



Article

# Chondrocytes from Osteoarthritis Patients Adopt Distinct Phenotypes in Response to Central T<sub>H</sub>1/T<sub>H</sub>2/T<sub>H</sub>17 Cytokines

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**Abstract:** Chronic low-grade inflammation plays a central role in the pathogenesis of osteoarthritis (OA), and several pro- and anti-inflammatory cytokines have been implicated to mediate and regulate this process. Out of these cytokines, particularly IFN $\gamma$ , IL-1 $\beta$ , IL-4 and IL-17 are associated with different phenotypes of T helper (T<sub>H</sub>) cells and macrophages, both examples of cells known for great phenotypic and functional heterogeneity. Chondrocytes also display various phenotypic changes during the course of arthritis. We set out to study the hypothesis of whether chondrocytes might adopt polarized phenotypes analogous to T<sub>H</sub> cells and macrophages. We studied the effects of IFN $\gamma$ , IL-1 $\beta$ , IL-4 and IL-17 on gene expression in OA chondrocytes with RNA-Seq. Chondrocytes were harvested from the cartilage of OA patients undergoing knee replacement surgery and then cultured with or without the cytokines for 24 h. Total RNA was isolated and sequenced, and GO (Gene Ontology) functional analysis was performed. We also separately investigated genes linked to OA in recent genome wide expression analysis (GWEA) studies. The expression of more than 2800 genes was significantly altered in chondrocytes treated with IL-1 $\beta$  [in the C(IL-1 $\beta$ ) phenotype] with a fold change (FC) > 2.5 in either direction. These included a large number of genes associated with inflammation, cartilage degradation and attenuation of metabolic signaling. The profile of genes differentially affected by IFN $\gamma$  (the C(IFN $\gamma$ ) phenotype) was relatively distinct from that of the C(IL-1 $\beta$ ) phenotype and included several genes associated with antigen processing and presentation. The IL-17-induced C(IL-17) phenotype was characterized by the induction of a more limited set of proinflammatory factors compared to C(IL-1 $\beta$ ) cells. The C(IL-4) phenotype induced by IL-4 displayed a differential expression of a rather small set of genes compared with control, primarily those associated with TGF $\beta$  signaling and the regulation of inflammation. In conclusion, our results show that OA chondrocytes can adopt diverse phenotypes partly analogously to T<sub>H</sub> cells and macrophages. This phenotypic plasticity may play a role in the pathogenesis of arthritis and open new therapeutic avenues for the development of disease-modifying treatments for (osteo)arthritis.



**Citation:** Pemmari, A.; Leppänen, T.; Hämäläinen, M.; Moilanen, T.; Moilanen, E. Chondrocytes from Osteoarthritis Patients Adopt Distinct Phenotypes in Response to Central T<sub>H</sub>1/T<sub>H</sub>2/T<sub>H</sub>17 Cytokines. *Int. J. Mol. Sci.* **2021**, *22*, 9463. <https://doi.org/10.3390/ijms22179463>

Academic Editor: Nicola Veronese

Received: 30 June 2021

Accepted: 6 August 2021

Published: 31 August 2021

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**Keywords:** chondrocyte; IL-1 $\beta$ ; IFN $\gamma$ ; IL-17; IL-4; RNA-Seq



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## 1. Introduction

Osteoarthritis (OA) is the most common form of arthritis. It has been estimated to affect up to a half of the elderly population, and therefore causes widespread disability and human suffering as well as an immense burden to healthcare systems [1]. Once thought as a mostly mechanical “wear and tear” disease, the chronic inflammatory component of osteoarthritis has been increasingly recognized during recent decades [2]. Constant low-grade inflammation in the joint contributes to pain, oxidative stress, increased catabolism, and the eventual breakdown of articular cartilage [3,4]. Despite intense research, no disease-modifying pharmacological treatments are currently available for OA [5], demonstrating that our understanding of the pathogenesis of the disease remains limited.

When comparing chondrocytes from OA patients with healthy cells, several changes in gene expression can be observed [6,7]. The potential causal roles of these changes in the pathogenesis of OA are currently largely unknown. However, some of them can be considered harmful (such as secretion of catabolic enzymes and proinflammatory cytokines) and others protective (e.g., the production of extracellular matrix [ECM] components) [8,9]. The changes in OA chondrocyte phenotype are thought to be caused by several physical and chemical factors, among them local proinflammatory cytokines [10].

The T helper ( $T_H$ ) cell is probably the most well-known example of a cell capable of adopting distinct phenotypes in response to environmental factors. The different  $T_H$  phenotypes, in turn, are associated with different cytokines. The  $T_H1$  phenotype drives inflammation and defense against intracellular pathogens. These cells are induced by interleukin 12 (IL-12) and produce mainly interferon gamma ( $IFN\gamma$ ) as an effector cytokine [11]. In addition, they induce macrophages to produce IL-1 $\beta$ , which in turn promotes the proinflammatory effects of  $T_H1$  cells [12].  $T_H2$  cells are induced by interleukins 2 and 4. They secrete various factors that promote humoral immunity and regulate inflammation, of which IL-4 is regarded as the central cytokine [11].  $T_H17$  cells are most closely associated with autoimmunity; they are induced by transforming growth factor beta (TGF $\beta$ ) along with several proinflammatory cytokines, such as interleukins 6, 21 and 23, and they produce IL-17 as the central effector [13].

The macrophage is another cell type with well-defined differential phenotypes. The so-called “macrophage polarization” has two main phenotypes analogous to  $T_H1$  and  $T_H2$ . The proinflammatory or “classically activated” M1 phenotype is associated with proinflammatory cytokines such as IL-1 $\beta$  and  $IFN\gamma$ , while the healing-promoting “alternatively activated” M2 phenotype is mainly linked to IL-4 [14]. The effects of IL-17 on macrophage phenotype have also attracted considerable interest. The M17 phenotype is not as well-defined as the M1 and M2 phenotypes; however, macrophages stimulated by IL-17 are characterized by the increased production of chemotactic and proinflammatory factors in the initial stages of the inflammatory response [15] and by the clearance of apoptotic cells and resolution of inflammation in the later phase [16].

Some authors have noted similarities between the variable functions and gene expression profiles of macrophages and chondrocytes in the setting of arthritis [17]. As another intriguing observation, major  $T_H1/2/17$  cytokines have been shown to play roles in the development of different forms of arthritis. Of the cytokines that have been implicated in the development of OA, IL-1 $\beta$  is probably the most prominent. It has been shown to decrease the anabolic activity in chondrocytes and promote their apoptosis [18]. It also induces the expression of the proteolytic enzymes of the matrix metalloproteinase (MMP) and a disintegrin-like and metalloproteinase with thrombospondin motifs (ADAMTS) families [19]. OA chondrocytes have been shown to upregulate the expression of IL-1 receptor (IL-1R) increasing their sensitivity to this cytokine [20]. Despite this, systemic treatment strategies specifically targeting IL-1 $\beta$  seem to have rather limited efficacy in OA [21], and none have reached clinical use.

Another major proinflammatory cytokine playing a role in the pathogenesis of arthritis is interleukin 17A (IL-17A) [22]. It promotes inflammation in concert with other proinflammatory cytokines [23], and its concentration in the synovial fluid correlates with radiographic severity of joint destruction [24]. In chondrocytes, it induces proinflammatory and catabolic factors and reduces proteoglycan synthesis [25–27]. Along with other proinflammatory cytokines, it also increases bone degradation by activating RANK ligand (RANKL) in osteoclasts [28]. In a murine model of collagen-induced arthritis, IL-17 deficiency has been shown to protect joints from the disease and IL-17 overexpression to exacerbate it [29,30]. Some functional gene expression analyses have actually implicated IL-17 signaling as a pathophysiological factor over IL-1 $\beta$ , the cytokine long known to drive OA [31].

In contrast to IL-1 $\beta$  and IL-17, the potential role of  $IFN\gamma$  as a causative factor in OA has attracted less interest. However, it has been found to be upregulated in chondrocytes by

proinflammatory cytokines [32] as well as to be present in OA synovial fluid [33]. Some gene variants that affect the development of OA, particularly those of T-cell immunoglobulin and mucin-domain containing-3 (TIM-3), exert their effects via the modulation of IFN $\gamma$  expression [34].

In the light of the above connections between the cytokines linked to major T helper cell/macrophage phenotypes and OA, it can be hypothesized that chondrocytes might also adopt phenotypes analogous to T<sub>H</sub>1/2/17 or M1/2/17 cells, and that these phenotypes might play a role in the development of OA. In the present study, we investigated the effects of the central T<sub>H</sub>1/2/17 cytokines on gene expression in OA chondrocytes. We sought to identify significantly differentially expressed genes and modulated pathways. The results were also compared to those of a recent genome-wide association study comparing degraded OA cartilage to preserved cartilage [35]. To our knowledge, this is the first study comparing the effects of the central T<sub>H</sub>1/2/17 cytokines on OA chondrocytes and to characterize the resulting phenotypes.

## 2. Results

### 2.1. Effects of IL-1 $\beta$ on Chondrocyte Phenotype

After normalization and correction for multiple testing, a total of 2822 genes were found to be differentially expressed in IL-1 $\beta$ -treated chondrocytes [in the C(IL-1 $\beta$ ) phenotype] versus controls in a statistically significant manner (FDR-corrected  $p$ -value < 0.05) and with a fold change (FC) 2.5 or more in either direction. Of these, 1092 were up- and 1730 downregulated. The list of the 20 most strongly upregulated genes contains several proinflammatory cyto- and chemokines, while the most strongly downregulated ones include several factors associated with regulation of gene expression, such as histone proteins (Table 1).

### 2.2. Effects of IL-17 on Chondrocyte Phenotype

Three hundred and eighty genes were differentially expressed in IL-17-treated chondrocytes [in the C(IL-17) phenotype] versus controls with FC > 2.5 in either direction, 314 of which were up- and 66 downregulated. Among the 20 most strongly upregulated genes were several associated with inflammation and chemotaxis, while the most strongly downregulated include genes involved in connective tissue development (Table 2).

### 2.3. Effects of IFN $\gamma$ on Chondrocyte Phenotype

After normalization and correction for multiple testing, a total of 548 genes were found to be differentially expressed in IFN $\gamma$ -treated chondrocytes [in the C(IFN $\gamma$ ) phenotype] versus controls in a statistically significant manner and FC 2.5 or more in either direction. Of these, 462 were up- and 86 downregulated. The 20 genes most strongly upregulated in C(IFN $\gamma$ ) cells included many associated with inflammation, antigen processing and presentation, and the regulation of proliferation. The most strongly downregulated genes included those involved in cell adhesion, proliferation and migration, and in Wnt signaling (Table 3).

### 2.4. Effects of IL-4 on Chondrocyte Phenotype

Twenty-six genes were upregulated by IL-4 with FC > 2.5 (Table S1). No genes were downregulated by IL-4 to a similar extent, but 10 genes were downregulated with FC < -1.5 (Table S2). In the C(IL-4) phenotype, the upregulated genes included those associated with the regulation of inflammation and TGF $\beta$  signaling as well as metabolism and cell adhesion, while several genes linked to cell proliferation were among the downregulated ones.

**Table 1.** Twenty most strongly up- and downregulated genes in interleukin 1-treated OA chondrocytes (IL1) relative to control (Co).

Gene	Name	Function	Mean (Co)	Mean (IL1)	Fold Change	adj. p
<i>IL6</i>	Interleukin 6	Inflammation	12.4	18,406.9	<b>3685.72</b>	$<1.0 \times 10^{-4}$
<i>CXCL1</i>	C-X-C motif chemokine ligand 1	Inflammation, chemotaxis	13.8	23,793.7	<b>3457.68</b>	$<1.0 \times 10^{-4}$
<i>IL1B</i>	Interleukin 1 beta	Inflammation	2.8	9575.7	<b>3332.44</b>	$<1.0 \times 10^{-4}$
<i>CXCL8</i>	C-X-C motif chemokine ligand 8	Inflammation, chemotaxis	329.5	855,146.3	<b>2968.9</b>	$<1.0 \times 10^{-4}$
<i>CXCL6</i>	C-X-C motif chemokine ligand 6	Inflammation, chemotaxis	2.8	4951.8	<b>2352.02</b>	$<1.0 \times 10^{-4}$
<i>CXCL5</i>	C-X-C motif chemokine ligand 5	Inflammation, chemotaxis	7.4	7352.4	<b>1239.8</b>	$<1.0 \times 10^{-4}$
<i>CXCL2</i>	C-X-C motif chemokine ligand 2	Inflammation, chemotaxis	3.9	4798.2	<b>1198.05</b>	$<1.0 \times 10^{-4}$
<i>CXCL3</i>	C-X-C motif chemokine ligand 3	Inflammation, chemotaxis	3.1	3154.6	<b>1130.76</b>	$<1.0 \times 10^{-4}$
<i>CCL20</i>	C-C motif chemokine ligand 20	Inflammation, chemotaxis	418	381,100.8	<b>1128.35</b>	$<1.0 \times 10^{-4}$
<i>IL36RN</i>	Interleukin 36 receptor antagonist	Regulation of inflammation	8.6	5863.8	<b>914.19</b>	$<1.0 \times 10^{-4}$
<i>ADORA2A</i>	Adenosine A2a receptor	Regulation of inflammation	5.5	1550.7	<b>641.44</b>	$<1.0 \times 10^{-4}$
<i>IL36G</i>	Interleukin 36 gamma	Inflammation	1.8	1065.5	<b>562.03</b>	$<1.0 \times 10^{-4}$
<i>EREG</i>	Epiregulin	Regulation of proliferation	31.9	13,697.7	<b>506.87</b>	$<1.0 \times 10^{-4}$
<i>CSF3</i>	Colony stimulating factor 3	Granulocyte-mediated inflammation	0.1	63.9	<b>300.02</b>	$<1.0 \times 10^{-4}$
<i>VNN1</i>	Vanin 1	T cell migration	9.2	2467.2	<b>273.35</b>	$<1.0 \times 10^{-4}$
<i>CCL5</i>	C-C motif chemokine ligand 5	Inflammation, chemotaxis	4.1	1134.2	<b>271.85</b>	$<1.0 \times 10^{-4}$
<i>C15orf48</i>	Chromosome 15 open reading frame 48	?	27.2	4669.1	<b>253.13</b>	$<1.0 \times 10^{-4}$
<i>CCL3</i>	C-C motif chemokine ligand 3	Inflammation, granulocyte activation	0.5	166.3	<b>242.88</b>	$<1.0 \times 10^{-4}$
<i>FCAMR</i>	Fc fragment of IgA and IgM receptor	Adaptive immunity, leukocyte migration	2.6	492	<b>213.45</b>	$<1.0 \times 10^{-4}$
<i>SERPINB7</i>	Serpin family B member 7	Endoproteinase inhibition	22.1	3747.9	<b>205.63</b>	$<1.0 \times 10^{-4}$
<i>HRCT1</i>	Histidine rich carboxyl terminus 1	?	105.8	4.1	<b>-38.85</b>	$<1.0 \times 10^{-4}$
<i>LSP1</i>	Lymphocyte specific protein 1	Regulation of neutrophil mobility	1749.6	58.1	<b>-31.39</b>	$<1.0 \times 10^{-4}$
<i>HIST1H3G</i>	Histone cluster 1 H3 family member g	Regulation of transcription	183.4	9.6	<b>-28.26</b>	$<1.0 \times 10^{-4}$
<i>ACTC1</i>	Actin, alpha, cardiac muscle 1	Heart muscle constituent	195.2	10.5	<b>-24.79</b>	$<1.0 \times 10^{-4}$
<i>NXPH3</i>	Neurexophilin 3	?	39.2	2.4	<b>-23.89</b>	$<1.0 \times 10^{-4}$
<i>SCN2B</i>	Sodium voltage-gated channel beta subunit 2	Cell adhesion and migration	167	8.7	<b>-22.19</b>	$<1.0 \times 10^{-4}$
<i>HIST1H1A</i>	Histone cluster 1 H1 family member a	?	908.5	47.2	<b>-21.2</b>	$<1.0 \times 10^{-4}$
<i>GDF10</i>	Growth differentiation factor 10	Skeletal system development	813.6	45.7	<b>-20.57</b>	$<1.0 \times 10^{-4}$
<i>LINC02593</i>	Long intergenic non-protein coding RNA 2593	?	68.3	3.4	<b>-20.53</b>	$<1.0 \times 10^{-4}$
<i>HIST1H3B</i>	Histone cluster 1 H3 family member b	Regulation of transcription	990.6	59.2	<b>-20.46</b>	$<1.0 \times 10^{-4}$
<i>TMEM26</i>	Transmembrane protein 26	?	403.7	21.4	<b>-19.3</b>	$<1.0 \times 10^{-4}$
<i>PHYHIP1L</i>	Phytanoyl-CoA 2-hydroxylase interacting protein like	?	22	1.6	<b>-19.19</b>	$<1.0 \times 10^{-4}$
<i>SARDH</i>	Sarcosine dehydrogenase	Mitochondrial metabolism	25.8	2.4	<b>-19.08</b>	$<1.0 \times 10^{-4}$
<i>HIST1H2BO</i>	Histone cluster 1 H2B family member o	Regulation of transcription?	234.4	12.7	<b>-18.99</b>	$<1.0 \times 10^{-4}$

Table 1. Cont.

Gene	Name	Function	Mean (Co)	Mean (IL1)	Fold Change	adj. p
<i>ID3</i>	Inhibitor of DNA binding 3, HLH protein	Regulation of transcription	676.5	45.8	−18.32	<1.0 × 10 <sup>−4</sup>
<i>HIST1H2AJ</i>	Histone cluster 1 H2A family member j	Regulation of transcription?	857	47.1	−18.12	<1.0 × 10 <sup>−4</sup>
<i>HIST1H1B</i>	Histone cluster 1 H1 family member b	Regulation of transcription?	736	50.6	−17.69	<1.0 × 10 <sup>−4</sup>
<i>MFAP2</i>	Microfibril associated protein 2	ECM organization	33	3.2	−17.52	<1.0 × 10 <sup>−4</sup>
<i>TNNT3</i>	Troponin T3, fast skeletal type	Muscle constituent	95.6	6.4	−17.51	<1.0 × 10 <sup>−4</sup>
<i>HIST1H2AL</i>	Histone cluster 1 H2A family member l	Regulation of transcription?	321.4	21.2	−17.32	<1.0 × 10 <sup>−4</sup>

Red = upregulated genes; blue = downregulated genes.

Table 2. Twenty most strongly up- and downregulated genes in interleukin 17-treated OA chondrocytes (IL17) relative to control (Co).

Gene	Name	Function	Mean (Co)	Mean (IL17)	Fold Change	adj. p
<i>SAA2</i>	Serum amyloid A2	Chemotaxis	5.5	659.2	<b>319.99</b>	<1.0 × 10 <sup>−4</sup>
<i>IL6</i>	Interleukin 6	Inflammation	12.2	1431.4	<b>250.15</b>	<1.0 × 10 <sup>−4</sup>
<i>SAA1</i>	Serum amyloid A1	Inflammation, chemotaxis	63.7	3520.0	<b>183.26</b>	<1.0 × 10 <sup>−4</sup>
<i>SAA2-SAA4</i>	SAA2-SAA4 readthrough	Chemotaxis?	2.9	216.7	<b>156.18</b>	<1.0 × 10 <sup>−4</sup>
<i>CXCL6</i>	C-X-C motif chemokine ligand 6	Inflammation, chemotaxis	2.8	276.4	<b>141.01</b>	<1.0 × 10 <sup>−4</sup>
<i>CXCL1</i>	C-X-C motif chemokine ligand 1	Inflammation, chemotaxis	13.6	1170.5	<b>136.48</b>	<1.0 × 10 <sup>−4</sup>
<i>VNN1</i>	Vanin 1	T cell migration	9.1	820.5	<b>84.13</b>	<1.0 × 10 <sup>−4</sup>
<i>CCL20</i>	C-C motif chemokine ligand 20	Chemotaxis	412.8	26,508.9	<b>73.49</b>	<1.0 × 10 <sup>−4</sup>
<i>TNFSF18</i>	TNF superfamily member 18	T cell survival	4.2	470.3	<b>73.05</b>	<1.0 × 10 <sup>−4</sup>
<i>IL36RN</i>	Interleukin 36 receptor antagonist	Regulation of inflammation	8.5	468.0	<b>69.09</b>	<1.0 × 10 <sup>−4</sup>
<i>VNN3</i>	Vanin 3	?	1.8	130.3	<b>66.35</b>	<1.0 × 10 <sup>−4</sup>
<i>ADORA2A</i>	Adenosine A2a receptor	Inflammation, phagocytosis	5.4	105.9	<b>64.74</b>	<1.0 × 10 <sup>−4</sup>
<i>CXCL2</i>	C-X-C motif chemokine ligand 2	Inflammation, chemotaxis	3.9	220.3	<b>55.90</b>	<1.0 × 10 <sup>−4</sup>
<i>CXCL8</i>	C-X-C motif chemokine ligand 8	Inflammation, chemotaxis	324.8	14,116.5	<b>48.18</b>	<1.0 × 10 <sup>−4</sup>
<i>C15orf48</i>	Chromosome 15 open reading frame 48	Mitochondrial respiration?	26.9	820.3	<b>46.34</b>	<1.0 × 10 <sup>−4</sup>
<i>PDZK1IP1</i>	PDZK1 interacting protein 1	Regulation of apoptosis	5.2	206.9	<b>41.18</b>	<1.0 × 10 <sup>−4</sup>
<i>NOS2</i>	Nitric oxide synthase 2	Inflammation	137.9	3370.2	<b>40.02</b>	<1.0 × 10 <sup>−4</sup>
<i>ODAPH</i>	Odontogenesis associated phosphoprotein	Enamel production	1.4	41.9	<b>37.29</b>	<1.0 × 10 <sup>−4</sup>
<i>SLC28A3</i>	Solute carrier family 28 member 3	Nucleoside transport	4.3	150.4	<b>35.34</b>	<1.0 × 10 <sup>−4</sup>
<i>CXCL5</i>	C-X-C motif chemokine ligand 5	Inflammation, chemotaxis	7.3	207.5	<b>34.25</b>	<1.0 × 10 <sup>−4</sup>

Table 2. Cont.

Gene	Name	Function	Mean (Co)	Mean (IL17)	Fold Change	adj. p
<i>ACTC1</i>	Actin, alpha, cardiac muscle 1	Cardiac muscle component	191.7	26.7	−8.14	$<1.0 \times 10^{-4}$
<i>TOX</i>	Thymocyte selection associated high mobility group box	T cell development	14.6	3.9	−5.66	0.0010
<i>TMEM26</i>	Transmembrane protein 26	?	396.3	69.8	−5.47	$<1.0 \times 10^{-4}$
<i>TNNT3</i>	Troponin T3, fast skeletal type	Muscle component	93.9	17.9	−5.28	$<1.0 \times 10^{-4}$
<i>TENT5B</i>	Terminal nucleotidyltransferase 5B	Regulation of cell proliferation	152.5	39.7	−4.81	$<1.0 \times 10^{-4}$
<i>TMEM26-AS1</i>	TMEM26 antisense RNA 1	?	32.0	14.4	−4.77	$3.8 \times 10^{-4}$
<i>RCAN2</i>	Regulator of calcineurin 2	Regulation of transcription	326.5	74.6	−4.74	$<1.0 \times 10^{-4}$
<i>OPRL1</i>	Opioid related nociceptin receptor 1	?	11.8	3.0	−4.51	0.0068
<i>CSRNP3</i>	Cysteine and serine rich nuclear protein 3	Regulation of apoptosis	59.7	19.7	−4.01	$<1.0 \times 10^{-4}$
<i>ASPN</i>	Asporin	Cartilage constituent	2011.2	505.2	−3.92	$<1.0 \times 10^{-4}$
<i>HRCT1</i>	Histidine rich carboxyl terminus 1	?	104.1	25.8	−3.85	$<1.0 \times 10^{-4}$
<i>AQP1</i>	Aquaporin 1 (Colton blood group)	Regulation of osmotic pressure, angiogenesis, apoptosis	42.9	13.4	−3.69	$<1.0 \times 10^{-4}$
<i>YWHAZP5</i>	YWHAZ pseudogene 5	?	10.2	3.2	−3.68	0.013
<i>MRAP2</i>	Melanocortin 2 receptor accessory protein 2	cAMP signaling	1295.9	376.5	−3.62	$<1.0 \times 10^{-4}$
<i>C1QTNF7</i>	C1q and TNF related 7	?	63.4	20.1	−3.54	$<1.0 \times 10^{-4}$
<i>MFAP2</i>	Microfibril associated protein 2	Connective tissue organization	32.4	8.7	−3.47	$<1.0 \times 10^{-4}$
<i>CLEC3A</i>	C-type lectin domain family 3 member A	Skeletal system development	847.3	264.6	−3.46	$<1.0 \times 10^{-4}$
<i>GREM1</i>	Gremlin 1, DAN family BMP antagonist	Regulation of connective tissue development	5141.6	1566.4	−3.41	$<1.0 \times 10^{-4}$
<i>CRISPLD1</i>	Cysteine rich secretory protein LCCL domain containing 1	Morphogenesis	946.1	280.2	−3.39	$<1.0 \times 10^{-4}$
<i>HRASLS5</i> (=PLAAT5)	HRAS like suppressor family member 5	Glycerophospholipid metabolism	12.8	3.6	−3.37	0.019

Red = upregulated genes; blue = downregulated genes.

Table 3. Twenty most strongly up- and downregulated genes in interferon gamma -treated OA chondrocytes (IFN $\gamma$ ) relative to control (Co).

Gene	Name	Function	Mean (Co)	Mean (IFN $\gamma$ )	Fold change	adj. p
<i>IDO1</i>	Indoleamine 2,3-dioxygenase 1	Regulation of T cell -mediated immunity	17.5	42,320.0	4643.74	$<1.0 \times 10^{-4}$
<i>LGALS17A</i>	Galectin 14 pseudogene	?	0.4	1065.1	1750.58	$<1.0 \times 10^{-4}$
<i>GBP1P1</i>	Guanylate binding protein 1 pseudogene 1	?	2.6	2838.8	1245.34	$<1.0 \times 10^{-4}$
<i>CXCL10</i>	C-X-C motif chemokine ligand 10	Chemotaxis	2.2	2065.2	1117.91	$<1.0 \times 10^{-4}$
<i>GBP5</i>	Guanylate binding protein 5	Inflammasome activation	1.4	1518.3	1112.44	$<1.0 \times 10^{-4}$
<i>CXCL9</i>	C-X-C motif chemokine ligand 9	T cell chemotaxis	1.1	1069.9	1033.80	$<1.0 \times 10^{-4}$
<i>GBP4</i>	Guanylate binding protein 4	Inflammation?	30.9	27,565.6	955.57	$<1.0 \times 10^{-4}$

Table 3. Cont.

Gene	Name	Function	Mean (Co)	Mean (IFN $\gamma$ )	Fold change	adj. p
<i>IFI44L</i>	Interferon induced protein 44 like	?	9.7	6185.8	<b>694.66</b>	$<1.0 \times 10^{-4}$
<i>GBP1</i>	Guanylate binding protein 1	Negative regulation of inflammation	124.3	54,562.1	<b>454.62</b>	$<1.0 \times 10^{-4}$
<i>HLA-DRA</i>	Major histocompatibility complex, class II, DR alpha	Antigen presentation	5.6	2338.3	<b>408.93</b>	$<1.0 \times 10^{-4}$
<i>HLA-DRB1</i>	Major histocompatibility complex, class II, DR beta 1	Antigen presentation	10.7	2430.7	<b>383.18</b>	$<1.0 \times 10^{-4}$
<i>CD74</i>	CD74 molecule	Antigen presentation	31.9	11,211.5	<b>353.35</b>	$<1.0 \times 10^{-4}$
<i>RSAD2</i>	Radical S-adenosyl methionine domain containing 2	Antiviral action	44.5	15,365.2	<b>338.82</b>	$<1.0 \times 10^{-4}$
<i>RARRES3</i>	Retinoic acid receptor responder 3	Phospholipid catabolism	33.1	8271.1	<b>286.40</b>	$<1.0 \times 10^{-4}$
<i>BST2</i>	Bone marrow stromal cell antigen 2	Antiviral action	10.1	2908.5	<b>285.04</b>	$<1.0 \times 10^{-4}$
<i>GBP6</i>	Guanylate binding protein family member 6	Inflammation	1.0	193.3	<b>273.26</b>	$<1.0 \times 10^{-4}$
<i>HLA-DRB5</i>	Major histocompatibility complex, class II, DR beta 5	Antigen presentation	4.4	825.4	<b>253.47</b>	$<1.0 \times 10^{-4}$
<i>HLA-DRB6</i>	Major histocompatibility complex, class II, DR beta 6 (pseudogene)	Antigen presentation?	0.3	125.7	<b>226.68</b>	$<1.0 \times 10^{-4}$
<i>APOL4</i>	Apolipoprotein L4	Lipid metabolism	2.6	500.8	<b>225.95</b>	$<1.0 \times 10^{-4}$
<i>IFIT2</i>	Interferon induced protein with tetratricopeptide repeats 2	Regulation of proliferation	96.2	20,648.8	<b>225.79</b>	$<1.0 \times 10^{-4}$
<i>TNFRSF10D</i>	TNF receptor superfamily member 10d	Inhibition of apoptosis	4135.1	501.9	<b>-7.65</b>	$<1.0 \times 10^{-4}$
<i>ARHGAP9</i>	Rho gtpase activating protein 9	?	10.7	2.4	<b>-5.27</b>	0.0028
<i>NANOS1</i>	Nanos C2HC-type zinc finger 1	Regulation of translation and cell migration	83.4	16.9	<b>-4.94</b>	$<1.0 \times 10^{-4}$
<i>SNORD108</i>	Small nucleolar RNA, C/D box 108	?	66.6	13.8	<b>-4.81</b>	$<1.0 \times 10^{-4}$
<i>FAM189A2</i>	Family with sequence similarity 189 member A2	?	13.6	4.3	<b>-4.39</b>	0.0033
<i>PWAR6</i>	Prader Willi/Angelman region RNA 6	?	34.0	7.9	<b>-4.32</b>	$<1.0 \times 10^{-4}$
<i>GABRA4</i>	Gamma-aminobutyric acid type A receptor alpha4 subunit	Synaptic transmission	2346.1	549.2	<b>-4.28</b>	$<1.0 \times 10^{-4}$
<i>CORO2A</i>	Coronin 2A	?	13.5	3.7	<b>-4.11</b>	0.020
<i>WFDC1</i>	WAP four-disulfide core domain 1	Regulation of proliferation	65.1	18.0	<b>-4.06</b>	$<1.0 \times 10^{-4}$
<i>PRSS35</i>	Serine protease 35	?	51.4	13.5	<b>-4.01</b>	$<1.0 \times 10^{-4}$
<i>SLC16A14</i>	Solute carrier family 16 member 14	Organic acid transport	40.2	13.3	<b>-3.98</b>	$<1.0 \times 10^{-4}$
<i>PWAR5</i>	Prader Willi/Angelman region RNA 5	?	359.7	91.4	<b>-3.93</b>	$<1.0 \times 10^{-4}$
<i>MTURN</i>	Maturin, neural progenitor differentiation regulator homolog	?	1857.1	519.7	<b>-3.63</b>	$<1.0 \times 10^{-4}$
<i>C1QTNF5</i>	C1q and TNF related 5	Cell adhesion	152.4	46.1	<b>-3.47</b>	$<1.0 \times 10^{-4}$
<i>LONRF2</i>	LON peptidase N-terminal domain and ring finger 2	?	206.8	59.5	<b>-3.46</b>	$<1.0 \times 10^{-4}$
<i>FGFR4</i>	Fibroblast growth factor receptor 4	Cell proliferation and migration	11.1	5.1	<b>-3.31</b>	0.045
<i>TRABD2B</i>	Trab domain containing 2B	Wnt signaling, proteolysis	14.2	5.5	<b>-3.29</b>	0.0014
<i>TNNT3</i>	Troponin T3, fast skeletal type	Muscle contraction	106.0	31.6	<b>-3.26</b>	$<1.0 \times 10^{-4}$
<i>NCALD</i>	Neurocalcin delta	Endocytosis	17.3	6.6	<b>-3.24</b>	0.029
<i>CDH2</i>	Cadherin 2	Cell adhesion	12.0	4.1	<b>-3.23</b>	0.0012

Red = upregulated genes; blue = downregulated genes.

### 2.5. Functional Gene Categories in Different Chondrocyte Phenotypes

Table 4 shows the Gene Ontology (GO) terms affected with a high significance (FDR-corrected  $p$ -value  $< 0.01$ ) by at least one studied proinflammatory cytokine (IL-1 $\beta$ , IFN $\gamma$  or IL-17). The C(IL-1 $\beta$ ) phenotype was involved in the activation of a wide range of inflammatory terms and pathways, along with those related to cell adhesion as well as extracellular matrix production and degradation. The T<sub>H</sub>17-associated cytokine IL-17 affected a partly overlapping, but smaller, set of inflammatory cytokines compared to IL-1 $\beta$ . The C(IFN $\gamma$ ) phenotype was quite distinct compared to the C(IL-1 $\beta$ ) and C(IL-17) phenotypes; several terms related to antigen processing and presentation were affected by this cytokine alone. Nitric oxide synthase biosynthetic process and chemotaxis were among the functions involved solely in the C(IL-17) phenotype. In addition, many high-level GO terms related to inflammation were affected by all of the three proinflammatory cytokines.

In C(IL-4) cells, no significantly affected GO terms were detected when analyzing the genes with FC  $> 2.5$  in either direction. When the FC threshold was lowered to 1.5, GO terms associated with cell division were among the significant ones (Table S3).

### 2.6. Comparing the Effects of Different Proinflammatory Cytokines

Next, we cross-compared the genes markedly upregulated (FC  $> 2.5$ ) in the C(IL-1 $\beta$ ), C(IFN $\gamma$ ) and C(IL-17) phenotypes to further characterize the differences and similarities between the resulting phenotypes. As shown in Figure 1A, a large portion (nearly 85%) of genes markedly upregulated in C(IL-17) cells were included in the large set of those similarly affected by IL-1 $\beta$ , but 45 genes were solely affected by IL-17, and the overlap of C(IL-17) and C(IFN $\gamma$ ) phenotypes was considerably smaller than that of C(IL-17) and C(IL-1 $\beta$ ). The intersection of genes upregulated by both IL-17 and IFN $\gamma$  was nearly completely contained in those upregulated by IL-1 $\beta$  (Figure 1A). Many central regulators of inflammation such as *IL6*, *PTGS2* (cyclo-oxygenase 2 or COX-2) and *NOS2* (inducible nitric oxide synthase or iNOS) were markedly upregulated by all the three T<sub>H</sub>1/T<sub>H</sub>17 cytokines, in line with the widespread activation of inflammatory pathways observed in the GO analysis (Table 5).

When comparing genes markedly downregulated (FC  $< -2.5$ ) by the three proinflammatory cytokines, the large ( $>1000$  genes) list of genes downregulated by IL-1 $\beta$  again contained a large proportion (85%) of those downregulated by IL-17 and a smaller amount (48%) of genes similarly affected by IFN $\gamma$  (Figure 1B). Genes downregulated by all of the three cytokines are presented in Table 6 and include, for example, those associated with cell proliferation and skeletal system development.

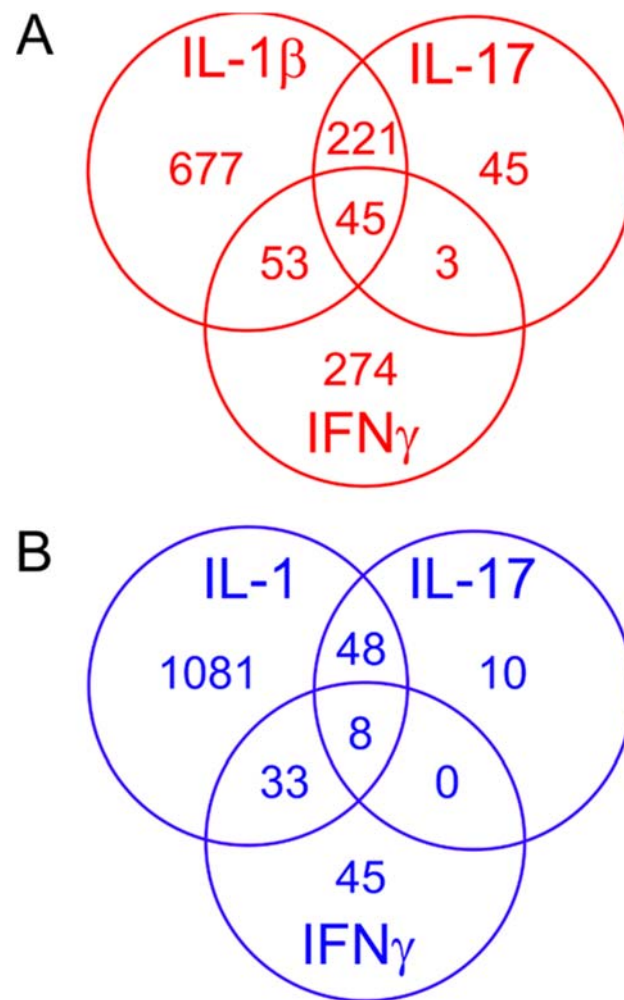
### 2.7. Effects of the Cytokines on Genes Differentially Expressed in Degraded and Preserved OA Cartilage

Some previous studies have investigated the differences in gene expression between degraded and preserved OA cartilage. Of these, the study by Almeida et al. [35] is probably the most comprehensive. To see whether the studied cytokines shift chondrocyte phenotype towards either degraded or preserved cartilage, we compared the differentially expressed genes in the phenotypes observed in the present study to those differentially expressed in the study by Almeida et al. [35] As a very large number (over 2300) of significantly differentially expressed genes were identified in that study, we focused on those 84 genes which were most strongly upregulated (FC  $> 2.5$  and FDR-corrected  $p$ -value  $< 0.01$ ) in the degraded cartilage. Of those 84 genes, 38 were significantly affected by at least one of the proinflammatory cytokines (IL-1, IL-17 or IFN $\gamma$ ) in our data. A large majority (30) of these 38 genes were also upregulated by IL-1 $\beta$ , showing that the cytokine shifts chondrocyte phenotype towards the one observed in the degraded cartilage. Several mediators of inflammation, such as *LIF*, *CCL20* and *TREM1*, were especially strongly upregulated. Only four of the 84 genes (namely *CLIC3*, *ERFE*, *SLC27A2* and *ANK3*) were downregulated by IL-1 $\beta$ .



**Table 4.** GO terms affected by different proinflammatory cytokines. Genes with FC > 2.5 in either direction were analyzed with DAVID, and the resulting lists were reduced with REVIGO. GO terms significantly affected (with FDR-corrected *p*-value < 0.05) by a cytokine are marked with an X.

Term	IL1	IL17	IFN $\gamma$	Term	IL1	IL17	IFN $\gamma$
Inflammatory response	X	X	X	Nucleosome assembly	X		
Immune response	X	X	X	Chromosome segregation	X		
Response to lipopolysaccharide	X	X	X	Protein heterotetramerization	X		
Chemotaxis	X	X	X	Wound healing	X		
Negative regulation of viral entry into host cell	X	X	X	Regulation of cell proliferation	X		
Negative regulation of type I interferon production	X	X	X	Cell migration	X		
Response to progesterone	X	X		Regulation of gene silencing	X		
Cell-cell signaling	X	X		Positive regulation of interleukin-12 production	X		
Angiogenesis	X	X		Odontogenesis	X		
Negative regulation of growth	X	X		Cellular response to mechanical stimulus	X		
Positive regulation of mitotic nuclear division	X	X		Peptidyl-tyrosine phosphorylation	X		
Negative regulation of cell proliferation	X	X		Collagen catabolic process	X		
Signal transduction	X		X	Positive regulation of cell division	X		
Response to virus	X		X	Positive chemotaxis		X	
Positive regulation of interleukin-6 production	X		X	Positive regulation of nitric-oxide synthase biosynthetic process		X	
Response to hydrogen peroxide	X		X	Acute-phase response		X	
Positive regulation of I-kappab kinase/NF-kappab signaling	X		X	Positive regulation of cytosolic calcium ion concentration		X	
Response to drug	X		X	Positive regulation of gtpase activity			X
Cellular response to zinc ion		X	X	Response to glucocorticoid			X
Response to toxic substance		X	X	Response to wounding			X
Tumor necrosis factor-mediated signaling pathway		X	X	Positive regulation of NF-kappab transcription factor activity			X
Cell division	X			Negative regulation of tumor necrosis factor production			X
DNA replication	X			Cellular response to organic cyclic compound			X
Telomere organization	X			Antigen processing and presentation			X
Positive regulation of gene expression	X			Antigen processing and presentation of peptide or polysaccharide antigen via MHC class II			X
Cell adhesion	X			Antigen processing and presentation of exogenous peptide antigen via MHC class I, TAP-independent			X
Extracellular matrix organization	X			Response to interferon-beta			X
Skeletal system development	X			Response to interferon-alpha			X
Sister chromatid cohesion	X			T cell costimulation			X
DNA replication initiation	X			Positive regulation of T cell mediated cytotoxicity			X
Cellular protein metabolic process	X			Defense response			X
Cell proliferation	X			Protein trimerization			X
Negative regulation of gene expression, epigenetic	X			Proteolysis			X
				Defense response to protozoan			X
				Positive regulation of peptidyl-tyrosine phosphorylation			X
				Protein polyubiquitination			X



**Figure 1.** Venn diagrams of genes markedly upregulated ( $FC > 2.5$ ) (A) or markedly downregulated ( $FC < 2.5$ ) (B) by IL-1 $\beta$ , IL-17 and IFN $\gamma$ . Red denotes up- and blue downregulated genes.

In the C(IFN $\gamma$ ) phenotype, 13 of the 84 genes associated with degraded cartilage (including *LIF* and *NGF*) were upregulated compared with control, but nearly as many (nine) were downregulated, including *TREM1*. This shows that the effects of IFN $\gamma$  on chondrocyte phenotype in relation to the degraded/preserved cartilage are more ambiguous than those of IL-1 $\beta$ .

In C(IL-17) chondrocytes, 25 of the 84 genes associated with degraded cartilage were upregulated compared to naïve chondrocytes (including *CCL20* and *IL11*), and none were significantly downregulated. Nine genes, including *IGFBP1*, *LIF* and *GPR158*, were upregulated in all three inflammatory phenotypes C(IL-1 $\beta$ ), C(IFN $\gamma$ ) and C(IL-17) and one (*ANK3*) was downregulated in all of them. (Figure 2 and Table S4).

**Table 5.** Genes upregulated by all studied proinflammatory cytokines with FC > 2.5. Shown are mean normalized expression levels in control (Co) and in C(IL1), C(IL17) and C(IFN $\gamma$ ) phenotypes, fold changes (FCs) for all comparisons vs. control and false discovery rate (FDR)-adjusted *p* values for them.

Gene	Name	Mean exp. (Co)	Mean exp. (IL1)	Mean exp. (IL17)	Mean exp. (IFN $\gamma$ )	FC (IL1 vs. Co)	adj. p (IL1 vs. Co)	FC (IL17 vs. Co)	adj. p (IL17 vs. Co)	FC (IFN $\gamma$ vs. Co)	adj. p (IFN $\gamma$ vs. Co)
<i>IL6</i>	Interleukin 6	12.8	18,406.9	1431.4	94.2	<b>3685.72</b>	$<1.0 \times 10^{-4}$	<b>250.15</b>	$<1.0 \times 10^{-4}$	<b>12.34</b>	$<1.0 \times 10^{-4}$
<i>IL36RN</i>	Interleukin 36 receptor antagonist	8.9	5863.8	468.0	36.7	<b>914.19</b>	$<1.0 \times 10^{-4}$	<b>69.09</b>	$<1.0 \times 10^{-4}$	<b>4.59</b>	$<1.0 \times 10^{-4}$
<i>ESM1</i>	Endothelial cell specific molecule 1	276.7	37,984.1	1373.5	1449.2	<b>157.25</b>	$<1.0 \times 10^{-4}$	<b>5.09</b>	$<1.0 \times 10^{-4}$	<b>4.70</b>	$<1.0 \times 10^{-4}$
<i>SAA2</i>	Serum amyloid A2	5.8	371.4	659.2	27.1	<b>149.11</b>	$<1.0 \times 10^{-4}$	<b>319.99</b>	$<1.0 \times 10^{-4}$	<b>8.73</b>	$<1.0 \times 10^{-4}$
<i>iNOS/NOS2</i>	Inducible nitric oxide synthase/Nitric oxide synthase 2	144.2	12,704.9	3370.2	3046.1	<b>131.22</b>	$<1.0 \times 10^{-4}$	<b>40.02</b>	$<1.0 \times 10^{-4}$	<b>30.16</b>	$<1.0 \times 10^{-4}$
<i>NOD2</i>	Nucleotide binding oligomerization domain containing 2	7.6	919.4	96.7	43.9	<b>116.73</b>	$<1.0 \times 10^{-4}$	<b>13.67</b>	$<1.0 \times 10^{-4}$	<b>5.61</b>	$<1.0 \times 10^{-4}$
<i>PTX3</i>	Pentraxin 3	184.4	18,888.7	4615.3	479.6	<b>113.19</b>	$<1.0 \times 10^{-4}$	<b>27.47</b>	$<1.0 \times 10^{-4}$	<b>2.60</b>	$<1.0 \times 10^{-4}$
<i>SAA1</i>	Serum amyloid A1	66.6	2188.7	3520.0	227.6	<b>94.66</b>	$<1.0 \times 10^{-4}$	<b>183.26</b>	$<1.0 \times 10^{-4}$	<b>6.46</b>	$<1.0 \times 10^{-4}$
<i>CD300E</i>	CD300e molecule	3.6	316.9	32.7	71.6	<b>72.79</b>	$<1.0 \times 10^{-4}$	<b>7.91</b>	$<1.0 \times 10^{-4}$	<b>17.15</b>	$<1.0 \times 10^{-4}$
<i>IL36B</i>	Interleukin 36 beta	11.3	466.3	80.1	39.1	<b>67.27</b>	$<1.0 \times 10^{-4}$	<b>9.65</b>	$<1.0 \times 10^{-4}$	<b>3.60</b>	$<1.0 \times 10^{-4}$
<i>TNFRSF1B</i>	TNF receptor superfamily member 1B	40.0	2370.7	525.8	118.9	<b>62.58</b>	$<1.0 \times 10^{-4}$	<b>14.66</b>	$<1.0 \times 10^{-4}$	<b>3.02</b>	$<1.0 \times 10^{-4}$
<i>TNFAIP6</i>	TNF alpha induced protein 6	1176.4	42,950.3	5512.4	4561.2	<b>36.87</b>	$<1.0 \times 10^{-4}$	<b>4.59</b>	$<1.0 \times 10^{-4}$	<b>3.59</b>	$<1.0 \times 10^{-4}$
<i>TMEM132A</i>	Transmembrane protein 132A	10.3	328.1	165.0	32.6	<b>33.90</b>	$<1.0 \times 10^{-4}$	<b>16.64</b>	$<1.0 \times 10^{-4}$	<b>3.18</b>	$<1.0 \times 10^{-4}$
<i>ICAM1</i>	Intercellular adhesion molecule 1	1415.2	42,657.2	4388.3	8524.5	<b>31.66</b>	$<1.0 \times 10^{-4}$	<b>3.15</b>	$<1.0 \times 10^{-4}$	<b>5.54</b>	$<1.0 \times 10^{-4}$
<i>C3AR1</i>	Complement C3a receptor 1	2.2	66.2	11.4	11.2	<b>28.15</b>	$<1.0 \times 10^{-4}$	<b>6.36</b>	$1.5 \times 10^{-4}$	<b>5.32</b>	$4.9 \times 10^{-4}$
<i>CLEC2B</i>	C-type lectin domain family 2 member B	5.3	145.0	48.5	20.6	<b>27.53</b>	$<1.0 \times 10^{-4}$	<b>9.35</b>	$<1.0 \times 10^{-4}$	<b>3.85</b>	$<1.0 \times 10^{-4}$
<i>COX-2/PTGS2</i>	Cyclooxygenase-2/Prostaglandin-endoperoxide synthase 2	1310.7	37,281.5	4678.6	5349.2	<b>26.96</b>	$<1.0 \times 10^{-4}$	<b>3.28</b>	$<1.0 \times 10^{-4}$	<b>3.57</b>	$<1.0 \times 10^{-4}$
<i>TLR2</i>	Toll like receptor 2	134.9	3348.9	782.0	371.4	<b>22.64</b>	$<1.0 \times 10^{-4}$	<b>5.02</b>	$<1.0 \times 10^{-4}$	<b>2.54</b>	$<1.0 \times 10^{-4}$
<i>CCL7</i>	C-C motif chemokine ligand 7	2.1	36.7	20.6	24.4	<b>20.66</b>	$<1.0 \times 10^{-4}$	<b>12.14</b>	$<1.0 \times 10^{-4}$	<b>10.56</b>	$<1.0 \times 10^{-4}$
<i>CCL2</i>	C-C motif chemokine ligand 2	150.4	2475.0	815.0	430.6	<b>19.42</b>	$<1.0 \times 10^{-4}$	<b>5.85</b>	$<1.0 \times 10^{-4}$	<b>2.61</b>	$<1.0 \times 10^{-4}$
<i>IRF4</i>	Interferon regulatory factor 4	23.5	400.1	94.9	114.2	<b>18.20</b>	$<1.0 \times 10^{-4}$	<b>4.62</b>	$<1.0 \times 10^{-4}$	<b>4.69</b>	$<1.0 \times 10^{-4}$
<i>CD274</i>	CD274 molecule	61.8	1048.8	350.1	3845.7	<b>17.56</b>	$<1.0 \times 10^{-4}$	<b>6.18</b>	$<1.0 \times 10^{-4}$	<b>60.08</b>	$<1.0 \times 10^{-4}$
<i>RBM47</i>	RNA binding motif protein 47	8.8	122.3	30.6	22.8	<b>14.96</b>	$<1.0 \times 10^{-4}$	<b>3.38</b>	$<1.0 \times 10^{-4}$	<b>2.67</b>	0.040
<i>CD38</i>	CD38 molecule	9.8	133.8	74.3	211.4	<b>14.81</b>	$<1.0 \times 10^{-4}$	<b>7.67</b>	$<1.0 \times 10^{-4}$	<b>20.76</b>	$<1.0 \times 10^{-4}$
<i>BDKRB1</i>	Bradykinin receptor B1	29.0	401.5	129.6	105.0	<b>13.95</b>	$<1.0 \times 10^{-4}$	<b>4.88</b>	$<1.0 \times 10^{-4}$	<b>3.19</b>	$<1.0 \times 10^{-4}$
<i>GCH1</i>	GTP cyclohydrolase 1	591.7	7968.7	2212.7	3584.2	<b>13.38</b>	$<1.0 \times 10^{-4}$	<b>3.90</b>	$<1.0 \times 10^{-4}$	<b>5.63</b>	$<1.0 \times 10^{-4}$
<i>LRRC38</i>	Leucine rich repeat containing 38	11.2	132.1	44.4	35.8	<b>11.59</b>	$<1.0 \times 10^{-4}$	<b>3.79</b>	$<1.0 \times 10^{-4}$	<b>2.98</b>	$<1.0 \times 10^{-4}$
<i>KIAA1217</i>	KIAA1217	15.3	157.8	55.1	109.1	<b>10.61</b>	$<1.0 \times 10^{-4}$	<b>3.80</b>	$<1.0 \times 10^{-4}$	<b>6.39</b>	$<1.0 \times 10^{-4}$
<i>SSTR2</i>	Somatostatin receptor 2	90.0	971.2	1549.7	340.1	<b>10.56</b>	$<1.0 \times 10^{-4}$	<b>16.11</b>	$<1.0 \times 10^{-4}$	<b>3.36</b>	$<1.0 \times 10^{-4}$
<i>DUSP5</i>	Dual specificity phosphatase 5	77.3	746.8	302.4	236.1	<b>10.54</b>	$<1.0 \times 10^{-4}$	<b>4.02</b>	$<1.0 \times 10^{-4}$	<b>2.90</b>	$<1.0 \times 10^{-4}$
<i>TYMP</i>	Thymidine phosphorylase	311.3	3020.1	1275.1	9324.0	<b>10.15</b>	$<1.0 \times 10^{-4}$	<b>4.24</b>	$<1.0 \times 10^{-4}$	<b>28.71</b>	$<1.0 \times 10^{-4}$
<i>GPR158</i>	G protein-coupled receptor 158	6.9	38.0	22.0	21.5	<b>9.98</b>	$<1.0 \times 10^{-4}$	<b>6.77</b>	0.0018	<b>5.55</b>	$7.6 \times 10^{-4}$
<i>PRLR</i>	Prolactin receptor	8.3	78.8	29.7	33.0	<b>9.93</b>	$<1.0 \times 10^{-4}$	<b>3.05</b>	0.0034	<b>3.92</b>	$<1.0 \times 10^{-4}$
<i>GSAP</i>	Gamma-secretase activating protein	122.2	1109.8	378.0	509.3	<b>9.18</b>	$<1.0 \times 10^{-4}$	<b>3.26</b>	$<1.0 \times 10^{-4}$	<b>3.74</b>	$<1.0 \times 10^{-4}$

Table 5. Cont.

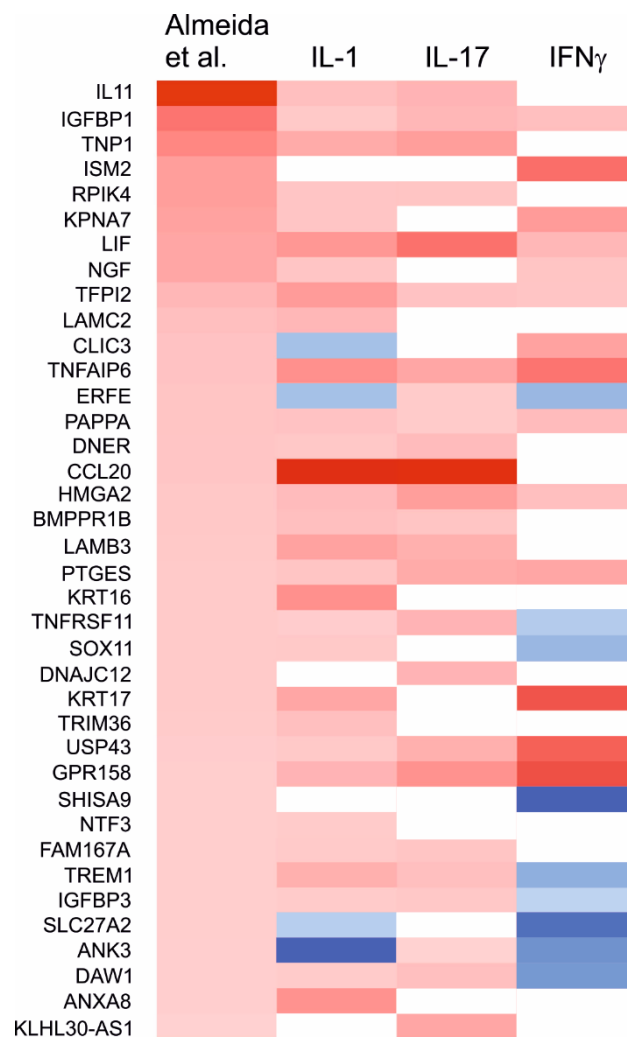
Gene	Name	Mean exp. (Co)	Mean exp. (IL1)	Mean exp. (IL17)	Mean exp. (IFN $\gamma$ )	FC (IL1 vs. Co)	adj. p (IL1 vs. Co)	FC (IL17 vs. Co)	adj. p (IL17 vs. Co)	FC (IFN $\gamma$ vs. Co)	adj. p (IFN $\gamma$ vs. Co)
<i>GPR39</i>	G protein-coupled receptor 39	15.4	110.6	39.1	41.4	<b>9.17</b>	$<1.0 \times 10^{-4}$	<b>3.24</b>	$1.7 \times 10^{-4}$	<b>2.71</b>	$<1.0 \times 10^{-4}$
<i>LYPD1</i>	LY6/PLAUR domain containing 1	10.5	71.5	28.7	27.7	<b>8.44</b>	$<1.0 \times 10^{-4}$	<b>3.31</b>	$5.6 \times 10^{-4}$	<b>2.62</b>	0.0023
<i>ODF3B</i>	Outer dense fiber of sperm tails 3B	34.6	261.0	106.0	773.8	<b>7.98</b>	$<1.0 \times 10^{-4}$	<b>3.28</b>	$<1.0 \times 10^{-4}$	<b>21.57</b>	$<1.0 \times 10^{-4}$
<i>SLC15A3</i>	Solute carrier family 15 member 3	16.3	119.4	54.7	607.4	<b>7.63</b>	$<1.0 \times 10^{-4}$	<b>3.45</b>	$<1.0 \times 10^{-4}$	<b>35.59</b>	$<1.0 \times 10^{-4}$
<i>HAL</i>	Histidine ammonia-lyase	6.2	44.1	28.7	47.4	<b>7.57</b>	$<1.0 \times 10^{-4}$	<b>4.71</b>	$<1.0 \times 10^{-4}$	<b>6.97</b>	$<1.0 \times 10^{-4}$
<i>DOCK4</i>	Dedicator of cytokinesis 4	44.0	306.8	144.9	139.2	<b>6.94</b>	$<1.0 \times 10^{-4}$	<b>3.21</b>	$<1.0 \times 10^{-4}$	<b>2.91</b>	$<1.0 \times 10^{-4}$
<i>RAB27B</i>	RAB27B, member RAS oncogene family	16.5	77.2	60.5	84.5	<b>5.98</b>	$<1.0 \times 10^{-4}$	<b>3.85</b>	$<1.0 \times 10^{-4}$	<b>5.62</b>	$<1.0 \times 10^{-4}$
<i>CH25H</i>	Cholesterol 25-hydroxylase	7.4	36.5	25.8	41.8	<b>4.41</b>	$<1.0 \times 10^{-4}$	<b>3.27</b>	0.022	<b>6.32</b>	$<1.0 \times 10^{-4}$
<i>USP43</i>	Ubiquitin specific peptidase 43	4.4	12.8	13.6	16.1	<b>3.94</b>	0.020	<b>3.41</b>	0.013	<b>4.50</b>	0.0091
<i>AC104966.1</i>	Ceruloplasmin (ferroxidase) (CP) pseudogene	16.5	47.6	57.3	53.7	<b>3.39</b>	$<1.0 \times 10^{-4}$	<b>3.79</b>	$<1.0 \times 10^{-4}$	<b>3.36</b>	$<1.0 \times 10^{-4}$
<i>KLK10</i>	Kallikrein related peptidase 10	14.0	37.1	33.0	43.1	<b>3.11</b>	0.022	<b>3.29</b>	0.0067	<b>2.65</b>	0.0028

Red = upregulated genes.

**Table 6.** Genes downregulated by all studied proinflammatory cytokines with FC  $< -2.5$ . Shown are mean normalized expression levels in control (Co), in C(IL1), C(IL17) and C(IFN $\gamma$ ) phenotypes, fold changes (FCs) for all comparisons vs. control and false discovery rate (FDR)-adjusted  $p$  values for them.

Gene	Name	Function	Mean exp. (Co)	Mean exp. (IL1)	Mean exp. (IL17)	Mean exp. (IFN $\gamma$ )	FC (IL1 vs. Co)	adj. p (IL1 vs. Co)	FC (IL17 vs. Co)	adj. p (IL17 vs. Co)	FC (IFN $\gamma$ vs. Co)	adj. p (IFN $\gamma$ vs. Co)
<i>SCN2B</i>	Sodium voltage-gated channel beta subunit 2	Sodium ion transport	170.8	8.7	65.9	63.7	<b>-22.19</b>	$<1.0 \times 10^{-4}$	<b>-2.59</b>	$<1.0 \times 10^{-4}$	<b>-2.90</b>	$<1.0 \times 10^{-4}$
<i>TNNT3</i>	Troponin T3, fast skeletal type	Skeletal muscle constituent	97.8	6.4	17.9	31.6	<b>-17.51</b>	$<1.0 \times 10^{-4}$	<b>-5.28</b>	$<1.0 \times 10^{-4}$	<b>-3.26</b>	$<1.0 \times 10^{-4}$
<i>MRAP2</i>	Melanocortin 2 receptor accessory protein 2	Metabolism?	1348.7	91.1	376.5	572.0	<b>-15.12</b>	$<1.0 \times 10^{-4}$	<b>-3.62</b>	$<1.0 \times 10^{-4}$	<b>-2.85</b>	$<1.0 \times 10^{-4}$
<i>WFDC1</i>	WAP four-disulfide core domain 1	Negative regulation of cell growth	60.1	6.1	34.9	18.0	<b>-12.06</b>	$<1.0 \times 10^{-4}$	<b>-2.68</b>	0.0019	<b>-4.06</b>	$<1.0 \times 10^{-4}$
<i>RANBP3L</i>	RAN binding protein 3 like	Nuclear export	654.8	74.6	284.8	280.0	<b>-9.40</b>	$<1.0 \times 10^{-4}$	<b>-2.54</b>	$<1.0 \times 10^{-4}$	<b>-2.60</b>	$<1.0 \times 10^{-4}$
<i>ASPN</i>	Asporin	Skeletal system development, negative regulation of TGF $\beta$ signaling	2094.0	206.3	505.2	837.5	<b>-8.28</b>	$<1.0 \times 10^{-4}$	<b>-3.92</b>	$<1.0 \times 10^{-4}$	<b>-2.77</b>	$<1.0 \times 10^{-4}$
<i>FGFR4</i>	Fibroblast growth factor receptor 4	Cell proliferation and migration	10.3	2.3	3.1	5.1	<b>-5.59</b>	$5.2 \times 10^{-4}$	<b>-3.12</b>	0.036	<b>-3.31</b>	0.045
<i>PTGER3</i>	Prostaglandin E receptor 3	Inflammation, cell death	494.1	173.6	162.3	188.8	<b>-2.69</b>	$<1.0 \times 10^{-4}$	<b>-3.03</b>	$<1.0 \times 10^{-4}$	<b>-2.82</b>	$<1.0 \times 10^{-4}$

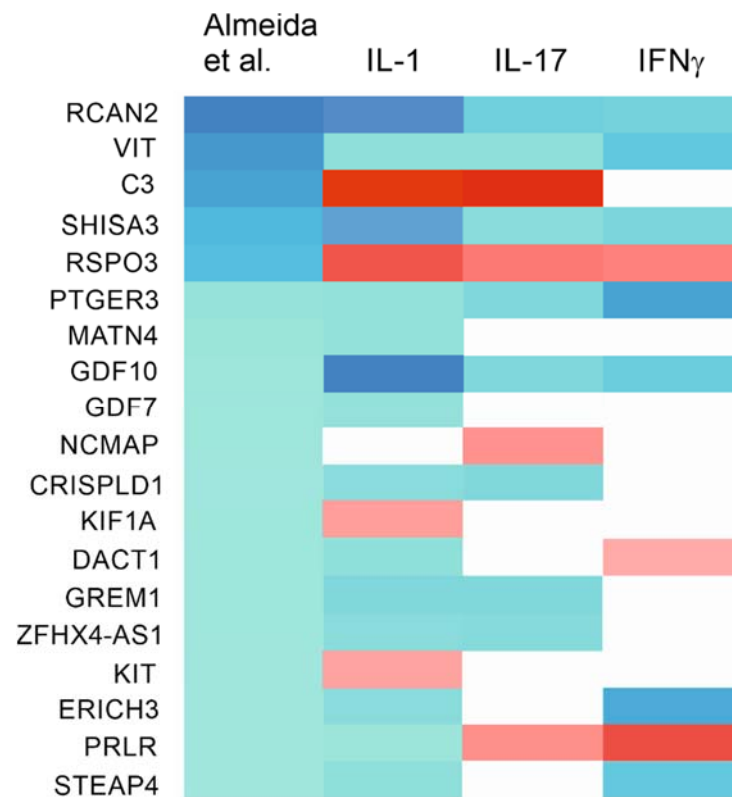
blue = downregulated genes.



**Figure 2.** Heatmap of genes markedly upregulated (FC > 2.5) in degraded cartilage in the study by Almeida et al. [35] and significantly affected by at least one studied proinflammatory cytokine. Upregulated genes are marked with red, downregulated with blue, and genes with no significant fold change with white.

In the study by Almeida et al. [35], 52 genes were associated with preserved rather than degraded cartilage (i.e., significantly downregulated in degraded cartilage with FC < −2.5). Of these, 19 were significantly affected by at least one of the proinflammatory cytokines in our data. In C(IL-1 $\beta$ ) cells, 13 of these 19 genes were significantly downregulated with *GDF10* displaying especially strong downregulation. In contrast, five of these genes were upregulated compared to control (including the especially strongly upregulated *C3* and *RSPO3*). This again shows that the net effect of IL-1 $\beta$  is to shift chondrocyte phenotype towards degraded cartilage. IFN $\gamma$  showed a directionally similar, but less pronounced effect: seven of the genes associated with preserved cartilage were significantly downregulated and three upregulated in the C(IFN $\gamma$ ) phenotype. In C(IL-17) cells, eight genes associated with preserved cartilage were down- and four upregulated; *C3* once again displayed especially strong upregulation. Five genes, including *PTGER3* and *GDF10*, were downregulated in all of the three chondrocyte phenotypes. On the other hand, *RSPO3* and *PRLR*, both downregulated in degraded compared with preserved cartilage, were upregulated by all of the three cytokines. These data indicate that the C(IL-1 $\beta$ ) and C(IL-17) phenotypes at least partly resemble the transcriptomic profile associated with degraded OA cartilage as identified by Almeida et al. [35]. In contrast, IFN $\gamma$  seems to have a smaller effect

on the genes directly linked to cartilage degradation in OA being instead characterized by the upregulation of genes associated with antigen processing and presentation. (Figure 3 and Table S5).



**Figure 3.** Heatmap of genes markedly downregulated ( $FC < -2.5$ ) in degraded cartilage in the study by Almeida et al. [35] and significantly affected by at least one studied proinflammatory cytokine. Upregulated genes are marked with red, downregulated with blue, and genes with no significant fold change with white.

Relatively few genes were significantly affected by IL-4 in our data, and none of them were markedly (with  $FC > 2.5$ ) associated with either degraded or preserved cartilage in the data of Almeida et al. [35]. However, looking at genes with a smaller proportional difference between degraded and preserved cartilage ( $FC > 1.5$  in either direction) produced several genes that were significantly affected by IL-4. Ten genes (including *DUSP5* and *COL7A1*) were upregulated in degraded cartilage and also upregulated in C(IL-4) cells. In contrast, one gene associated with degraded cartilage (*HMMR*) was downregulated by IL-4, and seven genes (including *COL14A1*) associated with preserved cartilage were upregulated by IL-4. (Table S6)

To demonstrate that naïve chondrocytes can be affected by the cytokines studied, we separately studied the expression of their receptors. As shown in Table S7, receptors for all studied cytokines were expressed in unstimulated OA chondrocytes at meaningful levels.

### 3. Discussion

Chondrocytes from OA patients were found to adopt distinct phenotypes in response to the central  $T_H1/T_H2/T_H17$  cytokines. The phenotype induced by the  $T_H1$  cytokine interleukin 1 (IL-1 $\beta$ ), the C(IL-1 $\beta$ ) phenotype, can be characterized by widespread, strong upregulation of inflammation and catabolism as well as downregulation of metabolic signaling. The effects of the  $T_H17$  cytokine IL-17 appear to be somewhat less widespread and partly overlapping those of IL-1 $\beta$ , with induction of inflammatory and chemotactic factors. The phenotype induced by the second  $T_H1$  cytokine interferon gamma (IFN $\gamma$ )

seems to be distinct from both C(IL-1 $\beta$ ) and C(IL-17) phenotypes, with a significant theme of antigen processing and presentation. The effects of the T<sub>H</sub>2 cytokine IL-4 were much more modest; some factors involved in the regulation of inflammation and TGF $\beta$  signaling were upregulated, while the downregulated genes were mostly associated with cell proliferation and migration.

In T cells, the T<sub>H</sub>1 phenotype drives inflammation and defense against intracellular pathogens (cell-mediated immunity) and is associated with the production of proinflammatory cytokines such as IFN $\gamma$  and IL-1 $\beta$  [36]. Conversely, T<sub>H</sub>2 cells promote humoral immunity, regulate inflammation and direct resolving and injury-healing responses [11]. Central T<sub>H</sub>2 cytokines are IL-4 and IL-13. A third relatively well-established population of T<sub>H</sub> cells is the T<sub>H</sub>17 phenotype. These cells produce IL-17, drive autoimmune reactions and activate neutrophils. This contrasts with T<sub>H</sub>1 cells that preferentially affect monocytes/macrophages, as well as T<sub>H</sub>2 cells that are associated with eosinophils, basophils and mast cells [37].

The central T<sub>H</sub>1/T<sub>H</sub>2/T<sub>H</sub>17 cytokines also induce loosely analogous macrophage phenotypes. Like T<sub>H</sub>1 cells, M1 or “classically activated” macrophages are induced by proinflammatory cytokines such as IL-1 $\beta$  and IFN $\gamma$  and promote inflammation by secreting further proinflammatory factors. M2 or “alternatively activated” macrophages are induced canonically by IL-4. In addition to functioning as antiparasite effectors, they attenuate inflammation, direct wound-healing processes and promote the resolution of inflammation. [38] IL-17 induces a less-studied macrophage phenotype characterized by increased chemotaxis and the production of proinflammatory factors such as cyclooxygenase 2 (COX-2), IL-6 and tumor necrosis factor alpha (TNF $\alpha$ ) [15,39] as well as resolution-promoting effects in the later phases of inflammation [16].

The chondrocyte phenotypes induced by different cytokines in our study can be considered analogous to T<sub>H</sub> cell and particularly macrophage phenotypes. IL-1 $\beta$  affects a very large number of genes and induces a phenotype characterized by the expression of inflammatory and matrix-degrading genes. The C(IL-17) phenotype appears likewise proinflammatory, but with a somewhat more limited repertoire of inflammatory genes. C(IFN $\gamma$ ) also appears to be a phenotype that is inflammatory, but is also characterized by genes linked to antigen presentation. The C(IL-4) phenotype is characterized by the expression of genes linked to TGF $\beta$  signaling and the regulation of inflammation.

The chondrocyte phenotypes induced by the T<sub>H</sub>1/T<sub>H</sub>2/T<sub>H</sub>17 cytokines appeared to be quite distinct as only 45 genes were markedly (FC > 2.5) upregulated and eight markedly downregulated (FC < -2.5) by all three proinflammatory cytokines, considering that hundreds of genes were up- and dozens downregulated to a similar extent by each of the three cytokines. The factors upregulated by all of the three proinflammatory cytokines (IL-1 $\beta$ , IFN $\gamma$  and IL-17) include the well-known inflammatory mediators *IL6*, nitric oxide synthase 2/inducible nitric oxide synthase (*NOS2/iNOS*) and prostaglandin-endoperoxide synthase 2/cyclooxygenase 2 (*PTGS2/COX-2*). On this list were also included, for example, pentraxin 3 (*PTX3*), toll-like receptor 2 (*TLR2*), chemokine (C-C motif) ligand 2 (*CCL2*), interferon regulatory factor 4 (*IRF4*) and prolactin receptor (*PRLR*). Pentraxin 3 (*PTX*) promotes inflammation by activating the classical complement pathway and by facilitating antigen recognition by mononuclear phagocytes [40], and it has been shown to be elevated in the serum and synovial fluid of patients with rheumatoid arthritis [41]. *TLR2* is a pattern recognition receptor mediating innate immune activation by microbial particles. In osteoarthritis, it is activated by hyaluronan and aggrecan fragments leading to the activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF- $\kappa$ B) signaling, which may contribute to OA progression and pain [42,43]. *CCL2* is a monocyte-attracting chemokine that has been linked to OA development and pain [44,45]. *IRF4* has recently been associated with cartilage destruction and pain in OA via the induction of *CCL17* [46]. Prolactin has been implicated to promote chondrocyte differentiation and attenuate apoptosis, and thus the upregulation of its receptor might promote cartilage survival [47,48].

Factors downregulated by all of the three proinflammatory cytokines include asporin (*ASP**N*) and prostaglandin EP3 receptor (*PTGER3*). Asporin belongs to the family of leucine-rich repeat proteins and is associated with cartilage matrix, also bearing a similarity to decorin [49]. The potential role of asporin in OA appears to be unclear; several studies have linked the protein to the development of the disease, where it might impair chondrogenesis by inhibiting TGF- $\beta$  signaling [50]. Polymorphisms of the asporin gene have also been linked to OA risk [51], even though the most recent meta-analysis failed to find evidence for this [52]. Prostaglandin E2 (PGE2)-induced *PTGER3* downregulation may contribute to cartilage inflammation and damage via NF- $\kappa$ B activation and IL-6 synthesis [53].

When the Gene Ontology (GO) terms significantly affected by the three different proinflammatory cytokines were studied, all three were found to affect those associated with inflammation. IL-1 $\beta$  was alone in significantly affecting several terms, such as cell adhesion, extracellular matrix metabolism and collagen catabolism, linking the chondrocyte phenotype induced by this cytokine to these functions. IL-17 solely affected nitric oxide synthase biosynthesis. This is intriguing, as the nitric oxide production is an important part of inflammatory response in chondrocytes [54]. The C(IFN $\gamma$ ) phenotype seems to be differentiated from others by activation of pathways related to antigen processing and presentation. Chondrocytes are not considered “professional” antigen-presenting cells, but they have, interestingly, been shown to present cartilage proteoglycans as antigens to CD8+ T cells, potentially contributing to local joint inflammation [55,56].

Previously published genome-wide expression analyses (GWEAs) have identified a number of differentially expressed genes between either damaged and intact OA cartilage or healthy and OA cartilage. These include genes involved in inflammation, skeletal system development, cell adhesion and monosaccharide metabolism [35,57–59]. When comparing our results to those of the comprehensive study by Almeida et al. [35], the C(IL-1 $\beta$ ) phenotype most closely resembled degraded OA cartilage, while IL-17 upregulated a smaller number of proinflammatory factors associated with degraded cartilage in that study. Accordingly, some genes associated with preserved as opposed to degraded cartilage were also downregulated by these proinflammatory cytokines. Most of these genes are linked to cartilage anabolism. The effects of IFN $\gamma$  and (especially) IL-4 on the genes identified by Almeida et al. [35] were more modest. It is important to note that the receptors for all cytokines studied were expressed at marked levels in our samples, which lends further validity to our results.

A potential limitation of the study is that whole thickness pieces of cartilage obtained from joint replacement surgery were used for chondrocyte isolation. Thus, the cells obtained are likely a mixture of chondrocytes from different layers of cartilage, and there might be some differences in the effects of cytokines between these groups. However, all chondrocytes can be expected to be exposed to cytokines diffused from the synovial fluid and/or produced by chondrocytes (in autocrine or paracrine manner). Thus, we think that the observed clear differences in the chondrocyte phenotypes in response to the major T<sub>H</sub>1/T<sub>H</sub>2/T<sub>H</sub>17 cytokines are relevant for further understanding of chondrocyte biology and OA pathophysiology. In future studies, cartilage layer-specific cell isolation methods or single-cell RNA-Seq could be considered to unravel possible zone-specific responses.

Another limitation of the study is that the chondrocytes used were obtained from OA joints; therefore, some of the detected effects of the cytokines might differ from those observed in healthy chondrocytes. Studying the effects of the cytokines on healthy chondrocytes would be an interesting avenue of future study; however, obtaining healthy primary human chondrocytes presents a practical challenge (compared to OA chondrocytes which can be obtained from joint replacement surgery). In the present study, we observed similarities between the C(IL-1 $\beta$ ) and C(IL-17) phenotypes and the gene expression profile of chondrocytes from degraded OA cartilage published by Almeida et al. [35]; C(IFN $\gamma$ ) and especially C(IL-4) bore less resemblance to that phenotype. This suggests that the cytokine-induced phenotypes observed in our data have relevance regarding OA pathogenesis.



In conclusion, OA chondrocytes, analogously to macrophages, can assume distinct phenotypes in response to the cytokines associated with the  $T_{H1}/T_{H2}/T_{H17}$  phenotypes of T helper cells. These results provide novel information on chondrocyte biology and the pathogenesis of OA with further insights into the development of disease-modifying drugs for (osteo)arthritis.

## 4. Materials and Methods

### 4.1. Cartilage and Cell Culture

Leftover cartilage pieces were collected from nine patients undergoing total knee replacement surgery in Coxa Hospital for Joint Replacement, Tampere, Finland. All patients fulfilled the American College of Rheumatology classification criteria for knee OA [60]. Patients with diabetes mellitus were excluded from the study to avoid potential confounding effects on chondrocyte metabolism [61]. The study was approved by the Ethics Committee of Tampere University Hospital, Finland, and carried out in accordance with the Declaration of Helsinki. Written informed consent was obtained from the patients. Chondrocyte isolation and culture was carried out as previously described [62]. To ensure an adequate yield of chondrocytes, all available cartilage was removed aseptically using a scalpel from the bony parts received from joint replacement surgery and cut into small pieces. The pieces were first washed with phosphate buffered saline (PBS). After that, they were incubated for 24 h in the presence of Liberase enzyme (Roche, Mannheim, Germany) 0.25 mg/mL, diluted in serumless Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich, St Louis, MO, USA) with glutamax-I containing penicillin (100 units/mL), streptomycin (100 µg/mL), and amphotericin B (250 ng/mL) (all three from Invitrogen, Carlsbad, CA, USA) at 37 °C. The resulting cell suspension was poured through a 70 µm nylon mesh and centrifuged for five minutes at 200 g. Cells were then washed twice and seeded on 24-well plates (0.2 million cells/mL) in DMEM supplemented with 10% heat-inactivated fetal bovine serum (Lonza) together with the aforementioned compounds. Confluent cultures were exposed to fresh culture medium alone, with 10 ng/mL IFN $\gamma$ , with 100 pg/mL IL-1 $\beta$ , with 50 ng/mL IL-17 or with 10 ng/mL IL-4, for 24 h. The concentrations used were chosen based on our preliminary experiments with cultured chondrocytes.

### 4.2. RNA Isolation and Sample Preparation

Culture medium was removed at the indicated time points and total RNA of the chondrocytes was extracted with GenElute Mammalian Total RNA Miniprep kit (Sigma-Aldrich). The sample was treated with DNase I (Fermentas UAB, Vilnius, Lithuania). RNA concentration and integrity were confirmed with the 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA).

### 4.3. Next Generation Sequencing and Data Analysis

Sequencing of samples was performed in the Finnish Institute of Molecular Medicine (FIMM) sequencing core, Helsinki, Finland, using the Illumina HiSeq 2500 sequencing platform. Sequencing depth was 20 million paired-end reads 100 bp in length. Read quality was first assessed using FastQC [63], and the reads were trimmed using Trimmomatic [64]. Trimmed reads were aligned to reference human genome with STAR [65]. Count matrices were prepared with the featureCounts program [66]. Differential expression was assessed with DESeq2 [67]. Gene expression levels were given as DESeq2-normalized counts, and genes with an average normalized count 10 or less across all samples were excluded from further analysis. For the purposes of further analysis, genes with a minimum of 2.5 fold change (FC) in abundance and FDR-corrected  $p$ -value < 0.05 were deemed biologically and statistically significant (unless otherwise indicated). Functional analysis was performed against the Gene Ontology (GO) database [68,69] using the DAVID tool [70], and REVIGO was used to reduce the resulting list [71].

#### 4.4. Statistics

For NGS data analysis, normalization was performed and differential expression studied using a negative binomial model implemented in DESeq2.

**Supplementary Materials:** The following are available online at <https://www.mdpi.com/article/10.3390/ijms22179463/s1>.

**Author Contributions:** Conceptualization, A.P., T.L., M.H., T.M. and E.M.; funding acquisition, A.P. and E.M.; investigation, A.P., T.L., M.H. and T.M.; methodology, A.P., T.L., M.H., T.M. and E.M.; project administration, T.M. and E.M.; resources, T.M. and E.M.; supervision, E.M.; validation, A.P., T.L. and M.H.; visualization, A.P.; writing—original draft, A.P.; writing—review and editing, A.P., T.L., M.H., T.M. and E.M. All authors have read and agreed to the published version of the manuscript.

**Funding:** This study was supported by grants from the Finnish Society of Rheumatology, Tampere Rheumatism Foundation, the competitive research funding of Pirkanmaa Hospital District and the Scandinavian Rheumatology Research Foundation. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

**Institutional Review Board Statement:** This study was approved by the Ethics Committee of Tampere University Hospital, Finland (ref# ETL R09116).

**Informed Consent Statement:** This study was approved by the Ethics Committee of Tampere University Hospital, Finland. Written informed consent was obtained from all subjects involved in the study.

**Data Availability Statement:** Complete gene expression data for all samples are available from the corresponding author upon reasonable request.

**Acknowledgments:** We wish to thank research coordinator Heli Kupari for her skillful assistance with the cartilage samples. We are also thankful to Meiju Kukkonen and Salla Hietakangas for their excellent technical assistance in the laboratory, as well as Heli Määttä for great secretarial help.

**Conflicts of Interest:** The authors declare that they have no competing interests.

## References

- Hunter, D.J.; Bierma-Zeinstra, S. Osteoarthritis. *Lancet* **2019**, *393*, 1745–1759. [[CrossRef](#)]
- Scanzello, C.R. Chemokines and inflammation in osteoarthritis: Insights from patients and animal models. *J. Orthop. Res.* **2017**, *35*, 735–739. [[CrossRef](#)] [[PubMed](#)]
- Greene, M.A.; Loeser, R.F. Aging-related inflammation in osteoarthritis. *Osteoarthr. Cartil.* **2015**, *23*, 1966–1971. [[CrossRef](#)] [[PubMed](#)]
- Robinson, W.H.; Lepus, C.M.; Wang, Q.; Raghu, H.; Mao, R.; Lindstrom, T.M.; Sokolove, J. Low-grade inflammation as a key mediator of the pathogenesis of osteoarthritis. *Nat. Rev. Rheumatol.* **2016**, *12*, 580–592. [[CrossRef](#)]
- Karsdal, M.A.; Michaelis, M.; Ladel, C.; Siebuhr, A.S.; Bihlet, A.R.; Andersen, J.R.; Guehring, H.; Christiansen, C.; Bay-Jensen, A.C.; Kraus, V.B. Disease-modifying treatments for osteoarthritis (DMOADs) of the knee and hip: Lessons learned from failures and opportunities for the future. *Osteoarthr. Cartil.* **2016**, *24*, 2013–2021. [[CrossRef](#)] [[PubMed](#)]
- Aigner, T.; Fundel, K.; Saas, J.; Gebhard, P.M.; Haag, J.; Weiss, T.; Zien, A.; Obermayr, F.; Zimmer, R.; Bartnik, E. Large-scale gene expression profiling reveals major pathogenetic pathways of cartilage degeneration in osteoarthritis. *Arthritis Rheum.* **2006**, *54*, 3533–3544. [[CrossRef](#)] [[PubMed](#)]
- Sandy, J.D.; Chan, D.D.; Trevino, R.L.; Wimmer, M.A.; Plaas, A. Human genome-wide expression analysis reorients the study of inflammatory mediators and biomechanics in osteoarthritis. *Osteoarthr. Cartil.* **2015**, *23*, 1939–1945. [[CrossRef](#)]
- Hardingham, T. Extracellular matrix and pathogenic mechanisms in osteoarthritis. *Curr. Rheumatol. Rep.* **2008**, *10*, 30–36. [[CrossRef](#)] [[PubMed](#)]
- Maldonado, M.; Nam, J. The Role of Changes in Extracellular Matrix of Cartilage in the Presence of Inflammation on the Pathology of Osteoarthritis. *BioMed Res. Int.* **2013**, *2013*, 284873. [[CrossRef](#)]
- Loeser, R.F.; Collins, J.A.; Diekman, B.O. Ageing and the pathogenesis of osteoarthritis. *Nat. Rev. Rheumatol.* **2016**, *12*, 412–420. [[CrossRef](#)]
- Zhu, J.; Paul, W.E. CD4 T cells: Fates, Functions, and Faults. *Blood, J. Am. Soc. Hematol.* **2008**, *112*, 1557–1569. [[CrossRef](#)] [[PubMed](#)]
- Dinarello, C.A. Immunological and Inflammatory Functions of the Interleukin-1 Family [Internet]. *Annu. Rev. Immunol.* **2009**, *27*, 519–550. [[CrossRef](#)]

13. Harrington, L.E.; Hatton, R.D.; Mangan, P.R.; Turner, H.; Murphy, T.L.; Murphy, K.M.; Weaver, C.T. Interleukin 17-Producing CD4+ Effector T Cells Develop via a Lineage Distinct from the T Helper Type 1 and 2 Lineages. *Nat. Immunol.* **2005**, *6*, 1123–1132. [[CrossRef](#)] [[PubMed](#)]
14. Kang, S.; Kumanogoh, A. The Spectrum of Macrophage Activation by Immunometabolism [Internet]. *Int. Immunol.* **2020**, *32*, 467–473. [[CrossRef](#)]
15. Raucci, F.; Saviano, A.; Casillo, G.M.; Guerra-Rodriguez, M.; Mansour, A.A.; Piccolo, M.; Ferraro, M.G.; Panza, E.; Vellecco, V.; Irace, C.; et al. IL-17-Induced Inflammation Modulates the mPGES-1/PPAR- $\gamma$  Pathway in Monocytes/Macrophages. *Br. J. Pharmacol.* **2021**. [[CrossRef](#)]
16. Zizzo, G.; Cohen, P.L. IL-17 Stimulates Differentiation of Human Anti-Inflammatory Macrophages and Phagocytosis of Apoptotic Neutrophils in Response to IL-10 and Glucocorticoids. *J. Immunol.* **2013**, *190*, 5237–5246. [[CrossRef](#)]
17. Minguzzi, M.; Cetrullo, S.; D'Adamo, S.; Silvestri, Y.; Flamigni, F.; Borzi, R.M. Emerging Players at the Intersection of Chondrocyte Loss of Maturational Arrest, Oxidative Stress, Senescence and Low-Grade Inflammation in Osteoarthritis. *Oxid. Med. Cell. Longev.* **2018**, *2018*, 3075293. [[CrossRef](#)]
18. López-Armada, M.J.; Caramés, B.; Lires-Deán, M.; Cillero-Pastor, B.; Ruiz-Romero, C.; Galdo, F.; Blanco, F.J. Cytokines, tumor necrosis factor-alpha and interleukin-1beta, differentially regulate apoptosis in osteoarthritis cultured human chondrocytes. *Osteoarthr. Cartil.* **2006**, *14*, 660–669. [[CrossRef](#)] [[PubMed](#)]
19. Daheshia, M.; Yao, J.Q. The interleukin 1beta pathway in the pathogenesis of osteoarthritis. *J. Rheumatol.* **2008**, *35*, 2306–2312. [[CrossRef](#)]
20. Martel-Pelletier, J.; McCollum, R.; DiBattista, J.; Faure, M.P.; Chin, J.A.; Fournier, S.; Sarfati, M.; Pelletier, J.P. The interleukin-1 receptor in normal and osteoarthritic human articular chondrocytes. Identification as the type I receptor and analysis of binding kinetics and biologic function. *Arthritis Rheum.* **1992**, *35*, 530–540. [[CrossRef](#)]
21. Jotanovic, Z.; Mihelic, R.; Sestan, B.; Dembic, Z. Role of Interleukin-1 Inhibitors in Osteoarthritis. *Drugs Aging* **2012**, *29*, 343–358. [[CrossRef](#)]
22. Yao, Z.; Painter, S.L.; Fanslow, W.C.; Ulrich, D.; Macduff, B.M.; Spriggs, M.K.; Armitage, R.J. Human IL-17: A novel cytokine derived from T cells. *J. Immunol.* **1995**, *155*, 5483–5486.
23. Miossec, P.; Korn, T.; Kuchroo, V.K. Interleukin-17 and Type 17 Helper T Cells. *N. Engl. J. Med.* **2009**, *361*, 888–898. [[CrossRef](#)]
24. Chen, B.; Deng, Y.; Tan, Y.; Qin, J.; Chen, L.-B. Association between severity of knee osteoarthritis and serum and synovial fluid interleukin 17 concentrations. *J. Int. Med. Res.* **2014**, *42*, 138–144. [[CrossRef](#)]
25. Martel-Pelletier, J.; Mineau, F.; Jovanovic, D.; Di Battista, J.A.; Pelletier, J.-P. Mitogen-activated protein kinase and nuclear factor  $\kappa$ B together regulate interleukin-17-induced nitric oxide production in human osteoarthritic chondrocytes: Possible role of transactivating factor mitogen-activated protein kinase-activated protein kinase. *Arthritis Rheum.* **1999**, *42*, 2399–2409. [[CrossRef](#)]
26. Pacquelet, S.; Presle, N.; Boileau, C.; Dumond, H.; Netter, P.; Martel-Pelletier, J.; Pelletier, J.-P.; Terlain, B.; Jouzeau, J.-Y. Interleukin 17, a nitric oxide-producing cytokine with a peroxynitrite-independent inhibitory effect on proteoglycan synthesis. *J. Rheumatol.* **2002**, *29*, 2602–2610.
27. Benderdour, M.; Tardif, G.; Pelletier, J.-P.; Di Battista, J.A.; Reboul, P.; Ranger, P.; Martel-Pelletier, J. Interleukin 17 (IL-17) induces collagenase-3 production in human osteoarthritic chondrocytes via AP-1 dependent activation: Differential activation of AP-1 members by IL-17 and IL-1beta. *J. Rheumatol.* **2002**, *29*, 1262–1272. [[PubMed](#)]
28. Nakashima, T.; Kobayashi, Y.; Yamasaki, S.; Kawakami, A.; Eguchi, K.; Sasaki, H.; Sakai, H. Protein Expression and Functional Difference of Membrane-Bound and Soluble Receptor Activator of NF- $\kappa$ B Ligand: Modulation of the Expression by Osteotropic Factors and Cytokines. *Biochem. Biophys. Res. Commun.* **2000**, *275*, 768–775. [[CrossRef](#)] [[PubMed](#)]
29. Nakae, S.; Nambu, A.; Sudo, K.; Iwakura, Y. Suppression of immune induction of collagen-induced arthritis in IL-17-deficient mice. *J. Immunol.* **2003**, *171*, 6173–6177. [[CrossRef](#)] [[PubMed](#)]
30. Chabaud, M.; Lubberts, E.; Joosten, L.; van den Berg, W.; Miossec, P. IL-17 derived from juxta-articular bone and synovium contributes to joint degradation in rheumatoid arthritis. *Arthritis Res.* **2001**, *3*, 168. [[CrossRef](#)] [[PubMed](#)]
31. Xu, Y.; Barter, M.J.; Swan, D.C.; Rankin, K.S.; Rowan, A.D.; Santibanez-Koref, M.; Loughlin, J.; Young, D.A. Identification of the pathogenic pathways in osteoarthritic hip cartilage: Commonality and discord between hip and knee OA. *Osteoarthr. Cartil.* **2012**, *20*, 1029–1038. [[CrossRef](#)] [[PubMed](#)]
32. Santangelo, K.S.; Nuovo, G.J.; Bertone, A.L. In vivo reduction or blockade of interleukin-1 $\beta$  in primary osteoarthritis influences expression of mediators implicated in pathogenesis. *Osteoarthr. Cartil.* **2012**, *20*, 1610–1618. [[CrossRef](#)] [[PubMed](#)]
33. Hoff, P.; Buttgerit, F.; Burmester, G.-R.; Jakstadt, M.; Gaber, T.; Andreas, K.; Matziolis, G.; Perka, C.; Röhner, E. Osteoarthritis synovial fluid activates pro-inflammatory cytokines in primary human chondrocytes. *Int. Orthop.* **2013**, *37*, 145–151. [[CrossRef](#)]
34. Li, S.; Ren, Y.; Peng, D.; Yuan, Z.; Shan, S.; Sun, H.; Yan, X.; Xiao, H.; Li, G.; Song, H. TIM-3 Genetic Variations Affect Susceptibility to Osteoarthritis by Interfering with Interferon Gamma in CD4+ T Cells. *Inflammation* **2015**, *38*, 1857–1863. [[CrossRef](#)] [[PubMed](#)]
35. de Almeida, R.C.; Ramos, Y.F.; Mahfouz, A.; den Hollander, W.; Lakenberg, N.; Houtman, E.; van Hoolwerff, M.; Suchiman, H.E.D.; Rodríguez Ruiz, A.; Slagboom, P.E.; et al. RNA sequencing data integration reveals an miRNA interactome of osteoarthritis cartilage. *Ann. Rheum Dis.* **2019**, *78*, 270–277. [[CrossRef](#)]
36. Chizzolini, C.; Chiccheportiche, R.; Burger, D.; Dayer, J.M. Human Th1 Cells Preferentially Induce Interleukin (IL)-1 $\beta$  While Th2 Cells Induce IL-1 Receptor Antagonist Production upon Cell/Cell Contact with Monocytes. *Eur. J. Immunol.* **1997**, *27*, 171–177. [[CrossRef](#)]

37. Weaver, C.T.; Elson, C.O.; Fouser, L.A.; Kolls, J.K. The Th17 Pathway and Inflammatory Diseases of the Intestines, Lungs and Skin. *Annu. Rev. Pathol.* **2013**, *8*, 477. [[CrossRef](#)] [[PubMed](#)]
38. Hume, D.A. The Many Alternative Faces of Macrophage Activation. *Front Immunol.* **2015**, *6*, 370. [[CrossRef](#)]
39. Alonso, M.N.; Wong, M.T.; Zhang, A.L.; Winer, D.; Suhoski, M.M.; Tolentino, L.L.; Gaitan, J.; Davidson, M.G.; Kung, T.H.; Galel, D.M.; et al. TH1, TH2, and TH17 Cells Instruct Monocytes to Differentiate into Specialized Dendritic Cell Subsets. *Blood J. Am. Soc. Hematol.* **2011**, *118*, 3311–3320. [[CrossRef](#)]
40. Diniz, S.N.; Nomizo, R.; Cisalpino, P.S.; Teixeira, M.M.; Brown, G.D.; Mantovani, A.; Gordon, S.; Reis, L.F.L.; Dias, A.A.M. PTX3 function as an opsonin for the dectin-1-dependent internalization of zymosan by macrophages. *J. Leukoc. Biol.* **2004**, *75*, 649–656. [[CrossRef](#)]
41. Sharma, A.; Khan, R.; Gupta, N.; Sharma, A.; Zaheer, M.S.; Abbas, M.; Khan, S.A. Acute phase reactant, Pentraxin 3, as a novel marker for the diagnosis of rheumatoid arthritis. *Clin. Chim. Acta* **2018**, *480*, 65–70. [[CrossRef](#)] [[PubMed](#)]
42. Liu, Y.-X.; Wang, G.-D.; Wang, X.; Zhang, Y.-L.; Zhang, T.-L. Effects of TLR-2/NF- $\kappa$ B signaling pathway on the occurrence of degenerative knee osteoarthritis: An in vivo and in vitro study. *Oncotarget* **2017**, *8*, 38602–38617. [[CrossRef](#)] [[PubMed](#)]
43. Miller, R.E.; Ishihara, S.; Tran, P.B.; Golub, S.B.; Last, K.; Miller, R.J.; Fosang, A.J.; Malfait, A.-M. An aggrecan fragment drives osteoarthritis pain through Toll-like receptor 2. *JCI Insight* **2018**, *3*, e95704. [[CrossRef](#)]
44. Raghu, H.; Lepus, C.M.; Wang, Q.; Wong, H.H.; Lingampalli, N.; Oliviero, F.; Punzi, L.; Giori, N.J.; Goodman, S.B.; Chu, C.R.; et al. CCL2/CCR2, but not CCL5/CCR5, mediates monocyte recruitment, inflammation and cartilage destruction in osteoarthritis. *Ann. Rheum. Dis.* **2017**, *76*, 914–922. [[CrossRef](#)]
45. Miller, R.J.; Malfait, A.M.; Miller, R.E. The Innate Immune Response as a Mediator of Osteoarthritis Pain [Internet]. *Osteoarthr. Cartil.* **2020**, *28*, 562–571. [[CrossRef](#)]
46. Lee, M.-C.; Saleh, R.; Achuthan, A.; Fleetwood, A.J.; Förster, I.; Hamilton, J.A.; Cook, A.D. CCL17 blockade as a therapy for osteoarthritis pain and disease. *Arthritis Res. Ther.* **2018**, *20*, 62. [[CrossRef](#)]
47. Adañ, N.; Guzmán-Morales, J.; Ledesma-Colunga, M.G.; Perales-Canales, S.I.; Quintanar-Stéphano, A.; López-Barrera, F.; Méndez, I.; Moreno-Carranza, B.; Triebel, J.; Binart, N.; et al. Prolactin promotes cartilage survival and attenuates inflammation in inflammatory arthritis. *J. Clin. Investig.* **2013**, *123*, 3902–3913. [[CrossRef](#)]
48. Ogueta, S.; Muñoz, J.; Obregon, E.; Delgado-Baeza, E.; García-Ruiz, J.P. Prolactin Is a Component of the Human Synovial Liquid and Modulates the Growth and Chondrogenic Differentiation of Bone Marrow-Derived Mesenchymal Stem Cells. *Mol. Cell. Endocrinol.* **2002**, *190*, 51–63. [[CrossRef](#)]
49. Lorenzo, P.; Aspberg, A.; Onnerfjord, P.; Bayliss, M.T.; Neame, P.J.; Heinegard, D. Identification and characterization of asporin, a novel member of the leucine-rich repeat protein family closely related to decorin and biglycan. *J. Biol. Chem.* **2001**, *276*, 12201–12211. [[CrossRef](#)]
50. Xu, L.; Li, Z.; Liu, S.-Y.; Xu, S.-Y.; Ni, G.-X. Asporin and osteoarthritis. *Osteoarthr. Cartil.* **2015**, *23*, 933–939. [[CrossRef](#)] [[PubMed](#)]
51. Zhu, X.; Jiang, L.; Lu, Y.; Wang, C.; Zhou, S.; Wang, H.; Tian, T. Association of aspartic acid repeat polymorphism in the asporin gene with osteoarthritis of knee, hip, and hand. *Medicine (Baltimore)* **2018**, *97*, e0200. [[CrossRef](#)]
52. Wang, J.; Yang, A.; Zhang, J.; Sun, N.; Li, X.; Li, X.; Liu, Q.; Li, J.; Ren, X.; Ke, Z.; et al. Genetic polymorphism in the asporin gene is not a key risk factor for osteoarthritis: Evidence based on an updated cumulative meta-analysis. *Exp. Ther. Med.* **2018**, *15*, 3952–3966. [[CrossRef](#)]
53. Wang, P.; Zhu, F.; Lee, N.H.; Konstantopoulos, K. Shear-induced interleukin-6 synthesis in chondrocytes: Roles of E prostanoid (EP) 2 and EP3 in cAMP/protein kinase A- and PI3-K/Akt-dependent NF- $\kappa$ B activation. *J. Biol. Chem.* **2010**, *285*, 24793–24804. [[CrossRef](#)]
54. Eitner, A.; Müller, S.; König, C.; Wilharm, A.; Raab, R.; Hofmann, G.O.; Kamradt, T.; Schaible, H.G. Inhibition of Inducible Nitric Oxide Synthase Prevents il-1 $\beta$ -Induced Mitochondrial Dysfunction in Human Chondrocytes. *Int. J. Mol. Sci.* **2021**, *22*, 2477. [[CrossRef](#)]
55. Kuhne, M.; Erben, U.; Schulze-Tanzil, G.; Köhler, D.; Wu, P.; Richter, F.J.; John, T.; Radbruch, A.; Sieper, J.; Appel, H. HLA-B27-Restricted Antigen Presentation by Human Chondrocytes to CD8+ T Cells: Potential Contribution to Local Immunopathologic Processes in Ankylosing Spondylitis. *Arthritis Rheum.* **2009**, *60*, 1635–1646. [[CrossRef](#)] [[PubMed](#)]
56. Brennan, F.R.; Mikecz, K.; Buzás, E.I.; Glant, T.T. Interferon-Gamma but not Granulocyte/Macrophage Colony-Stimulating Factor Augments Proteoglycan Presentation by Synovial Cells and Chondrocytes to an Autopathogenic T Cell Hybridoma. *Immunol. Lett.* **1995**, *45*, 87–91. [[CrossRef](#)]
57. Lewallen, E.A.; Bonin, C.A.; Li, X.; Smith, J.; Karperien, M.; Larson, A.N.; Lewallen, D.G.; Cool, S.M.; Westendorf, J.J.; Krych, A.J.; et al. The synovial microenvironment of osteoarthritic joints alters RNA-seq expression profiles of human primary articular chondrocytes. *Gene* **2016**, *591*, 456–464. [[CrossRef](#)]
58. Dunn, S.L.; Soul, J.; Anand, S.; Schwartz, J.-M.; Boot-Handford, R.P.; Hardingham, T.E. Gene expression changes in damaged osteoarthritic cartilage identify a signature of non-chondrogenic and mechanical responses. *Osteoarthr. Cartil.* **2016**, *24*, 1431–1440. [[CrossRef](#)] [[PubMed](#)]
59. Ramos, Y.F.M.; den Hollander, W.; Bovée, J.V.M.G.; Bomer, N.; Breggen, R.; Lakenberg, N.; Keurentjes, J.C.; Goeman, J.J.; Slagboom, P.E.; Nelissen, R.G.H.H.; et al. Genes Involved in the Osteoarthritis Process Identified through Genome Wide Expression Analysis in Articular Cartilage; the RAAK Study. *PLoS ONE* **2014**, *9*, e103056. [[CrossRef](#)] [[PubMed](#)]

60. Altman, R.; Asch, E.; Bloch, D.; Bole, G.; Borenstein, D.; Brandt, K.; Christy, W.; Cooke, T.D.; Greenwald, R.; Hochberg, M.; et al. Development of criteria for the classification and reporting of osteoarthritis: Classification of osteoarthritis of the knee. *Arthritis Rheum.* **1986**, *29*, 1039–1049. [[CrossRef](#)]
61. Mobasheri, A.; Vannucci, S.J.; Bondy, C.A.; Carter, S.D.; Innes, J.F.; Arteaga, M.F.; Trujillo, E.; Ferraz, I.; Shakibaei, M.; Martín-Vasallo, P. Glucose transport and metabolism in chondrocytes: A key to understanding chondrogenesis, skeletal development and cartilage degradation in osteoarthritis. *Histol. Histopathol.* **2002**, *17*, 1239–1267. [[PubMed](#)]
62. Koskinen, A.; Juslin, S.; Nieminen, R.; Moilanen, T.; Vuolteenaho, K.; Moilanen, E. Adiponectin associates with markers of cartilage degradation in osteoarthritis and induces production of proinflammatory and catabolic factors through mitogen-activated protein kinase pathways. *Arthritis Res. Ther.* **2011**, *13*, R184. [[CrossRef](#)] [[PubMed](#)]
63. Andrews, S. FastQC: A Quality Control Tool for High Throughput Sequence Data. 2010. Available online: <https://www.bioinformatics.babraham.ac.uk/projects/fastqc/> (accessed on 19 April 2021).
64. Bolger, A.M.; Lohse, M.; Usadel, B. Trimmomatic: A flexible trimmer for Illumina sequence data. *Bioinformatics* **2014**, *30*, 2114–2120. [[CrossRef](#)] [[PubMed](#)]
65. Dobin, A.; Davis, C.A.; Schlesinger, F.; Drenkow, J.; Zaleski, C.; Jha, S.; Batut, P.; Chaisson, M.; Gingeras, T.R. STAR: Ultrafast universal RNA-seq aligner. *Bioinformatics* **2013**, *29*, 15–21. [[CrossRef](#)]
66. Liao, Y.; Smyth, G.K.; Shi, W. featureCounts: An efficient general purpose program for assigning sequence reads to genomic features. *Bioinformatics* **2014**, *30*, 923–930. [[CrossRef](#)]
67. Love, M.I.; Huber, W.; Anders, S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol.* **2014**, *15*, 550. [[CrossRef](#)]
68. Ashburner, M.; Ball, C.A.; Blake, J.A.; Botstein, D.; Butler, H.; Cherry, J.M.; Davis, A.P.; Dolinski, K.; Dwight, S.S.; Eppig, J.T.; et al. Gene Ontology: Tool for the unification of biology. *Nat. Genet.* **2000**, *25*, 25–29. [[CrossRef](#)]
69. The Gene Ontology Consortium. Expansion of the Gene Ontology knowledgebase and resources. *Nucleic Acids Res.* **2017**, *45*, D331–D338. [[CrossRef](#)]
70. Huang, D.W.; Sherman, B.T.; Lempicki, R.A. Systematic and Integrative Analysis of Large Gene Lists Using DAVID Bioinformatics Resources. *Nat. Protoc.* **2009**, *4*, 44–57. [[CrossRef](#)]
71. Supek, F.; Bošnjak, M.; Škunca, N.; Šmuc, T. REVIGO Summarizes and Visualizes Long Lists of Gene Ontology Terms. *PLoS ONE* **2011**, *6*, e21800. [[CrossRef](#)]