Pro-resolving fatty acids and oxylipids in osteoarthritis and rheumatoid arthritis

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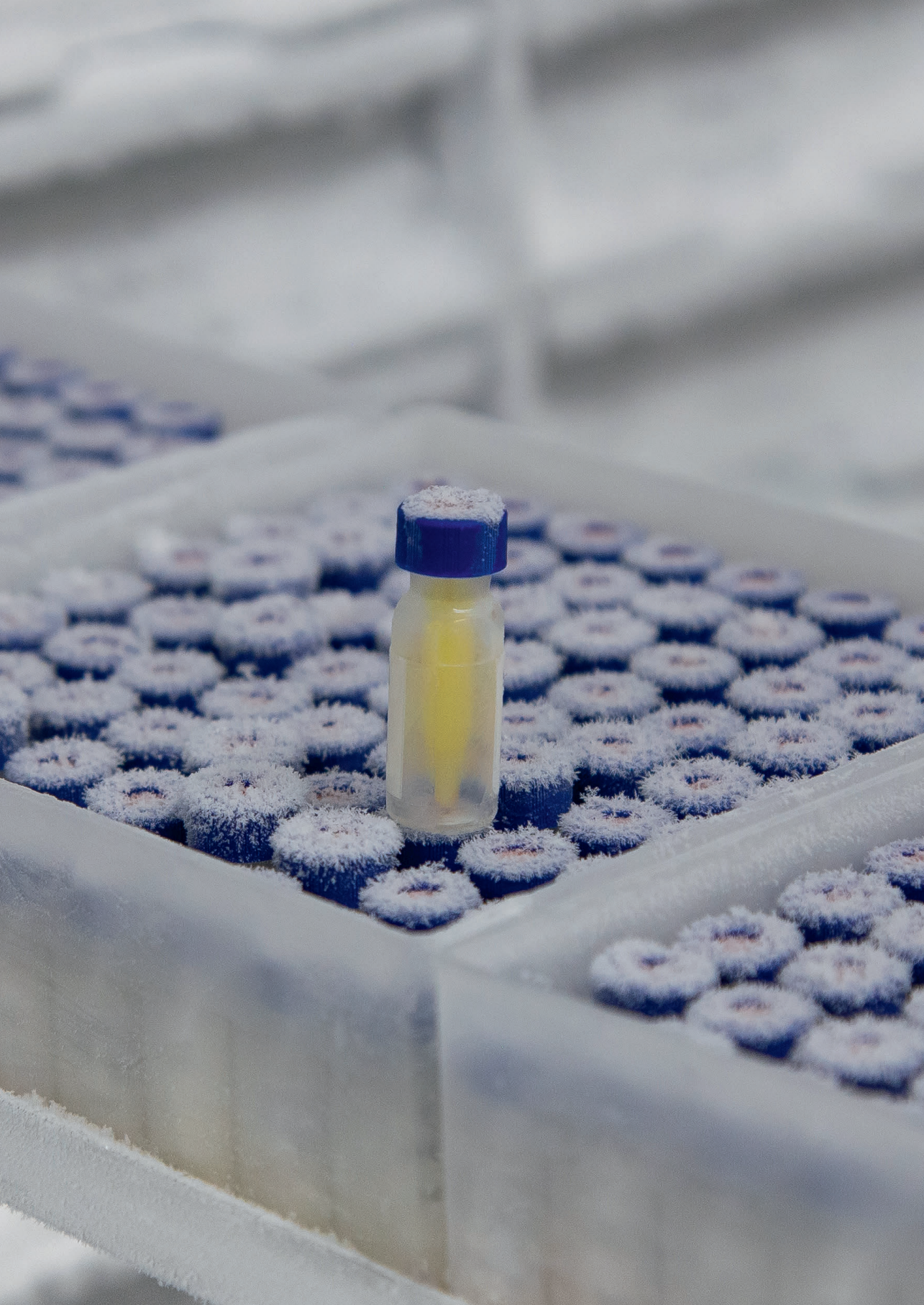

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Pro-resolving mediator treatment of high fat diet induced experimental osteoarthritis

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Abstract

Objective: Specialized pro-resolving mediators (SPM) are bioactive lipids derived from omega-3 and omega-6 fatty acids which have been ascribed pro-resolving capacity. Since inflammation is a well-established feature of osteoarthritis (OA), we investigated the correlation between inflammation and structural damage in experimental OA. Furthermore, we evaluated the effect of SPM treatment on structural damage. In addition, we investigated SPM receptor expression on human OA synovial cells and chondrocytes and the effect of various SPM on IL-6 production by human synovial cells.

Design: Male C57BL/6 mice were fed a high fat diet (HFD) and were either treated with 100 nM Lipoxin A4, Lipoxin B4 and Maresin-1, or the vehicle control. Inflammation was evaluated by in vivo imaging and at the end of the experiment, inflammation and structural damage were evaluated by histology. Synovial and cartilage tissue of 42 OA patients was used to evaluate SPM receptor expression by flow cytometry and IL-6 production by synovial fibroblasts and synovial macrophages upon incubation with SPMs.

Results: The HFD induced OA, indicated by cartilage damage and osteophyte formation, correlated with synovial inflammation, but SPM treatment in HFD induced experimental OA did not result in decreased joint pathology. Both human chondrocytes and synovial cells expressed a subset of SPM receptors and SPM decreased IL-6 production under certain experimental conditions, *in vitro*.

Conclusions: There was no effect of Lipoxin A4, Lipoxin B4 and Maresin-1 treatment on joint pathology in HFD induced OA. Further research is warranted to determine whether SPM have therapeutic potential in OA.

Introduction

Osteoarthritis (OA) affects the weightbearing joints such as knees, hips and spine but also smaller joints such as those in the thumb, fingers and toes. To date, there are not effective treatments available and patients are prescribed NSAIDs to control their pain symptoms. Inside the joints, OA is characterized by breakdown of cartilage, changes to the underlying bone structures and low-grade synovial inflammation. OA is a progressive disease of which the end stage is characterized by a complete disruption of the joint and at this stage the patients should undergo total joint replacement surgery.

There is emerging evidence that the chronic low grade inflammation in OA plays are role in its pathophysiology(1). Clinical studies show correlations between the presence of synovial inflammation and disease progression in knee and hand OA(2–4). In addition, synovial macrophages can induce cartilage destruction and bone remodeling in an experimental model of OA(5,6).

Next to local inflammation, systemic inflammation is also implicated in the development of OA as obesity strongly predisposes to OA(7).

Several local and systemic soluble inflammatory mediators are implicated to play a role in disease pathogenesis such matrix metalloproteinases, cytokines, adipokines and prostaglandins(8–11). However, results of clinical trials have been disappointing as neither of the trials improved structural damage outcome. It is proposed that new treatment options for OA should focus on treating the early phases of OA, to prevent structural damage. We hypothesize that these early phases of OA are characterized by chronic low-grade inflammation which should be targeted by pro-resolving mediators rather than strong anti-inflammatory mediators. Specialized pro-resolving mediators (SPM) are small bioactive lipid molecules derived from poly unsaturated fatty acids (PUFAs) and they are produced by a wide range of immune and tissue cells by the actions of LOX and COX enzymes. Many SPM are now identified and include lipoxins, maresins, resolvins and protectins. SPM are shown to accumulate during periods of acute inflammation to limit progression to persistent chronic inflammation in many experimental models including infection, asthma and inflammatory arthritis models(12–15). In particular, recently, SPM resolvin D1 (RvD1) was shown to ameliorate OA in a surgically induced model of OA(16).

In this study, we investigated synovial inflammation in vivo over time, as well as development of osteophyte formation and cartilage loss in the HFD induced experimental model of OA. In addition, we evaluated SPM receptor expression in human joint cells and

investigated the effect of SPM on IL-6 production in synovial cells. Finally, we treated the HFD animals with SPM to investigate whether they can limit structural damage.

Materials and Methods

Preparation of SPM

LXA4, LXB4 and Mar1 were purchased from Cayman Chemicals. RvE2 was a kind gift from dr. Makoto Arita (RIKEN, Yokohama, Japan). SPM were diluted in HPLC grade EtOH (Sigma-Aldrich) using a Hamilton syringe and glass vials (Agilent Technologies). For in vitro experiments, EtOH was evaporated using a gentle stream of nitrogen, appropriate medium was added and SPM were homogenized by 2 rounds of vortex (10 seconds) and sonication (10 seconds). For in vivo experiments, SPM were diluted in HPLC grade EtOH and added directly to sterile 0,9% NaCl and homogenized by 2 rounds of vortex (10 seconds) and sonication (10 seconds). These procedures were also performed with plain HPLC grade EtOH which was used as vehicle control.

Experimental OA

Male C56BL/6 mice were purchased from Charles River and were random assigned to the chow diet control group or high fat diet (HFD) diet groups. The HFD groups were fed with Rodent Diet with 60% kcal% fat (Research Diet) after an acclimatization period of three weeks and the treated and untreated mice were cages together. Both diets were provided ad libitum. The mice were treated every 7 days with a pool of SPM consisting of 100 nM LxA4, LxB4 and Mar1. The mice were injected i.v. with 100 μL per 30 grams of bodyweight. Every month, the mice were injected with IRDye®800CW 2-DG Optical Probe (Li-Cor), 10 nmol per 20 grams of bodyweight and the next day the mice were subjected to imaging using the IVIS Spectrum In Vivo Imaging System (PerkinElmer, Ohio, USA). After 6 months of HFD and SPM treatment, the mice were sacrificed and the knees were prepared for histology. The study was approved by the Institutional Animal Care and Use Committee (IACUC) in the USA and the Ethical Committee for Experimental Animal Experimentation of the LUMC.

Histology of mouse knees

After sacrifice, the knees were cut from the hind leg by cutting through the femur and tibia and fibula halfway of the bones. The skin, the fat and some muscle tissue was removed and the knees were fixed in 4% formaldehyde for 24 hours. The knees were then rinsed thoroughly for 10 minutes using distilled water and incubated with Kristensens Solution for 2 weeks to decalcify. Residual Kristensens solution was removed by washing the knees for 3 hours under running tap water and the knees were processed for histology. Tissue slides of 7 μM were stained with fast green (Klinipath) and safranine O (Sigma)

and of each knee, three sections were scored which were at least 140 μM apart. The cartilage was scored at 4 positions (femur lateral, femur medial, tibia lateral and tibia medial) and was given a score of grade 0 (no damage), grade 0.5 (faded Safranine O staining), grade 1 (superficial layer has frays), grade 2 (superficial layer has frays and clefts), grade 3 (<25% of the width of the tidemark is damaged), grade 4 (25-50% of the width of the tidemark is damaged), grade 5 (50-75% of the width of the tidemark is damaged) or grade 6 (>75% of the width of the tidemark is damaged). The total score of the knee is the sum of the 4 places with a maximum score of 24. At the same 4 locations, the osteophytes were counted and given a score (0-3) depending on the size of the osteophyte. Osteophyte scoring resulted in a score of the total number of osteophytes on the 4 locations combined with a maximum score of 4, as well as a total osteophyte score with a maximum of 12. Synovial inflammation was scored by grading the synovium thickness from 0-3 at 4 locations, with grade 0 (flat almost invisible synovium layer with flattened cells), grade 1 (more rounded cells), grade 2 (2-3 cell layers thick), grade 3 (dark purple staining and more than 3 cell layers thick). The total score of the knee is the sum of the 4 places with a maximum score of 12. Scoring was performed blinded by two observers which were blinded for the experimental groups and their score was averaged.

Human synoviocyte and chondrocyte isolation and culture

Synovium and cartilage were obtained as anonymized left-over material from total knee replacement surgery procedures and stored at 4 °C before processing the same day. Synovium was cut in small pieces and incubated in serum free IMDM medium (Lonza, Basel, Switzerland) supplemented with 1 mg/mL collagenase type II (Worthington biochemical corporation, Lakewood, NJ, USA) for 1.5 hours. Cartilage was cut into small pieces and incubated with 2 mg/mL Pronase (Roche) for 1.5 hours. Next, the cartilage was resuspended in DMEM/F12 medium with 0.75 mg/mL collagenase type II overnight. Digested tissue was separately filtered over a 70 μm cell strainer (Falcon, Corning Incorporated, Life Sciences, Durham, NC, USA) to obtain the synoviocytes and chondrocytes. The cells were washed 3 times before use. All digestion steps were performed at 37°C on a lab roller.

SPM receptor analysis

Isolated cells were stained with a different set of antibodies depending on the SPM receptor detection. For the detection of ERV1/ChemR23 (also known as ChemR23 or CMKLR1), isolated cells were stained using anti-CD31-AF647 (BD), anti-CD90-PE (Biolegend), anti-CD45-AF700 (Biolegend), anti-CD14-PECy (BD), anti-CD55-Biotin (BD), streptavidin-eFluor450 (eBioscience), anti-CMKLR1-FITC (AbD Serotec), anti-CD117-APC (BD), anti-CD3-PE (BD) and anti-CD19-APC-Cy7 (BD). For the detection of ALX/FPR2, isolated cells were stained using anti-CD31-FITC(BD), anti-CD90-APC

(Biolegend), anti-CD45-AF700 (Biolegend) or anti-CD45-FITC (BD), anti-CD14-PECy (BD), anti-CD55-Biotin (BD), streptavidin-eFluor450 (eBioscience), anti-CD117-APC (BD), anti-CD3-AF700 (BD) and anti-CD19-APC-Cy7 (BD). The anti-FPR2 antibody (Genovac) and the isotype for anti-FPR2 were labelled with PE using LYNX rapid R-Phycoerythrin (RPE) antibody conjugation kit (AbD Serotec) according to the manufacturer's protocol. CD31+ cells, CD55+ cells and CD90+ cells were gated from the CD45+ population. CD14+ cells, CD19+ cells and CD117+ cells were gated from the CD45+ population. For the detection of BLT1R, isolated cells were directly stained with anti-LTB4R-PE (BD). For the detection of DRV1/GPR32, isolated cells were directly stained with anti-GPR32 (Genetex) and subsequently with anti-rabbit IgG-FITC (Santa-Cruz). Isotypes for all antibodies were used to determine positivity. Dead cells were excluded using the Fixation and Dead Cell Discrimination Kit (Miltenyi) according to the manufacturer's protocol. Remaining red blood cells were lysed using lysisbuffer (BD) after staining, according to the manufacturer's protocol.

IL-6 production by synoviocytes

For effect on IL-6 production, freshly isolated synoviocytes were resuspended in IMDM supplemented with 10 % FCS, P/S and glutamax (2 x 10^6/0.5 mL) and incubated overnight at 37°C in a pre-warmed 24- wells plate. The next day, cells were washed with DMEM/F12 medium and incubated with SPM and 10ng/mL LPS overnight at 37°C using DMEM/F12 medium with 2% fatty acid free (FFA) BSA and penicillin/streptomycin (P/S). The next day, supernatant was collected and IL-6 production was quantified using a human IL-6 ELISA kit (R&D Systems) according to the manufacturer's protocol. For intracellular staining, 10 μg/mL Brefeldin A was added for 4 hours after O/N incubation with SPM. Alternatively, SPM were added after O/N incubation with DMEM/F12 medium with 2% FFA BSA, P/S for one hour. After 1 hour of incubation, Brefeldin was added for 4 hours. Subsequently, the cells were harvested using Accutase (Sigma), washed with PBS and stained with anti-CD45-AF700, anti-CD14-PECy7 and anti-IL-6-PE (eBioscience) for flowcytometry. Flowcytometry analysis was performed using FACSDiva™ Software (BD).

Statistical analysis

Wilcoxon signed rank tests, Mann-Whitney tests were performed sing Graphpad Prim 9.1.1 to evaluate significance.

Results

We used the HFD induced OA model to investigate the disease development in mice. Metabolic stress induced inflammation was shown to play a role in OA development in this model(17,18). We have fed male C57BL/6 mice a HFD that contained 35% lard

and we followed the mice for 6 months. These mice gained 3 times more weight than the control mice (Figure 1A). To investigate the course of inflammation in these mice, we visualized the inflammation *in vivo* using fluorescent 2-deoxy-glucose (2-DG). We could detect inflammation, as indicated by the strength of the 2-DG signal in the fore paws of the mice. The HFD mice showed significantly more inflammation in their fore paws after 4 months compared to their chow controls (Figure 1B). We could not detect any inflammation in the mice knees. To determine whether the HFD developed OA, we evaluated cartilage loss, osteophyte formation and synovial synovitis in the knees of these mice. We could not detect cartilage degradation in either of the groups, however there was a trend towards more inflammation in the HFD mice (Figure 1C). In contrast, all of the HFD mice developed osteophytes during these 6 months (Figure 1C). We next evaluated whether the osteophyte formation correlated with synovial inflammation in these mice. Indeed, there was a strong correlation between both osteophyte severity and osteophyte number but not with cartilage loss (Figure 1D).

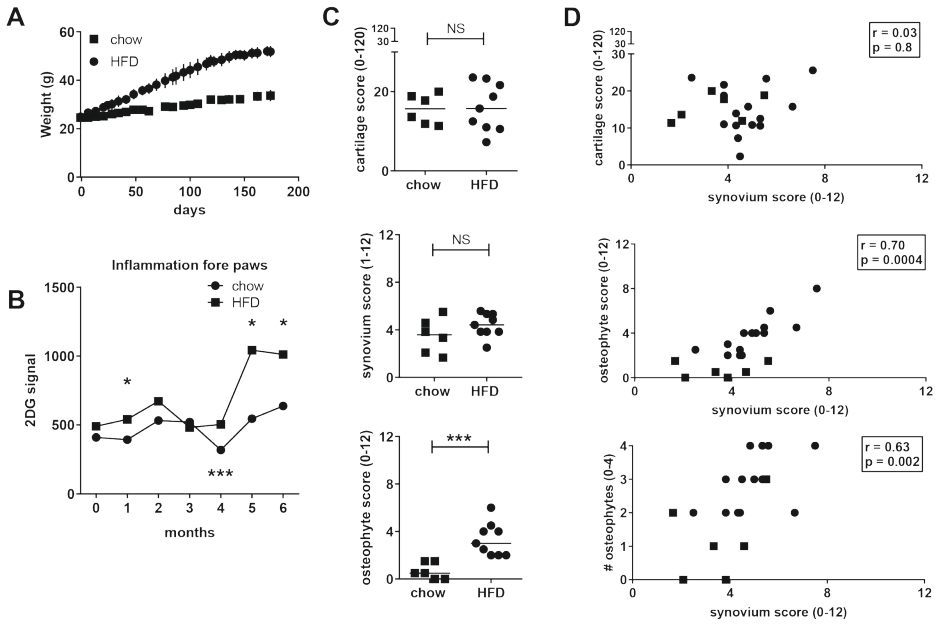


Figure 1. HFD induces inflammation which correlates with structural damage. A) Weight gain of the lean chow fed mice and HFD mice. Mean and SD are shown of n=6 chow and n=9 HFD mice. B) Mean quantified fluorescent 2-deoxyglucose signal in the fore paws of mice in vivo. C) Cartilage score, osteophyte score and synovium scores obtained by histology of the right knee. Each dot represents a mouse. n=6 chow and n=9 HFD mice. D) Correlations between synovium score and cartilage score, number of osteophytes an osteophyte score respectively. Scores are obtained by histology of the right knee. n=20 mice. Closed squares are chow mice and closed circles HFD mice. Statistical significance in this figure was obtained using Mann-Whitney *p<0.05, **p<0.01, ***p<0.001.

Expression of SPM receptors in human articular tissue

To determine candidate SPMs for treatment of experimental OA, we investigated SPM receptor expression on tissues of the human joint. We were able to evaluate the Lipoxin receptor (ALX)/N-formyl peptide receptor (FPR)-2 (ALX/FPR2), the Resolvin E1 receptor/ Chemerin receptor 23 (ERV1/ChemR23) also known as chemokine-like receptor 1 (CMKLR1), The leukotriene B4 receptor (BLT1) and the resolving D 1 receptor/G protein-coupled receptor 32 (DRV1/GPR32) expression on isolated chondrocytes and ALX/ FPR2 and ERV1/ChemR23 expression on cells isolated from synovium (synoviocytes), containing synovial immune cells and fibroblasts. We could detect the expression of the resolvin E1 and (partial) resolvin E2 receptor ERV1/ChemR23 on human chondrocytes (Figure 2A)(19). No expression of leukotriene B4 receptor BLT1 or resolvin D receptor DRV1/GPR32 was detected on chondrocytes. Synovium of 16 OA patients was tested for SPM receptor positivity. Only a small percentage of the CD31+ endothelial cells and fibroblasts showed expression of the ERV1/ChemR23. In contrast, expression of the Lipoxin A4/Resolvin D1 receptor ALX/FPR2 was only detected in CD45+ cells(20). Of the CD45+ cells, on average more than half of the CD14+ cells shower positivity for ALX/ FPR2. In addition, a small percentage of the mast cells, characterized by CD117 positivity showed expression of ALX/FPR2.

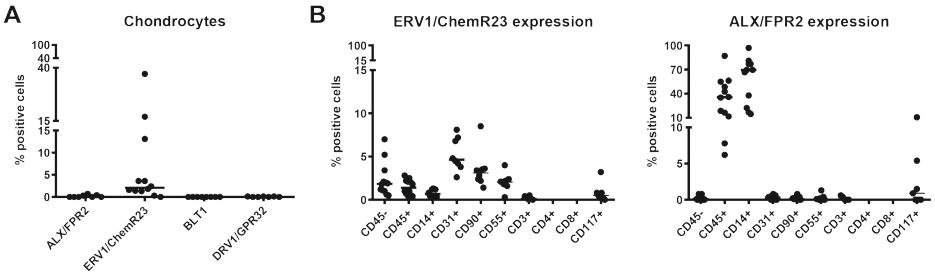


Figure 2. SPM receptor expression in chondrocytes and synovial cells. SPM receptor expression was determined using flowcytometry. A) Isolated chondrocytes, n=8 donors for ALX/FPR2, BLT1 and DRV1/GPR32 and n=12 donors for ERV1/ChemR23. B) Isolated synoviocytes, n=16 donors.

Effects of SPM on synoviocytes and macrophages

Since inflammation correlates with structural damage in the HFD mice model, and we detect expression of some of the SPM receptors, we investigated effects of a set of SPM on cells in the human synovium. We evaluated the effect of SPM on IL-6 production by synoviocytes including synovial fibroblasts and immune cells. Since synovium expresses ERV1/ and ALX/FPR2 we chose to incubate the cells with either LXA4, LXB4, Mar-1 or RvE2. Only with the lowest concentration (1 nM) we could detect an inhibition of IL-6 production by LXB4 and RvE2 in all the donors tested, although this was not significant ($p=0.06$, Figure 3A). We also investigated the effect of the pooled SPM, however

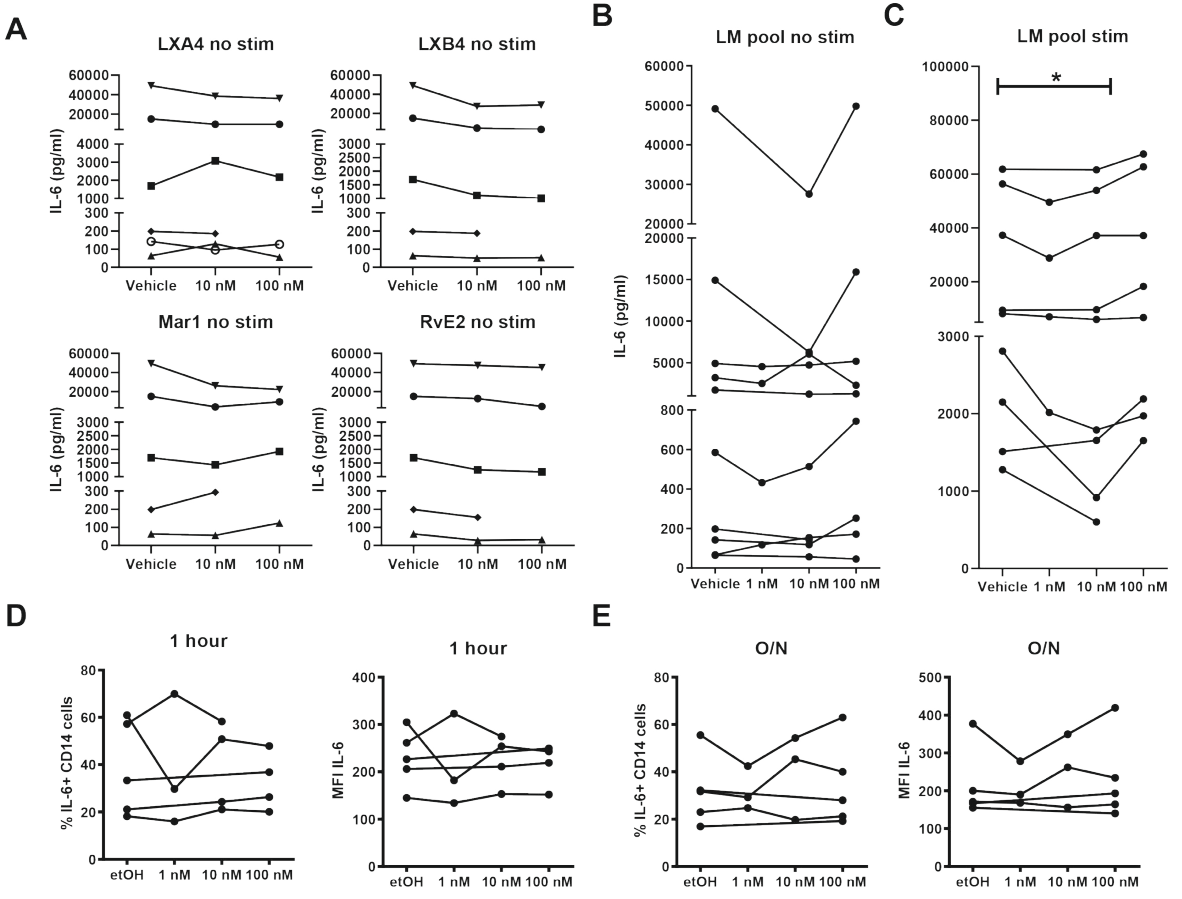
there was no difference in IL-6 production between the groups (Figure 3B). However when we stimulated the synoviocytes with LPS after the SPM stimulation to mimic the inflammatory environment, there was a significant inhibition of IL-6 production in the 10 nM concentration compared to the control group (Figure 3C). Next, we evaluated the effect of LXA4 on inflammatory cytokine IL-6 production As the CD14+ monocyte/ macrophage population expresses ALX/FPR2.We digested the synovium to obtain individual cells and incubated the cells either for 1 hour (figure 3D) or overnight (Figure 3E) before we added brefeldin A for 4 hours. Neither of the experiments showed an effect on intracellular IL-6 levels evaluated using numbers of IL-6 MFI (Figure 3) or number of IL-6 positive cells (data not shown) in the CD14+ population.

Treatment of HFD mice with pro-resolving mediators

Based on the receptor expression profile on human joint tissue cells and the previous reports that show macrophages are involved in osteophyte formation, we decided to treat the mice with agonists of the ALX/FPR2 receptor, Lipoxin A4 and Maresin-1[5]. In addition, Lipoxin A4 and Maresin-1 were detected in synovial fluid of arthritis patients and Lipoxin A4 was shown to attenuate adipose inflammation and produced by synovial tissue(21–23). Lipoxin B was added to complete the pool of SPM based on its yet unidentified role in inflammation and the most promising effect on synoviocytes (p=0.06 using 10 nM LXB4). Mice were treated weekly with 100 nM of Lipoxin A4, Lipoxin B4 and Maresin-1. After 6 months of treatment, there was no effect of these SPM on structural damage to the knee joints (Figure 4B-D).

> **Figure 3.** Effect of SPM on IL-6 production by synoviocytes. A-C) Isolated synoviocytes were incubated with SPM O/N and IL-6 production was determined in the supernatant by ELISA. Synoviocytes were either stimulated with individual SPM (A), a pool of SPM containing LXA4, LXB4, Mar1 and RvE2, or a pool of SPM containing LXA4, LXB4, Mar1 and RvE2 and LPS. Each line represents a donor, n=4-10 donors. D) Isolated synoviocytes were either incubated for 1 hour (A) or overnight (O/N) (E) with different concentrations of LXA4. IL-6 positivity and IL-6 mean fluorescence intensity (MFI) on CD14+ cells was determined by flowcytometry. Each line represents a donor, n=5 donors. Statistical significance in this figure was obtained using Wilcoxon signed rank test, *p<0.05, **p<0.01, ***p<0.001.

Figure 3.


A
LXA4 no stim
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RvE2 no stim
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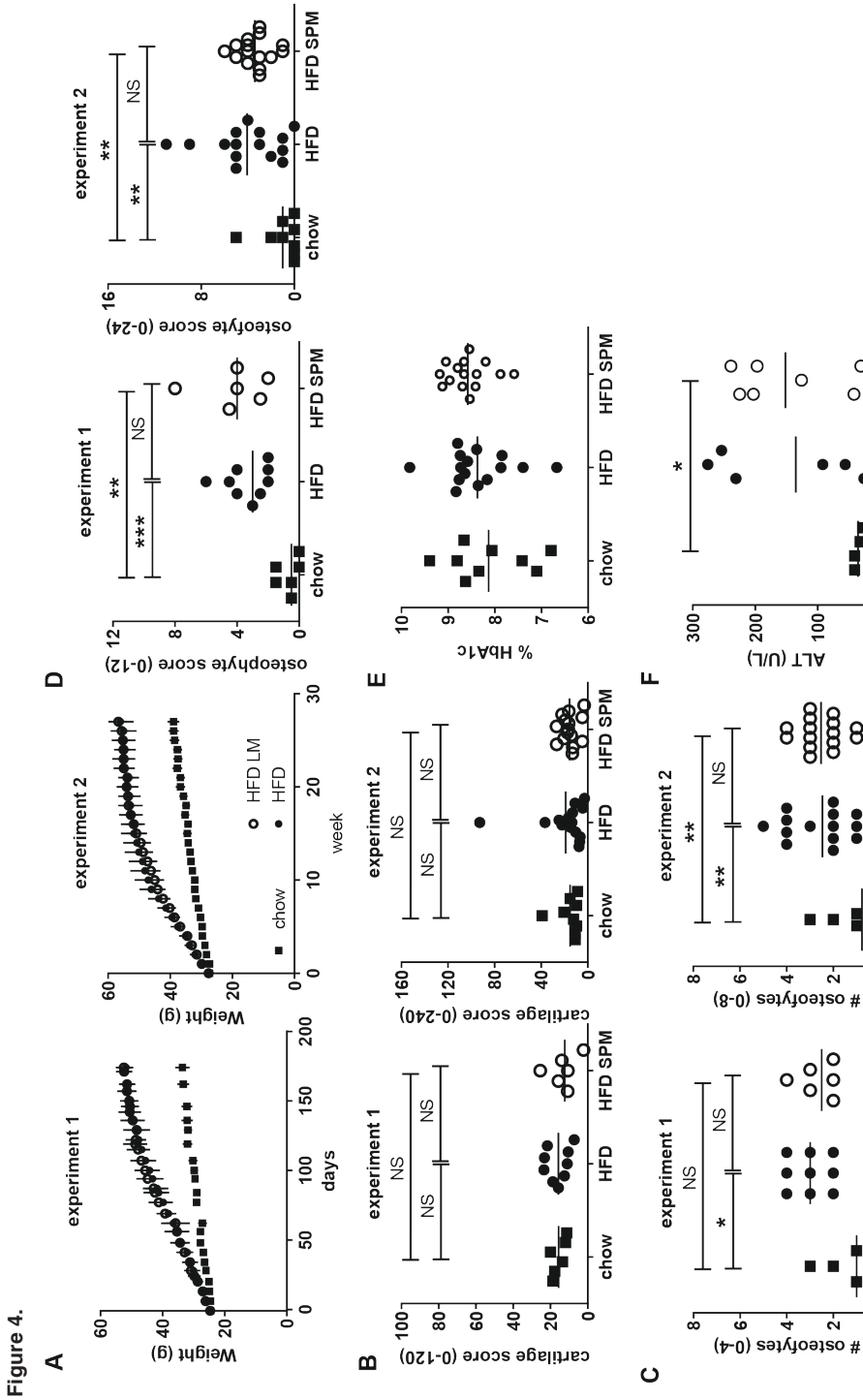
A
experiment 1
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E
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Figure 4.

< Figure 4. Pro-resolving mediator treatment of experimental OA. A) Weight gain of the chow control and HFD mice of two independent experiments. Mean and SD are shown of n=6 chow control, n=9 HFD untreated (vehicle control) and 6-9 HFD treated (SPM, 100 nM Lipoxin A4, Lipoxin B4 and Maresin-1) mice for experiment 1. In experiment 2, means are shown for n=9 chow control and 15 HFD mice of both the untreated and treated group. B) Cartilage score, C) osteophyte number and D) osteophyte score of two experiments. For experiment 1, scores of the right knee are shown. For experiment 2, mean scores of the left and right knee combined are shown. E) HbA1c and F) ALT levels were measured in serum of mice from experiment 2. Statistical significance for B-D was established using Mann-Whitney *p<0.05, **p<0.01, ***p<0.001.

No effects of treatment metabolic inflammatory factors

Although we could not detect any effect on structural damage in the knees of the mice, we wanted to explore whether the SPM attenuated the overall inflammatory status of the mice. We investigated the systemic blood levels of HbA1c as a marker of glycemic control in mice but there was no difference between the groups (Figure 4E)(24). Blood alanine aminotransferase (ALT) levels are a marker for liver damage and shown to be increased in HFD mice and lowered by the administration of Lipoxin A4(23). Indeed we detected an increase in ALT levels in the HFD treated mice, however the SPM treatment did not significantly alter ALT levels (Figure 4F).

Discussion

There is a stressing need for novel therapeutics to treat OA patients which limit structural damage progression. SPM were shown to limit inflammatory processes and promote resolution of inflammation and tissue repair(25).We show that human chondrocytes, synovial macrophages and synovial fibroblasts express SPM receptors ERV1/ChemR23 and ALX/FPR2. In addition, our results show a slight decrease of IL-6 production by synovial cells after treatment with a pool of SPM targeting ERV1/ChemR23 and ALX/FPR2 receptors, however only in the specific condition were the cells we additionally triggered with LPS. The individual SPM could not elicit the same response although LXB4 and RvE2 showed a similar trend as the pool of SPM. Treatment the mice with HFD induced experimental OA however did not prevent the development of structural damage.

We show for the first time that synovial inflammation is correlated with structural damage in HFD induced experimental OA(26). This correlation is also demonstrated for human OA, although whereas some reports show a good correlation, other show a moderate or no correlation, depending on the joints or patient population studied, emphasizing the heterogeneous disease we call OA(27–30). We were unable to show that the inflammation preceded the development of structural damage in the knees as the in vivo imaging technique was likely not optimized to detect (low grade) inflammation in the knees compared to the mouse paws which do not have much tissue or hair surrounding the joints.

We show that a small percentage of human chondrocytes are positive for the chemoattractant chemerin, and SPM Resolvin E1 and E2 receptor ERV1/ChemR23. It was shown before that all chondrocytes stained positive for ERV1/ChemR23, however this was after weeks of culture in which they might have upregulated the receptor(31). Fibroblast-like synoviocytes (FLS) from RA patients were shown to produce IL-6 upon ERV1/ChemR23 signaling via chemerin stimulation (32). It is proposed that the response to either inflammatory chemerin and pro-resolving RvE1 via ERV1/ChemR23 is tightly regulated and RvE1 might play a role in the resolution of inflammation in RA(33,34). We show that in OA patients there is a trend, but no significant effect of RvE2 on IL-6 production, which acts through the same receptor. It remains to be shown whether RvE1 might have a more potent effect.

In contrast to the chondrocytes and FLS, the CD14+ macrophages express the lipoxin A4 receptor ALX/FPR2. Macrophages are shown to be important mediators in OA disease development in human and experimental OA(35). However, we could not detect an effect of lipoxin A4 or resolvin D1 on macrophage IL-6 production in contrast to previous results. Sun at al showed decreased IL-6 production in OA synovial macrophages after RvD1 incubation. However, the contradictive results might easily be explained by the differences in experimental set up as we show that slight changes to the experimental protocol can change the outcome. Indeed, some studies reported the need for a specific experimental set up before an effect of LXA4 or aspirin triggered LXA4 could be seen on phagocytosis by THP-1 cells or neutrophils respectively(36,37). Our in vitro findings emphasize that the effect of SPM is not robust and provokes the thought how these SPM can cause a robust effect during inflammation.

Weekly treatment of mice with a pool of LXA4, LXB4 and Mar-1 did not limit the development of structural damage. A possible explanation could be that the cells in the joint to not respond to the SPM, as we also observe for the human cells. However, it could also be that the admission route should be altered. Recently it was shown that intra-articular injection RvD1 reduced the severity of mechanically induced experimental OA(16). In addition, the administration route might also explain why we could not reproduce the LXA4 induced lowered ALT levels, since LXA4 was previously shown to lower ALT levels after intra peritoneal injection.

In conclusion, we show for the first time that inflammation is correlated with structural damage in the HFD induced experimental OA model. We show expression of SPM receptors ERV1/ChemR23 and ALX/FPR2 joint tissues of OA patients, however only in one of a large variety of conditions and cell types tested, we could detect an effect of SPM on inflammatory IL-6 production by synoviocytes. Furthermore, weekly treatment

of mice with a pool of lipoxins and Maresin-1 did not prevent structural damage in HFD induced experimental OA.

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Author contributions

Hilde Brouwers: Conception and design, Collection and assembly of data, Analysis and interpretation of the data, Drafting of the article, Final approval of the article

Joanneke Kwekkeboom: Administrative, technical, or logistic support, Collection and assembly of data

Kosaku Murakami: Collection and assembly of data

Simone Perniola: Collection and assembly of data

Margreet Kloppenburg: Provision of study materials or patients, Critical revision of the article for important intellectual content

Rene Toes: Critical revision of the article for important intellectual content

Andreea Ioan-Facsinay: Obtaining of funding, Conception and design, Critical revision of the article for important intellectual content, Final approval of the article

Role of the funding source

The study sponsors had no role in the study design, in the collection, analysis and interpretation of data; in the writing of the manuscript; and in the decision to submit the manuscript for publication.

Competing interests

None of the authors have competing interests

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