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

C. Cherifi †, A. Latourte † ‡, S. Vettorazzi §, J. Tuckermann §, S. Provot †, H.-K. Ea † ‡, A. Ledoux ‖, J. Casas ¶ #, O. Cuvillier ‖, P. Richette † ‡, A. Ostertag †, E. Hay †, M. Cohen-Solal † ‡ *

† *Université de Paris, BIOSCAR Inserm U1132, Paris, F-75010, France*
‡ *Service de rhumatologie, AP-HP, Hôpital Lariboisière, Paris, F-75010, France*
§ *Institute of Comparative Molecular Endocrinology, University of Ulm, ZC 89081, Germany*
‖ *Institut de Pharmacologie et de Biologie Structurale, Université de Toulouse, CNRS, UPS, Toulouse, F-31000, France*
¶ *RUBAM, Department Biological Chemistry, IQAC-CSIC, Jordi Girona 18-26, Barcelona, S-08034, Spain*
# *Networking Biomedical Research Centre (CIBEREHD), Madrid, S-28706, Spain*

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SUMMARY

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^{LysMCre} 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^{LysMCre} mice or wild type mice treated with $S1P_2$ 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/$S1P_2$ 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^{LysMCre} 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.

Introduction

Cartilage remodeling is a physiological process that is disrupted in osteoarthritis (OA), leading to a loss of cartilage matrix[1]. OA is one of the leading causes of disability worldwide and its prevalence is steadily increasing[2–4]. No disease-modifying drug is currently available for OA, making the OA management a therapeutical challenge. Cartilage loss involves multiple pathophysiological mechanisms responsible for cartilage damage[5–7], including enhanced chondrocyte catabolism[8]. Changes occur in all tissues in OA joints[9], including subchondral bone alterations that promote or sustain the development of OA[10]. In this regard, there is evidence for a crosstalk between cartilage and subchondral bone leading to progressive cartilage destruction[11–13]. The increased subchondral bone resorption observed at early stages of OA is mediated by

* Address correspondence and reprint requests to: M. Cohen-Solal, Inserm U1132, Université de Paris, Hôpital Lariboisière, 2 rue Ambroise Pare, Paris, 75010, France. Tel.: 33-149956358; Fax: 33-149958452.
E-mail address: martine.cohen-solal@inserm.fr (M. Cohen-Solal).

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osteoclasts (OCs)[14], and anti-resorptive therapies targeting OCs prevent OA in murine models[15,16]. Although subchondral bone OCs precursors may directly contribute to chondrocyte catabolism, the factors involved in this crosstalk, particularly the role of the OC secretome in cartilage breakdown, remain elusive.

Sphingosine 1-phosphate (S1P), a downstream metabolite of ceramide, is a lipid mediator involved in a number of pathophysiological conditions and disorders including inflammation and autoimmune diseases[17]. The balance between levels of ceramide and S1P has been considered a switch determining whether a cell proliferates or dies[18]. S1P is synthesized by two sphingosine kinase isoforms, sphingosine kinase 1 (SphK1) and SphK2, and its degradation is also controlled by S1P phosphatases or S1P lyase[19,20]. Besides the intracellular role of S1P, most of its biological effects are explained by its binding to a family of five G protein-coupled receptors, $S1P_{1\text{-}5}$, that mediate specific effects according to the predominant subtypes expressed in cells[21]. In bone, S1P regulates bone remodeling: S1P lyase-deficient mice, which have high circulating S1P levels, show a high bone mass phenotype[22]. S1P is released by osteoblasts[23] and OCs[24] and contributes to the coupling of OCs and osteoblasts[24,25]. S1P mobilizes OC precursors in the blood and the bone microenvironment via a local gradient, thereby regulating bone homeostasis[25–27]. Furthermore, increased S1P secretion in OCs triggered osteoblast differentiation in a paracrine manner[28].

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

Materials and methods

Animal experimentation and ethics

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

OA was induced by meniscectomy (MNX) of the right knee in 10 week-old mice as described previously[29]. Briefly, knee joint instability was induced surgically in the right knee by medial partial meniscectomy. Surgery was performed under a binocular magnifier (X15) using a Sharpoint microsurgical stab knife. Mice were placed in dorsal position, knee flexed and right foot taped. After skin incision, the medial femoro-tibial ligament was cut, a short incision of the medial side of quadriceps muscle was performed, the knee capsule was cleaved and the patella was sub-luxated laterally. After section of the meniscotibial ligament, the medial meniscus was gently pulled out and ¾ of its anterior horn removed. Then, the patella was replaced, the quadriceps muscle and the skin plan sutured. Control animals underwent sham surgery (ligament visualization but not dissection) and done by a single trained operator for all experiments. Ketamine/xylazine mix and buprenorphin was administered to sedated mice, and a second injection of buprenorphin was given the day after surgery. *Sphk1 lox/lox* and *LysM-Cre* mice were generated as described[30]. SPHK1 was specifically deleted in monocyte/OC lineages by crossing *Sphk1 lox/lox* mice to *LysM-Cre* mice (mixed background).

In experiments using neutralization of S1P, C57Bl/6 mice received after anesthesia an intra-articular injection of 2 or 4 μg anti-S1P monoclonal antibody kindly provided by LPath (San Diego, USA;[31]) once a week ($n = 8$) or IgG as a control ($n = 7$) for 6 weeks from the day after the DMM. To inhibit $S1P_2$ signaling, JTE013, 4 mg/kg, was administered to C57Bl/6 mice by intra-articular injection in the knee ($n = 8$) once a week[32]. All animals were sacrificed at week 6.

Osteoclast (OC) culture

RAW264.7 monocyte cells were cultured in 6-well plates (400,000 cells/well). On day 2 and 4, differentiation was induced by treating cells with the receptor activator of nuclear factor kappa-B ligand (RANKL, 30 or 50 or 100 nM; Peprotech, France). At day 5, differentiated OCs were washed 3 times with phosphate buffered saline 1X and fresh medium was added. Finally, OC and RAW264.7 cell conditioned medium (OC-CM and RAW-CM, respectively) was collected on day 6 and stored at −80°C.

To generate primary OCs, bone morrow isolated from $Sphk1^{flox}$ or $SphK1^{LysMCre}$ mice at age 10 weeks were differentiated, treated with macrophage-colony stimulating factor alone (MCSF, 100 nM; Peprotech, France) or both MCSF and RANKL for 5 days. Monocyte and OC conditioned medium (CM) was collected under the same conditions as described above.

Culture of primary chondrocytes

Primary chondrocytes were harvested and cultured as previously reported[29,33]. Briefly, articular cartilage from the tibia plateau, femoral condyle and femoral head was obtained from 5- to 6-day-old mice. After matrix digestion with Liberase (Roche, France), chondrocytes were plated in 12-well plates (200,000 cells/well) and harvested in Dulbecco's modified Eagle's medium (DMEM, Gibco Life Technologies, France) with 10% decomplemented fetal bovine serum (FBS), 2% L-glutamine and 1% penicillin/streptomycin until confluence. Chondrocytes were then stimulated with Oc-CM or interleukin 1β (10 nM) for 24 h, with or without 5μM of the S1P2 inhibitor JTE013, or 10 nM of the $S1P_1/S1P_3$ antagonist VPC23019, 10μM JNK inhibitor SP600125 or 1, 2, 3 or 6μM S1P (all R&D Systems, France). To investigate signaling pathways, cells were pretreated with JTE013 for 1 h, and then stimulated with OC-CM for 5, 10 or 15 min. Chondrocyte supernatant was collected to evaluate the release of proteoglycans and MMPs in the medium.

Transfection of primary chondrocytes

At 80% confluence, chondrocytes and macrophages obtained from *Sphk1* floxed mice were transfected with adenovirus expressing Cre recombinase (200 multiplicity of infection, Vector Biolabs, US) to induce recombination. The control virus without the inserted gene (*Mock*) was a control. Small interfering RNA (siRNA) targeting $S1P_2$ (50 nM, Santa Cruz Biotechnology) was used to induce $S1P_2$ deletion in chondrocytes primed with interferin (Polyplus-transfection, Illkirch, France). Experiments were performed twice.

Culture of cartilage explants

Femoral head cartilage explants from 10-week-old male mice were cultured in DMEM with 10% FBS, 3 explants per well. After 24 h of culture, explants were stimulated with Oc-CM with or without JTE013 for 48 h. Explants were removed, fixed in paraformaldehyde (PFA) (4%) and decalcified with EDTA (0.5 M) for 24 h

at room temperature and prepared for cryosectioning. Results are from 3 separate experiments performed in triplicate.

Human explants

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

Histology

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

Immunohistochemistry

Paraffin-embedded sections were stained for SphK1 (kindly provided by Dr. Stuart Pitson, Centre for Cancer Biology, University of South Australia, Adelaide, Australia)[34], sphingomab mouse anti-S1P monoclonal antibody (provided by Lpath, San Diego, USA), MMP13 (Abcam, UK) and the aggrecan monoclonal antibody to C-terminal neoepitope NITEGE (Thermo Scientific, France). Heat-induced antigen retrieval involved use of citrate buffer (pH 6.0) at 70°C for 4 h, followed by digestion with proteinase K (Sigma, France). Sections were then blocked with 3% H_2O_2 for 10 min, and nonspecific binding sites were blocked with 3% normal horse serum and 2% bovine serum albumin for 30 min. Primary anti-SphK1 (1:250), anti-MMP13 (1:100), anti-NITEGE (1:100) or anti-S1P (1:100) antibody was added at 4°C overnight and revealed with ImmPRESS staining reagent (Vector Laboratories, France) and the diaminobenzidine substrate kit for peroxydase (Vector Laboratories, France). S1P staining was revealed by using the Vector M.O.M immunodetection kit.

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

Statistical analysis

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

Results

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

We first investigated S1P expression in undamaged and OA joint tissues in mice. The expression of S1P was noted in chondrocytes of calcified cartilage in sham and OA mice, along the subchondral bone surface and in bone marrow [Fig. 1(A)]. The mRNA expression of *Sphk1*, the enzyme that produces S1P, was increased in subchondral bone in the early stages of OA [Fig. 1(B)]. *Sphk1* mRNA expression was increased concomitant with OC differentiation [Fig. 1(C)], in parallel with the expression of *tartrate-resistant acid phosphatase* (*Trap*), another marker of OC formation (Supplemental Fig. 1(A)). By contrast, the expression of *Sphk2* remained unchanged in OA mice and with OC differentiation (Supplemental Fig. 1(B) and (C)). SPHK1 enzymatic activity was significantly higher in OCs than monocytes (RAW264.7 cells), Fig. 1(D)). To identify the mechanism by which OC secretome could enhance the matrix metalloproteinase (MMP) activity, primary murine chondrocytes were cultured in the presence of CM from monocytes (RAW-CM) or differentiated OCs (Oc-CM). Compared with the secretome of monocytes, the OC secretome significantly upregulated the expression of *Mmp3* and *Mmp13* in chondrocytes in a dose-dependent manner (Fig. 1(E) and (F) and Supplemental Fig. 1(D) and (E)). These findings are reproduced with exogenous S1P that promoted the expression of matrix metalloproteinase 3 (MMP3) and MMP13 in chondrocyte cultures at both the protein and mRNA levels and mainly observed at high doses (Supplemental Fig. 1(F–H)).

OC secretome activates chondrocyte catabolism via $S1P_2$ and mitogen-activated protein kinase signaling.

Because OCs inhibition reduces cartilage metabolism in mice[10,16] and produce S1P, we assessed whether osteoclastic S1P could contribute to chondrocyte catabolism. Primary murine chondrocytes express $S1P_{1,2,3}$, but not $S1P_4$ *and* $S1P_5$ mRNA [Fig. 2(A)]. Oc-CM significantly triggered the mRNA expression of only $S1P_{1,2,3}$. To identify the role of S1P in the pro-catabolic effects of osteoclast secretome, chondrocytes were cultured with Oc-CM and the antagonist of $S1P_2$ (JTE013) or $S1P_1/S1P_3$ (VPC23019)[22,28]. The chondrocytic expression of *Mmp3 and Mmp13* induced by Oc-CM was significantly abrogated by JTE013 [Fig. 2(B)], but not affected by VPC23019 (Supplemental Fig. 2(A)). $S1P_2$ knockdown decreased the protein levels of MMP3 and MMP13 upon Oc-CM stimulation [Fig. 2(C)]. In addition, the inhibition of MMPs with JTE013 was not observed when chondrocytes were primed with interleukin 1β (IL-1β), a pro-inflammatory cytokine known to activate cartilage catabolism (supplement Fig. 2(B) and (C)), which supports the specific mediation of $S1P_2$ in chondrocytes.

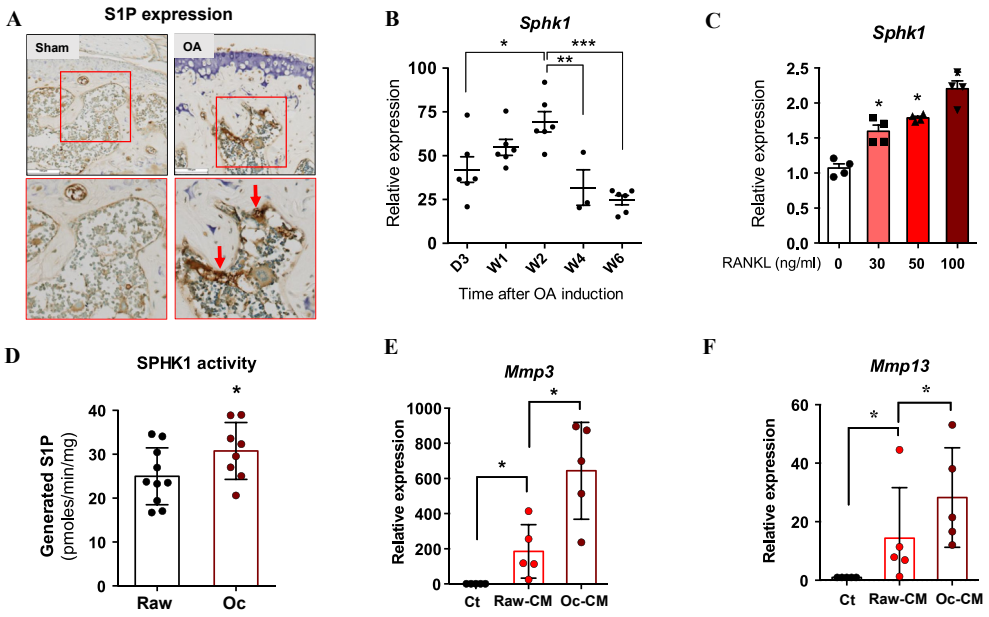

Fig. 1

Osteoclast (OC) secretome activates the expression of matrix metalloproteinase 3 (MMP3) and MMP13 in chondrocytes. A, Representative immunostaining for S1P in articular cartilage of medial tibia in sham-treated mice and mice with osteoarthritis obtained 6 weeks after surgery (OA). Scale bar 100 μm. **B,** Quantitative RT-PCR analysis of sphingosine kinase 1 (*Sphk1*) level in the mouse tibia plateau on day 3 and week 1, 2, 4, and 6 after OA induction in mice (n = 3–6 mice) and C, in monocytes (RAW264.7 cells) and OCs differentiated from RAW264.7 cells cultured with 30, 50 or 100 ng RANKL for 5 days ($n = 4$). D, SPHK1 enzymatic activity quantified in whole lysates of monocytes (RAW264.7 cells) and OCs cultured with 100 ng RANKL ($n = 8$–10). **E-F,** Quantitative RT-PCR analysis of *Mmp3* and *Mmp13* levels in mouse primary chondrocytes treated for 24 h with conditioned medium (CM) from RAW264.7 cells (RAW-CM) or osteoclasts (Oc-CM) ($n = 5$). Error bars indicate mean ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

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To investigate whether $S1P_2$ promoted Oc-mediated cartilage breakdown, we further tested the pharmacologic inhibition by JTE013 in cartilage explants. Oc-CM induced a loss of proteoglycan, revealed by decreased safranin-O staining and increased NITEGE staining [Fig. 2(D)]. Oc-CM enhanced the release of proteoglycan in the explant cultures [Fig. 2(E)] and the mature forms of MMP3 and MMP13 by chondrocytes [Fig. 2(F)]. The catabolic activities induced by Oc-CM were blunted by the $S1P_2$ inhibitor JTE013, suggesting the contribution of S1P in Oc-induced cartilage catabolism.

To better delineate the pathway that drives activation of catabolic enzymes in chondrocytes, we assessed the JNK pathway with Oc-CM stimulation. Indeed, this pathway has been shown to mediate the inflammatory response to $S1P/S1P_2$[35,36]. Here, the JNK-dependent pathway was highly activated in chondrocytes primed with Oc-CM [Fig. 2(G)]. The inhibition of $S1P_2$ by JTE013 slightly reduced JNK phosphorylation. Furthermore, the induction of MMPs by Oc-CM was reduced by treatment with the JNK inhibitor SP600125 [Fig. 2(H)], which suggests that $S1P_2$/JNK signaling mediates the effects of Oc-CM in chondrocyte catabolism.

$S1P_2$ mediates cartilage catabolism and OA in mice

Based on the catabolic effects of $S1P_2$ in chondrocytes and cartilage explants, we investigated the impact of $S1P_2$ inhibition in mice. JTE013 was injected intra-articularly in mice with OA triggered by MNX. The severity of cartilage lesions shown by OA score was significantly lower in mice treated with JTE013 than with vehicle [Fig. 3(A)]. JTE013 administration also reduced the number of MMP13-positive chondrocytes [Fig. 3(B)], but did not affect the OA-associated synovitis, which suggests that inhibiting $S1P_2$ affects mainly cartilage, but not the synovium [Fig. 3(C)]. Together, these results show that activation of the $S1P_2$ pathway promoted cartilage loss in OA.

Sphk1 deletion in myeloid cells reduces cartilage breakdown

Given the production of S1P by OCs, we then determined the specific contribution of OC-derived S1P in OA. SphK1-positive cells, which produce S1P, were located along the bone surface in sclerotic subchondral bone in both human and mouse joints [Fig. 4(A)]. We therefore investigated the effect of SphK1 in OA mice. *Sphk1*-floxed mice ($Sphk1^{fl/fl}$) were crossed with LysM-Cre mice to delete *Sphk1* specifically in OCs ($SphK1^{LysMCre}$). In these mice, the expression of Sphk1 in primary osteoclasts was abolished in $SphK1^{LysMCre}$ mice compared to $Sphk1^{fl/fl}$ mice, but not the Sphk2 expression [Fig. 4(B)]. Histology revealed a significantly lower OA score in $SphK1^{LysMCre}$ than $Sphk1^{fl/fl}$ mice [Fig. 4(C)] along with a lower number of MMP13-expressing cells [Fig. 4(D)]. The bone volume and number of OCs were similar in $SphK1^{LysMCre}$ and control mice in both subchondral and trabecular bone (Supplemental Fig. 3(A–C)). Therefore, the protection of cartilage damage observed in $SphK1^{LysMCre}$ mice is independent of OC differentiation

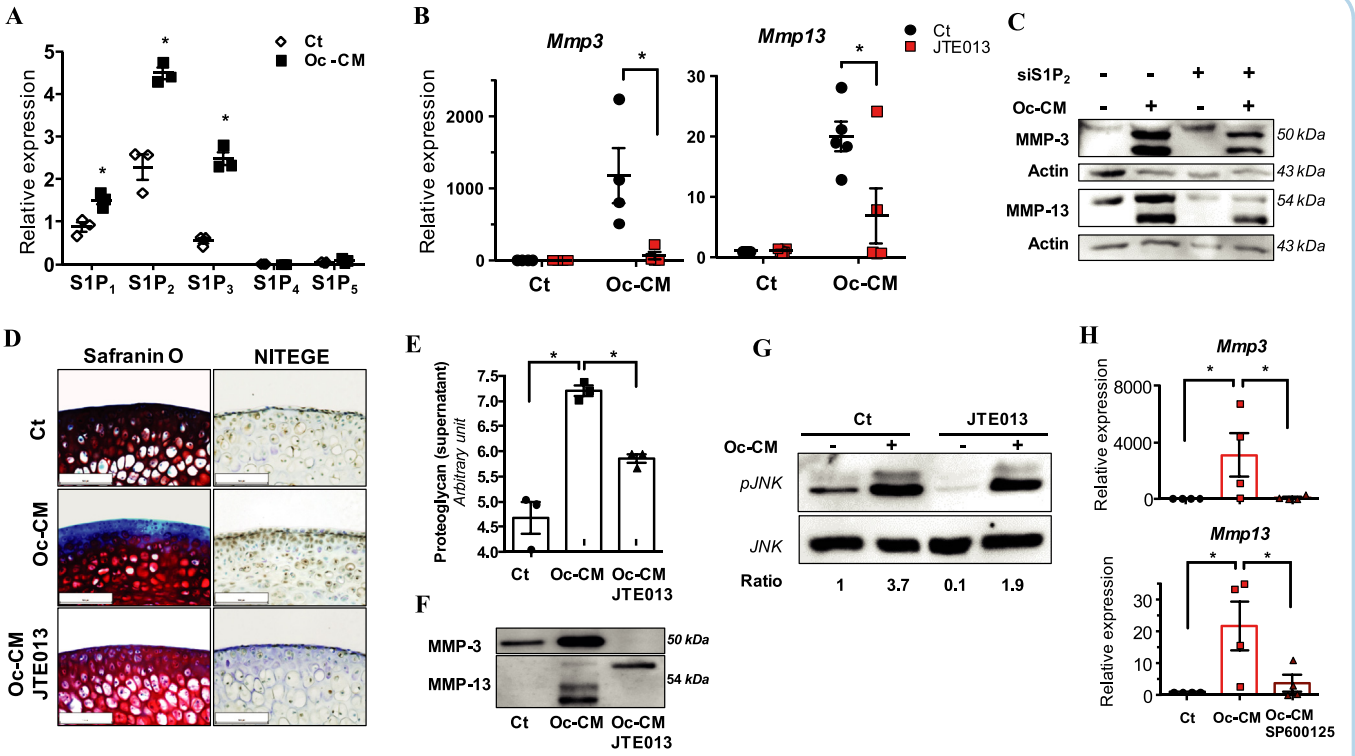

Fig. 2

OC secretome activates chondrocyte catabolism via $S1P_2$ and JNK signaling. Quantitative RT-PCR analysis of **A,** S1P receptors in mouse primary chondrocytes treated with OC-CM for 24 h ($n = 5$) and **B,** *Mmp3* and *Mmp13* levels in mouse primary chondrocytes treated with OC-CM with or without the $S1P_2$ inhibitor JTE013 (5μM), Ct being the controls ($n = 4–5$). **C,** Western blot analysis of MMP3 and MMP13 (upper) and actin (lower) levels in whole lysates of primary chondrocytes with or without treatment with 50 nM siRNA targeting $S1P_2$ ($n = 2$). **D,** Representative safranin-O staining (left) and NITEGE immunohistochemistry (right) of cryosections from mouse femoral-head cartilage explants treated with Oc-CM with or without JTE013 (5μM) for 72 h. Explants from 3 experiments ($n = 3$ femoral heads per condition). **E,** Quantified 1,9-dimethylmethylene blue assay of proteoglycan content in supernatant of cartilage explants ($n = 3$). Representative Western blot analysis of **F,** MMP3 and MMP13 levels in the supernatants. **G,** phospho-JNK and JNK levels in whole lysates of primary chondrocytes cultured with Oc-CM for 15 min in the presence or not of JTE013, 5μM ($n = 3$). **H,** Quantitative RT-PCR analysis of *Mmp3* and *Mmp13* levels in mouse primary chondrocytes cultured with Oc-CM for 24 h in the presence or not of the JNK inhibitor SP600125, 10μM ($n = 4$ in triplicate). Error bars indicate mean ± SEM. *$p < 0.05$.

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and bone remodeling, but to the secretion of S1P. Finally, $SphK1^{LysMCre}$ mice had no reduction of synovial inflammation (Supplemental Fig. 3(D)).

Given that SPHK1 is also expressed in human and mouse OA chondrocytes [Fig. 5(A)], the S1P-promoting secretion of MMPs could be generated by chondrocytes or osteoclasts. To this end, we tested the OC secretome in chondrocytes that do not produce S1P by generating *Sphk1*-deficient primary chondrocytes. We generated *Sphk1*-deficient primary chondrocytes by inducing *Sphk1* deletion by recombination in flox-*Sphk1* chondrocytes with cre-adenovirus [Fig. 5(B)] which were cultured with OC secretome. The loss of *Sphk1* in chondrocytes did not affect the ability of the OC secretome to induce the expression of *Mmp3* or *Mmp13* [Fig. 5(C)], which suggests that the osteoclastic, but not the chondrocytic S1P, activated the production of MMP3 and MMP13.

The S1P neutralizing antibody sphingomab protects mice against OA and synovial inflammation

Despite the reduction in OA observed in $SphK1^{LysMCre}$ mice, we investigated whether S1P released in synovial fluid may also contribute to cartilage damage since S1P is expressed in synovial tissues of patients with OA and arthritis[37]. Indeed, S1P levels were detected in synovial fluid of individuals with OA, the levels being as high than in patients with rheumatoid arthritis (Supplemental Fig. 4). The S1P released in the joint cavity could be involved in the regulation of chondrocytes in superficial layer of AC. Therefore, we investigated S1P blockade in mice by using sphingomab, a S1P-neutralizing monoclonal antibody injected intra-articularly. Sphingomab alleviated the severity of OA lesions at 2 and 4 μg in a dose-dependent manner [Fig. 6(A)] and reduced the number of

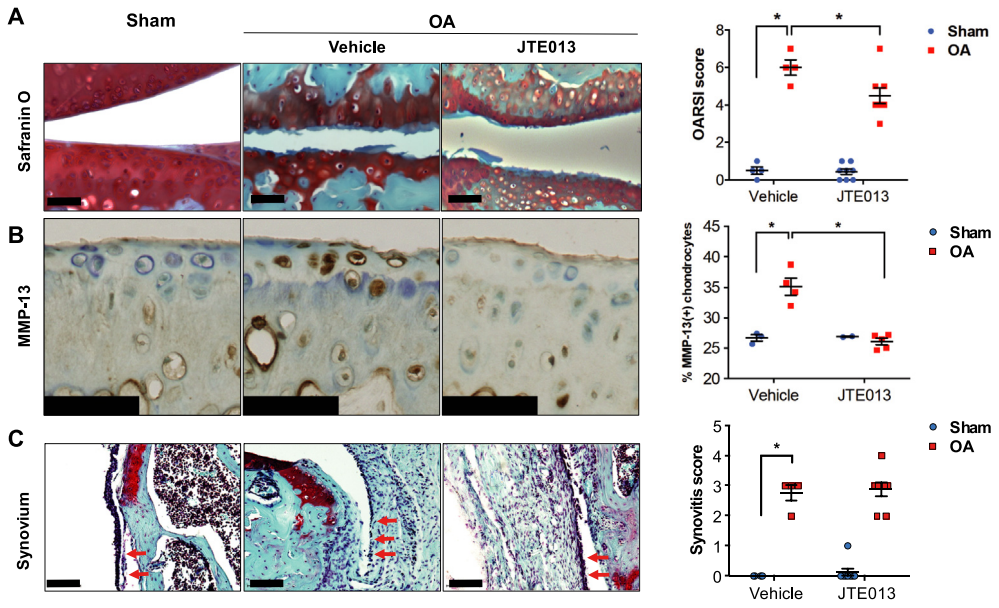

Fig. 3

S1P$_2$ mediates cartilage catabolism and OA. A, Representative safranin-O staining of articular cartilage and quantification of OA score in mice 6 weeks after OA induction or in mice with sham surgery. JTE013 (5 mg/kg once per week) was administered by local injection for 6 weeks ($n = 6–8$) and vehicle was injected in the control group ($n = 4$). **B,** Representative immunostaining for MMP13 in cartilage of medial tibia plateau in sham or OA mice treated locally with vehicle or JTE013 (5 mg/kg once per week). Quantification was done in the entire articular cartilage. **C,** Representative images of synovial tissue after OA induction. *$p < 0.05$. Scale bar: 100 μm.

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MMP13- and NITEGE-expressing cells [Fig. 6(B) and (C)]. In addition, sphingomab dose-dependently reduced synovial inflammation [Fig. 6(D)], which suggests that S1P blockade contribute to reduce inflammation. Together, these data showed that neutralization of S1P is required for the prevention of cartilage degradation and synovial inflammation in OA.

Discussion

Here, we show that OC-derived S1P controls the secretion of MMPs by chondrocytes via S1P$_2$ signaling, thereby promoting cartilage extracellular matrix breakdown. S1P regulates bone remodelling through various cell types and functions[38]. By using genetically modified mice and various pharmacological approaches, we found that suppressing myeloid-derived S1P blunted cartilage catabolism and the development of OA. Consistently, mice lacking *Sphk1* in myeloid cells showed less severe OA lesions. These results suggest that osteoclastic S1P may contribute substantially to cartilage damage and further highlights the potential contribution of the OC niche in subchondral bone to cartilage breakdown in a direct or indirect manner. Previously, it was found that myeloid S1P regulates bone remodelling by increasing the number of OCs and their attraction toward the bone surface[27,39] and promotes osteoblast mobility and differentiation[24,28]. Here, the lack of myeloid *Sphk1* had no effect on subchondral OC number or bone structure in OA mice, which may suggest that myeloid S1P is involved in cartilage breakdown. Indeed, deletion of myeloid *Sphk1* does not affect the osteoclast number and the subchondral bone resorption, but this effect might be mediated by a lower osteoblast function induced by a lower local secretion of S1P. However, osteoclast secretome may modify the activity of subchondral bone marrow cells which in turn impact the chondrocyte metabolism. It has been suggested that the circulation from bone to cartilage is mediated by local blood flow. Another hypothesis is that S1P produced by the osteoclast circulate to the cartilage through the subchondral bone. Indeed, the diffusion of small molecules from bone to cartilage is possible through the subchondral bone although, or may be driven by microcracks[40], in particular for the molecules which molecular weight is under 400kD[41], including S1P.

Unlike in mice, the chondroprotective effect of antiresorbing drugs in humans led to conflicting results[42–45], partly because of the heterogenity of the OA profile. The mechanisms underlying the chondrocyte protection by the inhibition of osteoclast function in OA are not limited to inhibiting resorption of bone matrix[10,16], but also mediated by OC-secreted factors in a paracrine manner. Such an effect involves S1P$_2$, a major S1P receptor in murine chondrocytes, as was previously reported in human osteoarthritic chondrocytes[46–49]. Here, the inhibition of S1P$_2$ alone, but not S1P$_1$ or S1P$_3$, limited the activation of catabolic genes in chondrocytes. This is consistent with the cartilage protection with S1P$_2$ blockade by JTE and siRNA or S1P neutralization with sphingomab in mice. Moreover, OC secretome stimulates the JNK pathway, that may mediate S1P signalling[26,30]. More importantly, the JNK inhibition reduced MMP expression, suggesting that JNK inhibition may result in milder cartilage damage. Indeed, high concentration of exogenous S1P is required to upregulated MMPs expression that suggests the involvement of other signalling pathways. In addition to the catabolic effect, S1P could influence chondrocyte anabolism. It has been reported that S1P increased the proliferation and the survival of chondrocytes[50]. In a model of osteochondral defects in rats, the administration of chondrocytes treated with recombinant acid ceramidase promoted the production of glycosaminoglycan as well

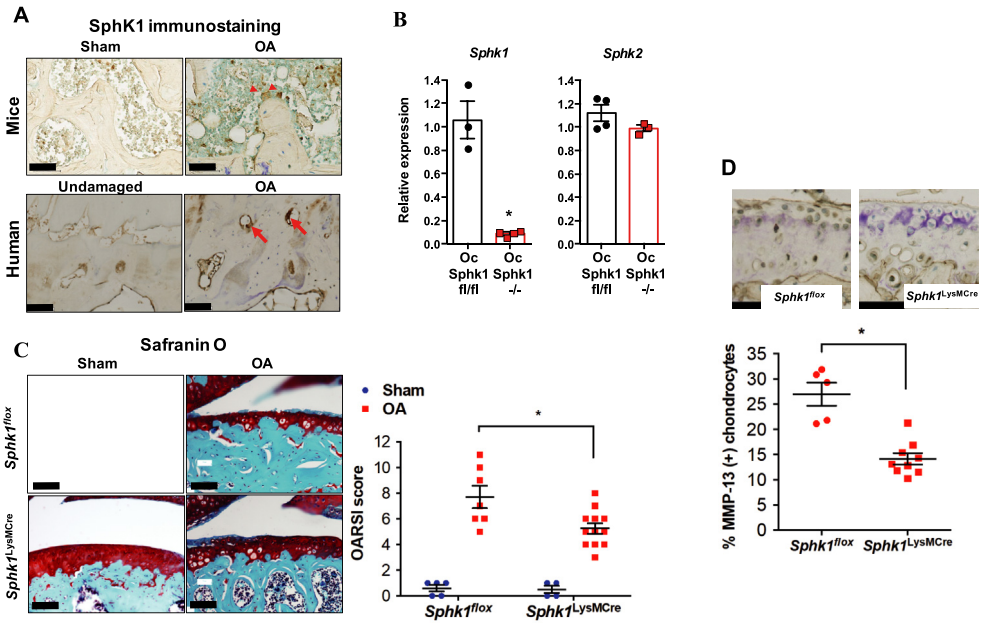

Fig. 4

***Sphk1* deletion in myeloid cells reduces cartilage breakdown. A,** Representative immunostaining for SphK1 in subchondral bone of medial tibia in sham or OA mice (upper panel) and undamaged and OA joints of humans (lower panel) ($n = 3$ per group). Scale bar: 100 μm. Quantitative RT-PCR analysis of **B,** *Sphk1* and *Sphk2* levels in mouse primary OCs from bone marrow lacking *Sphk1* (Oc $Sphk1^{-/-}$) or not (Oc $Sphk^{fl-fl}$) ($n = 3$–4).**C,** Representative safranin O staining of articular cartilage and quantification of OA score for medial femorotibial joints in $Sphk1^{flox}$ or $Sphk1^{LysMCre}$ 6 weeks after OA induction or sham surgery ($n = 5$–12). **D,** Representative immunostaining for MMP13 in articular cartilage of medial tibia joints with OA from $Sphk1^{flox}$ or $Sphk1^{LysMCre}$ mice and quantification of MMP13-positive chondrocytes by immunostaining ($n = 5$–9). Error bars indicate mean ± SEM. *$p < 0.05$.

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as the gene expression of aggrecan and collagen 2 and the cartilage repair[51]. Therefore, it might be possible that a dual action of local S1P concentration occurred here promoting chondrocyte catabolism or activating anabolism, thereby disrupting the homeostasis of chondrocyte balance.

The inflamed synovium induced by OA was not reduced in $Sphk1^{LysMCre}$ or JTE013-treated mice. This suggests that $S1P_2$ has little function in the migration of myeloid cells and shows the lack of a direct effect of JTE013 in synovial inflammation. Therefore, the cartilage catabolism is likely more driven by local S1P than by the

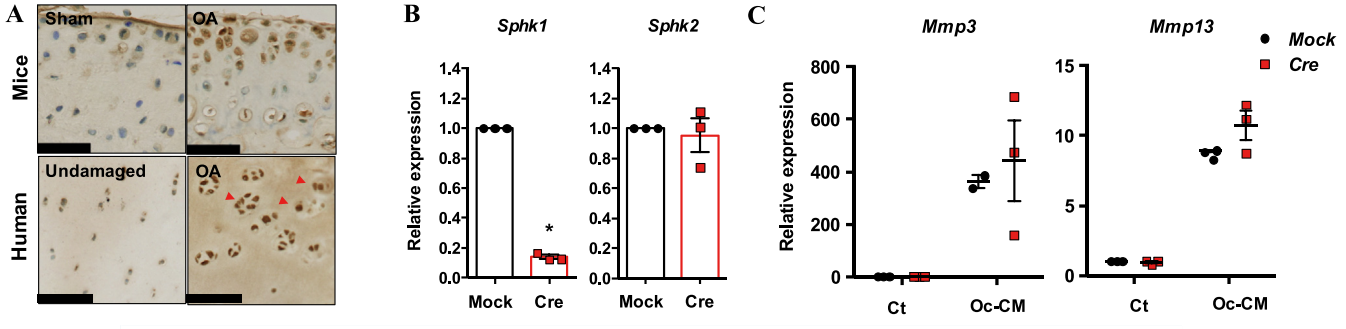

Fig. 5

Lack of autocrine effect of SphK1 in chondrocytes. A, Representative immunostaining of SPHK1 in articular cartilage of medial tibia in sham and OA mice (upper panel) and undamaged and OA human samples ($n = 3$). Scale bar: 100 μm. Quantitative RT-PCR analysis of **B,** *Sphk1* and *Sphk2* levels in mouse primary chondrocytes lacking *Sphk1* induced by empty virus (*Mock*) or (*Cre*) ($n = 3$–4 experiments) and **C,** *Mmp3* and *Mmp13* levels in mouse primary chondrocytes with or without *Sphk1* and cultured with Oc-CM ($n = 2$–3 experiments).

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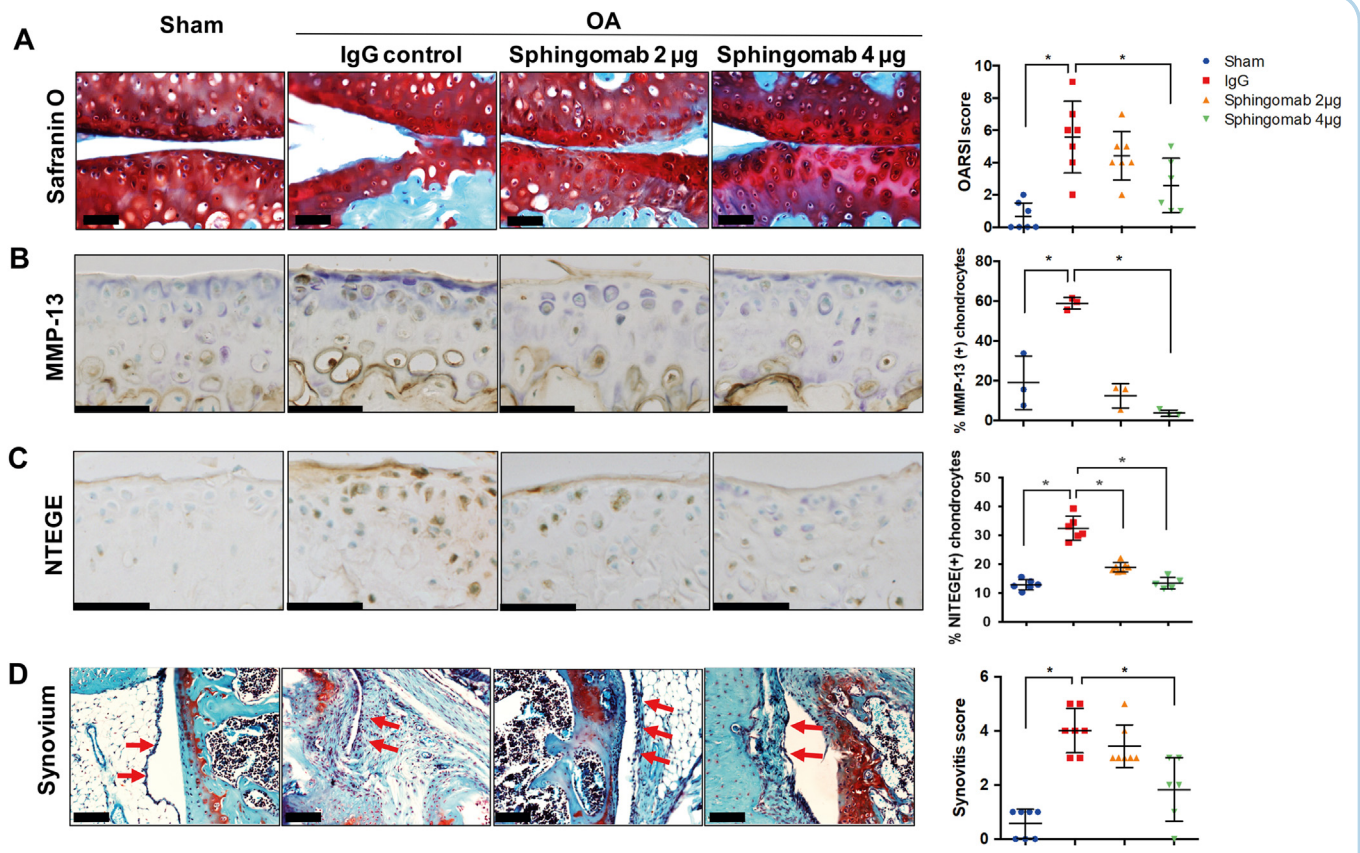

Fig. 6

Sphingomab protects mice against OA. **A,** Representative safranin O staining of articular cartilage and quantification of OA OARSI score in medial femoro-tibia joints from mice 6 weeks after OA induction or sham surgery. The neutralizing monoclonal antibody sphingomab was injected locally in joints (2 and 4 µg once per week for 6 weeks), and IgG (4 µg) was a control ($n = 7$ mice per group). **B,** Representative immunostaining and quantification of chondrocytes positive for MMP13 in medial tibia joints from sham or OA mice. **C,** Representative immunostaining of NITEGE in medial tibia joints from OA mice. **D,** Representative images of synovial tissue staining in knees 6 weeks after OA and sphingomab administration. Scale bar 100 µm *$p < 0.05$.

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synovial inflammation. S1P has been reported to be involved in the pathogenesis of several inflammatory diseases, such as lung inflammation and rheumatoid arthritis[52]. However, $S1P_1$ is the major receptor regulating the chemo-attraction and proliferation of myeloid cells and also the production of prostaglandin E2[53]. This effect is consistent with the suppression of inflammation with a specific $S1P_1$ antagonist[54]. Hence, blockage of $S1P_2$ may have modified the subchondral bone microenvironment to produce S1P. The neutralization of S1P with the antibody sphingomab was the only treatment that prevented synovial inflammation in addition to cartilage loss, which suggest the participation of S1P to the inflammatory aspect of OA. The lack of impact in the synovium of *Sphk1*-knockout mice or JTE013-treated mice could be due to SphK2, the other enzyme involved in maturation of S1P. However, myeloid cells maintained the expression of *SphK2*, as shown by the stable mRNA expression in the subchondral plate during the progression of OA and in *Sphk1*^{LysMCre} mouse-derived OCs. Despite a possible redundancy of SphK1 and SphK2[55], our results show that deletion of *Sphk1* in myeloid cells was sufficient to promote cartilage protection, and likely each enzyme could drive the maturation of S1P in specific tissues.

Because the effects of S1P are mostly mediated by its extracellular signaling, most drugs modulate S1P receptor activity such as Fingolimod[56], despite a lack of receptor specificity. We choose to block S1P in the joint cavity based on the hypothesis that S1P could target different tissues within the joint. S1P levels were detectable in the synovial fluids of OA and were at the same levels than those in people with rheumatoid arthritis, likely because synovial fluid production occurs in individuals with inflammatory flares, a condition associated with the production of synovial fluid. Given that articular cartilage is not vascularized, diffusion of molecules to the cartilage matrix is optimized when administered in synovial fluid. We cannot rule out that S1P produced by macrophages within the joint cavity could contribute to cartilage damage, in particular by using the *Sphk1*^{LysMCre} mouse model. It is therefore possible that S1P produced by macrophages within the joint cavity could contribute to cartilage damage. A local route allows for enhanced local concentration of the antibody and better diffusion in cartilage to reach chondrocytes. As a proof-of-concept for targeting S1P in OA, S1P was neutralized in synovial fluid by intra-articular injection. Indeed, sphingomab prevented both cartilage lesions and synovial inflammation in OA mice, in contrast to $S1P_2$ inhibition and

Sphk1 deletion in myeloid cells, which reduced cartilage lesions without affecting the synovium. The prevention of lesions in the whole joints with sphingomab might be related to the direct neutralization of a ubiquitous source of S1P, the reduction of local concentration of S1P and the subsequent binding to receptors, whereas JTE013 is restricted to inhibiting $S1P_2$. This observation addresses the human relevance towards osteoarthritic patients. Sphingomab has been developed as a humanized biotherapy and tested in phase two trial in a renal cell carcinoma setting (NCT01762033). No effect on progression-free survival was observed and was not further developed to the phase 3. There was however a favorable safety profile[57,58]. Our data provide insights in OA conditions that will allow repurposing the drug. Because of the effect on both cartilage and the synovium, the anti-S1P biotherapy could be better oriented to patients with inflammatory OA forms and administered intra-articularly to allow a reduction in joint inflammation. Clinical trials could be developed to assess the potential effect of anti-S1P in OA and better define the population who will best benefit of the treatment.

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

Author contributions

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

Conflict of interest

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

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Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.joca.2021.06.001.

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