

Bio-orthogonal Click Chemistry Methods to Evaluate the Metabolism of Inflammatory Challenged Cartilage after Traumatic Overloading

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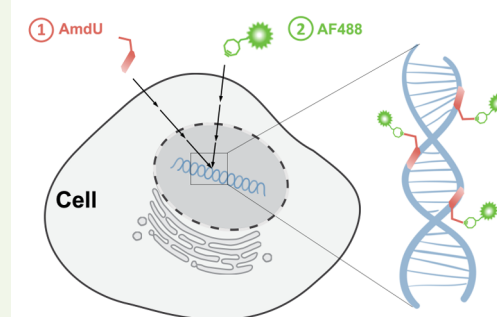


Supporting Information

ABSTRACT: During traumatic joint injuries, impact overloading can cause mechanical damage to the cartilage. In the following inflammation phase, excessive inflammatory cytokines (e.g., interleukin-1 β (IL-1 β)) can act on chondrocytes, causing over-proliferation, apoptosis, and extracellular matrix (ECM) degradation that can lead to osteoarthritis. This study investigated the combined effects of traumatic overloading and IL-1 β challenge on the metabolic activities of chondrocytes. Bovine cartilage explants underwent impact overloading followed by IL-1 β exposure at a physiologically relevant dosage (1 ng/mL). New click chemistry-based methods were developed to visualize and quantify the proliferation of *in situ* chondrocytes in a nondestructive manner without the involvement of histological sectioning or antibodies. Click chemistry-based methods were also employed to measure the ECM synthesis and degradation in cartilage explants. As the click reactions are copper-free and bio-orthogonal, i.e., with negligible cellular toxicity, cartilage ECM was cultured and studied for 6 weeks. Traumatic overloading induced significant cell death, mainly in the superficial zone. The high number of dead cells reduced the overall proliferation of chondrocytes as well as the synthesis of glycosaminoglycan (GAG) and collagen contents, but overloading alone had no effects on ECM degradation. IL-1 β challenge had little effect on cell viability, proliferation, or protein synthesis but induced over 40% GAG loss in 10 days and 61% collagen loss in 6 weeks. For the overloaded samples, IL-1 β induced greater degrees of degradation, with 68% GAG loss in 10 days and 80% collagen loss in 6 weeks. The results imply a necessary immediate ease of inflammation after joint injuries when trauma damage on cartilage is present. The new click chemistry methods could benefit many cellular and tissue engineering studies, providing convenient and sensitive assays of metabolic activities of cells in native three-dimensional (3D) environments.

KEYWORDS: IL-1 β , chondrocyte, proliferation, synthesis, proteoglycan, collagen

Click Chemistry to Visualize Proliferation



INTRODUCTION

Traumatic joint injuries are becoming increasingly common in young populations, potentially leading to high rates of post-traumatic osteoarthritis (PTOA) and permanent disability.^{1,2} In joint injuries, blunt overloading imparted on the cartilage can compromise the mechanical integrity of the cartilage extracellular matrix (ECM).³ Acute inflammation following traumatic joint injuries can last for 2–4 weeks, associated with elevated levels of pro-inflammatory cytokines.⁴ Synovial fluid interleukin-1 β (IL-1 β) concentrations can increase by 70-fold 24 h after knee injury⁵ and remain elevated for months to years after the initial injury.⁴ Inflammation is a widely accepted risk factor for the initiation of PTOA. The cytokines can act on chondrocytes, causing cell hypertrophy, over-proliferation, apoptosis, and elevated catabolic activities.⁶

Treatment strategies for acute joint inflammation vary in clinical practice, given individual physicians' clinical impressions and experience.⁷ Both nonpharmaceutical treatment options (PRICE, i.e., protection, rest, ice, compression, and elevation)⁸ and pharmaceutical options, such as nonsteroidal

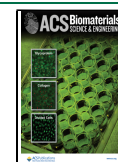
anti-inflammatory drugs (NSAIDs) and intraarticular cortisone injections, are widely adopted.^{9–11} It typically takes 2–4 weeks to ease the acute inflammation, and thus, there is a delay before any repair surgeries can be performed. For traumatically damaged cartilage, the duration of exposure to pro-inflammatory cytokines appears to be critical for cartilage health.¹² To date, the combined effects of traumatic overloading and acute inflammation on cartilage degradation have not been fully elucidated.

Evaluation of the adverse effects of pro-inflammatory cytokines on the function of chondrocytes and the turnover of cartilage ECM often requires the measurement of cell

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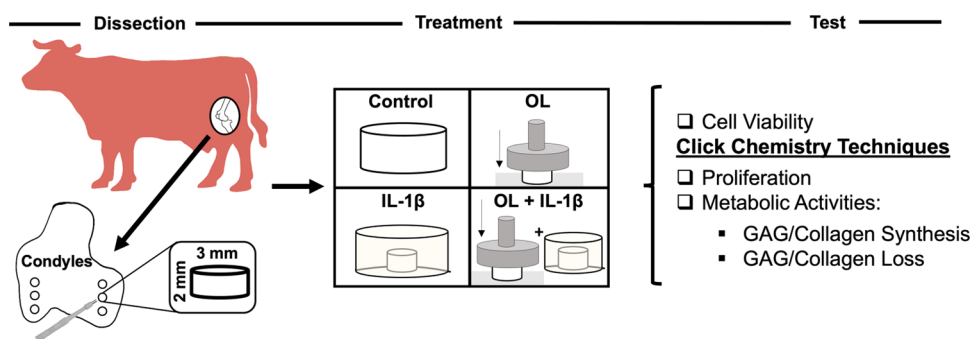


Figure 1. Experimental Design. Cartilage explants were harvested from calf femoral condyles and the patellar groove. Samples were randomly assigned to four groups: uninjured control, IL-1 β challenged (1 ng/mL), impact overloaded (50% compressive strain in 1 s), and impact overloaded followed by IL-1 β challenge. Each animal joint contributed an identical number of samples to each group, and experiments were repeated on at least three batches of joints ordered at different times from the abattoir. Following 24 h IL-1 β treatment, cartilage explants were assessed for cell viability and, using new click chemistry-based techniques, cell proliferation and GAG/collagen synthesis. GAG/collagen loss during *in vitro* culture was tracked during continuous IL-1 β inflammatory challenge.

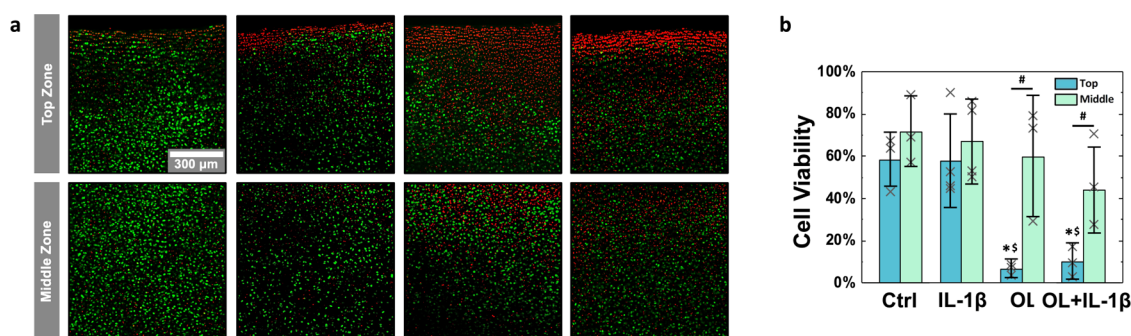


Figure 2. Cell viability in cartilage explants ($n = 3$). (a) Live/Dead staining of the control, overloaded, IL-1 β challenged, and overloaded plus IL-1 β challenged cartilage (green: live; red: dead). (b) Quantification of Live/Dead staining images. Overloading significantly reduced cell viability in the top zone of cartilage explants compared to the control or IL-1 β group and to their respective middle zones (*vs ctrl, \$vs IL-1 β , #between top and middle zones, $p < 0.05$).

viability, proliferation, protein synthesis, and the loss of ECM. Typical chemical assays for detecting proliferating cells include immunohistological BrdU staining or autoradiography of [3 H]thymidine uptake.^{13,14} Cartilage ECM protein assays often rely on colorimetric detection of sulfated glycosaminoglycan (sGAG, by dimethylmethylene blue [DMMB] assay) and collagen (hydroxyproline assay),^{15,16} which reflects the GAG or collagen content of the probed samples. The tests can become labor intensive when tracking cartilage degradation, requiring repetitive measures of the protein content released in the culture media during *in vitro* culture. To quantify the newly synthesized GAG or collagen in cartilage, isotopic labeling techniques are used such as [35 S] methionine labeling,^{17–19} although precautions are needed for the safety of the procedures.

In this study, new click chemistry-based techniques were developed to quantify the cell proliferation, GAG and collagen synthesis, and longitudinal loss of GAG and collagen contents in cartilage explants. Previously, copper-catalyzed click chemistry reactions have been used to detect DNA and RNA synthesis in monolayer cell cultures.^{20,21} The protocols used in this study benefit from strain-promoted azide–alkyne cycloaddition (SPAAC) reactions, or copper-free click chemistry, that can label chondrocyte DNA or ECM proteins.²² An azide-modified small-molecule agent is introduced into the culture media, which is subsequently taken by the cells and incorporated into the targeted DNA or newly synthesized protein. By “clicking” a dibenzocyclooctyne (DBCO)-modified

fluorophore to the azide group, the targeted molecules are readily detectable through the fluorescent tag. The click chemistry reactions are copper-free, so they are bio-orthogonal, *i.e.*, not interfering with the native biochemical activities. This allows the continued test and tracking of ECM proteins for several weeks. Using these new methods, this study quantified the combined effects of traumatic overloading and inflammatory challenge on chondrocytes in cartilage, including the influence on cell proliferation, ECM protein synthesis, and ECM degradation.

MATERIALS AND METHODS

Tissue Harvest and Preparation. Juvenile cartilage explants were harvested from the central region of the femoral condyle heads and patellar groove of freshly sacrificed knee joints from calves (1–2 months old), with mixed left or right side and gender (Green Village, NJ).²³ Cylindrical samples (diameter = 3 mm, thickness = 2 mm) were isolated using a biopsy punch and a custom-designed cutting tool that preserved the articular surface.²³ Harvested samples were cultured in chondrogenic media (DMEM supplemented with 1% ITS, 50 μ g/mL L-proline, 0.9 mM sodium pyruvate, and 50 μ g/mL ascorbate 2-phosphate) at 37 $^{\circ}$ C, 5% CO $_2$ for 48 h before any further experiments.^{23,24}

Cartilage explants were randomly assigned to one of four experimental groups: uninjured control, impact overloaded (OL), IL-1 β (1 ng/mL) challenged, and impact overloaded followed by IL-1 β challenge (OL + IL-1 β). Traumatic overloading of the explants was performed in an unconfined compression setup on an Instron Micro Tester (S848, Instron, Norwood, MA) in which a 50% strain

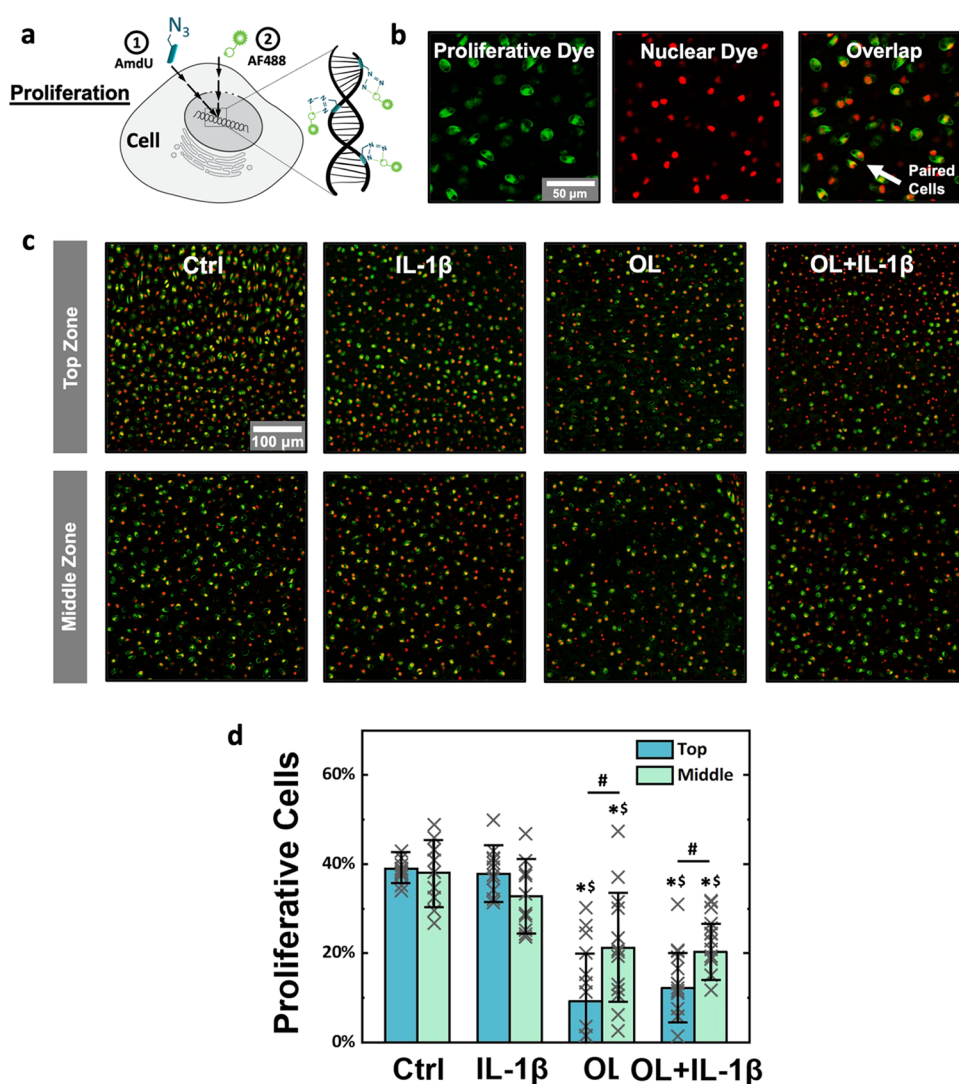


Figure 3. Proliferation of chondrocytes in cartilage explants ($n = 6$). (a) Click chemistry method for the labeling and visualization of proliferating chondrocytes. Azide-modified AmdU was added into culture media and incorporated into the proliferating DNA of cells instead of its natural analog thymidine. The fluorescent dye AF488 can click onto the azide groups in the labeled DNA. (b) Representative confocal images of proliferative cells stained by click chemistry (green), nuclei of all cells by BioTracker Red 650 Nuclear Dye (red), and the overlay image. (c) Confocal images (20X) of chondrocyte proliferation following IL-1 β challenge and impact overloading in the top and middle zone cartilage explants. Many paired cells immediately after division were labeled, especially in the top zone. (d) Quantified proliferation normalized to the number of total cell nuclei (*vs ctrl, $p < 0.001$; \$vs IL-1 β , $p < 0.05$; # between zones, $p < 0.05$).

was applied in 1 s and held for 15 s.²⁵ IL-1 β challenged cartilage samples were cultured with 1 ng/mL bovine recombinant IL-1 β (RBOIL1BI, ThermoFisher) supplemented in media. In animal joints, IL-1 β can reach 0.2 ng/mL immediately after joint fracture, associated with TNF- α at 1–4 ng/mL, IL-1 α at 0.3 ng/mL, and IL-6 at 0.1 ng/mL.²⁶ Here, 1 ng/mL IL-1 β was adopted to simulate the overall inflammatory challenge for chondrocytes following joint injuries. For the impact overloaded group, samples were overloaded and then cultured in chondrogenic media, and for the co-treatment group, samples were overloaded first and then cultured in IL-1 β supplemented media (Figure 1). After 24 h IL-1 β challenge, cell viability in cartilage ($n = 3$ explants) was examined using Live/Dead staining (ThermoFisher Scientific, Waltham, MA). Cartilage explants were bisected into hemicylinders, stained for 30 min with 2 μ M Calcein AM and 4 μ M ethidium homodimer, and washed thoroughly in DPBS before confocal imaging of cells inside the explant on a Zeiss LSM510 (Figure 2).

Proliferation Labeling. Proliferation of *in situ* chondrocytes was determined using a copper-free click chemistry-based assay ($n = 6$ explants). During the 24 h culture immediately after overloading,

cartilage explants in all groups were supplied with 10 μ M azide-modified nucleoside 5-(azidomethyl)-2'-deoxyuridine (AmdU; Sigma-Aldrich, St. Louis, MO) in the culture media. The membrane-permeable AmdU can be incorporated through endogenous metabolic phosphorylation into the proliferating DNA of cells instead of its natural analog thymidine (Figure 3a).^{20,27} Cells that entered the S-phase of the cell cycle during the 24 h culture will have azide-labeled DNA, which can subsequently be detected by a SPAAC reaction with a DBCO-modified fluorophore. After AmdU administration, cartilage explants were washed thoroughly with PBS to remove the excess AmdU molecules. Samples were then fixed in 3.7% formaldehyde for 24 h and underwent a cellular permeabilization treatment with 0.5% Triton X-100 for 20 min. Afterwards, the samples were bisected into hemicylinders to provide a flat surface for imaging and stained with fluorescent dye AF488 DBCO for 2 h (10 μ M; Alexa Fluor 488 DIBO analog, Click Chemistry Tools, AZ). AF488 DBCO, which is a water-soluble, green-fluorescent probe for copper-less detection of azide-tagged molecules, can “click” onto the azide-labeled proliferating DNA in cells.

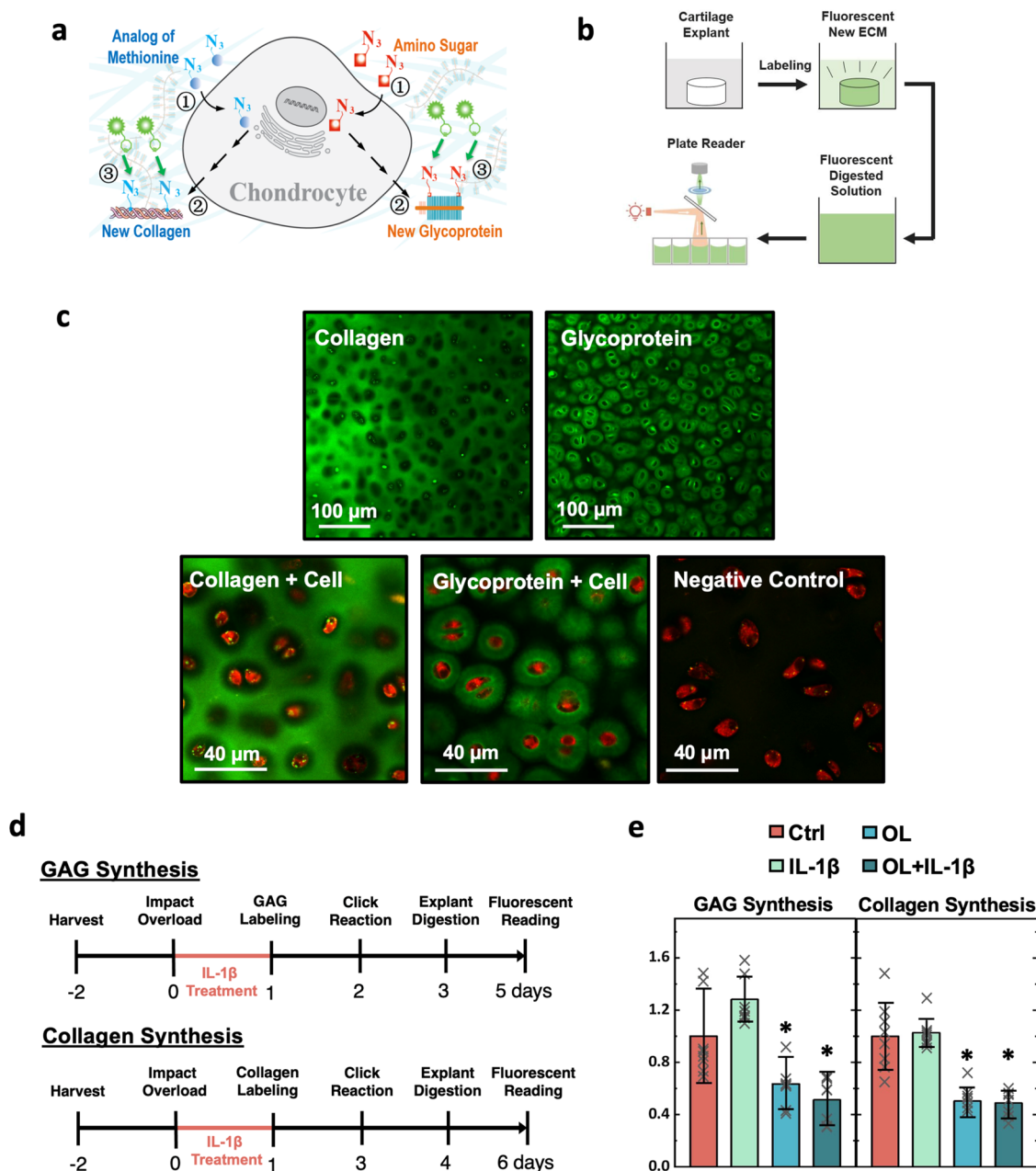


Figure 4. GAG and collagen synthesis in cartilage explants ($n = 8$). (a) Click chemistry method used to label newly synthesized glycoprotein (GAG) or collagen within the cartilage. A methionine analog with an azide group (AHA) was added into the culture media so the molecules could be used by chondrocytes for the synthesis of new collagen (left side of the cell). Fluorescent dye was attached to the azide-labeled new collagen through click reactions. Replacing the methionine analog with an azide-modified amino sugar (GAL), the newly synthesized GAG can be labeled (right side of cell). In this study, each explant was labeled for either GAG or collagen but not both. (b) Experimental protocol used to quantify the newly synthesized GAG/collagen within the cartilage explant. The click chemistry-labeled cartilage was digested, and the fluorescent intensity of digestion media was measured by a fluorescent plate reader. (c) Representative confocal images of fluorescently labeled new collagen and GAG (green) in cartilage explants. New collagen was evenly dispersed through the ECM but not the PCM. Glycoproteins were concentrated around the cell and formed a halo surrounding the chondrocytes (red). Negative control received the same treatment without metabolic labeling. (d) Timeline of experiments for measuring newly synthesized GAG and collagen in four groups of samples. (e) Synthesis of GAG in 24 h and collagen in 48 h. Synthesis was normalized to explant weight and then to the control group (*vs control, $p < 0.05$).

After click chemistry-based proliferation labeling, cartilage explants were counter-stained with BioTracker Red 650 Nuclear Dye (2X; Sigma-Aldrich, St. Louis, MO). Cells in cartilage were imaged with a 20X objective on a Zeiss LSM510 confocal microscope at a focal plane $\sim 50 \mu\text{m}$ below the cross sectional surface, to avoid cells that were damaged during the preparation and preceding cutting steps. For each sample, images of both the top zone (top 1 mm) and middle zone (bottom 1 mm) were recorded. All fluorescent images were processed and quantified in ImageJ (NIH, Bethesda, MD).²⁸ The

proliferation of each region was defined as the ratio between AmdU positive cells (green) and the number of all nuclei (red). Due to the nature of focal planes in confocal imaging, only cells with proliferative staining and a corresponding nuclear stain were counted as positively proliferating cells.

Synthesis of GAG and Collagen. Newly synthesized GAG or collagen in cartilage explants was detected and labeled using a copper-free click chemistry-based assay. In brief, to label the newly synthesized GAG, cartilage explants were cultured for 24 h in

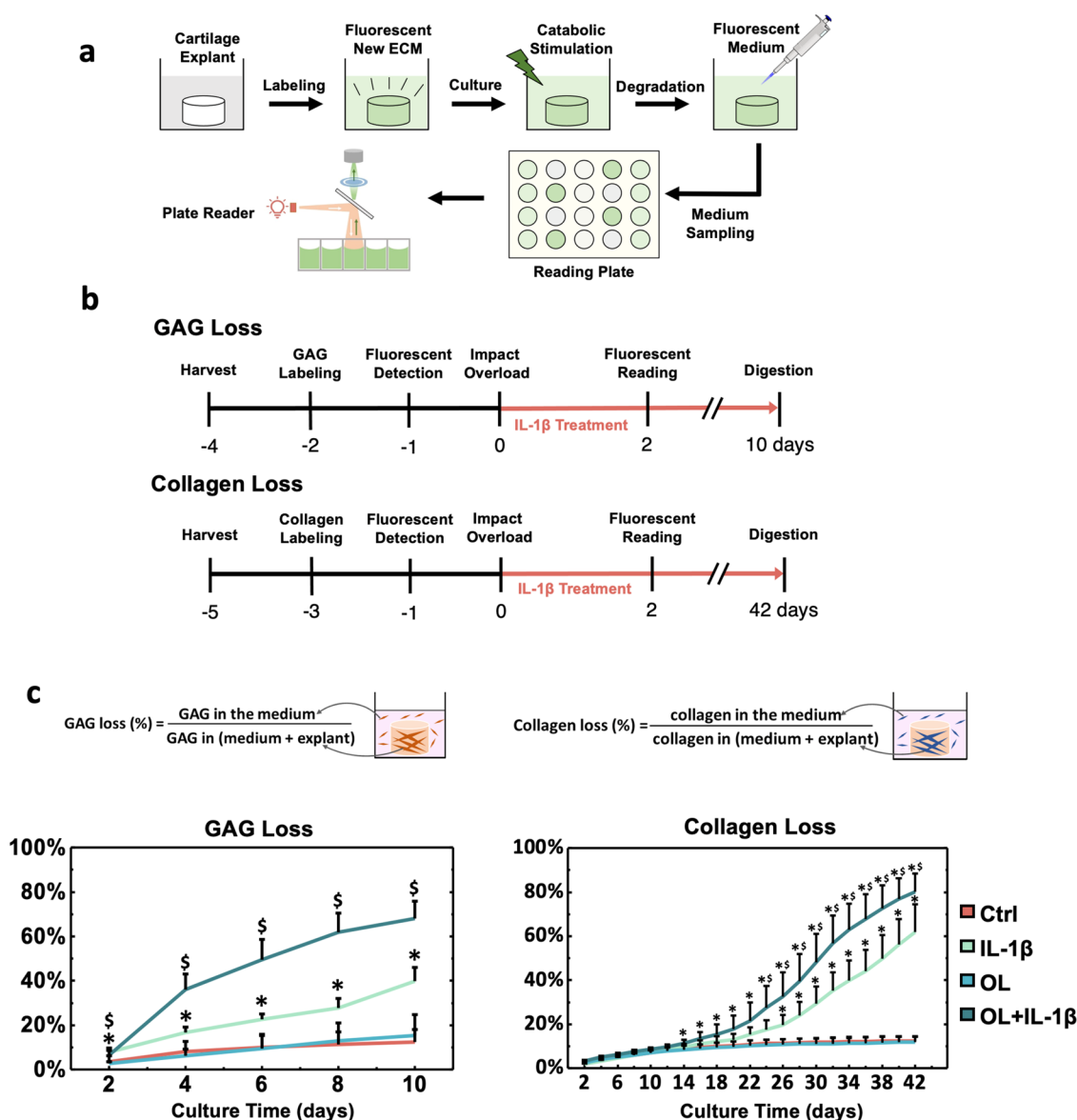


Figure 5. Longitudinal loss of GAG and collagen contents from cartilage explants ($n = 8$). (a) Experiment protocol for the click chemistry labeling of cartilage explants, and the longitudinal measurement of GAG and collagen loss. Cartilage explants with labeled newly synthesized GAG and collagen were cultured *in vitro* as usual. The fluorescent intensity of culture media was read every other day to quantify the GAG or collagen content released from the cartilage to the media. (b) Timeline of experiments for tracking the GAG or collagen loss during *in vitro* culture. GAG loss was tracked for 10 days, while collagen loss was measured for 42 days due to their distinctive release kinetics. (c) Cumulative GAG and collagen loss over the culture period. Loss of GAG or collagen into the media was normalized to the total amount of GAG/collagen loss plus the amount left in the explant (*vs control, $p < 0.01$; \$vs IL-1 β , $p < 0.001$).

media containing the azide-modified monosaccharide *N*-azidoacetyl-galactosamine-tetraacylated (GAL; 30 μ M; Click Chemistry Tools, AZ). GAL, an unnatural monosaccharide, can be utilized by chondrocytes for the synthesis of new GAGs (Figure 4a). The monosaccharide is used by the cells' biosynthetic pathways and incorporated into cell-surface O-linked glycoproteins.²⁹ To label the new collagen, samples underwent a 4 h methionine starvation in depletion medium (DMEM-LM supplemented with 1% ITS, 50 μ g/mL L-proline, 105 μ g/mL Leucine, 0.9 mM sodium pyruvate, and 50 μ g/mL ascorbate 2-phosphate) to reduce competition by methionine. Samples were then cultured for 48 h in media containing the azide-modified methionine analog, L-Azidohomoalanine (AHA; 30 μ M; Click Chemistry Tools, Az), which can be used in place of methionine during the synthesis of new collagen peptides, similar to [³⁵S]-methionine radiolabeling (Figure 4a). AHA can be taken up by cell amino acid transporters and used as a substrate by methionyl tRNA synthetase for charging onto its tRNA.³⁰ Collagen synthesis was

labeled for a longer time due to its slower synthesis rate than GAG. After GAL or AHA feeding, cartilage explants were washed thoroughly with culture media to remove excessive GAL or AHA molecules. The newly synthesized GAG or collagen in the cartilage explant was then conjugated with the fluorescent dye AF488 DBCO (30 μ M) via a copper-free click chemistry reaction. Each explant was labeled for a single target, *i.e.*, newly formed GAG or collagen. To verify the copper-free click chemistry method employed here was bio-orthogonal, cartilage samples treated with AHA, GAL, and/or AF488 DBCO were further cultured for 7 days and then examined with Live/Dead (ThermoFisher) staining. No extra cell death was noted in the samples compared to the control (Figure S1 in the Supporting Information). Therefore, the click chemistry-labeled cartilage could be cultured further, and the turnover of labeled ECM components can be tracked longitudinally under various experimental conditions.

In this study, immediately following overloading and 24 h IL-1 β challenge, cartilage explants in all four groups were labeled for GAG

($n = 8$) or collagen ($n = 8$) synthesis using the click chemistry method (Figure 4b,d). After the click chemistry reaction, the samples were enzymatically digested with papain (125 $\mu\text{g}/\text{mL}$) at 37 $^{\circ}\text{C}$. The amount of newly synthesized GAG or collagen in each explant was quantified as the fluorescent intensity of the digested media using a fluorescent plate reader (Gemini EM; Molecular Devices, San Jose, CA) and normalized to the explant weight obtained immediately after tissue harvest.

Degradation of GAG and Collagen in the ECM. To track ECM degradation, the release of either GAG ($n = 8$) or collagen ($n = 8$) contents from the cartilage explant to the culture media was measured. First, the newly synthesized GAG or collagen was labeled in the cartilage explant with the copper-free click chemistry method (Figure 5a,b). Then, explants underwent overloading damage and/or continuous IL-1 β challenge, with a follow-up culture for either 10 days (to track GAG loss) or 42 days (to track collagen loss), in accordance with the different GAG and collagen loss kinetics.¹⁵ Loss of pre-labeled GAG or collagen contents from the cartilage ECM introduced fluorescence to the culture media. The culture media were changed every other day, and the old media were read with a fluorescent plate reader. At the end of the explant culture, samples were digested with papain, and the fluorescence of the digestion media was obtained. The total amount of GAG or collagen was represented by the sum of longitudinal readings of media and the reading of digested solution, which was used to normalize the longitudinal GAG or collagen loss data points for each sample.

Statistical Analysis. All data are presented as means $\pm$ standard deviation. Viability and proliferation data from four groups and two zones were assessed via two-way ANOVA with Tukey post hoc tests to compare all means. For the synthesis of GAG and collagen contents, data were normalized to the control (uninjured) group, and one-way ANOVA followed by Tukey–Kramer post hoc comparison was applied to detect differences between groups. To test the significance of GAG or collagen loss from cartilage, one-way ANOVA with Tukey post hoc test was performed at each time point. All tests were conducted in R, and the significance level was set at $\alpha = 0.05$.

RESULTS

Chondrocyte Viability and Proliferation. *In situ* chondrocyte viability following traumatic overloading and/or 24 h inflammatory IL-1 β challenge was examined with Live/Dead staining (Figure 2). In control samples, cell death was noticed in the cartilage likely due to transitions from the *in vivo* to *post mortem* environments and trauma from joint dissection. As more dead cells were located close to the articular surface, cell viability of the top zone ($58.3 \pm 12.8\%$, mean $\pm$ standard deviation) tended to be lower than that of the middle zone ($71.5 \pm 16.5\%$), although the difference was not significant. Cell viability was not affected by 24 h 1 ng/mL IL-1 β challenge. In contrast, traumatic overloading, either followed with or without IL-1 β challenge, induced widespread cell death throughout the tissue depth. Most cells in the top layer were dead, with merely $6.4 \pm 4.3\%$ viability for the overloaded group and $9.9 \pm 8.4\%$ for the OL+ IL-1 β group (both $p < 0.05$ vs ctrl) and no significant difference between the two groups. Viability in the middle zones of the two OL groups was significantly higher than in their corresponding top zones ($p < 0.05$). Overall, there was no apparent difference in viability in the middle zone among the experimental groups.

Using the new click chemistry method, chondrocytes in cartilage explants that entered the S-phase of the cell cycle during the 24 h treatment period were labeled and imaged on a confocal microscope (Figure 3b). After transforming to *in vitro* culture, many chondrocytes in the calf cartilage entered the S-phase of the cell cycle during the 24 h, which could be related to the young age of the animals and the *in vitro* culture

environment.³¹ For example, many paired cells, which appeared to be immediately following cell division (Figure 3b,c), were labeled in the top layer. The percentages of proliferating cells in the top and middle zone cartilage were quantified separately for all groups (Figure 3d). In the top zone, control samples had a proliferation rate of $39.0 \pm 3.3\%$, which was not significantly affected by the 1-day IL-1 β treatment. Overloading alone reduced the proliferation rate to $9.3 \pm 10.5\%$ in the top zone ($p < 0.001$). Overloading plus IL-1 β treatment showed a similar decrease in proliferation in the top zone ($12.3 \pm 7.7\%$, $p < 0.001$ vs ctrl). In the middle zone, chondrocyte proliferation was $38.1 \pm 7.3\%$ for the control group, which was not affected by IL-1 β treatment but was significantly reduced by overloading ($p < 0.001$ vs ctrl), although not as drastically as in the top zone. In the two overloaded groups, due to the high percentage of dead cells close to the articular surface, proliferation in the top zones is significantly lower than in the corresponding middle zones ($p < 0.05$). To verify our click chemistry protocol, adult human cartilage (male, 58 years old) from the hip femoral condyle was used as a negative control. Few proliferative cells were revealed by the click chemistry staining (Figure S2 in the Supporting Information). Moreover, cartilage samples from the control group were prepared for Ki67 IF staining, which revealed a similar percentage of proliferative cells to the click chemistry method (Figure S3 in the Supporting Information).

Chondrocyte Metabolic Activities. Following traumatic overloading and/or IL-1 β challenge, the anabolic activities of chondrocytes in cartilage explants were measured and compared using the click chemistry methods. Newly synthesized GAG in 24 h was fluorescent-labeled. In the confocal image of *in situ* chondrocytes, new GAG molecules accumulated surrounding the cells after being synthesized and formed a fluorescent halo around the plasma membranes. The fluorescent intensity of the halo decreased from the membrane to the ECM direction. By digesting the tissue and measuring the fluorescent intensity of the digestion media, we compared the GAG synthesis among groups. Collagen synthesis was quantified with a similar protocol. Newly synthesized collagen molecules, unlike the new GAG, were dispersed evenly across the territorial/interterritorial ECM space in cartilage, with no distinct green halo surrounding the cells (Figure 4c). IL-1 β treatment at 1 ng/mL for 24 h had no significant effect on the GAG or collagen synthesis of *in situ* chondrocytes (Figure 4e). In contrast, overloading induced significant cell death, especially in the top zone, and the dead cells showed little GAG or collagen synthesis activities (Figure S4 in the Supporting Information). This reduced both GAG and collagen synthesis in the overloaded cartilage, which was close to only 50% of the control ($p < 0.05$).

The longitudinal loss of GAG or collagen contents from the cartilage was tracked for 10 or 42 days during *in vitro* culture, respectively, in accordance with their differing release kinetics.¹⁵ Compared to its prominent effects on proliferation and ECM synthesis, overloading alone showed no significant effects on the loss of GAG (11.7 ± 2.6 vs $10.5 \pm 0.7\%$ for ctrl, $p > 0.05$) or collagen (15.3 ± 9.4 vs $12.4 \pm 5.6\%$ for ctrl, $p > 0.05$) content from the explants in the following long-term culture (Figure 5c). In contrast, exposure to 1 ng/mL IL-1 β induced a significant loss of GAG and collagen contents. During IL-1 β challenge, GAG loss was evident immediately on day 2 of the treatment and culminated in a total release of $39.9 \pm 6.2\%$ after 10 days. In comparison to GAG loss, the loss of

collagen contents was significantly delayed, not evident until day 14 of the long-term culture. The total amount of collagen released in the 42-day culture was $55.6 \pm 10.9\%$. Although traumatic overloading alone induced little extra GAG or collagen loss, IL-1 β challenge after traumatic overloading drastically exacerbated the loss of both GAG and collagen contents ($p < 0.001$ vs the control). The total release of GAG reached $67.9 \pm 7.9\%$, while the total collagen release was $80.1 \pm 8.4\%$ for the OL+IL-1 β groups, significantly higher than the IL-1 β alone groups ($p < 0.001$).

DISCUSSION

New click chemistry-based methods were developed to visualize and quantify the cell proliferation, protein synthesis, and ECM degradation in cartilage explants during *in vitro* culture. For the first time, this study adopted the click chemistry-based proliferation labeling technique on native cartilage tissues. Intact proliferating chondrocytes were visualized in their native matrix, where cell morphology and DNA integrity are better preserved compared to sliced sections. Our proliferation staining protocol is based on the same principles of commonly used antibody methods, *i.e.*, BrdU, but the traditional histological operations were avoided, such as paraffin embedding, sectioning, and the use of antibodies. Commercial assay kits based on similar click chemistry technologies, such as Click-iT (Invitrogen), are now available for proliferation staining, but most of these kits are designed for monolayer cells.^{20,21} The click chemistry-based technique established in this study significantly increased the convenience and reduced the cost (by $\sim 90\%$ relative to commercial assays) of evaluating cell proliferation in cartilage tissues or three-dimensional (3D) neo-tissues.

Click chemistry-based methods for labeling GAG and collagen represent a sensitive new technique to measure both anabolic and catabolic activities of *in situ* chondrocytes. The methods are more convenient and cost-efficient than traditional chemical methods, such as isotopic labeling, DMMB assay for GAG content, and hydroxyproline assay for collagen content. Moreover, the ECM click chemistry methods are copper-free and bio-orthogonal,³² meaning that the click reactions occurring inside of chondrocytes barely interfere with the native biochemical behaviors of the cells, *i.e.*, the labeling of biomolecules in living cells has minimal cellular toxicity.³³ In this study, newly synthesized GAG or collagen molecules in cartilage explants during a 24–48 h period were visualized, quantified, and compared between groups. To track the GAG and collagen loss after click chemistry labeling, cartilage explants were cultured for 10 and 42 days after the labeling, respectively. The temporal profiles of GAG and collagen loss under inflammatory challenge match well with previous studies based on traditional chemical assays; IL-1 β -induced GAG loss saturates in 10 days and the collagen loss accelerates on day 14.^{15,34} Longitudinal measurements of GAG and collagen loss in this study are more convenient compared to the DMMB or hydroxyproline assays, as reading the fluorescent intensity of culture media every other day requires minimal effort. Benefiting from its bio-orthogonality, the procedure is highly adaptable to various physical or chemical treatment profiles on cartilage tissues or other cells in 3D constructs.

Using these techniques, the metabolism of inflammatory challenged cartilage following traumatic overloading was evaluated. IL-1 β inflammatory challenge alone had more influence on the catabolic activities than on the anabolic

activities of chondrocytes. Overexpression of inflammatory cytokines during the acute inflammation phase alone, although lasting only a few days to 2–4 weeks, can cause irreversible cartilage degeneration and initiate osteoarthritis.^{35–37} In this study, IL-1 β at 1 ng/mL induced a significant loss of GAG and collagen contents from the cartilage explants during continuous *in vitro* culture. There was a 40% loss of GAG during the 10-day culture and a 61% loss of collagen in 6 weeks. IL-1 β challenge showed no significant effects on the proliferation of chondrocytes, which could be related to the low dosage and short treatment time of IL-1 β .³⁸ In addition, the treatment reduced neither the GAG nor collagen synthesis. IL-1 β challenge has been shown to suppress the anabolic capability of chondrocytes, but most of these studies employed a much higher concentration than 1 ng/mL, usually at or above 10 ng/mL.^{39,40}

Traumatic overloading induced significant cell death in cartilage explants, affecting the overall metabolic and proliferative capabilities of the tissue. More widespread cell death was evident in the top zone than in the middle zone. Superficial zone cartilages can act as a protective layer for the rest of the tissue when subjected to mechanical injury.^{41–43} Due to its low proteoglycan content, superficial zone cartilage has a compressive modulus $\sim 10\%$ of the middle zone. The superficial zone strain and deformation therefore are several times higher than the middle zone causing significant cell death, in alignment with previous studies.^{44,45} The reduced number of live cells in the overloaded samples resulted in low proliferation and caused reduced synthesis of GAG and collagen in the top zone. However, despite the high number of dead cells, overloading alone induced similar ECM degradation to the control group. During serum-free *in vitro* culture of cartilage, loss of GAG and collagen is mainly due to the enzymatic degradation of ECM molecules, where enzymes and proteases are generated by the only cell population, chondrocytes, in the explant. Due to the high cell death in the overloaded samples, there were few metabolically active cells to produce proteases, which resulted in the nonelevated loss of ECM from the overloaded alone samples compared to the control.

Traumatic overloading exacerbated the catabolic effects of IL-1 β on cartilage. It could be because a damaged ECM with compromised mechanical integrity is easier to degrade or the cells that survived from overloading became more vulnerable or responsive to IL-1 β challenge with elevated catabolic activity or both. Either way, the ease of acute inflammation is essential for the protection of cartilage after joint injuries. According to the present data, severe inflammation for a few weeks following joint injury could induce significant and irreversible loss of GAG and collagen contents, which compromises the mechanical functions of cartilage and eventually leads to cartilage erosion. As acute inflammation after joint trauma is potentially controllable or at least modifiable,^{4,46} it could represent a feasible disease modification option for PTOA prevention.

A few limitations to this study should be discussed. First, bovine calf cartilage was used as an alternative to young healthy human cartilage. This is primarily due to the limited access to human tissues, but juvenile bovine tissues provide additional benefits such as the higher number of chondrocytes within the tissue and a superior proliferative rate and anabolic activity.³⁵ Second, IL-1 β was selected to induce inflammatory challenge. While IL-1 β is one of the most prevalent cytokines

present during animal joint inflammation,⁴ it cannot represent the overall effects of all pro-inflammatory cytokines, including TNF- α and the IL-1 α and IL-6 families.^{4,36} Third, apart from ECM degradation, this study primarily focused on the short-term responses of chondrocytes, i.e., 1–2 days following injury. While the acute inflammation phase could be as short as a few days in young animals,^{47,48} it can last for weeks in the human knee joint and then be sustained at a lower level for months to years after the initial injury.^{4,37} In addition, the longitudinal GAG and collagen loss measured in this study represent the loss of the newly synthesized proteins labeled by click chemistry. Although the data showed the turnover of a subpopulation of total GAG or collagen in the tissue, it enabled the comparisons between different groups and yielded insights into how cytokines and injurious mechanical stresses affect cartilage degradation.

CONCLUSIONS

Click chemistry-based techniques were developed to visualize cell proliferation in native cartilage. The method avoided tissue microsectioning and the use of antibodies, which improved efficiency and reduced the experimental cost. As the cells were labeled in their original 3D environment, the methods can also be applied for biomaterials on which histological sectioning is technically difficult. New click chemistry methods were also adopted to quantify the synthesis of GAG/collagen content in cartilage during *in vitro* culture. The highly sensitive method can be readily used for tissue engineering research, especially those involving cell culture in 3D scaffolds. The protein synthesis of cells can be quantified in a determined period while the tissue or scaffold is undergoing various chemical or physical stimulations. Using the new methods, this study evaluated the cell viability, proliferation, and anabolic and catabolic activities of *in situ* chondrocytes in cytokine challenged and traumatically damaged cartilage. The data offered new insights into the major causes of cartilage degeneration during the acute inflammation phase after joint injuries. Dead chondrocytes alone in cartilage cause little ECM degradation due to the lack of proteases. In contrast, metabolically active cells under cytokine challenge can cause severe loss of GAG and collagen in a short time. Such destructive effects of inflammation can be exacerbated significantly in mechanically damaged cartilage. Therefore, shortening of the acute inflammation phase could be essential to avoid irreversible degradation of cartilage after traumatic overloading.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acsbiomaterials.2c00024>.

Bio-orthogonal test of the click chemistry technique to tag proteins and glycoproteins in bovine cartilage (Figure S1); proliferation of human chondrocytes in osteoarthritic human hip cartilage with the click chemistry technique (Figure S2); Ki67 IF staining of juvenile bovine cartilage from the experimental control group (Figure S3); confocal images of click chemistry staining of newly synthesized GAG and collagen in the IL-1 β and OL + IL-1 β experimental groups (Figure S4) (PDF)

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Notes

The authors declare no competing financial interest.

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REFERENCES

- (1) Thomas, A. C.; Hubbard-Turner, T.; Wikstrom, E. A.; Palmieri-Smith, R. M. Epidemiology of Posttraumatic Osteoarthritis. *J. Athletic Train.* **2017**, *52*, 491–496.
- (2) Ackerman, I. N.; Kemp, J. L.; Crossley, K. M.; Culvenor, A. G.; Hinman, R. S. Hip and Knee Osteoarthritis Affects Younger People, Too. *J. Orthop. Sports Phys. Ther.* **2017**, *47*, 67–79.
- (3) Nishimuta, J. F.; Levenston, M. E. Response of Cartilage and Meniscus Tissue Explants to *in Vitro* Compressive Overload. *Osteoarthritis Cartilage* **2012**, *20*, 422–429.
- (4) Lieberthal, J.; Sambamurthy, N.; Scanzello, C. R. Inflammation in Joint Injury and Post-Traumatic Osteoarthritis. *Osteoarthritis Cartilage* **2015**, *23*, 1825–1834.
- (5) Sward, P.; Frobell, R.; Englund, M.; Roos, H.; Struglics, A. Cartilage and Bone Markers and Inflammatory Cytokines Are Increased in Synovial Fluid in the Acute Phase of Knee Injury (Hemarthrosis)—a Cross-Sectional Analysis. *Osteoarthritis Cartilage* **2012**, *20*, 1302–1308.
- (6) Lv, M.; Zhou, Y.; Polson, S. W.; Wan, L. Q.; Wang, M.; Han, L.; Wang, L.; Lu, X. L. Identification of Chondrocyte Genes and Signaling Pathways in Response to Acute Joint Inflammation. *Sci. Rep.* **2019**, *9*, No. 93.
- (7) Buckwalter, J. A.; Anderson, D. D.; Brown, T. D.; Tochigi, Y.; Martin, J. A. The Roles of Mechanical Stresses in the Pathogenesis of Osteoarthritis: Implications for Treatment of Joint Injuries. *Cartilage* **2013**, *4*, 286–294.
- (8) Bleakley, C.; Davison, G. Management of Acute Soft Tissue Injury Using Protection Rest Ice Compression and Elevation: Recommendations from the Association of Chartered Physiotherapists in Sports and Exercise Medicine (ACPSM) [Executive Summary] 2010.

- (9) Mehallo, C. J.; Drezner, J. A.; Bytowski, J. R. Practical Management: Nonsteroidal Antiinflammatory Drug (NSAID) Use in Athletic Injuries. *Clin. J. Sports Med.* **2006**, *16*, 170–174.
- (10) Fredberg, U. Local Corticosteroid Injection in Sport: Review of Literature and Guidelines for Treatment. *Scand. J. Med. Sci. Sports* **2007**, *7*, 131–139.
- (11) Scott, A.; Khan, K. M.; Roberts, C. R.; Cook, J. L.; Duronio, V. What Do We Mean by the Term “Inflammation”? A Contemporary Basic Science Update for Sports Medicine. *Br. J. Sports Med.* **2004**, *38*, 372–380.
- (12) van der Kraan, P. M. The Interaction between Joint Inflammation and Cartilage Repair. *Tissue Eng. Regen. Med.* **2019**, *16*, 327–334.
- (13) Iannone, F.; Bari, C. D.; Scioscia, C.; Patella, V.; Lapadula, G. Increased Bcl-2/P53 Ratio in Human Osteoarthritic Cartilage: A Possible Role in Regulation of Chondrocyte Metabolism. *Ann. Rheum. Dis.* **2005**, *64*, 217–221.
- (14) Heeley, J. D.; Dobeck, J. M.; Derice, R. A. [3H]Thymidine Uptake in Cells of Rat Condylar Cartilage. *Am. J. Anat.* **1983**, *167*, 451–462.
- (15) Li, Y.; Wang, Y.; Chubinskaya, S.; Schoeberl, B.; Florine, E.; Kopesky, P.; Grodzinsky, A. J. Effects of Insulin-like Growth Factor-1 and Dexamethasone on Cytokine-Challenged Cartilage: Relevance to Post-Traumatic Osteoarthritis. *Osteoarthritis Cartilage* **2015**, *23*, 266–274.
- (16) Lv, M.; Zhou, Y.; Chen, X.; Han, L.; Wang, L.; Lu, X. L. Calcium Signaling of In Situ Chondrocytes in Articular Cartilage under Compressive Loading: Roles of Calcium Sources and Cell Membrane Ion Channels. *J. Orthop. Res.* **2018**, *36*, 730–738.
- (17) Hermansson, M.; Sawaji, Y.; Bolton, M.; Alexander, S.; Wallace, A.; Begum, S.; Wait, R.; Saklatvala, J. Proteomic Analysis of Articular Cartilage Shows Increased Type II Collagen Synthesis in Osteoarthritis and Expression of Inhibin BA (Activin A), a Regulatory Molecule for Chondrocytes. *J. Biol. Chem.* **2004**, *279*, 43514–43521.
- (18) Nelson, F.; Dahlberg, L.; Laverty, S.; Reiner, A.; Pidoux, I.; Ionescu, M.; Fraser, G. L.; Brooks, E.; Tanzer, M.; Rosenberg, L. C.; Dieppe, P.; Robin Poole, A. Evidence for Altered Synthesis of Type II Collagen in Patients with Osteoarthritis. *J. Clin. Invest.* **1998**, *102*, 2115–2125.
- (19) Venkatesan, N.; Barre, L.; Benani, A.; Netter, P.; Magdalou, J.; Fournel-Gigleux, S.; Ouzzine, M. Stimulation of Proteoglycan Synthesis by Glucuronosyltransferase-I Gene Delivery: A Strategy to Promote Cartilage Repair. *Proc. Natl. Acad. Sci.* **2004**, *101*, 18087–18092.
- (20) Salic, A.; Mitchison, T. J. A Chemical Method for Fast and Sensitive Detection of DNA Synthesis in Vivo. *Proc. Natl. Acad. Sci.* **2008**, *105*, 2415–2420.
- (21) Jao, C. Y.; Salic, A. Exploring RNA Transcription and Turnover in Vivo by Using Click Chemistry. *Proc. Natl. Acad. Sci.* **2008**, *105*, 15779–15784.
- (22) Jewett, J. C.; Bertozzi, C. R. Cu-Free Click Cycloaddition Reactions in Chemical Biology. *Chem. Soc. Rev.* **2010**, *39*, 1272–1279.
- (23) Zhou, Y.; Park, M.; Cheung, E.; Wang, L.; Lu, X. L. The Effect of Chemically Defined Medium on Spontaneous Calcium Signaling of In Situ Chondrocytes during Long-Term Culture. *J. Biomech.* **2015**, *48*, 990–996.
- (24) Zhou, Y.; David, M. A.; Chen, X.; Wan, L. Q.; Duncan, R. L.; Wang, L.; Lu, X. L. Effects of Osmolarity on the Spontaneous Calcium Signaling of In Situ Juvenile and Adult Articular Chondrocytes. *Ann. Biomed. Eng.* **2016**, *44*, 1138–1147.
- (25) Loening, A. M.; James, I. E.; Levenston, M. E.; Badger, A. M.; Frank, E. H.; Kurz, B.; Nuttall, M. E.; Hung, H. H.; Blake, S. M.; Grodzinsky, A. J.; Lark, M. W. Injurious Mechanical Compression of Bovine Articular Cartilage Induces Chondrocyte Apoptosis. *Arch. Biochem. Biophys.* **2000**, *381*, 205–212.
- (26) Lewis, J. S.; Hembree, W. C.; Furman, B. D.; Tippetts, L.; Cattel, D.; Huebner, J. L.; Little, D.; DeFrate, L. E.; Kraus, V. B.; Guilak, F.; Olson, S. A. Acute Joint Pathology and Synovial Inflammation Is Associated with Increased Intra-Articular Fracture Severity in the Mouse Knee. *Osteoarthritis Cartilage* **2011**, *19*, 864–873.
- (27) Neef, A. B.; Luedtke, N. W. An Azide-Modified Nucleoside for Metabolic Labeling of DNA. *ChemBioChem* **2014**, *15*, 789–793.
- (28) Schneider, C. A.; Rasband, W. S.; Eliceiri, K. W. NIH Image to ImageJ: 25 Years of Image Analysis. *Nat. Methods* **2012**, *9*, 671–675.
- (29) Laughlin, S. T.; Agard, N. J.; Baskin, J. M.; Carrico, I. S.; Chang, P. V.; Ganguli, A. S.; Hangauer, M. J.; Lo, A.; Prescher, J. A.; Bertozzi, C. R. Metabolic Labeling of Glycans with Azido Sugars for Visualization and Glycoproteomics. In *Methods in Enzymology; Glycobiology*; Academic Press, 2006; Vol. 415, pp 230–250.
- (30) Dieck, S. t.; Müller, A.; Nehring, A.; Hinz, F. I.; Bartnik, I.; Schuman, E. M.; Dieterich, D. C. Metabolic Labeling with Noncanonical Amino Acids and Visualization by Chemoselective Fluorescent Tagging. *Curr. Protoc. Cell Biol.* **2012**, *56*, No. 7.
- (31) Akens, M. K.; Hurtig, M. B. Influence of Species and Anatomical Location on Chondrocyte Expansion. *BMC Musculoskelet. Disord.* **2005**, *6*, No. 23.
- (32) Sletten, E. M.; Bertozzi, C. R. From Mechanism to Mouse: A Tale of Two Bioorthogonal Reactions. *Acc. Chem. Res.* **2011**, *44*, 666–676.
- (33) Best, M. D. Click Chemistry and Bioorthogonal Reactions: Unprecedented Selectivity in the Labeling of Biological Molecules. *Biochemistry* **2009**, *48*, 6571–6584.
- (34) Lee, J. H.; Fitzgerald, J. B.; Dimicco, M. A.; Grodzinsky, A. J. Mechanical Injury of Cartilage Explants Causes Specific Time-Dependent Changes in Chondrocyte Gene Expression. *Arthritis Rheum.* **2005**, *52*, 2386–2395.
- (35) Anderson, D. E.; Johnstone, B. Dynamic Mechanical Compression of Chondrocytes for Tissue Engineering: A Critical Review. *Front. Bioeng. Biotechnol.* **2017**, *5*, No. 76.
- (36) Goldring, M. B. Osteoarthritis and Cartilage: The Role of Cytokines. *Curr. Rheumatol. Rep.* **2000**, *2*, 459–465.
- (37) Inoue, M.; Muneta, T.; Ojima, M.; Nakamura, K.; Koga, H.; Sekiya, I.; Okazaki, M.; Tsuji, K. Inflammatory Cytokine Levels in Synovial Fluid 3, 4 Days Postoperatively and Its Correlation with Early-Phase Functional Recovery after Anterior Cruciate Ligament Reconstruction: A Cohort Study. *J. Exp. Orthop.* **2016**, *3*, No. 30.
- (38) Schuerwegh, A. J.; Dombrecht, E. J.; Stevens, W. J.; Van Offel, J. F.; Bridts, C. H.; De Clerck, L. S. Influence of Pro-Inflammatory (IL-1 Alpha, IL-6, TNF-Alpha, IFN-Gamma) and Anti-Inflammatory (IL-4) Cytokines on Chondrocyte Function. *Osteoarthritis Cartilage* **2003**, *11*, 681–687.
- (39) Chadichristos, C.; Ghayor, C.; Kypriotou, M.; Martin, G.; Renard, E.; Ala-Kokko, L.; Suske, G.; de Crombrughe, B.; Pujol, J.-P.; Galéra, P. Sp1 and Sp3 Transcription Factors Mediate Interleukin-1 Beta down-Regulation of Human Type II Collagen Gene Expression in Articular Chondrocytes. *J. Biol. Chem.* **2003**, *278*, 39762–39772.
- (40) Shakibaei, M.; Schulze-Tanzil, G.; John, T.; Mobasheri, A. Curcumin Protects Human Chondrocytes from IL-1 β -Induced Inhibition of Collagen Type II and β 1-Integrin Expression and Activation of Caspase-3: An Immunomorphological Study. *Ann. Anat.* **2005**, *187*, 487–497.
- (41) Bartell, L. R.; Fortier, L. A.; Bonassar, L. J.; Cohen, I. Measuring Microscale Strain Fields in Articular Cartilage during Rapid Impact Reveals Thresholds for Chondrocyte Death and a Protective Role for the Superficial Layer. *J. Biomech.* **2015**, *48*, 3440–3446.
- (42) Bonnevie, E. D.; Delco, M. L.; Bartell, L. R.; Jasty, N.; Cohen, I.; Fortier, L. A.; Bonassar, L. J. Microscale Frictional Strains Determine Chondrocyte Fate in Loaded Cartilage. *J. Biomech.* **2018**, *74*, 72–78.
- (43) Ayala, S.; Delco, M. L.; Fortier, L. A.; Cohen, I.; Bonassar, L. J. Cartilage Articulation Exacerbates Chondrocyte Damage and Death after Impact Injury. *J. Orthop. Res.* **2021**, *39*, 2130–2140.
- (44) Schinagl, R. M.; Gurskis, D.; Chen, A. C.; Sah, R. L. Depth-Dependent Confined Compression Modulus of Full-Thickness Bovine Articular Cartilage. *J. Orthop. Res.* **1997**, *15*, 499–506.

- (45) Wang, C. C.-B.; Chahine, N. O.; Hung, C. T.; Ateshian, G. A. Optical Determination of Anisotropic Material Properties of Bovine Articular Cartilage in Compression. *J. Biomech.* **2003**, *36*, 339–353.
- (46) Grodzinsky, A. J.; Wang, Y.; Kakar, S.; Vrahas, M. S.; Evans, C. H. Intra-Articular Dexamethasone to Inhibit the Development of Post-Traumatic Osteoarthritis. *J. Orthop. Res.* **2017**, *35*, 406–411.
- (47) Bertuglia, A.; Pagliara, E.; Grego, E.; Ricci, A.; Brkljac-Bottegato, N. Pro-Inflammatory Cytokines and Structural Biomarkers Are Effective to Categorize Osteoarthritis Phenotype and Progression in Standardbred Racehorses over Five Years of Racing Career. *BMC Vet. Res.* **2016**, *12*, No. 246.
- (48) Ma, T.-W.; Li, Y.; Wang, G.-Y.; Li, X.-R.; Jiang, R.-L.; Song, X.-P.; Zhang, Z.-H.; Bai, H.; Li, X.; Gao, L. Changes in Synovial Fluid Biomarkers after Experimental Equine Osteoarthritis. *J. Vet. Res.* **2017**, *61*, 503–508.

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