Chondroitin Sulphate Attenuates Atherosclerosis in ApoE Knockout Mice Involving Cellular Regulation of the Inflammatory Response

Chondroitin sulphate (CS) has long been used to treat osteoarthritis. Some investigations have also shown that the treatment with CS could reduce coronary events in patients with heart disease but no studies have identified the mechanistic role of these therapeutic effects. We aimed to investigate how the treatment with CS can interfere with the progress of atherosclerosis. The aortic arch, thoracic aorta and serum were obtained from apolipoprotein E (ApoE) knockout mice fed for 10 weeks with high-fat diet and then treated with CS (300 mg/kg, \( n = 15 \)) or vehicle (\( n = 15 \)) for 4 weeks. Atheromatous plaques were highlighted in aortas with Oil Red staining and analysed by microscopy. ApoE knockout mice treated with CS exhibited attenuated atheroma lesion size by 68% as compared with animals receiving vehicle. Serum lipids, glucose and C-reactive protein were not affected by treatment with CS. To investigate whether CS locally affects the inflamed endothelium or the formation of foam cells in plaques, human endothelial cells and monocytes were stimulated with tumour necrosis factor \( \alpha \) or phorbol myristate acetate in the presence or absence of CS. CS reduced the expression of vascular cell adhesion molecule 1, intercellular adhesion molecule 1 and ephrin-B2 and improved the migration of inflamed endothelial cells. CS inhibited foam cell formation in vivo and concomitantly CD36 and CD146 expression and oxidized low-density lipoprotein uptake and accumulation in cultured activated human monocytes and macrophages. Reported cardioprotective effects of CS may arise from modulation of pro-inflammatory activation of endothelium and monocytes and foam cell formation.
Introduction

Atherosclerosis is a chronic inflammatory disease characterized by activation of the immune system and endothelium where tumour necrosis factor α (TNF-α) displays an essential role. In the evolution of the process, monocytes are recruited from the blood stream by the inflamed endothelium, and then infiltrate atherosclerotic lesions, differentiating into macrophages and ultimately into foam cells. Macrophages and foam cells deliver pro-inflammatory mediators such as TNF-α which participate decisively in the development and exacerbation of atherosclerosis.

Chondroitin sulphate (CS) is a glycosaminoglycan present in the extracellular matrix, especially in the cartilage, skin, blood vessels, ligaments and tendons. CS has demonstrated therapeutic immunomodulatory and anti-inflammatory effects and is actively used for the treatment of osteoarthritis. Several reasons have encouraged researchers to investigate whether CS might also be effective in the treatment of chronic inflammation in atherosclerosis. Administration of CS interferes with the pro-inflammatory response of activated murine macrophages but no studies have described the potential role of CS interfering with the formation of foam cells from macrophages in atherosclerotic lesions. In clinical trials in the early 1970s, CS was shown to reduce early coronary events and late mortality in 60 patients with coronary heart disease. While 70% of control patients suffered a cardiac event at 1 month, and 14 died after 6 years of follow-up, only 13 (10%) of the CS-treated patients presented an acute cardiac event and only 4 died. A few years later, a different clinical trial confirmed similar therapeutic effects of CS in mortality on atherothrombotic subjects. However, since then, the therapeutic use of CS has been restricted essentially to osteoarthritis. Indeed, no clinical trials have been designed following the current Consolidated Standards of Reporting Trials quality standards to demonstrate the potential therapeutic use of CS to prevent cardiovascular events. Recent evidence has opened again the debate of using CS to treat cardiovascular disease. A case-control study demonstrated that osteoarthritic patients chronically treated with CS show a low incidence of coronary events, but the cellular and molecular events promoting these protective effects are still not defined.

The key initiating process in atherogenesis is the subendothelial retention of lipoprotein particles. Local biological responses to these retained lipoproteins, including a chronic and maladaptive macrophage inflammatory response, promote subsequent lesion development. There are preferred sites for plaque formation in arteries. These are locations of the artery in which there is flow separation or recirculation, such as the aortic arch, or the ramifications of the subclavian arteries and the carotids. Disrupted flow patterns and, more specifically, flow re-circulation exacerbate low-density lipoprotein (LDL) internalization, cell adhesion molecules expression and monocyte adhesion to the endothelium. These events are early determinants for atherosclerosis progression. Ultimately, chronic inflammation and advanced atherosclerosis can promote cardiovascular events. In this scenario, we investigated whether CS controls the cellular and molecular events involved in the formation and progression of atherosclerotic lesions.

Materials and Methods

Animal Studies

Animal experiments were approved by the Animal Ethics Committee at Massachusetts Institute of Technology, Massachusetts, United States, and conformed to the National Institutes of Health (NIH) guidelines on the protection of animals used for scientific purposes. Thirty male 8 weeks old homozygous apolipoprotein E (ApoE) knockout (KO) mice (B6.129P2-Apoetm1Unc/Male) were purchased from Jackson Laboratory (Bar Harbor, Maine, United States), and 6 male 18 weeks old wild-type C57BL/6 mice were purchased from Charles River Laboratories (Wilmington, Massachusetts, United States). All animals were maintained in a temperature-controlled room (22°C) on a 12-hour light-dark cycle under institutional and NIH guidelines. After arrival, mice were continuously fed ad libitum with high-fat diet (Research Diets, New Jersey, United States), with 60% of the Kcal from fat and 0.72 mg of cholesterol per gram of food for a total of 10 weeks before starting the treatment. After that, mice were given 300 mg/kg of CS or saline solution by oral gavage daily for 4 weeks. All the mice were weighed before euthanasia. Mice were euthanized in a CO2 chamber. After verifying the death, animals were transferred to the surgical room. Then, the cava vein was isolated and catheterized with 26G catheter, and a blood sample was collected, clotted and the tube centrifuged at 1,600 × g for 15 minutes at 4°C. Serum was stored at −140°C for further analysis. The thorax was opened to expose the heart and a 26G catheter introduced into the left ventricle. A solution of phosphate-buffered saline (PBS) with 4% citrate was perfused for 10 minutes at 1 mL/min. Then, the animal was perfused with a solution of 10% formaldehyde in PBS with 4% citrate for 15 minutes. Liver, heart, both the lungs, kidneys, spleen, and fat tissue were collected for further analyses. Aorta was then isolated. Lipid profile and C-reactive protein were analysed in serum by Charles River Laboratories. Blood glucose was measured in a drop of blood using TrueTest disposable glucose strips and glucometer.

Oil Red Staining

A solution of 0.7% Oil Red O (Sigma-Aldrich, St. Louis, Missouri, United States) in 85% propylene glycol was prepared. Oil Red O was dissolved in a water bath at 100°C for 30 minutes, shaking the solution frequently, filtered with a Whatman filter and kept in an oven at 60°C for up to 1 week. To stain the whole aorta, a 2% Oil Red O solution was used. Animals were sacrificed by overexposure to CO2 and intracardially perfused (1 mL/min) with PBS 4% citrate for 3 minutes, then 10% formaldehyde in PBS for 15 minutes and PBS 4% citrate for 3 minutes, washed with propylene glycol 85% for 5 minutes and stained with 2% solution Oil Red O for 10 minutes. Afterwards, isolated aorta was incubated overnight in a 2% solution Oil Red O. The aorta was then
washed with distilled water until plaques were clearly visible and imaged with a stereo microscope. The size and number of atheroma plaques in aorta were measured using ImageJ.

**Tagging of CS and CS-Derived Disaccharides with Texas Red**
Chondroitin sulphate (BioIbérica S.A.U, Spain) at 2 mg/mL was tagged with Texas Red (Thermo Fischer Scientific, Waltham, Massachusetts, United States) using the 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide/N-hydroxysuccinimide (EDC/NHS) reaction (Thermo Fischer) at room temperature for 2 hours. Texas Red was added in a molar ratio of 4:1 with CS. The product was filtered using a 5-ml Amicon Ultra 3 KDa cut-off membrane (Millipore, Burlington, Massachusetts, United States) and 0.1 M sodium phosphate buffer. Tagged CS was then lyophilized overnight protected from light. After tagging the CS, part of the product was digested with chondroitinase ABC (Sigma-Aldrich) to obtain tagged disaccharides and then filtered through a 10-KDa cut-off membrane.

**Fluorescent Imaging of Texas Red-CS or Texas Red-Disaccharides**
To validate the fluorescence of tagged CS or tagged disaccharides, three control C57BL/6 wild-type or ApoE KO mice received Texas Red-CS or Texas Red-disaccharides or vehicle. The suggested dose for intravenous (i.v.) administration of CS is 45 mg/kg, extrapolated from the oral dose of 300 mg/kg and bioavailability of CS established in the literature by Ronca et al in 1998. Lyophilized Texas Red-CS and Texas Red-disaccharides were dissolved in saline and administered by tail vein injection. Aortic arch slices of 30 µm were obtained to assess the fluorescence location of the tagged molecules by Multiphoton microscopy Olympus FV-1000 MP (Olympus America Inc., Center Valley, Pennsylvania, United States). IVIS Spectrum-Bioluminescent and Fluorescent Imaging System (Xenogen, Hopkinton, Massachusetts, United States) was used for ex vivo analysis of Texas Red-CS and Texas Red-disaccharides in the whole aorta.

**Cell Culture**
Human aortic endothelial cells (HAECs) were grown on Endothelial Growth Medium-2 (EGM-2, Lonza, Walkersville, Maryland, United States) and the human monocye cell line THP-1 on Dulbecco’s modified Eagle medium supplemented with 10% foetal bovine serum. Human macrophages were obtained from incubation of THP-1 monocytes with phorbol myristate acetate (PMA) (100 ng/mL) for 2 days.

**In Vitro Proliferation Assay**
The effects of CS on the proliferation of HAEC were assessed using the CellTiter96 Aqueous One Solution Cell Proliferation Assay (MTS assay, Promega, Madison, Wisconsin, United States). Briefly, cells were seeded in pre-gelatinized 96-well plates at a cell density of 5 x 10^3 cells per well and incubated for 24 hours in the corresponding complete media. Then, cells were treated with TNF-α (5 ng/mL), TNF-α + CS (200 µg/mL) or PBS (as control) for 24 hours at 37°C. Just before the determination of cell viability, cells were washed with PBS and transferred into starvation medium. Cell proliferation was determined by adding 20 µL of MTS solution to each well. After 2 hours, the absorbance was measured at 490 nm using a Varioskan Flash spectrophotometer (Thermo Fisher Scientific). Cell viability was expressed as the absorbance of treated cells relative to cells treated with PBS (control cells). Each condition was performed in sextuplicate and reported as mean ± standard error of the mean (SEM).

**In Vitro Wound Closure Assay**
Human aortic endothelial cells were grown to confluence in 24-well plates in EGM-2 and treated with TNF-α (5 ng/mL), TNF-α + CS (200 µg/mL) or PBS (as control). A straight scratch was made on the HAEC using a P1000 pipette tip. The cells were then washed with PBS three times, and further cultured in EGM-2 containing TNF-α, TNF-α + CS or saline. After incubating for 0 and 24 hours, the gap width of scratch re-population was measured and recorded, and then compared with the initial gap size at 0 hour. The size of the denuded area was determined at each time point from the digital images using the ImageJ image processing software. The percentage of wounded area was calculated with the ratio: gap area/total area × 100.

**Gene Expression Analysis by Real-Time Polymerase Chain Reaction**
Total RNA from human cells was extracted using commercially available kits: RNeasy (Qiagen, Invitrogen, Paisley, UK). One microgram aliquot of total RNA was reverse transcribed using a complementary DNA synthesis kit (High-Capacity cDNA Reverse Transcription Kit, Applied Biosystems, Foster City, California, United States). Primers and probes for human gene expression assays were selected as follows: vascular cell adhesion molecule 1 (VCAM-1) (TaqMan assay reference from Applied Biosystems Hs01003372_m1), intercellular adhesion molecule 1 (ICAM-1) (TaqMan assay reference from Applied Biosystems Hs00164932_m1), ephrin-B2 (TaqMan assay reference from Applied Biosystems Hs00148932_m1), ephrin-B2 (TaqMan assay reference from Applied Biosystems Hs00187950_m1), CD146 (TaqMan assay reference from Applied Biosystems Hs00174838_m1) and glyceraldehyde 3-phosphate dehydrogenase TaqMan Pre-Developed Assay as an endogenous standard. Expression assays were designed using the TaqMan Gene Expression assay software (Applied Biosystems). Real-time quantitative polymerase chain reaction (PCR) was performed in duplicate and performed with a LightCycler-480 (Roche Diagnostics). A 10 µL aliquot of the total volume reaction of diluted 1:8 cDNA, TaqMan probe and primers and FastStart TaqMan Master (Applied Biosystems) was used in each PCR. The fluorescence signal was captured during each of the 45 cycles (denaturing 10 seconds at 95°C, annealing 15 seconds at 60°C and extension 20 seconds at 72°C). Water was used as a negative control. Relative quantification was calculated using the comparative threshold cycle (CT), which is inversely related to the abundance of mRNA transcripts in the initial sample. The mean CT of duplicate measurements was used to calculate ΔCT as the difference in CT for target and reference genes.
reference. The relative quantity of the product was expressed as fold induction of the target gene compared with the control primers according to the Eq. $2^\Delta\Delta CT$, where $\Delta CT$ represents $\Delta CT$ values normalized with the mean $\Delta CT$ of control samples.

### Uptake Assay of Dilox-LDL in Human THP-1 Macrophages

Human macrophages were obtained from incubation of THP-1 monocytes (5 x 10^4 cells in 24-well plates) with PMA (100 ng/mL) for 2 days. THP-1 human macrophages were incubated in the presence or absence of CS (200 µg/mL) for 24 hours and then treated with Dil-labelled oxidized LDL (ox-LDL) (5 µg/mL; Thermo Fisher Scientific). After 3 hours, cells were washed three times with PBS and the uptake of Dil-labelled ox-LDL was visualized with an epifluorescence microscope (Nikon Eclipse Ti, Kanagawa, Japan). Afterwards, cells were lysed by using a radioimmunoprecipitation assay (RIPA) buffer and the total fluorescence of each well was quantified using a Varioskan Flash spectrophotometer (Thermo Fisher Scientific).

### Ox-LDL Accumulation Assay in Human THP-1 Macrophages

THP-1 macrophages (5 x 10^4 cells/well) obtained as described above were seeded in 24-well black plates and incubated with or without the presence of CS (200 µg/mL) for 24 hours. Therefore, the cells were incubated with ox-LDL (50 µg/mL, Thermo Fisher Scientific) for 48 hours. THP-1 cells were stained with Hanks solution containing 2.5 µg/mL of the fluorescent probe Nile red (Sigma, St. Louis, Missouri, United States). Afterwards, cells were washed three times with PBS and the accumulation of lipids and ox-LDL was visualized with an epifluorescence microscope (Nikon Eclipse Ti). Cells were then lysed with RIPA buffer and the total fluorescence of each well was quantified using a Varioskan Flash spectrophotometer (Thermo Fisher Scientific).

### Statistical Analysis

Data are expressed as mean ± standard error. Statistical analysis of the results was performed by one-way analysis of variance, the Newman–Keuls test, the unpaired Student’s t-test or the log-rank (Mantel–Cox) test when appropriate. Differences were considered to be significant at a p-value of 0.05 or less. Data sampled from Gaussian populations were used for the calculation of Pearson’s correlation coefficient. A Pearson’s correlation coefficient (r) value of $> 0.75$ was considered to exhibit strong positive correlation, a value between 0.50 and 0.75 was considered as a moderate correlation, whereas value less than 0.5 was considered to demonstrate a weak correlation between two variables.

### Results

#### Administration of CS Reduces the Area of Atheroma Plaques in ApoE Knockout Mice

We used a genetically modified animal model of atherosclerosis (ApoE KO mice) additionally chronically fed with high-fat diet to create advanced atheromatous lesions. The number and the extension of atheroma plaques in ApoE KO mice treated with CS was visualized using Oil Red staining in the aortic arch and thoracic aorta, and digitally quantified using the ImageJ software (► Fig. 1A). As mechanical and rheological factors determine lesion number and localization, ApoE KO mice treated with CS showed the same number of atheroma plaques than mice receiving vehicle (► Fig. 1B). CS, however, did significantly reduce by threefold the total and maximum area of atheroma plaques (► Fig. 1C, 1D). These therapeutic effects of CS reducing the extension of atheroma plaques were accompanied by 100% survival of the animals treated with CS for 4 weeks as compared with 85% survival in the group of animals receiving vehicle (► Fig. 1E).

#### CS Treatment Does Not Modify Systemic Inflammation or the Circulating Levels of Glucose and Lipids

As CS is actively used to treat chronic inflammation in patients with osteoarthritis, we wondered whether CS could also reduce the systemic inflammation occurring in ApoE KO mice. We analysed the circulation levels of C-reactive protein, as a sensitive indicator of systemic inflammation. Our data show that the concentration of serum C-reactive protein was not modified in ApoE KO mice as a consequence of the treatment with CS as compared with mice receiving vehicle (► Fig. 2A). Thereafter, we analysed the serum levels of glucose in treated animals as an indication of the glucose metabolism and lipogenesis. There were no significant differences in serum glucose between ApoE KO mice treated with vehicle or CS, although CS-treated animals showed a trend to reduce glucose levels (► Fig. 2B). We further analysed serum markers of the lipid metabolism and transport in treated ApoE KO mice and we found no significant differences in the concentration of cholesterol, triglycerides, fatty acids, high-density lipoprotein or LDL (► Fig. 2C). Altogether these outcomes clearly demonstrate that reduction in atheroma plaque size by CS was not a consequence of systemic effects related to inflammation, glucose or lipid metabolism and transport, but a local effect in atherosclerotic lesions.

#### CS and Its Disaccharides Accumulate in Atheroma Plaques

To analyse the destination of CS and its digestion products (disaccharides) in the organism after administration, we labelled both CS and disaccharides with the fluorophor Texas Red. We first optimized the enzymatic assay using the enzyme chondroitinase ABC from Proteus vulgaris to mimic the digestion of CS in the gastrointestinal tract from CS to disaccharides. We digested different amounts of CS and enzyme and we measured the absorbance of formed disaccharides at 232 nm to track the generation of unsaturated bonds and disaccharides. The time needed to completely digest (95%) 1 mg of CS (amount needed for one mouse) is 28 minutes for 10 µU of enzyme, 14 minutes if 20 µU is used and 9 minutes when using 37.5 µU (► Supplementary Fig. S1, online only). We doubled the time to ensure that all the CS was digested. We injected intravenously both CS-Texas Red and CS-disaccharides-Texas Red to wild-type and ApoE KO mice at the same concentrations that are found in plasma after oral
administration in mice as previously described. When comparing ApoE KO mice with wild-type mice, CS-Texas Red and CS-disaccharides-Texas Red fluorescence was significantly higher in liver and lungs from ApoE KO mice being the heart the next tissue displaying the highest increase in fluorescence intensity (►Supplementary Fig. S2, online only). In the vascular tree, CS-Texas Red and CS-disaccharides-Texas Red accumulate mainly in the aortic arch of ApoE KO mice (►Supplementary Fig. S3, online only). We then performed tissue slices in aortic arches from APOE KO mice i.v. administered with tagged CS and disaccharides. We observed that both CS-Texas Red and CS-disaccharides-Texas Red were located and accumulated in the first layers of the areas of the aortic arch where wall thickening occurred, thus corresponding with atheroma plaques of the ApoE KO mice (►Fig. 3).

**CS Treatment Disrupts the TNF-α-Mediated Effects on Expression of Monocyte Attractants and Cell Migration in HCAEC**

Our in vivo outcomes pointed to a possible role of CS interfering locally with the inflammatory response of endothelium in atheroma plaques. For that reason, we analysed the effects of the treatment of CS in inflamed human coronary aortic endothelial cells (HCAECs). As expected, the incubation of these cells with TNF-α produced an increase in the expression of both monocyte adhesion molecules VCAM-1 and ICAM-1 (►Fig. 4A). These increased values were significantly attenuated when HCAECs were treated with CS (►Fig. 4A). Similarly, incubation of inflamed HCAEC with CS partially prevented the synthesis of the chemokine ephrin-B2 (►Fig. 4A). The anti-inflammatory effects of CS were not associated with endothelial cell (EC) proliferation, which was unaffected by CS treatment (►Fig. 4B). As CS is an extracellular matrix component secreted by pericytes to allow the growth of ECs, we wondered if CS might influence recovery of monolayer restoration in inflamed and wounded HCAEC. HCAEC cultures were grown to confluence and stimulated with TNF in the presence or absence of CS. A wound gap was created by scratching a straight line across the confluent monolayer of HCAEC. The gap healed completely within 24 hours in non-treated HCAEC (►Fig. 4C) but was retarded by TNF exposure and partially restored with CS (►Fig. 4C).

**CS Treatment Interferes with Inflammation-Mediated Up-Regulation of Monocyte/Macrophage CD36 and CD146 Receptors**

The finding that CS treatment reduces fat in atheroma plaques suggests that CS could be also involved in the
response of monocytes and macrophages to pro-inflammatory stimuli and subsequent differentiation into foam cells. Following this rationale, we activated the human monocyte cell line THP-1 with TNF-α or PMA in the presence or absence of CS and analysed the effects on the expression of CD36 and CD146, two key receptors involved in the transformation of macrophages into foam cells. \[18,19\]

TNF-α up-regulated expression of CD36 in monocytes and CS abolished this increase (►Fig. 5A). The transformation of monocytes into macrophages by PMA interestingly resulted in a 600-fold up-regulation of CD36 expression, and this increase was partially reduced (39% reduction; 609.6 ± 38.8 vs. 374.3 ± 43.5, \(p < 0.0001\)) when macrophages were treated with CS (►Fig. 5A). Similarly, expression of CD146 significantly rose in monocytes exposed to TNF-α, and this augmentation normalized when monocytes were also incubated with CS (►Fig. 5B). Macrophages obtained using PMA showed higher expression of CD146 than inflamed or non-inflamed monocytes, and this induction was partially reversed when macrophages were treated with CS (►Fig. 5B).

**CS Treatment Interferes with the Uptake and Accumulation of Ox-LDL in Macrophages and Foam Cells**

As CD36 and CD146 are scavenger receptors involved in the acquisition of lipids from the blood stream,\[19,20\] we wondered whether CS could also modulate macrophage uptake of ox-LDL, a critical factor associated with atherogenesis.\[21,22\] PMA-activated macrophages displayed a high uptake of fluorescent Dilox-LDL as visualized by fluorescence microscopy after 3 hours of incubation (►Fig. 6A). A lower intensity of fluorescent red signal was observed when macrophages were treated with CS (►Fig. 6A). We then lysed the cells and quantified the total fluorescence of Dilox-LDL taken up by macrophages using fluorescence spectrophotometry. We found a high amount of fluorescent Dilox-LDL in macrophages that was significantly reduced by the treatment with CS (►Fig. 6B). Afterwards, we analysed the effects of CS on lipid and especially ox-LDL accumulation in macrophages as a previous step to form foam cells. We treated macrophages with ox-LDL for 48 hours in the presence or absence of CS and highlighted all lipids using Nile Red fluorescent staining. Macrophages showed higher accumulation of lipids and ox-LDL than control cells as visualized with fluorescent microscopy, and this signal was weaker when cells were treated with CS (►Fig. 6C). Similarly, when we lysed the cells and quantified the fluorescence of accumulated lipids we also found a higher amount of lipids and ox-LDL in untreated macrophages that was significantly reduced in cells incubated with CS (►Fig. 6D). This impeded uptake and accumulation of ox-LDL and formation of foam cells in the presence of CS perfectly correlated with the

---

**Fig. 2** Treatment with chondroitin sulphate (CS) does not modify plasma levels of C-reactive protein, glucose or lipids in apolipoprotein E (ApoE) knockout (KO) mice. (A) Quantification of C-reactive protein in serum from ApoE KO mice receiving daily vehicle (\(n = 11\)) or CS (300 mg/kg, \(n = 15\)) by oral gavage for 4 weeks and high-fat diet since the day of arrival. (B) Quantification of blood glucose before euthanasia in ApoE KO animals receiving daily vehicle (\(n = 11\)) or CS (\(n = 15\)) for 4 weeks. (C) Quantification of serum lipid profile (cholesterol, triglycerides, fatty acids, high-density lipoprotein [HDL] and low-density lipoprotein [LDL]) in ApoE KO mice receiving vehicle (\(n = 15\)) or CS (\(n = 15\)). Values are mean ± standard error of the mean (SEM).
Fig. 3 Chondroitin sulphate (CS) and its digested products are specifically found in the sub-endothelial layer of atherosclerotic plaques after intravenous (i.v.) injection. Representative images of multi-photon fluorescence microscopy in sections of aortic arch (30 µm thick) from apolipoprotein E (ApoE) knockout mice 3 hours after i.v. injection of either CS chemically linked to the fluorophore Texas Red \( n = 3 \) or disaccharides from CS chemically linked to Texas Red \( n = 3 \) (scale bar = 100 µm).

Fig. 4 Chondroitin sulphate (CS) interferes with tumour necrosis factor \( \alpha \) (TNF-\( \alpha \))-mediated effects on expression of monocyte attractants and cell migration in human coronary artery endothelial cells (HCAECs). HCAECs were treated with phosphate-buffered saline (PBS) (Control, \( n = 6 \)), inflamed with TNF-\( \alpha \) (5 ng/mL, \( n = 6 \)) or inflamed and treated with CS (200 µg/mL, \( n = 6 \)) for 24 hours and gene expression of vascular cell adhesion molecule 1 (VCAM-1), intercellular adhesion molecule 1 (ICAM-1) and ephrin-B2 (A), as well as cell proliferation (B) was analysed (** indicates \( p \leq 0.0001 \), *** indicates \( p \leq 0.01 \) using a one-way analysis of variance [ANOVA]). (C) Representative images of light microscopy of HAEC grown to confluence in 24-well plates, scratched at time 0 hour (width of initial scratch highlighted with black arrows) and then treated with PBS (as control, \( n = 6 \)), with TNF-\( \alpha \) (5 ng/mL, \( n = 6 \)) or TNF-\( \alpha \) + CS (200 µg/mL, \( n = 6 \)) for 24 hours. The percentage of relative wound area is plotted on the right at time 0 and 24 hours (** indicates \( p \leq 0.0001 \) using a one-way ANOVA). Scale bar = 200 µm; original magnification, 40 × . Values are mean ± standard error of the mean (SEM).
Fig. 5 Chondroitin sulphate (CS) interferes with tumour necrosis factor α (TNF-α)-mediated up-regulation of monocyte and macrophage CD36 and CD146 receptors. The human monocyte cell line THP-1 was treated with phosphate-buffered saline (PBS) (Control, n = 6), or inflamed with TNF-α (5 ng/mL, n = 6) or inflamed and treated with CS (200 µg/mL, n = 6), or differentiated into macrophages with phorbol myristate acetate (PMA, 100 ng/mL for 2 days, n = 6) or differentiated with PMA and treated with CS (200 µg/mL, n = 6) for 24 hours, and the gene expression of CD36 (A) and CD146 (B) was quantified by real-time polymerase chain reaction (PCR) (*** indicates p ≤ 0.0001, ** indicates p ≤ 0.01, * indicates p ≤ 0.05 using a one-way analysis of variance [ANOVA]). Values are mean ± standard error of the mean (SEM).

Fig. 6 Chondroitin sulphate (CS) interferes with the uptake and accumulation of oxidized low-density lipoprotein (ox-LDL) in macrophages and foam cells. Human macrophages were obtained by differentiation of the human monocyte cell line THP-1 with phorbol myristate acetate (PMA, 100 ng/mL) for 2 days. (A) Representative images from fluorescence microscopy of THP-1 macrophages incubated with phosphate-buffered saline (PBS) (Control, n = 6), or ox-LDL complexed with DiI fluorescent dye (DiIox-LDL, 5 μg/mL, n = 6) for 3 hours or treated with CS (200 µg/mL) for 24 hours and then incubated with DiIox-LDL for 3 hours (n = 6) (scale bar = 50 μm). (B) Relative fluorescence units (RFU) quantified by fluorescence spectroscopy in cell lysates from THP-1 macrophages incubated with PBS (Control, n = 6), or DiIox-LDL for 3 hours (n = 6) or treated with CS (200 µg/mL) for 24 hours and then incubated with DiIox-LDL for 3 hours (n = 6) (*** indicates p ≤ 0.0001 using a one-way analysis of variance [ANOVA]). (C) Representative images from fluorescence microscopy for Nile Red staining of THP-1 macrophages incubated with PBS (Control, n = 6), or ox-LDL (50 µg/mL, n = 6) for 48 hours or treated with CS (200 µg/mL) for 24 hours and then incubated with ox-LDL for 48 hours (n = 6) (scale bar = 50 μm). (D) RFU quantified by fluorescence spectroscopy for Nile Red in cell lysates from THP-1 macrophages incubated with PBS (Control, n = 6), or ox-LDL for 48 hours (n = 6) or treated with CS (200 µg/mL) for 24 hours and then incubated with ox-LDL for 48 hours (n = 6) (** indicates p ≤ 0.0001 using a one-way ANOVA). Values are mean ± standard error of the mean (SEM). Original magnification of microscope images, 200 ×.
reduction of CD36 expression in macrophages treated with CS (►Supplementary Fig. S4, online only).

Discussion

Endothelium dysfunction of lesion-prone areas of the arterial vasculature is a significant contributor to the pathobiology of atherosclerotic cardiovascular disease.23 Haemodynamic forces such as re-circulation in arterial bifurcations influence the permeability of the endothelial barrier and expression of pro-inflammatory mediators and receptors that attract immune cells, which amplify the inflammatory response.24 Among all the pro-inflammatory cytokines, TNF-α displays a wide array of modulatory effects on endothelium and immune cells with a major impact on the pathogenesis and progression of atherosclerotic lesions in atherosclerosis.25 Indeed TNF-α induces foam cell formation in macrophages by stimulating the uptake of ox-LDL particles.26 Ox-LDL, in turn, contributes to plaque formation by promoting endothelial dysfunction, smooth muscle cells migration and proliferation and induction of platelet activation.27 Therefore, new therapies to interfere with TNF-α pro-inflammatory effects might well prevent atherogenesis and reduce the impact of atherosclerotic cardiovascular disease.

Chondroitin sulphate reduces the incidence of coronary events in patients with coronary heart disease.12,14 In this study, we examine how this clinical effect might have arisen in ApoE KO mice, a classic mouse model spontaneously developing atherosclerotic lesions that were further potentiated with a high-fat diet. We observed a drastic reduction in the area of arterial atherosclerotic plaques in ApoE KO mice treated with CS. Furthermore, ApoE KO mice treated with CS displayed a 100% survival compared with the 85% survival in animals receiving vehicle. These beneficial effects cannot be explained by a reduction in glucose, lipids or systemic inflammation. Moreover, we isolated CS and its digestion-derived disaccharides to the sub-endothelial space of atherosclerotic lesions in the aortic arch, suggesting that CS could interfere with the promotion of atherosclerosis by locally interacting with injured ECs and infiltrated monocytes in atheroma plaques. Our in vitro experiments showed that inflammation of HCAEC with TNF-α results in the up-regulation of the monocyte receptors VCAM-1, ICAM-1 and the chemokine and monocyte activator ephrin-B2. These three are important markers and mediators up-regulated in atherosclerosis and endothelial dysfunction.28,29 We show that CS interferes with the induction of these agents promoted by TNF-α in HCAEC. Continuous administration of TNF-α is also known to inhibit angiogenic sprouting and migration.30 Our results show for the first time that CS partially reverses the effect of TNF-α on EC migration and monolayer recovery. CS could help ECs in atherosclerotic lesions to re-structure and heal damaged areas of arteries. This together with the fact that we showed how CS treatment prevents the activation of monocytes to differentiate and migrate to the inflamed endothelium supports the hypothesis that CS treatment can reduce atherogenesis via a dual action on endothelium: by interacting with EC matrix and preventing TNF-α-mediated release of chemokines and cytokines, and by preventing deleterious effects on endothelial migration to heal wounds in atherosclerotic lesions.

Fig. 7 Schematic view illustrating the mechanistic role of chondroitin sulphate in atherogenesis. On the left, we highlight that chondroitin sulphate interferes with the pro-inflammatory response originated by tumour necrosis factor α (TNF-α) on endothelial cell expression of vascular cell adhesion molecule 1 (VCAM-1), intercellular adhesion molecule 1 (ICAM-1) and ephrin-B2, and on monocyte/macrophage expression of CD36 and CD146 oxidized low-density lipoprotein (ox-LDL) receptors, thereby reducing the uptake of ox-LDL and the formation of foam cells in atheroma plaques. On the right, we show that chondroitin sulphate additionally fosters endothelial cell migration and monolayer recovery of injured endothelium.
The accumulation and proliferation of lesional macrophages in atheromatous plaques is essential for disease progression and promotion of cardiovascular events.\textsuperscript{5,31} The activation state of local immune cells can exacerbate the extension of the damage.\textsuperscript{5} We found that human monocytes activated with TNF-\(\alpha\) are prone to increase expression of ox-LDL receptors CD36 and CD146. These two receptors are involved not only in the uptake of ox-LDL but also in the trans-endothelial migration of monocytes and foam cell formation.\textsuperscript{32,33} As CS obliterates the TNF-\(\alpha\)-mediated induction of both CD36 and CD146, this means that CS is actually reducing the ability of monocytes to migrate into atherosclerotic lesions and therefore dropping the number of precursors for potential foam cells. We also describe that differentiation of monocytes to macrophages is followed by much more pronounced up-regulation of both CD36 and CD146 than in activated monocytes. CS also reduces this induction in macrophages indicating a potential effect of CS on the formation of foam cells and the uptake speed and accumulation of ox-LDL in macrophages (\textsuperscript{5,31} Fig. 7).

The data presented herein add insight into how CS interferes with the progression of atherogenesis and support the rationale of considering CS as pharmacologic mediation of atherosclerosis initiation and progression for prevention of cardiovascular events.

**What is known about this topic?**
- Chondroitin sulphate is a glycosaminoglycan present in the extracellular matrix of blood vessels and its deficiency is associated with advanced atherosclerosis.
- Chondroitin sulphate reduces inflammation in mice with diet-induced obesity.

**What does this paper add?**
- This study is the first to provide insight into the cellular mechanisms by which chondroitin sulphate interferes with atherogenesis and opens new avenues in the management and prevention of atherosclerosis and cardiovascular disease.
- Chondroitin sulphate reduces atherosclerotic plaques in atherosclerotic ApoE knockout mice.
- Chondroitin sulphate interferes with the pro-inflammatory and atherogenic effects of TNF-\(\alpha\) on monocytes, macrophages and endothelial cells.

**Funding**
This work was supported by a grant from Spanish Ministerio de Economía y Competitividad (SAF2015–64126-R) and by Bioibérica and Fundacio Empreses IQS to M.B.; National Institutes of Health (R01 GM 49039) to E.R.E.; Ministerio de Economía (SAF2015–64126-R) to W.J.; European Association for the Study of Liver (EASL), Generalitat de Catalunya (PERIS SLT002/16/00341) and Beatriu de Pinos Program 2016 (BP-00236) to P.M.-L.

**Conflict of Interest**
None.

**Acknowledgments**
We acknowledge support provided by the David H. Koch Institute for Integrative Cancer Research at the Massachusetts Institute of Technology for providing access to multi-photon microscopy and IVIS Spectrum-Bioluminescent and Fluorescent Imaging System used for this study.

**References**
1. Roche VZ, Libby P. Obesity, inflammation, and atherosclerosis. Nat Rev Cardiol 2009;6(6):399–409
2. Nosalski R, Gузik Tj. Perivascular adipose tissue inflammation in vascular disease. Br J Pharmacol 2017;174(20):3496–3513
3. McKellar GE, McCartney DW, Sattar N, McInnes IB. Role for TNF in atherosclerosis? Lessons from autoimmune disease. Nat Rev Cardiol 2009;6(6):410–417
4. Libby P, Hansson GK. Inflammation and immunity in diseases of the arterial tree: players and layers. Circ Res 2015;116(2):307–311
5. Libby P. Inflammation in atherosclerosis. Nature 2002;420(6917):868–874
6. Hansson GK, Libby P. The immune response in atherosclerosis: a double-edged sword. Nat Rev Immunol 2006;6(7):508–519
7. Woolard RJ, Geissmann F. Monocytes in atherosclerosis: subsets and functions. Nat Rev Cardiol 2010;7(2):77–86
8. Tedgui A, Mallat Z. Cytokines in atherosclerosis: pathogenic and regulatory pathways. Physiol Rev 2006;86(2):515–581
9. du Souich P, Garcia AG, Vergès J, Montell E. Immunomodulatory and anti-inflammatory effects of chondroitin sulphate. J Cell Mol Med 2009;13(8A):1451–1463
10. Tan GK, Tabata Y. Chondroitin-6-sulfate attenuates inflammatory responses in murine macrophages via suppression of NF-\(\kappa\)B nuclear translocation. Acta Biomater 2014;10(3):2684–2692
11. Melgar-Lesmes P, Garcia-Polite F, Del-Rey-Puech P, et al. Treatment with chondroitin sulfate to modulate inflammation and atherogenesis in obesity. Atherosclerosis 2016;245:82–87
12. Morrison LM. Response of ischemic heart disease to chondroitin sulfate-A. J Am Geriatr Soc 1969;17(10):913–923
13. Morrison LM, Enrick N. Coronary heart disease: reduction of death rate by chondroitin sulfate A. Angiology 1973;24(5):269–297
14. Nakazawa K, Murata K. Comparative study of the effects of chondroitin sulfate isomers on atherosclerotic subjects. Z Alternforsch 1979;34(2):153–159
15. de Abajo FJ, Gil MJ, García Poza P, et al. Risk of nonfatal acute myocardial infarction associated with non-steroidal anti-inflammatory drugs, non-narcotic analgesics and other drugs used in osteoarthritis: a nested case-control study. Pharmacoepidemiol Drug Saf 2014;23(11):1128–1138
16. Tabas I, Williams Rk, Borén J. Subendothelial lipoprotein retention as the initiating process in atherosclerosis: update and therapeutic implications. Circulation 2007;116(15);1832–1844
17. Ronca F, Palmieri L, Panucci P, Ronca G. Anti-inflammatory activity of chondroitin sulfate. Osteoarthritis Cartilage 1998;6(6):519–527
18. Marcovecchio PM, Thomas GD, Mikulski Z, et al. Scavenger receptor CD36 directs nonclassical monocyte patrolling along the endothelium during early atherogenesis. Arterioscler Thromb Vasc Biol 2017;37(11):2043–2052
19. Luo Y, Duan H, Qian Y, et al. Macrophagic CD146 promotes foam cell formation and retention during atherosclerosis. Cell Res 2017;27(3):352–372
20. Ramos-Arellano LE, Muñoz-Valle JF, De la Cruz-Mosso U, Salgado-Bernabé AB, Castro-Alarcón N, Parra-Rojas I. Circulating CD36 and Chondroitin Sulphate Reduces Atheroma Melgar-Lesmes et al.
oxLDL levels are associated with cardiovascular risk factors in young subjects. BMC Cardiovasc Disord 2014;14:54
21 Di Pietro N, Formoso G, Pandolfi A. Physiology and pathophysiology of oxLDL uptake by vascular wall cells in atherosclerosis. Vascul Pharmacol 2016;84:1–7
22 Wu MY, Li CJ, Hou MF, Chu PY. New insights into the role of inflammation in the pathogenesis of atherosclerosis. Int J Mol Sci 2017;18(10):E2034
23 Gimbrone MA Jr, García-Cardena G. Endothelial cell dysfunction and the pathobiology of atherosclerosis. Circ Res 2016;118(04):620–636
24 Martorell J, Santomá P, Kolandaivelu K, et al. Extent of flow recirculation governs expression of atherosclerotic and thrombotic biomarkers in arterial bifurcations. Cardiovasc Res 2014;103(01):37–46
25 Boesten LS, Zadelaar AS, van Nieuwkoop A, et al. Tumor necrosis factor-alpha promotes atherosclerotic lesion progression in APOE-3-Leiden transgenic mice. Cardiovasc Res 2005;66(01):179–185
26 Hsu HY, Twu YC. Tumor necrosis factor-alpha -mediated protein kinases in regulation of scavenger receptor and foam cell formation on macrophage. J Biol Chem 2000;275(52):41035–41048
27 Gimbrone MA Jr, García-Cardena G. Vascular endothelium, hemodynamics, and the pathobiology of atherosclerosis. Cardiovasc Pathol 2013;22(01):9–15
28 Galkina E, Ley K. Vascular adhesion molecules in atherosclerosis. Arterioscler Thromb Vasc Biol 2007;27(11):2292–2301
29 Braun J, Hoffmann SC, Feldner A, et al. Endothelial cell ephrinB2-dependent activation of monocytes in atherosclerosis. Arterioscler Thromb Vasc Biol 2011;31(02):297–305
30 Sainson RC, Johnston DA, Chu HC, et al. TNF primes endothelial cells for angiogenic sprouting by inducing a tip cell phenotype. Blood 2008;111(10):4997–5007
31 Robbins CS, Hilgendorf I, Weber GF, et al. Local proliferation dominates lesion macrophage accumulation in atherosclerosis. Nat Med 2013;19(09):1166–1172
32 Park YM, Febbraio M, Silverstein RL. CD36 modulates migration of mouse and human macrophages in response to oxidized LDL and may contribute to macrophage trapping in the arterial intima. J Clin Invest 2009;119(01):136–145
33 Bardin N, Blot-Chabaud M, Despoix N, et al. CD146 and its soluble form regulate monocyte transendothelial migration. Arterioscler Thromb Vasc Biol 2009;29(05):746–753