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CARTILAGE

Decreased Peli1 expression attenuates osteoarthritis by protecting chondrocytes and inhibiting M1-polarization of macrophages

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Aims

Pellino1 (Peli1) has been reported to regulate various inflammatory diseases. This study aims to explore the role of Peli1 in the occurrence and development of osteoarthritis (OA), so as to find new targets for the treatment of OA.

Methods

After inhibiting Peli1 expression in chondrocytes with small interfering RNA (siRNA), interleukin (IL)-1β was used to simulate inflammation, and OA-related indicators such as synthesis, decomposition, inflammation, and apoptosis were detected. Toll-like receptor (TLR) and nuclear factor-kappa B (NF-κB) signalling pathway were detected. After inhibiting the expression of Peli1 in macrophages Raw 264.7 with siRNA and intervening with lipopolysaccharide (LPS), the polarization index of macrophages was detected, and the supernatant of macrophage medium was extracted as conditioned medium to act on chondrocytes and detect the apoptosis index. The OA model of mice was established by destabilized medial meniscus (DMM) surgery, and adenovirus was injected into the knee cavity to reduce the expression of Peli1. The degree of cartilage destruction and synovitis were evaluated by haematoxylin and eosin (H&E) staining, Safranin O/Fast Green staining, and immunohistochemistry.

Results

In chondrocytes, knockdown of Peli1 produced anti-inflammatory and anti-apoptotic effects by targeting the TLR and NF-κB signalling pathways. We found that in macrophages, knockdown of Peli1 can inhibit M1-type polarization of macrophages. In addition, the corresponding conditioned culture medium of macrophages applied to chondrocytes can also produce an anti-apoptotic effect. During in vivo experiments, the results have also shown that knockdown Peli1 reduces cartilage destruction and synovial inflammation.

Conclusion

Knockdown of Peli1 has a therapeutic effect on OA, which therefore makes it a potential therapeutic target for OA.

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Keywords: Peli1, Chondrocyte, Macrophage, Polarization, Toll-like receptor

Article focus

- This study investigated the role of Pellino1 (Peli1) in the pathogenesis and progression of osteoarthritis (OA) and its underlying molecular mechanisms.

Key messages

- Knockdown of Peli1 in chondrocytes with small interfering RNA (siRNA) produced anti-inflammatory and anti-apoptotic effects by affecting toll-like receptor and NF-κB signalling pathways.

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- Knockdown of Peli1 in macrophages suppressed m1-type polarization of macrophages.
- When applied to chondrocytes, Peli1 knockout conditioned macrophage medium also produced anti-apoptotic effects.

Strengths and limitations

- These findings may provide a new therapeutic target for the treatment of OA.
- The effect of Peli1 overexpression on OA needs to be further studied.
- Further transgenic animals and clinical studies are needed to better understand the pathogenesis of OA.

Introduction

Osteoarthritis (OA) is a serious degenerative disease that worsens with age. It is common in the elderly and is often accompanied by pain, stiffness, swelling, and activity disorders. Causes of OA include joint damage, diet, obesity, ageing, and genetics.[1] The crude incidence of OA increased by 102% in 2017 compared to 1990.[2] OA is expected to become the largest cause of disability in the general population by 2030.[3] Its occurrence and development are related to various mechanisms, including degradation and destruction of cartilage matrix, synovitis, osteophyte formation, subchondral bone changes, and macrophage activation.[1,4,5] Inflammatory changes and apoptosis of chondrocytes play an important role in the pathogenesis of OA.[6-8] While the molecular mechanism underlying OA remains elusive, it is imperative to further search for better therapeutic targets for OA to develop effective therapeutic options.

Macrophages result from the complete differentiation of the mononuclear phagocytic cell line. Macrophages are involved in the pathogenesis of many inflammatory diseases, for example Crohn's disease, hepatitis, rheumatoid arthritis, and OA.[9-13] The effect of macrophages in inflammatory diseases is mediated through macrophage polarization. Activation of macrophages can be divided into classical activation (M1) and alternative activation (M2) according to the different external microenvironments. M1 versus M2 macrophage polarization is a closely coordinated process. Lipopolysaccharides (LPS) and cytokines, particularly interferon-γ (IFN-γ), polarize macrophages towards the M1 type. M1 macrophages secrete lots of pro-inflammatory cytokines, including IL-1β, IL-6, IL-12, tumour necrosis factor-alpha (TNF-α), reactive oxygen species (ROS), and inducible nitric oxide synthase (iNOS), which promote tissue repair. IL-4, IL-10, IL-13, and glucocorticoids polarize macrophages toward M2 type. M2 macrophages secrete arginase-1 (Arg-1) and IL-10, which play an anti-inflammatory role and facilitate tissue healing.[12,14-16] It has been confirmed that the polarization of macrophages is of vital importance in OA. Thus, the treatment of OA by targeting the polarization of macrophages is a promising strategy.[12,17]

Peli was initially identified as a protein which interacts with the Pelle kinase domain, a direct homolog of IL-1 receptor (IL-1R)-associated kinases in the Drosophila melanogaster Toll signalling pathway.[18] Peli1 is a member of the Peli family of E3 ubiquitin ligases, which play a key role in a variety of immune cells. Peli1 regulates TLR, IL-1R, and T-cell receptor (TCR) signalling.[19,20] In myeloid differentiation primary response 88 (MyD88)- and TIR-domain-containing adapter-inducing interferon-β (TRIF)-dependent signalling pathways, Peli1 regulates ubiquitination of apoptotic protein inhibitors such as TNF receptor-associated factor 6 (TRAF6) and receptor-interacting protein (RIP),[19,21,22] ultimately regulating NF-κB activation and mitogen-activated protein kinase (MAPK) signalling.[23,24] In macrophages, Peli1 can promote M1-type polarization.[24] It has also been confirmed that Peli1 regulates obesity-induced glucose intolerance by enhancing M1 macrophage polarization through k63-linked interferon-regulatory factor 5 (IRF5) ubiquitination.[24-26] Peli1 also inhibits IL-10-mediated M2c polarization of macrophages.[27] However, the effect of Peli1 in OA has not been investigated so far.

Methods

Reagents and antibodies. Recombinant mouse IL-1β (# 211-11b) was acquired from PeproTech (USA). Mouse IL-1β, IL-6, and TNF-α enzyme-linked immunosorbent assay (ELISA) kits were acquired from Neobioscience (China). Lipofectamine 3000 reagent was acquired from Invitrogen (Thermo Fisher Scientific, USA). Rabbit anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody and secondary antibody were acquired from Proteintech (China). BCL2-associated X (BAX), B-cell lymphoma-2 (BCL-2), clv Caspase-3, t Caspase-3, iNOS, TNF receptor associated factor 6 (TRAF6), receptor-interacting protein (RIP), phosphorylated receptor-interacting protein (P-RIP), I kappa B kinase (IKK)-α, IKK-β, P-IKK-α/β, P-IKBα, IKBα, P65, P-P65, and CD86 were acquired from Cell Signaling Technology (USA).

Isolation and culture of primary chondrocytes. A total of 60 newborn male C57BL/6 mice (seven days old) were killed and disinfected. Cartilage was isolated from both knee joints of mice and cut into 1 mm pieces. Purified cartilage was absorbed with 0.2% trypsin for half an hour at 37°C, followed by 0.25% collagenase II for nine hours at 37°C. After resuspension and filtration, chondrocytes were grown in Dulbecco's Modified Eagle Medium (DMEM)/F12 (with serum and antibiotics), which was purchased from Boster (China) at 37°C with medium changes every two days. Before each experiment, the first- or second-generation cells were laid out on 10 cm plates and left to grow to about 70% confluency.

siRNA transfection. According to the manufacturer's instructions (Ribbio, China), Peli1-targeting siRNA and Lipofectamine 3000 transfection reagents were mixed with Opti-MEM culture media, and the mixture was then added to the chondrocyte culture medium. SiRNA concentration was 50 nM. The siRNA used had the following sense chain: Peli1, 5'-ACAAGATGGCTCCTTAATT-3';

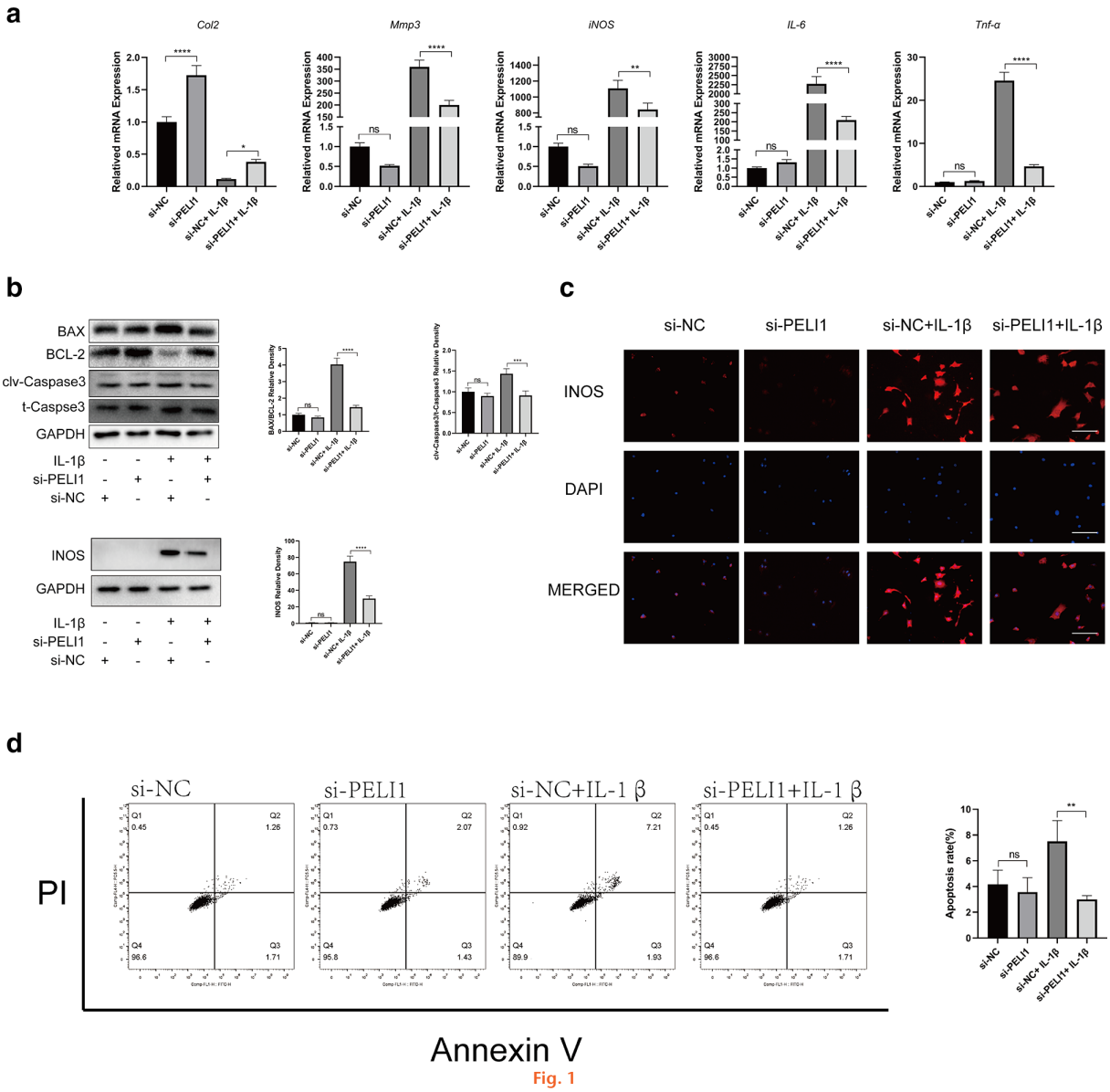

Fig. 1

Knockdown of Pellino1 (Peli1) reduces inflammatory and degenerative cytokine expression in chondrocytes. a) Reverse transcription quantitative polymerase chain reaction (RT-qPCR) detected the expression of chondrocyte destruction-related genes and gene products *COL2*, matrix metalloproteinase 3 (*MMP3*), inducible nitric oxide synthase (*iNOS*), interleukin 6 (*IL-6*), and tumour necrosis factor alpha (*TNF-α*) after Peli1 knockdown. b) Representative protein imprinting of iNOS, clv-Caspase3, t-Caspase3, BAX, and BCL-2 after 48 hours of small interfering RNA-normal contrast (si-NC) or si-Peli1 transfection of chondrocytes and subsequent treatment with IL-1β (5 ng/ml) for 24 hours. c) Representative immunofluorescence images of chondrocyte inducible nitric oxide synthase (iNOS); measuring scale = 100 μm. d) The apoptotic rate of cartilage cells was analyzed by annexin V-FITC/PI apoptosis analysis. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 compared with si-NC transfected or si-NC+ IL-1β-treated chondrocytes. mRNA, messenger RNA; NS, not significant.

TRAF6, 5'-CTTACAATTCTCGACCAG T-3'; RIP, 5'-GCCA AAUCUAAGCCAAAUGUATT-3'.

Flow cytometry. Annexin V-FITC apoptosis detection kit (Yi Sheng, China) was used to identify the cell apoptosis rate. In brief, chondrocytes were digested by trypsin without ethylenediaminetetraacetic acid (EDTA) and collected in a flow cytometry chamber at a concentration of about 1 × 10^5 cells/ml. Annexin V-FITC (5 μl) and PI (10 μl) were added to the flow tube under dark conditions, mixed, and kept to react for 15 to 30 minutes. The results were analyzed by flow cytometry. To differentiate M1 polarization, CD86 was selected to label the M1 phenotype, and phycoerythrin (PE)-conjugated anti-CD86 was used to evaluate macrophage subsets. The results were further analyzed by flow cytometry.

ELISA. The supernatant of RAW264.7 macrophages was collected 24 hours after LPS and siRNA intervention by centrifugation at 1,000 g for five minutes. Finally, IL-6, IL-1β, and TNF-α levels in the supernatant were detected using respective ELISA kits.

Animal models. OA was induced in 12-week-old male C57BL/6 J mice by DMM surgery.[28,29] A total of 60 mice

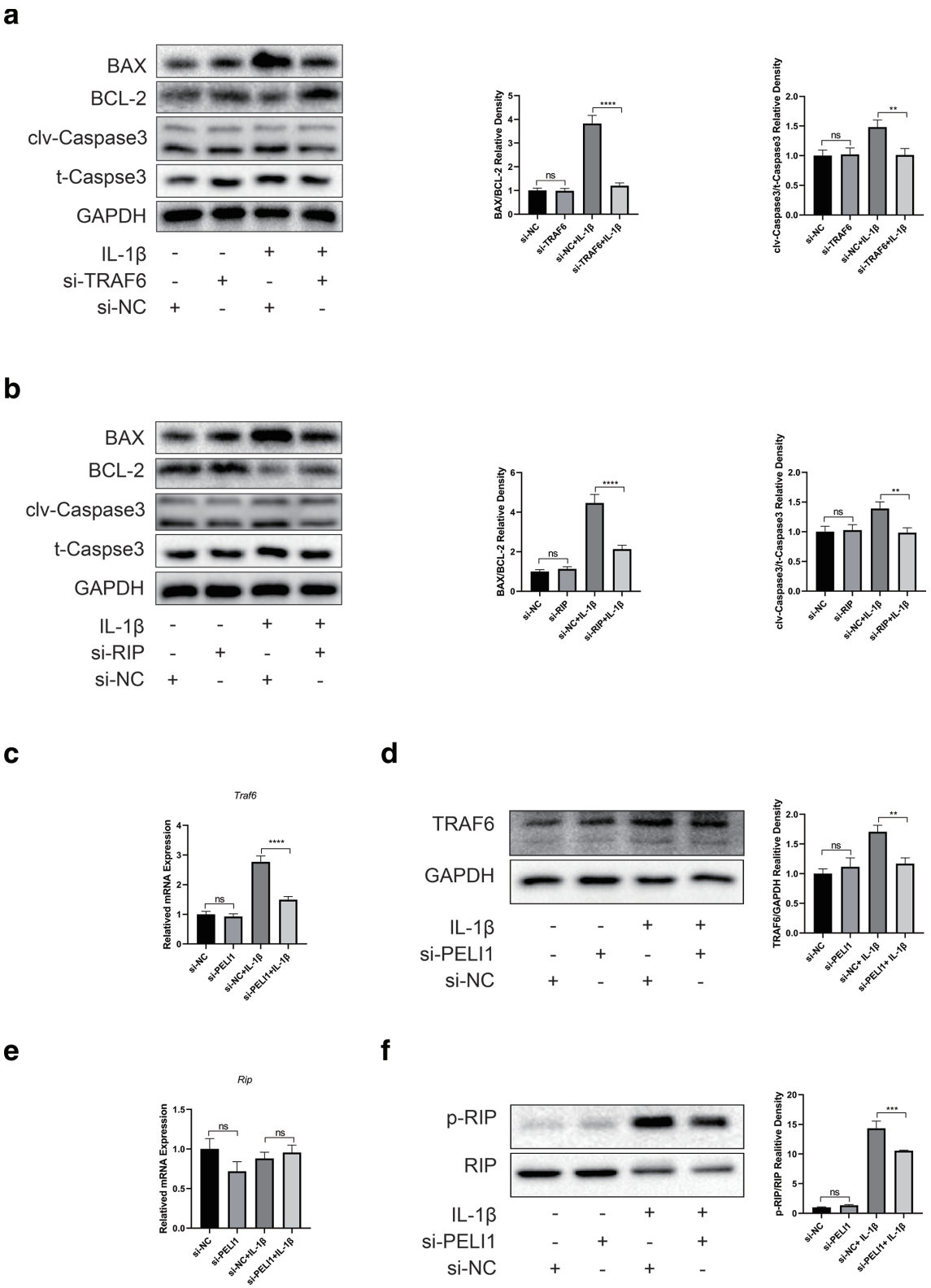

Fig. 2

Knockdown of Pellino1 (Peli1) inhibits toll-like receptor (TLR) signalling pathway in chondrocytes. a) Western blot showing that knockdown of TRAF6 could effectively inhibit chondrocyte apoptosis induced by interleukin (IL)-1β. b) Western blot showing that knockdown of RIP could effectively inhibit chondrocyte apoptosis induced by IL-1β. c) Reverse transcription quantitative polymerase chain reaction (RT-qPCR) showing that Peli1 knockdown affected the gene expression of *TRAF6*. d) Western blot showing that Peli1 knockdown affected the protein expression of TRAF6. e) RT-qPCR showing that Peli1 knockdown did not affect the gene expression of *RIP*. f) Western blot showing that Peli1 knockdown affected the protein expression of p-RIP/RIP. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 compared with si-NC transfected or si-NC+ IL-1β-treated chondrocytes. mRNA, messenger RNA; NS, not significant.

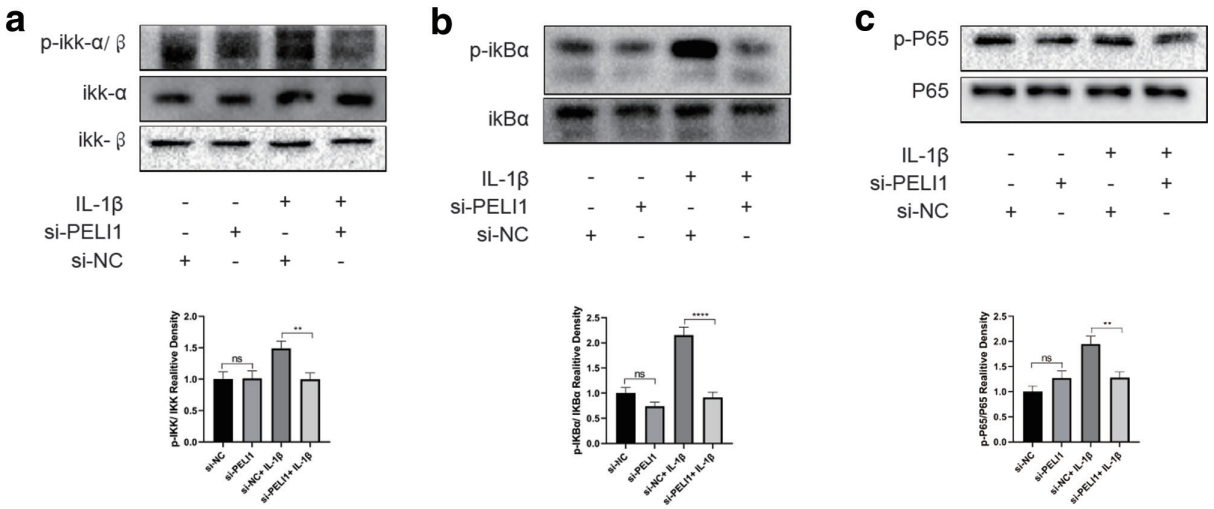

Fig. 3

Knockdown of Pellino1 (Peli1) inhibits nuclear factor kappa B (NF-κB) signalling in chondrocytes. a) Western blot showing that Peli1 knockout affected NF-κB signaling-related protein p-IKK/IKK. b) Western blot showing that Peli1 knockout affected NF-κB signaling-related protein p-IKBα/IKBα. c) Western blot showed that Peli1 knockout affected NF-κB signaling-related protein p-P65/P65. **p < 0.01, ****p < 0.0001 compared with si-NC transfected or si-NC+ IL-1β-treated chondrocytes.

were divided into four groups: 1) sham operation group: sham operation mice were administered adenovirus (AD)-SH control adenovirus (n = 15); 2) sham operation + AD-SH Peli1 group: sham operation mice were administered AD-SH Peli1 adenovirus (n = 15); 3) destabilized medial meniscus (DMM) group: DMM operation mice were administered AD-SH control adenovirus (n = 15); and 4) DMM + AD-SH Peli1 group: DMM operation mice were administered AD-SH Peli1 adenovirus (n = 15). After anaesthesia, in the experimental group, the medial meniscus-tibial ligament was severed. In the control group, only the joint capsule was opened, while other tissues remained intact. After wound healing, mice were injected intra-articularly with 10 µl AD-SH Peli1 or AD-negative adenovirus (1×10^9 plaque formation units (PFUs)) twice a week for two months.[30] The animal study was reviewed and approved by the Animal Care and Use Committee for Teaching and Research.

RNA extraction and RT-qPCR. RNA was extracted from chondrocytes using the OMEGA kit, which was acquired from Biotek (USA). We used the revert Aid First Strand cDNA Synthesis Kit to synthesize complementary DNA (cDNA), which was acquired from Toyobo (Japan). Then, SYBR Green Quantitative polymerase chain reaction (PCR) protocol was used to amplify the template to determine messenger RNA (mRNA) levels. All primer sequences used were as follows:

Peli1, 5'-TTTATCTCGAGCCCAGACGG-3', 5'-GTGC TTTGTACTGACTGCGTG-3';

Col2, 5'-GGCCAGGATGCCCGAAAATTA-3', 5'-CGCACCCTTTTCTCCCTTGT-3';

Mmp3, 5'-ACTCCCTGGGACTCTACCAC-3', 5'-GGTACCACGAGGACATCAGG-3';

iNOS, 5'-GACCCAGAGACAAGCCTAC-3', 5'-GTGAGCTGGTAGGTTCCTG-3';

Il-6, 5′-CAACGATGATGCACTTGCAGA-3', 5'-TGTG ACTCCAGCTTATCTCTTGG-3';

Tnf-α, 5′-CTCAGCGAGGACAGCAAGG-3', 5'-CTCAGCGAGGACAGCAAGG-3';

Cd14, 5'-TCGGATTCTATTCGGAGCC-3', 5'-GTTGCGGAGGTTCAAGAGT-3';

Cd80, 5'-ATGCAGGATACACCACTCC-3', 5'-AGAGTTGTAACGGCAAGGC-3'.

Expression levels of target genes were calculated using the 2–ΔΔCq method.

Western blot analysis. The chondrocytes were washed three times with phosphate-buffered saline (PBS). Cold radio-immunoprecipitation assay (RIPA) lysis buffer containing 1% protease and phosphatase inhibitors was then added, they were allowed to react on ice for about 20 minutes, after which total protein was extracted. The extract was subsequently centrifuged at 15,000 RPM and 4°C for 30 minutes to discard the precipitate. Protein concentration was measured by the bicinchoninic acid (BCA) method. Electrophoresis was carried out using sodium dodecyl-sulphate polyacrylamide gel electrophoresis (SDS-PAGE) gel (10% to 15%), and the bands were then transferred to a polyvinylidene fluoride (PVDF) film. The membranes were sealed with 5% BSA for one hour and then incubated with primary antibodies in shake flasks overnight at 4°C. Next, secondary antibodies were added, incubated for one to two hours, and contaminants were washed away with tris buffered saline with tween-20 (TBST). Finally, a visual analysis of the signal is performed.

Histological staining and analysis. After immersion in 10% paraformaldehyde for 48 hours, knee joints were decalcified with EDTA-buffered saline for 30 days. Tissue sections were inserted into paraffin, cut into sagittal sections 4 µm thick, and stained with haematoxylin and

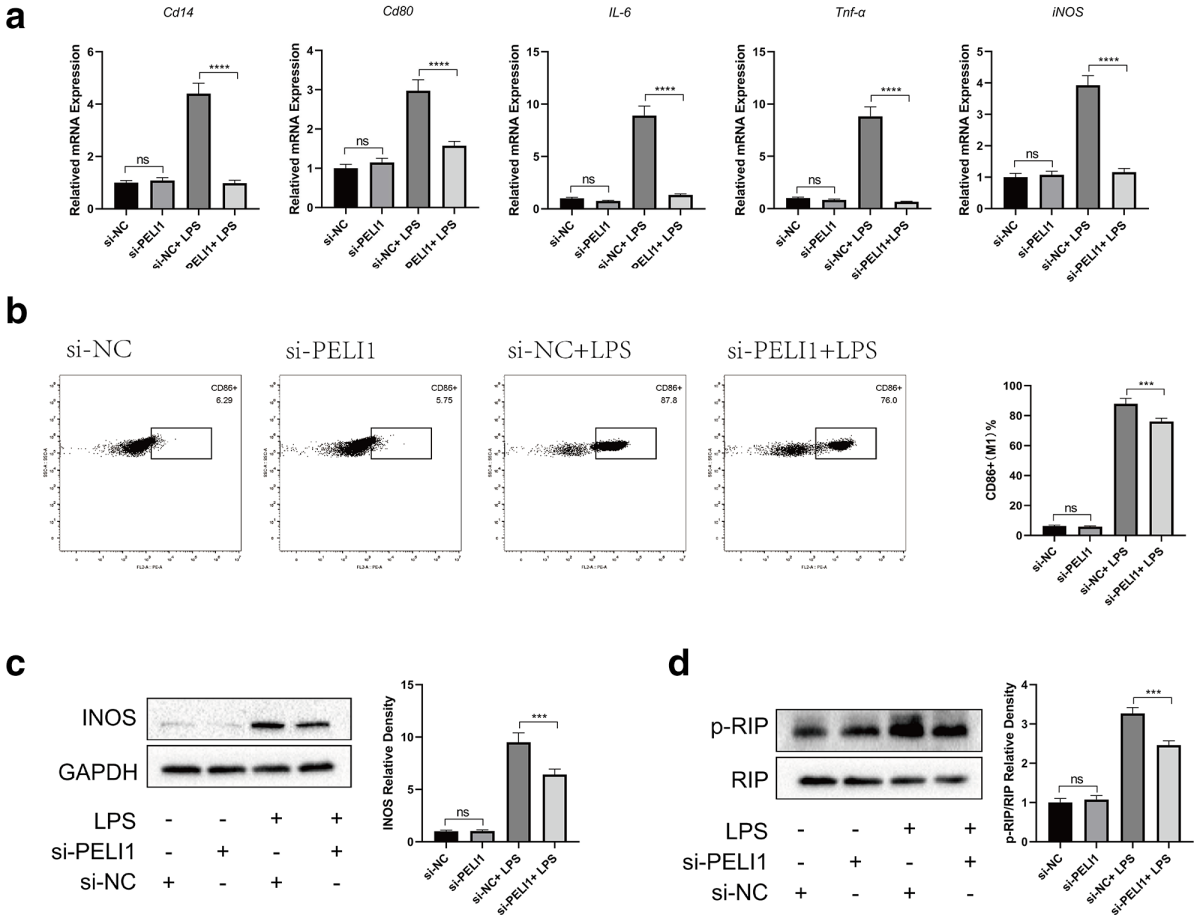

Fig. 4

Knockdown of Pellino1 (Peli1) inhibits M1 polarization of macrophages. a) Expression of macrophage M1-related genes *Cd14, Cd80, IL-6, TNF-α,* and inducible nitric oxide synthase (*iNOS*) was evaluated by reverse transcription quantitative polymerase chain reaction (RT-qPCR). b) Flow cytometry was used to evaluate macrophage M1-polarization by staining CD86. c) The expression of iNOS was evaluated by western blot. d) Evaluation of p-RIP/RIP expression by western blotting. ***p < 0.001, ****p < 0.0001 compared with si-NC transfected or si-NC+ LPS-treated RAW264.7 cells. GAPDH, glyceraldehyde 3-phosphate dehydrogenase; LPS, lipopolysaccharide; mRNA, messenger RNA; NS, not significant.

eosin (H&E) and Safranin O/Fast Green staining. The extent of damage to the knee joint was evaluated using the Osteoarthritis Research Society International (OARSI) score and synovitis score.[31,32] Images captured under a digital microscope were evaluated using immunohistochemical staining kits (DAB Kit, Invitrogen) and ImageJ software (National Institutes of Health, USA).

Immunofluorescence. Immunofluorescence staining analysis of chondrocyte iNOS. After fixation with 4% paraformaldehyde and infiltration with 0.2% Triton X-100, chondrocytes were incubated with primary iNOS antibody (1:200) overnight at 4°C. Cells were then incubated with anti-mouse and anti-rabbit secondary antibodies for one to two hours. Finally, images were taken with a fluorescence microscope.

Statistical analysis. Dates are expressed as mean and standard deviation (SD). Analyses were performed using Graphpad Prism v.5.0 (USA). Differences between groups were analyzed by independent-samples *t*-test, and comparisons between groups were explored by one-way analysis of variance (ANOVA) and Dunnett's post-hoc test. All experiments were performed in at least three independent biological replicates. A p-value < 0.05 was considered significant.

Results

Knockdown of Peli1 reduces inflammatory and degenerative cytokine expression in chondrocytes. Reverse transcription quantitative PCR (RT-qPCR) results showed that IL-1β significantly ($p < 0.001$) stimulated the mRNA levels of inflammatory cytokines *IL-6* and *TNF-α*, dramatically increased levels of the cartilage destructive factor matrix metalloproteinase 3 (*MMP3*) and *iNOS*, and markedly decreased the levels of protective factor collagen type 2 (*COL2*), suggesting that IL-1β may be a main pathological cause of cartilage destruction. Furthermore, we proved that Peli1 knockdown decreased the expression of *IL-6*, *TNF-α*, *MMP-3*, and *iNOS,* while increasing the expression of *COL-2* (Figure 1a). Thus, Peli1 is a possible therapeutic target to reduce inflammation. Knockdown efficiency of *Peli1* was demonstrated by RT-qPCR (Supplementary Figure a). Immunofluorescence and western blot showed

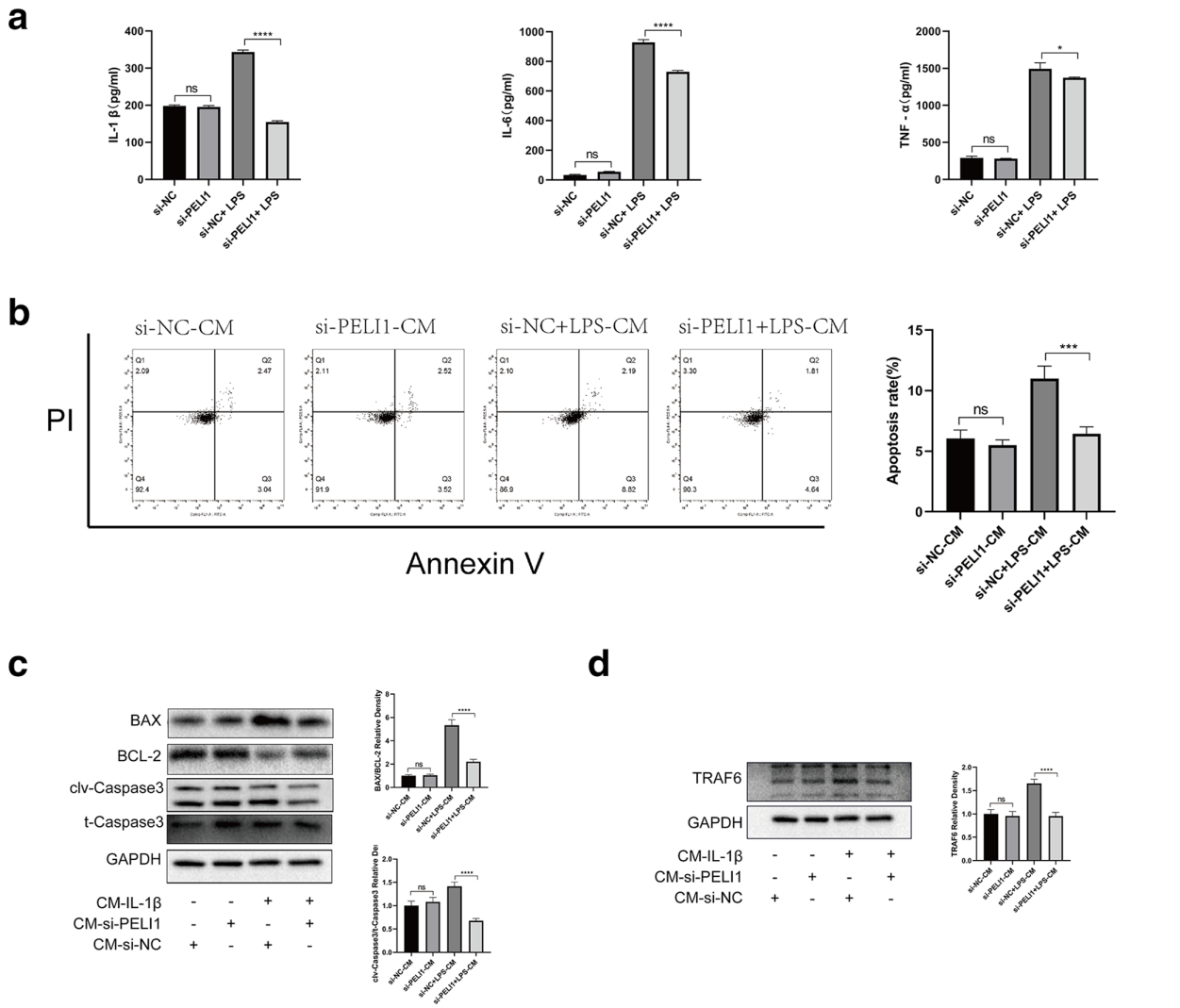

Fig. 5

Effect of macrophage conditioned medium on chondrocytes. a) Components of macrophage conditioned medium (CM), including interleukin (IL)-1β, IL-6, and tumor necrosis factor alpha (TNF-α), were tested by enzyme-linked immunosorbent assay (ELISA) kit. *p < 0.05, ***p < 0.001, ****p < 0.0001 compared with si-NC transfected or si-NC+ LPS-treated RAW264.7 cells. b) The apoptosis rate of chondrocytes stimulated by macrophage CM was analyzed by flow cytometry. c) Western blot shows the expression of BAX, BCL-2, clv-Caspase3, and t-Caspase3. d) Western blot analysis of TNF receptor-associated factor 6 (TRAF6) expression. ***p < 0.001, ****p < 0.0001 compared with si-NC-CM or si-NC+ lipopolysaccharide (LPS) CM group. GAPDH, glyceraldehyde 3-phosphate dehydrogenase; Peli1, Pellino1.

that Peli1 knockdown treatment (50 nmol/ml) had a positive influence on IL-1β-induced chondrocyte inflammation (Figures 1b and 1c). To explore the influence of Peli1 on IL-1β-induced chondrocyte apoptosis, Peli1 in chondrocytes was knocked down. Flow cytometry and western blot showed that Peli1 knockdown treatment (50 nmol/ml) reduced the apoptosis of IL-1β-induced chondrocytes (Figures 1b and 1d).

Knockdown of Peli1 inhibits TLR signalling pathway in chondrocytes. Activation of TLR has paramount importance in the pathogenesis of OA.[33,34] In our study, knockdown of TRAF6 and RIP (critical targets of the TLR signalling pathway) could effectively inhibit chondrocyte apoptosis induced by IL-1β (Figures 2a and 2b). Knockdown efficiency of TRAF6 and RIP were demonstrated by RT-qPCR (Supplementary Figures c and d). After knocking down Peli1 in chondrocytes, we detected the expression of TRAF6, receptor-interacting protein (RIP), and its phosphorylation in chondrocytes. After Peli1 siRNA transfection, the gene and protein expression of TRAF6 decreased in IL-1β-stimulated chondrocytes (Figures 2c and 2d). Furthermore, the gene expression of *RIP* did not change in IL-1β-stimulated chondrocytes (Figure 2e). However, the phosphorylated protein of RIP decreased in IL-1β-stimulated chondrocytes (Figure 2f). Overall, these results suggest that knockdown of Peli1 inhibits inflammation and apoptosis by inhibiting TLR signalling pathway.

Knockdown of Peli1 inhibits NF-κB signalling in chondrocytes. Immunoblotting analysis of chondrocytes revealed that IL-1β significantly activated the phosphorylation of

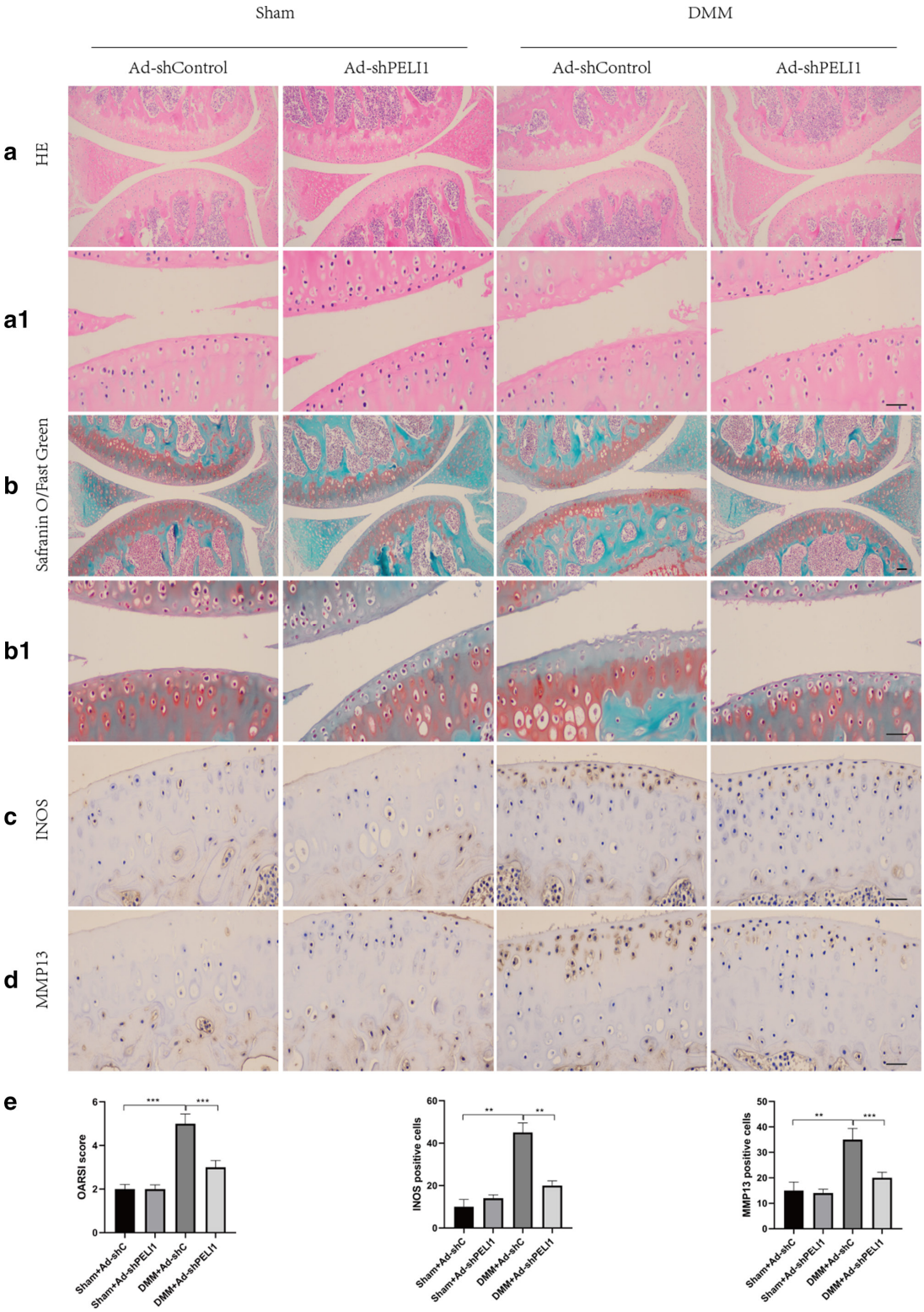

Fig. 6

Knockout of Pellino1 (Peli1) can reduce cartilage degradation in the destabilized medial meniscus (DMM) model. a) and a1) Haematoxylin and eosin (H&E) staining of cartilage of mice from adenovirus (AD)-SH control and AD-SH Peli1 groups two months after DMM surgery; measuring scale = 100 µm. b) and b1) Ferro O/solid green staining of cartilage of mice from AD-SH control and AD-SH Peli1 groups two months after DMM surgery. c) Inducible nitric oxide synthase (iNOS) immunostaining images of cartilage of mice from AD-SH Control and AD-SH Peli1 groups two months after DMM surgery. d) Matrix metalloproteinase 13 (MMP13) immunostaining images of cartilage of mice from AD-SH Control and AD-SH Peli1 groups two months after DMM surgery. e) Quantitative analysis of Osteoarthritis Research Society International (OARSI) score, and iNOS and MMP13 expression. **p < 0.01, ***p < 0.001 compared with Sham + AD SH Control or DMM + AD SH Control group. Measuring scale = 100 µm.

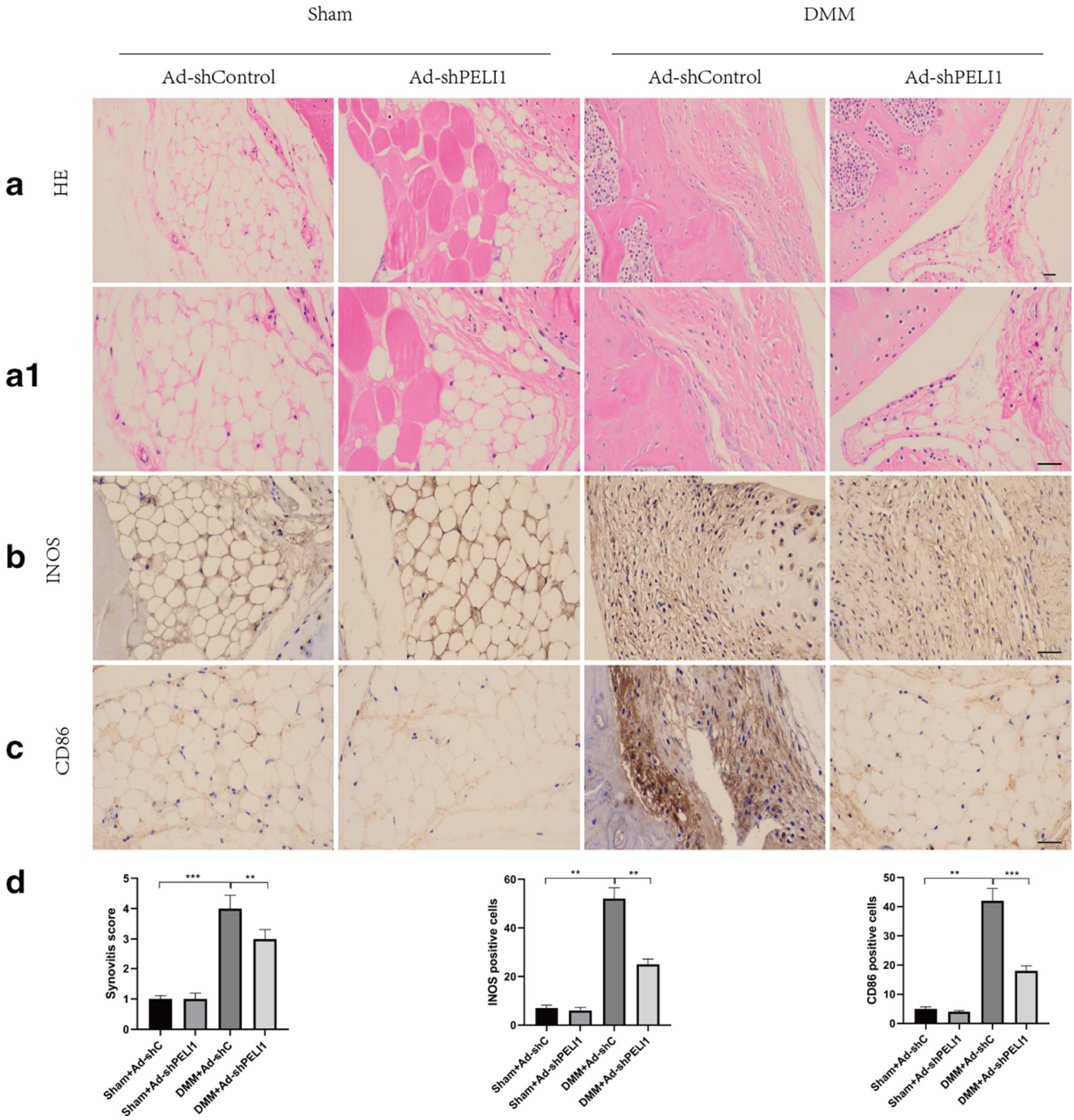

Fig. 7

Knockout of Pellino1 (Peli1) can reduce synovial inflammation in the destabilized medial meniscus (DMM) model. a) and a1) Haematoxylin and eosin (H&E) staining of synovium from AD-SH Control and AD-SH Peli1 mice two months after DMM surgery. b) Inducible nitric oxide synthase (iNOS) immunostaining images of synovium from AD-SH Control and AD-SH Peli1 mice two months after DMM surgery. c) CD86 immunostaining images of synovium from AD-SH Control and AD-SH Peli1 mice two months after DMM surgery. d) Quantitative analysis of synovitis score and iNOS and CD86 expression. **p < 0.01, ***p < 0.001 compared with Sham + AD SH Control or DMM + AD SH Control group. measuring scale = 100 μm.

IKK, IκBα, and p65 in NF-κB signalling ($p < 0.001$).[35,36] Phosphorylated IKK, IκBα, and p65 proteins were reduced in IL-1β-stimulated chondrocytes after PELI1 siRNA transfection (Figures 3a to 3c). Overall, these results indicate that knockdown of Peli1 inhibits inflammation and apoptosis by inhibiting the NF-κB signalling pathway.

Knockdown of Peli1 inhibits M1 polarization of macrophages. According to the results of RT-qPCR, knockdown of Peli1 decreased the expression of M1-related genes, namely *Cd14*, *Cd80*, *Il-6*, *TNF–α*, and *iNOS* (Figure 4a). Knockdown efficiency of *Peli1* was demonstrated by RT-qPCR (Supplementary Figure b). Western blot showed

that knockdown of Peli1 could reduce the protein level of iNOS in macrophages (Figure 4c). Flow cytometry revealed that the level of CD86 positive cells decreased after Peli1 was knocked down (Figure 4b). In addition, we found that the TLR signalling pathway is involved in the M1 polarization of macrophages. After knocking down Peli1, the phosphorylation level of RIP (an important target in the TLR signalling pathway) decreased (Figure 4d). These data show that knockdown of Peli1 inhibits the TLR signalling pathway, thereby inhibiting M1 polarization of macrophages.

Knocking down Peli1 prevents the apoptosis of chondrocytes by macrophage conditioned medium. The results of ELISA showed that LPS stimulated IL-1β, IL-6, and TNF-α increase in macrophages. This enables M1 macrophages to release a variety of pro-inflammatory factors and inflict the subsequent cartilage injury observed in OA.[37,38] After knocking down Peli1 in macrophages, levels of these pro-inflammatory factors decreased markedly (Figure 5a). This suggests a promising potential treatment for cartilage protection. Next, we used a macrophage conditioned medium to treat chondrocytes. Flow cytometry showed that Peli1 knockdown could decrease apoptosis in macrophage conditioned medium-induced chondrocyte apoptosis (Figure 5b). This result was further confirmed by western blotting (Figure 5c). Moreover, our study also proved that the above results were obtained by affecting the TLR signalling pathway (Figure 5d).

Knockout of Peli1 can reduce cartilage degradation in the DMM model. To investigate the effect of Peli1 in OA in vivo, a mouse OA model was established via DMM surgery. One week after the operation, AD-SH Control or AD-SH Peli1 adenovirus was injected every week into the joints of mice in the sham operation group and DMM group. Eight weeks after surgery, HE, Safranin O/Fast green, and immunohistochemical staining were performed to evaluate the histomorphological differences between these groups. The data showed that cartilage destruction occurred in the DMM group at two months after the operation, while the knockdown of Peli1 significantly reduced cartilage degradation and OARSI grade (Figure 6, parts a to b1). At the same time, iNOS expression increased significantly in DMM + AD-SH Control group while decreased in DMM+ AD-SH Peli1 group (Figure 6c). In addition, MMP13 expression increased significantly in DMM + AD- SH Control group while decreased in DMM+ shPELI1 group (Figure 6d).

Knockout of Peli1 can reduce synovial inflammation in the DMM model. DMM surgery can also lead to synovitis of the knee joint in mice. Three features of synovitis (enlargement of lining cell layer, cellular density of synovial stroma, leukocytic infiltrate) were evaluated and each feature was graded separately. As inflammatory changes are heterogeneous by nature, analysis was done at the site showing the strongest histopathological alterations. DMM surgery thickened the inner synovial cell layer, while Peli1 knockdown lowered the thickness of the inner synovial layer (Figures 7a and 7a1). Meanwhile, we found that the infiltration of pro-inflammatory M1 macrophages (iNOS, CD86 positive cells) increased in the synovium of the DMM + AD-SH Control group, but decreased in the synovium of the DMM + AD-SH Peli1 group (Figures 7b and 7c).

Discussion

OA is characterized by partial cartilage loss, synovial sac thickening, subchondral bone sclerosis, osteophyte formation, and structural changes in surrounding joints, ligaments, and muscles.[39-42] OA is an inflammatory immune-related disease accompanied by apoptosis and necrosis of chondrocytes. Currently, we have not found effective treatment methods for OA. Previous studies reported the involvement of Peli1 in inflammatory diseases, including central nervous system inflammation.[23] Meanwhile, Peli1 has been reported to be important in immune diseases, such as systemic lupus erythematosus (SLE).[43] Peli1 is also involved in the process of apoptosis.[44] In this study, we aimed to determine the association between Peli1 and OA. We used IL-1β to induce chondrocyte inflammation and apoptosis.[45] Through the study of chondrocyte function loss and function acquisition, we found that Peli1 has a significant influence on chondrocyte apoptosis and inflammation. Moreover, we found that Peli1 could significantly affect IL-6, TNF-α, MMP-3, aggrecan, and COL-2, indicating that Peli1 is of vital importance in cartilage degeneration.

TLR signalling pathway is very important in the pathogenesis of OA.[46,47] Researchers concluded that Peli1 promotes TLR signalling and proinflammatory cytokine production.[24] However, how Peli1 regulates TLR signalling has not been studied in chondrocytes. In our study, we found that in IL-1 β stimulated chondrocytes, Peli1 knockdown could reduce TRAF6 levels, inhibit the phosphorylation of RIP, and finally act on the NF-κB signalling pathway, resulting in anti-inflammatory and anti-apoptotic effects. However, other targets of the TLR signalling pathway need to be further studied.

There is increasing evidence that macrophages are essential cells in the progression of OA. M1 macrophages secrete a lot of pro-inflammatory cytokines, such as IL-1β, IL-6, and TNF-1α, worsening synovitis.[12,48] Enhanced inflammation promotes the accumulation of chondrocyte destructive enzyme MMP, leading to the degradation of extracellular matrix.[49,50] Therefore, macrophages can be regarded as new targets in OA treatment.

Pro-inflammatory cytokines by M1 macrophages includes IL-1 β, IL-6, TNF-α, IL-12, ROS, and iNOS, promoting the inflammatory process of OA.[11] Studies have found that Peli1 is closely linked to the M1 polarization of macrophages.[26,51] Our study found that LPS activated M1 macrophages by enhancing the phosphorylation of RIP and inducing the expression of M1 phenotypic markers, such as CD14, CD80, IL-6, TNF–α, and iNOS. However, knockdown of Peli1 significantly inhibited this pathway and reduced the secretion of pro-inflammatory cytokines by macrophages.

Therefore, the supernatant of macrophages obtained after knockdown of Peli1 can have anti-inflammatory and anti-apoptosis effects on chondrocytes.

We also found similar results in animal experiments: compared with DMM + AD-SH Control group, cartilage destruction and synovitis in DMM + AD-SH Peli1 group were decreased. This further proved that Peli1 is of vital importance in the regulation of OA.

OA is a complicated disease involving many types of cells. Most studies are limited by focusing on one cell type; in our study, we explored the effect of Peli1 on OA by studying its effect on macrophages and chondrocytes, and tried to explore OA pathogenesis from a larger perspective. However, there is still insufficient research on the interaction between macrophages and chondrocytes, which is also the direction of our future exploration.

In conclusion, our study determined that Peli1 plays an important role in the treatment of OA. Peli1 knockdown inhibited TLR and NF-κB signalling pathways to protect chondrocytes. At the same time, Peli1 knockdown attenuated the release of IL-1β, IL-6, and TNF-α by pro-inflammatory macrophages, thus protecting chondrocytes. Together, the results suggest that Peli1 may become a novel target for OA.

Supplementary material

Figures displaying the knockout efficiency of Pellino1 in chondrocytes and macrophages, and tumour necrosis factor receptor-associated factor 6 and receptor-interacting protein in chondrocytes, by reverse transcription quantitative polymerase chain reaction. An ARRIVE checklist is also included to show that the ARRIVE guidelines were adhered to in this study.

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