Complement biology in health and disease

Lubbers, R.

CHAPTER 2

COMPLEMENT COMPONENT C1Q IS PRODUCED BY ISOLATED ARTICULAR CHONDROCYTES

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As published in Osteoarthritis Cartilage. 2019 Oct 18. pii: S1063-4584(19)31228-2.

ABSTRACT

Objective: Inflammation and innate immune responses may contribute to development and progression of Osteoarthritis (OA). Chondrocytes are the sole cell type of the articular cartilage and produce extracellular-matrix molecules. How inflammatory mediators reach chondrocytes is incompletely understood. Previous studies have shown that chondrocytes express mRNA encoding complement proteins such as C1q, suggesting local protein production, which has not been demonstrated conclusively. The aim of this study is to explore C1q production at the protein level by chondrocytes.

Design: We analysed protein expression of C1q in freshly isolated and cultured human articular chondrocytes using western blot, ELISA and flow cytometry. We examined changes in mRNA expression of collagen, MMP-1 and various complement genes upon stimulation with pro-inflammatory cytokines or C1q. mRNA expression of C1 genes was determined in articular mouse chondrocytes.

Results: Primary human articular chondrocytes express genes encoding C1q, *C1QA*, *C1QB*, *C1QC,* and secrete C1q to the extracellular medium. Stimulation of chondrocytes with pro-inflammatory cytokines upregulated *C1QA*, *C1QB*, *C1QC* mRNA expression, although this was not confirmed at the protein level. Extracellular C1q bound to the chondrocyte surface dose dependently. In a pilot study, binding of C1q to chondrocytes resulted in changes in the expression of collagens with a decrease in collagen type 2 and an increase in type 10. Mouse articular chondrocytes also expressed *C1QA*, *C1QB*, *C1QC*, *C1R* and *C1S* at the mRNA level.

Conclusions: C1q protein can be expressed and secreted by human articular chondrocytes and is able to bind to chondrocytes influencing the relative collagen expression.

INTRODUCTION

Chondrocytes are the only cells present in the healthy cartilage matrix. They are responsible for the synthesis and turnover of the articular extracellular cartilage matrix (ECM). Originating from mesenchymal stem cells, chondrocytes are located in matrix cavities called lacunae. The two major ECM macromolecules produced by chondrocytes are collagen type 2 and aggrecan [1]. Healthy cartilage mainly consist of these two components, but in the development of osteoarthritis (OA) the composition of the cartilage changes. In OA the chondrocytes can dedifferentiate and thereby reverse to a fibroblast phenotype, and the chondrocytes can terminally differentiate to a hypertrophic phenotype. Dedifferentiation is characterized by expression of Collagen type 1 and decreased expression of ACAN and Collagen type 2. Whereas hypertrophy is characterized by the expression of Collagen type 10, runt-related transcription factor 2 (RUNX2) and matrix metalloproteinase 13 (MMP13) [2]. In the early stages of OA it has been shown that the cartilage content changes from mainly collagen type 2 to collagen type 1 [3]. Furthermore, chondrocytes that have been isolated from patients with OA also produced collagen type 10[4].

The complement system is an important part of the innate immune defence, which is able to remove dying cells, immune complexes and kill pathogens. The complement system can be activated via three pathways; the classical pathway, the lectin pathway and the alternative pathway. C1q is the recognition molecule of the classical pathway and together with the proteases C1r and C1s it forms the C1 complex [5]. C1q can activate the classical pathway (CP) by binding different ligands such as IgG and IgM antibodies, but also DNA, C-reactive protein (CRP) and lipopolysaccharides [5-7]. Following activation of the CP, a C3 convertase is generated which cleaves C3 into two functional fragments, C3b which functions as an opsonin and C3a which is a chemoattractant promoting further involvement of the innate and/or adaptive immune system. The final step in the CP is the formation of the membrane attack complex (MAC) which creates a pore on the target membrane inducing lysis (**Figure 1A**). While most of the complement proteins are made by the liver, an essential set of complement proteins is made by cells of the immune system [8, 9]. C1q producing cells originate from haematopoietic stem cells, such as macrophages, immature dendritic cells and mast cells [7, 10-12]. However, other reports suggest that decidual endothelial cells, microglia and osteoclasts can also produce C1q [13-15].

Previous studies described that matrix molecules like aggrecan, fibromodulin and osteoadherin can bind C1q and thereby activate the classical pathway of the complement system [16-18]. The cartilage oligomeric matrix protein (COMP) is a strong ligand for C1q. It is hypothesised that COMP interferes with the binding site of C1r and C1s resulting in inhibition of the classical pathway [19]. This interference is also reported for the cartilage fragments decorin and biglycan [20]. Taken into account the ability of

C1q to interact with matrix molecules, it is relevant to determine whether C1q is locally produced in the articular cartilage and whether they can cause detrimental changes in chondrocytes.

Proteomic data from synovial fluid from osteoarthritic patients shows the presence of complement components [21, 22]. Wang et al. described that complement is important in the pathogenesis of OA [23], as the presence of the MAC, which is the terminal product of the complement cascade, could be detected in the cartilage. Although the authors speculated that complement activation takes place outside the cartilage and then attacks damaged chondrocytes, it cannot be excluded that chondrocytes initiate complement activation leading to MAC deposition. Similarly, in a bovine cartilage model it was shown that terminal pathway complement components were detected upon degradation [24]. Additionally, in the early 90's it was already described that articular chondrocytes express mRNA for C1q and that deposits of the C1q molecule were present in the cartilage. However at that time, the source and function of the C1q was not clear [25]. Articular cartilage is avascular and relies on diffusion of molecules, the structure is heterogenous and the possibility to proteins to diffuse into the cartilage decreases with increasing molecular size [26, 27]. C1q exists out three different polypeptide chains: C1qA (27,5 kilo Dalton (kDa)), C1qB (25,2 kDa) and C1qC (23,8 kDa). From each chain six copies are required, resulting in a total of 18 polypeptide chainswhich assemble together (460 kDa) before the full C1q molecule is secreted by the cell (**Figure 1B**). OA causes disruption in the cartilage structure and therefore may become more permeable. The size limit of proteins that can move freely in cartilage is estimated to be around 65 kDa [25]. Diffusion of IgG (150 kDa) has been demonstrated but exhibited unexpectedly slow diffusion through the superficial region [27], C1q is an even larger protein with a total molecular mass of 460 kDa, which makes it unlikely that it can readily diffuse into cartilage.

In this study, we have investigated whether primary human articular chondrocytes are able to express and secrete C1q proteins. We have also determined if the expression of C1q is modulated under inflammatory conditions, frequently present in OA.

MATERIALS AND METHODS

Patient

Cartilage was obtained as left-over tissue from OA patients undergoing total knee-replacement surgery in the Alrijne hospital, Leiden. The study was approved by the local medical ethical committee.

Isolation of chondrocytes

Chondrocytes were isolated from cartilage obtained from total knee-replacement surgery. Cartilage pieces were incubated in 20 ml PBS with pronase (2 mg/ml, Roche)

for 90 minutes, followed by overnight incubation with collagenase type 2 (225 U/ml, Worthington) in 20 ml F12 DMEM culture medium (Gibco) supplemented with 1% penicillin and streptomycin. After digestion of the tissue, the chondrocytes were passed over a cell strainer (mesh width 70 µM) and were pelleted. The chondrocytes were cultured, without extra passages, for further stimulation purposes or directly lysed for Western blot analysis and RNA isolation. The purity of the chondrocytes upon isolation was determined by Flow Cytometry using CD14 (BD Biosciences cat#555399) and CD45 (BD Biosciences cat#555482) expression to check for contamination of hematopoietic cells.

Stimulation of chondrocytes

Primary chondrocytes were plated in a cell density of $0.5*10^5/cm^2$ in F12 DMEM culture medium (Gibco) supplemented with 10% BSA (Bovine Serum Albumin), L-glutamine, penicillin and streptomycin. Chondrocytes were stimulated for 24 hours with IL-1β (30 ng/ml, R&D systems), TNF-α (100 ng/ml, R&D systems), TGF-β (10 ng/ml, peprotech), LPS (100 ng/ml, Sigma) or C1q (1 / 10 / 100 µg/ml. Quidel) and RNA was isolated as described below. For western blot analysis primary chondrocytes were lysed and C1qA, C1qB, C1qC was determined as described previously [28]. For ELISA, lysates and culture supernatants of chondrocytes (unstimulated or stimulated with 30 ng/ml IL-1β) were harvested after 72h hours and C1q levels were determined as described before [28]. The supernatants were concentrated according to manufacturer's protocol (Amicon Ultra-0.5 Centrifugal Filter, Merck) before analysis by ELISA was performed.

ELISA

C1q levels were measured using an in-house developed ELISA. Maxisorp plates (nunc) were coated overnight with mouse anti-human C1q (2204), (Nephrology department, LUMC) in coating buffer (0.1M Na_2CO_3, 0.1M $NaHCO_3$, pH9.6). Plates were washed and blocked with PBS/1%BSA for one hour at 37°C. After washing, a serial dilution of a pool of normal human serum (NHS) was applied as a standard and samples were added in dilution buffer and incubated for one hour at 37°C. After washing, plates were incubated with rabbit anti-human C1q (Dako cat#A0136) for one hour at 37°C and for detection a goat anti-rabbit HRP (Dako cat#P0448) was used which was also incubated for one hour at 37°C. All washing steps were performed with PBS/1%BSA/0.05%Tween. The substrate was added to the plates using 2,2'-azino-bis-3-ethyl benzthiazoline-6-sulphonic acid (ABTS) and the signal was measured at an absorbance level of 415 nm using Biorad iMark Microplate Absorbance Reader.

Western Blot

Using western blot the composition of C1q was examined by detection of the three chains of the C1q protein. *Ex vivo* chondrocytes were lysed using NP-40 lysis buffer (Invitrogen, cat#FNN0021) in combination with a protease inhibitor cocktail (Sigma cat#P2714) according to manufacturer's protocol, next these lysates were applied in

reduced conditions. Proteins were separated by SDS/PAGE using Tris-glycine gels (Biorad cat#456-1033) under reducing conditions which were loaded with equal amounts lysed chondrocytes. Next proteins were transferred on a Trans-Blot Turbo Transfer pack: mini, 0.2 µM PVDF (Bio-Rad). The membrane was blocked one hour in PBS containing 0.05% Tween and 3% skimmed milk on room temperature. The rabbit anti-human C1q (Dako) was preincubated in C1q depleted serum (Quidel) for one hour. Next, the blot was incubated with the preincubated rabbit anti-human C1q overnight on 4°C. After washing with PBS/0.1% Tween the membrane was incubated with goat anti-rabbit HRP (Dako) for one hour at room temperature. Finally, the blot was washed and C1q was visualized using ECL Western Blotting Analysis system (GE Healthcare).

Intracellular flowcytometry staining C1q

Isolated chondrocytes were fixed and permeabilized by using the Cytofix/Cytoperm™ Fixation/Permeabilization Solution Kit (BD). Next, the chondrocytes were incubated with rabbit anti-human C1q-FITC (Dako) or with an isotype control rabbit Ig-FITC (Dako) for 20 minutes on ice in the dark. After incubation the chondrocytes were washed and measured on LSRII. Analyses were performed using FlowJo version 10.

Surface binding C1q chondrocytes

Chondrocytes were incubated for 45 minutes at RT with or without purified C1q in different concentrations (1 / 10 / 100 µg/ml). C1q incubation of chondrocytes was performed on a plate shaker at 600 rpm to prevent pelleting of the cells. After incubation the cells were washed and binding of C1q to the surface of chondrocytes was detected using rabbit anti-human C1q-FITC (Dako). Rabbit Ig-FITC (Dako) was used as isotype control. Samples were measured on LSRII and analyses was performed using FlowJo version 10.

qPCR Human chondrocytes

RNA was isolated from cultured chondrocytes using either RNeasy mini kit (Qiagen) or mirVana™ miRNA Isolation Kit (Invitrogen) and concentration was determined with the NanoDrop (NanoDrop Technologies). Subsequently, the RNA was treated with DNase I, Amplification Grade (Invitrogen) and cDNA was synthesized using superscript III (200U/µl, Invitrogen). The cDNA was diluted and qPCR was performed using SensiFast Sybr no-ROX (Bioline). Primers specific for *MMP1*, collagen type 1, type 2, type 10, *C1R*, *C1S*, *C3*, *C5* and the C1q genes *C1QA*/*B*/*C*, as well as the reference *RPL5* were used (see **Table 1** for primer sequences). The qPCR was performed on the real time PCR system (BioRad CFX-384) with an activation step of 3 min on 95°C (hot start polymerase activation), a melting temperature of 95°C for 5 seconds and at an optimized annealing temperature per primer for 5 seconds followed by an elongation step for 20 seconds on 72°C for 40 cycles. At the end of the protocol melting curves were performed from 65°C to 95°C to test specific binding of SensiFast Sybr.

Table 1. Human primers

Gene	5' Forward	3' Reverse
MMP1	CTGGCCACAACTGCCAAATG	ATTCTGTCCCTGAACAGCCC
Collagen type 1	CATGTTCAGCTTTGTGGACCTC	ATGGTACCTGAGGCCGTTCT
Collagen type 2	TCCTAAAGGAGCCCGAGGTGCC	CAGGAGCTTGGAGGCCGGGTT
Collagen type 10	CCCAGCACGCAGAATCCATCT	TTCCAGCCGGTCCAGGGATT
C1QA	CCAGGAAGAACCGTACCAGA	GACGATGGACAGGCAGATTT
C1QB	TCCAGATATGGAGGCCTGAC	TTCACTCAGCAGCATTCACC
C1QC	AAGGARGGGTACGACGGACT	GTAAGCCGGGTTCTCCCTTC
C1R	TCACAGTCCCCACGGGATAC	CCAGTGGAGAACCCAGTTGC
C1S	CTGCAGAGGGAGCGTCAA	TGGTAGGCTCAGCATAAACCC
C3	TGGCCAATGGTGTTGACAGA	GCGTAGACCTTGACTGCTCC
C5	TACCTTGCCTGTTGAAGCCC	CCAGGGAAAGAGCATACGCA
RPL5	TGGAGGTGACTGGTGATG	GCTTCCGATGTACTTCTGC

Immunofluorescence staining of C1q in chondrocytes

Freshly isolated chondrocytes were cultured on poly-d-lysine (Sigma 50 µg/ml) chamber slides (Thermo Scientific) and fixated and permeabilised using 1% paraformaldehyde and acetone. Cells were subsequently incubated with rabbit anti-C1q (Dako) or isotype control rabbit Ig (Dako) for one hour at RT. After washing, slides were incubated with goat anti-rabbit ALEXA 488 (Invitrogen) and ActinRed 594 (Applied bioprobes) for one hour at RT. Finally, the slides were covered with Vectashield containing DAPI (Vector laboratories) to stain nuclei and analysed on a confocal microscope (Leica SP8 confocal).

qPCR Mouse chondrocytes

Articular cartilage from tibia plateaus, femoral condyles were harvested from 5-6 days new born mice. The isolation protocol was adapted from literature [29, 30], in short, chondrocytes were seeded in 12-wells plates (200.000 cells/well) after matrix digestion with "liberase, Roche" (two digestions of 45 minutes with 0.1 mg/ml liberase and a third digestion with 0.025 mg/ml overnight). DMEM (Gibco Life Technomogies, France) supplemented with 10 % fetal bovine serum (FBS), 2% L-glutamine and 1% of antibiotic was used for this culture [30]. Isolated articular chondrocytes from several pups from the same litter were pooled, sample #1 is one pooled litter and sample #2 is a pool from another different litter. RNA was isolated using and cDNA synthesis was performed as previously described [31]. Next, real-time qPCR was performed with primers specific for mice *C1QA*/*B*/*C*, *C1R* and *C1S* (see **Table 2** for primer sequences). qPCR was performed on the real time PCR system (BioRad CFX-384) with an activation step of 3 min on 95°C (hot start polymerase activation), a melting temperature of 95°C for 5 seconds and at an optimized annealing temperature per primer for 10 seconds followed by an elongation

step for 15 seconds on 72°C for 40 cycles. At the end of the protocol melting curves were performed from 65°C to 95°C to test specific binding of SensiFast Sybr. After qPCR the products were analysed on agarose gel with Nancy-520 fluorescent DNA binding dye.

Table 2. Primers mouse

Gene	5' Forward	3' Reverse
C1QA	CCATCAGCAAAGGGCTGGAG	TACTAGGGTCATGGTCAGCA
C1QB	GACTTCCGCTTTCTGAGGAC	TGTGTCTTCATCAGCTCAGC
C1QC	GAC GTC TCT GTG ATT AGGCC	AGGGCCAGAAGAAACAGCAG
C1R	GAGGAGAATGGGACATCAT	GACACAGATGTTGGCATCGG
C1S	GTTTGGTCCTTACTGTGGTA	CCAAGGGTTCTTTTGCCCC

Statistics

Statistical analyses were performed using Graphpad Prism version 7. To compare relative expression levels or ratio the Wilcoxon test or Kruskall-Wallis test with Dunn's multiple comparisons test were used. To analyse the effect of increasing concentrations of C1q, compared to unstimulated, on relative expression the Friedman test with Dunn's multiple comparisons test was used, p-value's <0.05 were considered significant. In all graphs the mean with SEM is shown, or a representative figure.

RESULTS

Chondrocytes produce and secrete C1q protein

We isolated primary human chondrocytes from articular cartilage obtained during surgical procedures. First, we validated the purity of the isolated chondrocyte population, using flow cytometry. As shown in Figure 1A, chondrocytes do not express the hematopoietic marker CD45 and the myeloid marker CD14 (**Figure 1C**). Additionally, we used flow cytometry to analyse the presence of intracellular C1q after isolation. Indeed, we observed with intracellular staining that C1q is present in chondrocytes directly *ex vivo* (**Figure 1D**). To confirm these results, we subsequently let the chondrocytes adhere for at least one hour at 37°C on chamber slides followed by the analysis of the presence of C1q using confocal imaging. Also by microscopy we detected intracellular expression of C1q in the chondrocytes (**Figure 1E**). Finally, freshly isolated chondrocytes were lysed and protein extracts were prepared for western blot analysis to determine C1q expression. Also the lysates of the chondrocytes stained positive for C1q (**Figure 1F**). Collectively, these data indicate that chondrocytes express C1q protein.

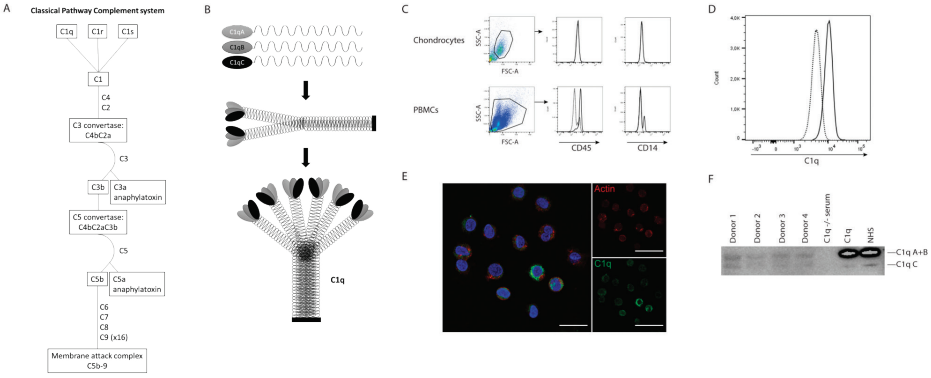

Figure 1. Chondrocytes express C1q protein

(A) Schematic presentation of the classical pathway of the complement system. (**B**) Schematic representation of the assembly of the C1q molecule. C1q is assembled from three different chains, A, B and C. (**C**) Flow cytometry of chondrocytes isolated from human cartilage compared to Peripheral blood mononuclear cells (PBMCs), dashed lines correspond with the isotype staining and solid lines to the antibody. (**E**) Flow cytometry of intracellular C1q in chondrocytes after isolation, the dashed line correspond with the isotype staining and the solid line to the antibody. (**E**) Cultured chondrocytes on chamber slides. C1q is visualized with ALEXA488. Actin is used as counterstaining at 594 nm. The nucleus is stained with DAPI, 630x magnification, the scale bars indicates 6.3 µm. (**F**) Western blot analysis of lysates of isolated chondrocytes from 4 different donors, purified C1q and normal human serum (NHS) serve as positive control whereas C1q-/- serum served as a negative control.

C1q mRNA expression is increased in the presence of OA relevant stimuli

Next, we aimed to determine whether C1q expression is modulated in an inflammatory milieu. To this end, mRNA expression of the C1q chains, *C1QA*, *C1QB* and *C1QC*, was determined in cells that were cultured with or without inflammatory stimuli that are thought to be relevant for OA, namely IL-1β, TNFα, and LPS and growth factor TGF-β [32]. Stimulation with these mediators resulted in differences in expression of *C1QA*, *C1QB* and *C1QC*, as analysed with Kruskall-Wallis (respectively: p=0.0282, p=0.0283 and p<0.0001). After adjusting for multiple comparisons the only remaining significant difference is IL-1β versus LPS in *C1QC* expression, probably due to low number of donors (n=3) (**Figure 2A-C**). Subsequently, we also wished to determine whether C1q-protein expression is also elevated by inflammatory stimuli. To this end, we stimulated chondrocytes with IL-1β, since this cytokine displayed the strongest effect on mRNA expression, albeit not statistically significant. After 72 hours, both the cell lysates and the culture supernatants were analysed. Although we detected C1q protein expression in both lysates and concentrated supernatant, we did not observe a difference between control and IL-1β stimulated chondrocytes (**Figure 2D**, **E**). Moreover, we noticed high variability in C1q production among donors.

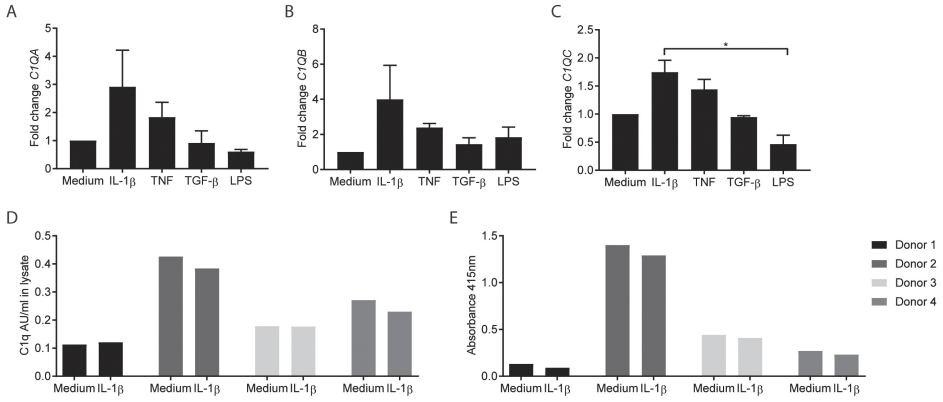

Figure 2. C1q after stimulation with pro-inflammatory cytokines

(A-C) Fold change expression after correction with *RPL5* of *C1QA*, *C1QB* and *C1QC*. Data represents mean and SEM of 3 different OA donors measured in duplicate after 24 hours stimulation (N=3). Differences in expression were analysed with Kruskall-Wallis, after adjusting for multiple comparison the individual significant adjusted p-values were plotted when p<0.05 (*). (**D**) Chondrocytes were stimulated with IL-1β for 72 hours, after stimulation the supernatant was collected and concentrated, additionally, the chondrocytes were lysed. Next, C1q was measured by ELISA. C1q measured in chondrocyte lysates by ELISA expressed as AU/mL in 4 donors. (**E**) Supernatant was collected and concentrated and C1q was measured and is expressed as the absorbance measured at 415nm. To compare relative expression levels Kruskall-Wallis test with Dunn's multiple comparisons test was used for statistical analysis and p-value's <0.05 (*) were considered significant.

Complement and matrix component expression in pro-inflammatory environment

Next, we sought to study the expression pattern of different collagens produced by chondrocytes upon stimulation with pro-inflammatory cytokines. TGF-β is known to increase the expression of Collagen type 1 in chondrocytes [33], although in our experiment the increase was only significant compared to IL-1β stimulation condition (**Figure 3A**). TGF-β also caused a slight non-significant upregulation in the expression of collagen type 2 and type 10 (**Figure 3B, C**). No effect was observed upon TNFα or LPS stimulation. IL-1β caused a trend towards decreased expression in all collagen types, type 1 (95% CI 0.33 – 0.52) type 2 (95%CI -0.03 – 0.20) and type 10 (95%CI -0.07 – 0.53). Also *MMP1* expression was determined for the different stimulations, we see differences between the stimulations, but these are not significant (**Figure 3D**). Furthermore, we investigated whether the expression of various complement components could be modulated by the inflammatory cytokines. *C1R* and *C1S* expression was analysed since these molecules in combination with C1q make one complete C1 complex, which activates the classical pathway of the complement system. C3 and C5 are further downstream in the complement system and important in the cascade for their ability to be cleaved into pro-inflammatory molecules. Stimulation with IL-1β, TNF and LPS seems to be accompanied by changes in the expression of *C1R*, *C1S* and *C3* (**Figure 3E**, **F** and **G** respectively), however, probably due to inter-donor variability, these changes

are not significant. *C5* expression seems to be unaffected by all different stimulations after 24 hours of stimulation (**Figure 3H**). Overall, the pro-inflammatory stimulation resulted not only in an upregulated expression of the C1q genes also in slightly enhanced expression of C1r and C1s, allowing the formation of the C1-complex.

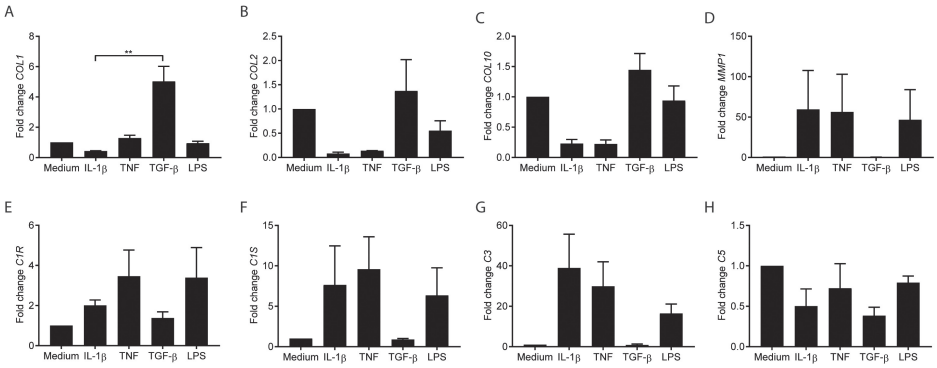

Figure 3. mRNA expression of complement and matrix genes after 24 hours stimulation (N=3). Fold change expression after correction with *RPL5* of (**A**) *COLA1*, (**B**) *COL2A1*, (**C**) *COL10*, (**D**) *MMP1*, (**E**) *C1R*, (**F**) *C1S*, (**G**) *C3* and (**H**) *C5*. Data represents mean and SEM of 3 different OA donors measured after 24 hours stimulation in duplicate. To compare relative expression levels Kruskall-Wallis test with Dunn's multiple comparisons test was used and and p-value's <0.05 were considered significant (p <0.01 **).

C1q can bind to chondrocytes

After showing that chondrocytes can produce C1q, we wished to assess the functional relevance of such local C1q production. First, we tested whether C1q protein was able to bind to chondrocytes. Chondrocytes were incubated with different concentrations of purified human C1q protein and surface binding was analysed by flow cytometry. We observed a dose-dependent binding of C1q to chondrocytes (**Figure 4A**). Next, we investigated whether C1q has a regulatory effect in chondrocytes on the mRNA expression of a set of complement genes (*C1QA/B/C*, *C1R*, *C1S*, *C3* and *C5*), different types of collagen (type 1, 2 and 10), *MMP-1*, *MMP13*, and on chondrocyte specific genes *SOX9* and *ACAN*. Chondrocytes were incubated for 24 hours with the same concentrations of C1q as used for flow cytometry, after which RNA was isolated and qPCR performed. Treatment with 100 µg/ml C1q induced significant decrease in *MMP13* expression (95% CI: 0.11 – 0.60), and a non-significant decrease in *COLL2* (95% CI 0.45 – 0.92) and *MMP1* (95% CI 0.30 – 0.60). In addition, we observed a non-significant increase in *C1QB* (95% CI: 1.012 – 2.12). *SOX9* and *ACAN*, two chondrocytes specific genes, were unaffected by the stimulation with C1q (**Figure 4B**). With lower concentrations of C1q no differences were observed (data not shown). It has been previously demonstrated that OA cartilage has a more dedifferentiate phenotype [34]. Therefore we analysed the ratio between expression of the genes encoding Collagen type 2 and 10, as an increased Collagen type 10 over Collagen type 2 expression which could be indicative

of hypertrophic chondrocyte differentiation. Stimulation of chondrocytes with 100 µg/ml C1q led to a significant increase in Collagen type 10 over Collagen type 2 expression. Overall, the data provide preliminary evidence that stimulation of chondrocytes by C1q affects expression of various genes (decreased *MMP13* and ratio change *COLL2*:*COLL10*) by the chondrocytes and could therefore affect the composition of the cartilage.

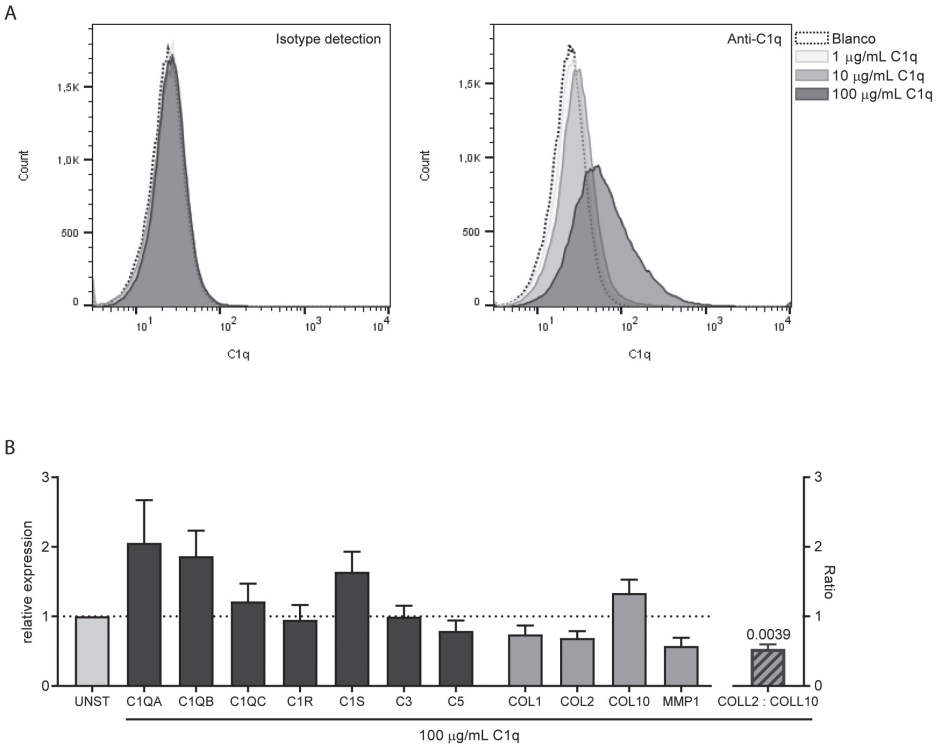

Figure 4. C1q effects on chondrocytes

(A) Representative figure for detection of C1q by flow cytometry on the surface of chondrocytes after incubation with an increasing concentration of C1q (n=3). (**B**) Chondrocytes were incubated in the presence of an increasing concentration of C1q (1 / 10 /100 µg/ml C1q) for 24 hours, after stimulation RNA was isolated and analysed for expression of both complement, matrix and chondrocyte specific genes. Stimulation and isolation was performed on biological triplicate and qPCR was performed with technical duplicate, mean with SEM are shown (n=9). Only the data from 100 µg/ml C1q stimulation is shown, on the right hand side the ratio of COL2:COL10 is presented. The dashed line at y=1 represents the unstimulated (UNST) level expression. The relative expression was analysed with Kruskall-Wallis test with Dunn's test for correction of multiple testing, the ratio was analysed with Wilcoxon-rank test. P-value's <0.05 were considered significant.

Mouse chondrocytes express all genes required to form the C1 complex
Finally, we aimed to demonstrate the presence of C1q transcripts in mouse articular chondrocytes. To this end, mRNA was isolated from articular mouse chondrocytes, from two different litters, and qPCR was performed for mouse *C1QA*, *C1QB*, *C1QC*, *C1S* and *C1R*. For both samples we observed expression and PCR product for all genes analysed. The PCR products were further analysed on agarose confirming the correct size of the bands and single bands. (**Figure 5**). These data indicate that, like human articular chondrocytes, mouse articular chondrocytes similarly express transcripts for the genes encoding the C1 complex, allowing further studies into the functional impact of C1 expression in experimental murine models.

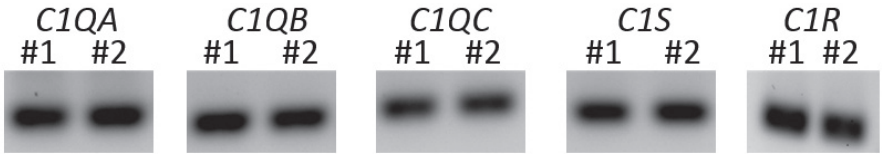

Figure 5. C1 expression in mouse articular chondrocytes
Articular chondrocytes from mouse pups that were 6 days old were collected from two different litters. Both samples are a pool of isolated articular chondrocytes from several pups from the same litter, sample #1 is one pooled litter and sample #2 is a pool from another litter. Agarose gel of PCR products from isolated mRNA analysed for *C1QA*, *C1QB*, *C1QC*, *C1S* and *C1R* expression.

DISCUSSION

The main cell types described to produce C1q are derived from haematopoietic stem cells [8]. Chondrocytes are derived from mesenchymal stem cells and are important in the production of extracellular matrix molecules to build up the cartilage. Wang et al. described that the dysregulation of complement system in the synovial joints has a central role in the pathogenesis of OA [23]. Also, in a transcriptomic approach complement genes were differentially expressed in OA knee cartilage [35]. Although in the early 90's, various complement components have been detected in cartilage including C1q mRNA, the presence of C1q at the protein level could at that time not be confirmed *in vitro*. Likewise, the possible function of C1q in cartilage was not addressed [25]. Here, we demonstrate that isolated chondrocytes indeed produce C1q protein.

To evaluate which factors can modulate the production of C1q by chondrocytes, we stimulated the chondrocytes with cytokines known to be present in the joint during inflammation [32]. The expression patterns of C1q genes were different compared to the expression of the collagen genes after TGF-β stimulation. As expected, the expression of the different types of collagen showed an increase of expression after stimulation of TGF-β [36]. In contrast, the expression of the C1q genes was mostly unaffected. Stimulation with IL-1β and TNF-α led to an increase in mRNA expression for the C1q

genes compared to unstimulated chondrocytes. However, an increase in C1q secretion at protein level was not observed in our experimental conditions, which could be due to a later translation. The production of C1q was very variable among donors, which may be related to the inflammatory state of the cartilage sample, or other unknown factors. Unfortunately, due to limitations in the samples; low numbers and the samples being obtained from left-over tissue, there is not enough information regarding patient demographics, to draw conclusions regarding C1q production and disease state.

C1q released by the chondrocytes could deposit on the cartilage or reach the synovium which is damaged by inflammation. However, C1q is a large 460 kDa protein and the size limit of proteins that can move freely in cartilage is estimated to be around 65 kDa [25]. Therefore, it is likely that C1q produced by the chondrocytes plays a local rather than systemic function. Conceivably, C1q would function in an autocrine setting in stimulating the chondrocytes, or alternatively C1q would be involved in local complement activation and / or cartilage biology, involving C1r and C1s directly outside the chondrocyte. We speculate that C1q and complement activation may be involved in the maintenance of a lacuna for the chondrocyte. In such a scenario the released C1q, possibly by the action of the enzymes C1r and C1s, may trigger the degradation of matrix molecules that are (too) close to the cell body of the chondrocytes.

Previous studies have demonstrated that the serine protease C1s can degrade collagen type 1 and type 2 when it is activated [37] and that it can play a role in the degenerative cartilage matrix in rheumatoid arthritis [38]. We also observed an increase in RNA expression of C1s and C1r after stimulation. In *ex vivo* chondrocytes we were also able to detect C1s in the lysates by Western blot (data not shown). This could indicate that under pro-inflammatory conditions, complement activation can occur both in the cartilage, as well as on the synovial surface of the cartilage. Together with the production of C1q and C3, it is conceivable that the production of different complement components by chondrocytes has a local function, contributing to cartilage degradation.

Next to a direct effect of C1q on complement activity via C1s on cartilage remodelling, C1q could also impact chondrocyte biology by binding C1q receptors. Several molecules have been coined to be C1q receptors [39], but which of these receptors would be relevant in the context of chondrocytes is unknown. The isolated chondrocytes represented a pure cell population as evidenced by FACS and as evidenced by cell culture. On the coverslips the cell population appears homogeneous. The cells may appear somewhat small as compared to their cultured and passaged counterparts, but this is likely the consequence of the fact that these are freshly isolated and non-passaged cells. We have shown that C1q can bind to the surface of chondrocytes but we currently have no insight into which receptors or ligands C1q is binding to. In a first study with a limited number of patients, we analysed the effect of C1q on the gene expression profile of chondrocytes. After incubation with 100 µg/ml C1q, a significant

change in the ratio between collagen type 2 and 10 expression levels was shown, in favour of more collagen type 10, suggesting that exposure to C1q (in high concentration) can contribute to a switch in the relative proportion of collagen expression, and tissue degrading enzyme(s). The concentration of C1q used to stimulate the chondrocytes (100 µg/ml) is roughly the same as the serum concentration of C1q, however if such a concentration is achievable locally in the cartilage is unclear. These data provide evidence that C1q can have functional effects on articular chondrocytes and provide the basis for future, more in-depth studies with larger cohorts of patients.

In our study we only used cartilage and chondrocytes derived from patients who underwent a total knee replacement due to OA, indicating that the cartilage could already be in inflammatory conditions. Therefore, for further investigations towards the role of complement in the cartilage it would be interesting to compare these results with healthy donors. The observation that also mouse primary chondrocytes express all genes required to make the C1 complex (*C1QA*, *C1QB*, *C1QC*, *C1R* and *C1S*) indicates that also experimental murine models can be employed to study the *in vivo* role of C1q in cartilage biology and pathology.

In summary, we show that isolated human articular chondrocytes express and secrete C1q protein, C1q is able to bind to chondrocytes and provide first evidence that C1q could induce changes in extracellular matrix molecule expression. Collectively, these data indicate a potential role in locally produced C1q in OA pathogenesis.

CONTRIBUTIONS

RL, RAvS, AIF and LAT designed the study and interpreted the data. RL, RAvS, JCK, NEWL, AMB, SM and CC collected and assembled the data. RJL, RM and AIF provision of study material or patients. RET, AIF and LAT obtained funding. All authors critically revised and approved the manuscript.

FUNDING

The authors wish to acknowledge the support of the IMI JU funded project BeTheCure, contract no 115142-2). L.T. was financially supported by a VIDI-grant from NWO-Zon-MW. R.T. was financially supported by a VICI-grant from NWO-Zon-MW. A. I-F. was financially contributed by The Dutch Arthritis Foundation (LLP-24).

CONFLICT OF INTEREST:

The authors report no conflict of interest.

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