

EXTENDED REPORT

Free fatty acids: potential proinflammatory mediators in rheumatic diseases

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ABSTRACT

Objectives Due to their role in inflammatory metabolic diseases, we hypothesised that free fatty acids (FFA) are also involved in inflammatory joint diseases. To test this hypothesis, we analysed the effect of FFA on synovial fibroblasts (SF), human chondrocytes and endothelial cells. We also investigated whether the toll-like receptor 4 (TLR4), which can contribute to driving arthritis, is involved in FFA signalling.

Methods Rheumatoid arthritis SF, osteoarthritis SF, psoriatic arthritis SF, human chondrocytes and endothelial cells were stimulated in vitro with different FFA. Immunoassays were used to quantify FFA-induced protein secretion. TLR4 signalling was inhibited extracellularly and intracellularly. Fatty acid translocase (CD36), responsible for transporting long-chain FFA into the cell, was also inhibited.

Results In rheumatoid arthritis synovial fibroblasts (RASf), FFA dose-dependently enhanced the secretion of the proinflammatory cytokine IL-6, the chemokines IL-8 and MCP-1, as well as the matrix-degrading enzymes pro-MMP1 and MMP3. The intensity of the response was mainly dependent on the patient rather than on the type of disease. Both saturated and unsaturated FFA showed similar effects on RASf, while responses to the different FFA varied for human chondrocytes and endothelial cells. Extracellular and intracellular TLR4 inhibition as well as fatty acid transport inhibition blocked the palmitic acid-induced IL-6 secretion of RASf.

Conclusions The data show that FFA are not only metabolic substrates but may also directly contribute to articular inflammation and degradation in inflammatory joint diseases. Moreover, the data suggest that, in RASf, FFA exert their effects via TLR4 and require extracellular and intracellular access to the TLR4 receptor complex.

INTRODUCTION

Around the beginning of the twenty-first century, obesity became a global epidemic.¹ Common comorbidities include cardiovascular and metabolic diseases. In this context, it could be shown that adipose tissue is also a source of various inflammatory mediators,²⁻⁴ which may in part account for the long-term comorbidities associated with obesity. Adipocytokines are one group of these inflammatory mediators; free fatty acids (FFA) released from adipose tissue may be another. Serum FFA levels are increased in obese individuals compared with lean individuals,⁵ and chronically elevated FFA levels in vivo have been shown to cause various detrimental effects, including impaired insulin sensitivity of muscles⁶ and liver,^{7,8}

endothelial dysfunction,⁹ hypertension¹⁰ and increased very low-density lipoprotein production.¹¹ In addition, FFA affect gene expression of adipocytes,¹² macrophages^{13,14} and monocytes¹⁵ in vitro.

Obesity is also a recognised risk factor for several arthritic diseases.¹⁶⁻²⁵ Increased mechanical stress on the joints is thought to be one causative factor specifically in osteoarthritis (OA) but is probably not the only one. In particular, articular fat has been shown to be a local source of inflammatory mediators³ and several adipocytokines have already been associated with rheumatic diseases.²⁶ However, adipocytokines may not be the only inflammatory mediators from adipose tissue. We therefore hypothesised that FFA may also be involved in the pathogenesis of rheumatic diseases. As synovial fibroblasts (SF) are not only key players in the pathophysiology of rheumatoid arthritis (RA)²⁷ but also of OA²⁸ and psoriatic arthritis (PsA),²⁹ we studied the effect of FFA on SF of different arthritides. We also analysed other cell types with relevance to the pathophysiology of rheumatic diseases, specifically chondrocytes³⁰ and endothelial cells.³¹

Toll-like receptors (TLRs) are pattern-recognition receptors that mainly, but not exclusively, recognise pathogen-associated molecular patterns and activate immunological responses. They are an important part of the innate immune system. Of these receptors, TLR4 is mainly known for recognising lipopolysaccharide (LPS) found on most gram-negative bacteria but also binds other ligands. FFA are one of the molecules that interact with and activate or inhibit TLR4.^{12,14,15} This is especially interesting as TLR4 has been shown to be involved in the pathophysiology of several rheumatic diseases, particularly RA.³² However, the nature of the interaction between FFA and TLR4 is still controversial.^{12,15,33,34} We hence investigated whether in RASf TLR4, as part of the innate immune system, is also involved in fatty acid signalling and whether FFA need to be internalised to exert their effect.

MATERIALS AND METHODS**Isolation of synovial fibroblasts**

Synovial tissue samples were obtained from synovial biopsy specimens from RA, OA and PsA patients (cf. online supplementary table) who were undergoing joint surgery. Normal synovial fibroblasts (NSF) from a healthy subject were obtained from the trauma surgery. All patients gave written informed consent and fulfilled the criteria of the



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American College of Rheumatology^{35 36} or the CASPAR criteria,³⁷ respectively. Following enzymatic digestion, primary SF were isolated^{38 39} and cultured in supplemented Dulbecco's Modified Eagle Medium (DMEM) as described below.

Cell culture

Primary SF were cultured in DMEM (PAA Laboratories, Cölbe, Germany) containing 10% heat-inactivated foetal bovine serum (FBS) (Sigma-Aldrich, Deisenhofen, Germany), 12.5 mM HEPES (PAA Laboratories), 100 U/mL penicillin/streptomycin (PAA Laboratories), subsequently called supplemented DMEM, and cultured for a maximum of 10 passages at 37°C in 10% CO₂. Human primary chondrocytes (PromoCell, Heidelberg, Germany) from knee joint cartilage were cultured in supplemented DMEM and passaged once before experimental use. Cell culture medium for human microvascular endothelial cells—human bladder microvascular endothelial cells (HBdMEC; PromoCell) and human macrovascular endothelial cells—human umbilical vascular endothelial cells (HUVEC; PromoCell)—consisted of DMEM containing 20% heat-inactivated FBS, 12.5 mM HEPES, 100 U/mL penicillin/streptomycin and Endothelial Cell Growth Medium MV SupplementMix (PromoCell). For harvesting or subculturing, cells were detached using trypsin-EDTA (PAA Laboratories).

Stimulation with FFA

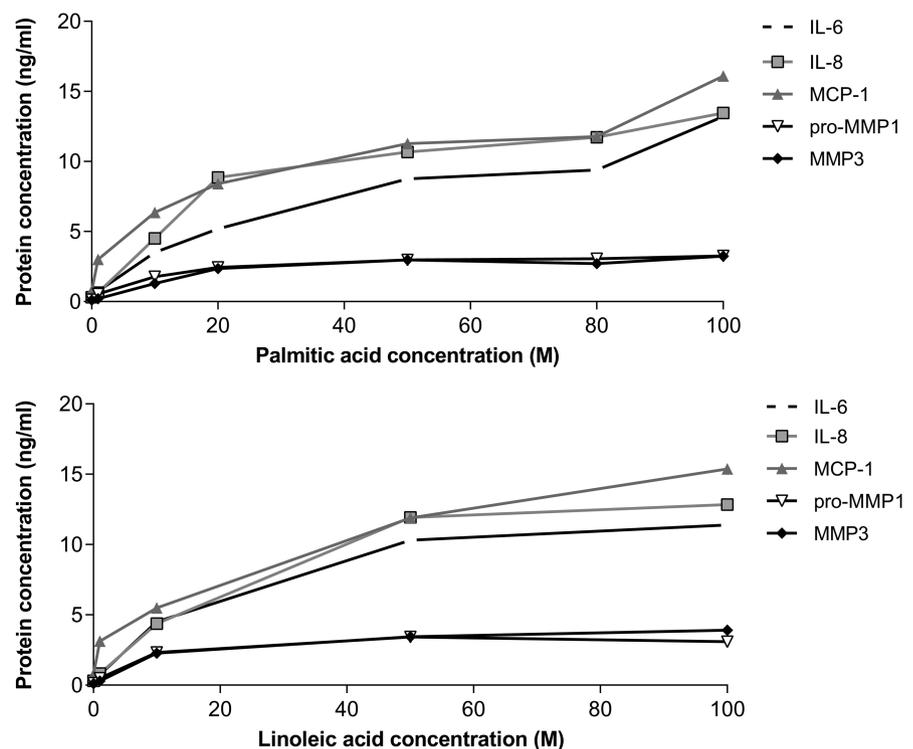
Cells were stimulated with FFA for 15 h as outlined below. SF and human primary chondrocytes were grown up to 70–80% confluency before stimulation. Human endothelial cells were stimulated at a confluency of 90–100%. All FFA were obtained from Sigma-Aldrich at the highest purity available (chemically synthesised, GC-purified). Saturated fatty acids included undecanoic acid (C11), lauric acid (C12), myristic acid (C14), palmitic acid (C16), margaric acid (C17) and stearic acid (C18). Unsaturated fatty acids included palmitoleic acid (C16:1, cis-9), oleic acid (C18:1, cis-9), linoleic acid (C18:2, n-6) and

eicosapentaenoic acid (C20:5, n-3). In vivo, FFA are mainly bound to serum albumin, which increases solubility and prevents cellular toxicity. Therefore, fatty acid/ bovine serum albumin complex solutions were prepared as follows (modified from Spector *et al*⁴⁰): fatty acids were dissolved in ethanol at 70°C to yield a concentration of 200 mM. This stock solution was then diluted 1:10 in 10% (w/v) bovine serum albumin (Carl Roth, Karlsruhe, Germany) at 55°C for 10 min. Fatty acid solutions (20 mM) were sterile filtered before use in stimulation experiments. Negative controls, that is, cells to which no FFA were added, were treated with the fatty acid solvent (= vehicle) to exclude effects mediated by the vehicle. Additionally, FFA solutions were analysed for LPS contamination by using a commercial LPS quantification assay (Pierce LAL Chromogenic Endotoxin Quantitation Assay, Thermo Fisher Scientific, Bonn, Germany) to show that the solutions (as used in the experimental settings) do not contain measurable amounts of LPS that would result in the observed effects. According to the results of this assay, the LPS concentration of 100 µM FFA in supplemented DMEM was below the detection limit of this assay (<0.1 endotoxin units [EU]/mL). Stimulation experiments with different LPS concentrations from 0.01 ng/mL up to 10 ng/mL showed that the concentrations required for inducing interleukin-6 (IL-6) secretion in rheumatoid arthritis synovial fibroblasts (RASf) were above the detection limit of the LPS quantification assay. Experimental replicates consisted of separate cell culture wells containing the same cells treated equally. Cell culture supernatants were collected and frozen at -20°C until further evaluation.

Inhibition of TLR4 signalling

TLR4 signalling was inhibited extracellularly by LPS-RS (InvivoGen, San Diego, USA) and intracellularly by CLI-095 (InvivoGen). Cells were preincubated for 2 h with the inhibitors before stimulation with FFA. According to the manufacturer's

Figure 1 Dose-dependent secretion of proinflammatory and matrix-degrading proteins by rheumatoid arthritis synovial fibroblasts (RASf) in response to stimulation with palmitic acid and linoleic acid. RASf (n=3) were stimulated with palmitic acid or linoleic acid at concentrations ranging from 1 to 100 µM. Supernatants were analysed for the levels of IL-6, IL-8, MCP-1, pro-MMP1 and MMP3 by immunoassay. MCP-1, monocyte chemoattractant protein 1.



instructions, LPS-RS was used at a final concentration of 100 ng/mL and CLI-095 at a final concentration of 1 μ g/mL.

Inhibition of fatty acid transport into the cell

Transport of fatty acids into the cells through CD36/fatty acid translocase (FAT) was inhibited by sulfosuccinimidyl oleate sodium (SSO) (Santa Cruz Biotechnology, Santa Cruz, USA). Cells were preincubated for 30 min with SSO at a concentration of 1 mM,⁴¹ and then SSO was removed by replacing the cell culture medium with SSO-free medium before stimulation with FFA as described above.

Quantification of protein levels

Cytokine, chemokine and MMP levels in cell culture supernatants were measured using commercially available ELISA (R&D Systems, Wiesbaden, Germany).

Statistical analysis

Data are presented as arithmetic mean \pm SEM. In order to assess the significance of differences, Student two-tailed t test was performed for pairwise comparisons. For multiple comparisons, ANOVA, including Tukey's posthoc test, was performed. p Values <0.05 were considered significant. Statistical calculations were performed using Microsoft Excel and GraphPad Prism.

RESULTS

Dose-dependent secretion of proinflammatory and matrix-degrading proteins by RASF in response to stimulation with palmitic acid and linoleic acid

RASF (n=3) were stimulated with palmitic acid, a saturated fatty acid, and linoleic acid, an unsaturated fatty acid, at concentrations ranging from 0 to 100 μ M. With both fatty acids, secretion of the proinflammatory cytokine IL-6, the chemokines IL-8 and monocyte chemotactic protein 1 (MCP-1), as well as the matrix metalloproteinases pro-MMP1 and MMP3, was induced in a dose-dependent manner (figure 1 and online supplementary figure S1 and S2). Effects were observed at concentrations above 1 μ M and reached a plateau at 50–100 μ M.

Analysis of the effects of 10 different fatty acids on the secretion of the proinflammatory cytokine IL-6 by RASF

Six saturated fatty acids (undecanoic acid, lauric acid, myristic acid, palmitic acid, margaric acid, stearic acid), two monounsaturated fatty acids (palmitoleic acid, oleic acid) and two polyunsaturated fatty acids (linoleic acid, eicosapentaenoic acid) were used to stimulate three different populations of RASF (n=3). Concentrations used for stimulation were 1, 10 and 100 μ M. All fatty acids induced the cytokine IL-6 in a dose-dependent manner. However, in most cases, only minor differences could be observed between the different fatty acids regarding their effect on the secretion of IL-6 by RASF (figure 2), and none of these were statistically significant. Also, for RASF, there was no characteristic response pattern for shorter fatty acids compared with longer fatty acids or saturated fatty acids compared with unsaturated fatty acids.

Differential responses of synovial fibroblast populations induced by stimulation with palmitic acid and linoleic acid

In order to investigate whether different types of SF respond differently to stimulation with FFA, we stimulated multiple populations of SF from RA patients (n=4), from OA patients (n=4) and from PsA patients (n=4) with palmitic acid (saturated fatty acid) (figure 3A) and linoleic acid (unsaturated fatty acid)

(figure 3B). One population (n=1) of SF from a healthy subject (NSF) was added as a reference. All examined SF populations responded to FFA with a dose-dependent increase in IL-6 secretion. However, rather than RASF, osteoarthritis synovial fibroblasts (OASF) and PsASF showing clearly different responses to

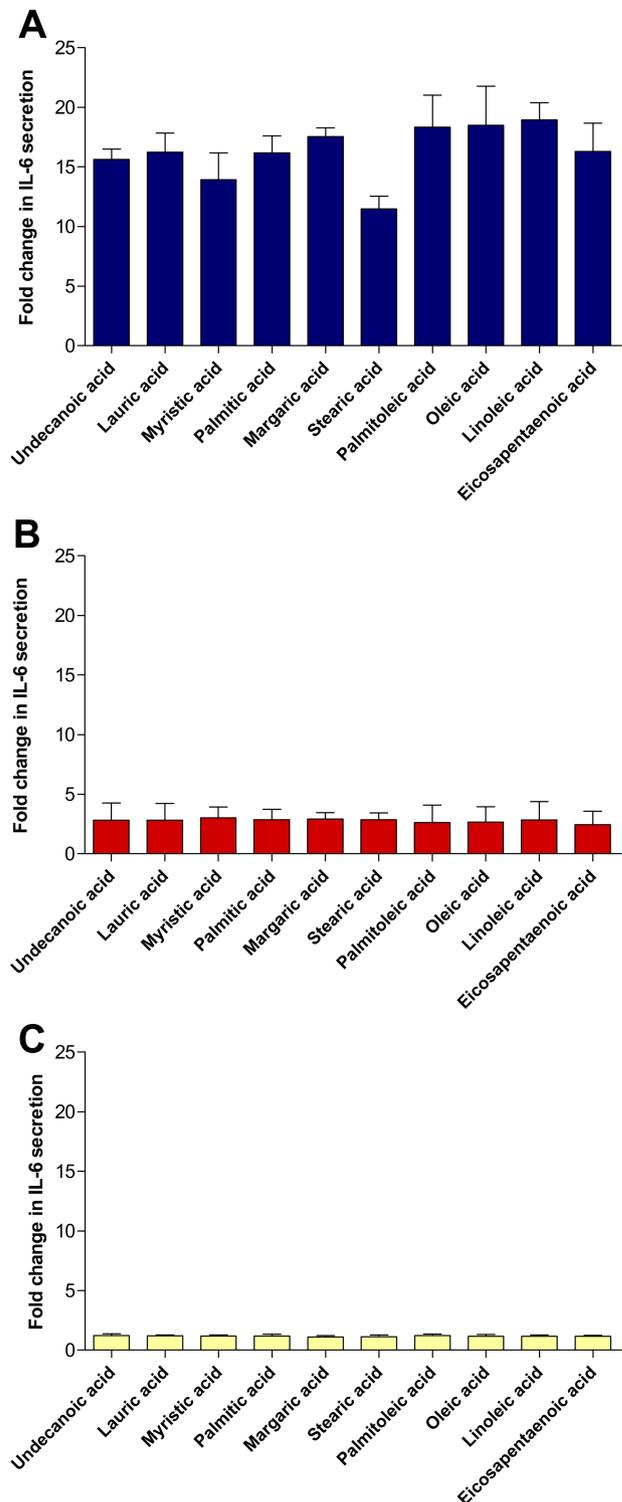


Figure 2 Similar effects of saturated and unsaturated fatty acids on the secretion of the proinflammatory cytokine IL-6 by RASF. Several saturated and unsaturated fatty acids were used for stimulating RASF (n=3) at 1, 10 and 100 μ M. IL-6 was quantified in the supernatants by immunoassay. Fold changes are relative to baseline secretion levels, that is, secretion levels of RASF treated with vehicle only.

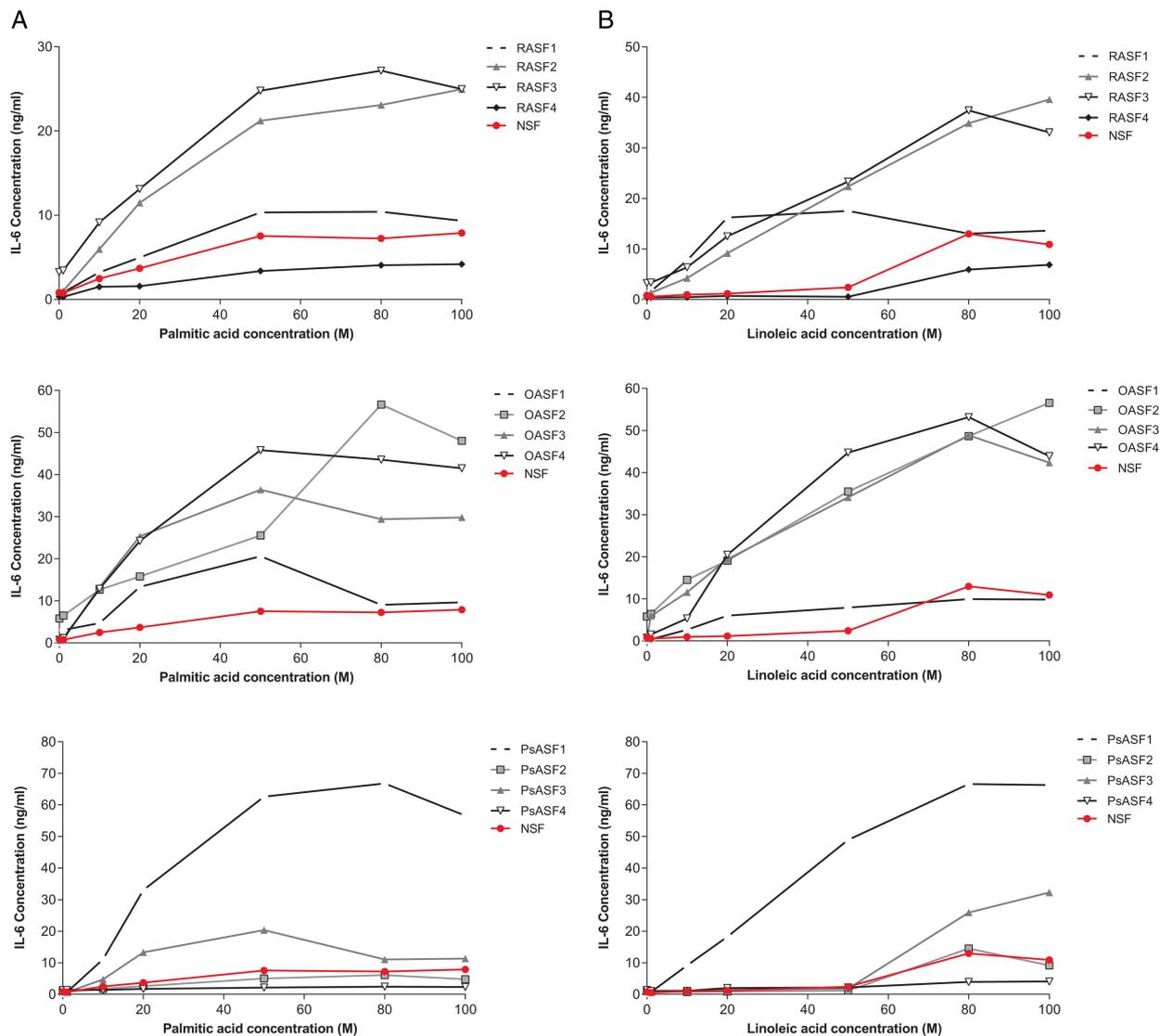


Figure 3 Differential responses of synovial fibroblast populations induced by stimulation with palmitic acid and linoleic acid. Multiple populations of synovial fibroblasts (SF) from rheumatoid arthritis patients ($n=4$), from osteoarthritis patients ($n=4$) and from psoriatic arthritis patients ($n=4$) were stimulated with palmitic acid (A) and linoleic acid (B) at concentrations ranging from 1 to 100 μM . One population ($n=1$) of SF from a healthy subject (NSF, normal synovial fibroblasts) was added as a reference and is shown in red. At the end of the stimulation, IL-6 levels in supernatants were analysed by immunoassay.

palmitic acid and linoleic acid, differences could mainly be observed between cell populations, that is, between cells from different patients (figure 3).

Differential responses of human chondrocytes induced by stimulation with different fatty acids

Human chondrocytes (from the knee joint of a healthy male donor, 70 years, Caucasian) were stimulated with 10 μM of six saturated fatty acids (undecanoic acid, lauric acid, myristic acid, palmitic acid, margaric acid, stearic acid) and four unsaturated fatty acids (palmitoleic acid, oleic acid, linoleic acid, eicosapentaenoic acid). In contrast to RASF, human chondrocytes showed different responses towards different fatty acids in regards to the induction of IL-6 secretion ($n=3$, experimental replicates) (figure 4). Palmitic acid had the strongest effect on human chondrocytes (11.6-fold \uparrow), while unsaturated fatty acids, especially polyunsaturated fatty acids, had only caused considerably weaker effects (2.5-fold \uparrow for linoleic acid, no significant effect for eicosapentaenoic acid).

Differential responses of human endothelial cells induced by stimulation with different fatty acids

Human macrovascular endothelial cells, HUVEC (from a single donor) (figure 5A), and human microvascular endothelial cells, HBdMEC (pooled from multiple donors) (figure 5B), were stimulated with 100 μM of a saturated fatty acid (palmitic acid), a monounsaturated fatty acid (oleic acid) and a polyunsaturated acid (linoleic acid) ($n=3$, experimental replicates). In both cell types, oleic acid had no significant effect on the induction of IL-6 secretion, while palmitic acid and linoleic acid only had a significant effect at a concentration of 100 μM . For endothelial cells, the polyunsaturated fatty acid linoleic was the strongest inducer of IL-6 secretion.

Abrogation of palmitic acid-induced cytokine secretion in RASF by extracellular and intracellular TLR4 inhibitors

It has previously been shown that TLR4 is involved in fatty acid-induced signalling.^{12 14 15 23} However, the actual interaction still has not been clearly defined. We therefore examined whether in SF

Figure 4 Differential responses of human chondrocytes induced by stimulation with different fatty acids. Human chondrocytes (knee joint of a healthy male donor, 70 years, Caucasian) were stimulated with saturated (grey bars) and unsaturated (white bars) fatty acids (n=3 experimental replicates) at a concentration of 10 μ M. Results for poststimulation IL-6 levels are shown as mean \pm SEM. *p<0.05; ***p<0.001; ns, not significant.

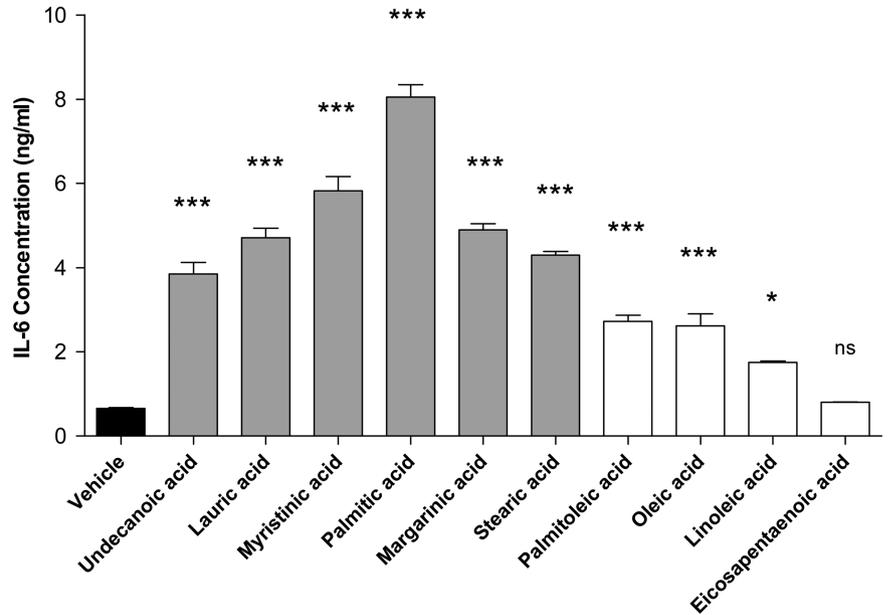
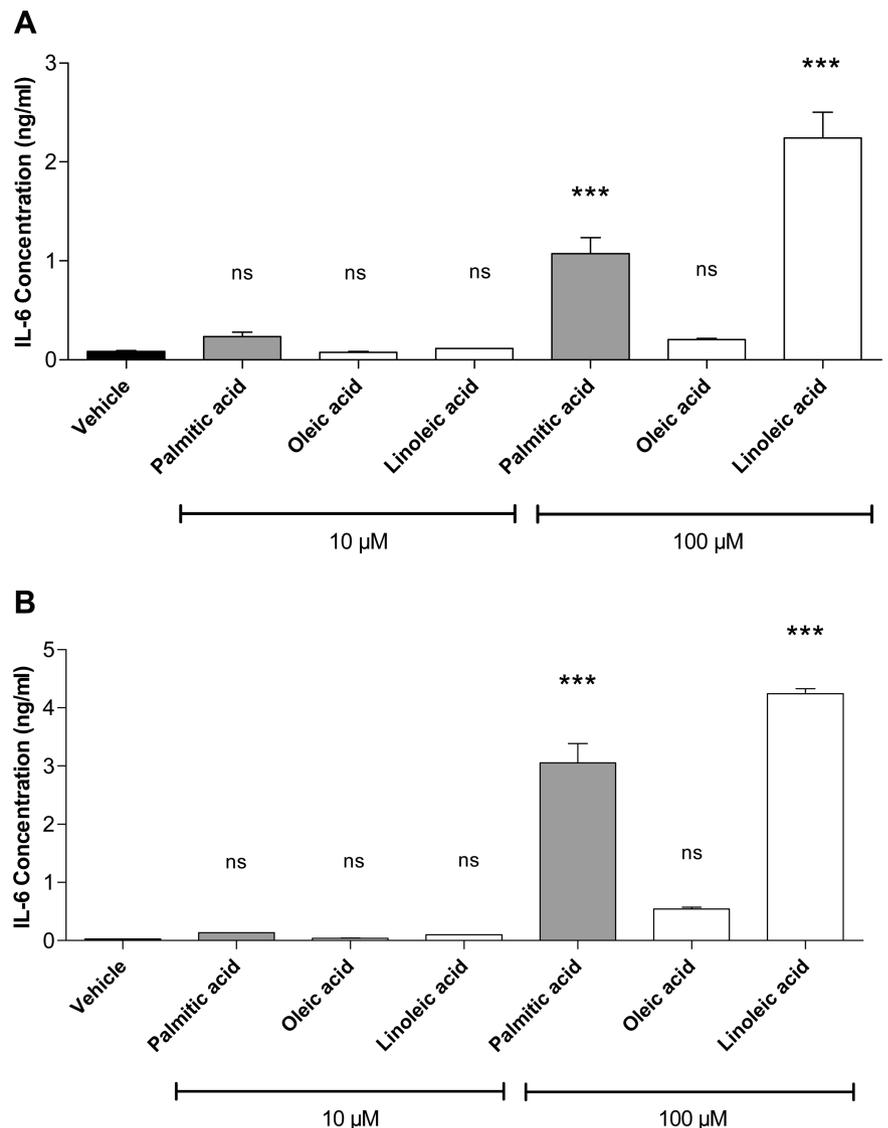


Figure 5 Differential responses of human endothelial cells induced by stimulation with different fatty acids. Human macrovascular endothelial cells, HUVEC (from a single donor) (A), and human microvascular endothelial cells, HBdMEC (pooled from multiple donors) (B), were stimulated with 100 μ M palmitic acid, oleic acid and linoleic acid (n=3 experimental replicates). IL-6 levels were quantified by immunoassay. Results are shown as mean \pm SEM. ***p<0.001; ns, not significant. HUVEC, human umbilical vascular endothelial cells; HBdMEC, human bladder microvascular endothelial cells.

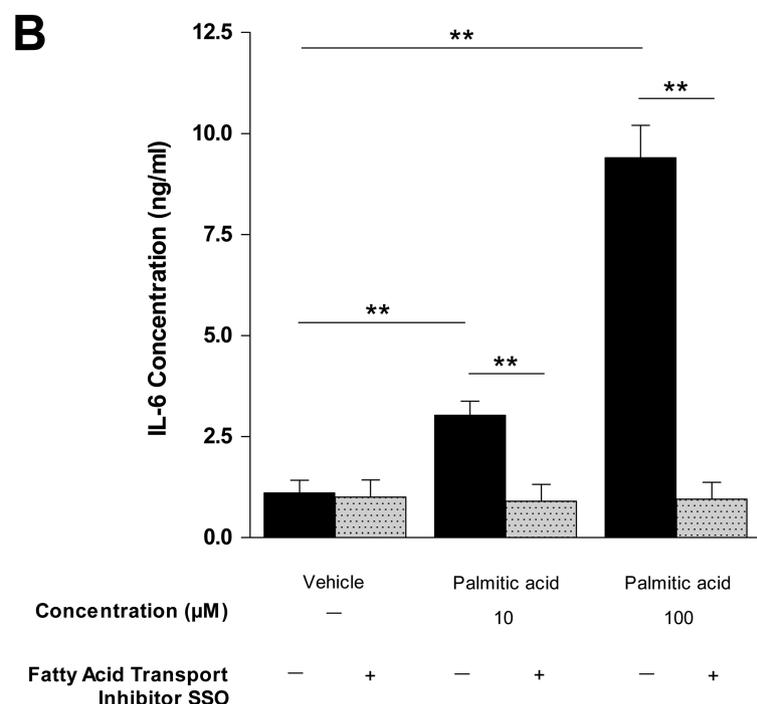
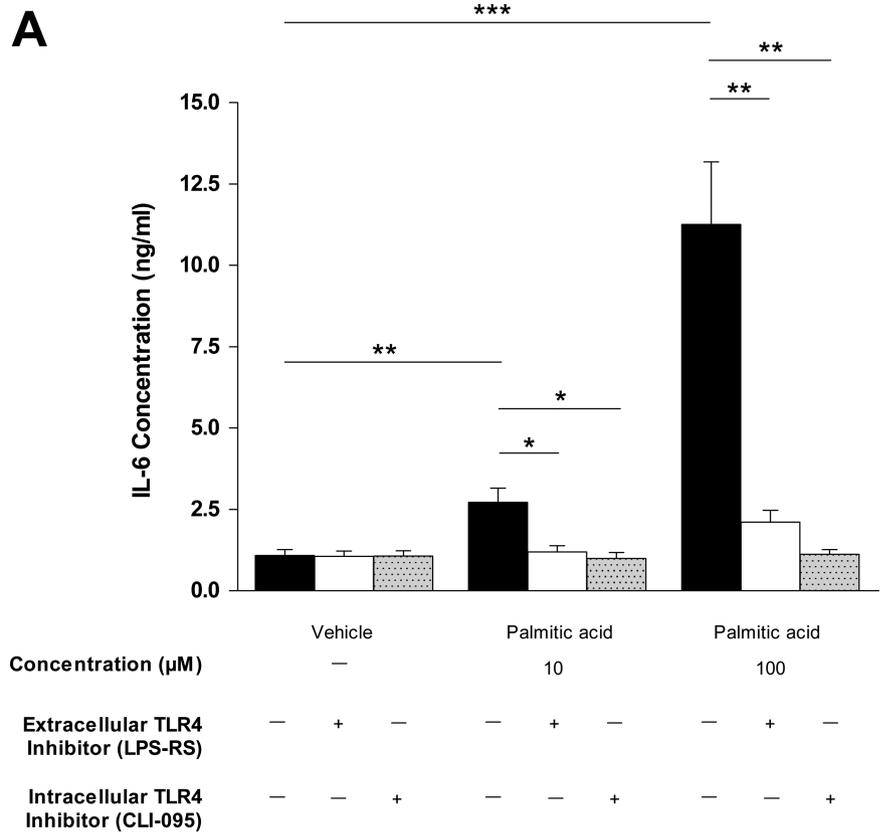


this interaction occurs intracellularly, extracellularly or both. LPS-RS is an extracellular inhibitor of TLR4,⁴²⁻⁴⁴ while CLI-095 is an intracellular inhibitor of TLR4.⁴⁵⁻⁴⁶ LPS-RS and CLI-095 completely abrogated the palmitic acid-induced IL-6 secretion by RASF (n=5) when cells were stimulated with 10 μ M palmitic acid (figure 6A). At a stimulation concentration of 100 μ M, palmitic acid-induced IL-6 secretion by RASF was significantly reduced by LPS-RS and again completely abrogated by CLI-095 (figure 6A).

Abrogation of palmitic acid-induced cytokine secretion in RASF by inhibition of the fatty acid transport molecule CD36/FAT

CD36/FAT is an important fatty acid transport molecule⁴⁷ that can be inhibited by SSO.⁴¹ Here, SSO was used to inhibit the uptake of palmitic acid into RASF, which completely abrogated the palmitic acid-induced secretion of IL-6 by RASF (n=3) at a stimulation concentration of both 10 and 100 μ M (figure 6B).

Figure 6 (A) Abrogation of palmitic acid-induced cytokine secretion in rheumatoid arthritis synovial fibroblasts (RASF) by extracellular and intracellular TLR4 inhibitors. RASF (n=5) were stimulated with 10 and 100 μ M palmitic acid. In parallel, TLR4 signalling was inhibited extracellularly by LPS-RS (white bars) and intracellularly by CLI-095 (grey bars). (B) Abrogation of palmitic acid-induced cytokine secretion in RASF by inhibition of the fatty acid transport molecule CD36/fatty acid translocase (FAT). RASF (n=3) were stimulated with 10 and 100 μ M palmitic acid. Fatty acid transport through CD36/FAT was inhibited by previous treatment with SSO (grey bars). Each diagram bars represent means (\pm SEM) of multiple cell populations (n=3). *p<0.05; **p<0.01; ***p<0.001; LPS, lipopolysaccharide.



DISCUSSION

In this study, we investigated our hypothesis that FFA contribute to a proinflammatory and prodestructive milieu that promotes the development and progression of arthritic diseases.

We could confirm this hypothesis in vitro by showing that in RASF FFA induce the proinflammatory cytokine IL-6, the chemokines IL-8 and MCP-1, as well as the matrix metalloproteinases pro-MMP1 and MMP3. The key proinflammatory cytokine IL-6 is also induced by FFA in OASF and PsASF, as well as other pathophysiologically important cell types of arthritic diseases, specifically chondrocytes and endothelial cells. The notion that adipose tissue in particular and not just increased body mass is responsible for the development and the symptoms of arthritis is supported by several observations. First, in humans, obesity also increases the risk of developing arthritis in non-weight-bearing joints such as the hands.^{20 24 48 49} Second, in mice, a high-fat diet-induced obesity caused OA and systemic inflammation in proportion to body fat, whereas increased mechanical joint loading by exercise did not promote but rather inhibit OA.⁵⁰ Last but not least, Toda *et al*⁵¹ found that particularly changes in body fat and not body weight are related to symptomatic relief of obese patients with OA after a weight control programme. Additionally, Gudbergesen *et al*⁵² showed that in OA patients symptomatic relief after weight loss was not due to increased muscle strength or improved knee joint alignment as neither of these was associated with the degree of symptomatic relief.

Our in vitro data suggest that besides adipocytokines FFA, which are increased in obese individuals compared with lean individuals,⁵ contribute to inflammation and joint destruction in arthritic diseases. Of note, SF from different patients with the same disease responded differently to stimulation with FFA regarding the induction of IL-6. One possible explanation for this finding could be the different expression levels of the receptor(s) for FFA. Assuming that TLR4 is the major receptor for FFA,^{12 14 15} RASF should show the strongest response to FFA as they express more TLR4 compared with OASF.⁵³ However, in our study, we observed that the response to fatty acids is rather dependent on the patient than on the arthritic disease.

In contrast, endothelial cells did not show a significant response to the low concentration (10 µM) of FFA. As lower levels of FFA are always present in serum, this is physiologically reasonable. At a higher concentration (100 µM), palmitic acid and linoleic acid caused a significant induction of IL-6, supporting the idea of a negative influence of FFA on the known long-term vascular damage in rheumatic diseases. For RASF, we observed similar effects for saturated and unsaturated fatty acids. Hence, at least for endothelial cells and RASF there seems to be no association between the degree of saturation of fatty acids and their effect on cells. Human chondrocytes, on the other hand, responded to saturated fatty acids more strongly than to unsaturated fatty acids. We assume that in different cell types saturated and unsaturated fatty acids have different modes of action, which could explain these differences.

We furthermore analysed whether TLR4, as part of the innate immune system, plays a role in FFA-induced signalling in RASF. It has already been established that FFA activate or inhibit certain cell types through TLR4.^{12 14 15} However, the exact nature of this interaction is still not quite clear: On the one hand, there is no direct binding of radiolabelled saturated FFA to TLR4 (more specifically, a soluble fusion complex consisting of the FLAG-tagged extracellular part of TLR4 fused to full-length MD-2 via a flexible linker);¹² on the other hand, the polyunsaturated fatty acid eicosapentaenoic acid blocks LPS binding to an

experimental LPS trap.¹⁵ Whether FFA mediate TLR4 activation by inducing its dimerisation is discussed controversially. Lee *et al*³³ found no effect on TLR4 dimerisation, while Wong *et al*³⁴ found that saturated fatty acid induced TLR4 dimerisation. In our study, we intended to elucidate whether FFA influence TLR4 signalling extracellularly and/or intracellularly in SF. For this purpose, we used an extracellular and an intracellular TLR4 inhibitor. LPS-RS is an underacylated form of LPS from *Rhodobacter sphaeroides*, which is capable of binding to the extracellular TLR4 coreceptor MD2.^{42–44} LPS-RS:MD2 complexes, however, are not able to trigger any signalling and thus block TLR4. CLI-095, also known as TAK-242, is a cell-permeable chemical that suppresses TLR4 signalling by blocking the signalling mediated by the intracellular domain of TLR4^{45 46} but not the extracellular domain. Additionally, we used SSO to inhibit fatty acid transport through CD36/FAT.⁴¹ Extracellular and intracellular TLR4 inhibition as well as fatty acid transport inhibition completely or nearly abrogated the IL-6 induction in RASF by palmitic acid. Our data therefore suggest that for TLR4 activation FFA need to interact with the TLR4 receptor complex extracellularly and intracellularly.

Taken together, the data show that that elevated FFA levels may be a hitherto unknown factor contributing to the increased risk of developing arthritis in obese individuals and the negative effects of obesity on most arthritic diseases.

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Contributors KWF had the main responsibility for the study, including design, experimental set-up, data analysis and interpretation, figures, and writing the paper. All authors were substantially involved in producing the final version of this paper.

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Competing interests None.

Patient consent Obtained.

Ethics approval Ethics Committee of the Justus-Liebig-University of Giessen.

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