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► **To cite this version:**

C. Cherifi, A. Latourte, S. Vettorazzi, J. Tuckermann, S. Provot, et al.. Inhibition of sphingosine 1-phosphate protects mice against chondrocyte catabolism and osteoarthritis. *Osteoarthritis and Cartilage*, 2021, 29 (9), pp.1335-1345. 10.1016/j.joca.2021.06.001 . hal-04202734

**HAL Id: hal-04202734**

**<https://hal.science/hal-04202734>**

Submitted on 22 Jul 2024

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## **Inhibition of sphingosine 1-phosphate protects mice against chondrocyte catabolism and osteoarthritis**

Chahrazad Cherifi<sup>1</sup>, Augustin Latourte<sup>1,2</sup>, Sabine Vettorazzi<sup>3</sup>, Jan Tuckermann<sup>3</sup>, Sylvain Provot<sup>1</sup>, Hang-Korng Ea<sup>1,2</sup>, Adeline Ledoux<sup>4</sup>, Josefina Casas<sup>5,6</sup>, Olivier Cuvillier<sup>4</sup>, Pascal Richette<sup>1,2</sup>, Agnes Ostertag<sup>1</sup>, Eric Hay<sup>1</sup> and Martine Cohen-Solal<sup>1,2</sup>

1- Université de Paris, BIOSCAR Inserm U1132, F-75010 Paris, France

2- Service de rhumatologie, AP-HP, Hôpital Lariboisière, F-75010 Paris, France

3- Institute of Comparative Molecular Endocrinology, University of Ulm, ZC 89081, Germany

4- Institut de Pharmacologie et de Biologie Structurale, Université de Toulouse, CNRS, UPS, F-31000 Toulouse, France

5- RUBAM, Department Biological Chemistry, IQAC-CSIC, Jordi Girona 18-26, S-08034 Barcelona, Spain

6- Networking Biomedical Research Centre (CIBEREHD), S-28706 Madrid, Spain

Corresponding author:

Pr Martine Cohen-Solal

Inserm U1132 and université de Paris

Hôpital Lariboisière, 2 rue Ambroise Pare, 75010 Paris

Phone: +33 149956358, Fax : +33 149958452

E-mail: [martine.cohen-solal@inserm.fr](mailto:martine.cohen-solal@inserm.fr)

**Running title:** Inhibition of sphingosine 1-phosphate protects against osteoarthritis

## Abstract

Objective: Cartilage loss observed in osteoarthritis (OA) is prevented when osteoclasts in the subchondral bone are inhibited in mice. Here, we investigated the role of the osteoclast secretome and of the lipid mediator sphingosine 1-phosphate (S1P) in chondrocyte metabolism and OA.

Materials and methods: We used *SphK1<sup>LysM<sup>Cre</sup></sup>* and wild type mice to assess the effect of murine osteoclast secretome in chondrocyte metabolism. Gene and protein expressions of matrix metalloproteinase (*Mmp*) were quantified in chondrocytes and explants by RT-qPCR and Western blots. *SphK1<sup>LysM<sup>Cre</sup></sup>* mice or wild type mice treated with S1P<sub>2</sub> receptor inhibitor JTE013 or anti-S1P neutralizing antibody sphingomab are analyzed by OA score and immunohistochemistry.

Results: The osteoclast secretome increased the expression of *Mmp3* and *Mmp13* in murine chondrocytes and cartilage explants and activated the JNK signaling pathway, which led to matrix degradation. JTE013 reversed the osteoclast-mediated chondrocyte catabolism and protected mice against OA, suggesting that osteoclastic S1P contributes to cartilage damage in OA via S1P/S1P<sub>2</sub> signaling. The activity of sphingosine kinase 1 (SphK1) increased with osteoclast differentiation, and its expression was enhanced in subchondral bone of mice with OA. The expression of *Mmp3* and *Mmp13* in chondrocytes was low upon stimulation with the secretome of *Sphk1*-lacking osteoclasts. Cartilage damage was significantly reduced in *SphK1<sup>LysM<sup>Cre</sup></sup>* mice, but not the synovial inflammation. Finally, intra-articular administration of sphingomab inhibited the cartilage damage and synovial inflammation.

Conclusions: lack of S1P in myeloid cells and local S1P neutralization alleviates from osteoarthritis in mice. These data identify S1P as a therapeutic target in OA.

## 1 Introduction

2

3 Cartilage remodeling is a physiological process that is disrupted in osteoarthritis (OA), leading to a  
4 loss of cartilage matrix <sup>1</sup>. OA is one of the leading causes of disability worldwide and its prevalence is  
5 steadily increasing <sup>2-4</sup>. No disease-modifying drug is currently available for OA, making the OA  
6 management a therapeutical challenge. Cartilage loss involves multiple pathophysiological  
7 mechanisms responsible for cartilage damage <sup>5,6,7</sup>, including enhanced chondrocyte catabolism <sup>8</sup>.  
8 Changes occur in all tissues in OA joints <sup>9</sup>, including subchondral bone alterations that promote or  
9 sustain the development of OA <sup>10</sup>. In this regard, there is evidence for a crosstalk between cartilage  
10 and subchondral bone leading to progressive cartilage destruction <sup>11-13</sup>. The increased subchondral  
11 bone resorption observed at early stages of OA is mediated by osteoclasts (OCs) <sup>14</sup>, and anti-  
12 resorptive therapies targeting OCs prevent OA in murine models <sup>15,16</sup>. Although subchondral bone  
13 OCs precursors may directly contribute to chondrocyte catabolism, the factors involved in this  
14 crosstalk, particularly the role of the OC secretome in cartilage breakdown, remain elusive.

15 Sphingosine 1-phosphate (S1P), a downstream metabolite of ceramide, is a lipid mediator involved in  
16 a number of pathophysiological conditions and disorders including inflammation and auto-immune  
17 diseases <sup>17</sup>. The balance between levels of ceramide and S1P has been considered a switch  
18 determining whether a cell proliferates or dies <sup>18</sup>. S1P is synthesized by two sphingosine kinase  
19 isoforms, SphK1 and SphK2, and its degradation is also controlled by S1P phosphatases or S1P lyase  
20 <sup>19,20</sup>. Besides the intracellular role of S1P, most of its biological effects are explained by its binding to a  
21 family of five G protein-coupled receptors, S1P<sub>1-5</sub>, that mediate specific effects according to the  
22 predominant subtypes expressed in cells <sup>21</sup>. In bone, S1P regulates bone remodeling: S1P lyase-  
23 deficient mice, which have high circulating S1P levels, show a high bone mass phenotype <sup>22</sup>. S1P is  
24 released by osteoblasts <sup>23</sup> and OCs <sup>24</sup> and contributes to the coupling of OCs and osteoblasts <sup>24,25</sup>.  
25 S1P mobilizes OC precursors in the blood and the bone microenvironment via a local gradient,  
26 thereby regulating bone homeostasis <sup>25-27</sup>. Furthermore, increased S1P secretion in OCs triggered  
27 osteoblast differentiation in a paracrine manner <sup>28</sup>.

28 Given the role of S1P in bone remodeling and because bone resorption increases cartilage  
29 metabolism, we hypothesized that S1P produced by osteoclasts or their progenitors could mediate  
30 cartilage catabolism and OA.

## 31 **Materials and Methods**

32

### 33 **Animal experimentation and ethics**

34 Mouse models are reported according to the principles of the ARRIVE guidelines. All animal protocols  
35 were approved by the local animal ethics committee (Apafis3817-2016012616163545). Only male  
36 mice were used for all the experiments. The sample size of mice per group was determined at the  
37 beginning of the experiments to achieve statistical power for the OA score. A number of 6-10 animals  
38 is required to this task. In experiments with genetic mice, littermates were used as controls and mice  
39 were randomly assigned to treatment groups. No animal was excluded except for technical reasons  
40 such as failure of tissue embedding or staining or poor RNA quality.

41 OA was induced by meniscectomy (MNX) of the right knee in 10 week-old mice as described  
42 previously <sup>29</sup>. Briefly, knee joint instability was induced surgically in the right knee by medial partial  
43 meniscectomy. Surgery was performed under a binocular magnifier (X15) using a Sharpoint  
44 microsurgical stab knife. Mice were placed in dorsal position, knee flexed and right foot taped. After  
45 skin incision, the medial femoro-tibial ligament was cut, a short incision of the medial side of  
46 quadriceps muscle was performed, the knee capsule was cleaved and the patella was sub-luxated  
47 laterally. After section of the meniscotibial ligament, the medial meniscus was gently pulled out and  $\frac{3}{4}$   
48 of its anterior horn removed. Then, the patella was replaced, the quadriceps muscle and the skin plan  
49 sutured. Control animals underwent sham surgery (ligament visualization but not dissection) and done  
50 by a single trained operator for all experiments. Ketamine/xylazine mix and buprenorphin was  
51 administered to sedated mice, and a second injection of buprenorphin was given the day after surgery.  
52 *Sphk1 lox/lox* and *LysM-Cre* mice were generated as described <sup>30</sup>. SPHK1 was specifically deleted in  
53 monocyte/OC lineages by crossing *Sphk1 lox/lox* mice to *LysM-Cre* mice (mixed background).

54 In experiments using neutralization of S1P, C57Bl/6 mice received after anesthesia an intra-articular  
55 injection of 2 or 4  $\mu$ g anti-S1P monoclonal antibody kindly provided by LPath (San Diego, USA; <sup>31</sup>)  
56 once a week (n=8) or IgG as a control (n=7) for 6 weeks from the day after the DMM. To inhibit S1P<sub>2</sub>  
57 signaling, JTE013, 4 mg/kg, was administered to C57Bl/6 mice by intra-articular injection in the knee  
58 (n=8) once a week <sup>32</sup>. All animals were sacrificed at week 6.

59

### 60 **Osteoclast (OC) culture**

61 RAW264.7 monocyte cells were cultured in 6-well plates (400 000 cells/well). On day 2 and 4,  
62 differentiation was induced by treating cells with the receptor activator of nuclear factor kappa-B ligand  
63 (RANKL, 30 or 50 or 100 nM; Peprotech, France). At day 5, differentiated OCs were washed 3 times  
64 with phosphate buffered saline 1X and fresh medium was added. Finally, OC and RAW264.7 cell  
65 conditioned medium (OC-CM and RAW-CM, respectively) was collected on day 6 and stored at -80°C.  
66 To generate primary OCs, bone marrow isolated from *Sphk1<sup>flox</sup>* or *SphK1<sup>LysMCre</sup>* mice at age 10 weeks  
67 were differentiated, treated with macrophage-colony stimulating factor alone (MCSF, 100 nM;  
68 Peprotech, France) or both MCSF and RANKL for 5 days. Monocyte and OC conditioned medium was  
69 collected under the same conditions as described above.

70

### 71 **Culture of primary chondrocytes**

72 Primary chondrocytes were harvested and cultured as previously reported <sup>29,33</sup>. Briefly, articular  
73 cartilage from the tibia plateau, femoral condyle and femoral head was obtained from 5- to 6-day-old  
74 mice. After matrix digestion with Liberase (Roche, France), chondrocytes were plated in 12-well plates  
75 (200,000 cells/well) and harvested in Dulbecco's modified Eagle's medium (DMEM, Gibco Life  
76 Technologies, France) with 10% decompemented fetal bovine serum (FBS), 2% L-glutamine and 1%  
77 penicillin/streptomycin until confluence. Chondrocytes were then stimulated with Oc-CM or interleukin  
78 1 $\beta$  (10 nM) for 24 h, with or without 5  $\mu$ M of the S1P2 inhibitor JTE013, or 10 nM of the S1P<sub>1</sub>/S1P<sub>3</sub>  
79 antagonist VPC23019, 10  $\mu$ M JNK inhibitor SP600125 or 1, 2, 3 or 6  $\mu$ M S1P (all R&D Systems,  
80 France). To investigate signaling pathways, cells were pre-treated with JTE013 for 1 h, and then  
81 stimulated with OC-CM for 5, 10 or 15 min. Chondrocyte supernatant was collected to evaluate the  
82 release of proteoglycans and MMPs in the medium.

83

### 84 **Transfection of primary chondrocytes**

85 At 80% confluence, chondrocytes and macrophages obtained from *Sphk1* floxed mice were  
86 transfected with adenovirus expressing Cre recombinase (200 multiplicity of infection, Vector Biolabs,  
87 US) to induce recombination. The control virus without the inserted gene (*Mock*) was a control. Small  
88 interfering RNA (siRNA) targeting S1P<sub>2</sub> (50 nM, Santa Cruz Biotechnology) was used to induce S1P<sub>2</sub>  
89 deletion in chondrocytes primed with interferin (Polyplus-transfection, Illkirch, France). Experiments  
90 were performed twice.

91 **Culture of cartilage explants**

92 Femoral head cartilage explants from 10-week-old male mice were cultured in DMEM with 10% FBS,  
93 3 explants per well. After 24 h of culture, explants were stimulated with Oc-CM with or without JTE013  
94 for 48 h. Explants were removed, fixed in paraformaldehyde (PFA) (4%) and decalcified with EDTA  
95 (0.5 M) for 24 h at room temperature and prepared for cryosectioning. Results are from 3 separate  
96 experiments performed in triplicate.

97

98 **Human explants**

99 Human cartilage samples were harvested from patients who underwent total knee replacement  
100 surgery. Samples were obtained in accordance with the guidelines and regulations of the French  
101 National Authority Legislation for the collection of human tissues. Collections were approved by the  
102 ethical committee of the institution. Informed consents were obtained from patients and stored in the  
103 medical record. Cartilage samples were collected from the femoral condyle at the posterior surface of  
104 the knees. We collected samples in a zone that appeared macroscopically undamaged defined by  
105 white and shiny cartilage without lesions and in a zone that appeared damaged defined by a  
106 discoloration with an irregular surface.

107

108 **Histology**

109 Mouse knee samples were fixed in 4% PFA for 24 h and decalcified in 0.5 M EDTA pH 8.0 for 10 days  
110 before being embedded in paraffin. Thirty serial sagittal sections of 5 µm were cut, and three were  
111 chosen at the upper, medium and lower levels every 50 µm from cartilage surface. OA scoring was  
112 performed after Safranin O-Fast Green staining, according to the OsteoArthritis Research Society  
113 International (OARSI) recommendations. For each experiment, the staining was performed in the  
114 same batch to allow comparisons and 3 sections per animal were counted. For each animal, the OA  
115 score was the highest score obtained at one of the three levels. The number of animals is indicated in  
116 legends. Histology scores were quantified with blinding by 2 experienced readers separately and  
117 adjudicated to achieve consensus.

118

119 **Immunohistochemistry**

120 Paraffin-embedded sections were stained for SphK1 (kindly provided by Dr. Stuart Pitson, Centre for  
121 Cancer Biology, University of South Australia, Adelaide, Australia)<sup>34</sup>, sphingomab mouse anti-S1P  
122 monoclonal antibody (provided by Lpath, San Diego, USA), MMP13 (Abcam, UK) and the aggrecan  
123 monoclonal antibody to C-terminal neoepitope NITEGE (Thermo Scientific, France). Heat-induced  
124 antigen retrieval involved use of citrate buffer (pH 6.0) at 70°C for 4 h, followed by digestion with  
125 proteinase K (Sigma, France). Sections were then blocked with 3% H<sub>2</sub>O<sub>2</sub> for 10 min, and nonspecific  
126 binding sites were blocked with 3% normal horse serum and 2% bovine serum albumin for 30 min.  
127 Primary anti-SphK1 (1:250), anti-MMP13 (1:100), anti-NITEGE (1:100) or anti-S1P (1:100) antibody  
128 was added at 4°C overnight and revealed with ImmPRESS staining reagent (Vector Laboratories,  
129 France) and the diaminobenzidine substrate kit for peroxydase (Vector Laboratories, France). S1P  
130 staining was revealed by using the Vector M.O.M immunodetection kit.

131

132 The details of the following methods will be found in the supplementary materials: measurement of the  
133 proteoglycan content in conditioned medium, quantification of SphK1 activity and S1P content,  
134 histomorphometry and microCT scanning of subchondral bone, analysis of gene expression and WB.

135

### 136 **Statistical analysis**

137 Data are expressed as mean ± SEM from at least 3 independent experiments unless otherwise  
138 indicated or as individual data points. Data distribution was evaluated on the basis of parameter  
139 characteristics, quantile-quantile (QQ) plots, and Shapiro-Wilk normality tests. Variances were  
140 compared by the Bartlett test. *T*-test or ANOVA was used taking into account equal variances. When  
141 different groups were compared by ANOVA, a Tukey-Kramer test was used for pairwise multiple  
142 comparisons or a Dunnett's test for multiple comparisons to a control group. In case of unequal  
143 variances, the Mann-Whitney or Kruskal-Wallis test was used. With comparison by Kruskal-Wallis test,  
144 multiple pairwise comparisons were then performed according to the Steel-Dwass-Critchlow-Fligner  
145 procedure. We used a specific mixed model when a random factor was used with missing data in the  
146 experimental design. This analysis involved using the package nlme 3.1-137 in R v3.5.1 (2018-07-02;  
147 R Foundation for Statistical Computing, Vienna, Austria; <https://www.R-project.org/>). All other  
148 statistical analyses involved using XLSTAT v2018.6 (Addinsoft 1995-2019). *P* < 0.05 was considered  
149 statistically significant.



150 **Results**

151

152 **OC secretome and exogenous S1P induce matrix metalloproteinase (MMP) expression in**  
153 **chondrocytes**

154 We first investigated S1P expression in undamaged and OA joint tissues in mice. The expression of  
155 S1P was noted in chondrocytes of calcified cartilage in sham and OA mice, along the subchondral  
156 bone surface and in bone marrow (Figure 1A). The mRNA expression of *Sphk1*, the enzyme that  
157 produces S1P, was increased in subchondral bone in the early stages of OA (Figure 1B). *Sphk1*  
158 mRNA expression was increased concomitant with OC differentiation (Figure 1C), in parallel with the  
159 expression of *tartrate-resistant acid phosphatase (Trap)*, another marker of OC formation  
160 (supplemental Figure 1A). By contrast, the expression of *Sphk2* remained unchanged in OA mice and  
161 with OC differentiation (supplemental figure 1B-C). SPHK1 enzymatic activity was significantly higher  
162 in OCs than monocytes (RAW264.7 cells), Figure 1D). To identify the mechanism by which OC  
163 secretome could enhance the MMP activity, primary murine chondrocytes were cultured in the  
164 presence of conditioned medium from monocytes (RAW-CM) or differentiated OCs (Oc-CM).  
165 Compared with the secretome of monocytes, the OC secretome significantly upregulated the  
166 expression of *Mmp3* and *Mmp13* in chondrocytes in a dose-dependent manner (Figure 1E-F and  
167 supplemental figure 1D-E). These findings are reproduced with exogenous S1P that promoted the  
168 expression of MMP3 and MMP13 in chondrocyte cultures at both the protein and mRNA levels and  
169 mainly observed at high doses (supplemental figure 1F-G-H).

170

171 **OC secretome activates chondrocyte catabolism via S1P<sub>2</sub> and mitogen-activated protein kinase**  
172 **signaling**

173 Because OCs inhibition reduces cartilage metabolism in mice <sup>10,16</sup> and produce S1P, we assessed  
174 whether osteoclastic S1P could contribute to chondrocyte catabolism. Primary murine chondrocytes  
175 express *S1P<sub>1,2,3</sub>*, but not *S1P<sub>4</sub>* and *S1P<sub>5</sub>* mRNA (Figure 2A). Oc-CM significantly triggered the mRNA  
176 expression of only *S1P<sub>1,2,3</sub>*. To identify the role of S1P in the pro-catabolic effects of osteoclast  
177 secretome, chondrocytes were cultured with Oc-CM and the antagonist of S1P<sub>2</sub> (JTE013) or  
178 S1P<sub>1</sub>/S1P<sub>3</sub> (VPC23019) <sup>22,28</sup>. The chondrocytic expression of *Mmp3* and *Mmp13* induced by Oc-CM  
179 was significantly abrogated by JTE013 (Figure 2B), but not affected by VPC23019 (supplemental

180 Figure 2A). *S1P<sub>2</sub>* knockdown decreased the protein levels of MMP3 and MMP13 upon Oc-CM  
181 stimulation (Figure 2C). In addition, the inhibition of MMPs with JTE013 was not observed when  
182 chondrocytes were primed with interleukin 1 $\beta$  (IL-1 $\beta$ ), a pro-inflammatory cytokine known to activate  
183 cartilage catabolism (supplement Figure 2B-C), which supports the specific mediation of *S1P<sub>2</sub>* in  
184 chondrocytes.

185 To investigate whether *S1P<sub>2</sub>* promoted Oc-mediated cartilage breakdown, we further tested the  
186 pharmacologic inhibition by JTE013 in cartilage explants. Oc-CM induced a loss of proteoglycan,  
187 revealed by decreased safranin-O staining and increased NITEGE staining (Figure 2D). Oc-CM  
188 enhanced the release of proteoglycan in the explant cultures (Figure 2E) and the mature forms of  
189 MMP3 and MMP13 by chondrocytes (Figure 2F). The catabolic activities induced by Oc-CM were  
190 blunted by the *S1P<sub>2</sub>* inhibitor JTE013, suggesting the contribution of *S1P* in Oc-induced cartilage  
191 catabolism.

192 To better delineate the pathway that drives activation of catabolic enzymes in chondrocytes, we  
193 assessed the JNK pathway with Oc-CM stimulation. Indeed, this pathway has been shown to mediate  
194 the inflammatory response to *S1P/S1P<sub>2</sub>*<sup>35,36</sup>. Here, the JNK-dependent pathway was highly activated  
195 in chondrocytes primed with Oc-CM (Figure 2G). The inhibition of *S1P<sub>2</sub>* by JTE013 slightly reduced  
196 JNK phosphorylation. Furthermore, the induction of MMPs by Oc-CM was reduced by treatment with  
197 the JNK inhibitor SP600125 (Figure 2H), which suggests that *S1P<sub>2</sub>/JNK* signaling mediates the effects  
198 of Oc-CM in chondrocyte catabolism.

199

### 200 ***S1P<sub>2</sub>* mediates cartilage catabolism and OA in mice**

201 Based on the catabolic effects of *S1P<sub>2</sub>* in chondrocytes and cartilage explants, we investigated the  
202 impact of *S1P<sub>2</sub>* inhibition in mice. JTE013 was injected intra-articularly in mice with OA triggered by  
203 MNX. The severity of cartilage lesions shown by OA score was significantly lower in mice treated with  
204 JTE013 than with vehicle (Figure 3A). JTE013 administration also reduced the number of MMP13-  
205 positive chondrocytes (Figure 3B), but did not affect the OA-associated synovitis, which suggests that  
206 inhibiting *S1P<sub>2</sub>* affects mainly cartilage, but not the synovium (Figure 3C). Together, these results show  
207 that activation of the *S1P<sub>2</sub>* pathway promoted cartilage loss in OA.

208

### 209 ***Sphk1* deletion in myeloid cells reduces cartilage breakdown**

210 Given the production of S1P by OCs, we then determined the specific contribution of OC-derived S1P  
211 in OA. SphK1-positive cells, which produce S1P, were located along the bone surface in sclerotic  
212 subchondral bone in both human and mouse joints (Figure 4A). We therefore investigated the effect of  
213 SphK1 in OA mice. *Sphk1*-floxed mice (*Sphk1<sup>fl/fl</sup>*) were crossed with LysM-Cre mice to delete *Sphk1*  
214 specifically in OCs (SphK1<sup>LysMCre</sup>). In these mice, the expression of Sphk1 in primary osteoclasts was  
215 abolished in SphK1<sup>LysMCre</sup> mice compared to *Sphk1<sup>fl/fl</sup>* mice, but not the Sphk2 expression (Figure 4B).  
216 Histology revealed a significantly lower OA score in SphK1<sup>LysMCre</sup> than *Sphk1<sup>fl/fl</sup>* mice (Figure 4C) along  
217 with a lower number of MMP13-expressing cells (Figure 4D). The bone volume and number of OCs  
218 were similar in SphK1<sup>LysMCre</sup> and control mice in both subchondral and trabecular bone (supplemental  
219 Figure 3A-B-C). Therefore, the protection of cartilage damage observed in SphK1<sup>LysMCre</sup> mice is  
220 independent of OC differentiation and bone remodeling, but to the secretion of S1P. Finally,  
221 SphK1<sup>LysMCre</sup> mice had no reduction of synovial inflammation (supplemental Figure 3D).  
222 Given that SPHK1 is also expressed in human and mouse OA chondrocytes (Figure 5A), the S1P-  
223 promoting secretion of MMPs could be generated by chondrocytes or osteoclasts. To this end, we  
224 tested the OC secretome in chondrocytes that do not produce S1P by generating *Sphk1*-deficient  
225 primary chondrocytes. We generated *Sphk1*-deficient primary chondrocytes by inducing *Sphk1*  
226 deletion by recombination in flox-*Sphk1* chondrocytes with cre-adenovirus (Figure 5B) which were  
227 cultured with OC secretome. The loss of *Sphk1* in chondrocytes did not affect the ability of the OC  
228 secretome to induce the expression of *Mmp3* or *Mmp13* (Figure 5C), which suggests that the  
229 osteoclastic, but not the chondrocytic S1P, activated the production of MMP3 and MMP13.

230

### 231 **The S1P neutralizing antibody sphingomab protects mice against OA and synovial** 232 **inflammation**

233 Despite the reduction in OA observed in SphK1<sup>LysMCre</sup> mice, we investigated whether S1P released in  
234 synovial fluid may also contribute to cartilage damage since S1P is expressed in synovial tissues of  
235 patients with OA and arthritis<sup>37</sup>. Indeed, S1P levels were detected in synovial fluid of individuals with  
236 OA, the levels being as high than in patients with rheumatoid arthritis (supplemental Figure 4). The  
237 S1P released in the joint cavity could be involved in the regulation of chondrocytes in superficial layer  
238 of AC. Therefore, we investigated S1P blockade in mice by using sphingomab, a S1P-neutralizing  
239 monoclonal antibody injected intra-articularly. Sphingomab alleviated the severity of OA lesions at 2

240 and 4 µg in a dose-dependent manner (Figure 6A) and reduced the number of MMP13- and NITEGE-  
241 expressing cells (Figure 6B-C). In addition, sphingomab dose-dependently reduced synovial  
242 inflammation (Figure 6D), which suggests that S1P blockade contribute to reduce inflammation.  
243 Together, these data showed that neutralization of S1P is required for the prevention of cartilage  
244 degradation and synovial inflammation in OA.

245

## 246 **Discussion**

247

248 Here, we show that OC-derived S1P controls the secretion of MMPs by chondrocytes via S1P<sub>2</sub>  
249 signaling, thereby promoting cartilage extracellular matrix breakdown. S1P regulates bone remodelling  
250 through various cell types and functions<sup>38</sup>. By using genetically modified mice and various  
251 pharmacological approaches, we found that suppressing myeloid-derived S1P blunted cartilage  
252 catabolism and the development of OA. Consistently, mice lacking *Sphk1* in myeloid cells showed less  
253 severe OA lesions. These results suggest that osteoclastic S1P may contribute substantially to  
254 cartilage damage and further highlights the potential contribution of the OC niche in subchondral bone  
255 to cartilage breakdown in a direct or indirect manner. Previously, it was found that myeloid S1P  
256 regulates bone remodelling by increasing the number of OCs and their attraction toward the bone  
257 surface<sup>27,39</sup> and promotes osteoblast mobility and differentiation<sup>24,28</sup>. Here, the lack of myeloid *Sphk1*  
258 had no effect on subchondral OC number or bone structure in OA mice, which may suggest that  
259 myeloid S1P is involved in cartilage breakdown. Indeed, deletion of myeloid *Sphk1* does not affect the  
260 osteoclast number and the subchondral bone resorption, but this effect might be mediated by a lower  
261 osteoblast function induced by a lower local secretion of S1P. However, osteoclast secretome may  
262 modify the activity of subchondral bone marrow cells which in turn impact the chondrocyte metabolism.  
263 It has been suggested that the circulation from bone to cartilage is mediated by local blood flow.  
264 Another hypothesis is that S1P produced by the osteoclast circulate to the cartilage through the  
265 subchondral bone. Indeed, the diffusion of small molecules from bone to cartilage is possible through  
266 the subchondral bone although, or may be driven by microcracks<sup>40</sup>, in particular for the molecules  
267 which molecular weight is under 400kD<sup>41</sup>, including S1P.

268

269 Unlike in mice, the chondroprotective effect of antiresorbing drugs in humans led to conflicting results  
270<sup>42-45</sup>, partly because of the heterogeneity of the OA profile. The mechanisms underlying the

271 chondrocyte protection by the inhibition of osteoclast function in OA are not limited to inhibiting  
272 resorption of bone matrix <sup>10,16</sup>, but also mediated by OC-secreted factors in a paracrine manner. Such  
273 an effect involves S1P<sub>2</sub>, a major S1P receptor in murine chondrocytes, as was previously reported in  
274 human osteoarthritic chondrocytes <sup>46-49</sup>. Here, the inhibition of S1P<sub>2</sub> alone, but not S1P<sub>1</sub> or S1P<sub>3</sub>,  
275 limited the activation of catabolic genes in chondrocytes. This is consistent with the cartilage protection  
276 with S1P<sub>2</sub> blockade by JTE and siRNA or S1P neutralization with sphingomab in mice. Moreover, OC  
277 secretome stimulates the JNK pathway, that may mediate S1P signalling <sup>26,30</sup>. More importantly, the  
278 JNK inhibition reduced MMP expression, suggesting that JNK inhibition may result in milder cartilage  
279 damage. Indeed, high concentration of exogenous S1P is required to upregulated MMPs expression  
280 that suggests the involvement of other signalling pathways. In addition to the catabolic effect, S1P  
281 could influence chondrocyte anabolism. It has been reported that S1P increased the proliferation and  
282 the survival of chondrocytes <sup>50</sup>. In a model of osteochondral defects in rats, the administration of  
283 chondrocytes treated with recombinant acid ceramidase promoted the production of  
284 glycosaminoglycan as well as the gene expression of aggrecan and collagen 2 and the cartilage repair  
285 <sup>51</sup>. Therefore, it might be possible that a dual action of local S1P concentration occurred here  
286 promoting chondrocyte catabolism or activating anabolism, thereby disrupting the homeostasis of  
287 chondrocyte balance.

288

289 The inflamed synovium induced by OA was not reduced in *Sphk1*<sup>LysMCre</sup> or JTE013-treated mice. This  
290 suggests that S1P<sub>2</sub> has little function in the migration of myeloid cells and shows the lack of a direct  
291 effect of JTE013 in synovial inflammation. Therefore, the cartilage catabolism is likely more driven by  
292 local S1P than by the synovial inflammation. S1P has been reported to be involved in the  
293 pathogenesis of several inflammatory diseases, such as lung inflammation and rheumatoid arthritis <sup>52</sup>.  
294 However, S1P<sub>1</sub> is the major receptor regulating the chemo-attraction and proliferation of myeloid cells  
295 and also the production of prostaglandin E2 <sup>53</sup>. This effect is consistent with the suppression of  
296 inflammation with a specific S1P<sub>1</sub> antagonist <sup>54</sup>. Hence, blockage of S1P<sub>2</sub> may have modified the  
297 subchondral bone microenvironment to produce S1P. The neutralization of S1P with the antibody  
298 sphingomab was the only treatment that prevented synovial inflammation in addition to cartilage loss,  
299 which suggest the participation of S1P to the inflammatory aspect of OA. The lack of impact in the  
300 synovium of *Sphk1*-knockout mice or JTE013-treated mice could be due to SphK2, the other enzyme

301 involved in maturation of S1P. However, myeloid cells maintained the expression of *SphK2*, as shown  
302 by the stable mRNA expression in the subchondral plate during the progression of OA and in  
303 *Sphk1<sup>LysM<sup>Cre</sup></sup>* mouse-derived OCs. Despite a possible redundancy of SphK1 and SphK2 <sup>55</sup>, our results  
304 show that deletion of *Sphk1* in myeloid cells was sufficient to promote cartilage protection, and likely  
305 each enzyme could drive the maturation of S1P in specific tissues.

306

307 Because the effects of S1P are mostly mediated by its extracellular signaling, most drugs modulate  
308 S1P receptor activity such as Fingolimod <sup>56</sup>, despite a lack of receptor specificity. We choose to block  
309 S1P in the joint cavity based on the hypothesis that S1P could target different tissues within the joint.  
310 S1P levels were detectable in the synovial fluids of OA and were at the same levels than those in  
311 people with rheumatoid arthritis, likely because synovial fluid production occurs in individuals with  
312 inflammatory flares, a condition associated with the production of synovial fluid. Given that articular  
313 cartilage is not vascularized, diffusion of molecules to the cartilage matrix is optimized when  
314 administered in synovial fluid. We cannot rule out that S1P produced by macrophages within the joint  
315 cavity could contribute to cartilage damage, in particular by using the *Sphk1<sup>LysM<sup>Cre</sup></sup>* mouse model. It is  
316 therefore possible that S1P produced by macrophages within the joint cavity could contribute to  
317 cartilage damage. A local route allows for enhanced local concentration of the antibody and better  
318 diffusion in cartilage to reach chondrocytes. As a proof-of-concept for targeting S1P in OA, S1P was  
319 neutralized in synovial fluid by intra-articular injection. Indeed, sphingomab prevented both cartilage  
320 lesions and synovial inflammation in OA mice, in contrast to S1P<sub>2</sub> inhibition and *Sphk1* deletion in  
321 myeloid cells, which reduced cartilage lesions without affecting the synovium. The prevention of  
322 lesions in the whole joints with sphingomab might be related to the direct neutralization of a ubiquitous  
323 source of S1P, the reduction of local concentration of S1P and the subsequent binding to receptors,  
324 whereas JTE013 is restricted to inhibiting S1P<sub>2</sub>. This observation addresses the human relevance  
325 towards osteoarthritic patients. Sphingomab has been developed as a humanized biotherapy and  
326 tested in phase 2 trial in a renal cell carcinoma setting (NCT01762033). No effect on progression-free  
327 survival was observed and was not further developed to the phase 3. There was however a favorable  
328 safety profile <sup>57,58</sup>. Our data provide insights in OA conditions that will allow repurposing the drug.  
329 Because of the effect on both cartilage and the synovium, the anti-S1P biotherapy could be better  
330 oriented to patients with inflammatory OA forms and administered intra-articularly to allow a reduction

331 in joint inflammation. Clinical trials could be developed to assess the potential effect of anti-S1P in OA  
332 and better define the population who will best benefit of the treatment.

333

334 In conclusion, we showed the contribution of myeloid-derived S1P in the development of cartilage  
335 damage in OA. However, only local neutralization of S1P by sphingomab biotherapy prevented  
336 cartilage and synovial lesions in mice. Therefore, the neutralizing antibody sphingomab could be  
337 considered as a local agent for treating OA.

338

### 339 **Acknowledgments**

340

341 The authors thank Alexandre Garcia for measurements of S1P.

342 The work was supported by the Sybil SP7 European project and the “Fondation de l’Avenir”. JT and  
343 SV received grants from the Deutsche Forschungsgemeinschaft within the collaborative research  
344 center SFB1149.

345

### 346 **Author Contributions**

347 MCS, EH and CC designed the study. CC, AL and EH conducted the cells and animal experiments. H-  
348 KE performed the OA surgery for all mice experiments. SV and JT produced the S1P flox mice and  
349 contributed to the experiment design. OC contributed to the design and provided the S1Pab for  
350 immunohistochemistry and sphingomab. AL performed the SPHK1 enzymatic assay. JC performed  
351 the S1P measurements in synovial fluid. AO performed the statistical analysis. CC, AL, PR, EH and  
352 MCS wrote the manuscript. All authors analyzed the data and reviewed critically the manuscript.

353

### 354 **Conflict of interest**

355 The authors report no conflict of interest in relation to the work.

356

357

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523 **Figures legends**

524

525 **Figure 1: Osteoclast (OC) secretome activates the expression of matrix metalloproteinase 3**  
526 **(MMP3) and MMP13 in chondrocytes**

527 **A**, Representative immunostaining for S1P in articular cartilage of medial tibia in sham-treated mice  
528 and mice with osteoarthritis obtained 6 weeks after surgery (OA). Scale bar 100  $\mu$ m. **B**, Quantitative  
529 RT-PCR analysis of sphingosine kinase 1 (*Sphk1*) level in the mouse tibia plateau on day 3 and week  
530 1, 2, 4, and 6 after OA induction in mice (n=3-6 mice) and **C**, in monocytes (RAW264.7 cells) and OCs  
531 differentiated from RAW264.7 cells cultured with 30, 50 or 100 ng RANKL for 5 days (n=4). **D**, SPHK1  
532 enzymatic activity quantified in whole lysates of monocytes (RAW264.7 cells) and OCs cultured with  
533 100 ng RANKL (n=8-10). **E-F**, Quantitative RT-PCR analysis of *Mmp3* and *Mmp13* levels in mouse  
534 primary chondrocytes treated for 24 h with conditioned medium (CM) from RAW264.7 cells (RAW-CM)  
535 or osteoclasts (Oc-CM) (n=5). Error bars indicate mean $\pm$ SEM. \* p<0.05, \*\* p<0.01, \*\*\* p<0.001

537

538 **Figure 2: OC secretome activates chondrocyte catabolism via S1P<sub>2</sub> and JNK signaling**

539 Quantitative RT-PCR analysis of **A**, S1P receptors in mouse primary chondrocytes treated with OC-  
540 CM for 24 h (n=5) and **B**, *Mmp3* and *Mmp13* levels in mouse primary chondrocytes treated with OC-  
541 CM with or without the S1P<sub>2</sub> inhibitor JTE013 (5  $\mu$ M), Ct being the controls (n=4-5). **C**, Western blot  
542 analysis of MMP3 and MMP13 (upper) and actin (lower) levels in whole lysates of primary  
543 chondrocytes with or without treatment with 50 nM siRNA targeting S1P<sub>2</sub> (n=2). **D**, Representative  
544 safranin-O staining (left) and NITEGE immunohistochemistry (right) of cryosections from mouse  
545 femoral-head cartilage explants treated with Oc-CM with or without JTE013 (5  $\mu$ M) for 72 h. Explants  
546 from 3 experiments (n=3 femoral heads per condition). **E**, Quantified 1,9-dimethylmethylene blue  
547 assay of proteoglycan content in supernatant of cartilage explants (n=3). Representative Western blot  
548 analysis of **F**, MMP3 and MMP13 levels in the supernatants. **G**, phospho-JNK and JNK levels in whole  
549 lysates of primary chondrocytes cultured with Oc-CM for 15 min in the presence or not of JTE013, 5  
550  $\mu$ M (n=3). **H**, Quantitative RT-PCR analysis of *Mmp3* and *Mmp13* levels in mouse primary  
551 chondrocytes cultured with Oc-CM for 24 h in the presence or not of the JNK inhibitor SP600125, 10  
552  $\mu$ M (n=4 in triplicate). Error bars indicate mean  $\pm$  SEM. \* p<0.05.

553

554 **Figure 3: S1P<sub>2</sub> mediates cartilage catabolism and OA**

555 **A**, Representative safranin-O staining of articular cartilage and quantification of OA score in mice 6  
556 weeks after OA induction or in mice with sham surgery. JTE013 (5 mg/kg once per week) was  
557 administered by local injection for 6 weeks (n=6-8) and vehicle was injected in the control group (n=4).  
558 **B**, Representative immunostaining for MMP13 in cartilage of medial tibia plateau in sham or OA mice  
559 treated locally with vehicle or JTE013 (5 mg/kg once per week). Quantification was done in the entire  
560 articular cartilage. **C**, Representative images of synovial tissue after OA induction. \* p<0.05. Scale bar:  
561 100 µm.

562

563 **Figure 4: Sphk1 deletion in myeloid cells reduces cartilage breakdown**

564 **A**, Representative immunostaining for SphK1 in subchondral bone of medial tibia in sham or OA mice  
565 (upper panel) and undamaged and OA joints of humans (lower panel) (n=3 per group). Scale bar: 100  
566 µm. Quantitative RT-PCR analysis of **B**, *Sphk1* and *Sphk2* levels in mouse primary OCs from bone  
567 marrow lacking *Sphk1* (Oc *Sphk1*<sup>-/-</sup>) or not (Oc *Sphk1*<sup>fl/fl</sup>) (n= 3-4). **C**, Representative safranin O staining  
568 of articular cartilage and quantification of OA score for medial femorotibial joints in *Sphk1*<sup>fl/ox</sup> or  
569 *Sphk1*<sup>LysMCre</sup> 6 weeks after OA induction or sham surgery (n=5-12). **D**, Representative immunostaining  
570 for MMP13 in articular cartilage of medial tibia joints with OA from *Sphk1*<sup>fl/ox</sup> or *Sphk1*<sup>LysMCre</sup> mice and  
571 quantification of MMP13-positive chondrocytes by immunostaining (n=5-9). Error bars indicate mean ±  
572 SEM. \* p<0.05.

573

574 **Figure 5: Lack of autocrine effect of SphK1 in chondrocytes**

575 **A**, Representative immunostaining of SPHK1 in articular cartilage of medial tibia in sham and OA mice  
576 (upper panel) and undamaged and OA human samples (n=3). Scale bar: 100 µm. Quantitative RT-  
577 PCR analysis of **B**, *Sphk1* and *Sphk2* levels in mouse primary chondrocytes lacking *Sphk1* induced by  
578 empty virus (*Mock*) or (*Cre*) (n=3-4 experiments) and **C**, *Mmp3* and *Mmp13* levels in mouse primary  
579 chondrocytes with or without *Sphk1* and cultured with Oc-CM (n=2-3 experiments).

580

581 **Figure 6: Spingomab protects mice against OA**

582 **A**, Representative safranin O staining of articular cartilage and quantification of OA OARSI score in  
583 medial femoro-tibia joints from mice 6 weeks after OA induction or sham surgery. The neutralizing

584 monoclonal antibody sphingomab was injected locally in joints (2 and 4  $\mu\text{g}$  once per week for 6  
585 weeks), and IgG (4  $\mu\text{g}$ ) was a control (n= 7 mice per group). **B**, Representative immunostaining and  
586 quantification of chondrocytes positive for MMP13 in medial tibia joints from sham or OA mice. **C**,  
587 Representative immunostaining of NITEGE in medial tibia joints from OA mice. **D**, Representative  
588 images of synovial tissue staining in knees 6 weeks after OA and sphingomab administration. Scale  
589 bar 100  $\mu\text{m}$ . \*  $p < 0.05$

