Alpha-1-antitrypsin reduces inflammation and exerts chondroprotection in arthritis

Magdalena K. Kaneva1 | Milind M. Muley2 | Eugene Krustev2 | Allison R. Reid2 | Patricia R. Souza1 | Francesco Dell’Accio1,3 | Jason J. McDougall2 | Mauro Perretti1,3

1The William Harvey Research Institute, The London School of Medicine, Queen Mary University of London, London, UK
2Departments of Pharmacology and Anaesthesia, Pain Management & Perioperative Medicine, Dalhousie University, Halifax, NS, Canada
3Centre for inflammation and Therapeutic Innovation, Queen Mary University of London, London, UK

Correspondence
Magdalena K. Kaneva, The William Harvey Research Institute, Queen Mary University of London, Charterhouse Square, London EC1M 6BQ, UK.
Email: kaneva.magdalena@gmail.com

Present address
Milind M. Muley, Neurosciences & Mental Health, Peter Gilgan Centre for Research and Learning (PGCRL), The Hospital for Sick Children, Toronto, ON, Canada

Funding information
This work was largely supported by the William Harvey Research Foundation through an unrestricted grant (MKK and MP), with additional support by operating grants from the Canadian Institute for Health Research and a CIHR team grant (PJT-153303; REACH; JJMcD) and the MRC (MR/R000956/1, FDA).

Abstract
While new treatments have been developed to control joint disease in rheumatoid arthritis, they are partially effective and do not promote structural repair of cartilage. Following an initial identification of α-1-Antitrypsin (AAT) during the resolution phase of acute inflammation, we report here the properties of this protein in the context of cartilage protection, joint inflammation, and associated pain behavior. Intra-articular and systemic administration of AAT reversed joint inflammation, nociception, and cartilage degradation in the KBxN serum and neutrophil elastase models of arthritis. Ex vivo analyses of arthritic joints revealed that AAT promoted transcription of col2a1, acan, and sox9 and downregulated mmp13 and adamts5 gene expression. In vitro studies using human chondrocytes revealed that SERPINA1 transfection and rAAT protein promoted chondrogenic differentiation through activation of PKA-dependent CREB signaling and inhibition of Wnt/β-catenin pathways. Thus, AAT is endowed with anti-inflammatory, analgesic, and chondroprotective properties that are partially inter-related. We propose that AAT could be developed for new therapeutic strategies to reduce arthritic pain and repair damaged cartilage.

Abbreviations: AAT, alpha-1-antitrypsin; ACAN, aggrecan; ADAMTS, a disintegrin and metalloproteinase with thrombospondin type 1 motif; ALK5, activin A receptor type II-like kinase; BIO, 6-bromoindirubin 3'-oxime; CFA, complete Freund’s Adjuvant; CMV, cytomegalovirus; COL2A1, type 2 collagen; CRE, cAMP response element; CREB, cAMP response element-binding protein; ECM, extracellular matrix; Fsk, forskolin; GFP, green fluorescent protein; GSK-3β, glycogen synthase kinase 3 beta; i.a., intra-articular; IgGs, immunoglobulins; HACs, human articular chondrocytes; His, histidine; IL, interleukin; ip, intra-peritoneal; ITS, insulin-transferrin-selenium; LPS, lipopolysaccharide; MMP, matrix metalloproteinase; NE, neutrophil elastase; NF-κB, nuclear factor kappa B; PKA, protein kinase A; RA, rheumatoid arthritis; RLU, relative luciferase unit; SERPINA1, serine protease inhibitor A1; Smad2/3, mothers against decapentaplegic homolog 2/3; SOX9, SRY-Box Transcription Factor 9; TGF-β1, transforming growth factor β1; TNF-α, tumor necrosis factor alpha; TNFR1/2, TNF-α receptors 1 and 2; WT, wild type.

Magdalena K. Kaneva and Milind M. Muley Co-first authors.
Jason J. McDougall and Mauro Perretti Share senior authorship.

This is an open access article under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited.

© 2021 The Authors. The FASEB Journal published by Wiley Periodicals LLC on behalf of Federation of American Societies for Experimental Biology.
1 | INTRODUCTION

Arthritis is a common health problem in the global population, affecting more than 350 million people, causing more disability than any other condition, including heart disease and diabetes. Rheumatoid arthritis (RA) is mainly characterized by joint inflammation and destruction of cartilage and the underlying bone tissues, leading to loss of motion in the affected joint. Despite the use of anti-inflammatory agents and disease modifying anti-rheumatic drugs, there are no treatments able to halt or reverse the ongoing destruction of the joint.

First isolated in 1955 and named after its ability to inhibit trypsin, α-1-Antitrypsin (AAT) was identified as a potent serine protease inhibitor, with highest affinity for neutrophil elastase (NE). Produced by hepatocytes and secreted in the blood, AAT concentrations rise ~4-fold during infection and inflammation, remaining elevated up to 7 days, suggesting a function in host protection. It is now reported that several cell types can produce AAT implying tissue-specific functions beyond AAT roles in acute inflammation.

Currently, intravenous infusions of human plasma-purified AAT preparations are used for the treatment of lung diseases associated with severe inherited AAT deficiency. But the pleiotropic properties of AAT provide a rationale for using this therapy outside of this disease. AAT therapy is beneficial in several experimental settings including transplant rejection, ischemia-reperfusion injury, graft-vs-host disease, autoimmune encephalomyelitis, preeclampsia, and inflamed pancreatic islets. Thus, AAT may be a more complex mediator of inflammation and host response than initially proposed.

In collagen-induced arthritis in mice, AAT as well as AAT gene therapy, significantly impacted disease onset and progression while in a model of gouty arthritis, AAT-Fc fusion protein reduced joint swelling and cellular infiltration. We have recently identified AAT as an abundant protein in resolving (end-of-acute inflammation) exudates, suggesting that it could be exerting tissue-protective and resolving actions. Initial analyses in experimental arthritis and in vitro on 3D chondrocyte cultures showed AAT to reduce joint inflammation and inhibit cartilage catabolism. However, the molecular mechanisms responsible for these effects were not studied and, more broadly, whether such macroscopic actions could be secondary to NE inhibition remains unclear. Here, we have expanded our knowledge on the biology of AAT and established at least some of the mechanisms underpinning its effects on chondrocytes, in conditions where NE and other serine proteases are unlikely to be present.

2 | MATERIALS AND METHODS

2.1 | Animals and models of inflammatory arthritis

C57BL/6 mice (22-30g: 10-12 weeks old) or male Wistar rats (250-450g: 8-15 weeks old), purchased from Charles River Laboratories, were maintained on a standard chow pellet diet with access to water ad libitum, and a 12-hour light-dark cycle. All animal experiments were approved by the local Animal Use and Care Committee in accordance with the United Kingdom Animals (Scientific Procedures) Act, 1986 and Canadian Council for Animal Care guidelines (http://www.ccac.ca/) and were approved by the Dalhousie University Committee on Laboratory Animals.

2.1.1 | KBxN serum-transfer arthritis

Male C57BL/6 mice (n = 5-6 per group) were administered 100 µL (ip) of arthritogenic K/BxN serum at day 0 and day 2 of arthritis, as described. On day 3, mice were administered either with vehicle (saline), or AAT (100 µg/mouse, ip, Abcam, ab91136, Cambridge, UK). Disease development was assessed through clinical score (0-3) of arthritis, where 0 equates no evidence of inflammation; 1, subtle inflammation of metatarsal phalangeal joints, individual phalanx evaluated; 2, edema on dorsal and ventral surface of paws; 3, major edema on all aspects of paws (maximum score 3 per paw, 12 per animal). Paw edema was measured by water plethysmometry (Ugo Basile SRL, Milan, Italy) and together with the cumulative disease incidence were also recorded daily. Mice were weighed daily for signs of cachexia.

2.1.2 | Complete Freund’s Adjuvant (CFA)-induced joint inflammation and pain

Further experiments were conducted to assess the effect of AAT on CFA-induced joint inflammation and pain. Male Wistar rats (n = 8-14/group) received injection of CFA (50 µL i.a.) into the right knee joint. For this group, AAT (100 µg:1 mL saline ip) was administered on day 1, 15 minutes before CFA injection on day 0, and on days 2, 4, and 6 post-CFA injections. Joint inflammation was assessed on day 21 and behavioral pain—at baseline and on days 1, 3, 7, 10, 14, 17, and 21 post-CFA injections.
2.1.3 | NE-induced knee inflammation

Mice (n = 8 per group) were anesthetized using isoflurane (2%-4%; 100% oxygen at 1 L/min), the right knee joint was shaved, and baseline knee joint diameter was recorded using a digital micrometer (Control Company, Friendswood, TX, USA), as described. Briefly, mice were injected with a mixture of saline (5 µL):NE (5 µg/5 µL), or AAT:NE (100 ng:5 µg in 10 µL), into the right knee. Post-injection, the knee was extended and flexed for 30 seconds to disperse the mixture throughout the joint. Vascular processes were monitored by intravital microscopy and laser speckle contrast analysis at 4 hours post-treatment. A separate cohort of mice was used to monitor mechanical hypersensitivity using von Frey hair algometry and weight-bearing deficits using dynamic weight-bearing apparatus.

2.2 | Histological and microscopy analyses

Mice were anesthetized and sacrificed by cervical dislocation at different times, related to the specific model applied, thus day 5 post-serum, 4 hours post-NE, or 21 days post-CFA injections. Knee joints were collected and fixed in 10% Neutral Buffered Formalin (v/v) and decalcified in 10% Formic Acid (v/v), followed by paraffin embedding. Coronal sections (5 µM) of the knee joints were stained with Hematoxylin and Eosin, or Toluidine Blue, according to the standard methods, to visualize the levels of joint inflammation and cartilage damage, respectively. Standard light microscopy was used to determine degree of synovitis, pannus formation, and cartilage damage; both rated from 0 (no disease) to 3, severe joint destruction, by blinded examiners. Percentage area toluidine blue positive was measured by ImageJ (NIH) by splitting each image into its RGB channels and quantifying the positive area after applying a threshold.

2.2.1 | Intravital microscopy

Intravital microscopy was used to assess leukocyte-endothelial interactions within the knee joint microvasculature, as described. Briefly, mean arterial pressure was measured on a differentially amplified BP monitor (BP-1; World Precision Instruments, Sarasota, FL, USA) which was connected to carotid artery cannula through an in-line pressure transducer (Kent Scientific Corporation, Torrington, CT, USA). Leukocytes were stained using Rhodamine 6G (0.05%; mice–0.06 mL, rats–0.12 mL) injected via the jugular vein. The knee joint microvasculature was accessed by removing a small piece of overlying skin (1.0 × 0.5 cm) and visualized using a Leica DM2500 microscope (Leica Microsystems Inc, Richmond Hill, ON, Canada; magnification 200x). Straight, branchless, post-capillary venules (d = 20-50 µm) were located on the knee joint capsule and three 1-min recordings were made for each time point using a BC-71 AVT camera (Horn Imaging, Aahen, Germany). Rolling and adherent leukocytes numbers were recorded and averaged.

2.3 | Nociception assays

2.3.1 | Measurement of mechanical hypersensitivity

von Frey hair filaments were used to assess ipsilateral hind paw mechano-sensitivity in mice. The 50% withdrawal threshold was calculated using Dixon’s up-down method. A filament was applied to the planter surface of the hind paw for 3s–a positive response was considered when the animal licked/shook the paw post-application of the von Frey hair filament. After a positive response, the next lower filament was applied. However, if an animal did not respond to a filament the next filament with a higher bending force was applied. The maximum cut-off for a mouse was 4 g and for a rat was 15 g bending force. After notice of the first response, four more responses were recorded. The 50% withdrawal threshold was calculated using the formula: 10[Xf + kδ]/10 000; where Xf = value (in log units) of the final von Frey hair used, δ = mean difference (in log units) between stimuli, and k = tabular value for the pattern of the last six positive/negative responses.

2.3.2 | Arthritic nociception

Dynamic weight bearing was used to measure spontaneous pain behavior in freely moving animals, as described. Briefly, animals were placed on a sensor pad (Bioseb DWB Mouse/Rat Sensor Pad, Vitrolles, France) inside a Plexiglass chamber with a video camera attached at the top (DFK22AUC03 camera, Imaging Source, Charlotte, NC, USA). A 4-5 min recording was made, during which hind limb weight-bearing changes were continuously monitored. Hind paw weight distribution was calculated using Bioseb software (v1.4.2.92) using the following formula:

% weight on ipsilateral paw = (weight borne by the ipsilateral paw / weight borne by the ipsilateral paw + weight borne by the contralateral paw) × 100.

2.4 | Cell culture and transfections

The immortalized chondrocyte C-28/I2 lines were purchased from Merck Millipore, cultured in EmbryoMax DMEM
supplemented with 4500 mg/L of Glucose, 2.25 g/L of Sodium Bicarb and L-Glut, without Sodium Pyruvate, 10% FCS (Merck Millipore, Hertfordshire, UK) and maintained at 5% CO₂. Human Articular Chondrocytes (HACs) were purchased from Cell-Applications Inc and maintained in Chondrocyte Growth Medium (Cell-Applications; Sigma-Aldrich, Poole, UK) at 5% CO₂. HACs were used after three passages maximum.

High-density micromass cultures were generated, as described. In some cases, prior to stimulations (as indicated in individual figure legends), the micromass were serum-starved for 24 hours in Phenol Red-Free DMEM/Ham’s F12 (1:1; Sigma-Aldrich, Poole, UK), supplemented with 1% of Insulin-transferrin-selenium G supplement (ITS; Invitrogen, Paisley, UK) to allow for collagen type 2 and aggrecan transcription. Some chondrocytes were transfected with JetPrime transfection reagent (Polyplus-transfection, Illkirch, France) and either SERPINA1-His-Bio (Addgene, Watertown, MA, USA) or eGFP (Addgene, Watertown, MA, USA) for 24 hours in the presence of 10% FCS and then, grown in 3D micromass cultures for additional 24 hours prior to experimentation, as indicated in individual figure legends.

HEK293 cells were maintained in DMEM supplemented with 10% of FCS and maintained at 5% CO₂.

2.4.1 | Luciferase reporter assays

Sub-confluent HEK293 cells, grown in monolayer in the presence of serum (10% FCS), were transfected with JetPrime, with > 90% transfection efficiency. For CREB signaling interrogation, cells were transfected with a luciferase reporter driven by reiterated consensus CRE-binding sites (CRE-firefly luciferase, Promega, Hampshire, UK), henceforth referred to as CREB Reporter, plus CMV-Renilla luciferase plasmid (in a ratio 1:100). For Wnt/β-catenin signaling interrogation, cells were transfected with TCF/LEF-firefly luciferase reporter vector (SUPER8XTOPFlash, referred to as TOPFlash) plus CMV-Renilla luciferase plasmid (in a ratio 1:100). Twenty-four hours after transfection, the medium was replaced, and the cells were treated for 24 hours as specified in individual figure legends. Luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega, Southampton, UK).

2.6 | Biochemical analyses

2.6.1 | Alcian Blue staining of sulfated glycosaminoglycans

Cartilage-specific sulfated glycosaminoglycans deposition was quantified, as previously described. Briefly, micromasses were fixed with 4% glutaraldehyde solution (v/v in ddH₂O) and submerged in Alcian Blue 8GX dye (1% in 0.1N HCl w/v; pH 0.2; Atom Scientific, Cheshire, UK) for 24 hours at room temperature (RT). Alcian Blue dye was extracted in guanidine-HCl (8 M; Sigma-Aldrich, Poole, UK) for 48 hours at RT. Concentration was quantified by interpolation of A₅₃₀ of the extracted dye with Alcian Blue standard curve and normalized to DNA content (ng/µg). DNA content was measured in the extracted dye solution by fluorescence (485/535nm) using the SYBR Green method, according to the manufacturer’s instructions (OriGene Technologies GmbH, Herford, Germany).

2.6.2 | Quantification of cytokine release

Plasma levels of mouse cytokines in cell-free supernatants were measured using a Ready-Set-Go ELISA Kits (eBioscience, Hatfield, UK).
2.6.3 | Western blot analyses

Western blot analyses were conducted for signaling pathways interrogation in chondrocytes. Antibodies and dilutions used are listed in Table 1. Briefly, stimulated cell monolayers were washed in ice-cold PBS and lysed in ice-cold RIPA Lysis buffer, supplemented with EDTA-free Protease Inhibitor cocktail (Sigma-Aldrich, Poole, UK) and Phosphatase Inhibitor Cocktail 2/3 (Sigma-Aldrich, Poole, UK) for 30 minutes on ice. Protein concentrations in cleared cell lysates were determined by bicinechonic acid protein assay (Pierce BCA Protein Assay Kit, Thermo Fisher Scientific, Paisley, UK). Samples (20 µg total protein) were prepared for SDS-PAGE on 10% (w/v) NuPAGE Bis-Tris gels (Invitrogen, UK) and transferred onto Nitrocellulose membranes (BioTrace, Pall Corporation, Port Washington, NY, USA). Blots were then blocked with 5% BSA in 0.1% TBS-Tween (TBS-T) and incubated with primary antibodies at the concentrations listed in Table 1 overnight at 4°C. For α-Tubulin immunoblotting, His-AAT or NE expression, membranes were blocked in 5% fat-free milk protein in 0.1% TBS-T (Blotto, Santa Cruz Biotechnology, Heidelberg, Germany). After four 5-minute washes in 0.1% TBS-T, some membranes were incubated for 45 minutes with secondary HRP-conjugated Immunoglobulins, and after further 3-5 washes, protein bands were visualized chemiluminescence (Luminata Forte; Merck Millipore, Hertfordshire, UK) using FluorChem E imaging system (Protein Simple, CA, USA). Measurements of band densitometry and quantification of protein expression were conducted using ImageJ (NIH). Phosphorylated protein expression was normalized to total protein levels and to α-Tubulin (loading control).

2.7 | Statistical analysis

All qPCR data are reported as Mean ± SEM unless otherwise indicated in individual figure legends. Significant differences in qPCR experiments were determined with the nonparametric Kruskal-Wallis ANOVA test, followed by Dunn’s multiple comparison test. AB staining and histology-staining experiments were analyzed using one-way ANOVA, followed by Dunnett’s multiple comparison posttest; Joint inflammation data (knee diameter, vascular conductance, and leukocyte trafficking) were analyzed using Student’s unpaired t test. Joint pain (von Frey, dynamic weight bearing) data were analyzed using one-way ANOVA test. All statistical analyses were performed using GraphPad Prism 5.0, (GraphPad Software, CA, USA). Values were considered significant, if \( P < .05. \)

3 | RESULTS

3.1 | Systemic AAT ameliorates cartilage damage in murine KBxN inflammatory arthritis

To complement our initial study with intra-articular delivery of AAT, we tested if systemic AAT could protect the arthritic joint. Following intra-peritoneal administration of AAT, while we observed minimal modulation of the clinical parameters of arthritis (Figure S1), histological analysis of knee sections revealed discernible proteoglycan loss from cartilage in arthritic joints: sulfated glycosaminoglycan content was reduced by > 30%, compared to vehicle-injected mice, an effect partially reversed by AAT (Figure 1A, B).

### Table 1 | Antibodies used for interrogation of signaling pathways

| Antibody | Dilution | Product code | Producer |
|----------|----------|--------------|----------|
| Rb anti-pSmad2 (Ser465/467) | 1:1000 | 3108 | Cell Signaling |
| Rb anti-Smad2 | 1:1000 | 5339 | Cell Signaling |
| Rb anti-pSmad3 (Ser423/425) | 1:1000 | 9520 | Cell Signaling |
| Rb anti-Smad3 | 1:1000 | 9523 | Cell Signaling |
| Rb anti-pCREB (Ser133) | 1:1000 | 9198 | Cell Signaling |
| Rb anti-CREB | 1:1000 | 4820 | Cell Signaling |
| Rb anti-β-Catenin | 1:1000 | 9562 | Cell Signaling |
| Ms anti-α-Tubulin (HRP) | 1:3000 | ab40742 | Abcam |
| Rb anti-6X His-Tag® (HRP) | 1:5000 | ab1187 | Abcam |
| Rb anti-AAT | 1:5000 | ab166610 | Abcam |
| Rb anti-NE | 1:1000 | ab68672 | Abcam |
| Goat anti-Rabbit Immunoglobulins/HRP | 1:2000 | P0448 | Dako |

Abbreviation: Rb, rabbit.
A marked accumulation of leukocytes occurred in the joints alongside pannus formation and synovial hypertrophy, all reduced by AAT (Figure 1C, D).

To test indications of potential phenotypic alterations in the joints of AAT-treated mice, transcription of key cartilage-relevant genes was monitored. On day 5 of arthritis, sox9 was downregulated in the arthritic paws, with col2a1 and acan expression being unaffected (Figure 2). The cytokine il1 and cartilage-destructive proteases adams5 and mmp13 gene products were all upregulated (Figure 2A). Following systemic delivery of AAT, sox9 was upregulated alongside its transcriptional targets, col2a1 and acan, and over-expression of mmp13 and adams5 was blocked. AAT reduced il1 gene expression and circulating serum levels of both IL-1β and IL-6, while IL-10 remained unaffected (Figure 2B).

3.2 | AAT ameliorates joint inflammation and pain in models of arthritis

We expanded these data by testing AAT in three other models. Persistent inflammation was observed within the knee joint microvasculature of CFA-injected rats during a 3-week time course (not shown). Prophylactic treatment with AAT decreased knee swelling at day 21 (Figure 3A). Intravital microscopy revealed that the number of rolling and adherent leukocytes was significantly reduced by AAT (Figure 3B, C). This treatment also affected other parameters including knee swelling, leukocyte kinetics, and mechanical hypersensitivity (Figure 3A-D). CFA injection induced a late alldynic response, causing a decrease in withdrawal threshold on days 7-21 (Figure 3D, F) and considerable weight-bearing deficits on days 1-17 (Figure 3E). AAT (100 µg ip; every 2 days) prevented mechanical hypersensitivity over the whole treatment (Day 0-7; Figure 3F). Although AAT treatment marginally improved weight-bearing deficits, the changes recorded did not reach statistical significance (Figure 3E).

Kaolin/Carrageenan i.a. injection increased joint swelling, vascular conductance, the number of rolling, and adherent leukocytes, all markers reduced by AAT (Figure S2A-D).

We and others have detected large amounts of NE complexed with AAT in synovial fluids of rheumatoid arthritis, and to a lesser extent in osteoarthritic samples (Figure S3A; Table S2). To establish if AAT anti-arthritic effects observed here related to NE-inhibition, AAT was pre-mixed with NE at a marked molar deficit, insufficient to cause enzymatic inhibition (100 ng AAT-5 µg NE). Following injection into the mouse knee joint, a much lower degree of proteoglycan loss was observed in the group receiving AAT (Figure 4A, B). Following NE, ~75% of joints presented mild synovitis, compared to ~25% of the mice receiving NE + AAT (Figure 4C, D). NE increased hind paw mechanical sensitivity by 75%,
and this was partially rescued by AAT (Figure 4F). Joint nociception following NE-injection was abrogated by AAT (Figure 4J), alongside 10% reduction in NE-induced knee swelling (Figure 4G), and ~60% in leukocyte rolling and adhesion (Figure 4D, E).

These results suggest that AAT prevents the development of pain behavior, joint inflammation, and cartilage degradation in arthritis at least partially through mechanisms outside of NE-inhibition.

### 3.3 AAT suppresses Wnt signaling and activates CREB signaling in chondrocytes

Next, we examined if AAT supported chondrogenic differentiation of chondrocytes in the absence of inflammatory stimuli. Unstimulated chondrocytes do not express endogenous NE (Figure 5A, Figure S3B), therefore, any function exerted by AAT in chondrocytes must be independent of its anti-inflammatory/anti-elastolytic effects. Indeed, both rAAT and AAT gene transfer promoted deposition of Alcian Blue-positive ECM (Figure 6A, E), and this coincided with upregulation of SOX9, COL2A1, and ACAN gene products (Figure 6B, F).

To gain information on the pathways directly engaged by AAT, we interrogated the Smad, Wnt/β-catenin, and protein kinase A(PKA)/cAMP response element-binding protein (CREB) signaling networks, all of which may have a role in chondrogenic differentiation. Initial experiments for Smad2/3 phosphorylation indicated lack of modulation upon AAT application (Figure S3).

Serpins, including AAT, have been reported to modulate Wnt signaling in various cell types. Using the Wnt reporter assay TOPFlash, we discovered that AAT potently inhibited the capacity of Wnt3A to induce β-catenin accumulation in chondrocytes, even in a polymerized
(non-inhibitory) form (Figure 5B, C, F). The nodal point of this inhibition occurred downstream of the Wnt receptors, because AAT still suppressed the Wnt reporter assay when activated in a ligand- and receptor-independent manner, by application of the glycogen synthase kinase 3 beta (GSK-3β) inhibitor 6-bromoiridin 3'-oxime (BIO) (Figure 5C, D).

Through interrogation of the PKA/CREB signaling pathway (Figure 6C, D, G, H), we could reveal that both rAAT and SERPINA1 transfection activated CREB signaling in chondrocytes: augmented CREB phosphorylation and activation of the CREB reporter assay were detected, with a boosted chondrocyte anabolism (Figure 6A, B, E, F). PKA inhibition abrogated AAT-induced CREB phosphorylation and activation of CREB-reporter assay (Figure 7B, C), and blocked the anabolic properties of AAT on chondrocytes, as quantified by normalized Alcian Blue staining (Figure 7A).

Overall, these findings demonstrate (i) a dissociation between AAT’s anti-inflammatory effects, potentially linked to NE inhibition, and its chondroprotective anabolic and anti-catabolic functions, and (ii) an induction of cartilage-protective pathways through a possible receptor-mediated signaling mechanism.

4 | DISCUSSION

Rheumatoid arthritis is a debilitating disease characterized by joint inflammation, pain, and structural damage. Patients affected by RA experience loss of function and pain, two major symptoms which markedly impact on quality of life. Despite recent successes in therapeutic developments, there is still a lack of new therapies that can target cartilage repair or prevent further damage if not reversing the disease.

We have recently identified AAT as a pivotal anti-arthritic molecule with anti-inflammatory and cartilage-protective effects when administered locally into murine arthritic joints.19 The results presented herein expand on these findings by demonstrating that systemic administration of AAT suppressed joint inflammation and cartilage damage in distinct models of rodent inflammatory arthritis and provided significant reduction in arthritic nociception. Crucially, AAT displayed independent anabolic effects on cartilage: it increased ECM production and upregulated expression of key chondrogenic genes in the absence of inflammatory stimuli or NE. These effects were associated with downstream inhibition of Wnt/β-catenin and activation of PKA/CREB signaling.
pathways in chondrocytes. Taken together, these data indicate that AAT is a versatile anti-arthritic agent that has the potential to promote cartilage repair and dampen down joint inflammation and pain.

Although the exact mechanisms underlying the therapeutics effect of AAT in arthritis in vivo remain to be further scrutinized, several hypotheses were considered here. The first one is centered on inhibition of inflammatory cytokine production. Various cytokines, including IL-1β, and TNF-α, play major roles in the pathogenesis of RA and strategies which block their activity have proven to be effective treatments. AAT-deficient individuals lack control over inflammatory mediators, including IL-1β, IL-6, IL-8, and TNF-α, and stimulation of peripheral blood mononuclear cells with AAT reduces their pro-inflammatory signature. Furthermore, Janciauskiene and colleagues reported that AAT’s inhibition of endotoxin-stimulated TNF-α and enhancement of IL-10 in human monocytes, are mediated by elevation of cAMP and activation of cAMP-dependent protein kinases. Additionally, in a model of gouty arthritis, AAT-Fc fusion protein was shown to reduce joint swelling and cellular infiltration through inhibition of protease-dependent processing/release of IL-1β. Building on our previous study with intra-knee injection of AAT, we observed that a single, low.

**FIGURE 4** AAT protects against NE-induced cartilage degradation, knee inflammation, and pain. C57BL/6 mice were injected with either PBS (5 µL) and NE (5 µg/5 µL), or AAT (100 ng/5 µL) and NE (5 µg/5 µL) into the right knee joint. After 4 hours, the knees were harvested, paraffin embedded, and multiple coronal sections (5-µm) were stained with H&E or Toluidine blue. A, Representative images (40 x) of knee joint microstructure with evident signs of proteoglycan depletion shown (red arrowheads); B, Cartilage integrity calculated from percentage area of cartilage positive for toluidine blue staining; C, Representative images (40x magnification) of histological sections from naive and arthritic joints stained with H&E; D, Histomorphometric analyses of joint sections. Scale bars: 200 µm. m, meniscus; F, femur; T, tibia. In separate experiments, treatment with AAT reduced G, knee diameter, H, vascular conductance, and the number of (E) rolling and (F) adherent leukocytes at 4 hours post-injection, compared to NE alone (yellow arrows indicate stained leukocytes). NE i.a. injection caused a significant secondary allodynia I, and weight-bearing deficits J, counteracted by AAT co-administration. Data presented as Mean ± SEM. *P < .05, **P < .01, ***P < .0001; Student’s unpaired t test; n = 8-14 (A-H), one-way ANOVA followed by Bonferroni’s posttest; n = 6-12 (I, J).
dose systemic injection of recombinant AAT is sufficient to reduce joint inflammation and cartilage proteoglycan depletion. AAT inhibited local IL-1β gene expression, circulating levels of the cytokine and reduced expression of Adams5 and MMP-13 genes within the joint. Simultaneously, AAT upregulated expression of the chondro-specific master transcription regulator Sox9 and its downstream targets collagen type II and aggrecan. AAT also inhibited proliferative synovitis and neutrophil infiltration into the synovial fluid and pannus development in the KBxN poly-arthritis model.

Since the major histological characteristics of inflammatory arthritis are proliferative synovitis and leukocyte infiltration, it is plausible that neutrophil proteases contribute to cartilage degradation and overall joint injury. Inflammatory stimuli activate neutrophils to release the contents of azurophilic granules, including NE, cathepsin-G, and proteinase-3, that proteolytically modify chemokine and cytokine activity, interact with cell-surface receptors and contribute to neutrophil migration by cleaving adhesion molecules. In particular, NE degrades elastin, collagens, proteoglycans, and other ECM components and its physiological activity is regulated by endogenous inhibitors, with AAT being the most potent and with highest affinity. It is well documented that NE activity and expression are increased in synovial fluids and synovia of patients with RA. Experimentally, the incidence of arthritis is reduced through the use of NE inhibitors, confirming its contributory role to disease development. To test this hypothesis, we applied AAT at a dose well below levels needed for enzymatic inhibition of NE: for complete NE neutralization to occur, a molar ratio of AAT:NE > 2:1 is needed. Consistent with previous studies where both recombinant NE and NE-rich RA synovial fluids degraded cartilage proteoglycans, here NE injection into murine knee joints caused rapid proteoglycan loss from the cartilage, accompanied by a generalized knee inflammation. Treatment with AAT at a dose less than 1/100 than that necessary to block NE, still prevented leukocyte infiltration, improved cartilage integrity, reduced synovial hyperaemia, and significantly ameliorated joint pain.

To investigate further whether the beneficial properties of AAT were distinct from NE inhibition, experiments were conducted with isolated chondrocytes, which do not express NE. These cells responded to both recombinant AAT stimulation and SERPINA1 transfection by activating biosynthetic pathways resulting in augmentation of anabolic gene expression, including the chondrogenic factor Sox9. Major

**FIGURE 5** AAT inhibits canonical Wnt-signaling. A, Neutrophil elastase (NE) expression was analyzed by Western blotting in lysates (25 µg) from unstimulated C28/I2s and primary neutrophils; rNE (2 µg) was used for positive control (full blot in Supplementary Figure S3B). B, D, Chondrocytes were stimulated for 6 hours with vehicle, Wnt3A (100 ng/mL) or BIO (20 µM) in presence or absence of AAT (10 µg/mL). Western blots show representative experiments out of three performed; α-tubulin was tested as loading control. C, F, HEK293 cells transiently transfected with TOPFlash and Renilla reporter plasmids were stimulated with either vehicle, Wnt3A (100 ng/mL) alone or in the presence of native or polymerized AAT (0.1-10 µg/mL). E, TOPFlash and Renilla transfected HEK293 cells were transfected with GFP or SERPINA1-His-Bio for 24 hours and then, stimulated with either vehicle, or BIO (20 µM) for further 24 hours. Luciferase activity standardized to Renilla luciferase activity was calculated to yield relative luciferase activity (RLU) then expressed as fold change over vehicle-treated cells as control. Statistical analyses were conducted with one-way ANOVA, followed by Bonferroni multiple-comparison posttest with *P < .05, **P < .001, ***P < .0001 vs respective control.
cartilaginous extracellular matrix components, aggrecan and collagen type II were also upregulated, indicating that in the absence of inflammation, AAT activates pathways involved in chondrogenic differentiation and might directly contribute to the cartilage protection observed in experimental arthritis.

Based on these results, we hypothesized that AAT could be interacting with some of the signaling networks controlling chondrogenic differentiation, including Smad2/3, PKA/CREB, and Wnt/β-catenin signaling. Early studies have indicated that impaired ALK5/Smad2/3 signaling is implicated in cartilage destruction in arthritis; however,
in the current study AAT did not activate either transcription factor. In contrast, the canonical Wnt pathway regulates multiple biological and pathological processes including angiogenesis and inflammation. Relevantly, it regulates chondrocyte differentiation in health and disease, and while moderate Wnt activity is essential for chondrocyte proliferation and maintenance of their typical characteristics, excessive activation results in chondrocyte hypertrophy, and expression of cartilage-degrading MMPs. Recently, the involvement of canonical Wnt/β-catenin signaling pathway in the pathophysiology of cartilage degenerative disease has attracted much attention: the arthritic joint is well known to express an abundance of Wnt ligands and overactivation of Wnt-signaling pathway is a major contributing factor toward the progression of both rheumatoid arthritis and osteo-arthritis. Therefore, Wnt inhibition may represent a promising strategy for disease modification in osteoarthritis and possibly RA and this notion is supported by successful early clinical studies. In the absence of Wnt ligands, cytoplasmic β-catenin is degraded by a complex composed of the constitutively active kinase GSK-3β, casein kinase I (CKI), adenomatous polyposis coli (PC1), and Axin. Upon Wnts engaging with their co-receptor complex (Frizzled and low-density lipoprotein-related protein (LRP5/6)) GSK-3β is inactivated. In this condition, β-catenin accumulates, translocates into the nucleus, and together with TCF/LEF transcription factors activates transcription of Wnt target genes.

**Figure 7** AAT stimulates chondrogenic differentiation through a PKA/CREB-dependent mechanism. A. HACs grown in 3D micromass cultures were serum starved for 24 hours and stimulated with Vehicle, AAT (10 µg/mL) or Fsk (30 µM) in presence or absence of H-89 (20 µM) for 24 hours. Representative images of micromasses stained with Alcian Blue for the detection of sulfated glycosaminoglycans and spectrophotometric quantification of guanidine-HCl-extracted Alcian Blue dye normalized to DNA content (µg/µl). B. CREB-phosphorylation as analyzed by Western blotting after 1h stimulation of monolayer HACs with Fsk or AAT at the indicated concentrations and presented as fold change over control. C. CREB-related gene activation was analyzed by reporter assay 24 hours after stimulations of HEK293 cells transiently transfected with CREB and Renilla reporter plasmids. Luciferase activity standardized to Renilla luciferase activity was calculated to yield relative luciferase activity (RLU) expressed as fold change over DMSO-treated controls. Data are Mean ± SEM (n = 3) and statistical analyses were conducted with two-way ANOVA, followed by Bonferroni posttest with *P < .05, **P < .01, ***P < .001 vs respective control.
Wnt-signaling has been implicated in the initiation and progressive deterioration of cartilage degeneration in arthritis, suggests that AAT inhibition of the canonical pathway in vivo could counteract cytokine-induced cartilage degradation.

This hypothesis is partially supported by the present study, where AAT significantly suppressed il1 transcription and IL1β plasma levels, and at the same time abrogated mmp13 transcription. Further investigations are required to confirm this mechanism of action.

cAMP is another important regulator of chondrocyte differentiation. Higher intracellular cAMP levels promote transcription of type II collagen and aggrecan. Extracellular stimuli activating adenylyl cyclase regulate the levels of cAMP, which activates PKA, which controls the expression of cAMP-inducible genes via phosphorylation of nuclear transcription factors like CREB. Elevation of cAMP activates pro-resolving pathways and reduces pro-inflammatory cytokine signaling in various cell types, including chondrocytes. For example, melanocortin receptor agonists, calcitonin, and the direct activator of adenylyl cyclase Forskolin reduce pro-inflammatory cytokines and promote chondrogenic processes through the elevation of intracellular cAMP. Our results demonstrate that AAT activates CREB in chondrocytes through a PKA-dependent pathway. Such an effect is in keeping with previous reports.

Phosphorylated CREB then undergoes nuclear translocation and binding to CRE sites on target genes, promoting deposition of matrix by the chondrocytes, as shown elsewhere. The engagement of the PKA/CREB pathway by AAT was confirmed with the PKA inhibitor H-89, which impaired downstream CREB signaling and AB-positive proteoglycan deposition. Although it is plausible that AAT-induced SOX9 upregulation is CREB-dependent, as CREB is a transcription factor regulating SOX9 promoter activation and Sox9 phosphorylation, the involvement of other pathways cannot be excluded at present.

The simultaneous CREB-activation and Wnt-inhibition, which was recently described to be a potent driver of chondrogenesis and cartilage regeneration, is a discovery central for understanding AAT’s mechanism of action in chondrocytes and one that, being the focus of ongoing endeavors, could prove to be central in driving AAT’s anti-arthritic properties.

In conclusion, we show here that in addition to its known anti-proteolytic and broad anti-inflammatory properties, AAT can independently function as an acute phase mediator of anti-nociception and chondroprotection. We partly elucidated the specific signaling pathways responsible for these functional outcomes post-AAT application, in vitro and in vivo. As such, these findings reveal the potential dual benefits of using AAT to preserve articular cartilage and alleviate arthritic nociception and inflammation.

ACKNOWLEDGMENTS
The authors acknowledge Dr Adrian Moore for his helpful advice on the project and on his contributions toward the first draft of the manuscript.

CONFLICT OF INTEREST
Professor Dell’Accio has received consultancy fees from Samumed and UCB Pharma for work not related to this study. Dr Krustev reports Investments in Gilead Sciences Inc and Intercept Pharmaceuticals Inc, not relating to the current work.

AUTHOR CONTRIBUTIONS
M.K. Kaneva, M.M. Muley, J.J. McDougall, and M. Perretti designed research; M.K. Kaneva and M.M. Muley performed research and analyzed data with contributions from E. Krustev, A.R. Reid, and P.R. Souza; F. Dell’Accio and J.J. McDougall provided new reagents/analytical tools; M.K. Kaneva and M. Perretti wrote the manuscript; M.K. Kaneva, M.M. Muley, F. Dell’Accio, J.J. McDougall, and M. Perretti provided significant intellectual input and reviewed initial drafts of the paper.

REFERENCES
1. Turino GM, Senior RM, Garg BD, Keller S, Levi MM, Mandl I. Serum elastase inhibitor deficiency and agrl-antitrypsin deficiency in patients with obstructive emphysema. Science. 1969;165(3894):709-710. http://dx.doi.org/10.1126/science.165.3894.709
2. Janciukasiene S. Conformational properties of serine proteinase inhibitors (serpins) confer multiple pathophysiological roles. Biochim Biophys Acta. 2001;1535(3):221-235. http://dx.doi.org/10.1016/s0925-4439(01)00025-4
3. Bergin DA, Hurley K, McElvaney NG, Reeves EP. Alpha-1 antitrypsin: a potent anti-inflammatory and potential novel therapeutic agent. Arch Immunol Ther Exp (Warsz). 2012;60(2):81-97. http://dx.doi.org/10.1007/s00005-012-0162-5
4. Travis J, Shieh BH, Potempa J. The functional role of acute phase plasma proteinase inhibitors. Tokai J Exp Clin Med. 1988:313-320.
5. Potempa J, Korzus E, Travis J. The serpin superfamily of proteinase inhibitors: structure, function, and regulation. J Biol Chem.
sulfide donor in acute monoarthritis. *AJP Regul Integr Comp Physiol.* 2008;295(3):R814-R820. https://doi.org/10.1152/ajpregu.90524.2008

22. Krustev E, Reid A, McDougall JJ. Tapping into the endocannabinoid system to ameliorate acute inflammatory flares and associated pain in mouse knee joints. *Arthritis Res Ther.* 2014;16. https://doi.org/10.1186/s13075-014-0437-9

23. Muley MM, Reid AR, Botz B, Bölcskei K, Helyes Z, McDougall JJ. Neutrophil elastase induces inflammation and pain in mouse knee joints via activation of proteinase-activated receptor-2. *Br J Pharmacol.* 2016;173(4):766-777. https://doi.org/10.1111/bph.13237

24. Chapman SR, Bach FW, Pogrel JW, Chung JM, Yaksh TL. Quantitative assessment of tactile allodynia in the rat paw. *J Neurosci Methods.* 1994;53(1):55-63. https://doi.org/10.1016/0165-0270(94)90144-9

25. Dixon WJ. Efficient analysis of experimental observations. *Annu Rev Pharmacol Toxicol.* 1980;20(1):441-462. https://doi.org/10.1146/annurev.pa.20.040180.002301

26. Philpott HT, O’Brien M, McDougall JJ. Attenuation of early phase inflammation by cannabidiol prevents pain and nerve damage in rat osteoarthritis. *Pain.* 2017;158(12):2442-2451. https://doi.org/10.1097/j.pain.0000000000001052

27. Veeman MT, Slusarski DC, Kaykas A, Louie SH, Moon RT. Zebrafish prickle, a modulator of noncanonical Wnt/Fz signaling, regulates gastrulation movements. *Curr Biol.* 2003;13(8):680-685. https://doi.org/10.1016/S0960-923X(03)00240-9

28. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2−ΔΔCT method. *Methods.* 2001;25(4):402-408. https://doi.org/10.1016/S1046-8073(00)00449-X

29. Schneider CA, Rasband WS, Eliceiri KW. NIH Image to ImageJ: 25 years of image analysis. *Nat Methods.* 2012;9(7):671-675. https://doi.org/10.1038/nmeth.2089

30. Momohara S, Kashiwazaki S, Inoue K, Saito S, Nakagawa T. Elastase from polymorphonuclear leukocyte in articular cartilage and synovial fluids of patients with rheumatoid arthritis. *Clin Rheumatol.* 1997;16(2):133-139. https://doi.org/10.1007/BF01694784

31. Potilinski MC, Ortíz GA, Salica JP, et al. Elucidating the mechanism of action of alpha-1-antitrypsin using retinal pigment epithelium cells exposed to high glucose. Potential use in diabetic retinopathy. *PLoS One.* 2020;15:e0228895.

32. Zhang B, Abreu JG, Zhou K, et al. Blocking the Wnt pathway, a unifying mechanism for an angiogenic inhibitor in diabetic retinopathy. *J Invest Dermatol.* 2014;134(6):1725-1734. https://doi.org/10.1038/jid.2014.40

33. McBride JD, Jenkins AJ, Liu X, et al. Elevated circulation levels of an antiangiogenic serpin in patients with diabetic microvascular complications impair wound healing through suppression of Wnt signaling. *J Invest Dermatol.* 2014;134(6):1725-1734. https://doi.org/10.1038/jid.2014.40

34. McIntees IB, Schett G. Cytokines in the pathogenesis of rheumatoid arthritis. *Nat Rev Immunol.* 2007;7(6):429-442. https://doi.org/10.1038/nri2094

35. Salgado E, Maneiro JR. New therapies for rheumatoid arthritis. *Med Clin (Barc).* 2014. https://doi.org/10.1016/j.medcli.2013.11.011

36. Malerba M, Ricciardolo F, Radaeli A, et al. Neutrophilic inflammation and IL-8 levels in induced sputum of alpha-1-antitrypsin PiMZ subjects. *Thorax.* 2006;61(2):129-133. https://doi.org/10.1136/thx.2005.043471
37. Pott GB, Chan ED, Dinarello CA, Shapiro L. α-1-Antitrypsin is an endogenous inhibitor of proinflammatory cytokine production in whole blood. *J Leukoc Biol*. 2009;85(5):886-895. https://doi.org/10.1189/jlb.0208145

38. Janciauskiene S, Larsson S, Larsson P, Virtala R, Jansson L, Stevens T. Inhibition of lipopolysaccharide-mediated human monocyte activation, in vitro, by α1-antitrypsin. *Biochem Biophys Res Commun*. 2004;321:592-600.

39. Janciauskiene SM, Nita IM, Stevens T. α₁-antitrypsin, old dog. *New Tricks J Biol Chem*. 2007;282:8573-8582.

40. Pham CTN. Neutrophil serine proteases: specific regulators of inflammation. *Nat Rev Immunol*. 2006;6(7):541-550. https://doi.org/10.1038/nri1841

41. Watanabe H, Hattori S, Katsuda S, Nakanishi I, Nagai Y. Human neutrophil elastase: degradation of basement membrane components and immunolocalization in the tissue. *J Biochem*. 1990;108:753-759.

42. Loebermann H, Tokuoka R, Deisenhofer J, Huber R. Human α₁-proteinase inhibitor. Crystal structure analysis of two crystal modifications, molecular model and preliminary analysis of the implications for function. *J Mol Biol*. 1984;177(3):531-557. https://doi.org/10.1016/0022-2836(84)90298-5

43. Moore AR, Appelboam A, Kawabata K, et al. Destruction of articular cartilage by alpha2 macroglobulin elastase complexes: role in rheumatoid arthritis. *Ann Rheum Dis*. 1999;58:109-113.

44. Kakimoto K, Matsukawa A, Yoshinaga M, Nakamura H. Suppressive effect of a neutrophil elastase inhibitor on the development of collagen-induced arthritis. *Cell Immunol*. 1995;165(1):26-32. https://doi.org/10.1016/cimm.1995.1183

45. James HL, Cohen AB. Mechanism of inhibition of porcine elastase by human alpha-1-antitrypsin. *J Clin Invest*. 1978;62:1344-1353.

46. Yasuhara R, Ohta Y, Yuasa T, et al. Roles of β-catenin signaling in phenotypic expression and proliferation of articular cartilage superficial zone cells. *Lab Invest*. 2011;91(12):1739-1752. https://doi.org/10.1038/labinvest.2011.144

47. Deshmukh V, Hu H, Barroga C, et al. A small-molecule inhibitor of the Wnt pathway (SM04690) as a potential disease modifying agent for the treatment of osteoarthritis of the knee. *Osteoarthr Cartil*. 2018;26(1):18-27. https://doi.org/10.1016/j.joca.2017.08.015

48. Kozhemyakina E, Lassar AB, Zelzer E. A pathway to bone: guidance molecules and transcription factors involved in chondrocyte geneic differentiation of chick limb bud mesenchymal cells. *Anim Cells Syst (Seoul)*. 2009;13:289-295. https://doi.org/10.1016/j.cellsig.2013.12.001

49. Goldman MB, Tsuchimichi K, Ijiri K. The control of chondrogenesis. *J Cell Biochem*. 2006;97(1):33-44. https://doi.org/10.1002/jcb.20652

50. Leonard CM, Fuld HM, Frenz DA, Downie SA, Massague J, Newman SA. Role of transforming growth factor-β in chondrogenic pattern formation in the embryonic limb: stimulation of mesenchymal condensation and fibronectin gene expression by exogenous TGF-β and evidence for endogenous TGF-β-like activity. *Dev Biol*. 1991;145(1):99-109. https://doi.org/10.1016/0012-1606(91)90216-P

51. Miao CG, Yang YY, He X, et al. Wnt signaling pathway in rheumatoid arthritis, with special emphasis on the different roles in synovial inflammation and bone remodeling. *Cell Signal*. 2013;25(10):2069-2078. https://doi.org/10.1016/j.cellsig.2013.04.002

52. Monteagudo S, Lories R. Cushioning the cartilage: a canonical Wnt restricting matter. *Nat Rev Rheumatol*. 2017;13(11):670-681. https://doi.org/10.1038/nrrheum.2017.171

53. Rabelo FDS, da Mota LHM, Lima RAC, et al. Wnt signaling pathway and rheumatoid arthritis. *Autoimmun Rev*. 2010;9(4):207-210. https://doi.org/10.1016/j.autrev.2009.08.003

54. Yazici Y, McAlindon TE, Fleischmann R, et al. A novel Wnt pathway inhibitor, SM04690, for the treatment of moderate to severe osteoarthritis of the knee: results of a 24-week, randomized, controlled, phase 1 study. *Osteoarthr Cartil*. 2017;25(10):1598-1606. https://doi.org/10.1016/j.joca.2017.07.006

55. Deshmukh V, O’Green AL, Bossard C, et al. Modulation of the Wnt pathway through inhibition of CLK2 and DYRK1A by lorericuvivin as a novel, potentially disease-modifying approach for knee osteoarthritis treatment. *Osteoarthr Cartil*. 2019;27(9):1347-1360. https://doi.org/10.1016/j.joca.2019.05.006

56. Nusse R, Clevers H. Wnt/b-catenin signaling, disease, and emerging therapeutic modalities. *Cell*. 2017;169(6):985-999. https://doi.org/10.1016/j.cell.2017.05.016

57. Aldonyte R, Jansson L, Janciauskiene S. Concentration-dependent effects of native and polymerised α₁-antitrypsin on primary human monocytes, in vitro. *BMC Cell Biol*. 2004. https://doi.org/10.1186/1471-2121-5-11

58. Hwang SG, Ryu JH, Kim IC, et al. Wnt-7a causes loss of differentiated phenotype and inhibits apoptosis of articular chondrocytes via different mechanisms. *J Biol Chem*. 2004;279(25):26597-26604. https://doi.org/10.1074/jbc.M401401200

59. Yuasa T, Otani T, Koike T, Iwamoto M, Enomoto-Iwamoto M. Wnt/b-catenin signaling stimulates matrix catabolic genes and activity in articular chondrocytes: Its possible role in joint degeneration. *Lab Invest*. 2008. https://doi.org/10.1038/labinvest.3700747

60. Yun K, Im SH. Transcriptional regulation of MMP13 by Lefl in chondrocytes. *Biochem. Biophys. Res. Commun*. 2007. https://doi.org/10.1016/j.bbrc.2007.10.121

61. Landman EBM, Miclea RL, van Blitterswijk CA, Karperien M. Small molecule inhibitors of WNT/b-catenin signaling block IL-1β- and TNFα-induced cartilage degradation. *Arthritis Res Ther*. 2013;15:1-11.

62. Kim K, Lee Y. Activation of CREB by PKA promotes the chondrogenic differentiation of chick limb bud mesenchymal cells. *Anim Cells Syst (Seoul)*. 2009;13:289-295.

63. Gonzalez GA, Montminy MR. Cyclic AMP stimulates somatostatin gene transcription by phosphorylation of CREB at serine 133. *Cell*. 1989. https://doi.org/10.1016/0022-8674(89)90013-5

64. Kaneva MK, Kerrigan MJ, Greco P, Curley GP, Locke IC, Getting SJ. Chondroprotective and anti-inflammatory role of melanocortin peptides in TNF-α activated human C-20/A4 chondrocytes. *Br J Pharmacol*. 2012;167.

65. Greco KV, Nalesso G, Kaneva MK, et al. Analyses on the mechanisms that underlie the chondroprotective properties of calcitonin. *Biochem Pharmacol*. 2014;91.

66. Gold M, Dolga AM, Koepke J, et al. A1-Antitrypsin modulates microglial-mediated neuroinflammation and protects microglial cells from amyloid-B-induced toxicity. *J Neuroinflammation*. 2014;11:1-11.

67. Mayr B, Montminy M. Transcriptional regulation by the phosphorylation-dependent factor creb. *Nat Rev Mol Cell Biol*. 2001. https://doi.org/10.1038/35085068

68. Juhász T, Matta C, Somogyi C, et al. Mechanical loading stimulates chondrogenesis via the PKA/CREB-Sox9 and PP2A pathways in chicken micromass cultures. *Cell Signal*. 2014. https://doi.org/10.1016/j.cellsig.2013.12.001
69. Eldridge SE, Barawi A, Wang H, et al. Agrin induces long-term osteochondral regeneration by supporting repair morphogenesis. *Sci Transl Med*. 2020;12:eaax9086.

**SUPPORTING INFORMATION**
Additional Supporting Information may be found online in the Supporting Information section.

**How to cite this article:** Kaneva MK, Muley MM, Krustev E, et al. Alpha-1-antitrypsin reduces inflammation and exerts chondroprotection in arthritis. *The FASEB Journal*. 2021;35:e21472. [https://doi.org/10.1096/fj.202001801R](https://doi.org/10.1096/fj.202001801R)