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# Early supplemental α2-macroglobulin attenuates cartilage and bone damage by inhibiting inflammation in collagen II-induced arthritis model

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## Abstract

**Objective:** To determine if early supplemental intra-articular A2M has a chondroprotective effect in CIA mice model.

**Methods:** DBA/1 mice were randomized into 4 groups (n =15/group) **:** 1) CIA + 1.2μg of A2M; 2) CIA + 0.8μg of A2M; 3) CIA + 0.4μg of A2M; 4) Vehicle + PBS. A2M was injected into right ankles and PBS was injected into the left ankles simultaneous as internal control at day 36, 43 and 50. The CIA inflammation clinical score and ankle thickness were recorded every other day starting on day 21 until sacrifice. Changes in inflammation were monitored by in vivo FMT. Inflammation, cartilage and bone damage were assessed with X-ray, histology and immunohistochemistry. Cartilage and inflammation-related gene expression was quantified by RT-PCR.

**Results:** All mice showed ankle inflammation on day 33. After day 43, lower clinical scores, ankle thickness and SHM scores in ATM-treated ankles compared with PBS-treated ankles. FMT data indicated that the inflammation markers MMPSense and ProSense were significantly elevated in the PBS-treated ankles than A2M-treated. Histology and X-ray analyses indicated that A2M administration resulted in lower levels of inflammatory infiltration and synovial hyperplasia, as

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Ethics approval and consent to participate

This study did not need consent from any individuals/patients because no humans were involved in this study. Ethical approval was needed for this study and it was approved by Shanxi Medical University (approved protocol 2015002).

**Disclosures:** None of the authors have a conflict of interest.

well as more typical cartilage and bone organization with increased COL II and Aggrecan staining when compared with PBS-treated ankles. In addition, RT-PCR showed that MMP-3, −9, −13, COL X and Runx2 were significantly less expressed in A2M-treated groups than PBS-treated animals.

**Conclusion:** Early supplemental intra-articular A2M exerts an anti-inflammatory effect and attenuates cartilage and bone damage in CIA model.

### Keywords

Inflammation; α2-macroglobulin; Fluorescence molecular tomography; CIA; Cartilage and bone damage

## Background

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by the destruction of joints, functional impairment, and disability [1]. The disease shows a steady progression of synovial hyperplasia and neovascularization, mixed mononuclear and granulocytic cellular infiltration, damage to articular cartilage, bone remodeling, and proliferation of both synovial and extra-articular fibroblasts [2, 3].

Imbalances between inflammatory and anti-inflammatory cytokines play a critical role in the initiation and progression of inflammatory arthritis.[4] Inflammatory cytokines, matrix metalloproteinases (MMPs), chemokines, and reactive oxygen play a major role in the pathological processes of RA [5]. A variety of rodent arthritis models have been used to study arthritis disease progression and the impact of promising new therapies. Type II collagen-induced arthritis (CIA) in DBA/1 mice is a useful model of RA, as it possesses many of the cell and humeral immunity characteristics found in human RA [6]. High levels of tumor necrosis factor-alpha. (TNFα), MMPs and other inflammation factors in the joint cause damage to cartilage, bone, and other joint tissues in these animal models [78–11]. Although several single inhibitors, including AMD3100-blocking SDF-CXCR4, anti-TNFα and IL-1 have been tested in animal studies [5, 12–15], only minor or partial inhibiting effects were observed. This may be because there is no single inhibitor that can repress most of the catabolic factors at play. Furthermore, the presence of neutralizing anti-drug antibodies is associated with lower serum levels of the anti-TNFα biologics, leading to lower efficacy and higher withdrawal rate in the RA patients [13]. Therefore, repressing all or majority inflammatory factors is critical in preventing cartilage and bone erosion in RA.

Alpha2-macroglobulin (A2M) is a 720KDa glycoprotein, and it is the most abundant proteinase inhibitor in the blood [16]. A2M binds and inhibits MMPs, plasmin [16], and all classes of endoproteases [17, 18]. Recent studies reported that A2M is a powerful inhibitor of many cartilage catabolic factors and that it can attenuate post-traumatic osteoarthritis (OA) cartilage degeneration [9, 19]. Studies have also shown that A2M inhibits a disintegrin and metalloproteinase with thrombospondin motifs-4 (ADAMTS-4), ADAMTS-5, ADAMTS-7, ADAMTS-12, IL-1 and MMP-13 activity [17, 18, 20–22].

Since RA is a chronic inflammatory disease that causes cartilage and bone damage, and A2M is a master inhibitor for most inflammation factors, we hypothesize that intra-articular

supplementation of A2M could potentially slow down or prevent cartilage and bone damage in a CIA mouse model by neutralizing these catabolic enzymes. To establish the functional role of A2M in RA treatment, we quantified the concentrations of cartilage catabolic factors and their gene expression after A2M supplementation. To assess the effects of A2M on inflammation, cartilage and bone damage *in vivo*, we evaluated clinical changes through serial documenting of inflammation clinical score and paw thickness. We used the *Sharp/van der Heijde method* (SHM) to assess radiograph changes. Changes in cartilage catabolic enzymes were monitored *in vivo* by fluorescence molecular tomography (FMT), a new advanced method for measuring certain catabolic proteases level *in vivo* [23]. Moreover, histological and immunohistochemistry analyses as well as RT-PCR were used to compare structural changes in cartilage, bone, and synovial membrane in A2M vs PBS-treated animals in a CIA mouse model. Our results indicated that A2M significantly reduced the redness and swelling of the inflammatory process, as well as cartilage and bone damage.

## Materials and Methods

### Induction of CIA and Clinical assessment of CIA

CIA model was induced using 2-month-old mice of DBA/1 strain as described previously [24]. Vehicle group was injected equal volume PSB at same time. Clinical scores were recorded starting on day 21 and every other day thereafter. The severity of the disease for each limb was recorded as described previously [14]. The experiment design can be found in Supplemental 1. (This study was conducted in full compliance with Shanxi Medical University ethics. Ethics board approval number is 2015002).

### Treatments with A2M

Sixty 8-week-old female mice (DBA/1) were randomized into 4 groups (n =15 per group)：1) CIA + 1.2μg of A2M (10μl); 2) CIA + 0.8μg of A2M (10μl); 3) CIA + 0.4μg of A2M (10μl); 4) 10μl PBS. Intra-articular A2M injection was made on the right ankle and an equal amount of PBS was injected on the left ankle simultaneous as internal control for groups 1, 2, 3 at day 36, 43 and 50. PBS was injected on both the left and right ankles simultaneous for control group 4. 10μl of PBS containing 0.04mg/mL, 0.08mg/mL, and 0.12mg/mL A2M were respectively used to treat animals in groups 1, 2, and 3 on day 36 and then weekly for 3 weeks. Treatment began on day 36 because on this day, the ankles of all CIA mice showed significant swelling and redness, but there was no obvious bone damage detected by X-ray.

### Radiography

Mice were anesthetized by chloral hydrate (300mg/kg, i.p.). X-rays of hind paws were taken with UltraFocus100 x-ray cabinet (Faxitron, Arizona, USA) and used to evaluate the severity of ankle joint bone damage before the animals were euthanized. Two independent investigators scored each radiograph under blinded conditions. Joint erosions and space narrowing were assessed using the modified SHM. We focused on talocrural joint, talo-navicular joint, and subtalar joint. The scored erosions with a maximum score of 10 per joint (5 for each side of the joint) as described previously [25, 26].

### Fluorescence molecular tomography (FMT)

FMT is a noninvasive and quantitative fluorescence-based technology with high molecular specificity and sensitivity for in vivo, 3-dimensional deep tissue imaging. It allows for the evaluation of disease progression at multiple time points without sacrifice of the animal [27–30], and for dynamic probing of biologic processes at different time points (from hours to days). ProSense 750 FAST (activated by proteases such as Cathepsin B, L, S, K, V and D) (10μl) and MMPSense 645 FAST (activated by MMP's including MMP 2, 3, 7, 9, 12, and 13) (10μl) (PerkinElmer, Massachusetts, USA) were given by tail intravenous on the day 33 and on every fourth day after (both fluorescent imaging agents clear from tissues after approximately 96 hours). FMT was performed 24h after tails intravenous injections. A total of six times points of FMT were recorded for each animal. The picomole concentrations of probes in the ankle joint were determined using region of interest analysis (ROI) by restricting the area of measurement from the distal-tibia to talus to isolate the joint space. Data are reported as means ± SEM. n = 7/group.

### Histologic assessment

Mice were sacrificed on day 57. Nine mice per group were used for histologic analysis as described previously [19]. The ankle joints were sectioned in the sagittal plane. Blocks were trimmed to expose cartilage as 0, and ten adjacent sections were collected at intervals of 0 μm, 200 μm, and 400 μm. Two serial 6-μm-thick sections from each interval were stained. The ankles were evaluated via histopathology and scored for inflammation and bone resorption as previously published [23, 31]. Cartilage degradation was quantified using the Osteoarthritis Research Society International (OARSI) grading system in mouse [32]. Three independent observers scored each section under blinded conditions, and the scores for all sections cut from the medial and lateral distal tibia were averaged within each joint.

### Quantification of inflammation, bone resorption and cartilage damage

Inflammation and bone resorption scores were assigned as described previously [23, 31]. Cartilage damage scores were assigned following OARSI scores for mouse [32]. All scores' detail can be found in Supplemental 2.

### Immunohistochemistry

Specimens were analyzed by immunohistochemistry using a Histostain-SP kit (ZYMED Laboratories/Invitrogen, Carlsbad, CA, USA) to detect type II collagen (COL II, 1:100, Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA, USA), type II collagen breakdown product (C12C, 1:100, IBEX Technologies, Mont-Royal, QC, Canada), type X collagen (COL X, 1:50, EMD Biosciences, Billerica, MA, USA), TNF-α (1:100, Affinity Bioreagents), and mouse matrix metalloproteinase-13 (MMP-13, 1:100, Santa Cruz Biotechnology), as described previously [19]. Photomicrographs were taken with a Nikon E800 microscope (Nikon, Melville, NY, USA) [33].

### Real-time quantitative PCR (qPCR)

Cartilage samples from 6 mice were dissected using a scalpel under dissection microscopy per group, and 2 mice pooled together as one sample (n=3), then ground with mortar and

pestle under liquid nitrogen. Primer pairs were as following: for MMP-3, GGC-TTC-AGT-ACC-TTC-CCA-GG (forward) and GCA-GCA-ACC-AGG-AAT-AGG-TT (reverse); for mouse MMP-9, TGG-GAC-CAT-CAT-AAC-ATC-AC (forward) and GAT-ACC-CGT-CTC-CGT-GCT (reverse); for mouse MMP-13, CTT-CTT-CTT-GTT-GAG-CTG-GAC-TC (forward) and CTG-TGG-AGG-TCA-CTG-TAG-ACT (reverse); for mouse COL X, GCC-AGG-AAA-GCT-GCC-CCA-CG (forward) and GAG-GTC-CGG-TTG-GGC-CTG-GT (reverse); for mouse runt-related transcription factor 2 (Runx2), ACT-TCC-TGT-GCT-CGG-TGC-T (forward) and GAC-GGT-TAT-GGT-CAA-GGT-GAA (reverse); for mouse Aggrecan, CAG-TGC-GAT-GCA-GGC-TGG-CT (forward) and CCT-CCG-GCA-CTC-GTT-GGC-TG (reverse); for mouse COL II, AGG-GCA-ACA-GCA-GGT-TCA-CAT-AC (forward) and TGT-CCA-CAC-CAA-ATT-CCT-GTT-CA (reverse) [34–37]. Relative transcript levels were calculated according to the equation $x=2^{-\Delta\Delta Ct}$, where $\Delta\Delta C_t = C_t\ E - \Delta C_t\ C$ ($\Delta C_t\ E = C_t\ exp - C_t 18S$, $\Delta C_t\ C$ _ $C_t\ C$ _ $C_t\ 18S$) [9].

### Statistical analysis

Datum is presented as the mean±standard error of the mean. Significance of FMT, ankle thickness and clinical score were analyzed using the Two-way ANOVA (Bonferroni's post-tests). One-way ANOVA (Bonferroni's Multiple Comparison Test) was performed to compare cartilage damage scores, X-ray and levels of mRNA in vivo for COL II, COL X, Aggrecan, MMP-3, MMP-9, MMP-13, and Runx2. P values less than 0.05 were considered significant. Statistical analyses were performed using GraphPad Prism software.

## Results

### 100% incidence of CIA in model on day 33

Clinical signs of the disease, i.e. periarticular redness and swelling (Fig. 1a) first appeared in hind paws between 27 and 33 days after induction, leading to a 100% incidence of CIA on day 33 after first immunization. Hind paw erythema and swelling were observed between days 21 and 57 in all CIA+PBS mice. Groups treatment with A2M significantly reduced redness and swelling of the inflammatory process (Fig. 1b). There was a significant difference in inflammation between the mice treated with A2M and the mice treated with PBS.

### Clinical scores indicated A2M relieved clinical symptoms

The main characteristic of RA is joint deformation due to severe inflammation and damage. Representative pictures of mice joints show significant differences in inflammation between A2M-and PBS-treated mice (Fig. 1a. 3B). CIA+PBS-treated mice developed edema and erythema that extended from the ankle to the entire leg, while A2M-treated mice exhibited milder symptoms. We observed significantly lower clinical scores and ankle thicknesses in A2M-treated mice compared with CIA+PBS-treated mice after day 43 ($P < 0.05$) (Fig. 1b). Mice treated with 1.2μg, 0.8μg and 0.4μg A2M had a mean clinical score of 2.2 (range from 1 to 3), 3.1 (range from 2 to 4), and 3.5 (range from 2 to 4), respectively, while mice treated with PBS had a mean clinical score of 3.9 (range from 3 to 4) ($P < 0.05$).

### Radiography indicated A2M prevented bone damage

We observed a significantly lower SHM (Erosions and Space narrowing) score in A2M-treated mice compared with CIA+PBS-treated mice (Fig. 2a). A2M-treated groups 1.2μg, 0.8μg and 0.4μg had mean SHM (Erosions) scores of 9.73 ± 1.24 (range from 1 to 16), 13.67 ± 0.81 (range from 8 to 19) and 16.93 ± 0.76 (range from 13 to 22). respectively. In contrast, SHM (Erosions) scores of CIA+PBS-treated mice were 24.27 ± 0.96 (range from 18 to 30). In addition, the mean SHM (Space narrowing) scores in A2M- 1.2μg, 0.8μg, 0.4μg and CIA +PBS-treated groups were 3.67 ± 0.27 (range from 1 to 5), 4.93 ± 0.32 (range from 3 to 7), 5.93 ± 0.33 (range from 4 to 9) and 8.53 ± 0.51 (range from 5 to 12), respectively (Fig. 2b).

### Mice treated with A2M had significantly less inflammation detected by FMT

In this study, intravenous injection of protease-activatable, pan-cathepsin (cathepsins are produced by inflammatory cells) fluorescent imaging probes ProSense750 FAST and MMPSense 645 FAST allowed the detection of activated inflammatory cells within the ankle joint of CIA mice. We built upon these earlier observations by assessing FMT imaging and quantification (in units of picomoles) of arthritic ankle joints starting on day 34 and until the end of this study.

FMT data indicated that concentrations of the proteases and MMPs were higher in the CIA +PBS ankles than that in the contralateral sides after A2M treatment within the same mouse. Tomographic quantification of ankle fluorescence in CIA+PBS- treated and CIA+A2M-treated animals showed significant differences in the ProSense750 FAST signal and the MMPSense645 FAST signal (Fig. 3A. 3B) in the ankle regions. Both ProSense750 FAST and MMPSense645 FAST levels in three of the A2M treatment groups and vehicle group were significantly lower than the levels in mice that underwent CIA+PBS treatment mice. We noticed that these levels in three of A2M treatment groups were still higher than that in the vehicle-treated control group.

### A2M attenuated inflammation infiltration and synovial hyperplasia as demonstrated by histologic evaluation.

Safranin O staining of histological specimens from A2M- and PBS-treated mice was significantly different. Histological assessment suggested that A2M administration maintained the level of inflammation in the joints low (Fig. 4a). Cartilage from mice that received the 1.2μg injection of A2M stained stronger for PG and had a more intact surface than that from mice that received 0.8μg and 0.4μg injection of A2M; however, it demonstrated weaker staining when compared with the vehicle group. Histological sections obtained from A2M-treated mice that received 1.2μg, 0.8μg and 0.4μg exhibited less synovial hyperplasia, more intact cartilage, typical bone organization, and uninflamed fat tissue. In contrast, joints in PBS-treated mice showed higher levels of inflammation, and displayed profound lymphocytic infiltration as well as neutrophils, large areas of fibrosis, several spots showing necrosis and obvious signs of inflammatory cellular infiltration. In these control samples, synovium was widely extended, and cartilage appeared eroded on the surface. Similarly, muscles and bones exhibited severe destruction, and fat tissue was not notable (Fig.4a). OARSI histologic grading system scores in all A2M-treated groups suggested mild degeneration (Mean ± SEM, 1.57 ± 0.28 in the 1.2μg group, 3.09 ± 0.33 in

the 0.8μg group and 4.56 ± 0.31 in the 0.4μg group), while in CIA+ PBS groups degeneration was scored as more severe (11.15 ± 0.31; P<0.05). Cartilage in the vehicle group received OARSI scores suggesting the least damage of all groups (0.15 ± 0.04; P< 0.05). In addition, inflammation scores in all A2M-treated groups were lower (1.44 ± 0.14 in the 1.2μg group, 1.74 ± 0.27 in the 0.8μg group and 2.19 ± 0.41 in the 0.4μg group) than in the CIA+ PBS treatment group (4.85 ±0.15). Moreover, bone scores in all A2M-treated groups were indicated of decreased bone damage (0.59 ± 0.31 in the 1.2μg group, 0.56 ± 0.26 in the 0.8μg group and 2.22 ± 0.38 in the 0.4μg group) when compared with the CIA + PBS treatment group (5.52 ± 0.41). (Fig. 4b).

### Immunohistochemistry of A2M-treated mice showed stronger COL II and Aggrecan staining and less MMP13, TNFα and C12C

Type II collagen staining in all three groups of A2M-treated mice was stronger than in the CIA+PBS-treated mice. In contrast, immunostaining (MMP13, TNFα and C12C) in the CIA +PBS group was stronger than in all A2M treatment groups. In addition, there was less immunostaining for MMP-13, TNFα and C12C in animals treated with 1.2μg of A2M than in those treated with 0.4μg and 0.8μg of A2M. This suggests a therapeutic effect proportional to the concentration of supplemental intra-articular A2M (Fig. 5).

### Supplemental A2M enhances mRNA levels of COL II and Aggrecan and reduces mRNA levels of MMP-3, −9, −13, COL X and Runx2.

Real-time qPCR results indicated that supplemental intra-articular A2M enhanced the levels of mRNA for COL II and Aggrecan, and suppressed those of mRNA for MMP3, MMP9, MMP13, Runx2, and COL X when compared with PBS-treated animals (Fig. 6). COL II and Aggrecan mRNA levels in mice that underwent CIA +PBS treatment were significantly lower than those in mice that underwent CIA and received 1.2μg and 0. 8μg of A2M. In contrast, mRNA levels of COL X, MMP3, MMP9 and MMP13 in all three A2M-treated groups were significantly lower than those in CIA+PBS-treated mice. Moreover, Runx2 mRNA levels in 1.2μg and 0.8μg groups were also significantly lower than those in the CIA +PBS group. These results suggest that A2M supplementation inhibits CIA inflammation and cartilage and bone damage in vivo by decreasing cartilage catabolic and inflammatory factors, in addition to inhibiting protease activity.

## Discussion

RA is a chronic inflammatory disease; imbalances between inflammatory and anti-inflammatory cytokines play a critical role in the initiation and progression of inflammatory arthritis. Several molecules have been reported to play a role in cartilage damage, bone resorption, and synovial inflammation; some such molecules include inflammatory cytokines, MMPs, chemokines, reactive oxygen and nitrogen intermediates, prostanoids and TNF-α [8, 38, 39]. Enhancing anti-inflammatory factors as a therapeutic means to reduce levels of inflammation may prevent joint damage and maintain joint function in inflammatory arthritis. Since A2M inhibits all classes of endoproteases, it could mitigate CIA progression by neutralizing inflammatory cytokines. Our results suggest that A2M can efficiently inhibit inflammation, attenuate cartilage damage and bone resorption. The balance of protease to

A2M in vivo may play an important role in mediating cartilage and bone destruction by catabolic enzymes in a CIA mouse model. Thus, inhibition of these inflammation molecules will likely slow or prevent the progression of arthritis.

High and low levels of A2M have been found in blood and synovial joints, respectively, due to the large molecular weight of A2M, which prevents its migration from blood into the synovial fluid (SF) [40, 41]. Thus, supplemental A2M may be a potential strategy to attenuate cartilage degeneration by reducing these catabolic enzymes induced by joint inflammation. FMT is a sensitive bio-imaging tool to evaluate inflammation and arthritis progression at multiple time points without having to sacrifice the animal. In this study, FMT data indicated that A2M specifically inhibits proteases and MMPs in a CIA model immediately after A2M injection when compared with PBS treatment. Higher doses of A2M were more efficacious in our experiments. A significant decrease in ProSense and MMPSense detected by FMT was observed immediately in the animals treated with A2M when compared with the animals treated with PBS in our CIA model.

Our histological and biochemical data further demonstrated that supplemental A2M not only attenuated cartilage and bone damage, but also inhibited catabolic factors. After day 33, the ankles of CIA mice showed obvious redness and swelling, and both the clinical scores and ankle thickness had significantly increased ($p<0.05$). We observed a significantly lower SHM (Erosions and Space narrowing) score in A2M-treated mice compared to CIA+PBS-treated mice on day 57. Our histological data further demonstrated that supplemental A2M can significantly attenuate cartilage damage and inhibit catabolic factor MMP-13 and TNFα activity, as well as also enhance cartilage matrix (i.e. COL II and Aggrecan) synthesis in a dose-dependent manner. Ankles treated with 1.2μg A2M had less inflammation and were better protected against damage when compared with those treated with low dose A2M. Real-time qPCR results indicated that supplemental intra-articular A2M enhanced the levels of mRNA for COL II and Aggrecan, and suppressed the levels of mRNA for MMP3, MMP9, MMP13, Runx2, and COL X compared with PBS treated animals.

The reason we started to treat the mice at day 36 is that we noticed all ankles of CIA mice showed significant swelling and redness, but no obvious bone damage as detected by X-ray on day 35. These findings suggested that early supplemental A2M given before bone changes may be critical to prevent cartilage and bone damage.

A2M can bind to IL-1β and TNFα directly, neutralize and reduce these enzymes activities [42–44]. Previous studies show that macrophages from RA SF can differentiate to fully mature osteoclasts in presence of TNFα/IL-1β, which results in bone loss in the RA joint [45, 46]. One explanation for A2M preventing bone resorption observed in this study is that A2M may binds to and inhibit TNFα and IL-1β inducing osteoclast formation and bone loss. In our future study we will further explore whether A2M prevents bone loss by blocking TNFα, IL-1β and osteoclast formation pathway.

In addition, we noticed that A2M enhanced cartilage matrix (i.e. COL II and Aggrecan) synthesis. Increased collagen and Aggrecan synthesis suggest that A2M may have cartilage repair functions and/or facilitate for cartilage matrix synthesis to proceed. This finding is

consistent with a previous report in which a high dose of A2M did not induce chondrocyte death rather than increase collagen II and Aggrecan production (9). These findings strongly indicate that A2M is a promising bio-inhibitor for catabolic proteases. Thus, early supplemental intra-articular A2M may provide chondral protection in RA by reducing the presence of local catabolic proteases. Our observations provide evidence that supplemental intra-articular A2M induces an anti-inflammatory mechanism and slows cartilage damage and bone resorption.

## Conclusion

Inflammation plays a critical role in the pathological process of arthritis. In this study, we demonstrate that early supplemental intra-articular A2M significantly attenuated cartilage and bone damage in a mouse CIA model by reducing the level of the inflammation, suggesting that supplemental A2M may be a novel therapy for arthritis.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Abbreviations

| | |
|---|---|
| **A2M** | Alpha-2- macroglobulin |
| **CIA** | Type II collagen-induced arthritis |
| **SHM** | Sharp/van der Heijde method |
| **COL** | Collagen |
| **RA** | Rheumatoid arthritis |
| **FMT** | Fluorescence molecular tomography |
| **ADAMTS** | thrombospondin motifs |
| **IHC** | Immunohistochemically analysis |
| **IL** | Interleukin |
| **MMPs** | Matrix metallopeptidases |
| **mRNA** | Messenger RNA |

| | |
|---|---|
| **OARSI** | Osteoarthritis Research Society International |
| **PBS** | Phosphate-buffered saline |
| **Agg** | Aggrecan |
| **ROI** | Region of interest |
| **SF** | Synovial fluid |
| **TNF** | Tumor necrosis factor |
| **RUNX** | Runt-related transcription factor |

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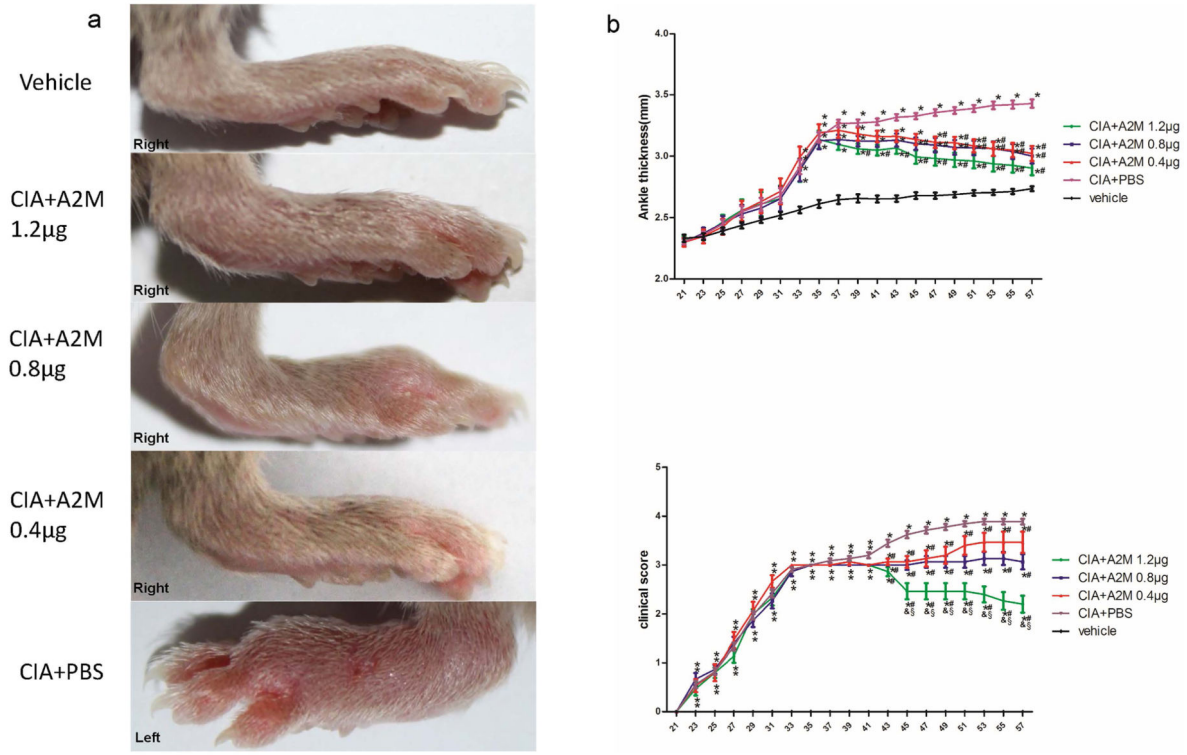



**Figure 1. A2M reduces paw thickness and clinical scores in CIA mice.**

**(a)** No clinical signs of CIA were observed in vehicle mice. **(b, c, d)** A significant reduction in the clinical signs of CIA was observed in mice treated with A2M. Higher A2M doses showed lower clinical signs of CIA. **(e)** Representative clinical signs of CIA: periarticular erythema and edema. **(f)** Average ankle thickness of hind paws: a significant difference between CIA+PBS and CIA+A2M treated mice at day 33 is shown. **(g)** A significant clinical score difference in hind paws after day 43. Values are expressed as mean ± SEM of 15 animals for each group. *P < 0.05 versus vehicle group. #P<0.05 versus CIA+PBS group. &P<0.05 versus CIA+A2M 0.4μg group.

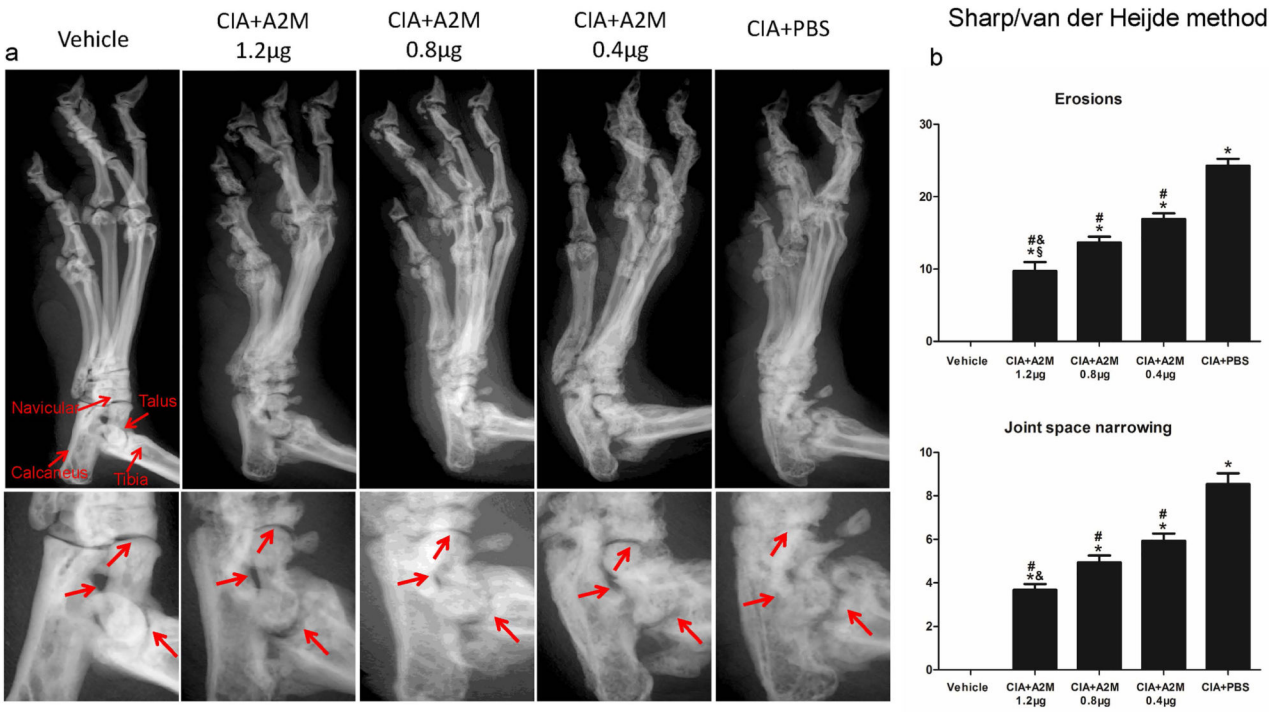
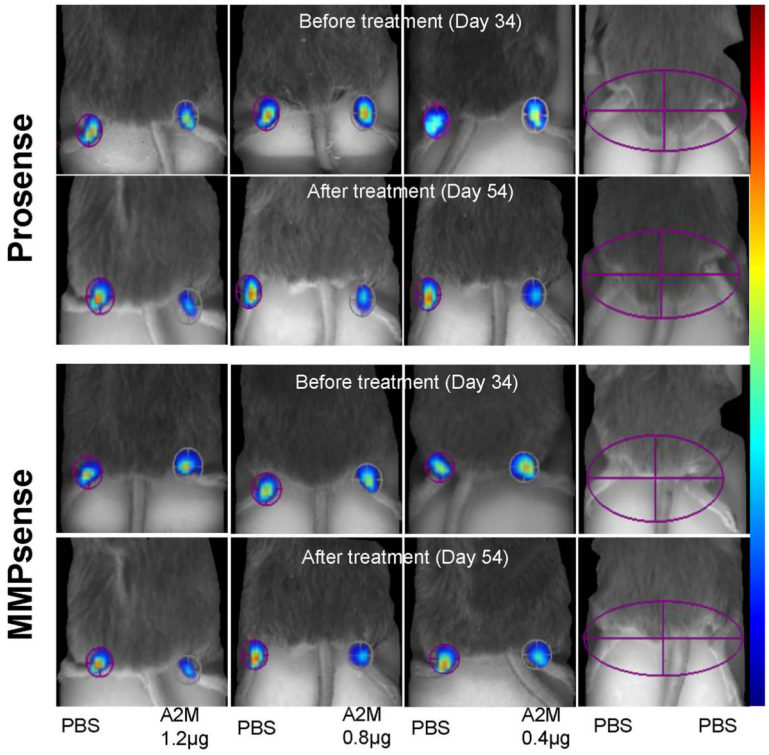
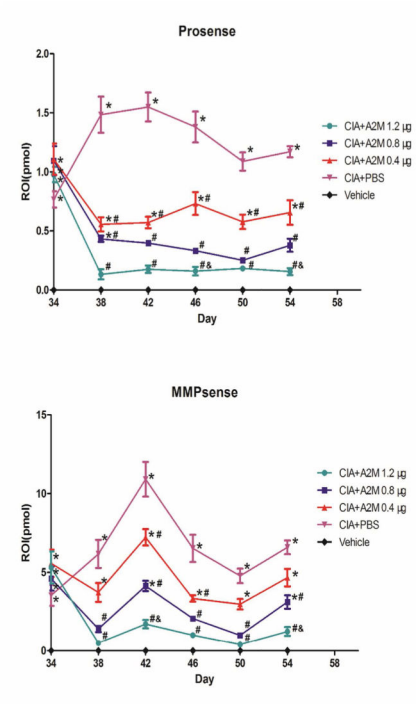



**Figure 2. Radiographic analysis indicated A2M prevented bone damage. X-rays were taken at day 57 in all mice.**

**(a)** No bone damage and joint space changes observed in vehicle group. **(b, c, d)** CIA+A2M treated mice demonstrated a significant reduction in erosion and joint narrowing. **(e)** Representative radiographic features in CIA: significant bone damage and joint space changes. **(f)** Average SHM score values of hind paws: a significant difference between CIA +A2M (only mild bone damage) and CIA+PBS treated groups is shown. Values are expressed as mean ± SEM of 15 animals for each group. *P < 0.05 versus vehicle group. #P<0.05 versus CIA+PBS group. &P<0.05 versus CIA+A2M 0.4μg group.



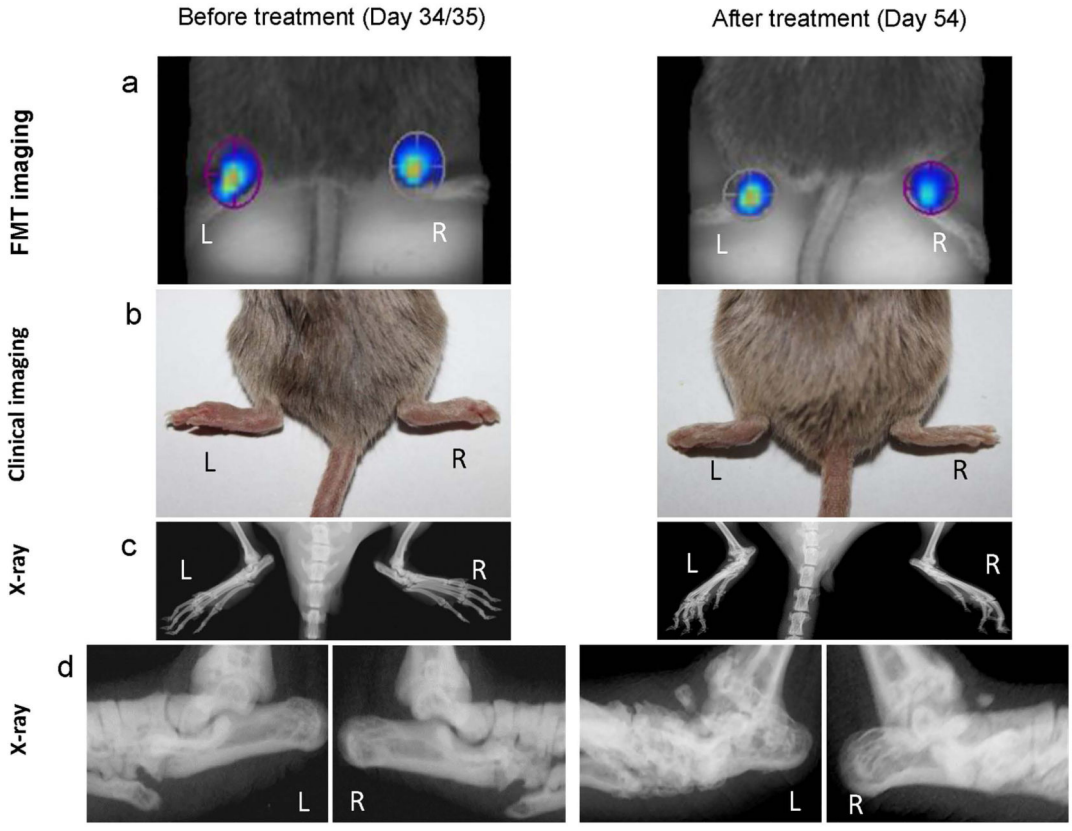



**Figure 3. A. Dynamic monitoring of inflammation with ProSense 750 and MMPSense 680 by FMT in CIA mice.**

**(a, b, c)** Representative FMT imaging of mice paws from each group. Left ankles (CIA +PBS) had a significantly higher signal than right ankles (CIA+A2M) after treatment. **(d)** No tomographic imaging signal was detected on the hind paws of a DBA/1 vehicle mouse. **(e)** Quantification of ProSense 750 FAST signal. **(f)** Quantification of MMPSense 680 FAST signal. Values are expressed as mean ± SEM of 7 animals for each group. *P < 0.05 versus vehicle group. #P<0.05 versus CIA+PBS group. &P<0.05 versus CIA+A2M 0.4μg group.

**3B. Representative imaging showing pathogenic changes before treatment (day 34) and after A2M treatment (day 54) in the same mice.** (a) A representative ProSence FMT imaging of mouse paws shows similar signal on right and left sides before treatment (day 34). At day 54, the left ankle, treated with PBS, had a significantly higher signal than the right ankle, treated with A2M. (b) Representative clinical signal of CIA observed in both ankles at day 34 (i.e. before treatment). A significant difference between PBS and A2M treatment at day 54 is shown. (c, d) X-rays showed no bone damage and/or joint space changes in either ankles at day 35, although inflammation was observed in both ankles. On the contrary, left ankles treated wevere bone resorption and space narrowing, but only mild bone damage was observed at day 54 on right ankles treated with A2M.

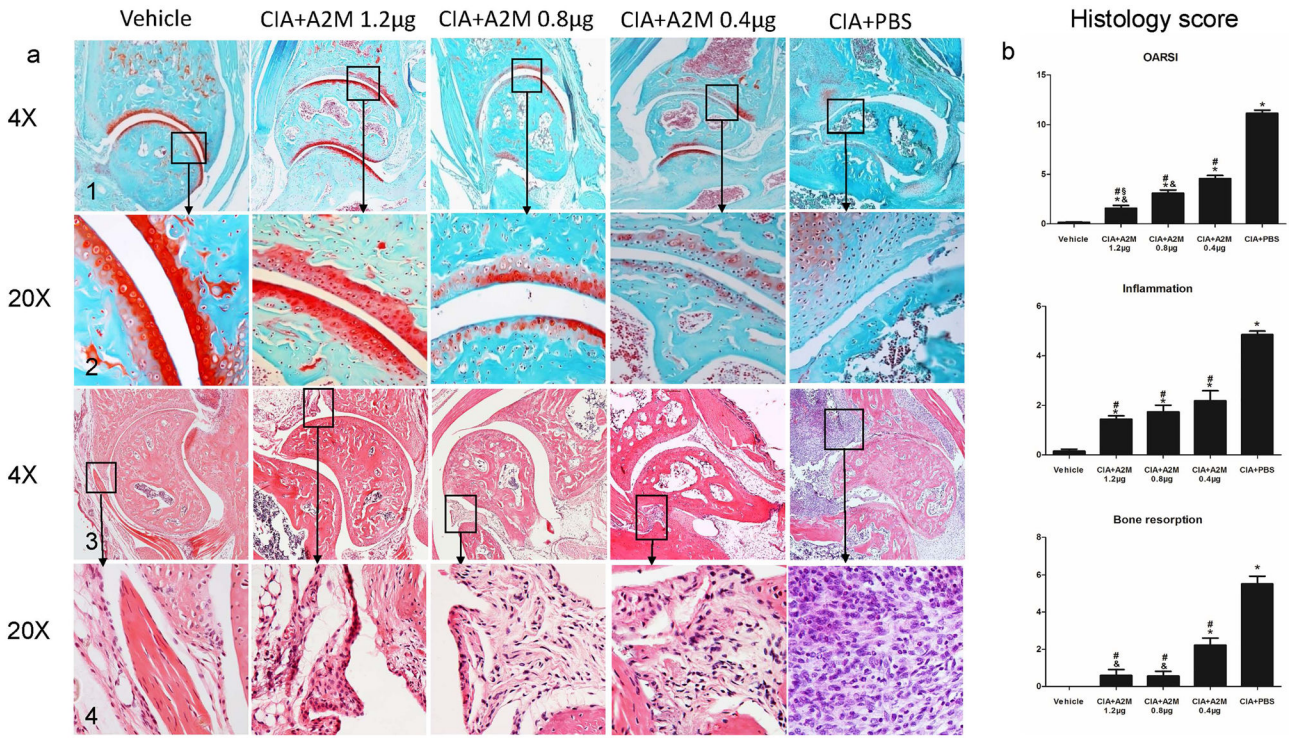
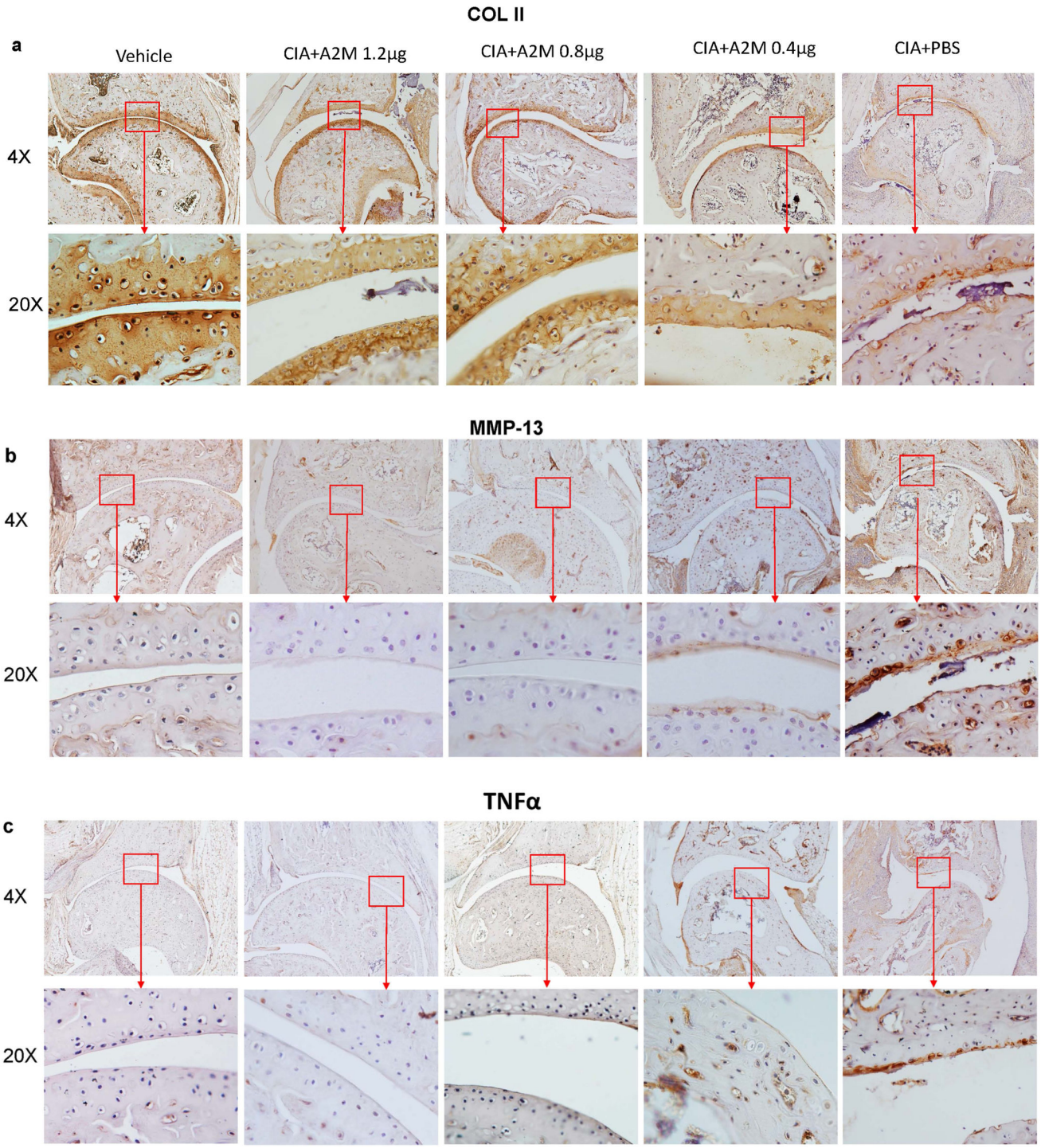



**Figure 4. Supplemental intra-articular injection of A2M attenuates cartilage degeneration and bone damage.**

**(a)** No abnormal synovial tissue, cartilage and/or bone were detected in the vehicle control animals. **(b)**The animals treated with 1.2μg A2M showed one or two layers of synovial cells, less bone damage and some area of cartilage loss in Safranin-O staining; **(c)** The animals treated with 0.8μg A2M, showed cartilage loss of Safranin-O staining and mild edema and inflammation in the joint space as compared with vehicle; **(d)** The animals treated with 0.4μg A2M group had moderate synovial thickening and inflammatory cells in the joint capsule, synovium, and joint space; **(e)** The animals treated with PBS group showed expansion of synovial and extra-articular tissues, obvious signs of edema and inflammatory cell infiltration, full-thickness defects in the cortical bone and marked loss of medullary trabecular bone; **(f)** Histology OARSI scores, inflammation scores, and bone resorption scores were significantly lower in animals treated with CIA+A2M when compared with animals treated with CIA+PBS. *P < 0.05 versus vehicle group. #P<0.05 versus CIA+PBS group. &P<0.05 versus CIA+A2M 0.4μg group. §P<0.05 versus CIA+A2M 0.8μg group.



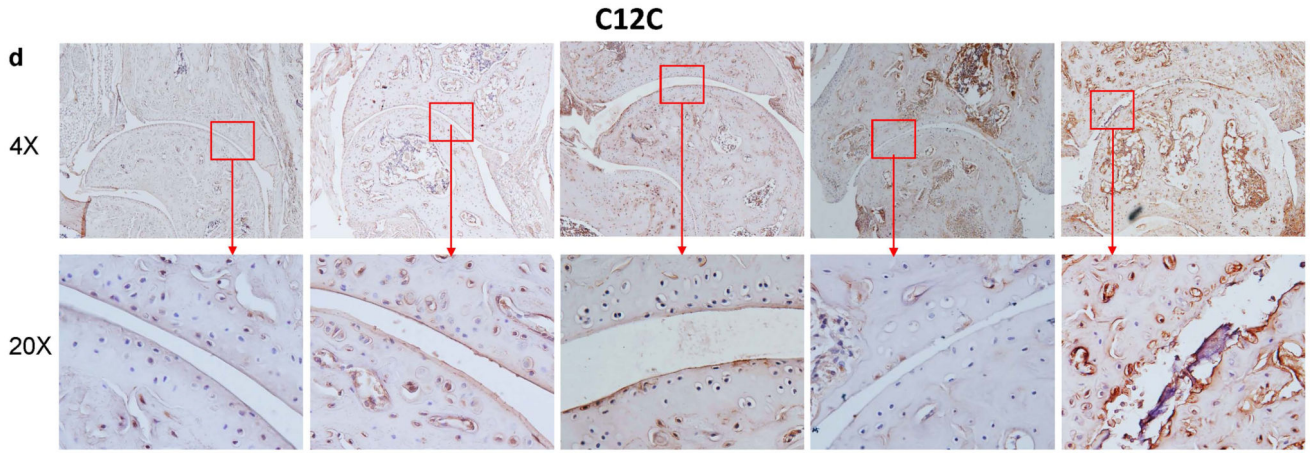



**Figure 5. Immunohistochemistry (IHC) analyses.**

(a) Strong staining for type II collagen was observed in mice treated by CIA+A2M compared with the CIA+PBS group. In contrast, MMP-13(b), TNF-α (c) and C12C (d) staining were significantly elevated in mice that underwent CIA+PBS treatment compared with the CIA+A2M treated mice.

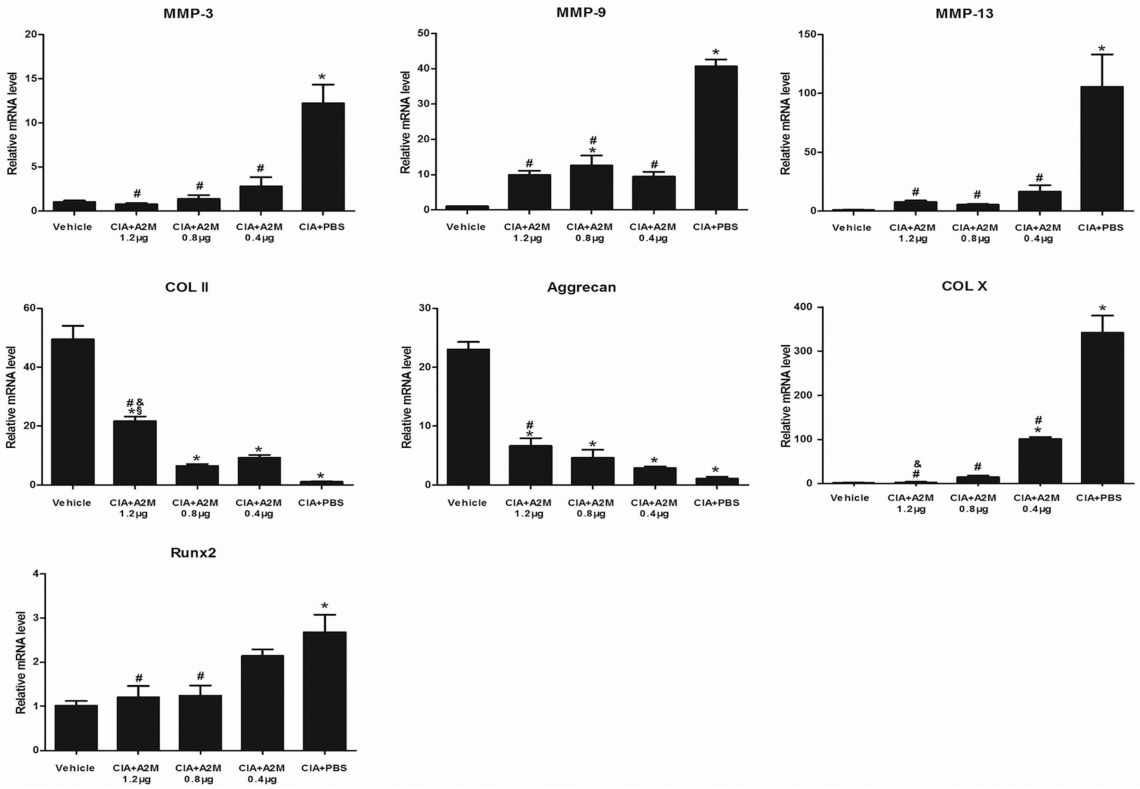



**Figure 6. Supplemental intra-articular A2M inhibits inflammatory factors and enhances anabolic metabolism in CIA mice.**

Levels of Aggrecan and COL II mRNA were higher in CIA mice that were treated with A2M compared to those treated with PBS. In contrast, these mRNA levels of MMP-3, MMP-9, MMP-13, Runx2 and COL X showed the opposite pattern. *P < 0.05 versus vehicle group. #P<0.05 versus CIA+PBS group. &P<0.05 versus CIA+A2M 0.4μg group. §P<0.05 versus CIA+A2M 0.8μg group.