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Suppression of experimental arthritis with self-assembling glycol-split heparin nanoparticles via inhibition of TLR4–NF-κB signaling

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Keywords

Heparin nanoparticles, Arthritis, Toll-like receptor 4, NF-κB, Inflammation

Abstract

It has been recently shown that Toll-like receptor4 mediated nuclear factor κB (TLR4–NF-κB) signaling plays a critical role in the pathogenesis of rheumatoid arthritis mediated by pro-inflammatory cytokines in arthritic synovium. Here we evaluate the therapeutic potential of glycol-split non-anticoagulant heparin/D-erythro-sphingosine nanoparticles (NAHNPs), which have shown strong inhibitory effect against TLR4 induced inflammation, in an experimental arthritis model. NAHNP significantly inhibited the production of pro-inflammatory cytokines such as TNF-α, IL-6 and IL-1β in lipopolysaccharide (LPS)-induced primary mouse macrophages and DC2.4 dendritic cell line. The nanoparticles were administered to type II collagen-induced arthritis (CIA) mice by intraarticular injections once per day starting from onset of the disease symptoms. Treatment with NAHNP had a potent suppressive effect in CIA mice, observed with a decrease in arthritis score and footpad swelling. The animals treated with NAHNP significantly reduced levels of IgG1 and IgG2a antibodies against bovine type II collagen. Levels of proinflammatory cytokines — e.g., TNF-α, IL-6 and IL-1β in knee joints and sera were significantly inhibited compared to control mice. Moreover, nuclear localization of RelA in knee joints was significantly inhibited in NAHNP treatment, indicating down-regulation of the NF-κB signaling pathway. In addition, histological examination revealed significant suppression of inflammatory cell infiltration, joint destruction and synovial proliferation in synovium compared with control mice. These results suggest that selective inhibition of TLR4–NF-κB signaling with lipid modified heparin derivatives composited to nanostructures provides an effective therapeutic approach to inhibit chronic inflammation in an animal model of rheumatoid arthritis.

1. Introduction

The involvement of toll-like receptors (TLRs) in the pathogenesis of rheumatoid arthritis is supported by an increasing number of studies [1–7]. Notably, expression of TLR4 is highly increased in the synovium of rheumatoid arthritis patients [8, 9] and TLR4 mutant mice are protected from experimental arthritis [10–12]. It is thought that extracellular endogenous ligands present in the arthritic joints activate TLR4 and contribute to maintaining inflammation [2, 3, 6, 13–15]. Recently, it was demonstrated that during arthritis, immune complexes containing citrullinated proteins greatly increase inflammation through a myeloid differentiation factor 88 (MyD88)-dependent pathway via TLR4 and activated Fcγ receptors [16]. Signaling activated by TLR4 ligands induces proinflammatory cytokine expression from TLR4-overexpressing cells such as macrophages, dendritic cells and fibroblasts in arthritic synovium [17]. Furthermore, TLR4 expressed on CD4+ T cells promotes autoimmune inflammation [18]. The generation of cytokines such as TNF-α, IL-6 and IL-1β regulated by transcription factor nuclear factor κB (NF-κB) is important in the pathogenesis of rheumatoid arthritis. Systemic inhibition of these cytokines with biologic drugs is effective as a short-term treatment but might also suppress the whole immune system and increase infection risk [19]. Evidence supports a role for TLR4 in the pathogenesis of rheumatoid arthritis, thus targeting the receptor of cell populations secreting distinct cytokines might be an effective approach to suppressing inflammation. We recently developed nanoparticles composed of heparins conjugated with D-erythro-sphingosine which blockade TNF-α production from Escherichia coli lipopolysaccharide (LPS)-induced macrophages. The conjugates form stable self-assemblies, and thus might be promising candidates for in vivo drug delivery and therapeutics [20]. Anti-inflammatory effect of heparin has been widely described in the literature although the mechanisms responsible for the effects are complex and incompletely understood [21]. The primary role of heparin as an anti-inflammatory agent was closely linked to its ability of binding and inhibiting proteins such as selectins and growth factors involved in inflammation and angiogenesis [22]. We found that unlike native heparin, heparins modified with lipids and composited to nanostructures act as selective TLR4 antagonists and have much greater anti-inflammatory activity. In vitro studies showed that glycol-split non-anticoagulant heparin/D-erythro-sphingosine nanoparticles (NAHNPs) suppressed the production of TNF-α from LPS-stimulated macrophages by inhibiting TLR4-mediated NF-κB signaling with an IC50 of 0.019 mg/mL. This suggests that the heparin nanoparticles can block the activation of TLR4-overexpressing primitive immune cells such as macrophages and dendritic cells in arthritic synovium which is a different target of heparin from that of the above-mentioned activity. In this context, the present study investigated the potential anti-inflammatory effect of NAHNPs in the collagen-induced arthritis model. First, we tested the inhibitory potency of NAHNPs on the production of proinflammatory cytokines from LPS-stimulated macrophages and dendritic cells. Next, we studied the therapeutic activity of these nanoparticles in a mouse model of

collagen-induced arthritis (CIA). We discuss these findings and potential benefits of these nanoparticles as a novel specific treatment for rheumatoid arthritis.

2. Materials and methods

2.1. Preparation of NAHNPs

Nanoparticles were prepared as described previously [20]. First, glycol-split non-anticoagulant heparin (NAH) was obtained by periodate-oxidation and borohydride-reduction of heparin (Product ID X1009996-8, Mw 14 kDa, Nacalai Tesque, Inc., Kyoto, Japan) according to the method of Conrad and Guo [23]. NAH (0.1 g) then was dissolved in formamide (5 mL) with 40 mg 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide followed by the addition of 5mL dimethyl formamide containing 5 mg D-erythro-sphingosine (LKT Laboratories, Inc., St. Paul, MN). The reaction mixture was stirred at room temperature for 24 h under a nitrogen atmosphere. Afterwards, the reaction mixture was precipitated in pure ethanol, centrifuged at 14,000 ×g for 10 min followed by decantation. This was repeated 3 times to remove any remaining unreacted D-erythro-sphingosine and reaction solvents. Precipitates were then dried in a vacuum and lyophilized as NAHNP. Nanoparticles were prepared by ultrasonication of NAHNP using a probe sonicator (UD-201, Tomy Seiko Co. Ltd., Japan) for 1min at 20 kHz and 50 W/cm2 in distilled water. For comparison, heparin was used without oxidative ring-opening reactions to synthesize native heparin nanoparticles (HPNPs). We used NAHNP and HPNP having the degree of D-erythro-sphingosine substitution of 4.2% and 6.3%, respectively, to keep their particle sizes comparable with each other (~150 nm) [20]. It should be noted that the particle size (or degree of D-erythro-sphingosine substitution) is not a critical determinant in anti-inflammatory effect in LPS-stimulated peritoneal-elicited macrophages [20].

2.2. Cell culturing and in vitro assay for cytokines

Mouse peritoneum-derived macrophages were received as described previously [24]. Briefly, macrophages were harvested from 5-week-old female ICR mice 4 days after intraperitoneal injection of 1 mL 2.9% thioglycolate medium (Nissui Pharmaceutical, Tokyo, Japan). Cells were washed and cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) (Flow Laboratories, Irvine, UK), penicillin G (100 U/mL), and streptomycin (100 μg/mL), and then seeded on 24-well plates (Becton Dickinson, Lincoln Park, NJ) at a density of 1.5 × 105 cells per well. Non-adherent cells were washed with phosphate buffered saline (PBS) after incubation for 2 h at 37 °C in a humidified atmosphere with 5% CO2. The remaining cells were cultured for 24, 48 or 72 h. After replacing the media with Opti-MEM® I Reduced Serum Medium (Gibco®, Life Technologies, Rockville, MD) cells were stimulated with LPS (Sigma-Aldrich, St Louis, MO) at final concentration of 20 ng/mL with or without

nanoparticles at final concentration of 0.5mg/mL and incubated for 24 h. The supernatants were assayed for cytokines. DC2.4 cells, a murine dendritic cell line, were grown in complete media RPMI 1640 as described above and were maintained at 37 °C in a humidified incubator with 5% CO2. Cells were maintained via weekly passage and utilized for experimentation at 80–90% confluency. Cells were stimulated with LPS (20 ng/mL) for 24 h in Opti-MEM® I Reduced Serum Medium with or without nanoparticles (0.5 mg/mL) and the supernatants were assayed for cytokines. Cytokines were quantified using mouse TNF-α, IL-6 and IL-1β ELISA kits (eBioscience, Inc., San Diego, CA) following the manufacturer's instructions. 2.3. Induction and assessment of arthritis Mice were obtained from Shizuoka Agricultural Cooperative Association for Laboratory Animals (Shizuoka, Japan). All animal experiments were performed in accordance with the Principles of Laboratory Animal Care as directed by the U.S. National Institutes of Health and the Guidelines for Animal Experiments of Kyoto University. Bovine type II collagen (4 mg/mL final concentration) (Elastin Products Co., Owensville, MO) was dissolved in 0.1 M acetic acid at 4 °C overnight and then emulsified with an equal volume of complete Freund's adjuvant containing 4 mg/mL Mycobacterium tuberculosis. Male 8-week-old DBA/1J mice were immunized subcutaneously at the base of the tail with 100 μL of the emulsion. At day 21 from primary immunization, mice were boosted intraperitoneally with 100 μL of bovine type II collagen emulsion prepared with incomplete Freund's adjuvant. NAHNP (20 mg/kg per animal) was administered by intraarticular injections to both knees once per day starting from day 28 until day 50 from primary immunization. Arthritis was assessed blindly using four paws per mouse using the following scores: 0, normal (no swelling); 1, mild/moderate erythema and swelling of paws and/or multiple digits; 2, swelling and severe erythema; and 3, swelling of all joints and ankylosis. Paw swelling was assessed by measuring the thickness of affected paws with calipers. Tissue TNF-α, IL-6 and IL-1β levels were measured using ELISA kits (eBioscience, Inc., San Diego, CA). Serum cytokines were measured using a flow cytometric bead array mouse inflammation kit (BD Biosciences, San Jose, CA).

2.4. Determination of anti-type II collagen immunoglobulins

Serum samples were stored in aliquots at −80 °C until they were used for ELISA assay. Ninety-six-well immunoplates (Nunc® Maxisorp, Thermo Scientific, Roskilde, Denmark) were coated overnight with 5 μg/mL of bovine type II collagen in PBS at 4 °C. Nonspecific binding was blocked with 2% of bovine serum albumin (BSA) in PBS at room temperature for 2 h. Diluted serum samples (in 0.2% BSA in PBS containing 0.05% Tween-20) were added and incubated for 2 h at room temperature. Serum dilutions were chosen after preliminary assays and ranged from 1:500 to 1:20,000 for IgG1, and from 1:5000 to 1:50,000 for IgG2a. The plates were then incubated with horseradish peroxidase-conjugated goat anti-mouse IgG1 or IgG2a (Abcam Inc., Cambridge, MA) for 1 h. 3,3′,5,5′-Tetramethylbenzidine

solution was used as a substrate and the optical density was measured using an ELISA reader (Eon Microplate Spectrophotometer, Biotek Instruments, Winooski, VT) at 450 nm. All incubations were made with a volume of 100 mL/well. Plates were washed 3 times with 250 mL PBS containing 0.05% Tween-20 between steps. The anti-type II collagen concentrations were determined by reference to standard curves generated using murine anti-bovine type II collagen IgG1 and IgG2a (Chondrex, Inc., Redmond, WA).

2.5. NF-κB activation assay

Nuclear RelA levels were measured using a TransAM® NF-κB p65 kit (Active Motif, Carlsbad, CA). Knee joints were dissected and cleaned from soft tissues. The tissue was homogenized in cold phosphate buffered saline (0.5 g tissue in 1 mL) and centrifuged at 14,000 ×g for 3min at 4 °C. The supernatant was then collected for analysis of cytokine levels. The remaining pellet was used for preparation of nuclear extracts using a TransAM® Nuclear Extract Kit (Active Motif, Carlsbad, CA) following the manufacturer's instructions. The samples were stored at −80 °C until analyzed. 2.6. Histology of knee joints for histological analysis, mice were sacrificed by cervical dislocation at day 50 after first immunization. Knee tissues were randomly collected, then fixed with 10% paraformaldehyde, decalcified in 5% formic acid and embedded in paraffin. Next, 7 μm sections were stained with hematoxylin and eosin (H&E). Histopathological changes were scored using the following parameters: inflammation — 0 (normal) to 4 (severe inflammation with necrosis and edema), and joint destruction—0 (normal) to 4 (severe extensive areas of cartilage ulcerations).

2.7. Statistics

GraphPad Prism 5.0 (GraphPad Software, Inc.) was used to perform statistical tests. Experiments involving two groups were analyzed by two-tailed, unpaired t test. Statistical analysis of in vivo data was performed by one-way ANOVA. P b 0.05 was considered statistically significant. Data are presented as means ± SEM for each group.

3. Results

3.1. Inhibition of LPS-induced production of cytokines from macrophages and dendritic cells

We recently demonstrated that NAHNP suppresses TNFα production in primary cultured mouse macrophages. Specifically, nanoparticles prepared from glycol-split heparin were more potent than those of prepared from native heparin in vitro [20]. Here, NAHNP was evaluated for its potential to inhibit IL-6 and IL-1β production from macrophages following LPS stimulation. Cells were challenged to LPS with or without NAHNP and supernatants quantified for cytokines. As expected, the nanoparticles significantly inhibited IL-6 and IL-1β levels (Fig. 1A). In a separate experiment, removal of NAHNP from the medium after 1-h

pretreatment but prior to LPS stimulation also resulted in significant suppression of cytokines after additional 24-h LPS exposure (data not shown). Besides macrophages, dendritic cells activated via TLR4 produce a variety of cytokines such as TNF-α, IL-6, and IL-1β. Therefore, we evaluated the anti-inflammatory effect of NAHNP using DC2.4 cells, a murine dendritic cell line. DC2.4 cells were stimulated with LPS and simultaneously treated with NAHNP. The addition of LPS resulted in increased production of TNF-α, IL-6 and IL-1β. NAHNP blocked the production of all cytokines tested (Fig. 1B). When not stimulated with LPS but only treated with NAHNP (0.5 mg/mL), neither macrophage nor DC2.4 cells produce TNF-α, IL-6 or IL-1β above their basal levels (data not shown).

3.2. Inhibition of arthritis

Mice immunized with bovine type II collagen (CII) to induce CIA received daily intraarticular injections of NAHNP (20 mg/kg in total to both knees) from disease onset (day 28). Nanoparticles prepared with native heparin/D-erythro-sphingosine conjugates (HPNP) were used for comparison. Long-term administration of HPNP ameliorated arthritis in some mice, which was not statistically significant compared to the CIA group (Fig. 2A), and induced knee hemorrhoids due to anticoagulation. NAHNP significantly (P b 0.05 compared with the CIA group) inhibited the development of arthritis (Fig. 2A) and reduced paw swelling (P b 0.01 compared with the CIA group) (Fig. 2B). 2-[(Amino-carbonyl) amino]-5-(4-fluorophenyl)-3-thiophene-carboxamide (TPCA-1), a selective IκB kinase 2 inhibitor, was applied as a positive control (20 mg/kg intraperitoneally [25]) (Fig. 2C). Quantification of major proinflammatory cytokines in knee tissue on days 35 and 45 revealed that TNF-α, IL-6 and IL-1β were markedly decreased (Fig. 3A). Nuclear localization of RelA in knee tissue extracts was significantly inhibited in NAHNP treated mice compared to controls (Fig. 3B). Synchronously with tissue cytokines, NAHNP decreased sera TNF-α, IL-6 and IL-1β levels on days 35 and 50 (Fig. 4). Humoral responses against CII were monitored throughout the study. In contrast to marked auto-antibody synthesis in CIA control mice, surprisingly, NAHNP-treated mice showed a rapid decrease of antibody titers from the first day of treatment (Fig. 5A and B) indicating a systemic effect despite local administration. The effect of NAHNP treatment on joint destruction was further confirmed with histological analysis of knee joints. Joints from NAHNP-treated mice showed a remarkable improvement in inflammatory cell infiltration, joint destruction and synovial proliferation compared with CIA controls (Fig. 6). Thus, the mechanism underlying NAHNP-induced suppression of CIA was likely to involve the complex inhibition of production of proinflammatory cytokines, i.e., TNF-α, IL-6 and IL-1β, induced by the TLR4-mediated NF-κB pathway and suppression of humoral responses during arthritis.

4. Discussion

We previously developed nanoparticles self-assembled from the amphiphilic conjugates of glycol-split non-anticoagulant heparin/D-erythro-sphingosine (NAHNP) and analyzed their stability for further in vivo drug targeting [20]. The nanoparticles ranged from110 to 160 nm. Interestingly, we found that these nanoparticles effectively blocked LPS-stimulated TNF-α production from macrophages in vitro. The anti-inflammatory effect was mediated through inhibition of the TLR4–NF-κB signaling pathway. The present study was initiated to examine the anti-arthritic activity of NAHNP, since inhibition of pro-inflammatory cytokines and transcription factors activated during inflammation is essential for effective disease-modifying anti-rheumatic drugs [19]. In vitro studies showed that NAHNP blocks the production pro-inflammatory cytokines from LPS-stimulated macrophages and dendritic cells by inhibiting TLR4-mediated NF-κB signaling. In rheumatoid arthritis, macrophages and dendritic cells overproduce proinflammatory cytokines, mainly TNF-α, IL-1β and IL-6, which is a pivotal event leading to chronic inflammation [4, 26]. These cytokines are controlled by a transcription factor NF-κB [27]. Analysis of nuclear extracts from arthritic synovial tissue revealed the presence of increased NF-κB DNA binding activity [28, 29].We found that NAHNP significantly inhibited most pro-inflammatory cytokines, including TNF-α, IL-1β and IL-6 in vivo. Broad-spectrum cytokine inhibition successfully reduced disease severity and cartilage destruction in CIA. Furthermore, NAHNP targeted TLR4 to suppress the nuclear localization of RelA in vivo. It was demonstrated that arthritis severity is associated with high humoral responses to antigens [30, 31]. Antigen-auto-antibody complexes induce cytokine production by macrophages through the synergistic activation of TLR4 and Fcγ receptors [16, 32]. Measurement of circulating autoantibodies in treated mice revealed that NAHNP inhibited IgG1 and IgG2a antibody isotypes. Our data supports the notion that antigen-immune complex-mediated inflammation in rheumatoid arthritis may be promoted by augmented specific signaling pathways via innate immune receptors such as TLR4–NF-κB [7, 16, 32–34]. Drug delivery systems such as liposomes [35–37], albumin microspheres [38], gelatin/chondroitin 6-sulfate [39] and polylactic acid/polyglycolic acid copolymer particles [40, 41] have been developed to inject into the articular joints. These particulate carriers are assumed to stay for long in the articular cavity and increase drug residence time following the intra-articular administration [42, 43]. It has also been known that particles can penetrate into the cartilage (b50 nm) [44] or be phagocytosed by macrophages [42] depending on their size. Heparin nanoparticles having a size of ~150 nm are supposed to reside long-term in the cavity as other particles can basically do; however, detailed pharmacokinetic investigation will be needed to clarify the distribution of these particles.

5. Conclusion

In conclusion, we show that hydrophobically modified glycol-split heparin nanoparticles exhibit anti-inflammatory effects and suppress an animal model of CIA. This effect is mediated through the inhibition of the TLR4–NF-κB signaling. Pharmacological activity of the nanoparticles was associated with suppression of complex pro-inflammatory cytokines in joints and sera, as well as suppressed levels of autoantibodies. Thus, selective inhibition of TLR4–NF-κB signaling with NAHNP provides an effective therapeutic approach to inhibit chronic inflammation in an animal model of rheumatoid arthritis. However, additional pharmacological and toxicological studies are required to evaluate whether the nanoparticles can be used in clinical settings.

Acknowledgments

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Conflict of interest

Authors declare no conflict of interest.

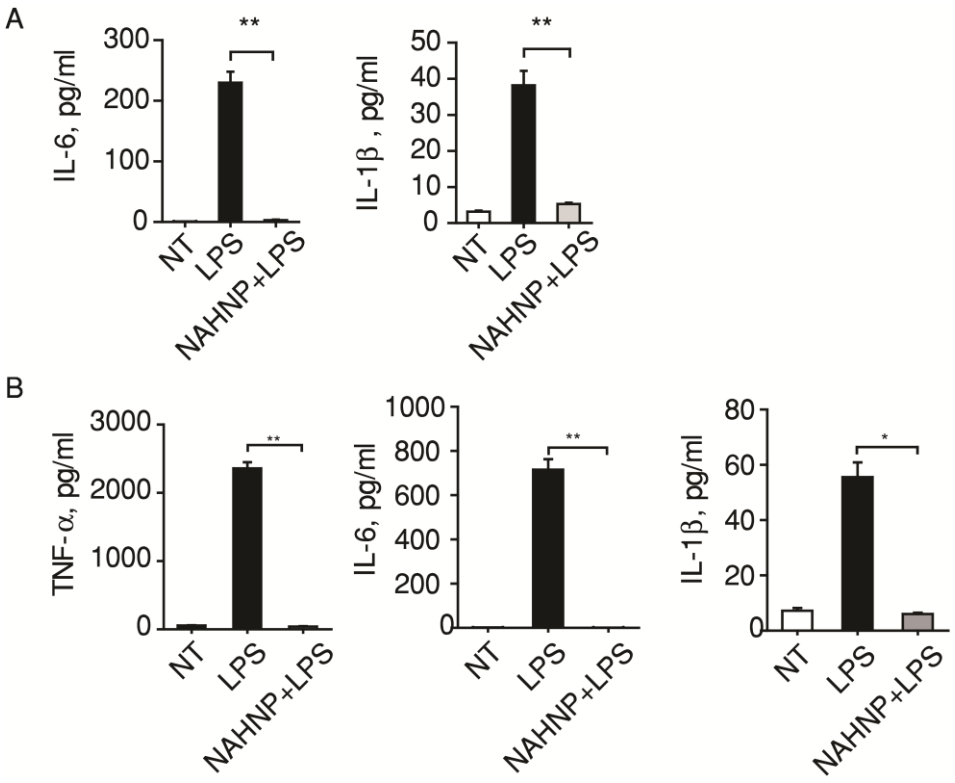

Figure 1 Effect of NAHNP on LPS-induced production of proinflammatory cytokines by primary macrophages and DC2.4 cells.

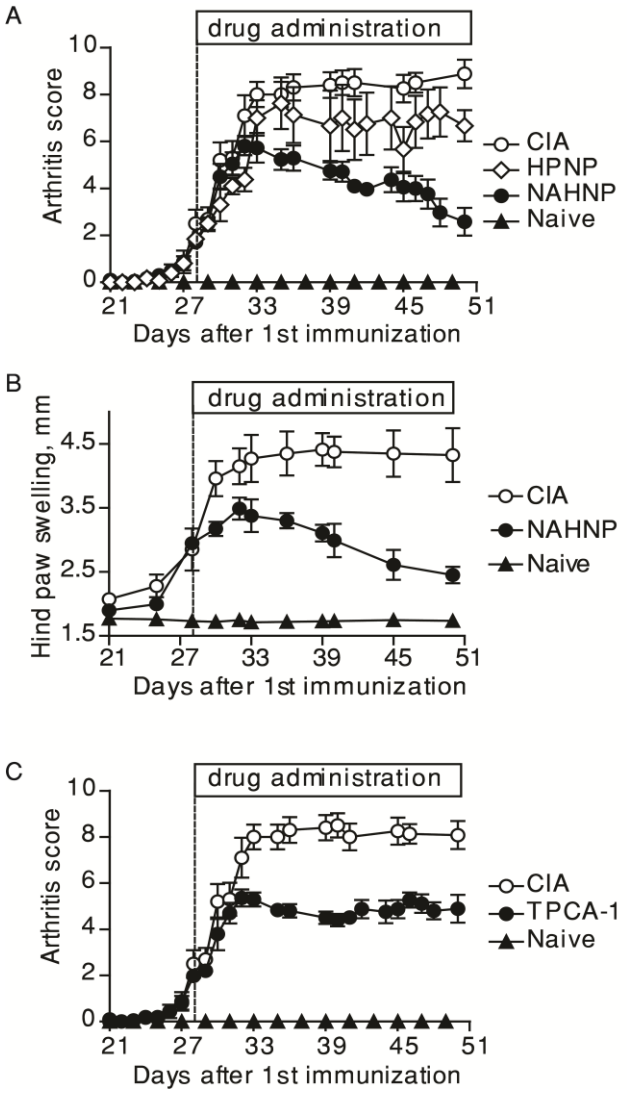

Figure 2 Therapeutic effect of NAHNP on CIA.

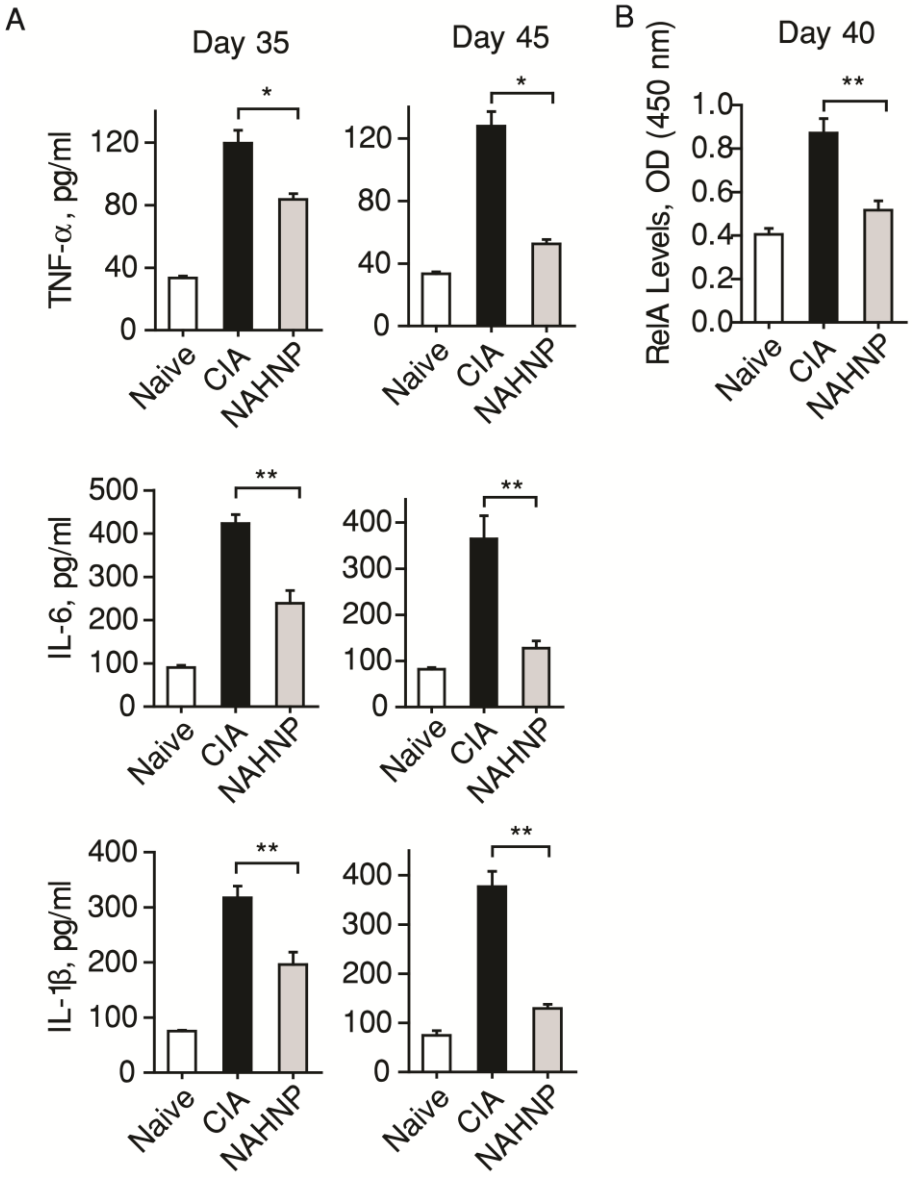

Figure 3 Effect of NAHNP on NF-κB activity and proinflammatory cytokine accumulation in knee tissue.

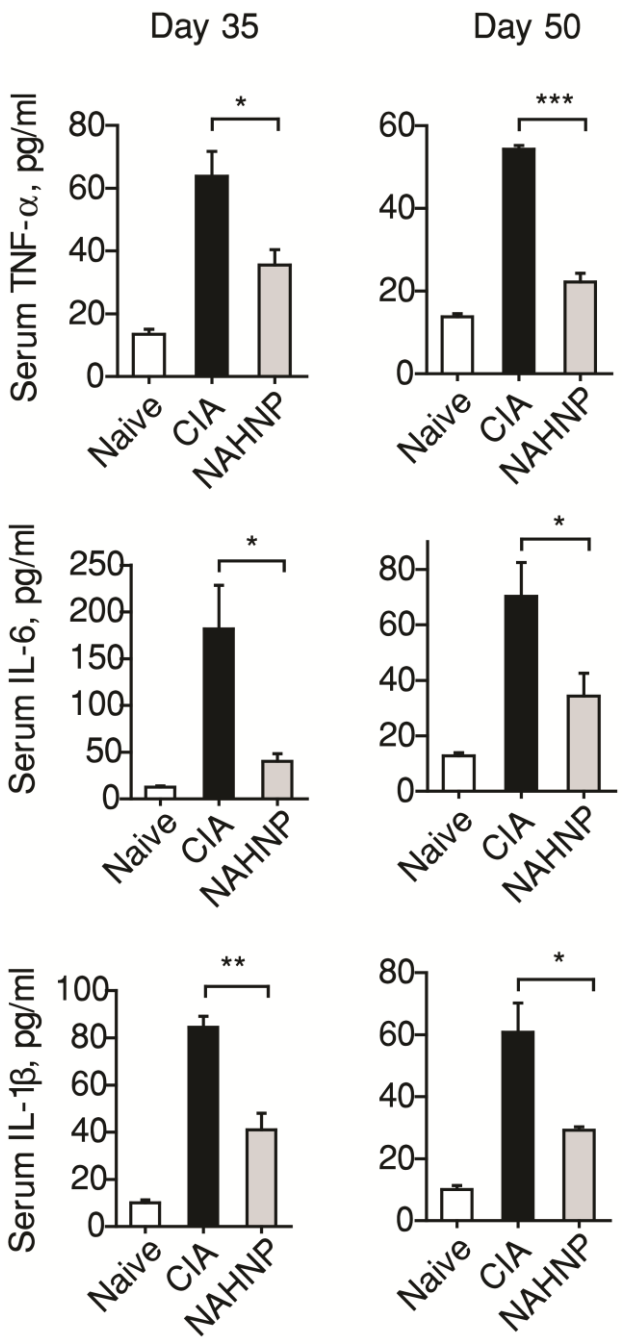

Figure 4 Effect of NAHNP on serum TNF-α, IL-6 and IL-1β on days 35 and 50 (n=8).

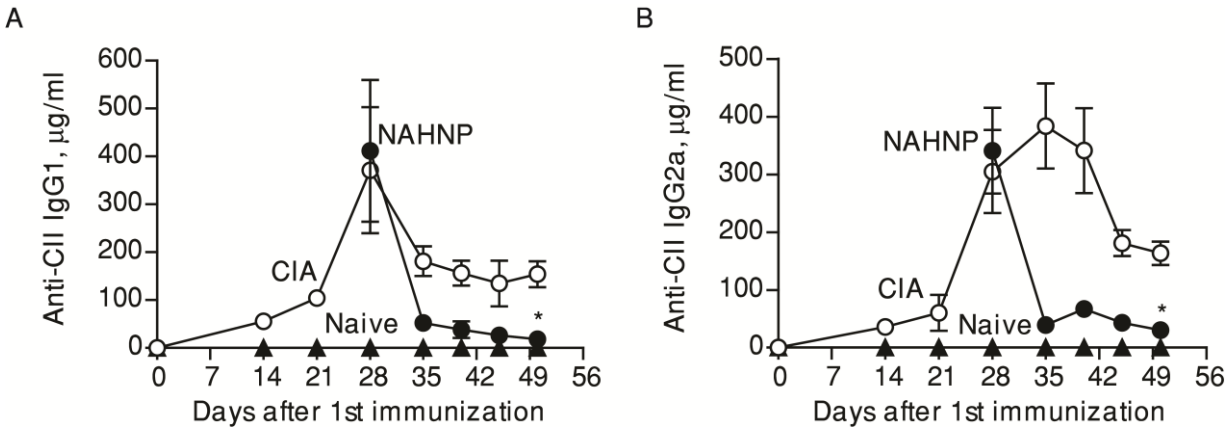

Figure 5 Effect of NAHNP on serum anti-collagen type II autoantibody isotypes IgG1 (A) and IgG2a (B) (n=10per group).

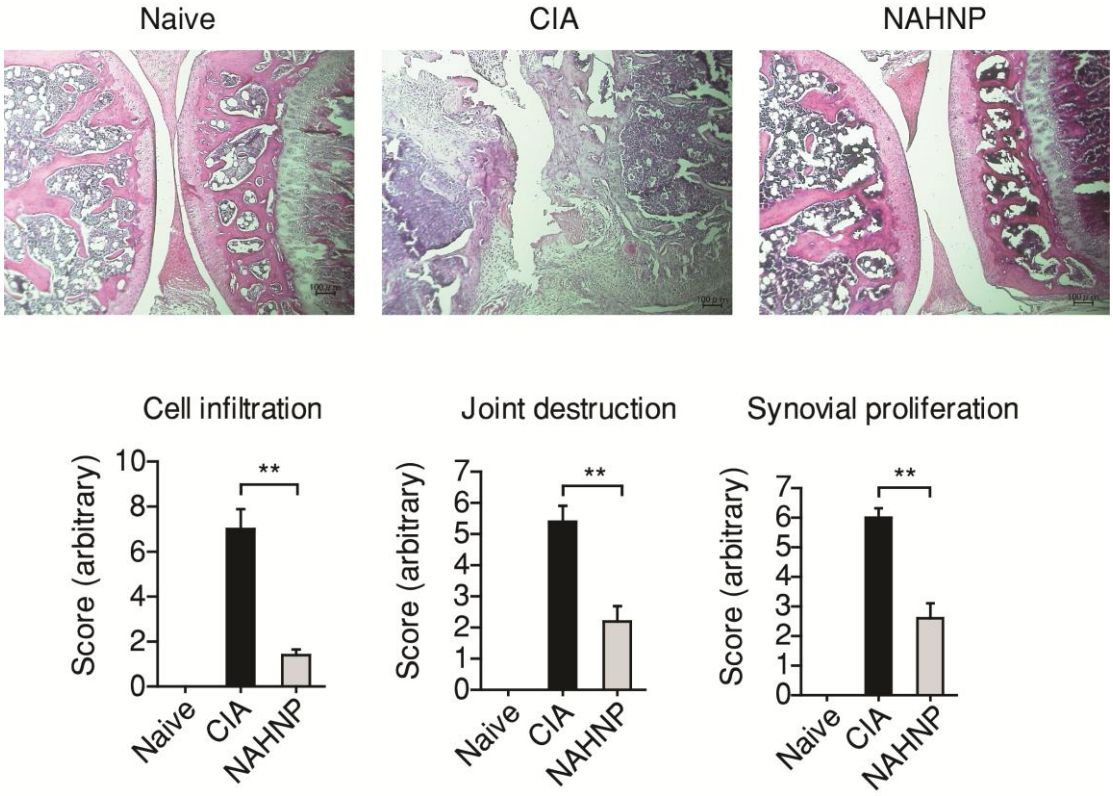

Figure 6 Effect of NAHNP on pathology of CIA mouse knee tissues.

Figure 1 Effect of NAHNP on LPS-induced production of proinflammatory cytokines by primary macrophages and DC2.4 cells. (A) Quantification of IL-6 and IL-1β from peritoneal macrophages isolated from ICR mice. Cells (1.5×10^5 cells per well) were seeded to 24-well-plates and were not treated (NT) or stimulated with LPS (20 ng/mL) for 24 h in the presence or absence of NAHNP (0.5 mg/mL). (B)DCs (1.5×10^5 cells per well) were seeded to 24-well-plates and were treated with 0.5 mg/mL of NAHNP and/or 20 ng/mL LPS. Supernatants were collected after 24 h and assayed for cytokines production. Data are shown as mean ±SEM (n=3). * $P\leq 0.05$, ** $P \leq 0.01$.

Figure 2 Therapeutic effect of NAHNP on CIA. Artistic severity score (A) and hind paw swelling (B) of male DBA/1J mice treated with NAHNP (20 mg/kg per animal) and HPNP (20 mg/kg per animal) by intraarticular injections to both knees once per day between days 28 and 50 (n=10). CIA-induced mice treated with saline. (C) Artistic severity score of mice treated with TPCA-1 (20 mg/kg) (n=8). Data represents mean ±SEM.

Figure 3 Effect of NAHNP on NF-κB activity and proinflammatory cytokine accumulation in knee tissue. Mice were killed at indicated days and knee tissues from naïve, CIA and NAHNP treated mice were dissected and homogenized. (A) Supernatants were analyzed for cytokines by ELISA (n=8). (B) Nuclear extracts were assayed for localization of RelA (2 knees per animal pooled, n=8 animals). Data represents mean ±SEM. * $P\leq 0.05$, ** $P\leq 0.01$.

Figure 4 Effect of NAHNP on serum TNF-α, IL-6 and IL-1β on days 35 and 50 (n=8). Serum samples from naïve, CIA and NAHNP treated mice were collected and assayed for cytokines as described in Methods. Data represents mean ±SEM. * $P\leq 0.05$, ** $P\leq 0.01$, *** $P\leq 0.001$.

Figure 5 Effect of NAHNP on serum anti-collagen type II autoantibody isotypes IgG1 (A) and IgG2a (B) (n=10per group). Data represents mean ±SEM. * $P\leq 0.01$ versus CIA group.

Figure 6 Effect of NAHNP on pathology of CIA mouse knee tissues. Top: H&E sections of normal joints (left) and joints of untreated (middle) or (right) NAHNP (20 mg/kg) treated CIA mice(day 50). Bottom: cell infiltration, joint destruction and synovial proliferation scores. Data represents mean ± SEM (n=5).

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