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RESEARCH ARTICLE

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Resveratrol counteracts IL-1β-mediated impairment of extracellular matrix deposition in 3D articular chondrocyte constructs

Sebastian Frischholz | **Oliver Berberich** | **Thomas Böck** | **Rainer H. Meffert** | **Torsten Blunk**

Department of Trauma, Hand, Plastic and Reconstructive Surgery, University of Würzburg, Würzburg, Germany

Correspondence
Torsten Blunk, Department of Trauma, Hand, Plastic and Reconstructive Surgery, University of Würzburg, Oberdürrbacher Straße 6, 97080 Würzburg, Germany.
Email: blunk_t@ukw.de

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Abstract

When aiming at cell-based therapies in osteoarthritis (OA), proinflammatory conditions mediated by cytokines such as IL-1β need to be considered. In recent studies, the phytoalexin resveratrol (RSV) has exhibited potent anti-inflammatory properties. However, long-term effects on 3D cartilaginous constructs under inflammatory conditions with regard to tissue quality, especially extracellular matrix (ECM) composition, have remained unexplored. Therefore, we employed long-term model cultures for cell-based therapies in an in vitro OA environment and evaluated effects of RSV. Pellet constructs made from expanded porcine articular chondrocytes were cultured with either IL-1β (1–10 ng/ml) or RSV (50 μM) alone, or a cotreatment with both agents. Treatments were applied for 14 days, either directly after pellet formation or after a preculture period of 7 days. Culture with IL-1β (10 ng/ml) decreased pellet size and DNA amount and severely compromised glycosaminoglycan (GAG) and collagen content. Cotreatment with RSV distinctly counteracted the proinflammatory catabolism and led to partial rescue of the ECM composition in both culture systems, with especially strong effects on GAG. Marked MMP13 expression was detected in IL-1β-treated pellets, but none upon RSV cotreatment. Expression of collagen type I was increased upon IL-1β treatment and still observed when adding RSV, whereas collagen type X, indicating hypertrophy, was detected exclusively in pellets treated with RSV alone. In conclusion, RSV can counteract IL-1β-mediated degradation and distinctly improve cartilaginous ECM deposition in 3D long-term inflammatory cultures. Nevertheless, potential hypertrophic effects should be taken into account when considering RSV as cotreatment for articular cartilage repair techniques.

KEYWORDS
articular chondrocytes, cartilage, cell-based therapy, extracellular matrix, IL-1β, inflammation, osteoarthritis, resveratrol

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1 | INTRODUCTION

Osteoarthritis (OA) as a joint affecting disease is an increasing challenge burdening not only the afflicted individual including major pain, joint dysfunction, and deformity (Robinson et al., 2016) but also health-care systems. As the most common form of arthritis, OA prevalence is estimated to be at least 19% in U.S. adults ≥45 years, and rising numbers over the next decades must be assumed (Lawrence et al., 2008; Wallace et al., 2017).

Therefore, in order to find therapeutic approaches, many studies focused on revealing the pathogenesis of this disease (Leung, Rainsford, & Kean, 2014; Sokolove & Lepus, 2013; Xia et al., 2014). Besides and related to risk factors such as age, obesity, traumatic injury, and mechanical overload, inflammation plays a crucial role in the multifactorial pathogenesis of OA (Blagojevic, Jinks, Jeffery, & Jordan, 2010; Lopes, Filiberti, Husain, & Humphrey, 2017; Robinson et al., 2016).

Indeed, OA does impair not only the articular cartilage itself but the whole joint including hypertrophy of the joint capsule and synovitis (Loeser, Goldring, Scanzello, & Goldring, 2012). This inflammation of the synovium results in high levels of plasma proteins, complement components, and cytokines in the synovial fluid affecting articular cartilage likewise (Pelletier, Martel-Pelletier, & Abramson, 2001; Sokolove & Lepus, 2013). This is related to enhanced cartilage loss, reduced mobility, and increased radiographic grades (Guermazi et al., 2011; Robinson et al., 2016).

Inflammatory cytokines such as IL-1β and TNFα have particularly been linked to cartilage catabolism and extracellular matrix (ECM) breakdown (Lopes et al., 2017; Sokolove & Lepus, 2013). Whereas systemically applied anti-IL-1β or anti-TNF therapies are proved for treatment of rheumatoid arthritis (RA), they have shown no satisfying value in OA clinical studies (Cohen et al., 2011; Verbruggen, Wittoek, Vander Cruyssen, & Elewaut, 2012). Instead, current treatments including common NSAIDs and joint replacement focus primarily on end-stage symptoms (Tonge, Pearson, & Jones, 2014). This points to a strong need for novel therapeutic approaches, such as combining both repair techniques and anti-inflammatory agents in early disease stages in order to block long-term progression of OA. In the present study, we therefore employed long-term model cultures for cell-based therapies in an OA environment, enabling investigations into the effects of anti-inflammatory agents.

Regarding inflammation in OA, the polyphenolic phytoalexin resveratrol (RSV) has been shown to have potent anti-inflammatory properties (Shen et al., 2012). Previous studies not only revealed that RSV is able to counteract IL-1β-mediated apoptosis of human articular chondrocytes in vitro but also discovered RSV-mediated signaling pathways (Csaki, Keshishzadeh, Fischer, & Shakibaei, 2008; Csaki, Mobasheri, & Shakibaei, 2009; Dave et al., 2008; Shakibaei, Csaki, Nebrich, & Mobasheri, 2008; Shakibaei, Mobasheri, & Buhrmann, 2011). In addition, RSV was reported to reverse IL-1β-induced down-regulation of collagen type II and aggrecan expression in mesenchymal stem cell (MSC)-derived chondrocytes in a 2-day experiment (Lei, Liu, & Liu, 2008). Whereas these studies investigated short-term effects and were carried out mostly in monolayer cultures, Sheu, Chen, Sun, Lin, and Wu (2013) characterized a hyaluronic acid/RSV hydrogel and analyzed long-term mRNA expression (collagen type I and II, matrix metalloproteinases [MMPs]) upon lipopolysaccharide (LPS)-induced inflammation. However, studies on the long-term effects of RSV on 3D constructs under inflammatory conditions with regard to tissue quality, especially ECM composition, have been lacking so far.

Thus, in order to further investigate the applicability of RSV for cell-based therapies in an inflammatory milieu, we focused on 3D porcine cartilage pellet constructs in a long-term culture setup of 14 days by employing IL-1β. Further, we conducted experiments with an additional preculture under noninflammatory conditions for 7 days prior to the culture period with IL-1β. Histological, immunohistochemical, and quantitative biochemical analyses of ECM composition as well as quantitative reverse transcription polymerase chain reaction (qRT-PCR) analyses were conducted.

2 | MATERIALS AND METHODS

2.1 | Isolation and culture of cells

Porcine articular cartilage was removed from the femorotibial joint of 4- to 5-month-old pigs, cut into pieces of 1-mm size, and digested with type II collagenase (Worthington, Lakewood, USA) as previously described (Blunk et al., 2002). The suspension was centrifuged (300 *g*, 10 min) and resuspended in expansion medium consisting of Dulbecco's Modified Eagle's Medium (DMEM) high glucose 4.5 g/L (Sigma-Aldrich, Munich, Germany) supplemented with 10% fetal bovine serum (FBS; Invitrogen, Karlsruhe, Germany) and 1% penicillin–streptomycin (100 U/ml of penicillin and 0.1 mg/ml of streptomycin; Invitrogen, Karlsruhe, Germany). Cells were then cultured in T175 cm^2 culture flasks (Greiner Bio-One, Frickenhausen, Germany) with a medium change every 2 days until cells reached 90% confluence after 5 days. Chondrocytes were passaged using trypsin-EDTA at 0.25% (Life Technologies, Karlsruhe, Germany), and first passage cells (P1) were utilized in the experiments.

2.2 | Pellet culture

Chondrocytes (P1) were suspended in seeding medium containing DMEM high glucose, 10% FBS, 1% penicillin–streptomycin, 50 μg/ml of ascorbic acid-2-phosphate (Sigma-Aldrich), 40 μg/ml of proline (Sigma-Aldrich), 10 mM of HEPES (Sigma-Aldrich), and 0.1 mM of nonessential amino acids (Thermo Fisher Scientific, Waltham, USA). Cells were seeded in 96-well plates (conical bottom; TPP, Trasadingen, Switzerland) at a density of 2×10^5 cells per well, centrifuged at 300 *g* for 5 min, and subsequently incubated overnight in an incubator (37°C, 5% CO_2, 21% O_2; IBS Integra Biosciences, Fernwald, Germany) in order to allow the formation of dense cell pellets. Subsequently, pellets were cultured in serum-free chondrocyte

medium containing DMEM high glucose, 1% penicillin–streptomycin, 50 μg/ml of ascorbic acid-2-phosphate, 40 μg/ml of proline, 100 μg/ml of sodium pyruvate (Sigma-Aldrich), and 1% insulin-transferrin-selenium-plus (ITS+ Premix, Corning, NY, USA).

2.3 | Experimental design

In two groups, pellets in serum-free chondrocyte medium were treated with IL-1β (BioLegend, London, UK) at concentrations of either 1 or 10 ng/ml simulating an inflammatory milieu. A third group received both IL-1β at 10 ng/ml and RSV (Sigma-Aldrich, Taufkirchen, Germany) at 50 μM as an anti-inflammatory stimulus. One additional group was treated with RSV only in order to evaluate effects on pellets under noninflammatory conditions. Pellets receiving serum-free chondrocyte medium only served as control. As shown in Figure 1, in one set of experiments, pellets immediately received treatments for 14 days, designated as "w/o preculture". In a second set, pellets were matured in serum-free chondrocyte medium for 7 days prior to culture with treatments for 14 days, designated as "with preculture".

2.4 | Biochemical analyses

Pellet size was determined by imaging and measurement using microscope BX51 plus DP71 camera and CellSense™ 1.16 software (Olympus, Hamburg, Germany). For biochemical analyses, pellets (nine pellets per condition were used, of which three per sample were pooled, resulting in $n = 3$) were washed with phosphate-buffered saline (PBS) and then digested in 0.375 ml of papain solution (Worthington, Lakewood, USA) at 3 U/ml for 16 h at 60°C (Böck et al., 2018).

DNA content of digested pellets was measured using Hoechst 33258 dye (Polysciences, Warrington, USA). Samples were analyzed using a fluorimeter at an excitation wavelength of 360 nm and an emission wavelength of 460 nm (Y. J. Kim, Sah, Doong, & Grodzinsky, 1988). In order to determine sulfated glycosaminoglycan (GAG) content, samples were incubated with dimethylmethylene blue dye and measured spectrophotometrically at 525 nm, using chondroitin sulfate as standard (Farndale, Buttle, & Barrett, 1986). Hydroxyproline content of pellets was measured after hydrolysis with hydrochloric acid and reaction with chloramine-T and *p*-dimethylaminobenzaldehyde (DAB) using a spectrophotometer at 560 nm (Woessner, 1961). A hydroxyproline to collagen ratio of 1:10 was used to calculate total collagen content (Hollander et al., 1994).

2.5 | Histology and immunohistochemistry

For histological and immunohistochemical analyses, pellets were initially fixed in 3.7% buffered formalin and embedded in Tissue-Tek O.C.T. Compound (Sakura Finetek, Zoeterwonde, Netherlands). Samples were cut into 5-μm-thick sections and collected on Super Frost® plus glass slides (R. Langenbrinck, Emmendingen, Germany). For histological evaluation of GAG deposition, sections were hydrated for 1 min, followed by immersion in Weigert's hematoxylin for 5 min, washing under running tap water for 5 min, and counterstaining in 0.02% fast green for 4 min, 1% acetic acid for 10 s, and 0.1% safranin-O for 6 min. After dehydration, sections were mounted with Entellan® (Merck, Darmstadt, Germany) (Stichler et al., 2017).

For immunohistochemical analyses, rehydrated sections were immersed in Proteinase K (Digest-All 4, Life Technologies, Karlsruhe, Germany) for 10 min, ensuring antigen retrieval. After being washed three times for 3 min with Tween 20 in Tris-buffered saline (TBST), sections were blocked with 1% bovine serum albumin in PBS for 20 min. Primary antibodies were diluted in antibody diluent (Dako, Hamburg, Germany) and incubated overnight at the following dilutions: for collagen type I (monoclonal IgG mouse ab6308, Abcam, Cambridge, UK) at 1:600, for collagen type II (polyclonal IgG rabbit ab34712, Abcam, Cambridge, UK) at 1:200, for collagen type X (monoclonal IgG mouse CloneX53, eBioscience) at 1:50, and for MMP13 (polyclonal IgG rabbit ab39012, Abcam, Cambridge, UK) at 1:100. Sections were then washed three times in TBST for 3 min. Secondary antibodies Alexa Fluor® 488 goat anti-mouse IgG (115-545-146) at 1:100 or Cy™3 goat anti-rabbit (111-165-003) at 1:100 (Jackson ImmunoResearch, West Grove, USA) were diluted and incubated in the dark for 2 h. Subsequently, slides were mounted with DAPI mounting medium ImmunoSelect® (Dako, Hamburg, Germany). As negative controls, equivalent concentrations of species-matched immunoglobulins on identically treated sections were used. Imaging was performed using a fluorescence microscope (Microscope BX51/DP71 camera, Olympus, Hamburg, Germany).

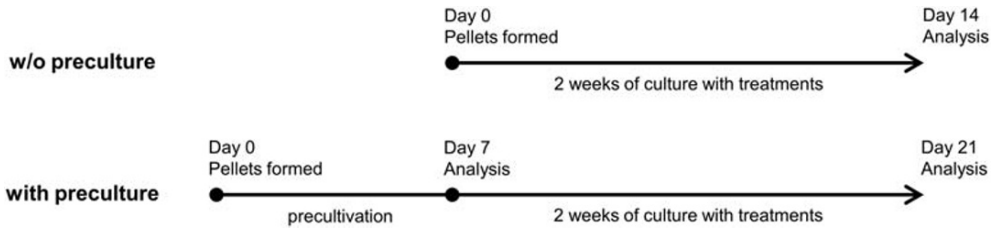

FIGURE 1 Experimental design: porcine articular chondrocytes were isolated and passaged, and pellets were formed. In one set, pellets immediately received treatments for 14 days, whereas in a second set, they were matured in chondrocyte medium for 7 days prior to culture with treatments for 14 days

2.6 | RNA isolation and qRT-PCR analysis

Pellets were homogenized in TRIzol® reagent (Life Technologies, Karlsruhe, Germany), and total RNA was extracted according to the manufacturer, whereby pellets were pooled (two per sample, with $n = 3$). Transcription into first-strand cDNA was performed using the ImProm™ reverse transcription system kit (Promega, Madison, USA). qRT-PCR analysis was performed with the Real-Time PCR Detection System CFX96T™ (Bio-Rad, Munich, Germany) using the Brilliant III Ultra-Fast SYBR® Green QPCR Master Mix (Agilent, Santa Clara, USA) with primer pairs for collagen type I, collagen type II, collagen type X, and beta-actin (Actb) as housekeeping gene (Table 1) (Gredes, 2008; customized by biomers.net GmbH, Ulm, Germany). The following cycling protocol was applied for all primers: 95°C for 3 min followed by 40 cycles at 95°C for 5 s and 60°C for 30 s, completed by increase in temperature from 65°C to 95°C in 0.5°C increments with 5 s per step. The mRNA expression levels of all genes were normalized to the housekeeping gene beta-actin for each group. The increase in expression levels for each gene was assessed using the $2^{-\Delta\Delta CT}$ method and further normalized to the control group receiving chondrocyte medium only.

2.7 | Statistical analysis

Statistical analysis was performed using GraphPad Prism, version 6.0 (GraphPad Software, La Jolla, USA). Data are presented as mean values ± standard deviation (SD). Statistical significance between groups was assessed by two-way analysis of variance followed by Tukey's post hoc test. Significant differences were marked as follows: $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$, $^{\Delta\Delta\Delta}p < 0.001$, and $^{\Delta\Delta}p < 0.01$.

3 | RESULTS

3.1 | Effects of IL-1β and RSV on pellet size and DNA content

Pellet constructs were cultured in chondrocyte medium supplemented with different concentrations of IL-1β (1 or 10 ng/ml), both IL-1β (10 ng/ml) and RSV (50 μM), or RSV (50 μM) alone for 14 days; cultures in only chondrocyte medium served as control (Figure 2). Treatment with IL-1β at 10 ng/ml led to a significantly lower pellet size and decreased DNA content, as compared with those of controls (Figure 2a). In contrast, treatment with IL-1β at 1 ng/ml resulted in only a slight reduction in pellet size and no significant difference in DNA content compared with those of controls. RSV counteracted the IL-1β-mediated reduction of both pellet size and DNA content; that is, it significantly increased diameter and DNA content (IL-1β: 1.45 ± 0.11 μg, IL-1β + RSV: 3.06 ± 0.10 μg of DNA; $p < 0.001$) even up to control levels (Ctrl: 3.24 ± 0.22 μg of DNA) (Figure 2a).

TABLE 1 Overview of used primers for qRT-PCR analysis

Gene	Primer sequence	
Collagen I	Forward	CCAACAAGGCCAAGAAGAAG
	Reverse	ATGGTACCTGAGGCCGTTCT
Collagen II	Forward	GCACGGATGGTCCCAAAG
	Reverse	CAGCAGCTCCCCTCTCAC
Collagen X	Forward	CACCAAGGCACAGTTCTTCA
	Reverse	ACCGGGAATACCTTGCTCTC
Actb (beta-actin)	Forward	AAGCCAACCGTGAGAAGATG
	Reverse	GTACATGGCTGGGGTGTTG

Abbreviation: qRT-PCR, quantitative reverse transcription polymerase chain reaction.

3.2 | RSV counteracts IL-1β-induced GAG depletion and MMP13 expression

With regard to GAG deposition, IL-1β at 1 ng/ml showed no significant effect; however, treatment with IL-1β at 10 ng/ml led to a massive depletion of absolute GAG content and GAG/DNA, as compared with that in controls (Ctrl: 92.04 ± 8.94 μg/μg, IL-1β: 8.35 ± 0.96 μg/μg of GAG/DNA; $p < 0.001$) (Figure 2a). Again, cotreatment with RSV counteracted the inflammatory stimulus and led to a partial rescue of GAG content with significant increases in total GAG as well as GAG/DNA (IL-1β: 8.35 ± 0.96 μg/μg, IL-1β + RSV: 43.44 ± 7.20 μg/μg of GAG/DNA; $p < 0.001$) (Figure 2a). Interestingly, RSV alone increased GAG/DNA content, as compared with that of controls (Ctrl: 92.04 ± 8.94 μg, RSV: 151.63 ± 4.31 μg/μg of GAG/DNA; $p < 0.001$).

Histological analysis confirmed the distinct decrease in size and the drastic GAG depletion for pellets receiving IL-1β at 10 ng/ml (Figure 2b). Also, well reflecting the quantitative data, a strong increase in GAG content for pellets treated with both IL-1β and RSV, as compared with IL-1β only, was clearly visible (Figure 2b).

Immunohistochemical staining for the prominent cartilage-degrading enzyme MMP13 showed a strong expression in IL-1β-treated (10 ng/ml) pellets, whereas, strikingly, in pellets cotreated with IL-1β and RSV, no MMP13 expression was observed (Figure 2c).

3.3 | RSV affects collagen content and composition

ptTreatment with IL-1β at 10 ng/ml, as compared with controls, significantly reduced absolute amounts of total collagen (Ctrl: 86.64 ± 7.12 μg, IL-1β: 25.29 ± 4.58 μg of collagen; $p < 0.001$) and collagen/DNA content (Ctrl: 26.69 ± 0.70 μg/μg, IL-1β: 17.38 ± 1.90 μg/μg of collagen/DNA; $p < 0.001$), whereas constructs treated with IL-1β at 1 ng/ml again showed no difference to controls (Figure 3a). Again, cotreatment of IL-1β-stimulated constructs with RSV significantly restored absolute collagen content (IL-1β: 25.29 ± 4.58 μg, IL-1β + RSV: 68.04 ± 10.16 μg; $p < 0.001$), whereas the increase of collagen/DNA was not statistically significant (IL-1β: 17.38 ± 1.90 μg/μg, IL-1β + RSV: 22.20 ± 2.64 μg/μg of collagen/DNA) (Figure 3a).

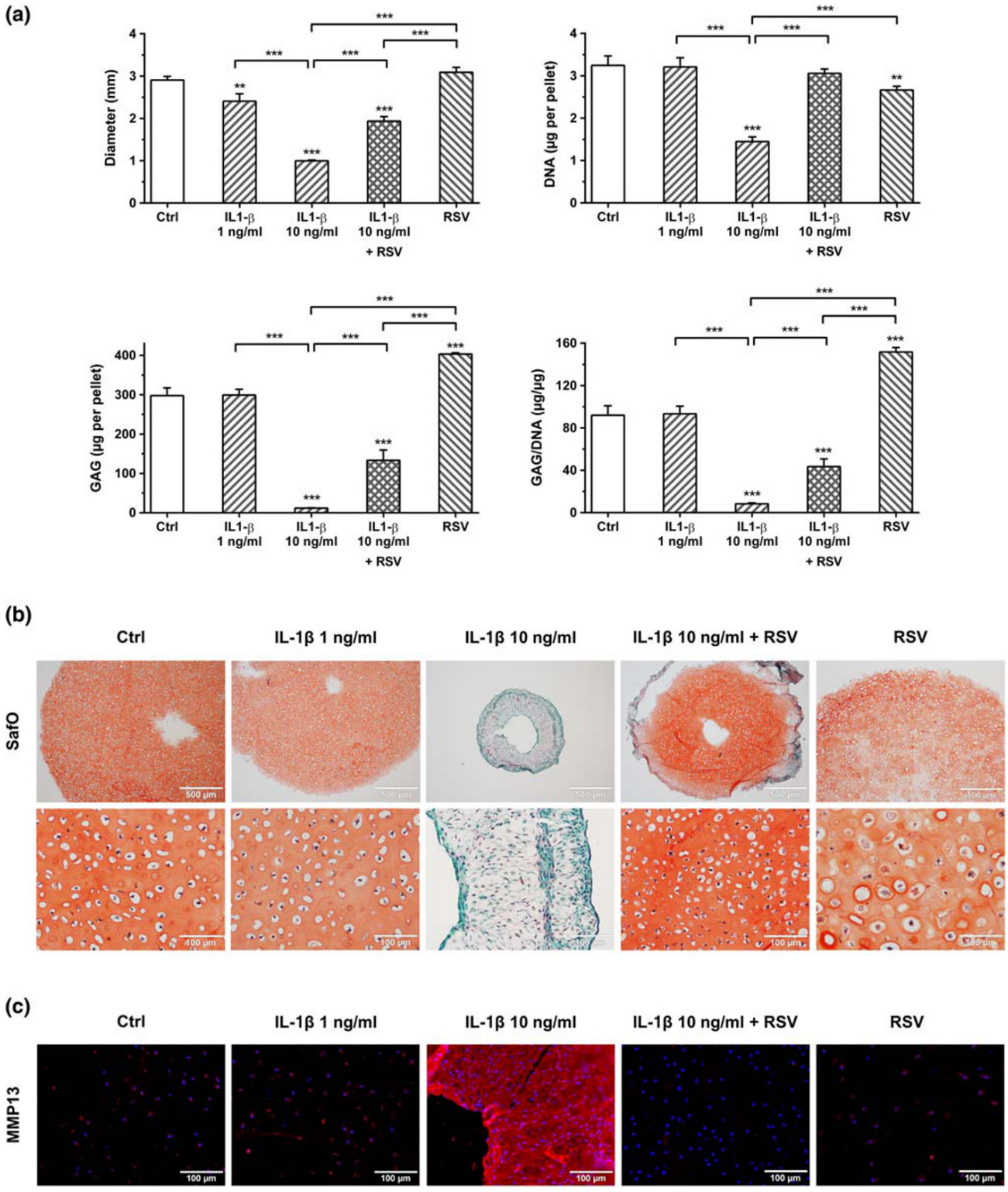

FIGURE 2 Construct analysis after 14 days of culture. (a) Pellet diameter and DNA content per pellet; glycosaminoglycan (GAG) deposition of chondrocytes, shown for absolute amounts of GAG (GAG/pellet) and normalized to DNA (GAG/DNA). Data are presented as means ± standard deviation (three pellets per sample were pooled, with $n = 3$). Asterisks above single bars represent statistical significance against control group, and above brackets between displayed groups; $^{***}p < 0.001$, $^{**}p < 0.01$. (b) Histological staining for GAG deposition with safranin-O. Scale bars represent 500 µm (upper) and 100 µm (lower). (c) Immunohistochemical staining for MMP13. Scale bars represent 100 µm [Colour figure can be viewed at wileyonlinelibrary.com]

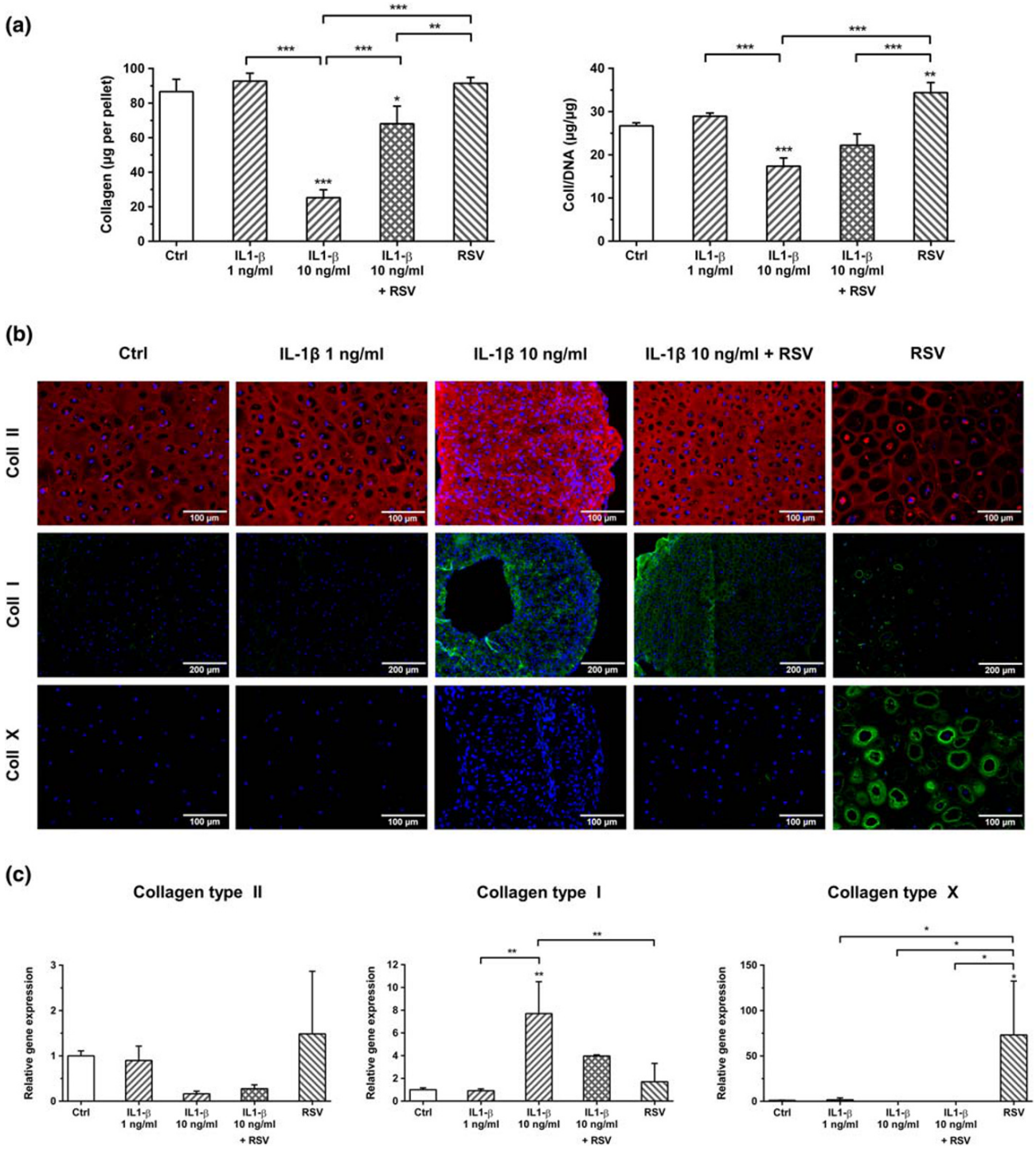

FIGURE 3 Collagen content after 14 days of culture. (a) Collagen production of chondrocytes, shown for absolute amounts of total collagen (collagen/pellet) and normalized to DNA (collagen/DNA). Data are presented as means ± standard deviation (three pellets per sample were pooled, with $n = 3$). (b) Immunohistochemical staining for collagen type II, type I, and type X. Scale bars represent 100 µm (upper, lower) and 200 µm (middle). (c) Gene expression of collagen type II, type I, and type X; qRT-PCR analysis at Day 14. Gene expression was normalized to beta-actin; the obtained values were further normalized to the values of the control group. Data are presented as means ± standard deviation (two pellets per sample were pooled, with $n = 3$). (a, c) Asterisks above single bars represent statistical significance against control group, and above brackets between displayed groups; $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$ [Colour figure can be viewed at wileyonlinelibrary.com]

Immunohistochemical analysis showed abundant collagen type II expression in all samples (Figure 3b). Staining appeared less distinct in samples treated with RSV only, which was likely due to the hypertrophic morphology of cells. Besides, qRT-PCR analysis at Day 14 normalized to the values of the control group showed no significant differences in gene expression of collagen type II (Figure 3c). Immunohistochemical staining for collagen type I, a marker for fibrous cartilage, was distinctly enhanced in samples treated with IL-1β (10 ng/ml) and also detected upon cotreatment with RSV (Figure 3b). Also in qRT-PCR analysis, a distinct increase of collagen type I expression was observed in pellets treated with IL-1β (10 ng/ml) alone, which was slightly reduced when cotreated with IL-1β plus RSV (Figure 3c). Interestingly, collagen type X, a marker for hypertrophic chondrocytes, was detected in pellets treated with RSV only, but in no other group including pellets cotreated with IL-1β and RSV (Figure 3b). Likewise, qRT-PCR analysis showed a significant increase of collagen type X gene expression in constructs treated with RSV only (75-fold compared with that of controls), but again in no other treatment group including pellets cotreated with IL-1β and RSV (Figure 3c).

3.4 | RSV improves IL-1β-impaired tissue composition also in the setup with preculture

In another set of experiments, pellet constructs were initially precultured in chondrocyte medium for 7 days prior to culture with treatments for 14 days in order to evaluate the impact of maturation on subsequent culture in an inflammatory milieu (Figure 1). After 21 days, pellets at IL-1β (10 ng/ml) exhibited significantly lower size and DNA content, as compared with those of controls, and did not exceed Day 7 levels (Figure 4a). Cotreatment with RSV counteracted this reduction and increased DNA content up to control levels (Figure 4a). With regard to both absolute GAG and GAG/DNA content, values after 21 days for pellets treated with IL-1β (10 ng/ml) were even significantly lower than baseline levels at Day 7 (IL-1β: 12.24 ± 1.05 μg/μg, Day 7: 56.55 ± 5.58 μg/μg of GAG/DNA; $p < 0.001$) (Figure 4a). Again, cotreatment with RSV strongly counteracted this loss of GAG content up to control levels (IL-1β + RSV: 99.31 ± 13.58 μg/μg, Ctrl: 89.38 ± 7.14 μg/μg of GAG/DNA) (Figure 4a), and this effect was likewise observed in histological staining (Figure 4b).

A similar pattern as observed for GAG, albeit not as drastic, was determined for absolute collagen and collagen/DNA, with a moderate reduction in pellets treated with IL-1β (10 ng/ml) and again rescue up to control levels after cotreatment with RSV (Figure 5a). Immunohistochemical staining showed abundant collagen type II expression in all samples, whereas only a faint staining at the construct edges for collagen type I and no signal for collagen type X were observed, with no obvious differences between the groups (Figure 5b). In qRT-PCR analysis, a low expression of collagen type II was observed in pellets treated with IL-1β (10 ng/ml), which was increased under cotreatment with RSV up to control levels. For collagen type X, again, a distinctly increased expression in pellets treated with RSV only was observed (Figure 5c).

When directly comparing the results from the two different culture regimes, that is, without and with preculture, upon IL-1β treatment (10 ng/ml), a more moderate decrease for DNA and collagen content was observed in the experiments with preculture. In contrast, treatment with IL-1β led to a strong decrease of total GAG and GAG/DNA in both experimental setups (data from Figures 2a and 4a, directly compared in Figure 4c). Interestingly, while a partial rescue of GAG content by the cotreatment with RSV was observed in the experiments without preculture, even a complete rescue of total GAG as well as GAG/DNA by the cotreatment with RSV was determined in the setup with preculture (Figure 4c).

4 | DISCUSSION

When aiming at repair and tissue engineering techniques for articular cartilage in inflammatory OA, it appears that anti-inflammatory drugs may delay advancement of deterioration by exhibiting beneficial effects at the defect site (Djouad, Rackwitz, Song, Janjanin, & Tuan, 2009; Makris, Gomoll, Malizos, Hu, & Athanasiou, 2015). In recent years, the polyphenol RSV has repeatedly been shown to counteract inflammatory signaling in chondrocytes (Shen et al., 2012). Thus, it appears as an attractive adjuvant treatment potentially enabling cell-based therapeutic approaches in OA. However, in vitro investigations so far have mainly focused on the elucidation of signaling pathways in short-term studies, and data in a 3D culture setting are scarce. Therefore, in this study, we examined the effects of RSV in model cultures for cell-based therapies in an OA environment, employing pellets made from expanded articular chondrocytes in an IL-1β-induced inflammatory milieu. For the first time, we show in a long-term in vitro culture potent effects of RSV on the ECM composition of 3D cartilaginous constructs.

In order to ensure comparability with other in vitro studies in the literature, the inflammatory cytokine IL-1β was applied in doses ranging from 1 to 10 ng/ml (Djouad et al., 2009; Im et al., 2012; Schulze-Tanzil, Mobasheri, Sendzik, John, & Shakibaei, 2004). In the present study, IL-1β exhibited a dose-dependent catabolic impact on both cell number and ECM content. Whereas for IL-1β at 1 ng/ml hardly any effects on the chondrocyte pellets were observed, IL-1β at 10 ng/ml led to drastic alterations with regard to reduced construct diameter and DNA content and strongly modulated ECM composition. These IL-1β-treated constructs showed a strong expression of the cartilage-degrading enzyme MMP13, were virtually depleted of GAG, had a reduced total collagen content, and showed distinct expression of collagen type I, a marker for fibrous cartilage.

In previous short-term studies mainly conducted in chondrocyte monolayer culture, increasing evidence has been found that RSV can suppress inflammatory signaling, with various pathways being under consideration (Shen et al., 2012). RSV has been reported to mediate antiapoptotic effects by inhibiting IL-1β-induced activation of caspase-3 and the cleavage of the DNA repair enzyme poly(ADP-ribose)polymerase (PARP) (Csaki et al., 2008; Shakibaei, John, Seifarth, & Mobasheri, 2007). In the same context, RSV has been

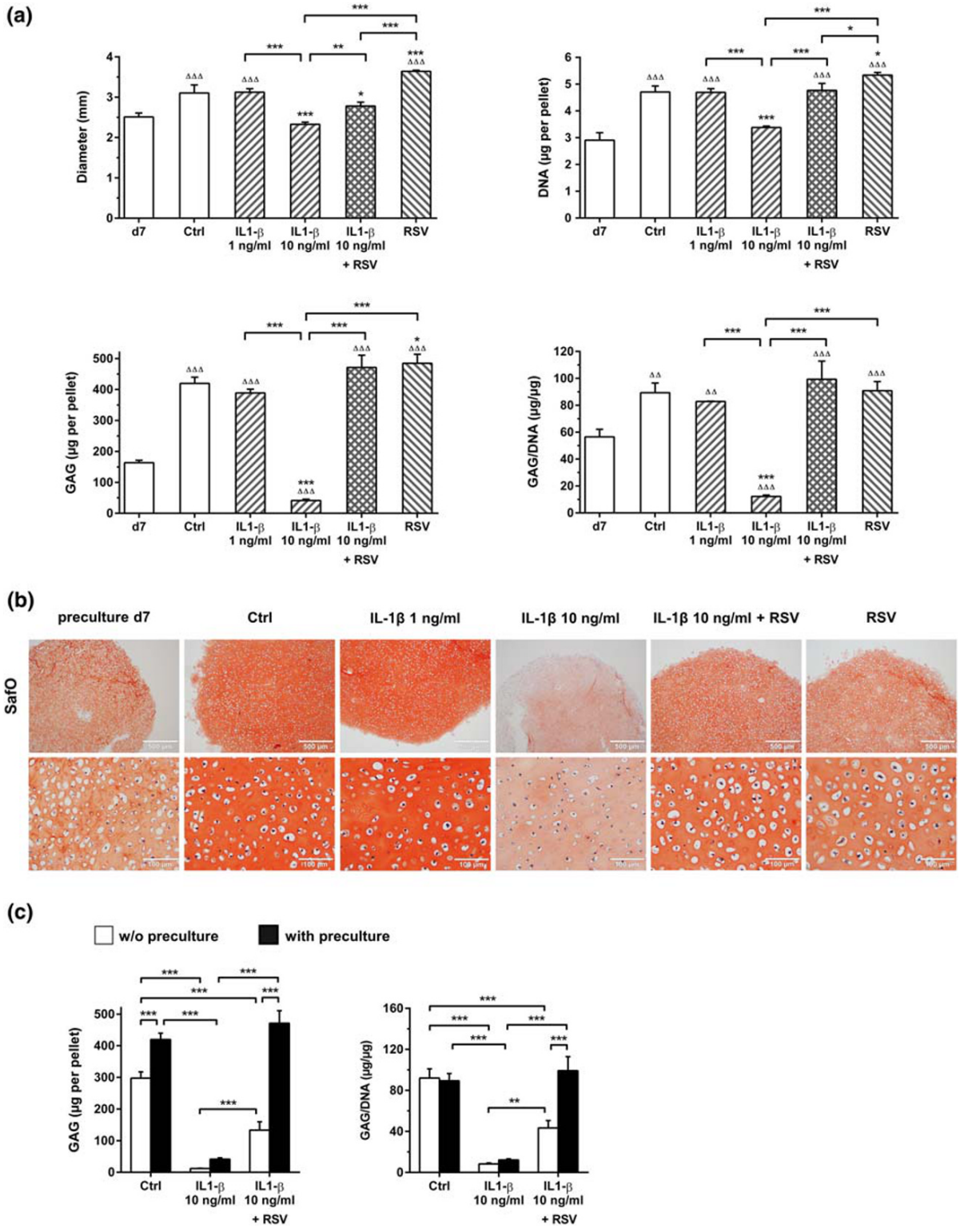

FIGURE 4 Construct analysis after 21 days of culture, that is, 7 days of precultivation in chondrocyte medium for all groups, followed by 14 days with different treatments. Data after 21 days are shown for all groups and compared with values at Day 7. (a) Pellet diameter and DNA content per pellet; glycosaminoglycan (GAG) deposition of chondrocytes, shown for absolute amounts of GAG (GAG/pellet) and normalized to DNA (GAG/DNA). Data are presented as means ± standard deviation (three pellets per sample were pooled, with $n = 3$). Asterisks above single bars represent statistical significance against control group after 21 days, above brackets between displayed groups; $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$. Triangles above single bars represent statistical significance between the respective treatment group after 21 days and control values at Day 7; $^{\Delta\Delta\Delta}p < 0.001$, $^{\Delta\Delta}p < 0.01$. (b) Histological staining for GAG deposition with safranin-O. Scale bars represent 500 µm (upper) and 100 µm (lower). (c) Comparison of experiments "without (w/o) preculture" and "with preculture" in terms of GAG deposition. Asterisks above brackets represent statistical significance between displayed groups; $^{***}p < 0.001$, $^{**}p < 0.01$ [Colour figure can be viewed at wileyonlinelibrary.com]

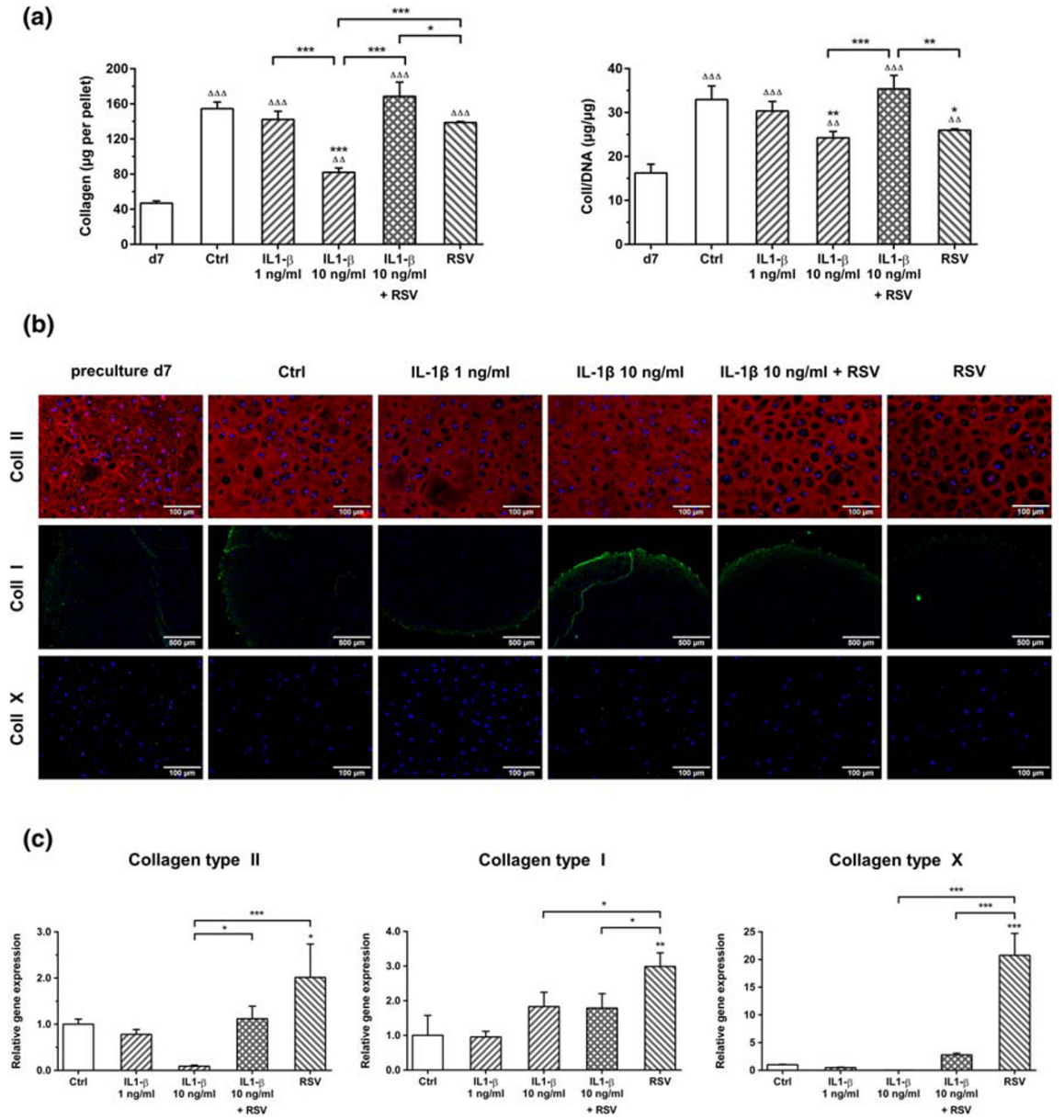

FIGURE 5 Collagen content after 21 days of culture, that is, 7 days of precultivation in chondrocyte medium for all groups, followed by 14 days with different treatments. Data after 21 days are shown for all groups and compared with values at Day 7. (a) Collagen production of chondrocytes, shown for absolute amounts of total collagen (collagen/pellet) and normalized to DNA (collagen/DNA). Data are presented as means ± standard deviation (three pellets per sample were pooled, with $n = 3$). (b) Immunohistochemical staining for collagen type II, type I, and type X. Scale bars represent 100 µm (upper, lower) and 500 µm (middle), respectively. (c) Gene expression of collagen type II, type I, and type X; qRT-PCR analysis at Day 21. Gene expression was normalized to beta-actin; the obtained values were further normalized to the values of the control group. Data are presented as means ± standard deviation (two pellets per sample were pooled, with $n = 3$). (a, c) Asterisks above single bars represent statistical significance against control group after 21 days, above brackets between displayed groups; $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$. Triangles above single bars represent statistical significance between the respective treatment group after 21 days and control values at Day 7; $^{\Delta\Delta\Delta}p < 0.001$, $^{\Delta\Delta}p < 0.01$ [Colour figure can be viewed at wileyonlinelibrary.com]

shown to counteract IL-1β-induced COX-2 activity and PGE_2 production in chondrocytes when cotreated for 24 h (Dave et al., 2008). Furthermore, RSV blocked IL-1β-induced nuclear translocation of NF-κB as well as NF-κB-regulated gene products involved in inflammation (COX-2, MMP-3, MMP-9, and VEGF) in human articular chondrocytes in experiments for up to 48 h (Csaki et al., 2009; Shakibaei et al., 2008). In the present study, we investigated if RSV impacted the general appearance and ECM content of 3D cartilaginous constructs under inflammatory conditions (IL-1β, 10 ng/ml) in a 14-day long-term culture setup. RSV was applied at 50 µM, a dose that has previously been shown to effectively stimulate expression of collagen type II and reduce IL-1β-mediated apoptosis in articular chondrocytes (Csaki et al., 2009; Liu et al., 2010; Maepa, Razwinani, & Motaung, 2016). Treatment with RSV under inflammatory conditions

indeed inhibited the drastic catabolic effects observed when pellets were cultured with IL-1β alone. RSV counteracted the strong reduction in construct diameter and DNA amount, and the marked upregulation of MMP13 expression elicited by IL-1β was inhibited by RSV. Cotreatment with RSV strongly increased absolute GAG content and GAG/DNA, as compared with IL-1β alone. Furthermore, total collagen content was increased as well, whereas immunohistochemical staining for undesired collagen type I was distinctly enhanced by IL-1β and still detected upon RSV cotreatment. The overall strong inhibition of IL-1β-induced catabolic effects by RSV was well in agreement with protein expression studies for up to 48 h showing that RSV inhibited IL-1β-induced down-regulation of aggrecan and collagen type II in monolayer culture (Liu et al., 2010; Shakibaei et al., 2007) as well as chitosan–gelatin scaffolds (Lei et al., 2008). Moreover, RSV has previously been shown to partly counteract mRNA expression of collagen type I as well as MMP1, MMP3, and MMP13 in chondrocytes under LPS-induced inflammatory conditions in a hyaluronic acid/RSV hydrogel (Sheu et al., 2013). Furthermore, it was recently reported that RSV incorporated in a nonseeded PLA-gelatin porous nanoscaffold promoted repair in a posttraumatic cartilage defect rat model, with expression levels being decreased for MMP13 and increased for collagen type II (Yu et al., 2018).

In a further approach within the present study, constructs were precultured for 7 days in chondrocyte medium before subjecting them to the IL-1β-mediated inflammatory environment for further 14 days. In general, results from this culture setup confirmed RSV as a potent treatment inhibiting the detrimental effects of the inflammatory cytokine. IL-1β led to distinctly reduced absolute GAG content and GAG/DNA, as compared with constructs after only 7 days of preculture, indicating ECM degradation. RSV counteracted degradation under inflammatory conditions and led to a strong rescue of GAG content, which was, interestingly, even more pronounced (up to control levels) in pellets receiving preculture compared with those without maturation. These findings additionally underlined the importance of specifically evaluating the point in time when exposing chondrocyte constructs to pathophysiological conditions. Future studies may further investigate constructs at different stages of development exposed to an inflammatory environment in combination with a potential adjuvant treatment such as RSV.

As a further control group in both experimental setups, that is, without and with preculture, RSV was investigated under noninflammatory conditions. RSV increased absolute GAG content and GAG/DNA, which was in accordance with results reported by Im et al. (2012) that RSV alone significantly increased GAG accumulation per cell in a long-term in vitro culture. Moreover, in the setup with preculture, collagen type II gene expression was upregulated by RSV, which also confirmed previous results obtained by Maepa et al. (2016) in porcine articular chondrocytes cultured in monolayers. In general, it has to be taken into account that in the present study, gene expression analyses were only conducted at Day 14 (without preculture) and Day 21 (with preculture) and may not reflect expression levels during the overall culture period, whereas protein expression determined by immunohistochemistry represents accumulation over time. In accordance with results found by H. J. Kim, Braun, and Dragoo (2014), we observed that RSV under noninflammatory conditions induced gene expression and protein accumulation of collagen type X, a marker for hypertrophy, in articular chondrocyte constructs. As hypertrophic development is rather to be avoided, this observation may potentially be critical for the use of RSV in cell-based therapy. Conceivably, a lower RSV dose may be associated with prevention of such side effects. Interestingly, under the inflammatory culture setups used in the present study, RSV (at 50 μM) counteracted IL-1β-mediated effects (at 10 ng/ml) but did not lead to collagen type X accumulation, which was further supported by qRT-PCR analysis. Therefore, further investigations concerning effects of RSV in different ranges of inflammatory stimuli (including IL-1β at 1 ng/ml) appear desirable in terms of possible therapeutic applicability.

In conclusion, in the present study, RSV was demonstrated to exhibit distinct effects on the ECM composition of 3D cartilaginous constructs in long-term inflammatory culture in vitro. RSV counteracted IL-1β-induced ECM degradation and led to partial rescue of deposition of cartilaginous matrix, especially GAG, impaired by IL-1β. Nevertheless, in future studies, potential hypertrophic effects should be taken into account when considering RSV as a cotreatment for articular cartilage repair techniques in OA.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ORCID

Sebastian Frischholz https://orcid.org/0000-0002-5210-5029

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