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Abstract

Introduction: Osteoarthritis (OA) is associated with the metabolic syndrome, however the underlying mechanisms remain unclear. We investigated whether low density lipoprotein (LDL) accumulation leads to increased LDL uptake by synovial macrophages and affects synovial activation, cartilage destruction and enthesophyte/osteophyte formation during experimental OA in mice.

Methods: LDL receptor deficient (LDLr−/−) mice and wild type (WT) controls received a cholesterol-rich or control diet for 120 days. Experimental OA was induced by intra-articular injection of collagenase twelve weeks after start of the diet. OA knee joints and synovial wash-outs were analyzed for OA-related changes. Murine bone marrow derived macrophages were stimulated with oxidized LDL (oxLDL), whereupon growth factor presence and gene expression were analyzed.

Results: A cholesterol-rich diet increased apolipoprotein B (ApoB) accumulation in synovial macrophages. Although increased LDL levels did not enhance thickening of the synovial lining, S100A8 expression within macrophages was increased in WT mice after receiving a cholesterol-rich diet, reflecting an elevated activation status. Both a cholesterol-rich diet and LDLr deficiency had no effect on cartilage damage; in contrast, ectopic bone formation was increased within joint ligaments (fold increase 6.7 and 6.1, respectively). Moreover, increased osteophyte size was found at the margins of the tibial plateau (4.4 fold increase after a cholesterol-rich diet and 5.3 fold increase in LDLr−/− mice). Synovial wash-outs of LDLr−/− mice and supernatants of macrophages stimulated with oxLDL led to increased transforming growth factor-beta (TGF-β) signaling compared to controls.

Conclusions: LDL accumulation within synovial lining cells leads to increased activation of synovium and osteophyte formation in experimental OA. OxLDL uptake by macrophages activates growth factors of the TGF-superfamily.

Introduction

Osteoarthritis (OA) is a common disease of unknown etiology. The association of OA with metabolic syndrome has long been established but the exact mechanism remains unclear [1,2]. The idea that obesity enhances OA development solely due to increased loading [3] is obsolete and more often studies show the association between obesity and OA development in non-weight-bearing joints [4-7].

Decreased levels of high-density lipoprotein (HDL) and increased levels of low-density lipoprotein (LDL) particles are, amongst other features, part of the metabolic syndrome [8]. In a comparative analysis of serological parameters, several studies demonstrated that OA patients have significantly higher serum levels of LDL compared to healthy controls [9,10]. Studies focusing on cardiovascular diseases, such as atherosclerosis, show pro-inflammatory capacities of LDL and modified LDL [11,12]. LDL particles form the main transport vehicle of cholesterol from the liver to the tissues. LDL can be oxidized in an inflammatory milieu and, therefore, high levels of LDL result in enhanced
oxidized LDL (oxLDL) levels in pathological conditions where free radicals are present [13,14]. OxLDL is taken up by macrophages via scavenger receptor class A, B (CD36) and E (lectin-like oxLDL receptor-1; LOX-1), resulting in a phenotype shift into a more inflammatory cell type [15-19].

A substantial population of OA patients develops a thickened lining layer comprising macrophages that exhibit an activated phenotype. Macrophages derived from biopsies with early OA produce elevated amounts of pro-inflammatory mediators [20]. Depletion of macrophages from OA synovium using anti-CD14-conjugated magnetic beads led to decreased levels of TNF-α, IL-1β, IL-6 and IL-8 [21]. In previous studies we have shown that synovial macrophages are crucial in the development of joint pathology in experimental OA. Selective depletion of lining macrophages using the clodronate-suicide technique prior to induction of collagenase-induced OA strongly inhibited development of cartilage destruction and osteophyte formation, probably regulated by a strong decrease in metalloproteinase (MMP)-3 and –9 expression [22].

Transforming growth factor-β (TGF-β) and bone morphogenetic proteins (BMP) are important growth factors involved in the formation of new cartilage or bone in ligaments (enthesophyte formation) or along the bone surface (ectopic bone formation or osteophyte formation) [23]. In previous studies we showed that multiple injections of members of the TGF-super family, such as TGF-β or BMP-2, directly into the knee joint of the mouse caused abundant enthesophyte/osteophyte formation [24,25]. Moreover, we postulated that local depletion of synovial macrophages prior to injections of these growth factors significantly inhibited new formation of cartilage/bone, suggesting that macrophage factors highly contribute to this process [26,27].

The presence of high levels of LDL in OA joints with an enhanced inflammatory environment may lead to uptake of oxLDL by synovial lining macrophages, thereby contributing to development of OA pathology. LDL receptor deficient (LDLr<sup>−/−</sup>) mice, which are generally used in atherosclerotic research [28], are unable to clear and metabolize cholesterol-rich intermediate and low density lipoproteins, causing hypercholesterolemia that can be enhanced by a cholesterol-rich diet [29]. In this study, we investigated the effect of increased serum LDL levels on OA development in experimental collagenase-induced OA. We focused on synovial thickening/activation, cartilage damage and enthesophyte/osteophyte formation in both LDLr<sup>−/−</sup> mice and mice receiving a cholesterol-rich diet.

**Methods**

**Animals**

Female mice homozygous for the Ldlr<sup>pm1Her</sup> mutation (LDLr<sup>−/−</sup>) and their wild type (WT) control C57BL/6 J were obtained from The Jackson Laboratory (Bar Harbor, ME, USA). Mice were 10 to 13 weeks old when used in the experiments, were housed in filter-top cages and received food and water *ad libitum*. Animal studies were approved by the Institutional Review Board (Animal Experiment Committee Radboud University Medical Center) and were performed according to the related codes of practice.

**Collagenase-induced osteoarthritis**

Mice were fed a standard diet or a cholesterol-rich diet (+0.15% cholesterol; AB Diets, Woerden, the Netherlands). Instability OA was induced by intra-articular injection of 1 unit bacterial collagenase (Sigma-Aldrich, St. Louis, MO, USA) into the right knee on days 84 and 86 after start of the diet [30]. On day 120, mice were weighed and sacrificed, after which both left and right knee joints were isolated and processed for histological analysis. Serum samples were obtained to determine cholesterol levels.

**Histological and immunohistochemical preparation**

Isolated knee joints were fixed in 4% buffered formalin and subsequently decalcified in formic acid and embedded in paraffin. Eight representative sections (7 μm) of each joint from various depths were stained with H&E or Safranin O-fast green for histological analysis. For immunohistochemistry, sections were deparaffinized and endogenous peroxidase activity was blocked using 1% H<sub>2</sub>O<sub>2</sub> in methanol. Next, sections were incubated in buffered citrate (pH 6.0) for antigen retrieval. Incubation with 0.1% Triton X-100 was used for cell membrane permeabilization, followed by incubation with rabbit anti-mouse apolipoprotein B (ApoB; Abcam, Cambridge, UK), rabbit anti-mouse S100A8 (kindly provided by Dr. Vogl, Institute of Immunology, University of Muenster, Germany), or normal rabbit immunoglobulin G (IgG) (R&D Systems, Minneapolis, MN, USA) as control. Subsequently, sections were incubated with the secondary antibody, biotinylated goat anti-rabbit IgG, and binding was detected using the ABC-HRP kit (Elite kit; Vector Laboratories, Burlingame, CA, USA). Peroxidase was developed with diaminobenzidine (DAB; Sigma-Aldrich) and sections were counter-stained with hematoxylin. Sections were randomly coded and scored in a blinded way by two independent investigators.

**Histological analysis**

Synovial thickening and immunohistochemical staining were measured using an arbitrary scoring system (0 to 3, where 0 = no thickening/staining and 3 = most observed thickening/staining). Three sections of various depths were scored per knee joint.

Cartilage damage in the tibial-femoral joint was scored using the OA score developed by Pritzker et al. [31], adapted by us for mice (from 0 = no damage, to 30 = maximal damage) [32]. Five sections were scored per knee joint.
Size of ectopic bone formation was measured by the use of image analysis (Leica Qwin, Leica Microsystems, Rijswijk, The Netherlands). Osteophytes and enthesophytes were manually circled by an investigator blinded for the experimental condition, after which the surface areas were calculated by Leica-software. The mean cross-sectional surface area in three sections per knee joint was determined, including knee joints without ectopic bone formation.

**Synovial wash-outs**
Synovium was isolated from murine knee joints 7 and 36 days after collagenase injection. Tissue was weighed and put in Roswell Park Memorial Institute medium (RPMI; Gibco, Invitrogen, Carlsbad, CA, USA), enriched with 48 μg/mL gentamicin (Centrafarm, Etten-Leur, The Netherlands) and 0.1% BSA (Sigma-Aldrich) for one hour at room temperature.

**Bone marrow derived macrophages**
Macrophages were differentiated out of bone marrow cells using 15 ng/mL recombinant murine macrophage colony stimulating factor (M-CSF; R&D Systems). For this, the mice from WT or LDLr<sup>−/−</sup> mice were removed and bone marrow cells were flushed with (D)MEM; Gibco) supplemented with 10% FCS (Thermo Scientific, Waltham, MA, USA), 1 mM pyruvate (Gibco) and 48 μg/mL gentamicin.

**Oxidized LDL preparation and macrophage stimulation**
OxLDL was prepared from a large batch of LDL which was first isolated by single-spin density-gradient ultracentrifugation from ethylenediaminetetraacetic acid (EDTA)-treated blood donated by healthy volunteers and frozen in 10 mM sulfate (Merck, Darmstadt, Germany) for 24 hours at room temperature. After 24 hours, the LDL was thawed and dialyzed for seven hours against saline in a Slide-A-Lyzer cassette (Pierce Chemical Company, Rockford, IL, USA). The pellet was reconstituted in RNAse free water and RNA was extracted using the single step RNA isolation method described by Chomczynski and Sacchi [34]. Isopropanol (Merck) was used for precipitation, after which the RNA pellet was washed twice with 75% ethanol. The pellet was reconstituted in RNase free water and subsequently treated with DNase (Invitrogen). RNA was reversed transcribed into complementary DNA (cDNA) using reverse transcriptase, oligo(dT) primers and dNTPs (Invitrogen).

Quantitative real-time polymerase chain reaction
RNA was extracted using the single step RNA isolation method described by Chomczynski and Sacchi [34]. Isopropanol (Merck) was used for precipitation, after which the RNA pellet was washed twice with 75% ethanol. The pellet was reconstituted in RNase free water and subsequently treated with DNase (Invitrogen). RNA was reversed transcribed into complementary DNA (cDNA) using reverse transcriptase, oligo(dT) primers and dNTPs (Invitrogen).

Quantitative real-time PCR (qPCR) was performed using StepOnePlus Real-Time PCR system and StepOne software v2.2 (Applied Biosystems, Foster City, CA, USA), under the following conditions: 10 minutes 95°C, followed by 40 cycles of 15 seconds 95°C and 1 minute 60°C. Data were collected during the last 30 seconds of each cycle. Product specificity was confirmed by assessment of dissociation-characteristics. The reaction was performed in a total volume of 10 μL, containing 3 μL diluted cDNA, 1 μL forward primer (5 μM), 1 μL reverse primer (5 μM) and 5 μL SYBR Green Master Mix (Applied Biosystems). The following primers were designed with Primer Express 2.0 (Applied Biosystems) and manufactured by Biologio (Nijmegen, the Netherlands): GAPDH: 5′-GGCAATTC AACGGGACA-3′ (forward) and 5′-GTATGGGGGTC TCCTCCTTG-3′ (reverse); IL-12p40: 5′-AGCTAACCAT CTCTGTTTGC-3′ (forward) and 5′-CCACCTCTAC AACATAGCGTTCTTCA-3′ (reverse); TGF-β1: 5′-GCA GTTGCTGAAAGCAAGGAA-3′ (forward) and 5′-AAGAG CAGTGAGCGCTGAATC-3′ (reverse); BMP2: 5′-GGCA GTTGCCATCAC-3′ (forward) and 5′-GCGGCCGCTGTT TTC-3′ (reverse); BMP7: 5′-CCGGTCCTCTGCAAGAC CA-3′ (forward) and 5′-AGTGCGTGCTGCTCCAATG-3′ (reverse); BMP8: 5′-AGCCAGGGAGGTTCTCCTTTCC-3′ (forward) and 5′-ATGTTGGTGATCAGGAGG-3′ (reverse). Relative expression levels were presented as ΔΔCt, being the threshold cycle (Ct) value corrected for GAPDH and unstimulated control.

CAGA-Luc and BRE-Luc reporter constructs
3T3 cells (ATCC, Manassas, VA, USA) were transduced with a CAGA-Luciferase reporter plasmid (CAGA-Luc) or a BMP responsive-luciferase reporter plasmid (BRE-Luc) for two and a half hours with a multiplicity of infection of 10. Both plasmids were kindly provided by Dr. Ten Dijk (Department of Molecular Cell Biology, Leiden University Medical Center, the Netherlands). After 24 hours, the transduction efficiency of CAGA-Luc was checked with fluorescence microscopy, confirming the presence of GFP that is constitutively active in this construct. The transduced cells were then stimulated overnight with macrophage supernatant or synovial wash-outs under serum-free conditions, after which luminescence was measured.
Statistical analysis
Statistical differences between two values were calculated using a student’s t-test or Mann Whitney U test depending on Gaussian distribution. Statistical differences between more than two values were tested using a one-way analysis of variance (ANOVA) or Kruskal-Wallis test, followed by a Bonferoni or Dunns post test, respectively. All analyses were performed using Graph Pad Prism 5 (GraphPad Software, La Jolla, CA, USA) and P-values less than 0.05 were considered to be significant. Data are depicted as mean ± standard error of the mean (SEM).

Results
A cholesterol-rich diet increased serum cholesterol levels and enhanced ApoB uptake by LDLr deficient synovial lining cells
To investigate the effects of enhanced serum cholesterol levels during pathogenesis of experimental collagenase-induced OA, both WT and LDLr−/− mice were fed a standard diet or cholesterol-rich diet for 120 days. A cholesterol-rich diet resulted in a 21% increase of body weight in WT mice (from 23.8 g ± 0.4 to 28.8 g ± 1.5; P = 0.05), but, in contrast, did not significantly affect the weight of LDLr−/− mice (Figure 1A). Serum analysis for cholesterol levels showed that a cholesterol-rich diet in WT mice increased serum LDL concentration by 128% (from 0.54 mmol/L ± 0.04 to 1.23 mmol/L ± 0.13; P = 0.006). In LDLr deficient mice, enhanced serum LDL concentration following a cholesterol-rich diet was even more pronounced (428% raise, from 7.33 mmol/L ± 0.46 to 38.73 mmol/L ± 3.11; P<0.0001; Figure 1B). Apart from enhanced serum LDL levels, local effects of a cholesterol-rich diet and LDLr deficiency were also investigated. Expression of ApoB, which is the main protein in oxLDL particles, was examined by immunohistochemistry. Both a cholesterol-rich diet and LDLr deficiency enhanced uptake of ApoB by the synovium. Particularly, cells in the lining layer accumulated ApoB within their cytoplasm. Mice deficient for the LDLr showed a significant increase in ApoB staining after receiving a cholesterol-rich diet, suggesting enhanced up-take of oxLDL (Figure 1C). These results suggest a positive relation between oxLDL accumulation by lining macrophages and serum LDL levels.

High serum cholesterol levels did not alter synovial thickening in WT mice on a normal diet (1.4 ± 0.2; Figure 2A). Synovial thickness, however, was not significantly higher in mice that received a cholesterol-rich diet, nor was it significantly higher in mice deficient for the LDL receptor. To study the contribution of the macrophage to lining layer activation, immunostaining with S100A8 was performed. S100A8 is expressed only by activated macrophages but not by fibroblasts [35]. WT mice showed an increased S100A8 expression after receiving a cholesterol-rich diet (from 0.43 ± 0.16 to 1.13 ± 0.35; P = 0.035). In LDLr−/− mice increased S100A8 expression after a cholesterol-rich diet did not reach significance (Figure 2B).

High serum cholesterol levels caused by a cholesterol-rich diet did not alter cartilage destruction in collagenase-induced osteoarthritis
Since we show that a cholesterol-rich diet is capable of activating macrophages, we further investigated whether high serum cholesterol levels affect cartilage damage. Overall cartilage destruction was mild (mean score WT mice without cholesterol-rich diet 6.1 ± 1.5; Figure 3). Although WT mice that received a cholesterol-rich diet showed marginal differences in cartilage damage compared to mice receiving a normal diet at the lateral side of the femur and both lateral and medial side of the tibia, these values did not reach significance. In line with this, no differences in cartilage damage were seen in LDLr deficient mice between the group receiving the normal diet and the group receiving the cholesterol-rich diet. Moreover, no differences between cartilage damage in WT mice on the normal diet and LDLr deficient mice on the normal diet were observed (Figure 3).

Increased serum cholesterol levels result in enhanced bone formation in ligaments during experimental osteoarthritis
Earlier studies have shown that synovial macrophages are crucial in mediating new formation cartilage/bone in ligaments and along the bone surface. Since formation of enthesophytes are a consistent and early feature of OA [36], leading to ligament rigidity and less functionality of the joint, we first examined the effects of high cholesterol levels on ectopic bone formation in collateral and cruciate ligaments. While only four out of ten WT mice with OA on a normal diet showed enthesophyte formation, there were eight out of ten WT mice on a cholesterol-rich diet that presented enthesophyte formation. In LDLr deficient mice on a normal diet, eight out of ten mice showed enthesophyte formation, whereas there were nine out of ten mice in the LDLr deficient group on a cholesterol-rich diet that showed this condition (Figure 4A). Both a cholesterol-rich diet and LDLr deficiency resulted in a significantly increased size of enthesophytes (fold increase...
of 6.7 and 6.1, respectively; *P<0.05). In contrast, the combination of LDLr$^{-/-}$ and a cholesterol-rich diet only showed a trend towards an increased total size of enthesophytes compared to WT mice on a normal diet; however, values did not reach significance (Figure 4B and C).

**High serum cholesterol levels result in increased osteophyte formation and presence of active TGF-β in collagenase-induced osteoarthritis**

Since enthesophyte formation in joint ligaments strongly correlates with osteophyte formation [23], we next investigated whether serum cholesterol levels affected osteophyte formation. We measured osteophyte size at the margins of the lateral and medial side of the tibial plateau and femoral condyles using image analysis. LDLr deficient mice, receiving a cholesterol-rich diet, showed significant enhancement of osteophyte formation at the lateral side of the tibial plateau, when compared to WT mice with a cholesterol-rich diet (fold increase 2.7; *P<0.01). Even more pronounced was the increase in osteophyte formation in LDLr deficient mice on a normal diet, compared to WT mice on the same diet (fold increase 5.3; *P<0.001; Figure 5A and B). Besides
Figure 2 Cholesterol-rich conditions lead to an activated synovium, rather than synovial thickening. Thickening of the synovial lining layer was determined in hematoxylin and eosine stained sections, using an arbitrary score between 0 and 3. All mice showed moderate synovial thickening with no significant differences between various groups (A). Immunohistochemistry, however, showed an increase in S100A8 expression in WT mice after receiving a cholesterol rich diet, suggesting enhanced macrophage activation (B). *P<0.05. n = 10 mice per group. WT, wild type.
increased osteophyte formation due to LDLr deficiency, a cholesterol-rich diet further increased osteophyte formation at the lateral site of the tibial plateau in LDLr deficient mice (fold increase 1.8; P < 0.05). At the medial side of the tibia, a cholesterol-rich diet resulted in increased osteophyte formation in WT mice (fold increase 4.4; P < 0.01). Thus, both a cholesterol-rich diet and LDLr deficiency cause increased osteophyte formation at the margins of the tibial plateau. At the margins of the femoral condyles, no significant differences in osteophyte formation were found between the various groups (data not shown).

In previous studies we have shown that TGF-β and BMPs are major growth factors involved in driving new formation of cartilage/bone [24,25]. We therefore tested synovial wash-outs of WT and LDLr−/− mice for the presence of active TGF-β using a CAGA-Luc assay (Figure 5C). Both on the 7th and 36th day after collagenase injection, levels of activated TGF-β were significantly higher in LDLr−/− mice on a cholesterol-rich diet, compared to WT mice on a cholesterol-rich diet.

OxLDL stimulated macrophages activate TGF-β in vitro
As ApoB accumulation in synovial macrophages correlated with increased ectopic bone formation in both WT and LDLr−/− mice, we investigated whether oxLDL stimulated macrophages are capable of producing anabolic factors that may be responsible for this process. For that reason, bone marrow derived macrophages were cultured with oxLDL for 24 hours. Staining with oil red O indeed showed a high uptake of oxLDL by macrophages, which did not alter cell viability (Figure 6A). Analysis of RNA expression did not show upregulation of important growth factors known to be involved in ectopic bone formation such as TGF-β, BMP2, -4 and -7, while IL12p40, a positive control for murine oxLDL-laden macrophages [37], did show a significant down regulation with a ΔΔCt of 2.0 ± 0.13 (Figure 6B). Next, we studied the presence of active TGF-β and BMP using specific bioassays. When supernatant of macrophages that were stimulated with oxLDL for 48 hours was functionally tested for TGF-β activity using the CAGA-Luc assay, we found a significant 2.9 fold increase in signal.
(P<0.05), while oxLDL alone did not increase TGF-β activity (Figure 6C). The BRE-Luc assay, which measures active BMP, did also show an increase in signal by supernatant of oxLDL-laden macrophages, albeit to a lesser extent (fold increase 1.8; P<0.05; Figure 6D).

Discussion

In the present study, we show that enhanced serum LDL cholesterol levels increase ectopic bone formation in mice with collagenase-induced OA. By using both a cholesterol-rich diet and LDLr deficiency, we demonstrated that an enhanced LDL cholesterol level in particular, rather than LDLr deficiency or a specific food element, is responsible for the enhancement of ectopic bone formation.

From previous studies we know that synovial lining cells are important for OA pathology. Selective elimination of synovial macrophages from the intimal lining of the knee joint prior to induction of collagenase-induced OA
ameliorated the development of both cartilage damage and ectopic bone formation. In mice that lack the LDLr, a cholesterol-rich diet led to a strong increase in ApoB staining within the lining layer. Every LDL particle contains a single ApoB molecule that can be used as a marker for LDL and oxLDL uptake [38]. The accumulation of ApoB in synovial lining cells of LDLr deficient mice suggests that lining cells in an OA joint take up oxLDL in a cholesterol-rich environment. Macrophages can phagocytize LDL via the LDLr which is suppressed when LDL serum levels rise, leading to regulation of LDL uptake into the cell [39]. Under inflammatory conditions, LDL is oxidized and phagocytized by scavenger type A and B receptors lacking this negative feedback loop [40]. A macrophage is a very
plastic cell type which can differentiate into activated M1 or suppressive M2 subtypes. Recently we found in in vitro studies that M2 macrophages express higher levels of SR-A and CD36 receptors compared to M1 macrophages and that enhanced uptake of oxLDL by M2 macrophages changed the suppressive character of this cell type into an M1-like macrophage releasing enhanced levels of IL-1 and IL-6 [16]. Although uptake of oxLDL in the lining layer of the LDLr⁻/⁻ mice receiving a cholesterol-rich diet was strongly enhanced, no elevation of S100A8 staining was observed. S100A8 is a member of the alarmin family which is particularly produced by activated macrophages but not by non-activated macrophages and has been described as a marker for macrophage activation [35]. The lack of S100A8 staining in LDLr⁻/⁻ mice suggests that uptake of large amounts of oxLDL by lining macrophages does not enhance the production of pro-inflammatory molecules and chemokines and, therefore, does not alter synovial thickening and cartilage destruction. In contrast, high cholesterol levels in WT mice induced only minor uptake of oxLDL by lining macrophages, but strongly enhanced S100A8 levels. This accumulation of S100A8 within the lining layer might explain the trend of increased cartilage destruction observed in WT mice on a cholesterol-rich diet. S100A8 production by synovial macrophages may be driven by moderate uptake of oxLDL or LDL, while excessive uptake of oxLDL may inhibit production of pro-inflammatory factors by synovial macrophages. Another explanation of the slight increase in cartilage damage observed in WT mice on a cholesterol-rich diet (Figure 3) is that it might also be due to the increased body weight (Figure 1A). Nevertheless, by using a cholesterol-rich diet rather than a high-fat diet, we minimized weight gain, preventing amelioration of the OA process via increased loading [41]. Lack of cartilage damage in cholesterol-rich conditions may also be explained by the

Figure 6 LDL- and oxLDL-laden macrophages enhance anabolic processes by activating TGF-β and to a lesser extent BMP, rather than increase production of TGF-β or BMP. Bone marrow derived cells were differentiated into macrophages, after which they were stimulated with 50 mg/mL oxLDL or an equal volume of PBS. Staining the cells with oil red O showed uptake of oxLDL (A). RNA expression did not show significant upregulation of TGF-β, BMP2, BMP4 or BMP7 (B); however, a functional TGF-β reporter gene luciferase (CAGA-Luc) assay did show increased presence of active TGF-β (C). Furthermore, the BMP responsive luciferase (BRE-Luc) assay also showed increased activity in supernatant of oxLDL stimulated macrophages, albeit to a lesser extent (D). UD = undetectable; *P<0.05 versus unstimulated control; **P<0.01; ns = no significant regulation. BMP, bone morphogenetic protein; LDL, low density lipoprotein; oxLDL, oxidized low density lipoprotein.
increased presence of active TGF-β which protects the cartilage from proteoglycan depletion and matrix breakdown, as described in an earlier study by Van Beuningen et al. [42]. From our own experience, we know that the experimental model which is used here is less severe in female mice compared to males. Although all mice in this experiment developed cartilage lesions, the OA scores were relatively low. It would be interesting to repeat our experiment in a more severe model, investigating whether increased cholesterol-associated cartilage damage is detected.

Apart from cartilage destruction, new formation of cartilage and bone is also observed in ligaments and locations along the bone surface during collagenase-induced OA. Focusing on ligaments (enthesophyte formation), we noticed that an increase in LDL levels by a cholesterol-rich diet markedly enhanced new cartilage and bone formation. OxLDL might directly induce proliferation and differentiation of stem cells present within the ligaments, or indirectly by enhancing growth factor production in synovium. Several studies have shown that oxLDL has a toxic effect on proliferation and differentiation of stem cells, and, in line with that, our own findings show that oxLDL (50 μg/mL) is highly toxic for stem cells (data not shown), whereas similar concentrations do not affect macrophage viability (Figure 6A) [43-47]. For that reason, we assume that enthesophyte formation may be more related to the effects of oxLDL on synovial lining activity. In previous studies in our lab it was found that intra-articular injection of the growth factors TGF-β and BMP2 resulted in formation of osteophytes [24,25]. Moreover, in the absence of synovial macrophages, injection of TGF-β was less able to induce new formation of cartilage and bone, suggesting that macrophages are crucial in mediating TGF-β effects [26]. Furthermore, we found that spheroid formation in a mesenchymal cell line was increased when these cells were co-cultured with macrophages, whereas total TGF-β levels in supernatant did not increase [27]. This fits with the present study where we show that there is increased activation of TGF-β, rather than production after stimulation of macrophages with oxLDL. Using supernatants of oxLDL stimulated macrophages, strongly elevated levels of active TGF-β and, to a lesser extent, BMP were measured. Also in vivo, LDLr<sup>−/−</sup> mice show an increased presence of TGF-β compared to WT mice, suggesting that this in vitro observation could very well reflect the in vivo mechanism. TGF-β was measured using a CAGA-Luc assay in which the CAGA promoter is coupled to a luciferase gene which becomes activated by active TGF-β via TGF-β receptor 1. TGF-β is released in an inactive form and can be activated by enzymes such as MMP2 and MMP9 [48]. These enzymes may be released either by activated synovial macrophages or by synovial fibroblasts in response to pro-inflammatory factors released by the macrophage. Recently, Ishikawa et al. demonstrated that apart from macrophages in rheumatoid arthritis fibroblast-like synoviocytes can also accumulate oxLDL via LOX-1, resulting in massive MMP production [49]. Studies are now in progress to define the factors involved in TGF-β activation. In addition to TGF-β activation, increased BMP activation was found after stimulation of macrophages with oxLDL. Enhanced BMP presence can also contribute to the observed bone formation, since previous studies showed that TGF-β is important for initiating osteophyte formation, whereas BMP are mainly involved at later stages in the process of endochondral ossification [50]. Also osteoarthritic chondrocytes have been shown to express LOX-1 and could, therefore, be affected by oxLDL directly [51]. Although our data support that (ox)LDL affects chondrocytes indirectly via synovial activation, it would be interesting to investigate how hypercholesterolemia could influence the chondrocytes directly and how this affects OA development.

We did not find osteophyte or enthesophyte formation in naïve knee joints of mice with an LDLr deficiency receiving a cholesterol-rich diet (data not shown). This would suggest that systemically enhanced LDL levels alone are not sufficient to induce joint pathology. Only when there are pro-inflammatory or damage associated stimuli present (that is, during OA or instability), may enhanced LDL levels result in increased osteophyte/enthesophyte formation.

**Conclusions**

This study shows for the first time that increased LDL cholesterol levels in an OA milieu are able to enhance ectopic bone formation. Our experimental data points towards a potential mechanism in which uptake of oxLDL by synovial lining macrophages results in activation of TGF-β and to a lesser extent BMP. Further research is needed in order to elucidate what factors in particular are responsible for the enhanced levels of active TGF-β and BMP, since direct production does not seem to increase according to mRNA expression levels. Nevertheless, this mechanism provides a firm step forward to unravelling possible factors affecting etiopathology of OA and osteophyte formation in particular.

**Abbreviations**

ApoB: Apolipoprotein B; BMP: Bone morphogenetic proteins; BRE-Luc: Bone morphogenetic protein responsive element luciferase reporter plasmid; BSA: Bovine serum albumin; CAGA-Luc: CAGA-box luciferase reporter plasmid; Ct: Threshold cycle; DAB: Diaminobenzidine; (D)MEM: (Dulbecco's) Modified Eagle's Medium; FCS: Fetal calf serum; GFP: Green fluorescent protein; HDL: High-density lipoprotein; H & E: hematoxylin and eosin; IL: Interleukin; LDL: Low-density lipoprotein; LDLr<sup>−/−</sup>: Low-density lipoprotein receptor deficient; LOX-1: Lectin-like oxidized low-density lipoprotein receptor-1; M-CSF: Macrophage colony stimulating factor; MMP: Metalloproteinase; OA: Osteoarthritis; oxLDL: Oxidized low-density lipoprotein; PBS: Phosphate-buffered saline; qPCR: Quantitative real-time polymerase chain reaction; TGF-β: Transforming growth factor-β; TNF-α: Tumor necrosis factor-alpha; WT: Wild type.
Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
WdM, AB and PvL were involved in conception and design of the study, acquisition and analysis of data, and interpretation of data. MH and BW were involved in performing animal experiments and acquisition of histological data. PvdK, LJ and WvdB were involved in conception and design of the study. All authors have been involved in revising the manuscript. All authors read and approved the final manuscript.

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