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DOI
10.1016/j.joca.2018.09.007

Publication date
2019

Document Version
Final published version

Published in
Osteoarthritis and Cartilage

Osteoarthritis and Cartilage 27 (2019) 118–128

Osteoarthritis and Cartilage

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ARTICLE INFO

Article history:
Received 22 February 2018
Accepted 13 September 2018

Keywords:
Osteoarthritis
C-reactive protein
Metabolic dysfunction
High-fat diet
Inflammation

SUMMARY

Objective: C-reactive protein (CRP) levels can be elevated in osteoarthritis (OA) patients. In addition to indicating systemic inflammation, it is suggested that CRP itself can play a role in OA development. Obesity and metabolic syndrome are important risk factors for OA and also induce elevated CRP levels. Here we evaluated in a human CRP (hCRP)-transgenic mouse model whether CRP itself contributes to the development of 'metabolic' OA.

Design: Metabolic OA was induced by feeding 12-week-old hCRP-transgenic males (hCRP-tg, $n = 30$) and wild-type littermates ($n = 15$) a 45 kcal% high-fat diet (HFD) for 38 weeks. Cartilage degradation, osteophytes and synovitis were graded on Safranin O-stained histological knee joint sections. Inflammatory status was assessed by plasma lipid profiling, flow cytometric analyses of blood immune cell populations and immunohistochemical staining of synovial macrophage subsets.

Results: Male hCRP-tg mice showed aggravated OA severity and increased osteophytosis compared with their wild-type littermates. Both classical and non-classical monocytes showed increased expression of CCR2 and CD86 in hCRP-tg males. HFD-induced effects were evident for nearly all lipids measured and indicated a similar low-grade systemic inflammation for both genotypes. Synovitis scores and synovial macrophage subsets were similar in the two groups.

Conclusions: Human CRP expression in a background of HFD-induced metabolic dysfunction resulted in the aggravation of OA through increased cartilage degeneration and osteophytosis. Increased recruitment of classical and non-classical monocytes might be a mechanism of action through which CRP is involved in aggravating this process. These findings suggest interventions selectively directed against CRP activity could ameliorate metabolic OA development.

Introduction

Osteoarthritis (OA) is a progressive joint disease of partially unknown aetiology that is characterised by focal loss of articular cartilage. In humans the most significant factor contributing to OA is overweight, leading to an OA phenotype here referred to as 'metabolic OA'. In recent years there has been increasing emphasis on the systemic, metabolic components involved in the development and progression of this OA phenotype[1–3]. There is mounting evidence that metabolic overload and related systemic inflammatory mediators are associated with OA development and progression. This led to the recognition of a clinically distinct OA phenotype, termed metabolic OA[4]. Especially the systemic low-grade chronic inflammation, which is strongly associated to metabolic overload[5], is thought to play an important role in the local development and progression of metabolic OA[3,4].

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https://doi.org/10.1016/j.joca.2018.09.007

Metabolic overload-induced systemic inflammation as seen in humans can be induced by prolonged high-fat diet (HFD) feeding in small animal models and has been shown to aggravate metabolic OA development in these models[6]. We have previously demonstrated in various obesity-prone mouse strains that HFD feeding alone does not necessarily lead to aggravated articular cartilage degradation and suggested that an additional trigger besides high-caloric feeding is necessary to evoke metabolic OA[7]. In a study by Gierman *et al.*[8], this human C-reactive protein-transgenic (hCRP-tg) strain was used to easily monitor the systemic inflammatory status of the mice via the general inflammation marker CRP. Aggravated OA pathology was observed in hCRP-tg male mice fed with a HFD (45 kcal% energy from fat) compared with chow diet. As these mice received a HFD without an additional trigger, it is conceivable that CRP itself might have triggered the aggravated OA pathology. Moreover, low-grade inflammation proved more important than mechanical overload in the development of HFD-induced metabolic OA in the hCRP-tg mouse[8]. Anti-inflammatory intervention with either cholesterol-lowering rosuvastatin or PPARγ agonist rosiglitazone showed significant suppression of both OA development as well as plasma CRP levels, supporting the importance of inflammation in metabolic OA pathogenesis.

CRP involvement has also been suggested in human OA pathogenesis. Systemic CRP levels are significantly elevated in OA patients compared with healthy controls and have been reported to relate with clinical features and radiographic severity[9–12]. The population-based Chingford study confirmed these findings and the authors suggested that CRP levels in early OA can be used as a predictive marker for disease progression[13]. In patients with advanced OA, systemic CRP levels reflected local joint inflammation[14] or pain[15] rather than radiographic OA. The latter finding was corroborated by a recent meta-analysis of 32 studies, where CRP levels were significantly associated with pain and decreased physical function but not radiographic OA[16]. In contrast, others demonstrated no association between CRP levels and OA severity after adjustment for body mass – including the follow-up of the Chingford study[17–20]. These contradictory results between studies have clouded the association between CRP levels and OA pathology.

In our present study, we aimed to elucidate the role of CRP in HFD-induced OA pathogenesis. Male hCRP-tg mice were compared with their wild-type male littermates on a HFD to infer whether the expression of CRP contributed to diet-induced OA aggravation. OA features like osteophyte formation and synovitis were determined to investigate involvement with different aspects of OA pathology. Systemic lipid profiles, blood immune cell populations and synovial macrophage subsets were evaluated to assess whether hCRP-tg mice had a more proinflammatory status relative to their wild-type littermates.

Methods

A detailed methods section is available in the Online Supplemental File.

Mice and experimental design

The experiment was carried out in male human CRP-transgenic (hCRP-tg, $n = 30$) and male wild-type littermates ($n = 15$) on a C57BL/6J background. Metabolic OA was induced by switching the diet of the mice from standard chow to a high-fat diet (HFD, 45 kcal % from fat; cat# D12451, Research Diets Inc., New Brunswick, USA) at 12 weeks of age. Both groups received the HFD for a consecutive period of 38 weeks. The experiment was approved by the institutional Animal Care and Use Committee of TNO and was in compliance with ARRIVE guidelines and European Community specifications regarding the use of laboratory animals.

Analysis of metabolic dysfunction and OA

Metabolic dysfunction was monitored at regular interval during the study period by assessing body weight gain, changes in body composition (EchoMRI LLC, Houston, TX, USA), fasted plasma total cholesterol, glucose and insulin levels. Fasted plasma CRP levels were determined by sandwich ELISA (cat.no. DY1707, R&D Systems, USA). Insulin resistance index (HOMA-IR) was calculated according to the equation proposed by Matthews *et al.*[21]: HOMA-IR = (glucose (mmol/L) × insulin (mU/L))/22.5.

Articular cartilage degradation, osteophyte formation and synovitis were scored on coronal 5 μm knee joint sections, stained with Haematoxylin, Fast Green and Safranin-O, according to OARSI histopathology initiative recommendations for the mouse[22]. For all grades, we report the sum of the medial and lateral compartments as the total score. Please see Supplemental Methods for more details.

Lipid and oxylipin analyses in fasted plasma

Samples were stored at −80°C before analysis. General lipid profiles were determined at time points $t = 0$ weeks, $t = 4$ days after HFD switch (peak in hCRP plasma levels) and $t = 38$ weeks. Oxylipin profiling was performed at $t = 0$, 14 and 38 weeks, due to deficient sample volumes at the intermediate blood collection at $t = 4$ days. Sample volumes of 5 μl and 50 μl were aliquoted from each fasted plasma sample for general lipid/free fatty acids (FFAs) analysis and oxylipin analysis, respectively. Please refer to the Supplemental Methods for a more detailed method description. The lipidomic datasets analysed during the current study are also stored in a phenotype database repository and are available by signing up via https://dashin.eu/interventionstudies/. After receiving credentials and logging in, the study can be accessed via https://dashin.eu/interventionstudies/study/show/39162914 or by searching the study code (hCRP_in_mouse) or study title ("Human C-reactive protein aggravates osteoarthritis development in mice on a high-fat diet").

Immunophenotyping of peripheral cell populations by flow cytometry

Peripheral myeloid and lymphoid populations were analysed by flow cytometry at three different time points ($t = 2/11/37$ weeks). Peripheral blood (5 drops/animal) was drawn via tail incision using lithium heparin-coated Microvette tubes. Cell-surface staining was performed with the myeloid and lymphoid antibody panels shown in Tables I and II. Pooled samples were used for unstained, single-stained and FMO controls to determine background levels of staining. Data were acquired with a 3-laser FACSCanto™ II flow cytometer (Becton Dickinson) (Supplemental Table S2) and analysed using FlowJo v10.2 (Treestar Inc., USA). Cell populations of interest were gated according to the gating strategies as depicted in Supplemental Figs. S3 and S4, to obtain their population frequencies. Baseline population frequencies were determined in age-matched hCRP-tg ($n = 5$) and wild-type ($n = 4$) males from later litters and were not included in the statistical analysis (shown here in grey). Detailed descriptions are included in the Supplemental Methods.

Immunohistochemical evaluation of macrophage subsets in the knee

Knee joints from both groups were stained with chromogenic triple-labelling immunohistochemistry (IHC) for M1 and M2

Table I
Antibody panel used for the analysis of peripheral myeloid subpopulations

Marker	Fluorochrome	Dilution	Clone	Manufacturer	Catalogue number
CCR2	FITC	1:100	475301	R&D Systems	FAB5538F
MHCII	PE	1:100	M5/114.15.2	BD Biosciences	557000
CD11c	PE/Cy7	1:100	N418	eBioscience	25-0114
GR1	PerCP/Cy5.5	1:100	RB6-8C5	BD Biosciences	552093
CD86	APC	1:100	GL1	BD Biosciences	558703
F4/80	APC/Cy7	1:200	BM8	BioLegend	123118
CD11b	Horizon V450	1:100	M1/70	BD Biosciences	560455

Abbreviations: FITC, fluorescein isothiocyanate; PE, phycoerythrin; Cy, cyanine; PerCP, peridinin chlorophyll protein complex; APC, allophycocyanin.

Table II
Antibody panel used for the analysis of peripheral lymphoid subpopulations

Marker	Fluorochrome	Dilution	Clone	Manufacturer	Catalogue number
CD8a	FITC	1:100	53–6.7	BD Biosciences	553031
CD4	PE	1:100	RM4-4	eBioscience	12-0043-83
CD62L	PE/Cy7	1:100	MEL-14	eBioscience	25-0621-82
CD45	PerCP/Cy5.5	1:100	30-F11	BD Biosciences	550994
CD25	APC	1:100	PC61.5	eBioscience	17-0251-81
CD3e	APC/Cy7	1:200	145-2C11	BD Biosciences	557596
CD19	Horizon V450	1:1000	1D3	BD Biosciences	560375

Abbreviations: FITC, fluorescein isothiocyanate; PE, phycoerythrin; Cy, cyanine; PerCP, peridinin chlorophyll protein complex; APC, allophycocyanin.

macrophage subsets, based on a previously described method for human tissue[23]. Primary antibodies targeted murine macrophages (F4/80, 1:100, MF48000 (BM8), Invitrogen), M1 macrophages (iNOS, 1:400, ab136918 (K13-A), Abcam) and M2 macrophages (CD206, 1:100, AF2535, R&D Systems). Quantification was performed in the lateral patellofemoral synovial lining of the knee joint. Digital images of the unmixed colour spectra for each chromogen in the region of interest (ROI) were obtained with a Nuance multispectral imaging system (40× magnification). Macrophage subtypes were analysed using ImageJ 1.51n image analysis software. Colocalization of two or the three markers was calculated from the areal overlap between positivity for F4/80 and CD206 or iNOS or both. Data are expressed as percentage positivity for a label or combination of labels within the ROI or within the F4/80$^+$ macrophage population. Please see Supplemental Methods for more details.

Statistical analysis

Statistical analysis was performed using Prism (v7.01, GraphPad Software, La Jolla, CA, USA) and IBM SPSS software (v25.0, IBM SPSS Inc., Chicago, IL, USA). Please refer to the statistical analysis section in the Supplemental Methods for a more detailed description.

Results

Increased cartilage degeneration and osteophytosis in hCRP-tg mice

To assess whether human CRP itself plays a role in the development of metabolic OA, male hCRP-tg mice were compared with their wild-type male littermates on a HFD. Chow controls were not included in our study, as previous work by our group[8] has shown that hCRP-tg male mice on a HFD had significantly higher OA grades than mice on chow.

Increases in body weight due to fat accumulation, plasma cholesterol and HOMA-IR reflect a state of metabolic dysfunction in both hCRP-tg and wild-type mice (Supplemental Fig. S1). Expression of CRP was validated at the protein level in fasted plasma from hCRP-tg males, whereas wild-type controls were negative. HFD feeding induced changes at the CRP protein level as observed before[8]. Directly after diet switch, HFD provoked a steep increase in CRP levels in hCRP-tg males followed by a gradual decrease over the course of the study (Supplemental Fig. S2(A)).

Human CRP-tg males showed increased articular cartilage fibrillations and vertical clefts with loss of surface lamina [Fig. 1(A) and (B)]. Cartilage erosion was only seen at the lateral knee compartments and almost exclusively occurred in the hCRP-tg group. Total OA scores demonstrated a significant difference between both groups, with a median [interquartile range] of 4.25 [3.72–5.44] in wild-type vs 6.00 [4.69–7.26] in hCRP-tg males, $P < 0.01$, $d = 0.818$, 95% CI [0.06–0.57] [Fig. 1(B)]. This difference in severity between groups was primarily visible at the lateral side of the knee joint with 2.38 [1.69–3.10] in wild-type vs 4.00 [2.82–5.00] in hCRP-tg, $P < 0.01$, $d = 1.066$, 95% CI [0.16–0.69].

Osteophyte formation was distinctly more present in hCRP-tg mice (3.00 [2.00–5.00]) compared with wild-type controls (2.00 [1.00–4.00]. Fig. 1(C)), $P = 0.03$, $d = 0.46$, 95% CI [−0.12 to 0.68]. Synovitis scores were comparable between both groups [Fig. 1(D)], with $P = 0.28$, $d = 0.10$, 95% CI [−0.32 to 0.44].

The relative individual induction of CRP levels at 4 days after diet switch did not associate with the individual OA grades at end point [Supplemental Fig. S2(B)], unlike observed before[8]. Individual OA grades reflecting cartilage degeneration did not associate to the individual osteophyte and synovitis scores [Supplemental Fig. S2(C) and (D)]. Changes in CRP levels within the hCRP-tg group showed no association to osteophyte or synovitis scores (data not shown).

Lipid metabolism comparable between genotypes

Strong diet-induced effects were observed for nearly all measured lipids, showing predominantly increased concentrations in plasma over time. Uni- and multivariate statistical analyses revealed no differences in general lipid and oxylipin profiles between hCRP-tg and wild-type males, neither per time point nor over time (data not shown). Furthermore, none of the measured lipids correlated with OA severity within the hCRP-tg group. Lysophosphatidylcholines (LPC) to phosphatidylcholines (PC) ratio, as a general indicator of inflammatory status and possible predictor of advanced knee OA in humans[24], dropped shortly after HFD switch (hCRP-tg: from 0.83 [0.62–1.10] to 0.70 [0.38–0.80]; wild-

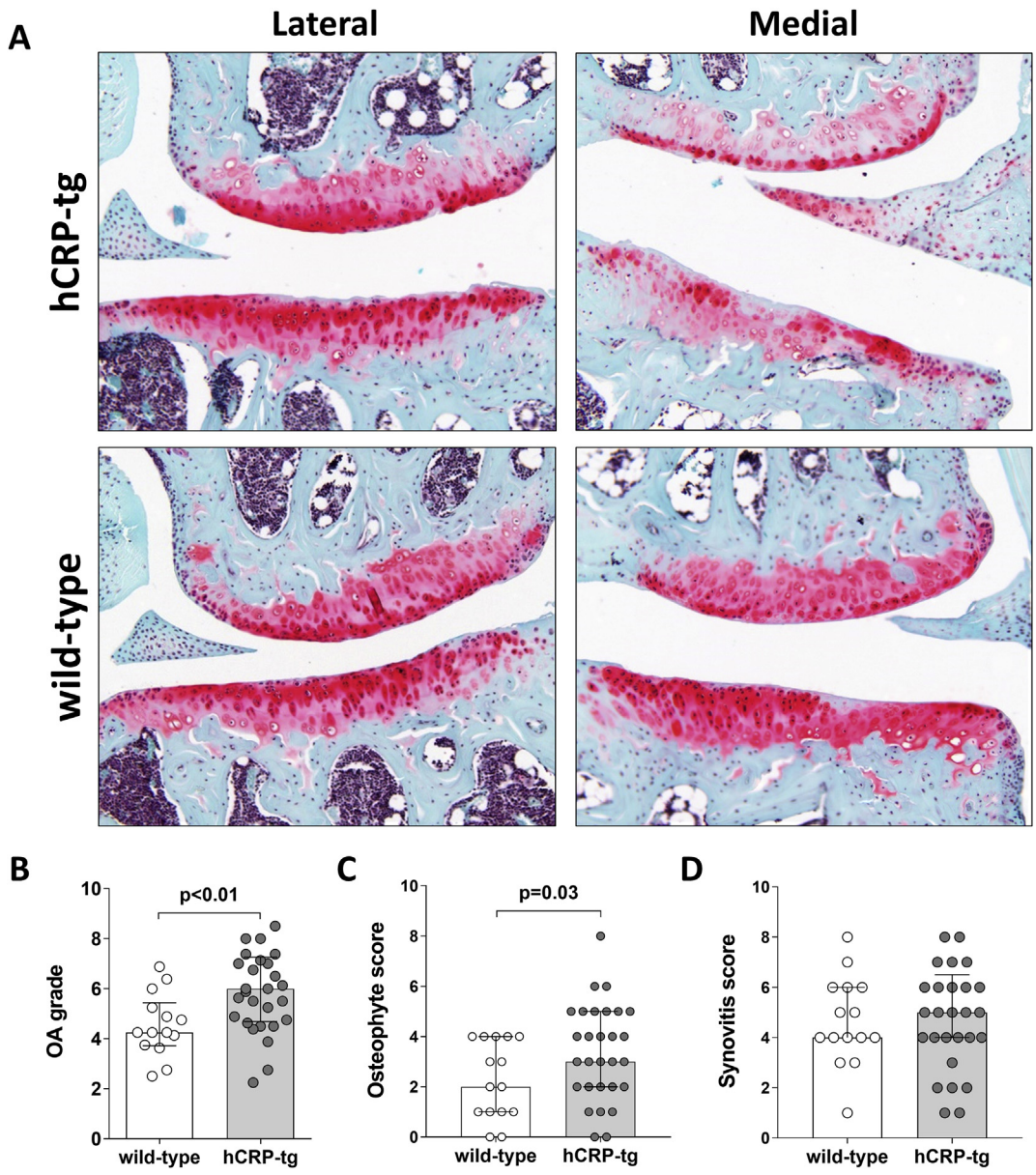

Fig. 1. HFD feeding aggravates OA progression in hCRP-tg mice. A) Representative coronal sections of the lateral and medial tibiofemoral compartments, stained with Fast-Green/Safranin-O, from hCRP-tg and wild-type littermates fed a HFD. Chow controls were not included in our study, but hCRP-tg males are known to not develop OA on a feeding regime of 38 weeks[8]. Magnification for all microphotographs was 40×. B) Summed histopathological scores for the tibiofemoral knee compartments of the hCRP-tg and wild-type groups. C) Total osteophyte scores showing the individual summed score for the tibiofemoral knee compartments for each animal per study group. D) Total synovitis scores showing the individual summed score for the tibiofemoral knee compartments for each animal per study group. B–D) Scoring was performed according to OARSI histopathology recommendations for the mouse[22]. Data are presented as group medians (indicated by bars) with IQR (error bars).

type: from 0.86 [0.75–0.93] to 0.69 [0.57–0.83]) and remained constant afterward. Genotypes demonstrated no major differences in individual PC or LPC levels, as represented by the PC/LPC pairs in Fig. 2(A). Similarly, the switch to HFD was directly reflected in a decline of the sums of omega-6 as well as omega-3 FFA as a percentage of total FFA in both groups. However, both groups showed significant increases in total omega-3 FFA at end-point, while total omega-6 FFA levels remained constant (data not shown). This was reflected in the omega-6/omega-3 FFA ratio, another lipid marker of general inflammatory state, which decreased significantly over time with no differences between groups (data not shown). Individual oxylipin changes were similar between genotypes. The arachidonic acid (AA) metabolite 5,6-epoxyeicosatrienoic acid (5,6-EET) and its stable hydrolysis product 5,6-dihydroxyeicosatrienoic acid (5,6-DHET) increased over time [Fig. 2(B)]. Oxidized linoleic acid (LA) metabolites showed a decrease over time, as represented by 13-hydroxyoctadecadienoic acid (13-HODE, Fig. 2(B)). The DHA-metabolite 17-hydroxy docosahexaenoic acid (17-HDHA) increased over time [Fig. 2(B)].

Increased monocyte activation in hCRP-tg mice on a HFD

Peripheral myeloid and lymphoid populations were analysed by flow cytometry at three different time points ($t = 2$, 11 and 37 weeks) to evaluate the direct and prolonged systemic effects of HFD feeding on immune status (Supplemental Figs. S3 and S4). Baseline population frequencies were determined in age-matched hCRP-tg ($n = 5$) and wild-type ($n = 4$) males from later litters and

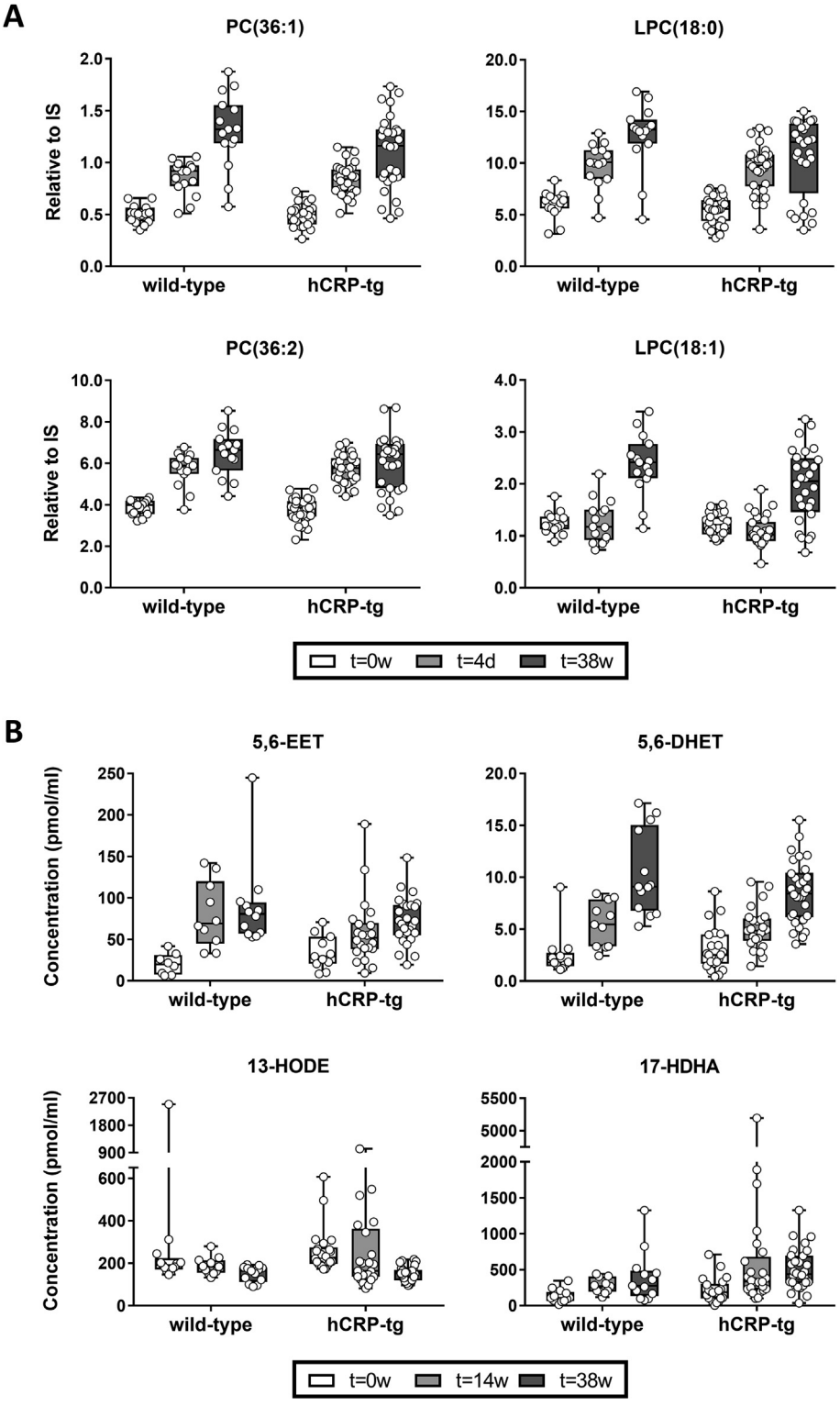

Fig. 2. Transgenic and wild-type hCRP males show similar plasma lipid profiles on a HFD. A) Representative PC/LPC pairs show increases in fasted plasma levels in both study groups during the HFD regimen. B) Representative oxylipins from the three most important oxylipin substrates (i.e., AA, LA and DHA) demonstrate distinct concentration changes in fasted plasma during the HFD regimen. Boxplots show boxes extending from the 25th to 75th percentiles containing the median (middle line), with error bars down to the minimum and up to the maximum value. The individual value for each animal is plotted as a dot superimposed on the graph. IS, internal standard; PC, phosphatidylcholine; LPC, lysophosphatidylcholine; EET, epoxyeicosatrienoic acid; DHET, dihydroxyeicosatrienoic acid; HODE, hydroxyoctadecadienoic acid; HDHA, hydroxy docosahexaenoic acid; AA, arachidonic acid; LA, linoleic acid; DHA, docosahexaenoic acid.

were not included in the statistical analysis (shown in Fig. 3(A) in grey).

HFD feeding induced similar immune reactivity within lymphocyte populations of hCRP-tg and wild-type males (data not shown), but triggered distinct changes in the circulating monocyte populations of these groups. Firstly, HFD feeding nearly doubled the total percentage of circulating CD11b^{+} monocytes in both groups (30.43 ± 5.26 at week 2 vs 53.85 ± 10.35 at week 37, Fig. 3(A)). Secondly, the hCRP-tg mice showed significant upregulation of activated non-classical GR-1^{low} monocytes expressing both the integrin CD11c and chemokine receptor CCR2 after 2 weeks of HFD feeding (Fig. 3(B), $P = 0.02$, $d = 0.82$, 95% CI [0.70–6.33]). Co-expression of the lymphocyte activation antigen CD86 on GR-1^{low}CCR2+ monocytes was elevated upon long-term HFD feeding, with hCRP-tg males showing a significantly increased co-expression compared with wild-type controls (Fig. 3(C) ($P = 0.02$, $d = 0.79$, 95% CI [0.73–8.07]) and Fig. 3(D) ($P = 0.02$, $d = 0.82$, 95% CI [0.79–7.39])). Correspondingly, percentages of GR-1^{low} monocytes without CCR2 expression were significantly decreased in hCRP-tg males compared with wild-types at 2 weeks (data not shown). Over time, hCRP-tg mice showed an increase in activated classical GR-1^{high}CCR2^{+}CD86^{+} monocytes compared with wild-type controls (Fig. 3(E), $P = 0.04$, $d = 0.71$, 95% CI [0.06–1.64]).

To evaluate the association between genotype and OA development on the changes in circulating immune cell populations, linear mixed modelling was used (Supplemental Fig. S5). In hCRP-tg mice the percentages of CD11b^{+} leukocytes were associated with lateral OA severity, while in wild-type littermates this correlation was inversed. Correspondingly, CD11b^{-} cell populations significantly decreased with increasing lateral OA severity in hCRP-tg mice and vice versa for wild-type controls. The hCRP-tg genotype was also significantly associated with higher percentages of both classical CD11b^{+}GR-1^{high} and non-classical CD11b^{+}GR-1^{low} monocytes with increasing lateral OA severity. Wild-type controls showed an inverse relationship between these monocyte subsets and lateral OA severity.

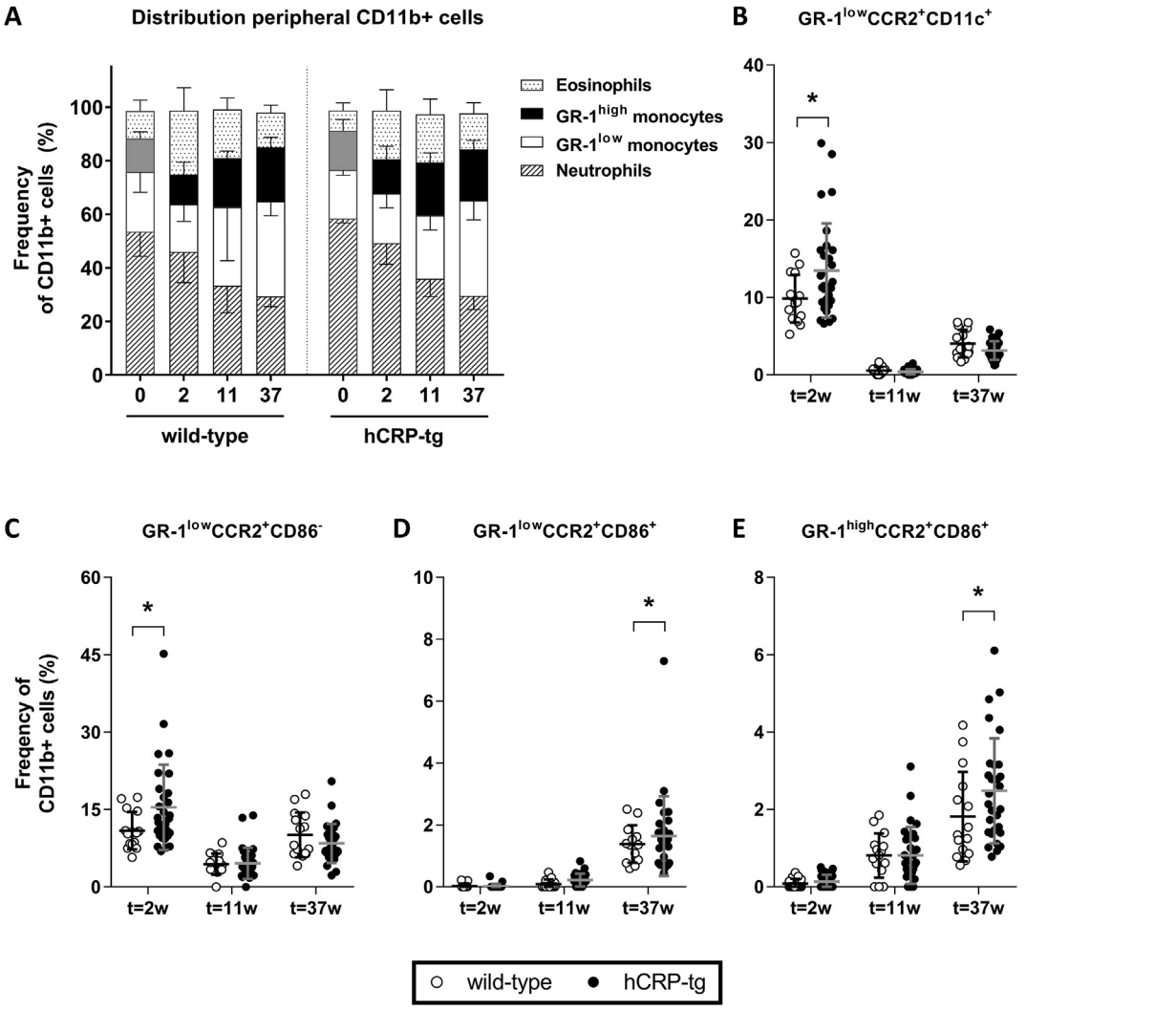

Fig. 3. Increased activation of classical and non-classical monocytes in hCRP-tg mice. A) Distribution of peripheral CD11b^{+} immune cells shows HFD-mediated monocytosis in both groups over time. Baseline population frequencies were determined in age-matched hCRP-tg ($n = 5$) and wild-type ($n = 4$) males from later litters and were not included in the statistical analysis (shown here in grey). B) GR-1^{low} non-classical monocytes showed an increase in CCR2 and CD11c expression upon 2 weeks of HFD feeding, which was significantly elevated in hCRP-tg compared with wild-type mice. C-D) GR-1^{low} non-classical monocytes expressing CCR2 upregulated the lymphocyte activation antigen CD86 after long-term HFD feeding (37 weeks), with hCRP-tg males showing higher co-expression of these activation markers compared with wild-type controls. E) GR-1^{high} classical monocytes co-expressing CCR2 and CD86 increase over time and were significantly more present in hCRP-tg compared with wild-type males at $t = 37$ weeks. Data are presented as group means (indicated by bars) with SD (error bars). *$P < 0.05$ compared with wild-type controls.

Human CRP has no effect on synovial macrophage accumulation and phenotype shift

As systemic CRP levels have been found to reflect local joint inflammation in patients with advanced OA[14], knee joints were evaluated for the local presence of M1 and M2 macrophage subsets in the synovial lining layer. Macrophages were detected by the pan-macrophage marker F4/80 and subsets were discriminated by colocalization of inducible nitric oxide synthase (iNOS) for M1 macrophages and the mannose receptor (CD206) for M2 macrophages.

Overall, both upon microscopical evaluation and image analysis, there were no significant differences found for synovial macrophage populations between genotypes [Fig. 4(A)]. Univariate testing hinted towards increased CD206 positivity and CD206/iNOS colocalization in knee joints from hCRP-tg males ($P = 0.122$ and $P = 0.183$ respectively, MWU-test; Fig. 4(B)).

Discussion

High CRP levels found in metabolic syndrome patients have been suggested to actively contribute to inflammatory morbidities

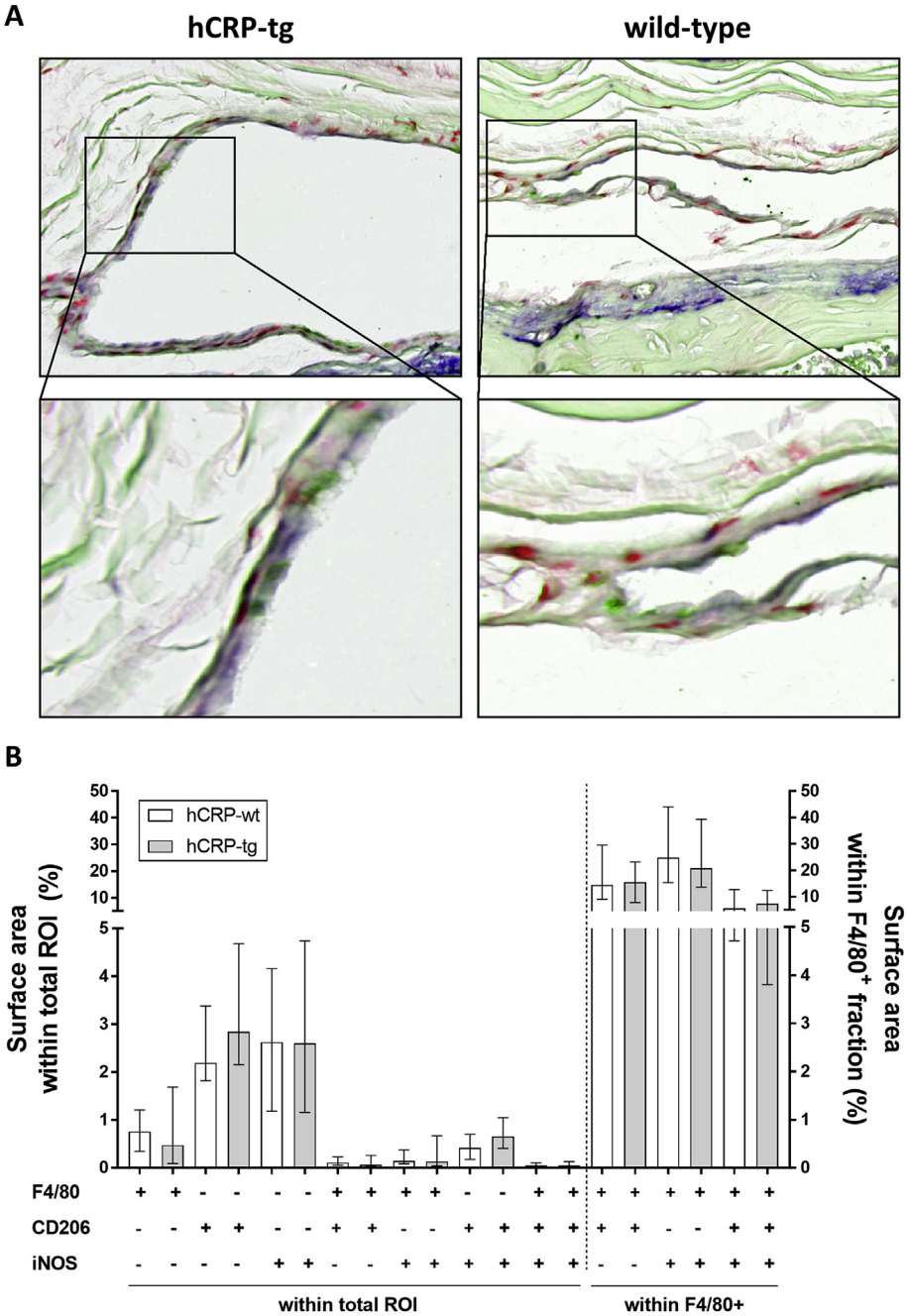

Fig. 4. Human CRP has no effect on synovial macrophage accumulation and subsets. A) Representative unmixed spectral images of the lateral patellofemoral synovial lining (ROI) showing pan-macrophage marker F4/80 (green), M1 subset marker iNOS (blue) and M2 subset marker CD206 (magenta) positivity. Magnification for the top microphotographs was 40×. B) Percentage of the surface area within the ROI or within the F4/80$^+$ cell fraction for hCRP-tg and wild-type mice, showing no CRP-induced shift in macrophage numbers and phenotypes. Data are presented as group medians (indicated by bars) with IQR (error bars).

and related increased cardiovascular and diabetic risks[25]. In the current study we demonstrate that hCRP-tg mice developed more severe OA compared with their wild-type controls under the same HFD regime. Although obesity-related low-grade systemic inflammation is recognized as a contributing factor in metabolic OA pathogenesis, associations between CRP levels and OA features rendered contradictory results between cohort studies. Here we show that hCRP-tg mice exhibit increased cartilage degradation and osteophytosis, but not increased synovitis. The latter is generally seen as a typical inflammatory component of OA, which may partly underlie the conflicting findings in the different cohort studies. Our study, in which the expression of CRP was the only variable, implicates CRP as an independent trigger to aggravate HFD-induced OA development.

The positive association between metabolic syndrome and CRP levels in humans[26] and the exacerbation of metabolic disorders in hCRP-tg mice[27,28] suggest CRP is more than merely an inflammation marker in metabolic disorders. In hCRP-tg mice, as in humans, the CRP protein is synthesized by hepatocytes only and is regulated at the transcriptional level[29]. Confirming previous observations[8], HFD feeding evoked a direct and prolonged rise in plasma CRP levels in hCRP-tg males from our study, indicative of a systemic inflammatory status. Possibly, CRP induces cascades that are not raised in wild-type C57BL/6J mice or amplifies the activation of inflammatory pathways that are not triggered by HFD alone.

While a widely used clinical marker of general inflammation, the physiological functions of human CRP remain to be fully elucidated and can be pro- or anti-inflammatory depending on the situation. One major route of action is the involvement of CRP in innate immunity through the opsonisation of pathogens or dying cells and subsequent activation and modulation of complement via binding to C1q[30]. Even though no added effect to synovitis severity was observed, an increased inflammatory pathway because of CRP signalling still seems likely as osteophyte formation is linked to inflammation[31,32]. It is well possible that CRP is driving a specific inflammatory pathway that is associated with osteophyte development. Angiogenesis at the osteochondral junction could be such a process, which has been demonstrated to lead to endochondral ossification and the formation of osteophytes[33]. Human CRP has been shown to upregulate vascular growth factor (VEGF) expression[34], a driver of neovascularization which is expressed by hypertrophic chondrocytes and synovial macrophages, and VEGF inhibition is suggested as a treatment strategy for OA[35]. Involvement of synovial macrophages is not supported by our results, but perhaps hypertrophic chondrocytes can play a role under the experimental conditions applied.

Inflammation is closely linked to lipid metabolism, a conjunction particularly noticeable in metabolic diseases. As obesity and metabolic syndrome are important risk factors for OA and induce elevated CRP levels in patients, we postulated that obese hCRP-tg males might demonstrate a more proinflammatory lipid profile compared to their controls. Indeed, HFD-induced effects were evident for nearly all lipids measured and indicated low-grade systemic inflammation. However, these changes were not significantly different between genotypes nor correlated to any of the observed OA features. It is possible that the added effect of CRP at the individual lipid level was overshadowed by the profuse systemic influence of the HFD itself.

Alternatively, it is arguable that CRP could have affected OA development locally. This is supported by de Visser *et al.* in a rat model of metabolic OA, where local changes in synovial fluid oxylipin concentrations were not equally translated into systemic changes for the same oxylipins measured in plasma[36]. A route through which CRP could be involved in lipid-driven local cartilage changes is via OA-induced upregulation and activation of phospholipase A2 (PLA2) enzymes by chondrocytes. PLA2 enzymes are able to dissociate pentameric CRP to its pathogenic monomeric (m)CRP subunits[37]. Increased PLA2 activity has been demonstrated in the synovial fluid of OA patients and animal models of OA[38–40]. Devoid of synovial fluid samples, we attempted to explore PLA2 activity in our lipidomic data from fasted plasma samples. Unfortunately, systemic lipid concentrations rendered some conflicting results concerning PLA2 activity. As PLA2 enzymes mainly convert PC to LPC, the decrease in systemic LPC/PC ratio in our HFD-fed mice suggests a lower activation of PLA2. Hydrolysis of PC to LPC by PLA2 enzymes produces a FFA, which is frequently the omega-6 fatty acid arachidonic acid (ARA)[41]. In this respect, the observed increase in omega-6/omega-3 FFA ratio substantiates decreasing PLA2 activity. However, the increases in ARA and its metabolites point to an increased activation of PLA2. Combined enzymatic activities of PLA2 enzymes and cyclooxygenase (COX)-2 generate prostaglandins from ARA and members of this pathway have been implicated in OA pathogenesis[42,43]. Although sample volumes were insufficient to measure prostaglandin E_2 (PGE_2) levels, we did observe an increase in prostaglandin PGD_2 concentration over time – further corroborating increased PLA2 activity. Clearly, the *in vivo* effects of the lipid metabolism on the inflammatory milieu are complex and circulating lipids do not provide the best read-out in our current model.

The physiological role of CRP may be just as complex, with its multiple active isoforms and manifold physiological functions in various biological systems. Even though CRP predates the adaptive immune system by millions of years, it was found to bridge innate with adaptive immunity by binding to Fcγ receptors on immune cells like monocytes[44]. Both hCRP-tg and wild-type mice showed expansion of the systemic monocyte fraction upon HFD feeding, consistent with previous findings[45]. However, we found that hCRP-tg monocytes expressed more activation markers like CD11c, CCR2 and CD86. The integrin CD11c was predominantly expressed by activated $CD11b^{+}GR\text{-}1^{low}CCR2^{+}$ monocytes, which are known to upregulate CD11c as an adhesive ligand during monocyte recruitment in shear flow[46]. This suggests that non-classical, patrolling $GR\text{-}1^{low}$ monocytes were more actively recruited to the tissues of hCRP-tg mice compared with wild-type controls. In addition, hCRP-tg mice showed an increase in activated $CD11b^{+}GR\text{-}1^{high}CCR2^{+}CD86^{+}$ classical monocytes, indicative of an enhanced inflammatory state. This latter observation might be linked to metabolic OA development, as the increased percentages of $CD11b^{+}$ immune cells and monocyte subsets in the hCRP-tg genotype were positively associated with increasing lateral OA severity. Wild-type controls showed inverse associations, strengthening the involvement of CRP in the activation of these immune populations.

The systemic activation of myeloid cells was not reflected in the knee joint, where synovial macrophage subsets were comparable between hCRP-tg and wild-type littermates. This is consistent with the similar synovitis scores found in both groups. Our findings add to recent reports on HFD-induced OA in mice on a C57BL/6J background, where macrophage depletion aggravated cartilage degeneration following injury[47] and resident adipose tissue macrophages retained their M2-like phenotype in the infrapatellar fat pad[48]. Together, these findings emphasize the persistence and therefore the importance of local macrophage populations in regulating homeostasis in the osteoarthritic knee joint. The combination of monocyte recruitment and unchanged resident macrophage populations in our study show similarities to observations in a mouse model of inflammatory arthritis[49]. Here, tissue-resident synovial macrophages showed no changes in phenotype or number and expressed markers of M2 polarization over the course of arthritis. However, the authors showed that non-classical monocytes recruited from circulation orchestrated the initiation and resolution

of joint inflammation by differentiating into M1 and M2 macrophages respectively. Perhaps the hint towards an overall increase in CD206 expression in the synovium indicates monocyte presence, as activated monocytes are able to upregulate CD206 expression[50]. This monocytic plasticity and the role of human CRP herein warrant further research in the context of metabolic OA.

Although the functional involvement of CRP in OA pathogenesis remains uncertain, our study shed some light on the biological processes involved. This uncertainty is inherent to the gain-of-function model we employed, as overexpression phenotypes often fail to faithfully reflect the physiological function(s) of a protein[51]. A loss-of-function model would have provided more straightforward interpretable results. However, unlike human CRP, mouse CRP is a minor acute-phase reactant and is synthesized in only trace amounts[52,53]. Human CRP, when transferred into mice, behaves as it does in man: its expression is highly inducible and tissue-specific[29]. We believe that this significant difference in the transcriptional control of CRP synthesis in humans and mice justifies the use of our model for studying human CRP. Still, extrapolation from mouse to man requires caution. Validation of our findings on peripheral monocyte subsets in obese OA patients with high and low CRP levels is required to confirm the role CRP in OA pathogenesis. An additional potential limitation of our study is that chow controls were not included in our study, as we focused on CRP involvement in OA and previous work by our group has shown that HFD feeding in hCRP-tg males led to severe OA development compared with chow-fed hCRP-tg males[8].

The present study implicates CRP as an independent trigger for the aggravation of metabolic OA by increasing cartilage degeneration and osteophytosis. Increased recruitment of classical and non-classical monocytes might be a mechanism of action through which CRP is involved in aggravating this process. Based on our data, involvement of CRP in lipid metabolism and synovial macrophage activation seems unlikely. Although the mechanism of action for CRP involvement in OA is not yet resolved, it is clear that we are selling CRP short when solely considering it a general systemic inflammation marker in metabolic OA. Our findings suggest that interventions selectively directed against CRP activity could ameliorate metabolic OA development.

Contributors

AEK, AMZ, IB and RS have designed the experiment. AEK, MT, SR and FH have carried out experimental procedures. AEK has been the primary person responsible for writing the manuscript. SR, DB, SM, NK, FL, IB, HW and RS were involved in drafting the work or revising it critically for important intellectual content. All authors approved the final version to be published.

Competing interests

The authors declare that they have no conflict of interest.

Sources of funding

This manuscript has received partial funding from the Netherlands Organization for Applied Scientific Research (TNO) and the European Union Seventh Framework Programme under grant agreement n◦305815 (EU FP7/2007-2013; HEALTH.2012.2.4.5-2; D-BOARD: Novel Diagnostics and Biomarkers for Early Identification of Chronic Inflammatory Joint Diseases). Funders did not have any additional role in the study design, data collection and analysis, decision to publish or preparation of the manuscript. Specific roles of the authors are described in the 'Contributors' section.

Acknowledgements

We thank Marjolein van Rotterdam for the technical and operational aspects of the animal facilities. We thank Sabina Bijlsma, Carina de Jong-Rubingh and Eric Schoen for their continued statistical support. We thank Jan Lindeman and Marlieke Geerts at the Leiden University Medical Center and for their help with the development of the triple chromogenic immunostaining for macrophages. We thank Anne Schwerk for her enthusiastic assistance concerning the histological part of the research. The authors acknowledge that due to space limitations only a subselection of references is included in this manuscript.

Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.joca.2018.09.007.

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