Supplemental Methods

Removal of endogenous fatty acids from commercial albumins

Commercially sourced albumins contain endogenous fatty acids reflecting those transported in the plasma from which they were isolated. To remove endogenous fatty acids from a non-contaminated BSA (BSA-3 was chosen), the method of Chen was used [Ref-S1]. Briefly, 7 g of BSA-3 was dissolved in 70 ml H₂O, and 3.5 g activated charcoal (Sigma 242276) was added. The pH of the mixture was then lowered to 3.0 by the addition of 0.1M HCl and stirred magnetically in an ice bath for 2 h. The slurry was then transferred to multiple 1.5 ml microtubes and centrifuged at 13,500 rpm for 20 minutes to pellet the activated carbon. Supernatants were then pooled and the pH of the resulting clarified solution was normalised to 7.0 by addition of 0.1M NaOH before sterile filtration (0.2 μm).

To create SFA/BSA complexes from freshly prepared FAF-BSA, a five-fold molar excess of each SFA was added to aliquots of FAF-BSA-3 and incubated at 37°C for 1 h. After complex formation, unbound SFAs were removed by passing each mixture through size-exclusion chromatography columns (GE Healthcare PD10), which concurrently permitted rebuffering in PBS. Eluates were then sterile-filtered (0.2 μm) and stored at 4°C for up to 2 days before use in assays.

Quantification of fatty acids in SFA/BSA complexes

Endogenous, or experimentally complexed, fatty acids were quantified in FAF-BSAs or BSA/SFA complexes using an enzymatic fatty acid quantification assay (Abcam, UK). Briefly, test and kit reagents were allowed to warm to room temperature, and an 8 point standard curve was prepared in a microtitre plate using free palmitate (C16:0) as calibrator. Samples were then diluted 1:10 in assay buffer and 50 μl of each was aliquoted to triplicate wells. 50 μl of reaction mixture was then added to all wells, mixed on a plate mixer, and incubated for 30 mins at 37°C in the dark. Finally, absorbance was measured at 570 nm using a colorimetric micro-plate reader.
Culture of 3T3-L1 adipocytes and C2C12 myotubes

3T3-L1 pre-adipocytes were cultured in DMEM/10% FCS, plated in 6-well plates and allowed to reach confluence over 7 days. Medium was then removed and replaced with IBMX differentiation medium (DMEM/10%FCS containing 0.5 mM 3-isobutyl-1-methylxanthine, 0.25 μM dexamethasone and 2 μg/ml bovine insulin) for three days, as described previously [Ref-S2]. Medium was then replaced carefully with DMEM/10% and cells were allowed to grow for a further week, after which time a high proportion of cells showed typical adipocyte morphology, including large lipid inclusions. C2C12 myotubes were cultured in DMEM/10% FCS as described previously [Ref-S3].

Real-time PCR for β-actin, IL-6, TNF-α and CCL-2 mRNA

Differentiated 3T3-L1 adipocytes and C2C12 myotubes were cultured in 6-well plates as described above. Medium was replaced with DMEM/1%FCS containing 100 mM each SFA or 1 mg/ml LPS and cultured for 3 h. RNA was then extracted (Qiagen RNEasy kit) and converted to cDNA (Stratagene Superscript). cDNA was then amplified using Quantace SYBR-green mastermix and Rotorgene (Corbett Life Sciences, UK) quantitative-PCR cycler acquiring over 40 cycles. Primers used were murine β-actin: (Forward - TTCTTTGCAGCTCCTTCGGCG; Reverse - TGGATGGCTACGTACATGGCTGGG), IL-6: (Forward - AACGATGATGCACTTGCAGA; Reverse - GAGCATTGGAAATTGGGGTA), TNF-α: (Forward - TCCCCAAAGGGATGAGAAGTTC; Reverse - TCACATGGGTTGAGCTCAG) and CCL-2: (Forward - GGCTCAGCCAGATGCAGTTAA; Reverse - CCTACTCATGGGATCATCTTGGT). Target gene message abundance was measured in triplicate and normalised to β-actin expression.
Preparation of common laboratory reagents for assay of TLR2/4 contaminants

Liquid laboratory reagents, such as sterile antibodies or molecular biology enzymes, were diluted directly 1:100 in DMEM/1% FCS. Solid reagents, such as casein or mouse chow, were resuspended in sterile PBS at a ratio of 1:10 (weight:volume), vortexed vigorously, inverted for 5 minutes and then centrifuged at 13,000 g for clarification. Supernatant was then sterile filtered before diluting 1:10 in DMEM/1%FCS. Each reagent was then applied to HEK-293 cells transfected with TLR2 or TLR4 and induction of NF-κB reporter measured as described in the main methods section.

Supplemental references:


Supplementary Table SI: Summary of control experiments performed in previous studies of SFA modulation of TLR-signalling

<table>
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<th>Authors</th>
<th>Reference</th>
<th>LAL assay</th>
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The procedures that were employed in each of the listed studies to control for potential contamination of reagents with bacterial molecules is presented. N indicates no respective control methodology was employed.
Supplementary figure I: Effect of reagents on HEK-293 CD14 transfectants in the absence of TLR co-transfection

HEK-293 cells were transfected with NF-κB sensitive reporter (pELAM) and CD14 but without TLR co-transfection. Induction of reporter was calculated relative to cells cultured in medium alone after 18 h challenge with 200 mM each SFA, 0.5 mg/ml BSA, 100 ng/ml each PAMP or 10 ng/ml IL-1β + 10 ng/ml TNF-α combined.
Supplementary figure II: Sensitivity of transfected HEK-293 cells to TLR-ligands

HEK-293 cells were transfected with NF-κB sensitive reporter (pELAM) and TLR2, TLR4/MD2 or TLR5. Respective positive control TLR-ligands Pam₃CSK₄ (A), LPS (B) or flagellin (C) were applied to cells at indicated concentrations and induction of reporter was calculated relative to cells cultured in medium alone after 18 h.

* P < 0.05 vs reporter levels in cells cultured in medium alone.
Supplementary figure III: Responses of TLR-transfectants to sodiated SFAs

HEK-293 cells were transfected with NF-κB sensitive reporter (pELAM) and TLR2, TLR4/MD2 or TLR5. Induction of reporter was calculated relative to cells cultured in medium alone after 18 h challenge with 200 μM of each saturated fatty acid in the sodiated form (C12:0, lauric acid; C14:0, myristic acid; C16:0, palmitic acid and C18:0, stearic acid), 100 ng/ml Pam₃CSK₄, LPS or flagellin (Pos).
Supplementary figure IV: Contaminating TLR-stimulants in fatty-acid free albumins

HEK-293 cells transfected with TLR2 (A), TLR4/MD2 (B) or TLR5 (C) were challenged with 0.5 mg/ml commercially sourced FAF-BSA-2 (Sigma A0281), FAF-human serum albumin (HSA-4, A1887), endotoxin-free FAF-BSA-5 (A8806) and positive controls 10 ng/ml Pam₃CSK₄, 1 ng/ml LPS or 100 ng/ml flagellin (Pos).

Activation of NF-κB-dependent reporter is presented as fold-induction relative to cells cultured in medium alone +/- SD. * P<0.05, ** P< 0.01 vs medium alone.
Supplementary figure V: Effect of SFAs on TLR-mediators in skeletal muscle cells and adipocytes

Differentiated 3T3-L1 adipocytes (left), or C2C12 skeletal myotubes (right) were challenged with 100 μM each SFA or LPS. IκBα degradation and p38 phosphorylation was measured by Western blot and expression of IL-6, TNF-α, CCL-2 mRNA was measured by real-time PCR relative to β-actin expression as described in the methods.
Supplementary figure VI: PBMC TNF-α production in response to SFAs

Human PBMC were separated from venous blood and cells were plated at a density of 1 x 10^6 / ml in six well plates. After challenge with vehicle (1% ethanol, EtOH), 100 μM of each saturated fatty acid (C12:0, lauric acid; C14:0, myristic acid; C16:0, palmitic acid and C18:0, stearic acid), or 100 ng/ml Pam₃CSK₄ (Pam3), LPS or flagellin (Flag) for 4 h, TNF-α release was measured in supernatants. * P < 0.05 vs vehicle treated cells.
Supplementary figure VII: Cytokine array of SFA vs LPS challenged PBMC

Human PBMC were challenged with medium alone, vehicle (1% ethanol), 100 μM mixed SFAs (equal proportions of C12:0, C14:0, C16:0 and C18:0), or 100 ng/ml LPS as control TLR-stimulant. Supernatant was decanted at 18 h and applied to antibody based cytokine arrays. Positive duplicate spots are identified as follows:

1) Positive control  5) Serpin E1  9) IL-6
2) IL-1Ra           6) GROα      10) MIP-1α
3) IL-8             7) CCL-1     11) MIP-1β
4) MIF              8) IL-1β     12) TNF-α

Not detected: C5a, CD40L, G-CSF, GM-CSF, sICAM-1, IFN-γ, IL1α, IL2, IL4, IL5, IL10, IL12p70, IL13, IL16, IL17, IL17E, IL23, IL27, IL32α, CXCL10, CXCL11, MCP-1, RANTES, SDF1, sTREM1
Supplementary figure VIII: Effect of SFAs on BLP- and LPS-induced TNF-α production

RAW macrophages were challenged with medium alone, 1 μg/ml Pam3CSK4 (A) or 100 ng/ml LPS (B) in the presence or absence of vehicle alone (EtOH) or 100 μM of each saturated fatty acid (C12:0, lauric acid; C14:0, myristic acid; C16:0, palmitic acid and C18:0, stearic acid). Secretion of TNF-α was measured at 4 h. Results shown are representative of at least three experiments.
Supplementary figure IX: Effect of SFAs on BLP- and LPS-induced TLR-stimulation

HEK-293 cells were transfected with NF-κB sensitive reporter (pELAM) and TLR2 (A) or TLR4/MD2 (B). After 72 h, transfected cells were challenged with medium alone, 100 ng/ml Pam₃CSK₄ (Pam3) (A) or 100 ng/ml LPS (B) in the presence or absence of vehicle alone (EtOH) or 100 μM of each saturated fatty acid (C12:0, lauric acid; C14:0, myristic acid; C16:0, palmitic acid and C18:0, stearic acid). Induction of reporter was calculated relative to cells cultured in medium alone after 18 h. Results shown are representative of at least three experiments.
Supplementary figure X: Presence of contaminating TLR-stimulants in common laboratory reagents

HEK-293 cells were transfected with NF-κB sensitive reporter (pELAM) and TLR2 (A) or TLR4/MD2 (B). Transfected cells were challenged with medium alone, 100 ng/ml Pam$_3$CSK$_4$ (Pam3) or 100 ng/ml LPS, 1:100 dilutions of commercially available restriction enzymes or azide-free antibodies, or sterile-filtered suspensions (1:10 weight /vol in PBS) of laboratory casein or mouse chow diluted 1:10 in DMEM/1%FCS.

* P < 0.05, ** P<0.01 vs cells cultured in medium alone.