


Suppression of Murine Osteoarthritis by 4-Methylumbelliferone

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ABSTRACT: Using in vitro models, we previously reported that 4-methylumbelliferone (4-MU) blocked many of the pro-catabolic features of activated chondrocytes. 4-MU also blocked safranin O loss from human cartilage explants exposed to interleukin 1 β (IL1 β) in vitro. However, the mechanism for this chondroprotective effect was independent of the action of 4-MU as a hyaluronan (HA) inhibitor. Interestingly, overexpression of HA synthase 2 (HAS2) also blocked the same pro-catabolic features of activated chondrocytes as 4-MU via a mechanism independent of extracellular HA accumulation. Data suggest that altering UDP-sugars may be behind these changes in chondrocyte metabolism. However, all of our previous experiments with 4-MU or HAS2 overexpression were performed in vitro. The purpose of this study was to confirm whether 4-MU was effective at limiting the effects of osteoarthritis (OA) on articular cartilage in vivo. The progression of OA was evaluated after destabilization of the medial meniscus (DMM) surgery on C57BL/6 mice in the presence or absence of 4-MU-containing chow. Mice fed 4-MU after DMM surgery exhibited significant suppression of OA starting from an early stage in vivo. Mice fed 4-MU exhibited lower OARSI scores after DMM; reduced osteophyte formation and reduced MMP3 and MMP13 immunostaining. 4-MU also exerted pronounced chondroprotective effects on murine joint cartilage exposed to IL1 β in vitro and, blocked IL1 β -enhanced lactate production in cartilage explants. Therefore, 4-MU is effective at significantly reducing the loss of proteoglycan and reducing MMP production both in vitro and in vivo as well as cartilage damage and osteophyte formation in vivo after DMM. © 2019 Orthopaedic Research Society. Published by Wiley Periodicals, Inc. J Orthop Res 2019.

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The glycosaminoglycan hyaluronan (HA) serves a primary role in retaining aggrecan proteoglycan in the extracellular matrix of cartilage; aggrecan that is necessary to maintain the hydrostatic, shock-absorbing properties of the tissue.^{1,2} Reversing deficits in HA, often associated with osteoarthritis (OA), has been a hypothetical strategy to preserve cartilage integrity and provide feedback signaling from the extracellular matrix to chondrocytes for a return to steady-state metabolism. Thus, it was somewhat counterintuitive when we observed previously that a chemical inhibitor of HA biosynthesis, 4-methylumbelliferone (4-MU), blocked the synthesis and production of MMP13, ADAMTS4, and TSG6 in human OA chondrocytes and bovine chondrocytes.³ Of more relevance, the addition of 4-MU to intact bovine or human knee OA cartilage explants provided “chondroprotection” of the tissues, reducing the release of sulfated glycosaminoglycan (sGAG) into the medium and providing a near-complete block in the loss of safranin O staining in the tissue. More intriguing was that 4-MU inhibition of MMP13 and other pro-catabolic markers did not require the inhibition of HA biosynthesis, being just as effective in chondrocytes following knock-down of *HAS2*, conditions where there was little HA production. More recently, we examined the reverse situation, overexpression of *HAS2* (*HAS2-OE*).⁴ We observed that *HAS2-OE* also

blocked the same pro-catabolic markers as 4-MU and promoted the retention of sGAG by chondrocytes. Surprisingly, the chondroprotective effects of *HAS2-OE* were not due to the enrichment of extracellular HA (outside-in signaling) but instead our data suggested induced changes in energy metabolism in the transduced chondrocytes.

Although intriguing, both the 4-MU and *HAS2-OE* studies were all performed using in vitro models including isolated human OA or bovine chondrocytes or cartilage explants treated with interleukin 1 β (IL1 β). These models are useful but likely do not fully represent OA in vivo.^{5–8} Questions arise as to whether the culture medium, serum, cytokines or normoxic conditions used in vitro truly reflect the environment of an OA joint in vivo. The destabilization of the medial meniscus (DMM) OA model in mice⁹ has become the standard, effective approach to model OA in vivo.^{10,11} With relevance to our studies, the DMM model displays progressive changes in articular cartilage, with less contribution by inflamed synovial tissues. 4-MU has proven effective in mouse models of inflammation and in cases where inflammation is dependent on HA biosynthesis.^{12,13} Thus as a primary aim, we explored whether 4-MU is effective in limiting the progression of OA in mice following induction by DMM.

The mechanism of action of 4-MU is complex. 4-MU is a low toxicity coumarin derivative that forms covalent glycoconjugates with D-glucuronic acid (GlcUA) via the transfer from UDP-GlcUA.¹⁴ The formation of these glycoconjugates restricts access of cytosolic UDP-GlcUA intermediates necessary for HA

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biosynthesis and serves as the primary mechanism for the 4-MU blockage of HA production.^{15,16} However, 4-MU also blocks the transcription of HAS2 mRNA^{3,17} providing an additional molecular dampener to HA biosynthesis. But in chondrocytes, 4-MU also blocks the transcription of a select set of genes activated after IL1 β treatment in addition to HAS2 namely, MMP13, ADAMTS4, and TSG6 with the modest enhancement of ACAN and COL2A.³ The mechanism for the selective transcriptional effects of 4-MU is as yet unknown.

As noted above, given that HAS2-OE also blocks the transcription of many of the same activated genes,⁴ some other non-canonical mechanism, in common to both 4-MU and HAS2-OE must be in play. Recent studies in human cancer cells demonstrate that experimental modulation of intracellular UDP-hexose pools exerts effects on cellular phenotype in large part by altering intracellular metabolism in addition to other concurrent effects on HA biosynthesis.¹⁸ HAS2-OE does give rise to changes in chondrocyte metabolism.⁴ If 4-MU and HAS2-OE are similar, 4-MU may provide a means to modulate the metabolomics of chondrocytes and thus contribute insight into how altering metabolism affects OA in the DMM model.

In this study, we examined knee joints from mice fed a diet of 5% 4-MU chow versus control chow and documented the effects on OA progression at various time points after DMM or sham surgery. 4-MU was effective at reducing features of OA in this in vivo model even at the earliest stage (2 weeks) when the most pronounced changes are observed primarily in the articular cartilage.

METHODS

Mice

All animal studies were approved by the Institutional Animal Care and Use Committee at East Carolina University. Male and female C57BL/6 mice (10-week old) and female CD-1 mice (10-week old) were purchased from Charles River (Wilmington, MA). All animals were housed in the same specific pathogen-free facility with 1 male or 3 females per microisolator cage on a 12-h light/dark cycle and provided food and water ad libitum.

Surgical Induction of OA

DMM surgery was performed in the right knees of 12-week-old male and female C57BL/6 mice after 2-week acclimation as previously described.⁹ After oral administration of Meloxicam at 5 mg/kg by 1 h prior to surgery, the mice were initially anesthetized with Isoflurane at a rate of 4–5% until reaching a surgical plane and then maintained at a 2–3% Isoflurane rate, with 0.8–1.0 L/min of O₂. Under sterile conditions, a 3-mm longitudinal incision was made over the distal patella to the proximal tibial plateau of the right knee and the joint capsule immediately medial to the patella tendon. The fat pad over the intercondylar area was dissected to expose the intercondylar region followed by transection of the medial meniscotibial ligament (MMTL). Then the joint capsule and the skin were closed with 8-0 nylon sutures. For sham surgery, performed on the right knee of an independent set of mice, the same procedure was performed except that the MMTL was

not cut. All procedures were conducted by one surgeon (S.T.). Meloxicam treatment continued for 2 days following surgery.

Chow Containing 4-MU

4-MU was pressed at 5% (w/w) into a modified ProLab[®] Isopro[®] RMH 3000 mouse chow with 2% chocolate flavor (4-MU chow; TestDiet, St. Louis, MO) and irradiated before shipment as previously described.^{19,20} This dose was determined to deliver approximately 150–250 mg/mouse/day using a previous study.²¹ Control chows with comparable formulas were ProLab[®] Isopro[®] RMH 3000 (no chocolate, no 4-MU) or RMH3000 chow with 2% chocolate flavor but no 4-MU (TestDiet, St. Louis, MO).

Treatment Groups

Male C57BL/6 mice were randomly assigned into 4 groups at 12 animals per group,^{22,23} DMM with control chow, DMM with 4-MU chow, Sham with control chow and Sham with 4-MU chow. In all groups, the mice were provided with control or 4-MU chow ad libitum the next day after surgery until euthanasia at 2, 4, and 8 weeks after surgery ($n = 12$ per group unless otherwise stated). To confirm any sex differences, the same experimental format was performed using female C57BL6 mice wherein the mice were euthanized at 4 weeks ($n = 10$ –12 per group). In an additional experiment, male C57BL/6 mice were randomly assigned into two groups, DMM with chocolate (C) control TestDiet[®] chow or, DMM with 4-MU TestDiet[®] chow, provided ad libitum from 1 week prior to surgery and for 2 weeks following surgery. The mice were euthanized at 2 weeks after surgery ($n = 12$ per group).

Histological and Immunohistochemical Evaluation of OA

For in vivo studies, at the time of euthanasia, the right knee joints were prepared for histology and immunohistochemistry. Briefly, the knee joints were fixed in 4% paraformaldehyde for 18 h. The samples were incubated in Decalcifying Solution (22050130; Richard Allan Scientific, San Diego, CA) for 1 week before embedding in paraffin. In a coronal plane, 8 μ m sections, that included the femoral condyles, menisci, and tibial plateaus, were cut from anterior to posterior aspects at 100 μ m intervals. These sections were stained with safranin O and fast green.³ Some adjacent sections were deparaffinized, rehydrated and antigen retrieval performed using 10 mM citrate buffer for 1 h at 60°C. These sections were incubated with rabbit primary antibodies against MMP13 (1:130, ab39012; Abcam, Cambridge, MA) or MMP3 (1:100, NB100-91878; Novusbio, Centennial, CO) overnight at 4°C, rinsed with phosphate-buffered saline, and incubated with reagents included in a rabbit *Vectastain* ABC-HRP kit (Vector Laboratories, Burlingame, CA). All sections were visualized using a Nikon Eclipse E600 microscope; images were captured digitally using a Retiga 2000R digital camera and processed using NIS Elements BR 1.30 imaging software (Nikon, Melville, NY). The percentage of MMP13 or MMP3 positive chondrocytes within equivalent areas of the medial compartment, at 2 weeks after DMM surgery, was determined.

Osteoarthritic cartilage changes in the medial tibial plateau were scored by two blinded observers (S.T. and Y.O.) using the OARSI Scoring System developed by Pritzker et al.²⁴ In brief, five safranin O/fast green stained serial sections around the anterior-midline region of each right knee joint were scored according to the formula: score (0–26) = grade (0–6.5 per 0.5) \times stage (0–4). The score of each mouse was then calculated by summing the scores of five sections (0–130). The area of medial tibia osteophytes was

measured using the 8-week after DMM surgery sections and presented as the average from five sections per knee.

Explant Culture of Mouse Knee Joints and Bovine Cartilage

Stock female, outbred CD-1 mice were used for tissue appropriation. For *in vitro* explant cultures, both left and right knee joints were harvested. Briefly, tibial-femoral joints were opened anteriorly but still articulated, placed into 12-well plates (one knee per well) and cultured for 2 days in 2.0 ml DMEM/F-12 medium (Mediatech, Manassas, VA) with 10% FBS (Hyclone, Pittsburgh, PA) followed by 10 days in serum-free medium without or with 3 ng/ml IL1 β (R&D Systems, Minneapolis, MN) and 1.0 mM 4-MU. Full-thickness cores (6 mm) of bovine articular cartilage were isolated from metacarpophalangeal joints of 18–24-month-old adult steers as described previously.^{3,25} These cores were also cultured and treated as the murine joints. The conditioned medium of each explant culture was collected, clarified by centrifugation and stored at –80°C until use. The explants were similarly processed for histology and immunohistochemistry in a similar fashion as described above.

Colorimetric Assays

Equal volumes of diluted (1:5) conditioned media from explant cultures were analyzed for released sGAG by DMMB assay as described previously^{3,26} and concentration determined by comparison with the standard curve of bovine chondroitin sulfate (Sigma Chem, St. Louis, MO). For measurement of lactate, equal volume aliquots of diluted (1:5) conditioned media of bovine cartilage explant cultures were assayed for L-lactate according to the manufacturer's instructions (Eton Bioscience, Research Triangle, NC) and concentration determined by comparison to a standard curve.

Western Blotting

Equal volumes of 10 \times concentrated conditioned medium were loaded into 4–12% NuPAGE[®] Novex[®] Tris-acetate gradient mini gels (Thermo Fisher Scientific, Waltham, MA). Following electrophoresis, the proteins were transferred to a nitrocellulose membrane. The membranes were blocked in TBS containing 0.1% Tween 20 and 5% non-fat dry milk (TBS-T-NFDM) for 1 h at room temperature, then incubated overnight with primary antibody against MMP13 (1:6,000; Abcam)³ or rabbit anti-G1-ITEGE³⁷³ (1:600; also detects mouse G1-VTEGE³⁷³)²⁷ in TBS-T-NFDM at 4°C. After rinsing and incubation with secondary antibody, immunoreactive bands were detected using chemiluminescence (Novex ECL; Thermo Fisher Scientific) followed by exposure of X-ray films and visualization/digitalization using a GelDoc with ImageLab software (Bio-Rad, Hercules, CA).

Statistical Analysis

All data in bar graphs are expressed as mean \pm standard deviation and OARSI Scoring System data are shown in box-plots. A two-tailed unpaired Student's *t* test or a Mann-Whitney *U* test were used for direct comparison between two groups. A *p*-value of less than 0.05 was considered significant. One, two, or three asterisks denotes a *p*-value <0.05, <0.01, <0.001, respectively.

RESULTS

Effects of 4-MU on Mouse Knee Explant Culture

Before beginning *in vivo* studies, the chondroprotective effects of 4-MU were examined in an *in vitro* murine OA model. Excised, opened, but still articulated mouse knee joints as shown in Figure 1B were treated with IL1 β in the absence or presence of 4-MU and then processed for histology and immunohistochemistry. Interestingly, safranin O staining of sections of mouse knee explants (Fig. 1A) revealed a substantial loss of safranin O in all layers of femoral and tibial cartilage following IL1 β treatment as compared with untreated explants. Co-incubation with 4-MU and IL1 β resulted in the marked retention of safranin O stained cartilage, providing morphology similar to that of untreated explants. Staining for MMP13 (Fig. 1C) and MMP3 (Fig. 1E) exhibited the highest protein expression in the medial compartment cartilage of IL1 β -treated explants. The expression was substantially reduced in explants co-treated with IL1 β and 4-MU, but not completely to the level observed in control joints. Quantification of MMP-positive cells is shown in Figure 1D and F. These data suggest that, like our previous observations in human and bovine cartilage explants,³ 4-MU is chondroprotective of murine joint cartilage degradation induced by IL1 β at least in this *in vitro* explant OA model.

The release of sGAG (namely aggrecan) into the culture medium of these murine explant cultures was also quantified (Fig. 2A). sGAG accumulation inversely paralleled the loss of safranin O staining shown in Figure 1A. 4-MU blocked the background release of sGAG in control conditions as well as the IL1 β -stimulated release of sGAG (Fig. 2A), confirming the histochemistry. The culture medium of these explants was also examined for the accumulation of degradation product of aggrecanase activity (G1-VTEGE domain of aggrecan) as well as released MMP13 protein (Fig. 2B). Both G1-VTEGE and MMP13 protein accumulation were enhanced in the medium of cultures treated with IL1 β . Importantly, the release of both proteins was blocked by co-incubation of 4-MU with IL1 β .

Using bovine cartilage explants under similar culture conditions as the murine explant model, accumulation of L-lactate in the medium was determined. IL1 β treatment resulted in enhanced release of lactic acid into the medium as compared with controls (Fig. 2C). More importantly, the IL1 β -enhanced lactate levels were significantly blocked by 4-MU. These results are not conclusive but reveal the wider scope of 4-MU action; inhibiting HA biosynthesis and cartilage degeneration as well as reversing metabolic imbalances associated with cytokine-activated chondrocytes.³

Effects of 4-MU on OA Development in Mice Following DMM Surgery

Mouse chow with 5% 4-MU (w/w) has been used successfully by investigators to deliver effective systemic doses that moderate a variety of functions.^{12,19–21} Figure 3A indicates the areas shown as representative images in Figure 3B–E. Safranin O stained histological sections of

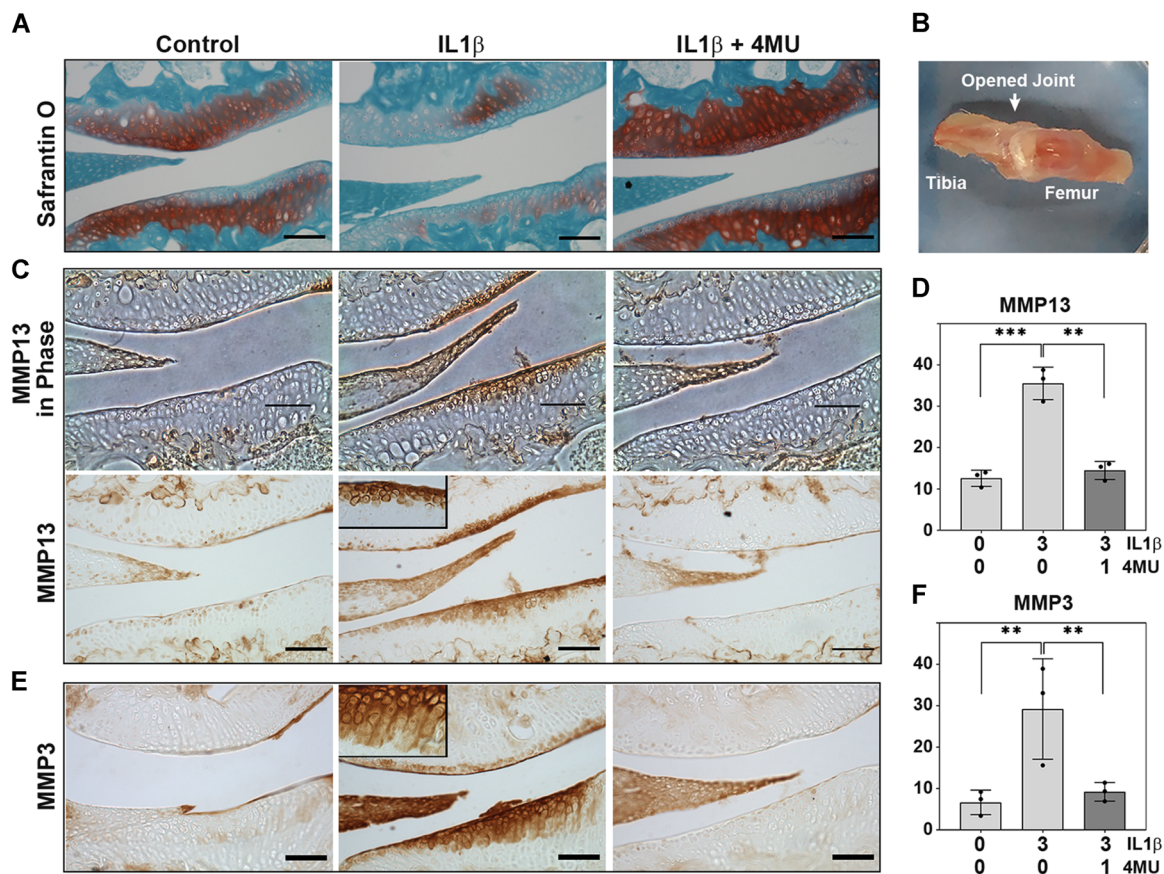


Figure 1. Articulated knee joints of female CD-1 mice (B: arrowhead) opened anteriorly, were cultured in serum-free media and treated without or with 3 ng/ml IL1 β with co-treatment with 0 or 1.0 mM 4-MU for 10 days. Shown are the representative images of sections following safranin O/fast green staining (A), MMP13 staining (C), and MMP3 staining (E). Bars = 100 μ m. From each animal, equivalent areas of the tibial plateau and femoral condyle cartilage in the medial compartment of the knee from each condition were analyzed for the number of clearly immunopositive chondrocytes imaged in brightfield (digitally enlarged examples are shown as insets in (C) and (E)). The percent of immunopositive chondrocytes was then calculated by comparison with the total number of chondrocytes within the same area as viewed in phase contrast (example images are shown for MMP13 stained images in (C)). This quantification of MMP13 (D) and MMP3 (F) positive chondrocytes (mean \pm standard deviation, $n = 3$ mice per group) is shown. ** $p < 0.01$, *** $p < 0.001$ (Student's t test). [Color figure can be viewed at wileyonlinelibrary.com]

sham-surgery mice, fed either control or 4-MU chow, exhibited no remarkable loss of stainable proteoglycan or morphological changes in articular cartilage at 2, 4, and 8 weeks after surgery (Fig. 3B and C). Although the sum of OARSI scores in all sham mice was comparatively low (between 0 and 12 points), slight increases were observed as time went on after sham surgery. However, no statistical differences were observed at 2, 4, and 8 weeks between control chow mice (sham) and 4-MU chow mice (sham) (Fig. 4A).

Significantly higher OARSI scores were observed in the articular cartilage of DMM mice compared with sham mice as early as 2 weeks after surgery (Fig. 4B). These results confirmed that the DMM surgery used in this study provided for progressive longitudinal changes in OA that could be evaluated and quantified histologically. Representative images of each condition highlight the main histological features (Fig. 3D and E). Focal discontinuity of the cartilage superficial zone and staining loss of safranin O in the mid zone was observed at 2 weeks in DMM mice fed control chow. Continued cartilage matrix loss occurring mainly in the

superficial zone was accompanied by much wider and deeper loss of safranin O staining, readily observed at 4 weeks after surgery. Furthermore, OA changes were most prominent at 8 weeks post-surgery, with observations of severe loss of matrix well into the deep zone (Fig. 3D). Reflecting these changes, the sum of OARSI scores of DMM mice consuming control chow increased continuously from 2 to 4 to 8 weeks (Fig. 4B). DMM mice consuming 4-MU chow displayed reduced changes in cartilage degeneration and OARSI scores at each time period evaluated. Discontinuity in the superficial zone was not severe at 2 weeks and cartilage matrix was preserved without severe loss of safranin O at 4 weeks in the 4-MU chow DMM mice. Also different from control chow DMM mice, destructive damages (clefs and delamination) were not observed at 8 weeks in the 4-MU chow DMM mice (Fig. 3E). Although a continuous increase of the sum of OARSI scores was observed during the time course in DMM mice consuming the 4-MU chow, there were significant decreases in these summed scores as with DMM mice on control chow. A few DMM mice on 4-MU chow were

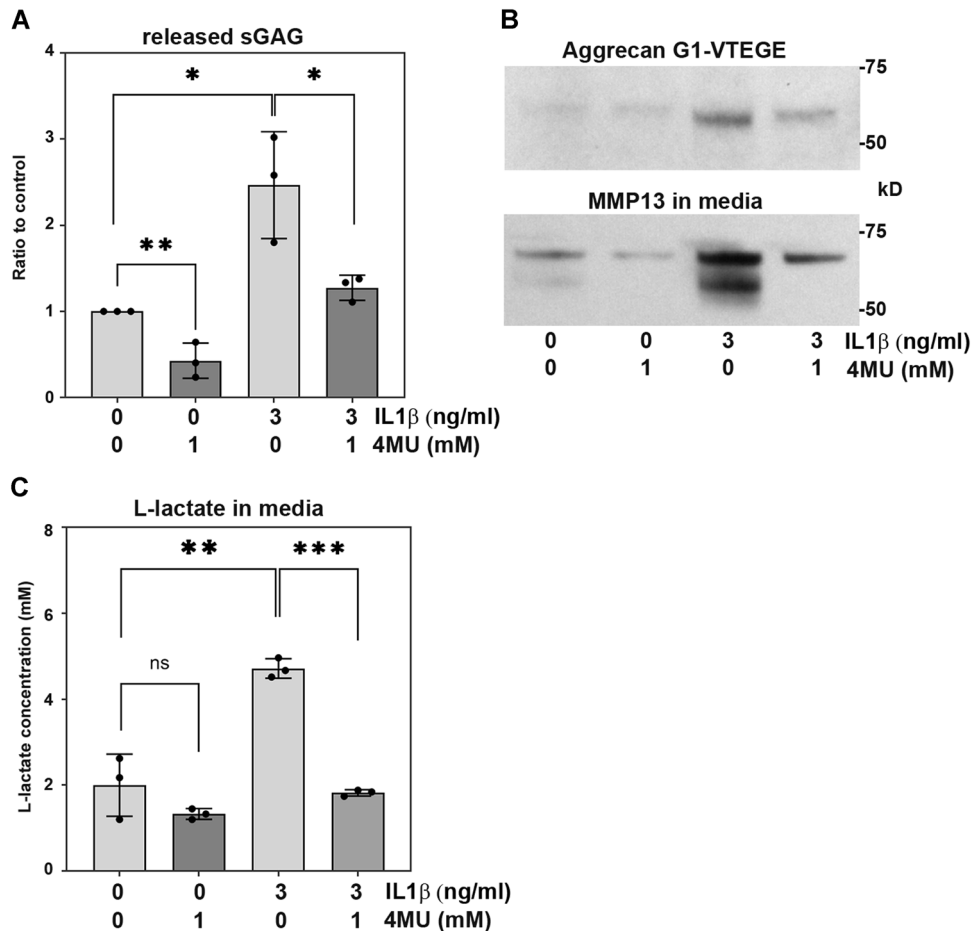


Figure 2. (A) The ratio of sulfated glycosaminoglycan (sGAG) content in the conditioned media of treated mouse knee joint explants (shown in Fig. 1), compared with untreated controls was calculated after using a dimethyl-methylene blue assay. Data represent the mean \pm standard deviation of three independent experiments. * $p < 0.05$, ** $p < 0.01$. (B) Equivalent volume aliquots of conditioned media from treated mouse explant cultures (shown in Fig. 1), were also processed for Western blotting to detect the aggrecan G1-VTEGE neopeptide and MMP13 protein. Shown is an example blot representative of three replicated, independent experiments. (C) The concentration of L-lactate in conditioned medium from bovine articular cartilage explants treated with interleukin 1 β (IL1 β) and 4-methylumbelliferone (4-MU) as indicated. ** $p < 0.01$, *** $p < 0.001$ (Student's t test).

distinct outliers (open circles). However, none of them exceeded the maximum score of DMM mice fed control chow (Fig. 4B). The significant difference at 2 weeks was $p = 0.038$; at both 4 and 8 weeks $p = 0.006$. Analysis of 4-week data on female mice was similar to males with the summed scores for DMM mice on 4-MU chow (median score 33.0) significantly lower than DMM mice on control chow (median score 47.8) with $p = 0.007$.

Osteophytes at medial tibia were not detected in sham mice at 8 weeks after surgery, but DMM mice with control chow showed the development of osteophytes. The size of osteophytes observed in DMM mice with 4-MU chow (Fig. 5A) was significantly reduced. The percentage of MMP13 (Fig. 5B) and MMP3 (Fig. 5C) positive chondrocytes was significantly lower in 4-MU fed mice than control mice at 2 weeks after DMM surgery.

Previous studies by other investigators suggested that a 1-week loading period may be required for mice on 4-MU chow to obtain maximal systemic levels of 4-MU.²¹ To explore whether this contributed to the

lower significant differences observed at 2 weeks as compared with those at 4 and 8 weeks, a 1-week pre-loading regimen was undertaken. Thus, mice were provided

4-MU chow or control chow containing chocolate flavor for 1 week prior to surgery and, for 2 weeks following DMM surgery. DMM mice fed chocolate-flavored control chow (Fig. 4C) showed similar OARSI scores as DMM mice fed control chow (Fig. 4B) by 2 weeks after DMM surgery. There was no significant difference between mice fed chocolate-flavored and unflavored control chows ($p = 0.67$). This result suggests that the chocolate flavoring, or other minor differences in the recipes of TestDiet control mouse chows, had no effects on OA progression after DMM surgery. More importantly, DMM mice fed 4-MU chow 1-week pre-surgery displayed lower summed OARSI scores and protection from cartilage damage as compared with DMM mice on chocolate-flavored control chow mice, 2 weeks after surgery (Fig. 4C). The significant difference was in the same range ($p < 0.05$) in the comparison of 2

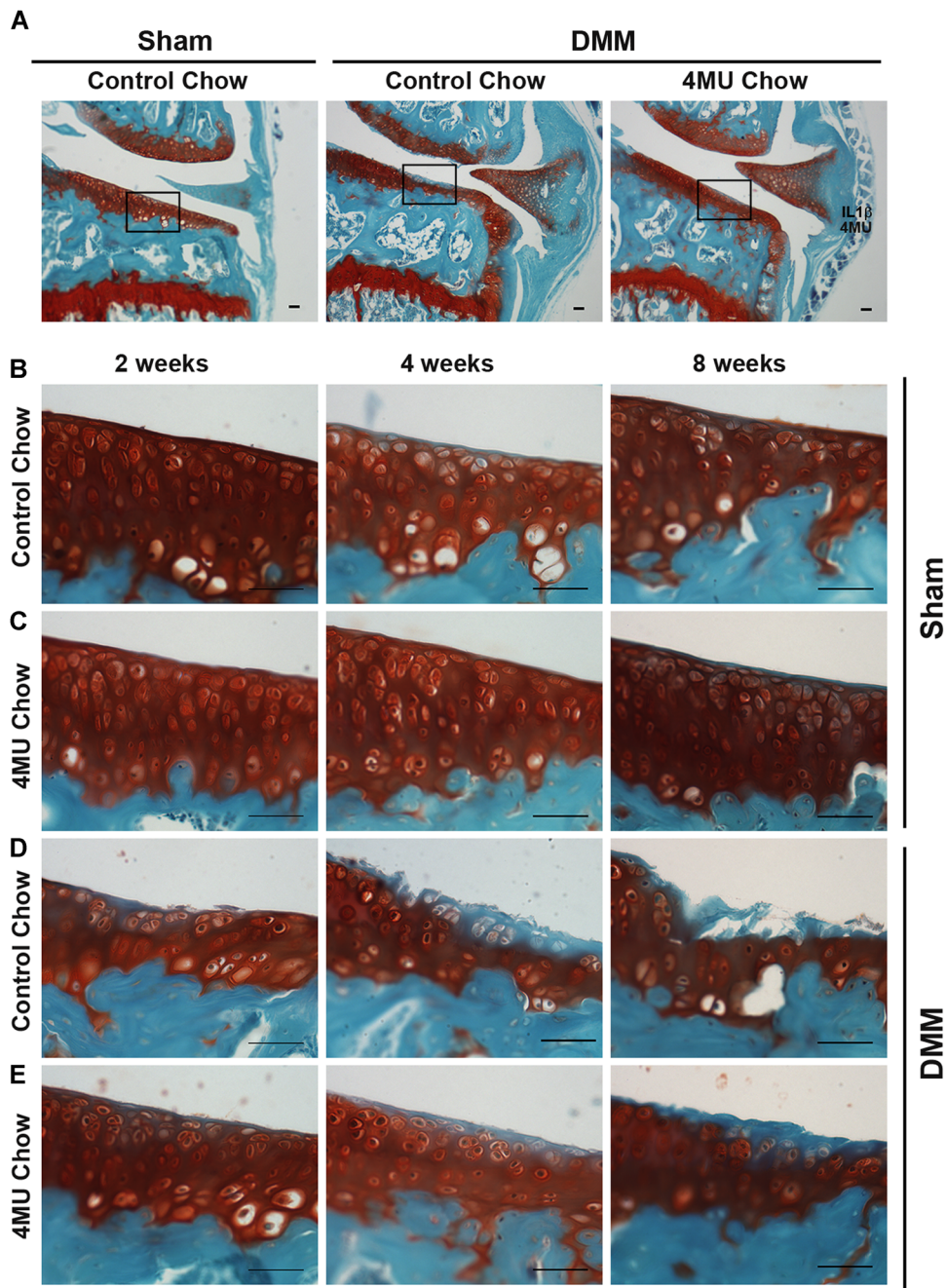


Figure 3. (A) Representative images of safranin O and fast green stained sections of the medial compartment of male C57BL/6 knee joints at 4 weeks after sham or destabilization of the medial meniscus (DMM) surgery. The middle area in the medial tibial plateau of mice (rectangle boxes in A) was selected to show representative images of osteoarthritic changes at 2, 4, and 8 weeks after sham surgery with control chow (B) or 4-MU chow (C) or DMM surgery with control chow (D) or 4-MU chow (E). Bars = 50 μ m. [Color figure can be viewed at wileyonlinelibrary.com]

weeks post-surgery as shown in Figure 4B. There was no significant difference between mice fed 4-MU chow without and with pre-loading ($p = 0.58$). Taken together, 4-MU contributes to the suppression of OA development from an early stage.

DISCUSSION

The primary result of this study was that the inclusion of 4-MU in the diet of mice limits OA damage to cartilage following DMM (Figs. 3 and 4). This suppression

was seen even at 2 weeks, the earliest time when OA changes are observable¹¹ and a time when MMPs are just becoming relevant (Fig. 5B). In later stages after DMM (8 weeks), osteophyte formation is clearly recognizable (Fig. 5A). Osteophyte size was also significantly reduced in DMM mice fed a 4-MU diet.

Many studies have demonstrated the *in vivo* usefulness of 4-MU in mouse models but primarily as an inhibitor of HA production^{28,29} and especially for models of inflammation dependent on HA

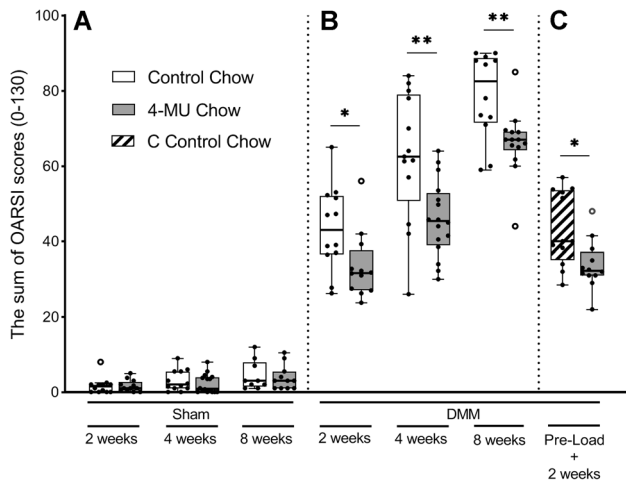


Figure 4. Histological evaluation of knee osteoarthritis of male C57BL/6 mice using the sum of OARSI scores²⁴ for five serial sections at the medial tibial plateau of each knee. Mice treated with control or 4-methylumbelliferone (4-MU) chow were evaluated at 2, 4, and 8 weeks after (A) sham surgery; sample numbers (control/4-MU) were 12/12; 12/16; 9/11, respectively or (B) destabilization of the medial meniscus (DMM) surgery; sample numbers (control/4-MU) were 12/12; 13/16; 12/14, respectively. (C) Chocolate control chow or 4-MU chow was provided to mice (sample numbers 12/12) from 1 week before DMM surgery and for 2 weeks following surgery. The evaluation was conducted at 2 weeks after DMM surgery. Data are shown with the Tukey box-plot with an overlay of individual scores as filled circles. Statistical outliers are depicted as open circles.

production.^{12,13,20,21,30} For example, Yoshioka et al.¹² observed that the application of 4-MU blocked the onset and severity of rheumatoid arthritis (RA) in a collagen-induced arthritis model (CIA). The primary effect of 4-MU was reduced synovitis related to a 4-MU-mediated reduction in HA production. However, they also observed the protection of articular cartilage from damage associated with the CIA model including reduced loss of safranin O staining and reduced MMP13 immunostaining. Our hypothesis is that chondroprotection in this CIA model includes direct effects of 4-MU on chondrocytes in addition to the reduction of inflammation of the synovium. Our goal was to examine the in vivo effects of 4-MU in an OA model more related to degenerative changes primarily in cartilage and less on the contribution of inflammation. Further supporting this effort, we chose to examine the earliest time points after DMM (namely 2, 4, and 8 weeks), and not include 12 weeks wherein synovial inflammation might become a factor. Additionally, the rescue of safranin O staining and reduction in MMP deposition by 4-MU in the DMM OA model in vivo (Figs. 3 and 5) closely matched the rescue of IL1 β -treated mouse cartilage in vitro model (Fig. 1)—the latter with no possibility of a contribution by inflammation.

Our previous study documented that while 4-MU does function as an effective HA biosynthesis inhibitor, it also exhibits another inhibitory potential completely independent of effects on HA production.³ 4-MU blocked the transcriptional expression of a select

group of pro-catabolic genes related to OA including MMP13 and ADAMTS4 even in the absence of ongoing HA production.³ Moreover, co-treatment of human or bovine cartilage explants (with no synovium) with 4-MU and IL1 β , provides a near-total block of safranin O staining loss. Although an interesting model, direct exposure of cartilage surfaces to IL1 β under serum-free and normoxic conditions in vitro may not represent authentic OA.⁵⁻⁸ DMM provides a more gradual and consistent model for OA¹¹ and, while all tissues of the joint are affected, initial changes occur primarily in the medial compartment articular cartilage and subchondral bone.

A pharmacokinetic study of 5% 4-MU/chow documented that 4-MU is readily metabolized in mice with a large proportion of 4-MU present in the circulation as glycoconjugates, lowering the effective dose of systemic free 4-MU.²¹ Interestingly, oral 4-MU was more effective at disease modification than the intraperitoneal injection of 4-MU. Thus, one limitation of our study is that the effective 4-MU dose available to the joint tissues of DMM mice was likely lower than the concentrations used in our previous in vitro studies.³ Nonetheless, although less dramatic than the chondroprotection observed in mouse joint explants exposed to IL1 β and 4-MU in vitro (Fig. 2), the effects of 4-MU in vivo, covering a period of months of natural, progressive degeneration, is the more relevant observation.

One potential limitation of this study: the pharmacokinetics study documented that even with chocolate additive, mice do not eat the 4-MU chow well in the first week, and lose weight in the first week followed by progressive regain of weight parallel to control animals.²¹ We observed a similar initial weight loss. Even with the progressive recovery, the weight of these animals was lower than control animals at every time point. In another study, mice leaner than controls showed the same DMM outcomes.²² When the OARSI scores shown in Figure 4 (4-week time point) of each individual mouse was normalized to its individual weight; the DMM mice fed 4-MU chow still exhibited a statistically-significant lower OARSI score (as a group $p = 0.040$) as compared with control chow DMM mice. Interestingly, female DMM mice with 4-MU chow lost less weight yet exhibited similar reduced OARSI scores as male mice; p -value = 0.009 as a group even after correcting individual female mice for weight loss. Most DMM mouse model studies primarily utilize male mice, as females (129S6/SvEv mice) were previously determined to be less susceptible to OA following DMM.³¹ We included female C57BL/6 mice at 4 weeks post-DMM for completeness of our study and found overall DMM OARSI scores were only slightly lower than in males.

Another possible limitation was that only the percentage of MMP13 or MMP3 positive cells per unit area were calculated for quantification of immunostaining shown in Figures 1C and E and 5B and C. It was more

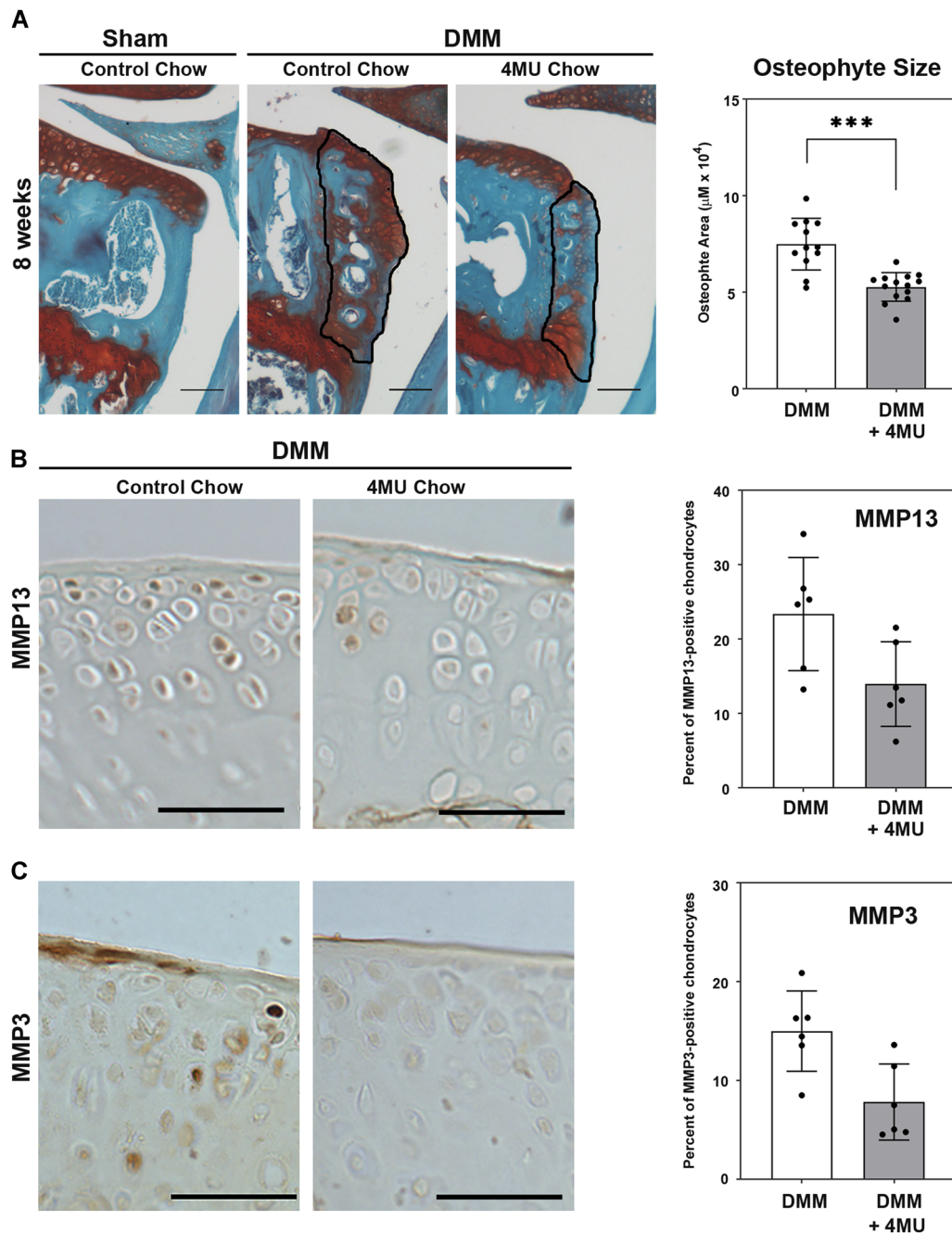


Figure 5. (A) Representative images of osteophyte morphology of male C57BL/6 mouse medial tibia (encircled areas outlined by black lines) 8 weeks after surgery. These areas were quantified to determine changes in osteophyte size (area in μm^2 , $n = 12$ mice per group). Bars = 100 μm . $***p < 0.001$ (Student's t test). Representative images of MMP13 (B) and MMP3 (C) staining of the medial tibia of destabilization of the medial meniscus (DMM) mice at 2 weeks after surgery and fed control or 4-methylumbelliferone (4-MU) chow. The percentage of positive chondrocytes was determined ($n = 6$ mice per group) as described in Figure 1. Bars = 50 μm . $*p < 0.05$ (Student's t test). [Color figure can be viewed at wileyonlinelibrary.com]

difficult to accurately quantify the MMP13 or MMP3 present in the cartilage extracellular matrix of pro-catabolically active cartilage. Thus, counting MMP-positive cells was a more stringent analysis but likely provided an under-estimate of the potential of 4-MU to block the accumulation of MMPs.

The mechanism for the transcriptional inhibition effects of 4-MU is currently unknown. What is known is that 4-MU blocks HA production by diminishing UDP-

hexose pools (UDP-GlcUA)¹⁴ and in some cells, disturbing UDP-hexoses affects energy metabolism which in turn, affects cell phenotype.¹⁸ Coincidentally, HAS2-OE also puts stress on intracellular UDP-hexose pools, inhibits the pro-catabolic phenotype of chondrocytes and reduces the rate of IL1 β -enhanced acidification of chondrocytes culture media (ECAR).⁴ In this study, 4-MU suppressed IL1 β -enhanced accumulation of lactic acid (Fig. 2C). In a separate recent study, we have

demonstrated that pro-catabolically activated chondrocytes exhibit an enhanced dependence of glycolysis for ATP production and, both 4-MU and HAS2-OE reverse this dependency, rescue their use of oxidative phosphorylation and rescue their deficits in p-AMPK.³² Moreover, these changes can be mimicked, even in cartilage explants, by glycolysis blocking compounds that have no direct effect on HA biosynthesis including 2-deoxyglucose or dichloroacetate.³² Thus, it appears that the blocking effects of 4-MU on the glycolytic pathway, driving a switch in energy metabolism, is the primary mechanism for reversing the pro-catabolic phenotype and providing chondroprotection.

AUTHORS' CONTRIBUTION

Conception and study design by S.T., S.I., D.P.O., C.B.K., and W.K. Acquisition, analysis, or interpretation of data by S.T., Y.O., C.B.K., and W.K. Drafting and critically revising the paper as well as reading/approval of the final submitted document by S.T., Y.O., S.I., N.I., D.P.O., C.B.K., and W.K.

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