

GDF11 antagonizes TNF- α -induced inflammation and protects against the development of inflammatory arthritis in mice

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ABSTRACT: Growth differentiation factor 11 (GDF11), a key member of the TGF- β superfamily, plays critical roles in various medical conditions. Recently, GDF11 was found to suppress the nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) signaling pathway and protect against inflammation. This study aimed to investigate the role of GDF11 in the development of rheumatoid arthritis (RA). We demonstrated that GDF11 treatment antagonized TNF- α -induced inflammation in macrophages. Moreover, GDF11 inhibited the development of arthritis in the collagen-induced arthritis and collagen antibody-induced arthritis models. Local gene transfer of GDF11 *via* adeno-associated virus exerted therapeutic effects, while local knockdown of GDF11 exaggerated inflammation in our collagen-induced arthritis model, as detected by expression levels of inflammatory biomarkers and the destruction of joint structures. Additionally, the results from both *in vitro* experiments and luciferase reporter gene mouse experiments implied that the NF- κ B pathway might play a critical role in the therapeutic effect of GDF11 in RA. This study presents GDF11 as a potential target for the treatment of inflammatory arthritis, including RA.—Li, W., Wang, W., Liu, L., Qu, R., Chen, X., Qiu, C., Li, J., Hayball, J., Liu, L., Chen, J., Wang, X., Pan, X., Zhao, Y. GDF11 antagonizes TNF- α -induced inflammation and protects against the development of inflammatory arthritis in mice. FASEB J. 33, 000–000 (2019). www.fasebj.org

KEY WORDS: rheumatoid arthritis • NF- κ B signaling pathway • synovitis • growth differentiation factor 11

Growth differentiation factor 11 (GDF11) is a key member of the bone morphogenetic protein subfamily of the TGF- β superfamily (1, 2). These proteins play critical roles in embryonic development, skeletal metabolism, and muscle

formation (3–5). GDF11 has attracted considerable attention from researchers because of its contradictory relationship with aging (6–9). For example, Poggioli *et al.* (10) reported that the administration of recombinant GDF11 (rGDF11) reversed cardiac hypertrophy in aging mice. Furthermore, GDF11 promoted the recovery of renal and cardiac function in elderly mice (11). Recent research suggested that GDF11 might be associated with inflammatory reactions (12). However, whether GDF11 is involved in inflammatory arthritis and the underlying mechanisms remains to be determined.

Rheumatoid arthritis (RA) is a chronic systemic autoimmune disease characterized by pain, swelling, inflammation of the synovial joints, and progressive cartilage damage (13). One percent of the global population has RA and experiences joint destruction, pain, and loss of function (14). Although the nosogenesis and underlying mechanisms of this disease are still not fully understood, it is accepted that inflammatory cytokines are critical for the induction of damage (13). Recently, the attenuation of inflammatory reactions has become a focus of research

ABBREVIATIONS: μ CT, micro-computed tomography; AAV, adeno-associated virus; BMDM, bone marrow-derived macrophage; CAIA, collagen antibody-induced arthritis; CIA, collagen-induced arthritis; COX-2, cyclooxygenase 2; GDF11, growth differentiation factor 11; GFP, green fluorescent protein; HE, hematoxylin and eosin; MMP-3, matrix metalloproteinase 3; NF- κ B, nuclear factor kappa-light-chain-enhancer of activated B cells; RA, rheumatoid arthritis; rGDF11, recombinant growth differentiation factor 11; si, small interfering; TNFR, tumor necrosis factor receptor; TRAP, tartrate-resistant acidic phosphatase; WT, wild type

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within the field of RA (15). It is widely believed that TNF- α plays an important role in the pathogenesis of inflammatory arthritis (15). The collagen-induced arthritis (CIA) mouse model and the collagen antibody-induced arthritis (CAIA) mouse model are often used in RA research (16, 17), and macrophages can be investigated in these models (18, 19).

Gene therapy is thought to be a potential method for targeted therapy (20), such as gene therapy using virus vectors (21, 22). Because of the safety and selectivity of adeno-associated virus (AAV), AAV was chosen as a carrier to investigate the function of various molecules in animal models (23). This virus has been used for the local delivery of GDF11 as an AAV vector (AAV-GDF11) to protect against atherosclerosis in mice (12). However, it is still unknown whether AAV-GDF11 affects inflammatory arthritis.

The purpose of this study was to determine whether GDF11 can antagonize the inflammatory response associated with RA and exert therapeutic effects on joint destruction; to study the underlying mechanisms involved; and to analyze the effects of local GDF11 knockdown or transgene expression mediated by AAV.

MATERIALS AND METHODS

Mice

Twelve-week-old male DBA/1J mice purchased from Vital River Laboratory Animal Technology (Beijing, China) were raised in accordance with institutional guidelines and with the approval of the Institutional Animal Care and Use Committee of Shandong University. Ten-week-old male wild-type (WT) BALB/c mice and BALB/c mice with nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) genetically engineered to express the firefly luciferase gene (NF- κ B-RE-luc) were provided by the National Resource Center of Model Mice (Nanjing, China) and maintained by the Institutional Animal Care and Use Committee of Shandong University. All animal experiments were approved by the Shandong University Animal Care and Use Committee (Shandong, China).

Generation and characterization of AAV

AAV9-GDF11, which induces elevated expression of the target protein, was produced and purified, and the vector genome titer was determined by GeneChem (Shanghai, China). The structure of the GV143 vector is shown in Supplemental Fig. S1A; the gene order was detected as described in Supplemental Table S1. Three GDF11 small interfering (si) RNA molecules were used to knock down GDF11 expression in 293T cells; their sequences are presented in Supplemental Table S2. After a Western blot assay, the most effective vector (61354-1) was selected to construct the AAV9-siRNA. The selected vector was GV478, whose structure was U6-MCS-CAG-EGFP, as shown in Supplemental Fig. S1B.

AAV injection and CIA model

The CIA model was constructed as previously described (13, 24, 25). First, bovine type II collagen (Chondrex, Seattle, WA, USA), and an equal volume of complete Freund adjuvant (MilliporeSigma, Burlington, MA, USA) was mixed well on ice to prepare an emulsion. Ten-week-old male DBA/1J mice were

divided into 4 groups ($n = 7$, 14 legs per group), as follows: AAV9-GDF11, AAV9-siGDF11, AAV9-GFP, and control groups. The AAV9-GDF11, AAV9-siGDF11, and AAV9-GFP groups were injected with AAV9-GDF11, AAV9-siGDF11, and AAV9-GFP, respectively, in the ankle joint and paw before CIA was established. After 14 d, on d 0 of model induction, the 3 groups were injected at the base of the tail with 200 μ l of the prepared emulsion. At the same time, the control group was injected an equal volume of PBS using the same methods. To initiate a secondary immune response, a second injection of vector was administered on d 21.

CIA model and rGDF11 injection

In the next step, 18 DBA/1J mice were divided into 3 groups ($n = 6$): a control group, a PBS group, and an rGDF11 treatment group. In the PBS group and the rGDF11 treatment group, primary immunization was performed as previously described, while the mice in the rGDF11 treatment group were injected with recombinant GDF11 (0.1 mg/kg) (PeproTech, Rocky Hill, NJ, USA) every 2 d. At the same time, the control group and the PBS group were injected with an equal volume of PBS using the same methods. A secondary immune response was induced in the PBS group and rGDF11 treatment group after 21 d, as previously described, while the control group was treated with PBS alone. At this time clinical arthritis scores were evaluated using a graded scale, as previously described (15). On d 42, the mice were anesthetized, and serum and tissues were collected for analysis.

CAIA model and recombinant rGDF11 injection

As described in the protocol of the CAIA kit, the mice in the PBS group and the rGDF11 treatment group were injected with a 150 μ l (1.5 mg) cocktail of collagen type 2 antibodies (Chondrex, Redwood, WA, USA) *via* the tail vein (26). At the same time, the mice in the control group were injected with an equal volume of PBS. On d 3, the mice in the PBS group and the GDF11 treatment group received an intraperitoneal injection of LPS (10 μ g), while the mice in the control group were injected with an equal volume of PBS. Then the mice in the treatment group were injected with recombinant GDF11 (100 μ g/kg) (PeproTech) every 2 d from d 3 to d 8. At the same time, the control group and PBS group were injected with an equal volume of PBS using the same methods.

In vivo imaging studies

After anesthesia by 10% chloral hydrate, the green fluorescent protein (GFP) signal at the injection sites in each mouse was measured using an optical imaging system (Bio-Rad, Hercules, CA, USA). For NF- κ B-RE-luc mice, the mice received an intraperitoneal injection of D-luciferin (150 mg/kg) (MilliporeSigma) after anesthesia. After 30 min, the fluorescence of the mice was measured by an optical imaging system (27).

Isolation and culture of bone marrow-derived macrophages

Bone marrow-derived macrophages (BMDMs) were isolated from WT mice as previously described (13, 15). Briefly, bone marrow cells were isolated from mouse femurs and tibias. After centrifugation at 1300 rpm for 3 min, red blood cell lysis was performed. The bone marrow cells were cultured in MEM (Gibco; Thermo Fisher Scientific, Waltham, MA, USA) containing fetal bovine serum (10%) (Gibco; Thermo Fisher), penicillin (1%) (HyClone, Logan, UT, USA), and streptomycin (1%) (HyClone).

Next, the bone marrow cells were differentiated into macrophages after stimulation with granulocyte-macrophage colony-stimulating factor (PeproTech) (40 ng/ml) for 8 d.

Nitrite production assay

The Griess assay was performed as previously described to determine whether GDF11 inhibits TNF- α -mediated inflammatory reactions in BMDMs (15). In brief, BMDMs were cultured in 96-well flat-bottom plates (1×10^5 cells per well) with 10 ng/ml TNF- α (R&D Systems, Minneapolis, MN, USA) for 72 h in the absence or presence of the indicated amounts of GDF11. The cell culture supernatants were collected and tested for NO using the Griess reaction.

Histopathologic examination of joints

After collection, paw samples were fixed in a formaldehyde solution for at least 7 d. Before paraffin embedding, the paw samples were submerged in 10% EDTA for decalcification until demineralization. Sections (5 μ m) were stained with hematoxylin and eosin (HE); the extent of synovitis, pannus formation, and bone/cartilage destruction was determined using a graded scale as previously described (15). To investigate osteoclast bone resorption activity in mice with CIA, the sections were stained using a tartrate-resistant acid phosphatase (TRAP) staining kit (Beyotime Biotechnology, Haimen, China). Then immunohistochemistry with the indicated biomarkers was carried out. Sections were pretreated with compound digestive juice (AR0022; Boster Biologic Technology, Wuhan, China) for 30 min at 37°C. Next, goat serum was used to block nonspecific proteins at room temperature for 30 min. The sections were then incubated with an anti-iNOS antibody (diluted 1:200; Santa Cruz Biotechnology, Dallas, TX, USA) and an anti-p-I κ B α antibody (1:200 dilution; Santa Cruz Biotechnology) for 1.5 h at 37°C. Detection was performed using an enhanced polymer detection system kit (Beijing Zhongshan Golden Bridge Biotechnology, Beijing, China) according to the manufacturer's instructions. The sections were observed by light microscopy, and images were collected with a digital data acquisition system (Thermo Fisher).

Real-time PCR

Total RNA was extracted from BMDMs, CIA mice, and CAIA mice in all groups with an RNeasy kit (Beyotime Biotechnology). RNA was reverse transcribed using an RT-for-PCR kit (Vazyme Biotech, Nanjing, China) according to the manufacturer's instructions. Real-time PCR was performed with SYBR Green I dye. The sequence-specific primers used in this experiment were synthesized by Sangon Biotech (Shanghai, China) and are listed in Supplemental Table S3. Each PCR product was verified by melting curve analysis, and all experiments were repeated at least 3 times.

Western blot analysis

Total protein was extracted from BMDMs and metatarsal joints from each experimental and control group using RIPA lysis buffer (Beyotime Biotechnology) mixed with 1% PMSF (Beyotime Biotechnology) and 1% protease and phosphatase inhibitor cocktail for general use (Beyotime Biotechnology). To destroy the 3-dimensional structure, the protein was heated at 100°C for 10 min with loading buffer (Thermo Fisher). Then, proteins were resolved on a 10% SDS-PAGE gel (Beyotime Biotechnology) and electroblotted onto a nitrocellulose membrane. After blocking in

5% nonfat dry milk in Tris-buffered saline with Tween 20 (10 mM Tris-HCl, pH 8.0; 150 mM NaCl; and 0.5% Tween 20), the protein blots were incubated with antibodies specific for iNOS (diluted 1:1000, ab3523; Abcam, Cambridge, United Kingdom), cyclooxygenase 2 (COX-2; diluted 1:1000; Santa Cruz Biotechnology), p-I κ B α (diluted 1:1000; Cell Signaling Technology, Danvers, MA, USA), and GDF11 (diluted 1:5000, ab124721; Abcam) for 1 h. After washing, a horseradish peroxidase-conjugated secondary antibody (1:2000 dilutions) was added, and the blots were detected using an ECL system.

Reporter gene assay

A reporter gene assay was performed as previously described, with modifications, to examine whether GDF11 inhibited the TNF- α -mediated transactivation of NF- κ B-dependent reporter genes (28). BMDMs were grown to 50% confluence and then transfected with 1 μ g of the p6XNF- κ B-Luc reporter plasmid and 1 μ g of the pSVGal plasmid (internal control) using the Lipofectamine 2000 reagent (Invitrogen; Thermo Fisher). Forty-eight hours after transfection, the cells were starved overnight and then stimulated for 6 h with 10 ng/ml TNF- α in the presence or absence of various doses of GDF11. Luciferase activity was then measured.

Statistical analysis

To compare various treatment groups, unpaired *t* tests (Mann-Whitney), paired *t* tests, and 1-way or 2-way ANOVA (where appropriate) were performed. For ANOVA, Bonferroni *post hoc* analysis was used to compare treatment groups. Statistical analysis was performed by GraphPad Prism 4.01 (GraphPad Software, La Jolla, CA, USA). Statistical significance was achieved when *P* < 0.05.

RESULTS

Recombinant GDF11 inhibits inflammatory reactions *in vitro*

GDF11 has been reported to protect against inflammation, and TNF- α plays an important role in inflammation (12, 14, 29). This finding prompted us to study the interaction between GDF11 and the TNF- α /tumor necrosis factor receptor (TNFR) signaling pathway. Coimmunoprecipitation was performed in BMDMs, and as shown in **Fig. 1A, B**, GDF11 interacted with TNFR1 and TNFR2. This result led us to further investigate the protective effects of GDF11 *in vitro*.

BMDMs were isolated from WT mice and then treated with TNF- α and 50 ng/ml rGDF11 for 12 h, followed by real-time PCR. Figure 1C–F shows that the levels of inflammatory cytokines, including IL-1 β , IL-6, iNOS, and COX-2, were enhanced after stimulation with TNF- α , and this effect was antagonized by rGDF11. Moreover, the conditioned medium was collected from all groups at 72 h and assayed by ELISA. Figure 1G, H indicates that the elevated levels of IL-1 β and IL-6 induced by TNF- α were significantly reduced after treatment with rGDF11. Next, mRNA and total protein were collected from BMDMs, and real-time PCR and Western blot analysis were performed to determine whether TNF- α -mediated oxidative stress was inhibited by rGDF11. As shown in **Fig. 1I–K**, TNF- α

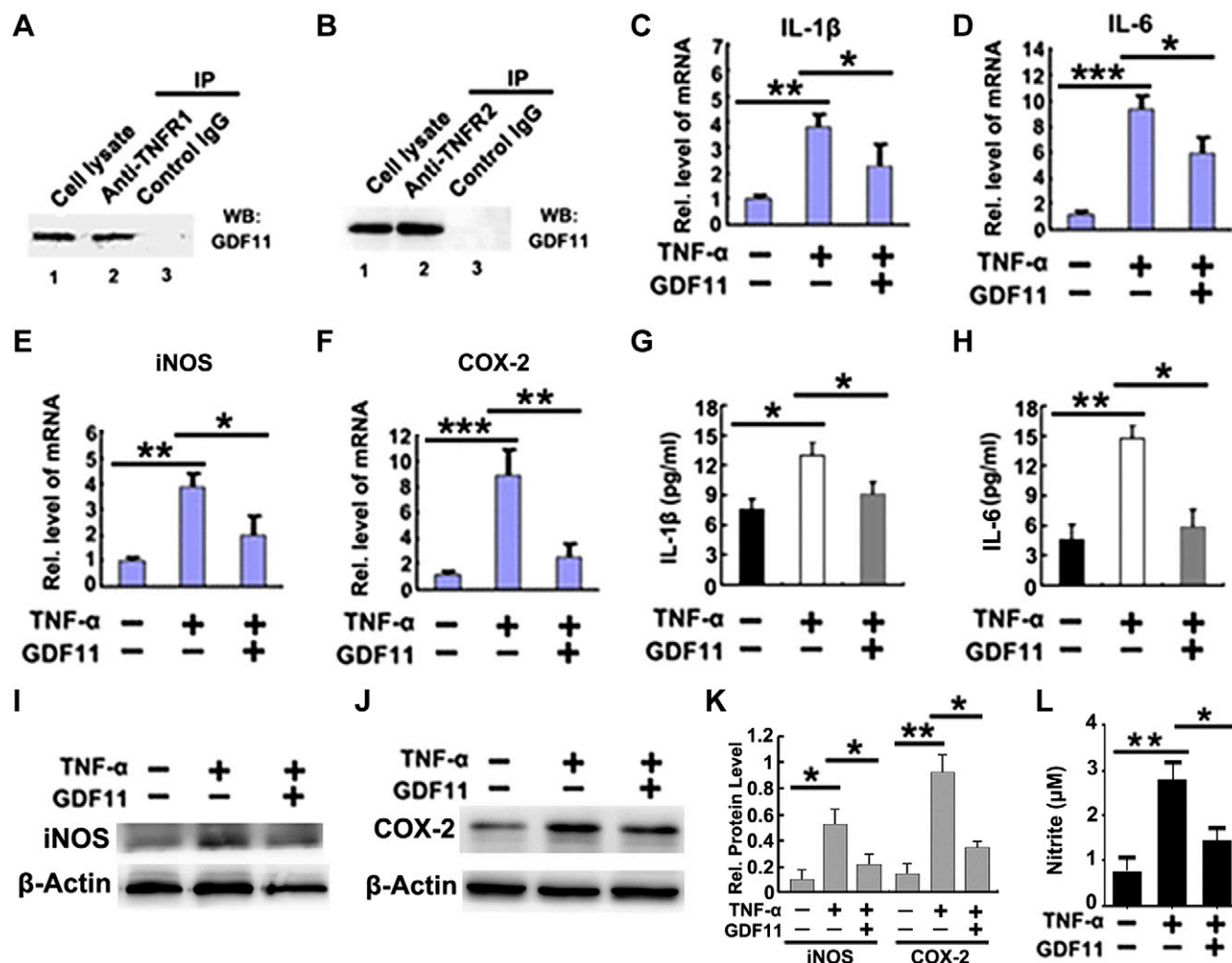


Figure 1. GDF11 inhibits inflammatory reaction induced by TNF- α in BMDMs. *A, B*) Interaction between GDF11 and TNF- α /TNFR signaling pathway was assayed by coimmunoprecipitation. *C–F*) mRNA levels of inflammatory cytokines, including IL-1 β , IL-6, iNOS, and COX-2, after stimulation with TNF- α in presence or absence of rGDF11 were tested by real-time PCR. *G, H*) Levels of circulating IL-1 β and IL-6 in serum were tested by ELISA. *I–K*) Expression of inflammatory cytokines, including iNOS and COX-2, was assayed by Western blot analysis. *L*) Level of NO released in conditioned medium was tested by Griess reagent. Values represent means $\pm$ sd. * P < 0.05, ** P < 0.01, *** P < 0.005 vs. vehicle control group.

markedly increased the expression levels of iNOS and COX-2, and this effect of TNF- α was significantly suppressed by the expression of GDF11. Additionally, Griess experiments were performed, and Fig. 1L shows that BMDMs produced less nitrite when stimulated by TNF- α in the presence of rGDF11. Collectively, these results demonstrate that rGDF11 suppressed TNF- α -induced inflammation *in vitro*.

Recombinant GDF11 protects against development of arthritis in CIA model

The CIA model is widely used in studies of RA (13). In this study, the CIA model was established in DBA/1J mice, and the paws of the rGDF11 treatment group showed attenuated swelling compared to those of the PBS group (Fig. 2A). Clinical scoring of arthritis in the CIA model indicated that rGDF11 significantly impeded the development of arthritis (Fig. 2B). Furthermore, the metatarsal joints of the mice were collected at the 42 d time point, and

histologic analyses were then performed. As shown in Fig. 2C, the severe synovitis, pannus, and erosion in the CIA model were diminished by GDF11 treatment, as indicated by a statistical grading system (Fig. 2D–F). Moreover, the synovium of the metatarsal joints and the serum were collected from each group, and real-time PCR, Western blot analysis, and ELISA were performed. The mRNA levels of inflammatory cytokines, including IL-1 β , TNF- α , IL-6, matrix metalloproteinase 3 (MMP-3), iNOS, and COX-2, in the CIA mouse model were markedly lower after treatment with rGDF11 (Fig. 2G–L). Furthermore, the circulating levels of IL-1 β , TNF- α , and IL-6 were greatly reduced in the CIA model after rGDF11 treatment (Fig. 2M–O). Moreover, rGDF11 significantly reduced the protein levels of iNOS and COX-2 in the joint tissue (Fig. 2P, Q).

Inflammatory bone destruction is known to be a feature of RA (30). In this study, the ankle joints were collected, and we then performed micro-computed tomography (μ CT). Figure 2R indicates that the severe bone damage in

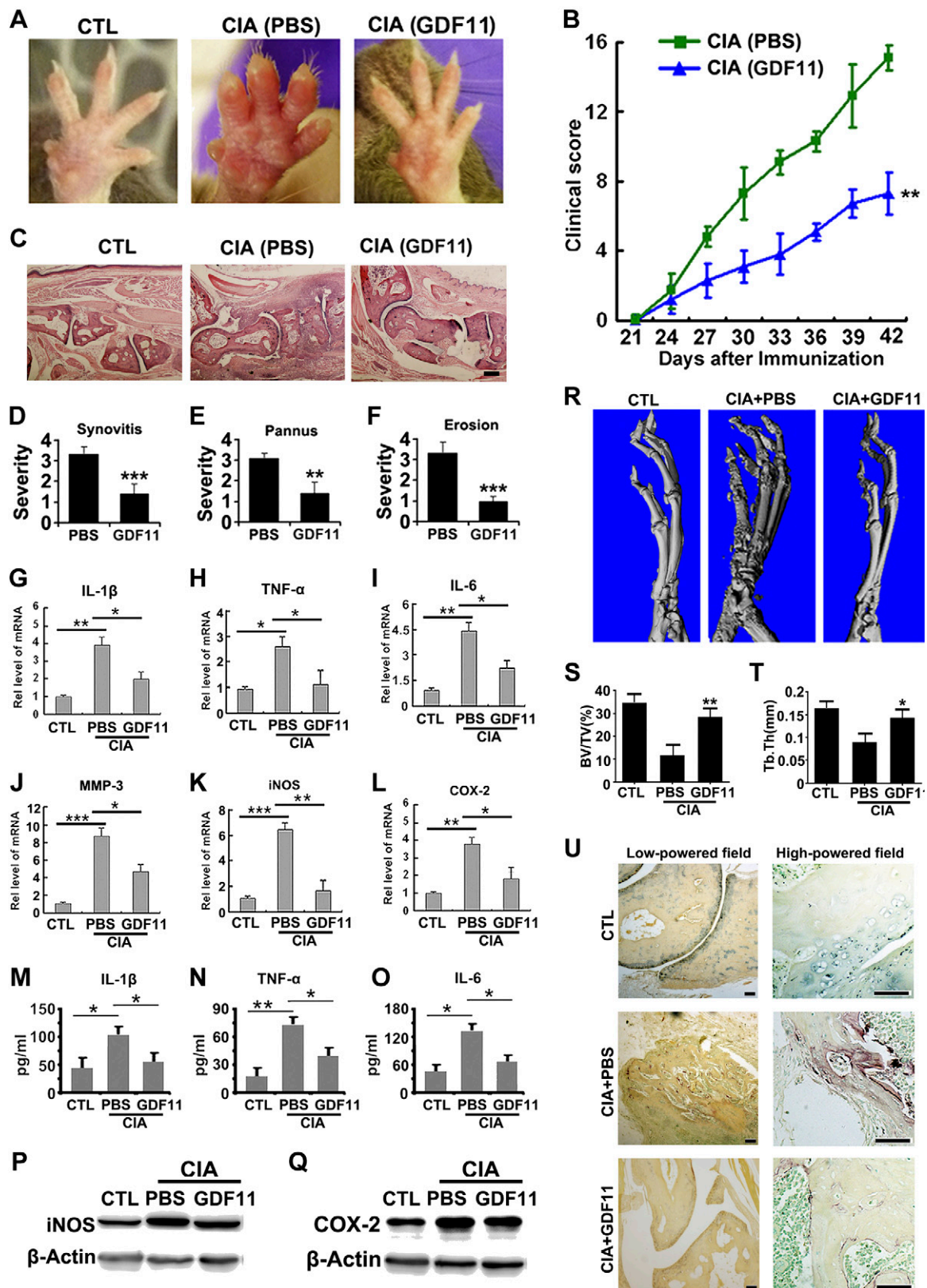


Figure 2. GDF11 protects against development of arthritis in CIA model. *A, B*) Swelling of hind paw is shown by photographs and clinical scores. *C–F*) Severity of synovitis, pannus, and erosion sites was measured through scoring system based on HE staining. Scale bar, 100 μ m. *G–L*) mRNA levels of inflammatory cytokines in joint, including IL-1 β , TNF- α , IL-6, MMP-3, iNOS, and COX-2, were measured by real-time PCR. *M–O*) Circulating levels of IL-1 β , TNF- α , and IL-6 in serum were measured by ELISA. *P, Q*) Expression of inflammatory cytokines, including iNOS and COX-2, was assayed by Western blot analysis. *R–T*) Bone volume fraction and trabecular thickness were assayed by μ CT and 3D reconstruction. *U*) Osteoclastogenesis was detected by TRAP staining, and low-power field and high-power field are both shown. Scale bars, 100 μ m. Values represent means $\pm$ SD. * P < 0.05, ** P < 0.01, *** P < 0.005 vs. vehicle control group.

the CIA model was alleviated by rGDF11 treatment. Similarly, Fig. 2S, T reveals that the CIA-mediated reduction of the bone volume fraction and trabecular thickness was reversed by treatment with rGDF11, as indicated by μ CT. Moreover, TRAP staining of the metatarsal joints was performed, and Fig. 2U indicates that inflammatory osteoclastogenesis was suppressed in the rGDF11 treatment group. Taken together, this set of experiments suggests that rGDF11 plays a protective role in the CIA model.

Recombinant GDF11 inhibits development of CAIA model

Further investigation of GDF11 function in inflammatory arthritis used the CAIA model established in 10 wk old WT BALB/c mice, with treatment with PBS or rGDF11. As shown in Fig. 3A, B, the severity of swelling in the hind paws of the mice was greatly improved after the administration of rGDF11, as indicated by photographs and clinical scores. On d 8, hind paws from each group were collected, and histologic analysis was performed. As shown in Fig. 3C–F, rGDF11 protects against synovitis, pannus, and erosion in the metatarsal joints.

Inflammatory biomarkers were assayed in the synovial tissue of the CAIA model. Real-time PCR revealed that the mRNA levels of TNF- α , MMP-3, iNOS, IL-6, and IL-1 β were significantly reduced in the rGDF11-treated group (Fig. 3G–K). Furthermore, immunohistochemistry and Western blot analysis were performed to measure iNOS in the synovial tissue. The rGDF11-treated group displayed lower levels of iNOS (Fig. 3L, M). In addition, the circulating levels of IL-1 β , IL-6, and TNF- α were significantly decreased in the CAIA mouse model after rGDF11 treatment (Fig. 3N–P). In summary, rGDF11 exhibited a protective effect against RA in the CAIA mouse model.

Local gene therapy with AAV-based GDF11 protects against development of arthritis in CIA model

AAV-GDF11 was established and delivered through intra-articular injection into the metatarsal joints in the CIA model to determine whether local delivery of GDF11 protects against inflammatory arthritis. AAV-GFP and AAV-GDF11 were injected into the joint 2 wk before the CIA model was induced. Figure 4A shows that local transgene expression was detectable before immunization, as indicated by optical bioluminescence. Additionally, Fig. 4B, C indicates that AAV-GDF11 attenuated severity of arthritis in the CIA model, as indicated by clinical score analysis. Moreover, the metatarsal joints were collected, and HE staining was performed. Figure 4D–G shows alleviated damage in the metatarsal joint in the AAV-GDF11 group, including reduced synovitis, pannus, and erosion scores.

Furthermore, synovial tissue and serum were collected, and real-time PCR, ELISA, and Western blot analysis were performed. As indicated in Fig. 4H–M, the mRNA levels of TNF- α , MMP-3, IL-6, IL-1 β , iNOS, and COX-2 were decreased in the AAV-GDF11 treatment group. Western blot

and immunohistochemistry results showed that the expression of iNOS was reduced in the AAV-GDF11 group (Fig. 4N, O). Moreover, we performed μ CT of the hind paws, and the AAV-GDF11 group showed less bone destruction, as indicated by the bone volume fraction and trabecular thickness (Fig. 4P–R). TRAP staining was performed, and inflammatory osteoclastogenesis was significantly inhibited in the presence of AAV-GDF11 (Fig. 4S).

Local knockdown of GDF11 via AAV-siGDF11 accelerates development of CIA

To determine whether insufficiency of GDF11 exaggerated the development of inflammatory arthritis, a CIA model was established, and AAV-siGDF11 was delivered into the hind paw *via* intraarticular injection 2 wk before CIA induction. Two weeks after injection, optical bioluminescence indicated that transfection of the siGDF11 transgene in the hind paw was successful (Fig. 5A). Moreover, both the mRNA levels and protein levels of GDF11 in the synovial tissue of the hind paws were reduced, as indicated in Fig. 5B, C. However, no significant difference in the circulating levels of GDF11 in the serum was observed (Fig. 5D). The severity of swelling in the AAV-siGDF11 group and AAV-GFP group was unchanged on d 42 after immunization, as indicated by clinical scores (Fig. 5E). Interestingly, however, we found that the AAV-siGDF11 group exhibited accelerated development of arthritis compared to the AAV-GFP group, especially between d 30 and d 36, which implied a significant difference in clinical scores (Fig. 5E, red arrow).

To confirm these findings, we repeated the experiment, and joint samples were collected on d 33 after immunization. Mice in the AAV-siGDF11 group again displayed more severe swelling (Fig. 5F). Moreover, HE staining was performed, and the AAV-siGDF11 group showed more severe synovitis, pannus, and erosion (Fig. 5G–J). The mRNA levels of IL-1 β , IL-6, TNF- α , MMP-3, COX-2, and iNOS in the synovial tissue were also significantly increased in the AAV-siGDF11 group, which indicated exacerbated local inflammatory conditions (Fig. 5K–P). Additionally, immunohistochemistry revealed that the knockdown of GDF11 led to enhanced expression of iNOS in the synovial tissue (Fig. 5Q).

GDF11 inhibits activation of NF- κ B signaling pathway both *in vitro* and *in vivo*

As previously described, NF- κ B is activated in inflammatory conditions and plays a critical role in RA (31). This information, together with the finding that GDF11 protects against inflammatory arthritis, led us to investigate the potential association between GDF11 and NF- κ B signaling in inflammation. BMDMs were cultured and stimulated with TNF- α for 1 h with or without rGDF11 treatment. As indicated in Fig. 6A, the expression of NF- κ B2, a critical component of the NF- κ B pathway, was suppressed by rGDF11. In addition, phosphorylation of I κ B α was reduced by rGDF11, as indicated by Western blot analyses (Fig. 6B). Moreover, cell immunostaining showed that the nuclear translocation of NF- κ B p65 was inhibited in the presence of rGDF11 (Fig. 6C, D). Additionally,

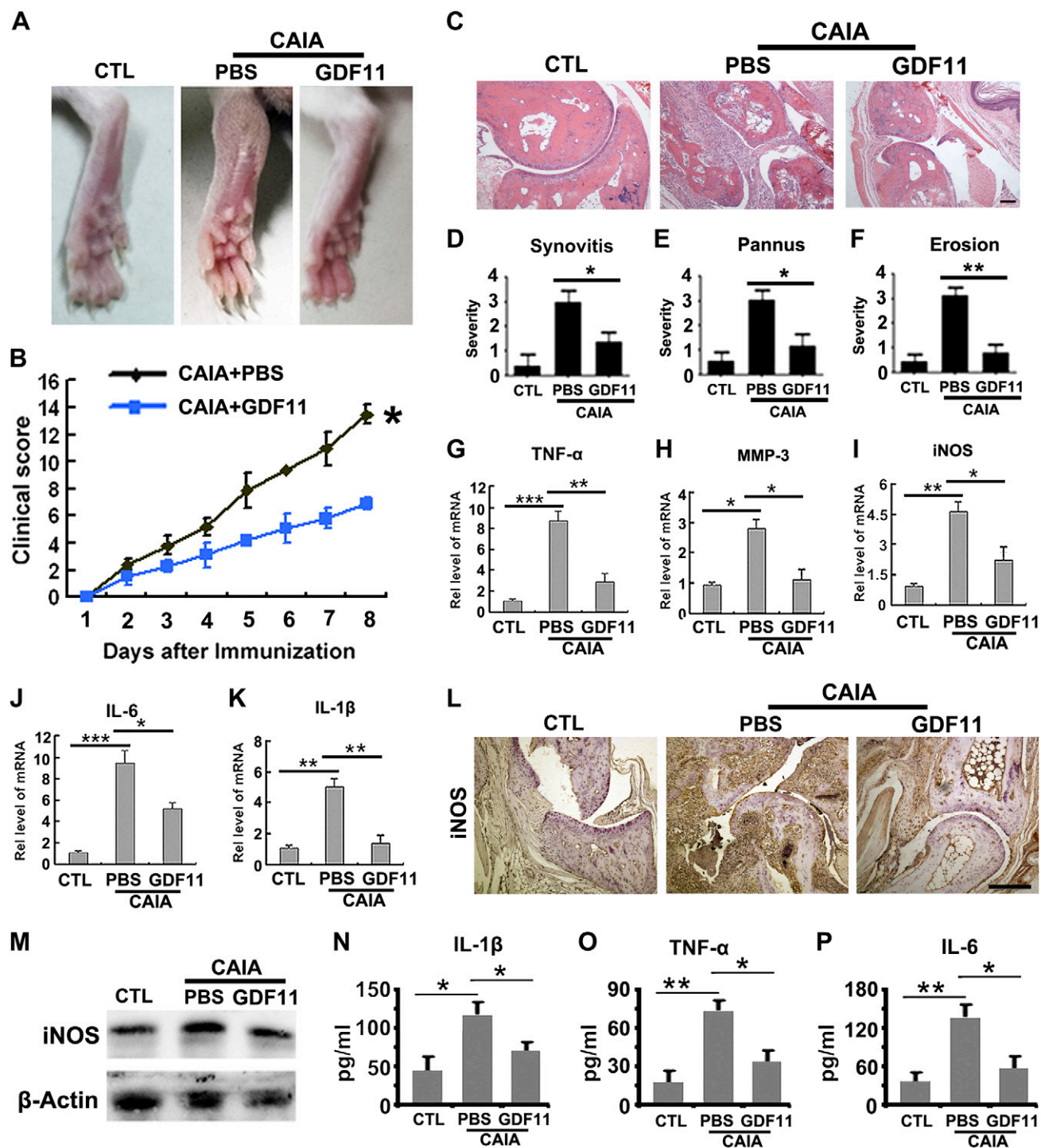


Figure 3. GDF11 inhibits development of CAIA model. *A, B*) Swelling of hind paw is shown by both photographs and clinical scores. *C–F*) Severity of synovitis, pannus, and erosion sites was measured through scoring system based on HE staining. Scale bar, 200 μ m. *G–K*) mRNA levels of inflammatory cytokines in joint, including TNF- α , MMP-3, iNOS, IL-6, and IL-1 β , were measured by real-time PCR. *L*) Level of iNOS in synovial tissue was assayed by immunohistochemical staining. Scale bar, 50 μ m. *M*) Synovial tissue was collected from each group, and expression of iNOS was assayed by Western blot analysis. *N–P*) Circulating levels of IL-1 β , TNF- α , and IL-6 in serum were measured by ELISA. Values represent means $\pm$ SD. * P < 0.05, ** P < 0.01, *** P < 0.005 vs. vehicle control group.

a reporter gene assay for NF- κ B signaling was performed, and rGDF11 repressed NF- κ B signaling in a dose-dependent manner (Fig. 6E).

To study the role of rGDF11 in the activity of the NF- κ B signaling pathway *in vivo*, the synovial tissues from the

CIA mice were collected, followed by real-time PCR for NF- κ B2 (Fig. 6F) and immunohistochemistry (Fig. 6G) and Western blot analysis for p-I κ B α (Fig. 6H). rGDF11 administration reduced the levels of these molecules in the CIA model. In addition, the CAIA model was established

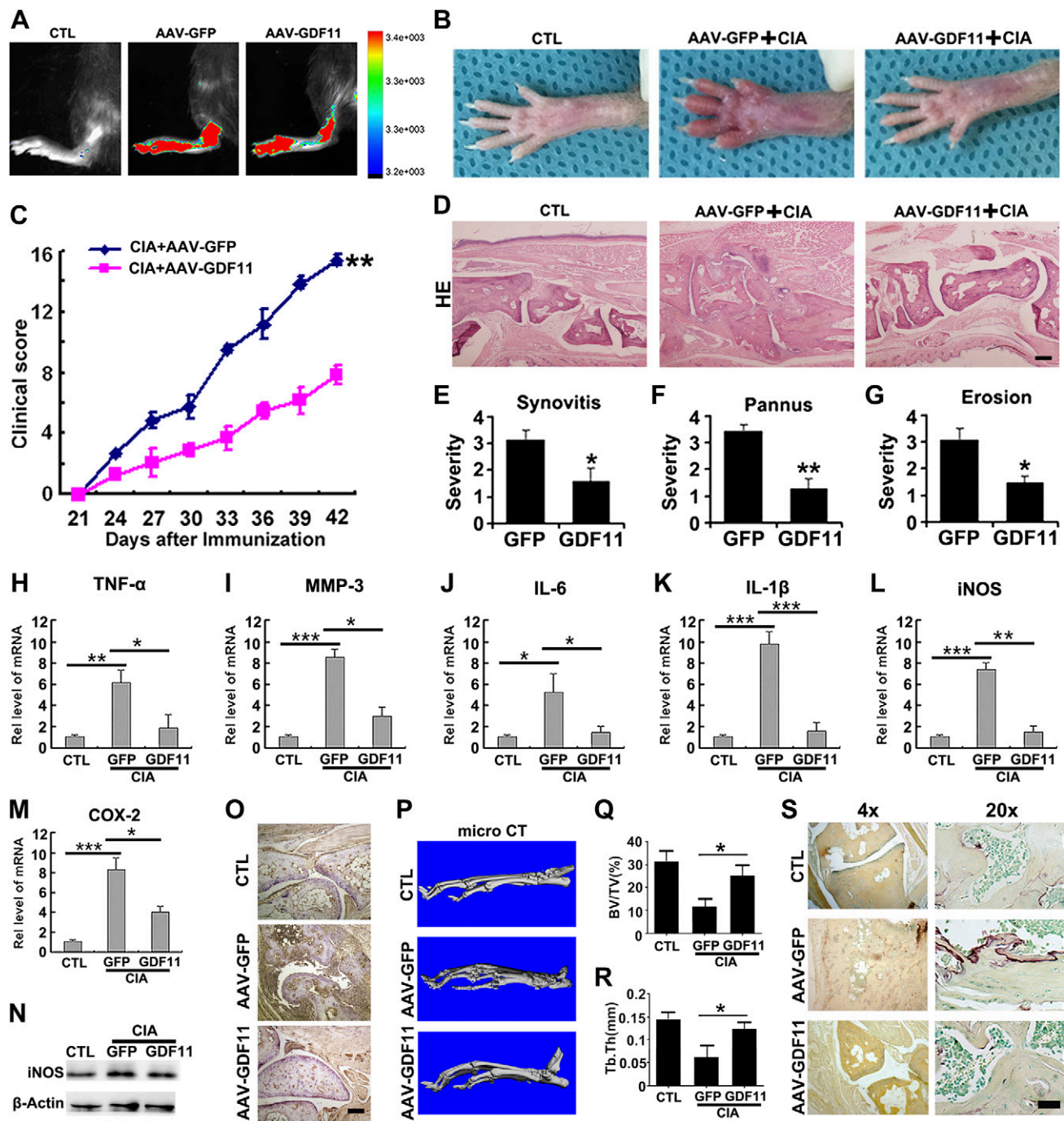


Figure 4. Local gene therapy with GDF11 mediated by AAV shows therapeutic effect in RA. **A**) Bioluminescent images of representative mice in AAV-GFP group and AAV-GDF11 group showed persistently high GFP signals at injection sites. **B, C**) Swelling of hind paw is shown by photographs and clinical scores. **D–G**) Severity of synovitis, pannus, and erosion sites was measured through scoring system based on HE staining. Scale bar, 200 μ m. **H–M**) mRNA levels of inflammatory cytokines in joint, including TNF- α , MMP-3, IL-6, IL-1 β , iNOS, and COX-2, were measured by real-time PCR. **N, O**) Expression of iNOS was determined by Western blot analysis and immunohistochemical staining. Scale bar, 50 μ m. **P–R**) Bone volume fraction and trabecular thickness were assayed by μ CT and 3D reconstruction. **S**) Osteoclastogenesis was shown by TRAP staining, and low-power field and high-power field are both shown. Values represent means $\pm$ SD. * P < 0.05, ** P < 0.01, *** P < 0.005 *vs.* vehicle control group.

in NF- κ B-RE-luc mice, and an optical bioluminescence assay was performed to further investigate the association between GDF11 treatment and NF- κ B signaling activity *in vivo*. The results indicated that rGDF11 treatment suppressed NF- κ B signaling in the CIA model, which was consistent with the severity of arthritis (Fig. 6I, J).

DISCUSSION

RA is a symmetrical, chronic, multijoint inflammatory disease that is characterized by inflammatory cell infiltration, synovial hyperplasia, and progressive joint destruction. In addition, progressive damage to the joint

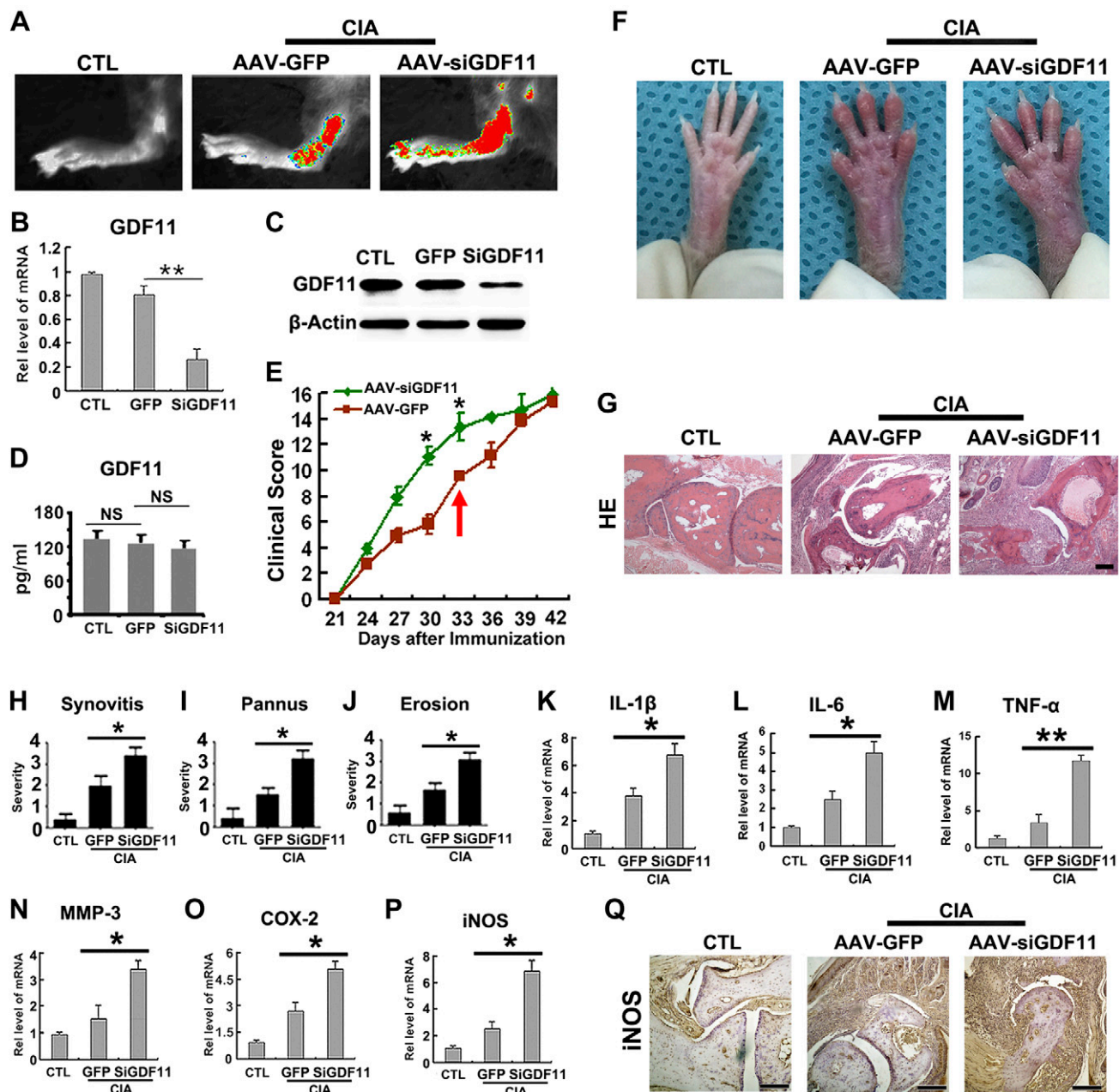


Figure 5. Local knockdown of GDF11 through AAV-siGDF11 accelerates development of CIA. **A)** Bioluminescent images of representative mice in AAV-GFP group and AAV-siGDF11 group showed persistently high GFP signals at injection sites. **B)** mRNA level of GDF11 in each group was assayed by real-time PCR. **C)** Expression of GDF11 was measured by Western blot analysis. **D)** Level of circulating GDF11 was assayed by ELISA. **E, F)** Swelling of hind paw is shown by photographs and clinical scores. **G–J)** Severity of synovitis, pannus, and erosion sites was measured through scoring system based on HE staining. Scale bar, 200 μ m. **K–P)** mRNA levels of inflammatory cytokines in joint, including IL-1 β , IL-6, TNF- α , MMP-3, COX-2, and iNOS, were measured by real-time PCR. **Q)** Expression of iNOS was assayed by immunohistochemical staining. Scale bars, 50 μ m. Values represent means $\pm$ SD. * P < 0.05, ** P < 0.01, *** P < 0.005 *vs.* vehicle control group.

structure caused by inflammation has been shown to be common in patients (32–34). However, treatments for RA are still lacking (32, 33). Antiinflammation and immunosuppression strategies have been the primary therapies for RA in clinical practice despite their adverse effects (35, 36). Therefore, inhibition of inflammatory reactions has become an alternative investigative focus for treating RA (37).

As previously described, TNF- α plays a critical role in the pathogenesis of inflammatory arthritis (38). By binding

its receptors, including TNFR1 and TNFR2, TNF- α accelerates the inflammatory reaction in RA (15). In this study, the potential interaction between GDF11 and TNFR1/TNFR2 was tested; macrophage experiments suggested that GDF11 might antagonize TNF- α -mediated inflammatory reactions *in vitro*. GDF11 is a key member of the TGF- β superfamily with multiple functions (3–5), and recent research suggested that GDF11 might be associated with inflammatory reactions (12). In the current study, different mouse models were established to determine

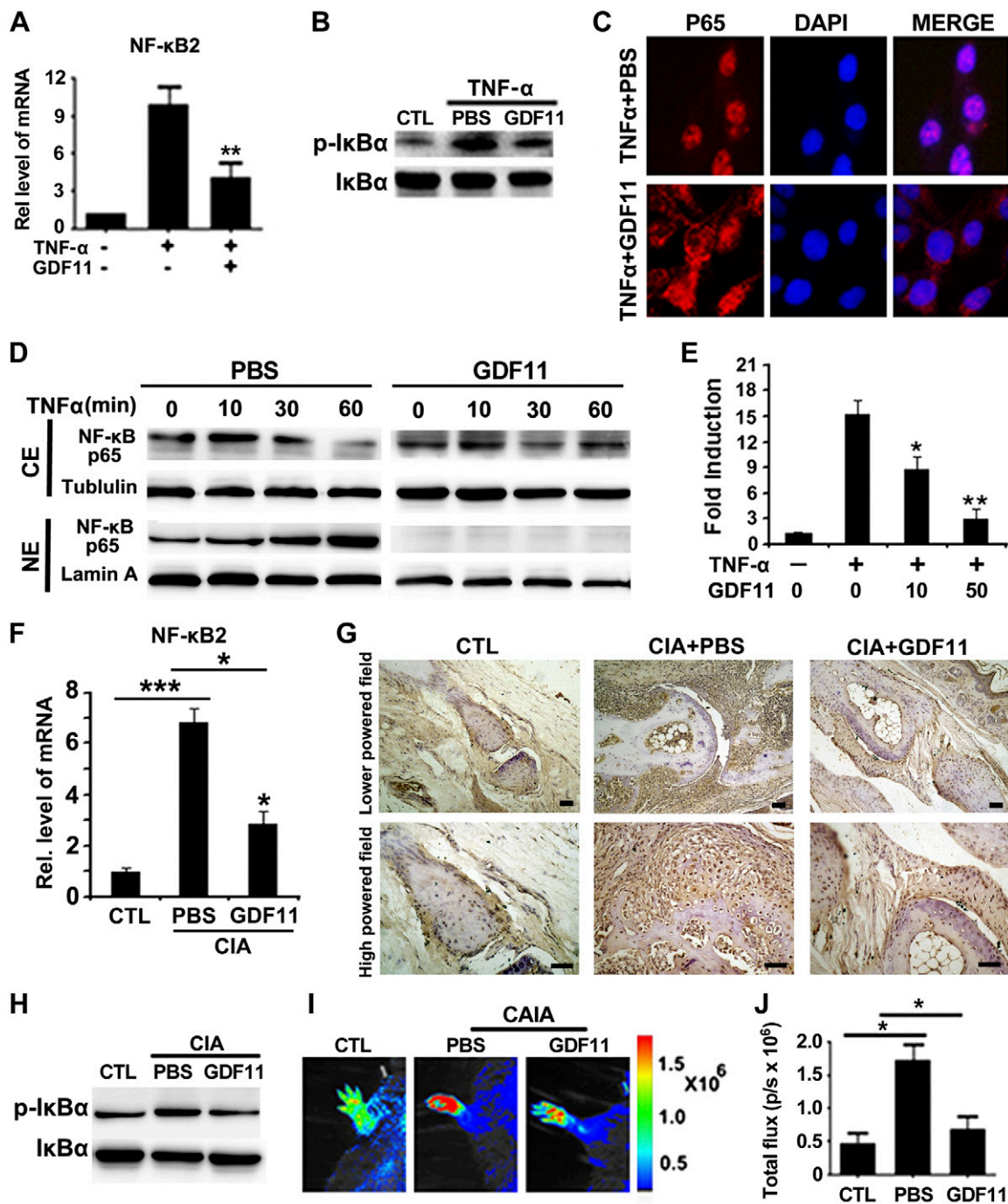


Figure 6. GDF11 inhibits NF-κB signaling pathway both *in vitro* and *in vivo*. BMDMs were incubated with TNF-α in presence or absence of rGDF11. *A*) mRNA level of NF-κB2 in BMDMs was tested by real-time PCR. *B*) Phosphorylation and expression of IκBα in BMDMs were determined by Western blot analysis. *C, D*) Nuclear translocation of NF-κB p65 in BMDMs was assayed by immunofluorescence and Western blot analysis. Tubulin and lamin are shown as loading controls. *E*) GDF11 inhibited TNF-α-induced NF-κB signaling in dose-dependent manner, as shown by reporter gene assay. *F*) mRNA level of NF-κB2 in CIA model was tested by real-time PCR. *G*) Immunohistochemistry for phosphorylated IκBα in articular cartilage of CIA mice in each group on d 42; low-power field and high-power field are both shown. Scale bars, 50 μm. *H*) Phosphorylation of IκBα in synovial tissue was also determined by Western blot analysis. *I, J*) CAIA model was established in NF-κB-RE-luc mice, and fluorescence of NF-κB was measured by optical bioluminescence. Values represent means ± SD. **P* < 0.05, ***P* < 0.01, ****P* < 0.005 *vs.* vehicle control group.

whether GDF11 has the potential to protect against inflammatory arthritis.

The CIA mouse model is a common model used in inflammation research that has a pathologic manifestation and nosogenesis similar to that of RA (34). In this study,

CIA was induced to investigate the effect of GDF11 on RA *via* the systemic delivery of rGDF11 and local intraarticular injection of AAV9-GDF11. Cartilage damage, synovial hyperplasia, pannus formation, and bone erosion are common features of inflammatory arthritis (36), and in this

study, exogenous GDF11 markedly attenuated all these parameters of arthritis in our CIA model.

Moreover, the levels of inflammatory cytokines and molecules associated with oxidative stress are commonly enhanced in the CIA model (39). In the current study, GDF11 diminished all tested inflammation- and oxidative stress-associated molecules, suggesting that GDF11 may protect against the development of arthritis. As reported previously, the CAIA model is also a commonly used model in RA research because it can avoid strain specificity (40). Characterized by progressive swelling of the joint and destruction of the joint structure, the CAIA model exhibited elevated levels of inflammatory and oxidative stress biomarkers. In the present study, the CAIA model was established in BALB/c mice, and rGDF11 treatment alleviated the arthritis phenotype and reduced the levels of inflammatory biomarkers in these mice.

TNF- α -induced activation of the NF- κ B signaling pathway is known to participate in various inflammatory conditions (41). NF- κ B signaling is activated in inflammatory arthritis (42). As previously described, NF- κ B has important effects on several inflammation-associated molecules, including iNOS and the COX-2 protein in RA, which may further activate NF- κ B to form a positive feedback loop (43). Recently, it was reported that GDF11 suppresses the NF- κ B signaling pathway and the production of downstream molecules in atherosclerosis (12). In this study, *in vitro* experiments, which revealed the phosphorylation of I κ B α and the critical component NF- κ B2, indicated that GDF11 antagonizes the TNF- α -induced NF- κ B signaling pathway.

NF- κ B-RE-luc mice are widely used to determine the activation of NF- κ B signaling during inflammation and other pathologic conditions (27). In this study, a CAIA model was established using NF- κ B-RE-luc mice, and an optical bioluminescence assay was used to determine whether GDF11 treatment markedly abolished the elevation of NF- κ B signaling in the paws in the CAIA model. The results imply that the protective role of GDF11 in inflammatory arthritis might be associated with the suppression of the NF- κ B signaling pathway.

To date, local gene therapy has not only been used in preclinical investigations of medication but also in clinical settings (20, 44). The most reliable and widely used gene vectors are lentivirus, adenovirus, and AAV. AAV has high transfection efficiency, superior safety, and low immunogenicity, and it is thought to be a strategy for the accurate delivery of targeted molecules in scientific research and clinical treatment (45). It has been reported that intraarticular injection of AAV-IL-38 exerted a protective effect against inflammatory arthritis (46), and AAV-GDF11 was used to investigate cardiovascular diseases (12). In the current study, the AAV9-GDF11 and AAV9-siGDF11 vectors were synthesized and delivered in the CIA model through intraarticular injection. The ankle joint and hind paw synovial tissue showed less swelling and joint destruction after GDF11 transgene delivery, while the local knockdown of GDF11 accelerated the inflammatory process in the hind paw in the CIA model, suggesting that

local modulation of GDF11 expression levels plays a role in the development of arthritis.

It is known that newborn GDF11 knockout mice cannot survive. Therefore, local knockdown of molecules *via* AAV has been performed in various studies (47). In this study, AAV9-siRNA was established and injected into the ankle joint, followed by induction of the CIA model. At d 42, no obvious difference in arthritis severity was observed between the GDF11 knockdown group and the AAV-GFP group. Interestingly, the knockdown of GDF11 in the hind paw led to accelerated development of arthritis. This effect might be associated with elevated local susceptibility to inflammation, which stems from the recruitment and accumulation of various types of immune cells. However, at a relatively late stage of CIA, this difference decreased. This phenomenon might be related to the enhancement of circulating GDF11 in the blood supply as a result of inflammation-induced capillary proliferation.

CIA and CAIA are the most frequently used animal models to investigate the effects of new therapeutics for RA. In the present study, we reported the potential involvement and underlying mechanisms of GDF11 in inflammatory arthritis. To demonstrate the direct relationship between GDF11 and RA in the clinic and to determine whether GDF11 plays a protective role in patients with RA, further preclinical and clinical studies will be performed in the future. In summary, GDF11 displayed antiinflammatory and therapeutic effects in mice with inflammatory arthritis, which may provide a therapeutic strategy for experimental and clinical investigations of RA. FJ

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AUTHOR CONTRIBUTIONS

W. Li, W. Wang, X. Pan, and Y. Zhao conceived and designed the experiments; W. Wang, Liang Liu, R. Qu, X. Chen, C. Qiu, J. Li, and Y. Zhao performed the experiments; J. Hayball, Long Liu, J. Chen, X. Wang, and Y. Zhao analyzed the data; and W. Li, X. Pan, and Y. Zhao contributed reagents, materials, and analysis tools.

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