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Stiffness- and Bioactive Factor-Mediated Protection of Self-Assembled Cartilage against Macrophage Challenge in a Novel Co-Culture System

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Abstract

Objective: Tissue-engineered cartilage implants must withstand the potential inflammatory and joint loading environment for successful long-term repair of defects. The work's objectives were to develop a novel, direct cartilage-macrophage co-culture system and to characterize interactions between self-assembled neocartilage and differentially stimulated macrophages. **Design:** In study 1, it was hypothesized that the proinflammatory response of macrophages would intensify with increasing construct stiffness; it was expected that the neocartilage would display a decrease in mechanical properties after co-culture. In study 2, it was hypothesized that bioactive factors would protect neocartilage properties during macrophage co-culture. Also, it was hypothesized that interleukin 10 (IL-10)-stimulated macrophages would improve neocartilage mechanical properties compared to lipopolysaccharide (LPS)-stimulated macrophages. **Results:** As hypothesized, stiffer neocartilage elicited a heightened proinflammatory macrophage response, increasing tumor necrosis factor alpha (TNF-α) secretion by 5.47 times when LPS-stimulated compared to construct-only controls. Interestingly, this response did not adversely affect construct properties for the stiffest neocartilage but did correspond to a significant decrease in aggregate modulus for soft and medium stiffness constructs. In addition, bioactive factor-treated constructs were protected from macrophage challenge compared to chondrogenic medium-treated constructs, but IL-10 did not improve neocartilage properties, although stiff constructs appeared to bolster the anti-inflammatory nature of IL-10-stimulated macrophages. However, co-culture of bioactive factor-treated constructs with LPS-treated macrophages reduced TNF-α secretion by over 4 times compared to macrophage-only controls. **Conclusions:** In conclusion, neocartilage stiffness can mediate macrophage behavior, but stiffness and bioactive factors prevent macrophage-induced degradation. Ultimately, this co-culture system could be utilized for additional studies to develop the burgeoning field of cartilage mechano-immunology.

Keywords

tissue engineering, immunology, cartilage, macrophage

Introduction

Key initial mediators of the immune response to tissue-engineered therapeutics are macrophages, which orchestrate the inflammatory and healing processes after injury, infection, and therapeutic implantation.[1] Macrophages can interact with tissue-engineered cartilages indirectly through cytokines released from those embedded in the synovium.[2,3] Macrophages have also been observed to directly interact with chondrocytes in tissue engineering studies through the formation of granular pannus tissue in a cartilage defect.[4-6] Depending on the healing or disease state, the spectrum of macrophage behavior and phenotype can polarize toward proinflammatory or anti-inflammatory states.[7] Biochemical

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signals identified as having polarizing effects include, for example, tumor necrosis factor alpha (TNF-α) and lipopolysaccharide (LPS), an outer membrane component of gram-negative bacteria;[8-10] these can activate macrophages toward a proinflammatory phenotype.[1,10] Conversely, interleukin 10 (IL-10) or a combination of interleukin 4 (IL-4) and interleukin 13 (IL-13) can drive macrophages toward an anti-inflammatory phenotype.[8,11] These phenotypic states are particularly important in cartilages such as hyaline articular cartilage, the knee meniscus, and the temporomandibular joint (TMJ) disc, which lack innate healing capacity.[12-14] Small defects that may emanate from wear-and-tear or traumatic injury can lead to inflammation and often result in osteoarthritis (OA), leading to pain and loss of joint function.[15,16] According to the Centers for Disease Control and Prevention, OA affects over 32 million people in the United States,[17] and this number is projected to rise up to 60% in the next 2 decades.[18] To prevent degenerative changes and to induce repair, these defects are often targets for surgical treatment, which alone can cause an immune response.[14]

Current approaches for repair of cartilage defects do not provide robust long-term solutions, in part because they encourage the development of mechanically inferior repair tissue, which can lead to further degeneration, OA, and inflammation, further activating macrophages and the immune response. Inflammation and immune cell activation can induce chondrocyte apoptosis and/or hypertrophy, all of which can impede healing.[15] In native cartilages, proinflammatory macrophages have been shown to enhance cartilage inflammation and resulting degeneration.[19] However, anti-inflammatory macrophage states have been shown to prevent extracellular matrix (ECM) degradation and to promote healing.[20,21] Characterization of these macrophage phenotypes in the context of tissue-engineered cartilages is limited. Thus, in addition to overcoming the challenge of mechanically inferior repair in future therapies, it is also necessary to consider the inflammatory immune response.

The effect of biophysical cues on macrophage polarization, such as material topography, applied mechanical forces, and ECM stiffness, has also been recently explored.[22-24] The ability of macrophages to sense biophysical cues, particularly ECM stiffness, has been shown to be a complex interplay between integrins, ion channels, transcriptional regulators, and the actin cytoskeleton.[22,25,26] Within the context of cartilage tissue engineering, ECM stiffness is of particular interest because repeated loading of the joint would require stiff neocartilage implants.[27] For example, transforming growth factor beta 1 (TGF-β1), chondroitinase ABC (C-ABC), and lysyl oxidase-like 2 (LOXL2) (termed TCL) treatment has been previously shown to increase matrix content and subsequent mechanical properties (e.g., stiffness) when applied to self-assembled neocartilage constructs.[28] Paradoxically, substrate stiffness has been previously shown to correlate positively with macrophage-induced proinflammatory responses.[29] For example, stiffer hydrogels elicit increased TNF-α and IL-1β levels,[30] which can lead to breakdown of the ECM.[19] This finding is in direct contradiction to the goal of cartilage tissue engineering, which is to produce neocartilage tissues with stiff ECM to withstand the loading environment of the joint. Characterizations investigating these conflicting conditions have not been previously performed on cartilage neotissue, representing a novel direction for advancing therapeutics for repair or replacement of articular cartilage. Thus, characterization of the interactions between macrophages and neocartilages meant to repair or replace native articular cartilage should be performed *in vitro* to inform strategies for more effective *in vivo* cartilage repair.

Toward translation of *in vitro* strategies for *in vivo* applications, tissue-engineered cartilages are fabricated with regulatory guidance in mind. For example, the Food and Drug Administration guidance on therapeutics intended to repair or replace articular cartilage of the knee indicates that therapeutics must be tested in a suitable large animal model.[31] Here, the Yucatan minipig was selected due to its similarities to humans in weight, anatomy, immunology, physiology, and bone biology.[32-36] In addition, costal chondrocytes from the rib cartilage have been previously identified as a cell source for tissue engineering applied to synovial joints because they can be harvested without further damaging diseased joints requiring treatment and can result in neocartilages mimicking articular cartilages of synovial joints.[37-42] Specifically, our group can extensively passage chondrocytes and redifferentiate cells back toward a chondrogenic phenotype using an aggregate culture.[43] Subsequent self-assembly of the rejuvenated costal chondrocytes results in a robust neocartilage construct.[44,45] These neocartilage constructs can then be used to investigate their interactions with macrophages.

Another reason to consider the immune response in developing new cartilage therapies is that allogeneic cell sources will be necessary to overcome the donor site morbidity and cell sourcing issues associated with autologous approaches.[6,46] An allogeneic approach increases the risk of an immune response,[47] despite various cartilages having been cited as immunoprivileged.[6,12,13] For example, recent work toward regeneration of the TMJ disc cartilage has shown that an allogeneic approach elicits a minor local immune response through some positive immunohistochemical staining for T cells, B cells, and macrophages, without exhibiting any systemic effects.[46] The study also demonstrated excellent disc healing as evidenced by more complete defect closure, less OA on adjacent articulating condylar surfaces, and increased repair tissue robustness, when compared to empty defect controls.[46] However, given

the likelihood that tissue-engineered cartilages will be produced from an allogeneic source, it is possible for an immune response to be mounted against the allogeneic implant, potentially affecting its mechanical integrity. Thus, this allogeneic approach warrants additional investigation. Within the field of cartilage tissue engineering, the potential interactions between neocartilage and immune system components have not been extensively studied.

In this study, a novel, direct co-culture system to explore the interaction between self-assembled neocartilage and macrophages is described. A direct co-culture system was selected to study the physical interaction between neotissue stiffness and cells that would likely occur *in vivo*, for example, through the formation of a granular pannus. Although synovial fibroblasts can also be found in the pannus tissue[48] and partially contribute to some of the inflammation seen during OA,[49] in this study, macrophages were investigated as the immune cell of choice due to the well-established effects of biophysical cues, specifically ECM stiffness, on macrophage phenotype.[22,50,51] The global objective was to characterize the interaction between macrophages in differentially activated states and neocartilage constructs formed under a variety of conditions. The objective of study 1 was to investigate the stiffness-mediated proinflammatory response of macrophages. It was hypothesized that stiffer constructs would polarize macrophages toward a proinflammatory phenotype and, thus, would cause a decrease in the mechanical properties of the constructs. Study 2 aimed to determine the protective effects of various bioactive factors against the potential degradation of neocartilage constructs under macrophage co-culture. It was hypothesized that neocartilage-specific bioactive factors (i.e., TCL treatment) would protect neocartilage during macrophage co-culture. It was also hypothesized that co-culture with IL-10-stimulated macrophages would result in improved neocartilage mechanical properties compared to those exposed to LPS-stimulated macrophages. The characterization of macrophage-neocartilage interactions performed here sets the stage for future studies spanning from mechanisms of neocartilage-macrophage interactions to immunomodulatory approaches for preclinical and clinical *in vivo* cartilage repair.

Materials and Methods

Isolation, Expansion, and Aggregate Rejuvenation of Chondrocytes

Costal cartilage from 3 Yucatan minipigs between 5 and 8 months of age (Premier BioSource) was obtained within 48 hours postmortem. All tissues used in this study were obtained from animals that were culled for reasons unrelated to this study. The cartilage was obtained from the entirety of the rib cage, minced into 1 mm^3 pieces, and digested using 0.4% w/v pronase (Sigma-Aldrich) supplemented with 3% fetal bovine serum (FBS; R&D Systems) for 1 hour at 37 °C followed by 0.2% w/v collagenase (type 2, Worthington Biochemical) supplemented with 3% FBS for 18 hours at 37 °C. Chondrocytes were filtered, counted, treated with ammonium-chloride-potassium (ACK) lysis buffer,[52] and washed with phosphate-buffered saline (PBS).

Costal chondrocytes were subsequently cultured in chemically defined chondrogenic medium (CHG) composed of Dulbecco's modified Eagle medium (DMEM, high glucose, GlutaMAX supplement; Gibco); 1% penicillin-streptomycin-fungizone (Lonza); 1% insulin, transferrin, and selenous acid+ (ITS+) premix (Corning); 1% nonessential amino acids (Gibco); 100 nM dexamethasone (Sigma-Aldrich); 50 µg/ml ascorbate-2-phosphate (Sigma-Aldrich); 40 µg/ml L-proline (Sigma-Aldrich); and 100 µg/ml sodium pyruvate (Sigma-Aldrich) supplemented with 2% FBS at a density of 2.5 million cells per T-225 flask. During monolayer expansion, culture was further supplemented with 1 ng/ml TGF-β1 (PeproTech), 5 ng/ml basic fibroblastic growth factor (bFGF; PeproTech), and 10 ng/ml platelet-derived growth factor (PDGF; PeproTech), termed TFP, which has been previously shown to increase proliferation and postexpansion chondrogenic potential.[42] Medium was exchanged every 3 to 4 days. Upon 90% confluence, cells were lifted and digested using 0.05% trypsin with 0.02% ethylenediaminetetraacetic acid (EDTA; Gibco) for 9 minutes followed by 0.2% w/v collagenase supplemented with 3% FBS for 40 minutes and frozen at passage 1 for downstream use. Cells were thawed for each experiment and expanded to passage 6 in CHG supplemented with TFP, as described above. Each passage had an approximate expansion factor of 4, which is calculated by dividing the final cell count by the initial seeding density. Doublings per passage, in this case 2 doublings, can be calculated by the following formula[43]: log(expansion factor)/log(2). This represents a cumulative expansion factor of 4096 as calculated by the following formula: expansion factor$^{\text{number of passages}}$. Toward addressing the issue of cell scarcity in donor cells for neocartilage tissue engineering, we employed passage 6 cells. The use of passage 6 cells is based on prior experiments optimizing efficient passaging and aggregate rejuvenation of chondrocytes toward creating a flat, robust construct.[14,43]

At passage 6, cells were placed into aggregate culture, termed aggregate rejuvenation,[43] with CHG containing 10 ng/ml TGF-β1, 100 ng/ml growth differentiation factor 5 (GDF-5; PeproTech), and 100 ng/ml bone morphogenetic protein 2 (BMP-2; PeproTech), collectively termed TGB. Aggregate rejuvenation has been previously shown to promote redifferentiation of cells toward a chondrogenic phenotype, specifically exhibiting high expression of collagen type II, aggrecan, and SRY-box transcription factor 9 and low expression of collagen type X and osteocalcin.[40] Cells

were plated on 1% agarose-coated plates at a density of 750,000/ml with medium changes every 3 to 4 days. Plates were kept on an orbital shaker at 50 revolutions per minute for 24 hours and subsequently cultured under static conditions. After 14 days of aggregate rejuvenation, cells were digested with 0.05% trypsin with 0.02% EDTA for 45 minutes followed by 0.2% w/v collagenase supplemented with 3% FBS for 2 hours. Cells were passed through a 70-μm filter for subsequent self-assembly.

Isolation of Bone Marrow–Derived Monocytes and Differentiation into Macrophages

Pelvises from 3 Yucatan minipigs between 5 and 8 months of age (Premier BioSource) were obtained within 6 hours postmortem. Costal cartilage and pelvises were not obtained from the same animals. Bones were cleaned of muscle and other soft tissues. Using a sterilized chisel and hammer, the bone marrow was exposed and rinsed from the pelvic bone cavity using RPMI-1640 (L-glutamine; Gibco). Resulting cells were passed through a 70-μm filter, spun down, and rinsed with PBS. Cells were treated with ACK lysis buffer and subsequently washed with PBS. Cells were plated at approximately 10 million cells per 100 mm diameter Petri dish in a chemically defined macrophage culture medium (MΦM) composed of RPMI-1640, and 1% penicillin-streptomycin (Gibco), supplemented with 10% FBS and 20 ng/ml granulocyte-macrophage colony-stimulating factor (GM-CSF; R&D Systems) to differentiate cells to bone marrow–derived macrophages. Recombinant porcine GM-CSF was selected for macrophage differentiation, as previously described.[53,54] Cells were fed every 3 to 4 days and lifted after 7 days and frozen for downstream use. As previously reported in another study by our group,[54] macrophage differentiation was confirmed through flow cytometry for cluster of differentiation 68 (CD68), immunofluorescence staining for CD68 and F4/80, and TNF-α secretion via an LPS dose response.

Formation of Self-Assembled Cartilage Constructs

After expansion and aggregate rejuvenation, chondrocytes underwent the self-assembling process.[44] Prior to seeding, nonadherent agarose wells were formed using 2% agarose (Fisher Scientific) in PBS and a negative mold to form the shape of 5 mm diameter cylindrical constructs, and CHG was exchanged on the wells 3 times prior to seeding. Chondrocytes were subsequently seeded at 2 million per well in 100 μl of CHG. Four hours after seeding, wells were topped off with another 400 μl of CHG. Medium was exchanged (500 μl) every day until day 3 when constructs were unconfined from agarose wells, transferred to untreated dishes, and fed with 2 ml medium every other day up to 27 days. Study 1 consisted of CHG only. Study 2 consisted of CHG coupled with TCL treatment which is TGF-β1 continuously until day 27 (10 ng/ml), C-ABC (Sigma-Aldrich) on day 7 for 4 hours, and LOXL2 (SignalChem) from days 14 to 27 to enhance engineered cartilage properties, as previously described.[55] Because C-ABC is used in this study both (1) as a culture supplement during the early period of tissue engineering culture and also (2) immediately before macrophage co-culture, the 2 different treatments are denoted as C-$ABC_{eng.}$ and C-$ABC_{comp.}$ to differentiate the culture additive and stiffness modulation, respectively. Briefly, C-$ABC_{eng.}$ (2 U/ml) consisted of activation with 50 mM sodium acetate (Sigma-Aldrich) and quenching with 1 mM zinc sulfate (Sigma-Aldrich). LOXL2 treatment consisted of 0.15 μg/ml of the enzyme coupled with 0.146 mg/ml hydroxylysine (Sigma-Aldrich) and 1.6 μg/ml copper sulfate (Sigma-Aldrich).[28]

Co-Culture of Macrophages and Self-Assembled Cartilage Constructs

After the self-assembling process, co-culture was initiated according to the steps illustrated in Figure 1. First, immediately prior to macrophage seeding, constructs were treated with C-ABC to modulate compressive stiffness via depletion of glycosaminoglycan (GAG) content (denoted as C-$ABC_{comp.}$ further). On day 27, constructs were treated with 0.0, 0.5, or 1.0 U/ml (1 ml/construct) of C-$ABC_{comp.}$ for 2 hours with activation and quenching, as described above. Macrophages were also thawed and cultured in MΦM overnight. On day 28, constructs were placed at the bottom of 2% agarose wells in 50 μl CHG. Based on previous work with biomaterials,[51,56] macrophages were then seeded at a density of 25,000 in 50 μl MΦM on top of constructs inside the agarose well to confine macrophages to the construct surface. Three macrophage donors were used separately in duplicate co-cultures for a total of 6 samples per co-culture condition. After 4 hours, a 1:1 mixture of CHG and MΦM was added to the co-culture system to sustain cell and tissue viability. Stimulation occurred the following day with 0.1 ng/ml of LPS in study 1 and either 1.0 ng/ml LPS or 10 ng/ml IL-10 in study 2. LPS stimulation concentrations were chosen based on a previous study.[54] Medium was exchanged with half the total volume every 3 days, and co-culture continued for 2 weeks. Both studies also included constructs cultured in 1:1 CHG:MΦM within agarose wells in the absence of macrophages (i.e., construct-only control), as well as 25,000 macrophages cultured in 1:1 CHG:MΦM within agarose wells without constructs (i.e., macrophage-only control).

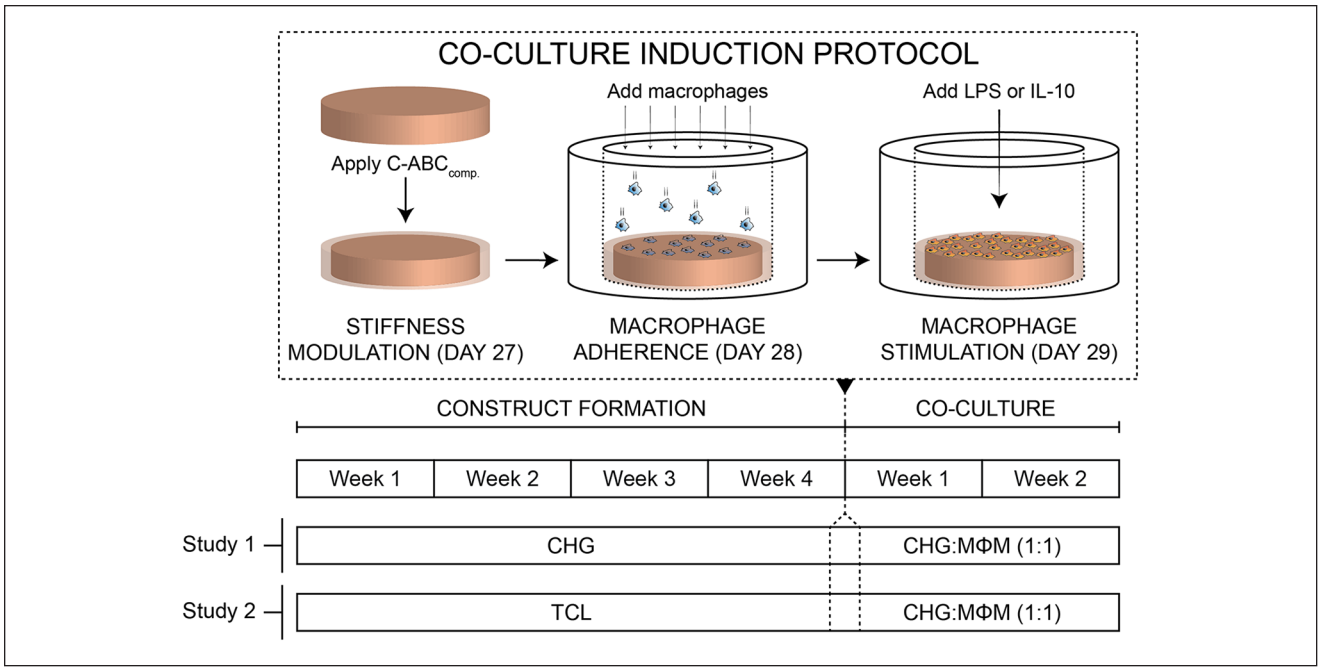

Figure 1. Stiffness is modulated via C-ABC$_{comp.}$, and macrophages are adhered and stimulated in a direct co-culture system. Constructs were cultured in either CHG only or with TCL treatment. After 27 days, compressive stiffness was modulated via C-ABC$_{comp.}$ application. Constructs were assayed for baseline properties (t = 0). The next day, macrophages adhered and were cultured in a 1:1 mix of CHG and MΦM medium, then stimulated with LPS in study 1 and LPS or IL-10 in study 2. Unstimulated macrophage controls, construct-only controls, and macrophage-only controls were also included. After 2 weeks of co-culture, constructs were assayed again (t = 2W). C-ABC$_{comp.}$ = chondroitinase ABC to modulate compressive stiffness; CHG = chondrogenic medium; TCL = transforming growth factor beta 1/chondroitinase ABC$_{eng.}$/lysyl oxidase-like 2; MΦM = macrophage medium; LPS = lipopolysaccharide; IL-10 = interleukin 10.

Sample Processing and Biochemical Analyses

Following culture, construct samples were weighed before and after lyophilization to obtain wet weight (WW) and dry weight (DW), and subsequently digested in papain for biochemical analysis. Total collagen (Col) content was measured via a modified hydroxyproline assay, as previously described.[57] GAG content was measured by a dimethylmethylene blue dye-binding assay kit (Biocolor).

Mechanical Testing

Constructs were analyzed under creep indentation and uniaxial tension to obtain compressive and tensile properties, respectively. As previously described,[28] constructs were trimmed into dog bone-shaped specimens and glued to paper tabs which were gripped to a uniaxial testing machine (Instron 5565). A pull-to-failure test was performed at 1% strain per second. Tensile Young's modulus and ultimate tensile strength (UTS) were determined using the force-displacement curves from a custom MATLAB (MathWorks) code. Creep indentation was performed on cylindrical pieces of construct, as previously described.[58] Briefly, 3-mm diameter punches from self-assembled cartilage constructs were indented with a flat, porous tip under a constant load. A linear biphasic model and finite element analysis were used to obtain aggregate modulus, permeability, and shear modulus from experimental curves.[59]

Histology

Construct samples were fixed in 10% neutral-buffered formalin for histological evaluation. Samples were subsequently processed, embedded in paraffin, and sectioned at 5 μm thickness. Samples were stained with hematoxylin and eosin (H&E) to show tissue and cellular morphology and Safranin O (Saf-O)/Fast Green to visualize GAG content.

Enzyme-Linked Immunosorbent Assays (ELISAs) for Cytokine Analysis

Medium for ELISAs was collected from sample wells either 24 or 48 hours after stimulation. Kits for TNF-α were purchased and used per the manufacturer's instructions (R&D Systems).

Table 1. Additional properties of CHG-treated constructs.

Time	Stiffness	Macrophage Condition	Construct WW (mg)	Col/WW (μg/μg)	Young's Modulus (MPa)	UTS (MPa)	Shear Modulus (kPa)	Permeability (10^{-15} m^4/Ns)
$t = 0$	Soft	None	15.443 ± 0.660^{C}	0.023 ± 0.002^{A}	1.825 ± 0.516	0.701 ± 0.094	66.8 ± 33.6^{B}	6.1 ± 2.2
	Medium		17.106 ± 0.693^{B}	0.017 ± 0.001^{B}	3.190 ± 2.695	0.515 ± 0.094	91.3 ± 11.3^{AB}	13.7 ± 19.5
	Stiff		18.781 ± 0.166^{A}	0.022 ± 0.002^{A}	2.048 ± 1.017	0.538 ± 0.115	157.7 ± 35.4^{A}	2.6 ± 1.6
$t = 2W$	Soft	None	18.635 ± 0.537^{a}	0.030 ± 0.002	2.357 ± 0.611	0.747 ± 0.279	68.9 ± 6.0^{a}	25.5 ± 11.7
		Unstimulated	15.012 ± 0.632^{b}	0.027 ± 0.002	2.508 ± 0.738	0.868 ± 0.257	31.8 ± 13.4^{b}	36.9 ± 54.2
		LPS	16.443 ± 1.049^{ab}	0.031 ± 0.002	2.488 ± 0.443	0.903 ± 0.143	42.7 ± 31.8^{ab}	60.0 ± 82.0
	Medium	None	20.288 ± 0.761^{a}	0.031 ± 0.004	2.092 ± 0.372^{ab}	0.779 ± 0.060^{ab}	102.2 ± 27.4^{a}	32.2 ± 13.4
		Unstimulated	16.182 ± 1.736^{b}	0.028 ± 0.003	2.113 ± 0.514^{b}	0.754 ± 0.215^{b}	44.9 ± 14.6^{b}	15.8 ± 14.4
		LPS	14.095 ± 1.991^{b}	0.028 ± 0.004	3.439 ± 1.136^{a}	1.147 ± 0.398^{a}	25.8 ± 6.4^{b}	14.1 ± 5.1
	Stiff	None	25.435 ± 2.600^{a}	0.024 ± 0.003	1.076 ± 0.392	0.389 ± 0.135	66.8 ± 14.9	76.4 ± 14.6^{a}
		Unstimulated	17.987 ± 1.321^{b}	0.025 ± 0.005	2.283 ± 0.858	0.697 ± 0.303	60.4 ± 18.4	20.3 ± 11.9^{ab}
		LPS	17.038 ± 1.353^{b}	0.025 ± 0.002	2.106 ± 0.964	0.697 ± 0.096	53.8 ± 14.5	11.4 ± 4.7^{b}

Significance is seen in construct WW among construct stiffnesses at $t = 0$, significantly increasing from soft to stiff groups. In addition, construct WW also decreases with the addition of macrophages to constructs, as seen in all groups. Statistics: Uppercase letters represent a connecting letters report from a one-way ANOVA with *post hoc* Tukey's HSD test comparing $t = 0$ properties among stiffnesses ($\alpha = 0.05$), $n = 3$ per group. Lowercase letters represent a two-way ANOVA with *post hoc* Tukey's HSD test comparing properties after 2 weeks of co-culture only within individual stiffnesses ($\alpha = 0.05$), $n = 3$-6 per group. Gray-shaded table cells indicate statistical comparisons. CHG = chondrogenic medium; WW = wet weight; Col = collagen; UTS = ultimate tensile strength; LPS = lipopolysaccharide; ANOVA = analysis of variance; HSD = honestly significant difference.

Statistical Analysis

All statistical analyses were performed using Prism 9 (GraphPad Software). Quantitative data including more than 2 groups were assessed using either a one-way or two-way analysis of variance (ANOVA) with a *post hoc* Tukey's honestly significant difference (HSD) test at a significance level of $\alpha = 0.05$. Two-way ANOVA factors and interactions were analyzed to determine the individual factor effects as well as any interactions between those factors. *P* values for ANOVA factors are capitalized. Significance among particular groups is illustrated by a "connecting letters report" with Latin characters (i.e., bars that do not share the same Latin character(s) are statistically significant), and *p* values for *post hoc* tests are lowercase. For two-way ANOVAs, *post hoc* Tukey's HSD tests were only used to compare groups within the dotted lines in each figure. For each set of quantitative data that only included 2 groups, a Student's *t* test was performed at a significance level of $\alpha = 0.05$.

Results

Study 1

C-ABC$_{comp.}$ modulates compressive stiffness in CHG-treated constructs. To modulate compressive stiffness of constructs, C-ABC$_{comp.}$ was applied at a concentration of 0.0 U/ml (stiff), 0.5 U/ml (medium), or 1.0 U/ml (soft). Directly following this treatment, constructs were evaluated to establish baseline properties ($t = 0$). Application of 1.0 U/ml (soft) and 0.5 U/ml (medium) of C-ABC$_{comp.}$ significantly decreased soft ($p = 0.0008$) and medium ($p = 0.02$) group WWs compared to the stiff group (**Table 1**). Similarly, GAG/WW significantly decreased by 28.0% in the soft group compared to the stiff group ($p = 0.02$) but was not significantly different from the medium group ($p = 0.15$) (**Fig. 2A**). As expected, this led to subsequent decreases in aggregate modulus values; compared to constructs from the

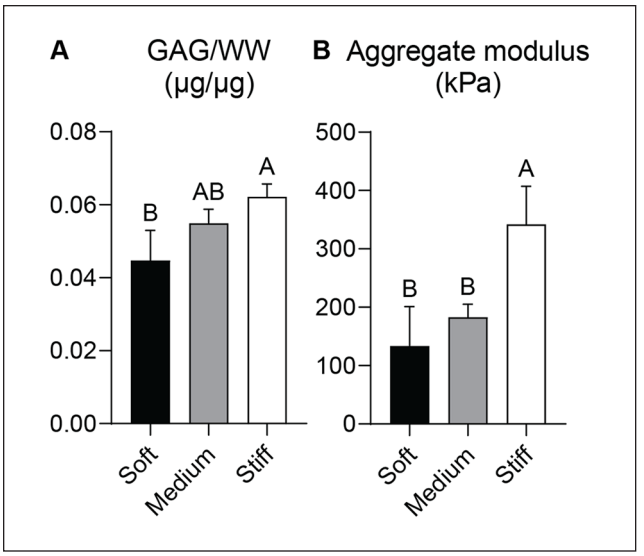

Figure 2. C-ABC$_{comp.}$ modulates the compressive stiffness of CHG-treated constructs. (**A**) GAG/WW increased across soft to stiff groups and decreased as higher C-ABC$_{comp.}$ concentrations were used to modulate compressive stiffness. (**B**) Similarly, aggregate modulus also trended higher from soft to stiff, as expected. Statistics: One-way ANOVA with *post hoc* Tukey's HSD, $\alpha = 0.05$, $n = 3$ per group. C-ABC$_{comp.}$ = chondroitinase ABC to modulate compressive stiffness; CHG = chondrogenic medium; GAG = glycosaminoglycan; WW = wet weight; ANOVA = analysis of variance; HSD = honestly significant difference.

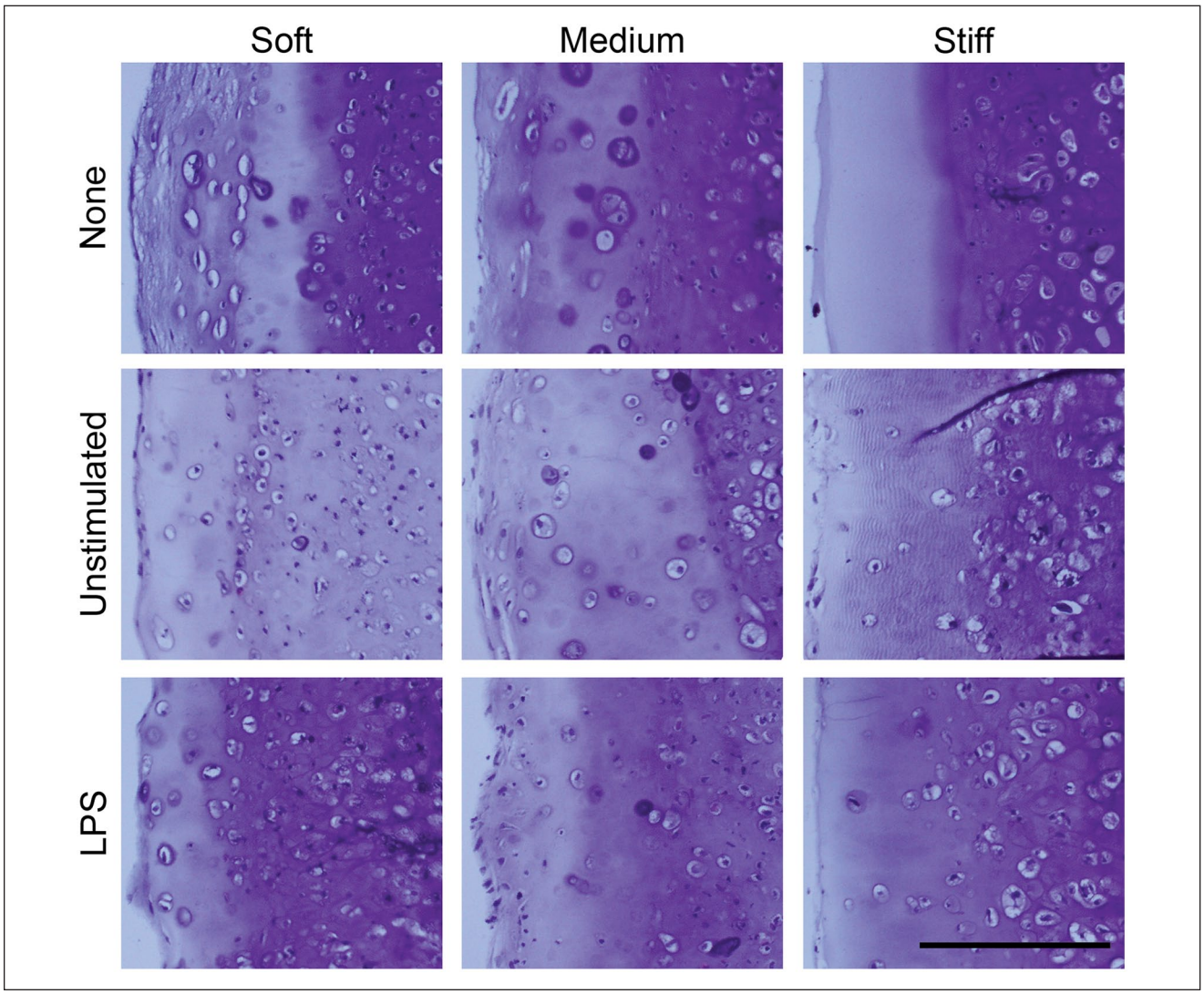

Figure 3. CHG-treated constructs of soft and medium stiffness differ in staining intensity. Following 2 weeks of co-culture, stiff constructs maintain cell morphology and tissue staining intensity in co-culture groups more than the soft and medium groups. Scale bar = 200 μm. CHG = chondrogenic medium; LPS = lipopolysaccharide.

stiff group (341.7 ± 65.6 kPa), constructs from the soft and medium groups exhibited significantly lower aggregate modulus values, 133.7 ± 67.2 kPa ($p = 0.009$) and 182.7 ± 22.4 kPa ($p = 0.03$), respectively (**Fig. 2B**). Tensile Young's modulus and UTS did not differ significantly among the groups at $t = 0$ (**Table 1**).

Stiff, CHG-treated constructs are protected from macrophage inflammatory challenge even in the presence of an elevated proinflammatory response. CHG-treated neocartilage cultured for 2 weeks in the presence of macrophages demonstrated differences in tissue morphological characteristics dependent on construct stiffness per H&E staining for general cellular and tissue morphology (**Fig. 3**). The unstimulated co-culture group for the stiff group did not appear to lose as much staining intensity relative to the corresponding construct-only control compared to the soft and medium groups. In addition, stiff construct-only controls did not exhibit cells near the construct edge, unlike the other construct-only control (none) groups. As an experimental factor, macrophage co-culture significantly decreased construct WW across all stiffnesses ($P < 0.0001$; **Table 1**). Similarly, the macrophage co-culture factor was also significant ($P = 0.006$) for GAG/WW (**Fig. 4A**), decreasing with macrophage application. For medium stiffness constructs, a significant decrease in GAG/WW was observed between the construct-only control (none; 0.066 ± 0.007 μg/μg) and LPS-stimulated macrophage co-culture (0.035 ± 0.018 μg/μg) ($p = 0.01$; **Fig. 4A**). Stiffness was a significant factor for Col/WW ($p = 0.001$; **Table 1**). Macrophage co-culture factor was not significant for Col/WW ($p = 0.34$; **Table 1**).

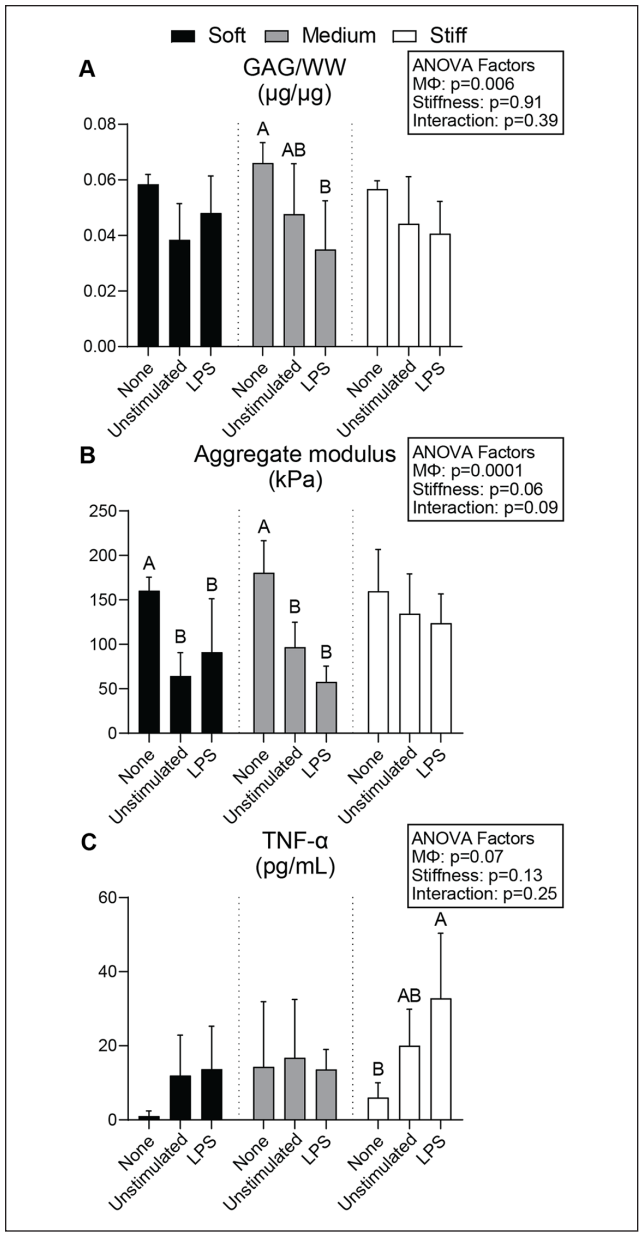

Figure 4. Soft and medium stiffness CHG-treated constructs suffer losses in aggregate modulus values despite no increases in TNF-α production. (**A**) GAG/WW was significantly affected by the MΦ factor, with significant decreases within the medium stiffness constructs between the construct-only control and LPS-stimulated co-culture group. (**B**) Trending with GAG/WW, aggregate modulus significantly decreased with the addition of macrophages (unstimulated or LPS-stimulated) in soft and medium stiffness constructs. (**C**) Conversely, after 48 hours of co-culture, only the stiff construct group had significant increases in TNF-α secretion between the construct-only control and the LPS-stimulated group. Statistics: Two-way ANOVA with *post hoc* Tukey's HSD among groups within a stiffness (dotted lines), $\alpha = 0.05$, $n = 3$-6 per group. CHG = chondrogenic medium; TNF-α = tumor necrosis factor alpha; GAG = glycosaminoglycan; WW = wet weight; MΦ = macrophage; LPS = lipopolysaccharide; ANOVA = analysis of variance; HSD = honestly significant difference.

Aggregate modulus values trended, as expected, with GAG/WW (**Fig. 4**). Interestingly, aggregate modulus for soft and medium groups significantly decreased from 160.3 ± 15.3 and 180.5 ± 36.1 kPa for construct-only controls to 91.2 ± 60.1 and 57.8 ± 17.7 kPa ($p = 0.04$ and $p = 0.001$), respectively, when co-cultured with LPS-stimulated macrophages (**Fig. 4B**). Significant changes in aggregate modulus for soft and medium groups also occurred when comparing construct-only controls with unstimulated macrophage co-culture groups, decreasing by 59.9% and 46.4% ($p = 0.002$ and $p = 0.02$), respectively (**Fig. 4B**). Construct-only controls (159.7 ± 47.2 kPa) in the stiff group did not differ from unstimulated (134.5 ± 44.8 kPa; $p = 0.60$) or LPS-stimulated (123.8 ± 32.9 kPa; $p = 0.36$) co-culture groups (**Fig. 4B**).

After 48 hours of stimulation, TNF-α levels significantly increased by 5.47 times in the stiff group when stimulated with LPS compared to construct-only controls ($p = 0.01$), but there were no significant differences when comparing either of those groups with unstimulated co-culture groups, although TNF-α levels in the LPS group trended 1.64 times higher than the unstimulated group ($p = 0.19$) (**Fig. 4C**). Interestingly, for soft and medium constructs, TNF-α levels did not differ significantly when the two macrophage co-culture conditions were compared against each other ($p = 0.97$ and $p = 0.90$; **Fig. 4C**). However, for the soft and medium groups, significant decreases in aggregate modulus values were observed when the constructs were exposed to either unstimulated ($p = 0.002$ and $p = 0.02$) or LPS-stimulated macrophages ($p = 0.04$ and $p = 0.001$) (**Fig. 4B**). Conversely, the stiff co-culture groups did not experience significant decreases in aggregate modulus ($p = 0.60$ and $p = 0.36$) compared to construct-only control (**Fig. 4B**).

Study 2

C-ABC$_{comp.}$ modulates compressive stiffness in TCL-treated constructs. Directly following C-ABC$_{comp.}$ treatment, TCL-treated constructs were evaluated to establish baseline properties ($t = 0$). As shown in **Figure 5C**, the soft group, which was treated with 1.0 U/ml C-ABC$_{comp.}$, had reduced ECM and GAG content at the periphery of the construct. Conversely, the stiff group (i.e., 0.0 U/ml C-ABC$_{comp.}$) exhibited intense matrix and GAG content all the way to the edge of the construct. As expected, the construct WW was also significantly higher in the stiff group compared to the soft group at $t = 0$ ($p = 0.03$; **Table 2**), although GAG/WW was not significantly different between the two stiffnesses ($p = 0.78$; **Fig. 5A**). Aggregate modulus for the stiff group (165.6 ± 20.7 kPa) was significantly higher ($p < 0.0001$) than the soft group (76.8 ± 18.6 kPa) (**Fig. 5B**). Thus, although C-ABC$_{comp.}$ did not change GAG/WW between the soft and stiff groups, it still had a significant effect on aggregate modulus, decreasing with higher concentrations of the enzyme. Additional properties are reported in **Table 2**.

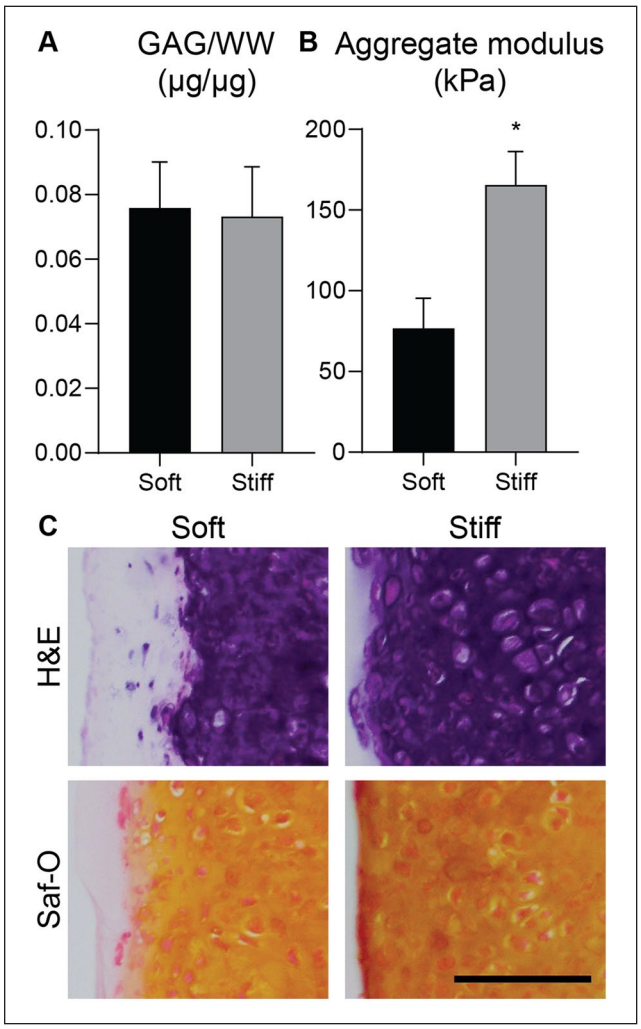

Figure 5. C-ABC$_{comp.}$ modulates the compressive stiffness of TCL-treated constructs. (**A**) GAG/WW does not differ significantly for TCL-treated constructs at $t = 0$. (**B**) Aggregate modulus significantly decreases with application of C-ABC$_{comp.}$. (**C**) Soft constructs show less intense and H&E and Saf-O staining due to C-ABC$_{comp.}$ application and only peripheral loss of GAG. Also, some cells are visible near the soft construct edge. Conversely, stiff constructs show intense Saf-O staining at the periphery indicating high GAG content, and cells are not present at the periphery of the construct. Scale bar = 100 µm. Statistics: Student *t* test, $\alpha = 0.05$. C-ABC$_{comp.}$ = chondroitinase ABC to modulate compressive stiffness; TCL = transforming growth factor beta 1/chondroitinase ABC$_{eng.}$/lysyl oxidase-like 2; GAG = glycosaminoglycan; WW = wet weight; H&E = hematoxylin and eosin; Saf-O = Safranin O.

TCL-treated constructs better withstand macrophage challenge. Saf-O staining of constructs illustrated variability in GAG content across macrophage donors as well as within the construct-only controls (none; **Fig. 6**). Compared to other conditions within stiffnesses, it appeared as though staining intensity was slightly diminished in the LPS-stimulated group. In the soft group, GAG/WW significantly decreased by 26.2% ($p = 0.02$), 33.8% ($p = 0.002$), and 31.8% ($p = 0.004$) for the unstimulated, LPS-stimulated, and IL-10-stimulated groups, respectively, when compared to the construct-only control (none) group (**Fig. 7A**). For the stiff group, no condition caused GAG/WW to change (**Fig. 7A**). However, in terms of aggregate modulus (**Fig. 7B**), in the soft group, only the LPS-stimulated group and construct-only control were significantly different from each other ($p = 0.04$); the LPS group had an aggregate modulus that was 43.8% of the construct-only control. This is in contrast to CHG-treated co-cultures from the soft and medium groups in study 1 which significantly decreased in aggregate modulus no matter the stimulation condition (**Fig. 4B**). For the soft group from study 2, the unstimulated group and IL-10 group had aggregate moduli that were 76.5% and 48.5% of the construct-only control, but these trends were not statistically significant ($p = 0.66$ and $p = 0.07$; **Fig. 7B**). For the stiff group, there were no significant differences in aggregate modulus, which were 186.0 ± 91.4, 173.0 ± 56.3, 144.0 ± 82.0, and 160.5 ± 65.3 kPa for the construct-only, unstimulated, LPS-stimulated, and IL-10-stimulated conditions, respectively (**Fig. 7B**). Additional data after 2 weeks of co-culture are presented in **Table 2**.

Co-culture with TCL-treated constructs suppress LPS-induced TNF-α production, and increased stiffness enhances the anti-inflammatory effect of IL-10-stimulated macrophages. Similar to study 1, there was a stiffness-mediated effect with increasing construct stiffness. There was a significant increase in TNF-α secreted by unstimulated, stiff co-cultures compared to the soft co-culture group ($p = 0.006$; **Fig. 8**). LPS stimulation increased the secretion of TNF-α overall. LPS-stimulated macrophages produced 117.6 ± 18.0 pg/ml TNF-α. Both soft and stiff constructs caused a significant 81.0% ($p < 0.0001$) and 76.4% ($p < 0.0001$) reduction in TNF-α levels compared to the LPS-stimulated macrophage-only control (**Fig. 8**).

When stimulating macrophages toward an anti-inflammatory phenotype, IL-10-stimulated macrophages secreted 15.6 ± 1.3 pg/ml TNF-α. When IL-10-stimulated macrophages were co-cultured with soft constructs, no significant difference in TNF-α levels was observed compared to macrophage-only control ($p = 0.48$; **Fig. 8**). However, stiff constructs significantly reduced the TNF-α secretion to 4.6 ± 2.9 pg/ml compared to macrophage-only controls ($p = 0.005$; **Fig. 8**).

Study 1 and Study 2 Comparison

TCL-treated constructs maintain or increase aggregate modulus compared to CHG-treated constructs after 2 weeks of co-culture. To compare the culture regimens of constructs and how construct mechanical properties change after macrophage co-culture, **Table 3** presents the percent changes of

Table 2. Additional properties of TCL-treated constructs.

Time	Stiffness	Macrophage Condition	Construct WW (mg)	Col/WW (μg/μg)	Young's Modulus (MPa)	UTS (MPa)	Shear Modulus (kPa)	Permeability (10^{-15} m^4/Ns)
$t = 0$	Soft	None	9.805 ± 0.470	0.031 ± 0.003	7.352 ± 3.323	2.097 ± 0.662	32.9 ± 7.9	4.9 ± 4.4
	Stiff		10.826 ± 0.612*	0.025 ± 0.003*	6.471 ± 1.038	1.703 ± 0.219	73.0 ± 11.9*	7.3 ± 6.9
$t = 2W$	Soft	None	13.100 ± 0.645[a]	0.034 ± 0.003	11.726 ± 4.720	2.994 ± 1.001	71.9 ± 26.8[a]	15.7 ± 16.5
		Unstimulated	11.678 ± 0.302[b]	0.039 ± 0.003	8.874 ± 1.932	2.395 ± 0.491	56.1 ± 15.9[ab]	11.4 ± 8.1
		LPS	11.783 ± 0.707[ab]	0.038 ± 0.004	7.803 ± 1.046	2.274 ± 0.503	38.0 ± 14.1[b]	10.5 ± 8.3
		IL-10	12.522 ± 0.656[ab]	0.038 ± 0.002	7.510 ± 1.1792	2.223 ± 0.711	41.2 ± 11.3[ab]	9.7 ± 6.2
	Stiff	None	15.455 ± 1.049[a]	0.032 ± 0.002	7.273 ± 2.142	2.081 ± 0.340	75.5 ± 26.5	29.0 ± 20.6[a]
		Unstimulated	13.435 ± 1.323[b]	0.031 ± 0.004	7.285 ± 0.987	2.066 ± 0.571	74.6 ± 20.9	19.9 ± 14.7[ab]
		LPS	13.462 ± 1.264[b]	0.033 ± 0.004	9.322 ± 3.323	2.313 ± 1.033	59.9 ± 24.0	8.8 ± 7.6[b]
		IL-10	14.229 ± 0.904[ab]	0.030 ± 0.004	9.560 ± 6.197	1.947 ± 0.402	70.1 ± 21.8	12.0 ± 12.6[ab]

Significance is seen in construct WW among construct stiffnesses at $t = 0$, similar to CHG-treated constructs. In addition, construct WW also generally decreases in both stiffnesses after 2 weeks of co-culture. Statistics: Asterisks (*) represent a Student's *t* test comparing $t = 0$ properties among stiffnesses ($\alpha = 0.05$), $n = 5$ per group. Lowercase letters represent a two-way ANOVA with *post hoc* Tukey's HSD test comparing properties after 2 weeks of co-culture only within individual stiffnesses ($\alpha = 0.05$), $n = 5$-6 per group. Gray-shaded table cells indicate statistical comparisons. TCL = transforming growth factor beta 1/chondroitinase $ABC_{eng.}$/lysyl oxidase-like 2; WW = wet weight; Col = collagen; UTS = ultimate tensile strength; LPS = lipopolysaccharide; IL-10 = interleukin 10; CHG = chondrogenic medium; ANOVA = analysis of variance; HSD = honestly significant difference.

aggregate modulus after 2 weeks of co-culture compared to baseline properties ($t = 0$). For CHG-treated co-culture groups, unstimulated and LPS-stimulated, all groups had a marked decrease from baseline properties at $t = 0$, ranging between 31.6 ± 9.7% and 68.2 ± 45.0%. In comparison, co-culture groups for TCL-treated constructs either maintained or increased the aggregate modulus compared to $t = 0$ controls, ranging from 87.0 ± 49.5% to 183.2 ± 46.4% of baseline values.

Discussion

Substantial progress has been made toward tissue-engineered cartilages with properties approaching or on par with native tissue.[45,60,61] While achieving biomimetic properties should be a part of the translational tissue engineering process, other factors must be considered to evaluate the potential success of a neocartilage implant. For example, as part of the innate immune response to surgical trauma, macrophages migrate to the treated area and would likely interact with the implant.[62] Due to the potentially deleterious effect macrophages can have on engineered tissue *in vivo*, the objective of this study was to develop a novel, *in vitro* direct co-culture model to study the interactions between differentially stimulated macrophages and self-assembled neocartilage. Two separate studies were conducted. Study 1 investigated the inflammatory response of macrophages to neocartilage of varying stiffnesses. The hypothesis for study 1 was confirmed; macrophages secreted more TNF-α, indicative of a proinflammatory phenotype, during co-culture with neocartilages of increasing stiffnesses. Interestingly, this response did not cause a reduction in construct mechanical properties compared to construct-only controls. However, over the 2-week co-culture period, aggregate modulus values decreased in all CHG-treated co-culture groups compared to $t = 0$ timepoints. Toward rescuing constructs that had a significant decrease in mechanical properties (i.e., the CHG-treated soft and medium groups) and investigating additional protection measures, study 2 was performed with bioactive factors that have been shown to increase the mechanical properties of neocartilage and polarize macrophages toward an anti-inflammatory phenotype. Macrophage polarization toward an anti-inflammatory phenotype did not improve neocartilage mechanical properties compared to LPS stimulation (i.e., proinflammatory phenotype). However, the neocartilage bioactive factors (i.e., TCL treatment) examined here prevented neocartilage mechanical degradation over time (i.e., with respect to $t = 0$) when compared to CHG-treated constructs, regardless of macrophage stimulation condition or construct stiffness.

This study showed that increasing neocartilage stiffness drives polarization of macrophages to an enhanced proinflammatory phenotype, but sufficiently stiff neocartilage may also be protected from macrophage-related deleterious effects. Interestingly, there was a significant increase in TNF-α production between construct-only controls and LPS-stimulated co-cultures in the stiff group (**Fig. 4C**). This stiffness-mediated effect on macrophage phenotype was not limited to proinflammatory macrophages. In study 2, IL-10-stimulated (i.e., anti-inflammatory phenotype) macrophages co-cultured with stiff constructs had TNF-α levels decrease by 62.5% compared to soft constructs exposed to the same co-culture condition (**Fig. 8**). This apparent stiffness-dependent macrophage behavior has been seldom examined on tissue substrates and has not been

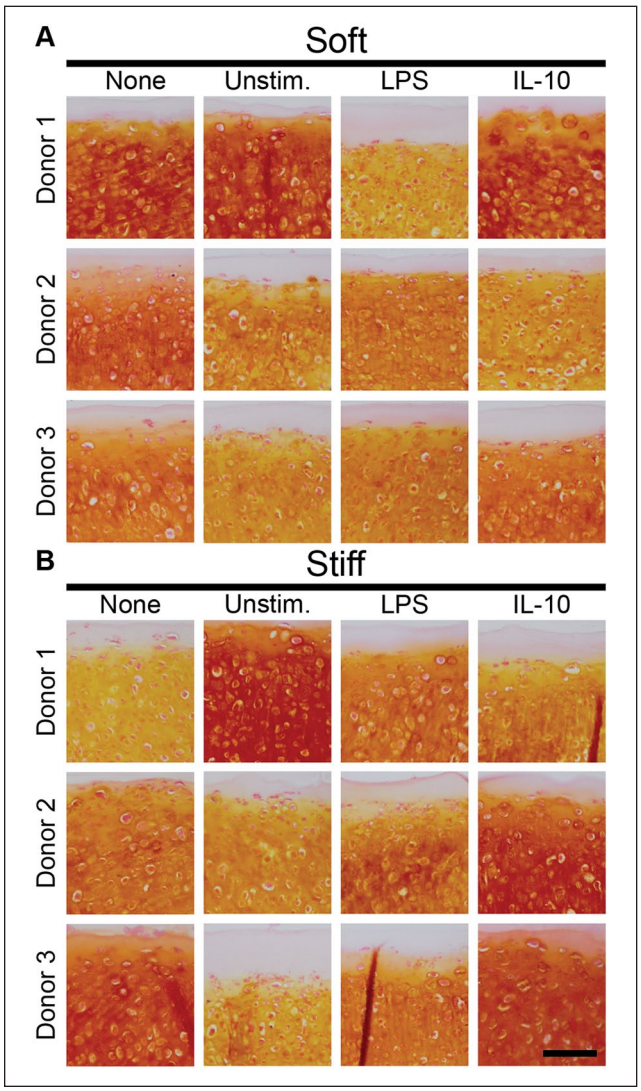

Figure 6. Saf-O staining after 2 weeks of macrophage co-culture shows donor-related variation, with diminished staining among LPS-stimulated co-cultures. Both construct-only controls and co-culture groups show variability between donors in both (**A**) soft and (**B**) stiff TCL-treated constructs. However, on average, staining is slightly diminished in some LPS-stimulated groups compared to construct-only controls. Scale bar = 100 µm. Saf-O = Safranin O; LPS = lipopolysaccharide; Unstim. = unstimulated; IL-10 = interleukin 10; TCL = transforming growth factor beta 1/chondroitinase ABCeng./lysyl oxidase-like 2.

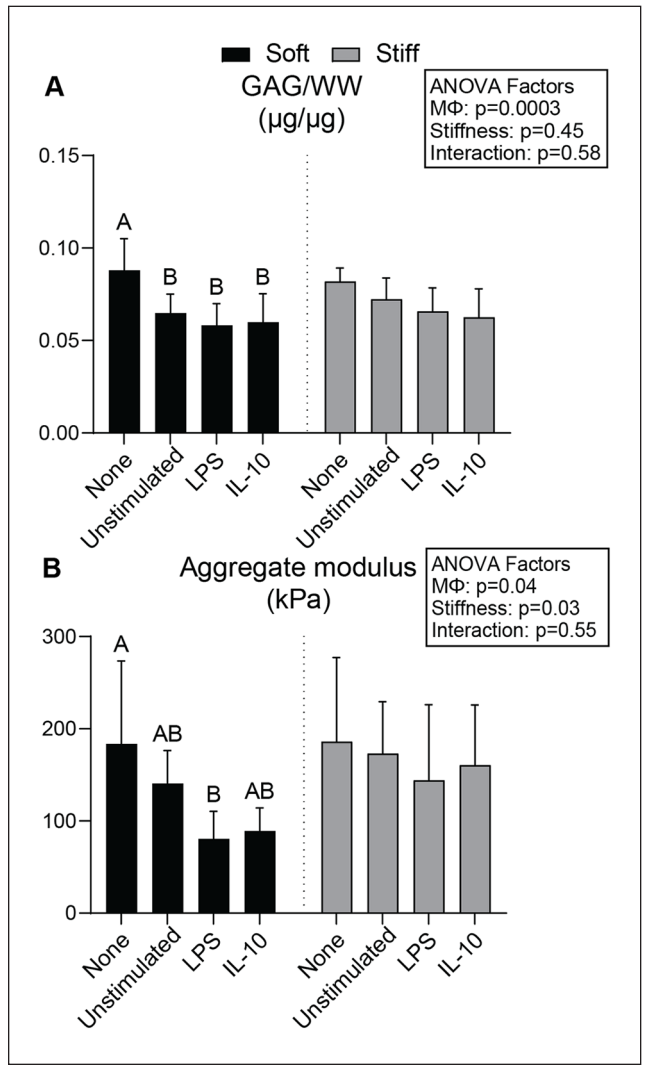

Figure 7. Aggregate modulus values of TCL-treated constructs only decrease in soft construct, LPS-stimulated co-cultures. (**A**) The construct-only control and macrophage co-cultures in the soft constructs are significantly different in GAG/WW, while the stiff group does not exhibit significant differences between groups. (**B**) Aggregate modulus also trends downward for the soft group when co-cultured with macrophages, but only the LPS-stimulated co-culture group is significantly different from the construct-only control. Stiff group aggregate moduli were largely unaffected by macrophage treatment. Statistics: Two-way ANOVA with *post hoc* Tukey's HSD among groups within a stiffness (dotted lines), $\alpha = 0.05$, $n = 5$-6 per group. TCL = transforming growth factor beta 1/chondroitinase $ABC_{eng.}$/lysyl oxidase-like 2; LPS = lipopolysaccharide; GAG = glycosaminoglycan; WW = wet weight; MΦ = macrophage; IL-10 = interleukin 10; ANOVA = analysis of variance; HSD = honestly significant difference.

previously examined on cartilage neotissue, but this has been well documented on less complex substrates such as hydrogels.[63] For example, it has been observed that when LPS-stimulated macrophages were seeded on polyethylene glycol (PEG) hydrogels of increasing stiffness, TNF-α and IL-1β expression increased alongside stiffness.[26,50,64] To our knowledge, this is the first time that modulation of macrophage phenotype using substrate stiffness has been shown for cartilage neotissue.

Proinflammatory cytokines such as TNF-α are known to have catabolic effects on native articular cartilage mechanical properties.[19] This is well established in the literature to be mediated through enzymatic degradation of the cartilage

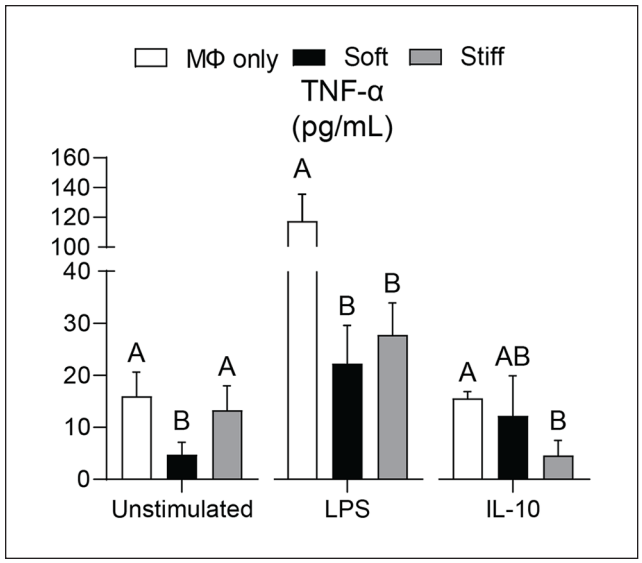

Figure 8. TNF-α secretion of TCL-treated construct co-cultures increases with stiffness, decreases with construct co-culture, and diminishes when macrophages are stimulated toward an anti-inflammatory phenotype. In unstimulated co-culture conditions, TNF-α production increases with stiffness. Similarly, TNF-α secretion also trends higher for stiff construct co-culture compared to the soft construct condition in the LPS-stimulated groups. Interestingly, construct addition significantly decreases the TNF-α production compared to macrophage-only controls in LPS-stimulated groups. For IL-10 stimulation, a decrease in TNF-α levels is seen with increasing construct stiffness. Statistics: One-way ANOVA with *post hoc* Tukey's HSD (for each stimulation condition), $\alpha = 0.05$, $n =$ 5-6 per group. TNF-α = tumor necrosis factor alpha; TCL = transforming growth factor beta 1/chondroitinase $ABC_{eng.}$/lysyl oxidase-like 2; MΦ = macrophage, LPS = lipopolysaccharide; IL-10 = interleukin 10; ANOVA = analysis of variance; HSD = honestly significant difference.

via matrix metalloproteinases (MMPs), which are upregulated when exposed to inflammatory cytokines such as TNF-α in multiple species, including the pig.[65-67] For example, as a result of age-related OA, advanced glycation end products (AGEs) can cause cartilage to become stiffer and more brittle.[68] As a result of AGEs and associated stiffening, it is likely that increased proinflammatory cytokines and MMPs could be secreted from macrophages within the joint, causing breakdown of the cartilage ECM.[69] However, contrary to this potential mechanism and the literature, stiff self-assembled neocartilage did not experience a drop in properties relative to its corresponding construct-only control after exposure to elevated TNF-α levels due to macrophage co-culture in study 1 (**Fig. 4**). The opposite was true for soft and medium stiffness co-cultured constructs in study 1; they experienced a significant decrease in aggregate modulus, without a corresponding increase in TNF-α secretion (**Fig. 4**). Similarly, we hypothesized that constructs co-cultured with macrophages stimulated with IL-10 might improve mechanical properties when compared to co-culture with LPS. However, there were no significant differences in aggregate modulus values between these co-culture conditions in either stiffness in study 2 (**Fig. 7B**). While the mechanism for this stiffness-mediated behavior is unclear, it was clear that additional factors, both neocartilage (i.e., TCL treatment) and macrophage (IL-10 stimulation) specific, for protection of soft neocartilage constructs against macrophage challenge were necessary.

Previous studies have investigated the use of TCL treatment for increasing robustness of neocartilage constructs. Here, TCL treatment prevents construct reductions in mechanical properties experienced by CHG-only treated neocartilage subjected to macrophage challenge. Compared to soft and medium CHG-treated constructs from study 1, which had a significant decrease in aggregate modulus no matter the co-culture condition (**Fig. 4B**), only the soft, TCL-treated constructs exposed to LPS-stimulated macrophages from study 2 significantly differed in aggregate modulus from the corresponding construct-only control (**Fig. 7B**). In addition, TCL-treated constructs co-cultured with macrophages in study 2 either maintained or increased their aggregate modulus compared to baseline values at $t = 0$ (**Table 3**). In comparison, CHG-treated constructs co-cultured with macrophages all experienced a reduction in aggregate modulus ranging from 31.6% to 68.2% of the corresponding values at $t = 0$ (**Table 3**). Macrophage co-culture reduced construct properties in CHG-treated constructs, but TCL treatment prevented such deleterious effects. When looking at constructs of similar baseline aggregate modulus values, the medium CHG-treated group corresponded to the stiff TCL-treated group. As the medium CHG-treated group experienced a marked drop in properties from baseline (31.6%-53.0%), whereas the stiff TCL-treated group did not (87.0%-104.5%), this suggests that an effect inherent to TCL treatment confers protection to constructs. While further exploration would be necessary to determine the mechanism responsible for this behavior, this is a significant finding toward developing future immunomodulatory approaches using tissue engineering techniques such as TCL treatment.

The mechanism of protection via TCL treatment is not fully known, but it may, in part, be due to TCL-treated constructs being less susceptible to macrophage infiltration compared to CHG-treated constructs. It is also known that TGF-β1 binds to cartilage ECM,[70] thus potentially suppressing the proinflammatory phenotype[71] of the co-cultured macrophages. Alternatively, it is possible that alterations in construct surface topology and ECM contents driven by TCL treatment alter the behavior and phenotype of macrophages.[63] LPS-stimulated macrophage TNF-α production was reduced by more than 4 times when co-cultured with TCL-treated constructs (**Fig. 8**), suggesting that TCL-treated constructs have an anti-inflammatory effect on

Table 3. CHG-treated constructs decrease aggregate modulus values compared to TCL-treated constructs which maintain or increase aggregate modulus values after 2 weeks of macrophage co-culture..

Study	Time	Stiffness	Macrophage Condition	Change from $t = 0$ (%)
Study 1: CHG-treated	$t = 2W$	Soft	None	119.9 ± 11.4
			Unstimulated	48.1 ± 19.7
			LPS	68.2 ± 45.0
		Medium	None	98.8 ± 19.7
			Unstimulated	53.0 ± 15.4
			LPS	31.6 ± 9.7
		Stiff	None	46.7 ± 13.8
			Unstimulated	39.4 ± 13.1
			LPS	36.2 ± 9.6
Study 2: TCL-treated		Soft	None	239.1 ± 117.2
			Unstimulated	183.2 ± 46.4
			LPS	104.8 ± 38.8
			IL-10	116.1 ± 32.4
		Stiff	None	112.3 ± 55.2
			Unstimulated	104.5 ± 34.0
			LPS	87.0 ± 49.5
			IL-10	96.9 ± 39.4

CHG-treated constructs drop from $t = 0$ baseline values of aggregate modulus across all stiffnesses when co-cultured with macrophages, ranging from 31.6% to 68.2% of original values. Conversely, TCL-treated constructs either maintain or increase from baseline values, ranging from 87.0% to 183.2%, when co-cultured with macrophages. Gray-shaded table cells delineate stiffnesses.
CHG = chondrogenic medium; TCL = transforming growth factor beta 1/chondroitinase $ABC_{eng.}$/lysyl oxidase-like 2; LPS = lipopolysaccharide; IL-10 = interleukin 10.

macrophage phenotype. This behavior could possibly be due to the increased collagen deposition as compared to CHG-treated constructs, because collagen gels and collagen type II have both been shown to promote an anti-inflammatory macrophage phenotype.[15,72] Ultimately, due to their stability, robustness, and potential anti-inflammatory effects, TCL-treated constructs should be used for future cartilage mechano-immunology studies.

In tissues such as neocartilage constructs, mechanical properties and biochemical content are inherently linked due to structure-function relationships. Here, we modulate compressive stiffness by removing GAG, which leads to changes in aggregate modulus values. Variations in GAG content as a result of $C\text{-}ABC_{comp.}$ treatment in the construct could contribute to changes in macrophage behavior. For example, it has been shown that individual GAGs can modulate macrophage phenotype by eliciting production of nitric oxide.[73] Because self-assembled neocartilage is a complex biological tissue, it would be challenging but potentially worthwhile to develop a way to decouple changes in stiffness from changes in ECM content and construct surface topology. Future studies may consider altering collagen content via collagenase or crosslinking via addition of exogenous lysyl oxidase in varying concentrations to modulate other mechanical properties (e.g., tensile).

The novel, direct co-culture model developed in this study investigated the interactions between macrophages and neocartilage constructs for the first time, and sets the stage for future investigations that foster the development of the nascent field of cartilage mechano-immunology. Future studies could include investigation of disease or injury modeling, biomolecular pathways that drive macrophage polarization and chondrocyte behavior, or anti-inflammatory macrophage-assisted cartilage tissue engineering. For example, the exact mechanism of TCL-mediated protection could be elucidated by using this co-culture system in future studies by extracting macrophages and chondrocytes, and exploring gene expression via single-cell RNA sequencing for each cell type. Furthermore, these *in vitro* studies could inform the development and engineering of neocartilage implants that minimize inflammation by tuning the mechanical properties of the neocartilages and, thus, modulate the macrophage-mediated immune response after implantation. Another example is combining this co-culture system with an *in vitro* integration system, where integration between excised native tissues and engineered cartilages is assessed over various co-culture times as an *in vitro* surrogate for healing potential *in vivo*. Eventually, large animal, orthotopic approaches in native cartilages will ultimately inform whether or not future immunomodulatory approaches will be feasible in humans. In conclusion, through the development of a novel, *in vitro* co-culture system, this study demonstrated that variable neocartilage stiffness can alter macrophage behavior, but that stiffness, as well as bioactive factor treatments (e.g., TCL treatment), can protect construct integrity in the presence of proinflammatory factors.

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Declaration of Conflicting Interests

The author(s) declared the following potential conflicts of interest with respect to the research, authorship, and/or publication of this article: Kyriacos A. Athanasiou and Jerry C. Hu are scientific consultants for Cartilage Inc.

Ethical Approval

Ethical approval was not sought for this study because the tissues from which cells were obtained were purchased from commercial vendors of animal specimens.

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