groups, whereas these pathological changes were alleviated in the PD + ND^{TLR9} + NIR group. Masson staining demonstrated that the collagen fibers in the PD + ND^{TLR9} + NIR group were denser and more organized. In contrast, the PD and PD + NIR groups displayed disorganized fiber structures (Fig. S15b, c).

Immunofluorescence staining revealed greater co-expression of MPO and cith3 in the PD and PD + NIR groups than in the blank group, whereas the PD + ND^{TLR9} + NIR group presented reduced signals, indicating the degradation of NETs at inflammatory sites (Fig. 9a, c and S16). ELISA further confirmed these observations, with the lowest NETs content in the serum of mice treated with ND^{TLR9} + NIR (Fig. 9d). Immunohistochemical staining for CTSK, myeloperoxidase (MPO), and Runx2 was performed to further evaluate osteoclast activity, NETs status and osteogenic potential in the periodontal tissues (Fig. 9b). The positive CTSK expression in both the PD and PD + NIR groups was significantly greater than that in the blank group, whereas this trend was reversed in the PD + ND^{TLR9} + NIR group, indicating the suppression of osteoclast function (Fig. 9e). The expression of MPO, a specific marker of neutrophils and NETosis, was significantly inhibited in the PD + ND^{TLR9} + NIR group (Fig. 9f). Similarly, Runx2 expression was

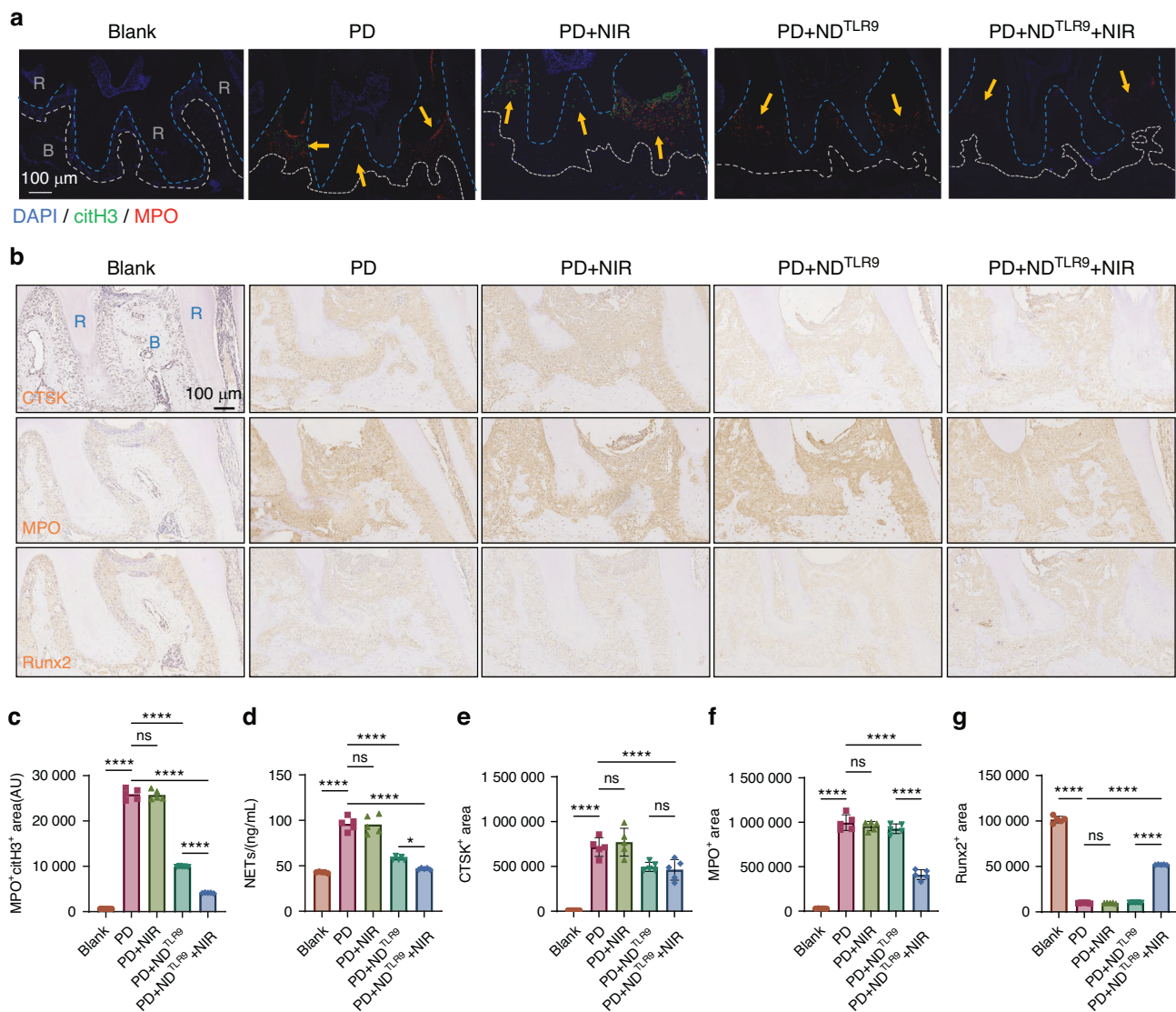


Fig. 9 In vivo NETs clearance and bone remodeling effects of ND^{TLR9}. **a** Immunofluorescence staining of citH3 and MPO in periodontal tissues. The blue dashed line indicates the root boundary, the gray dashed line indicates the alveolar bone boundary, and the yellow arrow highlights the periodontal tissue areas positive for MPO and citH3 expression. R indicates root, B indicates alveolar bone ($n = 5$). **b** Immunohistochemical staining of CTSK, MPO, and Runx2 in periodontal tissues ($n = 5$). **c** Quantitative analysis of the double-positive regions of citH3 and MPO expression ($n = 5$). **d** ELISA of NETs in serum ($n = 5$). **e** Quantitative analysis of CTSK positive area in periodontal tissues ($n = 5$). **f** Quantitative analysis of MPO positive area in periodontal tissues ($n = 5$). **g** Quantitative analysis of Runx2 positive area in periodontal tissues ($n = 5$). The data are shown as the mean $\pm$ SD. Statistical significance was determined by one-way ANOVA with Tukey's test: ns, $P \geq 0.05$; * $P < 0.05$; **** $P < 0.0001$. All experiments were independently repeated at least three times

lower in the PD group than in the blank group but was increased in the PD + ND^{TLR9} + NIR group, indicating increased osteogenic potential (Fig. 9g). The immunohistochemical and immunofluorescence results from the PD + ND^{TLR9} group suggested a relatively limited capacity to clear NETs, which was consistent with the in vitro findings.

DISCUSSION

In this study, we identified TLR9 as a critical receptor for NETs recognition by osteoclasts. On the basis of these findings, we developed a biomimetic photoresponsive nanodecoy (ND^{TLR9}) by coating TLR9-overexpressing cell membranes onto DNase I-loaded BPQDs. This design achieves three integrated functions: (i) TLR9-mediated targeting of NETs, (ii) NIR-triggered DNase I release for NETs degradation, and (iii) photothermal effects that

promote bone regeneration. To our knowledge, this is the first study to engineer TLR9-overexpressing membranes into a nanodecoy for simultaneous NETs clearance and periodontal bone repair.

Compared with existing NETs-targeting strategies, such as free DNase I (short half-life, no targeting)¹⁵ or non-specific nanocarriers,¹⁶ our system offers enhanced specificity via TLR9-mediated recognition and controlled drug release under NIR. Previous studies have used DNase I-coated nanoparticles or hydrogel microspheres for NETs scavenging,^{17,18} but they lack active targeting. Previous studies have shown that in pediatric systemic lupus erythematosus, NETs-DNA can be taken up by dendritic cells, leading to the production of TLR9-dependent type I interferon and the generation of autoantibodies against NETs-DNA.¹⁹ In addition, the treatment of human umbilical vein endothelial cells with NETs has been reported to promote

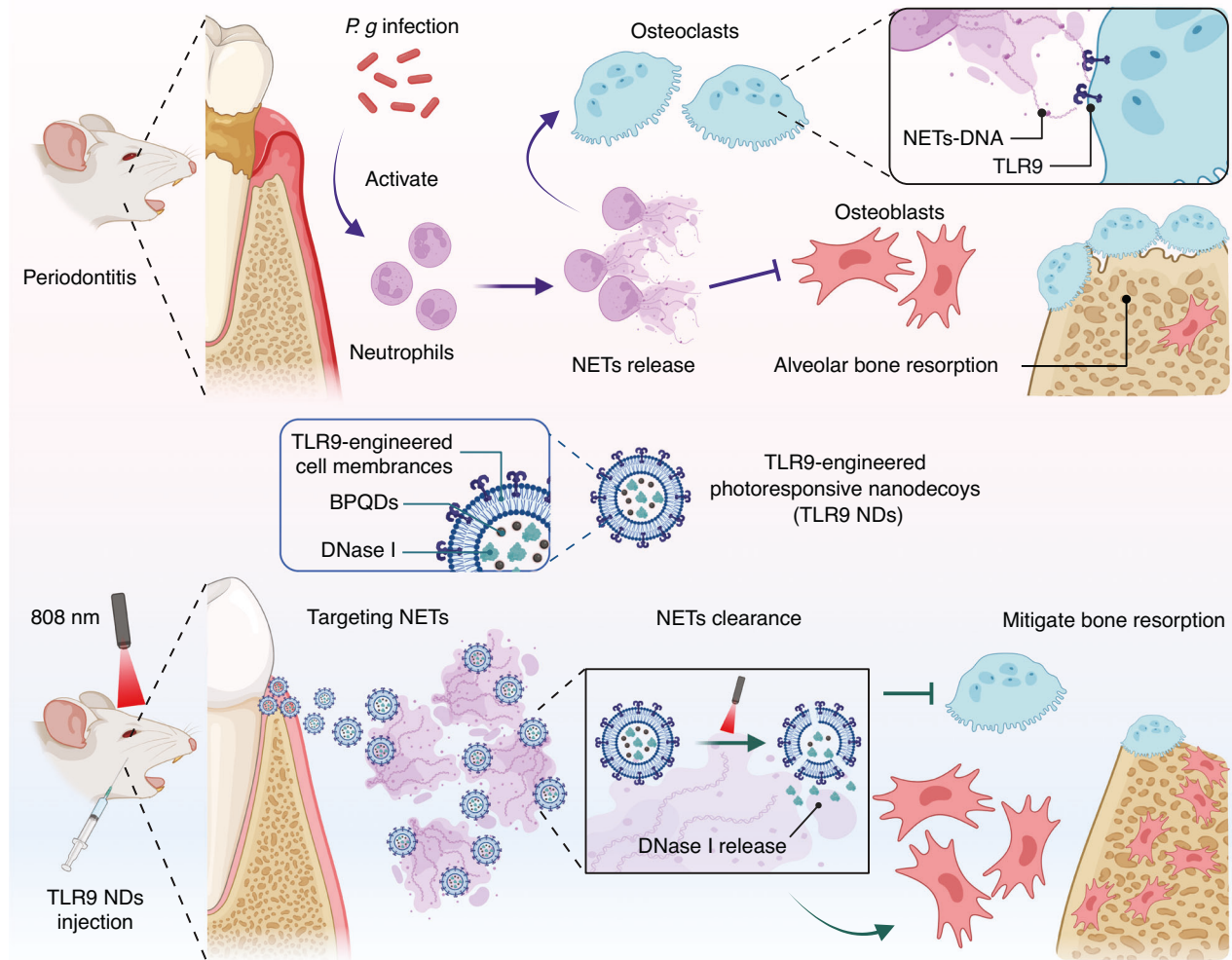


Fig. 10 Schematic illustration of TLR9-engineered photoresponsive biomimetic nanodecoys for the NIR-triggered targeting and clearance of NETs to alleviate alveolar bone resorption

endothelial-to-mesenchymal transition via TLR9 activation.²⁰ Collectively, these studies indicate that TLR9 is not only involved in NETs recognition but also exacerbates inflammatory responses.

Therefore, in line with our findings that TLR9 is an important receptor for NETs recognition by osteoclasts, a TLR9-biomimetic nanodecoy may block the contact between NETs and host cells, thereby mitigating the detrimental effects of NETs on host cells. Moreover, the protective encapsulation provided by the cell membrane coating can prevent DNase I from being degraded in vivo.²¹ The TLR9-overexpressing cell membrane endows the nanodecoys with enhanced NETs-targeting ability, while the incorporation of DNase I increases their NETs clearance potential.

Studies on the local application of photoresponsive nanomaterials in periodontitis are scarce. Therefore, the combination of laser irradiation with photothermal materials offers a multifunctional therapeutic strategy for the clinical management of periodontitis and represents a promising clinical application prospect.

Black phosphorus has attracted significant interest in biomedical applications because of its distinctive physicochemical properties.²² BPQDs exhibit broad visible-light absorption and a unique electronic structure, that results in high photothermal conversion efficiency and photosensitivity.²³ These characteristics enable pronounced photothermal and photodynamic effects, which have been exploited in tumor ablation therapies.²⁴ While BPQDs are known to possess both photothermal and photodynamic

properties, our study primarily demonstrated photothermal-mediated NETs clearance and DNase I release. The potential photodynamic contribution requires future investigation. Furthermore, the large specific surface area of BPQDs results in high drug-loading capacity.²⁵ BPQDs are also biodegradable, and breakdown in aqueous and oxygen-rich physiological environments into nontoxic phosphonate and phosphate ions.²⁶ Notably, phosphorus is an essential element for bone formation and is abundant in the human body.²⁷ Despite these advantages, the practical use of BPQDs is limited by challenges such as poor dispersibility and rapid degradation.²⁸ To address these issues, we first loaded DNase I onto BPQDs and then encapsulated the DNase I-BPQDs within TLR9-overexpressing cell membranes to form core-shell-structured nanoparticles. This design effectively increased the stability of both BPQDs and DNase I, shielding them from premature degradation.^{29–31} ND^{TLR9} exhibits outstanding targeting ability toward NETs and enhances the efficiency of NETs degradation by DNase I. ND^{TLR9} enables the rapid identification and elimination of NETs within a short time. Moreover, the system demonstrated favorable biosafety, supporting its potential as a nanomaterial for in vivo applications.

Our results showed that ND^{TLR9} combined with NIR irradiation mitigated osteoclast differentiation and promoted osteogenesis through multiple mechanisms. Notably, in our study, NIR irradiation alone did not increase ALP or ARS staining intensity

in the BMSCs. This may be attributed to the medium being changed every 2 days and the photothermal effects of ND and ND^{TLR9} lasting only 4 days under these conditions. Thus, the effective duration of NIR exposure was limited to 4 days. According to previous studies, the optimal intensity and duration of NIR irradiation required to promote osteogenesis are generally longer than those applied here.^{32–35} Therefore, the photothermal effects of NIR might be limited under our experimental conditions, which may not induce significant changes in later osteogenic stages. However, the release of DNase I plays a major role in NETs clearance and bone protection. We further evaluated the in vivo performance of ND^{TLR9}. The results demonstrated that ND^{TLR9} has a favorable photothermal effect and effectively inhibits alveolar bone resorption.

The clinical translation potential of ND^{TLR9} is supported by several factors. First, DNase I is already approved for pulmonary fibrosis, facilitating regulatory pathways.³⁶ Second, the use of an NIR laser is a minimally invasive adjunctive therapy in periodontics.³⁷ Third, local administration reduces systemic exposure. However, several challenges remain. The long-term safety of BPQDs degradation products needs further evaluation, although our short-term data revealed no overt toxicity except for a transient increase in renal phosphorus, which was cleared via urine and feces.

A limitation of this study is the incomplete elucidation of NETs endocytic pathways. Although our study revealed the colocalization of TLR9 with clathrin during NETs endocytosis, the specific pathways through which different cell types recognize and internalize NETs remain incompletely understood.^{38–41} A deeper investigation into these processes is crucial for developing targeted and precise strategies to modulate NETs. Our research revealed that after coculture with NETs, the expression of TLR9 on the cell membrane was upregulated. Previous studies have also reported that membrane-localized TLR9 can recognize DNA,⁴² supporting our results. However, whether TLR9 in endosomes originates from the cell membrane, lysosomes,⁴³ or both remains unclear. Our study does not directly prove the direction of TLR9 trafficking, and further investigations using live-cell imaging or endosomal fractionation are needed.

In summary, this study provides a proof-of-concept for the use of a TLR9-engineered photoresponsive nanodecoy for the treatment of periodontitis-associated bone loss. Thus, the current work represents an early-stage therapeutic strategy. The safety and effectiveness of ND^{TLR9}, as well as its specific mechanism, still require further confirmation through more animal models and in vitro experiments.

CONCLUSION

On the basis of the finding that osteoclasts recognize and internalize NETs via TLR9, thereby promoting osteoclast differentiation, in this study, a photoresponsive biomimetic nanodecoy system named ND^{TLR9} was developed. By mimicking the natural TLR9-DNA recognition mechanism and the controlled release of DNase I, this system enables targeted clearance of NETs. Additionally, ND^{TLR9} regulates osteoclast activity and exhibits osteogenic properties, thus facilitating bone regeneration. A schematic illustration of this study is presented in Fig. 10. This work provides valuable new insights for modulating the immune microenvironment and promoting periodontal bone regeneration in periodontitis therapy.

MATERIALS AND METHODS

Animals

6–8-week-old male C57BL/6 mice were purchased from SPF (Beijing) Biotechnology Co., Ltd. and housed in a specific pathogen-free (SPF) facility at the Animal Research Institute of

Beijing Stomatological Hospital. The mice were maintained under controlled conditions (25 °C, 12 h/12 h light/dark cycle) with free access to standard rodent chow and water. All the animals were allowed to acclimate for at least 1 week prior to the experiments. All procedures were approved by the Animal Care and Use Committee of Beijing Stomatological Hospital, Capital Medical University (Ethical Approval Number: KQYY-202502-002).

Preparation of *P. g* ultrasonic extract

P. g ATCC 33277 (Bio-Sci, China) was cultured anaerobically at 37 °C on blood agar plates (Guangdong Huankai Microbial, China) for 5–7 days. Bacterial colonies were harvested, washed twice with PBS, and resuspended in sterile deionized water. The suspension was subjected to ultrasonication on ice (300 W, 5 s on/5 s off pulses) for 30 min using an ultrasonic homogenizer. The lysate was subsequently centrifuged at 14 000 r/min for 15 min at 4 °C. The resulting pellet was weighed, resuspended in sterile deionized water, filtered through a 0.22-μm membrane, and stored at –80 °C until use.

Isolation and culture of neutrophils

Murine spleens were aseptically harvested from 6 to 8-week-old male C57BL/6 mice following euthanasia. Single-cell suspensions were prepared by mechanical dissociation through a 40 μm strainer. After centrifugation (300 × *g*, 10 min, 4 °C), erythrocyte-lysed splenocytes were resuspended in buffer and incubated with Anti-Ly-6G MicroBeads UltraPure (Miltenyi, Germany) for 10 min at 4 °C. The magnetically labeled cells were positively selected using a MACS column and separator (Miltenyi) according to the manufacturer's protocol. The enriched neutrophil fraction was collected by centrifugation (300 × *g*, 10 min, 4 °C). Purity was determined via flow cytometry, and cells exhibiting double positivity for Ly6G (127624, BioLegend, USA) and CD11b (101208, BioLegend) were identified as neutrophils (Fig. S17).

Collection of NETs

Purified neutrophils were resuspended in RPMI 1640 (Gibco, USA) supplemented with 10% fetal bovine serum (Front Biomedical, USA) and 1% penicillin–streptomycin (Gibco) and seeded into well plates. Cells were stimulated with 50 μg/mL *P. g* ultrasonic extract (based on the results of the preliminary dose experiment shown in Fig. S18) and incubated for 6 h⁴⁴ at 37 °C in 5% CO₂ to allow NETs formation. Following incubation, the culture supernatant was carefully collected and centrifuged at 4 °C and 300 × *g* for 10 min to remove cellular debris. Afterward, the samples were centrifuged at 4 °C and 1 000 × *g* for 10 min. The supernatant containing the released NETs was collected and stored at –80 °C for subsequent analysis.

Endocytosis of NETs by osteoclast precursor cells

RAW264.7 cells (CL-0190, Procell, China) are a commonly used murine macrophage-like cell line that can differentiate into osteoclasts upon RANKL stimulation. RAW264.7-derived osteoclasts with nuclei prelabeled with Hoechst (C1027, Beyotime, China), were seeded onto glass coverslips in 12-well plates. Sytox Green (S7020, Invitrogen, USA)-labeled NETs were added to the cultures and incubated for 6 h at 37 °C under 5% CO₂. After the medium was removed, the cells were further cultured for 1 h in DMEM supplemented with 75 nmol/L LysoTracker™ Red DND-99 (L7528, Invitrogen) to label the acidic compartments. The loading solution was then replaced with fresh medium. The cells were visualized using a confocal microscope (DMI6000, Leica, Germany). Images were captured and processed using Leica LAS AF software, and fluorescence quantification was performed with ImageJ software.

In another experiment, RAW264.7-derived osteoclasts were seeded onto glass coverslips in 12-well plates. NETs were added to the cultures and incubated for 6 h at 37 °C under 5% CO₂. After incubation, the cells were washed with PBS, fixed with 4% paraformaldehyde (Solarbio) for 30 min and blocked with 1% BSA (Sigma) for 1 h. The cells were subsequently incubated overnight

at 4 °C with a rabbit anti-TLR9 antibody (Bioss) and a CLTC monoclonal antibody (66487-1-Ig, Proteintech, USA) followed by a 1 h incubation at 37 °C with AF555-labeled donkey anti-rabbit IgG (H + L) (A0453, Beyotime) and AF488-labeled goat anti-mouse IgG (H + L) (A1089, Beyotime). After the final washes, the samples were mounted with anti-fade mounting medium containing DAPI (Appligen).

Immunofluorescence analysis of TLR9

RAW264.7-derived osteoclasts were transduced with a recombinant lentivirus (Genechem, China) at an MOI of 120 in the presence of HitransG A (Genechem). After 24 h, the medium was replaced with fresh complete medium. At 48 h post-transduction, cells were selected with 1 µg/mL puromycin for 5 days to establish stable lines. For the receptor recognition assay, wild-type and TLR9-knockout RAW264.7-derived osteoclasts were labeled with PKH26 membrane dye (D0030, Solarbio, China) and seeded into 12-well plates. Cells in the NETs + DNase I group were treated with NETs alongside 30 µg/mL DNase I (D8071, Solarbio) for 6 h. The cells in the NETs + ODN group were pretreated with 1 µg/mL of the TLR9 inhibitor ODN TTAGGG (HY-150751, MCE, China) for 1 h prior to the addition of NETs, followed by co-incubation for 6 h. After incubation, the cells were washed with PBS, fixed with 4% paraformaldehyde (Solarbio) for 30 min and blocked with 1% BSA (Sigma) for 1 h. The cells were subsequently incubated overnight at 4 °C with a rabbit anti-TLR9 antibody (bs-23640R, Bioss, China), followed by a 1 h incubation at 37 °C with AF488-labeled goat anti-rabbit IgG (H + L) (A0423, Beyotime). After the final washes, the samples were mounted with anti-fade mounting medium containing DAPI (C1211, Appligen).

TRAP staining

The cells were cultured in osteoclast induction medium (DMEM supplemented with 100 ng/mL RANKL) as described previously, and the medium was replaced every 2 days. After 7–10 days of induction, the cells were washed twice with PBS and fixed with a formaldehyde/acetone/citrate mixture for 30 s. The fixative was then removed by washing three times with deionized water. Staining was performed according to the manufacturer's protocol for the TRAP staining kit (Solarbio). Multinucleated TRAP-positive cells (containing three or more nuclei) were identified as osteoclasts. The cells were observed and counted under a light microscope (Nikon, Japan). Five randomly selected fields per well were analyzed, and the average number of osteoclasts was calculated.

RT-qPCR

Total RNA was extracted using a SteadyPure Quick RNA Extraction Kit (Accurate Biotechnology, China), and cDNA was synthesized from 1 µg of RNA by an Evo M-MLVRT Mix Kit (Accurate Biotechnology) according to the manufacturer's protocol. Real-time PCR was carried out for 40 amplification cycles, according to the manufacturer's instructions, using TB Green Premix Ex Taq II (Takara, Japan). The target genes are shown in Table S1.

Western blotting

Cultured cells were washed twice with ice-cold PBS and lysed on ice using RIPA buffer supplemented with a protease and phosphatase inhibitor cocktail (Appligen). Protein concentrations were determined by a BCA assay. Equal amounts of protein were denatured in 5x loading buffer (Appligen), separated by SDS-PAGE (NCM Biotech, China), and electrophoretically transferred to a PVDF membrane (Millipore). The membrane was blocked with 5% nonfat milk for 1 h at room temperature, and incubated overnight at 4 °C with a target-specific primary antibodies against CTSK (EP9403, Abcam, UK), Hsp90 (4874, CST, USA), GAPDH (2118, CST), and TLR9 (Bioss) and subsequently probed with an HRP-conjugated secondary antibody. Immunoreactive bands were visualized using enhanced

chemiluminescence (ECL) substrate (NCM) and imaged digitally for densitometric analysis.

Preparation of TLR9 nanodecoys

Compared with primary osteoclasts, RAW264.7 cells were selected as the cell membrane source for the nanodecoys because of their homology with osteoclasts, favorable culture characteristics, strong expansion capacity, and greater susceptibility to TLR9 overexpression via transfection. In contrast, primary osteoclasts are limited in yield and exhibit donor-to-donor variability, making them less suitable for reproducible large-scale membrane preparation. The membranes of TLR9-overexpressing RAW 264.7 cells were harvested. Cells were homogenized in HEPES buffer supplemented with 1% protease inhibitor. The lysate was centrifuged at 10 000 r/min for 5 min, after which the pellet was re-homogenized twice. The combined supernatants were subjected to ultracentrifugation at 110 000 × *g* for 1 h at 4 °C. The resulting membrane pellet was resuspended and stored at –80 °C. Because of the potential damage to cells and the loss of DNase I enzymatic activity caused by high temperatures, the concentration of BPQDs used in vitro was kept below 100 µg/mL.^{45,46} DNase I was conjugated to BPQDs via ultrasonication on ice (100 W, 5 s on/5 s off pulses) for 10 min. The DNase I-BPQDs were collected by centrifugation at 14 000 × *g* for 10 min. The TLR9-enriched membranes were self-assembled onto DNase I-BPQDs through electrostatic interactions aided by brief ultrasonication (100 W, 5 s on/5 s off pulses) for 4 min. The final nanodecoys (ND^{TLR9}) were purified by centrifugation at 12 000 × *g* for 10 min.

Characterization of TLR9 nanodecoys

The morphological characteristics of BPQDs, DNase I-BPQDs, ND and ND^{TLR9} were examined by transmission electron microscopy (JEM-2100Plus, JEOL, Japan). The particle size and zeta potential were determined via dynamic light scattering (DLS; Zetasizer Nano ZS90, Britain). The elemental composition was analyzed using energy-dispersive X-ray spectroscopy (EDS) in HAADF-STEM mode. The encapsulation efficiency and loading capacity of DNase I were calculated by measuring free and total DNase I concentrations with an activity detection kit (Beyotime). Optical properties were assessed with UV–vis–NIR spectroscopy (UV-3600, SHIMADZU, Japan), and the surface chemistry was characterized by X-ray photoelectron spectroscopy (XPS; K-Alpha, Thermo Scientific, USA). The photothermal effect was evaluated by irradiating samples with an 808 nm NIR laser (1.5 W/cm², 3 min; LASERWAVE, China) and monitoring the temperature with an infrared thermal imager (FOTRIC, China), with the data processed using AnalyziR software.

Evaluation of the cytotoxicity of TLR9 nanodecoys

Cell viability was assessed using the CCK-8 assay. RAW 264.7-derived macrophages were seeded into 96-well plates and incubated for 24 h. The medium was then replaced with fresh DMEM containing varying concentrations (10–70 µg/mL) of ND^{TLR9}. After 24 or 48 h of treatment, 10 µL of CCK-8 solution (Dojindo, Japan) was added to each well. The absorbance was measured at 450 nm using a microplate reader. In a separate experiment, cells were treated with DNase I-BPQDs, ND, or ND^{TLR9} (equivalent to 30 µg/mL DNase I), with or without NIR irradiation, for 24 h before viability assessment. Apoptosis was analyzed by flow cytometry using a FITC Annexin V Apoptosis Detection Kit (BD Pharmingen, USA). RAW 264.7-derived macrophages and BMSCs were seeded in 12-well plates and treated with DNase I-BPQDs, ND, or ND^{TLR9} for 72 h. The cells were then collected, stained according to the manufacturer's protocol, and analyzed by flow cytometry.

Cell targeting assay

Actin (C2201S, Beyotime)-labeled BMSCs and RAW 264.7-derived macrophages were seeded on 12-well plates and cultured for 24 h.

After they were incubated, the cells were treated with PKH26 labeled-ND^{TLR9} (50 µg/mL) for 24 h. After the incubation period, the cells were washed three times with PBS and fixed with 4% paraformaldehyde for 30 min. Finally, the samples were mounted using anti-fade mounting medium containing DAPI (Applygen). Images of the cells were obtained under a confocal microscope (Leica) to visualize and analyze the uptake of ND^{TLR9}.

NETs targeting and clearance effect evaluation

To assess targeting efficiency, neutrophils were first stimulated with *P. g* extract for 3 h to induce NETs formation. The medium was then replaced with fresh medium containing PKH67-labeled ND or ND^{TLR9} (50 µg/mL) and cocultured for 15–60 min. The cells were fixed, blocked, and incubated overnight with an anti-citrullinated histone H3 (anti-citH3) antibody (ab5103, Abcam). After being incubated with an AF555-conjugated secondary antibody (A0453, Beyotime), the samples were mounted with DAPI-containing medium (Applygen) and imaged by confocal microscopy (Leica) for targeted analysis.

To evaluate NETs clearance, NETs were induced by the following treatments: control, NETs, NETs + NIR, NETs + ND^{TLR9}, NETs + ND^{TLR9} + NIR, and NETs + ND + NIR. Posttreatment samples were processed for immunofluorescence as described above and imaged to assess clearance efficacy. NETs-positive areas were defined as regions that were double-positive for anti-citH3 and had a filamentous or web-like morphology. Quantification was performed using ImageJ with a threshold-based area measurement (same threshold for all groups). The fluorescence intensity was measured as the integrated density after background subtraction. A NETs ELISA kit (Enzyme-linked, China) was also used to evaluate NETs clearance efficacy. After the indicated treatments were applied for 1 h, cell culture supernatants from each group were collected. The samples were centrifuged at 300×*g* for 10 min to remove cell debris, and the resulting supernatants were subjected to ELISA according to the manufacturer's protocol. A standard curve was generated using known concentrations of NETs provided with the kit, and the NETs content in the supernatant of each group was calculated accordingly.

ALP staining

BMSCs were cultured in 12-well plates and treated as follows: control, NIR only, NETs, NETs + NIR, NETs + ND^{TLR9}, NETs + ND^{TLR9} + NIR, and NETs + ND + NIR. After 7 days of osteogenic induction, the samples were fixed in 4% paraformaldehyde for 30 min and then were washed 3 times with PBS. A BCIP/NBT ALP staining kit (Beyotime) was used to perform the staining procedure.

Alizarin red staining

BMSCs were cultured according to the conditions described for ALP staining. After 21 days of osteogenic induction, the cells were fixed in 4% paraformaldehyde for 30 min and then were washed with distilled water. The samples were subsequently incubated in the dark with 0.2% alizarin red S solution (Solarbio) for 30 min.

In vivo therapeutic effects

Male C57BL/6 mice were randomly allocated into five groups (*n* = 5): Blank, periodontitis, PD + NIR, PD + ND^{TLR9}, and PD + ND^{TLR9} + NIR. Periodontitis was induced by ligating the maxillary second molars with 5–0 silk sutures, followed by topical application of 1 × 10⁹ CFU *P. g* to the ligated sites once daily for three consecutive days. Afterward, the mice in the PD + ND^{TLR9} and PD + ND^{TLR9} + NIR groups received a local gingival injection of ND^{TLR9} (1 mg/mL, 100 µL per mouse). The PD and PD + NIR groups received an equivalent volume of PBS. The mice in the PD + NIR and PD + ND^{TLR9} + NIR groups were subjected to local NIR laser treatment (1.0 W/cm², 5 s) once daily for 7 days, after which the temperature was monitored by an infrared thermal imager. All the

mice were euthanized at the endpoint. Complete blood counts and blood biochemistry data were analyzed by the laboratory of Beijing Stomatological Hospital. The total phosphorus content in major organs was quantified using a Tissue Total Phosphorus Assay Kit (Bioss) to assess biodistribution. Organ morphology was evaluated by H&E staining. Alveolar bone resorption was quantified by micro-CT (resolution: 14.93 µm). Periodontal tissue sections were subjected to H&E and Masson staining for histopathological evaluation. Immunohistochemistry was performed to detect CTSK (Abcam), MPO (YM4746, Immunoway, USA), and Runx2 (ab192256, Abcam). Immunofluorescence costaining for citH3 and MPO was used to visualize NETs. Serum NETs levels were quantified using a commercial ELISA kit (Enzyme-linked, China).

Statistical analysis

All experiments were independently repeated at least three times. The data are presented as the mean ± standard deviation (SD). Statistical analyses between two groups were performed using Student's *t*-tests. Comparisons among multiple groups were performed using one-way analysis of variance (ANOVA). For heteroscedastic data, the Kruskal–Wallis test and Mann–Whitney *U* test were applied. *P* < 0.05 was considered to indicate statistical significance. All the statistical analyses were conducted using GraphPad Prism version 8.0 (GraphPad Software, USA) or Origin 2024 (OriginLab, USA).

DATA AVAILABILITY

All other relevant data supporting the key findings of this study are available within the article and its Supplementary Information files or from the corresponding author upon reasonable request.

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AUTHOR CONTRIBUTIONS

S.Y. Liu: Data curation, Formal analysis, Methodology, Visualization, Writing—original draft. Y.T. Liu: Funding acquisition, Visualization, Writing—review and editing. Y.Y. Chen: Data curation, Investigation, Visualization. Y. Jiao: Investigation, Methodology. R. Zhao: Formal analysis, Validation. Y.J. Wang: Investigation, Project administration. J.B. Zhang: Methodology, Validation. S. Zhang: Supervision, Validation. Y. Liu: Funding acquisition, Supervision, Writing—review and editing. L.J. Guo: Conceptualization, Funding acquisition, Supervision.

ADDITIONAL INFORMATION

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Competing interests: The authors declare no competing interests.

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