Targeted apoptosis of macrophages and osteoclasts in arthritic joints is effective against advanced inflammatory arthritis

Caifeng Deng, Quan Zhang, Penghui He, Bin Zhou, Ke He, Xun Sun, Guanghua Lei, Tao Gong & Zhirong Zhang

Insufficient apoptosis of inflammatory macrophages and osteoclasts (OCs) in rheumatoid arthritis (RA) joints contributes toward the persistent progression of joint inflammation and destruction. Here, we deliver celastrol (CEL) to selectively induce apoptosis of OCs and macrophages in arthritic joints, with enzyme-responsive nanoparticles (termed PRNPs) composed of RGD modified nanoparticles (termed RNPs) covered with cleavable PEG chains. CEL-loaded PRNPs (CEL-PRNPs) dually target OCs and inflammatory macrophages derived from patients with RA via an RGD-αvβ3 integrin interaction after PEG cleavage by matrix metalloprotease 9, leading to increased apoptosis of these cells. In an adjuvant-induced arthritis rat model, PRNPs have an arthritic joint-specific distribution and CEL-PRNPs efficiently reduce the number of OCs and inflammatory macrophages within these joints. Additionally, rats with advanced arthritis go into inflammatory remission with bone erosion repair and negligible side effects after CEL-PRNPs treatment. These findings indicate potential for targeting chemotherapy-induced apoptosis in the treatment of advanced inflammatory arthritis.
Rheumatoid arthritis (RA) is a chronic autoimmune inflammatory disease characterized by synovial inflammation and joint destruction1. Synovial inflammation is the dominant feature in the early stage of RA2–4. In addition to the inflammatory symptoms of joint swelling and synovial inflammation, obvious cartilage damages and bone erosion occur during the persistent progression of RA, which can eventually result in joint deformity and disability5–7. Patients with advanced RA suffer from the loss of physical function and low quality of life2,8. Therefore, a rational therapeutic approach that can alleviate synovial inflammation and reverse bone erosion is urgently needed for the treatment of advanced RA.

Macrophages and osteoclasts (OCs) have been demonstrated to play key roles in the pathogenesis of RA. The increase in the abundance of synovial macrophages is an early hallmark of rheumatic disease. Synovial macrophages from RA patients show distinct activation states and represent one potential key mediator of joint inflammation9,10. Additionally, studies have revealed large numbers of OCs at sites of arthritic bone erosion11–13. OCs are terminally differentiated cells with the unique ability to resorb bone matrix14–16. Notably, OCs in RA-affected joints can accelerate synovial inflammation through the production of pro-inflammatory cytokines17,18. Both OCs and synovial inflammatory macrophages express high levels of αvβ3 integrin. αvβ3 integrin has been demonstrated to play important role in activated macrophage-dependent inflammation and OC-dependent bone resorption19,20. Generally, the life spans of macrophages and OCs are precisely regulated by apoptosis to maintain immune homeostasis and bone function balance, respectively21–24. However, macrophages and OCs from RA joints show decreased apoptotic rates compared with those from healthy controls21,25,26. The insufficient apoptosis of macrophages and OCs in the RA joint contributes toward the persistent progression of joint inflammation and joint destruction. Accordingly, inducing the apoptosis of both macrophages and OCs in RA joints is a promising strategy for advanced RA therapy.

Current antirheumatic drugs, including glucocorticoids and biological antibodies, mainly target the macrophages-induced inflammatory response to reduce synovial inflammation,27. However, the application of glucocorticoids can result in severe side effects including bone loss and hyperglycemia28,29. While the frequent use of antibodies to block inflammatory cytokines can cause systemic immune suppression, thereby leading to a high risk of infections30,31. Furthermore, not all patients respond to antibody therapy and the benefits of using antibody therapy is short-lived21,30,32. Recently, Janus Kinase (JAK) inhibitors have been reported to have the potential to reverse the bone erosions in RA,33,34, yet JAK inhibition could lead to serious and opportunistic infections33,35. Celestal (CEL), a cytotoxic chemotherapeutic drug, can induce apoptosis in tumor cells and has been widely studied in cancer therapy36,37. In addition to its known efficacy in cancer treatment, CEL was previously shown in our laboratory to also treat glomerulonephritis by inducing the apoptosis of mesangial cells38. In a rat model of anti-Thy1.1 nephritis, the targeted delivery of CEL to the mesangial cells significantly increased its therapeutic efficacy and decreased its side effects38. Inspired by our previous findings, we hypothesized that selectively delivering CEL to both macrophages and OCs in RA joints may efficiently induce apoptosis in these cells, thus reducing synovial inflammation and reversing bone erosion in advanced RA.

In this study, we report the development of matrix metalloproteinase 9 (MMP9)-cleavable, polyethylene glycol (PEG)- and RGD peptide-modified poly (α, 1-lactide-co-glycolide) (PLGA) nanoparticles (termed “PRNPs”) for the targeted delivery of CEL to both OCs and macrophages in arthritic joints. PRNPs show high cellular uptake in both OCs and inflammatory macrophages derived from patients with late-stage RA via RGD-αvβ3 integrin interaction after responding to MMP9. In addition, CEL-loaded PRNPs (CEL-PRNPs) in the presence of MMP9 effectively induce the apoptosis of these cells. In adjuvant-induced arthritis (AIA) rats, PRNPs show an arthritic joints-specific distribution and target both OCs and macrophages within these joints. Further, the intravenous administration of CEL-PRNPs effectively relieves the ankle and paw swelling, restores the balance of bone function, and reverses bone erosion in the inflamed joints of AIA rats with advanced arthritis. Of note, CEL-PRNPs seem safe and induce negligible apoptosis in normal organs. In summary, CEL-PRNPs show great promise in promoting inflammatory remission and bone erosion repair in advanced inflammatory arthritis.

Results
Features of pathology in different stages of arthritis. AIA in the rat is a well-characterized model for assessing the stages of pathology in RA and exploring arthritis mechanisms39–41. Using micro-computed tomography (micro-CT) and histological assays, we investigated joint destruction and synovial inflammation in AIA rats. Micro-CT was used to scan the ankle joints of both normal rats and AIA rats. We found that, in contrast with the smooth bone surface and negligible bone erosion in ankle joints from rats with early-stage arthritis, AIA rats with advanced arthritis displayed rough bone surfaces, severe bone erosion, and significantly decreased bone mineral density (BMD) (Fig. 1a–c). Applying tartrate-resistant acid phosphatase (TRAP) staining of OCs further demonstrated that there were large numbers of OCs and areas of bone erosion in AIA rats within the advanced arthritis group (Fig. 1a). We also examined cartilage integrity using safranin O and toluidine blue staining to label the glycosaminoglycans (GAGs) in cartilage tissues42,43. The cartilage of AIA rats with early-stage arthritis remained intact and was comparable to that of the normal group. However, significant reductions of safranin O- and toluidine blue-positive areas were observed on the cartilage surface of AIA rats with advanced arthritis, indicating that a loss of GAGs and cartilage destruction occurred in advanced arthritis (Supplementary Fig. 1). We next investigated levels of synovial inflammation in AIA rats using immunohistochemical staining. Synovial macrophages with high potential to induce inflammation were identified as CD68+ cells44–46. Immunohistochemical images revealed that rats with disease induction showed increased infiltration of CD68+ macrophages into the synovium compared with the normal group (Fig. 1a). Notably, AIA rats with advanced arthritis showed an excessive abundance of CD68+ macrophages and synovial hyperplasia, consistent with the results of hematoxylin and eosin (H&E) assay (Fig. 1a; Supplementary Fig. 1). In addition to the increased accumulation of CD68+ macrophages, the augmented expression of MMP9 in the arthritic inflammatory environment was also observed during the induction of AIA (Fig. 1a). Previous studies also demonstrated macrophages and OCs promoted the high expression of MMP9 in the inflammatory microenvironment of RA47,48. Thus, these results revealed that there were excessive levels of CD68+ macrophages and OCs in the arthritic joints of AIA rats with advanced arthritis.

Preparation and characterization of CEL-PRNPs. PLGA nanoparticles exhibit good biocompatibility and high drug loading efficacy and have been widely employed as a drug delivery system49–51. Therefore, PLGA nanoparticles were adopted as the drug carrier in this work. We first modified PLGA with RGD peptide via the maleimide-thiol coupling reaction between Cys-RGD and Mal-PEG-PLGA and the chemical structure was
analyzed by proton nuclear magnetic resonance (1H-NMR) spectroscopy (Supplementary Fig. 2). Different PLGA nanoparticles were prepared as shown in Fig. 2a. Briefly, CEL-loaded PLGA nanoparticles (CEL-NPs) and CEL-loaded, RGD-modified PLGA nanoparticles (CEL-RNPs) were both prepared using an emulsion/solvent evaporation method. To obtain CEL-PRNPs, PEG2000-MMP9 cleavable peptide was linked to CEL-RNPs using the water phase reaction method. The particle sizes of CEL-NPs and CEL-RNPs were 155.7 ± 4.9 nm and 154.1 ± 4.6 nm, respectively. After covered with cleavable PEG2000, the particle size of CEL-PRNPs slightly increased to 162.2 ± 6.6 nm (Fig. 2b; Supplementary Table 1). The average zeta potentials of CEL-RNPs and CEL-PRNPs were −3.2 ± 0.6 mV and −5.3 ± 0.4 mV, respectively. The CEL encapsulation efficiencies of various developed PLGA nanoparticles were all close to 90% (Supplementary Table 1). The transmission electron microscope (TEM) images demonstrated that the morphologies of the prepared CEL-PRNPs were generally spherical and uniformly dispersed (Fig. 2c). The serum stability assay was conducted to investigate interactions between various developed PLGA nanocarriers and blood components. The average particle sizes remained nearly unchanged for CEL-NPs, CEL-RNPs, and CEL-PRNPs after 24 h of storage in 10% fetal bovine serum (FBS) at 37 °C (Fig. 2d; Supplementary Fig. 3), suggesting the good stability of these nanoparticles in serum.

**CEL-PRNPs increase apoptosis of O Cs and inflammatory macrophages.** To investigate whether PRNPs could target O Cs and pathogenic macrophages via RGD-mediated endocytosis, a cellular uptake study was performed. O Cs were established by using M-CSF- and RANKL-stimulated bone marrow macrophages (BMMs) and pathogenic macrophages were obtained by using lipopolysaccharide (LPS)-activated BMMs. The successful genesis of O Cs was confirmed using TRAP staining. Results showed that RANKL-induced O Cs stained red and were multinuclear, while the unstimulated BMMs stained yellow (Supplementary Fig. 4), suggesting that O Cs were efficiently generated via RANKL stimulation. To investigate the distribution behaviors of various prepared nanoparticles in O Cs and LPS-activated macrophages, the fluorescent probe, coumarin 6 (C6), was loaded into the nanoparticles. Confocal images of the O Cs and LPS-activated macrophages showed that RNP s conferred significantly
increased green fluorescence signal to these cells compared with NPs (Fig. 3a; Supplementary Figs. 5 and 6). However, this effect was not observed in the non-activated BMMs (Supplementary Fig. 7). These results suggested that the RNPs could selectively target both OCs and LPS-activated macrophages for cellular uptake. In the absence of MMP9, PRNPs showed remarkably reduced cellular uptake, whereas, in the presence of MMP9, this effect was reversed (Fig. 3a; Supplementary Figs. 5 and 6). This indicated that the PEG chains on the PRNPs could be cleaved by MMP9, thereby exposing the RGD peptide for the selective targeting of the OCs and LPS-activated macrophages. The results of quantitative cellular uptake analysis based on flow cytometry showed the same trends (Fig. 3d, e). Furthermore, the dual-targeting ability of PRNPs was also investigated on human OCs and inflammatory macrophages. Human OCs were obtained by using M-CSF- and RANKL-stimulated peripheral blood mononuclear cells from patients with late-stage RA. Human inflammatory macrophages were isolated by magnetic-activated cell sorting method from synovial tissues of patients with late-stage RA undergoing joint replacement surgery. As shown in Fig. 4a–c, the increased distribution of RNPs and PRNPs (in the presence of MMP9) were also observed on OCs and inflammatory macrophages derived from patients with late-stage RA. These results demonstrated that RNPs and PRNPs (in the presence of MMP9) could target both OCs and inflammatory macrophages through ligand–receptor interactions.

The inadequate apoptosis of macrophages and OCs in the rheumatoid inflammatory microenvironment is an important pathomechanism in synovial hyperplasia and joint destruction.23,25–27. To investigate whether CEL-PRNPs could effectively induce the apoptosis of OCs and inflammatory macrophages, their apoptotic profile was determined by flow cytometry assay. As shown in Fig. 3b, f, g, CEL-RNPs treatment significantly increased the apoptotic cell percentages in both OCs and LPS-activated macrophages compared with the CEL-NPs and CEL-PRNPs. However, in the presence of MMP9, treatment with the CEL-PRNPs resulted in high levels of apoptosis in both OCs and LPS-activated macrophages, demonstrating that CEL-PRNPs were MMP9-responsive. As mitochondrial dysfunction is also a hallmark of apoptosis, we further measured the mitochondrial membrane potential (a marker of mitochondrial dysfunction) in macrophages with the JC-1 dye.36 The JC-1 dye tends to aggregate (with red fluorescence) in normal mitochondria, and its color changes from red to green when the membrane potential collapses. Confocal images showed that both CEL-RNPs and CEL-PRNPs (in the presence of MMP9) resulted in significantly higher green fluorescence intensity signal compared with CEL-NPs (Fig. 3c), suggesting the severe disruption of the mitochondrial membrane in LPS-activated macrophages. Furthermore, CEL-RNPs and CEL-PRNPs (in the presence of MMP9) also triggered the higher rates of apoptosis among OCs and inflammatory macrophages derived from patients with late-stage RA, when compared with CEL-NPs and CEL-PRNPs (in the absence of MMP9) (Fig. 4e, f). These results proved that CEL-PRNPs had excellent ability to cause the apoptosis of OCs and inflammatory macrophages after responding to MMP9.

Fig. 2 Preparation and characterization of CEL-PRNPs. a Schematic illustration of CEL-PRNPs preparation. CEL celastrol, MMP9 matrix metalloproteinase 9, CEL-RNPs CEL-loaded RGD peptide-modified poly (ε-caprolactone-co-glycolide) (PLGA) nanoparticles, CEL-PRNPs CEL-loaded MMP9-cleavable polyethylene glycol (PEG)- and RGD peptide-modified PLGA nanoparticles. b Representative size distribution image of CEL-PRNPs (n = 3 independent samples). c TEM image of CEL-PRNPs (n = 3 independent samples). Scale bar = 100 nm. d The serum stability of CEL-PRNPs during 24 h incubation with 10% FBS at 37 °C and cumulative CEL release from CEL-PRNPs in PBS at 37 °C. Data represent mean ± SD (n = 3 independent samples).

PRNPs target both OCs and inflammatory macrophages in arthritic joints. We studied the biodistribution of the PRNPs in AIA rats with advanced arthritis using an in vivo imaging system and PRNPs loaded with DiD, a fluorescent probe. The in vivo fluorescence images showed that PRNPs significantly increased the DiD fluorescence distribution in inflamed joints compared with NPs and RNPs (Fig. 5a, d; Supplementary Fig. 8). Of note, the use of PRNPs remarkably reduced the DiD fluorescence distribution in the lung, liver and spleen compared with RNPs (Fig. 5a, e), which indicated that the nonspecific distribution of RNPs was avoided through the use of a surface coating.
comprising cleavable PEG chains, as αvβ3 integrins are also highly expressed in normal tissues. The ex vivo fluorescence images also demonstrated that PRNPs had the longest blood circulation time (Fig. 5a, e), which further suggested that the PEG2000-MMP9 cleavable peptide had been successfully linked to RNPs. Interestingly, PRNPs accumulation in inflamed joints at 24 h after intravenous injection was still as high as that in inflamed joints at 2 h (Supplementary Fig. 8). However, their accumulation in normal organs at 24 h was obviously less than that at 2 h (Supplementary Fig. 9). These results illustrated that PRNPs displayed the inflamed joint-targeting ability and extended retention in inflamed joints.

Next, AIA rats with unilateral inflamed joints were adopted to evaluate the selective accumulation of PRNPs in inflamed joints compared with normal joints. As shown in Fig. 5b, c, f, there were no significant differences in the distribution of free DiD between inflamed joints and non-inflamed joints. In contrast, DiD-loaded NPs, RNPs, and PRNPs showed a higher accumulation in inflamed joints than non-inflamed joints. As expected, PRNPs displayed the highest distribution in inflamed joints among the three nanoparticle types, which was consistent with the results obtained for AIA rats with bilateral inflamed joints (Fig. 5a, d).

The above results demonstrated that PRNPs increased drug distribution in arthritic joints. To investigate whether PRNPs could...
target both OCs and inflammatory macrophages in arthritic joints, the distribution of different DiD formulations in both types of cells was determined using the immunofluorescence staining method. Inflammatory macrophages and OCs were determined by immunofluorescence analysis of CD68 and CD51 (green fluorescence). As shown in Fig. 6, the DiD fluorescence distribution of PRNPs in the synovial joint was the highest among the three nanoparticle types, which was consistent with the results of in vivo and ex vivo imaging studies. In addition, DiD solution and DiD labeled NPs showed low levels of colocalization of the red (DiD) and green fluorescence, suggesting the nonspecific distributions in inflamed joints. Whereas, the DiD fluorescence of RNPs and PRNPs was mainly overlapped with the green fluorescence in synovial tissues. Therefore, the results proved that PRNPs could detach their PEG chains and transform into RNPs within inflamed joints.

CEL-PRNPs decrease the number of OCs and macrophages in arthritic joints. The above results showed that PRNPs exhibited good selectivity toward both OCs and inflammatory macrophages in arthritic joints. Furthermore, CEL-PRNPs (in the presence of MMP9) could more effectively induce apoptosis in both OCs and inflammatory macrophages due to the increasing cellular uptake. To demonstrate that PRNPs could realize the selective reduction of OCs and synovial macrophages at sites of inflammation in AIA rats with advanced arthritis, we measured the level of cellular apoptosis in the inflamed joints, the number of OCs and macrophages in the synovial joints, and the cytokine profiles in blood and joints.

AIA rats with advanced arthritis were randomly divided into five groups based on their treatment with either saline, CEL solution, CEL-NPs, CEL-RNPs, or CEL-PRNPs. Saline or 1 mg/kg CEL equivalents of CEL solution, CEL-NPs, CEL-RNPs, or CEL-PRNPs were intravenously injected into rats every other 2 days. Ankle joints were collected for TUNEL staining two days after the last treatment. Inflammatory macrophages and OCs were determined by immunofluorescence analysis of CD68 and CD51 (red fluorescence). As shown in Fig. 7a and Supplementary Fig. 10, free CEL solution led to a very low level of apoptosis in inflamed joints, whereas CEL-PRNPs triggered the highest level of apoptosis.
of apoptosis in inflamed joints among all of the treatment groups. Furthermore, apoptotic cells induced by CEL-PRNPs were mainly inflammatory macrophages and OCs (Supplementary Fig. 11).

To determine the abundance of OCs and synovial macrophages in inflamed joints, ankle joints, and blood were collected for TRAP staining, immunohistochemical staining, and enzyme-linked immunosorbent assays (ELISA) 2 days after the last treatment. OCs located in the areas of bone erosion are stained red when using TRAP staining assay. TRAP staining results showed that CEL-PRNPs significantly reduced the number of OCs in ankle joints and resulted in negligible bone erosion (Fig. 7b). ELISA results revealed that the serum TRAP in the
The balance of bone function. To convey advanced arthritis, RGDP peptide-modified polyethylene glycol (PEG)- and glycolide) (PLGA) nanoparticles, DiD-RNPs DiD-labeled RGD peptide.

The superabundant presence of OCs in RA severely disrupts the balance of bone resorption and formation, thereby demonstrating the recovery of bone function balance. In accordance with this, CEL-PRNPs promoted bone damage repair, as indicated by the significant accumulation of osteocalcin (OCN)-positive osteoblasts and the increased expression of alkaline phosphatase (ALP) in the arthritic joints.

Macrophages are the main producers of inflammatory cytokines including TNF and IL-1β in RA.

We proved that AIA rats following 17 days of disease induction had obvious bone erosion and significant loss of BMD.

CEL-PRNPs alleviated joint inflammation and bone erosion in rats. Finally, the therapeutic efficacy of CEL-PRNPs treatment was evaluated in AIA rats with advanced arthritis. The AIA rat developed severe swelling in the ankles and paws after 17 days of arthritis induction. Saline or various CEL-loaded PLGA nanoparticles were intravenously injected into rats (dose of 1 mg/kg for CEL). Anti-TNF, as one of the benchmarks in the treatment of RA, was also employed to treat AIA rats with advanced arthritis in this study. Free CEL and anti-TNF showed relatively low efficacy in decreasing the paw thickness and ankle diameters of AIA rats with advanced arthritis. In contrast, CEL-PRNPs showed higher efficacy in reducing swelling in ankle joints and paws compared with CEL-NPs and CEL-RNPs, yielding an ankle diameter and paw thickness closer to that of the normal group at the study endpoint.

To further illustrate that CEL-PRNPs could control inflammation and reduce cartilage destructions, the ankle joints of rats were sectioned for histological analysis at the study endpoint. H&E-stained sections from the saline group showed severe synovial hyperplasia, along with bone and cartilage destruction. The free CEL group displayed a limited effect in reducing these symptoms, while CEL-NPs and CEL-RNPs reduced synovial inflammation and decreased the loss of cartilage to some extent compared with the saline group. However, safranin-O and toluidine blue staining revealed that the GAG levels in CEL-NPs and CEL-RNPs were lower than those in the control AIA rats (AIA rats following 17 days of arthritis induction) (Supplementary Fig. 1), suggesting that the cartilage damage was insufficiently reversed and remained progressive. In contrast, H&E results showed mild synovial hyperplasia in the CEL-PRNPs group. Additionally, the CEL-PRNPs group had larger positive areas for safranin-O and toluidine blue staining, which were closer to those of the normal group (Fig. 8d; Supplementary Fig. 16). These results demonstrated that CEL-PRNPs effectively alleviated synovial inflammation and reduced cartilage destructions in AIA rats with advanced arthritis.

We proved that AIA rats following 17 days of disease induction had obvious bone erosion and significant loss of BMD. Using the micro-CT analysis, the inflamed ankle joints on day 29 after treatment were shown to exhibit rough bone surfaces and serious bone erosion in the saline group, with a significant

Fig. 6 PRNPs are selectively distributed in OCs and inflammatory macrophages in arthritic joints of rats with advanced arthritis. Confocal images showing the distribution of different DiD formulations in synovial macrophages (a) and OCs (b) in inflamed joints. Macrophages and OCs were determined by immunofluorescence analysis of CD68 and CD51 (green fluorescence), respectively. (Scale bar = 25 μm) (n = 3 independent animals). DiD 1,1′-dioctadecyl-3,3,3′,3′-tetramethyl indodicarbocyanine, 4-chlorobenzenesulfonate salt, DiD-NPs DiD labeled poly (D, L-lactide-co-ε-caprolactone) (PLGA) nanoparticles, DiD-RNPs DiD-labeled RGD peptide-modified PLGA nanoparticles, DiD-PRNPs DiD-labeled matrix metalloproteinase 9 (MMP9)-cleavable polyethylene glycol (PEG)- and RGD peptide-modified PLGA nanoparticles.
reduction in BMD and an increase in BS/BV compared with the normal group. Free CEL showed moderate efficacy in reducing bone erosion. Treatment with anti-TNF, CEL-NPs, and CEL-RNPs increased the BMD compared with free CEL but failed to repair the bone damage. Remarkably, the CEL-PRNPs group showed smooth bone surfaces and had a high BMD, closer to those of the normal group (Fig. 9a–d), demonstrating that CEL-PRNPs efficiently reversed bone erosion. The trabecular

**Fig. 7** CEL-PRNPs reduce the number of OCs and inflammatory macrophages in joints of rats with advanced arthritis. **a** TUNEL immunofluorescence staining in ankle joints from AIA rats receiving the indicated treatment (Scale bar = 200 μm) (n = 5 independent animals). **b** Immunohistochemical analyses of the TRAP-stained OCs and CD68-stained synovial macrophages in the joint tissues from rats receiving the indicated treatment (Scale bar = 100 μm) (n = 5 independent animals). **c** RANKL/OPG ratio in arthritic joints, IL-1β secretion in blood, and TNF secretion in blood from rats receiving the indicated treatment. Data represent mean ± SD (n = 5 independent animals). Statistical significance was determined by a two-sided Student’s t test. **d** Detection of IL-1β, TNF, OCN, and ALP expression levels in arthritic joints in different groups. Arthritic joints in different groups were stained with IL-1β, TNF, and OCN antibodies, respectively. ALP was stained light-dark in arthritic joints from different groups (Scale bar = 100 μm) (n = 5 independent animals). CEL celastrol CEL-NPs CEL-loaded poly (D, L-lactide-co-glycolide) (PLGA) nanoparticles, CEL-RNPs CEL-loaded RGD peptide-modified PLGA nanoparticles, CEL-PRNPs CEL-loaded matrix metalloproteinase 9 (MMP9)-cleavable polyethylene glycol (PEG)- and RGD peptide-modified PLGA nanoparticles, TUNEL TdT-mediated dUTP nick end labeling, TRAP tartrate-resistant acid phosphatase, RANKL receptor of activator of NF-kB ligand, OPG osteoprotegerin, IL-1β interleukin-1β, TNF tumor necrosis factor, OCN osteocalcin, ALP alkaline phosphatase.
parameters also confirmed that CEL-PRNPs treatment was the most efficient in increasing the trabecular number (Tb.N) and trabecular bone thickness (Tb.Th) while decreasing trabecular separation (Tb.Sp) among all of the treatment groups (Fig. 9e–g). Thus, these findings proved that CEL-PRNPs could effectively terminate the progression of bone damages and simultaneously repair bone erosion in a rat model of advanced RA.

Discussion

Apoptosis is the process of natural programmed cell death used to maintain organism homeostasis. For example, macrophages are the first line of defense against pathogens and are regulated by apoptosis to maintain immune homeostasis and protect the host against damage from excessive inflammation. OCs are terminally differentiated cells and known for their unique function in promoting bone resorption and the formation of resorption lacunae. The natural apoptosis of OCs helps to balance body bone-remodeling homeostasis to prevent OCs-mediated bone damage. However, macrophages and OCs are abnormally accumulated in RA joints. Furthermore, macrophages and OCs in arthritic joints are reported to exhibit the decreased apoptotic rates compared with those from healthy controls. Therefore, we proposed that enhancing the apoptosis of macrophages and OCs would be a promising treatment strategy for restoring immune homeostasis and bone function balance in arthritic joints.

Our previous study found that CEL could trigger the apoptosis of mesangial cells in glomerulonephritis. In the present study, we demonstrated the CEL-induced apoptosis of pathogenic macrophages and OCs (Figs. 3b and 4e–f). However, CEL treatment shows severe toxicity in normal organs due to its off-targeting activity. Indeed, significant CEL toxicity in the
heart, liver, and brain was also observed in our study (Supplementary Figs. 17 and 18). Thus, it is necessary to specifically induce the apoptosis of inflammatory macrophages and OCs in arthritic joints to treat RA. To realize cell-specific drug delivery in the treatment of RA, the drug delivery system should be able to target both the inflammatory sites and the relevant cells within these sites. The RGD peptide is a well-known ligand of the integrins that are highly expressed on the surfaces of pathogenic macrophages and OCs in RA synovium. We observed significant uptake of RGD-modified NPs (RNPs) by pathogenic macrophages (LPS-activated murine macrophages and human synovial macrophages) and OCs (Figs. 3a and 4a). However, integrins are overexpressed in many organs such as the liver and higher CEL toxicity in this organ (Fig. 5a; Supplementary Figs. 17 and 18). Furthermore, integrins are also highly expressed on the neovascularization endothelial cells within the inflammatory sites of RA, which could lead to the selective distribution of RGD-modified nanoparticles in endothelial cells. This selective uptake by endothelial cells would hinder these nanoparticles from reaching the inflammatory microenvironment in RA. Our results also revealed that RNPs and PRNPs (in the presence of MMP9) exhibited high uptake by TNF-activated HUVECs, which mimic neovascularization endothelial cells. However, as a result of their MMP9-responsive PEG chain modification, PRNPs showed decreased distribution in activated HUVECs (Supplementary Fig. 19). Therefore, PRNPs with their inflammatory microenvironment-responsive properties offer not only good...
in vivo safety but also enable the selective delivery of CEL-RNPs to the macrophages and OCs within the inflammatory microenvironment of arthritis.

Macrophages in the RA synovium produced cytokines, such as TNF and IL-1, to promote the progression of inflammation\(^{46,67}\). Thus, anti-cytokines are often applied in the clinical treatment of RA. In our rat RA model, we also found that anti-TNF treatment could significantly suppress the inflammation in early arthritis (Supplementary Fig. 20). However, the anti-TNF treatment showed reduced efficacy and limited bone protection in advanced arthritis (Figs. 8 and 9). What’s more, anti-TNF and anti-IL-1 therapies are clinically effective in only 40% of patients, and the disease recurs when treatment is stopped\(^{21,30,32}\). This might be due to synovial macrophages producing other inflammatory mediators besides TNF and IL-1, such as IL-6 and IL-15\(^{56,67}\). In addition, synovial macrophages have a prolonged life span due to their insufficient apoptosis\(^{56,67}\). In contrast with anti-TNF treatment, CEL-loaded nanoparticles can target the synovial macrophages as a whole to induce their apoptosis. Furthermore, our CEL-RNPs could selectively deliver CELs to inflammatory macrophages and OCs in arthritic joints to effectively inhibit the apoptosis of both cell types, thus significantly suppressing inflammation and terminating bone erosion (Figs. 8 and 9). Of note, the effective OCs depletion induced by CEL-RNPs efficiently restored the balance of bone function, as indicated by the reduced RANKL expression and decreased RANKL/OPG ratio (Fig. 7c; Supplementary Figs. 13 and 14). The recovery of bone function balance induced by CEL-RNPs promoted bone erosion repair by increasing ALP expression and the accumulation of OCN-positive osteoblasts (Fig. 7d).

Secondary osteoporosis occurs widely among RA patients because the increased number of OCs in RA severely disrupts the OC–osteoblast axis and enhances the bone resorption function of OCs\(^{12,76}\). Furthermore, glucocorticoids, one of the most common anti-inflammatory agents, are frequently applied in the treatment of patients with RA. Unfortunately, one severe side effect of glucocorticoids is osteoporosis induced by the prevention of calcium absorption\(^{29}\). Interestingly, the dual-targeting scaffold CEL-RNPs developed in this study could effectively increase the bone density, trabecular number, and bone thickness, and decrease the trabecular separation, thereby demonstrating the efficient control of osteoporosis in advanced arthritis (Fig. 9).

Such phenomena were mainly attributed to the enhanced OCs apoptosis induced by CEL-RNPs effectively restoring the bone function balance. Therefore, our findings might suggest a new direction in nanomedicine in the osteoporosis control of RA patients.

PLGA, PEG, and RGD have been approved in clinic application and the substrate peptide of MMP9 also consists of essential amino acids for humans. CEL-RNPs treatment showed good efficacy and led to negligible off-target apoptosis in rats. But further clinical translation, long-term toxicity of using PRNPs should be thoroughly evaluated in the future. In addition, knockout mice (αvβ3 integrin KO mice and MMP9\(^{−/−}\) mice) could be adopted to evaluate the efficacy of CEL-RNPs. The in vivo studies in knockout mice will be advantageous to the clinical translation of this developed drug delivery platform. In summary, we developed inflammatory macrophages and OCs dual-targeted strategy based on MMP9-responsive CEL-RNPs for advanced inflammatory arthritis treatment. CEL-RNPs were shown to efficiently target both inflammatory macrophages and OCs after responding to MMP9 in the inflammatory microenvironment of arthritis. Targeting of inflammatory macrophages promoted their apoptosis, thereby reducing inflammation in arthritic joints. Targeting of OCs promoted OC apoptosis and inhibited their osteoclastic function, consequently restoring the balance of bone function. Accordingly, CEL-RNPs efficiently controlled joint inflammation, reversed bone erosion, and prevented secondary osteoporosis. Taken together, CEL-RNPs realized the targeting of both synovial inflammation and bone erosion in advanced arthritis. This strategy for the selective apoptosis of inflammatory macrophages and OCs in arthritic joints shows great promise in inflammatory remission, bone erosion repair, and secondary osteoporosis prevention in advanced inflammatory arthritis.

**Methods**

**Materials.** CEL (Catalog # A0106) was obtained from Chengdu Must Biotechnology (Chengdu, China). Poly (ethylene glycol)-poly (l, l-lactide-co-glycolide 50/50) (PEG\(_{2000}\)-PLGA\(_{2000}\)) and maleimide-PEG\(_{2000}\)-PLGA\(_{2000}\) (Mal-PEG\(_{2000}\)-PLGA\(_{2000}\)) were obtained from the University of Electronic Science and Technology of China (Chengdu, China). Cys–RGD peptide and mPEG\(_{2000}\)-MMP9 cleavable Cys-peptide (mPEG\(_{2000}\)-GPLLAGQC) were custom-synthesized by GL Biochem (Shanghai) Ltd. (Shanghai, China). RANKL (Catalog # 315-11) and M-CSF (Catalog # 315-02) were obtained from PeproTech (Rocky Hill, USA). 1,1′-dioctadecyl-3,3,3′,3′-tetramethyl indocarbocyanine, 4-chlorobenzensulfonyl salt (DiD) (Catalog # M9379) was obtained from ChemBridge (San Diego, USA). LPS (Catalog # L4391) and human active MMP9 protein (Catalog # PF024) were purchased from Sigma Aldrich Co., LLC. (St. Louis, USA). Annexin V–FITC Apoptosis Detection Kit (Catalog # G8093-1) and TRAP Stain Kit (Catalog # D0023-1-1) were from Nanjing Keygen Biotech. Co., Ltd. (Nanjing, China). JC-1 molecular probe (Catalog # C2005) was acquired from Beyotime Institute of Biotechnology (Haimen, China). In Situ Cell Death Detection Kit (Catalog # 11684795910) for TUNEL assay was from Roche (Basel, Switzerland). ELISA kits to assay the levels of rat RANKL (Catalog # 11684795910) and OPG (Catalog # 11684795910) were from Nanjing Senbeija Biological Technology Co., Ltd. (Nanjing, China). ELISA kits to assay the levels of rat TNF (Catalog # KRC3011) and IL-1β (Catalog # BMS630) were purchased from Invitrogen (Shanghai, China).

**Patient samples.** Peripheral blood samples and synovial tissues were obtained from three female patients with late-stage RA (according to the American College of Rheumatology criteria) undergoing joint replacement surgery. All samples were collected from Xiangya Hospital of Central South University. Informed consent was obtained from all patients, and ethical approval was obtained from the Ethics Committee of the Xiangya Hospital of Central South University (approval No. 2019010305).

**Animals.** Healthy male Wistar rats (20 ± 2 g, 5 weeks old) and male C57BL/6 mice (20 ± 2 g, 6 weeks old) were obtained from Chengdu Dashuo Experimental Animal Co., Ltd. (Chengdu, China). Animals were housed in specific pathogen-free housing with a standard temperature of 22 ± 2 °C and a relative humidity of 55% (45–70%) in a 12:12 h light–dark cycle. All animal studies were conducted according to the requirements of the national act regarding the use of experimental animals (China) and complied with the guidelines evaluated and approved by the Animal Ethics Committee of Sichuan University.

**Cells.** The in vitro OC differentiation was carried out as previously described\(^{27}\). In brief, bone marrow cells were isolated from the tibiae of C57BL/6 mice and these cells were cultured with 30 ng/mL of M-CSF for 2 days and used as BMMs. To generate OCs, the BMMs were cultured in the presence of 100 ng/mL of RANKL and 30 ng/mL of M-CSF for 4 days. To obtain LPS-activated macrophages, BMMs were treated with 10 ng/mL of LPS for 48 h. All cells were cultured in RPMI-1640 medium containing 10% FBS and 100 U/mL of penicillin–streptomycin under 3% CO\(_2\) at 37 °C. Synovial tissue specimens were gained from patients with late-stage RA undergoing joint replacement surgery. The gained tissue specimens were washed, cut into small pieces and digested with collagenase. Tissue debris was removed by forcing the sample through a 70-µm cell strainer, thereby producing a cell suspension. Magnetic-activated cell sorting method was adopted to isolate the macrophages from synovium-derived cells. Synovial macrophages were isolated to a high percentage of purity (>95%) with the use of MACS CD14 MicroBeads (Miltenyi Biotech, Germany). Primary synovial macrophages were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) containing 10% FBS and 100 U/mL of penicillin–streptomycin under 3% CO\(_2\) at 37 °C.

Blood samples were obtained from patients diagnosed with late-stage RA. Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll–Paque (Miltenyi Biotech) density gradient centrifugation according to the manufacturer’s instructions. PBMCs were cultured in the presence of 100 ng/mL of RANKL and 30 ng/mL of M-CSF for 4 days. CEL-RNPs (MAL-PEG\(_{2000}\)-PLGA\(_{2000}\)) were cultured in RPMI-1640 medium containing 10% FBS and 100 U/mL of penicillin–streptomycin under 5% CO\(_2\) at 37 °C.
Human umbilical vein endothelial cells (HUVECs) were purchased from the Chinese Academy of Sciences Cell Bank for Type Culture Collection (Shanghai, China). HUVECs were cultured in RPMI-1640 medium containing 10% FBS and 100 U/mL of penicillin-streptomycin under 5% CO₂ at 37 °C. To activate HUVECs, cells were treated with cells with 50 ng/mL of TNF for 24 h.

**Synthesis and characterization of RGD-PEG<sub>2000</sub>-PLGA<sub>2000</sub>** RGD-PEG<sub>2000</sub>-PLGA<sub>2000</sub> was synthesized via the maleimide-thiol coupling reaction. Briefly, Mal-PEG<sub>2000</sub>-MAL (20 mg) and Cys-PEG<sub>2000</sub> (molar ratio = 1:2) were reacted in a solvent mixture comprising chloroform/MeOH (v/v = 2:1) with gentle stirring at room temperature for 12 h. The solvent was evaporated in a vacuum and the residue was re-dissolved with chloroform. The insoluble material (unreacted Cys-PEG<sub>2000</sub>) was filtered out, and the filtrate was evaporated in a vacuum to obtain RGD-PEG<sub>2000</sub>-PLGA<sub>2000</sub>. The successful synthesis of RGD-PEG<sub>2000</sub>-PLGA<sub>2000</sub> was confirmed by 'H NMR.

**Preparation and characterization of CEL-NPs, CEL-RNPs, and CEL-PRNPs.** CEL-NPs were prepared using an emulsion/solvent evaporation method. Briefly, PEG<sub>2000</sub>-MAL and CEL were dissolved in chloroform to form the oil phase. The resultant oil phase was then added into an aqueous phase. The mixture was emulsified by sonication with a probe sonicator (Ningbo Xinzhi Biotechnology Co. Ltd; Ningbo, China). CEL-NPs were then prepared after the chloroform was evaporated at low pressure. CEL-RNPs were prepared as indicated above using PEG<sub>2000</sub>-MAL-PEG<sub>2000</sub>-PLGA<sub>2000</sub> and RGD-PEG<sub>2000</sub>-PLGA<sub>2000</sub>, and CEL was dissolved in chloroform to form the oil phase. To obtain CEL-NPs, RGDPEG<sub>2000</sub>-GFLQLAGQC was conjugated with CEL-RNPs in PBS with pH 7.4 at room temperature for 4 h. Unconjugated PEG<sub>2000</sub>-GFLQLAGQC was removed by elution through a Sephadex G-75 column.

**Cellular uptake study.** BMMs, HUVECs, activated HUVECs, OCs, and macrophages derived from mice and patients were seeded in 12-well plates and were treated with C6-NPs, C6-RNPs, and C6-PRNPs (with or without 5 μg/mL of MMP9) in the serum-free medium. After a 1 h incubation, cells were collected, centrifuged, and then suspended in phosphate-buffered saline (PBS). The fluorescence intensity of C6 was measured by a flow cytometer (BD FACSCelesta, USA).

**AIA model.** The AIA model was developed according to a previous study but with slight modifications. In brief, healthy male Wistar rats (200 ± 20 g) were subcutaneously injected with Freund’s adjuvant (200 μL) containing 10 mg/mL of heat-killed mycobacteria (Chondrex, Washington DC, USA) into the base of their tails. Rats were observed daily for clinical signs of arthritis. The Lewis rats were sacrificed at the 21st day and 2 days after the last treatment. The animals were examined using in vivo imaging analysis of DiD fluorescence with a Caliper IVIS Lumina III In Vivo Imaging System (Perkin Elmer, USA). At the end of the experiment, rats were treated with free DiD, DiD-NPs, DiD-RNPs, or DiD-PRNPs via the tail vein. The biodistribution of DiD in the ankle joints was analyzed 24 h after administration using in vivo imaging analysis of DiD fluorescence with a Caliper IVIS Lumina III In Vivo Imaging System (Perkin Elmer, USA). At the end of this experiment, rats were sacrificed, their hind limbs and forelimbs were collected for ex vivo imaging of DiD fluorescence.

**Immunofluorescence staining.** Arthritic rats with advanced arthritis were intravenously injected with free DiD, DiD-NPs, DiD-RNPs, or DiD-PRNPs via the tail vein. Cell uptake studies were performed to prepare sections 24 h after administration. The prepared sections of 10 μm thickness were stained with rat anti-CD68 (Abcam, Cat#ab215221, 1/500 dilution) and anti-CDS1 (Abcam, Cat#ab79475, 1/500 dilution). DAPI was used for the nuclear stain. The fluorescent distributions in synovial joints were observed with a laser scanning confocal microscope (Leica TCS SP8 CARS, Germany).

**Cytokine assay.** AIA rats with advanced arthritis were as indicated above. Saline or various CEL-loaded PLGA nanoparticles were respectively intravenously injected into RA rats (dose of 1 mg/kg for CEL). Rats were harvested 2 days after the last treatment, and the rats were sacrificed and their articular cartilage were collected. The articular cartilage was sectioned and then sectioned for H&E, safranin O, toluidine blue, TRAP, CD68, MMP9, and MMP13 staining. These sections were observed by a light microscope and analyzed with Image J.

**Bone assessment and micro-CT analysis.** The ankle joints collected before the treatment and 2 days after the last treatment were fixed in 4% paraformaldehyde. Fixed ankle joints were then decalcified by daily changes of 15% (w/v) tetraethylammoniumtetracetic acid solution for 2 months. The decalcified joints were subsequently embedded in paraflin and then sectioned for H&E, safranin O, toluidine blue, TRAP, CD68, MMP9, RANKL, and MMP13 staining. These sections were observed by a light microscope and analyzed with Image J.

**Histology and immunohistochemical study.** Ankle joints collected before the treatment and 2 days after the last treatment were fixed in 4% paraformaldehyde. Fixed ankle joints were then decalcified by daily changes of 15% (w/v) tetraethylammoniumtetracetic acid solution for 2 months. The decalcified joints were subsequently embedded in paraflin and then sectioned for H&E, safranin O, toluidine blue, TRAP, CD68, MMP9, RANKL, and MMP13 staining. These sections were observed by a light microscope and analyzed with Image J.

**Safety evaluation.** To assess the in vivo safety of CEL-PRNPs, healthy male Wistar rats (200 ± 20 g) were intravenously administered with 1 mg/kg of CEL equivalents of CEL solution, CEL-NPs, CEL-RNPs, or CEL-PRNPs, respectively. An equal volume of saline was injected into the control rats. Rats were sacrificed 2 days after the last treatment, and the blood and major organs (heart, liver, spleen, lung, kidney, and brain) were collected for serum enzyme and histopathological analyses.
In addition, collected organs were sectioned for TUNEL staining and then analyzed with a laser scanning confocal microscope.

Statistical analysis. All quantitative parameters were presented as mean with standard deviation. For a two-group comparison, a Student's two-sided t test was performed for the statistical analysis. For multiple comparisons, the data were analyzed using a two-way analysis of variance (ANOVA). A significant difference was considered when the P value was less than 0.05.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

All data are available in the Article, Supplementary Information files, or from the corresponding author upon reasonable request. The source data underlying Figs. 1b, c, 2d, 3d–g, 4b–f, 5d–f, 7c, 8b, 9b–f, 10, 12, 14a–c, 15a–d, 16, 18a–d, 19b, and 20b, c and Supplementary Table 1 are provided as a Source Data file. Source data are provided with this paper.

Received: 18 January 2020; Accepted: 5 March 2021;
Published online: 12 April 2021

References

1. Smolen, J. S. et al. Rheumatoid arthritis. Nat. Rev. Dis. Prim. 4, 18001 (2018).
2. Yeo, L. et al. Expression of chemokines CXCL4 and CXCL7 by synovial macrophages de...
49. Fredman, G. et al. Targeted nanoparticles containing the proresolving peptide AC2-26 protect against advanced atherosclerosis in hypercholesterolemic mice. Sci. Transl. Med. 7, 273ra220–273ra220 (2015).

50. Liu, J., Zhang, R. & Xu, Z. P. Nanoparticle-based nanomedicines to promote osteoclast differentiation. Proc. Natl Acad. Sci. USA 107, 3117–3122 (2010).

51. Han, S. A., Lee, S., Seong, S. C. & Lee, M. C. Effects of CD14 macrophages and proinflammatory cytokines on chondrogenesis in osteoarthritic synovium-derived stem cells. Tissue Eng. Part A 20, 2680–2691 (2014).

52. Tu, J. et al. Synovial macrophages in rheumatoid arthritis: the past, present, and future. Mediat. Inflamm. 4, 1–8 (2020).

53. Liu, H. et al. Regulation of Mcl-1 expression in rheumatoid arthritis synovial macrophages. Arthritis Rheum. 54, 3174–3181 (2006).

54. Meng, J. et al. Catalpol suppresses osteoclastogenesis and attenuates osteoclast-derived bone resorption by modulating PTEN activity. Biochem. Pharm. 171, 113715 (2020).

55. Nishikawa, K. et al. Blimp1-mediated repression of negative regulators is required for osteoclast differentiation. Proc. Natl Acad. Sci. USA 107, 3117–3122 (2010).

56. Wang, Q. et al. Targeted delivery of low-dose dexamethasone using PCL-PEG micelles for effective treatment of rheumatoid arthritis. J. Control Release 230, 64–72 (2016).

Acknowledgements

This work was financially supported by the National Natural Science Foundation of China (No. 81872804) and Sichuan major science and technology project on biotechnology and medicine (2018SZDX0018). In addition, the authors would like to thank Dr. Li Chen from Analytical & Testing Center Sichuan University for her help with micro-CT scanning and analysis.

Author contributions

T.G. and G.L. conceived and planned the study. C.D. carried out the experiments, generated and analyzed data, created Figs. 2a and 8a and Supplementary Fig. 20a, and wrote the original paper. Z.Q., P.H., and K.H. helped with animal and cell studies. B.Z. collected human samples and helped with related experiments. X.S., T.G., and Z.Z. helped with paper editing.

Competing interests

The authors declare no competing interests.

Additional information

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41467-021-22454-z.

Publisher’s note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

© The Author(s) 2021